5. DISCUSSION
5. DISCUSSION

One of the major problems of effective treatment of infectious diseases, is the emergence of drug resistance among microbial populations. The resistance may be mediated by chromosome or by plasmids and the plasmids may also be transferred to other bacterial populations besides its own progeny. Dissemination of plasmid mediated resistance to bacterial populations has, thus, further made bacterial strains resistant to many antibacterial drugs. There are now an increasing number of case reports documenting the development of clinical resistance to newer and broad spectrum antibacterial drugs like fluoroquinolones (Norflox, Ciproflox, Ofloxacin etc.) in gram-ve bacilli such as Pseudomonas sp. and Salmonella sp. as reported by Howard et al, [1990] and Piddock et al, [1993]. Therefore, the prevailing drug resistance in pathogenic microorganisms associated with the use of antibiotics as well as their undesirable side effects have triggered the immense interest in drugs of plant origin. Plants are known to produce bioactive compounds in their natural course of life cycle as a part of metabolic process related to their survival under biotic and abiotic stresses of environment. Among these bioactive molecules, the compounds with antimicrobial activity may have the potential of being developed as plant based antimicrobial drugs and preparations therapeutic agents against infectious diseases.

The history of drug discovery implies that the ethnobotanical approach is the most productive of the plant surveying methods and the recent findings including that of ours confirm this hypothesis. Now, it is imperative to evaluate these herbs by modern scientific techniques in order to have better understanding of the therapeutic, phytochemical and toxicological aspects of these herbal drugs. Such studies may lead to novel chemotherapeutic agents that would be more effective specially in the areas where the drug resistance is faced, leading to a better control of infectious diseases. In the light of above facts, the present investigations have been undertaken to determine the antimicrobial potential of Indian medicinal plants. While collecting ethnobotanical information from the literature, it was observed that a number of medicinal plants are used by traditional herbalist in different parts of India and world over, to treat different ailments. This forms the basis of selection of medicinal plants in our investigation.

The study was conducted against several microorganisms selected on the basis of their pathogenicity. Some of them are opportunistic and some of them are causing the respiratory disorders, eye diseases and diarrhoea leading to high mortality rate in infants. While others are causing dysentry, boils, abscesses, vaginitis and candidiasis
etc. For example, gram+ve bacteria (Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis), gram-ve strains (Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Proteus vulgaris), a pathogenic yeast (Candida albicans) and three dermatophytes (Microsporum gypseum, Trichophyton rubrum and Epidermophyton floccosum) are chosen. Screening for the antimicrobial activity of each plant extracts at a comparatively lower concentration of 200 mg/ml was done against all the above organisms so that the plant extracts with a low potency can be detected easily. The results are very encouraging as 56 alcoholic extracts, 13 aqueous extracts and five hexane extracts from 82 plants showed antimicrobial activity against one or more test microorganisms. Table 6 & 7 represents the summary of the antibacterial and antifungal activity of the various extracts respectively against individual test organisms and table 8 displayed activity profile of ethanolic plant extracts.

It is to be noted that out of thirteen aqueous extracts, eight extracts (Holarrhena antidysenterica, Calotropis procera, Terminalia belerica, Terminalia chebula, Emblica officinalis, Plumbago zeylanica, Punica granatum and Withania somnifera) inhibited the growth of gram+ve as well as gram-ve bacteria, however, the rest five extracts of Onosma bracteatum, Terminalia arjun, Eclipta alba, Acacia catechu and Aloe barbadensis are active only against gram+ve bacteria. Also, out of thirteen aqueous extracts, only Acorus calamus extract showed anticandidal as well as antidermatophytic activity while the rest displayed only anticandidal activity. It is interesting to note that Onosma bracteatum, Terminalia arjun, Terminalia belerica, Terminalia chebula, Plumbago zeylanica, Punica granatum and Withania somnifera extracts showed antibacterial as well as anticandidal activity whereas extracts of Centratherum anthelminticum, Saussurea lappa, Abrus precatorius, Acacia arabica and Centella asiatica demonstrated only anticandidal activity. The most susceptible organisms towards aqueous extracts were gram+ve Staphylococcus aureus, Staphylococcus epidermidis and Bacillus subtilis with each having the inhibition percent of 21 (table-9).

