4.1. Introduction

With the ascendancy of science in the nineteenth century came the ability to synthesize plant parts and concentrate doses. Herb usage probably reached an all time low in the mid-twentieth century. But now, because of a greater concern about the side effects of synthetic drugs, an understanding of ecology and people's desire to take greater responsibility for their own health, herbal medicine is experiencing a remarkable revival. Pharmaceutical companies on the one hand recognize the value of herbs and are busy investigating worldwide herbal lore with unprecedented zeal, while on the other hand they wish to maintain their lucrative near monopoly over medicinal products (Bremness, 1988).

Angiosperms are storehouses of effective chemotherapeutants. Screening of these plants for a wide range of activities proves that these can be used for treating diseases like asthma, cholera, diarrhoea, dysentery, dermatological infections, intestinal disorders, recurrent fevers, ulcers, rheumatism and uremia. This has prompted researchers to study the antibacterial activity of ethanolic extract of medicinal plants against human pathogen bacteria isolate from diseased patients (Thomas, 1999).

In many developing countries most of the available medicines are obtained from medicinal plants, while in the western developed countries, plants mainly constitute raw materials for industrial processing or preparation of the pure chemical derivatives (Penso, 1980). Due to ever increasing need and cost of drugs, especially in developing countries, a need to search for low cost drugs from natural sources becomes imperative. Because the most rampant killer disease in developing countries are of microbiological
origin, research and development of antimicrobial therapeutics from plant origin could be invaluable (Gundidza and Gaza, 1993).

Need for new antimicrobial drugs have become apparent in the past few years, especially for the treatment of infections where microbial resistance to antibiotics has developed (Penna et al. 1997). As resistances toward prevailing antibiotics have become wide spread among bacteria and fungi, new class antimicrobial substances are urgently required (Darokar et al. 1998). Most of these drugs are referred to treat swellings, wound, purulent sores and venereal diseases (Gomes and Diniz, 1993).

A large number of Indian medicinal plants are regularly used as antibiotic agents by practitioners of Siddha, Ayurveda and Unani systems of medicine. In several plants antibacterial and antifungal activities were reported successfully. Ibrahim and Osman (1995) reported that ethanolic extracts of *Cassia alata* leaves were investigated for their antimicrobial activities on bacteria, dermatophytic fungi and non-dermatophytic fungi. *In vitro*, studies of this extract exhibited a high activity against various species of dermatophytic fungi, but a low activity against non-dermatophytic fungi. The minimum inhibitory concentration (MIC) of the extract revealed that *Trichophyton mentagorphytes*, *T. rubrum* and *Microsporum gypseum* had the MIC of 125 mg/ml, whereas *M. canis* had the MIC of 62.5 mg/ml.

Grosvenor (1995) reported that antibacterial assay of 114 species reported 82 % of the extracts tested were active against *Staphylococcus aureus*, while 35% were active against *Escherichia coli*. Antifungal activity was less dramatic, 19 % of the extracts inhibited *Saccharomyces cerevisiae*, while 20 % inhibited *Fusarium oxysporum*. The survey of relevant literature indicates that less than 30 % of these angiosperm species have previously been assayed for antimicrobial activity.
Ahmad et al. 1995 reported that about in 246 extracts from 82 traditionally used Indian plants were screened for a broad-spectrum antimicrobial activity. On the basis of screening, alcoholic extracts of (*Emblica officinalis*, *Terminalia chebula*, *T. belerica*, *Plumbago zeylanica* and *Holarrhena antidysenterica*) were selected for the determination of their antimicrobial potency in terms of their MIC agent several pathogenic and potentially pathogenic microorganisms.

Vijaya et al. (1995) reported that antibacterial effect of compounds extracted from *Camellia sinensis* L. and the methanol extract of *Euphoria hirta* L. were studied against dysentery causing *Shigella* spp. using the Vero cell line. Cytotoxic studies of the extract were performed using the cell line and the non-cytotoxic concentration of the extract was tested for antibacterial activity against the cytopathic dose of the pathogen. These extracts were found to be non-cytotoxic and effective antibacterial agents.

Valsara et al. (1997) reported that from the Indian traditional medicines, 78 plants were selected on the basis of their use in the treatment of infectious diseases. Different concentrations of ethanol extract were tested against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* agar dilution method.

