

Evaluation of the Efficacies of Selected Medicinal Plants on Pathogenic *Escherichia Coli* Strains

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Abstract: The medicinal plants are used in traditional treatments to cure variety of diseases for thousands of year. The aim of this study was to identify such plants with antimicrobial efficacy for controlling some pathogenic *Escherichia coli* strains. The locally available plants viz., garlic (*Allium sativum*), turmeric (*Curcuma longa*), crown flower (*Calotropis gigantea*), and Indian lilac (*Azadirachta indica*) were selected for the study. Ethanol extracts of these plants at two different doses (500- and 1000µg/disc) were examined against pathogenic *E. coli* strains using disc diffusion method as compared with standard kanamycin (30µg/disc.) Antibacterial activity was determined by measuring growth inhibition zones of *E. coli* strains. *E. coli* 119-EAE was more sensitive to all experimented extracts of medicinal plants except crown flower at the rate of 1000 µg/disc than other three strains. At the both concentration, the ethanol mixed extracts of crown flower and Indian lilac was most effective towards all *E. coli* strains. These extracts demonstrated the highest antibacterial activity against *E. coli* 119-EAE inhibiting its growth (21mm zone of diameter) at the dose of 1000µg/disc compared to standard antibiotic, kanamycin followed by Indian lilac and turmeric. The extracts of garlic exhibited the lowest inhibitory responses to all test organisms apart from *E. coli* 119-EAE strains. Furthermore, for in vivo challenge test of shrimps (*Penaeus monodon*), by using ethanolic mixed extracts of crown flower and Indian lilac the lowest average bacterial load of *E. coli* was observed to be 2.11×10^4 CFU/g at the concentration of 1000µg/µl in muscle of shrimp reducing from 3.09×10^4 CFU/g found without applying extracts as control. These results suggested that there was strong antagonistic activity of ethanolic mixed extract of crown flower and Indian lilac against the strain of *E. coli* and it can be used in favor of proper fish health management controlling the risk of contamination from pathogenic *E. coli* in shrimp as important source of drugs.

Keywords: Medicinal plants, Standard kanamycin, *Escherichia coli* Strains, Antibacterial activity, Shrimp.

1. INTRODUCTION

Most of *Escherichia coli* strains are harmless, but only a small percentage is pathogenic to humans. The widespread occurrence of waterborne infections of *E. coli* origin in humans has become one of the major health problems worldwide. Hitherto, several types of enterovirulent *E. coli* have been recognized as the aetiologic agents of various gastrointestinal infections in humans. There are four classes of enterovirulent *E. coli* collectively known as the EEC group. This group is comprised of enterohemorrhagic *E. coli* (EHEC), which produces Shiga-like toxin and attaching-effacing lesions; enterotoxigenic *E. coli* (ETEC), producing heat-stable or heat-labile enterotoxins; enteroinvasive *E. coli* (EIEC), that causes bacillary dysentery via invasion of intestinal epithelial cells or by cytotoxin and enterotoxin; and enteropathogenic *E. coli* (EPEC), whose mechanism of virulence is thought to be unrelated to the excretion of typical *E. coli* enterotoxins. Traditionally cultured shrimp farms in Bangladesh are highly susceptible to be contaminated with such pathogenic *E. coli* strains because of lack of proper sanitation near these farms whereas shrimp is the second most important source of export accounting for a yearly earning of over \$US 300 million. Shrimp production in Bangladesh has suffered production decline due to viral and bacterial diseases.

Control of fish disease is currently based almost entirely on chemotherapy applied in aquaculture for over 50 years [1] and it will entirely retain a role in the management of fish culture systems [2]. The customary use of antibiotic might lead to bacterial resistance against it and intensification of unacceptable residues in aquaculture products and environment. The resistant bacterial strains might have a great negative consequence on the therapy of fish diseases or human diseases and environment

