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Review Article

ISOLATION OF MICROORGANISM FROM STOOL SAMPLE OF DIARRHEA PATIENT AND EFFECT OF ANTIBIOTICS AND HERBAL EXTRACT

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ABSTRACT
The present investigation was based on study of stool sample collected from 15 children suffering from diarrhea, Cholera, Gastroenteritis. It includes biochemical & microbiological examination of bacteria which is considered to be etiological agent of enteric disease. After biochemical, microbiological morphology study and Gram staining technique and using PIB WIN soft ware mainly five types of pathogens *(E.coli, Vibrio cholerae, Enterobacter aerogenes, Klebsiella pneumoniae*) were identified and their percentage occurrence in the stool sample were determined. Percentage effectiveness or sensitivity of antibiotics disc (Amikacin 30 mcg/disc, Cefepime 30 mcg/disc, Chloramphenicol 30 mcg/disc, Ciprofloxacin 5 mcg/disc, Gentamycin 10 mcg/disc, Imipenem 10 mcg/disc, Tetracyclin 30 mcg/disc, Tobramycin 10 mcg/disc) & different conc. of herbal extract (10µl,20 µl ,30 µl) of *Ocimun sanctum, Aegle marmelos, Mentha spicata*) were observed on different isolated pathogens from stool sample, in addition these effects were compared.

Key *Words:* *Antibiogram, Crude Death Rate, Ethno-Pharmacology, Piperitone, Nitrofurantoin.*

INTRODUCTION
The enteric diseases are caused by the members of family Enterobacteriaceae and Vibrionaceae. These pathogens are named as enteric pathogens which belong to the genera that initiate infection by invading the intestinal epithelium. The enteric pathogens belonging to the family Enterobacteriaceae are predominantly facultative anaerobic bacterial flora of large intestine of human beings. These are generally non-spore forming, non acid fast and gram negative straight or curved rod. These organisms are classified based on their mode of use of lactose in MacConkey agar medium, the most popular medium for the isolation of fecal bacilli. The enteric disease causing members of family Enterobacteriaceae are *E.coli, Shigella, Salmonella*, Proteus, *Klebsiella pneumoniae, Citrobacter freundii, Enterobacter aerogenes*.The pathogens belonging to family Vibrionaceae are also responsible for enteric disease .The organism *Vibrio cholerae* causing cholera is characterized by Gram negative curved rod that actively motile. The vibrio colonies can be identified by the use of selective media like TCBS, MacConkey and blood agar. Socio-economically backward classes of population frequently receive the epidemics of diarrhea disease, accounting high morbidity and mortality among the people. The mortality rate due to diarrheal infection is a robust indicator of the overall health status of population. As per the SRS (sample registration system) data infant mortality was 91% in 2001 & 87% in 2002 due to diarrhea disease. The CRD (crude death rate) in Orissa in 2001 &2002 was 10.4 and 9.8% respectively. Data on severe diarrhea and gastroenteritis in children was 14.2% (as per Registration general of India, 1998 -2000).As per available report under the Orissa multi disease surveillance system 144672 diarrhea cases are registered in 2003 & 1,56,872 diarrhea cases in 2002. 15-40 % of all death among the children aged < 5years are due to diarrhea disease. The united nation said that no. of suspected cholera cases in Zimbabwe since August 2008 has claimed above 12600 with 570 deaths because of lack of water treatment, Brocken sewage pipe. As on 26thNov 2008 more than 3000 people have died from cholera. As it is being mentioned earlier world 25% death is caused by bacteria every year. It can be eradicated by developing sophisticated medical facilities and proper hygienic condition. Chemotherapeutic agent like antibiotics kills or stops the growth of susceptible microorganism. These drug include beta-lactame, aminoglycosidesand, fluroquinolones, Tetracyclins,

