In vitro immunomodulatory activity of aqueous Quercus infectoria gall extract

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ABSTRACT

Objectives: The present study reports the immunomodulatory potency of Q. infectoria gall extract in vitro. The aqueous extract was prepared and examined for its effects on cell proliferation, phagocytic activity, nitric oxide production, and cytokine synthesis by murine macrophages.

Methods: Proliferative and phagocytic activity and nitric oxide production of extract-treated and control cells were studied using proliferative assay, flow cytometry, and Griess reaction, respectively. An enzyme-linked immunosorbent assay was performed to determine the levels of pro- and anti-inflammatory cytokines in the macrophage culture.

Results: Treated macrophages had a higher proliferative rate and phagocytic activity as compared to untreated macrophages. The cell treatment with extract concentration of 64 μg/mL demonstrated a significant decrease in nitric oxide production (P<0.0001). An
increase in cytokine levels (IL-2, IL-5, IL-10, IL-17A, IL-23, TGF-β) was observed; however, this increase was not statistically significant.

**Conclusion:** This study suggests that gall extract possesses potential for augmenting immunomodulatory activity by cellular mediated mechanism and could play a role in the regulation of the innate immune response.

**Keywords:** *Quercus infectoria*, immunomodulation, macrophage, nitric oxide, proliferation, phagocytosis, cytokines

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**INTRODUCTION**

Augmenting the immune system and its response has now become part of modern medical practice, which can include preventive measures, optimization of wellness, and even self-medication for mild illness. The regulation of the immune system is apparently essential for the optimisation of the immune response. The mechanism of immunomodulation is highly complex because it requires activation, proliferation, and differentiation of immune cells such as macrophages, T-helper cells, B-cells among others. Macrophages and lymphocytes have the ability to guard and activate cellular defence with the help of molecular detectors such as toll-like receptors (TLRs). Specific antibodies are generated in response to antigens or toxins, for the purpose of initiating the destruction of foreign cells or organisms.

The immunomodulatory properties of plants are being studied with greater interest in recent years. This is due to a growing awareness regarding the need to modulate the immune system to achieve the desirable effects of infection prevention and the treatment of cancer and autoimmune diseases. A medicinal plant which has an immunomodulatory
effect could provide alternative substances or compounds for this purpose, especially in relation to host defence mechanism therapy.

The compounds responsible for therapeutic effects are usually plant secondary metabolites. Plant-derived products such as polysaccharides, lectins, peptides, flavonoids, and tannins have been used to modulate the immune response or immune system in various in vitro models. Immunomodulators could give effect to the human system by regulating immune properties such as phagocytic activity, cytokines release, and apoptotic activity in cells. The use of a plant extract as a supplement could enhance health and wellness, especially in people with immunity impairment.

*Quercus infectoria* is a popular medicinal plant traditionally used in the postpartum care and treatment of a variety of ailments. The galls of *Q. infectoria* are used as “jamu” (i.e., a health supplement). Pharmacologically, the galls of *Q. infectoria* have been documented to possess astringent, antibacterial, and antifungal properties in vitro. In addition, the gall extract has the ability to inhibit the function of macrophages or neutrophils and thus inhibit the release of inflammatory mediators. Tannin, constituting almost 50-70% of *Q. infectoria* galls extract is thought to be an important compound responsible for antimicrobial activity.

Previous studies on the antimicrobial properties of *Q. infectoria* gall extracts have largely focused on their efficacy against certain pathogenic bacteria and fungi. Improved knowledge of the impact of this bioactive compound on immune system is necessary. Antioxidant activity in plant materials, including *Q. infectoria* gall extracts, generally originates from their polyphenolic content. At present, there is a lack of experimental data on the potential benefits of the extract as an immunomodulator. If such
benefits could be demonstrated, *Q. infectoria* gall extract could be used as an alternative immunomodulator or as a supplement to minimise the side effects of cytotoxic drugs. The aim of this study was to explore the immunomodulatory potential of *Q. infectoria* gall water extract *in vitro*.

**METHODS**

**Plant material and extraction**

Galls of *Quercus infectoria* were purchased from a local herb shop in Kota Bharu, Kelantan. Identification of the gall was made based on physical appearance. Aqueous extract was prepared by immersing 100 g of *Q. infectoria* gall powder in 500 mL of sterile distilled water for 72 h in a 50 °C water bath. The mixture was then pre-filtered using a coffee filter and followed with filtration using Whatmann filter paper No 1. The filtrates were concentrated under reduced pressure using a rotary evaporator at a temperature of 80 °C. The resulting pellet was freeze-dried at -50 °C under vacuum until the pellet produced a fine crystal-like crude extract. The crude extract was stored in airtight jars at 4 °C until use. A hundred milligrams of crude extract powder was added to 4 mL of sterile distilled water. Serial dilution was performed in Roswell Park Memorial Institute (RPMI) 1640 medium to obtain a concentration of 64 µg/mL, 32 µg/mL and 16 µg/mL. The extract was freshly prepared prior to use.

**Cell line and culture preparation**

This study used a murine macrophage (*J774A.1*) cell line (ATCC, USA) maintained in a complete growth medium (RPMI 1640) [Addex Bio & Sigma, USA] containing 12% fetal bovine serum [Gibco, USA] and 1% penicillin/streptomycin/neomycin under standard
culture conditions (37 °C, 95% humidified air, and 5% CO₂). Cell passage was performed when the cell density reached 70-80% confluence. Cells were detached using a cell scraper and then aspirated into a 15 mL centrifuge tube. The flask was rinsed twice with pre-warmed growth medium and aspirated again into the 15 mL centrifuge tube. The medium containing cells was centrifuged at 1500 rpm for 5 min. The suspension was discarded and the pellet was re-suspended with 1 mL growth medium. The cell suspension was transferred into five new 25 cm² flasks containing 5 mL growth medium. For harvesting, the cells were transferred into a 15 mL centrifuge tube by scraping the cells and pelleted by centrifugation at 1500 rpm for 5 min. Cell pellets were washed and re-suspended in 200 µL of PBS or stored at -80 °C until use. Cell number and viability were assessed microscopically using trypan blue exclusion.

**Proliferative assay**

The effect of *Q. infectoria* gall extract on murine macrophage cell viability was evaluated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl-tetrazolium bromide) assay. In brief, cells were placed into the wells of a 96-well plate (2 x 10³ cell per well) and incubated at 37 °C in a CO₂ incubator for 24 h. The medium was then discarded and the wells received *Q. infectoria* gall extract in growth medium (64, 32 and 16 µg/mL) while a control well received medium only. The plate was incubated for 24, 48, and 72 h at 37 °C in a CO₂ incubator followed by the addition of 10 µL of MTT (5 µg/mL) solution into the wells. The plate was further incubated for 2 to 4 h. Thereafter, 100 µL of stop solution (DMSO) was added to each well to dissolve the formazan particles that had formed in live cells. The absorbance of each cell concentration (optical density, OD) at 570 nm was measured in a plate reader. The MTT solution (yellow) crystalized the viable cell and changed it to formazan. The colour of the solution then changed to purple. All tests were
performed in triplicate. The OD in untreated control cells was taken as 100% of viability. Cell viability was expressed as a percentage (%) compared to the control (absence of any extract).

**Phagocytic assay**

A phagocytic assay was performed according to the manufacturer’s instructions. Cells (1 x 10^5 cells/mL) were seeded in 6 wells and incubated overnight, followed by treatment with 64 μg/mL, 32 μg/mL, and 16 μg/mL of gall extract, as well as 10 μg/mL phytohemagglutinin (PHA) as a positive control. 100 μg/mL of latex beads-rabbit IgG-FITC solution (Cayman Chemical, USA) was then added into each well and left for 72 h at ambient temperature. The cells were harvested, transferred into a polystyrene tube and centrifuged at 1200 rpm at room temperature for 5 min. The supernatant was aspirated from each tube and 500 mL of assay buffer was added to cells. The tubes were centrifuged again and the supernatant was aspirated 2-3 times. The final solution was vortexed to ensure that all cells were suspended. Samples were analysed immediately by flow cytometry.

**Measurement of NO production**

Nitric oxide (NO) production was determined using a Griess reaction. Macrophage cells were placed into wells of a 24-well plate (1 x 10^5 cells/ well), which then received the aqueous extract (64 μg/mL, 32 μg/mL and 16 μg/mL); control wells received medium only. After 72 h of incubation at 37 °C, the cell-free culture medium was removed from each well. This material (100 μL) was then combined with an equal volume of Griess solution (1% sulfanilamide, 0.1% naphthyl ethylenediamine in 5% phosphoric acid) and incubated at room temperature for 10 min. Thereafter, the absorbance was measured at
570 nm in the plate reader and concentrations of NO were calculated by extrapolation from a standard curve prepared in parallel using sodium nitrite standards. These experiments were performed in triplicate.

**Cytokine assay**

A cytokine assay was performed according to the manufacturer’s instructions (multi-analyte ELISArray kit QIAGEN, USA) for the detection of murine macrophage TH1 (IL-2, IL-12, TNF-α and IFN-γ), TH2 (IL-4, IL-5, IL-6, IL-10 and IL-13), and TH17 (IL-17A, IL-23, TGF-β1) cytokines. In this assay, 5 x 10⁴ cells/mL cells were seeded in a 96-well plate and incubated overnight prior to treatment with 64 µg/mL concentration of extract. After 48 h of incubation, the culture supernatant was collected and assayed using a 96-well plate. After adding the stop solution, the colour changed from blue to yellow and the absorbance was measured at 450 nm within 30 min of stopping the reaction.

**Statistical analysis**

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software (version 22). Data obtained from at least three independent experiments are presented as the mean ± standard error (SE). Statistical evaluation of the results was performed by an independent t-test for proliferative and phagocytic assays and one-way ANOVA followed by a post-hoc Bonferroni test for the NO assay. Flow cytometry data were analysed using FCS Express 6 Plus Research Edition (De Novo) software. A P-value <0.05 was considered significant for all analyses.

**RESULTS**
**Proliferative activity**

An increase in macrophage proliferation was observed in extract-treated cells compared to untreated macrophages for all tested extract concentrations (Figure 1). Maximum percentage of proliferation was recorded at the concentration of 64 μg/mL at 72 h with a percentage proliferation rate of 154.2±0.05%.

**TABLE AND FIGURES**

![Figure 1. Proliferative activity of macrophages treated with different concentrations of aqueous *Quercus infectoria* gall extract after 24, 48, and 72 h of incubation. The optical density in untreated control cells was taken as 100% of viability. Asterisk (*) indicates that statistically significant differences (P<0.001) were observed between the percentage values of each concentration of extract-treated cells and untreated cells.](image-url)
Phagocytic activity

Flow cytometry analysis (Figure 2) visualized perturbations in phagocytosis of the cell population. The ability of the activated J774A.1 cells to phagocytose foreign particles was tested using fluorescent IgG-coated latex beads. Macrophages treated with *Q. infectoria* gall extract or PHA were expanded to the right lower quadrant, whereas the unstained macrophages were in the left lower quadrant of the dot plot, indicating that a macrophage increased its size during phagocytic activity. Flow cytometry analysis showed that without any treatment, only 55.1% of the cells phagocytosed the beads, while in treated macrophages with 16, 32, and 64 µg/mL of extract, the phagocytic activity increased to 60.8%, 60.9%, and 74.2%, respectively. However, the activity was not significantly different as compared to the negative control. Phagocytosis was higher for macrophages treated with PHA (92.6%) as compared to extract-treated macrophages (Figure 3).
Figure 2. Flow cytometry analysis of IgG-coated bead phagocytic activity of macrophages. A: unstained macrophages, B: untreated macrophages (negative control), C: treated macrophages with PHA (positive control), D: treated macrophages with 16 µg/mL, E: treated macrophages with 32 µg/mL, and F: treated macrophages with 64 µg/mL.
**Figure 3.** Phagocytic activity of macrophages treated with various concentrations of aqueous *Quercus infectoria* gall extract after 72 h. The percentage of cells that phagocytose beads is indicated. NC = negative control (untreated cells) and PC = positive control (cells treated with PHA). This graph was tabulated from flow cytometry analysis. Asterisks indicate significant differences (P<0.001) between PC and extract-treated cells at 16 µg/mL (*), and between PC and NC (**).
Nitric oxide (NO) production by macrophages

The results of NO production by macrophages treated with three different concentrations of extract are illustrated in Figure 4. A significant increase in NO production was observed for all treated groups in comparison to the untreated group. In contrast, cells treated with the highest concentration of extract (64 µg/mL) exhibited the lowest NO production (Figure 4).

