การเปรียบเทียบฤทธิ์ต้านการอักเสบของสารสกัดจากใบบัวบกในเซลล์แมคโครฟาจ
Comparison of Anti-inflammatory Effects of *Centella asiatica* (L.) Urb. Leaf Extract in Macrophages

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บทคัดย่อ

บัวบกเป็นสมุนไพรพื้นเมืองในเขตร้อนแห่งเอเชียที่ได้รับการคัดเลือกเพื่อส่งเสริมและพัฒนาให้เป็นหนึ่งในห้าผลิตภัณฑ์สมุนไพรยอดเยี่ยมของประเทศไทย เนื่องจากมีศักยภาพในการแข่งขันเชิงพาณิชย์ บัวบกมีสรรพคุณหลากหลาย โดยเฉพาะอย่างยิ่งช่วยแก้อาการช้ำในและต้านการอักเสบ การศึกษานี้มีวัตถุประสงค์เพื่อเปรียบเทียบฤทธิ์ต้านการอักเสบของสารสกัดใบบัวบกในเซลล์แมคโครฟาจ (RAW264.7) ที่ถูกกระตุ้นด้วยไลโปโพลีแซคคาไรด์ (LPS) โดยใช้เทคนิครีเวอร์สทรานสคลิปชั่น โพลีเมอเรสเชนรีแอคชั่น (RT-PCR) เพื่อดูการแสดงออกของยีนไซโคลออกซีจีเนส-2 (COX-2) อินเตอร์ลิวคิน -1 เบต้า (IL-1β) อินเตอร์ลิวคิน-6 (IL-6) อินดิวซิเบิลไนตริกออกไซด์ซินเทส (iNOS) ทูเมอร์เนโครซีสแฟคเตอร์อัลฟา (TNF-α) ผลการศึกษาเมื่อบ่มส่วนสกัดหยาบบัวบกที่สกัดด้วยเอทิลอะซิเตท (E1) เอทานอล (E2) 50% เอทานอล (E3) และ 80% เอทานอล (E4) กับเซลล์แมคโครฟาจนาน 24 ชั่วโมง พบว่า E3 มีความเป็นพิษกับเซลล์น้อยที่สุด รองลงมาได้แก่ E4, E2 และ E1 นอกจากนี้สารสกัดใบบัวบกส่วนสกัดต่าง ๆ ที่ความเข้มข้น 50-200 ไมโครกรัมต่อมิลลิลิตรสามารถยับยั้งการแสดงออกของ COX-2, IL-1β, IL-6, iNOS และ TNF-α ได้อย่างมีนัยสำคัญทางสถิติและใกล้เคียงกับยาอินโดเมทาซิน โดยพบว่าสารสกัด E1 และ E2 แสดงการยับยั้งการอักเสบที่ดีกว่าสารสกัด E3 และ E4 ผลจากการศึกษาในครั้งนี้เป็นรายงานฤทธิ์ต้านการอักเสบในระดับโมเลกุลของสารสกัดบัวบก ซึ่งเป็นข้อมูลที่ช่วยสนับสนุนการพัฒนาผลิตภัณฑ์ธรรมชาติและเภสัชภัณฑ์จากสมุนไพรบัวบกต่อไป

คำสำคัญ : ฤทธิ์ต้านการอักเสบ บัวบก ความเป็นพิษต่อเซลล์

Abstract

Bua-bog (*Centella asiatica* (L.) Urb) is a local herb in the tropical region of Asia that has been selected to promote and develop to be one of the five best herbal products in Thailand due to its commercial competitiveness. Bua-bog has many properties, especially anti-bruising and anti-inflammatory properties. The objective of this study were to compare the anti-inflammatory effect of Bua-bog extract in the LPS-stimulated macrophages (RAW264.7) by using reverse transcription-polymerase chain reaction (RT-PCR) to study the expression of COX-2, IL-1β, IL-6, iNOS and TNF-α. After 24 hr incubation with macrophage cells, the crude extract of Bua-bog extracted with ethyl acetate (E1), absolute ethanol (E2),

