Original Article

Comparative Analysis Of Synergetic And Antagonistic Effects Of Cinnamomum Verum And Amomum Subulatum With Antibiotic Against Urinary Tract Infection

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Abstract

As antibiotic resistance is increasing day by day, there is a need to develop alternative sources. Being a natural source, Cinnamomum verum and Amomum subulatum can be considered as an alternative therapy. The aim of the current study was to isolate E. coli and Shigella from Urinary tract infected Patients and evaluating the antimicrobial potential of the Cinnamomum verum, Amomum subulatum and antibiotics both synergistically and antagonistically. A total of 50 urine samples were collected from Patients admitted in Khyber Teaching Hospital, and processed using standard gram staining and biochemical test procedures. Furthermore, extract of Cinnamomum verum and Amomum subulatum weremade using standard protocol. Agar well diffusion method was used for the evaluation of antibacterial activity. Two bacteria were isolated i.e. E. coli (74%) and Shigella (26%). A total of 02 antibiotics were used in the current study i.e. Ciprofloxacin and Cefixime. Ciprofloxain showed highest zone against E. coli (27 mm) followed by Shigella (20 mm) whereas Cefixime showed (25 mm) zone against E. coli. Amomum subulatum showed highestzone against E. coli (23 mm) whereas Cinnamomum verum showed potent activity against Shigella (17 mm) followed by E. coli (14 mm). When the Cinnamomum verum and Amomum subulatum were used in combination with the antibiotics, it was noticed that the highest activity was shown by Amomum subulatum with Cefixime (32 mm) whereas (30 mm) zone ofinhibition was calculated when Cinnamomum verum was used in combination with Ciprofloxacin against E. coli. It is concluded from the present research study that the use of appropriate antibiotics in combination with Cinnamomum verum and Amomum subulatum extract are very essential to reduce the level of antimicrobial resistant and also very importantin treating several types of microbial infection because of its antimicrobial activities.

INTRODUCTION

Urinary tract infection (UTI) is nowadays a disease of much concern due to a variety of factor that leads to recurrent bacteremia, drug resistance, it is regarded as second most common organ infection in humans that accounts for almost 8 million visits of medical professional. Different forms of urinary tract infections occurs which depends on the severity of the disease and functional abnormality of the urinary tract. Women are showed to be more prone to UTIs due to structural anatomy of their reproductive and urinary organs, with many being at risk of acute non obstructive phyelopritis especially from those women that experienced recurrent infection. Because of the high rate of antibiotic consumption, a numberof natural remedies are in place to help in curing both complicated and uncomplicated UTIs which may serve as a breakthrough in overcoming the current state of antibiotic resistance (Bao et al., 2020). Urinary tract infections (UTIs) are a severe public health problem and are caused by a range of pathogens but most commonly by Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterococcus faecalis andStaphylococcus saprophyticus. Urinary tract infections(UTIs) due to Shigella

