Antioxidant and Mutagenic Activity of Herbal Tea Prepared from *Cosmos caudatus* Leaves at Different Maturity Stages

**Dian-Nashiela Fatanah, Noriham Abdullah*, Nooraain Hashim & Azizah Ab. Hamid**

**ABSTRACT**

Different maturity stages of *Cosmos caudatus* leaves have been used to prepare herbal tea were investigated for their effect on antioxidant activity and mutagenic activity. The analyses carried out were total phenolic content (TPC), total flavonoid content (TFC), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, β-carotene bleaching assay, oxygen radical absorbance capacity (ORAC) and *Ames Salmonella* mutagenicity. The results demonstrated that, *C. caudatus* herbal tea prepared from young leaves showed significantly highest antioxidant activity for all assays tested, followed by mixed leaves, mature leaves, old leaves and the lowest was in *C. caudatus* herbal tea from a commercial brand. Pearson’s correlation coefficient also demonstrated that TPC and TFC displayed a strong correlation with all antioxidant activity assays, showing that these compounds were the major contributors to the antioxidant activity in *C. caudatus* herbal tea. However, all studied *C. caudatus* herbal tea showed no mutagenic effects against *Salmonella typhimurium* tester strain TA98 and TA100 with and without S9 metabolic activation. Hence, it can be concluded that, different maturity stages could affect the antioxidant activity in *C. caudatus* herbal tea as it reduced the antioxidant activity as maturity increased, but did not give any effect on the mutagenic activity.

**Keywords:** Antioxidant activity; *Cosmos caudatus*; herbal tea; maturity; mutagenic activity

**INTRODUCTION**

Herbal tea is a tea brewed from the leaves, flowers, seeds, fruits and roots of plant species other than *Camellia sinensis* (Kara 2009). Nowadays, many countries especially Asia have varieties of herbal teas. In Malaysia, there are abundant of herbal teas produced by Small Medium Enterprise (SME) industry such as *misai kucing* (*Orthosiphon stamineus*) tea, *kacip fatimah* (*Labisa pumila*) tea, *kaca beling* (*Strobilanthes cripa*) tea, ginger (*Zingiber officinale*) tea and lemongrass tea. *Cosmos caudatus* or known as *ulam raja* is amongst of the herbs that are rich in potential health properties and has functionality to the consumer health and recently it is getting attention by the Malaysian herbal industries to be developed in tea form (Dian-Nashiela et al. 2015).

Traditionally, *C. caudatus* has been used to reduce body health, improving blood circulation, as anti-aging agent, strengthening bone marrow (because of high calcium content), to treat infection associated with pathogenic microorganisms and to promote fresh breath (Amna et al. 2013). It has been reported that, these beneficial health properties have been attributed to the higher antioxidant content in *C. caudatus* plant predominantly, a number of
proanthocyanidins that exist as dimers through hexamers, quercetin, chlorogenic acid, catechin, epicatechin, myricetin and naringenin (Shui et al. 2005). Nevertheless, usage of *C. caudatus* leaves at different maturity stages as raw material in herbal tea preparation could affect their antioxidant activity as well as their tolerance towards the mutagenicity level. Some current studies showed that, maturity stages could influences the antioxidant activity, biochemical compositions and physicochemical properties of the plants (Fawole & Opara 2013). In fact, there have been reports on increased global demands for herbal products that acts as energy boosters, detoxifiers, immune boosters and aphrodisiacs, thus, the evaluation of bacterial mutagenicity is important as an initial test for complex mixtures because of the possibility that, one or more of their components can be mutagenic (Ndhlala et al. 2010). Therefore, this present study was conducted to investigate the effects of antioxidant activity and mutagenic activity of herbal tea prepared from *C. caudatus* leaves at different maturity stages.

**MATERIALS AND METHODS**

**CHEMICALS**

All chemicals and standards were purchased from Sigma-Aldrich Chemie, Germany.

