Antioxidative Activity and Anti-inflammatory Effects on the Murine Macrophages of Methanol Extracts of Amphibians

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Abstract - Oxidative stress has been reported to be one of causes of neuritis. This study examined antioxidative activities of methanol extracts of six amphibian species known to be medicinal animals (*Rana catesbeiana, R. coreana, R. rugosa, R. dybowskii, R. nigromaculata,* and *Hyla japonica*) and investigated their effects of inhibiting nitric oxide (NO) production and cytotoxicity on the murine macrophage RAW264.7 cells. As inflammation is closely associated with reactive oxygen species, assays on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, xanthine oxidase inhibitory activity, superoxide anion radical scavenging activity and NO scavenging activity of the extracts of the six species were performed to investigate their antioxidative activity. The results obtained were as follows; All extracts showed antioxidative activity, and the activity of *R. dybowskii* was the highest in comparison among those. Anti-inflammatory effects of the extracts were also examined, the five extracts except that of *R. rugosa* did not show cytotoxicity for RAW264.7 cells at the maximal concentration (1,000 μg mL^{-1}). Selectivity index, meaning NO scavenging activity compared to cytotoxicity, showed the highest level in the extract of *R. dybowskii*. These results will be very useful basic data for future studies on prevention and treatment of human diseases to understand the biological roles of amphibian extracts throughout the antioxidative or anti-inflammatory pathways.

Key words : antioxidative activity, cytotoxicity, anti-inflammatory effect, Korean amphibians

INTRODUCTION

For useful biological resources, the interest in their use and the researches for them have focused on plants such as natural medicine and the interest in use and development of useful animals have been very low around the world. However, diversity and usefulness of animals is currently rethought and development of technologies in the agricultural and biological industries leads to confirmation of high possibility of using amphibians as a biological resource (Erspamer *et al.* 1986; Rinaldi 2002; Lu *et al.* 2008; Gomes *et al.* 2011; Wang *et al.* 2012). The use as a biological resource emphasizes the importance of gene pool and the use for productive, medical and dietary purposes. In particular, the use of amphibians for a medical purpose has been well known in both of Oriental and Western countries and in Korea it also has been utilized as a medicinal animal in traditional oriental medicine and fork remedies (Mor *et al.* 1994; Batista *et al.* 1999; Je *et al.* 2007; Qian *et al.* 2008; Cho *et al.* 2009; Jin *et al.* 2009). However, its effects and usage as a material for traditional oriental medicine shown in references and antient books were induced from experiences, fork remedies and

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traditional oriental medicine without a scientific analysis (Park and Lee 1998; Park *et al.* 2005).

Amphibians are usually called as 'wa' in traditional oriental medicine because it croaks well. In [Donguibogam] its cold characteristic was reported to control fissure and foods of children and in [Pen-Tsao-Kang-Mu] it was written to relieve diarrhea and pathology of fever. In traditional oriental medicine, it is used for nephropathy, diuresis, nutrition and flatulence, dried powder in warm honey water or boiled amphibian bodies by itself is eaten as a special efficient medicine of pulmonary tuberculosis, roborant and asthma. This study was conducted to provide necessary data for researches on antioxidants and anti-inflammatory materials and on their isolation and mechanism to prevent and cure diseases by investigating biological activity such as antioxidant activity and anti-inflammatory effect of methanol (MeOH) extracts of six amphibian species collected in Korea, which has been rarely studied and has been reported to be used as a fork remedy.

MATERIALS AND METHODS

1. Animals and preparation of extracts

Six amphibian species (*R. catesbeiana, R. coreana, R. rugosa, R. dybowskii, R. nigromaculata,* and *H. japonica*) were used for this study obtained from American Bullfrog Capture Operation Division (Jeongeup, Jeollabuk-do, Republic of Korea) and Frog Village (Muju, Jeollabuk-do, Republic of Korea). After lyophilization of animal specimens, samples were subsequently grinded, deposited on 500 mL 80% MeOH, and extracted three times by using a sonicator. And then, the supernatant was isolated and evaporated and after frozen drying it was used with diluted with 100 mg mL^{-1} 1 : 1 ethanol (EtOH) : phosphate-buffered saline (PBS) solution.

