

# Inhibitory effects of Indonesian temu kunci (*Boesenbergia rotunda*) rhizome extract on nitric oxide synthase production and on the kidneys of Wistar rats

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**Abstract.** Nitric oxide synthases (NOSs) are enzymes that function by generating nitric oxide (NO) from its substrate, L-arginine, in human tissues. There are three known isoforms of NOS, eNOS (endothelial NOS), nNOS (neuronal NOS), and iNOS (inducible NOS). iNOS is released in response to inflammation. The human iNOS inhibitor, S-ethylisothiourea (SEITU), has been found to attach to a Glu377 residue within the active-site cavity of the enzyme. The discovery of iNOS inhibitors is always challenging. *Boesenbergia rotunda* (*B. rotunda*) has been reported to reduce inflammation in an animal model. The present study examined the inhibitory effects of *B. rotunda* rhizome ethanolic extract (BRE) on iNOS using the fluorometric method and investigated the histopathological effects of higher concentrations of BRE (1,000, 2,000 and 4,000 mg/kg body weight) on the kidneys of male Wistar rats. Molecular docking simulation of quercetin and kaempferol towards iNOS was also performed. The phytochemical screening of BRE indicated the presence of polyphenolics, flavonoids, triterpenoids, and saponins. The *in vitro* experiment confirmed that BRE reduced NO production with a half-maximal inhibitory concentration value of 79.06 µg/ml. Both quercetin and kaempferol interacted with Glu377 by building hydrogen bonds. Furthermore, the animal experiment resulted in no mortality and no marked morphological changes in the rat kidneys at all concentrations, indicating the safety

of BRE as regards this organ. Taken together, BRE may be further developed as a safe anti-inflammatory drug candidate.

## Introduction

Inducible nitric oxide synthase (iNOS) is expressed in response to pathogenic stimuli and subsequently produces nitric oxide (NO) that plays role in cytoprotection. The over-expression of iNOS elevates the NO level and is implicated in the pathophysiology of complex multifactorial diseases, such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, rheumatoid arthritis and inflammatory bowel disease (1). The excess production of NO has also been detected in septic and cardiogenic shock (2).

iNOS consists of homodimeric proteins, of which monomers contain an N-terminal oxygenase domain (the catalytic site where the L-arginine substrate is oxidized and is converted into L-citrulline and NO) and a C-terminal reductase domain (3). The oxygenase and reductase domains are connected by a calmodulin-binding subunit. Moreover, the L-arginine substrate analogs have been proposed to bind to Glu371 and Trp366 residues in the catalytic site of the enzyme (4).

The discovery of plant-based anti-inflammatory drugs has been challenging. The well-known catechin contained in tea, epigallocatechin gallate, and proanthocyanidins in grape seed have exhibited potential use as NOS inhibitors (5). The human iNOS inhibitor, S-ethylisothiourea (SEITU), has been found to attach to a Glu377 residue within the active-site cavity of the enzyme (6).

Several *Boesenbergia* plants [*Boesenbergia thorelii* (*B. thorelii*) and *Boesenbergia longiflora* (*B. longiflora*)] have been investigated for their inhibitory effects on NO production using murine macrophage-like RAW264.7 cells. The ethanolic extracts of these plants have been shown to exert inhibitory effects against NO release with half-maximal inhibitory concentration (IC<sub>50</sub>) values of >100 for *B. thorelii* and 6.0 for *B. longiflora*, respectively, whereas the IC<sub>50</sub> of the water extracts

