TOTAL PHENOLIC CONTENTS AND ANTIOXIDANT SCAVENGING CAPACITY IN FREE AND BOUND EXTRACTS OF *CHRISTIA VESPERTILIONISIS* (DAUN RERAMA)

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ABSTRACT

Introduction: *Christia vespertilionisis* is a plant herb that has been used traditionally due to its benefits for potentially to treat some disease. It contain potential phytochemical compound that can be a component of drugs or natural products. This study was conducted to investigate the total phenolic content and its antioxidant radical scavenging activity on *C. vespertilionisis.* Series of test in determining the total phenolic content in three different types of extractions was conducted and its relationship with antioxidant scavenging activity was studied. **Methods:** The *C. vespertilionis's* powder was extracted with 80% methanol and hydrolyzed with HCl and NaOH. The total phenolic content was determined using Follin-Ciocalteu and DPPH assay was used for antioxidant activity. **Results:** Based on the test, the total phenolic content of all extracts was 9.13± 0.13 mg of GAE/g with insoluble bound extracts revealed a highest value of 4.94 ± 0.042 mg of GAE/g followed by soluble bound and free phenolic extracts with (3.16± 0.28 and 0.98 ± 0.07 mg of GAE/g respectively), ($p<0.05$). The antioxidant scavenging capacity extracts of IC_{50} showed that insoluble bound extracts showed lowest value (38.13 ± 0.65mg/ml) followed by soluble bound extracts and free phenolic extracts with (39.54 ± 0.77 and 42.59 ± 1.69mg/ml respectively). **Conclusions:** This study indicates that the extract of *C. vespertilionis* could be a good source of antioxidant especially on the bound extract.

KEYWORDS : *C. vespertilionisis,* TPC, antioxidant, DPPH assay

INTRODUCTION

Recently, research on medicinal plants are widely discovered and become global attention. They contain a vast range of scavenging free radical molecules such as phenolics compound, nitrogen compound and terpenoids which high in antioxidant activity, anti-inflammatory and antimicrobial properties (Catelle et

al.,1995; Cai et al., 2004). Previous study has shown that many chronic and degenerative diseases such as cardiovascular diseases, cancer, etc are caused by the oxidative stress imposed by reactive oxygen species (ROS) (Azizova, 2002; Young and Woodside, 2001). The formation of free radical will attack on biomolecules compound which will change the compound into a radical molecule and lead to chronic disease (Halliwell, 1989). Because of that, the role of antioxidant compound is important as defensive role by inhibiting or eliminating the generation of free radicals molecule. Many studies revealed that phenolic content has potential to treat free radical due to its antioxidant activity (Cai et al.,2004; Alothman et al.,2009).

Phenolic compounds are vital for plant defense which act as an antioxidant or antimicrobial agent to ensure the plants to survive (Chew et al., 2011). Its role as potent antioxidants and scavenger of free radical by donating hydrogen, reducing agents, metal chelators and singlet oxygen quencher (Chew et al., 2009). Other compound such as alkaloid, steroids, terpenes and saponins are some of phytochemical compound that normally found in plants. They have been categorized based on their biosynthetic pathway (Chew et al., 2011). These phytochemicals compounds exhibit other bioactivities such as anticarcinogenic, antimutagenic, anti-inflammatory and antimicrobial activity (Halliwell, 1989; Cai et al., 2004).

Statistically, 5-15% that includes 25000 species of higher plants in Malaysia have therapeutic potential. Recent studies conducted by Hamidun *et al*, (2015), found that species known as (*C.vespertilionis*) or also known as (Red Butterfly Wings) is has potential in cancer treatment in Malaysia. According to Hofer *et al*., (2013), *C. vespertilionis* is a new hope to treat neuroendocrine tumor.

