BIOMEDICAL AND ENVIRONMENTAL SCIENCES **15**, 215-222 (2002)

Silica Induced Early Fibrogenic Reaction in Lung of Mice Ameliorated by *Nyctanthes arbortristis* Extract

BHOLA NATH PAUL[*,1], ANAND PRAKASH[*], SIRISH KUMAR[*], AJAY K. YADAV[*], U. MANI[+], ASHOK K. SAXENA[*], ANAND PRAKASH SAHU[#], KEWAL LAL[+], AND KALYAN K. DUTTA[+]

[*]*Immunobiology Laboratory;*[+]*Inhalation Toxicology Laboratory;* [#]*Preventive Toxicology Laboratory; Industrial Toxicology Research Centre, M.G.Marg, Lucknow 226001, U.P. India*

Objective To investigate the pharmacological effect of *Nyctanthes arbortristis* (NAT) leaf extract in the prevention of lung injury induced by silica particles. **Method** Lung injury was induced in Swiss mice through inhalation exposure to silica particles (<5μ) using a Flow Past Nose Only Inhalation Chamber at the rate of -10 mg/m^3 respirable mass for 5 h. Lung bronchoalveolar lavage (BAL) fluid collected between 48 and 72 h was subjected to protein profiling by electrophoresis and cytokine evaluation by solid phase sandwich ELISA. Lung histopathology was performed to evaluate lung injury. **Results** Inhalation of silica increased the level of tumor necrosis factor-α (TNF-α), and of the 66 and 63 kDa peptides in the BAL fluid in comparison to sham-treated control. Pre-treatment of silica exposed mice with NAT leaf extract significantly prevented the accumulation of TNF-α in the BAL fluid, but the 66 and 63 kDa peptides remained unchanged. The extract was also effective in the prevention of silica-induced early fibrogenic reactions like congestion, edema and infiltration of nucleated cells in the interstitial alveolar spaces, and thickening of alveolar septa in mouse lung. **Conclusion** NAT leaf extract helps in bypassing silica induced initial lung injury in mice.

Key words: *Nyctanthes arbortristis;* Lung-injury; Silica; Mice prevention

INTRODUCTION

Deposition of silica particles in the lung of human beings or experimental animals leads to silicosis, a disease of progressive respiratory failure caused by a fibrotic reaction[1-3]. It has long been suspected that the phagocytosis of silica by pulmonary macrophages induces the secretion of fibrogenic factors[4]. Several potentially fibrogenic cytokines released by macrophages have been identified. These are interleukin-1(IL-1)[5], tumor necrosis factor-α (TNF-α)[6], platelet derived growth factor[7], basic fibroblast growth factor[8] and transforming growth factor-β(TGF-β)[9]. TNF-α plays an important role in silica-induced pulmonary fibrosis in mice because a single instillation of silica leads to a marked increase in the level of lung TNF messenger RNA lasting >70 days, while antibodies against TNF-α

[1] To whom correspondence should be addressed: Dr. B. N. Paul: C 802 Rohtas Apartment, Vikas Nagar, Lucknow 226022, Uttar Pradesh, INDIA; E-Mail: bholanathpaul@yahoo.com.

Biographical note of the first author: Assistant director, Immunobiology Division; Principal Investigator, DBT sponsored research project entitled "Screening of different plant extract for manipulation of cytokine network".

0895-3988/2002
CN 11-2816
Copyright ©2002 by CAPM

万方数据

block the development of silicosis. There are no obvious changes in IL-1α or TGF-β[1].

TNF-α has been postulated as a central mediator of fibrogenic lung disease caused by such diverse agents as bleomycin[10] and asbestos[11]. It is a multipotent cytokine, acting on one hand as a growth factor and on the other as an activator of gene expression. It controls diverse actions such as mucous production, expression of several types of interleukin, and fibroblasts attachment to collagen matrix. Despite a fairly complete understanding of the clinical and pathological features of fibrogenic lung disease, there is no effective therapeutic approach[11].

