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A randomized double blinded placebo controlled clinical trial for the evaluation of green coffee extract on immune health in healthy adults

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ABSTRACT

Background: The immune system functions to protect the host from a broad array of infectious diseases. Here, we evaluated the *in vitro* immunomodulatory effects of green coffee extract (GCE), and conducted a double-blinded, randomized and placebo-controlled trial among apparently healthy individuals.
Methods: We determined the levels and functions of inflammatory and immune markers viz., phospho-NF-κB p65 ser536, chemotaxis, phagocytosis, TH1/TH2 cytokines and IgG production. We also evaluated several immunological markers such as total leukocyte counts, differential leukocyte counts, NK cell activity, CD4/CD8 ratio, serum immunoglobulin, C-reactive protein (CRP) and pro-inflammatory cytokines (IL-6 and TNF-α).
Results and conclusion: GCE significantly inhibited LPS-induced NF-κB p65 ser536 phosphorylation, MCP-1-induced chemotaxis and significantly enhanced phagocytosis and IgG production. In addition, GCE modulated PMA/PHA-induced TH1/TH2 cytokine production. Clinical investigations suggested that the expression of CD56 and CD16 was markedly augmented on NK cells following GCE treatment. GCE significantly enhanced IgA production before and after influenza vaccination. Similarly, IL-6, TNF-α and CRP levels were significantly inhibited by GCE. Together, GCE confers several salubrious immunomodulatory effects at different levels attributing to optimal functioning of immune responses in the host.
Taxonomy: Cell biology, Clinical study, Clinical Trial.

1. Introduction

The immune system is categorized as innate and adaptive immune responses based on their functions,[1] and is modulated by a variety of factors that includes prior infections, immunization, and various other external stimuli.[2] Innate immune response provides the first line of defense against infection and is non-specific in nature to a pathogen. Innate immunity has no memory and does not confer long-lasting immunity in the host.[3] Innate immunity encompasses different components including physical and anatomical barriers, epithelial and phagocytic cell enzymes, phagocytes, inflammation-related serum proteins, surface and phagocyte granule antimicrobial peptides, cell receptors that can sense pathogens to signal a defensive response, and cells that release cytokines and inflammatory mediators. Once pathogenic invasion occurs, a signaling cascade is initiated that enhances an immune response resulting in the activation of defense mechanisms.[4–6]

Phagocytosis is considered one of the most important surveillance processes during innate immune responses in the host. Once phagocytes arrive at the site of pathogenic establishment, they ingest microbial pathogens within vacuoles called phagosomes. Following activation, the pathogens are often destroyed and presented to lymphocytes via MHC molecules to T cells.[6] Activated

Abbreviations: GCE, Green Coffee Extract; CA, Chlorogenic Acid; NF-κB, Nuclear Factor Kappa-light-chain-enhancer of activated B cells; TH, T Helper cell; Ig, Immunoglobulin; LPS, Lipopolysaccharide; MCP-1, Monocyte Chemoattractant Protein; IL, Interleukins; PMA, Phorbol 12-myristate 13-acetate; PHA, Phytohemagglutinin; IFN, Interferon; NK cell, Natural Killer Cell; CD, Cluster of Differentiation; DNA, Deoxyribonucleic Acid; TNF, Tumor Necrosis Factor.

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innate immune cells, have been shown to generate inflammatory molecules, such as cytokines (TNF-α and IL-1β), chemokines (MCP-1), C-reactive proteins, complement components and facilitate monocyte chemotaxis.[7–9] Recruitment of NK cells also occur that controls several types of tumors and microbial infections by limiting their spread and subsequent tissue damage.[10]

Adaptive immunity provides a specific immune response directed at an invading pathogen. Following exposure to a foreign organism there is an initial effector response that eliminates or neutralizes a pathogen. Subsequent re-exposure to the same organism induces a memory response with a more rapid immune reaction that eliminates the pathogen to prevent disease onset.[11] The cells of the adaptive immune system include antigen-specific T cells, which are activated to proliferate via the action of APCs and B cells, which differentiate into plasma cells to produce antibodies. CD4 (T helper) T cells play a paramount role in establishing and magnifying the immune response, and direct other cells to perform host defense tasks, and regulate the type of T helper responses that develop thereafter.[12] Th1 cells produce interferon-gamma (IFN-γ), interleukin (IL)-2, and tumor necrosis factor (TNF)-beta, which activate macrophages and attribute largely to development of cell-mediated immunity and phagocyte-dependent protective responses. By contrast, Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses.[13] B cells can recognize antigens directly, without the need for APCs, through unique antibodies expressed on their surface. The principal function of B cells is the production of antibodies against foreign antigens besides acting as APCs for effector T cells.[14]

The immune system is a tightly regulated network that is able to maintain a balance of immune homeostasis under normal physiological conditions. Normally, when challenged with a foreign antigen, specific and optimal immune responses are initiated to restore homeostasis. However, under pandemic circumstances, this balance is not maintained and immune responses either under- or over-react.[15] Nutrition is an essential regulator of immune homeostasis, and even minor deficiencies in certain micronutrients could disrupt the onset of optimal protective immune responses.[16] Polyphenols are well-known, pharmacologically active compounds with immunomodulatory functions.[17] Polyphenols naturally occur in several foods and beverages, such as coffee, teas, vegetables, and fruits.[18] Coffee is reportedly the world's second most consumed beverage after tea.[19] Green coffee is rich in phenolic compounds, which are known to protect against chronic degenerative disorders.[20] Chlorogenic acids (CAs) are the main dietary polyphenols present in green coffee beans. CAs are esters of cinnamic acids with quinic acid, and are naturally-occurring in many plants such as pear, apple, and potatoes.[21,22] Green coffee beans are rich sources of CAs, although significant amounts of the compound are lost during the roasting process.[23] Several animal studies suggest that CA has potent anti-diabetic, anti-obese, anti-lipidemic, anti-hypertensive functions, and enhances insulin resistance.[24–28] GCE and CA are reported to possess certain health-promoting properties, viz., anti-oxidant, anti-bacterial, and anti-inflammatory effects.[29–31] However, there is a paucity of data on their anti-inflammatory[32,33] and immunoregulatory credentials in the scientific literature. Here, we evaluated the *in vitro* effects of GCE on inflammation and immune responses in cellular models and via human clinical trials. In the current scenario, plant-based immunomodulatory therapeutics have attracted significant attention, and hence identification of immunomodulatory plant extracts and their active moieties may provide novel insights into their salubrious immune-boosting roles.

2. Experimental design and methods

Mouse leukemic macrophage cell line, RAW 264.7, cells were purchased from American Type Culture Collection (ATCC—TIB 71). The RAW 264.7 cells were cultured in complete Dulbecco's Modified Eagle's medium (DMEM) (Sigma Aldrich) with 10% fetal bovine serum (FBS, Gibco). The human monocytic cell line U937 (ATCC; CRL1593.2) was cultured in Roswell Park Memorial Institute medium (RPMI) 1640 medium (Sigma Aldrich) supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO_2. For inducing differentiation, the cells were seeded in a T25 flask and differentiation was initiated by addition of phorbol-12-myristate-13-acetate (PMA) to the culture medium to a final weight percentage of 50 ng/mL. Post-induction, the differentiated cells were allowed to grow normally for 24 h. Subsequently, the U937 cells were washed with complete culture media once and incubated in U937 culture medium for 48 h. The IM9 B lymphoblast cells (ATCC; CCL159) were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO_2. The Jurkat cells and IM9 B lymphoblast cells were cultured in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO_2 conditions. GCE was dissolved in dimethyl sulfoxide (DMSO) and used in dilution ratio of 1:1000 in the culture medium; DMSO final weight percentage was not higher than 0.1%. GCE (60% total chlorogenic acid) was purchased from Cymbio Pharma, Bangalore.

