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Full Length Research Paper

The effect of red ginger (*Zingiber officinale* Roscoe) extract on the growth of mastitis causing bacterial isolates

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Red ginger (*Zingiber officinale* Roscoe) a well known herb that was widely used as a remedy for various ailments in traditional medicine, belonged to the *Zingiberaceae* family. The red ginger had antibacterial properties. In the face of increasing bacterial resistance to various antibiotics and continuous efforts to look for new and safer antibacterial substances, the objective of this study was to assess the red ginger's antibacterial potentials for treating-mastitis. Mastitis was a common milking cows' disease that caused a tremendous economic loss to dairy farms. Several bacteria that is, *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Streptococcus agalactiae* were responsible for this loss. To achieve this objective 3 x 4 factorial experiment was implemented. Three bacterial isolates that is, *S. aureus*, *S. epidermidis,* and *S. agalactiae* were tested with the following each concentration of the red ginger extracts that is, 50, 25, 12.50, and 6.25%. The findings were as followed: (1) *S. epidermidis* was most affected by the red ginger extract, followed by *S. aureus* and *S. agalactiae,* (2) the higher the concentration of the red ginger extracts, the higher the bacterial growth inhibition effect, and (3) the growth inhibition effects of the red ginger extracts on *S. aureus*, *S. epidermidis,* and *S. agalactiae* isolates were highly significantly different at $\alpha < 0.0001$. Therefore, the red ginger the traditional remedy was effective in controlling the three mastitis causing bacteria's growth.

Key words: *Staphylococcus aureus*, *Staphylococcus epidermidis, Streptococcus agalactiae,* red ginger, traditional medicine, mastitis.

INTRODUCTION

Red ginger was a native plant to Southeast Asia. It belonged to the Zingiberaceae family (Vasala, 2001). Ginger had been used in traditional Indian and Chinese medicine for centuries to strengthen gastric and to treat a wide range of gastro intestinal (GI) disorders such as dyspepsia, to cure upper intestine ulcers including gastritis, and peptic ulcer disease (PUD), and therefore, to relieve stomach pain due to bacterial infection (Vasala, 2001; MDidea.com., 2009).

Further, ginger was also well-regarded for its ability to fight inflammation, to cleanse colon, to reduce spasms and cramps, and to stimulate circulation. So, it was well justified for the India's Ayurvedic and the ancient Chinese herbalists that had used ginger for 5,000 years as a medical panacea for curing various illnesses (Ghaly et al., 2009; MDidea.com., 2009; Silver, 2007).

Further study show that the ginger's constituents acted as strong antioxidants and effective antimicrobial agents that could heal sores and wounds of internal organs such as stomach and liver. In this relation, Mahady et al. (2005) pointed out that the primary factor associated with gastritis and peptic ulcer disease was the gram-negative bacterium -- *Helicobacter pylori (*HP). These HP infections were associated with chronic gastritis, gastric carcinoma and primary gastric B-cell lymphoma. Nanjundaiah et al. (2009) confirmed these findings, after experimenting with albino rats that were previously ulcer induced, infected with *H. pylori* and then treated with aqeous ginger extract orally.

Based upon the above evidence, Nanjundaiah et al. (2009) concluded that the aqeous ginger extract was able to protect the gastric mucosa from stress induced

mucosal lesions, inhibited gastric acid secretion, inhibited the growth of *H. pylori* and offered antioxidant protection against oxidative stress-induced gastric damages. So, Nanjundaiah et al. (2009) confirmed the medicinal properties and the popular use of ginger as described in both Ayurveda and Traditional Chinese Medicine literatures.

Next, Nwaopara et al. (2009) reported that: ginger had strong antibacterial and to some extent antifungal properties. *In vitro* studies had shown that active constituents of ginger inhibited multiplication of colon bacteria. Ginger inhibited the growth of *Escherichia coli, Proteus sp., Staphylococci, Streptococci and Salmonella*. Hence, ginger should have impact on the growth of *Bacilla cereus*, which mainly caused diarrhoea and nausea.

