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Immunosupressive Phenolic Constituents from *Hypericum montbretii* Spach.

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Abstract: The MeOH extract of aerial parts of *H. monbretii* Spach. have yielded quercetin (**1**), kaempferol (**2**), quercitrin (**3**), hyperin (**4**), myricitrin (**5**), rutin (**6**), I 3 II 8″ biapigenin **(7),** methyl chlorogenate (**8),** 5-*O*-*p*-coumaroylquinic acid (**9**), and chlorogenic acid (5-*O*-caffeoyl quinic acid) (**10**), mangiferin (**11**), and shikimic acid (**12**). Three compounds **1**, **2** and **4** were found to have an immunomodulating inhibitory activity on the oxidative burst response of human neutrophils, as well on mouse peritoneal macrophages. Effects of compounds on whole blood phagocytes activity was found to be with IC_{50} ranging between 8.0-12.2 µg/mL. Only compounds **1,** and **2** were found to interfere with T-cell proliferation, and IL-2 release in PHA activated peripheral blood mononuclear cells system. To the best of our knowledge, this is the first report of phytochemical and biological activity study on *H. montbretii* Spach.

Keywords: *Hypericum monbretii* Spach.; phenolics; immunomodulating inhibitory activity; reactive oxygen species; T-cell proliferation; IL-2 production.

1. Introduction

In recent years, the consumption of *Hypericum perforatum* (St. John's wort) derived products has increased exponentially, and presently it is one of the most consumed medicinal plants [1]. The commercially available *H. perforatum* derived products include phytopharmaceuticals, nutraceuticals, teas, tinctures, juices, and oily macerates [2]. Also, several brands of common food products, such as beverages and yoghurts also include St John's wort extract as additive. *H. perforatum* has a wide range of medicinal applications, including skin wounds, eczema, burns, diseases of the alimentary tract, and psychological disorders. Nowadays, its use in the treatment of mild to moderate depression has increased substantially [3,4]. Numerous papers, and reviews have been published on various aspects of uses, chemistry and pharmacology of *H. perforatum* [3,4,5].

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There is also considerable interest in the characterization of new constituents of genus *Hypericum* as a source of several of promising compounds with various biological activities, including cytotoxicity, antitumour, antimicrobial, and anti-inflammatory activities [6,7,8].

In previous work on *Hypericum montbretii*, the changes in hyperforin content and variation of bioactive substances during plant growth were investigated by HPLC method [9,10]. Antioxidant activities of some *Hypericum* species have been investigated as a comparative study with *H. perforatum*. In an antiradical activity assay, leaves and flowers of *H. montbretii* and leaves of *H. origanifolium* were the most active at the tested concentrations, exhibiting an activity comparable to that of the positive control BHT, but all of the extracts, with the exception of the leaves of *H. montbretii*, showed activity weaker than the leaves and flowers of *H. perforatum*, the reference plant [11]. In another study, phenolic constituents of 17 *Hypericum* species from Turkey including *H. montbretii* were analyzed using LC-MS-MS method [12]. From the aerial parts of *H. montbretii*, quercetin, myricetin, (+)-catechin, quercitrin, isoquercitrin, hyperoside, biapigenin and chlorogenic acid were isolated and identified by Sakar *et* al. [13]. However, our phytochemical study on *Hypericum montbretii* is the first report about immunomodulating inhibitory activity of its constituents. Previously, we have reported new cellular reactive oxygen species (ROS) release inhibitory benzophenone constituents of *Hypericum thasium* Griseb. [14].

ROS are generated by many redox processes which are normally involve in the metabolism of aerobic cells. These species are highly reactive, and harmful to the cells. If not eliminated, ROS can damage important molecules, such as proteins, DNA, and lipids. Cells express several defence mechanisms including antioxidant enzymes, and non enzymatic compounds that help to prevent the damaging effects of ROS [15]. However, these endogenous systems are often insufficient for complete elimination of ROS. Their excess has been implicated in the development of chronic diseases, such as cancer, arteriosclerosis, and rheumatism [16,17]. Oxidative stress can also play an important role in the development of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases [18,19].

In the present study, EtOAc, and butanolic extracts of the *Hypericum montbretii* were purified by different chromatographic methods such as combination of various column chromatographic techniques including reverse phase recycling HPLC. The phytochemical investigation on ethyl acetate extract led to the isolation of mainly phenolic compounds, except for shikimic acid. The structures of the isolated compounds were elucidated by spectroscopic techniques such as UV, IR, ^{1}H NMR, ^{13}C NMR, EI MS, HRFAB MS, and various 2D NMR techniques.