2.1. Cell viability

Cytotoxicity was determined by reduction of Thiazolyl Blue Tetrazolium Blue (MTT, Sigma) to formazan. Cells were seeded in 12-well culture plates for 24 h. The cells were treated with various concentrations of GCE and incubated for 24 h. After 24 h, the cells were analyzed for cytotoxicity by MTT assay. MTT (2 mg/mL in PBS, 100 μl) was added to each well. The cells were incubated at 37 °C for 30 min, and DMSO (100 μL) was added to dissolve the formazan crystals. The absorbance was measured at 560 nm using an ELISA plate reader (ThermoFisher, Germany).

2.2. Phospho-NF-κB p65 (RelA) ser536 assay

RAW264.7 macrophage cells were seeded into each well of 12-well plates until 80% confluent. NFκB p65 ser536 phosphorylation was stimulated with (1 μg/mL) lipopolysaccharides (LPS). Simultaneously, the cells were treated with GCE (50 μg/mL). Detection of NFκB p65 ser536 phosphorylation was done using a commercial ELISA kit (Cell Signalling Inc). Briefly, media was removed from the cells and rinsed with ice cold 0.1 M phosphate buffer saline (1X). Cells were lysed with 0.5 mL ice cold cell lysis buffer and incubated on ice for 5 min. The scraped cells were transferred to a tube and cells were lysed using probe sonication. The lysate was then spun at 14,000 RPM for 10 min at 4 °C and transferred to 96 well plates and incubated overnight at 37 °C. Next, washing of the wells was done with 1X wash buffer. To each well, 100 μL of NF-κB p65 detection antibody was added and the plate was incubated at 37 °C for an hour. The wells were subsequently washed again and 100 μL of horseradish peroxidase (HRP) -linked secondary antibody was added to each well. Later, the plate was incubated for 30 min at 37 °C. The wash step was repeated and 100 μL of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) buffer was added. Finally, the stop solution was added and absorbance read at 450 nm.

2.3. Chemotaxis assay

Human monocytic cell line U937 was grown to 90% confluence and starved overnight in serum-free media containing 0.2% bovine serum albumin (BSA) prior to setting up chemotaxis experiments. Chemotaxis assays with U937 monocytic cells were set up on 5 μm pore size QCM chemotaxis cell migration 96 well plates (Merck-Millipore). 50,000 cells per well (in 50 μL of serum free medium) were added to upper wells and chemotaxis was measured in response to serum free-medium and monocyte chemo attractant protein (MCP-1) containing medium added to bottom wells (150 μL volume). GCE (50 μg/mL) treated with cells in upper wells at the start of assay. Chemotaxis assays were carried out over a period of 24 h at 37 °C. The non-invaded cells were removed from the upper wells by wiping with cotton swabs followed by rinsing twice with phosphate buffered saline (PBS). Following the removal of cells from the upper wells, cells that had migrated to lower wells were labeled with CyQuant GR Dye and read with a fluorescence plate reader using 480/520 nm filter set.

2.4. Phagocytosis assay

The differentiated U937 monocytic cells were treated with GCE (50 μg/mL) and phagocytosis inhibitor. The phagocytic activity was determined by a commercial CytoSelect™ 96-Well Phagocytosis Assay kit (Cell Biolabs Inc; CBA-224). Zymosan suspension (10 μL) was added to each well and incubated for 2 h. The culture medium was removed and 200 μL of cold, serum-free medium added to each well. Fixation solution (100 μL) was then added to each well and incubated for 5 min at room temperature. The fixation solution was then removed and washed twice with 1X PBS. Prediluted 1X blocking reagent (100 μL) added of to each well and incubated the plate for 60 min at room temperature on an orbital shaker. The blocking reagent was washed three times with 1X PBS. Prediluted 1X permeabilization solution (100 μL) was added to each well, incubated 5 min at room temperature. The cells were washed once with 1X PBS. Hundred microliters of prediluted 1X detection reagent was added to each well and incubated for 60 min at room temperature on an orbital shaker. The detection reagent was removed and washed three times with 1X PBS. Detection buffer (50 μL) was added to each well and the plate was incubated for 10 min at room temperature on an orbital shaker. The readings were taken at 405 nm.

2.5. Live cell imaging of phagocytosis and NF-κB translocation study

To analyze role of GCE on clearance rate of pathogens (Zymosan particles, pHrodo™ Red Zymosan Bioparticles™ Conjugate, from Thermo Fisher Cat #P35364) live cell imaging using confocal microscope (LSM 710 NLO, Carl Zeiss) was performed. RAW264.7 macrophage cells were grown in special glass bottom confocal dishes and on achieving sufficient confluency, were challenged with zymosan particles alone and zymosan with GCE. The experimental dishes were placed on to the specialized incubation setup with temperature and humidified CO2 supply. With 40x/1.4 Oil DIC plan −Apochromat objective and 543 nm laser line with 2% of laser power, Zoom factor of 2, Master gain of 905, filter setup of MBS 458/543, Pinhole of 36 μm (1 AU) and bright field with the transmitted light. The imaging was continued for 16 h using time course and Z stacking imaging features. Later, the same were processed with ZEN 2010 software. Similarly, for the NF-κB translocation analysis, the RAW macrophages were seeded on cover-slides and cultured for overnight. The cells were incubated in the presence or absence of the green coffee extract and stimulated with LPS (1 μg/mL) for 90 min. Cells were fixed and immunofluorescence staining of NF-κB p65 was done using a rhodamine labeled specific antibody and imaged using confocal microscope.

2.6. TH1/TH2 cytokines

Human Jurkat cells were stimulated by the addition of 7.5 nM PMA (phorbol myristate acetate) and 5 μg/mL PHA (phytohemagglutinin). Simultaneously, the cells were treated with GCE (50 μg/mL). Cell-culture supernatants were collected at 24 h and stored at −20 °C. TH1/TH2 cytokine/chemokine concentration was analyzed by using a commercial Human Cytokine Antibody Array kit (Ray Biotech). According to manufacturer's instructions, the array membranes were incubated with blocking buffer followed by undiluted culture supernatants for 24 h. Later, the membranes were washed, incubated with biotin-conjugated antibody for 2 h, and later with HRP-conjugated streptavidin for 2 h. Subsequently, the membranes were incubated in detection buffer, and the signal was detected directly from the membrane using a chemiluminescence imaging system (BioRod XRS).

