

Original Article

Inhibitory effect of plant extract on tumor necrosis factor- α formation from carboxymethyllysine stimulated macrophages

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Abstract

Objective: Proteins modified with N^{ϵ} -carboxymethyllysine (CML), one of the advanced glycation end products (AGEs), activate macrophages and increase tumor necrosis factor- α (TNF α) production. In terms of preventive medicine, it is meaningful to suppress the production of TNF α which is involved in various lifestyle-related diseases. In this study, we examined the inhibitory effect of a plant extract on AGE-stimulated TNF α production of CML-stimulated cultured macrophages.

Methods: The mouse macrophage cell line RAW264.7 and, as a stimulator, human serum albumin (HSA) with CML modification (CML-HSA) was used. Test samples were extracts from the following 5 plants and also a mixture of the latter 4 plants: water chestnut (*Trapa bispinosa*), banabá (*Lagerstroemia speciosa*), Chinese blackberry (Tien-cha) (*Rubus suavissimus*), persimmon leaves (*Diospyros kaki*), and kuma bamboo (Kumazasa) (*Sasa veitchii*). TNF α concentration in the culture medium was measured by ELISA after CML-HSA stimulation with or without the pre-treatment of these test samples.

Results: TNF α production from RAW264.7 cells was significantly elevated 3 hours after adding CML-HSA to the medium ($p < 0.001$). Pretreatment with extracts of water chestnut, banabá, Chinese blackberry, persimmon leaf, and mixed plant extract inhibited the TNF α production by 14.6%-22.7%. Cell viability was not affected by the pre-treatment with these extracts.

Conclusion: The data that CML-HSA-stimulated TNF α production from macrophages was significantly reduced by pre-treatment with extracts of water chestnut, banabá, Chinese blackberry, persimmon leaf, or kuma bamboo, indicated that the components contained in these plants may have a favorable action against the glycative stress-induced inflammation.

KEY WORDS: glycative stress, advanced glycation end products (AGEs), inflammation, receptor for AGE (RAGE), N^{ϵ} -carboxymethyllysine (CML)

Introduction

N^{ϵ} -carboxymethyllysine (CML) is a representative advanced glycation end product (AGE). Several studies related to CML are available in the literature. CML is rich in the skin epidermal layer of which cell cycles are rapid¹⁾. Topical application of collagen modulated by CML induces apoptosis on human fibroblasts²⁾.

We have confirmed that, using the *in vitro* glucose/albumin reaction models, CML formation could be prevented by the extracts of banabá (*Lagerstroemia speciosa*), Chinese blackberry (Tien-cha) (*Rubus suavissimus*), persimmon leaf (*Diospyros kaki*), kuma bamboo (Kumazasa) (*Sasa veitchii*)³⁾, and the extract of water chestnut (Hishi) (*Trapa bispinosa*) inhibited the fluorescent AGEs⁴⁾.

CML, once modified by glycation, participates in inflammation mediated by white blood cells. The receptor for AGEs (RAGE), present on the membranes of macrophages,

can recognize and bind to the protein with modified CML. RAGE activates the cellular signals, through the reactions of reactive oxygen species (ROS) formation, MAP kinase activation and nuclear factor-kappa B (NF- κ B) activation, resulting in inflammatory cytokine formation⁵⁾. Tumor necrosis factor alpha (TNF α) is a major inflammatory cytokine. It is 17 kDa in molecular weight, forming a homotrimer structure, circulating in the blood, and shows inflammatory actions⁶⁾. TNF α is an important biomarker in the early phase inflammation and participates in leukocyte chemotactic factor secretion from the endothelium and bio-reactions like vascular endothelial damage and apoptosis induction⁷⁾. In preventive medicine, it plays a significant role to inhibit TNF α formation since TNF α participates in the pathogenesis and progression of lifestyle-related diseases. Earlier studies have assessed the inhibitory effect of plant-extract (*i.e.* fruits, spices) on the inflammation induced by AGEs^{8,9)}.

