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# Antibacterial activity of *Rubia tinctorum* extracts

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## Abstract

*Rubia tinctorum* a root belonging to family Rubiaceae was evaluated for the antibacterial activity against a number of bacteria. We determine antibacterial activity of methanol and chloroform extracts of *Rubia tinctorum* roots and two pure compounds isolated from the extract (namely alizarin and purpurin) against one gram positive and three gram negative bacteria using agar well-diffusion method. The minimum inhibitory concentration (MIC) was determined using micro dilution method. Extracts were dissolved in DMSO–$d_6$ to obtain a concentration of 10% (w/v), and the density of bacterial suspension was 1.5 × 108 cfu/ml. The results showed a significant and promising activity towards *Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E.coli*. Additionally, the minimum inhibitory concentration (MIC) was determined and illustrated that, the most susceptible organisms to Alizarin, purpurin, methanol and chloroform extract was *Klebsiella pneumonia* with MIC of (12.5 , 6.25 , 3.125 , 1.562) mg/mL respectively. Therefore, *R.tinctorum* extract could be considered as a promising source for effective antibacterial agent.

**Keywords:** Alizarin, purpurin, *Rubia tinctorum* roots extracts, antibacterial activity, minimum inhibitory concentration (MIC)

## 1. 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* roots or Madder is a plant belonging to family Rubiaceae. *R. tinctorum* 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 **(Sharifzadeh *et al.*, 2014)**. People from many parts of Serbia and other Balkan countries used this plant for treatment of bladder infections **(Rovcanin *et al.*, 2015)**. This plant has been used to dye textiles and as food colorant since ancient times. Furthermore the crude extract of *R. tinctorum* has been used as anti-inflammatory, antibacterial and antifungal agent **(Lajkó *et al.*, 2015)**. Madder roots are also reported as 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 **(Singh R *et al.*, 2004)**. Different biological effects of *Rubia* species have been reported as anti-cancer, anti-oxidant, antimicrobial and hepatoprotective effects **(Abd El-Mawla *et al.*, 2014)** , wound-healing, analgesic, antipyretic, antimicrobial, antiviral, antioxidant and antitumor activities **(Essaidi *et al.*, 2017**). *R. tinctorum* root extracts showed antibacterial activities against a wide range of pathogenic bacteria due to its production of anthraquinone pigments in its roots, as alizarin and purpurin which have been used for dying textiles since 2000 BC (**Kalyoncu *et al.*, 2006)** . This research focuses on the antibacterial activity of alizarin, purpurin methanol and chloroform extracts of *Rubia tinctorum* roots against gram positive and gram negative bacteria **(Ghafari *et al.*, 2018).**

## 2. Experimental:

### *2.1. Materials:*

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

### *2.2. Collection of plant material:*

*Rubia tinctorum* roots were purchased from the Egyptian market and identified by Faculty of Science, Suez Canal University. Roots were cleansed and ground using mechanical blender, the resulted powder was stored in airtight glass containers for future use.

### *2.3. Preparation of plant extracts:*

10gm of finely powdered madder roots was macerated with methanol (100 ml x 3), then left for 3 days, followed by filtration. The filtrate was concentrated under vacuum to give 2.5 gm dry extract. About 2 gm of the dry extract were suspended in 200 ml distilled water. This step was followed by fractionation with chloroform, then ethyl acetate (each 200 ml x 2). Each of these extracts was concentrated to dryness using a rotary evaporator **(Bobbarala *et al.*, 2009)**. After complete solvent evaporation, the extracts subjected to investigation (methanol and chloroform) were dissolved in 10% dimethyl sulphoxide (DMSO) (Merck (India) Ltd., Mumbai, India) to a final concentration of 50 mg/ml and stored at 5° in labelled sterile screw capped bottles for further use (10% w/v).

