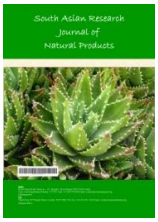

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Investigation of β-Lactamase Enzyme Inhibitory, Anti-Bacterial and Anti-Fungal Bio-Activities of *Dillenia retusa* Thumb: An Endemic Medicinal Plant to Sri Lanka

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Authors' contributions

This work was carried out in collaboration among all authors. Authors JKRRS, SKH, OVDSJW and MIC designed the study. Author HDSMP managed the literature searches, managed the analyses of the study, wrote the protocol and wrote the first draft of the manuscript. Author RM performed the statistical analysis of the study. All authors read and approved the final manuscript.

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ABSTRACT

Dillenia retusa (Dilleniacea) is a medicinal plant endemic to Sri Lanka with an immense bioactive potential. To date, no studies are reported on anti-microbial potential of this plant. The present study investigates anti-bacterial, β-lactamase enzyme inhibitory and anti-fungal bio-activities of the ethanol extracts of *D. retusa.*

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The leaves (DRL), fruits (DRF) and bark (DRB) of *D. retusa* were extracted with ethanol using cold extraction technique. Anti-bacterial properties of the extracts were investigated against five sensitive bacterial strains namely, *Shigella flexneri* (ATCC®12022), *Pseudomonas aeruginosa* (ATCC® 10145), *Escherichia coli* (ATCC® 25922), *Salmonella typhi* (ATCC® 14028), *Staphylococcus aureus* (ATCC® 6538) and four resistant bacterial strains namely, *Klebsiella pneumoniae* (MDR ATCC® 700603), *Escherichia coli* (MDR ATCC® 35218), *Salmonella enterica* (MDR ATCC® 700408) and *Staphylococcus aureus* (ATCC® MDR MRSA 252) using alamar blue micro-plate based assay. Anti-β-lactamase activity of the extracts were determined using β-lactamase enzyme inhibitory assay. Anti-fungal activity of the extracts was determined against five sensitive fungal strains namely, *Candida albicans* (ATCC® 14053), *Candida glabrata* (ATCC® 2001), *Aspergillus niger* (ATCC® 1015), *Microsporum canis* (ATCC® 10214) and *Trichophyton rubrum* (ATCC ®MYA-4438)) using agar-tube dilution method. Further, polyphenols and flavonoid contents of the extracts were determined. The bio-activities were statistically analyzed at $p<0.05$.

The extracts showed anti-bacterial activity against sensitive bacterial strains within the MIC range of 1.8-2.8 mg/mL and MBC range of 3-4.8 mg/mL and were found to be bactericidal. The extract of DRL showed the highest anti-β-lactamase activity (IC_{50}: 226.25 ± 10.53 µg/mL) in a dose-dependent manner. The antifungal activity of the extracts was detected within the range of 1.6-3.2 mg/mL and the highest anti-fungal activity was observed for DRB against *C. albicans* which may be attributed to the high polyphenol content of DRB.

The extracts were identified as novel potential sources of antimicrobial compounds which may impart even higher antimicrobial activities upon bio-activity guided fractionation and purification of extracts.

Keywords: β-lactamase enzyme; anti-bacterial; anti-fungal; Dillenia retusa.

1. INTRODUCTION

The use of medicinal plants and products in the management and treatment of acute and chronic infectious diseases has progressively increased in recent past [1]. Infectious diseases have become one of the prevalent health issue worldwide with the emergence of pathogenic bacteria and fungi. Multi drug resistant bacteria and fungi has caused high morbidity and mortality rates of the populations as a consequence of drug ineffectiveness and treatment failure [2]. Multi drug resistance has been now identified as a key challenge faced by the health care sector of Sri Lanka for the past few years. The abrupt emergence of gram negative and gram positive bacteria and extended spectrum of ß-lactamase producing bacteria continue to pose a challenge in Sri Lanka demarcating the importance of identifying new anti-microbial agents from potential sources such as medicinal plants. Promising anti-microbial properties of medicinal plants are well documented [3,4] and researchers still continue to explore medicinal plants to identify new chemical entities as promising anti-microbial therapeutic agents.

Dillenia retusa (Fig. 1) is a medicinal plants endemic to Sri Lanka. It has long been effectively used in the traditional system of medicine for treating a number of diseases in Sri Lanka [5]. Limited research has been carried out on this endemic plants and many more important bio-activities are yet to be investigated.

