ANTIMICROBIAL PROPERTIES OF *Gynura procumbens* (SAMBUNG NYAWA) LEAVES IN METHANOLIC AND ACIDIC EXTRACTS

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ABSTRACT

Introduction: *Gynura procumbens*, commonly known as *'sambung nyawa'* in Malaysia has been used traditionally as remedies for anti-inflammatory, anti-hyperlipidimic and anti-hyperglycemic. The purpose of the present study was to qualitatively evaluate the antimicrobial effects of *Gynura procumbens* extracts. **Methods:** The sample was extracted using methanol and acid hydrolysis. The antimicrobial effects of the sample were determined by disc diffusion method against two bacteria and two fungi namely *E. coli*, *S. aureus*, *C. albicans* and *S. cerevisiae*. **Results:** The results showed that the acidic extract of *G. procumbens* has positive reactions towards *E. coli*, *S. aureus* and *C. albicans* with the presence of zone of inhibition at the concentration of 150 mg/mL. Following the positive reaction, the minimum inhibitory concentration (MIC) of the acidic extract was then evaluated by broth dilution method. The MIC of *E. coli* and *S. aureus* were determined at concentration 37.5 mg/mL and 75 mg/mL for *C. albicans*. It indicated that acidic extracts at lower concentration could inhibit the bacteria, but high concentration of extracts was required in the inhibition of the fungi. **Conclusion:** It can be concluded that, the present study proves that there is potential of antimicrobial effects in *Gynura procumbens* leaves extracts.

Keywords: *G. procumbens*, antimicrobial, methanolic, acidic extract

INTRODUCTION

Plants have been widely used as medicines since thousands of years ago. Every culture on earth, through written or oral tradition, has relied on the vast variety of natural chemistry found in the healing plants for their therapeutic properties. According to the World Health Organization (2011), over three-quarters of the world population depend on the herbal medicines as a source of main healthcare. Recent study revealed that among 150 drugs consumed in the United States, about 57% of it consisted of minimum one active compound or one derived compounds which were from natural sources (Maridass, 2010). Current available drugs that are plant-based, plant peptides and proteins are considered as critical sources of biological compounds that exhibited bioactivities similar to synthetic drugs (Hew et al., 2013).

Gynura procumbens (G. procumbens) also known as *Sambung Nyawa* is from *Gynura* genus which is composed of more than 84 species, subspecies, and cultivars around the world (*Gynura procumbens*, 2009).

Saengsai (2003) stated that 5 species of *Gynura* have been identified in Thailand including *G.crepidoides*, *G.integrifolia*, *G.procumbens* or *G.sarmentosa*, *G.pseudochina* (Phakkat Kop) and *G.pseudo-china* (Waan Mahakaan).

According to Eswani et al., (2010), the medicinal plants can be defined as plants that have medicinal properties and were originated from forests. Bioactive secondary metabolites such as alkaloids, phenolics, sterols, flavonoids and terpenoids have huge potentials in medicinal uses, especially in improving the resistance against microorganisms (Ramawat et al., 2009). Continuous studies were conducted in searching for compounds that has antimicrobial characteristic as the antibiotic resistance had limited the use of antibiotics (Ranjan et al., 2012). The rapid increasing of antibiotic resistance has caused the use of plants as solution for the discovery of active antimicrobial agents (Hemaiswarya & Doble, 2009). Bacteria have developed resistance to antimicrobial drugs. The use of antimicrobial drugs has exerted the selective pressure that favours the growth of organisms that are resistant to the drug's action.

It is well studied that the concentration of phytochemicals such polyphenols play important roles as defence against plant pathogens and animal herbivore aggression and as response to various abiotic stress conditions. Therefore, the first aim of this study was to evaluate the antimicrobial effects of the *Gynura procumbens (Sambung nyawa)* plant extracts in two different fractions (free and bound extracts) by using disc diffusion method. This was followed by the second aim which to determine the minimum inhibitory concentration (MIC) of the extracts that can inhibit the microbial growth. Through these aims, we have identified the type of extract that could inhibit the microbial growth and have positive antimicrobial properties.