50% ethanol (E3) and 80% ethanol (E4) found that E3 was the least toxic to cells, followed by E4, E2 and E1. In addition, various extracts of Bua-bog at concentrations of 50-200 µg/ml were able to significantly inhibit the expression of COX-2, IL-1β, IL-6, iNOS and TNF-α and close to indomethacin drugs. It was found that E1 and E2 showed better inhibition than those of E3 and E4. The results of this study are a report on the anti-inflammatory effect at the molecular level of Bua-bog extract, which is the information to support the development of natural products and pharmaceutical products from this plant.

Keywords: Anti-inflammatory activity, *Centella asiatica* (L.) Urb, Cytotoxic effect

Introduction

Inflammation, especially chronic and unregulated inflammation, plays an important role in cancer development. Several pro-inflammatory mediators, such as tumor necrosis factor (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1β), and inflammatory-related enzymes including inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), released from immune and stromal cells surrounding tumor microenvironment can induce cell transformation and malignancy (Culig, 2011, pp. 308-314, Landskron *et al*., 2014, pp. 1-9). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced from the inflammatory process also involve with carcinogenesis (Landskron *et al*., 2014, pp.1-9). Suppressive effects of anti-inflammatory agents on pro-inflammatory mediator production can help to prevent and treat cancers (Todoric *et al*., 2016, pp. 895-905).

Centella asiatica (L.) Urb (Tiger Herbal, pennywort, gotu kola) is a perennial, creeper, faintly aromatic and a valuable medicinal herb of the family APIACEAE. It is widely distributed throughout tropical and subtropical regions of the world (Kirtikar & Basu, 1987, pp. 1193-1195). It is also well known for traditional drug formula using stem and leaves, aerial parts to decrease blood pressure, cure the fresh wound, heal bruised, and increase excretion of urine. The use of *C. asiatica* in food and beverages has increased over the years basically due to its beneficial functional properties. The fresh leaves taste slightly bitter and are eaten with northern food such as sour chopped meat salad or fried noodles. The green juice extracted from the leaves is very rich in vitamin A and is commonly taken for heat-burn after being boiled in water with sugar. The fresh plants have antipyretic, diuretic, antisyphilitic, astringent and expectorant activities, and can be used to soothe headaches or burns. In cosmetics, it is a magic herb that used as an ingredient in chapped lips protectant, in the oral gel for treating oral wounds, in dried skin lotions, in bath care products to improve skin cells (epidermis), and in hair tonic products to treat the dried head and skin problems. *C. asiatica*, has a wide range of biological activities desired for human health such as neuroprotective (Orhan, 2012, pp.1-8), cytotoxicity, insecticidal, phytotoxicity, antibacterial, anti-inflammatory, antifungal and antioxidant (Sultan *et al*., 2014, pp. 319-327). This plant should be further developed for the utilization as an ingredient in traditional medicine and cosmetics. Therefore, the objectives of this study were to compare the different solvent extracts of *C. asiatica* ethyl acetate (E1), absolute ethanol (E2), 50% ethanol (E3) and 80% ethanol (E4) extracts in *in vitro* for an anti-inflammatory effect.

Objective

To compare the different solvent extracts from *C. asiatica* for an anti-inflammatory effect.

Methodology

Plant material and extraction

C. asiatica was collected from Wattana Nakorn district, Sa Kaeo province, Thailand in February 2018. Fresh leaves of *C. asiatica* were washed, cut into small pieces and dried in an oven (Memmert, Germany) at 45°C. Ground-dried of *C. asiatica* was divided into 4 parts, 50 grams each, and then each part was immersed with ethyl acetate, absolute ethanol, 50% ethanol-water and 80% ethanol-water respectively for 5 days at room temperature. The crude extract was evaporated under reduced pressure to afford a brownish ethyl acetate (E1: 1.59 g), absolute ethanol (E2: 4.13 g), 50% ethanol-water (E3: 8.50 g) and 80% ethanol-water (E4: 12.30 g) extracts.

