ORIGINAL ARTICLE

In Vitro Antioxidant Properties and Methylglyoxal (MGO) Scavenging Effects of *Centella asiatica* Leaves in Water Extract

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ABSTRACT

Introduction: Advanced glycation end product (AGE) is formed by the non-enzymatic glycation, leading to free radicals' production and high oxidative stress. Accumulation of AGEs is associated with chronic diseases which are correlated with diabetes mellitus. *Centella asiatica* is a traditional medicinal herb used in Asia with numerous pharmacological effects such as antioxidant, wound healing, neuroprotective, and gastrointestinal treatment. This research was aim to evaluate the antioxidant, antiglycation, and phytochemical analysis of *C. asiatica* leaves (L.) water extract. **Methods:** The antioxidant activity of the plant extract was evaluated through 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl (OH) radicals, nitric oxide (NO), and chelating capacity assays. Total phenolic content (TPC) was performed to determine the presence of bioactive compounds, such as saponins, which have been extensively reported in previous studies. Butylated hydroxytoluene (BHT) and ethylenediaminetetraacetic acid (EDTA) were used as positive control in the antioxidant assays, while quercetin was used in the antiglycation assays. Antiglycation effects of *C. asiatica* L. were evaluated through Bovine serum albumin (BSA)-MGO, BSA-glucose, and MGO scavenging assay. The data was expressed in terms of IC_{50}. **Result:** The plant extract possessed significantly ($p < 0.001$) lower IC_{50} value compared to the BHT in DPPH and NO radical scavenging assay. It also showed stronger antiglycative effect with lower IC_{50} value compared to the quercetin in BSA-glucose and BSA-MGO assay. The TPC of *C. asiatica* L. was 3.20 mg/GAE/g at 100µg/ml. **Conclusion:** The antiglycation effects of C. asiaitca L. include the reduction of Amadori products formation, trapping α-dicarbonyl intermediates, and free radical scavenging. It is suggested that the antiglycation effects of *C. asiatica* L. maybe attributed by the presence of saponins and other phytochemicals. *Malaysian Journal of Medicine and Health Sciences* (2022) 18(6):183-192. doi:10.47836/mjmhs18.6.25

Keywords: *C. asiatica* L., AGE, MGO, antioxidant

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INTRODUCTION

The non-enzymatic glycation between reducing sugars and free amino groups or other carbonyl compounds is known as the Maillard reaction. During the early stage of glycation, reducing sugars react with a free amino group to form an unstable compound where the Schiff base undergoes rearrangement to form a steadier product which is Amadori products (1). The Amadori product further degrades into reactive dicarbonyl compounds such as methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG) (2). The reaction further produces AGEs in the late stage of the glycation. AGE is an irreversible compound created via oxidation, dehydration, and cyclization reactions (3). The accumulation of AGEs hinders the protein conformation where the mutation in the cells disrupt the development. Life-long accumulation of AGEs in the human body causes diabetic complications, aging, atherosclerosis, and other chronic complications (3).

Development of free radical, reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the human body are unavoidable as they can be formed endogenously with normal body metabolism while exogenous sources are mainly from diets, alcohol, and pollutants (3). Free radicals such as superoxide (O–2), hydroxyl (OH.), and peroxyl (ROO.) are utilised and generated throughout the glycation steps (3). OH radical is the most reactive oxidant among the ROS. It induces the oxidative degradation of deoxyribose sugar, lipids and protein, resulting in the formation of Schiff bases and Amadori products, and also generate various types of corresponding free radicals (4). Reactive dicarbonyl intermediates and free radicals are being formed

by the rearrangement of Amadori products during the intermediate stage of glycation. Imbalance ratio between free radicals and antioxidants lead to oxidative stress. Glycoxidation causes lipid peroxidation and autoxidation of aldose sugar, leading to the production of a low-molecular end product called malondialdehyde (MDA) (5). High amounts of MDA increase oxidative stress and amplify glycoxidation-induced damages. Further, high oxidative stress accelerates the AGEs formation, allowing more AGEs bind to the membrane receptor of AGE (RAGE). AGE-RAGE activates the tumor necrosis factor-alpha (TNF-α) and nuclear factor kappa-B (NF-κB), hence increases oxidative stress and thus create a pro-inflammatory status in endothelial cells (6).