2. Cell culture

Murine macrophage cell line RAW264.7 was obtained from Korean Cell Line Bank (Seoul, Republic of Korea) and was incubated at 37°C with 5% CO_2 conditions using Dulbecco's modified Eagle's medium (DMEM) including 100 units mL^{-1} penicillin-streptomycin and 10% fetal bovine serum (FBS). Subcultures were conducted every 3 ~ 4 days.

3. DPPH radical scavenging activity assay

To examine antioxidant activity of each sample, the Blois (1958) method measuring radical scavenging effect with DPPH (Sigma, USA) was used. DPPH solution was made by dissolving around 2 mg DPPH in 15 mL EtOH. After adding 6.25 mL dimethylsulfoxide (DMSO) to 12 mL of the solution, it was diluted with EtOH for absorbance of the control to be 0.94 ~ 0.97 at 517 nm wavelength and was shaken for 10 sec. In addition, 100 μL samples of each concentration dissolved in MeOH were put on 96 well plate and a same amount of 0.4 mM DPPH was added. After 10 min incubation at room temperature, absorbance was measured at 517 nm.

4. Xanthine oxidase inhibitory activity assay

The production of uric acid caused by xanthine/xanthine oxidase was measured with the increased absorbance at 290 nm (Cheng *et al.* 1998) and allopurinol (Sigma, USA) was used as the control. For the mixture, each samples of various concentrations, 0.5 mM xanthine and 1 mM EDTA were prepared in 200 mM phosphate buffer (pH 7.5) and 50 units μL^{-1} xanthine oxidase was added to induce production of uric acid. Xanthine oxidase inhibitory activity was presented with the decreased rate of absorbance of the produced uric acid.

5. Superoxide anion scavenging activity assay

The amount of superoxide anion formed by using phenazine methosulfate (PMS)/NADH system was measured at 517 nm with nitroblue tetrazolium reduction method (Fridovich 1970; Nishikimi *et al.* 1972; Liu *et al.* 1997). The mixture was prepared with each sample, 125 μM NADH and 63 μM NBT in 200 μL PBS (pH 8.4) and 8 μM PMS was added to provoke production of superoxide. Superoxide anion scavenging activity was shown with the decreased rate of absorbance of the produced superoxide.

6. NO scavenging activity assay

NO scavenging activity was analyzed by using sodium nitroprusside (SNP) forming naturally NO (Green *et al.* 1982; Marcocci *et al.* 1994). Each samples of various concentrations were added to 10 mM SNP and was incubated at 25°C for 3 hr. After the reaction, Griess solution [1% (w/v) sul-

fanilamide, 0.1% N-1-naphylethylen diamine in 2.5% (v/v) phosphoric acid] of a same amount with the mixture was added. It was at room temperature for 10 min and its absorbance was measured at 540 nm. NO scavenging activity was calculated with the amount of residual nitrite. The activity was presented with % of the scavenging activity at 500 μg mL^{-1}.

7. Cytotoxicity assay

RAW264.7 cells were put into 96 well microplates with 2×10^5 cells $well^{-1}$ by using the DMEM and were incubated for 18 hr. After each samples of different concentrations and 100 ng mL^{-1} lipopolysaccharide (LPS) (Sigma, USA) were added and incubated for 24 hr. 2 mg mL^{-1} 3-(4,5-dimehtylthiazol)-2,5-diphenyl-tetrazolium bromide (MTT) was added and was incubated for 1 hr and the media was removed. After the formazan sediment produced by reduction of MTT by adding 200 μL DMSO was dissolved and absorbance was measured at 540 nm with a microplate reader (Biotek, USA). By comparing the absorbance of each sample at each concentration with that of the untreated sample, cytotoxicity of the sample to RAW264.7 cells was evaluated.

8. NO production inhibition assay

RAW264.7 cells (2×10^5 cells $well^{-1}$) were put into 96 well plates and each samples of various concentrations were treated. After adding LPS (100 ng mL^{-1}), it was incubated for 24 hr. After mixing 100 μL supernatant of the media with 100 μL Griess solution they were incubated on 96 well plates for 10 min and absorbance was measured at 530 nm. The amount of NO was compared with that of sodium nitrite as a standard.

