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Research Article
Antimicrobial Activity of *Rubia cordifolia*: Methods to Determine Antimicrobial Activity

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Abstract

Objectives: The purpose of this study was to evaluate the antimicrobial effect of *Rubia cordifolia* root water and methanol extracts on various microorganisms using the agar well diffusion method and to evaluate the variants between the two techniques used in growing bacteria in the agar well diffusion method; the pour plate technique and the spreading technique. **Materials and Methods:** Water and methanol extracts of *R. cordifolia* roots were prepared and their antimicrobial effects on various microorganisms were evaluated by the agar well diffusion method. In agar well diffusion method, microorganisms were grown by either pour plate or spreading techniques. Statistical differences between the inhibition zones diameters resulted by using the spreading and the pour plate techniques were measured by ANOVA. **Results:** *Rubia cordifolia* root methanol extract showed antibacterial activity against all the three Gram-positive bacteria used in this study and four Gram-negative bacteria and showed antifungal activity against *Candida albicans*. Interestingly *R. cordifolia* root methanol extract showed activity against 3 out of 4 bacteria which showed resistance to all antibiotics used in this study. *Rubia cordifolia* root water extract showed antibacterial activity only against two Gram-positive bacteria. The study found that in agar well diffusion method using pour plate technique created significantly wider inhibition zone compared to the inhibition zone created by the spreading technique at similar concentrations of the extract. **Conclusion:** *Rubia cordifolia* root extracts showed antimicrobial effects. Using pour plate technique in agar well diffusion method is more sensitive in showing antimicrobial effectiveness than using spreading technique.

Key words: Agar well diffusion method, antimicrobial effect, pour plate technique, *Rubia cordifolia*, spreading technique

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The introduction of antibacterial drugs early 1930s and its highly effectiveness in controlling infectious diseases, made scientists optimist that infectious diseases could be controlled. There again the emergence of antibiotic resistant bacteria have proven this theory to be premature, the increasing numbers of antibiotic resistant bacteria[1] have worsen the patients outcome and increased the mortality all over the world[2,3]. A recent study by Lee *et al.*[1] found that bacteremia caused by antibiotic-resistant bacteria infections caused significantly higher mortality compared with non-antibiotic-resistant bacterial infections ($p<0.001$)[1]. Looking for new antimicrobial agents is a priority, one new/old approach is using medicinal plants. Many antimicrobial studies showed that plant crude extracts have bactericidal and fungicidal effects which could be a solution for the raising problems caused by the antibiotic-resistant bacteria[4-6].

Rubia cordifolia L., is a traditional Indian and Chinese medicinal plant which have been listed at the Chinese pharmacopoeia[6] in 2015. *Rubia cordifolia* is one of the 70 species that belong to the genus *Rubia*. *Rubia cordifolia* is widely distributed around the world, could be found in tropical Australia and tropical America, Western and Northern Europe, the Mediterranean and moderate temperature regions of Africa and Asia[7]. *Rubia cordifolia* is a perennial climbing plant, roots are long with a thin outer red layer while their stem are long with woody base[8].

Rubia cordifolia in traditional Chinese medicine is used to treat many diseases such as treating skin disease and cancer, as well as it's usage in skin care[8]. Moreover, *R. cordifolia* was proven to have anti-cancer[9], anti-inflammatory[10], antioxidant[11] and antimicrobial effects[12,13].

Antimicrobial activity of plant extracts is determined by different methods, the most widely used methods are the disk diffusion method, the broth or agar dilution method and the agar well diffusion method[5]. In the agar well diffusion method the microorganism being tested are either grown by the pour plate technique or by the spreading technique, these two methods of growing microorganisms may affect the antimicrobial activity results of a study.

The aim of this study was to evaluate the antimicrobial effect of *R. cordifolia* root water and methanol extracts on various facultative anaerobic microorganisms using the agar well diffusion method and to evaluate the variants between the two techniques used in growing bacteria in the agar well diffusion method; the pour plate technique and the spreading technique.

MATERIALS AND METHODS

Microbial organisms: Microorganisms used in this study were three Gram-positive bacteria (*Bacillus subtilis*, *Enterococcus faecalis* and *Staphylococcus aureus*), seven Gram-negative bacteria (*Acinetobacter baumannii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Salmonella enteritidis*) and *Candida albicans*. Microorganisms were obtained from Al-Balqa' Applied University, Zarqa College, microbiology laboratory.

