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

Sang-Bum Kim, Min-Ho Chang¹, Sang-Hyun Han² and Hong-Shik Oh²,*

Ohyun Middle School, Jeju 690-061, Korea
¹National Park Research Institute, Korea National Park Service, Namwon 590-811, Korea
²Department of Science Education, Jeju National University, Jeju 690-756, Korea

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...
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, 100μL 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-