

Modulation of NOX4 and MAPK Signalling Pathways by *Parkia speciosa* Empty Pods in H9c2 Cardiomyocytes Exposed to H₂O₂

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Gui *et al.*: Oxidative Stress Modulation by *Parkia speciosa*

Parkia speciosa, a plant that grows abundantly in Southeast Asia region, has been reported to exhibit antioxidant property. This study aimed to investigate the mechanism of antioxidant effects of empty pod extract of *Parkia speciosa* on hydrogen peroxide-induced oxidative stress in H9c2 cardiomyocytes. There were 4 groups of cardiomyocytes, the negative control, hydrogen peroxide, extract of *Parkia speciosa*+hydrogen peroxide and quercetin+hydrogen peroxide. Cardiomyocytes of groups 3 and 4 were preincubated for 1 h with 500 µg/ml of the ethyl acetate fraction of the *Parkia speciosa* extract or 1000 µM quercetin prior to exposure to 500 µM hydrogen peroxide for 1 h. Hydrogen peroxide significantly increased the protein expression of NADPH oxidase 4, superoxide dismutase 1 and p38 mitogen-activated protein kinase, in comparison to the negative control. NADPH oxidase 4 and superoxide dismutase 1 activities as well as reactive oxygen species level were also elevated. These elevations were significantly decreased in the pretreated groups. The *Parkia speciosa* extract group had comparable effects to that of quercetin. *Parkia speciosa* extract showed antioxidant property against hydrogen peroxide-induced oxidative stress in H9c2 cardiomyocytes by regulating mitogen-activated protein kinase and NADPH oxidase 4 signalling pathways.

Key words: *Petai*, NADPH oxidase, MAPK, polyphenol, superoxide dismutase

Oxidative stress is involved in the development of diseases, which include cardiovascular diseases^[1]. Increased oxidative stress in the cardiovascular system causes biochemical modification and oxidative injuries in the cells. There are many pathways involved in oxidative stress such as NADPH oxidase (NOX) pathways^[2] and phospho-p38 mitogen-activated protein kinase (MAPK)^[3]. Activation of NOX4 would increase reactive oxygen species (ROS) generation, in particular superoxide anion (O₂⁻), which then stimulates superoxide dismutase 1 (SOD1) to dismutase the anion^[4,5]. ROS acts as a second messenger that stimulates MAPK activity^[6]. Hydrogen peroxide (H₂O₂) is one of the signaling molecules that can permeate the cell membrane and activate NOX. NOX4, but not NOX1 and NOX2 is upregulated in cardiac cells with increased oxidative stress^[7]. Clinically, an increase in oxidative stress was reported in patients with ST-elevation myocardial infarction^[8].

Parkia speciosa Hassk, called as *petai papan* by the locals, of Leguminosae family, grows abundantly in

Southeast Asia region including Indonesia, Thailand, Philippines and Malaysia^[9]. Its empty pods with or without seeds are used in folk medicine to help control heart problems^[10]. The empty pods contain higher amounts of antioxidants than the seeds^[11], believed to be attributable to presence of quercetin, ellagic acid, gallic acid and catechin^[12,13]. The pods were also reported to possess antiinflammatory property^[13,14].

The antioxidant property of *P. speciosa* extract (PSE) has been reported in many studies owing to the presence of polyphenols^[15-17]. However, information on its antioxidant mechanism is still lacking. It might have a beneficial role in alleviating cardiovascular disease, thus, this study was conducted to further understand

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Accepted 23 September 2019

Revised 21 June 2019

Received 02 March 2019

Indian J Pharm Sci 2019;81(6):1029-1035

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its antioxidant mechanism based on MAPK and NOX signaling pathways in H9c2 cardiomyocytes that were induced by H₂O₂ as a model to enhance oxidative stress.

MATERIALS AND METHODS

Preparation of PSE:

Pods of *P. speciosa* plant were bought from a plantation in Bidor, Perak, Malaysia (Dec 2014). A voucher specimen (UKMB 40239) of the plant was located in the Universiti Kebangsaan Malaysia Herbarium (Bangi, Selangor, Malaysia) after its identification by a botanist. Preparation of ethyl acetate fraction from ethanol PSE was done according to a previously described method^[13]. The fraction was then kept at 4°. The yield based on dry weight of the pods was 0.938 %.