### 2.4. *Bacterial strains*:

In this study 4 bacterial strain were used, 1gram positive (*Staphylococcus aureus ATCC 25923*) and 3 gram negative (*Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853 and Klebsiella pneumonia ATCC 13883*). These strains were obtained from Infection control unit, Faculty of Medicine, Mansoura University. A series of morphological, physiological, and conventional biochemical tests were performed to identify the selected microorganisms. All the test strains were maintained on nutrient agar (Mueller-Hinton agar medium (MHA) at 4°C and sub-cultured on to Mueller Hinton Broth (MHB) for 24 h prior to testing. These bacteria served as test pathogens for antibacterial activity assay. Mueller Hinton Broth (MHB) was used for MIC preparation. Blood Sensitest Agar: Prepare and sterilise Oxoid Sensitest agar (CM409). Cool to 50°C and add defibrinated horse blood to a final concentration of 5%.6 mm diameter antibiotic discs supplied by Oxoid Pty Ltd (Thermo Fisher Scientific Australia amikacin (30 µg), Norfloxacin (10 µg) and meropenem (10 µg).

### *2.5. Antibacterial activity assay:*

Antibacterial activity of aqueous and solvent extracts was determined by agar well diffusion method according to National Committee for Clinical Laboratory Standards **(NCCLS, 1993)**. Inoculums were prepared by using 24 h plate cultures bacteria on 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. (**Ghafari *et al.*, 2018**). Inoculum containing $1.5 \times 10^8$ cfu/ml of each bacterial culture to be tested was spread on nutrient agar plates with a sterile swab moistened with the bacterial suspension. Subsequently, wells of 8 mm diameter were punched into the agar medium and filled with 100 µl (50 mg/ml) of plant extract and allowed to diffuse at room temperature for 2 h. The plates were then incubated in the upright position at 37° for 24 h. Wells containing the same volume of DMSO (10%) 4 types of extracts and distilled water served as negative controls while standard antibiotic discs of amikacin (30 µg), Norfloxacin (10 µg) and meropenem (10 µg) were used as the positive controls. After incubation at 37 ºC for 24 h, the diameters of the growth inhibition zones were measured in mm. Three replicates were carried out for each extract against each of the test organism as showed in **(Table1)**. Data were expressed as mean ± standard deviation.

**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 & Ruangviriyachi, 2014)** with a slight modification. Based on the preliminary screening, methanol & chloroform extracts that revealed potent antimicrobial activity were further tested to determine the minimum inhibitory concentration (MIC) for each bacterial sample. The MIC of these extracts was determined by broth dilution technique where the stocks of 50 mg/ml of the extracts were re-suspended in 10% DMSO to produce serial fold dilution in the range of (50 and 0.097) mg/ml. Each dilution was seeded with bacterial suspension ($1.5 \times 10^8$ cfu/ml). The plate was covered with a sterile sealer and incubated for 24h at 37∘C. after that we inoculated with a standardized inoculum from each well on blood agar medium and incubated for 24h at 37∘C for detection of growth or not. The MIC was considered as the lowest concentration of the extract that completely inhibits the bacterial growth **(CLSI *et al.*, 2015)**. All samples were tested in triplicates.

## 3. Results and Discussion:

The extract was evaluated for the activity at a concentration of 50 mg/mL and inhibition zone diameter in mm was applied as a criterion for the antimicrobial activity. **Table 1** shows displayed variable in vitro antimicrobial effects with inhibition zones in (mm) obtained after applying standard antibiotics susceptibility test as positive control.

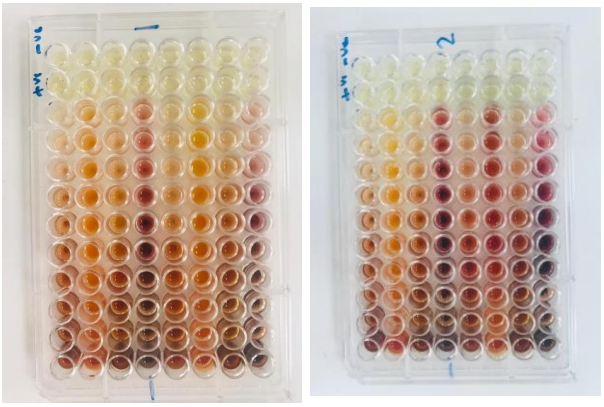

**Figure 1 : MIC of different types of extracts.**

The MIC of *R. tinctorum* extract against the most sensitive organisms was determined using micro-well dilution method in 96-well micro plates method.

