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Original Research Article

Synergistic Antibacterial Effect of Tea Leaves Extract and Penicillin Produced by *Penicillium chrysogenum* Isolated from Decaying Fruits and Vegetables

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ABSTRACT

There are indications that some herbal materials including tea, zinger, garlic, tulsi and neem can act as antibiotic resistance inhibitors. Combinations of some herbal materials and different antibiotics might affect the inhibitory effect of these antibiotics. The antimicrobial and resistance modifying potentials of herbal plants is due to of naturally occurring flavonoids and polyphenolic compounds. In this study it was evaluated that the combination of tea extract along with penicillin gave synergistic effect against various pathogenic bacterial strains. A total of 5 *Penicillium chrysogenum* isolated from different decaying fruit and vegetables. All the isolates were screened for penicillin production under different conditions. The production medium supplemented with 2% maltose as carbon source, at pH 6 it gave maximum zone of inhibition at suggested maximum production of antibacterial compound. 100M tea extract gave maximum zone of inhibition. The combination of penicillin with tea extract in ratio of 6:4, 5:5 and 4:6 gave maximum zone of inhibition showing optimum antibacterial effect, if used alone it does not yield such an effective result.

1. INTRODUCTION

Usually microbiologists distinguish two group of antimicrobial agents used in the treatment of infectious disease: antibiotics, which are natural substances produced by certain groups of microorganisms, and chemotherapeutic agents, which are chemically synthesized. In the therapeutic and pharmaceutical worlds, all these antimicrobial agents used for the treatment of infectious disease are termed as antibiotics, some antibiotics are prepared synthetically, but most of them are prepared by microbial biosynthesis [1]. Penicillin is a group of antibiotics sometimes abbreviated as PCN or PEN derived from Penicillium fungi. This group includes Penicillin G, Procaine penicillin, Benzathine penicillin and Penicillin V. Penicillin antibiotics are traditionally important since they are the first drugs that were effective against several serious diseases, such as syphilis, and infections caused by Staphylococci and Streptococci, ear, nose, throat infections, respiratory and urinary infections, prostrate infections and septicaemia. All penicillins are β-lactam antibiotics and are widely used for the treatment of bacterial infections usually caused G+, bacteria. Penicillin belongs to the group of beta-lactam antibiotics and is produced as secondary metabolites by specific actinomycetes and certain species of Penicillium [2].

Penicillium chrysogenum plays a significant role in the medical community as it produce an antibiotic whose mechanism of action is the inhibition of bacterial cell wall synthesis. Penicillin acts by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in G+ organisms [3]. *Penicillium chrysogenum* is a heterotrophic, brush like greenish fungus, commonly inhabitant in temperate and subtropical regions. Penicillium grows at its peak

during the spring and winter, but the mold has no seasonal variation. It is often referred to as medical mold. *Penicillium chrysogenum* is a cryophilic fungus form shiny bluish green colony surrounded by white mycelium [4]. The asexual spores or conidia originate from complexes known as conidiophores [5]. It can also be found on spoiled food products, soil but is mostly found in indoor environments especially in damp or water damaged surfaces such as damp building materials, walls, floor, paint, carpet mattress and upholstered furniture dust. This widely occurring mold grows abundantly on easily decomposable carbohydrates [6].

Antimicrobials have been tempered by the emergence of bacterial strains with resistance to antibiotic therapies. Antimicrobial resistance is not new but number of resistant organisms and the geographic locations affected by drug resistance [7]. Drug resistance presents an ever increasing global health threat that involves all major microbial pathogens and antimicrobial pathogens and antimicrobial drugs [8].

The multi drug resistant pathogens are capable of rapid and efficient horizontal transmission of genes encoding antibiotic resistance determinants [9]. There are indications that some herbal materials can act as antibiotic resistance inhibitors [10]. Combinations of some herbal materials and different antibiotics might affect the inhibitory effect of these antibiotics [11]. The antimicrobial and resistance modifying potentials of naturally occurring flavonoids and polyphenolic compounds have been reported in other studies [12].

2. MATERIALS AND METHODS

2.1 Material used

All the chemicals and media used were procured from Loba chemical and Hi media were of analytical grade. Media used were dehydrated and used as per manufacturer's direction. All the glassware likes Petri plates, flasks, test tubes and beakers etc. used were made of borosilicate. Laboratory isolates were used as test organisms.

