

Induction of resistance to respiratory tract infection with *Klebsiella pneumoniae* in mice fed on a diet supplemented with tulsi (*Ocimum sanctum*) and clove (*Syzygium aromaticum*) oils

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Background and purpose: The impact of diet and specific food groups on respiratory tract infections has been widely recognized in recent years. This study was conducted to study the effect of tulsi (*Ocimum sanctum*) oil and clove (*Syzygium aromaticum*) oil on the susceptibility of experimental mice to respiratory tract infection.

Methods: The effect of 2 different regimens of short-term (15 days) and long-term (30 days) feeding with tulsi oil and clove oil on the course of *Klebsiella pneumoniae* American Type Culture Collection 43816 infection in the lungs of mice was analyzed. The operative mechanisms of lipid peroxidation/nitrite production were studied by estimating their levels in bronchoalveolar lavage fluid (BALF). Bacterial colonization, malondialdehyde (MDA) and nitrite production in BALF, and tumor necrosis factor- α level in serum were assessed.

Results: The results showed that there was a significant decrease in bacterial colonization after short-term feeding with clove oil compared with the controls ($p < 0.05$). For tulsi oil-fed mice, the decrease in bacterial load was significant with long-term feeding ($p < 0.01$). The maximum decrease in MDA levels and increase in nitrite levels were noted with long-term feeding.

Conclusion: Dietary supplementation with tulsi and clove oils protects against bacterial colonization of the lungs.

Key words: Clove oil; *Klebsiella pneumoniae*; Ocimum; Pneumonia

Introduction

Higher plants have been used as sources of drugs to combat diseases for several thousand years [1,2]. However, there has been renewed scientific interest in plant extracts and their constituents for health care during the past 2 decades. This shift from synthetic chemical agents to plant-based products is primarily due to the frequent untoward effects seen with synthetic chemical agents [3]. In addition, under different stress conditions the body requires additional vitamins and/or minerals, which need to be supplemented in the diet [4]. Supplementation not only helps to overcome nutrient depletion but also decreases the risk for chronic diseases, with the added advantage of considerably reduced health costs [5].

Infections occur in the respiratory tract more frequently than in any other site. Most of these infections involve the upper respiratory tract and are caused by viruses. However, lower respiratory tract infections (LRTIs), particularly pneumonia, remain an important cause of morbidity and mortality for all age groups [6]. The classical cause of Gram-negative bacillary pneumonia is *Klebsiella pneumoniae*. Pneumonia caused by *K. pneumoniae* is particularly dangerous because, once it is established, it is extremely difficult to control. Mortality rates may reach or exceed 50%, even for treated patients [7-9]. Infections caused by *K. pneumoniae* can progress to shock and death if not treated early and aggressively.

Tulsi (*Ocimum sanctum*) is an erect, herbaceous plant found throughout India. Tulsi oil, distilled from the leaves and flowering shoots of the plant, contains sri tulsi (yield of oil, 0.2-0.3%), phenols (50.0-76.0%), and aldehydes (10.0-15.0%). The fatty acids present in tulsi oil include palmitic (6.9%), stearic (2.1%) oleic (9.0%), linoleic (66.1%), and linolenic (15.7%) acids [10].

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Clove (*Syzgium aromaticum*) is a conical ever-green tree. The oil distilled from clove buds (14-23%) is colorless or pale yellow, and contains free eugenol (70-90%), eugenol acetate (2-17%), and caryophyllene as its main constituents [11]. Some clinical studies have shown beneficial effects of tulsi [12], echinacea [13], and garlic and ginseng [14] supplementation in relation to upper respiratory tract infections (URTIs) and allergies. Therefore, this study was performed to evaluate the effects of tulsi and clove oil in the diet on lung colonization with *K. pneumoniae* American Type Culture Collection (ATCC) 43816 using an intranasal respiratory mouse model of acute pneumonia.

Methods

Bacterial strain

The standard strain of *K. pneumoniae* ATCC 43816, serotype O1:K1, obtained from the Department of Pediatrics, University of British Columbia, Vancouver, Canada, was used in this study. The organism was maintained on nutrient agar slants at 4°C.

