Int J Infect. 2017 April; 4(2):e38768. doi: 10.5812/iji.38768.

Published online 2016 August 6. **Research Article**

Antibacterial and Antibiotic-Potentiation Activities of *Levisticum officinale L.* Extracts on Pathogenic Bacteria

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Received 2016 April 26; **Revised** 2016 May 24; **Accepted** 2016 May 25.

Abstract

Background: Multidrug resistant (MDR) Gram-negative bacteria have resistance to many antimicrobial compounds by multiple mechanisms including reduced outer membrane permeability, and active efflux mechanisms by efflux pumps.
Objectives: The current study was planned to search the antibacterial activities of the ethanol and chloroform extracts of *Levisticum officinale* L., and their synergistic effects with ciprofloxacin against some Gram-negative pathogenic bacteria; also to analyze the extracts if they contain inhibitors of efflux pumps of the examined bacteria.
Materials and Methods: After grinding, the resulting powder of *Levisticum officinale L.* was extracted with 85% ethanol and chloroform by maceration method. The studied microorganisms included the reference strains of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli* and *Salmonella enteritidis*. Broth micro-dilution methods were used to determine the minimum inhibitory concentrations (MICs) of the extracts alone or in association with ciprofloxacin and phenylalanine-arginine-β-naphtylamide (PAβN) as an efflux pump inhibitor (EPI). The presumptive efflux activity was detected by the ethidium bromide (EB) well diffusion method.
Results: MIC determination indicated that the *Levisticum officinale L.* extracts inhibited the growth of all the studied bacteria within a concentration range of 3125 to 25000 μg/mL. The synergistic effects were noted between the *Levisticum officinale L.* extracts and ciprofloxacin on all tested bacteria. In *S. enertidis* and *E. coli* both extracts of *L. officinale*, but in *P. aeruginosa* and *A. baumannii* only ethanolic extract increased the amount of EB accumulation (i e, reduced efflux).
Conclusion: The overall results of the current study provided information for the possible use of the *Levisticum officinale L.* extracts to control bacterial infections caused by the examined bacteria.

Keywords: Antibacterial Activities, Gram-Negative Bacteria, Efflux Pumps, *Levisticum officinale L*

1. Background

Multidrug resistant (MDR) Gram-negative bacteria have resistance to many antimicrobial compounds by multiple mechanisms including reduced outer membrane permeability, and active efflux mechanisms by efflux pumps.

Efflux pumps in pathogenic bacteria and their roles in excreting the entered antimicrobial agents to outer vicinity of the cell is one of several mechanisms that lead to bacterial drug resistances (1).

Reports show that efflux-based systems may play a role in fluoroquinolone resistance in Gram-negative pathogenic bacteria, affecting hospitalized patients (2). The result of over expression of these pumps in pathogenic bacteria is the emergence of pathogenic strains that are clinically resistant to many antimicrobial agents such as ciprofloxacin (3).

Some compounds, such as phenylalanyl-arginyl-beta-naphthylamide (PAßN) are known as efflux pump inhibitors (EPIs) (4). PAßN selectively inhibit the efflux activities of a broad range of efflux pumps such as MexAB-OprM, MexEF-OprN, MexCD-OprJ and MexXY-OprM in *Pseudomonas aeruginosa* and AcrAB-TolC in some species of the Enterobacteriaceae (5).

It is reported that the extracts of some medicinal plants contain molecules that act as EPIs against the efflux pumps of bacteria (6).

Traditionally many medicinal plants are used in various parts of the world such as Iran to treat infections caused by pathogenic bacteria (7, 8).

Levisticum officinale L. is a perennial herb belonging to the Umbelliferae family, all parts of the plant are strongly aromatic, and its seeds, leaves and roots (fresh, powdered and as essential oils) are commonly used in foods and for their medicinal properties (9).

A report by Garvey et al. (10) indicates that extracts of some herbal medicinal plants contain inhibitors of efflux in Gram-negative bacteria, the most active compound falcarindiol, showed synergistic activity with ciprofloxacin extracted from *Levisticum officinale L.*

2. Objectives

The current study aimed to search the antibacterial activities of the ethanol and chloroform extracts of *L. officinale L.* in combination with ciprofloxacin as a fluoroquinolone representative against some Gram-negative pathogenic bacteria, and also analyze if the extracts contain inhibitors of efflux pumps of the examined bacteria.

3. Methods

Dried leaves and branches of *L. officinale L.* were purchased from local market in Shahrekord, Iran. After grinding, maceration method we used to prepare the extract. Briefly, 85% ethanol and chloroform were added to the powdered plant in conical flasks and left at room temperature for a period of two and three days for chloroform and ethanol solvents respectively. Daily filtration and refreshment of the solvent was followed for the ethanol extraction. But for chloroform solvent one stage filtration process was used. The collected filtrates were evaporated by incubating at 34°C, in the case of ethanol and in biolaminar safety hood in the case of chloroform solvents. All extracts were kept at 4°C until further investigations (11).

