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Abstract

The present study was carried out to identify antibacterial and anti-oxidant characteristics of traditional aqueous extracts derived from three traditional Chinese medicinal plants (*Scutellaria baicalensis*, *Coptis chinensis* and *Sonchus oleraceus*). It was indicated that the *S. oleraceus* showed the highest antibacterial efficacy, especially against *Staphylococcus aureus*. The minimum inhibitory concentration (MIC) of the *S. oleraceus* was 5.0 mg/mL what was in correlation with the high total phenolic and flavonoid contents and CUPRAC value, and MIC of both *S. baicalensis* and *C. chinensis* was 7.5 mg/mL. The rational pH of the working *S. oleraceus* was acidic, while the other two preferred to neutral or alkaline environment. The reasonable preservation temperature of *S. baicalensis* should not beyond 60°C, while the other two below 90°C. Meanwhile, *S. baicalensis* had significant anti-oxidant activity with the highest CUPRAC and ·OH scavenging activity. These results had provided useful information on further drug discovery.

Introduction

Currently, naturally derived products play an important role as a source of medicine (Rubio-Moraga et al., 2013). Plants are the sound source of the natural product in a most efficient way, their extracts have attracted a lot of interest due to large contributions to some important applications. Chinese traditional medicinal plants are important parts of Traditional Chinese Medicine (TCM), which are antibacterial and anti-oxidant and may play a significant role in the prevention and treatment of many diseases, and are considerably used for thousands of years not only in China, but all over the world (Liu et al., 2012; Sun et al., 2014; Zhang and Su, 2014). Despite the widespread use, there are many confusing aspects related to TCM, such as incorrect doses had led to the evolution of drug-resistance, which had limited their anthelmintic efficacy, e.g. Buchmann et al., (1992) revealed that extended exposure to subtherapeutic doses of mebendazole resulted in drug resistant parasite populations.

Based on traditional claims for the treatment of various disorders, the present study selected 3 traditional Chinese medicinal plants *Scutellaria baicalensis* Georgi, *Coptis chinensis* Franch and *Sonchus oleraceus*, which are widely distributed in the in northern China to evaluate the *in vitro* antibacterial activities and their optimal service conditions in a systematic and consistent manner. Furthermore, the study also aimed to determine their anti-oxidant contents, and measure their anti-oxidant activities. The results can provide scientific evidence supporting clinical application of these plants and serve as a starting point for new drug discovery from TCM.

Materials and Methods

Collection of Chinese medicinal plants

Three dried Chinese medicinal plant materials namely *S. baicalensis*, *C. chinensis* and *S. oleraceus* were purchased from Beijing Tong Ren Tang Chinese herbal



medicine shop, Beijing, China. These three medicinal plants were harvested and processed and naturally dried according to traditional procedures.

Preparation of aqueous extracts

The collected samples were cleaned, freeze-dried in a freeze dry system (Christ 1-4), and ground into a fine powder by a Kenwood Multi-Mill (Kenwood, Havant, UK). Dried plant samples were further air-dried in a ventilated oven at 40°C for 24 hours, then ground into a fine powder and passed through a sieve (24-mesh). Powdered sample (5 g) was extracted with 100 mL ultra-filtered water at 100°C for 30 min in a water bath shaker (Shaking Bath 5B-16) (Techne, Ltd., UK). The extract was filtered by a Millipore filter with a 0.45 µm nylon membrane under vacuum at 23°C, the filter residue was added the 50 mL water to operate heating circumfluence by Soxhlet's extraction equipment at 90°C for 2 times and 30 min per time, then the extract was filtered as the above method. All the filtrates were mixed and concentrated under reduced pressure in a vacuum rotary evaporator (R-201, Shanghai Shenshen, China) until all the solvents had almost evaporated. These extracts were eluted several times by PBS solution, and then adjusted the final volumes to 10 mL (the concentrations was 0.5 g/mL) in 10 mL volumetric flasks. The samples were stored at 4°C until use. Eight different concentrations (2.5, 5, 7.5, 10, 20, 40, 120 and 200 mg/mL) of each plant extract was prepared for screening the minimal inhibitory concentration (MIC) and antibacterial activities.

