

Full Length Research Paper

A new immunoregulatory agent from *Rosa damascena* reduces CD3 expression in human peripheral blood mononuclear cells (PBMCs)

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T-Lymphocytes (T-cells) play a dominant role in the initiation and maintenance of autoimmune inflammatory processes. They become activated by the presentation of antigen such as auto antigen. A main feature of T-cell activation is the production of cytokines. Though, uncontrolled T-cells activation through T-cell receptor (TCR) has been linked to numerous autoinflammatory and autoimmune pathologies. This study investigates the impact of methanol extracts from *Rosa damascena* on suppress T-cells activation. Peripheral blood mononuclear cells (PBMC) were treated by 10, 50,100 µg/ml of *R. damascene* methanolic extracts after activation of T cells with anti- CD3 and anti-CD28. The inhibitory role of methanol extracts of *R. damascena* to suppress the activated T-cells was measured using flow cytometric at three different time points (24, 48, and 72 h). The result showed that the methanol extraction from *R. damascena* petal parts can inhibit the activated T-cells in a dose dependent fashion as we observe reduced levels of cluster of differentiation of CD 3. It is concluded that, extracts of *R. damascena* could be used as a new agent (immunoregulatory) to suppress T-cell activity and treating variety of immune disorders with a low risk of side effects in future.

Key words: T-lymphocytes (T-cells), autoimmune inflammatory, *Rosa damascena*, (CD) 3.

INTRODUCTION

The proinflammatory activity of T-cells could be beneficial to the host during infection. Nevertheless, inappropriate T-cells activation has been associated with some autoimmune and autoinflammatory pathologies. Actually, clinical data shows that T-cells are linked to many autoimmune diseases including arthritis, multiple sclerosis, psoriasis, and lupus (Harrington et al., 2005). It

is evident from years of research and development of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, diclofenac sodium and ibuprofen which have had a measure of success in improving pain associated with autoinflammatory disorders and even reducing cellular/hormonal mechanisms involved in this process, through inhibition of initial steps in the biosynthesis

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pathway of prostaglandins (Albert et al., 2002).

With increased use of non-steroidal anti-inflammatory drugs, more side effects have been reported including ulceration, gastric injury, renal damage and cardiac abnormalities which limit their use (Dogne et al., 2006). Similarly Corticosteroids have effective anti-inflammatory activity but have no intrinsic analgesic properties (Coutinho and Chapman, 2011). They are only suitable for treating pain associated with inflammation (Tapiero et al., 2002). Therefore, there is strong interest in the development of new anti-inflammatory drugs, with possibly less side effects, based on natural products

Rosa damascena, also known as Damask rose (Kaul et al., 2000) has been used in traditional medicine for the treatment of several diseases (Boskabady et al., 2011). Herbal active components have various mechanisms of action; finding these active components and their mechanisms in the immune system is remarkably important (Megna et al., 2012). Some of herbal drug functions are characterized by their influence on lymphocytes (Wilasrusmee et al., 2002). *R. damascena* is a perennial erect shrub with aromatic light pink flowers, belongs to genus *Rosa* species (Rosaceae family) (Kaul et al., 2000).

R. damascena species is native to Damascus, Syria; grown in many countries all over the world for visual beauty and its use in production of fragrances (Boskabady et al., 2011). This plant is a rich source of vitamin C, flavonoids, tannins, carboxylic acids and myrcene (Schiber et al., 2005). In addition to its perfuming properties, it has been traditionally used in medicine to treat chest pains, relieve digestive problems, reduce fever and menstrual bleeding (Yassa et al., 2015). Valuable therapeutic applications of *R. damascena* in modern medicine have been reported as antimicrobial (Basim and Basim, 2003), antitumor (Zu et al., 2010), anti-depressant (Boskabady et al., 2006), anti-oxidant (Baydar and Baydar, 2013), and anti-inflammatory properties (Boskabady et al., 2011; Winther et al., 2005).

Though therapeutic and biological effects of *R. damascena* have been described, its effect on the immune system is not precisely investigated. These studies in traditional and modern medicine suggest that *R. damascena* has diverse effects on the immune system. Investigations over the last 20 years have shown that anti-CD3 drugs effectively treat autoimmune disease in animal models and have also shown promise in clinical trials.

The present study was undertaken to investigate whether alcoholic rose petals extracts has immunoregulatory suppressor effect on T-cell receptor (TCR) activities, as these cells are the major effector cells in cellular and humoral immune responses. Peripheral blood mononuclear cells (PBMCs) were the cells of choice in this study, as these cells are the key immune cell populations that can be easily used to measure the effects of drug treatment especially for researchers,

who prefer to use human samples.

MATERIALS AND METHODS

Plant material and preparation of extract

Flowers of *R. damascena* were collected early morning in April 2016, during the harvest season from Al Hada local farm (Taif-Saudi Arabia). The flowers were taxonomically identified and authenticated by Dr. Hadeer Darwesh, Biotechnology Department, Taif University. The total ethanol extracts was prepared by Soxhlet extraction method.

Fresh petal parts of the flowers were extracted with 100% methanol at ratio 8:1. The process of extraction carries on for 6 h at 60°C. The crude methanol extract was obtained after evaporating the ethanol to dryness at 40°C under reduced pressure. The crude extract was collected and stored at 20°C for further studies, and the percentage yield of the extract was calculated. 100% Dimethyl sulfoxide (DMSO) was used later to dissolve the dried extracts and prepare in different concentrations.

Antibody coating of the plate

24 well plates (Costar, Corning, NY) were pre-coated with 1 µg/ml of mouse anti-human CD3 mAb (BD Bioscience) in sterile (phosphate-buffered saline) PBS was prepared, a 50 µL of the antibody solution was dispensed to each well of the 24 well plate. The plates were incubated at 37°C for 2 h, and the solution was removed and rinsed twice with 200 µL of sterile PBS to remove all unbound antibody from each well before adding cells (Protocol was adapted from eBiosciences).

Human PBMCs separation and culture

Blood samples were obtained from healthy donors. The mean age of the patients was 49 years. 15 mL blood was drawn in a heparin tube (Becton Dickinson, Lincoln Park, NJ, USA). PBMC were isolated by mixed 1:1 with PBS and 20 mL histopaque was carefully carried out under layered in 50 mL tubes, followed by centrifugation at 1800 x g at room temperature for 20 min. The PBMCs were removed using 5 ml pipette, washed twice in Hank's Buffered Saline and cells were centrifuged at 400 g for 10 min.

The cell pellet was finally washed twice with RPMI 1640 (Gibco BRL, Uxbridge, UK) and re-suspended in medium consisting RPMI 1640, supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 25 mM HEPES buffer, 50 u/ml penicillin and 50 µg/ml streptomycin. (All from Sigma Chemical Co., Poole, UK).

T-lymphocyte activation and rose extracts treatments (effects of rose extracts on t-cell activation)

One (1) mm aliquots of the PBMC suspension at a concentration of 1×10^6 cells/mL were added to 24 well plates, pre-coated with anti-human CD3 mAb and for each condition, triplicate wells were used. Soluble anti-CD28 was added to cells at 1 µg/mL. Cells were treated with 10 µl of rose at various concentrations (0.5, 0.005, 0.00005 µg/ml) and plates were placed to humidified at 37°C, 5% CO₂ incubator. Cells were cultured at three different time points (24, 48, and 72 h). Control cells were incubated with 10 µl of RPMI 1640 for untreated cells, and with 10 µl of 100% DMSO.

Flow cytometric analysis

Immunofluorescence staining was performed according to the

method described by Beutner (1961). In brief, cells were harvested, washed in PBS containing 0.5% (v/v) BSA, and re-suspended in PBA buffer (PBA was prepared by mixing PBS and 0.5% BSA to 0.1% Sodium azide). Cells were then mixed with mouse anti-human CD25 PE-, CD4 FITC-, CD69 PCS-, and CD3 ECD-conjugated antibodies (BD Pharmingen) (R and D Systems, Abingdon, UK) and incubated on ice for 30 min in the dark.

Cells were then washed twice with PBS containing 0.5% BSA, re-suspended in 0.5% (w/v) paraformaldehyde fixative, and analysed by flow cytometry (FC500 flow cytometer, Beckman Coulter, Brea, CA).

