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Identification of the Antibacterial Chemical Constituents of *Amorpha fruticosa* L. by A Modified High-Throughput Screening Method

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An antibacterial high throughput screening (HTS) method is developed for the *Amorpha fruticosa* L. fruit extracts, which were tested against methicillin-resistant *Staphylococcus aureus* (MRSA). The chloroform extract was separated into 80 fractions *via* preparative high performance liquid chromatography (HPLC). The optimal fractions and tephrosin were tested against methicillin-resistant *Staphylococcus aureus* using high throughput screening method and the antibacterial activity of those selected bioactive fractions was further confirmed by MIC method. Tephrosin exhibited more excellent inhibition ratio than optimal fraction for methicillin-resistant *Staphylococcus aureus*. *Amorpha fruticosa* L. exhibited the strongest antibacterial activity and thus the optimal fraction was obtained.

Key Words: *Amorpha fruticosa* L., High throughput screening, Methicillin-resistant *Staphylococcus aureus*, Antibacterial activity.

INTRODUCTION

Amorpha fruticosa L. is a shrub plant belonging to genus *Amorpha* of the *Leguminosae* family. In the whole world, there exist about 25 species plants belonging to the genus *Amorpha*. But, the species *fruticosa* was the only plant species that had been introduced in China. This species of plants had a wide distribution in Liaoning Province of China[1]. Based on the theories describing the medicinal properties of this plant in the traditional Chinese Medicine, the fresh fruits of *Amorpha fruticosa* L. have been used in the treatment of carbuncle, eczema and burn[2]. In China, Record of Natural Resources of Chinese Materia Medica, effectiveness of dried fruits of *Amorpha fruticosa* L. could be clearly deciphered from the fact that it was used in the form of an ointment. This ointment was externally applied twice or thrice every day for healing infective wounds[3].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is especially troublesome in hospitals, where patients with open wounds, invasive devices and weakened immune systems are quite vulnerable to infection as compared to the general public. Therefore, it is needed to realize that there is an urgent requirement of having breakthrough discoveries of new types of antimicrobials: these antimicrobials should be able to combat infections caused by methicillin-resistant *Staphylococcus aureus*[4-10].

Medicinal plants are the richest source given the fact that many drugs for treating infectious diseases in China have been discovered and prepared using them[11]. We developed an antimicrobial high throughput screening method for plant drug discovery. Among these species, *Amorpha fruticosa* L. exhibited strong biological activity against MRSA. In this study, we expanded the horizons of our research study on the *Amorpha fruticosa* L. fruits extract by finding out its distinguishing properties using the analytical technique of HPLC. This technique enabled us to isolate the effective fractions of the crude extract. HPLC technique also made it quite possible for us to further characterize its properties including monomeric chemical substances[12].

EXPERIMENTAL

The fruits of *Amorpha fruticosa* L. were collected from Dalian City of Liaoning province, China in the month of July. The collection process was authenticated by Prof. Tingguo Kang, College of Pharmacy, Liaoning University of CMM. It must be noted that a voucher specimen has been deposited in

pharmacognosy laboratory under specimen number AF0017. The MRSA species were separated from Clinical Sample at the Dalian Medical University. These were then subsequently identified by Department of Microbiology according to NCCLC standard. Growth conditions employed in this process included a constant temperature of 37 ºC in an aerobic condition.

The samples were tested for activity through the high throughput screening procedure by adding 5 µL of sample to selected wells of a 96-well plate. This plate contained 95 µL of LB medium and 10^6 CFU/mL bacteria obtained from 3-5 h culture. The plates were then incubated in an aerobic shaker for 18 h at 37 ºC. After incubation, 5 µL of the thiazolyl blue tetrazolium bromide was added to each well. The live bacteria could crystallize the blue formazan, while the dead bacteria could not do the same. 4 h of incubation was necessary for the wells containing MRSA. 100 µL DMSO was added in each well; it was then shaken for 5 min. A reading using a Multiskan Ascant plate reader was recorded for the bacteria at 570 nm wavelength of monochromatic light. Wells with viable cell will typically range in blue colour giving an OD reading above 0.600. *In vitro* inhibitory rate testing of effective fractions can be calculated using the following formula:

$$\text{Inhibitory rate (\%)} = \frac{\text{Negative control OD} - \text{Sample OD}}{\text{Negative control OD}}$$

Sensitivity: Inhibitory rate > 70 %; middle sensitivity: 70 % > inhibitory rate > 50 %; insensitivity: < 50 %.

