5. DISCUSSION

The objective of the research work was to develop a novel oral formulation of lycopene and curcumin with enhanced bioavailability due to increased solubility and controlled release. The system comprises osmotic controlled release formulations of lycopene and curcumin through osmotically controlled asymmetric membrane capsule, with enhanced solubility using solid dispersion of drug.

Lycopene is the main carotenoid in tomatoes and tomato products and occurs also naturally in some other fruits and vegetables, guava, watermelon and pink grapefruit. Among the common dietary carotenoids, lycopene has the highest singlet oxygen capacity in-vitro. However, the extremely high lipophilicity (log \( P = 17.64 \)) of lycopene resulting in its extremely low aqueous solubility, which is significant barrier to its oral formulation and bioavailability.

Curcumin, a highly pleiotropic molecule with an excellent safety profile targeting multiple diseases with strong evidence on the molecular level, could not achieve its optimum therapeutic outcome in past clinical trials, largely due to its low solubility and poor bioavailability. Thus it was hypothesized that oral bioavailability of nutraceuticals can be enhanced by formulating them as osmotic drug delivery system.

5.1. Preformulation studies of Lycopene

The gift sample of lycopene was authenticated by various methods such as organoleptic, chromatographic and spectroscopic methods. The sample of lycopene exhibited similar colour, odour and texture as given in compendia. The melting point of lycopene was found to be 172±1.98°C which was within the range as reported in the literature (The Merck Index, 1996) confirmed identity of the drug. Thin Layer Chromatography was also performed and the solvent system used was mixture of 15 % v/v acetone in petroleum ether. The Rf value of the lycopene was found to be 0.89 ± 0.013 which was almost similar as reported in the literature (Sherma et al., 2003), proved the identity of the drug. The UV spectroscopic studies also confirmed the identity of drug as it gave similar values of \( \lambda_{\text{max}} \) (maximum absorbance wavelength) as given in literature (Naviglio et al., 2008). The authentication of the lycopene was further confirmed by IR spectroscopy. The IR spectrum of lycopene was analyzed to confirm the presence of various functional groups present in lycopene. The major peaks of functional groups and the finger print
region of sample drug were found to be similar as that of reference (Kamil et al., 2011) which confirmed the authenticity of lycopene.

The calibration curve of lycopene was prepared in phosphate buffer (pH 7.4), distilled water and 0.1 N HCl (pH 1.2). The plots between different concentrations of lycopene and absorbance were found to be linear in concentration range of 5-30 µg/mL with a regression coefficient of 0.9995 (phosphate buffer, pH 7.4), 0.9998 (distilled water), 0.9996 (0.1N HCl, pH 1.2) at 472 nm using UV spectrophotometer (Systronics, 2202). The HPLC method for lycopene was developed and validated. The developed method was found to be precise with LOD and LOQ of 0.00243µg/mL and 0.00738µg/mL respectively. The calibration curve of lycopene was found to be linear in concentration range of 0.5 to 3µg/mL. Solubility of lycopene was determined by equilibrium solubility method and it was found that solubility of lycopene was less in 0.1N HCl (pH 1.2) as compared to phosphate buffer (pH 7.4). The solubility of lycopene was modulated by forming inclusion complex with β-cyclodextrin.