C. asiatica (pegaga) is generally recognized as the Indian pennywort which belongs to the family of Apiaceae or Umbelliferae. It is an essential herbal medical herb used in several treatments and is used in Indian Ayurvedic medicine as a nerve tonic, in traditional Chinese medicine and African medicine (7). A wide variety of pharmacological effects from *C. asiatica* have been reported, including dermatological effect, antioxidant, anti-inflammation, wound healing, revitalizing the nerves and brain, treatment of gastrointestinal diseases, and coronary disease (8). Many researchers have reported that the primary phytochemical constituents of *C. asiatica* are saponins, which are also known as triterpenoids. Triterpene saponins contain sapogenins such as asiaticosides, brahmoside, asiatic acid and brahmic acid, which were found to be responsible for wound healing and vascular effects by increasing the secretion of interleukin (IL)-1β and inhibit excessive collagen produced at wound sites, respectively (9). Furthermore, (10) examined the saponins showed antiglycation activity against AGEs. Saponins are the diverse family of structure containing a steroid or triterpenoids aglycone (sapogenin) linked to oligosaccharide moieties. (11) stated that madecassoside isolated from *C. asiatica* protects the endothelial cells from oxidative stress-induced injury. Asiatic acid, another triterpene saponins from *C. asiatica* has been shown to have antioxidative effect against oxidative stress stimulated by tert-butyl hydroperoxide (t-BHP) in HepG2 cells (12).

Saponins have been a widely preferred antioxidant candidate used in pharmaceutical formulation and application, cosmetics and food. The detailed structured mechanism of free radical scavenging activity of saponins are still unclear, however many researchers hypothesized that it may be correlated to its structural composition (13). *C. asiatica* L. accumulates large amount of triterpenoid saponins called centelloids. Centelloid is composed of a hydrophobic triterpenoid (aglycone) linked to a hydrophilic carbohydrate moiety (glycone) (14). The sub-types of centelloid include ursane-family and oleanane family. Olenane-typed saponin shows inhibitory activity on aldose reductase and free radical scavenging effects (15). Furthermore, (16) reported ursane-typed saponins possess antioxidant and anti-inflammation properties by scavenging hydrogen peroxide and nitric oxide radicals, respectively. In addition, ursane- and olenane-typed saponins extracted from *C. asiatica* contribute neuroprotective effect by increasing the mRNA expression of antioxidant enzymes such as superoxide dismutase and catalase (17). Other than saponins, bioactive compounds such as catechin, apigenin, rutin, quercetin, and kaempferol have also been detected in the plant. *C. asiatica* L. also contains phenolic contents such as apigenin, rutin, quercetin, and kaempferol. Castilliferol-1 and castillicetin-2 are the specific flavonoids isolated from the plant (18).

Antioxidative activity plays an important role in performing antiglycation and avoid the process of Maillard reaction, by protecting endothelial cells from oxidative stress via free radical scavenging and trapping of dicarbonyl intermediates. *C. asiatica* has been extensively reported to have excellent antioxidant and anti-inflammatory activity for decades. Therefore, this research was aimed to investigate the antioxidant and antiglycation properties of *C. asiatica* and identify the phytochemicals in the plants which might be play the roles in the antiglycative process.

MATERIALS AND METHODS

Collection of *C. asiatica* Leaves (L.)

About 2 kg of C. asiatica leaves were bought from the Astaka wet market, Sitiawan Perak. The leaves were bought and identified by a botanist in University Sains Malaysia (USM) and specimen voucher (USH 3074) was deposited at that institute.

Water extraction of *C. asiatica* L.

C. asiatica L. water extract in powder form was obtained from 1 kg of air-dried *C. asiatica* leaves after 7 days. The dried leaves were ground into fine powder and mixed with 5 L of distilled water. The mixture was left aside for 24 hours for infusion with occasional shaking. The solution was filtered on the next day and the extract was obtained by evaporating with the freeze dryer. The extract was then kept in the desiccator until further used (19).

Total Phenolic Contents

Total phenolic contents of *C. asiatica* L. water extract was determined by utilizing the Folin-Ciocalteu's reagent (20). To 100 µl of sample (10, 50, 100 µg/mL), 0.5 mL of Folin-Ciocalteu's reagent was added with 0.4 mL of 7.5% (w/v) sodium carbonate. After centrifugation, the mixture was incubated in the dark for 30 mins. The absorbance of reaction mixture was measured at 765 nm using a 96 well plate reader. The total phenolic content of the sample extract was expressed as gallic acid equivalent (GAE) in mg/g of sample extract.

In Vitro Antioxidant Assays

DPPH Radical Scavenging Assay

DPPH radical scavenging assay was done to determine the antioxidant activity of *C. asiatica* L. in water extract based on the method by (21). One mL of sample (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/mL) was added to 1 mL of methanol solution of DPPH (0.1 mM) and was vortexed. The mixture was then incubated for 30 mins in the dark and the absorbance was measured at 517 nm against a blank using a 96 well plate reader. The negative control used was methanol with DPPH, and BHT was used as the positive control. The results were expressed in the form of IC_{50}. The percentage of DPPH free radical inhibition was calculated using [Equation 1] mentioned below:

$$\text{Percentage of Inhibition (\%)} = \frac{\text{(Abs control - Abs sample)}}{\text{Abs control}} \times 100\% \quad \text{[Equation 1]}$$

Where:
Abs control = Absorbance of the control
Abs sample = Absorbance of the sample
Meanwhile, the IC_{50} value of the negative control was calculated by using Cheburator Software which is installed in the 96 well plate reader.

