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PHARMACOLOGICAL AND BIOLOGICAL EVALUATION OF METHANOL EXTRACTS OF SELECTED CHOLISTANI PLANTS

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ABSTRACT

Cholistani plants are a rich source of bioactive compounds that are helpful in treating various ailments. The objective of the current investigation was to elucidate the pharmacological importance of four Cholistani plants including *L. indicum, E. granulata, C. prostratus,* and *H. crispum*. Methanolic extracts of these plants were subjected to antibacterial, antibiofilm, antioxidant, antidiabetic, and antiviral analysis. Disc diffusion assay was used for antibacterial activity and the MIC of active extracts was also calculated. *In vitro* antibiofilm assay was conducted against drug-resistant and drug-sensitive bacterial strains and % inhibition was calculated. DPPH assay was used for the evaluation of antioxidant potential. The antidiabetic potential was tested by α-glucosidase inhibitory assay. The Haemagglutination (HA) test was performed to assess the antiviral properties of these plants against the Avian Influenza Virus (H9N2). It was found that *E. granulata* prevailed in antibacterial potential with max ZoI against selected bacterial strains (16.5 mm versus *E. coli*. *C. prostratus*) surpassed all other plant extracts in terms of biofilm inhibition with up to 90% inhibition against *P. aeruginosa*. Considerable antioxidant potential was revealed by all examined plants in the order of *E. granulata*> *C. prostratus* > *L. indicum* > *H. crispum*. A substantial α-glucosidase inhibitory potential was detected in all the studied plants. All of the examined plants displayed significant (titer 0) antiviral activity. This study reveals that all of these plants have the potential to be employed as antibacterial, antioxidant, and antiviral agents. *In vitro* suppression of the α-glucosidase enzyme indicated that these plants are potent sources of antidiabetic compounds. In conclusion, all of the examined Cholistani plants are rich sources of pharmacological compounds and should be further researched for drug development.

Key words: *E. granulata*, *C. prostratus, L. indicum, H. crispum,* antibacterial, antioxidant, antibiofilm, antiviral:

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INTRODUCTION

The term 'Cholistan is derived from the Turkish word 'Chol' and this signifies the 'land of the desert'. It is also called the 'Rohi desert' that surrounds an area of 30km from Bahawalpur (Ahmad *et al*., 2012). The usage of medicinal plants as medications has dramatically grown in recent years. According to the global health organization, 65 to 80% of humans rely on plants for healing ailments owing to the expensive cost and adverse side effects of synthetic pharmaceuticals (Mesfin *et al*., 2013). Various components of plants such as seeds, roots, berries, leaves, bark, and flowers are employed by common practitioners for health care ever since ancient times (Mustafa *et al*., 2017).

L. indicum, a medicinal herb from the Cholistan desert, belongs to the Aizoaceae family and is known as "Baluka sag" and "jungli lonak" (Wariss *et al*., 2013). *Euphorbia granulata* Forssk, also known as Hazar Dani, is a prostrate, annual plant found worldwide (Webster, 2014). *Heliotropium crispum*, also known as kali Buri, is a medicinal desert plant used in herbal treatments and is

fed to cattle to promote lactation (Qureshi *et al*., 2010). *Convolvulus prostratus* Forssk, also known as "Hiran Booti," belongs to the morning glory family and is found in tropical, subtropical, and temperate climates (Ekka and Dixit, 2007). Plants contain phytochemicals like terpenoids, tannins, alkaloids, and flavonoids that have antibacterial properties (Bhalodia and Shukla, 2011). As antibiotic resistance and side effects increase, research on plants' antimicrobial role against resistant strains is gaining traction, as they are considered safe and efficacious (Algabr *et al*., 2022). Biofilm formation on medical devices poses significant challenges in medical research, particularly in urology, where it is a primary source of urogenital infections (Tenke *et al*., 2012). Biofilm-associated infections are difficult to cure, even in people with robust immune systems, as bacteria in biofilms may evade host defense. This has led to a search for new antimicrobial chemicals (Teanpaisan *et al*., 2017). A substance that considerably reduces or delays oxidative stress is known as an antioxidant molecule. Free radicles cause substantial oxidative damage to the body and are involved in causing various ailments (Venkatachalam *et al*., 2012). The modern world is concentrating on receiving antioxidants from natural sources as a health supplement (Kumar *et al*., 2022).

