MELYSA
Medical Laboratory Analysis and Sciences Journal
Volume 2 Issue 1, May 2020, page 1-9
ISSN: 2657-1471 (Online)
ISSN: 2656-937X (Print)
DOI: 1035584/melysa.v2i1.45

Antibacterial activity combined extracts of red ginger (Zingiber officinale var. Rubrum) and betel leaf (Piper betel L.) against Staphylococcus aureus and Escherichia coli

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ARTICLE INFO

Article history:
Received date:
2020 April 2nd
Revised date:
2020 April 19th
Accepted:
2020 May 13th
Published:
2020 May 14th

Keywords:
Red Ginger
Betel Leaf
Staphylococcus aureus
Escherichia coli
Antibacterial

ABSTRACT

The use of traditional medicine can be an alternative treatment with increasing antibiotic resistance. Red ginger (*Zingiber officinale* var. Rubrum) and betel leaf (*Piper betel* L.) are plants that can be used as an antibacterial. This study aimed to determine the antibacterial activity of combined extracts of red ginger and betel leaf. This experiment was carried out by making extracts of red ginger and betel leaf with maceration and tested the antibacterial activity. The antibacterial activity test used the paper disc diffusion method. The study was conducted with five different concentrations of extract 20%, 40%, 60%, 80%, and 100%. The results showed that the combination of red ginger and betel leaf extract at a concentration of 80% had the highest inhibitory area against *Staphylococcus aureus* (10.6 mm) and *Escherichia coli* (7.5 mm). The data obtained were statistically tested using One Way Anova with a significance level of > 0.05. The results of One-Way ANOVA analysis of the combination of red ginger and betel leaf extracts on *Staphylococcus aureus* had a significant value of 0.083, while those extracts on *Escherichia coli* had a significant value of 0.690. It can be concluded that the combination of red ginger and betel leaf extract at a concentration of 80% had a strong inhibitory level against *Staphylococcus aureus* and a moderate inhibitory level against *Escherichia coli*.

INTRODUCTION

The tendency of diseases caused by bacteria is still experienced by the population of Indonesia, including due to Staphylococcus aureus and Escherichia coli.[1] The World Health Organization (WHO) reports that there are about 10 billion infections existing around the world every year. Infectious diseases are also one of the most common causes of human death. The many types of division, classification, germ sensitivity pattern, and the discovery of new antibiotics often make it difficult for the clinician to determine the appropriate antibiotic choice when handling an infectious disease case.[2] Side effects of antibiotics can be allergic reactions. One alternative to avoid excessive side effects is the use of traditional medicines now more effectively.[3]

The plants that can used as an antibacterial are red ginger and betel leaf. The red ginger contains gingerol which has antibacterial activity.[4] The content of secondary metabolites in the ginger plants is mainly from the flavonoid, terpenoid, saponin and essential oil groups. The secondary metabolite compounds produced by the *Zingiberaceae*

plant generally can inhibit the growth of pathogens by damage mechanisms that occur in the structural components of bacterial cell membranes, including bacteria *Escherichia coli* and *Bacillus subtilis*, as well as several other microbes.[5] Red Ginger Extract (Zingiber officinale var. Rubrum) has an inhibition zone against Staphylococcus aureus of 15.83 mm and Escherichia coli of 14.22 mm at a concentration of 100% with the mechanism of damage that occurs to the component of structural bacterial cell membranes.[6]

The content of secondary metabolites in betel leaf plants include alkaloids, flavonoids, essential oils, and tannins. The results showed that the betel leaf extract on 50% concentration has a inhibitory area of 15 mm with a mechanism enzyme inactivation, protein denaturation, and damage to bacterial cells.[7] The combination of extracts from several plants put together has greater antibacterial inhibition compared to single plant extracts.[8] This study aimed to determine the antibacterial activity of the combination red ginger extract (Zingiber officinale var. Rubrum) and betel leaf extract (*Piper* bete/. L) against Staphylococcus aureus and Escherichia coli and to find out what the optimal concentration of the extracts on the bacteria.

MATERIALS AND METHODS

Materials

The tools used in this study were analytical scale (HWH DJ203A), blender (National), glass jars, aluminum foil, filter paper, glass funnels (Pyrex), stirring rods, paper discs (Oxoid), oven (Getra), porcelain cups, pycnometers (Pyrex), thermometer, test tube (Pyrex), waterbath (Thermostat water bath HH-6), petri dish (Normax), test tube (Pyrex), ose needle, autoclave (Alamerican), glassware (Pyrex), ruler (Microtop), cotton, incubation (Memmert), L rods, tweezers, flannelette, porcelain cup, bunsen (methylated light).

