ANTI-MYCOBACTERIAL ACTIVITY OF GARLIC (*ALLIUM SATIVUM*) AGAINST MULTI-DRUG RESISTANT AND NON-MULTI-DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

Emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) TB throughout the developing world is very disturbing in the present scenario of TB management. There is a fundamental need to explore alternative anti-TB agents. Hence natural plants should be investigated to understand their antimicrobial properties and safety. Garlic (*Allium sativum*) is one of natural plant which possesses variety of biological properties like anti-tumor, anti-hyperlipedemic and anti-microbial etc. The present study was evaluated for anti-bacterial activity of garlic against non-MDR and MDR isolates of *M. tuberculosis.* A total of 20 clinical isolates of MTB including 15 MDR and 5 non-MDR were investigated. Ethanolic extract of garlic was prepared by maceration method. Minimum inhibitory concentration (MIC) was performed by using 7H9 middle brook broth dilution technique. MIC of garlic extract was ranged from 1 to 3 mg/ml; showing inhibitory effects of garlic against both non-MDR and MDR *M. tuberculosis* isolates. Alternate medicine practices with plant extracts including garlic should be considered to decrease the burden of drug resistance and cost in the management of diseases. The use of garlic against MDR-TB may be of great importance regarding public health.

Keywords: TB, MDR, resistance, Garlic, Anti-bacterial activity.

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INTRODUCTION

Multi-drug resistant Tuberculosis (MDR-TB) is defined as an *in vitro* resistance of *Mycobacterium tuberculosis* (MTB) to at least rifampin and isoniazid (Skenders *et al*., 2005). Multi-drug resistance (MDR) has become a major concern to control TB particularly in the developing countries (Cohn *et al*., 1997). The development of mutations in different genes of mycobacterium leads to drug resistance and subsequent MDR-TB (Petrini and Hoffner, 1999). Management of MDR-TB entails intense chemotherapy for up to 2 years which is very damaging to a patient's health due to high levels of drug toxicity (WHO Report, 2008).

Because of treatment failure for MDR-TB, extensively drug resistant tuberculosis (XDR TB) emerged. It was originally defined as MDR-TB resistant to at least three of the six classes of second-line drugs used to treat patients with MDR-TB (Gandhi *et al*., 2006). Due to the increasing problem of antibiotic/drug resistance, WHO recommended exploring herbs or plants as alternative remedy for various bacterial infections. The antimicrobial properties of plants have been investigated by a number of researchers worldwide and the results are very promising (Cowan, 1999).

Garlic (*Allium sativum*) is natural plant being used as a food as well as folk medicine for centuries in all over the world (Rivlin, 2001). In 1996, Reuter *et al.* described garlic a plant with various biological properties like antimicrobial, anti-cancer, antioxidant, immuno-modulatory, anti-inflammatory, hypoglycemic, and anti-cardiovascular effects (Reuter *et al*., 1996). Different garlic extracts demonstrated activity against Gram-negative and Gram-positive bacteria including species of Escherichia, Salmonella, Staphylococcus, Streptococcus, Klebsiella, Proteus, Bacillus, clostridium, *Helicobacter pylori* (Cellin *et al*., 1996) and even acid-fast bacilli (AFB) such as MTB (Uchida *et al*., 1975). Allicin is thiosulfinate compound of garlic reported for its antibacterial activity. Allicin is proved to be anti-bacterial as it inhibits RNA synthesis (Feldberg *et al*., 1988).

According to Ayurvedic and Greek systems of medicine garlic is one of the established remedies for tuberculosis. In 1946 Rao *et al* firstly described the *in-vitro* garlic activity against *Mycobactrium tuberculosis* (Rao *et al*.,

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1946). A few studies have also been proving anti-mycobacterial activity of garlic against different species of mycobacteria (Gupta *et al*., 1955; Abbruzzese *et al*., 1987; Jain, 1998; Gupta *et al*., 1999; Bolton *et al*., 1982; Deshpande *et al*., 1993; Delaha and Garagusi, 1985).