In the present study, we observed TNF α formation when

cultured macrophages were stimulated by human serum albumin (HSA) modified with CML (CML-HSA), and assessed, using this model, the actions of 5 kinds of plant extracts (water chestnut, banabá, Chinese blackberry, persimmon leaf, and kuma bamboo), that are known to show a high activity preventing AGE formation in the *in vitro* reaction between glucose and albumin.

Methods

Plant extract preparation

Materials were provided from the following companies: water chestnut extract from Hayashikane Sangyo (Yamaguchi, Japan)¹⁰ and banabá, Chinese blackberry, persimmon leaf, kuma bamboo¹¹, and a mixture of these 4 leaves from Hikawa (Shimane, Japan)³. Each leaf 1.0 g was immersed in 40 mL distilled water at 80°C for 1 hour, followed by centrifugation, and then extracts were prepared. Powered water chestnut extract 0.6 g was dissolved in 40 mL distilled water.

Cell culture

Cultured macrophage, RAW264.7, was provided from Prof. Yukiko Minamiyama, Kyoto Prefectural University. As previously reported¹², RAW264.7 was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) added to 10% fetal bovine serum (FBS) (Nihon Biosciences, Tokyo, Japan) containing penicillin 10,000 units/mL, streptomycin 10 mg/mL and amphotericin B 25 μ g/mL at 37°C under the condition of 5% CO₂.

For the evaluation of CML-HSA-induced inflammation, cells, adjusted as 5×10^5 cells/mL, were cultured for 24 hours in 24-well plate dishes, followed by stimulation by 0.5~1.0 μ g/mL CML-HSA (CircuLex, Nagano, Japan). For the assessment of plant extracts (water chestnut, banabá, Chinese blackberry, persimmon leaf, and kuma bamboo), each sample, diluted with DMEM to the final concentration of 1.0~10 μ g/mL, was applied and incubated 24 hours before CML-HSA (0.5 μ g/mL) stimulation. The medium supernatant after CML-HSA stimulation was used for measurement of TNF α by enzyme-linked immunosorbent assay (ELISA).

Murine TNF α measurement

TNF α concentration in the cell culture medium was measured by ELISA as previously reported¹². Briefly, 0.8 μ g/mL mouse TNF-alpha antibody (R&D Systems, Minneapolis, MN, USA), diluted with 50 mM carbonate buffer (pH 9.6), was added to 96-well plates (Thermo Scientific, Waltham, MA, USA) and converted into solid phase. After 2-hour blocking of the reaction by 0.3% skim milk (Difco Laboratories, Detroit, MI, USA), each well was incubated for 1 hour with medium supernatant diluted with DMEM. Then wells were reacted with 0.1 μ g/mL mouse TNF α biotinylated antibody (R&D Systems) for 2 hours, followed by 30-minute standing with application of Pierce Streptavidin Poly-HRP Conjugate (Thermo). After 15-minute coloring reaction with TMB One Component HRP Microwell Substrate (Surmodics, Eden

Prairie, MN, USA), the reaction was stopped by H₂SO₄ (Wako Pure Chemical Industries, Osaka, Japan), and absorbance at 450 nm was measured by SpectraMax Paradigm Multi-Mode Detection Platform (Molecular-Devices Japan, Tokyo, Japan). TNF α concentration was calculated by the standard curve prepared by recombinant mouse TNF-alpha aa80-235 (R&D Systems) with known concentrations (15.6 pg/mL~1,000 pg/mL).

Cell viability assessment

When CML-HSA and plant extracts were applied to the Kit-8 (CCK-8; Dojindo, Kumamoto, Japan), in which viable cell numbers can be counted by colorimetry determination of water-soluble formazan reduced by intracellular dehydrogenase. This assessment was conducted parallel with TNF α measurement.