9. Statistical analysis

The results were presented with a mean and standard deviation and a statistical significance was analyzed with Student's t-test.

RESULTS AND DISCUSSION

1. Antioxidative activity of amphibian extracts

The antioxidant activities of MeOH extracts of *R. catesbeiana, R. coreana, R. rugosa, R. dybowskii, R. nigromaculata,* and *H. japonica* were presented in Table 1 and Fig. 1. DPPH free radical scavenging activity was increased with depending on the treated concentrations (Fig. 1A) and the IC_{50} values showing a concentration with 50% scavenging activity of each sample was the lowest in *R. dybowskii* by recording 1,570 μg mL^{-1} and the highest in *R. catesbeiana* (Table 1). Xanthine oxidase inhibitory activity of the amphibian MeOH extracts was measured with xanthine/xanthine oxidase system. Xanthine oxidase inhibitory effect at 625 μg mL^{-1} showed the highest level, 95.3% in *R. dybowskii* and the lowest level, 53.3% in *R. rugosa* (Table 1, Fig. 1).

Superoxide anion radical scavenging activity of the extracts showed low levels in all of the samples by recording less than 5% (Table 1). NO is an active species with a strong cytotoxicity and the production of much NO provokes indirect effects including nitrosation and nitration and oxidation to induce harmful effects. NO scavenging activity of the MeOH extracts of the six species of Amphibia was measured with the amount of nitrite by using SNP forming NO. At 2.5 mg mL^{-1} each sample showed 48 ~ 15% scavenging activity

Table 1. Comparison of antioxidative potential of MeOH extracts of amphibians

Sample	IC_{50} (μg mL^{-1})*			
	DPPH radical scavenging activity	Xanthine oxidase inhibitory activity**	Superoxide anion scavenging activity	NO scavenging activity***
R. catesbeiana	> 5000	71.2 ± 6.9	−	20.2 ± 2.2
R. coreana	1895 ± 31.2	81.9 ± 1.7	−	26.7 ± 0.1
R. rugosa	1763 ± 80.4	53.3 ± 9.3	−	15.6 ± 5.0
R. dybowskii	1570 ± 0.3	95.3 ± 10.3	−	48.1 ± 5.0
H. japonica	2687 ± 67.4	72.0 ± 4.9	−	35.1 ± 1.2
R. nigromaculata	2399 ± 74.6	65.3 ± 11.0	−	42.5 ± 0.4

*IC_{50} values were calculated from regression lines using seven different concentration in triplicate experiments.
**1.25 mg mL^{-1} scavenging activity (% control).
***2.5 mg mL^{-1} scavenging activity (% control).
− indicates < 5% radical scavenging activity in maximum concentration.

