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BIOMEDICAL ACTIVITY OF ZrO2 AGAINST *ESCHERICHIA COLI* ISOLATED FROM PREGNANT WOMEN WITH URINARY TRACT INFECTION

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ABSTRACT

Urinary tract infection (UTI) is one of the common bacterial infections during pregnancy. Its significance in pregnancy in view of its associated maternal and fetal morbidity and mortality has been widely evaluated. Colon infection caused by predominant organisms *E.coli* the most common pathogen in UTI and colon infections also serious troubles among pregnant women. So urine samples were collected from different clinics and Public Health Centre around rural area of Tirupur city. Totally 25 multidrug resistant *E.coli* was isolated from 17 mid stream urine samples and confirmed by using routine laboratory techniques. The isolates were subjected to four different antibiotic categories comprising 10 antibiotics. Strain No. MK04, MK08, MK09 and MK10 showed resistant against six antibiotics which showed 60% frequency followed by MK07 and MK05, showed resistant against five antibiotics which showed 50% frequency. Minimum resistant was recorded through the isolate MK01 showed 30% resistant against three antibiotics. Among 10 different antibiogram, 10 isolates showed multiple antibiotic resistant against the antibiotics tested. Currently, the metal oxide nanoparticles are the most promising tools applied as antimicrobial agents for human diseases. Different concentration of Zirconium oxide 50µl, 100µl and 150µl were used against *Eschericia coli*. In the present study metal oxide nanoparticles such as Zirconium oxide was used and it shows prominent antibacterial activity against UTI causing organism.

KEYWORDS: UTIs, Pregnant women, Antimicrobial drug resistance, ZrO2.

INTRODUCTION

Urinary tract infections (UTIs) are serious health problem affecting millions of people each year. It is one of the most common infections occurring in all age groups from neonates to old age. It is more common in females as compared to males, especially females of reproductive age group (15-50 years). Up to 60% women will develop UTI at sometimes in their lives.[1] However, UTI may be more serious during pregnancy because the microbes are more likely to travel to the kidneys.[2]

During pregnancy, the tendency of UTI increases partly due to the pressure of the gravid uterus on ureters causing stasis of urine flow and is also attributed to the hormonal and immunological changes during pregnancy.[3] Therefore, pregnant women should have a routine urine test through pregnancy.[4] UTI occurs approximately in 5-10% of all pregnancies[5] and it can be seen in three different forms in pregnancy: asymptomatic bacteriuria, and acute pyelonephritis.[4] The incidence of asymptomatic bacteriuria has been reported between 2-13% in pregnancy all over the world and if not treated, it will increase the frequency of premature delivery, neonates with low birth weight and is likely to cause acute pyelonephritis at a rate of 15-30%.[6] Numerous studies during the past 30 years have reported association between UTI during pregnancy and adverse outcomes.[7]

Normally women may be more susceptible to UTI because their urethral opening near the source of bacteria (e.g., anus, vagina) and their urethra is shorter, providing bacteria easier access to bladder. 50-80% women experience urinary tract infection at least once or twice in their lives.[8] UTI is also important complication of pregnancy. When it is associated with any structural and neurological fault of the urinary tract, often leads to death.[9] The rate of urinary tract infection usually they increase during pregnancy due to the pressure of gravid uterus on the ureters resulting in the stasis of urine flow and due to hormonal and immunological changes during normal pregnancy. The hormonal changes in pregnancy lead to decreased bladder tone, diminished peristalsis and dilatation of renal pelvis and ureter. Development of glycosuria seen in 70% pregnant women encourages bacterial growth in the urine. Women, who use a diaphragm, develop infections more often, and condoms with spermicidal foam may cause the growth of *E. coli* in the vagina, which may enter the urethra.