![Figure 4](image)

**Figure 4.** Nitric oxide production by macrophages treated with different concentrations of aqueous *Quercus infectoria* galls extract. The differences between the untreated and extract-treated cells were statistically significant (**P**<0.0001; *P*< 0.01).
Cytokine level

The absorbance values of treated macrophages showed higher levels of IL-2, TNF-α, IL-5, IL-10, IL-13, IL-23, TGF-β1, and IL-17A while lower values were observed for IL-12, IL-4, and IL-6. Overall, cytokine production by treated and untreated macrophages were statistically insignificant. The difference in the level of IL-13 produced by the treated and untreated macrophages was statistically significant (Figures 5, 6, and 7).

![Figure 5. TH1-related cytokine analysis of macrophages treated with aqueous *Quercus infectoria* gall extract (64 µg/mL) in comparison to untreated macrophages.](image-url)
Figure 6. TH2-related cytokine analysis of macrophages treated with aqueous *Quercus infectoria* gall extract (64 µg/mL) in comparison to untreated macrophages.
Figure 7. Cytokine analysis of macrophages treated with 64 µg/mL of aqueous *Quercus infectoria* gall extract. The absorbances observed for TH17-related cytokines (IL-6, IL-13, IL-17A, IL-23, TNF-α, and TGF-β1) are displayed in comparison to those observed for untreated macrophages.

**DISCUSSION**

The benefits of ethnomedicine cannot be ignored. For residents of developing countries, traditional medicine is preferred and can ultimately be more effective when providing aid. Recent studies show that traditional medicine is much more beneficial than once realized, and may be an important aspect in the future of medicine.\(^\text{20}\) The present study examined the aqueous *Q. infectoria* gall extract for its effects on J774.1A macrophage cell viability in an attempt to exclude any probable cytotoxic effects. The gall extract appears to be
non-toxic to the cells and is associated with the proliferation of macrophages (Figure 1). In a previous study, rectal administration of the aqueous extract showed low toxicity in rats.\textsuperscript{21}

The role of macrophages in inflammation is indisputable. They are one of the key players in initiation, development, and termination of inflammatory processes. The enhanced activity and proliferation of macrophages in the immune system depends not only on activation by a pathogen but can also be due to modulation of exogenous active compounds in the gall extract which possess anti-oxidant properties.\textsuperscript{22} Macrophages could undergo rapid \textit{in situ} proliferation in order to increase population density in the absence of any intruders in the tissue.\textsuperscript{23} The proliferation of macrophages is controlled by cytokine IL-4, which is dominant in TH2 inflammation.

Phagocytosis is one of the essential mechanisms in the inflammatory process. Phagocytic cells of the innate immune system accumulate in large numbers within the affected tissue or site of injury and restore tissue homeostasis. Macrophages not only phagocytose and kill infectious or foreign agents but are also important in clearing dead cells and restoring or mediating tissue remodelling during the late stages of inflammation.\textsuperscript{24} In this study, the phagocytic activity of macrophages increased in a dose-dependent manner following the gall extract treatment, though it was not as high as the activity produced by PHA-treated cells (Figure 3). Furthermore, the extract-treated cell size was larger compared to the size of untreated macrophages, as shown in the flow cytometry analysis (Figure 2). Another study also reported significant increased phagocytic activity and protective capacity of \textit{Q. infectoria} gall extract against butyl hydroperoxide (tBOOH)-stimulated rat peritoneal macrophages.\textsuperscript{10} The proliferation of macrophages also contributes to the enhancement of phagocytosis. Macrophages have the
capacity to kill microbes and tumour cells after an activation process by increasing their size, producing more pseudopods, and disrupting the plasma membrane of engulfed cells.\textsuperscript{25}

