Galician medical journal 2022
Vol. 29, Issue 1, E202215
DOI: 10.21802/gmj.2022.1.5

Research Article | **Internal Medicine**

Cytokine Response of CD4+ T-Lymphocytes with Red Rose (*Rosa Rosaceae* – Pierre de Ronsard) Extracts by *in Vitro* Evaluation

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Abstract

Background. Red rose extract is known to have anti-inflammatory and immune-modulation effects. In this study, the red rose extract was tested on CD4+T lymphocytes *in vitro*, and cytokine response was evaluated.

Materials and Methods. The red rose (*Rosa Rosaceae* – Pierre de Ronsard) extract used in this study was prepared and stored at -20°C until use. CD4+T-cells were seeded in 96-well plates at 313,500 cells/well in 100 μl cell culture medium in duplicate. One-half of the wells were used for biomarker screening in the culture medium, and the other half was used for cytotoxicity assay. Twenty-four hours after plating, the cells were treated in duplicate with 100 μl of the red rose extract diluted at 0.5%, 0.1%, 0.05%, 0.01% and 0.005% (v/v) in the cell culture medium or with culture medium only as control for 72 hours. Some other wells were allocated for untreated cells, and cells treated with the rose extract at 0.005% for 48-h incubation time.

Results. Several cytokines (GRO; IFN-γ; IL-1α, 6, 10; MCP-1; RANTES; TGF-β1; TIMP 1, 2; Ang1, Ang2; G-CSF; MMP-9; and VEGF R2) were elevated. Except for MMP-9, which had fold changes $>$ 2, other cytokines were minimally elevated at various concentrations and timing of rose extract treatment. None of the mentioned cytokines were less than 0.8-fold after treatment with the rose extract. Cytotoxicity assay revealed insignificant changes in the viability of T-cells.

Conclusions. There was a mild elevation in few inflammatory markers by CD4+ T-lymphocytes after *in vitro* treatment with the red rose extract (*Rosa Rosacea* - Pierre De Ronsard). Further *in vitro* and *in vivo* studies are required to evaluate the benefits of the red rose extract in immune regulation.

Keywords
Inflammation; Immune Response; Rose Extract; Pierre De Ronsard; Cytokine Levels; CD4+ T-Cells

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Introduction

Immune diseases are common in clinical practice [1, 2]. The incidence of immune-related dysfunction is seen in about 7.5% to 9.5% of the population [1]. Prevalence rates range from less than 5 cases per 100,000 people (e.g., chronic active hepatitis, uveitis) to more than 500 cases per 100,000 people (Grave disease, rheumatoid arthritis, thyroiditis) [2]. In the recent years, the incidence of autoimmune disorders continues to remain high [3]. Most non-communicable diseases, malignancies and infections also have some immunological background [4]. Positive immune modulation with reducing systemic inflammation has potential advantages as a therapy [5, 6]. However, current immunomodulators could reduce lymphocyte proliferation [7, 8] and function [9] which may lead to various side-effects. Some of the side effects include susceptibility to infections [10], worsening of metabolic parameters [11, 12], renal dysfunction [13], etc. T-cells play a key role in an immune response [14, 15] and discovering novel immunomodulators with no side effects and proliferation reducing remains relevant. Roses are widely used in medicine [16–19], however, the effects of red rose "Pierre de Ronsard" on T-cells are not well-documented.

This study was aimed to investigate the immunomodulation effect of the red rose (*Rosa Rosaceae* – Pierre de Ronsard) extract on CD4+ T-cells in vitro.