Data analysis

Statistical analysis was achieved using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) was used to analyze data. The p-values less than 0.05 were considered to indicate significant differences. Results are expressed as a mean \pm standard deviation (SD).

RESULTS

This study was undertaken to evaluate methanol *R. damascena* petal parts extracts effects on suppressing the activated T-lymphocytes (T-cells), by investigating the expression level of cluster of differentiation (CD) 3 that was expressed on the surface of T-cells. To investigate the likely inhibitory role of methanol extracts of *R. damascena* and their ability to suppress the activated T-cells flow, cytometric analysis was performed to investigate the expression level of CD3 T-cell co-receptor. The harvested cells were obtained after activation of T-cells and treated with different concentrations 100, 50 and 10 $\mu\text{g/ml}$ methanol extracts of *R. damascena*, analysed at three different time points (24, 48, and 72 h). The level of CD3 expression by the treated cells were compared with those of unactivated (untreated) and activated DMSO treated cells.

According to the results, the expression level of CD3 increased in control cells treated with 100% DMSO alone after 2, 48, and 72 h (Figures 1, 2 and 3, respectively) as compared to unactivated (untreated) cells, which showed significantly decreased in the expression of CD3 ($p < 0.0001$). However, the expression of CD3 decreased when the cells were treated with methanol extraction from *R. damascena* petal parts, demonstrating that the extracts could suppress the activated T cells in a dose-dependent manner significantly.

When the methanol extraction from *R. damascena* petal parts concentration rise to 100 $\mu\text{g/ml}$, the expression of CD3 being equally as unactivated (untreated) cells was significantly decreased ($p < 0.0001$) at all point in time when compared to cells treated with 100% DMSO alone. Treatment with 50 $\mu\text{g/ml}$ concentration result in significant difference in CD3 expression as compared with that of 100% activated DMSO treated cells (24 h $p < 0.05$, 48 h $P < 0.0001$ and 72 h

$P < 0.01$). Treatment with 10 $\mu\text{g/ml}$ concentration from *R. damascena* extracts at 24 and 72 h showed no significant difference in the expression of CD3 compared with 100% activated DMSO treated cells, but the expression of CD3 was significantly decreased at 48 h ($P < 0.01$) indicating that at this point in time, this concentration provide significant suppress to activated T-cells as compared to other time points.

In other words, the result suggested that the methanol extraction from *R. damascena* petal parts can inhibit the activated T-cells as shown in Figures 1, 2, and 3 compared with 100% activated DMSO treated cells alone, the activity of the methanol extraction from *R. damascena* petal parts in low dose is less effective. But with extracts concentration increasing to 100 $\mu\text{g/ml}$, it could significantly suppress the activated T-cells.

DISCUSSION

R. damascena is an ornamental plant, a member of Rosaceae family with more than 200 species and 18,000 cultivars around the world. Besides its perfuming effect, many pharmacological properties have been reported by several other researcher (Boskabady et al., 2011; Sedighi et al., 2014), which is of great interest in *R. damascena* because of its stated valuable effect in a variety of immune conditions and also for suppression of the immune system particularly lymphocytes.

R. damascena contains several compounds such as flavonoids, kaempferol, geraniol, citranellol, eugenol, linalool, nerol, myrcene and vitamin C that have applications in several therapeutic areas including HIV (Mahmood et al., 1996), diabetes (Gholamhoseinian et al., 2009), depression (Boskabady et al., 2006), cancer (Venkatesan et al., 2014), inflammation (Hajhashemi et al., 2010), and several infectious areas (Basim and Basim, 2003).

Exploration of the methanol *R. damascena* petal parts extracts effect on the expression of CD3, presented on the surface of activated lymphocyte (T cells) showed decreased in CD3 expression in a dose-dependent manner. Higher concentrations (100 and 50 $\mu\text{g/ml}$) of *R. damascena* petal parts extracts have a suppressive effect on activated T cells at all point in time (24, 48, and 72 h) as shown in Figures 1, 2, and 3 compared with 100% DMSO alone, the suppressive activity of the methanol extraction from *R. damascena* petal parts in low dose (10 $\mu\text{g/ml}$) is less effective.

