CHAPTER - 10

STUDIES ON ANTIMICROBIAL ACTIVITY

10.1. Introduction

Infectious diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality world wide[1]. During the past several years there has been an increasing incidence of bacterial and fungal infections. This fact coupled with the resistance to antibiotics and with the toxicity during prolonged treatment with several antimicrobial drugs has been the reason for an extended search for newer drugs to treat opportunistic microbial infection[2,3].

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased in India. According to World Health Organization[4] medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency[5]. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency[6-12]. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example the phenolic compounds which are part of the essential oils[13].

Historically, plants have provided a good source of anti-infective agents and many of them remain highly effective in the fight against microbial infections. Besides, they are cost effective and have fewer side effects[14,15]. The antiseptic properties of aromatic and medicinal plants and their extracts have been recognized since antiquity, while attempts to characterize these properties in the laboratory date back to the early 1900s[16,17]. Natural product contributes to a great extent to fight against pathogenic microorganisms. Many plants or their parts are used in food as spices and are thought to provide a natural preservation by inhibiting the microbial growth[18]. Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made

large contributions to human health and well-being. Their role is two fold in the development of new drugs, viz- they may become the base for the development of a medicine, and a natural blueprint for the development of new drugs or a phytomedicine to be used for the treatment of disease. The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha*, and related species, has been used for many years as and amoebicidal drug as well as for the treatment of abscesses due to the spread of *Escherichia histolytica* infections. Another important drug of plant origin with a long history of use is quinine. This alkaloid occurs naturally in the bark of *Cinchona* tree. Apart from its continued usefulness in the treatment of malaria, it can be also used to relieve nocturnal leg cramps. Currently, the widely prescribed drugs are analogs of quinine such as chloroquine. Some strains of malarial parasites have become resistant to the quinines; therefore antimalarial drugs with novel mode of action are required[19].

Similarly, higher plants have made important contributions in the areas beyond anti-infective, such as cancer therapies. Early examples include the antileukaemic alkaloids, vinblastine and vincristine, which were both obtained from the Madagascan periwinkle (*Catharanthus roseus* syn. *Vinca roseus*)[20]. Other cancer therapeutic agents include taxol, homoharringtonine and several derivatives of camptothein. For example, a well-known benzyl isoquinoline alkaliod, papaverine, has been shown to have a potent inhibitory effect on the replication of several viruses including cytomegalovirus, measles and HIV[21]. Most recently, three new atropisomeric naphthylisoquinoline alkaloid dimers, michellamines A, B, and C were isolated from a newly described species of tropical liana *Ancistrocladus korupensis* from the rainforest of Cameroon. The three compounds showed potential anti-HIV with michellamine B being the most potent and abundant member of the series. These compounds were capable of complete inhibition of the cytopathic effects of HIV-1 and HIV-2 on human lymphoblastoid target cell *in vitro*[22].

Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources. It was the discovery of penicillin that led to later discoveries of antibiotics such as streptomycin, aureomycin and chloromycetin. Though most of the clinically used antibiotics are produced by soil micro-organisms or fungi, higher plants have also been a source of antibiotics Examples of these are the bacteriostatic and fugicidal properties of *Lichens*, the antibiotic action of allinine in *Allium sativum* (garlic),

or the antimicrobial action of berberines in goldenseal *Hydrastis canadensis*[23]. Plant based antimicrobials represent a vast untapped source for medicines. Continued and further exploration of plant antimicrobials needs to occur. Plants based antimicrobials have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. Many plants have tropisms to specific organs or systems in the body. Phytomedicines usually have multiple effects on the body. Their actions often act beyond the symptomatic treatment of disease. An example of this is *Hydrastis canadensis*. Hydrastis not only has antimicrobial activity, but also increases blood supply to the spleen promoting optimal activity of the spleen to release mediating compounds[24].

Plants have supplied over 25% of prescription drugs used in human medicine and such pharmacologically active plants have also provided leads to natural pesticides. Himalayas has an extraordinarily rich flora and wide knowledge of indigenous medicinal plants is well documented. Accordingly, we are investigating the potential of Himalayan medicinal plants as a resource for new antimicrobial agent[25].

