

Research Article

Antibacterial, Antioxidant and Cytotoxicity Properties of Traditionally Used *Melastoma malabathricum* Linn Leaves

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Abstract: The study is aimed to evaluate the antibacterial, antioxidant and cytotoxicity properties of the Methanol extract of *Melastoma malabathricum* (MMML). The antibacterial of the Methanol extract of *M. malabathricum* leaves (MMML) was assessed against Gram-positive and Gram-negative bacteria by disk diffusion method, minimum inhibitory concentration and direct-TLC bioautography. In addition, the antioxidant activity was detected by DPPH radical scavenging activity and cytotoxicity property was determined by MTT assay. The data obtained from disk diffusion method and (MICs) values showed that the MMML possesses antibacterial activity against Gram-positive and Gram-negative bacteria with various values. In addition, direct TLC-bioautography revealed the presence of antibacterial components. The MMML exhibited higher antioxidant activity equal to standard antioxidant ascorbic acid accompanied with low cytotoxicity. Therefore, the MMML has potent antibacterial and antioxidant activities that are strongly associated with its ethno medicinal values.

Keywords: Antibacterial activity, antioxidant activity, cytotoxicity property, *Melastoma malabathricum*

INTRODUCTION

The use of Traditional Medicine (TM) or Complementary and Alternative Medicine (CAM) remains widespread around the world (Wangchuka *et al.*, 2011). Medicinal plants have been sources of both preventive and curative traditional medicine systems for new remedies (Kalayou *et al.*, 2012).

There is a growing interest in the antimicrobials plant origin in the pharmaceutical industry for the control of microbial pathogens. Even though the introduction of antibiotics improved the treatment of bacterial infections, the widespread emergence of antibiotics resistant strains of bacterial pathogens with the huge threat of drug resistance has led to the continuing search for useful natural antimicrobials. Plant extracts and bioactive constituents isolated from ethno medicinal plants are considered prolific resources for novel antibacterial substances with various structures and new mechanisms of action (Rios and Recio, 2005).

In many areas, particularly in the tropics, an abundance of medicinal plants offer people access to safe and effective products for use in prevention and treatment of varying ailments (Das *et al.*, 2010; Katovai *et al.*, 2012). *Melastoma malabathricum* Linn. (Melastomataceae) is one of the most important herbs or shrubs found in Malaysia and known to Malays as

“senduduk”. The plant has been used in traditional Malay medicine for alleviating diarrhoea, leucorrhoea, puerperal infection, dysentery; wound healing, post-partum treatment and haemorrhoids (Sirat *et al.*, 2010; Zakaria *et al.*, 2011a). The *M. malabathricum* has appreciable medicinal properties that have drawn the attention of the researchers in recent times. Many pharmacological studies have been carried out including antiviral (Nazlina *et al.*, 2008), antibacterial (Sunilson *et al.*, 2008; Zulaikah *et al.*, 2008; Choudhury *et al.*, 2011), antioxidant (Sirat *et al.*, 2010) and antinociceptive, anti-inflammatory and antipyretic activities (Zakaria *et al.*, 2011b).

The purposes of our study were to investigate antibacterial activity by disk diffusion method, minimum inhibitory concentration and direct-TLC bioautography, to evaluate antioxidant activity by scavenging capability of DPPH free radical and to assess the possible cytotoxicity property of the Methanol extract of *M. malabathricum* (MMML).

MATERIALS AND METHODS

Plant extract: *M. malabathricum* Linn leaves was purchased from Ethno Herbs Resources Sdn. Bhd (Malaysia) and identified by a botanist with the specimen voucher NI01 deposited in the Herbarium, Faculty of Science and Technology, Universiti

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Kebangsaan Malaysia. In preliminary extraction, 50 g of powdered plant leaves from *M. malabathricum* was treated with *n*-hexane to remove fats, waxes and chlorophylls. This is followed by extraction with Methanol (MeOH) according to Green (2004) using solvent to sample dry weight ratio of 10:1 (v/w). The powdered plant leaves were stirred vigorously in MeOH at ambient temperature for 72 h. After 24 h, the liquid was removed and filtered through Whatman No.1 filter paper. MeOH was again added to the powdered leaves. The method was repeated three times. The filtered extract was dried by rotary evaporator at 40°C to yield the Methanol extract of *M. malabathricum* Leaves (MMML).