Out of 56 ethanolic extracts, five extracts namely Emblica officinalis, Terminalia chebula, Terminalia belerica, Plumbago zeylanica and Holarrhena antidysenterica showed very strong antibacterial and antifungal activity with zones of inhibition > 20 mm. Whereas ethanolic extracts of Eclipta alba, Tamarindus indica, Embelia ribes, Piper nigirm and Ferula narthex displayed strong antibacterial activity with inhibition zones of 19 - 20 mm in which Eclipta alba, Tamarindus indica and Ferula narthex also showed moderate antifungal activity. Berberis aristata, Saussurea lappa, Aegle marmelos and Curcuma amada showed only strong antifungal activity with zones of
inhibition in the range of 19-20 mm. While Acorus calamus, Terminalia arjuna, Centratherum anthemum, Ocimum sanctum, Abrus precatorius, Cassia angustifolia, Cassia alata, Rubia cordifolia and Curcuma longa showed strong antifungal and moderate antibacterial activity. Cinnamomum tamala, Acacia arabica, Acacia catechu, Punica granatum and Withania somnifera showed strong antibacterial and antifungal activity with zones of inhibition ranging in 10-20 mm. The most susceptible organisms were gram+ve Staphylococci with inhibition percent of 88.4 when compared with that of 78.8% of Bacillus subtilis, 48% of Escherichia coli and Salmonella typhimurium, 55.7% of Pseudomonas aeruginosa and 34.6% of Proteus vulgaris (table-9).

Out of five hexane extracts, only Piper nigrum extracts showed strong antibacterial activity against both gram+ve and gram-ve bacteria followed by the extracts of Elettaria cardamomum. Piper longum extract was active against Escherichia coli, Ferula narthex extract was active against Staphylococcus aureus as well as Staphylococcus epidermidis and Zingiber officinale extracts was active against Bacillus subtilis, Staphylococcus aureus and Staphylococcus epidermidis. None of the fungi were inhibited by any of the hexane extracts. The most susceptible organisms were Staphylococci with inhibition percent of 7.6, followed by Bacillus subtilis with that of 5.7% and Escherichia coli with that of 3.8% whereas Pseudomonas aeruginosa, Proteus vulgaris and Salmonella typhimurium were not inhibited by any of the hexane extracts (table-9). Similar reports on antibacterial and antifungal activity with varying degree in their potency were reported by several other workers [Mariam, 1947; Kurup, 1956; Ikram & Haq, 1980; Dey & Das, 1988; Gundidza, 1990; Venkatanarayana et al, 1992; Belachew Desta, 1993; Mehta et al, 1993].

The difference in the potency of extracts in different reports may be due to different methods adopted for screening. The extent of antimicrobial activity follows a decreasing rank order of alcohol > aqueous > hexane. Maximum number of plant extracts showed activity in alcoholic extracts as compared to aqueous and hexane extracts. This suggests that ethanol would be a better solvent in an attempt to isolate the antibacterial as well as antifungal principles than aqueous and hexane. Similar findings in ethanolic extracts were also reported by Mehta et al, [1993] on antibacterial activity of ethanolic extracts of Emblica officinalis, Terminalia chebula and Terminalia belerica against Staphylococcus aureus, Salmonella typhimurium, Vibrio cholerae and Klebsiella pneumoniae while n-hexane and benzene extracts did not show any activity against above mentioned organisms. As can be seen from the table-6, several plant extracts showed antibacterial activity specially against gram-ve bacteria. This fact is interesting
as generally gram-ve organisms, particularly *Pseudomonas aeruginosa*, are more resistant than gram+ve one [Panizzi *et al.*, 1993; Stickler & King, 1992]. On the basis of outstanding performance of five ethanolic extracts i.e. those of *Emblica officinalis*, *Terminalia chebula*, *Terminalia belerica*, *Plumbago zeylanica* and *Holarrhena antidysenterica* against test bacteria and fungi as evident from (table 6, 7, & 8), only these were selected for their MIC determination, phytochemical screening and cytotoxicity studies. The several formulations based on selected plants extracts, were prepared and subjected to MIC determination as well.

