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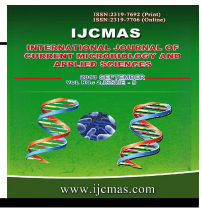

Original Research Article

In vitro Studies on antibacterial activity of aqueous extracts of spices and vegetables against *Bacillus licheniformis* strain 018 and *Bacillus tequilensis* strain ARMATI

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A B S T R A C T

The present study was conducted to investigate the antibacterial activity of commonly used spices and vegetables against two new strains isolated from poultry farm. Both the bacterial strains were found to be susceptible against the garlic (*Allium sativum*). Aqueous extracts of *Zingiber officinale*, *Allium cepa* and *Momordica charantia* were less effective as compared to *Allium sativum*. The strains were resistant to aqueous extracts of *Beta vulgaris*. The maximum zone of inhibition were found in *Bacillus licheniformis* strain 018 of 27 mm and 20 mm throughAgar well diffusion method and Agar well diffusion method respectively against *Allium sativum*. *Bacillus tequilensis* strain ARMATI were sensitive to aqueous extracts of garlic showing 18 mm and 17 mm of zone of inhibition through Agar well diffusion and Agar disc diffusion method respectively. Garlic was found more effective against *B. licheniformis* strain 018 compared to *B.tequilensis* strain ARMATI. These results suggest that *Allium sativum* is a potential spice for controlling these two bacterial strains.

K e y w o r d s

Spices extract; Vegetables extract; Antibacterial activity; Agar well diffusion method; Disc diffusion; Poultry farm bacteria.

Introduction

The natural products are found to be more effective with least side effects as compared to commercial antibiotics so they are used as alternative remedy for treatment of various infections (Tepe *et al.*, 2004). Vegetables, herbs and spices are an important part of the human diet. They have been used for thousands of years to enhance the flavour, colour and aroma of food. In addition to this, vegetables and spices are also used for preservation and medicinal value. Naturally present antimicrobial substances have been recovered from vegetables and spices. *Allium sativum* (Garlic) can be used as a spice in food and medicine (Ross *et al*., 2004). A bioactive compound in garlic that has antibacterial activity is allicin, waich is a volatile compound containing sulphur (Johnston, 2002).

Aqueous extracts of garlic also had antibacterial activity against bacteria that was found for aquaculture products including *Citrobacter freundii*, *E. coli*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* (Safithri *et al*., 2011). *Zingiber officinale* (Ginger), belonging to the family, *Zingiber aceae*is widely used as a spice and medicine. Ginger has been used as a spice and medicine in India and China since ancient times. Ginger has been used to treat digestive problems. Ginger has the capacity to eliminate harmful bacteria, such as *Escherichia coli*, responsible for most of the diarrhoea, especially in children (Azu and Onyeagba, 2007) .*Allium cepa* L (Onion) belongs to the family *Alliaceae*. It is also known as 'garden onion'. It is rich in proteins, carbohydrates, sodium, potassium and phosphorus. Onions have been reported to be an antibacterial, antifungal and has antihypertensive, hypoglycemic and antithrombotic activity (Lampe, 1999).