MATERIALS AND METHODS

Materials

Fresh *Gynura procumbens* leaves were obtained from the Kulliyyah of Pharmacy, International Islamic University Malaysia, Kuantan, Pahang. Other materials include Mueller Hinton Agar powder (MHA) (HI Media) Mueller Hinton broth powder (MHB) HI Media Sabouraud Dextrose Agar powder 4% (SDA) Merck, Germany Sabouraud Dextrose Broth powder (SDB) 2%Merck, Germany Tetracycline disc Oxoid, Basingstoke Nystatin disc Oxoid, Basingstoke Sulphuric acid 98% SYSTERM Methanol 99.8% R & M Chemicals Hydrochloric acid 37% Merck, Germany Nitrogen gas.

Sample preparation

The samples were washed under running tap water to remove any debris. The samples were kept in -80 °C freezer for one day before being dried in a freeze dryer. The samples were ground to fine powders by using a blender at room temperature and sealed in air-tight plastic bags and stored at -80 °C until further usage.

Phenolic acid extraction.

These following methods were adapted from Lin & Harnly (2007) methods.

a. Free Phenolic Extraction

Dried sample in powdered form (500 mg) was extracted with 20 mL methanol-water (60:40, v/v) using sonication for 60 mins at room temperature. The supernatants were dried using nitrogen gas.

b. Acid Hydrolysis Extraction

2.0 mL of the filtered free phenolic extract was mixed with concentrated HCl (37%, 0.4 mL) and was heated at 85 °C for 2 hours using water bath. Then, 1.6 mL of absolute methanol was added to the mixture and sonicated for 10 min. The extract was dried using rotavap and stored in -80°C for further usage.

Disc Diffusion Method

Disc diffusion method was done according to Shryock et al. (2002).The blank discs (6 mm in diameter) were filled with 50 µL of different concentration of extracts and were left dried. For the negative control, 50 µL of methanol for free phenolic extract and hydrochloric acid (for bound phenolic) was filled into the blank disc (6 mm in diameter) while for positive control, standard antibiotic discs (30 µg for tetracycline or 100 unit of nystatin) were used. After the discs were completely dried, they were placed over the plates that have been spread with the microorganisms (as shown in Table 1) by using sterile forceps. The plates were incubated at 37 °C for 24 hours for bacteria and at 30 °C for 48 hours for fungi. The diameter of inhibition zone around each discs were then measured by using a millimeter ruler.

Table 1: The list of test microorganisms and their strain

Test Microorganisms	Strain
Gram positive	
S. aureus	ATCC 25923
Gram negative	
E. coli	ATCC 25922
Fungi	
C. albicans	IMR C 523/11 A
S. cerevisiae	IMR S 617/06 B

a. Inoculums preparation

The inoculums for bacteria and fungi were prepared according to Shryock et al. (2002), with some modifications. The strains of microorganisms were subcultured to MHA plates for bacteria and SDA plates for fungi to obtain isolated colonies. After incubation period (24 hours for bacteria and 48 hours for fungi) in an incubator, three well-isolated colonies of the same morphological type were selected from the agar plate cultures respectively (Figure 1). The top of each colony was touched with a loop and then was transferred into a tube containing 25 mL of MHB for bacteria and 25 mL of SDB for fungi. Then, they were incubated at 37 °C for 24 hours for the bacteria and 30 °C for 48 hours for fungi.

Figure 1. The well-isolated colonies for the inoculum

In order to standardize the bacterial and fungal culture, the optical densities (OD) of the culture were verified using spectrophotometer in comparison with 0.5 McFarland Standard. The OD for the incubated bacteria and fungi were measured at 625 nm and 494 nm respectively using spectrophotometer. The range of OD for bacteria was 0.08-0.10 while the OD for fungi was 0.54-0.94. According to Rifai et al. (2005) and Shanthi and Nelson (2013), the acceptable range of OD for bacteria is 0.08-0.13 while for fungi is 0.5-2.5 when compared to 0.5 McFarland Standard to achieve microbial suspension containing 108 CFU/mL for bacteria and 106 CFU/mL for fungi.

