Daphne gnidium methanol leaf extract induces immune potential by activating monocytes and B-cells

Fadwa Chaabane (fadwachaabane@yahoo.fr)
University of Monastir
Mary Poupot
Paul Sabatier University
Jean Jacques Fournié
Paul Sabatier University
Fairouz Sioud
University of Monastir
Leila Chekir-Ghedira
University of Monastir

Research Article

Keywords: Daphne gnidium, immunomodulation, B lymphocyte, monocytes

Posted Date: February 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1388776/v1

Abstract

Modulation of the immune response in order to treat immune-mediated diseases has become a more and more common approach. Increasingly, studies have been conducted to examine products from plants for their ability to strengthen host immune defenses. In the present work, we investigated the immunompdulatory potential of a methanol extract from *Daphne gnidium* leaves on immune cells.

HPLC method was used for the tested extract analysis. PBMC was prepared from peripheral blood on a Ficoll-Paque density gradient and monocytes were purified from freshly prepared PBMC by negative selection using blocking agent and a mixture of biotinylated monoclonal antibodies. Proliferation of B lymphocytes and maturation of monocytes were studied by flow cytometry using appropriate anti body. Statistical significance was confirmed by *t* student test.

The obtained results indicated that this extract activated B-cells and monocytes by enhancing the expression of CD19 and CD14, respectively. Further, methanol extract, as well as daphnetin and apigenin-7-glucoside, enhanced the expression of CD86 by the monocytes. In addition, CD64 and HLA-DR expression were also promoted after methanol extract treatment. Conclusion: These data provide the first evidence of the potential utility of these natural products as part of a therapeutic arsenal to treat some diseases by enhancing the host defense responses.

Introduction

The immune system has the role of protecting the body from numerous types of foreign infectious agents. Many tissues and organs as well as other factors are implicated in this system. Many diseases like cancer are a consequence of the immune system dysfunction [1]. Besides, an impairment of immunological functions results in overwhelming the host by many pathogens that could cause secondary infections [2].

Modulating of the immune system to alleviate diseases that appear under the conditions of impaired immune response, involves induction of the expression of any part of the immune response [3].

Using medicinal plants as immunomodulators becomes an area of interest. In fact, medicinal plants could provide an alternative to chemotherapy by having immunomodulatory properties allowing improving the immunological response. Thus, plant-derived compounds such as lectins, peptides, phenolics, flavonoids, polysaccharides, tannins and saponins have been reported to modulate the immune system [4, 5].

Members of the *Daphne* genus (Thymelaeaceae) have been of interest due to their excellent medicinal values. Different classes of natural products have been isolated from these species, including flavonoids, coumarins and diterpenoids [6]. Some species are widely used to treat wound and bruises in folk medicine [7]. In fact, the powdered roots of *Daphne gnidium* have been used in the traditional medicine as an abortifacient and the bark has been used as a diuretic agent, to treat toothache and against hepatitis

[8, 9]. Recently, we have demonstrated that the methanol extract from *Daphne gnidium* has antigenotoxic and antioxidant activities *in vivo* [10]. We also reported an antitumoral and immunomodulatory potential of the aqueous extract *in vivo* [11].

The objective of the present investigation was to study the immunomodulatory activity of the methanol extract obtained from leaves of *Daphne gnidium*.

Materiel And Methods

Preparation of plant extract

Methanol extract was obtained by the Soxhlet apparatus. In fact, the powdered leaves (83g) were extracted with successively petroleum ether, chloroform, ethyl acetate and methanol (one liter each), to obtain respectively petroleum ether, chloroform, ethyl acetate and methanol extracts. Every cycle may be allowed to repeat many times for 6 hours. The methanol extract was tested in the present work.

Permission to collect plant material was given by Tunisian Ministry of Local Affairs and Environment. All methods were performed in accordance with the local and national regulations

Analysis of the methanol extract by HPLC/DAD

The aqueous extract was characterized by HPLC/DAD analysis using the following solvent gradient: solvent A 0.1 % formic acid; solvent B MeOH 0.1 % formic acid; here after solvent A percentages': t_0 98 %, t_2–12 90 %, t_{22} 85 %, t_{35} 75 %, t_{42} 60 %, t_{43-45} 55 %, with a flow rate of 0.8 ml/min. Identifications were completed by comparison of retention time (RT) and UV/vis spectra with a laboratory-built database and authentic samples.

Preparation of PBMC

Blood samples were obtained from healthy adult donors, peripheral blood mononuclear cell (PBMC) were purified on a Ficoll-Paque density gradient (Amersham Biosciences AB, Upsalla, Sweden) by centrifugation (800 g, 30 min, room temperature). PBMC were washed twice and diluted at 1.5 x 10^6 cells/ml in complete RPMI 1640 medium supplemented with penicillin and streptomycin (Cambrex Bio Science, Verviers, Belgium), 1 mM sodium pyruvate, and 10% heat-inactivated fetal calf serum (both Invitrogen, Paisley, UK).

