Chapter 3

Therapeutic studies of plumbagin: Antibacterial assay on MDR bacteria and anticancer assay on oral cancer cell lines
3.1 Review of literature

3.1.1 Therapeutic importance of medicinal plants

For thousands of years plants have been utilized as medicines (Samuelsson, 2004). Medicinal plants are the plants which contain the bioactive ingredients used to cure disease or relieve pain and these therapeutic properties are explored from a very long back (Okigbo et al., 2008, Himani et al., 2014). The medicinal properties of plants are mainly based on the phytochemicals which exhibits activities like, antioxidant, antimicrobial, antipyretic, anticancer, antifertility (Okigbo et al., 2008; Adesokan et al., 2008). Natural products derived directly from plants and many of its derivates are playing an important role as New Chemical Entities (NCEs) (Newman et al., 2003). Such medicinal plant originated NCEs are not only playing role as new drugs themselves but also acts as lead molecule for further optimization of new synthetic and combinatorial drug (Balunas and Kinghorn, 2005). By virtue of the sequencing of the human genome, numerous molecular targets have been identified which are crucial in various diseases (Richard & Dalia, 2004). Using high through-put screening assays directed towards these targets, known phytochemicals from medicinal plants can show selective and promising activity. Correlation between the secondary metabolites and their desired therapeutic activity should be known for the synthesis of new drugs to cure various diseases (Pandey et al., 2013).

Many of the traditionally used compounds isolated from medicinal plants were reported for showing the activity on new molecular targets. For example, indirubin which selectively inhibits cyclin-dependent kinases (Eisenbrand et al., 2004) and kamebakaurin, also inhibit NF-κB (Lee et al., 2002). Similarly, other known plant based drugs have shown activity on novel molecular targets, which has increased the interest in these familiar plant based compounds.

3.1.2 Examples of medicinal plant based therapeutics

Some of the early drugs used in therapeutics, isolated from medicinal plants were digitoxin, codeine, cocaine, quinine, and morphine. Few of these are used as drugs till today (Newman et al., 2000; Samuelsson, 2004). Plants contain a variety of phytochemicals with antimicrobial properties such as tannins, alkaloids and flavonoids, phenolic compounds, quinines, terpenoids etc. Some of the examples of plant based medicine used in pharmaceutical market are as follows. Galantamine, popular with trade name Reminyl is a phytochemical isolated from Galanthus woronowii is approved for the
treatment of Alzheimer’s disease. It binds and modulates nicotinic acetylcholine receptor (nAChR) and inhibits acetylcholinesterase (AChE) and thus slows down the process of neurological degeneration (Pirttila et al., 2004, Howes and Perry, 2011). Nitisinone, popular by its trade name Orfadin, is medicinal plant-based drug acting on hereditary disease tyrosinaemia (Frantz and Smith, 2003). Nitisinone is a modification of mesotrione which is a constituent of Callistemon citrinus (Myrtaceae). It mainly inhibits the enzyme 4-hydroxyphenylpyruvate dehydrogenase (HPPD) in humans which help in preventing tyrosine catabolism and thus avoid accumulation of toxic compounds in the liver and kidneys (Hall et al., 2001). Tiotropium (trade name Spiriva) is known for treatment of chronic obstructive pulmonary disease (COPD). Tiotropium is based on ipratropium, which is a derivative of atropine that has been isolated from Atropa belladonna L. and other members of the Solanaceae family (Dewick, 2002). It has been reported that Tiotropium showed more efficacy and long lasting effects than other available medications for COPD (Barnes, 2002). Calanolide A is a dipyranocoumarin natural product isolated from Calophyllum lanigerum var. Austrocoriaceum is an anti-HIV drug. It acts as a non-nucleoside reverse transcriptase inhibitor (NNRTI) of type-1 HIV and is effective against AZT-resistant strains of HIV (Buckheit et al., 1999; Yu et al., 2003).

3.1.3 Therapeutic properties of plumbagin

3.1.3.1 Traditional uses of Plumbago

The traditional Ayurvedic and Sidda medicine describes various medicinal properties of Plumbago and Plumbago was widely used in various Ayurvedic formulations. The roots form an important component of more than thirty ayurvedic formulations e.g. Kumaryasaway, Pippalyasaway, Yogarajaguggulu, Mahamasha massage oil, Hinguvachadi, Indukanta etc. (Durgaret et al., 1990; Binoy et al., 2014). Plumbago was used in India against fever, malaria, diarrhoea, dyspepsia, piles, and skin diseases including leprotic lesions (Gupta et al., 1993; Ankita & Nimali, 2015). ‘Amrita Bindu’ which is a salt-spice-herbal mixture was used as an antioxidant in India (Natarajan et al., 2006). In Taiwanese traditional medicine, Plumbago was reported for its use in anti-Helicobacter activity. In Nepal Plumbago was used as an antiviral medicine and also used as antifertility agent to control birth (Tiwari et al., 1982). Its use in South-Western Nigerian folk medicine against parasitic diseases, scabies and ulcers is reported (Gupta et al., 1993; Chaudhari and Chaudhari, 2015). In Java, the root is used in the veterinary medicine for expelling worms from horses.
Plumbago is widely used not only in Ayurveda, Siddha, Unani and Homeopathy but also in uncodified ethnic preparations of the rural folks (Timbadiya et al., 2015; Pandey et al., 2007). Roots of the plants are reported to possess many ethno-botanical uses such as treating oedema, piles, intestinal worms, skin disease, common wart, rheumatism, secondary syphilis etc. (Gebre-Mariam et al.; 2006, Giday et al., 2006). In Developing countries like India, China, and also other Asian countries, different parts of plants have been traditionally used in folk medicines for the treatment of rheumatoid arthritis, dysmenorrhoea and cancer. It was also used to treat a range of ailments including wounds, broken bones, and treatment of warts. A decoction of the aerial parts or roots of Plumbago auriculata is taken to treat black water fever. To get early healing, roasted and powdered roots are used into scarification over fractures and rubbed on the body to cure stitches. Some of the tribes in Satpuda forest region of Dhule and Jalgaon district of Maharashtra like Pawara Bhil and Pardhi tribes are known for consuming the root juice (Jain et al., 2010).

3.1.3.2 Uses of plumbagin in different therapeutics

3.1.3.2.1 Anti-inflammatory activity

Anti-inflammatory activity of Plumbago zeylanica and two other medicinal plants was checked in models of inflammation (Dang et al., 2011) and decrease in the protein concentration of the peritoneal exudates was reported. In another study, the inhibition of T cell proliferation by plumbagin was accompanied by a decrease in the levels of Con A (Concanavalin A) induced IL-2, IL-4, IL-6 and IFN-γ cytokines (Checker et al., 2009). This group had also postulated the immunomodulatory effects of plumbagin. For example, the compound inhibited T-cell proliferation in response to polyclonal mitogen Concanavalin A (Con A) by blocking cell cycle progression. It also suppressed expression of early and late activation markers CD69 and CD25, respectively, in activated T-cells. Plumbagin was found to exhibit therapeutic potential for its anti-allergic and anti-inflammatory effects for the first time in human PBMC (Peripheral blood mononuclear cells). Some of the allergen-specific immune responses like lymphocyte proliferation and cytokine secretion were studied in vitro using PMBC isolated from both allergic and non-allergic individuals. Plumbagin modulated levels of intracellular reactive oxygen species and glutathione and suppressed PHA induced activation of NF-κB in human PBMC (Kohli et al., 2011).
3.1.3.2.2 Anticancer activity

In cancer one or more cells the control of growth is lost which leads to either tumour formation (a solid mass of cells) cancer. It is one of the most dreadful diseases worldwide. Although extensive research is going on in this field, side effects of chemotherapy and radiotherapy etc are major concern in the treatment.

Plumbagin exhibited anticancer activity against many types of cancer including breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, myeloma, lung cancer, skin carcinoma, liver cancer, renal cancer, cervical cancer etc. (Padhye et al., 2010).

Plumbagin showed anticancer activity in human osteosarcoma (Yan et al., 2015). Melanoma cell lines (Bowes cell) and breast cancer cells line (MCF-7) showed response with IC$_{50}$ values of 1.39 and 1.28 µMol, respectively (Nguyen et al., 2004). In breast cancer studies, mechanism of action of plumbagin was inhibition of cell proliferation inducing cells to undergo G2-M arrest and autophagic cell death and reduced Cdc2 function (Kuo et al., 2006). Gomathinayagam and co-workers evaluated the anticancer effects of plumbagin against H460 and A549 NSCLC cancer cells and found that, plumbagin significantly inhibited the growth of H460 cells (Gomathinayagam et al., 2008). Plumbagin was found to down regulate signalling through EGFR in these cells. Furthermore, Kai-Hong Xu and his group examined in vivo effect of plumbagin and the results indicated that plumbagin injection for 3 weeks resulted to a 64.49% reduction of tumour volume compared with the control (Xu and Lu, 2010).