Study animals

Male and female LACA (Laboratory Animal Centre Albino) mice, aged 6 to 8 weeks and weighing 25 ± 5 g, were procured from the Central Animal House, Panjab University, Chandigarh, India. The animals were disease-free. Randomly selected groups of 8 mice were housed in propylene cages and had free access to an antibiotic-free diet (Hindustan Lever Limited, Mumbai, India) and water ad libitum. The temperature of the cages ranged from 18°C to 22°C, relative humidity was 55% to 65%, and the light/dark cycle was set at 6 am/6 pm.

Clove and tulsi oils

Clove and tulsi oils used in this study were obtained from Aggarwal's Pharmaceuticals (Delhi, India) and SK Products (Meerut, India). Stock solutions were prepared by emulsifying oils and gum acacia powder (5%) in distilled water. The oils were sterilized at 10 lb for 30 min before use.

Groups of 8 mice were fed on a standard laboratory chow diet with daily oral supplementation of 5% (w/v) tulsi oil 0.5 mL and 1% (w/v) clove oil 0.5 mL for 15 days (short-term feeding) and 30 days (long-term feeding). Control mice were fed on a standard laboratory chow diet and received daily oral normal saline 0.5 mL for 15 and 30 days.

Induction of acute pneumonia

Following short- and long-term feeding, pneumonia was induced in different groups of mice. The intranasal instillation method of Yadav et al was used [15]. Inoculum 50 µL containing 10^6 colony forming units (CFU)/mL of *K. pneumoniae* ATCC 43816 was instilled into the nasal opening of the mice and the animals were sacrificed on the third day postinfection.

Bacterial quantitation

For bacterial quantitation, the lungs of the mice were homogenized in sterile normal saline 1 mL. Serial 10-fold dilutions were made from the homogenate and 0.1 mL of the selected dilutions were plated out on nutrient agar and incubated overnight at 37°C. The following day, the colonies were counted and the bacterial load of *K. pneumoniae* in the lung specimens was calculated. Lung bacterial counts were calculated and reported as the bacterial count/g of lung tissue.

Bronchoalveolar lavage fluid extraction

Bronchoalveolar lavage fluid (BALF) extraction was performed following the standard method of Greenberger et al [16]. The trachea was exposed and intubated using a polyethylene catheter. BALF was performed by instilling phosphate buffered saline (PBS) containing ethylenediaminetetraacetic acid 5 mM in 1-mL aliquots. Approximately 2 mL of lavage fluid was retrieved per mouse.

Lipid peroxidation

The quantitative measurement of lipid peroxidation in the lung homogenates was measured according to the method of Wills [17]. The amount of malondialdehyde (MDA), an indicator of tissue damage following infection, was measured by reaction with thiobarbituric acid at 532 nm. The results were expressed as nmol of MDA/mg of protein using the molar extinction coefficient of chromophore.

Nitrite assay

Nitrite, an allied product of nitric oxide, concentration in BALF fluid was determined using a microplate assay method as described by Green et al [18]. Supernatant 100 µL and Griess reagent 100 µL (0.1% [w/v] naphthylethyldiamine dihydrochloride, 1% [w/v] sulfanilamide, 3% [v/v] hypophosphoric acid) was incubated for 10 min at room temperature and absorbance was measured at 545 nm. The nitrite concentration in the culture supernatants was calculated on the

basis of the standard curve obtained with the known concentration of sodium nitrite.

Quantitation of tumor necrosis factor- α

Tumor necrosis factor- α (TNF- α) in serum was estimated by the method of Kiener et al [19]. Two-fold serial dilution was made and 4×10^5 per mL L929 cell suspension containing actinomycin D was added to each well and incubated for 18 h at 37°C under 5% carbon dioxide in a humidified incubator. L929 cells were washed with PBS and 1% (w/v) crystal violet 50 μ L was added and removed from the wells by using cold tap water. 1% (w/v) sodium dodecyl sulphate 100 μ L was added to elute the stain from the cells. Absorbance of each well was read on the spectrophotometer at 590 nm. A graph of absorbance was plotted against serial dilutions to quantitate the units of TNF- α released.