Cells, adjusted to 5×10^5 cells/mL, were inoculated into 96-well plates followed by application of 100 μ L each test sample in the same manner as in the anti-inflammation evaluation. After 3 hours of incubation with CML-HSA, cells were reacted with 10 μ L CCK-8 for 1 further hour. Absorbance at 490 nm was measured by Spectra Max Paradigm Multi-Mode Detection Platform (Molecular-Devices Japan). The wells were prepared as follows: test sample (S), no-reagent-applied well as the negative control (C), and no-cell-inoculated well as the blank (B). Viability was calculated using the following equation

$$\text{Cell viability (\%)} = \frac{(\text{Abs. (S)} - \text{Abs. (B)})}{(\text{Abs. (C)} - \text{Abs. (B)})} \times 100$$

Anti-oxidative activity measurement

Anti-oxidative activity was evaluated by using i-Pack Oxystress TestTM (ARKRAY, Kyoto Japan)¹³. Results of anti-oxidative activity was expressed as ascorbic acid equivalent (mg/L) using ascorbic acid as the standard reagent.

Statistical analysis

Data were expressed as mean \pm standard deviation (SD). All statistical analyses were performed using SPSS II Statistics 22 (IBM Japan, Tokyo, Japan). Dunnett's test was used for intergroup comparison in the TNF α inhibition study and cell viability assessment. Differences were considered significant at a significance level of 5%.

Results

CML-HSA-stimulated TNF α formation in RAW264.7 cells

CML-HSA (0.5~1.0 μ g/mL) was applied onto RAW264.7 for 1 ~ 6 hours and the release of TNF α was measured by ELISA. After 3- and 6-hour stimulation, TNF α ($p=0.001$, 0.5 μ g/mL and $p < 0.001$, 1.0 μ g/mL) was significantly increased compared with control (**Fig.1**).

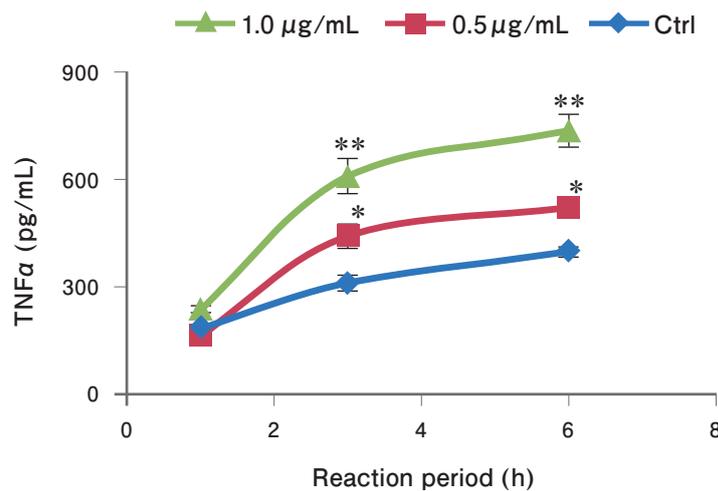


Fig 1. Changes of medium TNF α levels of the CML-HSA-stimulated macrophage.

Data are expressed as mean \pm SD. * $p < 0.01$, ** $p < 0.001$ vs. control by Dunnett's test, $n = 4$. The test culture cells, RAW264.7; stimulator, CML-HSA (0.5 $\mu\text{g}/\text{mL}$, 1.0 $\mu\text{g}/\text{mL}$); TNF α , tumor necrosis factor- α ; CML, N^{ϵ} -carboxymethyllysine; HSA, human serum albumin; SD, standard deviation.

