

In vitro immunomodulatory potential of macromolecular components derived from the aqueous extract of *ajowan* [*Trachyspermum ammi* (L.) Sprague]

Shruthi RR, Venkatesh YP & Muralikrishna G*

* Department of Biochemistry, CSIR-Central Food Technological Research Institute (CFTRI), KRS Road, Mysuru-570020, Karnataka State, India

E-mails: krishnagm2002@yahoo.com, gmk@cftri.res.in

Received 02 June 2016, revised 09 November 2016

Ajowan [*Trachyspermum ammi* (L.) Sprague] has proven medicinal and nutritive properties. The role of *ajowan* within the intricate network of interaction of cells of the immune system has also been quite studied. In this present study, the immunomodulatory properties of polysaccharide/polysaccharide-protein complexes (PPCs) have been assessed. *Ajowan* aqueous extract was precipitated with ethanol followed by dialysis. The resultant dialyzed solution was subjected to DEAE-Cellulose column chromatography by stepwise elution with sodium chloride (0-0.5M); 0.4 M NaCl eluate showed effective mitogenic activity towards splenocytes and hence named as, *ajowan* immunomodulatory component (ImC). This component of interest comprised of acidic polysaccharides, glycoproteins and associated bound phenolics. ImC induced proliferation of murine splenocytes effectively at the concentration of $1 \mu\text{g mL}^{-1}$ ($p < 0.005$). Pronase treated ImC (ImC-Pt) failed to elicit mitogenic effect towards splenocytes; however, it could activate peritoneal exudate cells for synthesis of nitric oxide and phagocytosis at the minimum concentration of 10 ng mL^{-1} ($p < 0.005$). From the above results it can be concluded that both protein and polysaccharide constituents of ImC have independent roles in its immunomodulation property, *i.e.*, protein on B-lymphocyte proliferation and carbohydrates on macrophage activation.

Keywords: Immunomodulation, *Ajowan* ImC, Arabinogalactan protein (AGP), Splenocytes, Macrophages, Cytokines

IPC Int. Cl.⁸: A61K 36/00, A01C 1/08, A61K 39/00, C07K, A61K 38/43

Macromolecular constituents such as, proteins and polysaccharides isolated from various plants documented in traditional medicine systems have been shown to exhibit a series of biological and therapeutic effects¹. From diverse studies, it has been established that these macromolecules mend the disease causing mechanisms by providing the necessary signals for protein targeting and also serve as a binding sites for toxins, hormones and other infectious agents such as bacteria and viruses involved in the pathophysiology of several disease conditions². Some of the bioactive plant polysaccharides present as polysaccharide-protein complexes (PPCs) have been used as promising therapeutic agents at pharmaceutical level in cancer therapy owing to their immunomodulatory attributes³. Protein immunomodulators are considered to be strong inducers of lymphocyte proliferation that play an important role in adaptive immune responses, whereas polysaccharides are generally considered as

classic T cell-independent antigens, that do not elicit cell-mediated immune response, and is short lived⁴. On the other hand, PPCs are considered as multi-cytokine inducers that can induce gene expression of various immunomodulatory cytokines and cytokine receptors thereby helping in maintenance of homeostasis without much of intense side effects like inflammation⁵. Thus, identification and characterization of such biologically active macromolecules helps in evoking new concepts that would lead to the discovery of new immunomodulators for clinical research. Of late, immune based therapies are gaining wider acceptance than monovalent approaches in treating several chronic disease conditions. Given that modulation of immune responses and enhancing the immunogenic potential of the system is believed to be one of the best disease preventive strategies, the identification of appropriate classes of bioactive molecules from herbal/natural sources to abate immunological complications that occur during the progression and pathogenesis of many diseases has spurred increasing

*Corresponding author

interest in alternative forms of medicinal systems such as those of *Ayurveda*, Traditional Chinese Medicine and *Unani*⁶. Several herbal constituents have the tendency to exert their beneficial effects mainly via modulating both humoral and cellular immune responses and exhibit their ability to regulate inflammatory processes by modulating cytokine expression, histamine release, lymphocyte stimulation, Ig class switching, phagocytosis so on and so forth. More often than not, the said herbal constituents tend to be secondary plant metabolites such as, glucans (polysaccharides), glycosides (saponins, PPCs), polyphenols, etc. Likewise, many herbal plant polysaccharides/PPCs have been studied for their immunomodulatory effects and have been demonstrated to boost up the immune system and render protection to the body against variety of diseases^{4,7}.