The depletion of TNF-α from the plasma of arthritic and soluble protein A treated mice following oral administration of water soluble fraction of the ethanol extract of *Nyctanthes arbortristis* (NAT) has been shown[12]. The decoction of the leaves of NAT is widely used in Ayurvedic medicine for the treatment of sciatica and arthritis[13-16]. As TNF-α is associated with lung injury by silica and other fibrogenic agents, NAT appears to be a potent candidate to ameliorate lung injury. Therefore, to evaluate the preventive efficacy of NAT extract in the early phase of pulmonary injury, a well-characterized murine model of silica induced lung injury was utilized. Here protection of mice from the initial fibroproliferative effects of inhaled silica particles by pretreatment with NAT leaf extract is shown.

MATERIALS AND METHODS

Preparation of Leaf Extract of Nyctanthes Arbortristis (NAT)

Details of plant identification and the preparation of water-soluble alcoholic extract were outlined elsewhere[12]. In brief, dried leaves were powdered and macerated with 95% ethanol, the extract filtered and the solvent evaporated. The residue was stirred vigorously with distilled water; the mixture was allowed to stand for 30 min and filtered. The residue was stirred twice more with water and filtered. The filtrate (in a pre-weighed container) was lyophilised and the residue was weighed and suspended in sterile distilled water.

Experimental Animals

Male Swiss mice at 4 - 5 weeks of age were used throughout the experiments. TNF-α in the BAL fluid of these mice was found < 23.4 pg/mL. Mice were provided with pellet food and water *ad libitum*, and were treated with 50 μg of plant extracts orally. This dose was found optimum for depleting plasma TNF-α level in arthritic mouse model[12]. Experiments were carried out in three different groups. One group of mice was exposed to silica particles through the nasal route. The other group received single oral administration of NAT extract 1 h before inhalation exposure to silica. Mice exposed to room air in exposure chamber for 5 h were treated as sham control. BAL fluid was collected between 48 and 72 h after inhalation exposure to silica. This time point was adapted from Liu and Co-workers[17,18] who had shown hypercellular and hypertrophic lesion with increased connective tissue matrix, macrophage and interstitial cells in B6129 mouse lung 48 h after chrysotile asbestos exposure. Swiss mice exposed to silica showed infiltration of nucleated cells in the interstitial alveolar spaces and conspicuous thickening of alveolar septa after 72 h.

Inhalation Exposure Conditions

Aerosol exposure to silica was carried out utilizing a Flow Past Nose Only Inhalation System (Dynamic Inhalation Chamber, IN-TOX, Albuquerque, New Mexico). The mice were exposed to crystalline silica (< 5 μm; a kind gift from Dr. V. Vallyathan, NIOSH, USA)

万方数据

at the rate of -10 mg/m^3 respirable mass for 5 h.

Lung Lavage Fluid

Mice subjected to BAL were anaesthetized by i.p. injection of sodium thiopentone (5mg/0.1mL/mice). BAL was performed by installation and withdrawal of a fixed volume of calcium and magnesium-free phosphate buffered saline, pH 7.4 (PBS). A total of 1 mL was instilled in each mouse. The effluent BAL fluid from each mouse was centrifuged at 4℃ for 10 min at 2 000 rpm and the supernatant was saved for cytokine analysis and protein profiling.

Cytokine Analysis

Solid phase sandwich ELISA Kit (R & D Systems, USA) was utilized for the evaluation of TNF-α, IL-1β and IL-4. The procedural details given in the technical brochure of R & D System were followed. In brief, TNF-α was assayed on microplates precoated with affinity purified polyclonal antibody specific for mouse TNF-α. One hundred μL of Standards (ranging from 750 to 23.4 pg/mL), controls, and samples was pipetted into the wells and incubated at room temperature for 2 h. The wells were washed 4 times and horseradish peroxidase linked polyclonal antibody specific for mouse TNF-α was added to the wells. Following a wash cycle to remove any unbound antibody-enzyme reagent, a substrate solution (hydrogen peroxide and tetramethyl-benzidine) was added to the wells. The enzyme reaction yielded a blue product that turned yellow on addition of stop solution. The optical density of the plate/wells was read at 450nm and at 540 nm (correction wavelength). Concentration of TNF-α was extrapolated from the standard curve of TNF-α. For evaluating IL-1β and IL-4, the procedural details remained the same as TNF-α except that the microplates were pre-coated with either polyclonal antibody specific for mouse IL-1β or monoclonal antibody specific for mouse IL-4; and the second antibody used was specific for mouse IL-1β or IL-4 respectively and was conjugated to horseradish peroxidase.

Histopathology

Lungs were harvested 72 h after exposure and reinflated by gentle injection of 4% phosphate buffered formalin fixative until they reached approximately normal anatomic volume. The pressure required for inflation was not measured, but was judged to be similar in all animals. Cubes of lung tissue were removed after several minutes fixation *in situ*, gently agitated in the fixative at room temperature for 24 h, and then washed and stored in 70% ethanol fixative. Tissue was embedded in paraffin, sectioned and stained with haematoxylin and eosin[19].

Lavage Protein Analysis

The protein profile of BAL fluids of NAT treated silica exposed mice, silica exposed mice and sham control mice was studied by Native PAGE[20] and SDS-PAGE (10%) under reducing condition following the method of Laemmli[21]. The gels were scanned in GEL DOCUMENTATION SYSTEM-1000 (Bio-Rad).

RESULTS

To elucidate the molecular mechanism of anti-inflammatory reaction induced by NAT

万方数据

leaf extracts, the effect of NAT on TNF-α in the BAL fluid of mice exposed to silica was studied. Oral administration of NAT leaf extracts did not elicit any behavioral changes in mice up to an observation period of 3h-post administration with the extract. No change in the body weight (14±1.4) of the experimental animals was observed till the day of sacrifice.

Histological Observation

The histopathological examinations of the sham control mice lungs showed normal pulmonary architecture with few inflammatory cells in the alveolar lumen (Fig.1b). The lung of mice exposed to silica showed edematous fluid in the alveolar spaces and infiltration of macrophages and nucleated cells in the alveolar lumen. The interstitial alveolar septum was thickened with the deposition of reticulin fibres. The bronchiolar epithelium was also thickened (Fig.1a). The lungs of silica exposed mice treated with plant extracts showed relatively fewer cells in the alveolar lumen and near normal alveolar septum in comparison to the silica exposed mice lungs (Fig.1c)

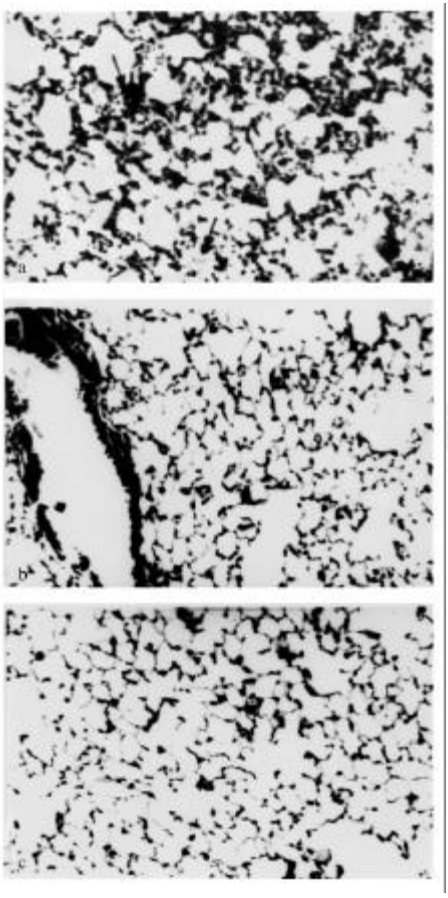

FIG.1.Early histopathological changes in the lung of mice 72 h after exposure to silica (5 μm). Changes in the lung architecture of silica inhaled mice at the rate of ~10mg/m^3 respirable mass (a), lung architecture of sham-control mice (b) and lung architecture of silica inhaled mice pretreated with 50 μg of NAT extract (c). Single arrow indicates infiltration of nucleated cells in the interstitial alveolar spaces leading to the thickening of alveolar septa and double arrows indicate edema.×262.5

万方数据

TNF-a Profile in BAL Fluids

Ten air-sham control mouse BAL samples were evaluated for detectable levels of mouse TNF-α. All the 10 mice had TNF-α < 23.4 pg/mL (Table 1). Greater abundance of TNF-α in the BAL fluid of mice exposed to silica was observed in 84% of the animals with a mean TNF-α level of 379 pg/mL. Remaining 16% of silica-exposed mice had TNF-α level < 23.4 pg/mL which was similar to air sham control animals. Pretreatment of mice with NAT leaf extract revealed TNF-α level < 23.4 pg/mL in 80% of individual mice while remaining 20% of the mice had a mean TNF-α level of 28.59 pg/mL.

