***In vitro* antioxidant, antiglycation, and MGO scavenging effects of *Leonurus sibiricus* water extract**

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Abstract. Recently, extensive research has been conducted to evaluate the inhibitory activity of different plant species on the advanced glycation end products (AGEs). *L. sibiricus* is a traditional herb that has been used for postpartum confinement meals in Sarawak, Malaysia and pharmacologically possess anti-hemorrhagic, antioxidant, anti-diabetic, and anti-cancer. The aim of this research was to evaluate the antioxidant, anti-AGEs, and preliminary biochemical screening of bioactive component present in *L. sibiricus* in water extract. Free radical scavenging activity of *L. sibiricus* was evaluated via DPPH, hydroxyl radical, nitric oxide, lipid peroxidation, chelating capacity, and total phenolic content was evaluated comparing with gallic acid. Inhibition of formation AGEs by *L. sibiricus* was evaluated using BSA-MGO, BSA-glucose and MGO scavenging assays. Flavonoids, phenols, saponins, alkaloids, phytosterol, and diterpenoids were identified in *L. sibiricus* extract. It also seems to inhibit early and late formation of AGE and MGO scavenging ability. *L. sibiricus* was able to inhibit the formation of early and late formation of AGE through the scavenging of the formation of reactive dicarbonyl intermediates and reduce the formation of methylquinoxaline adducts through the scavenge of MGO. The inhibition of AGEs formation by *L. sibiricus* maybe due its antioxidant property and the presence phytochemical bioactive constituents which has been previously reported to possess antioxidant and anti-AGE activity. Future research is ongoing to identify the adducts formed because of MGO scavenging by *L. sibiricus* using HPLC.

Keywords: AGEs, antioxidants, BSA-glucose, BSA-MGO, *L. sibiricus*

INTRODUCTION

Advanced glycation end products (AGEs) are formed when non-enzymatic glycation and oxidation happens between aldose sugar and modified amino groups and lipids. Formations of AGEs occur via endogenous and exogenous. Endogenous AGEs are the normal consequences of body metabolism while exogenous AGEs are derived from the non-enzymatic saccharification. This process is also known as Maillard reactions of reducing sugars on proteins or lipids when heat is applied (Yamagishi *et al.*, 2015). Early glycation

and oxidation processes induce the formation of unstable Schiff bases and Amadori products. Formation and rearrangement of Amadori products leads to the accumulation of reactive dicarbonyl intermediates (α -dicarbonyls or oxoaldehydes), such as methylglyoxal (MGO), glyoxal, and 3-deoxyglucosone (3-DG) (Qais *et al.*, 2019). In the advanced stage, free radicals and reactive dicarbonyl compounds will form into isomers, respectively with arginine and lysine residues of proteins to form AGEs (Brings *et al.*,

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2017). Oxidative stress, hyperglycemia, aging, smoking can promote the formation of AGEs, and vice versa (Vlassara and Uribarri, 2013). Interaction between AGEs and receptor of AGE (RAGE) also promote the productions of other free-radicals, such as hydrogen peroxide (H_2O_2), superoxide ion radicals (O_2^-), hydroxyl radical (OH^-), and nitric oxide (NO) (Cepas *et al.*, 2020). Elevation of oxidative stress due to free radical accumulation which will induce inflammatory mediators and damage protein molecules. Continuous formation and accumulation of AGEs also lead to diabetic complications, such as atherosclerosis, diabetic retinopathy, diabetic neuropathy, and diabetic nephropathy (Singh *et al.*, 2014).

Oxidative stress occurs when reactive oxygen species (ROS) and antioxidants are in imbalance state. ROS is one of the free radicals that is involved in molecular and cellular damaging mechanisms, which could lead to various diseases such as diabetes, inflammatory conditions, atherosclerosis, and neurodegenerative diseases (Di Meo *et al.*, 2016). Free radicals may attack free amino groups in long-lived proteins, such as collagen, resulting in the protein with altered covalent bonds. These modified proteins may further glycate to form AGEs (Vistoli *et al.*, 2013). Oxidative stress could also be generated during the second step of the polyol pathway, where sorbitol is converted into fructose by succinate dehydrogenase (Mathebula, 2015). Simultaneously, the oxidised NADPH or NAD^+ is converted by succinate dehydrogenase into NADH, a substrate for NADPH oxidase. NADPH oxidase is an enzyme involved in the electron transferring across the plasma membrane to the molecular oxygen (Tarafdar and Pula, 2018). The conversion of NADPH to NADH increases the generation of ROS as well as increasing flux of glucose through the polyol pathway, leading to the production of 3-DG and MGO, these reactive dicarbonyl intermediates or glycation agents are more reactive than reducing glucose in AGE formation (Ashraf *et al.*, 2015).

L. sibiricus is an herbaceous plant species native to China, Mongolia, and Siberia, it is also known as honeyweed or Siberian Motherwort. In Sarawak Malaysia, *L. sibiricus* is named as *Kacangma* which has been used as postpartum confinement dish for nursing women. It is effective in

activating blood circulation, postpartum haemorrhage, regulating menstrual irregularities, dysmenorrhea, and amenorrhea (Miao *et al.*, 2019). Sayed *et al.* (2016) reported that the phenolic contents found in *L. sibiricus* are mainly quercetin, rutin, verbascoside, chlorogenic acid and caffeic acid which have been previously reported to possess anti-glycation and antioxidant activity.