Bacteria and culture

Two bacteria were kindly provided by China general microbiological culture collection center, they are Gram-positive *Staphylococcus aureus* (*S. aureus*, ATCC25922) and Gram-negative *Escherichia coli* (*E. coli*, ATCC 25322). The strains were cultured at 37°C on plate count agar (PCA) medium (Sun et al., 2014).

Antibacterial assays

This assay was carried out on two bacterial cultures using an agar-well diffusion method (Sun et al., 2014), the reference bacterial strains used in this experiment were *S. aureus* and *E. coli*. Both bacterium were suspended in sterile water and diluted to $\sim 10^6$ CFU/mL. The suspension (100 µL) was spread onto the surface of PCA medium. Wells (4.6 mm in diameter) were cut from the agar with a sterile borer and 60 µL prepared medicinal plants extracted solutions above were delivered to them. Negative controls were prepared using PBS solution. The inoculated plates were incubated at 35°C for 24 hours. Antibacterial activity was evaluated by measuring the diameter of inhibition zone (DIZ) of the tested bacteria. DIZ was expressed in millimeters. All tests were performed in triplicate.

For determining the effect of different pH value to anti-

bacterial activity, the extracted samples (the selected concentration above) was adjusted pH value to 3, 5, 7, 9 and 11 using HCl or NaOH to keep 1 hour and then adjusted back to the primary value to test antibacterial activity, respectively.

For determination the effect of different temperature of to antibacterial activity, the extracted samples (the selected concentration above) were kept 1 hour under 30°C, 60°C, 90°C and 100°C on dry bath incubator and then cooled back to the room temperature to test antibacterial activity, respectively.

Anti-oxidant analyses

The anti-oxidant contents were determined using spectrophotometric methods. The total phenolic contents (TPCs) of three extracts were determined by Folin-Ciocalteu reagent method according to Terpinč Čeh et al. (2012) with some modifications. Exactly 0.1 mL of each appropriate dilutions of the filtered extracts (approximately 0.2 mg/mL) or gallic acid (10–100 mg/mL, as standard) was mixed with 0.5 mL of 10% Folin-Ciocalteu reagent, 0.4 mL of 7.5% sodium carbonate was added after 5 min. These mixtures were then incubated at 25°C for 40 min. The absorbance was recorded at 765 nm using a Shimadzu UV-1800 spectrometer. The TPC was obtained from a regression equation ($R^2 = 0.9996$), and expressed as mg gallic acid equivalents (GAE) per g dried extract.

The total flavonoid contents (TFCs) of three extracts were determined colorimetrically as described previously (Jia et al., 1999) with some modifications. Exactly 0.5 mL of the extracts was added to a tube containing 1.5 mL of distilled water. Then, 0.09 mL of 5% NaNO₂ solution was added to the mixture, and 0.18 mL of 10% AlCl₃·6H₂O solution was added after 6 min later. After 6 min, 0.7 mL of 4% NaOH solution was added, distilled water was added to bring the total volume to 3 mL. The absorbance was determined at 510 nm after 10 min. The total flavonoid content was determined by a rutin standard curve and expressed as mg rutin equivalents (RE) per g dried extract.

Anti-oxidant activities determination

The cupric reducing anti-oxidant capacity (CUPRAC) assay, a method for determination of total anti-oxidant capacity, was applied with some modifications (Celik et al., 2010). Sample mixture was performed by mixing 1 mL CuCl₂ (10 mM), 1 mL neocuproine solution (7.5 mM in 96% ethanol (v:v) and 1 mL NH₄Ac buffer (1.0 M, pH

= 7.0) in a test tube. Volumes of (x) mL extracted

sample (or standard) solution and ($1-x$) mL H₂O were added to the initial mixture so as to make the final volume 4.1 mL. The sample mixture was standing at room temperature for 30 min, and then the absorbance

of the final solution was read at 450 nm. A standard curve was prepared with gallic acid stock solution (1 mg/mL). Final results were given as mg gallic acid equivalents (GAE) per g dried extract.

