Discussion
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Tuberculosis is perhaps the most persistent human disease caused by an infectious bacterium, *Mycobacterium tuberculosis* from an ancient time. The death toll remains extremely high despite the introduction of modern multidrug chemotherapy in the 1960s and it was about 1.6 to 2 million fatalities annually. An increasing percentage of human clinical isolates which include drug-resistant or multidrug-resistant strains that worsen the ability to treat the disease in present scenario. According to the WHO report on Global TB control, there were 1.6 million deaths and 8.2 million new cases of tuberculosis globally in year 2006 (WHO Report 2009). Despite the global TB control efforts by WHO, the battle against this scourge of humanity is far from over and it constitutes serious challenge to human health irrespective of socio-economic barriers. Physiological peculiarities of tubercle bacillus, like ability to resist many antimicrobial agents and to persist in human tissues for infinite time periods make this pathogen the most successful parasite on this planet. Resurgence of TB despite the availability of effective chemotherapy for over half century has been attributed to the drug resistance and its deadly synergy with the HIV. Anti-tuberculosis drugs are a two-edged sword. On one side these drugs destroy *M. tuberculosis* while on other hand responsible for emergence of drug resistant bacteria against which same drugs become ineffective. Global surveillance has shown that the drug resistant tuberculosis is widespread and is now a threat to tuberculosis control programs in many countries.

Rifampicin is the backbone of modern anti-TB chemotherapy by virtue of being active against *M. tuberculosis* in exponential growth phase as well as possessing activity against nonreplicating persistent bacilli (Wayne and Hayes 1996). Rifampicin inhibits β subunit of DNA dependent RNA polymerase of *rpoB* gene. Any mutation in *rpoB* gene leads to rifampicin resistance, although mutations other than *rpoB* gene were also reported leading to rifampicin resistance (Ohno et al. 1996). Its standard dose in TB treatment is 10 mg/kg of body weight corresponding to 600 mg in most populations (Boogaard et al. 2009). But its short half life ($t_{1/2}$) of 3 h allow short periods of *M. tuberculosis* killing followed by the reoccurrence of bacterium between the doses.

Now a days, several medicinal herbs and natural molecules are used being seriously looked upon to combat many critical diseases and to treat nuisance arise due to drug resistance by virtue of their ability to promote host immunity. Plants such as *Viscum album*, *Tinospora cordifolia* and *Withania somnifera* have been reported for their
immunomodulatory activity (Kapoor 2000). Plant molecules are also responsible in modulating the drug resistance through inhibition of efflux mechanism of the mammalian and bacterial cells. Reserpine (Nefakh et al. 1991) epi catechin gallate (Gibbons et al. 2004) carnosic acid and carnosol (Oluwatuyi et al. 2004) are such plant derived molecules responsible for potentiating the effect of antibiotics through inhibition of bacterial efflux pumps (Stavri et al. 2007). In the same way, Piper longum an important medicinal plant is widely used in traditional medicine by many people in Asia and Pacific islands especially in Indian ayurvedic medicine. Piperine, a trans-trans isomer of 1-piperoyl-piperidine isolated from black pepper, is an inhibitor of human p-glycoprotein and CYP3A4 (Singh et al. 1986). It is also reported as a bioavailability enhancer and has potential immunomodulatory activity (Atal et al. 1981, 1985; Sunila and Kuttan 2004). Recently piperine has been reported as an inhibitor of NorA efflux pump of Staphylococcus aureus (Khan et al. 2006; Kumar et al. 2008).

The present study evaluates the role of piperine as a bioefficacy enhancer of rifampicin against M. tuberculosis infection. The preliminary in-vitro studies revealed that piperine does not have any antibacterial activity; however it potentiated the activity of rifampicin. It was evident from four to eight fold reduction in its MIC against susceptible as well as resistant M. tuberculosis. Rifampicin showed concentration dependent killing of M. tuberculosis during time kill studies. A combination of rifampicin (0.5 μg/ml) and piperine (25 μg/ml) exhibited bactericidal activity and showed more than 3-log reduction in CFU/ml in 8 days, whereas rifampicin alone exhibited the same effect at a higher concentration of 1 μg/ml.

