6. DISCUSSION

The use of chemotherapy to treat cancer is limited by the development of resistant cancer cell variants. Resistance can occur to individual cytotoxic drugs usually by alterations in the targets for these drugs, but can also occur to many different drugs with different chemical structures and different mechanism of action results the phenomenon called multidrug resistance (MDR) (Borst and Elferink, 2002).

Cervical carcinoma is the second most common cancer affecting women worldwide with an incidence ranging from 2.6 to 67.2 per 100 000 women in different countries (Parkin et al., 1999). Treatment for cervical cancer using chemotherapeutic drugs can be possible in early cancer stages, but the major obstacle in treatment failure is due to development of drug resistance.

The major impediment in cancer chemotherapy is multidrug resistance due to overexpression of ABC transporters. The overexpression of ABCB1 occurs in 40-50% of cancer patients and is associated with poor clinical outcome (Jamroziak et al., 2004). Reduced permeability of drugs was originally suggested to be a plausible mechanism for acquired resistance derived from CHO cell lines (Ling and Thompson, 1974).

The presence of intrinsic or acquired MDR is the major factor responsible for chemotherapy failure in cancer patients who undergoing chemotherapy. When patients with cancer are treated with cytotoxic agents, the pharmacological goal is to deliver as much as active drug as possible to the molecular target in the cancer cells, causing sufficient molecular damage to lead to cell death. The occurrence of drug resistance renders resistant not only to the drug used in the chemotherapy, but also to a broad spectrum of unrelated cytotoxic drugs.

The mechanism of drug resistance is characterized by a decrease in drug accumulation resulting from overexpression of energy-dependent multidrug efflux pump
known as multidrug transporter (ABC transporter). The major mechanism responsible to MDR in cancer cells is the overexpression of MDR-1 gene product resulting increased production of a 170,000 dalton p-glycoprotein in the plasma membrane associated with multiple drug resistance in drug resistance CHO cells (Ling and Thompson, 1974).

**Drug resistance cell lines**

Overexpression of p-gp has been well established as the cause of the MDR phenotype in many *in vitro* selected drug resistant cell lines. KB cell lines have been used to catalog the changes in specific protein synthesis associated with the development of multidrug resistance results in reduced drug accumulation which might be caused by activating efflux or decreasing uptake of anticancer drugs. KB-V1 cells were selected by subjecting KB cells in a step-wise fashion to increasing concentration of vinblastine. KB-V1 cells have been shown to express only p-gp at a higher level on their plasma membrane (Schoenlein *et al.*, 1992), but p-gp was not expressed in the drug sensitive cells. The level of p-gp in KB-V1 cell membrane is about 1% of total plasma membrane of drug resistant cell lines (Ambudkar *et al.*, 1992).

Likewise colchicine resistance clone KBChR8-5 with multidrug resistance is derived from the KB cell line (Fojo *et al.*, 1985). KBChR8-5 cells were selected by subjecting KB cell lines in a stepwise fashion to increase the concentration of colchicine (Akiyama *et al.*, 1985). The amount of purified p-glycoprotein thus obtained accounted for approximately 3 to 4% of the total plasma membrane protein in colchicine-resistant mutant cell line (Riordan and Ling, 1979).

**MDR-1 gene expression studies**

We analysed the presence of MDR1 gene in KB and KBChR8-5 cells by real time PCR. KBChR8-5 cells showed higher level of MDR1 gene expression at 7.1 fold increase in MDR1 when compared to KB cells, but KB cells showed very mild expression of MDR1 gene.
As KB and its stepwise derivative KBChR8-5 cell lines have been characterized extensively with respect to the phenomenon of drug resistance proving overexpression of MDR1 gene, so we decided to use KB and KBChR8-5 cell lines to assess the effect of curcuminoids on the cytotoxic activity and expression of MDR 1 gene, to see whether introduction of curcuminoids reduced the drug resistance of the cells.

**MDR modulators**

Many synthetic MDR modulators reverse the MDR phenotype *in vitro*. However, the efficacy of the compound in the animal studies and clinical trails has been disappointing due to dose limiting toxicity. First generations MDR modulators like verapamil, cyclosporin A are immunosuppressive agents are the most effective p-gp inhibitors *in vitro*, but they have limited clinical use.

Therefore it is necessary identifying natural compounds from plant origin that reverse the MDR phenotype, sensitize cancer cells to conventional chemotherapy without undesired toxicological effects. The dietary phytochemicals can overcome the limitation in clinical uses. The development of new chemosensitizers with higher selectivity, potency, dosage and less toxicity for a novel safety is yet effective inhibitor of these transporters (Ozben, 2006).

