CHAPTER-5
DISCUSSION

Antibiotic resistance has increased substantially in recent years and is posing an ever-increasing therapeutic problem (Guillemot 1999; Austin et al., 1999). Traditional methods currently that are being used to overcome antimicrobial resistance include: a) identification of new drugs, b) chemical modification of the existing drug (for example, semisynthetic penicillins like methicillin, and flucloxacillin), c) Use of a combination of two or more antibiotics with different mechanisms of action (Beringer, 1999). The antimicrobial activities in plants have been shown by the secondary metabolites, mainly to protect them from microbes. Plant-derived chemicals (phytochemicals) can be used in combination with traditional antibiotics for enhancing the activity of antibiotics (synergism) and making them more potent at low dosage. These synergistic combinations represent a rich source of pharmaceutical products with novel and multiple mechanisms of action that can overcome microbial resistance.

The mode of action of antibiotics comprise of inhibition of crucial life sustaining processes in the microorganism i.e., synthesis of cell wall material, DNA, RNA, ribosome and proteins. Herbal medicine and plant-derived therapeutic products in various forms have been used since years for the treatment of diseases in both Eastern and Western cultures. Herbs have been used in traditional systems of medicine like Ayurveda, Sidha and Unani. In the traditional background of medicine, India needs to increase its share in the world market (Dubey et al., 2004). In the modern era the treatment of diseases have been done by different medicinal plant formulations alone and in combination therapy with antibiotics or other plant formulations. The main idea of synergism is to develop combination therapy to formulate less toxic drug with low dosage. The aim of synergistic/additive interaction is to enhance the activity or efficiency of plant extracts/phytocompounds in addition of some other plant extracts/phytocompounds. These interaction studies may play key role in providing some clue to further investigate such interacting extract for novel compounds. In case of combination of medicinal plant extracts, some of the plant extracts enhance the potency of the real effective plant and some can reduce the toxic effects of the main medicinal herb, making the herbal combination safe for the human use (Miaorong and Jing, 1996). Epigallocatechin gallate synergizes the activity of β-lactams against
MRSA (methicillin resistant *S. aureus*) by direct binding to cell wall’s peptidoglycan (Zhao *et al.*, 2006). Ahmad and Aqil (2007) observed that crude extracts of some Indian medicinal plants; *Acorus calamus*, *Hemidesmus indicus*, *Holarrhena antidysenterica* and *Plumbago zeylanica* exhibit synergistic interactions with tetracycline and ciprofloxacin against multidrug-resistant enteric bacteria that produce extended spectrum β-lactamase (ESβL). There are no reports about the compounds, which act as bioactivity enhancers or behave as synergistic (Hemaiswarya *et al.*, 2008; Falagas *et al.*, 2005; Watanakunakorn, 1971).

The extracts of medicinal plants of Solan and Shimla were evaluated for antibacterial/synergistic activity in combination with erythromycin. The preliminary phytochemical screening of plants listed in Table 4.2 showed the presence of phenols, coumarins, tannins, alkaloids, glycosides, sterols, flavanoids, terpenoids and saponins. From the list of 73 medicinal plants (Table 3.1), 17 plants were categorized under three classes of synergism as; 1) Class I- increased zone of inhibition; 2) Class II- zone of inhibition same but making drug bactericidal; 3) Class III- zone of inhibition was decreased in combination, but making drug bactericidal (Table 4.4). The plant extracts of *Aegle marmelos*, *Curcuma longa*, *Tinospora cordifolia*, *Cymbopogon citrates*, *Allium cepa*, *Zingiber officinalis*, *Cinnamomum tamala*, *Pinus roxburghii*, *Piper nigrum*, *Withania somnifera*, *Punica granatum*, *Lawsonia alba*, *Colebrookea oppositifolia*, *Zanthoxyllum armatum*, *Terminalia arjuna*, *Syzygium aromaticum* and *Musa paradisiaca* were categorized under these classes against *E. coli* and *S. aureus*. From the list of plants (Table 4.4), *C. oppositifolia* was selected for further studies in which extract of leaf, inflorescence and bark were analyzed for antimicrobial and synergistic effect. Among different parts of plant of *C. oppositifolia* leaf extract has shown the best synergism with amoxyclyave and erythromycin, as methanolic extract of leaf does not possess any antibacterial effect alone. The bioguided solvent fractionation of methanol extract was done in different polar/non polar solvents and screened for antibacterial/synergistic effect with amoxyclyave and erythromycin. In which n- butanol and ethyl acetate fractions showed the synergistic activity. The volatile oil (leaf) of *C. oppositifolia* have shown synergism with erythromycin and amoxyclyave against bacterial strains of *S. aureus*, *K. pneumonia*, *S. pyrogenes*, *S. enterica*, *S. epidermidis*. The volatile oil (inflorescence) of *C. oppositifolia* has shown synergism against *S. pyrogenes*, *S. enterica* and *S. epidermidis* with erythromycin and amoxyclyave.
5.1 PHYTOCHEMICAL SCREEING AND ANTIBACTERIAL EFFECT

