Research Paper

Volume : 4 | Issue : 3 | March 2015 • ISSN No 2277 - 8179

Bactericidal effect of garlic extract on *Mycobacterium smegmatis*

Microbiology

KEYWORDS : Garlic extract - MicroRNA - *Corynebacteriumspp.* - *Mycobacterium smegmatis*

Souvik Roy — Assistant Professor, Post-Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata-700 016, India.

Aditi Nag Chaudhuri — Associate Professor and Head, Department of Microbiology, Lady Brabourne College, P-1/2, Suhrawardy Avenue, Kolkata-700 017, India.

ABSTRACT

Mycobacterium tuberculosis in India has developed alarming levels of multi-drug resistance (MDR). As a potential herbal alternative, minimal bactericidal concentration (MBC) of freshly-prepared crude aqueous garlic extract (AGE) against Corynebacterium spp. was found out to be 50 mg ml^{-1}, whereas that against Mycobacterium smegmatis, closely related to M. tuberculosis, was 60 mg ml^{-1}. 1.39 times and 1.06 times lesser amount of lipids extracted from cell walls of each crude AGE-treated bacterial culture strongly confirmed cell wall as the sub-cellular target. Also, Corynebacterial and Mycobacterial trehalose synthase activities were reduced by 2.2 and 1.31 times, and trehalose and mycolic acid contents diminished by 1.32 and 1.05 timesrespectively by crude AGE. Absence of microRNA-155 band on Tris-Borate-EDTA-urea gel from murine peritoneal macrophages infected with crude AGE-treated M. smegmatis, and fluorescence microscopic observation of green-colored viable macrophages from the same set further confirmed Mycobactericidal potency of crude AGE at 60 mg ml^{-1}.

INTRODUCTION

Tuberculosis (TB), a contagious disease caused by *Mycobacterium tuberculosis*, remains the single largest killer disease in the world. According to the 2013 global TB report of the World Health Organization (WHO), in 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease [1]. Among these, 170 000 deaths were exclusively from multi-drug resistant (MDR) – TB [1].

India is a developing country, with 75% of the poor living in rural areas[2]. *Mycobacterium tuberculosis* can thrive under conditions of such poor sanitation, malnutrition and illiteracy, and cause TB.The 2013 report of WHO says that the number of cases of TB in India is between 2.0 million - 2.4 million in 2012 (26% of the global cases), only to be followed by China and South Africa [1]. Here, everyday, more than 40 000 people become newly infected, more than 5 000 develop the disease, and there are two deaths every three minutes [1, 3]. In West Bengal, more than 150 000 fresh cases of TB occur every year, and thousands die of the disease [4].

The currently approved treatment for new cases of drug-susceptible TB includes a six- to nine-month long regimen of four first-line drugs - isoniazid, rifampicin, ethambutol and pyrizinamide [1]. But, unfortunately, India features as a leading country among the 27 high MDR-TB burden countries of the world [1]. In fact, the 2013 WHO's global TB report (based on data provided by the National TB programmes) has indicated that India has highest burden of MDR-TB patients[5]. Actually, the number of MDR-TB patients notified in India quadrupled from 4237 in 2011 to 16,588 in 2012 [6]. Indiscriminate sale of over-the-counter medicine, inadequate dose and / or wrong combination of drugs have lead to the emergence of MDR-TB here.

This emergence of MDR-TB throughout India is very disturbing in current scenario of TB management in India.Management of MDR-TB entails intense chemotherapy for up to two years which may be highly toxic to a patient [5]. Most of these drugs carry risks of side-effects, and patients hardly complete full course of medication due to various socio-economic reasons. Hence, in contemporary times, there has been an increased awareness about herbal drugs against MDR-TB.

Since ancient times, garlic (*Allium sativum*) enjoys a high reputation as a medicinal herb, and has evoked a lot of interest as a folk medicine. This use of garlic finds its mention in the *Ayurveda* and in several Greek medicinal texts. It is a cheap vegetable, popularly used in India - either smashed to a paste, or eaten raw, both involving severe damage to the plant tissues, releasing fresh allicin - its chief antimicrobial chemical.

Allicin in a freshly-prepared crude aqueous garlic extract (AGE) is stable only for about 16 hours [7]. A wide range of microorganisms, including several Gram-negative and Gram-positive bacteria, fungi, protozoa and viruses have been shown to be sensitive when incubated with crude AGE within this period of time [8]. However, reports on allicin's bacteriostatic and bactericidal effects against the chiefly pathogenic genera *Corynebacterium* and *Mycobacterium* – with a related cell wall structure-composition, is still lacking in India.