of fish farms [3]. As, antibiotics are always associated with side effects [4], using antimicrobial compounds from medicinal plants has more advantage because of its degradation nature [5]. This scenario actually brings human to new medical dilemma [6]. The natural products are found to be more effective with least side effects as compared to commercial antibiotics so that reason they are used an alternated remedy for treatment of various infections [7]. Medicinal plants having therapeutic properties exert beneficial pharmacological effects on the animal body ecofriendly and produce antioxidant and antimicrobial properties which save the host from cellular oxidation reactions and other pathogens [8, 9]. A scientific study to investigate the antibacterial activity of the medicinal plants, guava (*Psidium guajava*) against bacteria pathogenic for shrimp was initiated by Direkbusarakom and Aekpanithanpong (1992) [10] and turmeric (*Curcuma longa*) powder used in reducing the severity of white spot syndrome in shrimp reported by Rao (1996) [11]. Kraus *et al.* [12] reported that extract of different parts of Indian lilac (*Azadirachta indica*) possess fungicidal and bactericidal properties. Das *et al.* [13] tested the antibacterial activity of Indian lilac against *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *E. coli* and Myxobacteria. Externally garlic (*Allium sativum*) is used as disinfectant and it is applied to indolent tumours, ulcerated surface and wounds [14]. It was reported that extract obtained from garlic was also highly effective against two tested bacteria, *A. hydrophila* and *P. fluorescens* (MIC 0.6 mg/ml) [15]. The powdered root bark of *Calotropis gigantea*, commonly known as Crown flower is used to ease diarrhoea and dysentery, syphilis, coughs, asthma and fevers. Garlic, turmeric, crown flower and Indian lilac could be used as an alternative therapeutic measure against bacterial infection of fish [16]. But sporadic findings from the previous studies could not satisfy a suitable prevention and control measures. Considering the above aspects, the present study was conducted to investigate the therapeutic effect of medicinal plants on pathogenic *E. coli* strains and suggest a package in favor of proper health management in the aquaculture of Bangladesh by comparing the efficacies of selected antibiotic (kanamycin) and medicinal plants on common bacterial fish pathogens.

2. MATERIALS AND METHODS

2.1. Selection of Medicinal Plants

Four medicinal plants viz., garlic (*Allium sativum*), turmeric (*Curcuma longa*), crown flower (*Calotropis gigantea*), and leaves of Indian lilac (*Azadirachta indica*) and mixed of crown flower (*Calotropis gigantea*) and Indian lilac (*Azadirachta indica*) were applied for this study collected from the adjacent area of Khulna University, Khulna.

2.2. Extracts Preparation of Plants

Shade dried garlic, turmeric, crown flower, and leaves of Indian lilac and mixed of crown flower and Indian lilac was grinded into a coarse powder with the help of grinder and powder was stored in an airtight container and kept in a cool, dark and dry place. About 19.92 gm, 190 gm, 60 gm and 115.5 gm of powdered material of turmeric, Indian lilac, garlic and crown flower were soaked in 75 ml, 550 ml, 150 ml, 500 ml of 100% ethanol, respectively in glass container kept for a period of 15 days accompanying regular shaking and stirring. It was filtered using a piece of clean, white cotton plug and dried using rotary vacuum evaporator (Bibby RE200, Sterilin Ltd., U.K.) at 45°C to get the dried methanol extract. Then the crude extract was stored in a refrigerator at 4°C until the experiment commenced. The extracted powder was dissolved in sterilized distilled water to make 500µg/ml and 1000µg/ml solution. These mixtures were used to perform antibacterial assay.

2.3. Pathogenic Bacterial Strains

The following four pathogenic isolates of bacterial strains isolated from shrimp farms were used for the study: *E. coli* 119-EAE, *E. coli* 312-ST, *E. coli* 424-PHO, and *E. coli* 521-LT2. All these cultures were maintained on nutrient agar plates at 37°C according to the method described by Chowdhury and Muniruzzaman [17].

2.4. Antibacterial Activity Test

Antibacterial activity of ethanol extract of garlic, turmeric, crown flower, and leaves of Indian lilac and mixed of crown flower and Indian lilac was determined by disc diffusion method [18] being widely acceptable for the preliminary screening of antibacterial activity. Reference microorganisms from the stock were streaked onto nutrient agar plates and the inoculated plates were incubated overnight at 37°C. Small portion of the subculture was transferred into nutrient broth and incubated (2-4h) at 37°C until the growth reached log phase. Nutrient agar media seeded with standard

inoculum suspension was poured in Petri-dishes and allowed to solidify. Discs impregnated with extract (500µg/disc, 1000µg/disc), standard antibiotic disc (Kanamycin at 30 µg/disc) and blank (solvent ethanol) discs were placed on the Petri dishes and gently pressed to ensure contact with the inoculated agar surface and then finally incubated at 37°C for 18-24h. Triplicate plates were maintained for each organism.

