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ORIGINAL ARTICLE

Antioxidant Activity and Inhibitory Effect on Nitric Oxide Production of Rang Chuet (*Thunbergia laurifolia* Lindl.) Leaf Extracts in Lipopolysaccharide-Stimulated BV2 Microglial Cells

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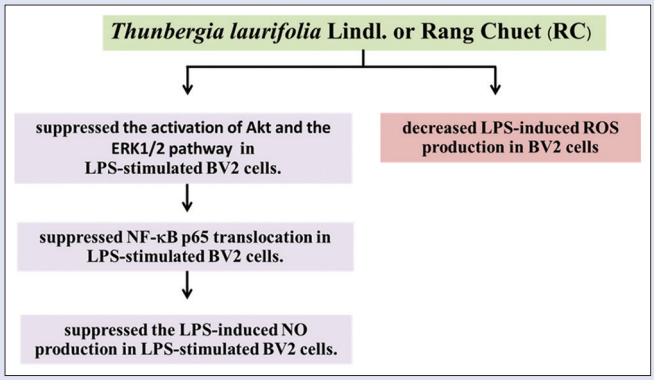
ABSTRACT

Background: Oxidative stress and neuroinflammation mediated by microglial activation play a significant role in the pathogenesis of neurodegenerative diseases. Therefore, negative regulators of microglial activation have been identified as potential therapeutic candidates for the treatment of such diseases. **Objectives:** The aim of this study was to investigate the effects of *Thunbergia laurifolia* Lindl. or Rang Chuet (RC) extracts on reactive oxygen species (ROS) and nitric oxide (NO) production in lipopolysaccharide (LPS)-activated BV2 microglia cells. **Materials and Methods:** BV2 cells were treated with LPS in the presence or absence of RC extract, and the levels of ROS and NO were measured using CM-H2DCFDA and Griess reagent assay, respectively. The nuclear levels of nuclear factor kappa B (NF-kB) p65 and the activation of Akt and the extracellular signal-regulated kinase 1/2 (ERK1/2) were detected using immunofluorescence and western blotting assay, respectively. **Results:** The treatment of BV2 cells with RC concentration-dependently suppressed the LPS-induced ROS production compared to the control group. RC treatment also significantly decreased LPS-induced NO production in a concentration-dependent manner. In addition, RC treatment also suppressed NF-kB p65 translocation and the activation of Akt and the ERK1/2 pathway in LPS-stimulated BV2 cells. **Conclusion:** Taken together, these results demonstrate that RC exerts anti-oxidative activity by suppressing ROS production and that it exerts antioxidative activity by suppressing NO in LPS-stimulated BV2 cells. The mechanisms by which RC suppresses NO and ROS production may occur through inhibition of Akt and the ERK1/2-mediated NF-κB pathway. The results suggest that RC may be useful in treating neurodegenerative diseases mediated by microglial cells.

Key words: Akt, BV2 microglia, extracellular signal-regulated kinase 1/2, nuclear factor kappa B, *Thunbergia laurifolia* Lindl

SUMMARY

- This study aimed to explore the anti-inflammation and antioxidative effects of *Thunbergia laurifolia* Lindl. or Rang Chuet (RC) extract in lipopolysaccharide (LPS)-stimulated BV2 microglial cells
- RC dose-dependently inhibited the production of nitric oxide and reactive oxygen species that were induced by LPS
- RC treatment exerted anti-inflammatory effects by suppressing nuclear level of nuclear factor kappa B p65 and the activation of Akt and extracellular signal-regulated kinase 1/2 pathway in LPS-stimulated BV2 cells.



Abbreviations used: AD: Alzheimer's disease; ALS: Amyotrophic lateral sclerosis; RC: Rang Chuet; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's modified Eagle's medium; ECL: Enhanced chemiluminescence; ERK1/2: Extracellular signal-regulated kinase 1/2; FBS: Fetal bovine serum; LPS: Lipopolysaccharide; MS: Multiple sclerosis; MSU: Mahasarakham University; NF-kB: Nuclear factor kappa B; NO: Nitric oxide; PD: Parkinson's disease; ROS: Reactive oxygen species; SD: Standard deviation.

