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Original Article

Evaluation of antimicrobial effects of *prosopis cineraria* leaves

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ABSTRACT

Antimicrobial resistance has become a serious problem, now a day. The objective of study was to evaluate the antimicrobial effects of leaves of *Prosopis cineraria* against a variety of bacterial and fungal strains. For this purpose, six bacterial (both gram positive and negative) and two fungal strains were selected. Crude plant extract was prepared in 70% hydro-alcoholic solution by simple extraction and it was observed that the leaves extract inhibited the growth of all tested microorganisms up to certain level at concentrations of 200, 400, 800 and 1600 µg/disc, by using disc diffusion method. Maximum zones of inhibition (mm) of leaves extract against *Eschericha coli, Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Klebsilla pneumonia* and *Pseudomonas auregenosa* were 25.7, 21.7, 24.7, 23.7, 26.7 and 26.3 mm respectively at concentration of 1600 µg/disc. Moreover, *Prosopis cineraria* leaves extracts in concentration of 1600 µg/disc remarkably inhibited the growth of *Cunninghamella echinulata* and *Aspergillus niger* species, with *zones* of inhibition of 15.7 and 24.3 mm, respectively. While, there was no inhibition of growth of microorganisms at low concentration (200 µg/disc) against these fungal strains. On the basis of results, it was concluded that plant extract significantly inhibited the growth of bacterial and fungal strains, further it was found that extract was more active against gram negative bacteria than that of gram-positive bacteria.

Key words: *Prosopis cineraria*, leaves extract, gram positive bacteria, gram negative bacteria, bacterial resistance

INTRODUCTION

Emergence of resistance has created a need to discover new antimicrobial agents. We are living in an era that may truly be called the post antibiotic era and this has become the bitter truths of our lives. In the twenty first century, microbes have developed resistance against all known antibiotics present to date and this has become a worldwide healthcare issue. [1] Due to extensive and unchecked use of antibiotic, multiple drug resistance has been developed against a large population of microbes.[2,3] So a need arises, that pushes us to create modification in already existing sources of antibiotic.[3] Emergence of resistance is directly related to the extensive unchecked use of antibiotics.[4] Resistance to antibiotic classes is increasing in a linear manner and complicated resistance mechanisms have developed multidrug-resistance (MDR) organisms and some "superbugs". Instead of so much need for development of new antimicrobials, research in this area has declined due to economical constraints in pharmaceutical industry, large academic research institutions and government sector. [1]

Instead of so much development has been taken place in science and technology in present era, still approximately 25% of the current medicines have their origin directly or indirectly from natural

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sources including plants. Since beginning of history, plants have been served as a rich source of bioactive metabolites which provide protection against number of diseases.[5] Plants are used as medicine since ancient times for the treatment of diseases and it is evident from literature that oldest known method for healing is the use of plants.[6] For discovering antimicrobial substances research has been done on lower plants , fungus and bacteria but higher plants has not been focused for discovering new chemical substances which can act as a source of antimicrobial agents and would be helpful to humanity. Secondary metabolites from higher plants have antimicrobial potential against microbes which attack plants.[7] At present acceptance of traditional medicine as an alternate to the chemical agents encourages the researchers to explore the plant sources as medicine, particularly due to emergence of the microbial resistance.[8] Medicinal plants are also considered as a valuable source of new medicinal entities which have therapeutic potential.[9] Compounds such as emetine, berberine and quinine which are derived from plants are very effective against the infectious microbes.[10] Plants proved to be a good source of anti-infective agents. Many secondary metabolites of plants like flavanoids, polyphenols, terpenoid, sesquiterpenes and tannins are effective against a number of microorganisms.[11]

Prosopis cineraria is locally known as Jand and belongs to family Leguminosae, which is second largest family of flowering plants. [12-13] It is considered as a tree or small shrub which is almost straight.[14] It finds its distribution to various locations of Gulf countries and also found in different locations of India like Rajasthan, Gujarat, Haryana, Uttar Pradesh and Tamil Nadu.[15]

Prosopis cineraria is extensively used as a protect-ant to avoid miscarriage in pregnant women who eat the mixture prepared with the sugar and pounded flowers of the plant. [14,16-17] Bark is beneficial in cases of rheumatism and also used for scorpion stings. Pods of the plant have astringent action. Ashes prepared by burning the leaves have hair removing properties when applied on skin with rubbing. [14, 18] Farmers eat the dry pods to get rid of the excessive thirst during summer season. When flowers are mixed with sugar in water, then they are utilized for skin diseases, prevention of boils and as blood purifier and for the cooling effects.[19] Dry bark is used to combat the muscle tumors, piles, asthma, bronchitis, leucoderma, dysentery, tonic, leprosy and as anthelmentic. This is equally effective for curing wandering mind as well as for its cooling effects and to heal scorpion and snake bites. [20] Wood of the plant is also used as fuel and for construction purposes as well as for agricultural implements. The plant is also used as a fodder for animals.[21] The fruit of the plant is used as a vegetable traditionally known as sangari. [16] Flowers of plants are used for the honey production and its bark has abortificent and laxative properties.[13]

MATERIALS AND METHODS

Collection and authentication of plant material: *Prosopis cineraria* leaves were collected in month of March from Cholistan Institute of Desert Studies (SIDS), the Islamia University of Bahawalpur. These were authenticated from a Botanist and a duplicate portion of the leaves was submitted at herbarium of the Faculty of Pharmacy and Alternative Medicine, the Islamia University of Bahawalpur and a herbarium voucher number PC-LE-03-12-036 was taken.

Preparation of ethanolic extract: Leaves of *Prosopis cineraria* were washed under running tap water and then with distilled water. Afterwards, leaves were dried under shade to a constant weight. Then that dried material was grinded into coarse powder by using electric grinder National, Japan. This powder was socked in seventy percent ethanol in a glass beaker for three days with occasional stirring. Then it was filtered first through two folds of muslin cloth and then from filter paper. Filtrate so achieved was placed in refrigerator Dawlance, Pakistan and marc was again macerated for three days and filtered as previously. Two filtrates were mixed and evaporated under reduced pressure and temperature by using rotary evaporator, Heidolph Laborota 4000 efficient, Germany and a semisolid paste was obtained finally. Its percentage yield was calculated as 12.53% and extract was preserved in freezer Haier, Pakistan at -20^{o}C until used for further experiment.

Bacterial strains: Antibacterial activity of *Prosopis cineraria* was evaluated against six bacterial strains, three of these were gram positive i.e. *Staphylococcus aureus, Bacillus subtilis* and *Salmonella typhi* while three ware gram negative bacteria i.e. *Eschericha coli, Klebsilla pneumonia* and *Pseudomonas auregenosa.* All these bacterial strains were clinical isolates and were collected from the Microbiology and Pathology department of the Quaid-e-Azam Medical Collage Bahawalpur.

Fungal strains: Two fungal strains were used for evaluation of antifungal activity i.e. *Cunninghamella echinulata* and *Aspergillus niger.*

Preparation of inoculums: All the bacterial strains were grown in the sterilized nutrient broth, Merck, Germany. The bacterial strains were grown

up to exponential phase for 18 hours at 37 ºC**.** Then these were adjusted to 1 X 10^5 colony forming units (CFU) by adding sterilized nutrient broth to it which matched with 0.5 McFarland turbidity standards.

Preparation of crude plant extracts discs: 10, 20, 40 and 80 mg of plant leaves extract was weighed by using analytical weighing balance and was dissolved separately in 70 % ethanol solution. 20 µl of each of this solution was added on each 6 mm filter paper discs so that each disc contains 200, 400, 800 and 1600 µg of crude plant extract. These discs were dried at room temperature. Similarly, discs of 70 % ethanol were prepared, which served as negative control of the experiment. Two standard antibiotics, Ciprofloxacin 5 µg and Gentamicin 10 µg discs were used as positive or standard control of the experiment which was purchased from Liofilchem, Italy.

Agar disc diffusion assay: Antibacterial activity of crude plant extracts was determined by using the methods as earlier used. [22,23] For antibacterial and antifungal assay of crude plant leaves extract, a standardized bacterial and fungal suspension in sterilized nutrient broth and Saboruad's dextrose agar was inoculated on the Muller Hinton agar and Sabourad's dextrose agar for antibacterial and antifungal assay respectively. A sterile swab was soaked in the bacterial or fungal culture from test tube and was moved up and pressed against the wall of tube to remove excessive liquid. These inocula of respective bacteria or fungus were spread on to the Muller Hinton agar or Saborad's dextrose agar respectively in a way that a uniform lawn of the bacterial or fungal growth was obtained after incubation. For this purpose, the swab was smoothly moved on entire surface of agar in one direction i.e., along x-axis. Then the plate was turned at 90º and the swab was moved along the rest of two directions i.e., along y-axis and z-axis. This ensures the uniform growth of the bacteria or fungus after incubation period. Then filter paper discs of each extract and negative control as well as discs of reference antibiotic or antifungal discs were placed at proper distance with flamed forceps. These discs were pressed smoothly so that proper contact with the agar had ensured. The plates that contain bacteria were incubated for 18-24 hours at 37 ºC and plates containing fungus ware incubated at 48 hours at 36 ºC. After incubation period the zones of inhibition were measured. Each assay was repeated thrice.

Determination of Minimum Inhibitory Concentration (MIC) by using 96-wells plates: 96-well plates under sterile conditions were used to determine antibacterial activity. The principle behind the method is that as number of bacterial cells increase during the lag phase of their life cycle the turbidity of the nutrient broth increases in which the bacteria are grown and therefore absorbance of the nutrient broth also increases.[24-26] For antimicrobial study, three gram positive (*Staphylococcus aureus, Bacillus subtili* and *Salmonella typhi)* and three gram negative bacteria *(Eschericha coli, Klebsilla pneumonia* and *Pseudomonas auregenosa*) were utilized. All the bacterial cultures were maintained on the nutrient broth solution and they were adjusted to 0.5 McFarland turbidity standards.[27] All test samples were dissolved in appropriate solvents. 20 µl of samples were pipetted into the wells at a concentration of 25-200 µg / ml. Then 180 µl of 12 hours old bacterial culture was added after suitable dilutions with nutrient broth into each well. Before incubation of the 96-wells micro plates the initial absorbance was adjusted among 0.12-0.19 at 540 nm. The final volume in each of the well was maintained to 200 µl. All bacterial cultures were incubated in incubator at 37 ºC with lids on micro titter plates for a period of 24 hours. Synergy HT Bio Tek® USA, micro plate reader was used to measure absorbance of the bacterial cultures before and after incubation period at 540 nm. The difference was taken as index of bacterial growth. Using the following formula percentage inhibition was calculated:

Percentage inhibition = (X – Y) 100 / X

Whereas: X = Absorbance of control with bacterial culture

Y = Absorbance of bacterial culture and test sample(s)

Results were calculated as mean of triplicate (n = 3 ± SEM). Ciprofloxacin and Gentamicin were taken as standard drugs for comparison, EZ – Fit5 Perrella Scientific Inc. Amherst USA software was used for calculation of the results, results were represented as MIC_{50}. Minimum inhibitory concentration (MIC) is that concentration of drug that inhibits any visible microbial growth. MIC_{50} – MIC_{90} are the concentrations which inhibit of 50% and 90% of bacterial growth.[7]

RESULTS AND DISCUSSIONS

Phytochemical analysis: Primary phytochemical analysis was performed by using standard qualitative methods and it was found that leaves of *Prosopis cineraria* contains tannins, flavanoids, saponins, glycos-ides, alkaloids and steroids. Primary qualitative phytochemical analysis of all ethanolic extracts of plant were performed by using standard methods which showed that they contained alkaloids, saponins, tannins,

pholobatannins, flavanoids, steroids and cardiac glycosides, these results were in accordance with the phytochemical analysis which was previously performed on this plant, the ethanolic and aqueous fractions prepared have alkaloids, glycosides, saponins, flavanoids and phenolic compounds.[28,29] *Prosopis cinerarium* is extensively used for the treatment of skin diseases, boils and as blood purifier.[19] Antimicrobial agents can be obtained from the plants as more than 1000 plants with antimicrobial activity have been reported so for.[30] Therefore, antimicrobial activity of *Prosopis cineraria* (L) Druce leaves extract was performed which was prepared in 70% ethanol. Antimicrobial activity was assessed at concentration of 200, 400, 800 and 1600 µg/disc, by using disc diffusion method. For this purpose, six bacterial strains were used, three of them were gram positive i.e. *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi* and three were gram negative i.e. *Eschericha coli, Klebsilla pneumonia* and *Pseudomonas auregenosa.* Two fungal strains were also used to assess antifungal activity i.e. *Cunninghamella echinulata* and *Aspergillus niger*. Ciprofloxacin 5 µg discs and gentamicin 10 µg discs were used as reference antibacterial agents and fluconazole at a dose of 400 µg /disc was used as reference antifungal agent. Leaves extract of *Prosopis cineraria* showed strong antimicrobial activity against all bacterial and fungal strains.

The leaves extract inhibited the growth of all organisms tested in varying degrees at concentration of 200, 400, 800 and 1600 µg/disc, by using disc diffusion method as indicated by their zones of inhibition in Table 1 and 2. Maximum zones of inhibition (mm) of leaves extract against *Eschericha coli, Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Klebsilla pneumonia* and *Pseudomonas auregenosa* were 25.7, 21.7, 24.7, 23.7, 26.7 and 26.3, respectively at concentration of 1600 µg/disc. Minimum zone of inhibition (mm) of this extract against *Eschericha coli, Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Klebsilla pneumonia* and *Pseudomonas auregenosa* were 7.33, 8.67, 9.67, 8.33, 21.7 and 9.66, respectively at concentration of 200 µg/disc.

Similar analysis of *A. nilagirica* leaf extracts were carried out on clinical bacterial pathogens. The analysis showed the presence of alkaloids, flavonoids, phenol, tannins and phlobatannins in leaves extract.[31] Almost all these secondary metabolites are also present in ethanolic extract of *Prosopis cineraria* leaves. These constituents could be potential alternatives to the traditional chemical control of clinical pathogenic bacteria. The antimicrobial activity of plant extracts might not be due to the action of a single active compound, but might be due to the synergistic effect of several compounds that are in minor proportion in a plant.[32]

In our study it has been found that among all the tested organisms, the gram-negative bacterial strain, *Klebsiella pneumonia* was found to be more susceptible to the leaves extract by showing zone of inhibition ranging from 21.7–29.3 mm. Gumgumjee *et al.,* reported that the most pronounced effect was shown by the ethanol extract of *Tamarindus indica* against *Klebsiella pneumonia.*[33]

It has been found that the alcohol is a better solvent for extraction of antimicrobial active substances compared to water and hexane.[34] Ethanol extracts in both studies might have higher solubility for phytoconstituents. These phytoconstituents may include alkaloids, glycosides or tannins which might be responsible for highest antibacterial activity against *Klebsiella pneumonia.*[33]