MATERIALS AND METHODS

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-deoxy-D-ribose 97% (Sigma-Aldrich, USA); acetic acid (R&M, UK); bovine serum albumin (BSA) (Sigma-Aldrich, USA); butylated hydroxytoluene (BHT), chloroform, ethylenediaminetetraacetic acid (EDTA) 99% (Sigma-Aldrich, USA); ferrozine 98% (Acros Organic, Belgium); Folin-Ciocalteu's phenol reagent (Merck, USA); gallic acid 98% (Acros Organic, Belgium); glacial acid (Riendemann Chmidt Chemical, Malaysia); hydrogen peroxide (System, Malaysia); iron (II), iron (III) chloride (Riendemann Chmidt Chemical, Malaysia); N-1-naphthyl ethylene diamine dihydrochloride (R&D, UK); potassium ferricyanide (Labjax Chemical Ltd, India); quercetin hydrate (Aldrich Chemistry, USA); Sodium Dodecyl Sulfate (SDS) (Bio-Rad, USA); sodium hydroxide (NaOH), sodium nitroprusside, sulfanilic acid (Merk, USA); Thiobarbituric Acid (TBA), Trichloroacetic Acid (TCA) (Fisher Scientific, USA)

Equipments

Vortex mixer (LMS Co., Ltd, Japan); Benchtop Centrifuge (Zentrifugen, Germany); pH meter (Mettler Toledo, Switzerland); UV-Vis spectrophotometer (SECOMAM, France); Water bath (Memmert, Germany); Electronic balance (Mettler Toledo, Switzerland); FLUOstar Omega Plate Reader (ThermoFisher, USA).

Collection of plant materials

The leaves of *L. sibiricus* were collected from Home Garden Sdn. Bhd. in Kuching, Sarawak. It was identified by a plant taxonomist in Sarawak

(Anon, 2021). The plant weighing 2 kg was air dried for one week.

Water extraction of *L. sibiricus* leaves

A total of 2 kg of *L. sibiricus* leaves were ground into powder and soaked in 10 litre of distilled water for 48 hours. With the use of Buchner Funnel Filtration Set and Grade 1 filter paper (11 μ m), the residues of *L. sibiricus* leaves were filtered and separated from the juice. Freeze dryer was used to evaporate the water content. The plant extract was stored in the freezer at -18°C to prevent the growth of mould.

In vitro antioxidants assay

DPPH radical scavenging assay

A DPPH radical scavenging assay method described by Mridula *et al.* (2021), with minor modification, was conducted to evaluate the antioxidant activity and quantify the antioxidants that are found in the plant extract. One millimetre of 0.1 mM DPPH dissolved in methanol solution was added to 1 ml of *L. sibiricus* water extract with different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μ g/ml) and vortexed. The reaction mixture was then incubated at room temperature for 30 minutes in the dark. After that, 96 well plate readers were used to measure the absorbance of the reaction mixture at 517 nm against the blank. Methanol was used as the negative control, while 1ml of BHT was used as positive control. The percentage of inhibition was calculated in terms of IC₅₀ value. The following equation [1] was used to determine the percentage of radical scavenging activity of each sample.

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

Where Abs: Absorbance.

Lipid peroxidation (LPO) assay

Lipid peroxidation assay detects the lipid peroxidation level in the system. Method of LPO assay described by Mridula *et al.* (2021) was conducted to evaluate the anti-lipid peroxidation of *L. sibiricus* water extract. One millimetre of *L. sibiricus* water extract (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μ g/ml) and 0.5 ml of 10% (v/v) egg homogenate were added. The reaction mixture was topped up with 1ml of distilled water followed by 5 μ l of 0.07 M FeSO₄. The reaction mixture was vortexed for 1 minute and incubated

for 30 minutes at room temperature in the dark. After the first incubation, 0.5 ml of 30% acetic acid, 1.5 ml of 0.8 % w/v TBA in 1% SDS, and 0.5 ml of 20 % TCA were added into the reaction mixture. The reaction mixture was then vortex and incubated for 60 minutes at 95°C. The mixture was cooled down for 5 to 6 minutes before adding 5 ml of butanol to each of the test tubes, and then centrifuged at 5000 rpm for 60 minutes. The supernatant was pipette out to measure the absorbance at 532 nm. BHT had been used as a positive control while distilled water was the negative control. The results of lipid peroxidation free radicals' inhibition were calculated via equation [1] and expressed in terms of IC₅₀ value.

Chelating capacity assay

The chelating capacity assay method described by Mridula *et al.* (2021) with minor modification has been conducted to identify the ability of *L. sibiricus* water extract in interfering the formation of ferrozine-Fe²⁺ complex *in vitro*. Firstly, 1 ml of FeSO₄ (0.1 mM) and 1 ml of ferrozine (0.25 mM) were added into 1 ml of *L. sibiricus* water extract (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μ g/ml). While the positive control used against the blank solution was EDTA. The reaction mixture was incubated for 10 minutes at room temperature, then the absorbance was measured at 562 nm with the use of 96 well plate readers. The % inhibition was calculated using equation [1].

