ANTIBACTERIAL ACTIVITY OF COW GHEE, URINE, AND MILK ON SOME PATHOGENIC ORGANISMS (*Escherichia coli* and *Staphylococcus aureus*)

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ABSTRACT

Background: Infectious diseases are the leading cause of death worldwide. *Staphylococcus aureus* and *Escherichia coli* are implicated in many infections such as gastroenteritis, urinary tract infections among others. Cowghee and urine are among the most appreciated natural substances known to mankind since ancient times and their medicinal application has been greatly mentioned in depth in Ayurveda. Cow milk is a healthy food, with bio-protective role and is easily digestible.

Aim: This study was aimed at determining the antibacterial activities of Ghee, urine and milk of cow on *Escherichia coli* and *Staphylococcus aureus.*

Methodology: Antibacterial activities of varying concentrations of Cow Ghee, urine and milk was determined against *E. coli* and *S. aureus* clinical isolates. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also determined. The dilutions were made using Dimethyl Sulfoxide, while the antibacterial assay was by modified ditch diffusion method. Antibacterial activities were assessed by determination of zones of inhibition (ZI).

Results: The clinical isolates were sensitive mostly at higher concentrations. Cow Ghee yielded zone of inhibition at a least concentration of 50% against *S. aureus* and 25% concentration against *E. coli.* While Cow urine produced ZI at least concentration of 50% for both the isolates. Cow milk did not have any activity against the isolates at all concentrations. Cow Ghee has MBC and MIC at 60% and 40% respectively for both of the isolates. For cow urine, the MBC was 80% for *S. aureus* and 60% for *E. coli.* while the MIC was 60% for *S. aureus* and 40% for *E. coli.*

Conclusion: This showed that cow ghee and cow urine has antibacterial bioactive components and are potential sources of antibacterials. As such the use of cow ghee can also be encourage not only for its organoleptic properties but also due its significant pharmacological advantages.

Key words: Cow ghee, Cow urine, Zone of inhibition and Antibacterials

INTRODUCTION

Cow Ghee, also used interchangeably with Butter Cream, best known in Hausa as "man shanu" refers to a complex biological dairy product, composed of milk fat and other minor components. Ghee is a natural fatty substance produced from milk (Jhaadav, 2017). It is one of the most valued as well as appreciated natural substance known to mankind since ancient times. Of all the natural foods rich in fats and protein, it is the most wholesome and delicious. Ghee is well known on the ancient countries subcontinent and it was produced in ancient Indian as far back as 1500BC. The major use of Ghee is frying and dressing foods, and it is considered as a sacred article in some religious rites (Jhaadav, 2017). The dietary habit of individual communities varies according to socio-economic factors, religion as well as traditional customs.

Precise information on variety of food consumption patterns of populations through application of appreciate methodology is often needed not only for assessing the nutritional status of people, but also for elucidating the relationship of nutrient intake with specific deficiency of certain nutrients. A well balanced diet is essential from early stages of life for proper growth, development, and healthy active life (API, 2012). People believe that Ghee is a rich source of all nutritive aspects. Also, it has good flavor with pleasant aroma besides being a source of certain essential fatty acids.

Ayurveda Pharmacopeia of India (2012) describes Ghee as cool agent which is capable of increasing mental power and physical strength. The medicine system has proposed its application in many health disorders. However, ignorance about the high fat consumption and its harmful effects may initiate health problem.

Cow urine has a great pharmacological importance and its medicinal application has been greatly mentioned in depth in Ayurveda. Cow urine is found to be effective against reversal of certain cardiac and kidney diseases, indigestion, stomach ache, oedema, skin disease etc. The cow urine distillate has been patented as an activity enhancer and availability facilitator for biomolecules including anti-infective and anticancer agents (Jhadav, 2017). Cow urine has certain volatile and non-volatile components which might have high antimicrobial activity. After photo activation and purification cow urine was found effective against certain drug resistant bacterial strains. Cow urine contains few essential components such as Nitrogen, Phosphorus, Pheromones, Potassium, chloride, calcium and urinary protein. Cow urine is also used by traditional healers in combination with herbs to treat fever, epilepsy and anaemia.

