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NIMBIDIN-INDUCED DOWNREGULATION OF TNF-α mRNA IN HUMAN MONOCYTES INFECTED WITH *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

Tuberculosis (TB) is spiraling out of control at an alarming rate where reports implicate TNF-α to proliferate MTB 85B in infected host during early stage of infection. Reactive oxygen species plays an important role augmenting TNF-α, which in turn proliferates MTB 85B in bacilli-infected monocytes. Thus, in view of the above, an attempt was made to probe the antioxidant, anti-inflammatory and antimicrobial effects of phytochemicals from plants having medicinal value in combating TB. Thus, we employed nimbidin, a mixture of tetranortriterpenes including nimbin and nimbinin from *Azadirachta indica* (Neem) to probe its antioxidant, antimicrobial and anti-inflammatory in MTB-infected human monocytes. Various techniques like 'real time' RT-PCR, cell culture, ELISA and enzyme assays were employed in the present study. We show the augmented expression of TNF-α mRNA in 24 h cultures of MTB-infected monocytes was suppressed by nimbidin in a dose dependent manner. The nimbidin -induced up-regulation of glutathione with simultaneous decrease in TNF-α expression supports the anti-inflammatory property of nimbidin. This suppression was mediated via inhibition of GPx activity and NF-κB pathway, because TNF-α mRNA was suppressed when glutathione or its precursor NAC as well as SN50, a known inhibitor of NF-κB, was present in cultures. The doses of nimbidin employed were non-toxic to host cells as revealed by RT-PCR of human housekeeping gene R18 as well as MTT cell viability assay. Thus, nimbidin may act as a potential adjunct in management of tuberculosis and inflammatory diseases.

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INTRODUCTION

Tumor necrosis factor-alpha (TNF-α)- a mediator of systemic inflammation, is a prototypic proinflammatory cytokine that contributes significantly to the development of immunopathology in various disease states (Chakravarty *et al*., 2008). TNF-α is an essential component of the innate defence mechanism of the host against pathogenic challenge. Unfortunately, it can also play a major role in the pathology of certain diseases, such as tuberculosis. This disease is a striking example of the role of TNF-α as a `double-edged sword', because apart from its role in controlling the Mycobacterium tuberculosis infection, it can also cause severe tissue damage (Amanda *et al*., 2009). TNF-α exhibits a very complex network of interactions and many of its activities are still not fully understood (Amanda *et al*., 2009). Depending on the production, TNF-α thus promotes containment or dissemination of *M. tuberculosis* and can contribute to both immune protection and pathology (Rook and Hernandez, 1996). Furthermore, tuberculosis (TB) is well documented to be a prominent opportunistic infection in HIV-1-infected subjects, which enhances HIV-1 replication, and that; it is associated with excess monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)-a activity in situ (Mayanja-Kizza *et al*., 2009). Stimulation of monocytes,

macrophages and dendritic cells with mycobacteria or mycobacterial products induces TNF-α production (Henderson *et al*., 1997). Thus, TNF-α has both adverse and beneficial effects in the human immune response (Tufariello *et al*., 2003). To limit the deleterious effects of TNF-α (Bekker *et al*., 2000), systemic production of TNF-α is downregulated (Friedland *et al*., 1997). In spite of this, anti-TNF-α therapy in the treatment of tuberculosis is associated with an increased risk of developing tuberculosis (Keane *et al*., 2002), mainly due to reactivation of a chronic infection (Keane *et al*., 2001). To avoid such therapy our laboratory is focused on identifying and exploring compounds from natural sources possessing antioxidant, anti-inflammatory and anti-microbial activity in the containment of TB. It's well documented that traditionally used plants for treating tuberculosis (TB) have not been evaluated in detail and hence a thorough study is required, where the outcome may help in finding its application in developing new anti-TB drugs (Gupta *et al*, 2017). The medicinal plants used in traditional medicine by various traditional/tribal healers and their knowledge about medicinal uses of plants which passes verbally from generation to generation without any documentation are also important criteria for plant based drug discovery program(Gupta *et al*, 2017). New anti-TB drug candidates are urgently required to ensure the generation of novel treatment regimens. Use of unexplored sources of plants and lead optimization strategies may also improve the efficiency of future anti-TB drug discovery (Gupta *et al*, 2017). In the present study, focus was on exploring the effect of *Azadirachta indica* extract's principle constituents like nimbidin a mixture of tetranortriterpenes including nimbin and nimbinin in MTB-infected monocytes, with special emphasis on TNF-α. Nimbidin seems to act as a potential agent to counteract the pathological effects of increased TNF-α production in tuberculosis infection. This may help in understanding the use of nimbidin as an adjunct in tuberculosis therapy as well as in its therapeutic use in inflammatory diseases.