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay described by Rahman *et al.* (2015) was conducted to estimate the scavenging activity of plant extract by generating the hydroxyl radicals using ascorbic acid-iron EDTA. To 1 ml of phosphate buffer (pH 7.4, 50 mM), 0.2 ml EDTA (1.04 mM), 0.2 ml FeCl₃·H₂O (20mM), and 0.2 ml of 60 mM deoxy-D-ribose were added. Next, 100 μ l of *L. sibiricus* water extract (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μ g/ml) was added into the mixture above. After incubation for 60 minutes at 37°C in a water bath, 2 ml of ice-cold TBA and 2 ml of TCA were added into the mixture. The reaction mixture was vortexed and incubated for another 15 minutes at 100°C. The positive and negative controls were BHT and distilled water, respectively. The reaction mixture was cooled down for about 15

minutes before measuring the absorbance at 512 nm and % inhibition was calculated using equation [1] mentioned above.

Nitric oxide radical scavenging assay

Nitric oxide is a free radical which can also interact with oxygen and reactive oxygen species (ROS) to generate reactive nitrogen species (RNS). Method described by Mridula *et al.* (2021) was used in this experiment. A volume of 0.5 ml *L. sibiricus* water extract (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml) and 1 ml of sodium nitroprusside (10 mM) were added into 0.5 ml of sodium phosphate buffer (pH 7.4, 0.1 M), then vortexed for 5 seconds. 1 ml of sulfanilic acid with 20% glacial acetic acid was then added into the mixture and incubated for 5 minutes before adding 1 ml of naphthalene. Lastly, incubate the mixture for 30 minutes at 25°C. In this assay, the blank was sodium phosphate buffer, while positive control is BHT. The absorbance of the reaction mixture was measured at the wavelength at 540 nm against blank and % inhibition calculated using equation [1].

Total phenolic content (TPC)

A volume of 1 ml *L. sibiricus* water extract (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml) was added with 0.5 ml of Folin-Ciocalteu's reagent followed by 0.4 ml of 7.5% (w/v) sodium carbonate according to the method described by Mridula *et al.* (2021). The reaction mixture was centrifuged at 3000 rpm for 10 minutes under room temperature, then incubated for another 20 minutes in the dark. The absorbance of the reaction mixture was measured at 765 nm. The total phenolic content of the sample extract (mg/ml) was expressed in gallic acid equivalent (GAE), where y is absorbance at 760 nm and x is total phenolic content in the different extracts. Gallic acid was the positive control in this test.

In vitro AGEs inhibition assay

BSA-MGO assay

Bovine serum albumin-methylglyoxal (BSA-MGO) system acts as a marker in the middle stage of oxidative cleavage products formation (Starowicz and Zieliński, 2019). According to the methods described by Sugiura *et al.* (2017), 1 ml of BSA (20 mg/ml) and 1 ml of MGO (60 mM) were dissolved separately in 0.1 M of sodium

phosphate buffer (pH 7.4). After that, 1 ml of *L. sibiricus* water extract (20, 40, 60, 80, and 100 µg/ml) is mixed with the BSA-MGO mixture. The mixture was vortexed for 5 seconds and incubated for 5 minutes at 38°C. After the first incubation, 0.5 ml (0.2 g/L) of sodium azide was added into the reaction mixture. The tubes are capped before incubating it for 7 days at 37°C in the dark. After 7 days, the absorbance of the reaction mixture was measured with the excitation wavelength at 370 nm and emission wavelength 420 nm. Reaction mixture without sample was served as negative control while quercetin was the positive control in this assay. The % inhibition of positive control and sample were calculated by using equation [2].

$$\%Inhibition = \frac{Flu\ negative\ control\ group - Flu\ test\ group}{Flu\ negative\ control\ group} \times 100\% \quad [2]$$

Where Flu: Fluorescence.

BSA-glucose assay

The use of BSA-glucose assay was to describe the early stage of AGEs formation (Starowicz and Zieliński, 2019). The procedure is similar as in the BSA-MGO assay, the only different is that the 0.2 M of sodium phosphate buffer was used to dissolve the BSA and glucose. The % inhibition of positive control and sample were calculated by using equation [2].

MGO scavenging assay

Methylglyoxal (MGO) is one of the carbonyl intermediates produced by the degradation of Amadori products during the middle stage of protein glycation. The MGO-scavenging method was described by Sugiura *et al.* (2017), this activity was carried out to determine whether *L. sibiricus* water extract has MGO-trapping capacity in the middle and advanced stage of AGEs formation. In the experiment, 0.125 ml of MGO and 0.125 ml of sample (20, 40, 60, 80, and 100 µg/ml) were added into 0.125 ml of phosphate buffer (pH 7.4, 50 mM). The reaction mixtures then incubated for 1, 2, 4, and 6 hours at 37°C. After incubation, 0.25 ml of o-phenylenediamine solution (50 mM) was added into the mixture, then left it for 30 minutes to 1 hour at 25°C to ensure the o-phenylenediamine to be derivatized. After that, the reaction mixture is quantified under wavelength 349 nm using POLARstar Omega Multidetector Plate Reader with FP and UV/Vis Spectrometer. Quercetin was being used as

positive control in this experiment while for negative control, quercetin or plant extract were excluded from the reaction mixture. The % inhibition of positive control and sample were calculated by using equation [2].

