Quercetin exhibits adjuvant activity by enhancing Th2 immune response in ovalbumin immunized mice

Article *in* Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie · April 2017

DOI: 10.1016/j.biopha.2017.03.067

8 authors, including:

Himanshi Tanwar
National Institute of Immunology
11 PUBLICATIONS **36** CITATIONS

SEE PROFILE

Lilly Ganju
Defence Research and Development Organisation
63 PUBLICATIONS **319** CITATIONS

SEE PROFILE

Biomedicine & Pharmacotherapy 90 (2017) 354–360

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Original article

Quercetin exhibits adjuvant activity by enhancing Th2 immune response in ovalbumin immunized mice

Divya Singh*, Himanshi Tanwar, Bindhya Jayashankar, Jyoti Sharma, Swetha Murthy, Sudipta Chanda, Shashi Bala Singh, Lilly Ganju

Defence Institute of Physiology & Allied Sciences (DIPAS), Delhi, India

ARTICLE INFO

Article history:
Received 10 January 2017
Received in revised form 12 March 2017
Accepted 22 March 2017

Keywords:
Quercetin
Flavonoids
Immunomodulators
Dendritic cells
Antibody titre

ABSTRACT

Quercetin, one of the most abundant of plant flavonoids, has been studied with a great deal of attention over the last several decades mainly for its properties in inflammation and allergy. In this study, we are reporting for the first time the *in vivo* immunostimulatory activity of quercetin in ovalbumin immunized Balb/c mice. Administration of quercetin (50 mg/kg body weight) along with ovalbumin antigen showed increased ovalbumin specific serum IgG antibody titres in comparison to the control group ($p < 0.05$). Quercetin administration not only showed predominance of Th2 immune response by increasing the IgG1 antibody titres, but also increased the infiltration of $CD11c^+$ dendritic cells in the mouse peritoneum and also increased LPS activated IL-1β and nitric oxide (NO) production by peritoneal macrophages. Expression of Tbx21, GATA-3 and Oct-2 proteins also enhanced in splenocytes of quercetin administered mice. Quercetin also did not cause any hemolysis in human RBCs. Overall, our findings strongly demonstrate the novel *in vivo* immunostimulatory and adjuvant potentials of quercetin.

1. Introduction

The discovery of novel plant compounds with immune system modulating activities has become an increasingly important area of research, particularly in the search for new-generation vaccine adjuvants [1]. There are many natural products, often extracted from plants which directly activate immune cells [2]. The discovery of such compounds could result in the development of new adjuvants for vaccines and drugs for the treatment of diseases such as allergy and cancer [3]. Flavonoids include a large group of low molecular weight polyphenolic secondary plant metabolites which can be found in fruits and vegetables, and plant derived beverages such as tea, wine and coffee [4]. They are polyphenolic compounds and display a variety of biological effects, such as antioxidation, anti-inflammation and protection against tumors [5]. In addition, flavonoids have been shown to regulate immune responses [6]. There are several subclasses in the flavonoid family. Quercetin, a typical member in the flavonol subclass, is one of the most common flavonoids in the diet. Its wide range of biological activities have been discussed for several decades. The immunomodulatory activity of quercetin has been investigated in NK cells, macrophages, mast cells, neutrophils, B cells, and T cells [7]. The effect of quercetin exhibiting antioxidant and anti-inflammatory properties has been extensively studied in the past few years [8–12]. Also, quercetin inhibits a broad spectrum of protein kinases by its capability to compete with the binding of ATP at the nucleotide binding site [13]. In addition, quercetin inhibited LPS induced DC activation [7]. Although, a number of such reports have confirmed the immunosuppressive actions of quercetin, the *in vivo* immunostimulatory and adjuvant activity of quercetin has not been explored and reported so far. In a recently published report, quercetin and its enzymatic derivative were shown to have immunostimulatory potential by increasing TNF-α and IL-6 production via increased nuclear translocation of NF-κB transcription factor [14].

Oral administration of quercetin has been reported to protect the irradiation-induced impairment of immune system [15]. Quercetin also increases Th1 response by *in vivo* administration and inhibits ova challenged asthmatic reactions [16]

Many of the plant derived extracts and compounds that are reported to have adjuvant activity with different antigens, are rich in flavonoids which may contribute to their adjuvant activity. Recently, the adjuvant property of supercritical extracts from

Abbreviations: LPS, lipopolysaccharide; DC, dendritic cells; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; NO, nitric oxide; RBCs, red blood Cells; ELISA, enzyme linked immunosorbent assay.

* Corresponding author at: Immunomodulation Laboratory, Defence Institute of Physiology & Allied Sciences, Lucknow Road, Timarpur, Delhi, 110054, India.
E-mail address: divyasingh2k8@gmail.com (D. Singh).

http://dx.doi.org/10.1016/j.biopha.2017.03.067
0753-3322/

seabuckthorn leaves in balb/c mice immunized with tetanus and diphtheria toxoids has been reported [17]. Therefore, in this study we have studied the *in vivo* immunomodulatory activity of quercetin in ovalbumin (Ova) immunized Balb/c mice that assisted in boosting the antigen specific humoral immune response.

The *in vivo* adjuvant potential of quercetin reported here is a novel finding that can be further explored for its intracellular mechanistic aspects and as an adjuvant candidate.

2. Materials and methods

2.1. Reagents

Culture RPMI 1640 medium, LPS *(Escherichia coli* O26:B6), Quercetin (>95%, powder), Ficoll-hypaque, Griess reagent, Dimethyl Sulfoxide (DMSO), 0.1 M DTT, cocktail of protease inhibitors, Tween-20, Bovine Serum Albumin (BSA) and 10% NP-40 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Protein assay reagent was purchased from Bio-Rad (USA). 1 M HEPES, 0.5 M EDTA, 0.1 M EGTA, 2 M KCl were obtained from Qualigens Fine Q5 Chemicals (India). Cytokine ELISA kits were purchased from eBiosciences (Minneapolis, MN, USA). Antibodies for western blotting were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Ammonium chloride (NH_4Cl), Potassium Chloride (KCl), sodium chloride (NaCl), Sodium carbonate (Na_2CO_3) Sodium bicarbonate potassium di hydrogen phosphate (KH_2PO_4), Di sodium hydrogen phosphate (Na_2HPO_4), sulfanilamide, naphthylene diamine, orthophosphoric acid, phosphate buffered saline, and ethyl alcohol were obtained from Qualigens Fine Chemicals, Mumbai, India. Purified Chicken Egg Ovalbumin (Ova) was purchased from Alpha Diagnostics, USA.

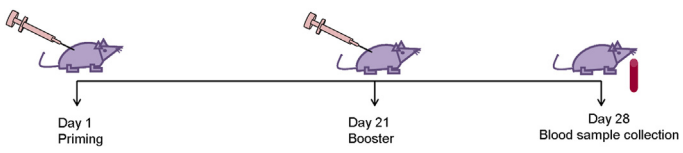
2.2. Experimental animals

Healthy, 8–12 week old Balb/c mice of either sex, weighing 25–30 g, were used for the experiments. The animals were maintained in the Experimental Animal Facility at DIPAS, under controlled environment of $25 \pm 1\,^{\circ}C$ and 12-h light-dark cycle. Handling of animals and all the experiments were performed in accordance with the regulations specified by the Institute's Animal Ethical Committee and conform to the National Guidelines on the care and use of laboratory animals, India.

2.3. Immunization protocol

Control group mice were primed intraperitonially (i.p.) either with Phosphate Buffered Saline (PBS) or Quercetin (25 or 50 mg/kg body weight) alone. Mice were given Ova (20 μg/mouse) (i.p) as antigen control. All the mice were primed on day one followed by a booster dose on day 21.

For evaluation of IgG titres, two different doses, Quercetin (25 mg/kg body weight) + Ova or Quercetin (50 mg/kg body weight) + Ova, were used. Blood samples were collected on day 28 for analysis of antibody titers in serum. Samples were stored at $-80\,^{\circ}C$ till analysed.



Flowchart for immunization schedule

2.4. Estimation of Ova-specific total IgG antibody and isotypes in serum

Antibody titers against Ova antigen were estimated in individual sera samples by indirect ELISA. Briefly, 96- well microtitre plates (Greiner, Germany) were coated with Ova (2 μg/ml) constituted in 0.01 M PBS (pH 7.2) and incubated overnight at $4\,^{\circ}C$. The plates were washed thrice with wash buffer [PBS containing 0.1% Tween-20 (PBST)] and blocked with 1% BSA for 1 h at $37\,^{\circ}C$. Plates were incubated with different dilutions (1:1000, 1:5000, 1:10000) of sera samples. The wells were washed thrice again with wash buffer and incubated for 2 h at $37\,^{\circ}C$. Horse Radish Peroxidase (HRP) conjugated rabbit anti-mouse IgG, diluted in PBST was added to the wells as secondary antibody. Plates were incubated at $37\,^{\circ}C$ for 1 h and washed thrice with PBST. The enzyme reaction was visualized with orthophenylene diamine substrate in citrate phosphate buffer with H_2O_2 as an oxidizing agent. The reaction was stopped after 10 min by addition of 50 μl of 2N H_2SO_4 and the absorbance was measured at 450 nm in an ELISA reader (Biotek, US).

2.5. Hemolytic assays

Heparinised blood samples were obtained from healthy individuals after getting informed consent. Samples were washed three times with 0.9% w/v NaCl, endotoxin free sterile saline solution by centrifugation at 1500 rpm for 5 min. Pellet was diluted in 0.5% saline solution for making the celll suspension. Equal volumes of 0.5 ml of the cell suspension and different concentrations of quercetin (1.5–0.187 mg/ml) were mixed in saline solution. Cell suspensions were incubated at $37\,^{\circ}C$ for 30 min, and centrifuged at 2000 rpm for 10 min. Hemoglobin in the supernatants was measured in 96-well microtitre plate by recording OD at 412 nm (Biotek Instruments, USA). Saline and distilled water were used as negative and positive controls respectively.

2.6. Ex vivo *stimulation of peritoneal macrophages*

After administration of booster on day 21, 4% thioglycollate was injected intra-peritoneally. Immunized mice were euthanized after 72 h and peritoneal macrophages and splenocytes were aseptically isolated as described elsewhere [18]. For the analysis of IL-1β production, peritoneal macrophages (1×10^6 cells ml^{-1}) were cultured in presence of LPS (1 μg/ml) for 48 h. Cell culture supernatants were harvested and stored at $-80\,^{\circ}C$ until used.

2.7. Measurement of NO

NO was measured as nitrite released from peritoneal macrophages. Cells from immunized mice were washed with PBS and cultured at a density of 2×10^6/ ml, in RPMI supplemented with 10% FCS and plated in 96-well culture plates. Culture was incubated overnight at $37\,^{\circ}C$ in an atmosphere of 5% CO_2 and 95% humidity. Thereafter, 50% of media from each well was aspirated and replenished with same amount of fresh media. LPS (1 μg/ml) was added to each well and further incubated for 48 h. To measure nitrite, 100 μl supernatant was mixed with an equal volume of the Griess Reagent at room temperature for 10 min. Absorbance was measured at 570 nm. NO estimation was carried out using standard curve plotted against known quantity of sodium nitrite. Results were represented in μM concentration obtained from mean absorbance of triplicate wells of at least three independent experiments.

2.8. Cytokine IL-1β estimation by ELISA

ELISA for IL-1β was performed as per the manufacturer's protocol (eBiosciences). Briefly, ELISA plate was coated with capture antibody and incubated overnight. Wells were washed using PBST 0.5% and incubated for 1 h after adding assay diluents then washed again. Samples and standards were added in the wells, incubated for 2 h and the plate was washed again. Then detection antibody was added and incubated for 1 h, after washing, avidin-HRP conjugate was added, incubated for 30 min and the plate was washed again. Finally substrate solution was added, incubated for 15 min, and then the reaction was stopped using stop solution. Absorbance was read at 450 nm using ELISA reader.

2.9. Flowcytometric evaluation of $CD11c^+$ DCs and intracellular $CD4^+$ IFN-γ^+/IL-4^+ cells

Peritoneal cells isolated from mice immunized with Quercetin and Ova were washed with PBS and stained with fluoresceinated $CD11c^+$ antibody (BD Biosciences) for 45 min in dark at room temperature. For intracellular staining, asceptically collected erythrocyte-depleted splenocytes (2×10^6 cells ml^{-1}) were cultured with or without Ova (50 μg/ml) for 24 h. Brefeldin A (BD Biosciences) was added to the culture medium, 2 h prior to the completion of 24 h. Cells were harvested, washed twice with PBS and stained with FITC conjugated CD4 antibody (BD Biosciences, US) for 45 min. Cells were permeablized and fixed with cytoperm and cytofix (eBiosciences) respectively and stained with fluoresceinated antibodies against IFN-γ and IL-4 (BD Biosciences). Further cells were washed with PBS and acquired and analysed on FACSCalibur™ (BD Biosciences) using Cellquest-Pro software.

2.10. Immunoblotting for expression analyses of proteins Tbx21, GATA-3 and Oct-2

Splenocytes and peritoneal cells isolated from the immunized mice were used for preparation of whole cell lysates as described earlier [19]. Equal quantities of protein samples (40 μg) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels following the method described by [20] and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, India).

The membranes were incubated for 1 h with 3% BSA in TBS buffer (0.1 M Tris–HCl, pH 7.4, 0.9% NaCl) to block non-specific binding followed by washing with $TBST_{20}$ (0.1% Tween-20 in TBS) and incubation with primary antibody (Sigma). β-actin (Sigma) was used as loading control. Antibodies against proteins Oct-2, Tbx21 and GATA-3 were used to probe the proteins resolved on PVDF membrane. Subsequently, the membranes were washed thrice, for 10 min with $TBST_{20}$ and incubated with secondary antibody horseradish peroxidase (HRP)–conjugated IgG (BD, Biosciences) against the primary antibody. The proteins were detected by chemiluminescence (Sigma).

2.11. Statistical analyses

Statistical significance was calculated by applying Independent samples *T*-test for comparing the control groups with the experimental groups. p value < 0.05 was considered to be significant.

3. Results

3.1. Enhancement of quercetin mediated antibody titres in ovalbumin immunized mice

Mice were immunized with quercetin doses of 25 and 50 mg/kg body weight, with and without Ova. Quercetin significantly enhanced dose dependent IgG titres at different serum dilutions in comparison to the IgG titres in Ova alone immunized mice (Fig. 1). However, the control groups immunized with PBS or quercetin alone (25 and 50 mg/kg body weight) did not increase the IgG titres ruling out the immunogenic activity of quercetin itself in the absence of Ova. Further, to analyse the IgG subtypes, we estimated the IgG1, IgG2a, and IgG2b antibodies in the mice sera

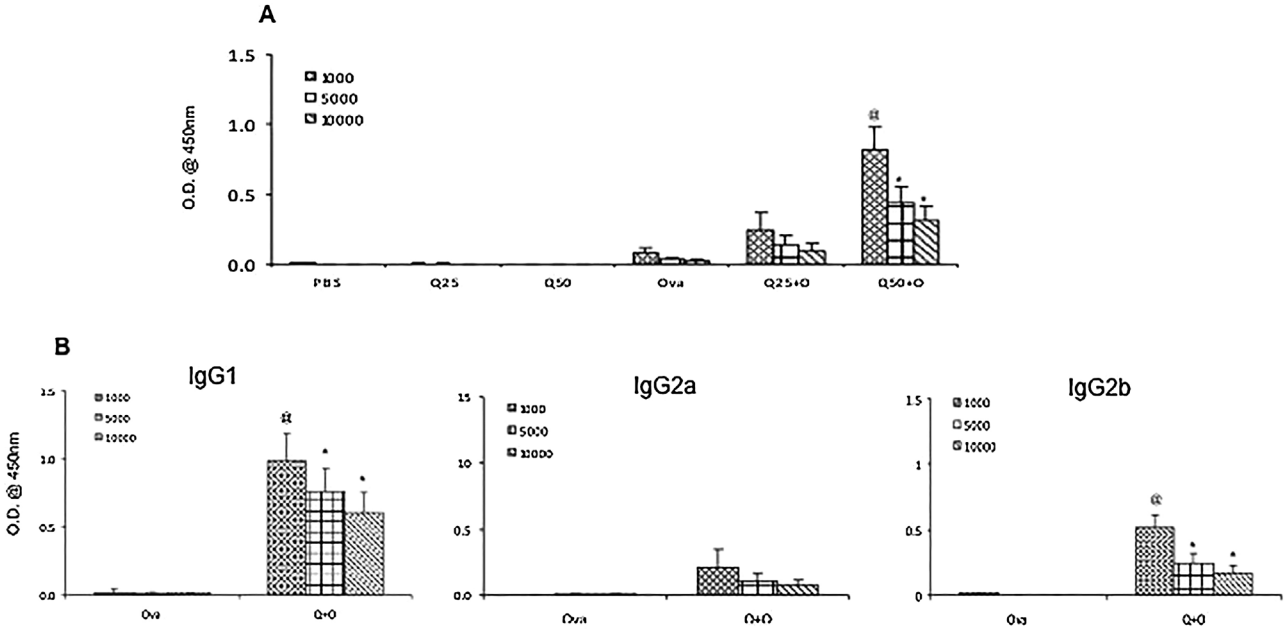


Fig. 1. (A) IgG titres in 1000, 5000 and 10000 times diluted sera samples. PBS: Phosphate Buffered saline (n = 6); Ova: ovalbumin (n = 9); Q25: Quercetin (25 mg/kg body wt.) (n = 6); Q50: Quercetin (50 mg/kg body wt.) (n = 6); Q25 + O: Quercetin (25 mg/kg bwt) + Ova (n = 6); Q50 + O: Quercetin(50 mg/kg bwt) + Ova (n = 9). Bars represent the mean O.D. values ± SEM. Independent samples *T*-test was employed for calculating statistical significance. $^{@}p < 0.01$ and $^{*}p < 0.05$ vs Ova. (B) IgG1, IgG2a and IgG2b titres in 1000, 5000 and 10000 times diluted sera samples (n = 6). Ova: ovalbumin Q50 + O: Quercetin(50 mg/kg bwt) + Ova. Bars represent the mean O.D. values ± SEM. Independent samples *T*-test was employed for calculating statistical significance. $^{@}p < 0.01$ and $^{*}p < 0.05$ vs Ova.

samples, where quercetin with Ova significantly increased IgG1 and IgG2b antibody titres. IgG2a titres did not show any statistically significant increase.

3.2. Hemolytic activity of quercetin

Quercetin was tested at 4 different doses (1.5– 0.1875 mg/ml) for its hemolytic activity in human blood samples and compared with distilled water as positive control and 0.5% saline as negative control. No significant increase in the percentage of hemolysis was observed in cells treated with all quercetin doses in comparison to the saline treated cells. Significant decrease ($p < 0.01$) in the percentage of hemolysis was observed for quercetin doses in comparison with positive control (Fig. 2).

3.3. Quercetin enhanced LPS mediated IL-1β and NO and production in peritoneal macrophages from the immunized mice

Peritoneal macrophages isolated from Quercetin + Ova immunized and mice treated with LPS, showed enhanced IL-1β and NO production in comparison to the LPS treated cells from Ova alone immunized mice. Untreated cells from both the groups showed no difference in IL-1β and NO production (Figs. 3 and 4).

3.4. Quercetin mediated recruitment of $CD11c^+$ DCs in peritoneum of immunized mice and increased $\%CD4^+IL\text{-}4^+$ cell population

Peritoneal cells isolated from Quercetin + Ova immunized mice were stained with fluorescent labelled antibody for CD11c, a cell surface expression marker for DCs. Quercetin administration significantly enhanced the recruitment of $CD11c^+$ cells in peritoneum as compared to that of Ova immunized mice (Fig. 5). To evaluate the Th1/Th2 immune response induced by quercetin, we analysed % intracellular IFN-γ and IL-4 producing $CD4^+$ cells in splenocytes by flowcytometry. Cells stimulated with Ova in Quercetin + Ova immunized mice showed 2-fold increase in $\%CD4^+IL\text{-}4^+$ cell population as compared to Ova alone immunized mice (Table 1).

3.5. Quercetin enhances Tbx21, GATA-3 and Oct-2 expression

Expression of transcription factors Tbx21, GATA-3 and Oct-2 in splenocytes isolated from Quercetin + Ova immunized mice in comparison to that in Ova immunized mice was determined by Western Blots. Immunization with Quercetin + Ova increased the expression of Tbx21, GATA-3 and Oct-2 proteins (Fig. 6).

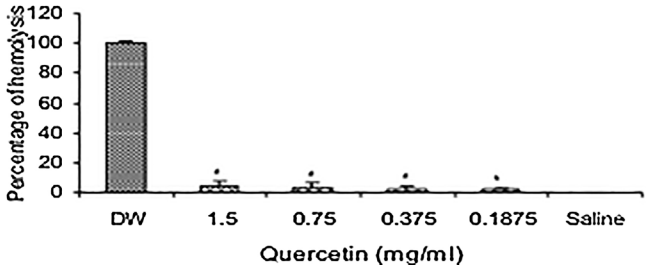


Fig. 2. Hemolytic activity of quercetin. Hemolytic activity of different concentrations of quercetin (1.5–0.1875 mg/ml) was measured in blood samples collected from healthy volunteers (n = 3). The graph represents the mean values of percentage of hemolysis ± SEM. Distilled water (DW) was used as positive control showing maximum hemolysis and saline was used as negative control which shows minimum hemolysis. One Way ANOVA Dunett's T3 test was applied to calculate significance between the hemolytic activity of different concentrations of Quercetin and positive control. p value < 0.05 was considered as significant.

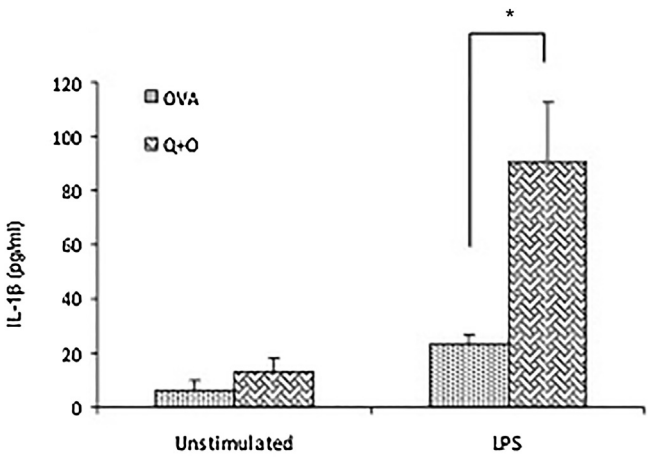


Fig. 3. Spontaneous and LPS induced IL-1β production in culture supernatants of peritoneal cells from immunized mice (n = 5). Q + O: mice immunized with Quercetin (50 mg/kg body wt.) + Ova; Ova: mice immunized with ovalbumin. *p < 0.05.

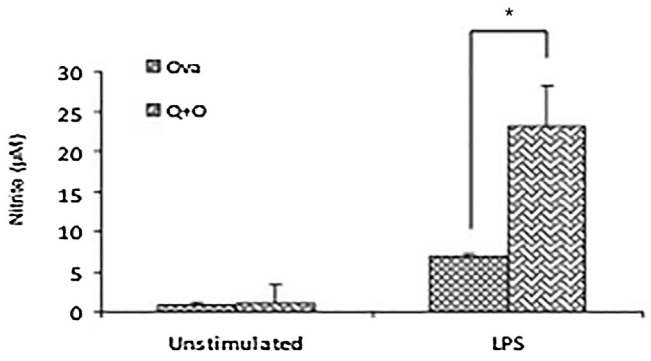


Fig. 4. Spontaneous and LPS induced NO production in culture supernatants of peritoneal cells from immunized mice (n = 6). Q + O: mice immunized with Quercetin (50 mg/kg body wt.) + Ova; Ova: mice immunized with ovalbumin. *p < 0.05.

4. Discussion

Among many of the different groups of natural products, flavonoids are a group of chemical entities of benzo-pyrone derivatives widely distributed in the plant kingdom. They are mainly classified as chalcones, flavan-3-ols, flavanones, flavones and flavonols, isoflavones, and bioflavonoids. Plant flavonoids are known to show anti-inflammatory activity *in vitro* and *in vivo* [10]. Quercetin, the most abundant of plant flavonoids, exerts a modulatory action on a variety of cells [8]. A number of studies have demonstrated the anti-inflammatory and immunosuppressive effects of quercetin both *in vivo* and *in vitro*. However, the literature is replete with studies showing *in vivo* immunostimulatory and adjuvant activity of quercetin except in a one very recent study where quercetin 3-O-xyloside activated murine macrophages to secrete TNF-α and IL-6 through up-regulation of the redox-dependent ASK1/MAPK/NF-κB signaling pathway [14]. Adjuvants are immunostimulating agents, molecules, compounds, or macromolecular complexes that boost the potency, quality, or longevity of specific immune responses to antigens with minimal toxicity [21]. Many natural products such as mineral salts, microbial products, emulsions, saponins, cytokines, polymers, microparticles, and liposomes show adjuvant properties [22]. In this study, we are reporting a novel finding demonstrating that quercetin when administered with ovalbumin significantly

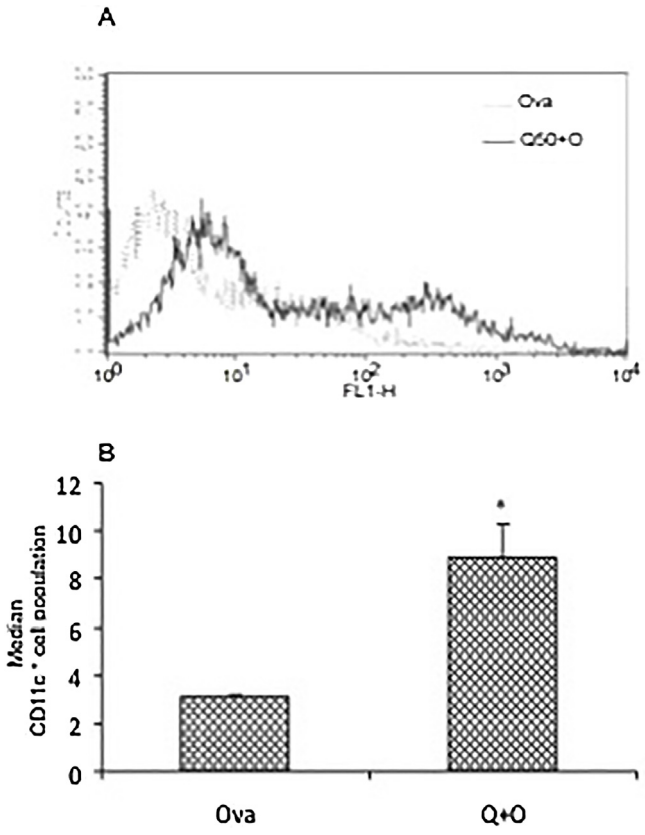


Fig. 5. (A) Representative histogram of CD11c cell surface expression in peritoneal cells from mice immunized with Quercetin with ovalbumin (Q + O) or without ovalbumin (Ova). (B) Graph shows average median values ± SEM (n = 5). $^{*}p < 0.05$ vs Ova.

enhanced antigen specific serum IgG titres in mice. However, quercetin when administered alone at two different doses, did not elicit any antibody titres (Fig. 1), clearly demonstrating its adjuvant nature.

Classical adjuvants, such as aluminum salts, generally induce a Th2-type immune response [23]. In our study, quercetin administration modulated the induction of Th2 type of immune response in Ova immunized mice with predominance of Th2 response as evident by the significant increase in Ova specific IgG1 antibody titres. IgG2a titres from Quercetin + Ova immunized mice were not found to be statistically significant. Further, it also increased the induction of IgG2b (Fig. 1).

One of the major drawbacks reported to be associated with plant derived molecules is the induction of hemolysis of RBCs which disqualifies them to be used for immunization purpose. In our study, quercetin showed insignificant hemolytic effect on human RBCs, wherein it can be considered safe for *in vivo* administration (Fig. 2). Adjuvants also induce the release of pro-inflammatory cytokines at the site of injection. Aluminium adjuvants induce inflammasome pathways to further activate production of cytokine IL-1β and IL-18 and can direct the humoral immune response [24]. In a study [25], it was shown that Th2 differentiation in mouse DCs was apparently directed by the specific secretion of IL-1β and IL-18. Our data demonstrates that there is a significant increase in LPS induced IL-1β production by the peritoneal macrophages of Quercetin + Ova immunized mice (Fig. 3). Induction of IL-1β suggests the *in vivo* immunostimulatory potential of quercetin in Ova-immunized mice which may further be explored by elucidation of the role of inflammasomes.

NO is a gaseous free radical molecule with pleiotropic functions in pathophysiology [26]. Many recent studies have reported the inhibitory role of quercetin on NO production by negative regulation of TLR-4 signalling and NF-κB pathways [27,28]]. Dietary supplementation of quercetin in mice inhibited LPS induced NO production by peritoneal macrophages [29]. In contrast, our data shows the increased production of LPS stimulated NO production by peritoneal macrophages from Quercetin + Ova immunized mice (Fig. 4). In a study, it was demonstrated that inflammasome-driven IL-1β production facilitated to trigger inducible nitric oxide synthase (NOS2)-mediated production of NO [30]. This supports our finding which could possibly be attributed to the quercetin induced production of IL-1β that induces increased nitric oxide synthesis. Our data is also supported by a recent finding [14], where Quer-xyl dose-dependently induced the inducible NOS expression and increased the production of NO.

Stimulation of the innate immune system defines and shapes the adaptive immune responses [31]. Immunostimulators and adjuvants induce recruitment of various innate immune cells such as monocytes and dendritic cells, to the site of injection, some of which then traffic the antigen to the draining lymph nodes to induce specific immune responses. Efficient antigen presentation and activation of DCs is important for the induction of adaptive immune response. Studies [23,32,33] have shown that intraperitoneal injection of Ova and alum led to uptake of antigen and maturation of DCs. In our study also, we have shown enhanced recruitment of $CD11c^{+}$ DC population in Quercetin + Ova immunized mice (Fig. 5) clearly demonstrating the adjuvant potential of quercetin.

The transcription factor, Oct-2 expression is restricted to B cells and neuronal cells and it has an ability to bind with high affinity to the conserved octamer DNA motifs found in Ig gene promoters and enhancers [34,35]. Increased Oct-2 expression appears to be responsible for the increased IgG titres by quercetin in this study (Fig. 6).

The differentiation of naive T-helper (Th) cells towards Th1 or Th2 cells is regulated by the transcription factors T-box 21 expressed in T-cells (T-bet/Tbx21) and GATA-binding protein-3 (GATA-3) [31]. GATA-3 plays a central role in regulating Th1 and Th2 cell differentiation. Upon interleukin (IL)-4 binding to its receptor, GATA-3 is induced through the action of Stat6 [36,37]. In

Table 1
Percent $CD4^{+}IFN\text{-}\gamma^{+}$ and $\%CD4^{+}IL\text{-}4^{+}$ cell populations in ovalbumin stimulated splenocytes from mice immunized with Quercetin + ovalbumin (Q + O) or with ovalbumin (Ova). Values represent the average % cell population ± SEM.

	$\%CD4^{+}IFN\text{-}\gamma^{+}$ cells		$\%CD4^{+}IL\text{-}4^{+}$ cells	
Mice (n=3) splenocytes	**Unstimulated**	**Ova Stimulated**	**Unstimulated**	**Ova Stimulated**
Ova immunized	4.88 ± 0.84	4.08 ± 0.08	1.43 ± 0.35	1.27 ± 0.16
Q + Ova immunized	4.39 ± 0.29	4.72 ± 0.52	1.58 ± 0.39	**2.55 ± 0.47** (2-fold increase)