### 5.2. Solid Dispersion of Lycopene

The solid dispersions of lycopene with β-cyclodextrin (LSDs) in different weight ratios (1:1 to 1:5) were successfully prepared by solvent evaporation method. The solubility of lycopene was significantly increased by forming solid dispersion with β-cyclodextrin, which may be due to conversion of crystalline form of lycopene in amorphous form, which was indicated by X-ray diffraction study. X-ray diffractogram of inclusion complex of lycopene revealed less intense peaks as compared with the X-ray diffractogram of lycopene, which may be due to the inclusion of drug into β-cyclodextrin cavity. The cytotoxicity of lycopene and solid dispersion of lycopene against MCF-7 cells were evaluated by MTT assay. The inhibitory rate of solid dispersion of lycopene to MCF-7 cells was higher than free drug which is due to the enhanced solubilization of solid dispersion of lycopene as compared to pure drug. Drug excipients compatibility study was carried out by using TLC and IR spectra analysis to determine the interaction between drug and carriers in physical mixture. There was no extra spot of polymer in case of physical mixture revealed compatibility of drug and polymer. Further the Rf value was also found to be same as that of drug in physical mixture. IR spectra analysis of drug, carrier and combination of drug and carrier confirmed compatibility of drug and carrier as there was no shift found in vibrational or stretching bands of key functional groups in physical mixture.
Measurement of the osmotic activity of the solid dispersion of lycopene was determined using a thistle tube. The final level of solution in tube was found to be the same as compared to the initial level of the solution, thus suggesting that the lycopene is osmotically inactive so there is need to incorporate osmogen into the formulation.

5.3. Characterization of Asymmetric Membrane Capsule

AMCs were prepared by dip coating method. AMCs were compared with conventional gelatin capsules (CGC) for appearance and geometric characterization. AMCs showed higher opacity as compared to CGCs and revealed high degree of similarity in dimensions with CGCs (p< 0.005). Capsule shell rupture force was determined and resulted in high rupture force (870.59 ± 2.15 g) for AMC suggested more rigidity of prepared AMC. The AMC had low moisture content and plasticizer content evident by high rupture force value indicated no influence of environmental conditions on AMC.

SEM photomicrographs of developed AMCs indicated outer dense region and inner porous region revealed the asymmetric nature of capsules which is desirable for osmosis. This might be due to incorporation of plasticizer (glycerol), which acted as pore former.

In-vitro release studies were performed for developed AMCs in USP-II dissolution apparatus. The best formulation was selected based on the optimization studies using central composite design. In-Vitro release kinetics for the AMCs of solid dispersion of lycopene was determined by fitting the dissolution data to various kinetic models and best model was selected based on goodness of fit (regression coefficient). Among the various formulations, F18 exhibited best pattern of zero order release with highest value of $r^2$ (0.9994).

5.4. Optimization Study

The effect of independent variables such as ethyl cellulose ($X_1$), glycerol ($X_2$) sodium chloride ($X_3$) over the dependent variables like cumulative % drug release ($Y_1$) and correlation coefficient of drug release ($Y_2$) was assessed by using central composite design. The results of in-vitro release suggested decrease in drug release with increase in the level of ethyl cellulose (EC). This might be due to the fact that with higher level of EC, the drug has to travel more path length. Due to this reason higher concentration of EC impedes the drug release. Glycerol and NaCl also affected the release rate in the manner that on increasing the level of Glycerol and NaCl, the release rate was found to increase due to fact that higher level of glycerol will result in more
porous structure of inner layer of AMCs and higher porosity of inner surface resulted in higher
drug release and in case of NaCl, higher osmotic pumping effect caused by high concentration of
osmogen leads to increase in drug release.
The correlation coefficient was found to increase with increase in the level of EC. Increase in the
level of glycerol caused decrease in correlation coefficient which might be due to more porous
inner structure of AMC resulted in irregular drug release. Increase in correlation coefficient with
increase in the level of NaCl which was followed by decrease in correlation coefficient might be
due to the reason that increase in level of NaCl provided adequate osmotic pressure making
hydration of core of capsule and resulted in gentle release profile.
On the basis of maximum value of % cumulative drug release ($Y_1$) and correlation coefficient of
drug release ($Y_2$), optimized formulation was selected. $F_{18}$ was selected as optimized formulation
having 85.63% of drug release and 0.9994 of correlation coefficient of drug. Further the $F_{18}$ was
subjected to analyze the effect of osmotic pressure and agitational intensity on in-vitro drug
release. Release studies of lycopene from AMC were performed to analyze mechanism of drug
release indicated that the release was highly dependent on osmotic pressure of release medium.
Lycopene release from AMC with no NaCl inside and outside showed uncontrolled release while
AMC with 100 mg of NaCl inside and 0 mg outside showed controlled release due to creation of
molar environment by osmogen (NaCl). The AMC with 100 mg of NaCl inside and 50 mg
outside exhibited slow release with plateau reaching in sixth hour might be due to decreased
osmotic gradient. All the results suggested osmotic pumping as a primary mechanism of drug
release from AMC.
Lycopene release from AMC was independent of agitational intensity of dissolution media as
there was non-significant difference in release of drug at different rpm ($p<0.01, f_2 = 98.17$).
In-vitro release studies were performed to analyze the effect of any defect in the asymmetric
membrane on the release kinetics of optimized formulation ($F_{18}$). In-vitro drug release profile of
intentionally defected formulation was compared with optimized $F_{18}$ and there was no significant
difference at $p<0.05$. Lycopene release from $F_{18}$ containing hole 2 mm in size is not different
from the control $F_{18}$ formulation.

