Journal of King Saud University – Science (2015) **27**, 302–311

King Saud University

Journal of King Saud University – Science

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ORIGINAL ARTICLE

Phytochemical screening and antimicrobial potentials of *Borreria* sps (Rubiaceae)

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Received 28 August 2014; accepted 2 December 2014
Available online 19 December 2014

KEYWORDS

Phytochemicals;
Rubiaceae;
Antibacterial;
Antifungal activity;
Zone of inhibition;
Disk diffusion assay

Abstract Successive hexane, acetone, ethanol and methanolic whole plant extracts of the *Borreria* sps were investigated for phytochemical screening and assessed for antimicrobial activity. Phytochemical analysis of *Borreria* sps extracts revealed the presence of phenolics, flavonoids and tannins. Among them, *Borreria laevicaulis* hexane extracts were found to be most effective showing the largest zone of inhibition against *Staphylococcus aureus* (22.15 mm) and *Candida albicans* (25.65 mm). Further studies indicated that the minimum inhibitory concentration of *B. laevicaulis* hexane extracts was found to be 62.5 μg/ml against *S. aureus* and 250 μg/ml against *C. albicans* and the zone of inhibitions was significantly higher than nystatin (positive control). Together, we provide new insights of the *B. laevicaulis* as a potential candidate for antimicrobial drug discovery using in vitro studies that might be useful to treat human infectious diseases and antibiotic resistant pathogens.

1. Introduction

Infectious diseases are fatal and life threatening throughout the world. The amplification of diseases is largely due to indiscriminate use of antibiotics (Avila et al., 2008). Recent studies have extensively addressed the dramatic increase of microbial resistance to antibiotics (Triyana, 2009; Kumar et al., 2006) and methods to treat them. Antimicrobial resistance evolved through mutation and genetic exchange systems which render the elimination of diseases becomes ineffective. Hence, there is an urge to continuously search for alternative sources including natural products. Traditional medicinal systems like Ayurvedic, Chinese Medicine, and Unani developed from plant resources have been used to treat various diseases. The isolation of bioactive compounds such as tannins, terpenoids, alkaloids, flavonoids etc. for potential drug discovery has been extensively reported (Choudhury et al., 2012; Taylor, 2013).

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Peer review under responsibility of King Saud University.

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http://dx.doi.org/10.1016/j.jksus.2014.12.001

Rubiaceae is well known for its medicinal values, used in the treatment of malaria, diarrhea, digestive problems, skin diseases, fever, hemorrhage, urinary and respiratory infections, headache, inflammation of eyes and gums (Conserva and Ferreira, 2012). Most species of Rubiaceae are normally grow as weeds in Malaysia due to their strong habitat adaptation ability. *Borreria exilis* (L.O. Williams) C.D. Adams is an annual herb distributed throughout the tropical countries, America and Africa (iNaturalist.org, 2013), used for treating headache (Conserva and Ferreira, 2012).

Borreria laevicaulis (Miq.) Ridl. is an annual or perennial herb naturalized along tropical Asia, Africa, Mauritius and East India. Traditionally, it is used as a poultice for headache; wounds healer; plant sap to treat eczema, worms and ringworm (Pravat and Prithwiraj, 2012). Leaf juice is applied for ringworm and eczema while the plant sap is used to treat the wound or lesion (Ebana et al., 1991). *Borreria latifolia* (Aubl.) K. Schum. is an annual herb that grows as a dominant weed on waste areas or agricultural fields and is normally distributed in India, Southeast Asia and Malaysia. It possesses antimicrobial properties against *Bacillus cereus*, *Bacillus megaterium* and *Pseudomonas aeruginosa* (Choudhury et al., 2012).

Borreria remotifolia DC is an annual herb widely distributed in tropical Asia, Africa, Australia and the Pacific Islands (FOC, 2013). Different plant parts have been used as antidotes to cure venomous stings and bites; roots as a medicinal are used to cure tetanus (Allabi et al., 2011). *Richardia brasiliensis* Gomes is an annual or perennial herb mainly distributed in the Southeastern United States, Asia, Midwest, South and Southeast of Brazil. It is traditionally used as an expectorant, antiemetic, diaphoretic, anti-inflammatory and in several treatments for hemorrhoids, coughs, bronchitis and headache (Hall et al., 2012). Phytochemical study revealed the presence of coumarin, flavonoids, steroids, terpenoids, alkaloids and resins in aerial parts of the plant (Morais et al., 2013). In the current study, we tested the phytochemical contents and antimicrobial activity of five *Borreria* sps against different pathogenic bacteria and fungal strains.