The aim of the present study was to evaluate anti-mycobacterial activity of Ethanolic garlic extract (EGE) against twenty clinical isolates of MDR and non-MDR *Mycobacterium tuberculosis* by using a recently discovered, most sensitive and rapid 7H9 middle brook broth dilution technique, which is a fluorescence based technique for detection of MTB.

MATERIALS AND METHODS

Prior to the start of the study, approval was obtained from the Ethical Committee, University of Health Sciences, Lahore, Pakistan.

Bacterial isolates

A total of 20 cultural isolates were investigated including 15 MDR and 5 non-MDR MTB. These isolates were provided by Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan and Gulab Devi Chest Hospital, Lahore, Pakistan.

Garlic extract preparation

One kilogram (kg) of garlic (small cloves) was obtained and processed to get powder. The powder was soaked in 70% ethanol (Merck, Pakistan) for one week. After filtration by Whatman No.1 filter paper, filtrate was passed through 0.45 µm (Millipore) diameter pore size filter membrane to remove any impurity. EGE was prepared by maceration method by processing filtrate for extraction by rotary evaporator (Heidolph apparatus) to evaporate ethanol at Pakistan Council of Scientific and Industrial Research (PCSIR) laboratories, Lahore by a standard procedure (Thakare, 2004). EGE was semisolid, brown to black in colour with pungent smell. It was stored at -20ºC till use.

Preparation and inoculation for culture

All the cultural isolates were stained by Zeihl Neelson (ZN) technique and morphology of Acid fast bacilli (AFB) was confirmed by microscopy.

Inoculation of 7H9 middle brook medium

MGIT tubes (7ml 7H9 middle brook broth) were labeled with specimen number. MGIT growth supplement/ PANTA in a quantity of 0.8 ml was added aseptically to each MGIT tube with the help of a micropipette (Eppendroff). Followed by it 0.5 ml of growth suspension was added. All the procedures handled under Class II biological safety cabinet (NuAire, Class II BSC). All 20 samples were processed in duplicate for confirmation of results. The inoculated MGIT tubes (7ml media + 0.8 ml growth supplement and 0.5 ml suspension) were then incubated at 37ºC ± 1 in BACTEC MGIT 960 analyzer.

Detection of positive growth

In case of positive growth the instrument signals green at the exact location of tube (fluorescence based principle), the tube was removed and observed for growth which appeared as granular floccules and not very turbid, however, in case of contamination it appeared heavily turbid. The visual finding for positive growth was also confirmed by using MGIT UV detector. ZN staining was performed for all the positive tubes to confirm the presence of AFB.

Specific identification of isolated M. tuberculosis

Specific Identification of *Mycobacterium tuberculosis* was performed by *p*- Nitro Benzoic acid (PNB) test in which 500µg/ml PNB was incorporated in MGIT tubes. Two tubes were labeled for each specimen, one as GC (growth control) without PNB and other with PNB. The growth of MTB was inhibited at 500µg/ml concentration of PNB.

Determination of anti-mycobacterial activity of garlic

Quality control

One of pure isolate of MTB was used as quality control provided by Department of Microbiology at Gulab Devi Chest Hospital, Lahore.

Preliminary Screening of Garlic Extract against *M. tuberculosis*

The activity of EGE was screened against MTB quality control strain. MGIT 7H9 middle brook tubes were inoculated with 0.5 ml of culture growth and different concentrations of garlic extract from 0.5 to 3.0 mg/ml in parallel to GC (lacking garlic extract). The tubes were incubated at 37ºC in MGIT 960 analyzer after bar-code scanning. The growth of MTB was inhibited in garlic containing tubes except at 0.5 mg/ml.

Determination of MIC for EGE

Stock solution and concentration of garlic extract

Garlic extract was dissolved in Di-methyl sulfoxide (DMSO) which has excellent solvating property (Bordwell, 1988). Final concentration of stock solution of EGE was 10 % (2g of EGE in 20 ml DMSO). The following concentrations 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/ml of the garlic extract were tested to determine its MIC against MDR and non-MDR isolates of MTB.

