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Full Paper

Effects of *Terminalia bellerica* Roxb. methanolic extract on mouse immune response in vitro

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Abstract: In this study, the effects of *Terminalia bellerica* methanolic extract (0.1, 1, 10, 100 and 500 μg/ml) on the mouse immune system were investigated in vitro. Phagocytic activity and lymphocyte proliferation were assayed. The results indicated the effect of the extract (500 μg/ml) on the stimulation of macrophage phagocytosis, through the production of superoxide anions and acid phosphatase, with a phagocytic index (PI) value of approximately 1.5 and 1.3, respectively. For the lymphocyte proliferation assay, the extract (500 μg/ml) with phytohemagglutinin exhibited maximal activation, with a stimulation index (SI) value of approximately 5.8. With concanavalin A, lipopolysaccharide, and pokeweed mitogen, similar activation (SI 4.5) of lymphocyte proliferation was observed. However, at low concentrations (0.1 μg/ml), *T. bellerica* extract with concanavalin A and pokeweed mitogen caused suppressant activity (SI 0.7). The results suggested that the effect of extract on T-lymphocyte proliferation occurred through the same mechanism as phytohemagglutinin, concanavalin A and B-lymphocyte proliferation through T-cell independent and T-cell dependent mechanisms, in manners similar to lipopolysaccharide and pokeweed mitogen respectively. It might be concluded that the methanolic extract of *T. bellerica* affected the mouse immune system, specifically both the cellular and humoral immune response in vitro, corresponding with its folklore applications. These results can be further applied to the treatment of human immune mediated diseases.

Keywords: *Terminalia bellerica* Roxb, in vitro immune response, lymphocytes, proliferation, macrophages, phagocytosis

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Introduction

Terminalia bellerica Roxb (Combretaceae) is a perennial herb mainly distributed in the tropical regions and commonly found in South-East Asia, including Thailand. It is one of the ingredients of "tripala", an Ayurvedic formulation that is believed to promote health, immunity and longevity [1]. This formulation, rich in antioxidants, is frequently used in Ayurvedic medicine to treat many diseases such as anemia, jaundice, constipation, asthma, fever and chronic ulcers [2]. The fruit of *T. bellerica* has been used to treat various ailments in the folklore medicine [3]. Antibacterial [4], antidiabetic, and antioxidant [5] activities of crude extracts of *T. bellerica* have been reported. The fruit is also reported to have purgative [6], cardiac depressant, hypotensive and chleretic effects [7]. Chemically, the presence of β-sitosterol, gallic acid, ellagic acid, ethyl gallate, chebulagic acid, mannitol, glucose, galactose, rhamnose, and fructose have been reported in the fruit of *T. bellerica* [8-9]. To investigate the immunomodulatory effects of the *T. bellerica* extract in vitro, phagocytic activity of mouse peritoneal macrophages on nitroblue tetrazolium dye reduction and lysosomal enzyme activity and proliferation of murine splenic lymphocytes using the MTT assay were assayed.

Materials and Methods

Plant material

Dried fruits of *T. bellerica* were collected in February 2007 and were authenticated by the botanist at the Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand. A specimen was prepared and deposited at the herbarium of Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand.

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nitroblue tetrazolium (NBT) dye, *p*-nitrophenyl phosphate (*p*-NPP), phytohemagglutinin (PHA), concanavalin A (Con A), lipopolysaccharide (LPS), pokeweed mitogen (PWM), dimethyl sulfoxide (DMSO), phorbol-12-myristate-13-acetate (PMA), zymosan A, and antibiotic-antimycotic solution (100 U penicillin, 100 μg streptomycin, and 0. 25 μg/ml amphotericin B) were purchased from Sigma-Aldrich (Germany). β – Mercaptoethanol and Triton-X were purchased from Fisher Scientific (UK). Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from GIBCO/BRL Invitrogen (Scotland).

Animals

Female ICR mice (5-6 weeks old) were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The animals were housed under standard conditions at 25±2 °C and fed standard pellets and tap water. The experiments were conducted under the surveillance of the Ethics Committee of Naresuan University, Thailand.

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Preparation of extracts

The dried fruits of *T. bellerica* were extracted by maceration in methyl alcohol for 24 h and then filtered. The filtrate was evaporated under reduced pressure until dry, and a yield of 31% (w/w of dried material) of the extract was obtained. To prepare extract concentrations of 0.1, 1, 10, 100 and 500 μg/ml, the extract was dissolved in 0.1% DMSO in phosphate buffer saline (PBS) solution. Insoluble material was removed by centrifugation and the extract solution was sterilised by passage through a 0.2 μm filter. 0.1% DMSO in PBS was used as control in all experiments.

Preparation of peritoneal mouse macrophages

Peritoneal macrophages were isolated following intraperitoneal injection of FBS as a stimulant [10]. Three days later, the peritoneal exudate was collected by peritoneal lavage with RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 100 U penicillin, 100 μg streptomycin and 0.25 μg/ml amphotericin B (complete RPMI; CRPMI). The exudate was centrifuged at 2000 rpm at 25 °C for 10 min, and the cells were washed twice and re-suspended in CRPMI medium. The cell number was adjusted to 1×10^6 cells/ml, and cell viability was tested by the trypan-blue dye exclusion technique.

Preparation of mouse splenocytes

Mice were sacrificed, and the spleens were removed aseptically. Single cells were prepared by mincing spleen fragments and pressing through a stainless 200-mesh screen in CRPMI medium. After centrifugation at 2000 rpm at 25 °C for 10 min, the cells were washed twice and re-suspended in CRPMI medium. The cell number was adjusted to 1×10^6 cells/ml, and cell viability was tested by the trypan-blue dye exclusion technique.

Nitroblue tetrazolium (NBT) dye reduction assay

The NBT dye reduction assay was carried out as previously described [11]. Macrophages (1×10^5 cells/well) were treated with the extract for 24 h at 37 °C in a 5% CO_2 humidified incubator. Cells were incubated with zymosan A (5×10^6 particles/well) and 3 mg/ml NBT dye. After incubation for 60 min, the adherent cells were rinsed vigorously with RPMI medium and washed four times with methanol. After air drying, 2 M KOH and DMSO were added and the absorbance was measured at 570 nm using a microplate reader (Bio-Tek Instrument Inc., USA). The phagocytic index (PI) was determined by the ratio of optical density of the test sample to that of the control.

Cellular lysosomal enzyme activity assay

The cellular lysosomal enzyme activity was used to determine the acid phosphatase activity in the phagocytes as previously described [12]. Macrophages (1×10^5 cells/well) were treated with the extract for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. The medium was removed by aspiration, and 0.1% Triton X-100, 10 mM *p*-NPP solution and 0.1 M citrate buffer (pH 5.0) were added to each well. The cells were further incubated for 30 min, and 0.2 M borate buffer (pH 9.8) was then added. The absorbance was measured at 405 nm using a microplate reader. The PI value was calculated as in the NBT dye reduction assay.

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Mitogen-induced splenocyte proliferation assay

The MTT technique was used to detect lymphocyte proliferation as previously described [13]. The optimum dose (5 μg/ml) of mitogen (PHA, Con A, LPS and PWM) was used as positive control. Briefly, splenocyte suspensions were treated with extract and mitogen for 48 h at 37 °C in a humidified 5% CO_2 atmosphere. Subsequently, 5 mg/ml MTT was added, and incubation continued for 4 h. The culture medium was removed by aspiration, and 0.04 M HCl in isopropyl alcohol and distilled water was added. The absorbance was measured at 570 nm using a microplate reader. The stimulation index (SI) was defined as the ratio of optical density of the test sample to that of mitogen.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± S.E. Statistical significance was analysed using the Student's *t*-test. P values less than 0.05 and 0.01 were considered significant.