spp. are rare, and Shigella sonnei UTIs are particularly unusual. We report a case of symptomatic UTI due to S. sonnei High recurrence rates and increasing antimicrobial resistance among uropathogens threaten to greatly increase the economic burden of these infections (Foxman. 2010). Herbs and herbal drugs have created interest among the people by its clinically proveneffects like immunomodulation, adaptogenic and antimutagenic. Also, the overuse of synthetic drugs, which results in higher incidence of adverse drug reactions, has motivated humans to return to nature for safe remedies. Herbal medicine (also Herbalism) is the study of pharmacognosy and the use of medicinal plants. Plants have been the basis for medical treatments through most of human (Sharma et al., 2009). The use of plant based drugs for treating various ailments is known to humans since thousands of years. There is evidence of spice trading between the Indian and Roman empiresfor their use in medicines, foods, perfumes etc. Plants and plant based products are the basis of many of the modern pharmaceuticals we use today for our various ailments. Since ancient time, naturally occurring plants have played an important role in the discovery of newtherapeutic agents. Almost all antibiotics are subjected to the problem of bacterial resistance. Therefore, newer herbal antibacterial compounds from plants and their semi synthetic derivatives to overcome the resistance are under investigation (Perveen et al ., 2019). Antibiotics for urinary tract infection are Ciprofloxacin which is a broad spectrum while Cefixime to which E. coli and Shigella are mostly resistive. Ciprofloxacin is animportant and commonly used member of the fluoroquinolone group of antibiotics. Ciprofloxacin inhibits DNA topoisomerase II and DNA topoisomerase IV activities, eventually leading to bacterial cell death. antibacterial action of ciprofloxacin by studying the effects of different antioxidant compounds on Ciprofloxacin susceptibility of Escherichia coli. Cefixime is active against Streptococcus pyogenes, S. pneumoniae, S. agalactiae and most strains of streptococci belonging to Lancefield group C. Pseudomonas aeruginosa is resistant to cefixime (Goswami et al., 2006). Cinnamomum verum is highly rich in nutrients and contains several medicinal properties. It is widely used in traditional medicinal system antispasmodic, carminative,antidiarrheal, antiemetic, analgesic and also for the treatment of influenza and common cold (Garg & Kumar. 2019). In recent years C. verum bark extracts and essential oils have been reported to exhibit significant antimicrobial activity against a wide variety of bacteria and fungi. These studies represent this plant as a valuable source of antimicrobial compounds therefore this study was designed to evaluate the antibacterial properties of the aqueous extract of C. verum bark against UTI causing bacteria (Zhang et al., 2016). Amomum subulatum provides many health benefits it has antiseptic and antispasmodic properties, anti-inflammatory properties, and is an antioxidant reservoir, a homeostasis agent and a blood pressure regulator. This tiny yet powerful black seed can help warm the respiratory tract (Nair. 2020). Black cardamom (Amomum subulatum) is commonly known as ‘Badi Elaichi’ is a tall, perennial, evergreen, herbaceous monocot plant. Black cardamom is well known spice, which has medicinal value Amomum subulatum seeds help combat cough, cold and sore throat. They help secrete stomach juices that promote digestion. This further prevents conditions like gastric ulcers, acidity. The seeds of Amomum subulatum the have antiseptic and anti-bacterial properties that protect against infections, further boosting the immunity system (Kaur et al., 2013). The aim of the current research was to check the synergetic effect of Cinnamomum verum and Amomum subulatum seeds along with antibiotics against microorganisms isolated fromUrinary tract infection.

MATERIALS AND METHODOS

The study was conducted at the Department of Health and Biological sciences AbasynUniversity Peshawar from November 2020 to August 2021.

Preapartion of Cinnamomum verum and Amomum subulatum extract

Fresh Cinnmomum verum and Amomum subulatum (seeds) were purchased from the local grain market. The spices were washed properly and then dried in a dry, dark place(for 15 days) and then grinded in to powder form through herb and spice grinder which was then kept in air tight bottles for preparation of different solvent extracts. The extract powder was then soaked for one week in distilled water and filtered through filter paper. After that the filtrate was dried in oven for seven days (Muhammad et al., 2020).

Collection of Urine Sample

For sample collection, midstream clean-catch method were followed. A total of 50 urine sample were collected from Khyber teaching Hospital Peshawar, Pakistan. After collection, the samples were brought to Microbiology

Research laboratory for further processing.

Samples processing

The collected samples were processed in the Microbiology laboratory at AbasynUniversity Peshawar. The samples were inoculated on different media i.e Nutrient agar and CLED Agar. The growth was observed after 24-28 hours incubation at 37°C.

Characterization of bacterial isolates

The species were inoculated on Nutrient agar plates and CLED agar plates. The streaked plates were incubated at 37°C for 24hr. The bacterial growth was later Gram stained. Characterization of bacterial isolates was based on standard microbiological methods. Identification of isolates was done on the basis of colony morphology and biochemical tests like Catalse, Oxidase, Coaglase, triple sugar iron, Urease, Citrate utilizationand Indole test.