Phytochemical analysis of PSE:

Phytochemical analysis of the PSE was conducted by the means of high-performance liquid chromatography (HPLC) according to a technique previously reported^[18] with some modifications^[13]. The chromatographic system used was Waters 2535 with a photodiode array detector (Waters 2998, Waters Corp., Milford, MA, USA). A C-18 reversed-phased column was used (5 µm, 4.6×250 mm; XBridge, Waters, Dublin, Ireland). The system was set at 300 nm. The mobile phases were 1 % aqueous acetic acid solution (A) and acetonitrile (B), with the following gradient at ambient temperature, 13 % B for the first 10 min, then changed to 20 % at 20 min, 30 % at 30 min, 50 % at 40 min and to 70 % at 60 min. At 80 min, the gradient was reduced to 20 % B. The flow rate was 0.5 ml/min. The obtained peaks were then compared against the retention time of catechin (30.8 min) and quercetin (47.1 min), the reference standards.

Cell culture and treatment:

H9c2 cardiomyocytes (ATCC, Manassas, Virginia, USA) were grown in Dulbecco's modified Eagle medium (DMEM) containing 1 % penicillin/streptomycin/amphotericin B solution (Gibco, Grand Island, NY, USA) and 10 % fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria) in a humidified incubator (5 % CO₂ and 95 % air) at 37°. Cells at passages 4-7 were used in the study. There were 4 groups of cardiomyocytes, which were H₂O₂ (500 µM), PSE (500 µg/ml)+H₂O₂ (500 µM), quercetin (1000 µM)+H₂O₂ (500 µM) serving as positive control^[19] and a negative control (0.1 % dimethyl

sulfoxide, DMSO in DMEM). The cells were pretreated for 1 h and exposure to H₂O₂ was for another 1 h. The extract was dissolved in 0.1 % DMSO which gave final concentration of 0.01 % DMSO in the culture.

Cell viability assay:

Seeded cells (3×10⁴ cells/well) were used to measure the effects of various concentrations of H₂O₂ (50-550 µM) on cell viability after 1 h using CellTiter 96 aqueous One Solution Proliferation Assay kit (Promega, Madison, Wisconsin, USA). The viability of the cells pretreated with various concentrations of the extract fraction (31.25-1000 µg/ml) for 1 h, and then treated with median inhibitory concentration (IC₅₀) of H₂O₂ for another 1 h, was also measured.

Western blot analysis:

The cells (1.2×10⁵ cell/ml) were seeded in T-75 flask. Extraction of protein samples from cultured cells and western blotting methods were according to previously described protocols^[20,21]. The primary antibodies used were NOX4, β-actin (Santa Cruz Biotech, Dallas, Texas, USA), SOD1 and p38 MAPK with 1:100, 1:1000, 1:1000 and 1:500 dilutions, respectively. The secondary antibodies were goat antirabbit IgG-HRP (Cell Signaling, Danvers, Massachusetts, USA) or goat antimouse IgG-HRP (Santa Cruz Biotech, Dallas, Texas, USA) at 1:3000 dilution. The intensity of the oxidative stress related proteins were determined relatively to the intensity of the β-actin and all results were analysed using Image-J software.

Preparation of cell lysate:

The cells (1.2×10⁵ cell/ml) were seeded in T-75 flask to obtain cell lysate. After being treated according to the groups, media in the culture flask were removed and the flask was rinsed with phosphate-buffered saline (PBS) solution twice. Then, the cells that attached to the surface of the flask were removed by using cell scraper. PBS solution containing the cells was centrifuged at 2500 rpm for 5 min. The cell pellet was resuspended in 1 ml of PBS solution, before being centrifuged again at 2500 rpm for 5 min at 4°. The resulting pellet was resuspended in 100 µl of lysis buffer containing protease inhibitor (Complete Mini EDTA-free; Roche, Mannheim, Germany) and dithiothreitol (0.154 mg/ml) in radioimmunoprecipitation assay buffer (RIPA buffer 1x; Sigma-Aldrich, St. Louis, Missouri, USA). The tube was vortexed every 5 min thrice. Then, the tube was centrifuged at 12 000 rpm for 15 min at 4°. The resulting supernatant was the cell lysate.