The medicinal plants of Solan and Shimla were studied for the phytochemical and antibacterial/synergistic activity. All plants listed in Table 4.4 have shown synergistic activity against *S. aureus* and *E. coli*. The extracts of *P. granatum* (flower, leaves) have shown the presence of all phytochemicals listed in Table 4.2, except phenol which was not detected in flower extract. The antibacterial activity was present in both parts of *P. granatum* against *E. coli* and *S. aureus*. The phytochemical, antimicrobial activity (Ahmad and Beg 2001), antiproliferative, anticancerous activities (Seeram *et al.*, 2005; Lansky and Newman 2007) and toxic effects (Vidal *et al.*, 2003) are well documented in *P. granatum*. In our observation *T. arjuna* (bark) have shown the absence of coumarins, sterols, glycosides, whereas Mandal *et al.* (2013) has documented the presence of phytosterol, lactones, flavonoids, phenolic, tannins and glycosides in *T. arjuna*. The anticancerous (Kandil and Nassar, 1998), pharmacognostical, phytochemical, pharmacological and clinical studies (Dwivedi and Udupa, 1989) are known in *T. arjuna* (bark). The protective effect of *T. arjuna* (bark) against cardiotoxicity (Singh, *et al.*, 2008) and antimicrobial activity (Perumal Samy *et al.*, 1998; Rani and Khullar, 2004) has been reported. *C. longa* (tuber) is known for anticancerous (Kuttan *et al.*, 1985), anti-inflammatory (Chainani-Wu, 2003), anti-HIV, anti-bacteria, antioxidant effects and nematocidal activity (Araujo and Leon, 2001; Ramsewak *et al.*, 2000). We observed the absence of phenol and terpenoids, whereas by Kumar *et al.* (2010) and Chatterjee *et al.* (2000) phenols and terpenoids are present in different parts of *C. longa*.

*M. fragrans* (aril, fruit) extracts was observed to be antibacterial against *E. coli* and *S. aureus*. *M. fragrans* (fruit) has shown the presence of coumarins, alkaloids, terpenoids and glycosides whereas the presence of flavanoids was also observed by Olaleye *et al.* (2006) in Nigerian plant samples. *M. fragrans* oil is reported for its broad spectrum antimicrobial activity against 25 different genera of bacteria including animal and plant pathogens, food poisoning and spoilage bacteria (Dorman and Deans, 2000). *A. cepa* (bulb) was also observed to be antibacterial against various pathogenic bacteria, documented by various research groups (Benkeblia, 2004; Hughes and Lawson, 1991; Abdou *et al.*, 1972; Dankert *et al.*, 1979). The absence of phenol, coumarins, alkaloids and terpenoids in ethanolic extract of bulb of *A. cepa*
were observed, whereas alkaloids have been reported in *A. cepa* by Ugwoke and Ezugwe (2010). In *T. cordifolia* (stem), we confirmed the presence of all phytocompounds listed in Table 4.2, which has been already reported in literature (Kavitha *et al*., 2011). The antibacterial activity of stem extract of *T. cordifolia* was observed against *E. coli* and *S. aureus*, which was reported by various research groups (Samy, 2005; Singh *et al*., 2010; Perumal Samy and Ignacimuthu, 2000). We observed the absence of phenol in *C. citrates* (oil) but in reports, the phenol was observed in leaf extract of this plant (Asaolu *et al*., 2009). The absence of terpenoids, saponins were observed, whereas Rajan *et al* (2011) has shown the presence of these phytoconstituents in *A. marmelos* fruit pulp. The antidiabetic, antihyperlipidaemic antioxidant properties were also reported in *A. marmelos* (Kamalakkannan *et al*., 2005). The antimicrobial activity of *A. marmelos* has been documented by many research groups against different microbes (Balakumar *et al*., 2011; Raja *et al*., 2009; Rajan *et al*., 2011). We also observed the antibacterial activity in this plant against *E. coli* and *S. aureus*.

In present study the antibacterial activity was detected in tuber extract of *Z. officinale*, *P. roxburghii* (leaves), *P. nigrum* (fruit), *W. somnifera* (leaves), *P. granatum* (flower) and *L. alba* (leaves) against *E. coli* and *S. aureus*. All these plants have been reported antimicrobial against five dermatophytes (*Trichophyton mentagrophytes, T. rubrum, Microsporum canis, M. nanum and Epidermophyton floccosum*) and three filamentous fungi (*Aspergillus niger, A. fumigatus and Mucor sp.*) and five strains of yeast (*Saccharomyces cerevisiae, Cryptococcus neoformans, Candida albicans, Ca. tropicalis and Torulopsis glabrata*) by Jantan *et al*., 2003. Similarly Chen *et al.* (2008) have reported five genus of Zingiberaceae plants of Taiwan, antibacterial against various food pathogens. Parihar *et al.* (2006) has reported antibacterial activity in leaves extracts of *P. roxburghii* against *A. tumefaciens* and *S. typhi*. Similarly the antimicrobial activity of *P. granatum* is well documented (Duraipandiyann *et al*., 2004; Holetz *et al*., 2002). The root and leaf extract of *W. somnifera* is reported for antibacterial activity against *E. coli* and *S. typi* (Arora *et al*., 2004). The anticandidal activity was observed in *L. alba* plant extract by Holetz *et al.* (2002). The volatile oil of *P. nigrum, S. aromaticum, M. fragrans* and *T. vulgaris* against 25 different genera of bacteria is reported by Dorman and Deans (2008).

The preliminary phytochemical screening of *Z. officinale* (tuber) and *P. roxburghii* (leaves) showed the presence phenols, flavonoids, alkaloids, glycosides, saponins, steroids,
terpenoids and tannin (Table 4.2) phytochemicals except terpenoids which were found absent in *Z. officinale* (tuber). If we compare this data with report by Kumar *et al.* (2011), the *Z. officinale* posses essential oils, phenolic compounds, flavonoids, carbohydrates, proteins, alkaloids, glycosides, saponins, steroids, terpenoids and tannin. *Z. officinale* was reported to be antibacterial against *Porphyromonas gingivalis*, *Porphyromonas endodontalis* and *Prevotella intermedia* (Park *et al.*, 2008). In the ethanolic extract of *P. nigrum* (fruit) the presence of phenols, coumarins, tannins, alkaloids, glycosides, sterols, flavanoids, saponins and absence of terpenoids was observed. According to Nahak and Sahu (2011), terpenoids were found to be present in *P. nigrum*. *Z. armatum* is reported antibacterial (Srivastava *et al.*, 2013), anthelmintic (Mehta *et al.*, 2012), antifungal, antilarvicidal, hepatoprotective and insecticidal (Singh and Singh, 2011). The phytochemicals reported in *Z. armatum* were sterols, triterpenes, volatile oils, coumarins, alkaloids, flavanoids, flavonic glycosides, saponins, tannins but in our study the extracts of *Z. armatum*, sterols were not detected.

