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Antioxidant Activity of Pitcher Extracts from Three *Nepenthes* Species

(Aktiviti Antioksidan Ekstrak Kendi bagi Tiga Spesies *Nepenthes*)

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ABSTRACT

Nepenthes, *locally known as 'periuk kera' in Malaysia, is a fascinating species due to uniqueness in their morphology in having pitcher organ for carnivorous diet. The pitcher plant has been used for cooking traditional delicacies and as traditional remedies to treat illness. Hence, this species might possess beneficial health properties. This study aimed to compare the antioxidant activity of the pitcher extracts from* Nepenthes ampullaria, Nepenthes rafflesiana *and their hybrid,* Nepenthes × hookeriana. *The samples were extracted using methanol:chloroform:water (3:1:1) via sonication assisted extraction and the extracts were subjected to three different antioxidant assays, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing power (FRAP) and total phenolic content (TPC). Extract from* N. ampullaria *exhibited the strongest radical scavenging activity (0.148 ± 0.04 mg/mL) with the highest ferric reducing power (0.009 ± 0.003 mg GA/mg dry weight) among the three species, whereas that of* N. rafflesiana *possessed the highest phenolic content (0.057 ± 0.017 mg GA/mg dry weight). However, the antioxidant capacities of the pitcher extracts were not significantly different (*p>*0.05) between the three species and were much lower than the gallic acid as a standard reference.*

Keywords: Antioxidants activity; DPPH assay; ferric reducing power assay; Nepenthes *sp.; total phenolic content*

ABSTRAK

Nepenthes, *lebih dikenali sebagai 'periuk kera' di Malaysia merupakan suatu spesies yang menakjubkan kerana keunikan morfologinya dalam mempunyai organ seperti kendi untuk diet karnivor. Tumbuhan berkendi ini telah digunakan dalam masakan tradisi dan juga sebagai ubat-ubatan tradisi untuk merawat penyakit. Oleh itu, spesies ini mungkin memiliki kandungan yang bermanfaat untuk kesihatan. Kajian ini bertujuan untuk membandingkan aktiviti antioksidan ekstrak kendi daripada* Nepenthes ampullaria, Nepenthes rafflesiana *dan hibrid mereka,* Nepenthes × hookeriana. *Setiap sampel telah diekstrak dengan menggunakan metanol:kloroform:air (3:1:1) melalui pengekstrakan dengan bantuan sonikasi dan telah diasai dengan tiga asai antioksidan yang berbeza, iaitu 2,2-diphenyl-1-picrylhydrazyl (DPPH), kuasa penurunan ferik (FRAP) dan jumlah kandungan fenolik (TPC). Ekstrak daripada* N. ampullaria *menunjukkan aktiviti pelupusan radikal yang terkuat (0.148 ± 0.04 mg/mL) dan mempunyai kuasa penurunan ferik yang tertinggi (0.009 ± 0.003 mg GA/mg berat kering) antara ketiga-tiga spesies ini, namun begitu* N. rafflesiana *mempunyai kandungan fenolik yang tertinggi (0.057 ± 0.017 mg GA/mg berat kering). Walau bagaimanapun, tiada perbezaan signifikan terhadap kebolehan antioksidan ekstrak kendi daripada tiga spesies yang dikaji dan ternyata jauh lebih rendah daripada asid galik yang merupakan piawai rujukan dalam kajian ini.*

Kata kunci: Aktiviti antioksidan; asai DPPH; asai jumlah kandungan fenolik; asai kuasa penurunan ferik; Nepenthes *sp.*

INTRODUCTION

Reactive oxygen species (ROS) could be defined as free radicals containing oxygen produced in living tissue (Diplock et al. 1998). These free radicals are highly reactive chemicals which have the potential to harm cells. ROS are constantly produced in the cells as the signalling components in stress responses, radiation, bacterial and viral toxin, smoking, alcohol and psychological or emotional stress in humans (Onoja et al. 2014). On the contrary, high levels of ROS are harmful and can lead to ageing and cell damage or development of cancer and some other diseases such as Alzheimer's disease, diabetes, atherosclerosis, neurodegenerative disease and cancer (Khalaf et al. 2008; Patel et al. 2010).