Briefly, our results have revealed that *R. damascena* ingredients in alcohol extraction suppress the activated T-cell. The suppressive properties of *R. damascena* in cell growth have been studied several times and it has been found that *R. damascena* could be used in inhibition of cancer cell development which induces the apoptosis and increases the expression of apoptotic protein (Elson, 1995).

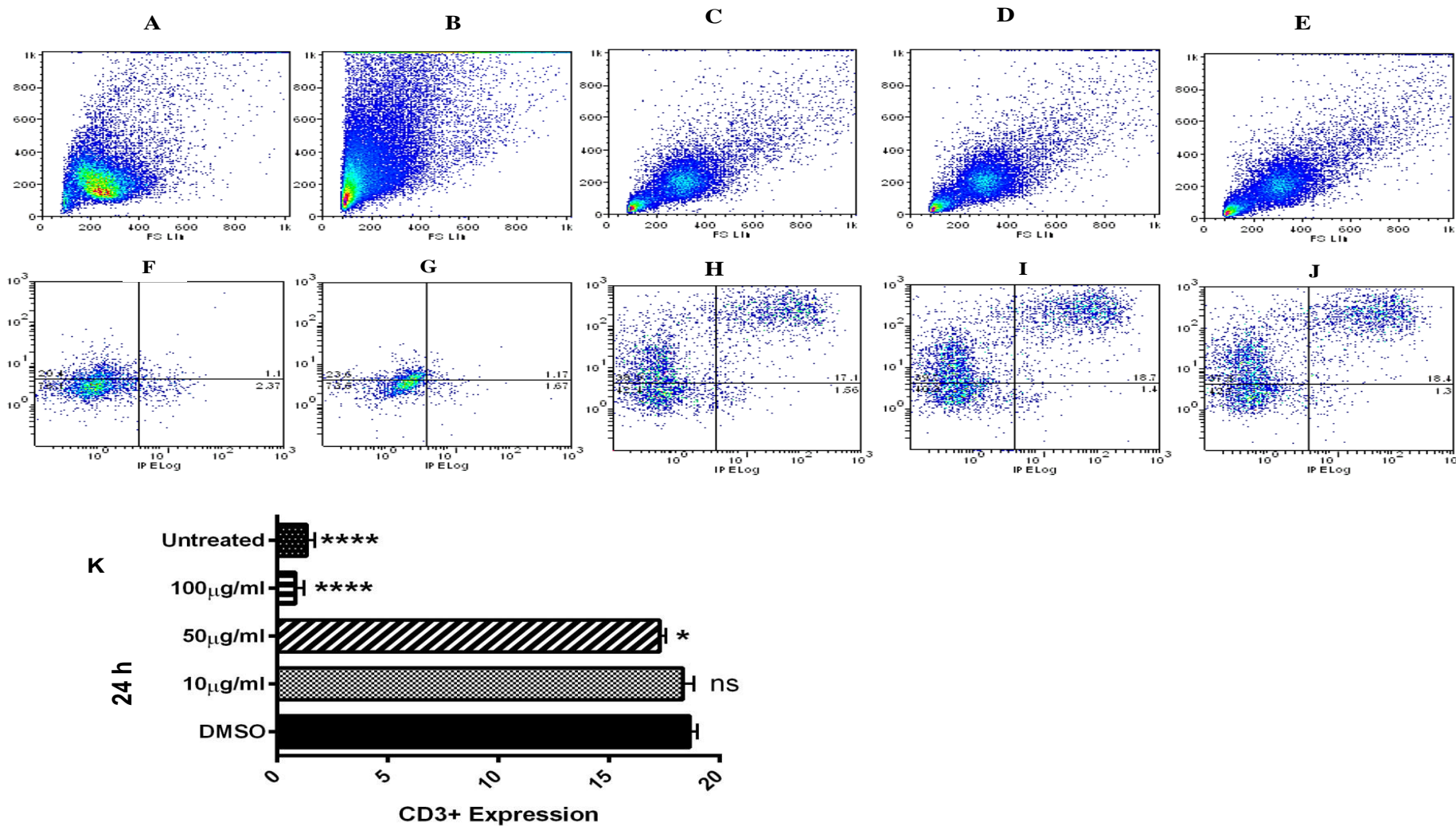


Figure 1. Assessments of the CD3 expression in PBMCs treated with methanol extracts of *R. damascena* petal parts by cytometric analysis after 24 h. (A, B, C, D and E) show the forward scatter (FSC) versus side scatter (SSC) dot blot. (F, G, H, I and J) y-axis shows PCS fluorescence log and the x-axis shows IPE log fluorescence. The cells were treated as follows: A & F untreated cells; B&G cells treated with 100 µg/ml methanol extracts of *R. damascena*, C&H cells treated with 50 µg/ml methanol extracts of *R. damascena*, D&I cells treated with 10 µg/ml methanol extracts of *R. damascena*, E&J) Negative control cells treated with DMSO. All treated cells were incubated for 24 h and then harvested and washed in PBS, and stained using conjugated antibodies [mouse anti-human CD3 PE-, and CD28 PCS (BD Pharmingen) (R&D Systems, Abingdon, UK)] for 30 min on ice in the dark. Cells were washed twice, and analysed by flow cytometry (FC500 flow cytometer, Beckman Coulter, Brea, CA). K) Multiple comparison of the suppress effects of different concentration of 100, 50 and 10 µg/ml methanol extracts of *R. damascena* compared to the effect of 100% DMSO treatment; data were analysed by One-way ANOVA and P values are shown where the difference between responses of different treatment relative to the control were determined to be statistically significant: ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. These results are representative of 3 independent repeats. FS Lin, forward scatter; SS Lin, side scatter.

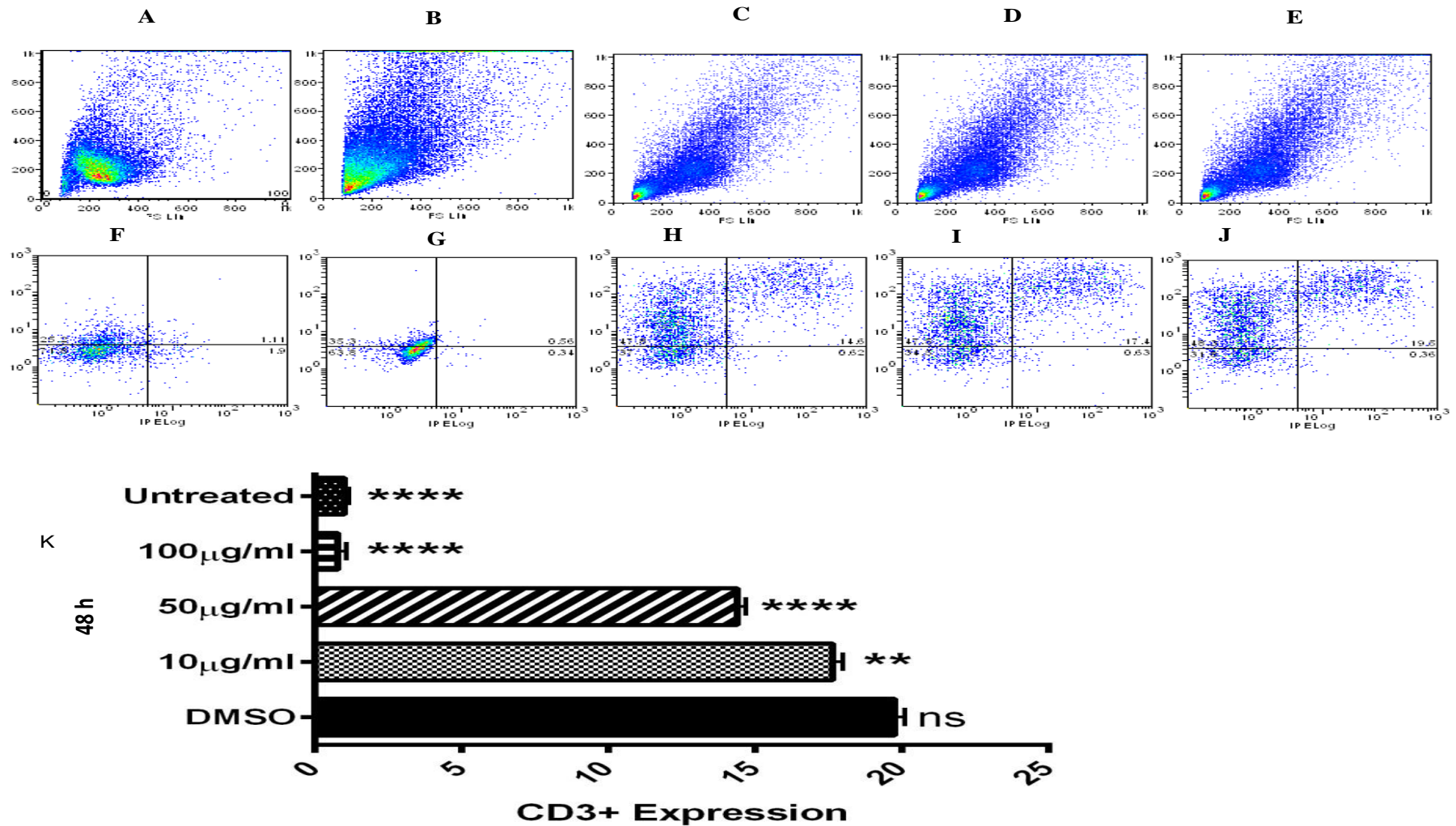


Figure 2. Assessments of the CD3 expression in PBMCs treated with methanol extracts of *R. damascena* petal parts by cytometric analysis after 48 h. (A, B, C, D and E) show the forward scatter (FSC) versus side scatter (SSC) dot blot. (F, G, H, I and J) y-axis shows PCS fluorescence log and the x-axis shows IPE log fluorescence. The cells were treated as follows: A & F, untreated cells; B&G, cells treated with 100 μ g/ml methanol extracts of *R. damascena*; C&H, cells treated with 50 μ g/ml methanol extracts of *R. damascena*; D&I, cells treated with 10 μ g/ml methanol extracts of *R. damascena*; E&J, negative control cells treated with DMSO. All treated cells were incubated for 48 h and then harvested and washed in PBS, and stained using conjugated antibodies [mouse anti-human CD3 PE-, and CD28 PCS (BD Pharmingen) (R&D Systems, Abingdon, UK)] for 30 min on ice in the dark. Cells were washed twice, and analysed by flow cytometry (FC500 flow cytometer, Beckman Coulter, Brea, CA). K) Multiple comparison of the suppress effects of different concentration 100, 50 and 10 μ g/ml methanol extracts of *R. damascena* compared to the effect of 100% DMSO treatment, data were analysed by One-way ANOVA and P values are shown where the difference between responses of different treatment relative to the control were determined to be statistically significant: ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. These results are representative of 3 independent repeats.