IL-1b and IL-4 Profile

About 80% of air-sham control group of mice had a mean value of 22.4 pg/mL IL-1β level (Table 1). Remaining 20% of the air-sham control mice had IL-1β level ~ 35 pg/mL. Inhalation exposure to silica did not alter the IL-1β in the BAL fluid in comparison to air-sham control values. Similarly, NAT extract did not have any effect on IL-1β level in the BAL fluid (Table 1). Several workers have attributed IL-4 as an anti-inflammatory cytokine[22, 23]. To explore the possibility of NAT leaf extract inducing IL-4, the level of IL-4 in the BAL fluid of mice exposed to silica was evaluated. However, this cytokine remained unaffected in all the groups. The level of the IL-4 remained < 7.8 pg/mL (Table 1).

TABLE 1

Cytokine Profiles in BAL Fluid of Mice Exposed to Silica and Treated With NAT Extract

Groups	TNF-α	IL-1β	IL-4
	(pg/mL)		
Silica inhaled mouse BAL (n =12)	379 .0 (84%) < 23.4 (16%)	33.6 (100%)	< 7.8 (100%)
NAT pretreated silica inhaled BAL (n =10)	< 23.4 (80%) 28.5 (20%)	36.8 (40%) < 7.8(60%)	< 7.8 (100%)
Air-sham control BAL (n =10)	< 23.4 (100%)	22.4 (80%) 35.0 (20%)	< 7.8 (100%)

Note.Figures in parentheses indicate the percentage of mice showing the corresponding cytokine level.

Protein Profile in BAL Fluids

BAL fluid drawn from different groups of animals was subjected to SDS-PAGE under reducing condition. Existence of a ~66 and 63 kDa protein was observed in BAL of all groups of animals including the sham-controls. A 2-folds increase in the level of the 66kDa peptide was seen in the silica-exposed mice, and NAT leaf extract treated silica exposed mice in comparison to air-sham control mice (Fig.2). Occasionally in silica exposed mice, a < 14.3 kDa was seen if substantial RBC leaked into the BAL fluid (not shown in Fig.1). In the native gel, however, the two peptides co-migrated at a position parallel to 66 kDa marker protein in a 10% polyacrylamide gel (Fig.3). However, the pattern of distribution of the proteins was similar to the SDS-PAGE.

万方数据

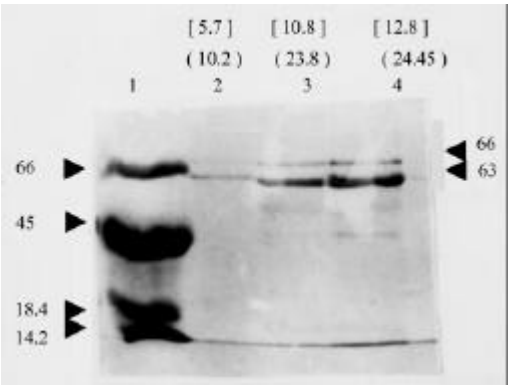

FIG.2.SDS-PAGE analysis of BAL fluid. Lane 1 represents molecular weight marker protein; Lanes 2, 3 and 4 represent BAL fluid from air sham control mouse, silica exposed mouse and NAT pre-treated silica exposed mouse respectively. 10μL of BAL fluid along with 5μL of sample buffer containing SDS and 2-mercaptoethanol was boiled for 2 min and loaded in each lane. Figures in parentheses show scanning data [uOD*mm2] of the 66 and 63 kDa peptides.

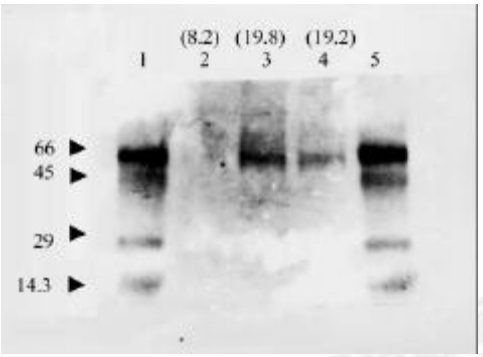

FIG.3.Native PAGE analysis of mouse BAL. Lane 1 and 5 represents molecular weight marker proteins. Lane 2, 3 and 4 represents BAL from air sham control mouse, silica exposed mouse and NAT pretreated silica exposed mouse respectively. 10μL of BAL fluid along with 5μL of sample buffer without SDS and 2-mercaptoethanol were loaded without boiling. Figures in parentheses shows scanning data [uOD*mm2] of protein band migrating parallel to the 66 kDa marker protein.

