Herbs from around the world have been traditionally used for centuries to strengthen the immune system as well as to cure various ailments. Renewed scientific interest in herbs and herbal products for health care has started during the last two decades. This shift from synthetic chemical agents to plant based products is primarily due to more frequent untoward effects seen with the former. *Emblica officinalis (amla)* has been known in Ayurvedic medicine for its tonifying, anti-ageing and immune enhancing properties as it provides a superior source of vitamin C. Each *amla* fruit contains up to 700 mg of vitamin C. Researchers have shown that 8.7 mg of natural vitamin C complex from *amla* is equivalent to 100 mg of the most commonly used synthetic version of it. *Amla* has been particularly indicated for anaemia, asthma, bleeding gums, diabetes, cold, chronic lung disease, hyperlipidaemia, yeast infections, scurvy and cancer. However, the precise

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**Protective efficacy of *Emblica officinalis* against *Klebsiella pneumoniae* induced pneumonia in mice**

A. Saini, S. Sharma & S. Chhibber

*Department of Microbiology, Panjab University, Chandigarh, India*

Received August 22, 2006

**Background & objectives:** *Emblica officinalis* (*amla*), which is a good source of vitamin C, has been shown to be beneficial due to its immune system enhancing property coupled with its tonifying and antiageing effect. The present study was conducted to evaluate the effect of *E. officinalis* feeding on the susceptibility of experimental mice to respiratory tract infection induced by *Klebsiella pneumoniae*.

**Methods:** The effect of short - (15 days) and long (30 days) - term feeding of *amla* in mice on the course of *K. pneumoniae* ATCC43816 infection in lungs was studied, in terms of bacterial colonization, macrophage activity, malondialdehyde (MDA) and nitrite production in bronchoalveolar lavage fluid (BALF). Tumour necrosis factor (TNF)-α level in serum was also assessed.

**Results:** Though there was a decrease in bacterial colonization after short-term feeding, it was not significant. On the contrary, the decrease in bacterial load was significant (*P*<0.05) on long-term feeding. The operative mechanisms in terms of lipid peroxidation, phagocytosis and nitrite production were studied by estimating their levels in bronchoalveolar lavage fluid (BALF). Maximum decrease in malondialdehyde (MDA) levels and increase in phagocytic activity and nitrite levels on long-term feeding was seen.

**Interpretation & conclusion:** These results suggest that dietary supplementation with *amla* protects against bacterial colonization of lungs on long-term feeding in experimental model. Further studies need to be conducted to understand the actual mechanism.

**Key words** *Amla* (*Emblica officinalis*) - herbal feeding - *Klebsiella pneumoniae* - pneumonia
role of amla in helping host against respiratory diseases has not yet been defined. Therefore, in the present study, an attempt has been made to study the protective role of amla in vivo in a mouse model of respiratory tract infection (RTI) via intranasal instillation. In community as well as in hospital acquired infections, Klebsiella pneumoniae has a special relevance. Therefore, an attempt was made to assess the antibacterial property of amla against K. pneumoniae ATCC 43816 as well as in vivo using RTI model in mice.

Material & Methods

Bacterial strain: The standard strain of Klebsiella pneumoniae ATCC 43816 of serotype O1:K1 obtained from Dr D.P. Speert, Department of Pediatrics, University of British Columbia, Vancouver, Canada was used in this study. Organism was maintained on nutrient agar slants, at 4°C.

Test animals: Both male and female LACA (Laboratory Animal Centre Albino) mice, 6-8 wk old, weighing 25 ± 5 g were procured from Central Animal House, Panjab University, Chandigarh. Animals in groups of eight were randomly housed in propylene cages and had free access to an antibiotic free diet (Hindustan Lever Limited, Mumbai) and water ad libitum.

Bactericidal effect of amla (E. officinalis) powder suspension against K. pneumoniae ATCC43816 was studied in nutrient broth supplemented with amla powder (5% w/v) suspension. Pure preparation of the herb “Amalaki” was used in this study (The Himalaya Drug Company, Bangalore, India). Nutrient broth alone acted as control.

