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Phytochemical and Antimicrobial Potential of Methanolic Leaf Extracts of *Acacia catechu* and *Lagerstroemia speciosa* against *Escherichia coli* O157:H7

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Abstract

Escherichia coli O157:H7 is a major foodborne pathogen causing severe health problems including diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS). It is found in fecal, contaminated food and produces Shiga toxins which usually cause hemorrhagic colitis in humans and finally lead to kidney failure. Keeping in view to remove the problems Mixture of the two plant extract syrup formulations was used to check antimicrobial efficacy. Qualitative and quantitative phytochemicals were analyzed from selected two plant leaf extracts of *Acacia catechu* and *Lagerstroemia speciosa*. Qualitative phytochemical analysis was used to observe the presence of tannins, flavonoids, phenolics, glycosidase, anthraquinone, and alkaloids as bioactive compounds. The Herbal syrup was prepared by mixing methanolic leaf extracts of the two plants at the concentration of 0.1g/ml which showed antibacterial activity by the disc diffusion method. The experiments showed positive results *in-vitro*. In the future, the formulated syrup could be tested on an *in-vivo* mouse model for oral testing.

***Key words*:** Antimicrobial activity, Bioactive compounds, *Escherichia coli* O157:H7, Phytochemical analysis, Herbal syrup

Escherichia coli is a Gram-negative, rod shape, facultative anaerobe of genus *Escherichia* commonly found in the lower intestine of warm-blooded organisms and harmlessly colonize into the gastrointestinal tract of human and animals as normal flora. However, some of the strains have evolved into pathogenic *E. coli* by acquiring virulence factors through plasmid, transposons, and bacteriophages, and pathogenicity was based on serogroup, clinical symptoms, or virulence factors [7], [14]. Among them, enterohemorrhagic *E. coli* (EHEC) is defined as pathogenic *E. coli* and is one of the Shiga toxin-producing strains that is a major food and a waterborne pathogen that causes diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans [1]. *E. coli* O157:H7 is the most frequently isolated serotype of EHEC from the ill person in the United States, Japan, and the United Kingdom (ji Youn Lim et al). This strain was first isolated in 1982 as a human pathogen associated with outbreaks of bloody diarrhea in Oregon and Michigan, U.S.A. [21]. Transmission for *E. coli* O157:H7 infections is via the consumption of contaminated foods and water [20], undercooked liquids, and by person-to-person through fecal shedding and account for an estimated 11% of infection. The production of Shiga toxins is a key factor contributing to the development of gastrointestinal illnesses, from watery diarrhea to hemorrhagic colitis and HUS.

The epidemiological investigation has identified cattle as the main reservoir for *E. coli* O157:H7 after tracing an outbreak of Shiga toxin enterohemorrhagic diarrhea into domesticated animals, particularly feedlot cattle. Ruminant animals on farms act as a natural reservoir of *E. coli* O157:H7. *E. coli* O157:H7 causes an estimated 75,000 foodborne illness cases annually in the United States [17]. A review of databases and studies from 10 out of 14 world health organization subregions showed the global incidence of *E. coli* to be 2.8 million cases per year [1]. This strain-induced HUS can result in systemic morbidities, such as acute renal failure, primarily in children.

Intimin (eaeA gene) is a key colonization factor, which plays significant a role in *E. coli* O157:H7 attachment to the host's microvilli of the intestine epithelial cells utilizing the fimbriae of the intestinal cellular walls [11]. Attached bacteria stimulate host cell actin polymerization accumulation, resulting in a raised attachment pedestal [22]. Large intestinal biopsy of hemorrhagic colitis specimens demonstrates cellular hemorrhage and edema characteristic of intestinal inflammation.

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Antimicrobial resistance represents a serious threat to human health across the globe. The cost of bringing a new antibiotic from discovery to market is high and the return on investment is low. Furthermore, the development of new antibiotics has slowed dramatically since the 1950s golden age of discovery. Plants produce a variety of bioactive secondary metabolites that could be used to fuel the future discovery pipeline. While many studies have focused on specific aspects of plants and plant's natural products with antimicrobial properties, a comprehensive review of the antimicrobial potential of the plants has never been attempted.