Dillenia retusa is one of the 100 known species of the genera Dillenia belonging to the family of Dilleniaceae. It is a deciduous tree mainly distributed in moist low country in Sri Lanka. The fruit of *D. retusa* has been traditionally used in hair care, the management of pain and in the treatment of fractures and dislocations [5]. The other species of this genus have been extensively used in the treatment of cancers, diabetes mellitus, diabetes, diarrhea, wounds and jaundice [6].

The medicinal plants belonging to the genus Dillenia are reported to possess number of pharmacological properties including anti-microbial properties against number of pathogenic bacterial and fungal strains [7]. Up to date, no any other study has been conducted to investigate anti-microbial properties of *D. retusa*, which is an important area of interest and needs to be exploited. Therefore, present study was conducted to investigate anti-microbial properties of the extracts of D. retusa in terms of β-lactamase enzyme inhibitory activity, anti-bacterial and anti-fungal activity.

Fig. 1. *Dillenia retusa* thumb

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Mullar Hinter Broth (MHB) and Sabouraud dextrose agar were purchased from Oxoid, UK. Ferric chloride, acetic anhydride, iodine in potassium iodide, sodium hydroxide, sodium hydroxide, copper sulphate, sulphuric acid, ethanol, dichloromethane, gelatin, dimethyl sulfoxide (DMSO), alamar blue, orfloxaxin, ampicillin, , micanazole, ampotericin B, β-lactamase, nitrocefin and clavulanic acid were purchased from sigma Aldrich, USA. All chemicals and reagents used in the experiment were of analytical grade.

2.2 Collection and Preparation of Plant Extracts

Leaves, bark and fruits of *D. retusa* were collected fresh from Gampaha District in June 2013, Sri Lanka. The voucher specimens of the collected plants were identified by a Senior Taxonomist and deposited at Herbal Technology Section, Industrial Technology Institute, Sri Lanka and The National Herbarium, Department of National Botanic Garden, Peradeniya, Sri Lanka (Voucher specimen no. DRL_001). Plant materials were air-dried under shaded and well-ventilated conditions (Relative humidity: 65% - 75%), at room temperature (25 ± 2 °C) for 72 h and ground to make coarse powder using a mechanical grinder [8]. Powdered plant materials (100 g) were soaked in ethanol (500 mL) overnight and stirred for 1 h using a mechanical stirrer at room temperature (25 ± 2 °C) followed by suction filtration through a celite bed, packed in a sintered funnel. The extraction was repeated for three times and combined filtrates were concentrated under reduced pressure at 40 °C using a rotary evaporator to obtain ethanol leaf extract (DRL), ethanol bark extract (DRB) and ethanol fruit extract (DRF) of D. retusa [8]. The solvent free extracts were stored in air-tight glass containers at -20 °C until used [9].

2.3 Phytochemical Screening of Extracts of Dillenia Retusa

The presence of alkaloids (Wagner's test and Dragendorff's test), flavonoids (Ferric chloride test), amino acid/ proteins (Biuret test), tannins (Gelatin test and ferric chloride test), sterols and triterpenes (Libermann-Buchard test) were qualitatively tested using respective standard screening methods [10]. Any change in colour or the precipitate formation was used as indicative of positive response to these chemical tests.

2.3.1 Determination of total polyphenol content of extracts of *Dillenia retusa*

The total polyphenolic content (TPC) of plant extracts was determined by a modified Folin-Ciocalteu method [11]. Plant extracts were tested within the assay concentration range of 50-500 µg/mL. Plant extracts were initially dissolved in DMSO and diluted in distilled water (20 µL) and added to ten times diluted folin-ciocalteu reagent (110 µL) followed by incubation with sodium carbonate solution (10% w/v, 70 µL) for 30 min at room temperature (25 ± 2°C). Absorbance was recorded at λ=765 nm. Results were expressed in terms of mg gallic acid equivalents (GAE)/g of extract using the calibration curve of Gallic acid, determined by linear regression (y=0.053x + 0.105, r2= 0.993).

2.3.2 Determination of total flavonoid content of extracts of *Dillenia retusa*

The total flavonoid content (TFC) of plant extracts was determined by the aluminium chloride method [12]. Plant extracts were tested within the assay concentration range of 50-500 µg/mL. Plant extracts, initially dissolved in DMSO and diluted in distilled water (100 µL) were incubated with AlCl3-methanol solution (2%, 100 µL) for 10 min Absorbance was recorded at λ=415 nm. Results were expressed in terms of mg quercetin equivalents (QE)/g of extract using the calibration curve of quercetin, determined by linear regression (y=0.033x–0.002, r^2= 0.999).