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of *B. thorelii* and *B. longiflora* were >100 µg/ml and 72.2 µg/ml, respectively (7). *Boesenbergia rotunda* (*B. rotunda*; Indonesian name, *temu kunci*) has been empirically used in the treatment of various conditions by applying the freshly ground rhizome topically on the inflamed site. This plant has been proven to contain quercetin, which belongs to a group of plant pigments known as flavonoids and polyphenolic compounds (8,9). A previous acute toxicity study revealed that the half-maximal lethal dose was >4,000 mg/kg body weight (BW) (10). A lower dose of this plant (500 mg/kg BW) has been shown to protect the liver from thioacetamide-induced hepatocirrhosis in rats (11). However, data on the inhibitory effects of *B. rotunda* rhizome ethanolic extract (BRE) on the iNOS enzyme and on the histopathology of the kidneys of male Wistar rats are limited. It was previously reported that panduratin A, hydroxypanduratin A and cardamonin, isolated from *B. rotunda* exerted an inhibitory effect on NO production with IC<sub>50</sub> values of 5.3, 13.3 and 24.7 µM, respectively (12).

## Materials and methods

**Study location.** The animal experiment was carried out at the Central Laboratory of Padjadjaran University (Sumedang, Indonesia). The *in silico* experiment was performed at the Center for Computational Research, Faculty of Pharmacy, Padjadjaran University.

**In silico experiment.** The hardware used was a personal computer with Windows 10 and RAM of 6 GB. The softwares used were LigandScout 4.1.4 (Padjadjaran University License), AutoDock 4.2.6 (The Scripps Research Institute; <https://autodock.scripps.edu/>) and BIOVIA Discovery Studio (<https://discover.3ds.com/discovery-studio-visualizer-download>; Academic License).

The crystal structures of human iNOS PDB ID: 3E7G (13) and PDB ID: 4NOS (14) were downloaded from the RCSB Protein Data Bank. LigandScout automatically activated the PDB algorithm and visualized the macromolecule view of the complex. The 3D structure of quercetin and kaempferol was obtained from the PubChem database (CID: 5280343 for quercetin and CID: 5280863 for kaempferol). The ligands were optimized, the stable conformations were selected, and converted to pdbqt format. Molecular docking simulation was performed using AutoDock 4.2.6.

**Plant materials.** The rhizomes were purchased from Lembang, West Java, Indonesia, and were taxonomically identified by a botanist at the Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University.

The plant samples were confirmed as *B. (L.) Mansf.* (Zingiberaceae). The characteristics of the plant samples matched those described in The IUCN Red List of Threatened Species (<https://www.iucnredlist.org/species/44392164/44540996>) (15).

**Instruments and glassware.** The instruments used were a Buchi® rotavapor R215 (<https://www.sigmaaldrich.com/ID/en/product/aldrich/z565490>), Buchi® heating bath B-491 (<https://www.sigmaaldrich.com/ID/en/product/aldrich/z563986>), Buchi® vacuum pump V-700 (<https://www.>

[sigmaaldrich.com/ID/en/product/sigma/z616834](https://www.sigmaaldrich.com/ID/en/product/sigma/z616834)), Buchi® distillation chiller B-741 (<https://www.sigmaaldrich.com/ID/en/product/aldrich/z565938>), Buchi® evaporator glassware, chemical glasswares Iwaki (<https://www.iwakiglassindonesia.com/en/home>), multimode reader Infinite M200Pro Tecan ([https://lifesciences.tecan.com/plate\\_readers/infinite\\_200\\_pro](https://lifesciences.tecan.com/plate_readers/infinite_200_pro)), and analytical balance (Mettler-Toledo).

**Chemicals.** The chemicals used were ethanol (96%; Brataco Chemika), hematoxylin (CAS Number 517-28-2; MilliporeSigma) and eosin (CAS Number 15086-94-9; MilliporeSigma).

**Preparation of BRE and phytochemical screening.** The extraction process was performed by modifying a previously described procedure (10). The rhizomes were cleaned from the soil, washed, thinly sliced, and sun-dried. The rhizome powder (500 g) was soaked in 95% ethanol for 24 h at 25–26°C. The extract was filtered and the residue was re-extracted for 2x24 h. The solvent was rotary-evaporated in a vacuum at 45°C and 80 rpm. The viscous extract of BRE yielded 17.24% w/w. The phytochemical screening was carried out by following a standard method (16).