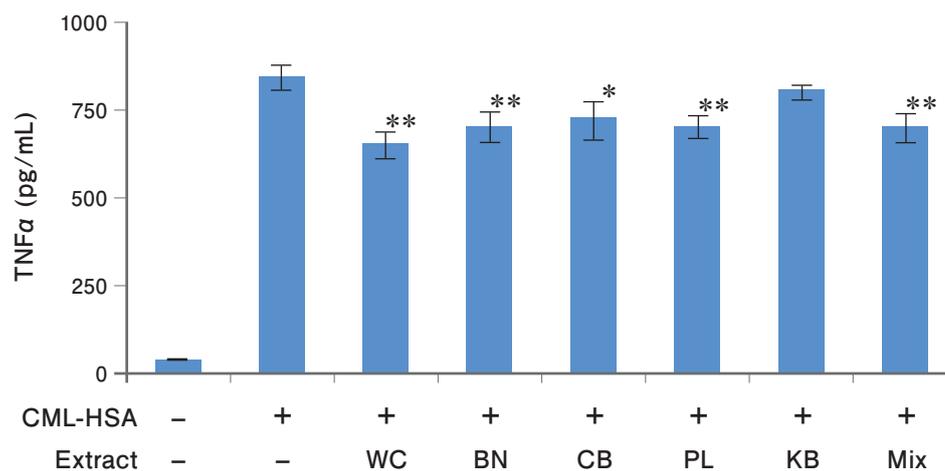


Fig 2. Inhibition by plant extracts on CML-HSA-induced TNF α release.

Data are expressed as mean \pm SD. * $p < 0.01$, ** $p < 0.001$ (1.0 $\mu\text{g}/\text{mL}$) vs. control (CML-HSA +, Extract -) by Dunnett's test, $n = 4$. The test culture cells, RAW264.7; stimulator, CML-HSA (0.5 $\mu\text{g}/\text{mL}$); WC, water chestnut; BN, banabá; CB, Chinese blackberry; PL, persimmon leaf; KB, kuma bamboo; Mix, mixture of BN, CB, PL and KB; TNF α , tumor necrosis factor α ; CML, N^{ϵ} -carboxymethyllysine; HSA, human serum albumin; SD, standard deviation.

Inhibition by plant extracts on CML-HSA-induced TNF α formation

The following extracts were reacted with RAW264.7 for 24 hours and diluted with DMEM, medium: water chestnuts (1.0 $\mu\text{g}/\text{mL}$), banabá and Chinese blackberry (10 $\mu\text{g}/\text{mL}$), and persimmon leaf and kuma bamboo (5.0 $\mu\text{g}/\text{mL}$). The TNF α concentration was measured after 3-hour stimulation with CML-HSA (0.5 $\mu\text{g}/\text{mL}$). TNF α production was significantly reduced by the pretreatment with water chestnut (22.7%, $p < 0.001$), banabá (17.0%, $p < 0.001$), Chinese blackberry (14.6%, $p = 0.001$), persimmon leaf (16.8%, $p < 0.001$) and mixed

extract (17.2%, $p < 0.001$), when compared with CML-HSA-stimulation without pretreatment (Fig. 2). The mixed extract (Mix) consisted of banabá, Chinese blackberry, persimmon leaf, and kuma bamboo.

Cell viability assessment

The influence on cell viability by CML-HSA and plant extracts was evaluated. The number of viable cells was significantly increased by CML-HSA stimulation compared with the control ($p = 0.018$). There was no significant change in viable cell numbers in the plant extract-pretreatment group

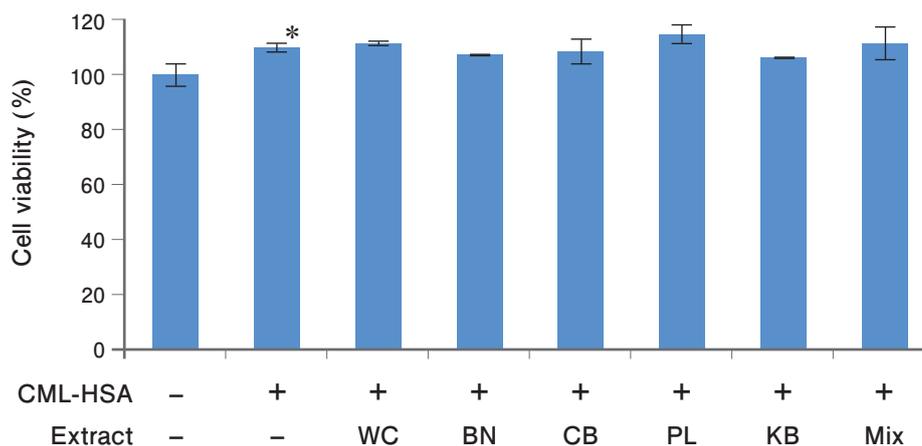


Fig 3. Evaluation of cell viability.