2.4 Anti-Bacterial Activity of Extracts of *Dillenia retusa*

2.4.1 Bacterial strains and culture conditions

The extracts were tested for anti-bacterial activity against five sensitive bacterial strains namely, Shigella flexneri (ATCC® 12022), Pseudomonas aeruginosa (ATCC® 10145), Escherichia coli (ATCC® 25922), Salmonella typhi (ATCC® 14028), Staphylococcus aureus (ATCC® 6538) and four resistant strains namely Klebsiella pneumoniae (MDR ATCC® 700603), Escherichia coli (MDR ATCC® 35218), Salmonella enterica (MDR ATCC® 700408) and Staphylococcus aureus (ATCC® MDR MRSA 252). The bacterial inocula were prepared by transferring isolated colonies from culture plates of respective bacterial strains in to MHB (4 mL), followed by 24 h incubation at 37 °C . The turbidity of inoculum was adjusted using 0.5 McFarland turbidity index (5×10^5 CFU/mL).

2.4.2 Micro-plate based alamar-blue assay

The assay was conducted according to the method described by Osaka et al. (2013) with some modifications [13]. Anti-bacterial activity of the plants extracts was tested in the assay concentration range of 0.15-2.4 mg/mL. The plant extracts, dissolved in DMSO (10 μL) were incubated with bacterial cells (5×10^5 CFU/mL, 7 μL) and final volume of the well was adjusted to 200 μL with MHB. The controls received abiotic MHB (193 μL) with plant extracts dissolved in DMSO (7 μL) at variable assay concentrations, abiotic MHB (200 μL) and MHB (183 μL) with bacterial cells (5×10^5 CFU/mL, 7 μL) and DMSO (10 μL).

After aseptic inoculation of micro-plates with bacterial cells and extracts, the plates were sealed using para films and incubated at 37 °C for 18-20 h. After the 20 h incubation, alamar blue (AB) solution (20 μL) was added to each well under minimum illumination. Plates were covered with Aluminium foil and re-incubated at 37°C with shaking at 80 rpm for 3 h and absorbance was measured at the wave lengths of 570 nm and 600 nm. Orfloxacin and Amphicillin were used as reference standards.

Reduction (%) of AB by extracts was calculated using following equation in comparison to the positive control and bacterial growth inhibition was calculated by subtracting % reduction of AB from 100.

$$[((\varepsilon_{ox})\lambda_2 \times A\lambda_1 - (\varepsilon_{ox})\lambda_1 \times A\lambda_2) / ((\varepsilon_{red})\lambda_1 \times A'\lambda_2 - (\varepsilon_{red})\lambda_2 \times A'\lambda_1)] \times 100$$

ε_{ox} = Molar extinction coefficient of the alamar blue oxidized form (blue)
A = Absorbance of the test samples
A'= Absorbance of the control
$\lambda 1$ = 570 nm
λ_2 = 600 nm

The AB Minimum inhibitory concentration (MIC) was determined by considering the lowest assay concentration of plant extracts giving rise to < 50 % reduction of AB, indicating purple/blue colour after 60 min upon addition of AB.

For the determination of Minimum bactericidal concentration (MBC), 50 μL aliquots of the micro-plate cultures of which did not show any colour change (Purple to pink) in MIC determinations were transferred to a fresh micro-plate and incubated with 150 μL aliquots of fresh MHB at 37 °C for 48 h was determined by selecting the lowest concentration of plant extracts at which <10 % reduction of AB was detected.

2.5 β-Lactamase Enzyme Inhibitory Assay

The assay was performed according to the method of Thammaiah et al. (2005) with modifications [14]. The beta-lactamase enzyme inhibitory activity of extracts was studied within the assay concentration range of 100-500 μg/mL. Plant extracts, initially dissolved in DMSO and diluted in Phosphate buffer (100 mM, pH 7.4, 55 μL) were incubated with β-lactamase enzyme solution (2.6 U/mL, 40 μL) in 96-well plates at room temperature for 20 min. The reaction was initiated by the addition of substrate solution of nitrocefin (2 mM, 5 μL). The change in absorbance was monitored for 15 min at the wavelength of 482 nm. Clavulanic acid was used as the reference standard.