MATERIALS AND METHODS

Chemicals

N-acetyl cysteine (NAC), SN50 - an inhibitor of NF-κB and its analogue SN50/M and monoclonal anti-TNF-a antibody were of Sigma–Aldrich (St. Louis, MO, USA). NADPH was from HiMedia and Ficoll-Paque was of Pharmacia (LKB Biotechnology Piscataway, NJ). Neem tablets having principle constituents like nimbidin - a mixture of nimbin and nimbinin, were purchased from Himalaya Drug Company, India. All other chemicals used were of the highest purity grade available. Blood was collected in heparinized syringes from healthy non-smoking volunteers of 20-40 years of age.

Preparation of Nimbidin for in vitro Culture

Nimbidin tablet was dissolved in dimethyl sulfoxide (DMSO) (0.1%) (Kaur *et al*., 2004) and prepared in double distilled water. Thereafter the supernatant was collected by centrifugation at 1500 rpm and was membrane (0.22 µm) filtered for in vitro uses.

Preparation of Mycobacteria and PBMC

Virulent laboratory-adapted *M. tuberculosis* ($H_{37}Rv$) was grown as described by us previously (Hasan *et al*., 2006, Wilkinson *et al*., 2001). Peripheral blood mononuclear cells (PBMC's) from blood (obtained from healthy, non-smoking volunteers of 20–40 years of age) were isolated as described by us previously (Hasan *et al*., 2006, Wilkinson *et al*., 2001, Islam *et al*., 2004).

Neem extract versus toxicity assessment of monocytes

The possible effect of nimbidin (0–1000 µg/ml) on monocytes viability was assessed by quantitative real-time RT-PCR analyzed by using human housekeeping gene i. e. 18S ribosomal RNA (R18) as described before (Hasan *et al*., 2006, Islam *et al*., 2004).

Cell culture and infection

PBMC ($0.5x10^6$ cells/well) were added in 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 by washing the plates (4X) with RPMI medium. Then, the adherent monocytes were cultured in RPMI supplemented with 2% serum, followed by overnight resting at 37°C, 5% CO_2, followed by washing (2X) the plates with RPMI medium. 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 nimbidin (0-1000 µg/ml), whereas some cultures received NAC (10 mmol/l), NADPH (0-10 nmol), SN50, SN50/M (100 µg/ml), Streptomycin (100 ug/ml), Rifampicin (100ug/ml) and Isoniazid (100 ug/ml). Cultures were then harvested after 24 hrs and cells were lysed in 0.5 ml of TRIZOL Reagent (Invitrogen, CA). Culture supernatants were stored at -70°C until use.

Isolation of total RNA/mRNA, reverse transcription (RT) and Quantitative real-time RT-PCR

Total RNA was isolated from uninfected as well as infected monocytes and subjected to reverse transcription as described by us previously (Hasan *et al*., 2006, Wilkinson *et al*., 2001, Islam *et al*., 2004). Real-time RT-PCR with internal fluorescent hybridization probes was employed to quantify host TNF-α gene transcription as described by us previously (Hasan *et al*., 2006, Islam *et al*., 2004). Human R18 housekeeping gene was employed to normalize gene expression. TaqManTM PCR primers and probes as well as target-specific RT primer for each assay were designed as described elsewhere (Hasan *et al*., 2006, Islam *et al*., 2004). The primer and probe sequences were used as reported by us previously (Hasan *et al*., 2006, Islam *et al*., 2004). The amplifications and conditions for PCRs were exactly the same as described by us earlier (Hasan *et al*., 2006, Islam *et al*., 2004). To assure lack of DNA contamination in the RNA samples, in some experiments, a duplicate tube of sample with no RT enzyme was included as control. DNA contamination remained negligible. In each sample, host 18S ribosomal RNA was used as internal control. Expression of TNF-α mRNA was corrected to internal control (host 18S rRNA) in the same sample and was expressed as copies of TNF-a in 10^{10} copies of R18 (equivalent to 1x 10^6 monocytes).

Determination of secreted TNF-α by ELISA

Secreted TNF-α in 24 hr culture supernatants of MTB-infected monocytes that were either untreated or treated with nimbidin was determined by ELISA Kits (R&D systems), according to manufacturer's specification as described by us previously (Hasan *et al*., 2006).

