Anti-inflammatory property of *Parkia speciosa* empty pod extract in human umbilical vein endothelial cells

Nor-Hidayah Mustafa¹, Azizah Ugusman², Juriyati Jalil³, Yusof Kamisah¹*  
¹Department of Pharmacology, Faculty of Medicine, UKMMC, Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, 56000 Cheras, Kuala Lumpur, Malaysia.  
²Department of Physiology, Faculty of Medicine, UKMMC, Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, 56000 Cheras, Kuala Lumpur, Malaysia.  
³Drug and Herbal Research Centre, Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia.

**ABSTRACT**  
*Parkia speciosa* Hassk, locally known as *petai papan*, is a common medicinal plant found in Southeast Asia. Its empty pods were reported to contain high concentrations of polyphenols, particularly quercetin. This study aimed to evaluate the anti-inflammatory properties of *P. speciosa* empty pod extract in human umbilical vein endothelial cells (HUVECs). The empty pods were extracted by sequential fractionation with absolute ethanol and ethyl acetate. HUVECs were divided into four groups. HUVECs were exposed to tumor necrosis factor-α (TNF-α, 10 ng/mL) in the presence (25 µg/mL) or absence of *Parkia speciosa* extract. Quercetin (125 µM) served as the positive control, while another group that was not exposed to TNF-α acted as the negative control. The protein expressions of the inflammatory mediators, which were NF-κB, COX-2 and VCAM-1, were significantly decreased in the *P. speciosa* and quercetin groups exposed to TNF-α. *P. speciosa* extract and quercetin also significantly reduced intracellular reactive oxygen species and nitric oxide levels as well as inducible nitric oxide synthase (iNOS) activity caused by TNF-α in HUVECs. In conclusion, *P. speciosa* empty pod extract exhibited potential anti-inflammatory properties against TNF-α-induced inflammation, possibly by modulating the NF-κB pathway. The effects were comparable to that of quercetin.

**INTRODUCTION**  
The pathogenesis of many diseases, such as atherosclerosis, hypertension and diabetic vasculopathy, involves inflammation (He *et al*., 2015). Inflammation leads to cellular and tissue injuries. Nuclear factor κB (NF-κB) signaling is believed to be featured in the inflammatory cascade (Hsu *et al*., 2013), resulting in an increase in vascular cell adhesion molecule-1 (VCAM-1) (Conde *et al*., 2017), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression as well as proinflammatory cytokines, like tumor necrosis factor-α (TNF-α) (Hsu *et al*., 2013). Elevated expression of iNOS will increase nitric oxide (NO) and reactive oxygen species production (Hsu *et al*., 2013). TNF-α has been employed in many studies to evoke inflammatory reactions in cultured cells (Cao *et al*., 2009; Qi *et al*., 2010). Many studies have shown that plants with polyphenol compounds possess anti-inflammatory properties (Diaz-Rivas *et al*., 2015; Tuzcu *et al*., 2017). *Parkia speciosa* Hassk or stink bean, a plant indigenous to Southeast Asia and locally known as *petai papan* from the family Leguminosae, was reported to contain high levels of polyphenol compounds, particularly in its empty pods (Kamisah *et al*., 2013; Ko *et al*., 2014).

It has been utilized in folk medicine to control hypertension (Lim, 2012). Previously, it was shown that there is prevention of hypertension in rats administered with N(G)-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase (Kamisah *et al*., 2017). Based on this, the current study was conducted to investigate the effects of *P. speciosa* empty pods on TNF-α-induced inflammation in human umbilical vein endothelial cells (HUVECs).
MATERIALS AND METHODS

Plant material and extraction method

Fresh empty pods of *P. speciosa* were collected from Bidor (Perak, Malaysia) in December 2014. Its voucher specimen (UKMB 40239) was authenticated by a botanist and deposited at the Herbarium of Universiti Kebangsaan Malaysia Bangi, Selangor, Malaysia. De-seeded and dried empty pods were finely chopped and ground to fine powder (1.2 kg) with a blender and then macerated and extracted (every 24 h) in absolute ethanol (3 x 4 L). The filtered extract was evaporated under vacuum using a rotary evaporator. The remaining crude extract was then sequentially partitioned three times with hexane and ethyl acetate. The resultant ethyl acetate extract was utilized for further study based on our preliminary work that showed its highest viability in HUVECs (unpublished data). The yield was 11.261%.