2.6 Anti-Fungal Activity of Extracts by Agar Tube Dilution Assay

Anti-fungal activity of plant extracts was determined using modified agar tube dilution method [15] against five human pathogenic fungal strains, namely, Candida albicans (ATCC® 14053), Candida glabrata (ATCC® 2001), Aspergillus niger (ATCC® 1015), Microsporum canis (ATCC® 10214) and Trichophyton rubrum

(ATCC® MYA-4438). Anti-fungal activity of the plant extracts was tested in the assay concentration range of 0.4 -2.4 mg/mL. Briefly, plant extracts, dissolved in DMSO (67 µL) were introduced to test tubes containing non-solidified medium Sabouraud dextrose agar (50°C), mixed well by gentle shaking and allowed to solidify in a slant position. After proper solidification, test tubes were inoculated with a piece of inoculum (Diameter: 4 mm) of cultures of test fungi (A. niger, M. canis and T. rubrum, C. albicans and C. glabrata) and incubated at 27 ^{o}C for 7 days. DMSO (67 µL) was used as the negative control and miconazole and amphotericin-B were used as the positive controls. The inoculated tubes were carefully examined for the growth of fungi during incubation. Growth inhibition (%) of fungi was determined measuring the linear length of fungal growth on the agar slant, starting from the place of inoculation at the bottom (mm) and compared with the controls.

2.7 Statistical Analysis

All analysis was carried out in triplicate and experimental results were expressed as mean ± standard error (SE), analyzed with one-way ANOVA. IC_{50} values were calculated using linear regression analysis. Pearson's correlation coefficient was used for the correlation analysis ($p<0.05$) (IBM SPSS Statistics 22.0).

3. RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Screening

The phytochemical screening tests revealed the presence of flavonoids, tannins and terpenoids in all three extracts. Alkaloids, sterols and amino acids were not detected in the extracts.

3.2 Total Polyphenol and Flavonoid Contents

Significant contents of polyphenols and flavonoids were detected in all three extracts. The bark extract showed the highest polyphenol content followed by leaf extract and fruit extract. Highest flavonoid content was detected in fruit extract followed by leaf extract and bark extract. The polyphenol and flavonoid content of all three extracts were significantly different from each other (Table 1).

3.3 Anti-Bacterial Activity by Micro-Plate Based Alamar-Blue Assay

Selection of a correct assay method is of high importance to produce data with greater accuracy and efficiency. Micro-plate based, colorimetric alamar blue assay was performed to investigate the anti-bacterial properties of the extracts. This method is proven to generate accurate, reproducible MIC's devoid of related dilution inaccuracies [16,17] to produce results (MIC/MBC) that could be compared with existing anti-biotics and within a group of bacterial strains [18]. The bacterial strains which showed >50% growth inhibitions were further studied for MIC and MBC. The extracts of D. retusa showed high growth inhibitions (>50 %) against S. flexneri (DRB), P. aerugenosa (DRL, DRF) and S. aureus (DRL). For above extracts, MIC's ranged from 1.8 to 2.8 mg/mL and MBC's ranged from 3.0 to 4.8 mg/mL (Table 2). Extracts showed a low inhibition against E. coli and showed no inhibition against S. typhi and other tested resistant bacterial strains at the tested assay concentrations (Table 3). Minimum inhibitory concentrations of Ampicillin were found to be 6.25, 6.25, 0.78, 100 and 0.19 µg/mL against sensitive strains of S. aureus, E. coli, S. typhi, P. aerugenosa and B. subtilis respectively and that of Orfloxacin was found as 100, 100, 0.19, 0.78 and 0.19 µg/mL respectively. The MIC of extracts were found to be significantly lower than that of standard drugs ($p<0.05$).

In general, gram negative bacteria become more readily resistant for inhibition over gram positive bacteria, due to the presence of the cell envelop with outer and inner membrane barrier, which may decline the passive penetration of hydrophilic molecules to reduces the interactions

Table 1. Total polyphenol and flavonoid contents of extracts of Dillenia retusa

Extract	Total polyphenol content (mg GAE/g)	Total Flavonoid content (mg QE/g)
Dillenia retusa (L)	394.15 ± 1.31^{a}	16.77 ± 0.38 ^{a}
Dillenia retusa (B)	827.92 ± 1.01^{b}	4.34 ± 0.05 ^{b}
Dillenia retusa (F)	37.03 ± 0.88 ^{c}	20.51±1.17 ^{c}

Data represented as mean ± SE (n=3). Mean within each column followed by the same letter are not significantly different at p<0.05; L: leaves, B: bark, F: fruit

with the cell targets [19,20]. Moreover, several mechanisms are proposed for bacterial resistance including prevention of reaching to target of action via reduced membrane permeability and increased efflux of inhibitors, modification and alterations of inhibitor targets, inactivation of inhibitors by enzymatic hydrolysis [21], that may have been caused for the observed low or no growth inhibition against the tested resistant bacterial strains at the tested concentrations of extracts (150 µg/mL, 600 µg/mL and 2400 µg/mL).