3.1.3.2.3 Antibacterial activity

Antimicrobial potential of plumbagin was reported against bacteria Staphylococcus aureus and fungus Candida albicans (Nair et al., 2016). Antibacterial activity of plumbagin is studied on number of pathogenic bacterial strains by group of researchers. A strong antibacterial activity of plumbagin against wild type E. coli strain AB1157 was reported with 99.9% got killed by plumbagin treatment. It was postulated that the antibacterial mechanism of plumbagin may be due to its toxicity generated by active oxygen species and may damage DNA via other a pathway besides H$_2$O$_2$ (Farr et al., 1985). Kamal and his co-workers (1995) conducted in vivo anti - S. aureus test in female mice and showed that plumbagin stimulated the response on oxygen radical released by macrophages and at high dose it had direct inhibitory activity against S. aureus. Anti-Helicobacter pylori activity of plumbagin was evaluated by Park and co-workers2006. Antibacterial activity was checked by on eight different bacteriaincluding gram-negative
bacteria like *E. coli* (MTCC 1195), *S. typhi* (MTCC 733), *K. Pneumoniae* (MTCC 2405), *Serratia marcescens* (MTCC 2645), *P. vulgaris* (MTCC 1771) and *P. aeruginosa* (MTCC 2642) and gram-positive bacteria like *S. aureus* (MTCC 1430) and *B. cereus* (MTCC 1272) by Jeyachandran and his co-workers (2006).

### 3.1.3.2.4 Cardio-tonic action
Cardio-tonic action of plumbagin was reported (Panichayupakaranant, 2002). When studied on guinea-pig, plumbagin exhibited a triphasic ionotrophic response in papillary muscles. Whereas plumbagin did not cause any positive inotropy under anoxic conditions, and the positive inotropic effects were inhibited by oxidative phosphorylation uncouplers (Itoigawa et al., 1991).

### 3.1.3.2.5 Anti-fungal activity
Plumbagin also shows antifungal activity. *P. indica* extracts exhibited antifungal activity against *Candida albicans*, *Microsporum* sp., *Blastomyces dermatitides*, and *Trichophyton* sp. (Dibyajyoti S & Swati, 2012). It has been reported that, hydro-alcoholic extract of roots with 80% ethanol possess potent antifungal activity against *A. niger* and *C. Albicans* (Valsaraj et al., 1997). Plumbagin had been reported as the active compound against *C. albicans* with MIC values 0.78 (Paiva et al., 2003).

### 3.1.3.2.6 Anti-parasitic activity
Paiva and co-workers (2003) also studied *P. indica* roots extract for macrofilaricidal property against *Setaria digitata*, which is a filarial parasite of cattle. It was observed that motility was completely inhibited in concentration range between 0.02 and 0.05 mg/mL. Anti-parasitic activity along with other pharmacological properties of *Plumbago zylanica* was reported (Du et al., 2012).

### 3.1.3.2.7 Antimalarial activity
It has been reported that plumbagin showed anti-*plasmodium falciparum* activity by inhibiting succinate dehydrogenase (SDH), which is a *P. falciparum* enzyme. It also inhibited *in vitro* growth of *P. falciparum* with IC50 value of 0.27 mM (Paiva et al., 2003). Mosquito larvicidal activity against fourth instar larvae of *Aedes aegypti* was checked by Sreelatha and her co-worker (2010) for all the napthaquinone derivatives from *Plumbago capensis* and found comparable with that of rotenone. It has been observed that among the
tested compounds, isoshinanolone and plumbagin showed excellent toxicity (Sreelatha et al., 2010).

3.1.3.2.8 Immunosuppressive activity
Apart from above said activities, plumbagin also exhibits immunosuppressive activity, which is also very important to understand the overall therapeutic potential of plumbagin. Plant containing plumbagin were evaluated in the treatment of chronic immunologically-based diseases. McKallip and his co-workers (2010) conducted a test and found out that plumbagin has significant immunosuppressive properties which were mediated by generation of ROS, up regulation of Fas, and the induction of apoptosis. Splenocytes from C57BL/6 mice cultured in vitro in the presence of 0.5 μM or greater concentrations of plumbagin significantly reduced proliferative responses to mitogens, including anti-CD3 mAbs, concanavalin A (Con A), lipopolysaccharide (LPS) and staphylococcal enterotoxin B (SEB). Treatment with the ROS scavenger, N-acetylcysteine (NAC), prevented plumbagin induced apoptosis, suggesting a role of ROS in these apoptosis (Roberts and Paul, 2006). Apart from these, plumbagin also exhibits anti-fertility activity, anti-hyperglycaemic activity, antioxidant activity, mutagenic activity, anti-inflammatory activity etc, which increases the therapeutic demand of plumbagin.

3.1.4 Antibacterial activity of medicinal plants on multi drug resistant (MDR) bacterial strains
Survey on global disease burden reported that, infectious diseases are responsible for 41% health problems, non-infectious diseases 43% and injuries accounts for 16% of health problems (Noumedem et al., 2013). After the discovery of antibiotics it was believed in medical fraternity that infectious diseases would be completely eradicated (Rosina et al., 2009). But, because of immense and inappropriate overuse of antibiotics, bacteria have developed resistance leading to the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms (Harbottle et al., 2006). Repeated overuse of the same antibiotics leads to development of different mechanisms for antibiotic resistance inside the same strain and consequently, infectious diseases are threatening as one of the leading causes of morbidity worldwide. Due to this rapid and global spread of resistant clinical isolates, there is great demand of new antimicrobial agents. However, the past record of resistance to newly introduced antimicrobial agents suggests that even life
expectancy of these newly developed antimicrobial drugs will be short (Coates et al., 2002). For this reason, herbal products are the subject of researcher’s attention, to develop new leads for better drugs against MDR strains of various microorganisms (Braga et al., 2005). As the alternative antimicrobial drugs are urgently needed reconsideration of the therapeutic use of ancient medications, such as plants is having paramount importance (Mandal et al., 2010).

Plants synthesises many secondary metabolites for their protection against microbial pathogen. This property of plant is studied in great details through in vivo and in vitro studies. Researchers have reported that various plant based drugs showed positive response in targeted screening against MDR bacteria such as MRSA, VRE, M. tuberculosis, enteric bacteria and others (Aqil & Ahmad, 2007; Rios & Recio, 2005). It was reported that acetone and ethanol extracts from fifteen plants exhibited significant activity against pathogens causing urinary tract infection (UTI) (Sharma et al., 2009). Antimicrobial activity of ethanol extracts of several Indian medicinal plants was reported by Aqil and co-workers (2005) on clinical isolates of S. aureus including B-lactamase producing MRSA and methicillin-sensitive S. aureus (MSSA). Naim and Tariq (2006) reported that oregano oil extracted from Origanum vulgare showed antibacterial activity against methicillin-sensitive and methicillin-resistant Staphylococcus aureus. It was reported that, Salmonella typhimurium multi drug resistant strains exhibited sensitivity against of Thymus vulgaris extracts.

Plumbago exhibits lots of antibacterial activities. Plumbago zeylanica showed anti-Helicobacter pylori activity with advantages like greater convenience and reduced cost for the treatment of H. Pylori (Wang & Huang, 2005). Plumbagin had been reported as the active compound against S. aureus with MIC and MBC values of 1.56 and 25µg/mL respectively (Paiva et al., 2003). Jamieson and his co-workers (1994) conducted tests in wild-type strain and mutated strains of Saccharomyces cerevisiae. The results showed that the mutated strain was 100 times more sensitive to plumbagin than its parent. Additionally, it effectively caused cell cycle arrest through modulation of multiple factors involved in regulation of cell cycle, and induced apoptosis through activation of caspase-3.

However, antimicrobial activity of plumbagin is not extensively studied on multi drug resistant bacteria till the objectives of present work were approved (2012). In the present study, we have evaluated antimicrobial potential of Plumbago auriculata extracts on multi drug resistant bacteria including E. coli MDR, P. aeruginosa MDR, S. aureus MDR, Klebsiella pneumoniae MDR and Citrobacter MDR. Above mentioned bacteria are
important causes of hospital born infections. Patients after getting recovery from the main
disease may get prolonged illness due to such infections.