Acacia catechu is a medicinal plant used for various purposes. It belongs to the Fabaceae family, which also includes soybeans, garden pea, peanuts, and alfalfa. This tree is distributed in the southern Himalayas of Pakistan, northern India, and Nepal, south to Andhra Pradesh in India, and east to Burma and Thailand. Some common names of this plant in different languages are "black catechu" in English, "Khair" in Gujarati, "Khayera" in Bengali, "Katha" in Hindi, "Kachin Amara" in Kannada, "Kath" in Kashmiri, etc. The bark of this plant is a strong antioxidant, astringent, anti-inflammatory, anti-bacterial, and anti-fungal in nature [15]. The extract of this plant is used to treat sore throats and diarrhea. Also useful in high blood pressure, dysentery, colitis, gastric problems, bronchial asthma, cough, leukorrhea, and leprosy.

Lagerstroemia speciosa commonly known as Banaba is a medium-sized to large deciduous tree of the family Lythraceous and it is distributed more or less throughout India, especially in Assam, Bengal, and the Deccan peninsula [19]. The prepared decoction of the leaves is used to treat diabetes mellitus in the Philippines [10]. The tree is clothed with 12-inch long, dark green, oblong, leathery leaves which turn attractively red before falling in winter [6]. The seeds are narcotic, leaves are purgative.

MATERIALS AND METHODS

Plants sample collection

Leaves of *Lagerstroemia speciosa and Acacia catechu* were collected from the roadside public garden located in Noida Sector 10, Uttar Pradesh.

Source of test organism

The strain of *Escherichia coli* O157:H7 was purchased from Hi-Media having the assassin number ATCC43888.

Preparation of methanolic plant extracts

Fresh leaves of *Acacia catechu* and *Lagerstroemia speciosa* were washed thoroughly under tap water. It is then cut into small pieces and dried in a hot air oven at 40°c for 20 minutes. After drying the plant samples, it was crushed using mortar and pestle. 1g of powdered plant sample was mixed with 10 ml of methanol and vortex 5 times in intervals of 5 minutes. It was then left overnight. Leaves extract was filtered using Whatman's filter paper. Crude extracts were transferred in clean vials. Vials were centrifuged for 15 minutes. Supernatants were transferred to another clean vial and pellets were discarded.

Reviving the purchased E. coli strain

E. coli O157:H7 which was bought from the market was revived by culturing on a selective media EMB (Eosin Methylene Agar). After 48 hours of incubation, the *E. coli* colonies appeared blue-black with a metallic green sheen caused by the large quantity of acid that is produced and that precipitates the dye onto the growth's surface.

Phytochemical analysis

Qualitative

Quantitative analysis was done to reveal the constituents of plant extract and also helpful in searching for bioactive compounds which are useful in the synthesis of useful drugs, shown in (Table 1).

Table 1 Shows the result of various phytochemicals present in both leaf extracts

Test	*Acacia catechu*	*Lagerstroemia speciosa*
Tannin		
Braymer's test	++	+++
Lead sub acetate test	+	+++
Flavonoids		
Alkaline reagent test	++	+++
Ammonia test	+	++
Saponin test	-	+
Anthraquinone	-	-
Phenolic compound		
Iodine test	-	+
Potassium dichromate test	-	-
Ferric chloride test	+++	+++
Glycosidase		
Keller-killani test	+	-
Alkaloids		
Dragendroff's test	+	++

(+++): High compound content; (++): Medium compound content; (+): Low compound content; (-): absence of compound

Test for tannins

Braymer's test: 1ml of plant filtrate was added to 3ml of distilled water and 3 drops of 10% ferric chloride was added. Blue-green indicates a positive result [22], [26].

Lead sub acetate test: 3 drops of lead sub acetate were added to 1ml of plant filtrate. A creamy gelatinous precipitate indicates a positive result [23].

Test for flavonoids

Alkaline reagent test: In 1ml of plant filtrate 2ml of 2% NaOH solution was added and followed by adding a few drops of HCI. When an intense yellow color becomes colorless with the addition of acid, it indicates a positive result [2], [4], [22].

Ammonia test: 5ml of dil. Ammonia solution was added to 1 ml of plant filtrate. Then conc. H_2SO_4 was added carefully by touching the wall of the test tube. The yellow color indicates a positive result [9].

