CHEMICAL AND MICROBIOLOGICAL STUDIES ON SOME MEDICINAL PLANTS

A Dissertation submitted in partial fulfillment of the requirement for the Degree of Master of Philosophy in Chemistry.

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Dedicated To
My Late
Nana Vaia &
Kuti Mama

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ICDDR,B	: International Center for Diarrheal Diseases Research in Bangladesh.
TLC	: Thin Layers Chromatography.
^{13} C-NMR	: Carbon (13) Nuclear Magnetic Resonance.
^{1}H-NMR	: Hydrogen (1) Nuclear Magnetic Resonance.
IR	: Infra Red
MIC	: Minimum Inhibitory Concentration
MBC	: Minimum Bactericidal Concentration
mm	: millimeter
Mg	: microgram
mg	: milligram
gm	: gram
ml	: milliliter
Soln.	: Solution
ppm	: parts per million
C.F.U	: Collony Forming Unit
mm	: millimicron
N	: Normality
Nm	: nano meter
Conc	: Concentration

ABSTRACT

Antibacterial activity of ten locally available medicinal plants, *Terminalia arjuna* (Arjun), *Rauvolfia serpentina* (Sharpagandha), *Holarhena antidysenterica* (Kurchi), *Aegle marmelos* (Bel), *Heliotropium indicum* (Hatisur), *Helicteres isora* (Atmora), *Syzygium cumini* (Kalojam), *Polygonum hydropiper* (Biskatali), *Spondias pinnata* (Amra), *Swertia chirata* (Chirata) were studied against ten gram negative & one garm positive bacteria. Methanol extract of the seeds of *Holarhena antidysenterica* was found to be active against *S.aureus;* Methanol extract of the bark of *Terminalia arjuna* was active against *Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Escherichia coli* and *S.aureus;* and Methanol extract of the roots of *Rauvolfia serpentina* was active against *S.aureus.* Extracts of the other plants showed no antibacterial activity.

Fractionation of Methanol extracts were carried out by solvent extraction and /or column chromatographic techniques. Antibacterial activity tests of different fractions were carried out against the same test organisms. Ethyl acetate fraction of methanol extract of *H.antidysenterica* was found to be active against *S.aureus;* Pet.ether-Ethyl acetate fraction (1:4) of *T.arjuna* was found to be active against *Shigella dysenteriae, Shigella flexneri, Shigella sonnei,Escherichia coli* and *S.aureus*; Pet.ether-Ethyl acetate fraction (2:1) of methanol extract of *R.serpentina* was active against *S.aureus*.

Antibacterial compound HA_1 then separated from the Ethyl acetate fraction of Methanol extract of *H.antidysenterica* with the help of preparative thin layer chromatography. Methanol extracts of *T.arjuna* and *R.serpentina* were then purified with the help of column chromatography followed by PTLC to yield compounds TA_3, and RS. Compound HA_1 was found to be active against S. aureus,TA_3 was found to be active against *Shigella dysenteriae, Shigella flexneri, Shigella sonnei,Escherichia coli* and *S.aureus;* and compound RS showed antibacterial activity against *S.aureus.*

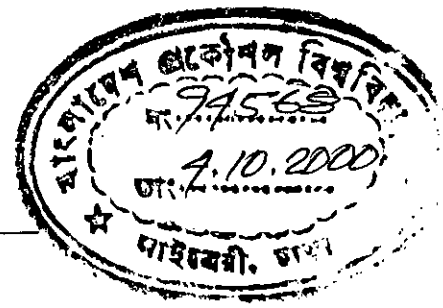
Three antibacterial compounds were then characterized by physical, chemical and spectroscopic analysis. Compound HA_1 was steroidal alkaloid with–C≡N group ;

compound TA_3 was flavone with ketone group; and compound RS was alkaloid with phenyl hydroxyl group, aryl ether group and carboxylic acid group.

Minimum Inhibitory Concentration (MICs) of compounds TA_3 for *Shigella dysenteriae* was 640 μg/ml, that of compound TA_3 for *S.flexneri* was 320μg/ml, that of compound TA_3 for *S.sonnei* was 960μg/ml, that of compound TA_3 for *E.coli* was 640μg/ml and that of compounds HA_1,TA_3 and RS for *S.aureus* were 320μg/ml,320μg/ml and 160μg/ml. respectively. The Minimum Bactericidal Concentration (MBC)of compound TA_3 *Shigella dysenteriae* was 790μg/ml, that of compound TA_3 for *Shigella flexneri* was 780μg/ml and that of compound TA_3 for *S.sonnei* was 1075μg/ml, that of compound TA_3 for *E.coli* was 775μg/ml, and that of compounds HA_1,TA_3 and Rs for *S.aureus* were 810μg/ml,730μg/ml and 670μg/ml respectively.

CHAPTER-1

General Introduction



CHAPTER-1

INTRODUCTION

1.1.GENERAL INTRODUCTION

The importance of plant and plant materials in human civilization is very great. Human being and other living organisms are to depend to a very great extant on the plant kingdom for many of their daily necessities of life. They provide human race with clothing and shelter, fuel, medicine, plants are unique in their ability to synthesize carbohydrates, fats and proteins which constitute three major food classes for human race. Apart from these , certain other substances such as glycosides, alkaloids , steroids ,vitamins , saponin , essential oils , resins , tannins and bitter principles etc. are produced within the plants by their metabolic activities. Some of these in regulated doses are effective remedies for many diseases. Plant and plant materials containing medicinal principles are usually classified as medicinal plants. The science of medicine is very old. At the very beginning the expanded knowledge of mankind tried to take care of themselves . 'Back of nature' is a slogan of course medicinal science is not out of this trend . Peoples of advanced countries like Germany, USA, France, Japan , China , now-a-days use herbal drugs instead of modern synthetic drugs for less side effects and more efficiency [1]. It is reported that 80% peoples of the present world get rid of various diseases by herbal medicines. The use of drugs obtained from the medicinal plants have increased not only in our country but also over the most developed ones too. In 1960, 47% of drugs prescribed by the physicians in the United States were from natural sources [2]. So, World Health Organization (WHO) has recently showed immense interest on herbal medicines and taken a master plan to develop herbal medicines greatly to ensure "Health for all by the year 2003" [3].

In spite of the tremendous advancement in medical science and technology, infectious diseases are a serious health problem particularly in the under privileged population in the remote rural areas in the developing countries. In the humid tropic land of Bangladesh diarrhea, dysentery, cholera, typhoid, pneumonia, diphtheria, malaria are major cause of

morbidity and mortality. The poverty, malnutrition, poor sanitation and lack of knowledge of personal hygiene are contributing tc the spread of these diseases. Many compounds either of natural origin or from programs of chemical synthesis are examined every year in the research for new structures with activity against microorganisms. The knowledge about the chemical constitutes of plants has advanced rapidly in recent years and the constitutes responsible for the specific physiological action of the plants as well as animals have in many cases been isolated purified and identified as definite chemical compounds. The availability of the medicinal plants needs the isolation, separation and characterization of physiologically active principles which are actually useful for the treatment of diseases. A systematic chemical study on these plants may led to the discovery of newer drugs having minimum or no drugs. The research on medicinal plants may not always produce new or potent drugs the background knowledge regarding the chemical structure and pharmacological action may open up newer possibilities for creative synthesis.

Bangladesh with its characteristics phytogeographic conditions is very rich in vegetation with potential sources worth exploiting. Therefore, in the present investigation first screening test was carried out for some medicinal plants of Bangladesh for their antibacterial activity against some bacterial species. Secondly, research work on separation and isolation of the antibacterial compounds and other compounds were carried out from the plant. Finally, an attempt was made to identify the active ingredients from the active plant materials.

1.2. STATUS OF DISEASES ESPECIALLY DIARRHEAL DISEASES CAUSED BY BACTERIA:

Global Problem:

Diarrheal diseases caused by a variety of bacterial, viral and parasitic pathogens remains a major public health problem in under developed and developing countries. A estimated 12,600 persons die each day from diarrheal diseases in under developing areas of the would [4].

In developing countries diarrheal diseases are the important cause of morbidity and mortality. Recent estimates by WHO indicate that in Africa, Asia (excluding china) and Latin America 750 million children aged less 5 years suffer from diarrheal annually, and 4-5 million of them die off diarrheal diseases.[5]

The commonest pathogens responsible for diarrheal diseases are rotavirus, followed by Enterotoxigenic *Escherichia coil, Vibrio cholerea, Shigela* sp and *salmonella* sp. Among all of the pathogens causes of diarrheal diseases *Shigellosis* is predominant. It is an important cause of morbidity and hospitalization in many developed and developing countries and remains an important health problem. Four Shigella sp. are available *Shigella dysenteriae, Shigella flexneri, Shigella sonnei* and *Shigella boydii.* All of this species are responsible for facially dysentery .The severity of the disease depends on *Shigella* sp.and their virulence[6]. Only 10-100 viable *Shigella* organisms are enough to produce a full-blown clinical attack, compared to more than 100,000 of *vibrio* cholera needed to initiate an attack of cholera.

Shigellosis is world wide in distribution and remains a major problem in countries which have lack effective sanitation and where malnutrition is common.[9] *Shigella sonnei* is most common in USA & Europe. In 1993 approximately 15000 cause of *Shigellosis* were reported in United States: *Shigella sonnei* was responsible for 66% children 10 yrs of age accounted for the greatest number case. In emerging nations *S.flexneri* and *S.dyenteriae* type-1 remain the most important pathogen.

In many parts of Europe & USA *S.Sonnei* is isolated more commonly, whereas in developing countries *S.flexneri* is the prominent isolate[4].

Diarrhoea genic *E.coli* are classified into a major categories ETEC,EPEC, EHEC & EIEC strains. Among these ETSC (Entrotoxigenic *Escherichia coli*) is the major cause of diarrhea in infants & young children in developing countries, and a leading cause of travelers diarrhea.

Diarrheal Problem in Southeast Asia[7]:

In southeast Asia, diarrhoeal diseases are associated with high morbidity and mortality, particularly among children under age 5. The magnitude of the diseases in some South-east Asian nations (Indonesia, Philippines, Thailand, Malaysia, Singapore and Bangladesh)was evaluated by analyzing data from these countries .In Indonesia morbidity rate were 430 per 1000 population per year of which 70%were children under 5. It was estimated that Indonesia in 1974 had about 50 million diarrhea causes and 60 million episodes in 1981 with 300,000-500,000 deaths. In the Philippines diarrhea ranked as the second morbidity cause for all age groups in 1974 (600 per 100,000) and as the second infant mortality cause (5 per 1,000). In Thailand 93786 diarrhea causes were hospitalized in 1980 forty percent of these were children under 5 years. Among these patients the morbidity rates associated with acute diarrhea, cholera and dysentery were, respectively, 458,03,0.09 and 56.44 per 100,000 people.

In Malaysia there were outbreak of EI To cholera and poliomyelitis in 1971 and in 1976 diarrhea was the number 5 cause of total hospital admissions and the number 9 cause of deaths in Malaysia.

In Singapore comparing 1975 to 1984 infant parental and neonatal mortality rates dropped respectively from 31.2, 26.2 and 19.1 per 1,000 births to 13.9,16.6 and 10.2 per 1,000 births. Also during these period there was a marked reduction in deaths due to pneumonia and diarrhea, from respectively 33% and 23% to 14% and 4%.

Diarrheal Problem In Bangladesh :

In Bangladesh morbidity rates were highest among and declined with age. Urban areas the attack rates for under age 5 children was 11-fold higher than for adults; but the difference was only 3-fold in rural areas. The overall attack rates implies a prevalence of 2.0% for the entire population, with the highest prevalence,4.5% for the under-5groups [7]. In a rural population of 100,000 the diarrheal attack rate was 85,000 (85.4%) episodes annually of which 37% were cholera in the under 5 groups. Mortality was highest in infants, followed by toddlers and old people.

Acute diarrhea creates a severe problem in Bangladesh. Probability of dying before age 5 in 1990/91 was around 155 per 1,000 within 1977 to 1990 it was 200 per 1,000 [8]. Among the diarrheagenic pathogens *Shigellosis* is predominant in this country alone and about 80-85% of bacillary dysentery takes place due to *Shigella* sp. In 1970 only 0.6% of all the diarrheal causes were associated with *Shigella*. This rates gradually 9% in 1972, 14% in 1973 and a peak of 20% in1974. During the early 1980's Shigella incidences among the diarrhoeal patients was about 10% and mortality among these incidences was 17% [9,6].

In Bangladesh infection caused by S.*flexneri* is most common [10,6].Among all the *Shigella*, infections (during 1976-87) approximately 71% were caused by *S.fiexneri* ,14% by *S.dysenteriae* type-I, 6% by *S. Boydii* and only 5% by *S.sonnei.*

1.3 DRUG RESISTANT MICROORGANISM: A SERIOUS PROBLEM IN THE MANAGEMENT OF BACTERIAL DISEASES.

In the later years owing to indiscriminate use of antibiotics, most of the strains of *Shigella, vibrio,Salmonella* and *Klebsiella* developed resistance to a number of drugs [12,13,14].e.g. Prior to 1970's the treatment of shigellosis was simple, Since most of the *Shigella* were susceptible to commonly available antibiotics such as chloramphenicol and tetracycline [12].

In Bangladesh, *Shigella* strains become resistant to tetracycline, chloramphenicol. Streptomycin and sulfonamides during 1975's.Ampicillin was then the drug of choice.[13,16]. In 1975, the first ampicillin-resistant strain of *Shigella dysenteriae* type-1 was isolated in Bangladesh [15].Co-trimoxazole was found very effective even in areas where ampicillin-rasistant strains had become predominant[17]. Resistance of *Shigella* to co-trimoxazole was reported in 1977 [17] and in the following years [15,18] and by1984 most strains become resistant to this drug[15].During 1982-84,99% of *Shigella dysenteriae* type-1 and 76% of *shigella flexneri* were reported multiply drug resistant [15].

According to the report of Matlab Diarrhoeal Treatment Centre, a field station of ICDDR,B almost 100% of the isolates of *Shigella dysenteriae* type-1 tested during 1994-

1995 were resistant to ampicillin, co-trimoxazole, trimethoprim-sulfametho-Xazole and nalidixic acid [19] .Nearly 50% of the isolates of *Shigella flexneri* were resistant to most currently used antibiotic mecillinam [19].

Vibrio cholera is completely resistant to Co-trimoxazole and tetracycline also contribute to the development of resistant strains [19]. *Salmonella* strains are usually resistant to ampicillin. It is found that 25% of *Salmonella* are resistant to ampicillin and 8% are resistant to chloramphenicol [20].Resistance to the most recently used antibiotic trimethoprim-sulfamethoxazole is also increasing. *Klebsiella* strains are also found to be resistant against sulphonamides, ampicillin, chloramphenicol, tetracycline etc. antibiotics [20] . A problem serious has been created in the treatment of bacterial diseases due to emergence of multiply drug resistant strains of *Shigella, Salmonella,Vibrio,Klebsiella etc.* These strains have been causing outbreak of Shigellosis and other bacterial diseases in recent year in Bangladesh [16,21] and in other developing countries [16].Hance, the development of antimicrobial drugs that will be active against resistant Strains of *Shigella, Salmonella,Vibrio,* and *Klebsiella* is a pressing need of the hour.

1.4 RATIONALE OF THE WORK :

Infectious diseases are a serious health problem in the developing countries like Bangladesh. In later years, owing to discriminate use of antibiotics, most of the strains of *Shigella ,Vibrio , Salmonella,,* and *Klebsiella* developed resistance to a number of drugs .Such as *Shigella* strains are commonly used antibiotics chemotherapeutic agents. e.g. ampicillin ,tetracycline ,chloramphenicol, streptomycin and cotrimoxazole [12,13,15].About 80% *Shigella* strains are now resistant to nalildixic acid, the drug of choice in *Shigella* infection [22]. The most recently used antibiotics such as mecilinam, pivmecilinam and ci-pro-floxacin also contributed to the development of resistant strains [23,16] . *Vibrio cholerae is* completely resistant to cotrimoxazole and resistant to tetracycline is also reported[19].

Salmonella strains are usually resistant to ampicillin and chloramphenicol. As many as 25% of *Salmonella* are resistant to ampicillin and 8% are resistant to chloramphenicol [20] .

Resistance to the most recently used antibiotic trimethoprim sulfamethoxazole is also increasing . *Klebsiella* strains are also found to be resistant against sulfonamides, ampicillin ,cephalosporins, chloranphenicol,tretracyclines and aminoglycocides [20].

But the development of resistant to these drugs has also been reported [16,23,24].The emergence of multiply drug resistance strains of pathogen has been causing outbreak of epidemic in developing countries[21,24]. Hence, more attention has been focused on the development of antimicrobial drugs those are active against these multiple drug resistant strains. It is , therefore ,a desperate need to search out new antibacterial agent from relief these killer diseases in perspective of Bangladesh.

Nature has endowed Bangladesh with enormous plant sources . Many of these plants grow wild in jungles, forests, gardens and many of them are found laying everywhere in the fertile region of the country. Some of these plants are used in Ayurvadic and Hekimi system of medicine prevalent in the country. The successful application of the plant and plant materials against various diseases fascinated chemists and biochemists to investigate these natural resources in the modern scientific way .

Higher cost, side effects and short of these drugs stated above led the researchers to find out a better drug and still research is going on. In the present research protocol, it was attempted to investigate plant products of indigenous origin, the plant products being the product of plant metabolism can easily be assimilated by human body and hence is less toxic. And also the deficiencies of the presently available antimicrobial agents together with the scientific interest and economic considerations have been drawn one attention to the traditional indigenous medicinal plants used in the infectious diseases.

Therefore, a study was made on some medicinal plants of local origin for their antibacterial activity and then separation and isolation of antimicrobial compounds from the active plants were carried out.

CHAPTER-2

SCREENING OF DIFFERENT PLANTS FOR THEIR ANTIBACTERIAL ACTIVITY

CHAPTER-2

SCREENING OF DIFFERENT PLANTS FOR THEIR ANTIBACTERIAL ACTIVITY

In the present research program for development of chemotheapeutic agents from Bangladeshi medicinal plants, ten plants were selected for testing antibacterial activity.

Table-1 : List of plants screened and parts used :

Sl. No.	Benglai Name	English Name	Botanical Name	Parts Used
1	Arjun	Arjuna Myro-balam	*Terminalia arjuna*	Bark
2	Sharpagandha	Snakeroot	*Rauvolfia serpentina*	Root
3	Kurchii	Kurchi	*Holarhena antidysenterica*	Seed
4	Bel	Wood apple	*Aegle marmelos*	Fruit
5	Hatisur	Heliotrope	*Heliotropium indicum*	Stem
6	Atmora	East Indian Screw Tree	*Helicteres isora*	Pods
7	Kalojam	Black Plum	*Syzygium cumini*	Bark
8	Biskatali	Water Pepper	*Polygonum hydropiper*	Stem
9	Amra	Wild Mango	*Spondias pinnata*	Bark
10	Chirata	Chirata	*Swertia chirata*	Stem

2.1 COLLECTION OF THE PLANTS :

Although all the plants are available all over Bangladesh, they were collected from a *Ayurveda* store situated at Chackbazar, Dhaka. All the selected plants are well known and so there was no chance of confusion. The identification was authenticated by comparing with the voucher specimens conserved at the Bangladesh National Herbarium .

2.2 CLEANING DRYING AND GRINDING :

The plants were first washed with water to remove the adhering dusts and then dried in the sunlight. The dried plants were then sliced into small pieces and ground in the grinding machine at 40-45 mesh size . The powdered plant materials were stored in air tight container .

2.3 EXTRACTION OF THE POWDERED PLANT MATERIALS :

2.3.1. Extraction with water

10 gm of each plant material (powder) was first extracted with water. The extraction was carried out in separating funnel with cold water for 2-days and then washed with water several times. The extract was stored in reagent bottle at 0^0C in refrigerator .

2.3.2. Extraction with methanol

The residue of the water extraction was then dried in the air and extracted with MeOH for 2 days. The MeOH extract was collected in reagent bottle and stored at 0^0C in refrigerator.

2.4 DETERMINATION OF ANTIBACTERIAL ACTIVITIES OF ALCOHOLIC & WATER EXTRACTS OF DIFFERENT PLANTS :

2.4.1.Organisms Employed for In Vitro Evaluation :

Ten gram negative and one gram positive bacterial pathogens, mainly responsible for diarrhoeal diseases in underdeveloped and developing countries were selected to determine their susceptibility to the different solvent extracts of ten plants specimens. Bacterial species were collected from International Center for Diarrhoeal Diseases Research, Bangladesh (ICDDR,B), Mohakhali, Dhaka.

Table-2 : **Organisms used in primary screening test :**

Sl.No.	Bacterial Species	ATCC No.
1	*Vibrio Choleriae*	J-1236
2	*Shigella dysenteriae-01*	AM-35858
3	*Shigella flexneri*	AM-36282
4	*Shigella sonnei*	AM-36858
5	*Shigella boydii*	AM-35193
6	*Shigella typhi*	AM-31173
7	*Salmonella paratyphi 'A'*	AM-30669
8	*Salmonella paratyphi 'B'*	AM-36387
9	Enteropathogenic *Escherichia coli*	ATCC-25922
10	*Klebsiella pneumoniae*	ATCC-23404
11	Staphylococus aureus	ATCC-25923

All the organisms were stored in Mueller-Hinton Slant at 0^0C in refrigerator.

2.4.2 CULTURE MEDIA USED

Mainly two types of culture media were used in this investigation. These were –

i. Mueller-Hinton Agar/ Broth Media.

ii. Nutrient Agar/ Broth Media.

Table –3 :Composition of Mueller-Hinton Broth Media (1000ml):

Ingredients	Amount, gm
Beef Extract (Infusion form)	: 20g
Acid Hydrolysate of Casein	: 17.5g.
Starch	:15g
Distill Water	:1000ml
p^H	:7.3 ±.2 at 25^0C

Table-4: **Composition of Mueller-Hinton Agar Media (1000 ml):**

Ingredients	**Amount, gm**
Beef Extract (Infusion form)	: 20g
Acid Hydrolysate of Casein	: 17.5g.
Starch	:1.5g
Bacto agar	:17.5g.
Distill Water	:1000ml
pH	:7.3 ±.2 at 25^0C

Table-5: **Composition of Nutrient Broth Media (1000ml) :**

Ingredients	**Amount, gm**
Bacto peptone	: 5g
NaCI	: 5g
Bacto yeast extract	:10g
Distill Water	:1000ml
p^H	:6.8 ± 0.2 at 25^0C

Table-6 : **Composition of Nutrient Agar Media (1000ml):**

Ingredients	**Amount, gm**
Bacto peptone	: 5g
NaCI	: 5g
Bacto yeast extract	:10g
Bacto agar	:20g
Distill Water	:1000ml
p^H	:6.8 ± 0.2 at 25^0C

Preparation of Agar/Broth Media:

Calculated amount of ingredients were taken in a conical flask . Distill water was added to it with constant stirring by means of a magnetic stirrer to make a clear solution . After dissolving the ingredients, p^H was adjusted using NaOH or HCl. Calculated amount of agar was then added to the solution and made up the final volume with distill water. Respective broth media (Mueller-Hinton or Nutrient)was produced at the same procedure without the addition of agar only.

2.4.3 APPARATUS USED :

Petridishes, screw cap test tubes, filter-paper discs, inoculating loops, autoclave, laminar flow, incubator, refrigerator , magnetic stirer, p^H meter, micropipette, sterile cotton, burner, etc.

2.4.4 STERILIZATION :

Sterilization of the media (Agar/Broth), petridishes, test-tubes, cotton swabs, filter-paper discs, and other necessary substance, were carried out in an autoclave . Sterilization was carried out at 126^0C and at 1.8 bar pressure for about 11 min.

