Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Antimicrobial Potential of Indigenous Medicinal Plants Against Multi Drugs Resistant Enterohemorrhagic *Escherichia coli* O157:H7 *Isolated From Canine* in Pattoki Tehsil, Punjab Province, Pakistan

Asfand Yar Khan[1], Mustofa Helmi Effendi[2], Wiwiek Tyasningsih*[3], Shakeeb Ullah[1], Imdad Ullah Khan[1], Muhammad Kamal Shah[1], Ali Zaman[1], Muhammad Muneeb[1] and Ghulam Shabbir[4]

[1]*Faculty of Veterinary and Animal Sciences, Gomal University, Dera Ismail Khan, Pakistan.*

[2]*Universitas Airlangga, Faculty of Veterinary Medicine, Division of Veterinary Public Health, Indonesia.*

[3]*Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia.*

[4]*Department of Theriogenology, Faculty of Veterinary Sciences, Pir Mehr Ali Shah Arid Agriculture University, Khushab Campus- Khushab.*

Abstract

BACKGROUND: Multiple drug resistant (MDR) Escherichia coli (*E. coli)* O157:H7 is a significant public health concern, particularly in canine puppies which are vulnerable to infections due to their developing immune system. **Aims:** This study was executed to screen the indigenous medicinal plants for their antimicrobial potential against MDR *E. coli* O157:H7 **Method:** *In-vitro* antibacterial activity of plants extracts were determined through anti-bacterial susceptibility, agar well diffusion, MIC and MBC assays. The plant with optimum antibacterial activity among said medicinal plants was selected for HPLC and *in-vivo* study (clinical symptoms, mortality, blood chemistry alterations, histopathological changes and growth performance). For *in-vivo* investigation, day old chicks (n=30) were randomly divided into three equal groups, i.e., G1; infected positive control, G2; negative control, and G3; treatment group (C. procera @ 4.3 mg/ml). **Results:** Overall, *3.75* % E. coli O157:H7 isolates showed MDR pattern exhibiting 26.6 % prevalence of *Stx-1* gene. *C. procera* showed highest ZOI (18.2 ± 1.8 mm), MIC 4.3 mg/ml and MBC values 62.2 mg/100 µl. The survival rate of birds for G3 was 80%. *C. procera* had a significant impact on birds feeding behavior and weight gain compared to control. Pathological lesions in G1 group revealed damaged cecum, and diffused congestion in liver while no histopathological changes were observed in G2 and G3 post-treatment. **Conclusion:** Methanolic extract of *C. procera* had a significant antibacterial effect and may be used as a substitute for the treatment of MDR *E. coli* O157:H7 infections.

Keywords: *E. coli* O157:H7, Multi drugs resistant, medicinal plants, *Calotropis procera*, antimicrobial activity.

Introduction

E. coli is a member of the enterobacteriacae family, a natural inhabitant of gastrointestinal tract of animals. Some strains of it like enterohemorrhagic *E. coli* (EHEC) O157:H7 is the most virulent, associated with diarrhea, causing hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [1]. E. coli O157:H7, worldwide recognize it as a zoonotic pathogen. Shiga toxins including Shiga toxin-1 and Shiga toxin-2 and hlyA are the major pathogenic factors involved with HC and HUS in Humans and Dogs [2]. Polymerase chain reaction is an accurate, sensitive and specific diagnostic assay used for bacterial identification and detection of virulent genes [3].

*Corresponding authors: Wiwiek Tyasningsih, E-mail: witya_kh@yahoo.com, Tel.: +628113443239

(Received 07 December 2024, accepted 04 March 2025)

DOI: 10.21608/EJVS.2025.342425.2543

Many scientists had reported that dogs may have carried antimicrobial-resistant *E. coli* strains with similar virulence factors to strains isolated from human beings, raising concerns about the potential for zoonotic diseases [4-7]. The emergence of MDR bacterial pathogens from dogs poses a significant threat to the health of pets and humans, allowing for the rapid spread of resistant strains [8-9].

Medicinal plants are an important aspect of traditional veterinary practices and have been a reliable source of botanicals in animals for several centuries [10]. Plant formulations are utilized in both traditional and folk medicine. The WHO estimates that 80% of populations in underdeveloped countries practice traditional medicine [11]. Medicinal plants have low antimicrobial resistance and are therefore used as alternatives to traditional medicines [12], and experimental studies have shown that medicinal plant products are the most cost-effective, biodegradable, environmentally safe, and effective natural antibiotics [13]. Several medicinal plants, including *Calotropis procera, Azadirachta indica, Melia azedarach, Withania coagulans* and *Nigella sativa* have proven potential antibacterial activity [14], these medicinal plants restrict bacterial growth by causing cytoplasmic bacterial leakage and affecting the function of the cell wall, cell membrane, and protein synthesis [15]. Despite the beneficial effects of these plant extracts, no research has been done on the antimicrobial properties of *Calotropis procera, Azadirachta indica, Melia azedarach, Withania coagulans* and *Nigella sativa* against MDR *E. coli* O157:H7 infections in canines in Pattoki tehsil, Punjab province, Pakistan. This study aims to study the prevalence of MDR E. coli serotype O157:H7 in canine bloody diarrheic puppies, their virulent Shiga-toxin detection and to evaluate the antibacterial activity of methanolic extracts of the aforementioned medicinal plants by using both in-vitro and in vivo techniques.

Material and Methods

Ethical Approval

The current study was approved by the University of Veterinary and Animal Sciences (UVAS), Lahore, Ethical Review Committee vide letter no. 949 dated 19-09-2019.

Samples collection

Four hundred (n=400) canine bloody diarrheic puppies, from different localities of (UVAS, Lahore, Ravi campus Pattoki), government hospitals and private veterinary clinics at Pattoki tehsil, Punjab province, Pakistan were screened for MDR *E. coli O157:H7,* samples were processed at the Department of Veterinary Medicine (DVM), UVAS, Lahore, Pakistan. Reference bacterial strain of *E. coli* O157:H7 *was* obtained from center of the advanced studies in vaccinology and biotechnology (CASVAB) Quetta, Baluchistan.

Identification and confirmation of E. coli O157:H7

The *E. coli* O157:H7 isolated from canine sample was initially identified based on EMB agar plates, biochemical test viz IMViC test, and CT-SMAC agar Plates but confirmation of *E. coli* O157:H7 was done through PCR. Extracted DNA was subjected to PCR by Targeting O157 Gene and H7 Gene using conditions as previously described [14].

Assessment of multi-drug resistance (MDR)

All the PCR confirmed E. coli O157:H7 isolates were tested for antibiotic sensitivity using disk diffusion method. A total of eight antibiotics were tested in five categories (Table 1.), aminoglycosides (gentamicin (GM); 10 µg, Tobramycin (TOB); 10 µg), tetracycline (tetracycline, TET, 30 µg), amide alcohol (chloramphenicol, C, 30 µg), quinolones (ciprofloxacin, CIP, 5 µg), and β-lactams (ampicillin, AMP, 10 µg; cefazolin, cefotaxime, 30 µg). The data were analyzed by using CLSI criteria (CLSI, 2023). The strain resistant to more than 3 antibiotics were classified as MDR strain.

