

Full Length Research Paper

Antimicrobial activity and phytochemical screening of various parts of *Ixora coccinea*

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Ixora coccinea L. (Rubiaceae) has been used traditionally for a variety of ailments and also cultivated for ornamental purposes. The present study investigated antimicrobial activity of methanolic extracts of various parts of *I. coccinea* and determined the chemical groups of the active constituents. Antimicrobial activity was assessed using agar disc diffusion, microdilution and thin layer chromatography (TLC) bioautography assays. Methanolic extracts of leaf, flower and stem of *I. coccinea* displayed good antimicrobial activity, with inhibition zone in the range of 6.7 to 11.3 mm. minimum inhibitory concentration (MIC) values for all three extracts ranged from 0.78 to 3.125 mg/ml. Leaf and stem extracts of *I. coccinea* showed broad-spectrum antimicrobial activity. Of interest, stem extracts had MIC values against *Staphylococcus aureus* that were only 62.4 times less potent than the vancomycin. Likewise, leaf and stem extracts displayed good antimicrobial activity of 62.4 and 31.2 times, respectively lesser than gentamycin against *Shigella flexneri*. Minimum bactericidal/bacteriostatic concentration (MBC) values for active extracts ranged from 0.78 to 6.25 mg/ml. TLC bioautography and phytochemical screening of the leaf and stem extracts showed that the antimicrobial activity of these extracts may be attributed to compounds belonging to terpenoid, flavonoid, coumarin, alkaloid and phenolic groups.

Key words: Antimicrobial, *Ixora coccinea*, minimum inhibitory concentration, minimum bacteriostatic/bacteriocidal concentration, bioautography.

INTRODUCTION

Plants are the oldest source of pharmacologically active substances and have provided humans with many medically useful compounds (Cordell, 1981). Plants produce a diverse array of secondary metabolites, many of which have antimicrobial activity. Hence, natural products in particular medicinal plants remain as a potential source of new antimicrobial agents (Cowan, 1999). The increasing prevalence of multidrug resistant strains of

bacteria and the recent appearance of strains with reduced susceptibility to clinically used antibiotics (Rubiaceae) is a small evergreen flowering shrub found throughout Asia (Latha and Pannikar, 1998). It has been raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Sieradzki et al., 1999). Therefore, there is a continuous search for substances from plant sources with

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proven antimicrobial activity. *Ixora coccinea* L. used traditionally for a variety of ailments and also cultivated for ornamental purposes. The leaves are used to treat diarrhoea, the roots are used to treat hiccup, fever, sores, chronic ulcers and skin diseases. The flowers have been used in catarrhal bronchitis and dysentery (Sivarajan and Balachandran, 1941). It is reported that this plant contains anthocyanins in flowers; methyl ester of palmitic, stearic, oleic and linoleic acids in root oil; octadecadienoic acid, saponins and tannins from root bark (Chopra et al., 1956; Grainge and Ahmed, 1988), alkaloids, flavonoids, saponins, sterols, terpenes and phenols (Annapurna et al., 2003). The aqueous extract of the *I. coccinea* demonstrated antinociceptive, anti-inflammatory and antitumor effects in mice (Rathnasooriya et al., 2005). Annapurna et al. (2003) has reported the antimicrobial activity of *I. coccinea* leaf extract based on disc diffusion method. However to date, there is no in depth antimicrobial evaluation done on this plant. With this in view, the present study was undertaken to evaluate in detail the antimicrobial activity of methanolic extract of various parts of *I. coccinea* against series of microorganisms and to identify the chemical groups of the bioactive constituents.

MATERIALS AND METHODS

Chemicals and reagents

Chloramphenicol was purchased from Acros Organics (New Jersey, USA). Gentamycin and amoxicillin were purchased from Sigma Aldrich (Steinheim, Germany) while vancomycin, miconazole and *para* iodinitrotetrazolium (INT) were purchased from Sigma (St. Louis, USA). Amphotericin B was purchased from Himedia (Mumbai, India). Penicillin G, gentamycin, chloramphenicol, amoxicillin and tetracycline impregnated discs were purchased from Oxoid (Hampshire, England). Muller Hinton agar, Muller Hinton broth, Potato Dextrose agar and methanol were purchased from Merck (Darmstadt, Germany) while potato dextrose broth was purchased from Lab Scan Analytical Sciences (Bangkok, Thailand). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Leicestershire, UK).