Antimicrobial Susceptibility Test

The antibiotic and extract susceptibility profile of species was determined by kirby bauer's disc diffusion method and well diffusion method. Isolated bacteria (E. coli andShigella) were inoculated on MHA plates with the help of sterile swab to create uniform bacterial lawn. Antibiotic discs (Ciprofloxacin 5µg and Cefixime 5µg) were placed over the agar plates with the help of sterile foreceps at equal distance. The plates were placed in incubator for 24 hours at 37°C. Zone of inhibition around antibiotic discs showed the resistance of bacteria to a particular antibiotic. While for checking synergetic effect ofCinnamomum verum and Amomum subulatum and antibiotics the isolated organisms were inoculated on to MHA plates. Each agar plate was divided by a marker pen into two halvesthe antibiotic discs were plated in one half and on the opposite side each antibiotic disc immersed in Cinnamomum verum and Amomum subulatum (100µg in 1ml of DMSO 100µg/ml) extract was plated opposite to the same antibiotic disc. At the center of agar, a sterile paper disc immersed in in Cinnamomum verum and Amomum subulatum was applied. MIC and MBC were also performed for antmicrobial activity of extract and antibiotics.

Minimum Inhibitory Concentration (MIC)

In microbiology, the minimum inhibitory concentration (MIC) is the lowest concentration of a chemical, usually a plants extract i.e Cinnamomum verum and Amomum subulatum which prevents visible growth of a bacterium or bacteria. MIC depends on the microorganism, the affected human being (in vivo only), and the antibiotic itself.The minimum inhibitory concentration, is the lowest concentration (in µg/mL) of an antibiotic that inhibits the growth of a given strain of bacteria. Agar dilution and broth dilution are the most commonly used techniques to determine the minimal inhibitory concentration of antimicrobial agents, including antibiotics and other substances that kill (bactericidal activity) or inhibit the growth (bacteriostatic activity) of bacteria (Kolarević et al., 2016).

Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration is the lowest concentration of anantibacterial agent required to kill a bacterium over a fixed, somewhat extended period, such as 18 hours or 24 hours, under a specific set of conditions. It can be determined from the broth dilution of MIC tests by sub culturing to agar plates that do not contain the test agent. The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculums by a pre-determined reduction such as ≥99.9%. The MBC is complementary to the MIC; whereas the MIC test demonstrates the lowest level of antimicrobial agent that greatly inhibits growth, the MBC demonstrates the lowest level of antimicrobial agent resulting in microbial death (Liu et al., 2020).

Optical density (OD_{600})

Optical density (**OD_{600nm}**) measurement of bacterial cultures is a common technique used in microbiology**.** Researchers have primarily relied on spectrophotometers to make these measurements, however the measurement is actually based on the amount of light scatteredby the culture rather than the amount of light absorbed. It is a commonly used in Spectrophotometry for estimating the concentration of bacteria or other cells

in a liquid as the600nm wavelength does little to damage or hinder their growth (Sutton. 2011).

RESULTS

A total of 50 urine samples were collected from KTH Peshawar. Among them, 27 were collected from female patients and 23 were collected from male patients as shown in figure 1.

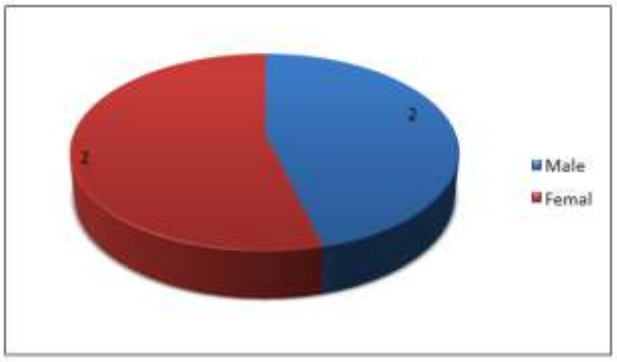

Figure .1 Show the gender wise Sample collection

After processing of these samples, 39 (78%) samples were positive for bacterial growth and 11 (22%) showed negative result for bacterial growth after 24 to 48 hrs of incubation period as shown in figure 2.

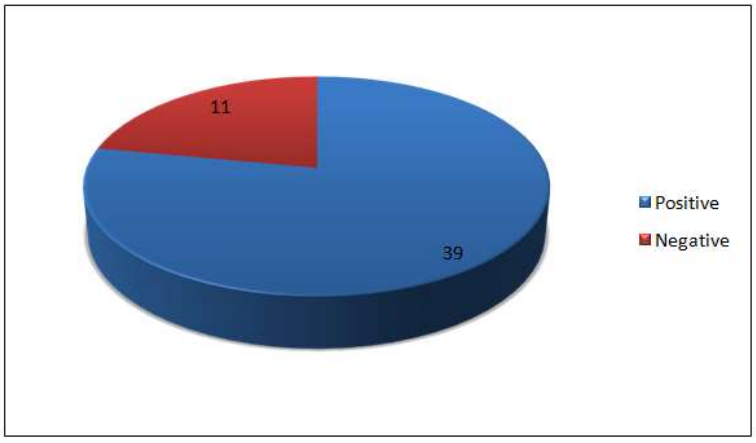

Figure 2. Show the Sample processing of negative and positive bacteria

A total of two bacterial species were identified i.e E. coli counted for (74%) and Shigella (26%) as shown in figure .3.

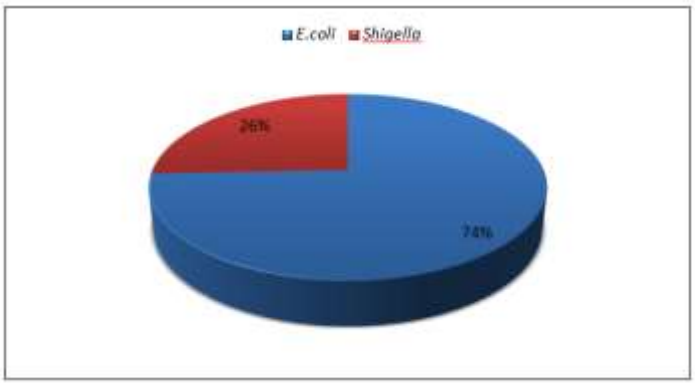

Figure .3 Percentage distribution of isolates among positive species.

Both E.coli and Shigella were grown on CLED and MacConkey Agar. E. coli showed pink color and Shigella

showed colorless colonies on MacConkey agar. Both were Gram negative Rod shaped bacteria.

Table 1. Biochemical tests of isolated bacterial species.

Isolates	Catalase	Oxidase	Coagulase	Urease	Indole	TSI test	Gas	H2S	Butt/ slope	Citrate	Identified Organisms
1	+	-	-	-	+	+	+	-	Yellow	-	E. coli
2	+	-	-	-	+	+	-	-	Yellow	-	Shigella

After cultural characteristics, a complete identification of these bacterial samples was carried out by performing biochemical tests. All the specimens were positive for TSI, Indole and Catalase test while showed negative result for Oxidase, Coagulase, Urease and Citrate test.

Table 2. CLSI Standard values of antibiotics showing resistance, susceptibility and intermediate values(CLSI .2017).

Antibiotics	Disk content	Against	Susceptible	Intermediates	Resistance
Ciprofloxacin	5µg	E. coli	≥21	16-20	≤15
Cefixime	5µg	E. coli	≥19	16-18	≤15
Ciprofloxacin	5µg	Shigella	≥21	16-20	≤15
Cefixime	5µg	Shigella	≥19	16-18	≤15

Table 3 . Zone of inhibition for just antibiotics

S.NO	Isolates	Ciprofloxacin	Cefixime
1	E. coli	27mm	25mm
2	Shigella	20mm	R

Different classes of antibiotics were used to determine the susceptibility profile of the isolated bacterial species. Ciprofloxacin showed (27mm) and Cefixime showed (25mm) zone of inhibition against E. coli. whereas Ciprofloxacin showed (20mm) and Cefixime showedno zone of inhibition against Shigella.