chloramphenicol, sulfonamide, these are effective against enterococci. But in recent year strain develops resistant to antibiotic has become international problems. Plants derived substances have become matter of great interest owing to their versatile application (Baris *et al.*, 2006). Medicinal plants are the richest bio-resources of drug of traditional system of medicine, modern medicine, food supplement and folk medicine, pharmaceutical intermediate and chemical entities of synthetic drug (Hammer *et al.*, 1999). A number of interesting outcome have been found with the use of mixture of natural product to treat disease, most notably the synergic effect of polypharmacological application of plant extract (Gibbons, 2003).The herbal extract obtained from *Aegle marmelos, Mentha spicata and Ocimum sanctum* can be used to treat enteric disease. Piperitone obtained from plant essential oil enhances the bactericidal activity of nitrofurantoin and furazolidone against bacteria belonging to family Enterobacteriaceae. *Mentha spicata & Mentha piperita* contain 40.12 % & 20.32% carvone respectively. Pure carvone and piperitone equally increases the bactericidal activity of nitrofurantoin. Ethanolics leaf extract are more effective towards the bacterial species. It contains shahidine an unstable oxazoline which is active against Gram +ve / Gram – ve both. Ethanolic leaf extract of *Ocimum sanctum* contain biologically active compound like urosolic acid, apigenin and luteolin which are responsible for antimicrobial activity. There are long term studies continuing, looking at long term effect of enteric pathogens, therefore there is a need of screening of new compound having antimicrobial activity against pathogens.

MATERIALS & METHODS

The Stool samples were collected from patients showing genuine symptoms of enteric disease like diarrhea and cholera as diagnosed by doctors of SCB medical college Cuttack. Gram stain, Antibiotics disc like (An, Cpm, C, Cf, G, I, T, Tb) for sensitivity test of isolated pathogens, differential media like MacConkey agar, TCBS, XLD, basal media NA (nutrient agar) ,media for antibiogram like MHA (Muller Hinton Agar), various chemical reagent for biochemical analysis, herbal extract of *Ocimum sanctum, Aegle marmelos, Mentha spicata* were used.

Method

The stool samples were collected from patient up to 10 years age group. The samples were brought to the laboratory in sterile container and to provide favorable condition to the microbe and to avoid drying up NSS solution was added to it.

Isolation & Identification of pathogens

All collected samples were streaked on basal media and also on the differential media like MacConkey, TCBS and XLD. All the plates were incubated at 37°c for overnight for further observation .The morphology of different isolated colonies was observed in different plates for identification and further confirmation to categories them as Gram positive/negative bacterial colonies were stained as follows using Gram's staining technique and observed under microscope (100 × objective). For further identification of isolates various biochemical test for pathogens were performed and result of biochemical test were analyzed through PIB WIN software and isolated microbes were identified.

Antimicrobial assay for antibiotics disc

The Muller Hinton agar was poured into the Petri plate and it was kept undisturbed for solidification. The plates were flooded with test inoculums and then the plate was dried. Antibiotics disc of various concentration were applied to the plates then plates were incubated at 37°c for 48 hours. The antibacterial activity was determined by measuring the diameter of the zone of inhibition surrounding bacterial growth colony. The microbial assay for the *Vibrio cholerae* was performed with Gentamycin, Tetracyclin, chloramphenicol, ciprofloxacin, Imipenem and for Enterobacteriaceae it was performed with Amikacin, ciprofloxacin, Tobramycin, Imipenem & Cefepime antibiotic disc diffusion method.

Herbal extract preparation

The leaves of corresponding plant *(Aegle marmelos, Mentha Spicata, Ocimum sanctum*) were collected and dried up completely at room temperature but in separation to each other. The dried leaves were grinded to produce fine powder. The powder was used extract preparation by dissolving it in a suitable

solvent and the complete process of extract preparation was performed in a soxhlet apparatus. The extraction was carried out for eight hours. The solution was then collected and condensed to separate the extract and solvent. All the extract were properly labeled and stored at 4°c for further use.

Antibacterial sensitivity test of herbal extract

The antibacterial sensitivity tests were performed by disc diffusion method. The various concentration of the extract was dissolved in its respective solvent. Sterile disc were prepared of 5 mm diameter and saturated with the extract solution. The discs were dried completely in the hot air for removal of solvent. The media MHA (Muller Hinton Agar) was poured into Petri plate & kept undisturbed then plate was flooded with test inoculums and plate was dried up to few minutes. Without further delay the dried discs were then applied to the plates were for overnight incubation at 37°c. Antibacterial activity was determined by measuring diameter of zone of inhibition of surrounding bacterial growth.

