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Changes in signal cross-talking in *Mycobacterium smegmatis* infected murine macrophages in presence of aqueous garlic extract

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Abstract

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis*, still continues to be the biggest infectious threat to mankind. The present havoc due to global resurgence of TB, and the emergence of multi-drug resistant (MDR)-TB in India makes the search for alternative anti-tubercular phytomedicines an absolute mandate. As a fresh preparation of crude aqueous garlic extract (AGE) was earlier found to be bactericidal against *M. smegmatis* having similar cell-wall structure-composition as the pathogenic *M. tuberculosis* at a minimal bactericidal concentration (MBC) of 60 mg/ml, and the same confirmed by biochemical assays and micro-RNA (mi-RNA) expression studies, the present work involves immuno-molecular studies on the differential expression of NF- κ B, a transcription factor involved in cellular immunobiological responses to foreign stimuli such as bacterial and viral antigens, in murine peritoneal-macrophages infected respectively with untreated, alive *M. smegmatis* (positive control) and *M. smegmatis* treated with crude AGE (at 60 mg/ml) (experimental) by SDS PAGE-Western Blot techniques. Significantly, a prominent band of NF- κ B was observed in the lane of macrophages infected with untreated, alive *M. smegmatis*, indicating up-regulation of NF- κ B gene expression there following the pathogen invasion, as compared to the macrophages infected with crude AGE-treated (at 60 mg/ml) *M. smegmatis*, thus confirming the mycobactericidal potency of aqueous garlic extract. Data obtained out of biochemical assays carried out for the determination of specific activities of inducible nitric oxide synthase (iNOS) and superoxide dismutase (SOD) enzymes from the same set of macrophage samples, which showed prominent induction of these two enzymes in case of macrophages infected with untreated, alive *M. smegmatis* with respect to macrophages infected with crude AGE-treated (at 60 mg/ml) *M. smegmatis*, also substantiated the same.

Keywords: aqueous garlic extract, *Mycobacterium smegmatis*, NF- κ B, SDS PAGE-Western Blot, inducible nitric oxide synthase, superoxide dismutase

1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, a small, aerobic, non-motile bacillus. It is the second-most common cause of mortality (after those due to AIDS) [1]. Roughly one-third of the global population is presently infected with TB [2], and every year, new infections crop up in at least 1% of the population [3]. According to the WHO report of 2015 on TB, an estimated 9.6 million people across the world developed active TB in 2014, which resulted in 1.5 million deaths (in 1.1 million HIV-negative and 0.4 million HIV-positive patients) [4]. In 2014, there were a slightly more than 6 million new TB cases worldwide (an increase of 6% from about 5.7 million in 2013) [4]. Also, in 2014, 3,59,000 new and relapse cases among children were reported, an increase of about 30% compared with 2013 [4]. Worldwide, 22 high-burden countries (HBCs) together experience 80% of TB cases, as well as 83% of deaths arising out of it [4, 5].

A number of risk factors make people vulnerable to TB infections, the most important of which is HIV-infection [6]. TB is also closely linked to both overcrowding and malnutrition [7], situations very common in India - a lower-middle-income country, where 75% of the poor live in rural areas, under conditions of poor sanitation, minimum of hygiene and overwhelming undernourishment [8]. The WHO report of 2015 on TB says that India is one of the HBCs of the world with regard to the number of TB patients, which was 23% of the global total in 2014 [4]. Out of a total population of 1295,292,000 in India, in 2014, there were 2,200,000 total cases of TB (about 40% of the Indian population was infected with the

TB bacteria, the vast majority of whom have latent, rather than active TB), including 1,10,000 HIV-positive cases of TB, accounting for a mortality of 2,20,000 TB-patients and 31,000 HIV-positive TB patients [4, 9]. Alarming, in 2014, public sector medical college hospitals in India alone reported 1,76,000 TB cases [4]. The number of detected TB cases in India increased from 23,162 in 2013 to 25,748 in 2014 [4]. The largest increases in the new and relapse TB cases among children were also reported from India (about 30,000 out of 3,59,000 global cases) [4].

