CHAPTER 8

ANTIMICROBIAL ACTIVITY

8.1. Introduction

The use of plants as source of medicine for the treatment of many diseases dated back to prehistory and people of all continents follow this old tradition. Despite the remarkable progress of synthetic organic chemistry in the twentieth century, over 25% of prescribed medicines in industrialized countries derived directly or indirectly from plants [1]. However, plants used in traditional medicine are still understudied, particularly in clinical microbiology [2].

Infectious diseases are responsible for one-quarter of all the deaths occur in the world, second to the cardiovascular diseases [3]. In the modern world filled with infectious disease due to poor hygiene and insufficient sewage distribution systems the advent of antibiotics was a boon to modern medicine. Myriad infectious diseases continue to kill millions of people every year, especially in developing nations. Many of these diseases are effectively treated with antibiotics or antimicrobial agents. However, the over use of antibiotics, as well as the prevalence of antibiotics introduced to human through the food chain, is contributing to a problem that may result in future plagues and uncontrollable epidemics.

Bacteria and microbes, like human being, are very adept at adapting. When continually exposed to the same antibiotic agent, microbes eventually develop a resistance to its killing effects. Emergence of multiple drug resistant strains has appeared as a real problem in the field of medical science. The primary cause of the development of resistance is occurrence of random mutations. Mutations may occur in genes responsible for conferring sensitivity against a drug. With a relative dearth of new antibiotics with novel mode of action we may find ourselves on the verge of a medical disaster. It is high time to revive the hidden wonders of plant molecules with the modern tools of target based screening to develop newer advanced generation drugs and antibiotics with novel modes of actions [4, 5]. Combinations of active molecules from natural sources like plants need to be systematically explored failing which the consequences are destined to be devastating and out of control for the human race in the new millennium. Among the most promising advances in the field of drug development is discovering new molecules

or novel uses of the already available compounds with known efficacy and without any side effects. Such active biomolecules combined with other antibiotics can kill the drug resistant bacteria and simultaneously check further development of resistance in the infectious microbes. Based on this information the present study was carried out to evaluate the antimicrobial activity of the methanol extract of leave of *C.oppositifolia* and root of *H.nepalense* as well as their isolated compound on different pathogenic bacterial strains by using *in vitro* and *in vivo* model system.

8.2. Materials and Methods

8.2.1. Plant materials

Methanol extracts of *Colebrookea oppositifolia* leaves and *Heracleum nepalense* roots as well as their isolated compounds (described in **Chapter 3**) were used as test drug in these experiments.

8.2.2. Microorganisms

A total of 257 bacterial strains belonging to different genera were tested in this study. The test organisms were obtained from Department of Bacteriology, Calcutta School of Tropical Medicine and Institute of Microbial Technology (IMTECH), Chandigarh, India. All the strains are of human origin and were isolated in our Institute, as well from different parts of India and abroad. Three multiresistant *Staphylococcus* strains (MRSC) (*Staphylococcus aureus* ML 275, *Staphylococcus aureus* NCTC 8530 and *Staphylococcus epidermidis* 865) were kindly provided by Prof. (Mrs.) Sujata Ghosh Dastidar, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

8.2.3. Media

8.2.3.1. Liquid media

8.2.3.1.1. 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.

8.2.3.1.2. 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

8.2.3.1.3. 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

8.2.3.2. Solid media.

8.2.3.2.1. 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

8.2.3.2.2. 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.

8.2.4. Preservation of bacterial cultures

All the strains of staphylococci, streptococci, bacilli, *E.coli*, klebsiellae, salmonellae, shigellae, citrobacter, *Pseudomonas* spp. and vibrios were preserved as stab slant cultures at a temperature of 4°C and also in freeze dried state. 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 bromoothymol blue lactose agar [6].

8.2.5. Standard antibiotics

The standard antibiotics used in the studies were amoxycillin (Lyka Labs, India) and gentamicin (Hindustan Antibiotics, Pimpri, Pune) obtained from the respective manufacturer.

8.2.6. Animals

Swiss adult albino mice weighing 20-25 g were used and provided with food (Chakan oil mill) and tap water. The mice exhibited genetic homogeneity and were susceptible to the infecting bacterium. The tests involved only male mice, as the female mice are reported to be more resistant to different infective organisms [8]. Batches each containing 20 mice were kept in standard stainless steel cages having solid bottom in well ventilated animal house with standard conditions of temperature (27°C±3°C), 12h/12h light/dark cycles.

The techniques used for collection of blood samples, injection as well as sacrifice of the animals, were approved by the Animal Ethical Committee of the institute.