RESULTS

Stool samples were collected from 15 diarrheal patients as diagnosed by the doctors. The samples were initially processed in the laboratory to explore the etiology agents, the results of which were shown in different tables according to different parameters. Age also affects distribution of enteric Diseases and it is found that percentage of occurrence of enteric disease in the age group 0-5 years has 80% while age above 5 years has 20 % of occurrence of enteric disease. That means the maximum numbers of patients are between the age group of 0-5 years (80%) followed by > 5 years age group (20%).Data taken of the 15 infected patient suffering from diarrhea disease and data reveals that chance of occurrence of enteric disease is in the ratio of 3:2 in the male and female respectively. Patients belonging to Lower Income Group (LIG) suffer more from enteric diseases. Result is found to be 10 (66.66%) of Lower Income Group followed by Middle Income Group (MIG) of 5 (33.33%) cases suffering from enteric diseases among 15 infected patient. For colony characterization of culture plate in the different growth medium were examined and it is found that culture grown in the nutrient medium mucous, translucent, circular tiny medium sized colonies and gram negative rods and culture grown on the TCBS were minute sizes, lightly mucoid, initially yellow colonies but color changes to green on further incubation, gram negative curved rods while colonies grown on XLD agar and Mac Konkey agar were appears yellow mucoid colony, even margin, large in size, elevated centre and purple pink, highly mucoid, circular colonies with elevated, gram negative straight rods respectively.

Bio chemical result were analyzed by PIB WIN soft ware and reflect that prevalence of Microorganisms isolated from stool samples were *Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes, Vibrio cholerae and Citrobacter freundii* .

Sensitiveness of antibiotics against microorganism isolated from stool sample

Different antibiotics has different percentage of effectiveness against different isolated pathogens .Percentage effectiveness of Amkacin, Tobramycin, cefepime, Imipenem and Ciprofloxacin against *E. coli* are 50, 20, 30, 70 ,53 respectively for *Klebsiella pneumoniae* percentage effectiveness were 53.33, 53.33, 20, 100, 10 respectively and 46, 20, 20, 75, 05 for *Enterobacter aerogenes* and percentage effectiveness of such antibiotic disc against *Citrobacter freundii* were 52, 20, 20, 70, 10 while effectiveness of Gentamycin, Chloramphenicol, tetracyclin, Imipenem and Ciprofloxacin against *Vibrio cholerae* were 26, 66.66, 10, 80, 10.

Antibiogram studies for Enterobacteriacae family

Antibiogram studies of different enteric pathogens belonging to the family enterobacteriacae towards various antibiotics and it is found that they are more susceptible to Imipenem and Amikacin. Antibiogram studies of Amikacin, Tobramycin, cefepime, Imipenem and Ciprofloxacin for enterobacteriacae family reveals that *E .coli* shows Intermediate effect against Amikacin and resistant for Tobramycin, cefepime while sensitive towards Imipenem and Ciprofloxacin.

Table 1: Biochemical Tests of pathogens for their identification

Biochemical test	***E.coli***	***Klebsiella pneumoniae***	***Citrobacter freundii***	***Enterobacter aerogenes***	***Vibrio cholerae***
Motility at 37^{o}C	Positive	Negative	Positive	Positive	Positive
Growth at 37^{0}C	Positive	Positive	Positive	Positive	Positive
Pigment	Positive	Positive	Positive	Positive	Positive
Macconkey growth	Positive	Positive	Positive	Positive	Positive
Catalase test	Positive	Positive	Positive	Positive	Positive
Oxidase test	Negative	Negative	Negative	Negative	Positive
Simmon's citrate test	Negative	Positive	Positive	Positive	Negative
Urease test	Negative	Positive	Positive	Negative	Negative
Hydrogen Sulphide test	Negative	Negative	Negative	Negative	Negative
Arginine dehydrolase	Positive	Negative	Positive	Positive	Negative
Lysine decarboxylase	Positive	Positive	Positive	Positive	Positive
Glucose acid test	Positive	Positive	Positive	Positive	Negative
Lactose test	Positive	Positive	Positive	Positive	Positive
Mannitol test	Positive	Positive	Positive	Positive	Positive
Amylase test	Negative	Negative	Negative	Negative	Negative
Methyl red test	Positive	Negative	Positive	Negative	Negative
Voges proskauer test	Negative	Positive	Positive	Negative	Positive
Indole test	Positive	Negative	Positive	Negative	Positive
Rhamnose test	Positive	Positive	Positive	Positive	Positive
Trehalose test	Positive	Positive	Positive	Positive	Positive

Klebsiella pneumoniae were sensitive towards Amikacin, Tobramycin and Imipenem and resistant towards Cefepime and ciprofloxacin. *Enterobacter aerogenes* shows intermediate effect towards

Amikacin and resistant towards Tobramycin, cefepime, Ciprofloxacin. Citrobacter freundii are sensitive towards Amikacin, Imipenem and resistant towards Tobramycin, cefepime, Ciprofloxacin.

