



## An *in vitro* study of the immunomodulatory effects of *Piper nigrum* (black pepper) and *Elettaria cardamomum* (cardamom) extracts using a murine macrophage cell line

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**Abstract:** Cardamom and black pepper have been used as spices in many different cultures of the world and the medicinal properties attributed to these are extensive. Although the immunomodulatory activities of many herbs have been studied, research related to possible immunomodulatory effects of various spices on macrophages is relatively scarce. Hence in this study, we have explored the potential immunomodulatory effects of black pepper and cardamom on macrophages. We show that black pepper and cardamom extracts act as potent modulators of the macrophages in a dose-dependent “see-saw” like manner. Our findings suggest that perhaps black pepper and cardamom could be used individually or synergistically (at appropriate concentrations) as candidates for developing potential therapeutic tools to regulate the responses of the immune system depending upon the type of disease.

**Keywords:** Immunomodulation; Black pepper; Cardamom; MTT assay; Doubling time

### I. INTRODUCTION

Monocytes and macrophages are the central cells of the innate immune system that arise from a common myeloid progenitor in the bone marrow [1]. Monocytes are circulating, short-lived cells that undergo spontaneous apoptosis on a daily basis [2]. It is only in response to differentiation factors that monocytes escape their apoptotic fate by differentiating into macrophages, which have a longer life span [3].

Of all the white blood cells (WBCs) that mediate the body’s immune response, the macrophages are the most plastic cells that are found in all tissues and exhibit great functional diversity [4],[5]. They have roles in development, homeostasis, tissue repair and immunity and can change their functional phenotype depending on the environmental cues they receive [4], [6]. Through their ability to clear pathogens and instruct other immune cells, macrophages not only have a central role in protecting the host but also contribute to the pathogenesis of inflammatory and degenerative diseases [7], [8].

Macrophages are cells that function in both innate and adaptive immunity [9]. They express a variety of pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin receptors, helicase RIG-like receptors and biosensor Nod-like receptors that recognize danger signals associated with invading pathogens, foreign substances (for example, silica and asbestos), and dead or dying cells [4], [10]. All these receptors have important roles in the activation of the innate immune response. They ‘instruct’ macrophages to engulf and destroy foreign particles and bacteria through the generation of a respiratory burst [6]. Macrophages function as antigen-presenting cells (APCs) and participate in the activation of the adaptive arm of the immune response [10]. These inflammatory cells produce large amounts of tumour necrosis factor (TNF), interleukin (IL)-12 and interleukin (IL)-23 and therefore are important drivers of antigen specific type I helper T cell responses [11]. T cell activation is therefore the hallmark of the initiation of the adaptive immune response [12]. Indeed it is now well known that APC maturation via CD40 ligation [13] and notch stimulation in T cells [14] is the connecting link between innate and adaptive immunity.

In recent years there has been a renewed interest in improving health and fitness through the use of more natural products [15]. Already, there is a great deal of interest in the use of herbal medicines that is based on the premise that plants contain natural substances that can promote health and alleviate illness [16] - [20]. Spices are important part of the human diet which are used to enhance the flavour, colour and aroma of food. In addition to boosting flavour, spices are also known for their preservative and medicinal value [21]. The role of Curcumin, a yellow pigment in the spice turmeric as an anti-inflammatory agent in promoting beneficial effects in arthritis,

allergy, asthma, atherosclerosis, heart disease, Alzheimer's disease, diabetes, and cancer has been extensively well reported [22], [23].

Considerable attention has been focussed on the use of biological response modifiers (BRMs) as an adjunct to the conventional treatment of cancer and for prevention of metastatic disease [24] – [26]. Because of their pleiotrophic effects on the immune system, the cellular mechanisms of their antitumor activity have been difficult to resolve. However, mechanisms that have been considered include augmentation of both cell mediated and nonspecific immunity [27]. With the hope of developing new drugs with no or less adverse effects much research has been focussed upon studying immune stimulating - biologically active substances like macrophage-activating polysaccharide [28], anti-complementary polysaccharide [29], B - cell proliferation activating materials [30] and anti - cancer substances [31]. In 1994, a study conducted by Wallace and Morahan [32] showed that in the Lewis lung (3LL) peritoneal carcinomatosis, the treatment with BRM MVE-2 retarded tumour growth by inducing the production of cytotoxic macrophages (m $\phi$ ). Recently, Nau et al (2014) [33] have suggested strategies to stimulate phagocytic microglial cells in the brain of immunocompromised patients to protect their central nervous system (CNS) from infections.