Research on microRNA (miRNA) is exploring rapidly as a new thrust area of biomedical research with relevance to TB [9]. Several studies have revealed altered gene expression profiles in macrophages from TB and healthy controls, which is possibly regulated by miRNAs [10].

So, in the current study, we have tried to evaluate the effectiveness of a herbal preparation based on garlic in controlling high rates of MDR-TB in India. The aim was to determine anti-Corynebacterial potency of crude AGE, and if any, its minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), to subsequently work on its mode of action down to possible molecular (miRNA) levels, including the use of an advanced tool like fluorescence microscope, and to extend the same studies to the antigenically-related, avirulent *Mycobacterium smegmatis*, which would establish its possible bactericidal activities against pathogenic *M. tuberculosis* also. This will hopefully open a new avenue of an effective and safer herbal alternative to the synthetic anti-TB drugs being traditionally used for decades in India, and would help decrease the burden of drug resistance, host side-effects and cost in the management of MDR-TB in our country.

MATERIALS AND METHODS

Bacteria and Culture Conditions

Pure culture of *Mycobacterium smegmatis mc26 strain* used was obtained from the Microbiology Department, Bose Institute, Kolkata. It was grown 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 [11].

Ten samples of *Corynebacterium* spp. were isolated from nasal vestibules of willing healthy human volunteers, and the isolated bacteria grown on selective tellurite blood agar plates for 24 hours at 37^{0}C. Following incubation, characteristic greyish-black colonies were identified by routine microbiological procedures and biochemical tests [12], and grown as pure cultures.

Experimental animal

A part of this study was conducted using 4-6 weeks-old, healthy male BALB/c mice (*Mus musculus*) of 18±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 space with 12-hour alternating light and dark cycles. For one week prior to 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.

Preparation of AGE

Fresh bulbs of garlic (*Allium sativum*) were purchased from a local daily market, 45 grams weighed and minced properly in cold, and homogenized using 300 ml of autoclaved, cold distilled water for 1-2 minutes in cold. The homogenate was then centrifuged in a High speed Centrifuge in cold at 3 000 rpm for 5 minutes to remove any debris [13] and make a homogeneous suspension of crude AGE stock (150 mg ml^{-1})**.**

Determination of the MIC and MBC of crude AGE

For MIC determination by broth-dilution technique, tubes of 14 ml sterile broth were inoculated with a loopful of the overnight grown pure culture of each isolated *Corynebacterium* sp. and *M. smegmatis*, and 1 ml of different concentrations of crude AGE – 40, 42, 44, 46, 48 , 50 and 55 mg ml^{-1} for *Corynebacterium* spp. and 40, 45, 50, 52, 54, 56, 58, 60 and 65 mg ml^{-1} for *M. smegmatis* were added, in parallel to a negative control tube (lacking garlic extract) in each case [14]. The tubes were incubated for 12 hours at 37°C**.**

MBC was determined by streak-plate method. 1 ml of different concentrations of the crude AGE - 48, 48.5, 49, 49.5 and 50 mg ml^{-1} for *Corynebacterium* spp. and 58, 58.5, 59, 59.5 and 60 mg ml^{-1} for *M. smegmatis*, was added to each 14 ml sterile, molten, cooled agar and poured into sterile petri-plates, excepting the negative control plate (lacking garlic extract) in each case [14]. Upon solidification, one loopful of the overnight grown pure culture of each isolated *Corynebacterium* sp. and *M. smegmatis* was streaked, and plates incubated for 12 hours at 37°C.

Comparison between the MBCs of crude AGE and lyophilized GE (LGE)

The freshly-prepared stock (150 mg ml^{-1}) of crude AGE was lyophilized in a Freeze Dryer availed at the Bose Institute, Kolkata, to make LGE. Experimental determination of MBC of LGE was then carried out with all the pure cultures of the isolated *Corynebacterium* spp. under identical assay conditions.

Confirmation of MBC of crude AGE and elucidation of its probable mode of action

Bacterial sub-cellular target site of crude AGE

Corynebacterial and Mycobacterial cell walls are lipid (mycolic acid)-rich. Chloroform-methanol extraction of lipids from the cell walls of both crude AGE-treated (Experimental) and untreated (Negative control) pure broth cultures was carried out [15]. Differences between the respective Negative controls and Experimentals give the amount of lipids (mycolic acid) extracted from the cell walls.