Trachyspermum ammi (L.) Sprague (TA) [syn. *Carum copticum* (L.)] commonly known as *ajowan/ajwain* represents a highly reputable grassy herb type which is classified as a member under the medicinally important, valued and well-known family of herbaceous plants, the Apiaceae^{8,9}. TA/*Ajowan* is notably acclaimed for its consolidated therapeutic/medicinal profile with the seeds and phytochemical constituents of *Ajowan* reported to exhibit a plethora of disease preventive properties, chiefly including biological effects such as, immunomodulatory/anti-inflammatory, blood pressure lowering, anti-calcifying and antioxidant activities¹⁰⁻¹⁴. The major active principles in TA/*Ajowan* thought to be responsible for these activities are thymol and carvacrol¹⁵⁻¹⁶. TA/*Ajowan* seeds have been implicated in modulating T cell responses, especially its pharmacological roles in treating inflamed lung airway in asthmatic cases of activated Th2 response, where, carvacrol contained in TA functions by balance shifting the immune response from the Th2 type back toward the Th1, through decrement of TNF- α , IL-1 β , IL-4, IL-17 and TGF- β cytokine expression but increased IFN- γ and FOXP3 expression. TA/*Ajowan* oil which has been exploited at a therapeutic level in effectively addressing gastric diseases and other respiratory ailments also been known to deliver its effects via immunomodulatory attributes. Thymol, the predominant essential oil constituent in TA/*Ajowan* oil has been known to stimulate peripheral blood mononuclear cell (PBMC)/lymphocyte proliferation, wherein, as a direct effect of TA/*Ajowan* oil, an

increase in the memory/activated CD8 α^+ T cell subsets which are believed play in favor of a wanted situation in immune responses has been observed¹⁷⁻¹⁸. However, there are no studies aimed at exploring the immunomodulatory effects of bioactive macromolecular composition comprising TA/*Ajowan* glucans and PPCs. In light of these, the present study has been focused on evaluating the immunomodulatory profile of such macromolecular components in TA/*Ajowan* using *in vitro* culture models.

Materials

E. coli lipopolysaccharide (LPS), concanavalin A (Con A), bovine serum albumin (BSA), RPMI-1640 medium, nonspecific protease (pronase E) and Histopaque-1083 were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Standard protein molecular weight markers, TMB/H₂O₂ (substrate for horseradish peroxidase) and DEAE-Cellulose were obtained from GeNei, Merck Specialties Pvt. Ltd., Mumbai, India. Fetal bovine serum (FBS), antibiotic solution (100X) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide A.R.] were obtained from Hi Media Laboratories Ltd., Mumbai, India. Tissue culture plates (96-well, gamma sterilized) were procured from Nest Biotech Co., Ltd., Jiangsu, China. All other chemicals and reagents used were of analytical grade. *Ajowan* was obtained from Pro Nature Organic Foods Pvt. Ltd., Bangalore, India.

Methods

Experimental animals

Spleen, thymus and myeloid lymph node (MLN) of BALB/c mice (5-6 weeks old, 23 $\pm$ 2 gm) were used for the isolation of splenocytes, thymocytes and MLN cells, respectively. Peritoneal exudate cells (PECs) of male Wistar rats (8-weeks-old) were the source of macrophages used for evaluation of nitric oxide (NO) release and phagocytosis assays. All experimental procedures involving the handling and caring of animals have been carried out in accordance with the Institutional Animal Ethical Committee (IAEC) guidelines.

Preparation and fractionation of *ajowan* extract

Ajowan was finely ground (100 gm) and extracted with distilled water (1 L) at 4 °C overnight, and the extract was subjected to precipitation using three volumes of absolute ethanol at 4 °C overnight. The ethanol precipitate was then reconstituted in a small

volume of 20 mM Tris-HCl buffer (pH 8.2) and dialyzed against the same buffer using 12-14 kDa cut-off dialysis membrane. The dialyzed sample was loaded on DEAE-cellulose column (3 × 25 cm) and stepwise-elution was carried out using 20 mM Tris-HCl buffer (pH 8.2) containing different molar concentrations of NaCl, i.e., 0.1 M (D1), 0.2 M (D2), 0.3 M (D3), 0.4 M (D4) and 0.5 M (D5) at a flow rate of 1 mL min⁻¹. Analysis of all the peak fractions of the components (D1, D2, D3, D4 and D5) for immunomodulatory activity was done by cell proliferation assay.

Biochemical analysis of the immunomodulatory component (ImC) of *ajowan*

D4 (0.4 M NaCl eluate) showed the highest immunostimulatory activity among the five DEAE-Cellulose components, and accordingly labeled as the immunomodulatory component (ImC) of *ajowan*. Protein concentration of ImC was determined by Bradford assay method using BSA as the standard¹⁹. DEAE-Cellulose fractionated components were run on 12 % SDS-PAGE and gels were separately stained with Coomassie brilliant blue R-250 and periodic acid-Schiff (PAS) staining to detect the presence of protein and glycoproteins, respectively. Carbohydrate content was measured by phenol-H₂SO₄ assay method using D-glucose as the standard. The uronic acid content was determined by MHDp method using glucuronic acid as standard²⁰⁻²¹. Total phenolics were measured by Folin-Ciocalteu method using ferulic acid as standard²². *Ajowan* ImC was subjected to pronase treatment to remove the proteins and dialyzed against distilled water using a 12-14 kDa cut-off membrane and lyophilized. The pronase-treated *ajowan* ImC is designated as ImC-Pt.