**NOS inhibitory activity assay.** The reagents were prepared per instructions written on the Fluorometric iNOS Inhibitor Screening kit (cat. no. K208-100, BioVision <https://www.biovision.com/documentation/datasheets/K208.pdf>) and the manufacturer's protocol was followed accordingly. The absorbance was measured (Ex/Em=360/450 nm) using a multimode reader Infinite M200Pro (Tecan Austria GmbH) and the samples were placed in a microplate 96-well U-bottom (BR701330-100EA; <https://www.sigmaaldrich.com/ID/en/product/aldrich/br701330>). The relative inhibition (%) was calculated by measuring the subtract background control (BC) reading from the enzyme control (EC) and inhibitor (S) using the following equation:

$$\% \text{ Relative Inhibition} = \frac{\Delta RFUEC - \Delta RFUS}{\Delta RFUEC} \times 100\%$$

The IC<sub>50</sub> was calculated using GraphPad Prism 8.4.3 software (GraphPad Software, Inc.). The results were compared to those of SEITU (S-ethylisothiouraea hydrobromide TCI CAS RN: 1071-37-0, product no. E0182 <https://www.tcichemicals.com/NZ/en/p/E0182>) a known iNOS inhibitor, and diphenylene iodonium chloride (DPI) included in the reagent kit (<https://www.biovision.com/documentation/datasheets/K208.pdf>), a nicotine amide dinucleotide phosphate oxidase (NOX) inhibitor.

**Animals.** A total of 20 male Wistar albino rats, aged 6–8 weeks, weighing 210–240 g, were acclimatized at 24°C under a 12-h light/dark cycle, 55% relative humidity, with food and water provided freely for 1 week. The animal handling, maintenance and euthanasia procedures were performed as approved by the Research Ethics Committee, Padjadjaran University, Indonesia (recognized by the Forum of Ethics Review Committee in Asia and Western Pacific Region). Euthanasia was performed using an initial concentration of 2% isoflurane with a slow flow rate (isoflurane Abbot USP, NDC: 10019-360-60) to prevent

