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Antioxidant, urobactericidal, and antibiotic modulating activity of a parasitic medicinal plant: *Cuscuta reflexa* Roxb.

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The therapeutic potential of age-old Indian herbal remedies needs immediate scientific validation against urogenital problems. This study investigates the antibacterial efficacy of the methanolic extract of *Cuscuta reflexa* Roxb. stem through multiple diffusion techniques and the zones of inhibition (ZOIs) are expressed in millimeters. The minimum inhibitory concentration (MIC) is found to be 1.25 mg/mL against *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa,* 2.5 mg/mL against *Proteus vulgaris*, and 0.625 mg/mL against *Staphylococcus aureus*. The MIC for Ciprofloxacin as a control is noted to be 0.062 µg/mL against *E. faecalis*, *P. vulgaris*, and *S. aureus*, and 1.25 µg/mL against *E. coli* and *P. aeruginosa*. *C. reflexa* extract exhibited substantial antibacterial activity against all tested strains, along with significant antibiotic-modulating activities specifically against *E. coli* and *E. faecalis*. The extract's ability to scavenge free radicals through the DPPH assay is indicated by an IC50 value of 212.61 µg/mL, with ascorbic acid as a control. The presence of phenols, tannins, glycosides, coumarin, flavonoids, saponins, steroids, and terpenoids is confirmed by qualitative tests. The total phenol and flavonoid contents are determined to be 22.54 ± 0.4 mg/g of Gallic acid equivalent and 113 ± 2.88 mg/g of Rutin equivalent, respectively. The vulnerating effect of *C. reflexa* extract on uropathogens secures its usefulness in pharmaceuticals

Key words: *Cuscuta reflexa* extract, antibacterial and antioxidant activities, phytochemical study, uropathogens.

INTRODUCTION

Scientific research must pay more attention to infectious diseases as they are the second leading cause of death worldwide. The widespread infectious bacteria have undergone multiple rounds of changes to adapt to their shifting environment, constantly producing new variants. These emerging variants of pathogenic strains frequently become more virulent and exhibit higher levels of antibiotic resistance. The severity of these variants can be mitigated by treating them with advanced antibiotics, supplemented with other antimicrobial compounds. This

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sources to control bacterial diseases. Nature has always been a valuable ally in the pharmacy field, as herbal drugs are comparatively time-tested and safer sources for human use. The constituent bioactive compounds, such as tannins, phenols, alkaloids, carbohydrates, terpenoids, steroids, and flavonoids, are synthesized through the primary or secondary metabolism of medicinal plants, providing definite physiological actions that can be widely used in human therapy (Akeel et al., 2014). Medicinal plants possess a large number of chemically and taxonomically diverse secondary metabolites that interact with other organisms in the environment, inhibiting bacterial or fungal growth *in vitro*. The antimutagenic and protective effects have been attributed to many classes of phytochemical compounds, mainly flavonoids and phenolic compounds, present in different parts of plants (Yamrutas et al., 2012). A revolutionary development in the medicinal field pertaining to microbe-induced diseases began in 1940, probably with the addition of phytochemical compounds. For treating urinary tract infections (UTIs), medicinal flora is frequently prescribed in the form of decoctions, powders, tablets, or ash. *Cuscuta reflexa* Roxb. was selected from the traditional healer's herbal list to search for a more potent and safer drug, as it possesses significant pharmacological and medicinal antibacterial properties.

C. reflexa, a member of the morning glory family, is frequently referred to as giant dodder and belongs to the Convolvulaceae family. These are parasitic plants found throughout temperate and tropical regions of the world, with the greatest species diversity in subtropical and tropical regions. These vines are thin, leafless, stem parasites that twine around a host plant. The flowers are small, bell-shaped, and white in color with yellow filaments (Noureen et al., 2019). Prior to contacting a host, seedlings of all species are self-sufficient, some yellow and some autotrophic. These evolved into holo-parasites from hemiparasites through the gradual reduction of the photosynthetic apparatus.

Previous phytochemical investigations of the plant revealed the presence of cuscutine, stigmasterol, kaempferol, dulcitol, myricetin, and coumarin in *C. reflexa* (Sharma et al., 2012; Manish et al., 2012; Anis et al., 2002). In general, the isolated compounds such as nitrogen (13.56%), aromatic compounds (7.88%), fluoro (28.40%), alkaloids (7.64%), silica (5.66%), phosphorus (16.31%), and chlorine compounds (6.26%) in *C. reflexa* as reported in GC-MS analysis are known to possess antimicrobial, antitumor, anticarcinogenic, and anti-inflammatory properties (Rai et al., 2016). Compounds like cuscutin, quercetin, amarbelin, amino acids, cuscutaline, scoparone, melanettin, hyperoside, aromadendrin, taxifolin, astragalin, myricetin, kaempferol, 3-O-caffeoylquinic acid, D-mannitol, myricetin 3-O-α-rhamnoside (Gupta et al., 2008), and dulcitol, laurotetanine (alkaloid) can cause convulsions and, in large quantities, may lead to death (Sharma, 2006). Dulcitol, sitosterol, carotenoids, flavonoids (Subramanian et al., 1963), violaxanthin, lutein, lycopene, carotene, and α-cryptoxanthin (Mukherjee et al., 2008), Choline kinase (Setty et al., 1972), benzofuran 2,3-dihydro-, 2-methoxy-4-vinylphenol, and 2-propenoic acid, 3-(4-hydroxyphenyl)-methyl ester (Bais and Kakkar, 2014) were also reported.

The GC-MS analysis of the methanolic extract of *C. reflexa* confirmed the presence of 2.46% lauric acid, 0.05% ester compounds, 0.05% alkanes, 0.08% phenolic compounds, 2.77% myristic acid, 4.15% plasticizer compounds, 2.27% palmitolic acid, 13.97% palmitic acid, 2.31% diterpene, 1.68% stearic acid, 5.19% monounsaturated fatty acids, 2.16% chlorine compounds, 11.6% steroids, 1.78% alkaloids, 3.56% triterpenes, and 39.27% amino compounds, along with 13.56% nitrogen, 7.88% aromatic compounds, 28.4% fluoro compounds, 7.64% alkaloids, 5.66% silica, 16.31% phosphorus, and 6.26% chlorine compounds (Rai et al., 2016).