Statistical analysis

The data were presented in mean $\pm$ standard deviation (SD) in triplicates ($n=3$). One-way ANOVA was conducted at the significant level of $p = 0.001$, 0.01 , and 0.05 using IBM SPSS Statistic software, while Multivariate ANOVA was carried out in MGO scavenging assay.

RESULTS

***In vitro* antioxidants capacity**

DPPH radical scavenging assay

The antioxidant capacity of *L. sibiricus* water extract was investigated via DPPH radical scavenging assay. *L. sibiricus* water extract showed very strong scavenging effect against DPPH radical with lower of $IC_{50} = 15.58 \pm 0.05$ $\mu\text{g/ml}$ compared to the negative control, $IC_{50} = 132.88$ $\mu\text{g/ml}$ ($p < 0.001$; $p = 1.55 \times 10^{-14}$). However, the DPPH radical scavenging effect of *L. sibiricus* water extract was significantly ($p < 0.001$; $p = 1.76764 \times 10^{-7}$) higher than the standard BHT with $IC_{50} = 3.71 \pm 0.27$ $\mu\text{g/ml}$ as shown in Figure 1A.

Lipid peroxidation assay

The lipid peroxidation inhibitory effect of *L. sibiricus* water extract was evaluated monitoring Malondialdehyde (MDA) formation. The IC_{50} value of *L. sibiricus* water extract showed significantly ($p < 0.001$; $p = 3.88 \times 10^{-12}$) lower than the negative control, which was 8.32 ± 0.001 $\mu\text{g/ml}$, and 12.04 $\mu\text{g/ml}$, respectively. Standard BHT showed significantly highest ($p < 0.001$; $p = 4.44 \times 10^{-6}$) inhibitory effect with the lowest $IC_{50} = 7.63 \pm 0.04$ $\mu\text{g/ml}$ as shown in Figure 1B.

Chelating capacity assay

The metal ion chelating capacity of *L. sibiricus* water extract was evaluated via chelating capacity assay as shown in Figure 2A. *L. sibiricus* water extract exhibited good metal ion chelation with lower $IC_{50} = 19.96 \pm 0.13$ $\mu\text{g/ml}$ compared to the

negative control with $IC_{50} = 104.39$ $\mu\text{g/ml}$. Standard EDTA had the strongest chelating capacity as it exhibited the lowest IC_{50} , 12.62 ± 0.04 $\mu\text{g/ml}$. There was a significant difference ($p < 0.001$; $p = 9.33 \times 10^{-8}$) between the positive control and *L. sibiricus* water extract; as well as between the *L. sibiricus* water extract and negative control ($p < 0.001$; $p = 4.13 \times 10^{-12}$).

Hydroxyl radical scavenging assay

The IC_{50} values of standard BHT, *L. sibiricus* water extract, and negative control are shown in Figure 2B. *L. sibiricus* water extract showed significantly stronger ($p < 0.001$) hydroxyl radical scavenging effect with lower $IC_{50} = 10.41 \pm 0.44$ $\mu\text{g/ml}$ compared to standard BHT ($p = 1.96 \times 10^{-7}$) and the negative control ($p = 3.67 \times 10^{-8}$). The IC_{50} value of BHT was 31.74 ± 0.24 $\mu\text{g/ml}$ while negative control was $IC_{50} = 38.77$ $\mu\text{g/ml}$.

Nitric oxide scavenging assay

The scavenging effect of *L. sibiricus* water extract against nitrite and nitric oxide radicals was evaluated through nitric oxide scavenging assay. *L. sibiricus* water extract showed significantly ($p < 0.001$; $p = 5.08 \times 10^{-11}$) lower IC_{50} value compared to the negative control, which was $IC_{50} = 3.15 \pm 0.12$ $\mu\text{g/ml}$ and $IC_{50} = 42.57$ $\mu\text{g/ml}$, respectively. While, there was a significant ($p < 0.001$; $p = 0.0002$) different between the BHT (1.79 ± 0.12 $\mu\text{g/ml}$) and the plant extract as shown in Figure 2C.

Total phenolic content (TPC)

The TPC assay was conducted to figure out the total amount of phenolic content in the *L. sibiricus* water extract. At 10 $\mu\text{g/ml}$, the TPC amount of *L. sibiricus* water extract was 6.12 mg GAE/g extract, followed by 9.60 mg GAE/g extract at concentration 50 $\mu\text{g/ml}$, and lastly 17.19 mg GAE/g extract at the highest concentration of 100 $\mu\text{g/ml}$. The results are shown in Table 1.

Table 1. Total phenolic content in *L. sibiricus* water extract.