The studied microorganisms including the reference strains of *P. aeruginosa* ATCC 9027 and *Acinetobacter baumannii* NCTC 13305 provided by B. Zamanzad and A. Gholipour (department of microbiology at Shahrekord medical school), *Escherichia coli* ATCC 25922 and *Salmonella enteritidis* RTCC 2465 provided by H. Motamedi (department of microbiology, college of basic science, Shahid Chamran University, Ahvaz, Iran), were kept in Lauria Bertani (LB) broth at 4°C and sub-cultured on appropriate agar plates 24 hours prior to antimicrobial tests. Mueller Hinton broth (MHB) was used for all the antibacterial assays. Ciprofloxacin (Cip) and phenylalanine-arginine-β-aphthylamide (PAβN) (manufactured by Sigma-Aldrich) were used as fluoroquinolone, microbial growth indicator and efflux pump inhibitor (EPI), respectively.

After a preliminary assay by tube-dilution method on the examined drugs against four standard bacterial strains, the MICs for drug combinations were determined following the double-serial micro-dilution method, according to guidelines of the clinical and laboratory standards institute (CLSI) (12). Briefly, the bacterial cultures were incubated aerobically at 37°C for 18 - 24 hours. The turbidity of the cultures was adjusted to 0.5 McFarland (1.5×10^8 CFU/mL) and then diluted in saline solution to obtain an inoculum of 5×10^5 CFU/well. The first well of each row in micro-plate was inoculated by four MIC of drug/drugs followed by double dilution in successive wells. The two last wells were considered as positive and negative controls.

The inoculated micro-plates were aerobically incubated by shaking for about 18 hours at 37°C. The lowest concentration that inhibits visible growth after incubation was defined as MIC. To verify synergistic activity of ciprofloxacin by the extracts, activity of ciprofloxacin in association with the extract was compared with that of ciprofloxacin plus PAßN (30 μg/mL in the prepared stock solution).

Interaction of drugs in combinations was calculated as the ratio of MICAntibiotic in combination/MICAntibiotic alone and the results were as follows: synergic (< 0.5), indifferent (0.5 to 4) or antagonist (> 4) (13, 14). All assays were performed in duplicate.

The effects on efflux pumps activity in synergistic drug combinations were evaluated by modification of Martins et al.'s protocol (15). Briefly, the examined strains were cultured as cross lines in four equals MHA plates and wells were created in cross lines using a sterile Burrell tip. of each MHA plate, one well was inoculated with 50 μL EB 6 mg/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). Inoculated plates were incubated overnight at 37°C. An increase in the amount of EB accumulated in the presence of an EPI such as PAβN indicated inhibition of efflux (15). The fluorescence evaluation of EB excitation was made by UV light employing by Gel Doc image analysis system (Bio-Rad, Hercules) and results were recorded.

4. Results

Ethanol and chloroform extracts of *L. officinale L.* were examined to detect synergy with ciprofloxacin and possible EPI effects. The positive control was PAβN whose antimicrobial activity was also assayed on the examined strains (Table 1).

The MICs of ethanolic and chloroform extracts and drug combinations against the examined bacteria are presented in Table 1. Of the investigated extracts, chloroform and ethanolic extracts showed the best activities against *S. enteritidis* and *E. coli*, respectively. By MIC determination, the extracts of *L. officinale L.* showed synergistic activity with ciprofloxacin against all the examined strains (Table 1).

An increase in the amount of EB, accumulated in the presence of an EPI such as PAβN indicates inhibition of efflux (12). To determine whether synergistic activity displayed by the extracts of *L. officinale L.* with ciprofloxacin, accumulation of EB in the presence and absence of 1 MIC

Table 1. Minimum Inhibitory Concentrations of Ciprofloxacin and Phenylalanine-Arginine-β-Aphthylamide in the Absence and Presence of *Levisticum officinale L*[a]

Combinati on Bacteria	Cip.	Eth.E. + Cip.	Eth.E.	Ch.E.+ Cip.	Ch.E.	PAβN	PaβN + Cip.
P. aeruginosa[b]	2	**0.25**	12500	2	2	3125	3.75
S. enteritidis[c]	0.0312	**0.00195**	50000	**0.0039**	12500	7.5	**0.0156**
E. coli[d]	0.0156	**0.00024**	50000	**0.00097**	6250	7.5	**0.0039**
A. baumannii[e]	8	1	25000	**4**	2500	3.75	8

Abbreviations: Eth.E, ethanol extract; Ch. E., chloroform extract; Cip., ciprofloxacin.
[a] Synergistic combinations are presented as bold numbers.
[b] *P. aeruginosa*, *P. aeruginosa.*
[c] *S. enteritidis*, *Salmonella enteritidis.*
[d] *E. coli*,*Escherichia coli.*
[e] *A. baumannii, Acinetobacter baumannii.*

of the chloroform and ethanol extracts of L. officinale was done for the tested strains.