An antioxidant molecule reduces or delays oxidative stress, which can cause various ailments. In type 2 diabetes, inhibiting the activity of glucosidase enzymes may lower hyperglycemia. Diabetic drugs that inhibit these enzymes slow down starch digestion in the small intestine, resulting in less glucose production and circulation (Briones and Bajaj, 2006). Commercial drugs for suppressing α-glucosidase have adverse effects and high costs (Adefegha and Oboh, 2012). Research is being conducted to develop innovative anti-glucosidase and anti-diabetic medicines with enhanced safety profiles for long-term treatment (Liu and Ma, 2017). The highly pathogenic avian influenza (HPAL) virus causes severe illness in domestic poultry, particularly H9N2, which is associated with morbidity in Pakistan and South East Asia (Wu *et al*., 2016). It is believed to be causing a new influenza pandemic in humans in the region. Herbal medications and plant extracts have shown strong antiviral benefits (Verma *et al*., 2008). Keeping in mind these challenges, present investigation was carried out to assess the antimicrobial, antibacterial, antioxidant, anti-viral, and anti-diabetic potentials of selected Cholistani plants.

MATERIALS AND METHODS

Specimen Collection: Selected plants were collected from herbarium of the Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur, Pakistan. Study was conducted from April 2021 till August 2021. Plant identification was done by taxonomist at CIDS and voucher numbers were issued to plants. *Limeum indicum* CIDS/IUB-0205/7, *Heliotropium crispum* CIDS/IUB-0502/23, *Convolvulus prostratus* CIDS/IUB-1103/46 and *Euphorbia granulata* CIDS/IUB-1602/60-B.

Extract preparation: The whole plants were collected, cleaned, and dried under shade for 10 days. The dried plants were converted into powder form and 10 g of each plant powder was dissolved in 100 ml of Methanol (MtOH) in an airtight container. The solution was kept at 37°C under constant shaking for 96 hrs. Later the samples were filtered through Whatman filter paper No. 1. The filtrates were subjected to a rotary evaporator at 45-50°C and after complete removal of solvent, the precipitates were collected, weighed, and dissolved in MtOH @ 500 mg/mL and 10 mg/mL (Stanković *et al*., 2016).

Antibacterial activity: Disc diffusion assay was performed for antibacterial evaluation against selected bacterial strains. The pre-soaked GF-1 grade filter paper discs of extracts and control were used. The bacteria were *inoculated* on agar plates, incubated for 40 min at 37 °C, and then discs were applied. The culture was again incubated at 37 °C overnight and the zone of inhibition (ZoI) was recorded by the standard method by CLSI guidelines. The pure solvent was used as the negative control. Ampicillin and Moxifloxacin were used as a positive control for normal and drug-resistant bacteria, respectively (Jahan *et al.*, 2018).

Minimum inhibitory concentrations (MIC): MIC was determined using the broth-dilution technique according to the protocol given by Wiegand *et al.*, (2008) with slight modifications. 50 µl of nutrient broth and 50 µl of fresh bacterial culture were added to wells of sterilized microtiter plate. 100 µl of plant extract and positive control drug were added to first well and serially diluted till 11^{th} well. 12^{th} well was used as negative control. Plate was incubated at 37°C overnight. Next day INT (Iodonitrotetrazolium chloride) (20 µl) was added to each well and plate was incubated at 37°C for 20-30min and color change was observed.

Antibiofilm assay: The antibiofilm potential of selected plant extracts was evaluated by the standard method given by Bazargani and Rohloff, (2016). OD_{630nm} was taken through an ELISA reader and % inhibition was calculated by the formula (%Inhibition = [(A0-AX)/A0] *100), where A0 is the absorbance of negative control and A_X is the absorbance of the sample.