Concentration of <i>L. sibiricus</i> water extract ($\mu\text{g/ml}$)	Total phenolic content of <i>L. sibiricus</i> water extract (mg GAE/g extract)
10	6.12
50	13.60
100	17.19

Figure 1. *In vitro* antioxidant activity of *L. sibiricus* water extract, positive control (BHT) and negative control (methanol and distilled water). (A) DPPH radical scavenging assay; (B) Lipid peroxidation assay. The IC₅₀ values of each compound were expressed in mean $\pm$ standard deviation (n=3). The data was statistically analysed by One-Way Anova. Alphabets A and B denoted that there was a significant difference ($p < 0.001$) between *L. sibiricus* water extract when compared to the controls, where ^A $p < 0.001$ vs positive control and ^B $p < 0.001$ vs negative control. While, C denoted that positive control was significantly different to the negative control (^C $p < 0.001$ vs negative control).

Figure 2. *In vitro* antioxidant activity of *L. sibiricus* water extract, positive control (EDTA and BHT) and negative control (distilled water and sodium phosphate buffer). (A) Chelating capacity assay; (B) Hydroxyl (OH) radical scavenging assay; (C) Nitric oxide (NO) radical scavenging assay. The IC₅₀ values of each compound were expressed in mean $\pm$ standard deviation (n=3). The data was statistically analysed by One-Way Anova. Alphabets A and B denoted that there was a significant different ($p < 0.001$) between *L. sibiricus* water extract when compared to the controls, where ^A $p < 0.001$ vs positive control and ^B $p < 0.001$ vs negative control. While, C denoted that positive control was significantly different to the negative control (^C $p < 0.001$ vs negative control).

Figure 3. *In vitro* antiglycation and AGEs inhibitory activity of *L. sibiricus* water extract, standard quercetin, and negative control. (A) BSA-MGO assay; (B) BSA-glucose assay; (C) MGO scavenging assay. The data was statistically analysed by One-Way Anova. ^ap < 0.001 vs positive control and ^bp < 0.001 vs negative control. While, C denoted that positive control was significantly different to the negative control (^cp < 0.001 vs negative control).

BSA-MGO assay

The AGEs inhibitory or antiglycation effects of *L. sibiricus* water extract was evaluated using BSA-MGO assay, BSA-glucose assay, and MGO scavenging assay. In BSA-MGO assay, *L. sibiricus* water extract showed the lowest IC₅₀ when compared to standard quercetin and the negative control, which was IC₅₀ = 3.74 ± 2.33 µg/ml, 7.34 ± 0.23 µg/ml, and 28.61 µg/ml, respectively. The inhibitory effect of *L. sibiricus* water extract was at par ($p > 0.001$, $p = 0.057$) with standard quercetin while significantly different ($p < 0.001$, $p = 5.04 \times 10^{-5}$) to negative control in BSA-MGO assay as shown in Figure 3A.

BSA-glucose assay

The AGEs inhibitory effect of *L. sibiricus* water extract in BSA-glucose assay was shown in Figure 3B. *L. sibiricus* water extract exhibited anti-AGE formation ($p < 0.001$; $p = 2.36 \times 10^{-7}$) effect with lower IC₅₀ = 2.25 ± 0.75 µg/ml compared to the negative control of IC₅₀ = 32.93 µg/ml. There was no significant difference ($p > 0.001$; $p = 0.55$) between the effect of the standard quercetin IC₅₀ = 1.05 ± 0.43 µg/ml and *L. sibiricus*.

MGO scavenging assay

MGO scavenging assay was conducted to evaluate the MGO trapping effect of *L. sibiricus* water extract. The overall IC₅₀ values of *L. sibiricus* water extract decreased with longer incubation hours as shown in Figure 3C. There was no significant difference in *L. sibiricus* water extract effect compared to standard quercetin ($p > 0.001$) throughout 1 ($p = 0.06$), 2 ($p = 0.72$), 4 ($p = 0.01$), and 6 ($p = 0.03$) hours. At 1 hour incubation, the IC₅₀ of *L. sibiricus* water extract was 11.72 ± 2.14 µg/ml, which was higher than the standard quercetin, IC₅₀ = 8.49 ± 0.10 µg/ml. At 2 hours incubation, the IC₅₀ of quercetin and *L. sibiricus* water extract were 6.64 ± 0.78 µg/ml and 7.16 ± 2.19 µg/ml, respectively. At 4 hours incubation, standard quercetin showed its lowest IC₅₀ in this assay, which was IC₅₀ = 2.10 ± 1.09 µg/ml; while the *L. sibiricus* water extract showed IC₅₀ = 4.15 ± 0.50 µg/ml. *L. sibiricus* water extract exhibited lower IC₅₀ value than the standard quercetin in MGO scavenging assay after 6 hours incubation, which was 3.18 ± 0.55 µg/ml and 8.29 ± 2.63 µg/ml respectively. Similarly, there was a significant difference ($p < 0.001$) between the *L.*

sibiricus water extract and negative control (IC₅₀ = 62.56 µg/ml) throughout 1, 2, 4, and 6 incubation hours ($p = 7.85 \times 10^{-12}$, 1.66×10^{-6} , 3.48×10^{-9} , 4.81×10^{-9} , respectively).

