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Antioxidant and Antibacterial Activities of Crude Extracts of *Homalium paniculiforum* Stem

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The present study reports antioxidant and antibacterial activities of crude extracts of *Homalium paniculiforum* stem using high-throughput assay. The crude extracts were prepared in two methods, accelerated solvent extraction and conventional solvent extraction, successively using petroleum ether, ethyl acetate, *n*-butanol and water. The EC_{50} values were calculated for DPPH and FRAP value in order to evaluate the antioxidant efficiency of all of the extracts. The MIC values were judged by visual for resazurin microtitre assay in order to evaluate the antibacterial ability of all of the extracts. In comparison, accelerated solvent extraction method can improve antioxidant and antibacterial ability of the extracts more or less. The conventional solvent *n*-butanol extract (EC_{50} = 47.99 mg/L, FRAP value = 434.8 ± 51.3 mol TE/g) exhibited highly striking 2, 2-diphenyl-1-picrylhydrazyl radical-scavenging activity, ability of chelating ferrous ions. The conventional solvent petroleum ether extract (EC_{50} > 200 mg/L, FRAP value = 26.6 ± 7.3 mol TE/g) exhibited the lowest. Resazurin microtitre assay show that the conventional solvent extract ethyl acetate and petroleum ether is more suitable for further study on chemical constituents with antimicrobial activity because of simply preparation and inhibition to all the strains.

Key Words: *Homalium paniculiforum*, Antioxidant activity, Antibacterial activity, Resazurin microtiter method.

INTRODUCTION

The flacourtiaceae is a large family consisting of some 93 genera and 1300 species found throughout the tropical and subtropical regions of the world and is still an under explored family with a diverse range of bioactive compounds[1]. Homalium is one of the largest genera with 200 species. There is still scant pharmacological and chemical information on the genus[2]. In the present investigation, homalium plant mainly contains alkaloids, phenolic glycosides, coumarins, triterpenes and other chemical composition, with antiviral, diabetes and other pharmacological activities[3-9]. *Homalium paniculiforum* is endemic forest tree growing up to 80-100 feet of Hainan Province and is used as Chinese traditional medicines in centuries for gonorrhea and as an astringent. There have been no reports of its antioxidant and antimicrobial activity.

Choosing the correct assay to assess the antioxidant and antimicrobial potential of extracts and compounds is important for generating high-quality data with the high accuracy, speed and efficiency, enabling the addition of potential new antioxidant and antimicrobial compounds and extracts to our armamentarium. In order to find if this folk medical use has a scientifically justified basis, rapidly screening to find the crude extract of highly bioactivity, the present study evaluate antioxidant activities of the extracts of the conventional solvent extraction and accelerated solvent extractions, different antioxidant tests were used, such as DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging and ferrous ions chelating activities, investigated the antibacterial activity by modified resazurin microtitre assay.

EXPERIMENTAL

The stem of *Homalium paniculiforum* were collected from Diaoluo Mountain forest of Lingshui County in September 2010, Hainan Province, China. A voucher specimen was deposited in the superior resources of Hainan Chemical Materials Application Technology Ministry of Education Key Lab, Hainan University. The plant materials were dried at room temperature and then powdered using a mixer-grinder.

Conventional solvent extracts: The dried and powdered stem of *Homalium paniculiforum* were exhaustively macerate in 95 % ethanol with reflux apparatus 3 times for 3 h. The liquid extract obtained was concentrated in vacuum at 40 ºC. The dry ethanol extract was dissolved in distilled water with different polar solvent extraction, dried and stored.

Accelerated solvent extracts: *Homalium paniculiforum* stem powder placed in 100 mL stainless steel timber extraction cell (bottom of the pool ahead of the installation of extraction

of cellulose membrane), temperature 120 ºC, static extraction time 5 min, 2 cycles for the fluid pressure of 1.034 MPa, 60 % of the pool to the volume of solvent rinse, N_2 purge time 100 s, separately with organic solvent such as petroleum ether, ethyl acetate, *n*-butanol and water extraction and then concentrated by vacuum distillation at 40 ºC, dried and stored.