The idea of stimulating macrophages with the help of easily available natural sources that forms a part of the regular diet would perhaps be helpful in assisting the body to mount a powerful defence against invading microbes. However the probability of such kind of effect to promote differentiation into alternatively activated macrophages cannot be ruled out [34]. It is now known that such altered macrophages are responsible for immune dysfunction [35] and tumor development [36]. In the tumour microenvironment, inhibition of the apoptotic program has shown to promote monocyte survival contributing to the accumulation of macrophages and the persistence of an inflammatory milieu. Signal transduction pathways such as the mitogen associated protein kinase (MAPK) pathways [37], phosphatidylinositol 3-kinase (PI3K)/Akt and anti apoptotic molecules have been identified to play a crucial role in determining monocyte life span by regulating gene transcription and inhibiting the apoptotic program [38]. Macrophages have known to be involved in various chronic and systemic inflammatory diseases like multiple and lateral sclerosis, rheumatoid arthritis and atherosclerosis [39] – [42]. In fact macrophage activation syndrome is responsible for causing a potentially fatal complication of systemic juvenile idiopathic arthritis with features of hemophagocytosis leading to coagulopathy, pancytopenia, and liver and central nervous system dysfunction [43].

Immunomodulation is an induced modification of immune responses by means of introducing natural or synthetic chemical substances that possess the ability to regulate the immune system. It is considered to be an invaluable tool for preventing as well as treating infectious as well as non-infectious diseases [44] – [46]. Many natural plants and herbs have a long history of medicinal uses and have been shown to possess immunomodulatory effects that are beneficial in fighting many diseases [47] – [50].

Several studies have demonstrated that black pepper extracts and its major constituents have diverse physiological effects on important organs like kidney and liver [51]. Continued oxidative stress due to generation of reactive oxygen species (ROS) has been shown to lead to chronic inflammation, which in turn could mediate chronic diseases like cancer, diabetes, cardiovascular, neurological and pulmonary diseases [52], [53]. Black pepper contains several antioxidants and is one of the most powerful antioxidants for preventing as well as curtailing oxidative stress. Its principle phytochemical, piperine is known to inhibit pro-inflammatory cytokines that are produced by tumour cells. Besides, black pepper also exhibits immunomodulatory properties by boosting the number of white blood cells (WBCs), thereby assisting the body to raise a powerful defence mechanism against invading microbes and cancer cells [54].

Cardamom, also known as “Queen of Spices” [55] is a well known aromatic spice used in Eastern, Arab and Scandinavian cuisines. Cardamom is known to play a wide range of health promoting roles. It has been used for teeth, gum and throat infection, as well as against lung congestion, pulmonary tuberculosis and digestive disorders [56]. Antimicrobial properties of cardamom extracts are also well documented [57] – [60]. Cardamom is one of the most common ingredients of Indian Ayurveda and Chinese traditional medicine [56]. Various studies suggest that cardamom extracts display anti-cancer activities [61] – [65]. Anti-inflammatory, antiproliferative, pro-apoptotic [64] and antioxidative [56] activities have been proposed as mechanisms underlying the anti-cancer properties of cardamom.

Culinary herbs [15] and spices possess a great potential in terms of preventing and treating various diseases which cannot be underestimated [66] – [68]. Very little is known about the potential immunomodulatory activities of commonly used spices and the molecular mechanisms of their effects remains either poorly understood or largely unidentified [69]. Although discriminate and proper use of herbal products and spices is safe and is known to provide therapeutic benefits, but their indiscriminate or excessive use can be unsafe and even dangerous [70]. In the present paper we have focussed on investigating the potential immunomodulatory activities of *Piper nigrum* (black pepper) and *Elettaria cardamomum* (cardamom) individually as well as synergistically on macrophages which are the most indispensable cells of the immune system [71]. We selected the murine tumour cell line P388D1 as our model system for carrying out *in vitro* experiments because they possess most macrophage characteristics – they adhere to glass and plastic, induce T-cells, are cytotoxic and

possess phagocytic properties [72]. Our findings suggest that both at lower as well as at higher concentrations *Elettaria cardamomum* and *Piper nigrum* demonstrate reciprocal immunomodulatory effects on the macrophage cells.

