CHAPTER - 4:
STUDIES ON THE ANTIMICROBIAL ACTIVITIES OF
ADIANTUM CAPILLUS-VENERIS L.
AND
ADIANTUM LUNULATUM BURM. F.
Advances in science, notably during the last two centuries, better understanding of human body, its physiology, led to the isolation of many active ingredients of herbs in pure form and formulated synthetic compounds with or without herbal extracts, obtaining the drugs mostly used in control of diseases (Chet, 1987, Lynch, 1990; Parihar et al., 2003). The use of drugs for curing various human ailments figured in ancient manuscripts; yet, a scientific study to determine their antimicrobial activity is comparatively new ((Parihar and Bohra, 2004). Now-a-days, a number of plants have been screened for their antimicrobial activities against various human and plant pathogenic organisms. During the last two decades the interest in herbal drugs of plant origin has increased in the developed as well as developing countries because herbal medicines have been reported to be safe and without any adverse side effects (Parihar and Bohra, 2004).

The herbal drugs used in traditional system of medicines have also been reported to possess antimicrobial effects (Chatterjee and Pakrashi, 1994). There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in the recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases (Aliero and Afolayan, 2006). This situation coupled with the undesirable side effects of certain antibiotics and the emergence of previously uncommon infections are serious medical problems (Marchese and Shitto, 2001). This has forced the scientists to search for new antimicrobial substances from various sources like medicinal plants (Aliero and Afolayan, 2006). Antifungal and antibacterial substances have been found in a number of plants and it is possible that such compounds provide protection against certain pathogenic organisms (Dawani et al., 2002; Parihar et al., 2004).

Most of the plants produce antimicrobial secondary metabolites either as part of their normal program of growth and development or in response to pathogen
attack resulting stress (Morrissey and Osbourn, 1999). The possibility, that these compounds may protect plants against diseases, has intrigued biologists since the early part of the 20th century (Link et al., 1929; Muller and Borger, 1940).

Maiden hair fern, *Adiantum capillus-veneris* L. and walking maiden hair fern *Adiantum lunulatum* Burm. f. of the family Adiantaceae generally occur in the mountainous regions throughout India; in plains they grow on rocks, inhabiting in shady places near swamps and on slopes of lower hills (Chandra, 2000). In traditional herbal medicinal systems *A. capillus-veneris* is used as expectorant, diuretic, febrifuge, as hair tonic, in chest diseases, in catarrhal infection, to treat hard tumours in spleen and it is anticancerous (Puri and Arora, 1961; Singh et al., 1989; Jain et al., 1992; Kumar, et al., 2003). *A. lunulatum* is used as antidiysenteric agent, effective against blood diseases, ulcer and erysipelas, in epileptic fits, in treatment of leprosy and other skin diseases, to cure bronchitis and asthma and as antidote in snake bites (Anand and Srivastava, 1994; Chatterjee and Pakrashi, 1994; Kaushik and Dhiman, 1995).

Antimicrobial activities of plant phenolics are well established (Harborne, 1994; Taiz and Zeiger, 1998). Antibacterial and antifungal activities of pteridophytic plants have been reported (Lall et al., 1964; Banerjee and Sen, 1980; Srivastava and Kediyal, 1984; Mittal et al., 2002; Sengupta et al., 2002; Parihar and Bohra, 2000, 2001, 2002a, 2002b, 2002c, 2002d, 2004; Parihar et al., 2002, 2003, 2004), though in comparison to angiosperms, these are very few in number. However, though the sporophytic plant parts were used and reported earlier as experimental material, there are no reports regarding the antimicrobial properties of fern gametophytes. Moreover, for studying antibacterial as well as antifungal assay, one method is not just sufficient because, there may be experimental errors. Taking all these parameters into consideration, the antibacterial and antifungal effect of the crude extracts and extracted phenols of the gametophyte and different parts of sporophyte of the two species of *Adiantum* viz. *A. capillus-veneris* and *A. lunulatum* were studied using different methods which were standardized and modified when necessary.
4.B: REVIEW OF THE LITERATURES

The use of medicinal plants in the treatment of infections is an age old practice and several natural products from plants are used for treatments of many diseases. Recently, the acceptance of traditional medicines as an alternative form of health care and the development of microbial resistance to the available antibiotics has led authors to investigate the antimicrobial activity of medicinal plants (Ingham, 1973; Bisignano et al., 1996; Lis-Balchin and Deans, 1996; Maoz and Neeman, 1998; Hammer et al., 1999; Nostro et al., 2000). Human infections constitute a serious problem and most frequent pathogens are microorganisms such as bacteria and fungi. On the other hand, the development of resistant strains of pathogenic bacteria to antibiotics currently in use is a serious problem of continuing concern to public health (Neu, 1992; Bisignano et al., 1999). The fungal infections in immunocompromised individuals have increased greatly in the recent years (Wheat, 1994; Dolande et al., 2002) and these infections include important risk factors such as: neutropenia, leucopenia, chronic administration of corticosteroid and other antifungal agents, hypertoxicity cutaneous reactions and tissue lesions (Pujol et al., 1996; Salvador et al., 2003).

Now-a-days, several antimicrobial drugs are available and particularly the antimycotic drugs have limited use due to number of factors such as low potency, poor solubility, emergence of resistant strains and drug toxicity (Siqueira and Guimaraes, 1984; Cohen, 1992; Neu, 1992; Bisignano et al., 1999). Therefore, search for discovery of new antimicrobial agents is necessary to stimulate research on new chemotherapeutic substances in the medicinal plants. Moreover, the increasing use of plant extracts in food, cosmetic and pharmaceutical industries suggests that, in order to find active compounds, a systematic study of medicinal plants is very important.

