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Full Length Research Paper

Inhibitory effect of *Cytisus nigricans* L. and *Cytisus capitatus* Scop. on growth of bacteria

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***In vitro* antibacterial activity of ethanol, ethyl acetate and acetone extract from *Cytisus nigricans* and *Cytisus capitatus* and their synergistic interaction with gentamicin and cephalexin were studied. Antibacterial efficacy of the extracts was defined by determining minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC). The values were in the range from 1.25 mg/ml to >20 mg/ml. The most sensitive bacteria were: *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* and *Pseudomonas aeruginosa* ATCC 27853. Herb-drug interactions tested by checkerboard method and expressed as fractional inhibitory concentration (FIC) index showed indifferent and synergistic effects. Synergism was observed only in combination between antibiotics and ethanol, ethyl acetate extract of *C. nigricans*. In the presence of sub-inhibitory concentrations (1/4 MIC, 1/8 MIC) of the extracts the activity of gentamicin and cephalexin were increased up to 16-fold. The highest amount of flavonoids was measured in acetone extracts while the highest amount of total phenols had ethanol extract of *C. nigricans* and acetone extract of *C. capitatus*.**

Key words: Antibacterial activity, *Cytisus nigricans*, *Cytisus capitatus*, herb-drug interaction, plant extract, total phenol and flavonoid content.

INTRODUCTION

During the last half of the 20th century, chemical ecology has become a recognized sub discipline. Understanding the chemical interactions between different plant species, and between plants and other organisms, has resulted in the discovery of bioactive compounds with potential uses for humans. Plants produce a diverse array of metabolites that are not involved in primary metabolism. These secondary metabolites determine our sensory perception of unique characteristics of plants: the pretty colors and smell the fragrances of flowers and fruits, the distinctive tastes of spices, vegetables and fruits. Moreover, all of the biological activities of plants that humans have used for medicinal reasons for hundreds of years can be attributed to secondary metabolites (Hartmann, 2008).

The increasing problem with bacterial resistance has led to systematic screening of plants as a source of bioactive compounds. Finding and testing of new antibacterial agents as well as development of new approaches could help to overcome the problem of bacterial resistance and less susceptible bacteria.

In recent years there have been many studies about the positive, synergistic, interactions between plant extracts or pure isolated compound with commonly used antibiotics against variety of microorganisms (Nostro et al., 2006; Nagoshi et al., 2006; Horiuchi et al., 2007; Stefanovic et al., 2009a, b). These findings support the possible use of phytocompounds together with antibiotics to increase their potency and avoid undesirable side effects (Kubo et al., 1996). Synergistic interaction between two agents, in which one agent enhances the action of the other and together, they act more effectively than a single agent. This could be a new approach to solve the problem of bacterial resistance and less susceptible bacteria.

Cytisus nigricans L. and *Cytisus capitatus* Scop. belong to family Fabaceae. This family is one of the largest and most economically important plant families. Plants are

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widely distributed in Europe, North Africa and West Asia.

C. nigricans L., syn. *Lembotropis nigricans* (L.) Griseb. (black-rooted broom) is an erect, deciduous, trifoliate, up to 1.5 m tall shrub with small, linear, palmate, mid-green leaves and erect, terminal raceme of deep yellow flowers. It grows in sunny, rocky place (Josifovic, 1972).

C. capitatus Scop. syn. *Cytisus supinus* L., *Chamaecytisus hirsutus* L. (round-headed broom) is 20 to 60 cm tall, evergreen to deciduous, trifoliate shrub. The sweetly fragrant yellow flowers, very attractive to bees, are organized in axillary clusters forming a shape of a round head. It prefers a humus rich, well drained, slightly moist soil under sunny conditions throughout the year (Josifovic, 1972). These plants represent native flora of Serbia.

Biological activities, as well as, the chemical components of these plants are almost unexplored. Only Hanganu et al. (2010) isolated several isoflavones, as a source of phytoestrogens, from hidroalcoholic extracts from species belonging to the Fabaceae family. The *C. nigricans* contained 270.6 ng/ml of ononin.