Determination of anti-inflammatory activity

Reagents

In this study, the chemicals were used Molecular Biology Agarose (Bio-Rad, Spain), 1kb DNA ladder (Promega, USA), Blue/Orange 6X Loading dye (Promega, USA), Primer (Proligo LLC, Boulder, co, USA), Tris base, Glacial acetic acid, EDTA (Ajax/Australia), Omiscript RT Kit (QIAGEN), TopTaq MasterMix kit (QIAGEN) and Novel Juice (GeneDirex). RNA extraction kit (GE Healthcare, UK) was used to extract total RNA from the cells. DMEM media (Invitrogen, USA), FBS (Invitrogen, USA), Penicillin-streptomycin (Invitrogen, USA), *Escherichia coli* lipopolysaccharides (LPS) (Sigma, USA), MTT (Invitrogen) also were used in this study.

Cell culture

The murine macrophage cell line, RAW264.7 cells, was purchased from PromoCell, Germany. The cells were cultured in DMEM media supplemented with 10% heat-inactivated calf serum (HyClone, USA) and 1% penicillin (100U/ mL)-streptomycin (100 µg/ mL) and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Cytotoxicity test

Macrophage RAW264. 7 cells were treated with various concentrations of extract and then incubated at 37 °C in the humidified atmosphere with 5% CO_2 for 24 hr. Cell viability was analyzed by using MTT assay (Mosmann, 1983, pp. 55-63; Sripanidkulchai & Junlatat, 2014, pp. 615-622) and the absorbance measured at 570 nm. The results were calculated for % inhibition and expressed as 50% inhibitory concentration.

Determination of inflammatory-related gene expression

The cells were overnight cultured in 12-well plate and treated with various concentrations of extract and positive control. After incubation at 37 °C in the humidified atmosphere with 5% CO_2 for 22 hr, the LPS was added then further incubated for 2 hr. Total RNA was extracted from the treated cells by using a GE Healthcare extraction kit. The first-strand cDNA was synthesized from total RNA (40 ng) with Omniscript reverse transcriptase kit. The primers were used for amplifying the respective fragments. Polymerase chain reaction (PCR) was performed by incubation of each cDNA sample with the primers, Taq polymerase, and deoxynucleotide mix. Amplification was completed for 30 cycles and the conditions for PCR amplification followed previous reports (Won *et al*., 2006, pp. 216-225, Sripanidkulchai *et al*., 2009, pp. 566-570). The PCR products were then analyzed on 1.5 % agarose gel, visualized by NovelJoice staining and RT-PCR product densities measured by Gel Documentation and System Analysis machine.

The inflammatory-related gene expressions were calculated for the relative mRNA expression level compared with β-actin.

Statistical analysis

All experiments were performed in triplicate and the results expressed as mean±S.D. One-Way ANOVA and multiple comparisons were used to analyze the significant different (P<0.05) by using SPSS software.

Results

Toxicity of the extracts on RAW 264.7 cells

The effect of E1, E2, E3, and E4 on the viability of RAW264.7 cells was determined using MTT assay. The cells were treated for 24 hr with various concentrations of the extracts at 0.00-1,000 µg/mL. Figure 1 shows toxic effects on RAW264.7 cells as IC_{50} value at 330±10, 420±30, 730±30, and >1,000 µg/mL of E1, E2, E4, and E3, respectively. The results suggested that E1 exhibited higher toxicity on the cells than that of the others. Based on these results, we evaluated the effect of these extracts on anti-inflammation at doses lower than its IC_{50} value.