**Turmeric**

Turmeric is widely used as a spice in South Asian dishes. In Ayurvedic practices turmeric has many medicinal properties. Curcumin is a major component of food flavoring turmeric and has been used as herbal medicine. Phase I clinical trials demonstrated curcumin is well-tolerated orally and no dose limiting toxicity was observed. Currently curcumin derivatives represent a well-established strategy to enhance metabolic stability and anti-proliferative activity against human cancer cells (Basile *et al*., 2009).
Collection of turmeric samples

In our study we have isolated curcumin and curcumin derivatives DMC, BDMC from raw turmeric rhizome to investigate the pharmacological role in multidrug resistance. The main turmeric producing states in India are Andhra Pradesh, Tamil Nadu, Orissa, Karnataka, West Bengal, Gujarat and Kerala. Maximum area under turmeric cultivation is in Andhra Pradesh followed by Tamil Nadu in the year 2006. In our study we collected turmeric rhizome from 5 different places in India such as Andhra Pradesh, Maharashtra, Assam, Tamil Nadu, Kerala.

A number of cultivars are available in India and are known mostly by the name of locality where they are cultivated. Some of the popular cultivars are Duggirala, Tekurpeta, Sugandham, Amalapuram, Erode, Allepey, Moovatupuzha and Lakadong. In our study we used five different cultivars of Curcuma longa L. from different places in India. Such varieties are Nizam variety, Rajapuri variety, Lakadong variety, Erode variety, Allepey variety.

Turmeric rhizome size, thickness, color and chemical composition varied in different varieties from different regions has been observed. These changes in chemical composition are due to agro-climatic conditions, cultivation practices, handling by small farmers, manuring, soil fertility, plant protection from insect and pests. These varieties are named by local names. The farmers tend to continue growing the varieties they have in a traditionally approach.

Evaluation of curcuminoids present in Curcuma longa L. collected from different places in India

Among five turmeric varieties analyzed Assam (Lakadong) variety showed optimum amount of total extracts. Curcumin (C) was found to be the major compound in the extracts of all varieties followed by demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC). Madhava Naidu et al., studied the percentage of composition of curcuminoids in four different turmeric samples (origin of turmeric samples – Tamil Nadu, Karnataka, North-East India, Kerala) determined by HPLC.
Curcumin was found to be the major compound in all of the tested turmeric samples. It was found that sample from North-East India Lakadong variety had greater amounts of total curcuminoids (Madhava Naidu et al., 2009). In our study among five varieties analyzed Assam variety showed optimum amount of total extracts. The presence of individual curcuminoids in Assam variety showed greater amount of each curcuminoids when compared with other varieties. The percentage of C, DMC, BDMC present in Assam extract were 23.9%, 10%, 6.1%. This shows Assam variety could be best turmeric variety for further isolation and separation of curcuminoids.

**Extraction of curcuminoids using various solvent systems**

Paramasivam et al., studied different solvent of varying polarities for extraction of curcuminoids from rhizome. Methanol was the most appropriate solvent for the maximum extraction of curcuminoids as the recoveries of curcuminoids were optimum in methanol. UV visible spectra were used for identification of curcuminoids (Paramsivam et al., 2009). In our study methanol extract showed high yield among other solvent extracts. Least yield of total extract were obtained in hexane extract, range of total extract obtained in other solvent extract are more or less same. Further all extracts were analysed in HPLC analysis for curcuminoids and percentage composition of individual curcuminoids present in the extract were observed. Curcumin was found to be the major compound in all of the tested extracts followed by demethoxycurcumin and bisdemethoxycurcumin. With the analysis of individual curcuminoids by HPLC shows optimum percentage yield of each curcuminoids in acetone extract were 22.8%, 14.2%, 6.5% for C, DMC, BDMC respectively. Among all other extracts the percentage of individual curcuminoids in acetone is higher whereas other extracts showed lower amount of individual curcuminoids, and hence acetone extract can be good source for the isolation of individual curcuminoids.

Curcuminoids in turmeric powder contain curcumin, Demethoxycurcumin and Bisdemethoxycurcumin in approximately 70-75%, 20-18% and 10-7% respectively (Pfeiffer et al., 2003). In commercially available curcumin powder DMC, BDMC was the minor compound. In order to recovery a consistent yield for isolating from crude,
individual compound should be high. Acetone extract showed individual compound at higher side than methanol and all other extract analyzed in HPLC.

