

Standardised Extracts of *Moringa Oleifera* and *Centella Asiatica* Enhanced the Antioxidant Activity, Learning and Memory Effects by Inhibiting Acetylcholinesterase Activity in D-Galactose Induced Ageing Rats

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ABSTRACT

The potential of *Moringa oleifera* Lam. (Moringaceae) and *Centella asiatica* (L.) Urban (Apiaceae) extracts (TGT-PRIMAAGE) in slowing the decline of memory and learning activity was investigated using D-galactose-induced ageing rat model. The extracts were profiled and standardised based on markers identified using LC/MS-QTOF. Toxicity study of the extract was done, and the rat did not show any sign of toxicity. The extract was orally administered to the rat and dose dependent (100, 500 and 1000 mg/kg) efficacy were investigated. The rats were subjected to Morris Water Maze whereby 3 parameters were studied (number of entry to platform, latency and novel object recognition).

Plasma was collected for the determination of catalase (CAT) activity and levels of malondialdehyde (MDA) and advanced glycation end products (AGEs). The activity of acetylcholinesterase (AChE), level of acetylcholine (ACh) and lipid peroxidation (LPO) were measured using the brain lysates. Significant improvement ($p < 0.05$) was seen in the memory and learning abilities in the aged rats that received medium and high dose of TGT-PRIMAAGE, and tocotrienol.

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Rats treated with TGT-PRIMAAGE had also shown improved CAT activity and resulted in reduced LPO. The level of ACh was found increased in parallel with the reduced AChE activity. The capabilities of learning and memory of the TGT-PRIMAAGE treated rats were enhanced via inhibition of AChE activity and subsequently increased level of ACh.

Keywords: Anti-ageing, antioxidant, *Centella asiatica*, D-galactose, *Moringa oleifera*

INTRODUCTION

Globally, the life expectancy of a human is on the rise with the number of more than 65-year-old elderly is expected to double between 2000 and 2050 (Jin et al., 2015). With the advancement in the health care system, the life span is increased; this should be accompanied by an increased in health span by slowing or limiting the speed of the ageing process. As a natural phenomenon, ageing however, has been associated with chronic diseases which include Alzheimer's, diabetes mellitus and cancers (Prasad et al., 2011). There are several theories underlying the mechanism of ageing such as increased reactive oxygen species (ROS) or oxidative stress caused by the uncontrolled production of free radicals and decreased antioxidant defences as one ages (Davalli et al., 2016). Oxidative stress had been claimed to cause ageing via accumulated damages of the macromolecules within the cell leading to cellular dysfunction and eventually death (Harman, 1956; Harman, 1972; Sohal & Weindruch, 1996). Therefore, therapy that limits the excessive or uncontrolled oxidative stress will aid in slowing ageing.

A high dose of exogenous D-gal-induced ageing rat model which was used in this study had been adopted by many studies as a useful animal ageing model. High dose D-galactose was shown to accelerate ageing in rodents due to the production of superoxide anions and oxygen-derived free radicals (Li et al., 2005; Chen et al., 2006; Parameshwaran et al., 2010; Haidar et al., 2015; Budni et al., 2017). In addition, D-gal changes the structures of peptides and proteins and caused the accumulation of advanced glycation end (AGE) products through non-enzymatic glycation (Wu et al., 2008).

The search for antioxidant resources which reduces the oxidative stress slows the aging process is an on-going interest of the scientific community. The continuous efforts include the discovery of natural products with antioxidative potentials. The *Moringa oleifera* Lam. leaves has been reported to possess high phenolic content and potent antioxidant properties which act via direct trapping of free radicals and metal chelation (Verma et al., 2009). The leaves of several cultivars of *Moringa* are valuable sources of polyphenols and are potential functional food and nutraceuticals (Nouman et al., 2016). On the other hand, the leaf of *C. asiatica* also possesses important phytochemicals that have an antioxidant

activity such as carotenoids and phenolic compounds (Singh et al., 2014). *C. asiatica* extract was reported to have abilities in reducing α -glucosidase activity and scavenge free radical (Dewi & Maryani, 2015).

In this study, we investigated the potential of a combination extract of *Moringa oleifera* Lam. and *Centella asiatica* Urb. in arresting the oxidative stress due to high dose D-galactose injected peritoneally to the rats. The effects in enhancing the capabilities of memory and learning were also studied.

MATERIALS AND METHODS

Sources and Identification of Plant materials

The leaves extracts of both the *Moringa oleifera* and *Centella asiatica* were obtained from The Borneo Moringa Sdn. Bhd. (Malaysia) in Tenom, Sabah, Malaysia. The leaves carried the voucher identification number of Bm_mo_191012_1 and Mtt_ca_150113_1 for *Moringa oleifera* and *Centella asiatica*, respectively. The identification was done by Sandakan Herbarium Sabah Forestry Department, Sabah, Malaysia. The combination of the leave extracts of *M. oleifera* leaves and *C. asiatica* leaves is referred to as TGT-PRIMAAGE and was prepared by The MitoMasa Sdn. Bhd. Malaysia.