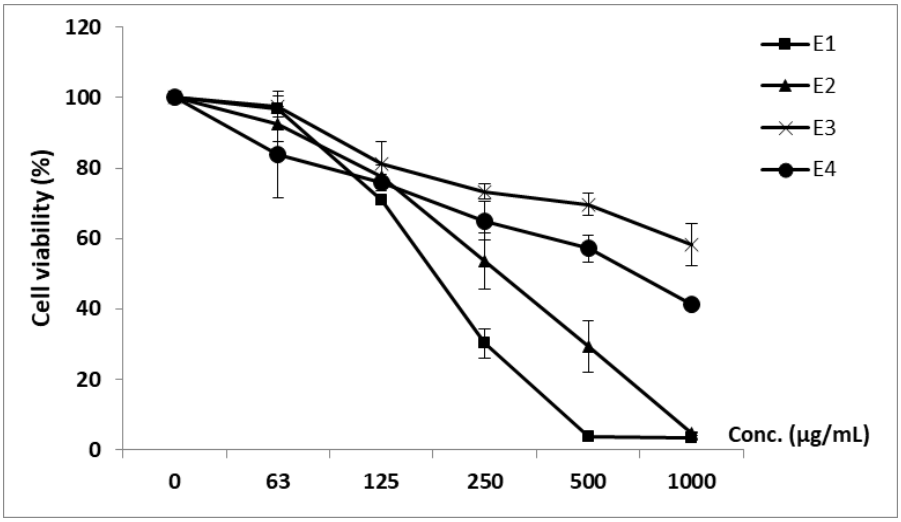

Figure 1: Effect of E1, E2, E3, and E4 on RAW264.7 cells viability.
Each value is a mean±SD compared to control from three individual experiments.

Effect of the extracts on the pro-inflammatory gene expression

The expression of the pro-inflammatory gene including COX-2, IL-6, TNF-α, iNOS, and IL-1β were not changed when treatment with the extracts alone but were up-regulated after treatment with LPS. After incubation for 22 hr with the extracts at 50-200 µg/mL concentration, it was found that all of the extracts could inhibit the expressions of these genes in the dose-dependent manner (Figure 2). Particularly at the concentration of 200 µg/mL, E1 exhibited the highest suppression percentage on IL-1β, IL-6 and TNF-α gene among sample test at 84% , 83% and 31% , respectively. On the other hand, E2 showed the highest suppression percentage on COX-2 and iNOS at 58% and 83%, respectively. Moreover, the suppression on these genes was similar to standard drug, indomethacin and aminoguanidine (Figure 3).