Inoculation of middle brook 7H9 broth for MIC of EGE

On day 1 MGIT tubes were labeled with the respective concentration of EGE. To the GC tube 0.8 ml growth supplement was added, followed by 0.5 ml of 1:100 dilution of 0.5 McFarland adjusted suspension. No garlic

was added to GC tube. The tubes for garlic concentrations were inoculated with 0.8 ml of OADC and then with measured volumes of EGE. To each labeled tube of respective garlic concentrations 500 µl of 0.5 McFarland adjusted growth suspension was added. The tubes were incubated at 37ºC by putting the carrier racks; designed for multi tubes to be analyzed with same conditions in side the MGIT 960 analyzer.

Interpretation of results

The BACTEC 960 instrument monitored the inoculated media and gave results within 4-13 days (when Growth Control reaches Growth Unit 400 or more) once the test was completed. The tubes were detected by MGIT UV detector and also stained by ZN technique to confirm whether organism present or inhibited.

STATISTICAL ANALYSIS

The data was analyzed by using computer software, Statistical Package for Social Sciences (SPSS) 16.0 version.

RESULTS

The results of inhibitory effect of EGE showed that different isolates of MDR and non-MDR MTB were inhibited at different concentrations of garlic extract ranging from 1.0-3.0 mg/ml. Most of MDR isolates were inhibited at 2.0 mg/ml of garlic extract while maximum inhibition of non-MDR was at concentration of 1.5 mg/ml (table 1). Table-2 represents MIC range of garlic extract on 20 isolates of MTB. MIC_{90} and MIC_{100} for MDR isolates were same as 3.0 mg/ml while for non-MDR isolates these were >2.5 and 3.0 mg/ml respectively.

Fig. 1 shows the cumulative percentage of MDR isolates of MTB inhibited at various concentrations of EGE. 100% MDR (n=15) were inhibited at 3.0 mg/ml. Fig. 2 shows the cumulative percentage of non-MDR isolates of MTB which was 100% at MIC of 3.0 mg/ml of garlic concentration.

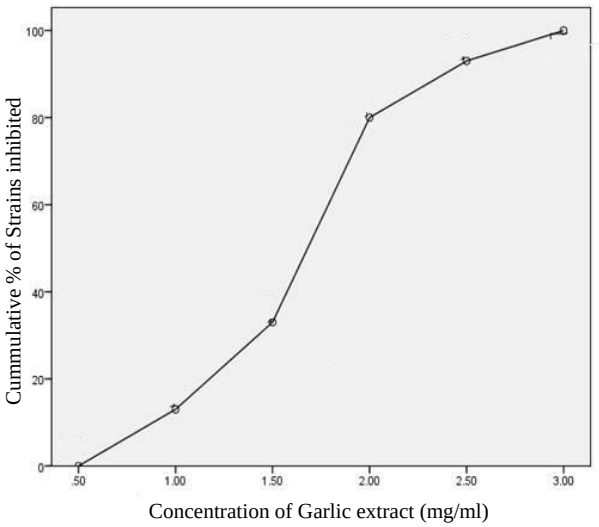

Fig. 1: Cumulative Percentage of Multi-drug resistant *M. tuberculosis* (n=15) inhibited at different concentrations of garlic extract

The cumulative percentage of MDR isolates of MTB which is 100%, 93%, 80%, 33%, and 13% at concentrations 3.0, 2.5, 2.0, 1.5, 1.0 mg/ml respectively.

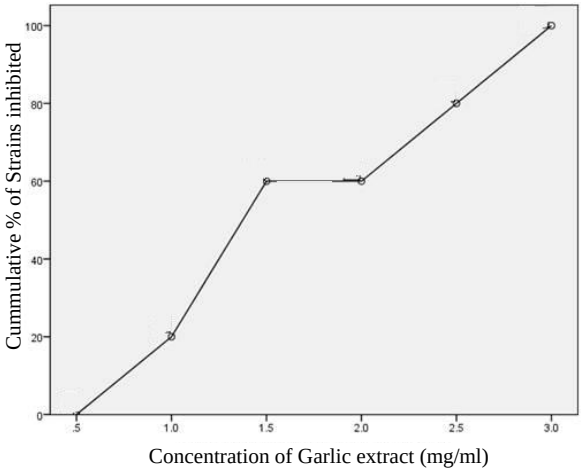

Fig. 2: Cumulative Percentage of Non-Multi-drug resistant *M. tuberculosis* (n=5) inhibited at different concentrations of garlic extract

The cumulative percentage of non-MDR isolates of MTB which is 100%, 80%, 60%, and 20% at concentrations 3.0, 2.5, 2.0 & 1.5, 1.0 mg/ml respectively.

