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# In vitro antibacterial activities of crude extracts of nine plants on multidrug resistance bacterial isolates of wound infections

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**Abstract.** *Prastiyanto ME, Dewi NMBA, Pratiningtias TD, Pratiwi NMR, Windayani A, Wahyunengsih E, Astuti, Amir E, Wardoyo FA. 2021.* In vitro *antibacterial activities of crude extracts of nine plants on multidrug resistance bacterial isolates of wound infections*. *Biodiversitas 22: 2641-2647.* Wound infections caused by bacteria is a become serious health problems, multidrug resistance bacteria (MDR) have increased this problem more severely, and therefore, antibacterial agents from natural biological sources are necessary to overcome these problems. This study examined the antibacterial activities of nine plants, i.e. garlic (*Allium sativum*), Solo garlic (*Allium sativum*), Java plum leaf (*Syzygium cumini*), Java plum fruit (*Syzygium cumini*), lime (*Citrus aurantifolia*), Kaffir lime (*Citrus hystrix*), Siamese weed (*Chromolaena odorata*), mangosteen (*Garcinia mangostana*) and bitter melon (*Momordica charantia*), against MDR bacteria isolated from wounds. The antibacterial activities were evaluated using agar well diffusion assay to determine the inhibition zones, and microdilution method to determine the value of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The best antibacterial activities were calculated as the most extensive inhibition zones with the smallest MIC and MBC values. Ethanol extracts from five plants (garlic, Solo garlic, Java plum (leaf), Kaffir lime and bitter melon) showed antibacterial activities against three MDR bacteria isolated from wounds. The bitter melon extract had the largest zones, 19.3 mm (methicillin-resistant *Staphylococcus aureus* [MRSA]), 10.6 mm (ESβL-producing *Escherichia coli*), and 13 mm (carbapenemase-resistant *Pseudomonas aeruginosa* [CRPA]) with the smallest MIC and MBC values against MRSA (3.12 and 25 mg/mL), ESβL- producing *E. coli* (12.25 and 50 mg/mL), and CRPA (6.25 and 25 mg/mL). This concludes that bitter melon has the potential to be developed as an antibacterial agent, particularly against MRSA strains, ESβL-producing *E. coli*, and CRPA that cause wound infections. Further in vivo research and the discovery of modes of action are needed to explain the antibacterial effects.

**Keywords**: CRPA, ESβL-producing *Escherichia coli*, In vitro antibacterial activities, MRSA, wound infection

## INTRODUCTION

Skin is an important organ that protects the body from damage and invasion of pathogenic bacteria (Xu et al. 2015). When the skin is damaged, the wound that exposes became prone to bacterial infection easily, which ultimately affects health. The wound may be healed in a few days or will develop for a long time and become chronic. A chronic wound is one of the most serious and fatal human problems (Han and Ceilleey 2017).

An infected wound may take longer time to recover, even may cause death in some cases (Liang et al. 2019). *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* are the bacteria commonly found in wounds (Pallavali et al. 2019). Antibiotics are commonly used to treat bacterial infections. However, the uncontrolled use of antibiotics contributes to the emergence of multidrug resistance (MDR) against many bacterial strains (Bologa et al. 2013). Patients infected with MDR bacteria may suffer from a prolonged disease that is difficult to treat and requires higher costs of treatment.

The Infectious Disease Society of America has considered the advent of several MDR bacteria, including those that are methicillin-resistant, extended-spectrum β-lactamase (ESβL)–producing-resistant, and carbapenemase-resistant, as a distinct challenge in management (Boucher et al. 2009). The burn wound infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) (Chopra et al. 2016), MDR-*P. aeruginosa* (Nasser et al. 2020) and *E. coli* (Nasser et al. 2020) increase mortality and morbidity. The prevalence of infections caused by MRSA, MDR-*P. aeruginosa* and *E. coli* have increased in recent years.