Preparation of plant extracts for antimicrobial assay: *Rubia cordifolia* roots were dried and grinded. Water and methanol extracts were prepared by macerating the grinded roots in water or methanol (1:30) and heated to 50°C for 24 h on a rotary shaker, the solution was then filtered trice through filter paper (Whatman No. 1) using a suction pump and centrifuged at 6000 rcf for 10 min. The supernatant of the water extract was then lyophilized while the supernatant of the methanol extract was evaporated in a rotavapor. The dry crude extract was then collected and kept in air tight bottle at -20°C until use.

Antimicrobial activity: The antimicrobial activity of the water extract and the methanol extract was evaluated by the agar well diffusion method using Mueller Hinton Agar No. 2 (MHA) (Thermo Scientific), microorganisms being tested were grown on MHA by either pour plate technique or spreading technique. Briefly, the microorganism was grown on MHA at 37°C overnight, a loop full of the growth was then inoculated into Mueller Hinton broth (Thermo Scientific) and incubated at 37°C on a rotary shaker until the turbidity of the growth was equivalent to the density of 0.5 McFarland standard. The microorganism was then either inoculated (0.25 mL) into molten MHA and poured into petri dishes (pour plate technique), or spread (0.1 mL) on the surface of MHA (spreading technique). Wells of uniform diameter (6 mm) were then made on the solidified agar. About 0.1 mL of plant extracts at the designated concentration (50, 25, 12.5, 6.25, 3.13 and 1.56 mg mL^{-1}) and the negative control (solvent without plant extract) were placed separately in each well. Erythromycin 15 mcg, nalidixic acid 30 mcg, penicillin 10 U and tetracycline 30 mcg disks (6 mm in diameter) were used as positive control (Thermo Scientific). Plates were then left at room temperature for 1 h to allow the solutions diffusion into the MHA, plates were then incubated at 37°C over night. Finally, the zones of inhibition were

Table 1: Antimicrobial activity (inhibition zone in mm) of *R. cordifolia* methanol extracts on different microorganisms

	Inhibition zone[a] (±SD[b])											
	Pour plate technique spreading technique											
Tested microorganism	50	25	12.5	6.25	3.13	1.56	50	25	12.5	6.25	3.13	1.56
Bacillus subtilis	18.3 (±1.0)	15.8 (±0.8)	13.3 (±2.9)	12.7 (±1.8)	10.8 (±2.9)	9.2 (±1.5)	14.1 (±0.9)	12.6 (±0.8)	10.4 (±0.5)	8.8 (±1.2)	8.8 (±1.0)	7.8 (±0.4)
Enterococcus faecalis	14.7 (±3.9)	13.3 (±2.6)	12.8 (±2.9)	11.4 (±3.4)	9.4 (±2.2)	8.4 (±2.1)	12.9 (±0.9)	10.6 (±0.5)	9.5 (±0.6)	7.0 (±1.4)	Nil	Nil
Staphylococcus aureus	18.9 (±0.8)	16.4 (±0.7)	12.9 (±1.0)	11.0 (±2.3)	Nil	Nil	17.0 (±1.2)	15.8 (±1.5)	11.3 (±1.5)	9.0 (±1.8)	Nil	Nil
Acinetobacter baumannii	16.5 (±1.2)	15.2 (±0.8)	14.5 (±1.2)	14.8 (±1.2)	14.4 (±0.9)	12.8 (±0.5)	12.2 (±1.9)	11.7 (±2.8)	10.8 (±2.4)	11.3 (±1.5)	11.0 (±1.1)	10.7 (±2.7)
Enterobacter aerogenes	12.2 (±0.8)	11.2 (±1.2)	9.7 (±0.8)	8.3 (±0.5)	8.5 (±0.8)	8.5 (±0.6)	9.0 (±0.8)	8.8 (±1.5)	8.7 (±1.5)	8.3 (±1.2)	8.7 (±1.5)	7.3 (±0.6)
Escherichia coli	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Klebsiella pneumonia	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Proteus mirabilis	13.6 (±1.0)	12.0 (±1.4)	11.0 (±1.7)	9.8 (±1.3)	8.7 (±0.6)	8.5 (±0.6)	13.8 (±1.8)	11.0 (±2.0)	10.0 (±1.6)	9.3 (±0.6)	8.7 (±0.6)	8.0 (±0.8)
Pseudomonas aeruginosa	13.8 (±1.3)	11.0 (±1.4)	11.8 (±1.1)	11.7 (±1.0)	12.2 (±3.3)	9.2 (±1.2)	9.7 (±1)	10.3 (±3.0)	9.0 (±3.5)	8.8 (±2.6)	Nil	Nil
Salmonella enteritidis	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Candida albicans	15.2 (±2.1)	13.8 (±1.2)	13.0 (±1.7)	12.2 (±0.8)	11.5 (±0.8)	11.0 (±0.8)	14.5 (±1.6)	13.2 (±1.8)	12.0 (±0.7)	12.4 (±0.5)	10.6 (±0.9)	9.8 (±2.3)

