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Effects of crude aqueous garlic extract on multi-drug resistant (MDR) *Mycobacterium smegmatis*

Souvik Roy and Aditi Nag Chaudhuri

Abstract
The present havoc due to a global resurgence of tuberculosis (TB), and a swift rise in multi-drug resistant (MDR)-TB and extensively-drug resistant (XDR)-TB cases in India makes search for novel anti-tubercular herbal medicines an absolute mandate. In this research work, the minimal bactericidal concentration (MBC) of freshly-prepared, crude aqueous extract of both multi-clove and single-clove varieties of garlic (*Allium sativum* L.) against MDR-*Mycobacterium smegmatis* (the bacterium sharing similar cell-wall structure-composition as the MDR-*M. tuberculosis*) was determined to be 10 mg/ml and 20 mg/ml respectively by broth-dilution-technique, and both values confirmed by spread-plate technique. Re-confirmation of the MBC was carried out through microscopic analyses after standard acid-fast staining, and biochemical determination of the specific activities of bacterial enzymes like catalase and NADH dehydrogenase. All these positive findings, once substantiated by successful human trials, would firmly establish the importance of green pharmacy and garlic in the treatment of DR-TB in India.

Keywords: Anti-tubercular herbal medicines, minimal bactericidal concentration, garlic, MDR-*Mycobacterium smegmatis*, acid-fast staining, catalase, NADH dehydrogenase

1. Introduction
Tuberculosis (TB), caused by the aerobic, acid-fast bacillus *Mycobacterium tuberculosis*, is presently a worldwide havoc. According to the 2016 World Health Organization (WHO) report on TB, there were about 10.4 million new cases of TB, and approximately 1.4 million TB deaths worldwide in 2015 [1, 2]. In addition, presently there has been an abnormal rise in the number of cases of drug-resistant TB (DR-TB), caused particularly by the multi-drug resistant (MDR)-strains of *M. tuberculosis*, together with the much notorious extensively drug-resistant (XDR)-strains [3]. In 2016, there were 5, 80,000 new cases of multi-drug resistant/rifampicin-resistant TB (MDR/RR-TB) globally, including 4, 80,000 (83%) new cases of MDR-TB. Out of these, there had been 2, 50,000 deaths due to MDR/RR–TB. Besides, 7579 cases of XDR-TB were also reported from 74 countries across the world in 2015 [1]. Alarming enough, India finds a place in all the three lists of the 30 high TB-burden, high TB/HIV-burden and high MDR TB-burden countries of the world, as prepared by WHO [1]. The number of TB cases in India was 28, 40,000 in 2015, leading to a mortality of 4, 80,000 HIV-negative TB-patients and 37,000 HIV-positive TB patients. Here, in 2015, the estimated new TB cases with MDR/RR-TB were 2.5%, whereas the estimated re-treatment TB cases with MDR/RR-TB were 16% of a total of 1, 30,000 cases [1, 2]. Also, in 2015, India recorded a confirmed number of 3048 cases of XDR-TB [1]. The major factors conducive for the rapid spread of DR-TB include any recent emigration from the lesser developed or developing countries, HIV-positive patients or who are at risk of acquiring HIV, and persons who have previously been treated with a regimen of drugs that did not include rifampicin (RIF) [4]. Indiscriminate use of medicine, over-the-counter availability, improperly-supervised treatment regime, inadequate dose, wrong prescription of wrong combination of drugs, and non-compliance leading to abrupt truncation of courses have all lead to the explosion of MDR-TB and XDR-TB here [3]. In developing countries like India, TB control programs are overwhelmed by the complexity of treating MDR-TB and XDR-TB infected people, as availability of sophisticated and efficient tools and therapies are limited [5]. In fact, when the anti-tubercular drugs are used singly, the resistant tubercle bacilli emerge rapidly and multiply. So, an effective management of DR-TB requires intense chemotherapy, which includes a 20-month long regime of multiple antimicrobials, as the fact that drug-resistance may be there in a patient's *M. tuberculosis* isolate, must be taken into account when selecting an effective therapy [4, 5, 6]. This precise multi-drug therapy (MDT) with four or five drugs depends entirely on the resistance pattern of the clinical isolate, and is not only time-consuming, but also costly [3]. This often results in poor compliance and high treatment drop-out [6]. Most of the allopathic medicines also carry

