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# SYNERGISTIC ACTIONS OF *RUBIA CORDIFOLIA* STEM EXTRACT WITH MEROPENEM AND POLYMYXIN B AGAINST DIFFERENT BACTERIA

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## ABSTRACT
Emerging global bacterial drug resistance is now included all life saving antibiotics. With no newer antimicrobials in the pipeline, human race stands critically regarding effective treatment of infectious diseases. This may lead to exponential rise of morbidity and mortality rates in infectious diseases as most of the critically ill patients are usually suffering from infectious diseases. Natural products of plants are a rich resource of newer antimicrobials. *Rubia cordifolia* – the extract of the stem of this plant, which we have studied in this paper, is a well known antimicrobial agent; however, there is no study on any synergistic effect of this extract with life saving antibiotics. Our aim in this study was to explore this area, which may help the mankind. Our study revealed definite synergistic action of this extract with meropenem and polymyxin B against *E. coli*, *Shigella* and *Salmonella* in disc diffusion tests, while MIC of the extract ranges between 0.39- 3.125 mg/ml against these bacteria. Thus the results of this study delineates a possibility of utilizing this extract in combination with these antibiotics, as this extract is nontoxic and used in some foods for a long time.

**KEYWORDS:** *Rubia cordifolia*, Meropenem, Polymyxin B, Synergy.

## INTRODUCTION
Antibiotics are a class of powerful drugs that have antimicrobial properties which promote bacterial destruction by killing or inhibiting their growth.[1] It is the most prescribed drug in the medicinal world. Although there are more than 100 antibiotics available throughout the world, the majority of them are broadly classified into eight groups – penicllins, cephalosporins, macrolids, fluoroquinolones, carbapenems, sulphonamides, tetrecyclins and aminoglycosides.[2]

But due to rapid mutation and genetic alteration of the bacteria most of them are becoming antibiotic resistant that has created a global medical challenge.[3] Antibiotic resistance is the ability of the bacteria to render themselves active against a series of antibiotic.[4] This ability have been observed more in gram negative bacteria than gram positive bacteria, due to the presence of an outer membrane that serve as the permeability barriers against antibiotics.[5] *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* are a few examples of antibiotic resistant gram negative bacteria. The threat keeps rising since no new antibiotics are discovered since 1990s and more bacteria have showed resistance to almost all available antibiotics.[6] According to WHO, 700,000 to several million people die per year due to multidrug resistance.[7, 8] In US, about 2 million people get infected by multi-drug resistant bacteria and 23,000 people die as a result.[9] This mortality rate has been found to be twice in India than US.[10]

Penicillins and Cephalosporins are those broad spectrum antibiotics that have been rendered inactive by innumerable bacteria. To avoid this, a new class of highly effective antibiotic known as carbapenems (Fig.1) have been developed and are reserved for known or suspected multidrug resistance bacterial infection.[11] Carbapenem is similar to penicillins and cephalosporins in the manner that they are all beta-lactam class of antibiotics but is superior to both of them in terms of effectiveness against antibiotic resistant bacteria.

Imipenem is the first clinically used carbapenem. But since it is hydrolysed by renal enzyme dehydropeptidase a more advanced form of the antibiotic was introduced commercially known as meropenem which is stable to mammalian dehydropeptidase.[11,12] Both of these antibiotics function against the bacteria by binding to the penicillin-binding protein and inhibiting the bacterial cell wall synthesis thus promoting bacterial degradation and death.[13] Both imipenem and meropenem work against

aerobic and anaerobic, gram-positive and gram-negative bacteria.[14] Although meropenem is more active against *Enterbacteriaceae* and less active against gram-positive bacteria, whereas imipenem is effective particularly against *Psudomonas aeruginosa* and *E*nterococcus species.[15,16] Meropenem is more resistant to breakdown by beta-lactamase producing bacteria and is administered to treat infections such as pneumonia, meningitis, intra-abdominal function, sepsis and anthrax.[17] Imipenem is used to treat infections such as endocarditis, pneumonia, urinary tract infections etc.[18] However, recent findings indicate emergence of carbapenem-resistant bacterial strains such as *Klebsiella pneumoniae*. To fight against this, another antibiotic Polymyxin B (Fig.1) is being used in resistant gram-negative infections. Polymyxins are cationic detergents that alter the cell membrane permeability resulting in water uptake and subsequent cell death.[19] But excessive and unmonitored use have created resistant strains against polymyxin B and thus new and cost effective measures must be taken to prevent further emergence of antibiotic resistant bacteria.

