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Antibiotic resistance pattern and biofilm forming ability of *ESBL*-carrying *Escherichia coli* and its susceptibility to *Hemidesmus indicus* (L.) R.Br. root extract in conjunction with amoxicillin

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Present investigation was conducted to evaluate biofilm forming ability of *ESBL*-carrying *E. coli* recovered from wild carnivores and its susceptibility to *Hemidesmus indicus* root extract. *E. coli* isolates were characterized for *ESBL* genes by PCR, antibiogram by disc diffusion test and biofilm formation by microtitre plate assay. In next part of study, methanolic *Hemidesmus indicus* root extract (MEH) was evaluated alone and in combination with amoxicillin for antibacterial and antibiofilm activity against *ESBL* and biofilm producing *E. coli*. A total of sixteen (53.33%) *ESBL* producing isolates were obtained. Among which 6.25% isolates were strong biofilm formers whereas 68.75% and 25% were moderate and weak biofilm producers, respectively. Amoxicillin in combination with MEH significantly inhibited growth and biofilm forming ability of *ESBL*-carrying *E. coli*. Amoxicillin and MEH showed additive antibacterial interaction and MEH synergized amoxicillin's antibiofilm activity against 50% isolates. Based on present findings it is recommended to use a combination of MEH and amoxicillin for therapeutic management of multidrug resistant *ESBL*-carrying *E. coli*.

Keywords: *Hemidesmus indicus* root, Methanolic extract, Amoxicillin, *E. coli*, Antibiotic resistance, Biofilm

Antimicrobial resistance (AMR) presents a big challenge globally. It has not only led to an increase in illness and deaths but also caused huge economic losses. According to a World Bank report, health costs are expected to exceed USD 1 trillion by 2050, which would significantly reduce GDP[1]. Global research on antimicrobial resistance reported that 1,042,500 deaths occurred in India during 2019 due to AMR[2]. Rising prevalence of AMR particularly in developing countries is due to increased colonization of people and animals with extended-spectrum β-lactamase (*ESBL*) producers[3]. *E. coli* is considered as the most common pathogens that carry *ESBL* genes[3]. The *ESBL* encoding genes varied between strains and mainly include *blaTEM*, *blaSHV*, and *blaCTX-M*[4,5]. Co-transmission of these *ESBL* genes between bacteria among human, animals and environment through food chain or some other direct contacts lead to its theatrical rise in incidence and quick spread[6]. *E. coli* can acquire antibiotic resistance determinants vigorously that makes bacteria unresponsive to newer antibiotic therapy also[7]. Biofilm not only protects the bacteria from host immune mechanism but also from antibiotics and enhances the rate of transfer of resistant genes between bacteria within biofilm[8]. Cellulose along with proteinaceous curli fibre provides cohesion and flexibility; and therefore acts as potential building blocks of *E. coli* biofilms[9,10]. Emergence of *ESBL*-carrying *E. coli* such as O157:H7 has become a noteworthy concern worldwide as its possible transmission to animals or human via food chain could flourish the organism′s virulence[11]. Therefore it becomes necessary to conduct multispectral surveillance of AMR covering human, animals, food chain and environment for absolute mitigation of AMR.

ESBL-producing organisms show remarkable resistance to β-lactams, such as penicillin and cephalosporins, and variable resistance to fluoroquinolones, tetracyclines, aminoglycosides, chloramphenicol, trimethoprim, and sulphonamides, whereas they are sensitive to carbapenems[3,12]. Emergence of new variants of *ESBL* showing resistance to carbapenem and colistin has eliminated almost all

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the options of antibiotic treatment[13,14]. Dramatic reduction in approval of newer antibiotics by FDA has been reported from 1983 to 2002 because newer drugs are insufficient to meet the present needs. In 2021, only six out of the thirty-two antibiotics justified the WHO priority pathogens list[15].Major pharmaceutical companies have abandoned this field due to regulatory uncertainties and low economic returns, with an assumption among them that resistance will develop to newly discovered antibiotics as well. Therefore clinicians may be advised for appropriate and judicious use of existing antibiotics. However, safer alternatives with both antibacterial and antibiofilm activity could be developed to potentiate the action of resistant antibiotics.