8.2.7. Preparation of impregnated discs of extract and standard antibiotics

The discs of 7.25 mm diameter were prepared by punching of Whatman No.1 filter paper 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 *C.oppositifolia* leaf and *H.nepalense* root were weighed and dissolved in 0.5 ml of dimethyl sulphoxide, as the extracts are not fully 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 solutions of the control antibiotics were prepared by dissolving the required amount of amoxycillin or diluting required amount of gentamicin in 10 ml of sterile distilled water separately to prepare two fold serially dilutions of the antibiotics (0–1000 µg/ml concentrations). All the stock solutions were then kept at 4°C and used for three months. For preparation of antibiotic impregnated discs 1.0 ml of the stock solutions of the antibiotic were added separately to each bottle of 100 discs. Each discs adsorbed 0.01 ml of the solution, so the entire 1.0 ml volume was adsorbed by the 100 discs, each giving the required two fold concentrations of 0–1000 µg/ml. The procedure was repeated for preparation of impregnated discs of the plant extracts and their isolated compounds. 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.

8.2.8. Antimicrobial activity of *C.oppositifolia* leaf

8.2.8.1. *In vitro* model

Microbial sensitivity tests were performed by disc diffusion method [7]. The nutrient agar plates, containing an inoculum size of 10^5-10^6 cfu/ml of bacteria were used. Previously prepared crude methanol extract (Concentration 128-2000 µg/ml) and isolated compound (Concentration 0-1000 µg/ml) discs were placed aseptically on sensitivity plates. The discs containing without test compound and standard antibiotics (Amoxycillin and Gentamicin) served as negative and positive controls respectively. All the plates were

then incubated at 37°C±2°C for 18 h. The sensitivity was recorded by measuring the clear zone of inhibition on agar plate around the discs.

The MICs were determined by the standard agar dilution method [8]. The crude methanol extract was dissolved in 0.5 ml of dimethyl sulphoxide, as they are not fully 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 – 128 μg/ml and subsequently increasing it by two fold concentration upto 2000 μg/ml. The concentrations of the tubes were mixed thoroughly, pH adjusted to 7.2 to 7.4 and poured into sterile Petri dishes. Bacterial cell suspensions were spot inoculated on the plates using a bacterial planter (10 μl). The final number of cfu inoculated onto the agar plates was 10^5 for all strains. The inoculated plates were then incubated at 37°C±2°C for 18 h. 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 Amoxycillin was served as negative and positive control respectively.

8.2.8.2. *In vivo* model

Salmonella typhimurium NCTC 74, naturally virulent to mice as well as sensitive to the extract and compound I, was selected as the challenge strain for this study. Virulence of this strain was enhanced by repeated mouse passages by administering intraperitoneally and recovering from the heart blood. The median lethal dose (MLD or LD_{50}) of the mouse-passaged strain was determined by using graded challenges on 5 groups of mice each having 6 mice and recording mortality up to 100 h [8]. The virulent strain, thus prepared, was preserved by freeze-drying. The basic assumptions behind this method is that the animals dying at a stated dose of the challenge would also have been killed by a greater dose of the challenges and conversely the animals surviving at a stated dose of the challenge would have also been survived at a smaller dose of the challenge. An accumulated value for the affected mice was obtained by adding the number of mice dying at a certain dilution to the number killed by lesser doses; a similar reverse addition was made for the survivors.

Experiments on the rate of mortality in Swiss albino mice with or without the methanol extract of *C.oppositifolia* leaf and compound I were carried out by challenging mice with 50 LD_{50} dose of the virulent strain of *Salmonella typhimurium* NCTC 74; which corresponded to 1.95×10^9 cfu in 0.5ml peptone water followed by mouse passaged. Reproducibility of challenge dose was ensured by choosing a fixed value of its optical density at 640nm in a calorimeter corresponding to a predetermined number of cfu on nutrient agar. For the test 05 groups of mice 20 in each group, each weighing 18 – 20 gm, kept in separate cages. Each mouse of the first group received 50 µg/g body weight dose of the extract in 0.1 ml of the stock solution by intraperitoneal (*i.p.*) injection. The second group received 128 µg/g body weight dose of the extract *i.p.* In the same way each mouse in the third and fourth group received 256, 512 µg/g body weight doses of the extract respectively. The control group (fifth group) was also challenged with the same organism and received 0.1 ml sterile saline in place of the extract. Similar procedure was carried out for the compound I with 05 groups of mice at doses of 25, 50, 100 & 200 µg/g body weight and 0.1 ml saline respectively. The protective capacity of the extract and the compound I was assessed on the basis of the following: (i) when both the infective challenge, as well as, the antibacterial test compounds doses are administered, (ii) when the test compounds alone were administered, (iii) and when the bacterial challenge plus 0.1ml normal saline (instead of test compounds) was administered. In a similar experiment 15mice were divided into 3 groups 5 in each, all of which received the bacterial challenged dose but group I and II received methanol extract and compound I respectively while group III received only normal saline 3h before the challenge. All animals of group I, II and III were autopsied 18 h after the bacterial challenge. Their spleen and liver was removed, homogenized in a glass homogenizer and preserved at -20°C for subsequent determination of cfu/ml counts. At the same time 0.2 to 0.4ml of heart blood samples were also collected aseptically from those animals, allowed to clot and analyzed for the size of bacteriaemia by clot culture method [9, 11].

