Toxicology International, *Vol 27(3&4), 193-201, 2020, DOI: 10.18311/ti/2020/v27i3&4/26169*

Original Research
ISSN (Print): 0971-6580
ISSN (Online): 0976-5131

Evaluation of the Antibacterial Activity of Isolated Anthraquinones from the Root of *Rubia cordifolia* Linn

Pottella Srinivasulu[1,2], Padarthi Pavan Kumar[2], Ch Aruna Kumar[2], J. Venkateswara Rao[3], S. Vidyadhara[3] and Janga Ramesh Babu[3]*

[1]Acharya Nagarjuna University, Guntur – 522510, Andhra Pradesh, India

[2]Department of Physiology and Pharmacology, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram – 522019, Andhra Pradesh, India

[3]Department of Pharmaceutics, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram – 522019, Andhra Pradesh, India; ramesh.janga11@gmail.com

Abstract

The present study was intended to screen the antibacterial activity of anthraquinones i.e. 2-Methyl Anthraquinone (MAQ) and Quinizarine (QNZ), isolated from roots of *Rubia cordifolia* Linn. Minimum Inhibitory Concentration (MIC) of isolated anthraquinones was evaluated against selected gram-positive [*Bacillus subtilis* (BS) and *Staphylococcus aureus* (SA)] and gram-negative [*Serratia marcescens* (SM) and *Escherichia coli* (EC)] bacterial strains. The MIC was determined by micro broth dilution method using Tryptone Soya Broth (TSB) in 96 well plates as well as by measuring zone of inhibition using nutrient agar medium by the cup-plate method. Absorbance values after micro broth dilution revealed the significant MIC of MAQ as 40 µg/mL against BS, 40 µg/mL of MAQ against SA and 80 µg/mL of MAQ against SM. Whereas, The MIC of QNZ was 20 µg/mL against EC. Percentage zone of inhibition after cup-plate method revealed the significant MIC of MAQ as 39.8% against BS when compared with vancomycin, 88.33% against SA when compared with linezolid, 53.37% against SM when compared with amikacin. Whereas, percentage zone of inhibition revealed the significant MIC of QNZ as 53.76% against EC when compared with ciprofloxacin. Based on the MIC results after both micro broth dilution and cup-plate method, we observed that MAQ has shown considerable antibacterial activity against selected bacteria.

Keywords: MIC, Micro Broth Dilution, Quinizarine, 2-Methyl Anthraquinone

1. Introduction

The most attractive area in the line of the discovery of new bioactive molecules is exploration of metabolites, as most of the metabolites proven to have antimicrobial activity. The plants, microbes and the symbiotic association of these two may produce a huge variety of metabolites. The plants may act as sheltered habitats of microorganisms, the sheltered habitats, habitats near to plant sources, i.e. soil, the roots of the plants on which microorganisms depend for their food and nutrition may have close contact. Thereby the root extracts may become the better source of secondary metabolites with antimicrobial activity[1–2].

Recently, there is much need to discover the new bioactive compounds as an important groups of antibiotics are in danger of losing their antimicrobial activities because of microbial resistance[3]. These situations provoke the minds of researchers to explore the plant and microbial worlds, as they can provide a huge range of complex and structurally different compounds, for new bioactive metabolites or compounds. The fact that a plant or microbial extract is believed to exhibits antimicrobial activity, to make it trustworthy, the researcher needs to estimate the therapeutic efficacy and spectrum of activity of the newly isolated compounds. A variety of laboratory methods can be used

*Author for correspondence

to evaluate or screen and compare the *in vitro* antimicrobial activity of an extract with that of the well established antimicrobial agents[4].

The most known and basic methods are disk-diffusion and broth or agar dilution methods. To study the antimicrobial activity of an agent in-depth, time-kill test and flow-cytofluorometric methods can be used, which provide information on the nature of the inhibitory effect (bactericidal or bacteriostatic) (time-dependent or concentration-dependent) and the cell damage inflicted to the test microorganism. A number of biological constituents including anthraquinones are believed to show some sort of antimicrobial activity[5].

The roots of *Rubia cordifolia* Linn[6] (RC) play important role in folk medicine such as antioxidant[7], anti cancer[8], urolithiasis[9], anti-hyperglycemic, nootropic activity[10] and antibacterial activity[11]. Anthraquinones are abundantly present in the RC[12–16] and believed to have antibacterial activity[17]. Several biological constituents including anthraquinones are believed to show some sort of antimicrobial activity. Hence, we isolated the anthraquinones named as 2-Methyl Anthraquinone (MAQ) and Quinizarine (QNZ) from the root of RC. Keeping in mind, the importance of drug resistance and utilization of plant based material for prevention of drug resistance; it has been decided - to evaluate the antibacterial activity of isolated anthraquinones against two gram-positive and two gram-negative bacterial strains.