**RAW MATERIALS COLLECTIONS AND SELECTION**

The fresh leaves of *C. caudatus* plant were planted and harvested in Durian Tunggal, Malacca, Malaysia at 8-week-old. The leaves were divided into 3 groups and classified as young leaves, mature leaves and old leaves. As method described by Dian-Nashiela et al. (2015), young leaves were selected from the first four tiers where the leaves are still tender, newly emerged and not attaining full expansion. Mature leaves are located at the middle part of the plant where the leaves are fully developed while old leaves are situated at the lower part of the plant and the leaves had showed initial sign of senescence. Mature leaves were selected between the fifth to eighth tiers and old leaves were selected starting from ninth tiers and above.

**SAMPLE PREPARATION**

Each group of *C. caudatus* leaves were prepared according to the normal procedure as being conducted for herbal tea preparation by Small Medium Enterprise (SME) industry in Malaysia. The leaves at different maturity stages were dried at 50°C for 8 h in cabinet dryer until constant weight. Then, the dried leaves of *C. caudatus* were processed in powder form based on Giao et al. (2009). The dried leaves were milled using ultra centrifugal mill at 8000 rpm and the milled leaves were then screened through different sieves sized ranged from 1 to 2 mm. After that, 2 g of the sieved leaves were collected and packed in a tea bag. Then, all the *C. caudatus* herbal teas were infused in 200 mL boiling distilled water for 3 min according to method suggested by Horžić et al. (2009). The infused *C. caudatus* herbal tea filtered through Whatman filter paper No. 41 prior to further analyses.

**DETERMINATION OF ANTI-OXIDANT ACTIVITY**

**TOTAL PHENOLIC CONTENT (TPC)**

The total phenolic content in *C. caudatus* herbal tea samples were determined by using the Folin-Ciocalteu assay (Harbourne et al. 2009). Accurately, 0.5 mL Folin-Ciocalteu reagent, 1.5 mL 7.5% sodium carbonate and 7.8 mL distilled water were introduced in a test tube containing 0.1 mL sample/standard. The solution was mixed thoroughly and allowed to stand for 2 h in a dark place. The absorbance was read at 765 nm and the results were expressed as mg gallic acid equivalent (mg GAE)/mL of herbal tea.

**TOTAL FLAVONOIDS CONTENT (TFC)**

The total flavonoid content was analysed according to method as described by Singh et al. (2012). One mL of *C. caudatus* herbal tea sample/standard was diluted with 4 mL distilled water, then 0.3 mL 5% sodium nitrate solution and 0.3 mL 10% aluminum chloride were added. The mixture of solution was kept for 5 min. After that, 2 mL of 1 M sodium hydroxide were added to the mixture and the mixture was vortexed thoroughly. The absorbance was measured at 510 nm using UV-VIS spectrophotometer. This was calculated as mg of quercetin (mg QE)/mL of herbal tea.

**FERRIC REDUCING ANTIOXIDANT POWER (FRAP)**

The FRAP assay for *C. caudatus* herbal tea samples was carried according to method of Deetae et al. (2012). The FRAP reagent was freshly prepared by mixing 300 mM acetate and glacial acetic acid buffer (pH 3.6), 20 mM ferric chloride and 10 mM TPTZ was made to 40 mM acetate and glacial acetic acid buffer (pH 3.6). Briefly, 0.1 mL sample/standard was mixed with 3 mL FRAP reagent and 3 mL distilled water. The mixture was incubated in the dark place at 37°C for 8 min and the absorbance was then read at 595 nm. The total antioxidant activity of *C. caudatus* herbal tea samples were determined against a standard of known FRAP value and was expressed as µM of trolox equivalent (µM TE)/mL of herbal tea.