The materials used in this study were red Ggnger (*Zingiber officinale* var. Rubrum), betel leaf (*Piper betel* L.), ethanol 96%, concentrated HCl, NaOH, HCl 2N, $FeCl_3$ 10%, chloroform, acetic acid, concentrated H_2SO_4 NaOH 1 N, iodine 0.1 ethanol 70%, HCl 2 N, major reagents, Wagner reagents, aquadest, nutrient agar, *Staphylococcus aureus*, and *Escherichia coli.*

Methods

1. **Determination of Plant**

   Determination of red ginger (*Zingiber officinale* var. Rubrum) and betel leaf (*Piper betel*. L) were carried out in the Laboratory of Biological Pharmacy at STIKes Bhakti Mandala Husada Slawi.

2. **Extraction**

   Fresh rhizome of red ginger and betel leaf were taken as much as 6 kg. Red ginger rhizome and betel leaves were washed by running water, cut, dried on sunlight covered with a black cloth, and blended. Then maceration was carried out for 5 days using ethanol solvent 96%.

3. **Extract Parameters**

   Non Specific Parameters

   a. **Dry Shrinkage**

      Weighed 1 g of the extract was put into a closed porcelain exchange which had previously been pressed and heated at 105^0 C for 30 minutes. Before weighing, the extract was spread in porcelain exchange, until it formed a layer 5– 10 mm thick. Put it in the oven, open the lid, dry it at 105^0 C until the weight was fixed, then the percentage was calculated.[9]

   b. **Water content**

      The moisture analyzer was set at 105^o C, and automatically checked when it was closed. 0.5 gram of extract was added and flattened in an aluminum bowl. After the lamp turned off, the extract weight was constant at 1 mg for 60 seconds and the screen will display the water content of the extract.[10]

   Specific Parameters

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To determine the organoleptic of the extract, it used the five senses to describe smell, shape, color and taste.[11]

4. Phytochemical Screening

a. Alkaloids

2 ml test solution was evaporated on a porcelain glass and added with 5 ml of 2 NHCl. The filtrate obtained was divided into 3 test tubes. The first tube was added with 3 drops of HCl 2 N functioning as a blank. The second tube was added 3 drops of Wagner reagent and the third tube was added 3 drops of major reagent. If sediment was formed in the addition of Wagner reagents and major reagents, identification showed the presence of alkaloids.[12]

b. Flavonoids

As much as 200 mg extract was added with 2 ml of aquadest and heated for 5 minutes. Then added NaOH formed an intense yellow color and HC which becomes colorless. It showed that it contained flavonoids.[13]

c. Tannins

A total of 500 mg of extract was added to 10 ml of aquadest, then allowed to stand for 15 minutes then filtered. Filtrate was added with 3 drops of 10% FeCl3 and color was formed. If it was formed blue and green colors, that indicated tannins.[14]

d. Saponins

A total of 500 mg of extract was filled into a test tube, then added 10 ml of hot water, cooled and strong shaken to form a froth as high as 1-10 cm for not less than 10 minutes.[15]

e. Terpenoids

200 mg extract was added with 2 ml of aquadest and then added 0.5 mL chloroform and 0.5 mL of acetic acid. Then 2 ml of concentrated sulfuric acid was added through the tube wall. Positive results showed reddish brown color.[15]

f. Essential Oils

100 mg extract was added with 1 ml of aquadest and then evaporated with a porcelain cup until a residue was obtained. Positive results were characterized by aromatic distinctive odors.[16]

g. Ethanol Free

A total of 5 ml extract was added 1 ml of NaOH 1 N and slowly (after 3 minutes) added 2 mL of Iodine 0.1 N. If Iodoform odor arose and yellow precipitate formed within 30 minutes, the material contained ethanol.

5. Thin Layer Chromatography (TLC)

Flavonoid compounds were identified by dissolving the sample in ethanol then spotted on GF254 silica gel. Elution was carried out with n-butanol: acetic acid: water (3: 1: 1). The plates were dried and observed in visible light, UV 254 and 366.[12]

6. Inhibition Test of Antibacterial Activity

The medium used was NA (Nutrient Agar) made by specified composition. The method used was the paper disc diffusion method. The disc paper was dipped in the test solution according to the concentration for 5 minutes then immersed in agar media containing bacteria. The concentrations used for the test solution were 20%, 40%, 60%, 80%, and 100%. The inhibition zone was measured after 24 hours incubation. Data obtained from the inhibition zone were statistically analyzed by ANOVA with a significance level of > 0.05.

