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# ANTIBACTERIAL ACTIVITY OF *RUBIA TINCTORUM* LINN. ROOT EXTRACTS

Rima Ghafari [*1], Najwa Mouslemanie [1] and Ream Nayal [2]

Department of Botany [1], Faculty of Science, University of Aleppo, Syria.
Department of Pharmacognosy [2], Faculty of Pharmacy, University of Aleppo, Syria.

**Keywords:**

*Rubia tinctorum* roots, Extracts, Antibacterial activity, Minimum inhibitory concentration (MIC)

**Correspondence to Author:**
**Rima Ghafari**

Master Student,
Department of Botany,
Faculty of Science,
University of Aleppo, Aleppo, Syria.

**E-mail:** mirago82@yahoo.com

**ABSTRACT:** This research aimed to determine the antibacterial activity of methanolic, aqueous, chloroformic and petroleum ether extracts of *Rubia tinctorum* roots against two gram positive and two gram negative bacteria using agar well-diffusion method. The minimum inhibitory concentration (MIC) was determined using microdilution method. Extracts were dissolved in DMSO to obtain a concentration of 10% (w/v), and the density of bacterial suspension was $1.5 \times 10^8$ cfu/ml. Results showed that methanolic extract showed antibacterial activity against both gram negative and gram positive bacteria with minimum inhibitory concentration ranging from 0.1562 to 0.3125 mg/ml, while aqueous extract showed no activity against the tested bacteria. However, chloroformic extract showed antibacterial activity only against *Escherichia coli* with minimum inhibitory concentration value of 2.5 mg/ml. But petroleum ether extract showed antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* with minimum inhibitory concentration ranging from 0.3125 to 1.25 mg/ml. But in conclusion, methanolic extract was the most effective against all tested bacteria. Further studies are needed for pharmaceutical purposes.

**INTRODUCTION:** The development of antibiotic resistance has forced scientists to search for new antibacterial substances from natural origin. The use of plant extracts and phytochemicals both with known antimicrobial properties, can be of great significance in therapeutic treatments. *Rubia tinctorum* Linn. or Madder is a plant of Rubiaceae family and originated from Syria, Lebanon, it's also originated from Ghafghaz and near East. Cultivation of Madder is prevailed in the world province for dye industry and extracting the drug components [1, 2, 3]. Common madder is one of the most important species belonging to this family which grows in the Mediterranean area [14].

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*R. tinctorum* Linn. has long been used in traditional medicine to cure various ailments, for instance Greek physicians have been used this plant as a diuretic and for treatment of jaundice, sciatica and paralysis. In Europe the plant was used for the treatment of rheumatic disorders. In the traditional medical texts of Iran, Makhzan-ol-advieh and Tohfat-ol-momenin, the plant was recommended for treatment of inflammatory disorders [4]. People from many parts of Serbia and other Balkan countries used this plant for treatment of bladder infections [5]. This plant has been used to dye textiles and as food colorant since ancient times. Furthermore the crude extract of *Rubia tinctorum* has been used as anti-inflammatory, antibacterial and antifungal agent [15].

Madder roots is a traditional herbal medicine used against kidney stones. At pH< 7, free anthraquinone preparations formed coloured, insoluble Ca and Mg complexes, which were deposited with urinary stones.

However, at pH 5-7, glycoside-bound anthraquinones formed soluble complexes, thus decreasing the amount of ionized Ca and Mg in the urine and preventing stone formation, a preparation from the roots of *R. tinctorum* is able to dissolve oxalates, phosphates and uric acid, which deposit in the kidneys and the urinary tracts as stones and sand [6]. Different biological effects of *Rubia species* have been reported as anti-cancer, anti-oxidant, anti-microbial and hepatoprotective effects [7], wound-healing, analgesic, antipyretic, antimicrobial, anti-viral, antioxidant and antitumor activities [16].

*Rubia tinctorum* root extracts showed antibacterial activities against a wide range of pathogenic bacteria and fungi, due to its production of anthraquinone pigments in its roots. One of these pigments is alizarin which has been used for dying textiles since 2000 BC [8]. This research focuses on the antibacterial activity of four different extracts of *Rubia tinctorum* roots against gram positive and gram negative bacteria.

## MATERIALS AND METHODS:

**Materials**: Mueller- Hinton agar (Merck, Germany). Nutrient agar (Merck, Germany). Microtitration plates (Citotest, China). Methanol, distilled water, chloroform, petroleum ether (Merck, Germany).

**Collection of Plant Material:** *Rubia tinctorum* roots were purchased from the local market and identified by the Department of Botany, Faculty of Science, Aleppo University. Roots were cleansed and ground using mechanical blender, the resulted powder was stored in airtight glass containers for future use.