Hydroxyl (OH) radical scavenging activity was determined according to the method of Smirnoff and Cumbes (1989) with some modifications. Briefly, 0.1 mL of samples (2–10 mg/mL) was mixed with 0.5 mL FeSO₄ (1.5 mM) and 0.15 mL sodium salicylate (20 mM), then added 0.25 mL H₂O₂ (6 mM) to start the reaction. The mixture was incubated at 37°C for 1 hour. The absorbance of the hydroxylated salicylate complex was recorded at 562 nm. The OH radical scavenging activity was calculated using the following equation:

$$\text{OH scavenging effect (\%)} = \frac{1 - (A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \times 100$$

where A_{sample}, A_{control} and A_{blank} was the absorbance of the sample test, the solvent control, and the reagent blank without sodium salicylate, respectively.

Statistical analysis

All measurements were replicated three times. Statistical analyses were performed with Data Processing System statistical software package using ANOVA followed by Duncan's multiple range tests to evaluate significant differences between different group means, different letter means at a significance level of p = 0.05.

Results

The range of concentrations tested was 0 to 200 mg/mL for three medical plants. There was a significant variation in the antibacterial activities (DIZ values) to the corresponding bacteria between *S. oleraceus* and other two medical plants extracts (Table I). For Gram-negative *E. coli*, the DIZ values of *S. oleraceus* ranged from 7.3 mm to 8.9 mm under the concentration of 5.0 mg/mL and 10.0 mg/mL, respectively. Under the concentration of 2.5 mg/mL, there was no obvious inhibition. However, under the concentration higher than 10 mg/mL, the *E. coli* was completely inhibited. So, the MIC of *S. oleraceus* for *E. coli* was 5.0 mg/mL, the corresponding minimum DIZ was 7.3 mm. Moreover, the MICs of *S. baicalensis* and *C. chinensis* extracts for *E. coli* were 7.5 mg/mL, and the DIZ values of *S. baicalensis* and *C. chinensis* were between 7.4 mm and 9.3 mm respectively.

For *S. aureus*, the antibacterial features of these three extracts were similar with those to *E. coli*, the MIC of *S. baicalensis*, *C. chinensis* and *S. oleraceus* was 7.5, 7.5 and 5.0 mg/mL, with the minimum DIZ 8.2 mm, 7.6 mm and 7.9 mm respectively. Just under the same concentration, the DIZ values to *S. aureus* were larger than to *E. coli*, indicating the antibacterial activities of

these three extracts to *S. aureus* were stronger than to *E. coli*.

The effect of different medical plant extract pH value on antibacterial activity was determined (Figure 1). The DIZ values of three extracts against bacteria were first increased and then decreased with pH values from 3 to 11 and the DIZ values of all extracts ranging from 6.3 mm to 12.8 mm, except for *S. baicalensis* against *S. aureus* which DIZ values decreased with increasing pH value, indicating the pH value of the working *S. baicalensis* against *S. aureus* should be around 3, while for *E. coli* it should be 5. The maximum DIZ value of *C. chinensis* against *E. coli* and *S. aureus* was 10 mm and 12 mm, which was under the pH value 9 and 7 respectively, indicating the pH value of the working *C. chinensis* against *E. coli* and *S. aureus* should be around 9 and 7. Moreover, the optimal pH value of *S. oleraceus* against both bacteria was approximately 7.

The effect of different medical plant extract temperature on antibacterial activity was determined (Figure 2). The DIZ values of these three medical plants extracts against *E. coli* increased along with the increasing treatment temperature, the turning point was around 90°C, and below 80°C the DIZ values increased