Significance of these bacteria in human pathogenesis can be explained as follows:

**S. aureus** is a gram positive bacterium from the family *Staphylococcaceae*. Staphylococcus infections may cause diseases either due to direct infection of *S. aureus* or due to the production of toxins. *Staphylococcus* can cause diseases like boils, impetigo, food poisoning, cellulitis, toxic shock syndrome, osteomyelitis, endocarditis, and it is a leading cause of community-acquired infections. It may lead to some lethal diseases such as pneumonia, meningitis, osteomyelitis, endocarditis and sepsis. Its infection sites are skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. *S. aureus* infection is main cause of postsurgical wound infections and is one of the five most common causes of hospital acquired infections (Yatin *et al.*, 2014). It has been reported that bloodstream infection (BSI) that is caused very often by *Staphylococcus aureus* can prolong hospitalization and increase hospitalization costs and risk of mortality (Das, 2016).

**E. coli** are gram negative bacteria from the family enterobacteriaceae. Although, most of the strains of *E. coli* are harmless and essential part of intestinal tract, some of them cause severe illness. *E. coli* is the predominant pathogen causing Urinary tract infection (UTI) (Sobel *et al.*, 2010). Some *E. coli* are pathogenic, causing diarrhoea, illness outside of the intestinal tract, urinary tract infections, respiratory illness, bloodstream infections etc. If UTI is caused by multi drug resistant (MDR) strain of *E. coli*, it may increases the expenditure of treatment, morbidity and death rate in developing countries like India (Al-jiffri *et al.*, 201).

**Pseudomonas aeruginosa** is type of gram negative bacteria from the family Pseudomonadaceae. It is the most prevalent cause of infections after burn injuries and outer ear infections known as otitis externa (Pane *et al.*, 2015). It may cause infections by developing colonies in medical devices e.g., catheters and spread by such contaminated equipment. *Pseudomonas* was reported as one of the cause for community acquired pneumonias (Kurai *et al.*, 2016) and may also be a common cause of "hot-tub rash", caused by lack of proper attention to water quality (Yu *et al.*, 2007). *P. aeruginosa* is a versatile pathogen with ability to cause various types of infections. It is reported by National Nosocomial Infections Surveillance system from 1986–2003 that *P. aeruginosa* is the second most infectious agent causing pneumonia (18.1%), the third
most infectious agent leading to urinary tract infection (16.3%) and the eighth most (3.4%) frequently isolated pathogenic microorganism from the bloodstream (Gaynes & Edwards, 2005). *P. aeruginosa* is considered as ideal “MDR pathogen” because of its ubiquity and it’s fundamentally developed antibiotic resistance mechanisms (Tartor & El-Naenaey, 2016).

*Klebsiella pneumoniae* is gram negative bacteria from the family Enterobacteriaceae. Generally, *Klebsiella* infects people with a weak immune system. Although it found in the normal flora of the mouth, skin, and intestines, *K. pneumoniae* can cause destruction to human lungs via inflammation and hemorrhage with cell death that sometimes produces thick, bloody, mucoid sputum (Chou et al., 2015). Outside the hospital it mainly causes pneumonia, as bronchopneumonia and also can cause bronchitis. Even after medications, it has a general death rate of about 50% and nearly 100% for alcoholic and bacteremia patients. *Klebsiella* had been reported as a cause of diarrhoea, thrombophlebitis, cholecystitis, bacteraemia, osteomyelitis, meningitis, wound infection, upper respiratory tract infection and septicaemia (Tarani et al., 2015).

*Citrobacter* is a gram negative bacterium from the family Enterobacteriaceae. *Citrobacter* occurs predominantly worldwide because it is a component of normal intestinal flora. It can cause infections of the urinary tract, may develops infant meningitis and sepsis. *Citrobacter* species have been described as the etiologic agents in cases of bacteraemia, meningitis, diarrhoea and brain abscess. *Citrobacter* can cause several infections including urinary tract infections, blood stream infections, sepsis in intra abdomen and pneumonia and other neonatal infection, such as meningitis, neonatal sepsis, joint infection or general bacteraemia (Ritu et al., 2015). *Citrobacter* infections are fatal sometimes with 33-48 % overall death rates (Pepperell et al., 2002). The most susceptible class for risk of infection are premature neonates and also debilitated or immunocompromised patients.

### 3.1.5 Oral cancer

Cancer is one of the most dreadful diseases and one of the most dominant causes of death in the 20th century and in 21st century, this rate further increasing worldwide (Dikshit et al., 2012). It is estimated that by 2020, there will be 15 million new cases of cancer and 10 million deaths will be due to cancer every year. Oral cancer includes cancer of the lip
and oral cavity (which includes gingival, buccal mucosa, hard palate, tongue and floor of mouth) and documented as the fifth most prevalent cancer worldwide (Subapriya et al., 2007). Thirugnana et al., (2013) reported that, there is a high prevalence of oral cancer in developing countries like India. Oral cancer is a major problem in the Indian subcontinent where it ranks among the top three types of cancer in the country (Elango et al 2006). Worldwide, every year there 300,000 cases of oral carcinoma and in India 20 cases of oral cancer per 100,000 populations are reported (Sambandham et al., 2013).

Tobacco and alcohol consumption are the main factors causing oral cancer. Smoking is the reason for 42% of deaths from oral cancer and heavy alcohol consumption accounts for 16% of the deaths. These percentages in developed countries are about 70% and 30%, respectively (Danaei et al., 2005). In Taiwan, India, and other neighbouring Asian countries the major factors causing oral carcinoma are smokeless tobacco products and betel quid with or without tobacco (Wen et al., 2010). Prevalently, infection occurs on tongue and floor of the mouth leading to formation of cancerous lesions. Pain is the most frequent symptom along with other symptoms like formation of painless patch in the mouth, development of painless lump in the mouth or neck, intricacy in chewing, swallowing, change in voice, blood in saliva, and tremendous weight loss.

Like other cancers, oral cancer patients are treated mainly with surgery and radiotherapy. Chemotherapy is also used to treat oral cancer, but it is usually in patients with metastasis (Wan et al., 2013). Although there is widely accepted leaning for the use of chemotherapy in combination with radiation therapy and surgery for patients with advanced oral cancer, limited evidence showing survival benefit to patients. In spite many developments combinatorial anti-cancer treatments, the 5-years survival rate is not more than 60% (Liu et al., 2012).

3.1.6 Plant based anticancer agents
Anticancer research is focussed towards the development of complementary and alternative medicine. Now a day’s researchers are interested in natural plant products with potential to prevent or cure the cancer without the side-effects which is major limitation seen with most other chemo-radio therapies. Several studies have been conducted on ethanomedicinal plants and herbs under a multitude of ethno botanical grounds which serve as a source of new leads for drugs without side effects. Hartwell has collected data for 3000 plants, which possess anticancer properties and subsequently can be used as potent anticancer drugs (Pandey, 2002). Several clinically useful anti-cancer agents are
plant products or their close derivatives like irinotecan, topotecan, etoposide, and paclitaxel (Cragg and Newman, 2005). Vinblastine and Vincristine isolated from *Catharanthus roseus* exhibits anticancer activity (Gueritte and Fahy, 2005). Camptothecin, a precursor to the semi-synthethetic drugs topotecan and irinotecan (Rahier et al., 2005) is isolated from, from the bark of *Camptotheca acuminata*. Huperzine A and galantamine (galanthamine) acting as acetylcholinesterase inhibitors have been approved for the treatment of Alzheimer’s disease and other neurodegenerative pathologies (Raves et al., 1997; Scott and Goa, 2000).

Anticancer activity of plumbagin on various cancers is well documented. As mentioned in section B3.1.3.2 plumbagin exhibits various biological activities and its effects on multiple signalling and apoptotic pathways. Plumbagin generate reactive oxygen species (ROS) which attributes for redox-cycling property of plumbagin which is very crucial in anticancer mechanism of plumbagin (Padhye et al., 2012). It has shown apoptosis in breast cancer cells lines (Ahmad et al., 2008). Various other mechanistic efforts ensured plumbagin as a potential anticancer agent against various types of cancers. It is documented that plumbagin inhibit the binding of p50 subunit of NF-κB in various cancer cells, which ultimately leads NF-κB-regulated survival mechanisms (Sandur et al., 2006).

Anticancer activity of the plumbagin was documented on breast cancer (Kuo et al., 2006), on prostate cancer (Aziz et al., 2008), ovarian cancer and cervical cancer cell lines was reported by Srinivas and co-workers (2004) and on liver cancer by Shih and co-workers (2009).