We investigated the effect of the compounds isolated from *Hypericum montbretii* on ROS production by using various professional phagocytes neutrophils, macrophages. In addition to this, the effect of these compounds on T-cell proliferation, and IL-2 production in response to the phytoheamaglutinin (PHA), was also investigated.

2. Materials and Methods

2.1. Plant Material

Hypericum montbretii Spach. (Guttiferae) aerial parts were collected from Derekoy, Turkey, in June 2000. A voucher specimen was deposited at the herbarium of Trakya University, Biology Department, Edirne, Turkey (EDTU 4394).

2.2. Extraction and Isolation

The whole air-dried ground plant material (488 g) was extracted at room temperature with MeOH for 15 days. The extract was concentrated *in vacuo*, and the residue was partitioned between H_2O and hexane, $CHCl_3$, EtOAc, BuOH, respectively. The EtOAc fraction (31 g) was separated into 10 subfractions by silica gel CC, eluted with different proportions of hexane/acetone, and acetone/methanol. Those fractions, containing similar components as judged by TLC, were combined and finally ten fractions were obtained. Sbfr. A was subjected to Si gel column, eluted with hexane/acetone (4:1) solvent system to obtain compound **1**. Subfr. B was subjected to preperative recycling HPLC. The use of ODS chromatography (H-80 column) on HPLC of subfr. B using H_2O-

MeOH (1:1) as a mobile phase at a flow rate of 4 mL resulted in the isolation of compounds **1** and **2**. Subfr. C was subjected to sephadex LH-20 column chromatography, and eluted with MeOH to obtain **3**. Subfr. D and E were subjected to polyamide column chromatography with $CHCl_3$/MeOH as the eluent to obtain compounds **4** and **5**, respectively. To remove the impurities, compounds **4** and **5** were rechromatographed on sephadex LH-20 column and eluted with water and increasing amount of MeOH. Subfr. F was loaded on to a sephadex LH-20 column, and eluted with water with increasing ratio of MeOH to obtain compound **6**. Compound **7** was obtained from subfraction G, when chromatographed on polyamide column, and eluted with $CHCl_3$ with increasing proportions of methanol. Also subfr. H was subjected to polyamide column chromatography by elution with step gradient $CHCl_3$/MeOH and further separation was carried out with recycling HPLC on ODS chromatography (L-80 column) by using H_2O-MeOH (1:1) as a mobile phase at a flow which resulted in the isolation of compound **8**. Similarly, like the other polar fractions, subfr. I was also loaded on a polyamide column. Compound **9** was obtained from last fraction by using $CHCl_3$ and increasing proportion of MeOH. 18 g of BuOH extract was subjected to Diaion HP-20 column chromatography with H_2O, 50% MeOH-H_2O and methanol as eluents (2 L each) and three fractions were collected. Fraction 1 was loaded on a charcoal column by using flash chromatography to remove the sugars from this fraction, and five subfractions obtained. Subfrac. 5 was subjected to preparative recycling HPLC (BP-120 column) and eluted with 100% H_2O to give compound **12**. Fraction 2 was loaded on a polyamide column, and eluted with $CHCl_3$: MeOH mixture (from 100% $CHCl_3$ to 20% MeOH/$CHCl_3$), followed by RP HPLC (H_2O: MeOH; 1:1, L-80 column, flow rate 4 mL/min) to yield compound **10**. Subfraction 3 was also rechromatographed over polyamide column by using mixture of $CHCl_3$: MeOH (from 100% $CHCl_3$ to 20% MeOH/$CHCl_3$) to give compounds **4**, and **11**.

2.3. Reagents and Chemicals for Biological Assays

Luminol (3-aminophthalhydrazide) was purchased from Research Organics (U.S.A.), while Hanks Balance Salts Solution (HBSS) were purchased from Sigma, Germany. Lymphocytes Separation Medium (LSM) and dextran were purchased from MP Biomedicals Inc., Germany. Zymosan-A (*Saccharomyces cerevisiae* origin) was purchased from Fluka (BioChemika, Buchs, Switzerland). Dimethyl sulphoxide (DMSO), ethanol, and ammonium chloride of analytical grades were purchased from Merck Chemicals, Darmstadt, Germany. The Luminometer used was Luminoskan RSL (Finland).