Leaves extract exerted inhibitory effect on the test organisms up to certain extent. Minimum inhibitory concentration which inhibited the growth of 50% and 90% bacteria i.e., IC_{50} and IC_{90} values for leaves extract were 11.27 µg/ml & 14.17 µg/ml for *K. pneumonia*, 12.14 µg/ml & 16.0 µg/ml against *E. coli*, 11.16 µg/ml & 18.11 µg/ml for *P. auregenosa, 12.0* µg/ml & 14.27 µg/ml for *S. aureus*, and 17.12 µg/ml & 17.14 µg/ml for *B. subtilis* and 13.11 µg/ml & 18.22 µg/ml for *S. typhi*. The results obtained in the agar diffusion plates followed the same trend with what was obtained in the minimum inhibitory tests. Gram-negative bacteria were more sensitive than Gram-positive bacteria due to the presence of secondary metabolites such as alkaloids, flavonoids and steroids.[35] This is not in agreement with previous reports that plant extracts are more active against Gram positive bacteria than Gram negative bacteria. These differences may be attributed to the fact that the cell wall in Gram positive bacteria is of a single layer, whereas the Gram-negative cell wall is multilayered structure.[36] At 1600 µg/disc concentration of *Prosopis cineraria* leaves extracts against *Cunninghamella echinulata zones* of inhibition were 15.7 mm and against *Aspergillus niger* 24.3 mm. While there is no growth inhibition at concentration of 200 µg/disc when, used during study against each strain. Hypothetical increase in the antifungal activity of any extract of *Prosopis cineraria* supposed to be found by increase in concentration (µg/ml). Nayan and Shukla reported the antifungal activity of leaves extract containing saponins, tannins, alkaloids, flavonoids, steroids and glycosides against *A.niger* with different

concentrations. This is showing increased antifungal activity with increase in concentration.[37]

CONCLUSION

On the basis of results it was concluded that plant extract significantly inhibited the growth of bacterial and fungal strains, further it was found that extract was more active against gram negative bacteria than gram positive bacteria.

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Table 1. Zone of inhibitions of *Prosopis cineraria* leaves extract and Ciprofloxacin against various strains of bacteria

No.	Strain of Bacteria	**Diameter of zone of inhibition** (mm) (Mean ± SEM)				
		Negative control	**Ciprofloxacin 5 µg**	***P. cineraria* 200 µg**	***P. cineraria* 400 µg**	***P. cineraria* 800 µg**	***P. cineraria* 1600 µg**
1	*E. coli*	7.33 ± 0.667	31.7 ± 1.45***	7.33 ± 0.88***	18.7 ± 1.20**	23.7 ± 0.882**	25.7 ± 1.20*
2	*P. auregenosa*	6.33 ± 0.333	33.7 ± 2.91***	9.667 ± 1.20**	19.7 ± 1.76*	24.7 ± 1.33*	26.3 ± 1.45^{ns}
3	*S. aureus*	6.33 ± 0.333	31.0 ± 1.53***	8.67 ± 0.88***	17.0 ± 1.53**	19.3 ± 1.45**	21.7 ± 2.40*
4	*K. pneumonia*	6.33 ± 0.333	34.3 ± 1.86***	29.3 ± 2.03***	21.7 ± 1.45**	21.7 ± 1.45**	26.7 ± 1.45*
5	*B. subtilis*	6.33 ± 0.333	36.7 ± 1.45***	9.67 ± 1.20***	17.7 ± 1.76**	21.3 ± 1.45**	24.7 ± 1.45**
6	*S. typhi*	6.33 ± 0.333	36.7 ± 1.76***	8.33 ± 0.88***	20.3 ± 1.45**	21.7 ± 1.76**	23.7 ± 1.76**

n = 3. P < 0.05 significant (*), P < 0.01 significant (**), and P < 0.0001 highly significant (***), ns = non significant

Table 2. Zone of inhibitions of *Prosopis cineraria* leaves extract and Gentamicin against various strains of bacteria

No.	Strain of Bacteria	**Diameter of zone of inhibition** (mm) (Mean ± SEM)				
		Negative control	**Gentamicin 10 µg**	***P. cineraria* 200 µg**	***P. cineraria* 400 µg**	***P. cineraria* 800 µg**	***P. cineraria* 1600 µg**
1	*E. coli*	7.33 ± 0.667	26.3 ± 1.45***	7.33 ± 0.88***	18.7 ± 1.20*	23.7 ± 0.882^{ns}	25.7 ± 1.20^{ns}
2	*P. auregenosa*	6.33 ± 0.333	29.0 ± 1.15***	9.67 ± 1.20***	19.7 ± 1.76*	24.7 ± 1.33^{ns}	26.3 ± 1.45^{ns}
3	*S. aureus*	6.33 ± 0.333	26.7 ± 1.45***	8.67 ± 0.88***	17.0 ± 1.53*	19.3 ± 1.45*	21.7 ± 2.40^{ns}
4	*K. pneumonia*	6.33 ± 0.333	8.33 ± 0.882***	21.7 ± 1.4^{5***}	24.7 ± 0.882*	24.7 ± 0.882^{ns}	26.7 ± 1.45^{ns}
5	*B. subtilis*	6.33 ± 0.333	31.3 ± 1.86***	9.67 ± 1.20***	17.7 ± 1.76**	21.3 ± 1.45*	24.7 ± 1.45*
6	*S. typhi*	6.33 ± 0.333	29.7 ± 2.03***	8.33 ± 0.88***	20.3 ± 1.45*	21.7 ± 1.76*	23.7 ± 1.76^{ns}

n = 3. P < 0.05 significant (*), P < 0.01 significant (**), and P < 0.0001 highly significant (***), ns = non significant