Antibacterial activity of herbal extracts against MDR E. coli O157:H7

Five plants (*Azadirachta indica (AI), Melia azedarach (MA) and Calotropis procera (CP), Nigella sativa (NS) and Withania coagulans* (WC) were collected. The seeds of NS, fruits of WC and leaves of AI, MA and CP were grinded to finely powdered form. Methanolic extracts (400 mg/ml) were prepared using the Soxhlet extraction method using methanol as the solvent. The extracts were then concentrated using a rotary evaporator. The extracted yield was measured and was preserved until of its use as described previously by [14].

The antimicrobial activity of the medicinal plants was evaluated using the well diffusion assay on Nutrient Agar plates, following previously established protocols by [16]. Proportionally, growing indicator bacteria were suspended in phosphate-buffered saline (PBS) to achieve a 0.5 McFarland standard. A volume of 0.5 ml of the pure culture was consistently swabbed onto sterile Nutrient Agar plates using sterile cotton swabs to form a confluent lawn of growth. Four wells of optimal size were aseptically created on the

inoculated plates, sealed, and filled with 100 µl of each medicinal plant extract (400 mg/ml). The plates were incubated at 37°C for 24 hours, and the activity against multidrug-resistant *E. coli* O157:H7 was evaluated by measuring the zones of growth inhibition around the wells.

The minimum inhibitory concentration (MIC) of the plant extracts was determined using the microbroth dilution method in 96-well microtiter plates, as described previously by [17]. A standardized bacterial inoculum was arranged, and two-fold serial dilutions of the plant extracts were performed in Mueller-Hinton Broth. Varying concentrations of the test agents were added to each well. Following incubation at 37°C for 24 hours, the optical density at 620 nm wavelenght was measured using an enzyme-linked immunosorbent assay (ELISA) reader.

MBC was measured through seeding 100µl sample from each Minimum inhibitory concentration test tube with a growth inhibitor, seeding on newly prepared Muller Hinton agar plate in aerobic condition for incubating at 37°C for 24 hours. Minimum bactericidal concentration data was recorded as the lowest concentrations of extracts that prevented the development of visible bacterial colonies on agar plates. The experiments were repeated three times.

High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) was used to evaluate phenolic acids, including gallic acid, sinapic acid, and caffeic acid, as well as flavanols such as myricetin, quercetin, and kaempferol, in the methanolic extract of *Calotropis procera*. Silica gel column chromatography was practiced to fractionate the most potent *C. procera* extract using solvents with increasing polarity. The analysis of phenolic acids and flavanols was regulated using an HPLC system (LC-110A) equipped with a CTO-10A column oven, an SPD-10A UV-Vis detector, and LC-10A data acquisition software, following a previously described method of [18].

Experimental design

MDR *E. coli* O157:H7 was cultured 24hr at 37 °C in lysogeny broth medium. Normal saline was added to LB culture to reach a concentration of 9×10^9 cells/ml (ID50). The therapeutic efficacy of *C. procera* was determined by rearing 30 (unvaccinated) 1-day old broiler chicks buy from commercial hatchery. The chicks were given a balanced commercial feed (21% and 18% CP respectively) until the end of the trial, as well as clean water was provided. The birds were reared in an open housing setup with strict hygiene standards. Chicks were divided into three groups, Group 1 was kept as a infected positive control, Group 2 was a non-infective negative control and G3 was a treatment group. G1 and G3 were maintained in one shed whereas birds from G2 (non- infected) were kept in separate shed with comparable housing and environmental circumstances. All chicks except of G2, received 1 ml of infected dose orally for 7 days after birth. Group 1 (infected but not treated) *E. coli* inoculated but treatment not given during trial period. G2 (negative control) received placebo only. Group1 was treated with *C. procera* at a concentration of 4.3 mg/ml,1ml orally for 5 days just 24 hours after the infection with MDR *E. coliO157:H7*for 5 days. Chicks were monitored daily for up to 21 days for clinical symptoms, mortality, blood chemistry alterations, and histopathological changes, and growth performance was assessed until day 22 when the experiment ended.

Haemato-biochemical parameters

The liver enzymes ALT (alanine transaminase), ALP (alkaline phosphatase), and AST aspartate transaminase were measured with commercially available kits. The Nut and Herrick technique was used for hematologic parameters such as red blood cells (RBC), the Drabkins method was used for hemoglobin determination [19-20].

Histopathological examination

Histopathology was used to assess the antimicrobial effects of C. *procera* on MDR E. coli O157: H7 in broiler chicks. After the end of experiment, remaining birds were sacrificed and the organs (liver and intestines) were collected in jars containing 10% buffered formalin and allowed a week to harden and were processed for histopathological investigation according to standard protocols.

Statistical analysis

SPSS version 22 was used for data analyzing. The data of Minimum inhibitory concentration, Minimum bactericidal concentration, bird FCR, LFT, and whole blood chemistry was analyzed by using a One-way analysis of variance (ANOVA).

Results

An overall 4.5 % (18/400) isolates we have isolated of *E. coli* O157:H7 from bloody diarrheic canine puppies through PCR from different localities of Pattoki tehsil, Punjab province, Pakistan, in them fifteen isolates 3.75 % (15/400) were shown to exhibit an MDR pattern. The isolates showed green metallic sheen colonies on EMB plates, smooth colorless colonies on CT-SMAC plates and PCR exhibited corrected band size of 259 and 625 bp of *rfb*O157 and *flic*H7 genes. PCR revealed that the most predominant virulence gene found in MDR. *E. coliO157:H7* isolates was Shiga toxin 1, The prevalence of the Shiga toxin 1 gene in the current study was 26.6 % (4/15).

Among five antibiotics category β lactam antibiotics exhibited the greatest resistance rate (86.6%, 13/15), followed by aminoglycosides (66.66%, 11/15), tetracyclines (80%, 9/15), and amide alcohols (53.3%, 8/15). Resistance to quinolone antibiotics was the lowest (40%, 6/15). Furthermore, the AMP (66.66%, 10/15), and tetracycline (80%, 12/15), gentamicin (60%, 09/15), had the highest resistance rates in eight antimicrobial agents. Ciprofloxacin had the lowest resistance rate (40 percent, 6 of 15). Of the 15 MDR strains, the most popular patterns included AMP/CFT/GM/TET, AMP/CFZ/CFT/TOB/C,and AMP/CFT/TOB/TET/C/CIP.