Certain chemical compounds believed to have anti-inflammatory, anti-cholesterol, anticancer and antioxidant properties. The flesh part of the onion contains flavonoids. Polyphenols are the important component of onion which differentiates it from other *Allium* species. *Beta vulgaris* L (Beet root) juice is considered powerful to prevent infectious and malignant disease. Beet root is a potential source of valuable water-soluble nitrogenous pigments, called betalains. Betalains have been extensively used in the modern food industry (Čanadanović-Brunet *et al*., 2011). Beetroot helps normalize the pH balance of the body and replenish the blood. *Momordica charantia* (Bitter gourd) often called 'bitter melon' belongs to the family *Cucurbitaceae*. The most important use of bitter gourd is, it reduces the blood sugar level and is very good for diabetic patients. It is also used as antiviral, antithelmintic, antimalarial and antimicrobic remedy (Grover and Yadav, 2004). *Momordica charantia* leaves are rich in phytochemicals which has free radicals scavenging activity. Bacteria present in the poultry farm are the causative agents of food poisoning, food spoilage, stomach pain, vomiting etc. *B. licheniformis* can cause food-borne gastro-enteritis, which is infection of the gut that can lead to a life threatening condition called septicaemia. Septicaemia is blood poisoning, and is classified as having a large amount of bacteria in the blood. *B.licheniformis*, although usually associated with the gut and gastrointestinal tract, can also cause distress in other parts of the body. It can cause ophthalmitis, which is the inflammation of the eye. Pathogenicity of *B.tequilensis*in human being is undetermined. Multi-drug resistant bacteria are present in chicken, apparently because of the use of antibiotics in poultry production, and are passing to people who work with, prepare or eat chicken, at some risk to their health. The present study was aimed to evaluate the potentiality of aqueous extracts of some of the commonly used spices and vegetables against the new bacterial strains isolated from poultry farm.

Materials and Methods

Sample Collection and isolation

Samples (surface soil) were collected from poultry farm of Guduvanchery, Tamilnadu (India). Soils 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 isolate was characterized by biochemical analysis using the tests prescribed in Bergey's Manual of Systematic Bacteriology. The Tryptone broth, MR-VP broth, Simmon's citrate agar and Christensen's agar medium were used for Indole test, Methyl Red test, Voges Proskauer 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^{TM}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^{TM}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^{TM}Elution Buffer. DNA was stored at -20ºC.

PCR amplification and sequencing

The 16S ribosomal RNA was amplified by using the PCR (ependorf ep.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.

Spices and Vegetables of interest

Most commonly used spices (*Allium sativum* and *Zingiber officinale*) and

vegetables (*Allium cepa, Beta vulgaris* and *Momordica charantia*) were purchased from the local market in Guduvanchery, Tamilnadu (India).

Aqueous Extract preparation of spices and vegetables

Fresh garlic (*Allium Sativum* L.) bulbs were peeled, weighed (10 g), and surface sterilized using 95%ethanol. The ethanol was allowed to evaporate in a sterile laminar flow chamber, and the garlic was homogenized aseptically with 10 ml of sterile double distilled water using a sterile mortar and pestle. The homogenized mixture was filtered through 8 layers of muslin cloth and centrifuged at 6000 rpm for 15 min. Supernatent was collected and kept at 4ºC for further use. Edible parts of fresh Onion (*Allium cepa)* were rinsed thoroughly in distilled water and air dried.10 grams were then blended and homogenized with sterile double distilled water in 1:1 ratio. The juice was then filtrated and squeezed.

The extract was stored at 4°C. In the same way equal weight of edible part of Zinger (*Zingiber officinale*), Beet root (*Beta vulgaris*) and Bitter gourd [*Momordica charantia* (excluding seed)] were weighed and sterilized with alcohol. Homogenized with sterile double distilled water in equal volume using mortar and pestle. Filtrates were centrifuged and supernatant were stored at 4ºC for further use.

Microorganisms Used

Bacillus licheniformis strain 018 (Accession no.-KC342225) and *Bacillus tequilensis* strain ARMATI (Accession no.-KC424491) isolated from poultry farm were used.

Antibacterial activity testing using Agar well diffusion assay

Both strains of bacteria were inoculated into 10 ml of sterile Nutrient broth in respective conical flasks, and incubated overnight at 37ºC in rotatory shaker. The cultures were swabbed on the surface of sterile Mueller Hinton agar (Hi-media) plates using a sterile cotton swab. 5 agar wells were prepared with the help of sterilized cork borer with radius 5 mm. Using a micropipette, 100µl of spices and vegetables aqueous extracts (supernatant) were added to the each well of the plate. The plates were incubated in an upright position at37ºCfor 24 hours. The diameter of inhibition zones measured in mm and the results were recorded.