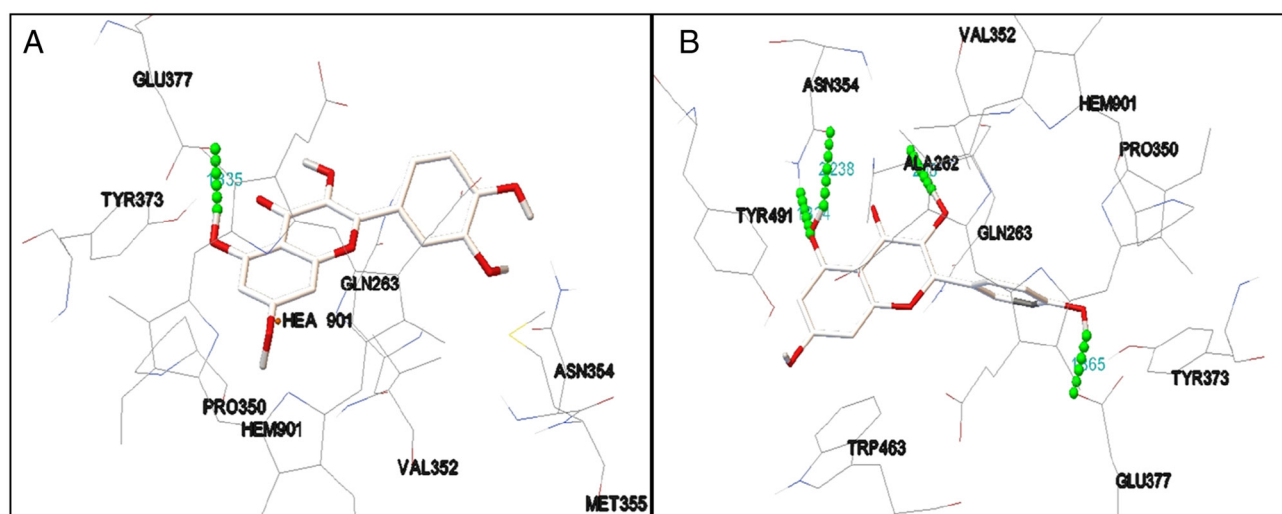


Figure 1. Interaction of (A) quercetin with a binding energy= $-7.5 \pm 0.08$ ,  $K_i=2.96$  kcal/mol; and (B) kaempferol with a binding energy= $-6.8 \pm 0.06$ ,  $K_i=9.62$  kcal/mol. Both quercetin and kaempferol interacted with Glu377 in the catalytic site of human inducible nitric oxide synthase by building hydrogen bonds (shown as green dotted lines).

sudden death, the concentration of isoflurane and the flow rate was increased gradually to a maximum of 4% under the supervision of a veterinarian.

**Histopathological analysis of the effects of BRE on the kidneys of male Wistar rats.** The present study was conducted by following the Regulation of the Indonesia National Agency of Drug and Food Control (<https://www.pom.go.id/new/home/en>).

The rats were randomly divided into four groups ( $n=5$ ) as follows: The normal control (treated with distilled water only) and three treatment groups of BRE (1,000, 2,000 and 4,000 mg/kg BW); the rats were administered a single oral administration. On day 21, the rats were sacrificed and their kidneys were dissected out, washed meticulously in 0.9 % sodium chloride solution, trimmed, fixation-processed in Bouin solution (cat no. HT10132, MilliporeSigma), embedded in paraffin wax (melting point,  $56-57^\circ\text{C}$ , Cas No. 8042-47-5, Merck KGaA), sectioned at a standard thickness ( $5-10\ \mu\text{m}$ ) using a microtome, stained with hematoxylin and eosin as per laboratory protocol (15 min), and observed under a light optical microscope (Primostar 3 binoculars; Carl Zeiss AG). The objects were captured using a digital camera (Infinity1®). The quantitative analysis of apoptotic vs. necrotic morphology was performed using time-lapse microscopy with Annexin and propidium iodide. Necrotic cells are characterized by cytoplasmic swelling, while apoptotic cells lose their membrane permeability hours later.

## Results and Discussion

**In silico analysis.** The *in silico* analysis revealed that quercetin (Fig. 1A) and kaempferol (Fig. 1B), both flavonols contained in BRE, interacted with Glu377, by building hydrogen bonds (at a distance of  $1.3\ \text{\AA}$  for both quercetin and kaempferol). Quercetin interacted with Glu377 in the catalytic site of human iNOS with a binding energy of  $-7.5 \pm 0.08$  and  $K_i=2.96$  kcal/mol, while kaempferol exhibited a binding energy of  $-6.8 \pm 0.06$  and  $K_i=9.62$  kcal/mol. Considering this, the binding affinity of

quercetin towards human iNOS was defined as stronger than that of kaempferol.

Moreover, both quercetin (cLog  $P=4.22$ ;  $MW=302.24$  atomic mass unit; hydrogen bond donors=5; hydrogen bond acceptors=7) and kaempferol (cLog  $P=4.40$ ; molecular mass=286.44 atomic mass unit; hydrogen bond donors=4; hydrogen bond acceptors=6) fulfilled the requirements of Lipinski's Rule of Five i.e., a molecule with a molecular mass  $<500$  Da, no more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, and an octanol-water partition coefficient Log  $P$ -value not  $>5$  (<http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp>). Lipinski's rules were originally formulated to support the development of oral bioavailable drugs (17).