Cell count and viability assay

Thymus, spleen and myeloid lymph node (MLN) were dissected from 6-week-old BALB/c mice aseptically and washed with complete RPMI-1640 medium (RPMI-1640 medium with 10 % FBS and 2.5 % antibiotics – streptomycin and tetracycline). To this, Histopaque-1083 was added in the ratio of 3:2 (v/v) and centrifuged at 100 × gm for 10 min. The whitish cell layer was aspirated and suspended in fresh RPMI-1640 medium; these cells devoid of RBCs were washed and finally suspended in complete RPMI-1640 medium. Percentage viability of thymocytes, MLN cells and splenocytes in the prepared cell suspensions was checked by Trypan

blue exclusion method using a Bright-Line® hemocytometer (Reichert Scientific Instruments, Buffalo, NY, USA)²³.

Mitogenic activity of *ajowan* extract and *ajowan* ImC

The mitogenic activity of *ajowan* ImC was carried out *in vitro* by MTT assay using 96-well tissue culture plates²⁴. To each well, 100 µL of RPMI-1640 complete medium containing the test sample at different concentrations (0.1 µg, 1 µg and 10 µg) were added followed by 100 µL of cell suspension (2 × 10⁵ cells per well). The culture plates were incubated in a CO₂ incubator (5 % CO₂) at 37 °C and 80 % relative humidity for 72 hrs. Cells in the absence of test sample represent control, and blank was carried out with complete medium. After 72 hrs of incubation, 20 µL of 6 mg mL⁻¹ MTT solution (MTT dissolved in PBS, filtered to remove any insoluble matter and filter-sterilized) were added and incubated for an additional 4 hrs under the same conditions. Next, the plate was centrifuged at 750 × gm at 4 °C for 15 min. The supernatant was removed and the blue formazan crystals were resolubilized in 100 µL of acidic isopropanol (0.7 N HCl in isopropanol) under agitation. After dissolving the crystals, the plates were read in a microtiter plate reader (Model 680, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 570 nm. Mitogenic activity is represented as proliferation index in comparison to control, whose absorbance at 570 nm is taken as 1.

Release of NO from rat PECs by *ajowan* ImC and ImC-Pt

Peritoneal exudates cells are tested for the release of nitric oxide (NO), as it refers to the quantitative measure for the activation of macrophages by test samples. Rat peritoneal exudate cells were isolated from adult male Wistar rats (8-week-old weighing 220–250 gm) as described²⁴. Cells were treated with RBC lysis buffer and washed thoroughly. One hundred microliters of cell suspension (2 × 10⁵ cells mL⁻¹) were added to the 96-well cell culture plate containing various concentrations of *ajowan* ImC (0.01, 0.1, 1, and 10 µg) in 100 µL complete RPMI-1640 medium and incubated at 37 °C in 5 % CO₂, 80 % humidified incubator for 48 and 72 hrs. After the incubation period, 100 µL of supernatant from each well were transferred to a fresh microtiter plate, and an equal volume of Griess reagent was added followed by incubation at 25 °C in the dark for 15

min. Calibration curve was prepared using sodium nitrite (NaNO_2). Absorbance of chromophoric azo-derivative was read in a microtiter plate reader at 540 nm, and the amount of NO released is represented in μM^{25} .

Effect of *ajowan* ImC and ImC-Pt on phagocytic activity of rat PECs

Phagocytic assay was performed according to the method described by Roy & Rai²⁶. Briefly, 1 mL of PECs (1×10^5 cells mL^{-1}) was flooded onto clean, sterile glass slides to which 10 μL of PBS containing *ajowan* ImC or ImC-Pt (0.01, 0.1, 1 and 10 μg) was added. The macrophages were allowed to adhere by incubating at 25 °C in a CO_2 incubator for 2 hrs; non-adherent cells were washed off with sterile PBS. Each slide is flooded with 0.3 % heat killed baker's yeast (*Saccharomyces cerevisiae*) prepared with PBS and phagocytosis was allowed to proceed at 25 °C. After 2 hrs, the slides were rinsed three times with PBS, fixed in methanol, and stained with differential Giemsa stain. The phagocytic index of the test sample was determined by considering the number of phagocytic macrophages in the control slide as 1.

Statistical analysis

All assays were performed in triplicates. The statistical analysis was done using Prism 5 software (Graph Pad, San Diego, CA). One way 'ANOVA' (analysis of variance) was done to determine the statistical significance. A p value of < 0.05 was taken as statistically significant.