[a]Inhibition zones presented are the average of three independent experiments each experiment was made in duplicate, inhibition zone diameter was measured including the diameter of the well (6 mm), [b]Standard deviation of the mean, Nil: No inhibition zones observed

measured from the base of the plate resting 5-7 cm above black flat surface and illuminated by reflecting light source[14]. Experiments were performed in duplicate and repeated independently three times.

Bauer-kirby disk diffusion test: Bauer-kirby disk diffusion test was used to measure sensitivity of bacteria to antibiotics[15]. Results were interpreted according to the clinical and laboratory standards institute (CLSI) document M100-S23[16].

Statistical analysis: Results presented are the mean of the three independent runs ±Standard Deviation (SD) of the inhibition zone diameter. Statistical differences between the inhibition zones diameter resulted by using the spreading technique compared to the inhibition zones diameter resulted by using the pour plate technique were measured by the analysis of variance (ANOVA). Fisher's exact test (two tailed) was used to examine which of *R. cordifolia* root extracts (methanol or water) had a greater antimicrobial effect.

The p-value ($p<0.05$) was defined as significant. The analyses were made using GraphPad Prism 5 software (San Diego, CA).

RESULTS AND DISCUSSION

Rubia cordifolia root methanol extract showed antibacterial activity against all the three Gram-positive bacteria used in this study (*B. subtilis*, *E. faecalis* and *S. aureus*) and four Gram-negative bacteria (*A. baumannii*, *E. aerogenes, P. mirabilis* and *P. aeruginosa*) and showed antifungal activity against *C. albicans*. While, *R. cordifolia* root methanol extract did not show antibacterial activity against three Gram-negative bacteria (*E. coli*, *K. pneumonia* and *S. enteritidis*) (Table 1). This is the first study to our knowledge that reported the antibacterial effect of *R. cordifolia* root methanol extract on *E. faecalis*, *A. baumannii*, *E. aerogenes* and *P. mirabilis*. A previous study by Basu *et al.*[13] found an antibacterial effect of *R. cordifolia* root methanol extract on the following Gram-positive and negative bacteria: *Bacillus cereus*, *Bacillus pumilus*, *B. subtilis*, *S. aureus* and *P. aeruginosa*, the study also found antibacterial effect on *Micrococcus luteus* and *Mycobacterium luteum* but the effectiveness was less pronounced when compared to other bacteria species included in the study[12].

Rubia cordifolia root water extract showed antibacterial activity against only two Gram-positive bacteria (*B. subtilis* and *E. faecalis*). While, *R. cordifolia* root water extract did not show antibacterial activity against one Gram-positive

Table 2: Antimicrobial activity (inhibition zone in mm) of *R. cordifolia* water extracts on different microorganisms

	Inhibition zone[a] (±SD[b])											
	Pour plate technique spreading technique											
Tested microorganism	50	25	12.5	6.25	3.13	1.56	50	25	12.5	6.25	3.13	1.56
Bacillus subtilis	12.5 (±2.6)	11.8 (±1.7)	10.0 (±1.6)	8.3 (±2.1)	7.7 (±1.5)	Nil	12.6 (±3.4)	9.8 (±0.8)	9.0 (±1.6)	8.1 (±2.0)	7.3 (±2.3)	Nil
Enterococcus faecalis	12.0 (±1.6)	9.7 (±1.5)	Nil	Nil	Nil	Nil	10.3 (±2.9)	8.3 (±2.6)	Nil	Nil	Nil	Nil
Staphylococcus aureus	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Acinetobacter baumannii	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Enterobacter aerogenes	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Escherichia coli	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Klebsiella pneumonia	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Proteus mirabilis	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Pseudomonas aeruginosa	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Salmonella enteritidis	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Candida albicans	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