**Separation of curcuminoids by TLC and Column chromatography**

In TLC method for separation of curcuminoids the Rf value of curcuminoids were 0.75, 0.55, and 0.27, for C, DMC, and BDMC respectively. Better resolution of Rf value showed that chloroform and methanol can be suitable solvent for the separation of compounds in column chromatography. Gupta *et al.*, studied different composition of mobile phase for separation of curcuminoids and desired resolution was achieved by using chloroform and methanol (95:5) as mobile phase the Rf obtained were 0.69, 0.44 and 0.29 for C, DMC, BDMC respectively (Gupta *et al.*, 1999). The consistent difference in Rf value is important role in separation column chromatography.

In column chromatography the separation is achieved by elution with chloroform and methanol with increasing polarity. The total curcuminoids present in the fractions were analysed using UV spectroscopy as 84%, 86%, 80.6% of C, DMC, and BDMC respectively. Since there was 10-15% loss of curcuminoids occurred, few fractions showed two curcuminoids in TLC therefore they are concentrated and rechromatographed on the column for further separation. There was an average of 8.8% loss of pigments on the column due to lack of separation of mixtures of pigments (Peret-Almeida., 2005). Further purification was achieved results in curcumin as bright yellow needle shaped crystals, DMC as light yellow crystals, BDMC as reddish orange color crystals.

**Characterization of curcuminoids**

The structure and purity of isolated curcuminoids were determined by GC-MS, FTIR, NMR.

**HPLC**

The purity of curcuminoids were analysed from HPLC, the curcuminoid mixture (as standard) showed three peaks with a retention time of 10.97, 12.33, 13.78 min
corresponding to C, DMC, BDMC respectively. The purified curcuminoids on HPLC analysis showed C, DMC, BDMC eluted at 10.81, 12.79, 13.03 min respectively. This demonstrated that the mixture has three curcuminoids and purity of each curcuminoid compared from standard showed in the range of 95-98% purity.

**GC-MS**

The main purpose of high resolution technique is to determine the precise mass of the ions. This can be achieved by using data system. The precise mass is determined by comparing the unknown mass of the sample peak with the known mass of a reference peak. The reference sample used here: Perfluorokerosene (PFK) (K.Biemann, 1962). Mass spectral analysis using double focusing analyzer afforded mass ions with m/z values of 368.31, 337.77, and 307.95 for C, DMC, and BDMC respectively. Identity of each compound was confirmed based on mass fragmentation pattern, retention times and retention indices compared from Mass spectral libraries. The molecular ion peak at m/z for C, DMC, and BDMC corresponded to the molecular formula C_{21}H_{20}O_{6}, C_{20}H_{18}O_{5}, C_{19}H_{16}O_{4} respectively.

**FTIR**

Molecular vibrations give rise to IR band and change in dipole moment of the molecule. The presence of stretch changes dipole moment resulting in IR band. The different absorption of varying stretch denotes the presence of functional group in the molecule. FTIR spectra are that confirm the structure of C, DMC, BDMC, the presence of O-H, C=O, C=O, =C-O-CH_{3}, C-OH stretch and aromatic stretch confirms the identity of curcuminoid structure. Only in BDMC shows absence of aliphatic C-H stretch corresponding to methoxyl group, thus three curcuminoids differs in structure.

**^{13}C NMR and ^{1}H NMR spectra**

In ^{13}C NMR the signal for methoxyl group is found in C and DMC occurred at 56.1. There is no signal at 56.1 in BDMC spectrum which denotes absence of methoxyl group in their structure.
In \(^1\text{H} \) NMR spectra the signals for aromatic and hepta-1,4,6-triene-3-one-5-ol moieties with their relative integral values are commonly found in all three curcuminoids. The signal for the methoxyl protons at 3.84 for methoxyl group was found in C and DMC and absent in BDMC. There are two methoxyl group present in C at 2\(^{nd}\) and 16\(^{th}\) position and DMC contains one methoxyl group at 2\(^{nd}\) position methoxyl group completely absent in BDMC.