Determination of NOX activity:

NOX activity assay was carried out using cell lysate following an established method^[22]. Briefly, 50 µg cell lysate was added into a reaction mixture (final volume of 200 µl) that consisted of 250 µg/l cytochrome C (Sigma-Aldrich, St Louis, Minnesota, USA), 100 µM NADPH (Sigma-Aldrich, St Louis, Minnesota USA), with or without 100 µM diphenyleneiodonium chloride (Sigma-Aldrich, St Louis, Minnesota USA). The mixture was incubated for 120 min at 37°. Then, the absorbance was measured at 550 nm in ELISA microtiter plate reader. The difference between the sample absorbance values at 0 and 120 min, was taken to calculate the NOX activity with the extinction coefficient 21 mmol/l/cm. It was expressed as percentage of control.

Determination of intracellular ROS level:

Cell-permeant fluorescent probe, 2',7'-dichloro dihydrofluorescein (DCFH-DA, Sigma-Aldrich, St Louis, Minnesota, USA)^[23] was used to determine ROS level in the cells (5×10^4 cell/ml). The absorbance was read at 485 nm excitation and 535 nm emission by utilizing a fluorescence plate reader (EnSpire Multimode Plate Reader, PerkinElmer, USA). ROS level was expressed as percentage of control.

Determination of SOD activity:

SOD activity was determined^[24-26] in the cell lysate. Lysate mixed with 50 mM PBS (pH 7.8), 201.06 mM L-methionine (Sigma-Aldrich, St Louis, Minnesota, USA), 1.71 mM nitro blue tetrazolium (Sigma-Aldrich, St Louis, Minnesota, USA), 1 % Triton X-100 (Sigma-Aldrich, St Louis, Minnesota, USA) and 106.28 µM riboflavin (Sigma-Aldrich, St Louis, Minnesota, USA) was placed in an illuminated aluminium box with two 20-watt Sylvania Arolux fluorescent lamps for 7 min. The absorbance was read at 560 nm by a spectrophotometer (Shimadzu, Kyoto, Japan). SOD activity was calculated as % inhibition of blue formazan formed from the reaction of nitro blue tetrazolium and superoxide produced. The enzyme activity was depicted as U/mg protein. The content of the protein was evaluated using Bradford reagent (Sigma-Aldrich, St Louis, Minnesota, USA).

Statistical analysis:

The experiments were conducted in triplicate. The data were expressed as mean±standard error of mean

(SEM). Shapiro-Wilk test was used to evaluate the normality of the data. For normally distributed data, one way analysis of variance (ANOVA) and Tukey's post-hoc test were applied, while for non-normally distributed data, Kruskal-Wallis test was conducted. P values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

P. speciosa empty pods has been reported to contain polyphenols^[12,13]. In the present study, the PSE showed 5 main peaks in the chromatogram (fig. 1). However, only quercetin was detected in the extract. The quercetin content in the extract was about 5.74 mg quercetin per 100 g dried extract. In our previous study, PSE ethyl acetate fraction contained the largest amount of quercetin compared to other fractions (ethanol and hexane)^[13]. Based on this, the ethyl acetate fraction was selected for further study and quercetin was chosen as the positive control.

H9c2 cardiomyocytes were cultured with increasing concentrations of H₂O₂ (50-550 µM) for 1 h. The exposure to H₂O₂ had decreased H9c2 cell viability concentration-dependently. The median inhibitory concentration (IC₅₀) value of H₂O₂ obtained was 500 µM (fig. 2A). Pretreatment with PSE (31.25 µg/ml to 1000 µg/ml) prior to exposure to 500 µM H₂O₂ for 1 h had increased cell viability concentration-dependently compared to non-pretreated group (0 µg/ml) that was exposed to H₂O₂ (p<0.05, fig. 2B). The cell viabilities in groups pretreated with 31.25, 62.5 and 250 µg/ml PSE were not different. The group pretreated with 500 µg/ml PSE had the highest cell viability, which was 80.08±0.41 %, while in the group pretreated with 1000 µg/ml PSE was 72.14±0.64 %. The concentration of 500 µg/ml of PSE was selected to be used in subsequent experiments. The significant reduction in cell viability after 1 h exposure of H₂O₂ showed cytotoxic effect of H₂O₂. The IC₅₀ of H₂O₂ obtained in the present study was similarly reported^[27]. The peroxide produces hydroxyl radicals in the presence on ferric ion, and superoxide anion which are toxic to cells and increases oxidative stress^[28], leading to cell death. The protective effects of PSE against cell death induced by H₂O₂ most probably owing to high content of flavonoid and phenolic compounds present in the empty pods^[15].