5.5. In-Vivo Pharmacokinetic Study

The pharmacokinetic parameters were determined by performing the in-vivo studies in rabbit
model. The mean pharmacokinetic parameters for lycopene and asymmetric membrane capsules
of lycopene are given in table 4.19 and mean plasma drug concentration vs. time profile for both administrations is presented in fig. 4.23. Results of in-vivo pharmacokinetic studies exhibited discernible difference in the plasma drug concentration between lycopene and asymmetric membrane capsules of solid dispersion of lycopene. The C_{max} was found to be 16.5 ng/mL and 105.32 ng/mL with oral administration of lycopene and F_{18} respectively after 12 h. The results showed a significant difference between the maximum plasma drug concentrations between lycopene and F_{18}. This may be due to the enhanced solubility and controlled release of drug from F_{18} formulation. There was significant or noticeable difference between AUC (area under the curve) of lycopene (414.311 ngh/mL) and F_{18} (1357.21 ngh/mL) which is due to the more absorption of lycopene from F_{18} formulation because of enhanced solubilization of drug from F_{18}. As evident from the results, the bioavailability of F_{18} was 3.28 fold that of pure lycopene. So therefore it is reasonable to conclude that asymmetric membrane capsules of solid dispersion of lycopene enhances the absorption and retention time of lycopene in-vivo significantly.

Level A in vitro- in vivo correlation was investigated by plotting the percent dissolved vs percent absorbed data. A good linear regression relationship (Level A) was observed between percent dissolved and percent observed with correlation coefficient (r^2) of 0.9775. Level A correlation represented a point to point correlation between in-vitro dissolution rate and in-vivo input rate, which meant that by using in-vitro dissolution profile of lycopene from asymmetric membrane capsules containing solid dispersion of lycopene, one can also predict their in–vivo performance.

5.6. Preformulation studies of Curcumin

The gift sample of curcumin was identified by various organoleptic, physicochemical and spectroscopic methods. The sample of curcumin possessed similar color, odor and texture as given in officials. The melting point of obtained sample was found to be 180±1.74°C which was within the range as reported in the literature (Merck Index, 1996) confirmed identity of the drug. Thin Layer Chromatography was also performed and the solvent system used was chloroform: methanol (9:1). The Rf value of the curcumin was found to be 0.91 ± 0.025 which was almost same as reported in the literature (Vedamurthy et al., 2010), confirmed the identity of the drug. The identification of the drug was further confirmed by its IR spectroscopy in which the spectrum was analyzed to confirm the presence of various functional groups present in curcumin.
The major peaks of functional groups and the fingerprint region of sample drug were found to be same as that of reference (Zhao et al., 2010) again confirmed the authenticity of sample drug.

The calibration curves of Curcumin were prepared in 0.1 N HCl (pH 1.2), distilled water and in phosphate buffer (pH 7.4). The graph between different concentrations of curcumin and absorbance were found to be linear in the concentration range of 5-30 µg/mL with a regression coefficient of 0.9998 (phosphate buffer pH 7.4), 0.9995 (distilled water), 0.9998 (0.1N HCl pH 1.2) at 432 nm using UV spectrophotometer (Systronics, 2202).