According to data from the government Revised National Tuberculosis Control Programme (RNTCP), out of a total population of 94,200,000 in West Bengal, 59,683 people have been diagnosed as smear-positive for TB in 2014 [9].

Although the number of TB deaths is unacceptably high, with a timely diagnosis and correct treatment, almost all people with drug susceptible (DS)-TB can be cured. The currently approved medication for new cases of DS-TB includes a six-month regimen of the four first-line drugs – isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) [10]. However, globally, an estimated 3.3% of new cases and 20% of previously treated cases had multi-drug resistant-TB (MDR-TB, which is defined to be caused by the TB bacteria that have developed resistance against INH and EMB - the two most powerful anti-tubercular drugs) in 2014 [4]. A shocking 4,80,000 new global cases of MDR-TB occurred in 2014 [4]. Out of these, an estimated 1,90,000 people died out of MDR-TB in 2014 alone [4].

Alarming, India ranks at the top among the 27 high MDR-TB burden countries of the world. In fact, the number of MDR-TB patients quadrupled in India in 2012 [11]. In 2014, in India, the estimated new TB cases with MDR-TB were 2.2%, while the estimated re-treatment TB cases with MDR-TB were 15% of their respective totals. The estimated MDR-TB among notified pulmonary TB cases in India was 71,000 (24,000 new cases and 47,000 re-treatment cases), and confirmed number of notified rifampicin-resistant/multi-drug resistant tuberculosis (RR/MDR-TB) cases were 25,748 (36% of the total estimated pulmonary RR/MDR-TB cases) in 2014 [4]. Indiscriminate use of medicine, over-the-counter sale, truncation of course, inadequate dose and natural resistance to drugs have all been conducive to the explosion of these MDR-strains of *M. tuberculosis* here.

Treatment of such DR-TB cases has become complicated and severely challenged in India. This is because, management of DR-TB requires intense chemotherapy, which consists of treatment with multiple antimicrobials over a span of 20 months [4]. This may cause poor compliance and high treatment drop-out [4]. Most of these medicines also carry risks of side-effects like hepatitis, hypersensitivity reactions, nausea and vomiting.

Hence, in recent times, there is being noticed an increased quest for alternative herbal drugs against MDR-TB. According to recent WHO reports, there are 2,50,000 higher plant species on earth, out of which 35,000 to 70,000 species have been used for medicinal purposes [12]. Herbal therapy that involves the use of medicinal plants for prevention and treatment of diseases, ranges from traditional and popular medicines of every country to the use of standardized and titrated herbal extracts.

India is a land of rich herbal heritage. So, Indian medicinal plants may be a possible source of novel mycobactericidal drugs, to which the deadly MDR-*M. tuberculosis* strains are not yet resistant. Medicinal plants mentioned in the *Ayurveda* (Indian traditional medicine system) and in Greek medicinal texts ages ago have thus been the matter of exhaustive studies, experimentation and clinical trials over the last couple of years. Crude plant extracts as antimicrobials are always a popular choice, since resistance in *M. tuberculosis* would not be easy for a wide array of phytochemicals present together.

Since ages, garlic (*Allium sativum*) is enjoying a high reputation as a medicinal herb. This clinical use of garlic is documented in the *Ayurveda* also. It is a cheap and regularly-used vegetable in India, which is either made into a paste, or chewed raw. The garlic plant tissues are severely damaged by both of these activities, releasing afresh its chief antimicrobial chemical – allicin ($C_6H_{10}OS_2$), an organo-sulfur compound of the name diallyl thiosulfinate.

In a freshly-prepared crude aqueous garlic extract (AGE), allicin remains stable only for about 16 hours [13]. When incubated with crude AGE, several Gram-negative and Gram-positive bacteria, fungi, protozoa and viruses are killed within this period of time [14].