Besides the conventional medicinal uses against jaundice, gout, headache, bilious disorders, flatulence, constipation, and other liver complaints, fevers, body pain, itching, migraines, chronic catarrh, amnesia, epilepsy, expectorants, prolonged fever, and constipation, *C. reflexa* is found to be anticonvulsant, muscle relaxant, antioxidant, antihypertensive, and cardiotonic (Rai et al., 2016). It exhibits many pharmacological activities such as antiepileptic, antitumor, anti-inflammatory, anticancer (Suresh et al., 2011), antibacterial, antiviral, antioxidant, hepatoprotective, hypoglycemic, relaxant and plasmolytic, α-glucosidase inhibition (Kaur, 2013; Paudel et al., 2013), and in androgen-induced alopecia (Pandit et al., 2008) and diuretic (Sharma et al., 2009).

The *C. reflexa* stems are often used in ethnomedicine as alternatives to treat different health issues when medical treatments are expensive. However, it is observed that relatively few urogenital disorders have been found to be healed by these plants. To evaluate its additional biological activity and phytochemical composition, this pharmacologically least explored species has been chosen. If the potential of the stems as a treatment for urogenital infections is confirmed, its constituent parts may be further separated and employed to create more potent antibiotics targeting the pathogens. This species is more common and abundant in Southern Odisha's tropical environment, which may make it easier to economically cultivate it in unsuitable land without affecting crop productivity.

MATERIALS AND METHODS

Bacterial strains

The present study focuses on evaluation of bactericidal potential of the methanolic extract of *C. reflexa* stem against the clinical isolates of *Enterococcus faecalis, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa* and *Staphylococcus aureus.* The test

Figure 1. *Cuscuta reflexa* Roxb. plant in its natural habitat.

strains used in the present study were isolated from the urine samples of patients suffering from urinary tract infections (UTI). They were kindly supplied by the Department of Microbiology, Maharaja Krushna Chandra Gajapati (MKCG) Medical College and Hospital, Berhampur, Odisha. The subcultures of the aforesaid pathogens are preserved at a low temperature in an aseptic condition, for further uses. Pathogens are subjected to 1 h activation in incubator at 37°C before each use.

Collection of plant materials

The *C. reflexa* stems are collected from Kandhaguda, Malkangiri, Odisha, India with geographical coordinate 18.3436°N and 81.8825°E on 2nd April 2021. The onsite plant picture is presented in Figure 1. The samples were authenticated by Prof. M. K. Misra, Taxonomist, Berhampur University and relevant guidelines for study on plant materials were followed. A voucher specimen was submitted to the departmental herbarium with a voucher number BOTBU2201.

Preparation of crude extract

The stems of *C. reflexa* was collected, thoroughly rinsed in distilled water and air-dried. These are chopped into small pieces, then grind into fine powder and are weighed as W_1. The powder is subjected to Soxhlet extraction in a ratio of 1:5 w/v of absolute methanol (approx. 210 mL) at 80°C for 72 h, followed by evaporation in water bath. The % of yield was calculated by using the following formula:

$$\% \text{ Yield} = W_1 / W_2 \times 1\ 00$$

where W_1 =The weight of the methanolic extract in grams, W_2=the weight of the initial dried sample, with minor modification of the standard formula for yield calculation (Anokwuru et al., 2011). The final crude methanolic extract was collected and preserved at a temp of 4°C, after noting the final weight as W_2. For the antibacterial study, fresh working concentration of the extract was prepared before each study. Aqueous solution of the crude sample was prepared by dissolving around 0.02 g of the crude extract in 1 mL of lukewarm water and stored in labelled Eppendorf tube for further use.

Qualitative phytochemical screening

The methanolic extract of stem of *C. reflexa* was subjected to preliminary qualitative screening, to ascertain the availability of the primary as well as secondary metabolites like alkaloids (Mayer's test, Wagner's test), anthocyanin, anthraquinones, carbohydrates (Benedict's test, Fehling's Test, Molisch Test), coumarin, emodin, flavonoids, glycosides (Liebermann's test, Acetic acid test), leucoanthocyanin, phenol compounds ($FeCl_3$ test, Lead acetate test, Potassium dichromate test), protein (Biuret test, Conc. HNO_3 test, Ninhydrin solution test), Saponin (Foam test with Water and $NaHCO_3$), steroid, tannin, terpenoid were screened qualitatively in *C. reflexa* extract via standard procedures (Trease et al., 1983; Sofowara, 1993; Harborne, 1998).

Quantitative analysis of phytochemicals

Standard procedures of Folin-Ciocalteu and Aluminium Chloride method (Singleton et al., 1965; Quettier-Deleu, 2000) were followed to plot calibration curves for total phenolic content (TPC) and total flavonoid content (TFC), which were quantified as mg/g Gallic Acid Equivalent and mg/g Rutin Equivalent, respectively, and calculated, by plotting respective standard calibration curves.

Determination of antioxidant activity

Using the DPPH assay, the methanolic extracts' capacity to scavenge free radicals was assessed (Braca et al., 2001) with little modification. Variable concentrations of solution of plant extracts such as 20, 40, 60, 80, 100, 200, and 500 µl were taken in a series

of test tubes and the volume was made up to 3 mL by addition of methanol, which were been combined with 1 mL of a methanolic solution containing 1, 1 Diphenyl-2-picrylhydrazyl radical (DPPH) radicals with ultimate 0.4 mM concentration. After 30 min of shaking and standing, the mixture was tested for absorbance at 517 nm. The standard utilized was Ascorbic acid. The following formula was used to determine the sample's percentage of DPPH decolorization.

% decolorization = (Abs. of control – Abs. of sample / Abs. of control) × 100

Modified Antibacterial susceptibility test (AST)

The antibacterial susceptibility of *C. reflexa* extract was investigated to study the effect of the *C. reflexa* extract against the clinical isolates of uropathogens like *E. faecalis, E. coli, P. vulgaris, P. aeruginosa* and *S. aureus,* by disc diffusion method, agar well diffusion, modified agar well diffusion method, modified AST where antibiotics were supplemented with *C. reflexa* extract and cfu/mL in spread plate method with variable concentrations. The resulting inhibition zones were recorded in mm, and compared.

Disc diffusion method

Around 6mm sized discs were loaded with 100, 200, 300 and 400µg concentrations of *C. reflexa* extract respectively. These discs were air dried and placed aseptically on nutrient agar plates, which were already swabbed with each of the activated uropathogens separately. These processed plates were incubated overnight at 37°C, and checked for zone of inhibitions (ZOI) in millimeter.

Agar well diffusion by swabbing

A single bacterial colony was activated in sterilized nutrient broth at 45°C for 15 min, which were subsequently swabbed on nutrient agar plates. 400, 800, and 1200 µg of *C. reflexa* extract was loaded respectively to three wells and diffusion of drug into the wells was assured at room temperature. The ZOIs in millimeters were noted down, after overnight incubation at 37°C.

Agar well diffusion by pour plate method

Aseptically, 100µl of activated culture was plated along with nutrient agar media. Three wells were made and loaded with 400, 800, and 1200 µg of *C. reflexa* extract to study the drug dependent ZOIs.