2.4. Chemiluminescence Assay

Luminol-enhanced chemiluminescence assay was performed, as described by Helfand [20]. Compounds were serially diluted to a final volume of 50 µL. To each well, 50 µL of a PMN suspension (1×10^7 cells/mL) and 50 µL luminol (7×10^{-5} M) solutions were added. The neutrophils were challanged by adding 50 µL of opsonized zymosan A (OPZ; final concentration: 200 µg/mL), and chemiluminescence emitted from each well was immediately measured as relative light unit (RLU) every 1 min for 0.5 sec during a 50-min period by using a Luminometer (Luminoskan RS Labsystem, Finland). Total integral levels were used to calculate the activity of test samples in relation to their corresponding controls (identical incubations without test sample). Experiments were performed in Hank's balanced salt solution (HBSS), buffered at pH 7.35 with $NaHCO_3$ and supplemented with 0.1% (w/v) gelatin to avoid cell aggregation (HBSS-gelatin).

2.5. T-Cell Proliferation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from heparinized venous blood of a healthy adult donor by Ficoll-Hypaque gradient centrifugation [21]. Cells were proliferated following a method reported by Nielsen et al. [22]. Briefly, 100 µL cells suspension (5×10^5/mL) was added in a 96 well round bottom tissue culture plate (Nalge Nunc. Inter., Roskilde, Denmark). Cells were stimulated with 1.25 µg/mL of phytohemagglutinin (Sigma). Various concentrations of compounds **1-12** were added to give final concentration of 3.1-50 µg/mL, each in triplicate. The plate was incubated for 72 hrs at 37 °C in 5% CO_2 incubator. Then cells were pulsed with (0.5 µCi/well) tritiated thymidine, and further incubated for 18 hours. Cells were harvested onto a glass fiber filter

(Cambridge Technology, USA) using cell harvester (SKATRON A.S. Flow Lab., Norway). The tritiated thymidine incorporation into the cells was measured by a liquid scintillation counter (LS 6500, Beckman Coulter, USA). CPM results were recorded after 120 seconds. The inhibitory activity of the compounds **1-12** on T- lymphocyte proliferation was calculated by using the following formula:

$$\text{Inhibitory Activity (\%)} = \frac{\text{Control Group (CPM) - Experimental Group (CPM)}}{\text{Control Group}} \times 100$$

2.6. Effect on Peripheral Blood Mononuclear Cells IL-2 Production

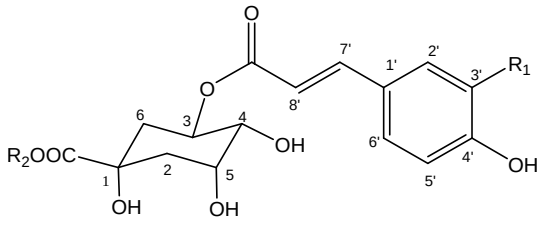
The levels of IL-2 cytokine production by mononuclear cells were monitored in the presence and absence of compounds, using the human cytokine kits (Diaclone, Besancon Cedex, France). Briefly, freshly prepared mononuclear cells (10^5/well) were cultured in 96-well microtiter plate in the presence or absence of 1.25 µg/mL PHA. Four different concentrations (1, 5, and 25 µg/mL) of compounds **1** and **2**, along with PHA were used in this assay. The plate was incubated at 37 °C for 18 hours. Then the supernatant was collected, and analyzed for IL-2 production.

2.7. Statistical analysis

All data are presented as mean ± standard deviation of three independent experiments. Statistical analysis for all the results is based on Student's *t*-test. Significance was attributed to probability values ($P < 0.05$, <0.005). The IC_{50} values were calculated by using Excel based program.

3. Results and Discussion

The concentrated MeOH extract prepared from the air-dried arial part of *H. monbretii* was extracted with hexane, chloroform, ethyl acetate, and butanol. Quercetin (**1**), kaempferol (**2**), quercitrin (**3**), hyperin (**4**), myricitrin (**5**), rutin (**6**), I 3 II 8″ biapigenin **(7)**, methyl chlorogenate (**8)**, and 5-*O*-*p*-coumaroylquinic acid (**9**), were isolated from ethyl acetate extract. Compounds isolated from BuOH fraction included chlorogenic acid (5-*O*-caffeoyl quinic acid) (**10**), mangiferin (**11**), and shikimic acid (**12**). Methyl chlorogenate (**8**) was also isolated from butanolic extract.