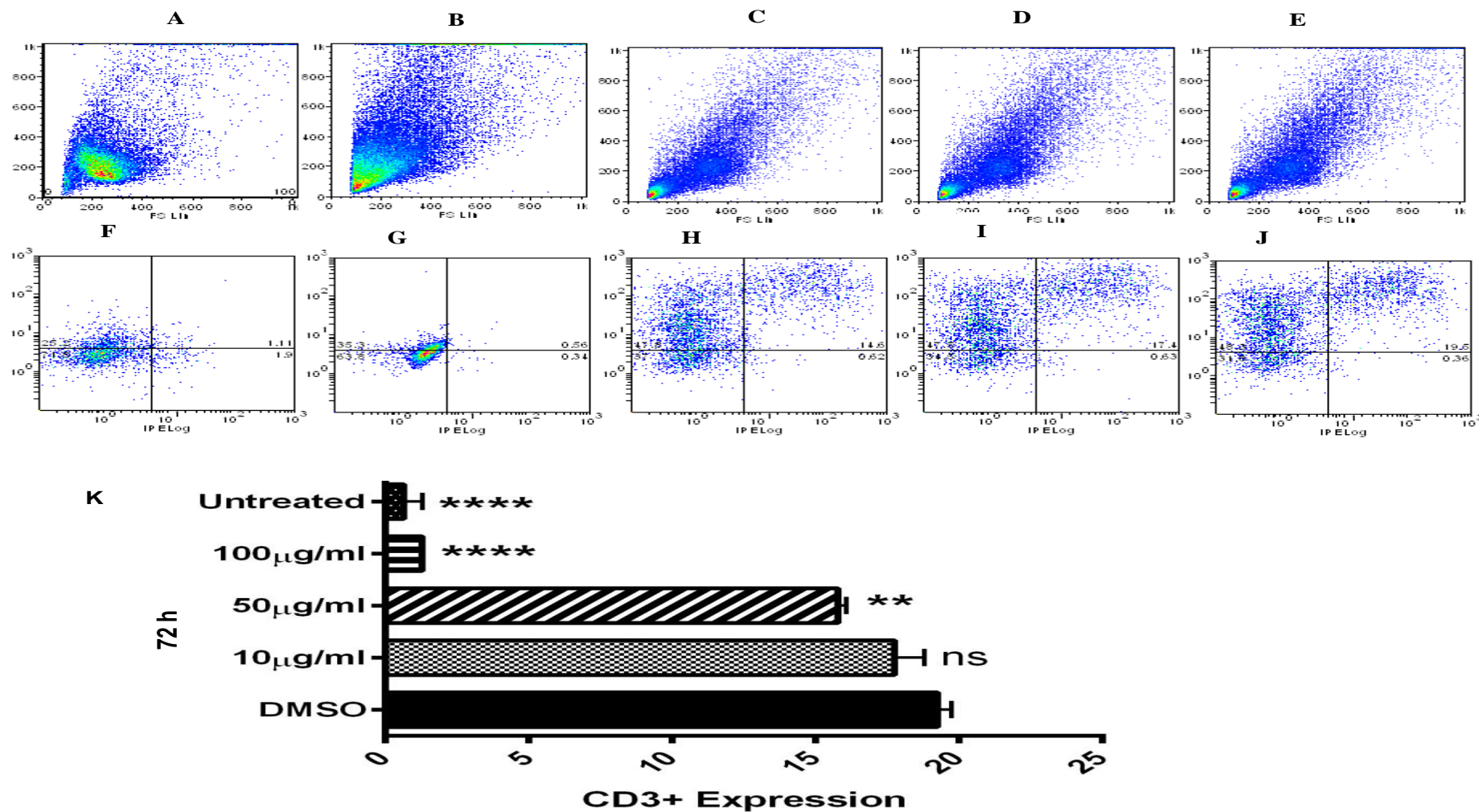


Figure 3. Assessments of the CD3 expression in PBMCs treated with methanol extracts of *R. damascena* petal parts by cytometric analysis after 72 h. (A, B, C, D and E) show the forward scatter (FSC) versus side scatter (SSC) dot blot. (F, G, H, I and J) y-axis shows PCS fluorescence log and the x-axis shows IPE log fluorescence. The cells were treated as follows: A & F, untreated cells; B&G, cells treated with 100 $\mu\text{g/ml}$ methanol extracts of *R. damascena*; C&H, cells treated with 50 $\mu\text{g/ml}$ methanol extracts of *R. damascena*; D&I, cells treated with 10 $\mu\text{g/ml}$ methanol extracts of *R. damascena*, E&J) Negative control cells treated with DMSO. All treated cells were incubated for 72 h and then harvested and washed in PBS, and stained using conjugated antibodies [mouse anti-human CD3 PE-, and CD28 PCS (BD Pharmingen) (R&D Systems, Abingdon, UK)] for 30 min on ice in the dark. Cells were washed twice, and analysed by flow cytometry (FC500 flow cytometer, Beckman Coulter, Brea, CA). K) Multiple comparison of the suppress effects of different concentration 100, 50 and 10 $\mu\text{g/ml}$ methanol extracts of *R. damascena* compared to the effect of 100% DMSO treatment, data were analysed by One-way ANOVA and P values are shown where the difference between responses of different treatment relative to the control were determined to be statistically significant: **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. These results are representative of 3 independent repeats.

Conclusion

It might be concluded that the methanol extracts of *R. damascena* petal parts was obtained through Soxhlet extraction method. The results from the *in vitro* proved that the extracts could suppress the activated T-cells, as we observe reduced levels of CD 3.

It is inferred that extracts of *R. damascena* can be used as a new agent (immunoregulatory) suppressor T-cell activities. Moreover, this could restore cellular immunity, which would have valuable applications as it offers an effective and safe strategy for treating variety of immune disorders, with a low risk of side effects in future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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