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EVALUATING THE ANTIBACTERIAL ACTIVITY OF AZADIRACHTA INDICA EXTRACTS ON ESCHERICHIA COLI AND SALMONELLA SPP.

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Abstract: The emergence of antibiotic-resistant bacteria has prompted the search for alternative sources of antibacterial agents. Azadirachta indica (neem) leaf extract has been used extensively as folk medicine for various diseases. This study aimed to evaluate the antibacterial activity of neem leaves ethanol extract against Escherichia coli and Salmonella spp. and to compare its activity with colistin sulphate. Ethanol extracts of varying concentrations (12.5%, 25%, and 50%) were prepared and tested against test organisms using agar diffusion method. The study showed significant antibacterial activity against E. coli and Salmonella spp. However, neem leaves extract was not active against all pathogenic bacteria tested. The extracts have activity against some gram-positive and gram-negative bacteria, and the largest zone of inhibition was recorded against Bacillus cereus. The study recommends the extensive studies of neem leaves to yield cost-effective antibiotics for the future.

Keywords: Antibacterial activity, Leaf extract, Colistin sulphate, Zone of inhibition, Azadirachta indica, Escherichia coli, Salmonella spp.

Introduction

The overuse and misuse of antibiotics have led to the emergence of antibiotic-resistant bacteria, posing a serious threat to public health. Scientists are searching for alternative sources of antibacterial agents to tackle this challenge. Azadirachta indica, also known as the neem tree, is a tree in the mahogany family, native to India, Burma, Bangladesh, Sri Lanka, Malaysia, and Pakistan, growing in tropical and semi-tropical regions. Its bark and leaf extracts have been therapeutically used as folk medicine in the control of leprosy, intestinal helminthiasis, respiratory disorders, constipation, and a general health promoter. This study investigates the antibacterial effect of the ethanolic crude extract of Azadirachta indica against Escherichia coli and Salmonella spp. Neem leaf extracts have been reported to have antibacterial activities in previous studies. [8] [8] [8] The present study aimed to evaluate the antibacterial activity of neem leaves ethanol extract and compare its activity with colistin sulphate, which is commonly used as an antibacterial agent. Ethanol extracts of varying concentrations were prepared and tested against test organisms using the agar diffusion method. This study recommends further extensive studies of neem leaves to yield cost-effective antibiotics for the future.

MATERIALS AND METHODS

2.1. Collection of plant materials

Fresh leaves of neem were collected from the garden of medicinal plants, Chittagong Veterinary and Animal Sciences University, during January to March, 2021.

2.2. Drying and grinding

Collected plants leaves were thoroughly washed in running tap water. Then the leaves were air dried for one week and then further dried in the oven at 55-60°C for 12 hrs. Dust was prepared by pulverizing the dried leaves with the help of a manual grinder. A 25-mesh diameter sieve was used to obtain fine dust and preserved them into airtight plastic container until being used.

2.3. Process of extract preparation

The powdered were mixed with ethanol and keep it for three day's. Then the sample was filtrated for three times to separate the ethanol and neem leaf extract. The obtained liquid extract was subjected to Rotary evaporator for 24 hrs at 50ºC. Within this 24hrs all the ethanol was evaporated.

2.3.1. Storage of extract

After collecting the extract it is storage at 4º C for few days.

2. 4. Antimicrobial activity testing

2.4.1. Test microorganisms

The disease causing strains of *E. coli* and *Salmonella spp* were obtained and used as test organisms.

2.4.2. Micro dilution assay

The minimum inhibitory concentration was defined as the lowest concentration of the compound to inhibit the growth of microorganisms. The minimum inhibitory concentration values were determined by broth dilution assay of micro dilution assay. Varying Concentrations of the extracts (500mg/ml, 400mg/ml, 300mg/ml, 200mg/ml, 100mg/ml and 50mg/ml) were prepared. 0.1ml of standardized test organism of Controls was equally set up by using solvents and test organisms without extract.

2.4.3. Agar disc diffusion method

This method is suitable for organism that grows rapidly over night at 35-37°C. The antibiotic (specific concentration) impregnated disc absorbs moisture from the agar and antibiotic diffuses in to the agar medium. The rate of extraction of the antibiotic from the disc is greater than the rate of diffusion. As the distance from the disc increases. There is a logarithmic reduction in the antibiotic concentration. Zone of inhibition of bacterial growth around each disc is measured and the susceptibility is determined.

2.4.4. Preparation of Mueller-Hinton Agar

Mueller-Hinton agar (MHA) preparation includes the following ways. Mueller-Hinton agar prepared from a commercially available dehydrated base (Hi-media, India) according to the manufacturer's instructions. Immediately after autoclaving, allowed it to cool in a 45 to 50°C water bath. Freshly prepared and cooled medium poured into glass, flat-bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. (This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100mm). The agar medium allowed cooling to room temperature.