Christia is an ornamental legume in the Fabaceae family consisting of 13 species in tropical Asia and five species found in China (Paula, 2007). Its distribution in tropical and subtropical Asia, includes Ryukyu Island, Taiwan, Indochina, Malaysia, Indonesia, Vietnam, Laos, Cambodia, Thailand, India, China and northern Australia. The genus is also naturalized in Fiji and on some Caribbean islands including St. Vincent, Martinique, Jamaica and St. Kitts (Locke and Heald, 1994). Christia spp. typically inhabits dry, grassy areas, sandy soils, and roadsides. The genus -1- Christia, commonly called the island pea (USDA, 2006), is a non-climbing perennial herb used as an ornamental in cultivated gardens in southeast Asia because of its uniquely shaped leaves, drought tolerance, and nitrogen-fixing ability (van Meeuwen et al., 1961; Ohashi, 1977). *C. vespertilionis* has been used traditionally to treat tuberculosis, snake bite and many more (Nguyen-Poplin et al., 2007).

METHODOLOGY

Christia vespertilionisis samples were bought from Pasar Tani Kuantan in dry form. The dried leaves were cut into small pieces and the samples were grounded into powder form with mechanical blender and were stored at incubator at 37°C.

Free Phenolic Extraction

The powdered samples were extracted by using Govardhan *et al.,* (2013) method with slightly modifications. The samples were prepared in triplicate. Each falcon tubes of 50ml containing 1gram of powdered sample was mixed with 20ml of 80% methanol. The samples were homogenized with Starsonic 60 Digit (34kHz, 175W) for 60 minutes at room temperature twice. The supernatant of the samples were filtered using filter paper (Whatmann No.1) and the residue was kept for insoluble bound analysis. The supernatant of the samples were evaporated to remove solvent (methanol) by using rotavap at 337mbar. The remaining samples were purified with diethyl ether three times. Two layers phase were formed, the organic phase of the samples were evaporated at 850mbar while the inorganic phase of the samples were

kept for bound soluble extraction. 10ml of absolute methanol were mixed with evaporated samples and kept at -80°C for free phenolic content analysis.

Soluble Bound Extraction

The inorganic phase of free phenolic extraction samples were added with 30ml of 2M sodium hydroxide (NaOH) and incubated for 2h at room temperature. After 2h, the samples were acidified with 4M of hydrochloric acid (HCl) until pH2 were achieved. The samples were then added with diethyl ether (DEE) three times for purification. The organic phase of the sample was evaporated with rotavapor at 850mbar. The remaining samples were added with 10ml of absolute methanol and kept at -80°C for further analysis.

Insoluble Bound Extraction

The residue of free phenolic extraction after sonication were diluted with 10ml of 4M sodium hydroxide (NaOH) and incubated for 1hr at room temperature. The samples were then acidified with 6M hydrochloric acid until pH2 was achieved. The samples were centrifuged at 4300 rpm for 10 minutes. The supernatant of the samples were collected and then 15ml of hexane were added to the samples. The samples were evaporated at 335mbar to remove the hexane. The remaining samples were purified with diethylether (DEE) three times. The organic phase of the samples was evaporated at 850mbar to remove the solvent. 10ml of absolute methanol was added to the samples and kept at -80°C for further analysis.

Total Phenolic Content Analysis

The total phenolic content of extracts will be measured by using the Follin-Ciocalteu method as described by Ismail *et al.,* (2010) with slightly modification. 10% of Folllin-Ciocalteu's phenol reagent was prepared by adding 10mg of gallic acid with 100ml of methanol. Then, 2.5g of sodium carbonate (Na_2CO_3) was diluted with 50ml of methanol. Gallic acid solution that used for standard was diluted by adding 100ml methanol with 2.5mg of gallic acid. A series of gallic acid solution was prepared. About 6µl gallic acid was transferred into each well, followed by 100µl Follin-Ciocalteu's phenol reagent and let it stand for 5 minutes. Then, 80µl Na_2CO_3 was added to each well and the microwell plate was incubated in dark room condition for one hour. After 1hour, the plate was read under microplate reader at 760nm. The steps were repeated by replacing standard gallic acid with the phenolic samples. The obtained results were expressed as mg Gallic acid equivalents (GAE) per gram of dried-weight (DW) of sample. The equation of standard curve was obtained y=1.8162x (R^2=0.9901), where x is the Gallic acid concentration in mg/ml and y is the absorbance reading at 760nm. The total phenolic contents were calculated by using this formula.