Many plants produce low molecular weight compounds which inhibit the growth of phytopathogenic fungi in vitro; these compounds may act as inhibitors
that are present constitutively in healthy plants (also known as phytoanticipins) or they may be synthesized (phytoalexins) in response to pathogen attack (Morrissey and Osbourn, 1999; Haralampidis et al., 2001). Sometimes fungi produce molecules that suppress plant defence responses (Kessman and Barz, 1986). So, from time to time there have been comprehensive reviews on antifungal compounds in plants (Mansfield, 1983; Kessman and Berz, 1986; Bennet and Wallsgrove, 1994; Grayer and Harborne, 1994; Ku, 1992, 1995; Osbourn, 1996a, 1996b).

Plants produce a wide variety of secondary metabolites many of which are antimicrobial (Lamb et al., 1989; Pomilio et al., 1992; Hain et al., 1993; Harborne, 1994; Bennet and Wallsgrove, 1994; Penna et al., 2001; Ku, 1995; Kombrink and Somssich, 1995; Qi et al., 1995; Berenbaum and Zangerl, 1996; Osbourn, 1996a; Zucci et al., 2000). Plant phenolics serving as antibacterial and antifungal agents are well established (Tomas-Barberan et al., 1990; Hain et al., 1993; Harborne, 1994; Kombrink and Somssich, 1995; Fernandez et al., 1996b; Szafer-Hajdrych et al., 1998; Weston et al., 1999; Ezzat, 2001; Lavermicocca et al., 2003; Paul et al., 2003; Salvador et al., 2002, 2003; Pretorius et al., 2003; Salvador-Hajdrych and Goslinka, 2004).

Use of ethnomedicines dates back to Pliny and Dioscorides (De Feo, 2003). But analyses of plants as antimicrobial agents were mainly confined to the higher groups, i.e. angiosperms (Dobrynin et al., 1976; Leven et al., 1979; Mahmoud et al., 1989; Ogunti et al., 1991; Schmitt et al., 1991; Abl-El-Nabi et al., 1992; Alade and Irobi, 1993; Shetty et al., 1994; Bisignano et al., 1996; Bagci and Digark, 1996; Drost-Karbowska et al., 1996; Lis-Balchin and Deans, 1996; Hammer et al., 1999; Perez et al., 1999; Costa et al., 2000; Mathekga et al., 2000; Baricevec et al., 2001; Bylka and Goslinka, 2001; Chattopadhyay et al., 2001; Ezzat, 2001; Afolayan et al., 2002; Juliana et al., 2002; Singh et al., 2002; Salvador et al., 2002, 2003; Obi et al., 2003; Paul et al., 2003; Pretorius et al., 2003; Alzadi and Yusoff, 2003; Adekunle et al., 2003; Bylka et al., 2004; Szafer-Hajdrych, 2004; Al-Burtamani et al., 2005; Wei et al., 2005; Kritzinger et al., 2005; Njenga et al., 2005; Aliero and Afolayan, 2006).
Plants as potent antibacterial agents have been reported earlier (Cherevatyi et al., 1980; Oloke et al., 1988; Meyer and Afolayan, 1995; Maurer-Grimes et al., 1996; Afolayan and Meyer, 1997; Rabe and van Staden, 1997; Maoz and Neemar, 1998; Mathekga and Meyer, 1998; Bisignano et al., 1999; Grierson and Afolayan, 1999; Perez et al., 1999; Nostro et al., 2000; Ramesh et al., 2001; Aljadi and Yusoff, 2003; Afolayan, 2003; Obi et al., 2003; Pretorius et al., 2003; Salvador et al., 2003; Parihar and Bohra, 2002b, 2002c, 2003a, 2003b, 2004; Bylka et al., 2004; Al-Burtamani et al., 2005; Kritzinger et al., 2005; Njenga et al., 2005; Aliero and Afolayan, 2006). Gram positive bacteria were found to be more susceptible to plant extracts than gram negative bacteria (Nostro et al., 2000; Bylka et al., 2004; Njenga et al., 2005). Nostro et al. (2000) noted the reason for the difference in sensitivity between gram positive and gram negative bacteria, which they ascribed as the difference in the cell membrane structure of the microorganisms; gram negative bacteria have an outer polypeptide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da (Nikaido and Vaara, 1985). Thus Scherrer and Gerhardt (1971) quoted that gram positive bacteria should be more susceptible by having only an outer peptidoglycan layer, which is not an effective barrier. The observations of Aliero and Afolayan (2006) were just the reverse of the previous observations; these authors noted that gram negative bacteria were more susceptible to the plant extracts in acetone than gram positive bacteria.

Plants as antifungal agents have also been reported (Kashmi and Trivedi, 1978; Droby et al., 1986; Oloke et al., 1988; Ku, 1992; Chauhan, 1993; Espinel-Ingroff et al., 1995; Maoz and Neemar, 1998; Perez et al., 1999; Costa et al., 2000; Fiori et al., 2000; Nostro et al., 2000; Ezzat, 2001; Afolayan et al., 2002; Portillo et al., 2002; Afolayan, 2003; Adekunle et al., 2003; Salvador et al., 2003; Bylka et al., 2004; Parihar et al., 2004; Al-Burtamani et al., 2005; Kritzinger et al., 2005; Njenga et al., 2005; Wei et al., 2005; Aliero and Afolayan, 2006). The antifungal compounds of the plants have certain degree of lipophilicity, which may determine toxicity by the interactions with the membrane constituents and their arrangements.
Antifungal agents also hamper fungal membrane synthesis (Lavermicocca et al., 2003).