In order to observe the antimicrobial potential of Five plants against fifteen MDR *E. coli* O157:H7 isolates initially various concentrations of extracts, ranging from 100mg/ml to 400mg/ml were tried out of which (400mg/ml) was selected for further testing as at this concentration all extracts showed bacterial inhibition by MIC as well as MBC. In terms of medicinal plants, the results fall in a sequence of Calotropis procera> *Azadirachta indica*> *Melia azedarach*> *Withiania Coagulans*> *Nigella sativa.* However, the Methanolic extracts showed the highest antibacterial activity at 400 mg/well concentration, their maximum inhibitory zone for *Calotropis procera, Azadirachta indica, Melia azedarach, Withiania Coagulans, Nigella sativa was* 18.2 ± 1.8, 15.1 ± 2.7, 13.5 ± 1.8, 10.4 ± 1.6, 9.9 ± 1.4 respectively. Results of the current study found that the *Calotropis procera had a greater* MIC value against MDR *E. coli* O157:H7 *as compared to other medicinal plants and were* 4.3 mg/ml, followed by *M. azedarach* was (5.8 mg/ml) concentration, *A. indica* extract was found to be 6.3 mg/mL. *W. coagulans* was 7.5 mg/ml and *N. sativa* was 7.5 mg/ml as shown in (Table 1).

Due to the highest results of *C. procera* as compared to other medicinal plants we have decided to perform HPLC of this particular plant to check various phenolic acid and flavonoids compound in it. In terms of phenolic acid The highest retention time of *Calotropis procera* was of Caffeic acid (8.247) followed by Synapic acid (6.780) while the lowest retention time was observed for Gallic acid (3.637) the percent area was highest in Gallic acid (85.4420) followed by Caffeic acid (13.8606) and Synapic acid (0.6974). while for flavonoids The highest retention time for Calotropis procera was of kaempferol (16.107) followed by Quercetin (9.753) while the lowest was seen for Myricetin (6.427), the percent area was highest in Myrecitin (83.5060) followed by Querecitin (9.5674) while the lowest percent area was seen in Kaempferol (6.9265).

The ability of methanolic extract of *C. procera* was evaluated to protect the broilers challenged with an infective dose of MDR *E. coli* O157:H7 infection. In G3 (control positive) hundred percent of the birds died after 18hr of MDR *E. coli* O157:H7 inoculation. On the contrary, in G2 (control negative) had the 100 % of the birds survived until the end of the experiment. G1 birds administered with *C. procera dose* (@ 4.3 mg/ml) after the experimental infection with MDR *E. coli* O157:H7 showed the survival rate of 80 %. Feeding behavior of this study shows, that the *C. procera* treated group improved the body weight of their birds when compared it to the control group.

The result of present study shows that birds administered with *C. procera* did not show significant differences ($p>0.05$) for ALT, ALP, AST, RBC, and Hb as shown in (Table 3)

Severe pathological lesions were reported in our study. G2 tissues were normal. While in G1 and G3 after experimental infection Integrity of glands and several parts of the caecum is affected, horse shaped monocytes and lymphocytes were seen in cecum, thickening of villi disrupted in the apical surface of intestines, diffused congestion and hepatitis were seen in the liver.

Discussion

In our study we have detected 4.5 % (18/400) isolates of *E. coli* O157:H7 from bloody diarrheic canine puppies through PCR from different localities of Pattoki tehsil, Punjab province, Pakistan [1]. A

total of fifteen isolates 3.75 % (15/400) of MDR were isolated from them. The isolates recovered from diarrheic canine puppies showed green metallic sheen colonies on EMB plates, smooth colorless colonies on CT-SMAC plates and PCR exhibited corrected band size of 259 and 625 bp of *rfb*O157 and *flic*H7 genes like the studies conducted on *E. coli* O157:H7 from canines [2,21].

The prevalence of the Shiga toxin 1 gene in the current study was 26.6 % (4/15), which was similar to the prevalence (43.7%) reported by [22]. Additionally, a low prevalence (1.98%) of the Shiga toxin-1 gene was demonstrated by [23]. The virulency of *E. coli* O157:H7 directly attributed to virulence factor (Stx-1 and Stx-2 genes), which destroy eukaryotic ribosomes and inhibit protein production in host tissues [24]. One reason for this change is that MDR *E. coli* O157:H7 is significantly more prevalent in stray dogs than in domestic dogs.

MDR *E. coli* O157:H7 with a broad range of antimicrobial resistance were detected in our study. In the current study, 15/18, 83.3 % of representative isolates were resistant to more than three classes of antimicrobials. Our results are higher than the previous study, which showed the detection rate of multidrug-resistant strains among E. coli isolates from hemorrhagic dogs was 58 % by [25] and 40.96% by [26]. While in-line with the study of [27], showed 72.7 % prevalence. The reduction in the detection rate of MDR strains might be attributed to the implementation of several policies by the relevant authorities in the country aimed at curbing antimicrobial resistance in animal-derived bacteria. However, resistant strains may have already been circulating in the dog population before the restrictions on antibiotic usage were enforced by the authorities, making the elimination of AMR strains challenging. The high detection rates of MDR *E. coli* O157:H7 observed in our study highlight the requirement of more viable techniques to control its occurrence and spread from companion animals. These findings emphasize the significance of strict supervision over antimicrobial usage in veterinary clinics to prevent the proliferation of pet-derived MDR bacteria.

The lowest concentration of the antibacterial agent required to inhibit pathogen growth is known as the MIC. High MIC values suggest poor antibacterial efficacy [28]. Our findings illustrated that methanol is an effective solvent for extraction. The antibacterial activity observed in our study showed improved results compared to a previous study of [29] on the MIC of methanolic extracts of C. procera leaves against E. coli. Current study found that the MIC value for MDR *E. coliO157:H7*was 4.3 mg/ml, while few previous studies reported MIC values 13.5 mg/ml and 28 mg/ml against *E. coli* [29]. Our findings were agreeing with the previous study who said that *C. procera* had a strong antibacterial activity against E. coli. The difference found in the results of this study might be attributed to a combination of factors, including climate, geography, circumstances, and the nature of the solvent employed for extraction [30]. The variance in outcomes is also determined by the type of extracted material. The current investigation found that the MIC activity of *M. azedarach* was (5.8 mg/ml) concentration. The results of this investigation contradict previous reports of [31] and [32], who reported 47.8 mg/ml and 32.5 mg/ml minimum inhibitory concentration for *M. azaderach* against *E. coli*. Our results are similar with previous reports, showing that methanolic extracts had stronger antibacterial activity than other extracts [33-34]. The difference in MIC values might be attributed to the difference in chemical composition between organs of plant. The MIC of *A. indica* extract against MDR *E. coliO157:H7*was found to be 6.3 mg/mL. In investigation done by [35], the MICs for aqueous extract was 10 mg/ml and 20 mg/ml for ethanolic extracts. However, a study by [36] observed that the MIC of *A. indica* methanolic extract against *E. coli* was found to be 83.3 ± 29.0 mg/mL. According to [37], *A. indica* petroleum ether extract has a MIC of 100 µg/mL against *E.coli*. This suggests that the polarity of the extraction solvent has a significant impact on *A. indica* activity. Our results for MIC for *W. coagulans* was 7.5 mg/ml were supported by [29,38] that it has a antimicrobial property, but exhibited moderate activity against E. coli [39]. The reason for this difference might be due to the Plant organs may have different chemical compositions, resulting in varying MIC values, which support our stud. A few earlier researchers [40-41] investigate that *N. sativa* seeds have good antibacterial activity in in-vitro studies, which is in consistent with our results. However, our findings do not agree with those of [42], who found that a methanol extract of *N. Sativa* was ineffective against *E. coli*. This variation might be attributed to differences in bacterial strain properties as well as solvent extraction methods.