RESULTS AND DISCUSSION

1. Determination of Plant

The results of the determination showed that the red ginger (*Zingiber officinale* var. Rubrum) and betel leaf (*Piper betel*. L) were really the desired plants in this study.

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2. Extraction

The extract yield obtained from the Red Ginger extract was 6.246%, while the Betel Leaf extract was 5.25%.

3. Extract Parameters

Specific parameters of red ginger extract were liquid extract, brownish yellow color, characteristic of ginger, spicy taste. Whereas in Betel Leaves in the form of thick extract, green color, the characteristic betel odor, a bit bitter and spicy taste.

Non specific parameters of dry shrinkage from red ginger extract were 2.919% and Betel Leaves were 4.743% (<10%).[6] The water content obtained from the extract of red ginger was 4% (<11%) and betel leaves by 1.8% (<10%).[10]

4. Phytochemical Screening

Phytochemical screening results of Red Ginger extract positively contain flavonoids, saponins, terpenoids, and essential oils. Betel leaf extract contains alkaloids, flavonoids, tannins, essential oils. Phytochemical screening of red ginger and betel leaf extracts is shown in Table 1.

Table 1. Results of Phytochemical Screening

Compound Group	Red Ginger	Betel Leaf
Alkaloids		+
Flavonoids	+	+
Saponins	+	
Tannins		+
Terpenoids	+	
Essential oils	+	+

5. Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was a separation technique using an adsorbent (stationary phase) in the form of a uniform thin layer overlaid on a flat plane surface in the form of glass plates, aluminum plates, or plastic plates, while the mobile phase was liquid, usually organic solvents and sometimes sometimes also water.[17]

Red ginger extract, betel leaf, and a combination of red ginger extract and betel leaf have the same Rf value with the quercetin Rf value. It means that red ginger extract, betel leaf, and a combination of red ginger extract and betel leaf contain flavonoid compounds. RF value of Red Ginger and Betel Leaf extracts is shown in Table 2.

Table 2. RF Value of Red Ginger and Betel Leaf Extracts

Extract	Compound Group	Rf Value	Rf Quercetin	Result
Red Ginger	Flavonoids	0.93	0.93	+
Betel Leaf	Flavonoids	0.87	0.87	+
Combination	Flavonoids	0.87	0.87	+

6. Inhibition Test of Antibacterial Activity

This study was conducted to determine the antibacterial activity of the combination of red ginger extract (Zingiber officinale var. Rubrum) and Betel Leaf (Sirih piper L.) against Staphylococcus aureus and Escherichia coli. This research was conducted by calculating the average diameter of inhibitory zones produced by a combination of extracts of the Red Ginger (*Zingiber officinale* var. Rubrum) and Betel Leaves (*Piper betel* L.)

Inhibition test was started by preparing the bacterial growth media, namely Nutrient Agar (NA) media, where this media contained nutrients that can support bacterial growth. Bacterial rejuvenation was carried out aseptically near the Bunsen fire by means of

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taking the culture before, ose must be heated first on the Bunsen fire. It aimed to kill other unwanted microorganisms.[18] After that, bacterial culture was taken, diluted, and flattened with L stems in NA media.

The method of testing this activity was carried out by the diffusion method using disc paper (disc diffusion). The advantage of the disc diffusion diffusion method was very easy to do because it was not complicated in its processing and efficient. It can be used to see the sensitivity of various types of microbes to antimicrobials at certain concentrations, did not require a lot of tools and materials while the loss cannot be known precisely the bacterial inhibition process or bacteriostatic inhibition because many factors affected, among others, the thickness of the media.

Paper discs with a diameter of 5 mm each were allowed to stand for 5 minutes, this was done to evaporate the ethanol solvent used to thin the combination of Red Ginger and Betel Leaves extract so that it was expected that in this antibacterial inhibition test it was a combination of Red Ginger extract and Betel Leaf. The solution was made in 5 concentrations with 20%, 40%, 60%, 80%, and 100% made from 100% stock solutions in a ratio of 1: 1.

Bacterial inhibitory activity was expressed based on the clear area appeared around the paper disc. The inhibition activity was grouped into 4 categories according to Davis Stout, namely the very strong category with a clear zone diameter of >20 mm, the strong category in a clear zone diameter of 10-20mm; while the clear zone diameter with a value of 5-10 mm was a moderate category, and the weak inhibitor was in a clear zone diameter <5 mm.[19]

The incubation time for bacteria used in rejuvenation was generally 24 hours with a temperature of 37^{o} C. Bacterial incubation was carried out for 24 hours because at that time the bacteria were in a logarithmic or exponential phase, at which stage the bacteria made a constant division and the number of cells increased. After experiencing a logarithmic or exponential phase, bacteria will experience a stationary or static phase.