The organisms that cause UTI during pregnancy are the same as those found in non pregnant women. *E. coli* is reported to be the major cause (85-95%) of urinary tract infection.[10] The other gram negative pathogens causing UTI are *Klebsiella* spp., *Proteus mirabilis* and

Pseudomonas aeruginosa, however, *Enterococci* and coagulase negative *Staphylococci* are the most frequently encountered gram positive bacteria in UTI.[11]

Escherichia coli present in the gastrointestinal tract as commensals provide the pool for initiation of UTI.[12] The organism is implicated in more than 90% of all uncomplicated cases of UTI.[13] The strains of *E. coli* causing UTI have been termed as Uropathogenic *E.coli* (UPEC).[14] In UPEC strains, virulence factors include the ability to adhere to uroepithelial cells, some O and K antigens, resistance to phagocytosis and to the bactericidal action of human serum.[15] Other factors known to contribute to the virulence of *E.coli* include the production of hemolysin, colicins, siderophores, cytotoxic necrotizing factor 1 (CNF 1) and cell surface hydrophobicity.[16] Adherence to uroepithelial cells is mediated by fimbrial and non-fimbrial adhesions.[17] These factors are generally lacking in the commensal or non-pathogenic *E.coli*. *Escherichia coli* caused UTI is a common infection encountered daily in medical practice and require correct diagnosis for proper management[18] and appropriate antibiotic use has unquestionable benefit. Different studies have shown that approximately 70 to 95% of community-acquired cases and about 30 to 50% of all nosocomial infections.[16] They also reported that these organisms were responsible for significant social and economic costs for both communities and public health resources.

Studies in developing countries have shown that UTI is usually present at the first antenatal visit and about 1% or less pregnent women develop bacteriuria after a negative screening in early pregnancy.[19] UTI in pregnancy contributes significantly to maternal and perinatal morbidity and mortality.[20] Maternal complications include obvious pyelonephritis in 25%-40% of patients as pregnancy advances among those with asymptomatic bacteriuria, and 1% - 2% in those without asymptomatic bacteriuria.[21] Other maternal complications include maternal anemia, hypertension, pre-eclampsia, chronic pyelonephritis, and occasionally, renal failure.[22] The fetus is at risk of prematurity, low birth weight, intrauterine growth restriction, and fetal death.

Resistance of antibiotics is yet another serious problem. This is due to overuse as well as misuse of antibiotics that resistance of antibiotics is increasing day by day.[23] UTI are usually treated with antibiotics including Nalidixic acid, Nitrofurantoin, Ofloxacin, Perfloxacin, Ciprofloxacin, Gentamycin, etc. In the last couple of years, there has been a lot of focus in scientific literature on inappropriate use of antimicrobial agents resulting in the spread of bacterial resistance.[24] The widespread and inappropriate use of antibiotics is recognized as a

significant contributing factor to the spread of bacterial resistance and the development of resistance to antimicrobial agents.[25] For most bacteria, there is evidence that increased usage of particular antimicrobials correlates with increased level of bacterial resistance to that agent. The emergence of antimicrobial resistance in the management of urinary tract infections is an important public health issue. While many antibiotics including Penicillin, Macrolides and Tetracyclines were very useful in the treatment of urinary tract infections in the past, the rates of bacterial resistance to antimicrobial agents has significantly increased and are increasing in many countries in recent times.[26] Despite the well-published concerns about the problems of inappropriate use of antimicrobial agents, or use of broad spectrum antibiotics, increasing resistance of bacteria causing urinary tract infections to antimicrobial agents remains a serious problem.

