STUDIES ON SOME ABSORPTION PROMOTERS OF HERBAL ORIGIN

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BY

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M. Pharm. (Herbal Drug Technology)

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This is to certify that the thesis entitled "Studies on Some Absorption Promoters of Herbal Origin" represents bonafide and genuine research work of Mr. Ashvin V. Dudhrejiya carried out unde my guidance and supervision. The work presented in this dissertation was carried out at the Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, India and upto my satisfaction.

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I hereby declare that thesis entitled "**Studies on Some Absorption Promoters of Herbal Origin**" is a bonafide and genuine research work carried out by me, under the guidance of **Prof. (Dr.) Navin R. Sheth,** Head, Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, India. The results presented in this dissertation are original and has not been submitted in part or full for any degree and diploma to any University.

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Dedicated

To

Almighty god

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Ashvin Dudhrejiya....

CONTENTS

A. ABBREVIATION

Abbreviation	Full name
5'-MHC	: 5'-Methoxyhydnocarpin
ABC	: ATP binding cassette
ADP	: Adenosine diphosphate
ANOVA	: Analysis of variance
ATP	: Adenosine triphosphate
AUC	: Area under the curve
BCS	: Biopharmaceutical classification system
BL	: Basolateral membrane
cGMP	: Cyclic guanosine monophosphate
CHEs	: Chinese herbal Promoters
CIMP	: Central Instistute of Medicinal and Aromatic Plants
CMC	: Carboxy methyl cellulose
CV	: Coefficient of variance
CYP	: Cytochrome P
DHB	: 6', 7'-Dihydroxybergamotin
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DSC	: Differential scanning calorimetry
EGCG	: Epigallocatechin gallate
FTIR	: Fourier transfer infrared
GFJ	: Grapefruit juice
GI	: Gastro intestinal
HAPs	: Herbal absorption promoters
H-DIs	: herb-drug interactions
HIV	: Human immunodeficiency virus
HPLC	: High pressure liquid chromatography
HTLV	: Human T-cell lymphotrophic viruses
i.v.	: Intravenous
IR	: Infra red
LDL	: Low density lipoprotein
LLOQ	: Lower limit of quantification

LOD	: Limit of detection
LQC	: Limit of quantification
mAU	: mili absorbance unit
mmol	: Mili mole
MDR	: Multidrug resistance
mg	: Milligram
min	: Minutes
ml	: Mililitre
MRP	: Multidrug resistance associated protein
mtr	: Meters
MTT	: dimethylthiazolyl diphenyltetrazolium bromide dye
NCE	: New chemical entity
nm	: Nanometer
NO	: Nitric oxide
OD	: Optical density
p.o.	: Per oral dose
PDE5	: Phospphodiesterase type 5
P-gp	: Para glycoprotein
PLGA	: Poly lactic acid glycolic acid
RH	: Relative humidity
Rho-123	: Rhodamine-123
RSD	: Relative standard deviation
SAR	: Structure activity relationship
SRB	: Sulforhodamine B
TCA	: Trichloro acetic acid
TEER	: Trans epithelial electric resistance
µg	: Microgram
µl	: Micro liter
µmol	: Micro mol
US-FDA	: United states- Food and drug administration
W/O/W	: water in oil in water

B. List of tables

C. List of figures

D. ABSTRACT

Poor absorption and bioavailability of drugs adopt challenges in efficient treatment of the patients. One of the widely used approaches to solve this issue is co-administration of absorption promoters. Synthetic absorption promoters have been found to be associated with mucosal damage and cytotoxicity. Herbal absorption promoters (HAPs) provide extremely promising alternative to the synthetic chemical counterparts in terms of safety and many times efficacy.

About 90 % of the absorption in gastro intestinal (GI) tract occurs in the small intestine. Mostly drugs are metabolized by cytochrome P (CYP) 450 and P-glycoprotein (P-gp) enzymes at intestinal cell wall. From these about 90 % of the drugs were metabolized by CYP 450 and more than 50 % of drugs were metabolized by CYP 3A4 which is the most abundant CYP 450 isoenzymes in humans and was responsible for the metabolism of the widest range of drugs. Some of these substrates i.e. amiodarone, carbamazepine, amitriptyline, nefazadone, sertraline, benzodiazepines, Ca^{+2} channel blockers, astemizole, terfenidine, buspirone, ciprofloxacin, lansoprazole, statins, and cisapride, sildenafil citrate, zidovudine, macrolide antibiotics, fluoxetine, fluvoxamine, grapefruit juice (GFJ), Indinavir, Itraconazole, ketoconazole and kitonavir etc. Some flavanoidal component i.e. silymarin, morin and naringin which are mostly found in fruits and plants and recognized as safe by FDA are and reported to interact with CYP 450 and P-gp and inhibit them and increases absorption of drugs.

In this research we selected three HAPs i.e. silymarin, morin and naringin. These selected herbal constitutes are studied on laboratory animals with selected modern drugs to check the effect of this herbal constituents on their permission through intestine. The major mechanism found for drug absorption retardation through intestine is CYP 450 and P-gp efflux pump so on basis of the mechanism drugs were selected. Atorvastatin calcium and sildenafil citrate which is reported to metabolize by CYP 450 while zidovudine and carvedilol phosphate reported to metabolize by the P-gp efflux pump. So in

present study these four allopathic drugs for with three HAPs for were studied for their effect on intestinal permission of selected drugs.

For the proving the compatibility study of selected HAPs with selected allopathic drug the Infra red (IR) spectroscopy and differential scanning colorimeter (DSC) analysis was performed and from these studies it was observed that there were not any major changes reported. So we can say that mixing of selected allopathic drugs with selected HAPs and can not cause any serious compatibility problems and HAPs i.e. silymarin, morin and naringin can be used together as additives with selected drugs i.e. atorvastatin calcium, sildenafil citrate, zidovudine and carvedilol phosphate.

The methods for analysis of selected drugs were developed on very sensitive, selective and precise instrument of HPLC binary system and analyzed by LC solution software approved by US-FDA and subsequently the methods were validated and results were found within the range in validation parameter. So this method is used for the analysis of selected drugs.

Atorvastatin in oral dose of 75 mg/kg when administered alone (control group) as well as with selected HAPs in oral dose of 100 µmol (test group) i.e. with naringin the C_{max} was increased significantly by 19.66 % in test group and with silymarin the C_{max} was increased significantly by 32.31 % in test group and the, while with morin the T_{max} remains 35 min in both control and test groups while C_{max} was decreased by 14.23 % in test group.

Sildenafil citrate in oral dose of 30 mg/kg when administered alone (control group) as well as with selected HAPs in oral dose of 100 µmol (test group) i.e. with naringin the C_{max} was decreased by 1.91 % in test group and with silymarin the C_{max} was increased significantly by 24.88 % in test group, while with morin the C_{max} was increased significantly by 11.09 % in test group.

Zidovudine in oral dose of 75 mg/kg when administered alone (control group) as well as with selected HAPs in oral dose of 100 µmol (test group) i.e. with

naringin the C_{max} was increased significantly by 60.49 % in test group and with silymarin the C_{max} was increased significantly by 28.32 % in test group, while morin was increased the C_{max} by 16.17 % in test group.

Carvedilol phosphate in oral dose of 60 mg/kg when administered alone (control group) as well as with selected HAPs in oral dose of 100 µmol (test group) i.e. with naringin the C_{max} was increased significantly by the 28.01 % in test group and with silymarin the C_{max} was increased significantly by the 31.95 % in test group, while with morin the C_{max} was increased significantly by 5.67 % in test group.

HAPs may exhibit a very high benefit to risk ratio. Proposed study will provide an alternative approach to reduce the therapeutic dose and thereby minimizing the side effects of drugs and decrease the dose of drug and cutting down the cost of therapy.

1.0 INTRODUCTION

The absorption of drugs from the oral route via GI track is a subject of interest and continuous investigation in pharmaceutical industry as good bioavailability implies that the drug is able to reach the systemic circulation by mouth. Oral drug absorption is affected by both drug properties and the physiology of the GI tract or patient properties, including drug dissolution from the dosage forms, the manner drugs interacts with aqueous environment and membrane, permeation across membrane and irreversible removal by first-pass organs such as the intestine liver and lung (Martinez et al., 2002).

Oral route is most preferred for drug delivery due to high patient compliance, non-invasive administration and low cost of therapy. Sufficient absorption of administered drugs from the GI tract is one of the prerequisites for successful therapy (Bohets et al., 2001). Drugs under biopharmaceutical classification system (BCS) class- I absorb well after oral administration and not pose bioavailability related problems; whereas drugs of classes II-IV (i.e. with low solubility, low permeability or both) pose challenges to the pharmaceutical development scientists in developing drug products with acceptable oral bioavailability. Low oral bioavailability of drugs also results in erratic pharmacokinetics (Aungst et al., 1996) and sometimes needs careful management of therapy. Several approaches are employed in alleviating oral bioavailability related problems. One such approach is the concomitant use of absorption promoters (Sharma et al., 1999). Recent *in vitro* studies (Aungst et al., 2000) have demonstrated that synthetic/chemical absorption promoters, such as sodium lauryl sulfate, palmitoylcarnitine, sodium caprate etc., when used at effective concentrations cause cytotoxicity or membrane damage. Limitations of these absorption promoters have suddenly increased interest in herbal absorption promoters (HAPs), once in dormant state.

According to the BCS drug substances are classified as follows (Amidon et al., 1995):

Class I - High Permeability, High Solubility

Example: Metoprolol

The bioavailability of drugs from this class is not having much problem as drug is solvated with fast rate and also absorbed well.

Class II - High Permeability, Low Solubility

Example: Glibenclamide

The bioavailability of those products is limited by their solvation rate.

Class III - Low Permeability, High Solubility

Example: Cimetidine

The absorption and hence bioavailability of drugs of this class is limited by the permeation rate but the drug is solvated very fast.

Class IV - Low Permeability, Low Solubility

Example: Hydrochlorothiazide

Those compounds have a poor bioavailability.

1.1 Bioavailability and drug absorption

Bioavailability is one of the essential tools in pharmacokinetics, it must be considered when calculating dosages for administration of non-intravenous routes. Bioavailability may define as measurement of the extent of active therapeutically drug that reaches the systemic circulation and is available at the site of action. By definition, when a drug is administered intravenously, its bioavailability is 100 %. However, when drug is administered via other routes (such as orally), its bioavailability decreases (due to incomplete absorption and first-pass metabolism).

1.1.1 Absolute bioavailability

Absolute bioavailability compares the bioavailability (estimated as area under the curve or AUC) of the active pharmaceutical drug in systemic circulation following non-intravenous administration (i.e. after oral, rectal, transdermal

and subcutaneous administration), with the bioavailability of the same drug following intravenous administration. The comparison must be consist of dose normalized if different doses are used; consequently, each AUC is corrected by dividing the corresponding dose administered. It is expressed as the letter F (Hoag and Hussain, 2001).

In order to determine absolute bioavailability of a drug, a pharmacokinetic study must be done to obtain a plasma drug concentration vs. time plot for the drug after both intravenous (i.v.) and non-intravenous administration. For example, the formula for calculating F for a drug administered by the oral route (p.o.) is given below.

$$F = \frac{[AUC]_{po} * dose_{IV}}{[AUC]_{IV} * dose_{po}}$$

Therefore, a drug given by the i.v. route will have an absolute bioavailability of 1 (F=1) while drugs given by other routes usually have an absolute bioavailability of less than one.

1.1.2 Relative bioavailability

This measures the bioavailability (estimated as area under the curve, or AUC) of a certain drug when compared with another formulation of the same drug (i.e. Formulation A and B), usually an established standard, or through administration via different route. When the standard consists of intravenously administered drug, it is known as absolute bioavailability.

$$relative\ \ bioavailability = \frac{[AUC]_A * dose_B}{[AUC]_B * dose_A}$$

1.1.3 Factors influencing bioavailability

The absolute bioavailability of a drug, when administered by an extravascular route, is usually less than one (i.e. $F<1$). Various physiological factors reduce the availability of drugs prior to their entry into the systemic circulation Such factors may include, but are not limited to (Robert, 2001):

- Physical properties of the drug (hydrophobicity, pKa, solubility).
- The drug formulations i.e. immediate release, modified release, delayed release, extended release, sustained release, excipient used, manufacturing methods etc.
- If the drug is administered in a fed or fasted state.
- Gastric emptying rate.
- Circadian differences.
- Enzyme induction/inhibition by other drugs/foods:
  Interactions with other drugs (e.g. antacids, alcohol, nicotine).
  Interactions with other foods (e.g. GFJ, pomello, cranberry juice).
- Transporters: Substrate of an efflux transporter (e.g. P-gp).
- Health of the GI tract.
- Enzyme induction/inhibition by other drugs/foods:
  Enzyme induction (increase rate of metabolism) e.g. phenytoin (antiepileptic) induces CYP1A2, CYP2C9, CYP2C19 and CYP3A4
  Enzyme inhibition (decrease rate of metabolism) e.g. GFJ inhibits CYP3A higher nifedipine concentrations.
- Individual variation in metabolic differences.
  Age: In general, drugs metabolized more slowly in fetal, neonatal, and geriatric populations.
  Phenotypic differences, enterohepatic circulation, diet and gender.
- Disease state: e.g. hepatic insufficiency and poor renal function.

Sufficient absorption of administered drugs from the GI tract is one of the prerequisites for successful therapy (Bohets et al, 2001).

1.2 Drug absorption through GI tract

Majority (approximately 90 %) of the absorption in the GI tract occurs in the small intestine, the surface of which has various projections (circular folds, villi and microvilli) that significantly increase the absorptive surface area of small intestine compared to other segments of GI tract. The enterocytes are the most abundant cells which account for majority of the absorption in the small intestine (Engman et al., 2003). The tight junctions present between the enterocytes and the lipophilic nature of the intestinal epithelium serve as a physical barrier for absorption of orally administered drugs (Jackson, 1987). Whereas the metabolizing enzymes, such as aminopeptidases, dipeptidyl peptidase-IV, isoenzymes of CYP450 superfamily, esterases, phenol sulfotransferase, UDP glucuronyltransferase and P-gp etc. expressed by the enterocytes serve as biochemical barrier (Hidalgo, 2001). Drug absorption across the intestinal epithelium may occur by one or more of five different transport pathways, shown in Figure 1.1.

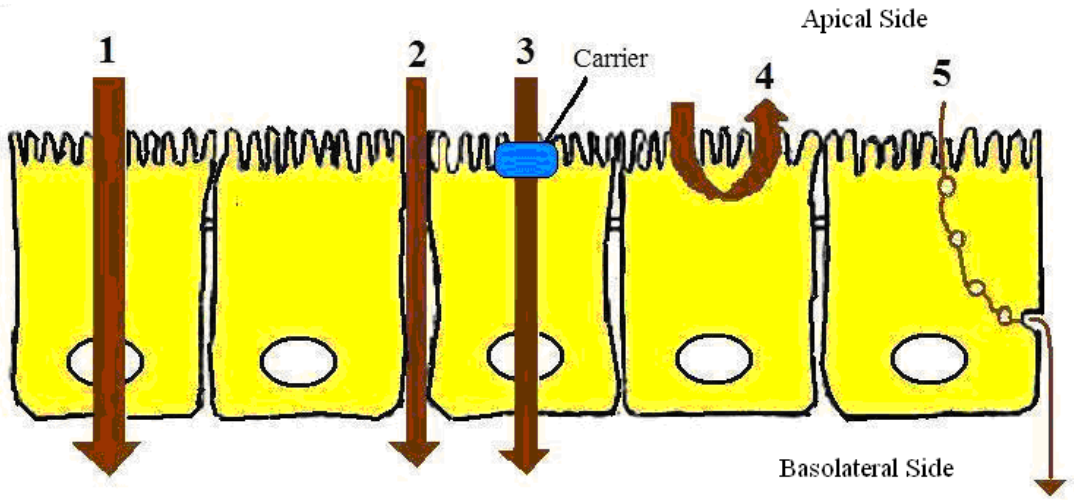


FIGURE 1.1: Schematic representation of routes and mechanisms of drug transport across the intestinal epithelium. 1. Passive transcellular route, 2. Passive paracellular route, 3. Carrier mediated transport, 4. Carrier mediate efflux and 5. Vessicular transport

1.2.1 Passive Transcellular Route

It involves the movement of solute molecules across the apical membrane through the cell cytoplasm and across the basolateral membrane (Hidalgo, 2001). Most drugs are relatively lipophilic and rapidly partition from the luminal

fluid into the cell membranes of the intestinal epithelium via this route. These drugs are normally rapidly and completely absorbed from the GI tract. The surface area of the transcellular route (i.e. cell membrane) is > 1000 fold larger (i.e. 99.9 % vs. 0.01 %) than that of the paracellular route (i.e. tight junctions), (Hidalgo, 2001; Pappenheimer and Reiss, 1987).

1.2.2 Passive paracellular route

The paracellular pathway is an aqueous, extracellular route across the epithelium. Many hydrophilic drugs and peptides partition poorly into the cell membranes and are subsequently not transported via the transcellular pathway. It is therefore generally assumed that these drugs are transported across the intestinal epithelium through the aqueous filled pores of the paracellular pathway (Fig. 1.1). These drugs are incompletely absorbed because the paracellular pathway occupies a very small surface area (0.01-0.1 %) of the total surface area of the intestine. Moreover, the apical and basolateral domains are separated by the tight junctions, providing a seal between adjacent epithelial cells that further restricts transport via this route (Jackson at el., 1987).

Evidence that the tight junctions can be modulated in response to various stimuli, such as hormones, nutrients etc., has stimulated investigations into ways to enhance the permeability by this route. The reversible opening of the tight junctions is of considerable interest in pharmaceutics since it shows a way by which the absorption of more hydrophilic drugs (that do not partition significantly into the cell membranes) canincreased (Artursson et al., 1990).

1.2.3 Carrier-mediated transport

A large numbers of transporters (carriers), expressed in the small intestinal mucosa are responsible for the absorption of nutrients and vitamins (Hidalgo et al., 2001). Structural analogs within a series of xenobiotics are able to utilize these transport pathways (Fig.1.1). These influx transporters can bind compounds that are dissolved in the intestinal fluid and translocate them across the apical membrane of enterocytes, thus facilitating drug absorption

process. Compounds that are substrates for the influx transporters exhibit intestinal absorption higher than expected from their diffusion across cell membranes (Raeissi et al., 1999; Artursson et al., 1991; Hidalgo et al., 1990).

1.2.4 Carrier-limited transport (apical efflux)

In contrast to the role of influx transporters, which can enhance intestinal drug absorption, efflux transporters mediate the extrusion of compounds from the cell cytoplasm to the intestinal lumen through a process known as apical efflux (Fig. 1.1). Two families of transporters, multidrug resistance (MDR) and multidrug resistance associated protein (MRP) (Marttin et al., 1995; Merkus et al., 1993), mediate apical drug efflux. P-gp the product of the MDR1 gene, is located on the apical membrane of normal enterocytes. It is the most studied member of the apical efflux transporters and has been shown to limit intestinal absorption of a large number of drugs. Here, saturation of the carrier could result in an increase in absorption of the drug (Phung-Ba et al., 1995; Hunter et al., 1993).

1.2.5 Vesicular transport

The vesicular transport (Fig. 1.1) includes fluid-phase endocytosis, receptor-mediated endocytosis and transcytosis. This complex route has a very low capacity and is only of relevance for highly potent macromolecular drugs that are active at low concentrations (Swaan et al., 1998). The membrane vesicles contain large amount of proteolytic enzymes which are responsible for significant degradation of most of the exogenous proteins. These reasons limit this pathway as general drug transport route (Lazarova et al., 1993; Heyman et al., 1982).

1.3 Oral bioavailability and herbal absorption promoters

Incomplete oral bioavailability has various causes e.g. poor dissolution or low aqueous solubility degradation of the drug in to the GI fluids poor membrane permeation and pre-systemic GI metabolism or hepatic metabolism. Out of various approaches used one most acceptable approach is incorporation of

absorption promoters for enhancement of bioavailability of otherwise poorly absorbed drugs.

Despite tremendous innovations in the drug delivery methods in the last few decades, the oral route still remains as most preferred route of administration for most new chemical entities (NCE). The oral route is preferred by virtue of its convenience, low costs, and high patient compliance compared to alternate routes. However, compounds intended for oral administration must have adequate aqueous solubility and intestinal permeability in order to achieve therapeutic concentrations. With the explosive growth in the field of genomics and combinatorial chemistry coupled with technological innovations in the last few years, synthesizing a large number of potential drug candidates is no longer a bottleneck in the drug discovery process. Instead, the task of screening compounds simultaneously for its clinical biological activity and biopharmaceutical properties (e.g. solubility, permeability/absorption, stability etc.) has become the major challenge. This has provided a great impetus within the pharmaceutical industry to implement appropriate screening models that have high capacity are cost-effective and highly predictive of *in vivo* permeability and absorption.

An absorption promoter can function by one or more of the following mechanisms:

i. Improve the permeant's solubility.
ii. Improve the permeant's diffusivity and permeability across the epithelium.
iii. Protect the permeant from pH, luminal and/or brush border enzymes.
iv. Protect the permeant from nonspecific binding sites.
v. Reduce the activity of or compete for uptake by efflux pumps in the apical side of intestinal membrane.

With various types of absorption promoters available, narrowing the selection requires a comparison of the benefits and risks of these agents. The three most important criteria for evaluating absorption promoters are effectiveness

of the absorption promoter, potential to cause toxicity and the mechanisms by which absorption is promoted. Certainly, the most obvious indicator of success associated with absorption promoter is the extent of bioavailability enhancement realized. Many studies have shown that the absorption enhancing effects of the synthetic absorption promoters, including surfactants, bile salts and fatty acids, are often accompanied by damage to the epithelial cell membrane when they are used at an effective concentration (Marttin et al., 1995). As a result, only a few formulations containing these absorption promoters are available in the market for clinical use.

HAPs are isolated components or extracts of plants and employed to promote absorption of several drugs and nutrients (Badmaev et al., 2000; Odou et al., 2005; Nabekura et al., 2005). The concentrations employed for the absorption promotion by HAPs are very low as compared to their concentrations which can cause toxicity. This concept of enhancement of drug bioavailability/ bioefficacy is not new but is based on clues from *Ayurveda* wherein it has been found that certain herbals, either individually or as a group, are repeatedly found along with the active medicament in large number of prescriptions recommended for a variety of diseases. The addition of the chosen herbs or part of it helped in the availability and absorption of the drugs. Earlier, the Ayurvedic Physicians used black pepper or Trikatu (1:1:1mixture of dried fruits of *Piper nigrum* and *Piper longum* and dried rhizomes of *Zingiber officinalis*) in several drug formulations to enhance drug activity and to minimize side effects. Out of the 370 compound formulations, 210 contain either *Trikatu* or its individual components (Hand Book of Domestic Medicine and Common Ayurvedic Remedies, 1979). In the last few years, the scientists have tried to explore the scientific basis of the use of the herbs prescribed by Ayurvedic physicians. Their efforts have resulted in better understanding of mechanism of action of HAPs, isolation and characterization of individual compounds and generating toxicity data. Improved understanding of HAPs has made it evident that these herbs were not included in the herbal formulations just for sake of rhyme but had definite role to play in promoting absorption and enhancing bioavailability.

In this research work we selected three HAPs i.e. silymarin, morin and naringin. This selected herbal constitutes are studied on laboratory animals with selected modern allopathic drugs to check the effectiveness of this HAPs for their permission through intestine. The major mechanism found for drug absorption retardation through intestine is CYP 450 and P-gp efflux pump so on basis of the mechanism drugs were selected. Atorvastatin and sildenafil which is reported to metabolize by CYP 450 while zidovudine and carvedilol phosphate reported to metabolize by P-gp efflux pump. So in present study these four allopathic drugs for with three HAPs for were studied for their effect on intestinal permission of selected drugs.

2. REVIEW OF LITERATURE

2.1 Anatomy and physiology of small intestine

The human small intestine is approximately 2-6 m and is loosely divided into three sections: duodenum, jejunum, and ileum, which comprise 5 %, 50 %, and 45 % of the length (Ganong et al., 1995). Approximately 90 % of all absorption in the gastrointestinal tract occurs in the small intestinal region. The intestinal surface of the small intestine has surface projections that increase the potential surface area for digestion and absorption. Macroscopic valve like folds called circular folds, encircling the inside of the intestinal lumen is estimated to increase the surface area of small intestine threefold. Villi increase the area 30-fold and the microvilli increase it by a factor of 600 Thus, such unique structures lead to a tremendous increase in surface area available for absorption in the small intestine

The major role of the small intestine is the selective absorption of major nutrients and to serve as a barrier to digestive enzymes and ingested foreign substances. The epithelial cells in the intestinal region are a heterogeneous population of cells that include enterocytes or absorptive cells, goblet cells that secrete mucin, endocrine cells, paneth cells, M cells, tuft and cup cells (Carr et al., 1984; Madara et al., 1987). The most common epithelial cells are the enterocytes or the absorptive cells. These cells are responsible for the majority of the absorption of both nutrients and drugs in the small intestine. It is polarized with distinct apical and basolateral membrane that are separated by tight junctions. Thus, bulk of absorption takes place in small intestine by mechanisms such as passive diffusion (paracellular and transcellular), carrier-mediated process (facilitated and active) and endocytosis. In the present study objective targeted, to perform solid state study of different drugs with different HAPs by FTIR and DSC technique to conduct the study regarding improvement of bioavailability of some poorly absorbed synthetic drugs using HAPs and to find out the effective concentration of selected HAPs.

2.1.1 CYP3A4 in intestine involve in drug metabolism

There is a first pass extraction progress when drugs orally absorbed, which are metabolized by the microsomal cytochrome superfamily of enzymes. The CYP P450 enzymes are responsible for the majority of oxidative metabolism of drugs. Although approximately 15 forms of CYP participate in metabolism of drugs, four forms i.e. CYP 3A4, CYP 1A2, CYP 2C9 and CYP 2D6 together account more than 90 % of oxidative metabolism of drugs. CYP 3A4 (P450 NF, P 450 3A4, Hpcn1) a major phase-I drug metabolizing monooxygenase, by itself participants in the metabolism of greater than 50 % of drugs that are metabolized oxidatively. CYP 3A4 also can metabolize inherent metabolite, aflatoxin B1,precarcinegen, etc (Dantzig et al., 1999). The site of such first pass oxidative metabolism has usually been considered to be the liver because of its site, its relatively high level of P 450 activity and its anatomic location relative to the site absorption. However, recent studies indicate that CYP 3A4 metabolic activity in the intestinal mucosa may substantially contribute to the over all first pass effect. Enzymes of the CYP 3A family are very abundant, according for approximately 30 % of hepatic CYP and greater than 70 % of small intestinal CYP (Wacher et al., 1998). The expression of CYP 3A4 in the intestine is site dependent that means the concentration of CYP 3A4 is different in different portion. Abnormally CYP3A4 is greatest in the duodenum and lowest in the ileum. That is to say, the upper small intestine (duodenum and proximal jejunum) serves as the major site for intestinal first pass metabolism of drugs (Paine et al., 1997). Because of the absorption of chemical inducers and the polymorphism of gene, it is possible for an individual to have relatively high liver CYP3A4 activity but present low intestinal CYP 3A4 activity or to have relativity low liver CYP3A4 activity while having relatively high intestinal CYP3A4 activity and the CYP3A4 levels is variable with 10 to 100 fold variations was reported (Lown et al., 1997; Lown et al., 1994).

Furthermore, despite hepatic and intestinal CYP3A4 appear to the some enzymes, having identical cDNAs and demonstrating almost identical Km values for midazolam 1’ hydroxylation, CYP3A4 in the liver and small intestine

are not coordinately regulated (Wacher et al., 1998). For some drugs, such as grapefruit juice (Lown et al., 1997), only influence the level of CYP3A4 in small intestine, but not affect that in liver, and other drugs, such as erythromycin (Ryoko et al., 2001) only increase activity of CYP 3A4 in hepatic, but not increase that in small intestine. All the facts above bring the complex and difficulty of the studies of CYP 3A4.

2.1.2 Effect of P-gp as a drug efflux pump involve in drug metabolism

In the intestine of human, P-gp is located almost exclusively within the brush border on the apical surface of the enterocyte, and other normal tissues such as the liver, brain, kidney and so on also express P-gp. P-gp is the product of the multidrug resistance gene MDR1 and belongs to a member of a large multigene family of ATP binding cassette (ABC) membrane transporters (Yumoto et al., 1999; Fischer et al., 1998; Zhang et al. 1998) and is composed of about 1280 amino acids. Although, CYP 3A4 in the intestine has an effect in the first pass elimination of oral drugs, more and more researches conclude that the intestinal P-gp probably plays a mojor significant role (Schinkel et al., 1997; Sparreboom et al., 1997; Masuda et al., 2000; Kim et al, 1998). Similar with CYP 3A4 the expression level of P-gp in each small intestine varies with maximal expression in ileum and moderate express in duodenum and jejunum and then a decrease in expression through the proximal and distal colon. In addition, the expression of P-gp does not show some relationship in the liver and the small intestine (Ryoko et al., 2001). In addition, the special relationship of P-gp in the plasma membrane and CYP 3A4 inside the cell on the endoplasmic reticulum suggests that P-gp may act to regulate exposure of substrates to metabolism by CYP 3A4. A substrate from P-gp is repeatedly circulates between the gut lumen and epithelial cell, leading to increased metabolism due to prolonged exposure to the metabolic enzyme, resulting in reduced absorption of the drug into blood (Zhang et al., 1998; Mayo et al., 1997). Moreover, when P-gp transfer drug from the cell, it also remove metabolites of drug from the cell interior, and lead to increasing of metabolism by CYP3A4 (Dantzig et al., 1999, Hochman et al., 2000).

2.1.3 Coordination of CYP3A4 and P-gp involve in drug metabolism

Recent studies have revealed that CYP 3A4 and P-gp not only function in a complementary fashion to reduce systemic exposure to drugs but there is also considerable to overlap in the drugs which interact with the two proteins (Wacher et al., 1995) Many important clinical drugs i.e. immunosuppressant agents, antibiotics, calcium channel antagonists, cancer chemotherapeutic agents, antiarrhythmics and so on, are metabolized by CYP 3A4 (Yumoto et al., 1999) and some of them are not only substrate or inhibition of CYP 3A4 but also the substrate or inhibition of P-gp. That is to say, when these drugs induce or inhibit CYP 3A4, they probably induce or inhibit P-gp at the same time. Recent studies shows expression of the CYP 3A4 and P-gp in human parental colon carcinoma cell line LS 180, and found the strong inducer of CYP 3A4, such as reserpine, rifampicin, phenobarbital etc. were also the powerful inducers of P-gp (Schuetz et al., 1996). Otherwise, it was also suggested that, although many P-gp inhibitors were potent inhibitors of CYP3A4, a varying degree of selectivity was present (Ohnishi et al., 2000). For example, GFJ can significantly reduce the content of CYP 3A4 but not the content of P-gp in human body (Wacher et al., 1998; Ohnishi et al., 2000; Fontana et al., 1998). However, some studies have also shown that GFJ compounds not only inhibit drug metabolism by CYP 3A4, but also enhance drug absorption by the inhibition of drug efflux via P-gp in a human colon carcinoma cell line, Caco-2 cells used as a model of intestinal absorption (Takanaga et al., 1997) and HMF, langeretin and nobiletin, which separated from the orange juice are all P-gp inhibitors, However none of them inhibited CYP3A4 (Takanaga et al., 2000). Although, there are striking overlaps of substrates and inhibitors between CYP3A4 and P-gp, an important finding was that no significant correlation exists between the ability of the compounds to inhibit P-gp and their ability to inhibit CYP3A4. Probably, the molecular recognition sites of P-gp and CYP3A4 differ in ways that result in differential effects of compounds at the two sites (Hall et al., 1999; Ohnishi et al., 2000).

2.2 CYP 450 enzyme system

The CYP P450 enzyme system consists of a superfamily of hemoproteins that catalyze the oxidative metabolism of a wide variety of exogenous chemicals clouding drugs, carcinogens, toxins and endogenous compounds such as steroids, fatty acids and the prostaglandins (Shimada et al., 1994). The CYP 450 enzyme family plays an important role in phase-I metabolism of many drugs. The broad range of clinical medicines undergoes CYP 450 mediated oxidative biotransformation is responsible for the large number of clinically significant drug interactions during multiple drug therapy. Clinical case reports or studies usually provide the first evidence of interaction between drugs. Several recent studies discuss such drug interactions at the molecular or enzyme level and therefore constitute an important link between clinical and experimental research. Central to this approach is an understanding of catalytic importance of individual CYP 450 iso-enzymes in particular metabolic pathways.

2.2.1 Nomenclature of CYP 450

Nomenclature of CYP 450 enzyme system has been established by CYP 450 nomenclature committee. The name CYP 450 family is derived from the fact that these proteins have a heme group and an unusual spectrum (Shimada et al., 1994). These enzymes are characterized by a λ_{max} of 450 nm in the reduced state in the presence of carbon monoxide. Naming a CYP450 gene include root symbol “CYP” for humans (“CYP” for mouse and Drosophila), an Arabic numeral denoting the CYP family (*e.g.* CYP 1, CYP 2), letters A, B, C indicating subfamily (*e.g.* CYP 3A, CYP 3C) and another Arabic numeral representing the individual gene/isoenzyme/isozyme/isoform (e.g. CYP 3A4, CYP 3A5). Of the 74 gene families so far described, 14 exist in all mammals. These 14 families comprise of 26 mammalian subfamilies. Each isoenzyme of CYP 450 is specific gene product with characteristic substrate specificity. Isoenzymes in the same family must have >40 % homology in their amino acid sequence and members of the same subfamily must have >55 % homology. In the human liver there are at least 12 distinct CYP 450 enzymes**.** At present it appears that from about 30 isozymes, only six isoenzymes from

the families CYP 450 1, 2 and 3 are involved in the hepatic metabolism of most of the drugs. These include CYP 1A2, 3A4, 2C9, 2C19, 2D6 and 2E1 (Nelson et al., 1996).

Isoenzymes and drug interactions: The advances in CYP enzyme system has made it possible to associate specific enzyme activity with the formation of a particular metabolite and in some cases to identify the major isoenzyme responsible for the total clearance of a drug. Individual isoenzymes involved in the metabolism of large number of important drugs. Induction or inhibition of these isoenzymes leads to clinically significant drug interactions. Individual isoenzymes and their drug interactions are as follows:

3A Isoenzymes: Members of the 3A subfamily are the most abundant CYP 450 enzymes in the liver and account for about 30 % of CYP proteins in the liver. High levels are also present in the small intestinal epithelium and thus makes it a major contributor to presystemic elimination of orally administered drugs. There is considerable interindividual variability in hepatic and intestinal CYP 3A activity (about 5-10 fold). Since 40 to 50 % of drugs used in humans involve 3A mediated oxidation to some extent, the members of this subfamily are involved in many clinically important drug interactions. 3A4 is the major isoenzyme in the liver. 3A5 is present in the kidneys. Inducers of 3A4 usually do not upregulate 3A5 activity. Noteworthy is high concentration of terfenadine, astemizole and cisapride when these drugs are taken concomitantly with azole antifungals or macrolide antibiotics or fluoxetine or fluvoxamine. High concentration of these drugs lead to life threatening cardiac arrhythmias like torsades de pointes and ventricular fibrillation. Terfenadine has been withdrawn in USA and the European Union. Fexofenadine, the active metabolite of terfenadine, is now marketed as a noncardiotoxic alternative to terfenadine. US-FDA is currently considering to contraindicate the use of selective serotonin reuptake inhibitors (SSRI) with the non-sedating antihistamines. Mibefradil, a newer calcium channel blocker, that preferentially blocks T-type calcium channels, has already been voluntarily withdrawn from the market because of large number of drug interactions. GFJ is often taken

at breakfast in the western countries when drugs are also often taken. GFJ having bioflavonoids mainly naringin and furacoumarin which cause mechanism based inhibition of presystemic elimination of a number of drugs and increase their bioavailability. An important interaction involving induction of CYP 3A is the reduction in efficacy of contraceptives taken orally by rifampicin and rifabutin, because of an induction of the CYP 450 3A mediated metabolism of estradiol and norethisterone, the components of oral contraceptives (Badayal et al, 2001).