H9c2 cells exposed to H₂O₂ have been used as a model for evaluating the effects of natural compounds on cardiovascular diseases that are caused by oxidative

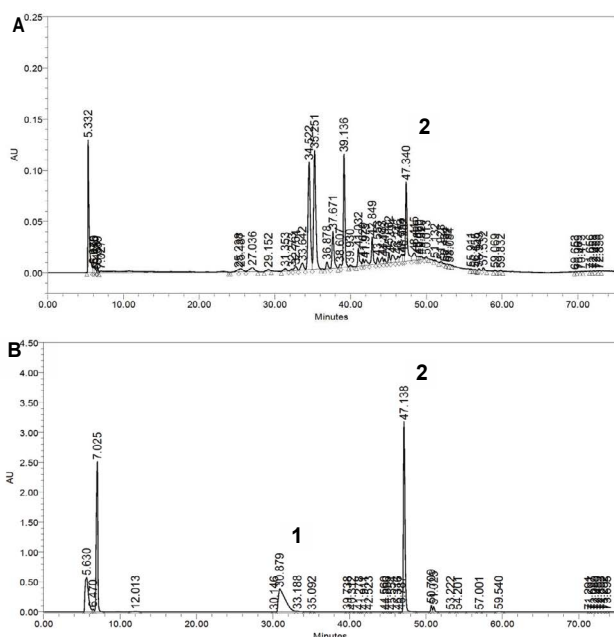


Fig. 1: Chromatography analysis
(A) *Parkia speciosa* extract (peak 2- quercetin based on (B) reference standards (peak 1- catechin at 30.8 min and peak 2- quercetin at 47.1 min) detected at 300 nm

stress^[5,25]. Oxidative stress enhances ROS production and increases the risk for cardiovascular disease^[26]. Overproduction of ROS is believed to contribute to the declining cardiac function in patients with cardiac disease^[8]. It is known that NOX is involved in one of the pathways in oxidative stress. Induction with H₂O₂ elevated the NOX4 protein expression significantly (1.17±0.02, p<0.05) compared to the negative control. PSE and quercetin pretreatments significantly blocked the elevation of the NOX4 induced by H₂O₂. In the negative control, PSE+H₂O₂ and quercetin+H₂O₂ groups, there was no detectable NOX4 expression was observed (fig. 3A). NOX activity was significantly more than two-fold higher (383.33±16.67 %, p<0.05) in H9c2 cells that were exposed to H₂O₂ than the negative control. PSE (183.33±16.67 %) and quercetin (150.00±28.87 %) pretreatments had reduced NOX activity significantly compared to H₂O₂ group (p<0.05). NOX activity was similar in both pretreated groups, which was also comparable to the negative control (fig. 3B). The ROS level was measured to confirm the presence of oxidative stress. Exposure to H₂O₂ significantly augmented intracellular ROS level (163.76±4.23 %) in H9c2 cells. Both PSE (92.85±0.98 %) and quercetin (94.34±2.87 %) groups that were treated with H₂O₂ had significantly lower intracellular ROS level than the negative control and H₂O₂ groups. In the pretreated groups, the ROS levels were similar (fig. 4).

Elevation of oxidative stress induced by H₂O₂ promotes NOX4 expression, and then its activity as seen in this study. NOX4 is the main isoform of NOX expressed in the cardiomyocytes and the major source of O₂^{•-} in the heart^[29,30]. Its activation involves p22^{phox} and polymerase delta interacting protein 2 (Poldip2)^[31]. When induced with H₂O₂, p22^{phox} forms a stabilizing complex with NOX4, followed by the translocation of Poldip2 to form an active complex^[31,32]. Then, NOX4 produces O₂^{•-} by transferring electrons from NADPH to