Ginger extract and its pungent compounds demonstrated greater antibacterial activity against a variety of bacteria species including *H. pylori*, *Staphylococcus aureus*, Pseudo*monas aeruginosa* and *E. coli*.

On the other hand, El-Shouny and Magaam (2009) reported that in comparison to thyme, black tea, green tea and cinnamon extracts, ginger extract obviously was the most effective antimicrobial agent against the multi-drug resistant *P. aeruginosa*. In dairy farm, mastitis was one of the common health problems found in dairy herds. Mastitis not only caused significant loss to the dairy cows' milk yields, but also affected the milk composition and quality. The subclinical mastitis more frequently occurred amongst the milking cows. The bacteria persisted in mammary glands and teat canals. In the mammary glands the bacteria produced toxins that destroyed cell membranes and directly damaged milk-producing tissue and therefore, reduced the quantity as well as the quality of milk produced. This undoubtedly led to considerable economics losses to the dairy farms (Baldassi et al., 1995).

Surveys accomplished among the milking cow herds demonstrated that 95% of the clinical mastitis cases in dairy cows were associated with the Gram-positive bacteria. However, incidences of mastitis associated with the Gram-negative bacteria such as *Coliform* were increasing (Clark et al., 1995). From recent studies, researchers concluded that the main bacteria involved in mastitis incidences were *Staphylococcus aureus*, *Staphylococcus epidermidis, Streptococcus agalactiae* and *Coliform* (Salasia et al., 2005; Baldassi et al., 1995).

Despite the general practice to treat bacterial infections including mastitis with antibiotics, Salasia et al. (2005) suggested that treating mastitis incidences amongst the dairy cows with antibiotics was no longer effective. This was based upon their research findings regarding 32 *S. aureus* isolates that were obtained from mastitic milk samples collected from Kaliurang, Boyolali, Baturaden, and Bantul in Yogyakarta and Central Java provinces. Their findings demonstrated that the *S. aureus* isolates were resistant to several commonly used antibiotics, such as Ampicillin, Erythromycin, Gentamycin, Oxacylin, and Tetracycline. According to Gur et al. (2006) microorganisms had developed resistance against many antibiotics due to the indiscriminate use of antimicrobial drugs. This created problems in the treatment of infectious diseases.

Antibiotic resistance had become a global concern (Westh et al., 2004). Since bacterial resistance to antibiotics was at an increasing rate, interest in discovering new natural antimicrobials was rising. Finding alternative antimicrobials to cure bacterial infections including mastitis in dairy cows was urgently needed. In this relation, finding plant products with antimicrobial properties for a possible application in food production as well as in human and animal health care to prevent the bacterial and fungal growth was emphasized (Lee et al., 2004; De Souza et al., 2005; Gur et al., 2006; Jasmine et al., 2007; Parekh and Chanda, 2007; Eyob et al., 2008; Kumaraswami et al., 2008; Oskay et al., 2008; Adekunle and Adekunle, 2009; Okigbo et al., 2009).

Moreover, there were some advantages of using antimicrobial compounds of medicinal plants, such as fewer side effects, better patient tolerance, less expensive, acceptance due to long history of use, and being renewable in nature (Gur et al., 2006). Parekh and Chanda (2007) further elaborated that higher plants represented a potential source of novel antibiotic prototypes. Numerous studies had identified compounds within herbal plants that were effective antibiotics. Traditional healing systems around the world that utilized herbal remedies were an important source for the discovery of new antibiotics (Samy and Gopalakhrisnakone, 2008). Besides, some traditional remedies had already produced compounds that were effective against antibiotic-resistant strains of bacteria.

Research showed that the antimicrobial activity of such plants was due to specific phyto chemicals or essential oils (Abed 2007; Lee et al., 2004; Oiye and Muroki, 2002). In this conjunction, Okigbo et al. (2009) disclosed that the extracts of three African tuberous plants, that is, *Zingiber officinale* Rosc., *Curcuma longa* L., and *Dioscorea bulbifera* L., contained tanins, phenols, saponins, alkaloids, flavonoids and steroids triterpenes. These biologically active chemical compounds were potent antimicrobials on three human pathogens – *E. coli, S. aureus, and C. albicans*. Furthermore, the potency of the extracts varied with the solvent of extraction and concentration of the plant extracts, said Eyob et al. (2008), Jagtap et al. (2009), Okigbo et al. (2009) and Elavazhagan and Arunachalam (2010).