2.4.5. Turbidity standard for inoculums preparation

To standardize the inoculums density for a susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used. The preparation procedure of $BaSO_4$ 0.5 McFarland standards as follows:

- A 0.5m1 aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaC1_2$. $2H_2O$) was added to 99.5ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. ▪ The correct density of the turbidity

standard verified by using a spectrophotometer with a one cm light path and matched curette to determine the absorbance. The absorbance at 625 nm should be 0.008 to 0.10 for the 0.5 McFarland standards.

- The Barium Sulfate suspension transferred in four to six ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculums.
- These tubes were tightly sealed and stored in the dark at room temperature.
- The barium sulfate turbidity standard vigorously agitated on a mechanical.
- Vortex mixer before each use and inspected for a uniformly turbid appearance.

2.4.6. Method

25 µl of inoculum was spread over the Mueller-Hinton medium, using sterile spreader. After few minute, four discs were made in each petri plate and loaded with 12.5%, 25%, 50% ethanolic extract and the remaining disc was colistin sulphate. (The diameter of this disc was 6 mm. The first three disc have different concentration of ethanol neem leave extract that was 12.5%, 25% and 50% respectively and the remaing disc was colistin sulphate that are collected from ®OXOID). After that, plates were incubated at 37°C for 24hrs. Antimicrobial activity was evaluated by measuring zone of inhibition by using Himedia zone scale.

RESULTS

In the CS test, ethanolic neem leaves extract shows various zone of inhibition against *E. coli* and *Salmonella sp*.

3.1 Antibacterial activity against *Salmonella sp*.

Table 1: Antibacterial activity of ethanolic neem leaf extract and colistin sulphate against *Salmonella sp.*

Concentration	Well diameter(cm)	Zone of inhibition(cm)	Inhibition length (cm)
12.5%	0.6	Resistance	Resistance
25%	0.6	1	0.4
50%	0.6	1.4	0.8
Colistin sulphate	0.6	2.1	1.5

Table 1 shows various zone of inhibition in different concentration of ethanolic neem leaf extract proportionally. In case of 12.5% extract concentration, MHA agar plate shows no zone of inhibition. In case of 25% extract concentration, MHA agar plate shows 1 cm zone of inhibition and length of inhibition is 0.4cm. In case of 50% extract concentration on inoculated MHA agar plate shows 1.4cm zone of inhibition and 0.8cm length of inhibition. In same agar plate colistin sulphate shows 2.1cm zone of inhibition and its length of inhibition was 1.5cm.

3.2 Antibacterial activity against *E. coli*

The table 2 shows various zone of inhibition in different concentration of ethanolic neem leaf extract propotionaly. In case of 12.5% extract concentration, MHA agar plate shows no zone of inhibition. In case of 25% extract concentration on MHA agar plate shows 1.2 cm zone of inhibition and length of inhibition is 0.6cm. In case of 50% extract concentration on inoculated MHA agar plate shows 1.4cm zone of inhibition and 1cm length of inhibition. In same agar plate colistin sulphate shows 1.7 cm zone of inhibition and 1.1cm length of inhibition.

Table-2: Antibacterial activity of ethanolic neem leaf extracts and colistin sulphate against *E. coli.*

Concentration	Well diameter(cm)	Zone of inhibition(cm)	Inhibition length(cm)
12.5%	0.6	Resistance	0
25%	0.6	1.2	0.6
50%	0.6	1.4	1.0
Colistin sulphate	0.6	1.7	1.1