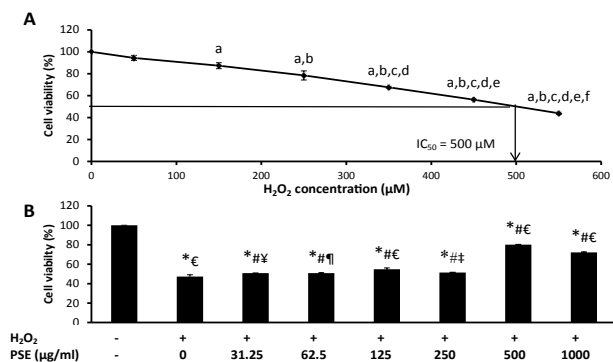


Fig. 2: Cell viabilities in H9c2 cardiomyocytes
(A) treated with various concentrations of H₂O₂ for 1 h and (B) pretreated with various concentrations of *Parkia speciosa* extract (PSE) for 1 h, followed by 1 h incubation with H₂O₂ (500 µM). Data are shown as mean±SEM (n=3). ^ap<0.05 vs. 0 µM, ^bp<0.05 vs. 50 µM, ^cp<0.05 vs. 150 µM, ^dp<0.05 vs. 250 µM, ^ep<0.05 vs. 350 µM, ^fp<0.05 vs. 450 µM. *p<0.05 vs. negative control, #p<0.05 vs. H₂O₂, [€]p<0.05 vs. other PSE concentrations, [¥]p<0.05 vs. other PSE concentrations except 62.5 µg/ml and 250 µg/ml, [¶]p<0.05 vs. other PSE concentrations except 31.25 µg/ml and 250 µg/ml, [‡]p<0.05 vs. other PSE concentrations except 31.25 µg/ml and 62.5 µg/ml

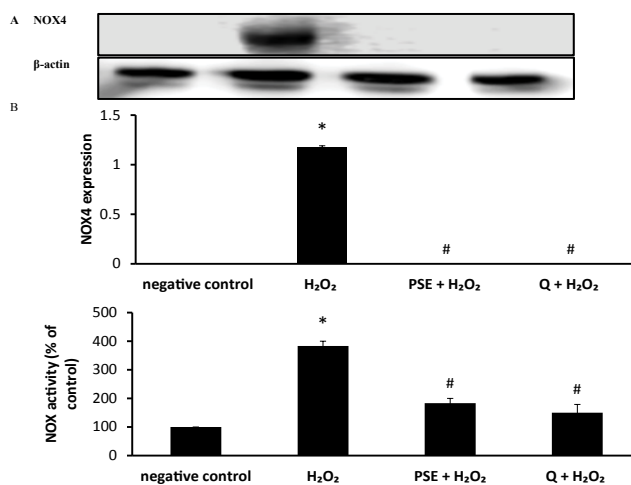


Fig. 3: Pretreatment with PSE and quercetin on NADPH oxidase in H9c2 cardiomyocytes exposed to H₂O₂
Effect of pretreatment with 500 µg/ml *Parkia speciosa* extract (PSE) and 1000 µM quercetin (Q) on (A) NADPH oxidase (NOX) expression and (B) NOX activity in H9c2 cardiomyocytes exposed to 500 µM of H₂O₂. Data shown as mean±SEM (n=3). *p<0.05 vs. negative control, #p<0.05 vs. H₂O₂

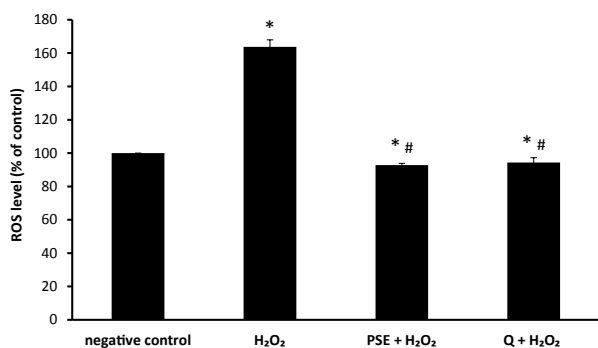


Fig. 4: ROS level in H9c2 cardiomyocytes pretreated with 500 µg/ml PSE and Q prior to exposure to H₂O₂

Reactive oxygen species (ROS) level in H9c2 cardiomyocytes pretreated with 500 µg/ml *Parkia speciosa* extract (PSE) and 1000 µM quercetin (Q) prior to exposure to 500 µM of H₂O₂. Data shown as mean±SEM (n=3). *p<0.05 vs. negative control, #p<0.05 vs. H₂O₂