Determination of DPPH radical-scavenging activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of the extracts were measured by improving published methods[10-15]. Each extract has 6 parallel experiments and 6 repeat testing and vitamin C, rutin, BHT were used as standard. Take sections of 40 mg crude extract with ethanol or methanol solution to get various concentrations: 4000, 2000, 1000, 500, 250 and 125 mg/L. From each concentration, 10 µL of extract was mixed with 190 µL of 0.105 mmol/L DPPH methanolic solution. The contents were kept in dark for 0.5 h and shaken vigorously by microplate reader with measuring at 515 nm. Methanol (190 µL) plus the extract solution (10 µL) was used as a blank, while methanol (10 µL) and the DPPH solution (190 µL) was used as a control. The scavenging activity was calculated using the formula of scavenging activity (%) = [1-(A_{515} of sample- A_{515} of blank)/A_{515} of control] × 100. Take the final concentration as the abscissa, fit exponential function, calculate average value and analysis error by Origin 8, get scavenge 50 % DPPH clearance rate (EC_{50}) of different samples.

Ability of chelating ferrous ions: Mix 2.5 mL $FeSO_4$ solution of series concentration 25, 100, 150, 200, 400, 500, 800 and 1000 µmol/L, 2.5 mL TPTZ solution of 10 mmol/L, 25 mL acetic acid-sodium acetate buffer of 300 mmol/L (pH = 3.6), add 170 µL the mixture into 96 microplate, read the absorbance at 593 nm in the xMark microplate reader with temperature 37 ºC[16]. Take different concentration of $FeSO_4$ solution as the abscissa, absorbance value as the vertical coordinates, draw the standard curve (Fig. 3a).

Mixture 2.5 mL $FeCl_3$ solution of 20 mmol/L, 2.5 mL TPTZ solution of 10 mmol/L and 25 mL acetic acid-sodium acetate buffer of 300 mmol/L (pH = 3.6) and then add 150 µL the mixture into 96-well microplate, read the absorbance at 593 nm in the xMark microplate reader with temperature 37 ºC (before reaction). Add 20 µL 500 mg/L extract solution, read again after 8 min. Calculate FRAP values by the standard curve according to the absorbance change values. All experiments were repeated four times.

Resazurin microtitre assay: Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays. It is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. Resorufin is further reduced to hydro-resorufin (uncoloured and nonfluorescent). A resazurin reduction test has also been used for decades to demonstrate bacterial and yeast contamination of milk.

To determine antimicrobial activities, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* ATCC 10231 were used. Briefly, the inoculum was prepared in MH medium, adjusted to a McFarland tube No. 1, then diluted 1:30. Two-fold dilutions of 10 mg/mL each crude extract (DMSO)and gentamycin sulfate (sterile water) were prepared in saline, the concentration range of test material used was 1000-7.815 mg/L. 120 µL MH medium was dispensed in each well of the sterile 96-well plate and 50 µL of each bacterial suspension was added to 20 µL serial two-fold dilutions of crude extract and then10 µL of resazurin solution 0.04 % (w/v) was added to each well. The plates were sealed in a plastic bag and incubated at 37 ºC for 2-8 h. A change in colour from blue (oxidized state) to pink (reduced) indicated the growth of bacteria and the MIC was defined as the lowest concentration of drug that prevented this change in colour[17,18]. Each MIC was determined in triplicate experiments.