Thus, new antibacterial agents from natural biological sources are required. Biological antibacterial agents can be obtained from honey (Panjaitan et al. 2018), mushrooms (Prastiyanto et al. 2020b; 2016), isolate bacteria from marine organisms (Asagabaldan et al. 2019), bacteriocins (Lestari et al. 2019), fruits (Prastiyanto et al. 2020d; Wahyuni et al. 2019), latex (Prastiyanto et al. 2020c) and seeds (Ilvani et al. 2019; Prastiyanto et al. 2020a). Many studies in the medical field reported the importance of traditional medicinal plants as alternatives of antimicrobial agents (Akhtar and Mirza 2015; Aumeeruddy-elalfi et al. 2015; Prastiyanto et al. 2021).

This study aimed to investigate the antibacterial potentials of traditional plants. Nine plants examined to study their antibacterial activities against resistant bacteria

isolated from wounds, such as methicillin-resistant *S. aureus* (MRSA), (ESβL)-producing *E. coli*, and carbapenemase-resistant *P. aeruginosa* (CRPA). The nine plants were garlic, Solo garlic, Java plum (leaf), Java plum (fruit), lime, Kaffir lime, Siam weed, mangosteen, and bitter melon.

## MATERIALS AND METHODS

### Plant materials and preparations of extracts

Sampling of nine healthy plants were carried out in the rainy season of December 2019. Different parts of the plants were washed with water to remove unnecessary materials, dried in the sun for seven days, ground and then stored in sterile airtight containers for further usages in the next processes. Plant extracts were prepared by maceration with 96% ethanol solvent. 200 g of ground plant parts were soaked in 600 mL of solvent for 24 hours at room temperature, protected from light and were later shaken. The solvent replacement was done every day until the solution was clear, in which no more active compounds were contained in the dry powder. The supernatant was filtered using Whatman No.1 filter paper. The maceration solutions were concentrated under reduced pressure using a rotary evaporator at 50 ˚C. The crude extracts were collected and allowed to dry at room temperature.

### Isolation, identification of bacterial strains and antibiotic sensitivity test

MDR bacteria were directly isolated from wound samples obtained from patients in Dr. Kariadi Hospital, Semarang, Central Java, Indonesia. All isolates were identified by biochemical tests using Vitek®MS (bioM´erieux, Marcy l'Etoile, France), following minimum inhibitory concentration (MIC) interpretive standards from the Clinical Laboratory Standard Institute M100-S25 (CLSI 2019).

### Antibacterial assay of plant extracts

*Agar well diffusion assay*

The antibacterial activities of various plant extracts were evaluated using a well-diffusion assay (Andleeb et al. 2020). MDR bacteria in a subculture on blood agar plate (BAP) media were incubated for 24 hours at 35 ± 2°C. The MDR bacterial colonies were dissolved in a normal saline solution with a turbidity equivalent to the 0.5 McFarland standard. 100 µL of each MDR bacterium was inoculated in Muller Hilton agar (MHA) by spreading the bacterium on the surface of the agar using a sterilized glass spreader. After five minutes of inoculation, the wells were prepared using a sterilized steel *cork borer* (1cm in diameter). Four wells were made on each plate and loaded with each plant extract (250, 500, 750, and 1000 mg/mL). All plates were then incubated aerobically at 35 ± 2 °C for 16-20 hours. Dimethyl sulfoxide (DMSO) was used as a negative control. Vancomycin and oxacillin were applied as positive controls for MRSA, ampicillin and meropenem for ESBL-producing bacteria, and meropenem and tetracycline were for CR bacteria. Antibacterial activities of the extracts were determined by measuring the diameters of the inhibition zones in mm against the tested organism.

*Determination of MIC and minimum bactericidal concentration (MBC) of the plant extracts*

MIC values of plant extracts were determined in 12-well sterile microplates using the broth microdilution method (CLSI 2018). Each test was carried out in triplicate. MHB (100 µL) was placed into the well and plant extract (100 µL) was put in the dilution series. 10 µL bacterial cell suspensions were placed in each well. Microplates were incubated aerobically at 35 ± 2°C for 16-20 hours. Oxacillin was used as positive controls for MRSA, ampicillin was applied for ESBL-producing bacteria, while meropenem was utilized for CR bacteria.

MIC was determined by selecting the lowest concentration of plant extracts that inhibited bacterial growth and was detected by the naked eye without any assistance from a particular device. Then, wells were sub-cultured using a 10 µL inoculating loop on to a 5% sheep BAP at (35 ± 2)°C for 16–20 hours of incubation. The lowest concentration of the extract that did not show any growth was defined as MBC (Yin et al. 2018).

