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Antimicrobial Activity of Cow Urine and *Azadirachta indica* and Phytochemical Analysis

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ABSTRACT

We have evaluated antimicrobial activity of neem leaves extract and cow urine extract on bacterial strains which are *Bacillus subtilis, Escherichia coli, Proteus vulgaris, Staphylococcus aureus. Pseudomonas aeruginosa* to assess the phytochemical analysis of neem leaves and cow urine. In this various studied extracts of cow urine and *Azadirachta indica* possessed antimicrobial activity against pathogens liked *Bacillus subtilis, Escherichia coli. Proteus vulgaris, Staphylococcus aureus* and *Pseudomonas aeruginosa.* The extracted Indian cow urine and *Azadirachta indica* demonstrated antimicrobial properties against all tested microorganisms. Given their abundance in nature, cow urine and *Azadirachta indica* offer a cost-effective and easily processable solution for a range of health issues, making them promising for future applications. The medicinal properties of these cow urine and *Azadirachta indica* can be exploited to formulate drugs for several diseases caused by antibiotic resistant pathogenic microorganisms.

Key words: *Antimicrobial activity, Azadirachta indica, Cow urine, Bacteria Test*

Introduction

India, known for its rich biodiversity, has a long tradition of using medicinal plants for healthcare. *Azadirachta indica* (Neem) is one such plant with significant antimicrobial properties against bacteria, yeasts, and fungi (Preethi and Lognathan, 2010). India also has a large cattle population, and cow urine has been traditionally valued for its medicinal properties. It is used in practices like panchagavya, which combines cow urine with other cow-derived substances for medicinal and agricultural purposes. The excessive use of antibiotics has led to drug-resistant strains, making alternative antimicrobial options important. Cow urine and Neem are explored for their synergistic antimicrobial activity. Neem has been extensively studied and found to have various medicinal properties, including antibacterial, anti-inflammatory, and immune-modulatory effects. Cow urine, known as Gou-Muttra, is considered in a Ayurveda, with potential benefits for diseases such as cancer, asthma, and allergies.

Materials and Methods

Cow urine collection

Cow urine was collected from a cow from a farm from kasegaon, Dist- Sangli.

Medicinal plants collection

Matused leaves of *Azadirachta indica* were collected from Kasegaon, Dist- Sangli, Maharashtra, India. Fresh and shade dried leaves were used for the study.

Preparation of Extract of *Azadirachta indica* leaves

Aqueous extraction 50 g of shade dried *A. indica* leaves, coarsely powdered and added with 150 ml of sterile distilled water and placed in a water bath at 80 °C for 1.5 h.

This solution was filtered through a filter paper and dried into powder by placing at 40 °C for 2 days.

Solvent extraction

These leaves were air dried in shade for a week and powdered mechanically. The 20 g of air dried plant material was suspended in 40 ml of solvent solution for 2 h.

The solvent extract was filtered through filter paper and kept overnight for evaporation at room temp. The extracts was dissolved in dimethyl sulphoxides to make final concentration and stored as stock solution. For solvent extraction method methanol, acetone. solvents were used.

Preparation of *Azadirachata indica* leaves extract in cow urine

200 g of *Azadirachata indica* fresh leaves were surface sterilized with 0.1 % mercuric chloride and rinsed with sterile distilled water thrice. These leaves were cut into small pieces and placed in an earthen pot contains 1 l of fresh cow urine collected from a single cow fed with the same type of feed throughout the study. The pot was incubated in a pit for 10 days. After 10 days incubation crude extracts was collected from the pot and filtered through the filter paper and condensed into a dry powder at 40 °C.

In vitro testing of extracts for antimicrobial activity

Testing for antibacterial activity

The cup-plate agar diffusion method was adopted to assess the antibacterial activity of the prepared extract 0.6 ml of standardized bacterial stock suspensions thoroughly mixed with 60 ml of sterile Muller Hinton agar. 20 ml of the inoculated agar were distributed into sterile petri dishes. The agar was left to set and in each plates three cups of 10 mm diameter were cut using a sterile cork borer no.4 and the agar disc were removed.

Alternate one cup was filled with 0.1 ml of extract, second cup was filled with antibiotic (positive control), and third cup was filled with distilled water (negative control). The sample were added into cup by using micro pipette and allowed to diffuse in a refrigerator for 2-4 min. After diffusion plates were incubated in the upright position at 37 °C for 24h. After incubation the plates were observed and zones of inhibition were measured in mm.

