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In-vitro Assessment of Biological Activities: Antifungal, Antibacterial, Antibiofilm, Antioxidant, and Cytotoxicity of *Clematis grata* Wall

Shingraf Zahid[1], Madiha Khalid[1*], Sadia Nazer[2], Tafail Akbar Mughal[2], Sadia Mumtaz[1], Nausheen Irshad[3] and Abdul Rauf[4]

1. Department of Biotechnology, Women University of Azad Jammu and Kashmir, Bagh, Pakistan
2. Department of Zoology, Women University of Azad Jammu and Kashmir, Bagh, Pakistan
3. Department of Zoology, University of Poonch, Rawalakot, Azad Jammu and Kashmir, Bagh, Pakistan
4. Department of Zoology, University of Azad Jammu and Kashmir, Pakistan

***Corresponding author e-mail:** madihaakhalid11@gmail.com

SUMMARY

Since prehistoric times, medicinal plants have been employed in traditional healing methods. Plants produce hundreds of different main and secondary metabolites. Several medicinal plants contain antibacterial, antifungal, analgesic, anti-inflammatory, and other effects, according to research. Clematis grata has piqued our interest due to its antibacterial properties. *Clematis grata Wall is* a climbing medicinal plant belonging to the *Ranunculaceae* family. Various phytochemicals including triterpene, saponins, alkaloids, flavonoids, lignans, steroids, coumarins, macrocyclic compounds, phenolic glycosides, and anemone in the aerial parts are present. The current study focused on the evaluation of the biological activities of plant stem extracts. This study investigated the In-vitro antioxidant, antibacterial, antifungal, antibiofilm and cytotoxic properties of *Clematis grata* stem extract was evaluated. Antibacterial activity via Agar well diffusion assay was carried out against two Gram-negative i.e. *Pseudomonas aeruginosa* and *Escherichia coli* and one Gram-positive i.e. *Staphylococcus aureus*, whereas antifungal activity via Disc diffusion method was analyzed against two pathogenic fungus species *Penicillium digitatum* and *Rhizopus stolonifers*. *Clematis grata* stem extract were used in different solvents n-hexane, methanol, and aqueous at the concentration of 0.2, 0.6 and 0.9 mg/ml. Maximum inhibition was recorded at 0.9 mg/ml concentration. Plant extracts had antibacterial activity, according to the findings. When tested using the DPPH assay, *Clematis grata* extracts showed significant antioxidant activity. The current study found that *Clematis grata* stem methanolic and n-Hexane extracts have high biological activity.

Keywords: Anti-biofilm, antifungal activity, cytotoxic assay

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INTRODUCTION

Because many historically used medicinal plants are present in developing countries, these countries are also on the same path and need to be explored (Kumari et al., 2019). Many ancient and modern societies, including Ayurveda, Yunani, and folk medicines, employ herbal medicines to treat a wide range of ailments (Husain et al.,

2023). These natural items are well-known for their capacity to act as both therapeutic and preventive agents and their low toxicity and bad effects. Local inhabitants have traditionally used several plants to treat various ailments (Kapoor et al., 2017). Several unique pharmacophores have been discovered as a consequence of research into the phytochemistry of medicinal plants. All plants include both primary and secondary metabolites. According to estimates, 70-80% of the poor world relies on traditional plant-based remedies due to the expensive expense of drugs (Aslam et al., 2016). Secondary metabolites are naturally created by plant components such as the roots, stem, bark, flowers, leaves, and seeds. Plant extracts are the primary source of pharmaceuticals for the creation of new therapies. The kind of solvent used and the extraction method considerably influence the variety of bioactive compounds found in plant extracts (Aljohny et al., 2021). Currently, nevertheless, therapeutic herbs are more frequently prepared and sold as botanical dietary supplements (BDS), which individuals take internally to maintain a healthy lifestyle and as a source of vital nutrients (Husain et al., 2021). The current COVID-19 epidemic represented a turning point in this trend and greatly boosted the acceptance, revenue, and applications of these goods (Ahmad et al., 2021). Controlling the bacteria that cause infections is necessary to safeguard humanity (Pattanayak et al., 2018; Kumari et al., 2019).

Because of increasing microbial drug resistance to currently available antimicrobial medicines, the incidence of microbial infectious illnesses and associated complications is steadily rising. These multidrug-resistant bacteria are linked to higher rates of morbidity and death and cause a variety of diseases around the world. Our society is greatly impacted by the increases in antibiotic resistance and the increased recurrence rates of such prevalent diseases. When compared to synthetic chemicals, plant-derived antimicrobials are one of the most fortunate sources and are harmless because of their natural nature (Mickymaray et al., 2019; Akhtar and Shareen, 2023).

Compounds derived from medicinal plants may provide fresh, easy-to-use methods of battling dangerous bacteria. The current state of affairs and prospects for medicinal plants' antibacterial activity are emphasized. There are challenges associated with the efficacy of medicinal plant extracts as antibacterial agents. Results from antimicrobial susceptibility tests used to assess the antibacterial activity of plant extracts might differ. While attempts have been made to increase the antibacterial activity of chemical compounds, several obstacles and challenges must be solved to produce novel antimicrobials from plant extracts (Vaou et al., 2021).