Cow milk is a healthy food because of low calorie low cholesterol and high micro nutrient, protein calcium, vitamin and plays an important role in meeting requirement of various essential nutrient. It contains vitamin A, vitamin B complex group and vitamin C. It possesses a rejuvenatory health protecting properties and is one of the best vitalizers. It has bio-protective role in human health and is easily digestible. It is found to be effective in curing fever, pain tumours, diabetes and weaknesses and importantly act as a medium to administer medicine. It delayed the processes involved in aging. Lactic acid bacteria are present in curd and buttermilk that produces antifungal metabolites, viz; cyclic dipeptides, phenyl lactic acid as well as proteinaceous compounds and 3-hydroxylated fatty acid (schnurer, 2010).

Microorganisms have survived for thousands of years by their ability to adapt to antimicrobials, via spontaneous mutation or by DNA transfer which enables some bacteria to oppose the action of certain antibiotics, rendering the antibiotics ineffective (Samaila, 2018). Microorganisms employ several mechanisms in attaining drug resistance/multi-drug resistance which include: modifying their glycoprotein cell wall, enzymatic deactivation of antibiotics, altering the cell wall permeability to antibiotics, or transforming the target sites of antibiotics, the use of efflux pumps and horizontal gene transfer (Chowdhury, 2012).

Many different bacteria now exhibit multidrug resistance, including staphylococci, Salmonellae, as well as numerous other gram-negative bacteria and *Mycobacterium tuberculosis*. The recent emergence of Extended Spectrum β-Lactamase -producing *E. coli* and *K. pneumoniae* has led to therapeutic failures and death when treating septicaemia with standard agents, like cefotaxime. (Brooks, 2015).

Antibiotic resistant bacteria are able to transfer copies of DNA that code for a mechanism of resistance to other bacteria even distantly related to them, which then are also able to pass on the resistance genes and so generations of antibiotics resistant bacteria are produced via horizontal gene transfer (Schweizer, 2015).

Taking into cognizance those facts, the need for development of new antimicrobials becomes evident.

Natural products are given presence in this quest due to their safety and effectiveness. However, cow Ghee, urine and milk had been reported to exert antibacterial activity (Jhadav, 2017) due to their ability to generate radical oxygen species which are harmful to bacterial cells. Likewise, cow Ghee, urine and milk are traditionally used to cure catarrh, bone problems and many other ailments (API, 2012). This property needs to be investigated for future exploitation.

There are limited reports of bactericidal as well as the bacteriostatic activity of cow Ghee, urine and milk in this area particularly those against bacteria, which have developed resistance to many antibiotics. Therefore, this study attempts to evaluate the antibacterial effect of cow ghee, urine and milk on *Staphylococcus aureus* and *Escherichia coli.* This research is a quest for new, non-toxic, bioactive compounds, which will be responsible for the antibacterial activity, and that can help towards ameliorating the global resistance menace.

MATERIALS AND METHODS

Study Area

This study was conducted in the microbiology department of Gombe State University, Gombe State North Eastern Nigeria.

Sample Size

A total of 5 Cow Ghee samples were collected from 5 different Ghee sellers within Gombe metropolis. Five urine samples were also collected from 5 different cows within Gombe metropolis and another five milk samples from different cow milk sellers were also collected. The total of fifteen samples were transported in a sterile container to the microbiology laboratory of Gombe State University.

Isolation and identification of test organisms

The organisms (*Staphylococcus aureus and Escherichia coli*) were collected from Microbiology Laboratory Gombe State University. The organisms were sub cultured on nutrient agar. The colonies were further subcultured on selective media to obtain pure culture (Cheesbrough, 2006)

Biochemical Tests

The organisms were subjected to the following tests;

Citrate Test

Using a sterile wire loop, the bacterial colonies were inoculated into the tube containing Simmons citrate medium by means of streak inoculation. The tube was incubated for 24hours at 37°C. Development of deep blue color within 24hours to indicated a positive results and absence of deep blue color indicates negative result. (Doughari *et al.*, 2009).

Coagulase Test

Half ml of blood was dispensed in a sterile test tube. Two hundred and fifty µl of overnight broth culture containing isolate was added to the diluted plasma in the test tube. The tube was incubated at 37OC for 4hours. Formation of clot was observed after 24 hours (Cheesbrough, 2006).