2.7. IgG production

IM9 human B lymphoblast cells were stimulated with Cowan I strain *Staphylococcus aureus* (SAC) (1:105, v/v) for 2 days. Then, the viable cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO2 for 7 days in the presence or absence of IL-2 (100U/mL), GCE (50 μg/mL). The amounts of IgG secreted in the culture supernatant were determined using a commercial Human IgG total ELISA Ready-SET-Go (eBioscience) as per the manufacturer's protocol.

2.8. Statistical analysis

Statistical analyses were performed in https://astatsa.com. All experiments were done in triplicates and repeated twice and data presented as mean ± SD. A one-way ANOVA Tukey's post-hoc test was used. p values ≤ 0.05 were accepted as statistically significant differences.

2.9. Randomized clinical trial (RCT)

2.9.1. Subjects and methods

The protocol also designed with Good Clinical Practice (GCP) and the Helsinki Declaration Standards, as well as per Indian GCP and Schedule Y (amended version 2005) and Indian Council of Medical Research codes. This study was approved by the Human Ethics Committee (CLINICOM, Bangalore, India) and registered in Clinical Trials Registry - India (CTRI/2014/12/005247).

2.10. Study population

Study subjects were healthy Indian men and women aged 18–55 years old were recruited at Family Dental and Health Care, Devanahalli Town, Bangalore Rural, India. The 70 volunteers were divided into 3 groups of males and females with 23:24:23 persons in respective group. Subjects included were healthy males and non-pregnant females between 18 and 55 years of age with no present medical illness and were not on any forms of medication or any herbal formulation, whatsoever. The subjects were excluded if they had deteriorating health status at the time of enrollment, rapid weight loss, terminal disease, significant chronic disease, immunodeficiencies, history of allergy, lactose intolerance, prior serious reaction to influenza vaccine, any vaccination within current

season, medication or supplement use that potentially could influence the immune system, and currently participating or having participated in another clinical trial during the last 3 months prior to the study initiation.

2.11. Randomization and intervention

A total of 90 volunteers were screened, and 70 (27 male and 43 female subjects) were found to satisfy all the eligibility criteria. On receipt of a signed informed consent, eligible subjects were randomly assigned to each arm using blocked randomization method in SAS (version 9.3). The overall study duration was 90 days consisting of 5 visits (Visit 1 to Visit 5) as explained below (Fig. 1). All healthy volunteers underwent screening procedures at Visit 1 (screening/baseline) after providing the consent. Subsequent to analyzing the laboratory reports, the eligible subjects were randomized at visit 2 and were assigned to Subject Randomization Number (SRN). Subjects were given the investigational product. Group 1: Twenty three subjects were randomized in the treatment who received 125 mg of GCE containing 75 mg/day of chlorogenic acid. Group 2: Twenty four subjects were randomized in the treatment who received 250 mg of GCE containing 150 mg/day of chlorogenic acid. Subjects were instructed to swallow one capsule twice daily, i.e., two capsules per day, one in the morning and one in the evening, with water, after food. Group 3: Twenty three subjects were randomized in the placebo group; subjects used placebo which contained no active herbal extract. The rationale for proposed study design is to use the live or inactivated pathogens or part of pathogens as vaccines to stimulate an adequate adaptive/acquired immune response and also to develop vaccine-specific antibodies.[34] This kind of response frequently used as a way to measure the immunomodulatory effect of certain drugs and dietary interventions.[35] It is also a validated model to assess the in-vivo functional capacity of the human immune system.[36] Tetanus and influenza vaccines have also been used in clinical trials to measure antibody response.[37] Therefore, vaccination would help to determine the efficacy of green coffee extract on cell mediated and humoral immune response in the present clinical study. Hence, during visit 3 of our clinical study subjects were vaccinated with influenza vaccine (AGRIPPAL S1, inactivated influenza vaccine (surface antigen) IP 2013/2014 season). Herbal extracts, probiotics and micronutrients have shown a enhanced response after an influenza vaccination challenge.[38–41] During 4th and 5th visits, the subjects had a physical examination, and adverse events and concomitant medications (if any) were reviewed and documented. Blood samples were collected and protocol compliance was assessed as per the visit schedule. Adverse events were monitored during the entire study period. The schematic diagram of study design is demonstrated in Fig. 1.

2.12. Routine laboratory analysis

Vital signs, including systolic and diastolic blood pressure, heart rate, respiration, temperature and complete physical examination such as weight, height, body mass index, general appearance, skin, head, neck, thyroid, eyes, ears, nose, throat, respiratory, cardiovascular, central nervous and musculoskeletal system were recorded at the study center. Venous blood was drawn and examined for RBC count, platelet count, haematocrit or PCV, haemoglobin, random glucose, total protein, albumin, blood urea nitrogen (BUN), serum glutamic-oxaloacetic transaminase (SGOT/AST), serum glutamic pyruvic transaminase (SGPT/ALT), alkaline phosphate, sodium, potassium and triglycerides. These blood parameters were performed using standard automated equipment.

2.13. Immunological studies

Venous blood was used for the measurement of immunological profiles including total leukocyte count (TLC), differential leukocyte count (DLC), NK cell activity (CD3%, absolute CD3 count, CD (both 16 and 56) percent, CD (both 16 and 56) absolute count), CD4/CD8 count, serum immunoglobulin levels (IgG, IgM, IgE and IgA), inflammatory status via CRP and pro-inflammatory cytokines (IL-6 and TNF-α), and immune homeostasis was measured by assaying the plasma levels of auto-antibodies (against DNA and thyroglobulin).

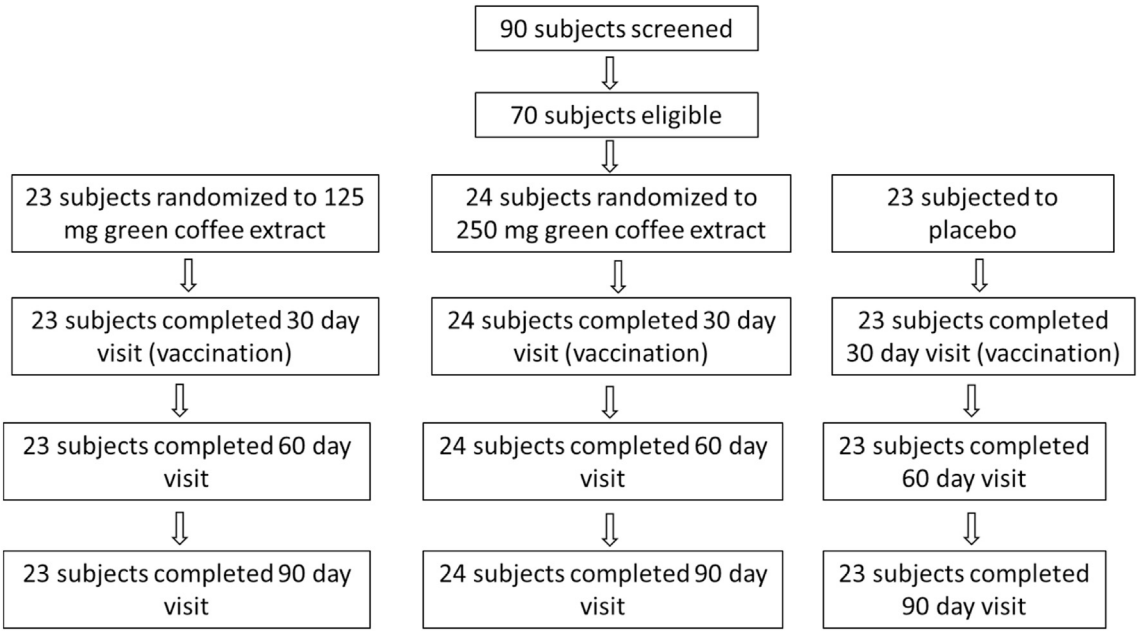

Fig. 1. Flow chart showing the distribution of trial participants.