Plants are a fantastic source of medicinal chemicals (Saeed et al., 2022; Umair et al., 2022; Imtiaz et al., 2023), and research on their potential uses in medicine is ongoing worldwide. Because of their overuse, improper prescription, and ongoing mutation, most dangerous bacteria have become resistant to antibiotics, which presents a serious risk to public health. Antimicrobial compounds that are resistant are proliferating at an unexpected rate, and illnesses are becoming more severe and violent, which makes them easier to spread. According to WHO reports, many infectious illnesses may prove to be incurable and uncontainable in the years to come (Dar et al., 2022).

Clematis grata locally called as "Bilri, Dhand and, Bohree Bail," *Clematis grata* Wall is a member of the broad family Ranunculaceae (Chawla et al., 2012). As

a diuretic, antimalarial, antidote for snake bites, anti-dysentery, and in the treatment of a variety of illnesses, the aerial parts of various *Clematis* species are particularly used in Europe and Eastern Asia. There have been anti-inflammatory, cytotoxic, antibacterial, anti-diabetic, and hepatoprotective pharmacological actions. These species should be investigated scientifically for their bioactive properties and potentially used as pharmaceuticals. Based on their historical application in the treatment of diabetes, *Clematis grata* (the whole plant), and Royleacinerea (D. Don) Bail, (the leaves and stem), were chosen (Gruenwald et al., 2000; Sidhu et al., 2015; Rattan, et al., 2023).

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL

Clematis grata Wall stem was collected in August and October from a village in Dherray, Bagh AJK, Pakistan. The plant was acknowledged by an ethnobiologist from the Department of Botany, Government Degree College Abbaspur Stem was rinsed with distilled water to remove dust particles. The plant sample was dried under shade at room temperature for 20 days. The dried plant was finely powdered after drying using a blender apparatus.

EXTRACT PREPARATION

Powder extraction from the stem was simply carried out through a conventional maceration process (Sapiun et al., 2020). The dried stem part of *clematis grata* was used for the preparation of powder extract in polar and non-polar solvents. By adding 30 g chopped powder of stem was separately extracted in 200ml distilled water, methanol and n-Hexane. The distilled water mixture was stirred on a magnetic stirrer overnight and then boiled at 60^{o}C for 20 minutes then cooled at room temperature and filtered by using Wattsman no 1 filter paper (Figure 1).

ANTIMICROBIAL ACTIVITY

Two antimicrobial activities antibacterial and antifungal of *Clematis grata* stem extracts were carried out against clinical bacterial pathogens and pathogenic fungi isolated from rotted foodstuff.

Bacterial Pathogens

The antimicrobial activity of polar and non-polar *clematis grata* stem extracts was assessed against the two Gram-negative that is *Pseudomonas aeruginosa* as well as *Escherichia coli* while one Gram-positive i.e. *Staphylococcus aureus*. Bacterial pathogens are isolated from various clinical samples like urine and blood.

Antibacterial assay

The agar well diffusion assay was used to appraise the bacterial activity of *C. grata* stem extracts by using previous methods with slight modification (Mughal et al., 2022). Broth culture was prepared and kept for 24 hours in a shaking incubator. Sterilized petri plates were labeled and nutrient agar mixed with overnight grown broth culture was poured into the Pertiplates. After solidification, three wells of 5 mm diameter were made in each plate with a 1 mL sterilized micropipette tip, and the

agar plug was removed with a sterilized needle. 20 µL of each prepared *C. Grata* stem extract concentration was poured into the wells separately. DMSO was taken as the negative control and Ciproflaxin was taken as positive control. All the plates were incubated for 24 hours. After incubation, the diameter of the zones of inhibition was measured in millimetres to determine microbial growth with the help of scale.

Figure 1: Extract preparation of *Clematis grata* stem via maceration process

Minimum inhibitory concentration (MIC)

The antibacterial activity of C. Grata stem extracts was assessed using the standard micro-dilution technique, which determines the lowest inhibitory concentration that inhibits bacterial growth. The lowest inhibitory concentration of C. Grata extracts was determined using the agar well diffusion technique. The antibacterial activity of extracts was examined at three different concentrations (0.2 mg/ml, 0.6 mg/ml, and 0.9 mg/ml). The MIC was defined as the lowest concentration that stopped the growth of test microorganisms (Nazer et al., 2020).

Isolation of Fungus Strains

Two fungal species were isolated in the laboratory *Penicillium digitatum* (Pd) from Orange peel and *Rhizopus stolonifers* (Rs) were isolated from bread (Figure 2).