Bacterial strains and growth condition:

Gram-positive bacteria: *Staphylococcus epidermidis* ATCC 12228, *S. aureus* ATCC 11632, Methicillin-Resistant *S. aureus* ATCC 43300 (MRSA), 11 clinical MRSA isolates, *Streptococcus pneumoniae* ATCC 10015, *Bacillus thuringiensis* ATCC 10792, *Enterococcus faecalis* ATCC 14506 and Gram-negative bacteria: *Pseudomonas aeruginosa* ATCC 10145 and 3 clinical *P. aeruginosa* isolates, *E. coli* ATCC 10536, *Shigella sonnei* ATCC 2993, *Proteus mirabilis* ATCC 12453, *P. vulgaris* ATCC 33420, *Vibrio parahaemolyticus* ATCC 12802, *Serratia marcescens* ATCC 13880, *Enterobacter aerogenes* ATCC 13048 and 3 clinical *Acinetobacter* isolates were kindly provided by the Microbiology Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The bacteria cultures were maintained in Brain Heart Infusion broth (BHIB; Oxoid, UK). Bacteria were cultured at 37°C for 24 h and then sub-cultured on BHI agar (Oxoid, UK) at 37°C for 24 h. For each experiment, bacteria were re-suspended in 0.85% saline to obtain the required densities equivalent to the McFarland 0.5 turbidity standard.

Disk diffusion method: Antibacterial activity of the MMML was determined by disk diffusion method according to CLSI (2006). Briefly, 100 mg/mL of the MMML was dissolved in 5% dimethylsulfoxide (DMSO, Merck, Germany). The surface of the Mueller-Hinton agar (MHA; Oxoid, UK) was inoculated using a 100 µL of 0.5 McFarland (10^8 colony forming unit CFU/mL) standardized inoculum suspension of bacteria and allowed to dry. Sterile filter paper disks (6 mm) were impregnated with 10 µL of each concentration, air dried, placed onto MHA and incubated at 37°C for 24 h. A negative control was prepared using disks loaded with 5% DMSO whereas vancomycin (5 µg/disk), ciprofloxacin (5 µg/disk) and tetracycline (30 µg/disk) were used as a reference antibiotic. The tests were performed in triplicate and the antibacterial activity was expressed as the mean of the inhibition zones diameter in millimeters (mm).

Minimum Inhibitory Concentration (MIC)

determination: Minimum Inhibitory Concentrations (MICs) were measured according to NCCLS (2000). Two-fold serial dilutions of 96-well containing 100 µL of Mueller-Hinton broth (MHB; Oxoid, UK) with various concentrations of the MMML were prepared. Each round bottom 96-well received 100 µL of bacterial suspension adjusted to 0.5 McFarland standards to get a final volume 200 µL/well. The negative control wells received only 200 µL of MHB. All tests were performed in triplicate. The MIC was recorded as the lowest concentration that produced a complete suppression of visible growth after 24 h. incubation at 37°C.

Minimum Bactericidal Concentration (MBC)

determination: To determine Minimum Bactericidal Concentrations (MBCs), an aliquot of 5 µL was withdrawn from wells with no bacterial growth and plated onto Nutrient agar (NA; Oxoid, UK) then incubated overnight at 37°C. The lowest concentration which showed no growth on the agar was defined as the MBC.