Interestingly, in the present study comparatively high inhibitions (> 50%) have been observed against two gram negative, bio-film forming bacterial strains, P. aeruginosa and S. flexnery indicating the efficacy of the extracts on gram negative bacterial growth inhibition.

To date, growth inhibitions against resistant bacterial strains are not reported for extract of any Dillenia species. Number of other species of the genus Dillenia have showed growth inhibitions against sensitive bacterial strains and most of them are found in agreement with the data of the present study. Previous literature data highlighted anti-bacterial properties of methanol leaf extract of D. indica (MIC: 0.31 – 20 mg/mL), against a range of bacterial strains including P. aerugenosa, S. aureus, E. coli and S. typhi [22]. In addition, several other species of genus Dillenia including Dillenia papuana, Dillenia pentagyna, Dillenia suffruticosa and Dillenia sumatrana have showed anti-bacterial properties against various bacterial strains including E. coli, B. subtilis, S. aureus, S. flexneri type-1 and P. aeruginosa [23,24,25,26].

The MIC index (MIC/MBC) was calculated to investigate the bacteriostatic (MIC/MBC >4) or bactericidal (MIC/MBC <4) nature of the extracts which showed high activities. The MIC index for the all tested extracts were found to be less than 4, implying the bactericidal nature of the tested extracts (Table 4). The Bactericidal nature of the extracts could be explained with the means of four proposed mechanisms namely, inhibition of cell wall synthesis, inhibition of bacterial protein and nucleic acid synthesis, disruption of microbial membrane structure and function and blockage of cellular metabolism via inhibition of key enzymes [27,28]. Further studies are needed to determine the exact bactericidal mechanism of each bacterial strain, which showed growth inhibitory activities.

As mentioned before, some of the tested bacterial strains, especially resistant strains were found to be inactive for the plant extracts even at higher concentrations. This may be due to the intrinsic resistance mechanisms of bacteria, which is exerted via a resistant outer membrane barrier and multi-drug resistant pumps by which the activity of plant extracts is prevented [29]. The nature of the solvent extracts, especially the type of phytoconstituents present in the extracts also have a direct impact on the anti-bacterial activity. In the present study ethanol has been used as the extraction solvent as it is a commonly used solvent in the extraction of anti-bacterial compounds [30] and due to safety and suitability in the formulation of topical anti-bacterial products. However, if targeting on single anti-bacterial compounds, anti-bacterial screening of different solvent extracts with different polarities is recommended as it allows to identify potent solvent extracts, that can be further purified by activity guided fractionation.

The types of phytoconstituents present in the extracts also determine the activities of the extracts against the growth of bacterial strains. The genus Dillenia is reported to have flavonoids and triterpenoids [7]. The phytochemical screening also revealed the presence of phytochemicals including polyphenols, flavonoids and tannins. Polyphenols, phenolic acids, and flavonoids are reported to pose broad spectrum anti-bacterial properties [31]. Polyphenolic tannins known to inhibit bacterial growth via iron deprivation, hydrogen bounding or non- specific interactions with vital proteins such as enzymes [32] Whereas flavonoids are known to inhibit bacterial growth by forming complexes with extracellular and soluble proteins and bacterial cell wall [33]. Therefore, the polyphenol and flavonoid compounds present in the extracts could be attributed for the observed anti-bacterial properties in the present study.

3.4 β-Lactamase Enzyme Inhibitory Activity

β-lactamase is a bacterial enzyme by which resistance is imparted via hydrolysis of β-lactam ring structure of β-lactam anti-biotics. In the present study, a known substrate of β-lactamase, nitrocefin has been used as the substrate for the enzyme, which undergoes hydrolysis to form a colour compound (brick red) and it was spectrophotometrically measured to access the enzyme activity [34].