The aims of this work were to explore the antibacterial activity of ethanol, ethyl acetate and acetone extract of *C. nigricans* and *C. capitatus* and their possible synergistic interaction with commonly used antibiotics (gentamicin and cephalexin), as well as, to determine a total phenol and flavonoid content in the extracts.

MATERIALS AND METHODS

General

The following reagents were used: Folin-Ciocalteu phenol reagent and aluminium chloride hexahydrate (from Fluka Chemie AG, Buchs, Switzerland), gallic acid and rutin hydrate (from Sigma Chemicals Co., St Louis, MO, USA). All solvents (ethanol, ethyl acetate, acetone, methanol) and sodium hydrogen carbonate were purchased from Zorka pharma, Sabac, Serbia, except dimethylsulfoxide (DMSO) was from Merck, Germany. Resazurin powder was obtained from Alfa Aesar, Germany. Nutrient media: Mueller-Hinton broth and Mueller-Hinton agar were purchased from Liofilchem, Italy. Antibiotics (gentamicin and cephalexin) were from Panfarma, Beograd and Serbia.

Plant material

Aerial parts of *C. nigricans* and *C. capitatus* were collected on Mt. Goc (Serbia) during the summer of 2009. Identification and classification of the plant material was performed at the Faculty of Science, University of Kragujevac. The voucher samples of *C. nigricans* (200915) and *C. capitatus* (200916) are deposited in the Herbarium of the Faculty of Science, University of Kragujevac.

Extraction

Dried, ground plant material was extracted by direct maceration with ethanol, ethyl acetate and acetone. Briefly, 30 g of plant material was soaked with 150 ml of solvent for 24 h at room temperature, after that the resulting extract was filtered through filter paper (Whatman no.1). The residue from the filtration was extracted again twice using the same procedure. The filtrates obtained were combined and then evaporated to dryness using a rotary evaporator at 40°C. The amounts of crude extracts obtained from *C. nigricans* were 3.9 g of ethanol extract, 2.24 g of ethyl acetate extract, 0.83 g of acetone extract and 3.45 g of ethanol extract, 1.61 g of ethyl acetate extract, 1.37 g of acetone extract from *C. capitatus*. Stock solutions of crude extracts were obtained by dissolving in DMSO and then diluted into Mueller-Hinton broth to achieve a concentration of 10% DMSO. Solutions of antibiotics were obtained by dissolving in a Mueller-Hinton broth.

Test microorganisms

The following bacteria were used: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and clinical isolate of *S. aureus* (PMFKg-B30), *Bacillus subtilis* (PMFKg-B2), *Enterococcus faecalis* (PMFKg-B22), *Klebsiella pneumoniae* (PMFKg-B26), *E. coli* (PMFKg-B32), *P. aeruginosa* (PMFKg-B28) and *Proteus mirabilis* (PMFKg-B29). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. Bacteria are stored in microbiological collection at the Laboratory of Microbiology (Faculty of Science, University of Kragujevac).

Inoculum preparation

Bacterial suspension were prepared from overnight cultures by the direct colony method. Colonies were taken directly from the plate and suspent into 5 ml of sterile 0.85% saline. The turbidity of initial suspension was adjusted comparing with 0.5 Mc Farland standard (0.5 ml 1.17% w/v $BaCl_2$ × 2H $_2O$ + 99.5 ml 1% w/v H_2SO_4) (Andrews, 2001). When adjusted to the turbidity of a 0.5 Mc Farland standard, a suspension of bacteria contain about 10^8 colony forming units (CFU)/ml. Ten-fold dilutions of initial suspension were additionally prepared into sterile 0.85% saline to achieve 10^6CFU/ml. The number of bacteria was confirmed by plate count.