Glutathione peroxidase assay

The activity of glutathione peroxidase (GPx) was measured as described elsewhere (Mates *et al*., 1999). Briefly, MTB-infected monocytes were co-cultured for 24 h with or without 10 mmol/l NAC, (0-10) nM NADPH, 100ug/ml SN50, 100 µg/ml SN50/M and 1000 µg/ml nimbidin. Thereafter, cells were scrapped, sonicated and centrifuged as described earlier (Mates *et al*., 1999), 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°C. The conversion of NADPH to NADP was evaluated using UV absorbance at 340 nm (Mates *et al*., 1999). GPx activity was calculated after subtraction of the blank value, as µmol of NADPH oxidized/min/mg protein (U/mg protein).

Statistical analysis

Results were analyzed by use of paired t-test and expressed as Mean ±SE of ten experiments. $P<0.05$ was considered statistically significant.

RESULTS

Toxicity determination by analyzing housekeeping gene and cell viability assay

Prior to any study, an attempt was made to ensure that the doses of nimbidin (0-1000 µg/ml) selected in this study were non-toxic to human monocytes. Thus, a dose-response effect of nimbidin on human house keeping R18 gene as well as cell viability assessment by MTT assay was done. As evident from MTT cell viability assay and 'real time' RT-PCR data (Fig. 1A & 1B), none of the nimbidin doses showed any toxic or adverse effect on cell viability and human house keeping R18 rRNA expression. It may be worthy in pointing out here that doses of nimbidin higher than 1500 ug/ml exhibited toxic effect to host cells as revealed by MTT assay (data not shown). Thus, nimbidin doses of 0-1000 ug/ml were non-toxic in our system and were employed in the present study.

Dose-response effect of neem extract host TNF-α gene expression in *M. tuberculosis*-infected monocytes

Contrary to the effect on human house keeping genes as shown above, nimbidin showed a dose dependent suppression in augmented TNF- α expression as evident from 'real time' RT-PCR (Fig. 1C). In comparison to control infected monocytes, an appreciable decrease in endogenous TNF-α mRNA was recorded by around 0.4 logs, 0.9 logs, 1.7 logs, 2.4 logs, 3.8 logs and 4.8 logs ($P<0.001$ for all) with treatments of 100, 200, 400, 600, 800 and 1000 µg/ml of nimbidin respectively (Fig. 1C). Similar observations were recorded in PCR products (Fig. 1D), and that, as evident from Fig. 1E, observations made at the protein level of TNF- α expression in culture supernatants correlated appreciably to the above TNF- α expressed at the gene level. The IC_{50} of nimbidin-induced TNF-α suppression in MTB-infected monocytes was ~600 µg/ml and 350 µg/ml at the gene and protein levels respectively. Uninfected normal healthy monocytes that were either untreated or treated with varying doses of nimbidin, exhibited no of negligible TNF- α expression.

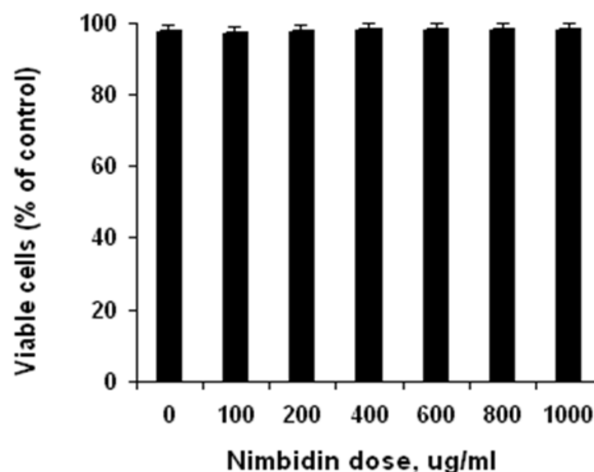


Figure 1A.

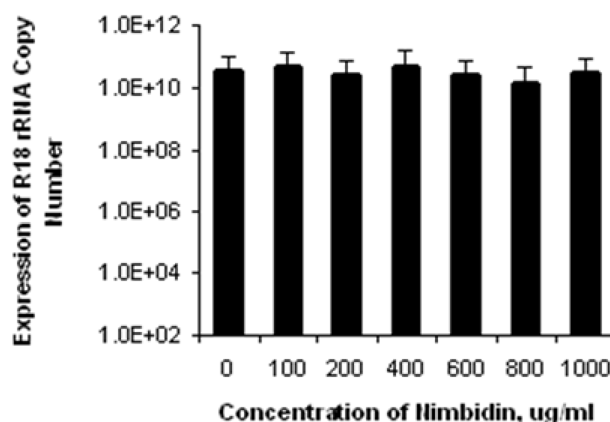


Figure 1B.

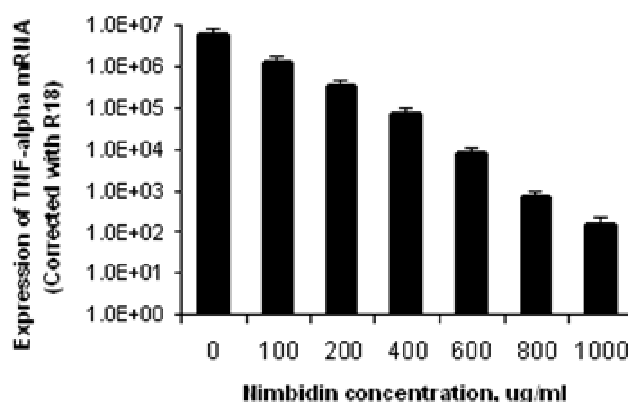
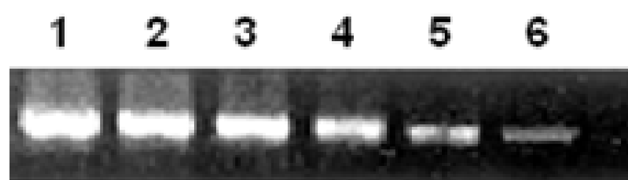


Figure 1C.



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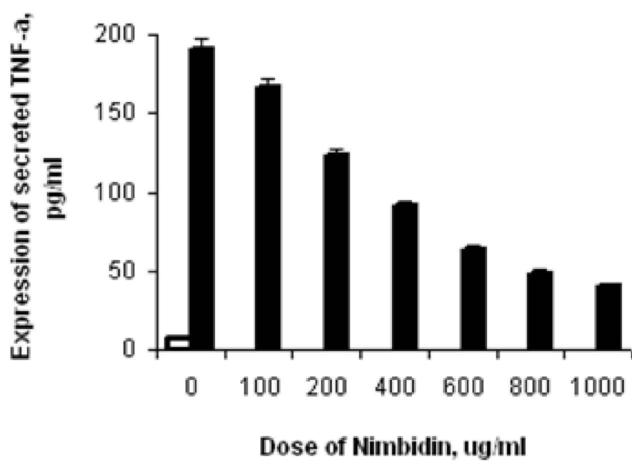

Figure 1E.

Fig. 1. Human monocytes were infected with *M. tuberculosis* $H_{37}Rv$ (1:1 bacterial/cell) and co- cultured with nimbidin (0-1000 µg/ml) for 24 hours. MTT cell viability assay for dose (0–1000 µg/ml) response effect of nimbidin (A), Thereafter, Real –time RT-PCR was carried out for monitoring the effect of nimbidin on expression of host (B) house keeping gene R18 (18S rRNA) and (C) TNF-alpha mRNA. The same for TNF-alpha mRNA (D) was assessed by PCR products, where the doses of nimbidin were as follows: Panel 1 (0 µg/ml), panel 2 (200 µg/ml), Panel 3 (400 µg/ml), Panel 4 (600 µg/ml), Panel 5 (800 µg/ml) and panel 6 (1000 µg/ml). Data represent mean ± SEM of 3 experiments (P<0.001 for all). Thereafter, a dose response effect of nimbidin on the expression of (E) secreted TNF-α in 24 hr culture supernatants of 1:1 MTB-infected human monocytes was carried out by ELISA. Empty bar: normal healthy uninfected monocytes; black bars: monocytes infected with 1:1 MTB. The results are mean (±SE) of 5 experiments (p<0.001)

Assessing the role of reactive oxygen species (ROS) on the expression of soluble TNF-alpha in 24 hr cultures of MTB-infected monocytes

Reactive oxygen species (ROS) plays an important role in augmenting TNF-alpha, and in turn, MTB 85B. Thus an attempt was also made to probe the effects of NADPH, a known positive modulator of ROS, on sTNF-alpha secretion in infected monocytes. As evident from (Fig. 2), uninfected healthy control monocyte cultures showed negligible expression of secreted TNF-alpha, whereas 24 hr MTB-infected monocyte cultures showed augmented expression of secreted TNF-alpha (188.69 pg/ml; P<0.001). Co-culturing of MTB-infected monocytes with varying doses of NADPH (0, 2, 4, 6, 8 and 10 nM) exhibited a further dose-dependent augmentation in secreted TNF-alpha expression.