Hydroxyl Radical Scavenging Assay

Hydroxyl radical scavenging assay conducted with modification (21). A volume of 0.2 mL EDTA and 0.2 mL of 20 mM FeCl3 6H2O were added to 1 mL of sodium phosphate buffer (50 mM, pH 7.4). The reaction mixture was vortexed, then 0.2 mL of deoxy-D-ribose (60 mM) and 100 µL of *C. asiatica* water extract (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/mL) were added. The mixture was then incubated for 60 mins under 37°C in a water bath. A volume of 2 mL TBA and TCA, respectively were added into the reaction mixture and it was incubated for another 15 mins at 100°C. The absorbance of the reaction mixture was measured at 512 nm using 96 well plates reader. BHT and distilled water were used as positive control and negative control, respectively. [Equation 1] and Cheburator Software were used to calculate the results, which were expressed in terms of IC_{50}.

Chelating Capacity Assay

Iron chelating activity of *C. asiatica* L. water extract was conducted based on their ability to interfere with the formation of ferrozine-Fe^{2+}complex. The chelating capacity assay was based on the method conducted by (20) with modification. To 1 mL of sample (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/mL). A volume of 1 mL of $FeSO_4$ (0.1 mM) and 1 mL of ferrozine (0.25 mM) were added. The mixture was incubated for 10 mins at room temperature, the absorbance was measured at 562 nm against a blank solution using a 96 well plate reader. EDTA was used as a positive control. The inhibition percentage of ferrous ion chelating capacity was calculated using [Equation 1] and Cheburator Software, which were expressed in terms of IC_{50}.

Nitric Oxide Radical Scavenging Assay

The scavenging capacity of *C. asiatica* L. water extract towards nitric oxide radical was conducted based on the methods by (20) with modifications. To 0.5 mL of sample (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/mL), 1 mL of sodium nitroprusside (10 mM) and 0.5 mL of phosphate buffer saline were added and vortexed for 5 s and was incubated in the dark at room temperature for 150 mins. Then 1 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) was added to 0.5 mL of the incubated mixture. The mixture was then vortexed and incubated again for 5 mins. One millimetre of 0.1% N-1-naphthyl ethylenediamine dihydrochloride was added and incubated at 25°C for 30 mins. The absorbance of the pink colour chromophore was measured at 540 nm using a 96 well plate reader. BHT was used as a positive control. The results of *C. asiatica* L. water extract was calculated by using [Equation 1] and Cheburator Software, which were expressed in terms of IC_{50}.

In vitro Antiglycation Assays

BSA-Glucose Assay

BSA-glucose assay represented the early stage of glycation where the proteins glycate with aldose sugar to form Schiff bases and Amadori products. According to (20), 1 mL of *C. asiatica* L. extract at concentrations of (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/mL) was added to solution containing 1 mL of BSA and glucose (500 mM) in 0.1M of phosphate buffer solution. The reaction mixture was incubated for 5 mins. After incubation, 0.5 mL of sodium azide was added to the reaction mixture and was incubated for another 7 days at 37°C. The absorbance was measured at an excitation wavelength of 370 nm and an emission wavelength of 440 nm after 7 days. [Equation 1] and Cheburator Software were used to calculate the results, which were expressed in terms of IC_{50}.

BSA-MGO Assay

BSA-MGO assay represents the advanced stage of glycation where the reactive diacrbonyl intermediates, such as MGO, glyoxal, and 3-DG glycate with the amino groups of protein to form AGEs. Based on (20), 1 mL of *C. asiatica* L. water extract (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/mL) was added with 1 mL of BSA along with 1 mL of MGO, phosphate buffer, and 0.5 mL of Sodium azide. The reaction mixture was incubated for 7 days at 37°C in the dark. The absorbance was measured at an excitation wavelength of 370 nm and an emission wavelength of 440 nm after 7 days. [Equation 1] and Cheburator Software were used to calculate the results, which were expressed in terms of IC_{50}.

MGO Scavenging Assay

Zero point one two five millilitres (0.125 mL) of phosphate buffer solution, were mixed with 0.125 mL of MGO and 1mL of *C. asiatica* L. water extract. The reaction mixtures were incubated for 1 hour, 2 hours, 4 hours, and 6 hours. After the incubation, 0.25 mL of o-phenylethylamine was added and incubated for 30 mins to 1 hour. The absorbances of MGO-induced AGEs adducts called methylquinoxaline were measured at 315 nm using 96 well plates reader (22). [Equation 1] and Cheburator Software were used to calculate the results, which were expressed in terms of IC_{50}.

Statistical Analysis

All data were presented as mean ± standard deviation (SD) in triplicates (n = 3) using the SPSS Software One-Way ANOVA. IC_{50} values of *C. asiatica* L. were considered statistically significant when ap < 0.001, bp < 0.01, cp < 0.05 vs BHT and EDTA (positive control); a*p < 0.001, b*p < 0.01, c*p <0.05 vs negative control.