Phytochemical analysis of *P. speciosa* empty pods

High-performance liquid chromatography (HPLC) analysis was conducted to identify the polyphenolic compounds present in the ethyl acetate fractions of *P. speciosa* empty pod ethanolic extract according to the method described by Komolafe *et al.* (2014) with certain modifications. Briefly, the modifications were the use of a chromatographic system (Waters 2535) equipped with a reversed-phase column C-18 (4.6 x 250 mm, 5μm; XBridge, Waters, Dublin, Ireland) and a photodiode array detector (Waters 2998) that was set at 254 and 365 nm. Samples were eluted with 1% aqueous acetic acid solution (A) and acetonitrile (B) at 0.5 mL/mL with the following gradient: 13% B from 0 to 10 min that was increased to 20%, 30%, 50%, 70% and back to 20% B at 20 min, 30 min, 40 min, 50 min, 60 min, and 80 min, respectively.

All chromatographic procedures were carried out at ambient temperature. The peaks obtained from the elution of the extract which was dissolved in HPLC grade methanol (10 mg/ml) were confirmed by comparing the retention time with those of reference standards (gallic acid, ellagic acid, catechin and quercetin).

Cell viability assay

HUVECs (Gibco, Grand Island, NY, USA) were grown in endothelial cell media and maintained in 1% endothelial cell growth supplement according to the manufacturer’s recommended protocol. Cells were passaged at confluence and only culture passages 3 to 6 were made use of in this study. 3-(4,5-Dimethylthiazol-2-yl)-5-[(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed with the CellTiter 96 Aqueous One Solution Proliferation Assay Kit (Promega, Madison, USA) to measure cell viability.

Cell culture treatment

HUVECs were pretreated with *P. speciosa* (25 μg/mL) extract or quercetin (125 μM) (Indra *et al.*, 2013) for 6 h before being exposed to TNF-α (10 ng/mL; R&D Systems, Abingdon, UK) and actinomycin D (1 μg/mL; Sigma-Aldrich, USA) (Zhou-Stache *et al.*, 2002) for another 16 h. Quercetin served as the positive control. Another group without any treatment served as the negative control group.

Western immunoblot analysis

The expression of targeted proteins, which were NF-κB p65 (Santa Cruz Biotech, USA), iNOS (Abcam, USA), COX-2 (Cell Signaling, USA), VCAM-1 (Santa Cruz Biotech, USA), and β-actin (Santa Cruz Biotech, USA) were determined by Western blot analysis with the respective primary antibodies (1:1000) and secondary antibodies (1:3000) (Xie *et al.*, 2000). Incubation with β-actin antibody was carried out as a comparative control.

Measurement of intracellular ROS and NO levels as well as iNOS activity

Intracellular ROS levels were measured via a fluorescent probe, 2′,7′ dichlorodihydrofluorescein (DCFH-DA; Sigma Co., USA) (Wen *et al.*, 2013). NO levels in the cell culture medium were measured with Griess reagent (Miranda *et al.*, 2001). Meanwhile, the activity of iNOS was determined according to the manufacturer’s assay kit protocol (Elabscience, China).

Statistical analysis

All experiments were carried out in triplicate. The data were expressed as mean ± standard error of mean (SEM). Normally distributed data were statistically analyzed through one way analysis of variance (ANOVA) followed by Tukey’s post-hoc test, while non-normally distributed data was evaluated by the Kruskal-Wallis test. *P* values < 0.05 were considered significant.

RESULTS

Phytochemical analysis

Only two standards (catechin and quercetin) appeared when eluted at 365 nm. There were six major peaks that appeared in the chromatogram of the extract. However, when compared with the reference standards, only quercetin was identified (Figure 1). All peaks of the reference standards (gallic acid, ellagic acid, catechin, and quercetin) appeared at 254 nm, but no peaks appeared in the sample (data not shown). The quercetin content in the extract was 5.84 mg per 100 g dry extract.

Viability percentage

TNF-α significantly reduced cell viability by approximately 50% when compared to the negative control (without TNF-α or extract). Preincubation with the extract at 12 μg/mL and larger concentrations significantly increased cell viability in HUVECs exposed to TNF-α. However, no difference was noted among the groups given the extract (Figure 2).
Fig. 1: Chromatogram of reference standards (A) (peak 1: catechin; peak 2: quercetin) and parkia speciosa extract (B) (peak 2: quercetin) at 365 nm.