2.4.5 PROCEDURE FOR THE DETECTION OF ANTIBACTERIAL ACTIVITY:

2.4.5.1 Preparation of Media Petridishes :

After sterilization, media and petridishes were transferred to the laminar flow from the autoclave. Hot media was poured into the moisture free plants to a uniform depth of 5mm.and allowed to solidify .The plates were incubated at 37^0C for 24 hours. The plates were then observed for the presence of any contamination. Fresh plats were used only in the tests.

2.4.5.2 Inoculation of Petridishes:

Fresh Muller-Hinton Agar plates (contamination) were streaked with the each test organism from pure cultures with the help of an inoculating loop in an aseptic condition. Inoculated plates were incubated at 37^0C for 24 hr. to obtain a clear growth of the test organisms. It was then stored at 4^0C and cultured organisms were used later whenever needed. After each 7-10 days intervals a fresh subculture for each test organism had to prepare and previous plates were discarded after sterilization .

2.4.5.3 Preparation of Filter-paper Discs :

Three types filter paper discs (5.5 mm dia.and 1.5mm thickness) sample discs ,blank discs ,and control discs were prepared for the investigation of antibacterial activity.

Sample Discs : A set of sterile uniform sized (mentioned above) filter paper discs were placed on a sterile petridishes and soaked with the solution of extracts by means of sterile pipettes, and discs were then allowed to dry at room temperature. The dried discs were then stored in vials at 0^oC.

Control Disc:

The sterilized discs were taken in sterilized blank petridishes. The discs were impregnated separately with methanol and water solvents in an aseptic condition Then the discs were dried in air and stored in freeze.

Blank Disc :

Blank discs were prepared in order to determined the antimicrobial activity of the used solvents in cases where water used as solvent. There was no need to use blank discs when organic solvents were used in dissolve the test materials.

STANDARD ANTIBOITIC DISCS:

The standard antibiotic discs were obtained from ICDDR,B. The standard antibiotic discs of ampicillin was used . The concentration of ampicillin used 10 µg/disc.

2.4.6 DETERMINATION OF ANTIBACTERAIL ACTIVITY :

The antibacterial activity of the extracts were determined by the discs diffusion method by measuring the diameter of the inhibitory zone in millimeter by a transparent scale.

2.4.6.1Preparation of Inoculum Suspension :

4-5 ml Muller–Hinton broth media were taken into screw cap test tubes and sterilized. Four to five similar colonies from a fresh subculture on agar plates were aseptically transferred to the broth in test tubes and incubated at 37^0C for 18-24 hr. until it becomes turbid enough and proper density of the inoculum suspension was achieved.

For instant use of organism 0.85% saline (NaCI) water is used as a suspension medium. 6-8 ml of saline water was taken into test tubes and sterilized . Test organisms were transferred into the tubes with a wire loop to a turbidity that is visually comparable with Mac. Farland 0.5 turbidity standard. The solution was then used as inoculum suspension.

2.4.6.2 Inoculation of test plates:

Sterile cotton swabs were dipped into the inoculum (saline test culture or broth test culture) and inoculum were removed from swabs pressing against the inside wall of the tubes. Contamination free Muller–Hinton agar plates were streaked by swabbing across the entire plates first in a horizontal direction and then in vertical direction to insure a heavy growth of the test organisms over the entire surface. In this way one or more plates were inoculated or seeded for each test organism.

2.4.6.3 PLACEMENT OF THE STANDARD AND SAMPLE DISCS ON THE INOCULATED TEST PLATES :

The antibiotic and samples discs were placed on the surface of the inoculated Muller – Hinton Agar plates with sterile needle tip. Each disc was pressed down gently to ensure complete contract with the agar surface .The discs were placed evenly so that they were no closer than 15 mm. From the edge of the plates and no two discs were closer than 24mm.from center to center. The plates were covered and inverted then placed in a incubator at 37^0C for 16 to 18 hrs.

2.4.6.4 MEASUREMENT OF THE ZONES OF INHIBITION OF THE PLATES :

After 16 to 18 hours incubation each plate was examined .The extracts which have antibacterial activity were grown zone of inhibition are shown in table-7 and the diameter of

the zones of inhibition including the diameter of the discs were measured by the naked eyes using a scale in terms of millimeter which are also shown in table-8, 9 & 10.

Table-7 : **In vitro activities of methanol and water extracts of different plants screened against ten gram negative and a gram positive bacteria :**

	Plants	Antibacterial Activity										
		S.dys.	S.son	S.flex	S.boy.	S.ty.	Sal.Par. "A"	Sal.Par. "B"	E.Col.	V.C	S.aur	K.pneu
1	*Holarhena antidysenterica*											
	*i.*MeOH-Ext.	-	-	-	-	-	-	-	-	-	+	-
	ii.H2O-Ext.	-	-	-	-	-	-	-	-	-	+	-
2	*Terminalia arjuna*											
	*i.*MeOH-Ext.	+	+	+	-	-	-	-	+	-	+	-
	ii.H2O-Ext.	+	+	+	-	-	-	-	+	-	+	-
3	*Rauvolfia serpentina*											
	*i.*MeOH-Ext.	-	-	-	-	-	-	-	-	-	+	-
	ii.H2O-Ext.	-	-	-	-	-	-	-	-	-	-	-
4	*Aegle marmelos*											
	*i.*MeOH-Ext.	-	-	-	-	-	-	-	-	-	-	-
	ii.H2O-Ext.	-	-	-	-	-	-	-	-	-	-	-
5	*Heliotropium indicum*											
	*i.*MeOH-Ext.	-	-	-	-	-	-	-	-	-	-	-
	ii.H2O-Ext.	-	-	-	-	-	-	-	-	-	-	-
6	*Helicteres isora*											
	*i.*MeOH-Ext.	-	-	-	-	-	-	-	-	-	-	-
	ii.H2O-Ext.	-	-	-	-	-	-	-	-	-	-	-
7	*Syzygium cumini*											
	*i.*MeOH-Ext.	-	-	-	-	-	-	-	-	-	-	-
	ii.H2O-Ext.	-	-	-	-	-	-	-	-	-	-	-
8	*Polygonum hydropiper*											
	*i.*MeOH-Ext.	-	-	-	-	-	-	-	-	-	-	-
	ii.H2O-Ext.	-	-	-	-	-	-	-	-	-	-	-
9	*Spondias pinnata*											
	*i.*MeOH-Ext.	-	-	-	-	-	-	-	-	-	-	-
	ii.H2O-Ext.	-	-	-	-	-	-	-	-	-	-	-
10	*Swertia chirata*											
	*i.*MeOH-Ext.	-	-	-	-	-	-	-	-	-	-	-
	ii.H2O-Ext.	-	-	-	-	-	-	-	-	-	-	-

(+) Organism susceptible to the extract; (-) Organism resistant to the extract.

Table–8: **Zone of inhibition exhibited by the methanol extract of *Holarhena anti-dysenterica* .**

Sl. No.	Bacterial Species	Zone of Inhibition (in mm)
1	*Staphylococcus aureus*	22

Table-9 : **Zone of inhibition exhibited by the methanol extract of *Terminalia arjuna* .**

Sl. No.	Bacterial Species	Zone of Inhibition (in mm)
1	*Shigella dysenteriae*	14
2	*Shigella flexneri*	13
3	*Shigella sonnei*	10
4	*Escherichia coli*	11
5	*Staphylococcus aureus*	18

Table-10: **Zone of inhibition exhibited by the methanol extract of *Rauvolfia serpentina.***

Bacterial Species	Zone of Inhibition (in mm)
Staphylococcus aureus	25

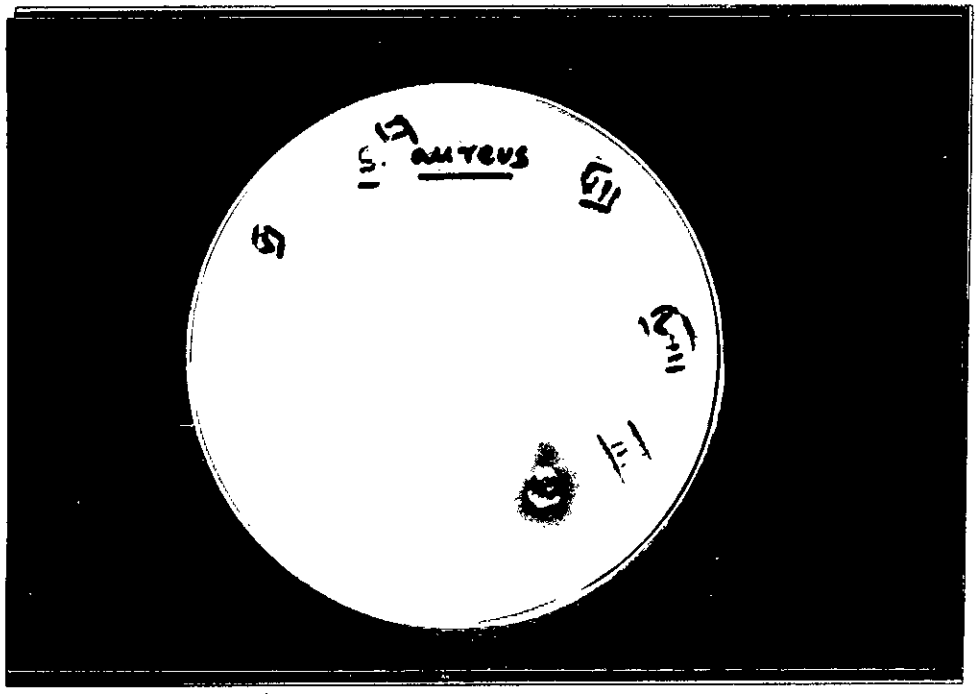


Figure-2.1 : Zone of inhibition exhibited by methanol extract of the seeds of *H.antidysenterica* aganist *Staphylococcus aureus* I-*H.isora*, II-Swertia chirata, III-*H.antidysenterica*,IV-A.m*armelos*, V-*S.pinnata*, VI-*H.indicum*.

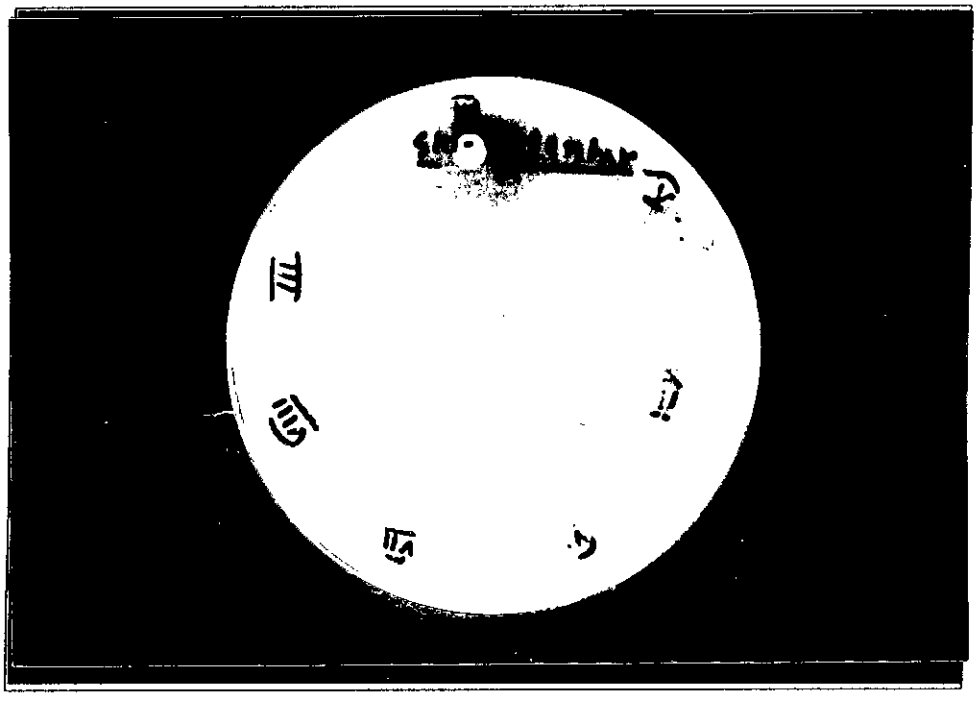


Figure- 2.2 : Zone of inhibition exhibited by methanol extract of the bark of *T.arjuna* aganist *Shigella dysenteriae*. I-*H.isora*, II-S.*chirata*, III-*T.arjuna* IV A. *marmelos*, V-*S. pinnata*, *V*I-*H.indicum*,

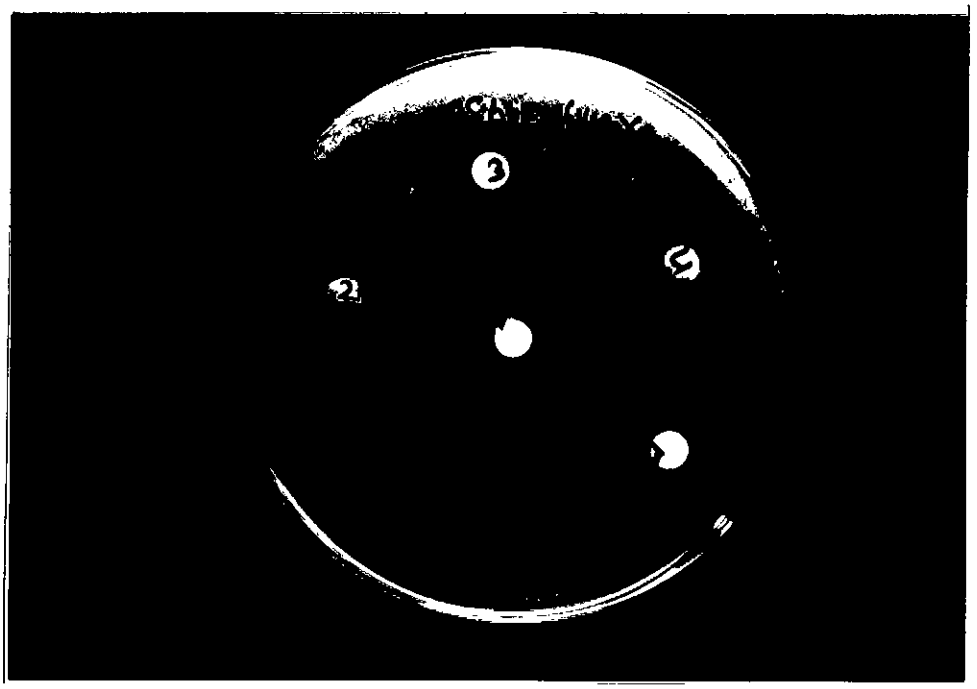


Figure-2.3 : Zone of inhibition exhibited by methanol extract of the bark of *T.arjuna* aganist *Shigella flexneri*.1-*H.indicum*, 2-S.*chirata*, 3-A. *marmelos*, 4-*R.serpentina*, 5-*H.isora*, 6-*T.arjuna*

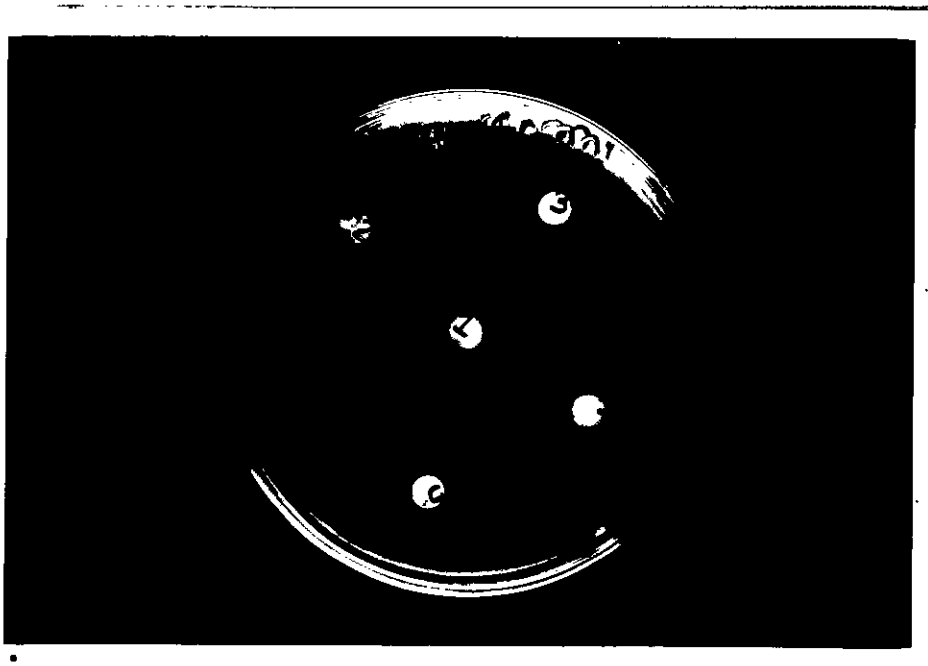


Figure-2.4 : Zone of inhibition exhibited by methanol extract of the bark of *T.arjuna* aganist *Shigella sonnei*.1-*H.indicum*, 2-S.*chirata*, 3-A. *marmelos*, 4-*R.serpentina*, 5-*H.isora*, 6-*T.arjuna*

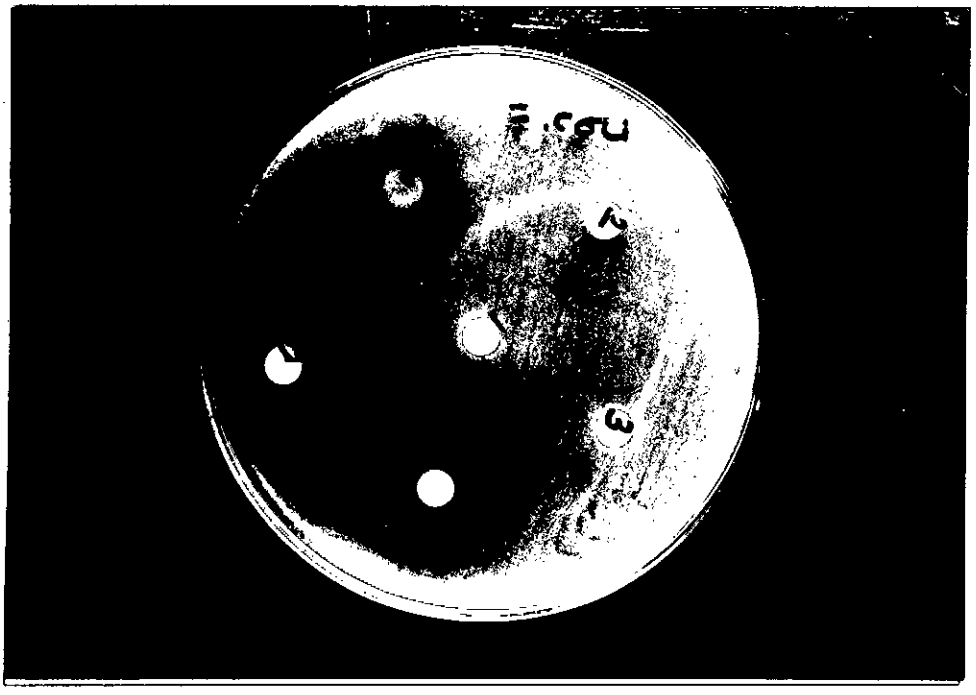


Figure-2.5 : Zone of inhibition exhibited by methanol extract of the bark of *T.arjuna* aganist *E.coli*.1-*H.indicum*, 2-S.*chirata*, 3-A. *marmelos*, 4-*R.serpentina*, 5-*H.isora*, 6-*T.arjuna*

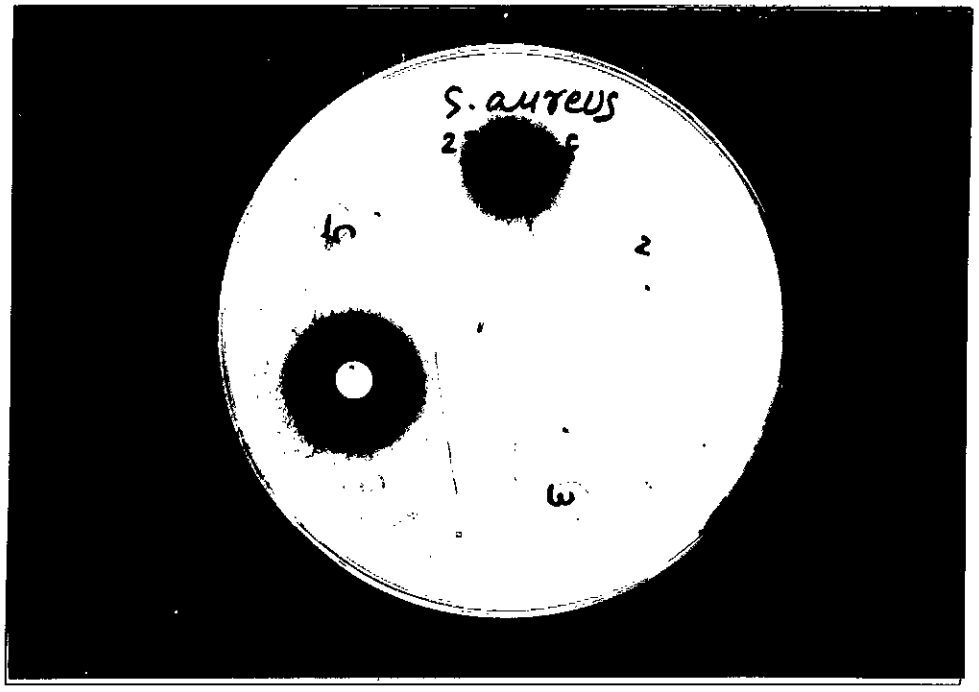


Figure-2.6 : Zone of inhibition exhibited by methanol extract of the bark of *T.arjuna* and *R. serpentina* aganist *S.aureus*.1-*H.indicum*, 2-S.*chirata*, 3-A. *marmelos*,4-*R.serpentina*, 5-*H.isora*, 6-*T.arjuna*

2.4.7 RESULTS & DISCUSSION :

Ten locally available plants were selected in screening test for the study of antibacterial activity . Plant materials were first extracted with water and then with methanol .The samples were first extracted with a very polar solvent e.g. water to get all components present in the samples .Some of the components were not soluble in water had the tendency to dissolve in methanol. Water and methanol extracts of the plants were tested for probable antibacterial activity against 10 gram negative and one gram positive bacteria. Among all the extracts methanol extracts of the seeds of *H.antidysenterica* ,the bark of *Terminalia arjuna* and the roots *of R.serpentina* showed a significant positive test against *Shigella dysenteriae type –01,Sh. flexneri, Sh. sonnei,E.Coli and S.aureus* .

It was found that the zones of inhibition of methanol extract of seeds of *H.antidysenterica* against *S.aureus strains was* 22mm. In case of the MeOH extract of the bark of *Terminalia arjuna* against *S. dysenteriae type –01,S.flexneri, S.sonnei ,E.coli and S.aureus* bacteria were 14 mm,13mm,10mm,11mm and 18mm.respectively.The zone of inhibition of the MeOH extract of the roots of *R.serpentina* against *S.aureus* were 25mm.The solvent extracts were crude but the zones of inhibition were high which indicated the stronger antibacterial activity of the bark of *T.arjuna.*

CHAPTER-3

LITERATURE SURVEY OF HOLARHENA ANTIDYSENTERICA, TERMINALIA ARJUNA & RAUVOLFIA SERPENTINA

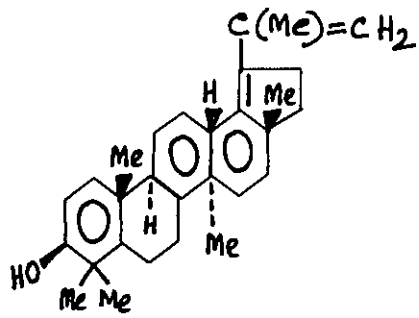
CHAPTER-3

LITERATURE SURVEY OF HOLARHENA ANTIDYSENTERICA, TERMINALIA ARJUNA & RAUVOLFIA SERPENTINA

3.1 LITERATURE SURVEY ON HOLARRHENA ANTIDYSENTERICA (KURCHI)

In the present research work on different medicinal plant extracts against various bacteria, the methanol and water extracts of the seeds of *H.antidysenterica* showed a strong antibacterial activity against *Staphylococcus aureus*. Therefore, through a literature survey on *H.antidysenterica* has been carried out. The literature survey on *H.antidysenterica* denotes that many workers have been separated various constituents, a few workers tested antihepatotoxic effects of the plant. But still now no research work has been carried out to separate and identify the compound (or compounds) which is active against shigellosis bacteria, k.*pneumoniae*,*Staphylococcus aureus*,*E.coli* etc.deadly bacterial strain.