Critical review of literature revealed that the potency of the plant inhibiting the growth of pathogenic bacteria remained unexplored. So the present study was designed to investigate the antimicrobial activity of the methanol extract of rhizomes of *K. rotunda* and leaves of *E. cannabinum* on different pathogenic bacterial strains by using *in vitro* model system.

10.2. Materials and methods

10.2.1. Plant materials

Methanol extracts of rhizomes of *K. rotunda* and leaves of *E. cannabinum* were subjected as test drug in these experiments.

10.2.2. Drugs and chemicals

The standard antibiotic ciprofloxacin used in this study was obtained as a gift sample from Ankur Drugs, Himachal Pradesh. Dimethyl sulphoxide (DMSO) and Mueller Hinton Agar were obtained from Ranbaxy Fine Chemicals.

10.2.3. Microorganisms

A total of 80 strains belonging to seven different genera (*Staphylococcus Species, Salmonella Species, Shigella Species, Klebsiella Species, Pseudomonas, Vibrios, E. coli Species*) comprising both Gram positive and Gram negative were used as test organisms which were obtained from Dept. of Pharmaceutical Technology, Jadavpur University, Kolkata, India. They were used to evaluate the antibacterial activities of the methanolic extract of both the plants. All the strains were clinically isolated from human beings.

10.2.4. Media

10.2.4.1. Liquid media

- **Peptone water**

Peptone water having the following composition was used for the cultivation of bacterial strains as well as for spot inoculation.

Bacteriological peptone (Oxoid)	- 1.0%
Sodium chloride (Analar)	- 0.5%

The pH was adjusted to 7.2 to 7.4 and the volume was made up with distilled water.

- **Alkaline peptone water**

This alkaline medium used for the cultivation of *Vibrio cholerae*, was prepared as per the following composition:

Bacteriological peptone (Oxoid)	- 1.0%
Sodium chloride (Analar)	- 0.5%
pH adjusted to	- 8.5 to 9.0

- **Nutrient broth**

Bacteriological peptone (Oxoid)	- 1.0%
Beef extract (Oxoid)	- 0.5%
Sodium chloride (Analar)	- 0.5%
pH adjusted to	- 7.2 to 7.4

10.2.4.2. Solid media

- **Nutrient agar**

This medium was used to isolate pure cultures of Gram-positive bacteria. It contained the following ingredients:

Agar (Oxoid)	- 3.0%
Beef extract (Oxoid)	- 0.5%
Bacteriological peptone (Oxoid)	- 1.0%
Sodium chloride (Analar)	- 0.5%
pH adjusted to	- 7.2 to 7.4

- **Bromothymol blue lactose agar**

This medium consisted of the following ingredients:

Agar (Oxoid)	- 3.0%
Bacteriological peptone (Oxoid)	- 1.0%
Beef extract (Oxoid)	- 0.5%
Sodium chloride (Analar)	- 0.5%

The pH was adjusted to 7.2 to 7.4 and 1.25 ml of bromothymol blue was added per 100 ml of the medium. After sterilization, 1.0% lactose was added, steamed for 30 minutes and poured in sterile petri dishes. This medium was used to isolate pure cultures of Gram-negative bacteria.

10.2.5. Preservation of bacterial cultures

All the strains of *Staphylococci, E. coli, Klebsiellae, Salmonellae, Shigellae, Pseudomonas* spp. and *Vibrios* were preserved as stab slant cultures at a temperature of 4°C. All these strains were checked for purity and identified where necessary. Routine subculturing of the Gram-positive bacteria was carried out on nutrient agar and Gram-negative strains on bromothymol blue lactose agar[26].