2. Materials and methods

2.1. Collection of plant materials

B. exilis, *B. latifolia*, *B. laevicaulis*, *B. remotifolia* and *R. brasiliensis* were collected from various locations around Kelantan, Malaysia. All the plant samples were identified by a botanist, from the University of Malaysia, Kelantan.

2.2. Preparation of plant extracts

The fresh plant samples (whole plant parts) collected were washed individually under running tap water and dried in an oven at 40 °C for 3 days. The dried plant materials were ground into powder using an electrical blender. About 10 g of dry powdered plant material from each plant was extracted by soxhlation using various solvents like methanol, ethanol, acetone and hexane. Extracts were then concentrated using a rotary evaporator and the concentrated residual extracts were stored at 4 °C in a dry airtight container until further use.

2.3. Microbial culture, inoculum preparation

Pathogenic bacterial and fungal strains were tested for the antimicrobial activity using *Borreria* sps plant extracts. Tested strains included gram positive bacteria such as *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (clinical isolates); Gram negative bacteria such as *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Klebsiella pneumoniae* (clinical isolates), and fungi such as *Candida albicans* (clinical isolates), *Aspergillus niger* (clinical isolates). All American Type Culture Collection was obtained from the

Table 1 Qualitative analysis of phytochemicals from whole plant extracts of the *Borreria* sps.

		Phenolic	Alkaloids	Flavonoids	Tannins	Terpenoids	Saponins
		$FeCl_3$	Mayer	NaOH	Braymer	Salkowki	Foam test
Borreria exilis	Methanol	+	–	+	+	+	–
	Ethanol	+	–	+	+	+	–
	Acetone	+	+	+	+	–	–
	Hexane	–	+	–	–	–	–
Borreria laevicaulis	Methanol	+	+	+	+	+	+
	Ethanol	+	+	+	+	+	+
	Acetone	+	+	+	+	–	+
	Hexane	+	+	+	+	–	–
Borreria latifolia	Methanol	+	–	+	+	–	–
	Ethanol	+	–	+	+	–	–
	Acetone	+	–	+	+	–	–
	Hexane	+	–	+	+	–	–
Borreria remotifolia	Methanol	+	–	+	+	–	+
	Ethanol	+	–	+	+	–	–
	Acetone	+	–	+	+	–	–
	Hexane	–	+	+	–	–	–
Richardia brasiliensis	Methanol	+	+	+	+	–	+
	Ethanol	+	–	+	+	–	–
	Acetone	+	–	+	+	–	–
	Hexane	–	–	–	–	–	–

Note: +, indicates presence of phytochemicals; –, indicates absence of phytochemical.

Veterinary Research Centre at Perak and clinical isolates were obtained from the Hospital University Science of Malaysia, Kelantan. Cultures of bacteria were streaked on nutrient agar (NA) with an incubation hour of 18–24 h at 37 °C and fungi on potato dextrose agar (PDA) and incubated at 28 °C for 72 h.

2.4. Phytochemical screening

Crude plant extracts were subjected to preliminary screening for the presence of active secondary metabolites. Each plant extract was tested individually with specific chemical reagents