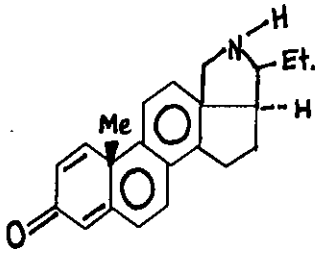
Ali et al.[25] isolated a new lupene-type triterpene, holarrhenol(I) from the leaves of *Holarrhena antidysenterica* wall.



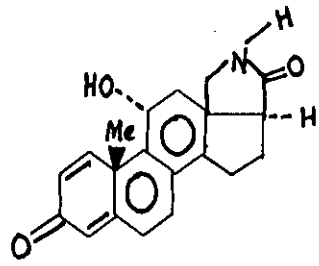
(I)

Its structure has been established as by spectral data analysis and chemical means.

Begum et al.[26] isolated three steroidal alkaloids, kurchilidine(I), kuchamide (II) and regholarrhenine of the conanine series from the bark of H.antidysenterica. I & II were new natural products while regholarrhenine was reported earlier from this source.



(I)



(II)

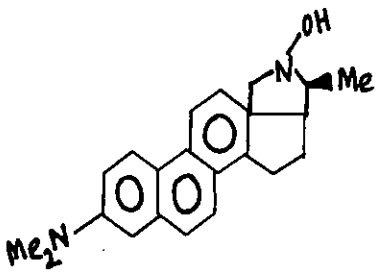
Thappa et al.[27] worked on Conessine, the steroidal alkaloid of *H.antidysenterica,* possesses a wide range of activities against four insects species viz. *Aedes aegypti*, *Dysdercus koenigu, Spodoptera litura* and *Pieris brassicae*.In D.koenigii the compd. inhibits the egg hatching of treated adults and nymphs. In A. Aegypti the larval developmental periods are extended, resulting in a high mortality rates. Such effects are produced at a very low dosages of 0.5 to10 ppm. Antifeedant activity was observed against larvae of S. litura and P. brassicae at conca. of 0.005 to 0.2% of Conessine.

Panda et al.[28] established Callus and suspension cultures of H.antidysenterica for the production of steroidal alkaloids, esp. Conessine. The doubling time and specific growth rate of cells in suspension culture were computed to be 47.5 h and 0.35/day, resp. A max . of 300mg alkaloids /100g dry cell wt.in 40 days and 130mg/100g dry cell wt. in 8 days were obtained in the cellus and suspension cultures, resp. Alkaloid production in suspension culture was combined growth and non-growth-associ. phenomenone. About 90% of the total alkaloids produced in the cell culture was conessine.

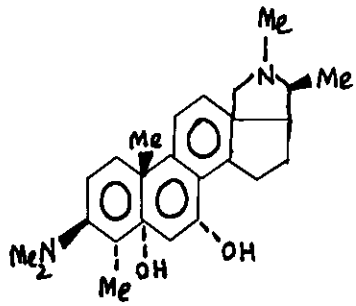
Dohnal et al.[29] found five alkaloids in 1-and 6-yr old callus tissues of H.antidysenterica ; two of them were identified as Conessine and Conimine. The alkaloid ext. inhibited the growth of *Shigella sonnei, Shigella flexneri and Salmonella enteritidis* strains, but not of S.typhi and S.paratyphi.

Bhutani et al.[30] isolated three new steroidal alkaloids, regholarrhenine D(I),E(II) and F(III),along with the none alkaloids kurcholessine, conessine, conessimine and isocone

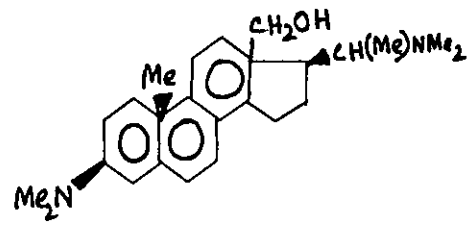
ssimine from the stem bark of *H.antidysenterica*. The structures were elucidated on the basis of spectral and chemical evidence. It was shown that the first alkaloid possesses the endo N-OH function in lieu of the endo N-Me function in Conessine. The second alkaloid was the C-7 stereoisomer of previously isolated alkaloid Kurcholessine. The third alkaloid was established as the 18-hydroxymethyl analog of Kurchessine.



(I)



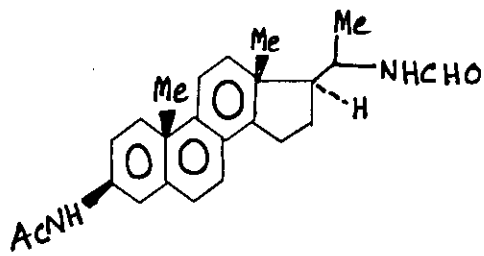
(II)



(III)

Dwivedi et al.[31] developed a turbidimetric method for the quantitative estimation of the total alkaloids of kutaj bark (H. antidysenterica) in crude medicinal preparations and in the body fluids of man and rat.

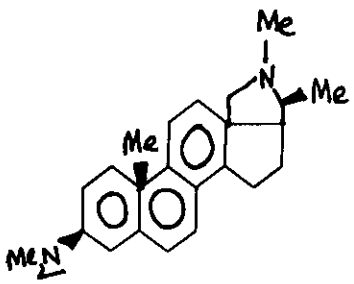
Siddiqui et al.[32] isolated a new alkaloid, provisionally named holarrifine (I) from the fresh,



(I)

undried bark of H. antidysenterica and structure was elucidated by chemical and structural studies using IR, mass, and ^{1}H- NMR spectrometry .

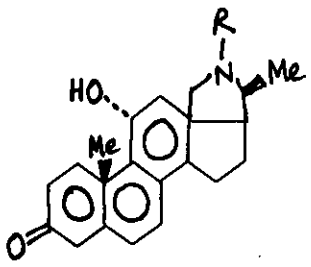
Richter et al.[33] worked on conessine (I), a steroid alkaloid present in bush–like plants of the genus holarrhena , delayed the next imaginal molting in last larval cockroach instars



(I)

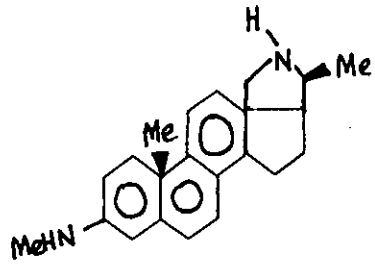
when ingested at concentration of 150 µg per larva per day. Molting interval was increased by 18 days , a 66% increase compared to control larva. I reduced ecdysteryd formation by the prothoracic glands to 2.77µg/gland/24h, which corresponds to that of inactive glands. Cannabalistic behavior was also induced by ingestion of I with a nearly 23% fatality rate in treated larvae.

Bhutani et al.[34] isolated three new steroidal alkaloids, namely regholarrhenine A(I), B(II), and C(III) from the stem bark of *H.antidysenterica* . collected at the flowering stage are



(I),R=Me

(II),R=H



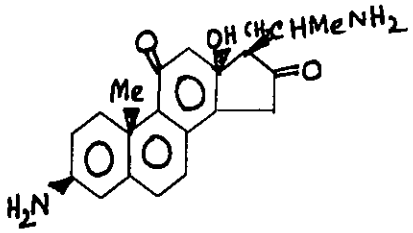
(III)

described. Structural studies on the basis of ^{1}H-NMR, ^{13}C-NMR, ORD,CD,and mass spectral and chemical evidences show that the first 2 alkaloids lack the C-3 amino function in the conenine structure ,but possesses a dienene system in ring A. In addition both contain a hydroxy group at C-11,but differ in the nature of the N in the heterocyclic ring.

III is the partially dimethylated enamine analog of conessine, where both the heterocyclic and C-3 nitrogens are secondary amines.

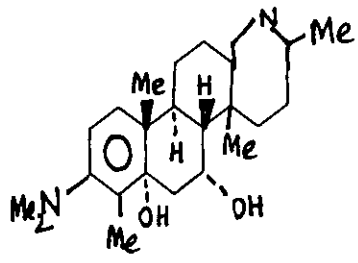
Bhutani et al.[35] studied on the alkaloid content of kurchi *(H. antidysenterica)* samples from 11 locations in India ranged 0.60-4.27,0.60-1.40,and 0,30-0.91% for stem bark, leaves and seeds ,respectively. In stem bark, maximum alkaloid contents occurred in April and October. Market samples contained much less of alkaloids than the wild population, possibly due to common adulterants *Wrightia tinctoria*, W.tomentosa, and *Ervatomia heyeniane* were 0.23,0.40,and 0.81% repectively.

Siddiqui et al.[36] isolated a new alkaloid, named holarricine (I) from the seeds of H. antidysenterica and elucidated as 11, 16-diketo-holarrhimine from chemical and spectral data.



(I)

Siddiqui et al. [37] isolated two new alkaloids provisionally named holacine (I) and holacimine from the bark of *H.antidysenterica* .The structure of I was elucidated through spectral studies and chemical reactions.



(I)

3.2 LITERATURE SURVEY OF TERMINALIA ARJUNA (ARJUN) :

In the present research work on different medicinal plant extracts against various bacteria, the methanol and water extracts of the bark of *Terminalia arjuna* showed a strong antibacterial activity against *Shigella dysenteriae* type-*01,Shigella flexneri, Shigella Sonnei,E.coli and Staphylococcus aureus*. Therefore, a through literature survey on *Terminalia arjuna* has been carried out .

Pettit, George R.et.al[38]. isolated the anticancer active compounds gallic acid,Ethyl gallate and the flavone luteolin. Luteolin has a well established record of inhibiting various cancer cell lines and was also found to exhibit specific activity against the pathoganic bacteria Neisseria gonorrhoeae.

Kusomoto,Ines Tomoco et.al[39] examined 39 crude drugs of Indian traditional medicine were subjected to screening their inhibitory effects on HIV type-1 protease (PR). The extract of the bark of *T.arjuna* inhibited the HIV–1 PR activity by more than 70% at a concentration of 0.2 mg\ml.

Karamsettey, -M.et.al[40] studied the effect of an aqueous extract of T.arjuna on rat atria and thoracic aorta .

Radhakrishnan ,-R.et.al[41] observed T. arjuna, an Ayurvedic cardiotonic , increases contractile force of rat isolated atria.

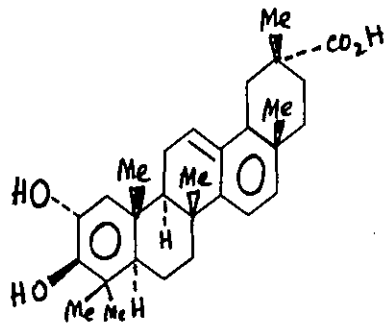
Dwivedi,- set.al[42] reviewed the pharmacognostical ,phytochemical, pharmacological and clinical studies on *T.arjuna*.The crude bark powder is cardioprotective and antiischaemic and possesses diuretic, prostaglandinenhancing and coronary risk factor modulating properties. The active principle is still unknown.

Lin-T-C.et.al[43] investigated the bark of T.arjuna led to the isolation and characterization of a rare complex –type tanin (10),in addition to nine well known hydrolyzable tanins :2,3-(5)-HHDP-D-glucose (1), 2,3-(5)-HHDP-6-O-galloyl-D-glucose (2),punicalin (3),punicalagin (4),terchebulin (5), terflavinc (6), castalagin (7), casuariin (8), and casu-

arinin (9).Chemical structures were established on the basis of spectroscopic and chemical evidences.

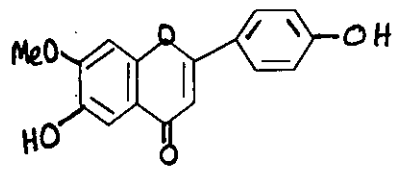
Tripathi,-V.K.et.al[44] isolated Arjunolitin, a triterpene glycoside from *T.arjuna*.

Ahmad, Mesbah U.et.al[45] isolated Terminoic acid (I), a new tryhydroxytriterpene carboxylic acid from bark of *T.arjuna*, along with 3 other non characterized compounds



(I)

Sharma et.al[46] isolated a new flavone designated as arjunolone (I) from the stem bark of *T.arjuna* , and elucidated it's structure on the basis of spectral data I was characterized as 6,4'-dihydroxy-7-methoxyflavone.

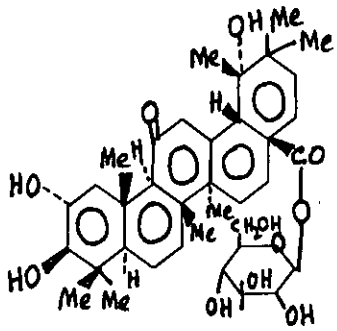


(I)

Madhusudanamna et.al[47] isolated flavon –3-ols from arjun *(T.arjuna)* bark.Arjun bark yielded 13.75% tanning contg.(+) catechol, (+)- gallocatcchol,epicatechol and epigallocatechol.

Blatia et.al[48]. opened a new source of oxalic acid from bark of *T.arjuna* .The digestion of water extracted bark of *T.arjuna* with 20% H_2SO_4 at 100^0C resulted in the isolation of oxalic acid (144-62-7) in 12-21% yield.

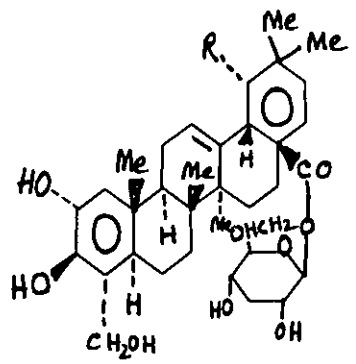
Tsuyuki et.al[49] isolated a new triterpene glucoside arjunglucoside III (I) from *T.arjuna* , as a β-D-glucopyranosyl 2α,3β,29α-trihydroxy-11-oxoolean-12-en-28-oate.



(I)

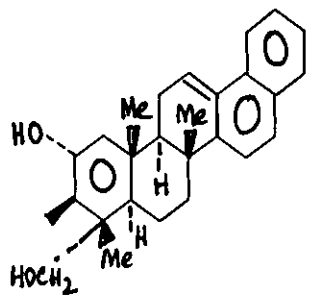
Ali et.al[50] worked on chemical investigation on *T.arjuna* bark.Extraction of bark of *T.arjuna* with rectified sprits gave a ppt. on concentration .The ppt. on extraction with petroleum ether ($60-80^0$) yielded β-sitosterol.The residue on further extraction with ether afforded acidic substance (4%), m.p.214-26^0. This substance on fractionation yielded 2 acidic triterpenoid compound B, m.p. 263-5^0. The residue was mainly tanin.

Honda et.al[51] isolated Arjungenin, a new triterpene from *T.arjuna*, was 2α,2β,19α,23-tetrahydroxyolean-12-en-28-oic acid. The structure of the two new triterpene glucoside arjunglucoside I and arjunglucoside II, isolated from the same plant, had structures I (R=OH) and II (R=H) respectively .



(I)

Honda et.al[52] isolated a new sapogenin, named arjungenin I from *T.arjuna* . It was shown to be 2α, 3β,19α,23-tetrahydroxy-olean-12-en-28-oic acid.



(I)

Cholabawalla et.al[53] studied on *T.arjuna,* an evaluation of the cardiotonic and other properties of *T.arjuna* .An alcoholic extract of the bark of *T. arjuna* ,a tree growing in Burma and Ceylon, contained CaO 0.33 %, MgO 0.078% and Al 2O3 0.076%. The powdered bark contained 38% CaO. The powered bark seemed to give relief to the symtomatic complaints of essential hypertension, but in the cases studied had no advantage over digitalis. The powder bark apparently had a diuretic and general tonic effect in cases of cirrhosis of liver.

3.3 LITERATURE SURVEY ON RAUVOLFIA SERPENTINA (SHARPAGANDHA)

In the screening test, it was found that, the methanol extract of the roots of *Rauwolfia serpentina* showed strong antibacterial activity against *Staphylococcus aureus*. Therefore, a literature survey on this plant specimen were carried out.

The literature survey on *Rauvolfia serpentina* revels that a little research work on the antibacterial activity of this plant has yet been carried out. A number of research work has been done on the separation of many alkaloids from the root of Rauvolfia serpentina. Uses of the root *R. Serpentina* in the treatment of hypertension and blood vessel protective and blood presser lowering substance from the plant has been identified.

Ashutosh Dutt et. al.[54] developed a method for the assay of total alkaloid and gave values for the stems ,leaves and roots . The pharmacologicpal properties of an alcohol extract of the roots of *R . serpentina* can not be competely accounted for on the basis of the alkaloids known to be present. Hence, a search for active material was made in the resin fraction. Powdered roots (100g) were extracted with alcohol. The extracted solids (4-13g) were leached with distilled water. The water insoluble residue (1.1-1.9g), free from alkaloids, was extracted with petroleum ether to remove oils. From the residue (0.75-1.0g) alcohol extracted 0.52-0.57g of active material which produced sedative and hypnotic effects in animal.

E.Schlitter et al[55] separated alkaloid serpentine from *R . Serpentina.* Sepentine (1) was isolated in 2-step procedure. The powdered extracted with NH_4OH and extracted with $(CH_2)_2Cl_2$; this extract contains the weak bases, mainly ajmaline. The root powder was dried, then percolated with MgOH containing 1% AcOH ; this extract contains the strong bases, mainly I with some ajmaline that was not removed by the $(CH_2)_2Cl_2$. All by products gave only oily salts and amorphous bases.

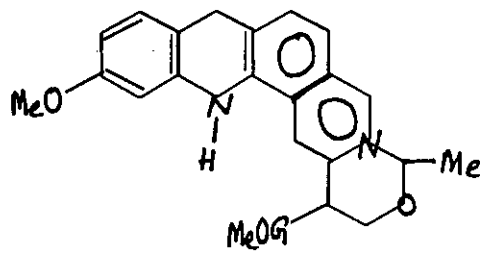
Sukumar Bose[56] isolated a new alkaloid rauwolfinine (I) from the root of *R. Serpentina.* I was isolated in quantity and its properties studied. It has the composition $C_{19}H_{26}O_2N_{29}$ (found:C, 72.71,72.73;H,7.85,8.04;N,8.6) no MeO or CH_2O_2 groups. 1N-Me (calculated,

for NMe, 4.77. Found 4.91),$[\alpha]^{82}D$-34.7^0. The UV spectrum shows max.at 249 and 292 mμ and min. at 226 and 272 mμ and resembles that of yohimbine, serpentine, and corynantheie in the far UV but not in the long region.I is a monoacidic base and forms a HCl salt, mp.195^0(decomposition).

Kurt Kramer et al.[57] worked on adrenolitic and central effects of extracts from R.serpentina. Intravenously rejected *R.serpentina* extracts, appreciably lowered the blood pressure of dogs and counter acted the effect of administered sympathomimetic drugs. In addition to the peripheral sympathicolytic effects the extracts showed central effects on the presser reflex of the heart. They had no direct effect on heart-lung prepns .

A.Stoll et al.[58] separated a new alkaloid from *R.serpentina*. Extraction of an aqueous suspension of the pulverized roots of *R.serpentina* with Et2O, followed by partial purification to remove faulty impurities, gave a total of 9.9gm of weakly basic alkaloid (I). From I was obtained 1.6gm of a $CHCl_3$, dissolving in 1% tartaric acid and reprecipitation with alkali, giving 0.21gm sarpagine, $C_{19}H_{22}0_2N_2$ (II), colorless needles from MeOH and EtOH, plates from Me_2CO, loses 1mole of crystallization at 130^{0}C decomposition above 320^{0}C (without melting). Equiv. Wt. 311, 309(calcd. 310-38); II HCl, colorless needles from EtOH, decompn. about 220^{0}C (both with melting).

E. Schlittler et al.[59] separated a new alkaloid reserpine from *R.serpentina*. Reserpine I, $C_{22}H_{26}04N_2$ m 238-9^0, $[\alpha]^{23}D$ −117±4^0 ($CHCl_3$), $[\alpha]^{22}D$ −95±4^0(pyridine), was found to contain 2 OMe, 1 CMe,1 active H, and no NMe groups.The following salts were prepared (m.p.given); HCL,244-6°,nitrate, 258-60°; perchlorate, 254.5°;Mel, about 240°C (decompn).The UV spectroscopy I has max. at 229mμ (∈ approx. 6200) and 298 mμ (∈=43000), it appeared to that of the alkaloid studied by popelak, et. al. (C.A. 48, 9626 h).the IR spectrum showed characteristic band at 3380,1703,& 1602 cm^{-1},and a double at 800-830 cm^{-1}.The following structural formula was proposed.



(I)

Bithika Rakshit et.al.[60] separated a new alkaloid chanrdrine from the roots of *R.serpentina*. A new alkaloid chandrine, was isolated from the roots of *R.serpentina* C25H30N2O8, m.p.230-1^0, picrate m.p.180^0. No experimental details were given.

Andrew Quevauviller et.al.[61] isolated a new alkaloid raugalline from *R. serpentina* and worked on its pharmacodynamics. The LD_{50} and LD_{100} if raugalline hydrochloride (l) determined by intravenous injection in mice were 33 mg\Kg & 40mg\Kg respectively. I has weak parasympatholytic properties (decrease of hypertension by excitation of the vagus nerve, suppression of reflex bradycardia, in vitro-antagonism with acetylcholenic contraction of the intestine and sympatholytic properties.

S.B. Penick et al[62] separated reserpine from *R.serpentina* is extracted with C_6H_6, the alkaloid obtained from the extract were dissolved in anhydrous MeOH and crystallization in the MeOH solution gave reserpine.

L.Cozzaroli [63] worked on the action of *R.serpentina* on the glycemic curves from glucose administration in normal and diabetic people. Observations on 30 patients are reported .The *R.serpentina* extract administration could cause a decrease of blood glucose and urine glucose and in (especially moderately) diabetic people.

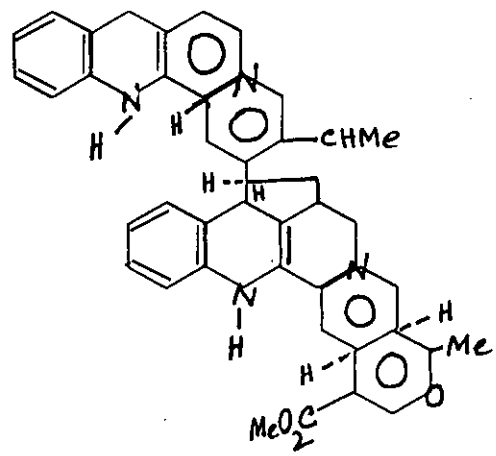
Urszula Rulkowska et al.[64] determined Reserpine in radix *R.* serpentina and in pharmaceutical II fluorometric method. A procedure for the determination of pure reserpine (I)

from crude alkaloid preparations based on UV flourescence measurements following TLC is described.