8.2.8.3. Determination of mode of action of the extract of *C.oppositifolia* & compound I

A multiresistant and highly sensitive strain *S.aureus* 8530 was cultured in nutrient broth overnight, 2ml of the culture was added to 4 ml of nutrient broth and incubated for 2 hours so that the culture could reach logarithmic growth phase. At this stage, the cfu count of the culture was determined and the extract as well as the compound I was added separately at a concentration higher than the MIC. The cfu counts were determined again at the time interval of 2, 4, 8 and 18 h [10].

8.2.9. Antimicrobial activity of *H.nepalense* root.

The antibacterial activity of methanol extract of *H.nepalense* root and its isolated compound II was determined as per the methods described above in **8.2.8** for *C.oppositifolia* leaf.

8.3. Results

8.3.1. Antimicrobial activity of *C.oppositifolia* leaf

8.3.1.1. *In vitro* model

The methanol extract of *C.oppositifolia* leaf exhibited a significant *in vitro* antimicrobial activity against 257 Gram-positive and Gram-negative bacteria including MRSC. All the three reference MRSC strains of bacteria were found to be sensitive between 256 and 1000 μg/ml concentration of the extract. The results of the antimicrobial spectrum of the leave extract presented in Table 8.1 showed that out of 257 bacteria, the growth of 171 isolates were inhibited by the extract at a concentration of 128 – 512 μg/ml. 79 isolates were resistant at <1000μg/ml, while remaining 07 isolates where resistant up to <2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 63 out of 78 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 94 were sensitive between 256-512 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the extracts was directed both against Gram-positive and Gram-negative bacteria. The isolated compound I was also tested for antimicrobial activity. The

result is presented in Table 8.2, which revealed that all the isolates were sensitive at 128-256 μg/ml concentration of the compound I. It was interesting to note that all the MRSC strains were susceptible to compound I at concentration of 128 μg/ml, while they are resistant to both the standard antibiotics used.

8.3.1.2. *In vivo* model

The result of the median lethal dose (MLD or LD_{50}) of the mouse passaged strain *S. typhimurium* NCTC 74 depicted in Table 8.3 show that in group I where the highest challenge dose was administered (cfu 5.0×10^9) corresponding to 130 O.D. reading, all the 06 animals died. In group II where the challenge dose contained 4.1×10^8 viable cells per ml with 112 O.D reading, 04 mice died. In the following two groups (III and IV), which contained 3.9×10^7 (O.D-95) and 2.5×10^6 (O.D-87) viable cells per ml respectively, only 03 and 02 mice died, whereas at the lowest challenge dose of 3.9×10^5 cfu ml^{-1} (O.D-76) in the group V no animal died. It was noted from the accumulated values that 100% of the animals died in group I, while 50% of the animals died in group III. Therefore MLD of *S.typhimurium* NCTC 74 was found to be 3.9×10^7 cfu/ml and 50 LD_{50} dose was calculated to be 1.95×10^9 cfu/ml for the same organism.

The protective capacity of *C.oppositifolia* and compound I against *S.typhimurium* NCTC 74 is presented in Table 8.4. The result showed that only 04 out of 20 mice were died in the bacterial challenge and actual test dose of the extract at 256 μg/g body weight, while no mice was died in a control group receiving only the extract. When the challenge dose of *S.typhimurium* NCTC 74 and the extract at a dose of 50 μg/g body weight level was administered, 10 mice were died, followed by 08 mice at 128 μg/g, 18 mice at 512 μg/g body weight in the test groups. In the control group where only bacterial challenge dose was administered 16 out of 20 mice were died. Further study with compound I revealed that only 02 out of 20 mice died in the bacterial challenged group along with a test dose of 100 μg of compound I per gram body weight of mice, while no mice died in the control group received only compound I. When compound I was administered at a dose of 50 μg/g body weight level with bacterial challenge, 06 animals died followed by 10 animals at dose of 25 μg/g and 12 died at 200 μg/g in the test groups. In the control group

which received only the bacterial challenge 15 out of 20 mice were died. The protection test turned out to be highly significant at ($p<0.01$ in Chi square test) 256,128 μg/g doses of the extract and 100, 50 μg doses of compound I compared with the control without the test compounds but with the bacterial challenge.

The results of *in vivo* activity of the extract and the compound I against *S.typhimurium* NCTC 74 in mice have been presented in Table 8.5. The viable counts of the test organism, *S. typhimurium* NCTC 74, in mice, which received the methanol extract and the challenge, yielded 1.4×10^3 to 3.4×10^5, 2.4×10^3 to 1.2×10^5 and 4.8×10^2 to 4.8×10^5 cfu ml^{-1} for spleen, liver homogenate and heart blood respectively when autopsied and tested after 18 hrs of administration. Similarly the endogenous cfu counts in the homogenate of mouse spleen and liver and heart blood on 18th h of post bacterial challenge with compound I found to be 1.8×10^3 to 1.5×10^4, 1.1×10^3 to 1.2×10^4 and 1.8×10^3 to 2.1×10^4 respectively. The groups which received the usual challenge dose without the methanol extract or compound I (control), yielded 1.0×10^7 to 2.0×10^8, 3.1×10^7 to 9.0×10^8 and 2.5×10^6 to 5.0×10^8 ranges of cfu counts of the organism in spleen, liver homogenate and heart blood respectively.

8.3.1.3. Determination of mode of action

The determination of mode of antibacterial action of the extract and compound I on multiresistant strain of *S. aureus* 8530 is presented in Figure 8.1. At the logarithmic growth phase of culture, at 6.4×10^8 cfu counts of the strain, 128 μg ml^{-1} (MIC value) of the extract and compound I was added separately. The cfu counts ml^{-1} in the culture with extract were determined and found out to be 2.5×10^8, 6.4×10^6, 7.0×10^5 and 3.0×10^2 at 2,4,8 and 18 h respectively. Similarly the cfu count in the culture with compound I was found to be 2.8×10^7, 4.2×10^5, 6.8×10^3, 5.9×10^1 at the same time intervals.

Table 8.1 *In vitro* antimicrobial spectrum of methanol extract of *C. oppositifolia*.

Bacterial Species	Number Of strain	MIC of leaf extracts (μg/ml)					MIC of amoxycillin (μg/ml)						
		128	256	512	1000	>2000	0.25	0.5	8	64	128	256	>1
E.Coli	70	03	25	21	20	01	-	07	03	03	05	15	3
Klebsiella spp.	12	-	-	03	09	-	-	-	-	-	-	02	1
Salmonella Spp.	18	-	01	06	11	-	-	-	-	-	01	04	[illegible]
Shigella spp.	34	-	02	10	20	02	-	-	-	-	02	10	2
Vibro cholerae	15	-	06	04	05	-	-	-	-	-	-	03	1
Citrobacter spp.	15	-	02	03	08	02	-	-	-	-	-	01	[illegible]
Pseudomonas aeruginosa	15	-	06	04	03	02	-	-	-	-	-	02	
Bacillus subtilis	06	1	03	02	-	-	-	-	04	01	-	01	
Staphylococcus aureus	62	19	30	10	03	-	01	27	12	13	-	09	
Streptococcus faecalis	10	4	06	-	-	-	01	05	04	-	-	-	
Total	257	27	81	63	79	07	02	39	23	17	08	47	1

Inoculum size used 10^5 cfu per spot for all the organisms except *S.aureus*, where the inoculum size per spot was 10^6 cfu. The results are the mean value of triplicate tests.

Table 8.2 The MIC of 15 sensitive bacteria against methanol extract of *C.oppositifolia* and Compound I.

Name of the Organism	MIC (μg/ml)				Diameter of zone of inhibition (mm) in methanol extract
	ME	CM I	Amoxycillin	Gentamycine	
E.Coli 832	512	512	0.50	0.25	+
E.Coli TG_1	512	512	0.50	>256	+
E.Coli 87.1	256	512	0.50	0.50	+
E.Coli HD_{10}	128	128	0.25	0.50	+
S. aureus NCTC 6571	128	128	0.50	1.0	++
S. aureus NCTC 8530	128	128	0.50	0.50	++
S. aureus Bang 44	512	512	8.0	1.0	+
S. aureus ML 275	128	128	0.50	1.0	++
S. epidermidis 865	128	128	0.50	0.50	++
Bacillus lichenfermis 10341	1000	1000	0.125	0.50	+
Bacillus subtilis 8241	128	128	0.50	256	++
S.typhimurium NCTC 74	256	512	8.0	>256	+
V.Cholerae 14033	128	128	8.0	>256	+
Klebsiella pneumoniae.	512	512	256	0.50	+
Pseudomonas aeruginosa	1000	1000	2.0	0.50	+

ME: Methanol extract of *C.oppositifolia*; CM I: Compound I; +: ≤ 10mm; ++: ≥12mm; inoculum size used 10^5 cfu per spot for all organisms except S.*aureus*, where 10^6 cfu where used. The results are means ± S.E.M (n=3).