Statistical analysis

The results were analyzed statistically by applying Student's *t* test.

$$t = \frac{|\bar{X}_1 - \bar{X}_2|}{S} \sqrt{\frac{n_1 n_2}{n_1 + n_2}}$$
 where \bar{X}_1 and \bar{X}_2 are the means of the 2 samples, n_1 and n_2 are the number of observations in the 2 samples, and *S* is the standard deviation.

$$S = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}$$
 where *S*₁ and *S*₂ are the standard deviations of the 2 samples.

$$S_1 = \sqrt{\frac{\Sigma(X_1 + \bar{X}_1)}{n_1 - 1}} \quad S_2 = \sqrt{\frac{\Sigma(X_2 + \bar{X}_2)}{n_2 - 1}}$$

Degree of freedom (df) = $n_1 + n_2 - 2$.

The probability for obtaining values for a given degree of freedom was determined by comparing the 't' value with probability (*p*), as follows: $p > 0.05$, non significant; $p < 0.05$, almost significant; $p < 0.01$, significant; and $p < 0.001$, highly significant.

Results

Effect of short- and long-term feeding

Bacterial load assessment showed that both tulsi oil and clove oil restricted *K. pneumoniae* colonization in the lungs of the experimental animals, sacrificed on day 3 postinfection, in terms of decreased CFU/gm of lung tissue. A significant decrease in *K. pneumoniae* load was observed with clove oil ($p < 0.05$) feeding

followed by tulsi oil supplementation for 15 days. The bacterial counts were significantly less in the oil-fed animals than in the control animals following 30 days of feeding ($p < 0.01$). Comparison of short- and long-term feeding resulted in a significantly lower *K. pneumoniae* bacterial load in the lungs of mice fed tulsi oil for 30 days ($p < 0.01$), whereas similar results were seen after 15 and 30 days of clove oil supplementation. Fig. 1 shows the effects of clove oil and tulsi oil feeding for 15 and 30 days on the establishment of acute pneumonia by *K. pneumoniae* in the experimental animals.

Malondialdehyde estimation

The maximum decrease in MDA levels was observed in animals fed on clove oil ($p < 0.05$). Comparison of short-term and long-term feeding showed that the greatest decrease in MDA levels was observed with long-term clove oil feeding. The results of the MDA estimation are shown in Fig. 2.

Nitrite determination

The nitrite levels in the uninfected control animals were almost negligible compared with the levels in the infected animals. Fig. 3 shows the production of nitrite in the lungs of the different groups of mice.

Tumor necrosis factor- α determination

The increase in TNF- α levels in tulsi oil-fed mice was greater with long-term feeding than with short-term feeding. The TNF- α levels were similar for both groups of clove oil-fed mice. Fig. 4 shows the results for TNF- α .

Discussion

Several studies have evaluated the role of plant-based products for protection against respiratory tract infections [20,21]. Shaughunglia, a combination herbal medicine, has been used for the treatment of acute respiratory tract infection. Chinese herbal medicine has also been proven to be effective for the treatment of persistent childhood pneumonia [22], and bronchitis and bronchial asthma [23].

Tulsi has been shown to be effective for bronchitis and bronchial asthma. The essential oil from its leaves has been shown to have antibacterial and antifungal activity against a number of organisms [24]. This study supports these observations, as protection against respiratory tract infections was observed in

