Study on Biological Active Components of Eurycoma Longifolia

Nik Nur Shamiha N. D.[1,3*], Siti Shukriyah S.[1,2], Ryuichiro S.[2], Yoshiaki S.[2], Jiyauddin Khan[1,3], Ibrahim A.[1,3]*, Mohd Nizam A. G.[1,3], Mohd Fadli Asmani[1,3] and Eddy Yusuf[3]

[1]*School of Pharmacy, Management and Science University, 40100 Shah Alam, Selangor Darul Ehsan, Malaysia*

[2]*Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan*

[3]*ICHLAS, Management & Science University, 40100 Shah Alam, Selangor, Malaysia*

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Abstract: Background and aims: Constant hyperglycemia in diabetic patient may lead to excess glycation and thus is believed to cause diabetic complications. Eurycoma longifolia (Simaroubaceae) is tested for inhibitory activity of advanced glycation end-products (AGEs) formation in vitro. Materials and methods: Three concentration of methanolic extract were tested together with bovine serum albumin in anti-CML antibody. HRP- conjugated anti-mouse IgG antibodies were introduced and sample were reacted with phenyldiamine dihydrochloride. Absorbance were read by using micro-ELISA and percentage of inhibition were calculated. Results: The calculated percentage of AGEs formation inhibition by E. longifolia root are -3.62 % (0.1 mg/mL), 58.38 % (1 mg/mL) and 92.28 % (10 mg/mL) as compared to aminoguanidine 5.55 % (0.1 mg/mL), 39.32 % (1 mg/mL), 72.92 % (10 mg/mL) as referring to the concentration. Since the biological activity was tested on the whole methanolic extract, the activity is suggested to be due to synergistic activity of the extract. Conclusion: New biological activity of E. longifolia methanolic extract which is inhibition of AGEs formation in vitro is seen. However, isolation of Fr.8-2, m/z:381 does not lead to any compound isolated related to the plant.

1 INTRODUCTION

This study focus on the antiglycation activity of *Eurycoma longifolia* (Simaroubaceae). To date, there is no known activity on inhibition of advanced glycation end products (AGEs) formation by *E. longifolia.* The plant is widely known for anti-tumor promoting activities, antischistosomal, plasmodicidal activities (Jiwajinda, S. *et al.,* 2002), potent antiulcer activity (Tada, H. *et al.,* 1991), helps to improves stress hormone profile and certain mood state parameters (Talbott, S. M., Talbott, J. A., George, A., & Pugh, M., 2013), cytotoxic activity (Kuo, P.C., Damu, A.G., Lee, K.H., & Wu, T.S., 2003), antibacterial action (Farouk, A., & Benafri, A., 2007) as well as antimalarial activity (Ang, H., Chan, K., & Mak, J., 1995). Chronic use of *E. longifolia* is said to increase the testosterone level in men.

AGEs formation lead to kaput protein. AGEs formation in normal healthy people may not be detrimental to their health compared to diabetic patient. It is believed that major complication of diabetes; nephropathy, neuropathy and retinopathy are augmented by AGEs formation as well as constant hyperglycemia. This study utilizes the method of evaluation based on a previous study conducted by Okada, Y., Ishimaru, A., Suzuki, R. and Okuyama, T. in 2004. Inhibition of AGEs formation is based on the carboxymethyl lysine level inhibition.

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By determining the biological activity, it may leads to isolation of compounds responsible for the said activity. This study suggest for further progress in formulating therapeutic agents to counter diabetic complications and thus improve the patients' quality of life and reduce the mortality rate among diabetic due to complications.