DISCUSSION

According to the results, antioxidants interrupt the propagation of free radicals through quenching hydroxyl radical (OH•) and hydrogen atom donation or electron transferring to the free radicals to produce non-radical molecules, concurring with the statement reported by Romera-Castillo and Jaffé (2015). *L. sibiricus* water extract showed effective scavenging effects against the free radicals. The *in vitro* antioxidant activity of *L. sibiricus* water extract maybe includes the quenching of hydroxyl radicals through the donation of electron and chelate ferrous ions at the early stage of Fenton reaction and inhibit the formation of malondialdehyde (MDA) (Figure 2A and 2B). Excess ferrous ions (Fe²⁺) react with oxygen and hydrogen peroxide (H₂O₂) to produce ferric ions (Fe³⁺) and OH• radicals via Fenton reaction (Xu, Wu and Zhou, 2020). OH• radical and Fe²⁺ induce oxidative degradation on carbonyl or carboxyl groups of deoxyribose sugar in DNA molecules, resulting in lipid peroxidation by damaging mono- and polyunsaturated fatty acids of phospholipid bilayer membrane (Solís-Calero *et al.*, 2015) (Figure 1B). Consequently, a low-weight molecular product called MDA is formed from the degradation of lipids and deoxyribose sugars will increase oxidative stress (Dorsey and Jones, 2017). In this reaction, Fe²⁺ also acts as a catalyst to enhance the oxidative potential of H₂O₂ and other ROS (Ameta *et al.*, 2018). Prevention of intracellular free radicals' production could be used as the treatments and management of free radical-induced diseases, such as cardiovascular diseases, cancer, inflammatory disease and so on (Maddu, 2019). Compounds with high phenolic content has been shown to possess high antioxidant activity. This experiment revealed high total phenolic content in *L. sibiricus* water extract which may reveal the reason for its high *in vitro* antioxidant activity (Table 1).

Quercetin was used as positive control in the antiglycation assay, which acted as a fine

substitute to aminoguanidine (AG). This is because quercetin potent in antiglycation than AG in all stages of glycation (Ashraf *et al.*, 2015). *L. sibiricus* water extract showed strong antiglycation or AGEs inhibitory effects in BSA-MGO, BSA-glucose and MGO scavenging assay (Figure 3A, 3B, and 3C). AGEs are the end products of non-enzymatic glycation between aldose sugars and proteins or lipids, which are catalysed by free radicals to synthesize Schiff bases and Amadori products. Reactive dicarbonyl intermediates from Amadori products react with the amino acids to form AGEs during the advanced stage of glycation. In BSA-MGO and BSA-glucose demonstrated the advanced stage and early stage of glycation, respectively. *L. sibiricus* water extract exhibited very strong antiglycation (inhibition of AGE formation), the effect was found be like standard quercetin (Figure 3A and 3B). Hence, suggesting it has similar antiglycation mechanisms as the quercetin, which are reducing the amount of Amadori products in the early stage, trapping and scavenge reactive dicarbonyl intermediates, and scavenge free radicals. ROS are produced during the process of AGEs formation too, the imbalance ratio of antioxidants and ROS induce oxidative stress, which may cause complications of diabetes, such as diabetes retinopathy, neuropathy, nephropathy, and strokes.

Sayed *et al.* (2016) reported that *L. sibiricus* contains flavonoids, chlorogenic acid and caffeic acid which have been previously reported to possess anti-glycation and antioxidant activity. Flavonoids such as quercetin and rutin play an important role in reducing the formation of Schiff bases and Amadori products through free radicals scavenging. The scavenging effect of flavonoid is contributed by its structure that composed of 2 benzene rings and more than 3 -OH groups, which allow them to donate hydrogen atom to free radicals. Thus, aldose sugar is protected from autoxidation and lead to the reduction of Amadori products formation (Yeh *et al.*, 2017). Phenolic compounds also found to scavenge and trap reactive dicarbonyl intermediates. Further, phenyl and benzene rings of chlorogenic and caffeic acid consist of hydroxyl groups that increase their electron density to transfer more electrons to free radicals (SUN *et al.*, 2015). Treml and Šmejkal (2016) reported phenyl or benzene rings with three or more -OH groups have better MGO

trapping ability than the ones with only 1 or 2 -OH groups.

L. sibiricus water extract has similar MGO scavenging effect as standard quercetin (Figure 3C). It scavenged and trapped MGO into adducts, however the adducts yet to be identified. Quercetin has reported to scavenge MGO into two adducts called Que-mono-MGO and Quer-di-MGO (Liu *et al.*, 2021). The research conducted by (Liu *et al.*, 2017) investigated that the quercetin-MGO adducts have free radical scavenging effects and further trapping the MGO into tri-MGO adducts. The hydroxyl groups in the A ring of flavonoids were suggested to be the active site of trapping MGO (Yang *et al.*, 2017); while (Liu *et al.*, 2017) found out that the hydroxyl groups in B ring of quercetin is the main active site of free radical scavenging.

L. sibiricus water extract may be because of the presences of numerous phytochemical bioactive components which has been previously reported to possess similar effect such as saponins, alkaloids, flavonoids, phenols, tocopherol and diterpenoids. Ashraf *et al.* (2015) reported that saponins possess chelating activity and could trap ferrous ions before the formation of ferrozine in Haber-Weiss and Fenton reactions. Saponins are also an antiglycation agent that is involved in various steps of glycation and AGEs formation. Research conducted by Chen *et al.* (2019) reported that saponins reduced the Amadori haemoglobin A1c (HbA1c) level in the early stage of glycation and scavenge free radicals in advanced stage of glycation. Sitarek *et al.* (2016) reported that flavonoids can be largely found in the leaves, stems and roots of *L. sibiricus* plant, which can modulate the antioxidant genes and thus repair and protect the DNA from oxidative damage. Flavonoids in *L. sibiricus* extract are also suggested to have antidiabetic effect as it can increase the insulin secretion and proliferation of insulinoma cells (Schmidt *et al.*, 2013).