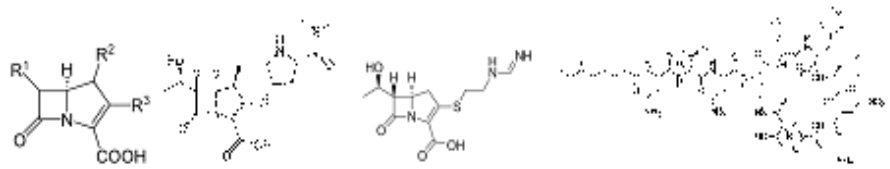


**Fig.1 Structures of some life saving antibiotics from acute infections**

Under this circumstances, different measures must be taken to increase the effectiveness of these drugs. Combination therapy has been found to be a better choice for treatment where different chemical components are being combined with the existing antibiotics to enhance their reactivity, thus promoting synergy. Furthermore, different plant based medicines are being used to fight against many infections and diseases causing pathogens. In recent times, medicinal herbs play a significant role in treating those diseases. The herbs are used as raw drugs, extracts or in combination with other drugs to enhance effectiveness of them. Herbs like turmeric, fenugreek, ginger, garlic have shown many active phytochemicals that have proved them to be potent pharmaceutical drugs. *Rubia cordefolia* is one of the medicinal plants that have been reported to contain multitude of chemical compounds that help in the treatment of different ailments. The plant has showed significant antibacterial properties against various organisms like *Staphylococcus aureus* and *Escherichia coli*.[20] Scientists are thinking of a new approach where this natural products are being combined with the known antibiotics to observe any synergistic activity against resistant bacteria. In our study we have applied this new approach to observe synergy against gram positive bacteria *Staphylococcus aureus* and four Gram negative bacteria- *E.coli*, *Salmonella* sp., *Klebsiella* sp. and *Shigella* sp.

*Rubia cordifolia*, commonly known as Indian madder or Manjistha, is a herbal plant that belongs to the coffee family Rubiaceae and genus *Rubia*. The genus *Rubia* contains about 70 different species that are distributed throughout the world, but in India, North-Western Ghat of Himalayas has been found to be their natural habitat.[21] The plant was originally used as an organic dye, but later found its immense popularity as pharmaceutical drug in the medicinal world, especially in Ayurveda. The plant is a perennial climber tree that can grow up to 12m long. The leaves are pointed, rough, ovate-lanceolate, 5-10cm in length, 2-3cm broad, 4-7 leaves whorled around the central stem and has tiny hooks for climbing. The flowers are small, whitish or yellowish, fragrant and blooms during August to October. The tree also bears fruits at that time and are minute, smooth, 4-6mm in diameter and red to blackish in colour. The stems are often rough, long, grooved, woody rhizomatous based.[22] The roots which are the most valuable part of the plant are often long, up to 12mm thick, cylindrical and red-rusty brown in colour.

The roots of the plant have multitude of medicinal properties and act against intestinal ulcers, rheumatism, tuberculosis, many skin disorders, bone fractures, burns etc. The plant is considered as an tonic, antitussive, antiseptic, antihelmintic, galactopurifier, ophthalmic normalizer and antioxidant.[23,24] The paste of the roots act as anti-inflammatory agent when applied externally over the wounds. The decoction of the roots are effective against jaundice, paralytic affections, amenorrhea, menstrual cycle irregularities, kidney stones and urinary troubles.[25] *R. cordifolia* is proved to be active against a number of cancer cell lines such as P388, L1210, L5178Y, 1316 melanoma, sarcoma-180, and Lewig lung cancer.[26] Chemical analysis showed the presence of bicyclic pepides such as RA-XIV, RA-XVI that exhibits the anti-cancer property.

The root extracts are active against *Staphylococcus aureus*. Scientific studies have showed that the root extract dissolved in different polar solvents inhibited both gram negative and gram positive bacteria.[27] The

chemical composition established the presence of emodin and Physcion, active antimicrobial agents.[28]

Apart from the medicinal values, the plant is used as a natural food colourant and as natural dye. The plant contains large amounts of quinones, mainly anthraquinone glycosides that provides the dye its colour. Alizarin (2-dihydroanthraquinone) or mordent red is the dye originally used for colouration and purpurin as a fast dye for cotton printing. Later synthetic 2-dihyroanthraquinone was discovered that replaced the use of *Rubia* as food colourant.[29]

## MATERIALS AND METHODS

### The bacterial strains

Five different bacteria – *Staphylococcus aureus* MRSA strain, *Salmonella typhi*, *Shigella dysenteriae*, *Klebsiella pneumoniae* and ESBL *E.coli* were isolated and freshly cultured in Mueller Hinton agar plates and incubated for overnight growth.

### Antibiotics

Three commercially purchased antibiotics discs of meropenem, imipenem and polymyxin B were used in our experiment.

### Extraction of *Rubia cordifolia* stems

The bark of the dried *R. cordifolia* stem was first removed using sterile scalpel blades and crushed in grinder. Then one gram of the grinded stem was weighed and mixed with 10ml of ethanol in a vortex for five minutes. After that centrifugation was performed and the separated supernatant was collected for future work.

### Preparation of filter paper discs containing *Rubia* extract

The *Rubia* extract of 1gm/10ml was used to soak 6mm size sterile Whatman no. 1 filter paper discs which were later incubated and dried at 37$^{o}$C and used in the experiment.

### Agar Diffusion test

Agar diffusion or commonly known as Disc diffusion method, which was introduced by Kirby–Bauer is one of the classic microbiological approach and is very frequently used. Due to its ease of execution, efficiency, and economic feasibility the disc process is so widely accepted for determining antimicrobial resistance around the globe.

With the test, the discs were impregnated with defined concentrations of different antimicrobial agents are placed onto the agar plates which were already inoculated with the test organisms. Upon incubation for 16–24 h at 35°-37 °C zones of growth inhibition around each of the discs were measured to the nearest millimetre. A lucid spherical zone of no growth in the immediate vicinity of a disc represents susceptibility to that antimicrobial. Reference tables, the size of zone can be related to the MIC and results recorded as whether the organism is susceptible (S), intermediately susceptible (I), or resistant (R) to that antibiotic.

Each bacterium was inoculated in two Mueller Hinton media containing agar plates in uniform layer. Then antibiotic discs and *Rubia* extract discs were placed in three sets - *R. cordifolia* with antibiotic in one set, only *Rubia* extract discs in second set and only antibiotic discs in the third set. These three sets were made for each of the antibiotics. After the placement of the discs the plates were incubated at 37$^{o}$C and observed after overnight incubation. Sensitivity zones were observed which were then measured and recorded for statistical analysis.

### Micro-plate Titre Assay: Determining MIC

In microbiology, the minimum inhibitory concentration (MIC) is the lowest concentration of a chemical, usually a drug, that will inhibit the growth of bacterium provided overnight incubation. MIC depends on the microorganism and the antibiotic used.

The test strains were chosen in their respective optimum growing phase and bacterial suspensions were prepared taking, McFarland turbidity standards (1 unit) in Mueller Hinton Broth. Using the multipipettor, 100µl of medium were dispensed into all wells of the microtitre plate (3 columns for each organism) and was labelled properly in the plate and the lid. 100ul of 1gm/10ml (Stock) *Rubia* extract was mixed into all the wells in row 1. Then the micropipettor was set at 100µl and the media and the extract were mixed in row 1 by sucking up and down for multiple times without splashing. Then 100 µl was withdrawn from row 1 and added to row 2, making row 2 a twofold dilution of row 1. This step was repeated up to row 6 in each case. 100µl from row 6 were discarded for maintaining identical volumes in all the wells. Then a base line OD was recorded at 620nm after 5min incubation at room temperature and then kept for Incubation at 37$^{o}$C. Finally the OD was taken after 24 hours of incubation in an ELISA Reader and the data were recorded.

## RESULTS

Statistical analysis after calculation of Mean, standard deviation and standard errors of different zones of inhibition in disc diffusion tests showed insignificant p values of the differences in most of the cases except *Shigella*, *Salmonella* and *E. coli* where p values of the differences in combinations were statistically significant. particularly with meropenem. Statistical analysis also showed that use of polymyxin B in combination with the extract is also beneficial in cases of *Salmonella* and *Shigella* infections. Thus synergy of antimicrobial action with the extract is evident with meropenem and polymyxin B.