Materials and Methods

CD4+T lymphocyte *In Vitro* Treatment

The red rose (*Rosa Rosaceae* – Pierre de Ronsard) extract from the rose petals used in this study was prepared [20] and stored at -20°C until use. Human peripheral blood CD4+ T-cells (Cell Applications, ref. 6902-50a, lot 3298) were seeded in 96-well plates at 313, 500 cells/well (950,000 cells/cm^2) in 100 μl cell culture medium (Cell Applications, ref. 615-250) in duplicate, with one half of wells used for biomarkers screening in the culture medium and the other half for cytotoxicity assay. Twenty-four hours after plating, the cells were treated in duplicate with 100 μl of the red rose extract diluted at 0.5%, 0.1%, 0.05%, 0.01% and 0.005% (v/v) in the cell culture medium or with culture medium only as control for 72 hours. Some other wells were used for untreated cells and cells treated with the rose extract at 0.005% for 48-h incubation time. After 48 and 72 hours, the corresponding wells were used for cytotoxicity assay and from the duplicate wells, the cell culture media were collected and stored at -80°C until biomarker screening assay. Biomarker screening assay was performed with Human Angiogenesis Antibody Array G-Series 1000 from RayBio®AAH-ANG-G1000.

Cytotoxicity Assay

After 48-h incubation time, cell viability was assessed using - Cell Counting Kit–8 (Dojindo EU Gmbh, ref. CK04). The kit uses WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) or MTT which produces a water-soluble formazan dye upon bio-reduction in the presence of an electron carrier, 1- Methoxy PMS (phenazine methosulfate). WST-8 (10% of the medium volume in wells) was added to the media for 4 hours at 37°C. During the incubation time, it is bio-reduced by cellular dehydrogenases to an orange formazan product that is soluble in the culture medium. Then, the amount of formazan produced is directly proportional to the number of living cells. The absorbance of formazan was measured at 450 nm, which enabled the calculation of viable cells percentage for each treatment compared to non-treated cells.

Profiling of Secreted Cytokines

The technique of Quantibody assay is similar to the method described in our previous studies [20]. During the incubation time, the volume of the medium varied differently in each well and the volume collected might be reduced. To normalize the profiling results, the cell culture medium was added to the different collected media to reach 200 μl before performing profiling assay. Then, the medium samples were tested undiluted on arrays.

Results

The results of cytotoxicity assay showed there was no strong effect on the viability of T-cells even though the viability went down until 87% for cells treated for 72 hours with 0.01% red rose extract. Indeed, such a decrease in the viability in one tested replicate is not statistically representative of the cytotoxicity effect of the red rose extract. Table 1 shows the results of cytotoxicity assay.

Interferon γ (IFN-γ), monocyte chemoattractant protein 1 (MCP-1), growth-regulated oncogene (GRO), regulated on activation, normal T-cell expressed and secreted (RANTES) and tissue inhibitor of metalloproteinases (TIMP), Angiopoietin-1 (Ang1) and matrix metallopeptidase 9 (MMP-9) were elevated. Except for MMP-9, which had fold changes > 2, other cytokines were mildly elevated at various concentrations and timing of red rose extract treatment (Table 2 and 3). GRO was mainly elevated at higher (0.5%) and lower (0.005%) concentrations. MCP-1 and RANTES showed an increasing trend with decreasing levels of red rose extract concentrations. Interleukin 6 (IL-6) showed a higher value in higher (0.5%) and lower dilutions (0.005%). The results also showed that cytokines IL-6, IL-10, IL-1α, transforming growth factor beta 1 (TGF-β1), TIMP-2, Ang2, granulocyte-colony stimulating factor (G-CSF), and vascular endothelial growth factor receptor 2 (VEGF R2) were enough detected to enable the calculation of one-fold change at least.

Discussion

Our study results showed cytokines - GRO, IFN-γ, IL-6, MCP-1, RANTES, TGF-β1, TIMP-1, TIMP-2, Ang1, Ang2, G-CSF, IL-10, IL-1α, MMP-9, and VEGF R2 - were enough detected to enable the calculation of one-fold change at least.

Our previous study results of red rose extract treatment on endothelial cells showed a reduction in cytokine levels and other inflammatory markers [20]. In the clinical scenarios, it could be advantageous, i.e., a reduction in the inflammation of endothelial cells when combined with a stimulatory effect on lymphocytes, which is uniquely observed in this study. Cytotoxicity assay in this study revealed insignificant changes with minimal reduction in the viability of cells. The absence of cytotoxicity of the red

Table 1. The results of cytotoxicity assay based on absorbance observed due to orange formazan product.