LCMS-based Profiles of TGT-PRIMAAGE

The active ingredients in each batch of the extracts of TGT-PRIMAAGE were profiled using LC/MS-QTOF for the purpose of standardising the extracts produced. The dried form of TGT-PRIMAAGE extract (1 mg/mL) were reconstituted in the mobile phase before being injected into LC-MS/QTOF (model 6520 Agilent Technologies, SA, USA) using a ZORBAX Eclipse Plus C18 column (100 mm x 2.1 mm x 1.8 μ m, Agilent Technologies, SA, USA) maintained at 40°C. The flow rate used was 0.25 mL/min with a linear gradient comprising solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) over 36 minutes from 5% to 95% of mobile phase (B). The total run time was 48 minutes for each analysis. Electrospray ionization (ESI) source were set with V Cap 4000 V, skimmer 65 V and fragmentor 125 V. The nebulizer was set at 45 psig and the flow rate of nitrogen drying gas was set at 12 L/min. The drying gas temperature was maintained at 350°C. The range of data collected in positive ESI mode was from 100 to 1000 m/z . Two reference masses were continuously injected; i.e. 121.0509 m/z ($C_5H_4N_4$) and 922.0098 m/z ($C_{18}H_{18}O_6N_3P_3F_{24}$) to monitor and ensure detection of the accurate mass of the compounds. Agilent Mass Hunter Qualitative Analysis B.05.00 software (Agilent Technologies, Santa Clara, CA, USA) was used for the processing and analysis of the MS data. The chromatographic profiles of TGT-PRIMAAGE was analysed based on the accurate mass data identified and the possible chemical formula for compounds were

annotated using METLIN databased year 2012. The widely accepted accuracy threshold for confirmation was established at 5 ppm with score more than 90.

Acute Toxicity Study

An acute toxicity test was carried out according to the stepwise procedure by Organization of Economic Cooperation and Development (OECD) guideline 423 for testing of chemical.

Experimental Study Using the D-Galactose-Induced Ageing Rats

The protocol of the study was reviewed and approved by the Research Committee on the Ethical Use of Animal UiTM (UiTM CARE: 91/2015). Adult male Sprague Dawley rats (200-360 gram) were caged separately (one rat per cage) with access to a standard rodent diet. They were fed with 400 gm of pellets and tap water *ad libitum* with 12-hour light and dark cycle in a room at controlled temperature and humidity. The standard rodent diet comprised crude protein, fibre and fat, moisture, ash, calcium and phosphorus at 21, 5, 3, 13, 8, 0.8, 0.4%, respectively. The rats were exposed to the procedure of acclimatization for 1 week to minimize the psychological pressure of the environment and handling stress.

The D-galactose induced ageing rat model was developed according to Haider et al. (2015). The rats (n=21) were assigned into 7 groups (each group comprised of 3 rats):

- i. Normal control
- ii. D-GAL: ageing rats administered with 300 mg/kg of D-Galactose
- iii. TE: D-GAL rats treated with 200 mg/kg of Tocotrienol
- iv. P-100: D-GAL rats served with low dose (100 mg/kg) of TGT-PRIMAAGE
- v. P-500: D-GAL rats served with medium dose (500 mg/kg) of TGT-PRIMAAGE
- vi. P-1000: D-GAL rats served with high dose (1000 mg/kg) of TGT-PRIMAAGE

All the rats (except the normal controls) were intraperitoneally injected with D-galactose (300 mg/kg) for 7 days to induce ageing and three groups were orally administered with TGT-PRIMAAGE at different doses. Tocotrienol (TE) were used as the positive control. The physical states of the rats were observed every day to check for abnormalities with respect to behaviours and death. The rats were then subjected to memory and learning test 24 hours after the last injection on day 7.

Memory and Learning Test

The rats were subjected to Morris Water Maze (MWM) for the evaluation on spatial memory performance of the rats. A circular pool with an appropriate size was used (diameter of 1.8 metre top, 1.4-metre bottom, 1.4-metre height). The temperature of the water was kept at 25±1°C. In this experiment, the time taken by the rats to find the hidden platform

(latency) and the numbers of entry to the platform zone were recorded and analyzed using Any-Maze (Version 8, USA).

Object recognition memory was assessed using a white painted open-field maze box with the size of (52 cm x 52 cm x 52 cm). The novel object preference ratio was determined as the ratio of the amount of time spent exploring novel object over the total time exploring both novel & familiar objects. All the rats were sacrificed using an overdose of ketamine, and the rats were incinerated at the end of the study.