Table 4. Zone of inhibition of Cinnamomum verum and Amomum subulatum seeds

S.NO	Isolates	Zone of inhibition	
1	E. coli	Cinnamomum verum	Amomum subulatum Seeds
		14mm	23mm
2	Shigella	17mm	Nil

Cinnamomum verum extract showed antibacterial activity against E. coli and Shigella in which greater antibacterial activity was showed against Shigella (17mm) followed by E. coli (14mm) whereas Amomum subulatum seeds extract showed greater antibacterial activity against E. coli (23mm) while Shigella showed resistance to Amomum subulatum seeds extract.

Table 5 . Synergistic effect of Cinnamomum verum and Amomum subulatum seeds

extract with antibiotics against the isolated bacteria.

S.NO	Extract + antibiotics	E. coli	Shigella
1	Cinnamomum verum + Ciprofloxacin	30mm	23mm

2	Cinnamomum verum + Cefixime	20mm	17mm
3	Amomum subulatumseeds + Ciprofloxacin	21mm	10mm
4	Amomum subulatum seeds + Cefixime	32mm	17mm

Cinnamomum verum + Ciprofloxacin showed (30mm) and Cinnamomum verum + Cefixime showed (20mm) zone of inhibition against E. coli. While Cinnamomum verum + Ciprofloxacin showed (23mm) and Cinnamomum verum + Cefixime showed (17mm) zone of inhibition against Shigella. Amomum subulatum seeds + Ciprofloxacin showed (21mm) and Amomum subulatum seeds + cefixime showed (32mm) zone of inhibition against E. coli. While Amomum subulatum seeds + Ciprofloxacin showed (10mm) and Amomum subulatum seeds + cefixime showed (17mm) zone of inhibition against Shigella.

Table 6. MIC and MBCof Cinnamomum verum and Amomum subulatum

S.NO	**Isolates**	**MIC and MBC(µg/ml)**			
1	E.coli	Cinnamomumverum	Cinnamomum verum	Amomum subulatum seed	Amomum subulatum seed
		MIC	MBC	MIC	MBC
		10	15	10	15
2	Shigella	15	20	15	20

Cinnamomum verum showed MIC (10 µg/ml) and MBC (15 µg/ml) against E. coli. While it showed MIC (15 µg/ml) and MBC (20 µg/ml) against Shigella. whereas Amomum subulatum seed showed MIC (10 µg/ml)and MBC (15 µg/ml) against E. coli. While itshowed MIC (15 µg/ml) and MBC (20 µg/ml) against Shigella.

Table 7. MIC and MBC of Antibiotics

S.NO	**Isolates**	**Ciprofloxacin(µg/ml)**	
1	E.coli	MIC	MBC
		10	15
2	Shigella	15	20

Ciprofloxacin showed MIC (10 µg/ml) and MBC (15 µg/ml) against E. coli. While itshowed MIC (15 µg/ml) and MBC (20 µg/ml) against Shigella.

Table 8. OD_{600nm} (MIC and MBC) of Cinnamomum verum and Amomum subulatum

S.NO	**Isolates**	**OD (MIC and MBC)**			
1	E.coli	Cinnamomumverum	Cinnamomum verum	Amomum subulatum seed	Amomum subulatum seed
		OD (MIC)	OD (MBC)	OD (MIC)	OD (MBC)
		0.525	0.475	0.404	0.381
2	Shigella	0.590	0.569	0.558	0.489

Optical density of Cinnamomum verum MIC (0.525) and MBC (0.475) against E. coliwhile it showed MIC (0.590) and MBC (0.569) against Shigella. Optical density of Amomum subulatum seed MIC (0.404) and MBC (0.381) against E. coli while it showed MIC (0.558) and MBC (0.489) against Shigella.