Direct TLC-bioautography: The TLC plates (6×6 cm) was spotted with 5 µL (100 mg/mL) of reconstituted MMML and then developed with mobile eluting solvent systems (analytical grade) Ethyl acetate: Methanol (9.5:0.5). Direct TLC-bioautography was carried out to detect bioactive constituents by modified method of Sgariglia *et al.* (2011). The plates were observed under Ultra Violet (UV) light at wavelengths of 254 and 365 nm (Camac Universal lamp TL-600). The eluted TLC plate 1 that served as a control was sprayed with cerium sulphate and heated for 2 min at 100°C to allow development of color whereas plate 2 was left to dry in the fume hood overnight to remove the eluent solvent system. The plate was thereafter sterilized for 20-30 min under UV light. Fully dried TLC plates were dipped for 5 min in the combination of MHB-MH-agar (90:10) adjusted to 0.5 McFarland standards of bacterial pathogens (Okusa *et al.*, 2010). In this study, two Gram positive-bacteria including *S. aureus* (ATCC 11632), MRSA (ATCC 43300) were used. The loaded plates were placed in a humid chamber and incubated at 37°C overnight. Plates were then stained with 5 mg/mL solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, France) and further re-incubated at 37°C for 3 h. Inhibitory zones depicted as clear areas against blue background where reduction of MTT to formazan did not occur indicated bacterial growth inhibition.

Antioxidant activity: Quantitative analysis of antioxidant activity was performed on the MMML using DPPH assay according to Miliauskas *et al.* (2004). The free radical capacities were established spectrophotometrically against 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH, Sigma, France). Antioxidant substances can donate hydrogen that reacts

with DPPH. The changes in color (from deep violet to light yellow) were measured at 515 nm on a UV/visible light spectrophotometer (Spectronic Genesys 8, Rochester, USA). An MMML solution was prepared by dissolving 5 mg/mL of dry extract in 1 mL of methanol. The DPPH (6×10^{-5} M) was prepared in methanol prior to use in a tightly sealed dark glass containers. Three ml of DPPH were mixed with 77 μ L MMML solution. The samples were kept in the dark for 15 min at room temperature and then the decrease in absorption was measured. Absorption of blank sample containing the same amount of methanol and DPPH solution was prepared and measured daily. Standard antioxidant, ascorbic acid (Sigma, France) was prepared by dissolving 5 mg/mL in methanol. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula:

$$\% \text{ Inhibition} = \left[\frac{(AB-AA)}{AB} \right] \times 100$$

where,

AB = Absorption of blank sample (t = 0 min)

AA = Absorption of tested extract solution (t = 15 min)

Determination of the cytotoxic activity of MMML:

Cytotoxicity assay was performed according to Raheel

et al. (2013) against Vero cells (African green monkey kidney cells). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% Foetal Bovine Serum (FBS). Cultures for the assay were prepared from confluent monolayer cells, seeded at a density of 2×10^4 cells/well in 96-well microplates flat bottom and incubated at 37°C overnight in a 5% CO₂ to allow attachment of the cells. The growth media on confluent Vero cells grown overnight was removed and replaced with 100 μ L (100 mg/mL) of MMML. It was followed by reconstitution in 5% Dimethylsulfoxide (DMSO) and preparation in growth media at various concentrations. After incubation at 37°C in a 5% CO₂ for 48 h, the viability of cells was determined by MTT assay based on the reductive cleavage of the 3- (4, 5-Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (yellow tetrazole) by mitochondrial dehydrogenase enzyme present in living cells to yield purple formazan crystals. The media was removed, 100 μ L of DMEM and 20 μ L of 5 mg/mL MTT dissolved in Phosphate Buffer Solution (PBS) were added to each well. The plates were re-incubated for 4 h under the same conditions. After incubation, MTT was removed and 100 μ L of DMSO was added to each well. Subsequently, the plates were gently rocked to dissolve the formazan crystals. Berberine chloride

Table 1: Antibacterial activity of the Methanol extract of *M. malabathricum* Linn leaves (MMML) against tested bacteria species, determined by disk diffusion method and Minimum Inhibitory Concentration (MIC)