Antibiogram studies for Vibrio cholerae

Antibiogram studies for Vibrio cholera were showed that it is more susceptible towards Chloramphenicol Imipenem while resistant towards Gentamycin, tetracyclin, Ciprofloxacin.

Percentage effectiveness of herbal extract on enteric pathogens

Effect of different concentration (10µl, 20 µl ,30 µl) of herbal extract of three plants namely *Aegle marmelos, Mentha spicata and Ocimum sanctum* were studied on enteric pathogens. Result shows that there is no measurable percentage effectiveness of 10 µl herbal extract of *Aegle marmelos* on isolated enteric pathogens causing diarrhea and 20 µl of extract has no effect on *Vibrio cholerae, E. coli, Citrobactor freundii* while on *Klebsiella pneumoniae and Enterobacter aerogenes* percentage effectiveness were 25 and 10 respectively. Effect of 30 µl of *Aegle* leaf extract on *Vibrio cholera, E. coli, Citrobactor freundii, Klebsiella pneumoniae* and *Enterobacter aerogenes* were 25, 0, 0,45, 43 respectively. Percentage effectiveness of 10 µl of herbal extract of *Mentha spicata* on Enteric pathogens (*Klebsiella pneumoniae* and *Enterobacter aerogenes*, *Vibrio cholera, E .coli, Citrobactor freundii*) were found to be 40, 45, 25, 43, 45 respectively while effect of 20 µl of herbal extract of *Mentha* were 60, 53, 30, 42, 40 respectively and 30 µl of herbal extract shows 45, 58, 52, 40, 45 effectiveness respectively. Percentage effectiveness of 10 µl of herbal extract of *Ocimum sanctum* on Enteric pathogens (*Klebsiella pneumoniae and Enterobacter aerogenes, Vibrio cholera, E. coli, Citrobactor freundii*) were found to be 10, 20, 22, 30, 30 respectively while effect of 20 µl of herbal extract of Ocimum were 45, 25, 22, 40, 20 respectively and 30 µl of herbal extract shows 65, 20, 42, 40, 48 effectiveness respectively.

Sensitivity of Enteric pathogens towards different concentration of herbal extract

Klebsiella pneumoniae, E. coli, Enterobacter aerogenes , Citrobactor freundii, Vibrio cholera were resistant towards 10 µl and 20 µl of herbal extract of *Aegle marmelos* while *Klebsiella pneumoniae, Enterobacter aerogenes* were intermediate towards 30 µl of *Aegle* extract and *E. coli, Citrobactor freundii, Vibrio cholera* were resistant. *Klebsiella pneumoniae, E. coli, Enterobacter aerogenes , Citrobactor freundii, Vibrio cholerae* were resistant towards 10 µl of herbal extract of *Ocimum sanctum* and 20 µl of extract have intermediate effect on *Klebsiella pneumoniae, E. coli* and *Enterobacter aerogenes , Citrobactor freundii, Vibrio cholera* were resistant while *Klebsiella pneumoniae* was sensitive for 30 µl of *Ocimum* extract and *E. coli, Citrobactor freundii, Vibrio cholera*e were intermediate towards 30 µl of *Ocimum* extract and *Enterobacter aerogenes* was resistant. *Klebsiella psneumoniae, E. coli, Enterobacter aerogenes, Citrobactor freundii* were showed intermediate effect towards 10 µl of extract of *Mentha* and vibrio cholera was resistant. Klebsiella pneumoniae, *Enterobacter aerogenes* were sensitive towards 20 µl of *Mentha* extract while *E. coli, Citrobactor freundii* were intermediate and *Vibrio cholerae* was resistant. *Klebsiella pneumoniae, E. coli, Citrobactor freundii* were intermediate for 30 µl of *Mentha* extract *Enterobacter aerogenes, Citrobactor freundii* were intermediate and *Vibrio cholerae* was resistant towards and *Enterobacter aerogenes, Vibrio cholerae* were sensitive.

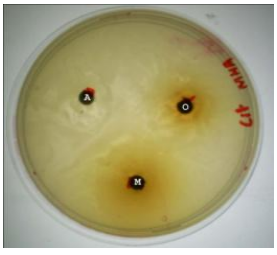

Figure 1: Effect of 10µl of herbal extract on *Citrobactor freundii*.