The results show that from 188.69 pg/ml (0 nM NADPH), the secreted TNF-α expression was up-regulated to 201.22 pg/ml, 222.34 pg/ml, 289.45 pg/ml, 344.21 pg/ml and 371.39 pg/ml with 2 nM, 4 nM, 6 nM, 8 nM and 10 nM of NADPH respectively (Fig. 2A). Next, GPx activity with respect to varying doses of NADPH was determined in *M. tuberculosis*-infected monocytes. Uninfected monocytes served as controls. At dose 0 µg/ml, GPx activity in uninfected control exhibited high GPx activity (81.32 U/mg protein) in comparison to *M. tuberculosis*-infected monocytes, which showed a much-suppressed GPx activity (23.87 U/mg protein) (Fig. 2B). Interestingly, *M. tuberculosis*-infected monocytes co-cultured with varying doses of NADPH (0–10 nM) exhibited a further enhanced linear decrease in GPx activity with dose.

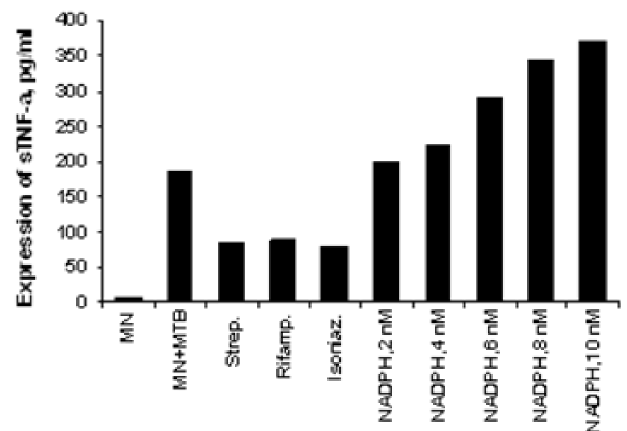

Fig. 2A. ELISA for modulation of expression of sTNF-alpha: Human monocytes infected with *M. tuberculosis* $H_{37}Rv$ (1:1 bacterial/cell) cultures received MN only, MN+1:1 MTB, Streptomycin (strep) (100 µg/ml), Rifampicin (rifamp) (100 µg/ml), Isoniazid (isoniaz) (100 µg/ml) as well as doses of NADPH (2-10 nM) in different bar diagram. Expression of sTNF–α was determined in culture supernatants at 24 hr. Data represent mean ± SEM of five experiments (p< 0.001)

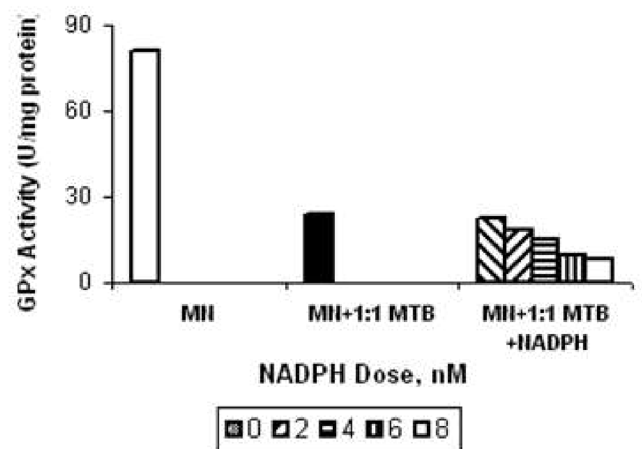

Fig. 2B. ELISA for modulation of GPx activity: Human monocytes were infected with *M. tuberculosis* $H_{37}Rv$ (1:1 bacterial/cell). Some cultures were not infected (MN only; empty bar) and served as control. Cultures then received (1:1) MN+MTB (black bar) as well as doses of NADPH (0-10 nM) in different bar diagram. GPx activity was determined in culture supernatants at 24 hrs. Data represent mean ± SEM of five experiments (p< 0.001)

GPx activity was recorded as 23.87, 18.44, 14.98, 9.22 and 7.18 U/mg protein with 0, 2, 4, 6, 8 and 10 nM of NADPH respectively (Fig. 2B).