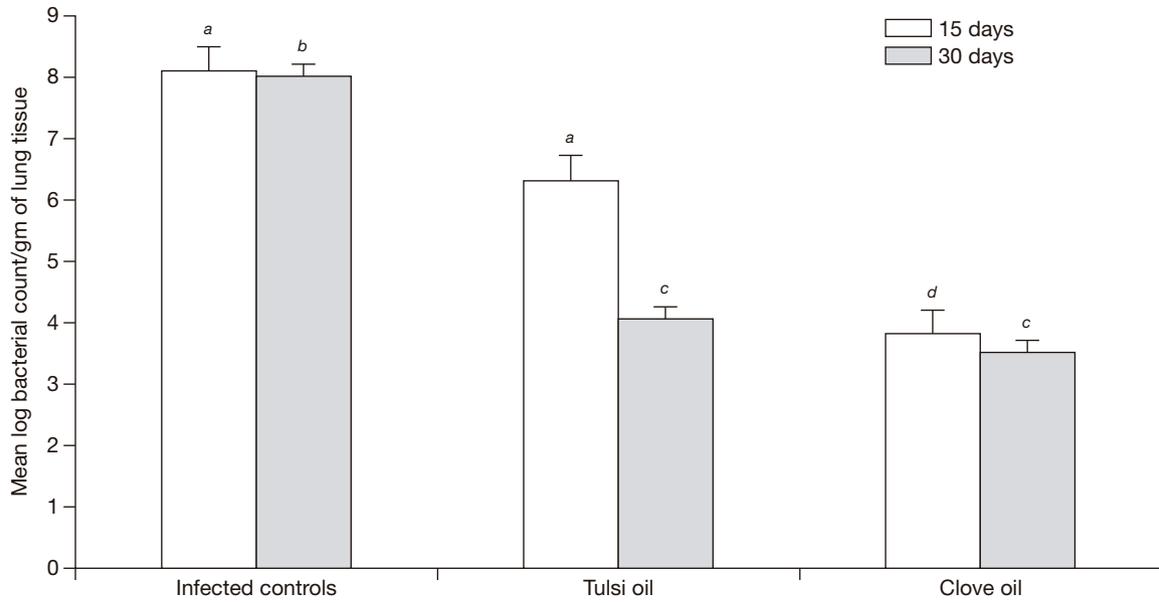


Fig. 1. Acute pneumonia following infection with *Klebsiella pneumoniae* in mice fed on tulsi oil or clove oil for 15 and 30 days.

^a $p > 0.05$ for mice fed for 15 days.

^b $p > 0.05$ for mice fed for 30 days.

^c $p < 0.01$ for mice fed for 30 days.

^d $p < 0.05$ for mice fed for 15 days.

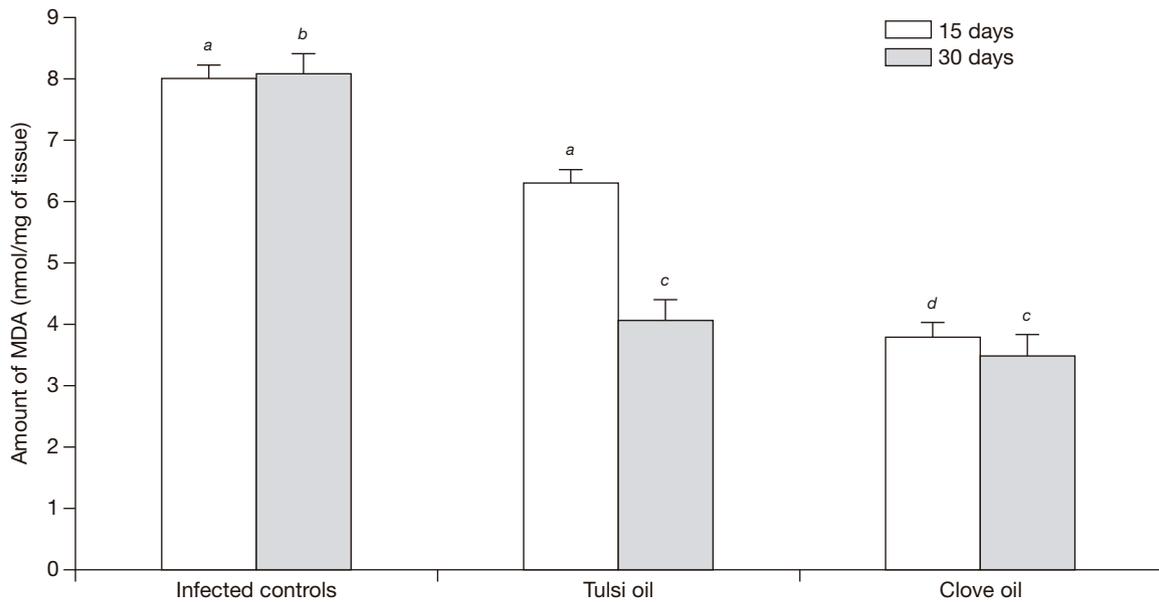


Fig. 2. Malondialdehyde (MDA) in lung homogenates following infection with *Klebsiella pneumoniae* in mice fed on tulsi oil or clove oil for 15 and 30 days.

