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The protective effect of *Boesenbergia rotunda* extract on cisplatin-exposed human embryonic kidney-293 cells by inhibiting the expression of kidney injury molecule-1, neutrophil gelatinase associated-lipocalin, NF-κB, and caspases

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ABSTRACT

Introduction: Acute kidney injury (AKI) is a major problem in platinum-based chemotherapy patients. *Boesenbergia rotunda* can induce the generation of osteoblast cells and significantly increase pancreatic antioxidant enzyme activities; therefore, this study aimed to investigate the cytotoxicity of cisplatin on human embryonic kidney-293 (HEK-293) cells and the protective impact of the ethanol extract of *B. rotunda* (EEBR) against such conditions.

Methods: Cytotoxicity was assessed using the CCK-8/WST-8 reagent, while the protective activity was assayed on 1 μg/mL cisplatin-exposed HEK-293 cells by quantifying the expression of nephrotoxicity biomarkers, e.g., kidney injury molecule-1 (Kim-1) and neutrophil gelatinase associated-lipocalin (NGAL), nuclear factor-kappaB (NF-κB), apoptotic caspase-3, and caspase-7 genes, in cisplatin-exposed HEK-293 cells.

Results: Cisplatin was confirmed as highly toxic against the HEK-293 cells (IC_{50} = 2.5145 μg/mL), whereas quercetin was of moderate toxicity (IC_{50} = 185.6225 μg/mL). EEBR revealed an IC_{50} = 40.0655 μg/mL. Moreover, EEBR concentrations of 5, 10, and 20 μg/mL confirmed its remarkable protective activity against cisplatin-exposed HEK-293 cells (P=0.031, 0.014, 0.046, respectively) compared to the cisplatin-treated cell lines without treatment. The quantitative real-time polymerase chain reaction (PCR) revealed that a higher concentration of EEBR significantly suppressed the expression of Kim-1, while lower concentrations of EEBR significantly inhibited NGAL and NF-κB genes. Higher concentrations of EEBR reduced the expression of caspase-3. All concentrations of EEBR stimulated the expression of caspase-7.

Conclusion: The significant protective activity observed in this study indicated that EEBR might be beneficial in protecting kidney cells against cisplatin.

Implication for health policy/practice/research/medical education:
The results of this study can be used as a reference for the scientification and development of *B. rotunda* rhizomes as a candidate for nephroprotective phytopharmaceuticals, particularly for patients with cisplatin chemotherapy.

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Introduction

Acute kidney injury (AKI) is a major problem in bedridden patients or in platinum-based chemotherapy patients. This disease is linked with elevated morbidity and mortality, prolonged hospitalization time, and long-term development of chronic kidney disease. A meta-analysis article reported studies on AKI incidence globally, mostly happening in hospital settings of high-income countries. The prevalence of AKI is 33.7% in children and 21.6% in adults (1).

AKI is marked by one of these parameters (*a*) an elevation in serum creatinine by 0.3 mg/dL within 2 ×

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24 hours; (*b*) an elevation in serum creatinine to 1.5x within 7 d; (*c*) a reduction in urine volume by 0.5 mL/kg/h for minimum 6 hours (2). The molecular pathways associated with the onset and progression of kidney injury are not completely understood. AKI might be caused by cellular stress and loss of structure, which eventually leads to cell death either through apoptosis or necrosis. Various inflammation-related markers for AKI have recently been described. Kidney injury molecule-1 (Kim-1) and neutrophil gelatinase associated-lipocalin (NGAL) have been introduced among those biomarkers (3,4). Activation of caspases occurs approximately a half day after cisplatin treatment *in vitro* (5), and suppressing the activity of caspase reduced cisplatin-induced apoptosis (6,7). A previous study reported that *Boesenbergia rotunda* could stimulate the generation of osteoblast cells (8) and significantly increase pancreatic antioxidant enzyme activities (glutathione, superoxide dismutase, and catalase) (9). Considering the antioxidant property and the activity of *B. rotunda* in inducing cells growth, our present study aimed to investigate the cytotoxicity of cisplatin on human embryonic kidney-293 (HEK-293) cells and the protective impact of the ethanol extract of *B. rotunda* (EEBR) against such conditions by quantifying the expression of Kim-1, NGAL, nuclear factor-kappaB (NF-κB), caspase-3, and caspase-7 genes in cisplatin-exposed HEK-293 cells.