Biel et al[65] worked on reserpine alkaloid – antidepressant composition for treating hypertension. The little compns. contain, for example, resperpine –1,imipramine –5,strach USP 57, lactose USP 73, talc USP 9, and stearic acid 6 mg. The reserpine imipramine, starch and lactose powders are slugged, then granulated, mixed with the talc and stearic acid and tableted .

Khaleque et al[66] isolated of β-sitosterol, sucrose, and to alkaloids from the dry roots of *R. serpentina*. Powder dry roots of *R.serpentina* (8.85 Kg) extracted 3 times with petroleum ether , the combined extracts concentrated , the residual mass (19.63g) taken up in petroleum ether, the product (1g) which separated, filtered and chromatographed 20 times over neutral Al 2 O3 ,and the first fractions gave β-sitosterol (I), mp.132-4^0, identified by mixed m.p with authentic I; from the mother liquors of the above crystns.was obtained α_2 –sitosterol m.p. 151-3^0, identified by the mixed m.p.

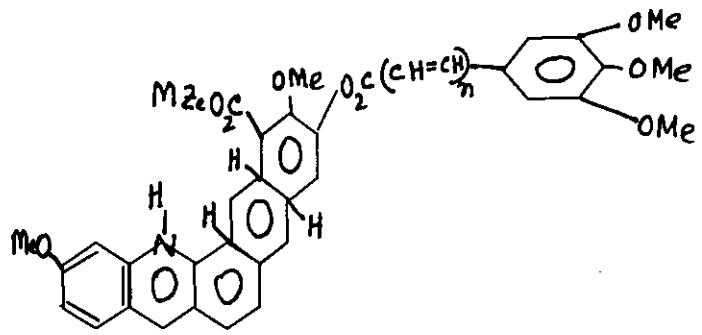
Hiroshi et al.[67] worked on X-ray crystallographic determination of the structure of the alkaloid serpentine. This structure of the serpentine from *R.serpentina* was detd. by X-ray



(I)

crystallography of its dihydrobromide dihydrate.

Hartman et al.[68] determined reserpine and rescinnamine in alkaloid extracts and pharmacological preparations by direct quantitative photometry on TLC.

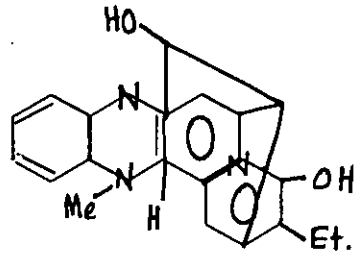


I. n=0

II. n=1

A sensitive and selective method for quant. method for the deten. of reserpine (I) [50-55-5] and alkaloid extracts and pharm. prepns.is desired.

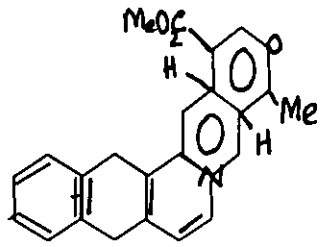
Tukhtasinov et al[69] extracted strain of *R.serpentina* tissue culture with 90% aq. iso-PrOH, after evaporation, the ppt. was dissolved in MeOH and then in tertaric acid (solution) alkalized, treated with C_6H_6, and recrystallised to give ajmaline (I) [4360-12-7].



(I)

Belganskik et al. [70] isolated serpentine and alstonine from *R.serpentina* . Alstonine (I) [47485-83-6] and serpentine (II) [18786-24-8] were extdt. in the pure form from *R.serpentina* by a process in which the methanolic exts. were evaporated to a dryness then taken up by H_2O at p^H 7.0-7.5, extd. by $CHCl_3$,where II was recovered in aqueous phase and I recovered in the other phase by extraction with MeCOEt. Cancer cells and

tumors were killed upon administration of a freshly prepared mixture of I & II and cyclophosphamide [50-18-0].



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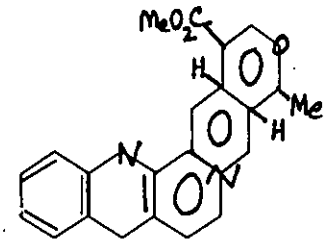
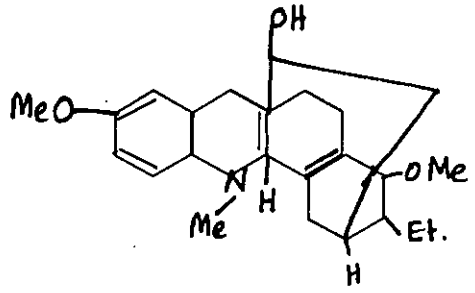


(II)

Siddiqui et al.[71] isolated a new dihydro indole alkaloid named sandwicoldine from *R.serpentina* and elucidated its structure through chemical and spectroscopic studies.



(I)

Siddiqui et al.[72] isolated a new heteroyohimban alkaloid ajmalicidine from the roos of *R.serpentina* of Thai origin. Its structure was elucidated as 1-carbomethoxy-17α - hydroxymethoxy-16,17-dihydro ajmalicidine through chemical and spectral studies.

Polz et al.[73] isolated two novel indole alkaloids raumacline and Nb-methylraumacline from the cell cultures of *R.serpentina*. The structure of the new alkaloids were determined by spectroscopic methods and chemical synthesis from ajmaline.

Chapter – 4

SEPARATION AND ISOLATION OF ANTIBACTERIAL COMPOUND(S) FROM HOLARHENA ANTIDYSENTERICA

CHAPTER – 4

SEPARATION AND ISOLATION OF ANTIBACTERIAL COMPOUND(S) FROM HOLARHENA ANTIDYSENTERICA (KURCHI).

4.1.1 INTRODUCTION

In the primary screening test of this investigation it was found that methanol and water extracts of the seeds of *H.antidysenterica* showed a significant antibacterial activity against *Staphylococcus aureus.* Therefore the present section was focused on separation and isolation of antibacterial compound(s) present in the plant. In this connection the seeds of *H.antidysenterica* was serially extracted with four different organic solvents with increasing polarity to distribute the compounds present in the plant into different solvents according to their solubility properties. The antibacterial activity of these different solvent extracts was carried out against the bacterial species found susceptible to the extract in the screening test.

The active extract found was then chemically fractionated into different fractions by solvent extraction with different solvents to make the separation of the antibacterial compounds easier from the crude active extracts. Antibacterial activity of different fractions was then carried out against the test organisms.

The antibacterial compounds were then separated and isolated from the active fraction with the help of preparative thin layer chromatography (PTLC).

4.1.2 ABOUT THE PLANT : Holarhena antidysenterica

Family Name : Aposynaceae
Common Names :

Botanical Name	: Holarhena antidysenterica wall
English Name	: Kurchi
Bengali Name	: Kurchii
Part of the Plant Used	: Seed

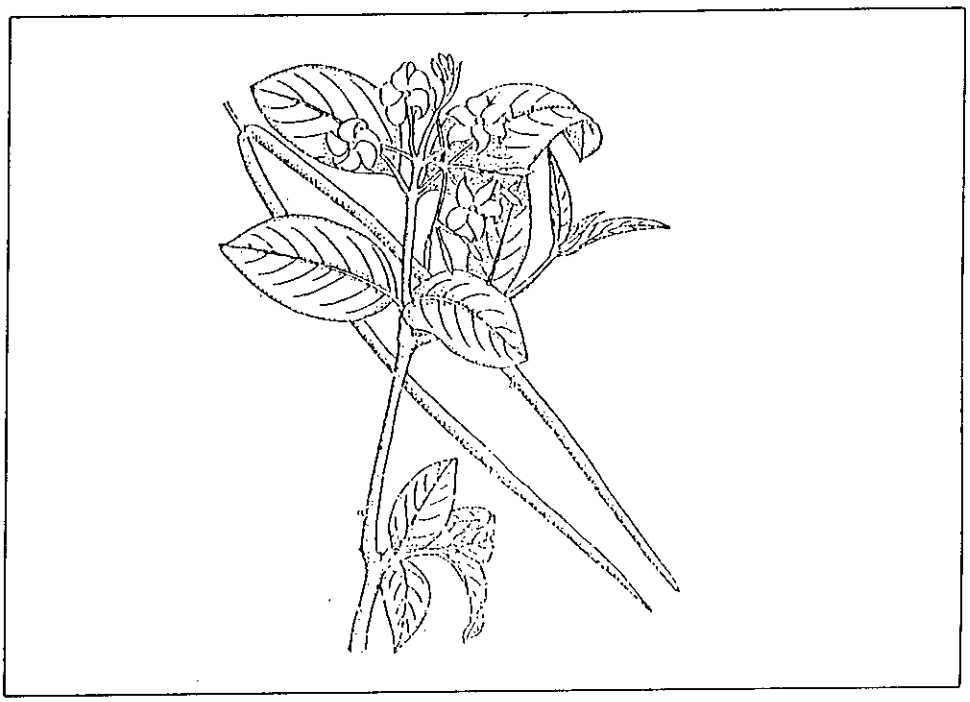


Fig: 4.1: Photograph of the plant of *H.antidysenterica*.

Identifying Character and Natural Odor[74]

It is a moderate tree of 20/25 feet hight. The length and weidth of it's leaves is 6-7 inch and 1.5 to 2.5 inch respectively. Oval shaped Bota tends to narrow gradually. It's leaves drop-out in spring. In summer new leaves grown & a lot of white flowers smell with a little bite are seen in the rainy season.

Habitant and Therapeutic Uses of *H.antidysenterica*[75]

The bark is stomachic, astrringent, powerful antidysenteric, antidiarrhoeal, febrifuge and anthelmintic. The seeds are considered to possess similar properties as the bark. Leaves are used in chronic bronchitis.

Availability : Forests of Dhaka, Chittagong and Mymensingh of Bangladesh.

4.1.3 SUCCESSIVE EXTRACTION OF THE SEEDS OF HOLARHENA ANTIDYSENTERICA AND ANTIBACTERIAL ACTIVITY OF DIFFERENT EXTRACTS

4.1.3.1 EXTRACTION OF THE SEEDS OF HOLARHENA ANTIDYSENTERICA WITH DIFFERENT SOLVENTS OF INCREASING POLARITY .

Clean, dry seeds of *H.antidysenterica* ground in grinding machine. Ground seeds with 40 mesh was first extracted with petroleum ether and then successively with ethyl acetate, MeOH and H_2O with increasing polarity. 1200 gm of ground seeds was extracted with 2.5 L pet. ether which was carried out in room temperature. Extract was filtered out and the residue was extracted again with 2.5 L pet. ether . The two extracts were combined and stored in a refrigerator.

The residue obtained after extraction with pet. ether, was air dried and then again extracted with 2.5 L ethyl acetate in room temperature. The extract was separated by filtration. The residue was again extracted with 2.5 L ethyl acetate. Two extracts were combined and stored.

The residue obtained after extraction with ethyl acetate, was air dried and was extracted with methanol and finally with water in the same procedure.

The extraction scheme of the seeds of *H.antidysenterica* with different solvents of increasing polarity is shown in figure 4.2.

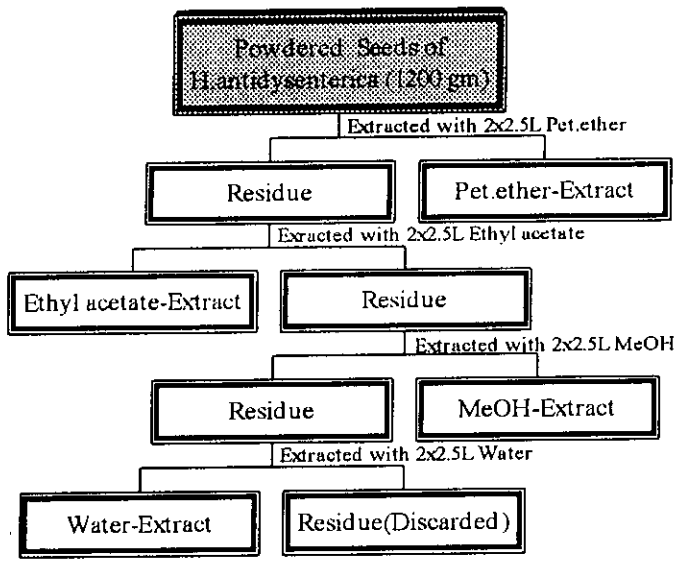


Fig-4.2: Extraction scheme of the seeds of *H.antidysenterica* with different solvents.

Solvents from pet. ether, ethyl acetate and methanol extracts were evaporated under reduced pressure by means of Rotary Vaccum Evaporator and finally dried to solid. The percentage yields of each of the extracts were shown in Table-11.

Table-11 : Percentage yield of the different solvent extract of the seeds of *H.antidysenterica.*

Solvent	Powdered Seeds (gm)	Quantity of Solvent in Liter	Weight of Dried Extract (gm)	Yield (%)
Pet. ether	1200	5	123	10.25
Ethyl acetate		5	28	2.3
Methanol		5	256	21.3
Water		5	-	-

4.1.3.2:DETERMINATION OF ANTIBACTERIAL ACTIVITY OF DIFFERENT SOLVENT EXTRACT OF THE SEEDS OF HOLARHENA ANTIDYSENTERICA.

Pet.Ether-Ext.,Ethyl Acetate-Ext., MeOH-Ext., and water-Ext. of the seeds of *H. antidysenterica.* were investigated for their antibacterial activity against *Staphylococcus* aureus as the seeds of *H.antidysenterica* was found to be active against the test organism in the primary screening test.

Procedures for the detection of antibacterial activities:

For the detection of antibacterial activities of different extracts, the Kirby-Bauer technique was followed as described in chapter-2.

Four sets of filter paper discs(5.5mm dia &1.5mm thickness)were impregnated separately with four different extracts and dried. The test organisms were inoculated on Mueller Hinton (M.H) agar plates and were incubated at 37^0C for 24 hours. Four to five similar Colonies from the agar plates were transferred to 3 ml of M.H. broth in test tubes and incubated for 4 to 6 hours. The cultures were diluted in saline to match the $BaSO_4$

\standard[76]. Sterile cotton swabs were dipped into inoculum and inocula were removed from swabs pressing against the inside wall of the tubes. Mueller Hinton agar plates streaked by swabbing across the entire plate three times, each time at 60^0 angle to the previous streaking. The discs were placed on the seeded plates with sterile forceps and each disc was gently pressed down to ensure proper contact with the agar. The plates were incubated for 24 hours at 37^0C. The antibacterial activities were expressed by measuring zone of inhibition in mm (Table-13).

***Table-12*: Antibacterial activities exhibited by the different solvent extracts of the Seeds of *H.antidysenterica*.**

Bacterial Species	Activity			
	Pet.ether-Ext.	Ethyl acetate-Ext	MeOH-Ext.	H_2O-Ext.
Staphylococcus aureous	-	-	+	+

(+) : Susceptible to the extracts ; (-): Resistant to the extracts.

***Table-13*: Zone of inhibition exhibited by MeOH- Ext. of *H.antidysenterica* against the test organisms and comparison with standard ampicillin.**

Bacterial Species	Zone of Inhibition	
	MeOH- Ext.	Standerd Antibiotic
Staphylococcus aureous	18 mm	16 mm

4.1.4 FRACTIONATION OF THE CRUDE METHANOL EXTRACT WITH DIFFERENT SOLVENTS.

Methanol extract of the seeds of *H. antidysenterica* was found to be active against *Staphylococcus aureus.* As the methanol extract was in crude form, separation of active compound (s) from it was very much difficult. Hence, the fractionation of crude methanol extract was carried out by extraction with different solvents (Hexane, $CHCl_3$,Et.acetate) to make the separation of the antibacterial compounds easier from the crude fraction.

4.1.4.1 MATERIALS AND METHODS :

Sample:

Crude methanol extract was dried to solid in a rotary vacuum evaporator at reduced pressure.

Solvents:

n-Hexane, Chloroform , Ethyl acetate etc.

Apparatus:

Separating funnel, Beaker, 100 ml measuring Cylinder, Reagent bottles etc.

Procedure:

1 gm of dried extract was dissolved in 90 ml methanol and 10 ml distilled water was added to make the volume up to the 100 ml. It was then transferred into a separating funnel. 100 ml n-Hexane was added to it and shacked mildly, and allowed for setting. The solution was separated into two fractions, Hexane fraction as upper layer and aqueous fraction as lower layer. Hexane fraction was separated out and collected. Aqueous fraction was recycled to the funnel and again extracted with 100 ml Hexane, in the same way. Extraction was carried out for 3 times. Three Hexane fractions were combined and dried. 12.5 ml distilled water was then added to the remaining aqueous fraction into the separating funnel. 100 ml $CHCl_3$ was added to the funnel. Shacked mildly and allowed for setting. Lower layer was collected as $CHCl_3$- fraction. Remaining aqueous fraction again extracted with 100 ml $CHCl_3$. The process was repeated for 3 times, and three $CHCl_3$- fractions were collected, combined and dried.

Into separating funnel 16 ml distilled water was added to the remaining aqueous fraction. The total volume was extracted with 100 ml Et.acetate for 3 times. In each case Et.acetate fractions were separated as lower layer. Three fractions were combined and dried.

The remaining volume in funnel was collected as aqueous fraction.

A typical flow scheme for the solvent extraction of crude methanol- extract is given below :

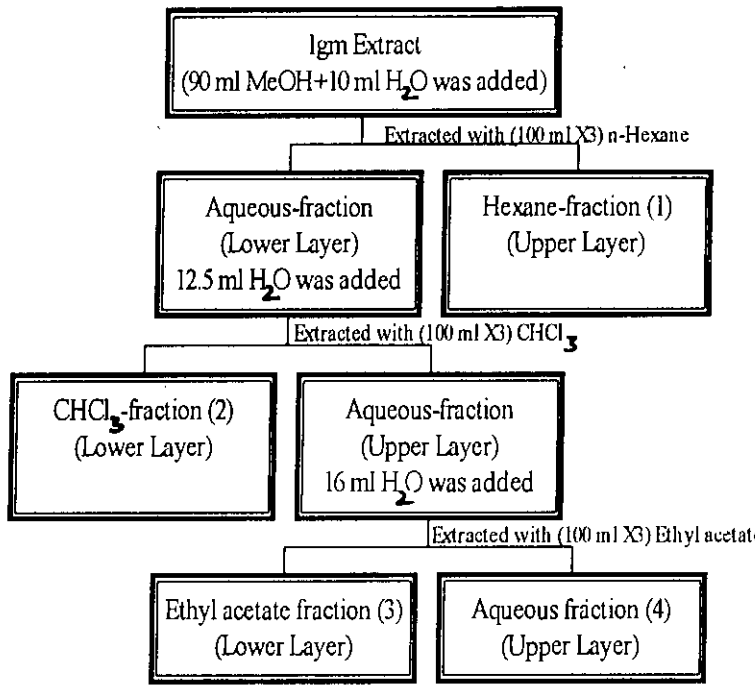


Fig:- 4.3: Flow scheme for the solvent extraction of crude methanol extract of *H.antidysenterica.*

4.1.5 MICROBIOLOGICAL INVESTIGATION OF FOUR FRA TIONS OBTAINED BY THE FRACTIONATION OF MeOH-EXTRACT.

The antibacterial activity of these fractions (Hexane-fraction, $CHCl_3$-fraction, Ethyl acetate-fraction and aqueous-fraction) obtained by the fractionation of methanol extract were carried out against the test organism *Staphylococcus aureus*. Activity was tested by disc diffusion method described in chapter-2. The result obtained was listed in Table-14.

***Table-14* : Susceptibility of the test organisms to the fraction obtained from the solvent extraction of MeOH-extract.**

Bacterial Species	Antibiotic activity			
	C_6H_{12}-fraction	$CHCl_3$-fraction	Ethyl acetate-fraction.	Aq-fraction
Staphylococcus aureous	-	-	+	+

(+) : Susceptible to the organism ; (-): Resistant to the organism.

4.1.5 SEPARATION & ISOLATION OF THE ANTIBACTERIAL-COMPOUND(S) FROM THE ETHYL ACETATE FRACTION OBTAINED BY THE FRACTIONATION OF CRUDE METHANOL EXTRACT.

Aqueous-fraction and Et.Ac.-fraction obtained by the solvent extraction of crude MeOH-extract of the seeds of *H.antidysenterica* was found to be active against *S.aureus*. But Et.Ac. fraction showed a significant antibacterial activities than Aq.-fraction. Antibacterial compounds from the ethyl acetate fraction were then separated by preparative thin layer chromatography (PTLC).

To do this, first a suitable solvent system have had to developed to run the PTLC plate to obtained a good resolution of the compounds present in the crude. It was carried out by running the plate with different solvent systems. Separated compounds were microbiologically tested whether it exhibited activity against test organisms or not.

4.1.5.1 APPARATUS USED :

TLC plate (20×20cm),TLC jar, Spreader machine, Spotter, UV-light(254nm & 350nm), Oven, Silica coated Aluminium plates.

4.1.5.2 DEVELOPMENT OF A SUITABLE SOLVENT SYSTEM:

It is of prime importance to select a suitable solvent to obtain a good resolution of the compounds present in the crude fraction. The choice of the solvent is influenced by the nature and solubility of the mixture but other things being equal, it is often better to choose a solvent of indifferent eluting power.

With the aim of finding out a good developing solvent a number of TLC plates were run with different solvent system. Employed solvent system and their separation behavior are listed in the Table-15 below:

Table-15: Different solvent systems employed for the analysis of Ethyl acetate-fraction on thin layer chromatography (TLC):

Sl.No.	Solvent System	Ratio	Resolution
1	Et-Ac: Pet.ether	1:1	-
2	Et-Ac: Pet.ether	3:1	-
3	Et-Ac: $CHCl_3$	1:1	-
4	Et-Ac: $CHCl_3$	4:1	-
5	Et-Ac	100%	-
6	Et-Ac: MeOH	3:1	+
7	Et-Ac: MeOH	3:2	+ +

(-), No Separation; (+), Separation; (+ +), Good Separation;(Et.-Ac) Ethyl acetate.

Among these solvent system listed above a good resolution of the compounds present in the Et.-Ac.-fraction was found with Et.-Ac.: MeOH (3:2). With this developing solvent separate and distinct spot on the middle of the plate and another spot on the base line were found.

Detection of the spots:

The spots were identified by :

i) Under short wave UV light (254nm) or

ii)By development with Phospho- Molebdenum developing reagent.

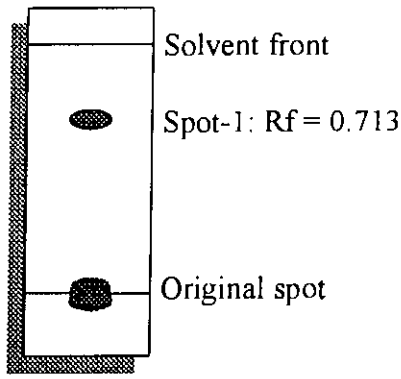


Fig:- 4.4: Thin Layer Choromatogram obtained by TLC of Et.Ac.fraction with Et.Ac-MeOH (3:2).

4.1.5.3 PREPARATIVE THIN LAYER CHROMATOGRAPHY (PTLC) FOR THE SEPARATION OF THE COMPOUNDS PRESENT IN THE ETHYL ACETATE FRACTION.

The compounds present in the Et.Ac.-fraction were separated by performing PTLC with the developing solvent Et.Ac.: MeOH (3:2).

Parameters:

Plate	: Aluminium Plate (20×20 cm)
Adsorbent	: Silica gel (60 G)
Thickness	: 0.75μm.
Developing solvent	: Et.Ac.: MeOH (3:2)
Development	: Ascending, One dimensional.

Development of the plate :

Activated plate was first spotted with Et.Ac.-fraction and allowed to dry. The plate was then placed in TLC jar containing 50 ml of developing solvent Et.Ac.-MeOH (3:2).The plate was allowed to run to the desired solvent front. It was then taken out of the jar and dried by blow drier.

Detection of the Resolute compounds:

The chromatogram was examined by Phospho-Molebdenum developing reagent .Two bands were found. Of them one separated and distinct band was marked on the plate.

