




## *Ex-vivo* Immune-stimulating Activity of *Scutellaria baicalensis* and Its Major Flavonoids on Human Immune Cells

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**Abstract:** This study investigates the activation of human immune cells (T, B, and NK cells, and granulocytes) by the ethanolic and aqueous extracts of *Scutellaria baicalensis* root (SBR) and to identify the compounds responsible for such an effect. The cell activation was determined by expression of the cell surface glycoprotein CD69 (cluster of differentiation 69), as measured by flow cytometry. The content of the main flavonoids in SBR extracts was estimated by high performance liquid chromatography with a diode array detector. NK cells were substantially activated by the SBR ethanolic extract (25–200 µg/mL), whereas B cells were activated on a lower scale. NK cells were slightly activated by the SBR aqueous extract (100–200 µg/mL). Wogonoside and baicalin, two major flavonoids present in the SBR extracts, exhibited the highest activation effect on NK cells. The content of baicalin, wogonoside, and baicalein accounted for 201.2, 47.7, and 3.4 mg/g in the ethanolic extract; and for 150.4, 33.6, and 4.6 mg/g in the aqueous extract, respectively. This study shows the immune-stimulating properties of SBR extracts and identifies their active compounds. This data may support the potential utilization of SBR for strengthening the human immune system against bacterial and viral infections.

**Keywords:** *Scutellaria baicalensis*; immune-stimulation; CD69; flavonoids; baicalin; baicalein; wogonoside.  
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### 1. Plant Source

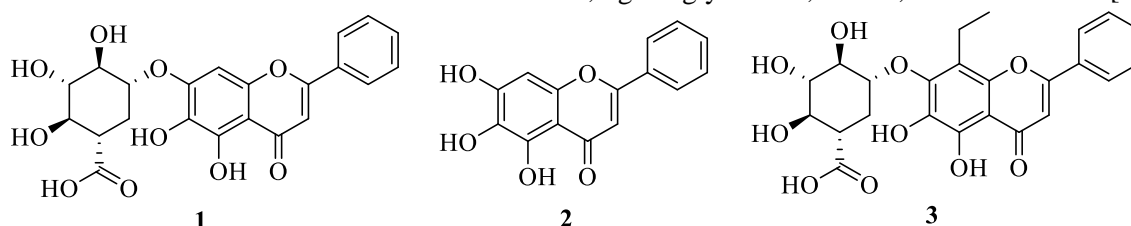
The roots of *Scutellaria baicalensis* Georgi (Lamiaceae) were collected in the Experimental Garden of the Faculty of Horticulture, Mendel University in Brno (the Czech Republic). The voucher

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specimen (No. AL-748) was verified and deposited by Prof. Lubomír Opletal in the herbarium of the Faculty of Pharmacy in Hradec Králové (the Czech Republic).

## 2. Previous Studies

*Scutellaria baicalensis* Georgi (Baikal skullcap) is a perennial herb native to east Asia. Its root is commonly used in traditional Chinese Medicine [1,2]. More than forty flavonoids have been reported as the main active secondary metabolites in *Scutellaria baicalensis* roots (SBR) [2]. They are predominantly represented by baicalin (**1**), accounting up to 20% in the root, and its aglycon baicalein (**2**) (Figure 1) [3]. Other flavonoids present in SBR include wogonoside (**3**) (Figure 1) and its aglycon wogonin, norwogonin, scutellarin and its aglycon scutellarein, oroxylin A, and skullcapflavone I and II. SBR also contains small amounts of essential oils, lignan glycosides, sterols, and amino acids [3,4].



**Figure 1.** Chemical structures of baicalin (**1**), baicalein (**2**), and wogonoside (**3**)

Besides exhibiting a wide range of biological activities [1], SBR has been suggested as a promising immune-modulating agent [5–13]. Indeed, SBR extract was shown to modulate mice immunity through both the regulation of type 1 and type 2 helper T cells, and the inhibition of the production of interferon-gamma (IFN- $\gamma$ ), nitric oxide (NO), interleukin 12 (IL-12), and tumor necrosis factor alpha (TNF- $\alpha$ ) [5]. This extract also normalized the count of T-lymphocytes, immunoglobulin A, and granulocytes in patients undergoing antineoplastic chemotherapy [6,7]. Furthermore, a SBR extract enriched in baicalein and wogonin has been reported to upregulated innate antiviral immunity by modulating cytokine production and stimulating human leukocyte resistance [8]. In another study [9], a SBR extract standardized to baicalin was found to induce apoptosis in leukocytes of children diagnosed with acute lymphocytic leukemia. Baicalin is the most abundant secondary metabolite present in SBR and several studies have been mainly focused on this compound to ascertain its involvement in the immune-modulating activities of SBR. In fact, previous investigations have demonstrated that baicalin stimulates the production of INF- $\gamma$  in T-lymphocytes and natural killer (NK) cells, as revealed by a study on mice *in vivo* and on human blood leukocytes *in vitro* [10]. It has also been reported that baicalin stimulates the proliferation of T- and B-cells [11], and may promote regulatory T cells differentiation and upregulate the inhibitory function of regulatory and effector T cells [12]. However, not only flavonoids are responsible for the claimed immune-modulating effect of SBR. For instance, a water-soluble polysaccharide glucan (SbRP-1) from SBR showed an immune-stimulating effect on the azathioprine model of immunosuppression [13]. Therefore, it is conceivable that the effect of SBR and its components on immunity may differ depending on the method or model used to study this property. SBR-based phytopreparations are mostly available as ethanolic tinctures or herbal teas, which have not been extensively evaluated for their immune-stimulating effects. In addition, no study has determined the contribution of SBR flavonoids to this bioactivity.