2. Materials and Methods

Quinizarine (QNZ; CAS number: 81-64-1) and 2-Methyl Anthraquinone (MAQ; CAS number: 84-54-8) and Di Methyl Sulfoxide (DMSO) were procured from Sigma Aldrich, India. Peptone, meat extract B, agar and other culture components includes *Bacillus subtilis* (BS) [Gram positive; MTCC – 1134], *Staphylococcus aureus* (SA) [Gram positive; MTCC –1144], *Serratia marcescens* (SM) [Gram negative; MTCC – 86] and *Escherichia coli* (EC) [Gram negative; MTCC – 1089] were procured from HiMedia Laboratories, India. All standard compounds Vancomycin (VM), Linezolid (LZ), Amikacin (AK) and Ciprofloxacin (CF) received as a gift sample from Alkem Laboratories, Mumbai, India. All the bacterial pure cultures were procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India.

3. Determination of MIC

3.1 Preparation of Test and Standard Samples

Test samples were prepared by using DMSO as a solvent. Concentrations of the test (MAQ and QNZ) and standard [Vancomycin (VM), Linezolid (LZ), Amikacin (AK) and Ciprofloxacin (CF)] compounds were prepared using 10X as dilution factor such as from 10 to 100µg/mL. The DMSO was used as control along with test compounds.

3.2 Preparation of Tryptone Soya Broth (TSB)

Accurately weighed the components such as Pancreatic digest of casein (1.7 g), Papaic digest of soya bean meal (0.3 g), Sodium chloride (0.5 g), Dextrose (0.25 g) and Dibasic potassium phosphate (0.25 g), were used to prepare 100 mL of TSB. Casein and soya bean helping the growth of microorganisms by providing the amino acids and peptides. Whereas, dextrose as carbohydrate and dibasic potassium phosphate as a buffer for maintaining the pH of the medium. After preparation of TSB, the medium was adjusted to pH-7.2 ± 0.3. Taken 5 mL of TSB into each of the test tubes and sterilized by autoclaving at 121^{0}C, 15lbs pressure for 15 minutes[18].

3.3 Preparation of Inoculum

The Pure cultures of *Staphylococcus aureus* (SA) and *Escherichia coli* (EC) were cultured in the sterile nutrient broth before the day of assay. The pellet formed was shaken and used as the test organism.

3.4 Broth Culture Method

The grown colonies from the inoculum were contacted with a loop and transferred into a TSB medium. The medium was incubated at 30-35°C for three days until a match with a 0.5 McFarland standard. If necessary, the culture was diluted with distilled water. The comparison of prepared suspension of culture with that of McFarland standard was done by photometric method at 625nm UV spectrophotometry[19–20].

3.5 Procedure for Determination of the Minimum Inhibitory Concentration

The lowest concentration of a test substance that killed all the microorganisms present in a given sample under standard conditions is called the Minimum Inhibitory Concentration (MIC). Various concentrations of the test and standard compound such as 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/mL were prepared using suitable solvents. The 96 well Microtitre Plate (MTP) was used for analysis and labeled the plate as per the number of samples, then added 100 µL of broth medium into each well Then, added 5 µL of inoculum to the respective columns in the well, (1.5 x 108 CFU/mL) for this assay. Later, added the test, standard compounds and blank to the assigned wells of MTP. Afterwards, incubated the MTP at 37°C in an incubator and observed the growth after 24 hours. After getting growth, the MTP scanned using ELISA plate reader and calculated the MIC.

4. Determination of antimicrobial activity

4.1 Preparation of Standard and Test Samples

The standard and test samples were prepared by using DMSO as a solvent. The concentrations of MAQ and QNZ compounds were 10 to 90, 100, 200, 400, 600, 800, 1000 µg/ml for testing antibacterial activity, whereas, Vancomycin (VM), Linezolid (LZ), Amikacin (AK) and Ciprofloxacin (CF) as 100 µg/ml were used as standard and DMSO was used as control.

4.2 Inoculum Preparation

The following bacterial pure cultures were used for evaluation of antibacterial activity: *Bacillus subtilis* (BS) [Gram positive; MTCC – 1134], *Staphylococcus aureus* (SA) [Gram positive; MTCC –1144], *Serratia marcescens* (SM) [Gram negative; MTCC – 86] and *Escherichia coli* (EC) [Gram negative; MTCC – 1089]. The above bacterial pure cultures were subcultured 24 hours before the test day. The fully grown cultures were used in the test on the day of the assay.

4.3 Preparation of Nutrient Agar Medium

As per the Indian Pharmacopeia, composition of nutrient agar medium[13–14] for 100 ml is peptone (1 gm), meat extract (1 gm), sodium chloride (0.5 gm), agar (1.2 g). Taken the accurate and calculated quantities of each of the ingredients and dissolved in some quantity of distilled water, warmed gently to dissolve all the ingredients and made up to the quantity sufficient. Checked and maintained the pH at 7.2. The nutrient agar medium was sterilized by autoclaving at 121^{0}C, 15 lbs pressure for 15 minutes.

4.4 Cup-plate Method[21]

The antimicrobial activity of our test sample is going to compare with that of the standard antibiotic against a test microorganism. Prepared and sterilized the nutrient agar medium by autoclaving, 21 ml of base layer poured into a sterile Petri dish, allowed to solidify. After that 4 ml of seeded agar medium (medium inoculated with the 24 hr old culture of the test organism) placed uniformly on the base layer, kept aside for solidification. For each sample kept duplication of plates, in each Petri dish made two cups by using sterile stainless steel borer, filled those cups with standard and test samples respectively. Kept the Petri dishes in the freezer for 2 hr and shifted to the incubator at 37^{0}C for 18 hr. After incubation period diameters measured and the results were tabulated.