DPPH activity (Antioxidant activity): The antioxidant activity of all extracts was checked by DPPH assay with the method given by Brand-Williams *et al*., (1995) . Ascorbic acid was used as the positive control. Color change of dye was observed and OD_{517} was recorded.

Radical scavenging potential was calculated by %RSA= [(A0-AX)/A0] *100 (where A0 is the absorbance of negative control and A_X is the absorbance of the sample).

α-glucosidase inhibitory activity: In vitro α-glucosidase inhibitory activity was carried out by the standard method presented by Phan *et al.*, (2013). The activity was measured in the form of % inhibition by the formula (% Inhibition= [(OD_0-OD_S)/OD_0] *100), where OD_0 is the absorbance of negative control and ODs is the absorbance of the sample.

Antiviral activity

Inoculation of viruses in chicken embryonated eggs: In 9-10 days old chicken embryonated eggs, and selected viral strains were cultured through the chorio-allantoic route. The eggs were incubated at 37°C for 48-72 hrs. The allantoic fluid was collected and exposed to Haemagglutination (HA) to assess the titers of the virus.

Haemagglutination (HA) test: HA test was conducted by the method given by Killian, (2008). Red dots at the bottoms of the wells show negative results while uniform reddish color shows positive results.

Statistical analysis Data presented as mean ± SEM. One-way ANOVA was used for the analysis of data using Graph pad prism version 8.0.1. Dunnett's multiple comparison test was used and values were compared with positive control where ($P \leq 0.05$)were considered significant (Ross *et al.*, 2017).

RESULTS

Antibacterial activity: In terms of antibacterial activities MtOH extracts of all chosen Cholistani plants, exhibited strong antibacterial capability against selected bacterial strains. *L. indicum* was most effective against *K. pneumoniae* with a 14.5mm ZoI and MIC 390.62 µg/µl. It was least effective against *P. aeruginosa* (8mm ZoI) with notable effects against other strains as well. When *E. granulata* was evaluated for antibacterial properties, it was shown to be most efficient against *E. coli* with 16.5mm ZoI and MIC 1562.5 µg/µl. *E. granulata* was also shown to be particularly efficient against *S. aureus* and *K. pneumoniae* with 16 mm ZoI which was practically equivalent to positive control. The least inhibitory potential was seen against *S. aureus* MDR (7.5mmZoI) (7.5mmZoI). *H. crispum* exhibited maximum and minimum antibacterial potential against *P. aeruginosa* and *P. aeruginosa* MDR with 12 mm (MIC 1562.5 µg/µl) and 8.5mm ZoI. *C. prostratus* revealed generally strong antibacterial potential with 16mm ZoI (MIC 3125 µg/µl) against *P. aeruginosa* MDR and *S. aureus* (Table 1).

Antibiofilm activity: Antibiofilm potential of selected Cholistani plants was evaluated against drug sensitive and drug resistance strains of *S. aureus* and *P. aeruginosa.* In case of MDR strains *E. granulata* was most effective against *S. aureus* MDR with 84% inhibition whereas *C. prostratus* was highly effective against *P. aeruginosa* MDR with 79% suppression of biofilm development. When tested against drug sensitive strains, *C. prostratus* was the most efficient in preventing the growth of *S. aureus* biofilm, however *H. crispum* and *L. indicum* also showed significant suppression. *C. prostratus* also outperformed all other plant extracts in its ability to suppress the formation of *P. aeruginosa*'s biofilm by 90%, which was even more effective than the positive control (Table 2).

Antioxidant Activity: The antioxidant potential of selected plants was observed by DPPH assay and %RSA was calculated for each plant. It was observed that *E. granulata* possessed the highest scavenging potential i.e., 82%. Other plants, in the following order: *C. prostratus* > *L. indicum* > *H. crispum*, showed the ability to scavenge radicals: 57%, 53%, and 51.34%RSA, respectively. The ascorbic acid was used as positive control (Fig 1).