2.2 Collection of sample

10 different samples were collected from spoiled fruits and vegetables for the isolation of *Penicillium chrysogenum*.

2.3 Isolation of *Penicillium chrysogenum*

Sabouraud's glucose agar was added in 100 ml distilled water as per manufacture's re-commendation. The fruiting bodies of mold were grasped from the sample and spot inoculated on Sabouraud's glucose agar plates with the help of loop. The plates were incubated in an inverted position for 7days at 25 -28°C [13].

2.4 Characterization of fungal isolates

2.4.1 Microscopic examination

A drop of Lacto-phenol cotton blue dye was poured on the glass slide and fungus was placed over it with the help of needle. The slide was examined under low and high power objective after covering it with cover slip [13].

2.4.2 Antimicrobial activity

Muller Hinton agar plates were prepared for testing the antimicrobial activity of *Penicillium chrysogenum*. 10 µl of 24 hours broth culture of Staphylococcus aureus, *Pseudomonas aeruginosa*, *Bacillus subtilis* and Escherichia coli was evenly spread using sterile spreader on each plate respectively. Wells of 6 mm in diameter were made aseptically on each agar plate with the help of cork bore. The mold was taken from the isolation plates and placed in the wells. The plates were incubated at 37°C for 24 hours. Clear zone around the well indicated the production of antibacterial compounds and selected for further study.

2.5 To check the production of Penicillin

2.5.1 Penicillin production using different carbon sources

The Erlenmeyer's flasks were taken for the preparation of production media. All the three production media consisted of 0.68 mol/L KH_2PO_4, 0.242 mol/L NH_4Cl, 0.06 mol/L phenyl acetic acid, 0.79 mol/L K_2SO_4, 0.05 mol/L $MgSO_4.7H_2O$, 6 mol/L ammonia and 2 mol/L citric acid. The carbon source for media 1 was 2g/100mL maltose, media 2 was 2g/100mL dextrose and 3 was 2g/200mL lactose. The flasks along with the production media were sterilized at 15 psi and 121°C for 20 mins. The flasks were transferred to the clean air laminar flow chamber and spores of *Penicillium chrysogenum* were inoculated. After the inoculation the flasks were

kept in a shaking incubator at 25°C and 50 rpm for 3-5 days [4].

2.5.2 Penicillin production on different pH

Four production media were prepared by taking 2g/100mL of maltose, 0.68 mol/L KH2PO4, 0.242 mol/L NH4Cl, 0.06 mol/L phenyl acetic acid, 0.79 mol/L K2SO4, 0.05 mol/L MgSO4.7H2O, 6 mol/L ammonia and 2 mol/L citrate.

The production media were sterilized at 121°C and 15 psi for 20-45 mins. The flasks were allowed to cool at room temperature in laminar air flow chamber. The pH of flask 1 was set at 5, flask 2 at 6, flask 3 at 7 and flask 4 at 8 and spore inoculation was done. The flasks were maintained at 25°C and 50 rpm for 3-5 days in a shaking incubator.

2.6 Characterization of potential Penicillin producer

Muller Hinton agar plates were prepared and 10 µl bacterial cultures viz. Staphylococcus aureus, *Pseudomonas aeruginosa*, *Bacillus subtilis* and Escherichia coli were spreaded over the plates respectively. Three wells were made on each plate and 20 µl of the prospective penicillin from each flask 1, 2 and 3 was poured into the wells. Plates were kept in an incubator for 24 hours at 37°C [4].

2.7 Antimicrobial activity of tea extract

2.7.1 Tea extraction

20 grams of tea was weighed and added to 100 ml of distilled water. The mixture was boiled for 5mins and allowed to cool [14]. Tea extract was suspended in cold phosphate buffer saline (PBS) at 75M, 50M and 25M concentrations. The suspension was centrifuged at 2500 rpm for 10 minutes.

2.7.2 Antimicrobial assay

Muller Hinton agar plates were inoculated with 10 µl of broth culture of test organisms and evenly spread with bent sterile glass rod. Three wells were punched on each agar plate using sterile cork bore. 20 µl of tea extract from each concentration was added into each well. The plates were incubated at 37°C for 24 hours [15].