Comparison between the activities of Trehalose Synthase (TS) enzyme in both crude AGE-treated and untreated Corynebacterium spp. *and M. smegmatis*

Trehalose synthase (TS) enzyme activities from both crude AGE-treated (Experimental) and untreated (Negative control) pure broth cultures of each bacterium were determined [16]. In this study, trehalose synthase (TS) enzymatic activity was measured in terms of trehalose phosphorolysis activity, in terms of NADPH generated (trehalose + inorganic phosphate [P_i] →D-glucose + α-D-glucose-1-P). The amount of the α-D-glucose-1-P produced was determined by coupling TS activity with the activities of phosphoglucomutase(α-D-glucose-1-P→α-D-glucose-6-P) and glucose-6-phosphate-1-dehydrogenase (α-D-glucose-6-P + $NADP^+$→6-phospho-D-gluconolactone + NADPH + H^+) [16]. The amount of this NADPH produced was measured by $\Delta O.D._{340}$ over a 1 minute time-interval**,** and is proportional to TS activity.

Trehalose and Mycolic acid contents in both crude AGE-treated and untreated Corynebacterium spp. *and M. smegmatis*

Trehalose contents in both crude AGE-treated (Experimental) and untreated (Negative control) pure cultures of each bacterium were estimated. Differences in trehalose contents between the respective Negative controls and Experimentals reflect relative differences in their mycolic acid contents.

First, lipid extraction was carried out for both the crude AGE-treated (Experimental) and untreated (Negative control) sets of each sample of *Corynebacterium* sp. and *M. smegmatis*. Then, for each Experimental and Negative control set, trehalose content was estimated using UV-Vis spectrophotometer at 620 nm, against a suitable blank [17].

miRNA assay

miRNA assay is carried out in order to re-confirm Mycobactericidal potency of crude AGE. Following an intra-peritoneal 1ml 1% sterile starch injection, the BALB/c mice were maintained properly for 4 days, their peritoneal monocytes isolated, and 3 million cells / well were added to a 6-well adherent plate. It was then cultured in a 5% CO_2 incubator at 37^{0}C for 48 hours to permit monocyte to macrophage differentiation. The top viscous layer of each well was discarded, and the adherent monolayer washed with normal Phosphate Buffered Saline (PBS), pH 7.2. Then infection with untreated *M. smegmatis* (Negative control) was given to the macrophages in a well in the ratio of macrophage: bacteria 1:10, and incubated for 12 hours at 37^{0}C in a 5% CO_2 incubator. Then the top viscous layer was discarded, and the macrophage monolayer was re-washed with ice-cold PBS, pH 7.2. Then entire contents of the well were scrapped off, transferred to a sterile Eppendorf tube and the step repeated twice [18]. The same steps were followed for macrophage infected with crude AGE-treated *M. smegmatis* (Experimental), except that, to the bacterial infection given to the macrophages, crude AGE (at its MBC) was added in a 1:1 ratio before incubation.

Then miRNA was isolated from these macrophages by following the standard protocol provided in the Ambion Pure Link miRNA Isolation Kit supplied by Invitrogen BioServices India Pvt. Ltd [19]. After isolation, the miRNA samples from each set were resolved on a standard 15% Tris-Borate-EDTA (TBE)-urea gel, and

Volume : 4 | Issue : 3 | March 2015 • ISSN No 2277 - 8179

observed against a control lane of standard RNA size-markers.

Observation of Macrophages under Fluorescence Microscope

In order to substantiate the Mycobactericidal efficacy of crude AGE, murine peritoneal macrophages cultured were studied under fluorescence microscope in three sets - without any treatment (Control), after being infected and incubated with *M. smegmatis,* and after being infected and incubated with *M. smegmatis*, treated with crude AGE at its MBC.

Staining macrophages with an acridine orange-ethidium bromide fluorescent dye mixture and observing them under fluorescence microscope allows the determination of apoptosis based on nuclear morphology and membrane integrity [20].

Acridine orange is a cell-permeable dye that penetrates cells and intercalates DNA to appear green (viable cells). Ethidium bromide enters only non-viable (apoptotic) cells that exhibit disrupted membrane integrity, and fluoresce orange. Thus, the nuclei in viable cells fluoresce green, while the nuclei in non-viable cells fluoresce orange [21].

The macrophages in each of the three cases were cultured and collected as described for miRNA isolation. Then, those macrophages were mixed in a 1:1 ratio with a 1:1 acridine orange (5 mg ml^{-1})-ethidium bromide (3 mg ml^{-1}) dye solution (half-diluted with PBS, pH 7.2) and immediately observed under a fluorescence microscope.