α- glucosidase Inhibitory assay: According to the antidiabetic potential of MtOH extracts of selected plants was checked by α- glucosidase inhibitory assay, *C. prostratus* and *E. granulata* were found to be most effective with 82% and 81% inhibitions respectively. Whereas, *L. indicum* (73%) and *H. crispum* (61%) showed considerable inhibition potential values. Acarbose was used as positive control and exhibited 95% inhibition against α- glucosidase (Fig 2).

Antiviral activity: MtOH extracts of selected plants were subjected to an HA (Hemagglutination) test for antiviral potential evaluation against AIVH9N2. *E. granulata* and *C. prostratus* were equally effective with 0 titers (<log2). In the case of other plants, *H. crispum* was more effective than *L. indicum* with 4 (8 logs) and 8 (7 logs) titers respectively (Table 3).

Table 1 Antibacterial activities of MtOH extracts of selected plants against drug sensitive and MRR strains.

Bacterial Strains	Plants	ZoI (mm)	MIC(µg/µl)
	L. indicum	11.5	-
E. coli	*E. granulate*	16.5*	1562.5
	H. crispum	10	3125

	C. prostrates	9.5	3125
	Ampicillin	17.5**	12500
	Pooled SEM=0.82		
	L. indicum	14.5	390.62
	E. granulate	16*	-
K. pneumoniae	*H. crispum*	10.5	-
	C. prostrates	12.5	390.625
	Ampicillin	16**	195.3
	Pooled SEM=0.698		
	L. indicum	10	-
	E. granulate	12*	-
P. vulgaris	*H. crispum*	10.5	390.62
	C. prostrates	5	781.25
	Ampicillin	13.5**	195.3
	Pooled SEM=0.954		
	L. indicum	8	-
	E. granulate	14*	562.5
P. aeruginosa	*H. crispum*	12	1562.5
	C. prostrates	13	-
	Ampicillin	15**	6250
	Pooled SEM=0.30		
	L. indicum	10	-
	E. granulate	7.5	-
S. aureus MDR	*H. crispum*	11	1562.5
	C. prostrates	11.5*	3125
	Moxifloxacin	16.5**	390.62
	Pooled SEM=0.74		
	L. indicum	13.5	390.625
	E. granulate	16*	3125
S. aureus	*H. crispum*	9	195.312
	C. prostrates	13	-
	Ampicillin	16.5**	6250
	Pooled SEM= 0.698		
	L. indicum	11	781.25
	E. granulate	8.5	-
P. aeruginosa MDR	*H. crispum*	8.5	-
	C. prostrates	16*	3125
	Moxifloxacin	10.5**	390.62
	Pooled SEM=3.5		

*indicates max ZoI
** indicate ZoI of positive control
Pooled SEM is calculated by adding log of sum of SEMs.

Table 2 Antibiofilm potential of MtOH extracts of different plants against selected bacterial strains. Data is presented as % inhibition± SEM.

Plant	*S. aureus*	*S. aureus* MDR	*P. aeruginosa*	*P. aeruginosa* MDR
	% Inhibition± SEM			
*Positive control	89.80±0.219	86.50±0.45	84.45±1.337	89.63±0.060
L. indicum	73.52±1.56	72.61±0.9	83.34±1.27	61.33±1.58
E. granulata	67.61±0.40	84.27±0.65	79.00±1.5	60.73±1.35
C. prostratus	81.60±0.21	83.79±0.25	90.09±0.039	79.64±0.51
H. crispum	74.49±1.73	76.922±1.39	74.55±1.2	72.18±0.418

* *positive* control= Ampicillin (Drug sensitive strains) and Moxifloxacin (MDR strains)

