Main antimicrobial components of *Tinospora capillipes*, and their mode of action against *Staphylococcus aureus*

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Abstract In this investigation, the antibacterial modes of action of Radix Tinosporae, its major single components, and nine antibiotics with different targets or modes-of-action on *Staphylococcus aureus* were studied. Metabolic profiles of cultures treated with different medicines were acquired by HPLC/ESI-MS. After HPLC-MS data pretreatment, those profiles acquired were reduced into several MS vectors. Then statistical processing by principal components analysis was carried out upon those vectors, two conclusions could be drawn: (1) the antibacterial mode of action of Radix Tinosporae is similar to that of rifampicin and norfloxacin, which act on nucleic acid; (2) its active components playing main antimicrobial roles on *Staphylococcus aureus* might be alkaloids, such as palmatine and jatrorrhizine.

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1. Introduction

*Tinospora capillipes* Gagnep, a member of the Menispermaceae family, is a special plant native in South China. Its rhizome (Radix Tinosporae, R.T.) has been used in traditional Chinese medicine (TCM) for thousands of years. The book *Gang mu shi yi* [1] recorded it could detoxify arsenous oxide. The book *Guangxi traditional medicine* [2] tells that R.T. could cure gastroenteritis and remit stomach ache. The folk medicine used it in the treatment of bacillary dysentery, and also used it as an anticarcinogen. However, the mechanism is unknown, which poses challenges to the pharmaceutical and agrochemical industries [3–5]. Nowadays, high-throughput methods, such as metabonomics, are introduced to solve this problem [6–8]. Metabonomics is an emerging analytical technology [9], which represents the end of ‘omics’ hierarchy [10]. It is defined as a system approach to investigate the metabolic consequences of patho-physiological or genetic modification in a multivariate and dynamic manner [11]. A ‘metabolic profile’ is derived from a series of specific analytical tests run in combination and used as a diagnostic aid [12]. The metabolic profile could provide some useful information about metabolism and mechanism of action [13].

In this paper, with a view to investigating the antibacterial mode of action of R.T., *Staphylococcus aureus* (*S. aureus*) was selected as our research target, in respect that it is not only a typical Gram-positive coccus but also a common medicinal coccus [14].

The metabolic profiles of the *S. aureus* cultures treated with R.T., its major chemical components (columbin, palmatine, tinoside, and jatrorrhizine), and nine kinds of antibiotics (Table 1), were obtained by using HPLC/ESI-MS. After data pretreatment, those profiles acquired were reduced into several MS vectors containing 950 *z* values. Principle component analysis (PCA) was performed on those vectors. With the help of this approach, we try to investigate the antibacterial mode of R.T. and to identify its active components, using *S. aureus* as target.

2. Materials and methods

2.1. Chemicals

All authentic reference compounds (columbin, palmatine, tinoside, jatrorrhizine, chloromycin, streptomycin, achaemycin, rifampicin, cefotaxime, vancomycin, norfloxacin, erythromycin, and lincomensin standards) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (China). Chloroform, *n*-hexane, acetone, and ethanol were of analytical grade obtained from Merck (Darmstadt, Germany). Methanol and acetonitrile were HPLC-grade obtained from SDS (Peyrin, France). Ultrapure water was generated by the Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Extracting different apolar parts in R.T

The dried material (10 g) was pulverized finely and passed through an 80 mesh sieve, and then placed in a Soxhlet extraction apparatus. The material was refluxed with 100 mL of ethanol-water (95:5 v:v) for 24 h. The ethanol and water were removed from the extracted by evaporation at a temperature not exceeding 60 °C. The residue (I) was sequentially extracted by 20 mL *n*-hexane, 20 mL chloroform, and 20 mL acetone. After volatilization of each of the solvent (the obtained residues are named as II, III, IV in extracted order), I, II, III, and IV were redissolved in methanol and stored at 0 °C until use.

2.3. HPLC conditions

For identification of the major components in R.T., their retention times were compared with those of the authentic standards (columbin, palmatine, tinoside, and jatrorrhizine). A reversed-phase high performance liquid chromatography system (RP-HPLC, HP 1100, Agilent) with a photodiode array detector was used. A C18 column (Waters Symmetry C18, 150 × 3.9 mm, 5 μm) was used for the stationary phase at 40 °C with a flow of 1.0 mL min⁻¹. The mobile phase consisted of (A) water and (B) acetonitrile, was first filtered through a membrane of 0.22 μm, and then degassed ultrasonically. The mobile phase underwent a gradient elution procedure of 20% B in 10 min, 20–50% B in 15 min, 50–60% B in...
Table 1 Modes of actions of selected known antibiotics

<table>
<thead>
<tr>
<th>Drug/class</th>
<th>Function inhibited</th>
<th>Molecular target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloromycetin</td>
<td>Protein synthesis</td>
<td>50S ribosomal subunit</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Protein synthesis</td>
<td>30S ribosomal subunit</td>
</tr>
<tr>
<td>Achromycin</td>
<td>Protein synthesis</td>
<td>30S ribosomal subunit</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Protein synthesis</td>
<td>50S ribosomal subunit</td>
</tr>
<tr>
<td>Lincolmestin</td>
<td>Protein synthesis</td>
<td>50S ribosomal subunit</td>
</tr>
<tr>
<td>Norfloxacine</td>
<td>DNA replication/ transcripion</td>
<td>Gyrase and topoisomerase</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Transcription</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>Cefatxaime</td>
<td>Peptidoglycan synthesis</td>
<td>Transpeptidases and carboxpeptidases</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Peptidoglycan synthesis</td>
<td>Cell wall peptidoglycan SYNTHESIS</td>
</tr>
</tbody>
</table>

10 min, 60–80% B in 10 min, 80% B in 5 min. The injected volume was 20 μL.

