Azadirachta indica induced suppression of *Mycobacterium tuberculosis* secreted proteins in human monocyte

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Introduction:

Tuberculosis is the most important mycobacterial infection in humans, being the second major infectious cause of death and killing around two million people every year [1]. With the emergence of multidrug-resistant TB (MDR-TB) as well as AIDS-associated TB, this killer parasite is spiraling out of control [2]. Thus, in view of it, focus has now shifted in exploring new novel natural compounds that may combat tuberculosis [3, 4]. Therefore, in the present study, we probed the anti-tuberculosis effect of *Azadirachta indica* (Neem), which has attained worldwide prominence in recent years owing to its medicinal properties. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex having wide range of pharmacological properties. It has been known to possess antioxidant and anti-inflammatory properties. It is hoped that the data of the present study may help in understanding the potentiality of neem extract in employing it as a valuable adjunct in TB treatment.

Methods:

1. Preparation of Mycobacteria:

Virulent laboratory-adapted *M. tuberculosis* ($H_{37}Rv$) were grown in Middlebrook 7H9 broth (HiMedia, India) supplemented with Middlebrook ADC enrichment fluid (HiMedia) or

modified Souton medium [5] at 37°C in 5% CO_2. Midlogarithmic mycobacterial cultures (14 days) were harvested and quantified by use of a colony-forming unit assay, as described elsewhere [6]. Aliquots of the stock were kept at 70°C. The viability of the stock remained >99% at 1 year.

2. Preparation of PBMC, cell culture and infection:

Peripheral blood mononuclear cells (PBMCs) from blood were isolated as described by us previously [3]. PBMC ($0.5x10^6$ cells/well) were added onto 12-well tissue culture plates in complete RPMI-1640 medium, and were subsequently incubated at 37°C, 5% CO_2 for 1-2 hrs for adherence. Thereafter, non-adherent cells were removed and the adherent monocytes were rested overnight at 37°C, 5% CO_2. Thereafter, monocytes were infected with *M. tuberculosis* at 1:1 (bacteria: cell) in 30% autologous unheated serum for 90 min. at 37°C, 5% CO_2, and subsequently washed for 4 times complete medium. Cells harvested at this time point were considered as time zero after infection (t_0). Other cultures received RPMI-1640 medium with 2% autologous serum. As per experimental design, cultures immediately after infection received varying concentrations of neem extract (0-1000 ng/ml)., NAC (10 mM), SN50 (100 ug/ml) or SN50M (100 ug/ml). Cultures were then harvested after 24 hrs and cell protein lysates were prepared as described earlier [3,4].

3. Measurement of secreted TNF-α and MTB 85B protein in culture supernatant by ELISA:

The amount of soluble secreted TNF-α protein in various culture supernatants were determined by use of a commercial ELISA Kit (R & D systems), according to the

manufacturer's specifications. The cut off or lower limit of sensitivity was 4.4 pg/ml. ELISA was carried out to determine secreted MTB 85B protein in culture supernatants as described by us previously [4].

4. Glutathione peroxidase assay:

The activity of glutathione peroxidase (GPx) was measured as described elsewhere [13]. Briefly, MTB-infected monocytes were co-cultured for 24 h with varying concentrations of neem extract (0-1000 ng/ml). Thereafter, cells were scrapped, sonicated and centrifuged as described earlier [3 and references within], and the supernatants were subjected to GPx activity determination. The GPx activity was quantified in 100 μl of each sample, with continuous photometric monitoring of oxidized glutathione (GSSG) at 37^{o}C. The conversion of NADPH to NADP was evaluated using UV absorbance at 340 nm [3]. GPx activity was calculated after subtraction of the blank value, as lmol of NADPH oxidized/min/mg protein (U/mg protein).

Results:

Dose response effect of neem extract on soluble TNF-alpha and MTB Ag85B protein expressions in monocyte cultures infected with MTB

We observed neem extract-induced suppression in secreted TNF-α and MTB Ag85B protein expressions in MTB-infected monocyte cultures were dose dependent (Figs. 1 & 2, P<0.001 for all). Neem extract at a maximum dose of 1 ug/ml exhibited a down-regulation in secreted TNF-α and 85B proteins to the extent of 4.7-folds (Fig. 1, P<0.001) and 4.3-folds (Fig. 2, P<0.001) respectively.

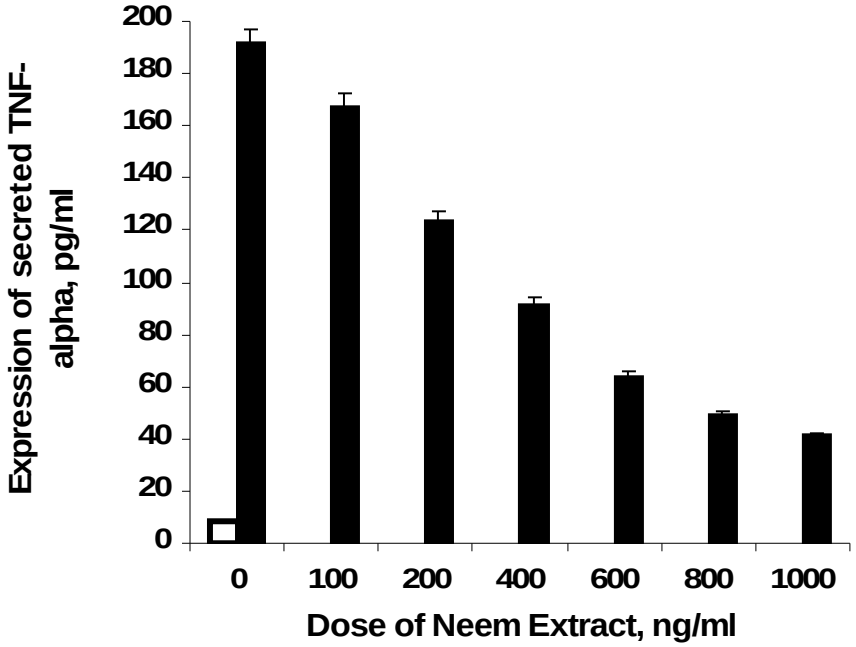

Fig. 1. Dose response effect of neem extract on the expression of secreted TNF-alpha in 24 hr culture supernatants of 1:1 MTB-infected human monocytes. Empty bar: normal healthy uninfected monocytes; black bars: monocytes infected with 1:1 MTB. (n=6, P<0.001 for all).

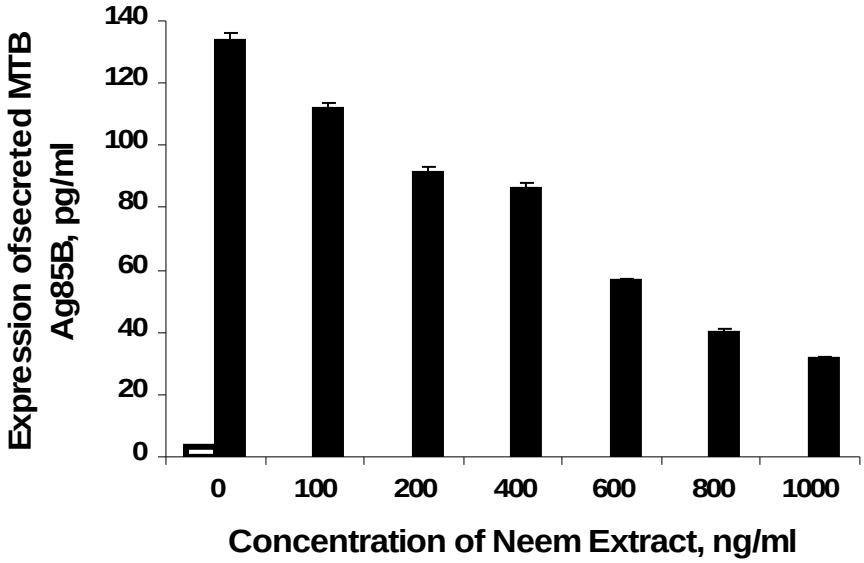

Fig. 2. Dose response effect of neem extract on the expression of secreted MTB Ag85B in 24 hr culture supernatants of 1:1 MTB-infected human monocytes. Empty bar: normal healthy uninfected monocytes; black bars: monocytes infected with 1:1 MTB. (n=6, P<0.001 for all).