^a $p > 0.05$ for mice fed for 15 days.

^b $p > 0.05$ for mice fed for 30 days.

^c $p < 0.05$ for mice fed for 30 days.

^d $p < 0.05$ for mice fed for 15 days.

animals fed on tulsi and clove oil. The protection was assessed on the basis of bacterial numbers and MDA levels in the lungs of the animals. The decreased lung colonization by *K. pneumoniae* matched the low level

of MDA in the lung homogenates. As MDA is an indicator of tissue destruction [17,25], this result suggests that consumption of tulsi and clove oil not only provided protection in terms of reducing bacterial

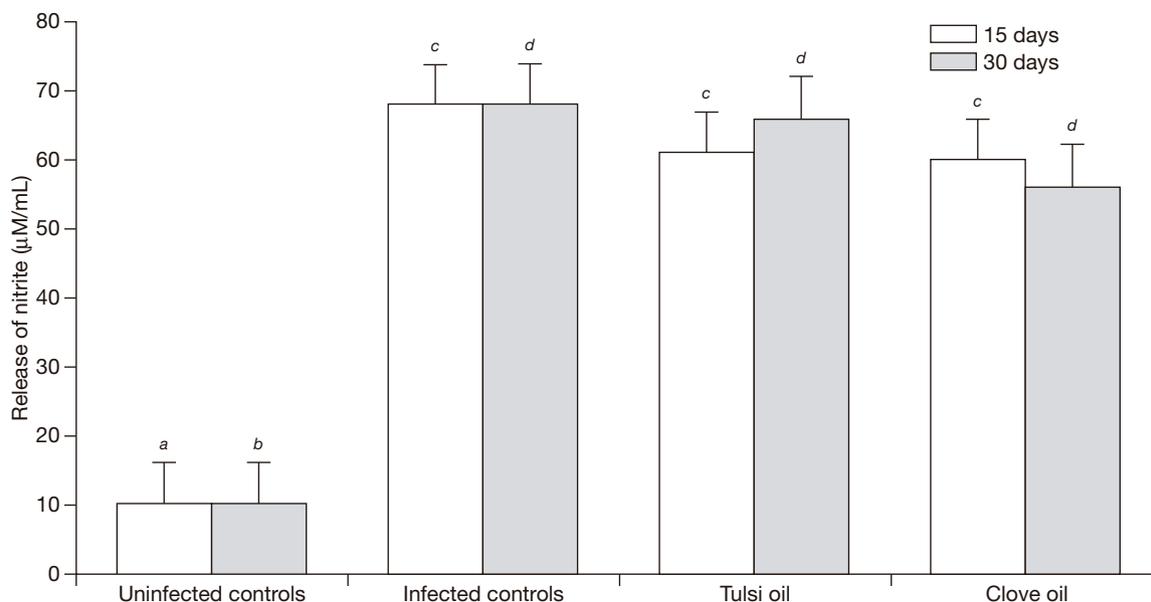


Fig. 3. Nitrite levels in bronchoalveolar lavage fluid following infection with *Klebsiella pneumoniae* in mice fed on tulsi oil or clove oil for 15 and 30 days.

^a $p > 0.05$ for mice fed for 15 days.

^b $p > 0.05$ for mice fed for 30 days.

^c $p < 0.01$ for mice fed for 15 days.

^d $p < 0.01$ for mice fed for 30 days.