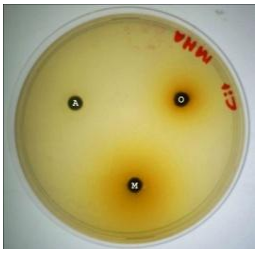

Figure 2: Effect of 20µl of herbal extract on *Citrobactor freundii.*

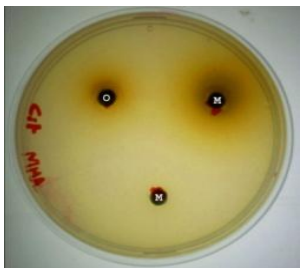

Figure 3: Effect of 30µl of herbal extract on *Citrobactor freundii.*

Figure 4: Effect of 10µl of herbal extract on *E. coli.*

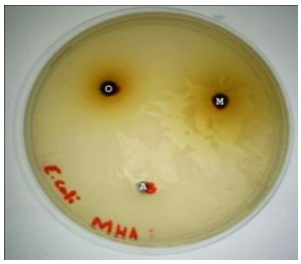

Figure 5: Effect of 20µl of herbal extract on *E. coli*

Figure 6: Effect of 30µl of herbal extract on *E. coli*

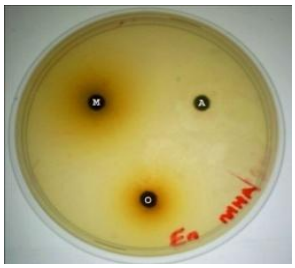

Figure 7: Effect of 10µl of herbal extract on *Enterobacter aerogenes.*

Figure 8: Effect of 20µl of herbal extract on *Enterobacter aerogenes.*

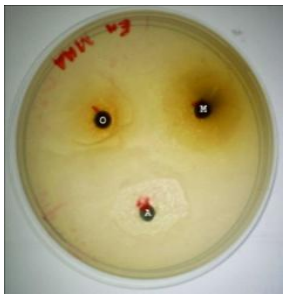

Figure 9 Effect of 30µl of herbal extract on *Enterobacter aerogenes.*

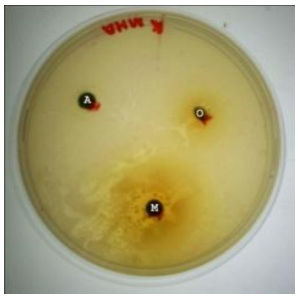

Figure 10: Effect of 10µl of herbal extract on *Klebsiella* pneumoniae.

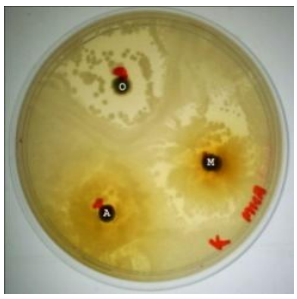

Figure 11: Effect of 20µl of herbal extract on *Klebsiella pneumoniaee*.

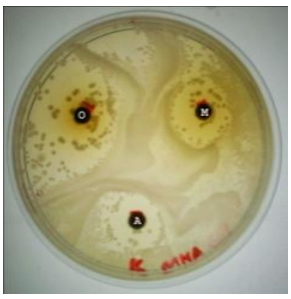

Figure 12: Effect of 30µl of herbal extract on *Klebsiella pneumoniaee.*

Figure 13: Effect of 10µl of herbal extract on *Vibrio cholerae.*

Figure 14: Effect of 20µl of herbal extract on *Vibrio cholerae*.