Fig. 2: Effects of various doses parkia speciosa (PS) extracts (3-50 µg/mL) on cell viability using MTS assay after being exposed to TNF-α. Bars represent mean ± SEM (n=3), *P < 0.05.
Protein expressions of NF-κB p65, iNOS, COX-2, and VCAM-1

Stimulation with TNF-α elevated NF-κB p65 protein expression in HUVECs compared to the negative control. *P. speciosa* and quercetin treatments significantly reduced NF-κB p65 protein expression versus the TNF-α-induced group. The expression of NF-κB p65 in the *P. speciosa* group was significantly higher than the negative control and quercetin groups (Figure 3A). Further, TNF-α incubation rose iNOS protein expression in HUVECs. With this, both *P. speciosa* and quercetin groups had significantly lower iNOS protein expression in cells exposed to TNF-α than the TNF-α alone group (Figure 3B). The protein expression of COX-2 in HUVECs was significantly elevated in cells incubated with TNF-α compared to the negative control. Pretreatments of *P. speciosa* and quercetin significantly mitigated the effect of TNF-α on COX-2 protein expression. The *P. speciosa* group, but not the quercetin group, had significantly higher COX-2 protein expression than the negative control. The expression of the extract group was significantly greater than the quercetin group (Figure 4A).

VCAM-1 protein expression was significantly elevated in TNF-α-induced group compared to the negative control. The expression of VCAM-1 in both treated groups (extract and quercetin) was significantly reduced. Moreover, no difference was evident between the extract and quercetin groups (Figure 4B).

![Fig. 3](image-url)

**Fig. 3:** Effects of *parkia speciosa* extract (PS, 25 µg/mL) and quercetin (125 µM) on NF – Kb (A) and inducible nitric oxide synthase (Inos) (B) protein expression in TNF-α-activated (10 ng/ml) HUVECs. The data were expressed as means ± SEM (n=3), *P <0.05 vs control, #P <0.05 vs TNF-α.
Intracellular iNOS activity, NO and ROS levels

Exposure to TNF-α significantly elevated iNOS activity and NO levels (P< 0.05). Pretreatments of *P. speciosa* extract and quercetin similarly diminished the elevation of both parameters. HUVECs treated with TNF-α had significantly higher levels of intracellular ROS than the negative control. Pretreatment of the extract or quercetin significantly reversed the effect of TNF-α on intracellular ROS levels. No significant differences with regards to ROS levels were observed among the extract, quercetin, and negative control groups (Table 1).

**DISCUSSION**

Chromatographic analysis showed there to be the presence of quercetin in the extract. Ko et al. (2014) also reported the components of the phytochemical present in the *Parkia speciosa* empty pod extract. We attempted to analyze the phytochemical content in the extract via their method but failed.

They had described the presence of gallic acid, catechin, chlorogenic acid, quercetin, ellagic acid, kaempferol, vanillic acid, and epicatechin within their extract. Site and time of collection (season) could affect the composition of phytochemicals in plants. In particular, they analyzed ethanol extract without further fractionation through HPLC while in our study, we analyzed the ethyl acetate fraction of the ethanolic extract. This might explain the discrepancy in our findings. A recent study also demonstrated the presence of quercetin in *Parkia speciosa* empty pod methanol extract (Kamisah et al., 2017).

This study was a preliminary investigation with the objective of screening the anti-inflammatory of the *P. speciosa* empty pod extract in HUVECs. To the best of our knowledge, there is no single study that has reported the anti-inflammatory properties of this plant. The concentration of *P. speciosa* extract at 25 µg/ml was chosen based on its highest cell viability in HUVECs co-incubated with TNF-α and the use of quercetin as the positive control was because of its presence in the extract. TNF-α

**Table 1: Inflammatory parameters measured in HUVECs pretreated with *P. speciosa* (PS, 25 µg/ml) or quercetin (125 µM) and exposed to TNF-α (10 ng/ml).**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>TNF-α</th>
<th>TNF-α + PS</th>
<th>TNF-α + Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS activity (ng/ml)</td>
<td>0.23 ± 0.03</td>
<td>3.40 ± 0.10*</td>
<td>2.68 ± 0.02#</td>
<td>2.24 ± 0.30#</td>
</tr>
<tr>
<td>NO level (µM)</td>
<td>3.16 ± 0.17</td>
<td>17.53 ± 0.16*</td>
<td>10.24 ± 0.25#</td>
<td>6.83 ± 0.20#</td>
</tr>
<tr>
<td>ROS level (% of control)</td>
<td>100.0 ± 0.0</td>
<td>182.2 ± 1.7*</td>
<td>120.0 ± 5.4#</td>
<td>97.7 ± 6.9#</td>
</tr>
</tbody>
</table>

