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Validate antibacterial mode and find main bioactive components of traditional Chinese medicine *Aquilegia oxysepala*

Yan Yu, Zhi-biao Yi and Yi-Zeng Liang*

Research Center of Modernization of Chinese Medicines, Central South University, Changsha 410083, PR China

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Abstract—Traditional Chinese medicines have been used for thousands of years and are still being used as one of the regular treatments for many diseases. However, their mechanisms were still unknown. In this investigation, a possible procedure combining metabonomics and principal component analysis to investigate antibacterial modes of action and find main antimicrobial component in traditional Chinese medicine, *Aquilegia oxysepala*, is developed. Metabolic profiles of *Staphylococcus aureus* treated with nine antibiotics of known modes of action and with *A. oxysepala* were acquired by HPLC/DAD/ESI-MS. After statistical processing by principal components analysis on metabolic profiles, two conclusions could be drawn: (1) the target of *A. oxysepala* may be similar to that of lincolmensin, erythromycin, chloromycetin, streptomycin, and acheomycin, whose targets are protein; (2) its bioactive component playing main antimicrobial roles on *S. aureus* may be maguoflorine.

Aquilegia oxysepala, a member of the *Ranunculus L.* species, has been used in traditional Chinese medicine (TCM) for thousands of years. Its major compounds include genkwanin, apigenin, maguoflorine, and berberine (Fig. 1). The experience of folk medicine shows that it has especially effectiveness for the treatment of gynopathy, such as irregular menstruation and metrorrhagia. However, the components in a single TCM may be numerous, even saying nothing of TCM preparations. Furthermore, some of them are not known to us. It is very difficult to separate, identify, and quantify exhaustively the components in complex TCMs. Many analytical methods for TCM have been established, but only a few components can be separated and quantified. All these are the great handicaps to validation of the mechanism of TCM and its main antimicrobial components.[1–3] On the other hand, there are numerous substances in cell, such as protein, nucleic acid, and low-weight metabolites, and so on, hence, one might prefer to find out the site of action of the drug rather than conduct exhaustive separation. All these pose challenges to the pharmaceutical and agrochemical industries. The aim of this paper was to try to solve this problem from the other direction. Metabonomics combined with principal component analysis (PCA) was used to validate possible antibacterial mode and then to try to find the antimicrobial components of the traditional Chinese medicine.

Staphylococcus aureus was selected as our research target, since it is not only a typical Gram-positive coccus but also a common medicinal coccus.[4] *Aquilegia oxysepala*[5] and its major chemical components (genkwanin, apigenin, maguoflorine, and berberine) (see Figs. 1 and 2), and nine antibiotics with known mode of action (Table 1), were added into cultures. After 24 h growth, cultures were harvested[6] and intracellular metabolites were extracted[7] and analyzed by HPLC/DAD/ESI-MS. Then PCA was performed on the metabolic profiles acquired. With the help of this method, we tried to investigate the antibacterial mode and find the antimicrobial components of TCM on *S. aureus* as well.

Figure 3 depicts the most informative parts of HPLC profiles of controls, cultures treated with rifampicin, and cultures treated with norfloxacin. The obvious differences could be found between these metabolic profiles, hence allowing for classification of drugs according to their metabolic profiles. This provided the basis to classify the metabolic profiles by PCA and to find out the possible mode of action of TCM.

Keywords: Aquilegia oxysepala; Antibiotics; Antibacterial mode of action; Metabolic profiles; Principal component analysis.
* Corresponding author. Tel.: +86 731 882 2841; fax: +86 731 882 5637; e-mail: yizeng_liang@263.net

