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# *In vitro* Interaction of Certain Antimicrobial Agents in Combination with Plant Extracts Against Multidrug-resistant *Pseudomonas aeruginosa* Strains

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**Abstract:** This study has been carried out to evaluate the possible *In vitro* interaction between ethanolic extracts of *Rus coriaria* (seed), *Sacropoterium spinosum* (seed) and *Rosa damascena* (flower) and certain known antimicrobial drugs including oxytetracycline HCl, penicillin G, cephalexin, sulfadimethoxine as sodium and enrofloxacin. Synergy testing of these extracts and antibiotics was carried out against 3 multidrug-resistant *Pseudomonas aeruginosa* strains using microdilution method. Results of this study showed that there is a decrease in the MIC in case of combination between ethanolic plant extracts and test antimicrobial agents. The most interesting result that the combination between *R. coriaria* and these antibiotics, which showed a high decrease in MIC and a strong bactericidal activity against these strains. These results may indicate that combinations between *R. coriaria* extract and these antibiotics could be useful in fighting emerging drug-resistance P. aeruginosa, this may due to that *R. coriaria* extract contain natural inhibitors working by different mechanisms or inhibiting efflux pumps. Now we have experiments underway leading to the identification of the active molecules present in *R. coriaria*. Further, *in vivo* experiments are needed to confirm Pseudomonal protection.

**Key words:** Synergism · *R. Coriaria* · S. *spinosum* · *R. damascene* · Medicinal plants · Antimicrobial agents · *P. aeruginosa* · Palestine

## INTRODUCTION

There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases, appearance of undesirable side effects of certain antibiotics, as well as the increasing development of resistance to the antibiotics in current clinical use [1]. Therefore, actions must be taken to control the use of antibiotic, to better understand the genetic mechanisms of resistance and to continue studies to develop new drugs. There are different approaches to cure and control the infection caused by the MDR strains of bacteria. One of these by isolation of active phytochemicals that can help to prevent the spread of infection. An other method is to formulate new synergistic combinations using different commercially available antibiotics, or to combine an antibiotic with active phytochemicals having antimicrobial properties. Several *In vitro* studies have reported synergistic effects with significant reduction in the MICs of the antibiotics, resulting from the combination of different antibiotics with different crude plant extracts against *S. aureus* strains [2-7] and emerge as the real sources of potential resistance modifying agents [8,9]. In addition to that, synergistic effects have been reported against Gram-negative bacteria [10-15]. The ability of plant extracts to potentiate antibiotics has not been well explained. It is predicted that inhibition of drug efflux and alternative mechanisms of action could be responsible for the synergistic interactions between plant extracts and antibiotics [16,17].

*Pseudomonas aeruginosa* causes nosocomial infections as a result of its ubiquitous nature, ability to survive in moist environments and resistance to many

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antibiotics and antiseptics. A main problem is the emergence of multidrug-resistant *P. aeruginosa* strains resistant to different antimicrobial agent classes. Infections caused by this microorganism are often severe, life threatening and difficult to treat because of the high frequency of an emergence of antibiotic resistance during therapy [18]. Perhaps, this high degree multidrug resistance related to the presence of antibiotic efflux systems which provide resistance to multiple antimicrobial agents [19].

There are little data on synergy between extracts of *Rus coriaria*, *Sacropoterium spinosum* and *Rosa damascena* and antibiotics [7,20]. The purpose of the present work was to establish synergy between ethanolic plant extracts of *R. coriaria* (seed), S. *spinosum* (seed) and *Rosa damascena* (flower) and certain known antimicrobial drugs such as oxytetracycline HCl, penicillin G, cephalexin, sulfadimethoxine as sodium and enrofloxacin using microdilution method against 3 multidrug-resistant *P. aeruginosa* strains. Thereby, throwing the light on the potential role of the phytochemicals in increasing the effectiveness of antibiotics.