Measurement of Catalase (CAT) Activity in Plasma

Fifty (50) μ l of plasma samples were incubated with 1.0 ml of substrate (65 μ mol per ml H_2O_2 in 60 mmol/l phosphate buffer, pH 7.4) at 37°C for 60 seconds. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol/l ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) and the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm (Microplate Reader, POLARstar Omega, BMG LabTech, Germany) against blank. Three measurements were done for each sample. Serum CAT activity was calculated following the equation below:

$$\text{Serum CAT activity (kU/L)} = \frac{[A(\text{Blank 1}) - A(\text{Sample})]}{[A(\text{Blank 2}) - A(\text{Blank 3})]} \times 271$$

Where:

Blank 1 = Control, Blank 2 = Standard, Blank 3 = Reagent Blank, and 271 = Constant.

Measurement of Advanced Glycation End Products (AGEs) in Plasma

AGEs was measured using the quantitative sandwich enzyme immunoassay technique with an antibody specific for AGEs which had been pre-coated onto a microplate (Cusabio Biotech Co., Ltd, China). A concentration curve over the range from 3.9 μ g/ml to 250 μ g/ml was calibrated using the standards provided by the manufacturer. Plasma samples were diluted 20-fold with sample diluent provided. AGEs present in the samples were bound by the immobilized antibody while the unbound substances were removed and biotin-conjugated antibody specific for AGEs was added to the wells. Avidin conjugated Horseradish Peroxidase (HRP) and its substrates were added to the wells subsequent to removal of unbound avidin-enzyme. The optimal density of the colour developed was determined within 5 minutes by the use of microplate reader that was set to 450 nm (Microplate Reader, POLARstar Omega, BMG LabTech, Germany).

Lipid Peroxidation in Plasma

Thiobarbituric acid reactive substances (TBARS) which are the by-products of lipid peroxidation were measured according to the protocol provided by the supplier (Cayman's TBARS Assay, Cayman Chemical, Ann Arbor, MI). Briefly, 100 μ L of each of the plasma

sample was mixed with sodium dodecyl sulphate (SDS) solution and 4 mL of colour reagent. The colour reagent contains TBA, acetic acid and sodium hydroxide. The mixture was boiled for 1 hour and the reaction was stopped by leaving the mixture on ice for 10 min. The mixture was centrifuged at 2000 x g at 4°C for 10 min. The supernatant was subjected to spectrometry measurement at 530 nm using the Microplate Reader (POLARstar Omega, BMG LabTech, Germany).

Acetylcholine (ACh) Abundance in Brain Lysates

The level of acetylcholine (ACh) in the brain tissues of the rats were measured using EnzyChrom™ Acetylcholine Assay (BioAssay System, CA) in accordance with the manufacturer's instruction. ACh was hydrolyzed by acetylcholinesterase to choline which was oxidized by choline oxidase to betaine and H₂O₂. The resulting H₂O₂ reacted with a specific dye to form a pink coloured product. Twenty (20) µL of the supernatant from brain tissue lysates was mixed with 80 µL of working reagent and the intensity of colour change proportional to the acetylcholine concentration was measured at 570 nm (Microplate Reader, POLARstar Omega, BMG LabTech, Germany).

Acetylcholinesterase (AChE) in Brain Lysates

QuantiChrom™ Acetylcholinesterase Assay (BioAssay System, CA) was used to measure the concentration of AChE. Briefly, 200 µL of the reaction mixture containing 10 µL of the supernatant from the brain homogenate of each rat and 190 µL of the working reagent in the 96-well plate was prepared. The intensity of the colour change was measured at 412 nm at 2 time points; after 1 minute and after 9 minutes using microplate reader (POLARstar Omega, BMG LabTech, Germany). The activity of AChE was the difference of absorbance values between the samples and the standard.

Lipid Peroxidation in Brain Lysates

The procedure was the same as described in 2.4.1.3. In this assay, 100 µL of the supernatant from brain tissue lysates was used instead of plasma.

Statistical Analysis

The data collected were tabulated and analyzed using IBM SPSS ver. 22.0 (IBM Co., Armonk, NY, USA). The study parameters were presented as mean ± standard error and compared between different experimental groups using one-way analysis of variance (ANOVA), followed by post-hoc Tukey's test. The data was considered as statistically significant with p-value of less than 0.05.

RESULTS AND DISCUSSION

Standardised Compounds Profiled in TGTPRIMAAGE

The LCMS profile of the extract starting from retention time 7.5 to 13.5 minutes revealed the presence of 10 phenolic compounds (Figure 1; Table 1). The top ten most abundantly identified compounds were phenolic compounds, one of the compounds (brucine) belonged to alkaloids groups; two compounds (2'-Hydroxygenistein 7-(6''-malonylglucoside) and Iriflogenin 4'-O-glucoside) were isoflavonoids and the rest were flavonoids. These compounds were used as markers for standardisation of the batches of TGT-PRIMAAGE.