Bioassay with plant extracts are so far carried out by using different solvent extracts of plants like acetone extract (Afolayan et al., 2002; Parihar and Bohra, 2003b; Bylka et al., 2004; Kritzinger et al., 2005; Njenga et al., 2005; Aliero and Afolayan, 2006), ethanolic extract (Adekunle et al., 2003; Obi et al., 2003; Salvador et al., 2003; Bylka et al., 2004; Kritzinger et al., 2005), hexane extract (Baricevec et al., 2001; Salvador et al., 2003), ethyl acetate (Pretorius et al., 2003), methanolic extract (Njenga et al., 2000; Ezzat, 2001; Ramesh et al., 2001; Aliero and Afolayan, 2006) etc. Different authors had shown that alcoholic extracts were more effective than aqueous extracts (Parihar and Bohra, 2004; Adekunle et al., 2003; Obi et al., 2003; Parihar et al., 2003, 2004; Aliero and Afolayan, 2006).

So far 27 species of ferns were described to be antimicrobial of which only 19 are used in India (Parihar and Bohra, 2003b). Ethnobotanic importance of pteridophytic plants has been described by various researchers all over the world (Asolkar et al., 1992; Chatterjee and Pakrashi, 1994). The graceful primitive vascular plants, pteridophytes are distributed in various geoclimatic conditions throughout the world (Kumar et al., 2003). Ferns are represented by more than 10,000 species throughout the world (Dixit, 1984). 1,100 species of pteridophytes belonging to 19 genera are so far reported from India (Chandra, 2000; Dixit, 1984). The pteridophytes are known to man for more than 2000 years for their medicinal values; Theophrastus (327 – 287 B.C.) and Dioscorides (50 A.D.) had referred the medicinal attributes of certain ferns (Kumar et al., 2003). The medicinal use of Marsilea minuta L. and Adiantum capillus-veneris L. had been mentioned by Sushruta and Charaka in their Samhitas (Singh, 1999) thus revealing the importance of ferns since ancient times.

Antimicrobial activity of the extracts from different plant parts have been studied (Okeke, et al., 2001; Afolayan, 2003). Salvador et al. (2003), while studying the antimicrobial activity of Alternanthera maritima found that the aerial parts were more effective than the roots.
Different sporophytic plant parts were analysed separately by Ramesh et al. (2001), Parihar and Bohra (2001, 2002a, 2002d, 2003b, 2004), Parihar et al. (2003, 2004). The extensive review of literatures revealed that though sporophytic plant parts of the ferns have been used as antimicrobial agents but no work has been done on the fern gametophytes in this respect.
4.C: MATERIALS AND METHODS

Collection of plant materials:
The sporophytic plant body of *Adiantum capillus-veneris* L. and *Adiantum lumulatum* Burm f. were collected from the experimental garden of the Botany Department of Burdwan University in three different seasons viz., summer (March to June), rainy (July to October) and winter (November to February). 100 mg of the sporophytic plant body of each of the species in each season were taken and washed thoroughly in running water for two to three times and then in distilled water. The plant materials were then taken in between the folds of blotting paper and kept at 35°C for 30 minutes to allow the excess water to evaporate. Crude extracts of the sporophytic plant body were prepared according to the methods followed by Guha *et al.* (2004).

Culture of gametophytes:
Gametophytes were raised and cultured in Modified Moore’s medium (Kato, 1969) with 2 % agar. They were grown at a temperature of 24° C ± 1° C, with 70 % to 80 % relative humidity and 16 h light / 8 h dark photoperiod with light source from cool white fluorescent lamp of 2000 Lux intensity. These cultured gametophytes were collected, blotted dry in between the folds of blotting paper at 35°C for 30 minutes. The gametophytes were now ready for preparation of crude extracts.

Preparation of crude extracts:
100 mg of fresh tissue were taken and crushed in mortar and pestle along with 80 % concentration of boiled ethanol. The ethanolic mixture was centrifuged at 4000 rpm for 10 minutes. The supernatant was taken and the volume was made to 5 ml by the same concentration of ethanol. 4 ml of distilled water was added to this alcoholic extract and placed in a hot plate with temperature fixed at 40°C to evaporate the alcohol. When the alcohol was completely evaporated, the crude extract thus comes in water solution with a concentration of 2.5 % volume / volume.
Apart from preparation of gametophytic and whole sporophytic crude extracts, different parts of the sporophyte like rhizome, rachis, immature laminar units, young fertile laminar units, mature fertile laminar units and spore dehisced laminar units were also taken into consideration. The different sporophytic plant parts were very cautiously separated and washed thoroughly, blotted dry and crude extraction was made following the methods adapted previously in this chapter.

**Extraction of free phenols:**
Free phenols were extracted and estimated as per the methods of Bray and Thorp (1954) with slight modification as described in the previous chapter (Chapter 3.1).

**Antimicrobial study:**
Antimicrobial tests were performed by using crude extracts and extracted phenols from the gametophytes and sporophytes as well as the different parts of the sporophyte of *Adiantum capillus-veneris* and *Adiantum lunulatum*.

