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Evaluation of the Antioxidant and Antibacterial Activities of Various Solvent Extracts from *Passiflora wilsonii* Hemsl.

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ABSTRACT

This study describes the antioxidant, antibacterial activities of five different solvents [methanol, aqueous ethanol (ethanol-water, 70:30, v/v), acetone, ethyl acetate and water extracts of *Passiflora wilsonii* Hemsl.]. The results obtained in this study have considerable value with respect to various solvent extracts from *Passiflora wilsonii* Hemsl., showed varying degrees of antioxidant activity in different test systems in a dose-dependent manner. All solvent extracts of *Passiflora wilsonii* Hemsl. exhibited scavenging activities toward 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), superoxide anion and hydroxyl radicals. On the other hand, the ethanol fraction showed the highest antioxidant and antibacterial activities in most instances. This study verified that the ethanol extract have strong antioxidant and antibacterial activities which were correlated with its high level of flavonoids.

Key words: *Passiflora wilsonii* Hemsl., solvent extracts, antioxidant activities, antibacterial activities

INTRODUCTION

China is an immense country in relation to traditional medicinal herbs. There is also a long history related to the study and use of medicinal plants. Traditional Chinese medical science is a precious cultural heritage for China and the world. Because of the vast expanse of land and complicated natural environment, there have rich resources and a great variety of medicinal plants (Li *et al.*, 2006; Lee *et al.*, 2008).

Passiflora wilsonii Hemsl., a traditional folk medicine, has been used in the minority of Yunnan province. It was born in the bush elevation of 1700-2500 m, distributed in Yunnan and Tibet. In the past several years, according to scientific reports have described the properties of *Passiflora wilsonii* Hemsl. Li *et al.* (2004, 2007) reported the isolation of seven known triterpenoids and steroids compounds from this plant, their structures were determined on the basis of chemical and spectral data evidences as 4-oxo-3, 4-seco-A(1)-norfriedelan-2-oic acid, 2-hydroxyl-3, 4-seco-friedelan-3-oic acid ethyl-ester, sitost-4-en-3-one, 3-(2, 4-dihydroxyphenyl)-2-propenoic

acid, glycerol 1-octadecanoate, 1-octacosanol, 24R-ethyl-5 α -cholestane-3 β and 6 α -diol. A new phytosterone compound 24R-ethyl-5 α -cholestane-3 β and 6 α -diol-23-one also was reported. This is the first report on the isolation of these compounds from *Passiflora wilsonii* Hemsl. Yu *et al.* (2003) also reported that six compounds were isolated from *Passiflora wilsonii* Hemsl. including glut-5-en-3 β -ol, maslinic acid, pachysandiol, friedelan, ergosterol epidioxide and β -sitosterol. Besides, no more reports have been conducted to the pharmacological activities of *Passiflora wilsonii* Hemsl.

In recent years, a huge number of medicinal plants have attracted a great deal of scientific and public interest with regard to their potential uses in folk medicine and as sources of natural antioxidant and antibacterial agents (Rawat *et al.*, 2011). Consequently, the antioxidant and antibacterial activities of plant extracts have formed the basis of many applications (Yesil-Celiktas *et al.*, 2007; Aneja *et al.*, 2011).

However, very little information exists currently in scientific literature on the antioxidant and antibacterial activities of *Passiflora wilsonii* Hemsl. extracts. The present

study was conducted to evaluate the effects of various extracting solvents on the antioxidant and antibacterial activities of *Passiflora wilsonii* Hemsl. extracts by employing various *in vitro* test models.

MATERIALS AND METHODS

Plant materials and chemicals: The sample of *Passiflora wilsonii* Hemsl. was collected from the fully mature plants grown in Yunnan Province in 2012, identified by Dr. Xuelian Wei (Weishi Chinese herbal medicine Tang, Yizhou). 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Ruibio, Germany), Tris (hydroxymethyl) aminomethane (Tris) (Amresco, USA), 1,1-Diphenyl-1-picrylhydrazyl (DPPH) (TCI, Japan). All the other solvents and chemicals used were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd (SCRC) (China) and Xiya Reagent (China) used without further purification.