Most of the compounds found in the extract were flavonoid which belonged to the family of flavonoids glycosides. The compounds have been associated with a broad spectrum of health-promoting effects and are an indispensable component in a variety of medicinal applications including enhancing memory function by regulating proteins such as the cAMP response element-binding protein (CREB), involved in the expression of important gene related to memory (Krishnaveni, 2012). Standardisation of the extraction procedure are fundamentally important to produce batches of extracts with similar profiles of compounds and therefore ensure more consistent efficacy for routine use either as supplement or for alternative health promoting and maintenance therapy.

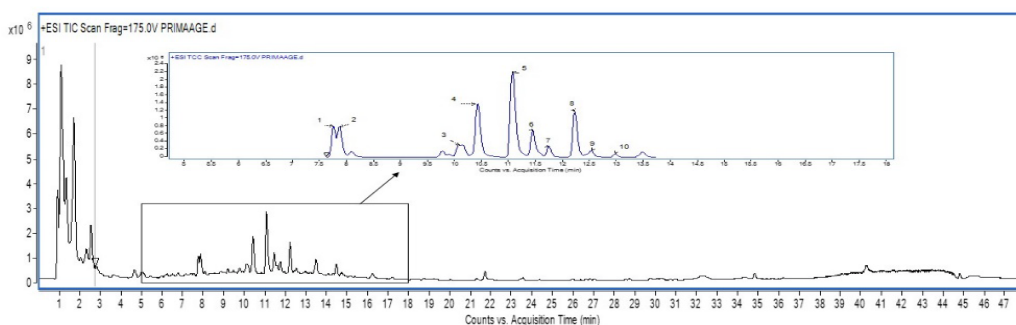


Figure 1. LC/MS Q-TOF total ion chromatogram (TIC) of TGT-PRIMAAGE extract

Acute Toxicity Study

Administration of the extract up to the maximum dose did not result in mortality or change in the behaviour of the animals. Thus, there is no evidence of acute toxicity of TGT-PRIMAAGE in rats.

Memory and Learning Tests

The number of platform entries achieved by the rats treated with medium dose (P-500) and high dose (P-1000) of TGT-PRIMAAGE was 3.89 ± 0.29 and 4.22 ± 0.40 , respectively

Table 1
Ten most abundantly identified phenolic compounds identified in TGT-PRIMAAGE

Peak number	Retention Time (min)	(M+H) ⁺ m/z	Error (ppm)	Score	Molecular Formula	Predicted LC-MS/MS Fragmentation – 10V	Predicted compound	Class
1	7.753	395.1965	-1.1	95.14	C23 H26 N2 O4	115.0393, 163.0576, 259.0799, 295.1021, 356.1156	Brucine	Alkaloid
2	7.868	595.1676	-3	94.12	C27 H30 O15	107.0494, 131.0536, 252.1118, 345.1078, 412.1453, 523.6995	Kaempferol 3-O-glucosyl-(1->2)-rhamnoside	Flavonoids
3	10.084	611.1611	-0.32	97.67	C27 H30 O16	107.0492, 213.1163, 262.0930, 386.0628, 480.0304, 577.1481	Isorhamnetin 3-O-[b-D-xylopyranosyl-(1->6)-b-D-glucopyranoside]	Flavonoids
4	10.427	465.1027	-0.06	98.75	C21 H20 O12	153.1252, 189.0756, 315.1791, 382.7752, 433.1265	Myricitrin	Flavonoids
5	11.068	551.1047	-2.99	94.45	C24 H22 O15	103.0423, 207.1605, 303.0478, 417.1183, 477.2124, 519.0181	Quercetin 3-O-(6-O-malonyl-β-D-glucoside)	Flavonoids
6	11.445	449.1088	-1.69	94.38	C21 H20 O11	107.0478, 208.1529, 287.0570, 356.9702	astragalin	Flavonoids
7	11.727	507.1133	-0.37	96.56	C23 H22 O13	107.0500, 163.0398, 211.1667, 287.0737, 341.1373, 428.4636, 499.1238	Quercetin 3-(6"-acetylglucoside)	Flavonoids

Table 1 (Continued)