5. Statistical Analysis

All the results were represented as mean± S.E.M. The statistical significance between the means of various groups was analyzed by unpaired two-tailed students t-test and statistically significant when $p<0.05$.

6. Results

6.1 Determination of MIC

Results of micro broth dilution revealed the MIC of MAQ as 40 µg/mL against BS, 40 µg/mL against SA, 80 µg/mL against SM and 20 µg/mL against EC. As well as, MIC of QNZ was 40 µg/mL against BS, 60 µg/mL against SA, 90 µg/mL against SM and 40 µg/mL against EC. The MIC values of both test compounds were shown in Table 1.

Table 1. MIC of test samples against selected bacterial strains

S. No	Concentration (µg/ml)	BS			SA			SM			EC		
		MAQ	QNZ	VM[a]	MAQ	QNZ	LZ[b]	MAQ	QNZ	AK[c]	MAQ	QNZ	CF[d]
1	10	+	+	-	+	+	-	+	+	+	+	+	-
2	20	+	+	-	+	+	-	+	+	-	+	-	-
3	30	+	+	-	+	+	-	+	+	-	+	-	-
4	40	-	-	-	-	+	-	+	+	-	-	-	-
5	50	-	-	-	-	+	-	+	+	-	-	-	-
6	60	-	-	-	-	-	-	+	+	-	-	-	-
7	70	-	-	-	-	-	-	+	+	-	-	-	-
8	80	-	-	-	-	-	-	-	+	-	-	-	-
9	90	-	-	-	-	-	-	-	-	-	-	-	-
10	100	-	-	-	-	-	-	-	-	-	-	-	-

(+: Absence of anti-bacterial activity; -: Presence of anti-bacterial activity). a: Vancomycin (VM; 5ug/mL); *Bacillus subtilis* (BS), *Staphylococcus aureus* (SA), *Serratia marcescens* (SM) and *Escherichia coli* (EC) b: Linezolid (LZ;4ug/mL); c: Amikacin (AK; 5ug/mL); d: Ciprofloxacin (CF; 1ug/mL).

6.2 Antibacterial Activity

To determine MIC by cup-plate method, different concentrations (100, 200, 400, 800 and 1000 µg/ml) of test compounds screened against BS, SA, SM and EC, zone of inhibition were compared with Vancomycin (VM), Linezolid (LZ), Amikacin (AK) and Ciprofloxacin (CF), respectively. The results of percentage of the zone of inhibition of different concentrations was found to be 39.8, 46.6, 47.57, 47.57 and 47.57% for MAQ and 35.89, 38.46, 41.88, 41.02 and 42.73% for QNZ against BS when compared with VM. As well as, the percentage of the zone of inhibition of different concentrations was found to be 88.33, 90, 95, 96.66 and 98.33% for MAQ and 70.37, 71.6, 72.83, 74.07 and 83.95% for QNZ against SA when compared with LZ. Besides, the percentage of the zone of inhibition of different concentrations was found to be 53.37, 58.98, 72.09, 76.77 and 77.71% for MAQ and 47.72, 63.72, 63.12, 63.12, 70.05 and 72.36% for QNZ against SM when compared AK. The percentage of the zone of inhibition of different concentrations was found to be 50.98, 54.9, 55.88, 56.86 and 59.8% for MAQ and 53.76, 56.98, 65.59, 68.81 and 67.74% for QNZ against EC when compared with CF. The zone of inhibition in diameter (mm) shown in Figures 1-8, tabulated in Table 2 and the percentage of inhibition is tabulated in Table 3.

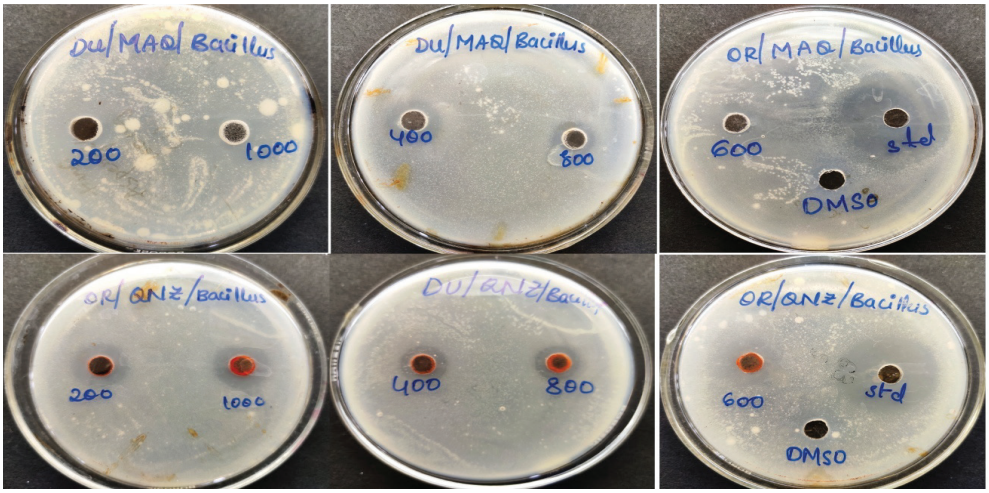


Figure 1. Zone of inhibition by isolated anthraquinones from *Rubia cordifolia* Linn. against *Bacillus subtilis.*

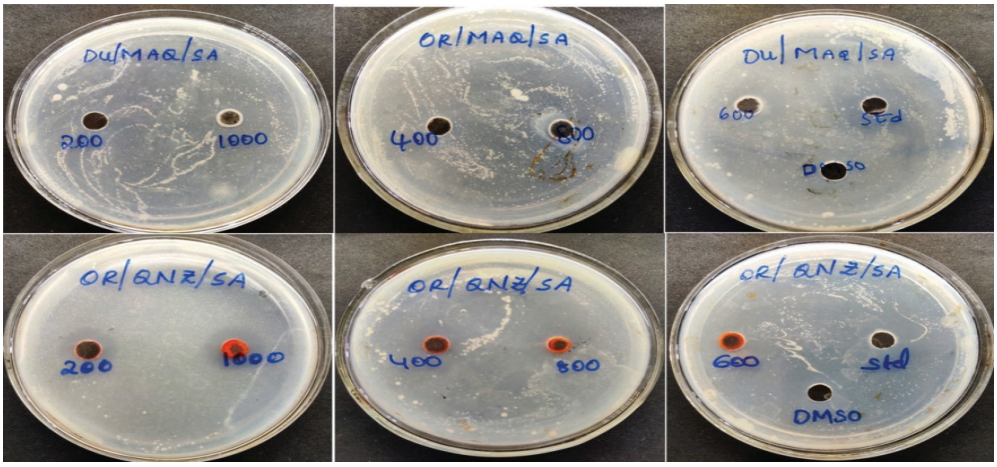


Figure 2. Zone of inhibition by isolated anthraquinones from *Rubia cordifolia* Linn. against *Staphylococcus aureus.*

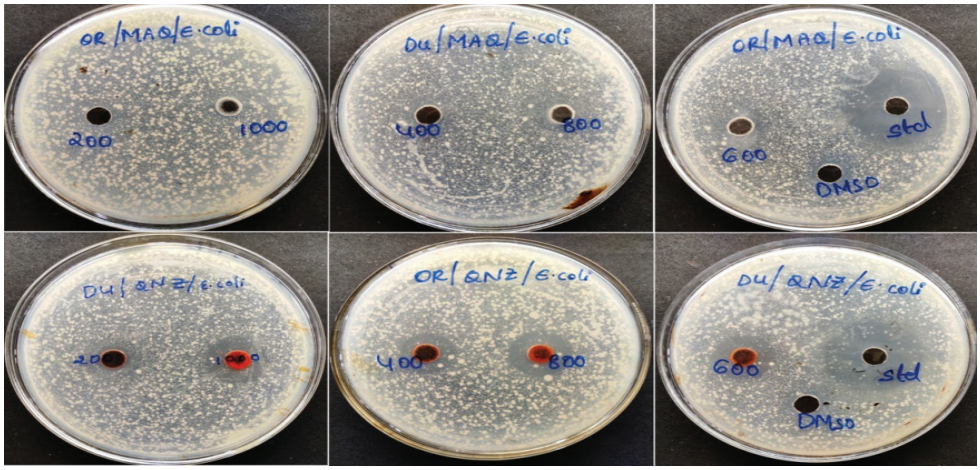


Figure 3. Zone of inhibition by isolated anthraquinones from *Rubia cordifolia* Linn. against *Escherechia coli.*

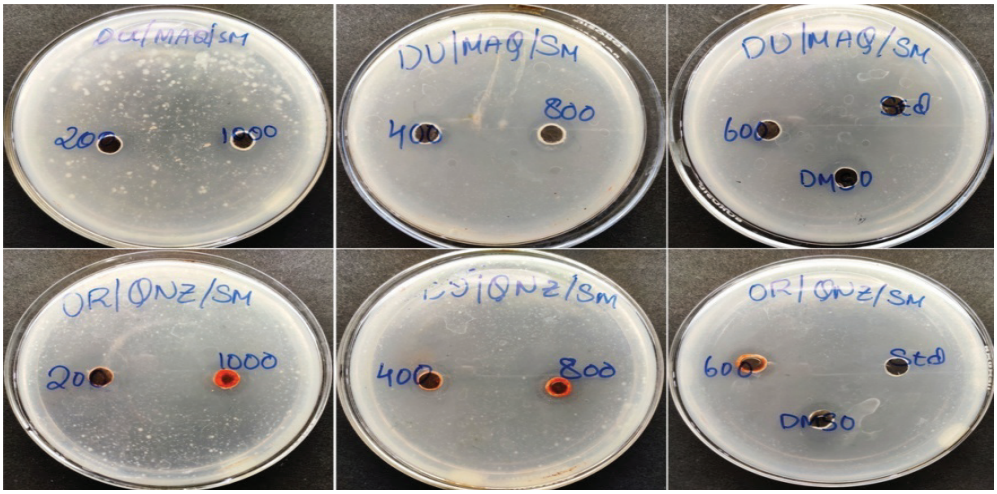


Figure 4. Zone of inhibition by isolated anthraquinones from *Rubia cordifolia* Linn. against *Serratia marcescens.*

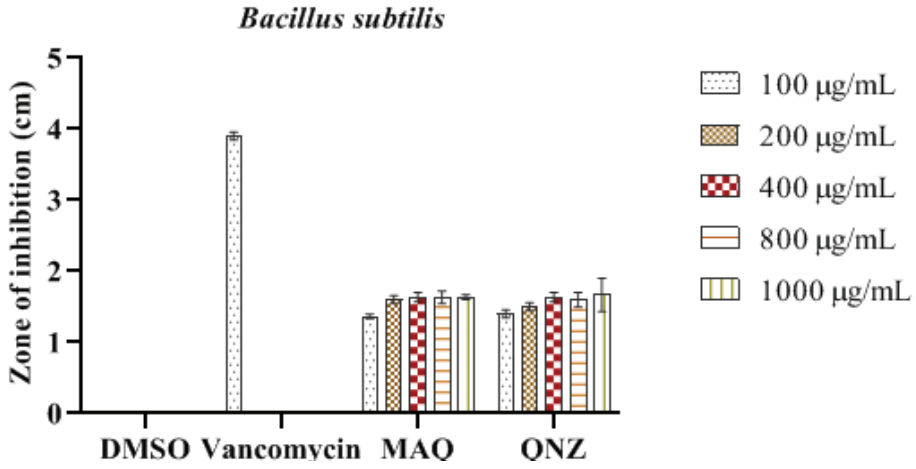


Figure 5. Effect of test compounds on zone of inhibition (cm) against *Bacillus subtilis.*

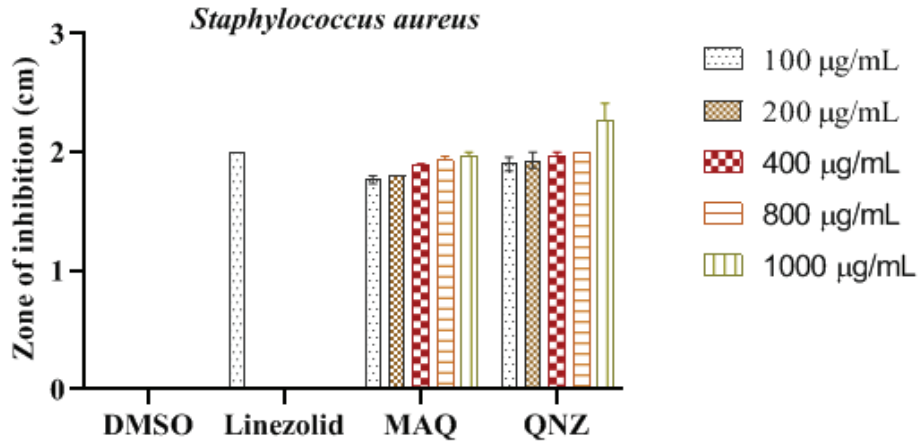


Figure 6. Effect of test compounds on zone of inhibition (cm) against *Staphylococcus aureus.*

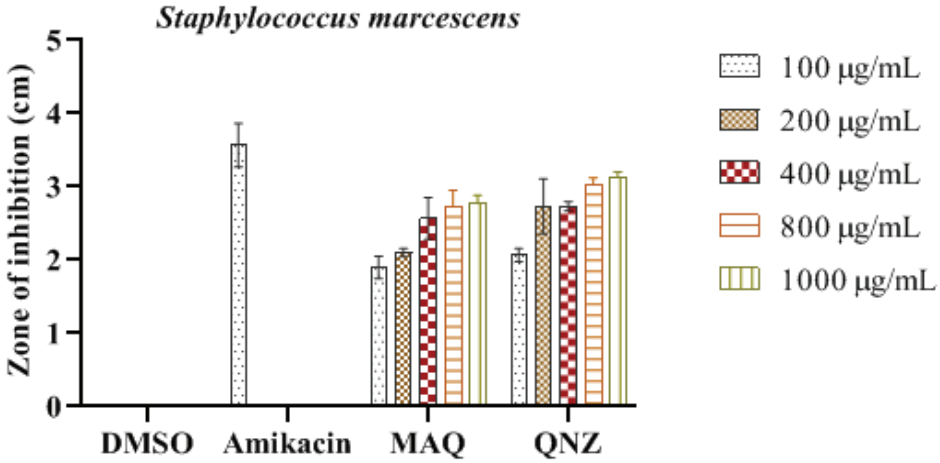


Figure 7. Effect of test compounds on zone of inhibition (cm) against *Escherechia coli.*