Table 8.3 Median lethal dose (LD_{50}) of *Salmonella typhimurium* NCTC 74.

Batch	cfu/ml	Optical density (O.D)*	No of animals		Mortality	Accumulated values from mortality data				LD_{50}	50 LD_{50}
			Dead	Survived		Died (D)	Survived (S)	Mortality ratio ** [D/(D+S)]	Percent [D×10/ D+S]		
I	5.0×10^{9}	130	06	0	6 / 6	15	0	15 / 15	10%		
II	4.1×10^{8}	112	04	02	4 / 6	09	02	09 / 11	81.81%		
III	3.9×10^{7}	95	03	03	3 / 6	05	05	05 / 10	50%	3.9×10^{7} cfu/ml	1.95×10^{9}
IV	2.5×10^{6}	87	02	04	2 / 6	02	09	02 / 11	18.18%		
V	3.9×10^{5}	76	0	06	0 / 6	0	15	0 / 15	0%		

* : Readings were taken in Klett Summerson Colorimeter at 640 nm.

**: Mortality ratio was calculated by the formula of Reed and meunch.

Table 8.4 Determination of *in vivo* protective capacity of methanol extract of *C.oppositifolia* and compound I

Group	Test Groups[a]		Control Groups[b]		Test groups[c]		Control groups[d]	
	Extract (μg/g)	Mice died	Extract (μg/g)	Mice died	Compound I (μg/g)	Mice died	Compound I (μg/g)	Mice died
I	50	10	50	0	25	10	25	0
II	128	08[e]	128	0	50	06	50	0
III	256	04[f]	256	0	100	02[f]	100	0
IV	512	18	512	3	200	12[e]	200	2
V	Bacterial challenge dose (1.95 × 10^9)	16	-	-	Bacterial challenge dose (1.95 × 10^9)	15	-	-

a. Received challenge dose of 1.95 × 10^9 cfu/ml of *S.typhimurium* NCTC 74, 3h after administration of the extract.

b. Received only the extract and saline but no challenge dose.

c. Received challenge dose of 1.95 × 10^9 cfu/ml of *S.typhimurium* NCTC 74, 3h after administration of the compound I.

d. Received only the compound I and saline but no challenge dose.

e. $P < 0.05$

f. $P < 0.01$ according to the chi-square test, after elimination of the effect due to the extract and compound I.

Table 8.5 *In vivo* antimicrobial spectrum of the methanol extract of *C.oppositifolia* and compound I against *S.typhimurium* NCTC 74 in mice [a].

Group	Time of Sampling (hr)	Mouse Number	Treated with	cfu/ml Count in		
				Liver	Spleen	Heart blood
Group I	18	1	Methanol Crude extract of *Colebrookea oppositifolia* (256μg/g)	2.4×10^3	1.8×10^5	4.8×10^5
		2		6.8×10^4	3.4×10^5	1.5×10^5
		3		1.2×10^5	3.4×10^3	4.8×10^2
		4		5.0×10^4	1.4×10^3	4.8×10^3
		5		1.2×10^4	1.45×10^5	4.8×10^2
Group II		1	Compound I (100μg/g)	1.2×10^4	1.5×10^4	2.1×10^4
		2		3.0×10^3	1.8×10^3	1.8×10^3
		3		3.5×10^3	6.4×10^3	3.8×10^4
		4		1.1×10^3	2.5×10^3	3.6×10^4
		5		1.4×10^3	2.3×10^3	4.3×10^3
Group III		1	Sterile Saline	1.0×10^7	3.1×10^7	5.0×10^8
		2		1.5×10^7	4.5×10^7	2.5×10^6
		3		1.9×10^8	9.0×10^8	3.0×10^7
		4		2.0×10^8	8.3×10^8	5.0×10^8
		5		2.5×10^7	3.3×10^7	6.3×10^7

a. Variable counts between 3 groups significant, $P < 0.001$ (Student 't' test).