risks of side-effects like hepatitis, hypersensitivity reactions, dark urine, fever, jaundice, loss of appetite, nausea and vomiting. Therefore, in recent times, there has been an increased quest for alternative herbal drugs against MDR-strains and XDR-strains of *M. tuberculosis*. The magnitude of the MDR-TB and XDR-TB infections in mostepicentres necessitates the urgency of finding novel drugs that could easily overcome the problem of drug resistance [5]. According to recent WHO reports, there are 2,50,000 higher plant species on earth, out of which 35,000 to 70,000 species are used for medicinal purposes. In India, the number of medicinal plants, both indigenous and introduced, has been estimated to be between 3,000 to 3,500 species of higher plants [7]. Garlic (*Allium sativum* L.), a species in the onion genus *Allium*, is novel in this respect. When a garlic cell is broken by chopping, chewing, or crushing, then only the pyridoxal-5-phosphate (P5P)-dependant enzyme alliinase/alliin-lyase (E.C.4.4.1.4) stored in the cell vacuoles break down sulfur-containing allin stored in large quantities in the cytosol to produce the therapeutically useful, fresh allicin [8, 9]. When incubated with crude AGE, several Gram-negative and Gram-positive bacteria, fungi, protozoa and viruses are killed [8]. However, from multiple literature survey, it has been found that any kind of reported documentation on the effectiveness of crude AGE against *M. tuberculosis* and its DR-varieties are still lacking in India. So taking the above situation into consideration, the same author group has earlier experimentally validated the clinical efficacy of crude aqueous garlic extract (AGE) against wild-type (WT) *M. smegmatis* (used here as an alternative to the pathogenic WT *M. tuberculosis*) [10, 11]. The present research work deals with ascertaining the mycobactericidal efficacies, if any, of crude aqueous extracts prepared from both the commonly-used and cheap multi-clove, and the lesser-used and costlier single-clove varieties of garlic against the MDR-strain developed artificially from the WT *M. smegmatis* mc^{2}6 strain (used here as an alternative to the pathogenic MDR-*M. tuberculosis*), determination and confirmation of their minimal bactericidal concentration (MBC), and biochemical determination of the specific activities of bacterial enzymes like catalase (CAT) and NADH dehydrogenase (Ndh).

2. Materials and methods

2.1 WT *Mycobacterium smegmatis* mc^{2}6 strain and culture conditions

The pure culture of WT *M. smegmatis* mc^{2}6 strain used in this study, kindly provided by the Department of Microbiology, Bose Institute, Kolkata, was susceptible to the first-line anti-tubercular drugs – INH, RIF, ethambutol (EMB) and pyrazinamide (PZA). It was allowed to grow in sterile Middlebrook 7H9 (MB7H9) broth, supplemented with 0.5% glycerol and 0.25% bovine serum albumin (BSA) at 37 °C for 24 hours, with continuous shaking [12]. Post-incubation, the pure culture of WT *M. smegmatis* mc^{2}6 strain was confirmed by standard microbiological procedures and biochemical tests [13-17].

2.2 Antibiotic used for the development and preservation of MDR-*M. smegmatis* mc^{2}6 strain

Each antibiotic (R-Cinex) capsule (purchased from Lupin Ltd.), required for the artificial development of MDR-*M. smegmatis* mc^{2}6 strain and its preservation, contained two active ingredients – INH and RIF in 2:3 ratio (300 mg:450 mg).

2.3 Development of MDR-*M. smegmatis* mc^{2}6 strain and culture conditions

For the artificial development of MDR-*M. smegmatis* mc^{2}6 strain, first the R-Cinex antibiotic stock (200 µg/ml) was prepared. Dilutions were then performed to make the effective antibiotic concentrations vary as 5, 10, 15, 20, 25, 30, 60 and 100 µg/ml, to which 10 µl of the WT *M. smegmatis* mc^{2}6 strain culture were added in each case. In order to confirm that MDR-*M. smegmatis* mc^{2}6 strain have successfully been developed in presence of 5 to 100 µg/ml of the antibiotic, spread-plate technique was performed with nutrient agar (NA) plates. 100 µl of MDR-*M. smegmatis* mc^{2}6 strain culture taken from each of the three selected culture tubes having 25, 60 and 100 µg/ml concentrations of the antibiotic was spread on the respective plate, and incubated for 24 hours at 37 °C.

2.4 Acid-fast staining to observe and confirm MDR-strain

To observe and confirm the MDR-strain developed, acid-fast staining was performed according to the standard protocol.

2.5 Preparation of crude AGE (multi-clove and single-clove) stock

Fresh bulbs of both the commonly-used and cheap multi-clove, and the lesser-used and costlier single-clove varieties of garlic (*Allium sativum* L.) were purchased from a local daily market, and homogeneous suspensions of crude AGE stock (100 mg/ml) from each variety were prepared [10, 11].

2.6 Determination and confirmation of the MBC of crude AGE (multi-clove) against MDR -strain

The MBC of the freshly-prepared crude AGE (multi-clove) stock (100 mg/ml) against MDR-strain was determined by broth-dilution technique, using crude AGE (multi-clove) concentrations of 1, 2, 3, 5, 10 and 20 mg/ml and 10 µl of the MDR-strain in each case. In order to confirm the actual MBC of crude AGE (multi-clove) against MDR-strain, spread-plate technique was carried out with NA plates. 100 µl of MDR-strain culture taken from each of the six culture tubes (in presence of the antibiotic solution – 100 µg/ml, and 1, 2, 3, 5, 10 and 20 mg/ml concentrations of crude AGE, multi-clove) was spread on the respective plate, and incubated for 16 hours at 37 °C.

2.7 Determination and confirmation of the MBC of crude AGE (single-clove) against MDR-strain

The MBC of the freshly-prepared crude AGE (single-clove) stock (100 mg/ml) against MDR-strain was similarly determined by broth-dilution technique, according to the same protocol as determined for crude AGE (multi-clove), using crude AGE (single-clove) concentrations of 1, 3, 5, 10 and 20 mg/ml and 10 µl of the MDR-strain in each case. The results were similarly confirmed by spread-plate technique.

2.8 Comparison of specific activities (ΔOD/min/mg protein) of CAT and Ndh enzymes in crude AGE (multi-clove)-treated MDR-strain, untreated WT-strain and untreated MDR-strain

2.8.1 Preparation of bacterial cell-lysates

First, 1 ml of each of the pure cultures of crude AGE (multi-clove)-treated MDR-strain, untreated WT-strain and untreated MDR-strain was centrifuged at 1000 rpm for 10 minutes at 4^0C in the high speed Cold Centrifuge, and the respective supernatants discarded [18]. Each of the pellets was then suspended in 1-2 ml 1X ice-cold phosphate-buffered saline (PBS), pH 7.4, containing 1 mM ethylene diamine tetra acetate (EDTA). The respective cell-pellet suspensions were

then sonicated on ice, twice for 30 seconds, each punctuated by a time-interval of 30 seconds, with the help of Hielscher-Ultrasound Technology ultrasonic processor (Model number: UP200S with S40 at stand). The sonicated cell-pellet suspensions were again centrifuged at 8000 rpm for 15 minutes at 4^0C in the high speed Cold Centrifuge. The respective supernatants were then collected, aliquoted for use in this assay, and stored on ice till required [19].

2.8.2 Determination of the protein content (mg) of the respective bacterial cell-lysates by Lowry (Folin-Ciocalteau) method

The protein content (mg) of the respective bacterial cell-lysates was determined by the standard Lowry (Folin-Ciocalteau) method [20]. The OD readings were then taken with the UV/VIS spectrophotometer (Hitachi U-2900 UV/VIS spectrophotometer 200V; Model number: 2J1-0004) at 660 nm (OD_{660}) against the Blank (B), and the values plotted in the Standard Curve of BSA, from which the protein content (mg) of the respective bacterial cell-lysates was calculated.

2.8.3 Assay of CAT enzyme

The respective CAT assays were performed according to the standard protocol, and the ΔOD readings over a span of 15 seconds were then taken with the UV/VIS spectrophotometer at 240 nm (ΔOD_{240}) against the Blank (B) [19].