Modified agar well diffusion method

For a comparative inhibitory potential test, a nutrient agar plate was streaked with all five strains of activated bacteria with 80 0µg of *C. reflexa* extract loaded well at the center. This plate was incubated overnight at 37°C, after proper drug diffusion into the well.

Antibiotic modulating activity test

Standard antibiotic discs of AMC 30 (Amoxycillin and Clavulanic Acid-30), NIT 300 (Nitrofurantoin 300), CIP 5 (Ciprofloxacin 5), CIF 5 (Cefixime 5), S 10 (Streptomycin 10) were aseptically placed on nutrient agar plates, swabbed with the activated strains. The ZOI were measured after overnight incubation. This AST was considered as standards for comparison with another plate of *C. reflexa* extract treated antibiotics. The differences in the inhibition zones presents the levels of supplementary and complementary potentials of *C. reflexa* extract for the traditional antibiotics, whichmay help us to determine whether the components of the drug are directly effective against a pathogen or they can be added to the traditional antibiotics for the enhanced effects.

Colony forming units per mL (Cfu/mL) determination in control and *C. reflexa* extract treated bacteria by spread plate method

Three test tubes with 3 mL of nutrient broth each were inoculated with 100 µl of activated culture. Of these, first one was treated as a control, second one was added with 100 µg/mL of *C. reflexa* extract and third one was treated with 600µg/mL of *C. reflexa* extract. All three were incubated for 4 h at 37°C. Aliquots of these cultures were subjected to two rounds of serial dilution (10 µl in 990 µl). Aliquots of this diluted bacterial suspension were spread on sterilized agar nutrient plates and the cfu/mL was calculated by counting the colonies after overnight incubation.

Determination of minimum inhibitory concentration (MIC)

The MIC analysis was performed via broth microdilution techniques according to CLSI guidelines (National Committee for Clinical Laboratory Standards) procedures for aerobic testing, with 96-well microtiter plate with some modifications (Bazargani and Rohloff, 2016; Julianti et al., 2017; Tripathy et al., 2023). The stock extract solution was prepared by dissolving 250mg of drug in 1ml of Luria Bertani broth. Around 5 ml of sterile broth were inoculated with 10µl of bacterial strains at log phase after 6–7 h activation at 37°C, to get 500 times dilution and to ensure the concentration of bacterial suspensions approximately up to 10^6 cfu/mL.

In the first column wells of a microtiter plate, media were taken, in second 200µl bacterial culture, in third column, the positive control 0.5mg of Ciprofloxacin and 5mg of *C. reflexa* extract in 200µl of broth was taken in the first row in duplicate. These were subjected to serial half fold dilution and then 100 µl of bacterial suspension was added to each well to make the final volume of 200µl/well. *C. reflexa* extract without bacteria served as blank. Each plate was wrapped loosely with parafilm to prevent dehydration and finally incubated at 37°C for 20-24 h. Finally, 40 µl of MTT (3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was added at a concentration of 0.2mg/ml to each of the well and incubated at room temperature for 30 min. The optical densities of microplates were taken in Microplate reader at 595 nm. Bacterial growth was observed as purple coloration of the wells. The concentration of the wells with lowest colorations, of each bacterium was referred to as the MIC value.

RESULTS

Extract preparation

The crude extract was chocolaty brown in colour, soluble in Luke-warm water. Around 15.15 g of crude extract residue was obtained after solvent (methanol) removal, by taking 41.97 g of dried stem sample, which presents 36.09% of yield.

Phytochemical analysis and antioxidant assay

C. reflexa extract was tested positive to phytochemical components like carbohydrates, coumarins, flavonoids,

Table 1. *In vitro* antibacterial activity of methanolic crude extract of *Cuscuta reflexa* against different uropathogenic bacteria through disc diffusion method.

Zone of inhibition (ZOI) measured in mm				
Variable	**100 µg**	**200 µg**	**300 µg**	**400 µg**
E. faecalis	0	0	8±0.66	9±0.33
E. coli	0	0	9±0.33	10±0.88
P. aeruginosa	0	7±0.33	7±0.66	8±0.33
P. vulgaris	0	7±0.33	8±0.33	10±0.66
S. aureus	0	0	9±0.66	11±0.57

Values represent the average ± SEM of triplicate sets of experiments.

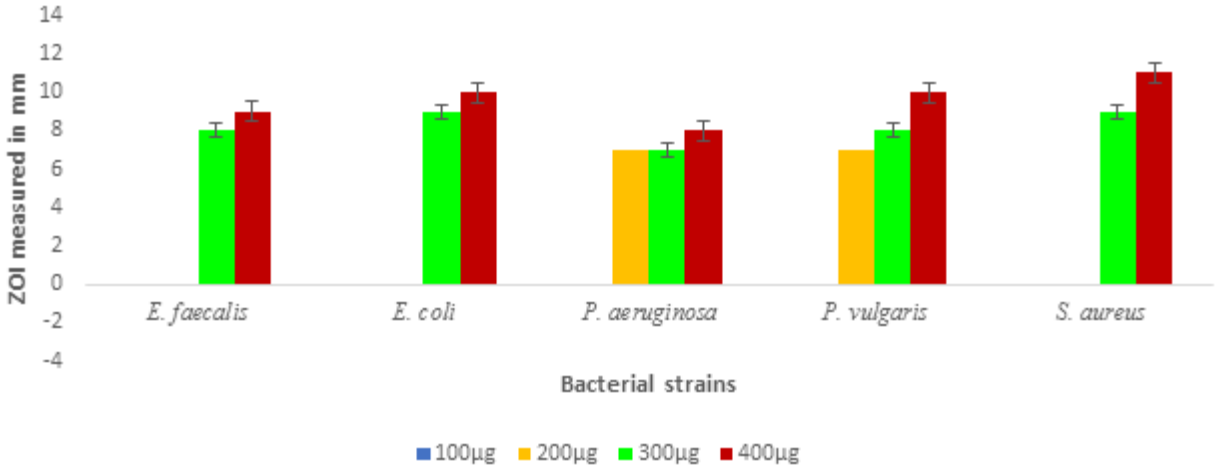


Figure 2. Graphical presentation of comparative inhibitory potential of methanolic crude extract of *Cuscuta reflexa* (*C. reflexa* extract) against different uropathogenic bacterial strains by disc diffusion method.

glycosides, phenols, tannins, saponins, steroids and terpenoids, but alkaloids, anthraquinones, anthocyanins, emodin, leucoanthocyanins and proteins were absent in qualitative analysis. The total phenol content (TPC) of *C. reflexa* extract was quantified as 22.84 ± 0.4 mg/g of Gallic acid equivalent (GAE) and the total flavonoid content (TFC) was 113 ± 2.88 mg/g of Rutin equivalent (RE). The IC_{50} value of *C. reflexa* extract was calculated as 212.61 µg/mL with Ascorbic acid as the standard.