Results

Analysis of *ajowan* extract and fractionation by DEAE-cellulose chromatography

Ethanol precipitation of *ajowan* aqueous extract followed by dialysis and lyophilization yielded 8.4 gm solid from 100 gm *ajowan* powder. The protein content was found to be ~1.8 gm, total sugar ~1.5 gm, uronic acid ~0.48 gm (all per 100 gm *ajowan* dry powder) and very low amount of phenolic content (0.2 mg). The amount of proteins and carbohydrates was comparatively lesser than the said amount in the literature, as smaller peptides and oligomers of below 12 kDa were removed during dialysis and only macromolecules (> 12 kDa) retained were taken for the experiments. The resolubilized ethanol precipitate of *ajowan* was fractionated on DEAE-Cellulose as shown in Fig.1. The resulting 5 components (D1 to D5) were analyzed for protein and total sugar content and assessed for immunomodulatory activity (Fig. 2); out of which only component D4 (0.4 M NaCl eluate) showed significant proliferative activity towards murine splenocytes and accordingly designated as the immunomodulatory component (ImC). This fraction of interest was quantified for protein, total sugar content and bound phenolics. Protein and carbohydrates constituted only 3.3 and 2 %, respectively of the yield with negligible amount of phenolics. SDS-PAGE of ImC showed 4 prominent bands and PAS staining confirmed the presence of a couple of glycoproteins (Figs. 3 a&b). The uronic acid content of ImC represents ~50 % of total carbohydrate content. The pronase treated ImC (ImC-

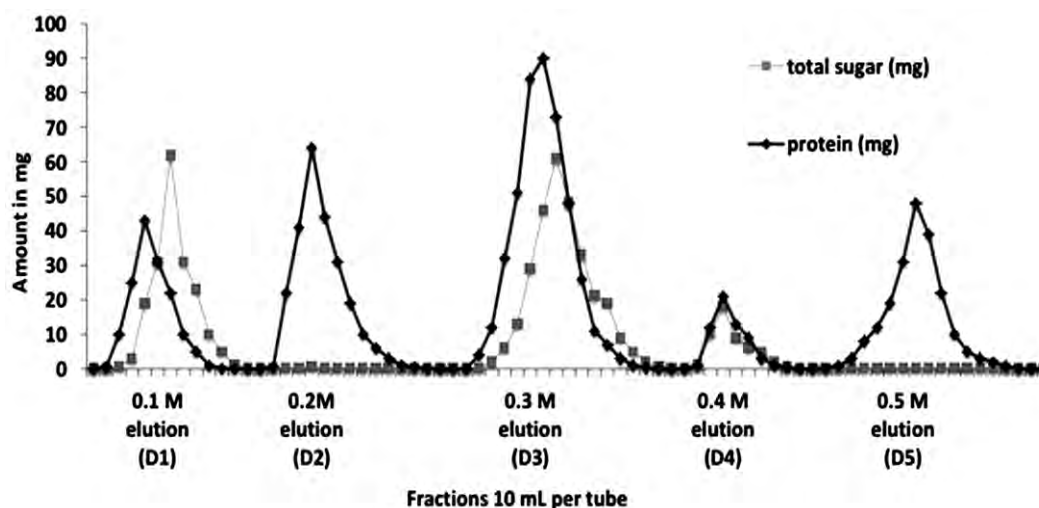


Fig. 1—DEAE-Cellulose chromatography of ethanol precipitate of *ajowan* aqueous extract. Buffer: 20 mM Tris-HCl buffer (pH 8.2) step elution using increasing concentration of NaCl (0.1- 1.0 M)

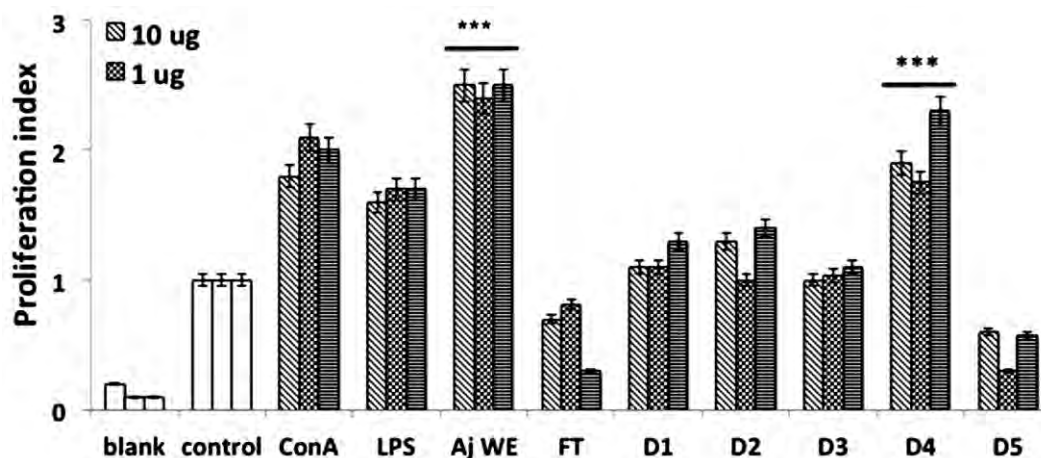


Fig. 2—Mitogenic activity of *Ajowan* components. *Ajowan* aqueous whole extract (AjWE), DEAE-Cellulose components (D1-D5) and flow through (FT) were assessed for proliferation activity at different concentrations (0.1 to 10 μ g) using murine splenocytes (2×10^5 cells per well), incubated at 37 °C for 72 hrs. ConA and LPS were used as positive controls. Each value is represented as mean $\pm$ SD, n=3. Level of significance *** $p < 0.005$ compared to untreated cells (control).