Test for saponin

1ml of plant filtrate was added to 9 ml of distilled water and shaken vigorously. Bubble formation indicates a positive result.

Test for anthraquinones

Ammonium hydroxide test: 200µl of plant filtrate was added in 1ml of isopropyl alcohol and 200µl of conc. Ammonium solution was added. Red coloration formation after 2 minutes indicates a positive result [3], [16], [24].

Test for phenolic compounds

Iodine test: In 1ml of plant filtrate, a few drops of iodine solution were added. A transient red color indicates a positive result [25].

Potassium dichromate test: In 1ml of plant filtrate few drops of potassium dichromate solution were added. A dark color indicates a positive result [8].

Ferric chloride test: In 1ml of aqueous plant filtrate few drops of 5% ferric chloride solution were added. Dark green/bluish-black color indicates a positive result [18], [23].

Glycosidase test

Keller-Killani test: In 1ml of plant filtrate 1.5ml of glacial acid was added. Then, 1 drop of 5% ferric chloride was added, and conc. H_2SO_4 was added along the side of the test tube. A blue color solution in the acetic acid layer indicates a positive result [22], [12].

Alkaloids test

Dragendroff's test: In a few ml of plant filtrate, 1-2 ml of Dragendroff's reagent was added. A reddish-brown precipitate indicates a positive result [24], [22].

Quantitative

Quantitative analysis was done to calculate the concentration or amount of desired bioactive agents present in the plant's parts extract, shown in (Table 2-4).

Table 2 Estimation of tannin by Van Burden and Robinson method

Tubes	Distilled water (in ml)	Tannic acid (in ml)	Reagent (in ml)		
Blank	5	0	2		
T_1	4.9	0.1	2		
T_2	4.8	0.2	2	Incubation for	O.D at
T_3	4.7	0.3	2	10 minutes	605nm
T_4	4.6	0.4	2		
Sample 1	4.5	0.5	2		
Sample 2	4.5	0.5	2		

Table 3 Determination of total flavonoids

Tube	Distilled water (in ml)	Conc. QC (in µl)	10%$AlCl_3$	1M NaOH	
Blank	7.4	0	0.3	2	
T_1	7.2	200	0.3	2	
T_2	7.0	400	0.3	2	O.D at
T_3	6.8	600	0.3	2	510nm
T_4	6.6	800	0.3	2	
Sample 1	6.9	500	0.3	2	
Sample 2	6.9	500	0.3	2	

Table 4 Estimation of total phenolic content by Folin Ciocalteu's method

Tube	Distilled water (µl)	Gallic acid (µl)	FC reagent (in ml)	7.5% $NaCO_3$ (in ml)	
Blank	500	0	2.5	2	
T_1	499	1	2.5	2	
T_2	490	10	2.5	2	O.D at
T_3	480	20	2.5	2	765nm
T_4	470	30	2.5	2	
Sample 1	460	40	2.5	2	
Sample 2	450	50	2.5	2	

Antimicrobial activity of plant extract

Plant extracts were poured into a sterile petri plate and left overnight for drying. Then dimethyl sulfoxide (DMSO) was added to the dried plant extract sample and mixed properly. The antimicrobial activity was checked by the disc diffusion method where NAM media is used. For the preparation of NAM media, all the reagents were measured and mixed in distilled water, pH was checked and adjusted. 1.5g of Agar was added and autoclaved. Meanwhile, Petri plates were washed, wiped with 70% ethanol, and kept under LAF for 10 minutes. After autoclaving, the media was poured into a sterile petri plate and left for solidification. After solidification, the fresh nutrient broth was spread onto nutrient agar media (NAM) media using a cotton swab. Petri plates were divided into four sections. Meanwhile, 1 ml of ciprofloxacin antibiotic and 9 ml of distilled water were mixed. Whatman's 6 mm filter paper disc was dipped in ciprofloxacin and placed in the center of the NAM plate as a positive control. The disc was dipped in plant extract samples (*A. catechu* and *L. speciosa*) having a concentration of 0.1g/ml which was placed at the center of another two sections of NAM media. A plain disc was placed at the center of the third section of NAM media as a negative control. The plate was incubated at 37°C for overnight. The zone of inhibition was measured.