Table 2. Anti-bacterial activity of plant extracts against sensitive bacterial strains

Extract	Test bacterial strain (Sensitive)/Assay concentration of plant extracts (ug/mL)														
	EC			ST			SF			PA			SA		
	150	600	2400	150	600	2400	150	600	2400	150	600	2400	150	600	2400
Dillenia retusa (L)	NI	7.90 ± 0.25	25.60 ± 0.38	NI	NI	NI	NI	7.61 ± 0.23	23.22 ± 0.74	NI	13.32 ± 0.44	58.40 ± 1.54	NI	24.15 ± 0.95	57.45 ± 1.13
Dillenia retusa (B)	NI	17.85 ± 0.30	20.08 ± 1.00	NI	NI	NI	2.60 ± 0.14	38.71 ± 1.39	65.09 ± 0.82	12.98 ± 0.42	23.37 ± 0.51	45.06 ± 0.58	NI	NI	NI
Dillenia retusa (F)	NI	15.61 ± 0.37	22.87 ± 0.52	NI	NI	NI	NI	2.23 ± 0.09	8.98 ± 0.18	13.34 ± 0.29	45.91 ± 1.35	77.96 ± 0.57	NI	NI	NI

Anti-bacterial activity is given as % growth inhibition at each tested concentration.
Results are given as Mean ± SE (n=3), Standard drugs (orfloxaxin and ampicillin showed >90% inhibitions at all tested concentrations.
EC: Escherichia coli; ST: Salmonella typhi; SF: Shigella flexneri; PA: Pseudomonas aeruginosa; SA: Staphylococcus aureus
L: leaves, B: bark, F: fruit; NI: no inhibition

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extracts of Dillenia retusa

Extract	Test bacterial strain/ MIC and MBC (ug/mL)											
	EC		ST		SF		PA		SA		SA (MDR)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Dillenia retusa (L)	> 5	> 5	> 5	> 5	> 5	> 5	2.0	4.4	2.8	4.8	> 5	> 5
Dillenia retusa (B)	> 5	> 5	> 5	> 5	2.0	3.0	> 5	> 5	2.2	3.4	> 5	> 5
Dillenia retusa (F)	> 5	> 5	> 5	> 5	> 5	> 5	1.8	3.0	> 5	> 5	> 5	> 5

MIC mg/mL: Minimum Inhibitory Concentration, MBC mg/mL: Minimum bactericidal concentration
L: leaves, B: bark, F: fruit
EC: Escherichia coli; ST: Salmonella typhi; SF: Shigella flexneri; PA: Pseudomonas aeruginosa; SA: Staphylococcus aureus
MDR: Multi drug resistant

Table 4. MIC index of active extracts of Dillenia retusa

Extract	Test bacterial strain			
	SF	PA	SA	SA (MDR)
Dillenia retusa (L)	-	0.45	-	-
Dillenia retusa (B)	0.67	-	0.65	-
Dillenia retusa (F)	-	0.6	-	-

SF: Shigella flexneri; PA: Pseudomonas aeruginosa; SA: Staphylococcus aureus, MDR: Multi-drug resistant, MDR: Multi drug resistant Staphylococcus aureus
L: leaves, B: bark, F: fruit