Till the objectives of present study were approved (2012), the anti-cancer effects of plumbagin were not previously investigated thoroughly on oral cancer. In the present study, we have checked anticancer activity of plumbagin on three oral cancer cell lines.
3.2 Material and methods for therapeutic activity studies

3.2.1 Material and methods to check antibacterial activity of plumbagin against pathogenic bacteria and their multi drug resistant (MDR) strains

The antimicrobial assay was performed against following bacterial species and their multi drug resistant bacterial strains. All ATCC bacterial strains and clinical isolates of multi drug bacterial (MDR) strains were provided by Department of Microbiology, Dr. D. Y. Patil Medical College, Hospital and Research Centre, Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune.

3.2.1.1 ATCC control strains of bacteria used to check antibacterial activity

ATCC control strains of bacteria used are enlisted as follows

1) Escherichia coli ATCC25922
2) Pseudomonas aeruginosa ATCC27853
3) Staphylococcus aureus ATCC25923
4) Klebsiella pneumoniae ATCC700603

3.2.1.2 Multi Drug Resistant (MDR) bacteria used

Clinical isolates of multi drug bacterial (MDR) strains used are enlisted as follows

1) Escherichia coli MDR strains
   1-U1815- Collected from urine sample.
   2-P1189- collected from pus sample.

2) Pseudomonas aeruginosa MDR strains
   1- P44- Collected from pus sample.
   2- F831- Collected from fluid sample.

3) Staphylococcus aureus MDR strains
   1- B48- Collected from blood sample.
   2- F204- collected from fluid sample.

4) Klebsiella pneumoniae MDR strains
   P290- Collected from pus sample.

5) Citrobacter MDR F182 strain (collected from fluid sample)
3.2.1.3. Composition of various media used for antimicrobial activity

Table 3.1 Composition of MacConkey agar

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone (Pancreatic digest of gelatin)</td>
<td>17 g</td>
</tr>
<tr>
<td>Proteose peptone (meat and casein)</td>
<td>3 g</td>
</tr>
<tr>
<td>Lactose monohydrate</td>
<td>10 g</td>
</tr>
<tr>
<td>Bile salts</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Neutral red</td>
<td>0.03 g</td>
</tr>
<tr>
<td>Crystal Violet</td>
<td>0.001 g</td>
</tr>
<tr>
<td>Agar</td>
<td>13.5 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>Add to make 1 Liter</td>
</tr>
</tbody>
</table>

Final pH - 7.1 +/- 0.2 at 25°C. Source: [http://himedialabs.com/](http://himedialabs.com/)

Table 3.2 Composition of Blood agar

<table>
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<th>Ingredients</th>
<th>Concentration</th>
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<tr>
<td>Peptone</td>
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</tr>
<tr>
<td>Beef extract/yeast extract</td>
<td>0.3%</td>
</tr>
<tr>
<td>Agar</td>
<td>1.5%</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5%</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Add to make 1 Liter</td>
</tr>
<tr>
<td>Sheep Blood</td>
<td>5%</td>
</tr>
</tbody>
</table>

Final pH - 7.2 to 7.6 (7.4) at 25°C. Source: [http://himedialabs.com/](http://himedialabs.com/)

Table 3.3 Composition of MH (Mueller Hinton) agar

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Concentration in (g/L)</th>
</tr>
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<tbody>
<tr>
<td>Beef Extract</td>
<td>2.00 g</td>
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<tr>
<td>Acid Hydrolysate of Casein</td>
<td>17.50 g</td>
</tr>
<tr>
<td>Starch</td>
<td>1.50 g</td>
</tr>
<tr>
<td>Agar</td>
<td>17.00 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000 mL</td>
</tr>
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</table>

Final pH - 7.3 ± 0.1 at 25°C  Source: [http://himedialabs.com/](http://himedialabs.com/)
Table 3.4 Composition of Brain heart infusion broth

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf brain, infusion from</td>
<td>200.000</td>
</tr>
<tr>
<td>Beef heart, infusion from</td>
<td>250.000</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>10.000</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.000</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.000</td>
</tr>
<tr>
<td>Disodium phosphate</td>
<td>2.500</td>
</tr>
</tbody>
</table>

Final pH - 7.4±0.2 at 25°C  Source: [http://himedialabs.com/](http://himedialabs.com/)

3.2.1.4 Single colony isolation and inoculum preparation

Single colony was obtained using streak plate method. Streaking was performed and plates were incubated for 24 hrs at 37°C. For isolation of single colony media used was MacConkey agar and Blood agar. Bacterial strain preserved in nutrient agar at 4°C. Loop full of it inoculated in nutrient broth and incubated at 37°C overnight then checked suspension after 24 h.

3.2.1.5 Preparation of antibiotic discs

Preparation of antibiotic discs of control plumbagin (SiPB) and extracted plumbagin (ExPB)

Antibiotic discs of control plumbagin from Sigma Aldrich (SiPB), of 6mm in diameter were prepared by using Whatman filter paper no.1 to find out the zone of inhibition against different bacteria. Stock solution of control plumbagin (SiPB) was prepared with concentration 10 mg/mL. It is standardized that 140 disc absorbed 1mL of solution. Accordingly, discs of different concentration i.e. 20 µg/disc, 50 µg/disc, 100 µg/disc, 150 µg/disc, 200 µg/disc, 250 µg/disc were prepared.

Antibiotic discs of extracted plumbagin (ExPB) were prepared in the same way as that of SiPB. These discs with different concentration were used against above mentioned bacterial species.
3.2.1.6 Protocol for disc diffusion test for SiPB and ExPB (Miles and Amyes Laboratory control of antimicrobial therapy, 1996)

**Media used:** MH (Mueller Hinton) agar

Disc diffusion assay method was performed to determine bacterial inhibition by control plumbagin from Sigma (SiPB) and extracted plumbagin (ExPB). For the antimicrobial assay, single pure colony of bacteria and is obtained by using streak plate technique as mentioned above. Single colony was then inoculated in normal saline water and its turbidity was compared with 0.5 McFarland standards. Once inoculum was ready, with the help of cotton stick inoculums was lawn onto MH (Mueller Hinton) agar plate. Discs with respective drug concentration of particular drug were placed on plate. Plates were incubated at 37°C for 24 hrs. After 24 hrs zone of inhibition (ZOI) were noted. Each concentration was checked in triplicate against all the bacterial strain and mean of each with standard deviation is considered and graphs are plotted having concentration per disc on X-axis and Zone of inhibition (ZOI) on Y-axis.

3.2.1.7 Protocol for MIC (Minimum inhibitory concentration) assay

The minimum inhibitory concentration can be defined as the lowest concentration of the plant extracts against the test bacteria and determined as inhibition the colour change in the bacterial suspension in the set of tubes (Salar *et al.*, 2012).

The minimum inhibitory concentration (MIC) was determined for control plumbagin from Sigma (SiPB) and extracted plumbagin (ExPB). The MIC range was selected for experiment is 512µg/mL to 1µg/mL. 10mg/mL of plumbagin was used as a stock solution. Initially working dilution was prepared. A pure culture of single microorganism was grown in brain heart infusion broth. The culture inoculums were standardized using standard microbial techniques to have turbidity nearly equal to 0.5 McFarland turbidity standards. The antimicrobial agent was diluted multiple times with two fold dilutions, through a sterile diluents Mueller Hinton broth. After the dilution performed, from the inoculum loop full of culture were inoculated into each test tube. The serially diluted antimicrobial agent which was inoculated with respective bacteria was incubated at 37°C for 24 hrs.

One tube was kept as positive growth control i.e. broth + organism without drug to confirm that growth of bacteria occurs in broth if drug was not added. One tube as negative control containing only broth, providing that broth itself was not showing any growth.
After incubation, the series of dilution tube were observed for microbial growth, indicated by turbidity. The last tube in the dilution series that did not demonstrate growth corresponds with minimum inhibitory concentration (MIC) of the antimicrobial agent. Each experiment for both control plumbagin from Sigma (SiPB) and extracted plumbagin (ExPB) was repeated three times.

**Figure 3.1: Dilutions for MIC assay**
3.2.2 Material and methods for testing anticancer activity of plumbagin against oral cancer cell lines

The experiments to analyse the effect of plumbagin on the survival of oral cancer cells were carried out by ‘Anticancer Drug Screening Facility, (ACTREC), Kharghar, Navi Mumbai-410210’, using Sulphorhodamine B (SRB) colorimetric assay for cytotoxicity screening following methods of Vichai and Kirtikara, 2006.

3.2.2.1 Material used for cytotoxicity assay

i) Oral cancer cell lines used:
The cell lines were selected from the cancer cell line collection of ‘Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai, INDIA.