DISCUSSION

The present study elucidated the biological effect of NAT leaf extract on the initial reaction of murine lung injury by silica particles and supports the notion of NAT extract as an herbal preventive drug. Inhalation exposure to silica in mice was reported to enhance the TNF-α in BAL fluid of mice[1]. It is shown here that NAT treatment reduces the TNF-α level in the BAL fluid of silica exposed mouse and the lung architecture is maintained to normal ones. In contrast, IL-1β appears to have no role in the initial lung reactions induced by silica in mouse and therefore the role of NAT leaf extract in this proinflammatory cytokine cannot be gauged.

It was further demonstrated that IL-4 plays an insignificant role in the prevention of initial reaction of lung fibrosis by NAT leaf extract. The presence of elevated level of 63 and

万方数据

65 kDa peptides in both the NAT leaf extract pretreated silica exposed and only silica exposed mice in comparison to air sham control group indicates that NAT leaf extract plays no role in altering the level of these peptides in BAL fluids. The mechanism by which NAT leaf extract prevents the silica induced initial reaction of lung fibrosis is not yet known. One of the possible mechanisms by which this can be achieved is that NAT leaf extract interferes in the cyclooxygenase- prostaglandin E_2 (PGE_2) pathways to prevent the TNF-α induction. The PGE_2 has been convincingly demonstrated to inhibit production of mature TNF-α protein activity and down regulate the level of mRNA for TNF-α in macrophages[24]. The leaves of *Nyctanthes arbortristis* have been found to contain tannic acid, methyl salicylate, amorphous glucosides, mannitol, ascorbic acid, carotene and traces of volatile oil[25, 26]. Acetyl salicylic acid, otherwise known as aspirin, acts by inhibiting prostaglandin synthesis. Presence of salicylate in the NAT extract suggests that it can interfere in the formation of prostaglandin E_2 and thereby reduce the level of TNF-α.

ACKNOWLEDGEMENTS

We are extremely grateful to Dr. P.K.Seth, Director, ITRC for his keen interest in the project. We thank Dr. Suresh Chand Srivastava for his valuable suggestion, Sri Brij Mohan Pandey and Sri Hari Ram for their technical assistance and Sri Kisan Lal for photography and art work. This work was supported in part by the Department of Biotechnology, New Delhi, grant No. BT/Pro892/PB/17/042/97.