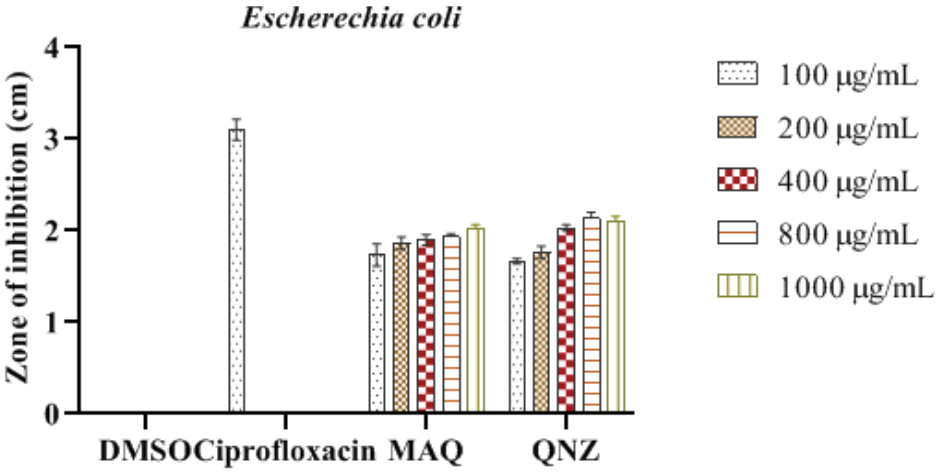


Figure 8. Effect of test compounds on zone of inhibition (cm) against *Staphylococcus marcescens.*

Table 2. Effect of isolated anthraquinones against gram positive and negative bacteria

Organism	Compounds	Zone of inhibition (cm)						
		100 µg/mL	200 µg/mL	400 µg/mL	800 µg/mL	1000 µg/mL	Std @100 µg/mL	DMSO
BS	MAQ	1.36 ± 0.033	1.6 ± 0.057	1.63 ± 0.066	1.63 ± 0.88	1.63 ± 0.033	3.9 ± 0.057[a]	0
	QNZ	1.4 ± 0.057	1.5 ± 0.057	1.63 ± 0.066	1.6 ± 0.1	1.66 ± 0.23		
SA	MAQ	1.4 ± 0.057	1.5 ± 0.057	1.63 ± 0066	1.6 ± 0.23	1.66 ± 0.233	2 ± 0[b]	0
	QNZ	1.76 ± 0.033	1.8 ± 0	1.9 ± 1.57	1.93 ± 0.033	1.96 ± 0.033		
SM	MAQ	1.9 ± 0.15	2.1 ± 0.057	2.56 ± 0.28	2.73 ± 0.21	2.76 ± 0.12	3.56 ± 0.29[c]	0
	QNZ	2.06 ± 0.088	2.73 ± 0.37	2.73 ± 0.066	3.03 ± 0.088	3.13 ± 0.066		
EC	MAQ	1.73 ± 0.12	1.86 ± 0.066	1.9 ± 0.057	1.93 ± 0.033	2.03 ± 0.033	3.1 ± 0.11[d]	0
	QNZ	1.66 ± 0.033	1.76 ± 0.066	2.03 ± 0.033	2.130.066	2.1 ± 0.057		

The results were represented as mean± S.E.M. The statistical significance between the means of various groups was analyzed by unpaired two-tailed students t-test and statistically significant when p< 0.05. BS: *Bacillus subtilis*; SA: *Staphylococcus aureus*; SM: *Serratia marcescens*; EC: *Escherechia coli*; MAQ: 2-methyl anthraquinone; QNZ: quinizarin; DMSO: Dimethyl sulfoxide. a: Vancomycin (VM; 5ug/mL); b: Linezolid (LZ;4ug/mL); c: Amikacin (AK; 5ug/mL); d: Ciprofloxacin (CF; 1ug/mL).

Table 3. The percentage of inhibition by isolated anthraquinones from *Rubia cordifolia* Linn. against gram positive and negative bacteria

Organism	Compounds	Zone of inhibition (cm)						
		100 µg/mL	200 µg/mL	400 µg/mL	800 µg/mL	1000 µg/mL	Std @100 µg/mL	DMSO
BS	MAQ	39.8	46.6	47.57	47.57	47.57	100[a]	0
	QNZ	35.89	38.46	41.88	41.025	42.735		
SA	MAQ	88.33	90	95	96.66	98.33	100[b]	0
	QNZ	70.37	71.6	72.83	74.07	83.95		
SM	MAQ	53.37	58.98	72.09	76.77	77.71	100[c]	0
	QNZ	47.72	63.12	63.12	70.05	72.36		
EC	MAQ	50.98	54.9	55.88	56.86	59.8	100[d]	0
	QNZ	53.76	56.98	65.59	68.81	67.74		

The results were represented as mean± S.E.M. The statistical significance between the means of various groups was analyzed by unpaired two-tailed students t-test and statistically significant when p< 0.05. BS: *Bacillus subtilis*; SA: *Staphylococcus aureus*; SM: *Serratia marcescens*; EC: *Escherechia coli*; MAQ: 2-methyl anthraquinone; QNZ: quinizarin; DMSO: Dimethylsulfoxide. a: Vancomycin (VM; 5ug/mL); b: Linezolid (LZ;4ug/mL); c: Amikacin (AK; 5ug/mL); d: Ciprofloxacin (CF; 1ug/mL).