Antibacterial activities

Disc diffusion

The zone of inhibitions resulting due to loading of different concentrations of *C. reflexa* extract like 100, 200, 300, and 400 µg, against each of the strain, presents its in-vitro antibacterial potential. These ZOIs are presented in Table 1 and graphically depicted in Figure 2.

From Table 1 and Figure 2, it can be noted that, in disc diffusion, a low conc. of 100 µg of *C. reflexa* extract per disc do not execute any potential inhibition against all the test strains. At the same time, 200 µg/disc of the same plant extract is effective only against the *P. aeruginosa*, *P. vulgaris* with 7±0.33 and 7±0.33 mm of inhibition each. At 300 µg/disc the *E. coli* and *S. aureus* are inhibited up to 9±0.33 and 9±0.66 mm, respectively. A maximum ZOI of 11±0.57 mm is noted against *S. aureus* at 400 µg/disc followed by 10±0.88 and 10±0.66 mm against *E. coli* and *P. vulgaris.* Doses less than 200 µg/disc are not effective against the bacterial strains, whereas the higher doses are better effective in inhibiting and the extract had a dose dependent effect.

Agar well diffusion

The antibacterial activity of *C. reflexa* extract was screened *in-vitro* using swabbing and pour-plate method of the agar well diffusion against each of the five strains, at different concentrations of the extract. The comparative results of both the methods respectively are presented in

Table 2. *In vitro* antibacterial screening of methanolic crude extract of *Cuscuta reflexa* against different uropathogenic bacteria through agar well diffusion method.

Variable	Zone of inhibition (ZOI) measured in mm					
	Swabbing method			Pour plate method		
	400 µg	800 µg	1200 µg	400 µg	800 µg	1200 µg
E. faecalis	0	23±0.33	26±0.33	16±0.33	18±0.57	20±0.57
E. coli	0	12±0.66	14±0.33	17±0.33	18±0.88	23±0.33
P. vulgaris	0	14±0.57	16±0.33	17±0.57	18±0.66	21±0.57
P. aeruginosa	14± 0.57	16±0.88	18±0.88	0	12±0.33	17±0.66
S. aureus	0	11±0.33	12±0.57	16±0.33	21±0.57	24±0.88

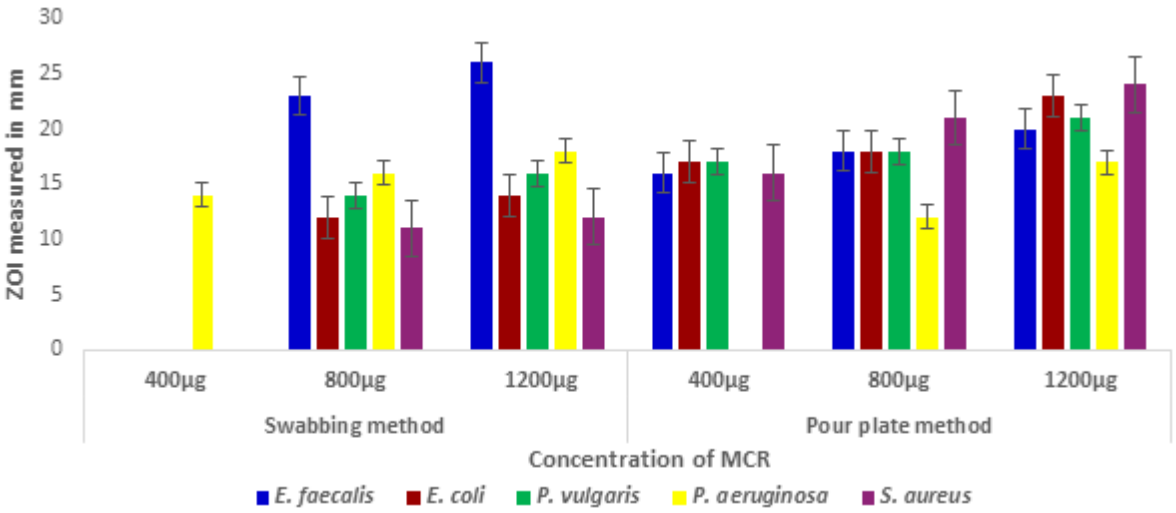


Figure 3. Graph presenting comparative inhibitory potential of methanolic crude extract of *Cuscuta reflexa* against different uropathogenic bacterial strains by agar well diffusion method.

Table 2 and plotted graphically in Figure 3.

From the agar well diffusion results, presented in Table 2 and Figure 3, it is noted that, in the swabbing method, *E. faecalis* is the comparatively more vulnerable bacterial strain when treated with different doses *of C. reflexa* extract. At concentrations of 800 µg and 1200 µg per well, *E. faecalis* is inhibited up to 23±0.33 and 26±0.33 mm, respectively. A ZOI of 14±0.57 mm is noted against *P. aeruginosa* at a concentration of 400 µg/well, 16±0.88 mm is recorded at 800 µg/well, and 18±0.88 mm at 1200 µg/well against *P. aeruginosa*. *P. vulgaris* is the next vulnerable bacterial strain with ZOIs of 14±0.57 and 16±0.33 mm at 800 and 1200 µg of the drug per well, respectively. In contrast, 400 µg of *C. reflexa* extract has no significant potential to inhibit the bacterial strains except for *P. aeruginosa*. In the pour plate agar well method, *S. aureus* is better inhibited up to 21±0.57 and 24±0.88 mm at concentrations of 800 and 1200 µg per well, followed by ZOIs of 18±0.88 and 23±0.33 mm for E. coli at the same concentrations. *P. vulgaris* is inhibited up to 21±0.57 mm by *C. reflexa* extract at a concentration of 1200 µg/well. Hence, it can be said that inhibition by *C. reflexa* extract in agar well diffusion is dose-dependent

Antibiotic modulating activity of crude extract of *C. reflexa*

The outcomes of antibiotic sensitivity of the bacterial strains towards conventional antibiotic discs were tested. The action of *C. reflexa* extract-loaded antibiotic discs was also measured, and the difference was noted down, representing the supplementary and complementary effects of the extract. The percentage of the supplementing effect is calculated and presented in Table 3 and Figure 4.

