THE EFFECTS OF CURCUMIN AND VITAMIN D COMBINATION AS INHIBITOR TOWARD SALMONELLA TYPHI BACTERIA GROWTH IN VIVO

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ABSTRACT

Objectives: The prevalence of typhoid fever was reportedly high, especially in the Asian continent, as many as 80% of cases came from slums in Bangladesh, China, Indonesia, Laos, Nepal, Pakistan, and Vietnam. Due to many cases of antibiotic resistance in typhoid fever, various efforts have been made by combining antibiotic therapy or active compounds with adjuvants and herbs. Curcumin is an active compound found in many herbal plants, especially in the Asian Continent. Curcumin has an antimicrobial effect, presumably due to its ability to bind Vitamin D receptors (VDR) as a potential ligand. This condition increases the expression of cathelicidin antimicrobial peptides and eradicates bacteria. Vitamin D will definitely bind to VDR as well; on this basis, this study wants to prove the effect of the combination of curcumin and Vitamin D therapy in inhibiting the growth of Salmonella typhi.

Methods: This study is a true experimental pre- and post-test design using colony calculation method to investigate the effectiveness of curcumin and Vitamin D in suppressing the growth of S. typhi bacteria in peritoneal fluid male mice strain balb/c. Mice were divided into five groups randomly, namely, the negative control groups, Group I (curcumin 200 mg/BB/day), Group II (curcumin 400 mg/BB/day), and Group III (curcumin 200 mg/BB/day and Vitamin D 200 IU/day), and the positive control groups (antibiotic levofloxacin). The intervention was carried out for 5 days. After the 5th day, mice were then maintained for 3 weeks to determine the amount of colony growth in the post-intervention period.

Results: The comparison of the results between each group gave significance in the average number of bacterial colonies of intraperitoneal fluid. Each group gave a significant difference of <0.05. Curcumin has an activity as an antimicrobial, the higher the dose, the greater the number of bacteria inhibited growth. After curcumin therapy, 200 mg/kg and 400 mg/kg for 5 days, a decrease in the number of bacterial colonies in the intraperitoneal fluid was found. This study concluded that curcumin has an antimicrobial effect on S. typhi. The groups with combination therapy of Vitamin D and curcumin intervention also gave the same results.

Conclusion: Based on the results of this study, the combination of curcumin and Vitamin D is able to inhibit the growth of S. typhi bacteria, even up to 30 days after infection.

Keywords: Curcumin, Vitamin D, Colony count, Salmonella typhi.
In this study, a combination of curcumin and Vitamin D was carried out on Salmonella-induced mice. This is based on research by Wang et al., which shows that 1,25-dihydroxyvitamin D (Vitamin D3) can induce gene expression of two antimicrobial peptides, namely, cathelicidin and beta-2-defensins [16].

Due to the high number of cases on antibiotic resistance in typhoid fever, various efforts have been made by combining antibiotic therapy or active compounds with adjuvants and herbs. However, the research is not able to prove that this combination is useful for treating typhoid fever. Further researches are still needed to investigate the possible effects of this combination. This research is an exploratory study to ascertain the effect of curcumin combined with Vitamin D on S. typhi as a modality in inhibiting the growth of S. typhi bacteria.

Curcumin compounds
Curcumin is the main pigment found in Curcuma longa turmeric plants. Curcumin is commonly used as colorant for food. Besides, curcumin is also widely used traditionally for the treatment of skin diseases, respiratory related diseases such as sinusitis, asthma, phlegm thinners, treatment related to the digestive tract, abdominal pain, urinary tract infections, rheumatism, and hepatitis and treatment of women after childbirth [17].

Curcumin is a curcuminoid compound which is a yellow pigment in ginger and turmeric rhizome. This compound belongs to the phenolic group. The isolated curcuminoid is yellow or orange-yellow, and tastes bitter. Curcuminoid has a distinctive smell and is not toxic. The solubility of curcumin is very low in water and ether, but soluble in organic solvents such as ethanol and glacial acetic acid. Curcumin is stable in acidic conditions, but unstable under alkaline conditions and light. In alkaline conditions with pH above 7.45, 90% of curcumin is degraded to form a by-product in the form of trans-6-(4-hydroxy-3-methoxyphenyl)-2,4-di-o-coso-5-hexenal (majority), vanillin, furfural acid, and ferulol methane. With the presence of light, curcumin is degraded to vanillin, vanillic acid, ferrale aldehyde, furfural acid, and 4-vinylguaiacol [18].