2D6: This isoenzyme represents <5 % of total CYP proteins. It has aroused great interest because of its large number of substrates (30-50 drugs) and its genetic polymorphism. It is also called debrisoquine hydroxylase after the drug that led to the discovery of its genetic deficiency. Many psychotropic, antiarrhythmic and β-adrenergic receptor blocker drugs are substrates as well as inhibitors of 2D6.

IA2: This is the only isoenzyme affected by tobacco. Cigarette smoking may lead to threefold increase in CYP 1A2 activity. Theophylline is metabolised in part by 1A2, which explains why smokers require higher doses of theophylline than non-smokers. Alcohol inhibits metabolism of caffeine, a substrate of CYP 1A2. Alcohol has been reported to mask the CYP 1A2 inducing potential of smoking. Exposure to polyaromatic hydrocarbons found in charbroiled food can also induce this isoenzyme (Crewe et al., 1992). This isoenzyme also causes metabolic activation of procarcinogens to carcinogens e.g. aromatic and heterocyclic amines.

2C9: S-warfarin and phenytoin, both involved in large number of drug interactions, are metabolised mainly by CYP 2C9 (Rettie at el., 1992) St. John's wart a herbal antidepressant has been reported to decrease levels of warfarin by induction of CYP 2C9.

2C19: This iso-enzyme also exhibits genetic polymorphism. CYP 450 2C19 is involved in major metabolism of a number of clinically important drugs i.e.

omeprazole, diazepam, anti-depressants, anti-malarials etc. (Lamba at el., 1998).

2E1: This iso-enzyme is involved with metabolism of low molecular weight toxins, fluorinated ether, volatile anesthetics, procarcinogens etc. This iso-enzyme is inducible by ethanol and is responsible in part for metabolism of acetaminophen. The metabolite of acetaminophen formed is highly reactive and hepatotoxic. Alcohol dependant patients may be at increased risk of acetaminophen hepatotoxicity because of induction of 2E1 by alcohol (Raucy at el., 1989). Isoniazid has biphasic effect on 2E1 activity, a direct inhibitory effect immediately after its administration followed by an inducing effect because of CYP protein stabilization (Nelson et al., 1996). Knowledge of substrates, inhibitors and inducers of CYP isoenzymes assists in predicting clinically significant drug interactions.

2.3 P- glycoprotein

2.3.1 Structure, Tissue distribution and physiological function of the P-glycoprotein

P-gp is a type of ATPase, and an energy-dependent trans-membrane drug efflux pump which belongs to members of ABC drug transporters. P-gp, a 1280 amino acid long (molecular weight 170 kDa) glycoprotein, is expressed as a single chain containing two homologous portions of equal length, each containing six transmembrane domains and two ATP binding regions separated by a flexible linker polypeptide region between the Walker A and B motifs (Varma et al, 2003; Ambudkar et al. 1999).

Immunohistochemical analysis using monoclonal antibody provided evidence for localization of P-gp in a wide range of tissues, particularly in columnar epithelial cells of lower GI tract, capillary endothelial cells of brain and testis, canalicular surface of hepatocytes and apical surface of proximal tubule in kidney. Due to selective distribution at the port of drug entry and exit, P-gp has been speculated to play a major physiological role in absorption, distribution and excretion of xenobiotics. Overall P-gp function as a

biochemical barrier for entry of xenobiotics and as a vacuum cleaner to expel them from the brain, liver, etc. and ultimately from systemic circulation (Thiebut . et al, 1987).

P-gp is mainly described in cancer research as being a resistance factor to chemotherapies, also called MDR for multidrug resistance. P-gp globally confers a chemoresistance to cells for numerous xenobiotics by actively pumping these molecules outside cells, and consequently decreasing their intracellular concentrations. This ability to extrude xenobiotics gives this protein some physiological properties of protection and detoxification. For example, knock out mice (mdr1a−/−) are described as being more sensitive to ivermectine and as developing a central neurotoxicity, while wild-type animals are healthy (Balayssac et al., 2005; Schinkel et al., 1994).

The anatomical localization of P-gp in various tumors (where it confers the MDR phenotype) and at the apical membrane of polarized cells in several normal human tissues with excretory (liver, kidney, adrenal gland) and barrier (intestine, blood brain barrier(BBB), placenta, blood testis and blood ovarian barriers functions suggests for P-gp a physiological role in detoxification and protection of the body against toxic xenobiotics and metabolites by secreting these compounds into bile, urine, and the intestinal lumen and by preventing their accumulation in the brain, testis, and fetus (Fig. 2.2) (Beijnen et al., 2007; Thiebut et al., 1987).

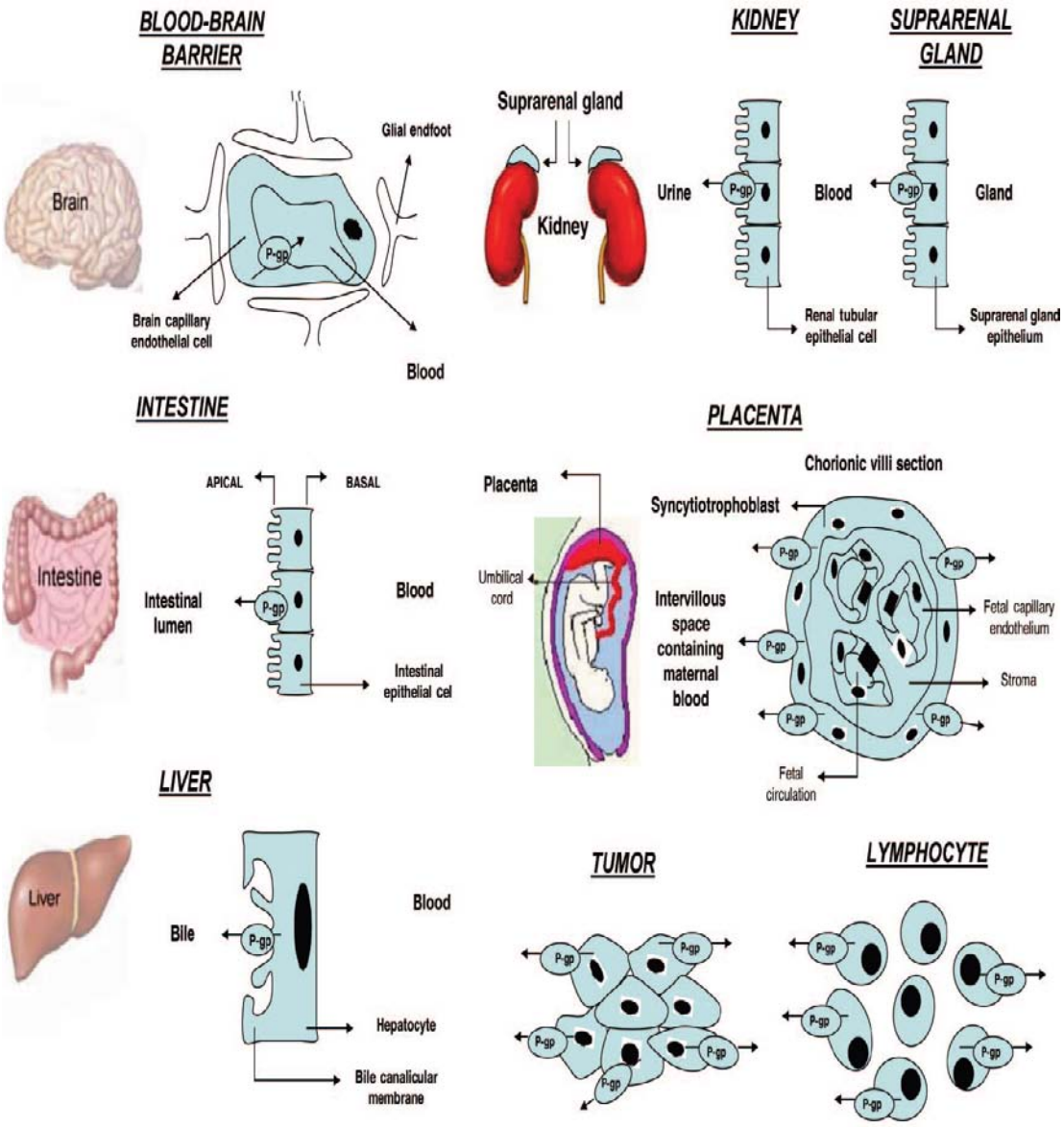


FIGURE 2.1: Schematic representations of the main sites of localization of P-gp in the body

2.3.2 Mechanism of drug efflux by P-gp

Various models were proposed to explain the mechanism of xenobiotic extrusion by P-gp, however, the exact site of substrate interaction with the protein is not well resolved. The three prevalent models pore model, flippase model and hydrophobic vacuum cleaner (HVC) model, explains the efflux mechanism to certain extent (Fig. 2.1). Among these HVC model has gained wide acceptance in which P-gp recognizes substrates embedded in the inner

leaflet of plasma membrane and transported through a protein channel. Recently, reported that three-dimensional conformation of P-gp changes upon binding of nucleotide to the intracellular nucleotide-binding domain (Higgings and Gottesman , 2003; Varma et al., 2003)

In the absence of nucleotide, the two transmembrane domains form a single barrel with a central pore that is open to the extracellular surface and spans much of the membrane depth, while upon binding nucleotides, the transmembrane domains reorganize into three compact domains that open the central pore along its length in a manner that could allow access of hydrophobic drugs directly from the lipid bilayer to the central pore of the transporter. ATP binding and hydrolysis was found to be essential for functioning of P-gp, where one molecule of drug is effluxed at the expense of two molecules of ATP. The catalytic cycle of P-gp, which expands the opportunity for the development of P-gp inhibitors, comprises of two cycles where drug and nucleotide binding sites coordinately function to efflux out the substrates by an ATP driven energy-dependent process.

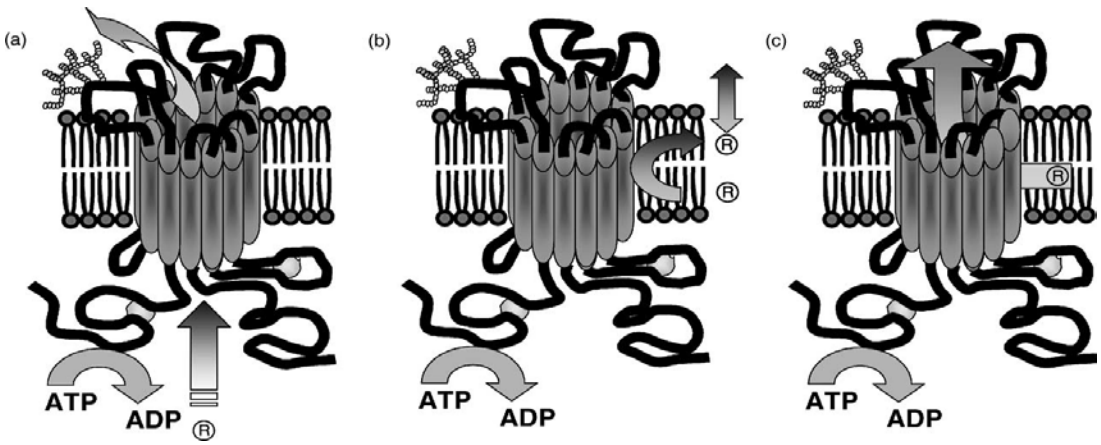


FIGURE 2.2: Models proposed to explain the mechanism of drug efflux by P-gp (a) Pore model (b) Flippase model and (c) hydrophobic vacuum cleaner model

In pore model, drugs associate with P-gp in the cytosolic compartment and are transported out of the cell through a protein channel. In Flippase model, drugs embed in the inner leaflet of the plasma membrane, bind to P-gp within

the plane of membrane and are translocated to the outer leaflet of the bilayer from which they passively diffuse into extracellular fluid. The hydrophobic vacuum cleaner model combines the features of ‘pore’ and ‘Flippase’ models.

The drug and ATP initially binds to the protein at their own binding sites, where nucleotide hydrolyses to ADP yields energy for the extrusion of drug. The release of ADP from nucleotide binding site ends the first catalytic cycle followed by a conformational change that reduces affinity for both substrate and nucleotide. Further, the second catalytic cycle starts by hydrolysis of another molecule of ATP and released energy is utilized to reorient the protein to its native conformation. Subsequent release of ADP completes another catalytic cycle, bringing P-gp molecule back to the original state, where it again binds to both substrate and nucleotide to initiate the next cycle (Sauna et al., 2001; Varma et al., 2003)

2.3.3 P-glycoprotein inhibitors

P-gp is found on the different barriers that limit the passage of xenobiotics into the organism. P-gp is described as an important mechanism decreasing the bioavailability of oral drugs by limiting intestinal absorption. Moreover, P-gp can directly eluate drugs from the general circulation into the bile and intestinal lumen via hepatocytes and enterocytes, respectively. Thus it can be supposed that compounds able to interact with P-gp activity could induce modifications of the bioavailability for numerous concomitantly administered drugs. Compounds able to modulate or inhibit P-gp activity would increase xenobiotic levels and also P-gp substrates (Aungst, 1999; Suzuki and Sugiyama, 2000; Schinkel and Jonker, 2003). Based on the specificity and affinity, P-gp inhibitors are classified to three generations. (Hunter and Hirst, 1997)

i. **First-generation** inhibitors are pharmacological actives, which are in clinical use for other indications but have been shown to inhibit P-gp. These include calcium channel blockers i.e. verapamil, immune-suppressants like cyclosporine-A, antiestrogens like tamoxifen, anti-

hypertensives, quinidine, reserpine, yohimbine and toremifena. The usage of these compounds is limited by their toxicity due to the high serum concentrations achieved with the dose that is required to inhibit P-gp.

ii. **Second-generation** madulator agents that lack in the pharmacological activity of the first-generation compounds and usually possess a higher P-gp affinity. However, inhibition of two or more ABC transporters leads to complicated drug–drug interactions by this class of compounds, which include non-immunosuppressive analogues of cyclosporine A, PSC 833; D-isomer of verapamil, dexverapamil; and others such as biricodar (VX-710), GF120918 and MS-209.

iii. **Third generation** P-gp blockers are under development, however, primarily with the purpose to improve the treatment of multidrug resistant tumours and to inhibit P-gp with high specificity and toxicity. Modulators such as LY 335979, OC 144093 and XR 9576 are identified to be highly potent and selective inhibitors of P-gp with a potency of about 10 fold more than the first and second-generation inhibitors.

P-gp can be inhibited by:

(i) Blocking drug binding site either competitively, non-competitive or allosterically.

(ii) Interfering ATP hydrolysis.

(iii) Altering integrity of cell membrane lipids.

Although most of the drugs inhibit P-gp function by blocking drug binding sites, presence of multiple binding sites complicate understanding as well as hinder developing a true, conclusive Structure activity relationship for substrates or inhibitors. Compounds inhibiting ATP hydrolysis could serve as better inhibitors, since they are unlikely to be transported by P-gp, and these kind of agents will require at low dose which is well desirable to use locally at gut lumen. Quercitin, a naturally occurring flavanoid, has been proposed to block P-gp function by an unknown mechanism but in general by interfering ATPase activity (Shapiro and Ling, 1997; Drori et al., 1995). Following Tab.

2.1 shows the few therapeutic agent that are have its known P-glycoprotein inhibition activities (Linardi and Natalini, 2006)

Table 2.1 : Therapeutic agent that are P-glycoprotein inhibitors

Class of Drug	Name of Drugs
Antiarythmics	Amiodarone, atorvastatin, simvastatin, quinidine, verapamil, amiodarone, carvedilol, nicardipine.
Anticancer drugs	Actinomycine D, doxorubicin, vinblastine
Antibiotics	Erythromycin, Itraconazole, ketoconazole
Antidepressants	Paroxetine, sertraline, desmethylsertraline, Fluoxetine, Saint John's Wort
Proton pump inhibitors	Esomeprazole, lansoprazole, omeprazole, pantoprazole
Opioids	Methadone, pentazocine, fentanyl, loperamide, asimadoline
Immunosuppressants	Cyclosporine A, tacrolimus, sirolimus.
Steroids	Dexametasone, aldosterone, progesterone, hydrocortisone, corticosterone.
Miscellaneous	Bromocriptine, chlorpromazine, tamoxifen, grapefruit juice, ivermectin, phenothiazines, colchicine, itraconazole.

Commonly used pharmaceutical surfactants are emerging as a different class of P-gp inhibitors, which act by altering integrity of membrane lipids. The change in secondary and tertiary structure is found to be the reason for loss of P-gp function due to disturbance in hydrophobic environment by surfactants. Series of study was observed that the change in fluidity of cell membrane facilitates influx of P-gp substrates by surfactants like polyethylene glycol, cremophor EL and Tween 80, demonstrated in Caco-2 cell line. Surfactants seem to be better choice since they were already approved for routine use in pharmaceutical formulations. However, until now it has been tested at *in vitro*

level, which should be further evaluated by animal or human studies. (Hugger et al.,2002; Audus et al., 2002)

2.3.4 Herbal modulation of P-gp

P-gp is vulnerable to inhibition, activation, or induction by herbal constituents. This was demonstrated by using an ATPase assay, purified P-gp protein or intact P-gp expressing cells, and proper substrates and inhibitors. Curcumin, ginsenosides, piperine, some catechins from green tea, and silymarin from milk thistle were found to be inhibitors of P-gp, while some catechins from green tea increased P-gp mediated drug transport by heterotropic allosteric mechanism, and St. John's wort induced the intestinal expression of P-gp *in vitro* and *in vivo*. Some components (e.g., bergamottin and quercetin) from GFJ were reported to modulate P-gp activity. Many of these herbal constituents, in particular flavonoids, were reported to modulate P-gp by directly interacting with the vicinal ATP-binding site, the steroid-binding site, or the substrate-binding site. Some herbal constituents (e.g., hyperforin and kava) were shown to activate pregnane X receptor, an orphan nuclear receptor acting as a key regulator of MDR1 and many other genes (Zhou et al., 2004). The modulation of P-gp activity and expression by these herb constituents may result in altered absorption and bioavailability of drugs that are P-gp substrates. This is exemplified by increased oral bioavailability of phenytoin and rifampin by piperine and decreased bioavailability of indinavir, tacrolimus, cyclosporine, digoxin, and fexofenadine by coadministered St. John's wort. However, many of these drugs are also substrates of CYP 3A4. Thus, the modulation of intestinal P-gp and CYP 3A4 represents an important mechanism for many clinically important herb drug interactions.

Twenty-nine phenolic compounds, isolated from four *Clusiaceae* species (Garcinia and Calophyllum) and classified into four chemical classes (coumarins, chroman acids, xanthones and benzophenones), were tested on a human erythroleukemic cell line (K562/R7) overexpressing the P-gp in order to evaluate their activity as inhibitors of this protein. Among the twenty-nine natural products tested, only six of them exhibited moderate modulation of the

P-gp-mediated daunorubicin efflux. The class of coumarin presented the greatest number of active derivatives in this test (Raad et al., 2007).

The extent of herb-drug interactions (H-DIs) is still largely unknown, although the use of herbal medicines among the general public is increasing. The theoretical and clinical evidence for some important H-DIs is presented, together with an explanation of pharmacodynamic interactions and pharmacokinetic effects involving P-gp and CYP 450 enzymes. Currently, research is being undertaken to identify potential interaction problems by means of *in vitro* and *in vivo* experimental methods; these, together with the most important H-DI's are summarized. The most commonly reported herb in H-DIs is St John's wort. It has proven enzyme-inducing properties and should be avoided in patients taking oral contraceptives, anticoagulants, immune suppressants, anti-neoplastics, protease inhibitors, digoxin, fexofenadine, statins, omeprazole, verapamil and other antidepressants (Williamson, 2006).

The effects of citrus flavonoids on P-gp mediated drug efflux was examined in human intestinal Caco-2 cells. The cellular accumulation of rhodamine-123 was measured using 10 citrus flavonoids for preliminary screening. Among the flavonoids tested, diosmin significantly increased the accumulation of rhodamine-123 in Caco-2 cells. In the bidirectional transport of digoxin, diosmin increased the apical-to-basal (A-to-B) transport but decreased the basal-to-apical (B-to-A) transport in both concentration- and time-dependent manners. The digoxin transport ratio (B-A/A-B) was estimated to be 2.3 at a concentration of 50 µM of diosmin, which was significantly lower than the 15.2 found in the control. The apparent Ki values for P (app,A-B) and P (app,B-A) were 16.1 and 5.7 µM, respectively. These results demonstrated that diosmin effectively inhibited the P-gp mediated efflux in Caco-2 cells. Diosmin is one of the main components in citrus fruits, and the intake of food supplements containing this compound may potentially increase the absorption of drugs able to act as P-gp substrates (Yoo et al., 2007).

The chemical structure and physical chemical properties (with SAR analysis) of the limonoids and a number of known P-gp substrates are also provided. The limonoids selected lie within the 3D (MW, Clogp, Hb) parameter-frame-setting of known P-gp substrates so could potentially be substrates themselves. Based on phytochemical and structure activity relationship (SAR) analyses of available literature data, it is suggested that limonoids found in citrus fruits and other Chinese herbs, when consumed in moderate amount are beneficial to health and may have preventive effect on carcinogenesis. However, due to potential interactions of limonoids with a wide range of drugs via P-gp and other drug metabolizing enzyme systems, it is advisable not to take citrus juices with therapeutic drugs simultaneously (Lien et al., 2002).

2.4 HAPs: Silymarin, naringin and morin

The effect of flavonoids on P-gp mediated cellular efflux and to determine the molecular mechanism of the flavonoid-drug interaction. Studies were conducted in the sensitive and multidrug resistant human breast cancer cell lines MCF-7 and MDA435/LCC6 and examined the effects of the flavonoids biochanin A, morin, phloretin, and silymarin on daunomycin (DNM) accumulation and doxorubicin cytotoxicity. The potential mechanism involved in the interaction was evaluated by determining flavonoid effects on 1) P-gp ATPase activity, 2) [3H]azidopine photoaffinity labeling of P-gp, and 3) cellular P-gp levels. Biochanin A and silymarin potentiated doxorubicin cytotoxicity in P-gp positive cells. Biochanin A and phloretin stimulated, whereas morin and silymarin inhibited P-gp ATPase activity, confirming that these flavonoids interact with P-gp. In conclusion, biochanin A, morin, phloretin and silymarin all inhibited P-gp mediated cellular efflux and the mechanism of the interaction involved, at least in part, a direct interaction. The findings of this study indicate a potential for significant flavonoids drug interactions with the P-gp substrates (Zhang and Morris, 2003).

The flavonoids are present in fruits, vegetables and beverages derived from plants (tea, red wine), and in many dietary supplements or herbal remedies including *Ginkgo biloba*, soy Isoflavones, and milk thistle. This review will

summarize the current literature regarding the interactions of flavonoids with ABC efflux transporters, mainly P-gp, MRP1, MRP2 and BCRP and discuss the potential consequences for flavonoid and the drug transport interactions (Zhang and Morris, 2003).

GFJ was an inhibitor of the intestinal CYP 450 3A4 system, which is responsible for the first-pass metabolism of many medications. Through the inhibition of this enzyme system, GFJ interacts with a variety of medications, leading to elevation of their serum concentrations. Most notable are its effects on cyclosporine, some 1,4-dihydropyridine calcium antagonists, and some 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CO-Enz-A) reductase inhibitors. In the case of some drugs, these increased drug concentrations have been associated with an increased frequency of dose-dependent adverse effects. The P-gp pump, located in the brush border of the intestinal wall, also transports many cytochrome P-450 3A4 substrates, and this transporter also may be affected by grapefruit juice. *In vitro* data show that grapefruit juice activates P-gp in intestinal cell monolayers (Garvan and James, 2000).

The effect of silymarin on the pharmacokinetics of osuvastatin in systems overexpressing OATP1B1 or BCRP transporters and in healthy subjects. The concentration-dependent transport of rosuvastatin and the inhibitory effect of silymarin were examined *in vitro* in OATP1B1-xpressing oocytes and MDCKII-BCRP cells. Based on the concentration dependency of rosuvastatin transport in the OATP1B1 and BCRP overexpression systems, rosuvastatin is a substrate for both transporters. Silymarin inhibited both OATP1B1- and BCRP-mediated rosuvastatin transport *in vitro* (K_i 0.93 µmol and 97 µmol respectively). However, no significant changes in AUC, half-life, V_d/F, or Cl/*F* of rosuvastatin were observed in human subjects following pretreatment with silymarin. Silymarin does not appear to affect rosuvastatin pharmacokinetics *in vivo*, suggesting that silymarin, administered according to a recommended supplementation regimen, is not a potent modulator of OATP1B1 or BCRP *in vivo* (Deng et al., 2008).

The effect of naringin on the bioavailability and pharmacokinetics of paclitaxel after oral administration of paclitaxel or its prodrug coadministered with naringin to rats. Paclitaxel (40 mg/kg) and prodrug (280, 40 mg/kg paclitaxel equivalent) were coadministered orally to rats with naringin (1, 3, 10 and 20 mg/kg). The bioavailability of paclitaxel coadministered as a prodrug with or without naringin was remarkably higher than the control. Paclitaxel prodrug, a water-soluble compound concerning with its physicochemical properties, passes through the GI mucosa more easily than paclitaxel without obstruction of P-gp and CYP 450 in the GI mucosa. Oral paclitaxel preparations which is more convenient than the i.v. dosage forms could be developed with a prodrug form with naringin (Choi and Shin, 2005).

Some well-know flavonoids from vegetables and fruits were tested for their potential ability to modulate the function of P-gp in the multidrug resistant (MDR) human cervical carcinoma cell line, KB-V1. The data demonstrated that kaempferol and daidzein stimulated vinblastine sensitivity of KB-V1 cells ($P<0.05$) and revealed that the inhibitory concentration at 50 % growth (IC50) of vinblastine was decreased markedly in the presence of these flavonoids in a dose dependent manner. Kaempferol also increased the intracellular accumulation, and reduced the efflux of rhodamine 123 (Rh123), which is known to be a good substrate of P-gp in KB-V1 cells. These findings provide evidence that the flavonoid, i.e. kaempferol, could reverse the vinblastine resistant phenotype by inhibiting P-gp activity in KB-V1 cells, and the ability to affect the P-gp activity could be of relevance to the chemosensitization of this flavonoid towards anticancer drugs (Khantamat et al., 2004).

The metabolic breakdown of albendazole by mucosal CYP 3A4 enzymes was studied by coadministering albendazole (10 mg/kg) with GFJ. Concentrations of albendazole sulfoxide (ABZSX), the active metabolite of albendazole, were compared with those after albendazole was administered with water, a fatty meal or GFJ plus cimetidine (10 mg/kg). In comparison to water, maximum ABZSX concentration (C_{max}) was enhanced 6.5 fold by a fatty meal (from 0.24 ± 0.09 mg/l to 1.55 ± 0.30 mg/l; mean ± SD; $P < 0.001$) and 3.2 fold by the

GFJ (from 0.24 ± 0.09 mg/l to 0.76 ± 0.37 mg/l; P<0.031). When GFJ was combined with cimetidine, C_{max} was significantly lower than with GFJ alone (0.41 ± 0.29 mg/l and 0.76 ± 0.37 mg/l respectively; P<0.022). The area under the concentration-time curve from 0 to infinity followed a comparable pattern. Half-life ($T_{1/2}$) was 8.8 ± 4.2 hr and 8.2 ± 4.3 hr after administration with water or a fatty meal (P<0.001). GFJ shortened $T_{1/2}$ by 46 % (P<0.026). We hypothesize that albendazole is metabolized by CYP 3A4 enzymes in the intestinal mucosa. This process can be inhibited by the GFJ. The cimetidine decreased albendazole bioavailability (Nagy et al., 2002).

2.5 Herbal Absorption Promoters (HAPs)

HAPs are isolated components or extracts of plants and employed to promote absorption of several drugs and nutrients. The concentrations employed for the absorption promotion obtained by HAPs are very low as compared to their concentrations which can cause toxicity. This concept of enhancement of drug bioavailability is not new but is based on clues from Ayurveda wherein it has been found that certain herbals, either individually or as a group, are repeatedly found along with the active medicament in large number of prescriptions recommended for a variety of diseases. The addition of the chosen herb or part of it helped in the availability and absorption of the drugs.

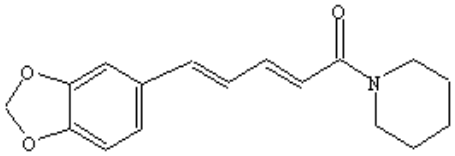
In the last few years, the scientists have tried to explore the scientific basis of the use of the herbs prescribed by *Ayurvedic* physicians. Their efforts have resulted in better understanding of mechanism of action of HAPs, isolation and characterization of individual components and generating toxicity data. Improved understanding about HAP has made it evident that these herbs were not included in the herbal formulations just for sake of rhyme but had definite role to play in promoting absorption and enhancing bioavailability (Badmaev et al., 2000; Odou et al., 2005; Nabekura et al., 2005). The following example highlights the modulating effects of herbal constituents on P-gp arising from *in vitro* and *in vivo* studies and the potential role in herb-drug interactions.

2.5.1 Alkaloid

Alkaloids are a major class of compounds present in plants. They are used for a variety of disorders. Few alkaloids known to possess absorption promoting activity are described below:

2.5.1.1 Piperine

Piperine (1-Piperylpiperidine), a pungent alkaloid, is the major constituent present in black (*Piper nigrum* Linn) and long pepper (*Piper longum* Linn) which was used in spices and herbal medicines. Piperine was reported to have antidiarrheal, anti-inflammatory, hepatoprotective, chemopreventive, immunomodulating, anticonvulsant, and antioxidant activities. A clinical study in healthy volunteers showed that coadministration of piperine (5 mg/day) for 21 days increased the AUC of coenzyme Q10 (120 mg) by approximately 30 % (Badmaev et al., 2000). Thus, piperine may be used as a bioavailability enhancer of drugs in humans. Animal studies indicated that piperine enhances drug absorption and bioavailability by inhibiting certain metabolizing enzymes, by modulating P-gp efflux by producing a local thermogenic action and by modulating membrane dynamics and forming apolar complex with drugs and solutes (Bhardwaj et al., 2002; Badmaev et al., 2000; Khajuria et al., 1998) as shown in (Tab. 2.2)



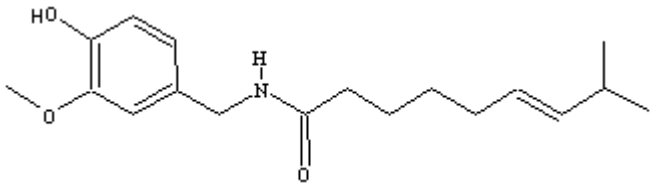
Piperine

Table 2.2: Drugs showing enhanced absorption/bioavailability on co-administration of Piperine

Absorption promotion Mechanism	Drugs	Reference
Inhibition of CYP 450 isoenzymes and other drug metabolizing enzymes	Propranolol and Theophylline	Bano et al., 1991
	Curcumin	Shoba et al., 1998
	Verapamil	Bhardwaj et al., 2002
	Vasicine and Sparteine	Atal et al., 1981
	Amoxicillin trihydrate	Hiwale et al., 2002
	Cefotaxime sodium	
	Nimesulide	Gupta et al., 2000
	Pentobarbitone	Mujumdar et al., 1990
	Aflatoxin B_1	Allameh et al., 1992
Inhibition of P-gp mediated drug efflux	Phenytoin	Velpandian et al., 2001; Bano et al., 1987
	Rifampicin	Zutshi et al., 1985
	Digoxin and Cyclosporine	Bhardwaj et al., 2002

2.5.1.2 Capsaicin

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is a pungent component present in chilli peppers and related plants of capsicum species. This alkaloid is reported to cause significant increase in bioavailability of theophyline in rabbits (Bouraoui et al., 1988). Capsaicin has been proposed to inactivate CYP 450 2E1 by irreversibly binding to the active sites of the enzyme. The enhanced accumulation was attributed to inhibition of P-gp (Surh et al., 1995).

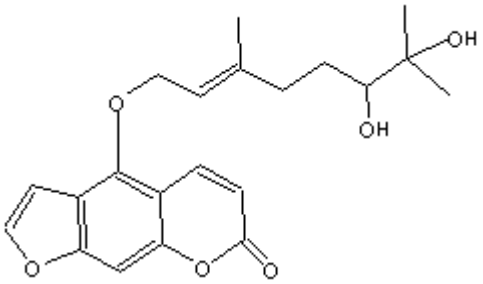


Capsaicin

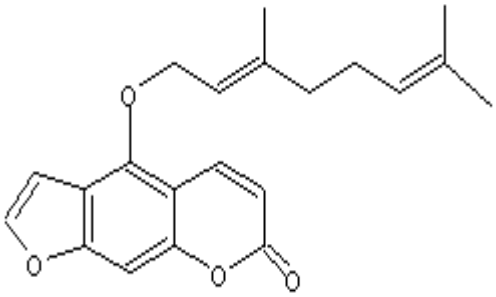
2.5.1.3 Furanocoumarins:

6', 7'-dihydroxybergamottin and Bergamottin:

6', 7'-dihydroxybergamottin (DHB) and its more lipophilic analog, bergamottin are the furanocoumarins present in GFJ and seville orange juice (Malhotra et al., 2001; Gray, 1983). DHB is a potent inhibitor of CYP450 3A4 enzymes (Bailey et al., 1998; Schmiedlin-ren et al., 1997; Lown et al., 1997). Lipophilic furanocoumarins also inhibits P-gp mediated drug transport and hence can increase the absorption and bioavailability of P-gp substrates (Table 2.3).



6', 7'-Dihydroxybergamottin



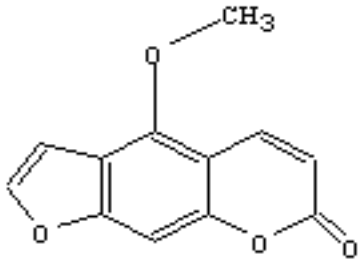
Bergamottin

Table 2.3: Drugs showing increased absorption/bioavailability on consumption of GFJ

Absorption promotion mechanism	Drugs	Reference
Inhibition of P-gp mediated drug efflux	Diltiazem	Christensen et al., 2002
	Talinolol	Spahn-Langguth at el., 2001
	Vinblastine	Takanaga et al., 1998
	Rhodamine-123, Fexofenadine and Saquinavir	Tian et al., 2002
	Digoxin	Xu et al., 2003
	Felodipine	Lown et al., 1997
	Saquinavir	Honda et al., 2004

2.5.1.4 Bergapten

Bergapten (5-Methoxypsoralen), a furanocoumarin present in Seville orange juice in addition to 6',7'- dihydroxybergamottin and bergamottin were also found to be a mechanism-based inhibitor of CYP 450 3A4. Relative to the control, bergapten (10 μmol/l) inhibited significantly the CYP 450 3A4 activity in cultured intestinal epithelial cells by 34 %. The bioavailability of felodipine was increased by 76 % after Seville orange juice ingestion compared to common orange juice (Malhotra et al, 2001).