The minimum inhibitory concentration (MIC) studies of all the five alcoholic extracts indicates that *Emblica officinalis* showed the lowest MIC of 800 ng/ml, followed by *Terminalia chebula* 950 µg/ml, *Holarrhena antidysenterica* 1000 µg/ml, *Plumbago zeylanica* 3000 µg/ml, and *Terminalia belerica* 4000 µg/ml, against one or the other bacterial population (table 11 and figure 1 to figure 8). *Plumbago zeylanica* showed the lowest MIC of 4000 ng/ml, against *Candida albicans* followed by *Emblica officinalis* (7000 µg/ml) and *Terminalia belerica* (7000 µg/ml), *Holarrhena antidysenterica* (8000 µg/ml) and *Terminalia chebula* (9000 µg/ml). It seems that none of the authors have reported the minimum inhibitory concentrations of the crude plant extracts, chosen for this work.

The antimicrobial screening and MIC study indicate that gram+ve bacteria, particularly *Staphylococcus aureus* and *Staphylococcus epidermidis*, excepting *Bacillus subtilis*, are most sensitive and readily inhibited by plant extracts than gram-ve bacteria (*Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus vulgaris*). There is a morphological basis for the difference in susceptibilities. *Escherichia coli* and other gram-ve bacteria, excepting halobacteria, have an outer membrane composed mainly of lipopolysaccharide molecules, as suggested by the strong resistance of wild type strains to hydrophobic antibiotics. The outer membrane also acts as a selective barrier to hydrophilic molecules [Nikaido and Vaara, 1985] with an exclusion limit of around 600 Da for sugars and peptides [Decad and Nikaido, 1976 and Payne and Gilvarg, 1968]. Gram+ve bacteria lack this outer membrane but have a very much thicker peptidoglycan layer which is not as effective permeability barrier to hydrophilic solutes as its exclusion limit is approximate 10⁶ Da [Scherrer and Gerhardt, 1971]. *Bacillus subtilis* is not as sensitive as *Staphylococcus aureus* and *Staphylococcus epidermidis* because they have the ability to form highly resistant resting stages called spores and constitute some of the most resistant forms of life.

Amongst all the test organisms, *Candida albicans* is the most resistant form as
evident from table 11 and figure 8. The interesting feature of *Candida albicans* is its ability to switch colony morphologies. It switches heritably, reversibly and at a high frequency ($10^{-2}$ to $10^{-4}$) among at least seven colony phenotypes or between white and opaque colour phenotypes. Colony morphology switching may cause variation in susceptibility to drugs and hence in the pathogenicity [Datta, 1992].

Twenty five different types of formulations based on selected plant extracts were also used in this study. The reason behind the testing of the formulations was to remain faithful to the traditional system of medicine as in the Ayurvedic and Unani systems, the formulations are also prescribed for a variety of ailments. It was intended to further testify the efficacy of traditionally prescribed as well as new formulations of different combinations of five selected plant extracts in order to find out the potential combined effect (Synergistic effect). Out of 25 formulations, ten formulations showed good synergistic activity possibly due to interaction of two or more drugs which produce a combined effect than their separately measured individual effects against the microbial population, leading to a significant reduction in the MIC in comparison to the MICs of individual extracts.

The comparative evaluation of all the ten formulations indicates that formulation no. 11, 21 & 24 are the most effective against the pathogenic *Staphylococcus aureus* strain and showed synergistic effect leading to fairly reduced MIC of 400 $\mu$g/ml in comparison to the MICs of individual extracts. (table 12 figure 9). Similarly, against gram-ve *Escherichia coli*, the formulation no 1 & 22 showed a highly reduced MIC of 550 $\mu$g/ml compared to the MICs of formulation constituents (table 12 figure 10). Formulations no. 10, 21 & 23 found to be the most effective formulations against *Candida albicans* with reduced MICs of 900, 1000 and 1000 $\mu$g/ml respectively in comparison to the MICs of individual constituents as shown in table 12 figure 11. It is interesting to note that all the ten formulations showing strong synergistic effect, have *Emblica officinalis* as a common constituent. Further, the ethanolic extract of *Emblica officinalis* when tested separately showed the lowest MIC against bacterial population.