- AW13516
- AW 8507
- KB

Out of the above mentioned cell lines, AW13516 and AW 8507 were derived from Indian patients.

ii) Media used: RPMI 1640 media containing 10% fetal bovine serum (FBS) and 2mM/l glutamine

iii) Trypsin EDTA 0.25% (wt/vol) was used for trypsinisation of cells.

iv) Solvent used to dissolve drugs: DMSO

v) Sulphorhodamine B (SRB - 0.4 % (w/v) in 1% acetic acid),

vi) TCA 10% (wt/vol)

vii) Tris base was used for removal of bound dye.

viii) Drugs tested:

Plumbagin extracted from in-vitro plant cultures (ExPB) and the Sigma plumbagin (SiPB) were checked at different concentrations mentioned below. Adriamycin was used as a Positive control.

- Test Drug: SiPB & ExPB
- Positive control: Adriamycin.

Drug concentrations checked: $10^{-7}$M, $10^{-6}$ M, $10^{-5}$ M and $10^{-4}$ M.
3.2.2.2 Protocol for SRB assay

Three oral cancer cell lines were used viz., AW13516, AW 8507 and KB. Standard Plumbagin from Sigma (SiPB) and extracted plumbagin (ExPB) were dissolved in DMSO and were diluted to give different concentrations viz., $10^{-7}$M, $10^{-6}$M, $10^{-5}$M, and $10^{-4}$M. Adriamycin was used as a positive control compound.

**Cell culture and treatment**

1) The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2mM L-glutamine. From the culture flasks of selected oral cancer cell lines, medium was removed and cells were washed with PBS. Then cells were trypsinized using 0.25% (wt/vol) trypsin–EDTA. Cell count was done using trypan blue solution and adjusted to seeding density ($1.9 \times 10^4$ cells per well)

2) For present screening experiments, cells were inoculated in 96well microtiter plates with 90 µl/well at appropriate plating densities, depending on the doubling time of individual cell lines. After cell inoculations, the microtiter plates were incubated at 37°C, in 5% CO$_2$, 95% air and 100% relative humidity for 24 hrs prior to addition of plumbagin.

3) After 24 hrs, cells from one plate of each cell line were fixed in-situ, with 100 µl of 10% (wt/vol) TCA addition to each well and incubated at 4°C for 1 hr. Then plates were washed and allowed to dry. It represents a measurement of the cell population for each cell line at the time of addition of the drug.

**Endpoint measurement**

1) In another plate, drugs were added with concentration $10^{-7}$M, $10^{-6}$M, $10^{-5}$M, and $10^{-4}$M. For each experiment, Adriamycin was used as a positive control compound, with similar concentrations.

2) After the addition of drug, the plates were incubated at standard condition for 48 hrs.

3) Then the assay was terminated by the addition of cold TCA. Cells were fixed in-situ by gentle addition of 50 µl of 10% (wt/vol) TCA and incubated for 1hr at 4°C.

4) The supernatant was discarded; the plates were washed 5 times with tap water and dried. Sulphorhodamine B (SRB) solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells and plates were incubated for 20 mins at room temperature.

5) After staining, unbound dye was removed by washing 5 times with 1% acetic acid. Then the plates were air dried.
6) Bound dye was subsequently eluted with 10 mM Tris base and absorbance was read on an Elisa plate reader at wavelength of 540 nm.

Calculations:
Values of Percent control growth, Total growth inhibition (TGI) and LC$_{50}$ were determined using the following calculations given by Vichai and Kirtikara, 2006

1) Percent control growth:
Percent control growth was expressed as the ratio of average absorbance of the test well to the average absorbance of control wells × 100.

$$\text{% of control cell growth} = \frac{\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{day0}}}{\text{mean OD}_{\text{control}} - \text{mean OD}_{\text{day0}}} \times 100$$

The dose responsive parameters were calculated for each test article.

2) Total growth inhibition (TGI):
TGI, i.e. drug concentration resulting in total growth inhibition was calculated from

$$\text{mean OD}_{\text{Sample}} = \text{mean OD}_{\text{day0}}$$

3) LC$_{50}$: is the concentration of drug resulting in a 50 % reduction in the measured protein at the end of the drug treatment as compared to that of the beginning indicating a net loss of the cells following treatment.

$$\frac{\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{day0}}}{\text{mean OD}_{\text{day0}}} \times 100 = -50$$
3.3 Results and discussion for therapeutic activity studies

3.3.1 Results and discussion for antibacterial activity of plumbagin against pathogenic bacteria and their multi drug resistant (MDR) strains

Multi drug resistance of bacteria causes great therapeutic challenges and economic loss in the treatment of infectious diseases which is a largest threat for the success of today’s antimicrobial agents. Fewer side-effects, cost effectiveness and capabilities to overcome MDR strains etc are the advantages of herbal drug because of which research in this area is of paramount importance (Kuldeep et al., 2014).

In the present work antimicrobial activity of plumbagin was checked on several bacterial control ATCC strains and their clinical isolates which are MDR strains. The antimicrobial assays were performed at Department of Microbiology, Dr. D. Y. Patil Medical College, Hospital and Research Centre, Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune following the protocol as discussed in section 3.2.1.6, given by R. S. Miles and S. G. B. Amyes, (1996) from Laboratory control of antimicrobial therapy. In the present study it was observed that both SiPB and ExPB showed antimicrobial activity in dose dependent manner i.e. exhibited maximum activity at highest concentration (250 µg/disc), which was in accordance to the reports by Ahmad and co-workers (2012), who reported the increased antimicrobial effect of Holoptelea integrifolia leaves with higher concentration against all the four bacterial strains.

3.3.1.1 Isolation of single colony

Isolation of pure and single colony is crucial step in studies with bacterial culture. Single colony was obtained using streak plate method and it was incubated for 24 hrs at 37°C. For, isolation of single colony media used was MacConkey agar and Blood agar. Some of the single colonies isolated are depicted in Figures 3.2, 3.3 & 3.4. From these isolated single colonies, respective inoculums and plates were prepared for antimicrobial assay.

![E. coli single colony for MDR strains](image)

**Figure 3.2: E. coli single colony for MDR strains** a) P1189, b) U1815
3.3.1.2 Results for disc diffusion Assay

Disc diffusion test were performed using Protocol 3.2.1.6. For all the bacterial species (including control ATCC strains and the (MDR) multi drug resistant Strains) both control plumbagin from Sigma (SiPB) and extracted plumbagin (ExPB) were checked with five concentration viz, 20 µg/disc, 50 µg/disc, 100 µg/disc, 150 µg/disc, 200 µg/disc, 250 µg/disc. All the bacterial strains showed different sensitivity for all the drugs and their respective concentration. Each experiment was repeated three times and mean of the results are plotted in graphs with standard deviation.

A) Disc diffusion assay for S. aureus

Disc diffusion assay for SiPB and ExPB was checked for 3 strains of S. aureus with different concentration

For SiPB:

As shown in Figure 3.5 and Figure 3.6, ATCC 25923 strains of S. aureus and MDR B48 showed very good sensitivity against control plumbagin SiPB. Zone of inhibition (ZOI) was 20 mm for ATCC 25923 strain at 20µg/disc. Gradual increase in the concentration of drug per disc increased ZOI (Figure 3.6), which was 42 mm for 250 µg/disc. Similarly for
S. aureus MDR B48, zone of inhibition (ZOI) was 25 mm for the concentration 20mg/disc. Further increase in SiPB concentration resulted in increase in the ZOI (Figure 3.6). ZOI was 42 for 250µg/disc SiPB. However, S. aureus MDR F204, showed poor sensitivity for SiPB. Zone of inhibition was zero at concentration 20mg/disc, which could not go higher than 9 mm even at the concentration 250 µg/disc.