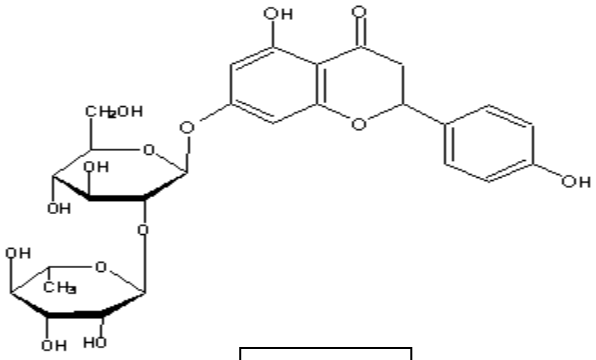
Bergapten

2.5.2 Flavonoids

Flavonoids, classified into four subgroups namely flavone, flavonol, flavonone and isoflavonone, are low molecular weight polyphenolic compounds that usually exist in plants as secondary metabolites. Flavonoids modulate P-gp by directly interacting with the vicinal ATP-binding site, the steroid binding site or the substrate-binding site. They are also able to inhibit CYP 450 metabolizing the enzymes (Conseil et al., 1998; Hodek et al., 2002; Doostdar et al., 2000).

2.5.2.1 Naringin

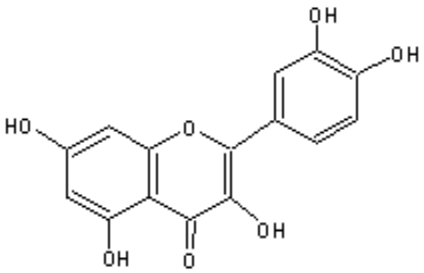
Naringin (7-(2-O-(6-deoxy- α - L- mannopyranosyl)- β- D-glucopyranosyloxy) - 2, 3-dihydro-4', 5, 7-trihydroxyflavone) is the most prevalent flavonoid in GFJ. Naringin has been reported to possess the ability to inhibit the P-gp efflux pump (Conseil et al, 1998; Takanaga et al., 1998; Bailey et al., 1993). It also has been reported to inhibit selectively intestinal CYP450 3A, which is the main subfamily of the CYP450 (Hodek et al., 2002; Doostdar et al., 2000) It was reported that naringin increased the bioavailability of paclitaxel (a P-gp substrate) and its prodrug after oral administration (Choi and Shin, 2005).



Naringin

2.5.2.2 Quercitin

Quercitin (2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-chromen-4-one), a water soluble plant pigment. Quercitin is also found in *Ginkgo biloba, Hypericum perforatum* (St. John's Wort), *Sambucus canadensis* (Elder), and many others. Quercitin acts as a natural modulator of P-gp efflux pump (Conseil et al., 1998, Chieli et al. 1995).



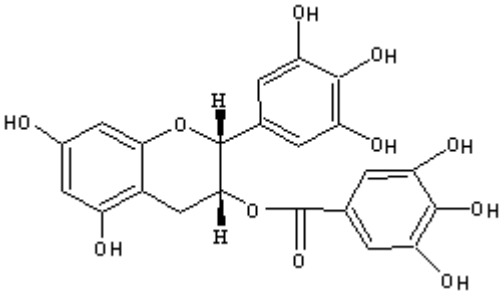
Quercitin

Co-administration of paclitaxel or its prodrug with quercitin in rats led to a significant improvement in bioavailability as compared to control (Choi et al., 2004). Quercitin inhibite P-gp mediated Hoechst 33342 efflux and enhanced its accumulation in multidrug resistant CHRC5 cells (Shapiro and Ling, 1997).

2.5.2.3 Catechins

Green tea (*Camellia sinensis*) a common beverage worldwide, contains many polyphenolic compounds. A typical brewed green tea beverage contains 30 to 42 % catechins by dry weight (Balentine et al., 1997). Six catechins present in green tea, the most abundant being (-)-epigallocatechin gallate (EGCG)

followed by (-)-epicatechin gallate, (-)-epigallocatechin, (-)-epicatechin, (-)-catechin gallate and (+)-catechin (Chu and Juneja, 1997).

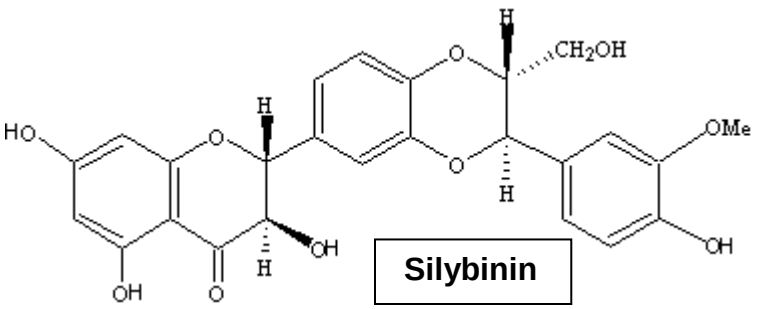


Epigallocatechin gallate

Catechins were shown to inhibit P-gp activity *in vitro* using several marker substrates. Among the catechins present in green tea, EGCG was the most potent inhibitor of P-gp (Jodoin et al., 2002).

2.5.2.4 Milk Thistle (Silymarin)

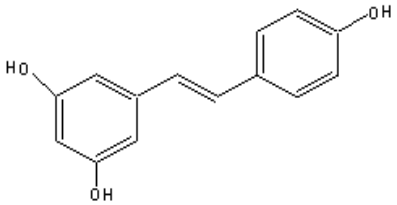
Milk thistle **(***Silybum marianum***)** is one of the most commonly used herbal medicines. It contains mainly contains Silymarin, a mixture of various flavonolignans (Barreto et al, 2003; Wallace et al, 2003). Silymarin is composed of mainly silybinin (50-80 %), with small amounts of other flavonolignans such as silychristin and silydianin (Kvasnicka et al, 2003). silymarin increased daunomycin accumulation in P-gp positive cells, but not P-gp negative cells, in a flavonoid concentration and P-gp expression level-dependent manner. Silymarin potentiated doxorubicin cytotoxicity in P-gp positive cells, while it cab be inhibited the P-gp ATPase activity and azidopine photoaffinity labeling of P-gp, suggesting a direct interaction with the P-gp substrate binding (Zhang and Morris, 2003). These findings indicated that silymarin and its metabolites inhibited P-gp mediated cellular efflux, raising a potential for significant drug interactions with P-gp substrates.



Silybinin

2.5.2.5 Resveratrol

Resveratrol (trans-3, 5, 4'-trihydroxystilbene) is a polyphenol obtained in abundance from *Vitis vinifera*, labrusca, and muscadine grapes and in small quantities from eucalyptus, spruce, and lily. It has been shown to cause mechanism-based inactivation of CYP 450 3A4 and may cause clinically relevant drug interactions with CYP 450 3A4 substrates (Chan and Delucchi, 2000).

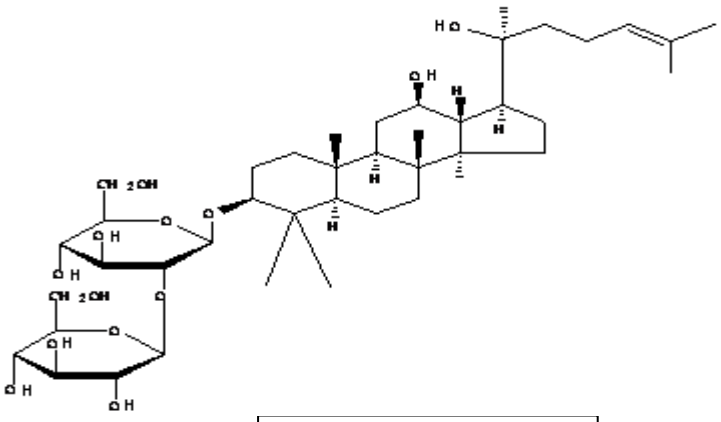


Resveratrol

2.5.3 Glycosides

2.5.3.1 Ginsenosides

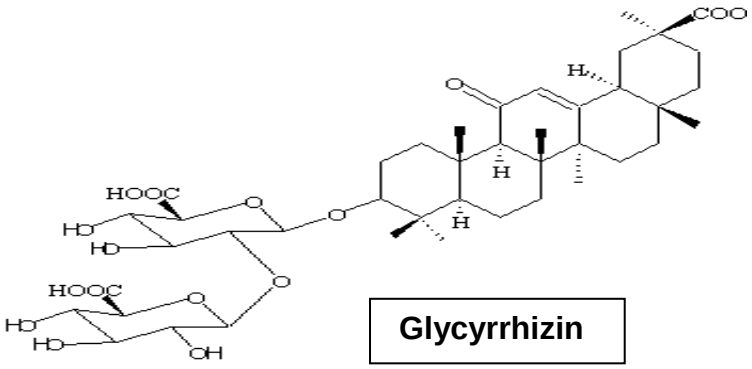
Ginseng *(Panax ginseng)* major constituents include ginsenosides (panoxosides), sterols, flavonoids, peptides, vitamins, polyacetylenes, minerals, β-elemine, and choline (Deyama et al., 2001; Han et al., 2001). Ginsenosides are considered the major pharmacologically active constituents, and approximately 12 types of ginsenosides were isolated and structurally identified. Ginsenoside Rg3 promoted accumulation of Rho-123, inhibited vinblastine efflux, and reversed the resistance to doxorubicin and vincristine in multidrug resistant KBV20C cells in a dose-dependent manner (Kim et al., 2003).



Ginsenoside Rg3

2.5.3.2 Glycyrrhizin

Glycyrrhizin a chief constituent (6-10 %) of Liquorice (*Glycyrrhiza glabra)*, is a triterpenoid saponin glycoside and is a potassium and calcium salt of glycyrrhizinic acid. Glycyrrhizinate dipotassium has been reported to enhance the *in vivo* transmucosal absorption of antibiotics and insulin (Tanaka et al., 1992; Mishima et al., 1989).



Glycyrrhizin

2.5.4 Fatty acids

Drug absorption has been increased by a wide variety of long chain fatty acids (Williams and Barry, 2004). The mechanisms by which fatty acids increase intestinal absorption have been shown to involve both paracellular and transcellular routes (Aungst et al, 1996). These lipoidal absorption promoters may enter the lipid bilayer structure in intestinal brush border membrane and disrupt the configuration of the lipid region (Wang et al., 1994; Muranishi, 1990).

The effects of various fatty acids on cefoxitin absorption from rat intestinal loops *in situ*, using emulsion vehicles containing 10 % fatty acid. The rank order of absorption enhancement was lauric acid > palmitic acid > caprylic acid > oleic acid (Palin et al., 1986). Oleic acid, linoleic acid and linolenic acid as absorption promoters into the oily phase of insulin W/O/W multiple emulsion. Strong hypoglycemic effects were observed with the emulsions containing these absorption promoters with more pronounced effects in the colon than in the ileum (Morishita et al., 1998).

2.5.5 Oils

2.5.5.1 Peppermint oil

Peppermint oil significantly enhanced oral bioavailability of cyclosporine in rats (Wacher et al., 2002). Peppermint oil is an effective inhibitor of cyclosporine metabolism *in vitro*. Inhibition of cyclosporine metabolism in the small intestine and possibly on first-pass through the liver are potential means by which peppermint oil may improve cyclosporine bioavailability *in vivo*. Similar finding in the case of felodipine and peppermint oil co-administration (Dresser et al., 2002).

2.5.5.2 Sunflower oil

Significant improvement in bioavailability of moxidectin in rabbits when co-administered with sunflower oil. The enhanced moxidectin bioavailability was attributed to greater extent of intestinal lymphatic transport in presence of sunflower oil (Bassissi et al., 2004).

2.5.6 Miscellaneous

2.5.6.1 Rosemary extract

Rosemary extract has been reported to cause a substantial increment in the intracellular accumulation of doxorubicin and vinblastine and inhibit the efflux of these P-gp substrates in drug resistant MCF-7 human breast cancer cells. Rosemary extract directly binds to P-gp binding site and exerts its action via competitive inhibition of substrate binding (Plouzek et al., 1999).

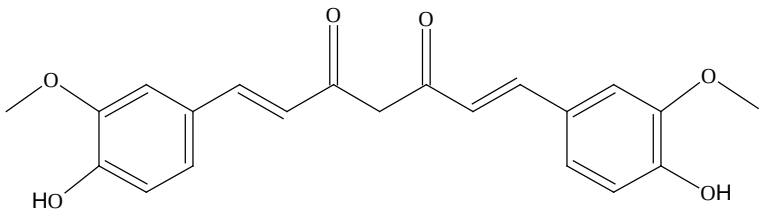
2.5.6.2 Chinese herbal enhancers

Chinese herbal enhancers (CHEs) are used in over 30-75 % Chinese medicines since thousands of years. Toxicities of CHEs are expected to be much mild than synthetic agents (Hu, 2004). The C_{max} and AUC of simvastatin increased 39 and 23 times, respectively, after oral administration simvastatin with HUCHE 015 enhancer in rats. Co-administration of HUCHE 011 with paclitaxel has been reported to cause a significant increase in intracellular accumulation of Rho-123 *in vitro* in HCT-15 cell lines and bioavailability of paclitaxel in rats. These effects of HUCHE 011 due to the

inhibition of P-gp. CHEs may act as enzyme inhibitors and efflux inhibitors, in the case of CYP 450 and P-gp substrates, carriers for drug thereby facilitating their transport and catalyst (Hu, 2004).

2.5.6.3 Curcumin

Curcumin (5-hydroxy-1, 7-bis (4-hydroxy-3-methoxy-phenyl)-hepta-1, 4, 6-trien-3-one) is a natural polyphenol found in rhizomes of *Curcuma longa* (turmeric). In primary cultures of rat hepatocytes as well as in human cervical carcinoma cell line KB-V1 expressing high levels of P-gp, curcumin increased Rho-123 accumulation and inhibited its efflux in a dose-dependent manner (Zhou et al., 2004).



Curcumin

curcumin inhibited verapamil stimulated ATPase activity and the photoaffinity labeling of P-gp with the prazosin analog iodoarylazidoprazosin in a concentration-dependent manner, indicating direct interaction of curcumin with P-gp and possible binding to the same site as other agents such as prazosin and verapamil (Anuchapreeda et al., 2002).

2.5.6.4 Ginger

Dried rhizomes of *Zingiber officinale* are reported to increase the bioavailability of certain drugs by promoting rapid absorption from the GI tract or by protecting the drug from being metabolized or oxidized in first passage through the liver after absorption. Absorption of sulfaguanidine increased in male rats on co-administration of ginger (Sakai et al., 1986). Accumulation of daunorubicin and Rho-123 increased in KB-C2 cells on co-administration of 6-gingerol due to P-gp inhibition (Nabekura et al., 2005).

2.5.6.5 St. John's Wort

St. John's wort (*Hypericum perforatum*) is one of the most common herbal medicines used in the therapy of depression. Several studies indicate that St. Johns wort induces the intestinal P-gp *in vitro* and *in vivo* (Durr et al., 2000; Hennessy et al., 2002; Perloff et al., 2001). Treatment of LS-180 intestinal carcinoma cells with St. John's wort or hypericin at 3–300 mmol caused a four- to sevenfold increase in the expression of P-gp (Perloff et al., 2001). Cells chronically treated with St. John's wort had decreased accumulation of rhodamine 123. Oral administration of St. John's wort for 14 days in healthy volunteers resulted in 1.4 fold increase in P-gp expression (Durr et al., 2000). The probe substrates of P-gp, fexofenadine and cyclosporin were found to have increased clearance in healthy subjects treated with St. John's Wort (Dresser et al., 2003)

2.6 Cytotoxicity study by using cell lines

The SRB assay is used for cell density determination, based measurement of cellular protein content. The method described here has been optimized for the toxicity screening of compounds to adherent cells in a 96-well format. After an incubation period, cell monolayersfixed with 10 % (w/v) trichloroacetic (TCA) acid and stained for 30 min, after which the excess dye is removed by washing repeatedly with 1 % (v/v) acetic acid. Then the protein bound dye is dissolved in 10 mmol Tris base solution for OD determination at 510 nm using a microplate reader. The results are linear over 20 fold range of cell numbers and sensitivity is comparable to those offluorometric methods. The method not only allows a large number of samples to be tested within a few days, but also requires only simple equipment and inexpensive reagents. The SRB assay is therefore an efficient and highly cost-effective method for screening.

2.6.1 SRB assay

SRB assay, which was developed in 1990, remains one of the most widely used methods for *in vitro* cytotoxicity screening (Skehan, P. et al., 1990). The assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue culture plates by the TCA. SRB is a bright pink dye

of aminoxanthene with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions, and dissociate under basic conditions (Lillie et al., 1977). As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass. The strong intensity of SRB staining allows the assay to be carried out in a 96-well format. The assay can detect densities as low as 1,000–2,000 cells per well, and with a signal-to-noise ratio of 4.83 at a density of 5,000 cells per well. This level of sensitivity is comparable to those of fluorescent dye-staining methods and is superior to those of other protein staining methods using conventional dyes (Skehan, et al., 1990; McCaffrey et al., 1988). Results from the SRB assay exhibit a linear dynamic range over densities of 7,500–180,000 cells per well, corresponding to B1-200 % confluence. Furthermore, the SRB method has proven to be practical, because after the TCA-fixed and SRB-stained cell monolayers are dried they can be stored indefinitely. Color extracted from SRB stained cells is also stable. With its high level of sensitivity, adaptability to the 96 well format and endpoint stability, the SRB assay is well suited to large-scale screening applications as well as research.

This assay has been widely used for drug-toxicity testing against different types of the cancerous and non cancerous cell lines (Monks et al., 1991). Other cell-growth assays have been employed to assess drug efficacy against both intracellular pathogens and host cells simultaneously in co-cultures (McLaren et al., 1983; Kleymann et al., 2004) and it is possible to use SRB method for this purpose. An antiviral assay using SRB has been developed at our facility for the screening of natural compounds against herpes simplex virus type 1 (HSV-1), (Pittayakhajonwut et al., 2005). In addition, the SRB method has also been shown to be effective for *in vitro* testing of cancer cell sensitivity to radiation and for the study of interactions between radiotherapy and chemotherapy with sensitivity comparable to the standard of clonogenic assay (Griffon et al., 1995; Pauwels et al., 2003). The effectiveness of the SRB assay is frequently compared to that of another method using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT assay requires cellular metabolic activity to convert the

colorless tetrazolium to the purple-colored formazan dye (Plumb et al., 1989) therefore it detects only viable cells, whereas the SRB method does not distinguish between viable and dead cells. This difference, however, does not compromise the ability of the SRB assay to detect cytotoxic effects of a drug.

Studies undertaken by several groups showed that results from the SRB assay correlated well with those of the MTT assay, although the inhibitory concentration (IC) values of compounds tested using the SRB method were slightly higher 50 (Rubinstein et al., 1990; Haselsberger et al., 1996; Perez et al., 1993). However, the SRB assay has several advantages over the MTT assay. For example, some compounds can directly interfere with MTT reduction without having any effects on cell viability (Plumb et al., 1989), while SRB staining is rarely affected by this type of interference. Furthermore, SRB staining is independent of cell metabolic activity; therefore, fewer steps are required to optimize assay conditions for specific cell lines than in the MTT assay (Keepers et al., 1991). The application of the SRB assay is limited to manual or semiautomatic screening due to the multiple washing and drying steps, which, at present, are not amenable to automation. This method nevertheless provides an efficient and sensitive tool for screening, especially for use in less well-equipped laboratories. The protocol presented here has been slightly modified from the original SRB assay described by Skehan et al., because we found that the concentrations of TCA and SRB required for the fixation and staining of several cell lines could be decreased, therefore reducing the health risks to technicians and the burden of toxic waste of the disposal.

The method is suitable for ordinary laboratory purposes and for very large-scale applications, such as the National Cancer Institute's disease-oriented *in vitro* anticancer-drug discovery screen, which requires the use of several million culture wells per year. Cultures fixed with TCA were stained for 30 min with 0.4 % (w/v) sulforhodamine B (SRB) dissolved in 1 % acetic acid. Then unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with the 10 mmol in to the buffered Tris base [tris

(hydroxymethyl) aminomethane] for determination of the optical density in a computer-interfaced, 96-well microtiter plate reader. The SRB assay results were linear with the number of cells and with values for the cellular protein measured by both Lowry and Bradford assays at the densities ranging from sparse sub-confluence to multilayered supra-confluence. The signal to noise ratio at 564 nm was approximately 1.5 with 1,000 cells per well. The sensitivity of the SRB assay compared favorably with sensitivities of several fluorescence assays and was superior to those of both the Lowry and Bradford assays and to the those of 20 other visible dyes. The SRB assay provides a colorimetric end point that is nondestructive, indefinitely stable, and visible to the naked eye. It provides a sensitive measure of the drug induced cytotoxicity is useful in quantitating clonogenicity, and is well suited to high-volume, automated drug screening. SRB fluoresces strongly with the laser excitation at 488 nm and it can be measured quantitatively from the single cell level by static fluorescence cytometry (Anne et al., 1991).

3. OBJECTIVES

Oral route is most preferred for drug delivery due to high patient compliance, noninvasive administration and low cost. Drugs of classes II-IV (with low solubility, low permeability or both) pose challenges to the pharmaceutical development scientists in developing drug products with acceptable oral bioavailability. Low oral bioavailability of the drugs also results in erratic pharmacokinetics and sometimes needs careful management of therapy. One such approach is the concomitant use of absorption promoters. Recent *in vitro* studies have demonstrated that the synthetic absorption promoters such as sodium lauryl sulfate, palmitoyl carnitine, sodium caprate etc., when used at effective concentrations produced any cytotoxicity or the membrane damage. Limitations of synthetic absorption promoters suddenly increased interest in herbal absorption promoters. HAPs provide promising alternative to synthetic counterparts in terms of safety and many times in the efficacy of intestinal wall. Majority (approximately 90 %) of the absorption in the GI tract occurs in the small intestine.

Several of the HAPs have been shown in to possess enormous potential to improve the oral bioavailability of poorly absorbed drugs without causing local or systemic toxicities. Several *in vitro* and *in vivo* studies and few clinical studies with HAPs have exhibited a very high benefit to risk ratio. These findings have renewed interest of the pharmaceutical scientists in exploring established and newer herbal origin compounds for possible application in enhancement of bioavailability of otherwise poorly absorbed drugs. Improved oral bioavailability due to the concomitant administration of HAPs reduce the therapeutic dose of the drug thereby minimizing side effects and cutting down on the cost of therapy. This can be panacea in addressing the problems of absorption/bioavailability of drugs presently posed in effective drug delivery. Efforts should be made to add to the list of HAPs acting through various mechanisms in promoting absorption not only through oral route but also through other routes of drug administration. However, role of these absorption promoters can only be settled after acute and chronic toxicological studies

followed by clinical evaluation of the pharmaceutical products containing HAPs on case to case basis.

In this study we have investigated the three HAPs i.e. silymarin, naringin and morin which are natural flavanoids it was studies with drugs which are metabolized by the first pass metabolism intensively particularly by enzyme CYP 450 and its various subtract and P-gp efflux mechanism and having a very poor bio-availability i.e. sildenafil citrate, atorvastatin calcium, zidovudine and carvedilol phosphate.

The objectives of the present investigation are:

i. To study the absorption promotion activity of selected isolated chemicals component of herbal origin.
ii. To develop suitable sensitive analytical methods for selected drugs in blood plasma and in presence of HAPs.
iii. To study detail mechanism by which the selected HAPs enhance the absorption.
iv. To find out the effective concentration of selected HAPs.
v. To study the toxicity of selected HAPs in its effective concentration.

4. MATERIALS AND METHODS

All the common chemicals were used were of analytical grade and glasswares were of borosilicate glass. The details of important chemicals are as under.

4.1 Reagents and Materials

- Atorvastatin Calcium (Gift sample from Strides Arco ltd, Bangalore)
- Sildenafil Citrate (Gift sample from Zydus Research Center, Ahmedabad)
- Zidovudine (Gift sample from Strides Arco ltd, Bangalore)
- Carvedilol Phosphate (Gift sample from Zydus Research Center, Ahmedabad)
- Silymarin (Gift sample from Cadila Pharmaceutical Company)
- Morin (Purchased from Sigma chem., USA)
- Naringin (Purchased from Sigma chem., USA)
- Methanol (HPLC grade – Rankem)
- Acetonitrile (HPLC grade – Rankem)
- Ammonium acetate (Merck)
- Ortho Phosphoric Acid (Ultrapure-Merck)
- Wattman filter paper no. 1
- Triple distill water
- HCT-15 cells (Procured from National Cancer Institute, Frederick, USA)
- MCF-7 cells (Procured from National Cancer Institute, Frederick, USA)
- SRB dye (Sigma chem., USA)
- Sodium Carboxy Methyl Cellulose (Himedia)
- Saline solution

4.2 Apparatus and Instruments

- Shimadzu Prominence UFLC system
- Sonicator (Equitron)
- Precision balance (swisser Insrument Gandhinagar)
- Analytical balance (shimadzu,Japan)
- pH meter (Elico, Ahmedabad)

- Cooling Centrifuge (Remi ,vasai)
- Deep freez (-20°c) (Remi,vasai)
- Vortexer (Genei, Banglore)
- Micro pipettes (eppendroff) (2-20 µl, 10-100µl, 100-1000 µl)
- Volumetric flask of 10 ,25 , 50, and 100 ml
- Syringe (2.0 mL)
- Oral Canula

4.2.1 Softwares

- SAS, version 9.1
- Maglon software
- Graph pad
- Prism 5
- Micro Soft office excell

4.3 HPLC Analytical method development and Validation

The drug analysis i.e. sildenafil citrate, zidovudine, atorvastatin calcium and carvedilol phosphate were performed on high performance liquid chromatography (HPLC) for high accuracy and for each drug the analytical method was developed and validated according the analytical parameters recommended for HPLC. Typical analytical performance characteristics that should be considered in validation of the method were performed for the all the drugs selected for the current study i.e sildenafil citrate, zidovudine, atorvastatin calcium and carvedilol phosphate. The analytical methods for atorvastatin calcium were mentioned as below.

4.3.1 Typical Analytical Parameters for HPLC Analysis

4.3.1.1 System suitability

Replicate injections of standard preparation used in standard curve solution are compared to ascertain whether requirement for precision are met. Five replicate injections of analyte are used to calculate the relative standard

deviation (RSD) if the requirement is 2 % or less and data from six replicate injections are used if RSD requirement is more than 2 %.

2ml of drug solution (100 µg/ml) was taken in 10 ml volumetric flask and diluted up to mark with methanol. 20 µl of this solution (20 µg/ml) was injected six times in to HPLC system and analyzed at 230 nm and RSD was calculated.

4.3.1.2 Linearity

The linearity of analytical method is its ability to elicit test results that are directly proportional to concentration of analyte in sample within given range. The linearity range of analytical method is interval between upper and lower level of analyte including level that been demonstrated to be determining with precision and accuracy using method.

The linearity is expressed in terms of correlation co-efficient of linear regression analysis. The linearity of response for drug was assessed by analysis of eight independent levels of calibration curve in range of 100-50000 ng, in terms of slope, intercept and correlation coefficient values.

4.3.1.3 Precision

The precision is measure of either the degree of reproducibility or repeatability of analytical method. It is indication of random error. Precision of analytical method is usually expressed as a standard deviation, relative standard error or co-efficient of variance of series of measurement. The value of % CV should be not more than 5 %.

4.3.1.4 Reproducibility

Variations of result within same day and amongst days are called as reproducibility. It includes following parameter:

4.3.1.4.1 Intraday reproducibility

A variation of result within same day is called intraday variation. It was determined by repeating calibration curve as describe by three times on same day and % CV was calculated.

4.3.1.4.2 Interday reproducibility

Variation of result amongst day is called interday variation. It was determined by repeating calibration curve daily for 3 different days as and % CV was calculated.

4.3.1.5 Accuracy

Accuracy of analysis is determined by systemic error. It is defined as closeness of agreement between actual (true) value and analytical value. Accuracy may expressed as % recovery by analysis of known added amount of analyte. It is measure of exactness of analytical method. It is determined by calculating recovery by standard addition method.

Accuracy data of drug:

Pre-analyzed drug solution (A) = 100 µg/ml

Standard drug solution (B) = 100 µg/ml

Sample solution was prepared from combination of above solution.

Solution was injected (10 µg/ml) in to HPLC system and analyzed at suitable wavelenght. The difference in area of each sample solution (sample no. 2 to 5) to sample solution (sample no. 1) obtained was used to calculate the % recovery.

4.3.1.6 Limit of detection

It is the lowest concentration of an analyte in a sample that can be detected but not necessarily quantified under the stated analytical conditions. Limit of detection (LOD) is commonly used to substantiate that analyte concentration is above or below a certain level different concentration of standard drug solution were used and minimum detectable limit was found.

1 ml of drug (100 µg/ml) was taken in 10 ml volumetric flask and diluted up to mark with methanol. Aliquots of the solution (1, 2, 3, 4, 5, 6, 7, 8 ml) were transferred into series of 10 ml volumetric flask and diluted with methanol up to mark. 20 µl of each solution were injected in to HPLC system and analyzed at 230 nm and find the lowest concentration that was detectable.

4.3.1.7 Limit of quantification

It is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental condition. The limit of quantification (LOQ) is commonly used for impurities and degradation assay of bulk and drug product.

4.3.1.8 Specificity

The specificity of an analytical method is ability to measure accurately an analyte in presence of interferences like synthetic precursor, excipient, degradant or matrix component. It was determined by spiking 20 µl of solution containing suitable concentration of test drug and 20 µl of internal standard. Internal standard drug solution were added in to the 80 µl rat blood plasma and mixed well. Then added 300 µl of the acetonitrile and vortexed for 2 min. centrifuged at 12000 rpm for 10 min at 4 ^{o}C. Taken out supernant and injected to HPLC.

4.3.1.9 Recovery

Six replicates of standard (STD) 1 and STD 5 were analyzed by following the procedure for preparation of standard solution and compared with same concentration level STD samples processed by following the procedure for extracted sample preparation.

4.3.1.10 Chromatographic system for HPLC Analysis

- Work station : Class – VP 10 Shimadzu.
- Binary solvent delivery system : SCL-10 Avp Shimadzu solvent Delivery module
- Universal injector : Shimadzu 7D Rheodyne 7725i, 100 µl injector.
- Detector : Photodiodarry Detector (SPD-10 M 10 Avp)
- Integration of data : Lc Solution software
- Syringe : Hamilton syringe 25 µl.

4.3.2Atorvastatin Calcium

4.3.2.1 Solution and sample preparation

♦ **Mobile phase buffer solution (20mmol potassium dihydrogen phosphate)**

Weigh accurately 680.45 mg of potassium dihydrogen phosphate in 250 ml triple distilled water, and pH adjusted to 3.10 using the orthophosphoric acid. The solution was filtered by using wattman filter paper (No. 1) and sonicated for 10 min and then used.

♦ **Mobile phase**

Mobile phase buffer solution: Methanol (20:80)

♦ **Drug stock solution**

Accurately weighed quantity of atorvastatin calcium equivalent to 10 mg. atorvastatin was transferred in to 10 ml volumetric flask and dissolved in 5 ml of methanol and diluted with methanol up to mark to give stock solution having a strength of 1 mg/ml. Appropriate dilution of stock solution were made with distilled water to obtain working solutions from 1.0 -188.0 µg/ml of atorvastatin by serial dilution method.

- **Internal standard stock solution**

  Accurately weighed quantity of atorvastatin calcium equivalent to 10 mg. atorvastatin was transferred in to 10 ml volumetric flask and dissolved in 5 ml of methanol and diluted with methanol up to mark to give stock solution having a strength of 1 mg/ml. Transferred 1.75 ml of stock solution into 25 ml volumetric flask and dissolved in 10 ml distilled water and diluted with distilled water up to mark to give stock solution having a strength of 70 µg/ml.

- **Preparation of atorvastatin calcium suspensions**

  A suspension of atorvastatin calcium of concentration 20 mg/ml. Sodium CMC (1 % w/v) was used as suspending agents. The vehicle sodium CMC (1 % w/v) in distilled water] was used as negative control.

4.3.2.2 Preparation of calibration curve

Aliquots of the drug tock solution (1, 2, 3, 4 and 5 ml) were transferred in to series of 10 ml volumetric flask and diluted with methanol up to mark. 20µl of each solution were injected in to HPLC system and analyzed at 230 nm. Calibration curve was obtained by plotting respective peak area against concentration.

4.3.2.3 Chromatographic condition

- Pumping mode : Binary
- Mobile phase : 20 mMol Potassium Dihydrogen phosphate (pH-3.30 adjusted with Ortho-phosphoric acid) Buffer : Methanol (20:80)
- Column : Phenomax C18, (250 x 4.6 mm i.d., 5µm)
- ʎmax : 230 nm
- Injection volume : 20 µl
- Run time : 10 min
- Flow rate : 1.0 ml/min

4.3.3 Carvedilol

4.3.3.1 Solution and sample preparation

- **Mobile phase buffer solution (30 mMol ammonium formate, pH-4.00)**

  Weigh accurately 472.95 mg of ammonium formate in 250 ml triple distilled water, and pH was adjusted to 4.00 using formic acid. The solution was filtered using Wattman filter paper (No. 1) and sonicated for 10 min and then used.

- **Mobile phase**

  Mobile phase buffer solution: Methanol (25:75)

- **Drug stock solution**

  Accurately weighed quantity of carvedilol phosphate equivalent to 10 mg. carvedilol was transferred in 10 ml volumetric flask and dissolved in 5 ml of methanol and diluted with methanol up to mark to give stock solution having a strength of 1 mg/ml. Appropriate dilution of stock solution were made with distilled water to obtain working solutions from 0.5-208.0 µg/ml of carvedilol by serial dilution method.

- **Internal standard stock solution**

  Accurately weighed quantity of diclofenac sodium equivalent to 10 mg. Diclofenac was transferred in to 10 ml volumetric flask and dissolved in 5 ml of methanol and diluted with methanol up to mark to give stock solution having a strength of 1 mg/ml. Transferred 1.50 ml of stock solution into 25 ml volumetric flask and dissolved in 10 ml distilled water and diluted with distilled water up to mark to give stock solution having a strength of 70 µg/ml.

- **Preparation of carvedilol suspensions**

  A suspension of carvedilol of concentration 22 mg/ml. Sodium CMC (1 % w/v) was used as suspending agents. The vehicle sodium CMC (1 % w/v) in distilled water] was used as negative control.

4.3.3.2 Chromatographic condition

- Pumping mode : Binary
- Mobile phase : 30 mmol ammonium formate Buffer (pH-4.00 with formic acid): Methanol (25:75)
- Column : Phenomax C18, (250 x 4.6 mm i.d., 5µm)
- Injection volume : 20 µl
- Run time : 7.50 min.
- Flow rate : 1.2 ml/min.

4.3.3.3 Preparation of calibration curve

Aliquots of the drug stock solution [0.01, 0.02, 0.04, 0.160, 0.320 ml (from 50 µg/ml), 0.320,0.64, 1.28 and 2.08 ml (from 1000 µg/ml) were transferred into series of 10 ml volumetric flask and diluted with distill water up to mark which gives concentration range 0.500-208.00 µg/ml. 20 µl of each solution were injected in to HPLC system and analyzed. Calibration curve was obtained by plotting respective peak area ratio against concentration.

4.3.4 Zidovudine

4.3.4.1 Solution and sample preparation

- **Mobile phase**

  Methanol : Triple distill Water (70 : 30)

- **Drug Stock Solution**

  Accurately weighed quantity of zidovudine 10 mg was transferred to 10 ml volumetric flask and dissolved in 5 ml of triple distill water and diluted with Triple distill water up to mark to give stock solution having a strength of 1 mg/ml

4.3.4.2 Chromatographic condition

- Pumping mode : Binary
- Mobile phase : Methanol : Water (70 : 30)
- Column : Phenomax C18, (250 x 4.6 mm i.d., 5µm)
- λ_{max} : 266 nm
- Injection volume : 20 µl
- Run time : 5 min
- Flow rate : 1.0 ml/min

4.3.4.3 Preparation of calibration curve

Aliquots of the drug stock solution (1, 2, 3, 4 and 5 ml) were transferred into series of 10 ml volumetric flask and diluted with Triple distill water up to mark. 20 µl of each solution were injected in to HPLC system and analyzed at 266 nm. Calibration curve was obtained by plotting respective peak area against concentration.

4.3.4 Sildenafil citrate

4.3.4.1 Solution and sample preparation

- **Mobile phase buffer solution**

  It was prepared by mixing 500 ml of 30 mMol ammonium acetate, and the pH was adjusted to 6.00 using 1 N sodium hydroxide solution. The solution was filtered using Wattman filter paper (No. 1) and sonicated for 10 min and then used.

- **Mobile phase**

  Mobile phase buffer solution : Acetonitrile (35:65)

- **Drug stock solution**

  Accurately weighed quantity of sildenafil citrate 10 mg was transferred in to 10 ml volumetric flask and dissolved in 5 ml of methanol and diluted with methanol up to mark to give stock solution having a strength of 1 mg/ml

- **Preparation of suspensions**

  A suspension of sildenafil citrate of concentration 3 mg/ml. Sodium CMC (1% w/v) was used as a suspending agents. The vehicle [sodium CMC (1% w/v) in distilled water] was used as negative control.

4.3.4.2 Chromatographic condition

- Pumping mode : Binary
- Mobile phase : Acetonitrile: 30 mMol ammonium acetate buffer, (pH- 6.00 with 1 N NaOH) 65:35
- Column : Phenomax C18, (250 x 4.6 mm i.d., 5µm)
- λ_{max} : 230 nm
- Injection volume : 20 µl
- Run time : 10 min
- Flow rate : 1.0 ml/min

4.3.4.3 Preparation of calibration curve

Aliquots of the drug stock solution (1, 2, 3, 4 and 5 ml) were transferred into series of 10 ml volumetric flask and diluted with methanol up to mark. 20 µl of each solution were injected in to HPLC system and analyzed at 230 nm. Calibration curve was obtained by plotting respective peak area against concentration.

4.4 *in vivo* bioavailability study

4.4.1 Atorvastatin calcium

Healthy wistar albino rats (250–300 g) had free access to normal standard chow diet and tap water. Animals were fasted for 24 hr prior to the experiments and were given water freely. Each rat was anaesthetized by inhalation of diethyl ether. Drug suspension was prepared by adding atorvastatin calcium (30 mg/kg) to the 1% CMC solution (3 mg/mL). In the present study, rats were divided in to following groups as mentioned below. In each group rats (n = 6 per each treatment) were given orally a 30 mg/kg of

atorvastatin calcium along with selected HAPs i.e. silymarin, morin and naringin in concentration of 100 µm in each dose.

Group i: Rats were given orally a 30 mg/kg of atorvastatin Calcium with silymarin (100 µm) subsequent to atorvastatin calcium administration

Group ii: Rats were given orally a 30 mg/kg of atorvastatin Calcium with morin (100 µm) subsequent to atorvastatin calcium administration

Group iii: Rats were given orally a 30 mg/kg of atorvastatin calcium with (100 µm) subsequent to atorvastatin calcium administration

Group iv: Rats were given orally a 30 mg/kg of atorvastatin calcium without any subsequent administration

Blood samples were withdrawn from the retro orbital plexus according to a predetermined time schedule at 0.5, 1, and 2 hr post dose and collected in EDTA containing tubes. Blood samples were centrifuged at 12000 rpm for 10 min and the plasma was removed and process for extraction and analyzed by HPLC.

4.4.2 Procedure for Extracted sample preparation

- 0.5 ml of the spiked plasma in the eppendrof tube was taken.
- 50 µl of TCA was added in the same eppendrof tube and vortex the samples thoroughly for 3 min.
- The sample was then centrifuged at 12000 rpm for 10 min at 4 ^{o}C
- 300µl of supernant was transferred in to another pre-labeled eppendrof tube.
- The sample was injected into HPLC system.

4.4.3 Data processing

The chromatograms were acquired by using Class VP, version 1.6 supplied by HPLC manufacturers. The calibration curve was plotted as the peak area ratio (drug) on Y-axis vs. the nominal concentration of drug on the X-axis. The

concentrations of the unknown samples were calculated by using the linear regression equation with 1/x2 weighing factor.

4.4.4 Pharmacokinetics and statistical analysis

Pharmacokinetic and statistical analysis for plasma concentration vs. time profile of atorvastatin calcium was performed on the data obtained from male wistar rat.

4.4.4.1 Pharmacokinetic parameters

Pharmacokinetic parameters i.e. T_{max}, C_{max} were calculated using plasma concentration vs. time profile (Actual time of the sample collection) data of atorvastatin calcium in individual wistar rat by using the graph pad software. Pharmacokinetic parameters were calculated by non-compartmental analysis using graph pad software. The peak level C_{max} and time to reach peak level T_{max} was estimated from the plasma concentration time profile data.

4.4.4.2 Statistical Analysis

SAS, version 9.1 was used for the statistical analysis of the pharmacokinetic parameters i.e. C_{max}, T_{max}. etc.

4.4.4.3 Analysis of variance

The untransformed and ln-transformed pharmacokinetic parameters i.e. C_{max}, was subjected to Analysis of Variance (ANOVA). ANOVA model was included sequence, subject (sequence), period and formulation effects as factors. Difference between the treatments, period of treatment, sequence of dosing was considered statistically significant if probability values of the respective effects (p-values) were < 0.05.

4.4.5 Carvedilol

4.5.5.1 Blood sample collection and processing

Male wistar rats (250–300 g) had free access to normal standard chow diet (Jae and Chow, Korea) and tap water. Animals were fasted for 24 h prior to the experiments and were given water freely. The protocol of the experiment

was approved by the Institutional Animal Ethical Committee (IAEC) as per the guidance of the Committee for The Purpose of Control and Supervision of Experiments on Animals (CPCSEA), (SU/DPS/IAEC/9006). The each rat was anaesthetized by inhalation of diethyl ether. Drug suspension was prepared by adding Carvedilol Phosphate (60 mg/kg) to the 1 % Sodium CMC Solution. In the present studythe rats were divided into the following groups:

Group i: Rats were given orally a 60 mg/kg of carvedilol phosphate with silymarin (100 µm) subsequent to carvedilol phosphate administration

Group ii: Rats were given orally a 60 mg/kg of carvedilol phosphate with morin (100 µm) subsequent to carvedilol phosphate administration

Group iii: Rats were given orally a 60 mg/kg of carvedilol phosphate with morin (100 µm) subsequent to the carvedilol phosphate administration

Group iv: Rats were given orally a 60 mg/kg of the carvedilol phosphate without any subsequent administration

Blood samples were withdrawn from the retro orbital plexus according to a predetermined time schedule at 0, 20, 50, 95, 155, 220, 280 and 340 min, and collected in EDTA containing tubes. Blood samples were centrifuged at 12000 rpm for 10 min and the plasma was removed and process for extraction and analyzed by HPLC.

4.5.5.2 Extraction procedure for sample preparation

- The spiked plasma 0.1 ml of sample was taken in the eppendrof tube.
- 20µl of internal standard (Diclofenac sodium, 60 µg/ml) and 300 µl methanol was added in same eppendrof tube and vortex the samples thoroughly for 2 min.
- The samples was then centrifuged at 12000 rpm for 10 min at 4 ^{o}C.
- The supernant transferred in to another prelabelled eppendrof tube.
- Inject 20 µl the sample into HPLC system.

4.5.5.3 Pharmacokinetics and statistical analysis

Pharmacokinetic and Statistical analysis for plasma concentration Vs time profile of Sildenafil citrate was performed on the data obtained from wistar albino rat.

4.5.5.3.1 Pharmacokinetic parameters

Pharmacokinetic parameters i.e. T_{max}, C_{max} were calculated using the plasma concentration vs. time profile (actual time of the sample collection) data of the carvedilol phosphate in the individual wistar rats using graph pad software. Pharmacokinetic parameters were calculated by non-compartmental analysis using Graph pad software. The peak level C_{max} and time to reach peak level T_{max} was estimated from the plasma concentration time profile data.

4.5.5.3.2 Statistical analysis

SAS, version 9.1 was used for the statistical analysis of the pharmacokinetic parameters i.e. C_{max}, T_{max} etc.

4.5.5.3.3 Analysis of variance

The untransformed and ln-transformed pharmacokinetic parameters Cmax, was subjected to Analysis of Variance (ANOVA). ANOVA model was included sequence, subject (sequence), period and formulation effects as factors. Difference between the treatments, period of treatment, sequence of dosing was considered statistically significant if probability values of the respective effects (p-values) were < 0.05.

4.5.6 Sildenafil citrate

4.5.6.1 Blood sample collection and processing

Male wistar albino rats (250–300 g) had free access to normal standard chow diet (Jae and Chow, Korea) and tap water. Animals were fasted for 24 hr prior to the experiments and were given water freely. Each rat was anaesthetized by inhalation of Diethyl ether. Drug Suspension was prepared by adding Sildenafil citrate (30 mg/kg) to the 1 % CMC Solution (3 mg/mL). Following mentioned groups are selected for the study as mentioned below:

Group i: Rats were given orally a 30 mg/kg of sildenafil citrate with silymarin (100 µm) subsequent to the sildenafil citrate administration

Group ii: Rats were given orally a 30 mg/kg of sildenafil citrate with morin (100 µm) subsequent to the sildenafil citrate administration

Group iii: Rats were given orally a 30 mg/kg of sildenafil citrate with (100 µm) subsequent to sildenafil citrate administration

Group iv: Rats were given orally a 30 mg/kg of the sildenafil citrate without any subsequent administration

Blood samples were withdrawn from the Retro orbital plexus according to a predetermined time schedule at 0.5, 1, and 2 hr post dose and collected in the EDTA containing tubes. Blood samples were centrifuged at 12000 rpm for 10 min. and the plasma was removed and process for extraction and analyzed analyzed by HPLC.

4.5.6.2 Procedure for extracted sample preparation

- 0.5 ml of the spiked plasma in the eppendrof tube was taken.
- 50 µl of TCA was added in the same eppendrof tube and vortex the samples thoroughly for 3 min.
- The sample was then centrifuged at 12000 rpm for 10 min at 4 ^{o}C.
- 300µl of supernant was transferred in to another pre-labeled eppendrof tube.
- The sample was injected into HPLC system.

4.5.6.3 Data processing

The chromatograms were acquired by using Class VP, version 1.6 supplied by HPLC manufacturers. The calibration curve was plotted as the peak area ratio (drug) on Y-axis vs. the nominal concentration of drug on the X-axis. The concentrations of the unknown samples were calculated by linear regression equation with 1/x2 weighing factor.

4.5.6.4 Pharmacokinetics and statistical analysis

Pharmacokinetic and statistical analysis for plasma concentration Vs time profile of Sildenafil citrate was performed on the data obtained from wistar albino rat.

4.5.6.4.1 Pharmacokinetic Parameters

Pharmacokinetic parameters T_{max}, C_{max} were calculated using plasma concentration vs time profile (actual time of sample collection) of the sildenafil citrate in individual male wistar rats using the graph pad software. Pharmacokinetic parameters were calculated by non-compartmental analysis using graph pad software. The peak level C_{max} and time to reach peak level T_{max} was estimated from the plasma concentration time profile data.

4.5.6.4.2 Statistical Analysis

SAS, version 9.1 was used for the statistical analysis of the pharmacokinetic parameters i.e. C_{max}, T_{max}. etc.

4.5.6.4.3 Analysis of variance

The untransformed and ln-transformed pharmacokinetic parameters C_{max}, was subjected to ANOVA. The ANOVA model was included sequence, subject (sequence), period and formulation effects as factors. Difference between the treatments, period of treatment, sequence of dosing was considered statistically significant if probability values of the respective effects (p-values) were < 0.05.

4.5.7 Zidovudine

4.5.7.1 Blood sample collection and processing

Male wistar albino rats (250–300 g) had free access to normal standard chow diet (Jae and Chow, Korea) and tap water. Animals were fasted for 24 hr prior to the experiments and were given water freely. Each rat was anaesthetized by the inhalation of diethyl ether. Drug suspension was prepared by adding the zidovudine (75 mg/ kg) in to the 1 % CMC solution (3 mg/ml). Following mentioned groups are selected for the study as mentioned below:

Group i: Rats were given orally a 75 mg/kg of zidovudine with silymarin (100 µm) subsequent to the zidovudine administration

Group ii: Rats were given orally a 75 mg/kg of zidovudine with morin (100 µm) subsequent to zidovudine administration.

Group iii: Rats were given orally a 75 mg/kg of zidovudine with (100 µm) subsequent to zidovudine administration.

Group iv: Rats were given orally a 75 mg/kg of zidovudine without any subsequent administration

Blood samples were withdrawn from the retro orbital plexus according to a predetermined time schedule at 0.5, 1, and 2 hr post dose and collected in EDTA containing tubes. Blood samples were centrifuged at 12000 rpm for 10 min and the plasma was removed and process for extraction and analyzed analyzed by HPLC.

4.5.7.2 Procedure for extracted sample preparation

- 0.5 ml of the sample spiked plasma in the eppendrof tube was taken.
- 50 µl of TCA was added in the same eppendrof tube and vortex the samples thoroughly for 3 min.
- The sample was then centrifuged at 12000 rpm for 10 min at 4 ^{o}C.
- 300µl of supernant was transferred in to another pre-labeled eppendrof tube.
- The sample was injected into HPLC system.

4.5.7.3 Data processing

The chromatograms were acquired by using Class VP, version 1.6 supplied by HPLC manufacturers. The calibration curve was plotted as the peak area ratio (Drug) on Y-axis Vs the nominal concentration of drug on the X-axis. The concentrations of unknown samples were calculated by the linear regression equation with 1/x2 weighing factor.

4.5.7.4 Pharmacokinetics and statistical analysis

Pharmacokinetic and statistical analysis for plasma concentration vs. time profile of zidovudine was performed on the data obtained from male wistar rat.

4.5.7.4.1 Pharmacokinetic parameters

Pharmacokinetic parameters i.e. T_{max}, C_{max} were calculated by using the plasma concentration vs time profile (Actual time of sample collection) data of zidovudine in individual animal using graph pad software. Pharmacokinetic parameters were calculated by non-compartmental analysis using graph pad software. The peak level (C_{max}) and time to reach peak level (T_{max}) was estimated from the plasma concentration time profile data.

4.5.7.4.2 Statistical analysis

SAS, version 9.1 was used for the statistical analysis of the pharmacokinetic parameters i.e. C_{max}, T_{max}. etc.

4.5.7.4.3 Analysis of variance

The untransformed and ln-transformed pharmacokinetic parameters C_{max}, was subjected to ANOVA. ANOVA model was included sequence, subject (sequence), period and formulation effects as factors. Difference between the treatments, period of treatment, sequence of dosing was considered statistically significant if probability values of the respective effects (p-values) were < 0.05.

4.6 *In vitro* cytotoxicity of selected herbal absorption promoters against human cancer cell lines

4.6.1 Cell lines preparation

For the current study three cell lines were selected for the study i.e. HCT-15 for expression of CYP 450 activity & MCF 7 for the expression of P-gp expression (P. Skehan et al., 1990). The human cancer cell lines were procured from National Cancer Institute, Frederick, U.S.A. Cells were grown in tissue culture flasks in complete growth medium (RPMI-1640 medium with 2mmol glutamine, pH 7.4, supplemented with 10 % fetal calf serum, 100 µg/ml

streptomycin and 100 units/ml penicillin) in a carbon dioxide incubator (37 °C, 5 % CO_2, 90 % RH). The cells at subconfluent stage were harvested from the flask by treatment with trypsin [0.05 % in PBS (pH 7.4) containing 0.02 % EDTA]. Cells with viability of more than 98 % as determined by trypan blue exclusion, were used for determination of cytotoxicity The cell suspension of 1 x 10^5 cells/ml was prepared in complete growth medium (Monks et al., 1991)

4.6.2 Preparation of stock solution of selected HAPs

The stock solution of selected HAPs i.e. silymarin, morin and naringin were prepared in water to obtain strength of 2 x 10^{-2} mol. The stock solutions were serially diluted with complete growth medium containing 50 µg/ml of gentamycin to obtain working test solutions of required concentrations.

4.6.3 SRB (Sulforhodamine B dye) assay for measurement of *In vitro* cytotoxicity study

In vitro cytotoxicity against two human cancer cell lines was determined (Monks et al., 1991) using 96-well tissue culture plates. The 100 µl of cell suspension was added to each well of the 96-well tissue culture plate. The cells were allowed to grow in carbon dioxide incubator (37 °C, 5 % CO_2, 90 % RH) for 24 hr. Test materials of selected HAPs i.e. silymarin, naringin and morin were added in concentration of 25 µmol, 50 µmol, 100 µmol & 200 µmol strength and 5-flourouracil (FU) and tamoxifen in concentration of 10 µg/100 µl strength as positive in complete growth medium (100 µl) were added after 24 hr of incubation to the wells containing cell suspension. The plates were further incubated for indicated time period in a carbon dioxide incubator. The cell growth was stopped by gently layering TCA (50 %, 50 µl) on top of the medium in all the wells. The plates were incubated at 4 ^oC for 1 hr to fix the cells attached to the bottom of the wells. The liquid of all the wells was gently pipetted out and discarded. The plates were washed five times with distilled water to remove TCA, growth medium low molecular weight metabolites, serum proteins etc. and then it was air-dried completely. The plates were stained with Sulforhodamine B dye (0.4 % in 1 % acetic acid, 100 µl) for 30 min. The plates were washed five times with 1% acetic acid and then air-

dried. (Skehan et al., 1990). The adsorbed dye was dissolved in Tris-HCl Buffer (100 μl, 0.01 mol, pH 10.4) and plates were gently stirred for 10 min on a mechanical stirrer. The optical density (OD) was recorded on enzyme linked immune sorbent assay (ELISA) reader at 540 nm on GENESIS Work station and TECAN multi-plate reader by using Maglon software.

The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. % growth in presence of test material was calculated considering the growth in absence of any test material as 100 % and in turn percent growth inhibition in presence of test material was calculated.

4.7 *In vitro* enzyme assays

P-gp-ATPase enzyme activity and two subtract of CYP 1A1 (Benzopyrene Hydroxylase) and CYP 450 3A4 (Erythromycin demethylase) were selected for the study and its activity was measured by *in vitro* assays as mentioned below.

4.7.1 *In vitro* Pgp-ATPase activity

4.7.1.1 Animals

Healthy wistar rats (150–200 g body weight) of both sexes were maintained in regulated environmental conditions (well-ventilated with >10 air changes/h; 12 h light/dark photoperiod; temperature 28 ± 2 ºC; relative humidity, 60 ± 10%), according to CPCSEA guidelines. Animals were fed with standard pelleted diet and water was provided *ad libitum*. Seven days after acclimatization, the animals were used.

4.7.1.2 Preparation of intestinal membrane fractions

Crude intestinal membrane fractions were prepared from jejunal mucosa of rats as described earlier (Yumoto et al., 2001). Crude membrane fractions were prepared from the intestinal mucosa and liver of rats. The luminal contents of whole small intestine were thoroughly washed out with a sufficient amount of ice-cold saline, and the intestine was divided into two parts of the

same length. The mucosal surface of the lower half of intestine was scraped off with a cover glass. The intestinal mucosa collected and the isolated liver were homogenized in a buffer containing 250 mM sucrose and 5 mM HEPES-Tris (pH 7.4) with a tissue homogenizer. The homogenates were centrifuged at 3,000 rpm for 10 min. The supernatants were then centrifuged at 24,000 rpm for 30 min. The pellets containing the crude membrane fractions were resuspended in 50 mmol mannitol and 20 mmol HEPES-Tris (pH 7.5). The suspension samples were used immediately after preparation. Protein determination was done by the method of described earlier (Lowry et al, 1951) using bovine serum albumin as standard.

4.7.1.3 Measurement of ATPase activity

P-gp-dependent ATPase activity was measured by determining vanadate-sensitive release of inorganic phosphate (Pi) from ATP as reported earlier (Hrycyna et al., 1998). In brief, membrane preparations (15–20 µg protein/assay) were pre-incubated for 3 min in the absence and presence of 10 mm *ortho*-vanadate in an assay buffer containing (in mm) Tris 100.0 (pH 7.5), NaN3 10.0, EGTA 4.0, ouabain 2.0, DTT 4.0, KCl 100.0 and $MgCl_2$ 20.0 (final volume 100 µl). An addition of 5.0 mm ATP was made in all incubations which were allowed to continue for 30 min. The reaction was stopped by adding 5 % (w/v) sodium dodecyl sulphate and the released Pi was measured as described in the procedure. In one set of identical incubations test substances were also added. Vanadate-sensitive activity in the presence and absence of a test substance was calculated as the difference between ATPase activity obtained in the presence and absence of sodium *ortho*-vanadate. Test compounds of selected HAPs i.e. silymarin, naringin and morin were added in the concentration of 10 µmol, 30 µmol and 100 µmol strength and prepared in 10 % DMSO. All incubations were done in triplicate.

4.7.1.4 Stastical analysis

The results were stastically analyzed by one way ANOVA test by using Dunnett's test i.e. control group vs. test compound's group. The graphical representative was also performed by using % change vs. control.

4.7.2 CYP 450 1A1Enzyme: Benzopyrene hydroxylase activity

Microsomal Benzopyrene Hydroxylase (AHH) activity, AHH activity in tissue assayed by the procedure described below. (Nebert et al.; Wettenberg et al.). The incubation mixture contained in 1.0 ml:50 µmol of Tris-HCl, pH 7.6, 0.5 µmol of NADPH, 3 µmol of $MgCl_2$, 0.50 to 2.0 mg of cellular protein and 100 mµmol of substrate, Benzo[a]pyrene, added in 0.040 ml of methanol. The reaction was terminated on ice by addition of 4.0 ml of an Acetone: Hexane (1:3) mixture. After shaking at the 37 °C for 10 min, 1.0 ml of organic layer was extracted in to the 2.0 ml of 1 N NaOH and fluorescent of this extract was then immediately measured at 398 nm excuitation and 522 nm emission (Weibel et al., 1975).

4.7.2.1 Reagent preparation for assay mixture (1ml)

The assay mixture required to prepare was 1 ml and for getting the required concentration in final assay mixture and volume required in test and control assay are mentioned in below Tab.4.1

TABLE 4.1: Reagent preparation for assay mixture for CYP 1A1 (1 ml)

Addition	**Final Concentration**	**Control Assay**	**Test Assay**
Tris.HCl	50 mM	560 µl	560 µl
$MgCl_2$	3 mM	100 µl	100 µl
Microsomes	~1 mg	100 µl	100 µl
Test compounds (HAPs) In 10% DMSO	-	-	50 µl
Vehicle 10 % DMSO		50 µl	-
Preincubate for 5 min in at 37 ^oC			
Benzo[a]pyrene	100 µmol	40 µl	40 µl
NADPH	0.5 mmol	100 µl	100 µl
Acetone hexane mixture	-	4.0 ml	-
Incubate for additional 15 min at 37 ^oC			
Acetone Hexane mixture	-	-	4.0 ml

Test compounds of selected HAPs i.e. silymarin, naringin and morin were added in concentration of 10 µmol, 30 µml and 100 µmol strength and prepared in 10 % DMSO. 1.0 ml of organic layer was extracted with 2.0 ml of 1N NaOH, and the fluorescence of the aqueous phase measured immediately at 398 nm excitation / 522 nm emission wavelength.

4.7.2.2 Stastical analysis

The results were stastically analyzed by one way ANOVA test by using Dunnett's test i.e. control group vs. test compound's group. The graphical representative was also performed by using % change vs. control.

4.7.3 CYP 450 3A4 Enzyme: Erythromycin demethylase

Erythromycin *N*-demethylation was determined by the earlier reported method (Tu and Yang et al., 1983). Erythromycin was incubated at 37 °C for 15 min with 1 mg (HL 24493) of human liver microsomes or for 30 min with 1 mg of microsomes from human B-lymphoblast cells in the presence of a NADPH-generating system as described above. Reactions were quenched with 0.05 ml of 25 % $ZnSO_4$ and 0.05 ml of 0.3 N $Ba(OH)_2$. After the samples were vortexed and centrifuged at 14,000 3 g for 10 min, 0.35 ml of supernatant was transferred to another tube and then mixed with 0.15 ml of a concentrated NASH reagent (15 g of ammonium acetate and 0.2 ml of acetyl acetone in 18 ml of 3% acetic acid). The mixture was incubated at 56°C for 30 min and transferred to a 96-well plate. Samples were analyzed by measuring absorbance at 405 nm with Ceres UV 900 HDi spectrophotometer to determine the formation of formaldehyde (Wang et al., 1997).

4.7.3.1 Reagent preparation for assay mixture (1ml)

The assay mixture required to prepare is 1ml and for getting the required concentration in final assay mixture and volume required in test and control assay are mentioned in below Tab. 4.2

TABLE 4.2: Reagent preparation for assay mixture for CYP 450 3A4 (1ml)

Addition	**Final Concentration**	**Blank**	**Control**	**Test**
Buffer	100 mmol	710 µl	710 µl	660 µl
$MgCl_2$	6 mmol	50 µl	50 µl	50 µl
Erythrpmycin	1 mmol	50 µl	50 µl	50 µl
Test compound in 10 % DMSO		-	-	50 µl
Microsomes	1 mg/ assay	40 µl	40 µl	40 µl
Preincubate for 5 min in at 37 ^oC				
NADPH		-	50 µl	50 µl
Incubate for 30 min at 37 ^oC				
$ZnSO_4$	25%	50 µl	50 µl	50 µl
$Ba(OH)_2$	0.3 N	50 µl	50 µl	50 µl
Centrifuge at 14000 rpm for 10 min and collect supernatant				
Supernatant		350 µl	350 µl	350 µl
NASH reagent		150 µl	150 µl	150 µl
Incubate at 56 ^oC for 30 min: Read OD at 405 nm (Plate reader spectrophotometer)				

Test compounds of selected HAPs i.e. silymarin, naringin and morin were added in concentration of 10 µmol, 30 µmol and 100 µmol strength and prepared in 10 % DMSO. Samples were analyzed by measuring absorbance at 405 nm with plate reader spectrophotometer to determine the formation of formaldehyde.

4.7.3.2 Stastical analysis

The results were stastically analyzed by one way ANOVA test by using Dunnett's test i.e. control group vs. test compound's group. The graphical representative was also performed by using %change vs. control.

4.8 Primary compatibility study of Herbal absorption promoters (HAPs) with drugs

4.8.1 Infrared spectroscopic study

Sample consisting of HAPs i.e. silymarin, morin, naringin and drugs i.e. sildenafil citrate, zidovudine, atorvastatin calcium and carvedilol phosphate were mixed in equal proportion were mixed geometrically and analyzed for fourier–transformed infrared (FTIR) spectroscopy.

FTIR spectra were obtained on Shimadzu FTIR-8400 spectrophotometer using the KBr disk method (2 mg sample in 200 mg KBr). The scanning range was 450 to 4000 cm^{-1} and the resolution was 1 cm^{-1}.

4.8.2 Differential scanning calorimetry

Sample consisting of HAPs i.e. silymarin, morin, naringin and drugs i.e. sildenafil citrate, zidovudine, atorvastatin calcium and carvedilol phosphate were mixed in equal proportion were mixed geometrically and analyzed for DSC.

DSC curves were obtained by a differential scanning calorimeter (DSC 60, TA-60WS, Shimadzu, Japan) at a heating rate of 10°C/min from 30 to 300°C in an air atmosphere.

5. RESULTS

5.1 High Performance Liquid Chromatography (HPLC) method Validation

5.1.1 Atorvastatin Calcium

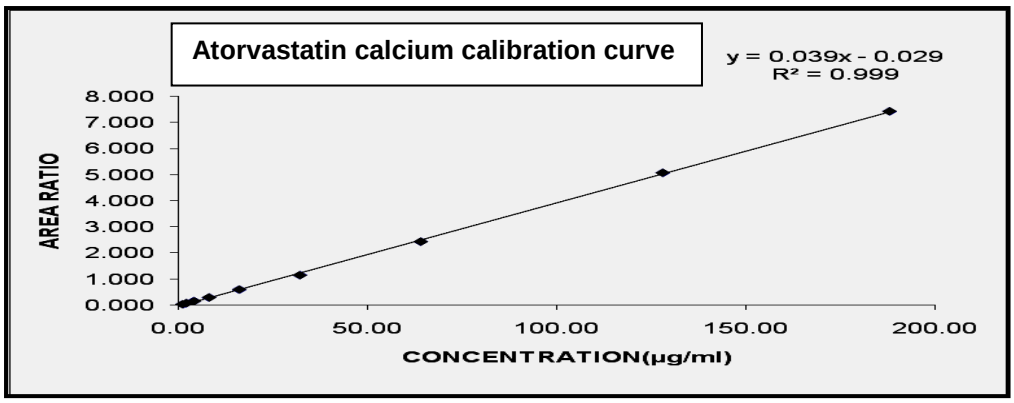
The various parameters were used to validate the HPLC methods for validation of atorvastatin calcium i.e. system suitability, linearity, accuracy and precision, intraday accuracy and precision, interday accuracy and precision, recovery, limit of detection, limit of quantification and specificity.