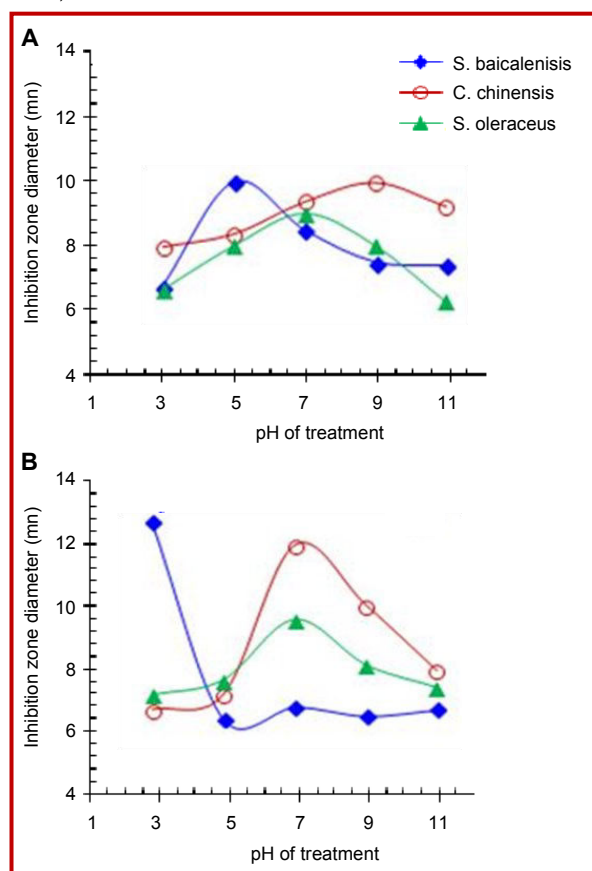


Figure 1: Antibacterial activity of three Chinese medicinal plants after different pH values treatment against (a) *Escherichia coli* and (b, below) *Staphylococcus aureus*, respectively

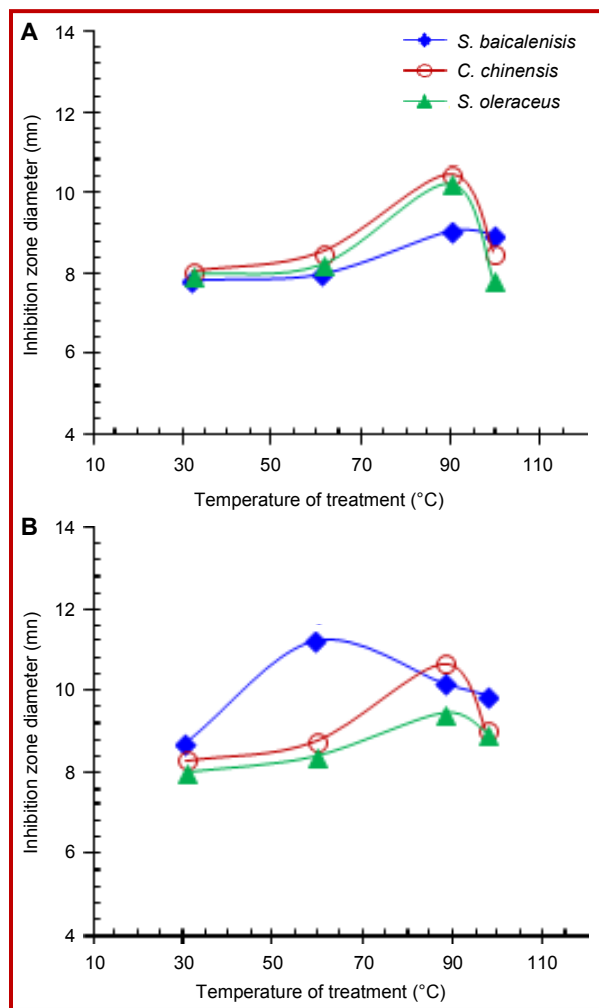


Figure 2: Antibacterial activity of three Chinese medicinal plants after different temperature treatment against (a) *Escherichia coli* and (b, below) *Staphylococcus aureus*, respectively

gradually with the value about 8 mm, but decreased dramatically beyond 90°C. And the *C. chinensis* and *S.*

oleraceus against *S. aureus* as well, with the optimal treatment temperature 90°C. However, the changing trend of DIZ values of *S. baicalensis* against *S. aureus* was increased and then decreased with the increasing treatment temperature, and the maximum DIZ value was 11.4 mm under the treatment temperature 60°C.

The comparative evaluation of the anti-oxidant compounds of the three Chinese medicinal plants extracts was based on the contents of total phenolic content (TPC) and total flavonoid (TFC) (Table II).