Gadhi et al. (1999) reported that several fractions of the methanolic extract of the rhizome and the leaves of *Aristolochia paucinercis* Pommel. were screened for antibacterial activity using the agar dilution method against bacterial strains. The MIC estimate showed that it was most active against *Clostridium perfringens*, *C. difficile*, *Enterococcus luteus* and *Bacillus subtilis*. 
Thapliyal et al. (2000) tested crude extracts of *Rauwolfia tetraphylla* L, *Andrographis paniculata* Nees, *Piper longum* L., *Terminalia arjua* Wt. & Am., *Terminalia catappa* L. and *Plumbago zeylanica* L. against fungal pathogens viz., *Trichosporium vesiculosum* Butler, *Macrophomina phaeolina* (Tassi) G. Ooid and *Aspergillus flavus*. 100 % fungal growth inhibition after 72 hours was observed by *Rauwolfia tetraphylla*, *Andorgraphis paniculata* and *Piper longum* against all the pathogens. But only very less fungal spore growth inhibition was observed by other three plant extracts.

Radha et al. (2000) reported that *Syzygium travancoricum* extract from air dried leaves using hexane, chloroform and ethyl acetate were tested against fungi like *Candida albicans*, *Aspergillus terreus*, *Fusarium oxysporum* and *Pestalotiopsis* sp. Hexane extract exhibited considerable bioactivity than other solvents. Preliminary TLC analysis of this hexane extract revealed many spots testing positive to spray reagents for terpenes.

Chicory is one of important medicinal plants used to cure many diseases. The whole plant is medicinally used due to the presence of several bioactive compounds like coumarins, sesquiterpenes, flavonoids and alkaloids as described in the early chapter. As mentioned in the previous chapters, several studies on antifeedent (Rodriguez et al., 1976; Rees and Harborne, 1985; Takasugi et al., 1985), postcoital contraceptive (Keshri et al., 1998), antiulcerogenic (Ahmad et al., 1998), antiinflammatory (Ki et al., 1999), antihepatotoxic (Gadgoli and Mishra, 1997; Mitra et al., 1998) and anticancer (Hughes and Rowland, 2001) properties of *C. intybus* have been conducted to find their role in pharmacology.

In 1990, Monde et al. isolated an antifungal compound called cichoralexin from the leaves of *Cichorium intybus*. Nishmura et al. (1999) also isolated another antifungal
sesquiterpenoid, 8α-angeloyloxycichoralexin, in addition to cichoralexin and 10α-hydroxycichopumilide from chicory and tested for their antifungal activities against various plant pathogenic fungi. Yusuf et al. (2002) tested the petroleum ether and methanolic extracts of nine wild plant species for their antimycotic activity against eight phytopathogenic fungi. In this study they observed C. intybus inhibited the spore germination of two fungi.

However, antimicrobial activity of this plant against human pathogenic microorganisms is still unexplored. Hence, an attempt was made to test the efficacy of the ethanolic extract of in vivo root and leaf, callus and in vitro root and leaf against some human pathogenic bacteria and fungi.

In this present study, the efficacy test was made against five human pathogenic bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella penumoniae, Escherichia coli* and *Salmonella typhae*) and three human pathogenic fungi (*Candida albicans, Aspergillus flavus* and *Aspergillus niger*).

**4.1.1. Characteristics of the bacterial pathogens**

**4.1.1.1. Staphylococcus aureus**

*Staphylococcus aureus* are gram positive cocci. Morphologically, they are spherical and arranged characteristically in grape like clusters. They are aerobes and facultative anaerobes. They grow on ordinary media like nutrient agar. Sheep blood agar are recommended for isolating *Staphylococcus aureus*. Most of the acute pyogenic infections are caused by these organisms. The diseases may be classified as cutaneous, deep infection and food poisoning.
4.1.1.2. *Psedomonas aeruginosa*

*Psedomonas aeruginosa* is aerobic, non sporing gram negative bacillus. It is slender and actively motile by a polar flagellum. It grows on ordinary media. It shows beta haemolysis on blood agar and in MacConkey agar it produce non lactose fermenting colonies. It is one of the most important agent causing nosocomial infections, wounds, burns and chronic ulcers of the skin.

4.1.1.3. *Klebsiella pneumoniae*

*Klebsiella pneumoniae* is medically important gram negative non motile capsulated bacillus. It grows well on ordinary media. They are widely distributed in nature, occurring both as commensals intestine and saprophytes in soil and water. *Klebsiella pneumoniae* produces large and usually mucoid colonies on nutrient agar. The colonies on blood agar and MacConkey agar are as like on nutrient agar. Most strains are lactose fermenting. This species can be found as a commensal in the mouth and upper respiratory tract, and also in moist environment and in hospitals. This species causes chest infection, severe pneumonia, urinary tract infection, septicemia and meningitis.