Syrup formulation and its effect on E. coli O157:H7

A hot process was used to formulate a syrup in which 1 ml of plant extract and 6g of sucrose were added to 25 ml of distilled water. The mixture was mixed thoroughly and was heated in a water bath at not more than 70°C till it was reduced to 5ml with a thick consistency. The syrup was then cooled and stored at 4°C. The effect of syrup was determined by the disc diffusion method on NAM media. NAM media was prepared and poured into a sterile petri plate. After solidification, a fresh nutrient broth (*E. coli* O157:H7) was spread on nutrient agar media using a cotton swab. Meanwhile, the Petri plate was divided into three sections for placing the disc of the sample, positive control, and negative control. Dip the Whatman's 6mm filter paper disc in formulated syrup and place it on the center of one section; one1611 was dipped into ciprofloxacin for positive control and one plain disc for negative control was placed at the center of the second and third sections respectively. The plate was incubated overnight at 37°C. The zone of inhibition was measured.

RESULTS AND DISCUSSION

The cultured *E.* coli O157:H7 strain appears black-brown in color with a metallic green color when grown in EMB media as shown in (Fig 1).

Fig 1 *Escherichia coli* growth on the EMB agar

Methanolic extract of both *Acacia catechu* and *Lagerstroemia speciosa* shown in (Fig 2A-B) exhibits antimicrobial activity against *E. coli 0157:H7* but the strain was more inhibited by the *L. speciosa* extract having a zone of inhibition of 9 mm in diameter and *A. catechu* having the zone of inhibition of 7mm. The antimicrobial activity was shown in (Fig 2C). While formulated syrup shown in (Figure IV) exhibits antimicrobial activity having a diameter of 10 mm on the NAM plate shown in (Fig 2D).

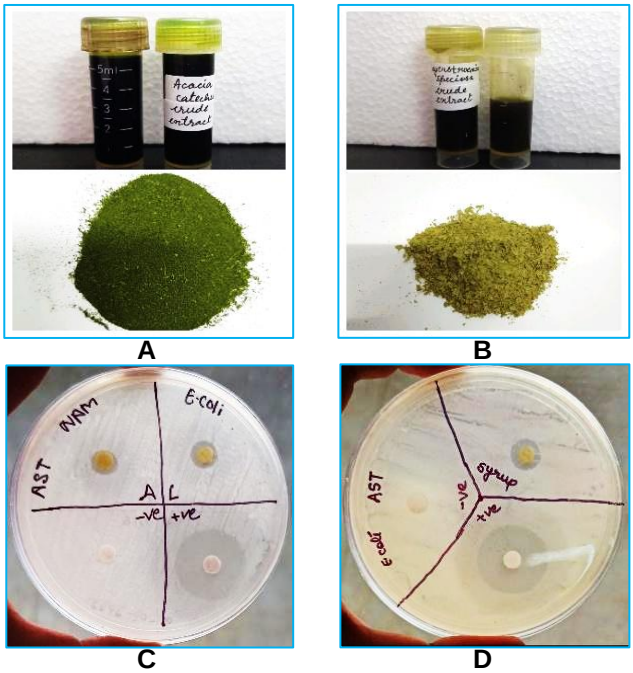


Fig 2 (A) *Acacia catechu* leaves extract (B) *Lagerstroemia speciosa* leaves extract (C) Antimicrobial activity of methanolic plant's leaves extract (D) Antimicrobial activity of formulated mixture(syrup)

When extracts are tested for various qualitative phytochemicals, the tests show the presence of tannins, flavonoids, phenolic compounds, glycosidase, and alkaloids shown in (Table 1). The results of all tests done for qualitative analysis of various phytochemicals were shown in (Figure III). Absorption was taken at 605nm, 510nm, and 765nm for quantitative analysis of tannin, flavonoid, and, phenolic compounds respectively as shown in (Tables 2-4) and (Graph 1-3) and the concentrations of phytochemicals was calculated using the formula y=mx ± c.