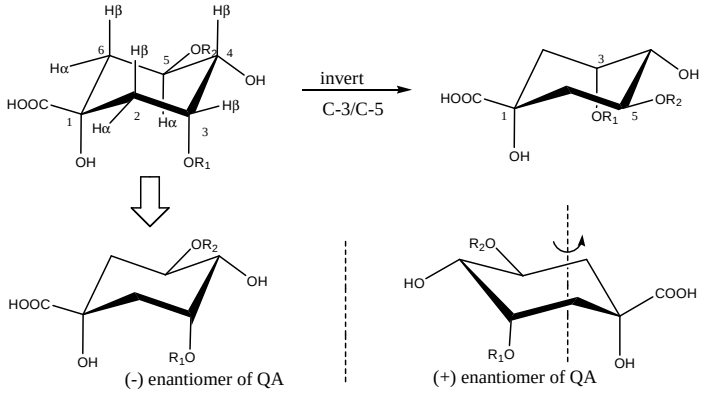
R_1: OH, R_2: CH_3: Methyl chlorogenate (**8**)
R_1: H, R_2: OH; 3-O-p-Coumaroyl quinic acid (**9**)
R_1: OH, R_2: OH; Chlorogenic acid (**10**)

Figure 1. The structures of the compounds **8**, **9**, and **10**.

Although all compounds are known, it is useful to describe the structure elucidation of quinic acid derivatives **8**, **9**, and **10**, briefly (Fig. 1) here, since there is stereochemical confusion about their structures in the literature. This is because of the formula drawing of (+)-enantiomer of naturally occuring (-)-quinic acid turning out to be identical to the structure obtained after inversion of the chirality at both centers C-3 and C-5 (Fig. 2) [23]. This situation caused wrong nomenculature as well as the difficulty of the distinction of absolute structure. A clear distinction can be made by the

coupling pattern of most downfield C***H***OH resonance. If caffeoyl moeity is attached as *equatorial*, H-5 would be observed with two large diaxal and one small coupling constant, on the other hand it would be observed with three small coupling constants in case of H-3, when caffeoyl moeity is *axial*.

Compound **8** showed a molecular ion peak $[M-H]^-$ at *m/z* 367 in FAB MS (-) ve corresponding to molecular formula $C_{17}H_{20}O_8$. The ^{1}H-NMR spectrum showed distinct signals at δ 7.52 (1H, d, *J* = 16.0 Hz, C-7′) and 6.22 (1H, d, *J* = 16.0 Hz, C-8′) for the *trans* olefinic protons. The aromatic region proton signals showed an ABX system at δ 7.05 (1H, d, *J* = 1.61 Hz, H-2′), 6.92 (1H, dd, *J* = 8.2 Hz, *J* = 1.6 Hz, H-6′), 6.79 (1H, d, *J* = 8.2 Hz, H-5′). Some additional upfield signals for the quinic acid protons were also observed in the ^{1}H-NMR spectrum. It showed three oxymethine signals at δ 5.30 (1H, ddd, *J* = 11.6 Hz, *J* = 7.4 Hz, *J* = 4.2 Hz, H-5), 4.16 (1H, t, *J* = 2.7 Hz, H-3), and 3.76 (1H, dd, *J* = 7.4 Hz, *J* = 2.7 Hz, H-4). Since the most downfield C*H*OH resonance was observed at δ 5.30 as ddd (*J* = 11.6 Hz, *J* = 7.4 Hz, *J* = 4.2 Hz), it was concluded that the caffeoyl moiety attached to C-5 position of quinic acid was *equatorial* [24]. The HMBC experiment of the compound showed that there were correlations between methyl ester carbonyl, and H-2, H-6 methylene protons. Another HMBC correlation was observed between H-5 and caffeoyl carbonyl carbon.



Chlorogenic acid: R_1=H, R_2=*Trans*-caffeic acid
Neochlorogenic acid: R_1=*Trans*-caffeic acid, R_2=H
Quinic acid: R_1=R_2=H

Figure 2. The formula of (+)-enantiomer of naturally occuring (-)-quinic acid found to be identical to the structure obtained after inversion of the chirality at both centers C-3 and C-5 (revised from Pauli *et* al.)

Compound **9** was obtained as an amorphous white substance with the molecular formula $C_{10}H_{18}O_8$. This compound exhibited a molecular ion peak $[M-H]^-$ at *m/z* 337 in negative FAB-MS. In the ^{1}H-NMR spectrum of the compound, two aromatic proton signals were observed at δ 7.45 (2H, d, *J* = 8.4 Hz, H-2′ and H-6′) and 6.79 (2H, d, *J* = 8.4 Hz, H-3′ and H-5′). Two *trans* olefinic protons were appeared as an AB system at δ 6.28 and 7.61 (1H, d, *J* = 15.9 Hz) indicating the presence of an (*E*)-*p*-coumaric acid moiety. Quinic acid protons showed similar signal pattern as observed for compound **8** [25].