Peak number	Retention Time (min)	(M+H) ⁺ m/z	Error (ppm)	Score	Molecular Formula	Predicted LC-MS/MS Fragmentation – 10V	Predicted compound	Class
8	12.222	535.1087	-1.3	96.72	C ₂₄ H ₂₂ O ₁₄	137.0596, 201.0952, 303.0526, 338.2431, 386.1955, 435.9733, 508.7124	2'-Hydroxygenistein, 7-(6"-malonylglucoside)	Isoflavonoids
9	12.525	565.1188	-0.3	98.07	C ₂₅ H ₂₄ O ₁₅	127.0379, 239.1246, 287.0555, 329.0668, 400.9877, 448.9877, 503.0624	Isorhamnetin, 3-(6"-malonylglucoside)	Flavonoids
10	12.913	491.1184	0.72	94.44	C ₂₃ H ₂₂ O ₁₂	107.0480, 129.0552, 205.0949, 248.1645, 355.1419, 394.2155, 463.3346	Iriflogenin, 4'-O-glucoside	Isoflavonoids

(Figure 2). There was no significant difference in the number of platform entries achieved by these 2 groups of rats when compared with the rats that received tocotrienol (3.45 ± 0.40 ; Tukey HSD, $p > 0.05$). However, a significant difference was observed for the behaviour of the rats treated with low dose versus medium and high doses of TGT-PRIMAAGE (1.56 ± 0.11 ; Tukey HSD, $p < 0.05$).

For the escape platform (latency), rats induced with the D-galactose group took the longest time (30.84 ± 2.38 seconds) to reach the platform while the rats that received high dose TGT-PRIMAAGE took a shorter time (7.71 ± 0.61 seconds); which was significantly different (Tukey HSD, $p < 0.05$). There were also no significant difference in the latencies compared between the rats treated with high dose TGT-PRIMAAGE with (i) the normal control (6.30 ± 1.00 seconds; Tukey HSD, $p < 0.05$) and (ii) the rats that received tocotrienol (11.10 ± 3.30 seconds; Tukey HSD, $p < 0.05$). Rats treated with a low dose of TGT-PRIMAAGE had similar latency with the D-galactose induced ageing rats without treatment (30.73 ± 7.02 vs. 30.84 ± 2.38 , Tukey HSD, $p > 0.05$).

The differences in the ability to recognize novel object between the D-galactose induced ageing and the normal rats were statistically significant (Figure 2; 28.34 ± 1.87 vs 62.64 ± 5.86 ; Tukey HSD, $p < 0.05$). The rats treated with medium (72.41 ± 1.45) and a high dose of TGT-PRIMAAGE (83.62 ± 1.13) had a significant improvement (Tukey HSD test, $p < 0.05$) in the ability to recognize novel object compared to the D-Galactose group (28.34 ± 1.87). The improved ability was similar for the rats treated with medium and high dose of TGT-PRIMAAGE and tocotrienol (57.17 ± 11.93 ; Tukey HSD, $p > 0.05$). Interestingly, the rats treated with high dose TGT-PRIMAAGE had a higher percentage of time spent near the novel object, which indicates enhanced memory and learning ability as compared to the normal group (83.62 ± 1.13 vs. 62.64 ± 5.86 ; Tukey HSD test, $p < 0.05$).

The Morris water maze (MWM) is one of the most frequently used platforms to investigate spatial learning and memory in laboratory rats. The MWM had been used in the validation of rodent models for neurocognitive disorders and the evaluation of possible neurocognitive treatments (D'Hooge and De Deyn, 2001). MWM was used to test for spatial learning that relied on distal cues to navigate from the start locations around the perimeter of an open swimming arena to locate a submerged escape platform. Spatial learning is assessed across repeated trials and reference memory is determined by a preference for the platform area when the platform is absent (Vorhees and Williams, 2006). In addition, non-spatial object recognition memory task tests were conducted to study the memory for previously explored objects (Clark et al., 2000). The task, which requires no deprivation or punishment, is based on the rats' innate tendency to preferentially explore novel versus familiar objects. Task performance depends on an intact hippocampal function (Clark et al., 2000). Ageing tends to cause an impairment in the visual object recognition memory (Pilotti et al., 2003). Based on the tests conducted, rats treated with a medium and a high

dose of TGT-PRIMAAGE had gained better learning and memory abilities compared to the rats that were not treated. In fact, the rats treated with high dose TGT-PRIMAAGE showed significantly higher improvement in the latency and novel object recognition tests compared to the rats treated with tocotrienol.