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INTRODUCTION

It is well known that neuroinflammation and oxidative stress are associated with the pathogenesis of many diseases, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and Parkinson's disease (PD).[1-5] Numerous studies have shown that microglia, the resident immune cells of the central nervous system, play an important role in this process.[2-6] Chronic microglial activation producing reactive oxygen species (ROS), pro-inflammatory mediators such as nitric oxide (NO), proteases, and pro-inflammatory cytokines such as tumor necrosis factor alpha and interleukin 1 beta, all of which have been reported to endanger neuronal population.[7-9] Therefore, attenuation of microglial activation can be a therapeutic approach in the prevention and treatment of neuroinflammation as well as neurodegenerative diseases.

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In the last two decades, the use of natural products and functional foods as medicine has considerably increased due to it is well known that natural products have few side effects compared to synthetic drugs.[10] Therefore, investigation a natural product that can reduce neuroinflammation mediated by microglial cells is an important. *Thunbergia laurifolia* Lindl., commonly known as Rang Chuet (RC). It is classified as Acanthaceae family. RC is local Thai plant that found in the North. Major constituents of *T. laurifolia* include apigenin, caffeic acid, and other flavonoids and chlorophyll derivatives with antioxidant activity. In Thai traditional medicine, leaves of *T. laurifolia* (L.) are used as an antidote for poisons and drugs including the treatment of drug addiction.[11,12] The plant has also been reported to have anti-inflammatory, antidiabetic, and antipyretic properties.[13,14] Although many medical properties of *T. laurifolia* (L.) have been reported, to our knowledge, its molecular mechanisms on microglial activation have not yet been identified. The aim of this study was to examine the effects of RC on NO and ROS production as well as the transcription factor that regulates pro-inflammatory gene expression. The molecular mechanisms of RC on lipopolysaccharide (LPS)-induced microglial activation were also investigated.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, penicillin-streptomycin, fetal bovine serum, and all of the other cell culture reagents were from Hyclone (South Logan, UT). LPS, dimethyl sulfoxide and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT), were purchased from Sigma (St. Louis, MO). The Griess reagent kit for nitrite determination was from Molecular Probes (Eugene, OR) and the ROS detection kit was from Calbiochem (La Jolla, RC). Antibodies against total and phosphor (p)-Akt, total and *P* extracellular signal-regulated kinase 1/2 (ERK), and p-nuclear factor kappa B (NF-κB) p65 (Ser563) were from Santa Cruz Biotechnology (Santa Cruz, RC, USA). The BRC protein assay kit, chemiluminescence, and nitrocellulose membranes were obtained from Pierce (Rockford, IL, USA).

Plant material and extraction

Leaves of *T. laurifolia* (L.) were obtained from Udon Thani Province, Thailand, and identification was performed by Mahasarakham University, Faculty of Medicine, Division of Applied Thai Traditional Medicine. A herbarium specimen number. MSUT_7233 was deposited at the Faculty of Sciences. The extraction of *T. laurifolia* (L.) was described in a previous method.[15]

Cell culture and treatments

Cultured of BV2 microglial cell line was described in a previously method.[15] To begin the experiment, cells were plates overnight. To detect cell viability assay, after overnight, the medium was removed and replaced with new medium containing specified concentrations of RC ranging from 0 to 300 μg/ml in the presence or absence of LPS. To detect the NO and ROS production as well as nuclear levels of NF-kB p65 and the activation of Akt and ERK1/2, the medium was removed and replaced with fresh medium in the presence of LPS with or without the specified concentrations of RC.