Materials and Methods

Cells and chemicals

HEK-293 cell lines (ATCC® CRL-1573™) were the collection at the Cell and Molecular Biology Laboratory, Faculty of Pharmacy, Universitas Padjadjaran. Chemicals were cisplatin (CAS 15663-27-1-Calbiochem), quercetin (CAS reg. no. 117-39-5, Sigma-Aldrich, Saint Louis, USA), Cell Counting Kit-8 (CCK-8): water-soluble 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium (WST-8) (product code: CK04-11, Dojindo Europe), Eagle's minimum essential medium (EMEM) produced by ATCC, penicillin-streptomycin (Gibco™ 670087), and non-essential amino acid (NEAA) 1% (Gibco).

Cell culture

The HEK293 cell line was grown at 37°C with 5% CO2 in EMEM (supplemented with penicillin-streptomycin, 1% NEAA, 10% and fetal bovine serum, (FBS), 1%).

Cytotoxicity assay

The cytotoxicity of EEBR against the HEK-293 cells was assessed using the CCK-8/WST-8 reagent. EEBR was dissolved in 1% DMSO in EMEM. The solution was further prepared to concentrations of 10, 20, and 40 µg/mL in DMSO 1%. The IC_{50} was determined using GraphPad Prism 8.4.2.

Furthermore, the cytotoxicity of cisplatin and quercetin was also evaluated using the same procedure. Quercetin solutions were prepared to concentrations of 8, 16, and 32 µg/mL, while cisplatin solutions were 1.56, 3.13, and 6.25 µg/mL, respectively, in DMSO 1%.

Cytoprotective activity assay

The cytoprotective activity assay was done by adopting an earlier method (10) with limited modifications. The assay was performed on eleven groups, which were (*a*) negative control (cisplatin, 1 µg/mL); (*b*) quercetin, 2 µg/mL; (*c*) quercetin, 4 µg/mL; (*d*) quercetin, 8 µg/mL; (*e*) quercetin, 16 µg/mL; (*f*) quercetin, 32 µg/mL; (*g*) EEBR, 2.5 µg/mL; (*h*) EEBR, 5 µg/mL; (*i*) EEBR, 10 µg/mL; (*j*) EEBR, 20 µg/mL; (*k*) EEBR, 40 µg/mL, respectively, in three replicates.

A total of 50 µL (containing approximately 5×10^3 cells) of HEK-293 cells were added to each well. Cells were incubated (temperature 37°C, 5% CO2) for 24 hours. After that, single layers (cells) were removed from the supernatant, then the cells were washed with DMEM, and 50 µL of EEBR in 1% DMSO (2.5, 5, 10, 20, and 40 µg/mL) was added. The mixture was incubated under the same conditions for 1 hour, then added 50 µL of cisplatin (1 µg/mL) solution. On the next day, the medium was removed, and 10 µL of CCK-8/WST-8 reagent was added and further incubated for another 2 hours. After that, 100 L of hydrochloric acid (HCl) was added to stop the reaction, and the absorbance was measured at 450 nm.

Real-time RT-PCR analysis of Kim-1, NGAL, NF-κB, caspase-3, and caspase-7 expression

mRNA was isolated from HEK-293 cell samples with RiboZol™ RNA Extraction Reagent (US & Canada) and purified using the ethanol precipitation method as per described by the manufacturer's protocol.

The primers for

- Kim-1 gene: (forward primer: CTGCAGGGAGCAATAAGGAG, reverse primer: ACCCAAAAGAGCAAGAAGCA);
- NGAL gene: (forward primer: ACCACGGACTACAACCAGTTCGC, reverse primer: CAGTTGTCAATGCATTGGTCGGTG);
- NF-κB gene: (forward primer: ACCTTTGCTGGAAACACACC, reverse primer: ATGGCCTCGGAAGTTTTCTTT);
- caspase-3 (forward primer: AAAGGATGACTGGGAGTGG, reverse primer: ATGACGACCTGGAACATCG);
- caspase-7 (forward primer: TCATCTCATCCCTTCTCTGGA, reverse primer: TACATTTGCCCATCTTCTCG);
- β-actin (forward primer: GGAAATCGTGCGTGACATTAAA, reverse primer: GCGGCAGTGGCCATCTC).