O₂ and O₂⁻[33]. The effect of H₂O₂ on NOX4 expression was totally blocked by the PSE and quercetin. It was similarly demonstrated[34] that showed reduction of NOX4 expression by ZYZ-772, a quercetin metabolite from *Zanthoxylum bungeanum* in H9c2 cells. On the other hand, the NOX activity was not similarly blocked in both groups (PSE and quercetin) because the activity measured was not NOX4-specific, but measured total activity of other NOXs as well (NOX1, NOX2, NOX3, NOX5, DUOX1 and DUOX2). The protective effect of PSE was most likely afforded by its quercetin content. It is postulated that PSE and quercetin inhibited Poldip2 translocation to p22^{phox} in the membrane, as well as inhibited translocation of cytosolic subunit (p40^{phox}, p47^{phox} and p67^{phox}) to the membrane and hence, the formation of active oxidase to form O₂⁻[35].

In H₂O₂-treated H9c2 cells, SOD1 protein expression was significantly increased (1.34±0.08, p<0.05). The increment was significantly attenuated in the groups pretreated with PSE (0.82±0.07) and quercetin (0.20±0.08, p<0.05). Quercetin pretreatment had significantly diminished SOD1 protein expression, which expression was significantly smaller than the negative control group and PSE-pretreated group (p<0.05). The protein expression observed between the negative control group and PSE-pretreated group was not different (fig. 5A). Incubation with H₂O₂ significantly elevated SOD activity (127.01±10.73 U/mg protein) in the cells compared to the negative control (81.97±5.60 U/mg protein). The SOD activity was decreased to 69.86±6.87 U/mg protein in the PSE-pretreated and 49.46±5.78 U/mg protein in the quercetin-pretreated groups, compared to the H₂O₂ group (p<0.05). The activity of the enzyme was not

different in both pretreated groups, and comparable to the activity of the negative control group (fig. 5B).

Upregulation of NOX4 and elevated NOX activity in the present study increased production of ROS in the cells, which was mitigated by PSE and quercetin. O₂⁻ is importantly involved in the pathogenesis of many diseases, like hypertension and atherosclerosis[36]. Quercetin in the PSE might be neutralizing ROS by donating electrons or hydrogen atoms. Produced O₂⁻ is subsequently converted to H₂O₂ by SOD[33]. Therefore, increased production of the anion following exposure to H₂O₂ had increased the expression and activity of the SOD1, as observed in the present study, in turn to increase the synthesis of functional SOD enzyme to convert O₂⁻ anion to less reactive H₂O₂[4], which is then converted into water and oxygen by catalase and glutathione peroxidase[37]. SOD is the primary defense mechanism and plays more important role in detoxifying ROS than catalase and glutathione peroxidase[4].

Reduced formation of ROS by both PSE and quercetin had reduced the demand to increase SOD1 synthesis in the cells, as proven by decreased expression and activity of the antioxidant enzyme. Quercetin had stronger effect on SOD1 downregulation than PSE, possibly attributable to its higher concentration (1000 µM) than in the latter (2.12 µM). However, its effect on the SOD activity was comparable with PSE, which might be due to the post-translational modification that leads to decreased SOD enzyme synthesis by the quercetin.

p38 MAPK is one of the pathways known to be involved in oxidative stress. Therefore, the protein expression of MAPK was determined to confirm the involvement of the kinase in the protective effect of PSE. H₂O₂ induction in H9c2 cells significantly (p<0.05) increased p38 MAPK protein expression in comparison to the negative control group (fig. 6). p38 MAPK protein expression was significantly reduced in with PSE- (0.07±0.02) and quercetin-pretreated groups compared to the H₂O₂ group (p<0.05). PSE-pretreated group had significantly greater p38 MAPK protein expression than the negative control. Quercetin-pretreated group had comparable protein expression with the negative control group and significantly lower protein expression than PSE-pretreated group. No p38 MAPK expression was detected in the quercetin-pretreated and negative control groups.