Plant material and extraction

Fresh plant materials of *I. coccinea* (leaves, flowers and stem) were collected from Penang, Malaysia. A voucher specimen (Collection No. 11038) has been deposited at the Herbarium of School of Biological Sciences, Universiti Sains Malaysia. The plant parts were washed with water to remove dirt prior to the drying process at 40°C for a week. The dried plant materials were powdered (100 gm) and extracted repeatedly with methanol (500 ml) by maceration for a week. Fresh methanol was replenished every two days. The extracts were filtered and the filtrates were concentrated *in vacuo* using rotary evaporator at 45°C. The concentrated extracts were subjected to freeze drying to obtain dry powdered extracts.

Microorganisms and media

Test microorganisms were obtained from the School of Biological Sciences and School of Pharmaceutical Sciences, Universiti Sains

Malaysia, Penang, Malaysia. The test microorganisms used in this study were: *Bacillus cereus* (ATCC 10876), *Streptococcus pneumoniae* (ATCC 6303), *S. aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (laboratory strain), *S. flexneri* (ATCC 12022), *Enterobacter aerogenes* (laboratory strain) and *Saccharomyces cerevisiae* (laboratory strain). The strains were maintained and tested on Muller Hinton agar (bacteria) and potato dextrose agar (fungus). For the antimicrobial tests, strains were grown overnight in Muller Hinton agar (bacteria) and Potato Dextrose agar (fungus) in an anaerobic chamber at 37°C under atmosphere consisting 10% CO₂, 10% H₂O and 80% N₂.

Antimicrobial evaluation

Agar disc diffusion assay

The antimicrobial activity of different parts of *I. coccinea* was initially evaluated by agar disc diffusion assay (Bauer et al., 1966). 20 ml Muller-Hinton agar for bacteria or potato dextrose agar for fungi, sterilized in a flask and cooled at 45 to 50°C, were transferred to petri dishes (diameter of 90 mm) and allowed to solidify. Inoculum were prepared by mixing a few microbial colonies with Muller Hinton broth (bacteria) or potato dextrose broth (fungi) and incubated at 37°C for 3 h to get an approximately standard 0.5 Mac Farland solution. Then, inoculum suspensions were streaked over the surface of the media on each plate using sterile cotton swab to ensure the confluent growth of the organism. For antimicrobial testing, a 50 mg/ml stock solution of each extract (leaf, flower and stem) was prepared in methanol. Whatman filter paper No.1 discs of 6 mm diameter were used in this assay. Each sterile disc was impregnated with 20 µl of various extracts of *I. coccinea* (concentration 1 mg/disc) and then placed onto the agar plates which had previously been inoculated with the test microorganisms with sterile forceps and pressed gently to ensure contact with the inoculated agar surface. Amoxicillin (30 µg/disc), penicillin G (10 µg/disc), vancomycin (10 µg/disc), chloramphenicol (30 µg/disc), gentamycin (10 µg/disc), tetracycline (30 µg/disc) and miconazole (30 µg/disc) were used as positive controls while methanol (20 µl) was used as the negative control.

Finally, the inoculated plates were incubated at 37°C for 18 h and at 28°C for 48 h for the bacteria and fungi, respectively. The diameters of the inhibition zones were measured in millimeters. All measurement was carried out in triplicate. Extracts showing inhibition zones of more than 8 mm were selected for subsequent determination of MIC, MBC and minimum fungicidal concentration (MFC).