RESULTS

Total Phenolic Contents (TPC)

Total Phenolic Contents of *C. asiatica* L. water extract was compared with gallic acid equivalent (GAE) in mg/g of the sample extract. Table I displays the total phenolic content of three different plant extract concentrations. At 10 µg/mL, the total phenolic content of *C. asiatica* L. water extract was 1.44 mg/GAE/g extract followed by 2.38 mg/GAE/g extract at 50 µg/mL and 3.20 mg/GAE/g extract at 100 µg/mL.

Table I: Total Phenolic Contents of *C. asiatica* L. water extract

Concentration of *C. asiatica* L. water extract (µg/ml)	Total phenolic content of *C. asiatica* L. water extract (mg/GAE/g)
10	1.44
50	2.38
100	3.20

Where GAE: gallic acid equivalent

In vitro antioxidant assay

DPPH Radical Scavenging Assay

Referring to the Fig.1(A) on DPPH radical scavenging assay, *C. asiatica* L. showed a statistically significant of p < 0.001 with IC_{50} of 1.18 ± 0.10 µg/ml, compared to negative control (57.28 ± 0.02 µg/mL). and BHT (0.95 ± 0.17 µg/mL).

Hydroxyl Radical Scavenging Assay

C. asiatica L. water extract exhibited a hydroxyl radical scavenging activity with an IC_{50} of 22.84 ± 1.09 µg/mL, IC_{50} of BHT was 28.41 ± 0.18 µg/mL and IC_{50} for negative control was 38.77 ± 0.0001 µg/mL. Hydroxyl radical scavenging activity *C. asiatica* L. water extract was significantly (p < 0.001) better hydroxyl radical scavenging activity compared to the BHT and negative control. Fig. 1(B).

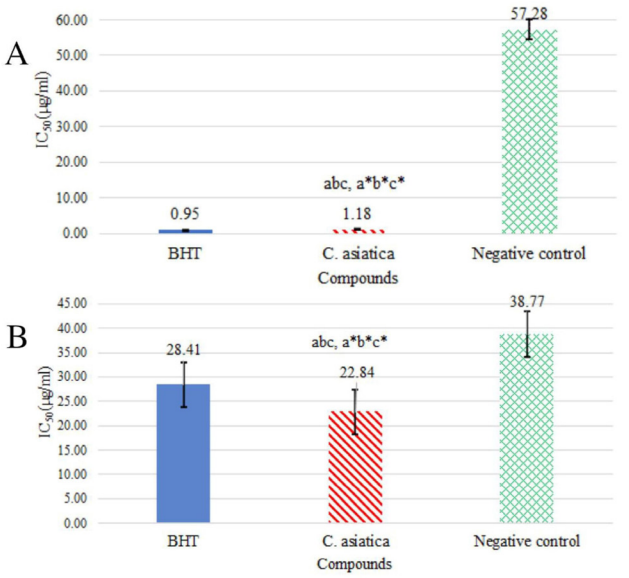

Figure 1: IC_{50} of *C. asiatica* L. water extract, positive control (BHT/EDTA), and negative control in antioxidant assays. DPPH radical scavenging assay (A) and hydroxyl radical scavenging assay (B). The IC_{50} were expressed in terms of mean ± standard deviation (SD) (n = 3).The data was statistically analysed by One-way ANOVA using SPSS Software. Alphabet a, b, and c denote the significant level of 0.001, 0.01, and 0.05, respectively. IC_{50} of *C. asiatica* L. water extract with alphabet labelling indicates it has statistically significant difference comparing to the controls. (*): plant extract compared to negative control.

Chelating Capacity Assay

IC_{50} of *C. asiatica* L. water extract was significantly lower than the negative control, which was 8.89 ± 0.25 µg/mL, and 23.27 ± 0.006 µg/mL, respectively in Fig. 2(A). The plant extract exhibited weaker chelating effect compared to EDTA which has a lower IC_{50} 4.89 ± 0.15 µg/mL obtained. Meanwhile, the plant extract was still considered effective in chelating as its IC_{50} was significantly (p < 0.001) lower when compared to the negative control.

Nitric Oxide (NO) Radical Scavenging Assay

C. asiatica L. water extract showed significantly (p < 0.001) stronger NO radical scavenging effect compared to BHT and negative control with the lowest IC_{50} value obtained (0.71 ± 0.12 µg/mL); while the IC_{50} of BHT was 1.67 ± 0.15 µg/mL and the negative control was 42.57 ± 0.001 µg/mL. Fig. 2(B)

In Vitro Antiglycation Assays

BSA-Glucose Assay

IC_{50} of *C. asiatica* L. was 6.44 ± 0.45 µg/mL, which was significantly (p < 0.001) lower and has stronger antiglycation effect compared to the quercetin (15.08 ± 1.79 µg/mL) and negative control (32.93 ± 0.24 µg/mL) in this glucose-induced BSA glycation assay. Fig. 3(A).