## MATERIALS AND METHODS

**Plant Material and Extract Preparation:** The plant materials used in this study consisted of *R. coriaria* (seed), S. *spinosum* (seed) and *R. damascena* (flower), which are growing in Palestine. The fresh plant materials were dried in an open air protected from direct exposure to sunlight. Approximately of 30-50 g of dried plant materials were separately powdered and were extracted with 200-300 ml of 80% ethanol as describe previously [21]. Extracts were filtered through Whatman No. 2 filter paper under vacuum and concentrated to dryness at 37°C. Then, 100 mg of the dry residue was dissolved in 1 ml of sterile distilled water.

**Bacterial Strains:** Three strains of multidrug-resistant *P. aeruginosa* were isolated from clinical samples (urine, surgical wound and ear swab) have been used in this study. These strains were resistant to different antibiotics as ampicillin, cefuroxime, cefotaxime, gentamicin, amikacin, erythromycin, clindamycin, ofloxacin, nalidixic acid, norfloxacin, ciprofloxacin and amoxicillin-clavulanic. In addition, *Bacillus subtilis* ATCC 6633 was included as a reference strain.

**Antimicrobial Drugs:** Five drugs were evaluated for synergism assays. These included oxytetracycline HCl (10%), enrofloxacin (10%), sulfadimethoxine as sodium (40%), cephalexin (0.15%) and penicillin G (penicillin G procaine 900000 and penicillin G sodium 300,000 U). All these drugs were produced by Jerusalem Pharmaceutical CO. Balsam branch except penicillin G was produced by Birzeit-Palestine Pharamaceutical Co. These drugs were diluted to a final concentration 200 U/ml fror penicillin G; 50 µg/ml for oxytetracycline HCl, cephalexin and enrofloxacin; and 100 µg/ml sulfadimethoxine.

**Antimicrobial Tests:** Minimum inhibitory concentration (MIC) of antibiotics as well as plant extracts were determined by the microdilution method as described by Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards, NCCLS) [22]. The antibiotic was serially diluted in Mueller Hinton broth. Plant extracts solution were separately added into wells in a final concentration 1.5 mg/ml, then bacterial inoculum size of $10^5$ CFU/ml was added to each well. Controls without plant extracts, without bacterial inoculum or with plant extracts only were also included in the experiment. Each plant extract was run in duplicate. The test plates were incubated at 37°C for 18 h. The MIC was taken as the minimum concentration of the dilutions that inhibited the growth of the test microorganism. For bactericidal activity detection, 100 µl were spread on agar plate and incubated at 37°C for 18 h.

## RESULTS

Our results showed that there is a decrease in the MIC in case of combination between ethanolic plant extracts of *R. coriaria*, *S. spinosum* and *R. damascena* and different antimicrobial agents (oxytetracycline HCl, penicillin G, cephalexin, Sulfadimethoxine as sodium and enrofloxacin) against 3 test strains of P. aeruginosa using microdilution method. This implies that these plant extracts increased the antibacterial activity of the antibiotics against the test strains of *P. aeruginosa* and showed synergistic interaction. The most interesting result that the combination between *R. coriaria* and these antibiotics, which showed a high decrease in MIC and a strong bactericidal activity against these strains.

Table 1: Minimum inhibitory concentration of antibiotics alone, plant extracts alone and in combination against 3 clinical isolates of P. aeruginosa using microdilution method