$$\text{TPC}\left(\frac{\text{mg}}{\text{g}}\right) = \frac{\left(\text{concentration of gallic acid}\left(\frac{\text{mg}}{\text{ml}}\right) \times \text{volume of sample used (ml)}\right)}{\text{mass of the extract (g)}}$$

Equation 3.1

DPPH Assay

About 2.5mg of gallic acid was diluted with 100ml of methanol and used for standard. Then, a series of gallic acid concentration dilution was prepared before mixing the solution. 2.0mg of DPPH was weighed and diluted with 50ml of methanol. 100µl of gallic acid and 100µl DPPH was transferred into the well. The microwell plate was kept in dark for about 30 minutes. Methanol was used as positive control. After 30 minutes, the absorbance of the solution was measured at 517nm with microplate reader using Tecan

microplate reader. The reading were repeated by replacing gallic acid standard with phenolic samples. The percentage inhibition of DPPH radical scavenging activity was calculated using the formula of:

$$\text{DPPH Scavenging Activity (\%)} = \left(\frac{\text{Abs control} - \text{Abs samples}}{\text{Abs control}}\right) \times 100$$

Equation 3.2

Absorbance control represent the absorbance reading without sample and absorbance samples represent the absorbance with samples. IC^{50} values were calculated to find the samples concentration required to reduced 50% absorbance of DPPH by plotting graph of percentage inhibition of DPPH scavenging activity against samples concentration.

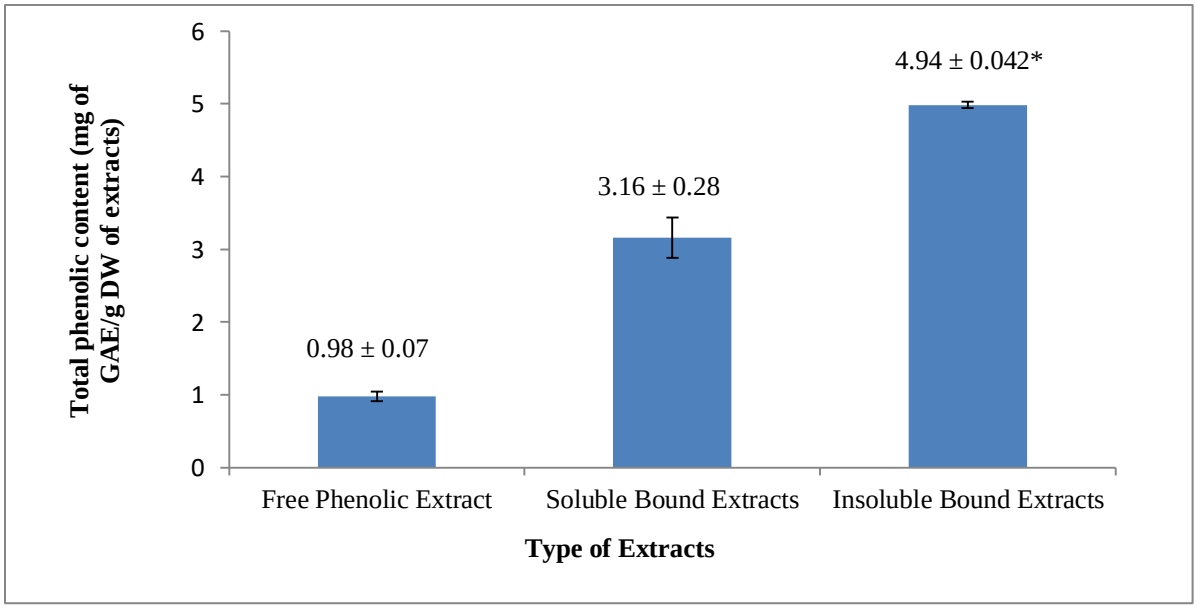
Statistical analysis

All experiments were conducted in triplicates and data reported are mean ± standard deviation. All data of samples were analyzed by using Statistical Package for Social Science (SPSS) version 23.0. Comparison of difference of total phenolic contents and the DPPH radical scavenging capacity phenolic compound in free, bound soluble and bound insoluble extraction were analyzed with one-way ANNOVA and Turkey Test at 95% confidence.