BSA-MGO Assay

C. asiatica L. water extract showed stronger antiglycation

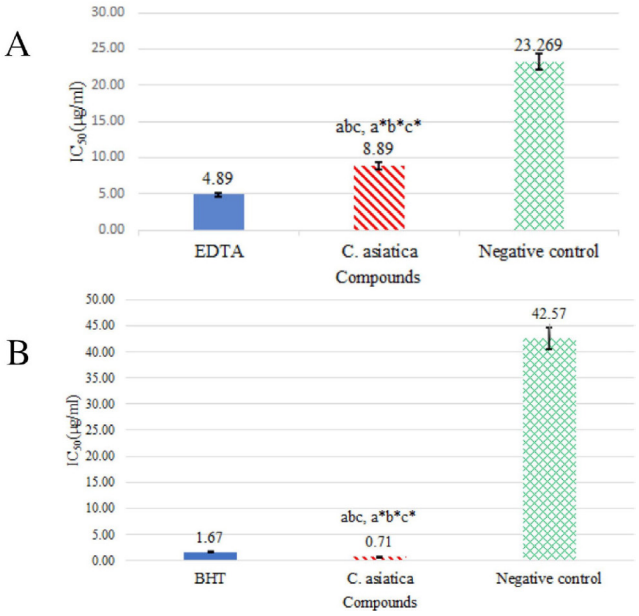

Figure 2: IC_{50} of *C. asiatica* L. water extract, positive control (BHT/EDTA), and negative control in antioxidant assays. Chelating capacity assay (A) and nitric oxide radical scavenging assay (B). The IC_{50} were expressed in terms of mean ± standard deviation (SD) (n = 3). The data was statistically analysed by One-way ANOVA using SPSS Software. Alphabet a, b, and c denote the significant level of 0.001, 0.01, and 0.05, respectively. IC_{50} of *C. asiatica* L. water extract with alphabet labelling indicates it has statistically significant difference comparing to the controls. (*): plant extract compared to negative control.

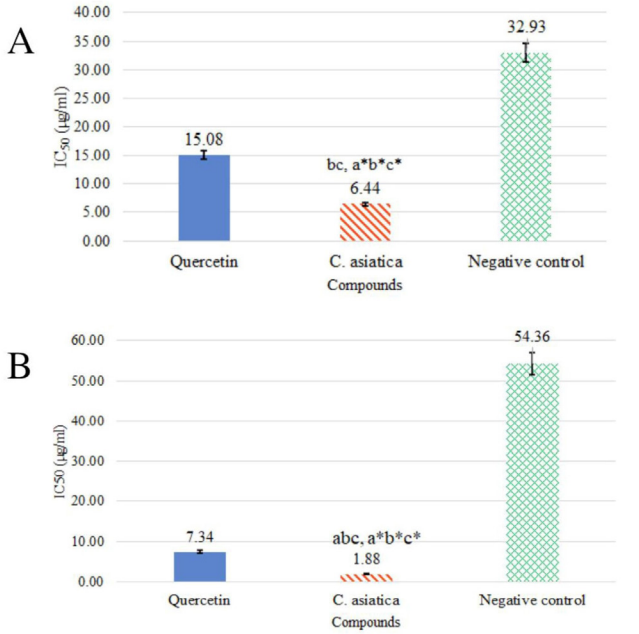

Figure 3: IC_{50} of *C. asiatica* L. water extract, positive control (quercetin), and negative control in antiglycation assays. (A) BSA-glucose assay and (B) BSA-MGO assay. The IC_{50} were expressed in terms of mean ± standard deviation (SD) (n = 3). The data was statistically analysed by One-way ANOVA using SPSS Software. Alphabet a, b, and c denoted the significant level of 0.001, 0.01, and 0.05, respectively. IC_{50} of *C. asiatica* L. water extract with alphabet labelling indicates it has statistically significant different comparing to the controls. (*): plant extract compared to negative control.

effects compared to quercetin and negative control, which can be referred in Fig. 3(B). The plant extract exhibited the lowest IC_{50} (1.88 ± 0.01 µg/mL), which showed better antiglycation effect than quercetin (7.34 ± 0.001 µg/mL) and negative control (54.36 ± 0.05 µg/mL).