**STUDY OF ANTIBACTERIAL ACTIVITY:**
The antibacterial activities of the crude extracts and extracted phenols of the gametophytic and sporophytic plant parts of *A. capillus-veneris* and *A. lunulatum* were measured following two methods: Agar cup assay and Tube dilution technique. The antibacterial activities of the plant extracts were tested against two bacterial strains taken from pure culture, viz. *Bacillus subtilis* AR-2 (Gram positive) and *Escherichia coli* XL-1 Blue (Gram negative).

**Pure culture technique:** Bacterial strains used for the experiments were taken from pure culture following the methods mentioned in Prescott *et al.* (2002). Here a loop full of each of the bacterial strains were taken and added to 10 ml of sterilized distilled water and mixed thoroughly in a vortex mixture. Then 1 ml of solution was taken and added to 9 ml of sterilized distilled water. This dilution procedure was
followed upto $1 \times 10^{-4}$. From the dilution series of $10^{-4}$, 0.1 ml was taken and streaked on Petri dish containing nutrient agar. This was incubated at $35^\circ C \pm 2^\circ C$ for 48 h to 72 h. Now, from the end of the streak, a loop full of culture was taken and mixed thoroughly in sterilized distilled water. Then the dilution series was done and Petri dish was streaked as before. From the end of the streak, a loop full of inoculum was taken and inoculated on 10 ml of nutrient broth. The broth was incubated in Eyla shaker with 15 rotations / minute at a temperature of $35^\circ C \pm 1^\circ C$ for 24 h. 0.1 ml of the broth solution was taken and inoculated in 9.9 ml of nutrient broth and incubated in shaker as before and the process was continued for four dilutions. Now from the final broth solution, 0.1 ml solution was taken, streaked on Petri dish, incubated and loop full of inoculum was taken from its end and added to fresh nutrient broth. From this broth each of 0.1 ml was taken and streaked on nutrient agar slants. The slants were incubated at $35^\circ C \pm 2^\circ C$ temperature for 48 h to 72 h. These were then sealed tightly and preserved in refrigerator. The slants were then taken for antimicrobial assay.

**Agar cup assay:** Agar cup assay was performed following the methods mentioned in Tortura *et al.* (2001). Loop full of bacteria from slant cultures were transferred to tubes containing 9 ml of sterilized nutrient broth and incubated on rotary shaker for 24 h at $35^\circ C \pm 2^\circ C$ with 15 rotations / minute. From these tubes, each of 1 ml of broth cultures were transferred to sterilized Petri dishes. 20 ml of molten agar ($45^\circ C$ temperature) was poured in Petri dishes and thoroughly shaken so that the inoculum gets completely mixed with the nutrient agar. The plates were allowed to solidify. Then they were chilled at $10^\circ C$ temperature for 1 h. Cups of 6 mm diameter were made in the plates in a systematic manner with the help of a cork borer. 0.1 ml of crude extracts of the sporophytic and gametophytic plant body and their extracted phenols were applied in separate cups and incubated at $37^\circ C$. After 24 h, the diameter of the bacterial inhibition zones or the hallow zones formed due to bacterial lysis were measured. Distilled water was used as control. For each analysis, 6 replicates were made.
Tube dilution technique: This technique was performed according to the methods mentioned in Tortura et al. (2001). Here, loop full of bacteria from the slant cultures were transferred to 9 ml of sterilized nutrient broth and incubated in rotary shaker for 24 h at 35°C ± 2°C temperature, with 15 rotations / minute. To these 9 ml of nutrient broth containing tubes, 0.5 ml of the bacterial inoculum and 0.5 ml of the plant extracts were added. The tubes were kept in incubator – shaker for 24 h at 37°C ± 2°C. Afterwards, the turbidity was measured at 540 nm in Beckman DU spectrophotometer. Distilled water was taken as control. All the experimental sets were having 6 replicates for each.

Composition of nutrient broth:

Beef extract : 1 g  
Yeast extract: 2 g  
Peptone: 5 g  
NaCl: 5 g  
Distilled water: 1 L

Composition of nutrient agar: 1 L Nutrient broth + 1.5 g Agar.

STUDY OF ANTIFUNGAL ACTIVITY:

The antifungal activities of the crude extracts and extracted phenols of gametophytic and sporophytic plant parts of A. capillus-veneris and A. lunulatum were measured using the following methods:

1. Agar disc method
2. Liquid culture method
3. Suspension culture method

The plant extracts were tested against the fungal strains, viz. Aspergillus niger and Rhizopus stolonifer. In all the three experiments, control sets were maintained and distilled water was taken as control.
**Agar disc method:** This was done following the methods mentioned by Alam (2004). 20 ml of potato-dextrose-agar (PDA) medium was poured in sterilized Petri dishes along with 1 ml of plant extracts. The extract was mixed thoroughly with the PDA medium by shaking in clockwise and anticlockwise directions. The plates were allowed to solidify. Then they were chilled for 1 h at 10°C temperature. 5 mm diameter cups of PDA were removed from the centre of the Petri plates with the aid of cork borer. The centres were then filled with the same diameter of mycelial discs taken from young cultures (7 days old from the day of inoculation). The Petri dishes were wrapped with black paper and incubated at 28°C ± 1°C for 14 days. Colony growths were measured on the basis of linear dimensions as well as by mycelial dry matter weight.