**Cytotoxicity of curcuminoids on MDR cells**

MTT proliferation assay was used to determine the relative cytotoxicity of curcuminoids in KB and KBChR8-5 cell lines. Although three forms of curcuminoids interact with p-gp expressing cells and results in cytotoxicity in the concentration range \( \text{IC}_{50}\) values 5-25\(\mu\)M. Chearwae et al., studied cytotoxic activity of three curcuminoids on KB-V1 and its parental cell lines results in \( \text{IC}_{50}\) range 25-90\(\mu\)M cytotoxicity of each compound on both cell lines are nearly equal. Curcumin showed greater cytotoxic effect (Chearwae et al., 2004). In our study BDMC showed greater cytotoxic activity The \( \text{IC}_{50}\) value of curcuminoids on MDR subline was nearly equivalent to that of parental cell line. This suggest that if curcuminoids enters cell through multidrug transporter there should be significant difference in \( \text{IC}_{50}\) values of resistant and parental cell lines but \( \text{IC}_{50}\) value of KBChR8-5 and KB cells do not posses much difference in cytotoxicity which implies that curcuminoids may not be transported by multidrug transporter and block the efflux pump for cytotoxic activity instead it may enter through other process to induce anticancer activity.

**Assessment of collateral sensitivity**

Collateral sensitivity is assessed *in vitro* by determining the cytotoxicity (\( \text{IC}_{50}\)) of a compound against a parental line relative to its MDR subline. It is calculated as the ratio of compounds \( \text{IC}_{50}\) for parental cell divided by its \( \text{IC}_{50}\) for MDR cells. A RR value >1 indicates that the compound kills MDR cells more effectively than parental cells (Bradley et al., 1989; Hall et al., 2009). The RR value for C and DMC was <1 and BDMC was 1.5 which indicates BDMC kills KBChR8-5 cells more effectively than parental KB cells. MDR cell population is collaterally sensitive to BDMC. RR value for
C and DMC were <1 indicating that cells are resistance to that drug relative to parental cell lines. Relative drug resistance of KBChR8-5 cells for curcuminoids was calculated by ratio of IC₅₀ for MDR cells divided by IC₅₀ for parental cell. The relative drug resistance was 1.04 and 1.17 times more resistant to C and DMC respectively than parental cell line.

**MDR-1 gene expression studies on curcuminoids treatment in KB cell lines**

Limtrakul *et al.*, demonstrated in preliminary studies of the effect of purified curcuminoids on the expression of MDR-1 gene in KB-V1 cells indicated that BDMC had more potent inhibitory effect on MDR-1 expression (Limtrakul *et al.*, 2004). Though curcuminoids showed cytotoxicity at varying levels the expression of MDR 1 gene may vary with treatment of curcuminoids. KB cells were treated at concentration of two fold less than IC₅₀ values. Effect of crude curcuminoids mixtures and pure curcumin, DMC, BDMC on MDR1 gene expression on KB-V1 cells showed potent inhibitory effect of MDR-1 gene expression at 5µM (Limtrakul *et al.*, 2004). In our study though KB cells showed very mild expression in untreated cells, but study on the effect of curcuminoids on KB cells for MDR-1 gene expression was done, the KB cells were treated at 5µM and each curcuminoids showed varying inhibitory effect. The KB cells on treatment with BDMC showed significant inhibitory effect on MDR-1 gene studied by real time PCR.

**MDR-1 gene expression studies on curcuminoids treatment in KBChR8-5 cell lines**

Drug resistance studies were evaluated with cell lines which have greater expression of MDR-1 gene, KBChR8-5 is MDR overexpression cells. Since KB cells shows mild expression of MDR, the effect of curcuminoids on MDR gene has been studied in KBChR8-5 cell lines which is derived from parental KB cells. In the vinblastine resistant cell line KB-V1 cells the expression of MDR1 mRNA was studied on KB-V1 cells after treatment with 5µM of crude curcuminoids, pure curcumin and Bisdemethoxycurcumin were shown to be decreased by 13%, 30%, 49% but Demethoxycurcumin showed no significant level of decrease of MDR-1mRNA (Limtrakul *et al.*, 2004). Tetrahydrocurcumin the major metabolite of curcumin inhibited all three major ABC transporter (Chearwae *et al.*, 2007). In our study colchicine resistant
KBChR8-5 cells on treatment 5µM of each curcuminoids results in different range of inhibition of MDR 1 gene. Curcumin showed most effective inhibitory level than other two curcuminoids, DMC showed 0.94 fold, BDMC showed 1.01 fold, but curcumin 1.47 fold decreases in MDR-1 expression. Treatment of curcumin at 5µM showed significant inhibitory effect with level of fold change at 1.47% inhibition. This result suggests that curcumin inhibits MDR1 gene expression but not directly involved in cytotoxicity when compared with other two curcuminoids. It may help in reduced production of p-gp pump which may enhance the susceptibility of tumor cells to apoptosis induced by anticancer drugs. Curcumin can be used as MDR modulators or reversing agents can be used in combination of anticancer drugs (vinblastine, vincristine, doxorubicin) to circumvent drug resistance which can subvert the cell’s defense on MDR cells.