## 3. Present Study

In the present study, the capacity of two SBR extracts and their main flavonoids to activate human immune cells was investigated *ex vivo* by measuring the expression of CD69 (cluster of differentiation 69) on the surface of human immune cells. The content of the main flavonoids in the SBR extracts was determined by high performance liquid chromatography with a diode array detector (HPLC-DAD). It is known that upon activation with a variety of stimuli (like IL-2, TNF- $\alpha$ , cell

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antigens, or viral infections), human immune cells (T, B, and NK cells, monocytes, neutrophils, and eosinophils) express CD69 transmembrane glycoprotein on their surface, which functions as a signal-transmitting receptor and helps co-stimulate further activation and proliferation. CD69 is one of the earliest activation markers [14,15]. Anti-human CD69 antibodies can be fluorescently marked; therefore, it is possible to measure their fluorescence intensity by flow cytometry. In this way, the activation of immune cells was possible to be estimated (in Supporting Information). This method has been used to evaluate the immune-stimulating properties of medicinal herbs [16,17]. As seen in Figure 2C, the SBR ethanolic extract led to a substantial increase in the number of activated NK cells, but a slight increase in the number of activated total lymphocytes (Figure 3C) after 24 h of treatment. The SBR aqueous extract afforded a slight increase in the number of activated NK cells and total lymphocytes (Figures 2D, 3D). The CD69 expression on activated NK cells and total lymphocytes in response to SBR treatment was shown as a shift to the right in the representative histograms (Figures 2C–D, 3C–D). The B and T cells (figures not shown) exhibited only a negligible increase in their number in response to ethanolic SBR treatment. As shown in Tables 1 and 2, the stimulation index of ethanolic and aqueous SBR extracts (25–200  $\mu\text{g/mL}$ ) on the tested immune cells (as described in Supporting Information) confirmed these observations. The SBR ethanolic extract exerted a better stimulation index on the immune cells than the SBR aqueous extract (Tables 1,2). However, the two studied SBR extracts showed almost no effect on granulocytes (Tables 1,2). The slight stimulation index on B cells in response to SBR extracts treatment was similar to that observed over total lymphocytes. As shown in Table 3, wogonoside, followed by baicalin were the most active flavonoids over the tested immune cells. Except T cells, all tested immune cells were activated by these two compounds. Baicalin (**1**) is the  $\beta$ -D-glucuronic acid-conjugated form of baicalein (**2**); in other terms, baicalein is the aglycone part of the molecule. Therefore, it is conceivable that glucuronic acid may play a fundamental role in the activation effect showed by baicalin. Surprisingly, baicalin (**1**) and wogonoside (**3**) (another flavonoid glycoside conjugated with  $\beta$ -D-glucuronic acid) were the most active compounds in this study, thus suggesting the vital role of glucuronic acid in the immune-stimulation effect exerted by these two compounds. This led us to hypothesize that unlike the aglycones, the increased hydrophilicity of **1** and **3** may facilitate these compounds to solubilize better in the assay aqueous media, thereby favoring their access to cells for exerting their effect.

The HPLC analysis of the SBR extracts (Figure S1) confirmed the presence of the flavonoids **1**, **2**, and **3**. The SBR ethanolic extract contained 201.2 and 47.7 mg/g of **1** and **3**; respectively, while the SBR aqueous extract was found to contain 150.4 and 33.6 mg/g of **1** and **3**, respectively (Table S1). Baicalein was the minor flavonoid in the two tested extracts (Table S1). Baicalin has been earlier reported as the major flavonoid in the SBR, while its aglycon baicalein is present at lower concentrations [3]. A previous study has reported wogonoside as the second most abundant flavonoid in SBR [3]. In the present study, the detected amounts of flavonoids in SBR extracts were consistent with those reported in the literature [3,18]. Other flavonoids in SBR represent less than 1.0% (w/w) [3]. In the current investigation, the immune-stimulating activity of the SBR ethanolic extract might be attributed to baicalin and wogonoside.