2 MATERIALS AND METHODS

2.1 Materials

E. longifolia sliced, dried roots were procured from Malaysia, 99.5 % Methanol (Wako Pure Chemical Industries, LTD, Lot: DSH3947), 99.9 % Methanol (Wako Pure Chemical Industries, LTD, Lot: DSJ0787), 99.7 % Methanol (Wako Pure Chemical Industries, LTD, Lot: DSF3711), ethyl acetate (Kanto Chemical Co. Inc. Lot: 51B1137), 99.0 % 1-butanol (Wako Pure Chemical Industries, LTD, Lot: DSR6754), 99.0 % Chloroform (Wako Pure Chemical Industries, LTD, Lot: DSF3237), 99.8 % Chloroform D + Silver Foil (Cambridge Isotope Laboratories, Inc., Lot: PR-27572/04086CL1), dimethyl sulfoxide, bovine serum albumin, phosphate buffer solution, glucose, PBS containing 0.05% Tween 20, 0.5% gelatin in 100 mL coating buffer, HRP-conjugated anti-mouse IgG antibodies, 1,2-phenyldiamine dihydrochloride, 100 µL of 1 M sulfuric acid.

2.2 Apparatus

Evaporating flask, mantel heater, rotary evaporator (EYELA NVC, No: 038006204), desiccator with silica gel, TLC ODS plate, TLC silica gel plate. UV light transmitter, microtube, micropipette, 96-well plate, micro-ELISA plate reader, HPLC-RI detector (Waters 600 Pump, Waters 600 Controller, Shodex RI-201H Refractive Index Detector), MPLC Micro pump KPW-20 (Kusano, Kagakukikai Co.), Advantec Fraction Collector (CHF122SC), H-NMR Varian (Agilent, 400 Hz), EI-MS (JEOL), HPLC ODS-4151-N column (Senshu Pak, 10 x 150 mm, No: 1110201H), MPLC column (MERCK, LiChroprep Si 60 (40-63 µm), No: 540087666).

2.3 Preparation of Methanolic Extract

Methanolic extract of the roots of E. Longifolia (200 g) was obtained by extraction with MeOH (5.4 L) three times under reflux for 3 hours. The solvent was evaporated *in vacuo* to give MeOH extract (6.74 g).

2.4 Isolation of Components from Methanolic Extract

The methanolic extract was suspended in water, then extracted with EtOAc and n-BuOH, sequentially. Each soluble portion was evaporated *in vacuo* to give EtOAc (1.07 g) and n-BuOH (1.79 g) fractions, respectively. The EtOAc fraction was chromatographed on a prepacked silica gel column (LiChroprep Si60 (40-63 µm) Merck Co. serial number: 540087666, 140987) eluting with $CHCl_3$ to give 15 fractions. **Fr.8**, **Fr.9**, and **Fr.10** was further purified with HPLC-RI (Detector: RI-201H, SHODEX, Column: ODS-4151-N; size: 10 x 150 mm; number: 1110201H, Senshu Scientific Co. Ltd.) detector. **Fr.8** (0.0123 g) was further purified with HPLC-RI detector using MeOH to provide four fraction **Fr.8-1** (0.0001 g) **Fr.8-2** (0.0006 g) **Fr.8-3** (0.0001 g) and **Fr.8-4** (0.0001 g). **Fr.9** (0.0148 g) was further purified with MeOH-H_2O mixture (MeOH : H_2O = 10 : 1) to gives four fraction **Fr.9-1** (0.0014 g) **Fr.9-2** (0.0010 g) **Fr.9-3** (0.0014 g) and **Fr.9-4** (0.0007 g). **Fr.10** (0.0074 g) was further purified with MeOH-H_2O mixture (MeOH : H_2O = 10 : 1) to gives three fraction **Fr.10-1** (0.0002 g) **Fr.10-2** (0.0003 g) **Fr.10-3** (0.0001 g).

2.5 Inhibition Test on AGE Formation in vitro

BSA was incubated with 200 mmol/L glucose in both presence and absence of test compound for 7 days in 0.1 M of phosphate buffer (pH 7.4) at 37 °C. After incubation, coating buffer, blocking buffer and anti-CML antibody were introduced to the cell. HRP-conjugated anti-mouse IgG antibodies was treated to the cells. 1 M sulfuric acid was used to stop the reaction. The level of inhibition is measured by calculating the level of CML measured by CML-specific micro-ELISA plate reader at 492 nm (SpectraMax PLUS 190PC ROM v1.23). Percentage of inhibition was calculated as in following equation:

Inhibition (%) = [1-(A_s – A_b)/(A_c – A_b)] x 100,

where A_s is the CML level in the incubated mixture with sample, A_c is the CML level in the incubated mixture without sample, and A_b is the CML level in the incubates mixture without sample and glucose that served as blank control.