## RESULTS AND DISCUSSION

### Extract yield

Ethanol extracts from nine plants were estimated to determine the extract yields (Table 1). Bitter melon showed the highest results, showing that its constituents were relatively polar.

### Tested microorganisms

The results of the identification and test of bacterial sensitivity to antibiotics are presented in Figure 1. The results reveal that the bacteria isolated from the wounds were *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and they were resistant to several antibiotics. *S. aureus* was found resistant to oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, clindamycin, tetracycline, and rifampicin. *E. coli* showed resistance against ampicillin, ampicillin-sulbactam, piperacillin-tazobactam, cefazolin, ceftazidime, ceftriaxone, cefepime, aztreonam, amikacin, gentamicin, and ciprofloxacin. Whereas, *P. aeruginosa* was observed resistant to ampicillin, ampicillin-sulbactam, piperacillin-tazobactam, cefazolin, ceftazidime, cefepime, aztreonam, amikacin, gentamicin, ciprofloxacin, meropenem and tigecycline. The bacteria isolated from the wound samples were methicillin-resistant *St. aureus* MRSA, (ESβL)-producing *E. coli* and carbapenemase-resistant *P. aeruginosa* (CRPA).

**The antibacterial activities**

*Agar well diffusion assay*

The antibacterial activities of the nine extracts were tested in vitro by agar well diffusion assay against three resistant bacteria causing wound infections. The antibacterial activities were determined by measuring the diameters of the inhibition zones in mm concerning MRSA, ESβL-producing *E. coli*, and CRPA (Figure 2).

Of the nine plant extracts at various concentrations (250, 500, 750, and 1000 mg/mL), the extracts of garlic, Solo garlic, Java plum (leaf), Kaffir lime and bitter melon showed inhibition zones in the three tested bacteria (Figure 3), and the bitter melon extract had the largest zone. The extracts of Java plum (fruit), Siam weed and mangosteen did not show any inhibition zones against ESβL-producing *E. coli*, but demonstrated inhibition zones on MRSA and CRPA. Meanwhile, lime extract only indicated an inhibition zone on MRSA. The inhibition zones of nine extracts of the three test bacteria disclosed inhibition zone diameters of 6-19.3 mm (MRSA), 3.9-10.6 mm (ESβL-producing *E. coli*), and 3.9-13 mm (CRPA). 1000 mg/mL bitter melon extract indicated the largest inhibition zone diameters of the three assessed bacteria of 19.3 mm (MRSA), 10.6 mm (ESβL-producing *E. coli*), and 13 mm (CRPA). The bitter melon extract also demonstrated a diameter of inhibition zone greater than the antibiotic control.

**MIC and MBC**

MIC of nine extracts was tested in vitro by the microdilution method for three resistant bacteria isolated from wounds (Table 2). The extracts of garlic, Solo garlic, Java plum (leaf), Kaffir lime and bitter melon showed MIC values between 3.12 and 25 mg/mL for MRSA, ESβL-producing *E. coli*, and CRPA. Among the five extracts, bitter melon presented the lowest MIC values against MRSA (3.12 mg/mL), ESβL-producing *E. coli* (12.25 mg/mL), and CRPA (6.25 mg/mL). This result was lower than the value of antibiotic control.

MBC from nine extracts was tested in vitro by the microdilution method for three resistant bacteria isolated from wounds (Figure 4). The extracts of garlic, Solo garlic, Java plum (leaf), Kaffir lime and bitter melon showed MBC values for MRSA, ESβL-producing *E. coli*, and CRPA. The extracts of Java plum (fruit), Siam weed and mangosteen did not show any MBC values for ESβL-producing *E. coli*, but demonstrated inhibition on MRSA and CRPA. However, lime extract only presented MBC values on MRSA. The MBC values ranged from 25 to 50 mg/mL.