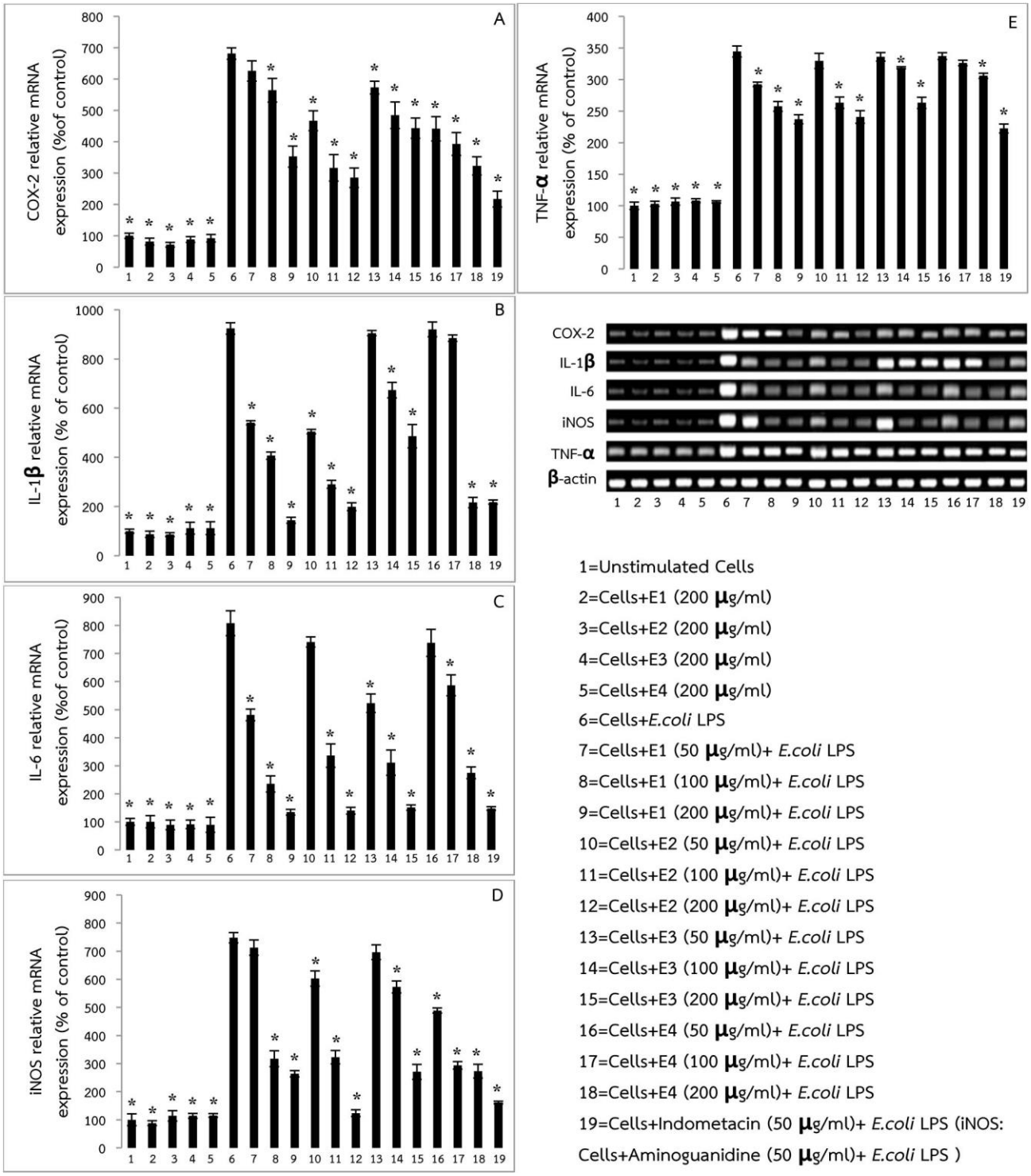

Figure 2: Inhibitory effect of plant extracts on mRNA expression of COX-2 (A), IL-1β (B), IL-6 (C), iNOS (D) and TNF-α (E) compared with β-actin mRNA expression. Values were expressed as mean±SD (n=3). (*Significant difference from LPS treatment alone, P<0.05)

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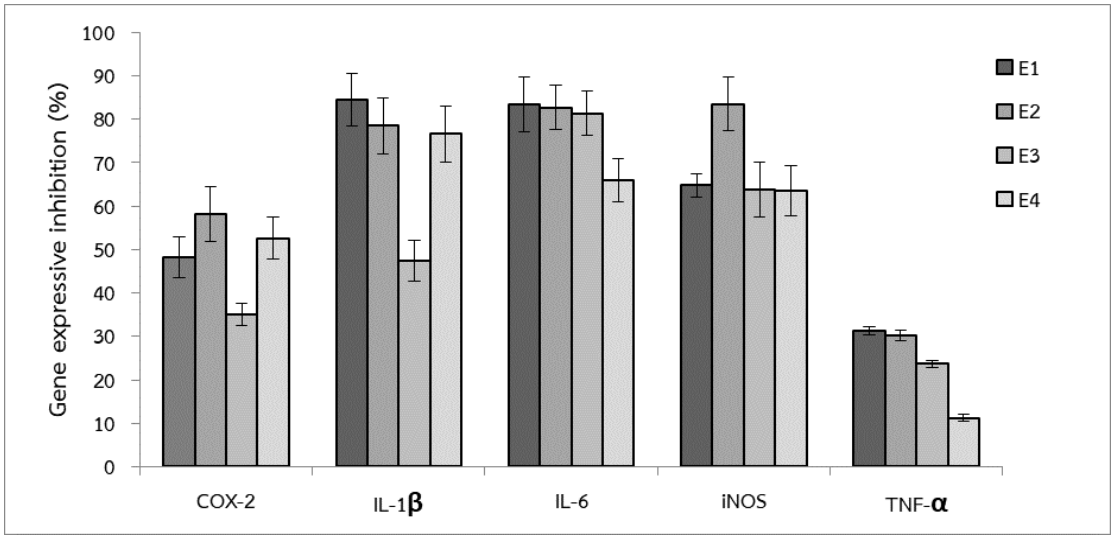