RESULT

Total Phenolic Content (TPC)

Figure 1 shows that the total phenolic content of *C. vespertilionisis* in free phenolic, soluble bound and insoluble bound extracts. The total phenolic content in *C. vespertilionis* was 9.08 ± 0.13mg of GAE/g samples.



*The mean difference is significant at the 0.05 level (ANOVA test)

Figure 1
The total phenolic content of *C. vespertilionisis* in free phenolic, soluble bound and insoluble bound extracts.

Based on the result, the highest phenolic extracts was seen in the insoluble bound extract (4.94 ± 0.042 mg of GAE/g sample) followed by soluble bound extracts (3.16 ± 0.28 mg of GAE/g sample) and free phenolic extracts (0.98 ± 0.07 mg of GAE/g sample) ($p<0.05$). Within group, it was significantly difference between free phenolic with soluble bound extracts, free phenolic with insoluble bound extracts and soluble bound extracts with insoluble bound extracts ($p<0.05$).

Antioxidant Radical Scavenging Capacity

The percentage of radical scavenging capacity (I%) for three different sample extracts (free, soluble bound and insoluble bound phenolic) are presented in Table 1. Based on the result, at the concentration of 50mg/ml, the extract showed highest percentage of inhibition in radical scavenging capacity where soluble bound extract is the highest (59.76 ± 1.31%), followed by insoluble bound extract and free phenolic extract where the percentage are (54.99 ± 1.25% and 54.52 ± 1.49% respectively) ($p< 0.05$) significance difference. The result showed that the scavenging activity increased with the increase of the concentration of the extracts.

Table 1
Antioxidant radical scavenging capacity

Concentration (mg/l)	**Radical Scavenging Capacity (% inhibition)**		
	Free phenolic Extract	Soluble bound Extract	Insoluble bound Extract
2.5	8.26 ± 2.38	6.81 ± 1.71	24.22 ± 5.93
3.35	15.98 ± 3.43	14.83 ± 6.03	29.44 ± 3.49
5	18.20 ± 2.84	18.68 ± 6.94	35.14 ± 4.00
10	25.48 ± 3.47	23.44 ± 6.58	43.05 ± 1.38
50*	54.52 ± 1.49	59.76 ± 1.31	54.99 ± 1.25

Values are expressed as mean of triplicates % ± SD, (n=3)
*indicated significance difference ($p<0.05$)

IC_{50} values of the samples were calculated based on the equation from the graph of each dilution. Table 2 shows IC_{50} values of *C. vespertilionis* extracts for free phenolic, soluble bound and insoluble bound. Insoluble bound extract showed the highest antioxidant properties (38.13±0.65), followed by soluble bound and free phenolic extracts (39.54 ± 0.77 and 42.59 ± 1.69 mg/ml, respectively) ($p<0.05$).

Table 2

IC_{50} of free phenolic, soluble bound and insoluble bound extracts in *C.Vespertilionisis*

Extracts	**Mean IC^{50} (mg/ml)**
Free phenolic	42.59 ± 1.69
Soluble bound	39.54 ± 0.77
Insoluble bound	38.13 ± 0.65*

Values are expressed as mean of triplicates mg/ml ± SD, (n=3)
* indicated significant difference ($p<0.05$)

Based on the analysis of the mean for the inter-relationships between three samples, the results showed that there were significantly differenced ($p < 0.05$) between free phenolic and bound soluble extracts. In fact, free phenolic and soluble bound extracts also showed a significant difference ($p<0.05$).