*Different from control (P< 0.05); #different from TNF-α group (P< 0.05).
increased NF-κB p65 protein expression in HUVECs as similarly reported in other studies (Cao et al., 2009; Profumo et al., 2016). NF-κB protein complex is involved in several cellular responses to stimuli, such as free radicals, stress, and cytokines (Donato et al., 2015), associated with activation of adhesion molecules, chemokines, and cytokines (Valen et al., 2001). Exposure of cells to TNF-α causes phosphorylation of the NF-κB inhibitory protein, IκB, which mediates its translocation to the nucleus and regulates the expression of numerous genes and proteins involved in inflammation (Lawrence, 2009). Activation of the NF-κB pathway activates the gene that encodes iNOS protein (Aktan, 2004), which is observed as enhanced iNOS protein expression and activity leading to increased NO production as per our study. P. speciosa and quercetin pretreatments had significantly decreased these parameters. The protective effects of the extract were most likely attributable to its polyphenolic content, especially that of quercetin. The effects of quercetin seemed to be more prominent earlier in the pathway (NF-κB) than at the later stages (iNOS). Quercetin has been described to inhibit NF-κB (Indra et al., 2013) and iNOS expression (Garcia-Mediavilla et al., 2007).

Activation of iNOS would further elevate NO (Sarah et al., 2014) and ROS production (Cook, 2006) in TNF-α-induced cells as similarly observed during the state of stress (Kamisah et al., 2016). Increased production of NO may couple with ROS in the cells to form peroxynitrite radicals (Cook, 2006), which promote further damage to these cells. Both P. speciosa and quercetin reduced TNF-α-induced NO levels but inhibited ROS production. The greater impact of both pretreatments on ROS could be also attributed to their potent antioxidant properties (Kamisah et al., 2013; Zhu et al., 2017) in addition to their anti-inflammatory properties. Our recent study indicated that in vivo supplementation of P. speciosa reduced NADPH oxidase enzyme in hypertensive rats (Kamisah et al., 2017). This enzyme is a source of free radicals, especially superoxide anion (Klima et al., 2013). Quercetin was also reported to attenuate NADPH oxidase expression (Xiao et al., 2017).

COX-2 protein expression is upregulated in TNF-α-induced cells. COX-2 is an enzyme for prostaglandin synthesis that is responsible in inflammation (Li et al., 2014). Upregulation of the enzyme by NF-κB is partially responsible for inflammatory responses (Hsu et al., 2013). The extract and quercetin in our study exhibited decreasing the expression of NF-κB-associated downstream inducible enzymes, like iNOS and COX-2, as well as via upstream mechanisms of NF-κB by diminishing ROS and NO production (Hsu et al., 2013). Quercetin was demonstrated to inhibit COX-2 expression (Garcia-Mediavilla et al., 2007).

NO may also modulate endothelial cell adhesion molecule expression in cultured endothelial cells in vitro by various mechanisms. The upregulated expression of cell adhesion molecules on endothelial cells will alter the adhesive properties of vasculature, which implies a signal for HUVECs activation (Kriegstein and Granger, 2001). VCAM-1 is constitutively expressed in the vascular endothelium when induced by proinflammatory cytokines (Qi et al., 2010). In the current study, VCAM-1 expression was downregulated by P. speciosa and quercetin in TNF-α-induced HUVECs, which was consistent with previous work (Bhaskar et al., 2016). VCAM-1 plays an important role in the early development of atherosclerosis (Bhaskar et al., 2016). Therefore, our findings suggest that P. speciosa extract may be beneficial for attenuating early progression of atherosclerosis. Anti-inflammatory properties of P. speciosa extract in the work presented here could be further explored in future studies in diseased states.

**CONCLUSION**

*Parkia speciosa* empty pod extract treatments attenuate TNF-α-induced inflammatory responses in HUVECs by blocking the activation of NF-κB p65, which leads to a reduction in iNOS, COX-2, and VCAM-1 expression as well as ROS and NO production. The effects were comparable to that of quercetin.

**ACKNOWLEDGMENTS**

The authors would like to acknowledge the financial support from the Universiti Kebangsaan Malaysia (UKM) grant (AP-2014-013) and technical help from Ms. Manali Haniti Mohd Zahid, Puan Nurul Hafizah Abas, En Fadhulliah Zuhair Japar Sidik, Puan Juliana Abdul Hamid, and Ms. Nurul Akmal Muhammad.

**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

**REFERENCES**


How to cite this article: