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# Antimicrobial Spectrum of Red Piper Betel Leaf Extract (*Piper crocatum* Ruiz & Pav) as Natural Antiseptics Against Airborne Pathogens

**Sri Agung Fitri Kusuma[1]*, Rini Hendriani[2], Aryo Genta[1]**

*[1]Department of Pharmaceutical Biology, [2]Department of Pharmacology,*
*Faculty of Pharmacy, Padjadjaran University, Sumedang, West Java, Indonesia 45363*

**Abstract**

**Aim:** The present study was performed to investigate the antimicrobial activities of red piper betel leaf ethanol extracts as natural antiseptics against some airborne pathogens as follows: *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and *Candida albicans.*

**Methods:** The extraction of dried piper betel leaves was prepared using a maceration method. The antimicrobial activities of the extract were tested using the agar diffusion method, then followed by the determination of minimal inhibition concentration (MIC) test which was conducted using macrodillution method. Whereas the determination of minimum bactericidal concentration (MBC) was done by subculturing the overnight incubation of MIC result onto Mueller Hinton Agar medium surface. The minimal inhibitory time required of each tested microbial was done by incubating the test medium at the time range of 1.5-6 min, followed by subculturing it onto MHA using the streak plate method.

**Results:** The results showed that the ethanol extract of red piper betel leaves has antibacterial and antifungal activity against all tested microbes. The value of MIC and MBC was ranging as follows: *E. coli* and *P. aeruginosa* (2.5-5%w/v), *S. aureus* (5 - 10% w /v), *C. albicans* (1.25 - 2.5% w/v). The contact time required for 20%w/v extract concentration for inhibiting bacterial growth (1.5 min) and *C. albicans* (0.75 min).

**Conclusion:** It can be concluded that the ethanol extract of red piper betel is highly potent as natural antiseptics against airborne pathogens with an effective time of minimum inhibitory.

**Keywords**: *Piper crocatum*, leaves, antibacterial, airborne.

## INTRODUCTION

Airborne transmission poses a major challenge to the control of human pathogens [1]. Airborne transport has been linked to the transmission of a variety of pathogenic fungi, bacteria, and viruses [2,3,4,5]. Another study stated that from a total of 400 samples of four daily used objects (100 of each) such as: computer keyboards, computer mice, elevator buttons and shopping cart handles. 95.5% of the total samples collected were contaminated with mixed bacterial growth. Potential pathogens isolated from all specimens were: *Staphylococcus aureus*, Pseudomonas spp. and Gram negative bacilli [6]. From another study stated that the rank order of skin pathogens was: *Staphylococcus aureus* (45.9%), *Pseudomonas aeruginosa* (10.8%), Enterococcus spp. (8.2%), *Escherichia coli* (7.0%), Enterobacter spp. (5.8%) and Klebsiella spp. (5.1%) [7]. From open-air markets, banks, filling-stations, supermarkets, residential homes and hostels, found the same bacteria contamination, but only one fungus, *Candida albicans* (13%), was isolated [8]. These data show that airborne contaminants spread through the hands can be a source of infection that is meaningful. Thus the use of antiseptics on the skin of the body and hands are expected to control the further infection.

The widespread use of antiseptics has encouraged the development of microbial resistance to antiseptics that had been circulated. Cases such resistance are as follows: *Salmonella enterica* to benzalkonium chloride, *Pseudomonas aeruginosa* to polyquaternium, MRSA (*methicillin resistant Staphylococcus aureus*) against quaternary ammonium [9], and *Candida albicans* against cetylpyridinium chloride [10]. Five strains of *Pseudomonas aeruginosa* have been reported to be resistant against chlorhexidine gluconate and benzalkonium [11]. It encourages the search of new antiseptic candidates derived from natural materials. One of the plants that are empirically used as an antiseptic is red piper betel leaves (*Piper crocatum* Ruiz & Pav.).

Red piper betel leaves have a more fragrant aroma than the green betel leaves. Owned fragrant aromas of red betel are one of the advantages that can be used as a natural fragrance in antiseptic preparations. In addition, red piper betel (*Piper crocatum* Ruiz & Pav) leaves contain active compounds such as: flavonoids, polyphenols, tannins and essential oils [12]. The chemical constituents could play role in the antimicrobial effect of the ethanol extract of red piper betel leaves. This study was conducted to observe the antibacterial activity of the ethanol extract of the red piper betel leaves against some airborne pathogens.

## MATERIALS AND METHODS

### Materials

The plant material used in this study is red piper betel leaves (*Piper crocatum*) from Bogor. The plant material has been determined in the Laboratory of Plant Taxonomy Department of Biology, Faculty of Mathematics and Natural Sciences, University of Padjadjaran, Bandung - Sumedang Highway Km 21 Jatinangor Sumedang, West Java.

The microbes that were used is *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa 15442*, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 10231. The culture media that were used are Mueller-Hinton Agar (MHA-Oxoid), Mueller-Hinton Broth (MHB-Oxoid), Sabouraud Dextrose Agar (SDA-Oxoid) and Sabouraud Dextrose Broth (SDB-Oxoid).

**Preparation of Leaves Extract**

The dried leaves were grounded using a mortar and pestle into fine powder. The powdered leaves, then were extracted by using a maceration method using ethanol 70% as the solvent for 72 h successively with intermittent shaking every 2 h. Then the extracts were evaporated using a rotary evaporator at 50 $^0$C, then continued to evaporate on a water bath until dried extract with constant weight was obtained. The extract was stored in a refrigerator at 4 ºC until time of use. The percentage yields (w/w) of the extracts were calculated using the formula below [13]:

(Weight of extract ÷Weight of starting plant material) × 100%

**Phytochemical Analysis**

Phytochemical screening was done by using Fansworth method to determine the containment of alkaloids, flavonoids, tannins, Quinones, phenolics, saponins, steroids, triterpenoids, monoterpenoids and sesquiterpenoids in the ethanol extract of red piper betel leaves [14].

**Preparing Bacterial Suspension**

The bacterial suspension was prepared by transferring a loopful of inoculum into normal saline (0.9%) under aseptic conditions from the stock culture maintained at 4$^0$C. The density of each microbial suspension was adjusted to equal that of $10^8$cfu/ml (standardized by 0.5 McFarland standard) [15]. A 0.5 McFarland standard was prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate ($BaCl_2.2H_2O$), with 9.95 ml of 1% sulfuric acid ($H_2SO_4$) in a test tube with constant stirring [16].

**Antibacterial activity**

The leaf extracts were tested for antibacterial activity using the agar diffusion method. The 20 µL bacterial suspensions with 0.5 McFarland in turbidity were inoculated into a sterile petri dish containing the volume of 20 ml MHA medium. The mixture of bacterial suspension and agar was homogenized until it became solid. The media is then perforated to make holes for storing the extract. The extract used in the test are 10, 20, 40, and 60% w / v using the solvent DMSO. Volume 50 µl of every extract concentration was populated and poured into each hole. The tested media then was incubated for 24 h at temperature 37 $^0$C. The extract was tested in triplicates. After that, we observed the diameter of the zone of inhibition around the holes.

**Antifungal activity**

The leaf extracts were tested for antifungal activity using the agar diffusion method. The 20 µL fungal suspensions with 0.3 McFarland in turbidity were inoculated into a sterile petri dish containing the volume of 20 ml SDA medium. The mixture of fungal suspension and agar was homogenized until it became solid. The media is then perforated to make holes for storing the extract. The extract used in the test are 10, 20, 40, and 60% w / v using the solvent DMSO. Volume 50 µl of every extract concentration was populated and poured into each hole. The tested media then was incubated for 48 h at room temperature. The extract was tested in triplicates. After that, we observed the diameter of the zone of inhibition around the holes.

**MIC and MBC determination**

Determination of MIC of extracts was done using macrodillution method. The extract, then serially two fold diluted to a concentration of 10%, 5%, 2.5%, 1.25%, 0.625%, 0.3125% and 0, 15625%w/v using a liquid medium (MHB) for bacterial growth and SDB medium for fungal growth. The volume of 10 µL standardized cell bacterial suspensions ($1x10^6$ cfu/ml), and fungal suspensions ($1x10^3$ cfu/ml) was put into each tested concentration. The liquid media, then were incubated for 20 h with the temperature at 37$^o$C (bacteria) and for 48 h at room temperature (fungal). The lowest concentration of the tested extracts, requisite for inhibiting the growth of each microbe was considered as the MIC. Minimum bactericidal concentration (MBC) was determined from the MIC range using the spread plate method. Solid media in Petri dishes were sub-cultured from tubes without growth and incubated. The petri dishes were observed macroscopically. The highest dilution that yielded no microbe colony on a solid medium was taken as MBC.