Preparation of extracts: *Passiflora wilsonii* Hemsl. was dried under shade and ground to a fine powder (40 mesh) in a grinding mill (High speed grinder, Zhongnan Pharmaceutical Machinery Factory, Changsha) and the powdered drug material (50 g) was separately extracted with 750 mL of five different solvents [methanol, aqueous ethanol (ethanol-water, 70:30, v/v), acetone, ethyl acetate and water] for 2 h at 60°C. The fraction after filtration was dried under reduced pressure at 50°C to get the crude dried fraction. Concentrated extracts were frozen overnight and freeze-dried with vacuum, the freeze-dried extracts were stored in refrigerator at -18°C until used for the evaluation of the antioxidant and antibacterial activities.

Determination of total flavonoids: The amount of total flavonoids in the extracts was measured spectrophotometrically following the method previously described by Ozyurek *et al.* (2014). The 0.5 mL extract was mixed with 2 mL of distilled water and subsequently with 0.15 mL of 5% NaNO₂ solution. After 5 min, 0.6 mL of 10% AlCl₃ was added and allowed to stand for 6 min, 2 mL of 4% NaOH was added to the mixture and the volume was made up to 5 mL using distilled water and then mixed thoroughly and allowed to stand for another 15 min. Absorbance was read at 510 nm against the water blank and flavonoid content was expressed as mg rutin equivalent.

Evaluation of antioxidant activity

Reducing power: The ability of *Passiflora wilsonii* Hemsl. extracts to reduce Fe³⁺ was determined using the method described by Tian *et al.* (2009).

DPPH radical scavenging activity: 1,1-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was evaluated by measuring the scavenging activity of the

Passiflora wilsonii Hemsl. extracts on the stable free radical DPPH as a reagent according to the method described by Tukappa and Londonkar (2013).

$$\text{DPPH radical scavenging activity (\%)} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100$$

where, A_{sample} was the absorbance of the mixture of the test sample and DPPH reagent after reaction; A_{blank} was the absorbance the mixture of methanol and DPPH reagent after reaction. Ascorbic acid was used as a standard for this study.

ABTS radical scavenging activity: Determination of the 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity was based on the procedure described by Heo *et al.* (2014) with some modifications. The solution consisting of 7 mmol of ABTS and 2.45 mmol potassium persulfate (1:0.5 v/v) was left to stand for 12-16 h in the dark at room temperature. Absorbance of the reactant was later adjusted to 0.700±0.020 with methanol at a wave length of 734 nm. The 4.0 mL of the diluted solution was mixed with 1.0 mL of the various concentrations (20-200 µg mL⁻¹) of *Passiflora wilsonii* Hemsl. extracts, or methanol as a blank. After a 7 min reaction, the absorbance was measured at 734 nm. The scavenging activity of ABTS free radical was calculated by the equation below and expressed as the percentage of inhibition rate of free radical scavenging compared with the blank:

$$\text{ABTS radical scavenging activity (\%)} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100$$

where, A_{sample} was the absorbance of the mixture of the test sample and ABTS reagent after reaction; A_{blank} was the absorbance the mixture of methanol and ABTS reagent after reaction. Ascorbic acid was used as a standard for this study.

Hydroxyl radical scavenging activity: The hydroxyl radical assay was determined according to the method described by Chien *et al.* (2013) with some modifications. A 1.0 mL sample extracts solution (20-200 µg mL⁻¹) was mixed with 1.0 mL of 5 mmol L⁻¹ salicylic acid solution (dissolved in absolute ethanol), 1.0 mL of 5 mmol L⁻¹ ferrous sulphate solution and 2.0 mL of distilled water. Subsequently, 1.0 mL of 5 mmol L⁻¹ H₂O₂ was added to start the Fenton reaction at 37°C for 30 min, or deionized water as a blank. The absorbance of the mixture was measured at 510 nm. The hydroxyl radical scavenging effect was calculated with the following equation:

$$\text{Hydroxy radical scavenging activity (\%)} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100$$

where, A_{sample} was the absorbance of the mixture of the test sample and reaction solution; A_{blank} was the absorbance of the

blank reaction in which the sample was replaced by deionized water. Ascorbic acid was used as a standard for this study.