## II. MATERIALS AND METHODS

### A. Plastic ware

Tissue culture grade plastic ware, disposable sterile pipettes (2ml, 5ml and 10ml capacity), 96-well plates, pipette tips, 35mm dishes, microfuge vials and cryovials were purchased from Tarsons Products Private Limited (Kolkata, India). 0.22 $\mu$ m pore size syringe filters were purchased from Moxcare Products Inc. (Haryana, India).

### B. Reagents

Roswell Park Memorial Institute (RPMI)-1640, Penicillin, Streptomycin and Trypan Blue were from HiMedia (Mumbai, India); Fetal Bovine Serum (FBS), 3, (4-5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Lipopolysaccharide (LPS) isolated from *Escherichia coli* 055:B5 and L-glutamine were from Sigma-Aldrich (St. Louis, MO, USA).

### C. Preparation of aqueous crude extracts

The aqueous extracts for black pepper and cardamom were prepared as mentioned by Majdalawieh and Carr, 2010 [69]. Briefly, whole dry seeds of black pepper and cardamom were purchased from the local market of Pune, Maharashtra, India. They were washed, dried and ground in liquid nitrogen. After complete evaporation of liquid nitrogen, 12.5ml of double distilled H<sub>2</sub>O was added to the 25 grams of ground spice and stirred overnight with a magnetic stirrer to allow extraction. The crude spice extracts were centrifuged at 12,000g for 20 minutes at room temperature. The supernatants were then harvested and subjected to rotatory evaporation, after which a stock concentration of 25mg/ml of each extract was prepared. The extracts were sterilized using 0.22  $\mu$ m pore size syringe filters.

### D. Cell culture

The murine macrophage like lymphoma cells P388D1 were procured from National Centre for Cell Science (NCCS), Pune, Maharashtra, India. The cells were maintained in RPMI-1640 supplemented with 10% (v/v) inactivated fetal bovine serum (FBS), 100units/ml of penicillin, 100 $\mu$ g/ml of streptomycin and 3% L-glutamine. The cells were incubated at 37<sup>0</sup>C in a humidified atmosphere containing 5% (v/v) carbon-di-oxide (CO<sub>2</sub>). The medium was changed every 2 days and the cells were serially passaged biweekly.

### E. Treatment of murine macrophage cells with aqueous extracts

The murine macrophage cell line was serum starved for 24 hours. After 24 hours of serum starvation the cells were collected in complete medium, and viable cell counts were taken using trypan blue dye exclusion method. 5 X 10<sup>4</sup> P388D1 cells were seeded in triplicates of 96-well plates or in triplicates of 35mm dishes. The plates/dishes were incubated for 24 hours at 37<sup>0</sup>C at 5% CO<sub>2</sub>. After 24 hours of incubation, the old medium was decanted and all the plates/dishes were replenished with fresh RPMI-1640 medium supplemented with 10% FBS. The cells were then treated with varying concentrations (as represented in the graphs) of aqueous extract(s) of either black pepper or cardamom or both and subjected to *in vitro* assays.

### F. Cell proliferation assay using MTT reagent

MTT assay was carried out to assess whether or not the extracts of black pepper and/or cardamom would stimulate proliferation of the macrophage cell line. After incubating the cells with the extracts for 48 hours, 10 $\mu$ l of MTT reagent (5mg/ml) was added in each well. The cells were further incubated with the MTT reagent for 5 hours at 37<sup>0</sup>C after which the reaction was terminated by adding extraction fluid (0.04N Hcl in isopropanol). The extraction fluid dissolved the formazon crystals, thereby generating a colored reaction product, the intensity of which was measured at 570nm-630nm using EON<sup>TM</sup> Microplate Spectrophotometer from BioTek Instruments, Inc (Vermont, USA).

