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Screening of antibacterial properties of Garlic (*Allium sativum* L.) extracts against multidrug resistant diabetic foot ulcer bacteria

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Abstract
Diabetes mellitus has one of the important complications of foot ulcer. Occasionally it bring into being lower extremity of toes amputation. The pus samples were carefully collected from diabetic foot ulcer and processed in the laboratory. Most of the bacteria were isolated from foot ulcer the pus sample showed multiple drug resistance. In order to keep away from repeated use of commercial antibiotic for foot ulcer, alternative methods of herbal medicine were tested in this study. The result of the present study showed that the methanolic extract of *Allium sativum* (Garlic) have excellent antibacterial activity against foot ulcer microflora. Highest zone of inhibition was observed on 300µg of methanolic extract of *Allium sativum*. The presence of active components like flavonoids, alkaloids, steroids and triterpenes in the methanol extract of *A. sativum* that gave excellent antibacterial activity against foot ulcer microflora.

Keywords: Allium sativum, antibacterial, steroids, flavonoids etc.

Introduction
Bacterial infections in diabetic patients have threatened the life of millions of people around the world during the last decade. Lehto *et al.* (1996) [17] reported that the occurrence of amputation was 5.6% in men and 5.3% in women. High fasting plasma glucose at baseline examination and the duration of diabetes were associated with a twofold risk for amputation. Diabetic patients are prone to ulcerations of the lower extremities, commonly complex by bacterial infection and are then dependent upon their caregivers for preservation of their limbs without the dreaded outcome of amputation (Armstrong, 2011) [3]. The proportion of diabetic persons and a history of foot ulceration are understandably higher than the proportion with an active ulcer; 3.1 to 11.8% of persons with diabetes, or 12.9 million to 49.0 million persons worldwide and 1.0 million to 3.5 million in the United States alone, have a history of foot ulceration (http://www.diabetesatlas.org).
Multiple drug resistance has urbanized nowadays due to the haphazard use of commercial antibiotics which leads to side effects on human beings. So, there is a stable need for new authoritative therapeutic agents. Recently, much attention has been paid to take out biologically active compounds isolated from plants used in herbal medicines. Garlic is principally used as an herb to improve many food dishes in various cultures. It contains many substances which studies have shown to act together to prevent disease and age-related conditions (Anonymous, 2009) [1]. Garlic *(Allium sativum)* belongs to the *Allium* family Liliacea. Borek (2001) [5] reported that Garlic strengthened the immune system. It also has ability to reduce blood cholesterol level and inhibition of cholesterol synthesis (Piscitelli *et al.*, 2002) [24]. Garlic has long been known to have antibacterial, antifungal (Rakshit and Ramalingam, 2010) [25], anticancer (Yoshida *et al.*, 1987) [30] and antiviral properties (Pan *et al.*, 1985) [22]. Garlic contains at least 33 sulfur compounds, pungent odor and a lot of its medicinal effects are responsible for the presence of sulfur compounds (Jangam and Badole, 2014) [10]. One of the most active compound present in Garlic is allicin (diallyl disulfide) which is active only when crushed or cut. In the year of 1940's, Allicin, was first chemically secluded and also recognized for its antimicrobial effects against many viruses, bacteria, fungi and parasites (Papu *et al.*, 2014) [23].

Garlic consumption associated with lot of the health benefits have been attributed to the presence of thiosulfinates and organosulfur compounds (Naznin *et al.*, 2008) [20]. Meriga *et al.* (2012) [19] reported that the bulb extracts of garlic exhibited strong antioxidant activity. Hence, the present study aimed to evaluate the efficacy of antimicrobial activity of methanolic garlic extract against diabetic foot ulcer microflora.

Materials and Methods

1. Case Study on Diabetes mellitus patients

Diabetes can cause many serious long-term complications which may cause erectile dysfunction of organs and poor healing of wounds. A total number of 30 patients with foot ulceric diabetes mellitus were selected for the study. Pus sample were collected from the patients affected with foot ulcer.

2. Collection of samples

The pus sample from diabetic foot ulcer were carefully collected by sterile cotton swab and dipped in Ames transport medium from Shifa hospital, Tirunelveli, Tamil Nadu, India. The pus samples were swabbed on Nutrient agar plates and incubated at 37ºC for 24 hours. After incubation, the individual colonies having different colony morphologies were streaked on different selective agar plates such as Eosin Methylene Blue agar, Mannitol Salt agar, MacConkey agar, Blood agar, Salmonella Shigella agar, Pseudomonas isolation agar by quadrant streak method and the plates were incubated at 37ºC for 24 hours. The isolated bacteria were subjected to Bergy"s manual biochemical identification.