| | MIC (µg/ml) | | | Minimum fold reduction of |
|---|---|---|---|---|
| Antibiotic[a]/plant extrat | Strain1 | Strain 2 | Strain3 | inhibitory concentration |
| *R. coriaria* | 3.125 X $10^3$ | 3.125-1.563 X $10^3$ | 1.563 X $10^3$ | |
| S. *spinosum* | 6.5 X $10^3$ | 12.5-6.5 X $10^3$ | 12.5 X $10^3$ | |
| *R. damascena* | 25 X $10^3$ | 25-12.5 X $10^3$ | 12.5 X $10^3$ | |
| ENR | >25 | >25 | >25 | |
| *R. coriaria* + ENR | <0.0122 | <0.0122 | <0.0122 | >2000 |
| S. *spinosum* + ENR | 1.563 | 0.39 | 0.195 | >64 |
| *R. damascene* + ENR | 3.125 | 3.125 | 3.125 | >64 |
| OT | >25 | >25 | >25 | |
| *R. coriaria* + OT | <0.0244 | <0.0244 | <0.0244 | >1024 |
| S. *spinosum* + OT | 6.25 | 6.25 | <0.0244 | >4 |
| *R. damascene* + OT | 6.25 | 6.25 | 6.25 | >4 |
| CL | >25 | >25 | >25 | |
| *R. coriaria* + CL | <0.0122 | <0.0122 | <0.0122 | >2000 |
| S. *spinosum* +CL | 0.0488 | 0.195 | 1.563 | >16 |
| *R. damascene* CL | 0.0488 | 0.195 | 1.563 | >16 |
| P (Unit) | >100 | >100 | >100 | |
| *R. coriaria* + P | <0.0488 | <0.0488 | <0.0488 | >2000 |
| S. *spinosum* + P | 12.5 | 1.563 | 0.195 | >8 |
| *R. damascene* + P | 12.5 | 3.125 | 1.563 | >8 |
| SDM | >50 | >50 | >50 | |
| *R. coriaria* + SDM | <0.195-0.0977 | <0.0244 | <0.0244 | >256 |
| S. *spinosum* + SDM | 6.25 | 6.25 | <0.0244 | >8 |
| *R. damascene* + SDM | 6.25 | 0.78 | 3.125 | >8 |

[a]P, Penicillin G; CL, Cephalexin; SDM, sulfadimethoxine as sodium; ENR, Enrofloxacin; OT, Oxytetracycline Hcl

Minimum fold reduction of inhibitory concentration and change in MIC of antimicrobial agents are presented in Table 1.

## DISCUSSION

Many studies have shown that active efflux can be a mechanism of resistance for almost all antibiotics [23]. The majority of the efflux systems in bacteria are non-drug-specific proteins that can recognize and export a broad range of chemically and structurally unrelated compounds from bacteria without drug alteration or degradation [24]. Antibiotic efflux is a major mechanism of antibiotic resistance in *P. aeruginosa* due to Mex efflux proteins. Resistance to β–lactams and non-β-lactam antibiotics has been attributed to efflux by the MexAB-OprM pump [25]. Other Mex efflux proteins mediating multidrug resistance have also been identified in *P. aeruginosa* [26]. Efflux pump inhibitors combined with antibiotics strategy is an effective way to solve the problem caused by resistant bacteria [27]. The majority of plant derived antimicrobial compounds generally have higher MICs than bacterial or fungal produced antibiotics, thus limiting their therapeutic potential [28].

The plant extracts tested in this study, especially *R. coriaria* extract with oxytetracycline HCl, penicillin G, cephalexin, Sulfadimethoxine as sodium, or enrofloxacin showed a powerful bactericidal activity to three test strains of *P. aeruginosa* and combinations have obvious synergistic activity. These results may indicate that *R. coriaria* extract contain natural inhibitors working by different mechanisms or inhibiting efflux pumps.

In conclusion, the results of this study were encouraging, although clinical controlled studies are needed to define the real efficacy and possible toxic

effects *in vivo*. However, it is hard to predict synergistic effects *in vivo* on the basis of the presented *in vitro* evidence alone because it is difficult to estimate the *in vivo* concentration of active ingredients. Now we have experiments underway leading to the identification of the active molecules present in *R. coriaria*. Here we recommended the evaluation of the exact drug-plant ratio at which the interaction in maximal between the plant extract and antimicrobial drug. A wider study with increase in the number of drugs, increase number of clinical isolates, are also necessary in order to establish the mode of action against the *P. aeruginosa* isolates and the mechanism of synergy, which is fundamental to development of pharmacological agents to treat diseases by *P. aeruginosa* using medicinal plants. Our results revealed that the combined use of plant extracts and antibiotics could be useful in fighting emerging drug-resistance problem and *in vivo* experiments are needed to confirm pseudomonal protection using these combinations.

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