2.14. Statistical analysis

All experimental data were recorded on case report forms, verified and independently monitored. Statistics for all immunological parameters and repeated measure ANCOVA was performed for the primary variables using baseline as covariates if data are measurable, else *t*-test was performed between the treatments by software SAS® for Windows 95/NT (Version 9.3 or higher, SAS Institute, Cary, North Carolina, USA).

3. Results

3.1. GCE exposure resulted in a dose-dependent decline in the viability of RAW264.7 cells

Cytotoxicity studies are the key initial steps to determine potential toxicity involving plant extracts, or other bioactive compounds isolated from plants. Cytotoxicity of GCE was analyzed in RAW264.7 macrophage, human monocytic cell line U937, IM9 B lymphoblast cells and Jurkat cells. The results indicated (Fig. 2a) that GCE exposure resulted in a dose-dependent decline in cell viability as compared to untreated control cells after 24 h of treatment. We found that 50 μg/mL of GCE was non-toxic to the cells although cytotoxicity was noticed from 100 μg/mL onwards. Hence 50 μg/mL dosage was selected for further *in vitro* studies. Cells such as human monocytic cell line U937, IM9 B lymphoblast cells and Jurkat cells showed similar trend in cell viability hence data was shown only for RAW264.7 macrophages.

3.2. Experimental GCE exposure led to inhibition of LPS-induced NF-κB p65 (RelA) ser536 phosphorylation in RAW264.7 cells

LPS is an exogenous pyrogen that can trigger the onset of inflammation by binding to TLR4-CD14-MD-2 complex expressed on inflammatory cells via the LPS-binding protein. Next, we set out to investigate if GCE had any anti-inflammatory role against LPS-induced NF-κB p65 ser536 phosphorylation in mouse RAW264.7 cells (Fig. 2b). Treatment of the cells with LPS (1 μg/mL) stimulated a peak in NF-κB p65 ser536 phosphorylation by 90 min when compared with control ($p < 0.01$). The present study showed statistically significant inhibition of GCE on LPS-induced NF-κB p65 ser536 phosphorylation when compared with LPS treated group ($p < 0.01$). Then, we examined whether GCE could inhibit NF-kB translocation from cytoplasm into nucleus. We measured the level of NF-kB p65 in the nucleus after stimulated with LPS in RAW macrophage cells in the presence or absence of GCE in order to detect whether NF-kB were blocked. We clearly showed that NF-kB p65 translocated in LPS stimulated cells but not in the nucleus of control cells. LPS stimulation led to an increase in NF-kB p65 levels in the nucleus, while treatment of GCE restrained this effect (Fig. 2d). Together, we found that GCE had an anti-inflammatory role as shown by its ability to inhibit NF-κB p65 (RelA) ser536 phosphorylation in RAW264.7 cells.

3.3. GCE treatment significantly inhibited MCP-1-induced monocyte migration during inflammation

Chemotactic migration of inflammatory cells represents a prerequisite to initiation of inflammation. Given that migration of monocytes is critical to magnify inflammation and tissue damage, next, we investigated the effect of GCE on MCP-1-induced chemotactic migration of human monocytes. The cellular anti-inflammatory response was measured by inhibition of MCP-1-induced chemotactic migration. The monocytes were treated with MCP-1 and GCE, and subsequently the cellular migration was measured (Fig. 2c). Our data demonstrated that MCP-1 exposure led to significant chemotactic migration of monocytes as compared with control ($p < 0.01$) whereas, GCE significantly inhibited the migration when compared with the MCP-1-treated group ($p < 0.01$). Together, we proposed that GCE exposure inhibited MCP-1-induced monocytic migration during inflammation.

3.4. GCE treatment enhances phagocytic activity in human monocytes

Phagocytic immune cells play a central role in innate immunity

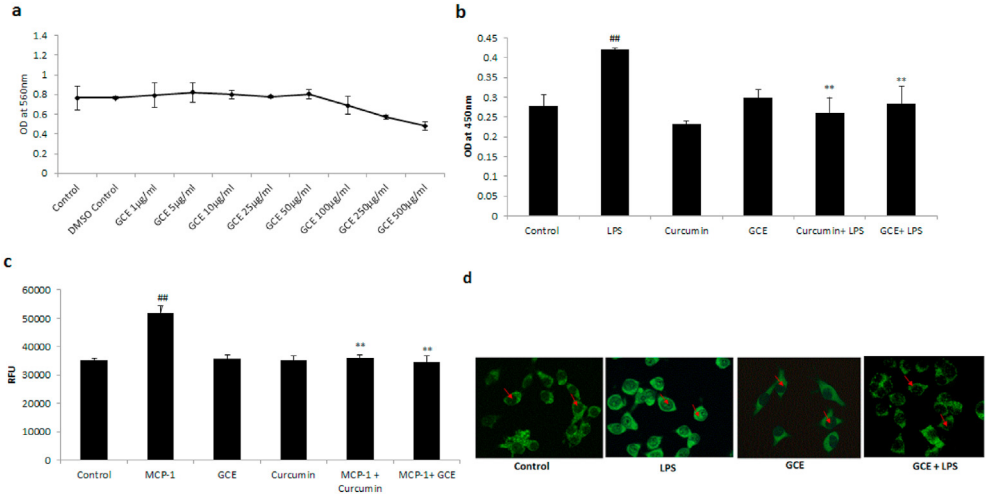

Fig. 2. GCE on cell viability and anti-inflammatory response. **a)** RAW 264.7 cells were treated with 1 μg/mL to 500 μg/mL of GCE for 24 h. **b)** Effect of GCE (50 μg/mL) on LPS induced NFkB p65 ser536 phosphorylation in RAW 264.7 macrophages. The symbols represent statistical significance : ##, ** = $p < 0.01$. ## Significant differences vs. control, ** Significant differences vs. LPS. **c)** Effect of GCE (50 μg/mL) on MCP-1 induced chemotaxis in human monocytes. The symbols represent statistical significance : ##, ** = $p < 0.01$. ## Significant differences vs. control, ** Significant differences vs. MCP-1. **d)** Inhibition of LPS induced NF-κB translocation into nucleus by the Green coffee extract. Control: NF-κB p65 was located in the cytoplasm in control group (red arrow). LPS: NF-κB p65 translocated to nucleus after stimulated by LPS (red arrow). GCE & GCE + LPS: GCE attenuated the translocation of NF-κB p65 to nucleus (red arrow). These were observed by immunofluorescence using confocal microscope (LSM 710 NLO, Carl Zeiss).

by eliminating pathogenic microbes and cancerous cells. Further, phagocytosis contribute to adaptive immune response by presenting antigens to immune cells. Next we sought to evaluate the *in vitro* role of GCE exposure of human monocytes to their phagocytotic activities (Fig. 3a). We found that the phagocytic activity of human monocytes in the presence of zymosan was significantly increased when compared with unstimulated control cells ($p < 0.05$). Similarly, the cells treated with GCE presented enhanced phagocytosis of zymosan particles when compared with control cells exposed to zymosan ($p < 0.01$). To confirm the phagocytic activity, we conducted live cell imaging investigation that clearly indicated that GCE exposure significantly enhanced pathogenic clearance (Fig. 3b). These results indicated that GCE was able to induce phagocytic activity and hence we proposed that GCE could enhance pathogenic clearance.