**DPPH (2,2-DIPHENYL-1-PICRYLHYDRAZYL) RADICAL SCAVENGING ASSAY**

The DPPH assay was performed according to procedure as described by Nuengchamnong and Ingkaninan (2010). Accurately, 0.1 mL of sample/standard was mixed with 2.9 mL 0.05 mM DPPH in methanol and incubated in the dark at room temperature for 30 min. The radical scavenging activity of *C. caudatus* herbal tea/standard was measured as a decrease in the absorbance of DPPH using UV-VIS spectrophotometer where methanol was used as blank.
**B-CAROTENE BLEACHING ASSAY**

The antioxidant activity of all *C. caudatus* herbal tea samples is based on the \(\beta\)-carotene bleaching assay method developed by Velioglu et al. (1998). The \(\beta\)-carotene (0.2 mg in 1 mL chloroform), linoleic acid (0.02 mL) and Tween 20 (0.2 mL) were transferred into a round bottom flask. Chloroform was removed at room temperature under vacuum at reduced pressure using rotary evaporator. Following evaporation, 50 mL of distilled water was added to the mixture and then shaken vigorously to form emulsion. About 2 mL aliquots of the emulsion were pipette into test tubes containing 0.2 mL of the ethanol/comboination of BHA/BHT standard/*C. caudatus* herbal tea samples and immediately placed in water bath at 50°C for 120 min and was read at 470 nm. Antioxidant activity (AA) was expressed as percent of inhibition relative to the control.

**OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC) ASSAY**

For ORAC assay, the antioxidant activity of *C. caudatus* herbal tea samples were analysed according to Khairusy et al. (2012) method. About 25 µL of sodium phosphate buffer (as a blank)/standard/samples were added into 96-well plate containing 150 µL of fluorescein working solution. The 96-well plate was then placed in the multi-mode microplate reader and incubated for 5 min at 37°C. After the incubation period, 25 µL of AAPH solution was added to all of the experimental wells. The excitation and emission were set at 495 and 528 nm, respectively. The relative fluorescein intensity was monitored and recorded every 2 h. Then, the final ORAC value was calculated using the net area under the decay curves and was expressed as µmol of trolox equivalent (µmol TE)/mL of herbal tea.

**DETERMINATION OF MUTAGENIC ACTIVITY**

**AMES SALMONELLA ASSAY**

The Ames *Salmonella* assay was performed using method suggested by Mortelmans and Zieger (2000). The procedure involves adding the buffer or S9 liver metabolic activation, the histidine dependent bacteria (about 10^9) and test chemical to 2 mL of top agar containing biotin and trace amount of histidine (0.05 mM each). The mixture was then gently mixed and poured on glucose minimal (GM) agar plates. When the top agar solidified, the plates were incubated in an inverted position at 37°C inside an incubator for 48 h at time which the histidine revertant colonies were counted. The colonies were counted using automatic colony counter.

### TABLE 1. Antioxidant content and antioxidant activity of *C. caudatus* herbal tea prepared at different maturity stages

<table>
<thead>
<tr>
<th>Herbal tea sample</th>
<th>TPC (mg GAE/mL herbal tea)</th>
<th>TFC (mg QE/mL herbal tea)</th>
<th>FRAP (µM TE/mL herbal tea)</th>
<th>DPPH (µg/mL)</th>
<th>(\beta)-carotene (Antioxidant activity, %)</th>
<th>ORAC (µmol TE/mL of herbal tea)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young leaves</td>
<td>66.30 ± 5.20a</td>
<td>203.22 ± 15.90a</td>
<td>502.21 ± 21.18a</td>
<td>1055.37 ± 42.38a</td>
<td>77.89 ± 6.38a</td>
<td>3.65 ± 0.15a</td>
</tr>
<tr>
<td>Mature leaves</td>
<td>36.55 ± 2.77b</td>
<td>124.55 ± 6.22b</td>
<td>332.00 ± 8.81b</td>
<td>1409.99 ± 103.17b</td>
<td>65.89 ± 3.00b</td>
<td>2.18 ± 0.18b</td>
</tr>
<tr>
<td>Old leaves</td>
<td>18.38 ± 2.46c</td>
<td>72.24 ± 3.04c</td>
<td>239.18 ± 19.19c</td>
<td>2408.84 ± 365.36c</td>
<td>56.01 ± 3.76c</td>
<td>0.92 ± 0.09d</td>
</tr>
<tr>
<td>Mixed leaves</td>
<td>46.79 ± 1.32b</td>
<td>149.32 ± 2.84b</td>
<td>373.36 ± 12.30b</td>
<td>1362.19 ± 217.39d</td>
<td>62.72 ± 4.38b</td>
<td>3.20 ± 0.02b</td>
</tr>
<tr>
<td>Commercial brand</td>
<td>7.39 ± 0.91e</td>
<td>17.20 ± 1.07e</td>
<td>84.17 ± 5.84e</td>
<td>7033.00 ± 612.25e</td>
<td>44.12 ± 2.66d</td>
<td>0.62 ± 0.06e</td>
</tr>
</tbody>
</table>

Values are expressed as mean and standard deviation. Means with different letter within a column are significantly different \((p<0.05)\).
in which the 8-week-old *C. caudatus* plant give most powerful antioxidant activity compared to 10-week-old and 12-week-old of this plant. This is because the better sunlight exposure on young leaves than on mature and old leaves (Fernando et al. 2013) might aids most of new biosynthesis, simultaneously produce higher antioxidant in young leaves (Menichini et al. 2011). Due to strong antioxidant activity, it leads to the formation of macromolecular compounds with the stronger radical scavenging power which may be attributable to the increased resonance delocalisation and higher stability of the aryloxyl radicals incurred by hydrogen bonding (Farhooosh et al. 2007). For these reasons, Müller et al. (2013) believed that, total phenolics and flavonoids that prevailed during the early maturity stages possess a great ability to scavenge light-induced reactive oxygen species (ROS). Other than that, Barros et al. (2007) believed that, the reduction of antioxidant activity is due to the aging process, stimulates the formation of ROS and cause extensive production of ROS, which are then neutralised by the phenolic compounds, resulting in the lowering of their content and antioxidant activity. Since the mature and old leaves possess inadequate antioxidant activity and/or owing overproduction of ROS, this equilibrium is hampered favouring ROS surge that culminates in oxidative stress, eventually senescence of plant tissues (Sreelatha & Padma 2009).

Nevertheless, at a physiological level, the combination of many phytochemicals even at low concentrations, provides the molecules that display additive and very often synergistic effects in their antioxidant properties (Blasa et al. 2010). This is proven from the combination of *C. caudatus* leaves from three different maturity stages, where *C. caudatus* herbal tea prepared from mixed leaves showed highest antioxidant activity than mature and old leaves singly. This effect most probably due to depolymerisation and intermolecular of H-bonding reaction as well as might be due to the easier accessibility of the phenolic-OH (Celik et al. 2010). On the other hand, *C. caudatus* herbal tea from commercial brand recorded the lowest antioxidant activity than other *C. caudatus* herbal teas. This high variation in the antioxidant activity is due to the manufacturing conditions employed by the company, the differences in the composition of teas, that is, the company uses different leaf to stem ratios (Zielinski et al. 2014) and also because of the geographical origin as well as agronomic situation (Shahidi & Naczk 2004).

**PEARSON’S CORRELATION COEFFICIENT**

The correlation analyses by using Pearson’s correlation coefficient were conducted to determine the interrelationship between antioxidant content (TPC and TFC) with antioxidant activity of four independent assays (FRAP, DPPH, β-carotene and ORAC) of *C. caudatus* herbal tea. It was observed that (Table 2), FRAP, β-carotene and ORAC assay had positively strong correlation with TPC and TFC while DPPH scavenging assay showed negative correlation with TPC and TFC. This is in agreement with study by Barros et al. (2007) who reported that, samples with higher antioxidant content showed higher antioxidant activity and lower IC₅₀ values while the sample with lowest antioxidant content exhibited lower antioxidant activity and IC₅₀, thus produced negative correlation between DPPH with TPC and TFC. Therefore, from these correlations, it indicated that, phenolic and flavonoid compounds might be the major contributor in *C. caudatus* herbal tea samples. It has been highlighted that the contribution of phenolic and flavonoid compounds to *in vitro* antioxidant activity of herbs (Andarwulan et al. 2010).

**TABLE 2. Pearson’s correlation coefficient (R) between antioxidant activity of *C. caudatus* herbal tea prepared from leaves at different maturity stages**

<table>
<thead>
<tr>
<th></th>
<th>FRAP</th>
<th>DPPH</th>
<th>β-carotene</th>
<th>ORAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC</td>
<td>0.9967 ± 0.002ᵃ</td>
<td>−0.9224 ± 0.058ᵇ</td>
<td>0.978 ± 0.030ᵃ</td>
<td>0.984±0.015ᵃ</td>
</tr>
<tr>
<td>TFC</td>
<td>0.9980 ± 0.002ᵃ</td>
<td>−0.9288 ± 0.060ᵇ</td>
<td>0.986 ± 0.017ᵃ</td>
<td>0.993±0.008ᵇ</td>
</tr>
</tbody>
</table>

Values are expressed as mean and standard deviation. Means with different letter within a column are significantly different (p<0.05)

**MUTAGENIC ACTIVITY**

In terms of mutagenicity, all *C. caudatus* herbal tea samples were investigated by using *Salmonella typhimurium* tester strains TA98 and TA100 in the absence and presence of the S9 metabolic activation. Tables 3 and 4 tabulated the number of revertants/plate in *S. typhimurium* strains and the mutagenic index (MI) respectively, after treatment with all *C. caudatus* herbal tea samples. From the results obtained, none of these *C. caudatus* herbal teas were detected any sign of mutagenicity towards the *S. typhimurium* strain TA98 and TA100 for the assay with and without S9 metabolic activation since the average revertants numbers did not satisfy the criteria for mutagenicity. According to Ndhlala et al. (2010), if there were no notable increase in the number of revertants and the number of revertants were not equal or not greater than two times of the positive control or there were no reduction in the number of revertant colonies to levels far below the negative control, then, the samples can be classified as non-toxic. On the other hand, the mutagenic index (MI) also displayed the values lower than that of potential mutagenicity. Santos et al. (2011) remarked that, sample is considered mutagenic potential when the MI is equal to or greater than two for at least one of the tested doses.

It should be noted that, the high antioxidant activity in all *C. caudatus* herbal tea samples could give protection against the mutagenic effect. This is because in general, the antioxidant activity of plant extracts is associated with group of compounds such as phenolic acids and flavonoids which could acts as stabilising agent of scavenging radicals (Fernando et al. 2013). This had been proven by few studies, for examples, Marnewick et al. (2000) reported...
that, phenolic compounds from water extracts of herbal teas dramatically decreased the mutagenicity of a variety of genotoxic and carcinogens while Saraç and Şen (2014) found that, extract of *Liquidambar orientalis* Mil var. *orientalis* which possessed higher antioxidant activity did not exhibit any mutagenic effect in the mutagenicity assay performed with *S. typhimurium* TA98 and TA100.

**CONCLUSION**

Hence, it can be concluded that, as the maturity of *C. caudatus* leaves used to prepare herbal tea increased, the antioxidant activity decreased significantly with the *C. caudatus* herbal tea prepared from young leaves possessed highest antioxidant activity. The strong Pearson’s correlation coefficient showed that, phenolic and flavonoid compounds are the major contribution to the antioxidant activity in *C. caudatus* herbal tea. Nevertheless, none of *C. caudatus* herbal teas studied induced any increased in the number of revertants, demonstrating the absence of mutagenic activity.

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Dian-Nashiela Fatannah, Noriham Abdullah* & Noorain Hashim Faculty of Applied Sciences Universiti Teknologi MARA 40450 Shah Alam, Selangor Darul Ehsan Malaysia

Noriham Abdullah* Malaysia Institute of Transport (MITRANS) Universiti Teknologi MARA 40450 Shah Alam, Selangor Darul Ehsan Malaysia

Noriham Abdullah* Malaysia Institute of Transport (MITRANS) Universiti Teknologi MARA 40450 Shah Alam, Selangor Darul Ehsan Malaysia

Abd. Hamid, Azizah Department of Food Sciences Faculty of Food Science and Technology Universiti Putra Malaysia 43400 UPM Serdang, Selangor Darul Ehsan Malaysia

*Corresponding author; email: noriham985@salam.uitm.edu.my

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