Evidence found through research show that the ginger active ingredients that contributed to its antimicrobial properties were likely resided in its volatile oils, which comprised of approximately 1 to 3% of its weight. Oonmetta-aree et al. (2006) listed essential oils (bisabolene, phellandrene, citral, borneol, citonellol, etc.),

oleoresin (gingerol, shogaol), phenol, vitamins and minerals as the ginger ingredients.

Then, MDIdea.com. (2009) described that the primary constituents of Ginger root were: (1) essential oil included: zingiberene, zingiberole, camphene, Cineole, borneol, bisabolene, Cineole, phellandrene, citral, borneol, citronellol, geranial, linalool, limonene, zingiberol, zingiberene, camphene, (2) Phenol included: gingerol and zingerone; (3) oleoresins included: gingerol and shogaol; (4) Proteolytic enzyme: zingibain, and (5) Others included: mucilage, protein, Vitamin B6, Vitamin C, Calcium, Magnesium, Phosphorus, Potassium, Sulphur, Linoleic acid, and vegeto matters such as gum, starch, lignin, asmazone, acetic acid, and acetate of potash.

In line with the above evidence, the objective of this research was to assess the red ginger's (*Zingiber officinalis* R.*)* antimicrobial potentials as an alternative treatment to the mastitis infections amongst the local milking cows.

MATERIALS AND METHODS

Material

Methanol was used as a solvent to produce the four concentrations of the red ginger extracts required for this study. Then, the Mueller-Hinton (MEU) blood agar and the broth media were also required as the growth media for the three bacterial isolates that were tested in this study. In addition to this, the blood agar media were used as the purification control.

Isolates of *S. aureus*, *S. epidermidis* and *S. agalactiae* were collected from mastitic milk samples. Five to ten milliliters of milk specimens were aseptically taken from udders of each clinically mastitis ridden local milking cows by strip milking technique. The cows were raised in small farms in the vicinity of Cisarua, Cibinong, Cimanggis, Cijeruk, Ciawi and Cipaku villages, District of Bogor, West Java. The mastitic milk samples were stored in tightly closed sterile bottles that were kept in an ice cooled insulated box during transportation from the fields to the BALITVET lab in Bogor. The mastitic milk specimens were collected 15 times over a three-month period. These specimens were later used for the bacterial verification.

The collected mastitic milk specimens were then cultivated in the Mac Conkey blood agar media plates at the BALITVET laboratory at Bogor. The inoculated blood agar plates were incubated at 37°C for 24 h. Next, the size, the shape, the color and the surface of the bacterial colonies grown in the blood agar media plates were microscopically verified to determine their shapes and gram characteristics. Afterward, several sugar and other biochemistry tests were performed to determine the genus and the species of the bacteria. The tests were accomplished according to the Cowan and Steel method (1973).

Extracting the red ginger

The red ginger rhizomes used in this study were bought from a market in Bogor. The rhizomes were air dried away from sun shine. The dried red ginger rhizomes were finely ground to produce the red ginger powder. Methanol was then added to the red ginger powder. In order to homogenize the red ginger liquid, the mixture was shaken for one hour. Agitation was required to accelerate the dilution of the red ginger's active compounds in the methanol solvent. The mixture was kept for 24 h. Then, the liquefied red ginger was filtered by paper filter. Next, the obtained methanol solution contained the red ginger active compounds was poured into a Florentine tube. The tube was then placed in a rotary evaporator to evaporate the methanol solvent at 40°C at 140 to 160 rpm, and at 15 to 20 lbs of pressure.

Sterile aquadest was added to the obtained extract so as to make four concentrations of the red ginger extracts, that is 50, 25, 12.50 and 6.25%. Next, 15 ml of each concentration was dropped at sterile paper disks (Bauer et al., 1966). Each disk was then laid at the MEU blood agar media that had been previously inoculated with each of the three bacterial isolates. These media were incubated for 24 h at 37°C. The bacterial growth inhibition zones were then observed and measured. The size of the growth inhibition zones indicated the effectiveness of the red ginger extracts in controlling the bacterial growth.