Determination of minimum inhibitory concentration (MIC)

The MIC of *I. coccinea* extracts were determined by serial two-fold dilution method as described by Eloff (1998). The extracts were initially dissolved in 50% DMSO to make up a concentration of 50 mg/ml. Then stock solution was serially diluted two-fold by using Muller Hinton broth (MHB) as diluents. Each well was inoculated with 100 µl of suspension containing 1.5×10⁸ CFU/ml (equivalent to McFarland 0.5) of the culture. Final concentration of extracts and positive controls ranged from 25 to 0.006 and 0.4 to 0.00039 mg/ml, respectively. The 96 well plates were incubated at 37°C for 18 h for bacteria and at 28°C for 48 h for fungus. Amoxicillin, chloramphenicol, vancomycin and gentamycin (for bacteria) and miconazole (for fungus) served as positive controls, respectively while 50% DMSO was used as negative control. Microbial growth was evaluated by addition of 50 µl of 0.2 mg/mL of freshly prepared solutions of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl-tetrazolium bromide) or INT (*para* iodinitrotetrazolium) dissolved in water into each of the microplate wells. The covered microplates

Table 1. Zone of inhibition (mm) by various extracts of *I. coccinea* and reference antibiotics.

Strain	Mean zone of growth inhibition (mm) ^a (n=3)									
	<i>I. coccinea</i> extracts (1mg/disc)			Reference antibiotic (µg/disc)						
	Leaf	Flower	Stem	AMX 30	PEN G 10	VC 10	CMC 30	GM 10	MICO 30	M
Gram positive bacteria										
<i>Bacillus cereus</i>	7.0±1.0	7.0±0	6.0±0	12.7±0.6						-
<i>Streptococcus pneumoniae</i>	9.0±0	8.3±0.6	8.3±0.6		28.7±0.6					-
<i>Staphylococcus aureus</i>	8.0±0	9.0±0	9.0±0			13.0±0				-
Gram negative bacteria										
<i>Escherichia coli</i>	6.0±0	6.7±0.6	8.7±0.6				19.7±0.6			-
<i>Klebsiella pneumonia</i>	8.7±0.6	6.0±0	8.7±0.6					19.0±0		-
<i>Shigella flexneri</i>	8.8±0.6	8.0±0	9.0±0				20.3±0			-
<i>Pseudomonas aeruginosa</i>	8.0±0	9.0±0	9.0±0				20.0±0			-
<i>Enterobacter aerogenes</i>	10.0±0.5	8.0±0	9.0±0					29±0		-
Fungi										
<i>Saccharomyces cerevisiae</i>	11.0±0	10.0±0	11.3±0.6						12.7±0.6	-

^a Diameter of inhibition zone including disc diameter of 6.0 mm. Values for growth inhibition are presented as mean±SD; '-' no inhibition zone observed; AMX, amoxicillin; PEN G, penicillin G; VC, vancomycin; CMC, chloramphenicol; GM, gentamycin; MICO, miconazole; M, methanol.

were incubated further for 30 min. MIC was defined as the lowest drug concentration of extract inhibiting growth of microorganism and there was no colour changes observed in the wells from yellow to purple for MTT and colourless to pink for INT.

Determination of MBC and MFC

MBC or MFC was defined as the lowest concentration of extracts that showed complete inhibition of colonies of microorganisms on agar plates. A loopful of each bacterial or fungal culture in each microplate well was inoculated to the culture medium agar plate and incubated under same conditions as described earlier (Shin et al., 2004). Each assay was carried out in triplicate.

Bioautography

Bioautography was performed using bacterial cultures (*S.*

aureus and *S. flexneri*) which were found to be the most susceptible to leaf and stem extracts following method described by Masoko and Eloff (2005). The developed TLC plates were carefully dried and inoculated with fine spray of the concentrated suspension of actively growing microorganisms containing 10⁹ cfu/ml approximately. The plates were incubated overnight at 37°C and then sprayed with 2 mg/ml solution of INT and further incubated for 2 to 3 h. The presence of white spots indicates presence of compounds that inhibited the growth of tested microorganisms.