Short- and long- term feeding of amla powder to mice: Group of eight mice were fed on standard lab chow diet with daily supplementation of 0.5 ml of 5 per cent (w/v) amla powder suspension administered orally for 15 (short-term) and 30 (long-term) days. Control mice were fed on standard lab chow diet and received 0.5 ml normal saline orally for 15 and 30 days.

Induction of acute pneumonia by intranasal route: Following feeding for short and long term, pneumonia was induced in different groups of mice. Intranasal instillation method of Yadav et al was followed, a 50 µl inoculum containing 10^6 cfu/ml of K. pneumoniae ATCC 43816 was instilled into the nasal opening of mice and the animals were sacrificed on third post infection day.

Bacterial quantitation: For bacterial quantitation, standard plate count method was used. Lungs were homogenized in 1 ml sterile normal saline. Serial ten fold dilutions were made from the homogenate and 0.1ml of selected dilutions were plated out on nutrient agar and incubated overnight at 37°C. The following day, colonies were counted and bacterial load of K. pneumoniae in lung specimens was calculated. Lung bacterial counts were calculated and reported as the bacterial count per gram of lung tissue.

Broncho-alveolar lavage (BAL) fluid extraction: BAL was performed following a standard method. Trachea was exposed and intubated using a polyethylene catheter. BAL was performed by instilling phosphate buffer saline containing 5 mM EDTA in 1 ml aliquots. Approximately, 2 ml of lavage fluid was retrieved per mouse.

Lipid peroxidation: The quantitative measurement of lipid peroxidation in the BAL samples was done according to the method of Wills. The amount of malondialdehyde (MDA) formed was measured by reaction with thiobarbituric acid at 532 nm. The results were expressed as nmol of malondialdehyde/mg protein using the molar extinction coefficient of chromophore.

Phagocytosis: Phagocytosis was performed according to the method of Allen et al. Bacteria were harvested and suspended in phosphate buffer saline so as to obtain the optical density corresponding to 10^6 cfu/ml. Alveolar macrophages were obtained and their suspension was made in RPMI 1640 (High Media Laboratories Ltd., Mumbai). For uptake of bacteria, normal mouse serum, macrophage cell suspension (10^6 cells/ml) and bacterial suspension (106 cfu/ml) was taken, vortexed and incubated at 37°C under 5 per cent CO2 atmosphere. Aliquots were taken regularly after 0, 30, 60 and 90 min of incubation and centrifuged. The viable count of bacteria in the supernatant was determined by plating appropriate serial dilutions on nutrient agar plates. The results were expressed as percentage viable bacteria taken up by the macrophages at respective sampling time interval. For intracellular killing, bacterial suspension (10^6 cfu/ml) was mixed with normal mouse serum and kept for 30 min at room temperature. Macrophages (10^6cells/ml) were added to above bacterial suspension, incubated and centrifuged. The cells were lysed at different time intervals. The colony forming units (cfu) were counted after overnight incubation at 37°C.

Nitrite assay: Nitrite concentration in BAL fluid was determined using a microplate assay based on the method of Green et al; 100 µl of supernatant with an
equal volume of Griess reagent [0.1%(w/v) naphthylethylendiamine dihydrochloride, 1% (w/v) sulphanilamide, 3% (v/v) hypophosphoric acid] was incubated for 10 min at room temperature and absorbance at 545 nm was measured. Nitrite concentration in culture supernatants was calculated on the basis of the standard curve obtained with known concentration of sodium nitrite.

Quantitation of tumour necrosis factor (TNF-α): TNF-α in serum was estimated by the method of Kiener et al. Two-fold serial dilution of mouse serum was made and 4 x 10^5 per ml L929 cell (Central Research Institute, Kasauli) suspension containing actinomycin D (A9415 Sigma Aldrich, New Delhi) was added to each well and incubated for 18 h at 37ºC under 5 per cent CO2 in humified incubator. L929 cells were washed with phosphate buffer saline and 50 µl of 1 per cent (w/v) crystal violet was added. 100 µl of 1 per cent (w/v) sodium dodicyl sulphate was added to elute the stain from cells. Absorbance of each well was read on spectrophotometer at 590 nm. A graph of absorbance was plotted against serial dilutions to quantitate units of TNF-α released.