The N-terminal catalytic oxygenase of human iNOS consists of the heme Glu377 residue and the amino acids at positions 369-372. In the complexes between iNOS and its inhibitors, the inhibitors imitate the guanidinium group of the substrate of the enzyme, by building hydrogen bonds to Glu377 and pi-pi stacking with heme (14). Another molecular docking study reported that their tested ligand, namely andrographolide, interacts with residues Trp372, Glu377, and Arg199 with a strong binding affinity (18). Furthermore, L-arginine, the substrate of NOS, has been reported to establish a salt-bridge interaction with the conserved carboxylate of Glu377 in the catalytic site of iNOS. In addition, this substrate builds hydrogen bonds with the amide carbonyl of Trp372 (19). Another previous study on synthesized N-(3-hydroxy-3-(pyridine-3-yl)propyl)imidamide, indicated a tight hydrogen bonding of the imidamide moiety of this compound to Glu377 residue in the catalytic site of iNOS (20), which confirms the critical role of Glu377.

**Phytochemical screening of BRE.** The phytochemical screening of BRE confirmed the presence of flavonoids, saponins, polyphenols, and triterpenoids (data not shown). Similar to these results, a previous study by Jing *et al* (21) reported that polyphenols, including caffeic acid, coumaric acid, chlorogenic

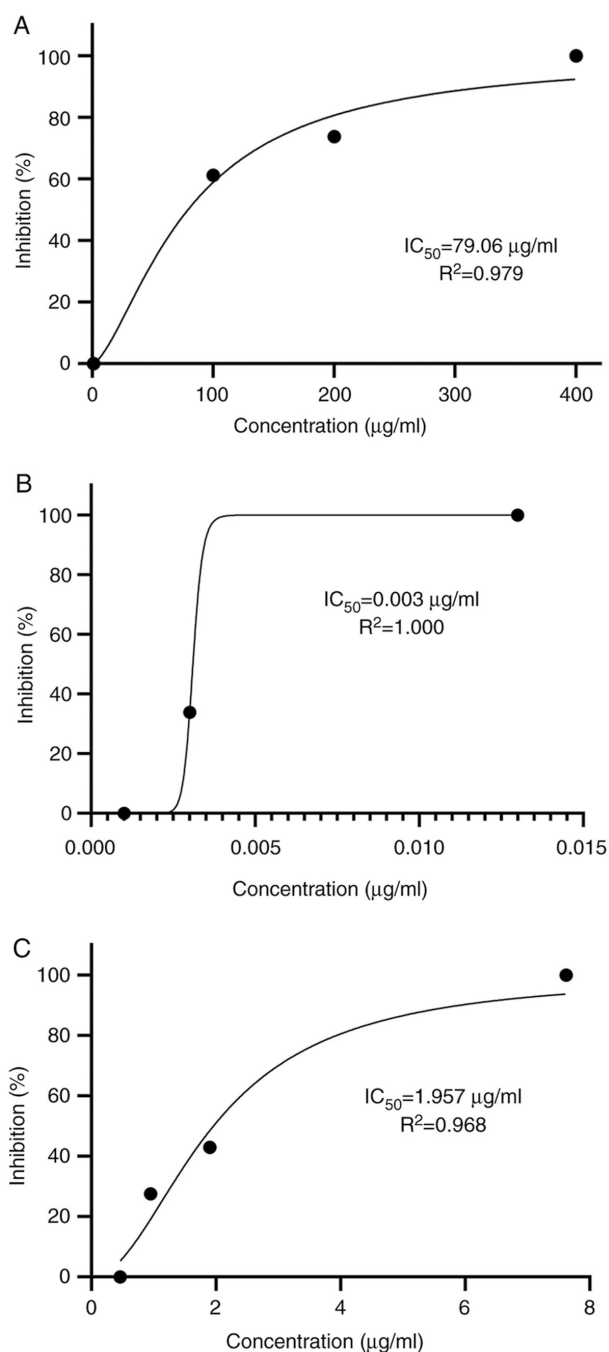


Figure 2. Inhibitory activity of (A) BRE ( $IC_{50}=79.06 \mu\text{g/ml}$ ); (B) DPI ( $IC_{50}=0.003 \mu\text{g/ml}$ ); (C) SEITU ( $IC_{50}=1.957 \mu\text{g/ml}$ ) against iNOS by *in vitro* study.

acid, hesperidin, kaempferol, naringin and quercetin, were contained in *B. rotunda* rhizomes. The presence of quercetin in BRE has also been previously reported (9).