2.5. Determination of Zone of Inhibition

After proper incubation, the antibacterial activity of the test agents was determined by measuring the diameter of zone of inhibition in term of millimeter with calibrated digital slide calipers [18, 19] and assessed against a panel of four pathogenic bacterial strains at the dose of 500 µg/disc and 1000 µg/disc and the results were compared with the activity of standard kanamycin (30 µg/disc).

2.6. In Vivo Challenge Test of Ethanolic Extract

In-vivo challenge test of ethanolic extract of garlic, turmeric, crown flower and leaves of Indian lilac and mixed of crown flower and Indian lilac on the experimental shrimps was done with the following steps.

2.6.1. Experimental Setup

In total, 44 shrimps with an average weight of 50±3 g were randomly distributed into 11 aquaria each (4 x 4 x 4 cm) containing 4 shrimp individual. The experiment was set up with a completely randomized design in treatments. Eleven treatments were used with five at 500 µg/µl and five for 1000 µg/µl: Control (T_C), extract was not included and shrimps were injected with pathogenic *E. coli*, (T₁) 500 µg/µl garlic extract was included and shrimps were injected with pathogenic *E. coli*, (T₂) 500 µg/µl turmeric extract was included and shrimps were injected with pathogenic *E. coli*, (T₃) 500 µg/µl crown flower extract was included and shrimps were injected with pathogenic *E. coli*, (T₄) 500 µg/µl leaves of Indian lilac extract was included and shrimps were injected with pathogenic *E. coli*, (T₅) 500 µg/µl mixed of crown flower and Indian lilac extract was included and shrimps were injected with pathogenic *E. coli*. (T₆) 1000 µg/µl garlic extract was included and shrimps were injected with pathogenic *E. coli*, (T₇) 1000 µg/µl turmeric extract was included and shrimps were injected with pathogenic *E. coli*, (T₈) 1000 µg/µl crown flower extract was included and shrimps were injected with pathogenic *E. coli*, (T₉) 1000 µg/µl leaves of Indian lilac extract was included and shrimps were injected with pathogenic *E. coli* and (T₁₀) 1000 µg/µl mixed of crown flower and Indian lilac extract was included and shrimps were injected with pathogenic *E. coli*.

2.6.2. Preparation of Injecting Solution and Method of Injection

Bacterial colony of one strain of *E. coli* was collected with isolating loop and mixed (1 loop full in 1 ml) properly with Alkaline Saline Peptone Water (ASPW) in sterilized test tubes and further used for administration. One ml of 500 and 1000 µg/disc ethanolic extract of garlic, turmeric, crown flower, and leaves of Indian lilac and mixed of crown flower and Indian lilac was mixed with 1ml bacterial solution and kept in ice box. Shrimps were injected with mixture of extract and bacterial solution at the ventral region of 2nd abdominal segment at 45° angle with the vertical axis of the shrimp body.

2.6.3. Sampling Strategy

Experimentally injected shrimps were sacrificed to collect organ of muscle. Then samples were collected at 24 hours after injection with a 3 hours interval. Two (2) shrimps at each time were sacrificed from each group of experiment. All the samples were marked properly and preserved in deep freeze (-20° C) for further laboratory analysis.

2.6.4. Enumeration of *E. coli* Load in Muscles with and without Extract

At first enrichment of stock solution of each sample was done. Then obtained solution was serially diluted and cultured in Macconkey agar media at 37°C for 24 h ± 3 hours and *E. coli* enumeration was done.

3. RESULTS AND DISCUSSION

3.1. Results

The garlic, turmeric, crown flower, and leaves of Indian lilac and mixed of crown flower and Indian lilac were tested for the antibacterial activity against pathogenic *E. coli* bacterial strain at a concentration of 500µg/disc and 1000µg/disc through screening the zone of inhibition of the plant extracts and its comparison with standard antibiotic kanamycin (30µg/disc).