### G. Growth Curve experiment:

These experiments were performed in order to examine whether or not treatment of the P388D1 cells with the aqueous extracts of black pepper and/or cardamom would lead to altered cell-cycle kinetics. Cells were harvested after every 24 hours for a period of 5 days and subjected to trypan blue dye exclusion method in order to assess the viable cell counts.

### H. Statistical analysis:

The data were analyzed by one-way repeated measure analysis of variance (One-Way RM ANOVA). The mean and SEM values of various assays were used for plotting the error bar graphs, using the Sigma Plot software (version-12.0). Level of significance was denoted as follows \*P $\leq$  0.05, \*\*P $\leq$  0.01 and \*\*\*P $\leq$  0.001.

### III. RESULTS

#### A. Aqueous extract of black pepper stimulates proliferation of macrophages at higher concentrations

Based on previous studies [27], [54], [69], we hypothesized that aqueous extract of black pepper would enhance the proliferation of macrophages. To test this hypothesis, we cultured P388D1 cells with aqueous extracts of black pepper at various doses (0.01, 0.1, 1, 5, 10, 25, 50, 100, 250µg/ml) for 48 hours. The cultured P388D1 cells were then subjected to MTT assay. As shown in Figure 1, the proliferation of the macrophage cell line was enhanced in a dose-dependent manner (from lower to higher concentrations) till 100µg/ml. However at 250µg/ml the extract may perhaps have exerted toxicity upon the cells which explains the sudden drop in the percent survival of the cells at this particular concentration.

#### B. Aqueous extract of cardamom stimulates proliferation of macrophages at lower concentrations

Previous studies have also shown that cardamom possess immunostimulatory properties [69]. Hence we hypothesized that cardamom, just like black pepper, would stimulate the macrophages to proliferate in a dose-dependent manner. To test this hypothesis, we cultured P388D1 cells with aqueous extracts of cardamom at various doses (0.01, 0.1, 1, 5, 10, 25, 50, 100, 250µg/ml) for 48 hours and subjected the cultured cells to MTT assay. As shown in Figure 2, treatment of P388D1 with aqueous extracts of cardamom also led to enhanced proliferation of the macrophages in a dose-dependent manner. However, the dose-dependent increase in proliferation was in the reverse order (from higher to lower concentration) as compared to black pepper. Together these data (Figure.1 and Figure.2) suggest that if black pepper and cardamom can individually stimulate macrophages and allow them to proliferate at certain concentrations; then at certain other concentrations they can also induce inhibitory stimuli and thus suppress their proliferation. Noteworthy is that while the concentration at which black pepper allowed maximum proliferation of the murine macrophages was at the higher end (100µg/ml), cardamom caused maximum proliferation of cells at the lowest concentration (0.01µg/ml). Our data indicate that black pepper and cardamom extracts act as potent modulators of the macrophages in a dose-dependent “see-saw” like manner.

#### C. Synergistic stimulatory and inhibitory effects of aqueous extracts of black pepper and cardamom on proliferation of macrophages

To determine whether the aqueous extracts of black pepper and cardamom had any synergistic effects on the macrophage cell line, we cultured the P388D1 cells with a combination of aqueous extracts of both black pepper and cardamom. In our previous experiments we had seen a “see-saw” like effect of the aqueous extracts of black pepper and cardamom individually at lower and higher concentrations. Therefore, in these set of experiments we decided to evaluate the immunomodulatory effects of both the extracts used in combination at favourable concentrations (at concentrations that caused proliferation) and non-favourable concentrations (at concentrations that caused inhibition). It was observed (Figure 3) that when the macrophage cells were exposed to a combination of favourable concentrations of aqueous extracts of *Piper nigrum* (PN) (100µg/ml) and *Elettaria cardamomum* (EC) (0.01µg/ml), it led to significantly greater proliferation (187%) of P388D1 cells as compared to the effect of each extract individually at favourable concentrations (92% for PN and 124% for EC). Similarly when the cells were exposed to a combination of non-favourable concentrations of aqueous extracts of PN (0.01µg/ml) and EC (100µg/ml), it led to a significant reduction in proliferation (24%) of macrophage cells as compared to the effect of each extract individually at non-favourable concentrations (40% for PN and 42% for EC). When the extracts of PN and EC were used in a combination at favourable and non-favourable concentrations (100µg/ml of PN+EC and 0.01µg/ml of PN+EC), it was observed that cardamom exhibited a more dominant buffering effect on the murine macrophage cells than black pepper. When both were used at a concentration of 100µg/ml at which PN stimulated the cells to proliferate (92%) and EC inhibited proliferation of cells (42%), the percent survival of cells fell down to 72%. Similarly, when both black pepper and cardamom were used at a concentration of 0.01µg/ml at which EC induced proliferation (124%) while PN inhibited proliferation (40%), the percent survival of the cells rose to 98%. However it was observed that at a concentration of 250µg/ml, both PN and EC in combination proved to be highly toxic to the cells, which may perhaps support that indiscriminate or excessive usage of spices can indeed be unsafe and even dangerous [70]. Together, these data indicate that not only do aqueous extracts of black pepper and cardamom significantly enhance the proliferation of macrophages, but they also interact cooperatively to further augment macrophage proliferation.