MGO Scavenging Assay

The MGO scavenging effects of the *C. asiatica* L. water extract and quercetin expressed in terms of IC_{50} have been shown in Fig. 4. Throughout the incubation hours, the IC_{50} values of different concentrations of *C. asiatica* L. were significantly ($p < 0.001$) lower and exhibited stronger MGO scavenging effect compared to the negative control (60.34 µg/mL). The IC_{50} of *C. asiatica* L. water extract (8.19 ± 0.14 µg/mL) was at par ($p > 0.001$) with the quercetin (8.25 ± 0.21 µg/mL). This indicated that the *C. asiatica* L. water extract exhibited better MGO scavenging effect than the quercetin at the first hour of incubation. At the second hour of incubation, there was a significant ($p < 0.001$) drop of IC_{50} value in quercetin 4.20 ± 0.02 µg/mL compared to *C. asiatica* L. water extract (11.05 ± 0.97 µg/mL). From 4 to 6 hours of incubation, it showed that IC_{50} values of the quercetin and *C. asiatica* L. water extract have further decreased. The IC_{50} of *C. asiatica* L. water extract has decreased from 8.89 ± 0.35 µg/mL (4 hours) to 8.00 ± 0.49 µg/mL (6 hours). While, the IC_{50} of quercetin was significantly ($p < 0.001$) lower than the plant extract, which has decreased from 3.67 ± 0.23 µg/mL (4 hours) to 3.25 ± 0.06 µg/mL (6 hours). However, the *C. asiatica* L. water extract exhibited stronger MGO scavenging effect with longer incubation hours. Hence, with longer incubation hours, the *C. asiatica* L. water extract exhibited stronger MGO scavenging effect.

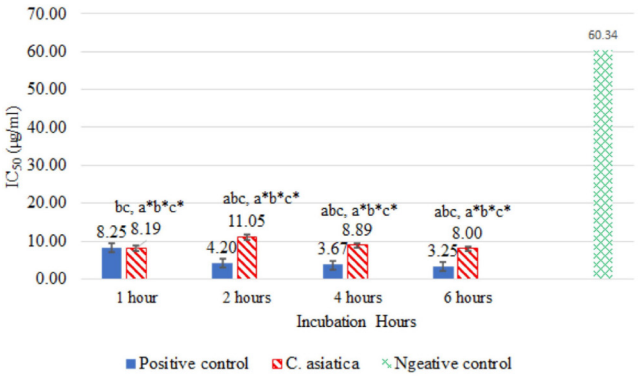

Figure 4: IC_{50} of *C. asiatica* L. water extract, positive control (quercetin), and negative control in MGO scavenging assays. The IC_{50} were expressed in terms of mean ± standard deviation (SD) (n = 3). The data was statistically analysed by One-way ANOVA using SPSS Software. Alphabet a, b, and c denoted the significant level of 0.001, 0.01, and 0.05, respectively. IC_{50} of *C. asiatica* L. water extract with alphabet labelling indicates it has statistically significant different comparing to the controls. (*): plant extract compared to negative control.

DISCUSSION

C. asiatica L. water extract exhibited effective free scavenging activity against DPPH radicals, hydroxyl radicals, NO radicals, as well as metal ions chelating. The free radical scavenging capabilities of plant extract is usually due to the presence of phenolic compounds and their ability to donate hydrogen ions. The TPC values of *C. asiatica* L. water extract was considered low, as it ranged from 1.44 mg/GAE/g to 3.20 mg/GAE/g. The possible reason for this circumstance was the water is not suitable solvent to extract TPC from C. asiaticsa L. According to the research done by (23), they discussed that water did not show strong capability of increasing TPC. However, the experiment observed that saponin was majorly extracted instead of phenolic compounds. Folin-Ciocalteu reagent is used to obtain a crude estimate amount of phenolic compounds present in the plant extract. Poor sensitivity and specificity of the Folin reagent has been reported by numerous researchers (24). Other phenolic compounds, other substances can be oxidised by the reagent too (24). This could be explained the low correlation between total phenolic contents and antioxidant activity of *C. asiatica* *C. asiatica* L. water extract. It also suggested that non-phenolic compounds also responsible for antioxidant activity. (25) reported that *C. asiatica* leaves contain high concentrations of madecassic acid, madecassosides, asiatic acid, and asiaticoside. These are the significant triterpenes compounds are classified as saponins, they have been used as the biomarker components for quality assessment of *C. asiatica* as well. This could be explained *C. asiatica* L. possessed effective antioxidant activity with lower IC_{50} values even though less TPC was detected.

Through standard phytochemical screening, saponins were found in the majority of a 100 g/mL water extract of *C. asiatica* L., whereas other phytochemicals such flavonoids and alkaloids were discovered in less. A stable persistent forth in the mixture of *C. asiatica* L. powder with distilled water indicated that there is the presence of saponins. A froth of emulsion was observed when a few drops of olive oil was added to the mixture (26). The major antioxidant and antiglycation activities of *C. asiatica* L. water extract may be attributed majorly by saponins, as well as the combined effect of other phytochemicals.

