

## ORIGINAL PAPER

# Regulation by Phloroglucinol of Nrf2/Maf-Mediated Expression of Antioxidant Enzymes and Inhibition of Osteoclastogenesis via the RANKL/RANK Signaling Pathway: In Silico study

Agus Hadian Rahim<sup>1</sup>, Bambang Setiawan<sup>2</sup>, Firli Rahmah Primula Dewi<sup>3</sup>, Zairin Noor<sup>4</sup>

<sup>1</sup>Department of Orthopaedics and Traumatology, Hasan Sadikin General Hospital, Medical Faculty Padjadjaran University, Bandung, West Java, Indonesia

<sup>2</sup>Research Center for Osteoporosis, Department of Medical Chemistry and Biochemistry, Medical Faculty Lambung Mangkurat University, Banjarmasin, South Kalimantan, Indonesia

<sup>3</sup>Malang In Silico Club, Malang, East Java, Indonesia

<sup>4</sup>Research Center for Osteoporosis, Department of Orthopaedics and Traumatology, Ulin General Hospital, Medical Faculty Lambung Mangkurat University, Banjarmasin, South Kalimantan, Indonesia

Corresponding author: Bambang Setiawan. Research Center for Osteoporosis, Department of Medical Chemistry and Biochemistry, Medical Faculty Lambung Mangkurat University, Banjarmasin, South Kalimantan, Indonesia. Jl. Veteran, Banjarmasin, South Kalimantan, Indonesia. Handphone: +6281351111307. E-mail: ganes79setiawan@gmail.com

## ABSTRACT

**Introduction:** Phloroglucinol is an antioxidant compound with many positive effects on health. The purpose of this study was to determine the role of phloroglucinol in osteoclastogenesis via the RANKL/RANK signaling pathway and the activity of the transcription factor Nrf2. **Material and methods:** Analysis was performed *in silico* using the primary method of docking by the use of Hex 8.0 software and Haddock web server. Analysis of interactions was then performed to determine interactions between the ligand and its receptors by using the software LigPlus and LigandScout 3.1. **Results:** Results indicated that phloroglucinol compound was thought to inhibit osteoclastogenesis via three mechanisms: inhibiting RANKL-RANK interaction, sustaining the RANKL-OPG bond, and increasing the activity of the transcription factor Nrf2.

**Key words:** *in silico*, Nrf2, osteoclastogenesis, phloroglucinol, RANKL, RANK.

## 1. INTRODUCTION

Formation and maintenance of bone tissues are regulated by two main mechanisms, bone formation by osteoblasts and bone resorption by osteoclasts (1). Osteoblasts are derived from primitive multipotent mesenchymal stem cells capable to differentiated into bone marrow stromal cells, chondrocytes, muscle cells, and adipocytes (2). Osteoclasts are multinuclear cells derived from the fusion of mononuclear hematopoietic precursor cells (3). Development and differentiation of both types of cell are regulated very strictly by various endogenous substances, including growth factors, cytokines, and hormones.

During bone resorption, osteoclasts will act along with macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- $\kappa$ B (NF- $\kappa$ B) ligand (RANKL) (4), osteoclast differentiation factor (5) and osteoprotegerin ligand (6). RANKL is an essential cytokine for osteoclasts differentiation expressed by osteoblasts, activated T-cells and stromal cells (7). In case of a binding of RANKL and its receptor RANK, a protein family of TNF receptor-associated factor (TRAF) will recruit activated RANK, leading to activation of the transcription factor NF $\kappa$ B and mitogen-activated pro-

tein kinase (MAPK) (8).