Figure 3.5: Zone of inhibition (mm) for SiPB a) S. aureus ATCC 25923 b) S. aureus MDR B48 and c) S. aureus MDR F204

![Figure 3.5](image)

<table>
<thead>
<tr>
<th>Concentration (µg/disc)</th>
<th>ZOI (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20µg/disc</td>
<td>0</td>
</tr>
<tr>
<td>50 µg/disc</td>
<td>5</td>
</tr>
<tr>
<td>100 µg/disc</td>
<td>10</td>
</tr>
<tr>
<td>150 µg/disc</td>
<td>15</td>
</tr>
<tr>
<td>200 µg/disc</td>
<td>20</td>
</tr>
<tr>
<td>250 µg/disc</td>
<td>25</td>
</tr>
</tbody>
</table>

Figure 3.6: Disc diffusion assay for S. aureus ATCC and MDR strains against SiPB & ExPB
For ExPB:
Extracted plumbagin (ExPB) showed very good sensitivity on ATCC 25923 strains of *S. aureus* and MDR B48 as shown in Figure 3.6 and Figure 3.7. For ATCC 25923 strain, Zone of inhibition (ZOI) was 9 mm at concentration 20 µg/disc. Gradual increase in the concentration resulted in increased ZOI (Figure 3.4), which was 31 mm for 250 µg/disc. Similarly, for *S. aureus* MDR B48 zone of inhibition (ZOI) was 12 mm for the concentration 20 µg/disc. Further increase in the concentration of ExPB per disc resulted in increase in the ZOI (Figure 3.6). It was 38 mm when concentration was reached to 250µg/disc. However, ExPB also showed poor activity for *S. aureus* MDR F204. At concentration 20 µg/disc, ZOI was zero which could not go higher than 10 mm even at the concentration 250 µg/disc.

![Figure 3.7](image1.png)

**Figure 3.7: Zone of inhibition (mm) for CRPB a) S. aureus ATCC 25923 b) S. aureus MDR B48 c) S. aureus MDR F204**

Previously it was reported that *S. aureus* ATCC 25923 and its two MDR including MRSA showed sensitivity towards methanol extracts from medicinal plants like tulsi, lemongrass, aloe-vera, oregano and rosemary (Praveen and Shramishtha, 2012). Earlier it was reported that Plumbagin showed antimicrobial activity on ATCC strains of *S. aureus* and *C. albicans*. It was postulated that, production of reactive oxygen species (ROS) by plumbagin in *S. aureus* and *C. albicans* might be the cause of antimicrobial activity of plumbagin for these microorganisms (Sweatha *et al.*, 2016).

**B) Disc diffusion assay for E. coli**

For SiPB:
As shown in Figure 3.8 and Figure 3.9, control plumbagin from Sigma (SiPB) showed very moderate sensitivity with zone of inhibition (ZOI) size in between 10 mm to 12 mm on the ATCC 25922 strain of *E. coli* as well as multi drug resistant strains (MDR) strains of *E. coli* U1815 for all the concentration ranging from 20 µg/disc to 250 µg/disc. Same
effect was there for MDR P1189. However this strain showed ZOI 14 mm at concentration 250 µg/disc.

**For ExPB:**

For extracted plumbagin (ExPB) zone of inhibition (ZOI) was zero for all the three strains for the concentration 20 µg/disc to 100 µg/disc as shown in Figure 3.8 and Figure 3.10. For the concentration 150 µg/disc all three strains showed ZOI 10 mm. Further increase in concentration showed moderate ZOI increase for *E. coli* ATCC 25922 strain and MDR U1815. However, ZOI reached up to 15 mm for MDR P1189 at concentration 200 µg/disc.

![Disc diffusion assay for E. coli](image)

**Figure 3.8: Disc diffusion assay for *E. coli* ATCC and MDR strains against SiPB & ExPB**

This indicates that the clinical isolate from pus sample MDR P1189 of *E. coli* is having more sensitivity than *E. coli* ATCC 25922 strain and MDR from urine U1815 for both control plumbagin SiPB as well extracted plumbagin ExPB.

![Zone of inhibition for SiPB](image)

**Figure 3.9: Zone of inhibition for SiPB a) *E. coli* ATCC25922 b) *E. coli* MDR U1815 and c) *E. coli* MDR P1189**
Antimicrobial activity of ethanol extract of lemongrass was reported observed on the clinical isolates of *E. coli* and *K. Pneumonia* (Praveen and Sharmishtha, 2012). Alcoholic extract of *P. zeylanica* (14%) showed curing of plasmid pUK651 from *E. coli* to confirm the antimicrobial activity of it on clinical isolate of *E. coli*. However Paiva and co-workers (2003) reported *Plumbago* extracts was ineffective against the *E. coli* and *S. typhimurium*.

C) Disc diffusion assay for *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* typical prototype of multi drug resistant bacteria and generally exhibits resistance to drugs.

**For SiPB:**

As shown in Figure 3.11 & 3.12 control plumbagin from Sigma (SiPB) showed poor activity on all the strains of *P. aeruginosa* including ATCC and MDR P44 and F81. No zone of inhibition (ZOI) was observed for the concentration 20 µg/disc to 150 µg/disc. ZOI was observed when concentration was increased further in between 7 mm to 10 mm, which are not considerable zone of inhibition in size for antimicrobial activity.

**For ExPB:**

Extracted plumbagin ExPB also showed similar activities on all the strains of *P.aeruginosa* including ATCC and MDR P44 and F81. No zone of inhibition was observed for the concentration ranging from 20 µg/disc to 150 µg/disc as shown in Figure 3.11. Negligible ZOI were observed with further increase in the concentration of ExPB. Size of ZOI was from 8 mm to 10 mm, (Figure 3.13) which are not significant to support antimicrobial activity of the compound.
Figure 3.11: Disc diffusion assay for *P. aeruginosa* ATCC and MDR strains against SiPB & ExPB

Figure 3.12: Zone of inhibition (mm) for SiPB for a) *P. aeruginosa* ATCC 27853 b) *P. aeruginosa* MDR P44 and c) *P. aeruginosa* MDR F831

Figure 3.13: Zone of inhibition (mm) for ExPB for a) *P. aeruginosa* ATCC 27853 b) *P. aeruginosa* MDR P44 and c) *P. aeruginosa* MDR F831
P. aeruginosa have shown a typical resistance towards both SiPB and ExPB. In gram-negative bacteria, outer membrane composed of phospholipids and lipopolysaccharides acts as a barrier to avoid the entry and action of most of the antimicrobial agents through cell envelope. Richa & Ekta (2014) reported that Pseudomonas aeruginosa and Proteus vulgaris are showing less activity against Plumbago extracts. Jeyachandran and co-workers (2009) reported moderate to low activity of plumbagin and Plumbago extracts on Pseudomonas aeruginosa (MTCC 2642). Antibacterial activity of the alkaloid extracts from medicinal plant C. citrinus and V. adoensis was demonstrated on S. aureus and P. aeruginosa (Donald et al., 2016).

D) Disc diffusion assay for Klebsiella pneumoniae

For SiPB:

As shown in Figure 3.14 and Figure 3.15, Klebsiella pneumoniae ATCC 700603 control strain and MDR strain P290 showed poor response towards control plumbagin SiPB. At the concentration 20 µg/disc, there was no zone of inhibition. From 50 µg/disc ZOI of 8 mm and 7 mm were observed for ATCC and MDR P290 strains respectively, but could not increase than 10mm even with concentration 250 µg/disc for both the strains.

Figure 3.14: Disc diffusion assay for Klebsiella pneumoniae ATCC and MDR strains against SiPB & ExPB
For ExPB:
Extracted plumbagin (ExPB) also showed similar response for both *Klebsiella pneumoniae* ATCC 700603 control strain and MDR strain P290, as shown in Figure 3.14 and Figure 3.16. There were no zones of inhibition with the concentration 20 µg/disc to 100 µg/disc. For ATCC strain, at 150 µg/disc 8mm ZOI was observed and for 250 µg/disc 10 mm. Even less was recorded for the MDR strain; for ATCC 7mm ZOI at 200 µg/disc and 8 mm for concentration 250 µg/disc. These ZOI values are insignificant which indicate that both ATCC and MDR P290 strains of *Klebsiella* are not sensitive to extracted plumbagin ExPB.

![Figure 3.15: Zone of inhibition for SiPB a) Klebsiella pneumonia ATCC 700603 and b) Klebsiella pneumonia MDR P290](image)

![Figure 3.16: Zone of inhibition for ExPB a) Klebsiella pneumoniae ATCC 700603 and b) Klebsiella pneumonia MDR P290](image)

Ethanol extracts of medicinal plant *Terminalia chebula* and *Ocimum sanctum* showed antibacterial activity against *Klebsiella pneumonia* (Anjana et al., 2009) Moderate activity of *Plumbago* extracts was reported by Jeyachandran and co-workers (2009) on *Klebsiella pneumoniae* (MTCC 2405).
E) Disc diffusion assay for *Citrobacter* MDR F182

Disc diffusion assay was carried out for multi drug resistant strain of *Citrobacter* F182, which showed good sensitivity for both control plumbagin from Sigma (SiPB) and for the extracted plumbagin (ExPB) as shown in Figure 3.17 and 3.18.