Therefore, antioxidants are used as health supplements to protect and prevent such damages from ROS. Antioxidant molecules or known as the free radical scavengers are chemicals that could interact with free radicals, chelating metals and performed as oxygen scavengers (Onoja et al. 2014). These antioxidants are available naturally in plants or as synthetic compounds. Several synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytouluene (BHT), tertiary butylated hydroxyl quinone (TBHQ) and gallic acid esters are also available commercially. These synthetic antioxidants are widely used in food industry to prevent food oxidation and increase shelf life, but there are certain concerns on their safety and toxicity (Carocho & Ferreira 2013; Patel et al.

2010; Reddy & Grace 2016). Hence, there has been an increasing interest and demand for natural antioxidants, especially from industries related to cosmetics, foods and pharmaceuticals on bioactive compounds from natural products and medicinal plants to substitute synthetic antioxidants (Barros et al. 2007; Thao et al. 2016).

Nepenthes species is a tropical carnivorous pitcher plant from the family of Nepenthaceae. The plant is native and highly distributed in Southeast Asia of Peninsular Malaysia, Singapore, Sumatera and Borneo (Adam & Hamid 2007; Moran et al. 2010). The *Nepenthes* plant is fascinating to study because of their carnivorous diet with unique pitcher organ growing from the tip of leaf (Wang et al. 2009). Furthermore, hybridisation that is extensive in *Nepenthes* could also lead to variations and unique phytochemical production (Lätti et al. 2011). Traditionally, the *Nepenthes* plant is commonly used as folk medicine to regulate the menstrual cycle, ease child birth, relieve asthma, treat eye inflammation, gastric ulcer, jaundice, high blood pressure and also as an astringent (Aung et al. 2002; Sanusi et al. 2017; Thao et al. 2016; Van Thanh et al. 2015). Collectively, the pitcher has also been used to cook traditional delicacies (Schwallier et al. 2015).

Previously, the leaf extract of the *Nepenthes* plant has been reported to contain many beneficial bioactivity properties, including antibacterial, antifungal, antimalarial, antiosteoporotic, and antidiabetic (Likhitwitayawuid et al. 1998; Shil et al. 2010; Shin et al. 2007; Van Thanh et al. 2015). In contrast, the chemical analysis of the pitcher tissue is still lacking with no previous report on its antioxidant activity. It is interesting to study the pitcher since the tissue is elongated from the leaf and only unique to *Nepenthes* species. In this study, the pitcher extracts from three related lowland *Nepenthes* species, namely *Nepenthes ampullaria, Nepenthes rafflesiana* and their hybrid, *Nepenthes* × *hookeriana* were compared on their antioxidant activity via 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, ferric reducing power (FRAP) assay and total phenolic content (TPC).

Materials and Methods

Pitcher sampling and phytochemical extraction

Mature pitchers of three lowland *Nepenthes* species, *N. ampullaria*, *N. rafflesiana* and *N.* × *hookeriana* were sampled in the morning between 9 and 11 am, about 7 days after the pitcher opening from a terrace at Universiti Kebangsaan Malaysia (2°55'12.7"N, 101°46'59.7"E). Each pitcher represents a biological replicate, with 5 biological replicates and 5 technical replicates in this study. The collected samples were lyophilised and 10 mg of dried samples were extracted with 200 µL of methanol: chloroform: water (3:1:1) via sonication for 15 min according to Rosli et al. (2017). Filtered extracts through a 0.22 µm PTFE membrane were stored at -80°C.

