

Article

Cytotoxicity and antimicrobial activity of *Dialium ovoideum thwaites*, an endemic plant in Sri Lanka

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Abstract: This study was aimed exploring the cytotoxicity and antimicrobial activity of the leaves *D. thwaites* is an endemic plant to Sri Lanka. The plant is known for its nutritional and medicinal applications where especially the leaf decoctions are being used to wash skin wounds in indigenous medicine. The brine shrimp lethality assays was performed to evaluate normal toxicity and it gave LC₅₀ value greater than 1000 µg/ mL showing that the plant extracts are non-toxic to the normal cells. The agar-well diffusion assay was performed to assess the antimicrobial activity, and strains of bacteria; *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and Methicillin Resistant *Staphylococcus aureus* (MRSA) and fungus; *Candida albicans* were used as test organisms. The results indicated that all the extracts are active against *Staphylococcus aureus* (Gram Positive) with maximum inhibition shown for methanolic and aqueous extracts. When it was tested against MRSA both aqueous and methanolic extracts gave similar inhibitions. The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined as 6.25 mg/mL and 100 mg/mL respectively with *S. aureus* whereas no inhibition observed by any of the extract against Gram negative bacteria and *Candida albicans*. Thus, this study revealed the leaves of *D. thwaites* possesses strong anti-bacterial activity against *S. aureus* and MRSA. The results confirmed the efficacy of using *D. thwaites* as the natural antimicrobial agent and suggested to develop the leaves into value added products to be used in topical applications as antiseptic solutions or ointments.

Key words: *Dialium ovoideum thwaites*; antimicrobial; antifungal; cytotoxicity; methicillin resistant

1. Introduction

The antibiotic resistance (ABR), multidrug resistant (MDR) strain in pathogenic bacteria and the evolution of new strains of disease causing agents, are of great concern to the global health community (Manandha *et al.*, 2019; Giamarellou, 2010). Microbial infections on skin are a frequently occurred health problem as the skin serve as an ecosystem with diverse habitats which the support a wide range of micro-organisms (Grice and Segre, 2011). The pathological situation named “impetigo” is a common bacterial skin infection usually observed on the superficial epidermis and caused by *Staphylococcus aureus*. This infection accounts for 50% to 60% of all bacterial skin infections and topical antibiotic creams such as mupirocin and fusidic acid are prescribed as the treatments (Green *et al.*, 2012). Meanwhile *Staphylococcus aureus* had also started to develop antibiotic resistance since the introduction of penicillin in 1940 and this situation causes the difficulties to treat such diseases (Boswihi and Udo, 2018). As a solution to penicillin resistance, a semi-synthetic penicillinase resistant penicillin called methicillin had been introduced but shortly *Staphylococcus aureus* has begun to develop methicillin resistance too (Breyre and Frazee, 2018). This methicillin resistant *Staphylococcus aureus* (MRSA) has been reported to cause many complications and difficult-to-treat diseases including abscess, purulent cellulitis and even severe skin and soft tissue infections (SSTI) (Kasote *et al.*, 2015) while the number of efficient antibiotics against them are challenging. Therefore, finding potential alternative compounds that can

be effective in complex situation of microbial infections, through medicinal plants with ethnomedical uses would be the most promising solution as medicinal plants are the sources for potential novel pharmacological active compounds.

The Sri Lankan Ayurvedic system of medicine has been a very long history and been used about 550 to 700 species out of over 3000 vascular plants and a quarter of which is endemic (Yasapalitha and Rupasinghe, 2016). *Dialium ovoideum thwaites* is such an endemic plant species to Sri Lanka found in the semi-dry zone of the country and is being extensively used in Ayurvedic medicine in the form of leaf decoctions to wash wounds and to treat skin infections (Ayurvedic medicinal plant of Sri Lanka, 2019). It is noteworthy that only a very limited number of scientific studies had been reported of this plant and, therefore in our previous study was carried out to reveal phytochemical, proximate and antioxidant properties of the plant (Bulugahapitiya *et al.*, 2019). Therefore, this an extended study aimed at determining normal toxicity of and assessing antimicrobial activity of the leaves extract of the plant.

2. Materials and Methods

2.1. Plant material

Leaves were collected from Wellawaya area, Monaragala district, Sri Lanka and were authenticated. Cleaned leaves were blotted and shade dried for 21 days. The dried leavers were ground to fine powder using a blender and were stored in sealed zip-lock bags at 4 °C until usage.

2.2. Preparation of extracts

The methanolic extract was prepared by macerating 160 g of dried powder in 600 mL of pure methanol for four days with frequent agitation. The macerated solution was filtered and concentrated under vacuum. 10 g of crude was suspended in 150 mL of distilled water and was sequentially extracted with 50 mL of each; n-hexane, chloroform and ethyl acetate thrice (50×3) and combined fractions was concentrated under *vacuum*.

The aqueous extract was prepared by Soxhlet extraction method and was concentrated under high vacuum. All fractions were stored at 4 °C until test was performed.