Nitric oxide (NO) is an important biological mediator and regulator of inflammation. In this study, extract-treated macrophages displayed a significant increase in NO production compared to untreated cells. Interestingly, it was observed that the production of NO decreased in a dose-dependent manner, indicating that a higher concentration of gall extract would suppress NO generation (Figure 4). This finding further supports the idea that \textit{Q. infectoria} has anti-inflammatory properties. The extract inhibits various functions of macrophages and neutrophils related to the inflammatory response, including NO synthesis. Inflammatory macrophages constantly express inducible NO synthase (iNOS) to produce a large amount of NO. \textit{Quercus infectoria} gall extract is not only capable of inhibiting the generation of NO in macrophages, but also effectively scavenges free radicals induced by oxidative stress.\textsuperscript{9, 26}

TH1 cytokines (IFN-\(\gamma\) and IL-2) are usually predominant in innate immunity, typically against intracellular bacteria and protozoa, whereas TH2 cytokines play an important role in allergic disease and are typically against extracellular parasites including helminths. The TH2 effector cytokines are IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25. This study has demonstrated that the extract had the capacity to reduce IL-4, IL-6, and IL-12 gene expression. These cytokines are important in the inflammatory process. IL-4 is a prototypic immunoregulatory cytokine. Like many cytokines, it can affect a variety of target cells in multiple ways. IL-4 has an important role in regulating antibody production, haematopoiesis and inflammation, and the development of effector T-cell responses. When acting on macrophages (a cell type critically involved in inflammation),
IL-4 induces alternative macrophage activation, which acts as an anti-inflammatory and stimulates normal tissue repair.\textsuperscript{27}

IL-6 is a proinflammatory cytokine involved in the generation and propagation of inflammation. It is also involved in acute phase reactions and is important to the development of specific cellular and humoral immune responses, including end-stage B cell differentiation, immunoglobulin secretion, and T-cell activation.\textsuperscript{28} In addition, IL-6 is the main switch in the transition between acute and chronic inflammation and a key factor in the recruitment of monocytes to the area of inflammation. IL-12 plays an important role in the activities of natural killer cells and T lymphocytes. As IL-12 is involved in TH1 development, it is believed to represent an important link between innate and adaptive immunity.\textsuperscript{29}

This study indicated that \textit{Q. infectoria} gall extract has anti-inflammatory potential. TNF-\textalpha cytokine levels increased as a result of treatment in contrast to results reported in another study in which TNF-\textalpha decreased significantly in extract-treated macrophages.\textsuperscript{10, 30} Increases in cytokine levels (IL-2, IL-5, IL-10, IL-17A, IL-23, TGF-\textbeta) were observed in this investigation but were not statistically significant. This study showed that IL-13 synthesis was significantly higher in extract-treated cells compared to a control. IL-13 has a role in inhibiting pro-inflammatory cytokines and chemokine production \textit{in vitro}. It also acts as an anti-inflammatory cytokine, playing a unique role in the induction and maintenance of IgE production.\textsuperscript{29} TGF-\textbeta is a multifunctional cytokine involved in cell growth and differentiation.\textsuperscript{31} In the presence of IL-6, TGF-\textbeta drives the differentiation of TH17 cells. This action promotes further inflammation and increases autoimmune conditions.\textsuperscript{32} TH17 is a major helper cell involved in mucosal immunity and
inflammation. It produces IL-17 and other cytokines which are good at fighting extracellular pathogens and fungi.

CONCLUSION

The extract of *Q. infectoria* gall is non-toxic and has a potential role in enhancing the proliferation and phagocytosis of macrophages, reducing NO production in a dose-dependent manner, and regulating cytokine levels in macrophages. This extract has the capacity to modulate the inflammatory mode of macrophages via a reduction in iNOS and NO levels, as well as IL-4, IL-6, and IL-12 cytokines while enhancing the production of IL-13 and other cytokines. Further detailed studies are required to identify the active constituents and their mechanisms for this effect both *in vitro* and *in vivo*.

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Disclosure

The authors declare that we have no conflicts of interest associated with this publication.

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