It is observed from Table 3 and Figure 4 that *C. reflexa* extract complements the activity of antibiotic discs of AMC 30 by 27 mm, CIP 5 by 29 mm, CFM 5 by 18 mm, and S10 by 25 mm. Additionally, it supplements NIT 300 against *E. faecalis* by 10 mm, which is a 62.5% increase in inhibition. Here, complementary effect means the

Table 3. *In vitro* antibiotic modulating activity of crude extract of *C. reflexa.*

Bacterial strain	Difference between the zone of inhibition (ZOI) of conventional antibiotic disc and *C. reflexa* extract (**200 µg**) **supplemented antibiotic discs expressed in terms of mm**														
	AMC 30+MCR	AMC 30	Difference	NIT 300+MCR	NIT 300	Difference	CIP 5+MCR	CIP 5	Difference	CFM 5+MCR	CFM 5	Difference	S 10+MCR	S 10	Difference
E. faecalis	27	0	27	26	16	10	29	0	29	18	0	18	25	0	25
E. coli	12	0	12	17	0	17	33	0	33	21	0	21	25	15	10
P. vulgaris	0	0	0	8	8	0	40	37	3	15	12	3	20	20	0
P. aeruginosa	0	0	0	12	12	0	41	36	5	14	14	0	26	24	2
S. aureus	10	10	0	20	20	0	33	32	1	18	18	0	15	8	7

AMC 30 - Amoxycillin and Clavulanic Acid-30, NIT 300 - Nitrofurantoin 300, CIP5 - Ciprofloxacin 5, CFM 5 - Cefixime 5, S10 - Streptomycin 10.

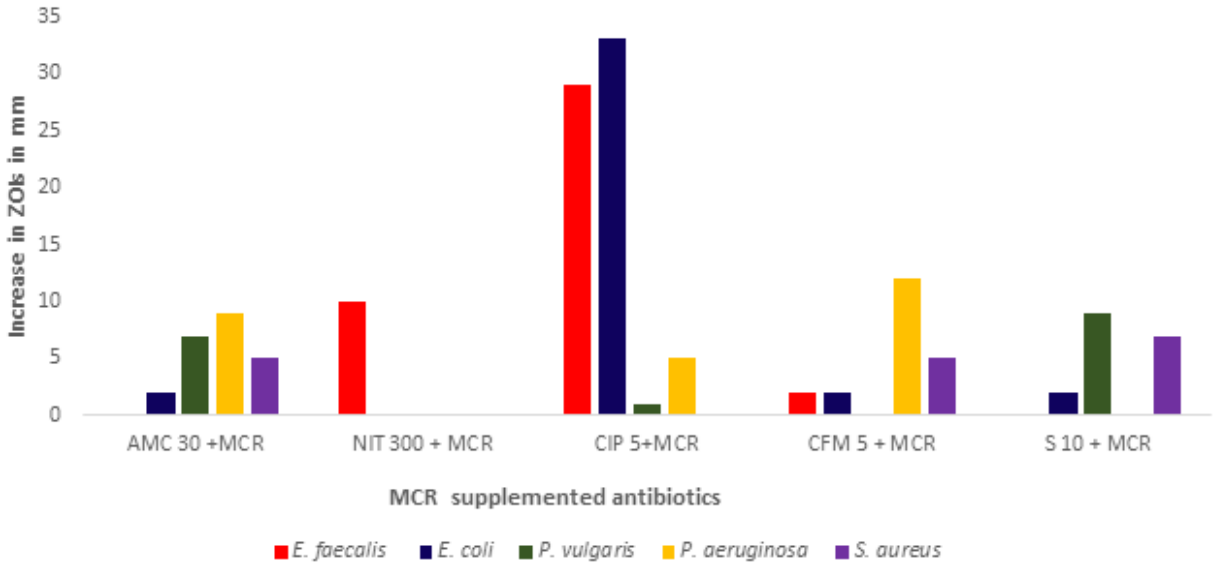


Figure 4. Graph presenting the antibiotic modulating activity of methanolic crude extract of *Cuscuta reflexa* (*C. reflexa* extract) against different uropathogenic bacterial strains by modified antibiotic sensitivity test.

antibiotic becomes active upon addition of *C. reflexa* extract, which was not effective alone against the bacterial strains. Similarly, an extra zone of inhibition was noticed with S10 against *E. coli*, which is around 33.3% more than the antibiotic's activity alone. CIP5 and CFM 5 are supplemented by *C. reflexa* extract against *P. vulgaris* by 3 mm each, representing 8.1 and 25% of extra inhibition, respectively. The supplementary effect indicates the additional inhibition due to *C. reflexa* extract. It also enhances the antibacterial activity of CIP 5 and S10 against *P. aeruginosa* by 5 and 2 mm, resulting in a 13.8 and 8.3% increase in inhibition, respectively. Moreover, it supplements the inhibition of antibiotics like CIP 5 and S10

Table 4. *In vitro* antibacterial activity screening of methanolic crude extract of *Cuscuta reflexa* against different uropathogenic bacteria by modified agar well diffusion method.

Name of strain	*E. faecalis*	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*S. aureus*
ZOI (mm)	7.33 ± 0.33	9.0 ± 0.57	12.0 ± 0.57	6.33 ± 0.33	13.66 ± 0.66

Zone of inhibition (ZOI). Values represent the average ± SEM of triplicate sets of experiments.

Table 5. Cfu/mL in 10^7 is determined in wild and methanolic crude extract of *Cuscuta reflexa* (*C. reflexa* extract) treated bacteria at log phase by spread plate method.

Variable	Control	Low dose (100 µg/mL)	% of increase in inhibition in terms of cfu/mL at 100 µg/mL	High dose (600 µg/mL)	% of reduction in cfu/mL at 600 µg/mL
E. faecalis	11.56±0.4	4.52±0.04	60.89	2.1±0.14	81.83
E. coli	18.12±0.32	14.04±0.12	22.51	9.7±0.18	46.46
P. aeruginosa	4.2±0.16	4.1±0.1	2.38	3.96±0.04	5.71
S. aureus	21.76±0.4	9.68±0.08	55.51	6.14±0.26	71.78

Values represent the average ± SEM of triplicate sets of experiments.