**Determination of mycelial dry matter weight:** Mycelial dry matter weights were measured following the methods mentioned by Medda (1991). Here the mycelia mass were transferred to copper nets, which were immersed in boiling distilled water to remove the agar from its surface thus leaving the mycelia only in the nets. The mycelia were then collected on pre-weighed filter papers. It was oven dried at 100°C for 2 h and the dry weight was then determined.

**Slight modification of the agar disc method:** In agar disc method, at the time of mycelial disc inoculation from the young cultures, in order to avoid falling of spores in the medium, sterilized filter papers with centres cut into circular patterns were taken. The diameters of the circular cuts were slightly greater than the cups made by the cork borer. After opening the Petri dish, just before the mycelial disc inoculation, the filter papers were aseptically placed over the medium. Then the mycelial discs were inoculated, the filter papers were removed and the Petri dishes were closed.

**Liquid culture method:** This was done following the methods mentioned by Mukhopadhyay and Nandi (1997). Here single mycelial discs of the test strains were inoculated in 250 ml Erlenmeyer flask containing 100 ml of liquid medium.
(potato-dextrose) and 4 ml of plant extract. The flasks were incubated at 28°C ± 1°C for 8 days. The mycelia mass were collected by filtration through pre-weighed G-4 sintered glass filters. The filtrate was oven dried at 100°C for 2 h and the dry weight was then determined.

**Suspension culture method:** This was done following the method mentioned by Calam (1986). This method was same as liquid culture method; only here the flasks were incubated in rotary shaker at 28°C ± 1°C temperature and shaking at 150 RPM. Dry weight was measured like that of liquid culture method.

**Potato dextrose medium (cf. Bandopadhyay, 2000):**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato extract</td>
<td>200-250 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 L</td>
</tr>
</tbody>
</table>

**Preparation of potato extract:** Potatoes were cut into small pieces. 200 g to 250 g of potatoes were taken and boiled in 700 to 800 ml of distilled water for 45 minutes. Then the water extract was collected and the diced potatoes were removed. Now the water extract was made to 1 L by adding distilled water. Dextrose was then added.

**Potato dextrose agar medium (cf. Bandopadhyay, 2000):**

1 L of potato-dextrose medium + 20 g Agar.

**STATISTICAL ANALYSIS:**

Each of the experimental results was undertaken with replicates of 10 along with control sets. All the results were statistically analyzed for L.S.D. following the two factor analysis of variance method using Fisher’s L.S.D. procedure at P = 0.05 (Zar, 1974). In case of suspension method, for the measurement of mycelial discs,
standard deviations of each data were calculated using the following formula (cf. Acharyya Choudhury and Gupta, 1976).

\[ \text{Standard deviation (S.D.)} = \sqrt{\left( \frac{\sum d^2}{N-1} \right)} \]

Where \( d \) = deviation of class value from the mean;
\( N \) = total number of observations;
\( \sum \) = Summation.
4.D: RESULTS

In between the species of *Adiantum*, *A. capillus-veneris* showed better bactericidal property than *A. lunulatum* (Figs. 4.D.F1 and 4.D.F2). In between the gram positive bacteria (*Bacillus subtilis*) and gram negative bacteria (*Escherichia coli*), *B. subtilis* showed more susceptibility to the plant extracts (Figs. 4.D.F7 and 4.D.F8). Among the different sporophytic plants of both the species of *Adiantum*, immature laminar units possess the highest bactericidal property (Figs. 4.D.F7 to 4.D.F10). Both the crude extracts and extracted phenols from the gametophytes caused greater bacterial lysis than that of the sporophytic extracts (Figs. 4.D.F1 and 4.D.F2). In case of agar cup assay, the greater the effect of the plant extracts, the bigger were the hallow zones (Plates: 4.D.P1 and 4.D.P2). In tube dilution technique, the optical density increases with the increase in turbidity and it was found to be highest in control (Figs. 4.D.F2, 4.D.F9 and 4.D.F10). The optical density was least in the tubes containing extracted phenols from the gametophytes, thus indicating maximum bacterial lysis.

