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Antioxidant and antimicrobial activities of the extracts from *Sophora flavescens*

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Abstract
An indigenous herb, *Sophora flavescens*, has been widely used as a traditional medicine in Taiwan. The main goal of this study was to determine the antibacterial and antioxidant activities of various extracts, including 95% ethanol crude extracts, ethyl acetate fraction, and aqueous fraction, from *Sophora flavescens*. Antioxidant activity was determined by the methods of DPPH radical scavenging test, trolox equivalent antioxidant capacity (TEAC), and reduction capacity. In addition, the contents of total phenolics and flavonoids were determined. Antibacterial activity was performed by disk diffusion method, minimum inhibition concentration (MIC), minimum bactericidal concentration (MBC), time-killing curve and synergy effect. The clinical antibiotic isolates, including gram-positive and gram-negative pathogens were used for antimicrobial activity assay. Experimental results showed that the extracts of ethyl acetate exhibited a higher antioxidant activity and antioxidant activity among all the extracts. *In vitro*, the ethyl acetate extracts presented a significant antibacterial activity against oxacillin-resistant *S. aureus* with MIC value of 0.025 mg/mL and MBC of 0.04 mg/mL. The extracts of ethyl acetate scavenged DPPH radicals and TEAC with values of 178 ppm (IC_{50}) and 78 g trolox / 100 g DW, respectively. A higher content of both total phenolics and flavonoids were found in the ethyl acetate extracts which correlated with a better biological activities compared with other extracts. These results reveal that the extracts of ethyl acetate from *Sophora flavescens* could be developed as a potential natural antioxidant and antibacterial agent.

Keywords: *Sophora flavescens*, antioxidant activity, antibacterial activity

1. Introduction
Many recent studies have indicated that Chinese medicinal plants contain a wide variety of natural antimicrobial substances, such as terpenes, flavonoids and phenolic compounds [1]. Researchers have been interested in biologically active compounds isolated from Chinese medicines to inhibit the spread of pathogenic microorganisms. Multiple resistances in human pathogenic microorganisms have globally developed, however, and caused serious nosocomial infections. Among the nosocomial drug-resistant strains, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, were the most prevalent isolates in Taiwan [2]. The recent emergence of drug-resistant strains are deeply worrisome and highlights the urgent need for novel antibacterial agents. From this point of view, it is important to identify new sources of safe and inexpensive antimicrobial substances that occur naturally.
As age or live face increased pressure, *in vivo* oxidation - reduction reactions will be losing its balance; the dynamic balance between anti-oxidation system and the free radical mechanism is no longer present. Lots of medical studies have reported that the excessive reactive oxygen species triggered a number of age-related diseases, such as atherosclerosis, hypertension, diabetes, accelerated aging of the human body and caused cancer or genetic mutations. Antioxidant substitutes are thus bringing attention for the medical profession [3].
An indigenous herb, *Sophora flavescens*, is a species of evergreen shrub ranging throughout the temperate regions of Asia. This plant has a slow rate of growth, with adult species reaching about 1.5 meters in height. It keeps its leaves all year, and blooms yellow flowers during July and August. The roots and barks of *S. flavescens* are harvested in autumn and dried prior to use in topical and internal preparations. Asian traditional medicine systems consider the roots of this species possessing the following properties: antibacterial, anthelmintic, astringent, diuretic, and tonic. In traditional Chinese medicine, this herb is specifically directed towards addressing situations of "damp heat" and "wind Qi-stagnation" [4]. This study is designed to determine the antimicrobial and antioxidant activities of extracts and fractions from *S. flavescens*.

2. Materials and methods

2.1 Material

Sophora flavescens herbs were bought from local Chinese herbal stores in Kaohsiung area. The plant was verified by Department of Traditional Chinese Medicine at Kaohsiung Medical University. The clinical antibiotic resistant strains used in this research were isolated from patients' blood and phlegm from Chia-Yi Christian Hospital in Taiwan. The standard strains for comparison, included *A. baumannii* ATCC 19606, *S. aureus* ATCC 6538P, *P. aeruginosa* ATCC 29260, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25257, were purchased from Food Industry Research and Development Institute Bio-resources Collection and Research Center in Taiwan.

