

## Antimicrobial Activity of Kaempferia Galanga Rhizome against Biofilm of Vibrio Cholerae Outbreak from Limbang Sarawak

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### ABSTRACT

Kaempferia galangal rhizome is one of the traditional medicinal plant species in Zingiberaceae family and well known as “cekur” in Borneo. Rapid emergence of multidrug resistance of *V. cholerae* biofilm has increased the failure of antibiotic treatment in recent years. This research was designed to demonstrate the antimicrobial activity of *K. galangal* rhizome extract against twenty six ( $n=26$ ) *V. cholerae* biofilm which were isolated from outbreak in Limbang, Sarawak in 2016. *K. galangal* rhizome was analyzed by methanol extraction and tested against biofilm of *V. cholerae*. The susceptibility of *V. cholerae* towards *K. galangal* rhizome was evaluated using disk diffusion method which showed a maximum zone of inhibition of 12.0 mm at 1000 mg/mL concentration. As a result, the MBEC<sub>50</sub> of *V. cholerae* was between 125 mg/mL to 250 mg/mL while more than 90% biofilm eradication (MBEC<sub>90</sub>) was achieved by 500 mg/mL extract concentration. Extract-treated cell showed change in the morphology of *V. cholerae* by destruction of cell wall. *K. galangal* rhizome extract acts as a potent antibiofilm agent with dual actions by preventing and eradicating the biofilm of *V. cholerae*.

#### Keywords:

*K. galangal* rhizome, *vibrio cholerae*, antimicrobial activity, minimum biofilm eradication concentration, biofilm

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## 1. Introduction

According to a study conducted by the World Health Organization [20], antibiotic therapy has faced difficulties due to misuse and overuse of antimicrobials causing several life threatening infectious diseases. Infections caused by resistant bacteria will affect the treatment, prolonged illness, lead to expensive health-care resources and increased risk of worse clinical outcomes and death. The increasing failure of the chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of antimicrobial agents from local edible plants against microorganisms [15].

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Biofilm is defined as a community of microbial cell found in a wide range of ecosystem that is attached to the surface and embedded in the extracellular polymeric substances [7]. According to Costerton [4], bacteria that included in structure of biofilm are commonly resistant to most of the current antimicrobial therapies. Earlier studies by Centers for Disease Control and Prevention [2] showed that plant extracts have been used as a main weapon to control formation of biofilm by interfering with microbial metabolism or by facilitating their adherence to the surface.

In 2016, the cholera outbreak has been reported in Limbang, Sarawak on March 6 by Borneo Post [12]. Even though 111 cases were reported after March 6, two of the cases were a cause for concern, as one of the patients is pregnant while the other is suffering from a medical ailment. Cholera is one of an acute diarrhoeal disease which is potentially epidemic. It is also classified under life-threatening caused by toxigenic *V. cholerae* strains. *V. cholerae* is a rod-shaped facultative anaerobe which is gram-negative. This bacterium enters the human body by ingestion of contaminated water or food and sometimes by faecal materials and resided in human gut. Referring to Finkelstein [9], an individual who is affected by *V. cholerae* will have symptoms like watery stools followed by vomiting that would result in hypoglycemic shock then acidosis. If untreated immediately this will lead to severe dehydration and finally causes death within hours.

*Kaempferia galangal* rhizome is an aromatic ginger belongs to Zingiberaceae family that is locally known as sand ginger or “cekur”. The extract have been used in traditional Chinese medicine as a decoction or powder for indigestion, cold, abdominal pains, headache and toothache. Rhizome of this plant is widely cultivated throughout Southeast Asia. Choudury et al. [3] stated that *Kaempferia galangal* rhizome contains cineol, borneol, 3-carene, camphene, kaempferol, kaempferide, cinnamaldehyde, p-methoxycinnamic acid, ethyl cinnamate, and ethyl p-methoxycinnamate as a phytochemical compounds.