2. Activity of selected commercial antibiotics against the isolated pathogens

Assay of antibiotic activity against isolated diabetic foot ulcer microflora was performed by Kirby-Bauer disc diffusion method. The Mueller-Hinton agar plates were prepared and the organism was swabbed over it using a sterile cotton swab. The antibiotic discs were placed on the surface of the agar plates and then, the plates were incubated at 37ºC for 24 hrs. After incubation, the zone of inhibition was measured (mm).

3. Preparation of aqueous garlic extract

Fresh Garlic (*Allium sativum*) was purchased from local market, Thoothukudi, Tamil Nadu. Identity and verification of the sample was done by Department of Botany, Kamaraj College, Thoothukudi.

50g of peeled garlic was weighed and washed with sterile distilled water by soaking for 5 minutes and then it was soaked in 95% ethanol for 3 minutes to make the surface sterilization. Then the garlic was dried for 10 minutes to evaporate the ethanol. Then the dried garlic was crushed in sterile mortar and pestle by adding 0.5ml of distilled water. After mashing the garlic will be in a paste form, 50g of each ground material was soaked in 500ml of methanol for atleast 72 hours with frequent shakings. The samples were then filtered through Whattman No: 1 filter paper. A stock solution of 0.1 g/ml in dimethyl sulfoxide (DMSO) was made the extract. The extract was kept at 4^0C, until used.

4. Antibacterial activity of methanolic extract of *A. sativum* against diabetic foot ulcer microflora

Antibacterial activity of methanolic extract of *A. sativum* against diabetic foot ulcer microflora using agar well diffusion method.

The pathogens isolated from diabetic foot ulcer sample was inoculated into 10ml of sterile nutrient broth and incubated at 37ºC for 24 hours. Different concentrations of methanol extract of *A. sativum* i.e., 300µg, 200µg, 100µg, 40 µg/well were used for the assay. The colony forming units (CFU/ml) of 0.1ml suspension of the pathogens were adjusted to 5×10^5 CFU/ml. A suspension of test organism (0.1ml) was swabbed on the surface of Muller Hinton Agar medium (MHA) by using the sterile cotton swab. After that, a sterile cork borer (5 mm diameter) was used to made wells in the seeded Muller-Hinton agar. Then, each extract (different concentrations were dissolved in 50µl of DMSO) was poured into wells separately and allowed to diffuse at room temperature. Equal volumes of 10% DMSO was served as negative control. The plates were incubated in an upright position at 37ºC for 24 hours. The diameter of inhibition zones was measured in mm and the results were recorded (Indu *et al.*, 2006) [14].

4.1 Fractionation

Collected methanolic extracts of 5ml *A. sativum* was fractionated with column chromatography under reduced pressure over silica gel. These fractions were collected and evaporated to dryness in desiccators and stored in a refrigerator until used for the antimicrobial screening and phytochemical analysis.

5. Phytochemical Screening

The methanolic extracts of *A. sativum* were screened for the presence of secondary metabolites using the procedure of Sofowora (1993) [29]. Twenty millilitres of each extract was measured into the test tubes and concentrated by evaporating the extractant in a water bath. Tests were carried out for carbohydrates, reducing sugars, tannins, polyphenols, lipids, flavonoids, ketones, alkaloids, steroids and triterpenes.

6. Determination of the minimum inhibitory concentration (MIC)

MIC of the methanolic extracts of *A. sativum* was carried out according to modified methods of resazurin microtube assay by Sarker *et al.* (2007) [27]. One milileter of sterile Muller Hinton Broth was transferred in to each test tube. The methanolic extract of *A. sativum* was dissolved in 10 per cent DMSO to obtain 1000 mg/ml stock solution. Different concentrations of methanol extract of *A. sativum* (Fraction I) i.e., 500µg, 250µg, 125µg, 62.7, 31.25, 15.8 and 7.8µg/well were used for the assay. A volume of 50 µl of methanol extract from stock solution was added into the first tube. After fine mixing of the (methanolic extracts of *A. sativum*) extracts and 50 µl of broth solution was transferred to the second tube and in this way, the serial dilution procedure was continued to a twofold dilution to obtain concentrations like 500 to 7.812 mg/ml of the extract in each tube.

7 Minimum Bactericidal Concentrations (MBC)

The MBC of the methanolic extracts of *A. sativum* was determined by plating 100µl of samples from each MIC assay tube with growth inhibition into freshly prepared MHA medium and the plates were incubated at 37^0 C for 24 h bacteria. The MBC values were recorded as the lowest concentration of the extracts that did not permit any visible bacterial colony growth on the agar plate during the period of incubation.