Compound **10** was obtained as a pale yellow amorphous material. It exhibited UV and IR absorptions characteristic of phenolic moiety. The molecular ion peak $[M-H]^-$ at *m/z* 353 was observed in FAB MS (-) ve. The ^{1}H-NMR spectrum of **10** showed similar signals to those of compound **8**. The only difference between the two compounds was that absence of methoxy signal in ^{1}H-NMR spectrum of compound **10**. Similarly, the site of (*E*)-caffeoyl moiety at the quinic acid was deduced from HMBC experiment showing the correlation between H-3 (δ 5.31, ddd) of quinic acid, and carbonyl carbon (δ 168.6, C-9) of caffeic acid moeity. Therefore the structure of **10** was identified as 5-*O*-caffeoyl quinic acid (chlorogenic acid) [26].

During the phagocytosis process, reactive oxygen species (ROS) are generated. This process involves the intial formation of superoxide by the NADPH oxidase, and the subsequent generation of the bactericidal agents which include hypochlorous acid, and peroxy nitrates. Formation of ROS by phagocytes is needed to defend against the bacterial infection which in laboratory environment can

react with luminal, and emit a detectable light. Luminol is known to interact with all ROS, including hydrogen peroxide, hydroxy radical, hypochorous acid, and to a less extent with superoxide [27].

Effects of the isolated compounds on oxidative burst (ROS) production in human was studied and the amount of the ROS generated in each system was monitored by chemiluminescence technique, with the use of luminol as an enhancer, and opsonized zymosan-A (SOZ) as an activator. The effect of the test compounds on whole blood, isolated neutrophils or murine macrophages, in response to zymosan activation, is shown in Figures-3-A, 3-B, and 3-C, respectively.

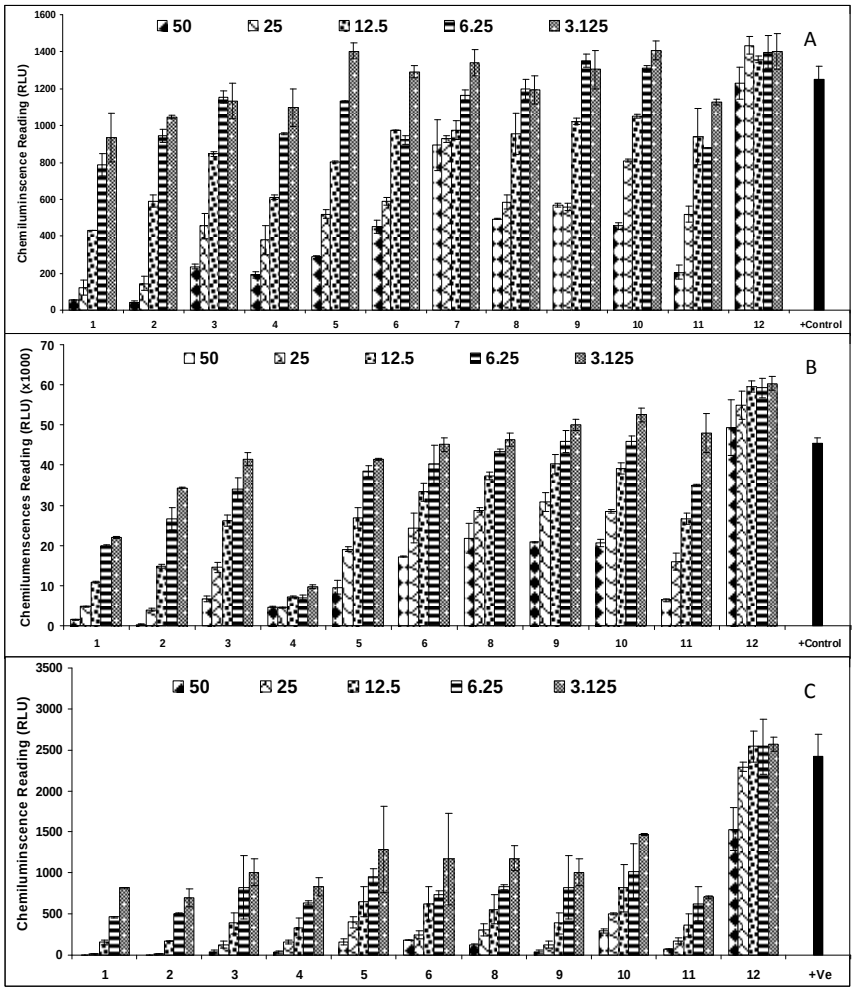


Figure 3. Dose-dependent effect of compounds **1-12** on ROS production. The ROS luminol dependant chemiluminescence respons of compounds on oxidative burst was monitored using human whole blood (A), human neutrophils (B), and mouse peritoneal macrophages (C). The compounds activity was compared with the untreated samples (control/+ve) in the chemiluminescence (CL) assay. Each plot and error bar represents readings ± SD of three repeats.