Antifungal assay

Antifungal activity was carried out via Disc diffusion previously used method with slight modifications (Gizaw et al., 2022). Two fungal species *Penicillin Digitatum and Rhizopus stolonifers* were isolated in the laboratory from rotted bread and orange on PDA media. For the antifungal assay three concentrations of plant extract 0.2, 0.6, and 0.9 mg/mL were prepared and vortexed for proper dissolving. Fungus culture was grown for 45 hrs in broth media mixed with freshly autoclaved PDA media and poured in labeled Petri plates. Three discs impregnated with extract were dried used these discs placed at equal distances on the Petri plates. Petri dishes were set aside at room temperature in the laminar flow. DMSO was taken as the negative control and fluconazol antifungal drug was taken as positive control. Incubation of Petri dishes in

the incubator was done for 30-45 hours. After incubation diameter of zones of inhibitions was measured in mm with scale. The test was run in triplicate, and the mean and standard deviation were calculated. The growth sensitivity tests expressed as 0 for no sensitivity, 1-5 mm for low sensitivity,>5-10 mm for moderate sensitivity and >10-25 mm for the highest sensitivity.

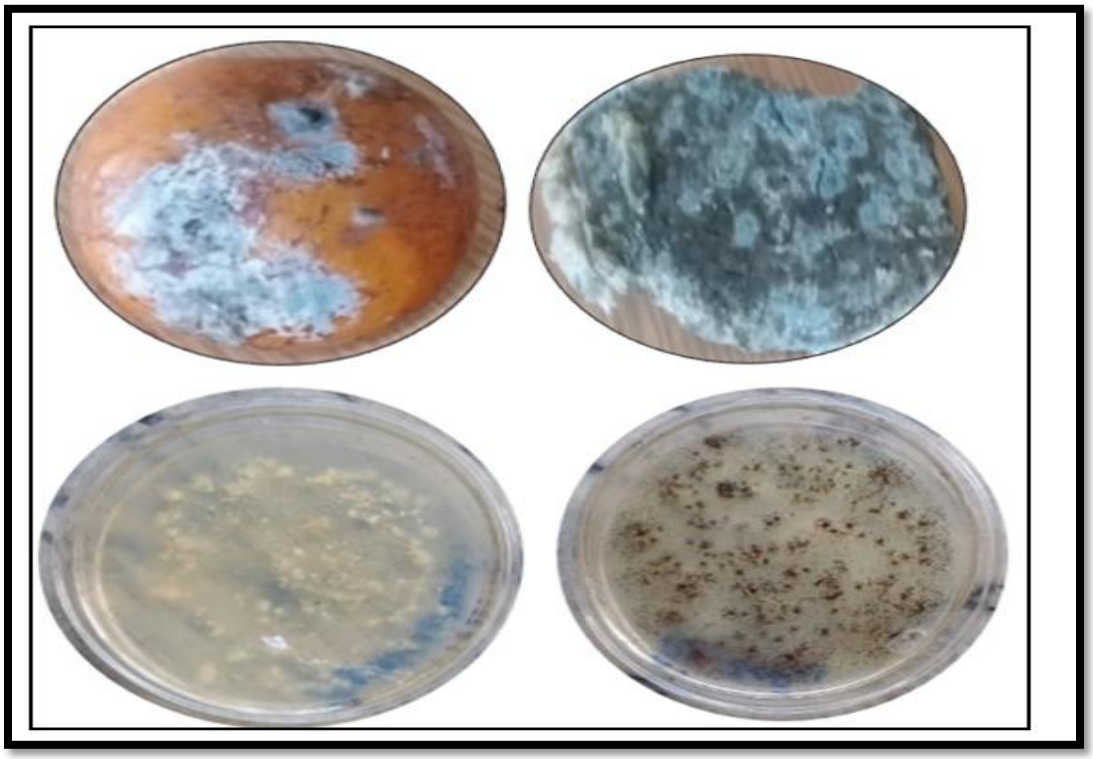

Figure 2: Isolation of fungus strains of *Penicillium digitatum* (Pd) and *Rhizopus stolonifers* (Rs)

Antibiofilm assay

A modified crystal violet (CV) test was used to assess anti-biofilm activity. Bacterial pathogens such as *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (100 l) were cultured in Nutrient broth medium (2 mL) with 30 l of each C. grata extract separately and Chloramphenicol (1 disc 10 g) as a positive control, and incubated overnight at 37ºC. As a negative control, broth culture was employed (Kawsud et al., 2014). The broth medium was discarded the next day, and 125 µl of CV (0.1 per cent) was added and then incubated at room temperature for another 10- 15 minutes. CV was discarded and tubes were dried. Following the staining with CV, acetic acid (30%) was also added to 100 ml dH2O to solubilize the CV and left at room temperature for 10-15 minutes. Using a spectrophotometer, the solubilized CV was determined at 550 nm. The control was 30% acetic acid in dH_2O (Nazer et al., 2020).

Antioxidant assay Diphenypicrylhydrazyl (DPPH) radical-scavenging activity

The DPPH assay was used to determine the antioxidant potential of *C. grata* extract. The stock solution was prepared by dissolving DPPH in Methanol and kept in the

dark at room temperature for 30 minutes. Sample preparation was carried out as DPPH was added into each *C. grata* stem extract and standard solution separately, into each sterilized labelled test tube and placed in the dark at room temperature for 30 minutes (Dutra et al., 2022). Absorbance was recorded at 520nm. The sample's radical scavenging was reported as the percentage of free radicals inhibited by *C. grata* extract. Percentage inhibition was measured using the formula below. Ascorbic Acid was used as a Positive control.