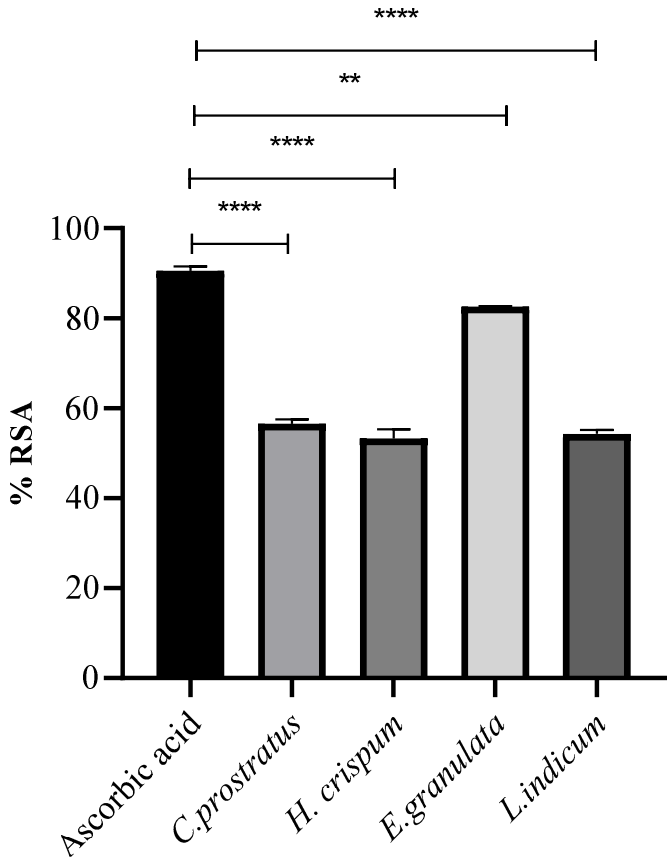

Fig. 1 %Radical scavenging activity of Methanolic extracts of selected plants.
Values are presented as %RSA± standard error. ****(P<0.0001) and **(P=0.0137).

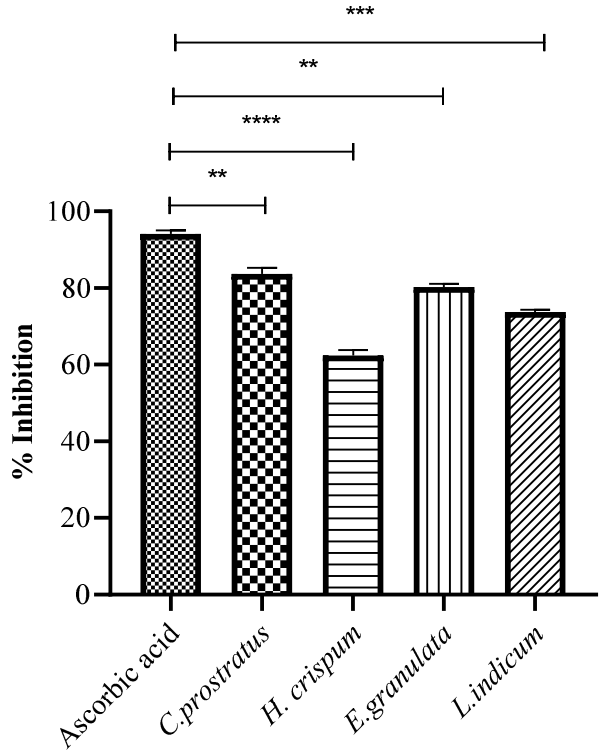

Fig 2 α- glucosidase Inhibitory potential of Methanolic extracts of selected Cholistani plants.
Values are presented as %Inhibition± SEM. ****(P<0.001), ***(P<0.002) and **(P<0.0011).

Table 3 Antiviral activity of MtOH extracts of selected plants against AIVH9N2.