Indole-Production Test

Peptone water was dispensed in 5 millilitre amounts in test tubes and sterilized by autoclaving for 15 min at 121°C (Cheesbrough, 2014). The organisms were inoculated in the broth for 48 hours. Kavocs reagent was then added, and the tube was shaken gently and allowed to stand. Production of a red colour indicates that cultured organisms were Indole positive (Doughari *et al.,* 2011).

Catalase Test

A glass slide was used for the test. Three drops of hydrogen peroxide were dropped onto the centre of the glass slide. The cultures of the bacteria to be tested were applied onto the glass slide using an applicator stick. The appearance of bubbles indicated a positive test while the absence of the bubbles indicated a negative test (Brooks, 2013).

Oxidase Test

A filter paper was impregnated with 0.3 millilitre of 1% P-aminodimethylalaniline oxalate (Gaby and Hadley reagent) and 0.2ml of 1% of nephthanol. The bacteria to be tested were then smeared on the filter. Color change was subsequently observed. A deep blue coloration indicates a positive reaction, while a colourless one indicates negative (Cheesbrough, 2006).

Media Preparation

The following media were prepared:-

Nutrient Agar

This medium was used for culturing the organisms before confirmation. Twenty eight gram of nutrient agar powder was dissolved in one (1) liter of distilled water following the manufacturer's instructions. The agar suspension was kept in an autoclave to sterilize at 121 °C for 15 minutes. The sterilized agar suspension was brought out to cool about 45 °C (checking hand tolerance level). Following aseptic techniques, the nutrient agar was poured into the bottles and placed in slopping position to form a slant. The slant was kept in an incubator for 24hours to observe any growth indicating contamination, before the assay organisms are inoculated on it for preservation.

Eosin-Methylene Blue Agar

Eosin-Methylene Blue Agar was used for sub-culturing suspected *Escherichia coli* strains. It was prepared according to manufacturer's instructions. Accordingly, 3.7g was dissolved in 100ml of distilled water using a 250ml conical flask, mixed thoroughly, and heated until it had completely dissolved. The conical flask was covered with foil paper properly. The mixture was then set in the autoclave for 121 °C for 15 minutes. After sterilization it was allowed to cool, and then poured into five Petri dishes and allowed to solidify (Cheesbrough, 2006).

Mannitol Salt Agar

Mannitol salt agar was for sub-culturing *Staphylococcus aureus.* It was prepared according to manufacturer's instructions. 6.66g was dissolved in 60ml of distilled water using 250ml conical flask and mixed thoroughly until completely dissolved. The conical flask was covered with foil paper. The mixture was then set in the autoclave for 121°C for 15 minutes. After sterilization, it was allowed to cool, then poured into the petri dishes and allowed to solidify (Cheesbrough, 2006).

Mueller-Hinton Agar

Mueller-Hinton Agar (MHA) was the media used for sensitivity testing. It was prepared according to manufacturer's instructions, (38g in 1000ml). Accordingly, 11.4g was dissolved in 300ml of distilled water using a 500ml conical flask and covered with a foil paper completely. It was autoclaved at 121°C for 15minutes. After sterilization, it was allowed to cool and poured into 15 Petri dishes and allowed to solidify (Cheesbrough, 2006).

Preparation of Standardized Inoculum

McFarland standard was prepared and used for standardization. A 1% solution of Sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99ml of water and mixed well. A 1% solution of barium chloride was prepared by dissolving 0.5g of dehydrated Barium chloride in 50ml of distilled water. A 0.5 McFarland standard was prepared by adding 0.6ml of 1% Barium chloride to 99.4ml of Sulphuric acid solution. Bacteria to be tested were emulsified in normal saline, and the turbidity was adjusted by comparing the turbidity of the bacteria inoculums to the McFarland Standard till the turbidity matches. (CLSI, 2012)