![Disc diffusion assay for Citrobacter MDR F182](image)

**Figure 3.17: Disc diffusion assay for *Citrobacter* MDR strains F182 against SiPB & ExPB**

For both SiPB and ExPB, zone of inhibition was 12 mm at lowest concentration 20 µg/disc. Further increase in the concentration of drug resulted in the gradual increase in ZOI as shown in Figure 3.17. At concentration 250 µg/disc ZOI for SiPB was 24 mm and for ExPB ZOI was 25 mm. These values indicate that, clinical isolate of *Citrobacter* – MDR F182, is sensitive for both SiPB and ExPB.

![Zone of inhibition for Citrobacter MDR F182 for a) SiPB and b) ExPB](image)

**Figure 3.18: Zone of inhibition for *Citrobacter* MDR F182 for a) SiPB and b) ExPB**

There are reports of antimicrobial activity of *A. acuminata* extracts and some other medicinal plants were checked against *Citrobacter* species (Monali *et al*., 2015). But interestingly, till the objectives of the present project were approved, there were no reports on systematic studies of antimicrobial activity of *Plumbago* extracts on *Citrobacter*.
species. We for the first time reporting the antimicrobial activity of plumbagin on multi drug resistant *Citrobacter*.

### 3.2.1.2 Results for minimum inhibitory concentration (MIC).

MIC values were found using the protocol 3.2.1.7. The last tube in the dilution series that does not demonstrate growth corresponds with minimum inhibitory concentration (MIC) of the antimicrobial agent.

**For SiPB:**

As depicted in Table 3.5 MIC values were obtained for all ATCC and multi drug resistant strains of all the bacteria. MIC values indicated that SiPB is exhibiting strong antimicrobial activity on *Staphylococcus aureus* ATCC and MDR B48. *Citrobacter* MDR F182 also exhibited good sensitivity. It was followed by both ATCC and MDR P290 strains of *Klebsiella pneumoniae* and *Escherichia coli* ATCC. Very less activity was observed for one isolate of *Staphylococcus aureus* MDR F204 and both MDR isolates of *E. coli* U1815 and P1189. However, all strains of *Pseudomonas aeruginosa* ATCC and MDR P44 and F 831, exhibited resistance for SiPB.

#### Table 3.5: MIC assay for SiPB

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MIC for SiPB</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> ATCC</td>
<td>64µg/mL</td>
</tr>
<tr>
<td>B48 (<em>Staphylococcus aureus</em> MDR)</td>
<td>64µg/mL</td>
</tr>
<tr>
<td>F204 (<em>Staphylococcus aureus</em> MDR)</td>
<td>256µg/mL</td>
</tr>
<tr>
<td><em>Citrobacter</em> MDR F182</td>
<td>128µg/mL</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> ATCC</td>
<td>128µg/mL</td>
</tr>
<tr>
<td>P290 (<em>Klebsiella pneumoniae</em> MDR)</td>
<td>128µg/mL</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC</td>
<td>128µg/mL</td>
</tr>
<tr>
<td>U1815 (<em>E. coli</em> MDR)</td>
<td>256µg/mL</td>
</tr>
<tr>
<td>P1189 (<em>E. coli</em> MDR)</td>
<td>256µg/mL</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC</td>
<td>Resistant</td>
</tr>
<tr>
<td>P44 (<em>Pseudomonas aeruginosa</em> MDR)</td>
<td>Resistant</td>
</tr>
<tr>
<td>F831(<em>Pseudomonas aeruginosa</em> MDR)</td>
<td>Resistant</td>
</tr>
</tbody>
</table>
For ExPB

MIC values for ExPB were also having somewhat similar pattern as that of SiPB. *Staphylococcus aureus* ATCC and isolate MDR B48 showed the highest antimicrobial sensitivity also for ExPB as shown in Table 3.6. *Citrobacter* MDR F182 also exhibited good antimicrobial activity. It was followed by both ATCC and MDR P290 strains of *Klebsiella pneumoniae* and *Escherichia coli* ATCC.

Table 3.6: MIC assay for ExPB.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MIC for ExPB</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> ATCC</td>
<td>128µg/mL</td>
</tr>
<tr>
<td>B48 (<em>Staphylococcus aureus</em> MDR)</td>
<td>128µg/mL</td>
</tr>
<tr>
<td>F204 (<em>Staphylococcus aureus</em> MDR)</td>
<td>512µg/mL</td>
</tr>
<tr>
<td><em>Citrobacter</em> MDR F182</td>
<td>128 µg/mL</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> ATCC</td>
<td>256µg/mL</td>
</tr>
<tr>
<td>P290 (<em>Klebsiella pneumoniae</em> MDR)</td>
<td>256µg/mL</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC</td>
<td>256µg/mL</td>
</tr>
<tr>
<td>U1815 (<em>E. coli</em> MDR)</td>
<td>512µg/mL</td>
</tr>
<tr>
<td>P1189 (<em>E. coli</em> MDR)</td>
<td>512µg/mL</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC</td>
<td>Resistant</td>
</tr>
<tr>
<td>P44 (<em>Pseudomonas aeruginosa</em> MDR)</td>
<td>Resistant</td>
</tr>
<tr>
<td>F831(<em>Pseudomonas aeruginosa</em> MDR)</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

Very low activity was observed for one isolate of *S. aureus* MDR F204 and both MDR isolates of *E. coli* U1815 and P1189. However, all strains of *Pseudomonas aeruginosa* ATCC and MDR P44 and F 831, exhibited resistance for SiPB as shown in Table 3.5 and 3.6.

Minimum inhibitory concentration (MIC) data matches with the observations from disc diffusion assay depicted as zone of inhibition (ZOI), which ensure the pattern of antimicrobial activity of all the microorganisms against SiPB and ExPB. From both MIC and disc diffusion assay results, it can be concluded that *Staphylococcus aureus* exhibited the best sensitivity among all the tested bacteria. *S. aureus* is the leading cause of hospital born infections which may be fatal to patient even after getting recovery from main
disease. *Staphylococcus aureus* ATCC 25923 and clinical isolate from blood sample MDR B48 showed maximum sensitivity than clinical isolate from fluid sample F204. *Citrobacter* is emerging as one of the important pathogenic bacterium from the family Enterobacteriaceae. *Citrobacter* MDR also showed considerable sensitivity with ZOI 25 mm against both SiPB and ExPB. *Citrobacter* is the infectious agent causing urinary tract infections, blood stream infections, and sepsis in intra abdomen to immunocompromised people. There are very less reports for antimicrobial activity against *Citrobacter*. It makes strong antimicrobial activity of plumbagin against *Citrobacter* very remarkable.

Moderate activity was exhibited by *Klebsiella pneumoniae* ATCC 700603 and its clinical isolate from pus sample MDR P290. Moderate activity was also exhibited by control strain of *Escherichia coli* ATCC 25922 and its clinical isolates MDR U1815 and clinical isolate from pus sample P1189. However, all strains of *Pseudomonas aeruginosa*, including *P. aeruginosa* ATCC 27853 and its clinical isolates from pus sample MDR P44 and fluid sample F831 showed complete resistance towards both SiPB and ExPB.

Similar results were reported by Jeyachandran and co-workers indicating that the root extracts of *Plumbago zeylanica* exhibited strong antimicrobial activity against *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus*. The activity was moderate against *Klebsiella pneumoniae*, *Serratia marcescens* and *Bacillus subtilis*. However it was recorded very low effective against *Proteus vulgaris* and *Pseudomonas aeruginosa*. Praveen and Sharmishtha, (2012) carried out assays and postulated that the extracts of *P. auriculata* may lead to the formation of antibiotics against multi-drug resistant strains for hospital induced infections and also for community.

From the present study, we have concluded that *P. auriculata* have great potential as antimicrobial drug against MDR clinical isolates. Furthermore, in a few cases like *S. aureus* and *Citrobacter* both SiPB and ExPB were active against MDR bacteria with very low concentrations like 20 µg/disc indicating minimization of the possible toxic effects. These microorganisms can spread the disease through a carrier if proper care is not taken in hospital born infections. This study would suggest development of hygiene maintenance products like sanitizer, hand wash and soap to keep patients free from hospital born infection using extracts of *Plumbago* and also development of stable and biologically active compounds which can be employed in the formulation of antimicrobial agents against multi drug resistant bacteria.
3.3.2 Results and discussion for anticancer activity of plumbagin against oral cancer cell lines

As mentioned in section 3.1.3.2.2, anticancer activity of plumbagin is reported for many cancers like breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, myeloma, lung cancer, skin carcinoma, leukemia, liver cancer, renal cancer, cervical cancer etc (Padhye et al., 2012). In 2011, Manu et al. have reported that plumbagin inhibits the expression of CXCR4 in oral adenosquamous carcinoma cells (CAL27). Interaction of CXCR4 with its ligand CXCL12 is a critical process in metastasis that accounts for more than 90% of cancer related cell deaths including oral cancer. But the effect of plumbagin as a therapeutic compound in oral cancer has not been extensively studied till the objectives of present study were approved (2012).