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH radical-scavenging-activity was determined by the method of Ahmad et al. (2014) with slight modifications. Briefly, extraction solvents were dried out from the extracts using vacuum concentrator (RVC 2-18, CHRIST, Germany). The dried extracts were diluted with methanol with initial concentration of 1 mg/mL and were placed into a 96-well microplate (100 µL). The absorbance of the sample was measured at 490 nm (Abs 1) using microplate reader (iMark™ Microplate Absorbance Reader, BioRad, United States of America). Then, 0.5 mM DPPH radical (Sigma-Aldrich, United States of America) in methanol solution (100 µL) was added. As the mixture was incubated at 37°C for 30 min, the absorbance (Abs 2) was measured again. Gallic acid was used as a positive control with initial stock concentration at 1 mg/mL. Inhibition concentration to scavenge 50% of DPPH radicals (IC_{50}) values were determined according to DPPH scavenging activity (SA) and the SA was calculated using the formula:

$$\text{Scavening Activity (\%)} = \left[1 - \frac{(\text{Abs } 2_{\text{sample}} - \text{Abs } 1_{\text{sample}})}{(\text{Abs } 2_{\text{control}} - \text{Abs } 1_{\text{control}})}\right] \times 100$$

Ferric reducing power (FRAP) assay

The ferric reducing power was determined according to Kuda and Yano (2009) using 25 µL of methanol diluted dried sample (1 mg/mL), 25 µL of phosphate buffers (pH 6.6) and 50 µL of 0.1% potassium ferricyanide mixed in a 96-well microplate. Briefly, the mixture was incubated at 37°C for 60 min before adding 25 µL of 10% TCA and 100 µL of distilled water and the absorbance was measured at 655 nm (Abs 1). Then, 25 µL of 0.1% ferric chloride was added and the absorbance was measured again (Abs 2). The reducing power was calculated as formula:

$$\text{Reducing Power} = (\text{Abs2}_{sample} - \text{Abs1}_{sample}) - (\text{Abs2}_{control} - \text{Abs1}_{control})$$

Total phenolic content (TPC)

Sample extracts with the concentration of 1 mg/mL (10 µL) was mixed with 100 µL of 10% Folin-Ciocalteu reagent (Merck, United States of America). After 5 min, the solution was mixed with 100 µL of 7.5% Na_2CO_3 solution and the mixture was left to stand for 60 min. The absorbance was then recorded at 650 nm. Gallic acid was used as standard. The total phenolic content was as gallic acid equivalents by reference to linear equation of the standard curve ($y = 3.2032x + 0.0574$, $R^2 = 0.998$) with the initial concentration of 1 mg/mL (Clarke et al. 2013).

Statistical analysis

Statistical analysis was performed using Statistical Packages for the Social Sciences (SPSS) 17.0 software. Significance of differences between each sample was analysed using one-way analysis of variance (ANOVA)

and the correlation between the antioxidant assays were analysed using Pearson correlation analysis.

Results and Discussion

DPPH ASSAY

According to Shian and Abdullah (2012), antioxidants can be classified into two main groups; primary and secondary antioxidants. The antioxidant molecules that could react and scavenge the free radicals are usually regarded as primary antioxidants. Hence, DPPH assay is used to measure the ability of primary antioxidants from samples in scavenging DPPH radicals, through donating hydrogen atom or electron, thus changed the free radicals into a stable product (Azlim Almey et al. 2010; Mehta & Gowder 2015; Shian & Abdullah 2012). Lower IC_{50} values correspond to higher antioxidant activity, which mean lower concentration needed to scavenge 50% of DPPH radicals (Maisuthisakul et al. 2007). The DPPH radical scavenging activity ability of the pitcher extracts of three *Nepenthes* plant species are shown in Table 1. In the DPPH analysis, although there were insignificant differences (p>0.05), *N. ampullaria* showed the highest DPPH scavenging activity (0.148 ± 0.04 mg/mL), followed by *N. rafflesiana* (0.176 ± 0.05 mg/mL) and *N.* × *hookeriana* (0.234 ± 0.09 mg/mL) (Figure 1).