Determination of Minimum Inhibitory Concentration (MIC)

Minimal inhibitory concentration (MIC) of *G. procumbens* was determined using 96-well microplate by observing the lowest concentration of samples that give effect against bacteria and fungi activities. Firstly, 200 µL of 150 mg/mL sample solution was transferred into well A. Followed by transferring 100 µL from well A into well B. This two fold serial dilution was continued until well H. 100 µL from well H was removed so that all wells contain only 100 µL of sample solutions. Next, well B until H was filled with 100 µL of test organisms. Addition of 100 µL of test organisms into the extracts further diluted the extract concentration by two-fold. So, the final concentration of the mixture of the extracts and the test organisms was half of the extract concentrations prepared earlier. The well number 5, 9, 10 and 11 served as duplicate and triplicate of each sample, respectively. The well number 2 and 6 were filled with positive control which was 100 µL of antibiotic (tetracycline for bacteria and nystatin for fungi) and 100 µL of the test organism. Well number 4 and 8 were filled with 100 µL Mueller Hinton Broth (MHB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungi. Next, well number 3 and 7 were filled 100 µL of negative control which was methanol. Then, the microplate was covered with a parafilm and incubated for 24 hours at 37 °C for bacteria and 48 hours at 30 °C for fungi. After incubation, all the wells were examined based on the turbidity of the solution. All the bacteria and fungi were standardized first to the turbidity of 0.5 McFarland standards by using spectrophotometer. The range of OD for bacteria was 0.08-0.10 while the OD for fungi was 0.54-0.94.

RESULTS

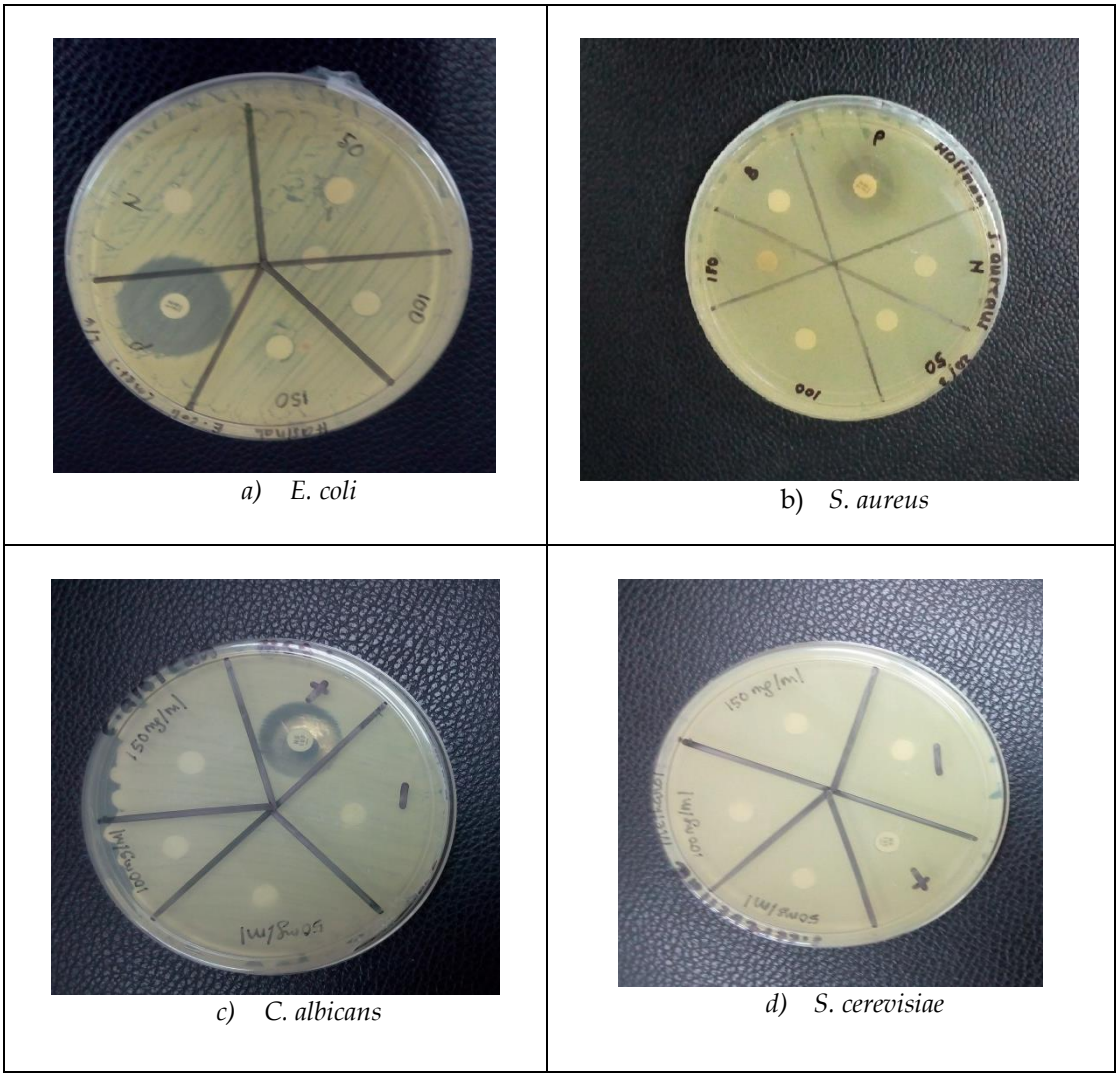
Antimicrobial activities of methanolic extract using disc diffusion method

