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Antibacterial activities and ciprofloxacin potentiation of *Melissa officinalis* extracts against some gram negative pathogenic bacteria

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ABSTRACT

Background and aims: In bacteria, using inhibitors of efflux pumps (EPIs) is one of several strategies to combat with bacterial resistance. It is well documented that most medicinal plants especially those with antimicrobial properties are composed of elements (EPIs) that disturb the efflux pumps of bacteria.

The current work was designed to evaluate antibacterial activities of ethanol and chlorophorm extracts of *Melissa officinalis* along with the synergistic effects of the extracts with ciprofloxacin against some gram-negative pathogenic bacteria. We also examined the inhibitory effects of the extracts on efflux pumps.

Methods: Minimum inhibitory concentrations (MICs) of the extracts alone or in association with ciprofloxacin or phenylalanine arginine β- naphtylamide (PAβN) were determined using broth micro dilution method. Effects of the extracts on efflux pumps of the examined bacteria were detected by using ethidium bromide in well diffusion assays.

Results The extracts from *M. officinalis* showed antibacterial activities against all examined bacteria in a range of 3125 to 25000 µg/mL as determined by MIC determination.

The extracts from *M. officinalis* showed synergistic effects with ciprofloxacin on *Salmonella enteritidis* and *Escherichia coli.* In *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* PAβN had no effect on MIC of ciprofloxacin but the association of extracts decreased it. In *S. enertidis* and *E Coli,* both extracts of *M. officinalis* increased the amount of ethidium bromide accumulation (i.e. reduced efflux).

Conclusion: The overall results show that associations of fluoroquinolones with extracts of *M. officinalis* may potentiate the antibacterial effects of fluoroquinolones.

Keywords: Antibacterial activities, *Melissa officinalis*, Gram-negative bacteria, Efflux pumps

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Original article

INTRODUCTION

Multiple mechanisms including active efflux pumps are used by multidrug resistant (MDR) gram-negative bacteria to resist against antimicrobial compounds.

Efflux pumps role in excreting entered antimicrobials out of the cell is one of several mechanisms that lead to resistance against antibacterial drugs [1].

It is reported that efflux pumps are involved in fluoroquinolone resistance among the gram negative pathogenic bacteria that affect hospitalized patients [2]. Over expression of these pumps in pathogenic bacteria can lead to emergence of resistant pathogenic strains that resist against many antimicrobials such as ciprofloxacin [3].

A well known chemical efflux pump inhibitor compound (EPI) is phenylalanyl arginyl beta naphthylamide (PAßN) [4]. In some species of the Enterobacteriaceae, PAßN inhibit the efflux activities of AcrAB-TolC, but in *P. aeruginosa*, MexAB-OprM, MexEF-OprN, and MexCD-OprJ, pumps are inhibited [5].

Reports show that extracts of some medicinal plants may inhibit efflux pumps of bacteria and can be considered as EPIs [6].

Among the medicinal plants, *Melissa officinalis* traditionally is used for the treatment of headache, hypersensitivities, digestion disorders, and sedative properties.

This plant contains various compounds such as phenolic acids which in addition to physiological activities, show antibacterial, antiviral, and antifungal effects [7].

The aim of the present work was to search the antibacterial activities of the ethanol and chlorophorm extracts of *M. officinalis* and also to evaluate if these extracts have synergistic effects with ciprofloxacin as a fluoroquinolone representative. Also, it was attempted to analyze the extracts to see if they contain inhibitors of efflux pumps of some gram-negative pathogenic bacteria.

METHODS

Dried leaves and branches of *M. officinalis* were purchased from a local market in Shahrekord and transferred to Shahrekord University for taxonomical identification at the Botany Department.

The plant pieces were collectively powdered and extracted with ethanol 85% and chloroform for 48 h at 25°C. After filtration, the extracts were concentrated under vacuum (8). All extracts were kept at 4°C for further investigations.

Bacterial Cultures and Materials

The examined microorganisms including standard strains of *Pseudomonas aeruginosa* ATCC 9027 and *Acinetobacter baumannii*, NCTC 13305 kindly prepared by Dr. B.