Test bacteria	Plant extract MMML		Susceptibility of bacteria			
	Zones of inhibition (mm)		Antibiotics			
Gram-positive bacteria	500 μ g/disk	1000 μ g/disk	Vancomycin 5 μ g	MIC mg/mL	MBC mg/mL	SI = CC ₅₀ /MIC
<i>Staphylococcus epidermidis</i> ATCC 12228	10±0	15±0.58	19±0	1.56±0	3.13±0	2.4
<i>Staphylococcus aureus</i> ATCC 11632	16.67±0.58	19±0	17±0	1.56±0	3.13±0	2.4
MRSA ATCC 43300	16.67±0.58	18.67±0.58	16±0	1.56±0	3.13±0	2.4
Clinical MRSA isolates						
M01	17±0	20±0	17±0.58	0.78±0	1.56±0	4.81
M02	17.33±0.58	19.33±0.58	16±0	0.78±0	1.56±0	4.81
M03	17.33±0.58	19±0	17±0	0.78±0	1.56±0	4.81
M04	16.33±0.58	19.67±0.58	17±0.58	0.78±0	1.56±0	4.81
M05	17±0	20.33±0.58	17±0.58	0.78±0	1.56±0	4.81
M06	17.33±0.58	20.33±0.58	17±0	1.56±0	3.13±0	2.40
M07	16.33±0.58	18.33±0.58	15±0	0.78±0	1.56±0	4.81
M08	16.33±0.58	19±0	17±0.58	0.78±0	1.56±0	4.81
M09	17±0	19±0	17±0.58	1.56±0	3.13±0	2.40
M10	16.67±0.58	19.33±0.58	17±0	0.78±0	1.56±0	4.81
M11	16±0.58	18±0	16±0	3.13±0	6.25±0	1.20
<i>Streptococcus pneumonia</i> ATCC 10015	12±0.58	15±0	17±0.58	3.13±0	6.25±0	1.20
<i>Bacillus thuringiensis</i> ATCC 10792	15±0.58	19±0	22±0	0.78±0	0.78±0	4.81
<i>Enterococcus faecalis</i> ATCC 14506	10±0	15±0.58	25±0	1.56±0	3.13	2.40
Gram-negative bacteria			Ciprofloxacin 5 μ g			
<i>Pseudomonas aeruginosa</i> ATCC 10145	14.33±0.58	19±0	15±0.58	1.56±0	3.13	2.40
Clinical <i>P. aeruginosa</i> isolates						
P01	16.33±0.58	20±0	14±0	0.78±0	1.56±0	4.81
P02	14±0	16.33±0.58	16±0.58	3.13±0	6.25±0	1.20
P03	13.33±0.58	15.33±0.58	15±0	1.56±0	3.13±0	2.40
Clinical <i>Acinetobacter</i> isolates			Tetracyclin 30 μ g			
A01	16±0	19±0.58	18±0	1.56±0	3.13±0	2.40
A02	17±0.58	19±0	18±0	1.56±0	3.13±0	2.40
A03	16±0.58	20±0.58	18±0.58	0.78±0	1.56±0	4.80
<i>E. coli</i> ATCC 10536	8±0	12±0.58	22±21	3.13±0	6.25±0	1.20
<i>Shigella sonnei</i> ATCC 2993	11±0	13±0	-	0.78±0	0.78±0	4.81
<i>Proteus mirabilis</i> ATCC 12453	16±0	18±0.58	12±0	0.78±0	0.78±0	4.81
<i>P. vulgaris</i> ATCC 33420	11±0	14±0	12±0	0.78±0	0.78±0	4.81
<i>Vibrio parahaemolyticus</i> ATCC 12802	15±0.58	19±0	-	0.78±0	0.78±0	4.81
<i>Serratia marcescens</i> ATCC 13880	7±0	13±0.58	18±0	12.5±0	2.5±0	0.30
<i>Enterobacter aerogenes</i> ATCC 13048	8±0	12±0.58	22±0.58	3.13±0	6.25±0	1.20

SI: Selectivity index = CC₅₀/MIC; The values are the means of replicates±standard deviation

(Sigma, France) was used as positive control. The Optical Density (OD) of each well was measured at wavelength of 540 nm by an ELISA reader (CDS, India). Cytotoxicity was expressed as 50% Cytotoxic Concentration (CC₅₀) of constituents that inhibit the growth of cells by 50% when compared to untreated cells. The percentage of cell viability was measured as follows:

$$\text{Viability} = \frac{(\text{Treated cells} - \text{Blank})}{(\text{Untreated cells} - \text{Blank})} \times 100$$