Rapid development of resistant bacteria and the dearth of a suitable therapeutic option have prompted physicians and researchers to seek better alternatives. Plant-derived substances/ /extracts could be accepted to offset the adverse consequences of multidrug resistance as an alternative or in accompaniment to antibiotics against bacteria. *Hemidesmus indicus* is a lacticiferous plant of the family *Asclepiadacae* and is known for numerous biological activities including antibacterial and anttibiofilm property[16,17]. Traditionally, it has been used in the treatment of venereal, skin and urinary tract associated diseases. Indian subcontinent is the rich source of this plant and is locally called as 'Anantmoola'[18]. The roots of this plant provide variety of phytochemicals having different pharmacological action[19]. The current study has been conducted with the hypothesis that, the use of *Hemidesmus indicus* root extract in combination with antibiotics would facilitate the diffusion of antibiotics into the biofilm matrix. Thereby bacteria would encounter optimal dose of antibiotics and combined action of antibiotics with MEH would aid in complete eradication of bacteria[20]. Therefore, the present investigation was initially carried out to characterize *ESBL*-carrying *E. coli* which was followed by discovering the antimicrobial and antibiofilm potential of methanolic extract of roots of *Hemidesmus indicus* (MEH) against *ESBL*-carrying *E. coli*.

Materials and Methods

E. coli culture

Present study used *E. coli* culture (n=30) isolated from feces of wild carnivores. Isolates were maintained at department of wildlife health and forensic, DSVC Kamdhenu Vishwavidyalaya, Anjora, Durg, Chhattisgarh, India.

PCR detection of *ESBL* genes by multiplex

Boiling and snap chilling method was used to isolate genomic DNA from *E. coli*[21]. *ESBL* encoding genes viz. *blaCTX-M* (593 bp), *blaTEM* (445 bp), and *blaSHV* (747 bp) were amplified and analyzed by multiplex PCR as earlier described[22].

Antibiogram and serotyping of *ESBL*-positive *E. coli*

Disc-diffusion method determined antibiotic sensitivity pattern of *ESBL*-positive *E. coli* and result was analyzed according to guidelines of clinical and laboratory standards of institute[23]. *ESBL*-positive *E. coli* isolates were serotyped using antisera against O- and H-antigen at CRI, Himachal Pradesh, India.

Biofilm production by *ESBL*-positive *E. coli*

Biofilm production was measured qualitatively through the expression of cellulose and curli fibres on congo red indicator (CRI) agar[10]. The *E. coli* was streaked on CRI agar plate, and it was then aerobically incubated for 2-48 h at 37ºC. The morphology of the colonies was recorded, and the expression of the components of extracellular matrix (ECM) was found out in relation to the morphology of the colonies. Brown, dry and rough (BDAR) morphotypes demonstrate curli fibre expression whereas pink dry and rough (PDAR) colonies were suggestive of cellulose expression. Red, dry and rough (RDAR) colonies indicated co-expression of curli and cellulose; and smooth and white indicated absence of curli as well as cellulose.

Using a microtitre plate test, the amount of biofilm formed by *E. coli* was quantified as strong, moderate, weak, and non-biofilm former[16,17].

Methanolic extract of roots of *Hemidesmus indicus* (MEH)

In a mixer grinder, 10 g of the *Hemidesmus indicus* (Fig. 1) root were ground. After stirring with methanol (1:10) for 24 h the root powder was centrifuged and then supernatent was dried at 50-55 ºC. The dried extract was weighed, suspended in dimethyl sulphoxide (100 mg/mL) and filtered (0.33 µm filter). Filtrate was stored in refrigerator until it was needed.

Phytochemical screening

Extraction was done for phytochemical analysis using methanol as solvent. Further extract was screened for the presence or absence of sterols, flavonoids, phenolic compounds, tannins, saponins, alkaloids, proteins, carbohydrates, tannins, lignin and terpenoids as per the previously described protocols[24].

Fig. 1 — *Hemidesmus indicus* (Anantmool) plant.

Aoxicillin

Aqueous solution (100 mg/mL) of amoxicillin (HIMEDIA, Purity ≥97.50%, Batch No.0000411190) was prepared and diluted as needed.