Our data demonstrated that the treatment of phagocytes with compounds inhibited the production of ROS. The preliminary screenings on whole blood phagocytes oxidative burst activity showed moderate inhibitory potential of compounds **1**, **2**, and **4** with IC_{50} ranges between 8.0-12.2 µg/mL (Table 1). However, very low inhibitory signals were observed with compounds **3**, **5**, **6**, **8**, **9**, and **11**. Further investigation was carried out by monitoring effects of all the isolated compounds on oxidative burst of isolated polymorphonuclear cells (PMNs) and peritonal macrophages by using SOZ activator. Figures-3-B, and -3-C showed a comparison of neutrophil and macrophages oxidative burst activity in the presence of varying concentrations of compounds. On neutrophils activity, compounds **1** and **4** exerted a significant, and prominant dose dependant inhibition with IC_{50} value less than 3 µg/mL, while compound **2**, as well as compounds **3**, **5**, and **11**, showed a moderate inhibitory effect

(IC_{50}= 9 to 13.7 µg/mL). The rest of the compounds showed only weak inhibitory activity. Interestingly, when these compounds were tested on peritoneal macrophages all, compounds showed a strong suppression of ROS production, except compound **12**.

The exact mechanism through which this inhibitory activity is maintained is not clear. However this could be due to the ability of compounds to block the complement receptors, consequently inhibiting NADPH oxidase, a cell membrane associated enzyme. As zymosan-A activates the phagocytes by binding with the complement receptor type 3 on the cell surface [28]. Figure 4 shows compounds **1** and **2** inhibit the stimulatory action of PHA on the T-cell proliferation with IC_{50} = 5.9 and 4 µg/mL, respectively). However compounds **3**, and **8** showed weak inhibition, but no effect was observed with the remaining compounds of the series (Table 1).

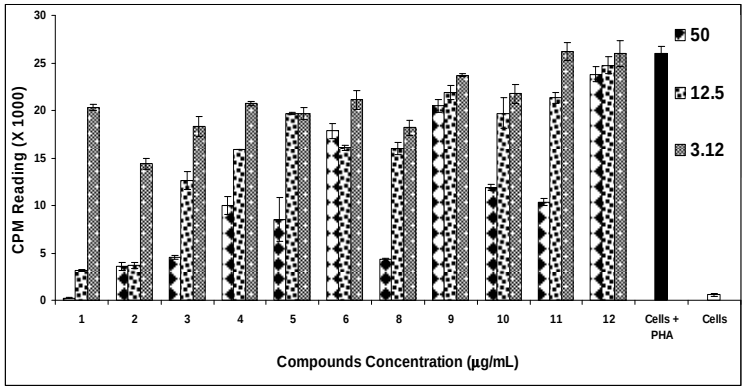


Figure 4. Effect of compounds on phytohemagglutinin (PHA) T-cell proliferation. The bar graph represents effects of various concentrations of the test compounds **1–12** after 72 h incubation with peripheral blood mononuclear cells at 37 ºC. Effect of compounds on T-cell proliferation response is compared with non-proliferated (cells) and proliferated (cells + PHA) cells. Each bar represents the mean value of triplicate reading ± SD.

Table 1. The effect of compounds **1-12** on oxidative burst ROS production on human whole blood, isolated neutrophils, and peritoneal macrophages. Reading represents readings ± SD of three repeats. + ve control = Sodium diethyldithiocarbamate ($3H_2O$).