3.5. Exposure of GCE balances TH1/TH2 cytokines production in human T cells

There are two main subsets of T lymphocytes, which are distinguished by the presence of cell surface molecules known as CD4 and CD8. CD4 expressing cells are known as T helper cells that produce a variety of cytokines. This subset is further subdivided mainly into TH1, TH2 and TH17, and the cytokines they produce are known as TH1-type cytokines, TH2-type cytokines and TH17 cytokines, which includes both pro- and anti-inflammatory cytokines. The human T cells (Jurkat) were induced with PHA/PMA for an hour and treated with GCE for 24 h. The TH1/TH2 cytokines and other inflammatory mediators such as Eotaxin-1, Eotaxin-2, G-CSF, GM-CSF, ICAM-1, IFN-γ, I-309, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-6 sR, IL-7, IL-8, IL-10, IL-11, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-16, IL-17, IP-10, MCP-1, MCP-2, M-CSF, MIG, MIP-1α, MIP-1β, MIP-1 delta, RANTES, TGF-β1, TNF-α, TNF-β, sTNFRI, sTNFRII, PDGF-BB and TIMP-2 by antibody array (Fig. 3c). The qualitative analysis study clearly showed that GCE inhibits the PMA/PHA-induced cytokines viz., GM-CSF, IFN-γ, IL-1α, IL-4, IL-12 p70, MCP-1, MIG. However, GCE enhances the cytokines such as G-CSF, IL-2, IL-15, IL-17, MCP-2, IL-8, RANTES, and PDGF-BB in the presence of PMA/PHA exposure. A balanced release of TH1 and TH2 cytokines by GCE is central to optimal host immune responses.

3.6. Increased the production of IgG in human B lymphocytes was evident after treatment with GCE

Humoral immunity plays a significant role in strengthening immune responses, and protects the body from infection by extracellular pathogens and cancers. IgG is the most common circulating antibody, which also triggers complement protein cascade and enhances the effectiveness of phagocytosis by opsonization. Hence, we next examined the efficiency of GCE on antigen-specific IgG production by IM9 human B lymphocytes (Fig. 3d). Our data demonstrated that SAC and IL-2 significantly enhanced the production of IgG when compared with control ($P < 0.01$). Similarly, GCE significantly increased IgG production ($P < 0.05$) when compared to SAC and IL-2 treated cells. Together, we concluded that exposure of IM9 human B lymphocytes with GCE could enhance humoral immune responses.

3.7. General and safety observations with human subjects in randomized clinical study

Safety of an investigational product is of paramount importance in randomized clinical trials. The present clinical study clearly showed that there were no significant differences in body weight, height or BMI in GCE-treated subjects when compared with the placebo group. Similarly, there were no significant differences observed after treated with GCE in blood parameters and biochemical analysis such as RBC count, total leukocyte count (TLC), differential leukocyte count (DLC), platelet count, haematocrit or PCV, haemoglobin, random glucose, total protein, albumin, blood urea nitrogen (BUN), SGOT(AST), SGPT (ALT), alkaline phosphates, sodium, potassium and triglycerides. Hence, the present study clearly indicated that GCE is a safe herbal ingredient for likely human consumption.

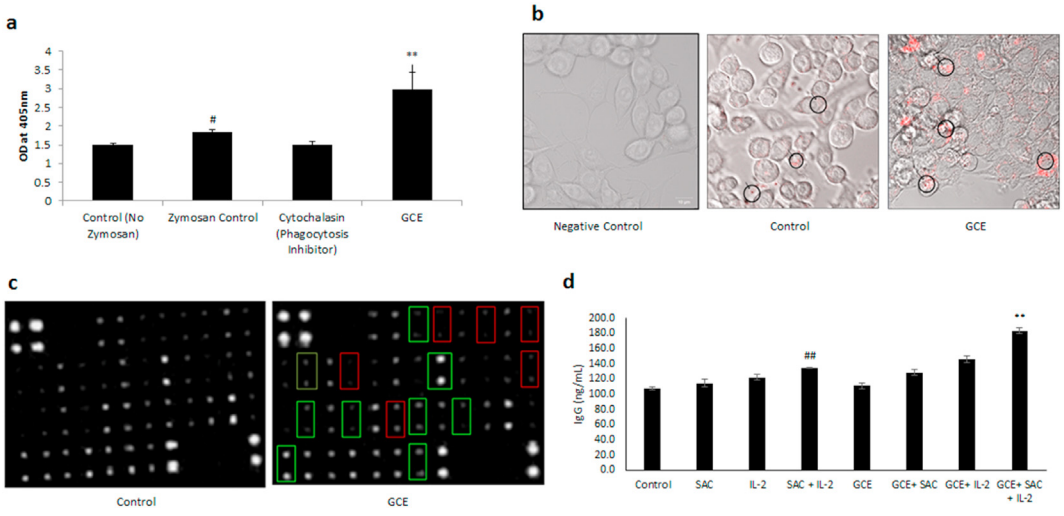

Fig. 3. GCE on immune responses. **a.** Effect of GCE (50 μg/mL) on phagocytic activity in human monocytes. The symbols represent statistical significance : # = $p < 0.05$, ** = $p < 0.01$. # Significant differences vs. control, ** Significant differences vs. zymosan control. **b**. Live cell imaging of phagocytic activity. Negative control: RAW cells alone (nullify the background auto fluorescence of the cells produced using 543 nm laser line). Control: RAW cells challenged with Zymosan particles alone. GCE: RAW cells challenged with Zymosan particles with GCE (50 μg/mL). Fluorescence of Zymosan particles was captured in the cells, shown in red colour in the images (encircled with black arrows indicated in image). **c.** Effect of GCE (50 μg/mL) on TH1/TH2 cytokine production in PHA/PMA induced in human T cells. Control: PHA/PMA induced control T cells; GCE: GCE treated with PHA/PMA induced T cells. Green color indicates enhancement of cytokines and Red color indicates down regulation of cytokines. **d.** Effect of GCE (50 μg/mL) on IgG production in human B cells. The symbols represent statistical significance : ## = $p < 0.01$, ** = $p < 0.01$. ## Significant differences vs. control, ** Significant differences vs. SAC + IL-2.

3.8. GCE intervention enhanced the functional efficacy of NK cells

NK cells are classified as group one innate immune cells and swiftly respond to a wide variety of pathological challenges. NK cells are cytotoxic lymphocytes expressing CD56 and CD16 surface proteins, capable of killing tumor and virus-infected cells without prior antigen exposure. In the present study, we have analyzed GCE on CD16/CD56 expression for NK cell activity with and without vaccination. There was a significant difference in circulating CD16/CD56 NK cells after treating with 125 mg GCE before vaccination (30th day; $p < 0.01$) when compared with the baseline and placebo groups. GCE (125 mg) treatment after vaccination (90th day) also showed a significant ($p < 0.05$) improvement in circulating CD16/CD56 NK cells when compared with the baseline. In addition, 250 mg GCE treatment showed significant ($p < 0.05$) enhancement in NK cell activity at the 30th day when compared to the baseline (Fig. 4a). Hence, the circulating CD16/CD56 NK cells positively correlated with the activation of innate immune responses.

3.9. GCE treatment did not alter the ratio of CD4/CD8 T cells

T helper (CD4) and T cytotoxic cells (CD8) play an essential role in efficient specific antimicrobial responses. Next, we analyzed the ratio of CD4/CD8 markers expression to study the efficacy of GCE on CMI responses. There were no significant differences in CD4/CD8 ratio after treatment with GCE (Fig. 4b). However, both 125 and 250 mg GCE treatments showed that there was a mild enhancement (10.04%) observed in CD4/CD8 ratio at 90th day when compared to placebo. CD4/CD8 ratios between 1.5 and 2.5 are generally considered normal. Hence, we concluded that GCE did not alter the CD4/CD8 ratio, and therefore GCE appers to strengthen the CMI responses.

3.10. GCE treatment modulates IgA production

IgA is a one amongst the five primary immunoglobulins (IgA, IgD, IgE, IgG and IgM) that plays a critical role in mucosal homeostasis and resistance against infection. IgA inhibits bacterial and viral adhesion to epithelial cells in the gastrointestinal, respiratory and genitourinary tracts. Here, we studied the role of GCE on the production of IgA before and after and influenza vaccination (Fig. 4c). Our results clearly showed that significant ($p < 0.05$) improvement in IgA production on treatment with 250 mg GCE was evident on day 90 of vaccination when compared with the baseline. Similarly, 250 mg GCE at 90th day showed an enhancement in IgA production when compared with 60th day 250 mg GCE treatment. Based on the clinical study results, we concluded that GCE may strengthen mucosal immune responses.

3.11. GCE exposure enhances IgG production

IgG is the most abundant serum antibody and plays a central role in the humoral immune response providing protection from blood pathogens. IgG plays crucial role in opsonization and neutralization of pathogens, and activation of the classical complement cascade. Our investigations showed that GCE did not alter IgG production when compared with placebo (Fig. 4d). Similar trends were observed with IgM and IgE production when compared with placebo (data not shown). However, there was an effect observed in IgG production when compared to the baseline. Intervention of GCE may involve in the modulation of humoral immune responses.

3.12. Treatment with GCE inhibited the production of IL-6 production

IL-6 is a pleotropic cytokine produced by inflammatory and immune cells in response to infections. The pro-inflammatory cytokine IL-6 is a biomarker of inflammation (Fig. 5a). The levels of IL-6 showed significant ($p < 0.05$) enhancement in the placebo group at 90th day when compared with day 1 of the placebo group. The present clinical study with healthy volunteers showed significant ($p < 0.05$) reduction in IL-6 cytokine level after treatment with GCE (250 mg) at 90th day when compared with the placebo group. Similar trend was observed in 125 mg GCE treatment at 90th day although this was not statistically significant. Suppression of IL-6 production by GCE is suggestive that the compound may act as an anti-inflammatory agent.

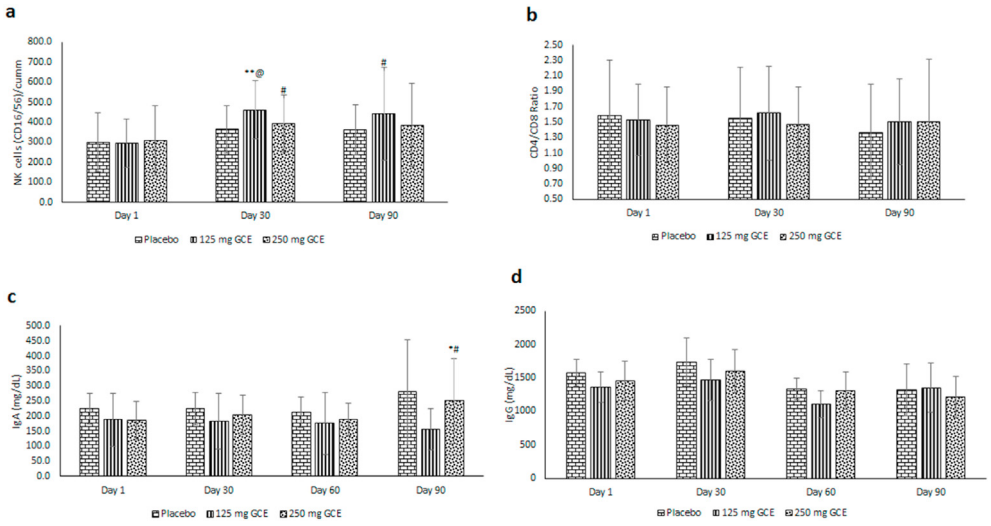

Fig. 4. GCE on immune responses in clinical study. **a.** Effect of GCE (125 and 250 mg) on NK cell activity. The symbols represent statistical significance : # = $p < 0.05$, ** = $p < 0.01$, @ = $p < 0.05$. **, # significant differences vs. baseline, @ significant differences vs. placebo. **b.** Effect of GCE (125 and 250 mg) on CD4/CD8 ratio. **c.** Effect of GCE (125 and 250 mg) on IgA production. The symbols represent statistical significance : #, * = $p < 0.05$. *significant differences vs. baseline, # significant differences vs. 60th day GCE. **d.** Effect of GCE (125 and 250 mg) on IgG production.