#### D. Synergistic stimulatory effects of aqueous extracts of black pepper and cardamom at favourable concentrations results in an increased cell yield of P388D1 cells by reducing their doubling time

Since at favourable concentrations of aqueous extracts of PN (100µg/ml) and (EC) (0.01µg/ml), the proliferation of macrophages enhanced in a synergistic fashion, we were keen to find out whether the upsurge in proliferation at these favourable concentrations would also lead to altered cell cycle kinetics. In order to



examine this issue we carried out a growth curve experiment. The serum-starved P388D1 cells were seeded in complete growth medium in continuous presence of the aqueous extracts and viable cell counts were taken at indicated time points using trypan blue dye exclusion method. It was clearly seen (Figure 4) that the P388D1 cells treated with a combination of favourable concentrations of aqueous extracts of both black pepper and cardamom, PN=100µg/ml + EC=0.01µg/ml, entered the log phase at an earlier time point as compared to macrophages treated with only PN=100µg/ml or only EC=0.01µg/ml. The time interval taken by the PN=100µg/ml + EC=0.01µg/ml treated macrophages to double (doubling time) in the first 24 hours was 13 hours as compared to 54 hours and 64 hours of cells treated with aqueous extracts of only PN and only EC respectively at favourable concentrations. The doubling time in the next 24 hours of cells treated with a combination of favourable concentrations of aqueous extracts of black pepper and cardamom was reduced by more than 50% (26 hours) as compared to those treated with only black pepper (113 hours) and only cardamom (44 hours). These data indicated that the treatment of P388D1 cells with a combination of extracts at favourable concentrations led to their enhanced growth due to shortening of their doubling time. We also looked at the effect of aqueous extracts of black pepper and cardamom on the growth kinetics of the P388D1 macrophage cells at unfavourable concentrations in combination and individually. Noteworthy was that the cells barely survived till 72 hours at which >90% of cells were found to be dead (data not shown).

#### IV. DISCUSSION

The main focus of many researchers is to explore new drugs to promote optimal immune function. Natural products have had a long history of medicinal properties and since then many active constituents of plants have been identified and isolated to treat certain medical conditions [73], [74]. A variety of them contain different phytochemicals with biological activity that can provide therapeutic effects. They have been shown to help reduce high blood cholesterol, provide protection against cancer and even stimulate the immune system [75].