Alizarin had the highest activity on *Klebsiella pneumonia* (MIC 12.5 mg/mL) followed by *Pseudomonas aeruginosa* (MIC 25 mg/mL) whereas the extract possessed the lowest activity on both *Staphhylococcus aureus* and *E. coli* (MIC 50 mg/mL), also purpurin had the highest activity on *Klebsiella pneumonia* (MIC 6.25 mg/mL) whereas the extract possessed the lowest activity on *staphhylococcus aureus, E. coli* and *Pseudomonas aeruginosa* (MIC 12.5 mg/mL) (**Figure 2 &3)**.

On the other hand *R. tinctorum* methanol extract had the highest activity on *Klebsiella pneumonia* and *Staphhylococcus aureus* (MIC 3.125 mg/mL) whereas this extract possessed the lowest activity on both *E.coli and Pseudomonas aeruginosa* (MIC 25 mg/mL)**,** while *R. tinctorum* chloroform extract had the highest activity on *Klebsiella pneumonia* (MIC 1.5625 mg/mL) followed by *staphhylococcus aureus* (MIC 3.125 mg/mL) then *E. coli* (MIC 12.5 mg/mL), whereas the extract possessed the lowest activity on both *pseudomonas aeruginosa* (MIC 25 mg/mL) **(Figures 4 & 5)** .

**Minimum Inhibitory Concentration Values**:

The MIC against the most sensitive organisms was determined using modified agar well diffusion method. The obtained results as shown in table 1 were consistent with those obtained from the previous antibacterial test in the present study. Alizarin, purpurin, methanol and chloroform extract of *R. tinctorum* had the highest activity on *Klebsiella pneumonia* (MIC 12.5, 6.25, 3.125, 1.562) mg/mL respectively. Then *Staphhylococcus aureus* (MIC 50, 12.5, 3.125, 3.125) mg/mL respectively. Whereas they possessed the lowest activity on both *E. coli* and *Pseudomonas aeruginosa.*

## 4- Conclusion

In the present study, we have evaluated the antibacterial activity of **a**lizarin, purpurin, methanol and chloroform extract of *R. tinctorum* that showed activity against both gram negative and gram positive bacteria, our observations revealed that they could be a promising lead for the developments of effective therapeutic agents especially against *Klebsiella pneumonia* followed by *Staphylococcus aureus*. Therefore, a future bioassay guided phytochemical study is need to isolate the bioactive compound then assess their activities as effective antibacterial agents.

**Table 1 Antimicrobial activity of *R. tinctorum* extract against tested bacteria**

| | Zone of inhibition in (mm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Alizarin** | **Purpurin** | **Methanol extract of *R. tinctorum*** | **Chloroform extract of *R. tinctorum*** | **Amikacin** | **Norfloxacin** | **meropenem** |
| | **50 mg/ml** | | | | **30 µg** | **10 µg** | |
| ***S. aureus*** | **10.1** | **13** | **19** | **13** | **15** | **NA** | **10** |
| ***E. coli*** | **11** | **15** | **14.3** | **10.1** | **27** | **13** | **30** |
| ***K. pneumonia*** | **19.2** | **21** | **21** | **17** | **23** | **15** | **28** |
| ***P. aeruginosa*** | **16** | **11.5** | **9** | **8.3** | **17** | **30** | **25** |

The test was done using the diffusion agar technique, well diameter: 8.0 mm, 100 µl was tested, Positive control for bacteria: Amikacin (30 µg/ml), Norfloxacin (10 µg/mL) and meropenem (10 µg/mL) *NA: No activity.