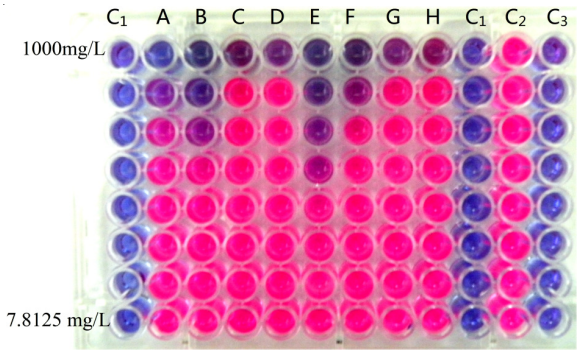

Fig. 1. Plates after 4 h in modified resazurin assay. [Pink colour indicates growth and blue means inhibition of growth; the test organism was *Staphylococcus aureus*; C1, sterility control (Gentamycin sulphate in serial dilution + broth + saline + indicator), no bacteria; C_2, control without drug (saline + broth + bacteria + indicator); C_3, positive control (Gentamycin sulfate in serial dilution + broth + bacteria + indicator); A-H, test crude extract (in serial dilution in wells 1-8 + broth + bacteria + indicator)].

RESULTS AND DISCUSSION

Determination of DPPH radical-scavenging activity: Conventional solvent extraction with petroleum ether, ethyl acetate, *n*-butyl alcohol, water and the extracts were written as MSO, EtOAc, NBA and W, respectively. Accelerated solvent extraction with petroleum ether, ethyl acetate, *n*-butyl alcohol, water and the extracts were written as ASE-MSO, ASE-EtOAc, ASE- NBA and ASE-W.

Both conventional solvent extracts and accelerated solvent extracts displayed striking DPPH radical scavenging activities (Fig. 2, Table-1), but the antioxidant activity of all the extracts are lower than all the standard substance. The accelerated solvent extracts showed a more powerful radical-scavenging activity than conventional solvent extracts, except extract by *n*-butanol. The extract NBA showed the highest antioxidant activity (EC_{50} = 47.99 mg/L), while the extract MSO showed the lowest antioxidant activity (EC_{50} > 200 mg/L).

Ability of chelating ferrous ions: As shown in Fig. 3, the results of the ferrous ion-chelating assay were more or less matched to those of DPPH radical-scavenging assays. The ability of chelating ferrous ion of the extract NBA and ASE-EtOAc are stronger than the standard substance BHT. The extract NBA showed the highest ability of chelating ferrous ion (FRAP value = 434.8 ± 51.3 mol TE/g), while the extract MSO showed the lowest ability of chelating ferrous ion (FRAP value = 26.6 ± 7.3 mol TE/g).

TABLE-1
EC_{50} VALUE OF EXTRACTS AND STANDARD SUBSTANCE

Conventional solvent extracts	EC_{50} (mg/L)	Accelerated solvent extracts	EC_{50} (mg/L)	Standard substance	EC_{50} (mg/L)
MSO	>200	ASE-MSO	196.49	Vc	4.59
EtOAc	90.46	ASE-EtOAc	63.24	BHT	12.11
NBA	47.99	ASE-NBA	162.52	Rutin	3.41
W	154.00	ASE- W	119.46	–	–