Minimum inhibitory concentration (MIC) determination

MIC was measured by microdilution susceptibility test in microtitre plate following CLSI recommendations23. Both amoxicillin, and MEH were tested in triplicate. Test substance was diluted two-fold in sterile saline and then E.coli culture (109 CFU/ mL) was added to each well. One column was retained as the negative control (BHI broth only) and one as positive control (E.coli culture without test substance). Plate was incubated for 24 h at 37 ºC and read spectrophoto-metrically in ELISA reader at 620 nm. The test substance′s lowest concentration that completely inhibited bacterial growth i.e. lowest concentration with an optical density (OD) value comparable to negative control was designated as the MIC and recorded.

Minimum biofilm inhibitory concentration (MBIC) Determination

MBIC was determined by microtitre plate assay[17]. 100 µL of test compound was added in triplicate to the first well of three successive columns. Along the column, the test compound was two-fold diluted in sterile normal saline. Next, 100 µL of *E.coli* cultured in glucose supplemented brain heart infusion broth, was introduced into every well. To each of the first three wells of the columns, was added to each well. One column included positive control (untreated *E.coli* culture) and one column as negative control/blank (sterile media only). After that, the cells were left to grow into biofilm in the wells for at 37˚C. After 24 h, cells non-adhered to wells were poured out and the biofilm that had attached to the well′s wall and bottom was triple washed with 200 µL of PBS (pH 7.2). After fixation with sodium acetate (2%), adherent bacteria were stained by crystal violet (0.1%). Wells were then washed twice in PBS (pH 7.2). Stained crystal violet was released by glacial acetic acid (33%). Plate was read in an ELISA reader at 492 nm and OD value was recorded. The test compound′s minimum concentration required to demonstrate a 50% decrease in the biofilm development in comparison to control was identified as $MBIC_{50}$. Biofilm inhibition (%) was calculated as below.

$$\text{Biofilm inhibition}(\%) = \frac{OD_{control} - OD_{test}}{OD_{control}} \times 100$$

Estimation of antimicrobial activity of MEH in combination with amoxicillin

Combined effect of amoxicillin and MEH against *ESBL*-carrying *E.coli* was assessed by checkerboard assay[17]. FIC_{index} (Fractional inhibitory concentration index), and $FBIC_{index}$ (Fractional biofilm inhibitory concentration index) were determined. If the value of FIC_{index} or $FBIC_{index}$ is less than or equal to 0.5, it is considered as synergistic interaction whereas additive if the value lies between 0.5 and 4, and antagonistic if the value is more than 4[25].

Statistical analysis

One-way factorial ANOVA was used to evaluate the data, and variations between groups were compared. When *P*-values were less than 0.01 or 0.05, the data was deemed significant.

Results

***ESBL* genes and antibiogram profile**

Distribution of *ESBL* genes among *E.coli* isolates is shown in Table 1. *ESBL* genes were detected in 16 isolates (53.33%). Isolates exhibited higher prevalence of *blaCTX-M* (53.33%) followed by *blaTEM* (50%) while *blaSHV* was found in only one isolate. *ESBL*

producers *E.coli* showed multiple drug resistance. Results of disc diffusion test revealed that 100% isolates were resistant to penicillin and amoxicillin followed by ceftriaxone (93.75%), cefpodoxime (87.5%), tetracycline (62.5%), oxytetracycline (56.25%), gentamicin (37.5%), norfloxacin (37.5%), enrofloxacin (31.25%), chloramphenicol (18.78%), amoxicillin - clavulanate (12.5%) and least resistant to ceftriaxone-tazobactum (6.25%).

E.coli serogroups

O7 (n=4) and O17 (n=3) were predominant serogroups followed by O119 (n=2). Serogroup of remaining isolates were O157, O26, O88, O84, O120, O28, and O11.

Biofilm forming ability of isolates

E. coli isolates exhibited three types of morphotype on CRI agar viz. RDAR, BDAR; and PDAR morphotypes (Table 2). None of isolates produced smooth and white colony. PDAR morphotype (56.25%) was predominantly expressed followed by BDAR (31.25%) and RDAR (12.5%) morphotypes. Cellulose was predominantly expressed (56.25%) at 37 °C by *E. coli* isolates. Using microtitre plate test, each of the 16 *E.coli* isolates produced biofilm (Table 2). Biofilm producing *E. coli* was categorized quantitatively as strong (6.25%), moderate (68.75%) and weak (25%) biofilm formers.