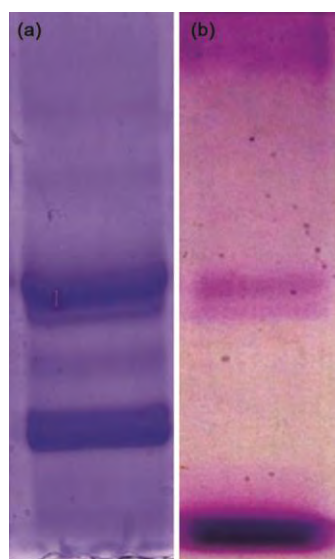


Fig. 3 a-b; (a)—SDS-PAGE (12 %, reducing condition) of 0.4 M NaCl eluate (ImC) of DEAE-Cellulose chromatography & Fig. 3(b)—Periodic acid-Schiff (PAS) staining of ImC on 12 % SDS-PAGE (reducing condition).

Pt) showed significant drop (> 50 %) in total carbohydrate content upon removal of protein.

Mitogenic activity of *ajowan* components on immune responder cells

The significant mitogenic activity of ImC was seen towards murine splenocytes. Hence, this component was further analyzed for its response towards other two lymphoid tissues, viz., thymus (source of T cells) and myeloid lymph node or MLN (source of myeloid or granular precursor cells) along with spleen (source of B and T lymphocytes). The stimulatory activity

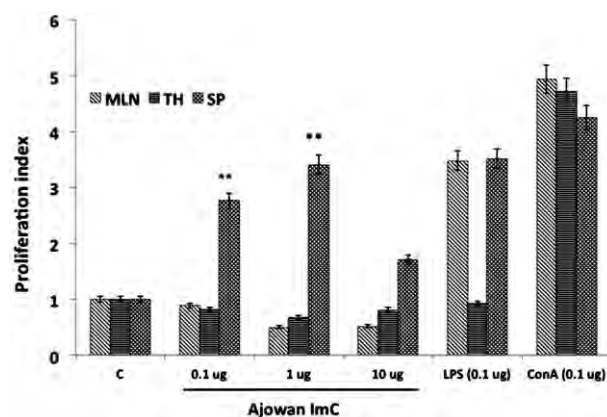


Fig. 4—Mitogenic activity of *ajowan* ImC. The mitogenic activity of *ajowan* ImC was assessed for proliferation activity at different concentrations (0.1 to 10 μ g) towards murine splenocytes (SP), thymocytes (TH) and myeloid lymph node (MLN) cells (2×10^5 cells per well), incubated at 37 °C for 72 hrs. Con A and LPS were used as positive controls. Each value is represented as mean $\pm$ SD, n=3. Level of significance ** $p < 0.01$, * $p < 0.05$ compared to untreated cells (control, C).

was seen towards splenocytes only, and the other two cell types (thymocytes and MLN cells) did not respond to the ImC fraction as shown in Fig. 4. LPS and ConA were used as reference positives; Component ImC showed ~2.5 and 3.5-fold increase in mitogenic activity at 0.1 and 1 μ g, respectively, compared to untreated cells. The pronase-treated ImC component (ImC-Pt) was also examined for immunostimulatory activity and it failed to induce splenocyte proliferation singly (Fig. 5). This experiment clearly explains the importance of *ajowan* protein components in the mitogenic activity of ImC component.