Preparation of Concentration of Cow Ghee, urine and milk

Different percentage concentrations of the cow Ghee, urine and milk were prepared constituting of 20%, 40%, 60%, 80%, and 100%, for the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests. For the sensitivity test, 4 concentrations were prepared: 12.5%, 25%, 50%, and 100% using sterile DimethylSulfoxide (DMSO) as a diluent. These were achieved by dissolving the respective volumes in the DMSO. 10ml of the sample (Ghee, urine and milk) were used for the 100% concentration. Subsequently, 8mls sample (Ghee, urine and milk), was dissolved in 2mls DMSO for the 80% concentration, 6mls of the sample (Ghee, urine and milk) in 4mls of DMSO, for the 60% concentration, 4mls sample (Ghee, urine and milk) in 4ml DMSO, for the 40% concentration, 2mls of the sample (Ghee, urine and milk) in 8mls DMSO, for the 20% concentration.

For the 25% concentration, 2.5mls of the sample (Ghee, urine and milk) were dissolved in 7.5mls of DMSO and for the 75% concentration, 7.5mls of the sample (Ghee, urine and milk) were dissolved in 2.5mls of DMSO. The 50% and 100% concentrations were prepared as described above for the MIC and MBC discs (Ghareeb, 2018).

Antibacterial Susceptibility Test

The agar well technique was employed according to Mohammed (2016). Mueller-Hinton agar was prepared according to manufacturer's instruction. The media was aseptically poured into the sterile disposable petri dishes. A sterile cotton swab was dipped into the standardized bacterial suspension and evenly inoculated on the surface of the Mueller-Hinton agar plates. The plates were then allowed to dry for 5 minutes. Wells were made using sterile cork borer and various concentrations of the samples (Ghee, urine and milk) were added into the respective wells. Ciprofloxacin antibiotics discs (500mg tablet dissolved in 1ml of distilled water) were placed on each plate in order to serve as positive control. Sterile DMSO served as negative control. The plates were incubated for 24hours at 37°C. Then the zones of inhibitions were examined and the diameter of the zone was measured (Mohammed, 2016).

Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration was determined through the tube/broth dilution technique by preparing various concentrations of the samples (Ghee, urine and milk) using DimethylSulfoxide. The dilutions made were 100%, 80%, 60%, 40%, and 20%. They were then incorporated into test tubes containing 2 ml nutrient broth, after which 0.1 ml of standardized inocula of the test organisms (*Staphylococcus aureus and Escherichia coli*) were added to each of the test tubes and incubated at 37°C for 24hours; then observed for the least concentration without turbidity. Tubes containing broth and inocula without extract served as negative control (Mohammed, 2016).

MIC was then recorded as the lowest concentration of the samples (Ghee and urine) that inhibits bacterial growth (no visible growth or turbidity).

Minimum Bactericidal Concentration (MBC)

The MBC was determined using the method of Mohammed (2016). The tubes in each set which did not show any growth during the MIC were used. A loopful of the content from each tube was streaked unto fresh nutrient agar plates and incubated for 24 hours at 37°C. After the incubation period, the plates were examined for the presence or absence of growth. Minimum bactericidal concentration (MBC) was recorded as the lowest concentration of sample (Ghee, urine and milk) at which no colony was formed.

Ethical consideration

Ethical approval (appendix 1) for the study was obtained from the ethics committee, Gombe State University.

Data analysis

The data generated from the study were entered in to Microsoft Excel and analysed using statistical software, Statistical Package for Social Sciences version 21 (SPSS, 2006). Results are presented in text, table and figures where necessary. Data are expressed as percentages. Significance was inferred at $p \leq 0.05$.

RESULTS

Organisms for assay were provided by the Microbiology Laboratory, GSU, and confirmed by colonial morphology, biochemical characteristics and Gram's stain. The results of the confirmation process revealed the colonial morphology of the isolates on nutrient agar and on selective media, and the results of microscopy and biochemical tests, which were used to identify the organisms (Table 1).

Table 2 and Table 3 showed that the zone of inhibition of cow Ghee on *Staphylococcus aureus* and *Escherichia coli* occurred mostly at significantly high percentage concentrations.