Figure 3: The expressive inhibition of the extracts (200 µg/ml) on COX-2, IL-1β, IL-6, iNOS and TNF-α gene in RAW264.7 cells. Values were expressed as mean±SD (n=3).

Discussion

With nutritional and medicinal properties, *C. asiatica* was reported to have several pharmaceutical effects such as anti-tumor, anti-inflammation, anti-virus and immunomodulation. Abundant bioactive substances were found in *C. asiatica* such as terpenoids, phenolics, flavonoids, and miscellaneous, which is found in the ethanolic and aqueous extracts of *C. asiatica* (Gray *et al.*, 2018, pp. 161–194). In this study, *C. asiatica* ethyl acetate (E1), absolute ethanol (E2), 50% ethanol (E3), and 80% ethanol (E4) extracts showed strong anti-inflammatory activity by suppression of the LPS-induced pro-inflammatory mediator gene expressions including IL-1β, IL-6 and TNF-α, and inflammatory-related enzymes including COX-2 and iNOS. Moreover, E1 and E2 could reduce these gene expressions at a similar or a better degree than the positive control suppressors, indomethacin and aminoguanidine. In this research, indomethacin and aminoguanidine were used as standard substances. Indomethacin is a nonsteroidal anti-inflammatory drug (NSAID) commonly used as a prescription medication to reduce fever, pain, stiffness, and swelling from inflammation. It works by inhibiting the production of prostaglandins, endogenous signaling molecules known to cause these symptoms. It does this by inhibiting cyclooxygenase, an enzyme that catalyzes the production of prostaglandins (Uehara *et al*., 2016, pp.847-852). Aminoguanidine has been identified as one of the first iNOS selective inhibitors. Aminoguanidine is over 50-fold more effective at inhibiting the enzymatic activity of iNOS than endothelial or neuronal NOS (Corbett & McDaniel, 1996, pp. 31-30). The suppressive effect on pro-inflammatory mediator genes might due to its chemical contents including terpenoids (asiatic acid, asiaticoside) and flavonoids (quercetin, quercitrin, kaempferol) (Yasurin *et al.*, 2016, pp. 1-9).

In LPS-exposed macrophages, reactive molecules such as hydroxyl free radicals, hydrogen peroxide and nitrogen monoxide are produced and involved in cellular signaling for pro-inflammatory gene expressions. These pro-inflammatory mediators and inflammatory-related enzymes including TNF-α, IL-1β, IL-6, COX-2 and iNOS also play roles in oxidative stress-induced inflammation (Fangkrathok *et al.*, 2013, pp. 631-637). This inflammatory environment can harm surrounding cells and if this harmful occurring in long term it may lead to carcinogenesis. Consumption of vegetables and herbs can be an

alternative way in illness prevention. The data from other studies suggest that *C. asiatica* can also be a good anti-inflammatory source for illness prevention and treatment.

