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Analysis of bioactivity of leaf extract of *Piper methysticum* Forst F. as inhibitors of bacterial activity of *Salmonella paratyphi*

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ABSTRACT

Piper methysticum Forst F. is categorised as a yearly plant that has been used by the peoples in Merauke, Papua Province of Indonesia for medicinal, social activity, and cultural purposes. P. methysticum contains chemical compound of Alkaloid (Piperidine, pipermethysticine), flavonoid, resin (kava lactone), kavain, tannin, saponin, anthraquinone, and a little volatile oil. This plant could effectively function as anti-fungal, antibiotic, antiseptic, antimicrobial medication. Since the 20th century, kava has become popular among western people as supplementary herb which could cure anxiety and insomnia. The scientist today have been searching for new drugs for treatment of human diseases caused by pathogenic bacteria. One of the most threatening bacteria is the genus Salmonella, especially Salmonella typhi that causes typhoid fever, Salmonella paratyphi that is responsible for paratyphoid fever, and Salmonella typhimurium that causes typhoid like disease in mice. It is already known that Piper methysticum (Wati) extract posseses antimicrobial activity against Salmonella typhi, Salmonella typhimurium, and Escherichia coli. But there was no information of its ability to inhibit Salmonella paratyphi activity. Here, we show that there is Piper methysticum inhibition on Salmonella paratyphi activity. This result was taken from the agar diffusion method and standard viable plate count method. Zone of inhibitions were observed by agar diffusion method with diameters ranging from 10 to 14.5 mm in size for Piper methysticum leaf extract with concentration of 1140 mg/mL, and 8.5 to 12 mm in size for Piper methysticum leaf extract with concentration of 500 mg/mL. Standard viable plate count gave reduction number of bacteria colonies approximately 15 % to 77 % for Piper methysticum concentration of 50 mg/mL. These results show that Piper methysticum leaf indeed posses antibacterial activity against Salmonella paratyphi and has great potential in the development of new drugs for treatment of paratyphoid disease.

Keywords: Bioactivity, leaf extract, *Piper methysticum* Forst F, bacteria of *Salmonella paratyphi*, Papua-Indonesia

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INTRODUCTION

One of the research topics that continue to be done and considered the most challenging currently in various parts of the world is a new *drug discovery* research. Continuing this research related to the human need to combat various diseases as well as to protecting human health. New medicines needed to compensate for the human needs in preventing and fighting disease. Although many drugs currently used, but still needed improvement in the quality of treatment and suppression of drug prices to be affordable by the whole society.

Stages of *drug discovery* in general starting from the search of materials that have the potential to be developed into drugs [1]. Further analysis is viewed mutations in human DNA in the coding region and the non-coding region [2-5]. The main source of the material derived from natural or synthetic materials. Although the technology has been developed natural materials synthesis (*synthesis of natural products*), but still there are compounds that can not be

synthesized and must be taken directly from nature, either from land or sea. Most of the existing drugs derived from plants or microbes, some of which undergo chemical modifications to produce an effective drug in accordance with the desired [6]. One example of a disease that many use drugs like this is cancer, where most of the medicine in the world is derived from natural materials [7].

Many studies have been done, including by conducting screening were randomized to the various sources of natural ingredients to find a compound having bioactivity. The materials have long been used as traditional medicine for its own interest to give this test. Active compound derived, were tested for biological (*bioassays*) to determine the type of biological activity possessed. After isolation, purification and the elucidation of the structure, and then conducted a series of clinical trials before it can be approved as a drug to be distributed to the market [8].

Opportunity to discover new drugs is very large in countries with abundant natural resources and biological diversity. Indonesia is one country with those categories. In addition, the fact that the Indonesian nation since ancient times have used a variety of traditional medicines derived from nature. Source of traditional medicine is very interesting to study further and developed into modern medicine.

One source of natural materials that have the potential as a drug is *Piper methysticum* plant. Based on the results of previous studies, *Piper methysticum* plants are known to have many beneficial properties for health. In addition, the plant is not *Piper methysticum* foreign goods for the people of southern Papua, where almost all people in the southern province of Papua recognize *Piper methysticum* leaves as customary intoxicating drink. On the basis of empirical facts, then leaves *Piper methysticum* taken as a source of natural ingredients in this study to examine further benefits.