Against *Staphylococcus aureus,* it produces a zone of inhibition at the lowest concentration of50% and *Escherichia coli* produces zones at the lowest concentration of 25%. Highest activity at the 100% concentrations was found in *Escherichia coli* with an 18mm zone, against the Ciprofloxacin standard having 22mm. Lowest activity was found in *Staphylococcus aureus*, with 10mm zone of inhibition at 50% concentration, compared to the Ciprofloxacin standard having 20mm.
Table 4 and Table 5 showed that the zone of inhibition of cow urine on *Staphylococcus aureus* and *Escherichia coli* occurred mostly at significantly high percentage concentrations also. Against *Staphylococcus aureus* produces a zone of inhibition at the lowest concentration of50% and *Escherichia coli* produces zones at the lowest concentration of 50% also. Highest activity at the 100% concentrations was found in *Escherichia coli* with a 10mm zone, against the Ciprofloxacin standard having 22mm. Lowest activity was found in *Staphylococcus aureus* and *Escherichia coli*, with 9mm zone of inhibition at 50% concentration, compared to the Ciprofloxacin standard having 20mm.

Table 1: Identification of Test Organisms

Characteristic	***Staphylococcus aureus***	***Escherichia coli***
	Colonial Morphology on Nutrient Agar	
Appearance and Size	Circular, pinhead colonies	Circular/Round and small
Margin and Elevation	Entire margins, convex elevation	Entire, slightly raised
Texture, pigmentation and transparency	Smooth, brown appearance	Creamy and mucoid
	Selective Media	
	Mannitol Salt Agar	Eosin-Methyl Blue Agar
Form and size	Circular, medium sized colonies	Small and round
Margin, Pigmentation, Texture and Transparency	Yellow pigmented	Entire colonies that possess a greenish-metallic sheen.
Gram's Stain	Gram positive cocci in bunches seen.	Gram negative rods, singly and in pairs seen.
Catalase	+	+
Oxidase	-	-
MR	NA	NA
VP	NA	NA
Indole		+
Citrate		-
Urease	NA	NA
Coagulase	+	NA

Key: NA = Not carried out, + = Positive, - = Negative, MR = Methyl Red, VP = Voges-Proskauer.

Table 2: Zones of Inhibition (ZI) of Cow Ghee on *Escherichia coli*

Concentration	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
100%	17mm	17mm	16mm	18mm	12mm
50%	12mm	12mm	10mm	12mm	11mm
25%	11mm	9mm	0mm	11	0mm
12.5%	0mm	0mm	0mm	0mm	0mm
Positive Control (Ciprofloxacin)	23mm	22mm	21mm	22m	21mm
Negative Control (DimethylSulfoxide	0mm	0mm	0mm	0mm	0mm

Table 3 Zone of inhibition of cow Ghee on *Staphylococcus aureus*

Concentration	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
100%	14mm	12mm	12mm	12mm	12mm
50%	10mm	11mm	0mm	11mm	11mm
25%	0mm	0mm	0mm	0mm	0mm
12.5%	0mm	0mm	0mm	0mm	0mm
Positive Control (Ciprofloxacin)	23mm	22mm	21mm	22m	21mm
Negative Control (DimethylSulfoxide	0mm	0mm	0mm	0mm	0mm

Table 4 Zone of inhibition of cow urine on *Escherichia coli*

Concentration	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
100%	10mm	9mm	9mm	10mm	9mm
50%	10mm	0mm	0mm	9mm	9mm
25%	0mm	0mm	0mm	0mm	0mm
12.5%	0mm	0mm	0mm	0mm	0mm
Positive Control (Ciprofloxacin)	23mm	21mm	22mm	22m	22mm
Negative Control (DimethylSulfoxide	0mm	0mm	0mm	0mm	0mm

Table 4.5 Zone of inhibition of cow urine on *Staphylococcus aureus*

Concentration	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
100%	9mm	0mm	10mm	0mm	9mm
50%	0mm	0mm	9mm	0mm	0mm
25%	0mm	0mm	0mm	0mm	0mm
12.5%	0mm	0mm	0mm	0mm	0mm
Positive Control (Ciprofloxacin)	21mm	19mm	20mm	20mm	21mm
Negative Control (DimethylSulfoxide	0mm	0mm	0mm	0mm	0mm