Antibacterial activity testing using Agar disc diffusion assay (Kirby- Bauer method)

Each of the culture was swabbed on the surface of each sterile Mueller Hinton agar (Hi-media) plates using a sterile cotton swab. For agar disc diffusion method, the disc (6 cm) was saturated with 25µl of each of the spices and vegetable extracts (supernatant). After that the disc was allowed to dry and introduced on the upper layer of the agar plate. The plates were incubated overnight in upright position at 37ºC. Microbial growth was determined by measuring the diameter of the zone of inhibition.

Result and Discussion

In the present study the organisms isolated from poultry farm were identified as new strains of *Bacillus* species according to morphological (Table 1), biochemical characteristics (Table 2), 16SrRNA gene sequencing and phylogenetic tree (Figure-1.

Determination of antimicrobial activity

Antimicrobial properties of spices and vegetables were studied against the new strains of *Bacillus* species isolated from poultry farm by Agar well diffusion method and Agar disc diffusion method (the two most commonly used method to determine antimicrobial susceptibility.

Agar well diffusion method

Zone of inhibition varied (10 mm to 27 mm) between different *Bacillus* species strains. Among all spices and vegetables tested, *Allium sativum* were found to be active against both new strains of *Bacillus* species that have been isolated. *Allium sativum* was showing maximum and minimum zone of inhibition of 27 mm and 18 mm against *Bacillus licheniformis* strain 018 and *Bacillus tequilensis* strain ARMATI respectively. *Bacillus licheniformis* strain 018were found to be resistant against aqueous extracts of *Zingiber officinales* and *Momordica charantia***.** *Beta vulgaris* were showing very light zone of inhibition against *Bacillus licheniformis* strain 018 indicating ineffective to this strain. *Bacillus tequilensis* strain ARMATI were found to be susceptible against all the aqueous extract of spices and vegetables except *Alium cepa* and *Beta vulgaris* with maximum zone of inhibition of 18 mm against the aqueous extract of *Allium sativum* and minimum zone of inhibition of 10 mm against *Momordica charantia* (Table 3 and Figure 1).

Agar disc diffusion method

Allium sativum was showing inhibitory effect against both the new strains of *Bacillus* species that have been isolated. *Allium sativum* was showing maximum and minimum zone of inhibition of 20 mm and 17 mm against *Bacillus licheniformis* strain 018 and *Bacillus tequilensis* strain ARMATI respectively. These strains were found resistant to aqueous extracts of *Zingiber officinale, Momordica charantia*, *Allium cepa* and *Beta vulgaris* (Table.4 and Figure 2).

Phylogenetic tree

The 16S rRNA sequence obtained was subjected to BLAST search using in the NCBI data base. BLAST search result showed that *Bacillus licheniformis* strain 018 and *Bacillus tequilensis* strain ARMATI have 99% similarity to the isolate *Bacillus sp*. A phylogenetic tree was constructed based on neighbor-joining method (Figure 3).

Durairaj *et al*., (2009) reported that aqueous garlic extract (AGE) has the potential of a broad spectrum of activity against both Gram (+) and Gram(-) bacteria. In our study AGE has potential to inhibit the growth of poultry farm bacteria i.e. Gram (+) bacteria. But we can see the variation in the size of the inhibition zone shown by AGE. This may be due to the lipid content of the membrane of the bacteria and the permeability of allicin and other garlic constituents.

The antibacterial activity of garlic is widely attributed to allicin. Allicin interferes with RNA production and lipid synthesis. All these things contribute to the bacteria can not grow in the presence of allicin or AGE. Onyeagba *et al.,* (2004) reported that the crude extract of garlic and ginger applied singly and in combination did not exhibit any *in vitro* inhibition on the growth of

Table.1 Morphological characteristics of the microorganisms

Characteristics	Result
Gram staining	+
Motility	+

Table.2 Biochemical test results of microorganisms

Tests	*B.licheniformis* strain 018	*B.tequilensis* strain ARMATI
Indole	_	_
Methyl Red	_	_
VogesProskaver	_	_
Citrate utilisation	+	+
Catalase	+	+
Urease	+	_