Table 3. Inhibition Zone of Red Ginger Extratct, Betel Leaf Extract, and Combination of extracts against *Staphylococcus aureus*

Concentration	Red Ginger Extract	Betel Leaf Exctract	Combination
20 %	3.8	4.6	5.3
40 %	7.5	11.8	7.6
60 %	5.1	7.8	9.8
80 %	6	8	10.6
100 %	5.5	8.3	10.1

Based on the results of Table 3 it can be concluded that the concentration of the combination of red ginger and betel leaf extracts was directly proportional to its antibacterial activity. The higher the extract concentration, the more zone diameter of inhibition against Staphylococcus aureus.

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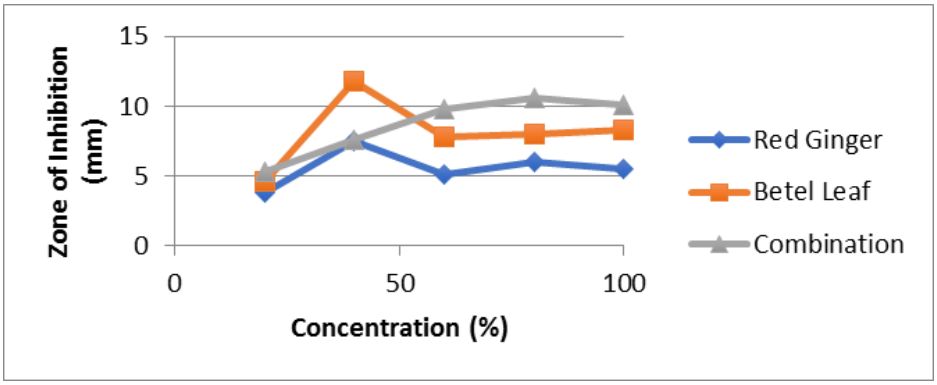

Figure 1. Graph of Inhibition Zone of Red Ginger Extratct, Betel Lea Extract, and Combination of extracts against *Staphylococcus aureus*

Figure 1 showed that the increased concentration of the extract of Red Ginger, Betel Leaves, and the combination of the extract will also increase the inhibitory zone to the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria. Based on the category according to Davis Stout at a concentration of 20% a single extract of Red Ginger (3.8 mm) and Betel (4.6 mm) had inhibition with a weak category and a combination of extracts with a concentration of 20% (5.3 mm), 40% (7.6 mm), and 60% (9.8 mm) had inhibition in the medium category, whereas at concentrations of 80% (10.6 mm) and 100% (10.1 mm) had inhibition with a strong category against *Staphylococcus aureus*.

Table 4. Inhibition Zone of Red Ginger Extratct, Betel Lea Extract, and Combination of extracts against *Escherichia coli*

Concentration	Red Ginger Extract	Betel Leaf Exctract	Combination
20 %	4.5	5.6	5.8
40 %	6.5	5	5
60 %	4.6	7	6.3
80 %	4.8	7.5	7.5
100 %	9.8	7	11

Based on the results from Table 4 it can be concluded that the higher the concentration the greater the inhibitory power obtained from the combination of the extract of Red Ginger and Betel Leaves.

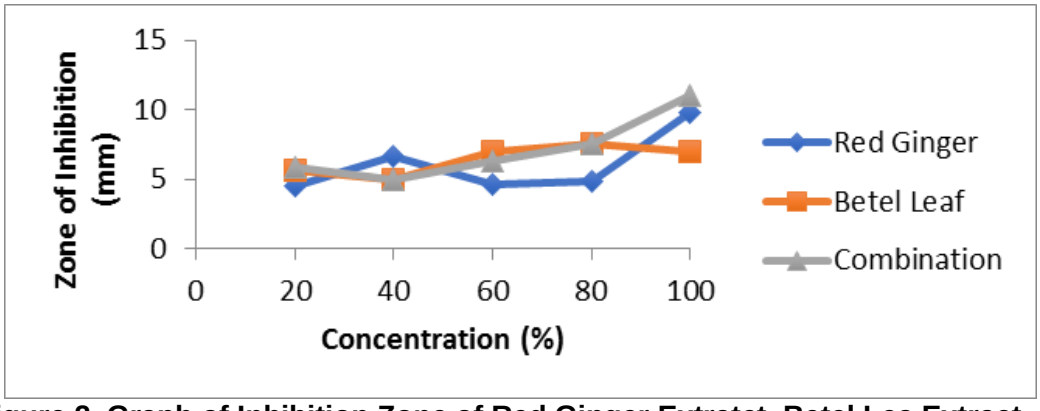

Figure 2. Graph of Inhibition Zone of Red Ginger Extratct, Betel Lea Extract, and Combination of extracts against *Escherichia coli*