Antibacterial assay

Antibacterial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by using microdilution plate method with resazurin (Sarker et al., 2007). Briefly, the 96-well microplate was prepared by dispensing 100 µL of Mueller-Hinton broth into each well. A 100 µl from the stock solution of tested extract (concentration of 40 mg/ml) was added into the first row of the plate. Then, two-fold, serial dilutions were performed by transferring 100 µl of solution from one row to another, using a multichannel pipette. The obtained concentration range was from 20 to 0.156 mg/ml. Ten microlitres of each 10^6 CFU/ml bacterial suspension was added to wells. Finally, 10 µl of resazurin solution was added. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37°C for 24 h. MIC was defined as the l owest concentration of tested compound that prevented resazurin color change from blue to pink.

Cephalexin was used as positive control. Solvent control test was performed to study an effect of 10% DMSO on the growth of microorganism. It was observed that 10% DMSO did not inhibit the growth of microorganism. Moreover, because of the two-fold serial dilution assay the final concentration of DMSO used in the experiment was 5% and lower. Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Minimum bactericidal concentration was determined by plating 10 µL of samples from wells, where no indicator color change was recorded, on Mueller-Hinton agar. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum bactericidal concentration.

MIC index

The MIC index (MBC/MIC) was calculated for each extract and positive control drug to determine whether an extract has bactericidal (MBC/MIC≤4) or bacteriostatic (>4 MBC/MIC<32) effect on growth of bacteria (Cutler et al., 1994).

Combination assay

The synergistic interactions were evaluated by checkerboard method (Satish et al., 2005). Briefly, the series of twofold dilutions of gentamicin and cephalexin concentration from MIC to 1/32 MIC and the series of twofold dilutions of ethanol, acetone and ethyl acetate extracts concentration from MIC to 1/32 MIC were prepared. The 36 different combinations were tested for one bacterium. Hundred microlitres of Mueller-Hinton broth were added into 36 wells of 96-well microplate. 50 µl of each dilutions of extract were added horizontally into six rows and 50 µl of each dilutions of antibiotic were added vertically into six columns. The final volume was 200 µl. Because the final volume is four times as great as the volume of each antimicrobial, the antimicrobial concentrations used in the stock solutions are four times greater than the desired final concentrations. Each well contained unique combination of plant extract and antibiotic concentration. The plate was inoculated with 10 µL of the bacterial suspension prepared as those used for MIC assay. Finally, 10 µl of resazurin solution was added. The plate was incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of antimicrobial agents in combination that prevented resazurin color change from blue to pink. Each test included growth control, solvent control and sterility control.

In vitro interactions between antimicrobial agents were determined and quantified by calculating the fractional inhibitory concentration (FIC) index using the following formula: FIC index = (MIC of plant extract in combination/MIC of plant extract alone) + (MIC of antibiotic in combination/MIC of antibiotic alone). Interpretation of the FIC index (FICI) was as follows: FICI ≤ 0.5 synergy; FICI > 0.5 - 4 indifference and FICI > 4 antagonism (Satish et al., 2005; White et al., 1996). The action of antimicrobial agents is considered to be:

- Synergistic if their joint effect is stronger than the sum of effects of the individual agents.
- Indifferent if their joint effect is equal to the effect of either individual agent.
- Antagonistic if their joint effect is weaker than the sum of effects of the individual agents or weaker than the effect of either individual agent.

If the MIC of any agent alone occurred at the highest concentration tested, the FIC index was considered not determinable and synergy could not be assessed. Where more than one combination resulted in a change in the MIC value of the extract or antibiotic, the FIC index was expressed as the average of the FIC index values.

Total phenol content

Total phenol content in the plant extracts was measured using spectrophotometric method (Singleton et al., 1999). The methanol solution of the extract in concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanol solution of the extract, 2.5 ml of 10% Folin-Ciocalteu reagent dissolved in water and 2 ml of 7.5% $NaHCO_3$. The blank was prepared containing 0.5 ml of methanol, 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of 7.5% of $NaHCO_3$. The samples were incubated in the thermostat at 45°C for 45 min. The absorbance was measured using spectrophotometer at λ_{max} = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and a calibration curve was created. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration curve; then the content of phenolics in the extracts was expressed in terms of gallic acid equivalent, GAE (mg of GAE/g of extract).