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control - Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Cytotoxic Assay through MTT assay

Cytotoxicity Evaluation Using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) was used to estimate the viability of the bacterial cells the human bacterial pathogens *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Staphylococcus aureus* were cultured in freshly prepared nutrient broth medium (4ml) at 37ºC for overnight. After 24 hours, overnight bacterial cell culture (100 µl) was poured in freshly ready nutrient broth medium (1 ml) along with 100 µl of each sample in triplicate with control and blank (*C. grata* stem extract and Chloremphenicol) then incubated at 37ºC for 4 h. For the decrease response, 10 µl for MTT was added and the mixture was incubated at 37ºC (without shaking) for 2 to 4 hours with an opened tube cap. The formation of formazan crystals during this reaction (observed purple colour) was observed, and DMSO (500 µl) was added. The absorbance was recorded at 570 nm through a spectrophotometer. DMSO was taken as a control (Nazer et al., 2020).

RESULTS

From the results it was observed that *C. grata* stem n-Hexane extract at the concentration of 0.9 mg/ml had a remarkable zone of inhibition against *E.coli* (20±3.3 mm) and *S.aureus* (16.6±2.2 mm) as shown in Table 1 and Figure 3. Methanolic extract (0.9 mg/ml) of *C. grata* showed moderate zones against *E.coli* (5.6±2.8 mm) and *S. aureus* (3±0.6 mm) and minimum zones were recorded against *P. aeruginos* (3±2 mm). Aqueous extract of *C. grata* stem showed moderate antibacterial effect at the concentration 0.9 mg/ml against *E. coli* (5.6±2.2 mm) and *P. aeruginos*a (4±0.6 mm). Antibiotic (Ciprofloxacin 10µg) used as control showed moderate zones against *E.coli* (6.6 ±0.57 mm), *S. aureus* (6±0 mm) and *P. aeruginosa* (9±0 mm). DMSO did not affect all the tested pathogens. 0.2 as well as 0.6 mg/ml concentrations of all extracts did not show any potential or effective antibacterial activity against all the tested pathogens (Table 1).

Antifungal activity of *C-grata* stem extracts (n-Hexane, methanol, aqueous) at the concentrations of 0.2 mg/ml, 0.6 mg/ml, 0.9 mg/ml through the Disc diffusion method. Results showed that *C. grata* stems n-Hexane extract at the concentration of 0.2 mg/ml extract have minimum zones of inhibition were recorded against *Penicillium digitatum* (*Pd*) (3.33±0.44 mm) and *Rhizopus stolonifers* (*Rs*) (4.33±0.44 mm) as shown in Table 2 and Figure 4. Methanol and n-hexane extracts of *C. grata* at 0.9 mg/ml concentration were found to be most effective against *Pd* (9.6±0.44 mm)

and *Rs* (8 ±2 mm) respectively. *C. grata* aqueous extract at the concentration of 0.9 mg/ml was found less effective against *Pd* (6.33 ±2.44 mm) and *RS* (6±0.66 mm) respectively. Results of the present study showed that in comparison with *C. grata* stem crude extract in different solvents standard antifungal drug fluconazole showed minimum inhibitory effect against *Pd* (3.3±1.11mm) and *Rs* (3±0.66 mm).

The antioxidant capacity of *C. grata* stem extract was assessed using the DPPH free radical scavenging test. Table 3 indicated that n-Hexane at all doses (0.10 mg/mL, 0.15 mg/mL, and 0.5 mg/mL) displayed good antioxidant activity (24.31%, 61.04%, and 71.21%, respectively) across all *C. grata* stem extracts. Methanolic stem extract demonstrated potential free radical scavenging activity at all doses tested (57.81%, 64.54%, and 78.68%, respectively). The highest antioxidant activity was found in aqueous stem extract at 0.10 mg/mL, 0.15 mg/mL, and 0.5 mg/mL (68.51%, 76.17%, and 81.88%, respectively). Ascorbic acid had percentage inhibition values of 94%, 95%, and 96%. Plant extracts have much higher antioxidant efficacy than ascorbic acid.

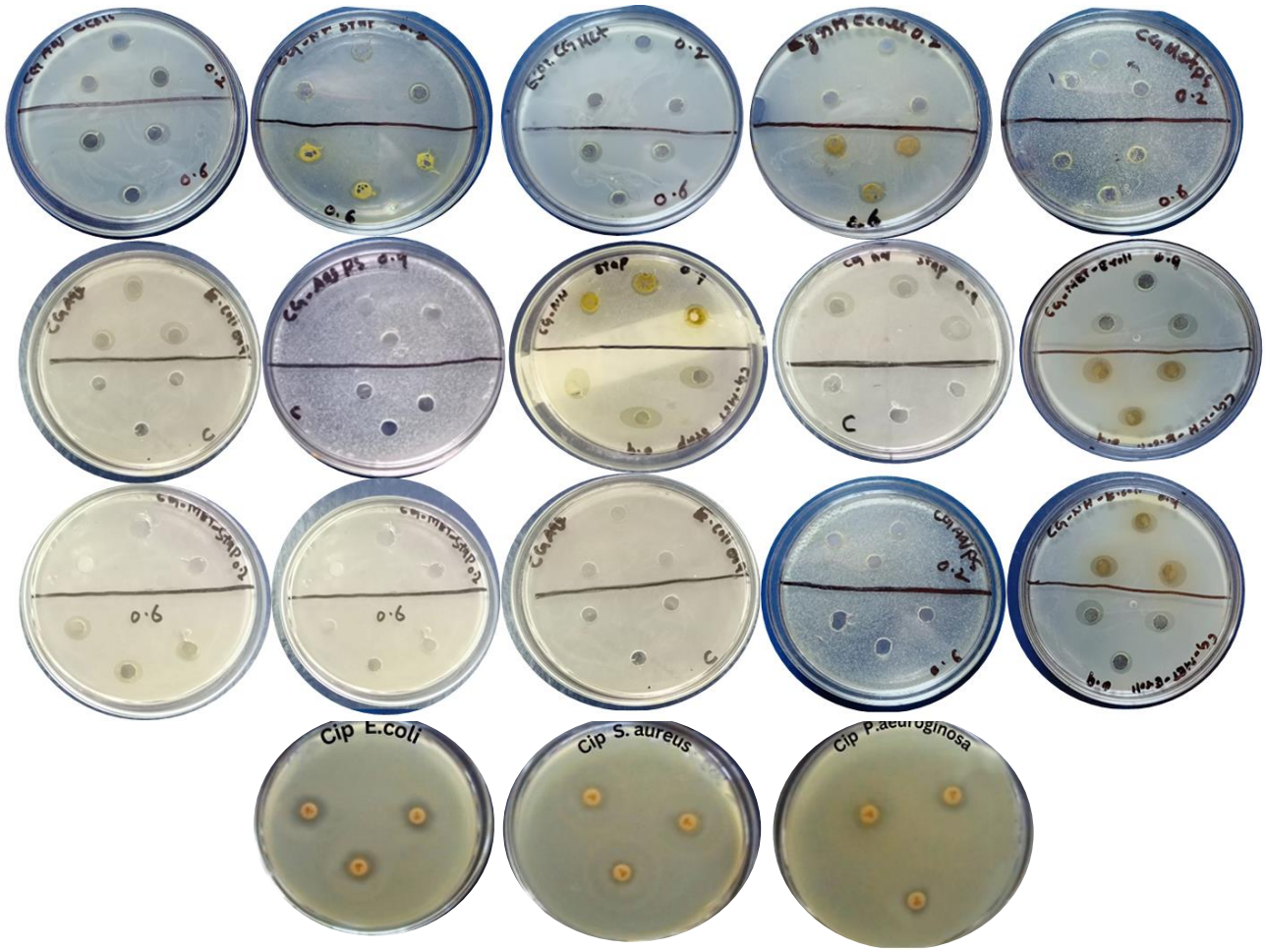

Figure 3: Antibacterial activity of *C. grata* stem extract at different concentrations (0.2, 0.6, 0.9 mg/mL) and ciprofloxacin (10 µg) against *E. coli, S. aureus, P. aeruginosa.*

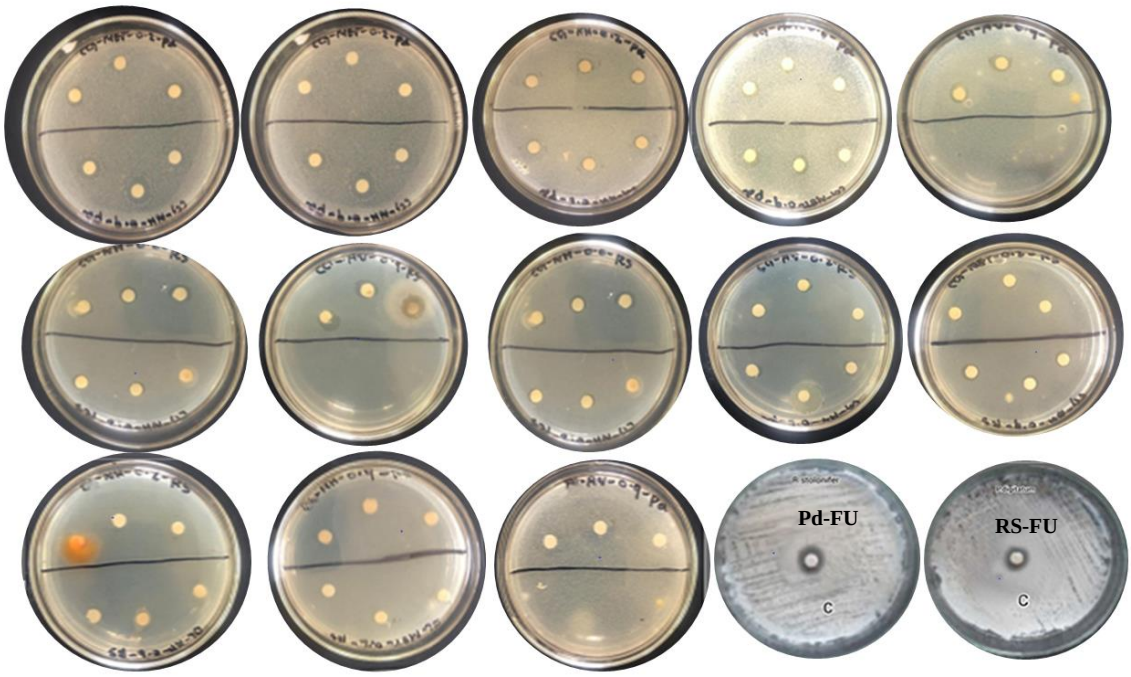

Figure 4: Anti-fungal activity for *C. grata* stem extract with three different concentrations (0.2 mg/mL, 0.6 mg/mL, 0.9 mg/mL) against *Penicillin digitatum* and *Rhizopus stolonifer* and antifugal activity of Fluconazol against *Penicillin digitatum* (Pd-Fu) and *Rhizopus stolonifer* (Rs-fu).

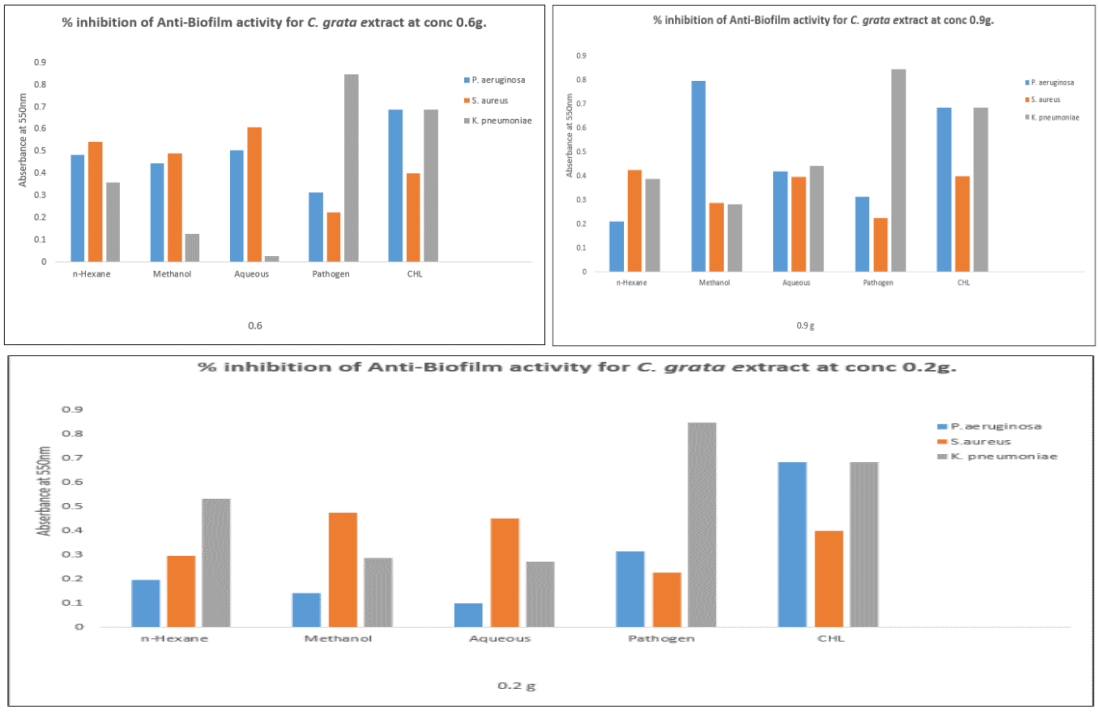

Figure 5: Anti-biofilm Activity for *C. grata* stem extracts with three different concentrations (0.2 mg/mL, 0.6 mg/mL, 0.9 mg/mL) against pathogens *(P. aeruginosa, S. aureus* and *K. pneumonia).*

C. grata n-Hexane, aqueous and methanol at 0.2 mg/mL extracts have shown a maximum inhibition against *P. aeruginosa* biofilm formation. *C. grata* methanolic and aqueous extracts at the concentration of 0.6 mg/mL showed maximum resistance towards biofilm formation of *K. pneumoniae.* At 0.9 mg/ml *C. grata* n-Hexane, aqueous and methanol extract showed minimum biofilm formation against *P. aeruginosa* and *S. aureus.* In comparison with all plant extracts chlorempheicol showed minimum inhibition of biofilm formation against all tested bacterial pathogens (Figure 5).