3.1.1. Antibacterial Activity Test

The ethanol extract of garlic, turmeric, crown flower, Indian lilac and mixed of crown flower and Indian lilac at the dose of 500µg/disc and 1000µg/disc showed antibacterial activity against all the experimental bacterial strains of *E. coli* isolated from shrimp farm. The inhibitory zone formed by standard antibiotic discs of kanamycin (30µg/disc) against all test organisms, *E. coli* strains was more or less same constituting mean value of 20 mm.

At the both concentration, the ethanol mixed extracts of crown flower and Indian lilac was most effective towards all *E. coli* strains compared to other experimental medicinal plant extracts. It showed higher inhibitory effects at the concentration of 1000µg/disc than the low concentration (500µg/disc). These mixed extracts was the highest inhibitory responded extracts against *E. coli* 119-EAE creating 21mm and 20 mm zone of inhibition at the doses of 1000µg/disc and 500µg/disc respectively followed by Indian lilac (19mm) at the dose of 1000µg/disc whereas in case of the remains three *E. coli* strains it acted as the medium inhibitory responded plants at the both concentration (Figure 1&2).

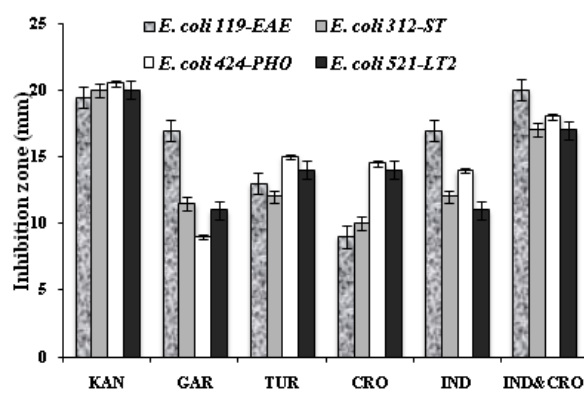


Fig1. Antimicrobial activity of medicinal plant extracts (500µg/disc). Values are expressed as mean ± standard deviation of the three replicates. KAN indicates Kanamycin; Gar indicates garlic; TUR indicates turmeric; CRO indicates crown flower; IND indicates leaves of Indian lilac; IND&CRO indicates mixed of crown flower and Indian lilac

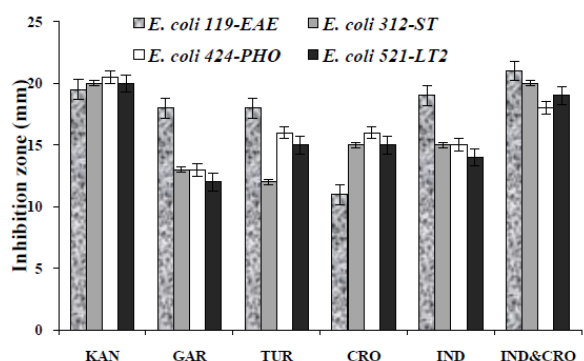


Fig2. Antimicrobial activity of medicinal plant extracts (1000µg/disc). Values are expressed as mean ± standard deviation of the three replicates. KAN indicates Kanamycin; Gar indicates garlic; TUR indicates turmeric; CRO indicates crown flower; IND indicates leaves of Indian lilac; IND&CRO indicates mixed of crown flower and Indian lilac

In contrast to turmeric and crown flower, the rest two effectively inhibited the growth of *E. coli* 119-EAE among four bacterial strains and showed the similar effects screening a zone of 17mm at 500µg/disc concentration. Extracts of garlic, turmeric and Indian lilac demonstrated medium to low inhibitory effects against *E. coli* strains 312-ST, 424-PHO, and 521-LT2 (Fig 1). At the higher concentration extract of crown flower showed the lowest activity index presenting inhibitory zone of 11mm against *E. coli* 119-EAE whereas extract of garlic exhibited low activity against all experimental strains of *E. coli* except strain 119-EAE having 18mm zone of inhibition. The maximum inhibitory effects of turmeric and Indian lilac as high inhibitory responded herbs against *E. coli* 119-EAE were observed to be 18mm and 19mm of zone of diameter respectively at the higher concentration. In case of *E. coli* 424-PHO, the zone of growth inhibition created by both turmeric and crown flower was equal (16mm) at 1000µg/disc (Fig 2).