Superoxide radical scavenging activity: The scavenging ability of superoxide radical was measured by the method described by Kannadhasan and Venkataraman (2013) with some modifications. The 4.5 mL of 50 mmol L⁻¹ Tris-HCl buffer (pH 8.2) was mixed with 2.0 mL sample extracts solution at various concentrations (20-200 µg mL⁻¹). The solution was incubated at room temperature, then 0.4 mL of 25 mmol L⁻¹ pyrogallol at the same temperature was added to the solution and the mixture was shaken rapidly. The absorbance was measured at 325 nm, against blank (water instead of samples). The scavenging effect was calculated with the following equation:

$$\text{Superoxide radical scavenging activity (\%)} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100$$

where, A_{blank} is the change speed of absorbance of the control group in the superoxide radical generation system and A_{sample} is the change speed of absorbance of the sample. Ascorbic acid was used as a standard for this study.

Antibacterial activity: The *Passiflora wilsonii* Hemsl. extracts were individually tested against a set of common pathogenic microorganisms, including two Gram-positive bacteria: *Staphylococcus aureus* 26003 and *Streptococcus mutans* 32400 and three Gram-negative bacteria: *Pseudomonas aeruginosa* 10104, *Shigella sonnei* 51592 and *Escherichia coli* 44102. The pure bacterial were obtained from Guangdong Microbiology Culture Center (GIMCC). The agar disc diffusion method was employed for the determination of antibacterial activities of the various concentrations and various solvent extracts (Khurram *et al.*, 2012). The test extracts were dissolved in sterile water to a concentration of 100 mg mL⁻¹. An overnight culture of the tested bacteria in tryptose broth was diluted to about 10⁵ CFU with the same broth and inoculated with an inoculating device onto agar containing serial two fold dilutions of the test compounds. The organisms were incubated at 37°C for 24 h. The Minimum Inhibitory Concentrations (MIC) of the extract for each test microorganism were considered the agar plate with the lowest concentrations without growth.

Statistical analysis: Data for antibacterial and antioxidant activity are expressed as Mean±SD for analysis performed in triplicate and the results were expressed as Mean±SD. Analyses of variance were performed by ANOVA test and a value of p<0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Total flavonoids content: It is well known that flavonoid compounds contribute directly to the biological activity of

Table 1: Total flavonoid content of the different extracts of *Passiflora wilsonii* Hemsl.

Extracts	Total flavonoids (mg rutin equivalent/g)
Ethanol	40.2±1.1 ^a
Aqueous ethanol	37.6±1.2 ^b
Acetone	31.5±0.8 ^c
Ethyl acetate	24.4±0.8 ^d
Water	18.5±0.7 ^e

Values (Mean±SD, n = 3) in the same column followed by a different letter are significantly different (p<0.05)

plant materials (Bao *et al.*, 2005). Therefore, the total flavonoids content in the five different solvent extracts were determined. As shown in Table 1, there were differences in total flavonoids contents of the different extracts.

The highest levels of total flavonoids content were found in ethanol extract (40.2±1.1 mg of GAE/g dried extract) followed by the aqueous ethanol, acetone and ethyl acetate extracts, while total flavonoids content of the water extract was the lowest (16.5±0.7 mg of GAE/g dried extract). This result also showed that the ethanol extract was better for flavonoid extraction for *Passiflora wilsonii* Hemsl. The rich-flavonoid plants could be a good source of antioxidants that would help to increase the overall antioxidant capacity of an organism and protect it against lipid peroxidation (Sharififar *et al.*, 2009).

Antioxidant activity: Radical scavenging activities are very important due to the deleterious role of free radicals in biological systems. Several in vitro tests were adopted to evaluate the antioxidant activity of various solvents extracts at different concentrations and the results were compared to ascorbic acid used as positive control. The ethanol extract showed the highest antioxidant activities in test models, this result implies that reducing power may be directly related to total flavonoids.