FRAP ASSAY

The secondary antioxidant molecules are active in suppressing radical formation and preventing oxidative damage (Shian & Abdullah 2012). Furthermore, the secondary antioxidants also known as the metal chelator and oxygen scavenger. Hence, ferric reducing power (FRAP) assay is suited for determining the secondary antioxidant capability in the samples, by observing the change in absorbance and the colour change from yellow ferric ion (Fe^{3+}) to green-blue ferricyanide (Fe^{2+}) complex according to the reducing power (Figure 2) (Zulkefli et al. 2013). As shown in Table 1, *N. ampullaria* displayed the highest ferric reduction power (0.009 ± 0.003 mg GA/mg dry weight), with the highest gallic acid equivalent concentration compared to the other two species albeit not statistically significant.

TPC ASSAY

According to Ainsworth and Gillespie (2007), phenolic compounds are outstanding oxygen radical scavengers due to their low electron reduction potential than the oxygen radicals and the phenoxyl radicals are less reactive than the oxygen radicals. Thus, the phenolic compounds could scavenge intermediates of reactive oxygen without promoting further oxidative reactions. In total phenolic content (TPC) assay, the concentration of the phenolic groups can be determined from reaction with the Folin-

TABLE 1. Antioxidant properties of pitchers from different species of *Nepenthes*. Each value represents mean ± SD of 5 independent biological replicates

Sample	DPPH (mg/mL) (IC_{50})	Total phenolic content (mg GA/mg of dry weight)	FRAP value (mg GA/mg dry weight)ht)
Nepenthes ampullaria	0.148 ± 0.04	0.031 ± 0.019	0.009 ± 0.003
Nepenthes × *hookeriana*	0.234 ± 0.09	0.032 ± 0.012	0.007 ± 0.002
Nepenthes rafflesiana	0.176 ± 0.05	0.057 ± 0.017	0.008 ± 0.002
Gallic acid	0.005 ± 0.001	-	-

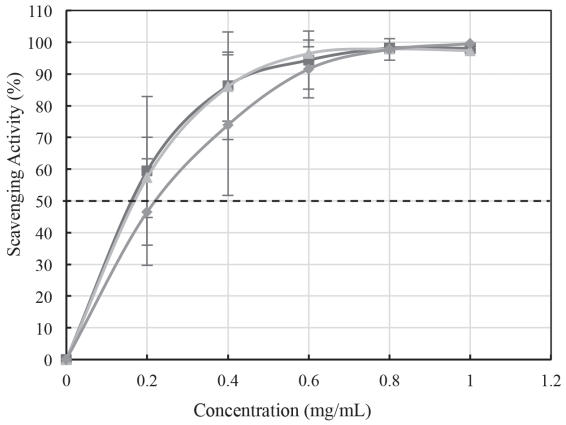

FIGURE 1. DPPH scavenging activity of pitchers from different species of *Nepenthes*. Each value represents mean ± SD of 5 independent biological replicates. The markers represent different species, ■ : *N. ampullaria*, ▲ : *N. rafflesiana* and ◆ : *N.* × *hookeriana*