Rapid emergence of multidrug resistance of biofilm of *V. cholerae* has increased the failure of antibiotic treatment in recent years [16]. For the past two decades, many studies have concentrated exclusively on identification of new antibiotic from various medicinal plants extract [1]. Recently, uncontrolled use of antibiotics in treatment of disease has become a major health concern [10]. Thus, this study was conducted to demonstrate the antimicrobial activity of *Kaempferia galangal* rhizome extract over biofilm of *Vibrio cholerae* strain which caused the outbreak in Limbang, Sarawak.

## 2. Methodology

### 2.1. Sample Preparation

The plant of *Kaempferia galanga* was collected from a local area in Samarindah, Kuching in September 2016. The sample was washed thoroughly with distilled water then shade dried at room temperature for 7 days. The sample was grounded to fine powder by using a grinder in the lab of Resource Science and Technology Faculty, UNIMAS.

### 2.2 Methanol Extraction

Twenty (n=20) g of powdered sample was added into 150 mL of 96% methanol in conical flask. The conical flask was placed on shaker machine for 30 days. After that, the extract was filtered using filter paper and evaporated in oven at 45°C. The dried extract was dissolved in Dimethyl sulfoxide (10% DMSO) and stored in refrigerator until further analysis [8].

### 2.3 Bacterial Strains and Preparation of Inoculums

A total of twenty six (n=26) *V. cholerae* strains isolated from cholera outbreak in 2016 from Limbang, Sarawak were used. By using aseptic technique, isolated *V. cholerae* strain was transferred into a 1.5 mL microcentrifuge tube which contained 1 mL of tryptic soy broth (TSB). The culture was thoroughly mixed by vortexing. The turbidity of suspension was adjusted to 0.5 McFarland Standards. McFarland Standards was prepared by mixing barium chloride with sulfuric acid (McFarland Standards 0.5: 0.05 mL 1% barium chloride with 9.95 mL 1% sulfuric acid).

#### 2.4 Disk Diffusion Test

The extract was tested to determine their antibacterial activities by using the standard paper blank disc diffusion assay as described by Dalsgaard *et al.*, [5]. Then, methanol extract was diluted to concentration 10 mg/mL, 100 mg/mL and 1000 mg/mL with 10% DMSO solution [6]. For the preparation of bacterial inoculum, isolated colony of *V. cholerae* was suspended in 2 mL of sterile saline. The turbidity of suspension was adjusted with 0.5 McFarland standard [10]. *V. cholerae* culture was swabbed onto a Mueller-Hinton Agar (MHA) medium using sterile cotton swab. Sterile filter paper discs (6 mm in diameter) loaded with 20  $\mu$ L of extract were then allowed to dry and placed on the agar plates of inoculated testing bacteria. The plates were incubated at 37°C for 24 hours and observed for zone of inhibition in millimeter [17]. The experiments were performed in triplicates. Chlorhexidine (CHX) (1,1-hexa-methylenebis(5-p-chlorophenyl) biguanide) was used for standard control and was dissolved in sterile-distilled water to get 10 000  $\mu$ g/mL (1% stock solution).

#### 2.5 The Determination of Minimum Biofilm Eradication Concentration (MBEC)

The antimicrobial activity of *Kaempferia galangal* rhizome extract in the biofilm was also examined using the minimum biofilm eradication concentration (MBEC) assay. Briefly, 200  $\mu$ L ( $10^6$  cfu/mL) of *V. cholerae* strain was inoculated into the 96-well microtiter plate (flat bottom) and incubated without agitation for 24 hours at 37°C. Column 1 served as negative control (bacteria-free) while column 2 was positive control (untreated *V. cholerae*). After incubation, the medium was discarded by pipetting it out. Then, each well of micro-titer was filled with 100  $\mu$ L of TSB. *K. galangal* rhizome extract was then added to biofilms in two-fold dilutions serially from 500 mg/mL to 0.156 mg/mL and incubated for 24 hours in appropriate conditions at 37 °C.

After incubation, the medium was discarded and non-adherent cells were removed thoroughly by washing the biofilm thrice with sterile distilled water. The plate was inverted and dried for 30 minutes. Percentage eradication was calculated by using the equation  $[1 - (A_{570} \text{ of the test} / A_{570} \text{ of non-treated control})] \times 100$ . The MBEC value was defined as the concentrations that showed 50% and 90% eradication of biofilm formation. PBS was used as the positive, non-treated and blank controls, respectively. Proceeded by MBEC where the biofilm at the bottom of wells were scratched with metal loop and spread over the surface of tryptic soy agar (TSA).