So, in the current study, we have tried to confirm the effectiveness of a herbal preparation based on garlic in controlling high rates of MDR-TB in India. The same research group have earlier shown that freshly-prepared crude AGE can be potentially bactericidal against *M. smegmatis*, the bacterium sharing similar cell-wall structure-composition as the pathogenic *M. tuberculosis*, at a minimal bactericidal concentration (MBC) of 60 mg/ml [15].

Macrophage-mediated innate immune response functions as the first line of host defense against *Mycobacterium* – an intracellular pathogen. So, in order to substantiate the mycobactericidal potency of crude AGE through immunological studies, the aim of the present research work was to use immuno-molecular tools like sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)-Western Blot (Immunoblot) in order to show that expression of nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells or, NF- κ B - a transcription factor involved in cellular immunobiological responses to foreign stimuli such as bacterial and viral antigens, is up-regulated in macrophages by untreated, alive *M. smegmatis* infection as a part of its defense mechanism, in comparison to macrophages infected with crude AGE-treated (at its MBC of 60 mg/ml) *M. smegmatis*.

The immunological basis of this mode of macrophage defense through NF- κ B includes complex cell-signalling cascades [16]. These may, in turn, recruit interleukin-1 receptor-associated kinase (IRAK) 2 and 4, leading to the activation of NF- κ B [16]. This activation has numerous consequences, including the production of inflammatory mediators, regulation of phagocytosis, and connecting the phagosomal pathway with autophagosomes, thereby inducing rapid mycobacterial killing within infected macrophages [16]. So, present studies on differential expression of NF- κ B in case of macrophages infected with untreated, alive *M. smegmatis* (positive control) and crude AGE-treated (at 60 mg/ml) *M. smegmatis* (experimental), when compared with only macrophages (negative control), were expected to validate the mycobactericidal efficacy of crude AGE.

Macrophages can phagocytose and eliminate intracellular *Mycobacterium* by multiple bactericidal mechanisms, including generation of bactericidal free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), in the process activating programmed cell death (PCD) [17]. Following activation, mediated by receptors like TLRs or, exposure to appropriate proinflammatory cytokines like IL-1, TNF- α and IFN- γ , macrophages express high levels of inducible nitric oxide synthase enzyme (*i*NOS or, NOS2) or, cytokine-inducible NOS [18, 19]. *i*NOS is a type of enzyme in the family of nitric oxide synthases (NOSs) [19]. NOSs (EC 1.14.13.39) produce nitric oxide (NO), a potential RNS, from L-arginine, according to the stoichiometry: L-arginine + 3/2 NADPH + 1/2 H⁺ + 2O₂ \rightleftharpoons L-citrulline + NO + 2H₂O + 3/2 NADP⁺ [19]. As opposed to the critical calcium-dependent regulation of constitutive NOS (*c*NOS) enzymes (neuronal NOS or, *n*NOS and endothelial NOS or, *e*NOS), the inducible isoform *i*NOS is calcium-insensitive. The *i*NOS promoter is activated by interferon regulatory factor 1 (IRF1) and NF- κ B [19]. Upon antigenic stimulation, *i*NOS produces large amounts of NO as its defense [18, 19]. It is synthesized by many cell types in response to cytokines and is an important factor in the response of the body to attack by parasites (both protozoa and worms), bacterial and fungal infections, and tumorigenic growth [19]. As the induction of the high-output *i*NOS usually occurs in an oxidative environment, as a part of the oxidative burst of macrophages against the invading pathogen, NO reacts with superoxide radicals (O₂⁻), a ROS, leading to the formation of the lethal RNS - peroxynitrite (ONOO⁻) [18, 19].