The extents of synergism as measured in terms of synergism index (%) varies from 116.67% to 112.53% in the ten possible formulations based on two plant extracts against the three test organisms viz. *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Not all the formulations show synergism leading to the reduction in MICs as evident from table-21. The positive values of synergism index is indicative of synergism whereas negative value indicates antagonistic effect. By careful examination of table-21 it may be inferred that the formulation based on EO&TB (formulation no. 2),
EO&HA (formulation no. 4) and PZ&HA (formulation no. 10) showed highest synergistic effect against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* respectively. Whereas formulations based on EO&PZ (formulation no.3) stood second in terms of synergism index against all three organisms. Whereas the formulations based on TC&PZ (formulation no. 6) showed strong antagonistic effect on the MIC and hence, least effective against *Staphylococcus aureus* and *Escherichia coli*. Whereas as TC&TB (formulation no. 5) showed strong antagonistic effect on MIC against *Candida albicans*. It is therefore, deduced that the most effective and economic formulations will be those against a particular microorganism which showed higher synergism index. Where as least effective and uneconomic formulations will be those of lower synergism index. The five ethanolic plant extracts and formulations, showing outstanding antimicrobial activity showed no cytotoxicity as evident from table 19 & 20.

**RESULTS VALIDATING USE**

Traditionally, the fruit extracts of *Emblica officinalis*, *Terminalia chebula*, and *Terminalia belerica* are applied for the treatment of sore throat, cough, respiratory disorders, skin diseases, boils and abscesses, eye disorders, diarrhoea, dysentry, vaginitis etc. as reported by [Lyenger, 1985, Chopra, 1992; Behl, 1993 and Bakhru, 1995]. Studies show that the plants are active against some of the organisms responsible for above infections. Therefore, there is already enough support for validation of use for preparations derived from *Emblica officinalis*, *Terminalia chebula* and *Terminalia belerica* against eye infections, boils, abscesses and other wound infections. All the three plants are broad spectrum antibacterials and are strogly inhibitory to the gram+ve *Staphylococcus aureus* which is a major cause of blepharitis, conjunctivitis, boils, wound infections and abscesses. These active plants could provide potential alternatives to the antibiotics. The results also justify the traditional use of these plants on infections involving *Staphylococcus aureus*. The plants also inhibit the gram-ve bacteria, *Escherichia coli*, *Salmonella typhimurium* which sometimes cause severe gastro-intestinal infections validating their use in diarrhoea, dysentry and stomachache as traditional medicine. Similarly, *Plumbago zeylanica* root extract which is used in open abscesses, other skin infections and diarrhoea suppresses established concentration of gram+ve (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and gram-ve (*Escherichia coli* and *Salmonella typhimurium*). This justifies the use of *Plumbago zeylanica* as a traditional medicine in above mentioned infection conditions. The situation is quite similar with *Holarrhena antidysenterica* traditionally used in various gastro-intestinal infections such as diarrhoea and dysentry. It also strongly inhibits the growth
of *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* justifying its use in many antidiarrhoeal preparations. *Pseudomonas aeruginosa*, one of the resistant bacteria to antibiotics amongst gram-ve population is a causative agent of a number of infections such as suppurative otitis, some localised infections of wounds and bedsores, eye infections and urinary tract infections, infections in burns and infantile diarrhoea [Ananthanarayan, 1990]. It is observed that all the five alcoholic plant extracts are very effective against *Pseudomonas aeruginosa*. The extracts may be explored to treat the infections caused by *Pseudomonas aeruginosa*. *Candida albicans*, which is associated with the pathogenesis of oral thrush (in children and elderly patients) provides some scientific rationale for the use of the extract as an oral wash and in the treatment of candidal oral thrush by some traditional herbalist [D'souza, 1993]. The studies on antimicrobial evaluation of formulations can further suggests the composition of most economic and most potent preparations for the treatment of particular infectious conditions.