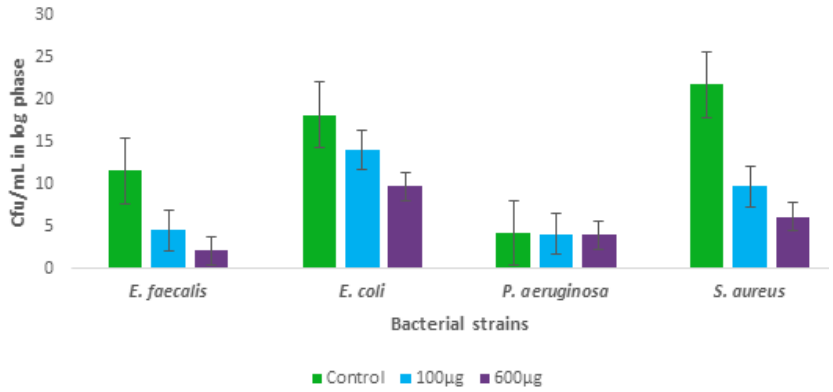


Figure 5. Graph presenting the in vitro antibacterial activity of methanolic crude extract of *Cuscuta reflexa* (*C. reflexa* extract) at 100 µg/mL (low) and 600µg/mL (high) dose against different uropathogenic bacteria at their exponential growth phase in spread plate method.

against *S. aureus* by 1 and 7 mm, corresponding to a 3.1 and 87.5% increase in ZOIs, respectively.

Modified agar well diffusion method

The relative vulnerability levels of bacterial strains towards the *C. reflexa* extract were simultaneously studied using the modified agar well diffusion method, and the ZOIs were measured in mm. These values are presented in Table 4. The modified agar well diffusion method helped overcome variations in inhibition potential due to different working conditions. The comparative inhibition rate of *C. reflexa* extract was studied simultaneously on multiple pathogenic bacterial strains at a particular concentration of *C. reflexa* extract. The vulnerability of *S. aureus* and *P. vulgaris* was evidenced by ZOIs of 13.66 ± 0.66 mm and 12.0 ± 0.57 mm, respectively.

Colony forming units per mL (Cfu/mL) determination in control and *C. reflexa* extract treated bacteria by spread plate method

The colony forming units per mL at 10^7 were determined in wild bacteria and taken as the control. The cfu/mL of bacterial strains treated with 100 and 600 µg/mL doses of *C. reflexa* extract were determined using the spread plate method and are presented in Table 5 and graphically displayed in Figure 5. These values were compared to assess the dose-specific effects of *C.* reflexa extract. The growth of *E. faecalis* was restricted by 60.89% upon treatment with 100µg/mL and further enhanced by

81.83% when treated with 600µg/mL of *C. reflexa* extract. Similarly, the growth of *E. coli* was inhibited by 22.51 and 46.46% upon treatment with 100 and 600 µg/mL of *C. reflexa* extract, respectively. A marginal inhibition of 2.38 and 5.71% was observed against *P. aeruginosa*. However, against *S. aureus*, a substantial increase in inhibition of 55.51 and 71.78% was noted upon increasing the concentration of *C. reflexa* extract from 100 to 600 µg/mL.

Minimum inhibitory concentration of *C. reflexa* extract against different uropathogenic bacteria

The minimum inhibitory concentration of *C. reflexa* extract was found to be 1.25 mg/mL against *E. faecalis, E. coli* and *P. aeruginosa*, 2.5 mg/mL against *P. vulgaris* and 0.625 mg/mL against *S. aureus*. The MIC for Ciprofloxacin as a control was noted to be 0.062 µg/mL against *E. faecalis, P. vulgaris, S. aureus* and 1.25 µg/mL against *E. coli* and *P. aeruginosa*.

DISCUSSION

Due to the high expense of allopathic medications, the inaccessibility of these medications in remote locations, and the widespread perception of no negative side effects of natural compounds, has recreated an interest in herbal therapy for a variety of health issues (Raza et al., 2015). From medical point of view, the natural pharmacologically active compounds including important essential and trace elements in the plant extract ensures the relief from different UTIs, which can be incorporated into the clinical medicines. So, extensive in vitro pharmacological investigations of traditional medicinal plants offer an incredible chance to discover broad spectrum phyto-drugs.

The preliminary phytochemical screening of alcoholic extract showed the presence of phytochemicals such as alkaloids, flavonoids, carbohydrates, glycosides, phytosterols, phenolics, tannins, each of which have been known to have antioxidant properties (Ranjan et al., 2020). Its ethanolic extract was found to possess phytochemicals like alkaloids, carbohydrates, glycosides, flavonoids, tannins, phenolic compounds and steroids (Patel et al., 2014). Whereas in the present study, phytochemicals like carbohydrates, coumarins, flavonoids, glycosides, phenols, tannins, saponins, steroids and terpenoids are confirmed. The variation in phytochemicals in *C. reflexa* is found to be region dependent (Dokuparthi et al., 2014). The ethyl acetate fraction of the ethanolic extract of the stem of *C. reflexa* showed high antioxidant activity (Yadav et al., 2000). In the present study, the IC_{50} value of *C. reflexa* extract is calculated to be 212.61 µg/mL with ascorbic acid as the standard. The free radical scavenging activity posed due to the antioxidant activity of flavonoids (Sawi et al., 2010), terpenoids (Wang et al., 2019), saponin (Chen et al., 2014), phenolics (Pourreza, 2013) and tannins (Amarowicz, 2007) present in different plant extracts.

Little is known about the antimicrobial potential of the extract of *C. reflexa*. Moreover, the conclusions have been deduced from single methods such as disc or agar well diffusion. Under variable working conditions, the rate of inhibition of the same drug at the same concentration has shown variations. To overcome this issue, the present study utilized multiple diffusion methods, supplemented antibiotics with *C. reflexa* extract, and determined cfu/mL to obtain a more conclusive therapeutic understanding of the *C. reflexa* extract. Additionally, the minimum inhibitory concentration of the *C. reflexa* extract was also determined. According to the study, S. *aureus* was found to be the most vulnerable strain, followed by *E. coli.* With a better diffusion of 1200 µg of *C. reflexa* extract in agar well, *E. faecalis* was significantly inhibited by the *C. reflexa* extract.

The ethanolic extract of *C. reflexa* was screened against *Bacillus subtilis*, *S. aureus*, *E. coli* and *Salmonella* Typhi bacteria at 200, 300, 400 and 500 µg/mL, respectively and highest antibacterial activity is found at 500 µg/mL against *E. coli* with a ZOI of 24.6±0.24 mm (Patel et al., 2014). In the present study, a ZOI of 11±0.57, 10±0.88 mm was recorded against *S. aureus* and *E. coli* respectively at 400 µg in disc diffusion (Table 1 and Figure 2). Crude ethanolic extract showed inhibitory activity against *E. coli* and *S. sonnei* (Ayesha et al., 2011). *C. reflexa* collected from different seasons showed antimicrobial activity against *S. aureus, Staphylococcus epidermidis, E. coli, Micrococcus luteus, P. aeruginosa* (Sharma et al., 2013). On a contrary, at a concentration of 1200 µg of *C. reflexa* extract, *E. faecalis* was inhibited to maximum by 26±0.33 mm by swabbing method whereas *S. aureus* inhibited up to 24±0.88 mm by pour plate method of agar well diffusion (Table 2 and Figure 3). Similarly, in modified agar well diffusion, the *S. aureus* was found the most vulnerable to *C. reflexa* extract (Table 4). Among the five bacterial strains, *S. aureus* was found to have least MIC of 0.625 mg/mL, followed by 1.25 mg/mL for *E. faecalis, E. coli* and *P. aeruginosa.* The highest MIC of 2.5 mg/mL was observed against *P. vulgaris.* A MIC of 0.062 µg/mL is determined for Ciprofloxacin taken as positive control against *E. faecalis, P. vulgaris, S. aureus* and 1.25 µg/mL against *E. coli* and *P. aeruginosa*.