2.2 Preparation of ethanol extracts and partition

The dry herbs were ground to powder and mix with 95% ethanol at 1 : 5 ratio, then rotary extraction overnight in 37 ℃, 200 rpm. The extracts were then filtered and concentrated in vacuum at 40 ℃. The extraction procedure was repeated twice. The resulting extracts were suspended in water and partitioned with ethyl acetate.

2.3. Antimicrobial analysis

(a) Disc diffusion method: The petri dish was prepared with a base layer of Muller Hinton (MH) agar (10 mL) and a top layer of 0.75 % MH agar (5 mL), then inoculated with 50 µL of each bacterial suspension (10^5 cfu/mL). Paper discs (8 mm in diameter) were impregnated with 30 µL of herbal extracts (3 mg/disc), and placed on the inoculated plates, then incubated at 37 ℃ for 14 hours. The diameters of the inhibition zones (DIZ) were measured [5].

(b) Minimum Inhibition Concentration (MIC), Minimum Bactericidal Concentration (MBC): Different volumes of natural herbal extracts (0.2 g/mL) were added into LB broth medium, and in the same volume of DMSO as a negative control group. The bacterial culture incubated for 14 to 16 hours were serial diluted into 10^7 CFU/mL, then 50 µL of the diluted culture broth were added to the natural herbal LB broth (final bacterial concentration 10^5 CFU/mL) and incubated at 37 ℃ for 12 hours. After 12 hour incubation, 100 µL culture medium were transferred and eventually spread on MH agar plates. The culture plates were then incubated at 37 ℃ for 12 hours, and counted for the number of colonies from different extract concentrations. The minimum inhibitory concentration (MIC) is defined as the extract concentration if 99% bacterial concentration was reduced; the extract concentration, reduced 99.9% of bacterial concentration is defined as a minimum bactericidal concentration (MBC).

(c) Synergy effect: The synergy effect is determined by double disc diffusion method, in which 1 g of herbal extracts was dissolved in 1 mL of DMSO (1 g/mL), and various concentrations of different antibiotics, included Ampicillin (Amp), Cephalosporin (CEP), Clindamycin (CC), Erytromycine (Em), Gentamycin (Gm), Kanamycin (KM), Piperacillin (PIP), Streptomycin (Sm), Trimethoprim / sulfamethoxazole (SXT) and Tetracycline (Tc), were used for the determination of synergy effect. The combined effect within the antibiotics and extracts was determined based on the shape of inhibition zone between antibiotics and extracts [5].

(d) Time-killing curve: A single colon of test strain was inoculated in 5 mL LB broth containing Ampicillin (50 mg/mL) and incubated at 37 ℃ for 14 to 16 hours. 50 µL of the overnight culture (10^7 CFU/mL) with 2 times of the MIC extracts were added into 5 mL LB broth containing Ampicillin (50 mg/mL) and incubated at 37 ℃, 200 rpm shaking incubator. During the incubation, the bacterial culture was taken at 0, 1, 2, 3, 4, 6, 8, 10, 12, and 24-hour time point and transfer to MH plates for an overnight incubation; the number of colonies were counted after the incubation.

2.4. Antioxidant activity analysis

(a) 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay: The herbal extracts were diluted with methanol and then reacted with the DPPH solution. The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark. The absorbance at 517 nm of the reaction solution was measured by spectrophotometer. Inhibition of the DPPH radicals was calculated using the following equation:

$$1\% = [1 - \frac{X1 - X2}{X3}] \times 100\%$$

Where X1 is the absorbance of the tested sample (in a DPPH solution), X2 is the absorbance of the tested samples without DPPH and X3 is the absorbance of the control sample (which contains all reagents except for the test samples). The IC_{50} value represents the concentration of the tested sample that caused 50% inhibition [6].