One of the transcription factors responsible for the regulation of differentiation and function of osteoblasts is Nrf2 (nuclear factor E2 p45-related factor 2). The transcription factor Nrf2 has a basic well-conserved structure of leucine zipper. Under normal conditions, Kelch-like ECH-associated protein 1 (Keap1), which is a cysteine-rich cytoplasmic protein, binds to Nrf2 in the cytoplasm via interaction of its double glycine-rich domain with the hydrophilic region of Neh2 domain of Nrf2 (9). Oxidative stress can lead to modification of Keap1 cysteine residues, detaching Nrf2 from Keap1, which prevents Nrf2 from proteosomal degradation and leads Nrf2 to translocate into the nucleus (10). In the nucleus, Nrf2 associates with small Maf proteins and form heterodimers which then binds to the antioxidant-responsive elements (ARE) to induce the expression of several genes coding for phase II detoxification enzymes or antioxidant enzymes, including glutathione (GSH) S-transferase, superoxide dismutase (SOD) 2, thioredoxin (TRX) 1, Trx reductase (TrxR) 1, glutathione reductase 1, glutathione peroxidases 2 and 3, peroxiredoxins (Prx's) I and VI, and sulfiredoxin (Srx) (11-13).

There are an increasing number of studies reporting the

role of free radicals in the case of post-menopausal osteoporosis. There are close relationships among oxidative stress, antioxidant levels and bone mineral density (14, 15). Hydrogen peroxide is believed to be the reactive oxygen species (ROS) responsible for bone resorption due to estrogen deficiency (16). H<sub>2</sub>O<sub>2</sub> is found to stimulate bone resorption by osteoclasts directly via induction of various cytokines and growth factors (17). ROS is also found to increase or decrease the expression of receptor activator of NF- $\kappa$ B ligand osteoprotegerin/OPG in osteoblasts. In addition, ROS is also found to inhibit osteoblast differentiation (18) and induce apoptosis of osteoblasts (19).

ROS production in activated osteoclasts occurs via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) and is thought to have an important role in bone resorption (20). A study reported that RANKL may mediate stimulation of osteoclast precursors to form ROS via the signaling pathways involving TRAF6, Rac1 and Nox1 (21). Several types of antioxidants, including ascorbic acid, lycopene, curcumin, coenzyme Q10 and  $\alpha$ -lipoic acid, are found to inhibit bone resorption by osteoclasts through inhibiting ROS formation (22, 23).

Phloroglucinol derivatives are secondary metabolites commonly found in plants of the families Myrtaceae, Guttiferae, Euphorbiaceae, Aspidiaceae, Compositae, Rutaceae, Rosaceae, Clusiaceae, Lauraceae, Crassulaceae, Cannabinaceae and Fagaceae. This compound constitutes one component of phlorotannin present in abundant amounts in *Ecklonia cava* (brown algae). Several *in vitro* and *in vivo* studies indicated that this compound has protective effects on H<sub>2</sub>O<sub>2</sub>-induced oxidative stress (24, 25). In addition, phloroglucinol has extensively been studied with regard to its pharmacological activity and toxicity, such as in cases of chronic and acute toxicity, the relaxant properties of smooth muscle cells, as well as its effect on the rectosigmoid motility in patients with irritable bowel syndrome (26). The purpose of this *in silico* study was to analyze the role of phloroglucinol compound in RANK-RANKL signaling pathways and in the activation of the transcription factors NRF2 and NF $\kappa$ B.

## 2. MATERIAL AND METHODS

### 2.1. Nucleotide sequence and protein structure retrieval

Phloroglucinol component structure (C<sub>6</sub>H<sub>6</sub>O<sub>3</sub>, CID: 359) was obtained from PubChem Open Chemistry Database. Sequences of the proteins NRF2 (GI: 693842), Maf (GI: 223590080), NF $\kappa$ B p65 (GI: 62906901), NF $\kappa$ B p50 (GI: 21542418), IKK $\alpha$  (GI: 317373368), IKK $\beta$  (GI: 14285497), I $\kappa$ B $\alpha$  (GI: 126682), I $\kappa$ B $\beta$  (GI: 57015399), RANK (GI: 19924309), RANKL (GI: 2612922) and OPG (GI: 2072185) were obtained from sequence databases of the National Center for Biotechnology Information (NCBI), the United States National Library of Medicine (NLM), National Institutes of Health (NIH) (<http://www.ncbi.nlm.nih.gov>).