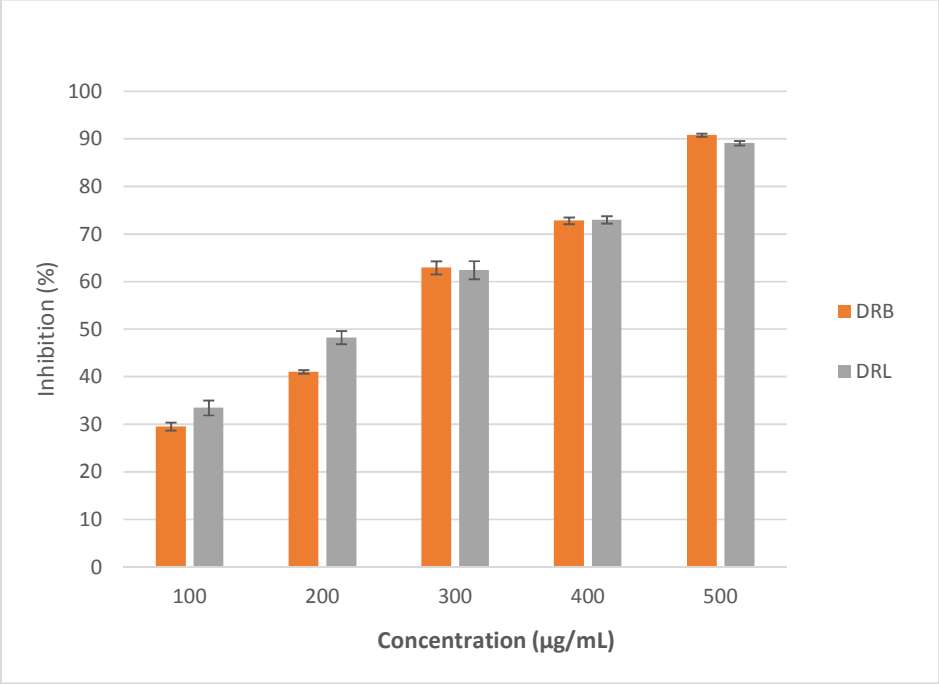
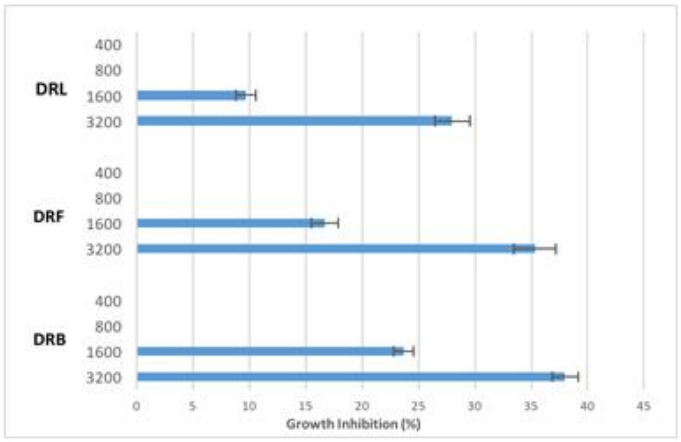


Fig. 2. Dose-response studies of β-lactamase enzyme inhibitory activity of extracts of *Dillenia retusa*

DRB: Dillenia retusa bark and DRL: Dillenia retusa leaves



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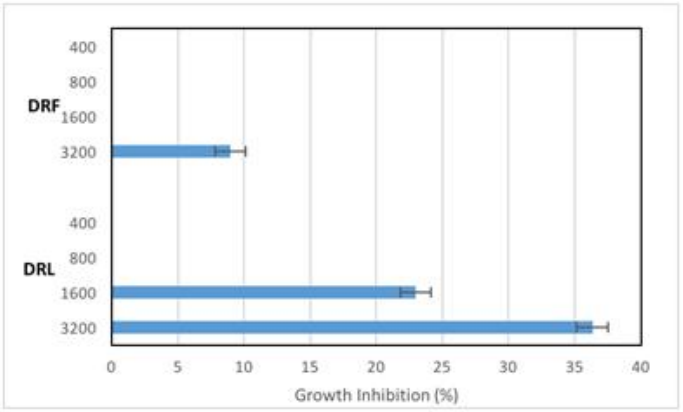

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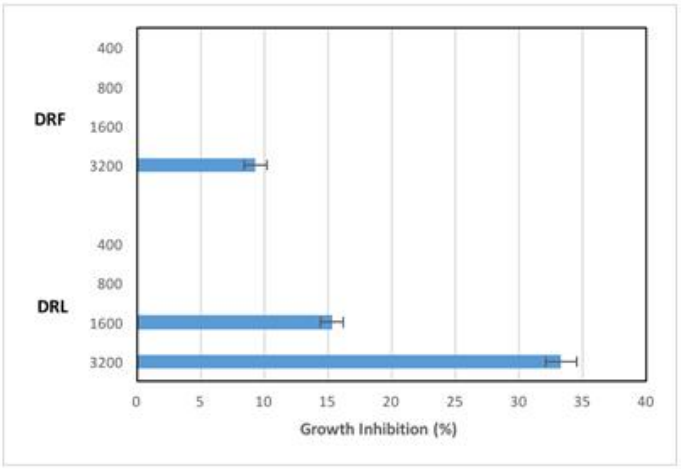

C

Fig. 3. Growth inhibition (%) of Candida albicans (A), Aspergillus niger (B) and Candida glabrata (C) at tested concentrations (400, 800, 1600, 3200 μg/mL) of ethanol extracts of Dillenia retusa.
The standard drugs miconazole and amphotericin-B showed >90% inhibitions at the same tested concentrations
DRB: Dillenia retusa bark, DRL: Dillenia retusa leaves, DRF: Dillenia retusa fruits