Results and Discussion

Immune activation is an effective and protective approach against emerging infectious diseases [14], and alternative medicine is becoming more popular for the treatment of these illnesses. *T. bellerica* has been used as a traditional remedy in Thailand [3] and various pharmacological activities, such as antioxidant [5], antibacterial [4], anti-HIV, antimalarial, and antifungal activities [15] have been reported. Among the array of medicinal properties attributed to it, a significant one is its therapeutic immunomodulating activity. Thus, this in vitro study was undertaken to determine whether an extract of *T. bellerica* fruit has any immunomodulatory activity.

It is well known that macrophages play a significant role in the defense mechanism against host infection and proliferation of tumour cells. The modulation of macrophage antitumour properties by various biological response modifiers is an area of interest for cancer chemotherapy [16].

As shown in Figure 1, macrophages treated with 500 μg/ml of *T. bellerica* extract stimulated NBT dye reduction, with the maximum PI value of about 1.5. After treatment, lysosomal activity was enhanced, with the PI value being approximately 1.3 compared to the control. The results indicated that the extract not only activated oxidative burst reduction, but also stimulated acid phosphatase production.

Although the phagocytic activity of the *T. bellerica* extract has never been reported, the superoxide anion production response of gallic acid, an active component isolated from *T. bellerica* extract, has been discussed. Previous reports of a slight increase of reactive oxygen species (ROS) production in macrophage RAW 264.7 cells in response to gallic acid [17] were consistent with our results. However, the suppressant effect on macrophage chemiluminescence [18] and the inhibition of superoxide scavenging of gallic acid [5] were also presented. Therefore, the compounds responsible for phagocytic activity may be either gallic acid or other phytochemicals present in *T. bellerica*.

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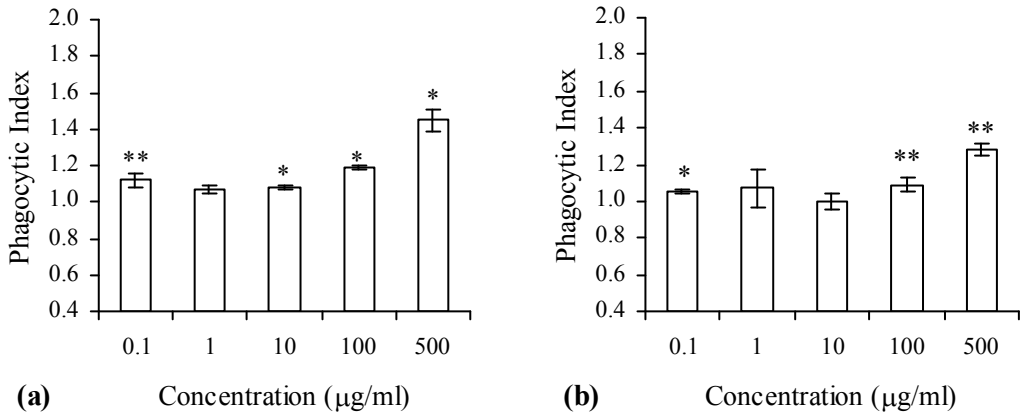

Figure 1. Effects of *T. bellerica* methanolic extract on in vitro phagocytosis response of mouse macrophages: (a) nitroblue tetrazolium (NBT) dye reduction, (b) lysosomal enzyme activity. Each value represents the mean ± S.E. of triplicate experiments, compared to the control. (**P*<0.01, ***P*<0.05)

The release of nitric oxide, superoxide anion, hydrogen peroxide and lysosomal enzyme from activated macrophages is an enzyme-controlled process. So it is possible that the extract may induce some alteration in the mechanism of activation of the related enzyme such as phosphotyrosine phosphatase, which could result in an increase of O_2^- [19]. Moreover, the effect of the extract at high concentrations on the stimulation of phagocytic activity may be due to a significant amount of active ingredients contained in *T. bellerica* extract. These observations suggest a possible application of *T. bellerica* to the treatment of microbial infections and cancer.