Studies on antibacterial agents from natural ingredients are important efforts, particularly in recent times, due to the increasing level of antibiotic resistance among pathogenic bacteria. Abuse of antibiotics usage has been considered the major cause of the increasing antibiotic resistance against bacteria. The effort in this study focused on the use of widely available plants. Nine plants were used in this investigation to evaluate the antibacterial activities against MDR bacteria, including methicillin-resistant *S.aureus*, ESBL-producing *E. coli*, and CRPA, isolated from wounds. These results are consistent with the previous research reports that *S. aureus*, *E. coli*, and *P. aeruginosa* are the most common bacteria found in infected wounds (Manzuoerh et al. 2019; Petkovs˘ek et al. 2009).

The nine plant extracts appeared to have inhibition zone diameters ranging from 6 mm to 19.3 mm, with the most significant results were shown by the bitter melon extract. The bitter melon extract had the largest diameters of the inhibition zones in the three tested bacteria, 19.3 mm (methicillin-resistant *S. aureus*), 10.6 mm (ESβL-producing *E. coli*), and 13 mm (CRPA), and greater diameters of inhibition zones than the antibiotic control.

The extract of bitter melon was proven to have lowest MIC and MBC values, against MRSA (3.12 and 25 mg/mL), ESβL-producing *E. coli* (12.25 and 50 mg/mL), and CRPA (6.25 and 25 mg/mL). This provides evidence that bitter melon ethanol extract shows antibacterial activities against methicillin-resistant *S.aureus* strains, ESβL-producing *E. coli*, and CRPA. The extract displays broad-spectrum antimicrobial activities (Khan and Omoloso 1998; Mwambete 2009). Although, testing of the groups contained in bitter melon was not performed in this research, some other studies have confirmed that bitter melon contains flavonoids, alkaloids, and terpenoids (Kumar et al. 2010; Leelaprakash et al. 2011; Annapoorani and Manimegalai 2013).

**Table 1.** The extract yield

| Plants | Scientific name | Part of plants | Yield (%) |
|---|---|---|---|
| Garlic | *Allium sativum* Linn | Tuber | 1.11 |
| Solo garlic | *Allium sativum* Linn | Tuber | 0.63 |
| Java plum | *Syzygium cumini* (L) Skeels | Leaf | 10.30 |
| Java plum | *Syzygium cumini* (L) Skeels | Fruit | 13.21 |
| Lime | *Citrus aurantifolia* Swingle | Rind | 11.20 |
| Kaffir lime | *Citrus hystrix* DC. | Rind | 14.12 |
| Siam weed | *Chromolaena odorata* (L) RMKing & H.Rob. | Leaf | 9.50 |
| Mangosteen | *Garcinia mangostana* L | Rind | 13.10 |
| Bitter melon | *Momordica charantia* Descourt | Fruit | 28.60 |

**Table 2.** The MIC values of nine plant extracts against MRSA, ESBL-producing *E. coli* and CRPA (mg/mL)

| Extract and control | Tested bacteria | | |
|---|---|---|---|
| | MRSA | ESBL-*E.coli* | CRPA |
| Garlic | 12.5 | 25 | 12.5 |
| Solo garlic | 25 | 25 | 12.5 |
| Java plum (leaf) | 25 | 25 | 12.5 |
| Java plum (fruit) | 25 | - | 25 |
| Lime | 25 | - | - |
| Kaffir lime | 12.5 | 25 | 12.5 |
| Siam weed | 25 | - | 25 |
| Mangosteen | 12.5 | - | 25 |
| Bitter melon | 3.12 | 12.5 | 6.25 |
| Oxacillin | 4 | - | - |
| Ampicillin | - | 32 | - |
| Meropenem | - | - | 16 |

The antibacterial activities of plants can be related to phytochemical compounds which can protect the human body against microbial infection. The most important phytochemicals are flavonoids, alkaloids, and terpenoids (Kumar et al. 2013). Flavonoids (Khalid et al. 2019) and terpenoids (Broniatowski and Mastalerz 2015) have been recognized to show strong antibacterial activities. The mechanism of antibacterial activities of flavonoids, alkaloids, and terpenoids in bitter melon has not been identified. However, phytochemical compounds can inhibit bacterial growth by damaging bacterial cell walls (Abuga et al. 2020). Bitter melon is proven to be potentially developed as an antibacterial agent, especially for MDR strains from wounds. Further *in vivo* research and the investigation of modes of action are essential to explicate the antibacterial effects so that potential clinical drugs and health products can be advanced. This study can provide novel information about the benefits of bitter melon as a natural source of the antibacterial agent against MDR bacteria.