For the purpose of measuring Selectivity Index (SI), selective index of MMML was calculated as follows:

$$\text{Selectivity index (SI)} = \frac{\text{CC}_{50}}{\text{MIC}}$$

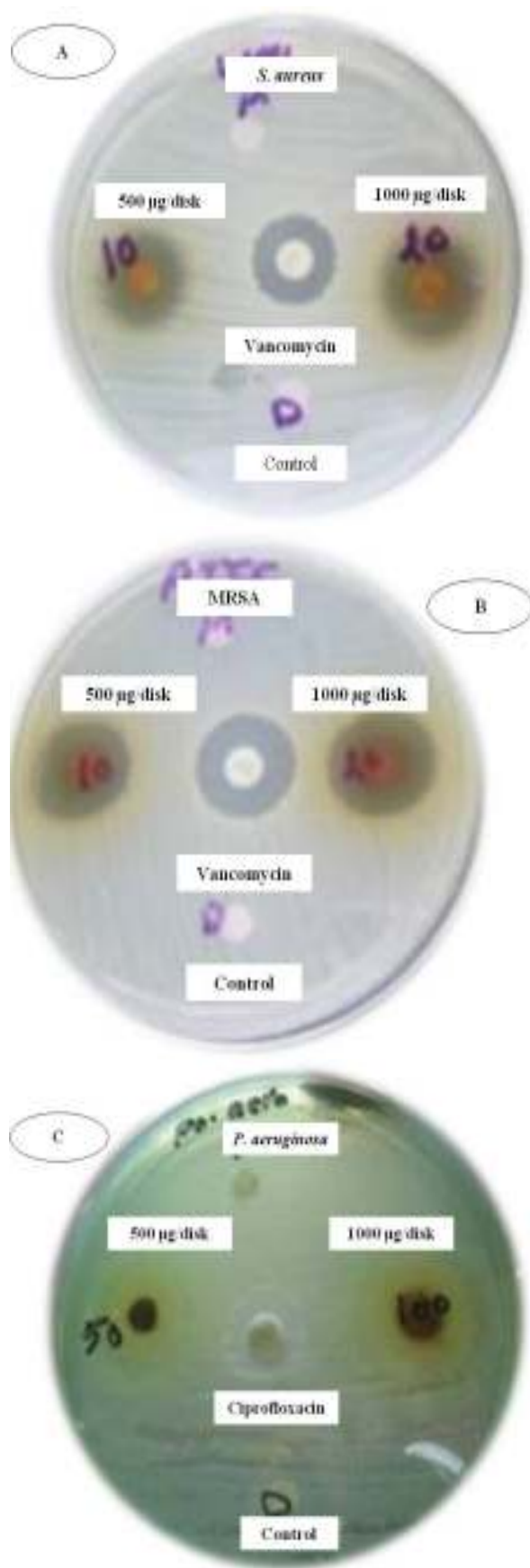
RESULTS AND DISCUSSION

Evaluation of antibacterial activity: The zones of inhibition, Minimum Inhibitory Concentration (MIC) values and Minimum Bactericidal Concentration (MBC) values of the MMML are shown in the Table 1. In general, the MMML showed antibacterial activity against all tested Gram-positive and Gram-negative bacteria with large zones of inhibition diameter for *S. aureus*, MRSA, *P. aeruginosa* and *V. parahaemolyticus* at concentration 1000 µg/disk (Fig. 1).

In order to obtain more quantitative and precise results for the antibacterial activity, MIC and MBC values were evaluated. The MMML exhibited antibacterial activity with MIC values between 0.78±0 to 25±0 mg/mL against all tested pathogenic bacteria (Table 1). Differences in antibacterial activity were observed between various pathogenic bacteria. Some of the clinical isolates of MRSA were observed to be more sensitive to MMML with MIC less than 1 mg/mL. Compounds from plants are usually classified as “antimicrobial” on the basis of susceptibility tests that produce minimum inhibitory concentrations in the range of 100 to 1000 µg/mL that are still higher than those of typical bacterial and fungal antibiotics which is between 0.01 to 10 µg/mL (Tegos *et al.*, 2012).