Effect of methanol leaf extract on proliferation of PBMC

Aliquots of PMBC (2 x 10^5 cells) were seeded into 96-well plates in final volumes of 200 µl/well and then treated with the methanol extract. After 8 days of incubation cells were centrifuged at 1500 rpm, re-suspended in phosphate-buffered saline (PBS), and then stained for 15 min at 4 °C in darkness using mouse anti-human fluorochrome conjugated antibodies. Identification of different type of lympohcytes was done using flow cytometry. Lymphocytes B were characterized as $CD3^+$ /$CD19^+$ cells using mouse

anti-human, fluorochrome-conjugated monoclonal antibodies, specific for CD19 Apc-cy 7 and CD3 pacific blue. Monocytes were identified as $CD3^-/C14^+$ using mouse anti-human, fluorochrome-conjugated monoclonal antibodies, specific for CD14 PC7 and CD3 pacific blue.

Monocytes purification

Monocytes were purified from freshly prepared PBMC by negative selection, using blocking agent and a mixture of biotinylated monoclonal antibodies (anti-body mix) against monocytes, according to manufacturer instructions (Invitrogen). Purity of the monocytes was assessed by flow cytometry on LSR-II cytometer (BD Bioscience). Monocytes were characterized as $CD3^-/CD14^+$ cells using mouse anti-human, fluorochrome-conjugated monoclonal antibodies specific for CD14-PC5 and CD3-FITC.

Effect of methanol extract, daphnetin, luteoline-7-glucoside and apigenin-7-glucoside on monocytes maturation

Monocytes were cultured at a final concentration of 10^6 cells/ml in complete RPMI medium, supplemented with 10% SVF (Invitrogen), 1mM sodium pyruvate (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Cambrex biosience, Rockland, ME,USA). Cells were treated at the same day and for 48h by 1µg/ml of the tested extracts and 10 µM of the tested molecules.

Monocytes were then washed with PBS and stained by anti-human, fluorochrome-conjugated monoclonal antibodies specific for HLA-DR-FITC, CD86-PE, CD64-PC5 and CD14-PC7 (BD Biosciences).

Blood samples were collected and processed following the standard ethical procedures of declaration of Helsinki, after obtaining written, informed consent from each donor and local ethical committee approval for the study (Comité de Protection des Personnes Sud-Ouest et Outremer II, Bordeaux University Hospital; ethical committee agreement 2013/57).

Results

Composition of the methanol extract

Daphnetin and two flavone glycosides were identified by HPLC/DAD analysis in methanol extract. Retention times were observed at RT 17,3 min, 30,89 min and 34,4 min for daphnetin, apigenin-7-glucoside and luteolin-7-glucoside respectively (Figure 1).

Effect of *Daphne gnidium* leaf extract on proliferation of PBMC

Incubation of human PBMC for 8days with the methanol extract of *Daphne gnidium* stimulated proliferation of resident B-cells and monocytes. The percentage of B-cells evaluated by flow cytometry ($CD19^+$ cells) increased from ≈ 8% (control cell level) to ≈ 20%, when cells were treated with the extract at 1 µg/ml (Figure 2). The percentage of monocytes ($CD14^+$ cells) increased significantly from < 1% (control cell level) to ≈ 4% due to the methanol extract (Figure 3).

Effect of extract on monocyte activation

CD86, CD64, and HLA-DR expression was analyzed by flow cytometry to determine the level of monocytes activation. Treatment of monocytes for 48h with the methanol extract at 1µg/ml, and daphnetin and apigenin-7-glucoside at 10 µM, increased significantly the level of CD86 expression (Figure 4). In addition, CD64 and HLA-DR expressions were increased compared to levels in untreated cells due to the extract (Figures 5 and 6). Figure 7 shows the adherent cells which appear after incubation with the methanolic extract. These adherent cells should be monocytes after activation and differentiation into macrophages.

Discussion

The use of traditional medicine to modulate the immune response and treating certain disease has been described and several active ingredients derived from plants have been identified and purified [12, 13].

In the present study the methanol extract from *Daphne gnidium* leaves showed a stimulatory effect on immune cells. In fact, our results revealed that the tested extract at 1µg/ml significantly increased B lymphocyte activity. Thus, the incubation of the lymphocytes in the presence of the studied extract increased the expression of CD19 estimated by a comparison with lymphocytes incubated without stimulators. This indicates that the methanol extract possesses mitogenic activity. B lymphocytes are implicated in the humoral immunity and they proliferate into immunoglobulin (Ig) secreting lymphocytes after activation [14]. In the light of these results, we can conclude that the methanol extract from *Daphne gnidium* leaves activate the humoral response.

Certain herbal medicinal ingredients affect the peripheral lymphocyte proliferation and serum antibody titer [15]. Stimulating the humoral immunity was reported with many plants used in traditional medicines [16]. The immunoenhancing activity observed with the tested extract can be attributed to flavonoides. In fact, authors reported that cianidanol and the O-methyl-derivative have an immunoenhancing effect on T and B cell functions [17]. Besides, Al-Abdaly [18] demonstrated significant increase of lymphocyte in mice treated by flavonoides from *crataegus oxyacantha*. However, apigenin-7-glucoside identified in the methanolic extract by HPLC-DAD (data not shown) showed no significant effect on PBMC proliferation. Thus, we hypothesize that PBMC proliferation inducing effect observed with *Daphne gnidium* extracts should be attributed to other unidentified flavonoids or to a mix of their different constituents.