#### 2.6 Scanning Electron Microscopy (SEM)

A scanning electron microscopy (SEM) observation was performed to examine the morphology changes of *V. cholerae* after treated with several concentrations of *K. galangal* rhizome extract. After 8 hours of incubation at 37 °C in an appropriate condition, the bacterial pellet was collected and fixed overnight in 2.5 % glutaraldehyde at 4 °C. Then, the bacterial pellet was washed with PBS and dried for 15 minutes before being dehydrated by absolute ethanol for another 15 minutes. The samples

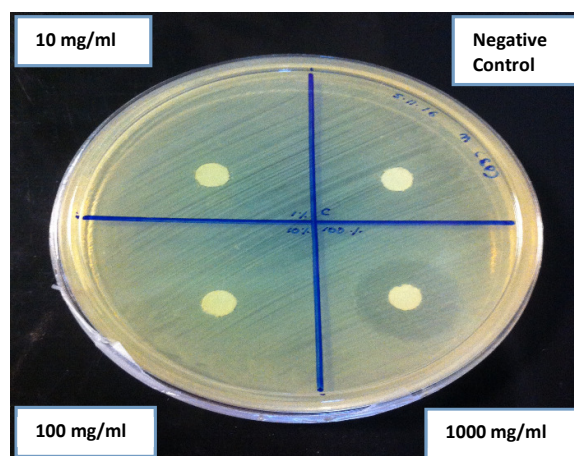
were subsequently dried by a critical point drying method and coated with gold. The microbial morphology was observed with a field emission SEM.

### 2.7 Statistical Analysis

The data obtained after measuring the diameter zone of inhibition was subjected to one-way analysis of variance (ANOVA) to determine the differences among the detergents defined at  $p < 0.05$ . The corresponding variables would be more significant  $< 0.05$ , if the absolute F ratio is larger and the p-value becomes smaller. All measurements were carried out in triplicates and the results were reported as the mean  $\pm$  standard deviation (SD) of independent trials. Data analysis was carried out using the Minitab 16 statistical package (Minitab Inc., State College, PA, USA).

## 3. Results and Discussion

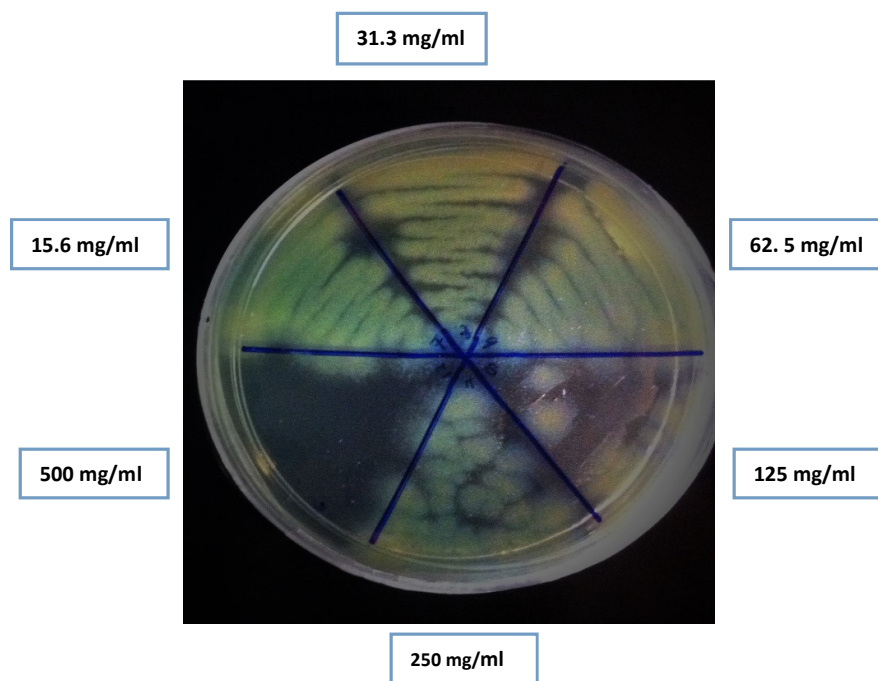
The result of antimicrobial activity was determined by measuring the diameter of zone of inhibition in millimeter (mm). The *K. galangal* rhizome extract was tested under *in vitro* conditions by agar disc diffusion at different concentrations against *V. cholerae*. The antimicrobial potential of extract was assessed in terms of zone of inhibition of bacterial growth. At concentration 1000 mg/mL, the highest zone of inhibition  $12.00 \pm 0.05$  (mm) was observed against *V. cholerae*. In the case of 100 mg/mL and 10 mg/mL, there was no zone of inhibition observed. Therefore, the methanol extract does not inhibit the growth of *V. cholerae* at 100 mg/mL and 10 mg/mL as no clear zone was observed around the filter paper disk as shown in Figure 1. Overall, all the *V. cholerae* strains appeared most sensitive towards *K. galangal* rhizome extract at 1000 mg/mL concentration.