The chemical structure of curcumin consisting of curcumin, demethoxycurcumin, and bisdemethoxycurcumin is shown in Fig. 1.

Effect of antimicrobial curcumin
In a study conducted by Ramprasad and Sirsi, it was shown that curcumin has antibacterial properties, especially against Micrococcus pyogenes var. aureus. Phenols and their derivatives in turmeric have been shown to have bacteicidal and bacterialidic properties so they are often used as disinfectants. Phenol compounds in curcumin function as an antimicrobials by denaturing cell proteins and damaging cell membranes.

This compounds act actively against vegetative bacterial cells, but not against bacterial spores [19]. Curcumin presumed to have a structure similar to nortohydroguaiaretic (NDGA) compounds which have strong antibacterial properties. Research by Shih and Harris reported that NDGA at a concentration of 1000 ppm had a strong effect of lethality on Escherichia coli [20]. According to Wimarsil et al. [2015], turmeric inhibits Gram-positive rod-shaped bacteria because of its curcumin content [21].

Research by Fauzah and Zulfit use five groups of E. coli bacteria with concentrations of Curcuma domestica 50%, 25%, 12.5%, 6.25%, and 3.12%, and one cup as a positive control and one cup as a negative control carried out for 1 month. The test results showed significant differences occurred in all test groups. This means that the use of turmeric extract can inhibit the growth of E. coli bacteria. The concentration of turmeric extract, which affects the growth of E. coli bacteria is 12.5%, 25%, and 50% due to the active content of curcumin and essential oils [22].

Another study was conducted by Cut, to determine the antibacterial effect (inhibitory concentration) of turmeric extract on the growth of Bacillus sp. and Shigella dysenteriae bacteria. The effectiveness of turmeric extract test at a concentration of 15%, 30%, 50%, 75%, and 100% was found to get the concentration of turmeric extract to inhibit the growth of Salmonella bacteria in the category of the weak inhibitor [23]. The similar result obtained in research by Yuliati, it was found that turmeric extract had more effective antibacterial activity on Bacillus sp. bacteria than against S. pyogenes, although the difference was not significant [24].

A study conducted by Tyagi et al. which aimed to investigate the antibacterial activity of curcumin toward four groups of bacteria, namely, Gram-positive (Staphylococcus aureus and E. faecalis) and Gram-negative (E. coli and Pseudomonas aeruginosa). The results proved membrane leakage in Gram-negative and Gram-positive bacteria on the exposure of curcumin [25]. A study by Rai et al. showed that curcumin could inhibit bacterial cell proliferation by inhibiting the dynamics of the FtsZ assembly (bacterial protofilament), which polymerized to form Z rings (Z-ring) in the middle of the cell that triggers bacterial cell division/proliferation. The assembly and stability of the FtsZ protofilament have been shown to play an important role in bacterial cytokinesis. As a result, curcumin inhibits the formation of a cytokinetic Z-ring, which will inhibit bacterial proliferation [26].

In vivo studies show that administering curcumin doses of 100 mg/kg, protects mice from pneumonia caused by S. aureus, including methicillin-resistant strains (methicillin-resistant S. aureus), with a target of the a-hemolysin S. aureus protein [27]. Another in vivo study of mice induced with S. typhi, after 3 days of administering curcumin at a dose of 200 mg/kg, there were many bacterial colonies which were distal to the ileum. However, the number of colonies was reduced after 5 days of curcumin administration [28].

S. typhi
Salmonella has a variation of lengths. Most isolates are motile with peritrix flagella. Salmonella can grow easily on a simple medium, but almost never ferments lactose and sucrose. This bacteria does not produce gas on sugar fermentation. They generally produce H₂S. This organism is able to survive in frozen water for a long period. Salmonella is resistant to certain chemicals (e.g., brilliant green, sodium tetraethionate, and sodium deoxycholate) which inhibit other enteric bacteria; thus, the addition of the substance to the medium is useful for isolating salmonella from feces [29].

S. typhi is a Gram-negative bacterial stem, which does not form a spore, is motile, and has capsules, and is flagella (moves with hair like). These bacteria can live at pH 6–8 at a temperature of 15–41°C (optimal temperature 37°C). These bacteria cannot survive at a temperature of 54.4°C for 1 h and 60°C for 15–20 min. These bacteria also cannot survive pasteurization, boiling, and chlorination [30].