The crude extracts and the extracted phenols from the gametophyte as well as the different sporophytic plant body of *A. capillus-veneris* and *A. lunulatum* were found to be bioactive against the two fungal strains, viz. *Aspergillus niger* and *Rhizopus stolonifer*. In between the two species of *Adiantum*, *A. capillus-veneris* was found to be better antifungal agent than *A. lunulatum* (Fig. 4.D.F3 to 4.D.F6). Gametophytes of both the species of *Adiantum* were found to be more potent antifungal agents than the sporophytes (Figs. 4.D.F3 to 4.D.F6). Among the different sporophytic plant parts, immature laminar units of both the species of *Adiantum*, showed the highest fungistatic property (Figs. 4.D.F.11 to 4.D.F.18). In suspension culture method, the mycelial discs of the controlled cultures were smaller in size in comparison to the mycelial discs of the cultures with plant’s crude extract or extracted phenols [Diameter of mycelial discs of *R. stolonifer* was 1.52 ± 0.043 mm and of *A. niger* was 1.61 ± 0.032 mm in control culture sets, whereas the diameter of mycelial discs of *R. stolonifer* was 6.66 ± 0.71 mm and *A. niger* was
5.03 ± 0.84 mm in cultures with extracted phenols. (These readings were not given in tabular form and only these four readings were noted and is well evidenced from Plate: 4.D.P5).
FIGURE- 4.D.F1: Effect of sporophytic and gametophytic extract of *A. capillus-veneris* and *A. lunulatum* on area (sq. mm) of hallow zones in agar cup assay. a. Crude extract on *B. subtilis*; b. Crude extract on *E. coli*; c. Extracted phenol on *B. subtilis*; d. Extracted phenol on *E. coli*. (L. S.D. at 5%: a. 20.34; b. 33.46; c. 25.42; d. 19.86).
FIGURE- 4.D.F2: Effect of sporophytic and gametophytic extract of *A. capillus-veneris* and *A. lunulatum* on optical density (at 540 nm) of the bacterial tubes in tube dilution method. a. Crude extract on *B. subtilis*; b. Crude extract on *E. coli*; c. Extracted phenol on *B. subtilis*; d. Extracted phenol on *E. coli*. (L. S.D. at 5 %: a. 0.011; b. 0.016; c. 0.021; d. 0.017).
FIGURE- 4.D.F3: Effect of sporophytic and gametophytic extract of *A. capillus-veneris* and *A. lunulatum* on the area of growth zones (sq. cm.) of fungal cultures in agar disc method. a. Crude extract on *A. niger*; b. Crude extract on *R. stolonifer*; c. Extracted phenol on *A. niger*; d. Extracted phenol on *R. stolonifer*. (L. S.D. at 5%: a. 3.48; b. 2.52; c. 1.96; d. 1.43).
FIGURE- 4.D.F4: Effect of sporophytic and gametophytic extract of *A. capillus-veneris* and *A. lunulatum* on the mycelial dry weight (mg) in agar disc method. a. Crude extract on *A. niger*, b. Crude extract on *R. stolonifer*, c. Extracted phenol on *A. niger*, d. Extracted phenol on *R. stolonifer*. (L. S.D. at 5 %: a. 4.78; b. 3.18; c. 3.02; d. 2.86).
FIGURE- 4.D.F5: Effect of sporophytic and gametophytic extract of *A. capillus-veneris* and *A. lunulatum* on the mycelial dry weight (mg) in liquid culture method. 
a. Crude extract on *A. niger*; b. Crude extract on *R. stolonifer*; c. Extracted phenol on *A. niger*; d. Extracted phenol on *R. stolonifer*. (L. S.D. at 5%: a. 5.16; b. 6.32; c. 3.18; d. 3.52).
FIGURE- 4.D.F6: Effect of sporophytic and gametophytic extract of *A. capillus-veneris* and *A. lumulatum* on the mycelial dry weight (mg) in suspension culture method. a. Crude extract on *A. niger*; b. Crude extract on *R. stolonifer*; c. Extracted phenol on *A. niger*; d. Extracted phenol on *R. stolonifer*. (L. S.D. at 5 %: a. 4.36; b. 3.96; c. 2.21; d. 3.18).
FIGURE- 4.D.F7: Effect of the extracts of different sporophytic plant parts of *A. capillus-veneris* on area of hallow zones (sq. mm) on *B. subtilis* and *E. coli* cultures in agar cup assay. a. Crude extract; b. Extracted phenol. (L. S.D. at 5 %: a. 5.62; b. 2.82).

**Abbreviations**: IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
FIGURE- 4.D.F8: Effect of the extracts of different sporophytic plant parts of *A. lunulatum* on area of hallow zones (sq. mm) on *B. subtilis* and *E. coli* cultures. a. Crude extract; b. Extracted phenol. (L. S.D. at 5 %: a. 3.67; b. 4.13).

**Abbreviations:** IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
FIGURE- 4.D.F9: Effect of the extracts of different sporophytic plant parts of *A. capillus-veneris* on O. D. (at 540 nm) of bacterial tubes in tube dilution technique with *B. subtilis* and *E. coli* cultures. a. Crude extract; b. Extracted phenol. (L. S.D. at 5 %: a. 0.015; b. 0.016).

**Abbreviations:** C = Control, IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
FIGURE- 4.D.F10: Effect of the extracts of different sporophytic plant parts of *A. lunulatum* on O. D. (at 540 nm) of bacterial tubes in tube dilution technique with *B. subtilis* and *E. coli* cultures. a. Crude extract; b. Extracted phenol. (L. S.D. at 5 %: a. 0.018; b. 0.017).

**Abbreviations:** C = Control, IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
FIGURE- 4.D.F11: Effect of the extracts of different sporophytic plant parts of *A. capillus-veneris* and *A. lunatum* on the area (sq. cm) of growth zones of *A. niger* in agar disc method. a. Crude extract; b. Extracted phenol. (L. S.D. at 5 %: a. 3.26; b. 2.03).

Abbreviations: C = Control, IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
**FIGURE 4.D.F12**: Effect of the extracts of different sporophytic plant parts of *A. capillus-veneris* and *A. lunulatum* on the area (sq. cm) of growth zones of *R. stolonifer* in agar disc method. a. Crude extract; b. Extracted phenol. (L. S.D. at 5%: a. 3.98; b. 2.64).

**Abbreviations**: C = Control, IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
FIGURE- 4.D.F13: Effect of the extracts of different sporophytic plant parts of *A. capillus-veneris* and *A. lunulatum* on the mycelial dry weight (mg) of *A. niger* in agar disc method. a. Crude extract; b. Extracted phenol. (L. S.D. at 5%: a. 5.87; b. 3.03).