2.8.4 Determination of specific activities (ΔOD/min/mg protein) of CAT from the respective bacterial cell-lysates

From the ΔOD/15 second readings obtained in the respective CAT assay experiments, ΔOD/min was calculated, the specific activities (ΔOD/min/mg protein) of CAT determined from the respective samples, and compared.

2.8.5 Assay of Ndh enzyme

The respective Ndh assays were performed according to the standard protocol, and the ΔOD readings over a span of 5 minutes were then taken with the UV/VIS spectrophotometer at 340 nm (ΔOD_{340}) against the Blank (B) [21].

2.8.6 Determination of specific activities (ΔOD/min/mg protein) of Ndh from the respective bacterial cell-lysates

From the ΔOD/5 minute readings obtained in the respective Ndh assay experiments, ΔOD/min was calculated, the specific activities (ΔOD/min/mg protein) of Ndh determined from the respective samples, and compared.

3. Statistical analysis

All results were expressed as mean ± SEM for individual experiment. Each experiment was performed three times (n=3), and the mean value from all set of those experiments was presented. Student's t-test was performed as applicable in each case, and the values were found to be significant at 5% probability level. Windows Microsoft Excel 2007 software was employed for computation, data analysis and graphics.

4. Results & Discussion

4.1 Development of MDR-*M. smegmatis* mc^{2}6 strain

Post-incubation, bacterial growth was observed in all the culture-tubes, with growth intensity gradually being decreased from 5 to 100 µg/ml (Fig. 1). In this way a pure culture of MDR-*M. smegmatis* mc^{2}6 strain was developed.

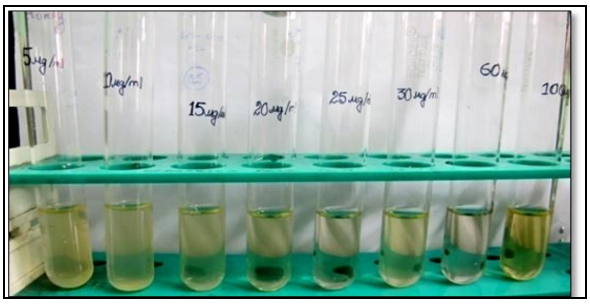

Fig 1: Observation of the growth of MDR-*M. smegmatis* mc^{2}6 strain in increasing concentrations of the antibiotic R-Cinex.

During confirmation by spread-plate technique, following incubation, it was found that the bacterial growth and the number of isolated colonies gradually decreased with increasing concentrations of the antibiotic - from 25 to 60 to 100 µg/ml, from being a lawn of bacterial growth to being 90 and 70 respectively (Fig. 2). Thus the MDR-*M. smegmatis* mc^{2}6 strain was confirmed to have been successfully developed artificially.

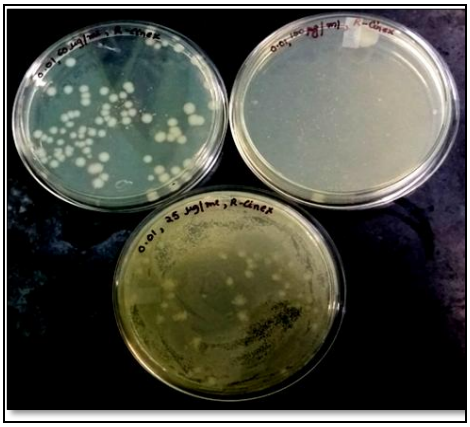

Fig 2: Observation of NA plates (with three different antibiotic concentrations: 25, 60 and 100 µg/ml) for confirmation of development of MDR-*M. smegmatis* mc^{2}6 strain.

4.2 Acid-fast staining to observe and confirm MDR-strain

Microscopic observation (450X) revealed pale-red colored, short bacterial cells, heavily distorted in their thick rod shapes in the presence of the antibiotic at a high concentration of 100 µg/ml, appearing in single, pair or cluster arrangement (Fig. 3a). This was in marked contrary to the microscopic observation (450X) of pale-red colored, medium-sized, thick rod-shaped bacterial cells in single, pair or cluster arrangement, after standard acid-fast staining of the WT *M. smegmatis* mc^{2}6 strain (Fig. 3b).