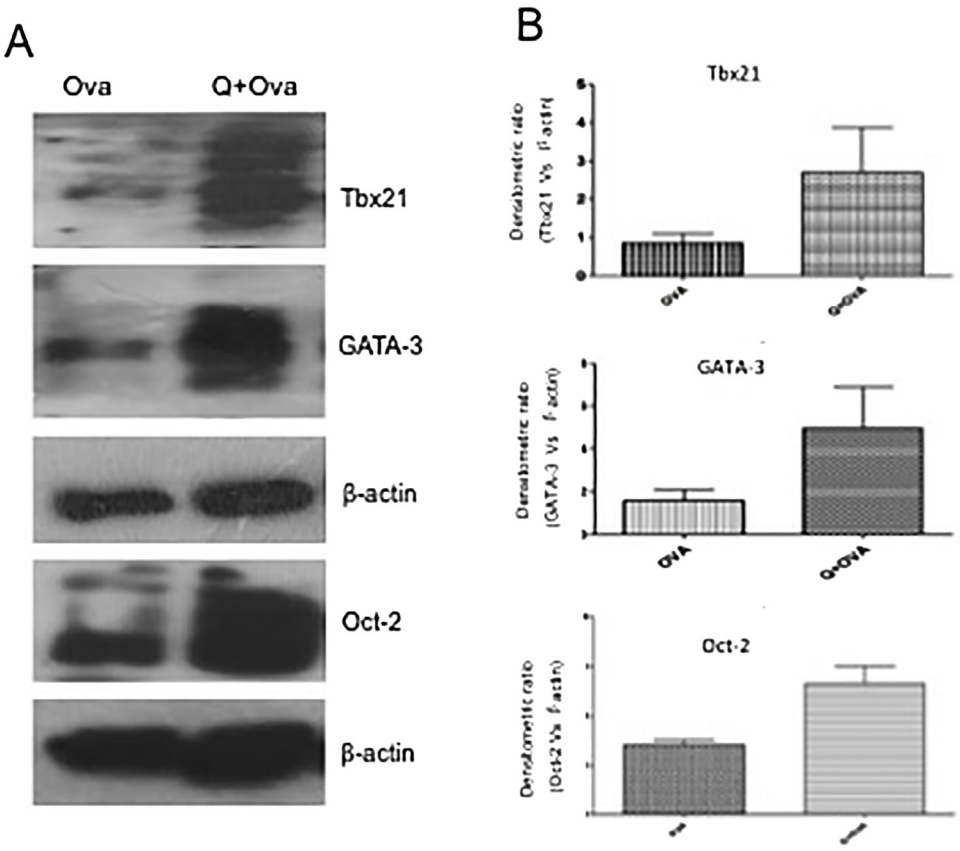


Fig. 6. (A) Expression profile of Tbx21, GATA-3 and Oct-2 in murine splenocytes. Blots are representative of 3 independent samples from mice immunized with Quercetin + Ova (Q + Ova) or with ovalbumin alone (Ova). β-actin was used as loading control. (B) Graphs represent densitometric ratio of protein expression vs β-actin (n = 3).

our study, we observed enhanced expression of both GATA-3 and Tbx21 in splenocytes of Quercetin + Ova immunized mice as compared to ova immunized group (Fig. 6).

In a study [16], the role of quercetin in regulating Th1/Th2 cytokine production, T-bet and GATA-3 gene expression was studied in Ova-induced asthma model in mice where quercetin reduced the increased levels of IL-4 production in Ova immunized mice and increased IFN-γ, Th1 cytokine production in quercetin administrated mice. Our results have shown 2 fold increase in % $CD4^{+}IL\text{-}4^{+}$ cells in quercetin + Ova immunized mice compared to ova immunized mice (Table 1) that corroborates the Th2 skewed response shown by quercetin.

Overall our findings of this study have shown a new aspect of quercetin activity that will provide new insight into the immunopharmacological role of quercetin in terms of its activity as an adjuvant. The potential adjuvant activity of quercetin can further be studied with different vaccines and antigens and in combination with other flavonoids also for generation of desired immune response. This will help in broadening the current perspectives in our understanding of the functions of quercetin and its various derivatives.

Conflict of interest statement

The authors report no conflict of interest.

Acknowledgement

This work was funded by Defence Research & Development Organization (DRDO), Ministry of Defence, India.

References

[1] P.V. Licciardi, J.R. Underwood, Plant-derived medicines: a novel class of immunological adjuvants, Int. Immunopharmacol. 11 (2011) 390–398.
[2] D. Hagan, New Generation Vaccine Adjuvants, Encyclopedia of Life Sciences, John Wiley & Sons, 2007.
[3] J.R. Ladinoa, A.G. Rossa, A.W. Crippsb, D.P. McManusc, R. Quinnd, Natural products and the search for novel vaccine adjuvants, Vaccine 29 (2011) 6464–6471.
[4] S. Chirumbolo, The role of quercetin, flavonols and flavones in modulating inflammatory cell function, Inflamm. Allergy Drug Targets 9 (2010) 263–285.
[5] D.W. Lamson, M.S. Brignall, Antioxidants and cancer III: quercetin, Altern. Med. Rev. 5 (2000) 196–208.
[6] R.J. Nijveldt, E. Nood, D.E.C. Hoorn, P.G. Boelens, K. Norren, P.A.M. Leeuwen, Flavonoids: a review of probable mechanisms of action and potential applications, Clin. Nutr. 74 (2001) 418–425.
[7] R.Y. Huang, Y. Yu, W. Cheng, C. OuYang, E. Fu, C. Chu, Dendritic cell activation and function immunosuppressive, J. Immunol. 184 (2010) 6815–6821.
[8] S. Chirumbolo, M. Marzotto, A. Conforti, A. Vella, R. Ortolani, P. Bellavite, Bimodal action of the flavonoid quercetin on basophil function: an investigation of the putative biochemical targets, Clin. Mol. Allergy 8 (2010) 13.
[9] C.C. Chuang, K. Martinez, G. Xie, A. Kennedy, A. Bumrungpert, A. Overman, W. Jia, M.K. McIntosh, Quercetin is equally or more effective than resveratrol in attenuating tumor necrosis factor-{alpha}-mediated inflammation and insulin resistance in primary human adipocytes, Am. J. Clin. Nutr. 92 (2010) 1511–1521.
[10] H.P. Kim, K.H. Son, H.W. Chang, S.S. Kang, Anti-inflammatory plant flavonoids and cellular action mechanisms, J. Pharmacol. Sci. 96 (2004) 229–245.
[11] S.J. Kim, J.Y. Um, J.Y. Lee, Anti-inflammatory activity of hyperoside through the suppression of nuclear factor-κB activation in mouse peritoneal macrophages, Am. J. Chin. Med. 39 (2011) 171–181.
[12] S. Bhaskar, V. Shalini, A. Helen, Quercetin regulates oxidized LDL induced inflammatory changes in human PBMCs by modulating the TLR-NF-κB signaling pathway, Immunobiology 216 (2011) 367–373.
[13] P.A. Ruiz, A. Braune, G.H. lzlwimmer, L. Quintanilla-Fend, D. Haller, Quercetin inhibits TNF-Induced NF-kB transcription factor recruitment to proinflammatory gene promoters in murine intestinal epithelial cells, J. Nutr. 137 (2007) 1208–1215.
[14] J. Lee, J.W. Choi, J.K. Sohng, R.P. Pandey, Y.M. Park, The immunostimulating activity of quercetin 3-O-xyloside in murine macrophages via activation of the