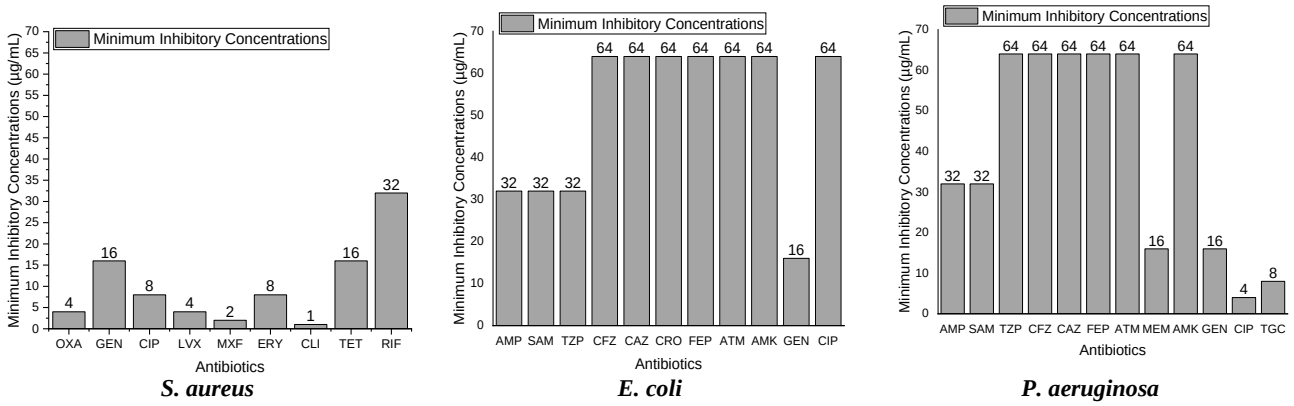


**Figure 1**. The results of identification and sensitivity to bacterial antibiotics isolated from wounds. OXA: Oxacillin; GEN: Gentamicin; CIP: Ciprofloxacin; LVX: Levofloxacin; MXF: Moxifloxacin; ERY: Erythromycin; CLI: Clindamycin; TET: Tetracyclin; RIF: Rifampicin; AMP: Ampicillin; SAM: Ampicillin-sulbactam; TZP: Piperacillin-tazobactam; CFZ: Cefazolin; CAZ: Ceftazidime; CRO: Ceftriaxone; FEP: Cefepime; ATM: Aztreonam; AMK: Amikacin; MEM: Meropenem; TGC: Tigecycline