2.8 Antimicrobial assay with combination of green tea extract and penicillin

2.8.1 Stock solution of green tea extract and penicillin

Stock solutions were prepared in such a way that the mixture contains from ten parts of penicillin and zero parts of tea extract to zero parts of penicillin to ten parts of tea extract. Test tubes were taken and 10 ml of penicillin was added to the first test tube. 9 ml of penicillin and 1 ml, 8 ml penicillin and 2 ml tea extract, 7 ml of penicillin and 3 ml of tea extract, 6 ml of penicillin and 4 ml tea extract, 5 ml of penicillin and 5 ml of tea extract, 4 ml of penicillin and 6 ml of tea extract, 3 ml of penicillin and 7 ml of tea extract, 2 ml of penicillin and 8 of tea extract, 1 ml of penicillin and 9 ml of tea extract and 10 ml of tea extract was taken in the test tubes respectively.

2.8.2 Antimicrobial assay

The surface of Muller Hinton agar plates was inoculated with 10 µl of bacterial cultures. Three wells were aseptically punched on each agar plate using a sterile cork bore. 20 µl of each stock solution was placed in the wells. The plates were incubated at 37°C for 24 hours [15].

3. RESULTS AND DISCUSSION

3.1 Isolation

Samples from citrus fruit waste were inoculated on Sabouraud's glucose agar plates and fungal strains PC1, PC2, PC3, PC4 and PC5 having greyish-blue green colour were isolated after 7 days of incubation.

3.2 Characterization of *Penicillium chrysogenum*

3.2.1 Morphological characterization

Greyish-green colonies having velvety or cottony appearance were found. The colony diameter was 4 mm to 5 mm (Fig.1). The reverse for colonies was yellowish cream. The morphological characters of fungal colonies were found to be similar with that of *Penicillium chrysogenum*. Similarly, [13] has been reported that the fungal strain isolated from

citrus fruits and vegetables having green colour and reverse yellow was *Penicillium chrysogenum*.

3.2.2 Microscopic examination

Lacto-phenol cotton blue dye was used for microscopic examination of *Penicillium chrysogenum*. After staining, it was observed that the isolated fungal strain was brush shaped. Stipe was long and smooth. The branched conidiophores were arising from septate mycelium (Fig.2). *Penicillium chrysogenum* was confirmed after studying the microscopic characters of fungal isolates. Singled cell spores or Conidia were developed at the end of Sterigmata. It has been reported earlier that microscopic features of *Penicillium chrysogenum* were terverticillate penicilli and smooth, spherical to elliptical conidia [13].

3.3 Antimicrobial activity of *Penicillium chrysogenum*

Antimicrobial assay was applied for determining the antimicrobial potential of isolated *Penicillium chrysogenum*. The effect of *Penicillium chrysogenum* on different bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli* was observed and zone of inhibition formed was measured (fig.3&4).

3.4 Antimicrobial activity of potential Penicillin

During the antimicrobial testing of *Penicillium chrysogenum* isolates against Staphylococcus aureus the zone of inhibition formed by isolate PC1 was 8mm, PC2 was 7 mm, PC3 was 7.5 mm, PC4 was 8mm and PC5 was 7.5. In case of *Bacillus subtilis*, PC1 produced inhibition zone of 6mm, PC2 formed 6.5 mm, PC3 produced zone of 6mm, PC4 formed 7 mm and PC5 formed 6.5mm.

On the culture of *Pseudomonas aeruginosa*, zone of inhibition formed by PC1 was 7.5mm, PC2 was 7mm, PC3 was 7.5mm, PC4 was 6.5mm and PC5 was 7mm (Fig.4). In case of *Escherichia coli*, the zone of inhibition formed by isolate PC1 was 8mm, PC2 was 8.5mm, PC3 was 8mm, PC4 was7.5mm and PC5 was 7.5 mm.

All the isolates of *Penicillium chrysogenum* showed antimicrobial activity and are considered to be highly effective against microorganisms. Thus, these isolates can be used for the production of Penicillin. It has been shown in a similar work that cultural extracts of *Penicillium chrysogenum* exhibited antibacterial effects on *E. coli*, *B. subtilis*, *P. aeruginosa* and *Klebsiella pneumoniae*. The degree of inhibition on the growth of bacterial isolates varied [16]. Penicillin was produced from *Penicillium chrysogenum* at variable pH and different carbon sources. Diffusion method was used to analyse the antimicrobial activity of Penicillin. The zone of inhibition produced by Penicillin was measured to determine its effectiveness against microorganisms.