The HPLC method for curcumin was developed and validated. The developed method was found to be precise with LOD and LOQ of 0.00194µg/mL and 0.00659µg/mL respectively. The calibration curve of curcumin was found to be linear in concentration range of 0.5 to 3µg/mL. Solubility of curcumin was determined by equilibrium solubility method and it was found that solubility of curcumin was less in 0.1N HCl (pH 1.2) as compared to phosphate buffer (pH 7.4). The solubility of curcumin was modulated by forming inclusion complex with β-cyclodextrin.

**5.7. Solid Dispersion of Curcumin**

The solid dispersions of curcumin with β-cyclodextrin (CSDs) in different weight ratios (1:1 and 1:2) were successfully prepared by solvent evaporation method. The solubility of curcumin was significantly increased by forming solid dispersion with β-cyclodextrin, which may be due to conversion of crystalline form of curcumin in amorphous form, which was indicated by X-ray diffraction study. X-ray diffractogram of inclusion complex of curcumin revealed less intense peaks as compared with X-ray diffractogram of curcumin, which may be due to the inclusion of drug into β-cyclodextrin cavity. The cytotoxicity of curcumin and solid dispersion of curcumin against MCF-7 cells were evaluated by MTT assay. The solid dispersion of curcumin showed enhanced cytotoxicity against MCF-7 cells as compared to the free drug which may be due to the improved solubility of curcumin by forming inclusion complex with β-cyclodextrin. Drug excipients compatibility study was carried out by using TLC and IR spectra analysis to determine the interaction between curcumin and β-cyclodextrin in physical mixture. There was no extra spot of polymer in case of physical mixture revealed compatibility of drug and polymer. Further the Rf value was also found to be same as that of drug in physical mixture. IR spectra analysis of curcumin, β-cyclodextrin and combination of both confirmed compatibility of drug and carrier as
there was no shift found in vibrational or stretching bands of key functional groups in physical mixture.

5.8. Characterization of Asymmetric Membrane Capsule

SEM photomicrographs of developed AMCs indicated outer dense region and inner porous region revealed the asymmetric nature of capsules which is desirable for osmosis. The pores in the membrane were created due to incorporation of plasticizer (glycerol), which acted as pore former.

*In-vitro* release studies of asymmetric membrane capsules of solid dispersion of curcumin were performed in 2 groups as per the $2^3$ factorial design. In group 1, incorporation of higher amounts of osmogen (AMC 3) resulted in the highest release due to creation of high osmotic pressure. When the pore former (glycerol) was at a higher concentration (AMC 3), the release from this formulation was slightly higher may be due to the fact that higher amount of glycerol resulted in increased pore formation on the membrane surface during dissolution. When ethyl cellulose concentration was at higher (AMC 2), the release of curcumin from the asymmetric membrane capsule was constrained as compared with AMC 1 formulation. The decreased *in vitro* release from AMC 2 might be due to the increase in diffusional path for the curcumin to transverse before being released into the dissolution medium. In second group, when pore former as well as the osmotic agent were at higher concentrations (AMC 7), there was an increase *in vitro* release with faster achievements of $t_{50\%}$ (5.9 h). This effect might be due to the combination of increased pore formation on the capsule membrane and increased solubility of curcumin within the formulation thereby helping in faster release of curcumin.

5.9. Optimization Study

The release profiles of Curcumin from all formulations in the dissolution medium were statistically compared with release rate profiles of the theoretical formulation (extra design checkpoint batch), which was obtained by using the polynomial equation. The statistical significance was tested at $P < 0.05$. The best formulation among the non-significant pairs of formulations was found to be AMC 6, which gave the value of $f_2$ (similarity factor) as 90.68.