Urinary tract infections are typically treated with medications called antibiotics or antimicrobials that have antagonistic effect on the bacteria. The selective pressure of the antimicrobials selects those strains that are resistant to the applied antimicrobials causing the resistant strains to multiply and spread. Therefore, multidrug resistant (MDR) organisms are frequently found in urinary tract infection.[27] Antimicrobial therapy of UTI caused by *E. coli* is often impaired due to the resistance to commonly- used antimicrobial agents.[28]

There is a large reservoir of resistant genes, in bacterial genomes and in extra-chromosomal pieces of DNA (plasmids) that encode different mechanisms of drug resistance.[29] The transmission of antibiotic resistance, often to several drugs simultaneously, from one bacterium to another is attributed to plasmids. Understanding antibiotic resistance patterns and molecular characterization of plasmids is epidemiologically useful.[30]

The infectious diseases remain one of the greatest challenges to global health. The diagnosis of UTI is very difficult for the elder people because of the asymptomatic bacteriuria.[31] So there is an urgent need to produce the new antibacterial agents from different sources. During the past decades, the nanoparticles are attracting a great deal in biological and pharmaceutical applications.[32] Moreover, the metal oxide nanoparticles have good antibacterial activity and antimicrobial formulations comprising nanoparticles could be used as an effective bactericidal agent.[33]

Nanotechnology has already used in broad field of life, such as cosmetics, sunscreen, textiles, paints, electronics, materials engineering, agriculture, optics and industry. Moreover, the

most important nanotechnology applications that hold the expectations of providing great benefits for humanity in the future are medicine.[34]

Moreover, nanotechnology is considered to be the knowledge of the future with several opportunities for applications. The main reason for the increased interest into nanotechnology in medicine is the expectation of using the nanoparticles and nano-sized machines in different fields such as imaging, drug delivery, biosensors and cancer phototherapy. Nanotechnology developments are also of great interest in the field of microbiology especially synthesis of nano-sized drug particles with tailored physical and chemical properties creates an important approach for coping with microbial infection.[35]

Therefore, there is an acute need for more effective and long term solutions to this ever growing problem.[36] One of the promising efforts to address this challenging and dynamic pattern of infectious diseases is the use of nanotechnology. Nano technological applications in medicine have yielded a completely new field of technology that is set to bring momentous advances in the fight against a range of diseases.[37] In comparison to the conventional antibiotics, nano structured antimicrobial agents help in reducing the toxicity, overcoming resistance and lowering the cost. In addition, nanosized drug carriers are also available, which can efficiently administer the antibiotics by improving the therapeutics and pharmacokinetics of the drug. Nanotechnology also assists in development of fast, accurate and cost effective diagnostics for the detection of pathogenic microbes.

Recently, Zirconia (Zirconium dioxide, ZrO_2) finds many applications in various fields of industry. ZrO_2 nanoparticles with antimicrobial activity when embedded and coated the surface can find immense applications in water treatment, synthetic textiles, biomedical and surgical device, food processing and packaging. Moreover, the composite preparations using ZrO_2 and polymers can find better utilization due to the enhanced antimicrobial activity.

The multi resistant pathogens due to antigenic shift are ineffectively managed with current medications. This resistance to medication by pathogens has become a serious problem in public health and therefore mandating the need to develop new bactericides and virucides. Zirconium oxide nano particle (ZrO_2), having a long history of general use as an antiseptic and disinfectant, are able to interact with disulphide bonds of the glycoprotein / protein contents of microorganisms, viruses, bacteria, fungi. The ZrO_2 nanoparticles change the three dimensional structure of proteins by interfering with S-bonds and block the functional

operation of the microorganisms. Nevertheless, studies related with metal oxide nanoparticles against urinary tract infections pathogens are too limited. Hence, the present study has been made an attempt to find out the potential nanoparticles against urinary tract infection (UTI) pathogens isolated from pregnancy women.

MATERIALS AND METHODS

Sample Collection

Urine samples were collected from different age group of pregnant women from different clinics and PHCs (Primary Health Centre) from rural area of Tirupur. Urine samples were collected in sterile universal container containing 0.1 ml of Boric acid using transport media. It was place in ice pack box and was brought to the laboratory of Microbial Technology, PG and Research Department of Zoology, Chikkanna Govt. Arts College, Tirupur – 641602. After reaching the laboratory the samples were brought to room temperature and processed with in 1 hour.