Mutant prevention concentration (MPC) has been proposed as a new measure of antibiotic potency by which the ability to restrict selection of resistant mutants is evaluated. To be clinically useful, the MPC must be below the Cmax in serum or tissue at the site of infection. Thus, use of a combination of MPC and pharmacokinetic parameters provides a way to compare antibacterial agents for their potential ability to restrict the selection of resistant mutants (Sindelar et al. 2000). The most significant finding of this study is the ability of piperine to prevent the emergence of rifampicin resistant mutants at a clinically achievable concentration of rifampicin (2 μg/ml). These results are of significant importance because rifampicin’s monotherapy in M. tuberculosis leads to emergence of drug resistance (Gumbo et al. 2007) as the reported MPC of rifampicin against M. tuberculosis is >80 μg/ml (Dong et al. 2000).
Another interesting aspect of study was the extended PAE of rifampicin when tested in combination with piperine. The PAE of a drug against *M. tuberculosis* might be a putative pharmacodynamic parameter that would be of huge help in the design of an optimal dosing schedule for an antimicrobial agent (Vogelman *et al.* 1988). Previous studies showed rifampicin’s prolonged PAE but there is always a difference in PAE due to its variable $C_{\text{max}}$ and also PAE less than 24 h is insignificant for anti-tuberculosis drugs since most of the chemotherapeutic regimens for tuberculosis are given daily or on alternate days. (Chiu *et al.* 2001) In the present study rifampicin exhibited a concentration dependent PAE of 24 h, 48 h and 72 h in presence of piperine at 25 µg/ml. Accumulation and efflux of ethidium bromide is a good indicator of involvement of efflux pumps in the resistance mechanisms in the bacteria, particularly in the Gram positive bacteria such as *Staphylococcus aureus* (Khan *et al.* 2006; Kumar *et al.* 2008). However, uptake of ethidium bromide by slow-growing mycobacteria is very sluggish (Mailaender *et al.* 2004) and interpretation of the uptake experiments is complicated by the high amount of surface-adsorbed compounds (Danilchanka *et al.* 2008). This problem is even more pronounced for antibiotics which are the substrates of drug efflux pumps (De Rossi *et al.* 2006). Since efflux is the only known mechanism of resistance for ethidium bromide, reversal in MIC of ethidium bromide by any substance is an indication that the substance probably working as an inhibitor of bacterial efflux pumps (Brenwald *et al.* 1998; Jones *et al.* 2003). It was observed that there was an increase in the MIC of ethidium bromide in the *M. tuberculosis* rif (rifampicin resistant mutant) and piperine reversed the MIC of ethidium bromide thus approving the role of piperine as an efflux pump inhibitor in *M. tuberculosis*. Drug efflux pumps are major contributors to drug resistance in human pathogens and cancer cells. Drug resistance in bacteria including some species of *M. tuberculosis* has been associated with membrane-located efflux pumps that prevent cytosolic accumulation of drugs. The genome sequence analysis of *M. tuberculosis* H37Rv has revealed 20 Open Reading Frames as putative efflux pumps (Cole *et al.* 1998). One of the ORF designated as Rv1258c has been reported to be overexpressed under rifampicin pressure as well as in the clinical isolates resistant to rifampicin (Jiang *et al.* 2008; Siddiqi *et al.* 2004). In this study also qRT-PCR analysis of putative efflux pump (Rv1258c) in *M. tuberculosis* rif showed 3.6-fold increase in the transcription level of this gene as compared to that in *M. tuberculosis* H37Rv (wild type) which relates to the findings of earlier investigators.
Since piperine could not restore the initial MIC of rifampicin in *M. tuberculosis* rif to the value of MIC in *M. tuberculosis* H37Rv (wild type). We investigated the mutations in *rpoB* gene which is the target site of rifampicin. It was reported previously that 80% of mutations occurs at 531 (TCG-TTG, Ser-Leu) base pair involved in high level of resistance (Valle et al. 2001). We also found the previously reported mutation in *M. tuberculosis* rif. Similar mutation was also found in the clinical isolates of *M. tuberculosis* as shown in results (Table 4.8).