The effect of the methanolic extract on the maturation of monocytes was investigated. Our results revealed that this extract increased the expression of CD64, CD86 and HLA-DR antigens leading to more mature phenotypes of monocytes. Thus, the studied extract enhance the monocytes differentiation into macrophages which play the role of antigen-presenting cells (APCs). In fact, macrophages are involved in the capture of infectious agents, senescent and apoptotic cells [19].

The ligation between macrophages and T cells is strengthened by adhesion molecules on macrophages like B7–1 (CD80) and B7–2 (CD86). A decrease in the expression of certain markers involving the

absence of costimulatory signals leads to the failure of T cell activation and apoptosis [20]. The effect obtained with the methanol extract on CD86, can be ascribed to the presence of daphnetin and apigenin-7-glucoside which enhances CD86 expression.

As CD64 is essential for the uptake of immune complexes [21], we have tested the effect of the methanolic extract, on its expression. The extract was able to induces maturation of monocytes through increasing the expression of CD64.

Daphnetin, luteolin-7-glucoside and apigenin-7-glucoside enhance only slightly the expression of HLA-DR (data not shown). However, they may altogether in the methanolic extract induce a synergetic effect on the expression of HLA-DR, as this extract stimulates the expression of this marker.

Several studies reported the effect of plant on dendritic cell maturation. In fact, Lu et al. [22] showed that *Antrodia camphorata* water extract, enhance CD86 and HLA-DR expression. Besides, Hochu-ekki-to, a traditional Japanese herbal medicine, up regulate CD80, CD83, and CD86 expression [23].

On the other hand, Wang et al. [24] showed that *Areca nut* extracts inhibit monocyte maturation and decrease significantly the expression of CD86 [24].

Conclusion

Based on the present data it may be concluded that *D. gnidium* leaves can impart immunomodulatory effects through stimulation of lymphocyte B and monocyte maturation. The pharmacological-active ingredients in the methanolic extract from *Daphne gnidium* and the signaling pathways involved in the modulation of both lymphocytes and monocytes responses, remain to be further elucidated.

Declarations

Acknowledgements

The authors acknowledge the financial support from the Ministry of Higher Education and Scientific Research of Tunisia.

Conflict of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

References

1. Patwardhan B, Kalbag D, Patki PS, Nagsampagi BA. Search of immunomodulatory agents: a review. Indian Drugs 1990; 28: 348–358.

2. Rao CS, Raju C, Gopumadhavan S, Chauhan BL, Kulkarni RD, Mitra SK. 1994 Immunotherapeutic modification by an Ayurvedic formulation Septilin; Indian J Exp Biol. 1994; 32:553–558.
3. Alamgir M, Uddin SJ. Recent advances on the ethnomedicinal plants as immunomodulatory agents. Ethnomedicine: A Source of Complementary Therapeutics. Debprasad Chattopadhyay. 2010; 227–244 ISBN.
4. Ielpo MT, Basile A, Miranda R. **Immunopharmacological properties of flavonoids**. Fitoterapia. 2000; **71**:S101.
5. Lee GI, Young H, Rakimn A **et al**. **Inhibitory Effects of Oriental Herbal Medicines on IL-8 Induction in Lipopolysaccharide-Activated Rat Macrophages**. Planta Med 1995; **61**: 65–26.
6. Li Sh H, Wu LJ, Yin HY. Chemical and pharmacological advances of the study on Zushima. Zhongguo zhongyao zazhi. 2000; 27: 401–403.
7. Qi XJ, Li N, Shan YF, Peng YH. Quantitative analysis of syringin from Zu Shima by HPLC. Zhong Cao Yao 2005; 36: 1243–1244.
8. Borris RP, Blasko P, Cordell GA. Ethnopharmacologic and phytochemical studies of the Thymelaeaceae. J Ethnopharmacol. 1998; 24: 41–91.
9. Bellakhdar J, Claisse R, Fleurentin J, Younos C. Repertory of standard herbal drugs in the Moroccan pharmacopoeia. J Ethnopharmacol. 1991; 35: 123–143.
10. Chaabane F, Mokdad-Bzeouich I, Sassi A, et al. Genoprotective and neuroprotective effects of *Daphne gnidium* leaf methanol extract, tested on male mice. Drug Chem Toxicol. 2015; 18:1–6.
11. Chaabane F, Mustapha N, Mokdad-Bzeouich I **et al**. <bi>In vitro</bi> **and** <bi>in vivo</bi> **anti-melanoma effects of** <bi>Daphne gnidium</bi> **aqueous extract via activation of the immune system**. **Tumor Biol** 2015; **doi** 10.1007/s13277-015-4492.
12. Agarwal SS, Singh VK. Immunomodulators: A review of studies on Indian medicinal plants and synthetic peptides: Part I. Medicinal plants. Proc Indian Natl Sci Acad 1999; 65:179–204.
13. Dua PR, Shankar G, Srimal RC, et al. Adaptogenic activity of Indian Panax pseudoginseng. Indian J Exp Biol. 1989; 27: 631–634.
14. Hayashi EA, Granato A, Paiva LS, Bertho AL, Bellio M, Nobrega A. TLR4 Promotes B Cell Maturation: Independence and Cooperation with B Lymphocyte-Activating Factor. J Immunol. 2010; 184: 4662–4672.
15. Kong X, Hu Y, Rui R, Wang D, Li X. Effects of Chinese herbal medicinal ingredients on peripheral lymphocyte proliferation and serum antibody titer after vaccination in chicken. Int Immunopharmacol. 2004; 47: 975–82.
16. Azuma I. Immunostimulants Now and Tomorrow. Japan Scientific Societies Press, Tokyo 1987.
17. Brattig NW, Diao GJ, Berg PA. **Immunoenhancing effect of flavonoid compounds on lymphocyte proliferation and immunoglobulin synthesis**. Int J Immunopharmacol. 1984; **6**: 205–215.
18. Al-Abdaly A. Effect of flavonoids extracted from hawthorn (crataegus oxyacantha) on some hematological parameters of female mice. Al-Anbar J Vet Sci. 2011; 4: 145–151.