**NOS inhibitory activity assay.** The percent relative inhibition curve for BRE (Fig. 2A) revealed an  $IC_{50}$  value of  $79.06 \mu\text{g/ml}$ , which is categorized as a low inhibition (22) of NO production. However, compared to that of DPI ( $IC_{50}=0.003 \mu\text{g/ml}$ ) (Fig. 2B) and SEITU ( $IC_{50}=1.957 \mu\text{g/ml}$ ) (Fig. 2C), the inhibitory activity of BRE towards iNOS was weaker.

As is currently recognized, DPI is a potent inhibitor of NOX and thus blocks the production of excess reactive oxygen

Table I. Results of the quantitative analysis of the kidney tissues of male Wistar rats using time-lapse microscopy.

Rat group	Quantitative analysis of the kidney tissue (per 1,000 cells)		
	Normal	Apoptotic	Necrotic
Normal	873	54	73
BRE at 1,000 mg/kg BW	858	58	84
BRE at 2,000 mg/kg BW	849	71	80
BRE at 4,000 mg/kg BW	838	64	98

The results indicated that all doses of BRE were safe as proven by only small numbers of apoptotic and necrotic cells detected. BRE, *Boesenbergia rotunda* rhizome ethanolic extract; BW, body weight.

species (ROS) in the cell (23). The inhibition of iNOS by quercetin and kaempferol, in pure forms, has also been reported. An *in vitro* study on a human hepatocyte-derived cell line supplemented with quercetin or kaempferol (5 to  $200 \mu\text{mol/l}$ ) confirmed a significant concentration-dependent decrease in iNOS production (24). As also previously reported, a small amount of quercetin is present in the *B. rotunda* rhizome (9).

**Histopathological effects of BRE on the kidneys of male Wistar rats.** The main objective of evaluating the safety of any herbal drugs is to identify the nature and significance of toxicity and to establish the exposure level at which this toxicity is observed (25). The acute toxicity of the BRE has previously been evaluated in normal healthy Wistar rats for 21 days (10) and 72 h (26). In these studies, the rats were subjected to a single dose of 1,000 mg/kg BW to 4,000 mg/kg BW (10), 2,000 mg/kg BW (26) and 5,000 mg/kg BW (11) of BRE, respectively. As also previously reported, all the rats in these tests remained alive and did not manifest mortality or any visible signs of toxicity (10,11,26).

The quantitative observation of the kidneys, represented by the microscopic observation of normal cells, apoptosis and necrosis, is presented in Table I. Only small numbers of apoptotic and necrotic cells are detected during observation (calculated per 1,000 cells). The microscopic examination of the kidneys revealed similar results in all tested doses of BRE that confirmed its safety.

Research on the clinical toxicity of quercetin and kaempferol has indicated that these compounds were considered safe for humans within the accepted daily intake limit. Both quercetin and kaempferol rapidly undergo phase 1 and 2 biotransformations in the liver and circulate as methyl, glucuronide, and sulfate metabolites, which can be measured in the blood and urine of the participants (27).