Plants	HA Titer	Log reduction	$IC_{50\ \mu g}$
L. indicum	8	7	500
E. granulate	0	<log2	125
C. prostrates	0	<log2	125
H. crispum	4	8	250
Virus control	1024	0	
Amantadine	0	<log2	125

*Log reduction= [log2 (control=10)-log2 Sample]

DISCUSSION

The desert offers a range of plants that are helpful to fight against numerous disorders (Malik *et al*., 2015). Antibiotic resistance among microbial strains is fostered by the widespread use of antibiotics in clinical treatment, agriculture, and veterinary care (Hussain *et al.*, 2015). This has led to the investigation of novel antimicrobial agents, mainly among plant extracts, in order to produce new medications that overcome the disadvantages of antibiotics (Bereksi *et al*., 2018). In the present work, the antibacterial potential of MtOH extracts of four Cholistani plants *E. granulata, C. prostratus, L. indicum*, and *H. crispum* was examined. *E. granulata* dominated antibacterial activity but other plants also shown great potential, suggesting that these plants may become a suitable alternative to antibiotics (Ihtesham *et al*., 2019; Indrianingsih *et al*., 2021).

Due to various therapeutic issues connected with conventional antibiotics, there has been a rise in interest in utilizing plant extracts for the treatment of biofilm-related disorders. Herbal plants are excellent source of alternative remedies for a number of ailments (Wijesundara and Rupasinghe, 2019). In the current investigation *C. prostratus* displayed the strongest antibiofilm potential, whereas *L. indicum*, *E. granulata*, and *H. crispum* showed potential in varied order. (Chouhan *et al.*, 2023) also reported that *C. prostratus* possessed great antibacterial potential against selected bacterial strain. The efficiency of these Cholistani plants against MDR strains implies that these plants are the significant source of antimicrobial agents against biofilm-forming resistant pathogens (Song *et al*., 2018).

Antioxidants act as a key defense against the substantial oxidative damage caused by the reactive oxygen species in the body (Venkatachalam *et al*., 2012). Extensive studies are being performed to examine the antioxidant potential of medicinal plants. According to the present research, *E. granulata* exhibited the greatest antioxidant potential of all other selected plants investigated, with 82% RSA. Al-Robai *et al.*, (2023) conducted DPPH assay of *E. granulata* and reported that is a potent source of antioxidant compounds. *C. prostratus*, *L. indicum*, and *H. crispum* have also exhibited strong antioxidant potential.

Diabetes is a prominent cause of death and morbidity globally. Antidiabetic medicines are more effective in the early stage of diabetes and are often used in combination with other drugs. Therefore, individuals are inclined toward the usage of herbal medications for diabetes (Elbashir *et al*., 2018). In the present investigation, *C. prostratus* and *E. granulata* displayed maximum inhibitory potential but *L. indicum* and *H. crispum* were also regarded as effective (Arshad *et al*., 2021). A similar study was conducted where hydro-alcoholic extract of *Euphorbia Hirta* exhibited maximum inhibition of alpha- glucosidase enzyme (Sheliya *et al.*, 2016).

Due to unsatisfactory and restrictive therapy alternatives, there is an ever-increasing demand for antiviral drugs. Consequently, medicinal plants, with a wide range of phytochemicals, have substantial potential to solve this problem (Zitterl-Eglseer and Marschik, 2020). All of the selected Cholistani plants tested positive for antiviral activity against AIVH9N2, notably *E. granulata* and *C. prostratus*, highlighting the significance of these plants as natural sources of antiviral drugs.

Conclusion: Cholistan plants *Limeum indicum*, *Heliotropium crispum*, *Convolvulus prostratus* and *Euphorbia granulata* are a rich source of antibacterial, antibiofilm, antioxidant, antidiabetic, and antiviral compounds. Additionally, these medicinal plants have proved their worth as a low-cost and feasible alternative drug source to present medicines.

Author's Contribution: Mirza Imran Shahzad, Fatima Sadiq and Hadeeqa Habib conceived and designed the study. Muhammad Abdullah identified the selected plants. Kiran Fatima and Muneeza Mustafa executed the antibacterial activity. Haleema Saeed and Maryam Shafiq evaluated the antioxidant potential of selected Cholistani plants. α-glucosidase assay was performed by Saba Ajmal and Tamawul Noor. Antiviral activity of the selected plants was performed by Irfan Saeed and Sajid Hussain. Hina Ashraf and Fatima Sadiq checked the antibiofilm potential of selected Cholistani plants. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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