3.5 Effect of carbon sources

The antimicrobial potential of penicillin was determined on different test organisms by antimicrobial assay. In case of maltose, the zone of inhibition was 8 mm in case of Staph aureus, *Bacillus subtilis* was 9 mm, *Pseudomonas aeruginosa* was 8.5 mm and *Escherichia coli* was 8 mm.

When dextrose was used as a carbon source, the inhibition zone formed by Penicillin was 6.5 mm on plates inoculated with *Staphylococcus aureus*, 7.5 mm in *Bacillus subtilis*, 7 mm in *Pseudomonas aeruginosa* and 6 mm in *Escherichia coli*. Zone of inhibition in case of *Staphylococcus aureus* was 4.5mm, *Bacillus subtilis* was 6.5 mm, *Pseudomonas aeruginosa* was 6 mm and *Escherichia coli* was 5 mm when lactose was used as carbon source (Fig.5)

Maximum zone of inhibition was formed by maltose followed by dextrose and lactose respectively. Maltose enhanced the production rate of Penicillin and can be considered as competent carbon source. Dextrose showed less productivity as comparison to maltose. The effect of carbon source was least observed in production media containing lactose. The results showed that carbon source is the one of main factors for determining growth of Penicillium and play an important role in Penicillin production.

It has been reported that different production media showed varied range of growth of Penicillum. Lactose media showed less zone of inhibition which reflected that Penicillin production was unstable and the optimum production of Penicillin

was obtained in maltose which formed highest zone of inhibition [4].

3.6 Effect of pH

The antimicrobial activity of penicillin at different pH was observed by measuring the zone of inhibition formed by Penicillin. At pH 5, the zone of inhibition was 7mm in case of Staphylococcus aureus, *Bacillus subtilis* was 8mm, *Pseudomonas aeruginosa* was 7.5mm and Escherichia coli was 7mm. At pH 6, the Penicillin produced inhibition zone of 9mm on plates inoculated with S. aureus, 9mm in B. subtilis, 8.5mm in P. aeruginosa and 8mm in E. coli. Zone of inhibition in case of S. aureus was 6.5mm, B. subtilis was 7mm, P. aeruginosa was 6mm and E. coli was 5.5mm at pH 7. When the pH was set at 8, the zone of inhibition of formed in case of S. aureus was 4mm, B. subtilis was 5mm, P. aeruginosa was 4.5mm and E. coli was 4mm (Fig.6).

The maximum antimicrobial activity of Penicillin was observed when produced at pH 6 and minimum antimicrobial activity was observed at pH 8. With the increase and decrease in the pH, there was decrease in the effectiveness of Penicillin against bacteria. It has been reported in earlier work that the pH of production media was adjusted at 6 throughout the process of production. Experimental results presented inhibited Penicillin production with change in pH [17]. Thus, the optimum pH of the production of Penicillin was 6.

3.7 Antimicrobial activity of tea extract

Dilutions of tea extract were prepared for determining its antimicrobial effect at different concentrations. Antimicrobial activity of tea extract was calculated by observing the zone of inhibition formed by tea extract on Muller Hinton agar plates inoculated with Staphylococcus aureus. In case of 100M concentrate tea extract, inhibition zone of 6mm was obtained. Zone of inhibition was 4.5mm in 75M, 3mm in 50M and 2.5mm in 25M concentrated tea extract (Fig.7)

Tea extract showed inhibitory effect on S. aureus but the degree of effectiveness varies with the change in concentration. The 100% concentration tea extract was most effective against bacteria and 25% was having least antimicrobial potential among all the concentrations. In a similar work, it was reported that with the decrease in concentration, the antimicrobial potential of tea extract also decreases [15]. It has been reported that all the tea extracts from the different solvents were a potential source of antimicrobial substance for drug development for use against potential pathogens [18].

3.8 Antimicrobial activity of combination of tea extract and penicillin

The combined effect of tea extract and Penicillin was seen on Muller Hinton agar plates inoculated with Staphylococcus aureus. The antimicrobial activity of stock solutions from 10 parts of Penicillin and zero parts of tea extract to zero parts of Penicillin and 10 parts of tea extract was observed and zone of inhibition was measured (Table-1).