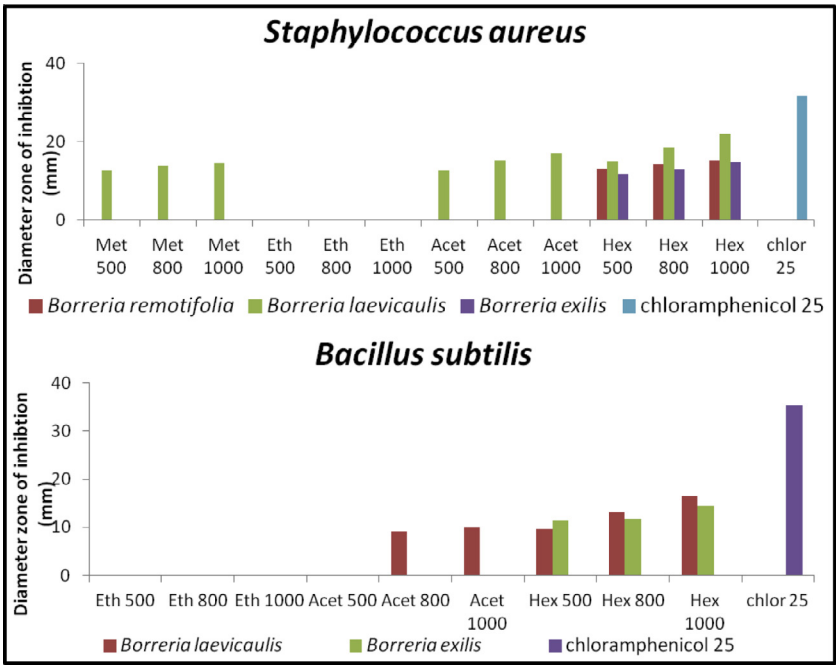

Figure 1 Anti-bacterial activity of Borreria sps crude extracts by disc diffusion assay. Histogram showing quantification of zone of inhibition (mm) displayed against *S. aureus* and *B. subtilis* using different concentrations (μg/disk) along with positive control (chloramphenicol).

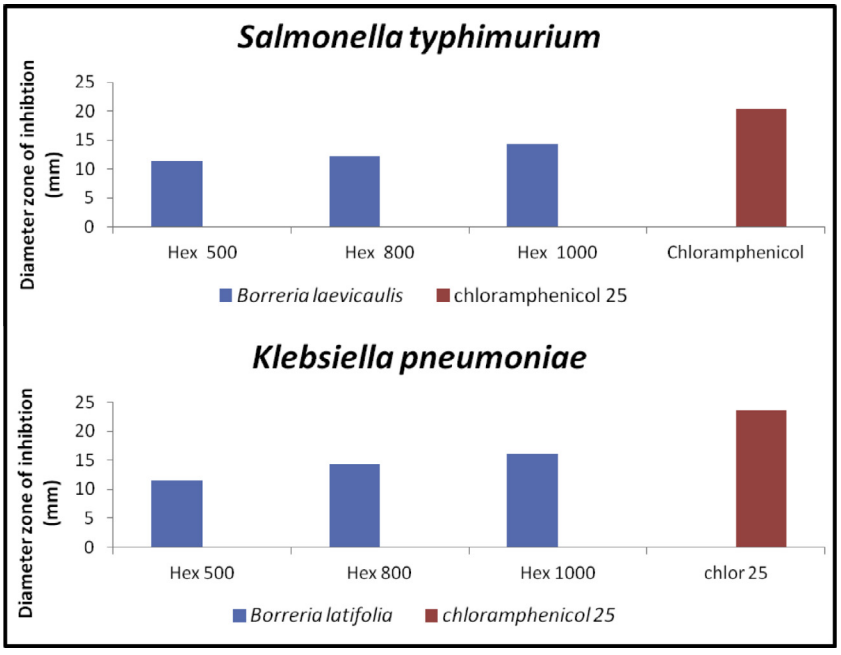

Figure 2 Anti-bacterial activity of hexane extracts of *Borreria* sps by disc diffusion assay. Histogram showing quantification of zone of inhibition (mm) displayed against *S. typhimurium*, *K. pneumoniae* using different concentrations (μg/disk) along with positive control (chloramphenicol).

according to standard procedures (Thamaraiselvi et al., 2012; Wangchuk et al., 2011; Rajesh et al., 2013; Raaman, 2006). Visible color change or precipitate formation was taken into consideration for the presence (+) or absence (–) of particular active constituents.

2.5. Anti-microbial screening

2.5.1. Zone of inhibition

The zone of inhibition was determined by the disk diffusion method according to the Kirby-Bauer method (Bauer et al.,