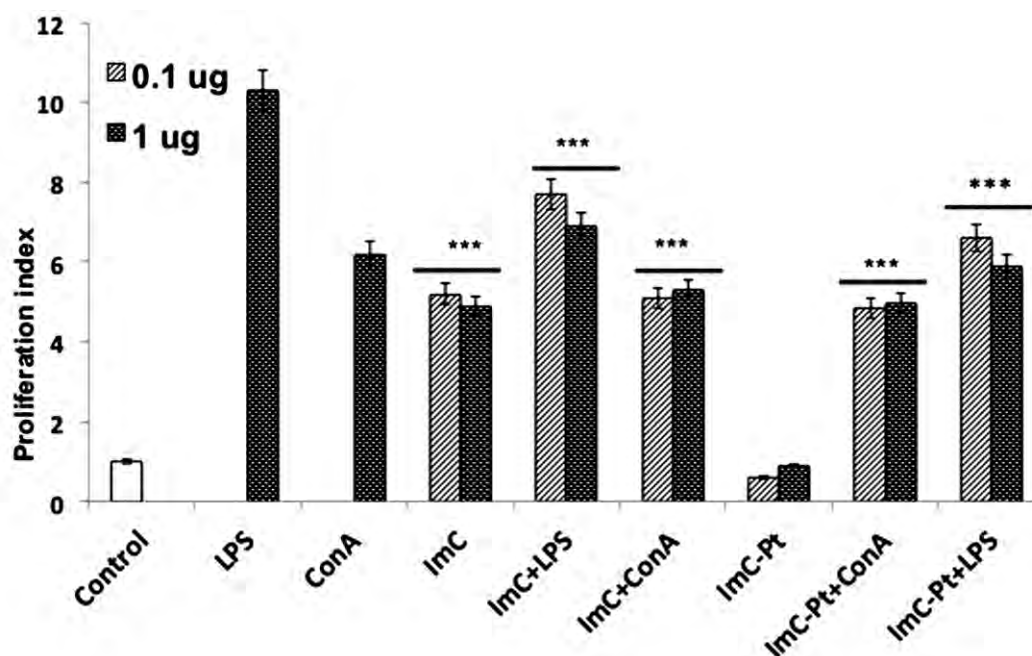


Fig. 5—Effect of *ajowan* ImC and ImC-Pt on murine splenocytes. Both the samples were incubated with splenocytes in the presence of ConA and LPS (100 ng and 50 ng, respectively) for 72 hrs. Each sample was tested in triplicates and represented as mean $\pm$ SD. *** $p < 0.005$ compared to control.

Activation of PECs (source of macrophages) by ImC and ImC-Pt

Nitric oxide (NO) release can be used as a quantitative index of macrophage activation. In the present study, it is seen that both ImC and pronase-treated component (ImC-Pt) stimulated macrophages to produce NO. The release of NO from rat peritoneal exudate cells, upon incubation with ImC is shown in Fig. 6a. ImC and ImC-Pt components showed 4 and 3-fold increase in NO release, respectively, compared to untreated cells at four different concentrations (0.01, 0.1, 1, and 10 μ g) measured at 72 hrs. The differences in NO production between the components, LPS and the controls were statistically significant ($p < 0.01$). Overall, ImC and ImC-Pt produced a similar magnitude of response compared to LPS, indicating that the protein portion is not involved in the activation of macrophages.

Effect of ImC on the phagocytic activity of PECs

The engulfment of ghost yeast cells by macrophages was significantly higher compared to control for both ImC and ImC-Pt (Fig. 6b). The phagocytic index increased by 2 to 2.5-fold ($p < 0.005$) at 0.01, 0.1, 1 and 10 μ g of *ajowan* ImC (D4) and ImC-Pt.

Discussion

Spices have been an integral part of culinary preparations and many are being celebrated for their medicinal properties well before culinary usage. Spices not only excite the taste buds but also constituted with impressive health benefits. Over the past few years, immunomodulatory macromolecules have been studied from different spices, for example, the mannose specific lectins ASA I and ASA II proteins from garlic, immunomodulatory fructans and mannose specific lectin from onion and macromolecules from guduchi^{24,25, 27, 28,29}. *Ajowan* is one of the spices for which studies related to immunomodulation are limited to small molecules like carvacrol, carvone and volatile oils (thymol, cymene, terpinene, pinene, etc.). One of the components of DEAE- cellulose column (component D4 or ImC) showed significant immunostimulatory property. This component induced morphological changes (blasting) followed by proliferation of the splenocytes (B lymphocyte-rich cells), whereas the proliferation activity was marginal with thymocytes and MLN cells. This differential effect of ImC on different immune responder cells shows the specificity towards the receptors of the lymphocytes. Thymocytes are rich with cytotoxic T cells (CTL/ T_c) and different types of helper T cells (T_H). ImC had no

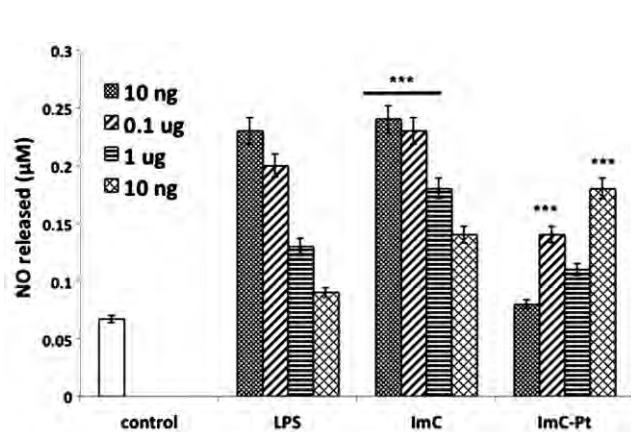


Fig. 6(a)—Effect of ImC and ImC-Pt on macrophage activation. ImC and ImC-Pt were tested (at different concentrations of 0.01 to 10 µg) for nitric oxide release by peritoneal exudate cells. Cells were incubated for 72 hrs at 37 °C, 80 % relative humidity and 5 % CO₂ and was quantified for nitrite using Griess reagent. LPS was used as positive control. Each value is represented as mean ± SD, n=3. Level of significance; *** $p < 0.005$, ** $p < 0.01$ compared to control.