### 2.2. 3D Structure modeling of DNA and proteins and bioactive components

3D structure modeling of NRF2, Maf, NF $\kappa$ B p65, NF $\kappa$ B p50, IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , RANK, RANKL and OPG is predicted using the SWISS-MODEL web server (26,) with

the homology modeling method. The 3D structures of the proteins were then validated by using Ramachandran plot analysis. Conversion of \*.sdf file into \*.pdb file of phloroglucinol was performed by using the OpenBabel software (29).

### 2.3. Computational docking

Docking simulation of RANK-RANKL, RANK-OPG, Maf-NRF2, Maf NRF2-promoter region of GSH reductase and NF $\kappa$ B-IKK was performed using the software HEX 8.0 (30) and the web server High Ambiguity Driven Biomolecular Docking (Haddock) (31). Docking protocol consists of three phases of visualization: rigid-body energy minimization, semi-flexible refinement and finishing refinement in explicit solvent. Upon the completion of each phase, docking conformations were then scored and sorted based the scoring function to facilitate the selection of the best conformation to be used at the next phases.

### 2.4. Analysis of Inter-Protein interactions

Results of docking analysis would then be visualized using the software LigPlot+ (32) and LigandScout 3.1 (33). Analysis of interactions between a protein with another protein was carried out to determine the bonds formed, including hydrogen bonding, hydrophobic bonding and van der Waals bonding. Additionally, pharmacophore analysis was performed to determine the residues directly involved during the interaction. Furthermore, energy-minimization analysis was carried out to improve the structure and shape of the molecules during the interaction.

## 3. RESULTS

### 3.1. Phloroglucinol inhibits RANKL-RANK interaction

Receptor activator of nuclear factor- $\kappa$ B (RANK) is a transmembrane protein which is a receptor of RANKL. The RANKL-RANK bond will stimulate the formation and activity of osteoclasts, one of which being through the NF $\kappa$ B signaling pathway. Results of the docking analysis showed that the normal energy for RANKL-RANK bonding was -763.32 kJ/mol. The presence of phloroglucinol, which is an antioxidant compound, was thought to inhibit RANKL-RANK interaction as seen from the higher energy required (-728.22 kJ/mol) for interacting. Despite the change in binding energy between the normal condition without phloroglucinol and the condition with the presence of phloroglucinol, analysis of interaction position showed that there is no change in the position of RANKL-RANK interaction. RANK amino acids directly interacting with phloroglucinol were Asp148, Thr149, Gln144 and Leu i43 with a hydrophobic bond. In conclusion, phloroglucinol caused changes in binding energy but did not cause a change in the position of the interaction.

### 3.2. Phloroglucinol sustains RANKL-OPG interaction

Osteoprotegerin (OPG) is a decoy receptor for RANKL. RANKL-OPG interaction would inhibit the formation and activity of osteoclasts since it blocks RANKL-RANK interaction. In normal conditions, the energy required for RANKL-OPG interaction is -67.13 kJ/mol. The presence of phloroglucinol would reduce the energy required for