As the crystal structure information is not available of Rv1258c, 3-D structure of this protein was predicted based on homology modelling using in-silico approach. The docking studies revealed one of the binding pockets, having greater affinity for piperine and reserpine. Within this active site, piperine also showed H-bonding with Arg-141 among several other amino acid residues involved in interactions and this involvement of Arg-141 apparently seems to be contributing to the inhibitory activity of piperine. In brief, the in silico docking experiments supported our in-vitro results and suggestive role of piperine as inhibitor of mycobacterial putative efflux pump (Rv1258c). Piperine’s role as an inhibitor of NorA efflux pump in *S. aureus* has been established. (Khan et al. 2006; Kumar et al. 2008). To our knowledge, this is the first attempt to study the sensitivity of a *Mycobacterium tuberculosis* efflux protein to piperine.

The other aspect of the study was to evaluate the immunomodulatory effect of piperine. The in-vitro results revealed that piperine at 1 μg/ml concentration, increased the T cell proliferation and its differentiation into Th1 sub-population. This polarization of T cells into the Th1 cells was accompanied by secretion of Th1 like cytokines such as IFN-γ and IL-2 by these cells. The overall effect of these cytokines is to recruit macrophages into the inflammatory site and promote phagocytic activity. Since T-cells play an important role in regulating the immune responses by being responsible for cell mediated immunity in a balanced immune system. Piperine was further tested in-vivo at a dose of 10 mg/kg in mice infected with *M. tuberculosis*. It activated and differentiated T cells into a Th1 sub-population as evident from the increased population of CD4⁺/CD8⁺ lymphocyte sub population. There was subsequent increase in the secretion of Th1 cytokines such as IFN-γ and IL-2 by these cells. However, there was no effect on Th2 cytokines such as IL-4. The Th1 cytokines are associated with the generation of cell-mediated immunity and resistance to intracellular parasites, whereas Th2 cytokines favour the induction of humoral immunity and resistance to extracellular parasites (Mosmann and Sad 1996;
Stevens et al. 1988). IL-2 stimulates NK-cell proliferation and activation for the production of IFN-γ (He et al. 2004). IFN-γ is responsible for recruiting macrophages into the inflammatory site and promotes phagocytic activity. The protective role of IFN-γ in *M. tuberculosis* or *Mycobacterium leprae* infection is well established and it has been effectively demonstrated that IFN-γ knockout mice are more susceptible to *M. tuberculosis* (Condos et al. 1997; Kaplan et al. 1989; Xing et al. 2001). Probably due to this Th1 cytokines mediated protection by piperine, the proliferation of infection was controlled and there was no increase in bacterial CFU load (Figure 14.4). The increase in the level of Th1 cytokines was related to the corresponding increase in the mRNA transcripts of IFN-γ and IL-2 (with no increase in IL-4 mRNA transcript) in the lung tissues of the infected mice. The light microscopy of the sections of lung of the animal group treated with pipeline 10 mg/kg showed well formed epitheloid cell granuloma.

In order to investigate the effect of pipeline on the therapeutic outcome of rifampicin treatment, we treated the *M. tuberculosis* infected mice with the combination of pipeline and rifampicin. There was marked improvement in the therapeutic outcome of rifampicin (10 mg/kg) when combined with piperine at (10 mg/kg). The most important finding of this study was that the combination of higher dose of rifampicin (20 mg/kg) and piperine reduced the bacterial load in lungs below the detection limit within 4 weeks, whereas rifampicin alone at the same dose could achieve it in 8 weeks (Table 4.9). These findings indicate that such a combination could potentially reduce the treatment time of this infection.

The immunomodulatory properties of piperine are little reported. There has been one report on the immunomodulatory and anti-cancer properties of piperine in tumor bearing mice. The immunomodulatory potential of piperine was measured through haematological parameters, increase in bone marrow cellularity, increased antibody titer and increased plaque forming cells in spleen (Sunila and Kuttan 2004). Piperine with long history of medicinal use has been experimentally evaluated for its multiple biological activities such as anti-metastatic, anti inflammatory, heptoprotective and antithyroid (Koul and Kapli 1993; Pradeep and Kuttan 2002). Piperine favourly respond to splenocytes comprising of both B and T cells (Pathak and Kandelwal 2007a). Furthermore piperine also mitigate the adverse effect of cadmium on cytokines(IL-2, IFN-γ) and cell proliferative mitogenic response (Pathak and Khandelwal 2007b). In our study, we have for the first time reported the detailed immunomodulatory activity of
piperine with reference to its protective immunity mediated through Th1 cytokines and its enhancement of the activity of rifampicin. Piperine in combination with rifampicin might improve its therapeutic efficacy in immuno-compromised TB patients.