REFERENCES

1. Piguet, P.F., Collart, M.A., Grau, G.E., Sappino, A.P., and Vassalli, P. (1990). Requirement of tumor necrosis factor for development of silica induced pulmonary fibrosis. *Nature* **344**, 245-247.
2. Dauber, J.H., Rossman, M.D., Pietra, G.G., Jimenez, S.A., and Daniele, R.P. (1980). Morphologic and Biochemical abnormalities produced by *intra tracheal* instillation of quartz in to guinea-pig lungs. *Am. J. Path.* **101**, 595-612.
3. Bowden, D.H. (1987). Macrophages, dust and pulmonary diseases. *Exp. Lung. Res.* **12**, 89-107.
4. Heppleston, A.G., and Styles, J.A.(1967). Activity of macrophage factor in collagen formation by silica. *Nature* **214**, 521-522.
5. Schmidt, J.A., Mizel, S.B., Cohen. D., and Green, I. (1982). Interleukin-1: A potential regulator of fibroblast proliferation. *J. Immunol.* **128**, 2177-2182.
6. Sugarman, B.J., Aggarwal, B.B., Figari, I.S., and Paliadino, M.A. (1985). Recombinant Tumor Necrosis Factor-α: Effect on proliferation of normal and transformed cells *in vivo*. *Science* **230**, 943-945.
7. Ross, R., Raines, E.W., and Bowen-Pope, D.F. (1986). The Biology of Platelet-derived Growth Factor. *Cell* **48**, 155-169.
8. Folkman, J. and Klagsbrun, M. (1987). Angiogenic factors. *Science* **235**, 442-447.
9. Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, K.M., Roche, N.S., Wakefield, L.M., Heine, U.L., Liotta, L.A., Falanga, V., Kehrl, J.H., and Fauci, A.S. (1986). Transforming Growth Factor type β: Rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. *Proc. Nat. Acad. Sci*. (USA) **83**, 4167-4171.
10. Piguet, P.F., Collart, M.A., Grau, G.E., Kapanci, Y., and Vassalli, P.(1989). Tumor Necrosis Factor/Cachectin plays a key role in bleomycin induced pneumopathy and fibrosis. *J. Exp. Med.* **170**, 655-663.
11. Brass, D.M., Hoyle, G.W., Poovey,H.G., Liu, J-Y, and Brody, A.R., (1999). Reduced tumor necrosis factor-α and transforming growth factor-β_1 expression in the lung of inbred mice that fail to develop fibroproliferative lesions consequent to asbestos exposure. *Am. J. Path*. **154**, 853-862.
12. Paul, B.N. and Saxena, A.K. (1997). Depletion of tumor necrosis factor-α in mice by *Nyctanthes arbortristis*. *J. Ethnopharmacol.* **56**, 153-158.
13. Kirtikar, K.R. and Basu, B.D. (1935). In *Indian Medicinal Plants* (2nd ed.,L.M.Basu Ed.), pp.1526-1528. Allahabad.
14. Sri Gulabkumaverba Ayurvedic Society (1949). In *Charak Samhita*, Vol.**V**., pp.23-24. Ayurvedic Mudranalaya, Jamnagar.
15. Chopra, R.N., Chopra, I.C., Handa, K.L., and Kapur, L.D. (1958). In *Chopra's Indigenous Drugs of India* (2nd

万方数据

ed.), pp. 408. U.N.Dhar and Sons Private Ltd., Calcutta.
16. Nadkarni, K.M. (1976). In *Indian Meteria Medica* , Vol.**1**, pp.857. Bombay Popular Prakasan, Bombay .
17. Liu, J.Y., Morris, G.F., Lei, W.H., Corti, M., and Brody, A.R. (1996). Up regulated expression of TGF-α in bronchiolar-alveolar duct regions of asbestos-exposed rats. *Am. J. Pathol*. **149**, 205-217.
18. Liu, J.Y., Morris, G.F., Lei, W.H., Hart, C.E., Lasky, J.A., and Brody, A.R. (1997). Rapid activation of PDGF-A and –B expression at sites of lung injury in asbestos-exposed rats. *Am. J. Respir. Cell Mol. Biol.* **17**, 129-140.
19. Sahu, A.P. and Lal, K. (1995). Effect of bleomycin and *Staphylococcus* protein A on lungs of mice. *Ind. J. Exp. Biol*. **33**, 734-738.
20. Bollag, D.M., Rozycki, M.D., and Edelstein, S.J. (1996). In *Protein Methods* , pp.155-170. Wiley Liss, NY.
21. Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
22. Abbas, A.K., Murphy, K.M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature* **383**,787-793.
23. Xu, H., Dilulio, N.A., and Fairchild, R.L .(1996). T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production. IFN-γ producing (TCI) effector $CD8^+$ T cells and IL-4/IL-10 producing (TH_2) negative regulatory $CD4^+$ T cells. *J .Exp. Med*. **183**, 1001- 1004.
24. Petlipher, E.R., and Whittle, B.J.R.(1992). Prostaglandins: In *Encyclopedia of Immunology*, Vol. **3** (I.M. Roitt and P.J. Delves, Eds.), pp.1279-1282. Academic Press, San Diego.
25. Saxena, R.S., Gupta, B., Saxena, K.K., Singh, R.C., and Prasad, D.N. (1984). Study of anti-inflammatory activity in the leaves of *Nyctanthes arbortristis* Linn.- an Indian medicinal plant. *J. Ethnopharmacol*. **11**, 319-330.
26. Zaheer, S.H., Prasad, B., Chopra, R.N., Santapan, H., and Krishnan, M.S. (1966). In *The Wealth of India*. Vol. **7**, pp. 69-79. Publications and Information Directorate, CSIR, New Delhi.

(Received November 29, 2001 *Accepted May* 10, 2002*)*

万方数据