Phytochemical Analysis

Phytochemical analysis was performed by both qualitatively and quantitative for *Azadirachta indica* and cow urine (Abdullahi *et al.,* 2020).

Phytochemical analysis of *Azadirachta indica*

The extract were analyzed by the following procedures to test for the presence of the alkaloids, saponins, tannins, terpenoids, flavonoids, glycosides, volatile oils and reducing sugars.

Tannins

To a portion of the extract with water 3-4 drops of 10% ferric chloride solution was added. A blue colour was observed for gallic tannins and green colour for catecholic tannins.

Reducing sugars

To 0.5 ml of plant extract. 1ml of water and 5-8 drops of Fehling solution was added and heated over water bath. Brick red precipitate indicated the presence of reducing sugar.

Glycosides

25 ml of dilute sulfuric acid was added to 5 ml extract in a test tube and boiled for 15 min. cooled and neutralized with 10% NaOH, then 5 ml of Fehling solution added. Glycosides shared brick red precipitate.

Alkaloids

2 ml of extract was measured in a test tube to which picric acid solution was added. An orange coloration indicated the presence of alkaloids.

Flavanoids

4 ml of extractsolution was treated with 1.5 ml of 5 % methanol solution. The solution was warmed and metal magnesium was added. To this solution 5-6 drops of concentrated hydrochloric were added and red colour was observed for flavonoids and orange colour for flavones.

Volatile oils

2ml of extract was shaken with 0.1 ml dilute NaOH and a small quantity of dilute hydrochloric acid. A

white precipitate was formed the volatile oils.

Terpenoids

4 mg of extracts was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulfuric acid was added slowly and red violet colour was observed for terpenoids.
Physicochemical analysis of cow urine : (Abdullahi *et al.*, 2020).

Quantitative phytochemical analysis was carries out for cow urine extracts.

Powdered fractions were dissolved in DMSO and standard procedures were followed to identify the constitutes

Test for alkaloids

Mayers test

1 mL of the filtrate 3 drops of diluted hydrochloric acid and Mayers reagent were added drop by drop on the sides of the test tubes. A creamyor white precipitate indicate the positive results.

Test for carbohydrates

Benedict's test

0.5 ml of the extract, 0.5 ml of the Benedict's reagent was added together. The mix ture was heated on boiling water bath for 2 min. A red color precipitate indicate the presence of sugar.

Test for saponins

The extract was diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for five min 2 cm layer of foam indicated the presence of saponins.

Test for tannins

About 0.5 mg of dried powder sample were boiled in 20 mL of water in test tubes, then filtered. Few drop of 1% ferric chloride were added and observed for brownish green or blue-black coloration for tannins.

Test for terpenoids

5mL of the extracts was mixed with 2 ml of chloroform and concentrated sulfuric acid to form a layer. A reddish brown coloration in the interface showed the presence of terpenoids.

Test for flavonoids

5 mL of diluted ammonia solution was added to a portion of aqueous extract followed by the addition of concentrated sulfuric acid. Appearance of yellow coloration indicated the presence of flavonoids.

Test for glycosides

1 mL of the extracts, 1 ml of alpha naphthol and chloroform was added along the sides and observed for the development of violet color which indicated the presence of glycosides.

Test for anthroquinone

1 mL of extract, 1 ml of diluted sulfuric acid were added and allowed to stand for some time to develop color. Development of pink color showed the presence of anthroquinone.

Test for quinine

1 mL of extract, 1 mL of concentrated sulfuric acid was added and allowed to stand for some time to develop color. Development of red color showed the presence of quinine.

Test for coumarin

1 mL of extract, 1 mL of 10% NaOH was added and allowed to stand for some times. Development of yellow color showed the presence of coumarin.

Test for phenol

4 drops of alcohol and ferric chloride solution were added to 1 ml of the extract and allowed to stand for few minutes. Development of yellow color showed the presence of phenol.