10.2.6. Preparation of impregnated discs of extract and standard antibiotics

The filter paper discs of 6mm diameter were prepared by using Whatman filter paper No.1 and were sterilized by dry heat at 160°C for an hour in batches of 100 in screw capped Bijou bottles. The dried extract (semisolid) of *K. rotunda* rhizome and *E. cannabinum* leaves were weighed and dissolved in 0.5ml of (DMSO), as the extracts are not completely soluble in water, and then diluted in sterile distilled water to make the required stock solutions. For each extract three stock solutions were prepared. Similarly the stock solution of the standard antibiotic was prepared by dissolving the required amount of ciprofloxacin. All the stock solutions were then kept at 4°C and used for three months. For preparation of antibiotic impregnated discs 1.0ml of the stock solution of the antibiotic was added separately to each bottle of 100 discs. Each discs adsorbed 0.01ml of the solution, so the entire 1.0ml volume was adsorbed by the 100 discs, each giving the required two fold concentrations of 0-500µg/ml. The procedure was repeated for preparation of impregnated discs of the plant extracts. The discs were used in wet condition and for further use they were stored at 4°C, as the discs can retain their moisture and potency for at least 3 months in the screw capped bottles.

10.2.7. Antimicrobial activity of extract of *K. rotunda* rhizome (*In vitro* model)

Microbial sensitivity tests were performed by disc diffusion method[27]. Petriplates were prepared aseptically by pouring 20ml of Mueller Hinton Agar and allowed to solidify. Plates were dried and 0.1ml of standardized inoculums suspension was poured and uniformly spread. The excess inoculum was drained and the inoculum was allowed to dry for 5 minutes, then the discs were placed in the inoculated agar. Ciprofloxacin (5µg/disc) was used as positive control and 5% DMSO was used as negative control in these assays. The inoculated plates were incubated at 37^0C for 24 hours. The zones of inhibitions observed were measured in millimeters after 24h. Each assay in this experiment was repeated three times. The sensitivity was recorded for a zone of clearance around the discs. The MICs were determined by the standard agar dilution method[28].

The crude methanol extract was dissolved in 0.5ml of DMSO, as they are not completely soluble in water, and then diluted by sterile distilled water to make solution. The drug solution was then added to the molten nutrient agar in different tubes to give final

concentrations of 0-500µg/ml. The contents of the tubes were mixed thoroughly, pH adjusted to 7.2 to 7.4 and poured into sterile Petri dishes. The cultures were diluted to 10^6 colony forming units (CFU), spot inoculated using a bacterial planter (10µl) on the nutrient agar sterile Petri discs and incubated at 37°C±2°C for 24h to determine the MIC of the extracts. The lowest concentration of the plate, which did not show any visible growth after incubation, was considered as MIC. The agar plate containing only sterile distilled water and ciprofloxacin was served as negative and positive control respectively[29,30].

10.2.8. Antimicrobial activity of extract of *E. cannabinum* leaves (*In vitro* model)

The antimicrobial activity of methanol extract of *E. cannabinum* leaves was determined as per the methods described above in 10.2.7 for *K. rotunda.*

10.3. Results

Out of seven different pathogenic Gram-positive and Gram-negative strains, the significant results were obtained with one Gram-positive i.e. *S. aureus* NCTC 6571 and two Gram-negative bacteria i.e. *Shigella dysentariae-2* and *Klebsiella pneumoniae 14.* Table 10.1 and 10.2 represents that the MIC of ciprofloxacin is 10µg/ml whereas the MIC of *K. rotunda* and *E. cannabinum* varies from 100 to 500µg/ml. From table 10.3, it is revealed that the diameters of the individual zone of inhibition of *K. rotunda* and ciprofloxacin against *S. aureus* NCTC 6571 were 6.1mm and 13.2mm respectively, which have been enhanced to 6.3 and 13.7mm respectively when both the disc was placed in the same culture. In the test of combined effect of crude extract and antibiotic, the surface areas of zones of inhibition were increased by 6.66% for *K. rotunda* and 7.72% for ciprofloxacin. It is also seen that in case of *Shigella dysentariae-2*, the diameters of zone of inhibition of *K. rotunda* and Ciprofloxacin were 9.4 and 15.0mm. These diameters were increased to 9.7 and 15.6mm with the percentage increase of 6.48 and 8.16 respectively when the discs were placed on the same culture. Similarly, when the discs of *K. rotunda* and ciprofloxacin were placed on plates flooded with *Klebsiella pneumoniae 14*, a notable increase in diameter of zones of inhibition were observed independently. These on combination showed an increase in diameter and the percentage increase data were found to be 6.35 for *K. rotunda.* and 8.10 for ciprofloxacin. From table