ASK1/MAPK/NF-κB signaling pathway, Int. Immunopharmacol. 31 (2015) 88–97.
[15] J.H. Jung, J.I. Kang, H.S. Kim, Effect of quercetin on impaired immune function in mice exposed to irradiation, Nutr. Res. Pract. 4 (2012) 301–307.
[16] H.J. Park, C.M. Lee, I.D. Jung, J.S. Lee, Y.I. Jeong, J.H. Chang, S.H. Chun, M.J. Kim, I. W. Choi, S.C. Ahn, Y.K. Shin, S.R. Yeom, Y.M. Park, Quercetin regulates Th1/Th2 balance in a murine model of asthma, Int. Immunopharmacol. 9 (2009) 261–267.
[17] B. Jayashankar, D. Singh, H. Tanwar, K.P. Mishra, S. Murthy, S. Chanda, J. Mishra, R. Tulswani, K. Misra, S.B. Singh, L. Ganju, Augmentation of humoral and cellular immunity in response to Tetanus and Diphtheria toxoids by supercritical carbon dioxide extracts of *Hippophae rhamnoides* L. leaves, Int. Immunopharmacol. 44 (2017) 123–136.
[18] B. Jayashankar, K.P. Mishra, L. Ganju, S.B. Singh, Supercritical extract of Seabuckthorn Leaves (SCE200ET) inhibited endotoxemia by reducing inflammatory cytokines and nitric oxide synthase 2 expression, Int. Immunopharmacol. 20 (2014) 89–94.
[19] B. Jayashankar, K.P. Mishra, M.S.Y. Kumar, K. Udayasankar, K. Misra, L. Ganju, S. B. Singh, A supercritical CO2 extract from seabuckthorn leaves inhibits pro-inflammatory mediators via inhibition of mitogen activated protein kinase p38 and transcription factor nuclear factor-κB, Int. Immunopharmacol. 13 (2012) 461–467.
[20] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[21] C.R. Alving, K.K. Peachman, M. Rao, Steven G. Reed, Adjuvants for human vaccines, Curr. Opin. Immunol. 24 (2012) 310–315.
[22] S. Awate, L.A. Babiuk, G. Mutwiri, Mechanisms of action of adjuvants, Front. Immunol. 114 (2013) 1–10.
[23] W. Wang, M. Singh, Selection of adjuvants for enhanced vaccine potency, World J. Vaccin. 1 (2011) 33–78.
[24] S.C. Eisenbarth, O.R. Colegio, W. O'Connor, F. Sutterwala, R.A. Flavell, Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants, Nature 453 (2008) 1122–1126.
[25] A. Sokolovska, S.L. Hem, H. HogenEsch, Activation of dendritic cells and induction of CD4(+) T cell differentiation by aluminum-containing adjuvants, Vaccine 25 (2007) 4575–4585.
[26] C. Yang, J. Yuk, E. Jo, The role of nitric oxide in mycobacterial infections, Immune Netw. 9 (2009) 46–52.
[27] E.B. Byun, M.S. Yang, H.G. Choi, N.Y. Sung, D.S. Song, S.J. Sin, E.H. Byun, Quercetin negatively regulates TLR4 signaling induced by lipopolysaccharide through Tollip expression, Biochem. Biophys. Res. Commun. 431 (2013) 698–705.
[28] C.H. Kang, Y.H. Choi, S.K. Moon, W.J. Kim, G.Y. Kim, Quercetin inhibits lipopolysaccharide-induced nitric oxide production in BV2 microglial cells by suppressing the NF-κB pathway and activating the Nrf2-dependent HO-1 pathway, Int. Immunopharmacol. 17 (2013) 808–813.
[29] A.A. Qureshi, X. Tan, J.C. Reis, M.Z. Badr, C.J. Papasian, D.C. Morrison, N. Qureshi, Inhibition of nitric oxide in LPS-stimulated macrophages of young and senescent mice by δ-tocotrienol and quercetin, Lipids Health Dis. 10 (2011) 239.
[30] D.S. Lima-Junior, D.L. Costa, V. Carregaro, L.D. Cunha, A.L. Silva, T.W. Mineo, F.R. Gutierrez, M. Bellio, K.R. Bortoluci, R.A. Flavell, M.T. Bozza, J.S. Silva, D.S. Zamboni, Inflammasome-derived IL-1β production induces nitric oxide-mediated resistance to *Leishmania*, Nat. Med. 19 (2013) 909–915.
[31] T. Ebensen, C.A. Guzmán, Immune modulators with defined molecular targets, Hum. Vaccin. 1 (2008) 13–22.
[32] M. Kool, V. Pétrilli, T. DeSmedt, A. Rolaz, H. Hammad, M. vanNimwegen, I.M. Bergen, R. Castillo, B.N. Lambrecht, Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome, J. Immunol. 181 (2008) 3755–3759.
[33] M. Kool, T. Soullié, M. vanNimwegen, M.A.M. Willart, F. Muskens, S. Jung, H.C. Hoogsteden, H. Hammad, B.N. Lambrecht, Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells, J. Exp. Med. 205 (2008) 869–882.
[34] L.M. Corcoran, F. Koentgen, W. Dietrich, M. Veale, P.O. Humbert, All known in vivo functions of the Oct-2 transcription factor require the C-terminal protein domain, J. Immunol. 172 (2004) 2962–2969.
[35] D.S. Latchman, The Oct-2 transcription factor, Int. J. Biochem. Cell Biol. 28 (1996) 1081–1083.
[36] H. Chakir, H. Wang, D.E. Lefebvre, J. Webb, F.W. Scott, T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3, J. Immunol. Methods 278 (2003) 157–169.
[37] M. Zhou, W. Ouyang, The function role of GATA-3 in Th1 and Th2 differentiation, Immunol. Res. 28 (2003) 25–37.