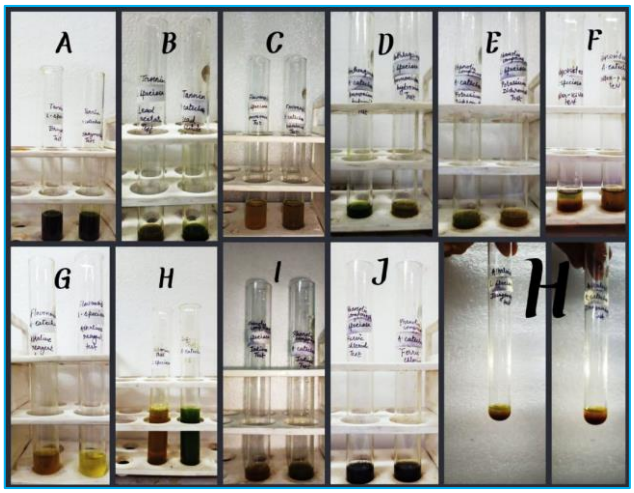


Fig 3 Results of (A) Braymer'test (B) Lead sub acetate test (C) Ammonia test (D) Ammonia hydroxide test (E) Potassium dichromate test (F) Killer-Killani test (G) Alkaline reagent test (H) Saponin test (I) Iodine test (J) Ferric chloride test (H) Dragendroff's test

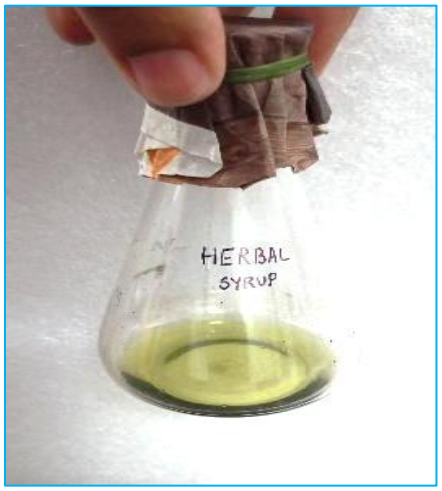


Fig 4 Formulated herbal syrup

Table 5 Total quantitative estimation of tannin

Tubes	Concentration (mg/ml)	Optical density
Blank	0	0.00
T_1	0.02	0.045
T_2	0.04	0.090
T_3	0.06	0.166
T_4	0.08	0.224
S_1	0.121	0.338
S_2	0.178	0.500

Table 6 Quantitative estimation of flavonoid

Tubes	Concentration (mg/ml)	Optical density
Blank	0	0
T_1	0.02	0.253
T_2	0.04	0.402
T_3	0.06	0.636
T_4	0.08	0.584
S_1	1.0	0.695
S_2	0.067	0.543

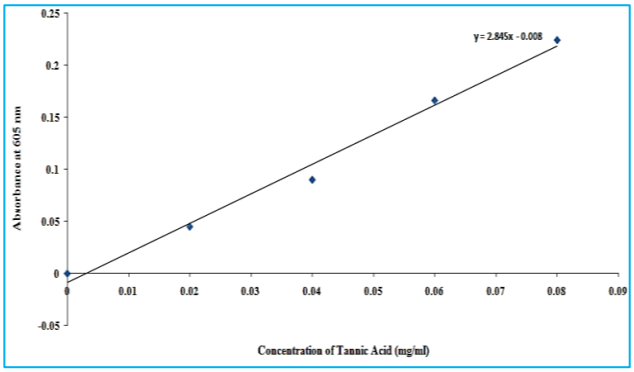
Table 7 Quantitative estimation of phenolics (Gallic acid)

Tubes	Concentration (mg/ml)	Optical density
Blank	0	0
T_1	0.002	0.345
T_2	0.02	0.641
T_3	0.04	0.792
T_4	0.06	1.118
S_1	0.071	0.992
S_2	0.088	1.216

