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Short communication

A TLC bioautographic assay for the detection of nitrofurantoin resistance reversal compound

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Abstract

A simple TLC bioautographic method was developed for detection of antibiotic resistance reversal agents. In this study, the retention factor values of the components of some essential oils not previously shown to have any antibacterial activity were evaluated on nitrofurantoin supplemented agar media. The active component of *Artemisia annua*, *Artemisia dracunculus* and *Eucalyptus globulus* essential oils was piperitone which increased the antibacterial activity of nitrofurantoin against *Enterobacter cloacae*. Piperitone was not detected in the essential oil of *Humulus lupulus* and we could not observe any clear areas in this bioautographic method.

Keywords: Bioautographic method; Antibiotic resistance; Essential oils; Piperitone

1. Introduction

Resistance to antimicrobial agents by pathogenic bacteria has emerged in recent years and is a major health problem. Consequently, there has been increasing interest in the use of inhibitors of antibiotic resistance for combination therapy [1,2]. These types of compounds have potential in decreasing the effective dose of antimicrobial drugs for therapy. *In vitro* experiments have shown that natural products and some of their components decrease the minimum inhibitory concentration (MIC) of antibiotics for different microorganisms [3–8]. The use of antibiotic resistance reversal agents for the degradation of bacterial resistance is going to be an issue of considerable importance, thus a chromatographic method should be developed and tested for detection of this group of compounds in complex plant matrices. Also such assays are particularly important to avoid the time consuming isolation of known substances or inactive ones. TLC bioautographic methods combine chromatographic separation and in situ activity determination facilitating the localization and target-directed isolation of active constituents in a mixture. Traditionally, bioautographic technique has used growth inhibition of microorganisms to detect anti-microbial components of extracts chromatographed on a TLC layer. This methodology has been considered as the most efficacious assay for the detection of anti-microbial compounds. The objective of this paper was to develop a simple TLC bioautographic method for detection of antibiotic resistance reversal agents. In this method, the R_f value of components which have not previously shown any antibacterial activity is detected against the resistant strain on antibiotic supplemented agar media.

Recently, piperitone, a volatile component of *Mentha longifolia* var. *chorodictya*, has been reported to increase the antibacterial activity of nitrofurans drugs [7]. *M. longifolia* var. *chorodictya* named commonly in Iran as Poneh Sorkhabadi, and grown in north part of Iran and Afghanistan. In this investigation, the synergistic activity of the essential oils from four common plants wormwood, tarragon, hops and Tasmanian blue gum was tested using agar dilution method and compared with the essential oil of *M. longifolia* against *Enterobacter cloacae*. Using this bioautographic method the active compounds of the samples which increased the antibacterial effect of nitrofurantoin was localized on a chromatogram and identified by GC–MS analysis.

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2. Experimental

2.1. Materials

Natural product essential oils, including *Artemisia annua, Artemisia dracunculus*, *Eucalyptus globulus, Humulus lupulus* and *M. longifolia* were obtained from Prof. Farsam of the Department of Medicinal Chemistry at our Faculty and Dr. M. Bagheri from Azarbaijan Medicinal Plant Center, Ardabil, Iran. *dl*-piperitone(3-methyl-6-(1-methylethyl)-2-cyclohexen-1-one) was provided by Charabot Grasse (France).

2.2. MIC determination

The MICs of nitrofurantoin in the presence and absence of essential oils (1 μl/ml) were studied by conventional agar dilution methods using *E. cloacae*. Different concentrations of nitrofurantoin were included in Muller-Hinton agar plates. The MIC was defined as the lowest concentration of antibiotic where bacterial growth was not detected. The test strain used in this study was obtained from Shariati University Hospital, Tehran (Iran). The identity of this strain was confirmed using conventional methods. Polyethylene glycol (PEG) 4000 at a concentration of 1% (w/v) was used to facilitate oil diffusion in all of the media used in this study.