**Fig. 1.** Disk diffusion of *Kaempferia galangal* rhizome extract against *V. cholerae* at several concentration

At 500 mg/mL concentrations *Kaempferia galangal* rhizome extract, biofilm of *V. cholerae* was completely eliminated as shown in Figure 2. The biofilm of *V. cholerae* was completely susceptible to 500 mg/mL extract concentration whereas 50 mg/mL and 5 mg/mL concentration is susceptible over the biofilm of *V. cholerae*. Based on the observation of streaked plate, biofilm of *V. cholerae* was totally inhibited at 500 mg/mL concentration because there was no visible growth of bacteria. At 125 mg/mL and 250 mg/mL concentration of methanol extract, growth of biofilm was reduced by half. The concentrations of *Kaempferia galangal* rhizome extract to eradicate for more than 50% biofilm

formation (MBEC<sub>50</sub>) of *V. cholerae* ranged between 125 mg/mL to 250 mg/mL. And for more than 90% biofilm eradication (MBEC<sub>90</sub>) was done by 500 mg/mL concentration. PBS could completely (100%) inhibit or remove biofilm formation of *V. cholerae*.

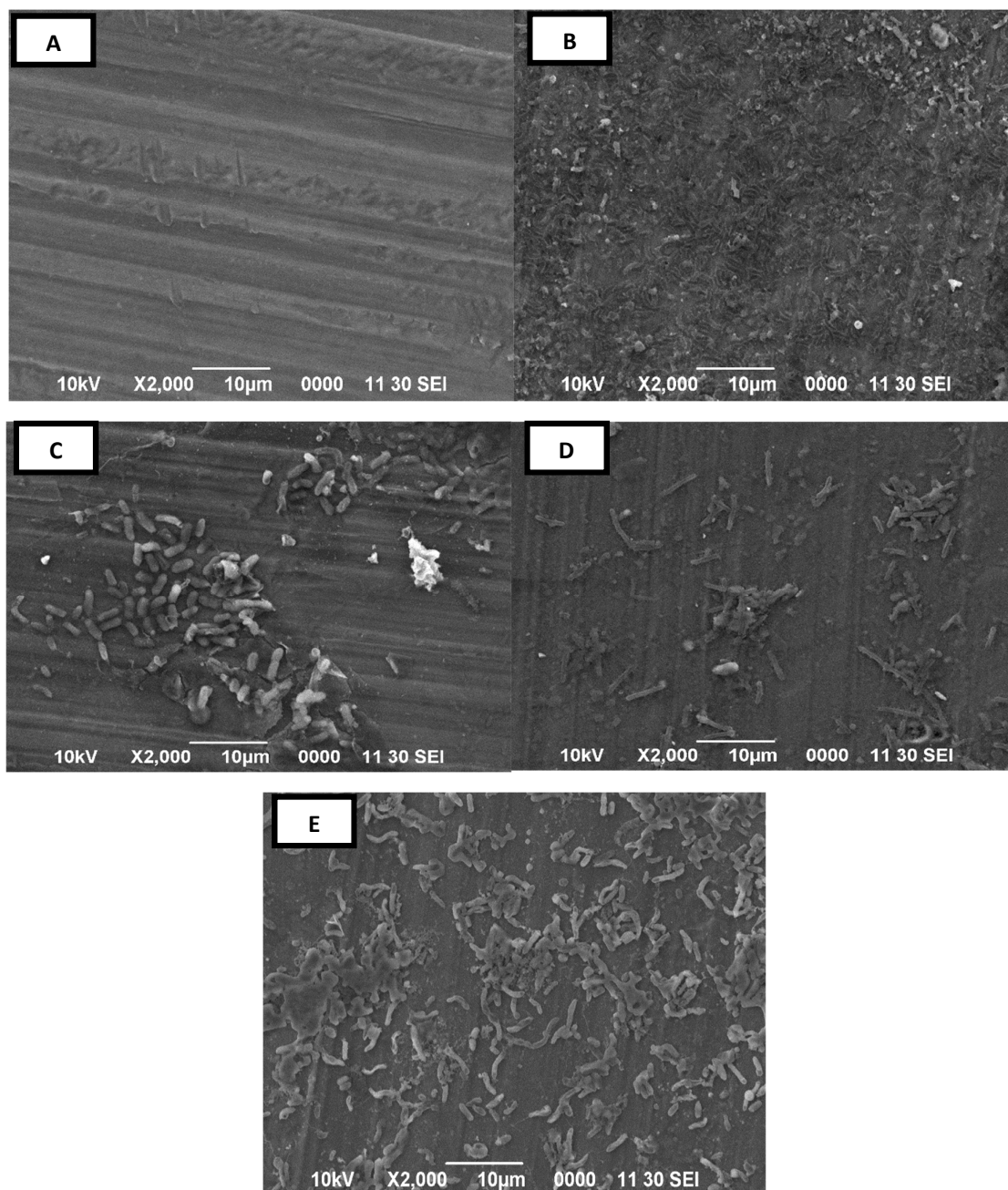


**Fig. 2.** Minimum biofilm eradication concentration (MBEC) of *V. cholerae* after treated by 500 mg/mL *Kaempferia galangal* rhizome extract

In Figure 3, the change in morphology of *V. cholerae* biofilm was observed using SEM after treating with several concentrations of *K. galangal* rhizome extract in comparison with the positive control. Bacterial cells of *V. cholerae* in positive control showed a regular and smooth rod shaped surface of bacterium as shown in Figure 3 (B). Refer to Figure 3 (C and D), the shape or structure of the bacterium is still unchanged after treated with 10 mg/mL and 100 mg/mL. In other word, the rod shape was maintained at 10 mg/ml and 100 mg/mL. At 1000 mg/mL concentration, the shape of the bacteria have changed or lost their original shape or structure when treated with methanol extracts which was shown clearly in Figure 3 (E). A distorted and irregular cell wall structure was reported at Figure 3 (E).