TABLE 5.1: System suitability of atorvastatin calcium

Sr. No.	AREA (mAU)		Area Ratio
	Atorvastatin Ca.	Diclofenac Na.	
1	684181	152364	4.49
2	700451	155640	4.50
3	698996	155075	4.51
4	701376	154022	4.55
5	698688	154081	4.53
6	696738	154236	4.52
		N	6
		Mean	4.517
		SD	0.023
		% CV	0.517

TABLE 5.2: Back calculated concentration of calibration standards from calibration curve of atorvastatin calcium

LINEARITY (n=6)	Concentration (µg/ml)								
	CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	CS-8	CS-9
	1.000	2.000	4.000	8.000	16.000	32.000	64.000	128.000	188.000
Mean (n=6)	1.022	2.000	3.980	7.888	15.821	31.165	63.242	125.836	191.287
% Nominal conc.	102.18	99.99	99.49	98.60	98.88	97.39	98.82	98.31	101.75
SD	0.039	0.064	0.126	0.242	0.370	1.190	2.527	3.329	3.509
% CV	3.85	3.22	3.17	3.06	2.34	3.82	4.00	2.65	1.83



Straight line equation	:	y = 0.039 x Conc. – 0.029
Correlation coefficient (r)	:	0.9995
Slope	:	0.039
Intercept	:	-0.029

FIGURE 5.1: Calibration curve for atorvastatin calcium

TABLE 5.3: Intraday accuracy and precision of atorvastatin calcium

Concentration	LLOQ (1.00 µg/ml)	LQC (3.00 µg/ml)	MQC (24.00 µg/ml)	HQC (158.00 µg/ml)
N	6	6	6	6
Mean	1.060	3.137	24.667	157.588
SD (n=2)	0.022	0.018	0.461	0.953
% CV	2.07	0.58	1.87	0.60
% Nominal conc.	105.957	104.558	102.781	99.739

TABLE 5.4: Interday accuracy and precision of atorvastatin calcium

Concentration	LLOQ (1.00 µg/ml)	LQC (3.00 µg/ml)	MQC (24.00 µg/ml)	HQC (158.00 µg/ml)
N	30	30	30	30
Mean	1.092	3.168	24.549	159.429
SD (n=6)	0.027	0.030	0.637	4.668
% CV	2.46	0.96	2.59	2.93
% Nominal conc.	109.238	105.614	102.287	100.904

TABLE 5.5: Recovery of atorvastatin calcium

Concent-ration	LQC (3.000 µg/ml)		MQC (24.000 µg/ml)		HQC (158.000 µg/ml)	
	Aqueous Sample	Extracted Sample	Aqueous Sample	Extracted Sample	Aqueous Sample	Extracted Sample
Mean (n=6)	344297	266283	1166977	949579	2357307	1917789
SD	13406	1873	27020	13288	30719	11472
% CV	3.89	0.70	2.32	1.40	1.30	0.60
% Recovery	77.341		81.371		81.361	

TABLE 5.6: Recovery variability across QC levels of atorvastatin calcium

QC LEVELS	% Recovery
LQC	77.34
MQC	81.37
HQC	81.36
Mean	80.02
SD	2.322
% CV	2.90

TABLE 5.7: Recovery of diclofenac sodium

Concentration	Diclofenac Na (70 µg/ml)	
	Aqueous Sample	Extracted Sample
Mean (n=18)	171057	131163
SD	2949	3295
% CV	1.72	2.51
% Recovery	76.678	

TABLE 5.8: Summary of validation parameters of atorvastatin calcium

Sr. No.	Parameter	Results
1	Linearity Range Co-relation Coefficient Slope Intercept	1.000 µg-188 µg 0.9995 0.039 -0.029
2	Precision (% CV) Interday Intraday	 LLOQ-2.46, LQC-0.96, MQC-2.59, HQC-2.93 LLOQ-2.07, LQC-0.58, MQC-1.87, HQC-0.60
3	Limit of Detection	0.500 µg
4	Limit of Quantification	1.000 µg
5	Accuracy (% Recovery) Atorvastatin Diclofenac Sodium	 80.02 %, % CV-2.90 76.678 %
6	Specificity	Specific
7	Retention time Atorvastatin calcium Diclofenac sodium	 6.20 ± 0.5 8.92 ± 0.5

5.1.2 Carvedilol phosphate

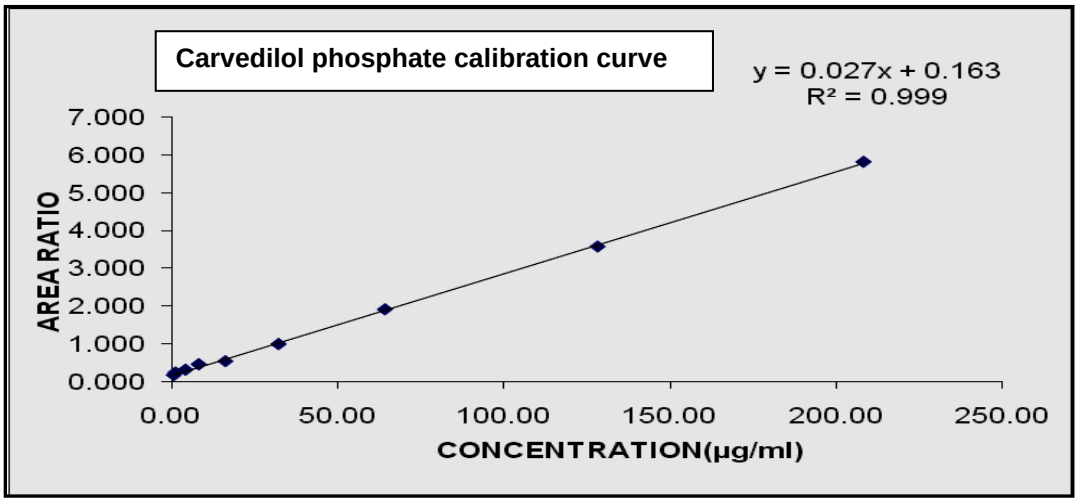
The various parameters were used to validate the HPLC methods for validation of carvedilol phosphate i.e. system suitability, linearity, accuracy and precision, intraday accuracy and precision, interday accuracy and precision, recovery, limit of detection, limit of quantification, specificity,

TABLE 5.9: System suitability of carvedilol phosphate

Sr. No.	AREA (mAU)		
	Carvedilol	Diclofenac Na	Area Ratio
1	85167	113849	0.75
2	85604	112628	0.76
3	86414	116732	0.74
4	85508	115863	0.74
5	86489	115752	0.75
6	85535	117860	0.73
		N	6
		Mean	0.743
		SD	0.012
		% CV	1.551

TABLE 5.10: Back calculated concentration of calibration standards from calibration curve of carvedilol phosphate

LINEARITY	Concentration (µg/ml)								
	CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	CS-8	CS-9
	0.500	1.000	4.000	8.000	16.000	32.000	64.000	128.000	208.000
Mean (n=6)	0.498	0.917	4.023	8.381	16.040	29.430	64.678	127.218	208.494
% Nominal conc.	99.50	91.69	100.58	104.76	100.25	91.97	101.06	99.39	100.24
SD	0.009	0.022	0.087	0.281	0.072	0.895	0.306	1.234	1.388
% CV	1.71	2.41	2.16	3.35	0.45	3.04	0.47	0.97	0.67



Straight line equation	:	y = 0.027 x Conc. +0.163
Correlation coefficient (r)	:	0.9996
Slope	:	0.027
Intercept	:	0.163

FIGURE 5.2: Calibration curve for carvedilol phosphate

TABLE 5.11: Intraday accuracy and precision of carvedilol phosphate

Concentration	LLOQ (0.500 µg/ml)	LQC (0.750 µg/ml)	MQC (24.00 µg/ml)	HQC (96.00 µg/ml)
N	6	6	6	6
Mean	0.496	0.748	23.919	96.445
SD (n=6)	0.008	0.013	0.161	1.275
% CV	1.66	1.74	0.67	1.32
% Nominal conc.	99.183	99.722	99.663	100.464

TABLE 5.12: Interday accuracy and precision of carvedilol phosphate

Concentration	LLOQ (0.500 µg/ml)	LQC (0.750 µg/ml)	MQC (24.00 µg/ml)	HQC (96.00 µg/ml)
N	30	30	30	30
Mean	0.481	0.739	24.171	95.948
SD (n=6)	0.014	0.020	0.337	1.702
%CV	2.88	2.76	1.39	1.77
%Nominal conc.	96.183	98.593	100.714	99.946

TABLE 5.13: Recovery of carvedilol phosphate

Concentration	LQC (0.750 µg/ml)		MQC (24.00 µg/ml)		HQC (96.00 µg/ml)	
	Aqueous Sample	Extracted Sample	Aqueous Sample	Extracted Sample	Aqueous Sample	Extracted Sample
Mean (n=6)	5726	4912	85710	75470	315490	273177
SD	144	147	809	568	2626	6676
% CV	2.51	2.99	0.94	0.75	0.83	2.44
% Recovery	85.78		88.05		86.81	

TABLE 5.14: Recovery variability across QC Levels of carvedilol phosphate

QC LEVELS	% Recovery
LQC	85.78
MQC	88.05
HQC	86.59
Mean	86.81
SD	1.151
% CV	1.33

TABLE 5.15: Recovery of diclofenac sodium

Concentration	Diclofenac Na (60 µg/ml)	
	Aqueous Sample	Extracted Sample
Mean (n=6)	121105	100355
SD	11656	3550
% CV	9.62	3.54
% Recovery	82.86	

TABLE 5.16: Summary of validation parameters of carvedilol phosphate

SR. No.	Parameter	Results
1	Linearity range Co-relation coefficient Slope Intercept	0.500 µg-208.000 µg 0.9996 0.027 0.163
2	Precision (% CV) Intraday Interday	 LLOQ-1.66, LQC-1.74, MQC-0.67, HQC-1.32 LLOQ-2.88, LQC-2.76, MQC-1.39, HQC-1.77
3	Limit of detection	0.250 µg
4	Limit of quantification	0.500 µg
5	Accuracy (% Recovery) Carvedilol Diclofenac sodium	 86.81 %, % CV-1.33 82.86 %
6	Specificity	Specific
7	Retention time Carvedilol phosphate Diclofenac sodium	 2.78 ± 0.5 8.32 ± 0.5

5.1.3 Sildenafil citrate

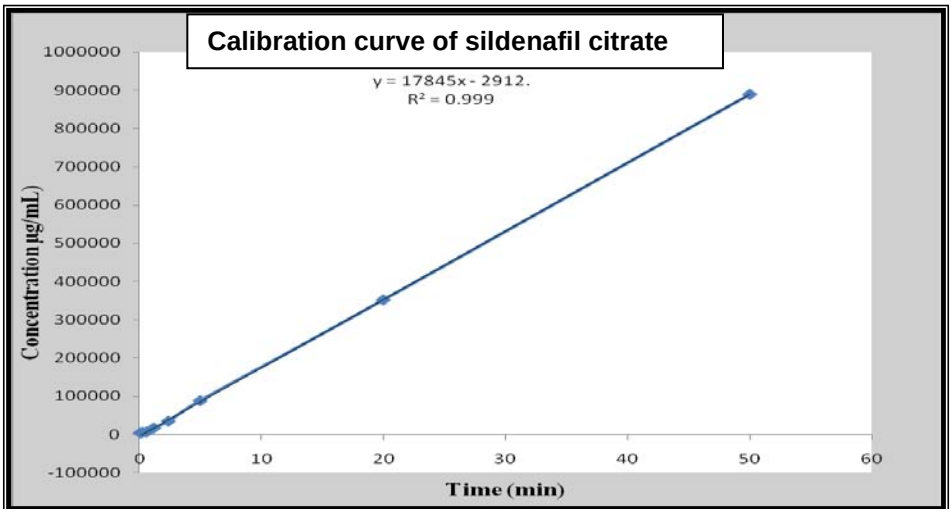
The various parameters were used to validate the HPLC methods for validation of sildenafil citrate i.e. system suitability, linearity, accuracy and precision, intraday accuracy and precision, interday accuracy and precision, recovery, limit of detection, limit of quantification, specificity.

TABLE 5.17: Precision data for replicate injection of sildenafil citrate

Sr. no.	Retention time	Peak area (mAU)
1	5.33	454668
2	5.33	453225
3	5.33	454110
4	5.32	455263
5	5.33	452148
6	5.33	452197
	n	6
	Mean	453601.83
	SD	1294.88
	% CV	0.86

TABLE 5.18: Calibration data for sildenafil citrate

Sr. No.	Conc. of sildenafil citrate (µg/ml)	Mean Area ± SD (n=6)	% CV
1	0.1	004110.60 ± 00043.53	1.06
2	0.3	005184.01 ± 00155.04	2.99
3	0.6	006093.60 ± 00154.94	2.54
4	1.2	016792.40 ± 00145.55	0.87
5	2.4	034556.07 ± 00586.73	1.70
6	5.0	088044.12 ± 00449.59	0.51
7	20	352208.60 ± 05494.92	1.56
8	50	890154.40 ± 11724.92	1.32



Straight line equation	Y =117845 x Conc. – 2912
Correlation coefficient (r)	0.999
Slope	17845
Intercept	2912

FIGURE 5.3: Calibration curve for sildenafil citrate

TABLE 5.19: Intraday precision data for sildenafil citrate

Sr. no	Conc. of sildenafil citrate (µg/ml)	Mean area ± SD (n=6)	% CV
1	0.1	004110.60 ± 00043.53	1.06
2	0.3	005184.01 ± 00155.04	2.99
3	0.6	006093.60 ± 00154.94	2.54
4	1.2	016792.40 ± 00145.55	0.87
5	2.4	034556.07 ± 00586.73	1.70
6	5.0	088044.12 ± 00449.59	0.51
7	20	352208.60 ± 05494.92	1.56
8	50	890154.40 ± 11724.92	1.32

TABLE 5.20: Interday precision data for sildenafil citrate

Sr. no	Conc. of sildenafil citrate (µg)	Mean area ± SD (n=6)	% CV
1	0.1	004209.49 ± 0152.44	3.62
2	0.3	005259.68 ± 0153.03	2.91
3	0.6	006203.50 ± 0162.12	2.61
4	1.2	016765.77 ± 0249.96	1.49
5	2.4	034553.59 ± 0262.30	0.76
6	5.0	088168.28 ± 0324.04	0.37
7	20	352327.90 ± 3303.69	0.86
8	50	892792.16 ± 5035.35	0.56

TABLE 5.21: Recovery data for sildenafil citrate

Conc. of sildenafil citrate Taken (µg/ml)	Conc. recovered (mean) ± SD (µg/injection) (n=6)	Accuracy ± CV (%)
STD 8	49.87 ± 2.14	99.74 ± 0.24
STD 4	1.17 ± 3.46	98.33 ± 1.46

TABLE 5.22: Summary of validation parameters

r. No.	Parameters	Results
1	Linearity range	0.1-50 µg
2	Co-relation coefficient	0.999
3	Precision % CV Interday Intraday	 LLOQ-0.37, LQC-3.61 LLOQ-0.51, LQC-2.99
4	Repeatability	0.29 %
5	Limit of detection	0.1 µg
6	Limit of duantification	0.1 µg
7	Accuracy (% Recovery)	99.74%, CV-0.83 %
8	Specificity	Specific

5.1.4 Zidovudine

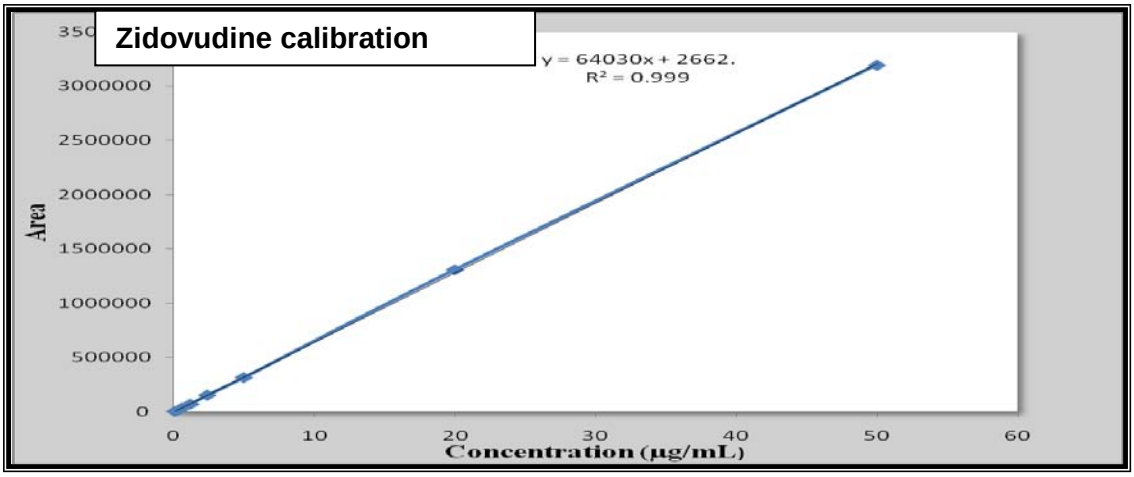
The various parameters were used to validate the HPLC methods for validation of zidovudine i.e. system suitability, linearity, accuracy and precision, intraday accuracy and precision, interday accuracy and precision, recovery, limit of detection, limit of quantification, specificity.

TABLE 5.23: Precision data for replicate injection of zidovudine

Sr. NO.	Retention time (min)	Peak area (mAU)
1	3.36	1321039
2	3.36	1317146
3	3.36	1336097
4	3.37	1315986
5	3.36	1320061
6	3.36	1334145
	n	6
	Mean	1324079
	SD	8771.61
	% CV	0.66

TABLE 5.24: Calibration data for zidovudine

Sr. no	Conc. of zidovudine (µg/ml)	Mean Area ± SD (n=6)	% CV
1	0.1	0007408.63 ± 000103.02	1.39
2	0.3	0015293.40 ± 000274.23	1.79
3	0.6	0039986.04 ± 001616.64	4.04
4	1.2	0075354.20 ± 002755.47	3.36
5	2.4	0157036.47 ± 007153.23	4.56
6	5.0	0318604.48 ± 014268.97	4.48
7	20	1310639.18 ± 029247.25	2.23
8	50	3193729.25± 153794.83	4.82



Straight line equation	Area=64030 x Conc. + 2662
Correlation coefficient (r)	0.999
Slope	64030
Intercept	2662

FIGURE 5.4: Calibration curve for zidovudine

TABLE 5.25: Intraday precision data for zidovudine

Sr. no.	Conc. of zidovudine (µg/ml)	Mean area ± SD (n=6)	% CV
1	0.1	0007408.63 ± 000103.02	1.39
2	0.3	0015293.40 ± 000274.23	1.79
3	0.6	0039986.04 ± 001616.64	4.04
4	1.2	0075354.20 ± 002755.47	3.36
5	2.4	0157036.47 ± 007153.23	4.56
6	5.0	0318604.48 ± 014268.97	4.48
7	20	1310639.18 ± 029247.25	2.23
8	50	3193729.25± 153794.83	4.82

TABLE 5.26: Interday precision data for zidovudine

Sr. no.	Conc. of zidovudine (µg/ml)	Mean area ± SD (n=6)	% CV
1	0.1	0007347.38 ± 000051.00	0.69
2	0.3	0015474.67 ± 000161.23	1.04
3	0.6	0038882.07 ± 00572.07	1.47
4	1.2	0073580.00 ± 001838.45	2.50
5	2.4	0152746.12 ± 003010.78	1.97
6	5.0	0320903.14 ± 013040.03	4.06
7	20	1324899.30 ± 028514.79	2.15
8	50	3232159.74 ± 158357.91	4.90

TABLE 5.27: Recovery data for zidovudine

Conc. of zidovudine taken (µg/ml)	Conc. recovered (mean) ± SD (µg/injection) (n=6)	Accuracy ± % CV
STD 8	49.87 ± 2.14	98.87 ± 0.26
STD 4	1.17 ± 3.46	99.53 ± 1.16

TABLE 5.28: summary of validation parameters of zidovudine

Sr. No.	Parameters	Results
1	Linearity Range	0.1-50 µg
2	Co-relation Coefficient	0.999
3	Precision % CV Interday Intraday	 LLOQ-0.69, LQC 4.90 LLOQ-1.39, LQC-4.82
4	Repeatability	0.66 %
5	Limit of Detection	0.1 µg
6	Limit of Quantification	0.1 µg
7	Accuracy (% Recovery)	99.70, CV-0.71 %
8	Specificity	Specific

5.2 *In vivo* bioavailability study

5.2.1 Atorvastatin calcium

5.2.1.1 Effect of silymarin as absorption enhancer

The method was applied to a bioavailability study for examining the influence of the co-administration of silymarin on the absorption of findings of the pharmacokinetic studies were shown in Tab. 5.29 and plasma concentration profile at different time intervals was depicted in Fig. 5.5

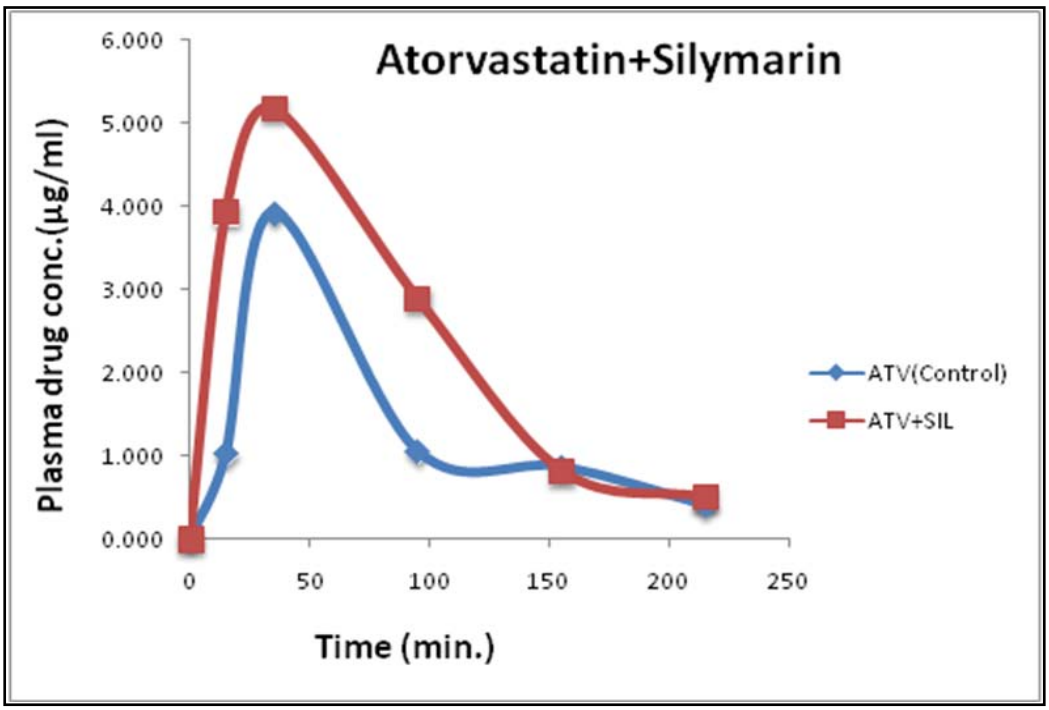


FIGURE 5.5: Mean plasma concentration-time profiles of atorvastatin calcium following oral (75 mg/kg) administration to rats in the presence and absence of silymarin (mean ± SEM, n=6). ♦ Control (atorvastatin calcium 75 mg/kg, oral). :: ■ Subsequent administration of 100 µm of silymarin with atorvastatin calcium

TABLE 5.29: Mean pharmacokinetic parameters of the atorvastatin calcium following oral (75 mg/kg) administration to rats in the presence and absence of silymarin (mean ± SEM, n=6).

Parameters	Atorvastatin (75 mg/kg) (control)	Atorvastatin (75 mg/kg) + Silymarin (100 µmol)	% Increase of Bioavailability
T_{max} (min)	35	35	32.31%
C_{max} (µg/ml)	3.921 ± 0.098	5.188 ± 0.035***	

****p*<0.001, significantly different from control (Atorvastatin calcium)

5.2.1.2 Effect of naringin as absorption enhancer

Same as above the method was applied to a bioavailability study for examining the influence of the co-administration of naringin on the absorption of atorvastatin calcium. Findings of the pharmacokinetic studies were shown in Tab. 5.30 and the plasma concentration profile at different time intervals was depicted in Fig. 5.6.

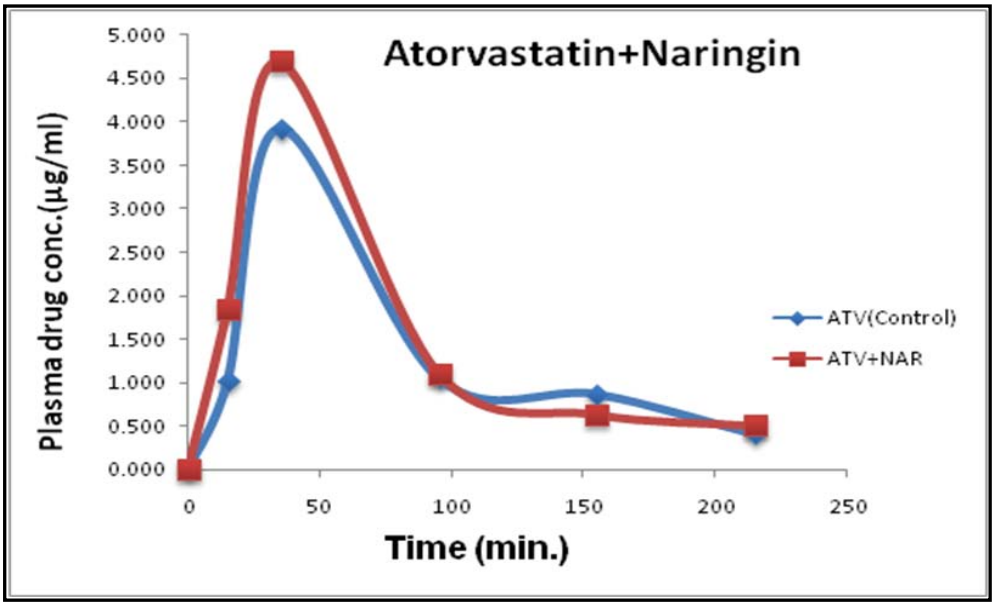


FIGURE 5.6: Mean plasma concentration-time profiles of atorvastatin calcium following oral (75 mg/kg) administration to rats in the presence and absence of naringin (mean ± SEM, n=6). ♦ : Control (atorvastatin calcium 75 mg/kg, oral). ■ : Subsequent administration of 100 µm of naringin with atorvastatin.

TABLE 5.30: Mean pharmacokinetic parameters of atorvastatin calcium following oral (75 mg/kg) administration to rats in the presence and absence of naringin (mean ± SEM, n=6).

Parameters	Atorvastatin (75 mg/kg) (control)	Atorvastatin (75 mg/kg) + Naringin (100 µmol)	% Increase of Bioavailability
T_{max} (min)	35	35	19.66%
C_{max} (µg/ml)	3.921±0.098	4.692±0.0298***	

***p<0.001, significantly different from control (Atorvastatin calcium)

5.2.1.3 Effect of morin as absorption enhancer

Same as above the method was applied to bioavailability study for examining the influence of co-administration of morin on the absorption of atorvastatin calcium. Findings of the pharmacokinetic studies were shown in Tab. 5.31 and the plasma concentration profile at different time intervals was depicted in Fig. 5.8.

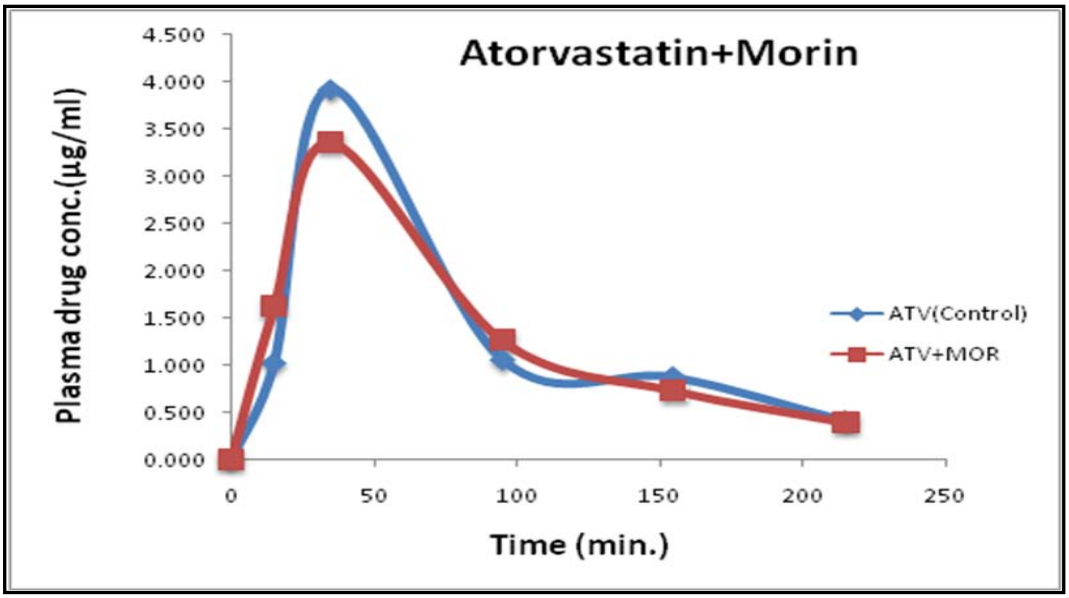


FIGURE 5.7: Mean plasma concentration-time profiles of atorvastatin calcium following oral (75 mg/kg) administration to rats in the presence and absence of morin (mean ± SEM, n=5). ◆: Control (Atorvastatin calcium 75 mg/kg, oral). ■: Subsequent administration of 100 µm of morin with Atorvastatin.

TABLE 5.31: Mean pharmacokinetic parameters of atorvastatin calcium following oral (75 mg/kg) administration to rats in the presence and absence of morin (mean ± SEM, n=6).

Parameters	Atorvastatin (75 mg/kg) (control)	Atorvastatin (75 mg/kg) + Morin (100 µmol)	% Increase of Bioavailability
T_{max} (min)	35	35	-14.23%
C_{max} (µg/ml)	3.921 ± 0.098	3.363 ± 0.011	

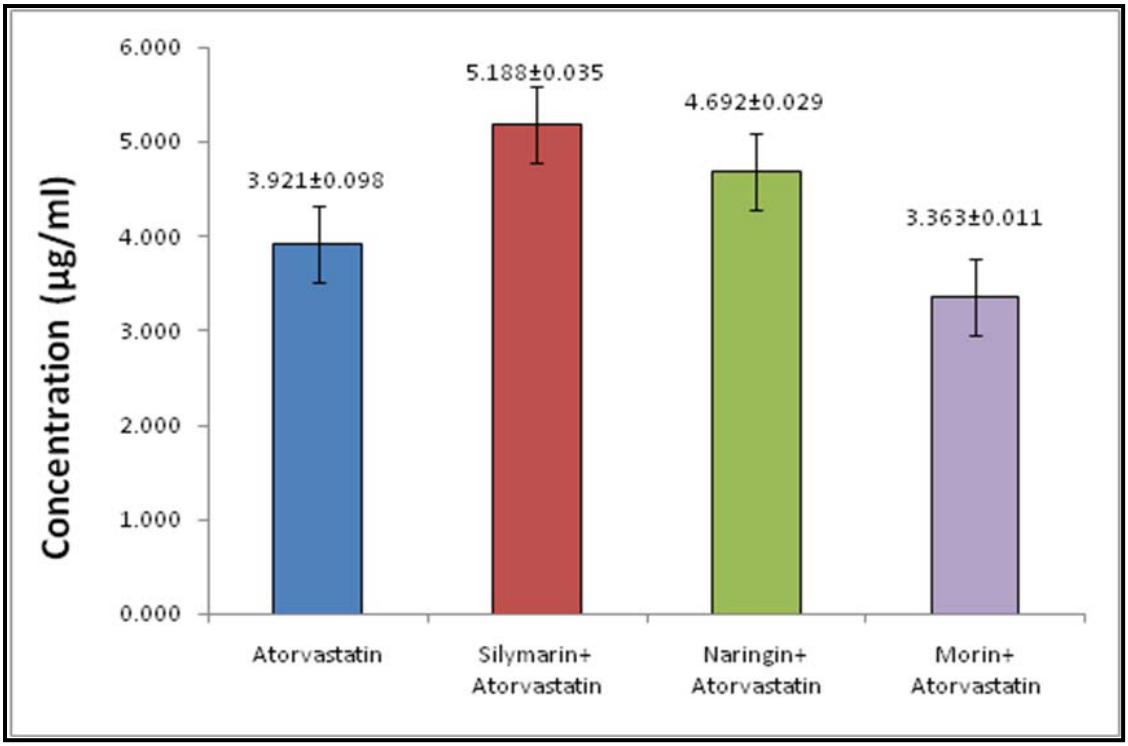


FIGURE 5.8: Comparison of atorvastatin calcium with HAPs

From the experimental analysis the results indicates that the silymarin and naringin significantly increase the concentration of drug in rat blood plasma while morin not increase significantly the concentration of drug in rat blood plasma, as compare to control (Atorvastatin Calcium).

5.2.2 Carvedilol Phosphate

5.2.2.1 Effect of silymarin as absorption enhancer

The method was applied to a bioavailability study for examining the influence of the co-administration of silymarin on the absorption of carvedilol. Findings of the pharmacokinetic studies were shown in the Tab. 5.32 and the plasma concentration profile at different time intervals was depicted in Fig. 5.9.

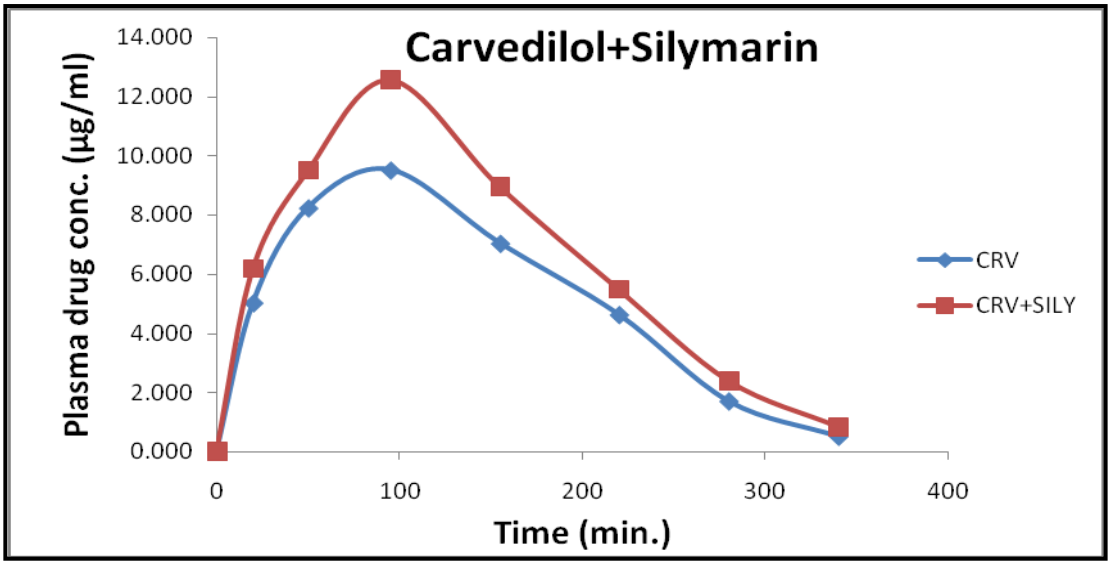


FIGURE 5.9: Mean plasma concentration-time profiles of carvedilol following oral (75 mg/kg) administration to rats in the presence and absence of silymarin (mean ± SEM, n=6). ♦Control (Carvedilol 60 mg/kg, oral) . ■ Subsequent administration of 100 µm of silymarin with carvedilol phosphate

TABLE 5.32: Mean pharmacokinetic parameters of carvedilol following oral (60mg/kg) administration to rats in the presence and absence of silymarin (mean ± SEM, n=6).

Parameters	Carvedilol (60 mg/kg) (control)	Carvedilol (60 mg/kg)+ Silymarin (100 µmol)	% Increase of Bioavailability
T_{max} (min)	95	95	31.95
C_{max} (µg mL^{-1})	9.517±0.290	12.558±0.305***	

***p<0.001, significantly different from control (Carvedilol)

5.2.2.2 Effect of naringin as absorption enhancer

Same as above the method was applied to a bioavailability study for examining the influence of the co-administration of naringin on the absorption of carvedilol. Findings of the pharmacokinetic studies were shown in Tab. 5.33 and the plasma concentration profile at different time intervals is depicted in Fig. 5.10.

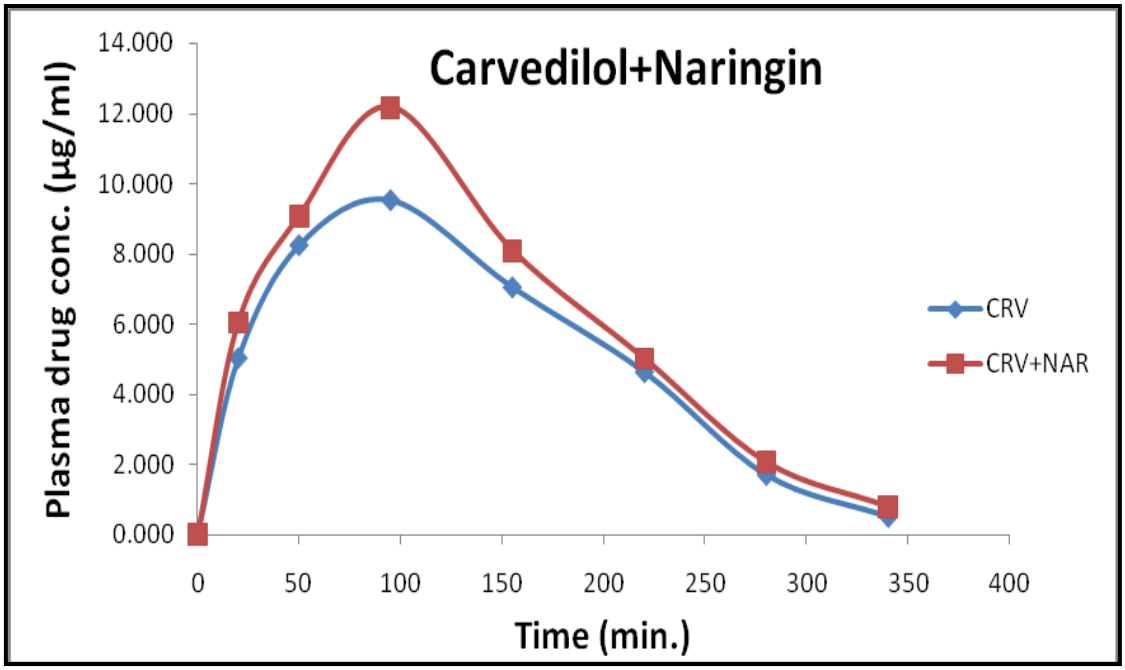


FIGURE 5.10: Mean plasma concentration-time profiles of carvedilol following oral (60 mg/kg) administration to rats in the presence and absence of naringin (mean ± SEM, n=5). ♦: Control (Carvedilol 60 mg/kg, oral). ■: Subsequent administration of 100 µm of naringin with carvedilol phosphate

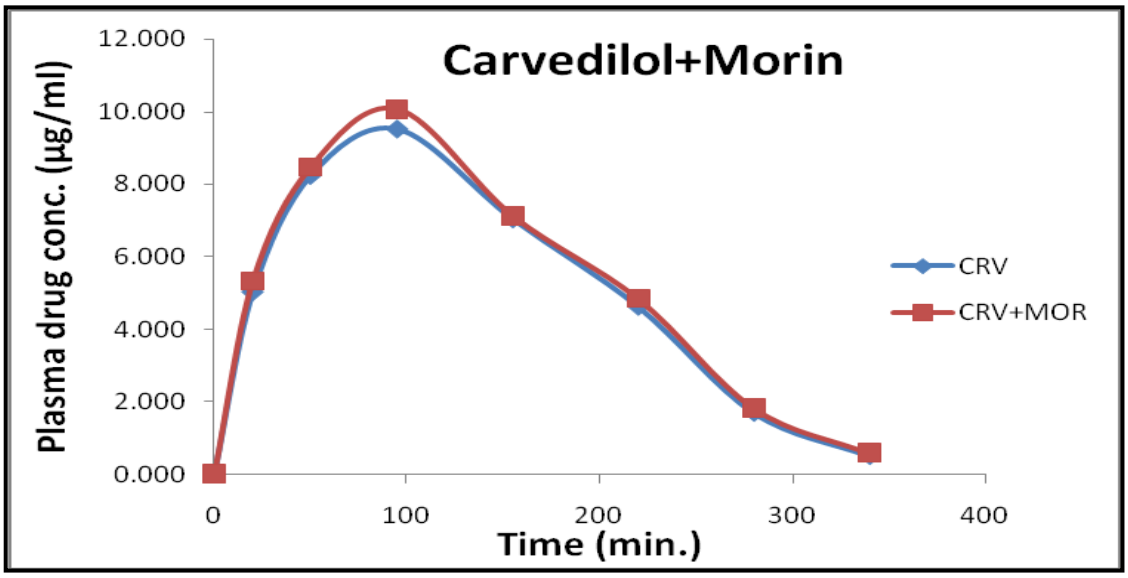
TABLE 5.33: Mean pharmacokinetic parameters of carvedilol following oral (60 mg/kg) administration to rats in the presence and absence of naringin (mean ± SEM, n=6).