Because of the important role played by NO in immune defense mechanisms in mammals, we wish to study, with the help of biochemical assay, the differential specific activity levels of *i*NOS enzyme in macrophages under different situations of infections - by untreated, alive *M. smegmatis* and crude AGE-treated (at 60 mg/ml) *M. smegmatis*, against a minimum induction in case of only macrophages (negative control, NC). From the assay results, when the infectious agents are alive, and when incubated with crude AGE, we expect an alteration in *i*NOS activities. Such results are also expected to corroborate with the results of the SDS PAGE-Western Blot analyses of NF- κ B expressions from the same set of samples. The results should provide some clues as to the efficiency of crude AGE as a potent mycobactericidal agent.

Superoxide radical (O₂⁻), a by-product of oxygen metabolism, is one of the main toxic ROS generated due to the respiratory burst within the macrophages [18]. Superoxide dismutase (SOD, EC 1.15.1.1) enzymes alternately catalyze the dismutation of the O₂⁻ into either ordinary molecular oxygen (O₂) or hydrogen peroxide (H₂O₂), thus detoxifying the O₂⁻ into one of these two less damaging species [18, 20]. H₂O₂ generated is also damaging, but is degraded by other enzymes such as catalase [18, 20].

Hence, through biochemical assay, we expect a marked induction of SOD, the enzyme responsible for removal of O₂⁻, and a consequent enhancement in its specific activity as a survival strategy of infectious agents, like untreated, alive *M. smegmatis*, when compared against minimum specific activity values in case of crude AGE-treated (at 60 mg/ml) *M. smegmatis* and only macrophages (negative control, NC). The results would help us to confirm the

antitubercular potency of crude AGE against *M. smegmatis*, and in turn against *M. tuberculosis*.

2. Materials and Methods

2.1 Chemicals, Reagents, Media and Antibodies

All chemicals, reagents, media and antibodies (primary and enzyme-conjugated secondary) used in this study were purchased from Merck Specialities Private Limited, India and Sisco Research Laboratories Private Limited, India. All dehydrated media, media base and supplements were procured from HiMedia Laboratories, India.

2.2 Equipment

The electrophoresis and transfer equipment for SDS-PAGE and Western Blot were procured from Bio Rad Laboratories India Pvt. Ltd.

2.3 Bacteria and Culture Conditions

Pure culture of *M. smegmatis* mc²⁶ strain used in the study was obtained from the Microbiology Department, Bose Institute, Kolkata. It was allowed to grow in Middlebrook 7H9 (MB7H9) broth, supplemented with 0.5% glycerol and 0.25% Bovine Serum Albumin (BSA) for 24 hours at 37 °C with continuous shaking [21].

2.4 Experimental animal

This study was carried out using 4-6 weeks-old, healthy male BALB/c mice of 16±2 g weight, obtained from Indian Institute of Chemical Biology (IICB), Kolkata. All experimental animals were maintained in cages on standard mice chow and water *ad libitum* in a climate-controlled, light-regulated animal house with 12-hour alternating light and dark cycles. For one week before experimentation, the animals were acclimatized at a room temperature of 25-28 °C under a 12 hour light/dark cycle and 50-70% relative humidity in the animal house. Ethical Approval regarding the use was given by the Institutional Animal Ethics Committee, University of Calcutta (Registration no. 885/ac/05/CPCSEA), registered under "Committee for the Purpose of Control and Supervision of Experiments on Laboratory Animals" (CPCSEA), Ministry of Environment and Forests, Government of India.

2.5 Preparation of crude AGE

Fresh bulbs of garlic (*Allium sativum*) purchased from a local daily market were divided into separate cloves. The cloves were peeled to obtain edible portions, 6 grams of them weighed and minced properly in cold, and homogenized in a sterilized glass homogenizer using 100 ml of autoclaved, cold distilled water for 1-2 minutes in cold [22]. The homogenate was then centrifuged in a high speed Cold Centrifuge at 3000 rpm for 5 minutes to remove any debris [22]. This homogeneous suspension of crude AGE stock (60 mg/ml) was collected in a sterile vial and stored at 4 °C (for only a maximum of 16 hours), till being serially diluted for the work [13, 22].