Table 1 — Distribution of *ESBL* genes among *E.coli* (n=16)

Co-existence of *ESBL* genes	Number of isolates	Distribution
blaCTX-M , *blaTEM* & *blaSHV*	01	6.25%
blaCTX-M & *blaTEM*	14	87.5%
blaCTX-M	01	6.25%

Phytoconstituents of MEH

Phytochemical analysis of methanolic extract of *Hemidismus indicus* root revealed a rich array of bioactive compounds, including flavonoids, alkaloids, tannins, glycosides, sterols, phenolics, and proteins. The extract's complex phytochemical profile, elucidated through specific chromogenic reactions, underscores its vast therapeutic potential, with putative applications in antioxidant, anti-inflammatory, and antimicrobial domains.

MIC of MEH and amoxicillin

MIC of MEH and amoxicillin against *E. coli* was 70±8.16 µg/mL, and 9.06±1.18 µg/mL, respectively (Table 3, Fig. 2). Amoxicillin showed significantly lower MIC than that of MEH (P<0.05). Amoxicillin and MEH combination yielded lower MIC (P<0.05) as compared to amoxicillin and MEH alone (Table 3; Fig. 2). Interaction between MEH and amoxicillin against *E.coli* was additive (FIC_{index} >0.5-4).

$MBIC_{50}$ of MEH and amoxicillin

$MBIC_{50}$ of MEH and amoxicillin against *E. coli* was 250±32.27 µg/mL, and 106.25±18.75µg/mL, respectively (Table 3, Fig.2). Inhibition of biofilm was relatively higher by amoxicillin than MEH (P<0.05). Amoxicillin and MEH combination showed significantly lower $MBIC_{50}$ (P<0.05) than amoxicillin and MEH alone (Table 3, Fig.2). Antibiofilm interaction between amoxicillin and MEH against 50% of isolates was synergistic ($FBIC_{index}$<0.5) and for remaining isolates interaction was additive.

Discussion

An increase in interactions between people, domestic animals, and wildlife could lead to the spread of infectious pathogens between different

Table 2 — Biofilm-forming ability of *ESBL*-carrying *E. coli*

Characteristics of *E.coli*	Number of *ESBL*-carrying *E. coli*		
	n = 05	n = 09	n = 02
Morphotype on CRI agar	BDAR	PDAR	RDAR
Expression of cellulose/curli	Curli	cellulose	curli & cellulose
Intensity of biofilm formation	weaker (n=2) to moderate (n=3)	weaker (n=2) to moderate (n=7)	moderate (n=1) to strong (n=1)

Table 3 — MIC, $MBIC_{50}$, FIC index and FBIC index of MEH and amoxicillin against *E.coli* (n=10)

Test compound	MIC (µg/mL)	$MBIC_{50}$ (µg/mL)	FIC index (Antibacterial interaction)	FBIC index (Antibiofilm interaction)
MEH	70 .± 8.16	250±32.27	0.75 - 1.25* (n=10)	0.56-0.63* (n=5) 0.31-0.38** (n=05)
Amoxicillin	9.06 ± 1.18	106.25±18.75		
MEH (+ amoxicillin)	35 ± 4.08	93.75±19.21		
Amoxicillin (+ MEH)	3.44 ± 0.65	8.20±1.36		

[*Additive interaction between MEH and amoxicillin; **Synergistic interaction between MEH and amoxicillin]