Parameters	Carvedilol (60 mg/kg) (control)	Carvedilol (60 mg/kg)+ Naringin (100 µmol)	% Increase of Bioavailability
T_{max} (min)	95	95	28.01
C_{max} (µg mL^{-1})	9.517±0.290	12.183±0.110***	

****p*<0.001, significantly different from control (Carvedilol)

5.2.2.3 Effect of morin as absorption enhancer

Same as above the method was applied to bioavailability study for examining the influence of the co-administration of morin on the absorption of Carvedilol. Findings of the pharmacokinetic studies were shown in TABLE 5.34 and the Plasma concentration profile at different time intervals is depicted in Figure 5.11.



FIGUE 5.11:Mean plasma concentration-time profiles of Carvedilol following oral (60 mg/kg) administration to rats in the presence and absence of morin (mean ± SEM, n=6). ♦: Control (Carvedilol 60 mg/kg, oral). ■Subsequent administration of 100 µm of morin with carvedilol

TABLE 5.34: Mean pharmacokinetic parameters of Carvedilol following oral (75 mg/kg) administration to rats in the presence and absence of Morin (mean ± SEM, n=6).

Parameters	Carvedilol (60 mg/kg) (control)	Carvedilol (75 mg/kg)+ Morin (100 µmol)	% Increase of Bioavailability
T_{max} (min)	95	95	5.67
C_{max} (µg mL^{-1})	9.517±0.290	10.057±0.145**	

**p<0.01, significantly different from control (Carvedilol)

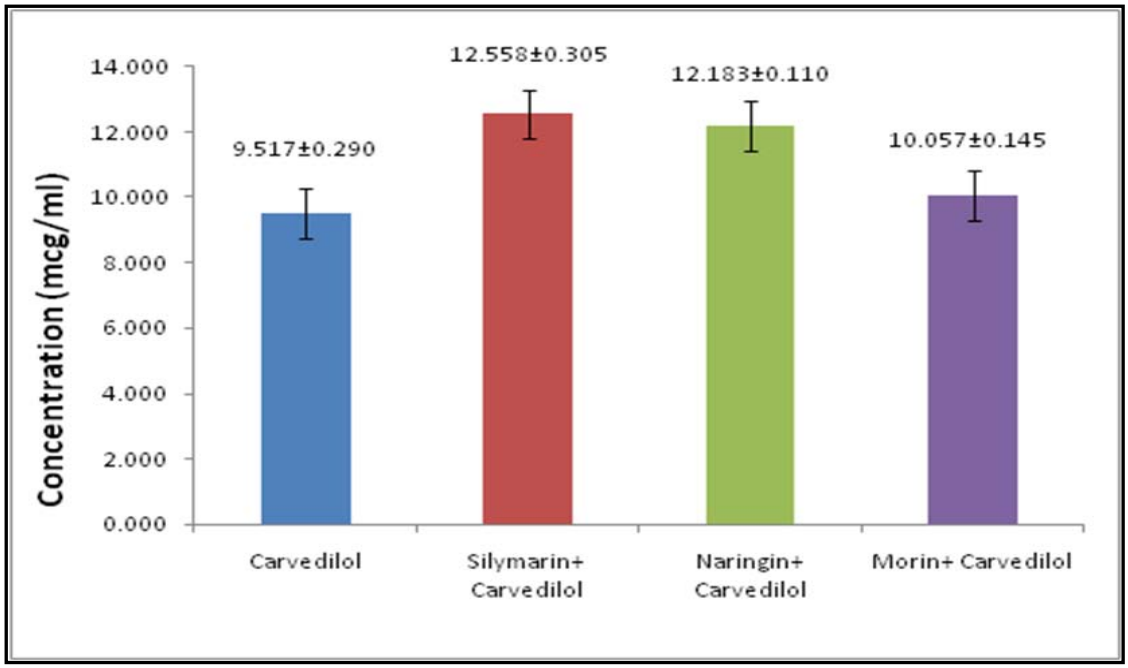


FIGURE 5.12: Comparison of carvedilol HAPs

From the experimental analysis the results indicates that the Silymarin, Naringin and Morin significantly increase the concentration of drug in rat blood plasma, as compare to control (Carvedilol).

5.2.3 Sildenafil citrate

5.2.3.1 Effect of silymarin as absorption enhancer

The method was applied to a bioavailability study for examining the influence of the co-administration of silymarin on the absorption of sildenafil citrate. Findings of the pharmacokinetic studies were shown in Tab. 5.35 and the Plasma concentration profile at different time intervals is depicted in Fig. 5.13.

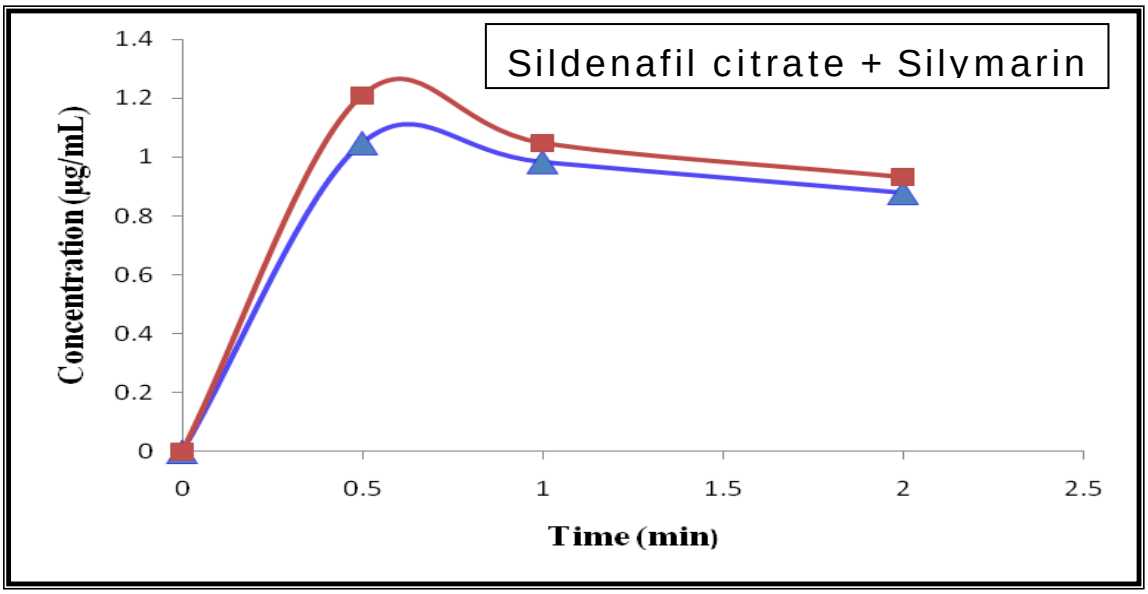


FIGURE 5.13: Mean plasma concentration–time profiles of sildenafil citrate following oral (30 mg/kg) administration to rats in the presence and absence of silymarin (mean + S.D., n = 5). ♦: Control (Sildenafil citrate 30 mg/kg, oral) ■: Subsequent administration of 100 µm of silymarin

Table 5.35: Mean pharmacokinetic parameters of sildenafil citrate following an oral (30 mg/kg) administration of sildenafil citrate to rats in the presence and absence of silymarin (mean ± SD, n = 6)

Parameters	Sildenafil Citrate (30 mg/kg) (control)	Sildenafil Citrate (30 mg/kg) + Silymarin	% Increase of Bioavailability
T_{max} (min)	40	40	24.88 %
C_{max} (µg/ml)	1.049 ± 0.881	1.310 ± 0.765**	

**p<0.01, significantly different from control (Carvedilol)

5.2.3.2 Effect of morin as absorption enhancer

Same as above method, the method was applied to a bioavailability study for examining the influence of the co-administration of morin on the absorption of sildenafil citrate. Findings of the pharmacokinetic studies were shown in the Tab. 5.36 and the Plasma concentration profile at different time intervals is depicted in Fig. 5.14.

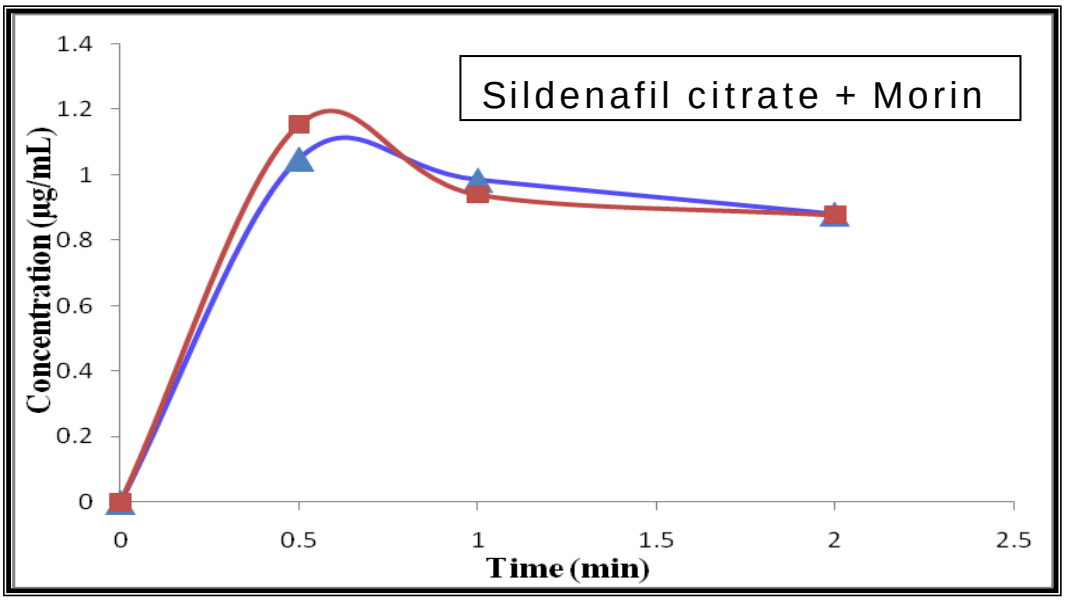


FIGURE 5.14: Plasma concentration–time profiles of sildenafil citrate following oral (30 mg/kg) administration to rats in the presence and absence of morin (mean ± SD, n = 6). ♦ :Control (Sildenafil citrate 30 mg kg^{-1}, oral); ■Subsequent administration of 100 µm of morin.

TABLE 5.36: Mean pharmacokinetic parameters of sildenafil citrate following an oral (30 mg/kg) administration of Sildenafil to rats in the presence and absence of morin (mean ± SD, n = 6)

Parameters	Sildenafil citrate (30 mg/kg) (control)	Sildenafil citrate (30 mg/kg) + Morin	% Increase of Bioavailability
T_{max} (min)	40	40	11.09 %
C_{max} (µg/ml)	1.049 ± 0.881	1.154 ± 0.347 **	

**p<0.01, significantly different from control (Carvedilol)

5.2.3.3 Effect of naringin as absorption enhancer

Same as above methods, the method was also applied to a bioavailability study for examining the influence of the co-administration of naringin on the absorption of sildenafil citrate. Findings of the pharmacokinetic studies and the plasma concentration profile at different time intervals was depicted in the Tab. 5.37 and Fig. 5.15 respectively.

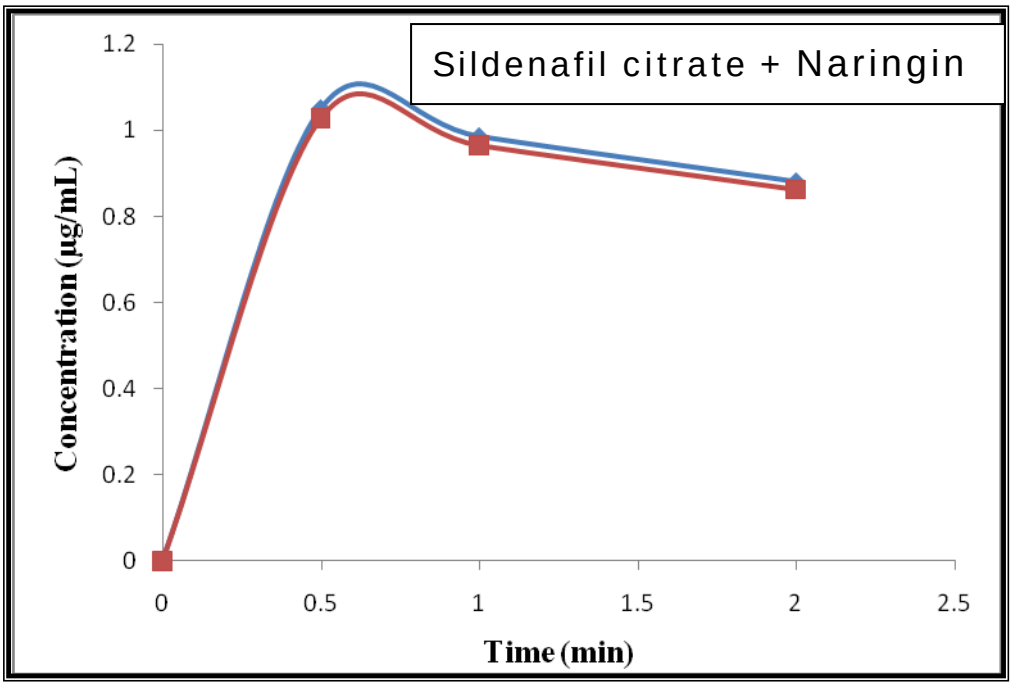


FIGURE 5.15: Mean plasma concentration–time profiles of sildenafil citrate following oral (30 mg/kg) administration to rats the presence and absence of naringin (mean ± SD, n = 6). ♦: Control (Sildenafil citrate 30 mg/kg, oral); ■: Subsequent administration of 100 µm of naringin.

TABLE 5.37: Mean pharmacokinetic parameters of sildenafil citrate following an oral (30 mg/kg) administration of sildenafil to rats in the presence and absence of naringin (mean ± SD, n = 6)

Parameters	Sildenafil citrate (30mg/kg) (control)	Sildenafil citrate (30mg/kg) + Naringin	% Increase of Bioavailability
T_{max} (min)	40	40	-1.90%
C_{max} (µg/ml)	1.049 ± 0.881	1.029 ± 0.245*	

**p*>0.05, significantly not different from control (Carvedilol)

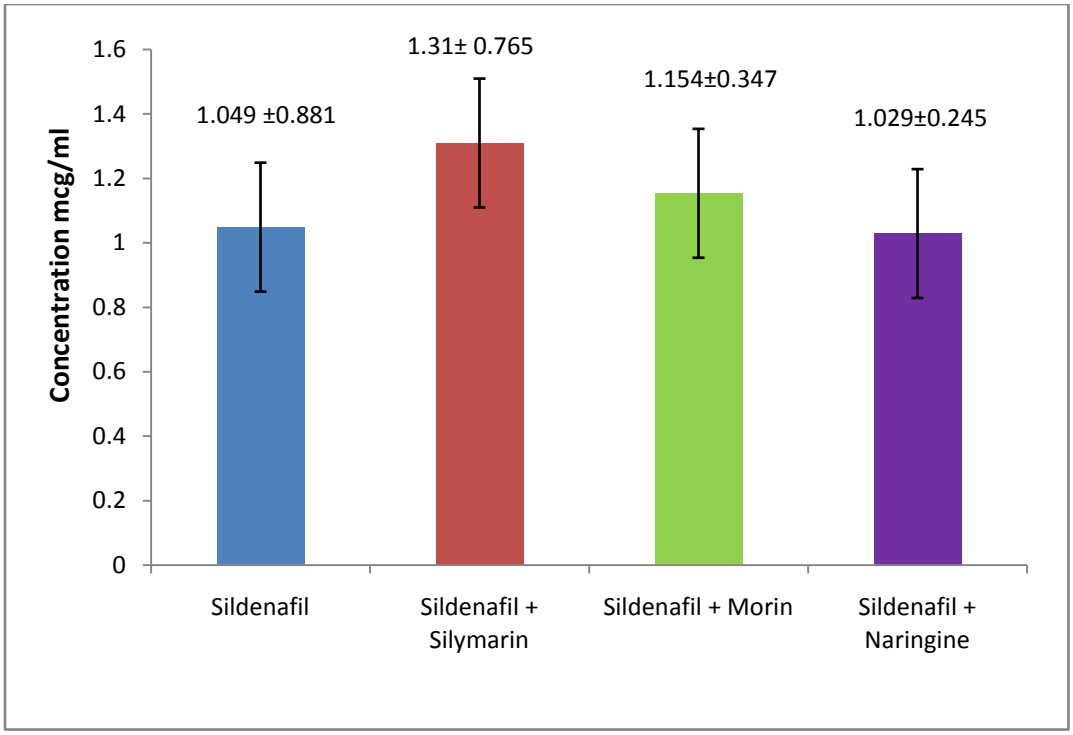


FIGURE 5.16: Comparison of sildenafil citrate with HAPs

5.2.4 Zidovudine

5.2.4.1 Effect of naringin as absorption enhancer

Same as above methods, the method was applied to a bioavailability study for examining the influence of the co-administration of Naringin on the absorption of Zidovudine. Findings of the pharmacokinetic studies and the plasma concentration profile at different time intervals was depicted in Tab. 5.38 and Fig. 5.17 respectively.

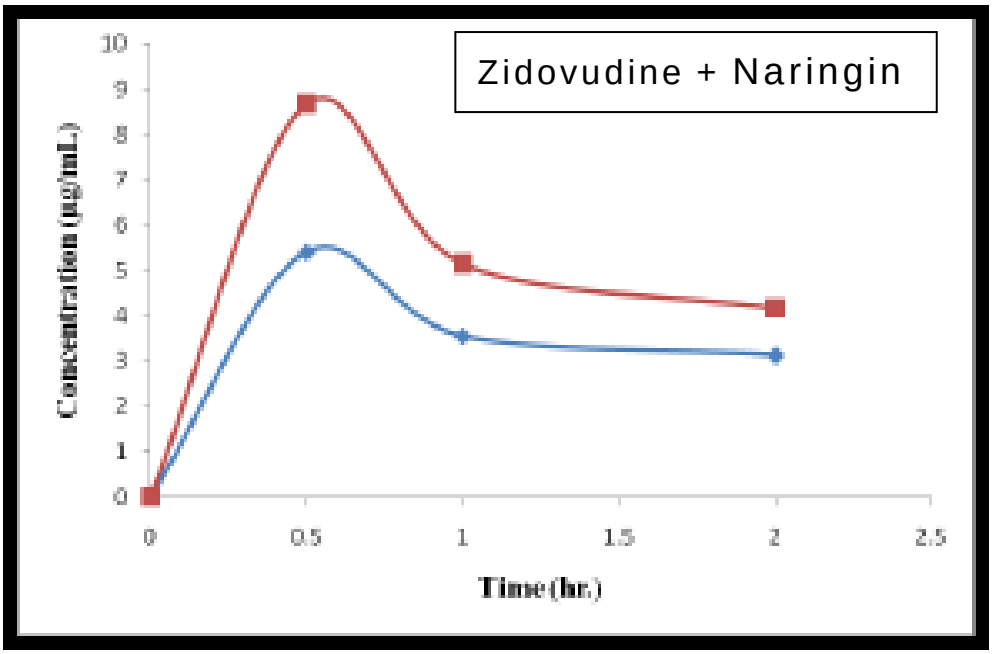


FIGURE 5.17:Mean plasma concentration–time profiles of zidovudine following oral (75 mg/kg) administration to rats in the presence and absence of naringin (mean ± S.D., n = 6). ♦Control (Zidovudine 75 mg/kg, oral); ■: Subsequent administration of 100 µm of naringin.

TABLE 5.38: Mean pharmacokinetic parameters of zidovudine following an oral (75 mg/kg) administration of zidovudine to rats in the presence and absence of naringin (mean ± SD, n = 6)

Parameters	Zidovudine (75mg/kg) (control)	Zidovudine (75mg/kg) + Naringine	% Increase of Bioavailability
T_{max} (min)	35	35	60.49%
C_{max} (µg/ml)	5.407± 0.557	8.678 ± 0.231**	

***p*<0.01, significantly different from control (Zidovudine)

5.2.4.2 Effect of silymarin as absorption enhancer

Same as above methods, for examining the influence of the co-administration of silymarin on the absorption of zidovudine in bioavailability study this method was used. Findings of the pharmacokinetic studies and the Plasma

concentration profile at different time intervals is depicted in Tab. 5.39 and Fig. 5.18 respectively.

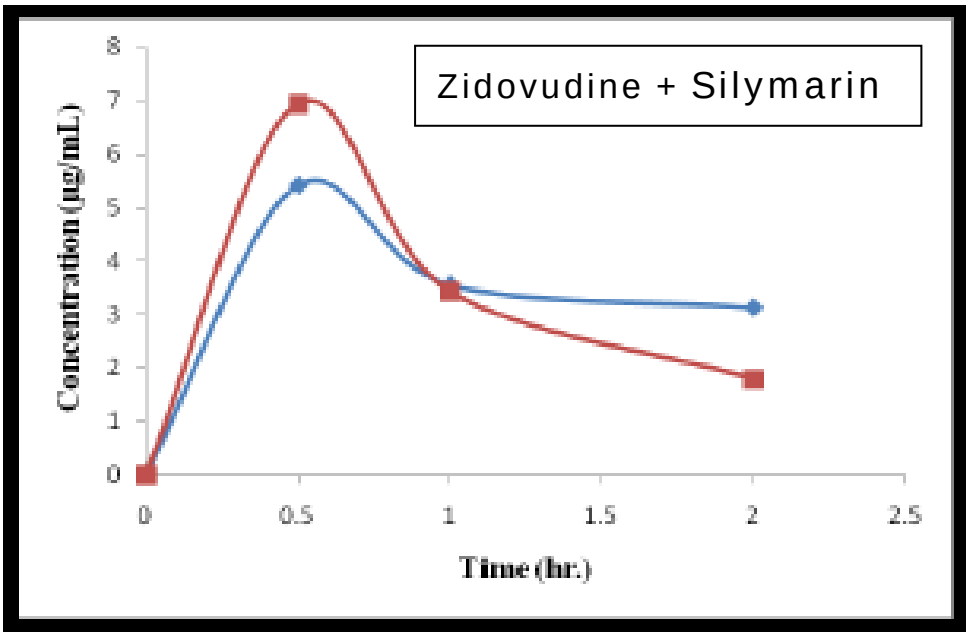


FIGURE 5.18: Mean plasma concentration–time profiles of zidovudine following oral (75 mg/kg) administration to rats in the presence and absence of silymarin (mean ± SD, n = 6). ♦: Control (Zidovudine 75 mg/kg, oral) ■: Subsequent administration of 100 µm of silymarin

TABLE 5.39: Mean pharmacokinetic parameters of zidovudine following an oral (75 mg/kg) administration of zidovudine to rats in the presence and absence of silymarin (mean ± SD, n = 6)

Parameters	Zidovudine (75mg/kg) (control)	Zidovudine (75mg/kg) + Silymarin	% Increase of Bioavailability
T_{max} (min)	35	35	28.31 %
C_{max} (µg/ml)	5.407± 0.557	6.938 ± 0.433**	

**p<0.01, significantly different from control (Zidovudine)

5.2.4.3 Effect of morin as absorption enhancer

As like above methods, this method also applied for examining the influence of the co-administration of morin the absorption of zidovudine in bioavailability study. Findings of the pharmacokinetic studies and the plasma concentration profile at different time intervals was depicted in Tab. 5.40 and Fig. 5.19 respectively.

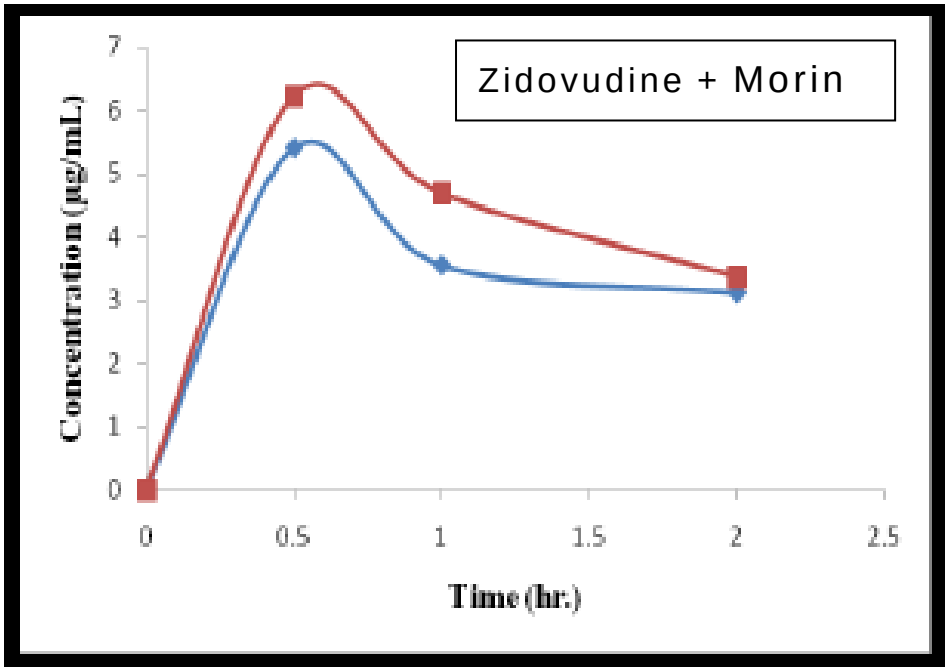


FIGURE 5.19: Mean plasma concentration–time profiles of zidovudine following oral (75 mg/kg) administration to rats in the presence and absence of morin (mean ± SD, n = 6). ♦ : Control (Zidovudine 75 mg/kg, oral); ■ : Subsequent administration of 100 µm of morin.

TABLE 5.40: Mean pharmacokinetic parameters of zidovudine following an oral (75 mg/kg) administration of zidovudine to rats in presence and absence of morin (mean ± SD, n = 6)

Parameters	Zidovudine (75 mg/kg) (control)	Zidovudine (75 mg/kg) + Morin	% Increase of Bioavailability
T_{max} (min)	35	35	16.17%
C_{max} (µg/ml)	5.407 ± 0.557	6.219 ± 0.453**	

**p<0.01, significantly different from control (Zidovudine)

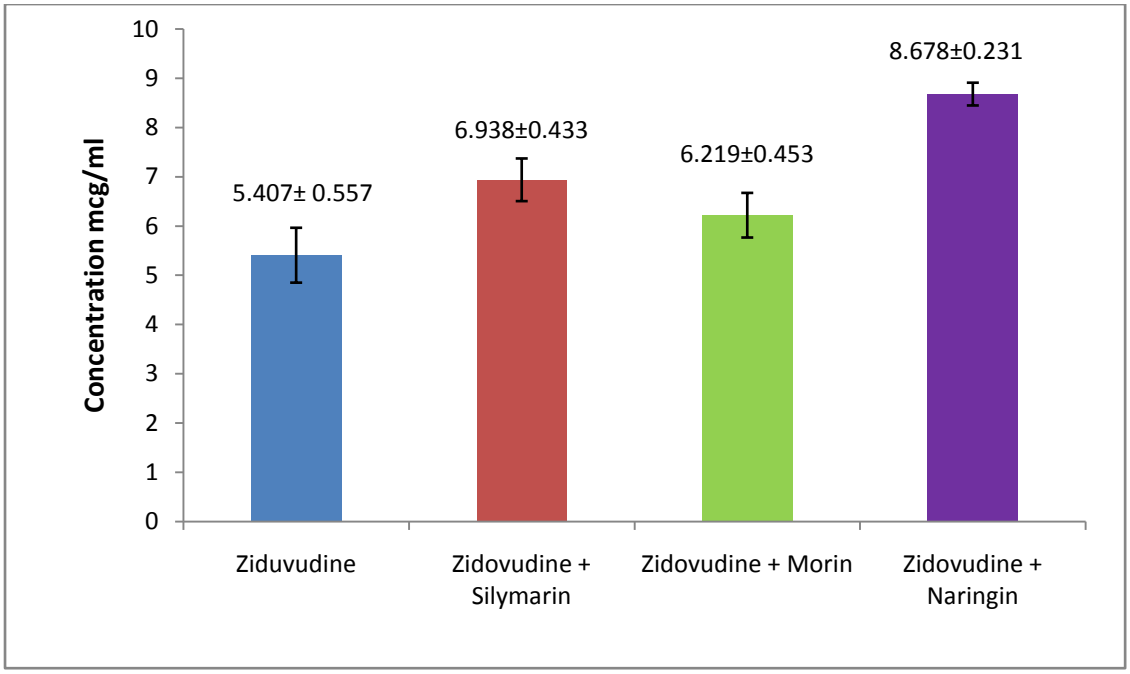


FIGURE 5.20: Comparison of zidovudine HAPs

5.3 *In vitro* cytotoxicity studies by SRB assay

The three HAPs named silymarin, morin and naringin were selected for the study of its cytotoxicity against colon cancer cell line (HCT-15) and breast cancer cell line (MCF-7) by SRB assay method.

5.3.1 *In vitro* cytotoxicity of HAPs against human cancer cell lines

The three HAPs named silymarin, morin and naringin were selected for the study. *In vitro* cytotoxicity against two human cancer cell lines was determined (Monks et al., 1991) using 96-well tissue culture plates. The 100 µl of cell suspension was added to each well of the 96-well tissue culture plate. The cells were allowed to grow in carbon dioxide incubator (37 °C, 5 % CO_2, 90 % RH) for 24 hr.

TABLE 5.41: *In vitro* cytotoxicity of HAPs against human cancer cell lines

Treatment	Conc. (µmol)	% Growth inhibition			
		HCT-15	MCF-7	HCT-15	MCF-7
Incubation Time		24 hr		48 hr	
Silymarine	25	8	10	0	1
	50	13	15	9	12
	100	0	8	10	18
	200	7	25	10	28
Morine	25	10	8	0	0
	50	3	11	6	0
	100	1	17	0	9
	200	0	19	0	19
Naringin	25	5	15	9	5
	50	13	8	0	6
	100	20	14	0	11
	200	19	2	0	0
5-FU	10	42	53	72*	92*
Tamoxifen	10	39	67	91*	89*

* If % growth of cell was inhibited more than 70% than results are believed to be significant and compound was cytotoxic

5.4 *In Vitro* Enzyme Activity

5.4.1 *In vitro* Pgp-ATPase activity

P-gp-dependent ATPase activity was measured by determining vanadate-sensitive release of inorganic phosphate (Pi) from ATP as reported earlier (Hrycyna et al., 1998).

TABLE 5.42: *In vitro* Pgp-ATPase activity of silymarin

Parameters	Control	Silymarin 10 µmol	Silymarin 30 µmol	Silymarin 100 µmol
Mean	0.584	0.376*	0.305333*	0.301**
SD	0.118013	0.089939	0.083201	0.026058
SEM	0.068137	0.051928	0.048037	0.015045

*P<0.05 & ** P<0.01, Significant different from control (Dunnett's test)

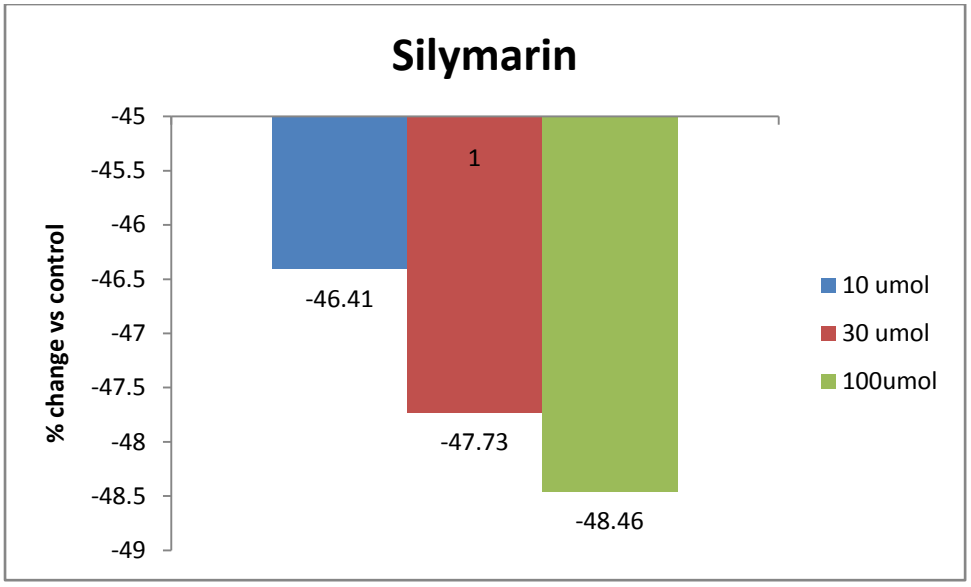


FIGURE 5.21: *In vitro* Pgp-ATPase activity of silymarin

TABLE 5.43: *In vitro* Pgp-ATPase activity of naringin

Parameters	Control	Naringin 10 µmol	Naringin 30 µmol	Naringin 100 µmol
Mean	0.584	0.202667***	0.176***	0.11***
SD	0.118013	0.032716	0.032078	0.022068
SEM	0.068137	0.018889	0.018521	0.012741

*** P<0.0001, highly significant different from control (Dunnett's test)

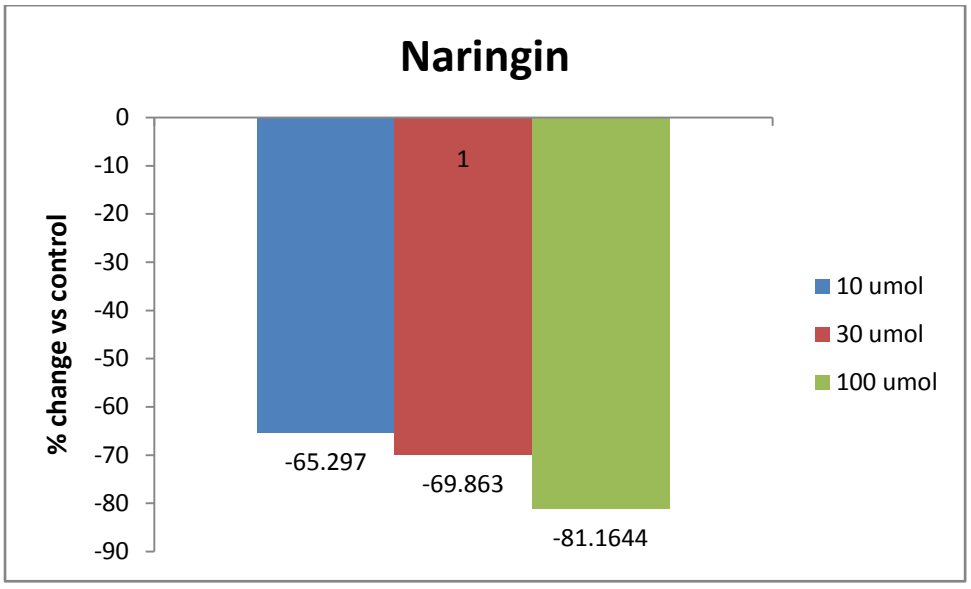


FIGURE 5.22: *In vitro* Pgp-ATPase activity of naringin

TABLE 5.44: *In vitro* Pgp-ATPase activity of morin

Parameters	Control	Morin 10 µmol	Morin 30 µmol	Morin 100 µmol
Mean	0.584	0.241333***	0.169667***	0.133667***
SD	0.118013	0.034443	0.035247	0.050143
SEM	0.068137	0.019886	0.02035	0.028951

*** P<0.001, Highly significant different from control (Dunnett's test)

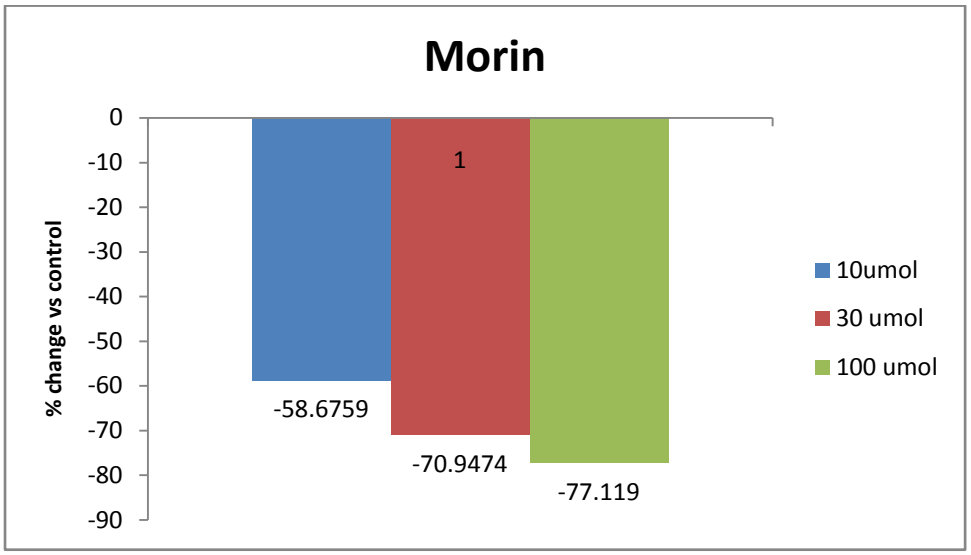


FIGURE 5.23: *In vitro* Pgp-ATPase activity of morin

5.4.2 *In vitro* CYP 450 Enzyme studies

Two measure subtract of CYP 1A1 (Benzopyrene Hydroxylase) and CYP 450 3A4 (Erythromycin demethylase) were selected for the study and its activity was measured.