The studies of MIC values by microdilution method of the extract showed different MIC values with different bacteria. Thus with *Salmonella* MIC value was the lowest at 0.39mg/ml, it was moderate with

*Staphylococcus* (3.125 mg/ml) and relatively high (6.25 mg/ml) with *E. coli* and *Klebsiella*.

| Treatment> Organism | M (mm) | M+E (mm) | I(mm) | I+E(mm.) | PB(mm) | PB+E (mm) | E (mm) |
|---|---|---|---|---|---|---|---|
| *S. aureus* 1 | 25 | 28 | 26 | 34 | 08 | 10 | 06 |
| *S. aureus* 2 | 24 | 30 | 24 | 28 | 10 | 13 | 06 |
| *E. coli* 1 | 24 | 30 | 26 | 30 | 11 | 13 | 10 |
| *E. coli* 2 | 24 | 30 | 20 | 26 | 12 | 14 | 12 |
| *K. pneumoniae* 1 | 11 | 11 | 17 | 17 | 13 | 14 | 08 |
| *K. pneumoniae* 2 | 10 | 8 | 13 | 14 | 12 | 12 | 08 |
| *S. dysenteriae* 1 | 20 | 27 | 18 | 22 | 13 | 20 | 08 |
| *S. dysenteriae* 2 | 22 | 25 | 18 | 25 | 14 | 16 | 08 |
| *S. typhi* 1 | 22 | 26 | 20 | 24 | 17 | 22 | 10 |
| *S. typhi* 2 | 16 | 22 | 14 | 18 | 16 | 18 | 08 |

**Table 1: Showing results of disc diffusion tests. M-Meropenem, I-Imipenem, PB-Polymyxin B, E-Rubia extract.**

| Bacteria | Base Line Reading.(620 nm) | | | | Reading after Incubation.(620 nm) | | | |
|---|---|---|---|---|---|---|---|---|
| Conc.RC | OD | Mean | SD | SEM | OD | Mean | SD | SEM |
| *Staphylococcus aureus.* | | | | | | | | |
| | Base Line Reading. | Mean | SD | SEM | Overnight Incubation | Mean | SD | SEM |
| 12.5 mg/ml | 0.226<br>0.234<br>0.241 | 0.2336 | 0.0075 | 0.0043 | 0.263<br>0.270<br>0.285 | 0.2726 | 0.0112 | 0.0064 |
| 6.25 mg/ml | 0.140<br>0.148<br>0.148 | 0.1453 | 0.0046 | 0.0026 | 0.215<br>0.231<br>0.228 | 0.2246 | 0.0085 | 0.0049 |
| 3.125 mg/ml | 0.136<br>0.130<br>0.137 | 0.1343 | 0.0037 | 0.0021 | 0.269<br>0.268<br>0.286 | 0.2743 | 0.0101 | 0.0058 |
| 1.5625 mg/ml | 0.112<br>0.114<br>0.116 | 0.1140 | 0.0020 | 0.0011 | 0.317<br>0.314<br>0.313 | 0.3146 | 0.0020 | 0.0012 |
| 0.781 mg/ml | 0.108<br>0.109<br>0.118 | 0.1116 | 0.0055 | 0.0031 | 0.316<br>0.289<br>0.304 | 0.3030 | 0.0135 | 0.0078 |
| 0.39 mg/ml | 0.092<br>0.094<br>0.097 | 0.0943 | 0.0025 | 0.0014 | 0.289<br>0.271<br>0.294 | 0.2846 | 0.0121 | 0.0069 |
| *Klebsiella pneumoniae.* | | | | | | | | |
| 12.5 mg/ml | 0.227<br>0.270<br>0.256 | 0.2510 | 0.0219 | 0.0126 | 0.271<br>0.251<br>0.257 | 0.2596 | 0.0102 | 0.0059 |
| 6.25 mg/ml | 0.151<br>0.151<br>0.147 | 0.1496 | 0.0023 | 0.0013 | 0.198<br>0.204<br>0.176 | 0.1926 | 0.0147 | 0.0085 |
| 3.125 mg/ml | 0.110<br>0.139<br>0.139 | 0.1293 | 0.0167 | 0.0096 | 0.205<br>0.286<br>0.285 | 0.2586 | 0.0464 | 0.0268 |
| 1.5625 mg/ml | 0.110<br>0.110<br>0.116 | 0.1120 | 0.0034 | 0.0020 | 0.323<br>0.346<br>0.377 | 0.3486 | 0.0271 | 0.0156 |
| 0.781 mg/ml | 0.109<br>0.109<br>0.109 | 0.1090 | 0.0000 | 0.0000 | 0.425<br>0.482<br>0.478 | 0.4616 | 0.0318 | 0.0183 |