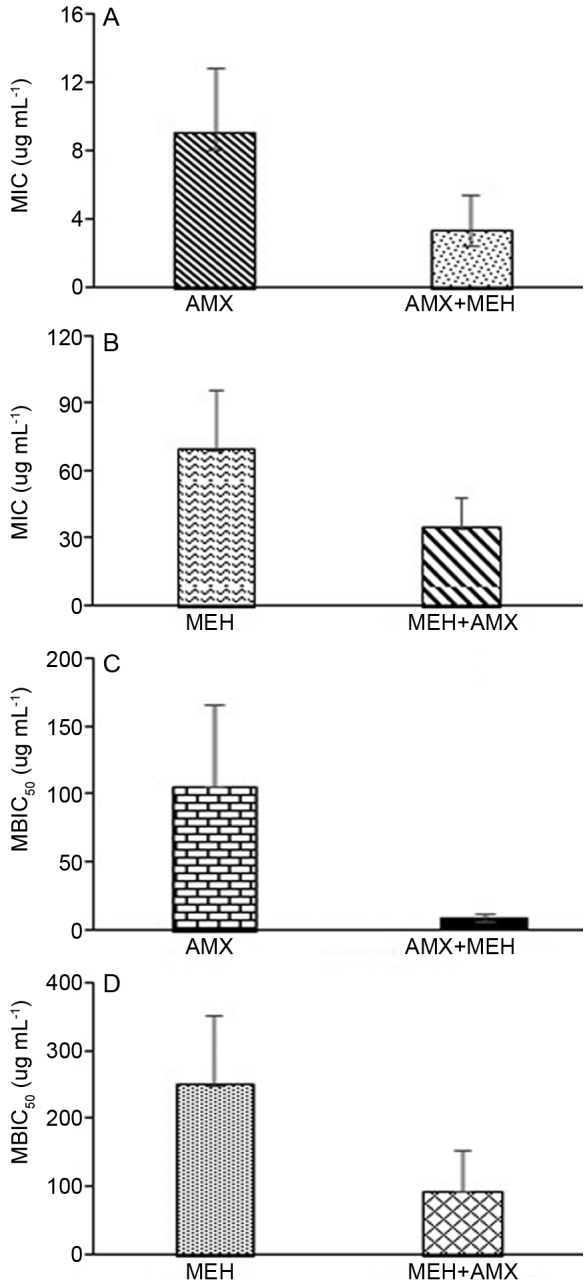

Fig. 2 — Assessment of antimicrobial activity of methanolic extract of *Hemidesmus indicus* root (MEH) and amoxicillin (AMX) against *E.coli* (n=10). (A): MIC (µg mL^{-1}) of AMX alone was significantly higher (P = 0.001) than that in combination with MEH. (B): MIC (µg mL^{-1}) of MEH alone was significantly higher (P = 0.001) than that in combination with AMX. (C): $MBIC_{50}$ (µg mL^{-1}) of AMX alone was significantly higher (P = 0.0001) than that in combination with MEH. (D): $MBIC_{50}$ (µg mL^{-1}) of MEH alone was significantly higher (P = 0.001) than that in combination with AMX.

species. Emergence of *ESBL* positive *E.coli* along with its ability to exist in complex communities called 'biofilm' makes treatment very challenging.

In accordance with these findings, earlier workers also reported a higher prevalence of *E. coli* carrying *blaCTX-M* and *blaTEM* genes[5,26]. On retrospective analysis it is observed that *blaTEM* and *blaSHV* were more prevalent during 1980s whereas currently *blaCTX-M* has the highest global circulation[26,27]. Cent percent resistance of *E.coli* to ceftriaxone, penicillin and amoxicillin could be associated with co-expression of both *blaCTX-M*, and *blaTEM* genes as previously reported[28]. β-lactam resistance in *E.coli* is primarily gained through acquisition and expression of *blaTEM* and *blaCTX-M* genes coded on chromosomes that encodes *ESBL*[7]. Present study corroborates earlier observations that showed simultaneous resistance of *ESBL* producing bacteria to many classes of antibiotics viz. β-lactams, aminoglycosides, fluoroquinolones, tetracycline, chloramphenicol and trimethoprim/sulfamethoxazole[3,12]. Circulation of *ESBL* genes in wildlife might be contributed by extensive use of β-lactam antibiotics in food and pet animals[6]. Occurrence of *ESBL* producing bacteria in wildlife underscores the magnitude of 'One Health' approach and recommends widespread surveillance of *ESBL* genes across various sectors.