2.3. Detection method

A TLC bioautographic method was used to detect active components. Piperitone and *M. longifolia* essential oil were used as positive controls in all experiments. After application of the sub-inhibitory content of essential oils (10 μl) and piperitone (3 μl) on a silica gel 60 F_{254} plate (Merck, Darmstadt, Germany), thin layer chromatography (TLC) was developed using toluene-ethyl acetate (93:7) as the eluent system [9] and dried for complete removal of solvents. The plates were either visualized using sulfuric vanillin [9] or biologically (bioautography) to evaluate the activity of the different essential oils. An inoculum of *E. cloacae* in the 0.1% triphenyl-tetrazolium chloride containing Muller-Hinton agar media (with or without 30 μg/ml nitrofurantoin) was distributed over TLC plates, and the plates were incubated at 35 °C for 24 h. Triphenyl- tetrazolium chloride was supplied by Aldrich Chemical Co., UK. Inhibition zones were observed as clear areas against a red colored background. Preparative TLC plates with a thickness of 1 mm were prepared using the same stationary and mobile phases as above, with the objective of isolating the components of essential oils that enhanced the antibacterial activity of nitrofurantoin against the test strain. These areas were scraped from the plates, and the substance eluted from the silica with ethanol. Eluted samples were further purified using the above preparative chromatography method. Finally, the components were identified by gas chromatography–mass spectrometry (GC/MS) on a ThermoFinnigan ThermoQuest instrument (Applied Science Uk) using a DB-1 capillary column 30 m × 250 μm × 0.1 μm). Helium was used as carrier gas at a constant flow rate of 1.5 ml/min. Electron impact mass spectra were obtained at 70 eV, the instrument scanning from 40–300 amu. Scan rate was 2.5 per second. The oven temperature was initially 50 °C (isother-

Table 1
Enhancement of bactericidal activity of nitrofurantoin for *Enterobacter cloacae* by various essential oils[a,b]

Selected essential oils	Synergistic MICs (μg/ml)	MIC reduction
Mentha longifolia	25	11×
Artemisia annua	70	3.9×
Artemisia dracunculus	50	5.5×
Eucalyptus globules	30	6.1×
Humulus lupulus	125	2.2×

[a] Tested at a sub-inhibitory concentrations of 1 μl/ml.
[b] Nitrofurantoin MIC for *Enterobacter cloacae* was 275 μg/ml.

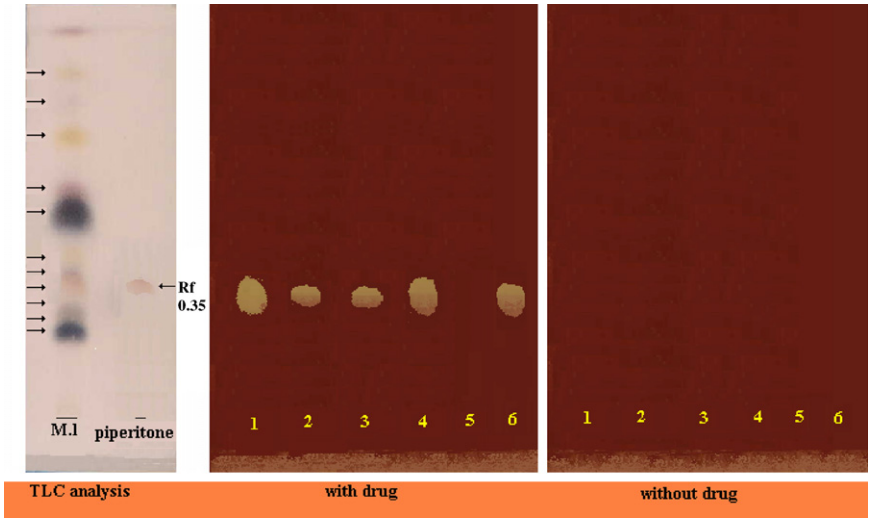

Fig. 1. Detection of active compounds of some essential oils involved in augmentation of nitrofurantoin activity by bioautography and thin layer chromatography methods. TLC plates, composed of Merk Silica gel 60 F_{254}, received 10 μl of the following essential oils: *Mentha longifolia* (1), *Artemisia annua* (2), *Artemisia dracunculus* (3), *Eucalyptus globulus* (4), *Humulus lupulus* (5). Piperitone (3 μl) was applied as reference control (line 6). The panels demonstrate the result in the presence (with drug) and absence (without drug) of nitrofurantoin.