Isolation of URO Pathogens from UTI Samples

Isolation of bacteria

The aseptically collected samples were serially diluted from 10^{-1} to 10^{-9} and the dilutions 10^{-4} to 10^{-6} were plated on to the Eosin Methylene Blue agar, MacConkey agar and XLD agar and incubated at 37ºC for 24 hrs. After incubation the individual colonies with different morphology were picked using sterile incisor and grown in nutrient broth and it was incubated at 37ºC for 24 hrs. Further it was plated to check for purity.

Identification of *E. coli*

Identification of the isolated bacterial pathogens was done on the basis of grams staining, morphology, and biochemical characters. (Indole, Methyl Red, Voges Proskauer, Citrate, Carbohydrate fermentation test, Starch Hydrolysis, EMB, XLD, TSI, Catalase, Oxidase, Urease, Motility).

Antibiotic Sensitivity Assay

The following antibiotic discs: Ampicillin, Gentamicin, Tetracycline, Co-trimoxazole, Amoxycillin, Amikacin, Cefodoxime, Cloxacillin, Ofloxacin, Ciprofloxacin were used for antibiotic sensitivity assay.

Preparation of Inoculum

Selected colonies were inoculated into nutrient broth then incubated at 37^{o}C for 12 hrs. These cultures were used for the experiment.

Antibiotic Susceptibility Test

The susceptibility of *E.coli* isolates to selected commonly prescribed antibiotics was performed using Kirby Bauer disc diffusion method. In this study following antibiotic impregnated disc of known concentrations was used: Ampicillin (AMP, 10mcg), Gentamicin (GEN, 10mcg), Tetracycline (TET, 30mcg), Co-trimoxazole (COT, 25mcg), Amoxycillin (AMX, 30mcg), Amikacin (AK, 30mcg), Cefodoxime (CFD, 10mcg), Cloxacillin (COX, 5mcg), Ofloxacin (OF, 5mcg), Ciprofloxacin (CIP, 30mcg) purchased from Himedia, Mumbai were applied by using the sterile forceps on the seeded with MHA plates in aseptic condition. Then the plates were incubated in vertically at 37ºC for 24 hrs.

Antibacterial Activity of ZrO_2

The antibacterial activity of the ZrO_2 nanoparticles was performed by using well diffusion method. About 20 ml of sterile molten Mueller Hinton agar was poured into the sterile petriplates. Triplicate plates were swabbed with the overnight culture of pathogenic bacteria *E.coli.* Different concentrations of nanoparticles (50µl, 100µl, 150µl) were prepared with Dimethyl sulfoxide (DMSO)**.** The isolates were selected for antibacterial assay which showed above 50% antibacterial activity against the antibiotics tested: Ampicillin, Gentamicin, Tetracycline, Co-trimoxazole, Amoxycillin, Amikacin, Cefodoxime, Cloxacillin, Ofloxacin, Ciprofloxacin. The solid medium was gently punctured with the help of cork borer to make a well. Finally the nanoparticle samples with the concentration: 50µg, 100µg, 150µg were added from the stock into each well and incubated for 24h at 37±2^{o}C. After 24 hrs of incubation, the zone of inhibition was measured and expressed as millimeter in diameter.

RESULT AND DISCUSSION

In this present study urine samples were collected from different clinics and PHC (Public Health Centre) from rural area of Tirupur city. The *Escherichia coli* was isolated from the samples. Further, the strains were confirmed by gram staining, standard biochemical test. The strains were gram negative bacilli, Indole positive, MR - positive, Voges Proskauer – negative, Citrate negative [Fig. 2], Oxidase negative, Starch hydrolysis negative, positive results were observed in case of Catalase, Nitrate reduction [Fig.2], Motility positive, Triple sugar iron agar [Fig.2]. Selective media like Eosin Methylene Blue and XLD agar media

were used to isolate the *E.coli*. It showed metallic sheen color colony and yellow color colony respectively. These colonies were isolated and stored for further experiment [Fig. 1].