The study protocol was approved by the institutional animal ethics committee of Panjab University, Chandigarh.

Statistical analysis: Results were analysed statistically by applying students’ t test, and P<0.05 was considered significant.

Results

Growth of K. pneumoniae ATCC 43816 in nutrient broth alone and in presence of amla powder was recorded. Decline in growth was observed when nutrient broth was supplemented with amla powder suspension (5 % w/v). The broth became sterile after 8 h of steady decrease in bacterial numbers showing bactericidal effect of amla powder on K. pneumoniae (Fig. 1).

The effect of amla powder feeding on the establishment of acute pneumonia by K. pneumoniae in experimental animals was assessed. The bacterial counts in the lung homogenates were less in the groups of mice fed on amla powder supplementation along with normal diet as compared to mice fed on normal diet without any supplementation. A significant decrease in bacterial load was observed only on long-term (30 days) feeding of amla powder (P<0.05) (Table I).

The extent of tissue destruction in experimental mice was assessed on the basis of MDA estimation. Following infection, maximum damage was observed in control infected group, as MDA levels were the highest in this group (8.1 nmoles/mg protein) significantly (P<0.01) higher than that in uninfected control mice (0.7 nmoles/mg protein). A non significant decrease in MDA levels (6.5 n moles/mg protein) was observed in animals following amla powder feeding. On long-term feeding of amla powder, significant decrease in MDA levels (3.1 nmoles/mg protein) was observed (P<0.05).

Alveolar macrophages obtained from animals fed on amla powder had higher rate of engulfment of bacteria as compared to macrophages from animals fed on normal diet. Alveolar macrophages obtained from the groups fed on amla powder for 30 days showed maximum uptake of bacteria (Table II). Similar, trend in intracellular killing of K. pneumoniae ATCC 43816 was observed by alveolar macrophages obtained from groups fed on amla powder (Table III). The alveolar macrophages killing was maximum in the group fed on amla powder for 30 days.

The result on production of nitrite in the lungs of mice fed on amla powder suspension for 15 and 30 days

<table>
<thead>
<tr>
<th>Groups of mice</th>
<th>Mean log bacterial count/g of lung tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected control mice</td>
<td>0.0</td>
</tr>
<tr>
<td>Infected control mice</td>
<td>8.1*</td>
</tr>
<tr>
<td>Mice fed on amla powder preparation for 15 days, Infected</td>
<td>6.5</td>
</tr>
<tr>
<td>Mice fed on amla powder preparation for 30 days, Infected</td>
<td>3.1†</td>
</tr>
</tbody>
</table>

*P<0.01 compared to uninfected control mice
†P<0.05 compared to infected control mice

Fig. 1. Bactericidal effect of amla powder (5%w/v) suspension on Klebsiella pneumoniae ATCC43816 on addition to nutrient broth.
and infected subsequently with *K. pneumoniae* ATCC 43816 showed that 68 µM nitrite was released following infection as the nitrite levels in the normal uninfected (control) animals were almost negligible (0.7 µM/ml). Maximum release of nitrite was observed in the bronchoalveolar lavage fluid of animals fed on amla powder (P<0.05) for 30 days.

Fig. 2 shows the comparative levels of TNF-α achieved in the serum of animals after infection in groups given short-and long-term feeding of amla powder. Increase in the amount of TNF-α was seen in the serum of control animals following infection. In case of uninfected and infected amla powder fed group no significant change in serum TNF-α level was seen on short-and long-term feeding.

**Discussion**

Decline in growth was observed when nutrient broth was supplemented with amla powder suspension. Antibacterial effect of amla (*Emblica officinalis*) on Gram-negative and Gram-positive organisms has been studied earlier. The possible reason for this effect can be attributed to the presence of flavonoids in amla. Flavonoids are the phenolic structures and their antimicrobial activity is probably due to their ability to form complex with extracellular and soluble proteins, or with bacterial cell walls which disrupts the microbial membranes.