The aim of the present study was to open up a new approach in the development of natural drugs with immunomodulatory effects at the *in vitro* level. Since controlled proliferation is a universal method of screening natural products [76], we carried out *in vitro* assays to determine the proliferation of macrophages in response to variable concentrations of aqueous extracts of black pepper and cardamom. Our results provide experimental evidence demonstrating that aqueous extracts of black pepper and cardamom are potentially capable of modulating the function of macrophages. Exposure of P388D1 cells to high concentrations of black pepper extract led to enhanced proliferation of these cells (maximum being at 100µg/ml), whereas when exposed to low concentrations of extract of black pepper, the cells displayed greatly reduced proliferative activity (minimum being at 0.01µg/ml) (Figure 1). On the other hand treatment of P388D1 cells with aqueous extracts of cardamom stimulated proliferation at lower concentrations (minimum being at 0.01µg/ml) and inhibited their proliferation at higher concentrations (maximum being at 250µg/ml) (Figure 2). However, when the macrophages were treated with a combination of aqueous extracts of both black pepper and cardamom at favourable concentrations, it not only stimulated their proliferation (Figure 3), but it led to a remarkable increase in the number of cells (Figure 4). This suggests that at favourable concentrations the aqueous extracts of black pepper and cardamom are capable of promoting proliferation in macrophages perhaps because at these concentrations the active constituents of black pepper and cardamom activate the proliferative signalling pathways in the macrophages, thereby producing such a potent stimulatory effect. Studies have already shown that piperine, an active alkaloid component of black pepper enhances the proliferation of murine splenocytes [77] and eugenol, an active component of cardamom enhances the cell-mediated lymphocyte proliferation *in vitro* [78]. Therefore just as these active compounds in black pepper and cardamom may be acting synergistically to promote proliferation of the macrophages at favourable concentrations, similarly at non-favourable concentrations these active compounds may be augmenting inhibitory effects upon each other. Nevertheless, such findings have tremendous therapeutic applications for neural degenerative diseases [39], inflammatory diseases [5], progression of tuberculosis [79] and HIV pathogenesis [80] and many more where macrophages are responsible for aggravating the medical conditions.

The immunomodulatory effects of macrophages with regards to production of pro-inflammatory cytokines IL-6 and TNF- $\alpha$  in response to aqueous extracts of black pepper and cardamom have been widely investigated by Majdalawieh and Carr in 2010 [69]. They showed that at concentrations of 1, 10, 50 and 100µg/ml, aqueous extracts of black pepper enhanced the release of IL-6 and TNF- $\alpha$  from the BALB/c splenocytes. These results were consistent with their findings using *in vitro* proliferation assay using [<sup>3</sup>H]thymidine incorporation that these four doses of aqueous extracts of black pepper also stimulated the splenocytes to proliferate. In our present study, we have used two doses (0.1µg/ml and 0.01µg/ml) below 1 µg/ml and one dose (250µg/ml) above 100µg/ml. Likewise Majdalawieh and Carr (2010) [69], we also got maximum proliferation of cells at a concentration of 100µg/ml of extract of black pepper. A concentration of aqueous extract of 250µg/ml proved to be toxic to the macrophages (Figure 1). Therefore, we propose that perhaps we would also get a similar profile of pro-inflammatory cytokines IL-6 and TNF- $\alpha$  of macrophages in response to favourable and non-favourable concentrations of extracts of black pepper, indicating that at these concentrations black pepper indeed promotes

(or not) macrophage pro-inflammatory responsiveness. With respect to cardamom however our results were varying from that of Majdalawieh and Carr (2010) [69]. Although similar to our experiments, theirs too displayed a dose-responsive effect of aqueous extracts of cardamom – our data showed an increase in macrophage proliferation from higher to lower concentrations (Figure 2), whereas their data showed increase in splenocyte proliferation from lower to higher concentrations. Furthermore, their findings did not show any significant effect of aqueous extract of cardamom alone at any doses on the release of pro-inflammatory cytokines IL-6 and TNF- $\alpha$  by the splenocytes and macrophages. On the contrary, they observed a dose-dependent inhibition of IL-6 and TNF- $\alpha$  release when splenocytes and macrophages were treated with the aqueous extract of cardamom. In our set of experiments, we have seen that aqueous extract of cardamom displays exactly opposite effects as compared to extract of black pepper. Aqueous extract of cardamom caused proliferation of macrophage cells at lower concentrations and imparted an inhibitory stimulus at higher concentrations. Based on our results we propose that perhaps higher concentrations of extract of cardamom may impede macrophage pro-inflammatory responsiveness, which explains why Majdalawieh and Carr (2010) [69] observed a dose-dependent inhibition of IL-6 and TNF- $\alpha$  release by splenocytes at any of the doses (1, 10, 50 and 100 $\mu$ g/ml). In agreement with these results, it has been proposed that eugenol leads to inhibited secretion of the proinflammatory mediators IL-1b and IL-6 [81], inhibitory NO synthase and NO [82], [83] and cyclooxygenase-2 [84]. Interestingly, oral administration of the aqueous extract of cardamom is accompanied by a significant reduction in cyclooxygenase-2 and inhibitory NO synthase expression in murine models of colon cancer [64]. Moreover, cardamom has been shown to have anti-inflammatory activity against acute carrageenan-induced plantar edema in albino rats [85].