Penicillinase enzyme from Bacillus cereus, a constitutive periplasmic enzyme was used as the enzyme in the assay. It is a mixture of two types of β-lactamases, β-lactamase I and II. According to the recent classification system of β-lactamases, the enzyme mixture belongs to group 1 (class C) and group 2 (classes A and D), which are considered as inhibitor resistant, broad and extended spectrum β-lactamases with a serine residue at the active site of the enzyme [35]. When reacted with clavulanic acid, a potent β-lactamase inhibitor in clinical use, the enzyme activity was markedly inhibited (IC_{50}:2.33 ± 0.19 μg/mL) in a dose dependent manner (1-30 μg/mL), Therefore, it was confirmed the enzyme belongs to the class of serine-β lactamases not the metallo-betalactamases [34,36].

Of the four extracts tested, extract of DRL showed the highest β-lactamase enzyme inhibitory activity (IC_{50}: 226.25 ± 10.53 μg/mL) followed by that of DRB (IC_{50}: 238.81 ± 1.72 μg/mL). The extract of DRF showed no β-lactamase enzyme inhibitory activity at the tested assay concentrations (100, 500 and 1000 μg/mL). The inhibitory activity of the extract was found to be significant ($p<0.05$) and dose-dependent within the range of tested concentrations (Fig. 2). Previous studies suggest polyphenols including flavonoids may have the potential to serve as inhibitors of β-lactamases [37]. Therefore, the presence of flavonoid and polyphenolic compounds may have a contribution in the inhibitory action of β-lactamase enzyme. However, studies on β-lactamase enzyme inhibitory activities of individual phytoconstituents are limited to date. Therefore, further investigations are essential to identify the phytoconstituents with inhibitory potential.

3.5 Anti-Fungal Activity of the Extracts

The extracts of *D. retusa* were tested within the concentration range of 400–3200 μg/mL.

The extracts have showed anti-fungal activities against C. albicans, A. niger and C. glabrata and no activity was observed against M. canis and T. rubrum.

The extract of DRL has showed moderate anti-fungal activity against C. albicans, A. niger and C. glabrata at 3200 μg/mL whereas the extract of DRF has showed moderate anti-fungal activity against C. albicans and comparatively low anti-fungal activities against A. niger and C. glabrata at 3200 μg/mL. The extract of DRB has showed moderate anti-fungal activity against C. albicans at 3200 μg/mL and showed no anti-fungal activity against any other tested fungal species at the tested concentrations (Fig.3).

Among the plant extracts of other Dillenia species, the highest fungal growth inhibitory activity is reported for non-polar leaf extracts of D. indica against a range of fungal strains including A. niger and C. albicans at 0.4 mg/disc treatment of extracts [22] whereas the bark and leaf extracts of D. pentagyna (3 mg/ disc) and D. suffruticosa (1 mg/disc) also have showed fungal growth inhibitory activities against A. niger and C. albicans [24]. As per the findings of these reported studies on anti-fungal activity of other Dillenia species, the activity is observed at >0.4 mg which is further supported by the findings of the present study.

When compared with standard drugs, the anti-bacterial, anti-fungal, anti-β-lactamase activities of the extracts of D. retusa is comparatively low. That may be due to the crude nature of the extracts, which may have a potential to impart better anti-microbial activities upon fractionation and purification. Moreover, further studies on different solvent extracts of D. retusa will also provide a better picture of the spread of anti-microbial potential of D. retusa.

4. CONCLUSION

The extracts of D. retusa have exhibited anti-bacterial, anti-β-lactamase enzyme inhibitory and anti-fungal properties which are comparable with some of the reported bio-activities of the extracts of other related Dillenia species. The observed bio-activities may be attributed to major chemical compound groups including flavonoids, tannins and terpenoids which are known to impart antimicrobial activity via several mechanisms including inhibition of nucleic acid synthesis, biofilm formation, membrane function, extracellular microbial enzymes and microbial metabolism and cell wall synthesis. However, further purification and compound isolation is needed to confirm the bio-active compounds and compound isolation is under progress aiming further investigations of bio-activities at compound level. To our knowledge, we report the in vitro anti-bacterial, anti-β-lactamase and anti-fungal bio-activities for the extracts of D. retusa for the first time, which has filled the gaps and updated the bio-activity profile of the genus Dillenia.

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DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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