The phytochemical screening of *C. oppositifolia* showed the presence of all the phyto-constituents like tannin, phenol, flavanoid, coumarin, alkaloid, terpenoid, saponin, sterol and glycosides in leaves and bark extract, whereas sterols were not detected in the inflorescence extract. The inflorescence extract of *C. oppositifolia* showed the absence of sterols, whereas in leaf and stem extract sterols were present. Mahadhvan *et al.*, (2011) have documented the presence of phenols, tannins, flavanoids, saponins, proteins and amino acids in the different polarity solvent extract. Similarly, Mahapatra *et al.*, (2013) isolated flavonoid from ethyl acetate fraction of methanolic extract of leaves of *C. oppositifolia*, exhibited distinct antimicrobial activity when tested against 185 bacteria belonging to both Gram positive and Gram negative.

### 5.2 SYNERGISTIC EFFECT BETWEEN EXTRACTS OF MEDICINAL PLANTS AND ANTIBIOTICS

In this section of discussion the synergistic effect of traditional medicinal plants of Solan and Shimla was discussed in combination with erythromycin, amoxyclave against *S. aureus* and *E. coli*. *C. citrutes* (oil) and *Z. officinale* (rhizome) has shown class I synergism against *S. aureus* and *E. coli* with erythromycin. The class I synergistic effect was observed by *S. aromaticum* (flower bud) against *E. coli* with erythromycin. Also class II synergism with erythromycin was
observed by this plant against *S. aureus*. The synergistic effect of *C. citrates*, *S. aromaticum* and *Z. officinale* was reported for various antimicrobial drugs by Betoni et al. (2006) and Satyan et al. (2011). *T. cordifolia* (stem) has shown the class I synergism against *S. aureus* and *E. coli*, whereas in *A. marmelos* (fruit) has shown class I synergism against *S. aureus*. There is no report on synergistic activity of *A. marmelos* in literature, whereas the synergistic effect of leaf extracts of *Tinospora cordifolia* with nalidixic acid was observed against *K. pneumoniae* and *B. cereus* (Ranjan et al., 2012). The class I synergism was shown by flower extract of *P. granatum* against *E. coli* and *S. aureus* with erythromycin. The compound extracted from *Punica granatum* in combination with fluconazole showed a synergistic interaction against *Candida albicans* and *Candida parapsilosis* (Endo et al., 2010). Also synergistic interaction between *Punica granatum* extract and antibiotics (chloramphenicol, gentamicin, ampicillin, tetracycline and oxacillin) against 30 clinical isolates of methicillin-resistant and sensitive *Staphylococcus aureus* was reported by Braga et al. (2005). In present study, the class I synergistic activity was also observed in *C. longa* (rhizome) against *S. aureus* and *E. coli*. In previous study *C. longa* extract was reported synergistic to clotrimoxazole, ampicillin-A, cloxacillin, chloramphenicol (Sagdic et al., 2005), which reports, no synergism was observed in the *C. longa* extract (Satyan et al., 2011) against resistant pathogens. The class III synergism was observed in *T. arjuna* (bark) against *S. aureus* but not against *E. coli*. The synergistic effect of *T. arjuna* was reported by Pathania et al. (2013) against various multidrug resistant bacteria. *A. cepa* (bulb), *M. fragrans* (fruit) and *C. tamala* (bark) have shown the class I synergism against *S. aureus* and *E. coli*. The synergistic effect of *A. cepa* (oil) with ketoconazole against *Trichophyton* species have been studied by Pyun and Shin (2006).