Compounds	**Oxidative burst (IC_{50} values, µg/mL)**		
	Whole Blood	**Neutrophils**	**Macrophages**
1	8.5 ± 0.5	<3.1	<3.1
2	11.7 ± 1.3	9.0 ± 1.9	<3.1
3	19.0 ± 2.8	13.7 ± 0.4	<3.1
4	12.2 ± 0.3	<3.1	<3.1
5	19.5 ± 1.2	12.5 ± 0.7	<3.1
6	23.6 ± 1.5	24.9 ± 5.0	<3.1
7	>50	NS	NS
8	24.3 ± 2.6	25.0 ± 7.0	<3.1
9	22.7 ± 0.8	>50	8.2 ± 1.7
10	36.0 ± 1.6	41.8 ± 3.2	5.9 ± 1.7
11	20.2 ± 4.7	13.3 ± 2.7	<3.1
12	>50	>50	>50
+ ve control	5.8 ± 1.7	0.3 ± 0.1	8.2 ± 1.9

PHA is a mitogen for T lymphocytes [29]. It binds to *N*-acetylgalactosamine glycoproteins expressed on the surface of T cells then activates the cells to proliferate. Thus, T cells were the major proliferating cells in the activated PBMC when PHA is used. This proliferation was significantly suppressed with the compounds **1**, and **2**. As it is known that the interaction of T-cells with antigens or mitogens initiate a cascade of genes expression, such as IL-2 and IFN-γ mRNA that induce the resting T-cells to enter the cell cycle and culminates in expression of the high affinity receptor for IL-

2 and secretion of IL-2 [30]. In response to IL-2, the activated T-cells progress through the cell cycle, proliferation, and differentiating into memory cells or effecter cells. Our results indicated that the compounds **1** and **2** suppressed IL-2 production in PHA-activated PBMC cultures (Fig. 5). Interleukin-2 is an important immune modulator cytokine [31]. Although IL-2 is involved in the immune response, an increase in IL-2 activity causes various immunological problems [32]. To overcome these problems, efforts have been made to inhibit the IL-2 function.

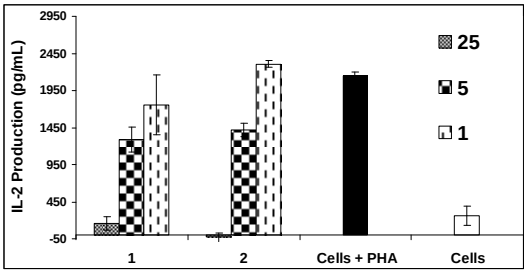


Figure 5. Effect of compounds **1** and **2** on IL-2 production from peripheral blood mononuclear cells (PBMC). PBMC were proliferated with PHA (5 g/mL) for 18 h with or without three different concentrations (1, 5 and 25 µg/mL) of the compounds. Supernatant from each concentration was screened with ELISA for cytokine production.

A number of assays were employed to investigate the immunomodulating effects of compounds isolated from *Hypericum montbretii* on different immune cells. Our results have clearly demonstrated that compounds **1**, **2**, and **4** have immunomodulating inhibitory activity on the oxidative burst response of human neutrophils, as well on mouse peritoneal macrophages. On the other hand, only compounds **1** and **2** were found to interfere with the T-cell proliferation (Table 2). The effect of these compounds on T-cell proliferation was further confirmed by suppression of IL-2 production. Compounds **1**, **2**, and **4** have clearly exerted immunomodulatory activity on cellular immune response in our bioassay.

Table 2. The 50% inhibitory dose of the tested compounds **1-12** on the T-cell proliferation. Each reading represents readings ± SD of three repeats.

Compounds	T - Cell Proliferation (IC_{50}, µg/mL)
1	5.9 ± 0.1
2	4.0 ± 0.2
3	11.8 ± 3.6
4	28.1 ± 3.7
5	33.8 ± 3.6
6	>50
7	NS
8	17.9 ± 1.9
9	>50
10	43.9 ± 0.6
11	38.0 ± 0.9
12	>50

Methyl chlorogenate (**8**): $[\alpha]_D$: +25.4 (*c* 1.00, MeOH). IR (KBr): 3416, 1735, 1693, 1604, 1521 cm^{-1}. UV/Vis λ_{max} (MeOH) nm (log ε): 220 (3.80), 242 (4.32), 298 (3.24), 330 (3.03). ^{1}H NMR (CD_3OD, δ, 400 MHz, ppm): 2.01 (1H, dd, *J*=13.4 and 6.4 Hz, H-6'ax), 2.10-2.21 (3H, m, H_2-2′ and H-6'eq), 3.68 (3H, s, H-8'), 3.73 (1H, dd, *J*=7.7 and 4.4 Hz, H-4'), 4.13 (1H, t, *J*=3.1 Hz, H-5'), 5.27 (1H, ddd, =11.6, 7.4, and 4.2 Hz, H-3'), 6.20 (1H, d, *J*=16 Hz, H-8), 6.76 (1H, d, *J*=8.2 Hz, H-5), 6.92 (1H, dd, J=8.2, and 1.6 Hz, H-6), 7.03 (1H, d, *J*=1.6 Hz, H-2), 7.52 (1H, d, *J*=16 Hz, H-7). ^{13}C NMR (CD_3OD, δ,100 MHz, ppm): 37.2 (C-2, C-6'), 52.1 (C-8'), 69.8 (C-5'), 71.2 (C-4'), 72.1 (C-3'), 75.9 (C-1'), 113.9 (C-2), 114.9 (C-5), 116.0 (C-6), 122 (C-8), 127 (C-1), 146 (C-7), 147.8 (C-3), 149.3 (C-4), 168.3 (C-9),