5.4.3 CYP450 1A1Enzyme: Benzopyrene Hydroxylase Activity

Microsomal benzopyrene hydroxylase (AHH) activity, AHH activity in tissue assayed as results was shown as below;

TABLE 5.45: *In vitro* CYP450 1A1 enzyme activity of silymarin

Parameters	Control	Silymarin 10 µmol	Silymarin 30 µmol	Silymarin 100 µmol
Mean	617.33	620.67†	614.67†	616†
SD	58.011	17.67	45.08	17.52
SEM	33.49393	10.20	28.03	10.12

†P>0.05, non significant different from control (Dunnett's test)

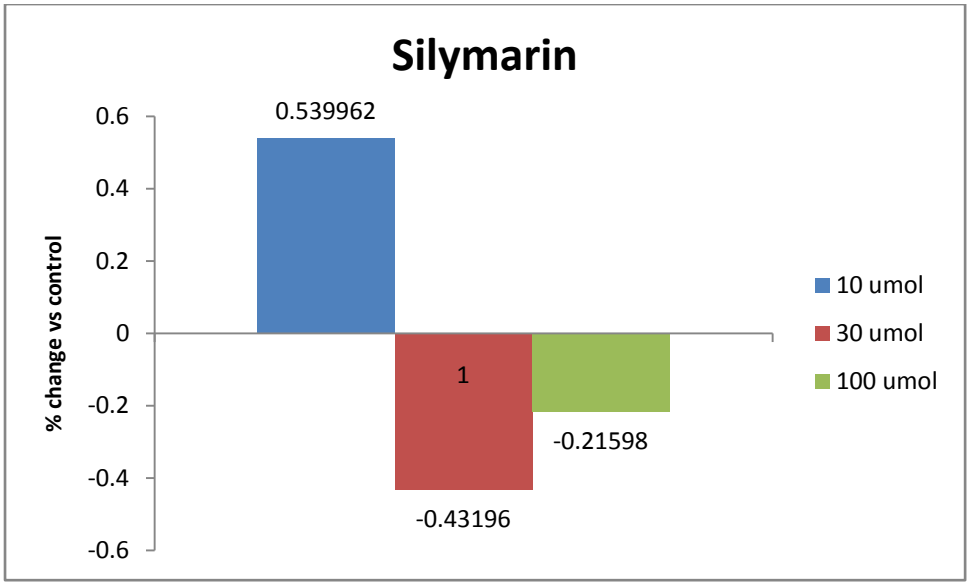


FIGURE 5.24: *In vitro* CYP450 1A1 enzyme activity of silymarin

TABLE 5.46: *In vitro* CYP450 1A1 enzyme activity of naringin

Parameters	Control	Naringin 10 µmol	Naringin 30 µmol	Naringin 100 µmol
Mean	617.33	490.66†	402.00**	343.33**
SD	58.01	72.29	75.90	41.48
SEM	33.49	41.74	43.82	23.95

†P>0.05, non significant different from control (Dunnett's test)
**P<0.05, Significant different from control (Dunnett's test)

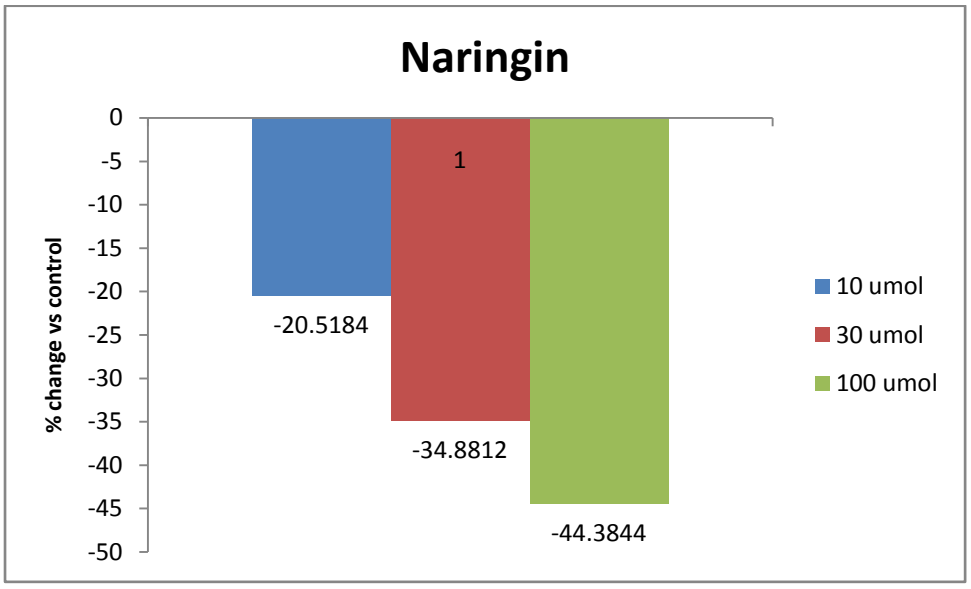


FIGURE 5.25: *In vitro* CYP450 1A1 enzyme activity of naringin

TABLE 5.47: *In vitro* CYP450 1A1 enzyme activity of morin

Parameters	Control	Morin 10 µmol	Morin 30 µmol	Morin 100 µmol
Mean	617.33	580.33†	512.67†	465.68†
SD	58.01	39.11	104.51	81.82
SEM	33.49	22.58	60.34	47.24

†P>0.05, non significant different from control (Dunnett's test)

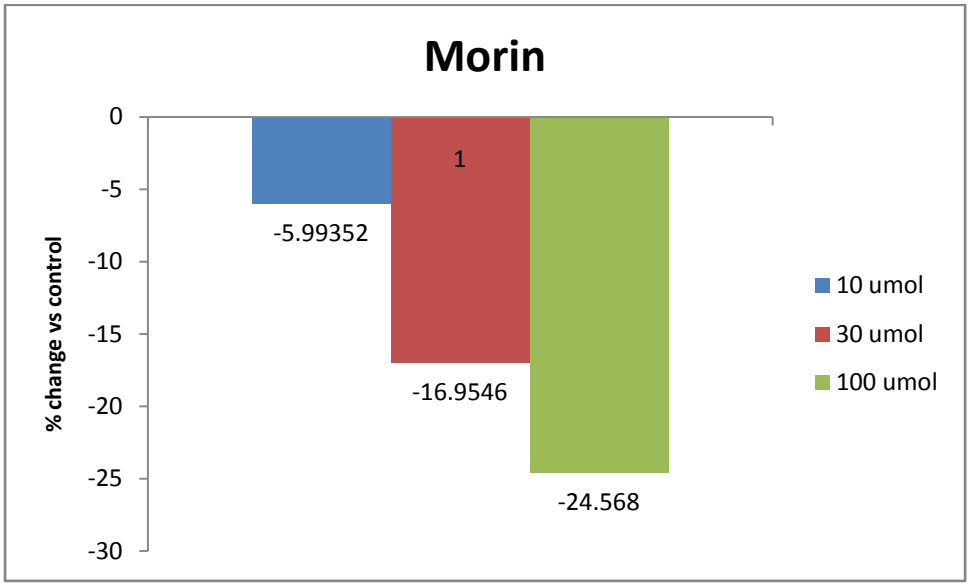


FIGURE 5.26: *In vitro* CYP450 1A1 enzyme activity of morin

5.4.3 CYP 450 3A4 enzyme: Erythromycin demethylase

Erythromycin N-demethylation was determined by the earlier reported method (Tu et al., 1983).

TABLE 5.48: *In vitro* CYP 450 3A4 enzyme activity of silymarin

Parameters	Control	Silymarin 10 µmol	Silymarin 30 µmol	Silymarin 100 µmol
Mean	0.0438	0.0546†	0.0669**	0.05687**
SD	0.0006	0.0097	0.0076	0.0064
SEM	0.0003	0.0056	0.0044	0.0037

†P>0.05, non significant different from control (Dunnett's test)
**P<0.05, Significant different from control (Dunnett's test)

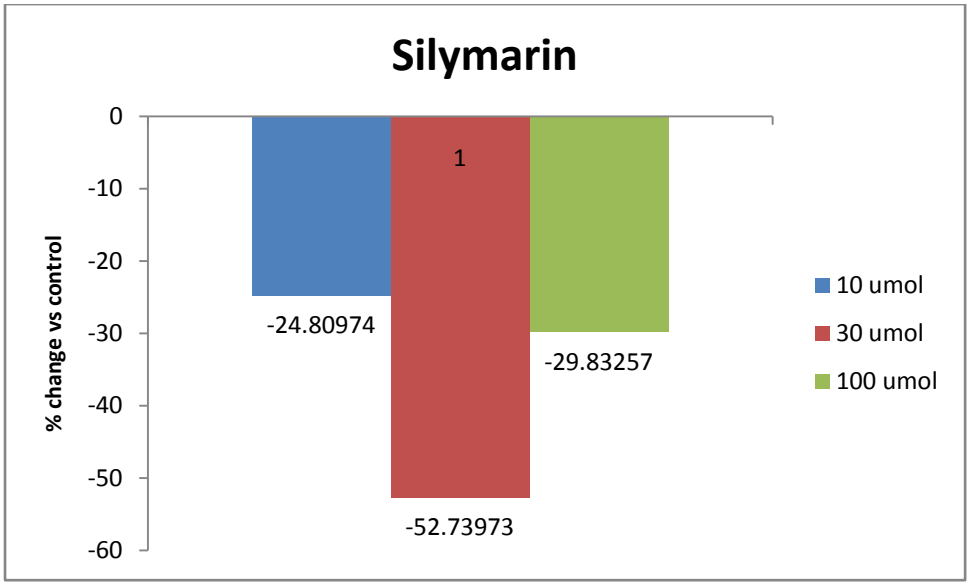


FIGURE 5.27: *In vitro* CYP 450 3A4 enzyme activity of silymarin

TABLE 5.49: *In vitro* CYP 450 3A4 enzyme activity of naringin

Parameters	Control	Naringin 10 µmol	Naringin 30 µmol	Naringin 100 µmol
Mean	0.0438	0.0494†	0.0544†	0.0481†
SD	0.000651	0.0082	0.0062	0.0038
SEM	0.0003	0.0047	0.0035	0.0022

†P>0.05, non significant different from control (Dunnett's test)

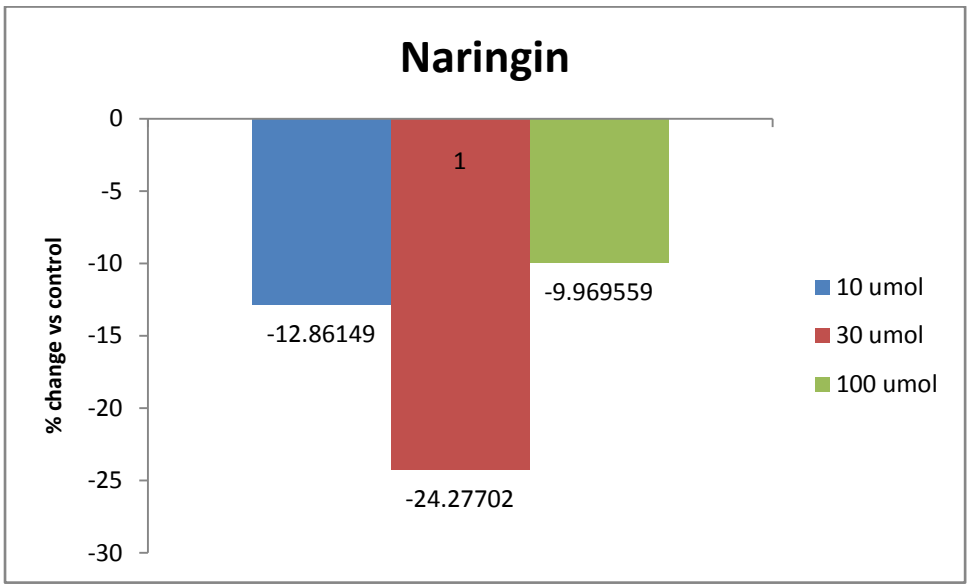


FIGURE 5.28: *In vitro* CYP 450 3A4 enzyme activity of naringin

TABLE 5.50: *In vitro* CYP 450 3A4 enzyme activity of morin

Parameters	Control	Morin 10 µmol	Morin 30 µmol	Morin 100 µmol
Mean	0.0438	0.0469†	0.0534†	0.0533†
SD	0.000651	0.0103	0.0088	0.0076
SE	0.0003	0.0059	0.0051	0.0044

†P>0.05, non significant different from control (Dunnett's test)

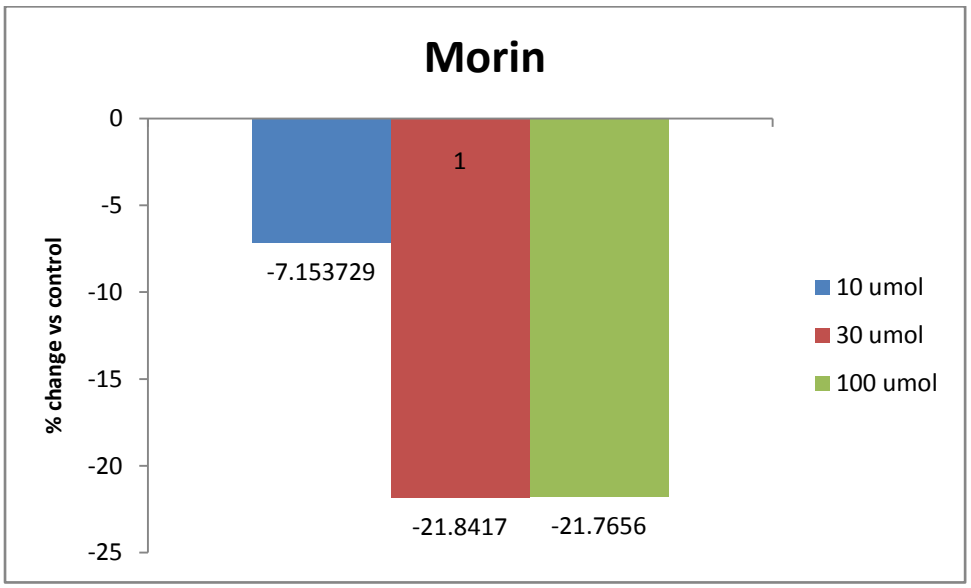


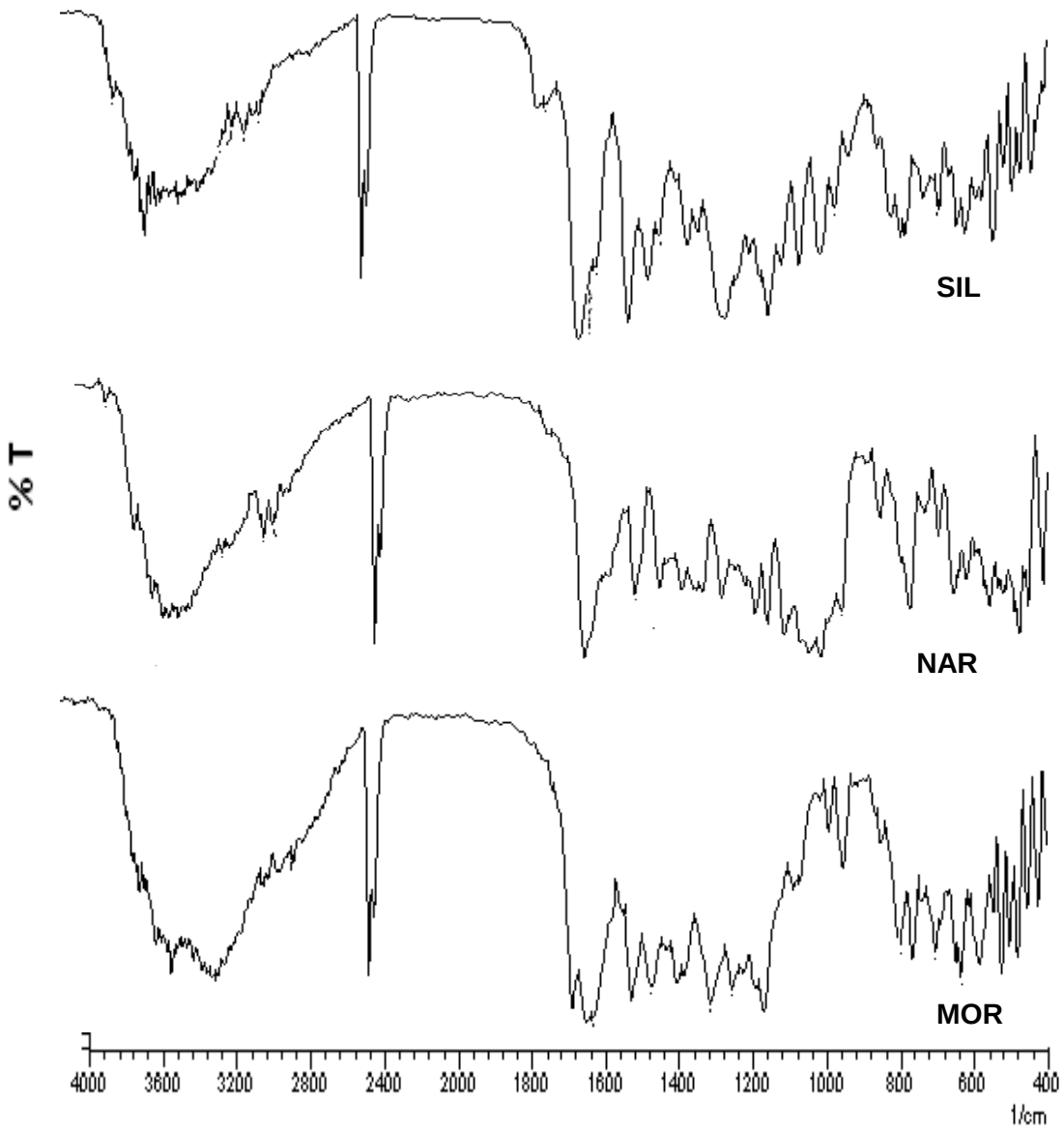
FIGURE 5.29: *In vitro* CYP 450 3A4 enzyme activity of morin

5.5 Primary compatibility study of herbal absorption promoters (HAPs) with drugs

In this study the IR spectra and DSC spectra were taken of selected drugs i.e. atorvastatin calcium, carvedilol phosphate, sildenafil citrate and zidovudine with each of the selected HAPs i.e. silymarin, naringin and morin along with individual spectra of each drug and HAPs. This will give a conformation about the compatibility of selected HAPs with the selected drugs and about the physical and chemical change occur due to interaction between them.

5.5.1 Infrared spectroscopic studies

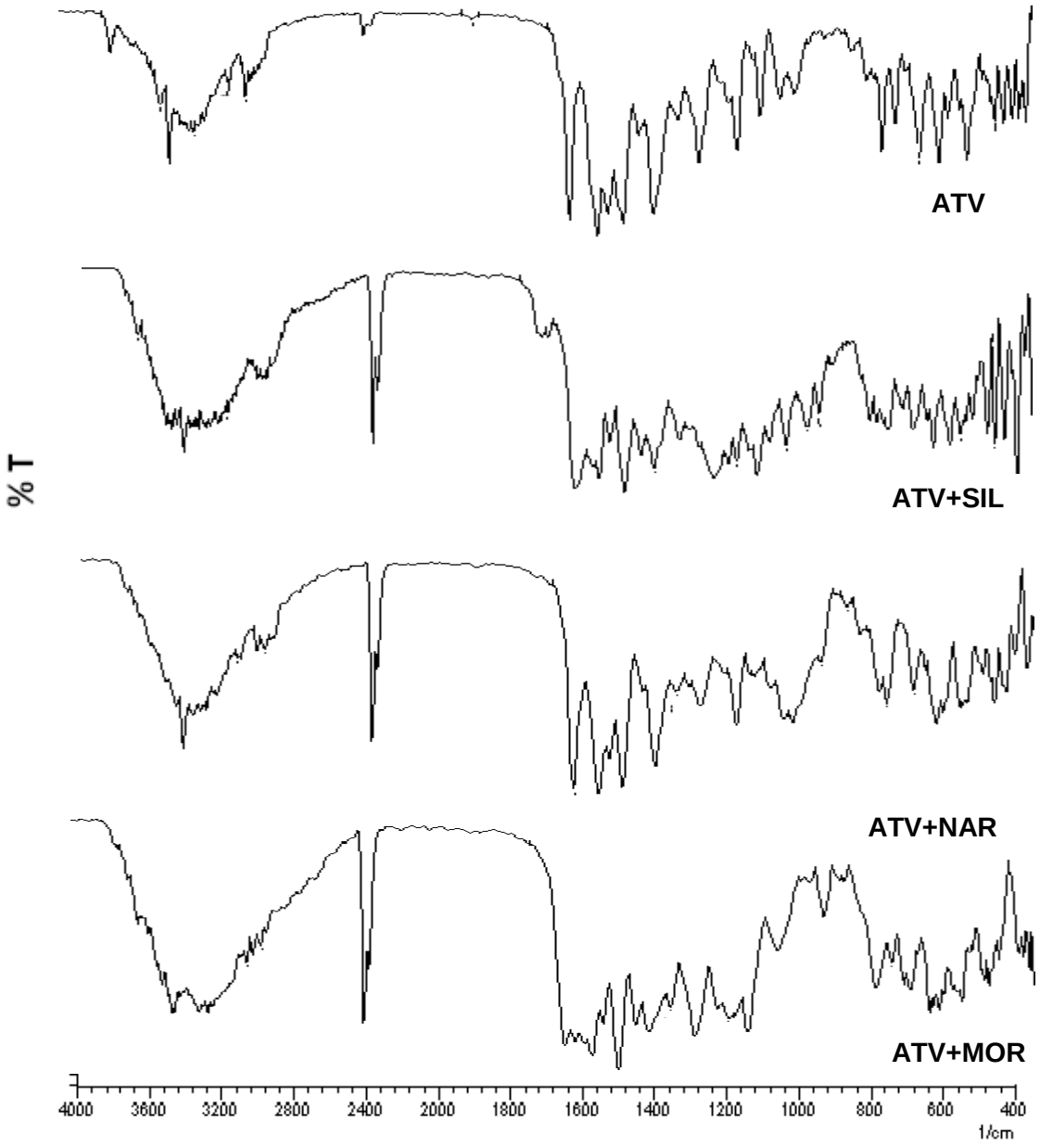
5.5.1.1 Herbal absorption promoters



SIL= Silymarin NAR= Naringin MOR= Morin

FIGURE 5.30: FT-IR spectra of HAPs (i.e. silymarin, naringin and morin)

5.5.1.2 Atorvastatin calcium

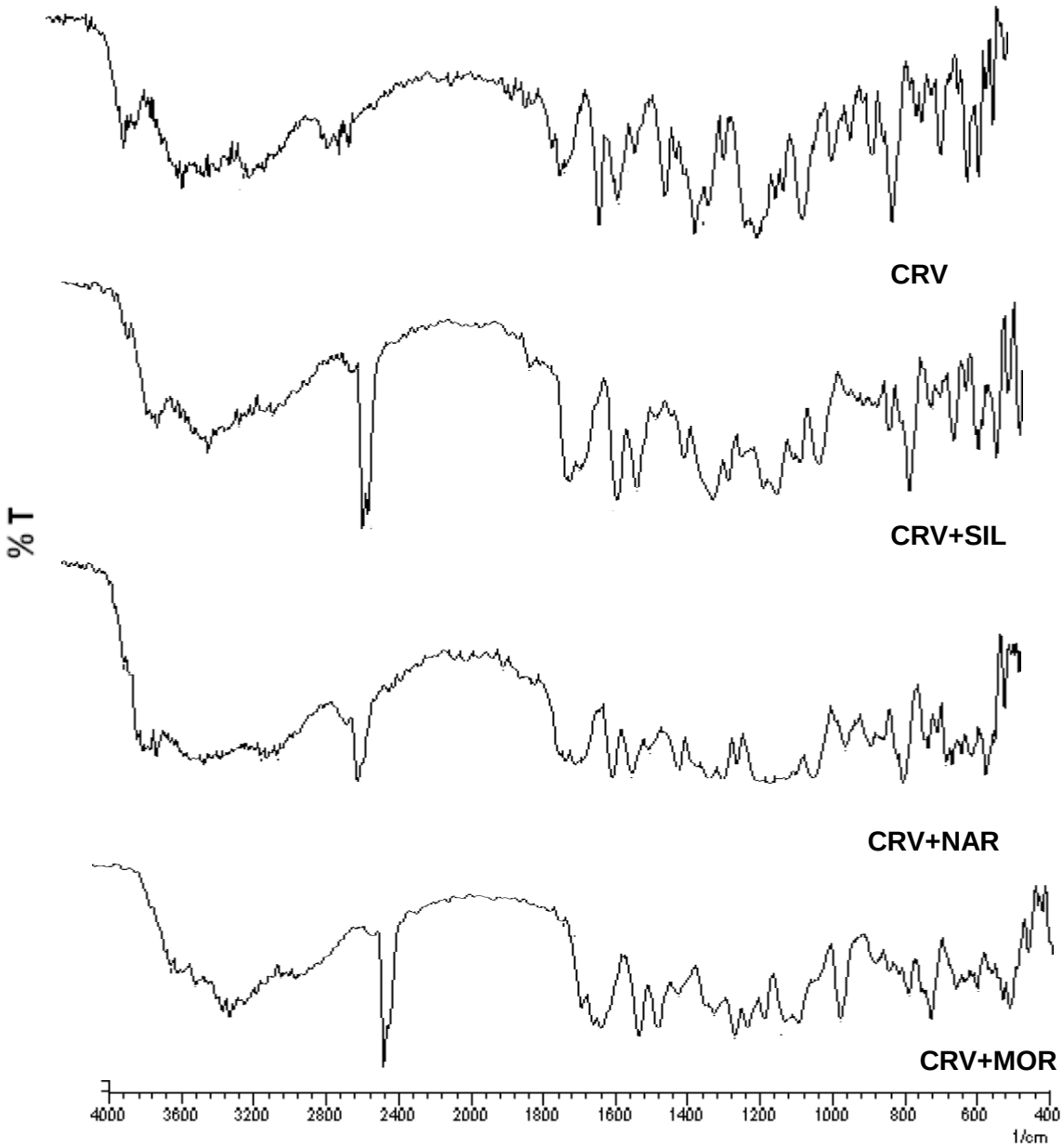


ATV=Atorvastatin ATV+SIL= Atorvastatin + Silymarin

ATV+NAR= Atorvastatin + Naringin ATV+MOR= Atorvastatin + Morin

FIGURE 5.31: FT-IR spectra of atorvastatin and atorvastatin with HAPs (i.e. silymarin, naringin and morin)

5.5.1.3 Carvedilol phosphate



CRV=Carvedilol

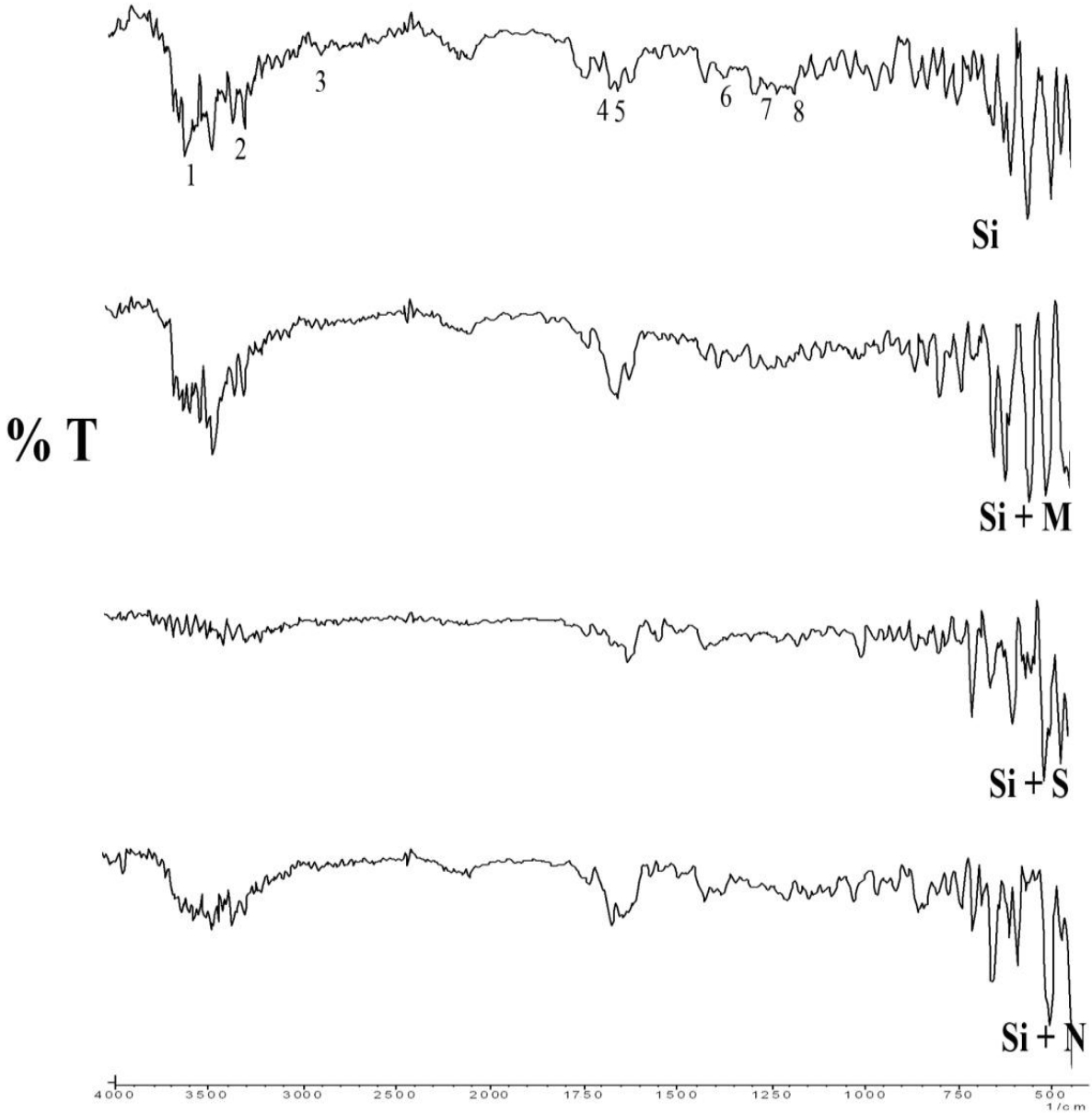
CRV +NAR= Carvedilol +Naringin

CRV +SIL= Carvedilol +Silymarin

CRV +MOR= Carvedilol +Morin

FIGURE 5.32: FT-IR spectra of carvedilol and carvedilol with HAPs (i.e. silymarin, naringin and morin)