Data are expressed as mean \pm SD. * $p = 0.018$, CML-HSA stimulation vs. control (CML-HSA -, Extract -), by Dunnett's test, $n = 5$. The test culture cells, RAW264.7; stimulator, CML-HSA (0.5 $\mu\text{g}/\text{mL}$); WC, water chestnut; BN, banabá; CB, Chinese blackberry; PL, persimmon leaf; KB, kuma bamboo; Mix, mixture of BN, CB, PL and KB; TNF α , tumor necrosis factor α ; CML, N^{ϵ} -carboxymethyllysine; HSA, human serum albumin; SD, standard deviation.

compared with the CML-HSA-stimulated group without pretreatment (**Fig. 3**).

Anti-oxidative activity measurement

Anti-oxidative activity of 4 test samples were as follows: banabá 2,243 mg/L, Chinese blackberry 1,882 mg/L, persimmon leaf 604 mg/L and kuma bamboo 200 mg/L. Each result is expressed as ascorbic acid equivalent.

Discussion

In this study, using cultured macrophage cells, RAW264.7, stimulated by CML-HSA as a glycation stress, TNF α formation was measured and the anti-inflammatory actions of plant extracts were assessed. The following receptors are known to bind to AGEs as a ligand on the cell surface in addition to RAGE: macrophage type-I and type-II class A scavenger receptors (MSR-A), CD36-SR-BI and LOX-1 (lectin-like oxidized low density lipoprotein receptor-1) which belong to the MSR-B family, galectin-3 complex, FEEL1-2 (fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1-2), megalin, and toll-like receptor 4 (TLR4)¹⁴. These receptors play roles in inflammation reaction, degeneration and removal of AGEs, or cell proliferation. RAGE participates in inflammation, recognizes proteins with CML modification or glyceraldehyde-derived AGEs (toxic AGEs; TAGE) which are known to be cytotoxic¹⁵. Mediated by NF- κ B activation, RAGE induces inflammatory cytokines (*i.e.* TNF α , interleukin [IL]-1 β , IL-6), matrix metalloproteinase (MMP), vascular endothelial growth factor (VEGF) and adhesion factors (*i.e.* intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1])¹⁶. The CML-HSA-stimulated TNF α formation observed in this study (**Fig. 1**) may be mainly due to the RAGE actions. Precise analysis is necessary for further investigations to determine

the specific contributions of each receptor.

CML accumulation induces chronic inflammation in tissue. Released inflammatory cytokines are reported to play a pathogenetic role in Alzheimer's disease¹⁷, age-related macular degeneration¹⁸, cancer¹⁹, and bronchial asthma²⁰, and also to inhibit activation of Sirtuin 1²¹ which is known as a longevity gene.

In addition to these disorders, "locomotive syndrome," which has recently attracted attention in preventive medicine, has been suggested in relation to glycation stress, and glycation stress may reduce the bone quality by forming pathological cross-links between bone collagen fibers²². Inflammatory cells infiltrated into the synovium induce synovitis, which plays a significant role in the pathogenesis of rheumatoid arthritis and knee osteoarthritis²³. The TNF α produced in the synovium induces the expression of the transcription factor CCAAT/enhancer-binding protein (C/EBP) β , through the induction of inflammatory mediators (*i.e.* prostaglandins) and vascular endothelial growth factor (VEGF), and enhances inflammation and causes articular swelling. Furthermore, TNF α activates MMP-3 and MMP-13, cartilage matrix degrading enzymes, and finally causes the degeneration and destruction of cartilage²⁴. In addition, TNF α induces the expression of receptor activator of nuclear factor kappa-B ligand (RANKL), essential for the differentiation of osteoclasts, and accelerates bone resorption, thus increasing the risk of osteoporosis developing²³. On the other hand, since the half-life of cartilage collagen is long, CML-modified proteins are abundant in tissue²⁵. It has been suggested that AGE deposits in the joint cartilage tissue may participate in the pathogenesis and progress of osteoarthritis of the knee²⁶. It is possible that the CML-modified protein-induced inflammatory substance in the cartilage may be involved in the pathogenesis of locomotive syndrome and in the hypofunction of locomotive organs in a similar manner as in the synovium.