19. Aderem A, Underhill DM. **Mechanisms of phagocytosis in macrophages**. Annu Rev Immunol 1999; **17**:593–623.
20. Doetze A, Satoguina J, Burchard G, Rau T, Fleischer B, Hoerauf A. Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T h3/Tr1-type cytokines IL-10 and transforming growth factor but not by a Th1 to Th2 shift, Int Immunol. 2000; 12: 623–630.
21. Daeron M. **F_c receptor biology**. Annu Rev Immunol. 1997; **15**:203–234.
22. Lu M-C, Hwang SL, Chang FR, et al. Immunostimulatory effect of Antrodia camphorata extract on functional maturation of dendritic cells. Food Chem. 2009; 113:1049–1057
23. Nabeshima S, Murata M, Hamada M, Chong Y, Yamaji K, Hayashi J. Maturation of monocyte-derived dendritic cells by Hochu-ekki-to, a traditional Japanese herbal medicine. Int Immunopharmacol. 2004; 4: 37–45.
24. Wang CC, Chen TY, Wu HY, Liu TY, Jan TR. Areca nut extracts suppress the differentiation and functionality of human monocyte-derived dendritic cells. J Periodont Res. 2012; 47: 198–203.

Figures

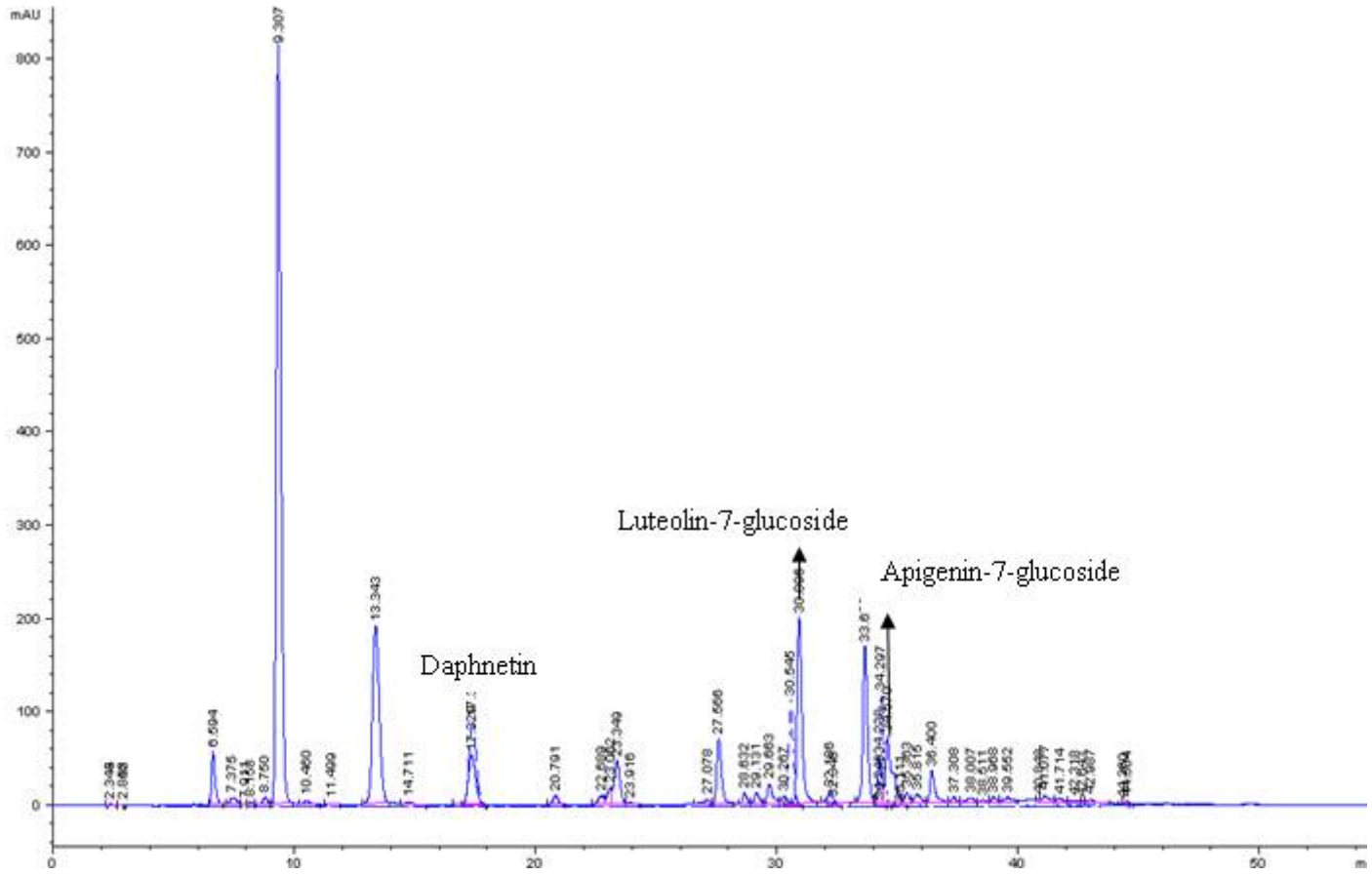


Figure 1

HPLC analysis of methanol extract

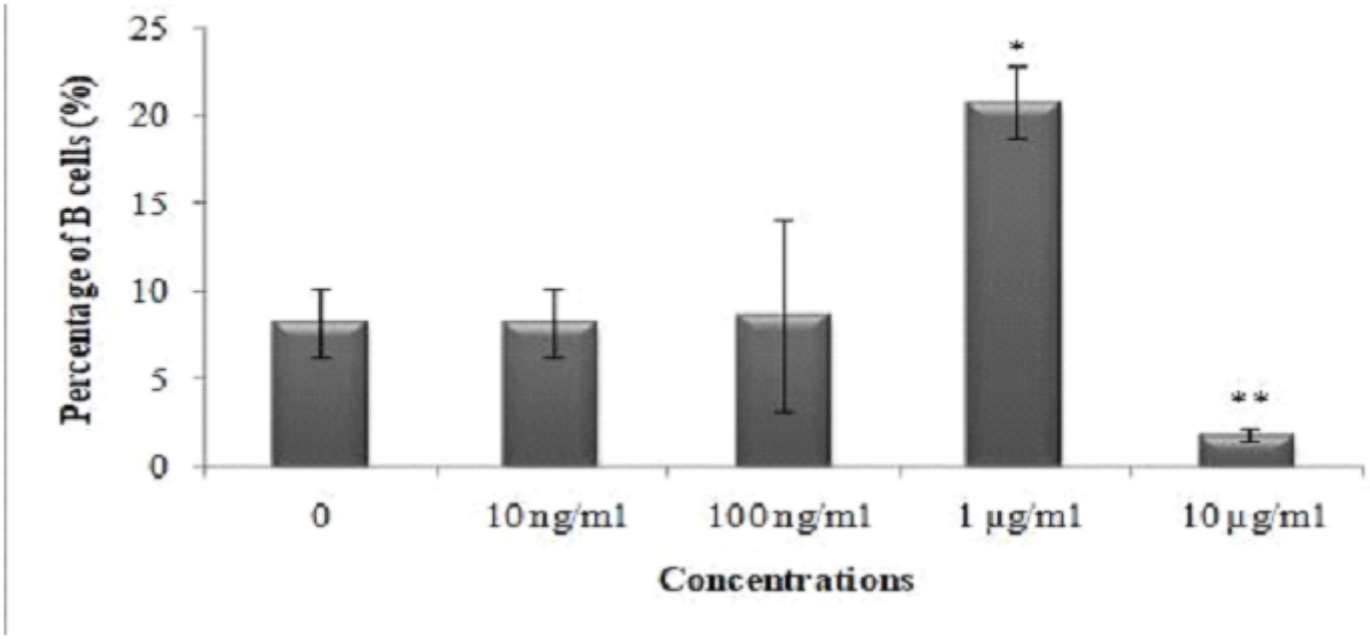


Figure 2

Effect of the methanol extract (1 µg/ml) from *Daphne gnidium* leaves on B-lymphocyte proliferation after 8 days of treatment. Values shown are means ± SD of three separate experiments. *Value significantly from negative control ($p < 0.05$).