2.6 Immunobiological studies on the differential expression of NF- κ B using SDS PAGE-Western Blot (Immunoblot)

Immunobiological studies were carried out using SDS PAGE-Western Blot (Immunoblot) to look for the differential expression of NF- κ B in murine peritoneal

macrophages, with an aim to confirm the mycobactericidal potency of crude AGE at its MBC of 60 mg/ml.

2.6.1 Sample preparation

Healthy BALB/c mice were intra-peritoneally injected with 1 ml 1% sterile starch solution, and maintained properly in the animal house for 4 days. Then they were sacrificed using mild chloroform anaesthesia, and their peritoneal monocytes were isolated by peritoneal lavage and infusing the peritoneal cavity with ice-cold sterile phosphate-buffered saline (PBS), pH 7.4. The monocytes were then added to DMEM high glucose medium (Dulbecco's Modified Eagle's Medium) in a 6-well adherent plate at a concentration of 3 million cells/well, and cultured in a 5% CO₂ incubator at 37 °C for 48 hours to allow for the differentiation of monocytes into macrophages. Post-differentiation, the viscous layer from the top of four wells was discarded (leaving two wells undisturbed), and the adherent monolayer of macrophages washed with normal PBS, pH 7.4. Two sets, in duplicates, were prepared – one for macrophages infected with untreated, alive *M. smegmatis* (positive control) and the other for macrophages infected with crude AGE-treated (at its MBC of 60 mg/ml) *M. smegmatis* (experimental). For the positive control and experimental sets, infections with untreated, alive *M. smegmatis* and crude AGE-treated (at its MBC of 60 mg/ml, added in a AGE: bacteria ratio of 1:1, and pre-incubated for 12 hours at 37 °C in a 5% CO₂ incubator) *M. smegmatis* were given to the macrophages in their respective wells in the ratio of macrophage: bacteria 1:10, and incubated for 12 hours at 37 °C in a 5% CO₂ incubator. Following incubation, the top viscous layers were again discarded, and the respective macrophage monolayers were re-washed with ice-cold PBS, pH 7.4. Then the adherent cell population of each of the wells were scraped, transferred to sterile Eppendorf tubes, and the step repeated twice [23]. Following centrifugation at 320 rpm for 15 minutes at 4 °C, the respective cells were re-suspended in ice-cold extraction buffer containing 50 mM Tris-HCl, pH-7.5, 50 mM EGTA, anti-protease mixture consisting of 0.33 mM leupeptin, 0.2 mM phenyl methyl sulfonyl fluoride (PMSF), 0.35 mM antipain, 0.24 mg of chymostatin/ml, 0.35 mM pepstatin, and 4.8 trypsin inhibitor units of aprotinin/ml, and 50 mM β-mercaptoethanol. The respective macrophage-containing suspensions were sonicated at 4 °C and centrifuged at 3400 rpm for 10 minutes at 4 °C to remove the nuclear fraction. The supernatants obtained were used for spectrophotometric (750 nm) quantification of protein contents by the standard Folin-Ciocalteu method, before performing SDS PAGE-Western Blot with them [23, 24, 25]. Bovine serum albumin (BSA) was used as the standard in this assay. The same steps, excepting pre-infection with bacteria, were followed for a third set, also prepared in duplicate in the two remaining wells of the 6-well adherent plate, for only macrophages (negative control).

2.6.2 SDS PAGE-Western Blot (Immunoblot)

Western Blot (Immunoblot) to study the differential expression of NF-κB was performed with the respective samples after addition of sample buffer to them, by following the standard protocol of SDS-10% PAGE to separate the proteins in the macrophage-lysates, transferring (blotting) to nitrocellulose (NC) membrane, blocking the NC membrane overnight with 3% BSA in Tris-saline buffer,

pH 7.5, treating it with primary antibody against NF-κB and the corresponding enzyme (alkaline phosphatase, AP)-conjugated secondary antibody, and visualizing immunoreactive bands after developing color in the NC membrane using NBT (nitro-blue tetrazolium chloride) - BCIP (5-bromo-4-chloro-3'-indolyl phosphate p-toluidine salt) as a chromogenic substrate for AP [24].