Alkaloid has been widely found in aerial parts of *L. sibiricus* plant, especially leonurine which have been reported to stimulate repair and protective effects against cellular DNA oxidative damage by scavenging free radicals and inhibits the formation of ROS (Yang *et al.*, 2018). Sitarek *et al.* (2016) reported the phenols in *L. sibiricus* showed antiglycation mechanisms through free radical scavenging, inhibiting the formation of

ROS, interrupting the dicarbonyl intermediates formation and metal ions chelating. Phytosterol interrupts the non-enzymatic glycation reaction between the protein and reducing sugar, thus suppressing the formation of Amadori products and AGEs (Sheng *et al.*, 2016).

CONCLUSION

L. sibiricus water extract possesses *in vitro* antioxidant and antiglycation activity through scavenging or trapping of reactive dicarbonyl intermediates, scavenging free radicals, and reduce oxidative stress. This effect may be due to the presence of bioactive phytochemicals in *L. sibiricus* water extract such as saponins, alkaloids, flavonoids, phenols and phytosterol which have been previously reported to possess antioxidant and antiglycation activities. Further research is ongoing to identify the adducts formed because of scavenge of MGO by *L. sibiricus* water extract.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest in this study.

REFERENCES

- Ameta, R., K. Chohadia, A., Jain, A., & Punjabi, P. 2018. Fenton and photo-Fenton processes. *Advanced Oxidation Processes for Waste Water Treatment*: 49-87.
- Anon, 2021. Kacangma. [online] Doa.sarawak.gov.my. Available at: <<https://doa.sarawak.gov.my/page-0-0-181-Kacangma.html>> [Accessed 1 July 2021].
- Ashraf, J., Ahmad, S., Rabbani, G., Hasan, Q., Jan, A., Lee, E., Khan, R., Alam, K., & Choi, I. 2015. 3-Deoxyglucosone: A potential glycation agent accountable for structural alteration in H3 histone protein through generation of different AGEs. *PLoS ONE* 10(2): e0116804.
- Brings, S., Fleming, T., Freichel, M., Muckenthaler, M., Herzig, S., & Nawroth, P. 2017. Dicarbonyls and advanced glycation end-products in the development of diabetic complications and targets for intervention. *International Journal of Molecular Sciences* 18(5): 984.
- Cepas, V., Collino, M., Mayo, J., & Sainz, R. 2020. Redox signaling and advanced glycation endproducts (AGEs) in diet-related diseases. *Antioxidants* 9(2): 142.
- Chen, W., Balan, P., & Popovich, D. 2019. Review of ginseng, anti-diabetic, studies. *Molecules* 24(24): 4501.
- Di Meo, S., Reed, T. T., Venditti, P., & Victor, V. M. 2016. Harmful and beneficial role of ROS. *Oxidative Medicine and Cellular Longevity* 2016: 1-3.
- Dorsey, B. & Jones, M. 2017. Healthy components of coffee processing byproducts. *Handbook of Coffee Processing By-Products*: 27-62.
- Liu, G., Xia, Q., Lu, Y., Zheng, T., Sang, S., & Lv, L. 2017. Influence of quercetin and its methylglyoxal adducts on the formation of α -dicarbonyl compounds in a lysine/glucose model system. *Journal of Agricultural and Food Chemistry* 65(10): 2233-2239.
- Liu, P., Yin, Z., Chen, M., Huang, C., Wu, Z., Huang, J., Ou, S., & Zheng, J. 2021. Cytotoxicity of adducts formed between quercetin and methylglyoxal in PC-12 cells. *Food Chemistry* 352: 129424.
- Narendra, M. & Emad, S. 2019. Diseases related to types of free radicals, antioxidants. IntechOpen. Available from: <https://www.intechopen.com/books/antioxidants/diseases-related-to-types-of-free-radicals>.
- Mathebula, S. 2015. Polyol pathway: A possible mechanism of diabetes complications in the eye. *African Vision and Eye Health* 74(1).
- Miao, L., Zhou, Q., Peng, C., Liu, Z., & Xiong, L. 2019. *Leonurus japonicus* (Chinese motherwort), an excellent traditional medicine for obstetrical and gynecological diseases: A comprehensive overview. *Biomedicine & Pharmacotherapy* 117: 109060.
- Mridula, S., Siddiqui Masroor, W., Xavier, M., Tan, W., Chan, H., Chirara, K., & Okechukwu, P. 2021. Antioxidant and anti-advanced glycation end products formation properties of palmatine. *Journal of Pharmacy & Pharmacognosy Research* 9(3): 366-378.
- Qais, F., Khan, M., Althubiani, A., Al-Ghamdi, S., & Ahmad, I. 2019. Understanding biochemical and molecular mechanism of complications of glycation and its management by herbal medicine. *New Look to Phytomedicine*: 331-366.
- Rahman, M., Islam, M., Biswas, M., & Khurshid Alam, A. 2015. *In vitro* antioxidant and free radical scavenging activity of different parts of *Tabeaia pallida* growing in Bangladesh. *BMC Research Notes* 8(1).
- Romera-Castillo, C., & Jaffé, R. 2015. Free radical scavenging (antioxidant activity) of natural dissolved organic matter. *Marine Chemistry* 177: 668-676.
- Sayed, M., Alam, M., Islam, M., Ali, M., Ullah, M., Shibly, A., Ali, M., & HasanOlive, M. 2016. *Leonurus sibiricus* L. (honeyweed): A review of its phytochemistry and pharmacology. *Asian Pacific Journal of Tropical Biomedicine* 6(12): 1076-1080.
- Schmidt, S., Jakab, M., Jav, S., Streif, D., Pitschmann, A., Zehl, M., Purevsuren, S., Glasl, S., & Ritter, M. 2013. Extracts from *Leonurus sibiricus* L. increase insulin secretion and proliferation of rat INS-1E insulinoma cells. *Journal of Ethnopharmacology* 150(1): 85-94.
- Sheng, Z., Dai, H., Pan, S., Ai, B., Zheng, L., Zheng, X., Prinyawiwatkul, W., & Xu, Z. 2016. Phytosterols in banana