METHODS
This research is a true experimental pre- and post-test design using colony calculation method to investigate the effectiveness of curcumin.
and Vitamin D in suppressing the growth of *S. typhi* bacteria in peritoneal fluid male mice strain balb/c.

**Procedure**

**Preparation and adaptation for animal experiment**

This stage is for the preparation and adaptation of animals experiment. Mice are placed in cages made of wire with a floor size of 30 cm x 50 cm x 15 cm. The density of each cage contains six tails. Mice are given 300 g/day/head of food (feed), enough drink, and the cage is cleaned every day. To maintain a stable environmental atmosphere, mice are placed in a room with sufficient air circulation according to room temperature at standard conditions (28±2°C) with 50±10% humidity. The room light is also maintained with 12 h cycle the light is turned on and 12 h extinguished. This procedure is carried out for 1 week.

After that, the randomization is carried out. All mice were grouped into five groups randomly, namely, the negative control group, Group I (curcumin 200 mg/BB/day), Group II (curcumin 400 mg/BB/day), and Group III (curcumin 200 mg/BB/day and Vitamin D 200 IU/day), and positive control group (antibiotic levofloxacin). The intervention was conducted for 5 days. After the 5th day, mice were then kept for 3 weeks to determine the number of colonies in the post-intervention period.

**Induction of *S. typhi***

In this stage, all mice were induced with intraperitoneal *S. typhi* of 0.2 ml/105 ml/CFU.

**Addition of curcumin**

Curcumin in this research was purchased from Merck curcumin for synthesis, with the chemical formula \[4-(\text{OH})-3-(\text{CH}_3\text{O})\] \(\text{C}_6\text{H}_3\text{CH=CHCO}_2\text{CH}_2\). This is also known as 1,7-Bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, turmeric yellow, diferuloylmethane. The curcumin dose per day given is 200 mg/kg BB mice, 400 mg/kg BB mice, and 400 mg/kg BB plus Vitamin D. Curcumin therapy is given 3 days after induction by Salmo nella bacteria. Curcumin is dissolved in distilled water and given through the nasogastric sonde for 5 days.

**Addition of Vitamin D**

Vitamin D in this research was purchased from Merck (colecalciferol and cholecalciferol). The dose of Vitamin D used in this study is in accordance with the dose of Vitamin D recommended for adults aged 19–50 years, which is 200 IU/day. Calculation of the dose given to mice is obtained by multiplying it by a conversion factor of 0.0026. Group III was given a dose of 0.52 IU of Vitamin D/day for 5 days. Vitamin D is dissolved in distilled water and given through the nasogastric sonde.

**Addition of levofloxacin**

Levofloxacin was obtained from Kimia Farma, Pharmaceutical, Indonesia. Dose 750 mg of levofloxacin given to mice was obtained from multiplication with a conversion factor of 0.0026. Based on the result, the positive control group was given a dose of levofloxacin 1.95 mg/day. Antibiotics are dissolved in distilled water and given through the nasogastric sonde for 1 week.

**Sampling of peritoneal liquid**

Mice are fixed in the supine position; the abdomen is cleaned with 70% alcohol and injected with 0.8–1 mL NaCl into the peritoneal cavity. Then, keep it still for 1 min while being shaken slowly. Peritoneal fluid is removed from the peritoneal cavity with a supine position; then, the liquid is aspirated with spoilted as much as 0.5 mL. The sampling of peritoneal fluid was carried out 3 times on the 3rd day, after the mice were induced by *S. typhi*, the last day of the intervention and 3 weeks after the intervention.

**Culture and calculation of colonies number**

The pour plate method is a technique for growing microorganisms in gelatin as media by mixing the gelatin liquid with a bacterial culture stock (gelatin) so that the cells are spread evenly and quietly on the surface or inside of gelatin [31].

This method was carried out by diluting peritoneal fluid samples of 0.5 mL into 4.5 mL of physiological salt (0.9% NaCl). Dilution is done 3 times so that the culture obtained is not too dense or overflow the cup (too dense culture will interfere with the observation). About 1 mL of the suspension was poured into sterile Petri dishes, followed by pouring warm sterile nutrient media (45°C) and then tightly closed and incubated for 1–2 days at 37°C.