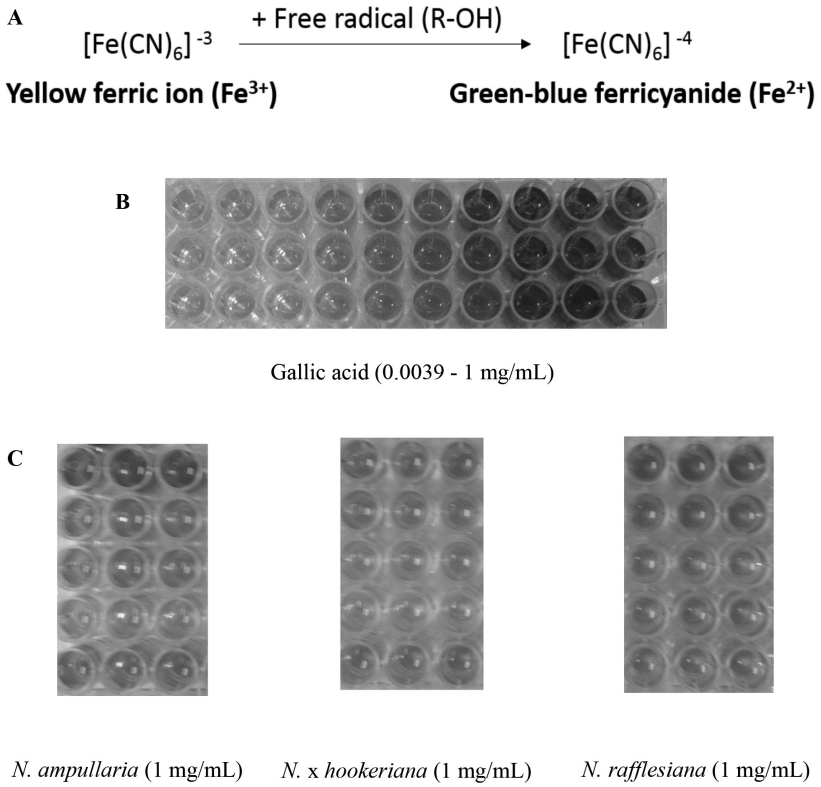

FIGURE 2. Reduction of ferric ion in FRAP assay. A. Reaction of ferricyanide ion with free radicals. B. Colour differences of assay's solution due to different concentrations of gallic acid (2-folds dilution). The standard was evaluated triplicates. C. Colour changes of assay's solution when introduced with 1 mg/mL pitcher extracts. Each pitcher extract represents by 5 independent biological replicates (vertical) and 3 technical replicates (horizontal)

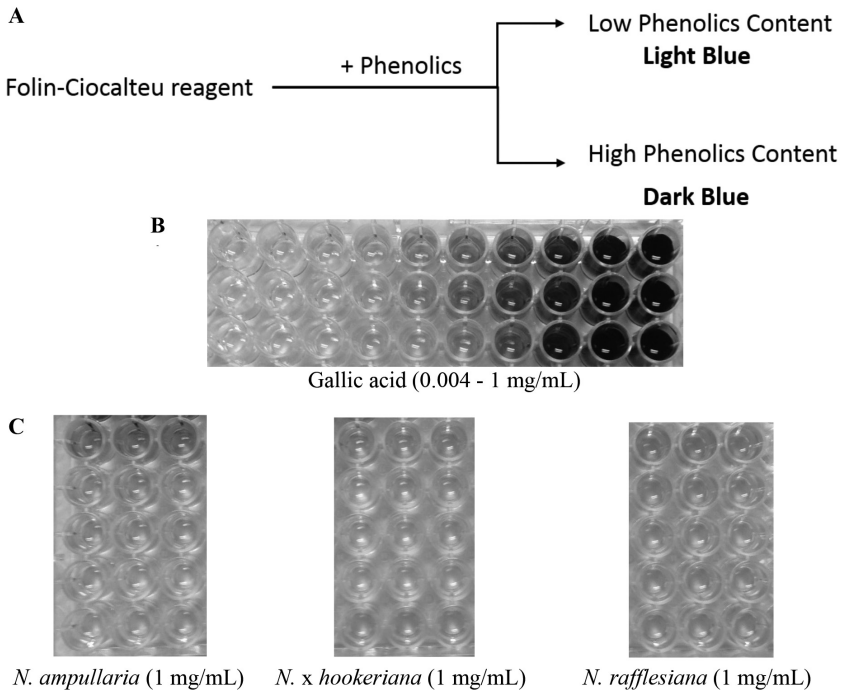

FIGURE 3. The colour changes of Folin-Ciocalteu reagent after introduced with phenolics. A. Reaction of Folin-Ciocalteu reagent with phenolics. B. Colour differences of Folin-Ciocalteu reagent due to different concentrations of gallic acid (2-folds dilution). The standard was evaluated triplicates. C. Colour changes of Folin-Ciocalteu reagent when introduced with 1 mg/mL pitcher extracts. Each pitcher extract represents by 5 independent biological replicates (vertical) and 3 technical replicates (horizontal)