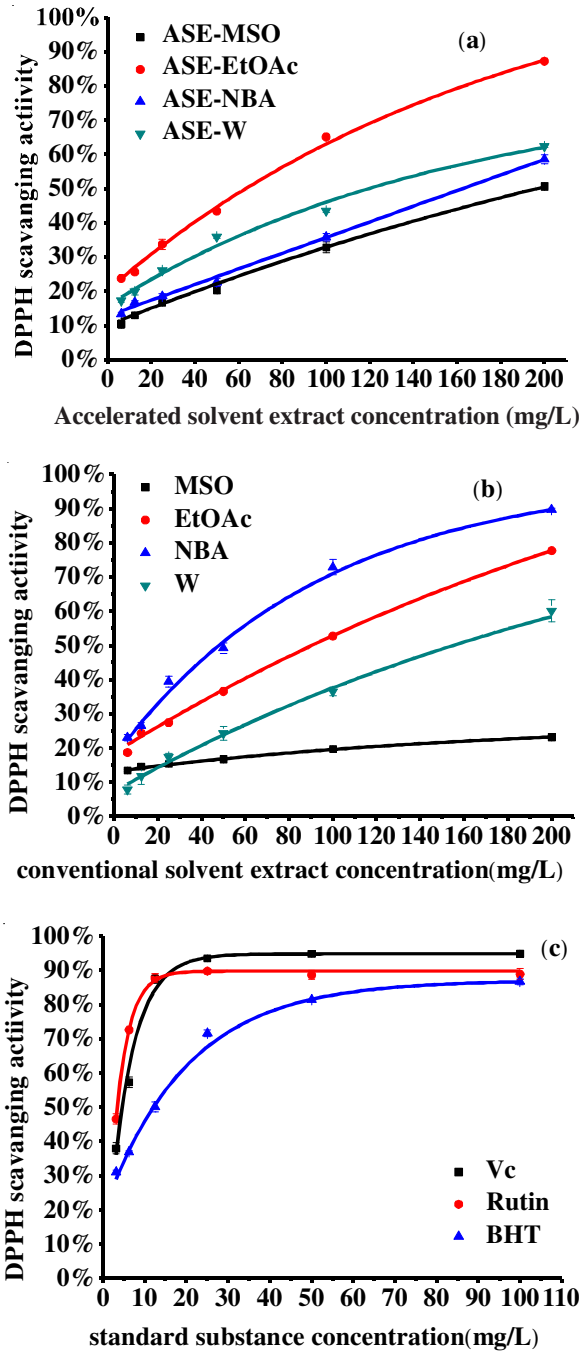

Fig. 2. DPPH radical-scavenging activities of extracts (a, b) and standard substance (c)

Resazurin microtitre assay: This modified resazurin assay has been showed that the extract ASE-MSO has stronger bacteria inhibition than other extracts (Table-2), but it has no obvious inhibition to *Escherichia coli*. Extracts of ethyl acetate with two methods, have certain inhibition to all the bacteria and the difference is not significant. Extracts of petroleum

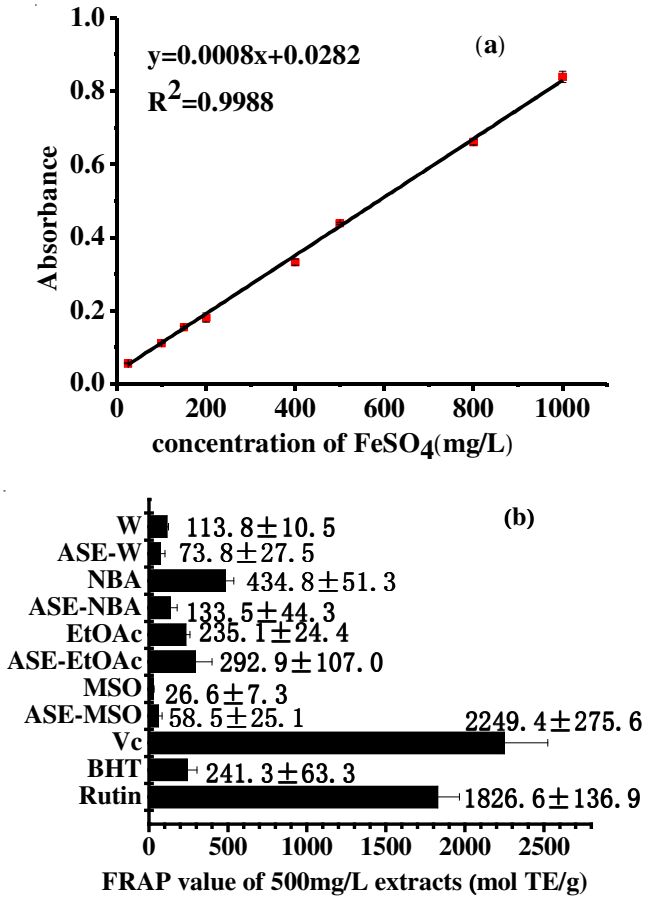

Fig. 3. Standard curve of $FeSO_4$ (a) and diagram of FRAP value (b)

ether, ethyl acetate, have stronger antibacterial ability than extracts of *n*-butanol and water. In comparison, accelerated solvent extraction method can improve the extract antibacterial ability more or less.