**Table 2: Growth patterns showing MIC values of the extract with *Staphylococcus aureus* and *Klebsiella pneumoniae*.OD-Optical density, SD-Standard deviation, SEM-Standard error of mean, Conc. RC-Concentration of *Rubia cordifolia*.**

| Bacteri | Base line reading.(620 nm) | | | | Reading after incubation.(620nm) | | | |
|---|---|---|---|---|---|---|---|---|
| Conc. I | OD 3 sets | Mean | SD | SEM | OD | Mean | SD | SEM |
| *E. coli* | | | | | | | | |
| 12.5 | 0.228 | | | | 0.215 | | | |
| mg/ml | 0.184 | 0.1950 | 0.0291 | 0.0168 | 0.224 | 0.2133 | 0.01484 | 0.00857 |
| | 0.173 | | | | 0.195 | | | |
| 6.25 | 0.133 | | | | 0.214 | | | |
| mg/ml | 0.115 | 0.1290 | 0.0124 | 0.0072 | 0.121 | 0.166 | 0.4657 | 02689 |
| | 0.139 | | | | 0.163 | | | |
| 3.125 | 0.122 | | | | 0.362 | | | |
| mg/ml | 0.118 | 0.1213 | 0.0030 | 0.0017 | 0.319 | 0.324 | 0.03576 | 0.02065 |
| | 0.124 | | | | 0.291 | | | |
| 1.5625 | 0.122 | | | | 0.445 | | | |
| mg/ml | 0.103 | 0.1150 | 0.0104 | 0.0060 | 0.431 | 0.41233 | 0.450 | 0.02598 |
| | 0.120 | | | | 0.361 | | | |
| 0.781 | 0.111 | | | | 0.602 | | | |
| mg/ml | 0.098 | 0.1056 | 0.0068 | 0.0039 | 0.556 | 0.61067 | 0.05948 | 0.03434 |
| | 0.108 | | | | 0.517 | | | |
| 0.39 | 0.092 | | | | 0.674 | | | |
| mg/ml | 0.083 | 0.0896 | 0.0058 | 0.0033 | 0.638 | 0.61933 | 0.06601 | 0.03811 |
| | 0.094 | | | | 0.546 | | | |
| *Salmonella typhi.* | | | | | | | | |
| 12.5 | 0.240 | | | | 0.248 | | | |
| mg/ml | 0.211 | 0.2180 | 0.0194 | 0.0112 | 0.237 | 0.23833 | 0.00907 | 0.00524 |
| | 0.203 | | | | 0.230 | | | |
| 6.25 | 0.142 | | | | 0.164 | | | |
| mg/ml | 0.146 | 0.1450 | 0.0026 | 0.0015 | 0.180 | 0.1813 | 0.0180 | 0.0104 |
| | 0.147 | | | | 0.200 | | | |
| 3.125 | 0.195 | | | | 0.225 | | | |
| mg/ml | 0.130 | 0.1526 | 0.0366 | 0.0211 | 0.198 | 0.2070 | 0.0155 | 0.0090 |
| | 0.133 | | | | 0.198 | | | |
| 1.5625 | 0.138 | | | | 0.151 | | | |
| mg/ml | 0.128 | 0.1260 | 0.0131 | 0.0075 | 0.152 | 0.1493 | 0.0037 | 0.0021 |
| | 0.112 | | | | 0.145 | | | |
| 0.781 | 0.138 | | | | 0.188 | | | |
| mg/ml | 0.113 | 0.1213 | 0.0144 | 0.0083 | 0.187 | 0.1760 | 0.0199 | 0.0115 |
| | 0.113 | | | | 0.153 | | | |
| 0.39 | 0.114 | | | | 0.199 | | | |
| mg/ml | 0.093 | 0.0996 | 0.0124 | 0.0071 | 0.244 | 0.2076 | 0.0328 | 0.0189 |
| | 0.092 | | | | 0.180 | | | |

**Table 3: Growth patterns showing MIC values of the extract with *E. Coli* and *Salmonella typhi*. OD-Optical density, SD-Standard deviation, SEM-Standard error of Mean. Conc.RC-Concentration of *R. cordifolia***