Phytochemical screening

The leaf and stem extracts of *I. coccinea* were found to have antibacterial effect, thus were selected for phytochemical screening by TLC. TLC was performed on aluminium-backed TLC plates (Merck, Silica gel 60 F₂₅₄). Plates (10 × 5 cm) were loaded with 10 spots (20 mg/ml) of each extract. The prepared plates were developed with

chloroform: methanol (9.5: 0.5). The components were visualized under visible and ultra violet light (254 and 365 nm), respectively. The developed plates were sprayed with the following reagents for detection of respective chemical groups: natural products reagent for flavonoid (Wagner et al., 1984), aluminium chloride for phenolic compounds, methanolic potassium hydroxide for coumarins (Harbone, 1973) and Dragendorff's reagent for alkaloids and Liebermann-Burchard reagent for terpenoids (Krebs et al., 1969).

RESULTS

Methanolic extracts of various parts of *I. coccinea* were evaluated for antimicrobial activity on nine pathogenic microorganisms by using agar disc diffusion assay and microdilution assay. The growth inhibition zones measured in agar disc diffusion assay are presented in Table 1. The disc

Table 2. Minimum inhibitory concentration (MIC) of various extracts of *I. coccinea* and reference antibiotics.

Strain	Minimum inhibitory concentration (mg/ml), n=3							% DMSO not inhibiting microorganisms
	<i>I. coccinea</i> extracts				Reference antibiotic			
	Leaf	Flower	Stem	AMX	VC	GM	MICO	
Gram positive bacteria								
<i>Streptococcus pneumoniae</i>	1.56 (500×)	3.125 (1000×)	0.78 (250×)	0.003125				12.5
<i>Staphylococcus aureus</i>	1.56 (125×)	12.5 (1000×)	0.78 (62.4×)		0.0125			25.0
Gram negative bacteria								
<i>Klebsiella pneumoniae</i>	1.56 (2000×)	3.125 (4000×)	1.56 (2000×)			0.00078		12.5
<i>Shigella flexneri</i>	1.56 (62.4×)	1.56 (62.4×)	0.78 (31.2×)			0.025		12.5
<i>Enterobacter aerogenes</i>	1.56 (125×)	3.125 (250×)	1.56 (125×)			0.0125		25.0
Fungi								
<i>Saccharomyces cerevisiae</i>	1.56 (250×)	1.56 (250×)	0.78 (125×)				0.00625	25

AMX, amoxicillin; VC, vancomycin; CMC, chloramphenicol; GM, gentamycin; MICO, miconazole; Values given in bracket is the number of times the extract is less potent than the reference antibiotics.

diffusion results showed the *I. coccinea* extracts have potential antimicrobial activity against all tested bacteria strains and fungi *S. cerevisiae* with zone of inhibition between 6.7 to 11.3 mm. However their antimicrobial effects were found to be less potent than the reference antibiotics. Methanol used as negative control showed no inhibitory effect against the microorganism's growth. In subsequent experiments, the MIC, MBC and MFC values of the *I. coccinea* extract were determined against selected susceptible microorganisms. The results are summarized in Tables 2 and 3. The MIC, MBC and MFC values of the test extracts of *I. coccinea* ranged from 0.78 to 6.25 mg/ml.

In general, stem extract of *I. coccinea* was found to be most active extract followed by the leaf extract. Flower extracts was found to be the least active. Interestingly, the stem extract was found to have good antimicrobial activity against

S. aureus and *S. flexneri* with MIC value of only 0.78 mg/ml. By comparing MIC values, the stem extract was found to be only 62.4 times less potent than vancomycin for *S. aureus* and 31.2 times less potent than gentamycin for *S. flexneri*. The stem extract of *I. coccinea* was also found to have good antimicrobial activity against *S. cerevisiae*. DMSO showed no toxic effect at 25% for *E. aerogenes*, *S. cerevisiae* and *S. aureus* and at 12.5% for the rest of the microorganisms. Based on MIC results, MBC and MFC were determined for the active extracts against selected microorganisms. Antimicrobial substances are considered as bacteriostatic agents when the ratio MBC/MIC > 4 and bactericidal agents when the ratio MBC/MIC ≤ 4 (Gatsing et al., 2009). The summary of the microbicide and microbistatic effects were given in Table 4. In the present study, leaf and stem extract showed the ratio MBC/MIC ≤ 4 for most of the test microbes,

suggesting that these extracts may act as bactericidal agent on these strains. Stem extract showed MBC/MIC ratio of more than 4 on *S. aureus* and *S. flexneri* which may be classified as bacteriostatic agent.