**Plant Extraction:** 10 g of finely powdered madder roots was macerated with 100 ml of either methanol or distilled water or chloroform or petroleum ether. The mixtures were then stirring at room temperature for 24 h. The mixtures were filtered through filter paper and the extracts were concentrated to dryness using a rotary evaporator. Sample solutions were prepared by dissolving extracts in dimethyl sulfoxide (DMSO) at 100 mg/ml (10% w/v).

**Bacterial Strains:** In this study two gram positive (*Staphylococcus aureus*, *Bacillus subtilis*) and two gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*). These strains were obtained from microbiology laboratory, Department of Botany, Faculty of Science, University of Aleppo. Nutrient agar medium was used for maintenance the bacterial strains, and Mueller-Hinton agar medium was used to investigate the antibacterial activity.

**Agar Well-Diffusion Test:** Agar well-diffusion test was carried out using Mueller-Hinton agar medium (Merck). Inoculums were prepared by using 24 h plate cultures of two gram positive and two gram negative bacteria on nutrient agar medium. Those colonies were suspended in 5 ml saline 0.85%, and turbidity was compared to 0.5 McFarland standard to produce a bacterial suspension of $1.5 \times 10^8$ cfu/ml. The suspension was loaded on sterile cotton swab that was rotated several times and pressed firmly against the inner sides of the tube then the dry surface of Mueller-Hinton agar plate was inoculated by the swab over the entire sterile agar surface. Then 5 mm in diameter holes were made in the agar, solutions of 10 % extracts in DMSO were added to the holes at different volumes (25, 50, 75, and 100) µl alongside with DMSO as negative control. In addition, commercial antibiotics, *i.e*. ampicillin (10 µg), norfloxacin (10 µg), clarithromycin (15 µg), cefixime (5 µg), amikacin (30 µg) and meropenem (10 µg) were used as positive control to determine the sensitivity of the tested bacteria. Inoculated plates were incubated at 37 ºC for 24 h and antibacterial activity was evaluated by measuring the inhibition zone diameter [9].

**Determination of Minimum Inhibitory Concentration (MIC):** Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism after 18 - 24 h. The extracts minimum inhibitory concentration values were determined by using micro-well dilution method in 96-well micro plates, according to the method of Saosoong *et al*., with a slight modification [10]. The tested bacteria were grown on nutrient agar medium at 37 ºC for 24 h and 0.5 McFarland ($1.5*10^8$ cfu/ml) turbidity was prepared in sterile saline. Extracts were dissolved in DMSO to obtain concentration of 10% (w/v). To the 96-well microtitration plate, 50 µl of distilled water was added to the wells except wells in the third column, then 50 µl of bacterial solutions were added to the first and third columns and 50 µl of extracts solution was added to the

second and third columns. 50 µl of extract solution was added to the forth column, after mixing well, 50 µl of this mixture was transformed to the next column and the whole process was repeated until the last column to obtain a series of known decreasing concentration: (2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, and 0.039) mg/ml. Then 50 µl of bacterial solution was added to columns containing decreasing concentrations of extracts, plates were incubated at 37 ºC for 24 h, after that 20 µl of a 0.05% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) was added to these columns and the plate was stood for 2 h more.

Since the slight yellowish tetrazolium salt is reduced to a red coloured product by biologically active organisms, the inhibition of the growth can be detected when the solution in the wells remains clear after incubation with TTC. The lowest concentration of each extract showing no visible growth was recorded as the minimum inhibitory concentration [11].

**Statistical Analysis:** All measurements were performed in triplicate (n=3) and were expressed as mean ± SD. Data was analyzed using SPSS16.0 for windows (SPSS, Inc. Chicago, IL, USA).

## RESULTS AND DISCUSSION:

**Extraction Yield:** The percentage yield values of the extracts are shown in **Table 1**, the yield varied from 49.97 to 1.086%, the highest yield was for methanolic extract due to the high polarity of methanol, while petrolic ether extract showed the lowest yield because of its low polarity.

**TABLE 1: YIELD AND PERCENTAGE YIELD OF PLANT EXTRACTS**

| Solvent | Yield (g) | Percentage (%) |
|---|---|---|
| Methanol | 4.9977 | 49.97% |
| Distilled water | 4.535 | 45.35% |
| Chloroform | 0.3033 | 3.033% |
| Petroleum ether | 0.1086 | 1.086% |

**Standard Antibiogram Assay: Table 2** shows inhibition zones in (mm) obtained after applying standard antibiotics susceptibility test. Three of standard antibiotics (Ampicillin, Clarithromycin and Cefixime) had no activity against the tested bacteria, while meropenem showed the highest inhibition zones against bacterial strains.