Table 1: Inhibition of *M. tuberculosis* with garlic extract

Isolates of MTB (number)	Concentrations of garlic (mg/ml)					
	0.5	1.0	1.5	2.0	2.5	3.0
MDR (n=15)	Nil	02	03	07	02	01
Non-MDR (n=05)	Nil	01	02	Nil	01	01

Table 2: Minimum inhibitory concentrations of garlic extract against *M. tuberculosis* (n=20)

Isolates of MTB (number)	MIC of garlic (mg/ml)			
	Range	MIC_{50}	MIC_{90}	MIC_{100}
MDR *M. Tuberculosis* (n=15)	1.0-3.0	> 1.5	3.0	3.0
Non-MDR *M. Tuberculosis* (n=05)	1.0-3.0	> 1.0	>2.5	3.0

DISCUSSION

This study demonstrated that the garlic extract inhibited all MDR (n=15) and non-MDR (n=5) isolates of MTB at a concentrations ranging from 1.0-3.0 mg/ml. Garlic has showed the same effects against both MDR and non-MDR isolates. It might be due to the difference in mechanisms of action of garlic; because the other drugs have single mode of action but garlic has been reported to have multi-factorial mechanisms due to various constituents that confer their effects at simultaneously (Bergner, 1996).

Our results are in accordance with studies already done. In one of the previous studies, garlic extract inhibited six isolates of MTB at concentration of 1.34-2.68 mg/ml and Mycobacteria other than tuberculosis (MOTT) at 1.34-3.35 mg/ml (Delaha and Garagusi, 1985). Another study demonstrated the garlic activity against MOTT at MIC of 1.0-3.0 mg/ml (Abbruzzese *et al*., 1987). However in contrast to this study, Rao et., al showed the inhibition of MTB at 2 mg/ml but he used only a single isolate (Rao *et al*., 1946). Deshpand *et al*., reported MIC of 1.0 mg/ml for aqueous garlic extract against MOTT (Deshpande *et al*., 1993).

The possible explanations for this difference in results among the studies might be due to various species of garlic which differ in concentration of active constituents, as the garlic cropped in China may have twice allicin as much as in Europe or United States (Lawson *et al*., 1991). On the other hand we have evaluated 20 isolates of MTB, while in previous studies only few isolates were studied, in which three isolates at 1.0 mg/ml, five at 1.5 mg/ml, while in aggregation 75% of MTB were inhibited at 2.0 mg/ml. The difference in inhibition of MTB isolates may confer presence of different genetic strains in of MTB in the studied samples.

A group of scientists also reported activity of purified allicin as anti-tuberculous agent against isolates of MTB with low MIC (Ratnakar and Murthy, 1996) but the present study was assumed that the crude extract retains its inhibitory activity due to various constituents against which resistance development might be difficult (Rabinkov *et al*., 1998).

It is worthwhile to develop new techniques and the guidelines for standardization of techniques implied for plant extracts analysis so that inter-study outcome can be safely measured up. It is also a need of hour to investigate extracts of allium species of different geographical locations for the most active ingredients responsible for their antibacterial activity.

CONCLUSION

This study demonstrated that the garlic extract has showed its effectiveness against clinical isolates of MDR *M. tuberculosis*. It is worthwhile to utilize garlic as natural supplement with other standard ATT. It is corresponding that substitute medicines practices with plant extracts including garlic as a means of decreasing the burden of drug resistance and reducing the cost of management of diseases would be of public health importance.

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