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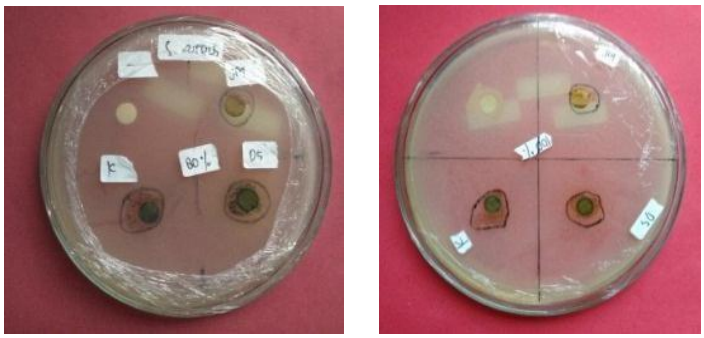
Figure 2 showed that the concentration of 20% (4.5 mm) of Red Ginger rhizome extract had a weak inhibition category and Betel Leaves extract (5.6 mm) had a medium inhibition power category. At a concentration of 40% a single extract of Red Ginger (6.5 mm) and Betel (5 mm) had inhibition with a moderate category, while at a concentration of 60% (4.6 mm) and 80% (4.8 mm) in rhizome extract Red Ginger had a weak inhibitory power and in betel leaf extract with a concentration of 60% (7 mm), 80% (7.5 mm), and 100% (7 mm) had a medium inhibitory power. Concentrations of 20% (5.8 mm), 40% (5 mm), 60% (6.3 mm), and 80% (7.5 mm) in the combination of extracts had moderate inhibition, while at a concentration of 100% (11 mm) had a strong inhibitory category against *Escherichia coli*.

The clear zone seen around paper discs was an area that was not overgrown by bacteria and looked clearer than the surrounding area (Figure 3). Bioactive compounds on paper disks diffuse with nutrient agar and provide inhibitory zones (clear zones) that can inhibit bacterial growth.[19] Red Ginger and Betel Extract can inhibit bacterial growth because it contained chemical compounds. Chemical compounds suspected to have antibacterial activity in Red Ginger were flavonoids, essential oils, terpenoids, and saponins, whereas in Betel are alkaloids, flavonoids, tannins, and essential oils.



(a) **(b)**

Figure 3. (a) The clear zone at 80% concentration in inhibiting *Staphylococcus aureus* bacteria, (b) The clear zone at 100% concentration in inhibiting *Escherichia coli* bacteria

The mechanism of alkaloids as an antibacterial was by disrupting the constituent components of peptidoglycan in bacterial cells so that the cell wall layers were not formed intact and cause cell death.[20] The mechanism of flavonoids as antibacterial was to form complex compounds with extracellular and dissolved proteins that can damage bacterial cell membranes and were followed by the release of intracellular compounds. While the mechanism of essential oils as an antibacterial was inhibiting or killing microbial growth by interfering with the process of cell wall formation, so that the cell wall was not formed. The mechanism of tannin as an antibacterial was to inhibit the reverse transcriptase enzyme and DNA topoimerase so that bacterial cells cannot be formed.[21]

The mechanism of saponins as an antibacterial was by causing leakage of proteins and enzymes in the cell. Saponins can diffuse through the outer membrane and cell walls of the vulnerable and then bind to the cytoplasmic membrane so that it disrupted and reduced the stability of the cell membrane. This caused the cytoplasm to leak out of the cell resulting in cell death. Antimicrobial agents that interfere with cytoplasmic membranes were bactericidal. Based on the calculation results of the One Way ANOVA analysis, the combination of Red Ginger and Betel Leaf extracts on *Staphylococcus aureus* had a significant value of 0.083> 0.05 while *Escherichia coli* had a significant value of 0.690 > 0.05.

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CONCLUSIONS

The combination of Red Ginger extract (Zingiber officinale var. Rubrum) and Betel Leaf (Piper betel L.) with 96% ethanol solvent showed antibacterial activity with a strong category at a concentration of 80% on the growth of *Staphylococcus aureus* and 100% against *Escherichia coli*. The optimal concentration for *Staphylococcus aureus* was at an 80% concentration with a resistance of 10.6 mm and for *Escherichia coli* at a concentration of 100% with an inhibition of 11 mm.

ACKNOWLEDGEMENT

Acknowledgments are conveyed to appropriate parties, especially to STIKes Bhakti Mandala Husada Slawi as laboratories facilitator.

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