**PHYTOCHEMICAL SCREENING OF ETHANOLIC EXTRACTS OF SELECTED PLANTS**

The phytochemical analysis of all the five ethanolic extracts of plants showed the presence of biologically active compounds such as phenolic compounds, tannins, glycosides in *Emblica officinalis*, *Terminalia chebula* and *Terminalia belerica*; saponins, and flavonoids in *Plumbago zeylanica*; steroids and alkaloids in *Holarrhena antidysenterica*.

*Emblica officinalis* fruits extracts which mainly contains tannins such as gallic acid, phyllembelin and ellagic acid. Phyllembelin has been identified as ethylester of gallic acid (3, 4, 5. trihydroxybenzoic acid) or as ethylgallate. These compounds are known to be associated with the antimicrobial activity of *Emblica officinalis* fruit extract as reported by [Khanna *et al*, 1973; Jain, 1974; Saxena *et al*, 1994]. The fruit extract of *Terminalia chebula* on phytochemical screening indicates the presence mainly of tannins such as chebulagic acid (ellagitannins), ellagic acid, gallic acid and syringic acid (3,5-methoxy-4 hydroxybenzoic acid) [Bhaumik *et al*, 1989]. The antimicrobial activity of *Terminalia chebula* may be attributed to be due to the presence of gallic and syringic acids [Marhuenda, 1987; Saxena *et al*, 1994]. Phytochemical analysis of *Terminalia belerica* showed the presence of tannins, glycosides and triterpenoids such as methylester of arjungenin, methylester of tomentosic acid, bellericagenin B, bellericaside B, belleric acid, arjunglucoside I and bellericoside. These are known to be associated with the antimicrobial property of the fruits of *Terminalia belerica* as reported by [Nandy
Phytochemical analysis of *Plumbago zeylanica* root extracts showed the presence of flavonoids and saponins, but it is also known to be containing a naphthoquinone (5-hydroxy-2 methyl-1,4-naphthoquinone) known as plumbagin which is associated with the antimicrobial property of *Plumbago zeylanica* [Krishnaswamy, 1980; Purushothaman, 1985; Hamsaveni, 1986]. Phytochemical analysis of alcoholic extracts of the bark of *Holarrhena antidysenterica* showed the presence of an alkaloids and steroids. The bark of *Holarrhena antidysenterica* contains a steroidal alkaloid as a major constituents known as conessine as an active constituent. Previous reports suggests that conessine is responsible for antimicrobial activity, especially antitubercular activity, as reported by [Chopra, 1980]. The presence of all the bioactive compounds those might be responsible for antimicrobial activities are supported by our UV and IR spectroscopic data as detailed in table 17 and figure 16 - 25. The structural formulae of some of the active compounds are given in table 18.

Phenolic compounds are widely distributed in the plant kingdom. These include simple phenols, coumarine flavonoids and complex phenols such as tannins [Singh, 1991]. The broad spectrum antibacterial and antifungal properties are attributed to the phenolic compounds [Clark, 1981; Simeray et al, 1982; Marhuenda, 1987; Iyer & Williamson, 1991; Gnanaguru et al, 1992]. Tannins have been shown to form irreversible complexes with proline rich proteins [Hagerman & Butler, 1981] which would result in the inhibition of cell wall and protein synthesis. This property may explain the mechanism of action of the plant extracts having tannins as a major constituent. Shah & Qadry [1993] suggested that tannins can be used as antiseptic on skin and mucous membrane, they precipitate the protiens leading to starvation of microorganisms as protein is not available in food. Tannins are used as healing agents in inflammation, leucorrhoea, gonorrhoea, piles, burns etc. Phenol group is responsible for the antiseptic activity. Tannins are also used in gastro-intestinal diseases like diarrhoea.