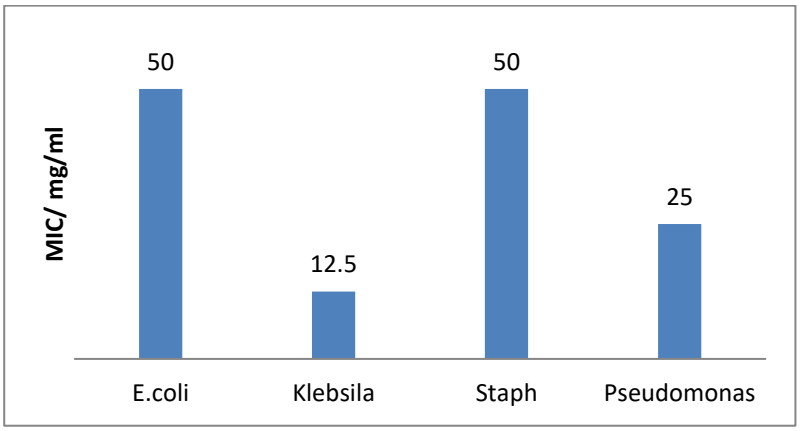


**Figure 2: Minimum inhibition concentration (MIC) of alizarin against selected pathogens**

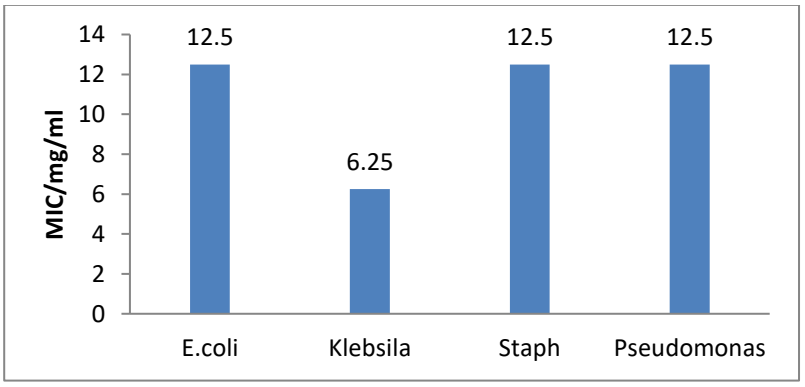


**Figure 3: Minimum inhibition concentration (MIC) of purpurin against selected pathogens**

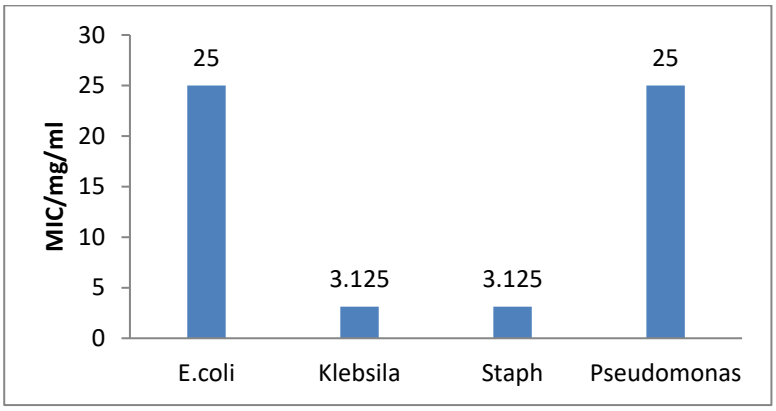


**Figure 4: Minimum inhibition concentration (MIC) of *R. tinctorum* methanolic extract against selected pathogens**

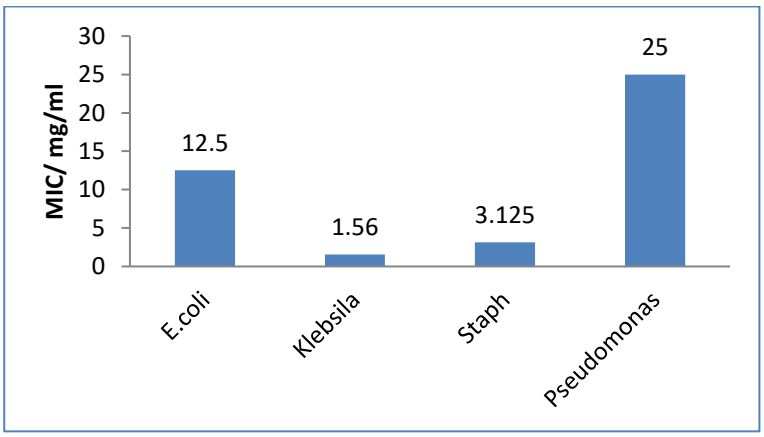


**Figure 5: minimum inhibition concentration (MIC) of *R. tinctorum* chloroform extract against selected pathogens**

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