In *S. enertidis* and *E. coli*, both extracts of *L. officinale L.* increased the amount of EB accumulation (i e, reduced efflux). In *P. aeruginosa* and *A. baumannii* only the ethanolic extract showed synergy with ciprofloxacin and EB accumulation, but PAβN did not reduce the MIC of ciprofloxacin (Table 1).

5. Discussion

There is continued clinical pressure for novel approaches to combat antibiotic-resistances and identify new antimicrobials to treat resistant bacterial infections. Screening plants for natural products with efflux pump inhibiting properties was a successful approach (15, 16).

Therefore, the present study examined *L. officinale* ethanolic and chloroform extracts to evaluate the suggested efflux inhibition activities.

It is reported that the examined bacterial strains tested with a combination of *L. officinale* extracts; ciprofloxacin and PAβN all contain multidrug resistance efflux pumps (3, 15).

It appears that extracts of *L. officinale L.* inhibited the growth of all tested bacterial strains within a concentration range of 3125 - 25000 μg/mL. The lowest MIC value (3125 μg/mL) was obtained with the chloroform extract of *L. officinale L.* against *P. aeruginosa*.

Reports show that other plant extracts contain compounds that inhibit efflux pump activity or have antibacterial activity (17). It is also reported that essential oil of a Corsican plant, *Helichrysum italicum*, reduced the MIC of chloramphenicol against *Enterobacter aerogenes*, *A. baumannii* and *P. aeruginosa* (17).

In terms of antibiotic-potentiation activity of ciprofloxacin, the chloroform and ethanolic extracts of *L. officinale L.* showed the best activities against *S. enteritidis* and *E. coli,* respectively. As ciprofloxacin is a substrate of many bacterial efflux pumps (13), many reports indicate activity of other plant extracts that synergized with this agent (18).

In the presence of ciprofloxacin, significant increase of *L. officinale* extracts activity was noted against *S. enteritidis* and *E. coli* compared with that of PAβN plus ciprofloxacin; it shows that at least one active compound of this plant, acting inside the bacterial cell could be the powerful substrate of efflux pumps of *S. enteritidis* and *E. coli*. Since ciprofloxacin is a substrate of many bacterial efflux pumps (1), many reports indicate the activity of other plant extracts that synergized with this agent (18).

The observation is suggesting that the association of *L. officinale L.* extracts and fluoroquinolones could be helpful to fight against infections due to *S. enteritidis* and *E. coli*. The synergistic effects of *L.officinale* extracts with different antibiotics noted on other bacteria are also suggesting that some of their constituents can act as efflux pump inhibitors (19).

PAβN had no effect on MIC of ciprofloxacin in *P. aeruginosa* and *A. baumannii*, but the association of extracts decreased it, this may imply that the extracts of *L. officinale L.* may also act by damaging cell membrane or cell wall of the bacteria and thereby facilitate the penetration of ciprofloxacin into bacterial cell (20, 21).

Samiee et al. (22) reported that the methanolic extract of *L. officinale L.* is rich in monoterpenes compounds that damage cell biomembranes.

Also, some reports show that falcarindiol a fraction of the chloroform extracts of *L. officinale L.*, and some other medicinal plants strongly inhibit the growth of different species of bacteria (10, 19). In addition to its antibacterial property, this polyacetylene exhibits various biological activities such as antifungal and cytotoxic properties (19).

However, more detailed studies on active constituents

and phytochemical properties of *L. officinale L.* are suggested to evaluate the mechanisms of its antibiotic potentiation.

Overall, it can be suggested that the association of *L. officinale* extracts and fluoroquinolones could be helpful to fight against infections based on the tested strains. The synergistic effects of *L. officinale* extracts with different antibiotics noted on other bacteria also suggest that some of their constituents can act as efflux pump inhibitors (10).

5.1. Conclusion:

The present investigation provides primary information for the possible use of *L. officinale L.* in association with fluoroquinolones to combat at least some Gram-negative pathogens.

Footnotes

Authors' Contribution: A. Ebrahimi designed and supervised the study also wrote the MS, A. Eshraghi did the examinations, M. Mahzounieh supervised the study, SH. Lotfalian did technical supports.

Funding/Support: The current study was granted by veterinary college of Shahrekord University for MSc thesis research project.

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