Cell treatment	48-h incubation time		72-h incubation time	
	Absorbance (450 nm)	Untreated cells, %	Absorbance (450 nm)	Untreated cells, %
Untreated cells	0.758	100%	0.754	100%
0.5% rose extract	-	-	0.813	108%
0.1% rose extract	-	-	0.709	94%
0.05% red rose extract	-	-	0.698	93%
0.01% red rose extract	-	-	0.655	87%
0.005% red rose extract	0.73	96%	0.723	96%

Table 2. Results of secreted cytokine profiling: fold changes of treated cells vs. untreated cells at both incubation times for AAH-ANG-G1 microarray. Target not detected (-).

	0.005% 48h vs. untreated cells 48h	0.5% 72h vs. untreated cells 72h	0.1% 72h vs. untreated cells 72h	0.05% 72h vs. untreated cells 72h	0.01% 72h vs. untreated cells 72h	0.005% 72h vs. untreated cells 72h
Angiogenin	-	-	-	-	-	-
EGF	-	-	-	-	-	-
ENA-78	-	-	-	-	-	-
bFGF	-	-	-	-	-	-
GRO	0.92	1.84	1.17	1.05	1.27	1.61
IFN-γ	0.96	1.19	1.25	1.23	1.15	1.21
IGF-I	-	-	-	-	-	-
IL-6	0.96	1.55	1.12	1.12	1.13	1.21
IL-8	-	-	-	-	-	-
Leptin	-	-	-	-	-	-
MCP-1	1.12	1.08	1.33	1.08	1.39	1.75
PDGF-BB	-	-	-	-	-	-
PIGF	-	-	-	-	-	-
RANTES	0.92	-	0.8	1.14	1.45	1.63
TGF-β1	0.93	1.05	1.07	0.97	1.01	1.13
TIMP-1	0.84	1.04	1.24	1.04	1.11	1.23
TIMP-2	-	-	1.2	-	1.05	1.16
Thrombopoietin	-	-	-	-	-	-
VEGF	-	-	-	-	-	-
VEGF-D	-	-	-	-	-	-

Notes: EGF – endothelial growth factor; ENA–78 – epithelial neutrophil activating peptide; bFGF – basic fibroblast growth factor; GRO – growth–regulated oncogene; IFN–γ – Interferon γ; IGF–I – insulin–like growth factor 1; IL – Interleukin; MCP–1 – monocyte chemoattractant protein 1; PDGF–BB – platelet–derived growth factor–BB; PIGF – placental growth factor; RANTES – regulated on activation, normal T cell expressed and secreted; TGF–β1 – transforming growth factor–β1; TIMP – tissue inhibitor of metalloproteinases; VEGF – vascular endothelial growth factor.

Table 3. Results of secreted cytokine profiling: fold changes of treated cells vs. untreated cells at both incubation times for AAH-ANG-G2 microarray. Target not detected (-).

	0.005% 48h vs. untreated cells 48h	0.5% 72h vs. untreated cells 72h	0.1% 72h vs. untreated cells 72h	0.05% 72h vs. untreated cells 72h	0.01% 72h vs. untreated cells 72h	0.005% 72h vs. untreated cells 72h
Ang1	0.72	0.69	1.3	1.29	1.35	1.18
Ang2	0.68	0.94	1.01	1.05	0.92	0.79
Angiostatin	-	-	-	-	-	-
Endostatin	-	-	-	-	-	-
G-CSF	1.04	-	-	-	-	-
GM-CSF	-	-	-	-	-	-
I-309	-	-	-	-	-	-
IL-10	1.14	-	-	-	-	0.94
IL-1α	1.05	1.17	1.09	1	0.96	0.93
IL-1β	-	-	-	-	-	-
IL-2	-	-	-	-	-	-
IL-4	-	-	-	-	-	-
I-TAC	-	-	-	-	-	-
MCP-3	-	-	-	-	-	-
MCP-4	-	-	-	-	-	-
MMP-1	-	-	-	-	-	-
MMP-9	-	2.68	1.81	1.71	2.62	2.23
PECAM-1	-	-	-	-	-	-
Tie-2	-	-	-	-	-	-
TNF-α	-	-	-	-	-	-
u PAR	-	-	-	-	-	-
VEGF R2	0.98	1.13	1.08	0.96	0.93	0.84
VEGF R3	-	-	-	-	-	-