Ciocalteu reagent, as indicated by the blue colour intensity in the reaction mixture (Figure 3) (Shian & Abdullah 2012). Deeper blue solution indicates higher phenolic concentration. Despite of the insignificant differences (p>0.05), TPC assay showed that the *N. rafflesiana* had the highest phenolic content (0.057 ± 0.017 mg GA/mg dry weight) whereas, *N. ampullaria* and *N.* × *hookeriana* showed similar phenolic content (Table 1).

RELATIONSHIP BETWEEN DPHH, FRAP AND TPC

In order to correlate the antioxidant capacities (DPHH, FRAP) with the TPC, the linear correlation coefficients (r) were calculated for the three plant extracts analysed. Figure 4 shows the relationship between the three antioxidant assays of all the five biological replicates. According to Table 2, our findings showed a significant positive correlation (r=0.671, p<0.01, R^2=43%) between FRAP and TPC, compared to significant negative correlations between DPPH with TPC (r=-0.671, p<0.01, R^2=45%). Such high r value suggested that higher phenolic content correlated with higher value of FRAP and lower value of IC_{50} for DPPH. Hence, the results are in agreement with findings by Clarke et al. (2013) who reported that extracts of plants from Malaysian rainforest displayed a high antioxidant behaviour in both DPPH and FRAP assay and were identified with high phenolic content. Phenolic compounds exhibit strong antioxidant activities due to their ability to act as reducing agents, hydrogen donor and singlet oxygen quenchers (Huda-Faujan et al. 2009). Therefore, the significant negative correlations between DPPH with FRAP (r=-0.801, p<0.01, R^2=62%) provide insights that antioxidants could lead to a lower DPPH IC_{50} value and higher FRAP value.

Previous study on antioxidant activity of leaf extracts from other species of *Nepenthes*, namely *N. khasiana* and *N. bicalcarata* exhibited decent DPPH scavenging activity by having 10-fold lower of IC_{50} values compared to the pitcher extracts (Ismail et al. 2015; Sanusi et al. 2017). These findings showed that the leaf extracts possessed a greater antioxidant activity compared to the pitcher extracts. Furthermore, *N. bicalcarata* leaf extract was also reported to be more active in scavenging DPPH radicals than butylated hydroxytoluene (BHT), a potent synthetic antioxidant that is widely commercialised in foods and pharmaceuticals industries (Ismail et al. 2015; Yehye et al. 2015). Therefore, the leaf extracts might possess higher abundance of metabolites that are responsible for antioxidant activity compared to the pitcher extracts, albeit that pitcher is developed from the leaf. The abundance of those metabolites might be contributed by some external factors such as the growing environment