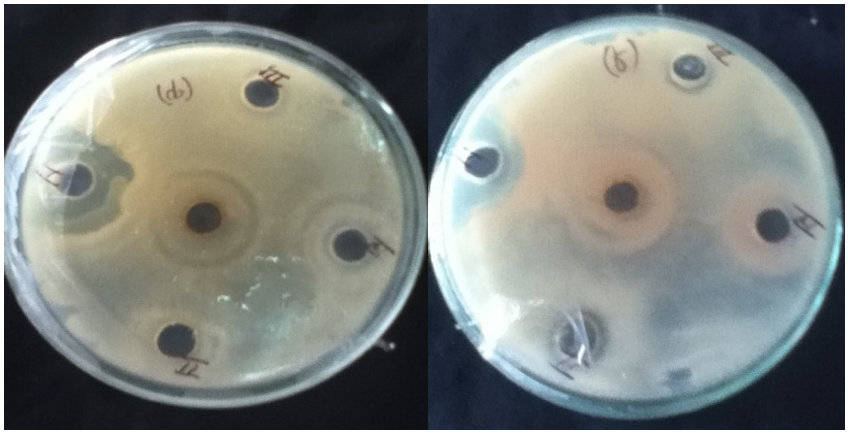
Table.3 Measurement of zone of inhibition (in mm) by Agar well diffusion method

Bacteria	*Allium sativum*	*Zingiber officinales*	*Momordica charantia*	*Allium cepa*	*Beta vulgaris*
Bacillus licheniformis strain 018	27 mm	-	-	18 mm	-
Bacillus tequilensis strain ARMATI	18 mm	11 mm	10 mm	-	-

Table.4 Measurement of zone of inhibition (in mm) by Agar disc diffusion method

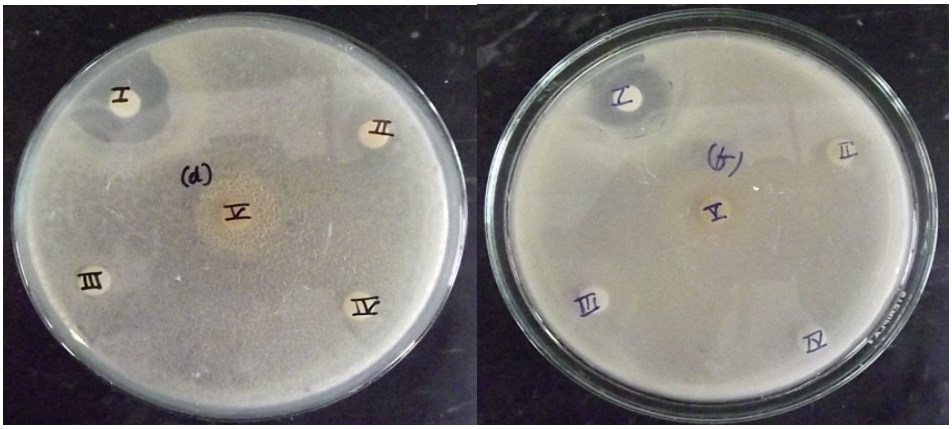
Bacteria	*Allium sativum*	*Zingiber officinales*	*Momordica charantia*	*Allium cepa*	*Beta vulgaris*
Bacillus licheniformis strain 018	20 mm	-	-	-	-
Bacillus tequilensis strain ARMATI	17 mm	-	-	-	-

Figure.1 Antimicrobial activity of spices and vegetables against the microorganism tested.