The antimicrobial activities of *G. procumbens* methanolic extract against four selected microorganisms are summarized in Table 2. The screening of antimicrobial assay using disc diffusion method showed that *G. procumbens* leaves of methanolic extract showed no inhibition zone against selected microorganisms at the concentration of 50 mg/mL, 100 mg/mL and 150 mg/mL (Figure 2). All inoculums used were in the range of the standard. It is really important to ensure the uniform lawn growth, as a smaller inoculum size may produce falsely large inhibition zones while a bigger inoculum size may produce falsely smaller zones. For negative control, 50 µL of methanol was filled into the blank disc (6 mm in diameter) while for positive control, standard antibiotic discs (30 µg for tetracycline or 100 unit of nystatin) were used.

Table 2 Diameter zone of inhibition of disc diffusion test (methanol extract)

Organisms	Diameter zone of inhibition (mm)				
	Concentration of methanol extract (mg/ml)				
	50	**100**	**150**	**Positive control**	**Negative control**
E.coli	NI	NI	NI	24	NI
S.aureus	NI	NI	NI	20	NI
C.albican	NI	NI	NI	19	NI
S. cerevisiae	NI	NI	NI	NI	NI

NI = no inhibition



a) E. coli

b) *S. aureus*

c) C. albicans

d) S. cerevisiae

Figure 2. Zone of inhibition of methanol extracts. Note: p/+=positive control, n/-= negative control