RESULTS AND DISCUSSION

Determination of the MIC and MBC of crude AGE

Post-incubation, growth of all 10 samples of the isolated *Corynebacterium* spp. was observed in all tubes with 40, 42, 44, 46 and 48 mg ml^{-1} concentrations, and growth of *M. smegmatis* was observed in all tubes with 40, 45, 50, 52, 54, 56 and 58 mg ml^{-1} concentrations of crude AGE. No growth was however observed in the garlic tubes with 50 mg ml^{-1} and 55 mg ml^{-1} in case of all samples of *Corynebacterium* spp., and 60 mg ml^{-1} and 65 mg ml^{-1} in case of *M. smegmatis*. Hence, the MIC of crude AGE lay between 48 and 50 mg ml^{-1}, and between 58 and 60 mg ml^{-1} respectively.

During MBC determination, growth of *Corynebacterium* spp. and *M. smegmatis* was found to be present in all plates with 48, 48.5, 49 and 49.5 mg ml^{-1} and 58, 58.5, 59 and 59.5 mg ml^{-1} concentrations of crude AGE respectively, with no growth in the garlic plate with 50 mg ml^{-1} and 60 mg ml^{-1}. Hence, the crude AGE exhibited a MBC of 50 mg ml^{-1} and 60 mg ml^{-1} respectively.

Comparison between the MBCs of crude AGE and LGE

The MBC obtained in case of the LGE was 2.4 times more (120 mg ml-1) than that determined for the crude AGE (50 mg ml-1) against the pure cultures of all the samples of the Corynebacterium spp. (Fig. 1). This suggests that stability of allicin achieved through inter-molecular H-bonds between its reactive oxygen atom and surrounding water molecules has been strongly compromised, due to the removal of water by lyophilization.

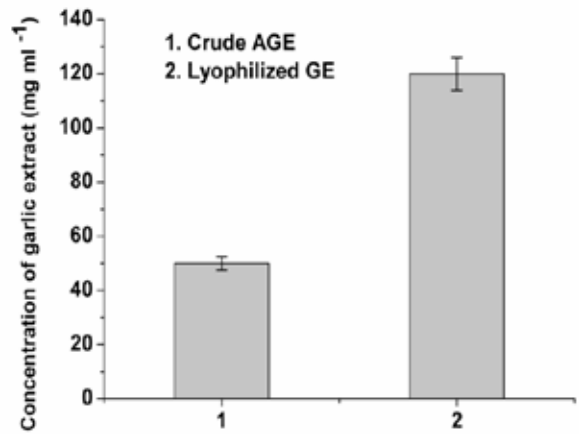


Figure-1: Comparative analysis of the MBCs of crude AGE and LGE

Confirmation of MBC of crude AGE and elucidation of its probable mode of action

Bacterial sub-cellular target site of crude AGE

The amount of lipids (mycolic acid) obtained by chloroform-methanol extraction from the cell walls of the Experimental and Negative control sets of *Corynebacterium* spp. and *M. smegmatis* (Fig. 2 and Fig. 3) are summarized in Table 1 and Table 2. As Corynebacterial and Mycobacterial cell walls are highly enriched with lipids, the observations suggested that the crude AGE degraded the cell wall lipid contents in the treated sets.

Table-1: Chloroform-methanol extraction of lipids (mycolic acid) in case of crude AGE-treated and untreated *Corynebacterium* spp.

Parameter	Corynebacterium spp.			
Lipids (mycolic acid) extracted (g mg-1 protein)	Crude AGE-treated (50 mg ml-1)	Untreated	Decrease (times)	Decrease (%)
	1.074	1.4926	1.39	28.05

Table-2: Chloroform-methanol extraction of lipids (mycolic acid) in case of crude AGE-treated and untreated *Mycobacterium smegmatis*.

Parameter	Corynebacterium spp.			
TS activity (amount of NADPH produced in mg min-1 mg-1 protein)	Crude AGE-treated (50 mg ml-1)	Untreated	Decrease (times)	Decrease (%)
	0.8	1.76	2.2	54.54

"Figure 2 about here".

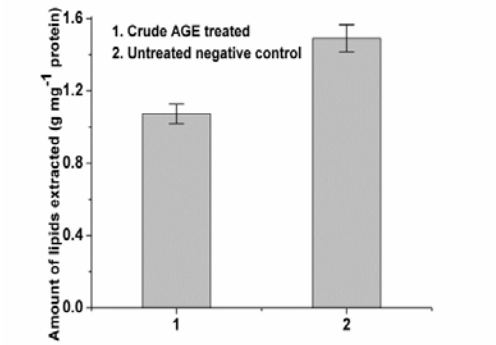


Figure-2: Comparison of lipids extracted from the cell walls of Experimental and Negative control sets of *Corynebacterium* spp.