The presence of a significant number of phytocompounds in this study highlights methanol's

effectiveness as a solvent and proved to be a better phytocompound for extracting phenolic and flavonoid compounds from the leaves of C. procera. Our findings align with those of [43], which illustrated the strong antibacterial activity of C. procera leaf extracts against E. coli. Additionally, the results of this study are consistent with prior research of [44] that reported high phenolic substances in the methanolic extracts of C. procera leaves. [45] observed that the methanolic leaf extracts of *Calotropis procera* were rich in phenolic and flavonoid content in terms of gallic acid and quercetin respectively. While a similar finding was observed in our study we had found Gallic acid, Synapic acid, Caffeic acid as a phenolic compound, and Myricetin, Quercetin, and Kaempferol as a flavonoid from the methanolic extracts of leaves of *C. procera. C. procera* can be used as an alternative medicine in the case of drug- resistant pathogens.

Feeding behavior of this study shows that the *C. procera* treated group improved the body weight of their birds when compared to control group. Authors of the present study agreed with the findings documented by [46] who demonstrated that leaves extract of *C. procera* had the higher level of carbohydrate, ash and protein.

The findings of this study uncover that the administration of *C. procera* to birds did not result in significant changes ($p>0.05$) in alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), red blood cell (RBC) count, or hemoglobin (Hb) levels. These results are in contrast with an earlier study of [47], which reported adverse effects of *C. procera* leaves on albino rats.

Severe pathological lesions were reported in our study. G2 tissues were normal. While in G1 and G3 after experimental infection Integrity of glands and several parts of the caecum is affected, horse shaped monocytes and lymphocytes were seen in cecum, thickening of villi disrupted in the apical surface of intestines, diffused congestion and hepatitis were seen in the liver. Our findings on infected liver birds are consistent with those of [48] and [47], who concluded that the liver may absorb high concentrations of Shiga toxins that pass via hepatic portal system. In this study results of Histopathological examination were consistent with those of [1], who demonstrated the intestinal inflammatory response can be seen to be caused by bacteria that survive in the intestinal environment and colonize the gut.

Conclusion

In conclusion, the methanolic extract of *C. procera* illustrated promising antimicrobial activity against multidrug-resistant (MDR) *E. coli* O157:H7 compared to other medicinal plants tested (*A. indica*, *M. azedarach*, *W. coagulans*, and *N. sativa*). However, due to the misuse of antibiotics, MDR *E. coli* O157:H7 strains have developed resistance against numerous antibacterial agents. These discoveries suggest that *C. procera* holds potential as a candidate for the development and improvement of novel drugs to treat MDR *E. coli* O157:H7 infections. In the future, as more and more attention are being paid to the spread of AMR in dogs, further studies may require to know the mechanism of action, side effects, and safety indicators of these five plants before the development and application of plant-based drugs against MDR E. coliO157:H7infections.

Acknowledgments

The Authors acknowledge the Professor. Dr. Muhammad Avais for helping in conducting the study and permission of work in their molecular diagnostic laboratory, the authors are also grateful to the staff of department of veterinary medicine, UVAS, Lahore.

Conflict of interest

The author declares that they have no conflict of interest in this work.

Funding statement

This study didn't receive any funding support.

TABLE 1. Antimicrobial resistance pattern of MDR *E. coli* O157:H7 (n=15)

Category of antimicrobial	No of resistant isolates (%)	Antibiotics		No of resistant isolates (%)
B-lactams	13 (86.6)	Penicillin	Ampicillins (Amp)	10 (66.66)
		Ist /2nd generation cephalosporin	Cefazolin (CFZ)	8 (53.33)
		3rd/4th generation cephalosporin	Cefotaxime (CFT)	7 (46.66)
Aminoglycosides	11 (66.66)	Gentamycin (GM)		9 (60)
		Tobramycin (TOB)		7 (46.66)
Tetracyclines	12 (80)	Tetracycline (TET)		12 (80)
Amide alcohols	8 (53.3)	Chloramphenicol (C)		8 (53.3)
Quinolone	6 (40)	Ciprofloxacin (CIP)		6 (40)

TABLE 2. The Scientific names of the species along with their families and other relevant information

Botanical name/variety	Family name	Common name	Part used	Voucher No.
Azadirachta indica	Meliaceae	Neem	Leaves	GC. Herb. Bot. 3668
Melia azedarach	**Meliaceae**	Bakain	Leaves	GC. Herb. Bot. 3669
Calotropis procera	Apocynaceae	Aak	Leaves	GC. Herb. Bot. 3672
Nigela Sativa	Ranunculaceae	Kalonji	Seeds	GC. Herb. Bot. 3670
Withania coagulans	**Solanaceae**	Paneer bootie	Seeds	GC. Herb. Bot. 3671

TABLE 3. *In vitro* antimicrobial activity of methanolic extracts of Five medicinal plants determined by agar well diffusion assay, minimum inhibitory concentration and minimum bacterial concentration against MDR *E. coliO157:H7.*

Plants	Strain	MIC	MBC
A. indica	MDR E. coli O157:H7	6.3^{a}	89.7^{b}
M. azedarach	MDR E. coli O157:H7	5.8^{a}	72.2^{b}
W. coagulans	MDR E. coli O157:H7	7.5^{a}	93.5^{b}
N. sativa	MDR E. coli O157:H7	10^{a}	10.5^{b}
C. procera	MDR E. coli O157:H7	4.3^{a}	62.2^{b}

Common letters (a-a, b-b) showed non-significant differences at P>0.05, whereas Different letters (a-b) within a column showed significant differences when P<0.05.

TABLE 4. Comparison of growth performance of healthy and treated birds P value (p>0.05)

	Initial body weight	Final body weight	Weight gain	Feed intake	FCR
Group 2	37.03 ± 1.21	670.01 ±1.31	490.12 ± 2.02	910.11 ± 32.13	1.73 ± 0.01
Group 3	37.01 ± 1.00	603.16 ±1.04	430.32 ± 1.01	860.11 ± 11.00	1.61 ± 0.05
p-value	0.5414	<0.05	<0.04	0.02	0.01

TABLE 5. Blood and liver function profile of healthy and treatment group P value (p>0.05).