A significant difference in TPC and TFC content was observed among three plant extracts. The highest phenolic content was 185.3 mg GAE/g dried extract which was found in the plants of *S. oleraceus*, followed by *S. baicalensis*. The TPC content of *C. chinensis* was significantly lower than that of the other two plants. The TFCs of the evaluated extract were presented in Table II, the results indicated a significant difference among three species. For instance, the highest TFC was found in the extract of *S. oleraceus* also with 145.2 mg RE/g dried extract, followed by *C. chinensis*, while the *S. baicalensis* was the lowest with the value of 33.1 mg RE/g dried extract. While all the ratio of TPC in relation to TFC was greater than one, indicating the TPC was higher than TFC content, in the order of *S. baicalensis* > *S. oleraceus* > *C. chinensis* extract, and the *S. baicalensis* was obviously higher than the other two.

The comparative evaluation of the hydrophilic anti-oxidant properties of the three Chinese medicinal plants extracts was based on the CUPRAC value and hydroxyl ($\cdot\text{OH}$) radical scavenging activity (Table II).

In terms of the CUPRAC assay, the total anti-oxidant capacity of three different Chinese medical plants was shown in Table I. The greatest quantity of CUPRAC value was found in *S. baicalensis* (82.4 mg GAE/g dried extract), followed by *S. oleraceus* (76.5 mg GAE/g dried extract). The lowest amount of CUPRAC was determined in *C. chinensis* (42.7 mg GAE/g dried extract).

Table I

Antibacterial activity of three Chinese medicinal plants

Concentration (mg/mL)	Diameter of inhibition zone (DIZ) (mm) ^a					
	<i>Escherichia coli</i>			<i>Staphylococcus aureus</i>		
	<i>S. baicalensis</i>	<i>C. chinensis</i>	<i>S. oleraceus</i>	<i>S. baicalensis</i>	<i>C. chinensis</i>	<i>S. oleraceus</i>
2.5	-	-	-	-	-	-
5.0	-	-	7.3 ± 0.4	-	-	7.9 ± 0.4
7.5	7.4 ± 0.5	7.1 ± 0.8	8.4 ± 0.7	8.2 ± 0.6	7.6 ± 0.5	9.1 ± 0.8
10	7.9 ± 0.6	7.4 ± 0.6	8.9 ± 0.7	9.3 ± 0.6	8.0 ± 0.6	10.7 ± 0.6
20	9.3 ± 0.4	9.6 ± 0.3	+	12.1 ± 0.5	9.4 ± 0.6	+
40	+	+	+	+	+	+
120	+	+	+	+	+	+
200	+	+	+	+	+	+

^aAverage of three replicates ± SD; + indicates complete inhibition and - indicates no inhibition

Table I

Anti-oxidant contents and anti-oxidant properties of three Chinese medicinal plants

Plant species	TPC (mg GAE/g)	TFC (mg RE/g)	CUPRAC (mg GAE/g)	OH scavenging activity IC ₅₀ value (mg/mL)
<i>Scutellaria baicalensis</i>	176.5 ± 7.4 ^{ab}	33.1 ± 2.0 ^c	82.4 ± 3.1 ^a	0.2 ± 0.0 ^a
<i>Coptis chinensis</i>	85.6 ± 3.3 ^b	75.0 ± 4.0 ^b	42.7 ± 1.3 ^b	0.1 ± 0.0 ^b
<i>Sonchus oleraceus</i>	185.3 ± 10.4 ^a	145.2 ± 5.3 ^a	76.5 ± 4.3 ^{ab}	0.1 ± 0.0 ^b

Data represent means ± SD of three independent replications (n=3); abc, different letters indicate significant differences (p<0.05) among the 3 different plants and refer to each subset of data. TPC, TFC, GAE, RE, CUPRAC and IC₅₀ correspond to total phenolic content, total flavonoid content, gallic acid equivalent, rutin equivalent, cupric reducing anti-oxidant capacity and half maximal (50%) inhibitory concentration, respectively

The result of hydroxyl free radical-scavenging ability of the three medical plants was shown in Table II. For each plant, the IC₅₀ showing the concentration that has the ability to scavenge 50% of hydroxyl radical, which was evaluated. A lower IC₅₀ value corresponds to a larger scavenging activity. The IC₅₀ value of *S. oleraceus* and *C. chinensis* for eliminating hydroxyl radicals was 0.1 and 0.1 mg/mL, respectively, while the IC₅₀ value of *S. baicalensis* was 0.2 mg/mL, which indicated that the scavenging activity of *S. baicalensis* against hydroxyl radical was significantly lower than that of *S. oleraceus* and *C. chinensis*.