"Figure 3 about here".

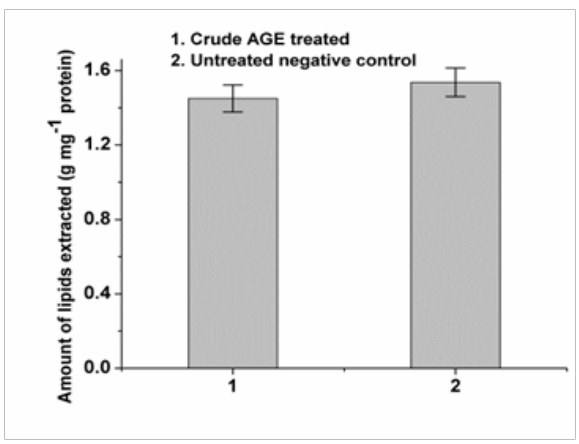


Figure-3: Comparison of lipids extracted from the cell walls of Experimental and Negative control sets of *M. smegmatis*

Comparison between the activities of Trehalose Synthase (TS) enzyme in both crude AGE-treated and untreated Corynebacterium spp. *and M. smegmatis*

In this study, TS enzymatic activity was measured in terms of trehalose phosphorolysis activity. As TS activity is directly proportional to the amount of NADPH produced (mg min^{-1} mg^{-1}protein), decreases in NADPH productions (Fig. 4 and Fig. 5) in both the treated cultures reflected reductions in the respective TS activities (Table 3 and Table 4). Thus, TS activity was reduced by crude AGE.

Table-3: TS activities (amount of NADPH produced) in cases of crude AGE-treated and untreated *Corynebacterium* spp.

Parameter	Mycobacterium smegmatis			
	Crude AGE-treated (60 mg ml-1)	Untreated	Decrease (times)	Decrease (%)
TS activity (amount of NADPH produced in mg min-1 mg-1 protein)	0.130	0.170	1.31	23.53

Table-4: TS activities (amount of NADPH produced) in cases of crude AGE-treated and untreated *Mycobacterium smegmatis.*

Parameter	Corynebacterium spp.			
Amount of Trehalose produced per gram of lipids (mycolic acid) per mg of protein (mM g-1 mg-1 protein)	Crude AGE-treated (50 mg ml-1)	Untreated	Decrease (times)	Decrease (%)
	4.03	5.34	1.32	24.72

"Figure 4 about here".

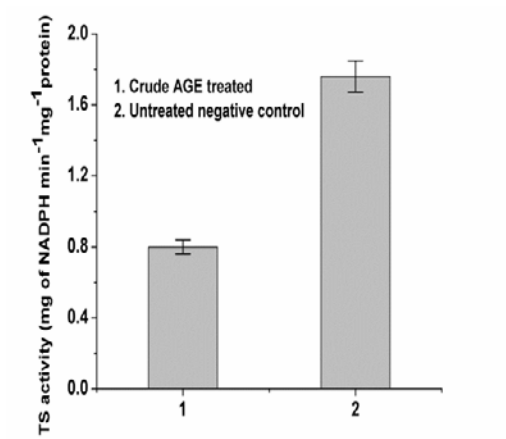


Figure-4: Comparison of TS activities (amount of NADPH produced) in case of Experimental *Corynebacterium* spp. and its Negative control

"Figure 5 about here".

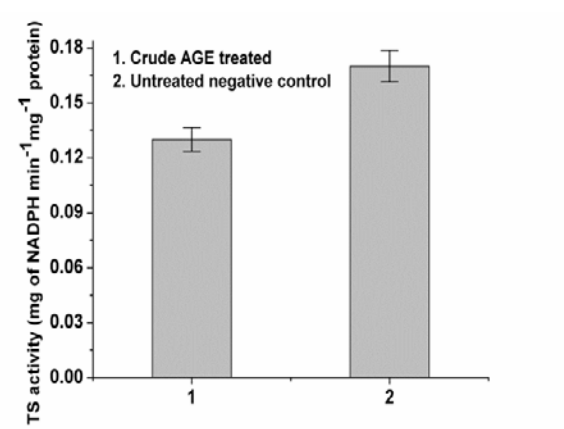


Figure-5: Comparison of TS activities (amount of NADPH produced) in case of Experimental *M. smegmatis* and its Negative control

Trehalose and Mycolic acid contents in both crude AGE-treated and untreated Corynebacterium spp. and M. smegmatis

The amount of Trehalose (mM g^{-1} mg^{-1} protein) extracted from

the bacterial cell walls of both crude AGE-treated (Experimental) and untreated (Negative control) pure cultures of each bacterium were estimated (Table 5 and Table 6). Since trehalose in the extracted lipids comes from mycolic acids, which are found as free lipids in the form of trehalose dimycolate (TDM), the differences in trehalose contents (Fig. 6 and Fig. 7) reflect relative differences in mycolic acid contents between the treated and untreated cultures. This also proved the anti-mycolic acid potency of crude AGE at its respective MBC (50 mg ml^{-1} and 60 mg ml^{-1}).