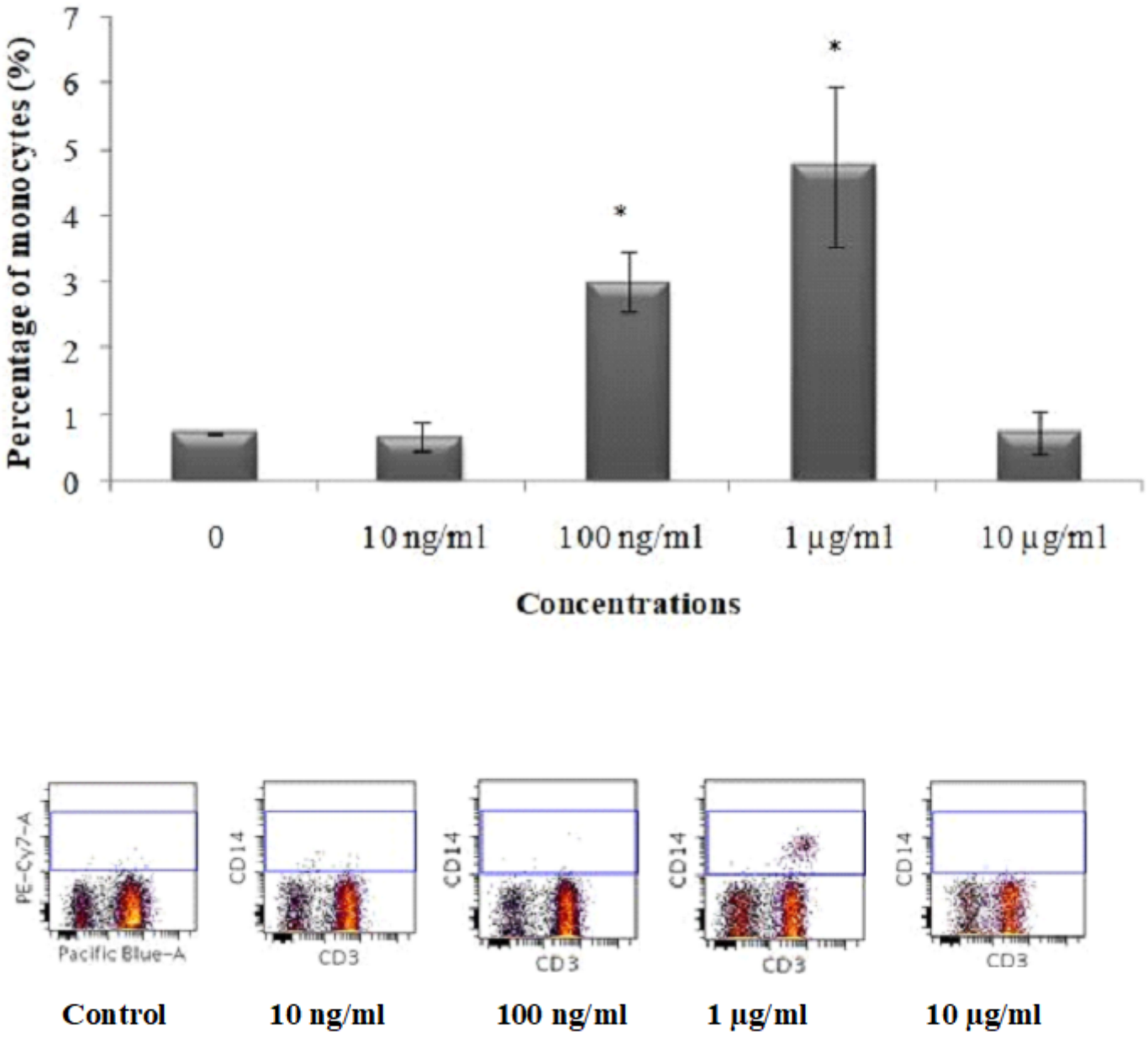


Figure 3

Effect of the methanol extract (1 µg/ml) from *Daphne gnidium* leaves on monocytes proliferation after 8 days of treatment. Values shown are means ± SD of three separate experiments. *Value significantly from negative control ($p < 0.05$).

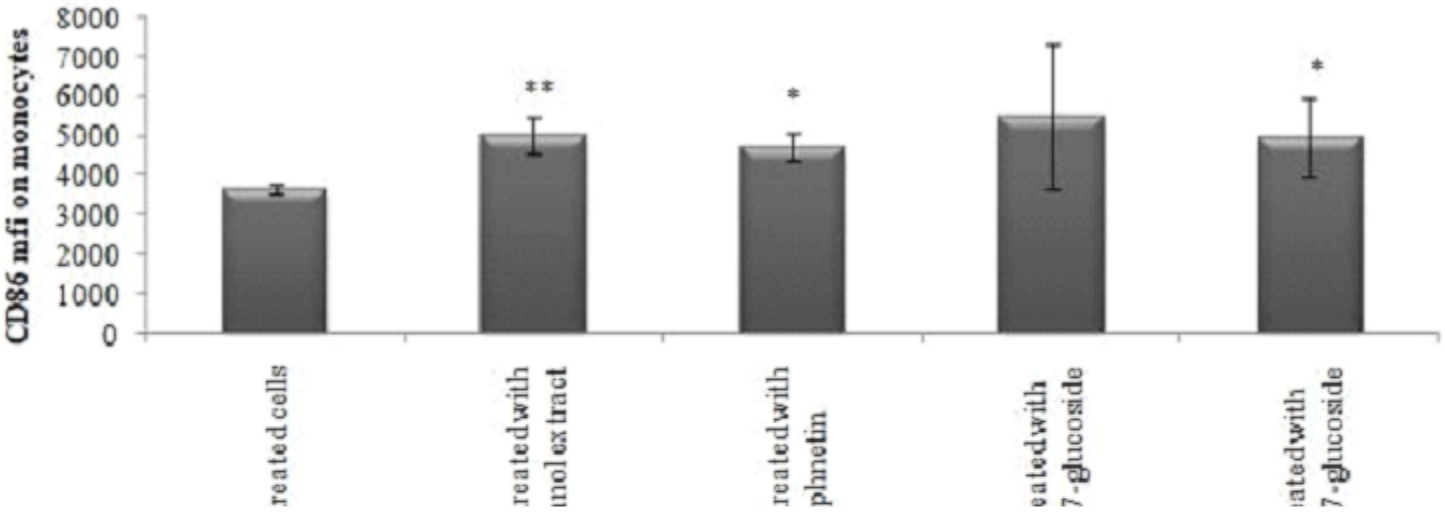


Figure 4

Methanol extract from *Daphne gnidium* leaves (1µg/ml), daphnetin, luteolin-7-glucoside and apigenin-7-glucoside (10 µM) promote CD86 expression after 2 days of treatment. Values shown are means ± SD of three separate experiments. Value significantly from negative control (*p < 0.05, **p < 0.01). MFI: Mean fluorescence intensity.