Table 3. Minimum Inhibitory concentration in µg / ml

No	Extract and Standard drugs	MIC	*E. coli*	*P. auregenosa*	*K. pneumonia*	*S. aureus*	*B. subtilis*	*S. typhi*
1	*P. cineraria* leaves extract	MIC_{50}	12.14±0.16	11.16 ±0.10	11.27±0.32	12.01±0.70	17.12±0.31	13.11±0.38
2	*P. cineraria* leaves extract	MIC_{90}	16.01±0.20	18.11±0.18	14.17±0.12	14.27 ±0.70	17.14±0.21	18.22±0.11
3	*Ciprofloxacin*	MIC_{50}	10.36±0.3	8.42± 0.123	9.31 ±0.18	8.21 ±0.02	11.21±0.1	10.89±0.11
4	*Ciprofloxacin*	MIC_{90}	8.21 ± 0.02	11.03 ± 0.10	12.34±0.22	10.12 ± 0.11	12.16±0.22	7.59 ± 0.11
5	*Gentamicin*	MIC_{50}	8.36 ± 0.12	9.42 ± 0.11	15.34±0.22	8.21 ± 0.02	7.59 ± 0.11	11.03±0.10
6	*Gentamicin*	MIC_{90}	11.21±0.12	12.19±0.21	11.11 ±0.08	13.42 ± 0.13	11.36±0.3	14.21±0.60

Table 4. Zone of inhibitions of *Prosopis cineraria* leaves extract standard against various strains of fungi

		Diameter of zone of inhibition (mm) (Mean ± SEM)					
No.	**Strain of Fungus**	**Negative control**	**Fluconazole 10 µg**	***P. cineraria* 200 µg**	***P. cineraria* 400 µg**	***P. cineraria* 800 µg**	***P. cineraria* 1600 µg**
1	*Aspergilus niger*	6.33 ± 0.333	25.0 ± 1.15***	6.33 ± 0.333***	18.3 ± 1.45*	20.7 ± 1.76ns	24.3 ± 0.882ns
2	*Cuminghamella echinulata*	6.33 ± 0.333	16.7 ± 0.882***	6.33 ± 0.333***	12.7 ± 1.45ns	15.3 ± 0.882ns	15.7 ± 0.882ns

n = 3. P < 0.05 significant (*), P < 0.01 significant (**), and P < 0.0001 highly significant (***), ns = non significant

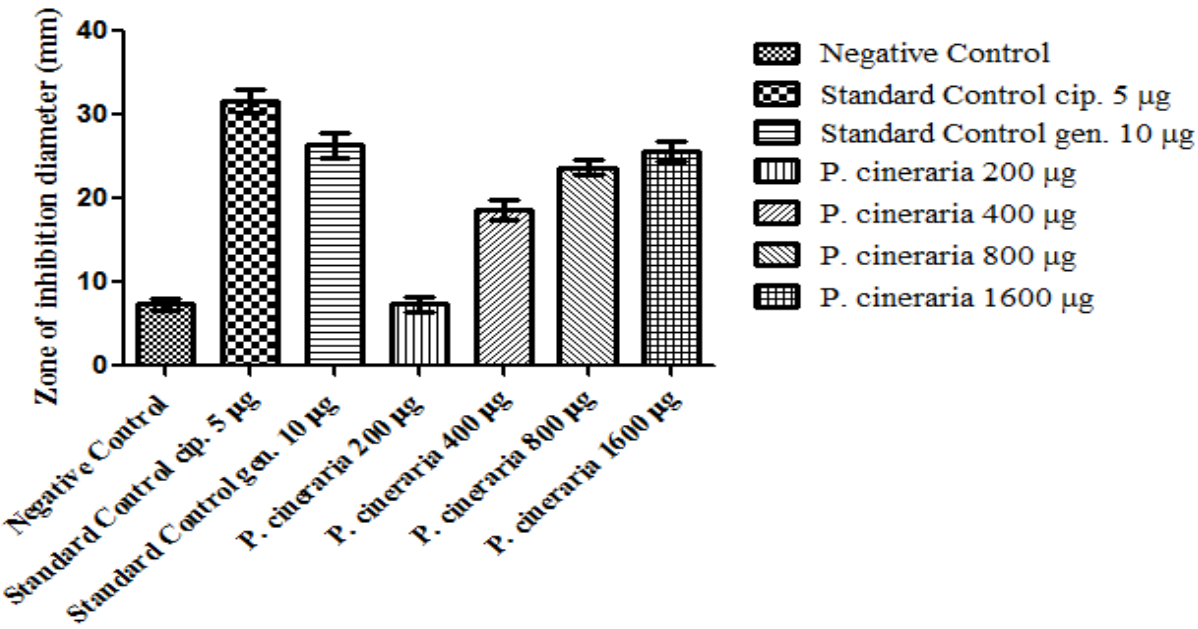

Figure 1. Effects of *Prosopis cineraria* crude leaves extract on *Escherichia coli*