Dose-dependent amelioration of glutathione peroxidase (GPx) activity by nimbidin in *M. tuberculosis* infected monocytes: NF-κB plays an important role in M. tuberculosis-infected monocytes

GPx activity with respect to varying doses of nimbidin was determined in *M. tuberculosis*-infected monocytes. Uninfected monocytes served as controls. At dose 0 µg/ml, uninfected control exhibited high GPx activity (79 U/mg protein) in comparison to *M. tuberculosis*-infected monocytes, which due to infection showed a much-suppressed GPx activity (33 U/mg protein). However, *M. tuberculosis*-infected monocytes co-cultured with varying doses of nimbidin (0–1000 µg/ml) exhibited a linear amelioration in GPx activity with dose. GPx activity in *M. tuberculosis*-infected monocytes was recorded as

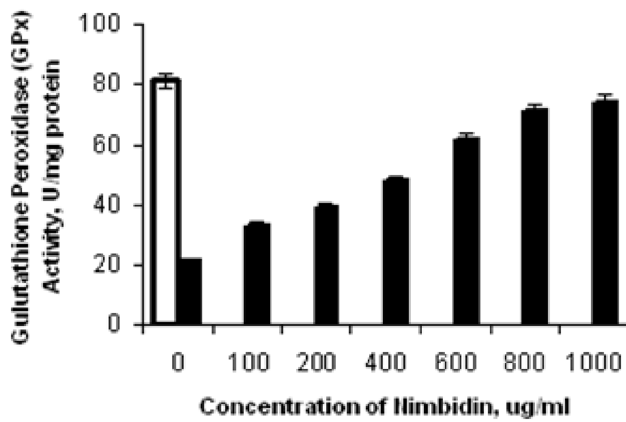

Fig. 3A. Modulation of glutathione peroxidase (GPx) activity: Human monocytes were infected with *M. tuberculosis* $H_{37}Rv$ (1:1 bacterial/cell). Uninfected monocyte cultures served as controls (empty bars). Cultures then received different doses (0-1000 µg/ml) of nimbidin. GPx activity was determined in culture supernatants at 24 hours. Data represent mean ± SEM of five experiments (P<0.001 for all)

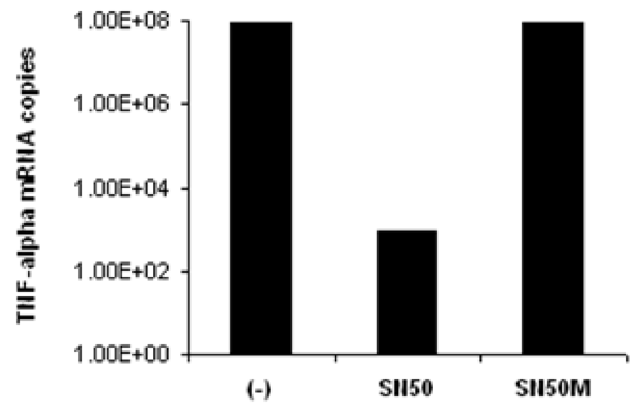

Fig. 3B. Effect of SN50 and SN50/M on the expression of TNF-α mRNA in MTB-infected monocytes after 24 hrs post infection. MTB-infected monocytes (-) that were devoid of SN50 served as control. The results are mean (±SE) of five experiments (p<0.001)

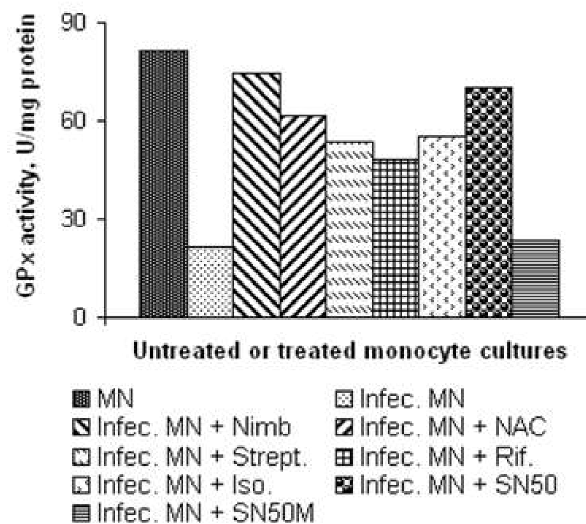

Fig. 4. Regulation of GPx activity in 24 hr cultures of MTB-infected monocytes with NAC (10 mM), Streptomycin (Strept.) (100 µg/ml), Rifampicin (Rif) (100 µg/ml), Isoniazid (Iso) (100 µg/ml), SN50 (100 µg/ml), SN50M (100 µg/ml), nimbidin (nimb) (1000 µg/ml) and NADPH (8 nM). Data represent mean ± SEM of five experiments (P<0.001 for all)