Cell viability assay

After 24 h of treatment, the cytotoxicity of RC was evaluated by MTT assay as previously reported.[15]

Nitrite assay

After 24 h of treatment, nitrite in the culture supernatants was measured as an indicator of NO production using Griess reagent according to the method described previously.[15] Briefly, 50 μL of the medium was plated in a separate 96 well plates and 50 μL of Griess reagent was added and incubated for 15 min. The absorbance was measured at 570 nm. The amount of NO was calculated using a sodium nitrite standard curve.

Intracellular reactive oxygen species assay

After 24 h of treatment, the presence of intracellular ROS was determined using CM-H2DCFDA as previously reported.[15]

Protein extraction and western blot analysis

After 24 h of treatment, cells were collected, and western blot analysis was performed by immunofluorescence staining according to previously reported.[15]

Immunofluorescence staining

After 4 h of treatment, the NF-κB nuclear translocation was performed by immunofluorescence staining according to previously reported.[15]

Statistical analysis

One-way ANOVA assay followed by the Bonferroni *post hoc* test was compared between control and treatment groups. $P \leq 0.05$ was considered statistically significant.

RESULTS

The effects of Rang Chuet on viability of BV2 cells

To investigate the cytotoxic effects of RC extract on BV2 cells, cells were treated with RC in different concentration with or without LPS. After 24 h of treatment, cell viability was evaluated by MTT assay. As shown in Figure 1, treatment with RC alone or with LPS did not show any cytotoxicity in BV2 cells at lower concentrations except at 300 μg/mL of treatment compared with the untreated control. Therefore, non-cytotoxic concentrations of RC were used in this study.

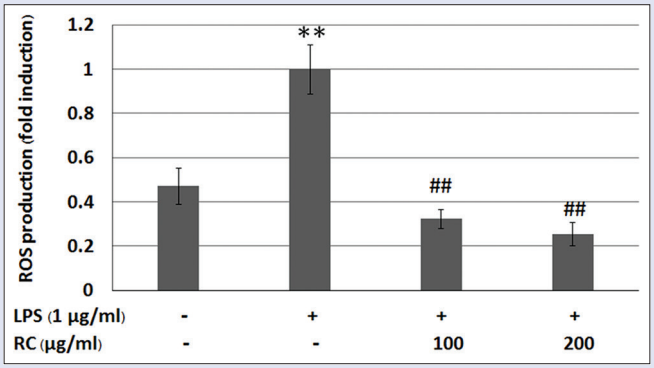

Figure 1: Effect of Rang Chuet on the viability of BV2 microglial cells. BV2 cells were incubated with Rang Chuet in the presence or absence of lipopolysaccharide. After 24 h of treatment, cell viability was measured by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Data represent mean ± standard error of mean ($n = 3$); *$P < 0.05$ when compared with control group; #$P < 0.05$ when compared with control lipopolysaccharide-treated group

Effect of Rang Chuet extracts on the production of nitric oxide in lipopolysaccharide-induced microglial activation

The Griess reagent assay was used to determine the effect of RC on NO production in LPS-induced microglial activation. As shown in Figure 2, in nonstimulated BV2 cells, a basal level of NO (4.35 ± 0.55 μM) was detected while LPS stimulation of the cells resulted in an increase in NO production and it is denoted as 46.1 ± 5.73 μM. Co-treatment with RC concentration dependently inhibited its induction.

Effect of Rang Chuet extracts on the production of reactive oxygen species in lipopolysaccharide-induced microglial activation

To investigate whether RC inhibits ROS production in LPS-induced microglial activation, the presence of intracellular ROS was determined using CM-H2DCFDA. The results show that stimulation of BV2 cells with LPS significantly increased in cellular ROS production compared to untreated control. RC treatment with LPS inhibited this induction significantly and concentration dependently, returning ROS production to levels seen in the control group [Figure 3].