*K. galangal* rhizome was chosen as sample of this study because limited research has been done on it. This research was designed to study the antimicrobial and antibiofilm activity of *K. galangal* rhizome extract as an alternative to the commonly used antibiotics. For the past two decades, many studies have been carried out from various medicinal plant as a novel antimicrobial and antibiofilm agents due to rapid emergence of multi-drug resistant [1]. Antimicrobial and antibiofilm activity of a plant was determined by bio-reactive phytochemicals which have potential to treat infectious diseases of human. It has been proven to be effective in treatment of long time illness such as diabetes and malaria.





**Fig. 3.** Scanning electron microscope images of *V. cholerae* treated with several concentrations; (A) Negative control [free bacteria] (B) Positive control [Untreated *V. cholerae*] (C) treated at 10 mg/mL (D) treated with 100 mg/mL (E) treated with 1000 mg/mL

Previous study by Kiuchi *et al.*, [13] on *K. galangal* rhizome over *V. cholerae* reported that  $12.3 \pm 0.05$  (mm) for methanol extract at 5 mg/mL. In this study, methanol solvent was found to have potent antimicrobial activity against *V. cholerae* strain. According to Elexson *et al.*, [8], methanol is the best solvent due to the consistent extraction result of antimicrobial substances from medicinal plants compared to other solvents, such as water, ethanol or hexane. In this study, there is no zone of inhibition was observed in this study at 10 mg/mL and 100 mg/mL concentration. In other word, methanol extract is more susceptible against *V. cholerae* at 10 mg/mL and 100 mg/mL.