2.3. Determination of normal toxicity using Brine shrimp assay

Brine shrimp (*Artemia salina*) lethality assay is commonly used to test the cytotoxic effect of bioactive chemicals such as bioactive natural compounds, toxin, pesticides etc. It is a preliminary toxicity screening used to test the normal toxicity of plant extracts as well (Sarah *et al.*, 2017; Kibiti and Afolayan, 2016). The test is based on the ability to kill a laboratory cultured larvae (nauplii) when exposing them to different concentration of plant extract in sea water within 24 hours time.

2.3.1. Hatching and preparation of brine shrimp

Artificial sea water was prepared by dissolving 114.0 g of NaCl in 3 L (38 g/L) of water. A spatula full of brine shrimp cysts were added and was incubated for 24 hours at room temperature along with continuous aeration and intensity of 60W bulb. The empty cysts and unhatched eggs were separated and the nauplii were pumped out to a new batch of artificial sea water.

2.3.2. Brine shrimp assay

A stock solution of 1000 ppm was prepared in artificial sea water with 0.2504 g of crude methanolic extract and 2 mL of DMSO. The dilution series was prepared with the concentrations (10 - 1000 ppm) while artificial sea water was used as the control. The test solution containing 2.50 mL of plant extract, 12 -15 nauplii in 10 mL sea water in test tubes were incubated for 24 hours at room temperature (triplicate). The solutions were quantitatively transferred on to petri dishes and the total and dead counts of nauplii were taken using a magnifying lens. The percentage mortality and the lethal concentration were determined.

$$\% \text{ Mortality} = \frac{\text{No. of dead nauplii}}{\text{Total No. of nauplii}} \times 100 \%$$

2.4. Antimicrobial assay

Antimicrobial assay for the plant extracts were performed using Agar well diffusion method in Mueller Hinton Agar (MHA) plates and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined according to the method described by Manandhar *et al.* (2019) with slight modifications.

2.4.1. Microbial culture used

E.coli (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10231) and a MRSA strain isolated from a clinical specimen were used as test microorganisms and cultures were obtained from Department of Microbiology, Faculty of Medicine, University of Ruhuna, Sri Lanka.

2.4.2. Antimicrobial assay with plant extracts

The Muller Hinton's Agar was prepared according to laboratory standards and were autoclaved at 121 °C for 15 minutes under 15 lb pressure. The sterilized nutrient broths were poured in to sterile petri dishes and were allowed to solidify in a refrigerator. The microbial suspensions were prepared in sterile saline and the concentrations were adjusted to 0.5 McFarland standard. Plant extracts of 200 mg/mL were prepared in 10% DMSO. Five wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer. Each well was loaded with 50 µL plant extracts in DMSO, positive control; 5 mg/mL of vancomycin, ceftriaxone and ceftazidime in DMSO for bacteria and fluconazole for fungi, and 10 % DMSO as negative control respectively. All the inoculated plates were incubated at 35 °C for 24 h and plates were observed for inhibition zone. The zone of inhibition (ZOI) were measured in mm at 18 h and 24 h.

2.4.3. Determination of MIC and MBC of plant extract

The broth microdilution method was used to determine the MIC. Twofold serial dilutions of aqueous and methanolic extracts (100 µL) were prepared directly in a well containing Mueller Hinton broth to obtain various concentrations. *S. aureus* suspension was prepared in MH broth and the concentration was adjusted to 0.5 McFarland standard. The amount of 100 µL of this bacterial suspension was added in to each well and Vancomycin was used as the positive control whereas 10% DMSO, bacterial suspension and MH broth as negative controls. The plate was covered with a sterile sealer and incubated for 24 h at 35°C. The MIC was considered as the lowest concentration of the extract that inhibits the bacterial growth in term of turbidity. In order to determine the minimum bactericidal concentration (MBC), all the serial dilutions of methanolic crude and aqueous fraction resulted from the MIC experiment was sub-cultured on blood agar and minimum concentration of the plant extract which required to kill all the bacterial colonies was measured within fixed time period (24 hours).

3. Results and Discussion

3.1. Preliminary cytotoxicity (Brin shrimp assay)

Brine shrimp lethality bioassay is a rapid general, bench top bioassay for bioactive compounds and extracts. This assay was first introduced by Michael *et al.* (1956) to test the toxicity of compounds and then it was further developed by many scientists. Meyer *et al.* (1982) has successively employed this lethality assay as a bioassay guide for active cytotoxic and antitumor agents. Accordingly, an extract or compound is considered as cytotoxic when the value of LC_{50} is ≤ 30 µg/mL. In this study, the methanolic crude of leaves of *D. thwaites* showed no toxicity towards brine shrimp larvae as lethal concentration (LC_{50}) is very large and even greater than 1000 ppm which is taken from the Figure 1 drawn y as percentage mortality x as log concentration for the dilution series of *D. thwaites*.