RT-PCR for Kim-1, NGAL, NF-κB, caspase-3, and caspase-7 genes was performed using Promega (GoTaq® 1-Step RT-qPCR System), and the reaction mixture contained GoTaq® qPCR master mixture 10 µL,

GoScriptTM RT Mix 0.4 µL, forward primer 2 µL reverse primer 2 µL, Cxr Dye 0.33 µL, magnesium chloride 1 µL, nuclease-free water 2.27 µL, template 2 µL, to a final volume of 20 µL. β-actin was used to normalize all of these genes. Gene expression was read by qPCR using T-Optical Therma Cycler-ENGL (Biometra, Germany). The fluorescent detection system employed BRYT Green® Dye as a DNA binder.

Statistical analysis

Parametric data were analyzed using one way ANOVA and post hoc Tukey test, while non-parametric data were analyzed using Kruskal-Wallis and post hoc Dunn's test (with IBM SPSS Statistics version 25.0 for Windows). *P* value <0.05 was considered statistically significant.

Results

The cytotoxicity effect of EEBR on HEK-293 cells

The cytotoxicity of EEBR on HEK-293 cells was evaluated by determining the survival growth rate of the cells. At variable concentrations, HEK-293 cells were treated with cisplatin (for the cytotoxicity assay of cisplatin) or quercetin (for the cytotoxicity assay of quercetin), or EEBR (for the cytotoxicity assay of EEBR), which resulted in cisplatin as highly toxic against the HEK-293 cells, whereas quercetin and EEBR were not toxic compared to cisplatin (IC_{50} value of cisplatin = 2.5145 µg/mL; IC_{50} value of quercetin = 185.6225 µg/mL, while IC_{50} value of EEBR = 40.0655 µg/mL, respectively) (Figure 1).

The protective effect of EEBR on cisplatin-exposed HEK-293 cells

Based on the high cytotoxicity of cisplatin, the concentration of cisplatin for toxicity inducer was affirmed at 1 µg/mL. When HEK-293 cells were incubated with cisplatin, elevated cell death was observed. Cisplatin 1 µg/mL inhibits the viability of HEK-293 cells (survival growth rate = 69.183%). Pre-treatment with quercetin or EEBR increased the % survival growth rate of the cells, thus, EEBR protects HEK-293 cells against the damage induced by cisplatin. The cytoprotective effect of EEBR on the cisplatin-exposed HEK-293 cell line is presented in Table 1.

EEBR reduced the expression of Kim-1, NGAL, NF-κB, and caspase-3 genes. The effect of EEBR on the expression of AKI biomarkers and apoptotic pathway proteins in the HEK-293 cell line is presented in Figure 2.

Cisplatin stimulated the expression of Kim-1, NGAL, NF-κB, and caspase-3 genes, while conversely inhibited the expression of caspase-7 (shown in the negative control group, treated with cisplatin only). Quercetin (positive control; 4 µg/mL) significantly suppressed the expression of Kim-1 and NF-κB genes; however,t not significantly reduced the expression of NGAL and caspase-3. All concentrations of quercetin increased the expression of caspase-7, although not significantly.

The highest concentration of EEBR (10 µg/mL) significantly suppressed the expression of Kim-1 and NF-κB genes, while lower concentrations of EEBR (2.5 and 5 µg/mL) significantly inhibited NGAL and NF-κB genes. EEBR (concentrations of 5 and 10 µg/mL) reduced the expression of caspase-3 and caspase-7, although not significantly, while, the lowest concentration of EEBR (2.5 µg/mL) significantly stimulated the expression of caspase-3 and caspase-7.