Zamanzad and Dr. A. Gholipour (Dept. of microbiology, Shahrekord Medical school), and *Escherichia coli* ATCC 25922 and *Salmonella enteritidis* RTCC 2465 kindly delivered by Dr. H. Motamedi (Dept. of microbiology, College of basic Science, Shahid Chamran University) were kept in Lauria Bertani broth (LB broth, Merck, Germany) at 4°C and sub cultured on appropriate agar plates 24 h prior to antimicrobial tests. Mueller Hinton Broth (MHB, Merck, Germany) was used for all the antibacterial assays. Ciprofloxacin (cip, Sigma, Germany) as fluoroquinolone representative and phenylalanine arginine β- naphtylamide (PAβN) (Sigma, Germany) as microbial growth indicator and efflux pumps inhibitor (EPI) were used, respectively.

Antimicrobial Testing

Tube dilution method was used for preliminary assaying of examined drugs against bacterial strains, and Double serial micro dilution method was used to determine the MICs for drug combinations according to guidelines of the Clinical and Laboratory Standards Institute [9]. Briefly, the cultures of the examined bacteria were incubated aerobically at 37 °C for 18- 24 h. The cultures turbidity were adjusted to 0.5 McFarland (1.5×10^8 CFU/ml) and then diluted in saline solution so as to obtain an inoculum of 5×10^5 CFU/ well. The first well of each 96 well micro plate row was inoculated with four MIC of drug/drugs (for each strain its own MIC was considered) and followed by double dilution in successive wells to detect any possible antagonistic or synergistic combinations. The two last wells were set up without drug/drugs and without bacteria as positive and negative controls, respectively.

Incubation of the inoculated micro plates was at 37 °C in aerobic and shaking conditions for about 18 h. After incubation, the lowest concentration that inhibits visible growth of the examined strain was defined as MIC. To verify synergistic activity of ciprofloxacin with the extracts, activity of ciprofloxacin in associations with extract was compared with that of ciprofloxacin plus PAßN (30 µg/ml in prepared stock solution), whose MIC was also determined.

Interaction of drugs in combinations was calculated by the ratio of $MIC_{\text{Antibiotic in combination}}/MIC_{\text{Antibiotic alone}}$ and the results were interpreted as follows: synergy (< 0.5), indifferent (0.5 to 4), or antagonism (> 4) [10, 11]. All examinations were performed in duplicate.

Efflux Pump Inhibition Assay

Effects on efflux pumps activity in synergistic drug combinations were evaluated by some modifications in the method of Martins et al. [12] so that we can use that method as well diffusion assay. Briefly, examined strains were cultured as cross lines in four equal place of MHA plates and wells were created in cross of lines using a sterile Burrell tip. Of each MHA plate, one well was inoculated with *50* µl ethidium bromide (EB) 6mg/L and *50* µl distilled water, two wells with synergistic ethanolic or chloroform extracts plus EB, and one with PAβN plus EB (in each case 1 MIC concentration and *50* µl volume). The

Table 1: Minimum inhibitory concentrations (MICs), (µg/mL.) of ciprofloxacin (CIP), and Phenylalanine arginine β-aphthylamide (PAβN) in the absence and presence of *M. officinalis* extracts against some gram negative
Bacteria*

Combination / Bacteria	Cip.	Eth.E. +Cip.	Eth.E.	Ch.E. + Cip.	Ch.E.	PAβN	PAβN+Cip
Ps. aeruginosa	1.98	0.992	12500	0.992	3125	3.75	1.98
S. enteritidis	0.0312	**0.0078**	25000	**0.0039**	12500	7.5	**0.0156**
E - coli	0.0156	**0.0019**	25000	**0.0039**	6250	7.5	0.0039
A. baumannii	8	4	25000	***8***	25000	3.75	*8*

*Synergistic combinations appeared as bold numbers.
*Eth.E. , Ch. E. and Cip. Stand for ethanolic extracts, chloroform extract and ciprofloxacin respectively

plates incubated overnight at 37 °C. EB accumulation in the bacterial cells in the presence of an EPI such as PAβN is due to inhibition of efflux pumps [12]. Evaluation of fluorescence from the excitation of EB by UV light was made with the use of a Gel doc and results were recorded.

RESULTS

Synergy of ethanol and chloroform extracts of *M. officinalis* with ciprofloxacin and their possible EPI effects were assayed on the examined strains. The positive control for EPI effects was PaβN, whose MIC was also determined on the tested bacteria (table 1).

The results of determination of the MICs of ethanolic and chloroform extracts alone and as their associations with ciprofloxacin against examined bacteria are shown in table 1.