It is known from previous studies that some plants *Piper methysticum* have a specific biological activity. However, no publication on leaves *Piper methysticum* ability to inhibit the growth of bacteria of *Salmonella paratyphi*. This study using of *Salmonella paratyphi* A, B, C, D, and E as the target bacteria, which aims to determine whether *Piper methysticum* leaf extract is also able to inhibit the bacteria. *Salmonella paratyphi* is a bacterial pathogen causing the disease paratyphoid fever. In developing countries such as Indonesia, the disease paratyphoid fever is common and becoming endemic cases. Because the source of drugs derived from his own country like leaves *Piper methysticum* is needed to reduce cases of paratyphoid fever.

Piper methysticum leaves used in this study was taken from one of the areas in the Papua province of Indonesia, which is the Merauke regency, where *Piper methysticum* leaves are then extracted with water solvent to obtain a extract powder of *Piper methysticum*. These extracts were tested for activity against the bacteria of *Salmonella paratyphi* A, B, C, D, and E with agar diffusion method. Then, also testing the count plate method to determine the number of bacteria growth can be inhibited by the *Piper methysticum* leaf extract.

MATERIALS AND METHODS

Culturing of the bacterium *Salmonella paratyphi*

Bacteria of *Salmonella paratyphi* as the target bacteria cultured in media *Luria Bertani* (LB) solid or liquid. Solid LB media made from a mixture bacto tryptone 1% (w/v), yeast extract 0.5% (w/v), NaCl 1% (w/v), and bactoagar of 1.5% (w/v), were completely dissolved in distilled water. For the manufacture of liquid LB medium, used the same mixture as the solid LB media but without bactoagar [9-11]. Both solid and liquid LB media, both then sterilized by autoclaving. Within Laminar Flow, solid LB media that has been sterilized and then poured into a 10 cm diameter petri dishes. Once hardened, the media were incubated at 37 °C without shaking overnight to determine whether there is contamination. Solid LB media uncontaminated then used to breed bacteria.

Bacterial cultures in solid media done in 2 ways, namely streaking (*streak plate technique*) or disseminated (*spread plate method*). In the etching method of bacteria, used stem ose to take a single bacterial colony of bacteria seed then was etched on the surface of solid media. As for the method spread of bacteria, the bacteria used stock liquid media captured with as many as 100 µL Eppendorf micropipette is then spread over the surface of a solid medium using a solid rod L. Media already inscribed or spread of bacteria and then incubated without shaking at 37 °C for 16-18 h.
Culturing the bacteria in a liquid medium is done by transferring a single colony of bacteria on solid media stock bacterium into a liquid medium using stem ose. This method is also known as inoculation. A liquid medium has been inoculated and then incubated at 37 °C with shaking using a *shaking incubator* for 16-18 h. The number of bacterial cells that grow in the liquid medium can be determined by measuring the absorbance or commonly called an *optical density* (OD), in which the OD 0.5 to 0.6 shows the bacterial count 10^6-10^8 cells/mL. Bacterial culture in liquid or solid media and then stored at 4 °C [12-14].

All the activities of breeding of bacteria, either in solid or liquid media media done in *Laminar Flow* with aseptic technique aims to minimize contamination. Disinfectant used is 70% alcohol that can kill the microorganisms. In addition to denature the protein and lipid membrane extract, alcohol can also cause dehydration and may even inactivate the virus.

Extraction of *Piper methysticum* leaves

To make *Piper methysticum* leaf extract used 56 grams of dried leaves of plants that have been pounded *Piper methysticum*. Then do a continuous extraction using a Soxhlet for about 9 h with water as much as 175 mL of solvent. *Piper methysticum* plant extract solution obtained is then dried in an oven 60-70 °C for 5 days, so that the resulting dry powder *Piper methysticum* leaf extract. The dry extract is stored at 4 °C.

Agar diffusion method

In the agar diffusion method was used sterile solid LB media. Bacteria cultured liquid medium taken as 100 µL using Eppendorf micropipette and spread over the surface of the solid media with the rods L. After pervasive, on the surface of solid media are then placed in sterile filter paper disc. The filter paper discs are made form spherical diameter of 5.5 mm and sterilized by autoclaving. Then on top of the filter paper disk dropped into each of 10 µL *Piper methysticum* leaf extract solution with a concentration of 500 mg/mL, 10 µL *Piper methysticum* leaf extract concentration of 1140 mg/mL, 10 µL ampicillin concentration of 1 mg/mL as a positive control, and 10 µL of distilled water as a negative control. Fig. 1 shows the position of the filter paper disc on a petri dish.