3.1.2. *In Vivo* Challenge Test of Shrimp (*P. Monodon*)

For *in vivo* challenge test, shrimp were injected with five different extracts of garlic, turmeric, crown flower and leaves of Indian lilac and mixed of crown flower and Indian lilac and challenged with *E. coli* through 24 hours observation. The antibacterial activity of these extracts was observed by comparing the average load of *E. coli* without and with extracts at 500µg/µl and 1000µg/µl concentration.

All the experimental extracts proved the antibacterial activity against pathogenic bacterial strains of *E. coli* inhibiting the growth of pathogenic bacterial strains of *E. coli* with more effectiveness at the concentration of 1000µg/ml than that of 500µg/ml of the extracts. Without applying of extract as control, the average load of *E. coli* isolated from shrimp was found to be 3.09×10^4 CFU/g. Compared to the control, significant reduction of the population of *E. coli* in shrimp were observed through the experimental period ranging from 2.62×10^4 - to 2.11×10^4 CFU/g.

When *in vivo* test was performed, turmeric showed the lowest activity in case of both concentration (T₂ & T₇) exhibiting the average load of *E. coli* of 2.53×10^4 CFU/g at the dose of 1000µg/µl in muscle of shrimp whilst best result of antagonistic effect against *E. coli* was found using mixed extract of crown flower and Indian lilac (T₁₀). These reduced the population of *E. coli* from 3.09×10^4 CFU/g found in control to 2.11×10^4 CFU/g at the concentration of 1000µg/ml within 24 hours of experimental period. However, after treatment with ethanolic extracts, there were considerable reductions of *E. coli* in shrimp at the highest concentration apart from use of turmeric (Table 1). In spite of observing very low effect of extract of turmeric compared to control to reduce the average load of *E. coli* in *in vivo* challenge test of this study the result was ultimately suited with another previous study [20].

Table1. *In-vivo* challenge test by comparing the average load of *E. coli* in shrimps without as control and with extracts in different treatments

Treatments	Average load of <i>E. coli</i> isolated from shrimp (CFU/g)
T _c	3.09×10^4
T ₁	2.39×10^4
T ₂	2.62×10^4
T ₃	2.41×10^4
T ₄	2.29×10^4
T ₅	2.19×10^4
T ₆	2.12×10^4
T ₇	2.53×10^4
T ₈	2.14×10^4
T ₉	2.12×10^4
T ₁₀	2.11×10^4

3.2. Discussion

Plant extract may contain active component that have the ability to complex with extracellular and soluble proteins of the microbial cell and/or to complex with bacterial cell walls and disrupt microbial membranes [21]. Some extracts may have ability to intercalate with DNA, form ion channels in the microbial membrane, and have competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors [22]. Deresse and Awole (2009) [23] also stated that garlic (*A. sativum*) has antimicrobial properties against *Salmonella*, *Shigella* and *E. coli* strain. In the present study, extracts of crown flower and Indian lilac among four medicinal plants used to observe the antibacterial activity exhibited the highest inhibitory responses against *E. coli* strains, especially 119-EAE at the highest concentration followed by Indian lilac. *E. coli* 119-EAE was most sensitive to all test extracts at the rate of 1000 µg/disc but crown flower whereas garlic demonstrated the lowest inhibitory responses to all test organisms apart from *E. coli* 119-EAE strains. Poonam *et al.* [24] found as same results using methanolic extracts against *E. coli* as the findings of the present study. Turmeric, crown flower and Indian lilac performed similar and medium inhibitory effects against *E. coli* 424-PHO and *E. coli* 521-LT2. *E. coli* 312-ST was low sensitive to all extracts except mixed extracts of crown flower and Indian lilac at high concentration. In addition, it is important to mention that *E. coli* 312-ST was more resistant to all extracts except mixed extracts of crown flower and Indian lilac. It was shown that the concentration of 1000 (µg/disc) of extracts was more effective as compared to that of 500 (µg/disc) (Table 2).