**Determination of minimum contact time**

This stage was aimed to determine the fastest time required by the extract of red piper betel leaves as a candidate antiseptic to kill bacteria and fungi. The timing of this contact was done by designing a series of concentrations of ethanol extract of red piper betel leaves as follows: 40, 20, 10, and 5%w/v. Microbial suspension was inoculated into each concentration of extract and incubated at room temperature using various time as follows: for bacteria (1.5; 3; 4.5 and 6 min) whereas for fungal (0.75; 1.5; 2.25 and 3 min). All microbial suspension, then inoculated into MHB media for bacteria, SDB media for fungal. Then all tested media were incubated based on the optimal condition for bacteria and fungal. The overnight incubation results were then streak using Ose on to the surface solid media, incubated and observed the colony growth.

## RESULTS

### Yield of the extract

The extraction of 573.76 g crude samples obtained 96.01 g extract, so the rendemen of the extract was 16,73%. As for the characteristics of the extract is viscous, green colored reddish, betel unique aroma and bitter taste.

### Phytochemical screening results

Based on the results of phytochemical screening, the extract of red piper betel leaves contains several secondary metabolites: alkaloids, flavonoids, polyphenols, quinones, and saponins.

### Antibacterial Activity Test Results

An ethanol extract of the red betel leaf was found to have antimicrobial activity against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa 15442*, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 10231. The result of the antibacterial activity test can be seen in table 1. The diameter of zone inhibition measured ranged from 10 to 17 mm for all tested bacteria and ranged from 14 to 16 mm for fungal strain [Figure 1].

### The result of MIC and MBC determination

The MIC value determined by broth dilution methods indicated that the antimicrobial activity of the ethanol extracts against the tested microbial was varied, presented in Table 2. The minimal inhibitory concentration ranged from 5 to 10%w/v for *S. aureus*, 2.5 to 5 %w/v for *E. coli* and *P. aeruginosa*, while for *C. albicans* ranged from 1.25-2.5 %w/v.

### The results of minimal contact time test

The results of the in vitro minimal contact time test are presented in Table 3. The data revealed that all tested microbial were killed in the extract concentration of 20%w/v in minimal contact time for 1.5 min.

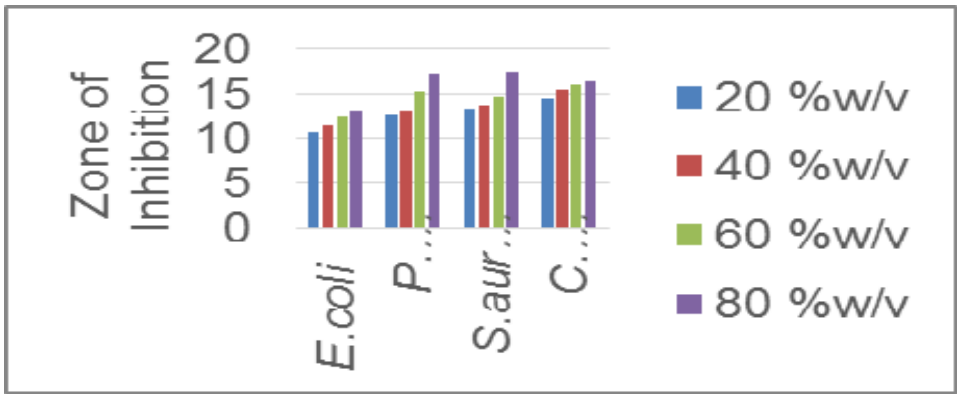


**Figure 1**: Antimicrobial activities of red Piper betel extracts

**Table 1: Antimicrobial activities of ethanol extracts of red piper betel leaves**

| Concentration (%w/v) | Diameter of Inhibitory Zone (mm) | | | |
|---|---|---|---|---|
| | *E. coli* | *P. aeruginosa* | *S. aureus* | *C. albicans* |
| 80 | 13.167±0.047 | 17.233±0.094 | 17.333±0.047 | 16.467±0.047 |
| 60 | 12.333±0.047 | 15.333±0.047 | 14.733±0.047 | 16.067±0.047 |
| 40 | 11.467±0.047 | 13.133±0.047 | 13.800±0.000 | 15.367±0.047 |
| 20 | 10.700±0.000 | 12.567±0.047 | 13.367±0.047 | 14.433±0.047 |
| Mean total | 11.916±0.924 | 14.566±1.854 | 14.808±1.538 | 15.583±0.772 |