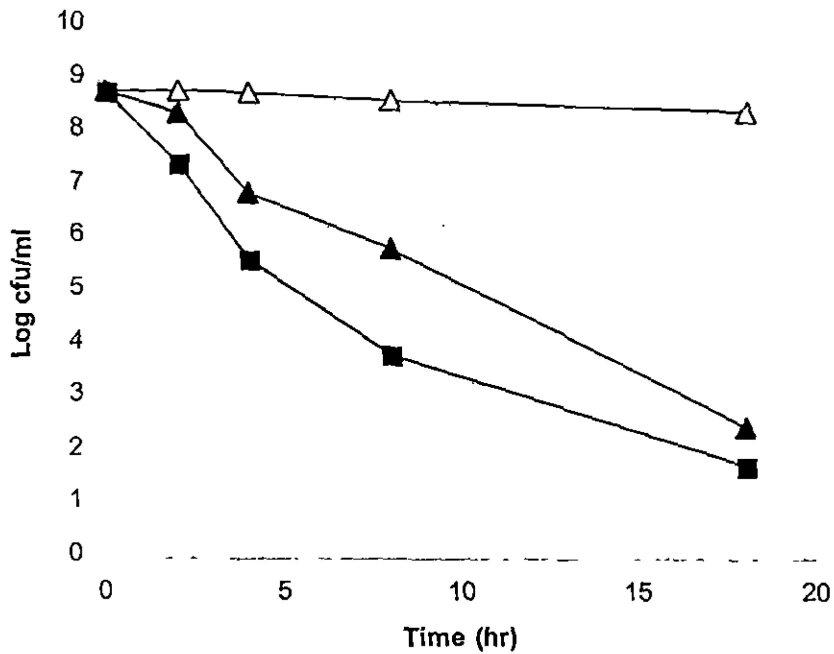

Figure 8.1 The mode of action of methanol extract and compound I of *C.oppositifolia* leaf on resistant strain of *S.aureus* 8530. (-Δ-): bacterial suspension in methanol (control); (-▲-): Methanol extract of the leave at 128 μg/ml; (-■-): Compound I at 128 μg/ml concentrations.

8.3.2. Antimicrobial activity of *H.nepalense* root.

8.3.2.1. *In vitro* model

The methanol extract of *H.nepalense* root exhibited a significant *in vitro* antimicrobial activity against 257 Gram-positive and Gram-negative bacteria including multiresistant *Staphylococcus* (MRSC) strains. All the three reference MRSC strains of bacteria were found to be sensitive within 1000 μg/ml concentration of the extract. The results of the antimicrobial spectrum of the root extract presented in Table 8.6 showed that out of 257 bacteria, the growth of 197 isolates were inhibited by the extract at a concentration of 128 – 512 μg/ml, 57 isolates were inhibited at a concentration of 1000μg/ml, while the remaining 03 isolates where inhibited at concentration >2000 μg/ml, the highest concentration of the extract tested. .The MICs tests revealed that 63 out of 75 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 120 were sensitive between 256-512 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the methanol extracts was directed both against Gram-positive and Gram-negative bacteria. The isolated compound II was also tested for antimicrobial activity. The result is presented in Table 8.7, which revealed that all the isolates were sensitive at 128-256 μg/ml concentration of the compound II. It was interesting to note that all the MRSC strains were susceptible to compound II at a concentration of 128 μg/ml, while they were resistant to the two standard antibiotics used.

8.3.2.2. *In vivo* model

The result of the median lethal dose (MLD or LD_{50}) of the mouse passaged strain *S. typhimurium* NCTC 74 was same as per the result depicted in *C.oppositifolia* leave (Discussed in **8.3.1.2**). The protective capacity of methanol extract of *H.nepalense* and compound II against *S.typhimurium* NCTC 74 is presented in Table 8.8. The result showed that only 3 out of 20 mice were died in the bacterial challenge and actual test dose of the extract at 128 μg/g body weight, while no mice was died in a control group receiving only the extract. When the bacterial challenge and extract at a dose of 50 μg/g body weight level was administered, 08 mice were died, followed by 05 at a dose of 256

μg/g, 12 mice at 512 μg dose per gram body weight in the test groups. In the control group 15 out of 20 mice were died which received only the bacterial challenge. Further study with compound II revealed that only 02 out of 20 mice died in the bacterial challenge and actual test dose of 100 μg/g body weight of mice, while no mice died in a control group which received only compound II. When bacterial challenge dose and compound II was administered at a dose of 50 μg/g body weight level, 04 animals were died followed by 06 at dose of 25 μg/g, 10 animals at 200 μg/g body weight level in the test groups. The protection test turned out to be highly significant at ($p<0.01$ in Chi square test) 128, 256 μg/g doses of the extract and 100, 50 μg/g doses of compound II compared with the control without the test compounds but with the bacterial challenge.