Reducing power: The reducing power of the extract Fe³⁺/ferricyanide complex to the ferrous form may serve as a significant indicator of its antioxidant activity. The reducing power was evaluated is based on the reduction of Fe³⁺ to Fe²⁺ in which the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each sample. Rising absorbance at 700 nm of the reaction mixture indicate an increase in reducing power (Junaid *et al.*, 2013).

As seen in Fig. 1, the absorbance at 700 nm of extracts increased with increase in concentration, means the reducing power increased with increase in concentration. At the concentration of 20 µg mL⁻¹ the absorbance at 700 nm of various solvent extracts (ethanol, aqueous ethanol, acetone, ethyl acetate and water) and standard was 0.232, 0.213, 0.168, 0.133, 0.106 and 0.654 but the absorbance at 700 nm of various solvent extracts (ethanol, aqueous ethanol, acetone, ethyl acetate and water) and standard sharply increased to 0.937, 0.845, 0.801, 0.669, 0.493 and 1.606 at the concentration of 200 µg mL⁻¹, respectively. In this study, the reducing power of those samples was in the following order: ascorbic acid>ethanol extract>aqueous ethanol extract>acetone extract>ethyl acetate extract> water extract.

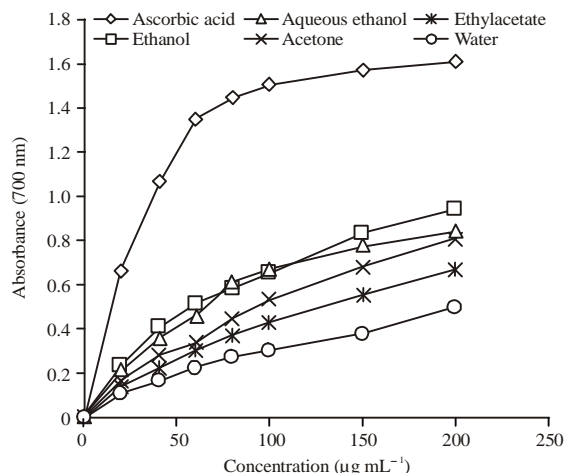


Fig. 1: Reducing power of various solvent extracts from *Passiflora wilsonii* Hemsl. and ascorbic acid. Values expressed as Means±SD (n = 3)

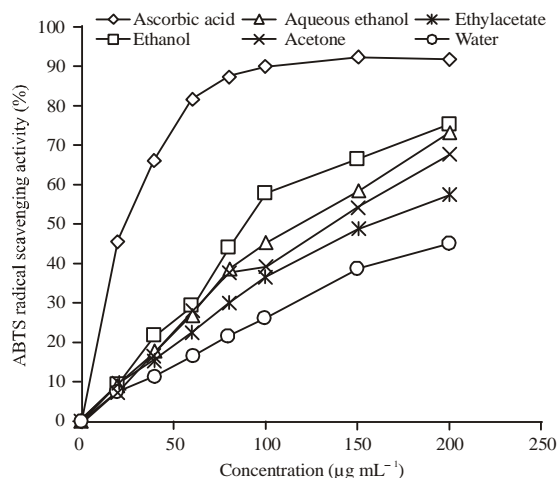


Fig. 3: ABTS radical scavenging activity of various solvent extracts from *Passiflora wilsonii* Hemsl. and ascorbic acid. Values expressed as Means±SD (n = 3)

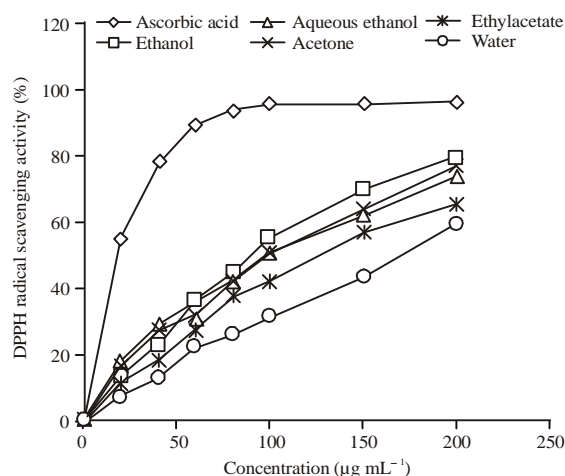


Fig. 2: DPPH radical scavenging activity of various solvent extracts from *Passiflora wilsonii* Hemsl. and ascorbic acid. Values expressed as Means±SD (n = 3)