S_1= *Acacia catechu* and S_2= *Langerstroemia speciosa*

Due to the rising prevalence of antibiotic resistance in microorganisms and the negative side effects of synthetic antibiotics, medicinal plants are now more widely used and regarded as clinically useful and safer in the treatment of bacterial infections. The results of the current study's antimicrobial activity test showed that methanolic extract of Lagerstroemia leaves and bark both significantly inhibited both bacteria and fungi, suggesting the presence of antimicrobial substances [15]. The presence of tannins, steroids/triterpenoids, and flavonoids in *L. speciosa* leaves was discovered during a previous phytochemical analysis [27]. Tannins have demonstrated antioxidant and protein-precipitating activities [22], this plant may have a significant impact on the treatment of infectious disorders. In response to microbial infection, plants are known to produce flavonoids, which disrupt metabolic processes (osmotic imbalance, ion channel modification and enzyme denaturation) of microorganisms [5]. The oxidized compounds may block enzymes by reacting with sulfhydryl groups or by interacting with proteins in a more general way. These are some of the processes hypothesized to be responsible for phenolic toxicity to microorganisms [3]. According to the current study, methanol is the best solvent to extract antimicrobial chemicals from leaves under experimental circumstances. Gram-negative organisms often have more complex cell walls than Gram-positive ones, which operate as a diffusional barrier and lessen the susceptibility of the latter to antibacterial drugs. Methanol extracts of A. catechu leaves have however shown some degree of inhibition against Gram-negative pathogens in spite of this permeability variation [13].

In future, these two plant extracts can be tested on mouse models for its efficacy in vivo to find how safe these extracts are for human use and eliminating diseases caused by the *E. coli* O157:H7.



$y = mx \pm c$

$x_1 = \frac{y \pm c}{m}$

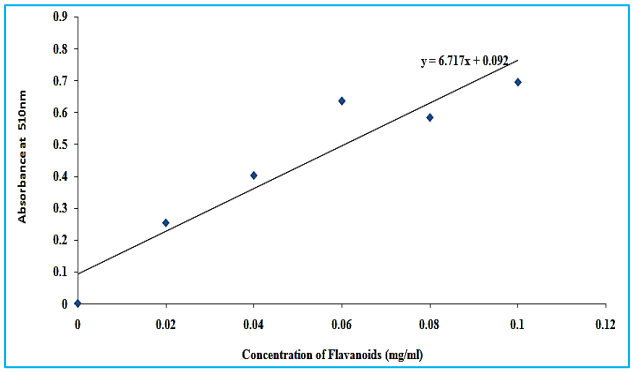
$x_{1=} \frac{0.338+0.008}{2.845}$

$x_1 = 0.242mg/ml$

From the above equation, we can obtain $x_{2=}$ 0.356mg/ml.

$x_{1=}$ *A. catechu* and $x_{2=}$ *L. speciosa*

Graph 1 Calibration curve for tannin



$y = mx \pm c$

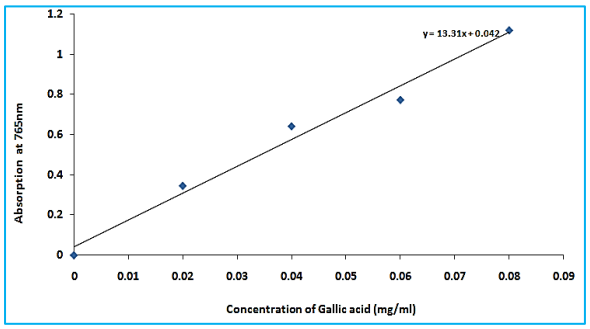
$x_1 = \frac{y \pm c}{m}$

$x_{1=} \frac{0.543-0.092}{6.717}$

$x_{1=}$ 0.134mg/ml

From the above equation, we can obtain $x_{2=}$ 0.14mg/ml

Graph 2 Calibration curve for flavonoids



$y = mx \pm c$

$x_1 = \frac{y \pm c}{m}$

$x_{1=} \frac{0.992-0.042}{13.31}$

$x_{1=}$ 0.142mg/ml

From the above equation, we can obtain $x_{2=}$ 0.176mg/ml

Graph 3 Calibration curve for phenolic compound

CONCLUSION

The understanding of plant extract and phytochemicals with known antimicrobial potential may be of wonderful cost in therapeutic use. Hence, in this study, the antimicrobial activities using methanolic extract of both plant leaves at different concentrations against human pathogenic organisms, the phytochemical constituents and bioactive compounds were conducted. Both methanolic plant extracts showed antimicrobial activities against test bacteria. methanolic extract of *Langerstroemia speciosa* showed the highest activities against *E. coli* O157:H7. The differences between the result may be due to different concentration of bioactive compounds in both the plant leaves extract. In a study by Nasrin F et.al methanolic leaves extract of *Langerstroemia speciosa* showed a good zone of inhibition (18±0.44) against *E. coli*.

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