**Table 1.** Stimulation index of the ethanolic extract of *Scutellaria baicalensis* on immune cells

Cell type	SI (stimulation index)				
	concentration ( $\mu\text{g/mL}$ )				
	25	50	100	200	PWM <sup>a</sup>
NK cells	6.7 $\pm$ 3.5	17.9 $\pm$ 10.4	15.5 $\pm$ 7.0	21.0 $\pm$ 9.4	39.7 $\pm$ 6.1
Total lymphocytes	2.6 $\pm$ 0.6	2.2 $\pm$ 1.2	5.4 $\pm$ 1.4	7.2 $\pm$ 2.3	26.0 $\pm$ 9.3
T cells	1.7 $\pm$ 0.1	1.2 $\pm$ 0.5	2.4 $\pm$ 0.2	2.8 $\pm$ 0.6	17.5 $\pm$ 6.5
B cells	2.6 $\pm$ 0.6	2.1 $\pm$ 1.2	5.2 $\pm$ 1.4	7.0 $\pm$ 2.3	25.5 $\pm$ 9.5
Granulocytes	1.5 $\pm$ 0.3	0.8 $\pm$ 0.0	0.6 $\pm$ 0.1	0.5 $\pm$ 0.1	7.9 $\pm$ 0.3

<sup>a</sup>PWM (positive control): pokeweed mitogen at a concentration of 10  $\mu\text{g/mL}$

**Table 2.** Stimulation index of the aqueous extract of *Scutellaria baicalensis* on immune cells.

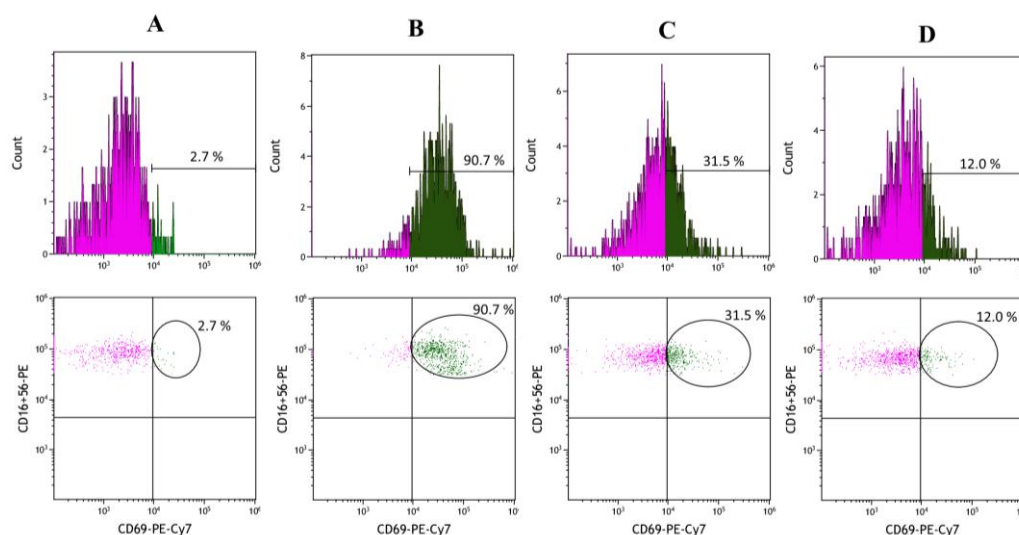
Cell type	SI (stimulation index)				
	concentration ( $\mu\text{g/mL}$ )				
	25	50	100	200	PWM <sup>a</sup>
NK cells	$1.2 \pm 0.1$	$2.7 \pm 1.3$	$4.5 \pm 2.0$	$6.9 \pm 2.5$	$39.7 \pm 6.1$
Total lymphocytes	$0.9 \pm 0.1$	$1.5 \pm 0.2$	$2.0 \pm 0.3$	$2.7 \pm 0.3$	$26.0 \pm 9.3$
T cells	$0.8 \pm 0.1$	$1.2 \pm 0.0$	$1.5 \pm 0.1$	$1.5 \pm 0.0$	$17.5 \pm 6.5$
B cells	$0.9 \pm 0.1$	$1.5 \pm 0.2$	$2.0 \pm 0.3$	$2.6 \pm 0.3$	$25.5 \pm 9.5$
Granulocytes	$0.5 \pm 0.1$	$0.8 \pm 0.1$	$1.2 \pm 0.1$	$2.4 \pm 0.0$	$7.9 \pm 0.3$