Our data indicate that both black pepper and cardamom act as potent modulators of the macrophages in a dose-dependent “see-saw” like manner and therefore have a great potential to serve as immunomodulatory agents. While at higher concentrations black pepper seems to play pro-proliferative, pro-inflammatory functions, cardamom manifests itself as a potent suppressor of inflammation; at lower concentrations there seems to be a complete role reversal.

## V. CONCLUSIONS

The molecular mechanisms underlying the immunomodulatory effects exerted by the aqueous extracts of black pepper and cardamom on cells of the immune system are still unknown, although elucidation of the specific signaling pathways involved in this immunomodulation is currently underway [69]. Several studies have shown that signaling pathways like MAPK [86] – [88], c-Jun amino terminal kinase (JNK) [89], [90] and PI3K/protein kinase B (PKB) [91], [92] stimulate macrophage proliferation. In future, we hope to examine the molecular mechanisms involved in the bidirectional effect of aqueous extracts of black pepper and cardamom at low and high concentrations. However our present findings suggest that black pepper and cardamom extracts act as potent modulators of the macrophages. Hence we anticipate that they may serve as potential molecular tools for developing new therapeutic targets individually or synergistically, in order to modulate inflammatory responses and prevent/treat various other types of diseases including cancer.

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## CONFLICT-OF-INTEREST DISCLOSURE

The authors declare no competing financial interests

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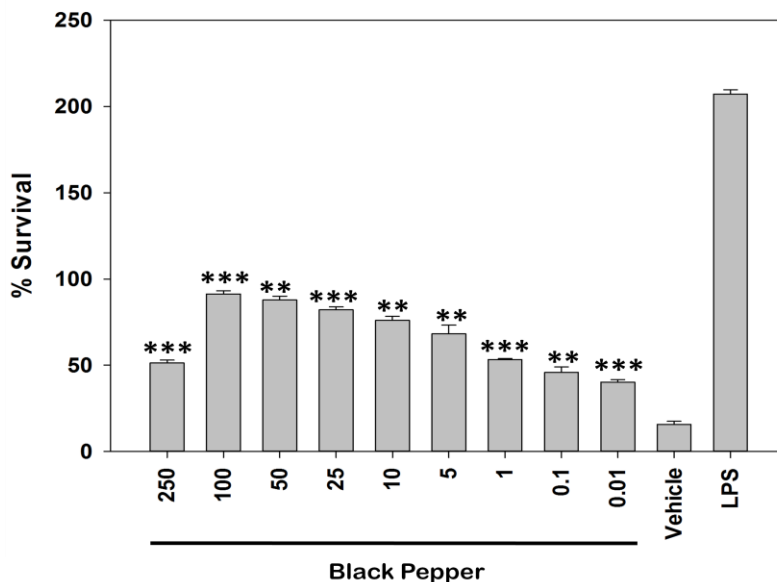
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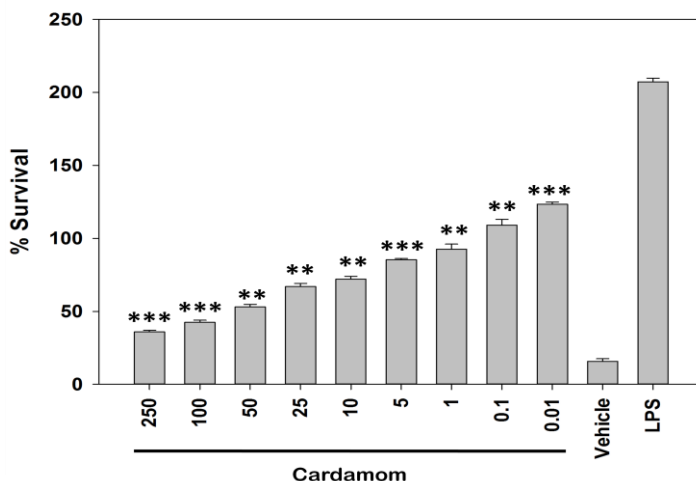
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### FIGURE CAPTIONS AND FIGURES