2.7 Biochemical assays to determine specific activities of iNOS-SOD enzymes

2.7.1 Determination of specific activity of iNOS enzyme

The assay was carried out according to the standard protocol, with minor modifications [26]. The substrate for the enzyme iNOS is 0.5 mM arginine. In this method, the respective macrophage-lysates acting as the source of the enzyme, were added directly to the cuvettes, each of which contained 100 mM MgCl₂, 100 mM Na⁺/K⁺ ferrocyanide and 100 mM HEPES, pH 7.4, along with the substrate. Following 3 seconds, the decrease in optical density (OD) reading at 420 nm was noted down, against a suitable Blank [27]. From the data obtained in this assay, and from the protein contents of the respective lysates determined spectrophotometrically at 750 nm, the specific activity of iNOS enzyme was calculated.

2.7.2 Determination of specific activity of SOD enzyme

The SOD assay was performed using the pyrogallol autoxidation method, after slight modifications [28, 29]. This simple, rapid, reliable and reproducible quantification method was performed with 2.6 mM pyrogallol in 10 mM HCl, in presence of 80 mM EDTA and 100 mM phosphate buffer, pH 8.5. After the addition of the respective macrophage-lysates acting as the source of the enzyme directly to the cuvettes, a lag period of 2 minutes was given to allow the steady state of oxidation of pyrogallol to be attained before taking the initial reading. The increase in OD reading was determined spectrophotometrically at 420 nm [27]. From the data obtained in this assay, and from the protein contents of the respective lysates determined spectrophotometrically at 750 nm, the specific activity of SOD enzyme was calculated.

3. Results and Discussion

3.1 SDS PAGE-Western Blot (Immunoblot)

Colorimetric detection in Western Blot depends on incubation of the NC membrane with a chromogenic substrate that reacts with the enzyme bound to the secondary antibody, which is in turn bound specifically to the primary antibody against the antigenic protein to be detected. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme, and stains the NC membrane at that particular area.

In our Western Blot analysis, the BCIP substrate was hydrolyzed by the AP conjugated to the secondary antibody to form an indoxyl intermediate. Then tautomerization occurred, which under alkaline conditions, resulted in dimerization to produce a dehydroindigo dimer product (5,5'-dibromo-4,4'-dichloro-indigo-white). The NBT was reduced to the insoluble NBT-formazan end product by the two reducing equivalents (hydrogen ions) generated by this dimerization. The resulting NBT-formazan was purple in color, allowing the easy detection of the immunoreactive bands on the membrane (Fig. 1).

Practically no NF-κB expression was found in case of only murine peritoneal macrophages (negative control). NF-κB expression was distinctly up-regulated in case of macrophages infected with untreated, alive *M. smegmatis* (positive control) as a part of its defense mechanism against the intracellular bacteria (NF-κB activates *i*NOS promoter), whereas NF-κB expression was negligible in case of macrophages infected with *M. smegmatis* treated with crude AGE (at its MBC of 60 mg/ml, experimental), confirming that crude AGE had killed *M. smegmatis*, as a result of which there was no effect of external stimulus, in the form of mycobacterial antigens, on macrophages.

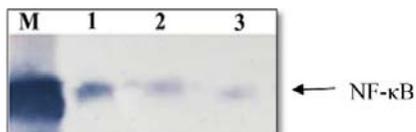


Fig 1: Detection of NF-κB by SDS PAGE-Western Blot. SDS PAGE-Western blot analysis of lysates of murine peritoneal macrophages shows prominent expression of NF-κB in macrophages infected with untreated, alive *M. smegmatis* (positive control, Lane-1), negligible in macrophages infected with *M. smegmatis* treated with crude AGE (at its MBC of 60 mg/ml) (experimental, Lane-2) and practically nil in case of only macrophages (negative control, Lane-3). M denotes the lane of standard protein marker (ladder). The blot represents the results of three independent experiments with identical results.