A high-throughput assay using a microplate reader with spectrophotometric detector and 96 well plates is easy to perform and has acceptable accuracy, precision and reproducibility, may be used for screening and investigating the potential natural drug. In the present study, antioxidant and antibacterial activities of the extracts of the conventional solvent extraction and accelerated solvent extractions of petroleum ether, ethyl acetate, *n*-butanol and water of *Homalium paniculiforum* stem, was examined with DPPH scavenging activity and FRAP, resazurin microtitre assay using high-throughput screening.

With regard to the EC_{50} values, the DPPH scavenging activity was in the following order: Rutin > Vc > BHT > NBA > ASE-EtOAc > EtOAc > ASE-W > W > ASE-NBA > ASE-MSO > MSO. Vitamin C, a strong antioxidant, exhibited the highest DPPH scavenging activity as anticipated. The ferrous ion-chelating ability was in the following order: Vc > Rutin > NBA > ASE-EtOAc > BHT > EtOAc > ASE-NBA > W >

TABLE-2
ANTIBACTERIAL ACTIVITY OF THE CRUDE EXTRACT FROM *Homalium paniculiforum*

Sample	MIC value (mg/L)				
	E. coli	*P. aeruginosa*	*S. aureus*	*B. subtilis*	*C. albicans*
MSO	1000	250	500	250	1000
EtOAc	1000	125	250	250	1000
NBA	>1000	>1000	1000	1000	>1000
W	>1000	>1000	1000	1000	>1000
ASE-MSO	>1000	125	250	250	500
ASE-EtOAc	500	125	500	250	1000
ASE-NBA	>1000	>1000	1000	500	>1000
ASE-W	>1000	>1000	1000	500	>1000
Time (h)	4	8	4	2	8

ASE-W > ASE-MSO > MSO. The ability of chelating ferrous ion of the extract NBA and ASE-EtOAc are stronger than the standard substance BHT. In comprehensive comparison, the convention solvent n-butanol extract showed the best antioxidative activity, conventional solvent petroleum ether extract exhibited the lowest antioxidative activity.

This modified resazurin assay enabled results to be comparable for the test material for different bacterial strains, the generation of an accurate MIC value, which can be compared to existing antibiotics, empowers us with the knowledge to decide whether the extracts and compounds are worth pursuing further in terms of antimicrobial potential. It has been showed that the extract ASE-MSO has stronger bacteria inhibition than other extracts, but it has no obvious inhibition to *Escherichia coli*. Extracts of ethyl acetate with two methods, have certain inhibition to all the bacteria and the difference is not significant. In comparison, accelerated solvent extraction method can improve the extract antibacterial ability more or less, extracts of petroleum ether, ethyl acetate, have stronger antibacterial ability than extracts of *n*-butanol and water, then the conventional solvent extract EtOAc and MSO is more suitable for further study on chemical constituents with antimicrobial activity because of simply preparation.

Conclusion

Accelerated solvent extraction method can improve antioxidative and antibacterial ability more or less, extract ASE-EtOAc, EtOAc has a content of antioxidative activity and antimicrobial activity. Extract MSO exhibited the lowest antioxidative activity, it is more suitable for studying on antimicrobial activity. Extract NBA showed the best antioxidative activity and lowest antimicrobial activity, extract W showed the lower antioxidative activity and lowest antimicrobial activity. In this study, we found that it is no certainly correlation with antioxidative activity and antibacterial ability.

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