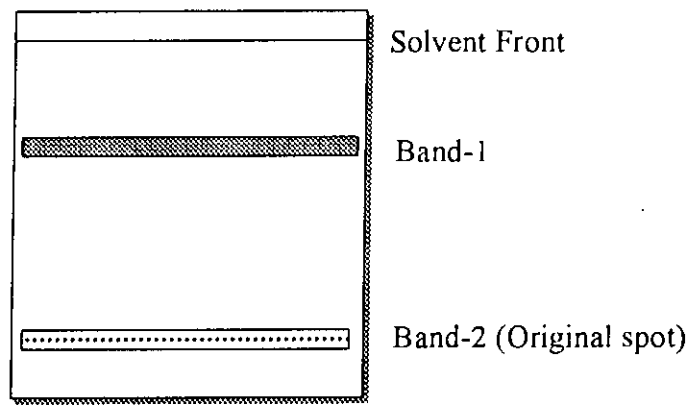


Fig: 4. 5: Chromatogram obtained by the PTLC of Et.Ac.-fraction with developing solvent Et.Ac-MeOH (3:2).

Elution of the Compounds from the TLC Plate:

The bands were scrapped off separately with a fine spatula,and marked as Band-1,and Band-2 as shown in figure-4.5. Band-1 & Band –2 were taken in two Conical flask and immersed in MeOH. Then these were filtered. These filtrates were evaporated by Rotary Vacuum Evaporator. By doing TLC it was found that a single spot was found in case of Band-1 & in case of Band-2 two spots were found.

4.1.5.4 ANTIBACTERIAL ACTIVITY OF ISOLATED COMPOUNDS

Two compounds (HA_1 & HA_2) separated from the Et.Ac-fraction PTLC technique were then microbiologically tested for their antibacterial activity against *Staphylococcus aureus*. The test was carried out following disc diffusion method [76] descrived in chapter-2 by measuring the zone of inhibition. The results of antibacterial activity are shown in Table-16.

***Table-16*: Antibacterial activity of the separated compounds(HA_1 & HA_2):**

Bacterial species	Zone of Inhibition(mm)	
	HA_1	HA_2
Staphylococcus aureous	21	-

It was evident from the Table-16 that, compounds HA_1 was found to be active against *S.aureus*; compound HA_2 exhibited no antibacterial activity.

4.2 SEPARATION AND ISOLATION OF ANTIBACTERIAL COMPOUND(S) FROM TERMINALIA ARJUNA (ARJUN).

4.2.1 INTRODUCTION:

In the primary screening test of this investigation it was found that methanol extract of the bark of *Terminalia arjuna* showed a significant antibacterial activity against *Shigella dysenteriae,Shigella flexneri, Shigella sonnei,E.coli* and *Staphylococcus aureus.*

Therefore the present section was proceeded on separation and isolation of antibacterial compounds present in this plant. In this connection the bark of T.arjuna was serially extracted with four different organic solvents with increasing polarity to distribute the compounds present in the plant into different solvents according to their solubility properties. The antibacterial activity of these different solvent extracts was carried out against the bacterial species found susceptible to the extract in the screening test.

The active extract found was then chemically fractionated into different fractions by extraction with different solvents to make the separation of the antibacterial compounds easier from the crude active extract. Antibacterial activity of different fraction was then carried out against the test organisms.

The antibacterial compound (s) were then separated and isolated fromthe active fraction with the help of preparative thin layer chromatography (PTLC).

4.2.2 ABOUT THE PLANT: *Terminalia arjuna*

Family Name : *Combretaceae*
Common Names :
Botanical Name : Terminalia arjuna(Roxb.) W&A.
English Name : Arjuna Myrobalam, Arjun.
Bengali Name : Arjun.
Part of the plant used : Bark.

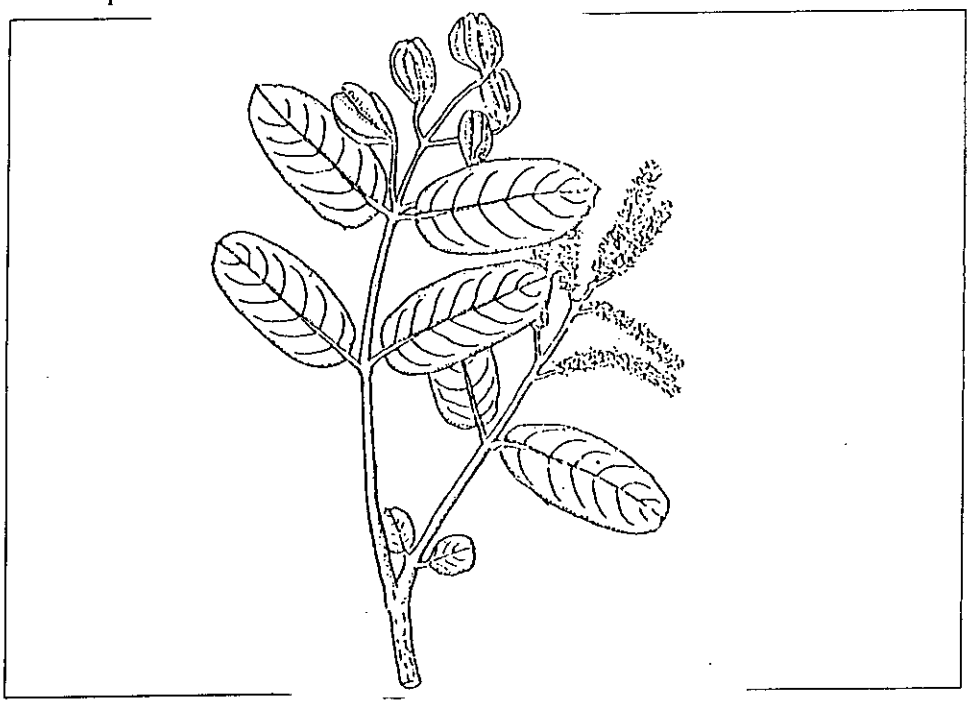


Fig: 4.6: Photograph of the plant *Terminalia arjuna*

Identifying character & natural odor[25]:

Terminalia arjuna (Family-Combretaceae) known as "Arjuna" in Indian Medicine,is a large tree with huge trunk and horizontally spreading branches. It normally attains the height of about 20 to 30 meters. The bark is smooth and pinkish gray from outside and reddish from inside. The wood is reddish white and heart wood is brown variegated with darker colored streaks. The leaves are usually sub-opposite 10-15 by 4-7 cm, oblong, obtuse or sub-cute, base rounded or cordate, often unequal sided, main nevers arcuate, 10-15 pairs and reticulate venation, 6-10 mm long petioles. Flowers, sessile in short axillary spikes or interminal panicles, bracteoles linear lanceolate shorter than the flowers, caducous, calyx glaberous. Teeth triangular, ovary quite glabrous, disk clothed with yellowish or reddish hairs and 3-5 cm long. Drupe fruits are 2.5-5 cm, ovoid or obovoid – oblong, fibrous –woody, glabrous, dark brown with five hard projection wings stariated with numerous curves veins.

Habitat and Therapeutic Uses[26] of *T.arjuna*

Bark is a cardic tonic, astringent and febrifuge. It is used in the treatment of red an swollen mouth, tongue and gums. It stops bleeding and pus formation in the gums . It is useful in dysentery , asthma, hypertension, wounds, skin eruptions, menstrual problem, pains and leucorrhoea. The twing are used by tribals to blisters and ulcers of the mouth.

Availability :

Terminalia arjuna is planted all over the country.

4.2.3 SUCCESSIVE EXTRACTION OF THE BARK OF TERMINALIA ARJUNA AND ANTIBACTERIAL ACTIVITY OF DIFFERENT EXTRACTS

4.2.3.1 EXTRACTION OF THE BARK OF TERMINALIA ARJUNA WITH DIFFERENT SOLVENTS OF INCREASING POLARITY

Clean, dry bark of *T.arjuna* ground in a grinding machine. Ground bark were first extracted with pet. ether and then successively with ethyl acetate, MeOH and H_2O with in-

creasing polarity. 1500 gm of ground bark was extracted with 2.5L pet.ether . Extract was filtered out and the residue was extracted again with 2.5L pet.ether. The two extracts were combined and stored in a refrigerator.

The residue obtained after extraction with pet. ether, was air dried and then again extracted with 2.5L ethyl acetate. The extract was separated by filtration. The residue was again extracted with 2.5L ethyl acetate. Two extracts were combined and stored.

The residue obtained after extraction with ethyl acetate, was air dried and was extracted with methanol and finally with water in the same procedure.

The extraction scheme of the bark of *T.arjuna* with different solvents of increasing polarity is shown in figure 4.7.

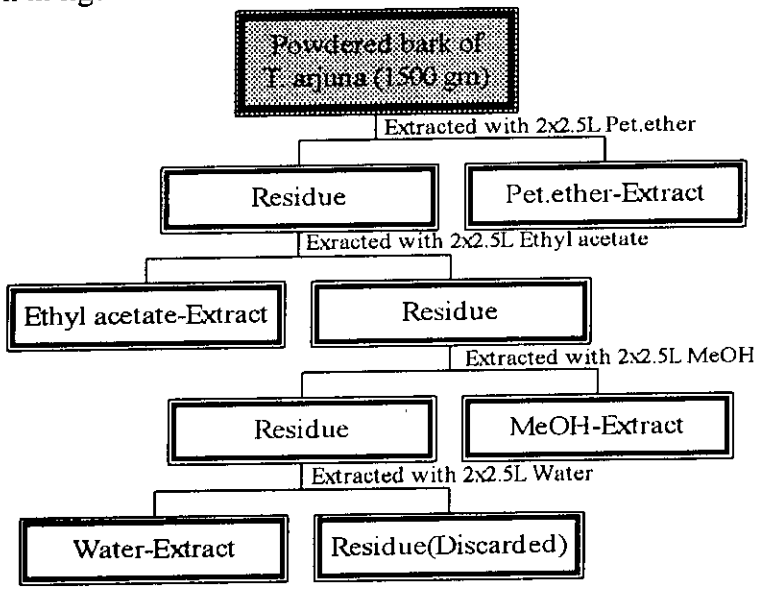


Fig- 4.7: Extraction scheme of the bark of *T.arjuna* with different solvents.

Solvents from pet. ether, ethyl acetate ,& methanol extracts were evaporated under reduced pressure by means of a Rotary Vacuum Evaporator and finally dried to solid.

The percentage yields of each of the extract are shown in Table-17.

Table-17: **Percentage yield of the different solvent extracts of the bark of *T.arjuna.***

Powdered Bark (gm)	Solvent	Quantity of Solvent (Liter)	Weight of dried Extract (gm)	Yield (%)
1500	Pet.ether	5	47	3.13
	Ethyl acetate	5	81	5.4
	Methanol	5	171	11.4
	Water	5	-	-

4.2.3.2 DETEERMINATION OF ANTIBACTERIAL ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF THE BARK OF TERMINALIA ARJUNA.

Pet.ether-exrtact, ethyl acetate- extract, methanol- extract and water-extract of the bark of the *T.arjuna* were investigated for their antibacterial activity against *Shigella dysenteriae,Shigella flexneri, Shigella sonnei,E.coli* and *Staphylococcus aureus* as the bark of *T.arjuna* was found to be active against these test organisms in the primary screening test.

Procedures for the detection of antibacterial activities:

For the detection of antibacterial activities of different extracts, the Kirby-Bauer technique[77] was followed as described in Chapter-2 and in Section -4.1.3.2.

Four sets of filter paper discs (5.5mm dia &1.5mm thickness)were impregnated separately with four different extracts and dried. The test organisms were inoculated on Mueller Hinton (M.H) agar plates and were incubated at 37^0C for 24 hours.Four to five similar Colonies from the agar plates were transferred to 3 ml of M.H. broth in test tubes and incubated for 4 to 6 hours. The cultures were diluted in saline to match the $BaSO_4$ standard [76].Sterile cotton swabs were dipped into inoculum and inocula were removed from swabs pressing against the inside wall of the tubes. Mueller Hinton agar plates streaked by swabbing across the entire plate three times, each time at 60^0 angle to the previous streaking. The discs were placed on the seeded plates with sterile forceps and each disc was gently pressed down to ensure proper contact with the agar. The plates

were incubated for 24 hours at 37^0C. The antibacterial activities were expressed by measuring zone of inhibition in mm (Table-19).

Table-18: Antibacterial activities exhibited by the different solvent extracts of the bark of *T.arjuna*.

Sl. No.	*Bacterial Strain*	Activity			
		Pet.Eth.-Ext.	EthylAcet.-Ext.	MeOH-Ext.	WaterExt
1	*Shigella dysenteriae*	-	-	+	+
2	*Shigella flexneri*	-	-	+	+
3	*Shigella sonnei*	-	-	+	+
4	*Escherichia coli*	-	-	+	+
5	*S. aureus*	-	-	+	+

(+) Susceptible to the extracts ; (-) Resistant to the extracts.

Table-19: Zone of inhibition exhibited by MeOH-extract against five micro orgainsms and comparison with standard ampicillin.

Sl. No.	**Bacterial Strains**	**Zone of Inhibition**	
		MeOH-Ext.	Standard Antibiotic
1	*Shigella dysenteriae*	14 mm	10 mm
2	*Shigella flexneri*	13 mm	11.5 mm
3	*Shigella sonnei*	10 mm	9 mm
4	*Escherichia coli*	11 mm	12 mm
5	*S. aureus*	18 mm	17 mm

4.2.4 FRACTIONATION OF THE CRUDE METHANOL EXTRACT BY COLUMN CHROMATOGRAPHIC TECHNIQUE.

Methanol extract (3.5 gm) was dissolved in minimum volume of methanol and was adsorbed on silica gel in the usual way. The adsorbed mass was then transferred on the top

of a silica gel column. The column was then eluted with pet.ether, mixture of pet.ether-ethyl acetate, ethyl acetate-methanol and finally washed with methanol. A number of colored bands were observed during the development of the column. Fraction of about 15 ml were collected in each test tube at regular intervals and checked on TLC plates. In all 153 collections were made. The detailed results of the chromatographic separation are shown in Table-20. Collections showing similar or almost similar TLC behavior were combined together and the total collections were combined to obtained six fractions, A_1-A_6.

Table-20: Column chromatographic separation of methanol extract of bark of *T.arjuna.*

Collection Nos.	TLC Examination	Yield & Observation
1-18	One spot with tail; R_f value=0.68 (PE 100%)	4 mg, One compound with impurity; Fraction-A_1
19-55	Two spots,R_f=0.72,& 0.64; (PE:EA=3:1)	309 mg, mixture of two compounds; Fraction –A_2
56-88	Four spots with tail; R_f=0.83,0.75,0.61,0.52 (PE:EA=1:4)	670 mg, mixture of four compounds ; Fraction –A_3
89-112	Two spots ;R_f=0.73, 0.59 (EA:MeOH= 4:1)	426 mg, mixture of two compounds ; Fraction-A_4
113-138	Three spots with tail ;R_f=0.84,0.71,0.63 (EA:MeOH= 1:1)	528 mg, mixture of three compounds; Fraction-A_5
139-153	Two spots with tail; R_f= 0.67,0.52	137 mg, mixture of two compounds ; Fraction-A_6

4.2.4.1 MICROBIOLOGICAL INVESTIGATION OF SIX FRACTIONS OBTAIN- ED BY CHROMATOGRAPHIC SEPARATION OF MeOH EXTRACT OF T.ARJUNA

The antibacterial activity of the six fractions (A_1,A_2,A_3,A_4,A_5 and A_6-fractions) obtained by the column chromatographic separation of methanol extract of *T.arjuna* were carried

out against *Shigella dysenteriae,Shigella flexneri, Shigella sonnei,E.coli* and *Staphylococcus aureus*.Activity was tested by disc diffusion method described in Chapter-2. The results obtained are presented in Table-21.

Table –21: Susceptibility of test organisms to the fractions obtained from the column chromatography of MeOH–extract.

Sl. No.	Bacterial Strains	Antibacterial Activity					
		A_1-frac.	A_2-frac.	A_3-frac.	A_4-frac.	A_5frac.	A_6-frac.
1	*Shigella dysenteriae*	-	-	+	-	-	-
2	*Shigella flexneri*	-	-	+	-	-	-
3	*Shigella sonnei*	-	-	+	-	-	-
4	*Escherichia coli*	-	-	+	-	-	-
5	*S.aureus*	-	-	+	+	+	-

(+) Susceptible to the organism; (-) Resistant to the organism.

Table-22: Zone of Inhibition obtained from A_3-fraction .

Sl.No.	Bacterial Strain	Zone of Inhibition(mm)
1	*Shigella dysenteriae*	15
2	*Shigella flexneri*	13.5
3	*Shigella sonnei*	10
4	*E. coli*	11.5
5	*S. aureus*	19

4.2.4.2. SEPARATION AND ISOLATION OF THE ANTIBACTERIAL COMPOUND FROM A_3-FRACTION

A_3-Fraction obtained by column chromatographic separation of crude MeOH-extract of the bark of *T.arjuna* was found to be active against *Shigella dysenteriae,Shigella flexneri,*

Shigella sonnei,E.coli and *Staphylococcus aureus*.Antibacterial compound from A_3-fraction was then separated by preparative thin layer chromatography (PTLC).

To do this, first a suitable solvent system have had to developed to run the PTLC plate to obtained a good resolution of the compounds present in the crude. It was carried out by running the plate with different solvent systems. Separated compounds were microbiologically tested whether it exhibited activity against test organisms or not.

4.2.4.2.1 : DEVELOPMENT OF A SUITABLE SOLVENT SYSTEM

Employed solvent system and their separation behavior are listed in Table-23.

Table-23: Different solvent systems employed for the analysis of A_3-fraction on thin layer chromatography (TLC).

SL.No.	Solvent System	Ratio	Resolution
1	PE	100%	-
2	PE:EA	1:1	-
3	PE:EA	1:2	-
4	PE:EA	1:3	+
5	PE:EA	1:4	+ +
6	EA	100%	-

(-) No separation ;(+) separation;(+ +) Good separation;

PE= Pet.ether,EA= Ethyl acetate.

Among these solvent system listed above a good resolution of the compounds present in the A_3-fraction was found with PE:EA (1:4). With this developing solvent four separate and distinct spots were found.

Detection of the spots:

The spots were identified by development with Phospho-Molebdenum developing reagent.

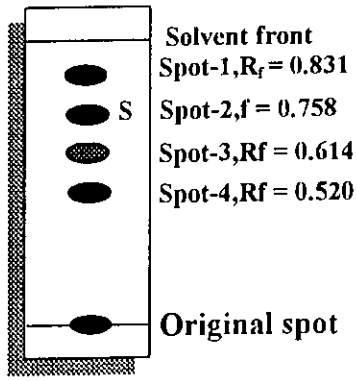


Fig:- 4. 8: Thin Layer Choromatogram obtained by TLC of A3-fraction with PE:EA (1:4).

4.2.4.2.2 PREPARATIVE THIN LAYER CHROMATOGRAPHY (PTLC) FOR THE SEPARATION OF THE COMPOUNDS PRESENT IN THE A_3-FRACTION

The compounds present in the A_3-fraction were separated by performing PTLC with the developing solvent Pet.ether-ethyl acetate (1:4).

Parameters:

Plate	: Aluminium Plate(20×20 cm)
Adsorbent	: Silica gel (60 G)
Thickness	: 0.75 μm
Developing solvent	: PE-EA (1:4)
Development	: Ascending, One dimensional.

Development of the plate:

Activated plate was first spotted with A_3-fraction and allowed to dry. The plate was then placed in TLC jar containing 50 ml of developing solvent PE-EA (1:4). The plate was

allowed to run to the desired solvent front. It was then taken out of the jar and dried by blow drier.

Detection of the resolute compounds :

The chromatogram was examined by Phospho-Molebdenum development reagent. A number of bands were found. Of them four fairly separated and distinct bands were marked on the plate.

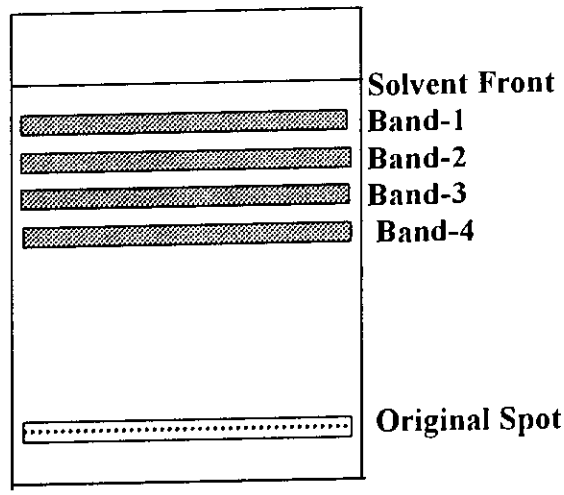


Fig: 4. 9: Chromatogram obtained by the PTLC of A3-fraction with developing solvent PE-EA (1:4).

Elution of the Compounds from the Separated Bands:

The bands were scrapped off separately with a fine spatula, and marked as Band-1,Band-2, Band-3 & Band-4 as shown in figure-4.9.

A plug of glass wool or cotton were placed into the bottom of the column and pressed gently. An uniform packing (3-4 inch) was made with methanol and poured into the column. Throughout the process the stop cock was kept open and some of solvent was allowed to flow out. In this way four separate columns were made for four bands.

Slurry of each band was made with small amount of methanol and poured into the columns. Each of the column was first run with 10 ml Pet.ether-Et.acetate(1:4) and then washed with 10 ml ethyl acetate- methanol (50:50).

All factions were then analyzed by TLC technique with PE-EA(1:4) as developing solvent. Results obtained are listed in Table-24.

Table-24: Results obtained by performing TLC of different fractions obtained from the elution of different bands.

Band	Fraction	No. of Spot	R_f Value
Band-1	1	1	0.831
	1′	1	0.831
Band-2	2	1	0.758
	2′	-	-
Band-3	3	1	0.614
	3′	1	0.614
Band-4	4	1	0.520
	4′	-	-

1,2,3 & 4 : Obtained by eluting with PE-EA(1:4).
1′,2′,3′ & 4′: Obtained by eluting with EA-MeOH (1:1).

From Table-24 it was evident that Band-1 contained a single compound and frction 1 & 1′ were combined. Similarly Band-2,band-3,Band-4 each contained a separate single compound. Therefore the compounds obtained from Band-1, Band-2, Band-3 &Band-4 were marked as TA_1,TA_2,TA_3 and TA_4 respectively.

4.2.4.2.3 ANTIBACTERIAL ACTIVITY OF THE ISOLATED FOUR COMPOUNDS

Four compounds (TA_1,TA_2,TA_3 & TA_4) separated from the A_3 fraction by PTLC technique were then microbiologically tested for their antibacterial activity against *Shigella dysenteriae,Shigella flexneri, Shigella sonnei,E.coli* and *Staphylococcus aureus*.. The test was carried out following disc diffusion method [76] described in Chapter-2 by measuring the zone of inhibition. The results of antibacterial activity are shown in Table-25.

Table-25 : Antibacterial activity of the separated four compounds.

Sl. No.	Baacterial Strain	Zone of Inhibition(mm)			
		TA_1	TA_2	TA_3	TA_4
1	*Shigella dysenteriae*	-	-	15	-
2	*Shigella flexneri*	-	-	13	-
3	*Shigella sonnei*	-	-	11	-
4	*E. coli*	-	-	12	-
5	*S. aureus*	-	-	20	-

It was evident from the Table-25 that compound TA_3 was found to be active against *Shigella dysenteriae,Shigella flexneri, Shigella sonnei,E.coli* and *Staphylococcus aureus*.. Compound TA_1,TA_2 & TA_4 did not show any antibacterial activity.