Fig. 1: Isolated colonies of *E. coli* from pregnancy women.

Fig.2: Biochemical characterization of *E.coli*

In this present study antimicrobial susceptibility testing was performed using Kirby – Bauer (1979) disc diffusion method using commercial antimicrobial discs [Fig. 3].

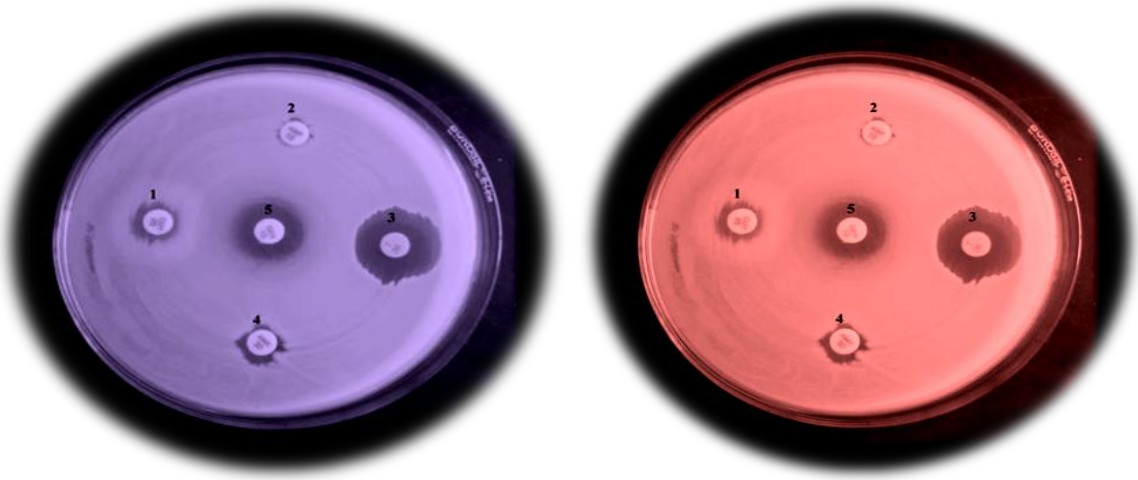

Fig.3: Antibiotic susceptibility test of *E. coli.*

1) Cefodoxime (CFD)	1) Cotrimoxazole (COT)
2) Ciprofloxacin (CIP)	2) Ampicillin (AMP)
3) Ofloxacin (OF)	3) Tetracycline (TET)
4) Amikacin (AK)	4) Amoxicillin (AMX)
5) Cloxacillin (COX)	5) Gentamycin (GEN)

Totally 10 antibiotic discs were used for this assay, among that Strain No. MK08, MK09 and MK10 showed maximum resistant of 60% and the antibiogram was AMP – COT – AMX – CPD – COX – CIP, AMP – COT – AMX – AK – CPD – COX AND AMP – GEN – COT – AMX – CPD – COX was recorded. Strains MK01 showed minimum resistant of 30% and the antibiogram AMP – COT – CPD was recorded.

The isolates were analyzed for antibiogram as described to determine the antibiotic susceptibility pattern along with the tendency of current resistance against widely used drugs. Totally, 10 different antibiogram were found in this study and the resistance was found against Ampicillin (50%), Gentamicin (4%), Tetracycline (2%), Co-trimoxazole (46%), Amoxycillin (48%), Amikacin (18%), Cefodoxime (50%), Cloxacillin (32%), Ofloxacin (0%), and Ciprofloxacin (2%).

Five strains MK05, MK07, MK08, MK09 and MK10 showed more than 50% percentage frequency against twenty five isolates of *Escherichia coli.*

The Maximum MAR index 0.60 was showed by MK08, MK09 and MK10 and minimum MAR index 0.30 was showed by MK01.

The medical application of nanoparticles is gaining popularity with an increasing number of nanoparticles based therapeutics currently in clinical development. We expect that with the introduction of safer nanomaterials together with novel engineering approaches that result in optimally designed nanoparticles, enter the clinic in future.