Results and Discussion

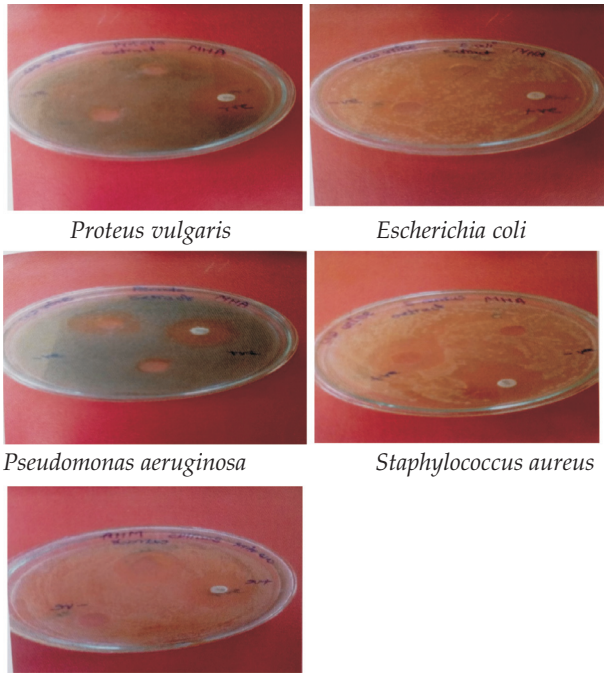
Effect of desi cow urine sample on microorganisms

The results of the effect of desi cow urine sample on the test microorganisms viz.; *Bacillus subtilis, Escherichia coli, Proteus vulgaris, Staphylococcus aureus, Pseudomonas aeruginosa*are presented in Table 1 and Photoplate 1.

It can be seen from Table 1 that, *Bacillus subtilis* showed the maximum zone of inhibition, i.e. 12 mm. The *Proteus vulgaris* showed the minimum 5 mm zone of inhibition. The *Escherichia coli* showed the 11 mm zone of the inhibition and the *Staphylococcus aureus* showed the 8 mm zone of the inhibition. The

Table 1. The effect of desi cow urine on the test organisms

Sr. No.	Test organism	Diameter of zone of inhibition of test organism (mm)
1	*Bacillus subtilis*	12 mm
2	*Escherichia coli*	11 mm
3	*Proteus vulgaris*	5 mm
4	*Staphylococcus aureus*	8 mm



Proteus vulgaris *Escherichia coli*

Pseudomonas aeruginosa *Staphylococcus aureus*

Bacillus subtilis

Photoplate 1. Showing the results of antimicrobial activity of cow urine extract against various test microorganism.

Pseudomonas aeruginosa showed the 9 mm zone of the inhibition.

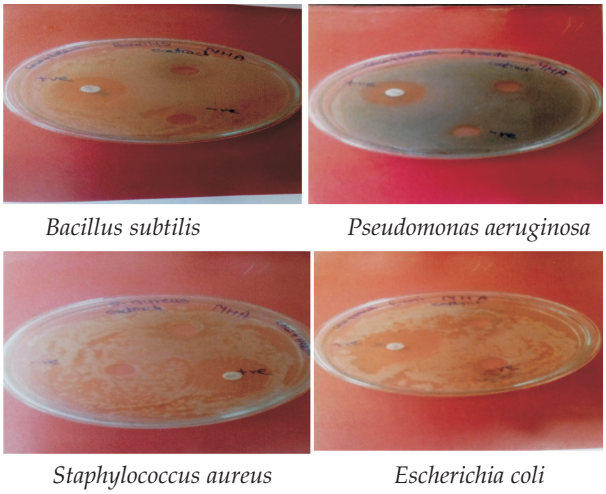
Effect of *Azadirachta indica* – cow urine extract

The result of *Azadirachta indica*- cow urine extract on different microorganisms viz.; *Bacillus subtilis, Escherichia coli, Proteus vulgaris. Staphylococcus aureus and Pseudomonas aeruginosa* are presented in Table 2. and Photoplate: 2.

It is clear from Table 2 that, *Azadirachta indica*-cow urine extract exhibited maximum antimicrobial activity against *Staphyloccocus aureus and Pseudomonas aeruginosa,* i.e.10 mm zone of inhibition. While moderate antimicrobial activity against *Escherichia coli* showed 5 mm zone of inhibition. Remeaning microorganisms, i.e. *Bacillus subtilis* showed 6 mm and *Proteus vulgaris* showed 7 mm zones of the inhibition

Table 2. The effect of *Azadirachta indica* – cow urine extract on test organism

Sr. No.	Test organism	Diameter of zone of inhibition of test organism (mm)
1	*Bacillus subtilis*	6 mm
2	*Escherichia coli*	5 mm
3	*Proteus vulgaris*	7 mm
4	*Staphylococcus aureus*	10 mm
5	*Pseudomonas aeruginosa*	10 mm



Bacillus subtilis *Pseudomonas aeruginosa*

Staphylococcus aureus *Escherichia coli*

Photoplate 2. Showing the results of antimicrobial activity of cow urine – *Azadirachta indica* extract against various test microorganism.