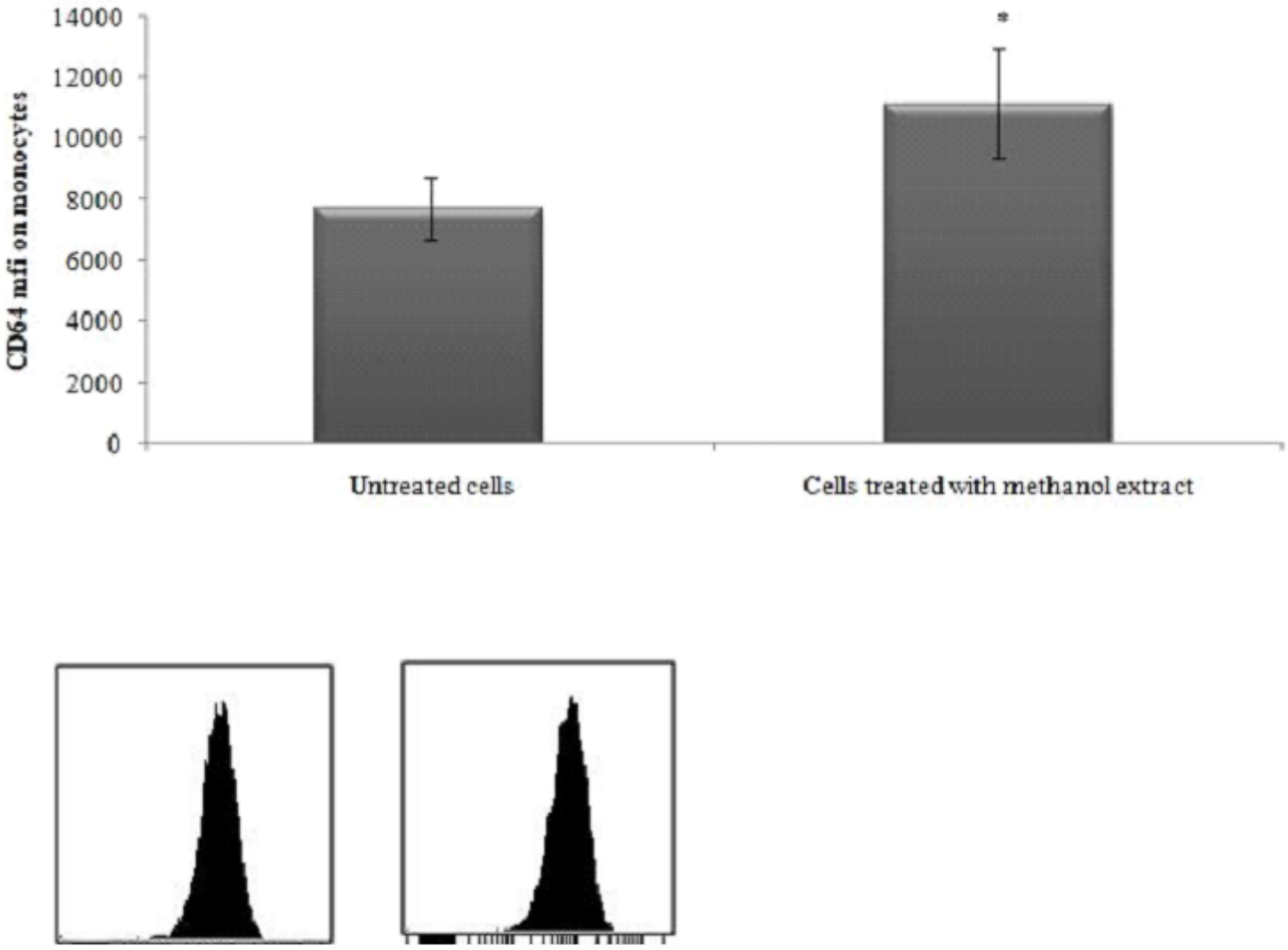


Figure 5

Methanol extract promotes CD64 expression after 2 days of treatment. Values shown are means ± SD of three separate experiments. *Value significantly from negative control ($p < 0.05$). MFI: Mean fluorescence intensity.