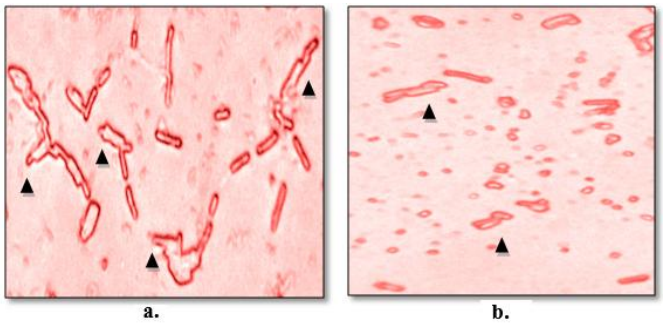

Fig 3: Microscopic observation (450X) after acid-fast staining of cells of: a. MDR-strain (indicated by black arrows); b. WT *M. smegmatis*mc^{2}6 strain.

4.3 Determination and confirmation of the MBC of crude AGE (multi-clove) against MDR-strain

Post-incubation, growth of the MDR-strain was found to be present in all tubes with 1, 2, 3 and 5 mg/ml concentrations of crude AGE (multi-clove). Bacterial growth intensity gradually diminished from 1 to 5 mg/ml of crude AGE. No growth was observed in the garlic tubes with 10 and 20 mg/ml ofcrude AGE (Fig. 4). Hence, the MBC of crude AGE (multi-clove) against the MDR-strain was observed to lie at 10 mg/ml.

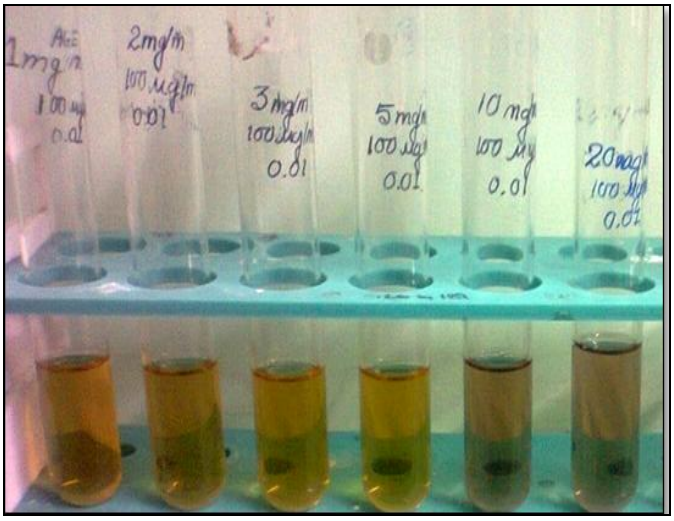

Fig 4: Determination of MBC of crude AGE (multi-clove) against the MDR-strain.

In the experimental confirmation of the MBC of crude AGE (multi-clove), growth of MDR-strain was found to be present in all NA plates with 1, 2, 3 and 5 mg/ml concentrations of the crude AGE. The number of bacterial colonies gradually decreased from 1 to 5 mg/ml of the crude AGE, with no growth observed in the garlic plates with 10 and 20 mg/ml of crude AGE (Fig. 5). Hence, the crude AGE (multi-clove) had a MBC of 10 mg/ml against the MDR-strain.

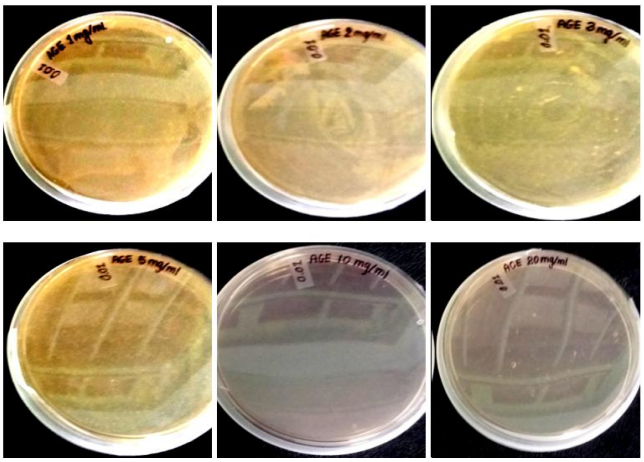

Fig 5: Confirmation of MBC (10 mg/ml) of crude AGE (multi-clove) against the MDR-strain.