In this study, the antibiotic modulatory activity of five important antibiotics, namely AMC 30 - Amoxicillin and Clavulanic Acid-30, NIT 300 - Nitrofurantoin 300, CIP 5 Ciprofloxacin 5, CFM 5 - Cefixime 5, and S10 – Streptomycin 10, was tested against common uropathogens. In some cases, antibiotic-resistant bacteria became sensitive when the antibiotic was supplemented with *C. reflexa* extract, or the extract enhanced its efficacy. Although *C. reflexa* extract was not

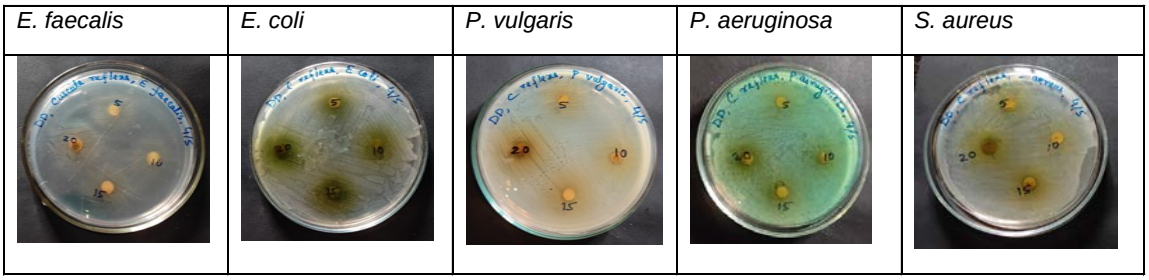


Plate 1. Plates of in vitro screening of antibacterial activity of methanolic crude extract of *Cuscuta reflexa* against different uropathogenic bacteria by disc diffusion method.

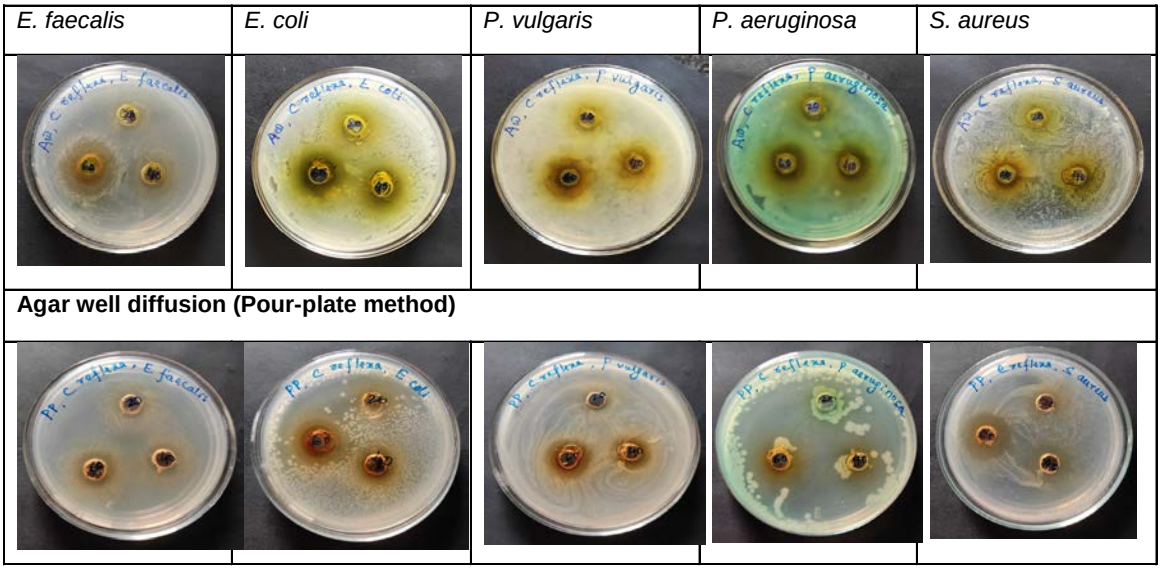


Plate 2. Plates of bacterial growth inhibitions study of methanolic crude extract of *Cuscuta reflexa* against different uropathogenic bacteria by agar well diffusion in swabbing and pour plate method.

very effective in modulating the effects of conventional antibiotics against *P. aeruginosa*, *P. vulgaris*, and *S. aureus*, significant antibiotic modulatory activity was exhibited by the *C. reflexa* extract against *E. coli* and *E. faecalis*, which are considered the most common uropathogens and causative agents for complicated and uncomplicated urinary tract infections. The drug was found to supplement the inhibition of CIP 5 the most against *E. faecalis* and *E. coli*. According to the present study, *C. reflexa* extract had a maximum supplemented zone of 7 mm (87.5%) against *S. aureus* with S10 and complemented the inhibition zone to a maximum of 33 mm against *E. coli* with CIP 5 (Table 3 and Figure 4).

The cfu/mL count through the spread plate method indicates significant inhibition of the aforementioned pathogens upon application of *C. reflexa* extract. The growth of *E. faecalis* was restricted by 60.89% upon treatment with 100 µg/mL and further enhanced by 81.83% with 600 µg/mL of *C. reflexa* extract. Similarly, 100 µg/mL of *C.* reflexa extract increased the inhibition of *E. coli* by 22.51%, whereas at a higher dose, it increased to 46.46%. *P.* aeruginosa showed an enhanced inhibition of 2.38 and 5.71% at 100 and 600 µg/mL of the drug, respectively. Against *S. aureus*, an increase in inhibition of 55.51 and 71.78% was noted upon increasing the concentration from 100 to 600 µg/mL (Table 5 and Figure 5).

Despite a number of reported pharmacological uses, this plant is poorly investigated for its antibacterial potential and clinical efficacies. Natural agents, considered a basic source of medicaments, are commonly used by pharmaceutical industries (Salehi et al., 2018). The bioactive components from this parasitic plant can therefore be utilized to synthesize formulations against bacterial infectivity. In vivo studies for the selected natural antioxidant compounds, either partially purified or in crude form, are also recommended to validate the computational results and may help in the formulation of natural antimicrobials with reduced toxicity and increased efficacy (Rehman et al., 2021) (Plates1 to 4).