	G3 Before Treatment	G3 After Treatment	p-value
RBCs	2.98 ± 2.20	4.20 ± 1.17	0.1
Hb	7.61 ± 0.02	11.51 ± 1.20	0.21
ALP	12.05 ± 1.01	18.60 ± 2.00	0.09
AST	8 .61 ± 2.20	10.61 ± 1.95	0.3
ALT	49.20 ± 8.01	58.09 ± 6.53	0.06

*RBC, red blood cell; Hb, hemoglobin; ALP, alkaline phosphatase; AST, asparate aminotransferase; ALT, alamine aminotransferase.

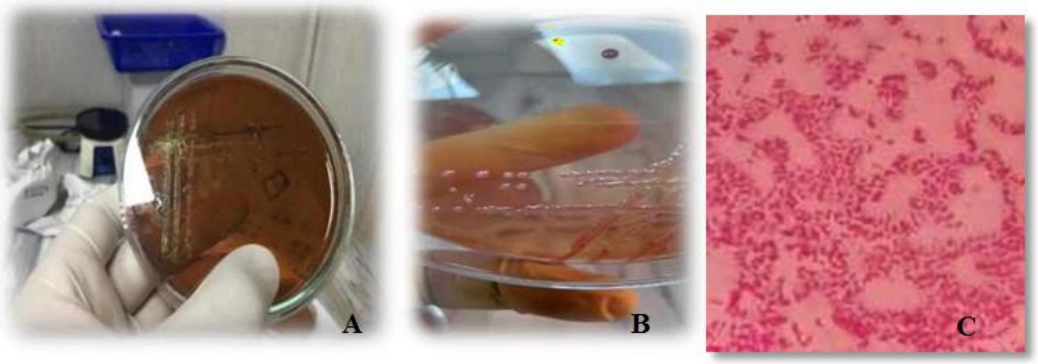

Fig. 1. A: Metallic sheen colonies of *E. coli* on EMB Agar B: Showing Smooth Colorless colonies of *E. coli O157:H7 on* (CT-SMAC) agar and C: Shows a Rod-shaped coco-bacilli pink in color representing the presence of *E. coli* under microscopic examination.

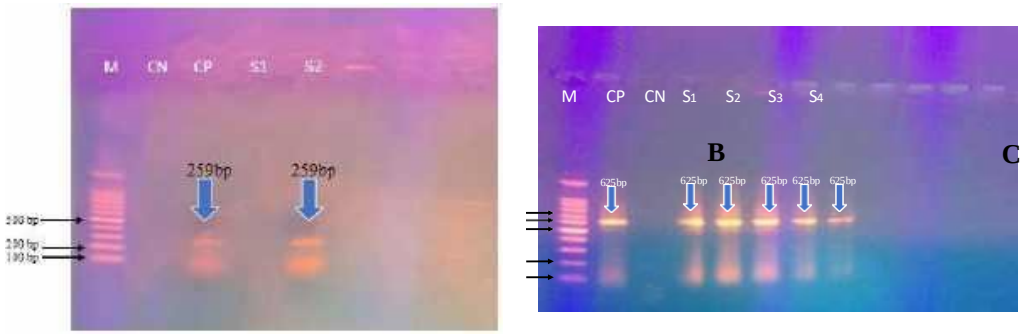

Fig. 2. The bands for genes O157 and H7 were visualized (M stand for DNA marker, CP for control positive, CN for control negative, and S1 and S2 for samples).

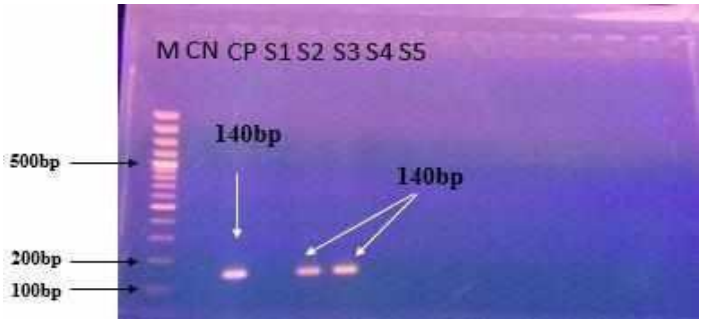

Fig. 3. The bands for Shiga toxin-1 gene was Visualized (M stand for DNA marker, CP for control positive, CN for control negative, and S1 and S2 for samples).

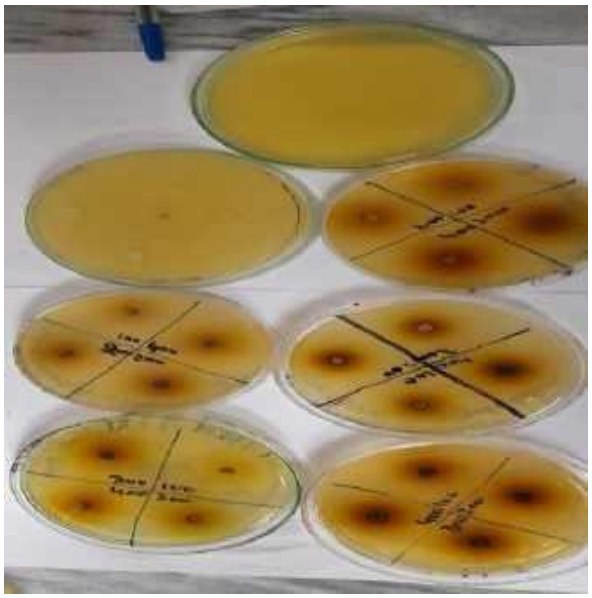

Fig. 4. Results of methanolic extracts of *Calotropis procera,* with control positive and negative plates