- (*Musa* spp.) flowers inhibit α -glucosidase and α -amylase hydrolyses and glycation reaction. *International Journal of Food Science & Technology* 52(1): 171-179.
- Singh, V., Bali, A., Singh, N., & Jaggi, A. 2014. Advanced glycation end products and diabetic complications. *The Korean Journal of Physiology & Pharmacology* 18(1): 1.
- Sitarek, P., Skala, E., Toma, M., Wielanek, M., Szemraj, J., Skorski, T., Bialas, A., Sakowicz, T., Kowalczyk, T., Radek, M., Wysockińska, H., & Śliwiński, T. 2016. Transformed root extract of *Leonurus sibiricus* induces apoptosis through intrinsic and extrinsic pathways in various grades of human glioma cells. *Pathology & Oncology Research* 23(3): 679-687.
- Solís-Calero, C., Ortega-Castro, J., Frau, J., & Muñoz, F. 2015. Nonenzymatic reactions above phospholipid surfaces of biological membranes: reactivity of phospholipids and their oxidation derivatives. *Oxidative Medicine and Cellular Longevity* 2015: 1-22.
- Starowicz, M. & Zieliński, H. 2019. Inhibition of advanced glycation end-product formation by high antioxidant-leveled spices commonly used in European cuisine. *Antioxidants* 8(4): 100.
- Sugiura, S., Minami, Y., Taniguchi, R., Tanaka, R., Miyake, H., Mori, T., Ueda, M., & Shibata, T., 2017. Evaluation of anti-glycation activities of phlorotannins in human and bovine serum albumin-methylglyoxal models. *Natural Product Communications* 12(11): 1934578X1701201.
- Sun, Y., Gu, J., Tan, X., Wang, C., Jia, X., Feng, L., & Liu, J. 2015. Curcumin inhibits advanced glycation end product-induced oxidative stress and inflammatory responses in endothelial cell damage via trapping methylglyoxal. *Molecular Medicine Reports* 13(2): 1475-1486.
- Tarafdar, A. & Pula, G. 2018. The role of NADPH oxidases and oxidative stress in neurodegenerative disorders. *International Journal of Molecular Sciences* 19(12): 3824.
- Treml, J. & Šmejkal, K. 2016. Flavonoids as potent scavengers of hydroxyl radicals. *Comprehensive Reviews in Food Science and Food Safety* 15(4): 720-738.
- Vlassara, H. & Uribarri, J. 2013. Advanced glycation end products (AGE) and diabetes: Causes and effects. *Current Diabetes Reports* 14(1): 453.
- Vistoli, G., De Maddis, D., Cipak, A., Zarkovic, N., Carini, M., & Aldini, G. 2013. Advanced glycooxidation and lipoxidation end products (AGEs and ALEs): An overview of their mechanisms of formation. *Free Radical Research* 47(sup. 1): 3-27.
- Yamagishi, S., Nakamura, N., Suematsu, M., Kaseda, K., & Matsui, T. 2015. Advanced glycation end products: A molecular target for vascular complications in diabetes. *Molecular Medicine* 21(S1): S32-S40.
- Yang, B., Choi, E., & Shim, S. 2017. Inhibitory activities of kaempferol against methylglyoxal formation, intermediate of advanced glycation end products. *Applied Biological Chemistry* 60(1): 57-62.
- Yang, L., Qiao, Y., Liu, G., Zhu, X., Luo, Y., Chen, J., Liu, X., Ma, Z., Shang, Y., & Gu, X. 2018. Effects of dietary supplementation with leonurine hydrochloride on growth performance, immune response, antioxidant capacity and blood parameters in male broiler chicks. *Journal of Applied Animal Research* 46(1): 1490-1495.
- Yeh, W., Hsia, S., Lee, W., & Wu, C. 2017. Polyphenols with antiglycation activity and mechanisms of action: A review of recent findings. *Journal of Food and Drug Analysis* 25(1): 84