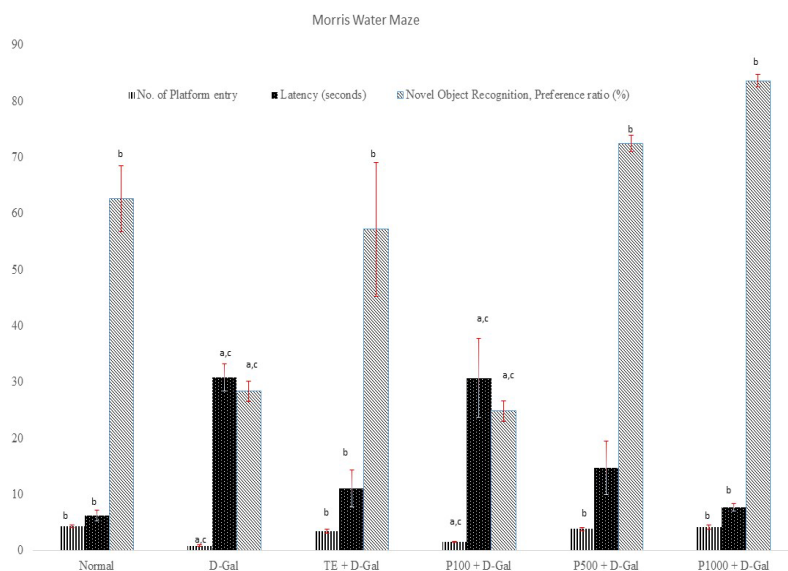


Figure 2. Cognitive activity of normal rats, untreated and pre-treated rats induced with D-galactose. * **Mean number of platform entries in Morris Water Maze test** with S.E.M in different group. a, $p < 0.05$: significantly different when compared to normal group (Tukey HSD test). b, $p < 0.05$: significantly different when compared to D-galactose (Tukey HSD test). c, $p < 0.05$: significantly different when compared to TE (Tukey HSD test). * **Mean latency to reach platform in Morris Water Maze test** with S.E.M in different group. a, $p < 0.05$: significantly different when compared to Normal group (Tukey HSD test). b, $p < 0.05$: significantly different when compared to D-Galactose group (Tukey HSD test). c, $p < 0.05$: significantly different when compared to TE (Tukey HSD test). * **Novel object preference ratio in novel object recognition test** with S.E.M in different group. a, $p < 0.05$: significantly different when compared to normal group (Tukey HSD test). b, $p < 0.05$: significantly different when compared to D-galactose group (Tukey HSD test). c, $p < 0.05$: significantly different when compared to TE (Tukey HSD test)

Catalase (CAT) Activity in Plasma

D-galactose induced a significant decrease of CAT activity in the rats as compared to the rats in the normal control (Figure 2, 57.28 ± 6.80 vs. 78.32 ± 4.26 kU/L; Tukey HSD, $p < 0.05$). Interestingly, CAT activities in all the three groups of rats treated with TGT-PRIMAAGE (low dose, 75.89 ± 2.91 kU/L; medium dose, 90.79 ± 0.95 kU/L; high dose, 94.44 ± 3.69 kU/L) were found to be elevated with significant differences compared to the D-Galactose group (Figure 3). The CAT activities increased as the doses of TGT-PRIMAAGE were increased. Furthermore, the catalase activity of the rats that received medium and high dose TGT-PRIMAAGE had significantly higher CAT activities compared to the rats that received tocotrienol (63.39 ± 1.90 kU/L; Tukey HSD, $p > 0.05$).

Catalase (CAT) is an enzyme involved in antioxidant defence by neutralising radicals produced by D-galactose (Bolzán et al., 1997). Current study showed that ageing rats had the lowest catalase activity while ageing rats supplemented with TGT-PRIMAAGE higher catalase activity. This is a useful indicator that TGT-PRIMAAGE is beneficial in slowing down the ageing processes by reducing the free radicals induced by D-Galactose.

Advanced Glycation End Products (AGEs) in Plasma

In the anti-glycation assay, the AGEs levels in the D-galactose induced rats were significantly elevated compared to the normal control (Figure 3, $294.97 \pm 16.67 \mu\text{g/mL}$ vs. $212.77 \pm 3.35 \mu\text{g/mL}$; Tukey HSD, $p < 0.05$). D-galactose induced ageing rats that received a medium ($248.67 \pm 2.98 \mu\text{g/mL}$) and high dose ($236.17 \pm 1.71 \mu\text{g/mL}$) of TGT-PRIMAAGE and tocotrienol showed significantly lower levels of AGEs compared with the ageing rats without treatment (Figure 3). Interestingly, rats that received tocotrienol ($233.18 \pm 2.99 \mu\text{g/mL}$) and high dose TGT-PRIMAAGE ($236.17 \pm 1.71 \mu\text{g/mL}$) had AGEs level similar with the normal control ($212.77 \pm 3.35 \mu\text{g/mL}$).

AGEs are the end results of a chain of chemical reactions involving an initial glycation reaction. AGEs originate from the non-enzymatic glycation reaction between sugars and protein, nucleic acids or lipid. AGEs were therefore higher in D-galactose induced ageing rats due to the higher glucose concentrations injected to the rats. High level of AGEs in serum is recognized as an ageing-related biomarker (Ramasamy et al., 2005). TGT-PRIMAAGE was found to reduce the level of AGEs. The high dose of TGT-PRIMAAGE and tocotrienol showed a similar reduction in the level of AGEs. Ascorbic acid and vitamins had been demonstrated previously to blocks glycation of erythrocyte haemoglobin by inhibiting *in vitro* lipid peroxidation and being regarded as both a glycation and AGE inhibitor (Vinson et al., 1996). In this study, TGT-PRIMAAGE had shown the ability to inhibit lipid peroxidation and reduced the levels of AGEs.