Selectivity Index (SI) calculated using the CC₅₀ and MIC values indicated that the highest selectivity index for MMML against the most clinical MRSA isolates, *Bacillus thuringiensis*, clinical *Pseudomonas aeruginosa* and *Acinetobacter* isolates, *Shigella sonnei*, *Proteus mirabilis*, *P. vulgaris* and *Vibrio parahaemolyticus* with SI equal to 4.8 while the lowest selectivity index for MMML was against *Serratia marcescens* with SI value 0.3.

It is worth to note, most medicinal plants produce many substances that are biologically active and working together catalytically and synergistically to increase the activity (Patwardhan, 2005). Furthermore, it has been well proven that the methanolic extract of *M. malabathricum* possesses antimicrobial activity



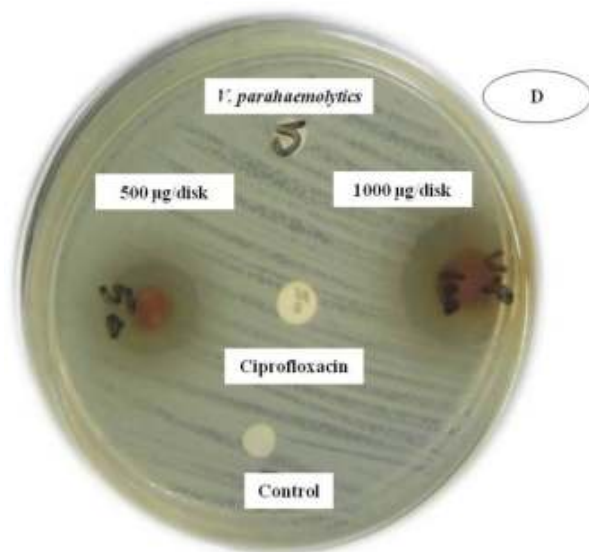


Fig. 1: (A) zones of inhibition of *Staphylococcus aureus*, (B) zones of inhibition of methicillin resistant *S. aureus*, (C) zones of inhibition of *Pseudomonas aeruginosa*, (D) zones of inhibition of *Vibrio parahaemolyticus* caused by the Methanol extract of *M. malabathricum* Linn leaves (MMML)

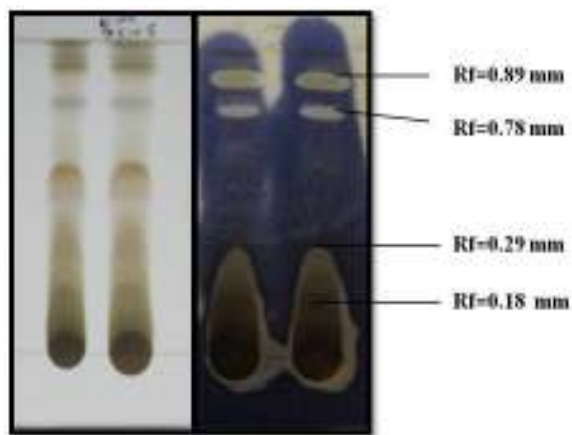


Fig. 2: Thin layer bioautographs of the Methanol extract of *M. malabathricum* Leaves (MMML) developed with ethyl acetate: methanol (9.5:0.5). In set, the right chromatogram was loaded with the bacteria MRSA and the one to the left without

and this activity was ascribed to flavonoids (Zakaria *et al.*, 2011a).

Direct TLC-bioautography: Direct TLC-bioautography was used to screen for the presence of varied bioactive components in MMML. Though, direct TLC-bioautography is not a quantitative method to determine antimicrobial activity, it is still a very useful method in indicating and isolating compounds with antimicrobial activity (Suleiman *et al.*, 2010). The