DISCUSSION

Total Phenolic Content (TPC)

The present research was aim to quantify the TPC and antioxidant, radical scavenging capacity (I%) of *C. vespertilionisis.* The plant was extracted into three different extracts (free phenolic content, soluble bound and insoluble bound) phenolic. All the phenolic extracts were extracted by 80% methanol. Govardhan, (2013) emphasized that type of solvent used in order to extracts phytochemical compounds was an important factor to produce high yield of phytochemical compound. Other than that, the extraction process also determines the quality of phytochemical compound analysis.

Previous study done by Su *et al.,* (2010) found that TPC of 80% methanol extracts in litchi pulp in free bound was slightly higher than bound phenolic about 190.69 ± 3.69 mg of GAE/g sample and 61.27 ± 1.78 mg of GAE/g sample respectively. This can be supported by identification and quantification done Nicoletti *et al.,* (2013) by using RP-HPLC which showed TPC in bound phenolic compound in durum wheat showed the highest phenolic content compared to free phenolic and insoluble phenolic with bound phenolic content contribute 61- 83.6% of total phenolic acid. The recovery of phenolic compound was discovered by increasing the extraction temperature (Bonolli et al.,2010 and Durling, 2007) which it will correspond to the release of bound polyphenol. These figures were much higher than those obtained in the present study. The observed differences could in part be related to the use of different varieties and standards for calculation

Furthermore, the used of solvent is merely important factor in extracting the phenolic compound (Govardhan et al., 2013). There is no specific universal solvent used to extract the phenolic compound from plants. In fact, selection the right amount of solvent affected the amount of extracted phenol (Xu et al, 2007). For example, Alothman *et al.,* (2013) showed that TPC of pineapple from 50%, 70% and 90% methanol extraction yield 39.0±1.42 mg of GAE/g sample, 51.3±1.92 mg of GAE/g sample, 35.6±1.16 mg

of GAE/g sample of phenolic content respectively. Thus, choosing the right solvent and suitable ratio for extracting phenolic compound contribute to the value itself.

Antioxidant Radical Scavenging Capacity

Stable DPPH scavenging activity is widely used to quantify and evaluate antioxidant activity. This technique is advantageous compared to other is due to require of short time in reducing the DPPH · form to DPPH non-radical by donating hydrogen atom (Dai and Mumper, 2010). Reduced DPPH will be determined by the reduction of absorbance at 517nm by the antioxidants. The physical change during evaluation of DPPH is the color turn from purple to yellow (Govardhan *et al*., 2013). The chemical structures of polyphenol that contain hydroxyl group are related to the antioxidant properties that scavenge the DPPH radical. Precisely, the hydroxyl group acted as the primary antioxidant.

The antioxidant radical scavenging capacities of all phenolic extracts of *C. vespertilionisis* from the analysis of IC_{50} in this study were ranked as follow: Bound insoluble phenolic extract (IPE) > Bound soluble phenolic extract (SPE) > Free phenolic extract (FPE). This trend suggested that the ability of the plant extract to act as a powerful radical scavenging activity was influenced by the extraction solvent used to extract the plant. This was supported by Verma *et al*., (2009), where acid hydrolysis treatment showed highest antioxidant activity compare with the extract without any treatment. Nevertheless, the use of acid hydrolysis treatment can decrease the content of the phenolic acids in the extract (Krygier *et al.,* 1982) but the addition of a metal chelator like (EDTA) during the alkaline hydrolysis treatment can help to prevent the degradation of phenolic acids (Nardini *et al*., 2002). Other factors might influence the antioxidant activity of the plant extract such as temperature, the concentration of the antioxidant and the pH used during extraction process (Gazzani *et al*., 1998). However, this study was only focusing on the total phenolic compound (free, soluble and insoluble) that presence in the sample extract thus it is not known whether others factor might influence the antioxidant activity of the plant. These reports further affirmed that the total phenolic content does influence the antioxidative capability of the plant extract.

CONCLUSION

Insoluble bound extracts showed greater percentage of phenolic extracts. Generally, the samples contain both free and bound phenolic content where the bound phenolic is much higher. Meanwhile, the antioxidant radical scavenging capacity in soluble bound extract was higher than free phenolic and insoluble bound extracts.

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