Lipid Peroxidation (MDA) in Plasma

MDA level was significantly higher in the D-galactose induced rats compared to the normal control (Figure 3, 627.90 ± 98.95 vs. $438.70 \pm 36.04 \text{ nmol/mL}$; Tukey HSD, $p < 0.05$). The effect of high dose TGT-PRIMAAGE was slightly higher than tocotrienol (507.16 ± 56.44 vs. $571.70 \pm 84.58 \text{ nmol/mL}$, Tukey HSD, $p > 0.05$) but no significant difference was observed. The results suggested dose-dependent reduction of the levels of MDA by TGT-PRIMAAGE (Figure 3).

MDA is a sensitive index for the lipid peroxidation induced by free radicals which may lead to oxidative deterioration of polyunsaturated lipids. Under the normal physiological conditions, only low levels of lipid peroxidation occur in the tissue of the body; while

excessive generation of free radicals leads to peroxidative changes that result in an increase in lipid peroxidation (Niki et al., 1993; Rikans & Hornbrook, 1997).

Ageing is associated with increased disruption of membrane lipids which leads to the formation of peroxide radicals. Lipids are a major component of living organisms and probably the first easy target of free radicals thus lipid peroxidation might play an important role in initiating and/or mediating some aspects of the ageing process (Praticò, 2002). In this study, the level of MDA was significantly higher (Tukey HSD, $p < 0.05$) in the D-galactose group as compared with the normal and the tocotrienol and TGT-PRIMAAGE treated groups. Interestingly, the medium and high dose of TGT-PRIMAAGE showed a better potential in arresting lipid peroxidation in both the blood and brain samples. A dose-dependent effect was observed, the higher the dose of TGT-PRIMAAGE the lower the MDA levels. TGT-PRIMAAGE had protected the cells against oxidative stress by reducing lipid peroxidation products as measured systemically and in the brain.

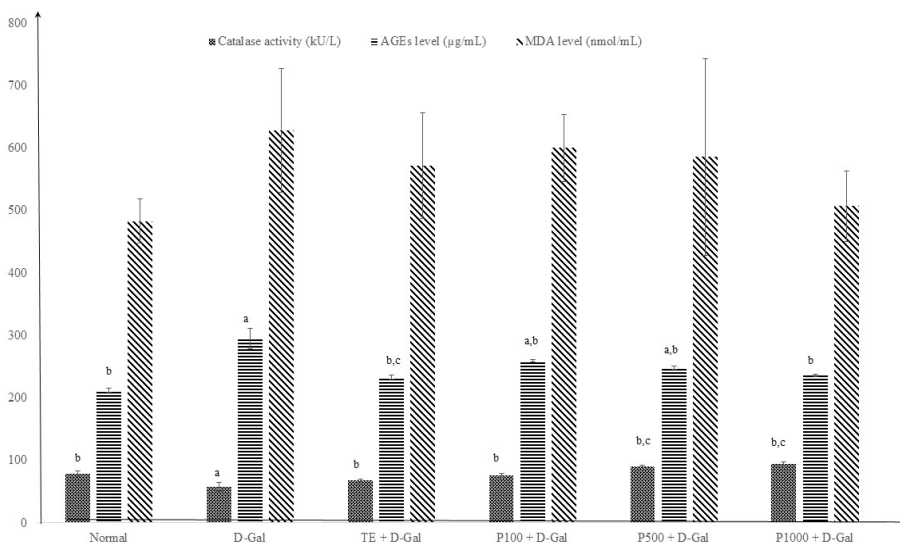


Figure 3. Catalase activity, AGEs and MDA levels in the plasma samples of the rats with different treatment. ***Mean value of assay.** a, $p < 0.05$ significantly different when compared to Normal group (Tukey HSD test). b, $p < 0.05$ significantly different when compared to D-Galactose group (Tukey HSD test) c, $p < 0.05$ significantly different when compared to TE (Tukey HSD test).

Lipid Peroxidation (MDA) in Brain Lysates

Similar to the trend observed for MDA level in the plasma samples, the MDA levels in the brain lysate was higher in the D-galactose induced rats compared to the normal control (Table 2, 12.64 ± 0.67 vs. 7.05 ± 0.34 nmol/mL; Tukey HSD, $p < 0.05$). The MDA levels

in the rats which received treatment of tocotrienol and 100 mg/kg of TGT-PRIMAGES were higher than those measured in the D-galactose induced ageing rats. Rats treated with medium (7.75 ± 0.56 nmol/mL) and high dose (7.52 ± 0.28 nmol/mL) of TGT-PRIMAAGE achieved significantly lower levels of MDA in the brain lysates compared to non-treatment group. The data is presented in Table 2.