Table2. Inhibitory responses of medicinal plants against four strains of *E. coli*

Extracts	Inhibitory response of herbal extracts on growth of bacterial strains in two different concentration							
	<i>E. coli</i> 119-EAE		<i>E. coli</i> 312-ST		<i>E. coli</i> 424-PHO		<i>E. coli</i> 521-LT2	
	500 *	1000*	500*	1000*	500*	1000*	500*	1000*
GAR	++	+++	+	+	+	+	+	+
TUR	+	+++	+	+	++	++	++	++
CRO	+	+	+	++	++	++	++	++
IND	++	+++	+	++	++	++	+	++
CRO&IND	+++	+++	++	+++	+++	+++	++	+++

+++ = High response, ++ = Medium response, + = Low response and * µg/disc

The efficacy test of medicinal plants viz., garlic, turmeric, crown flower and leaves of Indian lilac and mixed of crown flower and Indian lilac on pathogenic *E. coli* bacterial strains is essential to evaluate the antimicrobial effect of the extract. Herbal products like garlic (*A. sativum*), Indian lilac (*A. indica*), and turmeric (*C. longa*) are known to have antiviral and antibacterial activities [25, 26 and 27]. The antibacterial activity of Indian lilac against *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Escherichia coli* and *Myxobacteria* was tested as well [13]. Ethanol and methanol extracts of Indian lilac leaves created zone of inhibition of 15 and 14 mm respectively against *E. coli* [28] and the significant activity of the methanolic extract of the Indian lilac against *E. coli* showing an inhibitory zone diameter (2.0cm, 2.2cm, 2.4cm and 2.1cm) [24] supports the findings of the present study. A comparable experiment was conducted between commonly used antibiotics and the extracts of *A. indica* by disc diffusion method which had the similar effects on the bacteria [29]. It is suggested that Gram-positive organisms are more sensitive to *A. indica* than Gram-negative organisms because of its more complex nature of the cell wall [30]. The results of antimicrobial activity of *C. gigantea* against four microorganisms such as *E. coli* (15mm), *Vibrio mimicus* (15mm), *Vibrio parahaemolyticus* (15mm) and *Staphylococcus aureus* (9mm) [31] correlates with this study. The study was also carried out to investigate whether the administration of ethanolic extract of garlic, turmeric, crown flower, Indian lilac and mixed extract of crown flower and Indian lilac through injection is capable of protecting *P. monodon* from pathogenic bacteria *E. coli* and it was successfully proved that there was strong antagonistic activity of ethanolic mixed extract of crown flower and Indian lilac against the strain of *E. coli*. In case of garlic against *E. coli*, same observation was found at concentration of 2.26 mg/ml and reported that garlic extract has inhibitory effect on shrimp farm bacterial flora and may be applicable in controlling bacterial diseases of shrimp [32]. 2% olive leaves extracts (OLE) can control the microbial load (aerobic and coliforms bacteria) in PUD shrimp stored at 4°C and it was demonstrated that OLE might be beneficial to the fish processing industry as a natural preservative [33]. In other studies, *Labeo rohita* orally administered with extracts of *A. sativum* [34] as well as *Lates calcarifer* with extracts of *A. indica* [35] have shown in vivo efficacy when they were challenged against *A. hydrophila* and *Vibrio harveyi* respectively compared to control groups. The difference in the effect of these plant extracts within the organisms is that there are different antibacterial compounds in the plant extracts and that the compound that acted on one may not be the same as the one that acted on the others since antibacterial agents have different modes of action [36]. It is suggested that further work has to be done to examine the specific component(s) present in medicinal plants responsible for the effect on different bacterial strains.

4. CONCLUSION

The extracts of garlic, turmeric, crown flower and Indian lilac possessed good quality antibacterial activity confirming the immense potential of bioactive ingredients and may be used in primary health care as alternative medicine instead of antibiotics to avoid side effects. Mixed extracts of crown flower and Indian lilac exhibited remarkable biological control against *E. coli* strains. They can play a significant role in shrimp farming as an effective antibacterial agent to cure the bacterial disease caused by *E. coli* having cost-effective and eco-friendly. They can serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine for their some advantages with fewer side effects and relatively less expenses.

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