Total flavonoid content

The concentration of flavonoids in the plant extracts was determined using spectrophotometric method (Quettier et al., 2000). The sample contained 1 ml of methanol solution of the extract in concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was measured using spectrophotometer at λ_{max}= 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and a calibration curve was created. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration curve; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent, RUE (mg of RUE/g of extract).

RESULTS AND DISCUSSION

Antibacterial activity

The results of antibacterial activity of ethanol, ethyl acetate and acetone extract from *C. nigricans* and *C. capitatus* against 10 species of Gram-positive and Gram-negative pathogenic bacteria are presented in Tables 1 and 2. The extracts showed selective antibacterial properties and the activity depended both on the species of bacteria and on the type and concentration of extract.

Inhibitory effects of bacterial growth by the extracts from *C. nigricans* were in the range from 1.25 to > 20 mg/ml expressed as MIC values and in the range from 2.5 to > 20 mg/ml expressed as MBC values. The most sensitive bacteria were *P. aeruginosa* ATCC 27853 (MIC for ethanol extract was 1.25 mg/ml), *S. aureus* ATCC 25923 (MIC for acetone extract was 2.5 mg/ml) and *B. subtilis* (MIC for ethanol and acetone extract was 2.5 mg/ml). The ethyl acetate and acetone extract were not found effective against the growth of *E. coli.* According to the MIC index, 90% of the extracts showed bactericidal activity.

C. capitatus ethyl acetate and acetone extract showed better activity than ethanol extract. The MIC values of ethyl acetate and acetone extract were in the range from 1.25 to >20 mg/ml while for ethanol extract were in the range from 5 to >20 mg/ml. The MBC values of tested extracts were from 1.25 to >20 mg/ml. The most sensitive bacteria towards ethyl acetate and acetone extract of *C.*

Table 1. Antibacterial activity of ethanol, ethyl acetate and acetone extract from *C. nigricans.*

Species	Ethanol extract			Ethyl acetate extract			Acetone extract			Cephalexin		
	MIC*	MBC*	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC**	MBC**	MIC index
E. coli ATCC 25922	20	20	1	20	>20	n.d.	20	20	1	6.25	6.25	1
S. aureus ATCC 25923	5	20	4	5	20	4	2.5	10	4	6.25	6.25	1
P. aeruginosa ATCC 27853	1.25	5	4	5	5	1	10	10	1	> 1000	> 1000	n.d.
B. subtilis	2.5	20	8	5	5	1	2.5	2.5	1	12.5	12.5	1
S. aureus	20	20	1	20	20	1	20	20	1	1.56	1.56	1
E. faecalis	20	20	1	20	>20	n.d.	20	20	1	3.125	6.25	2
E. coli	20	20	1	>20	>20	n.d.	>20	>20	n.d.	1.56	1.56	1
K. pneumoniae	20	>20	n.d.	20	>20	n.d.	20	20	1	500	1000	2
P. aeruginosa	10	>20	n.d.	20	>20	n.d.	10	20	2	> 1000	> 1000	n.d.
P. mirabilis	10	10	1	20	>20	n.d.	20	20	1	> 1000	> 1000	n.d.

* Values are expressed in mg/ml; ** Values are expressed in μg/ml n.d. - not determined.

Table 2. Antibacterial activity of ethanol, ethyl acetate and acetone extract from *C. capitatus.*

Species	Ethanol extract			Ethyl acetate extract			Acetone extract			Cephalexin		
	MIC*	MBC*	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC**	MBC**	MIC index
E. coli ATCC 25922	20	20	1	20	20	1	20	20	1	6.25	6.25	1
S. aureus ATCC 25923	5	10	2	1.25	1.25	1	2.5	2.5	1	6.25	6.25	1
P. aeruginosa ATCC 27853	10	20	2	10	20	2	20	20	1	> 1000	> 1000	n.d.
B. subtilis	5	10	2	2.5	2.5	1	1.25	1.25	1	12.5	12.5	1
S. aureus	>20	>20	n.d.	>20	>20	n.d.	>20	>20	n.d.	1.56	1.56	1
E. faecalis	>20	>20	n.d.	10	10	1	20	>20	n.d.	3.125	6.25	2
E. coli	>20	>20	n.d.	>20	>20	n.d.	>20	>20	n.d.	1.56	1.56	1
K. pneumoniae	20	20	1	20	>20	n.d.	20	>20	n.d.	500	1000	2
P. aeruginosa	20	20	1	10	10	1	20	20	1	> 1000	> 1000	n.d.
P. mirabilis	20	>20	n.d.	10	20	2	20	20	1	> 1000	> 1000	n.d.

* Values are expressed in mg/ml; ** Values are expressed in μg/ml n.d. - not determined.

capitatus were Gram-positive bacteria: *S. aureus* ATCC 25923 and *B. subtilis*. No activity was recorded against clinical isolate of *S. aureus*, *E. coli* and *E. faecalis.* According to the MIC index, in more than 60% the extracts showed bactericidal activity. On the basis of literature data, this work represents

Table 3. Total phenol content and total flavonoid content of ethanol, ethyl acetate and acetone extract from *C. nigricans* and *C. capitatus.*

Plant	Plant extract	Total phenol content* (mg of GAE/g)	Total flavonoid content** (mg of RUE/g)
C. nigricans	Ethanol extract	86.31 ± 0.28	88.83 ± 0.18
	Ethyl acetate extract	40.51 ± 0.12	34.74 ± 0.95
	Acetone extract	49.57 ± 0.29	169.96 ± 1.25
C. capitatus	Ethanol extract	65.04 ± 0.25	73.01 ± 0.62
	Ethyl acetate extract	41.42 ± 0.15	91.61 ± 0.47
	Acetone extract	74.46 ± 0.29	168.11 ± 0.84

*Total phenolics are expressed as gallic acid equivalent/g of extract (GAE/g) ** Total flavonoids are expressed as rutin equivalent/g of extract (RUE/g). Values represent mean ± S.D.

the first results of antibacterial properties of these plants.

Plant extracts contain a vast source of different compounds (phenols, flavonoids, tannins, coumarins, alkaloids) which have an impact on growth and metabolism of microorganisms (Cowan, 1999). Since phenols and flavonoids significantly contribute to the overall antibacterial activity, it was reasonable to determine their total amount in the tested extracts. The total phenol and flavonoid content is shown in Table 3.

The highest amount of flavonoids was measured in acetone extracts, 169.96 mg of RUE/g of extract for *C. nigricans* and 168.11 mg of RUE/g of extract for *C. capitatus*, while the lowest content was measured in ethyl acetate extract of *C. nigricans* with 34.74 mg of RUE/g of extract. The most total phenolics were in *C. nigricans* ethanol extract, 86.31 mg of GAE/g. The lowest total phenol content was in ethyl acetate extracts of tested plants, 40.51 mg of GAE/g of extract for *C. nigricans* and 41.42 mg of GAE/g of extract.

Combining effects of extracts and antibiotics

In this work, possible joint activity of *C. nigricans* and *C. capitatus* extracts and two antibiotics, gentamicin and cephalexin, was evaluated. The experiment was done against pathogenic bacteria which are often cause by human infection and which often develop a resistance to antibiotics in clinical settings. Synergistic effect was detected only for extracts of *C. nigricans*. The results, expressed as FIC index, are presented in Table 4. For *C. capitatus*, indifferent effect in relation to all tested bacteria was observed.

