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Research Article

ANTIBACTERIAL ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF GARLIC AGAINST NEW STRAINS OF PATHOGENIC BACTERIA: AN *in vitro* STUDY

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ABSTRACT: The aqueous, ethanolic and methanolic extracts of garlic at different concentration were tested against two new strains of *Bacillus* species isolated from poultry farm. Among all the extracts of garlic tested, the ethanolic extracts showed increased inhibitory effect with maximum zone of inhibition of 21 mm against *Bacillus licheniformis* strain BIHPUR 0104. The aqueous extracts were more effective compared to methanolic extracts against *Bacillus subtilis* strain AK but methanolic extracts were showing more inhibitory effect than aqueous extracts against *Bacillus licheniformis* strain BIHPUR 0104. The ethanolic and methanolic extracts of garlic were active even at low concentration (5%) against *Bacillus licheniformis* strain BIHPUR 0104. Active compounds of garlic ethanol extracts were separated by Thin layer chromatography using Butanol:Ethanol (9:1 and 1:1) as eluting solvent and Rf values were calculated of the spots obtained. These results suggest that garlic is a potential spice to control pathogenic bacterial strain.

Key words- Disc diffusion method, Garlic, Poultry farm bacteria, thin layer chromatography.

INTRODUCTION

Herbs and spices have been used for their antimicrobial properties in preventing food spoilage and pathogenic diseases. Garlic (*Allium sativum*) had been used worldwide to fight bacterial infections as it exhibited a broad antibiotic spectrum against both Gram(+) and Gram(-) bacteria (Sadeghian A *et al*., 2002). A bioactive compound in garlic that has antibacterial activity is allicin, which is a volatile compound containing sulphur (Harris JC *et al*., 2001). Other bioactive compounds namely dialildisulphide and dialiltrisulphide have antibacterial activity (Avoto P *et al*., 2000). Garlic has been used since ancient times in India and China for a valuable effect on the cardiovascular disease (Gardner C *et al*., 2003). Garlic has also proposed to treat asthma, candidiasis, colds, diabetes and antibacterial effect against food borne pathogens like *Salmonella, Shigella* and *Staphylococcus aureus* (Taferi G *et al*., 2002). According to Ayurvedic and Greek system of medicine garlic is one of the established remedies for tuberculosis. A few studies have also been proved that garlic has antimycobacterial activity against different species of *Mycobacteria* (Gupta RL *et al*., 1999). Bacteria present in the poultry farm are the causative agents of food poisoning, food spoilage, stomach pain, vomiting etc. Infections attributed to *B. subtilis* include bacteremia, endocarditis, pneumonia and septicemia. There also have been several reported cases of food poisoning attributed to large numbers of *B. subtilis* contaminated foods. *B. subtilis* does produce the extracellular enzyme "subtilisin" that has been reported to cause allergic or hypersensitivity reactions in individuals repeatedly exposed to it. *B. licheniformis* is associated with food spoilage, poisoning and septicaemia (having a large amount of bacteria in the blood). The symptoms of infection through *B. licheniformis* include stomach pains, acute diarrhea, vomiting and ophthalmitis (inflammation of the eye). The present study deals with the antibacterial activity of different solvent extracts of garlic at different concentrations against the new strains of poultry farm bacteria and isolation of active biological compounds from the crude extracts by Thin layer chromatography.

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MATERIALS AND METHODS

Sample collection and isolation

Samples (surface soil) were collected from poultry farm were brought to the laboratory in aseptic condition. 1 gram of surface soil sample was suspended in 9 ml of saline and mixed vigorously to make uniform suspension. After that soil samples were serially diluted up to 10^{-5} and 0.1ml of aliquots were spread over nutrient agar plates from 10^{-4} and 10^{-5} dilution. The plates were incubated at 37ºC for 24 hours. Pure strains were picked out and purified by repeated streaking on nutrient agar slants. The culture was streaked on slants and kept in incubator at 37°C for 24 hours and were preserved in slants at 4±2°C.

Biochemical and morphological characterization

Purified isolates were characterized by biochemical analysis using Indole test, Methyl Red test, Voges Proskaver test, Citrate utilization test, Catalase test and Urease test. Gram staining and Motility test were performed under Morphological test.