(Fig. 5) Different concentration of nanoparticles Zirconium oxide 50µg, 100µg, and 150µg were prepared with Dimethyl sulphoxide (DMSO), well diffusion method was used; Zone of inhibition was recorded.

The strains MK04, MK05, MK07, MK08, MK09 and MK10 which showed more than 50% resistant against 10 antibiotics were used to test against Zirconium oxide Nanoparticles.

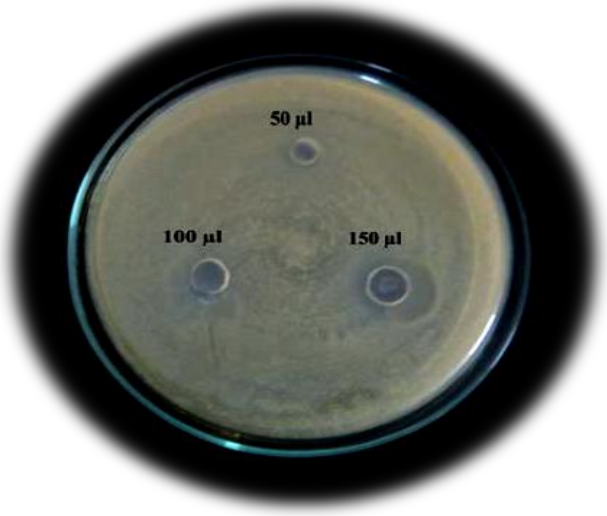

Fig. 5: Activity of Zro2 nanoparticles against of *Escherichia coli.*

Among the three concentrations of nanoparticles, maximum zone of inhibition 11mm, 16mm and 21mm was observed against the isolate MK10 at 50, 100 and 150µl concentration of nanoparticles. Minimum zone of inhibition 7mm, 10mm and 14mm was observed against the isolate MK09 at different µg concentration (50,100 and 150) of nanoparticles [Table: 1]

Table 1: Antibacterial activity of the ZrO_2 Nano particle against *E.coli.*

S. No	Strain. No	50µl	100µl	150µl
1	MK04	11mm	17mm	15mm
2	MK10	11mm	16mm	21mm
3	MK09	7mm	10mm	14mm
4	MK08	9mm	12mm	15mm
5	MK07	9mm	13mm	18mm
6	MK05	10mm	16mm	17mm

The inventor Kebira et al, (2009)[38] collected 3,341 urine samples were collected from in and out-patients attending Thika district hospital in Kenya between January and December 2008. The samples were cultured on Cystein Lysine Electrolytes Deficiency (CLED) media and the bacterial isolates recovered were tested against Trimethoprim-sulfamethoxazole, Cefuroxime, Augmentin, Nitrofurantoin, Nalidixic acid, Gentamycin, Cephaloxin, Norfloxacin, Ciproxin, Ceftazidime, Amikacin, Ofloxacin, Centriaxone, Perfloxacin, Ticarcillin, Pipril and Roceph using Kirby Bauer disc diffusion technique. Among the 3,341 samples examined, 24% had *Escherichia coli* isolates with 64% of them being from female patients compared to 36% that were from men aged above 21 years. In children aged >10yrs, boys had the highest prevalence (55%) compared to girls (45%). Those in age categories <21yrs had the more isolates (73%) followed by 5-10yrs (46%), 1-4yrs (16%), and the least 11-20yrs (5%). Upto

75% of the isolates were resistant to Trimethoprim-sulfamethoxazole; all (100%) were susceptible to Ticarcillin, Amikacin, Ofloxacin and Roceph; and 80% of the isolates were susceptible to Cephalexin, Ceftriaxime, Nalidixic acid, Gentamycin, Norfloxacin, Ciproxin, Ceftazidime/fortum and Centraixone. But in this present study 18% of *E.coli* strains were resistant to Amikacin, 4% resistant to gentamycin.