Lymphocyte transformation is an in vitro technique commonly used to assess cellular immunity in humans and other animals [20]. In this study, the proliferative response of lymphocytes, in terms of metabolic activity augmented by the *T. bellerica* extract, was assessed by a colorimetric MTT assay. Since the presence of mitogen in the system can affect the possible activation pathway of the extract [21], PHA and Con A were used for T cell activation whereas LPS and PWM were used to stimulate B cell proliferation through T cell-independent and T cell-dependent pathways respectively [10].

In the presence of PHA, the extract at 100 and 500 μg/ml stimulated the lymphocyte proliferation with the maximum SI value of about 5.8 (500 μg/ml), compared to treatment with PHA alone. Treatment with extract and Con A caused a dual response in a dose-dependent manner. The extract at low concentrations (0.1-10 μg/ml) caused suppression of lymphocyte proliferation, with a minimal SI value of about 0.7 (0.1 μg/ml), while the extract at high concentrations (100 and 500 μg/ml) led to activation, with a maximal SI value of 4.5 (500 μg/ml). With LPS, the extract at 100 and 500 μg/ml increased lymphocyte proliferation with a maximal SI value of 4.5 (500 μg/ml).

Moreover, a biphasic dose-dependent response was observed after treatment with the extract and PWM, similar to that observed after treatment with extract and Con A (Figure 2).

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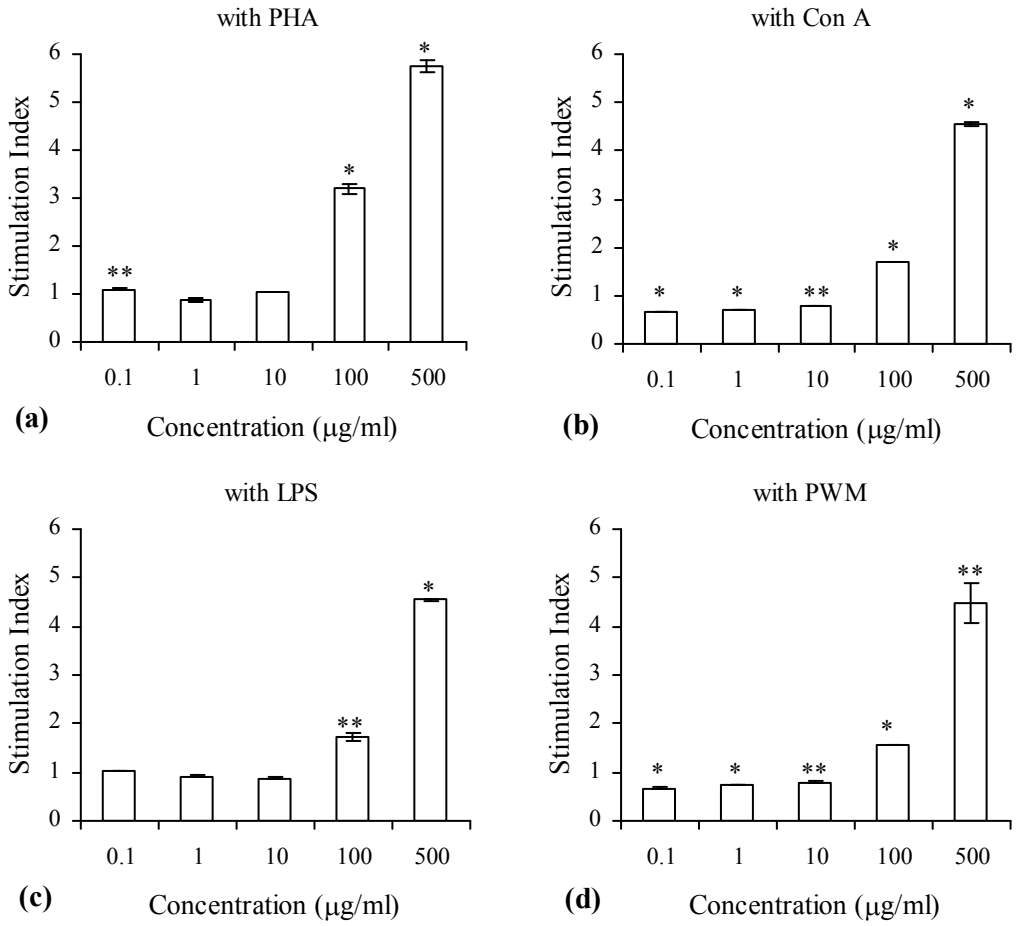