**Data analysis**

The results of the study were carried out by the Shapiro–Wilk normality test, to determine the normality of data distribution. Then, the Levene test is performed to determine the data variance. The normally distributed and homogeneous data are carried out by parametric one-way ANOVA test. Meanwhile, the data that were not normally distributed carried out with Kruskal–Wallis non-parametric tests. The test results showed significant data (p<0.05) followed by post hoc analysis to determine the differences between intervention groups.

**RESULTS**

The comparison of the results between each group gives a significance in the average number of bacterial colonies of intraperitoneal fluid. These results are shown in Table 1. Each group gives significance results <0.05. Curcumin has an activity as an antimicrobial, the higher the dose, the greater the number of bacteria inhibited growth. Fig. 2 shows the average results of calculation of the Salmonella bacterial colonies before therapy intervention, after intervention for 5 days and 3 weeks after therapy administrated.

**DISCUSSION**

After the Salmonella bacteria inside the body, it will go to the digestive tract, especially the ileum, and penetrate the blood vessels to spread systemically through circulation [32,33]. Most bacteria will be killed by stomach acid so that bacteria with such large amounts are needed to reach the intestine and can provide clinical manifestations [33].

Curcumin is an active compound that is widely known as an anti-inflammatory, anticancer, and recently, antimicrobial agent. The results of the study Guo et al. showed that the antimicrobial mechanism of curcumin through its ability to bind to the VDR as a potential ligand.

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Table 1: Characteristics of data on the number of bacterial colonies in each group

| Group          | n  | Pre-test Median | SD   | Post-test Median | SD   | Day 30 Median | SD   | p*      |
|----------------|----|-----------------|------|------------------|------|---------------|------|---------|
| Curcumin 200 mg/kg | 5  | 26.80           | 3.7  | 3.20             | 3.1  | 0.20          | 0.4  | 0.000   |
| Curcumin 400 mg/kg | 5  | 17.20           | 2.3  | 0.0              | 0    | 0.80          | 1.3  | 0.002   |
| Curcumin 200 mg/kg and 0.52 IU Vitamin D | 5  | 30.40           | 7.4  | 0.20             | 0.4  | 0.40          | 0.5  | 0.009   |
| Levofloxacin     | 5  | 22.00           | 6.1  | 1.20             | 1.8  | 0             | 0    | 0.010   |
| Aquades          | 5  | 21.80           | 6.4  | 6.00             | 2.8  | 1.20          | 1.3  | 0.003   |

*The mean difference is significant at the 0.05 level (ANOVA repeated test). SD: Standard deviation*
The bonds of curcumin and VDR will trigger the formation of CAMP to eliminate bacteria. In addition, curcumin can increase mRNA expression from CAMP so that it can increase the level of cathelicidin in the tissue [14]. Cathelicidin is a small peptide with some structural similarities with other antimicrobial proteins, such as defensins. Cathelicidin has a broad antibacterial spectrum toward Gram-positive, Gram-negative bacteria, fungi, and parasites. Cathelicidin damages bacterial membranes by changing membrane integrity, although some bacteria are known to have resistance to cathelicidin. The types of bacteria such as E. faecalis, S. pyogenes, Salmonella enterica, and P. mirabilis, can synthesize certain proteases to reduce cathelicidin expansion [15]. Nevertheless, this study proved that curcumin in still has an antimicrobial role against S. typhi.

After curcumin therapy, 200 mg/kg and 400 mg/kg for 5 days, a decrease in the number of bacterial colonies in the intraperitoneal fluid was found. This study concluded that curcumin has an antimicrobial effect on S. typhi. The group with combination therapy of Vitamin D and curcumin intervention also gave the same results. The number of bacterial colonies after 5 days of treatment showed a decrease in the number of bacterial colonies. Vitamin D can trigger the autophagy process toward Salmonella bacterial infection and modulate the inflammatory response that prevents the host from the detrimental effects of excessive inflammatory processes arising from infection [34]. Other studies proved the important role of Vitamin D in regulating innate immunity [35]. In response to pathogenic bacteria, the innate immune response triggers the production and release of antimicrobial peptides [16].

CONCLUSION

Based on the results of this study, a combination of curcumin and Vitamin D can inhibit the growth of S. typhi bacteria, even up to 30 days after infection.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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