33, 39, 43, 50, 60, 68 and 71 U/mg proteins with 0, 100, 200, 400, 600, 800 and 1000 µg/ml nimbidin respectively (Fig. 3A). Induction of TNF-α by cellular activation is mediated via NF-κB (Hasan *et al.*, 2007; Hasan *et al.*, 2006). It has been well documented that TNF-α induced nuclear translocation of NF-κB was inhibited in SN50 peptide-treated human monocytic cell lines as demonstrated in EMSA (Lin *et al.*, 1995). Thus, we employed SN50, an inhibitor of NF-κB, to assess the role of NF-κB in activation of gene expression in *M. tuberculosis*-infected monocytes. SN50 (100 µg/ml) was added to monocytes 3 minutes prior to *M. tuberculosis* infection. Control cultures did not receive SN50. At 24 hours, SN50 suppressed endogenous TNF-α mRNA expression in *M. tuberculosis*-infected monocytes by around 5.0 logs (P<0.001) in comparison to control cultures devoid of SN50 pre-treatment (Fig. 3B). To ensure that cellular inhibition was specific, we compared the effect of SN50 with its inactive analogue, SN50/M at the same concentration. As expected, SN50/M failed to show any effect on TNF-α mRNA expression (P<0.05) (Fig. 3B). Therefore, the increased expression of TNF-α in *M. tuberculosis*-infected monocytes is mediated mainly via NF-κB.

Nimbidin-induced regulation of glutathione peroxidase (GPx) activity in MTB-infected monocytes

Next, the comparative regulatory effects of nimbidin with known antioxidant N-Acetyl Cysteine (NAC) as well as NF-κB inhibitor SN50 and its control peptide SN50M, on the GPx activity in MTB-infected monocytes was probed. MTB-infected cultures were co-cultured for 24 hrs either with nimbidin (I000 µg/ml), NAC (10 mM), SN50 (100 µg/ml) or SN50M (100 µg/ml), and thereafter harvested. Uninfected monocyte cultures served as controls. As evident from (Fig. 4), uninfected cells showed a GPx activity to the order of 79 U/mg proteins whereas when the same cells were infected with 1:1 MTB, then the GPx activity was down regulated to 33 U/mg protein (Fig. 4). Interestingly, co-culturing with neem extract, NAC and SN50, showed amelioration in the GPx activities to the order of 72 U/mg proteins, 63 U/mg proteins and 70 U/mg proteins respectively (Fig. 4). On the other hand, SN50M failed to exert any amelioration effect (Fig. 4) Interestingly, amelioration capacity of non-toxic nimbidin was somewhat better than TB antibiotics like streptomycin, rifampicin or isoniazid (Fig. 4).

DISCUSSION

Previous studies show that infection of human alveolar macrophages with *M. tuberculosis* induce *M. tuberculosis* 85B and, TNF-α, both at mRNA and protein levels, which may be important to the immunopathogensis of disease (Islam *et al*., 2004). The initial interaction (both, phagocytic and non-phagocytic) of *M. tuberculosis* with mononuclear phagocytes gives rise to a cytokine profile that is dominated by TNF-α, a pro-inflammatory cytokine (Hasan *et al*., 2007, Lin *et al*., 1995, Means *et al*., 1999). TNF-α is present at the site of active *M. tuberculosis* infection in humans, regardless of the stage of mycobacterial infection (Hirsch *et al*., 2001). It is a pleiotropic cytokine, and its role in harmful or beneficial inflammatory processes is complex (Keane 2005). The expression of antigen 85B in *M. tuberculosis*-infected monocytes correlates positively with both the amount of secreted TNF-α and subsequent intracellular mycobacterial growth (Wilkinson *et al*., 2001). Attenuation of the biological activity of TNF-α has lately become an important therapeutic intervention in the management of a wide variety of chronic inflammatory diseases (Shanahan and Clair, 2002). However, clinical evidence indicates that neutralization of TNF-α is associated with an increased risk of opportunistic infections, including mycobacterial diseases (Dinarello, 2003). In view of this, modulation of TNF-α release is being proposed as the basis for novel therapeutic approaches (Warwick-Davies *et al*., 2001). Reports indicate that TNF antagonists namely infliximab and etanercept are effective in treating chronic inflammatory diseases by inhibiting TNF, but increase the risk of TB as a result of immunosuppression. Previous studies have shown that the risk of TB is greater in patients who received infliximab (Kim *et al*., 2009). Moreover, due to the clinical use of TNF antagonists is on increase, thus the incidence rate of TB may also increase. In view of it, it becomes mandatory that clinicians considering the use of TNF antagonists pay due attention to the prevention and control of TB and understand the mechanisms of action of the TNF antagonists (Kim *et al*., 2009). As mentioned above, since infliximab and other TNF antagonists-induced immunosuppression has been well documented, which in turn, increases the risk of TB, thus focus has now shifted to development of compounds from natural sources that have anti-oxidant and anti-mycobacterial activity, and devoid of any reported immunosuppression capability regarding tuberculosis. In light of the above, our study involves the incorporation of such a compound, namely, nimbidin from *Azadirachta indica* extract's, as the natural herbal component for tuberculosis management. The neem leaf exhibits immunomodulatory, anti-inflammatory, antibacterial, antiviral and antioxidant properties (Subapriya and Nagini, 2005). Furthermore, a neem compound azadirachtin has anti-tuberculosis, anti-viral and anti-bacterial property (Report of National Research Council, 1992). Based on these findings, we probed here the nimbidin-induced regulation of TNF-α in *M. tuberculosis*-infected human monocytes.