effect on proliferation of thymocytes and MLN cells (as showed in the data) either singly or in the presence of LPS/Con A (data not shown). It is well known that spleen contains mostly B-lymphocytes along with fewer cytotoxic T cells and monocytes, unlike thymus. Hence, the macromolecular components are acting mostly on B-lymphocytes. Hemagglutination assay using D4 component went negative with rabbit, mouse and human blood samples there by divulging the absence of lectin activity in the fraction of interest. Upon biochemical analysis, ImC revealed that it contained at least four proteins amongst which two are glycoproteins and colorimetric estimation (MHDP method) showed, that almost 50 % of the total carbohydrate is comprised of the acidic polysaccharides. The pronase-treated ImC (ImC-Pt) was also tested for the presence of acidic polysaccharides, but there was a significant loss in carbohydrate content upon protein digestion, which hinted the existence of macromolecules in complexes along with unbound molecules. ImC-Pt failed to activate murine splenocytes, but the proliferation activity remained unaffected with murine macrophages. Macrophage activation leading to NO production and phagocytosis was significant even after removal of the proteins from the immunomodulatory component ($p < 0.001$).

The ability of bioactive polysaccharides to modulate so many important immune cells is due to the structural diversity and variability of these

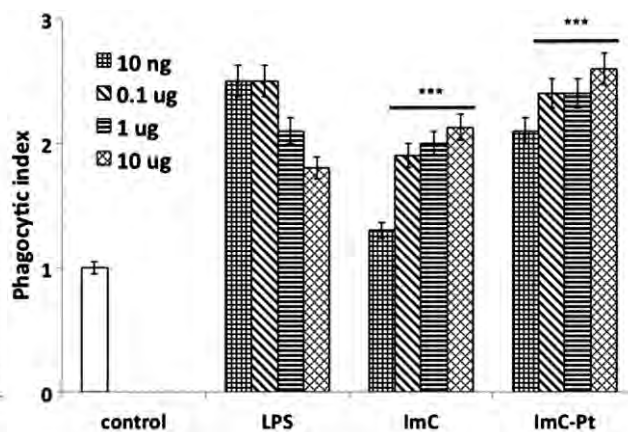


Fig. 6(b)—Effect of ImC and ImC-Pt on phagocytic activity of rat peritoneal macrophages. Dead yeast cells were incubated with sample treated macrophages (at different concentrations) and differential staining was done. The phagocytic index of the test sample was determined by comparing the number of phagocytic macrophages to that in the control slide. Level of significance *** $p < 0.005$ compared to control.

macromolecules. Among all macromolecules, polysaccharides are gifted with greatest potential for structural variability. This enormous potential of variability in polysaccharide structure facilitates the precise regulatory mechanisms of various cell-cell interactions in higher organisms. In conclusion, the results presented clearly indicate that *ajowan* ImC differentially activates B cells and macrophages, which play important roles in innate immune responses and production of antibodies. Moreover, it is also clear that the proteins of ImC play a significant role in the activation of splenocytes, whereas the polysaccharide portions are effective on peritoneal exudates cells. A detailed study of the structural characterization of polysaccharides responsible for macrophage activation will open the way to understand the immunomodulation caused by the popular spice *ajowan*.

Acknowledgment

Authors wish to thank Prof Ram Rajasekharan, Director, CSIR- CFTRI, Mysuru for his support and constant encouragement. They express their heartfelt gratitude to Prof Dipankar Nandi and Mr Shamik Majumdar, Department of Biochemistry, Indian Institute of Science (IISc), Bengaluru for their assistance in initial standardization of cell culture experiments.

One of the authors, Shruthi RR, would like to thank Council of Scientific and Industrial Research, for the grant of Research Fellowship.

Conflict of interest

All the authors don't have any objection for this submission. The work has not been published/ submitted or being submitted to another journal.