Effect of *Azadirachta indica* – methanol extract

The result of *Azadirachta indica* in methanol extract on different microorganisms viz.; *Bacillus subtilis, Escherichia coli, Proteus vulgaris, Staphylococcus aureus and Pseudomonas aeruginosa* are presented in Table 3. and Photoplate: 3.

It was observed from Table 3 that, *Pseudomonas aeruginosa* is more sensitive 17 mm zone of inhibition and the *Proteus vulgaris* is less sensitive 7 mm zone of inhibition. *Escherichia coli* shows 16 mm zone of

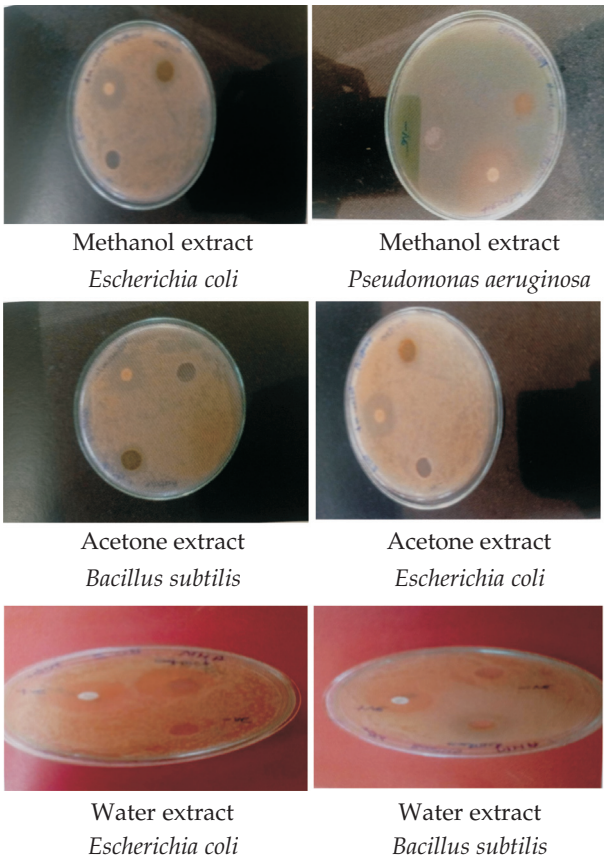
Table 3. The effect of *Azadirachta indica* in methanol extract on test organism

Sr. No.	Test organism	Diameter of zone of inhibition of test organism (mm)
1	*Bacillus subtilis*	12 mm
2	*Escherichia coli*	16 mm
3	*Proteus vulgaris*	7 mm
4	*Staphylococcus aureus*	12 mm
5	*Pseudomonas aeruginosa*	17 mm

inhibition. And *Bacillus subtilis* and *Stapylococcus aureus* shows the same, i.e. 12 mm zone of the inhibition against methanol extracts.

Effect of *Azadirachta indica* in aqueous extract:

The result of *Azadirachta indica* in aqueous extract on different microorganisms viz.; *Bacillus subtilis, Escherichia coli, Proteus vulgaris, Staphylococcus aureus and Pseudomonas aeruginosa* are presented in Table 4 and Photoplate: 3.



Methanol extract *Escherichia coli* | Methanol extract *Pseudomonas aeruginosa*

Acetone extract *Bacillus subtilis* | Acetone extract *Escherichia coli*

Water extract *Escherichia coli* | Water extract *Bacillus subtilis*

Photoplate 3. Showing the zone of inhibition of Methanol, Acetone, Water extracts of *Azadirachta indica* against various test microorganism.

The aqueous extracts of *Azadirachta indica* exhibited pronounced activity 16 mm against *Pseudomonas aeruginosa*. The *Staphylococcus aureus* shows 12 mm zone of the inhibition. *Escherichia coli* shows 11 mm and *Proteus vulgaris* shows 10 mm zone of the inhibition. The *Bacillus subtilis* shows less 7mmzone of inhibition.