Acetylcholinesterase Activity and Acetylcholine Levels in Brain Lysates

Non treated rats induced with D-galactose showed the highest level of acetylcholinesterase activity compared to the control and treatment groups (236.23 ± 6.85 U/L). There is a dose-dependent effect in a decreasing trend of the level of acetylcholinesterase activity in groups of rats treated with TGT-PRIMAAGE. The higher the dose of TGT-PRIMAAGE, the lower the level of acetylcholinesterase activity. Rats treated with the medium (141.44 ± 11.81 U/L) and high dose (138.63 ± 8.1 U/L) of TGT-PRIMAAGE had levels which are lower than the normal control (170.39 ± 5.33 U/L) and tocotrienol (148.88 ± 3.75 U/L) treated rats. The data is presented in Table 2.

The level of acetylcholine was the highest in the normal control, not induced with D-galactose (269.52 ± 22.32 μ M); while the level was the lowest in the non-treated D-galactose ageing rats (85.71 ± 6.23 μ M). The acetylcholine levels were similar among the rats treated with tocotrienol (151.43 ± 11.55 μ M), medium dose TGT-PRIMAAGE (142.38 ± 9.67 μ M) and high dose TGT-PRIMAAGE (152.86 ± 3.78 μ M). The data is presented in Table 2.

ACh is a cholinergic neurotransmitter that plays important role in regulating the cognitive functions of the brain. AChE hydrolyzes ACh into acetate and choline, resulting in the termination of synaptic transmission (Zugno et al., 2014). Anwar et al. (2012) reported that phenolic compounds decreased AChE activity in the cerebral cortex and striatum of adult Wistar rats resulting in higher ACh in the rats and better cognitive function. In this study, the memory-enhancing effect of TGT-PRIMAAGE was found to be accompanied by increasing cholinergic activity in the brain of the rats. The present study found that TGT-PRIMAAGE inhibited AChE activity and at the same time enhanced ACh in treated rats. AChE activity was inhibited in a dose-dependent manner for the rats that were treated with TGT-PRIMAAGE in comparison with the rats without treatment. AChE activity was also inhibited in the rats that received tocotrienol in comparison with rats induced by D-galactose. There were no significant differences in the inhibitory effect of AChE in the normal control and tocotrienol treated group on the AChE. The level of ACh was reduced in the D-galactose induced rats compared to the normal control rats. The levels of ACh increased as the doses of TGT-PRIMAAGE were increased.

Table 2

Lipid peroxidation (MDA), acetylcholinesterase activity and the level of acetylcholine in the brain lysates of the rats

	Normal	D-Gal	TE + D-Gal	P100 + D-Gal	P500 + D-Gal	P1000 + D-Gal
MDA (nmol/mL)	7.05±0.34 ^{b,c}	12.64±0.67 ^{a,b,c}	15.86±0.65 ^{a,b,c}	18.96±0.21 ^{a,b,c}	7.75±0.56 ^{b,c}	7.52±0.28 ^{b,c}
Acetylcholinesterase (U/L)	170.39±5.33 ^b	236.23±6.85 ^{a,c}	148.88±3.75 ^b	158.48±3.87 ^b	141.44±11.81 ^b	138.63±8.10 ^b
Acetylcholine (µM)	269.52±22.32 ^{b,c}	85.71±6.23 ^{a,c}	151.43±11.55 ^{a,b}	104.29±3.60 ^a	142.38±9.67 ^{a,b}	152.86±3.78 ^{a,b}

***Mean value of assay.** a, p<0.05 significantly different when compared to Normal group (Tukey HSD test), b, p<0.05 significantly different when compared to D-Galactose group (Tukey HSD test) c, p<0.05 significantly different when compared to TE (Tukey HSD test).

CONCLUSION

TGT-PRIMAAGE increased the antioxidant capacity by increasing CAT activity. With reduced oxidative stress, the levels of MDA and AGEs were reduced. The supplementation of TGT-PRIMAAGE to the ageing rats also showed improvement in memory and learning abilities by inhibiting acetylcholinesterase activity and increasing the level of acetylcholine.

ABBREVIATIONS

CAT – Catalase

D-gal – D-galactose

AGEs – Advanced glycation end products

MDA – Malondialdehyde

TE – Tocotrienol

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ETHICS STATEMENT

All the protocol was approved by the Research Committee on the Ethical Use of Animal UiTM (UiTM CARE: 91/2015 dated 1 July 2015).

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