4.3 SEPARATION AND ISOLATION OF ANTIBACTERIAL COMPOUND(S) FROM THE ROOTS OF RAUVOLFIA SERPENTINA (SHARPAGHANDA).

4.3.1 INTRODUCTION

In the primary screening test of this investigation it was found that methanol extract of the roots *of R.serpentina* showed a significant antibacterial activity against *Staphylococcus aureus.*

Therefore the present investigation was proceeded on separation and isolation of antibacterial compounds present in this plant. In this connection the roots of R. serpentina was serially extracted with four different organic solvents with increasing polarity to distribute the compounds present in the plant into different solvents according to their solubility properties. The antibacterial activity of these different solvent extracts was carried out against the bacterial species found susceptible to the extract in the screening test.

The active extract found was then chemically fractionated into different fractions by extraction with different solvents to make the separation of the antibacterial compounds easier from the crude active extract. Antibacterial activity of different fraction was then carried out against the test organisms.

The antibacterial compound(s) was/ were then separated and isolated fromthe active fraction with the help of crystallization .

4.3.2 ABOUT THE PLANT: *Rauvolfia serpentina*

Family : Apocynaaceae

Common Names :
Botanical name : Rauvolfia serpentina (L.) Benth. Ex Kurz.
English Name : Snakeroot
Bengali Name : Sarpagandha, Chotochand.
Parts of the plant used : Root

Fig: 4.10: Photograph of the plant *Rauvolfia serpentina* with root.

Identifying character & Natural Odor [74]:

Leaves in whorls of 3, thin, lanceolate, acute or acuminate, glabrous, bright green above, pale beneath. Flowers white, often tinged with violet, in many flowered irregular corymbose cymes, peduncles long bright red , calyx glabrous, light red, segments 2.5mm long, lanceolate, corolla long or rarely a little longer tube slender swollen a little above the middle. The inflorescence of this plant with red pedicles and calyx and white corolla is striking. The root is bitter, acrid, heating, sharp and pungent.

Habitat & Therapeutic Uses of R. serpentina[75]

The root is bitter tonic and possess well marked sedative properties. It acts also as febrifuge. Decoction of root is employed to increase uterine contractions and promotes expulsion of the roots. Root is a valuable remedy in high blood pressure; used for the treatment of mad and child crying; painful affections of bowels, insomnia, hypochondria and irritative conditions of the central nervous system. Rauvolfia is an important source of a number of active alkaloids like reserpine, ajmalicine etc. Rauvolfia has been employed for centuries for relief of various nervous disorders like anxiety, excitement, schizophrenia, insanity, insomnia and epilepsy. Roots extracts are valued in diarrhea, dysentery, cholera , colic and fever. The root is also used by the Mundas as a snake remedy and cures poisonous effects of scorpion-stin and snake-bite(Ayurveda).

Availability:

Rauvolfia serpentina is available in most of the places of Bangladesh.

4.3.3 SUCCESSIVE EXTRACTION OF THE ROOTS OF RAUVOLFIA SERPENTINA AND ANTIBACTERIAL ACTIVITY OF DIFFERENT EXTRACTS

4.3.3.1 EXTRACTION OF THE ROOTS OF RAUVOLFIA SERPENTINA WITH DIFFERENT SOLVENTS OF INCREASING POLARITY

Clean, dry roots of R.serpentina ground in a grinding machine. Ground roots were first extracted with pet. ether and then successively with ethyl acetate, MeOH and H_2O with

increasing polarity. 1500 gm of ground root was extracted with 2.5L pet.ether . Extract was filtered out and the residue was extracted again with 2.5L pet.ether. The two extracts were combined and stored in a refrigerator.

The residue obtained after extraction with pet. ether, was air dried and then again extracted with 2.5L ethyl acetate. The extract was separated by filtration. The residue was again extracted with 2.5L ethyl acetate. Two extracts were combined and stored.

The residue obtained after extraction with ethyl acetate, was air dried and was extracted with methanol and finally with water in the same procedure.

The extraction scheme of the roots of Rauvolfia serpentina with different solvents of increasing polarity is shown in figure 4.11.

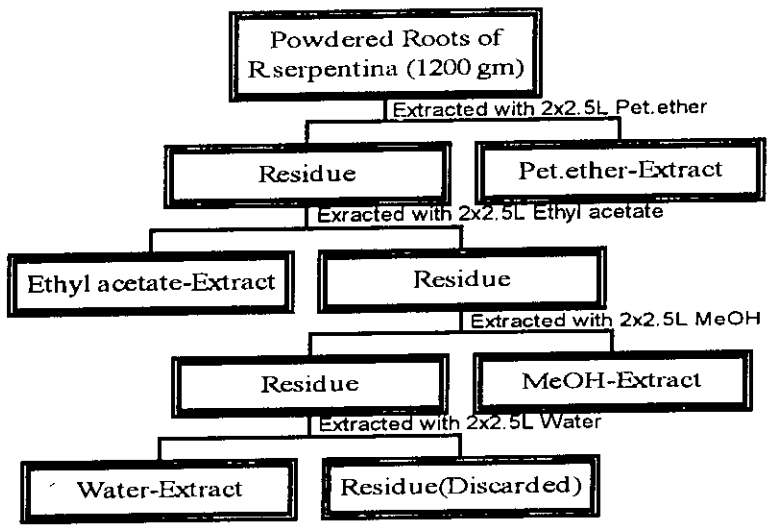


Fig:4.11:Extraction scheme of the roots of R. serpentina with different solvents.

Solvents from pet. ether, ethyl acetate ,& methanol extracts were evaporated under reduced pressure by means of a Rotary Vacuum Evaporator and finally dried to solid.

The percentage yields of each of the extract are shown in Table-26.

Table-26: **Percentage yield of the different solvent extracts of the root of *R.serpentina.***

Solvent	Powdered Root (gm)	Quantity of Solvent (Liter)	Weight of dried Extract (gm)	Yield (%)
Pet.ether	1200	5	58	4.83
Ethyl acetate		5	77	6.41
Methanol		5	139	11.58
Water		5	-	-

4.3.3.2 DETEERMINATION OF ANTIBACTERIAL ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF THE ROOTS OF RAUVOLFIA SERPENTINA

Pet.ether-exrtact, ethyl acetate- extract, methanol- extract and water-extract of the roots of R.serpentian were investigated for their antibacterial activity against *Staphylococcus aureus* as the roots of *R.serpentina* was found to be active against the above organism in the primary screening test.

Procedures for the detection of antibacterial activities:

For the detection of antibacterial activities of different extracts, the Kirby- Bauer technique [82] was followed as described in Chapter-2 and in Section -4.1.3.2.

The antibacterial activities were expressed by measuring zone of inhibition in mm (Table-28).

Table-27: Antibacterial activities exhibited by the different solvent extracts of the root of R.serpentina.

Bacterial Species	Activity			
	Pet.ether-ext.	Et.acetate-ext.	MeOH-ext.	H_2O-ext.
Staphylococcus aureus	-	-	+	-

(+) Susceptible to the extracts ; (-) Resistant to the extracts.

Table-28: Zone of inhibition exhibited by MeOH-extract against *Staphylococcus aureus* and comparison with standard ampicillin.

Bacterial Species	Zone of Inhibition	
	MeOH-extract	Standard Antibiotic
Staphylococcus aureus	25 mm	17 mm

4.3.4 FRACTIONATION OF THE CRUDE METHANOL EXTRACT WITH DIFFERENT SOLVENTS

Methanol extract of the roots of *R.serpentina* was found to be active against *S.aureus*. As the methanol extract was in crude form, separation of active compound (s) from it was very difficult. Hence the fractionation of crude methanol extract was carried out by column chromatography separation technique to make the separation of the antibacterial compound(s) easier from the crude fraction.

4.3.4.1 COLUMN CHROMATOGRAPHIC SEPARATION OF MeOH EXTRACT OF R.SERPENTINA

Methanol extract (4.5gm) was dissolved in methanol and absorved in small quantity of silica gel. The adsorved mass was completly dried under reduced pressure and carefully poured on the top of a column of silica gel of a column chromatographic unit. The column was then eluted with pet.ether, mixture of pet.ether-ethyl acetate, ethyl acetate-methanol and finally washed with methanol. Fractions of about 15ml were collected in each test tube at regular intervals and checked on TLC plates. In all 112 collections were made and by examining the TLC behavior four fractions R_1-R_4 were found. The detailed results of the chromatographic separation are shown in Table-29.

Table-29: Column chramatographic separation of MeOH extract of R.serpentina.

Collection Nos.	TLC Examination	Yield & Observation
1-26	Two spots, R_f= 0.78,0.64; PE=100%	93mg,mixture of two compounds, Fraction-R_1
27-61	Two spots, R_f= 0.63,0.48; PE:EA=2:1	1.3gm,mixture of two compounds Fraction-R_2
62-86	Three spots, R_f= 0.87,0.73,0.64; EA:MeOH=5:1	1.16 gm, mixture of three compounds , Fraction-R_3
87-112	Four spots, R_f=0.81,0.73,0.67, 0.51; EA:MeOH=1:1	1.08 gm, mixture of four compounds, Fraction-R_4

4.3.4.2ANTIBACTERIAL ACTIVITY CHECK FOR THE DIFFERENT FRACTIONS OF MeOH EXTRACT

The antibacterial activity of these four fractions (R_1,R_2,R_3 & R_4) obtained by the fractionation methanol extract, were carried out against *S.aureus*. Activity was tested by disc diffusion method described in Chapter-2. The results obtained are listed in Table –30.

Table-30: Susceptibility of the test orgainsm to the fraction obtained from methanol extract.

Bacterial Strain	Antibacterial Activity			
	Frac. –R_1	Frac. -R_2	Frac. -R_3	Frac. -R_4
S.aureus	-	+	-	-

(+) Susceptible to the organism ; (-) Resistant to the orgainsm.

Table-31: Zone of inhibition obtained from R_2-fraction.

Bacterial Strain	Zone of Inhibition of the R_2-fraction (in mm)
S.aureus	26

4.3.4.3 SEPARATION OF ACTIVE COMPOUND FROM R2-FRACTION BY CRYSTALLIZATION

The R_2-fraction was then concentrated in Vacuum Rotary Evaporator. The concentrated extract was then standed for few days for crystallization. Thus crystals were obtained. The crystals were separated by decantation from the mother liquer(designated as fraction-1). The crystals were then washed with pet.ether,ethyl acetate and methanol (the washing liquid was designated as fraction-2). Finally to get the pure crystals, the crystals were re-crystallized from MeOH solution. In the recrystallization the crystals were dissolved in hot MeOH and boiled for a while and then the solution was cooled at 0^0C by ice water . The crystals were then separated by decantation from mother liquer(designated as fraction-3). The pure crystals which were designated as the compound RS. The RS was dissolved in MeOH(designated as fraction-4).

4.3.4.4 ACTIVITY TEST FOR THE DIFFERENT FRACTIONS (FRAC.-1,FRAC-2, FRAC.-3 & FRAC.-4)

The activity of the four fractions was tested in vitro procedure and the results were shown in Table-32.

Table-32: Activity of fractions obtained from MeOH extract of *R.serpentina*.

Bacterial Strain	Antibacterial Activity			
	Frac-1	Frac-2	Frac-3	Frac-4
S.aureus	-	-	-	+

(+) Indicates Active; (-) Indicates Not Active.

From the observation it was found that the compound RS was active.

4.3.5 RESULTS & DISCUSSION

In the primary screening test it was found that, the methanol extracts of the seeds of *Holarhena antidysenterica*, the bark of *Terminalia arjuna* & the roots of Rauvolfia serpentina showed antibacterial activity against five bacterial species. These were *S. dysenteiae, S. flexneri, S. sonnei, E. Coli & S. aureus.* The powdered seeds of *H. antidysenterica* the bark of *T.arjuna* and the roots of *R.serpentina* were successively extracted with four different solvents such as pet. ether, ethyl acetate, methanol and water. Four extracts for each plant were then tested for probable antibacterial activities against the same five test organisms. From the experimental results (Table-12, Tab.-18 and Tab.-27) it was evident that, among four extracts only methanol extract and some extent water extract exhibited a significant antibacterial activity against the test organisms mentioned above. The zone of inhibitions of methanol extract of *H. antidysenterica* against *S.aureus* was 22 mm, and that of *T.ariuna* against *S. dysenteriae. S. flexneri, S. sonnei, E. Coli* and *S.aureus* were 14 mm, 13 mm, 10 mm, 11 mm and 18 mm respectively, and that of *R.serpentina* against *S. aureus* was 25 mm.

From comparison of the zone of inhibition exhibited by the methanol-extract and the standard antibiotic (Table 13, Tab.-19 and Tab.-28) against the test organisms, it was found that the samples had a comparable activity with standard antibiotic. As the samples were in crude form, therefore, it could be concluded that it would strongly active when the sample would be in pure form.

Crude MeOH-extract of the seeds of *H.antidysenterica* was separated into four different fractions: C6H12-fraction, CHCl3-fraction, Ethyl acetate and aq. fraction by the solvent extraction with Hexane, Chloroform, Ethyl acetate and water. These four fractions were microbiologically investigated for their any antibacterial activities by disc diffusion method[76]. Among these four fractions only Ethyl acetate-fraction showed significant antibacterial activity against *S.aureus.* Acqueous fraction showed antibacterial activity against same organism (Table-14). In this investigation next attempt was made taking

only Ethyl acetate-fraction to separate and isolate the antibacterial compound(s). The other three fractions were discarded.

Separation of compounds present in the Ethyl acetate- fraction, obtained by the solvent extraction of crude MeOH extract, were carried out by means of thin layer chromatographic technique. Two fair distinct spots were found which were detected under short-wave UV light (254 nm), and by spraying Vanillin H2SO4 and then heating at 115^0C for 15 min.

Preparative thin layer chromatography on silica gel 60G of the Ethyl acetate -fraction was then carried out with developing solvent (Ethyl acetate: MeOH= 3:2). The distinct band adsorbents scrapped off and the compounds were eluted. For each of the bands a single compound was found. From two bands two compounds were found which are shown in Table-16.

The separated two compounds were then microbiologically tested for their antibacterial activity, against the same test organism. Compound HA1 was found to be active against *S.aureus*. Compound HA2 showed no antibacterial activity.

The R_f value of the antibacterial compounds was then determined by thin layer chromatography. The R_f value of the compound HA_1 was 0.713 (solvent system EA:MeOH=3:2).

Crude MeOH-extract of the bark of *T. arjuna* was separated by column chromatographic techique. Six fractions were found which were marked as A1 to A6 fraction. Among these fraction only A3-fraction was active against *S.dysenteriae, S.flexneri, S. sonnei, E. coli and S.aureus*. The dried A3 fraction was separated by PTLC. Four bands were found these were marked as band-1, band-2, band-3 and band-4. Compound TA3 obtained from band-3 was active against five mentioned organisms. The Rf value of compound TA3 was 0.614 (solvent system PE:EA=1:4).

Crude MeOH-extract of the roots of *R.serpentina* was separated by column chromatography. Four fractions were found marked as R1 to R4-fraction. Among these fractions only R2-fraction was active against *S.aureus*. The concentrated R2-fraction was standed for few days for crystallization. Pure crystals were obtaind. This compound marked as RS . The Rf value of compound RS was 0.630 (solvent system PE:EA=2:1)

CHAPTER-5

CHARACTERIZATION OF THE ISOLATED ANTIBACTERIAL COMPOUNDS BY CHEMICAL & SPECTROSCOPIC STUDIES

CHAPTER-5

CHARACTERIZATION OF THE ISOLATED ANTIBACTERIAL COMPOUNDS BY CHEMICAL & SPECTROSCOPIC STUDIES

5.1 INTRODUCTION

Three compounds tentatively marked as HA_1, TA_3 and RS were separated by defferent seperating techniques in pure form obtained by the fractionation of crude methanol extract of the seeds of *H. antidysenterica*, the bark of *T.arjuna* and the roots of *R.serpentina*. Of these compounds HA_1 was found to be active against *S.aureus*; compound TA_3 was active against *S.dysenteriae, S. flexneri, S. sonnei, E.coli and S.aureus* & compound RS exhibited antibacterial activity was against *S aureus* .

Characteriztion of these three antibacterial compounds were then carried out with the help of chemical and spectroscopic analysis. In chemical analysis some group tests and test for alkaloid,flavone and steriod were carried out. Spectroscopic analysis was carried only with the help of IR-spectroscopy.

5.2. PHYSICAL TESTS

5.2.1. Color of the Compounds

Compounds	Color
HA_1	Color less
TA_3	Brownish
RS	Pale-Yellow

5.2.2 Solubility : Compound HA_1, TA_3 & RS were soluble in chloroform, ethanol, methanol, and acetone.

Melting Point :

Compounds	M.P(^{0}C)
HA_1	119-121
TA_3	195-197
RS	260-265

Rf Value of the compounds:

Compounds	R_f Value
HA_1	0.713(EA:MeOH=3:2)
TA_3	0.614(PE:EA=1:4)
RS	0.630(PE:EA=2:1)

5.3. CHEMICAL ANALYSIS

5.3.1 Test for unsaturation :

A little amount of the compounds were dissolved in ethanol taking in microtest tubes. 1 drop of 2% solution of potassium permenganate in ethanol was added to it. The mixtures were shaken and allowed to stand for a few minutes. The colour of the permenganate was discarged in each case. Therefore, the test was positive for each of the three compounds.

5.3.2. Test for Phenolic Hydroxyl Group

A little amount of the compounds were dissolved in dilute HCI taking in separate three test tubes. Few drops of ferric chloride solution were added to each of the three test tubes. Pink coloration for compound TA_3 & RS indicate the presence of phenolic-OH group, and no change of color in compound HA_1 indicate absence of phenolic group.

5.3.3. Test for Aldehyde & Ketone Groups

Preparation of Reagent : Dinitrophenyl hydrazine reagent was prepared by dissolying 3 gm of 2, 4-dinitrophenyl hydrazine in 15 ml of concentrated sulfuric acid. It was added

to 20 ml of distilled water and 70 ml of 95% ethanol with stirring. The solution was mixed thoroughly and filtered. The filtrate was collected and stored for use.

Test : 95% alcoholic (ethanolic) solution of the samples were added to the 2 ml of 2,4-dinitrophenyl hydrazine reagent. The solutions were then allowed to stand with occasional shaking. It was then observed for any precipitation. No precipitation was observed indicate the absence of aldehyde and ketone group in compounds HA_1 & RS. Precipitation in compound TA_3 that indicate presence of aldehyde/ketone group.

5.3.4. Test for Carboxylic acid Group (-COOH)

A little amount of samples were added to the 1 ml of sodium hydroxide solution. The solutions were shaken mildly to dissolves into the solution. The solubility of the samples were tested in 5% sodium bicarbonate solution. Carboxylic acid group caused effervescence due to the evolution of carbondioxide.

Compound RS gave positive test with carboxylic acid group and compounds HA_1 & TA_3 gave negative tests result indicated the absence of carboxylic acid group.

5.3.5. Test for Ester (-COOR) Group

Hydroxamic acid Test :

A little amount of sample was mixed with 1 ml of 0.2 N hydroxylammine hydrochloride in 95% ethanol and 0.2 ml of 6N aqueous NaOH solution was added to the mixture. The mixture was heated to boiling. The solution was then cooled and one drop of ferric chloride solution was added to it. No magenta color indicated the absence of ester group in all the compounds.

5.3.6. Test for Alkaloid

Preparation of Reagent :

Mayer's Reagent : 2.72gm of $HgCl_2$ was dissolved in 120 ml of distilled water and 10 gm of KI was dissolved in 20 ml of distilled water. The two solutions were mixed and diluted to 200 ml with distilled water.

Dragendorff's Reagent : 8 gm of bismath nitrate, $Bi(NO_3)_3 . 5H_2O$ was dissolved in 20 ml of nitric acid, HNO_3 (sp.gr. 1.18) and 27.2 gm of KI was dissolved in 50ml of distilled water. The two solutions were mixed and allowed to stand Potassium nitrate crystallized out and the supernetant was decanted off and made up to 100 ml with distilled weter.

Test : A few mg of the samples were taken on a watch glass and a few drops of dilute HCl was added to it with constant stirring. The clear supernatant liquid was treated with a few drops of Mayer's reagent and Dragendorff's reagent separately. For both reagents precipitation for the compounds HA_1 & RS indicated the presence of alkaloid, but no precipitation was observed in case of compound TA_3.

5.3.7. Test For Steroid .

A little amount of the Chloroform solution of the samples were taken in three separate test tubes and shaken mildly. Concentrated sulfuric acid was poured slowly by the side of the test tubes. Any colored ring at the junction of the two solutions indicated the presence of steroid.

Compound HA_1 gave positive test for steroid but compounds TA_3 & RS gave negative test for the steroid.

5.3.8 Test for Flavone :

Test : 4 ml of solution was taken in a test tube. Then 1.5 ml of 50% MeOH was added into this solution. This mixture was warm up and a small amount of metal Mg was taken into this warm solution. Then 5-6 drops of concentrated HCl was added in the test tube. Orange color reveled that TA_3 solution contained Flavone, in case of TA_3 but HA_1 and RS gave negative results.

5.4. SPECTROSCOPIC ANALYSIS

IR-spectrum for each of the three compounds were taken by KBr disc method and the bands obtained are discussed bellow :

Compound HA_1: Bands observed at the following wave number -

2910 cm^{-1} indicates C-H (Aliphatic) stretching.

2220 cm^{-1} indicates C≡N stretching.

1450 cm^{-1} indicates C-N stretching vibration.

710 cm^{-1} indicates meta-disubstituted compounds.

Compound TA_3: Bands observed at the following wave number -

3380 cm^{-1} indicates Hydrogen bonded –OH group

2950 cm^{-1} indicates C-H (Aromatic) stretching.

1700 cm^{-1} strong band indicates C=O stretching.

1615 cm^{-1} indicates Aromatic ring.

1270, 1190, 1105 and 1040 cm^{-1} strong bands indicate C=O stretching.

Compound RS : Bands at wave number -

3670 cm^{-1} indicates free bonded phenolic-OH group/or N-H stretching vibrations.

3350 cm^{-1} indicates Hydrogen bonded -OH group

3000 cm^{-1} indicates C-H (Aromatic) stretching.

2910 cm^{-1} indicates C-H (Aliphatic) stretching.

2850 cm^{-1} indicates C-H (Aliphatic) stretching.

2400 cm^{-1} indicates C-H stretching in carboxylic acid group.

1510 cm^{-1} indicates C-C (Aromatic) stretching.

1420 cm^{-1} indicates C-N stretching vibration.

1215 cm^{-1} indicates C-O (Aryl ethers) stretching.

770 cm^{-1} indicates N-H bending.