Pulmonary antioxidant defences are widely distributed in lungs and include both enzymatic and non enzymatic systems. The primary non enzymatic antioxidants are membrane bound vitamin C and vitamin E. *Amla* is the richest source of vitamin C and flavonoids and vitamin C, as an antioxidant, is very effective in inhibiting lipid peroxidation by scavenging reactive oxygen species and free radicals, thus preventing tissue damage. It has also been proposed that it can also act by protecting membranes against peroxidation by enhancing the activity of tocopherol, the chief lipid soluble chain breaking antioxidant.

Flavonoids also have well known antioxidant property. It is likely that both vitamin C and flavonoids may have contributed towards protection due to lower peroxidation levels in amla supplemented group. The decrease in MDA levels matched with reduced colonization of lungs with *K. pneumoniae*. These observations in relation to MDA level and lung bacterial load suggest protective role of *aml a* feeding against respiratory challenge with *K. pneumoniae*. Some clinical studies have shown beneficial effects of *tulsi*, echinacea, quercitin, garlic and ginseng supplementation in relation to upper respiratory tract infections and allergies. However, for comparison, *in vivo* studies in relation to *aml a* feeding are not available but reports on feeding of other herbal products are available. This approach is supported by the observation made by Telugu *et al* who have also

| Table III. Intracellular killing of *K. pneumoniae* ATCC 43816 by alveolar macrophages obtained from mice fed on amla powder for 15 and 30 days |
|---|---|---|
| Time (h) | Control | Amla powder fed group for 15 days | Amla powder fed group for 30 days |
| | Bacterial counts obtained in different groups | | |
| 0 | 3.7 × 10⁴ | 3.7 × 10⁴ | 3.7 × 10⁴ |
| 1 | 16.6 × 10⁴(39.85) | 12.7 × 10⁴(41.7) | 10.7 × 10⁴(54.4) |
| 2 | 14.3 × 10⁵(48.18) | 10.2 × 10⁵(53.6) | 8.2 × 10⁵(65.1) |
| 3 | 13.3 × 10⁵(51.8) | 7.1 × 10⁵(67.11) | 5.5 × 10⁵(76.5) |

Values in parentheses are percentage intra-cellular killing of bacteria by alveolar macrophages.
observed decreased lipid peroxidation levels in antioxidant (flavonoids from quercitin) supplemented group infected with influenza virus.

Effective host defence against lung bacterial infection is primarily dependent on the rapid clearance of microorganisms from the respiratory tract. Alveolar macrophages in the lungs constitute the first line of defence against infectious agents that reach lungs. The uptake and intracellular killing by the alveolar macrophages obtained from animals fed on amla powder showed better ability for uptake and killing the bacteria. The duration of amla powder feeding also affected the overall phagocytic ability of macrophages. This in turn might be due to the presence of tannins in amla which have been shown to have stimulatory effect on phagocytic cells28–30. The nitric oxide production was also found to be higher in groups fed on amla powder. Nitric oxide has been suggested to play a crucial role in antibacterial host defence against Klebsiella pneumoniae27. Therefore it appears that both activation of macrophage by tannins and their increased ability to produce nitric oxide might have contributed towards protection against K. pneumoniae mediated pneumonia in amla fed animals.

Innate immunity is recognized as a major contributor in protection against K. pneumoniae and TNF-α level has been shown to be an essential cytokine mediator28–30. Takashima et al30 observed an increase in TNF-α levels in the lungs and serum with the increase in bacterial counts in the lungs in pneumococcal pneumonia mouse model. In our study increase in serum TNF-α level was observed in control animals whereas a decrease was seen in amla fed mice. This indicated that amla feeding protects against K. pneumoniae mediated respiratory tract infection by keeping a check on the production of proinflammatory cytokine like TNF-α. While protective role of amla through antioxidant property and reduction in TNF-α appears to be playing an important role, it remains to be seen as to how cytokine network operation gets altered following administration of amla specially when given on long term basis.

References


Reprint requests: Prof. S. Chhibber, Basic Medical Sciences Building, Department of Microbiology, Panjab University, Chandigarh 160 014, India
e-mail: sanjaychhibber8@sify.com