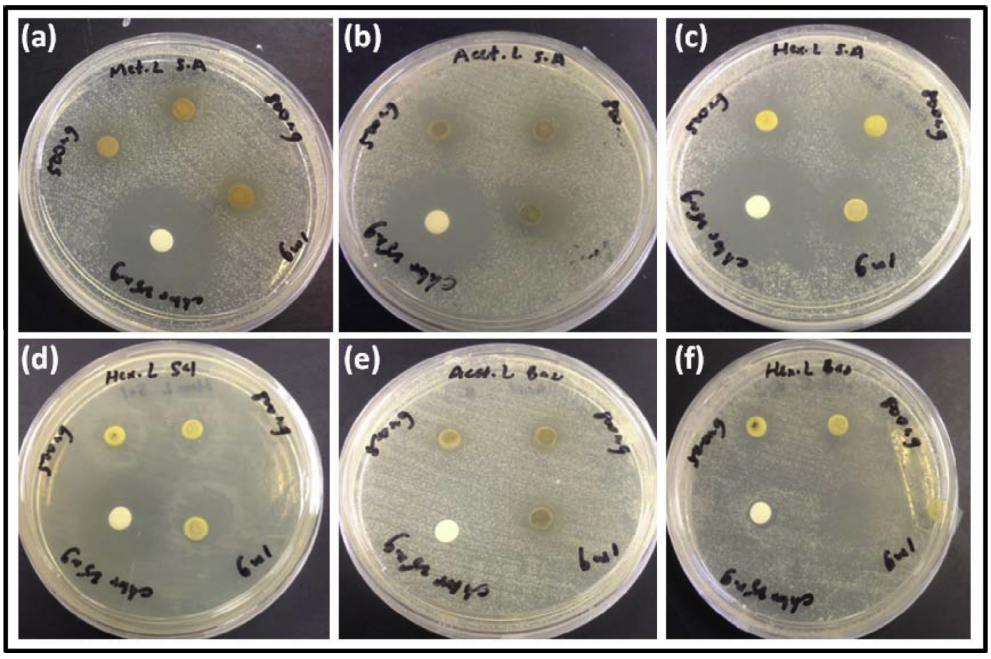

Figure 3 Anti-bacterial activity of *Borreria laevicaulis* by the disc diffusion assay. Zone of inhibition displayed on *Staphylococcus aureus* by (a) methanolic extracts (b) acetone extracts and (c) hexane extracts. Zone of inhibition displayed by (d) hexane extracts on *Salmonella typhimurium* (e) acetone extracts on *Bacillus subtilis* and (f) hexane extracts on *Bacillus subtilis*.

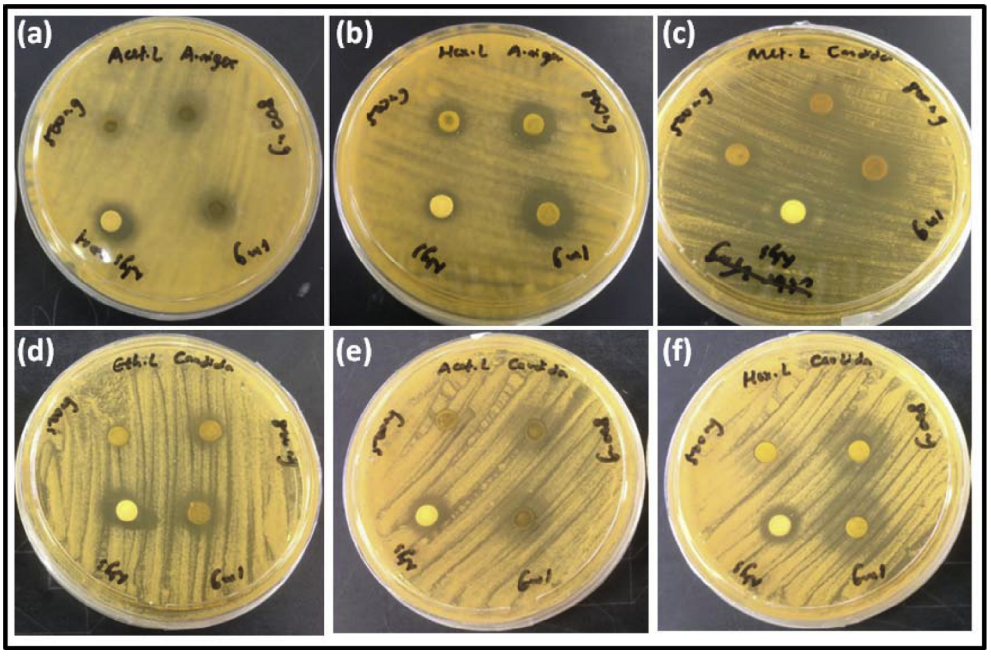

Figure 4 Anti-fungal activity of *Borreria laevicaulis* by the disc diffusion assay. Zone of inhibition displayed by (a) acetone extract (b) hexane extract on *Aspergillus niger*. Zone of inhibition displayed by (c) methanolic extracts (d) ethanolic extracts (e) acetone extract and (f) hexane extract on *Candida albicans*.

1966). Pre-loaded sterile disks (500, 800 and 1000 µg/disk) of each extract were dispensed onto the agar surface covered by test microorganisms at equal distance to each other, followed by incubation at 37 °C for bacteria at 16–18 h and 4 days at 28 °C for fungus. DMSO served as a negative control and standard antibiotics chloramphenicol (25 µg/disk) and nystatin (2000 units/disk) were used as positive controls for bacteria and fungus respectively. Antimicrobial activity was measured with a diameter zone of inhibition to the nearest millimeters (mm). All positive control disks were applied individually against each of the seven different test microorganisms (Barbour et al., 2004).