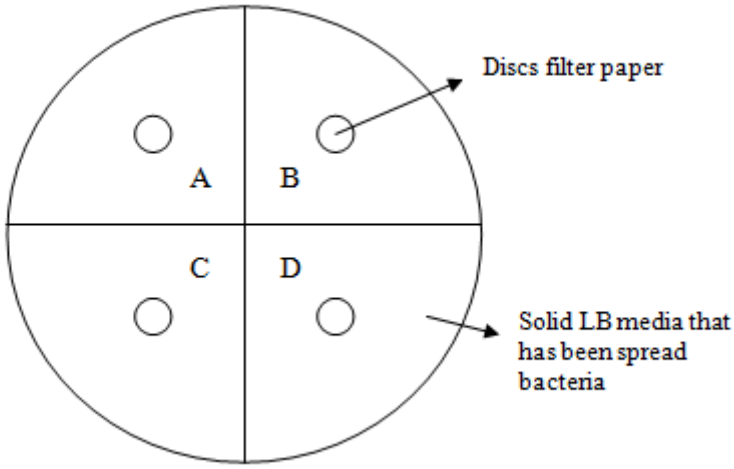

Fig. 1. Position the filter paper disc on a petri dish. Four discs of filter paper is placed in the middle of each part (A, B, C, and D) on a petri dish. Discs filter paper etched with 4 kinds of solution are: A few drops of extract *Piper methysticum* with a concentration of 500 mg/mL, and B droped of extract *Piper methysticum* with a concentration of 1140 mg/mL, C spilled ampicillin 1 mg/mL as a positive control, and D drops of distilled water as a negative control

The solution is dripped onto filter paper discs are left to infuse for about 30 min. Then done the incubation at 37 °C without shaking for 16-18 h. The results of the agar diffusion method demonstrated by the clear zone around the filter paper disc which is an area of inhibition of bacterial growth.

In the agar diffusion method, the leaf extract of *Piper methysticum* dripped on the paper disc was prepared by dissolving dry powder *Piper methysticum* leaves in boiling distilled water. The extract solution was stored at -20 °C. Ampicillin is used as a positive control was also stored at -20 °C.

Count plate method

At the plate count method used three kinds of solid media, namely solid LB media, solid LB medium containing *Piper methysticum* leaf extract, and solid LB medium containing ampicillin. Solid extract medium was prepared by adding 100 µL *Piper methysticum* leaf extract with a concentration of 50 mg/mL to the media before poured into the petri dish. While a solid medium containing ampicillin was made by adding 100 µL ampicillin concentration of 200 µg/mL to the media, which is also carried out before the media poured into a petri dish.

A total of 100 µL liquid bacterial culture media taken with a micropipette Eppendorf and propagated with stem L on the surface of a solid medium, either solid LB media without *Piper methysticum* dry leaf extract or ampicillin, solid LB medium containing *Piper methysticum* leaf extract, as well as the solid LB medium containing ampicillin, After absorbing the liquid medium, the three petri dishes were incubated at 37 °C for 16-18 h. The results of this count

plate method in the form of the number of single colonies of bacteria on each Petri dish [12]. The number of colonies on extract media compared to the number of bacterial colonies on media without extracts or ampicillin in order to get the percentage of bacteria that inhibited on solid medium containing the extract.

RESULTS AND DISCUSSION

In this discussion of the results and outlined the results of the analysis of antibacterial activity test *Salmonella paratyphi* contained in the *Piper methysticum* leaves. These results obtained with the observation zone of inhibition in agar diffusion method and the calculation of the percentage of inhibition of bacteria are undergo the results of counting plate method.