4.4 Determination and confirmation of the MBC of crude AGE (single-clove) against MDR- strain

Post-incubation, growth of MDR-strain was found to be present in all tubes with 1, 3, 5 and 10 mg/ml concentrations of crude AGE (single-clove). Bacterial growth intensity gradually diminished from 1 to 10 mg/ml of crude AGE. No growth was observed in the garlic tube with 20 mg/ml ofcrude AGE (Fig. 6). Hence, the MBC of crude AGE (single-clove) against the MDR-strain was observed to lie at 20 mg/ml.

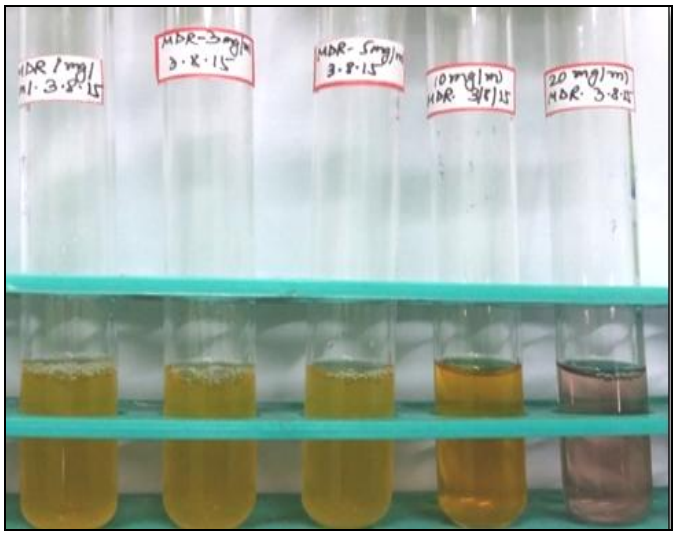

Fig 6: Determination of MBC of crude AGE (single-clove) against the MDR-strain.

In the experimental confirmation of the MBC of crude AGE (single-clove) against MDR-strain, growth of MDR-strain was found to be present in plates with 5 and 10 mg/ml concentrations of the crude AGE. The number of bacterial colonies gradually decreased from 5 to 10 mg/ml of the crude AGE, with no growth observed in the garlic plate with 20 mg/ml of crude AGE (Fig. 7). Hence, the crude AGE (single-clove) had a MBC of 20 mg/ml against the MDR-strain.

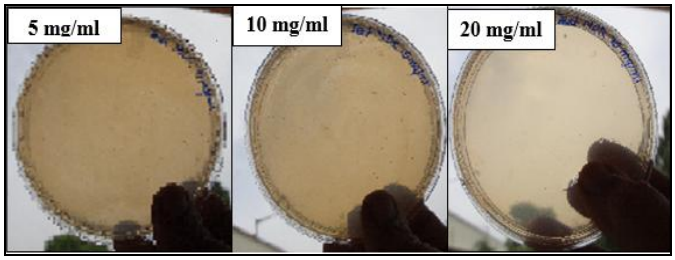

Fig 7: Confirmation of MBC (20 mg/ml) of crude AGE (single-clove) against the MDR-strain.

This observation of the MBC of crude AGE (single-clove) is socio-economically very significant, particularly in a developing country like India, as the crude AGE (of the commonly-used and cheap multi-clove variety) could kill MDR-strain at 10 mg/ml (half of that of the lesser-used and costlier single-clove variety, 20 mg/ml).

4.5 Comparison of specific activities (ΔOD/min/mg protein) of CAT and Ndh enzymes in crude AGE (multi-clove)-treated MDR-strain, untreated WT-strain and untreated MDR-strain

4.5.1 Determination of specific activities (ΔOD/min/mg protein) of CAT from the respective bacterial cell-lysates

From the respective ΔOD_{240}/15 second readings obtained in the respective CAT assay experiments, ΔOD/minute was calculated for each of them, and from the respective protein content (mg) determined from the Standard Curve of protein (BSA), the specific activities (ΔOD/min/mg protein) of CAT from the respective samples – crude AGE (multi-clove)-treated MDR-strain, untreated WT-strain and untreated MDR-strain were found out, and compared (Fig. 8).