Discoloration of purple DPPH radical solution into yellow indicates that the *C. asiatica* L. water extract was able to donate the hydrogen atoms to free radicals. The principle of DPPH procedure depends on the lowering of DPPH in the presence of a proton-releasing antioxidant. Result in Fig. 1A stated that the DPPH scavenging activity of *C. asiatica* L. water extract was fairly lower than the BHT. Early reports demonstrated the IC_{50} value of *C. asiatica* leaves in DPPH radical scavenging assay was in the range of 31.25 µg/mL (27). However, the IC_{50} value of *C. asiatica* L. water extract in this assay was far lower (1.18 ± 0.10 µg/mL) compared to the previous results. Research done by (28) showed the IC_{50} value of *C. asiatica* L. water extract was ranged at 8 µg/mL. The authors also mentioned that saponins are the main phytochemical constituent in the *C. asiatica* leaves water extract. Flavonoids from *C. asiatica* L. also played a part in DPPH radical scavenging. Results collected from (29) stated that castilliferol-1 and castillicetin-2 from *C. asiatica* L. exhibited good antioxidant activity in DPPH radical assay with the IC_{50} values of 23.10 and 13.30 µg/mL, respectively. This implies that saponin from the *C. asiatica* L. water extract has the proton-donating capacity that could serve as scavenger of DPPH radical and probably as a primary antioxidant.

The hydrogen atom donation ability of *C. asiatica* L. water extract could be observed in the other antioxidant assays too. In the NO scavenging activity, the phytochemicals from *C. asiatica* L. water extract possessed effective scavenging activity against NO radicals. In the present study, the plant extract possessed the lowest IC_{50} value (0.71 ± 0.12 µg/mL) among the antioxidant assays Fig. 2(B). It scavenged the NO radical which resulted in decrease in absorbance and the reduction of rhodamine chromophore (30). The scavenging activity of the plant extract is correlated to the presence of saponins, flavonoids, and alkaloids. Flavonoids and alkaloids play a role in NO suppression by competing with the oxygen species which subsequently interrupting the oxidative decomposition of NO into (NO^{2-}) and nitrates (NO^{3}-) (31). High concentration of nitrites increases oxidative stress as well as TNF-α levels, resulting in inflammation. Saponins present in the *C. asiatica* L. contribute to its anti-inflammation properties. (32) reported to downregulate the expression of PGE2 and NO metabolism in human endothelial cells. Inhibition of nitric oxide radicals by *C. asiatica* is responsible for certain pharmacological effects, such as neuroprotective, gastrointestinal protection, anti-diabetic, and anti-vascular leakage.

OH radicals contain the highest 1- electron reduction potential which primarily induce the cytotoxic effect in the human body (33). The OH radicals react with lipids, proteins, and deoxyribose sugar to form a variety of corresponding radicals, such as hydroxylcyclohexadienyl radicals, peroxyl radicals, lipid radicals, and so on (33). *C. asiatica* L. water extract portrayed the highest IC_{50} value (22.84 ± 1.089 µg/mL) in OH radical assay compared to the others. Nevertheless, the plant extract was expected to have high potency in scavenging OH radicals, as the result shown that *C. asiatica* L. water extract possessed lower IC_{50} value than the positive control. Researcher reported that the OH radical scavenging effect of the saponins were about 15.5% to 68.7% (34). The scavenging activity of *C. asiatica* L. water extract may be considerably more rely on the other phytochemicals such as flavonoids and alkaloids. Notwithstanding, it cannot deny the possibility of saponins in scavenging

OH radicals.

Flavonoids are the acclaimed potent scavenger of OH radical. The structural elements of flavonoid are responsible in OH radical scavenging, where its ring B and the double bond between C-3 OH group and C-4 carbonyl group will undergo hydroxylation (35). The presence of gallate and galacturonate moieties in the ring A or flavonoids also enhance the scavenging activity. Alkaloids such as β-carbolines and tetrahydro-β-carboline-3-cabolines are scavengers of OH radicals. The alkaloids are active against the hydroxylation of deoxyguanosine through scavenging of OH radicals to prevent oxidative damage of DNA (36). In the presence of phytochemicals, *C. asiatica* leaves is able to protect the human cells from lipid peroxidation, glucose autoxidation, and protein modification through OH radical scavenging.

Fenton reaction occurs when ferrous ions (Fe^{2+}) catalyse hydrogen peroxide (H_2O_2) to generate OH radicals and ferrozine ions (Fe^{3+}). Therefore, chelating capacity assay was carried out to identify the potential of *C. asiatica* L. water extract in chelating ferrozine ions which will induce lipid peroxidation and oxidative damage in mitochondria (37). MDA is a reliable biomarker to determine the extent of Fenton reaction and lipid peroxidation induced by OH radicals (38). It can be detected as a pink chromogen when reacted with TBA. Elevation of MDA level results in high oxidative stress and inflammation in endothelial cells. In the experiments, reduction of absorbance in both OH radical scavenging assay and chelating capacity assay indicated that the level of MDA was decreased and detected at lower ranged. Saponins, flavonoids, and alkaloids have been reported to chelate available Fe^{2+}ions and further inhibit the generation of OH radicals. Referring to Fig. 1(B) and 2(A), the *C. asiatica* L. water extract has successfully inhibited the formation of MDA through OH radical scavenging and Fe^{2+} ion chelating.