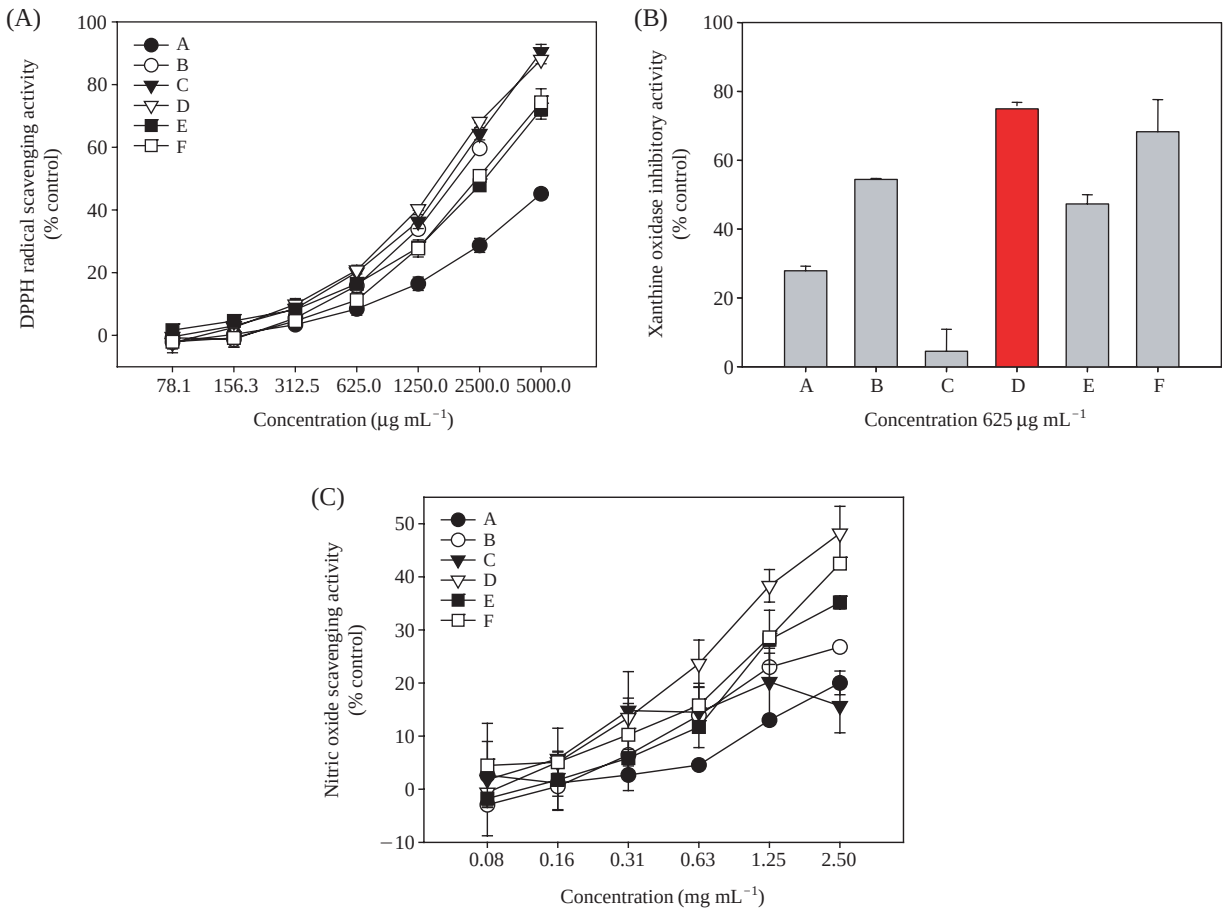

Fig. 1. Dose-dependent scavenging effects on DPPH radical, NO and xanthine oxidase inhibitory activity by amphibian extracts. The data indicate the mean±S.D. of triplicate experiments. A: *R. catesbeiana*; B: *R. coreana*; C: *R. rugosa*; D: *R. dybowskii*; E: *H. japonica*; F: *R. nigromaculata*.

and the scavenging effect of *R. dybowskii*, *R. nigromaculata* and *H. japonica* was found to be good by recording 48.1, 42.5 and 35.1%, respectively while that of *R. coreana*, *R. catesbeiana* and *R. rugosa* was relatively low by recording 26.7, 20.2 and 15.6%, respectively. Like previous studies on antioxidant activity of medicinal insect extracts revealing that the extracts of *Anomala albopilosa*, *Sympetrum eroticum* and *Anax parthenope* had high antioxidant activity (Kim *et al.* 2004; Yoon *et al.* 2007), this study also found that amphibians produced a superior antioxidant activity.

2. Anti-inflammatory activity of amphibian extracts

For vertebrates, infection and damage of capillary vessels of skin tissues provoke proliferation of synovial cells and increase of fibroblasts and macrophages along with saturation of lymphocytes inside of connective tissues. Interlukin secreted from the lymphocytes by these changes, actives effector lymphocytes and promotes various enzyme such as hyaluronidase, elastase and collagenase and inflammatory mediators like prostaglandin to trigger inflammation destroying connective tissues (Deby 1988; Shimizu and Wolfe 1990).

NO is a free radial produced from L-arginine with nitric oxide synthases (NOSs) (Palmer *et al.* 1988) and it shows many biological functions including body defence, signal transduction and a function as a secondary messenger of vasodilation (Monacada *et al.* 1991; Knowles and Mocada 1992; Nathan 1992). Constitutive NOS (cNOS) contains neuronal- and endothelial-NOS expressed in neurons and endothelial cells, respectively. The production of NO caused by cNOSs play a key role in managing homeostasis of a body (Kawamata *et al.* 2000). However, contrary to cNOSs, inducible NOS (iNOS) is presented on macrophages, vascular

smooth muscle cells, endothelial cells, hepatocytes and myocardial cells by following the stimuli of LPS, interferon-g, interleukin (IL)-1b and tumor necrosis factor (TNF)-α (Lee *et al.* 2000). The iNOS expressed on these tissues leads to inflammation, tumor (Nathan 1992), damage of tissues, gene variation and damage of nerves by producing much NO for a long time (Stuehr *et al.* 1991; Weisz *et al.* 1996). Like this, inflammation-related NO and prostaglandin E_2 are formed by iNOS and cyclooxygenase-2.

Macrophage is known to be related with homeostasis by affecting many host responses such as acquired immunity as well as innate immunity and it is very critical for body defense in the early infection by making NO and cytokine during inflammatory reaction (Higuchi *et al.* 1990). LPS existing on the outer membrane of gram positive bacteremia is reported to be an endotoxin and trigger sepsis and shock with