Ethanol and ethyl acetate extract of *C. nigricans* act synergistically with gentamicin and cephalexin. The FIC indices were in the range of 0.37 to 0.44. The combination of ethanol extract with gentamicin and cephalexin showed synergy in relation to *Pseudomonas aeruginosa* ATCC 27853, *B. subtilis* and *K. pneumoniae*. With *Enterococcus faecalis*, the combination of ethyl acetate extract with gentamicin and cephalexin demonstrated synergy. It was found that the presence of sub-inhibitory concentrations (1/4 MIC, 1/8 MIC) of the extracts modulated the activity of gentamicin and cephalexin by reducing the concentration of antibiotic; needed to inhibit the growth of bacteria. The concentration of antibiotics was decreased up to 16-fold. For *K. pneumoniae*, resistant to cephalexin, was interesting that ethanol extract increased the activity of antibiotic. Interaction between acetone extract of *C. nigricans* and antibiotics was indifferent.

Synergistic interactions are a result of a combined effect of active compounds from extracts and antibiotics. It seems that both active compounds, from extracts and antibiotics, directly or indirectly attach the same site on bacterial cell. Some authors suggest that phytocompounds disturb cell wall or increase permeability of the cytoplasmic membrane and thereby facilitate the influx of antibiotics, produce efflux pump inhibitors or inhibit penicillin-binding proteins (Zhao et al., 2001; Shiota et al., 2004; Sibanda and Okoh, 2007). However, the understanding of mechanism of synergy is fundamental to development of pharmacological agents against bacterial infection.

Conclusion

The results of this work indicate the potential antibacterial efficacy of *C. nigricans* and *C. capitatus* ethanol, ethyl acetate and acetone extract against tested pathogenic bacteria. The significant amount of phenols and flavonoids contribute in total biological activity of these plants. The detection of synergy between the extracts of *C. nigricans* and antibiotics, demonstrates the potential of this plant as a source of antibiotic resistance modiflying compounds.

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Table 4. Fractional inhibitory concentration (FIC) indices for the combinations between the extracts of *C. nigricans* and antibiotics.

Species	Antibiotic	MIC (μg/ml)	Ethanol extract	Ethyl acetate extract	Acetone extract
E. coli ATCC 25922	Gentamicin	0.39	1.41 (I)	1.41 (I)	1.41 (I)
	Cephalexin	6.25	1.56 (I)	1.56 (I)	1.35 (I)
S. aureus ATCC 25923	Gentamicin	0.19	1.12 (I)	1.32 (I)	1.42 (I)
	Cephalexin	6.25	1.24 (I)	1.12 (I)	1.24 (I)
P. aeruginosa ATCC 27853	Gentamicin	0.098	0.44 (S)	1.12 (I)	1.12 (I)
	Cephalexin	>1000	n.d.	n.d.	n.d.
B. subtilis	Gentamicin	3.125	0.375 (S)	1.29 (I)	1.29 (I)
	Cephalexin	12.5	0.375 (S)	1.29 (I)	1.29 (I)
S. aureus	Gentamicin	0.39	1.2 (I)	1.27 (I)	1.29 (I)
	Cephalexin	1.56	1.07 (I)	1.23 (I)	1.23 (I)
E. faecalis	Gentamicin	0.39	1.35 (I)	0.37 (S)	1.35 (I)
	Cephalexin	3.125	1.34 (I)	0.395 (S)	1.30 (I)
E. coli	Gentamicin	1.56	1.35 (I)	n.d.	n.d.
	Cephalexin	1.56	1.44 (I)	n.d.	n.d.
K. pneumoniae	Gentamicin	6.25	0.395 (S)	1.47 (I)	1.56 (I)
	Cephalexin	500	0.37 (S)	1.33 (I)	1.33 (I)
P. aeruginosa	Gentamicin	>1000	n.d.	n.d.	n.d.
	Cephalexin	>1000	n.d.	n.d.	n.d.
P. mirabilis	Gentamicin	>1000	n.d.	n.d.	n.d.
	Cephalexin	>1000	n.d.	n.d.	n.d.

I - indifference; S – synergism, n.d. - not determined.

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