The zone of inhibition produced by ten parts of Penicillin and zero part of tea extract (10:0) was 8mm. At 9:1, inhibition zone of 9mm was formed. Zone of inhibition was 10.5mm at 8:2 and 11mm at 7:3. Inhibition zone created was 12mm at 6:4, 5:5 and 4:6. At 3:7, 10mm of inhibition zone produced. 9mm of inhibition zone was formed at 2:8, 7.5mm at 1:9 and 6mm at 0:10 of Penicillin and tea extract.

Tea extract enhance the inhibitory effect of Penicillin against potential pathogens. With the addition of tea extract, the bacterial growth can be rendered up to greater extent. These results confirmed the synergistic effect of Penicillin and tea extract. Tea extract can be used to enhance the antimicrobial activity of Penicillin and as antibiotic resistance modifying agent. It has been reported that there was increase in antimicrobial activity with the increased concentration of penicillin while in case of tea extract, the antimicrobial potential was decreased with decreasing concentration of tea extract. Tea contains polyphenols which have antimicrobial activity against broad range of bacteria. Tea enhanced the activity of Penicillin in an additive way [14].

Synergistic intraction between the two antimicrobial agents means that their combined effect is stronger than the sum of effects of the individual agents. The zones of inhibition in antibiotic-plant extact plates were in the range of 0.5-11.5mm wider than the zones of inhibition in the control plates depending on the species of bacteria [19].

CONCLUSION

Our study concluded that the production medium supplemented with 2% maltose as carbon source, at pH 6 it gave maximum zone of inhibition at suggested maximum production of antibacterial compound. 100M tea extract gave maximum zone of inhibition. The combination of penicillin with tea extract in ratio of 6:4, 5:5 and 4:6 gave maximum zone of inhibition showing optimum antibacterial effect, if used alone it does not yield such an effective result.

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Fig.1. Showing isolates of *Penicillium chrysogenum*