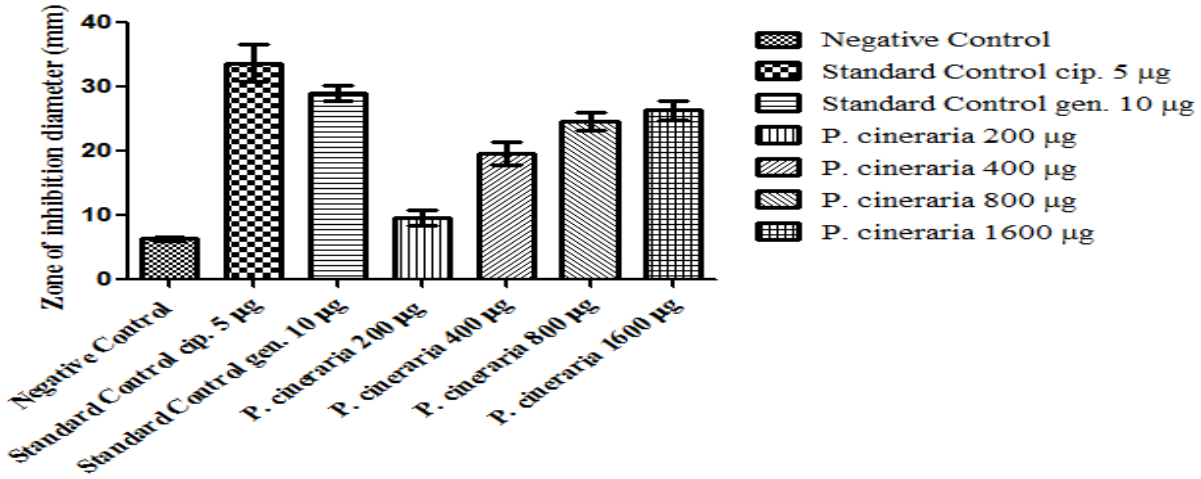

Figure 2. Effects of *Prosopis cineraria* crude leaves extract on *Pseudomonas aeruginosa*

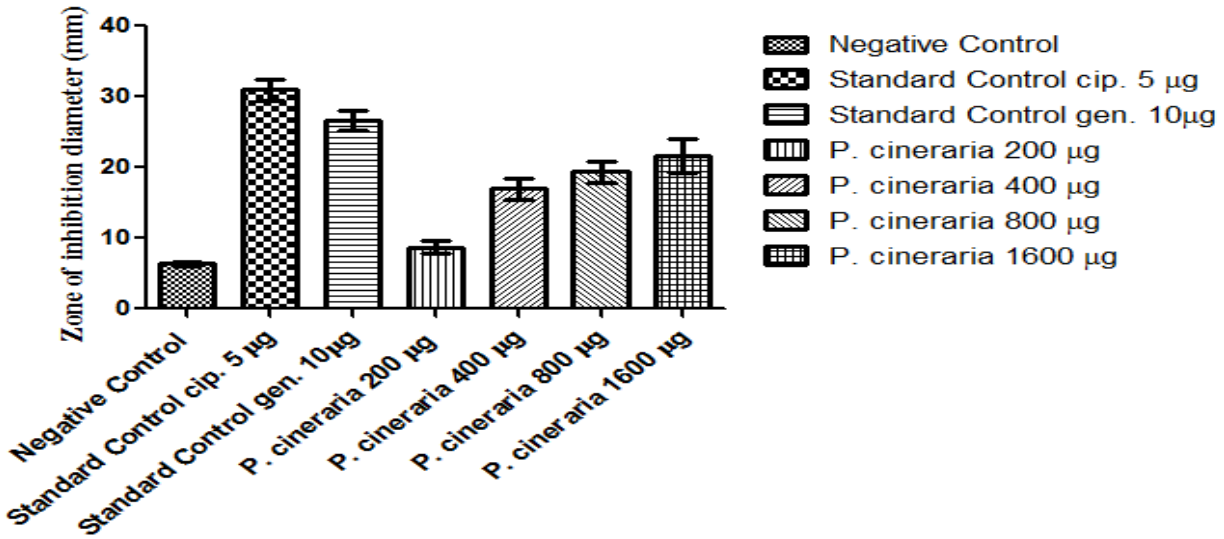

Figure 3. Effects of *Prosopis cineraria* crude leaves extract on *Staphylococcus aureus*