[a]Inhibition zones presented are the average of three independent experiments each experiment was made in duplicate, inhibition zone diameter was measured including the diameter of the well (6 mm), [b]Standard deviation of the mean, Nil: No inhibition zones observed

bacteria (*S. aureus*), all Gram-negative bacteria and *C. albicans* (Table 2). This is the first study to our knowledge that reported the antibacterial effect of *R. cordifolia* root water extract on *E. faecalis*. A study by Basu *et al*.[13] found an antibacterial effect of *R. cordifolia* root water extract on *B. subtilis* and *S. aureus*[12]. The conflict of results found between our group and Basu group concerning the *S. aureus* sensitivity to water extract could be related to the strain that was used, it seems that our group used a resistant *S. aureus* strain. A very extensive genetic variation in *S. aureus* was reported by Fitzgerald *et al*.[17], the group found that 22% of the *S. aureus* genome comprised of dispensable genetic material, 10 out of 18 large regions of difference were antibiotic resistance genes.

It is worth to note that a study by Mariselvam *et al*.[18] found that the silver nano-particles prepared by using *R. cordifolia* plant root water extract showed antibacterial effect to *P. aeroginosa*, *Plesiomonas shigelloides* and *Vibrio parahaemolyticus*[17].

Findings shown in Table 1 and 2 demonstrates that *R. cordifolia* root methanol extract had antibacterial activity at all three Gram-positive bacteria while affecting only 4 out of 7 Gram-negative bacteria. A study by Basu *et al*.[13] showed that *R. cordifolia* methanol extract had antibacterial activity at all six Gram-positive bacteria while affecting only 1 out of the 6 Gram-negative bacteria used in study[12]. This results indicate that *R. cordifolia* root methanol extract has a stronger antibacterial effect against Gram-positive bacteria compared to Gram-negative bacteria, it seems that the antibacterial compounds extracted from *R. cordifolia* cannot pass the outer membrane of the Gram-negative bacteria. Delaquis *et al*.[19] showed that Gram-negative bacteria had more resistance to dill, cilantro, coriander and eucalyptus essential oil fractions when compared to Gram-positive bacteria, Delaquis group concluded that the differences between the cell envelope of Gram-positive and Gram-negative bacteria made the access of the fractionated antibacterial compounds more restricted in Gram-negative bacteria[18].

Fisher's exact test (two tailed) was used to compare the antimicrobial effectiveness of *R. cordifolia* root methanol extract (8 out of 11 microorganism) to *R. cordifolia* root water extract (2 out of 11), the test showed that *R. cordifolia* root methanol extract had significantly higher antimicrobial effect ($p = 0.03$) compared to *R. cordifolia* root water extract. The organic solvent methanol seems to extract antimicrobial compounds more effectively than water does. The traditional way of using medicinal plants is by water extracting active compounds (boiling, soaking and chewing), this way of extracting can miss out many other active antimicrobial compounds. A study by Rabe and van Staden[20] found after studying the crude extracts of 21 different South African medicinal plants that the majority of the antibacterial activity was present in the methanolic extract rather than the water extract[19].

The antimicrobial activity of 4 different antibiotics which were used as positive controls are shown in Table 3. Four Gram-negative bacteria (*A. baumannii, K. pneumonia, P. mirabilis* and *P. aeruginosa*) showed resistance to all antibiotics used in this study. Interestingly *R. cordifolia* methanol extract showed activity against 3 out of 4 of these resistant bacteria (*A. baumannii*, *P. mirabilis* and *P. aeruginosa*), making it a potential antibacterial agent for the mentioned antibiotic resistant bacteria. *Rubia cordifolia* methanol even had antifungal effect against *C. albicans* which gives it an advantage as a wide spectrum

Table 3: Antimicrobial activity (inhibition zone in mm) of different antibiotics and the antibiotic sensitivity interpretation according to Kirby-Bauer method