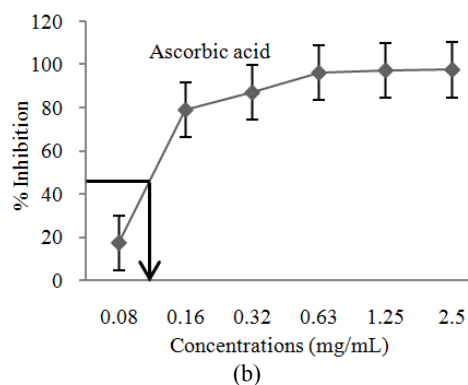
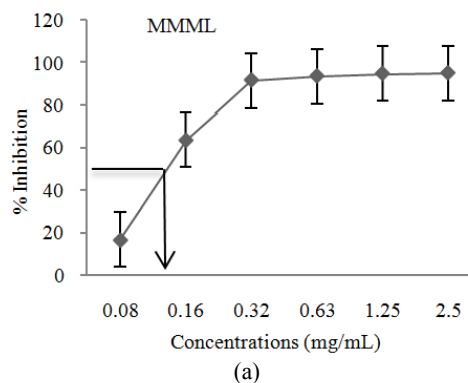


Fig. 3: Percentage inhibition of DPPH versus concentration of ascorbic acid (after 15 min)
 EC_{50} of MMML = 0.14 mg/mL; EC_{50} of ascorbic acid = 0.12 mg/mL

presence of clear areas against blue background on the TLC plates where reduction to formazan didn't occur after using MTT reflects inhibition of bacterial growth. The Retention factor (R_f) that represents the ratio of the distance moved by the compound from its origin to the movement of the solvent from the origin was measured. Figure 2 revealed the presence of constituents active against *S. aureus* and MRSA with R_f values 0.89, 0.78, 0.29 and 0.18 mm, respectively.

Antioxidant activity of *M. malabathricum* Linn leaves extract:

The majority of medicinal plants have been reported to have antioxidant activity (Aqil *et al.*, 2006). The DPPH assay was performed to quantify the antioxidant activity of the methanol extract of *M. malabathricum* Leaves (MMML) as shown in Fig. 3a and b. The antioxidant activity was determined by plotting percentage inhibition of the DPPH radical as a function of the MMML and ascorbic acid in mg/mL. Error bars (calculated from three repetitions) are present on graphs. With results shown in Fig. 3a and b, it should be taken into account that MMML had antioxidant activity equivalent to ascorbic acid. In Zakaria *et al.* (2011b), it was stated that the methanol extract of *M. malabathricum* leaves produced high antioxidant activity.

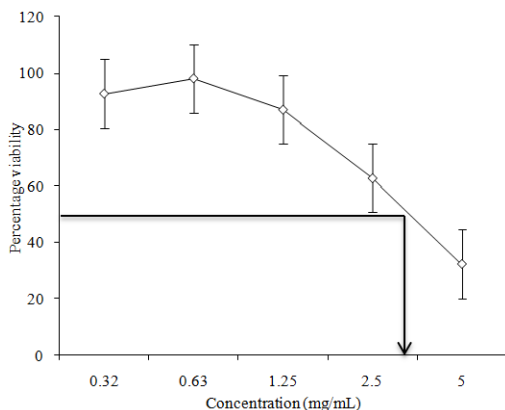


Fig. 4: Cytotoxicity assay of MMML showed percentage viability of vero cells using (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) MTT reagent. $CC_{50} = 3.75$ mg/mL

Cytotoxicity assay of MMML: *In vitro* cytotoxicity assay is important tool in assessment of various compounds and extracts not only because it reduces evaluation time but also it is less expensive. The cytotoxicity of the Methanol extract of *M. malabathricum* Leaves (MMML) was determined using monkey kidney of the Vero cells. The CC_{50} value of MMML was 3.75 mg/mL compared to Berberine chloride standard with CC_{50} value of 3.3 μ g/mL. Percent Vero cells viability based on MTT assay following exposure to the MMML is presented in Fig. 4. At the highest concentration 5 mg/mL, MMML exhibited deleterious effects on the viability of Vero cells. From this study, it can be concluded that MMML is not cytotoxic to Vero cells with CC_{50} equal to 3.75 mg/mL.

CONCLUSION

The MMML represents a source of multi-functional properties with potent inhibitory effects against Gram-positive and Gram-negative bacteria and high capacity to scavenge free radicals accompanied with low cytotoxicity. Therefore, the results showed that MMML is safe with potential antibacterial and antioxidant activity.

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