7. Discussion

Medicinal plants contain a lot of active constituents such as flavonoids, tannins, alkaloids, terpenoids and anthraquinones. Among all, anthraquinones are the potential molecules with antioxidant, antifungal, antibacterial, anti-cancer, laxative and anti-inflammatory activities. Anthraquinones are having the basic structure of anthracene and 9, 10-Dione. The MIC determined by micro broth dilution method and percentage of the zone of inhibition determined by nutrient agar medium. Two anthraquinones are isolated from the roots of *Rubia cordifolia* Linn. Two anthraquinone compounds were screened for antibacterial activity against two gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two gram-negative (*Serratia marcescens* and *Escherichia coli*) bacteria organisms. The Micro Broth Dilution (MBD) method results suggested that the MAQ inhibits predominantly gram-positive, especially BS, SA. The MIC values from the MBD for MAQ were 40 µg/mL against BS, SA and 80 µg/mL against SM, respectively as compared with that of standard MIC of VM (5 µg/mL), LZ (4 µg/mL) and AK (5 µg/mL). However, QNZ inhibits the gram-negative bacteria, especially EC at 20 µg/mL when compared with CF (1 µg/mL). Similarly, our source of anthraquinone i.e. methanolic extracts of RC at 600 µg/mL by broth dilution method; produced significant antibacterial activity against BS, SA, SM and EC, which supports our present study outcome[22–23].

MIC from the aqueous, chloroform, ethyl acetate, methanol extracts of the leaf and roots of RC against SA and EC, similar reports which ranges from 1.5-25 mg/mL[24]. Which is an additional support the MAQ and QNZ showed the better MIC when compared to the extract of *Rheum undulatum* (MIC = 125-250 µg/mL) contain anthraquinones like aloe-emodin, emodin and rhein against the *Staphylococcus* species[25]. The isolated compounds have shown activity against both gram-positive and negative bacteria than emodin, which is inactive against gram-negative bacteria[26]. MIC of MAQ showed predominant inhibition against gram-positive (BS and SA) and gram-negative (especially SA) when compared with standard drugs. Whereas, QNZ inhibits gram-negative (especially EC). These results are similar and strongly suggested that the anthraquinones as well as extracts from RC fight against gram-positive and gram-negative bacteria[27–29].

8. Conclusion

The study concludes that anthraquinones can be considered as potent antimicrobial agents against various pathogens. In our present study anthraquinones derivatives isolated from roots of *Rubia cordifolia* Linn shown significant results against BS, SA, SM and EC strains. So they can be selected to screen further and compare their antimicrobial activity with well-established antibiotics. Further studies are required to confirm their mechanism of action in future.

9. Acknowledgement

The authors are thankful to the management of Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Guntur, Andhra Pradesh, India.

10. References

1. Jain C, Khatana S, Vijayvergia R. Bioactivity of secondary metabolites of various plants: A review. Int J Pharm Sci. 2019; 10:494–04.
2. Othman L, Sleiman A, Abdel-Massih RM. Antimicrobial activity of polyphenols and alkaloids in middle eastern plants. Frontiers in Nicrobiology. 2019 15;10:911. PMid: 31156565 PMCid: PMC6529554. https://doi.org/10.3389/fmicb.2019.00911
3. Lopez-Jacome E, Franco-Cendejas R, Quezada H, Morales-Espinosa R, Castillo-Juarez I, Gonzalez-Pedrajo B, Fernandez-Presas AM, Tovar-García A, Angarita-Zapata V, Licona-Limón P, Martinez-Vazquez M. The race between drug introduction and appearance of microbial resistance. Current balance and alternative approaches. Current Opinion in Pharmacology. 2019 48:48–56. PMid: 31136908. https://doi.org/10.1016/j.coph.2019.04.016
4. Manandhar S, Luitel S, Dahal RK. In vitro antimicrobial activity of some medicinal plants against human pathogenic bacteria. Journal of TropicalMedicine. 2019 2019. PMid: 31065287 PMCid:PMC6466868. https://doi.org/10.1155/2019/1895340
5. Balouiri M, Sadiki M, Koraichi S, Ibnsouda. Methods for in vitro evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis. 2016 6(2):719. PMid: 29403965 PMCid: PMC5762448. https://doi.org/10.1016/j.jpha.2015.11.005
6. Deoda RS, Kumar D, Kadam PV, Yadav KN, Bhujbal SS, Patil MJ. Pharmacognostic and biological studies of the roots of *Rubia cordifolia* Linn. (Rubiaceae). Int J Drug Dev Res. 2011; 3(3):148–58.
7. Cai Y, Sun M, Xing J, Corke H. Antioxidant phenolic constituents in roots of *Rheum officinale* and *Rubia cordifolia*: Structure radical scavenging activity relationships. Journal of Agricultural and Food Chemistry. 2004 52(26):7884–90. PMid: 15612771. https://doi.org/10.1021/jf0489116