Culturing of the bacterium *Salmonella paratyphi*

Culturing the bacterium *Salmonella paratyphi* performed on solid media by scraping or spreading the seeds of bacteria and incubated at 37 °C for 16-18 h. Culturing is done to get stock of bacteria that are ready to be used in the next step. Bacteria that grow on solid media should be a single colony. This is because on the next steps to be taken to the single bacterial colonies were inoculated into a liquid medium, in which a single colony considered to be derived from a single cell. Colonies of bacteria that grow directly visually observed by eye. This bacterial stock stored at 4 °C, for a period save for 1 month. After that needs to be made more stock bacterium solid new media to provide a new sources of food for the bacteria.

Culturing the bacterium *Salmonella paratyphi* in liquid media is done by taking a single bacterial colony of bacteria stock solid media by using ose trunk and put it in a liquid medium. This work was done with aseptic technique. Then do the incubation at 37 °C with shaking which allows the aeration during the growth of the bacteria, ie for 16-18 h. Bacterial growth was observed with the occurrence of turbidity in a liquid medium. Bacterial liquid culture media is then used directly as a *fresh overnight culture* for further testing.

Extraction of *Piper methysticum* leaves

The extraction process is done until *Piper methysticum* leaf extract the form of dry powder that can be dissolved again with a certain concentration of the extract. Solvent extraction process using water is performed continuously using a soxhlet for 9 h, ie until the solution is clear colorless extraction approach. *Piper methysticum* leaf extract obtained is brown and then oven-dried at a temperature of 60-70 °C for five days. *Piper methysticum* leaf dry extract as much as 3.42 grams of solvent extraction results obtained as much as 24 mL. This dry extract is stored in a refrigerator at 4 °C to minimize the damage.

For further testing steps, dry leaf extract *Piper methysticum* then dissolved again with a certain concentration of 500 mg/mL and 1140 mg/mL for both agar diffusion method, as well as the concentration of 50 mg/mL for the purposes of calculating plate method. Dissolution of this dry extract using a solvent of water with a high temperature around 100 °C as it turns *Piper methysticum* leaf extract is dissolved well at high temperatures. While at low temperatures turns *Piper methysticum* leaf extract soluble only slightly.

Agar diffusion method

Agar diffusion method is the standard method to determine the presence or absence of the antibacterial activity of the compound. Bacteria new liquid culture media (*fresh overnight culture*) is spread on the surface of solid media. Later on it is placed a filter paper disc diameter of 5.5 mm and etched *Piper methysticum* leaf extract at a concentration of 500 mg/mL and 1140 mg/mL. On the other discs filter paper drip ampicillin 1 mg/mL and distilled water. The solution was dripped on the filter paper disk will diffuse into the agar. The diffusion has to be perfect, because the media is left for 30 min before it was incubated at 37 °C for 16-18 h.

After incubation, the clear zone around the visible disc of filter paper which indicates the inhibition of bacteria. Sample results agar diffusion method shown in Fig. 2 and Fig. 3. In the figure looks the clear zone around the filter paper discs were etched *Piper methysticum* leaf extract at a concentration of 1140 mg/mL. The clear zone is not overgrown with bacteria, while outside the clear zone bacteria to flourish. On filter paper discs etched with *Piper methysticum* leaf extract at a concentration of 500 mg/mL also contained clear zone is smaller, it is in accordance with the concentration difference *Piper methysticum* leaf extract is used.

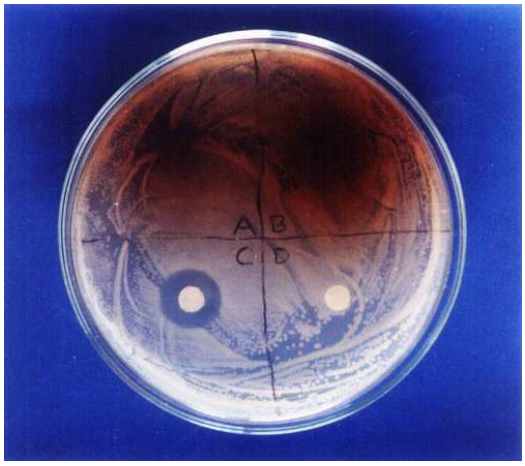

Fig 2. Examples of test results petri dish with agar diffusion method in bacteria *Salmonella paratyphi* A. A: *Piper methysticum* leaf extract at a concentration of 500 mg/mL, B: *Piper methysticum* leaf extract at a concentration of 1140 mg/mL, C: ampicillin concentration of 1 mg/mL, D: distilled water. Looks clear zone around the filter paper discs A, B, and C, while the filter paper discs D no clear zone

On filter paper discs etched with ampicillin also seen clear zone, which means that the positive control run well in which the antibiotic ampicillin is able to inhibit the proliferation of bacteria in a way inhibits formation of cell walls. While the negative control also goes well seen in the absence of clear zone around the filter paper disc drip with distilled water. This means that the inhibition of bacterial growth on the filter paper disk with leaf extract *Piper methysticum* not as solvent water, but by the compounds contained in the *Piper methysticum* leaf extract.