Genomic DNA isolation

2 ml of bacterial culture were centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded. 1 ml of UniFlex™ Buffer 1 and 10 µl of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 minutes at 37ºC in a water bath. To the lysed samples 1 ml of 1:1 phenol:chloroform were added and mixed well. The samples was centrifuged at 10,000 rpm for 15 minutes at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of UniFlex™ Buffer 2 were added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant was discarded. The pellet was air dried for about 10-15 minutes till the ethanol evaporate. The pellet was resuspended in 50-100 µl of UniFlex™ Elution Buffer. DNA was stored at -20ºC.

PCR amplification and sequencing

The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with *Taq* DNA polymerase and primers 27F (5` AGTTTGATCCTGGCTCAG 3`) and 1492R (5`ACGGCTACC TTGTTACGACTT 3`). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The same primers as above were used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http:// www.ncbi-nlm-nih.gov/. The DNA sequences were aligned and phylogenetic tree was constructed by using the Molecular Evolution Genetic Analysis (MEGA) software version 4.0. 16S rRNA sequence was then submitted to the GenBank, NCBI, USA.

Preparation of aqueous, ethanolic and methanolic extracts of Allium sativum

Garlic (*Allium sativum*) was purchased from local market of Nungambakkam, Chennai (India). *Allium sativum* bulbs (200 gm) were washed first by distilled water and then by 95% ethanol. *Allium sativum* bulbs were homogenized using sterile mortar and pestle. After that it was sieved through eight layers of sterile mesh cloth. These filtered extracts were centrifuged at 8000 rpm for 10 minutes. Supernatents were collected as 100% extracts. These extracts were stored at 4°C in refrigerator for further use. Extracts of *Allium sativum* were diluted to make different concentrations such as 75%, 50%, 25% and 5% by mixing with appropriate volume of sterile double distilled water. The ethanolic and methanolic extracts were prepared following the same procedure with the exception of solvent which was 95% ethanol and methanol instead of sterilized double distilled water.

Microorganisms used

Bacillus subtilis strain AK (Accession no.-KC414759) and *Bacillus licheniformis* strain BIHPUR 0104 (Accession no.-KC424492) isolated from poultry farm were used.

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Antibacterial sensitivity test using disc diffusion method

The microorganisms (*Bacillus subtilis* strain AK and *Bacillus licheniformis* strain BIHPUR 0104) grown in Nutrient broth were transferred to Mueller Hinton Agar plates with the help of cotton swabs. Sterile discs of 6 mm diameter were used for soaking the different solvent extracts of garlic at different concentrations. The soaked discs were transferred aseptically to the plates seeded with the microorganisms with the help of forceps. The petriplates were incubated in upright position at 37°C for 24 hours. After 24 hours zone of inhibition formed by different solvent extracts of garlic at different concentrations against the tested microbes were measured.

Separation of active fractions from ethanolic extracts of *Allium sativum*

The ethanolic extracts of *Allium sativum* were subjected to active compound separation by Thin layer chromatography (TLC) using silica gel as adsorbent. A line was drawn on the TLC plates at a distance of 2 cm from the base. The sample was spotted on with the help of capillary tube and it was allowed to dry. The plates were placed in two different jars having mobile phase (Butanol:Ethanol) with different ratios (9:1 and 1:1). After the solvent reaches more than half of the TLC plate it is taken out of the jar. The solvent run was drawn. The TLC plates were examined under the UV lamp and spots of the active compounds were circled with pencil. The spots were labeled and the distances from the origin were measured. The Rf values were calculated by the given formula

$$\text{Retention factor (Rf)} = \frac{\text{Distance travelled by solute from origin}}{\text{Distance travelled by solvent from origin}}$$

RESULTS

In this study the microorganisms isolated from poultry farm were identified as new strains of bacteria according to morphological, biochemical characteristics and 16S rRNA gene sequencing. The aqueous, ethanolic and methanolic extracts of garlic inhibited the growth of *Bacillus subtilis* strain AK and *Bacillus licheniformis* strain BIHPUR 0104. The maximum activity was noted by ethanolic extracts of garlic. Ethanolic extracts of garlic were found to be more effective compared to aqueous and methanolic extracts against *Bacillus subtilis* strain AK and *Bacillus licheniformis* strain BIHPUR 0104 with maximum zone of inhibition of 19.5 mm and 21 mm respectively (Table 1 and 2). Aqueous extracts of garlic was showing more zone of inhibition compared to methanolic extracts against *Bacillus subtilis* strain AK (Fig-a). Methanolic extracts of garlic was showing more inhibitory effect on *Bacillus licheniformis* strain BIHPUR 0104 compared to *Bacillus subtilis* strain AK at 5%,and 25% concentration. The ethanolic and methanolic extracts were active even at 5% concentration with minimum zone of inhibition of 8 mm against *Bacillus licheniformis* strain BIHPUR 0104 (Fig-d and f). These extracts were not showing any zone of inhibition at the same concentration against *Bacillus subtilis* strain AK (Fig-c and e). *Bacillus subtilis* strain AK and *Bacillus licheniformis* strain BIHPUR 0104 were found to be resistant to aqueous garlic extracts at 5% concentration. These two strains were susceptible at 25% concentration showing zone of inhibition of 11 mm and 10 mm (Fig-a and b). 100% garlic extracts were showing almost same zone of inhibition against both the strains. Separation of active compounds from the ethanolic extracts of garlic was detected by Thin layer chromatography by using Butanol:Ethanol as eluting solvent at different ratios (9:1 and 1:1) and Rf values were determined as 0.25, 0.24, 0.48 and 0.87 (Table 3).