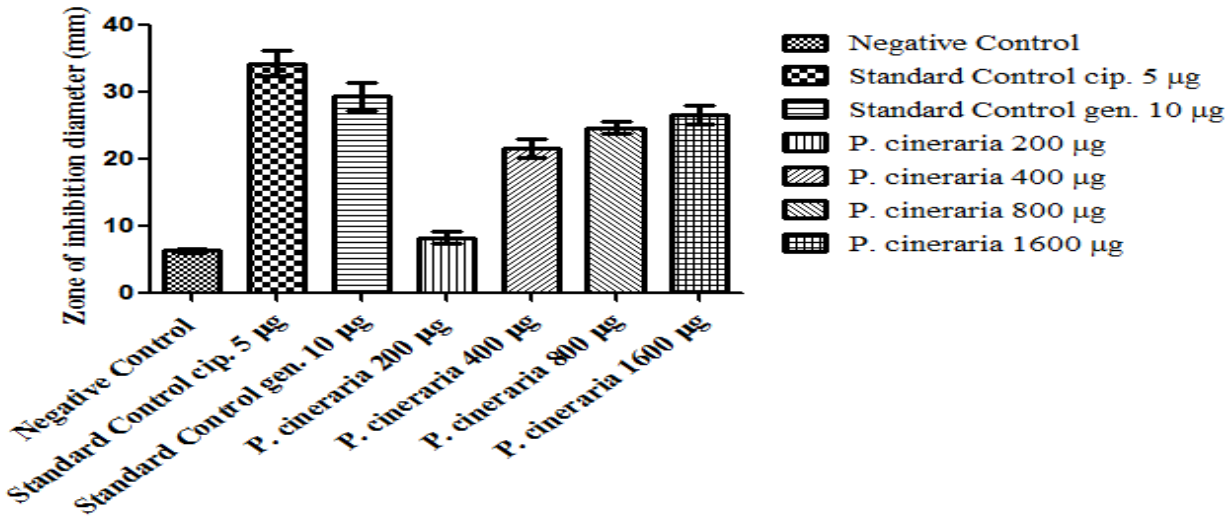

Figure 4. Effects of *Prosopis cineraria* crude leaves extract on *Klebsilla pneumonia*

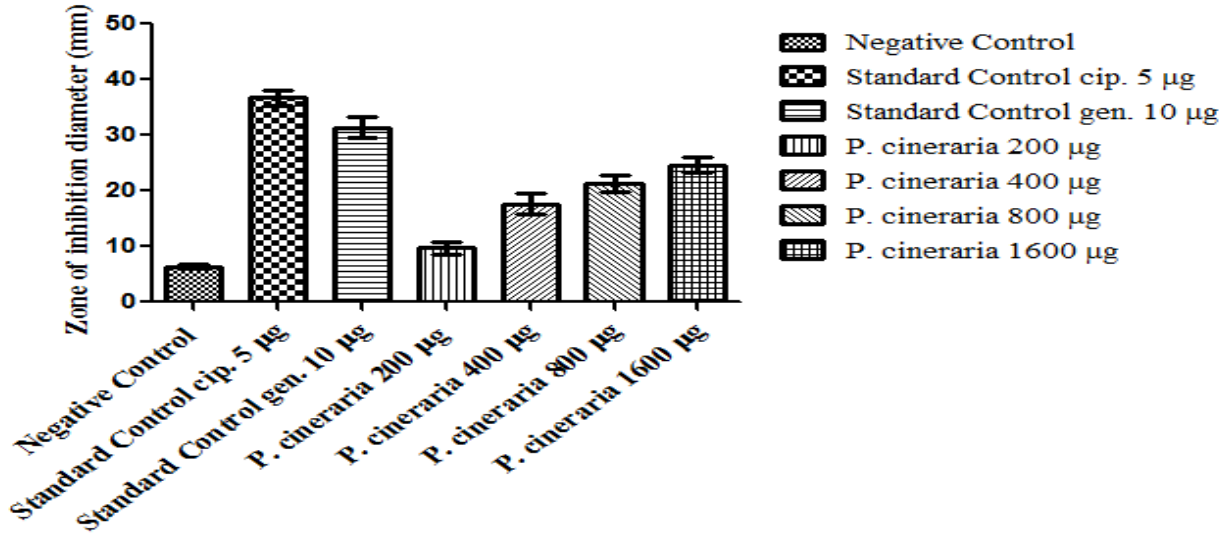

Figure 5. Effects of *Prosopis cineraria* crude leaves extract on *Bacillus subtilis*