2.5.2. Minimum inhibitory concentration (MIC)

MIC was carried out for each plant extract that displayed antimicrobial activity. Broth dilution method (Andrews, 2001) was applied to determine the lowest concentration of antimicrobial agent which inhibits the visible growth of test microorganisms being investigated. The MIC was performed by sterile Mueller Hinton Broth (MHB) for bacteria and sterile Sabouraud Broth (SB) for fungus respectively. As mentioned above DMSO served as a negative control and standard antibiotics chloramphenicol (25 µg/disk) and nystatin (2000 units/disk) were used as positive controls for bacteria and fungus respectively. The broth was incubated at 37 °C overnight for MHB and 28 °C for 72 h for SB. MIC values were determined by recording the end point of the tube showing no growth of microorganisms.

2.6. Statistical analysis

One way analysis of variance was used in the present study to analyze the data collected. The statistical data were expressed as mean ± standard deviation with $p < 0.05$ significance.

3. Results

3.1. Phytochemical screening

There has been a growing interest to identify Rubiaceae medicinal plants as therapeutic agents that can effectively interfere with disease causing microbes such as bacteria and fungi. Leading to its previous discoveries with a wide range of biological effects and in an effort to capitalize on its advantages, here we investigated the antimicrobial activity of *Borreria* sps by in vitro approaches. Phytochemical screening of soxhlet successive crude extracts viz., hexane, ethyl acetate acetone and methanolic extracts from the whole plant of the *Borreria* sps showed the occurrence of various secondary metabolites such as phenolics, alkaloids, flavonoids, tannins, saponins and terpenoids (Table 1). Among the five species of *Borreria*, *B. latifolia* contained phenolics, flavonoids, tannins; *B. remotifolia*, *B. exilis* and *R. brasiliensis* contained phenolics, alkaloids, flavonoids, tannins and terpenoids while *B. laevicaulis* contained all classes of chemical constituents.

3.2. Anti microbial screening

3.2.1. Zone of inhibition

The successive *Borreria* sps crude extracts viz. hexane, ethyl acetate, acetone and methanol were screened for antibacterial and antifungal activities by employing the disk diffusion method. The activity was recorded as a diameter zone of inhibition using the crude extract concentration ranging from 500 to 1000 µg/disk (Table 2). Hexane extracts of *B. remotifolia* exhibited activity against *S. aureus* while *B. exilis* exhibited activity on both *S. aureus* and *B. subtilis* (Fig. 1; Table 3). Hexane extract of *B. laevicaulis* exhibited significant antibacterial activity against *S. typhimurium*, *S. aureus*, *B. subtilis*

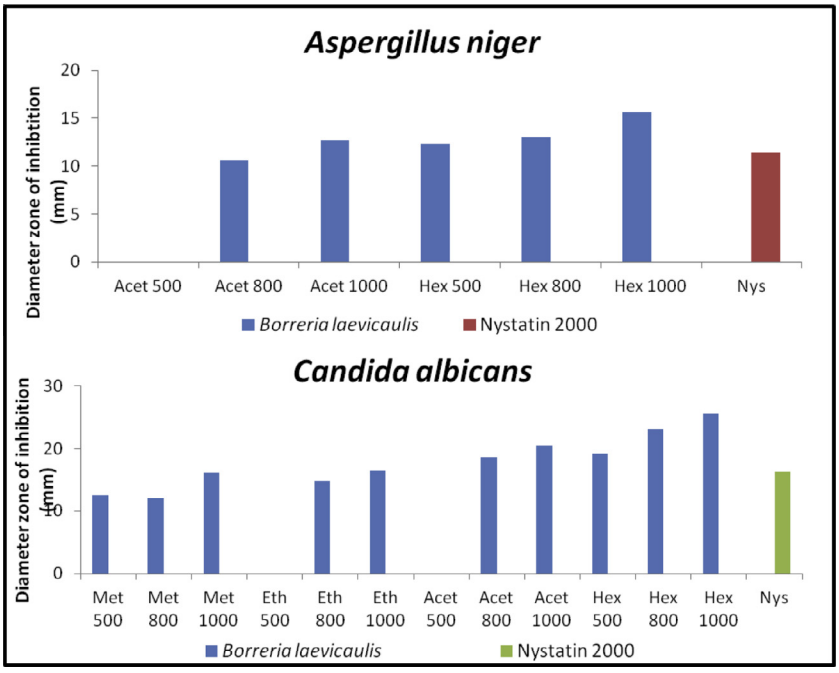

Figure 5 Anti-fungal activity of *Borreria* sps by disc diffusion assay. Histogram showing quantification of zone of inhibition displayed against *A. niger*, *C. albicans* using different concentrations (µg/disk) along with nystatin (2000 units/disk), positive control.