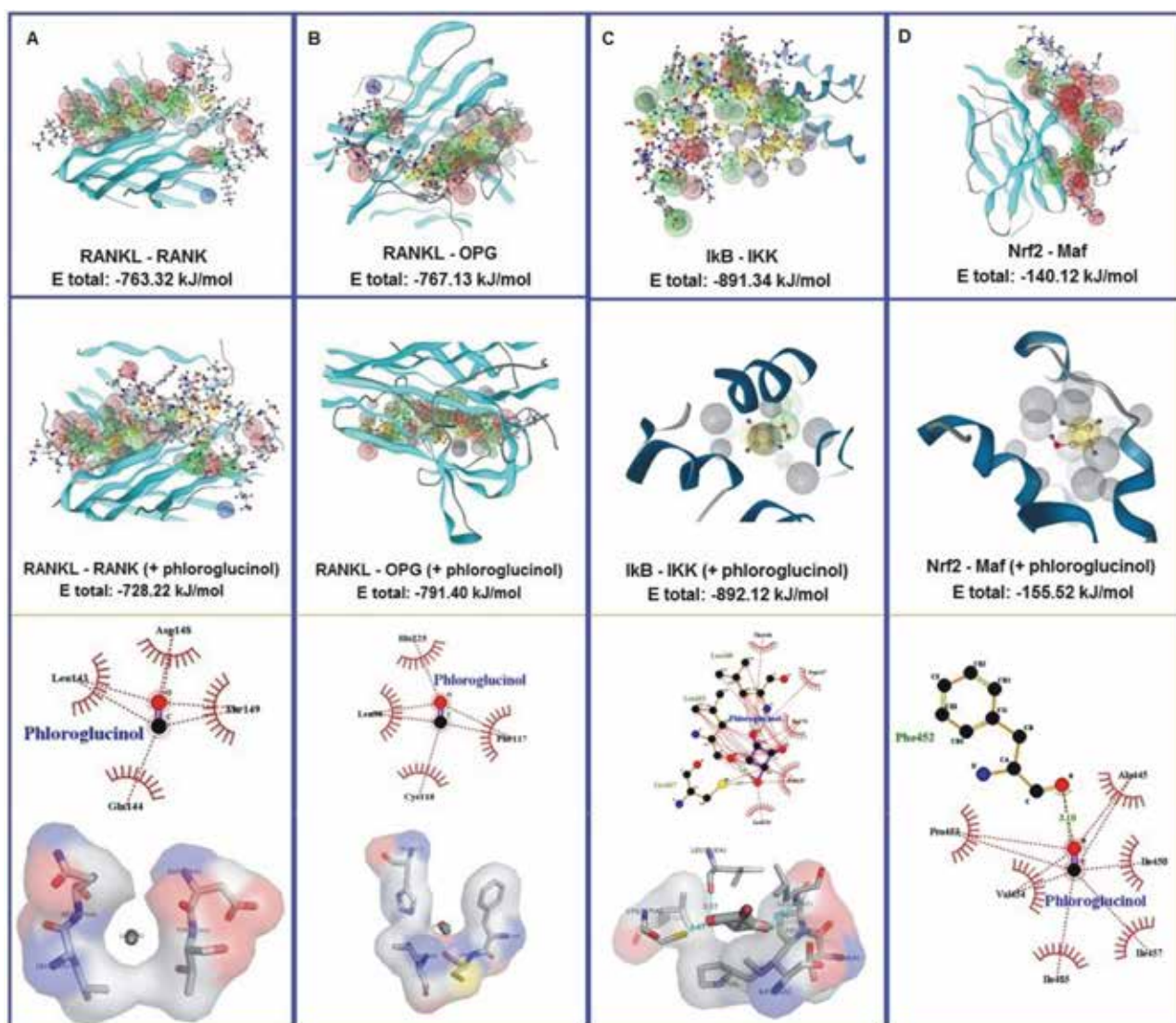


Figure 1. Bonding energies and interactions formed between phloroglucinol and various receptor proteins, including RANKL-RANK (A), RANKL-OPG (B), IκB-IKK (C), and Nrf2-Maf (D).

RANKL-OPG interaction (-791.40 kJ/mol); thus, it was suspected that the presence of phloroglucinol would sustain OPG-RANKL interaction. It was found that the amino acid residues directly interacting with phloroglucinol were His225, Leu 90, Cys118 and Phe117,

### 3.3. Phloroglucinol does not affect the activity of NFκB signal transduction

Activation of the transcription factor NFκB is one of the downstream signaling pathways of RANKL-RANK interaction. The purpose of the study was to determine the role of phloroglucinol in the classical NFκB signaling pathway, which involves a complex activity of IκB kinase (IKK) in phosphorylating the inhibitor of NFκB (IκB), leading to ubiquitin-dependent degradation of IκB and NFκB translocation to the nucleus. Analysis showed that the presence of phloroglucinol did not affect the activity of IKK in the degradation of IκB. IKK-IκB binding energy showed no significant difference between the presence and absence of phloroglucinol (-891.34 kJ/mol and -892.12 kJ/mol, respectively). This raised a presumption that phloroglucinol only inhibited osteoclastogenesis extracellularly through the inhibition of

RANKL-RANK interaction, but in the intracellular environment, phloroglucinol did not have the ability to inhibit the activity of NFκB.