(b) Determination of total phenolic concentration: An aliquot of 0.2 mL sample and gallic acid in ddH_20 were added to test tubes containing 1 mL Folin reagent and 1 mL sodium carbonate. After 2 hours reaction, the absorbance was determined at 760 nm. Gallic acid (0 - 250 ppm) was used for calibration of the standard curve that was $Y = 0.0095 \cdot X + 0.0009$ (where X = concentration of gallic acid equivalents expressed as milligrams of gallic acid per 100 g of dry weight of the plant material; Y = measured absorbance) and the correlation coefficient was $R^2 = 0.9999$.

(c) Determination of total flavonoids concentration: An aliquot of 1.0 mL sample and quercetin in methanol were added to test tubes containing 0.1 mL potassium acetate, aluminum nitrate and 3.8 mL methanol. After 40 min reaction, the absorbance was determined at 415 nm. The flavonoids were assessed by plotting the quercetin calibration curve (50 - 250 ppm) that was $Y = 0.0089 \cdot X + 0.0036$ (where X = concentration of quercetin equivalents expressed as milligrams of quercetin per 100 g of dry weight of the plant material; Y = measured absorbance). The correlation coefficient was $R^2 = 0.9976$.

(d) Trolox equivalent antioxidant capacity (TEAC) assay: An aliquot of 20 µL sample and different concentrations of trolox were added to 1 mL of 0.175 mM ABTS solution and then dark stand for 10 minutes. The absorbance was determined at 734 nm. The antioxidant capacity of the natural herbal extract was determined based on the calibration curve of the different concentrations of trolox. A trolox solution (final concentration 0 - 250 ppm) was used for calibration of

the standard curve that was Y = 0.0008·X＋0.6381 (where X = concentration of trolox equivalents expressed as milligrams of trolox per 100 g of dry weight of the plant material; Y = measured absorbance) and the correlation coefficient was R^2 = 0.9979.

(e) Reducing power test: An aliquot of 75 µL sample were added to test tubes containing 75 µL of 1% $K_3Fe(CN)_6$ and sodium phosphate buffer mixture, then incubated at 50 ℃ water bath. After cooling on ice, 75 µL of 10% TCA, 0.1% $FeCl_3$ and ddH_20 were added to 300 µL and completely mixed for 14 minutes, then determined the absorbance at 700 nm. The slope of the plot represents the reduction capacity of the test herbal extracts.

2.5. Statistical analysis
Data were expressed as mean ± SD. Independent-sample t-test was used for selected comparisons between samples. Alpha value was set *a priori* at P<0.05.

3. Results and Discussion
3.1. Disc diffusion method
A total of 15 strains, including 5 of the standard strains and 10 of the clinical antibiotic resistant isolates, were test for the antibacterial activity. The results of the disc diffusion method were shown in Table I. The extracts of ethyl acetate (EA) revealed a slightly higher antibacterial activity against the *S. aureus* strains and oxacillin-resistant *S. aureus* 287 with the diameters of inhibition zones (DIZ) between 16.7-18.4 mm among all of the test strains. Compared to the antibiotic, tetracycline, all of the extracts obtained from *Sophora flavescens* did not show significant antibacterial activity against the test strains.