Glutathione peroxidase activity:

Healthy control cultures showing appreciably high GPx activity was suppressed in MTB-infected monocytes (Fig. 3, P<0.001). Interestingly, with neem extract treatment of MTB infected monocytes, GPx activity ameliorated appreciably in a dose-dependent manner (Fig. 3, P<0.001). Regulation studies by employing SN50 exhibited the above suppression to be mediated via NF-kB (Fig. 4, P<0.001). These results clearly point to neem extract as a potent natural enhancer of GPx levels in monocytes infected with MTB.

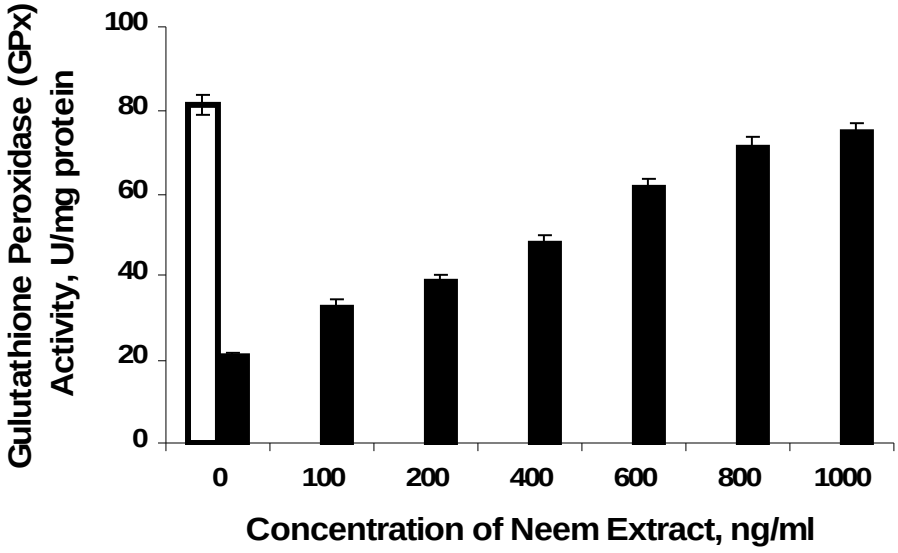

Fig. 3. Dose response effect of neem extract on GPx activity in 24 hr cultures of MTB-infected monocytes (Black bars). Uninfected monocyte cultures served as controls (empty bars). (n=6, P<0.001 for all).