Figure 2. Effects of *T. bellerica* methanolic extract with 5 μg/ml mitogen on the in vitro proliferation response of mouse lymphocytes: (a) with PHA, (b) with Con A, (c) with LPS, (d) with PWM. Each value represents the mean ± S.E. of triplicates, compared to mitogen alone. (**P*<0.01, ***P*<0.05)

Our investigation suggested that the *T. bellerica* extract affected T cell proliferation mainly through the same mechanism as PHA. The extract with LPS and PWM also affected B cell proliferation through T cell-independent and T cell-dependent mechanisms respectively. The results indicated that the extract affected cellular mediated immunity (CMI) rather than humoral mediated immunity (HMI). The extract inhibited PHA-induced human lymphocyte proliferation [22], but induced some enhancement of mouse splenic B-cells [23]. Since both inhibitory and stimulatory activities of gallic acid on lymphocyte proliferation have been previously reported, gallic acid might be responsible for this activity. Moreover, this circumstantiated the evidence that the same extract exerted a biphasic response, depending on the applied concentration. This was in agreement with the effects of other plants, which had either stimulatory or inhibitory activities on the immune response [24]. Besides gallic acid, there were other phytochemical compounds present in the fruit of *T. bellerica,* such as ellagic acid, ethyl gallate, chebulagic acid and β-sitosterol [8-9]. Three lignans and one flavan from the *T. bellerica* extract showed significant anti-HIV, antimalarial , and antifungal activity in vitro [15]

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Therefore, it is possible that other compounds contained in *T. bellerica* extract might be responsible for the immunomodulatory activity as well.

Conclusions

The present study has confirmed the traditional uses of *T. bellerica*, showing it has an immunomodulatory property that can differently alter both macrophage phagocytic activity and proliferation of splenic lymphocytes. Furthermore, the *T. bellerica* extract has immunosuppressant effects at low concentrations while stimulatory activity is observed at high concentrations. These results suggest a potential therapeutic application of this plant in the treatment of disease associated with the functions of phagocytes and lymphocytes. In vivo immunological assays and characterisation of the mechanism of action of the extract need to be further evaluated.

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References

1. G. C. Jagetia, M. S. Baliga, K. L. Malagi, and M. S. Kamath, "The evaluation of the radioprotective effect of Tripala (an ayurvedic rejuvenating drug) in the mice exposed to radiation", *Phytomedicine*, **2002**, *9*, 99-108.
2. T. Sandhya and K. P. Mishra, "Cytotoxic of breast cancer cell line, MCF 7 and T 47 D to triphala and its modification by antioxidants", *Cancer Lett.*, **2006**, *238*, 304-313.
3. N. Bunyapraphatsara, "Medicine plants (4)", Prachachon Publishing Ltd., Bangkok, **2000**, pp. 477-482.
4. I. Ahmad, Z. Mehmood, and F. Mohammad, "Screening of some Indian medicinal plants for their antimicrobial properties", *J. Ethnopharmacol.*, **1998**, *62*, 183-193.
5. M. C. Sabu and R. Kuttan, "Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property", *J. Ethnopharmacol.*, **2002**, *81*, 155-160.
6. M. D. Chakravarti and J. N. Tayal, "Preliminary examination of the fruits of *Terminalia belerica* Roxb", *Sci. Culture*, **1947**, *13*, 122.
7. H. N. Siddiqui, "Studies on *Terminalia belerica* Roxb: effect on bile secretion and pharmacodynamic properties", *Indian J. Pharmacol.*, **1963**, *25*, 297-302.
8. A. K. Nandy, G. Poddar, M. Sahu, P. Manto, and B. Shashi, "Triterpenoids and their glucosides from *Terminalia bellerica*", *Phytochemistry*, **1989**, *28*, 2769-2772.
9. L. R. Row and P. S. Murty, "Chemical examination of *Terminalia belerica* Roxb", *Indian J. Chem.*, **1970**, *8*, 1047-1048.
10. A. Manosroi, A. Saraphanchotiwitthaya, and J. Manosroi, "Immunomodulatory activities of *Clausena excavata* Burm. f. wood extracts", *J. Ethnopharmacol.*, **2003**, *89*, 155-160.
11. P. Rainard, "A colorimetric microassay for opsonins by reduction of NBT in phagocytosing bovine polymorphs", *J. Immunol. Methods*, **1986**, *90*, 197-201.