(d)=B. licheniformis strain 018 (f) =*B.tequilensis* strain ARMATI
(I=Allium sativum, II=Zingiberofficinale, III= Momordicacharantia, IV=Allium cepa,V=Beta vulgaris)

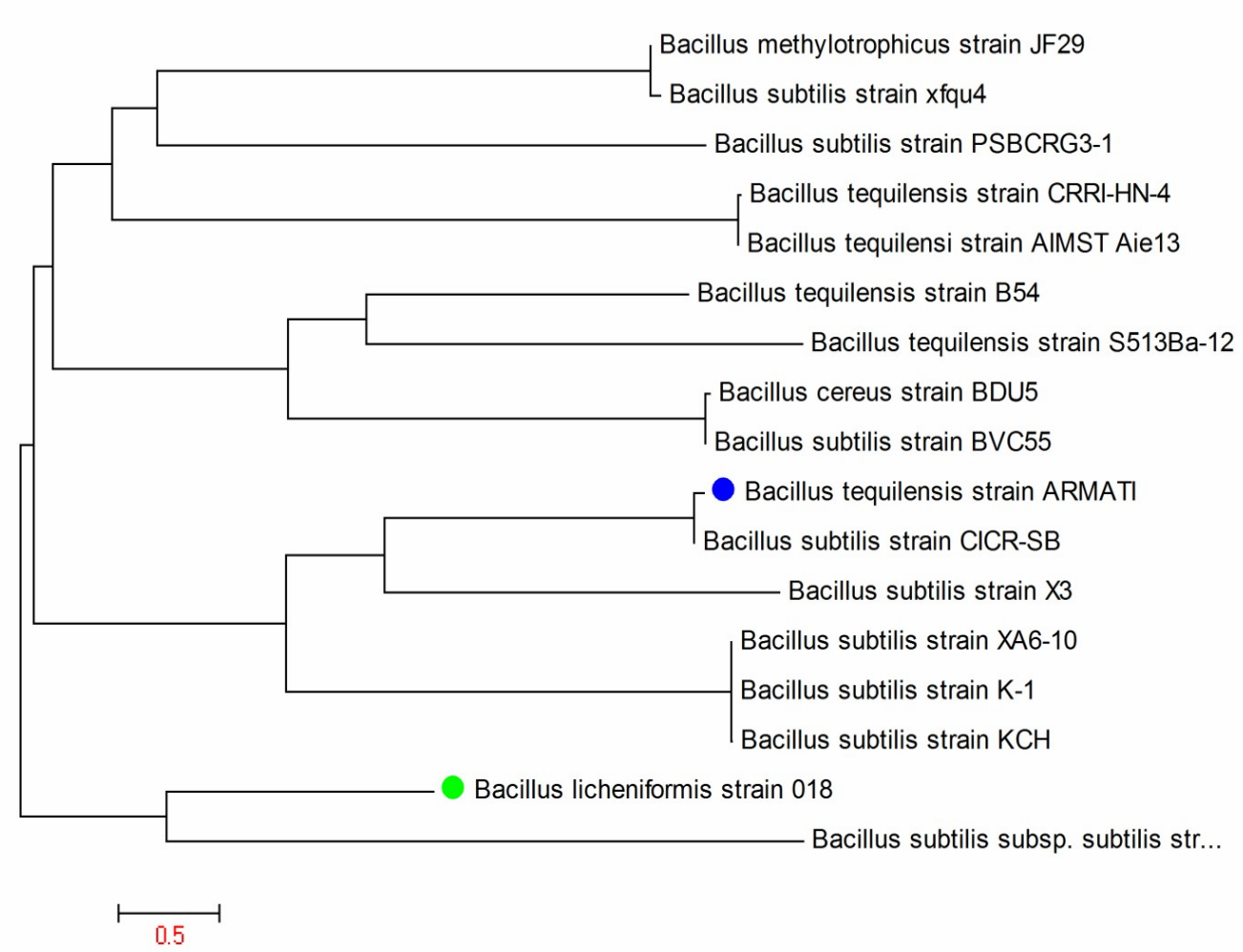
Figure.2 Antimicrobial activity of spices and vegetables against the microorganism tested.