	Antibiotic zone of inhibition[a] (±SD[b])-sensitivity interpretation (Kirby-Bauer method)			
Tested microorganism	E15	NA30	P10	TE30
Bacillus subtilis	11.0 (±0.8)-R	18.5 (±0.7)-I	Nil-R	22.0 (±0.7)-S
Enterococcus faecalis	12.5 (±5.3)-R	Nil-R	26.8 (±4.4)-S	15.5 (±2.9)-I
Staphylococcus aureus	20.5 (±0.7)-I	16.5 (±0.6)-I	17.0 (±1.0)-R	31.0 (±1.4)-S
Acinetobacter baumannii	Nil-R	Nil-R	Nil-R	11.4 (±1.9)-R
Enterobacter aerogenes	Nil-R	24.0 (±0.8)-S	7.5 (±0.6)-R	6.5 (±0.7)-R
Escherichia coli	Nil-R	20.5 (±2.1)-S	14.5 (±0.7)-R	21.5 (±0.6)-S
Klebsiella pneumonia	Nil-R	Nil-R	Nil-R	8.0 (±0.0)-R
Proteus mirabilis	Nil-R	Nil-R	Nil-R	Nil-R
Pseudomonas aeruginosa	Nil-R	Nil-R	Nil-R	10.0 (±1.0)-R
Salmonella enteritidis	Nil-R	26.0 (±1.2)-S	-	23.5 (±1.3)-S
Candida albicans	Nil-R	Nil-R	Nil-R	Nil-R

[a]Inhibition zones presented are the average of three independent experiments each experiment was made in duplicate, inhibition zone diameter was measured including the diameter of the disc (6 mm), [b]Standard deviation of the mean, E15: Erythromycin 15 mcg, NA30: Nalidixic acid 30 mcg, NV30: Novobiocin 30 mcg, P10: Penicillin 10 U, TE30: Tetracycline 30 mcg, Nil: No inhibition zones observed, I: Intermediate, R: Resistant, S: Susceptible

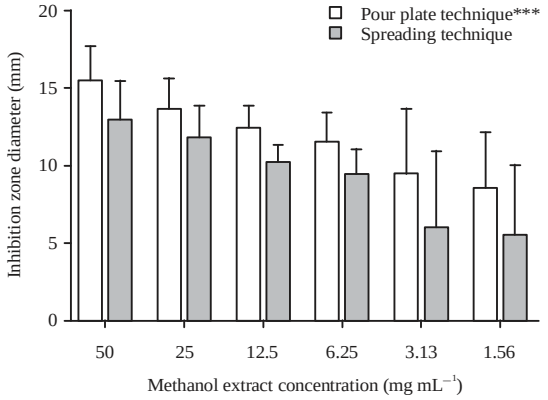

Fig. 1: Comparison between methanol extract of *R. cordifolia* antimicrobial activity on microorganisms at different concentrations using two techniques pour plate technique and spreading technique. Each column represent the average inhibition zone for different microorganisms (bacteria and *Candida albicans*) ±SD at a given methanol extract concentration, ***p = 0.0001

antimicrobial agent. It is worthy to note that a study by Sawhney *et al*.[21] found that *R. cordifolia* ethanol extract had an inhibitory effect on extended spectrum β-lactamase producing urinary *E. coli*[20].

We used *R. cordifolia* methanol extract effect (inhibition zone diameter) at different concentrations on different microorganisms (we did not include the microorganisms that methanol extract had no effect on them) to find which of the two techniques; pour plate or spreading gives a wider inhibition zones at similar concentration, it was found that using pour plate technique created significantly (p = 0.0001, ANOVA) wider inhibition zone compared to the inhibition zone created by using the spreading technique at similar concentration of the methanol extract (Fig. 1), this finding point out that using pour plate technique in agar well diffusion method is more sensitive in showing antimicrobial effectiveness than using spreading technique. This is the first study to our knowledge that shows the previous finding.

Pour plate and spreading techniques had similar trends in showing antimicrobial effectiveness either by using methanol extract or water extract, except when using methanol extract at concentrations 3.13 and 1.56 mg mL^{-1} on *E. faecalis* and *P. aeruginosa* bacteria, pour plate technique showed antibacterial effect while using the spreading technique no effect was shown (Table 1, 2). This result indicate that when using agar well diffusion to measure antimicrobial effectiveness of a compound (extract) at low concentrations, it is more relevant to measure effectiveness by using pour plate technique rather than using spreading technique. A study by Valgas *et al*.[22] found that well diffusion method is more sensitive than disc diffusion method in determining the antibacterial activity[21].

CONCLUSION

Rubia cordifolia root extract showed antimicrobial effects, interestingly *R. cordifolia* root methanol extract showed activity against 3 antibiotic resistant bacteria included in this study. It was also concluded that using pour plate technique in agar well diffusion method is more sensitive in showing antimicrobial effectiveness than using spreading technique.

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