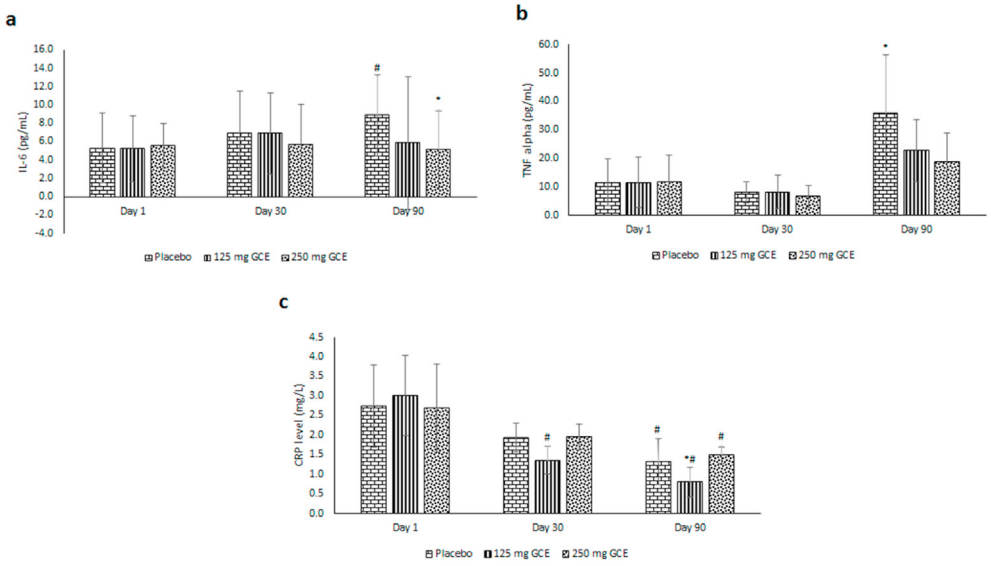

Fig. 5. GCE on inflammatory responses in clinical study. **a.** Effect of GCE (125 and 250 mg) on IL-6 production. The symbols represent statistical significance : * = $p < 0.05$, # = $p < 0.05$. *Significant differences vs. placebo (90th day), # Significant differences vs. placebo (day 1). **b.** Effect of GCE (125 and 250 mg) on TNF-α production. The symbols represent statistical significance : * = $p < 0.05$. *Significant differences vs. placebo (day 1). **c.** Effect of GCE (125 and 250 mg) on C- reactive protein. The symbols represent statistical significance : # = $p < 0.05$, * = $p < 0.05$. # Significant differences vs. baseline, * Significant differences vs. placebo.

3.13. TNF-α production was suppressed following treatment with GCE

TNF-α is a pro-inflammatory mediator that regulates many immunological as well as inflammatory functions. It is rapidly released following trauma and microbial infections. The levels of TNF-α was significantly ($p < 0.05$) enhanced in the placebo group on the 90th day when compared with day 1 of the placebo group (Fig. 5b). We examined whether the GCE was able to inhibit TNF-α secretion with pre- and post-vaccination conditions. Our clinical study showed that GCE was able to inhibit TNF-α on day 90 when compared to the placebo group although this was not statistically significant. Together, reduction in TNF-α production after treatment suggests that GCE may act as an anti-inflammatory herbal compound.

3.14. Exposure of GCE inhibited the C-reactive protein production

C-reactive protein is an acute-phase inflammatory mediator produced by the liver whose levels become elevated immediately following infection. CRP is regarded as a marker of systemic inflammation that predicts elevated risk of infection. Our current findings indicated that GCE (125 mg) significantly inhibited CRP ($p < 0.05$) on day 90 when compared with the placebo group (Fig. 5c). Similarly, a significantly decreased ($p < 0.05$) trend was observed in GCE (125 and 250 mg) treatment in the levels of CRP at days 30 and 90 when compared with the baseline. Hence, the present study revealed that GCE suppressed the production of CRP indicative of the anti-inflammatory role of GCE.

4. Discussion

One of the important functions of inflammation is to trigger the immune responses. However, prolonged inflammation can lead to impairment of proper immune function.[42] Nuclear factor κB (NF-κB) is a master regulator of inflammation and induces the expression of various pro-inflammatory genes including cytokines, chemokines and inflammasome.[43] NF-κB activation is tightly controlled by the inhibitor of κB (IκB), which normally interact with NF-κB and retained as inactive form in the cytosol and prevent it from entering the nucleus. Degradation of IκB bound to NF-κB enables it to enter the nucleus, where it binds to the promoters of pro-inflammatory genes.[44,45] In the present study, we analyzed Phospho-NF-κB p65 (RelA) ser536 to determine the anti-inflammatory efficacy and we found that GCE significantly inhibited LPS-induced NF-κB p65 (RelA) ser536 phosphorylation. Recent studies also indicates that bioactive compounds from green coffee extract, especially chlorogenic acid showed anti-inflammatory responses such as production of nitric acid and IL-1β by inhibiting JAK2/STAT3 activation in macrophages.[46,47] Furthermore, chlorogenic acid is an antioxidant, which has shown scavenging effect on reactive oxygen species, thereby decreasing oxidative stress in cells.[48,49] Reduction of oxidative stress will decrease the phosphorylation of NF-κB, thus reducing the levels of various inflammatory mediators and eventually reducing chronic inflammation in the host.[50] We have also analyzed the production of inflammatory cytokines such as TNF-α and IL-6. Our study clearly indicates that GCE potentially inhibited inflammatory cytokines which may be due to inhibition of NF-κB p65 (RelA) ser536 phosphorylation. Other inflammatory mediator CRP also plays an important role in inflammatory processes including the complement pathway, apoptosis, phagocytosis, nitric oxide release, and the production of pro-inflammatory cytokines, particularly interleukin-6 and tumor necrosis factor-α.[51]

Chemotaxis is a process wherein chemokines recruit leukocytes from the circulation to the site of infection and inflammation, however uncontrolled leukocyte migration leads to many inflammatory related diseases. Interference with chemokine and chemotactic functions are promising anti-inflammatory responses.[52] Chemotaxis is a most important activity of circulating monocytes. Chemokine receptor 2 (CCR2) is a dominant chemotaxis receptor in monocytes, which mediates chemotactic movement of monocytes in response to MCP-1.[53] We have analyzed the role of GCE on chemotactic migration of monocyte induced with Monocyte chemotactic protein-1 (MCP-1). MCP-1 induced chemotactic migration was inhibited by GCE, which may be due to the inhibition of CCR2 receptors of monocytes. Our results also

corroborated with earlier studies which reported that medicinal herbals has a potential role in inhibition of CCR2 receptors.[54] Another study revealed that active molecules from *Fallopia japonica* such as emodin and physcion were able to potentially inhibit CXCR4 receptor and chemotaxis migration.[55] GCE may act as an antagonist for CCR2 and CXCR4 receptors. In our study it was evident that GCE as an anti-inflammatory bioactive molecule inhibited LPS induced NF-κB p65 ser536 phosphorylation, MCP-1 induced chemotactic migration, IL-6, TNF-α and CRP levels in our clinical study subjects.

The immune system comprises of two lines of defence mechanism, they are innate and adaptive immunity. Innate immunity is a first line of defence against germs entering the body and it is a non-specific defence to encountering foreign pathogens. Innate immune response recruits immune cells to the sites of infection and inflammation through the production of chemokines, cytokines and other mediators. These proteins leads to the release of antibodies and other proteins which activate the complement system, opsonize foreign antigens and assist in phagocytosis.[56] In the present study, we sought to evaluate the *in vitro* immunomodulatory properties of GCE by analyzing innate immune responses by phagocytic activity and NK cell activity. Phagocytes have scavenger receptors, Fcγ Receptors (FcγRs), and complement receptors (CRs) which are involved in the uptake of foreign antigens.[57] Herbal compounds having the immunomodulatory property activate the complement pathway by generating complement receptor type 3 (C3b) and C3bi which binds to pathogens and assist in the phagocytosis. These complement bounded pathogens will be recognized by phagocytic immune cell receptors such as CR1 (CD35) and CR3 (CD11b) to start the phagocytosis process.[58] Moreover, herbal molecules stimulate the expression of Fcγ receptor on the phagocytic immune cells and then promotes the binding of opsonized pathogens to these receptors. The expression of Fcγ receptor such as FcγRIII (CD16) and FcγRII (CD32) are known to enhance the phagocytosis process.[59,60] The present study clearly indicates that GCE enhanced the phagocytic activity, which may be due to the stimulation of Fcγ and CR receptor.