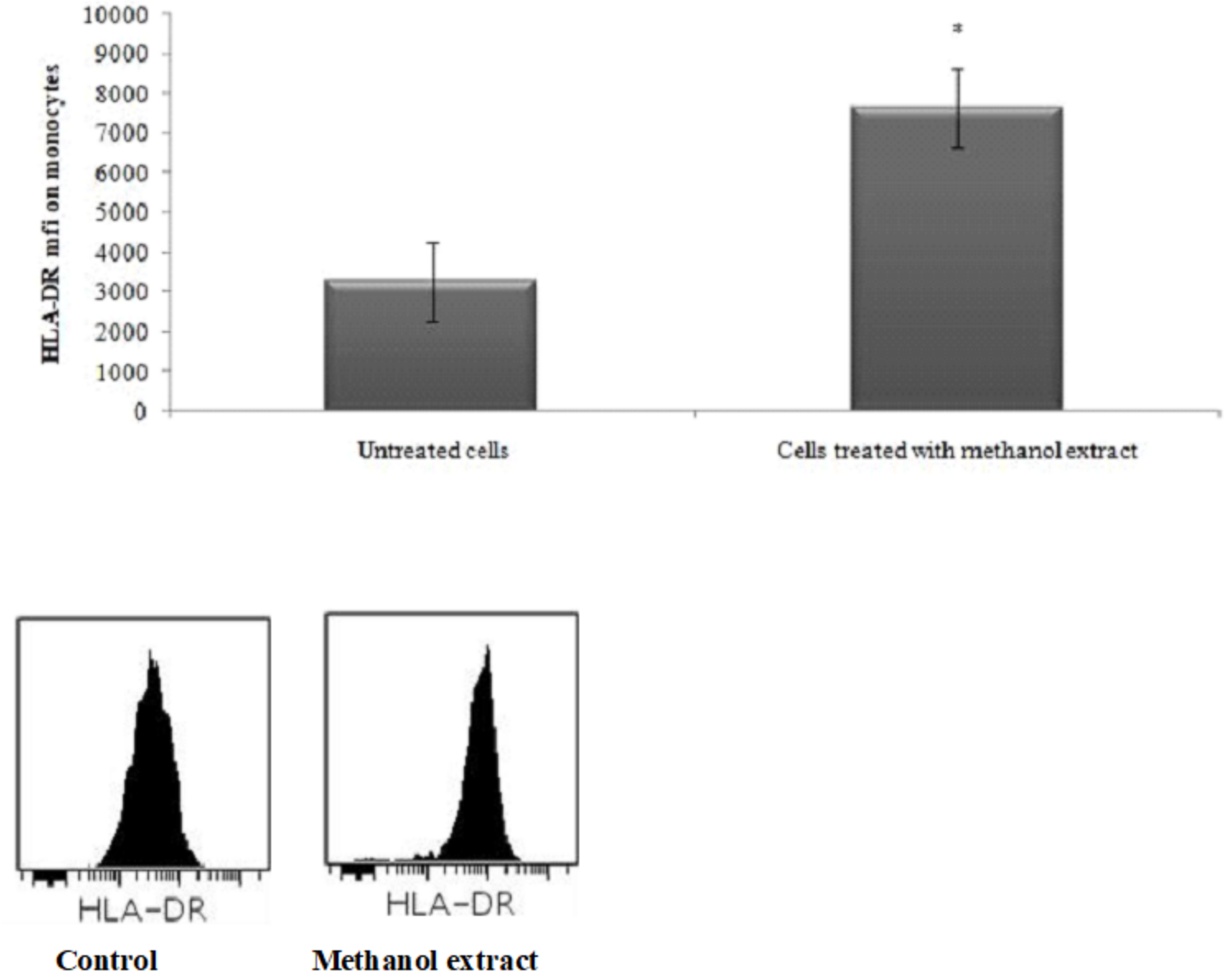
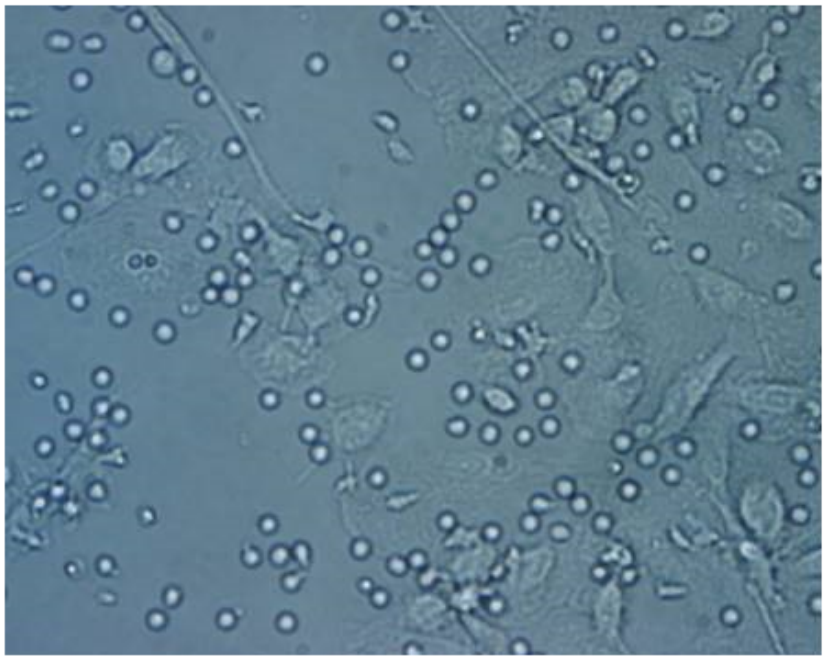


Figure 6

Methanol extract promotes HLA-DR expression after 2 days of treatment. Values shown are means ± SD of three separate experiments. *Value significantly from negative control ($p < 0.05$). MFI: Mean fluorescence intensity.

(A)



(B)

Figure 7

Metahnol extract enhances monocyte differenciation. **(A)** Untreated cells and **(B)** cells treated with methanol extract at 1µg/ml: we can see adherent cells which may be monocytes differentiated into macrophages.