3 RESULTS

Methanolic extraction of *E.longifolia* root (200 g) yielded 6.74 g of dried extract. Repeated extraction under reflux ensures complete extraction from the root. 20 mg of methanolic extract were subjected to inhibition of AGEs formation in vitro by measuring CML level using microELISA at 492 nm. Three reading were recorded for each concentration. The average reading is tabulated in Table 1.

Table 1: Average reading of absorbance of AGEs inhibition by microELISA.

Sample	Reading 1	Reading 2	Reading 3	Average reading
Blank	0.755	0.765	0.663	0.728
Control	2.592	2.280	2.287	2.386
AG 0.1	2.522	2.240	2.119	2.294
AG 1	1.657	1.758	1.787	1.734
AG 10	1.260	1.069	1.201	1.177
EL 0.1	3.146	2.220	1.972	2.446
EL1	1.344	1.683	1.226	1.418
EL 10	0.842	0.993	0.733	0.856

Upon calculating the average reading of each sample, the percentage of AGEs formation inhibition were determined by substituting the absorbance obtained by Micro-ELISA plate reader in the formula. -3.62 %, 58.38 % and 92.28 % of inhibition were calculated in *E. longifolia* with concentration of 0.1 mg/mL, 1 mg/mL and 10 mg/mL respectively. The percentage of inhibition of AGEs by *E. longifolia* compared to Aminoguanidine is as in Figure 1.

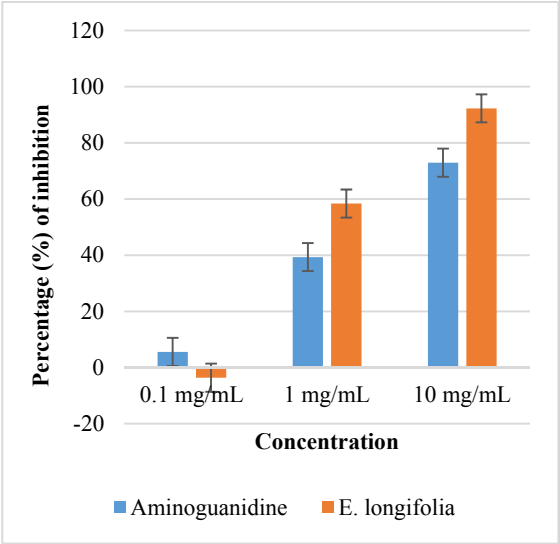

Figure 1: Comparison between percentages of inhibition of AGEs formation between aminoguanidine (positive control) and *E. longifolia* root extract.

4 DISCUSSION

Three concentration of *E. longifolia* root were tested; 0.1 mg/mL, 1 mg/mL, and 10 mg/mL. The calculated percentage of AGEs formation inhibition by *E. longifolia* root are -3.62 % (0.1 mg/mL), 58.38 % (1 mg/mL) and 92.28 % (10 mg/mL) as compared to aminoguanidine 5.55 % (0.1 mg/mL), 39.32 % (1 mg/mL), 72.92 % (10 mg/mL) as referring to the concentration. The activity of *E. longifolia* is suggested to match the activity of aminoguanidine. The percentage of inhibition is concentration dependent. Since the biological activity was tested on the whole methanolic extract, the activity is suggested to be due to synergistic activity of the extract. Due to the small amount of root, the isolated fractions yield are very small. Thus, we are unable to test the biological activity on each fraction. Based on EI-MS analysis of Fr.8-2, the molecular weight is suggested to be *m/z:* 381 as attached in Appendix. However, based on this data alone, we are not able to relate the finding to any compounds reported to be having relationship with the plant *E. longifolia.*

5 CONCLUSIONS

Methanolic extract of *E. longifolia* is suggested to be having the inhibitory activity of AGEs formation. The antiglycation activity is believed to be synergistic action between compounds in the extract. Further isolation of fractions however does not lead to identification of isolates. In the future study, it is strongly suggested that large amount of plant sample should be used and care attention to work procedure must be applied to prevent possible impurity to the components.

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APPENDIX