Generally, the chloroform extract showed a greater antibacterial activity against *P. aeruginosa* and *E. Coli*, (MIC, 3125 and 6250 µg/mL.) respectively. *S*ynergistic activities of the extracts combinations with ciprofloxacin on the examined strains were also recorded (table 1). However, this synergy was not shown against *A. baumannii*. No antagonistic effects were observed when combinations of the extracts with ciprofloxacin were assayed.

The presence of an EPI such as PAβN causes EB accumulation in the bacterial cells because of inhibition of the cell efflux pumps [12]. To make clear that synergistic activities of the extracts with ciprofloxacin were due to efflux pumps inhibition or other involved mechanisms, in synergistic cases, accumulation of EB in bacterial cells was evaluated in the presence and absence of 1 MIC of the chloroform or ethanolic extracts.

Both extracts of *M. officinalis* increased EB accumulation (i.e. reduced efflux) in *S. enertidis* and *E Coli*. In *P. aeruginosa* and *A. bumani*, there were no synergies with combinations of the extracts with ciprofloxacin, but PAβN combination also did not reduce the MIC of ciprofloxacine (table 1).

DISCUSSION

Combat with increased antibiotic resistance needs novel approaches for identifying new antimicrobials to treat resistant bacterial infections. One of the

successful approaches in last decade was plants screening for natural products having efflux pump inhibiting properties [12, 13].

In the current work, therefore, we examined possible antibacterial and efflux inhibition activities of *M. officinalis* ethanolic and chloroform extracts.

The bacterial strains were examined in this work with a combination of *M. officinalis* extracts, ciprofloxacin, and PaβN, which all possess efflux pumps causing multidrug resistance traits [12, 3].

We showed that extracts of M. *officinalis* inhibit the growth of all examined bacterial strains in concentration ranges of 3125 to 25000 µg/ml *(*table 1*).* The least MIC value (3125 µg/mL.) belonged to chlorophorm extract of *M. officinalis* against *P. aeruginosa.*

M. officinalis contains various compounds such as phenolic acids, which are known to possess many biological activities, including antibacterial, antiviral, and anti-fungal effects[7].

Based on the documented studies, extracts from some other plants also may inhibit efflux pump activity or have antibacterial activities [14]. One report shows that essential oil from *Helichrysum italicum* reduces the MIC of chloramphenicol against *Enterobacter aerogenes*, *A. baumannii,* and *P. aeruginosa* [14].

The best ciprofloxacin potentiation activities were recorded for chloroform and ethanolic extracts of *M. officinalis* against *S. enteritidis* and *E Coli,* respectively. Also, EB accumulation (i.e. reduced efflux) increased by the extracts in *S. enteritidis* and *E Coli* cells. Overall, our results imply that the extracts *of M. officinalis* contain active antibacterial compounds and might be powerful substrates of the examined bacterial efflux pumps. Ciprofloxacin is a substrate for many bacterial efflux pumps [1]; there are reports regarding synergistic activities of other plant extracts with this drug [15].

In *P. aeruginosa* and *A. baumannii,* PAβN did not decrease the MIC of ciprofloxacin, while the combinations of the extracts with the latter drug decreased it. This observation may indicate that the extracts from *M. officinalis* may also act by damaging bacterial cell membrane or cell wall, thereby facilitating the penetration of ciprofloxacin into bacterial cell [16, 17]. Lack of antagonistic effects of the extracts with ciprofloxacin is another promising advantage to consider the extracts as a candidate for fluoroquinolones potentiation.

However, safety of *M. officinalis* extracts needs a detailed study on active constituents and their phytochemical and toxicological properties.

Taking together, we can suggest that associations of fluoroquinolones with extracts of *M. officinalis* may potentiate the antibacterial effects of fluoroquinolones against the examined strains. The synergistic properties of *M. officinalis* extracts with other antibiotics that were noted on other bacteria also confirm that extracts of this herb can act as an efflux pump inhibitor [18].

CONCLUSIONS

The results of current work bring about primary information about the point that associations of fluoroquinolones with extracts of *M. officinalis* may potentiate the antibacterial effects of fluoroquinolones against at least some gram-negative pathogens.

CONFLICT OF INTEREST

The authors declared no competing interests.

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Authors' Contributions

All authors participated in the research design, performance, and analysis of the results.

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