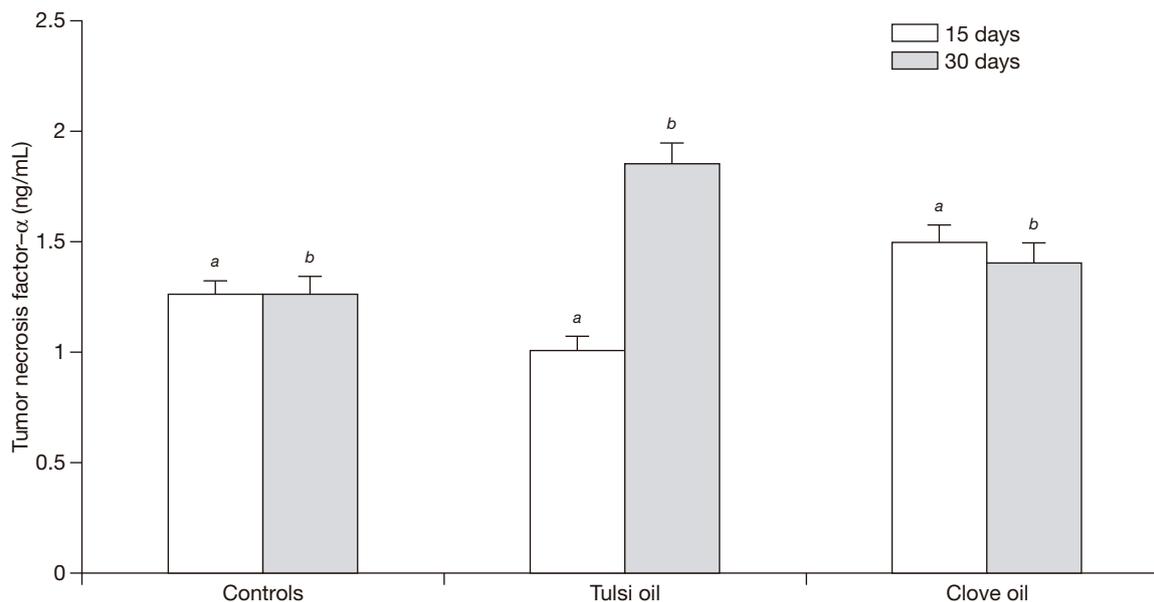


Fig. 4. Tumor necrosis factor- α in the serum of uninfected mice fed on tulsi oil or clove oil for 15 and 30 days.

^a $p > 0.05$ for mice fed for 15 days.

^b $p > 0.05$ for mice fed for 30 days.

numbers but also helped in protecting against tissue damage. The decrease in MDA level may be due to the presence of eugenol, which is a phenolic compound and is known to be present in both tulsi and clove oils [26,27]. The concentration of eugenol in tulsi oil has been shown to be 71%, whereas the concentration in

clove oil is higher (70-90%) [10]. Since eugenol is an inhibitor of lipid peroxidation, it is likely that its effect is to protect tissue from damage.

Effective host defense against bacterial lung infection is primarily dependent on the rapid clearance of microorganisms from the respiratory tract [28]. Alveolar

macrophages, which are the resident cells of lung tissue, are known to check bacterial colonization by production of nitric oxide [29]. Nitric oxide is known to play a critical role in the host defense against *K. pneumoniae* [30]. In this study, increased nitrate levels associated with increased macrophage phagocytic activity (data not shown) was seen in animals fed on tulsi and clove oil supplements.

Pulmonary infections caused by *K. pneumoniae* are associated with increased production of various cytokines that regulate lung host defense and inflammation [31]. TNF- α plays a dual role by upregulating adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and restricting microbial growth by amplifying innate immune resistance [32]. In this study, feeding with tulsi and clove oil increased TNF- α production. Similar results have been reported by other researchers. Hardardottir et al [33] and Blok et al [34] observed enhanced TNF- α production by murine resident peritoneal macrophages following fish oil intake. These researchers concluded that an acute increase in local cytokine levels is beneficial for enhancing resistance to infections [33,34]. Petursdottir et al observed that resident peritoneal macrophages obtained from fish oil-fed mice exhibited higher levels of TNF- α [35]. The increase in TNF- α level is suggestive of protection against bacterial infection. These observations indicate that tulsi and clove oil supplementation activates the immune system, which plays an important role in the clearance of infection from the lungs. However, the duration of supplementation with these oils is important, as it was observed that clove oil is effective after short-term feeding while the effect for tulsi oil was observed only after long-term feeding.

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