In current study, occurrence of O157 serogroup in faecal isolate of wild animal warrants immediate attention of researchers as it is most important serotype associated with enterohaemorrhagic *E. coli* and is responsible for fatal outcomes in human as well as animals[10]. Similarly, earlier study reported an increased incidence of O157 serogroup (12.64%) of *E. coli* isolated from feces[11]. Other important serogroup such as O26 is found to be associated with both enterohaemorrhagic and enteropathogenic *E. coli*, O119 with enteropathogenic *E. coli* and O88 is associated with STEC strains[15,29,30]. Serogroups O7, O11, and O17 are reported in patients with sepsis while O84 and O120 in faecal and meat isolate[31,32].

E. coli isolates of present study expressed cellulose predominantly (56.25%) as cellulose expression is higher among commensal faecal *E. coli* and is well expressed at 37 °C.[33] Biofilm formation could be correlated with presence of the curli and cellulose. Strong biofilm former isolate expressed both curli and fimbriae (Table 2). Extracellular matrix of the biofilm is constituted by curli and cellulose[34]. Curli are amyloid fibers that facilitate cell adhesion, cell aggregation, persistence, biofilm formation and thus contribute to pathogenesis[35]. As a second matrix component, cellulose

helps in cell aggregation and biofilm formation. Curli and cellulose act synergistically in bacterial cell attachment and maturation of biofilm. The difference in intensity of biofilm formation by *E. coli* could be due to variations in extracellular matrix components and the source of the sample. Production of either curli or cellulose affects the growth of biofilm[9,10].

MIC value of amoxicillin against *E. coli* isolates during present study is comparable to MIC value observed in earlier study[36]. Higher MIC value of amoxicillin could be attributed to the spectrum β-lactamase producer strain of *E. coli* in current study. β-lactamase is capable of hydrolysing and inactivating β-lactam antibiotics such as amoxicillin[37]. In a study, hydroethanolic root extract of *Hemidesmus indicus* was found to be more efficacious than ampicillin against *E. coli*[38]. MEH showed more or less similar MIC value against methicillin resistant *Staphylococcus aureus* whereas MIC value of amoxicillin against MRSA was less[17]. MEH exhibited antibacterial activity by virtue of various active ingradients such as alkaloids, polyphenols, flavonoids, tannins, glycosides, carbohydrates, saponins, terpenoids, and proteins[19,39]. MEH exerts antibacterial action all the way through damaging bacterial cell membrane[16,40].

Amoxicillin inhibited biofilm formation at concentration slightly higher than MIC value. Amoxicillin at sub-minimum inhibitory concentration compromised bacterial growth and induced biofilm formation that could aggravate development of resistance in commensal *E. coli* as well[41]. Thus, fractional diffusion of amoxicillin across biofilm matrix and induction of β-lactamases in matrix could be associated with the increased tolerance of bacteria towards amoxicillin. There is lack of study on antibiofilm effect of MEH against *E. coli*, however, antibiofilm potential of MEH was well documented for *Staphylococcus aureus*, *S. epidermidis,* methicillin resistant *S. aureus* and *Streptococcus pyogenes*[17,42]. *Hemidesmus indicus* root extract inhibit expression of adhesion genes leading to poor attachment of bacteria on the surface of cell thereby preventing further progression of biofilm formation[42].

Antibiotics are able to remove biofilm during the initial stage of illness, while tolerance develops against fully grown biofilms. To eliminate bacteria in mature biofilm, a large dose of antibiotic, as well as an increased duration of treatment, is required, which is practically not feasible due to drug toxicity. MEH inhibits biofilm formation by inhibiting cell adherence and thereby exposing the bacterial population to amoxicillin and immune cells, resulting in the efficient eradication of biofilm. Therefore, use of antibiofilm agent in association with antimicrobial drugs could increase overall efficacy of treatment against biofilm producing bacteria.

Conclusion

In current study it was observed that when used in combination with MEH; MIC and $MBIC_{50}$ of amoxicillin is reduced up to 62.03% and 92.28%, respectively. Synergistic effect of MEH on antibiofilm efficacy of amoxicillin against *E.coli* renders the use of MEH as an adjuvant to amoxicillin in treatment of *ESBL* and biofilm associated chronic *E. coli* infections. It may possibly lend a hand in upgrading the clinical efficacy of amoxicillin, which would be an economical choice for farmers in the near future.

Conflict of interest

The authors declare no competing interests.

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