To the best of our understanding, we show for the first time at the molecular level potent anti-inflammatory effects exerted by nimbidin on host mononuclear cells infected with *M. tuberculosis* as evidenced by a strong inhibition of the pro-inflammatory cytokine TNF-α. It's well known that *M. tuberculosis* induces high amounts of TNF-α (Means *et al*., 1999), and the success of intracellular growth of virulent *M. tuberculosis* has been in part attributed to the capacity to induce TNF-α. Interestingly, all the concentrations of nimbidin employed in the study failed to show any type of adverse/toxic effect on the cell viability and human housekeeping genes like R18, thereby demonstrating that the effect of nimbidin was not mediated by cellular death. Appreciable decrease in endogenous TNF α mRNA was recorded with treatments of nimbidin respectively. The IC_{50} of TNF-α suppression in MTB-infected monocytes by nimbidin to the order of ~600 µg/ml and 350 µg/ml at the gene and protein levels respectively clearly shows the appreciable anti-inflammatory effect of nimbidin in bacilli infected cells. Furthermore, as described by us previously, cellular signaling by TNF-α is mediated mainly through activation of NF-κB (Hasan *et al*., 2006, Islam *et al*., 2004). It was apparent from our results that the induction of TNF-α expression in *M. tuberculosis*-infected monocytes was mediated through activation of NF-κB, because mRNAs was suppressed in cultures receiving SN50. On the contrary, the inactive analogue of SN50 failed to show any effect. Thus, our results show that cellular activation is associated with augmentation of expression of TNF-α in *M. tuberculosis*-infected monocytes, and that, it is in agreement with our previous study (Hasan *et al*., 2006, Islam *et al*., 2004). Since a number of genes involved in inflammatory responses are regulated by NF-κB pathway, thus a high magnitude downregulation of the NF-κB pathway by nimbidin would predictably reduce the elaboration of NF-κB-mediated TNF-α mRNA expression. In addition, nimbidin exerted a higher degree of neutralizing effects than NAC on TNF-α-induced actions in *M. tuberculosis*-infected human monocytes. The exact mechanism underlying the antioxidant activity of nimbidin in MTB-infected monocytes still remains poorly understood. Anti-oxidant-induced enhancement of glutathione peroxidase activity has been reported (Brown *et al*., 1995, Perchellet *et al*., 1986). Decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanisms (Mesiter and Anderson, 1986). Here, we observed a decline in GPx activity in *M. tuberculosis*-infected monocytes that were untreated or treated either with NADPH or SN50/M, thereby concurring with earlier reports that substantial amounts of ROS are being generated in cells infected with *M. tuberculosis* due to cellular activation (Islam *et al*., 2004). Enhancement or amelioration of GPx activity in *M. tuberculosis*-infected monocyte cultures after addition of NAC, a precursor of the *in vivo* antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms (Mesiter and Anderson, 1986). Interestingly, the GPx activity was found to be further enhanced /augmented when various doses of nimbidin were co-cultured instead of NAC, thereby inferring nimbidin to be an effective natural antioxidant combating ROS which was generated due to stimulation by TNF-α of cellular activation in *M. tuberculosis*-infected mononuclear phagocytes. In continuation to the above, the nimbidin-induced suppression of TNF-α at both the gene and protein levels is indicative for the anti-inflammatory and elicitation of protective immune response properties of nimbidin. Furthermore, tuberculosis (TB) is the commonest co-infection among HIV-1-infected subjects worldwide, and its adverse impact on progression of HIV-1 disease is well documented (Mayanja-Kizza *et al*., 2009), thus, based on the results obtained, it appears strongly that nimbidin could be a valuable future adjunct in the management of tuberculosis. However, further in-depth investigations are required at the molecular level with nimbidin. Also, the present study provides a ray of hope for the use of unexplored sources of plants and lead optimization strategies that may also improve the efficiency of future anti-TB drug discovery as indicated by Gupta *et al*, 2017.

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