4.1.1.4. *Escherichia coli*

*Escherichia coli* is gram negative straight rod shaped bacterium arranged singly or in pairs. It is motile by peritrichous flagella. Four main types of clinical syndromes are caused by *E. coli*: urinary tract infection, diarrhoea or gastroenteritis, pyogenic infections and septicaemia.

4.1.1.5. *Salmonella typhi*

*Salmonella typhi* is a gram negative motile bacterium. It causes enteric fever and infections of the urinary tract.
4.1.2. Characteristics of the fungal pathogens

4.1.2.1. Candida albicans

*Candida albicans* is an ovoid or spherical yeast like fungus producing pseudomycelia by budding both in culture and in tissues. It is a normal inhabitants of the skin and mucosa. It causes candidosis (an infection of the skin) and moniliasis (an infection of the vagina).

4.1.2.2. Aspergillus flavus

*Aspergillus flavus* is an Ascomycetous fungus, grows rapidly producing aerial hyphae that bear characteristic conidial structures. Chains of spherical conidia are produced from the terminal vesicle of the long conidiophore. The colonies are velvety, yellow to green or brown. It produces the best known mycotoxin called 'aflotoxin'. It causes aspergillosis (pulmonary or disseminated).

4.1.2.3. Aspergillus niger

*Aspergillus niger* is also an Ascomycetous fungus bearing the characteristic conidial structures like *A. flavus*. It causes a very common disease called otomycosis. The symptoms are itching, pain and deafness.

4.2. Materials and Methods

4.2.1. Collection of culture

In the present, the following pure cultures of human pathogenic bacteria and fungi were collected from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh to test against the crude extracts of *Cichorium intybus*. The cultures were analysed before testing.
4.2.1.1. Bacterial cultures

*Staphylococcus aureus* 1144

*Pseudomonas aeruginosa* 2295

*Klebsiella pneumoniae* 2403

*Escherichia coli* 1574

*Salmonella typhi* 733

4.2.1.2. Fungal cultures

*Aspergillus niger* 2196

*Aspergillus flavus* 1783

*Candida albicans* 1637

4.2.2. Microbial analysis

4.2.2.1. Bacterial analysis

The samples were streaked on blood agar and chocolate agar. The blood agar plates were incubated aerobically and the chocolate agar plate in a carbon dioxide enriched atmosphere at 37°C. After 48 hours of incubation the colony morphology was observed.

From the overnight culture broth, a smear was prepared on a clean glass slide and gram stained. Then they were examined to know whether gram positive or gram negative.

The individual organisms were inoculated in 5 ml of sterile saline and kept for 24 hours incubation at 37°C. The turbidity shows the growth of organism in saline. The saline containing micro organisms are characterized by various biochemical tests, such as sugar fermentation, methyl red, voges proskauer, catalase paroducion, indole production, coagulase, etc.
4.2.2.2. Fungal analysis

Diagnosis can be established by microscopy and culture. Wet films or gram stained smears from lesions or exudates show budding gram positive cells. As *Candida* can be seen on normal skin or mucosa as well, only its abundant presence is of significance.

The laboratory diagnosis of fungal infections is made by microscopic examination of materials from the lesions and by morphological studies of fungus plates. Tissue specimens, such as skin scrapings, are generally examined as wet mounts after treatment with 10% potassium hydroxide. The alkali digests cells and other tissue materials, enabling the fungus elements to be seen clearly. The Periodic Acid Schiff (PAS) and methanamine silver stains are valuable methods for the demonstration of fungal elements in tissue sections.

4.2.3. Extraction Procedure

The *in vivo* grown plant parts like root and leaf and *in vitro* plant parts like callus, root and leaf were selected for this present study. These materials were washed and dried in shade and powdered.