Results

Among the 100 diabetic patients, only 10 patients were

affected with urinary tract infection. Majority of the 90 patients were affected with chronic diabetic disease. The chronic diabetic patients were possessed diabetic foot ulcer and finger amputations. A total of 30 patients pretentious with foot ulceric diabetes mellitus were selected for this study. Pus sample were collected from the patients affected with foot ulcer and inoculated into the nutrient agar plates. The colonies showing different morphologies were streaked on selective agar plates. The organisms thus isolated were identified based on morphological and biochemical characteristics using Bergy's manual (Table: 1).

Table 1: Microscopic examination and biochemical characteristics of the isolated pathogens

Tests	*Staphylococcus aureus*	*Citrobacter freundi*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Proteus vulgaris*
Gram's stain	+	-	-	-	-
Motility	Non-Motile	Motile	Motile	Motile	Motile
Indole production	-	-	-	+	+
Voges-proskauer	-	-	-	-	-
Methyl Red	+	+	-	+	+
Citrate	-	+	+	-	-
Urease	-	+	-	-	+
Catalase	+	+	+	+	+
TSI	A/A		K/K	A/A	K/A

+ = Positive, - = Negative, A = Acid, `K = Alkali

The pus samples of diabetic foot ulcer bacteria were identified to be *Staphylococcus aureus, Citrobacter freundi, Pseudomonas aeruginosa, Escherichia coli* and *Proteus vulgaris* (Table: 2)

Table 2: Pathogens isolated from Diabetic patients

Sl. No.	Diabetic foot ulcer bacteria
1	*Staphylococcus aureus*
2	*Proteus vulgaris*
3	*Citrobacter freundi*
4	*Pseudomonas aeruginosa*
5	*Escherichia coli*

Antibacterial assay of selected commercial antibiotics were tested against diabetic foot ulcer isolates. The results revealed that most of the organisms were found to be resistant against the antibiotics (Table: 3).

Table 3: Selected commercial drugs for Foot ulcer microorganisms

Organisms	Commercial Antibiotics						
	Streptomycin	Tetracyclin	Chloram phenicol	Oxacillin	Vancomycin	Ciprofloxacin	Methicillin
Staphylococcus aureus	-	-	-	-	-	-	-
Citrobacter freundi	-	-	++	-	-	-	+
Proteus vulgaris	-	+	-	-	+	-	++
Pseudomonas aeruginosa	-	-	+	-	-	+	+
Escherichia coli	-	-	-	-	-	+	-

- = Resistant. + = Intermediate. ++ = Sensitive

Methanol extract of *Allium sativum* was tested for their antibacterial activity against the diabetic foot ulcer bacterial isolates. The methanol extract of *A. sativum* was found to be effective inhibition against the diabetic the foot ulcer bacteria. Highest zone of inhibition was observed in the 300µg concentration against all pathogens. The zone of inhibition (mm) was observed on the different concentration of methanol extract of *A. sativum* against the diabetic the foot ulcer bacteria was showed in Figure 1

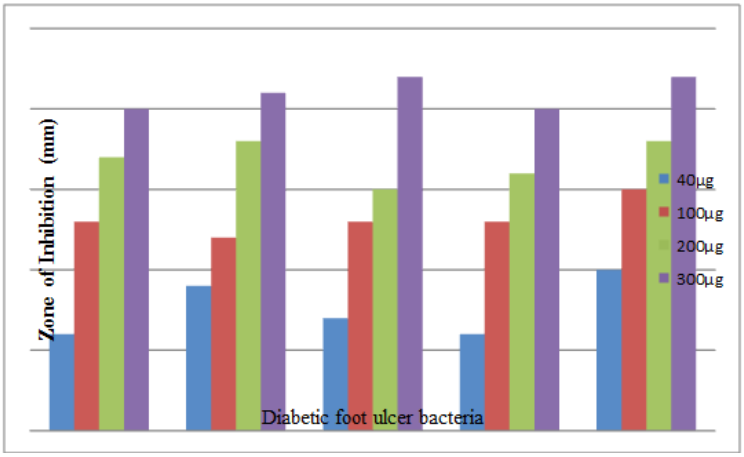

Fig 1: Antibacterial activity of methanol extract of *A. sativum* against diabetic foot ulcer bacteria (agar well diffusion method)

Methanol extracts of *A. sativum* showed only two fraction of column chromatography. Fraction I of methanol extracts of *A. sativum* showed excellent antibacterial activity against foot ulcer bacteria showed in Figure 2.