DPPH radical scavenging activity: The DPPH radical is a stable free radical which has been widely used to evaluate the free radical scavenging activities of natural compounds. Alcohol solution of DPPH has a characteristic absorption maximum at 517 nm. When DPPH ethanol solution is reduced, absorbance is decreased and the solution changes from purple to light yellow. The mechanism of scavenging DPPH radical is caused by the fact that natural compounds can transfer either an electron or a hydrogen atom to DPPH, so DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances (Sasikumar *et al.*, 2012).

In the present investigation, a comparison of various antioxidant activities of extracts and ascorbic acid (used as positive control) is shown in Fig. 2. The type of solvents had significant impact on DPPH values and DPPH radicals were

scavenged by the extract and ascorbic acid in a concentration dependent manner within the range of the given concentrations. DPPH radical scavenging abilities of these various solvent extracts sharply increased from 13.8, 17.2, 16.4, 10.9, 7.4-79.3, 73.4, 76.5, 65.1 and 59.4%, when the concentration was increased from 20-200 $\mu\text{g mL}^{-1}$, respectively. The results showed that among the solvent extracts analyzed for DPPH scavenging activity, all the extracts showed excellent percent inhibition of DPPH activity at the concentration of 200 $\mu\text{g mL}^{-1}$ but significantly lower than that of ascorbic acid (96.1%), especially ethanol extract of *Passiflora wilsonii* Hemsl. showed stronger DPPH scavenging activity rather than acetone, aqueous ethanol, ethyl acetate and water. The scavenging activity of water extract against DPPH was observed as the weakest among all the samples evaluated.

ABTS radical scavenging activity: The ABTS radical scavenging assay is one of the popular indirect methods of determining the antioxidative capacity of compounds (Shon *et al.*, 2003). The abilities of various solvent extracts from *Passiflora wilsonii* Hemsl., assayed to be scavenging the ABTS radical in comparison with ascorbic acid (used as positive control), are shown in Fig. 3.

All the tested samples exhibited effectual radical scavenging activity. As seen in Fig. 3, the extract had effective ABTS radical scavenging activity in a concentration-dependent manner (20-200 $\mu\text{g mL}^{-1}$). The DPPH radical scavenging abilities of these various solvent (ethanol, aqueous ethanol, acetone, ethyl acetate and water) extracts sharply increased from 9.4, 8.9, 7.3, 9.8 and 7.3-75.4, 73.8, 67.5, 57.3 and 45.1%, respectively. The scavenging effect of ascorbic acid (91.7%) was observed to be higher than that of various solvent extracts from *Passiflora wilsonii* Hemsl. There is a significant decrease in all the concentration of ABTS due to the scavenging capacity of all the extract concentrations. Also, the scavenging effect of the extract and

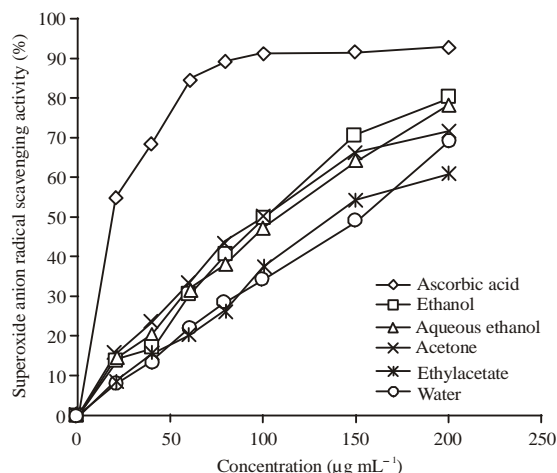


Fig. 4: Superoxide anion radical scavenging activity of various solvent extracts from *Passiflora wilsonii* Hemsl. and ascorbic acid. Values expressed as Means \pm SD (n = 3)

standards on the ABTS radical scavenging activity decreased in the order: Ascorbic acid>ethanol extract>aqueous ethanol extract>acetone extract>ethyl acetate extract>water extract which were 91.7, 75.4, 73.8, 67.5, 57.3 and 45.1% at the concentration of 200 µg mL⁻¹, respectively.