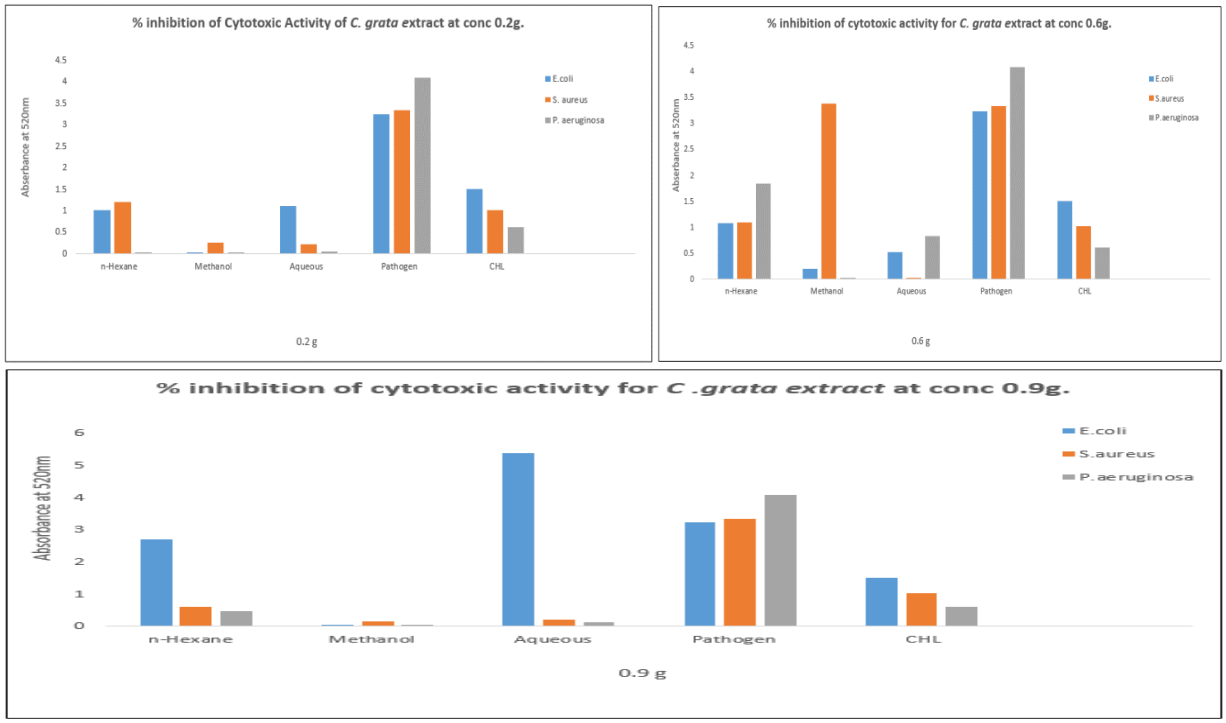

Figure 6: Cytotoxic activity for *C. grata* stem extracts with three different concentrations (0.2 mg/mL, 0.6 mg/mL, and 0.9 mg/mL) against pathogens *(P. aeruginosa, S. aureus* and *E.coli).*

Results of cytotoxic assay showed that stem extracts of *C. grata* (methanol, n-Hexane, aqueous) with conc's (0.2 mg/mL, 0.6 mg/mL, and 0.9 mg/mL) against tested pathogens (*E. coli*, *P. aeruginosa* and *S. aureus*) had remarkable cytotoxic potential as compared to standard drug chloramphenicolin (Figure 6).

DISCUSSION

In the current study, the antibacterial, antioxidant, antibiofilm, cytotoxic and antifungal activities were carried out. The agar well diffusion method was used to evaluate the antibacterial activity against different bacterial strains (*E. coli*, *P. aeruginosa* and *S. aureus*) at different concentrations (0.2 mg/mL, 0.6 mg/mL, 0.9 mg/mL) for *C. grata* stem extract (methanol, n-Hexane, aqueous). Among all the extracts the *C. grata* n-Hexane stem extract (0.9 mg/mL) showed the highest zone of inhibition (20±3.3 mm) against *E.coli*, (16.6±2.2 mm) against *S.aureus* and *P. aeroginosa* respectively. Methanolic extract (0.9 mg/mL) also had remarkable

potential against *E.coli* (5.6±2.8 mm). In comparison aqueous stem extract and ciprofloxacin shown less zone of inhibition against all tested bacterial pathogens (*E.coli, S.aureus, P.aeroginosa).*

The antioxidant potential of *C. grata* (n-hexane, methanol, and aqueous) extract showed good antioxidant efficacy. *C. grata* n-Hexane extract at conc. (0.10 mg/mL) showed 24.31%, *C. grata* methanol extract at conc. (0.15 mg/mL) showed 64.54% and maximum antioxidant potential was recorded for *C. grata* aqueous extract at conc. (0.5 mg/mL) 81.88%.

In previous research, the leaf extract of *C. grata* had the maximum zone of inhibition (ZOI) against *S. aureus* with an IC50 value of 11.55 g/mL, the acetone extract of the stem, with an IC50 value of 15.75 g/mL. The results showed the excellent antibacterial and antioxidant activity of the investigated plant extracts, allowing for further research and prospective application as a natural antibacterial and antioxidant agent (Kumari et al., 2019).