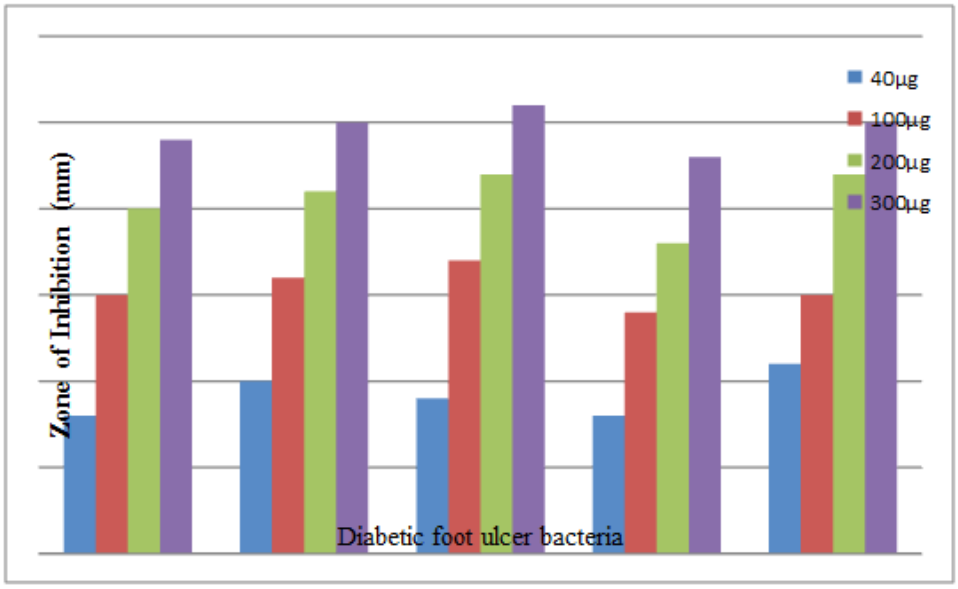

Fig 2: Antibacterial activity of methanol extracts of *A. sativum* (fraction I) against foot ulcer bacteria

Phytochemical screening results of *A. sativum* methanol extract of fraction I showed in Table 5 and 6. In the phytochemical analysis, the methanolic extracts of *A. sativum* were found to be carbohydrates, reducing sugars, lipids, flavonoids, ketones, alkaloids, steroids and triterpenes. Tannins and polyphenols were not detected in the *A. sativum* extracts under the conditions of this study.

Table 5: Phytochemical observation of *A. sativum* methanolic extracts

S. No	Phytochemical compounds	*Observation of A. sativum* methanol extracts.
1	Carbohydrates	Two layers of ethanol above and acid below were formed with a reddish brown ring at the junction of the two layers.
2	Reducing sugars	Brick-red colouration
3	Tannins/ Polyphenols	Golden precipitate instead of a bluish- black Colouration
4	Lipids	Red precipitate
5	Flavonoids	Orange colouration
6	Ketones	Yellow precipitate
7	Al kaloids	Orange and yellowish white precipitate for Mayer's and Dragendorff's reagent respectively.
8	Steroids and triterpenes	Greenish-blue and violet precipitates for steroids and triiterpenes respectively.

Table 6: Phytochemical Compounds of *A. sativum* methanol extracts.

S. No	Constituent	Methanolic extract of *A. sativum*
1	Reducing sugars	+ +
2	Tannins/ Polyphenols	---
3	Lipids	+ +
4	Flavonoids	+ +
5	Ketones	+ +
6	Alkaloids	+ +
7	Steroids and Triterpenes	+ +

++ Present, ---Absent

The MIC value of methanol extracts of *A. sativum* against *Pseudomonas aeroginosa*, *E.coli*, *Proteus vulgaris* and *Staphylococcus aureus* was 31.25µg/ml and MBC value was 62.7 µg /ml. Followed by the MIC value of *Citrobacter freundi* was 62.7 µg/ml and its MBC value was 125.4 µg /ml. MBC value of methanolic extracts of *A. sativum* showed doubled value of MIC. Table 7 showed the results of MIC and MBC value of *A. sativum* (Fraction I) methanol extracts against diabetic foot ulcer bacteria.