**Abbreviations:** C = Control, IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
**FIGURE- 4.D.F14:** Effect of the extracts of different sporophytic plant parts of *A. capillus-veneris* and *A. lunulatum* on the mycelial dry weight (mg) of *R. stolonifer* in agar disc method. a. Crude extract; b. Extracted phenol. (L. S.D. at 5%: a. 2.36; b. 1.92).

**Abbreviations:** C = Control, IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
FIGURE 4.D.F15: Effect of the extracts of different sporophytic plant parts of *A. capillus-veneris* and *A. lunulatum* on the mycelial dry weight (mg) of *A. niger* in liquid culture method. a. Crude extract; b. Extracted phenol. (L. S.D. at 5%: a. 10.45; b. 8.65).

**Abbreviations:** C = Control, IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
FIGURE- 4.D.F16: Effect of the extracts of different sporophytic plant parts of *A. capillus-veneris* and *A. lumulatum* on the mycelial dry weight (mg) of *R. stolonifer* in liquid culture method. a. Crude extract; b. Extracted phenol. (L. S.D. at 5 %: a. 9.87; b. 6.57).

**Abbreviations:** C = Control, IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
FIGURE 4.D.F17: Effect of the extracts of different sporophytic plant parts of *A. capillus-veneris* and *A. lunulatum* on the mycelial dry weight (mg) of *A. niger* in suspension culture method. a. Crude extract; b. Extracted phenol. (L. S.D. at 5%: a. 8.97; b. 6.68).

**Abbreviations:** C = Control, IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
FIGURE- 4.D.F18: Effect of the extracts of different sporophytic plant parts of *A. capillus-veneris* and *A. lunulatum* on the mycelial dry weight (mg) of *R. stolonifer* in suspension culture method. a. Crude extract; b. Extracted phenol. (L. S.D. at 5 %: a. 5.97; b. 3.23).

**Abbreviations:** C = Control, IL = Immature laminar unit, MFL = Mature fertile laminar unit, RA = Rachis, RH = Rhizome, SDL = Spore dehisced laminar unit, YFL = Young fertile laminar unit.
Plate: - 4.D.P1: Effect of the extracts from *Adiantum capillus-veneris* on bacterial culture plates. A. Extracted phenols on cultures of *Bacillus subtilis* AR-2. B. Crude extracts on cultures of *Escherichia coli* XLI-Blue. C. Extracted phenols on cultures of *Escherichia coli* XLI-Blue.

**Abbreviations:** 1- Rachis; 2- Rhizome; 3- Mature fertile laminar unit; 4- Spore dehisced laminar unit; 5- Young fertile laminar unit; 6- Immature laminar unit; B & E are the control sets.

Abbreviations: 1- Rachis; 2- Rhizome; 3- Mature fertile laminar unit; 4- Spore dehisced laminar unit; 5- Young fertile laminar unit; 6- Immature laminar unit; B & E are the control sets.
4.E: DISCUSSION

Crude extracts from the gametophytes and different parts of the sporophyte of both *Adiantum capillus-veneris* L. *Adiantum lunulatum* Burm. f. exhibited antimicrobial (both antibacterial and antifungal) properties. The extracted phenols cause greater bacterial lysis as well as show better antifungal property than the crude extracts. Phenols as antimicrobial agents are well established (Tomas Barberan *et al.*, 1990; Harborne, 1994). The weight / volume ratio of the plant material taken for crude extracts and extracted phenols were same (2.5 % volume / volume). Because the crude extracts were made with 80 % concentration of boiled ethanol, the total phenols of the plant material were pooled in that extract. With phenols, carbohydrate and amino acids were also present in the ethanolic extract. Moreover, some negligible amount of alkaloids and some unidentified compounds may also be present in the crude extracts. Carbohydrates, amino acids and the unidentified compounds in the crude extract perhaps antagonize the effect of phenols in the crude extracts, thus reducing its inhibitory potential than the effect of phenols alone. Total phenol content of *A. capillus-veneris* was more than that of *A. lunulatum* and the gametophytes contain more phenols than the sporophytes. Thus the presence of maximum content of phenols in the juvenile stage makes the gametophyte a stronger antimicrobial agent than the sporophytes (Guha *et al.*, 2004, 2005).

In the present observation, among the different parts of the sporophyte, immature laminar units possess the highest bactericidal property. This observation corroborates with the observations of Parihar *et al.* (2003), where they showed that the leaves of *Marsilea minuta* L. possessed the highest antibacterial activity than the stem or roots. Salvador *et al.* (2003) in their studies with the angiosperm *Alternanthera maritima* also showed that the aerial parts were more effective antibacterial agents than the roots. But all these observations contradicts with the observations of Parihar and Bohra (2003b, 2004) where they found rhizomes to be...
more effective than the leaves; though in these studies they had considered the same bacterial strain (*Salmonella typhi*) which may be sensitive to a particular phytochemical that may be present more in roots than in leaves.