Table 1: Disc inhibition zoon (DIZ) of the extracts from *Sophora flavescens*

Strains	DIZ （mm）			
	Crude (1000 mg/mL）	Ethyl acetate (100 mg/mL）	Water (100 mg/mL）	Tetracycline (7.5 mg/mL）
A. baumannii ATCC 19606	11.70 ± 0.40	9.50 ± 0.30	9.40 ± 0.70	28.60 ± 0.00
S. aureus ATCC 6538P	19.30 ± 0.80	18.40 ± 0.50	10.80 ± 0.00	37.00 ± 0.00
P. aeruginosa ATCC 29260	12.20 ± 0.30	—	—	23.00 ± 0.00
P. aeruginosa ATCC 27853	11.50 ± 0.00	—	—	20.80 ± 0.00
E.coli ATCC 25257	19.40 ± 0.50	—	—	27.90 ± 0.00
A. baumannii 814	11.80 ± 0.80	9.30 ± 0.40	9 .00 ± 0.00	17.10 ± 0.00
A. baumannii 817	12.50 ± 2.20	9.80 ± 0.30	9.30 ± 0.30	28.80 ± 0.00
S. aureus 908	17.60 ± 0.60	17.20 ± 1.10	11.10 ± 0.20	38.40 ± 0.00
S. aureus 985	17.80 ± 1.20	18 .00 ± 0.30	10.80 ± 0.10	20.50 ± 0.00
P. aeruginosa 717	13.80 ± 1.80	10.20 ± 0.60	—	34.90 ± 0.00
E.coli 9005UTI	12.10 ± 0.40	—	—	29.50 ± 0.00
Oxacillin-Resistant *S. aurens* 220	9.50 ± 0.00	9.30 ± 0.30	9.30 ± 0.30	27.30 ± 0.00
Oxacillin-Resistant *S. aurens* 287	17.40 ± 0.10	16.70 ± 0.30	11.30 ± 0.60	34.50 ± 0.10
Methicillin-Resistant *S. aurens* 2118	10.90 ± 0.40	10.30 ± 0.70	10.10 ± 0.40	31.40 ± 0.00
Methicillin-Resistant *S. aurens* 331	12 .00 ± 0.50	9.70 ± 0.10	9.30 ± 0.30	31.60 ± 0.60

Mean ± SD
-: no active

3.2. Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC)
Two of the test strains, *S. aureus* 985 and oxacillin-resistant *S. aureus* 287, were selected for the determination of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC). As shown in Table II, the ethyl acetate extracts presented a significant antibacterial activity against oxacillin-resistant *S. aureus* 287 with MIC value of 0.025 mg/mL and MBC of 0.04 mg/mL.

Table 2: Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts from *Sophora flavescens*

Strains	Extracts			
	Crude		Ethyl acetate	
	MIC (mg / mL)	MBC (mg / mL)	MIC (mg / mL)	MBC (mg / mL)
S. aureus 985	＞ 8.0	＞ 8.0	＞ 8.0	＞ 8.0
Oxacillin-Resistant *S. aurens* 287	0.08 ± 0.000	0.09 ± 0.014	0.025 ± 0.007	0.04 ± 0.000

Mean ± SD

3.3. Synergy effect
Drug combination effects were investigated by the disc diffusion method. As shown in Fig. 1, the active ethyl acetate fractions presented different synergistic interactions for the test strains (SA985 and ORSA287). For *S. aureus* 985, the ethyl acetate extracts did not show any synergistic effect with the eleven tested antibiotics. For oxacillin-resistant *S. aureus* 287, the ethyl acetate extracts presented synergistic effects with at least three different antibiotics, including Clindamycin, Cephalosporin and Tetracycline.