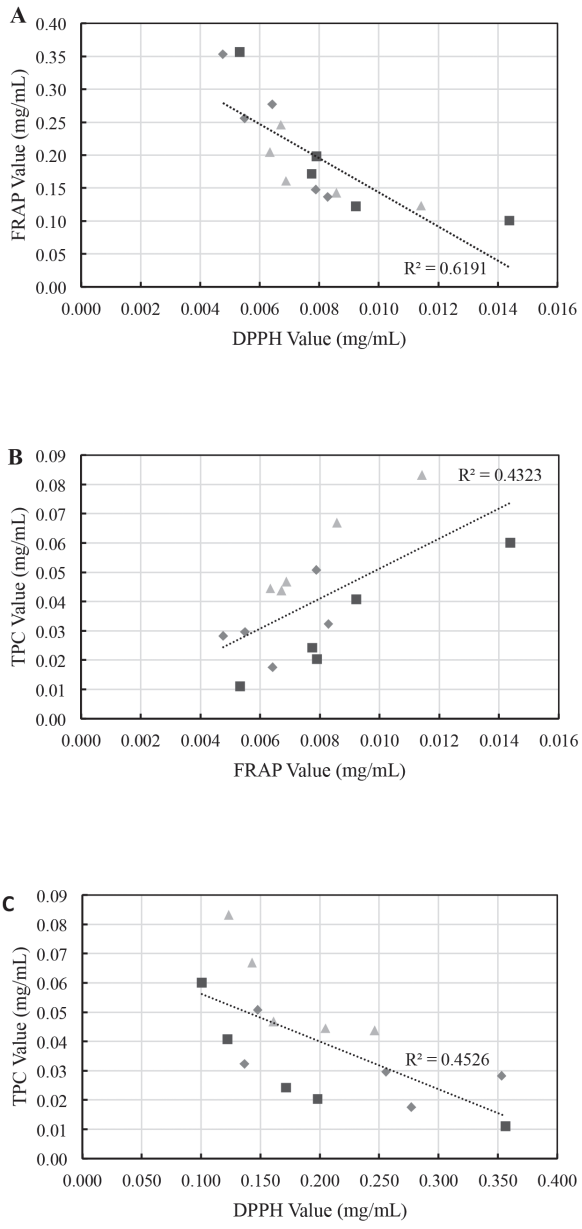

FIGURE 4. Correlation between antioxidant activity assays. A. Correlation between FRAP and DPPH IC_{50} value. B. Correlation between TPC and FRAP. C. Correlation between TPC and DPPH IC_{50} value. The markers represent different species, ■ : *N. ampullaria*, ▲ : *N. rafflesiana* and ◆ : *N.* × *hookeriana*

TABLE 2. The correlation of antioxidant activity assays and total phenolics content of pitchers from different species of *Nepenthes*

Pearson's r	DPPH	FRAP	TPC
DPPH	1.000	-0.801**	-0.671**
FRAP	-0.801**	1.000	0.672**
TPC	-0.671**	0.672**	1.000

** Correlation is significant at p<0.01

of samples (Goh et al. 2016; Kliebenstein 2004; Sampaio et al. 2016).

In the case for *N. ampullaria*, antioxidant activity was the highest among the three species based on DPPH and FRAP assays, but the phenolic content in the species was lower compared to *N. rafflesiana*. The results suggested that the antioxidant activity from pitcher extracts of *N. ampullaria* may be contributed by different classes of antioxidants molecules, such as terpenes which are also known for their excellent antioxidant activity (Öztürk 2012), or maybe resulted from the synergistic effect of abundant metabolites in the sample (Kopjar et al. 2016). Similar to phenolic, terpenoids also exhibit strong antioxidant capabilities (Adhikari et al. 2003). According to Grassmann (2005), essential oils that contain monoterpenes, diterpenes and sesquiterpenes indicate great antioxidant activity and terpenoids also show interesting synergistic actions with other antioxidants such as flavonoids. Therefore, antioxidant activity assay should not only focus on certain metabolite but also includes combination or mixture of secondary metabolites.

Conclusion

In conclusion, despite the lack of statistical significance, pitcher extracts from *N. ampullaria* showed potential as the best radical scavenger with the highest ferric reducing power among the three species, but the highest phenolic content was found in *N. rafflesiana*. The antioxidant activity of pitcher extracts appeared to be much lower than the leaf extracts in previous reports. We showed that higher phenolic content in pitcher extracts is indeed correlated with higher value of FRAP and lower value of IC_{50} for DPPH. This is the first report on the antioxidant activity of *Nepenthes* pitchers.

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