Table 1: Antibacterial activity of different solvent extracts of *Allium sativum* at different concentrations against *Bacillus subtilis* strain AK by disc diffusion method (in mm).

Concentration	Aqueous extracts	Ethanolic extracts	Methanolic extracts
5%	-	-	-
25%	11	13	10
50%	14	16	12
75%	16	18	15
100%	19	19.5	19

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Table 2: Antibacterial activity of different solvent extracts of *Allium sativum* at different concentrations against *Bacillus licheniformis* strain BIHPUR 0104 by disc diffusion method (in mm).

Concentration	Aqueous extracts	Ethanolic extracts	Methanolic extracts
5%	-	08	08
25%	10	14	12
50%	15	16	15
75%	17	19	17
100%	19	21	19

Table 3: TLC profile of ethanolic extracts of garlic by using mobile phase (Butanol:Ethanol) at different ratios

Mobile phase	No. of spots visible by UV	Rf values
Butanol:Ethanol (9:1)	1	0.25
Butanol:Ethanol (1:1)	3	0.24 0.48 0.87

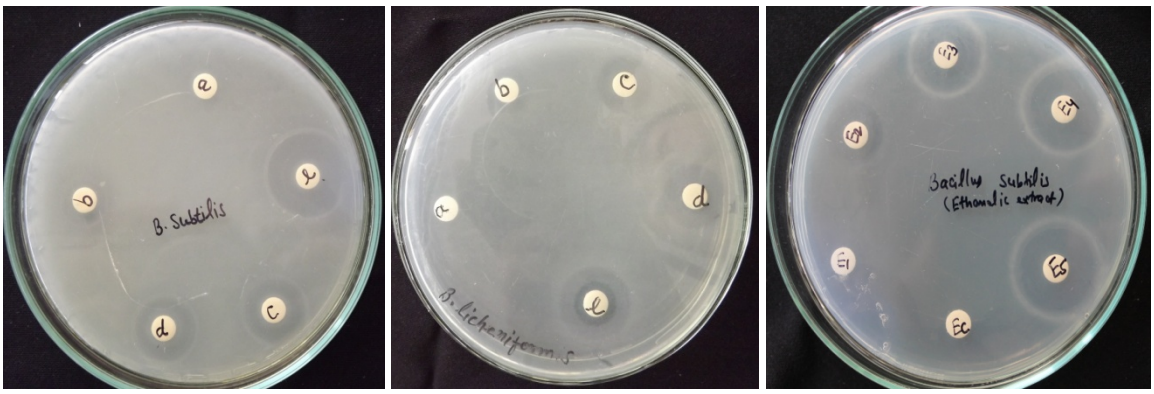

Fig: (a)= *B. subtilis* strain Fig: (b)= *B. licheniformis* strain Fig: (c)= *B. subtilis* strain