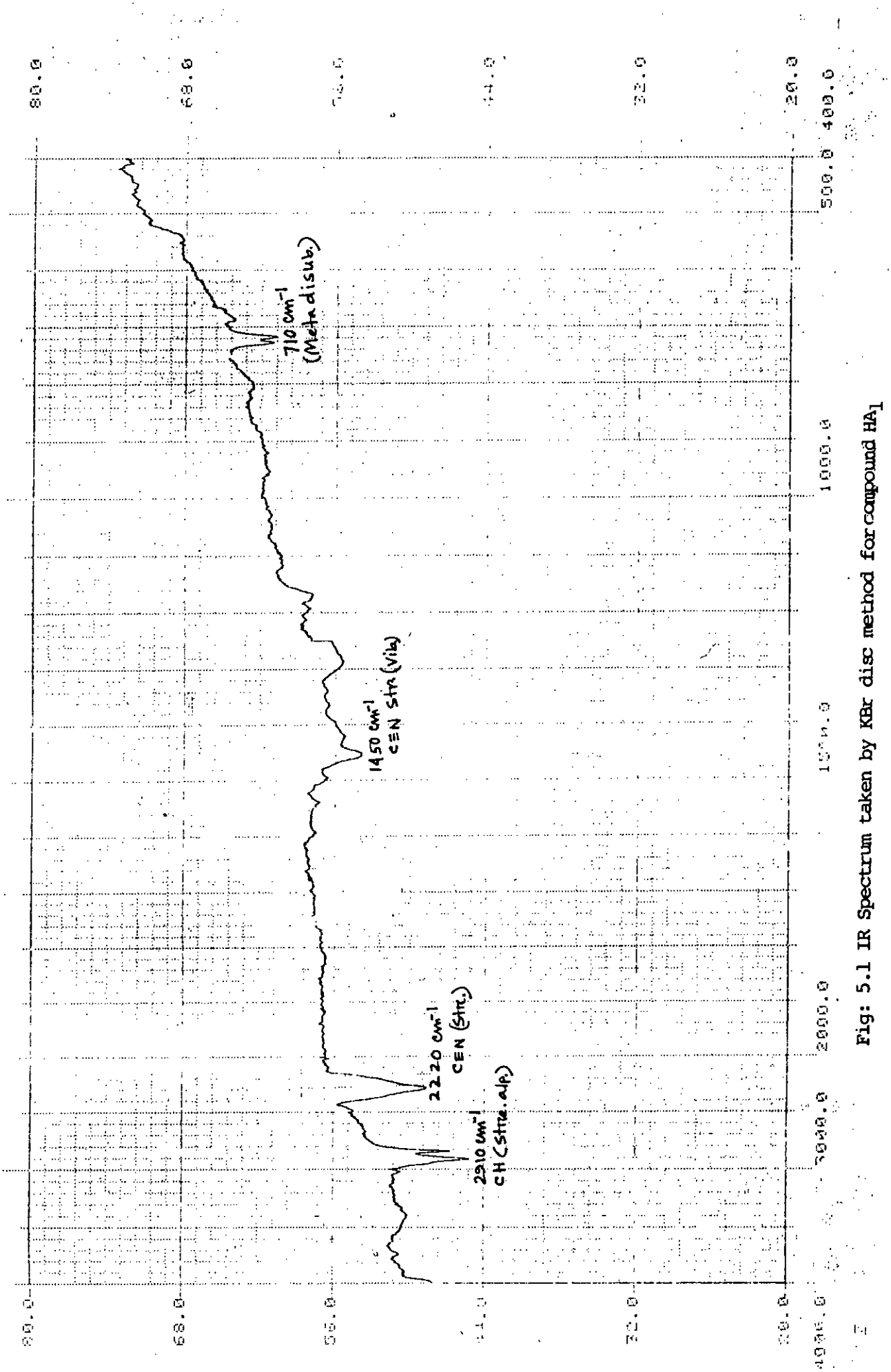


Fig: 5.1 IR Spectrum taken by KBr disc method for compound HA_1

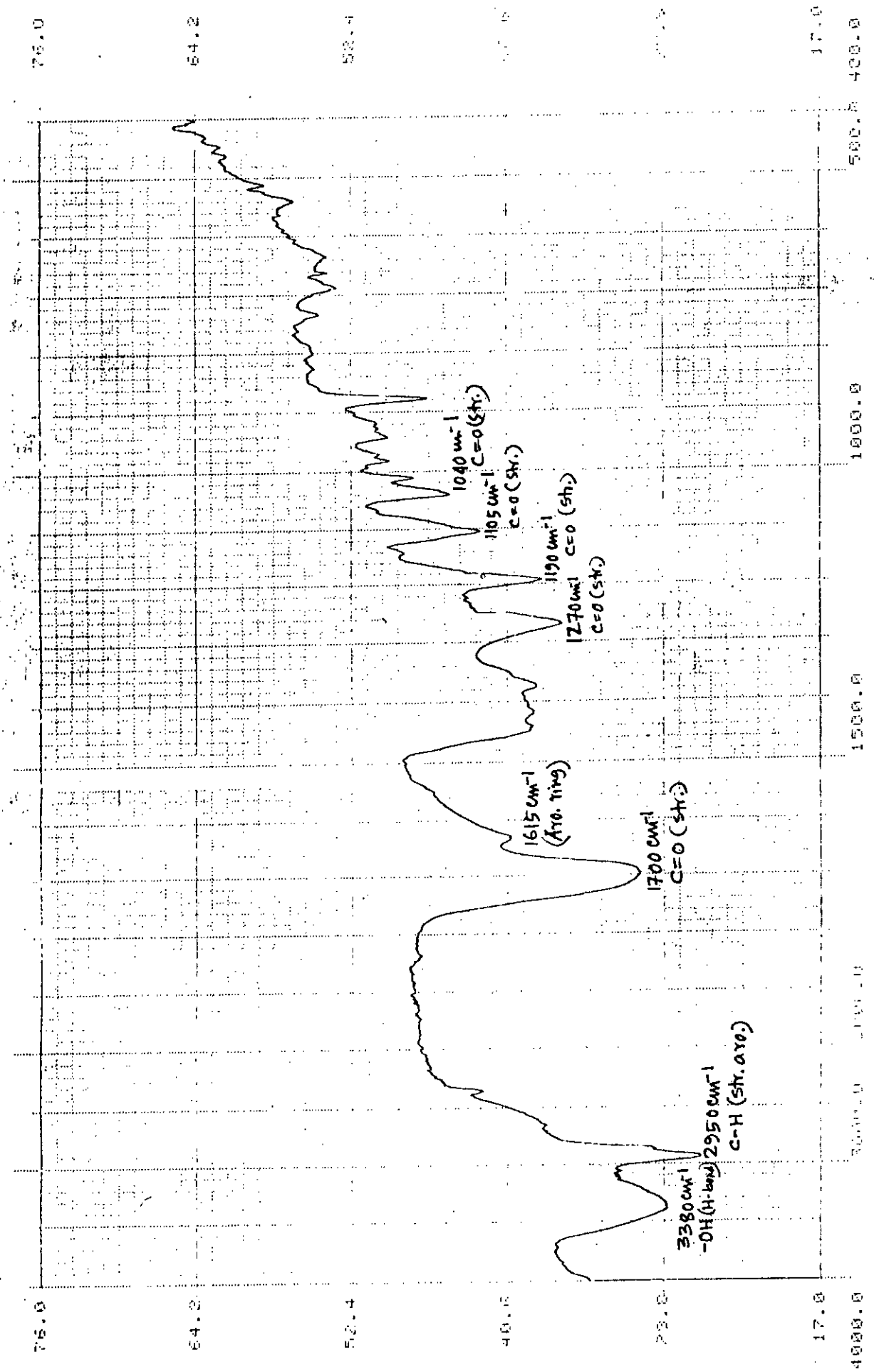


Fig: 5.2 IR Spectrum taken by KBr disc method for compound TA_3

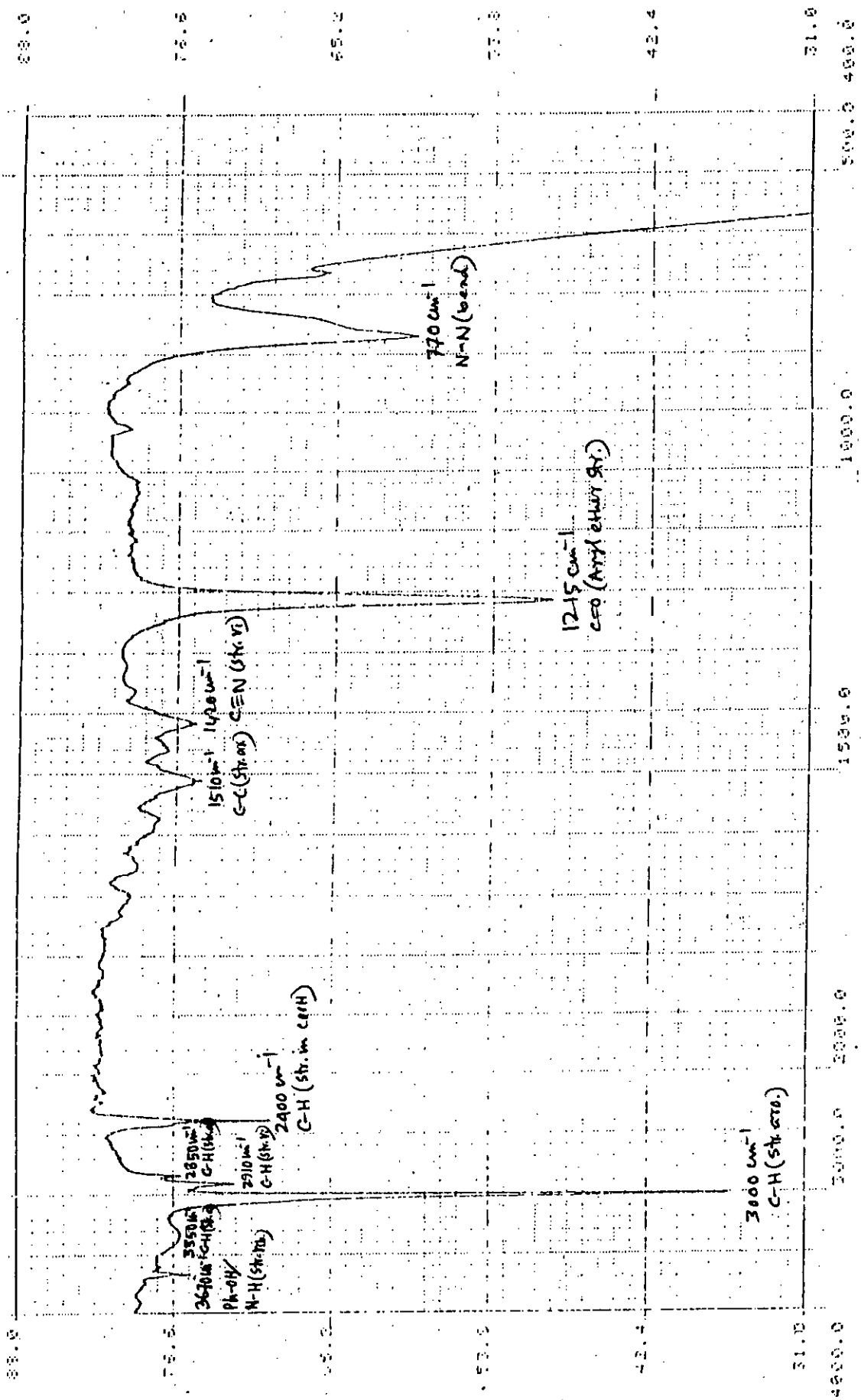


Fig: 5.3 IR Specturum taken by KBr disc method for compound RS.

5.5. RESULTS & DISCUSSION :

Compounds HA_1, TA_3 and RS were characterized by means of chemical and spectroscpic analysis. In chemical studies, the test for different functional groups (-OH group, – COOH group, -OOR group , >C=O group) and the test for steroid,flavone and alkaloid were carried out and in spectroscopic studies, IR-spectrum for three compounds were taken.

Color of the compounds HA_1 was colorless, TA_3 was brownish and RS was pale yellow. Compound HA_1 was steroidal alkaloid as it gave positive test results when treated with Dragendorf's/Mayer's reagent and steroid test. Compound RS was alkaloid according to alkaloid test. Compounds TA_3 and RS consists of phenolic hyroxy group as they gave positive $FeCl_3$ test. It was also evident from IR absorption band at 3300 cm^{-1} to 3650cm^{-1} for compound TA_3 and RS. From chemical analysis it was found that compound TA_3 gave positive test for aldehyde/ketone and compound RS gave positive test for carboxylic acid group. It was also evident from IR absorption band at 1700 cm^{-1} indicate TA_3 contain >C=O group and strong band at 1215cm^{-1} indicate that RS contain aryl ether group.

Therefore, it can be concluded that:

Compound HA_1 from seeds of Kurchi which was colourless compound with Melting Point 219-221^{0}C, R_f value 0.713 (solvent system EA:MeOH=3:2), containing steroid – alkaloid with C=N group including all peaks of IR spectrum were closely agreement with the repoted compound Conessine from Kurchi. So we could expected that the compound HA_1 was Conessine, a steroidal alkaloid.

Compound TA_3 from bark of Arjun which colour was brownish with Melting Point 195-197^{0}C, R_f value 0.614 (solvent system PE:EA=1:4), containing flavone with ketone and phenyl hydroxyl group including all peaks of IR spectrum were closely coinside with the repoted compound Arjunolone . So we could expected that the compound TA_3 was Arjunolone, a flavone.

Compound RS from roots of Sharpagandha which was colour was pale yellow with Melting Point 260-265^{0}C, R_f value 0.630 (solvent system PE:EA=2:1), containing alkaloid with phenyl hydroxyl, aryl ether, carboxylic acid group including all peaks of IR spectrum were closely agreement with the repoted compound Reserpine. So we could expected that the compound RS was Reserpine, an alkaloid.

CHAPTER-6

IN VITRO DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) & MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF THREE ANTIBACTERIAL COMPOUNDS ISOLATED FROM HOLARHENA ANTIDYSENTERICA, TERMINALIA ARJUNA, RAVOULFIA SERPENTINA.

CHAPTER-6

IN VITRO DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) & MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF THREE ANTIBACTERIAL COMPOUNDS ISOLATED FROM HOLARHENA ANTIDYSENTERICA, TERMINALIA ARJUNA, RAVOULFIA-SERPENTINA.

6.1. INTRODUCTION

The lowest concentration of the antibiotic which inhibits clearly the visible growth of the test organism is called the minimum inhibitory concentration (MIC) and the lowest concentration of the antibiotic needed for actual killing of the organisms (99.9% reduction in organisms from the original inoculum of approximately 10^5) is called the minimum bactericidal concentration (MBC). The MIC and MBC are used either to compare the susceptibility of a given strain against various antibiotics or to determine the bacteriostatic or bactericidal activity of a given strain against same antibiotic of varying concentration.

Three antibacterial compounds isolated from the seeds of *H. antidysenterica,* the bark of *T. arjuna* & the roots of *Ravoulfia serpentina.* Among these compounds HA_1 was found to active against *S. aureus,* compound TA_3 was active against *Shigella dysenteriae, Shigella flexneri, Sh. Sonni, E. coli & S. aureus.* and compound RS was found to be active against *S. aureus.* The in vitro determination of MICs and MBCs of these three antibacterial compounds were carried by Macrodilution Broth procedure for the test organisms susceptible to the compounds.

6.2. PRINCIPLE

A number of considerations are involved in selecting an appropriate agent to treat an infection of these factors the concentration of an antibacterial agents required to inhibit (becteriostatic) to kill (bactericidal) organisms in vitro and those attained in body fluids

are subject to direct measurement in the clinical laboratory. A variety of methods are available to determine the antibacterial activity of antibiotics. In this investigation the MIC and MBC were determined by Macrodilution Broth Procedure.

MICs were determined by making a sequence or series of decreasing concentrations or increasing dilution of the compounds in Muller-Hinton broth contained in test tubes. The test tubes were inoculated with the test organisms. The MBC were then be determined by subculturing from the tubes where growth has been inhibited.

6.3. MATERIALS & METHODS

6.3.1. CULTURE

Overnight broth cultures of *Shigella dysenteriae, Shigella flexneri, S. sonnei, Escherichia coli & S. aureus* at 37°C. Turbidity of actively growing cultures were adjusted with saline or broth to obtain a turbidity visually comparable to that of the turbidity standard (Mc. Farland 0.5 standard). Cultures were then further diluted (1:200) in broth. The inoculum thus prepared expected to contain 10^5 to 10^6 cells.

6.3.2. MEDIA & CHEMICALS

Mueller-Hinton agar/broth media, Normal saline water and Mc. Farland 0.5 turbidity standard etc.

6.3.3. EQUIPMENTS

Sterile screw cap test tubes, test tube rack, inoculating loop, 1 ml pipette or micropipette with sterile tips, beaker, marker, Bunsen burner, laminar flow, autoclave and incubator adjusted at 37°C.

6.3.4. PREPARATION OF SAMPLE SOLUTION

Calculated amount of the compound HA_1 was diluted into 9 different concentrations taking in 9 screw cap test tubes marked through X_1 to X_9. Each of the tubes contained 10 ml solution.

Table-33 : Preparation of sample solutions of compound HA_1 in varying concentration .

Tube No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9
Sample soln. (ml)	10	10	10	10	10	10	10	10	10
Conc. (µg/ml)	4000	3200	2400	1600	800	400	200	100	50

In the same way compounds TA_3 and RS were diluted into nine different concentrations.

6.3.5. PROCEDURE FOR THE DETERMINATION OF MICs & MBCs OF THE COMPOUNDS HA_1, TA_3 AND RS .

Ten sterile screw cap test tubes were taken and marked through 1 to 10. 2 ml of sample solutions was added to the each of the 1 to 9 test tubes from X_1 to X_9 respectively. 2 ml of Mueller-Hinton broth was added to the each Y_2 to Y_{10} test tubes. The last tube (Tube Y_{10}) receives no antimicrobial agent and serves as growth control,. to observe growth of the organism in the medium used. Inoculated each tubes (Y_1 to Y_{10}) with 1 ml. of the culture of the test organism. The final concentration of the sample solution were $1/5^{th}$ of the initial dilution series.

Table –34 : Dilution of sample solution to determine MIC & MBC by Macrodilution Broth Procedure.

Tube No.	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7	Y_8	Y_9	Y_{10}
Sample soln. (ml) From X_1 to X_9	4	2	2	2	2	2	2	2	2	0
Initial conc.(µg/ml)	4000	3200	2400	1600	800	400	200	100	50	0
Mueller-Hinton Broth (ml)	0	2	2	2	2	2	2	2	2	2
Bacterial suspension (ml)	1	1	1	1	1	1	1	1	1	1
Final Volume (ml)	5	5	5	5	5	5	5	5	5	5
Final conc.(µg/ml)	1600	1280	960	640	320	160	80	40	20	0

Determination of MICs :

All the test tubes (Y_1 - Y_{10}) were then incubated at 37°C for 24 hrs. After this period the test tubes were examined for visible growth or not and recorded growth as (+) and no growth as (-). The lowest concentration of the test tubes which showed no visible growth was marked as the minimum inhibitory concentration (MIC). In this way the MICs for *Shigella dysenteriae, Shigella flexneri. S.soni, Escherichia coli and S.aureus* were determined and the results obtained are listed in Table-36.

Table-35 : Visible growth of *S.aureus* for HA_1; of S.dysenteriae, Shigella flexneri, S. sonnei, E.coli & S. aureus for compound TA_3 and of S.aureus for compound RS.

Tube No.		Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7	Y_8	Y_9	Y_{10}
Conc. of Sample solution. (µg/ml)		1600	1280	960	640	320	160	80	40	20	0
Visible Growth	Comp-HA_1										
	S.aur.	-	-	-	-	-	+	+	+	+	+
	Comp-TA_3										
	S. dys.	-	-	-	-	+	+	+	+	+	+
	S.flx.	-	-	-	-	-	+	+	+	+	+
	S. son.	-	-	-	+	+	+	+	+	+	+
	E. coli.	-	-	-	-	+	+	+	+	+	+
	S. aur.	-	-	-	-	-	+	+	+	+	+
	Comp-RS										
	S. aur.	-	-	-	-	-	-	+	+	+	+

+, Visible growth; -, No visible growth.

Table -36: MICs of three antibacterial compounds for *S. dysenteriae, S.flexneri S.sonnei, E.coli & S.aureus.*

Bacterial Species	MIC (µg/ml)		
	Comp-HA_1	Comp-TA_3	Comp-RS
1. Shigella dysenteriae	-	640	-
2. Shigella flexneri	-	320	-
3. S.Sonnei	-	960	-
4. E. coli.	-	640	-
5. aureus.	320	320	160

Determination of MBCs :

The concentration which is bactericidal is then determined by measuring the viablity loss from the dilution series. It was done by subculturing the contents of each tube (determined from Table-35) into a series of Mueller-Hinton broth which do not contain any antibiotic or sample solution started from tube no Y_{10} worked down to tube no. Y_1. By remaining a loopful and inoculating it into 1 ml broth by sterilizing and cooling the loop between each transfer.

The test tubes were then diluted into 10 fold (1:10) dilution process. Ten microlitre of the each of the diluted sample was dropped on Mueller-Hinton plates (Marked through 1 to 10). The plates were then incubated at 37°C for 24 hrs. In this way the MBCs of compound HA_1 for *S.aureus*; of compound TA_3 for *S.dys., S.flx., S.son., E.coli & S.aur.;* of compound RS for *S.aur.* were determined.

Table-37 : Determination of the viability loss of Shigella dysenteriae for increasing concentration of compounds TA_3

Plate No.		1	2	3	4	5	6	7	8	9	10
Sample conc.(µg/ml)		1600	1280	960	640	320	160	80	40	20	0
No. of colony	Comp-TA_3	0	0	0	2	14	25	34	34+	+	+

Table-38 : Determination of the viability loss of *Shigella flexneri* for increasing concentration of compounds TA_3.

Plate No.		1	2	3	4	5	6	7	8	9	10
Sample conc.(μg/ml)		1600	1280	960	640	320	160	80	40	20	0
No. of colony	Comp-TA_3	0	0	0	2	13	27	39	39+	+	+

Table-39 : Determination of the viability loss of *Shigella Sonnei* for increasing concentration of compound TA_3.

Plate No.		1	2	3	4	5	6	7	8	9	10
Sample conc.(μg/ml)		1600	1280	960	640	320	160	80	40	20	0
No. of colony	Comp-TA_3	0	0	1	8	19	28	37	37+	+	+

Table-40 : Determination of the viability loss of *E. coli.* for increasing concentration of compound TA_3.

Plate No.		1	2	3	4	5	6	7	8	9	10
Sample conc.(μg/ml)		1600	1280	960	640	320	160	80	40	20	0
No. of colony	Comp-TA_3	0	0	0	2	14	27	38	38+	+	+

Table-41 : Determination of the viability loss of S.aureus for increasing concentration of compounds HA_1, TA_3 & RS.

Plate No.		1	2	3	4	5	6	7	8	9	10
Sample conc.(μg/ml)		1600	1280	960	640	320	160	80	40	20	0
No. of colony	Comp-HA_1	0	0	1	10	21	28	36	36+	+	+
	Comp- TA_3	0	0	0	1	14	27	35	35+	+	+
	Comp- RS	0	0	0	2	11	23	31	31+	+	+

As the number of colony is directly related to the concentration of the drug solution, the plotting of concentration of the sample solution against the number of colony obtained gave curve, which indicated the number of decreasing of bacterial growth with increasing concentration.

By extending the curve beyond the x-axis, a point was found on the axis, whose corresponding concentration indicated the minimum bactericidal concentration (MBC).