(d)=B. licheniformis strain 018 (f) = *B. tequilensis* strain ARMATI

(I=Allium sativum, II=Zingiberofficinale, III= Momordicacharantia, IV=Allium cepa,V=Beta vulgaris)

Figure.3 Phylogenetic tree obtained by Neighbor-joining analysis based on 16S rRNA gene sequences showing the phylogenetic position of strain ARMATI and 018. Scale bar, 0.5 estimated substitutions per nucleotide position**.**



test organisms (*Bacillus* species, *Staphylococcus aureus*, *E.coli*, *Salmonella* species). In our study aqueous extract of *Zingiber officinale* at high concentration was found to be active against *B. tequilensis* strain ARMATI. Ranjan *et al.*, (2012) reported that garlic can be used as food preservatives and thus the use of other chemical preservatives can be minimized, which could be beneficial for environment and consumer health, or a plastic for food preservation can be inverted using the antibacterial activity of garlic, the inner wall of the plastic coated with garlic. The onion bulbs contain numerous organic sulphur compounds including flavinoids, phenolic acids, saponins, cholesterol etc. The presence of these compounds may explain its antimicrobial activity. Benkeblia (2004) reported that in Algeria, red / purple onion exhibit better antibacterial activities as compared to yellow onion against *S. aureus* and *Salmonella enteritidis*. The zone of inhibition of extracts increased with increasing concentration of extracts

(Benkeblia, 2004). In this study aqueous extracts of *Allium cepa* were found to be active against *Bacillus licheniformis* strain 018 at high concentration only. So research should be continued as its antimicrobial herb against the poultry farm bacteria. Leelaprakash *et al.*, (2011) reported that *Momordica charantia* leaves are rich in phytochemicals which has free radicals scavenging activity. Recently researchers have found that *Momordica charantia* contains several proteins that inhibit HIV in vitro, these proteins known collectively as ribosome inactivating proteins (RIPs) are alpha-momorcharin, beta-momorcharin and MAP-30 (Momordica anti-HIV protein) (Yesilada *et al*., 1999). In this investigation, aqueous extract of edible parts of *Momordica charantia* were found to be active against *B.tequilensis* strain ARMATI strain only with minimum zone of inhibition i.e. poultry farm bacteria were found to be resistant to this vegetable. As research is still in progress, it is unclear which ingredient of this vegetable are having most antimicrobial activity. Extracts of beetroot showed some antimicrobial activity on *Staphylococcus aureus* and on *Escherichia coli* and also antiviral effect was observed (Rauha *et* al., 2000; Prahoveanu *et al*., 1986). In this study Beet root was ineffective against the poultry farm bacteria. *Bacillus licheniformis* strain 018 were showing very light zone of inhibition against beet root indicating this microbe resistant to this vegetable. More research should be done to know about the active compounds present in the beet root which has the antibacterial activity against the poultry farm microbes. Garlic extract, even at low concentration (25µl) is able to inhibit the growth of these strains, while other spices and vegetables which have potential to inhibit bacterial growth are effective at high concentration only. From this investigation, it is clear that among all spices and vegetables tested, garlic has good antimicrobial property against the bacteria that have been isolated from poultry farm.Garlic has shown better activity against *Bacillus licheniformis* strain 018as compared to *B.tequilensis* strain ARMATI. As *Bacillus licheniformis*is the major cause of diarrhoea, vomiting, food poisoning etc. so garlic may be a good source for the treatment of the people working in the poultry farm who might be affected from these microbes. As pathogenicity of *B.tequilensis* is unclear, but there is chance that new this new bacterial strain may be pathogenic to human being. If garlic is provided to those people as a raw food in their diet, they can be cured from the infection of this microbe also to some extent. The result of present study clearly indicates that the aqueous extract of spices and vegetables possess compounds with antimicrobial properties that can be further studied for their antimicrobial activity.

From this study and the previous reports it is clear that garlic satisfy all of the criteria for antibacterial agent as being cheap and safe. Filtrates of fresh garlic can be used to inhibit the growth of new strains of *Bacillus* species isolated from poultry farm. Thus use of fresh garlic filtrates may reduce the use of antibiotics in the poultry farm. Further investigation is required to know the antibacterial activity of these spices and vegetables. Also another study should be continued to isolate, identify and purify the active components of garlic and its use in experimental animals.

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