Table 2 Antimicrobial activity of *Borreria* sps *of* whole plant extracts.

Test sample	Extracts	Concentration (μg/disc)	Diameter zone of inhibition (mm)						
			Microorganisms						
			Escherichia coli	*Salmonella typhimurium*	*Klebsiella penumonia*	*Staphylococcus aureus*	*Bacillus subtilis*	*Aspergillus niger*	*Candida albicans*
Chloramphenicol		25	29.45 ±0.53	20.38^{a} ±0.03	23.20^{a} ±0.28	31.85^{a} ±0.64	35.33^{a} ±0.18		
Nystatin		2000 units/disc						11.42^{bc} ±0.11	16.25^{cd} ±0.07
DMSO			–	–	–	–	–	–	–
Borreria exilis	Hexane	500	–	–	–	11.75^{i} ±0.64	11.50^{de} ±0.14	–	–
		800	–	–	–	13.00^{gh} ±0.14	11.70^{cd} ±0.85	–	–
		1000	–	–	–	14.83^{ef} ±0.89	14.51^{b} ±0.69	–	–
Borreria laevicaulis	Methanol	500	–	–	–	12.70^{ghi} ±0.57	–	–	12.55^{fg} ±0.78
		800	–	–	–	13.79^{fg} ±0.69	–	–	12.10^{g} ±0.00
		1000	–	–	–	14.68^{ef} ±0.68	–	–	16.15^{cd} ±0.35
		800	–	–	–	–	–	–	14.84^{def} ±0.65
		1000	–	–	–	–	–	–	16.45^{cd} ±0.07
	Acetone	500	–	–	–	12.61^{hi} ±0.69	–	–	–
		800	–	–	–	15.21^{e} ±0.07	9.10^{f} ±0.14	10.65^{c} ±0.50	18.64^{bc} ±0.76
		1000	–	–	–	17.15^{d} ±0.07	10.08^{f} ±0.03	12.70^{bc} ±0.99	20.45^{b} ±0.07
	Hexane	500	–	11.35^{d} ±0.01	–	15.13^{e} ±0.18	9.60^{f} ±0.71	12.35^{bc} ±0.21	19.15^{b} ±0.07
		800	–	12.20^{c} ±0.00	–	18.64^{c} ±0.76	13.10^{cd} ±0.00	13.00^{b} ±0.00	23.21^{a} ±1.68
		1000	–	14.40^{b} ±0.28	–	22.15^{b} ±0.07	16.60^{b} ±0.71	15.63^{a} ±0.66	25.65^{a} ±0.78

–, represents no activity. Values are the mean of triplicates ± SE. Statistical analysis was found to be significant at $p < 0.05$. Zone of inhibition including the diameter of the disc. Chloramphenicol for bacteria; nystatin for fungi.