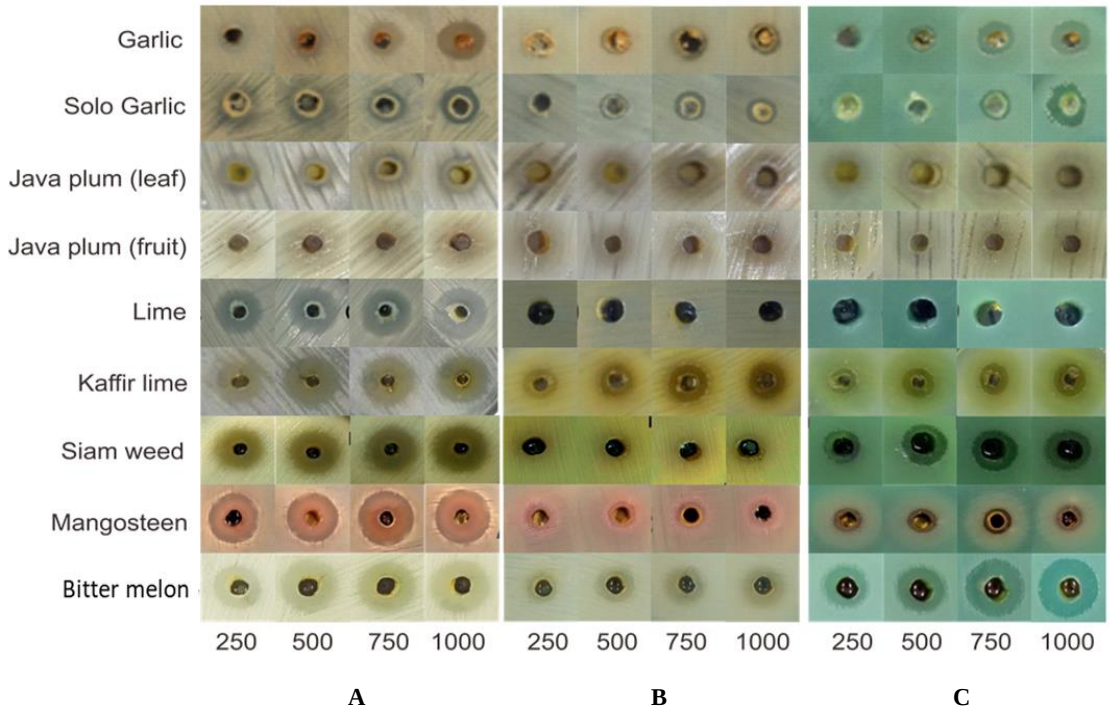


**Figure 2.** The inhibition zones of nine plants (250, 500, 750, and 1000 mg/mL) against MDR bacteria; A: MRSA; B: ESβL-producing *E. coli*; C: CRPA