The absence of zone of inhibition over *V. cholerae* represent the concentration is not sufficient to fight the pathogenicity of the bacteria. It shows that *K. galangal* rhizome extract have stronger antimicrobial activity at 1000 mg/mL concentration. The variation of antibacterial activity of our extracts might be due to changes and several adaptation to several climatical, ecological and geographical conditions. Based on Larsen *et al.*, [14], factors that may affect the composition of extracted product are soil composition, age of rhizome, vegetation cycle and effect of climate. Sunayana [18] claims that antimicrobial activity of *K. galangal* plant extracts was not purely dependent on only one main active chemical but to the combined action of additional other compounds include phenolic acids, alkaloids, flavanoids, terpenes, terpenoids and naphthoquinones.

At 500 mg/mL concentration of methanol extract, growth of biofilm was completely eliminated while at 250 mg/mL and 125 mg/mL concentration, the growth of biofilm was seen to be reduced into half. From the twenty six (n = 26) total of the *V. cholerae* isolates, the concentrations of *K. galangal* rhizome extract to eradicate for more than 50% biofilm formation (MBEC<sub>50</sub>) of *V. cholerae* ranged between 125 mg/mL to 250 mg/mL. And for more than 90% biofilm eradication (MBEC<sub>90</sub>) by 500 mg/mL concentration.

Based on the result obtained in this current study, higher concentrated methanol extract is needed to eradicate the biofilm of *V. cholerae* compared to other gram positive bacteria in previous study. This is because, gram negative bacteria are considered to be more resistant due to their outer membrane acting as a barrier to many environmental substances including antimicrobial substances. Moreover, they could stimulate the membrane-associated mechanisms of resistance via the expression of efflux pumps to control the antibacterial agents.

The antibiofilm is a desired property that expected from herbs extracts. In this study, *K. galangal* rhizome extract leaves extract has been revealed having dual actions in preventing and eradicating biofilm formation. It may be worth for considering the *K. galangal* rhizome extracts to replace chemical-based antimicrobial agents to divest cholera. However, it was reported on chemical-based antimicrobial agents developed chemical-based antimicrobial agents have proven side effects and complications in human body [10].

As revealed by SEM, biofilm of *V. cholerae* that treated by high extract concentration (1000 mg/mL) undergo irregular and distorted cell wall structure. This is because, high osmotic pressure on the cell causes swelling of bacteria cell and failure of cell membrane regulation. Moreover, *V. cholerae* is a gram negative bacteria which have thinner peptidoglycon layer causes may causes rapid alteration of bacterial shape. Meanwhile, low concentration exposure of extract does not revealed an irregular cell wall structure. All of the results above indicated that *V. cholerae* is more sensitive at 1000 mg/mL. Watnick [19] reported that formation of biofilm consist of two steps in which *V. cholerae* first adhere temporarily to the foreign-body surface and then accumulate to form a permanent, complex biofilm structure. The interaction between specific adhesions located on cell wall and extracellular matrix components deposit on the surface is vital for the primary attachment. The destruction of cell wall of bacteria by extract inhibited the growth of bacteria and lead to primary biofilm architecture.

#### 4. Conclusions

The results of this study indicates that *K. galangal* rhizome extract act as an antimicrobial and antibiofilm against biofilm of *Vibrio cholerae* and therefore suggest that the traditional use of this plant rhizome as an alternative way in antibiotic therapy of divesting cholera disease. This is due to the perception that plant extract is safe for consumption and do not give any side effects and complications in human body. However, these research findings require a further scope to identifying

and isolation of responsible phytochemical compounds which is useful to compact the diarrhoeal disease caused by *Vibrio cholerae*.

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