DISCUSSION

In the current research study, a total of two bacterial species such as E. coli and Shigella were isolated from urine sample which were collected from Khyber Teaching Hospital Peshawar, Pakistan, and check for antibiotics sensitivity as well as for synergetic effect of Cinnamomum verum and Amomum subulatum seeds with different class of antibiotics. Garg & Kumar. (2019) also proposed study that UTIs is one of the highlyprevalent infection worldwide while the incidence rate is high in developing countries than that of developed countries. In last few decades, a large number of uropathogens have been reported to exhibit drug resistance and multi drug resistance towards the commonly used antibiotics. Therefore this study was undertaken for studying the antimicrobial activity of aqueous extract of Cinnamomum verum against 5 UTI causing bacteria while the extract was found to inhibit the growth of all these bacteria including Pseudomonas aeruginosa, Proteus mirabilis, Enterobacter aerogenes, Klebsiella pneumonia, and Escherichia coli. Further the extract exhibited maximum relative percentage inhibition towards K. pneumoniae (95.06%) and best minimum inhibitory concentration towards E. coli (0.5 mg/ml). The results of the study emphasized on the strong antibacterial property of this plants and its potential application in antimicrobial drug discovery. This study also reported the Cinnamomum verum extract and its synergistic effectwith antibiotics against E.coli and Shigella as we concluded that most of the antibiotics i.e Ciprofloxacin showed greater combinatorial effect (30mm) against E. coli while the other antibiotic i.e Cefixime showed some synergestic effect (17mm) against Shigella and some antibiotics does not showed its effects i.e Shigella showed resistance to Cefixime. Cinnamomum verum could provide the body with invaluable nutritional ingredients as well asantioxidant substances and show high level of antimicrobial activity. Further the Cinnamomum verum extract exhibited maximum relative percentage inhibition towards E.coli (90%) and Shigella (95%) and also showed best minimum inhibitory concentration towards E. coli (10µg/ml) and against Shigella (15µg/ml) and also minimum bactericidal concentration against E. coli (15µg/ml) while against Shigella (20µg/ml). Shreya et al., (2015) reported that Cinnamon (Cinnamomum verum) is well known food spice but little do people know that Cinnamon has an enormous potential as an antimicrobial agent and is a powerful antioxidant. Its abundant value in treating disorders likediabetes inflammation, ulcers. Alzheimer's have already been proven. The objective of the present Research study was to find out the effect of Cinnamomum verum bark has any effect on urinary tract infection isolates and fungal isolates, in an attempt to replace chemical drugs with nature's products. Infected urine samples were used to isolate bacteria which were subjected to the antimicrobial assay of commercially available cinnamon oil and the extracts of cinnamon leaf and bark. During this study aqueous extract of C. verum was found to be effecting in inhibiting the growth of all test bacteria while the highest activity was found to betowards K. pneumoniae (19.33±1.52) followed by E. aerogenes (18.66±2.08), E. coli (18.33±1.52), P. aeruginosa (14.66±2.08), and P. mirabilis (13.66±0.57). Further the relative percentage inhibition of the plant extract towards UTI causing bacteria was evaluated. Aqueous extract of C. verum exhibited maximum RPI towards K. pneumoniae (95.06%) followed by E. aerogenes (91.8%), E. coli (80.88%), P. mirabilis (69.49%) and P. aeruginosa (68.75). The MIC of the extract was found to be towards E. coli (0.5 mg/ml), E. aerogenes (0.7 mg/ml), K. pneumoniae (1 mg/ml), P. mirabilis (2 mg/ml) and P. aeruginosa (2.5 mg/ml). This study also showed that ciprofloxacin is more effective in controllinginfection of different parts of the urinary tract due to less drug resistance. The present results also determined the Cinnamomum verum extract and its synergistic effect with antibiotics against E. coli and Shigella as we concluded that most of the antibiotics i.e Ciprofloxacin showed greater combinatorial effect (23mm) against Shigellawhile the other antibiotic i.e Cefixime showed some synergestic effect (17mm) againstShigella and some antibiotics does not showed its effects i.e Shigella showed resistance to Cefixime. Cinnamomum verum could provide the body with invaluable nutritional ingredients as well as antioxidant substances and show high level of antimicrobial activity. Further the Ciprofloxacin exhibited maximum relative percentage inhibition towards E. coli (80%) and Shigella (70%) and also showed best minimum inhibitory concentration towards E. coli (10µg/ml) and against Shigella (15µg/ml) and also minimum bactericidal concentration against E. coli (15µg/ml) while against Shigella (20µg/ml). Garg et al., (2016) demonstrated that Cardamom (Amomum subulatum) also knownas Badi ilaychi or brown ilaychi is a terrestrial, rhizomatous herb of Zingiberaceae family, distributed chiefly in Africa, tropical Asia. The aim of the present study was to explore the antibacterial potential of cardamom (Amomum subulatum) against the enteropathogenic and food-spoiler bacterial strains Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus pumilus. Bacterial cell membrane integrity was damaged and an increase in the absorbance at 260nm and 280nm was observed after incubation with cardamom extract. Enteropathogens Shigella species and E. coli are found to be sensitive to the extractsof Amomum subulatum. It is clear that the Amomum subulatum