Notes: Ang – Angiopoietin; G-CSF – granulocyte-colony stimulating factor; GM-CSF – granulocyte macrophage-colony stimulating factor; IL – Interleukin; I-TAC – interferon-inducible T-cell alpha chemoattractant; MCP – monocyte chemoattractant protein; MMP – matrix metallopeptidase; PECAM-1 – platelet endothelial cell adhesion molecule-1; Tie-2 – tyrosine-protein kinase; TNF-α – tumor necrosis factor α; u PAR - urokinase-type plasminogen activator receptor; VEGF - vascular endothelial growth factor.

rose extract, even in higher concentrations, has potential advantages to evolve as a therapy in the future. However, the results observed are preliminary and need further evaluation *in vitro*.

MMP-9 is involved in vascular remodeling of angiogenesis, wound repair, and neutrophil migration across the basement membrane [21, 22]. It is also regulated alongside with VEGF in angiogenesis, especially with hypoxia as a trigger [24–29]. At the same time, Ang1 is involved as the master regulator of angiogenesis [30, 31]. Das A. *et al.* [23] and Nowicki M. *et al.* [26] have found that angiopoietins increase MMP-9 and are closely regulated in the angiogenesis process. In our study, Ang1 was mildly elevated, especially in treatment with lower concentrations of the red rose extract – 0.05, 0.01, and 0.005 percent by 1.29, 1.35, and 1.18 times, respectively after 72 hours of treatment and together with increase in MMP-9 (1.71 – 2.68 times depending on the concentration), probably will have a superior influence of red rose extracts on angiogenesis. On other hand, TIMP-2 regulates MMP-9, and their balance is important for cells proliferation controlling [32]. Our study showed mild elevation of TIMP-2 accompanied with high elevation of MMP-9. These results confirm that the red rose extract does not affect balance between TIMP-2 and MMP-9. TGF-β1 plays a role of immunosuppressive cytokine through the inhibition of T-cell proliferation [33]. However, its level reduced after treatment with 0.005% (48 h) and 0.05% (72 h) extract and mildly elevated (1.01-1.13) in case of 72-h treatment with 0.005%, 0.01%, 0.1% and 0.5% concentrations.

A broad spectrum of cytokines is involved in the inflammation process. Thus, IFN-γ plays a key role in immune functions [34]; RANTES [37] and TIMP-1 [38] are inflammatory mediators in acute and chronic inflammation; MCP-1 [35] and GRO [36] are chemotactic cytokines, especially for mononuclear cells. These inflammatory markers (IFN-γ, MCP-1, GRO, RANTES, and TIMP-1) were observed to be elevated in this study and confirmed immunomodulation effect of the red rose extract on T-lymphocytes. Contrarily, these cytokines were reduced as compared to the controls in endothelial cells [20]. IL6, IL10, and IL1α serve as major regulators in immune responses and inflammation [39–41]. At the same time, inflammation and angiogenesis processes involve Ang2 [42] and VEGF R2 [43] as well. In our study, IL-6 was mildly elevated, IL-10 and IL-1α levels did not change significantly (Table 2 and 3). However, treatment of T-cells with the red rose extract at concentrations of 0.01 and 0.005% for 72 hours showed mild reducing in Ang2 and VEGF R2. G-CSF has a role in granulopoiesis and anti-inflammation [44], however, it was mildly increasing (1.04) after 48 hours of treatment with 0.005% red rose extract.