Discussion

In this study, we firstly evaluated the anti-oxidant contents, anti-oxidant properties and antibacterial activities of three traditional Chinese medicinal extracts (*S. baicalensis*, *C. chinensis* and *S. oleraceus*) from China using water as solvent solution, and also observed the effect of different pH value and temperature treatment on the antibacterial activity. The results suggested a practical potential for these three Chinese medicinal extracts application as an intervention strategy in mitigating micro-organisms infection to human beings, which was crucial for its rational usage in treating disease safely and excavating related new drugs.

Based on previous published studies to date, the most common methods of initial screening of various plant species for potential antimicrobial or anti-oxidant activities have been started with using crude alcohol extraction methods, such as ethanol or methanol extraction (Zhang et al., 2013; Irshaid et al., 2014; Khaled-Khodja et al., 2014). However, here in this study water extraction was used by reason of in the folk people usually decocts the medicinal herbs using water, also as the water extract showed the strongest antibacterial activity and with high content of total phenols (Wong et al., 2006; Ličina et al., 2013). Medicinal plants are antibacterial materials to bacteria, which have been used as sources of medicine in virtually all cultures. Although there are many reports of anti-oxidant capacity and antibacterial activity in TCM preparations, few systematic surveys exist. Both

of the three extracts tested in this study exhibited high antibacterial activity against both of the tested bacteria, and there was a highly positive relationship between the concentration of extract and antibacterial activity. To some extent, these results were similar to our previous study (Sun et al., 2014). Moreover, the antibacterial activities of three extracts under the same concentration against *S. aureus* were stronger than against *E. coli*, expressed as the DIZ values to *S. aureus* were larger than to *E. coli*. Our results suggested that Gram-positive bacteria were generally more sensitive to the herb extracts than Gram-negative bacteria, this was consistent with the previous studies on other spices and herbs (Ceylan and Fung, 2004; Lopez et al., 2005; Sun et al. 2014). A possible explanation for these observations may lie in the significant differences in the outer layers of Gram-negative and Gram-positive bacteria, Gram-negative bacteria possess an outer membrane and a unique periplasmic space not found in Gram-positive bacteria (Duffy and Power, 2001).

Further, this study has shown a link between the concentration of extracts and their antibacterial activity, and the present result indicated that the MIC values of *S. baicalensis*, *C. chinensis* and *S. oleraceus* to *E. coli* was 7.5, 7.5 and 5.0 mg/mL respectively, and those to *S. aureus* was similar with *E. coli*. Among three medicinal plants extracts, in general under the same concentration the *C. chinensis* displayed the lowest antibacterial activity to both bacteria, with the smaller DIZ values than the other two extracts. Importantly, a positive relationship between total phenol contents and anti-oxidant capacity and antibacterial activity was observed in these investigated plant extracts, similar to previous reports (Yildirim et al., 2001; Katalinic et al., 2006; Taguri et al., 2006). Moreover, the pH value of the working *S. baicalensis* extract against *E. coli* and *S. aureus* should be around 5 and 3, respectively, indicating this extract was more active under acid environment, while the other two extracts preferred to the neutral or alkaline environment. Antibacterial activity of three Chinese medicinal plants after different temperature treatment against two tested bacteria was shown in Figure 2, if the treated temperature beyond 90°C, all the antibacterial activity of extracts to both bacteria will be rapidly decreased except for *S. baicalensis* extract against

S. aureus which inflection point was 60°C. These results may provide a certain basis on the preservation and use of these three Chinese medicinal plants for us to some extent.