<sup>a</sup>PWM (positive control): pokeweed mitogen at a concentration of  $10 \mu\text{g/mL}$

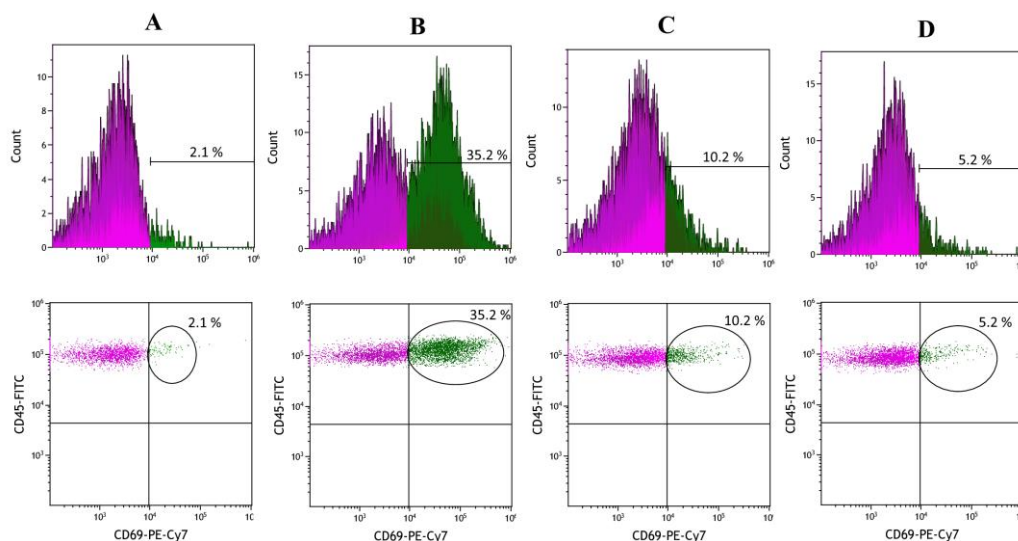
**Table 3.** Stimulation index of baicalin (1), wogonoside (3), and baicalein (2) on immune cells. The compounds were tested at  $100 \mu\text{g/mL}$ .

Cell type	SI (stimulation index)			
	Baicalin (1)	Baicalein (2)	Wogonoside (3)	PWM <sup>a</sup>
NK cells	$9.2 \pm 0.8$	$1.6 \pm 0.6$	$25.2 \pm 0.2$	$39.7 \pm 6.1$
Total lymphocytes	$2.7 \pm 0.1$	$1.0 \pm 0.3$	$3.4 \pm 0.9$	$26.0 \pm 9.3$
T cells	$2.0 \pm 0.2$	$0.8 \pm 0.2$	$2.1 \pm 0.8$	$17.5 \pm 6.5$
B cells	$3.0 \pm 0.0$	$1.1 \pm 0.4$	$4.1 \pm 1.6$	$25.5 \pm 9.5$
Granulocytes	$2.9 \pm 0.6$	$2.0 \pm 1.1$	$7.4 \pm 1.9$	$7.9 \pm 0.3$

<sup>a</sup>PWM (positive control): pokeweed mitogen at a concentration of  $10 \mu\text{g/mL}$



**Figure 2.** Representative histograms (upper side) and dot plots (lower side) showing the activation of NK cells in response to treatment with SBR extracts. **A:** Negative control, **B:** positive control, **C:** SBR ethanolic extract at  $200 \mu\text{g/mL}$ , and **D:** SBR aqueous extract at  $200 \mu\text{g/mL}$ . The pink color on the left represents the number of non-activated NK cells (no expression of antigen CD69 on their surface) and the green color on the right represents the number of activated NK cells

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**Figure 3.** Representative histograms (upper side) and dot plots (lower side) showing the activation of total lymphocytes in response to treatment with SBR extracts. **A:** Negative control, **B:** positive control, **C:** SBR ethanolic extract at 200  $\mu\text{g}/\text{mL}$ , and **D:** SBR aqueous extract at 200  $\mu\text{g}/\text{mL}$ . The pink color on the left represents the number of non-activated lymphocytes (no expression of antigen CD69 on their surface) and the green color on the right represents the number of activated total lymphocytes.

In conclusion, SBR stimulated substantially human NK cells, and a similar effect was exerted by **1** and **3**, the major flavonoids in the tested extracts. Accordingly, it is conceivable that these compounds may be the active principle responsible for the observed effect. Unlike the SBR aqueous extract, the ethanolic extract had a better ability to stimulate the expression of CD69, which correlated with its higher content of **1** and **3**.

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### Supporting Information

Supporting Information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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