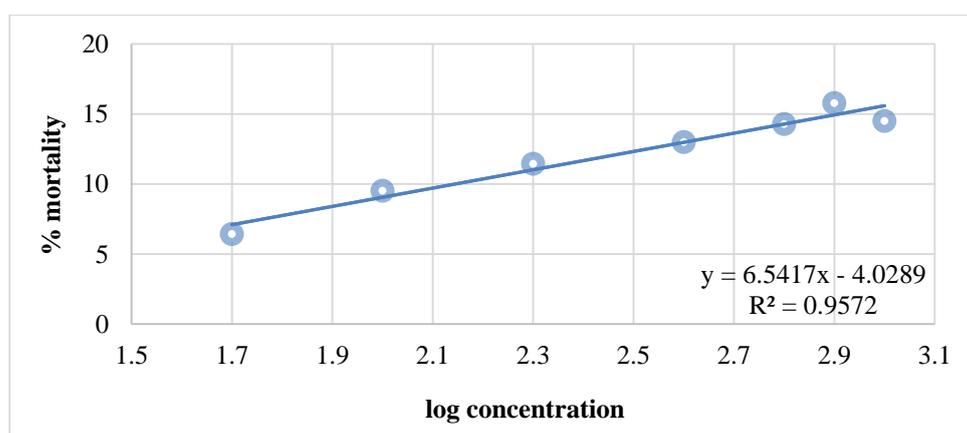


Figure 1. Percentage mortality versus log concentration for the dilution series of *D. thwaites*.

3.2. Antimicrobial Activity

In the preliminary screening, the methanolic crude and the four solvent extracts were tested against gram positive and negative bacteria and the common fungal strain; *C. albicans*. This study showed that all the extracts are inhibitory towards gram positive *S. aureus* while a significant antibacterial activity with respect to vancomycin positive control was shown by the methanolic crude and aqueous extract.

However at the same time it is noteworthy that any of the extracts did not show any inhibition towards the gram negative strains and fungal strain tested. The inhibitory zone diameters were measured after 18 h and 24 h of incubation and are summarized in Table 1.

Table 1. Diameters of inhibition zones for solvent extracts against *S. aureus* ATTC reference strain with 5 mg/mL vancomycin positive control (mm).

Extract /Control	<i>S. aureus</i>							
	After 18 h /mm			Mean /mm	After 24 h /mm			Mean /mm
	Trial 1	Trial 2	Trial 3		Trial 1	Trial 2	Trial 3	
Methanolic crude	19	18	18	18.3	20	19	19	19.3
Hexane fraction	12	12	12	12	13	13	13	13
CHCl ₃ fraction	14	14	14	14	14	14	14	14
Ethyl acetate fraction	14	14	14	14	14	14	14	14
Aqueous fraction	18	18	16	17.3	18	18	16	17.3
Vancomycin			24				24	
10% DMSO			0				0	

Further, inhibitory action of methanolic crude and aqueous crude were tested against clinically isolated MRSA along with vancomycin as the positive control. Surprisingly a significant inhibition was observed at 18h and 24h as given in the summary of diameter measurements in Table 2.

Table 2. Diameters zones of inhibition for solvent extracts against MRSA clinical isolate (mm).

Extract	MRSA –zone of inhibition (ZOI) in mm							
	After 18 h /mm			Mean /mm	After 24 h /mm			Mean /mm
	Trial 1	Trial 2	Trial 3		Trial 1	Trial 2	Trial 3	
Methanolic crude	16	17	16	16.3	16	17	17	16.3
Aqueous fraction	17	17	17	17	17	17	17	17
Vancomycin			25				25	
10 % DMSO			0				0	

3.3. Results of MIC and MBC determination

The effectiveness of the extracts in tested bacterial strains was determined by measuring the minimum inhibitory concentration (MIC) which was done by lowering the concentration of anti-bacterial agent/ plant extract on inhibition of bacterial growth. MIC was performed for only the organisms, *S. aureus*, which showed a zone of highest inhibition with aqueous and methanolic extracts of leaves in the antimicrobial assay by agar well diffusion method. In this study, MIC value of 6.25 mg/mL was observed for both methanolic crude and the aqueous extract. While the MIC value of vancomycin, a positive control was recorded to be less than 0.78 mg/mL. The minimum bactericidal concentration (MBC) is termed as the lowest concentration of antibacterial agent / plant extract required to kill all the bacterial colonies within fixed time period, and study showed that the concentration of 100 mg/mL of both aqueous and methanolic extracts to be the complete killing of bacterium whereas lesser the concentration more colonies occurred. Therefore, MBC value is reported as 100 mg/mL for both aqueous and methanolic extracts of *D. thwaites*.

4. Conclusions

The study confirmed that the leaf extracts of *D. thwaites* can be used as a potential anti-bacterial candidate against *S. aureus* and especially for MRSA infections which is world-wide health concern. The leaves have no significant antifungal action and no cytotoxicity. The minimum inhibitory concentration (MIC) for both aqueous and methanolic extract of leaves against *S. aureus* is 6.25 mg/mL whereas minimum bactericidal concentration (MBC) of leaves against *S. aureus* is 100 mg/mL. This study has provided a scientific validation for the use of leaves of *D. thwaites* in antimicrobial purposes in Sri Lankan indigenous medicine. As further advancement,

development of antibacterial agents from *D. thwaites* against *S. aureus* and MRSA as value added products such as disinfection solution and ointments are promising.

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Conflict of interest

None to declare.

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