10.4, it is shown that the percentages of zone of inhibition were increased to 3.42 for *E. cannabinum* and 4.60 for ciprofloxacin against *S. aureus* NCTC 8530. Similarly, for *E. cannabinum* and ciprofloxacin the increased percentage of zones of inhibition were noted as 3.88 in former and 4.04 in latter case for *Shigella dysentariae-2* and were found to be 3.96 % and 5.36 % respectively for *Klebsiella pneumoniae 14*. In fig. 10.1 it was observed that the crude methanol extract of *K. rotunda* exhibits better antibacterial potency against selected pathogenic Gram-positive and Gram-negative strains as compared to the extract of *E. cannabinum*.

10.4. Synergistic effect

Synergism between the compounds is said to occur when more inhibition results from the combination form than that of the individuals. This implies that one drug is affecting the microorganism in such a way that it becomes more sensitive to the inhibitory effects of the other. A total of seven bacterial strains belonging to both Gram-positive and Gram-negative were screened for synergistic effect by the combination of plant extracts with standard antibiotic ciprofloxacin. The combined effect of two agents was performed as described by Miles and Amyes[31] following the procedure adopted by Lamanna and Shapiro[32]. The discs were placed at suitable distances apart so that the respective agents would not diffuse into one another to produce a continuous range of concentrations in the initial period of inhibition. In synergism, the ridge of growth disappeared and the two circles merged to one forming a single asymmetric ellipse[33]. The synergistic effect from the association of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment.

The sensitivity tests of the microorganisms with respect to particular concentration (plant extract and antibiotic) were determined by measuring the diameters of zone of inhibition.

Table 10.1. Antibacterial spectra for MIC of Ciprofloxacin

Sl. No	Bacterial Species	No. of microorganism tested	MIC of CF ($\mu g.ml^{-1}$)			
			02	05	10	25
1.	*Staphylococcus Species*	14	-	01	10	03
2.	*Salmonella Species*	10	-	02	04	04
3.	*Shigella Species*	15	01	04	07	03
4.	*Klebsiella Species*	05	-	01	03	01
5.	*Pseudomonas*	06	01	01	04	-
6.	*Vibrios*	20	02	07	10	01
7.	*E. coli Species*	10	-	02	06	02

CF = Ciprofloxacin

Table 10.2. Antibacterial spectra for MIC of methanol extract of *Kaempferia rotunda* and *Eupatorium cannabinum*

Sl. No	Bacterial Species	No. of bacteria tested	MIC of KR ($\mu g.ml^{-1}$)					MIC of EC ($\mu g.ml^{-1}$)				
			50	100	200	500	750	50	100	200	500	750
1.	*Staphylococcus* Species	14	-	02	08	03	01	-	01	10	03	-
2.	*Salmonella* Species	10	01	02	05	02	-	01	01	04	03	01
3.	*Shigella* Species	15	01	03	08	02	01	01	02	07	04	01
4.	*Klebsiella* Species	05	01	01	02	01	-	-	01	02	01	01
5.	*Pseudomonas*	06	-	02	04	-	-	01	-	04	01	-
6.	*Vibrios*	20	03	06	08	02	01	02	05	07	05	01
7.	*E. coli Species*	10	-	02	02	06	-	01	-	05	03	01

KR = *Kaempferia rotunda*

EC = *Eupatorium cannabinum*

Table 10.3. Synergism between *Kaempferia rotunda* extract and ciprofloxacin by disc diffusion method