Note: Perforator diameter = 9 mm

**Table 2: MIC and MBC Result**

| Concentration (%w/v) | Colony Growth | | | |
|---|---|---|---|---|
| | *E. coli* | *P. aeruginosa* | *S. aureus* | *C. albicans* |
| 10 | - | - | - | - |
| 5 | - | - | + | - |
| 2.5 | + | + | + | - |
| 1.25 | + | + | + | + |
| 0.625 | + | + | + | + |
| 0.3125 | + | + | + | + |
| 0.15625 | + | + | + | + |

Notes: (+) = colony growth; (–) = no colony growth

**Table 3: Minimal contact time of the extract**

| Microorganism | Concentration (%w/v) | Time contact (min) | | | |
|---|---|---|---|---|---|
| | | 1.5 | 3 | 4.5 | 6 |
| *E. coli* | 40 | - | - | - | - |
| | 20 | - | - | - | - |
| | 10 | + | - | - | - |
| | 5 | + | - | - | - |
| *P. aeruginosa* | 40 | - | - | - | - |
| | 20 | - | - | - | - |
| | 10 | + | - | - | - |
| | 5 | + | - | - | - |
| *S. aureus* | 40 | - | - | - | - |
| | 20 | - | - | - | - |
| | 10 | + | - | - | - |
| | 5 | + | + | - | - |
| *C. albicans* | 40 | - | - | - | - |
| | 20 | - | - | - | - |
| | 10 | + | - | - | - |
| | 5 | + | + | - | - |

Notes: (+) = colony growth; (–) = no colony growth

## DISCUSSION

The plants are rich in a wide variety of secondary metabolites which were found to have *in vitro* antimicrobial properties [17]. The presence of phenolic compounds in the extract may attribute antimicrobial activity. Phenolic compounds are thought to be toxic to microorganisms, inhibiting the enzymes which are essential for the growth of microorganism [18]. Of all tested microbes, an ethanol extract of red betel leaf showed the highest inhibition against *C. albicans*.

The above results showed that the antibacterial and antifungal activities of the extracts increased linearly as the concentration of extracts increasing. Presumably this implies that higher concentrations may have a major impact on microbial growth inhibition capacity. This could be attributable to the polar nature of active antimicrobial agents. The extract of red piper betel leaves contains several antimicrobial secondary metabolites, which are all found in a more abundant amount in leaves of *P. crocatum.* Flavonoids had been reported to have a major antimicrobial activity because they can interact with the bacterial cell wall. Moreover, lipophilic flavonoids could disrupt the microbial cell membranes [19].

Interestingly, it was found that the extracts of red piper betel extracts required relatively lesser quantity for inhibiting the growth of fungal than bacterial. Such a difference in susceptibility between the eukaryotic cells of *C. albicans* and the prokaryotic cells of bacteria might be attributed to their difference in cell type. The most sensitive microbe inhibited by the extract was *C. albicans*. While for bacterial activity, *E. coli* and *P. aeruginosa* were more sensitive as compared with *S. aureus*. The extracts showed the lowest MIC values, this exhibited strong activity and a broad spectrum of action.

In another study reported that in order to be able to kill *C. albicans* required synthetic disinfectants i.e. alkaline glutaraldehyde at concentrations of 2% for 1 min. While 1% peroxygen, in the majority of cases, within 15 min to kill *S. aureus*, *P. aeruginosa* and *C. albicans* [20]. It demonstrates the power of red betel extract, which is also capable of killing all the microbes in just 1.5 minutes using 20% of extract concentration. This estimated the strength of this extract was 10 times lower than synthetic disinfectants.

## CONCLUSION

Based on this investigation, it can be concluded that the ethanol extract of red betel leaf could inhibit the growth of airborne pathogen. The most sensitive microbe was *C. albicans*. The extract was very effective against fungal rather than bacterial airborne contaminants. The present study improved the claimed uses of red piper betel leaves as empirically traditional medicine for treating various infectious diseases caused by the microbes.

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