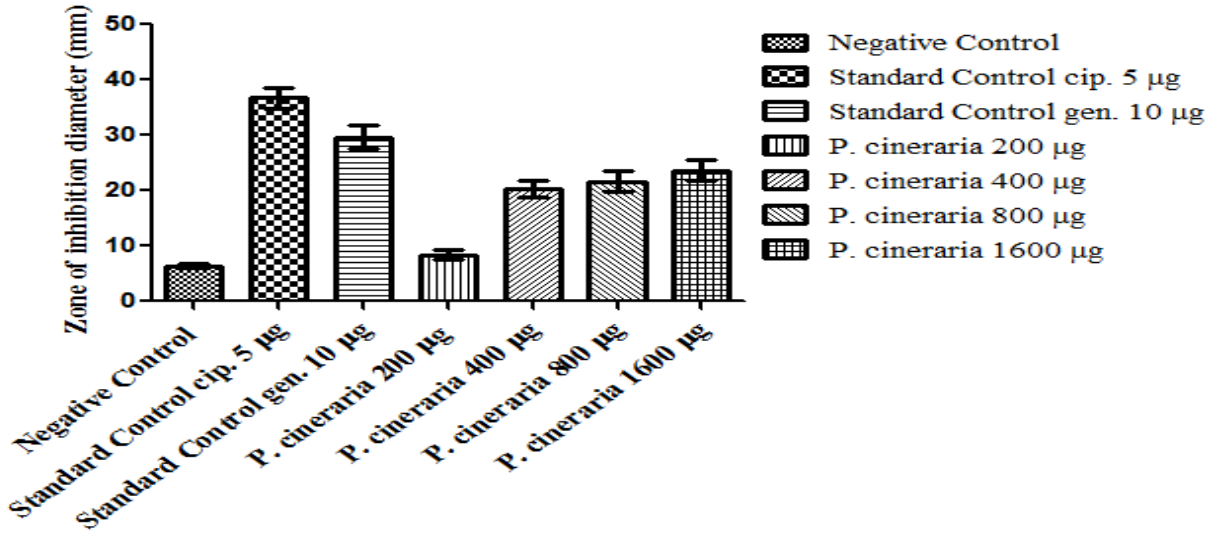

Figure 6. Effects of *Prosopis cineraria* crude leaves extract on *Salmonella typhi*

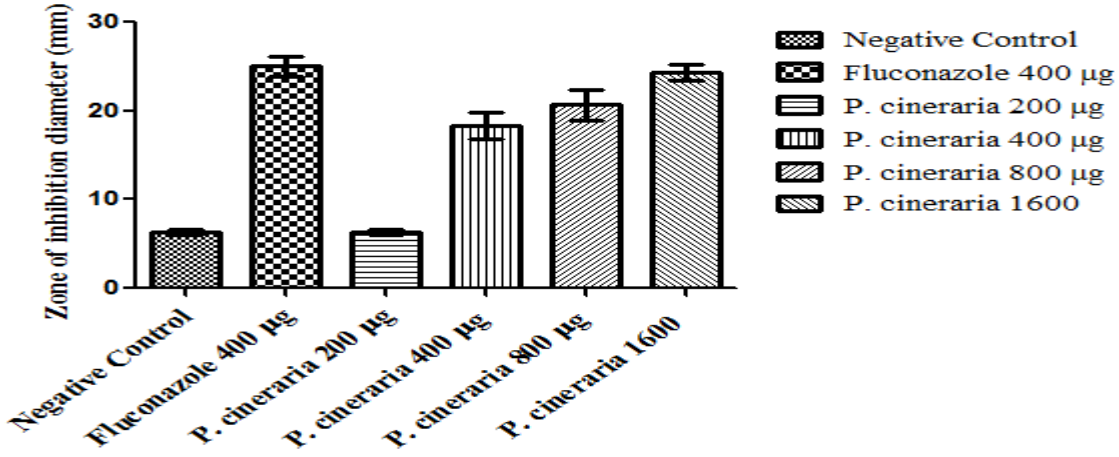

Figure 7. Effects of *Prosopis cineraria* crude leave extract on *Aspergillus niger*

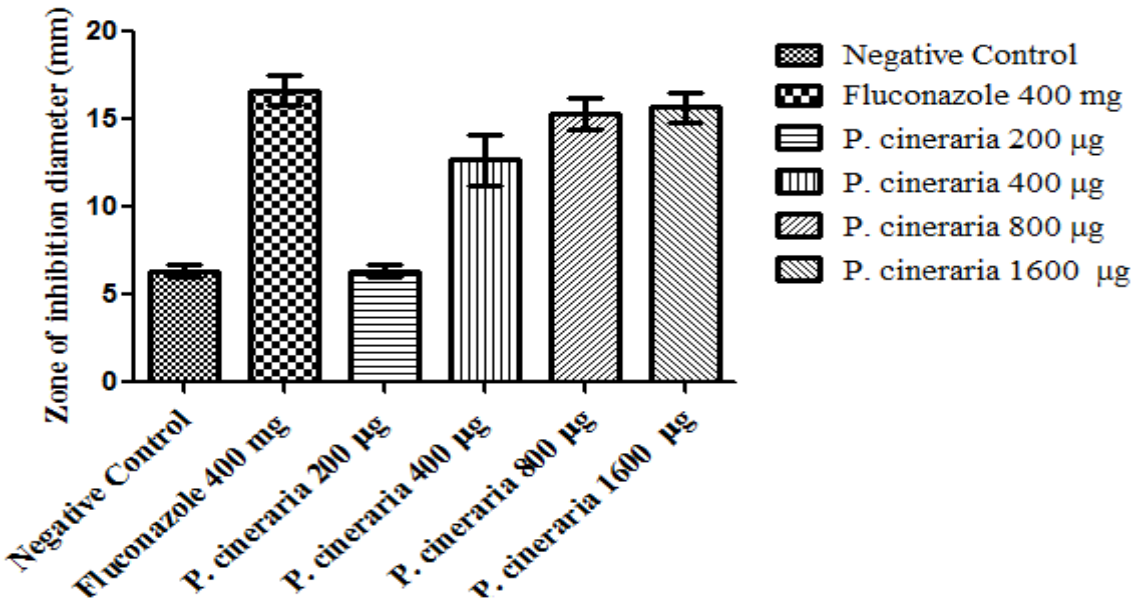

Figure 8. Effects of *Prosopis cineraria* crude leave extract on *Cunninghamella*

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