Design

This study was designed as a 3 by 4 factorial experiment. The first factor observed in the study was the type of bacteria used in the *in-vitro* tests. This factor had three levels, that is, *S. aureus*, *S. epidermidis* and *S. agalactiae*. The second factor was the concentration of the red ginger extracts. This factor had four levels, that is, 50, 25, 12.50 and 6.25% of the red ginger extracts. The observed dependent variable of this investigation was the diameter of the growth inhibition zone of each bacterial isolate tested.

Statistical analysis

The analysis of variance procedure was used to process the data concerning the diameter of the bacterial growth inhibition zones collected in this study. The purposes of this analysis were to determine: (1) the main effect of the first factor, that is, the type of bacteria tested, (2) the main effect of the second factor that is, the concentration of the ginger extracts, and (3) the interaction effect of both factors on the bacterial growth.

To further elaborate the results of the analysis of variance, the Duncan multiple range test (DMRT) procedure was employed to determine whether there were differences amongst the diameter means of the bacterial growth inhibition zones.

RESULTS AND DISCUSSION

Research results about the effect of four concentrations of the red ginger extracts on controlling the growth of *S. aureus*, *S. epidermidis* and *S. agalactiae* were shown by the size of each bacterium growth inhibition zone as follows.

Firstly, Table 1 demonstrated that the average growth inhibition zone of *S. epidermidis* was greater than that of *S. aureus.* Then, the average growth inhibition zone of *S. aureus* was greater than that of *S. agalactiae.* Secondly, Table 1 also pointed out that the higher the concentrations of the red ginger extracts, the bigger the size of the bacterial growth inhibition zones.

The main effect of type of bacteria tested on the growth inhibition zones

The analysis of variance show that the main effect of type

Table 1. The means of the bacterial growth inhibition zones (mm) by the treatment groups.

The bacteria	The concentration of red ginger extract (%)				Average
	50	25	12.5	6.25	
S. aureus	16.30	13.00	10.00	8.00	11.83
S. epidermidis	18.00	15.30	13.00	10.30	14.15
Str. agalactiae	14.00	12.00	10.00	6.00	10.25
Average	16.10	13.43	10.67	8.10	12.08

Table 2. The main effects of type of bacteria that is *S. aureus*, *S. epidermidis* and *S.agalactiae* on the bacterial growth inhibition zones

The bacteria	Diameter of growth inhibition zone (mm)	Level of significance*
S. aureus	11.83	b
S. epidermidis	14.15	a
Str. agalactiae	10.25	c

* Different alphabet code indicated a significant difference at $\alpha \leq 0.05$.

of bacteria tested, that is, *S. aureus, S. epidermidis,* and *S. agalactiae* isolates on the bacterial growth inhibition zones was highly significant at $\alpha \leq 0.01$. Further test results were presented in Table 2.

Table 2 shows that the size of three bacterial growth inhibition zones at MEU blood agar media, after being exposed to four different concentrations of red ginger extracts. In these *in vitro* tests, *S. epidermidis* produced the largest growth inhibition zone, followed by *S. aureus* and *S. agalactiae.* The test results also show that the two species of *Staphylococcus* produced larger growth inhibition zones than the *S. agalactiae.* Statistically, these differences were significant at $\alpha \leq 0.05$.

These findings demonstrated that the two species of *Staphylococcus* that is, the *S. aureus* and *S. epidermidis* were more sensitive to the red ginger extracts than the *S. agalatiae;* whereas the *S. epidermidis* was more sensitive to the red ginger extracts than the *S. aureus*. These findings were also confirmed by Nwaopora et al. (2009) and to some extent also by Oonmetta-aree et al. (2006).

Volk and Wheeler (1988) further explained the differences of the three bacteria species' sensitivity to the red ginger extracts. Accordingly, this was likely related to the size and the compositions of the bacteria's outer cell walls or capsules. These capsules protected the bacteria against threats from their external environment. Additionally, the type and the size of the bacterial colonies formed were also provided extra protection against environmental changes. These modes of protection made the bacteria were somewhat less vulnerable to the red ginger extracts' active substances.