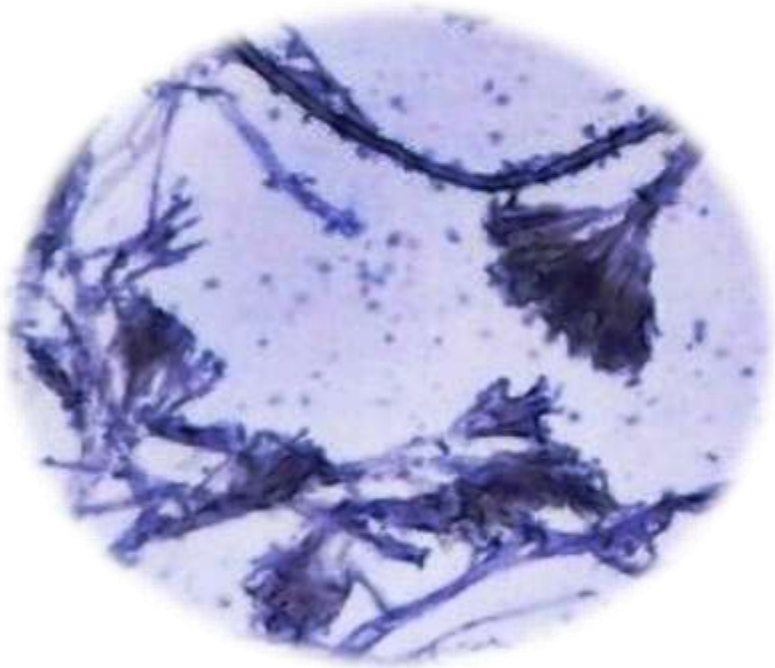

Fig.2. Showing microscopic view of *Penicillium chrysogenum*

Fig.3. Showing the antimicrobial activity of isolated *Penicillium chrysogenum*

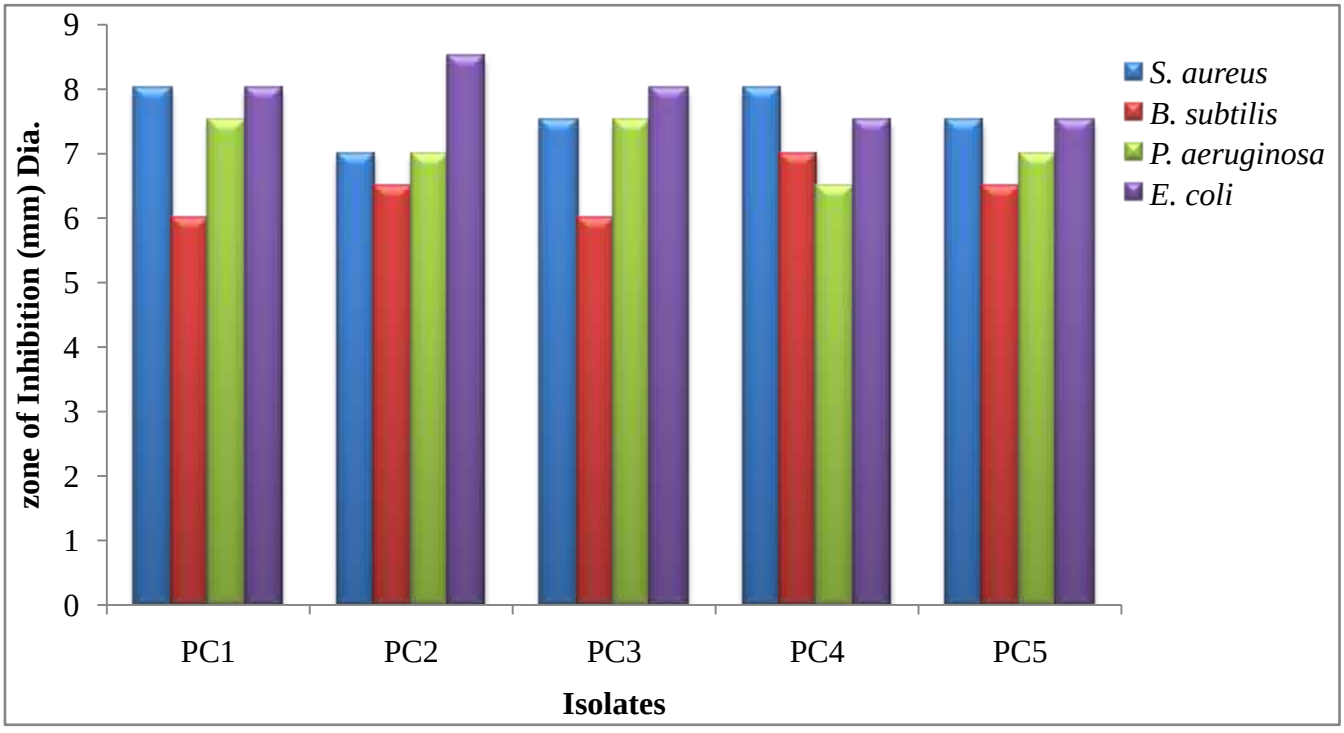

Fig.4. showing the antimicrobial activity of isolates of *Penicillium chrysogenum*