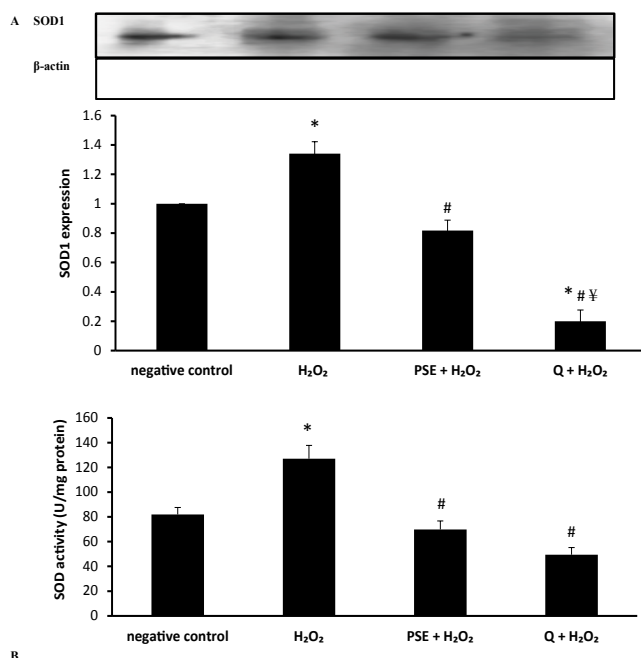


Fig. 5: Expression of SOD1 in H9c2 cardiomyocytes pretreated with PSE and quercetin prior to exposure to H₂O₂
The relative expression level of SOD1 (A) and activity (B) in H9c2 cardiomyocytes pretreated with 500 µg/ml *Parkia speciosa* extract (PSE) and 1000 µM quercetin (Q) followed by H₂O₂ exposure. Data shown as mean±SEM (n=3). *p<0.05 vs. negative control, #p<0.05 vs. H₂O₂, ¥p<0.05 vs. PSE+H₂O₂

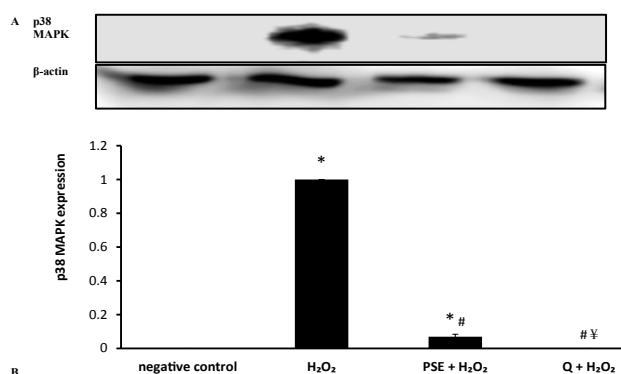


Fig. 6: Expression of p38 MAPK in H9c2 cells treated with PSE quercetin before exposure to H₂O₂
The relative expression level of p38 MAPK in H9c2 cells treated with 500 µg/ml *Parkia speciosa* extract (PSE) and 1000 µM quercetin (Q) for 1 h before exposure to 500 µM of H₂O₂ for 1 h. Data shown as mean±SEM (n=3). *p<0.05 vs. the negative control, #p<0.05 vs. H₂O₂, ¥p<0.05 vs. PSE+H₂O₂

ROS triggers phosphorylation and activation of p38 MAPK, evidenced by augmented expression of the kinase and such effect was attenuated by PSE and quercetin. H₂O₂ might cause oxidative modification of the intracellular kinase (MAP kinase kinase, MAP3Ks), which is involved in the first step for p38 MAPK activation^[38], or deactivation of MAPK phosphatases (MKPs)^[19]. Few other studies had previously shown that elevated ROS produced by

H₂O₂ activated p38 MAPK phosphorylation^[6,34]. PSE and quercetin could be triggering MKPs deactivation of MAPK by dephosphorylating residues of tyrosine and threonine at the MAPK activation site^[39]. More prominent inhibitory effect of quercetin on p38 MAPK expression could be due to its higher concentration.

The current *in vitro* study indicated the protective effects of PSE in impeding oxidative stress-associated cardiovascular diseases. It's *in vitro* beneficial effects needs to be further confirmed by *in vivo* studies, before its use as a supplement to reduce the risk of cardiovascular diseases. In conclusion, the current findings suggested that PSE acted as an antioxidant and protected H9c2 cardiomyocytes against H₂O₂-induced oxidative stress by modifying NOX4 and MAPK signalling pathways. Its effects were comparable to that of quercetin, but less effective in downregulating SOD1 and p38 MAPK. The beneficial effects of this plant could be most probably owing to the polyphenol content, particularly quercetin.

Acknowledgments:

The authors thanked technical aid provided by Encik Fadhlullah Zuhair Japar Sidik, Puan Juliana Abdul Hamid and Puan Nurul Hafizah Abas. This work was financially supported by Universiti Kebangsaan Malaysia (AP-2014-013).

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