The value of MIC was determined through careful observation of the wells which were devoid of any generated blue material. To further verify the MIC value, 10 µL bacteria of each hole was transferred to the LB culture plates, at 37 ºC for 24 h. Then, statistical counts of colony were observed[13,14].

Optimal fraction was purified using the technique of preparative HPLC and silica gel column chromatography. This helped the researchers of the study to yield effective compounds. Structures of these compounds were confirmed by comparison of their spectral data.

RESULTS AND DISCUSSION

Among these plant species, *Amorpha fruticosa* L. exhibited significant biological activity against MRSA. The *Amorpha fruticosa* L. extract was were fractionized with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH. The fractions were tested for MIC determination using the modified MTT method. The $CHCl_3$ fraction showed good antibacterial effects and. further purified by the analytical technique of HPLC into 80 fractions: It was subsequently tested again for antibacterial activity. The HPLC profile was shown in Fig. 1.

The eighty fractions were tested again for antibacterial activity. Among the fractions, it was found that six fractions (fraction D9, D10, D11, E2, E3 and E4) showed significant antibacterial activity. The results of each fraction's antibacterial activity *in vitro* are expressed in Fig. 2. The wells in this plate have been treated with MRSA and the *Amorpha fruticosa* L. Inhibitory rates of HPLC fractions D9, D10, D11, E2, E3 and E4 were determined. These were respectively reported as 58, 63, 71, 80, 62 and 66 % (1-80 reading left to right) through the technique. The results indicate that the antibacterial activity

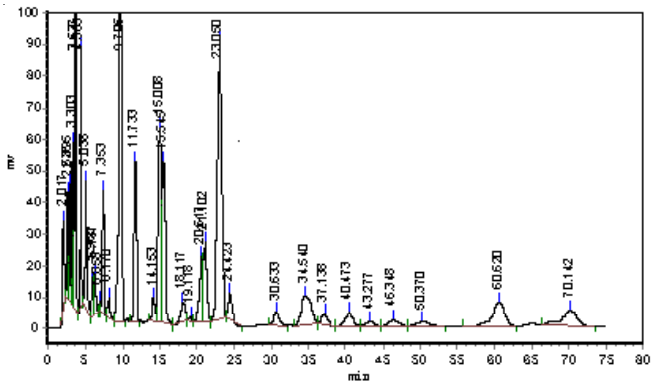

Fig. 1. HPLC Chromatograph of *Amorpha fruticosa* L. $CHCl_3$ fractions

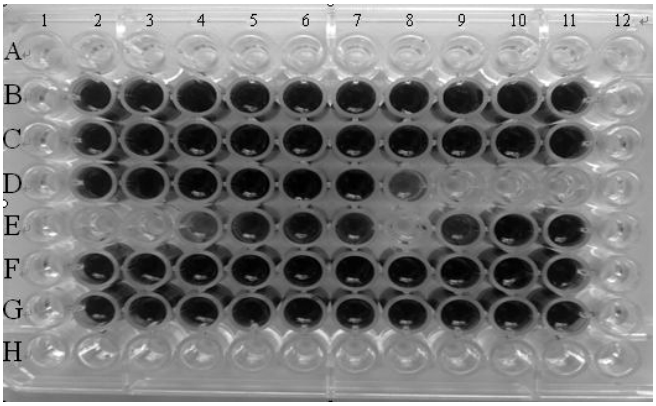

Fig. 2. High throughput screening results of the *Amorpha fruticosa* L. HPLC 80 fractions (B2-G11) against MRSA

was related to the content and distribution of effective ingredients. The modified MTT method suggests that fraction D11 and E2 manifest excellent inhibition. The MIC results of fractions D11 and E2 were estimated as 500 and 125 µM, respectively.

