Optimization of Conditions Affecting Direct Bioautography Assay for Antimicrobial Compounds

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Introduction

Purpose of this study was to optimize conditions affecting direct bioautography assay for antimicrobial compounds. Direct bioautography assay is fast, simple and cost effective method for screening of antimicrobial compounds from complex natural extracts. We used *Gloeophyllum trabeum* extract as a reference extract against the gram-negative bacteria *Escherichia coli* according to the method by Nagy et al. 2003.

Methods

The compounds were separated on TLC with selected eluents (Table 1.). Developed plates were visualized under UV (wavelength 254 nm) and then immersed in bacterial suspension (approximately 6 x 10^6 CFU/ml). After incubation the growth of bacteria was detected with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (2.5 mg/ml) (Hamburger & Cordell 1987). Substances inhibiting the growth of bacteria could be identified as white spots against a coloured background.

The factors affecting the growth of bacteria and colour formation on the plate were studied. The effects of eight different eluent compositions on TLC separation of compounds in *Gloeophyllum trabeum* extract and on the growth of bacteria were evaluated (Table 1.). In addition, effect of the density of bacteria in the immersion suspension was studied.

Number on TLC-plate (Fig. 1)	Eluent composition	Concentration of CH_3COOH
1.	chloroform	-
2.	MeOH	-
3.	chloroform : MeOH (65 : 35)	-
4.	chloroform : MeOH : CH_3COOH (65 : 35 : 5)	0.1%
5.	chloroform : MeOH : CH_3COOH (65 : 35 : 5)	0.5%
6.	chloroform : MeOH : CH_3COOH (65 : 35 : 5)	1%
7.	chloroform : MeOH : CH_3COOH (65 : 35 : 5)	2.5%
8.	chloroform : MeOH : H_2O (65 : 35 : 5)	-
9.	no development	-

Table 1. Different eluent compositions studied for separation of *Gloeophyllum trabeum* extract on TLC. The amount of acetic acid affected both the separation of the compounds as well as the growth of bacteria on the plate.

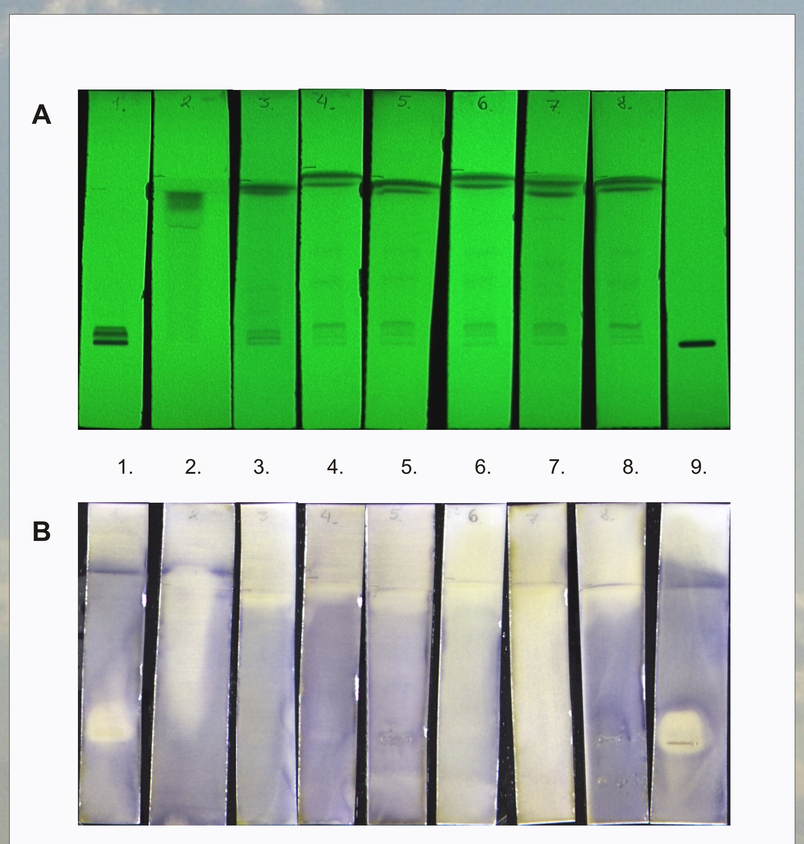

Figure 1. The effect of eluent composition on TLC separation (A) and the growth of bacteria (B). The TLC plates were developed and visualized under UV (wavelength 254 nm) (A). The same plates were immersed in bacterial suspension (approximately 6 x 10^6 CFU/ml) (B).

Results and Conclusions

Good separation of substances in the extract and deep colour formation was achieved when using eluent containing chloroform, MeOH and 0.1 % acetic acid (aq) at ratio of 65 : 35 : 5 (Fig. 1).

Separation of the compounds in the extract was improved when adding acid to the eluent (Fig. 1A). While the increased amount of acid in the eluent enhanced the separation it also impaired the colour formation (Fig. 1 B). That is thought to be consequence of acid's growth inhibiting effect on bacteria but it couldn't be compensated by increasing the amount of bacteria. However, the bacteria tolerated a small amount of acid in eluent (0.1 %) if the plates were dried thoroughly for the acid to evaporate. Consequently the TLC plates were left to dry for an hour and dried with a blow-drier after development.

The optimal density of bacteria in the suspension used for immersion of TLC plates, from 10^5 to 9 x 10^6 CFU/ml (Nagy et al. 2003), was achieved after 4.5 h incubation. At that time the bacteria are in the beginning of the logarithmic phase i.e. as metabolically active as possible to convert MTT to intensely coloured formazan.

When studying separated complex extracts it seemed that better contrast on coloured TLC plate clarified the reading of the results. Better contrast was achieved when using suspension containing rather higher than smaller amount of bacteria. Consequently the optimal concentration of bacteria for our purposes was decided to be from 5 x 10^6 to 5 x 10^7 CFU/ml. That could be reached after 4.5 - 5 hours incubation.

In conclusion the choice of the eluent (number 4 on Fig. 1 and Table 1.) was the best between a good separation and an acceptable colour formation i.e. growth of bacteria on TLC plate.

References

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