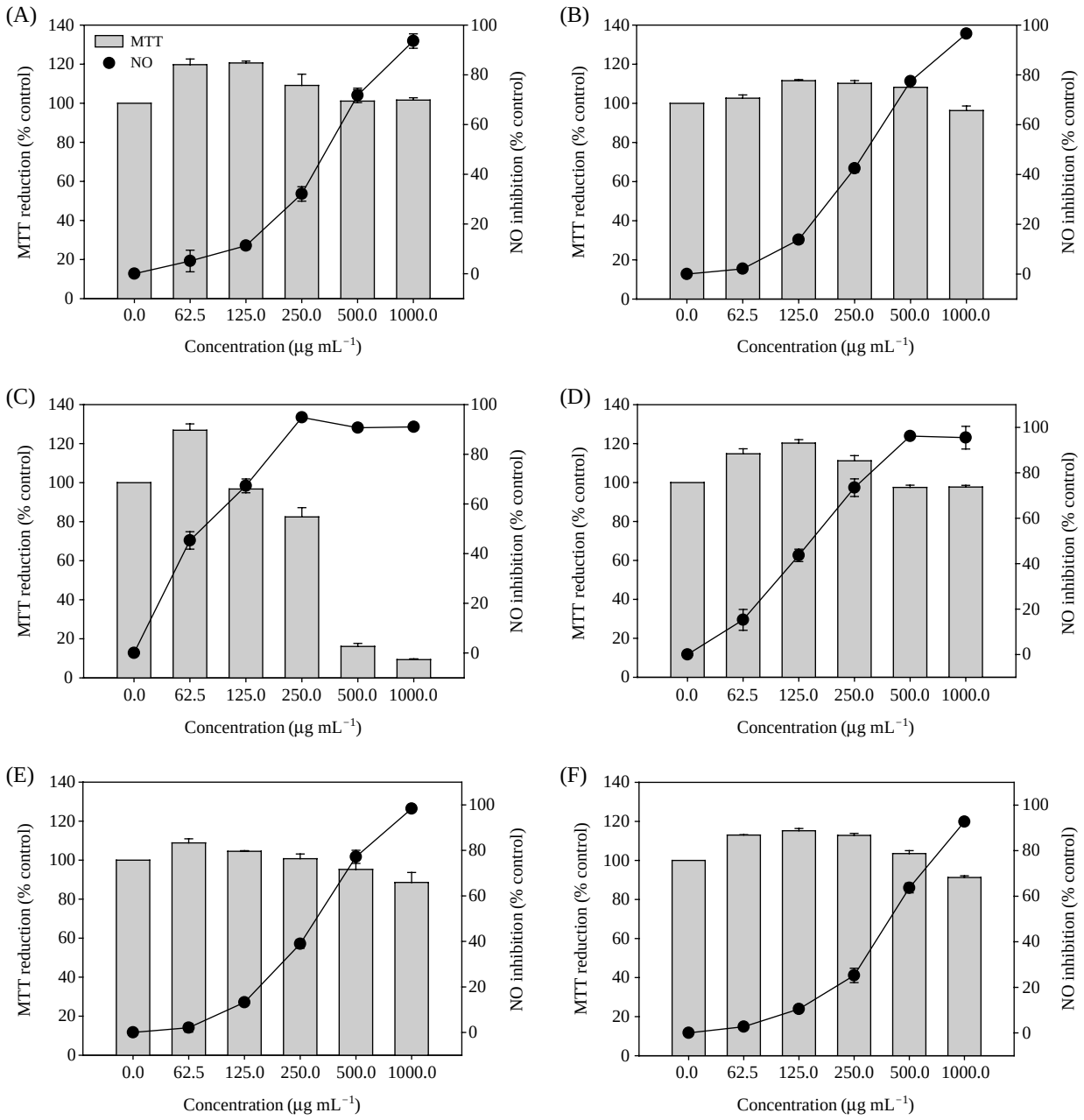

Fig. 2. Effects of MeOH extracts on the NO production in LPS-stimulated RAW264.7 cells. Cells were treated with LPS (100 ng mL^{-1}) alone or LPS plus the indicated concentrations of MeOH extracts for 24 hr. A: *R. catesbeiana*; B: *R. coreana*; C: *R. rugosa*; D: *R. dybowskii*; E: *H. japonica*; F: *R. nigromaculata*.

Table 2. Cell toxicity and the effects on LPS-induced NO production of the MeOH extracts in RAW264.7 cells

Sample	TC_{50}* (mg mL^{-1})	IC_{50}** (mg mL^{-1})	Selectivity index***
R. catesbeiana	＞1,000	341.8±0.1	2.92～
R. coreana	＞1,000	288.9±7.6	3.46～
R. rugosa	333.6±7.6	71.9±5.9	4.63
R. dybowskii	＞1,000	143.6±10.1	6.96～
H. japonica	＞1,000	303.5±14.5	3.29～
R. nigromaculata	＞1,000	397.8±20.0	2.51～

*TC_{50} is the concentration producing 50% toxicity in RAW264.7 cells.
**IC_{50} is the concentration producing 50% inhibition of NO production in RAW264.7 cells.
***Selectivity Index=TC_{50}/IC_{50}.

released from the outer membrane when bacteria die. LPS increases synthesis of pro-inflammatory cytokine such as TNF-α, IL-1b and IL-6 on macrophages like RAW264.7 cells or mononuclear cells, and particularly TNF-α and IL-1b induce expression of iNOS (Higuchi *et al.* 1990; Willeaume *et al.* 1995; McDaniel *et al.* 1996).

According to the results, MeOH extracts of *R. catesbeiana, R. coreana, R. dybowskii, R. nigromaculata,* and *H. japonica* did not show cytotoxicity to the maximal concentration, 1,000 μg mL^{-1} and TC_{50} of *R. rugosa* showing 50% cytotoxicity was observed to be 333.6 μg mL^{-1}. Selectivity index meaning NO scavenging activity compared to cytotoxicity was the highest in *R. dybowskii* by recording 6.96 and those of *R. rugosa, R. coreana, R. catesbeiana, R. nigromaculata* and *H. japonica* recorded 4.63, 3.46, 3.29, 2.92 and 2.51, respectively (Table 2, Fig. 2).

In conclusion, all MeOH extracts of the six species had antioxidant activity, and in comparison among those the activity of *R. dybowskii* was found to be the best by considering DPPH radical scavenging activity, xanthine oxidase inhibitory activity and NO scavenging activity. In addition, as *R. dybowskii* did not trigger cytotoxicity to 1,000 μg mL^{-1} and its selectivity index was also the highest (6.96) and its efficiency was observed to be the highest among the extracts. These results of this study are considered to be important basic data for studies on antioxidants and anti-inflammatory components through extraction of active ingredients from amphibians and on isolation and mechanism of active components to prevent or to treat diseases. In the future, *in vitro* and *in vivo* biological functions of sequential fractions of amphibians are needed to be examined and additional researches on possibility of an industrial use of EtOH or spirit extracts are also considered to be necessary.

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