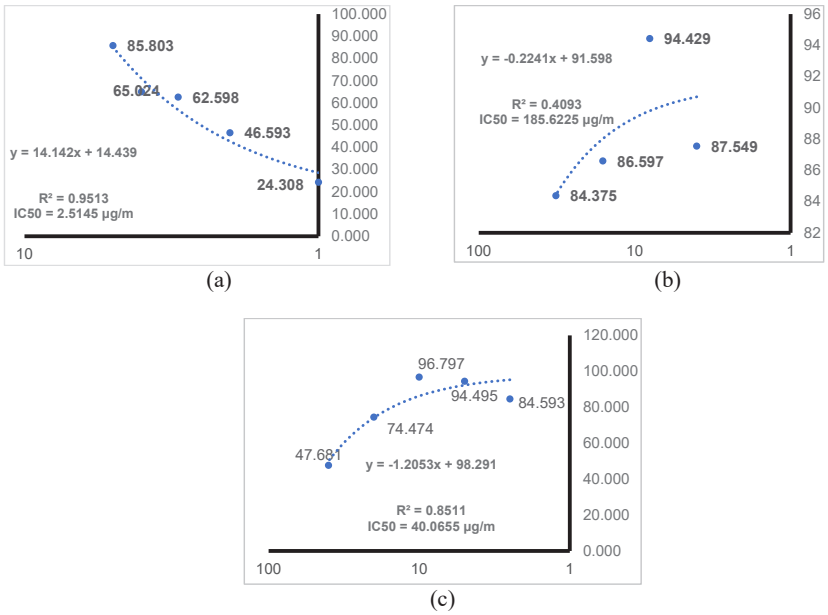

Figure 1. Effects of (a) cisplatin, (b) quercetin, and (c) EEBR on the survival growth rate of HEK-293 cells. The x-axis is the log concentration in µg/mL; the y-axis is the % survival growth rate.

Table 1. Cytoprotective effect of EEBR on cisplatin-exposed HEK-293 cells

Treatment group	Survival growth rate (%) ± SD	*P* value
Cisplatin 1 µg/mL	69.183±0.892	
Quercetin 32 µg/mL	67.879±0.057	0.758
Quercetin 16 µg/mL	74.739±0.000	0.538
Quercetin 8 µg/mL	78.241±0.602	0.218
Quercetin 4 µg/mL	75.420±1.000	0.488
Quercetin 2 µg/mL	73.090±2.423	0.590
EEBR 40 µg/Ml	64.746±0.081	0.538
EEBR 20 µg/mL	91.511±4.369*	0.046
EEBR 10 µg/mL	95.647±0.248*	0.014
EEBR 5 µg/mL	92.182±0.067*	0.031
EEBR 2.5 µg/mL	88.295±5.695	0.106

Non-parametric analysis with Kruskal-Wallis and Dunn's test. *indicates a significant difference (P<0.05) compared to the negative control group (cisplatin).

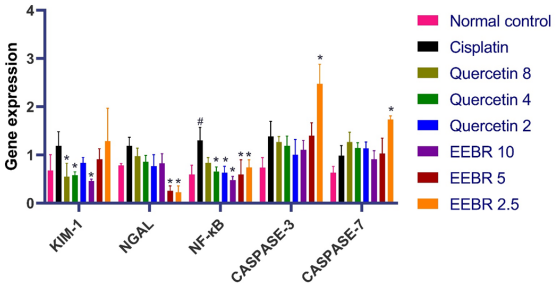

Figure 2. Effect of EEBR on the expression of Kim-1, NGAL, NF-κB, Caspase-3, and Caspase-7 genes. Parametric analysis with ANOVA and Tukey HSD, while non-parametric analysis with Kruskal-Wallis and Dunn's tests (P<0.05). *indicates a significant difference (P < 0,05) compared to the cisplatin-only (the negative control) group.

Discussion

Cisplatin was the first FDA-approved platinum compound for chemotherapy (11). Yet, in clinical trials, cisplatin despite its strong activity can induce damage to the DNA, produces reactive oxygen species (ROS), and ultimately induces nephrotoxicity. An excess of this drug causes hepatotoxicity and nephrotoxicity in both humans and experimental animals (12). It is crucial to understand the *in vitro* cytotoxicity of cisplatin, which was performed using the water-soluble CCK-8/WST-8, and the % survival rate of the cells was measured. CCK-8/WST-8 tetrazolium salt was reduced by dehydrogenases in the cells and produced a soluble orange color formazan compound. The level of formazan was equal to the number of living cells and was measured at 460 nm. When there is a lack of oxygen, e.g., due to a pathological condition, the HEK-293 cells will form spheroids and develop a necrotic core (13).