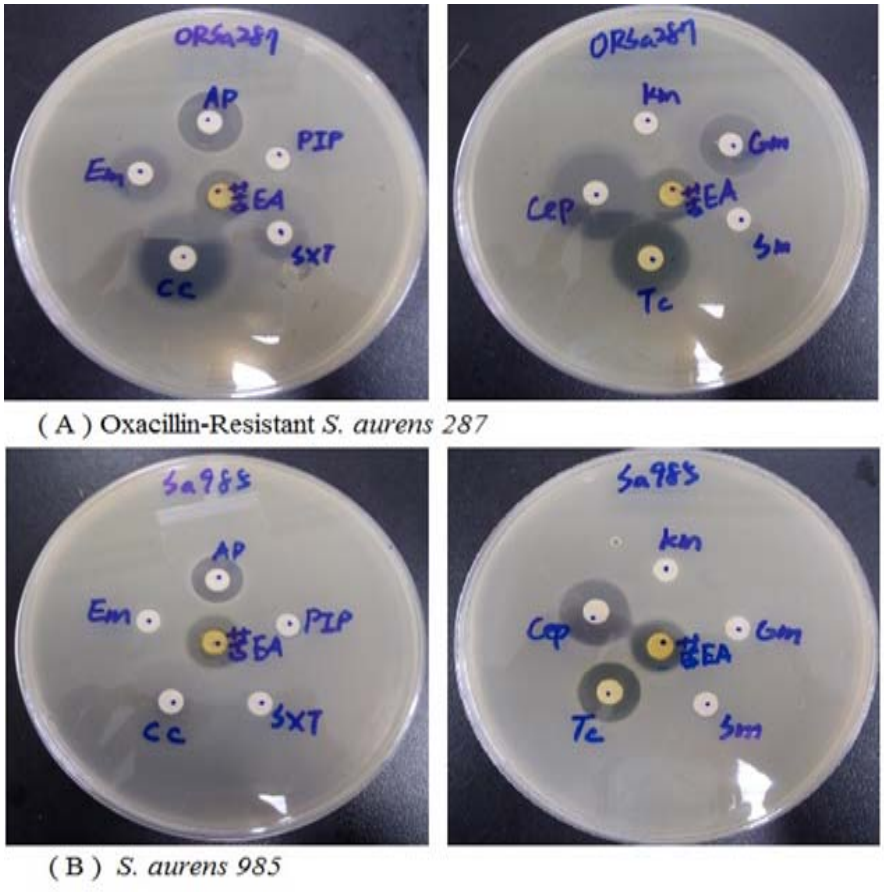

Fig 1: Synergy test using double disc diffusion method for the ethyl acetate fraction (EA) of *Sophora flavescens* and antibiotics, included ampicillin (Ap), cephalosporin (CEP), clindamycin (CC), erythromycin (Em), gentamycin (Gm), kanamycin (KM), piperacillin (PIP), streptomycin (Sm), trimethoprim / sulfamethoxazole (SXT) and tetracycline (Tc).

3.4. Time-killing curve

Due to their susceptibility, the time-killing curves of the clinical isolate (oxacillin-resistant *S. aureus* 287) were further analyzed. The results showed that all of the extracts from *Sophora flavescens* possessed antibacterial effect against ORSA 287 (Fig. 2); in which the ethyl acetate extracts revealed a strongly inhibition on the growth of ORSA 287. Within 3 hrs, the EA extracts showed potential effects of antibacterial activity as a steep decline in the CFU number of the test strain. The antibacterial effects lasted for 24 hours.

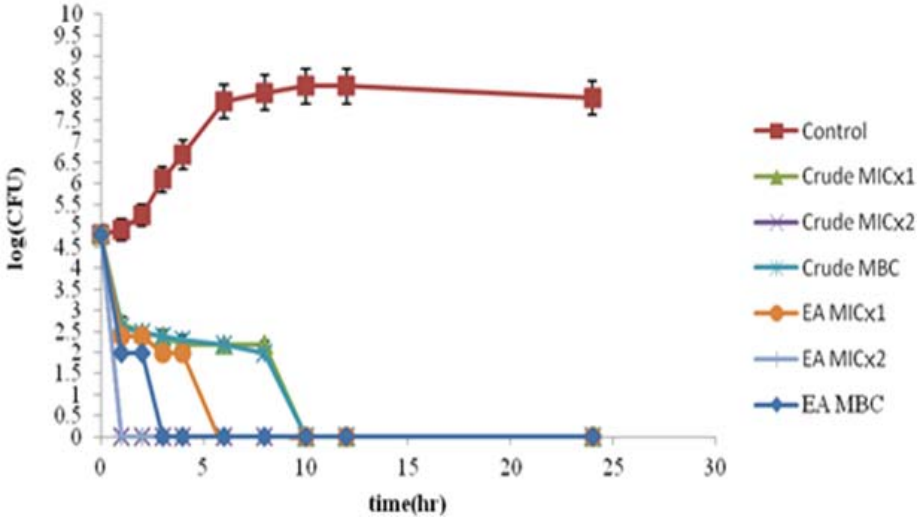

Fig 2: Effect of the extracts from *Sophora flavescens* on growth of the test strain oxacillin-resistant *S. aurens* 287. The curves represent viable cell counts of the test microorganism.