Quercetin used as positive control in the antiglycation experiment as it is a plant flavanol from the flavonoid group of polyphenols. Quercetin have been previously reported to show antiglycation activity through hydrogen atom donation, trapping α-dicarbonyl intermediates, and inhibiting or reducing the quantity of Amadori products (39). *C. asiatica* L. water extract was able to inhibit formation of AGE throughout the glycation process. Indeed, *C. asiatica* L. water extract portrayed better antiglycative activity in the early and intermediate stage of glycation based on the result shown in Fig. 3 (A) and 3 (B). In both BSA-MGO and BSA-glucose assay, *C. asiatica* L. showed strong antiglycation activity which was more efficient than the quercetin. This might be due to that *C. asiatica* L. water extract contains more than one phytochemical. The antiglycation mechanism of *C. asiatica* L. water extract was attributed by the combined effects of saponins and flavonoids.

Several researches have reported that saponins possess better antiglycation effects during the early stage of glycation in BSA-glucose (40). This may be due to the combination of antiglycation and antioxidant properties of saponins that protecting amino groups of proteins such as histidine, lysine, and arginine from the free radicals and α-dicarbonyl intermediates, during the early and late-stage of glycation (41). Experiments conducted by (23) showed that *C. asiatica* attenuated the formation of AGEs, Amadori products, and restricted the structural alterations of BSA subjected to glycation. These effects probably due to the antioxidant activities of *C. asiatica* mediated by the presence of flavonoids and triterpenoids (23). Similarly, research by (42) reported that the addition of *C. asiatica* extract was able to inhibit the formation of AGE adducts via free radical scavenging and curtailment of Amadori products and reactive dicarbonyl intermediates.

Demotion of IC_{50} in MGO scavenging assay demonstrated *C. asiatica* water extract also portrayed antiglycation effect during the advanced or late stage of glycation. Centella species capable to attenuate the MGO-induced glycation (43). The researchers also stated that Centella species has reduced the formation of MGO-glycation proteins. *C. asiatica* extract also showed an inhibition on the expression of Nε-Carboxymethyl-lysine (CML) in the MGO-induced and non-MGO induced assay (44). CML is one of the AGE adducts that is formed between the glycation of reducing sugar and lysine. Advanced stage of glycation is a stage where activation of RAGE, oxidative damage, and inflammatory reaction occur. *C. asiatica* has been shown as a promising candidate for AGE inhibitor in many research due to its efficiency in inhibiting the AGEs production. Molecular dynamic experiment conducted on *C. asiatica* showed that the bioactive compounds from *C. asiatica* had been proven to interrupt the AGE-RAGE interaction (45).

The researchers discovered that saponins, such as asiaticoside, madasiatic acid, and madecassic acid from *C. asiatica* showed the ability to bind with the RAGE to prevent inhibition and further glycation process in the adhesive cells. Madecassoside in *C. asiatica* extract successfully downregulate and inhibit the activation of inflammatory factors in the model rats. Furthermore, asiatic acid in the plant has significantly upregulating the expression of nuclear factor-erythroid factor 2-related factor 2 (Nrf2) (46). In vivo and in vitro experiments on *C. asiatica* extract also found that asiatic acid could decrease the levels of inflammatory factors in the body (47). In summary, madecassoside, asiatic acid, and madecassoside acid have been reported to carry out three main mechanisms in protecting endothelial cells from the oxidative damage, firstly is to reduce the level of pro-apoptotic level; secondly, decrease the levels of inflammation factors; and lastly is to increase the level of anti-oxidative stress factors (47, 48, 49).

Saponins are the major phytochemical that can largely been found in *C. asiatica* L. The antiglycation of *C. asiatica* L. water extract maybe partially due the combined effects of saponins, flavonoids, and alkaloids. Further research is necessary to elucidate the detailed antiglycation mechanism and identification of the other bioactive components present in *C. asiatica* L. water extract using analytical chemistry technique such as HPLC-MS.

CONCLUSION

C. asiatica L. water extract possess antioxidant and antiglycation properties. The antioxidant activity seems to be carried out through the donating hydrogen atom to the free radicals, diminishing the chance of generation of oxidative stress and preventing autoxidative degradation of lipids and deoxyribose sugar. The antiglycation activity *C. asiatica* L. water extract maybe through hydrogen atom donation, trapping of α-dicarbonyl intermediates, inhibiting or reduce the quantity of Amadori products. *C. asiatica* L. also can inhibit the expression of AGE adducts. Saponins maybe a major contributing bioactive present in *C. asiatica* L. water extract, this is due to saponins have been previously reported to inhibit early stage of formation of AGEs. Future research is necessary to elucidate the molecular mechanism of antiglycation activity and the bioactive component present in *C. asiatica* L. using analytical chemistry technique such as HPLC-MS.

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