In our study, cisplatin was proven to be highly toxic (IC_{50} value of cisplatin = 2.5145 µg/mL), meanwhile, quercetin is categorized as moderate toxicity in HEK-293 cells (IC_{50} value of quercetin = 185.6225 µg/mL), as per the criteria of cytotoxicity by the National Cancer Institute (NCI) of the US. The criteria of toxicity are described as follows: high cytotoxic if IC_{50}<20 µg/mL, moderate cytotoxic if IC_{50} is 21-200 µg/mL, weak cytotoxic if IC_{50} is 201-500 µg/mL, and not cytotoxic if IC_{50}>500 µg/mL (14).

In this study, cisplatin stimulated the expression of kidney injury biomarker genes (Kim-1 and NGAL), NF-κB, and apoptotic gene (caspase-3) in HEK-293 cells, which confirms its harmfulness to the cells. By stimulating the expression of apoptotic genes, the number of cell deaths will be considerably increased. Our findings also revealed that quercetin significantly suppressed the expression of Kim-1 and NF-κB genes in cisplatin-exposed HEK-293 cells, yet it did not significantly reduce the expression of NGAL and caspase-3. Quercetin also increased the expression of caspase-7. A previous study reported that quercetin was not harmful to normal cells, but it could inhibit the growth of cancer cells via different pathways (15). Quercetin strongly suppressed the viability of cancer cells, e.g., human leukemia U937 cells (16), ovarian carcinoma (SKOV3) cells (17), oral cancer cells (18), cervical cancer cells (19), and many more. This flavonol compound has revealed significant beneficial effects. At reasonable doses, it was reported to have no obvious toxic side effects on normal cells (20). The anticancer property of quercetin was predicted to result from cell cycle arrest at the G1 phase. This flavonol has been reported to induce a p21 CDK inhibitor associated with a the reduction of nuclear phosphoprotein pRb phosphorylation, which subsequently inhibited the G1/S cell cycle progression by restricting E2F1. Quercetin also stimulated a mild DNA injury and activated Chk2. Additionally, quercetin suppressed the cyclin B1 and CDK1, the key components of G2/M cell cycle progression (21).

EEBR protects the human embryonic kidney cells against cisplatin-induced damage as proven by its significant inhibition of the expression of Kim-1, NGAL, and NF-κB genes. Higher concentrations of EEBR reduce the expression of caspase-3, although not significantly compared to the cisplatin group. This kidney protective activity is predicted due to panduratin A and/or other constituents contained in the rhizome of this plant. As reported by previous studies, panduratin A attenuated colistin toxicity in human renal proximal tubular cells (RPTEC/TERT1) (22,23) and in mice (23). This compound blocked the activation of ERK1/2 and caspase 3 in cisplatin-treated mice (23). Furthermore, EEBR (doses of 1000 mg/kg and 2000 mg/kg) did not damage the kidney of Wistar rats as proven by histopathological examination (24).

Caspases are important mediators of apoptosis. In particular, caspase-3 and caspase-7 are the downstream effector caspases, which are activated in response to diverse cell death stimuli (25). Along with apoptosis, caspase-7 also functions in non-apoptotic roles, for example, in the continuation of the cell cycle. For that

purpose, it was described that the proteolytic activity of caspases influences the cell cycle proteins, in this case, caspases act as their substrates (26). During apoptosis, caspase-7 is activated at aspartate residue positions 23, 198, and 206, which produces the mature caspase-7 (27). Moreover, a previous study reported that by reducing the presence of caspase-7 cell growth was lessened due to a halt at the G2/M phase (28). By inhibiting Kim-1, NGAL, NF-κB, and apoptotic genes, EEBR can be proposed as a nephroprotective agent. To our knowledge, this is the first study to report that EEBR suppresses kidney injury biomarkers and apoptosis genes *in vitro*.

Conclusion

Taken together, our findings suggest that EEBR might be developed as a potential nephroprotective agent, particularly due to its inhibitory activity on the expression of kidney injury biomarker genes (Kim-1 and NGAL), inflammatory gene (NF-κB), and apoptotic caspase genes.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the first author upon reasonable request.

Authors' contributions

JL was principally responsible for the study design and contribution to the data interpretation, manuscript preparation and revision, and funds collection. DS contributes to the literature search, data collection, manuscript preparation, and statistical analysis. NMS and SAS contributed to data interpretation. All authors have read and approved the final manuscript for publication.

Conflict of interests

None to declare.

Ethical considerations

This work has been approved by the Ethical Committee of Padjadjaran University (Ethical approval No. 768/UN6.KEP/EC/2021).

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