The main effect of four concentrations of red ginger extract on the bacterial growth inhibition zones

As presented earlier in the findings, the higher the concentration of the red ginger extracts, the bigger the diameter of the bacterial growth inhibition zones. This finding was highly significant at $\alpha \leq 0.01$. Further accomplished statistical tests on the size of the bacterial growth inhibition zones were presented in Table 3.

According to Table 3, the first concentration of the red ginger extract, that is, 50.0%, produced the largest growth inhibition zone, followed by the second, the third and the fourth concentrations consecutively, 25.00, 12.50, and 6.25%. Further statistical tests accomplished on these findings pointed out that, the four bacterial growth inhibition zones produced by the four concentrations of the red ginger extracts were significantly different at $\alpha \leq 0.05$.

The above findings pointed out that the higher the concentrations of the red ginger extracts, the higher the bacterial sensitivities to the red ginger extracts as showed by the increased size of the bacterial growth inhibition zones. Eyob et al. (2008), Okigbo et al. (2009) and Jagtap et al. (2009) shared the same technique of increasing the concentrations of the active antimicrobial substance in the plant extracts to obtain better antimicrobial effects that was employed in this study. Therefore, the higher the concentrations of the antibacterial agents in the red ginger extracts, the larger the diameter of the bacterial growth inhibition zones obtained was evident.

The combined effects of the red ginger extract concentrations and the types of bacteria tested on the bacterial growth inhibition zones

The research findings demonstrated that statistically, the combined effects of the concentration of the red ginger extracts and the type of bacteria tested on the four bacterial isolate growth inhibition zones were also highly

Table 3. The main effect of the red ginger concentration increase on the bacterial growth inhibition zones.

The red ginger concentration	Diameter of growth inhibition zone (mm)	Level of significance*
50	16.11	a
25	13.44	b
12.5	10.62	c
6.25	8.11	d

* Different alphabet code indicated a significant difference at $\alpha \leq 0.05$.

Table 4. The combined effects of the red ginger extract concentrations and the type of bacteria on the bacterial growth inhibition zones.

Extract concentration (%)	Type of bacteria	Diameter of growth inhibition zones (mm)	Significance level*
50	*S. aureus*	16.3	b
	S. epidermidis	18.0	a
	Str. Agalactiae	14.0	cd
25	*S. aureus*	13.0	c
	S. epidermidis	15.3	bc
	Str. Agalactiae	12.0	c
12.5	*S. aureus*	10	fg
	S. epidermidis	13	d
	Str. Agalactiae	9	g
6.25	*S. aureus*	8	h
	S. epidermidis	10.3	f
	Str. Agalactiae	6.0	i

* Different alphabet code indicated a significant difference at $\alpha \leq 0.05$.

significant at $\alpha \leq 0.01$. Further statistical analyses accomplished on the averages of the four bacterial growth inhibition zones, using the DMRT yielded results as shown in Table 4.

Table 4 pointed out that the interaction effects of the red ginger extract concentrations and the types of bacteria tested produced different bacterial growth inhibition zones. Further observation showed that of the three bacterial isolates tested, *S. epidermidis* produced the largest growth inhibition zones at all concentrations of the red ginger extracts. These growth inhibition zones differed significantly at $\alpha \leq 0.05$ from the growth inhibition zones formed by the other two bacteria, that is, the *S. aureus* and the *S. agalactiae*.

Current literature provided four likely explanations to the above findings. The first was related to each species tolerance to the surface tension reducer agents. According to Volk and Wheeler (1988), naturally, bacteria had a three-layer of cell wall structured bond. This simple structured bond was made of: (1) cytoplasmic membrane, (2) thicker peptidoglycan membrane, and (3) varied outer membrane. Volk and Wheeler (1988) further mentioned that the cytoplasmic membrane was mainly composed of proteins and lipids that were vulnerable to the surface tension reducer agents.