**TABLE 2: INHIBITION ZONE OF STANDARD ANTIBIOTICS AGAINST TESTED BACTERIA**

| Standard Antibiotics and their main Groups | Code and conc. | Inhibition zone (mm) | | | |
|---|---|---|---|---|---|
| | | *S. aureus* | *B. subtilis* | *E. coli* | *P. aeruginosa* |
| Norfloxacin (Cephalosporin) | NX 10 mcg | 8 mm | 13 mm | 10 mm | 20 mm |
| Amikacin (Aminoglycosides) | AK 30 mcg | 16 mm | 15 mm | 18 mm | 18 mm |
| Meropenem (Carbapenems) | MEM 10 mcg | 20 mm | 27 mm | 18 mm | 28 mm |
| Ampicillin (Penicillin) | AM 10 mcg | - | - | - | - |
| Clarithromycin (Macrolids) | CLR 15 mcg | - | - | - | - |
| Cefixime (Fluoroquinolones) | CFM 5 mcg | - | - | - | - |

**Agar Well-diffusion Test:** Methanolic extract showed activity against all tested bacteria as shown in **Table 3**.

**TABLE 3: INHIBITION ZONE OF METHANOLIC EXTRACT AGAINST TESTED BACTERIA**

| Bacterial strain | Inhibition zone | | |
|---|---|---|---|
| | 50 µl | 75 µl | 100 µl |
| *S. aureus* | 10.3 mm ± 0.57 | 14.3 mm ± 0.57 | 15 mm ± 0.0 |
| *B. subtilis* | 10 mm ± 0.0 | 11 mm ± 0.0 | 13.3 mm ± 0.57 |
| *E. coli* | 15 mm ± 0.0 | 15 mm ± 0.0 | 17.3 mm ± 0.57 |
| *P. aeruginosa* | 11.6 mm ± 0.57 | 15.3 mm ± 0.57 | 18 mm ± 0.0 |

Values are expressed as mean ±SD (n=3)

Aqueous extract showed no activity against bacteria which differs from the results obtained from previous study done in Iran on *R. tinctorum* aqueous extract, which had activity against *B.*

*subtilis* with inhibition zone ≈10 mm [12], whereas this recent research results comply with another study done in turkey in which *R. tinctorum* aqueous extract showed no activity against *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* [8].

Chloroformic extract was active only against *E. coli*. The inhibition zone at 50 µl was 25.6 mm ± 0.57, this result differs from results obtained by Basu and his collegue research where *R. cordifolia* chloroformic extract did not show any activity against *E. coli*. This could be due to *Rubia tinctorum* roots richness with anthraquinones which are better extracted with chloroform rather than other solvents [13].

Petroleum ether extract showed activity at 50µl against *E. coli*, *P. aeruginosa* and *S. aureus* with inhibition zone of 10 mm ± 0.0, 17.6 mm ± 0.57, and 17 mm ± 0.0, respectively.

These results couldn't be compared to any other results because there is no researches about *R. tinctorum* petroleum ether extract. It is notable that inhibition zone diameters ranged from 10 to 15 mm for *S. aureus*, from 10 to 13mm for *B. subtilis*, for *E. coli* it ranged from 15 - 17 mm and 12 - 18 mm for *P. aeruginosa*. These results complies with other researches [8, 13].

**Minimum Inhibitory Concentration Values:** The MIC of methanolic, chloroformic and petroleum ether extracts are shown in **Table 4**.

**TABLE 4: MIC VALUES OF *R. TINCTORUM* EXTRACTS AGAINST BACTERIA (mg/ml)**

| Extract | Bacterial strain | MIC mg/ml |
|---|---|---|
| Methanol | *S. aureus* | 0.1562 |
| | *B. subtilis* | 0.1562 |
| | *E .coli* | 0.3125 |
| | *P. aeruginosa* | 0.3125 |
| Petroleum ether | *S. aureus* | 0.625 |
| | *E. coli* | 1.25 |
| | *P. aeruginosa* | 0.3125 |
| Chloroform | *E. coli* | 2.5 |

Methanolic extract MIC values were for gram positive bacteria less than values for gram negative bacteria. Both methanolic and petroleum ether extracts showed similar MIC values for *P. aeruginosa* (0.3125 mg/ml). Methanolic extract showed the lowest MIC values against *E. coli*, while the chloroformic extract showed the highest MIC value against *E. coli*.

**CONCLUSION:** In this study, antibacterial activity of *Rubia tinctorum* root extracts. Methanolic extract showed activity against both gram negative and gram positive bacteria, followed by petroleum ether extract which presented activity against the two gram negative bacteria and one of the gram positive bacteria, while chloroformic extract showed activity only against *E. coli* with the best inhibition zone among the extracts. While aqueous extract had no activity.

The best minimum inhibitory concentration was for methanolic extract against both gram negative and gram positive bacteria. Petroleum ether extract against *P. aeruginosa*. *Rubia tinctorum* root extracts activities were better than ampicillin, clarithromycin and cefixime.

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**CONFLICT OF INTEREST:** Authors declare that they have no conflict of interest.

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