Effect of Rang Chuet extracts on nuclear factor kappa B nuclear translocation in lipopolysaccharide-induced microglial activation

To investigated the inhibitory effect of RC in LPS-mediated activation of NF-κ B. In this study, the quantity of NF-κB p65 subunit in the nuclei of LPS-treated cultures of BV-2 microglia was determined by immunofluorescence staining assays. As shown in Figure 4, immunofluorescence microscopy revealed that NF-κB p65 was found mainly within the cytoplasm in the untreated control group. NF-κB p65 nuclear localization was induced when stimulating cells with LPS. The quantity of NF-κB p65 in the nuclei of LPS-treated cultures of BV-2 microglia was found to decrease following treatment with RC, indicating that RC treatment inhibits NF-κB activation in BV2 microglial cells.

Effect of Rang Chuet extracts on lipopolysaccharide-induced Akt and extracellular signal-regulated kinase 1/2 phosphorylation

Western blot analysis was used to determine the effects of RC on Akt and ERK1/2 phosphorylation in LPS-induced microglial activation. Akt phosphorylation level was significantly increased in LPS-treated cells. Treatment with 200 μg/ml of RC significantly inhibited LPS-induced Akt phosphorylation [Figure 5a]. As shown in Figure 5b, LPS stimulation also significantly induced ERK1/2 phosphorylation. RC treatment markedly reduced LPS-induced ERK1/2 phosphorylation.

DISCUSSION

Neuroinflammation and oxidative stress mediated by microglial activation have been associated with neurodegenerative diseases.[1,3] Therefore, inhibiting the activation of microglia is an important strategy for the prevention and treatment of these diseases. This study, LPS at 1 μg/mL was used to treated cells because this concentration could significantly induce microglial activation and did not affect cell viability.[15] We tested the effects of RC on pro-inflammatory mediator NO and oxidative stress in LPS-treated BV-2 cells and provide the first evidence that RC could significantly attenuate the release of pro-inflammatory mediators, NO as well as ROS production in LPS-stimulated microglial cells. We also demonstrated that RC treatment inhibited BV-2 microglial activation through NF-κB mediated PI3K/Akt and ERK signaling pathways.

NO is a pro-inflammatory mediator which has been reported to play an important role in the physiological process, such as vasodilation as well as host defense.[16] NO is generally considered to be undetectable in the healthy brain, but is strongly induction after brain injury or infection and neuroinflammation.[17] The neurotoxic effects of NO are due to a reaction with superoxide (O2•−), resulting in the formation of peroxynitrite (ONOO−), a potent oxidizing agent capable of mediating tissue and cellular injury. Excessive releasing of NO from activated microglial is associated with neuronal damage and correlates with the progression of neuroinflammation in AD, PD, MS, and ALS.[16,18] In the present study, we showed that LPS treatment resulted in significantly induced NO production and RC reversed this effect, demonstrating that RC could improve neuroinflammation caused by excessive NO.

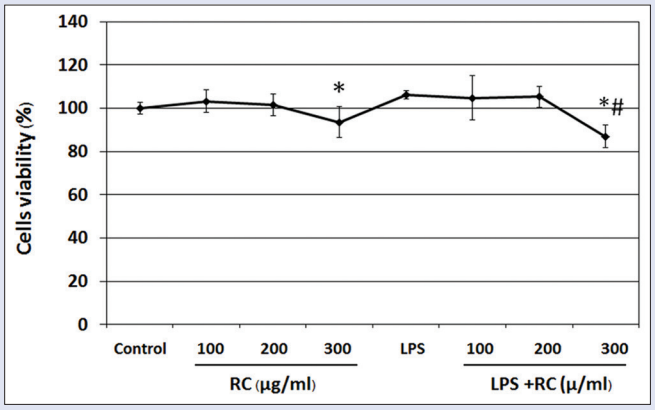

Figure 2: Effect of Rang Chuet on nitric oxide production induced by lipopolysaccharide in BV2 microglial cells. After 24 h of treatment, culture media were collected for the determination of nitric oxide production by the Griess reagent assay. Data represent mean ± standard error of mean (n = 3); $^{**}P < 0.01$ when compared with control group; $^{\#\#}P < 0.01$ when compared with lipopolysaccharide-treated group