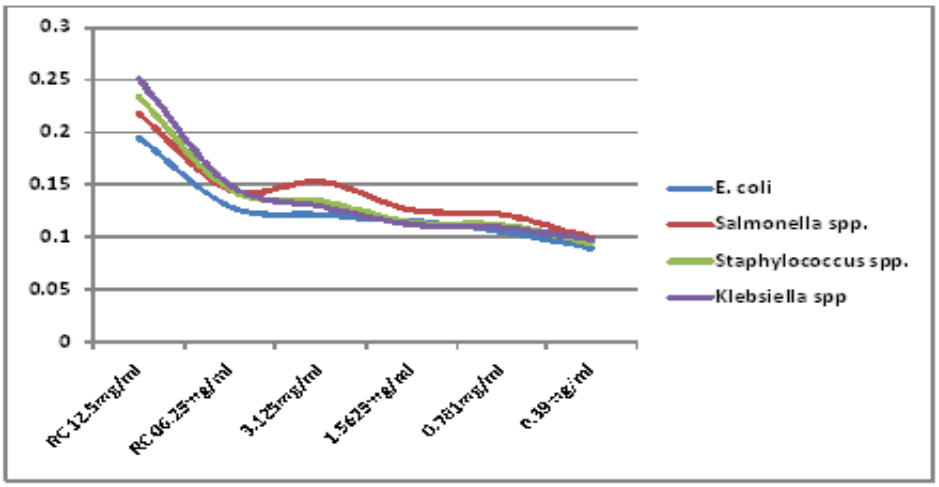


Fig.2: Baseline OD in different sets of MIC experiment.with decreasing concentration of extract. X axis: *Rubia* extract mg/ml, Y axis: OD values at 620 nm.

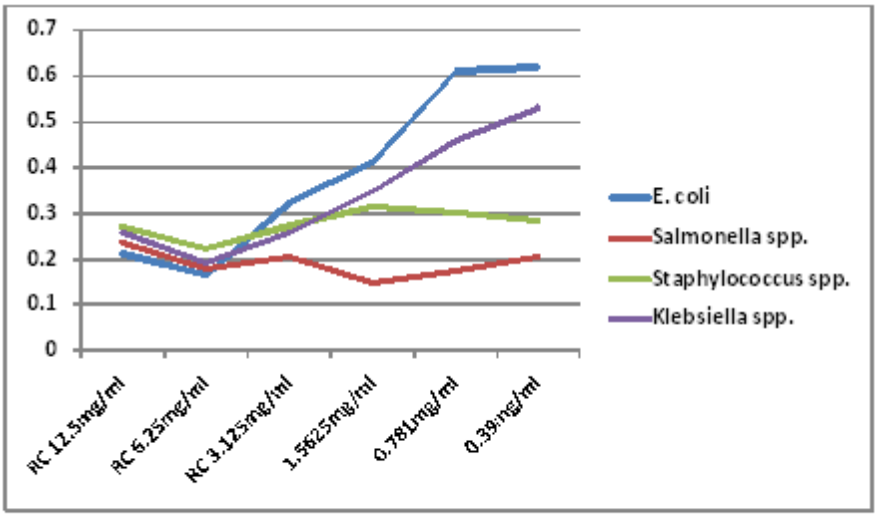


Fig.3: Growth patterns of different bacteria with decreasing concentration of extract. X axis: *Rubia* extract mg/ml, Y axis: OD values at 620 nm. (Reading after 24 hours of incubation)

## DISCUSSION

The results of this study indicated that *Rubia* extract has got direct antimicrobial action as well as it showed synergy with life saving antibiotics like meropenem and polymyxin B which appears very important in the emerging global bacterial drug resistance. *R.cordifolia* by virtue of its antimicrobial property has produced zones of inhibition in each case it was applied on several microorganisms. The drugs those were used i.e. imipenem, meromenem,polymyxin B have as well provided zones of inhibition as their characteristic antimicrobial nature which is already well established from past. But, in those cases where the plant stem extract was applied in a combination with the life saving drugs they showed some resonating results i.e. the diameter of the zones of inhibition were much higher than those cases when they were applied individually. It was observed that the gram negative microorganisms are more susceptible to this antimicrobial agent. Especially the growths of *E. coli*, *Shigella* spp. and *Salmonella* spp. were very well inhibited by the antimicrobial property of the *Rubia* extract. The gram negative microorganisms were found to be more susceptible towards this plant extract because of their cell wall composition , due to having thinner width and lesser content of peptidoglycan and teichoic acid in the outer surface. Apart from this, as gram negative organisms have a higher lipid and lipoprotein content (due to presence of outer membrane) and *R. cordifolia* extracts was also evaluated for antioxidant and lipid peroxidation inhibitory activity by 1, 1-diphenyl-2-picryl-hydrazyl and TBARs Thiobarbituric acid reactive substances, extract of *R. cordifolia* showed a significant inhibitory activity against Gram Negative bacterium growth. These investigations have revealed *R.cordifolia* as a promising anti-bacterial agent because it has exhibited inhibitory effects towards a range of bacteria and it has got a potent role of synergy action with life saving antibiotics.

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