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12. I. Suzuki, H. Tanaka, Y. Adachi, and T. Yadomae, "Rapid measurement of phagocytosis by macrophages", *Chem. Pharm. Bull.*, **1998**, *36*, 4871-4875.
13. T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays", *J. Immunol. Methods*, **1983**, *65*, 55-63.
14. C. J. Hackett, "Innate immune activation as a broad-spectrum biodefense strategy: prospects and research challenges", *J. Allergy. Clin. Immunol.,* **2003***, 112,* 686-694.
15. R. Valsaraj, P. Pushpangadan, U. W. Smitt, A. Adsersen, S. B. Christensen, A. Sittie, U. Nyman, C. Nielsen, and C. E. Olsen, "New anti-HIV-1, antimalarial, and antifungal compounds from *Terminalia bellerica*" *J. Nat. Prod.*, **1997**, *60*, 739-742.
16. N-S. Kang, S-Y. Park, K-R. Lee, S-M. Lee, B-G. Lee, D-H. Shin, and S. Pyo, "Modulation of macrophage function activity by ethanolic extract of larvae of *Holotrichia diomphalia*" *J. Ethnopharmacol.*, **2002**, *79*, 89–94.
17. V. Houde, D. Grenier, and F. Chandad, "Protective effects of grape seed proanthocyanidins against oxidative stress induced by lipopolysaccharides of periodontopathogens", *J. Periodentol.*, **2006**, *77*, 1317-1319.
18. P. R. Tam and R. D. Hinsdill, "Screening for immunomodulators: effects of xenobiotics on macrophage chemiluminescence in vitro", *Fundam. Appl. Toxicol.*, **1990**, *14*, 542-553.
19. M. C. Carreras, N. A. Riobo, G. A. Pargament, A. Boveris, and J. J. Poderoso, "Effects of respiratory burst inhibitors on nitric oxide production by human neutrophils", *Free Rad. Res.*, **1997**, *26*, 325-334.
20. M. Horsmanheimo, "Correlation of tuberculin-induce lymphocyte transformation with skin test reactivity and with clinical sarcoidosis", *Cellular Immunol.*, **1974**, *10*, 329-337.
21. A. Nakamura, K. Nagai, S. Suzuki, K. Ando, and G. Tamura, "A novel method of screening for immunomodulating substances, establishment of an assay system and its application to culture broths of microorganisms", *J. Antibiotic*, **1986**, *39*, 1148–1154.
22. A. Serrano, C. Palacios, G. Roy, C. Cespon, M. L. Villar, M. Nocito, and P. Gonzalez-Porque, "Derivatives of gallic acid induce apoptosis in tumoral cell lines and inhibit lymphocyte proliferation", *Arch. Biochem. Biophys.*, **1998**, *350*, 49-54.
23. Z. Q. Hu, M. Toda, S. Okubo, Y. Hara, and T. Shimamura, "Mitogenic activity of (-) epigallocatechin gallate on B-cells and investigation of its structure-function relationship", *Int. J. Immunopharmacol.*, **1992**, *14*, 1399-1407.
24. N. Kovačević, M. Čolić, A. Backović, and Z. Došlov-Kokoruš, "Immunomodulatory effects of the methanolic extract of *Epimedium alpinum* in vitro", *Fitoterapia*, **2006**, *77*, 561-567.