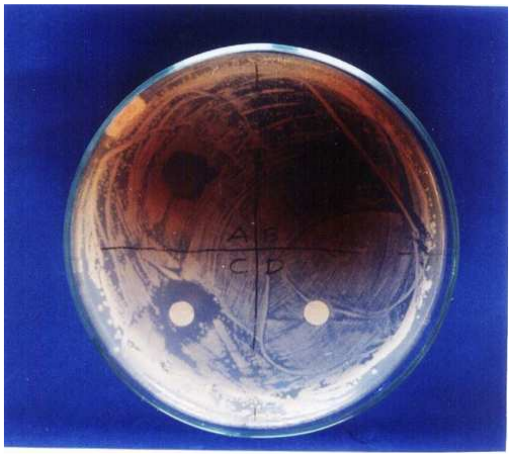

Fig 3. Examples of test results petri dish with agar diffusion method in bacteria *Salmonella paratyphi* B. A: *Piper methysticum* leaf extract at a concentration of 500 mg/mL, B: *Piper methysticum* leaf extract concentration of 1140 mg/mL, C: ampicillin concentration of 1 mg/mL, D: aquades, Looks clear zone around the filter paper discs A, B, and C, while the filter paper discs D no clear zone

The presence of clear zone around the filter paper disc shows the inhibition of bacterial growth in the area. Means in *Piper methysticum* leaves contained substances that have antibacterial activity *Salmonella paratyphi*. The results consistently seen that the higher the concentration of leaf extract *Piper methysticum* the greater the inhibition area. Inhibition area diameter of each type of *Salmonella paratyphi* can be seen in Table 1.

There are differences in the amount of the inhibition area diameter of each type of *Salmonella paratyphi*. This may be because the defense against attacks antibacterial compounds in each of these different types of *Salmonella paratyphi*. In addition, the amount of bacteria that is spread also vary. The thinner the layer of bacteria, the greater the inhibition area. Because the results of the agar diffusion method is only qualitative only, which indicates the antibacterial activity of *Salmonella paratyphi* types A, B, C, D, and E on the leaves of *Piper methysticum*. To find out how much ability to inhibit the activity of leaf *Piper methysticum* each type of bacterium *Salmonella paratyphi*, then performed a quantitative analysis by counting plate method.

Table 1. Diameter zone of inhibition using agar diffusion method

Types of bacteria	Diameter zona inhibisi			
	Piper methysticum leaf extract 500 mg/mL	*Piper methysticum* leaf extract 1140 mg/mL	Positive control	Negative control
S. paratyphi A	9	11	14	-
S. paratyphi B	12	14.5	15.5	-
S. paratyphi C	9	14	15	-
S. paratyphi D	9	13	16	-
S. paratyphi E	8.5	10	11,5	-

Count plate method

Count plate method was conducted to analyze the percentage of bacteria *Salmonella paratyphi* that inhibited. Bacterial culture is spread on the surface of the liquid medium containing solid media *Piper methysticum* leaf extract, solid media containing ampicillin, and the solid media without extracts or ampicillin. Then these three kinds of media were incubated at 37 °C for 16-18 h until seen a single bacterial colonies growing on the surface in order. This single bacterial colonies are counted manually. Bacterial colonies grown on solid media without extracts or ampicillin were more numerous than the bacterial colonies grown on extract solid media or on solid media containing ampicillin.

Number of single bacterial colonies on solid media extract compared with the number of single bacterial colonies on solid media without extracts or ampicillin, in order to get the percentage of colonies lost. Likewise, from the solid media containing ampicillin obtained percentage of colonies lost. The complete data of each type of *Salmonella paratyphi* shown in Table 2.