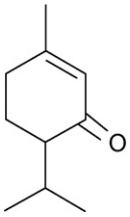

Fig. 2. The structure of piperitone (CAS Number 89-81-6).

mal, 5 min) and then was increased to 250 °C at 2.5 °C/min. The injector temperature was 250 °C. Identification of the components was made by comparison of their retention times and mass spectra with standards [7,10,11].

3. Results and discussions

The effects of essential oils on the bactericidal activity of nitrofurantoin were studied using the agar dilution method (Table 1). The results of these experiments showed that all samples reduced the MICs of nitrofurantoin against the test strain (2–11 times). Essential oils of *M. longifolia* and *E. globules* were most effective in increasing the antibacterial activity of nitrofurantoin against resistant Enterobacteria. The active components of essential oils involved in this process were detected using a bioautographic method. No selected essential oils showed any intrinsic antibacterial activity in absence of the drug (Fig. 1). By contrast, analysis of these volatile oils in nitrofurantoin containing agar media (30 μg/ml) allowed detection of components ($R_f = 0.35$) which were effective in enhancing nitrofurantoin activity (Fig. 1).These active components had $R_f = 0.35$ on TLC and UV $\lambda_{max} = 232.5$. They had the same R_f and λ_{max} values as the piperitone standard (Fig. 2). Piperitone was not detected in the essential oil of *H. lupulus* oil (data was not shown) and we could not observe any clear zone of inhibition against test strain in the presence of drug. The identification of the fractions as piperitone was further confirmed by GC/MS analysis. The mass spectroscopic data of the active fractions are as follow: MS (EI) *m*/*z* (%) 152 (M^+, $C_{10}H_{16}O$, 43), 153 (4), 69(15), 137 (53), 95(60), 82 (94), 110 (100).

To the best of our knowledge, based on a literature search, no studies have been conducted on the detection of antibiotic reversal agents using bioautographic technique. This bioautographic method can be conducted for screening of these agents in other plant or microbial extracts.

References

[1] T.E. Renau, S.J. Hecker, V.J. Lee, Ann. Rep. Med. Chem. 33 (1998) 121.
[2] G.D. Wright, Chem. Biol. 7 (2000) R127.
[3] S. Shin, Yakhak Hoeji 46 (2002) 203.
[4] S.M. Jaber, S.E. Farouk, M. Ilias, Phytother. Res. 18 (2004) 934.
[5] R. Giordani, P. Regli, J. Kaloustian, C. Mikail, L. Abou, H. Portugal, Phytother Res. 8 (2004) 990.
[6] M. Sato, H. Tanaka, T. Oh-Uchi, T. Fukai, H. Etoh, R. Yamaguchi, Phytother. Res. 18 (2004) 906.
[7] A.R. Shahverdi, F. Rafii, F. Tavassoli, M. Bagheri, F. Attar, A. Ghahraman, Phytother. Res. 18 (2004) 911.
[8] Y. Sakagami, M. Iinuma, K.G. Piyasena, H.R. Dharmaratne, Phytomedicine 12 (2005) 203.
[9] H. Wagner, S. Bladt (Eds.), Plant Drug Analysis: A Thin layer Chromatography Atlas, second ed., Springer-Verlag, Berlin Heidellberg, 1996, p. 166.
[10] Eight Peak Index of Mass Spectra, third ed., Mass Spectrometry Data Centre, Royal Society of Chemistry, The University of Nottingham, Nottingham, UK, 1983.
[11] R.P. Adams (Ed.), Identification of Essential Oil Components by GC–MS, Allured Publishing Corporation, Carol Stream, Illinois, 1995.