Conclusions

The present study demonstrated of comparing the anti-inflammatory effects of *C. asiatica* extract in Macrophages. The ethyl acetate (E1) and absolute ethanol (E2) extracts of this plant at concentrations of 200 µg/mL were able to significantly inhibit the expression of COX-2, IL-1β, IL-6, iNOS and TNF-α and close to indomethacin drugs, which are a non-steroidal anti-inflammatory drug. These results support the use of *C. asiatica* as a traditional medicine and can be a good candidate for natural product development. However, an *in vivo* study and its mechanism of action are needed for further study.

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References

Corbett, J. A. & McDaniel, M. L. (1996). The use of aminoguanidine, a selective iNOS inhibitor, to evaluate the role of nitric oxide in the development of autoimmune diabetes. *Methods, 10*, 21-30.

Culig, Z. (2011). Cytokine disbalance in common human cancers. *Biochim Biophys Acta*, *1813*, 308–314.

Fangkrathok, N., Junlatat, J. & Sripanidkulchai, B. (2013). *In vivo* and *in vitro* anti-inflammatory activity of *Lentinus polychrous* extract. *J Ethnopharmacol*, *147*, 631-637.

Gray, N. E., Magana, A. A., Lak, P., Wright, K. M., Quinn, J., Stevens, J. F., Maier, C. S. & Soumyanath, A. (2018). *Centella asiatica*–Phytochemistry and mechanisms of neuroprotection and cognitive enhancement. *Phytochem Rev., 17(1)*, 161–194.

Kirtikar, K. R. & Basu, B. D. (1987). Indian medicinal plants, Vol. II, Delhi: Dehra Dun and Periodical Experts. 1193-1195.

Landskron, G., De la Fuente, M., Thuwajit, P., Thuwajit, C. & Hermoso, M. A. (2014). Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res*, 1-19.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods*, *65*(1–2), 55–63.

Orhan, E. I. (2012). *Centella asiatica* (L.) Urban: From Traditional Medicine to Modern Medicine with Neuroprotective Potential. *Evid Based Complement Alternat Med*. *2012*, 1-8.

Sripanidkulchai, B., Junlatat, J., Wara-aswapati, N. & Hormdee, D. (2009). Anti-inflammatiory effect of *Streblus asper* leaf extract in rats and its modulation on inflammation-associated genes expression in RAW264.7 macrophage cells. *J Ethnopharmacol, 124,* 566-570.

Sripanidkulchai, B. & Junlatat, J. (2014). Bioactivities of alcohol based extracts of *Phyllanthus emblica* branches: antioxidation, antimelanogenesis and anti-inflammation. *J Nat Med*, 68, 615-622.

Sultan, R. A., Mahmood, S. B. Z., Azhar. I., Ahmed, S. W. & Mahmood, Z. A. (2014). Biological activities assessment of *Centella asiatica* (Linn.). *Journal of Herbs, Spices & Medicinal Plants*, 20,319–327.

Todoric, J., Antonucci, L. & Karin, M. (2016). Targeting inflammation in cancer prevention and therapy. *Cancer Prev Res*, *9*, 895-905.

Uehara, Y., Murata, Y., Shiga, S. & Hosoi, Y. (2016). NSAIDs diclofenac, indomethacin, and meloxicam highly upregulate expression of ICAM-I and COX-2 induced by X-irradiation in human endothelial cells. *Biochem Biophys Res Commun, 479,* 847-852.

Won, J. H., Im, H. T., Kim, Y. H., Yun, K. J., Park, H. J., Choi, J. W. & Lee, K. T. (2006). Anti- inflammatory effect of buddlejasaponinIV through the inhibition of iNOS and COX-2 expression in RAW264.7 macrophages via the NF-kappaB inactivation. *Br J Pharmacol, 148,* 216–225.

Yasurin, P., Sriariyanun, M. & Phusantisampan, T. (2016). Review: The Bioavailability Activity of *Centella asiatica. KMUTNB Int J Appl Sci Technol, 9*(1), 1–9.