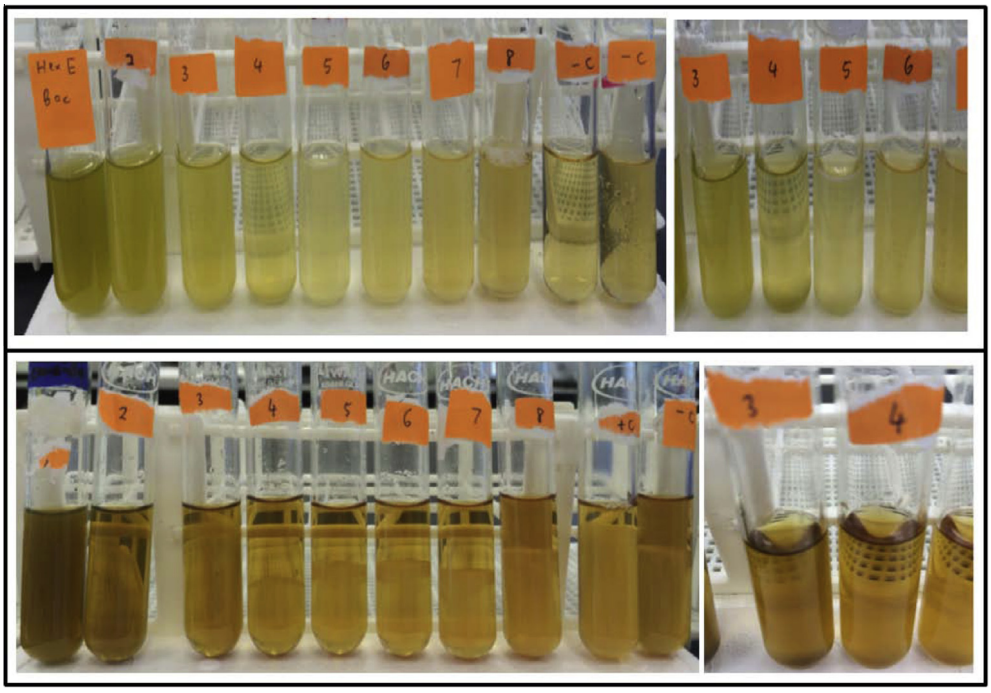

Figure 6 Minimum inhibitory concentration by hexane extracts of the *Borreria* sps. Top panel represents MIC displayed by *B. exilis*. Tube 4 showing the end point of clear solution and observed with turbid tube 5 onward against *B. subtilis*in Mueller Hinton Broth. Bottom panel represents MIC displayed by *B. laevicaulis*. Tube 3 showing the end point of a clear solution and observed with growing fungus in tube 5 onward against *C. albicans* in Sabouraud Broth.

(Fig. 2, Fig. 3) as well as antifungal activity against *A. niger* and *C. albicans* (Fig. 4). Out of these, maximum antibacterial (22.15 ± 0.07 mm) and antifungal (25.65 ± 0.78 mm) inhibition was observed in the hexane extract of *B. laevicaulis* at 1000 µg/disk against *S. aureus* (Figs. 2 and 3) and *C. albicans* respectively (Fig. 5). Altogether, among the different extracts, acetone and hexane extracts were found to exhibit maximum growth inhibition compared to methanolic and ethanolic extracts (Table 2; Figs. 1–5). These results suggest the antimicrobial activity was found to be stronger with non-polar fractions compared to polar fractions.

3.2.2. Minimum inhibitory concentration (MIC)

Next, we carried out MIC for each plant extract that only displayed antimicrobial activity. We observed minimum antibacterial (9.10 ± 0.14 mm) and antifungal (10.65 ± 0.50 mm) inhibition with an acetone extract of *B. laevicaulis* (800 µg/disk) against *B. subtilis* and *A. niger* (Table.2; Fig. 6). Lowest MIC for antibacterial activity was 62.5 µg/ml, on *S. aureus* of hexane extract of *B. laevicaulis*. Moreover, hexane extract of *B. laevicaulis* possessed a much lower MIC value of 250 µg/ml against *C. albicans* compared to others of 500 µg/ml (Fig. 6). Highest MIC value of 1 mg/ml was recorded on the hexane extract of *B. laevicaulis* against *S. typhimurium*.

Among the different species of *Borreria*, almost all tested plants except *R. brasiliensis* displayed growth inhibition against at least one strain of microorganism tested indicating the broad spectrum pharmacological activity of Rubiaceae (Table 3).

4. Discussion

Firstly, we show phytochemical screening of different species of *Borreria* crude extracts showing the occurrence of various secondary metabolites. Secondly, we demonstrate acetone and hexane extracts exhibit maximum growth inhibition compared to methanolic and ethanolic extracts suggesting that antimicrobial activity was found to be stronger with non-polar fractions compared to polar fractions. Third, we display *B. laevicaulis* as a potential candidate for antimicrobial drug discovery using in vitro studies to treat infectious diseases and antibiotic resistant pathogens. Conclusions drawn are based on several different analyses as described in our paper.

Hexane extracts induced highest zone of inhibition suggesting its antibacterial and antifungal activities. This can be explained with the active compounds which are responsible for antibacterial and antifungal activities of the extract reside in the non-polar fractions in relatively higher concentrations (Tadeg, 2004). Our observations are evidenced as shown by Onawumi et al., 2012; Nino et al., 2012. The cell wall of gram negative bacteria is more complex than gram positive bacteria which makes it more susceptible and impermeable (Kumar et al., 2006; Zaidan et al., 2005). Therefore, this explains why the gram negative bacteria were more resistant to antimicrobial compounds with its effective diffusion barrier. However, few plant species extracts still possess some degree of inhibition toward gram-negative bacteria.