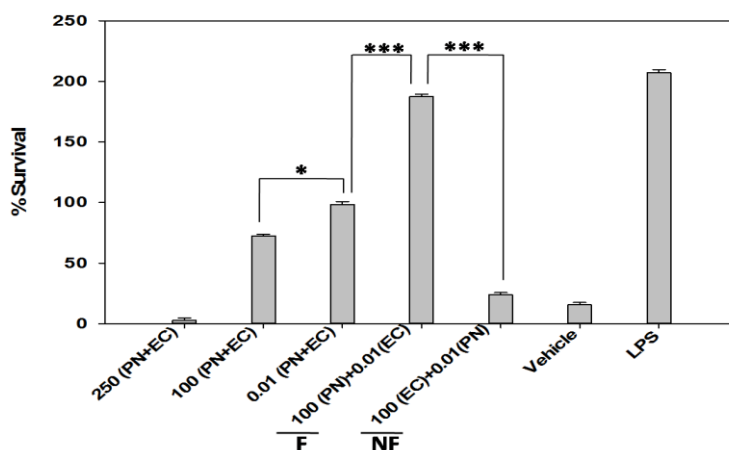
**Figure 1:** Dose responsive effect of aqueous extract of *Piper nigrum* (black pepper). The extract was used at concentrations of 0.01, 0.1, 1, 5, 10, 25, 50, 100 and 250µg/ml. After 48 hours of culturing macrophages with the extracts, the cells were subjected to MTT assay. It was observed that the percent cell survival increased in a dose-dependent manner with increasing concentrations of the extract, except at 250 µg/ml where it fell, probably due to induction of toxicity by the extract at this particular concentration. 10ng/ml LPS treated cells were used as positive control. Statistical significance was determined in comparison to non-treated murine macrophage cells. The data represent Mean ± SEM of three independent experiments.



**Figure 2:** Dose responsive effect of aqueous extract of *Elettaria cardamomum* (cardamom). The extract was used at concentrations of 0.01, 0.1, 1, 5, 10, 25, 50, 100 and 250µg/ml. After culturing P388D1 cells for 48 hours with the extracts, the cells were subjected to MTT assay. It was observed that the percent cell survival increased in a dose-dependent manner with decreasing concentrations of the extract. 10ng/ml LPS treated cells were used as positive control. Statistical significance was determined in comparison to non-treated murine macrophage cells. The data represent Mean ± SEM of three independent experiments.



**Figure 3:** Synergistic effect of aqueous extracts of black pepper and cardamom on proliferation of macrophages 48 hours post-treatment. The extracts of black pepper and cardamom were used in combination at concentrations of 100µg/ml, 0.01µg/ml and 250µg/ml. The cells were subjected to MTT assay after 48 hours of treatment with combination of extracts at favourable (F) and non-favourable (NF) concentrations as represented in the graph. It was observed that when treated with favourable concentrations of both black pepper and cardamom, the proliferation of cells enhanced as a result of their synergistic effect in which the stimulatory action of one extract was magnified in the presence of the other extract. However when exposed to non-favourable concentrations the percent cell survival fell down further due to synergistic inhibitory effects of black pepper and cardamom. 10ng/ml LPS treated cells were used as positive control. Statistical significance was determined by comparison between the linked bars. The data represent Mean ± SEM of three independent experiments.



**Figure 4:** Synergistic effect of aqueous extracts of black pepper and cardamom on cell yield of P388D1 cells. The extracts of black pepper and cardamom were used at concentrations of 100µg/ml and 0.01µg/ml in combination or individually. The cells were harvested after every 24 hours and live cell count was taken using trypan blue exclusion method. The P388D1 cells that were treated with a combination of favourable concentrations of aqueous extracts of black pepper and cardamom (PN=100µg/ml + EC=0.01µg/ml, dashed line with arrows) doubled rapidly as compared to the P388D1 cells treated with extract of only black pepper (PN=100µg/ml, dashed line with closed circles) or cells treated with extract of only cardamom (EC=0.01µg/ml, dotted line with open circles).