Plate 3. Single plate analysis of bacterial growth inhibitions study of methanolic crude extract of *Cuscuta reflexa* (MCR) against different uropathogenic bacteria *(E.f.= E. faecalis; E.c. = E. coli; P.v. = P. vulgaris; P.a. = P. aeruginosa; S.a. = S. aureus).*

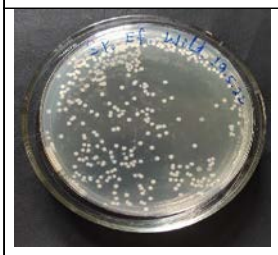
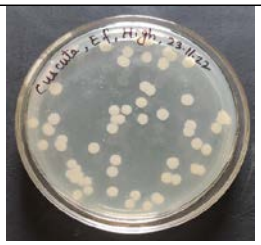
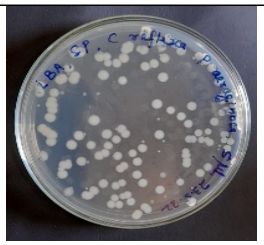
Control grown without extract	**Bacteria treated with low dose of extract**	**Bacteria treated with high dose of extract**
E. faecalis		
		
E. coli		
		
P.aeruginosa		
		
S. aureus		

Plate 4. *In vitro* antibacterial study of methanolic crude extract of *Cuscuta reflexa* treated at 100 µg/mL (low) and 600µg/mL (high) dose against different uropathogenic bacteria by spread plate method.

Conclusion

This piece of research represents an additional attempt to investigate the broad-spectrum antibacterial activities of increasing doses of *C. reflexa* extract. These activities can be applied against a wide range of infectious diseases, potentially aiding in combining the chemical composition of semi-synthetic antibiotics with the additional biotic potential of the *C. reflexa* extract. The isolation, identification, and purification of these phytochemicals, along with the investigation of their relevant antimicrobial, antioxidant, and enzyme inhibition potentials, as well as toxicological estimation, should be the future direction for research aimed at formulating novel therapeutics.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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Supplementary file

Plate 1. Plates of in vitro screening of antibacterial activity of methanolic crude extract of *Cuscuta reflexa* against different uropathogenic bacteria by disc diffusion method.

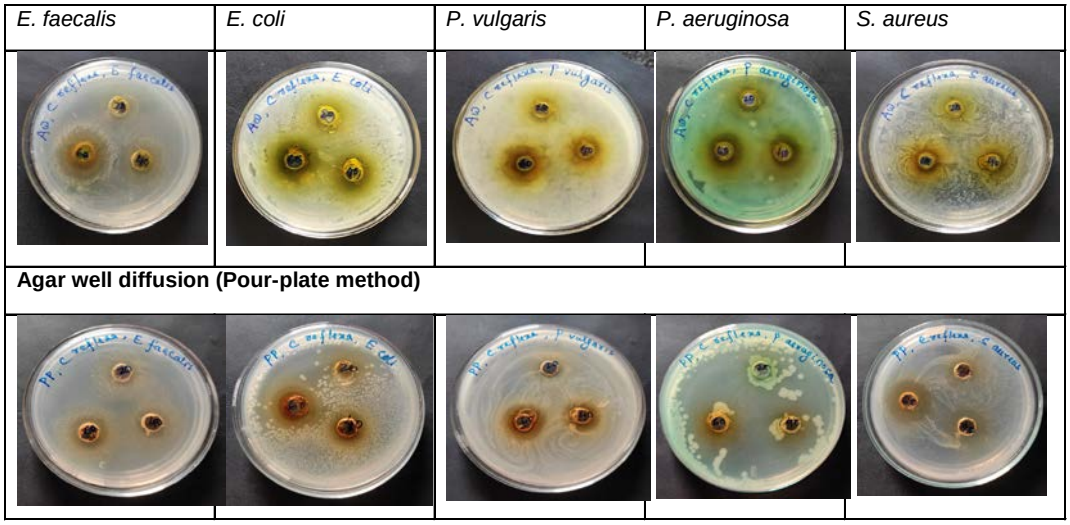


Plate 2. Plates of bacterial growth inhibitions study of methanolic crude extract of *Cuscuta reflexa* against different uropathogenic bacteria by agar well diffusion in swabbing and pour plate method.

Plate 3. Single plate analysis of bacterial growth inhibitions study of methanolic crude extract of *Cuscuta reflexa* (MCR) against different uropathogenic bacteria *(E.f.= E. faecalis; E.c. = E. coli; P.v. = P. vulgaris; P.a. = P. aeruginosa; S.a. = S. aureus).*

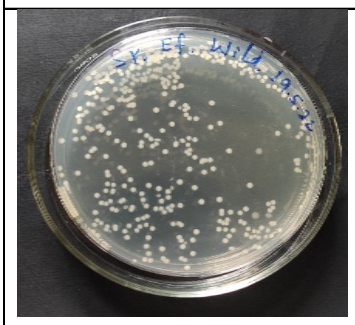
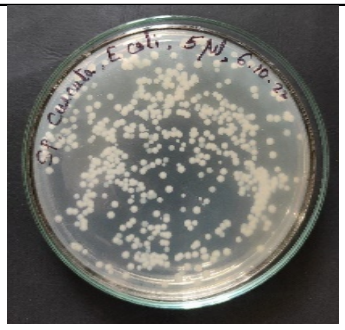
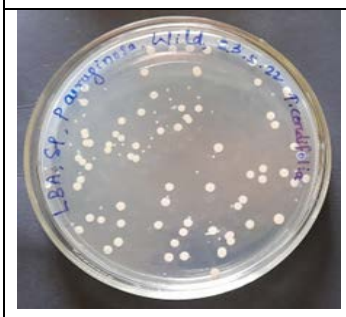
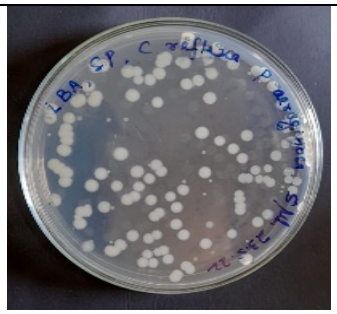
Control grown without extract	Bacteria treated with low dose of extract	Bacteria treated with high dose of extract
E. faecalis		
		
E. coli		
		
P.aeruginosa		
		
S. aureus		

Plate 4. *In vitro* antibacterial study of methanolic crude extract of *Cuscuta reflexa* treated at 100 µg/mL (low) and 600 µg/mL (high) dose against different uropathogenic bacteria by spread plate method.