The researcher Lamido – Zaria et al. (2010)[39] collected 260 samples comprising 65 urine samples each from pregnant women. The results showed that out of the sixty-five (65) urine samples collected from pregnant women attending the health institutions, 48 (73.9%) were positive for various species of bacteria which includes *Escherichia coli* 26 (40.0%), *Staphylococcus aureus* 10 (15.4%), *Proteus* species (15.4%) and *Klebsiella* species 2 (3.1%). The result of antibiotic sensitivity test on *E.coli* isolates showed that they are highly sensitive to the quinolone group of antibiotics such as Ciprofloxacin, Ceporex and Perflacin. The organism is intermediate sensitive to Streptomycin, Tarivid and Gentamycin but resistant to Augmentin, Nalidixic acid, Septrin and Ampicillin. Similar results were recorded in the study but total of 25 isolates of *E.coli* showed intermediate sensitive to Gentamycin but resistant to Ampicillin.

The reporter Sundaram Ravikumar et al. (2012)[40] stating that the antibacterial properties of the five metal oxide nanoparticles *viz*., Al_2O_3, Fe_2O_3, CeO_2, ZrO_2 and MgO against urinary tract pathogens namely, *Pseudomonas* sp., *Enterobacter* sp., *Klebsiella* sp., *Escherichia coli*, *Proteus morganii* and *Staphylococcus aureus* by well diffusion method. Different concentrations of the nanoparticles were analyzed by minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) techniques. Finally, the potential nanoparticle Al_2O_3 which showed maximum antibacterial sensitivity was also subjected for the time kill assay method. Among the nanoparticles, Al_2O_3 nanoparticle showed maximum sensitivity (16.00±0.21) mm against *E. coli*. None of the nanoparticles showed activity against *Pseudomonas* sp. The MIC results also revealed that, the Al_2O_3 nanoparticle showed maximum inhibition at the concentration of 5 µg/mL against *E. coli*, followed by 10 µg/mL against *Klebsiella* sp. and *P. morganii*, respectively. Moreover, the time kill assay revealed that, the bacterial growth was maximum inhibited at the concentration of 5 µg/mL from the 2nd hour. It can be concluded from the present findings that, the Al_2O_3 nanoparticle can be used as an alternative antibacterial agent for the urinary bacterial diseases after completing successful clinical trials. But, in this present investigation, ZrO_2 was used as nanoparticles

against the *E.coli* isolates which is isolated from different clinics and PHC (Public Health Centre) from rural area of Tirupur. Totally six isolates MK04, MK05, MK07, MK08, MK09 and MK10 of *E.coli* were tested against different ZrO_2 concentration (50µl, 100µl, and 150µl), as the zone of inhibition were rapidly increased from 50µl - 150µl concentration of nanoparticles. Among five isolates tested, maximum zone of inhibition 11mm, 16mm and 21mm was observed against the isolate MK10 and minimum zone of inhibition 7mm, 10mm and 14mm was observed against the isolate MK09.

The author Mrithunjai singh et al. (2008)[41] found that the nanoparticles increase chemical activity due to crystallographic surface structure with their large surface to volume ratio. They used silver ions and silver based compounds including silver nanoparticles, their research showed this effect was size and dose dependent and was more pronounced against Gram – negative bacteria than Gram – positive organisms. In the present investigation, ZrO_2 was used as nanoparticles and this showed activity against *E.coli* and the activity was dependent on dose.

CONCLUSION

At, presently the emergence of antibiotic resistance in the management of UTIs is a serious public health issue, particularly in the developing world where apart from high level of poverty, ignorance and poor hygienic practices, there is also high prevalence of fake and spurious drugs of questionable. For this reason in the last few years, the researchers most prospective to choose nanoparticle have emerged as a new source for the discovery of novel therapeutically active compounds. The study finally concluded that the nanoparticle proved as bioweapon for treating the urinary tract infection this may be applied as antibacterial agent in future.

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