Superoxide anion radical scavenging activity: Superoxide anion radical is considered as an initial free radical and formed from mitochondrial electron transport system. Superoxide anion which is a reduced form of molecular oxygen, has been implicated in the initiating oxidation reactions associated with aging such as ischemic reperfusion injury (Roginsky and Lissi, 2005). Thus, scavenge superoxide radical is necessary.

Figure 4 shows the percentage inhibition of superoxide radical generation from 20-200 µg mL⁻¹ concentration of ethanol extract, aqueous ethanol extract, acetone extract, ethyl acetate extract, water extract and ascorbic acid (used as positive control). In this study, the superoxide anion scavenging effects of various solvents were analyzed and the results are given in Fig. 4. The type of solvents had significant impact on superoxide anion values and Superoxide anion radicals were scavenged by the extract and ascorbic acid in a concentration dependent manner within the range of the given concentrations. The percentage inhibition of superoxide anion radical scavenging by 200 µg mL⁻¹ concentration of the ethanol extract was found as 79.8%, showed stronger Superoxide anion radical scavenging activity rather than aqueous ethanol, acetone, ethyl acetate and water. The superoxide radical scavenging activity of those samples was in the following order: ascorbic acid>ethanol extract>aqueous ethanol extract >acetone extract >water extract>ethyl acetate extract which were 92.4, 79.8, 78.2, 71.4, 60.9 and 68.7% at the concentration of 200 µg mL⁻¹, respectively.

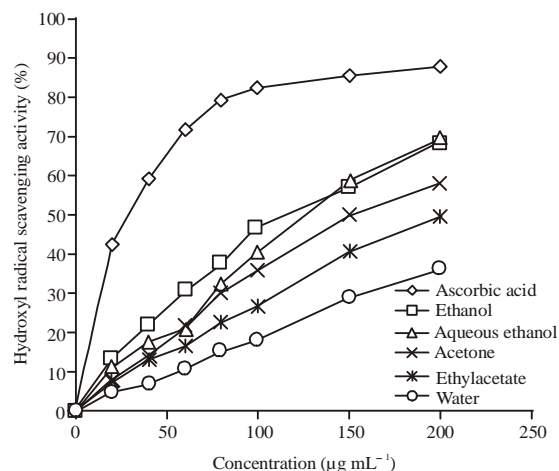


Fig. 5: Hydroxyl radical scavenging activity of various solvent extracts from *Passiflora wilsonii* Hemsl. and ascorbic acid. Values expressed as Means \pm SD (n = 3)

Hydroxyl radical scavenging activity: Among reactive oxygen species, Hydroxyl radical exhibits the strongest oxidative activity in terms of its very high redox potential and extremely fast kinetics. Hydroxyl radical is the most toxic radical known, as it can non-specifically oxidize all classes of biological macromolecules at virtually diffusion-limited rates (Tang *et al.*, 2013). Thus, hydroxyl radical scavenging activity is very important for evaluating the antioxidant activity of sample extracts. Hydroxyl radical scavenging activity of various solvent extracts from *Passiflora wilsonii* Hemsl. and ascorbic acid (used as positive control) were measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture and the results was shown in Fig. 5.

Hydroxyl radical scavenging effect of various solvent extracts from *Passiflora wilsonii* Hemsl. increased with concentrations. At concentration of 200 µg mL⁻¹, scavenging effects on hydroxyl radical were 68.2, 69.4, 57.9, 49.7, 36.2 and 87.6% for various solvent extracts (ethanol, aqueous ethanol, acetone, ethyl acetate and water) and ascorbic acid, respectively. Results indicated that ethanol and aqueous ethanol had strong capability of scavenging hydroxyl radical but significantly lower than that of ascorbic acid. The water extract had the lowest hydroxyl radical scavenging activity, with values only 36.2 at 200 µg mL⁻¹. So the hydroxyl radical scavenging activity of those samples was in the following order: ascorbic acid>aqueous ethanol extract>ethanol extract >acetone extract>ethyl acetate extract>water extract at the concentration of 200 µg mL⁻¹.