2.4. Bacterial strains and growth conditions

S. aureus CCTCC AB9105 was used in this study. All tests were performed in Mueller–Hinton broth (MHB). The growth of microbe culture was monitored by measuring optical density at 600 nm in a UV–Vis spectrophotometer. Microbe strain was cultured into 50 mL of MHB, and incubated in a shaker incubator overnight at 37 °C. The overnight culture was diluted to a concentration of approximately 10^6 CFU mL^-1 and used as the source of inoculums. Solutions of each drug and apolar extract were prepared with concentration ranging from 1.25 to 80000 μg mL^-1. 100 μL of the coccus inoculum was re-inoculated into MHB containing different concentrations of each drug (Table 2). The culture was again incubated overnight with shaking at 37 °C. The inhibition of each drug and apolar extract to cells growth was between the approximate ranges of 40–80% to ensure that any change in the metabolic profiles could be ascribed to the mode of action of the inhibitor and not changes in growth rate [15].

2.5. Extracting of metabolites from Staphylococcus aureus

Approximately 24 h after inoculation, when the majority of culture appeared to have entered stationary phase, samples of culture (5 mL) were quickly harvested with a 5 mL automatic Gilson pipette and released into 20 mL methanol-water solution (60% v/v) at −40 °C, resulting in a final methanol concentration of 50% (v/v) after quenching. The sample solutions were carefully released into the centre of the methanol solution to avoid freezing on the sides of the tubes. The biomass was separated from the quenching solution by centrifugation at 1000 rpm (relative centrifugal force 770 x g) for 20 min at 20 °C. After that, the pellet was resuspended in 10 mL cold (−40 °C) absolute methanol in a tube. Following rapid mixing, the tube was transferred into dry ice for 30 min, then thawed in an ice bath for 10 min and centrifuged (12000 rpm for 10 min at 4 °C, Relative centrifugal force 8000 x g). The supernatant was transferred to a new tube. To the extracted pellet, cold (−40 °C) methanol–water (50:50 v/v) was added to extract any metabolites left after the first extraction. The first and the second extracts were combined and concentrated for 6 h in vacuum at 4 °C [16]. The samples were stored at −40 °C until LC/ESI-MS analysis. This was done on every batch of culture with nine antibiotics, R.T. extracted by ethanol (I), different apolar parts in R.T. (II, III, IV), and the different components in R.T. (columbine, palmitine, tinoside, and jatrorrhizine).

2.6. HPLC-ESI-MS conditions

A Shimadzu LC system, consisting of a Shimadzu (Kyoto, Japan) LC-10ADvp solvent delivery pump, an FCV-10ALvp low pressure gradient unit, a DGU-14A degasser, a CTO-10Avp column oven, a SPD-M10Avp photodiode array detector, a 8125 Rhodyne manual injector (CA, USA), coupled to a LCMS-2010 single quadrupole equipped with electrospray ionization (ESI) probe were used in this study. The temperatures were maintained at 250, 250, 200 °C for the probe, CDL, and block, respectively. The voltages were set at 4.5 kV, −30 V, 25 V, 150 V and 1.5 kV for the probe, CDL, Q-array 1, 2, 3 bias, Q-array RF and detector, respectively. The flow rate of nebulizer gas was 4.5 L min^-1. Ions of selection monitoring were decided by positive scanning from m/z 100–1500. The separation was performed on a C18 (Thermo Hypersil-Keystone Hypepuny, 50 mm x 2.1 mm, 5 μm) analytical column, and the oven temperature was set at 40 °C. The mobile phase, consisting of (A) water and (B) methanol (containing 0.2% formic acid), was first filtered through a membrane of 0.22 μm and degassed ultrasonically. The mobile phase underwent a gradient elution procedure of 5-28% B in 20 min, 28–55% B in 20–30 min, 55–72% B in 30–50 min, 72–85% B in 50–70 min, 85–90% B in 70–90 min, 90–85% B in 90–110 min, 85–5% B in 110–140 min. The flow-rate was set at 0.2 mL min^-1. The injected volume is 5 μL.

2.7. Principle component analysis and pretreatment of data

PCA, which has been widely used for exploratory data analysis [17,18], may be a useful and convenient means for possible evaluation of modes of action. In this investigation, PCA was used to explore the intrinsic relationship between R.T. and nine kinds of antibiotics. The data of spectrophotograms for each sample at the determined retention time from 0 to 90 min (see supporting information) was picked up to perform PCA. Before PCA, a data pretreat is necessary. Each ESI-MS array was reduced into a single ‘active’ MS vector by summing the ion counts of a given m/z ratio over the total scan cycle. Thus, after this initial data reduction an ESI-MS spectrophotogram with MS range 50–1000 m/z would be reduced to a single vector having 950 values [19]. The m/z profiles of all samples obtained after preprocessing were shown as supporting information as well. Then PCA was performed on the MS matrices obtained. The loadings plots for the PCA analyses were provided as supporting information. All programs were coded in MATLAB 6.5 for windows.

3. Results and discussion

3.1. The major chemical components in R.T.

Typical HPLC profiles of samples I, II, III, and IV are shown in Fig. 3. Compared with Fig. 1b, c, d with Fig. 1a, we find that the extracts of n-hexane (II), chloroform (III), and acetone (IV) contained all main chemical components in R.T. (I) (Fig. 1a). Comparing the retention times and UV spectra with those of the authentic standards, the ester, columbine, was the major constituent in the n-hexane extract (II), the glycoside,
tinoside, in the chloroform extract (III), and the alkaloids, palmatine and jatrorrhizine, in the acetone extract (IV) (Fig. 1).

3.2. Antibacterial mode of action of R.T. and the major antimicrobial components

Fig. 2 depicts the MS vectors profiles of controls, cultures treated with norfloxacin and cultures treated with vancomycin. The obvious differences could be found between these profiles, hence, allowing the classification of drugs according to their MS vectors profiles. The PCA projection plots of all MS vectors profiles acquired (including control samples, the samples treated with I (○), the extracts II (△), the extracts III (□), the extracts IV (■), columbin (○), tinoside (●), palmatine (●), jatrorrhizine (●), and nine antibiotics) are shown in Fig. 3. One could see clearly that the
classification of all drugs and controls in Fig. 3 is very clear, though no two drugs produce exactly the same pattern of loadings. It should be pointed out that in the profiles either dose of drugs-treatment clusters together. Thus, a conclusion could be drawn that the difference between two doses of each drug is little in our investigation. Generally, the cultures treated with drugs of similar targets cluster together, at both treatment concentrations, and that those of different targets are distinguishable from each other in PCA projections (Fig. 3). For instance, cefataxime (●) whose target is on transpeptidases and carboxpeptidases, at both dose concentrations, formed a distinct cluster separate from the other antibiotics based on its different mode of action. Likewise, vancomycin (○, at both dose groups), whose target is on cell wall peptidoglycan, clustered separately as a group. Similarities in patterns are apparent among classes of drugs that affected the same site [20]. As we know from Table 1, lincomensin, erythromycin and chloromycetin have effects on 50S ribosomal subunit, and streptomycin and aecomycin act on 30S ribosomal subunit. In the projection plot (Fig. 3), one could see that the points of aecomycin (▲) and streptomycin (●) cluster together, lincomensin (●), erythromycin (▲) and chloromycetin (○) cluster together as well. The results of PCA support the hypothesis that modes of actions of a drug could be identified by the metabolic profiles acquired. This may provide the basis to classify the metabolic profiles by PCA and to find out the possible mode of action of TCM.

It is interesting that rifampicin (■), norfloxacin (□) and R.T. (○) cluster together. The targets of rifampicin and norfloxacin are on RNA polymerase, gyrase and topoisomerase IV. According to the principle of PCA, the mode of action of R.T. on S. aureus is therefore expected to be similar to rifampicin and norfloxacin. It may imply that the target of R.T. possibly is nucleic acid. The results from this figure are consistent with Table 1.

Moreover, from Fig. 3, one can clearly see that the points of the extracts III (●) and tinoside (○), the main component in III, are clustered as a single group, which distinguishes from any classification presented. This hints that the extracts III and tinoside have the similar mode of action, which is different from the mode of other drugs studied in this investigation. In the same way, the points representing the extracts IV (○) and it major compound columbin (◇) forms a single group, which means their mode are different with others. However, the points representing the profiles from the extracts IV (○) were close to that of the extracts of R.T. (I) (◇). An interesting observation was made with the points representing the profiles of palmatine (○) and jatrorrhizine (+), which are both components of the extracts IV, as they clustered close to those representing the extracts of R.T. (I, ◇). This finding implies that palmatine and jatrorrhizine, and possibly the components in IV, show a similar mode of action on S. aureus as the extracts of R.T. (I). It could be hypothesized that the synergistic action of alkaloids in R.T., rather than a single component, contributes to the main antimicrobial action on S. aureus.

4. Conclusion

In this paper, the MS vectors profiles of different cultures coccus treated by different antibiotics and by different extracts of R.T. were explored with the help of PCA in order to investigate the mode of action and to identify the main antimicrobial components of the R.T., based on comparison of the known modes of actions of several different antibiotics. Although the method is immature, the results obtained in this investigation provide some insight into the mode of action and possible mechanism of the compound under investigation. Further research will be required to validate the extract mechanism of this compound.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.07.056.

References