3.5. Antioxidant activities of the extracts from *Sophora flavescens*

The various extracts of *Sophora flavescens* were assayed for antioxidant activity using DPPH radical scavenging capacity, trolox equivalent antioxidant capacity (TEAC), and reducing power test. In addition, the contents of total phenolics and flavonoids were also determined. As shown in Fig. 3, the DPPH free radical scavenging capacity was increased with the concentration of extracts increased. Compared to the IC_{50} values of various extracts from *Sophora flavescens* (Table III),

the extracts of ethyl acetate (IC_{50} 178 ppm) showed the highest free radical scavenging ability than the crude extracts (IC_{50} 484 ppm) and water extracts (IC_{50} 927 ppm). The ethyl acetate extracts and crude extracts of *Sophora flavescens* revealed similar TEAC value of 78 g trolox / 100 g DW, followed by the water extracts of 43 g trolox / 100 g DW. Regarding to the reducing power assay, the extracts of ethyl acetate (0.7 abs/10^{-3}ppm, respectively) revealed a substantial higher reducing power than the crude and water extracts (0.2 and 0.1 abs/10^{-3}ppm, respectively) as shown in Table III. However, Compared to the antioxidants, Vitamin C and BHT (18.3 and 2.9 abs/10^{-3}ppm, respectively) revealed a highest reducing power among all of the test samples (Fig. 4); the various extracts of *Sophora flavescens* did not showed significant reducing power. The combined results from three different assays of antioxidant activity, the extracts of ethyl acetate possess the most significant antioxidant activity among the various herbal extracts. Polyphenolic and flavonoid compounds commonly found in plants, mainly of plant secondary metabolites are often related to plant antioxidant. Comparing the results shown in Table III, the highest concentration of phenolic content was found in the extract of ethyl acetate (36.49 g Gallic acid / 100 g DW), followed by the crude extracts (28.62 g Gallic acid / 100 g DW), and then the water extracts (20.72 g Gallic acid / 100 g DW). Same results were found in the total flavonoid content determination; the highest concentration of total flavonoids was found in the extract of ethyl acetate (4.68 g Quercetin / 100 g DW), followed by crude extracts (1.89 g Quercetin / 100 g DW), and then the water extracts (0.2 g Quercetin / 100 g DW). The phenolic and flavonoid contents showed a positive correlation with their antioxidant and antibacterial activities.

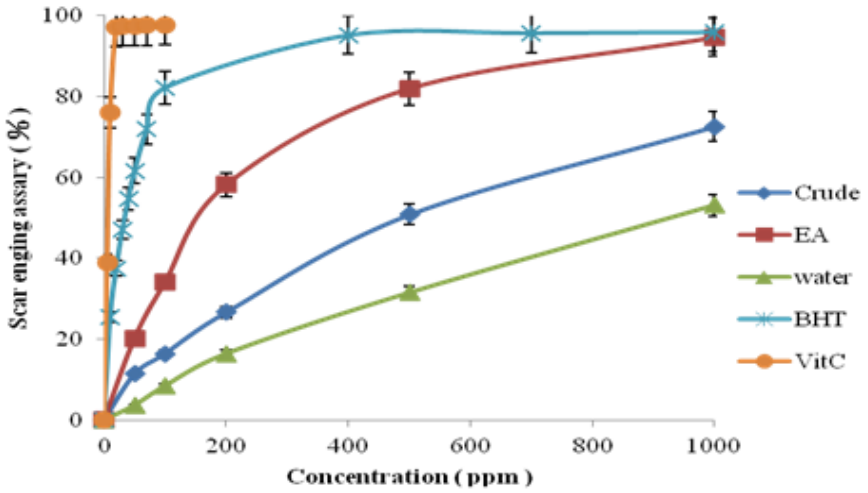

Fig 3: Free radical scavenging ability of various extracts from *Sophora flavescens* by DPPH assay.