Table 7: MIC and MBC of methanol extract of *A. sativum* (Fraction I) against foot ulcer bacteria

Foot ulcer Bacteria		MIC value in µg/ml							
		250.8	125.4	62.7	31.25	15.8	7.8	MBC value	MIC value
Pseudomonas aeruginosa	Growth on MHA plates	--	--	-	-	+++	+++	62.7	31.25
	Colour	Purple	Purple	Purple	Purple	Pink	Pink		
E.coli	Growth on MHA plates	--	--	--	-	++	+++	62.7	31.25
	Colour	Purple	Purple	purple	Purple	Pink	Pink		
Citrobacter freundi	Growth on MHA plates	--	--	+	+	+++	+++	125.4	62.7
	Colour	purple	Purple	Purple	pink	Pink	Pink		
Proteus vulgaris	Growth on MHA plates	--	--	--	-	++	+++	62.7	31.25
	Colour	purple	Purple	purple	Purple	Pink	Pink		
Staphylococcus aureus	Growth on MHA plates	--	--	--	-	++	+++	62.7	31.25
	Colour	purple	Purple	purple	Purple	Pink	Pink		

Discussion

Diabetes mellitus, a chronic disease mostly line of thinking to be uncommon in the developing world, now it has transpire as an important public health problem in Asia. Diabetes is the single most dangerous metabolic disease, widely recognized one of the most important causes of death and disability worldwide.

The present study revealed that among the 100 diabetic's patients, most of them have chronic diabetics. In the case study, surrounded by 30 diabetic's patients have foot ulcer. Armstrong (1997) reported that a diabetic foot ulcer lead up to 85% of non traumatic low extremity amputations. Approximately 3.4% of individuals having diabetes foot ulcers had deep infections, 15% developed foot ulcer during their lifetime.

Foot ulcer infections and their corollary cause substantial morbidity among diabetic patients. Most of the foot ulcer infections are mild to moderate in severity. In the similar, Sing (2005) reported that among the diabetes mellitus persons, the lifetime risk of estimated value of foot ulcer is to be 15%.

In the present study, the result showed that the isolated foot ulcer bacteria are mostly Gram positive cocci and Gram negative rods. Similar results was observed by Frykberg (2001) who stated that microbial infection should be anticipated in patients with a diabetic foot ulcer with variety of Gram positive cocci, Gram negative rods and anaerobic organisms were predominating.

My present study revealed that most of the isolated foot ulcer bacteria were highly resistant to various antibiotics. Some of the bacteria also showed methicillin, vancomycin resistant and produced of Extended-spectrum beta-lactamases (ESBL). Similarly, Goldstein *et al*. (1997) suggested that in the application of antibiotics deluded massive amount of developing resistance of diverse pathogenic bacteria. In the present work, the methicillin – resistant *Staphylococcus aureus* was isolated from foot ulcer sample. This was supported by Howden *et al.* (2005) reported that methicillin resistant *Staphylococcus aureus* was enormously present in the foot ulcer wounds. Keerthi Praba and Kumaresan (2014) reported that *Allium sativum* (garlic) extract showed better antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa. Staphylococcus aureus* was one of the major skin infections causing bacteria.

Garlic methanol extract showed highest zone inhibition against the foot ulcer pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris* and *Citrobacter freundi*). Similar results were identified by Iwalokum *et al.* (2004), who stated that the aqueous garlic extract has effective antimicrobial effects against 133 multi drug-resistant bacteria. Garlic possesses the power of draining the growth of pathogens (Gebreselema and Mebrahtu, 2013).

The result of phytochemical screening of garlic methanol extract had carbohydrates, reducing sugars, lipids, flavonoids, ketones, alkaloids, steroids and triterpenes. Cavallito (1945) reported that *A. sativum* might be an excellent wound healing property due to the presence of active components like flavonoids, alkaloids, steroids and triterpenes. Flavonoids are powerful water-soluble antioxidants and free radical scavengers, which prevent oxidation, cell damage and also have strong anticancer activity. They also lower the threat of heart diseases (Sofowora, 1993) [29]. Alkaloids recognized to possess analgesic, antispasmodic and bactericidal effects (Okigbo *et al.*, 2009) [21]. Ritota *et al*. (2012) [26] stated that during the HRMAS-NMR analysis, *A. sativum* extract indicated the presence of organosulphurs, allicin and some allyl-organosulphurs compounds. It was also confirmed by SPME-GC–MS.F

Conclusion

Multiple drug resistance has developed in these days for repeated use of commercial antibiotics for the treatment of infectious disease. In adding together this problem, all antibiotics are mostly associated with undesirable effects on the host. Therefore, there is a need to develop for extra antimicrobial drugs for the treatment of bacterial infections. From my results, it can be concluded that *A. sativum* methanol extracts have huge possible antimicrobial compounds that are active against foot ulcer bacteria. So that methanol extracts of *A. sativum* compounds can be of second-hand treatment of diabetic foot ulcer infections.

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