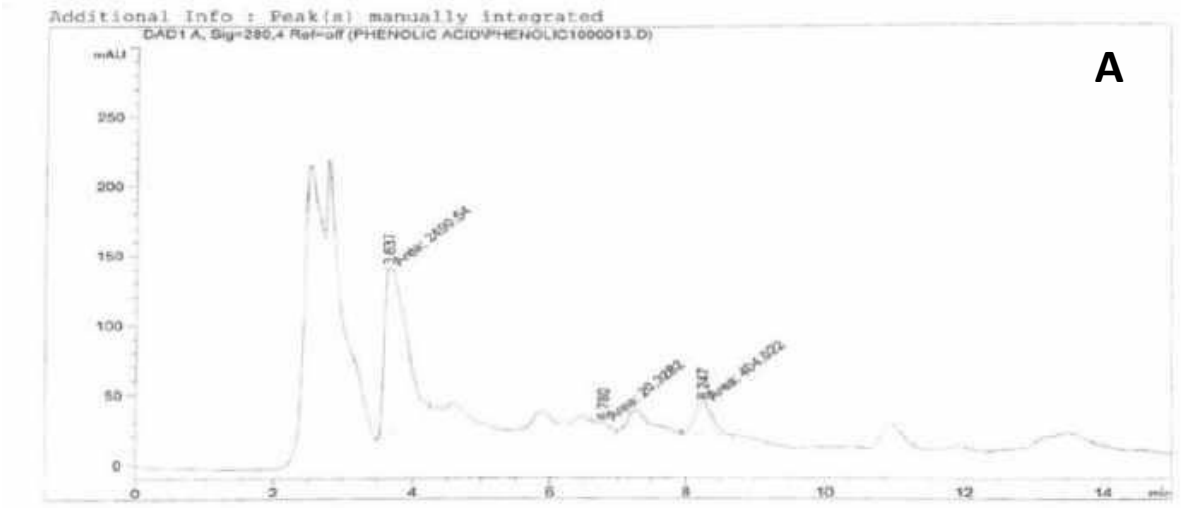

Fig. 5. Graph A shows phenolic contents in *C. procera* through HPLC

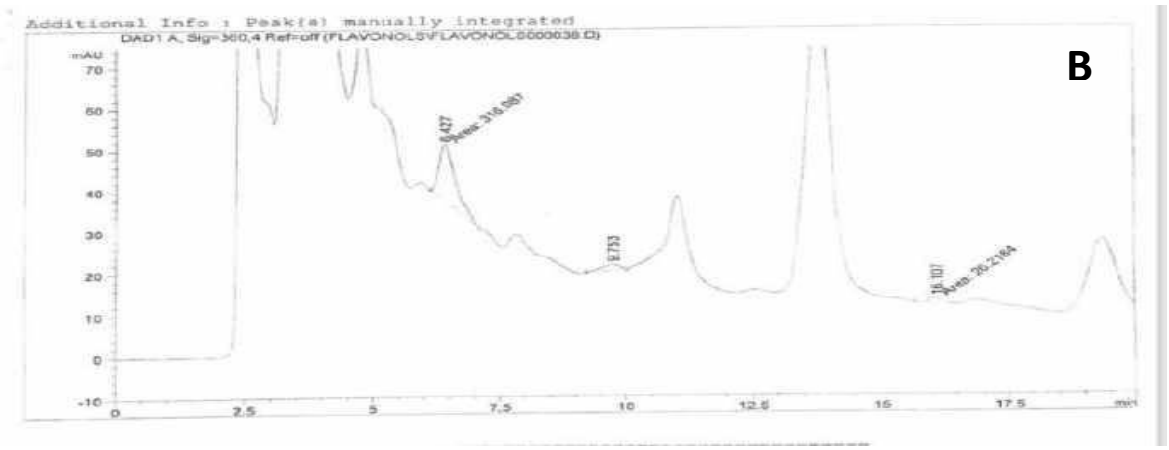

Fig. 6. Graph B represents flavanols contents in *C. procera* through HPL

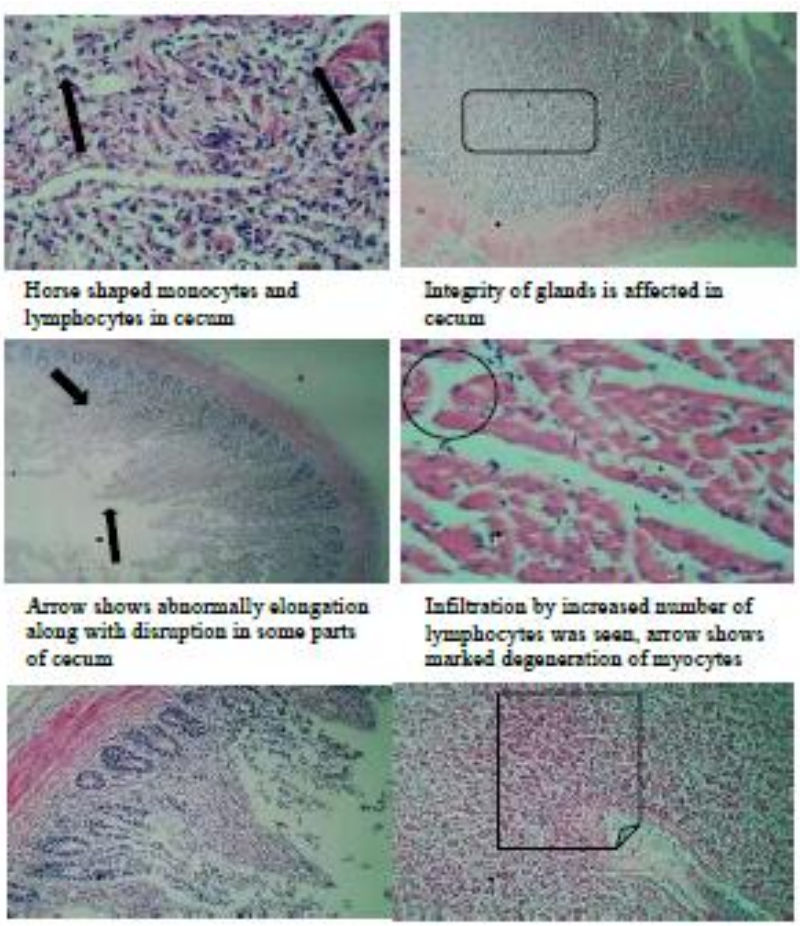

Fig. 7. Different slides shows pathological changes in G2 (Positive control) and G3 (Treatment group)

References

1. Khan, A.Y., Ahmad, S.S., Avais, M., and Ashraf, K. Molecular prevalence with associated risk factors and haemato-serum electrolyte analysis of *E. coli* O157: H7 in canine pups with diarrhea. *Pakistan Veterinary Journal*, **42**, 161-166 (2022). DOI: 10.29261/pakvetj/2022.009
2. Abdulrazzaq, K.M., Owain, M.S., Majeed, H.M., and Al-Hyani, O.H.H. Molecular detection of rfbO157, shiga toxins, and hemolysin genes for *Escherichia coli* O157: H7 from canine feces in Tikrit and Mosul cities, Iraq. *Iraqi Journal of Veterinary Sciences*, **35**, 325-329 (2021). DOI: 10.33899/ijvs.2020.126831.1392
3. Algammal, A.M., El-Tarabili, R.M., Alfifi, K.J., Al-Otaibi, A.S., Hashem, M.E.A., El-Maghraby, M.M., and Mahmoud, A.E. Virulence determinant and antimicrobial resistance traits of emerging MDR shiga toxigenic *E. coli* in diarrheic dogs. *AMB Express*, **12**, 34 (2021). DOI: 10.1186/s13568-022-01371-4
4. Kataoka, Y., Irie, Y., Sawada, T., and Nakazawa, M. A 3-year epidemiological surveillance of *Escherichia coli* O157: H7 in dogs and cats in Japan. *Journal of Veterinary Medical Science*, **72**, 791-794 (2010).
5. Bentancor, A., Rumi, M.V., Carbonari, C., Gerhardt, E., Larzabal, M., Vilte, D.A., Pistone-Creydt, V., Chinen, I., Ibarra, C., Cataldi, A., and Mercado, E.C. Profile of Shiga toxin-producing *Escherichia coli* strains isolated from dogs and cats and genetic relationships with isolates from cattle, meat, and humans. *Veterinary Microbiology*, **156**, 336-342 (2012). DOI: 10.1016/j.vetmic.2011.10.030
6. Wedley, A.L., Dawson, S., Maddox, T.W., Coyne, K.P., Pinchbeck, G.L., Clegg, P., Nuttall, T., Kirchner, M., and Williams, N.J. Carriage of antimicrobial-resistant *Escherichia coli* in dogs: Prevalence, associated risk factors, and molecular characteristics. *Veterinary Microbiology*, **199**, 23-30 (2017). DOI: 10.1016/j.vetmic.2016.11.017
7. Abad-Fau, A., Sevilla, E., Oro, A., Martín-Burriel, I., Moreno, B., Morales, M., and Bolea, R. Multidrug resistance in pathogenic *Escherichia coli* isolates from urinary tract infections in dogs, Spain. *Frontiers in Veterinary Science*, **11**, 1325072 (2024). DOI: 10.3389/fvets.2024.1325072
8. Vega-Manriquez, X.D., Ubiarco-López, A., Verdugo-Rodríguez, A., Hernández-Chiñas, U., Navarro-Ocaña, A., Ahumada-Cota, R.E., Ramírez-Badillo, D., Hernández-Díaz de León, N., and Eslava, C. Pet dogs' potential transmitters of pathogenic *Escherichia coli* with resistance to antimicrobials. *Archives of Microbiology*, **202**, 1173-1179 (2020). DOI: 10.1007/s00203-020-01828-9