Table-5: Amount of Trehalose produced in cases of crude AGE-treated and untreated *Corynebacterium* spp.

Parameter	Mycobacterium smegmatis			
Amount of Trehalose produced per gram of lipids (mycolic acid) per mg of protein (mM g^{-1} mg^{-1} protein)	Crude AGE-treated (60 mg ml^{-1})	Untreated	Decrease (times)	Decrease (%)
	1.703	1.79	1.05	4.86

Table-6: Amount of Trehalose produced in cases of crude AGE-treated and untreated *Mycobacterium smegmatis*.

Parameter	*Mycobacterium smegmatis*			
Amount of Trehalose produced per gram of lipids (mycolic acid) per mg of protein (mM g^{-1} mg^{-1} protein)	Crude AGE-treated (60 mg ml^{-1})	Untreated	Decrease (times)	Decrease (%)
	1.703	1.79	1.05	4.86

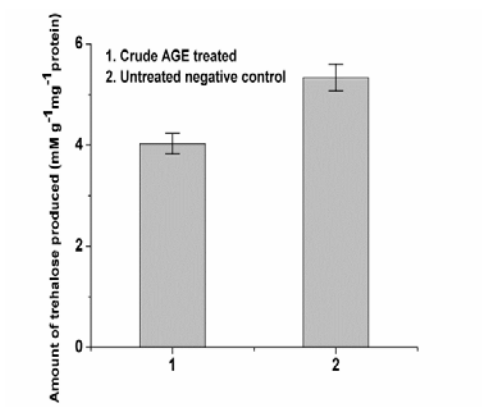


Figure-6 : Comparison of the Trehalose contents in case of Experimental *Corynebacterium* spp. and its Negative control

"Figure 7 about here".

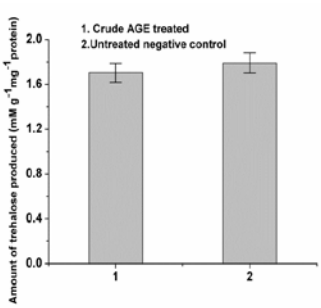


Figure-7 : Comparison of the Trehalose contents in case of Experimental *M. smegmatis* and its Negative control

miRNA assay

Earlier researches have shown that after an avirulent Mycobacterial infection, there is an increase in miRNA expression, particularly miR-155 [9, 10].

The electrophoretogram of the 15% TBE-urea gel (Fig. 8) showed multiple bands in the lane of the standard RNA size-markers (Lane A), one band (miR-155) in the lane of macrophages infected with untreated (Negative control) *M. smegmatis* (Lane G) and no band in the lane of macrophages infected with crude AGE-treated (Experimental) *M. smegmatis* (Lane H). This suggested that following *M. smegmatis* infection, miR-155 induction has taken place within the macrophages for carrying out the immunologic purposes of promoting maturation of mycobacterial phagosomes and decreasing the survival rate of intracellular *M. smegmatis*, at the same time re-confirming the Mycobactericidal potency of crude AGE at 60 mg ml^{-1}.

"Figure 8 about here".

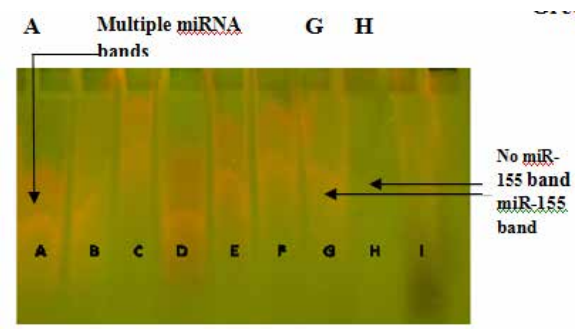
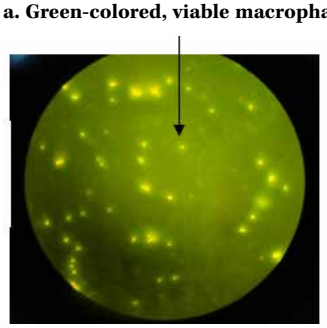