In the present study, the morphological appearance of the kidneys (Fig. 3) revealed no changes at all tested doses of BRE, which confirmed its safety as regards this organ. Moreover, the microscopic histopathological examination of the kidneys revealed normal results with BRE doses of 1,000 mg/kg BW (Fig. 4B) and 2,000 mg/kg BW (Fig. 4C) similar to that of the normal control group treated with distilled water (Fig. 4A). However, at a dose of 4,000 mg/kg BW (Fig. 4D), an irregular



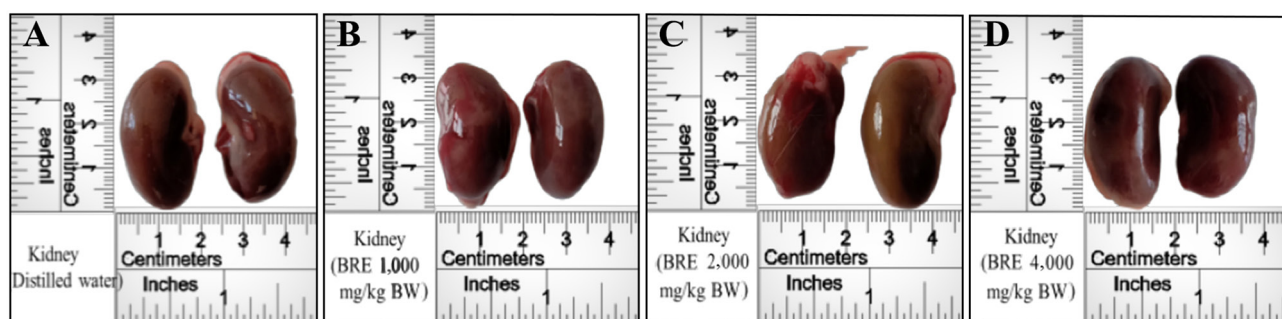


Figure 3. Morphological appearance of the kidneys on day 21 after a single administration of (A) distilled water in the normal control group; (B) BRE at 1,000 mg/kg BW; (C) BRE at 2,000 mg/kg BW; (D) BRE 4,000 mg/kg BW. BRE, *Boesenbergia rotunda* rhizome ethanolic extract; BW, body weight.

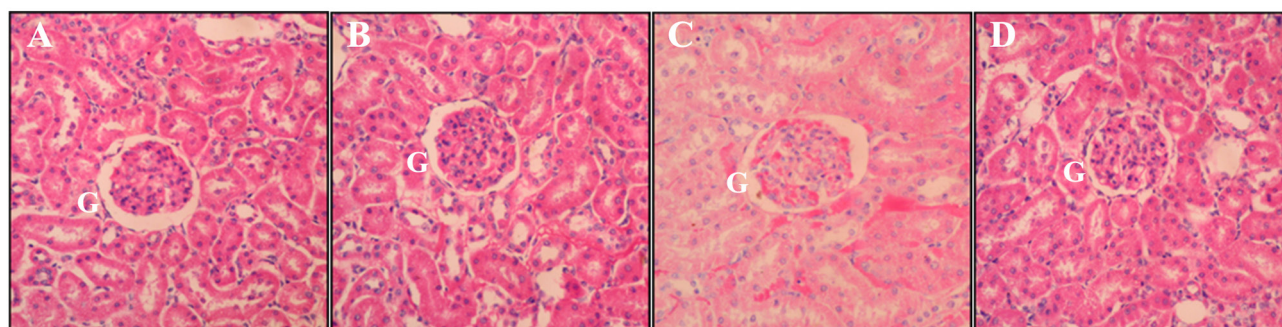


Figure 4. Microscopic histopathological examination of the kidney on day 21 after a single administration of (A) distilled water in the normal control group; (B) BRE at 1,000 mg/kg BW; (C) BRE at 2,000 mg/kg BW; (D) BRE at 4,000 mg/kg BW. The kidney tissues were subjected to hematoxylin and eosin staining. The staining was confirmed as homogenous and strictly followed the standard laboratory procedure. Magnification, x100. BRE, *Boesenbergia rotunda* rhizome ethanolic extract; BW, body weight; G, glomerulus.

shape of Bowman's capsule, which may indicate abnormal numbers of cells and glomerulosclerosis, was observed.