Phenolics exert antibacterial activity by injuring the cell membrane of bacteria, inactivating the enzymes and denaturing the membrane proteins (Boller, 1995; Tortura *et al*., 2001). The plant extracts were found to be more effective against gram positive *Bacillus subtilis* than the gram negative *Escherichia coli*. These observations were in support of the observations made earlier by many workers (Nostro *et al*., 2000; Bylka *et al*., 2004; Njenga *et al*., 2005). The difference in sensitivity between gram positive bacteria and gram negative bacteria was due to their morphological differences (Nostro *et al*., 2000). Gram negative bacteria have an additional outer membrane (phospholipidic membrane, which carries the structural lipopolysaccharide components) than the membranes present in gram positive bacteria. This additional membrane interferes with the penetration of antimicrobial agents, thus giving more protection to the gram negatives. This lipopolysaccharide component makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes (Nikaido and Vaara, 1985). Gram positive bacteria were thus more susceptible, having only outer peptidoglycan layer which is not an effective impermeable barrier (Scherrer and Gerhardt, 1971). Thus the inhibition zones of gram negative bacteria were smaller than gram positive bacteria (Figs. 4.D.F7 and 4.D.F8; Plates: 4.D.P1 and 4.D.P2). These observations were reverse of the observations of Aliero and Afolayan (2006). In their study with acetone and methanolic extracts of *Solanum tomentosum* against a wide number of bacteria, they found that the activities of acetone extracts affects more to gram negative bacteria than gram positive bacteria. While methanolic extracts were more effective on gram positive bacteria, water extract was less active than acetone or methanolic extracts. It would seem unlikely that the traditional healer (water extract) is unable to extract those compounds which are responsible for activity in the acetone or methanolic extract.

The application of crude extracts or extracted phenols from the plant materials to bacterial broth cultures inhibited the bacterial growth. Light passes more easily in
tubes with plant extracts than the tubes without plant extracts as observed from their optical densities (Figs. 4.D.F9 and 4.D.F10). For studying the antibacterial activity, two methods (agar cup assay and tube dilution technique) were taken into consideration. In agar cup assay, the diameter of the hallow zones were measured, whereas, in tube dilution technique, the turbidity of the bacterial cultures were measured spectrophotometrically. Thus, tube dilution technique, in conjunction with agar cup assay, gives a more scientific approach towards the efficacy of the investigation.

Extracts of several plant material show antifungal responses (Adekunle et al., 2003; Wei et al., 2005) but the assay of the antifungal compounds of the plants are not well known (Nostro et al., 2000). In the present observation, among the different parts of the sporophytic plant body of the two species of Adiantum, immature laminar units proved to be the best antifungal agent. These observations, however, do not corroborate with the observations of Parihar et al. (2004), who showed rhizome extracts to be better antifungal agents than the leaf extracts of Chelienthes albomarginata and Marsilea minuta on Aspergillus flavus; this may be due to the presence of a particular biochemical substance in rhizomes of these two species which antagonize the growth of fungi.

For studying the antifungal activities of the plant extracts, three different types of tests were performed to ensure the accuracy of results. The aims of these three tests were to give a multidirectional approach towards the study in order to minimize the error, which could otherwise have occurred with a single test. Of the two fungal strains, Rhizopus stolonifer is a plant pathogen, which damages fruits and vegetables during storage, shipping and marketing (Alexopoulos et al., 1996) while Aspergillus niger is pathogenic to human, causing a group of diseases called aspergilloses (Kwon-Chung and Bennet, 1992). A. niger also produces mycotoxins in cereals, peanuts etc. which when consumed causes kidney and liver damages and may ultimately lead to haemorrhage of lungs and brain (Griffin, 1994).

Measurement of the area of the fungal colony (Figs. 4.D.F11 and 4.D.F12 and Plate: 4.D.P3) as well as dry matter weight of the mycelial discs (Figs. 4.D.F13 and 4.D.F.14) showed the indication of the activity of the crude extracts and extracted
phenols of both the species of *Adiantum*. In case of liquid culture method, though the horizontal growths of mycelial masses were not much affected by the addition of crude extracts or extracted phenols, but the cultures showed positive response with decrease in vertical growth of the mycelial masses (Plate:4.D.P4) and dry weight of the mycelial biomass (Figs. 4.D.F5, 4.D.F15 and 4.D.F16).

In suspension culture method, the mycelial discs of the controlled cultures were smaller in size in comparison to that of the cultures with plant extracts or extracted phenols (Plate: 4.D.P5). Similar types of results were obtained by Lavennicocca *et al.* (2003) while studying the antifungal activity of phenyllactic acid against 19 fungal strains. Secondary metabolites of plants particularly phenols give defence against infection by disrupting the membrane as well as by preventing cell wall synthesis (Maher *et al.*, 1994; Taiz and Zeiger, 1998; Kucuk and Kivanc, 2003). The plant extracts or extracted phenols may be responsible for antagonizing the synthesis of fungal cell wall materials. Though the cell volume increased and the mycelial discs were bigger in size than the controlled ones, but without any increase in dry weight. In cultures without plant extracts, the mycelial dry weight was higher than that of the cultures with plant extracts (Figs. 4.D.F6, 4.D.F17 and 4.D.F18). A certain degree of lipophilicity determines the toxicity of plants antifungal agents by interaction with the membrane constituents and their arrangements (Tomas-Barberan *et al.*, 1990).

The adiantoid ferns comprise many species with biological activities which are used in nutrition or as alternate medicines (Rastogi and Mehrotra, 1995; Chatterjee and Pakrashi, 1994). But their exact quantification needs more elaborate work. Moreover, gametophytes as source material have been established (Guha *et al.*, 2004, 2005). Under *in vitro* conditions, gametophytes can be grown on simple medium and the maintenance of cultures is also very easy. But the use of gametophytes as antimicrobial agents can be successful only in large scale commercial conditions, so that the production cost can be minimized. So, all these things need further research to exploit them for the benefit of mankind.