Table 3: Antioxidant activity assay of the extracts from *Sophora flavescens*

Extracts	DPPH IC_{50} (ppm)	TPC (g Gallic acid /100 g DW)	Flavonoid (g Quercetin / 100 g DW)	TEAC (mmol Trolox / 100 g DW)	Reducing Power (abs / 10^{-3} ppm)
Crude	484.84 ± 16.54	28.62 ± 0.07	1.89 ± 0.20	78.05 ± 0.04	0.2
Ethyl acetate	178.50 ± 30.70	36.49 ± 0.09	4.68 ± 0.50	78.22 ± 0.00	0.7
Water	927.16 ± 38.76	20.72 ± 0.10	0.2 ± 0.03	42.72 ± 0.04	0.1
BHT	33.87 ± 1.89	-	-	-	2.9
Vit C	6.48 ± 2.54	-	-	-	18.3
Mean ± SD -: no detected					

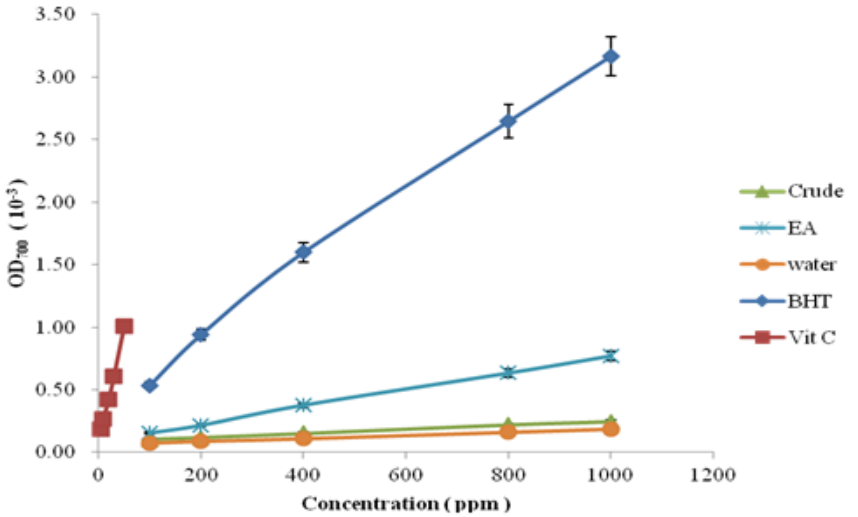

Fig 4: Reducing power determination of various extracts from *Sophora flavescens*.

4. Concussion

The use of herbal extracts as antimicrobial and antioxidant agents has two distinct advantages: the natural origin and the associated low risk. This means that they are less side effect for people and the environment, and that resistant pathogenic microorganism is less frequently developed. The present work shows the extracts of ethyl acetate from *Sophora flavescens* provide antimicrobial activities against oxacillin-resistant *S. aureus* strain. The antioxidant activity analyses have shown that the extracts of ethyl acetate have a good radical scavenging capacity. In addition, the experiment results revealed the ethyl acetate extracts of *Sophora flavescens* with a higher content of phenolic and flavonoid compounds, and have a better antioxidant capacity. Comprehensive experimental results showed that the extracts of *Sophora flavescens* present good anti-oxidation and anti-bacterial role, do have the opportunity to become a new generation of pharmaceutical antioxidants and antibiotics. In conclusion, the results of this study suggest the possibility of developing the extracts of *Sophora flavescens* as natural antioxidants and antimicrobials for the treatment of antibiotic resistant pathogens.

5. Acknowledgment

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