In humans, NK cells are divided into CD56dim and CD56bright. Around 90% of peripheral blood and spleen NK cells are CD56dim $CD16^{+}$ and express perforin61. *Rhaphidophora korthalsii* has been widely used in Chinese traditional medicine for cancer and skin disease treatment. Extract from *Rhaphidophora korthalsii* enhanced the CD56/CD16 expression which leads to production of immunomodulatory cytokines, proliferation and killing ability of NK cells.[62] Other studies also demonstrated that chlorogenic acid and other plant phenolic compounds enhances macrophage phagocytic activity and NK cell activity.[63,64] Similarly, other *in vitro* studies revealed that chlorogenic acid enhances T-cell proliferation after induction with influenza virus antigen through production of IFN-γ and IFN-α.[65,66] During viral infection, NK cells generate IFN-γ as a part of innate immune responses to inhibit viral replication and polarize T cell immunity.[67–69] Our study findings also corroborated with other studies on enhancement of CD16/CD56 expression after treated with GCE.

Adaptive immunity becomes active when pathogenic agents overpower the innate immunity making it ineffective in eliminating them and successful in establishing the infection. The adaptive responses are highly specific to the particular pathogen that induced them and provides long-lasting protection. The presence of an immunologic memory consisting of B and T lymphocytes that can recognize much more quickly and eliminate a specific pathogen during subsequent infections.[70] We have performed in-vitro analysis of TH1/TH2 cytokines and IgG production. Clinical study for CD4/CD8 ratio and immunoglobulins production. In our *in vitro* evaluation we found that GCE effectively modulated the TH1/TH2 cytokines and enhanced the IgG production. The antibody array analysis clearly showed that GCE inhibits the PMA/PHA (Phorbol myristate acetate and/or phytohemagglutinin) induced TH1/TH2 cytokines viz., GM-CSF, IFN-γ, IL-1α, IL-4, IL-12 p70, MCP-1, MIG. However, GCE enhanced the cytokines such as G-CSF, IL-2, IL-15, IL-17, MCP-2, IL-8, RANTES, and PDGF-BB in the presence of PMA/PHA exposure. Similar trends were observed in treatment with quercetin on TH1/TH2 cytokine production.[71] This speculation may be supported by the present observation showing the suppressive activity of TH1 pro-inflammatory cytokines and stimulatory activity of TH2 anti-inflammatory cytokines by GCE.

The IgG is the predominant isotype in the serum for primary humoral response and it is the main isotype in the succeeding humoral responses.[72] The present in-vitro study clearly showed that GCE enhanced the IgG production in the presence of IL-2 and activated with mitogen *Staphylococcus aureus* Cowan I (SAC). Our present finding is corroborated with earlier studies which revealed that plant extracts with high contents of polyphenols have a stimulatory effect on IgG production and such phenolic compounds are being used as vaccine adjuvants.[73,74] IgA plays an important role in mucosal immune response. Peyer's patches are the main inductive sites for IgA response. The study from Tauchi et al.,[75] revealed that traditional Chinese medicine Shosaiko was able to stimulate the Payer's patches to produce IgA in antigen challenged (sheep red blood cells) rats. Similarly, chlorogenic acid enhanced IgG production and secretory IgA in intestinal cells of weaned pigs.[76] In our clinical study, GCE showed significant enhancement in IgA production, which may be because of activation of B-cells in Payer's patches in the presence of antigens. In addition, another study also revealed that GCE was able to shift microbiota in human gut,[77] which may also be a possible reason for the enhancement of IgA production in GCE treated subjects.

In conclusion, GCE significantly inhibited LPS-induced NF-κB p65 ser536 phosphorylation and MCP-1-induced chemotactic migration. Innate and humoral immunity markers such as phagocytosis and IgG showed significant enhancement after treatment with GCE. Investigation of CMI markers such as TH1/TH2 cytokines showed that GCE inhibits PMA/PHA induced GM-CSF, IFN-γ, IL-1 alpha, IL-4, IL-12 p70, MCP-1, MIG production. GCE also showed activation of G-CSF, IL-2, IL-15, IL-17, MCP-2, IL-8 (CXCL8), RANTES and PDGF-BB. Collectively, our *in vitro* studies have shown that GCE possess anti-inflammatory and immunomodulatory properties. In human clinical study, immunological profiles such as NK cell marker, CD56 and CD16 surface protein expression showed significant enhancement after GCE treatment. GCE significantly enhanced the production of IgA before and after antigen challenges when compared with baseline. Similarly, inflammatory markers such as IL-6, TNF-α and CRP levels were significantly inhibited by GCE. However, the adaptive immune markers like CD4/CD8 ratio, IgG, IgM and IgE did not show any significant alteration when compared with the placebo. The collective results presented in this study clearly provided evidence that GCE can be a promising new therapeutic approach to reduce inflammation and necessitate beneficial modulation of immunological components in the host. Future direction for aiming to expand our current outcomes on how GCE mediates these effects are warranted.

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Ethics approval

We are confirming that the study was approved by CLINICOM (Independent Ethics Committee for Evaluation of Protocols for

Clinical Research), Bangalore. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This study was registered in Clinical Trials Registry - India (CTRI/2014/12/005247). The protocol also complied with Good Clinical Practice (GCP) and the Helsinki Declaration Standards, as well as per Indian GCP and Schedule Y (amended version 2005) and Indian Council of Medical Research codes.

Consent to participate

Written informed consent was obtained from each subject prior to initiation of the clinical study. The informed consent document (ICD) was translated into local language, Kannada. Investigator gave a description of any foreseeable risk/s and discomfort/s to the subject. Every subject had the right to withdraw from the study at any time without giving any reasons. Before participating in the study, the subject had signed and dated the ICD. If a subject was unable to comprehend and sign or leave their thumb impression on the ICD, they were excluded from the study. If the subject was illiterate, the consent was read and explained to the subject by the impartial witness/legal representative and their dated signature was taken apart from subject's left thumb impression on the ICD before entering into the study. A copy of the signed and dated ICD was provided to the subject. The Investigator kept the original signed copies of all consent forms in the study file.

Consent for publication

The authors are consent to publish this work.

Authors contributions

This clinical study has been sponsored by ITC Limited., and the randomized, double blinded, placebo controlled trial was conducted by an external CRO (Semler Research Centre Pvt Ltd). Data collection and analysis were performed by external CRO. It was also checked by Jeyaparthasarathy Narayanaperumal and Ganesh Gopal. The concept and design was done by Jeyaparthasarathy, Avin Dsouza, Amarnath Miriyala and Ganesh Gopal. *In vitro* study conception, design, data collection and analysis were performed by Jeyaparthasarathy Narayanaperumal and Ganesh Gopal. The experiments were performed by Jeyaparthasarathy Narayanaperumal, Avin Dsouza and Amarnath Miriyala. The first draft of the manuscript was written by Ganesh Gopal and Bhavna Sharma and all authors commented on versions of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no competing financial interest and also declare no conflict of interest. This work is financially supported by ITC Limited, INDIA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2022.01.007.

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