The fraction E2 was purified using the analytical technique of preparative HPLC. In this method, one known compound was yielded from the purified E2 fraction and the structure was confirmed by comparison of its spectral data with that of reported spectral characteristics. The compound was identified as tephrosin (Fig. 3). Based on the results obtained through HPLC analysis, it was found that the content of tephrosin is highest in fraction E2.

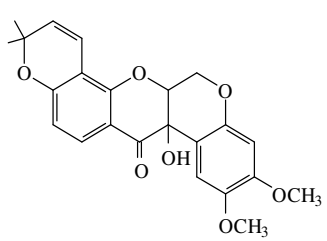

Fig. 3. Structure of tephrosin

Conclusion

We described a robust high throughput screening (HTS) method for rapid assay of antibacterial activity in a plant extract library. Thanks to the HTS method, *Amorpha fruticosa* L. was identified as a potential source for antibacterial discovery given

the fact that it exhibited a significant activity against MRSA[15]. It is a widely accepted fact that the factors of antibacterial effects of natural drug is related with drug factors (drug varieties, drug extraction, concentration, pH value, *etc.*), microbial factors (kinds and types of bacteria, concentration and physiological characteristics of bacterial suspension, culture time, *etc.*) and other factors (medium components and screening methods, *etc.*). Because our screening method was sensitive and cost-effective, we can certainly infer that the high throughput screening method is a useful tool for antibacterial discovery.

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REFERENCES

1. K.S. Hou, Chinese Seed Plants Dictionary. Beijing, China, Science Press, p. 26 (1982).
2. China Pharmaceutical University, Great Word Sea of TCM, Medical Science Publishing House, City, China, p. 861 (1997).
3. Chinese Herbal Medicine Companies, Chinese Medicine Resources Blog, Science Press, Beijing, China, p. 548 (1994).
4. S.R. Norrby, C.E. Nord and R. Finch, *Lancet. Infect. Dis.*, **5**, 115 (2005).
5. W.J. Witte, *Antimicrob. Chemother.*, **44(Suppl. A)**, 1 (1999).
6. M.E. Mulligan, K.A. Murray-Leisure, B.S. Ribner, H.C. Standiford, J.F. John, J.A. Korvick, C.A. Kauffman and V.L. Yu, *Am. J. Med.*, **94**, 313 (1993).
7. E.J. Gorak, S.M. Yamada and J.D. Brown, *Clin. Infect. Dis.*, **29**, 797 (1999).
8. H.E. Blok, M.R. Vriens and A.J. Weersink, *J. Hosp. Infect.*, **48**, 325 (2001).
9. M.M. MacKinnon and K.D. Allen, *J. Hosp. Infect.*, **46**, 216 (2000).
10. C. Theaker, S. Ormond-Walshe, B. Azadian and N. Soni, *J. Hosp. Infect.*, **48**, 98 (2001).
11. I. Francolini, P. Norris, A. Piozzi, G. Donelli and P. Stoodley, *Antimicrob. Agents Chemother.*, **48**, 4360 (2004).
12. S. Engel, P.R. Jensen and W. Fenical, *J. Chem. Ecol.*, **28**, 1971 (2002).
13. J. Zhao, L. Xu and Y. Zhang, *Nat. Prod. Res. Dev.*, **21(S)**, 34 (2009).
14. J. Tang, M. Tan, J. Zhao, Z. Wang and L. Zhou, *Nat. Prod. Res. Dev.*, **20**, 949 (2008).
15. L.A. Mitscher, Y.H. Park, A. Alshamma, P.B. Hudson and H. Thomas, *Phytochemistry*, **20**, 781 (1981).