Figure 15: Effect of 30µl of herbal extract on *Vibrio cholerae.*

DISCUSSION

Enteric diseases in children is a common illness and accounts for substantial proportion of consultation by doctors & health care centers. It is a major cause of morbidity & mortality in poor & developing countries. Several studies have been conducted with a view to establish the importance of different enteric bacteria in the etiology of acute diarrhea. However, the relative incidence of the pathogens varies from place to place & also in same geographic region from time to time.The present investigation was a hospital based study comprising of 15 children suffering from diarrhea, cholera & gastroenteritis. It includes the microbiological examination of bacteria which is considered to be the etiological agent of enteric diseases in children. The antibiogram study along with the Ethno-Pharmacological studies was carried out & the effectiveness was compared. The enteric diseases are more prevalent during the summer and rainy seasons. But rarely is noticed during winter. The reason behind this is the improper hygiene, contaminated food and drinking water as suggested in 1979 by Koornhof.The age wise distribution of patients suffering from enteric diseases. Among the cases studied maximum were between age group 0 – 5 yrs (80%) followed by 5 – 10 yrs (20%) which is supported by Desenclos *et al.,* (1998) who reported that children below 5 yrs of age were effected more than others. Griaster (1989) also reported that highest incidence of enteric diseases in the infant age group. The results were supported by Agarwal *et al.,* (1990). Behera *et al.,* (1979) who also observed that children below 5 yrs age are more affected than other. The sex-wise distribution of patients in the study states that out of 15 samples investigated the percentage of infection was found more in male (60%) than the female patients (40%).This data is supported by Ahmed *et al.,* 1995) according to him, the occurrence was more predominant in male than in female in the ratio of 3 : 2. The higher incidence of suffering in male babies may be due to lower body resistance as a result of certain genetic factors (Boris *et al.,*, 1964).The economic status of the family of patient (LIG & MIG) showed that incidence of enteric disease is 66.66% belong to lower income group followed by middle income group (33.3%). This finding is in accordance with Koornhof *et al.,* (1979) who reported that enteric diseases are particularly predominant in persons of low income group & maintaining improper hygiene. The colony & gram staining

characteristics of various microbial colonies isolated from the media plates. The media plates being NA, TCBS, XLD & MacConkey. It was found that most of the organisms were Gram negative bacilli. This data is supported by Bardhan *et al.*, (1998). Occurrence of different types of microbes in stool sample and microorganisms found in the stool sample are *E Coli, Klebsiella pneumoniae, Enterobacter aerogenes, Citrobacter freundii, Vibrio cholerae*. *E Coli* was found in larger proportion (66.66%) as supported by Levine *et al.*, (1993). Antibiogram studies of different GNB belonging to *Enterobacteriaceae* which were done using different antibiotic discs. Accordingly results were presented as resistant (R), sensitive (S), intermediate (I) using zone interpretative table supplied. Imipenem & Amikacin were found most susceptible to *Enterbacteriaceae.* This data is supported by Gonlugar (2004). According to Lockhart (2007) the rate of resistance to Ciprofloxacin has increased over the year. The antibiogram studies for *Vibrio cholerae* done by using antibiotic discs. Imipenem & Chloramphenicol were found to be more susceptible towards *Vibrio* supported by Clark RB (1992) & Grady (2002) respectively. Effect of various plant extracts on enteric pathogens. The maximum effect was noticed in case of *Mentha spicata* on *E Coli, Klebsiella* as supported by Priyabratta Pattnayak (2008). *Ocimum sanctum* showed intermediate effect on *E.Coli*, *Citrobacter*, *Enterobacter* as supported by Nair R, Chanda S (2006).

Conclusion

The strains were identified as *Vibrio cholerae, E.Coli, Klebsiella pneumoniae, Enterobacter aerogenes and Citrobacter freundii*. Certain strains showed antibiotic resistance towards specific antibiotics that were used. In order to find out the effectiveness of various plant extracts, the microbes were treated with plant extracts of various concentrations. The plant extract of highest concentration was more effective than the extract of same plant & having lower concentration .The antibacterial property of the solvent extracts of *Ocimum sanctum, Mentha spicata, Aegle marmelos* may be attributed to following reasons**,** the nature of biological active components whose activity can be enhanced in the presence of solvents and the stronger extraction capacity of solvents could have produced greater number of active components responsible for antibacterial activity. In recent times the biggest issue concerning the treatment of bacterial diseases is the development of antibiotic resistant strains. So more research is going on to find out the gene, plasmid or mechanism involved in deactivating the antibiotic. So the need of the hour is to start synthesizing new antibiotic compounds to overcome this antibiotic resistance problem. Also the various plant products should be tested against these pathogens & the key compound should be identified & isolated. Even though the plant products are not going to be that much effective as the antibiotics, but these are safe to use and any side effects related to prolonged use of antibiotics can be avoid.

Future aspect

The future aspects of this work include the screening of variety of medicinal plants possessing antimicrobial activity which can be effective against different pathogens. The preliminary bioinformatics analysis can be carried out for the identification of the genes responsible for antibiotic resistance. The gene has to be studied extensively because they may cause destructive effects if transferred to other related genera of human pathogenic bacteria. Antibiotic sensitivity and resistance patterns can be analyzed from time to time and this will help in effective medication against infectious pathogens.

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