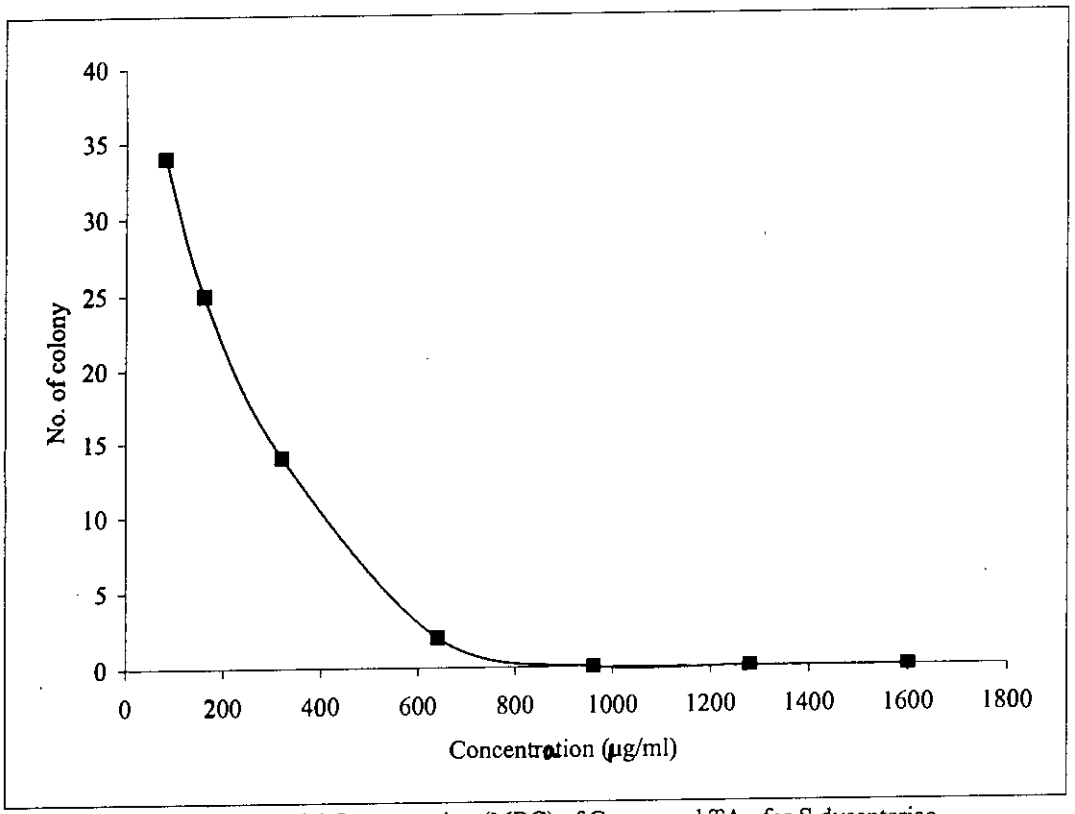


Fig: 6.1 Minimum Bactericidal Concentration (MBC) of Compound TA_3 for S.dysenteriae.

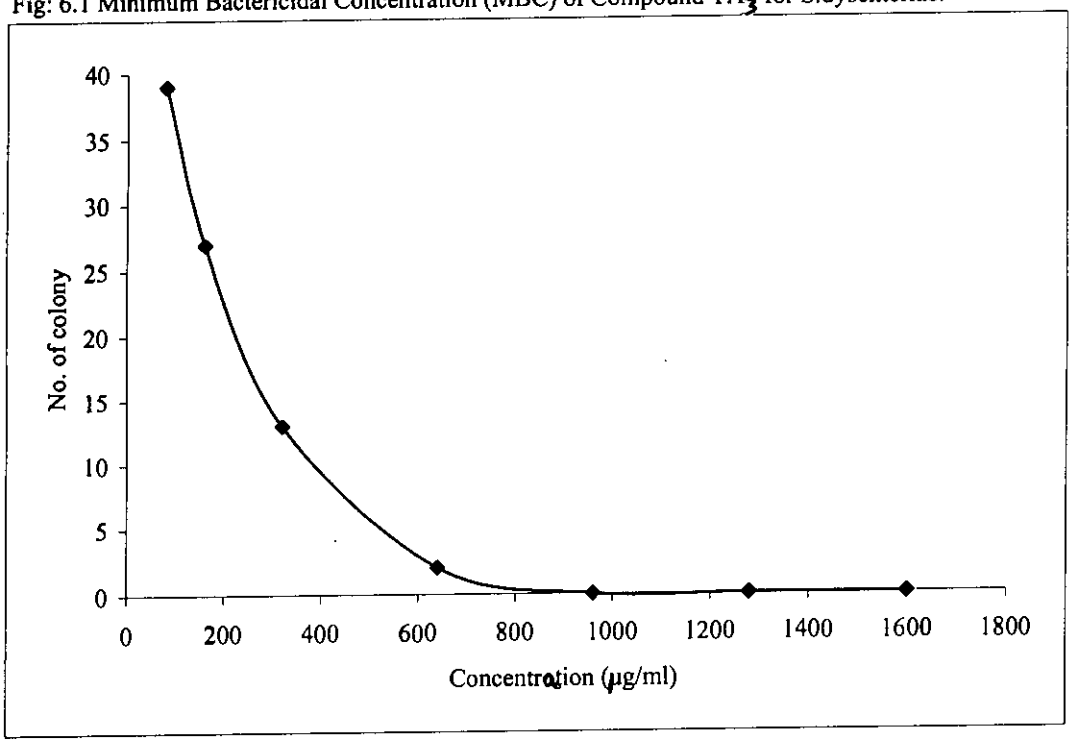


Fig: 6.2 Minimum Bactericidal Concentration (MBC) of Compound TA_3 for S.flexneri.

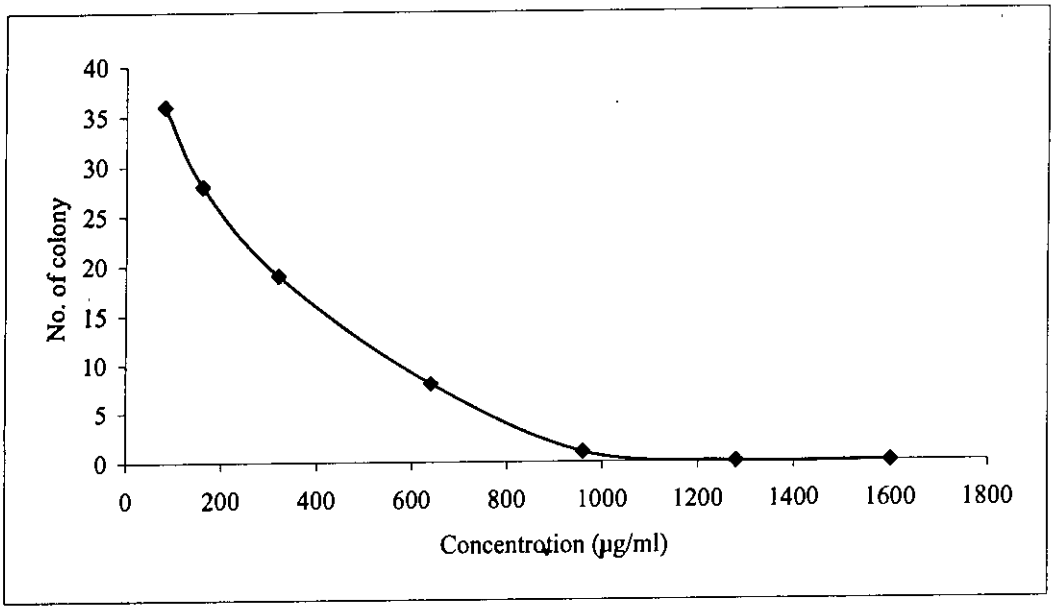


Fig: 6.3 Minimum Bactericidal Concentration (MBC) of Compound TA_3 for S.sonnei.

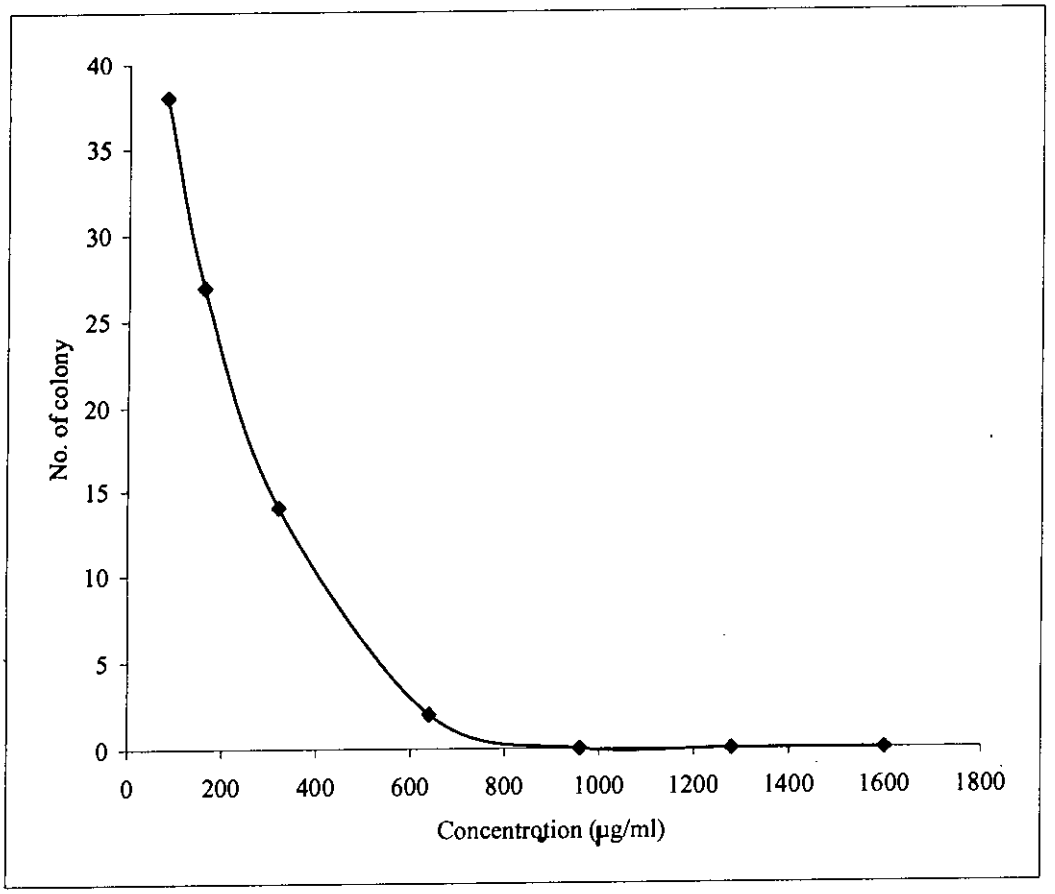


Fig: 6.4 Minimum Bactericidal Concentration (MBC) of Compound TA_3 for E.coli.

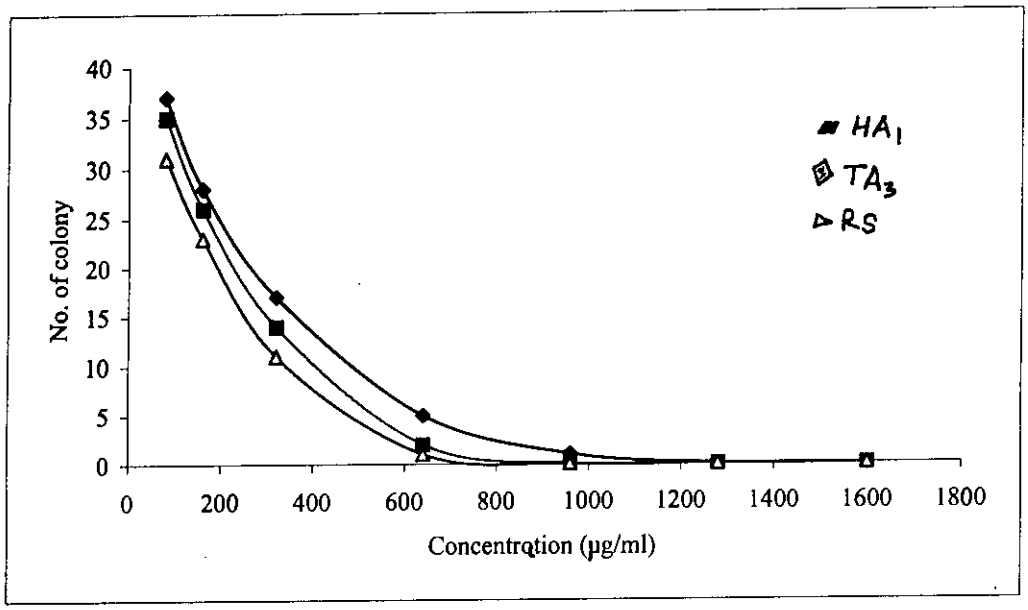


Fig: 6.5 Minimum Bactericidal Concentration (MBC) of Compound HA_1 , TA_3 and RS for S.aureus.

From figure 6.1 it was found that, the Minimum Bactericidal Concentration (MBC) of compounds TA_3 for *S. dysenteriae* was 790 µg/ml. From figure 6.2 it was found that the Minimum Bactericidal Concentration (MBC) of compound TA_3 for *S. flexneri* was 780 µg/ml. From figure 6.3 it was found that Minimum Bactericidal Concentration (MBC) of compound TA_3 for *S.sonnei* was 1080 µg/ml. From fig.6.4 it was found that Minimum Bactericidal Concentration (MBC) of compound TA_3 for *E.coli* was 775 µg/ml. Finally from figure 6.5 it was found that Minimum Bactericidal Concentration (MBC) of compounds HA_1, TA_3 & RS for *S.aureus* were 810, 730 and 670 µg/ml.

Table-42 : Minimum Bactericidal Concentrations (MBC) of compounds HA_1, TA_3 & RS.

Bacterial Species	MBC (µg/ml)		
	Comp-HA_1	Comp-TA_3	Comp-RS
1. Shigella dysenteriae	-	790	-
2. Shigella flexneri	-	780	-
3. Sh. sonnei	-	1080	-
4. E. coli	-	775	-
5. S.aureus	810	730	670

6.4. RESULTS AND DISCUSSION

The MICs and MBCs of compounds HA_1, TA_3 and RS isolated from the seeds of *H.antidysenterica*, the bark of *T. arjuna* & the roots of *Ravoulfia serpentina* were determined respectively. The MICs and MBCs of comopound HA_1 were determined for *S.aureus*, of compound TA_3 were determined for *Shigella dysenteriae, Shigella flexneri, Sh. sonnei, E. coli* and *S.aureus* and that of compound RS were determined for *S. aureus.*

It was evident from the Table-33 that, the visible growth of *S.aureus* for compound HA_1 was observed up to the concentration 320 µg/ml . There was no visible growth at concentration 640 µg/ml and above it. Therefore, the Minimum Inhibitory Concentration (MIC) of compound HA_1 for this test species was 640 µg/ml.

In the same way, the MIC of the compounds TA_3 and RS were carried out. The MIC of compound TA_3 for *Shigella dysenteriae* was 640 µg/ml, that of compound TA_3 for *Shigella flexneri* was 320 µg/ml, that of compound TA_3 for *S.sonnei* was 960 and that of compound TA_3 for E.coli was 640 µg/ml and that of compounds TA_3 & RS for *S.aureus* were 640 µg/ml, 160 µg/ml and 320 µg/ml respectively.

For the determination of MBCs of the compounds HA_1, TA_3 and RS for the same test organisms mentioned above, the viability loss of organisms with increasing concentration of the sample solution was measured (Table-37, 38, 39, 40 & 41). The number of colonies found were plotted against the corresponding concentration of the sample solution for each of the test organisms. The curves that were obtained indicated the nature of the relationship between the number of colony and the concentration of the sample solution. The MBCs were then determined by extending the curve beyond the X-axis. The concentration corresponding to the point at which the number of colony of the test organism were taken as Minimum Bactericidal Concentration (MBC) of that organism.

Therefore, from the figure 6.1, 6.2, 6.3, 6.4 and 6.5 it was found that the Minimum Bactericidal Concentration (MBC) of compound TA_3 for *Shigella dysenteria* was 790 µg/ml, that of compound TA_3 for *Shigflla flexneri* was 780 µg/ml, and that of compound TA_3 for S. sonnei was 1080 µg/ml, that of compound TA_3 for *E.coli* was 775 µg/ml and that of compounds HA_1, TA_3 and RS for *S. aureus* were 810 µg/ml, 730 µg/ml and 670 µg/ml respectively.

CHAPTER-7

SUMMARY

CHAPTER-7

SUMMARY

Diseases, decay and death have always co-existed with life. In spite of tremendous advancement of medical science and technology, infectious diseases are leading a serious health problem in the developing countries like Bangladesh. In the humid tropic land of Bangladesh diarrhea, cholera, typhoid, malaria, dyptheria , etc. are the major cause of morbidity and mortality. Only diarrhea is responsible for 200,000 deaths of children annually. The commonest pathogens responsible for diarrheal diseases are *Escherichia coli, Shigella spp.,Vibrio cholera , Salmonella spp.*, and rotavirus. Increased attention have been to these diseases due to emergence of multiply drug resistance strains which has made the problem more serious. Due to this high incidence of resistance of pathogenic organisms towards antibiotics, the development of new antibacterial agents from either of natural origin or from programs of chemical synthesis is of pressing need of time. Plants and plant materials have tried to relieve human ailments even from the prehistoric era. Still there is a desperate need of study on the antibacterial activities of plants to control these life threatening diseases in developing countries. In the present investigation, microbiological testing on some medicinal plants was carried out against eleven bacterial pathogens mainly responsible for diarrheal diseases for finding out new antibacterial compounds. The entire research work was carried out systematically through the following steps :

I. Microbiological investigation of different solvent extracts of the selected medicinal plants for their antibacterial activity.

II. Separation and isolation of antibacterial compounds from the *Holarrarhena antidysenterica, Terminalia arjuna & Rauvolfia serpentina*.

III. Characterization of isolated compounds.

IV. In vitro determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the isolated antibacterial compounds.

Making a brief study on the therapeutic uses of medicinal plants, ten locally available plants (*Holarrarhena antidysenterica, Terminalia arjuna, Rauvolfia serpentina, Swertia chirata, Aegle marmelos, Helicteres isora, Spondias pinnata, Heliotropium indicum, Syzygium cumini and Polygonum hydropiper)* were selected for primary screening and investigation. Water and methanol extracts of these plants were prepared and all of these extracts were microbiologically tested against ten gram negative bacteria (*Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Shigella boydii, Vibrio choleriae,Salmonella typhi, Klebsiella pneumoniae, Escherichia coli, Salmonella paratyphi "A",Salmonella paratyphi "B"*, and one gram positive bacteria (*Staphylococus aureous).*

Test was carried out following disc diffusion method of Bauer et al.(1996). The filter paper discs (5.5mm dia &1.5mm thickness) were impregnated separately with two extracts and dried. The test organisms were inoculated on Mueller- Hinton (MH) agar plates and were incubated at 37^0C for 24 hours. Four to five similar colonies from the agar plates were transferred to 3 ml of MH broth in test tubes and incubated for 4 to 6 hours. The cultures were diluted in saline to match the $BaSO_4$ standard (Bauer et al. 1996). Sterile cotton swabs were dipped into inoculum and inocula were removed from swabs pressing against the inside wall of the tubes. MH agar plates were streaked by swabbing across the entire plate 3 times, each time at 60^0 angle to the previous streaking. The discs were placed on the seeded plates with sterile forceps and each disc was gently pressed down to ensure proper contact with the agar. The plates were incubated for 24 hours at 37^0 C. The antibacterial activities were expressed by measuring zone of inhibition in mm. Among ten plant materials the methanol extracts of the seed of *H.antidysenterica,* the bark of *T. arjuna,* the roots of *R.serpentina* were found to be highly active against some deadly bacterial strains. The methanol extract of the seed of H.antidysenterica was highly active against *Staphylococus aureus* and the zone of inhibition was 22mm.The methanol extract of *T. arjuna* was found to be active against five bacterial species, *Shigella dysenteriae, Shigella flexneri, Shigella sonnei, E.Coli* and *Staphylococus aureus,* and the zone of inhibition were 14 mm,13mm,10mm,11mm & 18mm respectively.The Methyl extract of the roots of *R.serpentina* was found to be active against *Staphylococus aureus* and the zone of inhibitions was 25mm.

Separation and isolation of antibacterial compounds were then carried out from the seeds of *H.antidysenterica,* the bark of *T.arjuna,* the roots of *R.serpentina.* In this regard, each of the above plants was then serially extracted with petroleum ether (60-80^{0}C), ethyl acetate, methanol and finally water to have an idea about the nature and solubility of the compounds. All of the extracts were microbiologically tested by disc diffusion method against *Vibrio choleriae, Shigella dysenterica,* Sh.*flexneri, Sh. sonnei, Sh.boydii, Salmonella typhi, Salmonella parapythi* "A", *Salmonella paratyphi* "B",*Klebsiella pneumoniae,* and *Staphylococus aureus.* All of the methanol extracts were found to be active against some of the deadly pathogenic strain. The other extracts showed no antibacterial activity.

Fractionation of methanol extract of the seeds of *H.antidysenterica* was then carried out by solvent extraction. Four different fractions were obtained these were Hexane fraction, Chloroform fraction, Ethyl acetate-fraction and water fraction. These four fractions were microbiologically investigated for their any antibacterial activities. Among these four fraction only Ethyl acetate fraction showed strong antibacterial activity against *S.aureus.* Separation of compounds present in the Ethyl acetate fraction were carried out by means of Preparative Thin Layer Chromatography (PTLC). Isolated compound which was marked as HA_1 was physiologically active against *S.aureus.* Characterization of compound HA_1 was carried out by Physical, Chemical and Spectroscopic methods. Compound HA_1 (expected compound Conessine) was steroidal alkaloid with -C=N.group.

Fractionation of methanol extracts of *T.arjuna* was then carried out by Column Chromatographic technique. Six fractions were found marked as A_1 to A_6-fractions. Among these fractions only A_3 fraction was active against *Shigella dysenteriae, Sh.flexneri, Sh.sonnei, E coli, and S.aureus.* The dried A_3 fraction was then separated by PTLC. Four bands were found marked as band-1, band-2, band-3 and band-4. Compound TA_3 from band-3 was active against five mentioned organisms. Through Physical, Chemical and Spectroscopic analysis it was shown the compound TA_3 (expected compound Arjunolone)was flavone with ketone and phenyl hydroxyl group.

Fractionation of methanol extract of the roots of *R.serpentina* was then carried out by column chromatographic separation technique. The column was eluted with pet ether,

mixture of pet ether-ethyl acetate, ethyl acetate-methanol and finally washed with methanol. Fractions of about 15 ml were collected in each test tube at regular intervals and checked on TLC plates. In all 123 collections were made. Collections showing similar or almost similar TLC behavior were combined together and the total collections were combined to obtain four fractions, R_1-R_4. Four fractions were tested for their antibacterial activity .Fraction R_2 was active against S.aureus.

Fraction R_2 was then concentrated in vacuum rotary evaporator. The concentrate extract was then stand for few days for crystallization. Thus crystals were obtained. The crystals were separated by decantation from the mother liquor (designated as fraction-1). The crystal were then washed with pet ether , ethyl acetate and methanol (the washing liquid was designated as fraction-2). Finally to get pure crystal, the crystals were recrystallized from MeOH solution. In the recrystalization the crystals were dissolved in hot MeOH and boiled for a while and then the solution was cooled at 0^0C by ice water. The crystal were then separated by decantation from mother liquor (designated as fraction-3). The pure crystals (fraction-4) which was designated as the compound RS showed antibacterial activity against *S.aureus*. Through Physical,Chemical and Spectroscopic analysis it was seen that the compound RS (expected compound Reserpine) was alkaloid with phenyl hydroxyl group, aryl ether group and carboxylic acid group.

Minimum Inhibitory Concentration (MICs) of compounds TA3 for *Shigella dysenteriae* was 640 μg/ml, that of compuond TA3 for *S.flexneri* was 320μg/ml, that of compound TA3 for *S.sonnei* was 960μg/ml, that of compound TA3 for *E.coli* was 640μg/ml and that of compounds HA1,TA3 and RS for *S.aureus* were 320μg/ml,320μg/ml and 160μg/ml. respectively. The Minimum Bactericidal Concentration (MBC)of compound TA3 *Shigella dysenteriae* was 790μg/ml, that of compound TA3 for *Shigella flexneri* was 780μg/ml and that of compound TA3 for *S.sonnei* was 1075μg/ml, that of compound TA3 for *E.coli* was 775μg/ml, and that of compounds HA1,TA3 and Rs for S.aureus were 810μg/ml,730μg/ml and 670μg/ml respectively.

CHAPTER-8

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