Sl. No	Bacterial Strains	Diameter of the inhibition zone(mm)				% of increase of zone of inhibition	
		Single effects (A)		Combined effects (B)			
		KR* (X±SEM)	CF** (X±SEM)	KR* (X±SEM)	CF** (X±SEM)	KR* (X±SEM)	CF** (X±SEM)
1.	*S. aureus* NCTC 6571	6.1±0.062	13.2±0.056	6.3±0.014	13.7±0.031	6.66±0.021	7.72±0.028
2.	*S. aureus* NCTC 8530	6.3±0.021	14.2±0.045	6.5±0.015	14.7±0.036	6.45±0.019	7.16±0.056
3.	*S. typhi 59*	8.2±0.047	13.5±0.089	8.4±0.018	13.9±0.032	4.94±0.016	6.01±0.065
4.	*S. dysenteriae 2*	9.4±0.032	15.0±0.029	9.7±0.019	15.6±0.039	6.48±0.022	8.16±0.057
5.	*Klebsiella pneumoniae 14*	9.6±0.051	15.1±0.012	9.9±0.017	15.7±0.041	6.35±0.025	8.10±0.048
6.	*Vibrio cholera 14033*	6.8±0.032	10.9±0.061	7.0±0.021	11.3±0.029	5.97±0.018	7.47±0.053
7.	*E. coli-10*	6.6±0.035	13.5±0.045	6.8±0.016	13.9±0.033	6.15±0.024	6.01±0.049

SEM=standard error mean (n=3)

KR= *Kaempferia rotunda*, CF = Ciprofloxacin

(*) = 200 $\mu g.ml^{-1}$ in disc, (**) = 10 $\mu g.ml^{-1}$ in disc

Mean surface area of the inhibition was calculated as πr^2 on basis of their mean diameter (2r) and % increase was calculated as (B-A/A) x 100

Table 10.4. Synergism between *Eupatorium cannabinum* extract and ciprofloxacin by disc diffusion method

Sl. No	Bacterial Strains	Diameter of the inhibition zone (mm)				% of increase of zone of inhibition	
		Single effects (A)		Combined effects (B)			
		EC* (X±SEM)	CF** (X±SEM)	EC* (X±SEM)	CF** (X±SEM)	EC* (X±SEM)	CF** (X±SEM)
1.	*S. aureus* NCTC 6571	5.4±0.038	14.2±0.056	5.5±0.014	14.6±0.029	3.73±0.021	5.71±0.028
2.	*S. aureus* NCTC 8530	5.9±0.025	13.2±0.045	6.0±0.021	13.5±0.031	3.42±0.019	4.60±0.056
3.	*S. typhi 59*	4.9±0.056	13.5±0.089	5.0±0.074	13.8±0.039	4.12±0.016	4.49±0.065
4.	*S. dysenteriae 2*	5.2±0.024	15.0±0.029	5.3±0.025	15.3±0.041	3.88±0.022	4.04±0.057
5.	*Klebsiella pneumoniae 14*	5.1±0.042	15.1±0.012	5.2±0.031	15.5±0.052	3.96±0.025	5.36±0.048
6.	*Vibrio cholerae 14033*	5.5±0.56	10.9±0.061	5.6±0.033	11.1±0.036	3.66±0.018	3.70±0.053
7.	*E. coli-10*	5.3±0.084	13.5±0.045	5.4±0.041	13.8±0.054	3.80±0.024	4.49±0.049

SEM=standard error mean (n=3)

EC= *Eupatorium cannabinum*, CF = Ciprofloxacin

(*) = 200 $\mu g.ml^{-1}$ in disc, (**) = 10 $\mu g.ml^{-1}$ in disc

Mean surface area of the inhibition was calculated as πr^2 on basis of their mean diameter (2r) and % increase was calculated as (B-A/A) x 100