The results of *in vivo* activity of the extract and the compound II against *S.typhimurium* NCTC 74 in mice have been presented in Table 8.9. The viable counts of the test organism, *S. typhimurium* NCTC 74, in mice, which received the methanol extract and the challenge, yielded 2.1×10^2 to 2.8×10^4, 2.6×10^3 to 7.0×10^4 and 2.5×10^3 to 2.8×10^4 cfu/ml for spleen, liver homogenate and heart blood respectively when autopsied and tested after 18 h of administration. Similarly the endogenous cfu counts in mouse spleen and liver homogenate and heart blood on 18th hours of post bacterial challenge with compound II found to be 2.6×10^3 to 6.4×10^5, 5.2×10^3 to 6.4×10^4 and 1.2×10^3 to 3.8×10^4 respectively. The groups which received the usual challenge dose without the methanol extract or compound II (control), yielded 1.6×10^8 to 6.8×10^9, 3.9×10^8 to 4.8×10^9 and 5.2×10^8 to 7.2×10^8 ranges of cfu count of the organism in spleen, liver and heart blood respectively.

8.3.2.3. Determination of mode of action

The mode of antibacterial action was determined by selecting a multiresistant strain of *S. aureus* 8530 is presented in Figure 8.2. At the logarithmic growth phase of culture, at 6.4×10^8, cfu count of the strain 128 μg/ml (MIC level) of the extract and compound II was added separately. The cfu counts/ml in the culture with extract were determined and found to be 1.2×10^8, 1.4×10^6, 6.8×10^4 and 1.2×10^2 at 2,4,8 and 18 hours respectively.

Similarly the cfu count in the culture with compound II was found to be 3.4×10^6, 1.8×10^4, 1.4×10^2, 0.4×10^2 at the same time intervals.

Table 8.6 In *vitro* antimicrobial spectrum of *H.nepalense* root extract.

Bacterial Species	Number Of strain	MIC of leaf extracts (μg/ml)					MIC of amoxycillin (μg/ml)						
		128	256	512	1000	>2000	0.25	0.5	8	64	128	256	>1
E.Coli	70	08	12	21	28	01	-	07	03	03	05	15	[illegible]
Klebsiella spp.	12	01	02	04	05	-	-	-	-	-	-	02	[illegible]
Salmonella Spp.	18	02	04	06	06	-	-	-	-	-	01	04	[illegible]
Shigella spp.	34	07	09	12	06	-	-	-	-	-	02	10	[illegible]
Vibro cholerae	15	02	02	08	02	01	-	-	-	-	-	03	[illegible]
Citrobacter spp.	15	02	02	06	04	01	-	-	-	-	-	01	[illegible]
Pseudomonas aeruginosa	15	01	06	05	03	-	-	-	-	-	-	02	1
Bacillus subtilis	06	03	02	01	-	-	-	-	04	01	-	01	[illegible]
Staphylococcus aureus	62	29	20	10	03	-	01	27	12	13	-	09	
Streptococcus faecalis	10	04	06	-	-	-	01	05	04	-	-	-	
Total	257	59	65	73	57	03	02	39	23	17	08	47	1

Inoculum size used 10^5 cfu per spot for all the organisms except *S.aureus*, where the inoculum size per spot was 10^6 cfu. The results are the mean value of triplicate tests.

Table 8.7 The MIC of 15 sensitive bacteria against methanol extract of *H.nepalense* and Compound II.

Name of the Organism	MIC (μg/ml)				Diameter of zone of inhibition (mm) in methanol extract
	ME	EA	Amoxycillin	Gentamicin	
E.Coli 832	256	256	0.50	0.25	+
E.Coli TG_1	512	512	0.50	>256	+
E.Coli 871	256	512	0.50	0.50	+
E.Coli HD_{10}	256	256	0.25	0.50	++
S. aureus NCTC 6571	128	128	0.50	1.0	++
S. aureus NCTC 8530	128	128	0.50	0.50	++
S. aureus Bang 44	128	128	8.0	1.0	++
S. aureus ML 275	128	128	0.50	1.0	++
S. epidermidis 865	128	128	0.50	0.50	++
Bacillus lichenfermis 10341	512	512	0.125	0.50	+
Bacillus subtilis 8241	128	128	0.50	256	++
S.typhimurium NCTC 74	256	256	8.0	>256	+
V.Cholerae 14033	128	128	8.0	>256	++
Klebsiella pneumoniae.	256	256	256	0.50	+
Pseudomonas aeruginosa	512	1000	2.0	0.50	+

ME: Methanol extract of *H.nepalense*; EA: ethyl acetate fraction; +: ≤ 10mm; ++: ≥12mm; innoculum size used 10^5 cfu per spot for all organisms except S.*aureus*, where 10^6 cfu where used. The results are means ± S.E.M (n=3).

Table 8.8 Determination of *in vivo* Protective capacity of *H.nepalense* root extract and compound II.

