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

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*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 μg 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

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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 effects\(^1\). 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 conditions\(^2\). 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 attributes\(^3\). 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 lived\(^4\). 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 inflammation\(^5\). 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

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interest in alternative forms of medicinal systems such as those of Ayurveda, Traditional Chinese Medicine and Unani. 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.

*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. 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-calciﬁying and antioxidant activities. The major active principles in TA/Ajowan thought to be responsible for these activities are thymol and carvacrol. 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-1β, 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 alpha+ T cell subsets which are believed play in favor of a wanted situation in immune responses has been observed. 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 ± 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 standard. 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 MHDPI method using glucuronic acid as standard. Total phenolics were measured by Folin–Ciocalteau method using ferulic acid as standard. Ajowan ImC was subjected to pronase treatment to remove the proteins and dialyzed against distilled water using a 12-14 kDa cut-off dialysis membrane. The dialyzed sample was 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 described. Cells were treated with RBC lysis buffer and washed thoroughly. One hundred microliters of cell suspension (2 × 10⁶ cells per well) 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 of ajowan ImC was carried out in vitro by MTT assay using 96-well tissue culture plates. 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.

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min. Calibration curve was prepared using sodium nitrite (NaNO₂). 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.

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

Phagocytic assay was performed according to the method described by Roy & Rai. Briefly, 1 mL of PECs (1 × 10⁵ 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 CO₂ 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. 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 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 μg, 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.
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 μg) 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 μg 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. 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/Tc) and different types of helper T cells (Th). ImC had no
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 thereby 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 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.

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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.

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