9. Dazio, V., Nigg, A., Schmidt, J.S., Brilhante, M., Campos-Madueno, E.I., Mauri, N., Kuster, S.P., Brawand, S.G., Willi, B., Endimiani, A., and Perreten, V. Duration of carriage of multidrug-resistant bacteria in dogs and cats in veterinary care and co-carriage with their owners. *One Health*, **13**, 100322 (2021). DOI: 10.1016/j.onehlt.2021.100322

10. Sipahi, N., Kekeç, A.I., and Halaç, B. *In vitro* effect of some essential oils against multiple antibiotic-resistant bacteria from cats and dogs. *Pakistan Veterinary Journal*, **42**, 561-565 (2022). DOI: 10.29261/pakvetj/2022.055

11. Lee, C., Kim, S.Y., Eum, S., Paik, J.H., Bach, T.T., Darshetkar, A.M., Choudhary, R.K., Quang, B.H., Thanh, N.T., and Choi, S. Ethnobotanical study on medicinal plants used by local Van Kieu ethnic people of Bac Huong Hoa Nature Reserve, Vietnam. *Journal of Ethnopharmacology*, **231**, 283-294 (2019). DOI: 10.1016/j.jep.2018.11.006

12. Owolabi, A.O., Abah, K.A., and Oranusi, S.U. *In vitro* antimicrobial and antioxidant activity of *Carica papaya* and *Azadirachta indica* leaf and stem bark extracts on selected clinical isolates. *Indian Journal of Science and Technology*, **6**, 209-220 (2017).

13. Qureshi, S.A., Jahan, M., Lateef, T., Ahmed, D., Rais, S., and Azmi, M.B. Presence of gallic acid and rutin improves the hepatoprotective strength of *Withania coagulans*. *Pakistan Journal of Pharmaceutical Sciences*, **32**, 301-308 (2019).

14. Khan, A.Y., Ahmad, S.S., Avais, M., and Ashraf, K. *In-vitro* and *in-vivo* antimicrobial activity of five medicinal plants against virulent *Escherichia coli* O157: H7 strain harboring shiga toxin gene. *Pakistan Journal of Zoology*, **1-10** (2023). DOI: 10.17582/journal.pjz/20221009171005

15. Kebede, T., Gadisa, E., and Tufa, A. Antimicrobial activities evaluation and phytochemical screening of some selected medicinal plants: A possible alternative in the treatment of multidrug-resistant microbes. *PLoS One*, **16**, e0249253 (2021). DOI: 10.1371/journal.pone.0249253

16. Mohamed, H.G., Gaafar, A.M., and Soliman, A.S. Antimicrobial activities of essential oil of eight plant species from different families against some pathogenic microorganisms. *Research Journal of Microbiology*, **11**(1): 28 (2016).

17. Fon, E.F., Oko, A.O., Chia, P.N., and Yongabi, K.A. Efficacy of plasma micro broth dilution assay for antifungal susceptibility testing of *Candida albicans*. *African Journal of Microbiology Research*, **12**(31), 741-746 (2018.

18. Ullah, A., Anjum, A.A., Rabbani, M., Nawaz, M., Ashraf, M., Ijaz, M., Ali, A., Rashid, A., Najeeb, I., and Pervez, A. Phytochemical composition and *in vitro* activity of ethanolic extract of *Eucalyptus globulus* leaves against multidrug-resistant poultry pathogens. *Cellular and Molecular Biology*, **67**, 159-164 (2021). DOI: 10.14715/cmb/2021.67.1.24

19. Natt, M.P., and Herrick, C.A. A new blood diluent for counting erythrocytes and leucocytes of the chicken. *Poultry Science*, **31**, 735-738 (1952). DOI: 10.3382/ps.0310735

20. Balasubramaniam, P., and Malathi, A. Comparative study of hemoglobin estimated by Drabkin's and Sahli's methods. *Journal of Postgraduate Medicine*, **38**, 8 (1992). DOI: 10.4103/1858-5000.157502

21. Gannon, V., D'Souza, S., Graham, T., King, R., Rahn, K., and Read, S. Use of the Flagellar H7 Gene as a Target in Multiplex PCR Assays and Improved Specificity in Identification of Enterohemorrhagic *Escherichia coli* Strains. *Journal of Clinical Microbiology*, **35**, 656 (1997).

22. Okwu, M.U., Imade, O.S., Akpoka, O.A., Olley, M., Izevbuwa, O.E., and Usman, N.A. Comparative evaluation of the antibacterial effects of *Moringa oleifera* and *Nigella sativa* on some clinical bacterial isolates obtained from Igbinedion University, Okada, Nigeria. *Biyoloji Bilimleri Araştırma Dergisi*, **13**, 20-27 (2020).

23. Vračar, V., Potkonjak, A., Kosić, L.S., Lalošević, V., and Kozoderović, G. First molecular detection of shiga toxin-producing *Escherichia coli* in dogs from Serbia: A potential threat to human health? *Veterinaria Italiana*, **59**, 1-12 (2023). DOI: 10.12834/VetIt.2569.18469.2

24. Abd El-Baky, R.M., Ibrahim, R.A., Mohamed, D.S., Ahmed, E.F., and Hashem, Z.S. Prevalence of virulence genes and their association with antimicrobial resistance among pathogenic *Escherichia coli* isolated from Egyptian patients with different clinical infections. *Infection and Drug Resistance*, **13**, 1221-1236 (2020). DOI: 10.2147/IDR.S241073

25. Paula, C.J.S.D. and Marin, J.M. Multidrug-resistant Shiga toxin-producing *Escherichia coli* in dogs with diarrhea. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, **61**: 511-514 (2009).