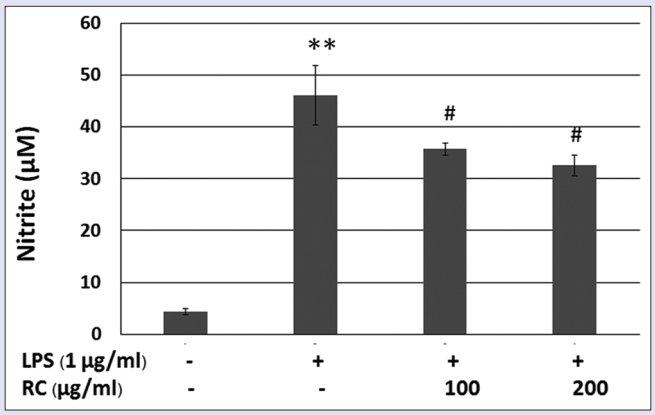

Figure 3: Effect of Rang Chuet on reactive oxygen species production induced by lipopolysaccharide in BV2 microglial cells. Data represent mean ± standard error of mean (n = 3); $^{**}P < 0.01$ when compared with control group; $^{\#}P < 0.05$ when compared with lipopolysaccharide-treated group

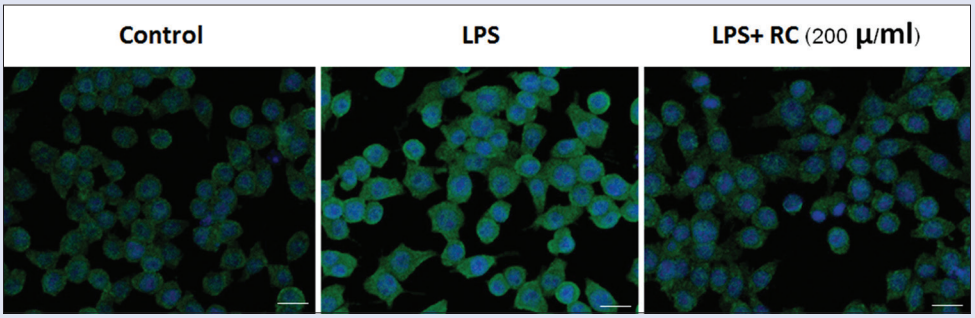

Figure 4: Effect of Rang Chuet on nuclear factor kappa B nuclear translocation in lipopolysaccharide-treated cultures of BV-2 microglia. BV-2 cells were treated with lipopolysaccharide in the presence or absence of Rang Chuet as indicated. After 4 h of treatment, cells were examined by immunofluorescence microscopy (magnification, 400; scale bars, 10 µm)