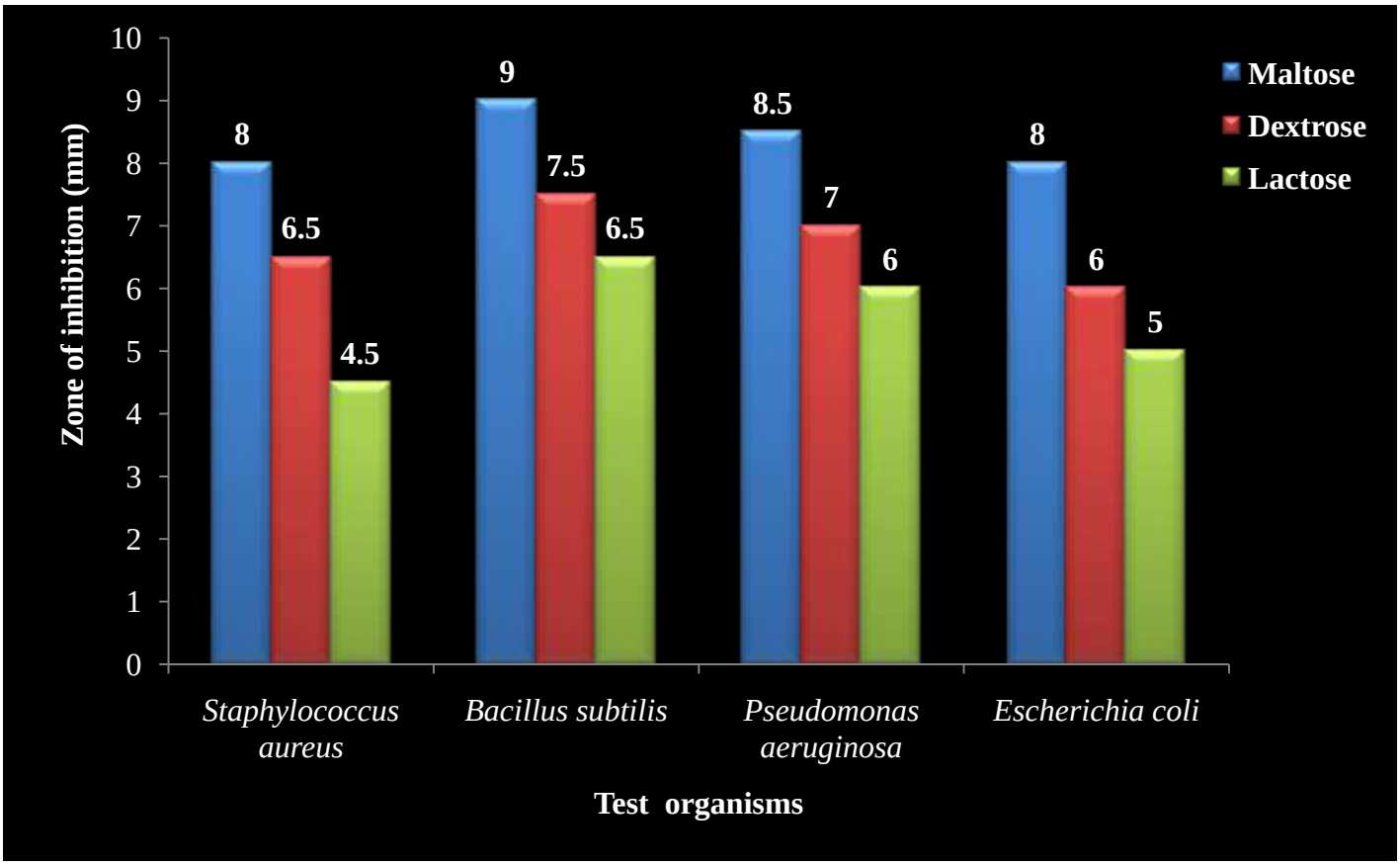

Fig.5. Showing the effect of different carbon sources on Penicillin production

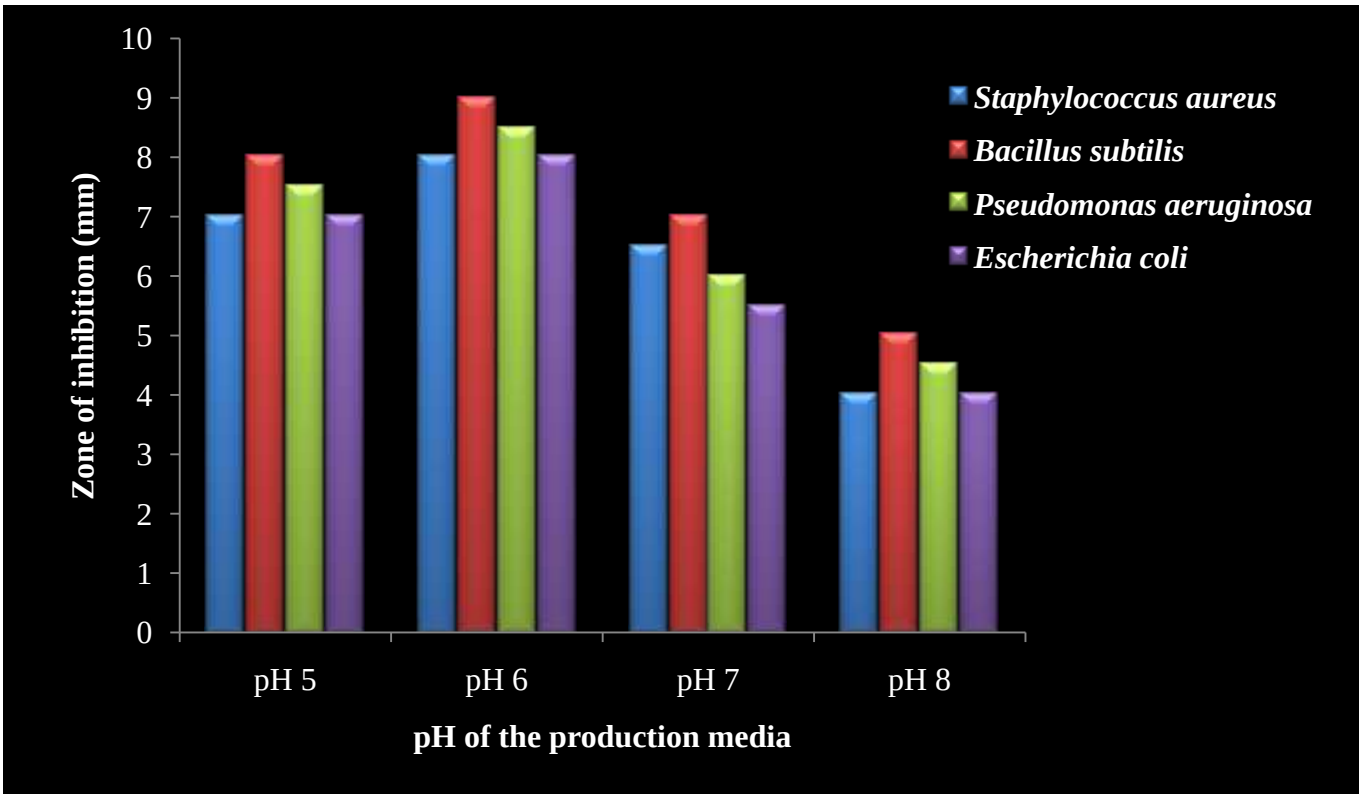

Fig.6. Showing the antimicrobial activity of potential Penicillin at different pH

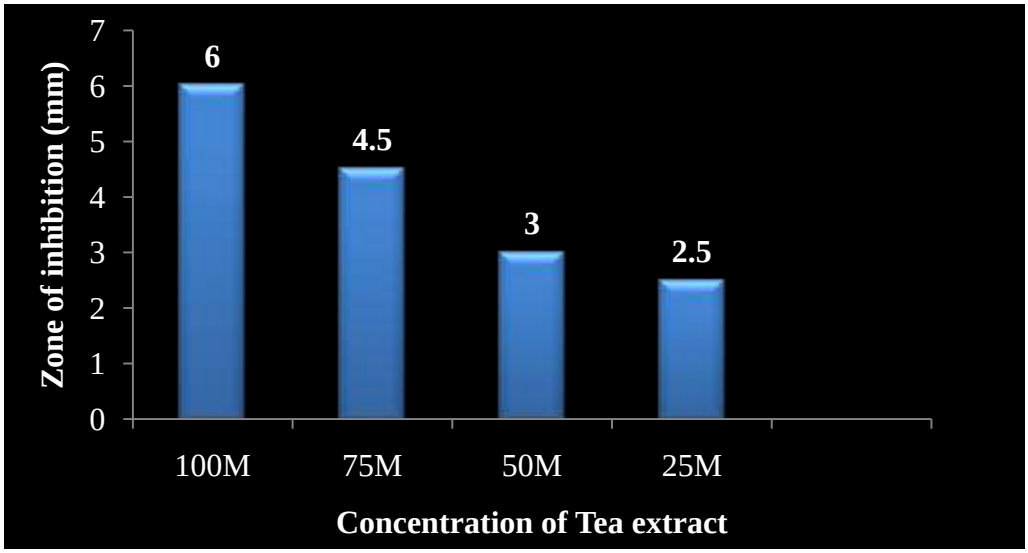

Fig.7. Showing the antimicrobial activity of tea extract at different concentration

Table 1. Showing the antimicrobial activity of combination of Penicillin and Tea extract

Penicillin : Tea extract	Zone of inhibition
10: 0	8
9:1	9
8:2	10.5
7:3	11
6:4	**12**
5:5	**12**
4:6	**12**
3:7	10
2:8	9
1:9	7.5
0:10	6

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