References

- Li TSC, The range of medicinal herbs and spices, In: *Handbook of Herbs and Spices*, 1st edn, edited by Peter KV, (Woodhead Publishing, Cambridge), 2006, 113–125.
- Virella G & Bierer BE, The introduction of an immune response: antigens, lymphocytes and accessory cells, In: *Introduction to Medical Immunology*, 4th edn, edited by Virella G, (Marcel Dekker Inc., New York), 1998, 49–69.
- Vane JR & Botting RM, Mechanism of action of anti-inflammatory drugs, *Scand J Rheumatol Supple*, 25 (102) (1996) 9–21.
- Ng TB, A review of research on the protein-bound polysaccharide (polysaccharopeptide, PSP) from the mushroom *Coriolus versicolor* (Basidiomycetes: Polyporaceae), *Gen Pharmacol*, 30 (1998) 1–4.
- Zhang M, Cui SW, Cheung PCK & Wang Q, Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity, *Trends Food Sci Tech*, 18 (2007) 4–19.
- Mahima, Rahal A, Rajib D, Shyma KL, Hari AS, Ruchi T, Amit KV, Amit K & Dhama K, Immunomodulatory and therapeutic potentials of herbal, traditional/indigenous and ethnoveterinary medicines, *Pak J Biol Sci*, 15 (16) (2012) 754–774.
- Pulok KM, Neelesh KN, Santanu B, Mukharjee D, Fernao CB & Motlalepula GM, Immunomodulatory leads from medicinal plants, *Indian J Tradit Knowle*, 13 (2) (2014) 235–256.
- Dwivedi SNMRP & Alava S, Phytochemistry, pharmacological studies and traditional benefits of *Trachyspermum ammi* (L.) Sprague, *Int J Pharmacol Life Sci*, 3 (2012) 1705–1709.
- Jeet K, Devi N, Thakur N, Sunil T, Lalit S & Thakur R, *Trachyspermum ammi*: A comprehensive review, *Int Res J Pharmacol*, 3 (2012) 133–138.
- Ram HN, Sriwastava NK, Makhija IK & Shreedhara CS, Anti-inflammatory activity of *Ajmodadi Churna* extract against acute inflammation in rats, *J Ayurveda Integr Med*, 3 (2012) 33–37.
- Anilakumar KR, Saritha V, Farhath K & Bawa AS, Ameliorative effect of ajwain extract on hexachlorocyclohexane-induced lipid peroxidation in rat liver, *Food Chem Toxicol*, 47 (2009) 279–282.
- Tabassum N & Ahmad F, Role of natural herbs in the treatment of hypertension, *Pharmacogn Rev*, 5 (2011) 30–40.
- Kaur T, Bijarnia RK, Singla SK & Tandon C, Purification and characterization of an anticalcifying protein from the seeds of *Trachyspermum ammi* (L.), *Protein Pept Lett*, 16 (2009) 173–181.
- Kaur T, Bijarnia RK, Singla SK & Tandon C, *In vivo* efficacy of *Trachyspermum ammi* anticalcifying protein in urolithiatic rat model, *J Ethnopharmacol*, 126 (2009) 459–462.
- Zarshenas MM, Moein M, Samani SM & Petramfar P, An Overview on *Ajwain* (*Trachyspermum ammi*) pharmacological effects: modern and traditional, *J Nat Rem*, 14 (2014) 98–105.
- Zachariah TJ, *Ajowan*, In: *Chemistry of Spices*, edited by, Parthasarathy VA, Chempakam B & Zachariah TJ, (CAB International, Wallingford, UK), 2008, 312–318.
- Vitali LA, Beghelli D, Biapa NPC, Bistoni O, Cappellacci L, Damino S, Bramucci M, *et al.*, Diverse biological effects of the essential oil from Iranian *Trachyspermum ammi*, *Arabian J Chem*, <http://doi.org/10.1016/j.arabjch.2015.06.002>
- Bairawa R, Sodha RS & Rajawat BS, *Trachyspermum ammi*, *Pharmacogn Rev*, 6 (2012) 56–60.
- Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem*, 72 (1976) 248–254.
- Dubois M, Gilles KH, Hamilton JK, Rebers PA & Smith F, Colorimetric method for determination of sugars and related substances, *Anal Chem*, 28 (1956) 350–356.
- Chitra M & Muralikrishna G, Bioactive xylo-oligosaccharides from wheat bran soluble polysaccharides, *LWT Food Sci Technol*, 43 (2010) 421–430.
- Ainsworth EA & Gillespie KM, Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent, *Nat Protocol*, 2 (2007) 875–877.
- Chandrashekar PM & Venkatesh YP, Identification of the protein components displaying immunomodulatory activity in aged garlic extract, *J Ethnopharmacol*, 124 (2009) 384–390.
- Aranha I, Clement F & Venkatesh YP, Immunostimulatory properties of the major protein from the stem of the Ayurvedic medicinal herb, guduchi (*Tinospora cordifolia*), *J Ethnopharmacol*, 139 (2012) 366–372.
- Clement F, Pramod SN & Venkatesh YP, Identity of the immunomodulatory proteins from garlic (*Allium sativum*) with the major garlic lectins or agglutinins, *Int Immunopharmacol*, 10 (2010) 316–324.
- Roy B & Rai U, Genomic and non-genomic effect of cortisol on phagocytosis in freshwater teleost *Channa punctatus*: an *in vitro* study, *Steroids*, 74 (2009) 449–455.
- Kumar VP, Prashanth KV & Venkatesh YP, Structural analyses and immunomodulatory properties of fructo-oligosaccharides from onion (*Allium cepa*), *Carbohydr Polym*, 117 (2015) 115–122.
- Prasanna VK & Venkatesh YP, Characterization of onion lectin (*Allium cepa* agglutinin) as an immunomodulatory protein inducing Th1-type immune response *in vitro*, *Int Immunopharmacol*, 26 (2015) 304–313.
- Kirthi S, Mishra NP, Singh J & Khanuja SPS, *Tinospora cordifolia* (Guduchi), a reservoir plant for therapeutic applications: A review, *Indian J Tradit Knowle*, 3 (3) (2004) 257–270.