8. Son JK, Jung SJ, Jung JH, Fang Z, Lee CS, Seo CS, MoonDC, Min BS, Kim MR, Woo MH. Anticancerconstituents from the Roots of *Rubia cordifolia*L. Chemical and Pharmaceutical Bulletin. 2008 56(2):213–6. PMid: 18239313. https://doi.org/10.1248/cpb.56.213
9. Divakar K, Pawar AT, Chandrasekhar SB, Dighe SB, Divakar G. Protective effect of the hydro-alcoholic extract of *Rubia cordifolia* roots against ethylene glycol induced urolithiasis in rats. Food and Chemical Toxicology. 2010 48(4):1013–8. PMid: 20079795. https://doi.org/10.1016/j.fct.2010.01.011
10. Patil RA, Jagdale SC, Kasture SB. Antihyperglycemic, antistress and nootropic activity of roots of *Rubia cordifolia* Linn. Indian J Exp Biol. 2006; 44:987–92.
11. Basu S, Ghosh A, Hazra B. Evaluation of the antibacterial activity of Ventilago madraspatana Gaertn., *Rubia cordifolia* Linn. and *Lantana camara* Linn.: Isolation of emodin and physcion as active antibacterial agents. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives. 2005 19(10):888–94. PMid: 16261521. https://doi.org/10.1002/ptr.1752
12. Tessier AM, Delaveau P, Champion B. New anthraquinones in *Rubia cordifolia* roots. Planta Medica. 1981 Apr; 41(4):337–43. PMid: 17401855. https://doi.org/10.1055/s-2007-971724
13. Dosseh C, Tessier AM, Delaveau P. New quinones in *Rubia cordifolia* L. Roots, III. Planta Medica. 1981 43(4):360–6. PMid: 17402060. https://doi.org/10.1055/s-2007-971524
14. Chang LC, Chavez D, Gills JJ, Fong HH, Pezzuto JM, Kinghorn AD. Rubiasins A-C, new anthracene derivatives from the roots and stems of *Rubia c ordifolia*.Tetrahedron Letters. 2000 41(37):7157–62. https://doi.org/10.1016/S0040-4039(00)01205-3
15. Miyazawa M, Kawata J. Identification of the key aroma compounds in dried roots of *Rubia cordifolia*. Journal of Oleo Science. 2006; 55(1):37–9. https://doi.org/10.5650/jos.55.37
16. Qiao YF, Wang SX, Wu LJ, Li X, Zhu TR. Studies on antibacterial constituents from the roots of *Rubia cordifolia* L. Acta Pharmaceutica Sinica. 1990; 25(11):834–9.
17. Ismail Y, Wedyan M, Al-zu'abe M, Abderrahman S. Antimicrobial activity of *Rubia cordifolia*: Methods to determine antimicrobial activity. Research Journal of Medicinal Plant. 2016; 10(8):457–62. https://doi.org/10.3923/rjmp.2016.457.462
18. MacFaddin JF. Media for the isolation-cultivation identification-maintenance of medical bacteria, vol. 1. Baltimore, MD: Williams and Wilkins; 1985.
19. Lapage SP, Shelton JE, Mitchell T. Methods in microbiology. Norris J, Rippons D (eds). 1970; 3.
20. Indian Pharmacopoeia, Govt of India. Delhi: The Controller of Publications; 1996.
21. Rose SB, Miller RE. Studies with the agar cup-plate method: I. A standardized agar cup-plate technique. Journal of Bacteriology. 1939 Nov; 38(5):525. PMid: 16560269 PMCid: PMC374540. https://doi.org/10.1128 JB.38.5.525-537.1939
22. Gorle AM, Patil SS. Evaluation of antioxidant and antiacne property of *Rubia cordifolia*. Der Pharmacia Sinica. 2010; 1(3):59–63.
23. Verma A, Kumar B, Alam P, Singh V, Gupta SK. *Rubia cordifolia* - A review on pharmaconosy and phytochemistry. International Journal of Pharmaceutical Sciences and Research. 2016 7(7):2720.
24. Kiang'U GAA. Antimicrobial and cytotoxicity activities of *Conyza bonariensis*, *Tribulus terrestris* and *Rubia cordifolia* growing in Arusha, Tanzania. Diss. NM-AIST, 2019.
25. Kosikowska U, Smolarz HD, Malm A. Antimicrobial activity and total content of polyphenols of Rheum L. species growing in Poland. Central European Journal of Biology 2010; 5. https://doi.org/10.2478/s11535-010-0067-4
26. Chukwujekwu JC, Coombes PH, Mulholland DA, Staden J Van. Emodin, an antibacterial anthraquinone from the roots of *Cassia occidentalis*. South African Journal of Botany. 2006; 72:295–7. https://doi.org/10.1016/j.sajb.2005.08.003
27. Li X, Liu Z, Chen Y, Wang LJ, Zheng YN, Sun GZ, Ruan CC. Rubiacordone A: A new anthraquinone glycoside from the roots of *Rubia cordifolia*. Molecules. 2009 14(1):566–72. PMid: 19169203 PMCid: PMC6253770. https://doi.org/10.3390/molecules14010566
28. Naidu KC, Lalam R, Bobbarala V. Antimicrobial agents from *Rubia cordifolia* and *Glycyrrhiza glabra* against phytopathogens of Gossypium. International Journal of PharmTech Research. 2009; 1(4):1512–8.
29. Sawhney R, Berry V, Kumar A. Inhibitory activity of *Rubia cordifolia* plant extract against ESBL producing Urinary E. coli isolates. Journal of Pharmacy Research. 2012 5(3):1328–30.