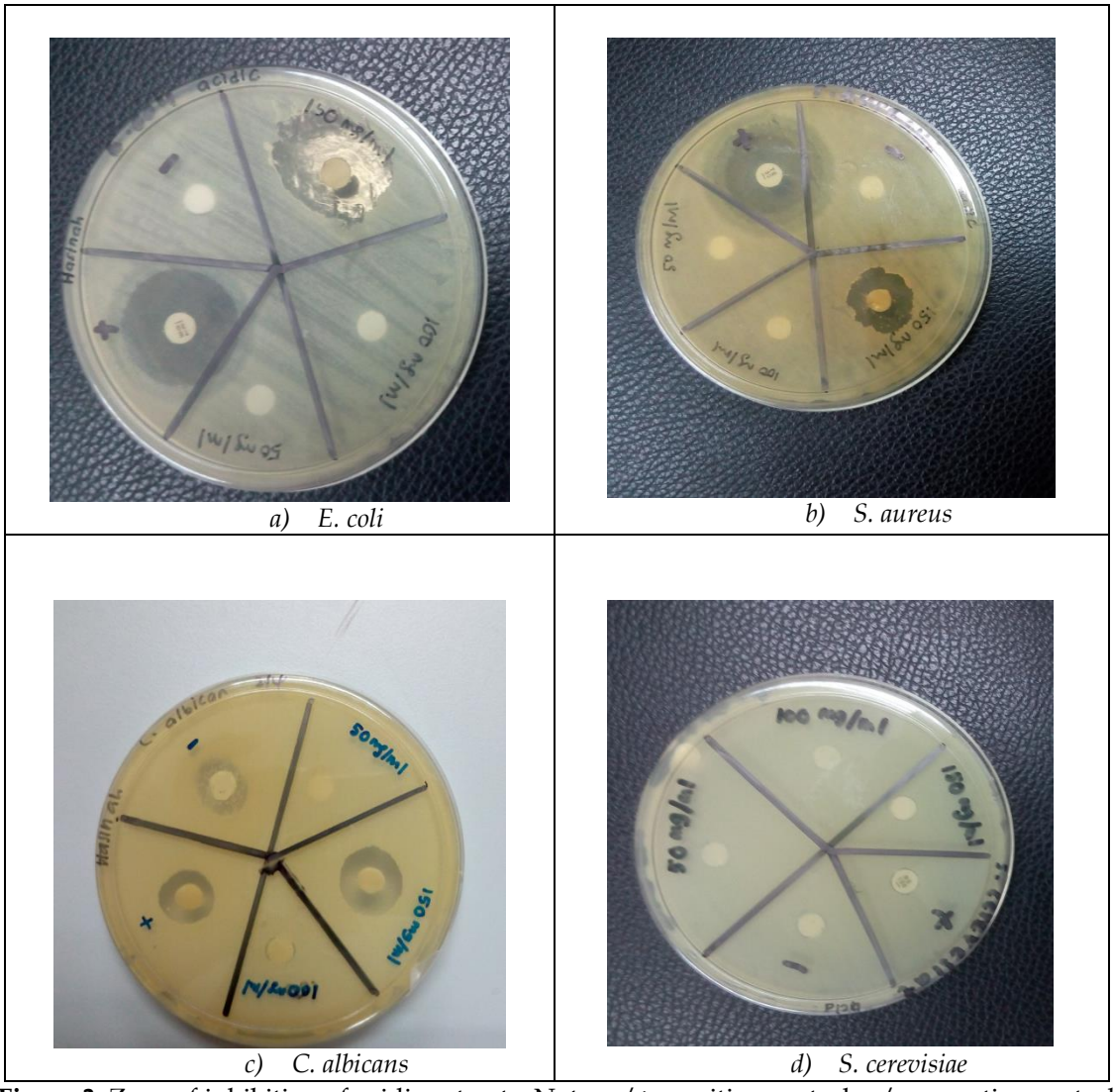
Antimicrobial activities of acidic extract using disc diffusion method

The antimicrobial activities of *G. procumbens* acidic extract against four selected microorganisms are summarized in Table 3. The acidic hydrolysis extract showed inhibition zone against *E. coli*, *S. aureus*, and *C. albicans* and no inhibition zone for *S. cerevesiae* at all concentrations (as shown in Figure 3). Theoretically, the *S. cerevisiae* should have an effect to the nystatin (positive control), however, the doxycycline hyclate and amphotericin B were reported to have better effects on it (Zhang et al. 2002; Salem et. al. 2015). The positive controls showed inhibition zone against *E. coli*, *S. aureus* and *C. albicans* compared to the negative control.