Table 2. Data from plate count method

Bacteria	Number of single colonies			% lost colonies		Ratio
	LB + ampicillin	LB	LB + leaf extract of *P. methysticum*	On LB + leaf extract *P. methysticum* (A)	On LB + appicillin (B)	(A):(B)
S. paratyphi A	82	181	52	71,27	54,70	1,30
S. paratyphi B	319	374	298	20,32	14,71	1,38
S. paratyphi C	245	283	65	77,03	13,43	5,74
S. paratyphi D	405	492	266	45,93	17,68	2,60
S. paratyphi E	355	579	489	15,54	38,69	0,40

The percentage of the lost colony of bacteria in media containing *P. methysticum* leaf extract is then compared to the percentage of bacterial colonies lost in media containing ampicillin. The results of this comparison are written in Table 2, where it turns out that comparison is not the same for each type of *Salmonella paratyphi*. This shows that there are differences in response or defense to each type of bacteria to antibacterial compounds contained in the leaves *P. methysticum*. From this research found that in *Salmonella paratyphi* A and *Salmonella paratyphi* B ratio values are similar. While *P. methysticum* leaf extract with a concentration of 50 mg/mL turned out to be the most effective in inhibiting the growth of *Salmonella paratyphi* C, which is nearly six times the ability of inhibition of bacterial activity by ampicillin 200 µg/mL. Meanwhile, the ability of the *P. methysticum* leaf extract in inhibiting the growth of bacteria Salmonella paratyphi E look less good, with only 0.4 times the ability of ampicillin.

From these results, the average ability of inhibition of bacterial growth by *P. methysticum* leaf is above the ability of ampicillin [15-22]. It should be noted that the leaf extract *P. methysticum* used to have a much higher concentration than ampicillin, which is 50 mg/mL while ampicillin concentration was 200 µg/mL. But this does not mean that the leaves *P. methysticum* less effective than ampicillin, because the leaf extract of *P. methysticum* contains a wide variety of compounds in which the active compound has the ability to inhibit the growth of bacteria is most likely the concentration is much smaller than the concentration of leaf extract *P. methysticum*, maybe even smaller than ampicillin concentrations used.

CONCLUSION

Dry powder leaves *P. methysticum* successfully obtained from continuous extraction results in this study. Demonstrated that the leaf extract solution *P. methysticum* of dry powder has good antibacterial activity of *Salmonella paratyphi* types A, B, C, D, and E. Inhibition of bacterial growth is seen through the test with two kinds

of methods. In the agar diffusion method, the result is a region of inhibition around the disc of filter paper that spilled *P. methysticum* leaf extract. In the area of inhibition of bacteria can not grow because it is sensitive to antibacterial substances in the leaves of *P. methysticum*. Inhibition area diameter disc filter paper with leaf extract *P. methysticum* 500 mg/mL is equal to 9 mm for *Salmonella paratyphi* A, C, and D, 12 mm for *Salmonella paratyphi* B, and 8.5 mm for Salmonella paratyphi E. On filter paper discs with *P. methysticum* leaf extract 1140 mg/mL inhibition area was 11 mm for *Salmonella paratyphi* A, 14.5 mm for *Salmonella paratyphi* B, 14 mm for *Salmonella paratyphi* C, 13 mm for *Salmonella paratyphi* D, and 10 mm for *Salmonella paratyphi* E. Through the plate count method: percentage obtained single colonies of bacteria *Salmonella paratyphi* lost on media written *P. methysticum* leaf extract, which amounted to 71.27% for *Salmonella paratyphi* A, 20.32% for *Salmonella paratyphi* B, 77.03% for *Salmonella paratyphi* C, 45.93% for *Salmonella paratyphi* D, and 15.54% for *Salmonella paratyphi* E. Comparison of percentage of single colonies were lost in the extract media with the percentage of single colonies were lost in the ampicillin media showed that the bacteria *Salmonella paratyphi* types A, B, C, D and E each have a different response or defense against *P. methysticum* antibacterial compounds in the leaves. For further research, it can be conducted to determine the active compounds in the leaves *P. methysticum* that can inhibit the growth of bacteria *Salmonella paratyphi* types A, B, C, D, and E. Furthermore *P. methysticum* leaves can be developed as materials for medicine for the disease paratyphoid fever.

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