**Co-incubation of curcumin with doxorubicin**

Polyphenolic compounds that occur ubiquitously in food of plant origin have the advantage of being natural dietary compounds that are nontoxic in animals have been studied for MDR reversing agents. Flavonoids like kaempferol and daidzein at a concentration of 30µM had a significant decrease of vinblastine IC₅₀ in the resistant KB-V1 cells. The reversing effects of these flavonoids were concentration dependent (Orawan Khantamat et al., 2004). ET-743 is a marine alkaloid can partially reverses the resistance to doxorubicin and vincristine with increased accumulation of these two drugs by down-regulating expression of P-gp (Atsuko et al., 2002). A major metabolite of curcumin called THC increased the sensitivity of vinblastine, mitoxantrone and etoposide in drug-resistant human cervical carcinoma cell line (Chearwae et al., 2007). Curcumin act as MDR modulator reduces the IC₅₀ of vinblastine about 5.6 fold at the concentration of 15µM. in KB-V1 cells (Chanmahasathien et al., 2011). Co-incubation of MDR modulators with anticancer drugs increases the sensitivity of chemotherapeutic agents.

Doxorubicin is a chemotherapeutic agent principally used for treatment of wide range of cancer like hematological malignancies, many types of carcinoma and soft tissue sarcoma. Curcumin reversed MDR of doxorubicin or daunorubicin in K562 / DOX cell line and expression of P-gp decreased in time dependent manner (Chang et al., 2006).
In resistant KBV20C cell line the cytotoxicity of vincristine and paclitaxel were partially restored by curcumin (Um et al., 2008). Colchicine resistant KBChR8-5 cells are also cross resistant to certain general drugs used in cancer chemotherapy. Therefore combination of curcumin with doxorubicin was studied. Cytotoxic assay was carried out in treatment of KBChR8-5 cells with doxorubicin alone and co-incubation of curcumin 5µM with varying concentration of doxorubicin. The IC$_{50}$ value of doxorubicin alone showed 10.68µM and co-incubation of curcumin with doxorubicin showed IC$_{50}$ value at 4.56 which implies that combination resulted in significant increase in cytotoxicity of doxorubicin in KBChR8-5 cells. This result suggested that curcumin inhibits MDR-1 gene expression and reduces p-gp levels in the cell and allows the accumulation of doxorubicin and results in cytotoxic activity by apoptosis induced by doxorubicin.

**Confocal microscopy**

Further followed by cytotoxic assays apoptotic cells were analysed by confocal microscopy. Salvine treated K562 / A02 cells displayed the dose-dependent morphologic changes of apoptosis including apoptotic bodies, nuclear condensation and cell shrinkage when stained with DAPI and observed under fluorescence microscope (Ze-Hong Miao et al., 2003). KBChR8-5 cells treated with pure doxorubicin and curcumin showed increased number of early and late apoptotic cells. There is a drastic change in number of apoptotic cells and morphological changes were observed in co-incubation of two drugs when stained with AO/EtBr. Orange labeled cells indicating a dead cell which implies co-incubation of drugs with increased cytotoxic effect has been observed microscopically. Whereas co-incubation of doxorubicin with curcumin resulted in increased number of cells that taken orange label indicating dead cells which implies co-incubation of drugs results in increased cytotoxic effect.

**Flow cytometry**

The cells undergoing cell apoptosis leads to cell cycle arrest. Analysis of Cell cycle distribution was done by flow cytometry. Curcumin induced G2/M arrest in HL-60 cells in dose dependent manner in the treatment of leukemia (Tzu-Wei Tan et al., 2006). In bladder cancer cells curcumin increased the percent of apoptosis significantly and
analysis revealed that 40µM curcumin arrested the cell cycle at the G2/M phase (Binqiang Tian et al., 2008). There is an increase in apoptosis in KBChR8-5 cells with curcumin (5µM) treated for 24 hours showed affecting G0-G1 stage; there is no significant difference in on further incubation at 48 hours. Co-incubation of curcumin (5µM) with doxorubicin (10µM) at 24 hours showed apoptotic cells remind high and arresting cell cycle at G2-M phase whereas on further incubation at 48 hours majority of cells undergoing apoptosis are accumulated in G2-M phase. Combination of drugs which implies as potent inhibitor of cell cycle at G2-M phase of KBChR8-5 cells thus acts as anti-MDR activity.