3.2 Determination of specific activity of *i*NOS enzyme

The results obtained in the biochemical assay for the determination of specific activity of *i*NOS enzyme from

murine peritoneal macrophages are summarized in Table 1. From the data obtained, it was observed that induction of *i*NOS enzyme had taken place appreciably in the set containing macrophages infected with untreated, alive *M. smegmatis* (0.147 units/sec/μg of protein), was negligible in macrophages infected with *M. smegmatis* treated with crude AGE at its MBC of 60 mg/ml (0.067 units/sec/μg of protein, which is 2.20 times and 54.42% lesser with respect to untreated, alive *M. smegmatis*-infected macrophages), and still lesser in case of only macrophages (negative control, NC, 0.051 units/sec/μg of protein) (Fig. 2).

The observation was in perfect accordance with the results of SDS PAGE-Western Blot analysis for the detection of NF-κB expression in the same 3 sets, which showed that the lysate of macrophage infected with untreated, alive *M. smegmatis* showed a prominent expression of NF-κB, which, in turn, had activated the *i*NOS promoter, accounting for a strong induction and subsequent good amount of specific activity of the *i*NOS enzyme in that set (0.147 units/sec/μg of protein). This is quite logical, as upon antigenic stimulation, *i*NOS produces NO in excess, that in turn, reacts with O₂⁻, forming peroxynitrite (ONOO⁻) as a response of the oxidative burst of macrophages to kill *Mycobacterium*.

As the specific activity of the *i*NOS enzyme (0.067 units/sec/μg of protein) was found to be much lesser (54.42%) in macrophages infected with *M. smegmatis* treated with crude AGE at 60 mg/ml, the data confirmed the mycobactericidal potency of crude AGE.

Table 1: Determination of specific activity of *i*NOS enzyme

Sl. No.	Samples	Specific activity of <i>i</i> NOS (units/sec/μg of protein)	Decrease with respect to (3) (times)	Decrease with respect to (3) (%)
(1)	Blank	0.000	-	-
(2)	Macrophage (negative control, NC)	0.051	-	-
(3)	Macrophage + <i>M. smegmatis</i>	0.147	-	-
(4)	Macrophage + <i>M. smegmatis</i> + AGE (60 mg/ml)	0.067	2.20	54.42

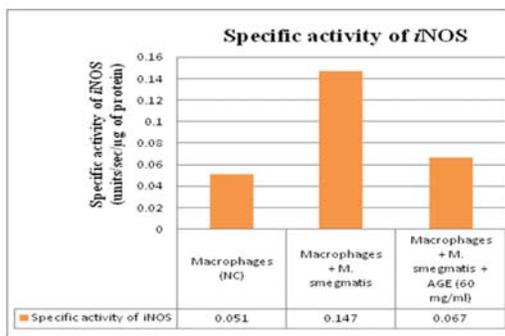


Fig 2: Comparative studies on the specific activities of *i*NOS enzyme from 3 different sets of lysates of murine peritoneal macrophages shows prominent specific activity of *i*NOS in macrophages infected with untreated, alive *M. smegmatis* (0.147 units/sec/μg of protein), negligible in macrophages infected with *M. smegmatis* treated with crude AGE (at its MBC of 60 mg/ml, 0.067 units/sec/μg of protein) and least in case of only macrophages (negative control, NC, 0.051 units/sec/μg of protein).