In this conjunction, gingerol and shogaol -- the pungent principles of the ginger oleoresin, were the probable responsible agents for the antibacterial property of ginger. Gingerol, one of the major phenollic compounds of the ginger oil, had certain surface tension reducer activity. MDIdea.com (2009) confirmed this property of gingerol that was able to create a detergent-like effect. So, gingerol and other ginger phenolic compound ruptured the bacterial outer cell membrane. As a result, there was a loss of cell permeability properties, which in turn caused leakage of cytoplasmic membrane and release of cell materials, including nucleic acid, metabolites and ions. In this case, higher ginger extract concentrations resulted in higher cell materials, including nucleic acid, critical molecules and ions loss (Oonmetta-aree et al. 2006).

The second explanation was related to the activity of red ginger crude extract compounds that contained lipophilic compounds soluble in ethanol, which was a

property of essensial oils. Most minor and major compounds of essential oils, according to Oonmetta-aree et al. (2006) were Acetoxychavicol acetate (ACA), p-coumaryl diacetate, palmitic acid acetoxyeugenolacetate, eugenol, β-bisabolene, β-farnesene and sesquiphelandrene. They were the phenolic compounds, the esther of weak acid, fatty acid, terpenes and others.

Since large number of different chemical compounds presented in the ginger crude extract, therefore, its mechanism of action could affect multiple target sites against the bacterial cells. In this case, Oonmetta-aree et al. (2006) mentioned that β-bisabolene, β-farnesene and sesquiphelandrene that were basically terpenes in the ginger's essential oils, had a mechanism of action that was similar to other terpenes and other phenolic compounds found in this crude extract. They involved in disruption of the cytoplasmic membrane and coagulation of the cell contents.

Besides, the essential oils contained in the ginger extract were able to induce the leakage of ions and other cell contents. The extract, therefore, affected the bacterial cytoplasmic membrane and induced the loss of nucleic acid and ions. In this conjunction, ACA, the main compound in this crude extract, was ester of acetic acid which had similar mechanisms in the bacterial cells. Acetate acted as other organic acids, having membrane gradient neutralization and denaturing of proteins inside the cell (Oonmetta-aree et al., 2006).

The third, according to Oonmetta-aree et al. (2006) antimicrobial activity of acetic acid was related to pH, and the undissociated form of the acid was primarily responsible for antimicrobial activity. When this ester was dissolved in solution, undissociated acetic acid could penetrate the cell membrane lipid bilayer of the bacteria and released protons into the cytoplasm, because the cell interior had a higher pH than the exterior.

Excess intercellular protons could acidify the cytoplasm and caused protein denaturation and energy loss due to activation of ATP-dependent proton pumps located in the cell membrane. The methyl ester was able to penetrate to the hydrophobic regions of the membranes and the carboxyl groups pass through the cell membrane, perturbing in the lowering of internal pH and denaturing of proteins inside the cell which coagulation of cell contents (Oonmeeta-aree et al., 2006).

The fourth, Volk and Wheeler (1988) explained that the phenolic compound and the proteolytic enzyme of the red ginger extract – Zingibain – precipitated the outer protein membranes, ruptured the cell wall, coagulated and caused loss of the cell contents and energy through cell wall leakages of *S. aureus, S. epidermidis* and *S. agalactiae*.

Through these mechanisms, whether singly or jointly, the mastitis causing bacteria tested in this study were destroyed by the red ginger extracts. In this way, the red ginger demonstrated the effectiveness of its antimicrobial properties on the three tested bacteria and, therefore, worth to consider as an alternative treatment to antibiotics to cure the mastitis incidences amongst the milking cows in Indonesia.

Conclusion

The red ginger extracts had the antimicrobial properties, effective in inhibiting the growths of three prominent mastitis causing bacteria, that is, *S. aureus*, *S. epidermidis,* and *Str. agalactiae*. The higher the concentration of the red ginger extracts, the more antimicrobial properties of the red ginger extracts, and the larger the diameter of the bacterial growth inhibition zones obtained. Of the three types of bacteria tested, *S. epidermidis* was the most sensitive to the red ginger extracts, followed by *Str. aureus* and *Str. agalactiae*, at all concentrations.

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