The class I synergism was also shown by extract of *Z. officinale* (rhizome), *P. roxburghii* (leaves), *P. nigrum* (fruit), *W. somnifera* (leaves), *P. granatum* (flower) and *L. alba* (leaves) against *E. coli* and *S. aureus* except *P. roxburxyhii* (leaves) in which it was observed only against *E. coli*. The eugenol essential oil exhibited the synergistic effect with fluconazole and amphotericin B against various MDR strains of *C. albicans* (Khan et al., 2012). The *S. aromaticum* oil and its active compound eugenol have shown synergy with fluconazole against *A. fumigatus* and *T. rubrum* (Khan and Ahmad, 2011). The essential oils extracted from *Z. officinale* and *M. fragrans* with nisin (bacteriocin of *Lactococcus lactis* subsp), showed synergistic effect against *Listeria monocytogenes* (Rahnama et al., 2012). Arora et al. (2004)
showed synergistic effect of *W. somnifera* extracts and Das *et al.* (2012) have shown synergism for *L. alba*. The piperine isolated from *P. nigrum*, in combination with rifampicin and isoniazid has shown strong synergism against *M. tuberculosi*s, the new drug formulation against tuberculosis and was named as ‘resorine’ which contains reduced dose (200mg) of rifampicin + isoniazid (300mg) + piperine (10mg) (Chawla, 2010). Class II synergism was observed in *Z. armatum* (leaves) against *S. aureus* and *E. coli*. No synergism was observed in *M. fragrans* (mace) extract in combination with erythromycin.

The extracts, solvent fractions and volatile oil of *C. oppositifolia* have shown the potent synergism with erythromycin, amoxyclyclae against *S. aureus* and *E. coli*. The volatile oils (inflorescence and leaves) extracted from *C. oppositifolia* have not shown antibacterial activity but surprisingly enhances the antibacterial activity of erythromycin and amoxyclyclae. The volatile oil (leaf) of *C. oppositifolia* have shown synergism with erythromycin and amoxyclyclae against bacterial strains of *S. aureus*, *K. pneumonia*, *S. pyrogenes*, *S. enterica*, *S. epidermidis*. The volatile oil (inflorescence) of *C. oppositifolia* has shown synergism against *S. pyrogenes*, *S. enterica* and *S. epidermidis* with erythromycin and amoxyclyclae. Class II synergism was observed by leaf oil of *C. oppositifolia* against *S. aureus*, inflorescence oil of *C. oppositifolia* against *S. aureus* and *S. sonnei*, making erythromycin bactericidal in combination. The bioguided fractionation of methanolic extract of *C. oppositifolia* was done by polar/ non polar solvents. The solvent fractions (n- butanol, ethyl acetate) of methanolic leaf extract of *C. oppositifolia* showed the class I synergism with amoxyclyclae and erythromycin against *S. aureus*. The ethyl acetate and n- butanol fractions have not shown antibacterial activity alone. The solvent fractions have not shown class I synergism against *E. coli*. Class II synergism was shown by n- butanol and ethyl acetate fraction, in which both of these fractions enhances the potency of erythromycin by making it bactericidal. Further analysis of volatile oil by GCMS showed the presence of α- pinene (23.85), caryophyllene (11.312) and δ- carene (13.642) as the major terpenoids. The synergistic interactions were observed in combination of α- pinene and δ- carene against *S. aureus* and *E. coli*, whereas no class-I synergistic effect was observed in δ- carene, α- pinene, caryophyllene in combination with erythromycin and amoxyclae against *E. coli* and *S. aureus*. Class II synergistic effect was observed by δ- carene in combination with erythromycin, as the combination mixture was observed bactericidal against *E. coli* and *S. aureus*, whereas erythromycin was bacteriostatic against these pathogens. Therefore it is believed that terpenoids
In the present study the synergistic effect of *C. oppositifolia* (solvent fractions and volatile oil) with erythromycin and amoxyclylave was first time reported. Previously, Ali *et al.* (2011) have isolated the acteoside from *C. oppositifolia* which alone was not antifungal but enhanced the activity of amphotericin B against *Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigates*.