Figure-8: 15% TBE-urea gel electrophoretogram of miRNA

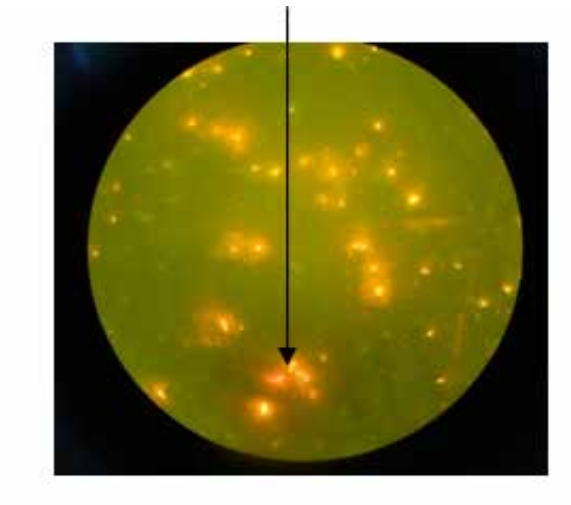
Observation of Macrophages under Fluorescence Microscope

In case of uninfected macrophages, green-colored viable cells were observed, due to penetration of only acridine orange into the cells (Fig. 9a). In case of macrophages infected with *M. smegmatis,* orange-colored, aggregated mass of non-viable (apoptotic) cells were observed, due to penetration of both ethidium bromide and acridine orange (Fig. 9b) This shows that due to induction of miR-155 expression in the infected macrophages, autophagic response has been increased. In case of macrophages infected with *M. smegmatis* treated with crude AGE at 60 mg ml^{-1}, green-colored, viable cells were observed, due to penetration of only acridine orange (Fig 9c) This indicated that as crude AGE is killing *M. smegmatis* at its MBC, the macrophages do not induce miR-155 expression. These observations re-established the Mycobactericidal potency of crude AGE.

a. Green-colored, viable macrophages



b. Orange-colored, aggregated mass of non-viable (apoptotic) macrophages



Green-colored, viable macrophages

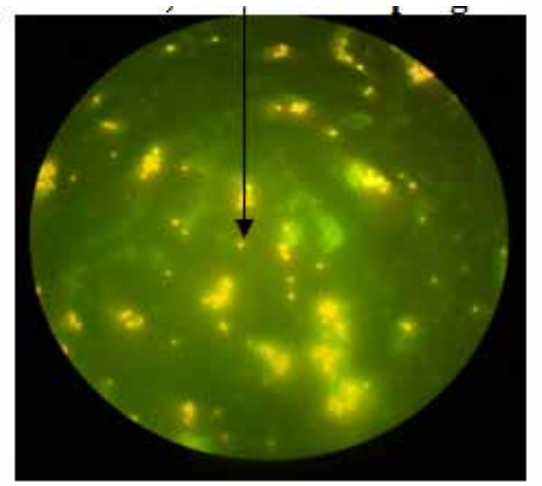


Figure-9: Macrophages stained by acridine orange-ethidium bromide dye-mixture, as observed under fluorescence microscope (400X)

CONCLUSION: All the above findings indicated that crude AGE can kill *Corynebacterium* spp. and *M. smegmatis*. The integrities of their lipid-rich cell walls were compromised, their TS activities were reduced, and their trehalose and mycolic acid contents were lowered by crude AGE. It thus confirmed the MBC of crude AGE against both the bacterial cultures, further substantiated by the fluorescence microscopic observation of viable macrophages infected with *M. smegmatis* treated with crude AGE at its MBC, and no expression of miR-155 in them. The present study thus suggests an alternative avenue for an effective, cheaper and safer natural therapeutic remedy of MDR-TB in India in the form of freshly-crushed garlic.