On the other side, phenolic compounds are widely distributed in various plant species (Li et al., 2006), they have received considerable attention. Phenolic anti-oxidants provided tremendous potential benefits because of their ability to scavenge active oxygen species and free radicals such as hydroxyl radicals as well as their ability to disrupt the free-radical chain reaction of lipid peroxidation (Bakirel et al., 2008). Moreover, antibacterial activity of polyphenols against both Gram negative and Gram positive bacteria have been reported in various plant species (Taguri et al., 2006; Padam et al., 2012). TPC was determined by Folin Ciocalteu method and the result was expressed in terms of mg GAE/g dried extracts. All the three extracts showed containing high contents of phenolic. This result demonstrated that these three medicinal plants rich in phenolic compounds. Many researches have stated clearly that phenolic has strong anti-oxidant activity (Fu et al., 2014). So, these three medicinal plants may be served as a new source of natural anti-oxidants. However, there was a significant difference in TPC among three species, *S. oleraceus* provided the highest value of TPC and *C. chinensis* showed the lowest among samples studied. Flavonoids are the most common and widely distributed group of plant phenolic compounds, which is ubiquitous in fruits and vegetables (Abu Baker et al., 2009). Total flavonoid was determined in the sample extracts by reaction with sodium nitrite, followed by the development of colored flavonoid-aluminium complex formation using aluminium chloride which can be monitored spectrophotometrically at 510 nm, and which was expressed in terms of mg RE/g dried extracts. *S. oleraceus* provided the highest value of TFC also, while *S. baicalensis* contained the lowest amount of TFC. By the way, *C. chinensis* showed the highest ratio of TFC in relation to TPC as compared to the other two samples.

However, it is difficult to separate and quantify individual anti-oxidants (i.e., parent compounds, glycosides, polymers and many isomers) due to their chemical diversity. Moreover, the total anti-oxidant power is often more meaningful to evaluate health beneficial effects because of the cooperative action of anti-oxidants. Therefore, it is desirable to establish a method that can measure the total anti-oxidant capacity level directly from plant extracts. CUPRAC method is a versatile, simple and low-cost anti-oxidant capacity assay for dietary polyphenols, vitamins C and E (Apak et al., 2008; Celik et al., 2010). In present work, *S. baicalensis* and *S. oleraceus* provided the higher CUPRAC value than *C. chinensis*, this trend was very similar with the result of TPC, while the TFC result of *S. baicalensis* was inconformity. It was coincident with the

previous report, CUPRAC and total polyphenols measurement results in the extracts of kiwifruit correlated very well ($r^2=0.81$), better than with other total anti-oxidant capacity assays (such as ABTS/TEAC). And the CUPRAC assay was probably the most consistent method of total anti-oxidant measurement in relation to Folin reagent-responsive TPC. The low correlation observed between CUPRAC results and flavonoids content was due to the nature of measurement technique. The $AlCl_3$ test for flavonoids does not measure those flavonoids that do not bear the characteristic chelating functional groups for Al binding (Park et al., 2006; Apak et al., 2008).

Anti-oxidants may play their protective role by different processes, therefore, it is pertinent to use several assays instead of only one to determine the anti-oxidant activity (Petlevski et al., 2013). In the present study, the *in vitro* anti-oxidant activities of three extracts were also determined using OH radicals scavenging assays. Among all the reactive oxygen species, hydroxyl radical is the most harmful free radicals, and mainly responsible for oxidative injury to biomolecules. The potential anti-oxidant activities and hydroxyl radical scavenging abilities have potentially beneficial implications in human health (Li et al., 2006; Fu et al., 2014). OH radical scavenging activities of three extracts were shown the activity of extract was compared using IC_{50} value, a smaller value corresponding to a higher anti-oxidant activity. *S. baicalensis* showed a significantly highest IC_{50} value than *C. chinensis* and *S. oleraceus*, they exhibited IC_{50} as 0.19, 0.11 and 0.12 mg/mL respectively, and no significantly difference was detected between *C. chinensis* and *S. oleraceus*. Results of above three radical scavenging assays demonstrated that extracts of these three plants exhibited relatively strong radical scavenging activities, and can serve as an effective radical scavenger.

In conclusion, the results from this study showed that aqueous extracts of three traditional Chinese medicinal plants possessed excellent anti-oxidant and antibacterial potential. Overall, the *S. oleraceus* extract showed the strongest antibacterial activity with the MIC value 5.0 mg/mL what is in correlation with high TPC, TFC and CUPRAC value, and the most rational pH value of the working antibiotics against bacteria should be around 7, the reasonable preservation temperature should below 90°C. On the other hand, *S. baicalensis* extract had significant anti-oxidant activity with the highest CUPRAC and OH scavenging activity.

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