MTT test was performed to assess the cytotoxic activities of *C. grata* stem extracts. The extracts at conc, 0.9 mg/mL *C. grata* n-Hexane had higher cytotoxic potential against *E.coli*, 0.6 mg/mL *C. grata* methanol had strong potential against *P. aeruginosa*, and 0.9 mg/mL *C. grata* aqueous had the best cytotoxic potential against *E.coli*. In comparison to the antibiotic chloremphenicol, C. grata extract showed potential cytotoxicity against all tested bacterial infections.

The *C. grata* extract has shown a significant effect on the suppression of biofilm formation. Among all extracts concentrations at 0.9 mg/mL the *C. grata* n-Hexane extract against *P. aeruginosa*, *C. grata* methanol extract (0.6 mg/mL) against *K. pneumoniae, C. grata* aqueous extract (0.6 mg/mL) against *K. pneumoniae* had shown significant reduction of the biofilm formation. In comparison positive control, very less inhibition of biofilm formation against *P. aeruginosa* and *K. pneumonia* was observed.

According to previous research, all the aqueous extracts have significantly reduced the biofilm biomass as compared to the untreated samples. Methanolic extracts of the plants *Betula pendula, Equisetum arvense, Herniaria glabra, Galium odoratum, Urtica dioica*, and *Vaccinium vitis-idaea* greatly reduce biofilm formation and lower virulence factors (Alam et al., 2020).

The Antifungal activity of the selected plant, *C. grata* stem extracts (n-hexane, methanol, and aqueous extracts) against fungus strains *Pencillium digitatum* (Pd) and *Rhizopus stolonifers* (RS) at conc, 0.2 mg/mL showed minimum zones of inhibition. At 0.9 mg/mL n-Hexane, methanolic, and aqueous extracts of *C. grata* maximum zones were recorded against PD (7.33±0.44 mm, 9.6±0.44 mm and 6.33± 2.44 mm) and RS (8±2 mm and 6± 0.66 mm) respectively. In comparison control fraction of (0.5 µg) Fluconazol (as an antifungal drug) fewer zones were observed as compared to *C. grata* plant extracts. Our findings are following previous research (Patil et al., 2017).

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Table 1: Anti-bacterial Activity Mean Deviation Calculation for *Clematis grata*

Clematis grata **Solvents used**	**Bacterial strain inhibition / Zone of inhibition (mm)**									
	N-Hexane			**Methanol**			**Aqueous**			**Ciproflaxin**
Extract Conc. **Tested pathogens**	0.2 mg/ml	0.6 mg/ml	0.9 mg/ml	0.2 mg/ml	0.6 mg/ml	0.9 mg/ml	0.2 mg/ml	0.6 mg/ml,	0.9 mg/ml	10µg
Escherichia coli	3±0.6mm	5±0mm	20±3.3mm	0±0mm	1.6±1.1mm	5.6±2.8mm	0±0mm	0 ±4mm	5.6±2.2mm	6.6±0.57mm
Staphylococcus aureus	1.6±0.4mm	3.6±1.1mm	16.6±2.2mm	0±0mm	2±0.6mm	3±0.6mm	0±0mm	1.6 ±0mm	4 ±0.6mm	6±0mm
Pseudomonas aeuroginosa	0±0mm	10±3.3mm	16.6±2.2mm	0±0mm	3±0.6mm	3 ±2mm	0±0mm	0 ±0mm	4±0.6mm	9±0mm

Table 2: Anti-fungal Activity Mean Deviation Calculation table for *C. grata.*

Clematis grata **Solvents Used**	**Fungal Strains inhibition / Zone of inhibition (mm)**									**Fluconazole**
	N-Hexane			**Methanol**			**Aqueous**			
Fungal Strains	0.2 g,	0.6 g,	0.9 g	0.2 g,	0.6 g,	0.9 g	0.2 g,	0.6 g,	0.9 g	**0.5µg**
Penicilluium digitatum	3.3±0.4mm	5.3 ±3.5mm	7.3±0.4mm	2.3±3.1 mm	3.3 ±1.1mm	9.6±0.4mm	3.3 ±0.4mm	3.3±0.9mm	6.3± 2.4mm	3.3 ±1.11mm
Rhizopus stolonifers	4.3±0.4mm	5±1.3mm	8±2mm	3±0.66mm	5±2mm	8±2mm	3±0.66mm	4±5.3mm	6± 0.6mm	3±0.66mm

Table 3: Anti-oxidant activity of *C. grata* stem and Ascorbic Acid.

Clematis grata	**% Inhibition and Extract Conc.**			**Ascorbic acid**		
Solvents Used	**0.10 mg/mL**	**0.15 mg/mL**	**0.5 mg/mL**	**0.10 mg/mL**	**0.15 mg/mL**	**0.5 mg/mL**
n- hexane	24.31%	61.04%	71.21%			
Methanol	57.81%	64.54%	78.68%	94 %	95%	97%
Aqueous	68.51%	76.17%	81.88%			