The various research groups are working on the synergistic effect of medicinal plants, their compounds and antibiotics, all over the world. The synergistic effect between pine oil, tetrasodium EDTA and sodium xylene sulfonate, against both Gram positive and Gram negative bacteria was observed by Spaulding *et al.* (1992). Ethanolic leaf extract of *Vangueria spinosa* in combination with doxycycline and ofloxacin was reported as synergistic against *S. aureus, E. coli, K. pneumonia* and *P. aeruginosa* (Chatterjee *et al.*, 2006). The extracts of *Psidium guajava, Rosmarinus officinalis, Salvia fruticosa, Majorana syriaca, Ocimum basilicum, Syzygium aromaticum, Laurus nobilis* and *Rosa damascena* were observed to be enhancing the activity of antimicrobial agents of different mechanisms (Adwan and Mhanna, 2008). Betoni *et al.* (2006) observed synergism in *Rhus coriaria, Psidium guajava, Lawsonia inermis, and Sacropoterium spinosum* with oxytetracyclin HCl, enrofloxaclin, gentamicin sulphate and sulphadimethoxin against *Staphylococcus aureus*. Some formulations are based upon the complete herbal combinations (Schulz *et al.*, 2001). Synergistic interaction was observed in combinations of extracts of *D. regia + Cichorium intybus, Acorus calamus + Holarrhena antidysenterica, Hemidesmus indicus + Acorus calamus* and *Plumbago zeylanica + Terminalia chebula* (Ahmad and Aqil, 2007).

**5.3 INTERACTION BETWEEN VOLATILE OIL/ TERPENOIDS AND THEIR COMBINATIONS WITH ANTIBIOTICS BY BIOPHYSICAL METHODS**

From the FTIR spectra of volatile leaf oil of *C. oppositifolia* and amoxicillin combination showed the function group change at 1599 and 1406 cm\(^{-1}\). Also the peak at 2259 cm\(^{-1}\) of leaf oil was disappeared in combination mixture with erythromycin. But if we compare our results of FTIR with HPLC of volatile oil (leaf, inflorescence) with antibiotics (erythromycin, amoxicillin/ amoxyclylave), no changes in retention times of their individual peaks were observed. Similarly UV- visible spectrometry and NMR studies proved that no complex formation was present but
they might be interacting with each other to enhance the activity of each other. The FTIR study of combination mixtures of volatile oils (α-pinene, δ-carene and caryophyllene) and antibiotics (erythromycin and amoxicillin/amoxyclave) was evaluated. The peak shift of amoxicillin was observed at 1599, 1774 and 2259 cm\(^{-1}\) in the combination mixture of α-pinene and amoxicillin. The peak shift was also observed at 1715 cm\(^{-1}\) in the combination mixture of α-pinene and erythromycin. Similarly peak modification was found at 1599 and 1774 cm\(^{-1}\) in the combination of caryophyllene and amoxicillin. The new peak appearance was observed at 1648 and 1248 cm\(^{-1}\) in δ-carene and erythromycin. Disappearance of peak was observed at 1599 and 1774 cm\(^{-1}\) in combination of caryophyllene and amoxicillin. The appearance and disappearance of peaks in the combination mixtures of antibiotics (erythromycin, amoxicillin) and terpenoids (α-pinene, δ-carene and caryophyllene) showed the modification of the functional groups present within them. The modifications of functional groups of these combinations could be the possible reason of synergism. The FTIR results were proved by UV-visible spectrometry as the change in the absorption spectra was observed in combination mixtures of antibiotics (erythromycin, amoxicillin) and terpenoids (α-pinene, δ-carene and caryophyllene), which shows some interaction among them but from NMR spectra we did not find any complex formation in these combination. HPLC chromatogram showed the disappearance of peak of erythromycin in the combination mixture of δ-carene and erythromycin combination at 2.8 min. For the justification of our results we have done in silico study to analyze the possible interaction between the terpenoids (α-pinene, δ-carene and caryophyllene) and antibiotics (erythromycin, amoxicillin). From docking study we observed best interaction among δ-carene+ amoxicillin and α-pinene+ erythromycin combination. So from the results it is concluded that due to some interactions may be present in these combinations, which could be the possible reason for synergism.

The synergistic effect may be due to complex formation of phytocompounds/extracts with antibiotics which leads to the more effective for particular species of microorganisms by inhibiting the crucial life sustaining processes and causing its lyses or death. Synergistic study in herbal medicine may lead to improved traditional formulations. It is the challenge to formulate new improved drugs with globally demanded drugs of Indian medicinal plants so that India can lead the world market of herbal drugs.