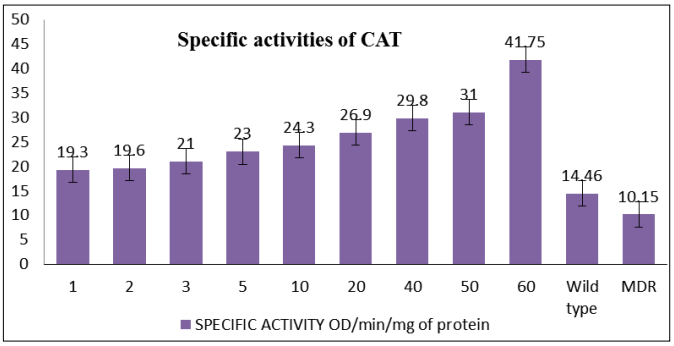

Fig 8: Bar graphs showing the respective specific activities (ΔOD/min/mg protein) of CAT.

Review of literature says that specific activity of CAT increase with increasing concentrations of AGE, in presence of H_2O_2 [22]. In this study also, it increased from 1 to 60 mg/ml of crude AGE in crude AGE (multi-clove)-treated MDR-strain. Literature says specific activity of CAT is lower in MDR-bacteria in comparison to the WT [22]. In this study also, it is lower in the untreated MDR-strain. These observations re-confirmed the mycobactericidal activity of crude AGE (multi-clove) against the MDR-strain.

4.5.2 Determination of specific activities (ΔOD/min/mg of protein) of Ndh from the respective bacterial cell-lysates

From the respective ΔOD_{340}/5 minute readings obtained in the respective Ndh assay experiments, ΔOD/minute was calculated for each of them, and from the respective protein content (mg) determined from the Standard Curve of protein (BSA), the specific activities (ΔOD/min/mg protein) of Ndh from the respective samples – crude AGE (multi-clove)-treated MDR-strain, untreated WT-strain and untreated MDR-strainwere found out, and compared (Fig. 9).

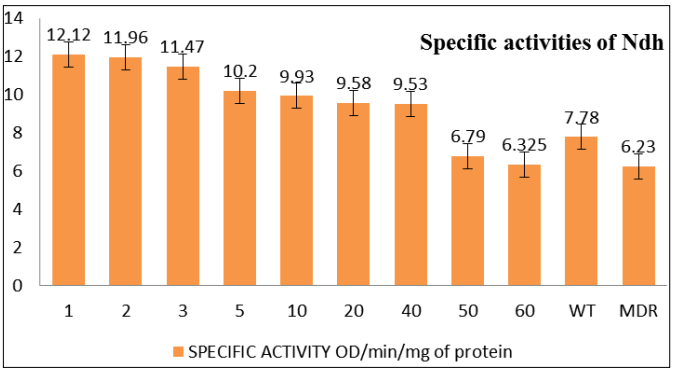

Fig 9: Bar graphs showing the respective specific activities (ΔOD/min/mg protein) of Ndh.

Review of literature says that specific activity of Ndh decrease with increasing concentrations of AGE, in presence of H_2O_2 [21]. In this study also, it decreased from 1 to 60 mg/ml of crude AGE in crude AGE (multi-clove)-treated MDR-strain. Literature says specific activity of Ndh is lower in MDR-bacteria in comparison to the WT [21]. In this study also, it is lower in the untreated MDR-strain. These observations re-confirmed the mycobactericidal activity of crude AGE (multi-clove) against the MDR-strain.

5. Conclusion

All the above experimental findings validated the mycobactericidal potency of crude AGE (multi-clove) against MDR-*M. smegmatis* mc^26 strain, thus opening a novel and relatively cheaper avenue for the treatment of MDR-TB in the form of an effective, safer and easily-available herbal alternative to the synthetic anti-TB drugs being traditionally used for decades in India.

6. Conflict of Interest

The authors declare that there is no potential conflict of interests among them regarding the publication of this paper.

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