The leaf and stem extract were further subjected to bioautography and phytochemical screening. Bioautography results demonstrated strong inhibition zones of these extracts against the growth of *S. aureus* and *S. flexneri* (Figure 1a and b; Table 5). There are few inhibition spots noted suggesting that more than one compound may be responsible for the observed antimicrobial effect. Interestingly, the active compounds (spots label A and D) of leaf extract which inhibited both microorganisms have almost same R_f values. The active compounds of leaf extract may belong to terpenoids, flavanoids, coumarins and alkaloids groups of compounds. As for the stem extract, spot B (R_f value of 0.39) and C (R_f value of 0.18),

Table 3. Minimum bactericidal/bacteriostatic concentration (MBC) and minimum fungicidal/fungi static (MFC) concentration of various extracts of *I. coccinea*

Strain	MBC and MFC (mg/ml), n=3					
	<i>I. coccinea</i> extracts			Reference antibiotic		
	Leaf	Flower	Stem	VC	GM	MICO
Gram positive bacteria						
<i>Staphylococcus aureus</i>	6.25 (250×)		6.25 (250×)	0.025		
Gram negative bacteria						
<i>Klebsiella pneumoniae</i>	6.25 (500×)		6.25 (500×)		0.0125	
<i>Shigella flexneri</i>			6.25 (62.5×)		0.1	
<i>Enterobacter aerogenes</i>	3.125 (16×)		3.125 (16×)		0.2	
Fungi						
<i>Saccharomyces cerevisiae</i>			0.78 (62.4×)			0.0125

VC, vancomycin; GM, gentamycin; MICO, miconazole. Values given indicate bracket is the number of times the extract is less potent than the reference antibiotics.

Table 4. Summary of the microbicide and microbiostatic effects of *I. coccinea* extracts on selected strains.

Extracts of various part of <i>I. coccinea</i>	Microbicide effect on specific strains	Microbiostatic effect on specific strains
Leaf	<i>Enterobacter aerogenes</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i>	-
Stem	<i>Saccharomyces cerevisiae</i> , <i>Enterobacter aerogenes</i> , <i>Klebsiella pneumoniae</i>	<i>Staphylococcus aureus</i> , , <i>Shigella flexneri</i>

Table 5. Summary of the bioautography of *I. coccinea* extracts against selected micro-organisms.

Extract	Microorganism	A	B	C	D	E	F
		Inhibition zone					
Terpenoid		+	+	+	+	+	+
Flavonoid		+	+	+	+	+	+
Coumarin		+	+	+	+	+	+
Alkaloid		+	-	-	+	-	-
Phenol		-	-	+	-	-	+
R _f value		0.37	0.39	0.18	0.33	0.38	0.16

Growth inhibition of microorganisms: '+' present; '-' absent.

which inhibited *S. aureus* were similar to the spot E (R_f value of 0.38) and F (R_f value of 0.16) which inhibited *S. flexneri*. These spots may consist of terpenoids, flavanoids, alkaloids, coumarins and phenolic compounds.

DISCUSSION

The antimicrobial activities of medicinal plants have been reported by many researchers (Cowan, 1999; Motamedi

et al., 2010; Shariff, 2001; Shin et al., 2004). Most antibacterial medicinal plants are active against gram-positive strains while few are active against gram-negative bacteria (Herrera et al., 1996; Meng et al., 2000; Scrivinasan et al., 2001). In the present study, antimicrobial activity of methanolic extracts various parts of *I. coccinea* was evaluated against series of micro-organisms. Among all three extracts of *I. coccinea*, leaf and stem extracts were found to be more active than flower extract. In particular, the leaf and stem extracts were active against few pathogenic microorganisms such