Table 3 Diameter zone of inhibition of disc diffusion test (acidic extraction)

Organisms	Diameter zone of inhibition (mm)				
	Concentration of acid extract (mg/ml)				
	50	**100**	**150**	**Positive control**	**Negative control**
E.coli	NI	NI	22	24	NI
S. aureus	NI	NI	19	20	NI
C. albican	NI	NI	11	14	NI
S. cerevisiae	NI	NI	NI	NI	NI

NI = no inhibition



a) E. coli *b) S. aureus* *c) C. albicans* *d) S. cerevisiae*

Figure 3. Zone of inhibition of acidic extracts. Note: p/+=positive control, n/-= negative control

Minimum Inhibitory Concentration (MIC) Test

By comparing with the positive control by using tetracycline for bacteria and nystatin for fungi, any clear well or no turbidity indicated no bacterial growth. There was only acidic extract that have shown positive

result in the disc diffusion method and *E. coli*, *S. aureus*, and *C. albicans* were selected for the MIC test. The highest concentration of the extract was 150 mg/mL and two-fold serial dilution was done for the extract to be analyzed. The lowest concentration was 1.1 mg/mL. As shown in Figure 4, starting from 37.5 mg/mL to 150 mg/mL of acid extract, the wells have shown no bacterial growth, indicated that 37.5mg/mL was the minimum inhibitory concentration. Tetracycline was selected as a positive control for bacteria whereas nystatin was selected for fungi. Both tetracycline and nystatin inhibited the bacterial growth in the microplates. Tetracycline is a broad-spectrum antibiotic produced by the streptomycin bacterium, indicated for the use against many bacterial infections while nystatin is a polyene antimycotic drug to which *Candida sp.* and *Cryptococcus* are sensitive (Nurul Ba'atun, 2007).

Conc. (mg/mL)	Ext. + *E.C*	Ext. + *E.C*	Ext. + *E.C*	Tet. + bacteria	Ext. + *S.A*	Ext. + *S.A*	Ext. + *S.A*	Met. + bacteria	MHB + bacteria
150	**X**	**X**	**X**	**X**	**X**	**X**	**X**	/	/
75	**X**	**X**	**X**	**X**	**X**	**X**	**X**	/	/
37.5	**X**	**X**	**X**	**X**	**X**	**X**	**X**	/	/
18.5	/	/	/	**X**	/	/	/	/	/
9.38	/	/	/	**X**	/	/	/	/	/
4.69	/	/	/	**X**	/	/	/	/	/
2.34	/	/	/	**X**	/	/	/	/	/
1.1	/	/	/	**X**	/	/	/	/	/

Notes: Ext. = Extract
Tet. = Tetracycline
Met.= Methanol
E.C = *E.coli*
S.A = *S. aureus*
MHB = Mueller Hinton Broth
X = No bacterial growth
/ = Bacterial growth/ turbid

Figure 4. Minimum inhibitory concentration of acid extraction on *E. coli* and *S. aureus*

Besides that, for *C. albicans*, the MIC ranged from 75 mg/mL to 150 mg/mL of acidic extract wells showed no bacterial growth (as shown in Figure 5). Therefore, it was indicated that 75 mg/mL was the minimum inhibitory concentration.

Conc. (mg/mL)	Ext. + *C. albicans*	Ext. + *C. albicans*	Ext. + *C. albicans*	Nys. + fungi	Met. + fungi	SDB +fungi
150	**X**	**X**	**X**	**X**	/	/
75	**X**	**X**	**X**	**X**	/	/
37.5	/	/	/	**X**	/	/
18.5	/	/	/	**X**	/	/
9.38	/	/	/	**X**	/	/
4.69	/	/	/	**X**	/	/
2.34	/	/	/	**X**	/	/
1.1	/	/	/	**X**	/	/

Notes: Ext. = Extract
Nys. = Nystatin
Met. = Methanol
SDB = Sabourad Dextrose Broth
X = No bacterial growth
/ = Bacterial growth/ turbid