Group	Test Group[a]		Control Group[b]		Test group[c]		Control group[d]	
	Extract (μg/g)	Mice died	Extract (μg/g)	Mice died	Compound II (μg/g)	Mice died	Compound II (μg/g)	Mice died
I	50	08	50	0	25	06	25	0
II	128	03[e]	128	0	50	04[f]	50	0
III	256	05[f]	256	1	100	02[e]	100	0
IV	512	12	512	2	200	10	200	2
V	Bacterial challenge dose (1.95 $\times 10^9$)	15	-	-	Bacterial challenge dose (1.95 $\times 10^9$)	15	-	-

a. Received challenge dose of 1.95 $\times 10^9$ cfu/ml of *S.typhimurium* NCTC 74, 3h after administration of the extract.

b. Received only the extract and saline but no challenge dose.

c. Received challenge dose of 1.95 $\times 10^9$ cfu/ml of *S.typhimurium* NCTC 74, 3h after administration of the compound II.

d. Received only compound II and saline but no challenge dose.

e. $P < 0.05$

f. $P < 0.01$ according to the chi-square test, after elimination of the effect due to the extract and compound II.

Table 8.9 *In vivo* antibacterial spectrum of methanol extract of *H.nepalense* and compound II against *S.typhimurium* NCTC 74 in mice [a].

Group	Time of Sampling (hr)	Mouse Number	Treated with	cfu/ml Count in		
				Liver	Spleen	Heart blood
Group I	18	1	Methanol Crude extract of *H.nepalense root* (128μg/g)	3.1×10^4	5.4×10^3	2.8×10^4
		2		2.6×10^3	2.8×10^4	5.8×10^3
		3		3.0×10^3	4.2×10^3	4.0×10^3
		4		7.2×10^3	1.4×10^3	3.2×10^3
		5		7.0×10^4	2.1×10^2	2.5×10^3
Group II		1	Compound II (100μg/g)	2.1×10^4	3.0×10^4	1.2×10^3
		2		6.4×10^4	4.2×10^4	2.4×10^3
		3		3.2×10^4	6.4×10^5	2.6×10^3
		4		5.2×10^3	3.2×10^3	3.8×10^4
		5		2.4×10^4	2.6×10^3	7.1×10^3
Group III		1	Sterile Saline	7.4×10^3	1.6×10^8	6.3×10^8
		2		4.8×10^9	4.4×10^8	5.2×10^8
		3		3.9×10^8	6.8×10^9	7.2×10^9
		4		5.4×10^8	6.4×10^9	4.4×10^9
		5		3.2×10^9	4.2×10^8	5.4×10^9

[a]: Variable counts between 3 groups significant, $P < 0.001$ (Student 't' test).

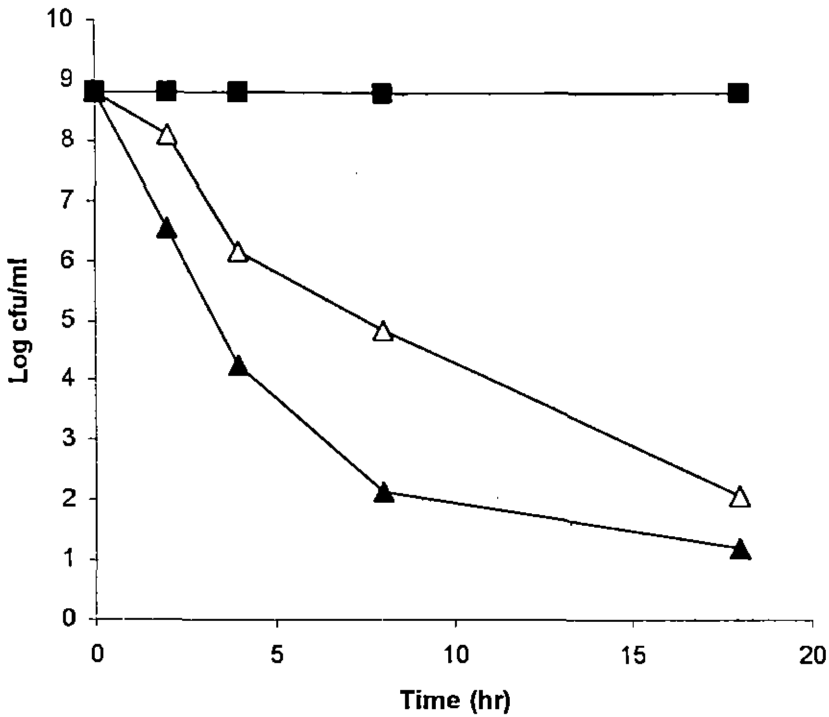

Figure 8.2 The mode of action of methanol extract and compound I of *H.nepalense* root on resistant strain of *S.aureus* 8530. (-■-): bacterial suspension containing methanol Control; (-Δ-): methanol extract of leave at 128 μg/ml; (-▲-): Compound I at 128 μg/ml.

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