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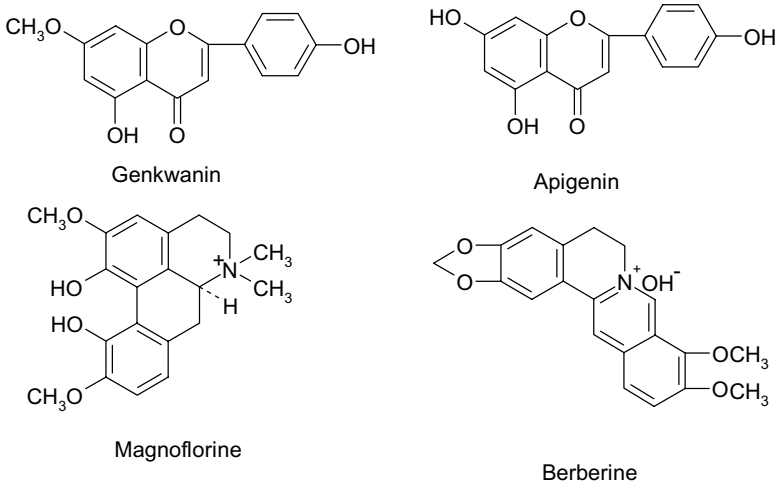

Figure 1. The structural formulae of genkwanin, apigenin, maguoflorine, and berberine.

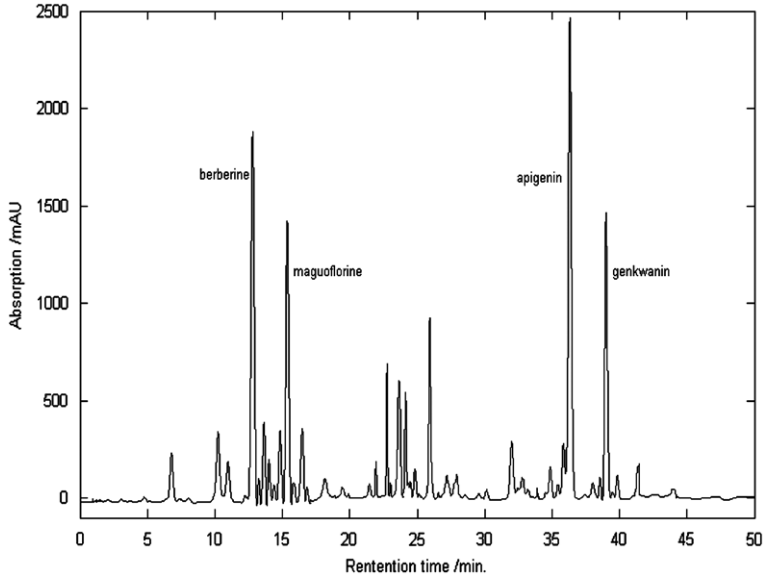

Figure 2. HPLC profile (detection method: DAD-UV, 254 nm) of *Aquilegia oxysepala*.

Table 1. Modes of action of selected drugs

Drug/class	Function inhibited	Molecular target
Chloromycetin	Protein synthesis	50S ribosomal subunit
Streptomycin	Protein synthesis	30S ribosomal subunit
Acheomycin	Protein synthesis	30S ribosomal subunit
Erythromycin	Protein synthesis	50S ribosomal subunit
Lincolmensin	Protein synthesis	50S ribosomal subunit
Norfloxacin	DNA replication/ ranscription	Gyrase and topoisomerase IV
Rifampicin	Transcription	RNA polymerase
Cefataxime	Peptidoglycan synthesis	Transpeptidases and carboxpeptidases
Vancomycin	Peptidoglycan synthesis	Cell wall peptidoglycan

Before multivariate analysis, two preprocesses for data pretreatment are necessary. First, peak tracking was conducted to extract spectral information from the data obtained from LC/DAD/MS. The spectral information from the components in each sample was then compared to validate whether a component in one sample was the same as in another sample.[8,9] The chromatograms after such a correction of retention time shift for both HPLC–UV and HPLC–MS together with a table of retention times, *m*/*z* values, and peak areas are provided as Supplementary information. After these two preprocesses, PCA was performed on a matrix consisting of the most informative peak clusters of chromatograms (HPLC–UV) for all the samples, including the cultures treated with *A. oxysepala*, several standards, such as genkwanin, apigenin, maguoflorine, and berberine, nine antibiotics, and controls, at the determined retention time (0–12.3 and 99.0–120.0 min)[10] (Fig. 4). All programs of PCA and other methods were coded in MATLAB 6.5 for Windows.

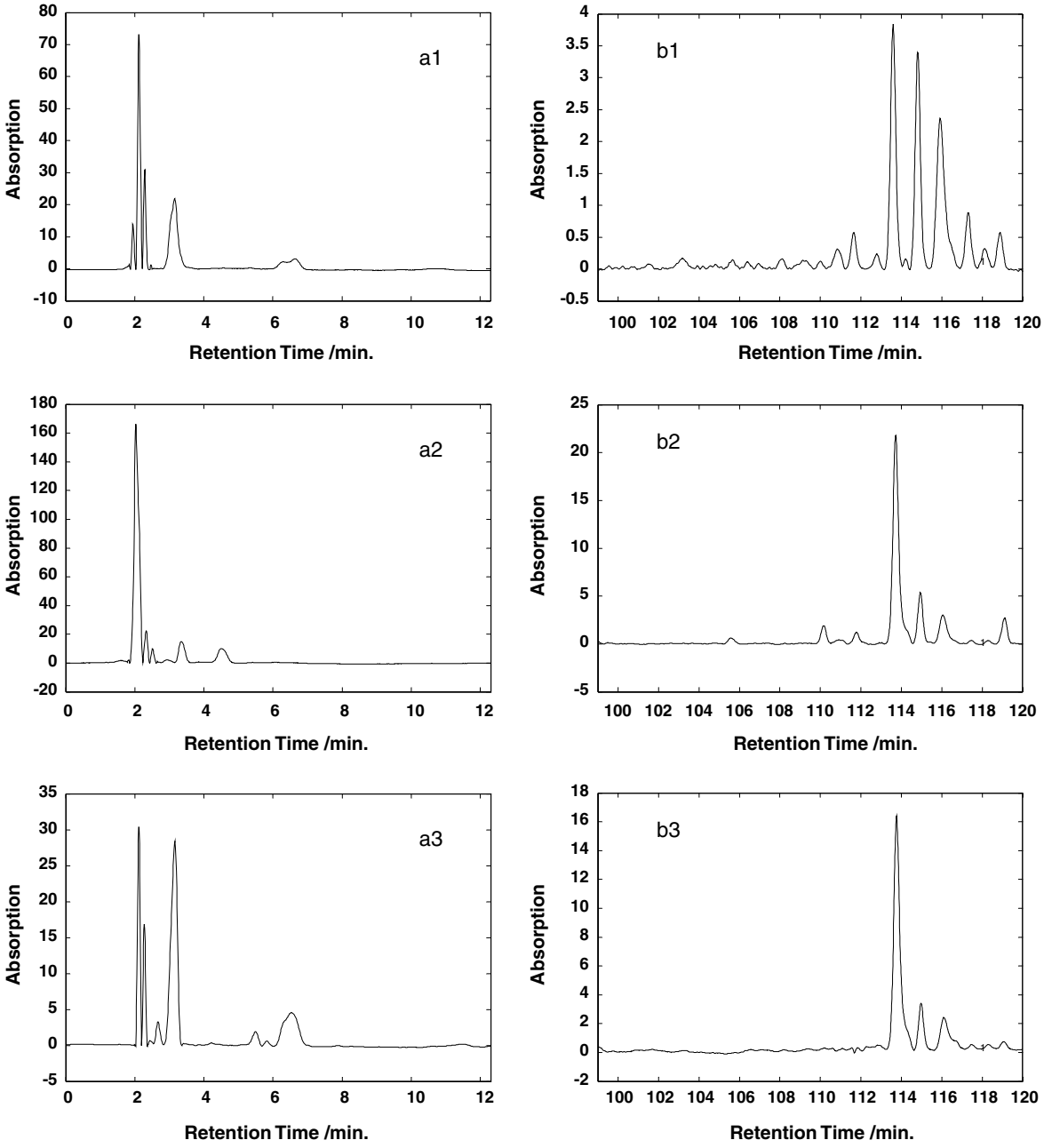

Figure 3. Typical HPLC profiles (detection method: DAD-UV, 254 nm) of controls at retention time 0–12.3 min (a1) and 99.0–120.0 min (b1), cultures treated with rifampicin at retention time 0–12.3 min (a2) and 99.0–120 min (b2), and cultures treated with norfloxacin at retention time 0–12.3 min (a3) and 99.0–120.0 min (b3).

From the points of Figure 4, one could see clearly that cultures treated with different drugs and controls (♦) are well-separated. It should be pointed out that the antibiotics clustered correctly according to their modes of action, at both treatment concentrations. 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.[11] Acheomycin (△), lincolmensin (×), erythromycin (▲), chloromycetin (☆), and streptomycin (★) cluster together. As we know from Table 1, lincolmensin, erythromycin, and chloromycetin have effects on 50S ribosomal subunit, streptomycin and acheomycin act on 30S ribosomal subunit. In a word, the mode of action of those five drugs is to inhibit protein synthesis. Besides, the points of rifampicin (■) and norfloxacin, whose targets are on RNA polymerase, gyrase, and topoisomerase IV, are gathered together. Although no two drugs produced exactly the same pattern of loadings, the classification of all drugs and controls in Figure 4

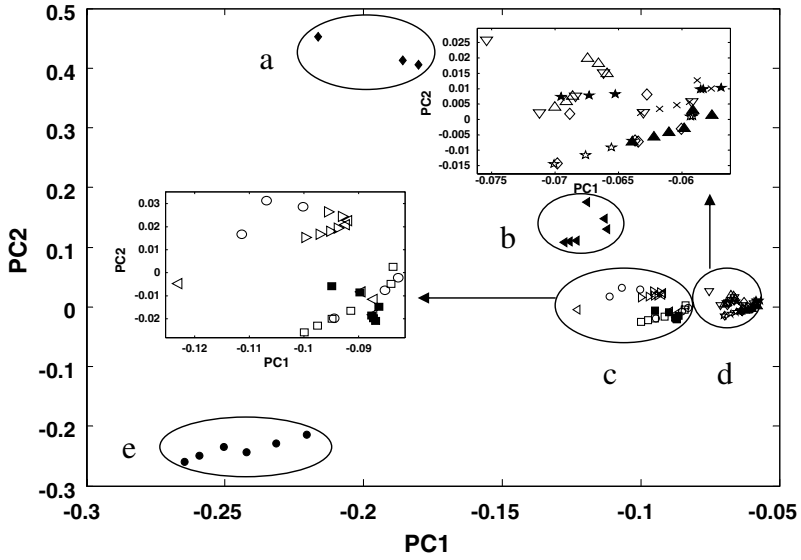

Figure 4. PCA projection of metabolic profile of controls and cultures treated with *Aquilegia oxysepala*, genkwanin, apigenin, maguoflorine, berberine, and nine antibiotics. Controls (♦), acheomycin (△), lincolmensin (×), erythromycin (▲), chloromycetin (☆), streptomycin (★), cefataxime (◀), vancomycin (•), rifampicin (■), norfloxacin (□), *A. oxysepala* (♦), genkwanin (○), maguoflorine (▽), apigenin (▷), and berberine (◁).

Table 2. Concentrations and sample numbers of drugs used in Fig. 4

Drugs used	Conc. (μg mL^{-1})	Sample symbols
Controls		♦
Norfloxacin	4, 2	□
Lincolmensin	16, 8	×
Cefataxime	0.25, 0.125	◀
Vancomycin	0.5, 0.25	•
Rifampicin	4, 2	■
Acheomycin	0.25, 0.125	△
Erythromycin	2, 1	▲
Chloromycetin	8, 4	☆
Streptomycin	16, 8	★
Aquilegia oxysepala	400, 200	◇
Berberine	40, 20	◁
Maguoflorine	200, 100	▽
Genkwanin	500, 250	○
Apigenin	500, 250	▷

is very clear. This may provide the basis to classify the metabolic profiles by PCA and to find out the possible mode of action of TCM.

The points of berberine (◁), genkwanin (○), apigenin (▷), rifampicin (■), and norfloxacin (□) are clustered together. That means the targets of berberine, genkwanin, and apigenin possibly are on nucleic acid as well. Anything more on the mechanism of action of berberine is documented by other works.[12–18] The PCA results supported the hypothesis that modes of actions of a drug could be identified by the metabolic profiles acquired.

The points of maguoflorine (▽) and *A. oxysepala* (♦) are clustered with acheomycin (△), lincolmensin (×), erythromycin (▲), chloromycetin (☆), and streptomycin (★). As we known from Table 1, lincolmensin, erythromycin, and chloromycetin have effects on 50S ribosomal subunit, streptomycin and acheomycin act on 30S ribosomal subunit. In a word, the mode of action of those five drugs is to inhibit protein synthesis. This may imply that the target of maguoflorine and *A. oxysepala* on *S. aureus* is possibly similar to that of lincolmensin, erythromycin, chloromycetin, streptomycin, and acheomycin, whose targets are protein.

The main antimicrobial component in TCM should play the main role on the target. That is, the mode of bioactive substance should be consistent with that of the whole medicine, though other chemical components also have effect on inhibiting growth of *S. aureus*. Hence, we reckon that the main antimicrobial substance in *A. oxysepala* on *S. aureus* might be maguoflorine.

This method is viable and feasible in finding preliminary results of antimicrobial mode of action and main antimicrobial components of traditional Chinese medicine. Although the method is immature, the results obtained by this method might provide some insight into the mode of action and possible mechanism of the compound under investigation.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.01.032.

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5. *Extracting components in Aquilegia oxysepala*. 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 was named *Aquilegia oxysepala*.
6. *Bacterial strains and growth conditions*. *Staphylococcus 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–visible spectrophotometer. Microbe strain was cultured into 50 mL MHB, and incubated in a shaker incubator overnight at 37 °C. The overnight culture was diluted to a concentration of approximately 10^8 cfu mL^{-1} and used as the source of inocula. Solutions of each drug and polar extract are prepared with concentration ranging from 1.25 to 80,000 μg mL^{-1}. After preparation, 1 ml solution was diluted with 9 mL MHB and the final concentrations of each drug and polar extract were shown in Table 2. At each passage, a volume of 100 μL of the coccus inoculum was re-inoculated into solutions containing different concentrations of each drug and the culture was again incubated overnight with shaking at 37 °C. The inhibition of each drug and polar extract to cells growth was between the approximate ranges of 40% and 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.
7. *Extraction of metabolites from Staphylococcus aureus*. Approximately 24 h after inoculation, when the majority of culture appeared to have entered stationary phase (growth curve figures shown in Supplementary information), the culture was centrifuged (Eppendorf centrifuge, 4000 rpm for 10 min at 4 °C). The supernatant was discarded and the pellet was suspended in a buffer (Tris–HCl, pH 7.4). Then, the suspension was centrifuged (4000 rpm for 10 min at 4 °C) and the supernatant was discarded again. This step was repeated three times. After that, the pellet was resuspended in 10 mL cold (−20 °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 (12,000 rpm for 10 min at 4 °C). The supernatant was transferred to a new tube. To the extracted pellet, cold (−20 °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. The samples were stored, at −40 °C, until LC/DAD/ESI-MS analysis. As this was done to every batch of culture with nine antibiotics, Henry Clematis Root extracted by ethanol, different components in Henry Clematis Root (genkwanin, apigenin, maguoflorine, and berberine).
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