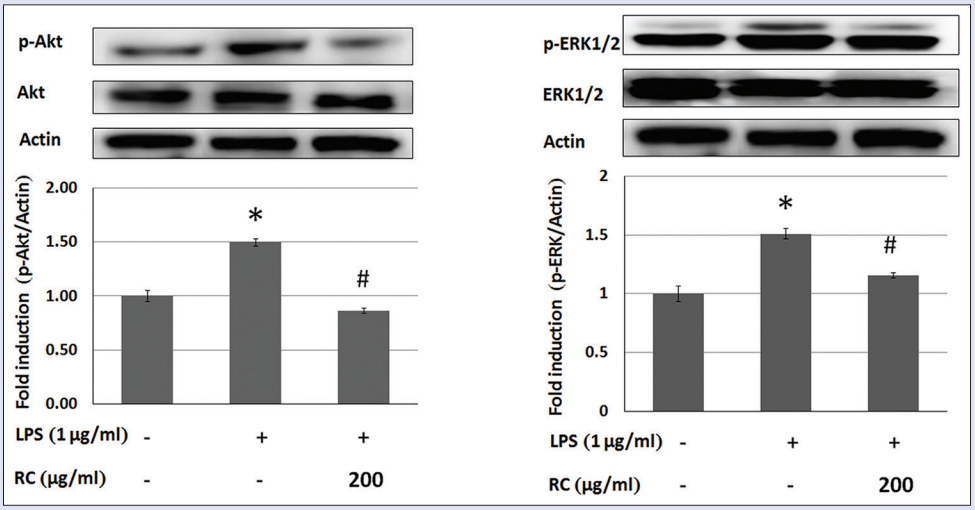

Figure 5: Effect of Rang Chuet on lipopolysaccharide-induced Akt (a) and extracellular signal-regulated kinase 1/2 (b) phosphorylation. After 24 h of treatment, total cell lysate was collected and levels of anti-Akt, p-Akt, anti-extracellular signal-regulated kinase 1/2 and p-extracellular signal-regulated kinase 1/2 were determined by western blot assay. Levels of p-Akt and p-extracellular signal-regulated kinase 1/2 were normalized to beta-actin. Histograms represent the fold increase of p-Akt and p-extracellular signal-regulated kinase 1/2 relative to that of the untreated control. These data represent mean ± standard error of mean ($n = 3$); $^{*}P < 0.05$ when compared with control group; $^{\#}P < 0.05$ when compared with lipopolysaccharide-treated group

ROS are highly reactive molecules. Although they have some essential roles in normal cell function, they are more associated with pathological effects. Increased ROS production causing protein, lipid, and DNA damage and it is considered to be an important mediator in the AD process.[19] ROS can also act as second messengers in intracellular signaling cascades that modulate inflammatory gene expression through activation of NF-κB, leading to enhanced neuroinflammation. In the present study, we observed that LPS treatment increased ROS levels, however RC treatment showed a significant decrease in ROS production compared to the LPS-treated group. This result suggests that RC may be an important role to inhibit the onset or the progression of some oxidative-related diseases. It has been reported that activating NF-κB, PI3K/Akt, and ERK signaling pathways induced by LPS stimulate inflammation.[20-25] Therefore, medicinal plants that inhibit the PI3K/Akt/NF-κB signaling pathways are strategy for the treatment of neuroinflammatory diseases. As a result, we found that RC decreased the nuclear level of NF-κB p65 in LPS-treated BV2 cells. This result suggests that RC may inhibit the production of NO and ROS by suppressing LPS-induced NF-κB activity. In addition, Akt phosphorylation in LPS-stimulated BV2 microglial cells was also markedly reduced by RC, suggesting that RC inhibits LPS-induced NF-κB activation through suppression of PI3K/Akt signaling pathway. Our results also demonstrated that LPS induced the phosphorylation of ERK1/2 and that RC markedly inhibited this effect. This result suggests that RC may also reduce the LPS-mediated pro-inflammatory response through the inhibition of ERK1/2 activation in BV2 microglial cells too.

CONCLUSION

Taken together, our results demonstrate that RC treatment significantly attenuates the release of pro-inflammatory mediators, NO as well as ROS in LPS-induced microglial activation. RC treatment also inhibits NF-κB activity through the prevention of NF-κB nuclear translocation. In addition, RC treatment also inactivates PI3K/AKT and ERK1/2 signaling pathway in LPS-induced microglial activation. These results indicate that RC, at non-toxic concentrations, exerts antioxidative and anti-inflammatory effects through AKT and ERK1/2 as well as inhibiting NF-κB signaling pathways. The data presented in this study suggest that RC could be a promising candidate for treating or preventing various neuroinflammatory diseases that are associated with microglial activation.

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Conflicts of interest

There are no conflicts of interest.

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