175.3 (C-7'). MS (EI, 70 eV): *m/z* (%) = 368.1[M^+] (4), 336 (6), 180.1 (44), 163.1 (caffeoyl), 89.1 (22), 55.1 (22). FAB (+) MS (rel.int. %): 369 (50), 277 (55), 185 (100), 163 (100).
5-O-p-Coumaroylquinic acid (**9**): $[\alpha]_D^{20}$: -52 (c 0.1, MeOH). IR (KBr): 3424, 1689, 1641, 1606, 1513, 1276, 1184, 1126, 1081, 969, 812 cm^{-1}. UV/Vis λ_{max} (MeOH) nm (log ε): 250 (3.50), 340 (4.25)
^{1}H-NMR (CD_3OD, δ, 400 MHz, ppm): 2.02-2.22 (4H, m, H-2' and H-6'), 3.72 (1H, dd, *J*=8.2 and 2.7, H-4'), 4.15 (1H, brs, H-5'), 5.32 (1H, ddd, *J*=3.5 Hz, H-3'), 6.32 (1H, d, *J*=15.9 Hz, H-8), 6.79 (2H, d, *J*=8.4 Hz, H-3 and H-5), 7.45 (2H, d, *J*=8.4 Hz, H-2 and H-6), 7.61 (1H, d, *J*=15.9 Hz, H-7). ^{13}C-NMR (CD_3OD, δ,100 MHz, ppm): 37.3 (C-6'), 38.3 (C-2'), 70.6 (C-3'), 71.0 (C-5'), 72.8 (C-4'), 78.5 (C-1'), 114.8 (C-8), 116.0 (C-3 and C-5), 126.3 (C-1), 130.3 (C-2 and C-6), 145.2 (C-7), 148.0 (C-4), 162 (C-9), 180.0 (C-7′). FAB MS (-) *m/z* (rel.int. %): 337 [M-H] (12), 275 (70), 255 (10), 183(100), 165 (20).
Chlorogenic acid **(10)**: $[\alpha]_D^{20}$: -24 (c 0.1, MeOH). IR (KBr): 3424, 1692, 1635, 1520, 1436, 1283, 1180, 1084, 1043, 979 cm^{-1}. UV/Vis λ_{max} (MeOH) nm (log ε): 331 (3.70), 297 (3.20), 245 sh (2.45), 222 (4.30). ^{1}H-NMR (CD_3OD, δ, 400 MHz, ppm): 2.01-2.23 (4H, m, H_2-2′ and H_2-6′), 3.71 (1H, dd, *J*=8.4, and 2.9 Hz, H-4′), 4.16 (1H, dt, *J*=5.2 and 2.9 Hz, H-5′), 5.31 (1H, ddd, *J*=8.9, 8.9, and 4.5 Hz, H-3′), 6.25 (1H, d, *J*=15.8 Hz, H-8), 6.76 (1H, d, *J*=8.2 Hz, H-5),6.94 (1H, dd, *J*=8.2 and 1.96 Hz, H-6), 7.03 (1H, d, *J*=1.96 Hz, H-2), 7.54 (1H, d, *J*=15.8 Hz, H-7). ^{13}C-NMR (CD_3OD, δ,100 MHz, ppm): 76.3 (C-1), 38.2 (C-2), 72.0 (C-3), 73.6 (C-4), 71.4 (C-5), 38.9 (C-6), 177.5 (C-7), 127.8 (C-1′), 115.2 (C-2′), 146.6 (C-3′), 149.4 (C-4′), 116.5 (C-5′), 123.0 (C-6′), 147.1 (C-7′), 115.2 (C-8′), 168.9 (C-9′). FAB (-) MS *m/z* (rel.int. %): 353 [M-H] (15), 275 (45), 183 (100), 165 (18).

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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