DISCUSSION

Many of the existing synthetic drugs cause various side effects. Hence, drug development plant based compounds could be useful in meeting this demand for newer drugs with minimal side effects. *A. indica* leaves possessed good antibacterial activity confirming the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care. The extract of *A. indica* when used as medicinal plant, could be useful for the growth inhibition of the carcinogenic bacterium, *Salmonellosis, E. coli, S. sobrinus*. The preliminary screening of antimicrobial activity was that both the aqueous and ethanol extracts of leaves of *A. indica* expressed antibacterial activity on at least one bacterium. Ethanol extract was the most effective against all the tested bacteria. Aqueous extract did not show any activity at low concentrations on *E. coli*. At other concentrations, its activity was found to be weaker in comparison to ethanol extract. On the basis of zone of inhibition, maximum activity was found at the concentration of 50% and in this case also ethanol extract showed better results. Aqueous extract was found to be less effective in antimicrobial activity in comparison to ethanol extract. Degree of susceptibility in descending order was for *E. coli* followed by *S. aureus*, *K. pneumonia* and *B. subtilis* at 50% concentration. Previous studies have showed that extracts of *A. indica* were found effective against *E. coli* and *S. faecalis* with fairly high degree of sensitivity (IZ=18-33 mm) to methanol extracts [8]. Methanol extract of *A. indica* showed fairly high degree of sensitivity (IZ=20-33 mm) to all tested bacteria, except *B. subtilis*, which was least susceptible to methanol extract [8]. Someone performed the antibacterial activity test using aqueous extract of neem leaf by Agar Well Diffusion Technique but the extract did not show any activity on *E. coli*. It might be due to aqueous extract of neem leaf which was tested in this study. Previous studies also represent that the alcoholic extraction of neem may have the antibacterial activity and aqueous extraction may not. For these above reasons, we used ethanol neem leaves extract which is similar to Koona et al. [8] and Ramesh et al. [9].

The extracts showed activity against some gram-positive and gram-negative bacteria. The largest zone of inhibition this plant was recorded against *Bacillus cereus* (28mm). These results are in accordance with [10]. Others investigator [11, 12] also found almost similar effect. On the contrary, Wiart et al. [13] and Vidyasagar et al. [14] reported that neem leaves have no inhibitory effect against *Salmonella typhi*.

In current study neem leaves extract was not active against all pathogenic bacteria. This study result is comparable to the report of Ramesh et al. [15]. Variable finding on antibacterial activity of neem leaves have been reported by many scientists [15, 16]. The current study is inconsistent with [14] who reported that extract of neem showed better inhibiting activity against *Escherichia coli* and *Salmonella typhi*. The antibacterial activity of *A. indica* might be due to presence of triterpenoids, phenolic compounds, carotenoids, steroids, valavinoids, ketones and tetra-triterpenoids azadirachtin. Earlier studies on *Azadirachata* claim that a spermicidal fraction of neem oil (NIM-76) is more effective as an antimicrobial agent as compared to the neem oil itself especially its effect is less on *E. coli.* Antibacterial activity of the extracts of *A. indica* was effective on *E. coli*. Few authors have reported antimicrobial activity of neem oil on *E. coli* with methanol but not with chloroform and hexane extracts as is influenced by pH of the final extract [17].

Ethanol neem leaf extract was used in this study because it is more effective. In case of *Salmonella*, we observed 17mm,14mm,12mm and no zone inhibition in disc diffusion method at Muller Hinton plate in respective antibiotic concentration (colistin sulphate disc made by company, 50% concentration of extract, 25% concentration of extract and 12.5% concentration of extract). This is similar to previous study. In case of *E.coli,* we have detected

21m, 14mm, 10mm and no zone inhibition at respective antibiotic concentration (colistin sulphate disc made by company, 50% concentration of extract, 25% concentration of extract and 12.5% concentration of extract)

The CS test of *Salmonella* of ethanol neem leaf extract by Agar disc diffusion method shows various zone of inhibition in different concentration of leaf extract proportionally. We used 6mm diameter disc and it shows 21mm, 14mm, 10mm and no zone inhibition at respective antibiotic concentration (colistin sulphate disc made by company, 50% concentration of extract, 25% concentration of extract and 12.5% concentration of extract).

In case of 12.5% ethanolic neem leaves extract we found no zone of inhibition for both microorganism, which is similar with Wiart et al. [13] and Vidyasagar et al. [14], who reported that neem leaves have no inhibitory effects against *Salmonella typhi* and this result is dissimilar with Jamkhand et al. [10] and Suresh et al., [11]. The ethanolic extract of neem leaves which have 25% concentration gives 1.4 cm Zone of inhibition against *Salmonella sp* and 1.2 cm Zone of inhibition against *E coli.* This result is contraindicated with Wiart et al. [13] in and Vidyasagar et al. [14]. Similar observation has also been conducted with Panchal *et al*., 2013, Jamkhande et al. [10] and Suresh et al. [11]. The disc prepare by ethanolic neem leaves extract that has 50% concentration shows 1.4 cm zone of inhibition for both micro-organism. The same findings were also reveled in a study by Ramesh et al. [9]: Jamkhande et al. [10] and Khan et al. *et al*., 2013 and this observation not supported by Wiart et al. [13] and Vidyasagar et al. [14].

CONCLUSION

From the results of the present study, it appeared that the ethanol extracts of neem have antibiotic effect against selected bacterial strains. In this research, neem laf extract was evaluated as a natural antibiotic against some microorganisms. In future, neam leaf extract should be use as natural antimicrobial which will be hepful for organic food production in livestock industry.

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