Anticancer activity of control plumbagin from Sigma (SiPB) and extracted plumbagin (ExPB) were carried out using Sulphorhodamine B (SRB) assay following the protocol mentioned in the section 3.2.2.2 on three oral cancer cell lines viz., AW13516, AW 8507 and KB. Adriamycin was used as a positive control compound. The experiments were carried out in ‘Anticancer Drug Screening Facility, ACTREC, Kharghar, Navi Mumbai-410210.’ Activity of SiPB was checked with all the three cell lines; however ExPB was checked against only KB cell line.

3.3.2.1 Anticancer activity of SiPB on human oral cancer cell line AW8507

The assay was carried out for SiPB and Adriamycin at different concentrations of drugs viz., $10^{-7}$M, $10^{-6}$M, $10^{-5}$M, and $10^{-4}$M and it was observed for growth inhibition of AW8507 cell line.

As indicated in Figure 3.19, it can be observed that percentage of control of cell growth was reduced with the increased concentration of drugs. For SiPB, reduction in growth was slow when concentration was increased from $10^{-7}$M to $10^{-6}$M. But when concentrations were increased further, there was a sudden fall in the percent control growth of cells. It was -80.8 for the concentration $10^{-4}$M, which indicated that only 20% of the cells were able to grow at this concentration. The positive control drug Adriamycin showed good activity even at $10^{-7}$M and was remarkable at $10^{-5}$M, showing -80.5% control growth which also indicated 20% cells were able to grow. Adriamycin is a known synthetic drug with some side effects. Similar results were obtained with SiPB with concentration of $10^{-4}$M.
3.3.2.2 Anticancer activity of SiPB on human oral cancer cell line AW13516
The assay was carried out at for SiPB and Adriamycin at different concentrations of drugs viz., $10^{-7}$M, $10^{-6}$M, $10^{-5}$M, and $10^{-4}$M and it was observed for growth inhibition of AW13516 cell line.

Figure 3.19: Growth curve for oral cancer cell line AW 8507 against SiPB

Figure 3.20: Anticancer activity of SiPB on oral cancer cell line AW13516
From the Figure 3.20, it can be observed that there was no reduction in % control growth of AW 13516 cell line by SiPB at the concentration $10^{-7}$M, $10^{-6}$M. But when the concentration of SiPB was increased further to $10^{-5}$M, % control growth suddenly reduced to -64.5 %; which means at this concentration only 35% cells were able to grow. From the Figure 3.20, it can be clearly said that SiPB and Adriamycin, both have controlled almost similar growth of DWD cell line at the concentration $10^{-5}$M and $10^{-4}$M. It indicates that control plumbagin from Sigma SiPB, exhibits the same anticancer activity as that of the known anticancer drug Adriyamycin.

3.3.2.3 Anticancer activity of SiPB on human oral cancer cell line KB

The assay was carried out at different concentrations of drugs viz., $10^{-7}$M, $10^{-6}$M, $10^{-5}$M, and $10^{-4}$M and it was observed for growth inhibition of KB cell line.

From the Figure 3.21, it can be observed that, there was no reduction in growth of KB cell line by SiPB at the concentration $10^{-7}$M, $10^{-6}$M and even very less reduction at the concentration $10^{-5}$M. But when concentration reached to $10^{-4}$M, growth was reduced up to -79% which indicated that at this concentration of SiPB, only 21% cells were able to grow.

![Figure 3.21: Anticancer activity of SiPB on oral cancer cell line KB](image)

Adriamycin exhibited steady reduction in growth from $10^{-7}$M, to $10^{-5}$M. At concentration $10^{-4}$M, it showed -76% growths that means 24% cells were able to grow. At the
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concentration $10^{-4}$M, SiPB exhibited better activity than that of the known anticancer drug Adriamycin.

### 3.3.2.4 Anticancer activity of ExPB on human oral cancer cell line KB

The assay was carried out for extracted plumbagin ExPB and Adriamycin at different concentrations of drugs *viz.*, $10^{-7}$M, $10^{-6}$M, $10^{-5}$M, and $10^{-4}$M and it was observed for growth inhibition of KB cell line.

![Growth Curve for ExPB: Human Oral Cancer cell line KB](image)

**Figure 3.22: Anticancer activity of ExPB on human oral cancer cell line KB**

From the Figure 3.22, it can be observed that when ExPB was used, there was slow reduction in growth, with increased concentration $10^{-7}$M, $10^{-6}$M, and $10^{-5}$M. However, when concentration reached to $10^{-4}$M, it reduced growth of cells -62%, which indicated that at this concentration only 38% cells were able to grow. Adriamycin reduced -77% growth indicating 33% cells were able to grow.

Though this reduction in growth of the cells is less, it is comparable with pure plumbagin (SiPB). SiPB had controlled cell growth 79% (Figure 3.22) and ExPB had controlled 62% growth. This may be because of some other impurities in the extracted plumbagin. Further purification may exhibit better activity of ExPB on oral cancer cells.
Statistical Analysis:
Each experiment was repeated three times and the ‘mean’ was plotted in the graph. For statistical analysis ‘student’s t’ test was used separately for each cell line for each drug. For all the experiments p value was observed less than 5 ($p<0.05$), which indicate the data was statistically significant. Plumbagin demonstrated significant inhibition ($p<0.05$) of cell growth in all the three cell lines.

3.3.2.5 Calculation of LC$_{50}$ and TGI values
From the above experiments, values of total growth inhibition (TGI) and LC$_{50}$ were determined (Vichai and Kirtikara, 2006) as shown in Table 3.7.

Table 3.7: LC$_{50}$ and TGI values of the drugs

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Drug Compound</th>
<th>µMolar drug concentrations calculated from graph</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LC$_{50}$</td>
</tr>
<tr>
<td>AW8507</td>
<td>SiPB</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>ADR</td>
<td>44.1</td>
</tr>
<tr>
<td>AW13516</td>
<td>SiPB</td>
<td>78.1</td>
</tr>
<tr>
<td></td>
<td>ADR</td>
<td>54.5</td>
</tr>
<tr>
<td>KB</td>
<td>SiPB</td>
<td>85.0</td>
</tr>
<tr>
<td></td>
<td>ADR</td>
<td>64.9</td>
</tr>
<tr>
<td>KB</td>
<td>ExPB</td>
<td>95.0</td>
</tr>
<tr>
<td></td>
<td>ADR</td>
<td>64.9</td>
</tr>
</tbody>
</table>

- LC$_{50}$ = LC$_{50}$ value is the concentration of drug causing 50% cell kill
- TGI = Total growth inhibition value is the concentration of drug causing total inhibition of cell growth

From the Table 3.7 and Figures from 3.16 to 3.19, it can be said that both plumbagin and extracted plumbagin have shown anticancer activities on oral cancer cell lines. Activity of extracted plumbagin was less than that of the Adriamycin and Sigma plumbagin. Extracted plumbagin showed remarkable activity with LC$_{50}$ value 95 and TGI value 66.4. Further
purification and combination may lead to development of a promising anticancer agent for oral carcinoma.

Novel therapies are much in demand in cancer research as resistance of cancer cells to drug-induced apoptosis is a major obstacle. In the present work we have examined the effect of control plumbagin from Sigma (SiPB) and our extracted plumbagin (ExPB) on oral cancer cells and reported the potential of plumbagin against this incapacitating condition. Our results demonstrated that increasing concentration of plumbagin both SiPB and ExPB (10^{-7}M to 10^{-4}M) inhibits growth of oral cancer cells (Figures 3.19-3.22). Growth was typically controlled by both the drugs at concentration 10^{-7}M. Moreover, plumbagin also induced cell death in these cells (LC_{50} values in Table 3.7), indicating its mechanism of action. We for the first time presented a direct report (Jyoti et al., 2015) demonstrating the efficacy of plumbagin as a potential drug candidate against oral cancer. However, at the same time Ono and co-workers (2015) have reported the anticancer activity of plumbagin on oral squamous cell carcinoma (OSCC) suppression. Use of our extracted plumbagin (ExPB) obtained by plant tissue culture techniques, in the formation of lead molecules for development of anticancer agent against oral cancer. It will also contribute to save the wild population of the plant and thus maintain the ecological balance.