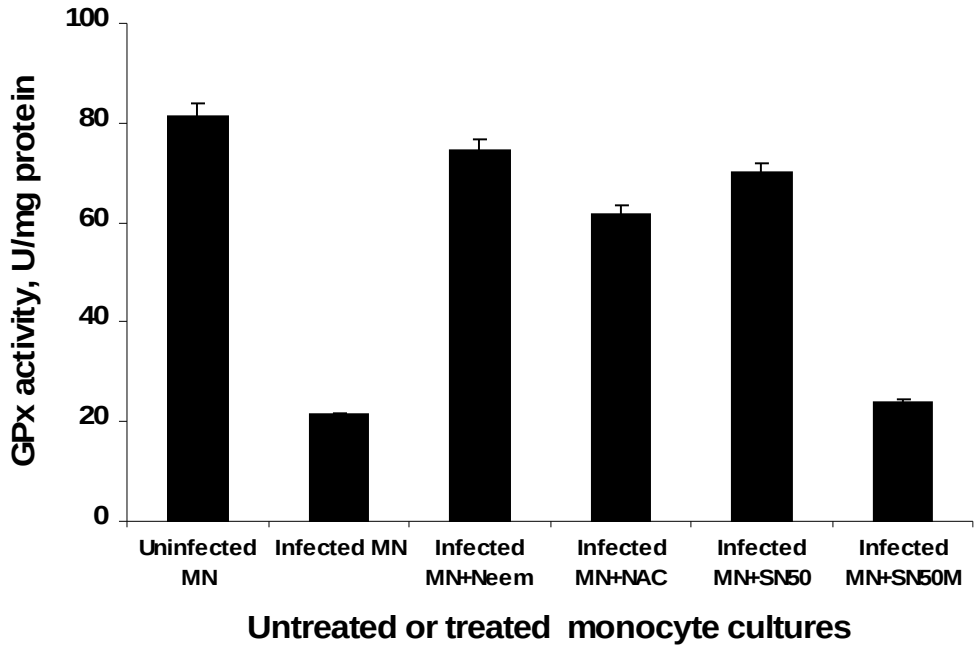

Fig. 4. Regulation of GPx activity in 24 hr cultures of MTB-infected monocytes with NAC (10 mM), SN50 (100 ug/ml), SN50M (100 ug/ml) and neem extract (1 ug/ml). (n=6, P<0.001 for all).

Conclusion:

1. The neem extract-induced suppression in secreted TNF-alpha and MTB Ag85B proteins in 24 hr culture supernatants of MTB-infected human monocytes was found to be dose-dependent.
2. Glutathione peroxidase activity was decreased after *M. tuberculosis* infection of monocytes.
3. Interestingly, glutathione peroxidase activity was augmented significantly with neem extract treatment in a dose-dependent manner.
4. The above-said suppression was mediated via NF-kB.

5. The results are indicative of neem extract- a natural antioxidant and an anti-inflammatory agent, may act as an adjunct in combating tuberculosis infection.

References:

1. WHO (2001) Global Tuberculosis Control, Geneva, Switzerland, *Report no. WHO/CDS/TB/2001.287.*
2. Toossi, Z; Wu, M; **Islam, N**; Teixeira-Johnson, L; Hejal, R and Aung, H. **(2004)** Transactivation of human immunodeficiency virus-1 in T-cells by Mycobacterium tuberculosis-infected mononuclear phagocytes. *J. Lab. Clin. Med., 144(2):108-15.*
3. Hasan, N., Yusuf, N., Toossi, Z. and **Islam, N. (2006)** Suppression of *Mycobacterium tuberculosis* induced reactive oxygen species (ROS) and TNF- α mRNA expression in human monocytes by allicin*. FEBS Letters 580: 2517-2522.*
4. Hasan, N., Siddiqui, M. U., Toossi, Z., Khan, S., Iqbal, J. and **Islam, N. (2007)** Allicin-induced suppression of *Mycobacterium tuberculosis* 85B mRNA in human monocytes. *Biochem. Biophys. Res. Comm., 355(2): 471-476.*
5. **Islam, N;** Kanost, RA; Teixeira-Johnson, L; Hejal, R; Aung, H; Wilkinson, RJ; Hirsch, CS; and Toossi, Z. **(2004)** The role of cellular activation and tumor necrosis factor alpha (TNF-alpha) in the early expression of *M. tuberculosis* 85B mRNA in human alveolar macrophages. *J. Infect. Dis. 15; 190:341-351.*
6. Wilkinson, RJ; DesJardin, LE; **Islam, N**; Gibson, BM; Wilkinson, KA; Poelman, D; Kanost, RA; Eisenach, KD and Toossi, Z. (**2001**) An increase in expression of a M. tuberculosis mycolyl gene (fbpB) occurs early after infection of human monocytes. *Mol. Microbiol, 39(3): 813-821.*