**Soxhlet extraction**: Powders were placed in the Soxhlet extractor separately. Organic solvent ethanol was used to extract the bioactive compounds of the medicinal plants. By extraction of the bioactive principle, the samples were flushed two times with respective solvents. Then the organic solvents were added into the Soxhlet extraction at the ratio into each of the sample to extract antimicrobial compounds. The collected extracts were concentrated and stored at 4°C until further use.
500 g of plant powder + sufficient organic solvent

\[\downarrow\]

Soxhlet extraction for 24 hour

\[\downarrow\]

extract recovered

\[\downarrow\]

removal of solvent

(distillation using rotary evaporator)

\[\downarrow\]

crude extract

\[\downarrow\]

dried at vacuum pump

(to removed water, oil and impurities)

\[\downarrow\]

test for antimicrobial activities

4.2.4. **Antibacterial Study** - Disc Diffusion Method (Bauer *et al.*, 1966)

Antibacterial activity of the extracts of different parts was determined by disc diffusion method. Five different concentrations (100 µg, 200 µg, 300 µg, 400 µg and 500 µg) of each extract were prepared.
4.2.4.1. Preparation of discs

Different concentrations of discs were prepared using sterile discs prepared from Whatman filter paper No.1. The work was done in two phases based on the concentrations of plant extract.

In the first phase 10 µg, 20 µg, 30 µg, 40 µg and 50 µg concentrated discs were prepared by placing one drop of plant extract.

1 mg dissolved in 1 ml of DMSO = 10 µg
2 mg dissolved in 1 ml of DMSO = 20 µg
3 mg dissolved in 1 ml of DMSO = 30 µg
4 mg dissolved in 1 ml of DMSO = 40 µg
5 mg dissolved in 1 ml of DMSO = 50 µg

Using 23 G needle in which 1 ml delivers 100 drops, ie., 1 drop = 10 µg. This procedure was repeated for higher concentrations in the second stage. Then the discs were incubated at 37°C for 1 hour for drying. These prepared discs were used for antibacterial studies.

4.2.4.2. Antibacterial testing

The filter paper discs of 6 mm in diameter were charged with appropriate concentrations of drugs (plant extract). The discs were allowed to dry in the cold condition (at 37°C for 30 minutes). The overnight broth was swabbed using sterile cotton swab on sterile Muller Hinton agar plates. Then different concentrations of dried extract discs (five per 10 cm plate) were placed with sterile forceps on Muller Hinton agar plates and incubated at 37°C for 24 hours. After 24 hours of incubation the degree of sensitivity was determined by measuring the zones of inhibition of growth around the
discs. Growth will be inhibited around discs containing extract to which the bacterium is susceptible but not around those to which it is resistant.

The diameter of the zone of inhibition is influenced by a variety of factors such as diffusibility of drug, the disc concentrations, the nature and composition of the medium, its thickness, presence of inhibitory or stimulatory substance, pH and time of incubation.

4.2.5. Antifungal Study - Agar Well Diffusion Method (Verpoorte et al., 1982)

In this technique 1 ml of the fungal spore suspension (grown for 3 days) in 10 ml of Sabourad's Dextrose Agar (SDA) broth was thoroughly mixed with 20 ml of molten SDA and poured into sterile Petri plates. When the agar was set, 5 wells of 6 mm diameter were made on each of the seeded plate. These holes were filled with 100 μl of testing samples. All these experiments were performed in duplicate. The Petri plates were incubated at 28°C for 7 to 8 days.

4.3. Results

4.3.1. Antibacterial Activity

The bacterial cultures were examined for the antibacterial activity of chicory. Inhibition zones were not observed in the cultures in 24 hours, i.e., the extracts of deifferent plant parts of chicory did not possess the antibacterial activity against the bacteria tested (Fig.4.1a-e).

4.3.2. Antifungal Activity

The fungal cultures were examined for the antifungal activity. Within 3 days all the three fungi selected for this study grew well in the culture medium and were not inhibited by the different extracts of chicory (Fig.1f-h).
Fig. 4.1. Antimicrobial activities of Cichorium intybus. a-e. bacteria; a. Staphylococcus aureus; b. Pseudomonas aeruginosa; c. Klebsiella pneumoniae; d. Escherichia coli; e. Salmonella typhi; f-h. fungi; f. Aspergillus niger, g. Aspergillus flavus; h. Candida albicans. (R - in vivo root; L - in vivo leaf; c - callus; r - in vitro root; l - in vitro leaf)
4. Discussion

The early studies showed that chicory is medicinally important to cure many diseases and also possesses several bioactive compounds including antifungal compounds. These compounds were tested for antifungal activities against some phytopathogens in 1990, (Monde et al., 1990; Nishmura et al., 1999; Yusuf et al., 2002). Yusuf et al. (2002) reported that *C. intybus* inhibited the spore germination of two fungi out of 8 fungi tested. There was no report for human pathogenic fungi so far. In the present study also the extract of various plant parts of chicory does not show any inhibitory activity against human pathogenic bacteria and fungi.