### 3.4. Phloroglucinol sustains the activity of transcription factor NRF2

Further analysis was performed to determine the role and activity of phloroglucinol in the intracellular environment. Nuclear factor E2 p45-related factor 2 (NRF2) is a transcription factor that regulates the expression of various type-2 detoxification genes. In addition, NRF2 is responsible for the regulation of differentiation and function of osteoblasts. When entering the nucleus, NRF2 binds to the protein Maf prior to binding to the promoter region of the target gene. *In silico* analysis showed that NRF2 bound to phloroglucinol was expected to more easily bind to Maf. It was shown by the reduction of energy needed for the interaction of the two proteins. This supported the presumed function of phloroglucinol compounds as an antioxidant, in which the compounds would sustain the production of antioxidant enzymes through increased NRF2 activity in the nucleus, as well as sustaining its functions in the differentiation of osteoblasts.

#### 4. DISCUSSION

The main purpose of the present study was to determine the role of phloroglucinol as an antioxidant compound and its contribution to osteoclastogenesis *in silico*. Results raised a speculation that phloroglucinol is capable of inhibiting osteoclastogenesis via inhibition of RANKL–RANK interaction and sustaining RANKL–OPG interaction. The bond of RANKL and its receptor RANK direct the activation of various NFκB–MAPK signaling pathways, including JNK, ERK and p38, which are found to play an important role in osteoclastogenesis (8, 34). NFκB and MAPK signaling pathways are found to be sensitive to ROS in response to various stimuli. Presence of antioxidant compounds is found to inhibit these signaling pathways in osteoblast precursors stimulated by RANKL (22, 23). Results of this study indicated that the antioxidant compound phloroglucinol as antioxidant compound could binding to IκB molecules via interactions with the residues Cys167 (3.07 Å), Leu163 (3.07 Å) and Leu148 (2.74 Å). However, results of the analysis of binding energy indicated that the presence of phloroglucinol did not affect the binding and phosphorylation of IKK molecules in IκB, since there was no change in binding energy required to allow for interaction.

Another finding of this study was that phloroglucinol could increase the activity of the transcription factor Nrf2 via its bond with the co-protein of the transcription factor Maf; thus it was expected to increase the production of antioxidant enzymes. Previous studies reported that Nrf2 signaling pathway has a crucial role in the regulation of RANKL-mediated osteoclastogenesis by controlling the intracellular ROS levels and regulating the expression of cytoprotective enzymes (35). There was a reduction in the ratio of Nrf2/Keap1 during osteoclastogenesis, leading to down-regulation of transcription of cytoprotective enzymes. Another study also reported an increase in ROS levels during RANKL-mediated osteoclastogenesis (25, 36). Furthermore, it was found that an increase in Nrf2 activity may reduce osteoclastogenesis, whereas a decrease in Nrf2 activity may induce osteoclastogenesis.

It was thought that the increase in Nrf2 activity due to the presence of phloroglucinol was mediated by the bonds of Nrf2 and Maf protein. Maf protein and Nrf2 will form heterodimers to induce ARE-dependent expression of the genes (26). However, another studies reported that when a Maf protein forms homodimers or heterodimers with other Maf protein, it would suppress Nrf2 transcription activity through a competitive binding with Nrf2 in the ARE binding site (38, 39).

These results of *in silico* analysis were predictive in nature; thus, further studies with experimental methods are needed to test the hypotheses generated by this study.

**CONFLICT OF INTEREST: NONE DECLARED.**

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