Antibacterial activity: The extracts were evaluated for the antibacterial activity using disc diffusion method and tested against six bacterial strains with streptomycin used as positive controls in the assay. The results of the antibacterial activity of methanol, aqueous ethanol, acetone, ethyl acetate and water extracts from *Passiflora wilsonii* Hemsl. are given in Table 2.

Table 2: Antibacterial activity of *Passiflora wilsonii* Hemsl. extracts using agar disc diffusion and MIC methods (MIC in $\mu\text{g mL}^{-1}$)

Bacterial species	Ethanol	Aqueous ethanol	Acetone	Ethyl acetate	Water extract	Streptomycin
<i>S. aureus</i> 26003	312.5	1250	625	1250	>5000	10.0
<i>M. luteus</i> 28001	625.0	1250	1250	2500	>5000	10.0
<i>B. subtilis</i> 63501	625.0	625	625	1250	>5000	5.0
<i>P. aeruginosa</i> 10104	1250.0	2500	1250	2500	>5000	5.0
<i>S. sonnei</i> 51592	1250.0	1250	1250	2500	>5000	2.5
<i>E. coli</i> 44102	625.0	1250	625	1250	>5000	10.0

As can be seen from Table 2, ethanol, aqueous ethanol, acetone, ethyl acetate and water extracts obtained from *Passiflora wilsonii* Hemsl. have been shown to be mild to moderately effective against most of the tested bacteria. The results were compared with those of Streptomycin as a standard antibiotic. The antibacterial activity of various solvent extracts from *Passiflora wilsonii* Hemsl. varied significantly ($p < 0.05$). The antimicrobial activity of the tested extracts showed different selectivity for each microorganism. The MIC of ethanol, aqueous ethanol, acetone and ethyl acetate extracts ranged from 312.5-1250 $\mu\text{g mL}^{-1}$, 625-2500 $\mu\text{g mL}^{-1}$, 625-1250 $\mu\text{g mL}^{-1}$ and 1250-2500 $\mu\text{g mL}^{-1}$, respectively. However, the results revealed that water extract were found to have lower activity against all tested bacteria. Ethanol extract showed higher activity against *S. aureus* and *M. luteus*, ethanol and acetone extracts showed higher activity against *E. coli* and ethanol, aqueous ethanol and acetone extracts showed excellent activity against *B. subtilis*. It was worth noting that all of the extracts showed greater potent antibacterial activity against Gram-positive bacteria than Gram-negative. However, all extracts were shown lower antibacterial activities against the tested bacteria which are comparable to that of the standard (Streptomycin).

CONCLUSION

This study presents the first attempt to evaluate the differences in biological activities among various solvent extracts from *Passiflora wilsonii* Hemsl., the results obtained in this study have considerable value with respect to various solvent extracts from *Passiflora wilsonii* Hemsl. showed varying degrees of antioxidant activity in different test systems in a dose-dependent manner. All solvent extracts of *Passiflora wilsonii* Hemsl. exhibited scavenging activities toward DPPH, ABTS, superoxide anion and hydroxyl radicals. Ethanol proved to be the most efficient solvent for extraction of antioxidants from *Passiflora wilsonii* Hemsl. as the related extract exhibited the stronger antioxidant capacity than aqueous ethanol, acetone, ethyl acetate and water in all the assays used. This result implies that reducing power may be directly related to total flavonoids. It possesses also an antibacterial activity against all test bacteria and especially against *S. aureus* and *M. luteus*, indicating an admirable potential of the related extracts for isolation of natural antioxidant and antibacterial agents. Therefore, it is important to consider the optimum technological conditions and

processing factors influencing activity and bioavailability of plant antioxidants and antibacterial. In addition, further purification of the active compounds and *in vivo* evaluation of antioxidant and antimicrobial activity along with toxicity studies of the extracts from *Passiflora wilsonii* Hemsl. are therefore suggested for further studies.

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