3.3 Determination of specific activity of SOD enzyme

The data obtained in the biochemical assay for the determination of specific activity of SOD enzyme from

murine peritoneal macrophages are summarized in Table 2. From the results obtained, it was seen that induction of SOD enzyme had been appreciable in case of macrophages infected with untreated, alive *M. smegmatis* (0.163 units/sec/μg of protein), negligible in macrophages infected with *M. smegmatis* treated with crude AGE at its MBC of 60 mg/ml (0.009 units/sec/μg of protein, which is 18.11 times and 94.48% lesser with respect to untreated, alive *M. smegmatis*-infected macrophages), and still lesser in case of only macrophages (negative control, NC, 0.008 units/sec/μg of protein) (Fig. 3). This is very important, as it justifies that induction of SOD enzyme has been done by AGE-untreated, alive *M. smegmatis* so as to detoxify the O₂⁻ generated as a result of respiratory burst in the macrophages, so as to prevent NO reacting with it and forming lethal ONOO⁻. Specific activity of the same drops as *M. smegmatis* is being treated and killed by crude AGE at 60 mg/ml. Hence, these results showing the 94.48% decrease in SOD specific activity (0.009 units/sec/μg of protein) in macrophages infected with *M. smegmatis* treated with crude AGE at 60 mg/ml also confirmed the mycobactericidal potency of crude AGE.

Table 2: Determination of specific activity of SOD enzyme

Sl.No.	Samples	Specific activity of SOD (units/sec/ μ g of protein)	Decrease with respect to (3) (times)	Decrease with respect to (3) (%)
(1)	Blank	0.000	-	-
(2)	Macrophage (negative control, NC)	0.008	-	-
(3)	Macrophage + <i>M. smegmatis</i>	0.163	-	-
(4)	Macrophage + <i>M. smegmatis</i> + AGE (60 mg/ml)	0.009	18.11	94.48

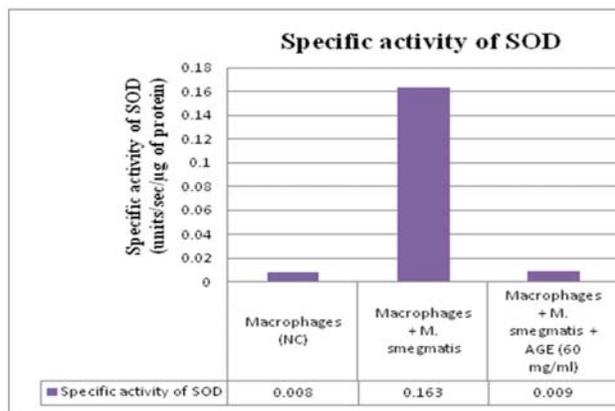


Fig 3: Comparative studies on the specific activities of SOD enzyme from 3 different sets of lysates of murine peritoneal macrophages shows prominent specific activity of SOD in macrophages infected with untreated, alive *M. smegmatis* (0.163 units/sec/ μ g of protein), much lesser in macrophages infected with *M. smegmatis* treated with crude AGE (at its MBC of 60 mg/ml, 0.009 units/sec/ μ g of protein) and even lesser in case of only macrophages (negative control, NC, 0.008 units/sec/ μ g of protein).

4. Conclusion

The results of our SDS PAGE-Western blot analysis, and also biochemical determination of the specific activities of iNOS and SOD enzymes are of great significance, particularly with respect to intracellular pathogens like *M. tuberculosis* and their MDR-strains, as this study confirms the mycobactericidal ability of crude AGE. Hopefully, this modality of treatment of MDR-TB - in the form of freshly-crushed garlic - would open a newer way of an effective, cheaper and safer herbal cure for TB, and help decrease the load of drug resistance, host toxicity and increasing cost in the management of MDR-TB in India. Since the standard antibiotic treatment regimen used for TB has become redundant against the MDR-TB strains of *M. tuberculosis*, the efficacy of garlic extract as a possible herbal remedy for the prevention of the disease would present a major solution to the already-paralysed prevailing treatment system.

5. Acknowledgement

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