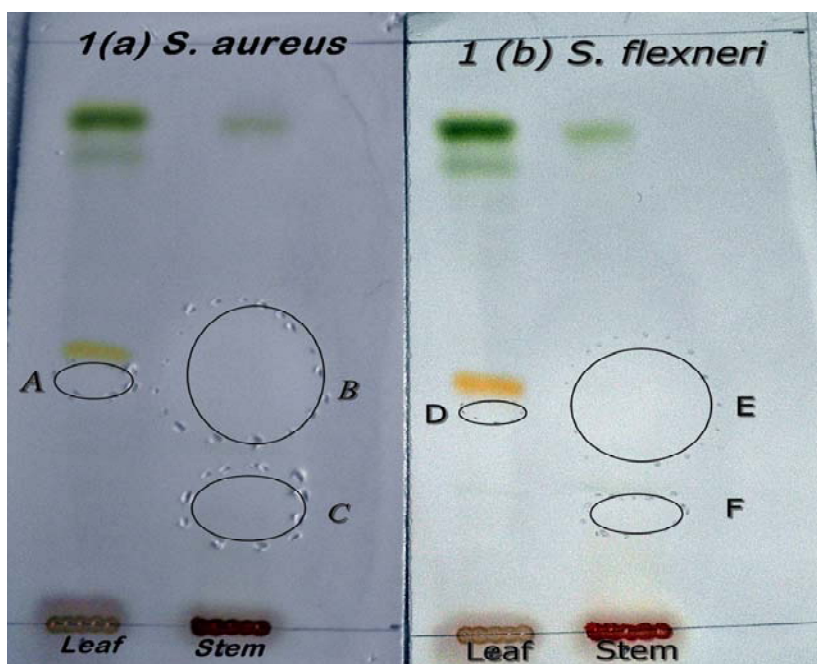


Figure 1. Bioautography of *I. coccinea* extracts against selected microorganisms. (Labelled spots indicate growth inhibition of microorganisms).

K. pneumonia, *S. aureus* and *S. flexneri*. Therefore, these extracts may be potential source leads for antimicrobial agents against these microorganisms. Annapurna et al. (2003) has previously reported on screening of antimicrobial activity of *I. coccinea* leaves extract using disc diffusion assay. The present findings were similar to the study whereby leaf extract was active against *S. aureus* with inhibition zone of 18 mm. Studies that used agar disc diffusion to detect antimicrobial activity of plant extract revealed poor accuracy and difficult nature of the method. According to Allen et al. (1991), low levels of antimicrobial activity of plant extracts are not detectable in agar disc diffusion method. MIC of *I. coccinea* extracts was carried out using microplate assay because this assay is based on correlation of bacterial growth with inhibitory effect of plant extract at certain concentration. This method has been identified as being more accurate than agar disc diffusion and less tedious (Eloff, 1998). *S. aureus* and *S. flexneri* was the most susceptible microorganisms against *I. coccinea* extracts (leaf and stem) with lowest MIC value 0.78 mg/ml.

In addition, these extracts were found to be bacteriostatic *S. aureus* and *S. flexneri*. According to Van and Viljoen (2009), as DMSO may exhibit antimicrobial efficacy, MIC values equivalent to or greater than that found for the DMSO control should be omitted from the data and considered not susceptible. In the present study, DMSO was found to have no inhibitory effect at the MIC values of the active extracts. Subsequently, the active constituents were identified by TLC bioautography and characterized by spraying with various reagents.

Bioautography is a useful technique for detecting bioactive compound(s) as well as indicator for separation technique during bioassay-guided isolation of active compounds (Masoko et al., 2007). For each extract, more than one active compound was identified. A group of compounds belonging to the terpenoids, flavanoids, alkaloids, coumarins and phenolics family may be responsible for the antimicrobial activity of stem and leaf extracts. These compounds are producing exciting opportunity for the expansion of modern chemotherapies against wide range of microorganisms (Lutterodt et al., 1999).

Conclusions

I. coccinea extracts have potential to be developed as antimicrobial agents, in particular against *S. aureus* and *S. flexneri*. Further studies on isolation and identification of the active principles and evaluation of possible synergism among these constituents for their antimicrobial activity are currently ongoing.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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