It is not surprising to note that *E. coli* in our study was the most resistant microorganism among all bacteria strains, despite the fact that *E. coli* developed multi drug resistance

Table 3 Antimicrobial activity of *Borreria* sps and *Richardia brasiliensis* whole plant extracts.

Test sample	Extracts	Concentration (µg/disc)	Diameter zone of inhibition (mm)						
			Microorganisms						
			Escherichia coli	*Salmonella typhimurium*	*Klebsiella pneumonia*	*Staphylococcus aureus*	*Bacillus subtilis*	*Aspergillus niger*	*Candida albicans*
Chloramphenicol		25	29.45 ±0.53	20.38[a] ±0.03	23.20[a] ±0.28	31.85[a] ±0.64	35.33[a] ±0.18		
Nystatin		2000 units/disc						11.42[bc] ±0.11	16.25[cd] ±0.07
DMSO			0	0	0	0	0	0	0
Borreria latifolia	Methanol/Ethanol/Acetone	500	–	–	–	–	–	–	–
		800	–	–	–	–	–	–	–
		1000	–	–	–	–	–	–	–
	Hexane	500	–	–	11.60[d] ±0.57	–	–	–	–
		800	–	–	14.24[d] ±1.05	–	–	–	–
		1000	–	–	16.10[bc] ±1.55	–	–	–	–
Borreria remotifolia	Methanol/Ethanol/Acetone	500	–	–	–	–	–	–	–
		800	–	–	–	–	–	–	–
		1000	–	–	–	–	–	–	–
	Hexane	500	–	–	–	13.20[gh] ±0.14	–	–	–
		800	–	–	–	14.30[ef] ±0.00	–	–	–
		1000	–	–	–	15.20[e] ±0.07	–	–	–
Richardia brasiliensis	Methanol/Ethanol/Acetone	500	–	–	–	–	–	–	–
		800	–	–	–	–	–	–	–
		1000	–	–	–	–	–	–	–

–, represents no activity. Values are the mean of triplicates ± SE. Statistical analysis was found to be significant at $p < 0.05$. Zone of inhibition including the diameter of the disc. Chloramphenicol for bacteria; nystatin for fungi.

toward different kinds of antimicrobial agents (List and Schmidt, 1989; Sader et al., 2002). On the other hand, *S. aureus* was the most susceptible bacteria of all the bacterial strains tested. Several reports suggest that *S. aureus* is the most common pathogen to cause skin infections (Satima et al., 1999; Jones et al., 2003). Thus, the fact that all selected plant species showing activity except *R. brasiliensis* and *B. latifolia* can be used for the treatment of skin infections. Although we observed no activity with *R. brasiliensis*, it does not mean that the plant lacks bioactive compounds. Doses employed to exhibit antimicrobial activity might be in low quantities (Mendonça-Filho, 2006; Stapleton et al., 2004; Ikigai et al., 1993; Brantner and Grein, 1994). This also suggests that usage of larger doses can overcome this problem Ogwal-Okeng et al., 2003; Rajeh et al., 2010. Further the dose employed and the types of bacterial and fungal strains used can be factors which influence its inhibitory effect (Tahiya et al., 2014).

5. Conclusion

Here, we show phytochemical screening of hexane, ethanol, acetone and methanolic extracts showed the occurrence of phenolics, alkaloids, flavonoids, tannins, saponins and terpenoids. We demonstrate acetone and hexane extracts exhibit maximum growth inhibition compared to methanolic and ethanolic extracts. Further we identify *B. laevicaulis* as a potential candidate for antimicrobial drug discovery based on our in vitro studies to treat infectious diseases and antibiotic resistant pathogens. Conclusions drawn are based on several different analyses as described in our paper. The present study is important as the search for new pharmacologically active compounds from plant extracts has led to the discovery of many clinically useful drugs. Nonetheless, the efficacy of these plant extracts needs to be validated in vivo. Importantly, as these extracts contain many compounds along with the active compounds they may cause side or toxic effects. Hence future directions should be focused on the isolation and identification of active compounds with antimicrobial activity rather than simply screening the plant crude extracts.

Acknowledgements

We would like to acknowledge Universiti Malaysia Kelantan for providing the facilities and laboratory assistance throughout the completion of this work. Sincere gratitude also goes to microbiology laboratory of Veterinary Research Centre and hospital Universiti Sains Malaysia for providing some microorganisms which were very helpful for this study. The authors are highly grateful to the UMK for their logistical support under Grant No. R/SGJP/A07.00/00710A/001/2012/000081).

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