*In-Vitro* release kinetics for the AMCs of solid dispersion of curcumin was determined by fitting the dissolution data to various kinetic models. While considering higher correlation
coefficient value (R), the release data seems to fit Zero-order model better. According to correlation coefficient value (R) of release models, AMC 6 seems to be the best formulation. *In-vitro* release data, for AMC 6 formulation further analyzed for exponent of drug release and $n = 0.4273$ confirmed that release of curcumin from AMC 6 formulation was fickian type.

The effect of osmotic pressure of dissolution media was determined performing the *in-vitro* release studies of optimized formulation (AMC 6) in presence of dissolution medium of different osmotic pressure. *In-vitro* drug release was found to depend upon osmotic pressure of media. As the osmotic pressure of the media increased, curcumin release from the asymmetric membrane capsules decreased. This is probably due to the decrease in osmotic gradient which suggested that the primary mechanism which governs the drug release from optimized formulation is osmotic pumping.

The effect of intentional defect on *in-vitro* drug release of curcumin release was also studied. *In-vitro* release profile of intentionally defected formulation was compared with optimized formulation using one-way ANOVA. The calculated F-value (1.019) and t-value (0.06058) was found to be less than tabulated F-value (4.22) and t-value (2.054), respectively and P value was 0.9522, which was not significant. Curcumin release from AMC6 containing hole 2 mm in size is not different from the control AMC6 formulation.

The effect of agitational speed on the release of curcumin was also analyzed. *In-vitro* release profile(s) at three different agitational speeds was compared using one-way ANOVA. The calculated F-value (0.002128) was found to be less than tabulated F-value (3.37), thus indicating that the change in agitational intensity do not have any significant effect on release profiles of asymmetric membrane capsules. This study describes the fact that the *in vitro* release from the asymmetric membrane capsules is independent of the hydrodynamic conditions of the body.

### 5.10. *In-Vivo* Pharmacokinetic Study

The pharmacokinetic parameters were determined by performing the *in-vivo* studies in rabbit model. The mean pharmacokinetic parameters for curcumin and asymmetric membrane capsules of curcumin were given in table 4.41 and mean plasma drug concentration vs. time profile for both administrations was presented in fig. 4.46. Results of *in-vivo* pharmacokinetic studies exhibited discernible difference in the plasma drug concentration between curcumin and
asymmetric membrane capsules of solid dispersion of curcumin. The $C_{\text{max}}$ was found to be 22.09 ng/mL and 170.58 ng/mL with oral administration of curcumin and AMC6 respectively after 12 h. The results showed a significant difference between the maximum plasma drug concentrations between curcumin and AMC6. This may be due to the enhanced solubility and controlled release of drug from AMC6 formulation. There was significant or noticeable difference between AUC (area under the curve) of curcumin (376.549 ngh/mL) and AMC6 (1547.88 ngh/mL) which is due to the more absorption of curcumin from AMC6 formulation because of enhanced solubilization of drug from AMC6. As evident from the results, the bioavailability of AMC6 was 4.11 fold that of pure curcumin. So, therefore; it is reasonable to conclude that asymmetric membrane capsules of solid dispersion of curcumin enhances the absorption and retention time of curcumin in-vivo significantly.

Level A in vitro- in vivo correlation was investigated by plotting the percent dissolved vs percent absorbed data. A good linear regression relationship (Level A) has been observed between percent dissolved and percent absorbed with correlation coefficient ($r^2$) of 0.9637. Level A correlation represented a point to point correlation between in-vitro dissolution rate and in-vivo input rate, which meant that by using in-vitro dissolution profile of curcumin from asymmetric membrane capsules containing solid dispersion of curcumin, one can also predict their in-vivo performance.

The stability studies of optimized formulations (AMC 6 and F$_{18}$) were performed in accordance to ICH Q1 A guidelines for 6 months to investigate the influence of humidity and temperature on appearance and in-vitro drug release of curcumin and lycopene. Capsule texture and color remained unchanged and AMC6 and F$_{18}$ showed comparable release profile after 6 months also, which proved the stability of asymmetric membrane capsules. Thus, suggesting that there was no problem of stability for asymmetric membrane capsules of curcumin and lycopene.