The AGEs-RAGE interaction is related to various lifestyle-related diseases. Therefore it is important to prevent TNF α production, which is produced in the early phase of

inflammation and influences the inflammatory response and injury. The common component in the anti-inflammation action of the 5 plant extracts in this study has not yet been reported. The following actions are indicated by the polyphenol included in each extract. Tannin is included in water chestnut peel extract which inhibits rat hind limb edema²⁷⁾. In a randomized controlled trial using banabá extract, production of the adhesion molecules ICAM-1 and VCAM-1, which are related to inflammation, was inhibited in patients with type 2 diabetes²⁸⁾. The main polyphenol of Chinese blackberry (Tiencha) is galloyl-oxygen-diphenyl (GOD) type ellagitannin; it is reported that Chinese blackberry extract prevents the release of leukotriene B4 from the rat peritoneal macrophage thus manifesting an anti-inflammation action²⁹⁾. The persimmon leaf flavonoid is effective for inflammatory disturbance in the nervous system in cases of ischemia reperfusion injury³⁰⁾. There is also a report that proanthocyanidin inhibits RAGE manifestation³¹⁾. In addition to its anti-inflammatory action, persimmon leaf proanthocyanidin improves hyperglycemia and dyslipidemia in the type 2 diabetes model mouse³²⁾. Ellagitannin in banabá extract possesses an insulin-like action and activates the glucose transport in adipocytes³³⁾. Therefore these components contribute to the improvement of glucose metabolism. Although the precise identification of the responsible ingredients was not undertaken in the present study, the results that these polyphenols prevent TNF α production and support disease prevention are considered to be worthy findings.

It's confirmed that the plant extracts used in this study indicate the inhibitory actions of *in vitro* AGE formation which is stronger than that of aminoguanidine (Fig. 2). It is not likely that each plant extract specifically inhibits the CML-HSA-stimulated TNF α formation. However, we can add the new action of AGE-induced inflammatory response prevention in addition to AGEs generation inhibition.

The anti-oxidative activity of the 4 extracts in the mixed plant extract, as measured by i-Pack Oxystress Test¹³⁾, showed an ascorbic acid equivalent of banabá 2,243 mg/L, Chinese blackberry 1,882 mg/L, persimmon leaf 604 mg/L and kuma bamboo 200 mg/L. According to "Standard Dietary Intake in Japan (2010)" guidelines announced by the Ministry of Health,

Labour and Welfare, the daily recommendation value of ascorbic acid is 100 mg/day for adults. The ascorbic acid equivalent of the mixed plant extract is markedly large compared with the recommended value. Since the oxidative stress is known to promote the glycative reaction and increase the AGE production in the skin or other tissues³⁵⁾, it is also important to control the oxidative reaction. These plant extracts may contribute to reduce glycative stress by acting as antioxidants.

Conclusion

In the present study model, employing TNF α formation from CML-HSA-stimulated macrophages as the index, some plant extracts inhibited TNF α formation, thus reducing inflammatory reaction. These actions of plant extracts may be useful since inflammation induced by glycative stress occurs in the body³⁴⁾. As the next step, we plan to study the correlation between CML and TNF α in the body and also the inhibitory effect of these extracts on CML and TNF α in a clinical study.

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Conflict of interest statement

The authors have no conflict of interest related to this study to declare.

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