NB: The antibiotic used (Ciprofloxacin) as a control. The concentration of antibiotic used was 16mg/disc. The NCLSI standard for Ciprofloxacin, for *S. aureus* and *E. coli*, as of 2016, is

≥ 21mm = Sensitive	16-20 mm = Intermediate	≤ 15mm = Resistant

Table 6 shows Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC)

The results of the minimum inhibitory concentration and the minimum bactericidal concentration results showed that for cow Ghee sample, the MBC was at 40% while the MIC was 20% observed for both *Staphylococcus aureus* and *Escherichia coli.*

Regarding the urine sample, the MBC of 80% was obtained for *Staphylococcus aureus* and MBC of 60% observed for *Escherichia coli*. The MIC of 60% observed for *Staphylococcus aureus*, whereas the MIC of 40% was observed for *Escherichia coli.*

No minimum inhibitory concentration or minimum bactericidal concentration was carried out on the milk sample.

Table 6: MIC and MBC (in percentage, %) of the Isolates

Isolates	MIC (%)		MBC (%)	
	Cow Ghee	Cow urine	Cow Ghee	Cow urine
E. coli	20	40	40	60
S. aureus	20	60	40	80

DISCUSSION

This study found out that the maximum zone of inhibition was highest in *Escherichia coli*, (18-13mm), with an average of 15mm. Thus, is in conformity with a finding from previous studies stating that cow Ghee was active against *E.coli* (Jhadav, 2017). It is notable also that against *Staphylococcus aureus,* a 10mm zone was produced.

The lowest zones of inhibition generated by the highest urine concentrations are found in *Staphylococcus aureus*, with zones ranging from 11-9 mm in the former, and 11-9mm in the latter, having an average of 10 mm in both. This is in line with the finding of Tan (2012), that *S. aureus* can easily develop resistance.

Observation revealed that the organisms exhibited resistance to cow milk only. This is in contrast to the report of Tailor (2010), that cow milk possesses antimicrobial substances such as lysozyme, lactoferin and peroxidase. This disparity could be attributed to the lower concentration of the milk used and to the mode of the milk preparation. Relatively low zone of inhibition values were obtained from the cow Ghee and urine compared to ciprofloxacin, a finding in agreement with previous findings (Choudhary, 2012).

This research found out that the minimum inhibitory concentration of cow Ghee against the isolates was 20%, while the minimum bactericidal concentration was 40%. For cow urine, the minimum inhibitory concentration was at 40% and minimum bactericidal concentration was 60% for *Escherichia coli*, and then minimum inhibitory concentration of 60% and minimum bactericidal concentration of 80% for *Staphylococcus aureus*. It is in agreement with Jadhav. (2017), who proposes the potentiality of the use of cow Ghee as a drug substitute.

CONCLUSION

This study revealed the antimicrobial activity of cow Ghee, urine and milk against the bacterial isolates. Higher concentrations of the cow Ghee elicited better zone formations than cow urine and cow milk shows no zone of inhibition at all. This study found out that the maximum zone of inhibition was highest in *Escherichia coli*, (18-13mm), with an average of 15mm. Thus is in conformity with a finding from previous studies stating that cow Ghee was active against *E. coli* (Jhadav, 2017). It is notable also that against *Staphylococcus aureus,* a 10mm zone was produced. The lowest zones of inhibition generated by the highest urine concentrations are found in *Staphylococcus aureus*, with zones ranging from 11-9 mm in the former, and 11-9mm in the latter, having an average of 10 mm in both.

Recommendations

This study hereby recommends the following:

1. Further research into the principles behind the antimicrobial activity of cow Ghee and cow urine should be undertaken;
2. In the future more research should be carried out on other bacterial species not covered by this research, e.g. *Bacillus subtilis, Pseudomonas aeruginosa,* etc.
3. The use of ciprofloxacin in therapy, is effective against both bacterial species, but attention had to be drawn to the slow resistance emerging against it;
4. Cow Ghee and urine should be tested against fungal isolates in further researches;
5. Sustained incorporation of cow Ghee in food products, both for family consumption and as an ingredient in industrial manufacturing processes, should be encouraged, in view of not only its desired organoleptic properties, such as flavor, taste and aroma, but also its proven antibacterial activity.

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