26. Teng, L., Feng, M., Liao, S., Zheng, Z., Jia, C., Zhou, X., Nambiar, R.B., Ma, Z. and Yue, M. A cross-sectional study of companion animal-derived multidrug-resistant *Escherichia coli* in Hangzhou, China. *Microbiology Spectrum*, **11**(2), e02113-22 (2023).

27. Ojo, O.E., Bello, A.O., Amosun, E.A. and Ajadi, R.A. Multidrug-resistant verocytotoxin-producing *Escherichia coli* O157:H7 in the feces of diarrheic and non-diarrheic dogs in Abeokuta, Nigeria. *Veterinarski Arhiv*, **84**(1), 63-73 (2014).

28. Marchetti, L., Buldain, D., Gortari Castillo, L., Buchamer, A., Chirino-Trejo, M., and Mestorino, N. Pet and stray dogs as reservoirs of antimicrobial-resistant *Escherichia coli*. *International Journal of Microbiology*, **2021**, 6664557 (2021). DOI: 10.1155/2021/6664557

29. Bilal, H., Ali, I., Uddin, S., Khan, I., Said, A., Rahman, M.U., Khan, A.M., Shah, A.B., and Khan, A.A. Biological evaluation of antimicrobial activity of *Calotropis procera* against a range of bacteria. *Journal of Pharmacognosy and Phytochemistry*, **9**, 31-35 (2020).

30. Ullah, I., Rizwan, M., Afridi, A.J., Ullah, I., Shah, S., and Afridi, P. Antimicrobial activity of *Calotropis procera* against pathogenic microorganisms. *The Sciencetech*, **5**, 122-130 (2024).

31. Kwon, T., and Cho, S.H. Draft genome sequence of Enterohemorrhagic *Escherichia coli* O157 NCCP15739, isolate in the Republic of Korea. *Genome Announcements*, **3**, 1-2 (2015). DOI: 10.1128/genomeA.00522-15

32. Touzout, S.N., Merghni, A., Laouani, A., Boukhibar, H., Alenazy, R., Alobaid, A., Alenazy, M., Ben-Attia, M., Saguem, K., and El-Bok, S. Antibacterial properties of methanolic leaf extracts of *Melia azedarach* L. against gram-positive and gram-negative pathogenic bacteria. *Microorganisms*, **11**, 2062 (2023). DOI: 10.3390/microorganisms11082062

33. Bakht, J., Bukhari, A., Iqbal, A., and Shafi, M. In vitro antimicrobial potential of samples from the leaves and fruits of medicinally important *Melia azedarach*: Antimicrobial activity of *Melia azedarach*. *Biological Sciences - Pakistan Journal of Scientific and Industrial Research*, **64**, 109-115 (2021).

34. Vidhyasri, G., and Prasanna, G. Phytochemical screening, *in vitro* antioxidant, antimicrobial, and anti-inflammatory activities of aqueous extract of *Melia azedarach*. *International Journal of Research*, **11**, 22-29 (2020).

35. Nigussie, D., Davey, G., Legesse, B.A., Fekadu, A., and Makonnen, E. Antibacterial activity of methanol extracts of the leaves of three medicinal plants against selected bacteria isolated from wounds of lymphoedema patients. *BMC Complementary Medicine and Therapies*, **21**, 1-10 (2021). DOI: 10.1186/s12906-020-03183-0

36. Salman, A. H., & Shwaish , M. M. Antibacterial activity of ethanolic extract of (Ammi visnaga) seeds against some types of bacteria. *Plant Science Today*, *11*(3). (2024). https://doi.org/10.14719/pst.4037

37. Shu'aibu, I., Hamman, J., Goje, L., Mu'inat, A., Jauro, H., and Kabiru, M. Phytochemical analysis and evaluation of bacteriostatic effect of neem (*Azadirachta indica*) leaves on some clinical bacterial isolates. *Journal of Harmonized Research in Applied Sciences*, **3**, 152-157 (2015).

38. Sareen, A., Sultana, S., Akbar, S., Dilshad, R., Naz, G., Akhtar, N., Mehboob, M.A., Tahir, H., and Nisar, R. *In vitro* evaluation of *Withania coagulans* for its thrombolytic, anti-oxidant, anti-bacterial, and cytotoxic potential. *Kurdish Studies*, **12(2)**, 6706-6723 (2024). DOI: 10.53555/ks.v12i2.2894

39. Zahid, R., Khan, J., Khuram, M., and Khan, M.A. Local medicinal plants of Pakistan in the fight against gastrointestinal tract pathogenic bacteria. *Pakistan Journal of Biotechnology*, **17**, 173-182 (2020). DOI: 10.34016/pjbt.2020.17.3.173

40. Carvalho, A.C., Barbosa, A.V., Arais, L.R., Ribeiro, P.F., Carneiro, V.C., and Cerqueira, A.M.F. Resistance patterns, ESBL genes, and genetic relatedness of *Escherichia coli* from dogs and owners. *Brazilian Journal of Microbiology*, **47**, 150-158 (2016). DOI: 10.1016/j.bjm.2015.11.005

41. Subramaniam, G., Sivasamugham, L.A., and Yew, X.Y. Antibacterial activity of *Cymbopogon citratus* against clinically important bacteria. *South African Journal of Chemical Engineering*, **34**, 26-30 (2020). DOI: 10.1016/j.sajce.2020.05.010

42. Sharma, K.R., and Kharel, R. Antibacterial, antidiabetic, and brine shrimp lethality activities of some selected medicinal plants from Kavrepalanchok District of Nepal. *Journal of Institute of Science and Technology*, **24**, 57-62 (2019). DOI: 10.3126/jist.v24i1.24629

43. Fawzi, E., Bahnass, M.M., and Attia, N.E.S. Antimicrobial activity of crude *Calotropis procera* extract with special reference to sheep salmonellosis. *Benha Veterinary Medical Journal*, **40**, 125-128 (2021). DOI: 10.21608/bvmj.2021.52847.1312

44. Mehmood, T., Arshad, H., Nawaz, S., Ullah, A., Hafeez, A., Anwar, F., Ahmad, M.M., and Iqbal, M. Pharmaceutical potential and phenolics profiling of leaves and bark of *Calotropis procera* in relation to extraction solvents. *Pharmaceutical Chemistry Journal*, **54**, 1-10 (2020). DOI: 10.1007/s11094-020-02250-7

45. Patel, H.V., Patel, J.D., and Patel, B. Comparative efficacy of phytochemical analysis and antioxidant activity of methanolic extract of *Calotropis gigantea* and *Calotropis procera*. *International Journal of Life Sciences Biotechnology and Pharma Research*, **5**, 107-113 (2014).