Bowman's capsule, a part of the kidney's nephron, encloses the glomerular capillary loops and is involved in blood filtration (28). The outer part of Bowman's capsule consists of parietal epithelial cells and squamous cells. The parietal epithelial cells can increase rapidly in numbers or decrease abnormally, which indicates the occurrence of disorders (29).

Notably, a previous animal study on the toxicity of other herbal drugs reported similar findings (30). The carcinogenic activity of turmeric oleoresin (major component 79-85% of curcumin) was observed in female B6C3F1 mice treated with a higher dose (10,000 ppm) than required for efficacy. That report was based on an increased incidence of hepatocellular adenomas in mice although no deaths were reported until the end of the study (30). However, a phase I clinical trial of turmeric oil in nine healthy volunteers for 3 months indicated no clinical, hematological, renal, or hepatotoxicity at 1 and 3 months; the serum lipid levels of the volunteers did not exhibit any significant changes, apart from one volunteer (reversible) (31). Experimental studies have proven that low concentrations of curcumin exert antioxidant effects, although higher concentrations of this compound increase the production of cellular ROS by a covalent reaction between the two conjugated  $\alpha,\beta$ -unsaturated carbonyls in the curcumin molecule with the thiol groups of cysteine residues of proteins (32).

Despite their advantages, when used for a long period of time, herbal drugs can cause herb-induced liver injury (33),

e.g., acute and chronic hepatitis, cholestasis, drug-induced autoimmunity, vascular lesions, and even hepatic failure and cirrhosis. An example is Senna (*Cassia angustifolia*), which was identified as a cause of hepatocellular necrosis and canalicular cholestatic hepatitis in a 77-year-old male with a history of long-term Senna intake for the treatment of chronic constipation (34). A previous review article study on Chinese herbal medicine reported that anthraquinones, *Stellera chamaejasme* flavonoids, and glycosides from herbs may cause acute and chronic kidney injury, as well as nephrolithiasis (35).

The present study examined the inhibitory effects of BRE on iNOS using the fluorometric method and investigated the histopathological effects of BRE on the kidneys of male Wistar rats. In conclusion, BRE inhibited the production of NO as confirmed by both the *in vitro* ( $IC_{50}=79.06 \mu\text{g/ml}$ ) and *in silico* (hydrogen bond interaction of quercetin and kaempferol with Glu377 similar to that of known iNOS inhibitors and substrate) experiments. During the length of the study, no rat mortality occurred and no marked morphological changes in the kidney of the rats were observed at all doses, with the exception of a slightly irregular shape of the Bowman's capsule at the dose of 4,000 mg/kg BW; this indicates the safety of BRE as regards this organ. However, further studies, e.g., on the cytotoxicity of BRE on kidney cell lines are required, and a phase I clinical trial in humans may also prove beneficial. Nonetheless, the present study provides evidence that BRE may be further developed as a safe anti-inflammatory drug candidate.

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## Availability of data and materials

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

## Authors' contributions

JL was principally responsible for the conception and design of the study. JL, IMP and RL supervised and monitored the project. AMR contributed to the animal handling, histopathological analysis, and in the *in vitro* iNOS inhibitory activity assay. SM and SJ participated in the *in silico* analysis. JL, IMP, RL, and SAS contributed equally to the acquisition, and interpretation of the reported data. JL and SM contributed to the writing and revision of the manuscript. AMR, SM, and SJ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

Animal handling, maintenance, and euthanasia procedures were performed as approved by the Research Ethics Committee (<https://kep.unpad.ac.id/>), Padjadjaran University, Sumedang, Indonesia (recognized by the Forum of the Ethics Review Committee in Asia and Western Pacific Region) document no. 1236/UN6.KEP/EC/2019.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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