Effect of *Azadirachta indica* in acetone extract

The result of *Azadirachta indica* in acetone extract on different microorganisms viz., *Bacillus subtilis, Escherichia coli, Proteus vulgaris, Staphylococcus aureus and Pseudomonas aeruginosa* are presented in Table 5. and Photoplate: 3.

Table 4. The effect of *Azadirachta indica* in the aqueous extract on test organism

Sr. No.	Test organism	Diameter of zone of inhibition of test organism (mm)
1	*Bacillus subtilis*	7 mm
2	*Escherichia coli*	11 mm
3	*Proteus vulgaris*	10 mm
4	*Staphylococcus aureus*	12 mm
5	*Pseudomonas aeruginosa*	16 mm

Azadirachta indica in acetone extract shows the maximum and same antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus* 18 mm zone of the inhibition. While *Proteus vulgaris* shows the minimum 15 mm zone of inhibition. The *Pseudomonas aeruginosa* showed the 16 mm zone of the inhibition. *Escherichia coli* showed the 17 mm zone of the inhibition.

Table 5. The effect of *Azadirachta indica* in acetone extract on test organism

Sr. No.	Test organism	Diameter of zone of inhibition of test organism (mm)
1	*Bacillus subtilis*	18 mm
2	*Escherichia coli*	17 mm
3	*Proteus vulgaris*	15 mm
4	*Staphylococcus aureus*	18 mm
5	*Pseudomonas aeruginosa*	16 mm

Results of Phytochemical analysis

The Phytochemical analysis was carried out for cow urine extracts. The Table 6 revealed that cow urine shows the presence of alkaloid, saponin, tannin, ter-

Table 6. Result of Phytochemical analysis of cow urine:

Sr. No.	Test	Result
1	Alkaloid	Present
2	Carbohydrate	Absent
3	Saponin	Present
4	Tannin	Present
5	Terpenoid	Present
6	Flavonoid	Present
7	Glycoside	Present
8	Anthraquinone	Present
9	Coumarin	Absent
10	Phenol	Present

Table 7. Results of Phytochemical analysis of neem tree leaves extracts

Sr. No.	Test	Solvent for extraction		
		Water	Methanol	Acetone
1	Tannin	Present	Present	Present
2	Reducing sugar	Present	Present	Absent
3	Glycosides	Present	Present	Present
4	Alkaloids	Present	Absent	Absent
5	Flavonoids	Present	Absent	Present
6	Volatile oil	Present	Absent	Absent
7	Terpenoids	Absent	Absent	Absent

penoid, flavonoid, glycoside, anthroquinone and phenol, but does not show the carbohydrate and coumarin.

The phytochemical analysis was carried out for Neem leaves solvent extracts.

It can be seen from Table 7 that neem leaves water extract showed the presence of reducing sugar, glycoside, alkaloid, Flavonoids, volatile oil while shows the absence of terpenoid.

The neem leaves methanol extract showed the presence of tannin, reducing sugar, glycoside while shows the absence of alkaloid, Flavonoids, volatile oil, The neem leaves acetone extract shows the presence of tannine, glycosides, flavonoids while showed the absence of reducing sugar, alkaloids, volatile oil and terpenoids.

Conclusion

Premature leaves of *Azadirachta indica* used as the starting material for preparation of methanol, aqueous and acetone extract. The extract was tested for their antimicrobial potential against the following organism *Bacillus subtilis, Escherichia coli. Proteus vulgaris, Staphylococcus aureus. Pseudomonas aeruginosa*. Among these *Escherichia coli, Stapylococcus aureus, Pseudomonas aeruginosa* showed higher zone of inhibition. While *Bacillus subtilis* and *Proteus vulgaris* showed comparatively less zone of inhibition. This study observes that all extracts of *Azadirachta indica* plant showed antimicrobial activity against all test organisms.

Phytochemical investigation of cow urine and *Azadirachta indica* extracts will definitely prove the presence of active phyto constituents like tannin, reducing sugar, glycoside, flavonoid, alkaloid, saponin, anthroquinone, which are the main constituents promoting antimicrobial activity Further research in this direction will help to develop proper formulation of cow urine and medicinal plant extracts to counter microbial infections, thus boosting the human immune system.

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