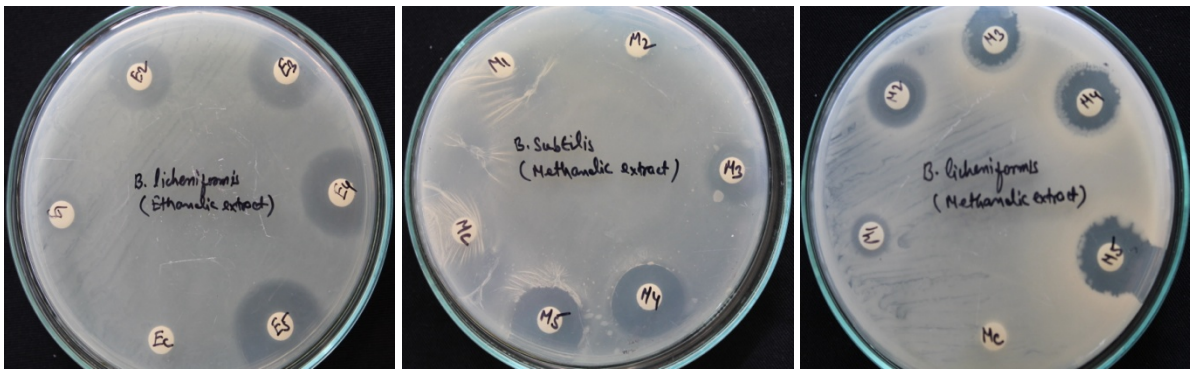

Fig: (d)= *B. licheniformis* strain Fig: (e)= *B. subtilis* strain Fig: (f)= *B. licheniformis* strain

(a, E1, M1= 5% Concentration ; b, E2, M2= 25% ; c, E3, M3= 50% ; d, E4, M4= 75% ; e, E5, M5=100%; Ec, Mc= Negative controls).

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DISCUSSION

De Boaer *et al* (2005) reported that successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. The result from the present study revealed that ethanolic extracts of the selected plant (garlic) were much better and active due to the better solubility of the active compounds in organic solvents. This finding support De Boer *et al* (2005) who demonstrated the better solubility of the active compounds in organic solvents. Antibacterial activity of aqueous, ethanolic and methanolic extracts of garlic against *Bacillus subtilis* strain AK and *Bacillus licheniformis* strain BIHPUR 0104 was increased as the extracts concentration increased. This results favour the findings of Safithri *et al* (2011). Mukhtar S *et al* (2012) reported that aqueous extracts of garlic was more effective as compared to ethanolic extracts against *Bacillus subtilis* DSM 3256 at all concentration. In our study ethanolic extracts of garlic (*Allium sativum)* were found to be more effective as compared to aqueous and methanoic extracts against *Bacillus subtilis* strain AK and *Bacillus licheniformis* strain BIHPUR 0104 at all concentration. The ethanolic extracts showed better results as compared to aqueous as being organic dissolves more organic compounds resulting in the release of greater amount of active antimicrobial components. This finding agrees with Cowan (1999). Karuppiah *et al* (2012) reported that the ethanolic extracts of garlic cloves showed large diameter of zone of inhibition against *Bacillus* species. In our study ethanolic extracts of garlic were found to be most effective against the new strains of *Bacillus* species isolated from poultry farm. Durairaj S *et al* (2009) reported that at 100% concentration of garlic extracts, the maximium zone of inhibition was observed against *Bacillus subtilis*. In this investigation 100% garlic extracts were showing almost same inhibitory effect against *Bacillus subtilis* strain AK and *Bacillus licheniformis* strain BIHPUR 0104. This study agrees with the finding of Durairaj S *et al.* Kanaki *et al* (2005) proposed that TLC method was found to be precise, specific and accurate for routine quality control of garlic and its formulation. In the present investigation, active compounds were detected from ethanolic extracts of garlic through TLC method. The result of present investigation revealed that garlic is a potential spice to inhibit the growth of *Bacillus subtilis* strain AK and *Bacillus licheniformis* strain BIHPUR 0104 isolated from poultry farm. The ethanolic extracts of garlic were found to be more effective compared to methanolic and aqueous extracts against both the strains. As both the strains of *Bacillus* species are pathogenic to human being so garlic can be a potential spice for the treatment of infections to the people who are continuously exposed to these microbes in the poultry farm. Garlic is a good spice as a substitute of antibiotics. New drugs can be synthesized from the active compounds of garlic as being effective and easily available. The drugs can be used for the treatment of the workers who might be infected from these two new strains of *Bacillus* species. Antibiotics were used for therapy, but continuous use of antibiotics against these bacteria may result as a resistant microorganisms. So garlic offers a new source of antibacterial agent. From this result it is clear that the medicinal value of garlic is compared to antibiotics.

CONCLUSION

It is concluded from this study that garlic extracts have antimicrobial activity against *Bacillus subtilis* strain AK and *Bacillus licheniformis* strain BIHPUR 0104. Since the introduction of antibiotics causes increase in the resistance of many bacterial pathogens. Hence garlic can be a potential spice as a substitute of antibiotics. Finally, studies on the mode of action and interaction with antibiotics of the garlic extracts should be identified. A detailed study is needed about the physiological role and antibacterial activity of garlic.

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