Figure 5. Minimum inhibitory concentration of acid extraction on *C. albicans*

DISCUSSION

Antimicrobial activities of Methanolic extract using disc diffusion method

Evaluation of antimicrobial activity of *G. procumbens* extracts was begun with antimicrobial screening by employing the disc diffusion test. The results of disk diffusion are considered as qualitative because it can only reveal the susceptibility of antimicrobials against the tested bacteria, which were described as susceptible, intermediate, and resistant correlated with diameter of the inhibition zone. The acceptable range of OD for bacteria was 0.08-0.13 OD while for fungi was 0.5-2.5 according to McFarland standard to achieve microbial suspension containing 108 CFU/mL for bacteria and 106 CFU/mL for fungi (Rifai et al., 2004; Shanti and Nelson, 2013). The highest concentration of the extract used was 150 mg/mL. The blank discs were filled with 50 µL of the different concentration of extracts and left dried before being placed over the plates. This was to ensure that the liquid from the extracts do not influence the growth of bacteria and also to ensure the discs would not move or slip from the original place.

The antimicrobial effect of plant extract varies from one plant to another in different studies carried out in different regions of the world. This may be due to many factors such as, the effect of climate, soil composition, age and vegetation cycle stage, on the quality, quantity and composition of extracted product, different bacterial strains (Masotti et al., 2003; Angioni, 2006). Polyphenols show antimicrobial activity against human pathogen (Daglia 2012). Low concentration of phenolic compounds in the extract might affect the antimicrobial properties. The active compounds in the crude extract only can possess their antimicrobial properties in a sufficient amount of concentration (Tiwari et al., 2011).

Antimicrobial activities of Acidic extract using disc diffusion method

Bound phenolic compounds in plants can be released through acidic, enzymatic, thermal and alkaline hydrolysis methods. A study done by Su et al., (2014) found that the acid hydrolysis method would be more suitable than the alkaline hydrolysis method for the extraction of bound phenolic compounds. As reported by Stefanovic et al., (2008), the degree of antimicrobial activity that exhibited from the samples depends on the species of microorganism tested, type and concentration of the extract. The selected microorganisms (*E. coli*, *S. aureus, C. albicans* and *S. cerevisiae*) used in this study possessed different ability to resist the antimicrobial agent. Each type of microorganisms has their own characteristics and ability to protect itself which make some of them resistant to certain antimicrobial compounds while others are not.

Minimum Inhibitory Concentration (MIC) Test

Minimum inhibitory concentration (MIC) test was performed if only the bacteria and fungi showed positive result in the disc diffusion test. This was demonstrated by the presence of inhibition zone in the inoculated agar plates. The lower the MIC, the higher the activity of the antibacterial agent against the organisms which means efficiency of the agent increases with the decrease of the MIC. In Figure 4, starting from 37.5 mg/mL to 150 mg/mL of acid extract, the wells have shown clearer appearance. It was indicated that 37.5mg/mL was the minimum inhibitory concentration due to no bacterial growth observed. Sule and Agbabiaka (2008) claimed in their study that the ethanolic extracts exerted inhibitory effect on the test organisms to different extent where *E. coli* MIC was 25 mg/mL when tested on bitter leaf extract. The minimum inhibitory concentration is much lower than what have been obtained in this study. This might be due to the different solvent used during the extraction of the plant. A study done by Mathur et al., (2011) showed that acidic extract of *Achyranthes aspera* leaf also had the effective minimum inhibitory concentration which was 0.3 mg/mL for *C. albicans*.

According to Bala et al., (2005) the MIC method was more reliable as it has the advantage of producing more define evaluation of the level of resistance in less sensitive strains. The acidic extract showed quite encouraging result where the inhibition of growth of *E. coli* and *S. aureus* can be observed starting from the concentration of 37.5 mg/mL until 150 mg/mL while for inhibition of *C. albicans* started from 75 mg/mL until 150 mg/mL. This can be determined by observing the 96-wells microplates for clear appearance and comparing the turbidity with the positive control. It was indicated that acidic extracts at lower concentration could inhibit the bacteria, but high concentration of extracts was required in the inhibition of the fungi.

CONCLUSION

It can be concluded that the present study proves that there is potential of antimicrobial effects in *G. procumbens* leaves extracts. For the future study, it is suggested to analyse the extracts in different type of Gynura species due to its different compounds characteristics.

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