REFERENCE

[1] World Health Organization (WHO), (2013), "Global tuberculosis report 2013." Web. http:// who.int/tb/publications/global_report/en/. Accessed 10 April 2014. | [2] Wikipedia, (2014), "Standard of Living in India." Web. http://en.wikipedia.org/wiki/Standard_of_living_in_India. Accessed 15 June 2014. | [3] Directorate General of Health Services, Ministry of Health and Family Welfare, (2013), "TBC India" Web. http://tbcindia.nic.in/rntcp.html. Accessed 2 February 2014. | [4] The Times of India Kolkata, (2003), "TB rates high in Bengal: WHO." Web. http://timesofindia.indiatimes.com/city/kolkata/TB-rates-high-in-Bengal-WHO/articleshow/41308700.cms. Accessed 16 October 2014. | [5] World Health Organization (WHO), (2008), "Global tuberculosis control - surveillance, planning, financing." Web. http://www.who.int/tb/publications/global_report/2008/en/.Accessed 1 June 2014. | [6] Mudur, G. S.,The Telegraph Calcutta India, (2013), "Drug-resistant TB challenge for India." Web. http://www.telegraphindia.com/1131025/jsp/nation/story_17492671.jsp#.VK4_YSuUe8s. Accessed 16 October 2014. | [7] Yu, T. H., and Wu, C. M. (1989), "Stability of allicin in garlic juice." | J Food Sci, 54, 977-981. | [8] Ankri, S., and Mirelman, D. (1999), "Antimicrobial properties of allicin from garlic." Microb Infect, 1, 125–129. | [9] Singh, P. K., and Singh, A.V., and Chauhan, D. S. (2013), "Current understanding on micro RNAs and its regulation in response to Mycobacterial infections." J of Biomed Sci, 20, 14. | [10] Harapan, H.,Fitra, F., Ichsan, I., Mulyadi, M., Miotto, P., Hasan, N. A., Calado, M., and Cirillo, D. M. (2013), "The roles of microRNAs on tuberculosis infection: Meaning or myth?" Tuberculosis, 93, 596-605. | [11] Chaudhuri, B., Sau, S., Datta, H. J., and Mandal, N. C. (1993), "Isolation, characterization, and mapping of temperature-sensitive mutations in the genes essential for lysogenic and lytic growth of the mycobacteriophage L1." Virology, 194, 166–172. | [12] Standards Unit, Evaluations and Standards Laboratory, Centre for Infections, (2008), "Identification of Corynebacterium species (National Standard Method)." Web. http://www.hemltd.ru/export/sites/HemLtd/publications/sections/Normativ/foreign/Infections/medicine/NHS034/article.pdf. Accessed 13 October 2014. | [13] Kilikdar, D., Mukherjee, D., Dutta, M., Ghosh, A. K., Rudra, S., Chandra, A. M., and Bandopadhyay, D. (2013), "Protective effect of aqueous garlic extract against lead-induced cardiac injury in rats."J Cell Tissue Res, 13(3), 3817-3828. | [14] Abdul, H., Muhammad, I.U., Muhammad, U., Shahid, H., Muhammad, A., and Khursheed, J. (2011), "Anti-mycobacterial activity of garlic (Allium sativum) against multi-drug resistant and non-multi-drug resistant Mycobacterium tuberculosis." Pak J Pharm Sci, 2(1), 81-85. | [15] Takayama, K., Hayes, B., Vestling, M.M., and Massey, R. J. (2003), "Transposon-5 mutagenesis transforms Corynebacterium matruchotii to synthesize novel hybrid fatty acids that functionally replace corynomycolic acid." Biochem J, 373, 465-474. | [16] Saito, K., Kase, T., Takahashi, E., Takahashi, E., and Horinouchi, S. (1998), "Purification and Characterization of a Trehalose Synthase from the basidiomycete Grifola frondosa." Appl Environ Microbiol, 64(11), 4340–4345. | [17] Mokrasch, Lewis, C. (1954), "Analysis of hexose phosphates and sugar mixtures with the anthrone reagent." J Biol Chem, 208, 55-59. | [18] Bhattacharyya, S., Ghosh, S., Dasgupta, B., Mazumder, D., Roy, S., and Majumdar, S. (2002), "Chemokine-induced leishmanicidal activity in murine macrophages via the generation of nitric oxide." J Infect Dis, 185, 1704–1708. | [19] Ambion, Life Technologies, (2012), "Pure Link miRNA Isolation Kit for rapid, efficient purification of small RNA molecules." Web. http://tools.lifetechnologies.com/content/sfs/manuals/purelink_microRNA_man.pdf. Accessed 1 June 2014. | [20] Jones, N. L., Islur, A., Haq, R., Mascarenhas, M., Karmali, M. A., Perdue, M. H., Zanke, B. W., and Sherman, P. M. (2000), "Escherichia coli Shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family." Am J Physiol Gastrointest Liver Physiol, 278, G811-19. | [21] Ching, J. C., Jones, N. L., Ceponis, P. J., Karmali, M. A., and Sherman, P. M. (2002), "Escherichia coli Shiga-like toxins induce apoptosis and cleavage of poly (ADP-ribose) polymerase via in vitro activation of caspases." Infect Immun, 70, 4669-4677.