extract has maximum inhibitory activity against E. coli with zone of inhibition 25 mm and showed weak activity against Shigella with mean zone of inhibition 7 mm. The antibacterial effects of theseproducts were compared with standard broad spectrum antibacterial drug (ciprofloxacin) having 26 mm zone size against E. coli. Cardamom extract inhibited the growth of all the bacterial strains tested here with MIC 6.24mg/ml for E. coli and 4.16mg/ml for other bacteria. The present research study revealed Amomum subulatum seeds and its synergistic effect with antibiotics against E. coli and Shigella as we concluded that most of the antibiotics i.e Ciprofloxacin showed greater combinatorial effect (32mm) against Shigella while the other antibiotic i.e Cefixime showed some synergestic effect (17mm) againstShigella and some antibiotics does not showed its effects i.e Shigella showed resistance to Cefixime. Amomum subulatum seeds could provide the body with invaluable nutritional ingridients as well as antioxidant substances and show high level of antimicrobial activity. Further the Amomum subulatum seeds extract exhibited maximum relative percentage inhibition towards E. coli (95%) and Shigella showed to the extract and also showed best minimum inhibitory concentration towards E. coli (10µg/ml) and against Shigella (15µg/ml) and also minimum bactericidal concentration against E. coli (15µg/ml) while against Shigella (20µg/ml). The studies conducted so far by various workers have shown that Cinnamomum verum and Amomum subulatum seeds has excellent medicinal properties and can be used affectively for medical applications. In this study we have tried to show its antibacterial properties in vitro and from the results obtained we can suggest that it may be possible togive the Cinnamomum verum and Amomum subulatum Seeds as a herbal medicine and also along with antibiotics in case of urinary tract infections i.e against E. coli and Shigella.

Conclusion

Isolation and identification of clinical pathogens are the two basic units of surveillance because they have a significant role in diagnosing the disease. This study helps us to know the the causative agents behind UTI infection. Furthermore, antibiotics sensitivity testing and synergetic effect of Cinnamomum verum and Amomum subulatum seeds alsohelps in determination of resistance and susceptibility patteren of isolated bacterial species from Urine sample. It is concluded from the present reaserch study that different in society, living style and geography are responsible for differences and bacterial species identified in particular samples it is concluded that medium, atmosphere, incubation period and nature of samples were considered as gold standard parameters in the detection of different pathogenic bacterial species i.e E. coli which was the most prevalent organism 74% and 26% Shigella.

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