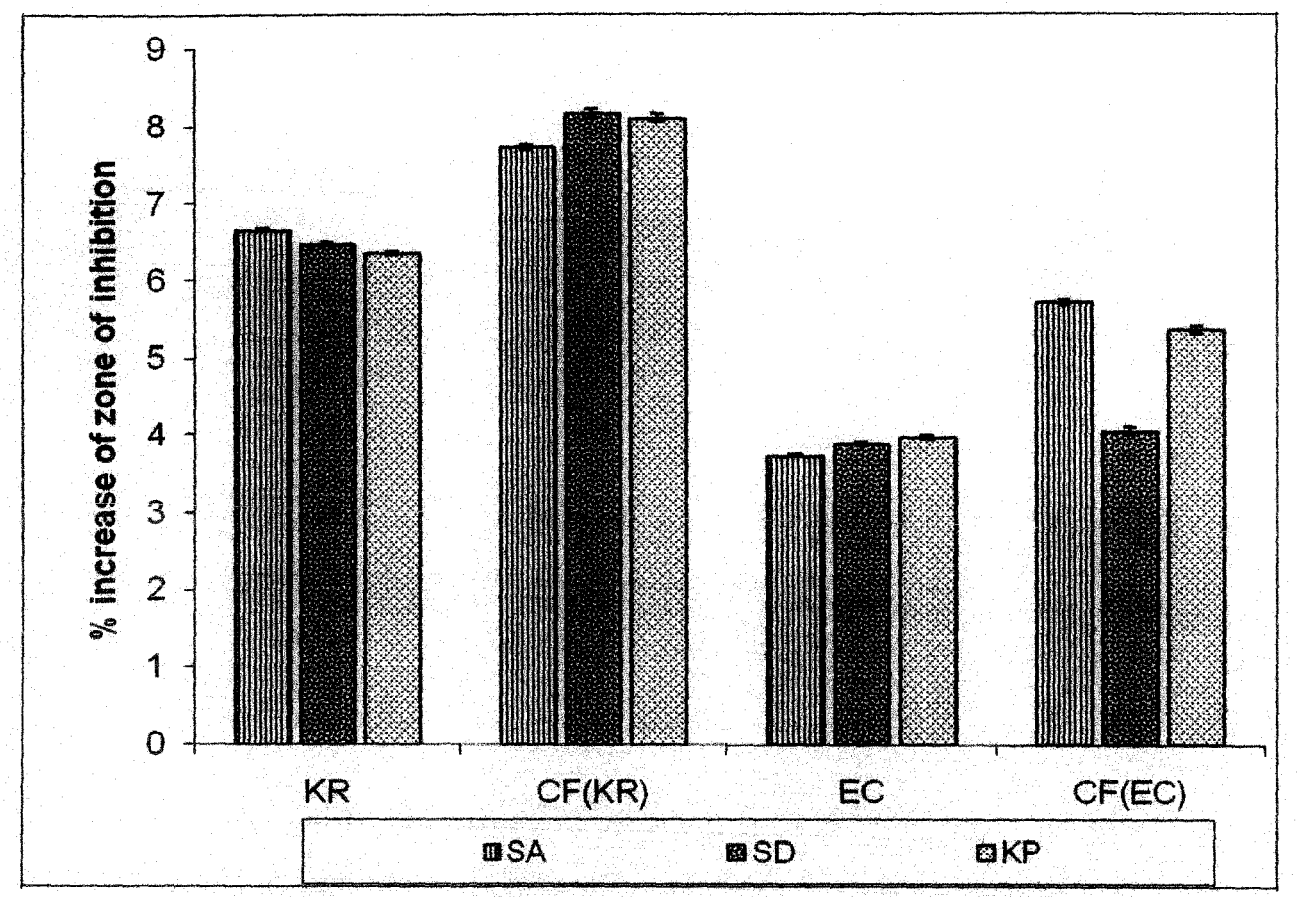

Fig 1: Synergism profile of plant extracts with Ciprofloxacin against SA, SD and KP

SEM = Standard error mean (n=3)

KR = *Kaempferia rotunda*, EC = *Eupatorium cannabinum*, CF = Ciprofloxacin

CF (KR) = % increase of ciprofloxacin with respect to *Kaempferia rotunda*

CF (EC) = % increase of ciprofloxacin with respect to *Eupatorium cannabinum*

SA = *S. aureus* NCTC 6571, SD = *S.dysenteriae-2*, KP = *Klebsiella pneumoniae 14*

10.5. References

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