Meanwhile, it is an important question of advantages or disadvantages of the red rose extract in comparison with other rose extracts/ingredients with immunomodulation effects [45–54]. Thus, the effect of *Rose odorata* was studied in the RAW 264.7 cell/monocyte-macrophage cell line, and reducing in TNF-α, IL-6, IL-1β was found [45]. Gruenwald J *et al.*, according to the systematic review results, have concluded that the ingredients of *Rosa canina* reduce inflammatory markers and cytokines *in vivo* [46]. Immunomodulation activity of Kushui Rose (*Rosa setate x Rosa rugosa*) was studied as well [47, 51]. It was found that WSRP-1β – a novel polysaccharide from Kushui Rose – has immunomodulatory activity by enhancing phagocytosis of macrophages, increasing production of reactive oxygen species (ROS), nitric oxide (NO), cytokines (IL-6, TNF-α), and activating nuclear factor kappa B (NF-κB) signaling pathway [47]. The results of our study showed immunomodulation potency of *Rosa Rosaceae* as well, as evidenced by elevation of MMP9, GRO, IFN-γ, MCP-1, TIMP-1 and TIMP-2 (Table 2, 3). Al-Oqail M *et al.* found cytotoxicity effect of *Rosa damascena* in cervical cancer HeLa cells [48]. In our study, IL-6 and IFN-γ were mildly elevated, but there were no changes in IL-10, TNF-α, and GM-CSF (Table 2, 3); cytotoxicity of the rose extract (Pierre de Ronsard) on CD4+ T lymphocytes by MTT assay was negligible.

Blossom and bee pollen from *Rosa rugosa* had effects of decreasing triglyceride (TG) levels in the sebaceous glands, decreasing the release of IL-1α and TNF-α, alleviating the release of MMP-2 and MMP-9, increasing the content of caspase-3, reducing the release of testosterone T, and increasing the release of estrogen E_2 [49]. Similarly, *Rosa webbiana* reduces TNF-α and NF-κB, which in fact can have antiepileptic, anti-apoptotic, and neuroprotective potentials [50]. Our study on T-cells showed that the red rose extract increased IL-1α levels, however, TNF-α levels were not changed.

Rugosic acid derived from *R. rugosa* has been shown to reduce NF-κB and the IL-6/ signal transducer and activator of transcription 3 (STAT3) axis in the acute lung injury model [52]. STAT3 is a critical signaling molecule that is involved in the formation of the tumor microenvironment through regulating downstream proinflammatory cytokines and factors promoting tumor growth, progression, and metastasis [53]. In our study, IL-6 was elevated, but the exact significance on the STAT3 axis needs to be evaluated by future studies in this context. *Rosa laevigata* extract has shown suppression effect of immunoglobulin E (IgE) and related cytokines in *in vitro* and *in vivo* model of allergic asthma [54]. IgE-associated cytokines like IL-5 and IL-13 were not studied in our study, whereas IL-4 levels were not changed with red rose extract treatment.

Limitations

This study is performed *in vitro*. Further extensive evaluation of the extract needs to be performed in the animal models, and the side-effect profile of the extract needs to be evaluated as well. The pharmacological activity, mechanism of action and chemical content of the *Rosa Rosaceae* (Pierre De Ronsard) extract should be evaluated in detail. In addition, the feedback response mechanism, which could exist in T-cells in the immune regulation process, needs to be studied *in vivo*.

Conclusions

In this study, red rose (*Rosa Rosaceae* – Pierre de Ronsard) extract treatment on CD4+ T-lymphocytes showed mildly

increased cytokines - GRO, IFN-γ, IL-6, MCP-1, RANTES, TGF-β1, TIMP-1, TIMP-2, Ang1, Ang2, G-CSF, IL-10, IL-1α, MMP-9 and VEGF R2 by at-least one-fold change. There was no significant fold change over 2 or lower 0.5, except for MMP-9 with a fold change, often > 2. A significant fold change variation between different concentrations of investigated extract and untreated cells were observed in RANTES levels, and to a lesser extent in MCP-1, IL-1α, and VEGF R2. Further studies are required to evaluate the actions of the red rose extract *in vitro* and *in vivo*.

Ethical Statement

No approval of local ethics committee was required to accomplish the goals of this study.

Conflict of Interest

The authors declare that no conflicts exist.

Financial Disclosure

The authors declared no financial support.

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Received: 2021-06-24

Revision Requested: 2021-09-27

Revision Received: 2021-11-26

Accepted: 2022-01-20