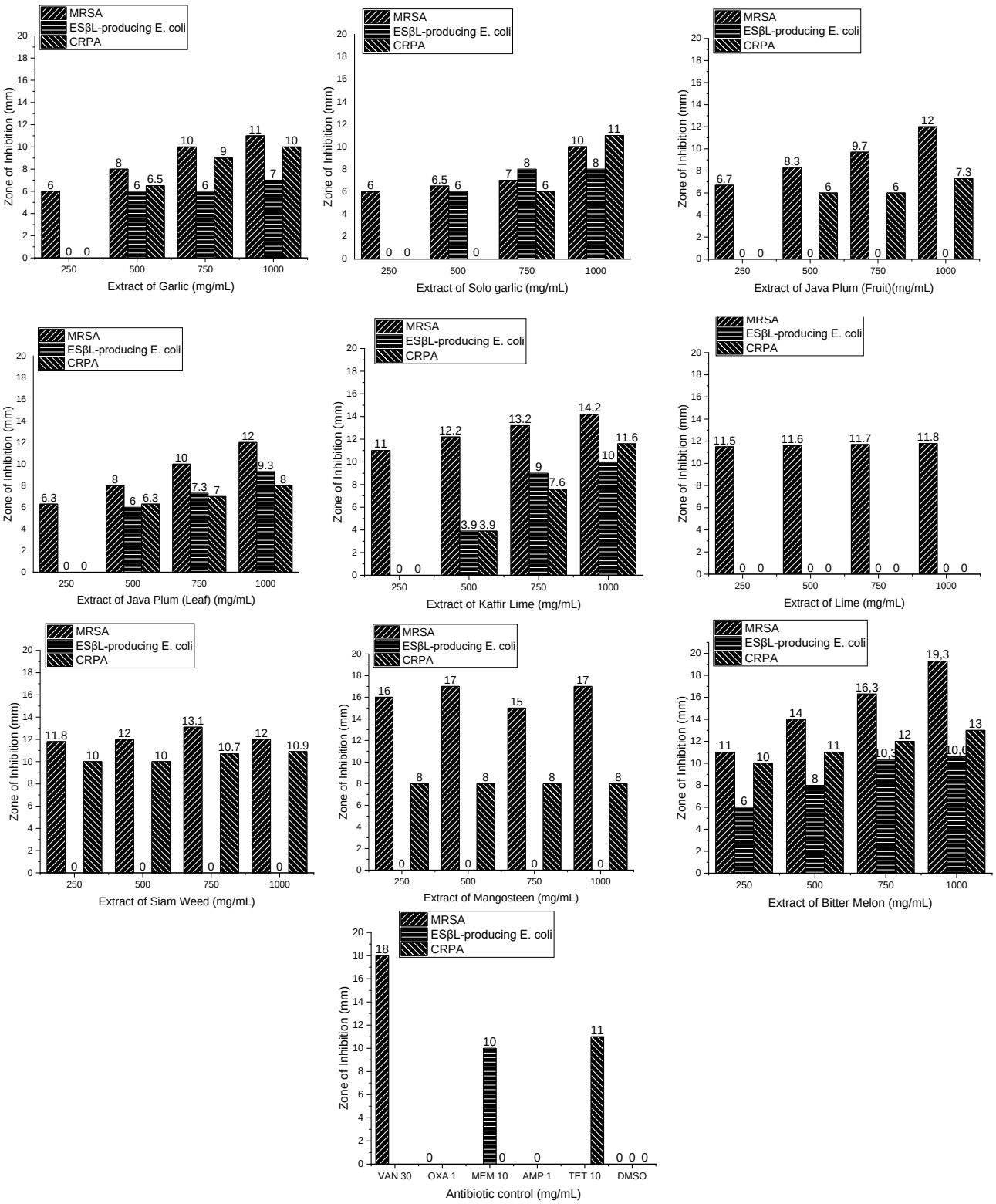


**Figure 3.** The diameters of the inhibition zones of nine plants. Vancomycin (VAN) and oxacillin (OXA) were used as positive controls for MRSA, ampicillin (AMP) and meropenem (MEM) for ESBL-producing *E. coli*, and meropenem (MEM) and tetracycline (TET) for CRPA.

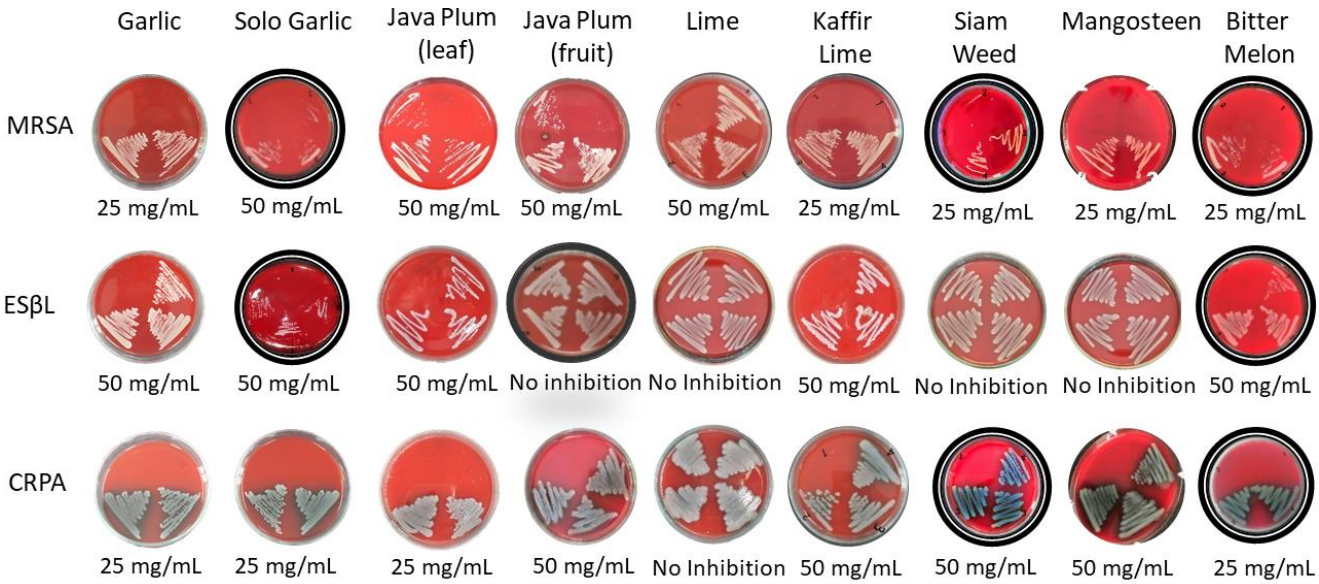


**Figure 4.** The MBC values of nine plant extract against MRSA, ESBL-producing *E. coli* and CRPA

In conclusion, the bitter melon has the potential to be developed as an antibacterial agent, particularly against methicillin-resistant *S. aureus* strains, ESβL-producing *E. coli*, and CRPA that cause wound infections. Further, *in vivo* research and the discovery of modes of action are needed to explain the antibacterial effects.

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