5.5.1.4 Sildenafil citrate



Si = Sildenafil citrate

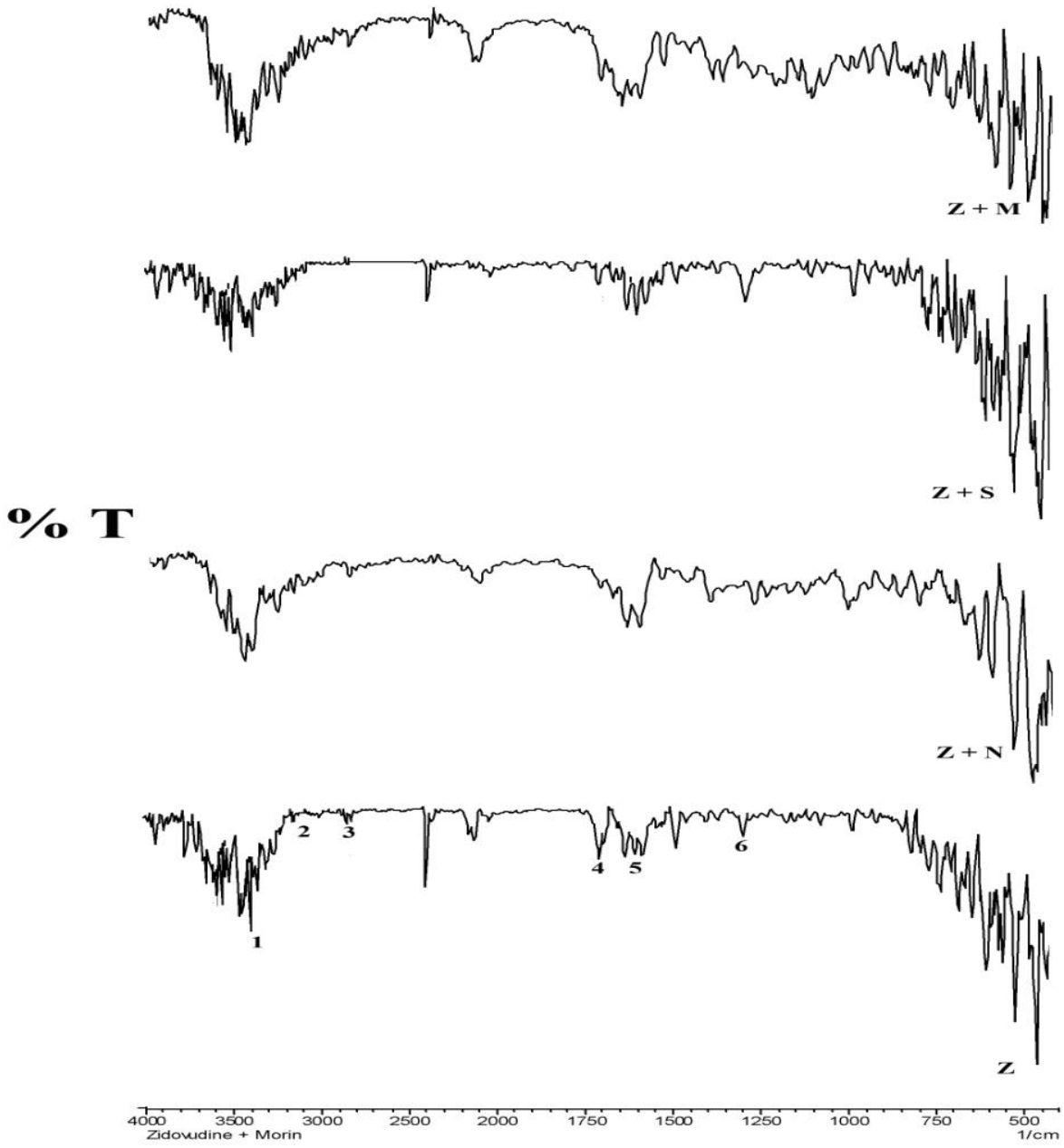
Si + S = Sildenafil citrate + Silymarin

Si + M = Sildenafil citrate + Morin

Si + N = Sildenafil citrate + Naringin

FIGURE 5.33: FT-IR spectra of sildenafil and sildenafil with HAPs (i.e. silymarin, morin and naringin)

5.5.1.5 Zidovudine



Z = Zidovudine
Z + S = Zidovudine + Silymarin
Z + M = Zidovudine + Morin
Z + N = Zidovudine + Naringin

FIGURE 5.34: FT-IR spectra of zidovudine and zidovudine with HAPs (i.e. silymarin, morin and naringin)

5.5.2 Differential scanning calorimetry

5.5.2.1 Herbal absorption promoters

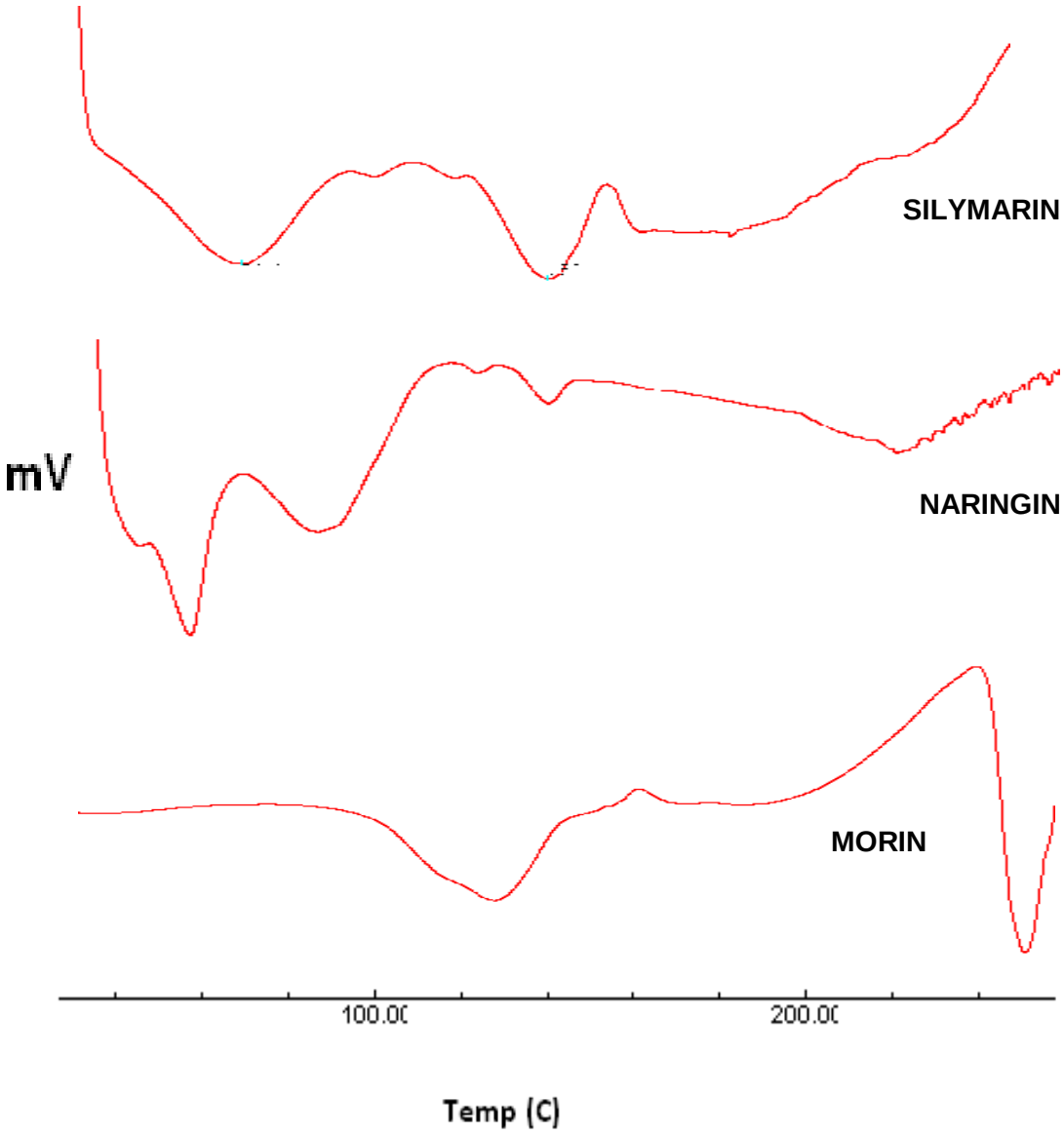
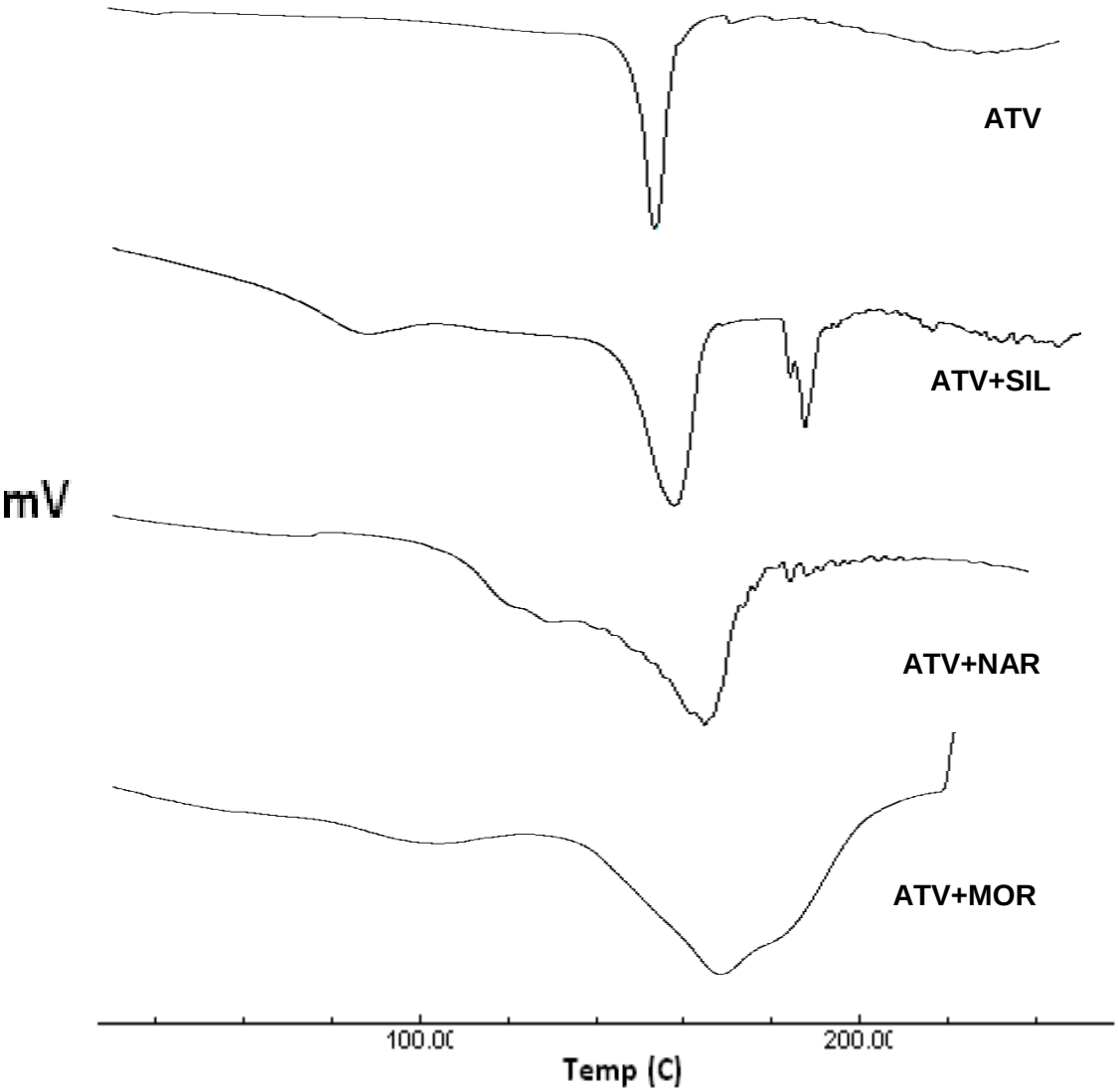


FIGURE 5.35: DSC thermogram of HAPs (i.e. silymarin, naringin and morin)

5.5.2.2 Atorvastatin calcium



ATV=Atorvastatin

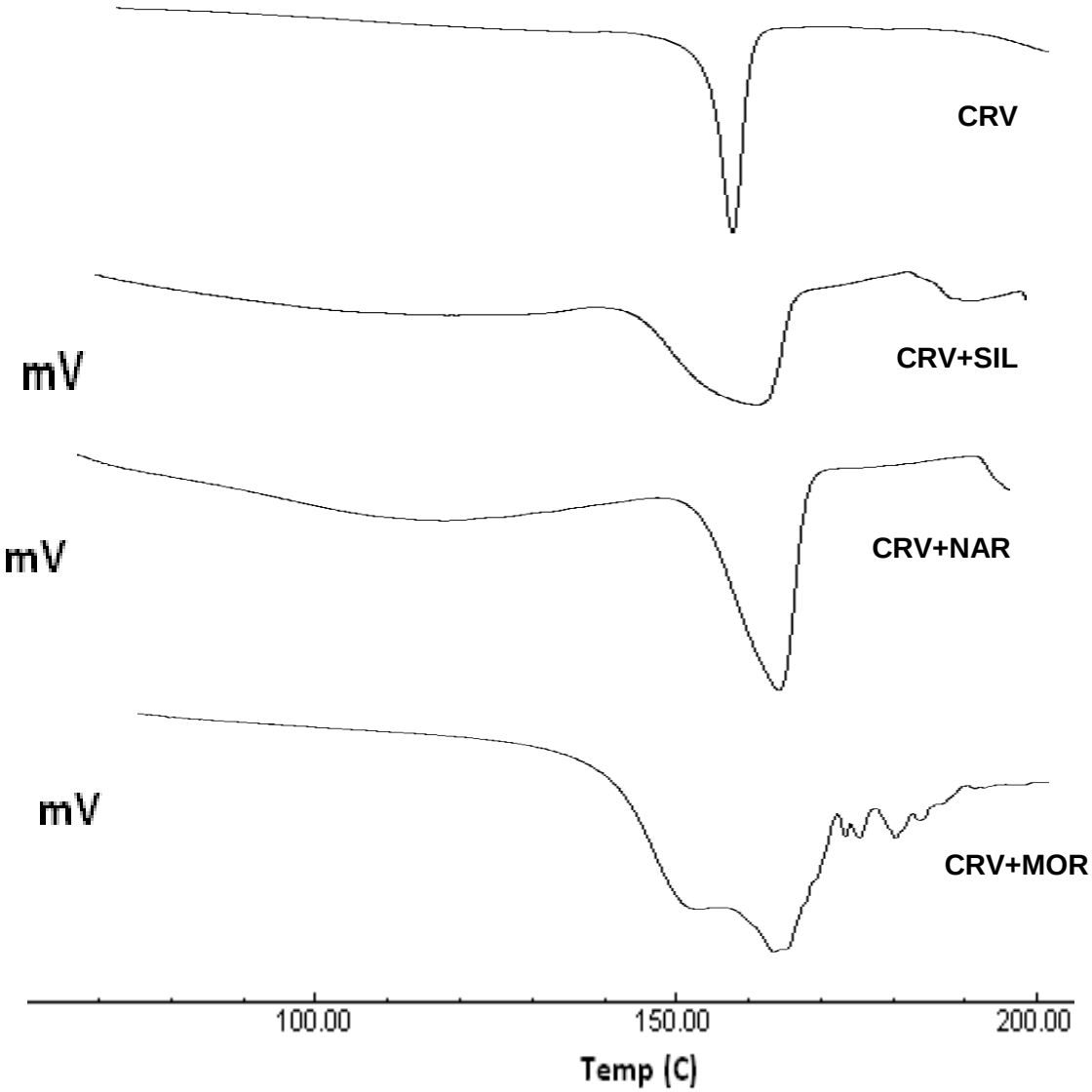
ATV+SIL= Atorvastatin + Silymarin

ATV+NAR= Atorvastatin + Naringin

ATV+MOR= Atorvastatin + Morin

FIGURE 5.36: DSC thermogram of atorvastatin and atorvastatin with HAPs (i.e. silymarin, naringin and morin)

5.5.2.3 Carvedilol



CRV=Carvedilol

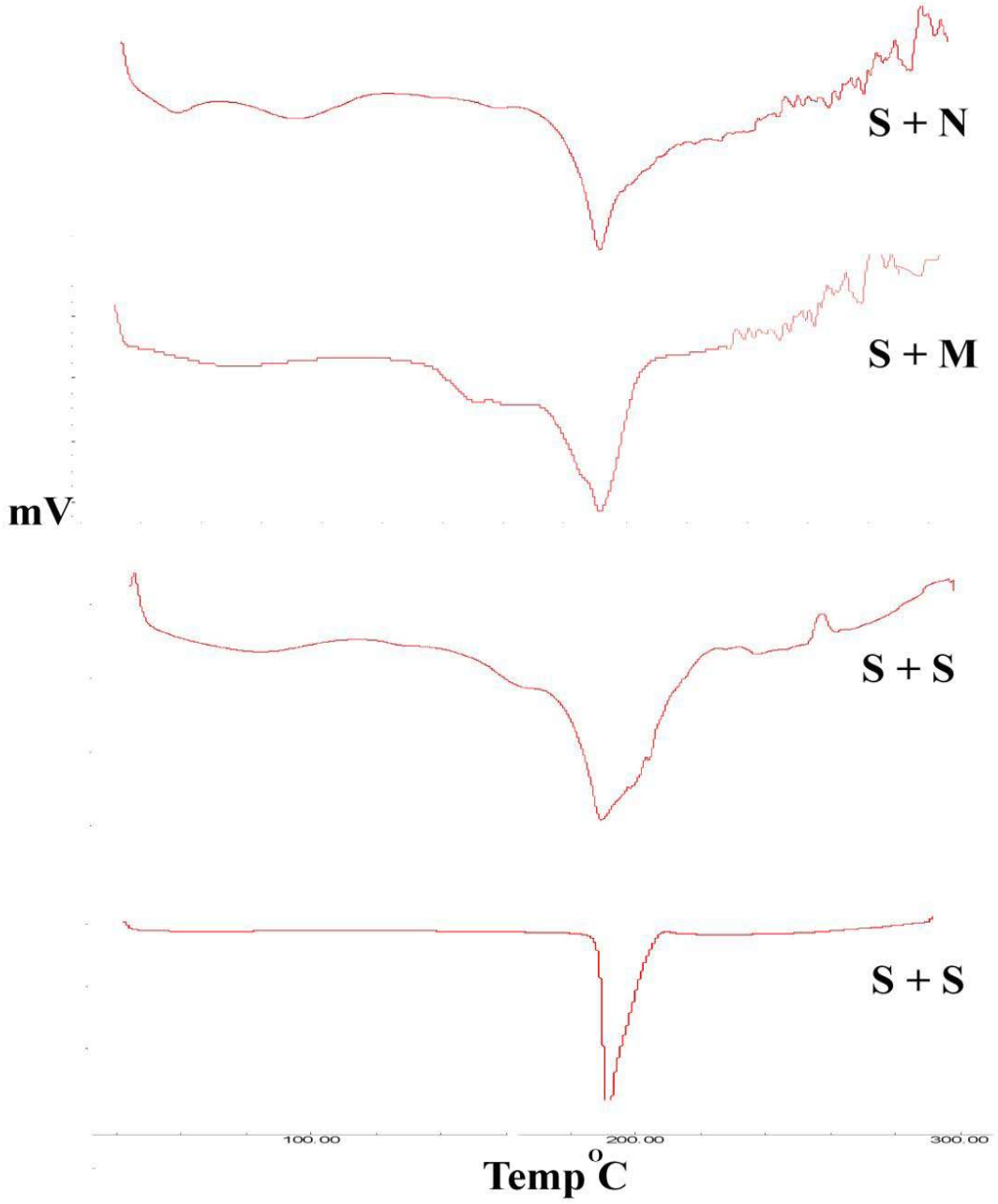
CRV + NAR= Carvedilol + Naringin

CRV + SIL= Carvedilol + Silymarin

CRV + MOR= Carvedilol + Morin

FIGURE 5.37: DSC thermogram of carvedilol and carvedilol with HAPs (i.e. silymarin, naringin and morin)

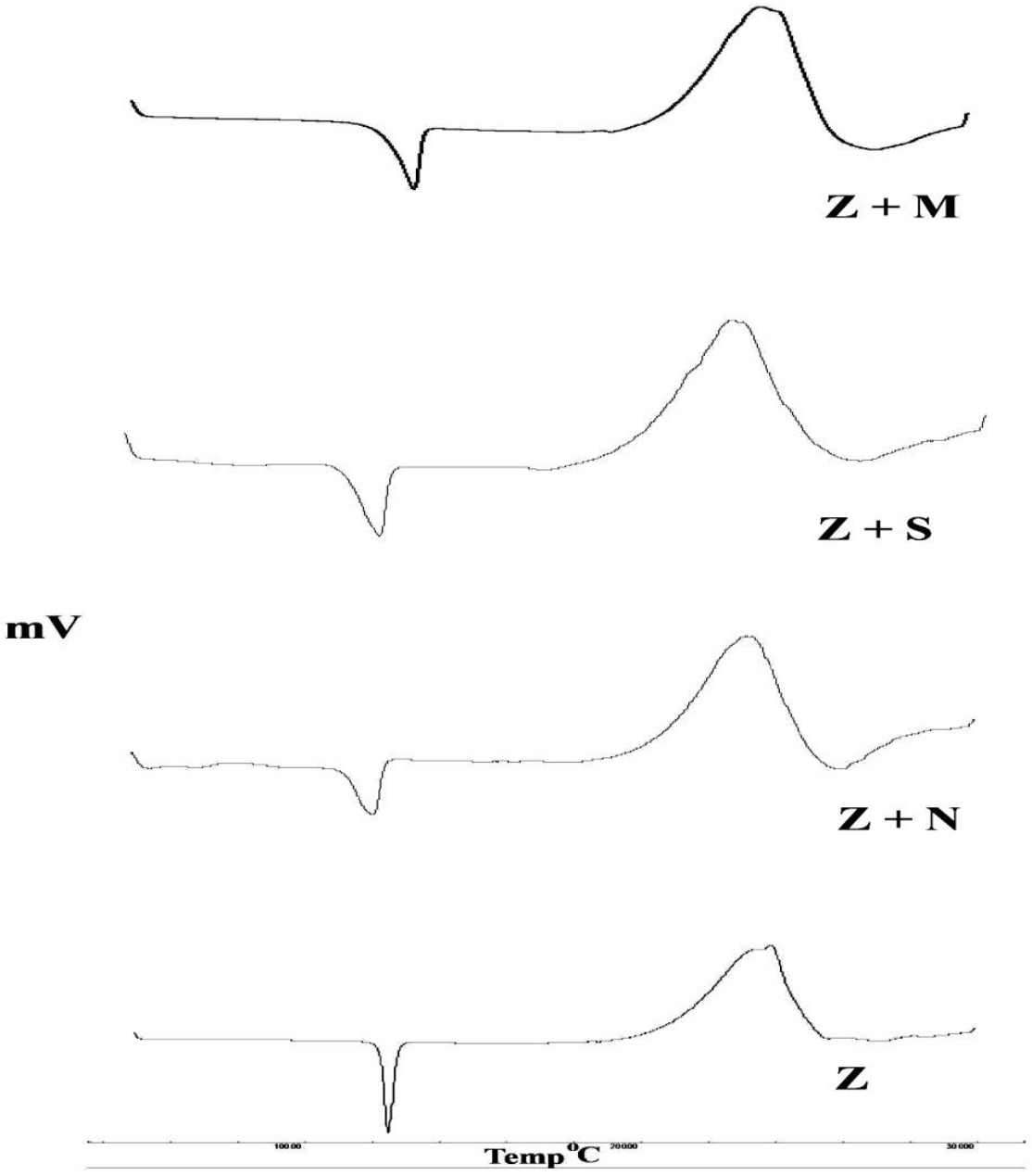
5.5.2.4 Sildenafil citrate



Si = Sildenafil citrate
Si + M = Sildenafil citrate + Morin
Si + S = Sildenafil citrate + Silymarin
Si + N = Sildenafil citrate + Naringin

FIGURE 5.38: DSC thermogram of sildenafil and Sildenafil with HAPs (i.e. silymarin, morin and naringin)

5.5.2.5 Zidovudine



Z = Zidovudine

Z + S = Zidovudine + Silymarin

Z + M = Zidovudine + Morin

Z + N = Zidovudine + Naringin

FIGURE 5.39: DSC thermogram of zidovudine and zidovudine with HAPs (i.e. silymarin, morin and naringin)

6. DISCUSSION

6.1 High Performance Liquid Chromatography (HPLC) Method Validation

6.1.1 Atorvastatin calcium

6.1.1.1 System suitability

For the precision of replicate of injection, % CV obtained was 0.517 % (Tab. 5.1) and in to system suitability the limit of % CV is not more than 5 %. So the % CV obtained from experimental analysis was within these criteria and on this basis method is precise for replicate of injection and suitable for system.

6.1.1.2 Linearity

The area ratio obtained of different concentration of atorvastatin calcium was used to plot a calibration curve of area ratio vs. concentration (µg/ml). Linearity range of atorvastatin calcium was found to be 1.0 - 188.0 µg with correlation co-efficient of 0.9995 (Fig. 5.1). The linearity equation is y =0.039X x –0.029, where X is concentration of atorvastatin calcium and Y is Area Ratio. Fig. 5.1 shows the calibration curve of atorvastatin calcium according to peak area ratio. The data with correlation co-efficient and % CV are given in Tab. 5.2. Which were within specified criteria so, this method is linear.

6.1.1.3 Accuracy and Precision

6.1.1.3.1 Intraday Accuracy and Precision

Data for intraday reproducibility were given in Tab. 5.3**.** The % CV obtained for intraday precision for atorvastatin calcium was found to be 0.58 - 2.07, which is less than 5 %. Nominal concentration was found 105.957 %, 104.558 %, 102.781 % and 99.739 % of LLOQ, LQC, MQC and HQC respectively which was within specified criteria which confirmed that method was precise.

6.1.1.3.2 Interday Accuracy and Precision

Data for interday reproducibility were given in Tab. 5.4**.** The % CV obtained for intraday precision for the atorvastatin calcium was found to be 0.96 - 2.93. This was less than the 5 %. Nominal concentration was found 109.238 %, 105.614 %,

102.904 % and 100.904 % of LLOQ, LQC, MQC and HQC respectively which was within specified criteria which confirmed that the method was precise. So the results of both above two experiments indicate that this method was precise on the basis of its reproducibility.

6.1.1.3.3 Recovery

The % recovery was found 77.34 %, 81.37 % and 81.36 % at the LQC, MQC and HQC respective QC level of the atorvastatin (Tab. 5.5). % mean recovery of the atorvastatin was 80.02 % and % CV within the QC level was 2.90 which was less than 5 % (Tab. 5.6). The % recovery of internal standard diclofenac sodium was 76.678 % (Tab. 5.7).

6.1.1.3.4 Limit of Detection

The detectable limit was 0.500 µg**.**

6.1.1.3.5 Limit of Quantification

The quantification limit was 1.000 µg.

6.1.1.3.6 Specificity

It resolves the peak of atorvastatin calcium (RT at 6.20 ± 0.5 min) and diclofenac sodium (RT at 8.92 ± 0.5 min) even in presence of herbal absorption enhancer or plasma (Fig. 5.1). In proposed method excipients or plasma did not interfere with analysis. This method was specific.

6.1.2 Carvedilol

6.2.2.1 System suitability

For the precision of replicate of injection % CV obtained was 1.551 % (Tab. 5.9), and in system suitability the limit of % CV not more than 5 %. So % CV obtained from the experimental analysis was within these criteria and on this basis method was precise for replicate of injection and suitable for system.

6.1.2.2 Linearity

The area ratio obtained of different concentration of carvedilol was used to plot a calibration curve of Area ratio vs. concentration (µg/ml). Linearity range of carvedilol was found to be 0.500-208.0 µg with correlation co-efficient of 0.9996 (Fig. 5.2). The linearity equation was y=0.027x +0.163 where X is concentration of carvedilol and Y is area ratio. Fig. 5.2 shows that the calibrations curve of carvedilol according to peak area ratio. The data with correlation co-efficient and % CV were given in Tab. 6.10. Which were within specified criteria so this method was linear.

6.1.2.3 Accuracy and precision

6.1.2.3.1 Intraday accuracy and precision

Data for intraday reproducibility were given in Tab. 5.11**.** The % CV obtained for intraday precision for carvedilol was found to be 0.67 - 1.74. This is less than 5 %. Nominal concentration was found 99.183 %, 99.722 %, 99.663 %, and 100.464 % of LLOQ, LQC, MQC and the HQC respectively which was within specified criteria. So this confirmed that the method was precise.

6.1.2.3.2 Inter day accuracy and precision

Data for interday reproducibility were given in TAB. 5.12**.** The %CV obtained for intraday precision for carvedilol was found to be 1.39 - 2.88. Which is less than 5%.% Nominal concentration was found 96.183, 98.593, 100.714, and 99.946 of LLOQ, LQC, MQC and HQC respectively which was within specified criteria. That confirmed that the method was precise. So, the results of above two experiments indicate that this method was precise on the basis of its reproducibility.

6.1.2.3.3 Recovery

The % recovery was found 85.78 %, 88.05 % and 86.59 % at LQC, MQC and HQC respective QC level of carvedilol (Tab. 5.13). % mean recovery of carvedilol was 86.81 % and % CV within QC level was 1.33 which was less than 5 % (Tab.

5.14). The % recovery of internal standard diclofenac sodium was 82.86 % (Tab. 5.15).

6.1.2.3.4 Limit of detection

The detectable limit was 0.250 µg**.**

6.1.2.3.5 Limit of quantification

The quantification limit was 0.500 µg.

6.1.2.3.6 Specificity

It resolves the peak of carvedilol (RT at 2.78 ± 0.5 min) and diclofenac sodium (RT at 8.32 ± 0.5 min) even in presence of herbal absorption enhancer or plasma (Fig. 5.2). In proposed method excipients or plasma did not interfere with analysis. This method is specific.

6.1.3 Sildenafil citrate

6.1.3.1 System suitability

For the precision of replicate of injection RSD obtained was 0.29 %. Tab. 5.17 and in system suitability limit of RSD is within ± 2 %. So this method was precise for replicate of injection and suitable for system.

6.1.3.2 Linearity

The peak area obtained at 230 nm of different concentration of sildenafil citrate was used to plot a calibration curve. Linearity range of sildenafil citrate was found to 0.1-50 µg with correlation co-efficient of 0.999 (Fig. 5.3). The linearity equation is Y=17845X – 2912, where X is concentration of sildenafil citrate and Y is peak area. Fig. 5.3 shows the calibration curve of sildenafil citrate according to peak area. The data of correlation co-efficient and % CV were given in the Tab. 5.18. The limit of correlation co-efficient and % CV was not less than a 0.99 and not more than 5 % respectively.

6.1.3.3 Accuracy and precision

6.1.3.3.1 Intraday reproducibility

Data for intraday reproducibility were given in Tab. 5.19. The % CV obtained for intraday precision for sildenafil citrate was found to be 0.541 - 2.99. The limit for % CV is not more than 5 %.

6.1.3.3.2 Interday reproducibility

Data for interday reproducibility were given in Tab. 5.20. The % CV obtained for interday precision for sildenafil citrate was found to be 0.37 - 3.61. The limit for % CV is not more than 5 %. So the result of both reproducible experiments indicates that this method is precise.

6.1.3.3.3 Recovery

The % recovery experiment revealed good accuracy. The % recovery of added sample was within ± 2 % (Tab. 5.21). This result clearly indicates that the method was accurate.

6.2.3.3.4 Limit of detection

The detectable limit was 100 ng.

6.1.3.3.5 Limit of quantification

The quantification limit was 100 ng.

6.1.3.3.6 Specificity

It resolves the peak of sildenafil citrate (RT at 5.33 ± 0.5 min) even in presence of herbal absorption enhancer or plasma. Fig. 5.3 in proposed method excipient or plasma did not interfere with analysis. This method is specific.

6.1.4 Zidovudine

6.1.4.1 System suitability

For the precision of replicate of injection, RSD obtained was 0.66 %. TAB. 5.23**,** and in system suitability the limit of RSD was within ± 2 %. So, this method was precise for replicate of injection and suitable for system.

6.1.4.2 Linearity

The peak area obtained at 266 nm of different concentration of sildenafil citrate was used to plot a calibration curve. Linearity range of zidovudine was found to be 0.1-50 µg with correlation co-efficient of 0.999 (Fig. 5.4). The linearity equation is Y = 64030X + 2662, where X is concentration of sildenafil citrate and Y is peak area. Fig. 5.4 shows the calibration curve of zidovudine according to peak area. The data with correlation co-efficient and % CV were given in Tab. 5.24. The limit of correlation co-efficient and % CV was not less than 0.99 and not more than 5 % respectively.

6.1.4.3 Accuracy and precision

6.2.4.3.1 Intraday reproducibility

Data for intraday reproducibility were given in Tab. 5.25. The % CV obtained for intraday precision for zidovudine was found to be 1.39 - 4.82. The limit for % CV is not more than 5 %.

6.1.4.3.2 Interday reproducibility

Data for interday reproducibility were given in Tab. 5.26. The % CV obtained for interday precision for zidovudine was found to be 0.69 - 4.90. The limit for % CV is not more than 5 %. So the results of above reproducible experiments indicate that this method was precise.

6.1.4.3.3 Recovery

The % recovery experiment revealed good accuracy. The % recovery of added sample was within ± 2 % (Tab. 5.27)**.** This result clearly indicates that the method was accurate.

6.1.4.3.4 Limit of detection

The detectable limit was 100 ng.

6.1.4.3.5 Limit of quantification

The quantification limit was 100 ng.

6.1.4.3.6 Specificity

It resolves the peak of zidovudine (RT at 3.36 ± 0.5 min) even in presence of herbal absorption enhancer plasma (Fig. 5.4). In proposed method excipient or plasma did not interfere with analysis. This method was specific.

6.2 *In vivo* bioavailability study

6.2.1 Atorvastatin calcium

6.2.1.1 Effect of silymarin as absorption enhancer

The method was applied to a bioavailability study for examining the influence of the co-administration of silymarin on to the absorption of atorvastatin. Findings of the pharmacokinetic studies were shown in Tab. 5.29 and plasma concentration profile at different time intervals was depicted in to the Fig. 5.5. It was observed that concomitant administration of silymarin and atorvastatin calcium increased significantly the bioavailability of atorvastatin calcium. Fig. 5.5 clearly suggests that there was no alteration in to the T_{max}. In other words, the onset of action of atorvastatin calcium remains unaltered but, the maximum concentration C_{max} increased significantly.

The mean plasma concentration–time profiles of atorvastatin calcium in the presence and absence of silymarin were characterized in rats and illustrated in

Fig. 5.5. The mean pharmacokinetic parameters of atorvastatin calcium were also summarized in Tab. 5.29.

Fig. 5.5 displays the atorvastatin plasma concentration–time profiles in the animals after orally administration of the atorvastatin suspension with silymarin solution. Silymarin increased the C_{max} of atorvastatin by approximately 32.31% from 3.921 to 5.188 µg/ml while there was no significant change in to the T_{max} of atorvastatin in the presence of silymarin. These results indicate that co-administration of atorvastatin with silymarin seems to increase the C_{max}.

6.2.1.2 Effect of naringin as absorption enhancer

Same as above the method was applied to a bioavailability study for examining the influence of the co-administration of naringin on the absorption of atorvastatin calcium. Findings of the pharmacokinetic studies were shown in Tab. 5.30 and the plasma concentration profile at different time intervals was depicted in Fig. 5.6. It was observed that concomitant administration of naringin and atorvastatin calcium increased significantly the bioavailability of atorvastatin calcium. Fig. 5.6 clearly suggests that there was no alteration in the T_{max}. In other words, the onset of action of atorvastatin calcium remains unaltered but, the maximum concentration C_{max} increased significantly.

The mean plasma concentration–time profiles of atorvastatin calcium in the presence and absence of naringin were characterized in rats and illustrated in Fig. 5.6. The mean pharmacokinetic parameters of atorvastatin calcium were also summarized in Tab. 5.30.

Fig. 5.6 displays the atorvastatin plasma concentration–time profiles in the animals after orally administration of the atorvastatin suspension with naringin solution. Naringin increased the C_{max} of atorvastatin by approximately 19.66 % from 3.921 to 4.692 µg/ml while there was no significant change in to the T_{max} of

atorvastatin in presence of naringin. These results indicate that co-administration of atorvastatin with naringin seems to increase the C_{max}.

6.2.1.3 Effect of morin as absorption enhancer

Same as above the method was applied to a bioavailability study for examining the influence of the co-administration of morin on the absorption of atorvastatin calcium. Findings of the pharmacokinetic studies were shown in Tab. 5.31 and the plasma concentration profile at different time intervals was depicted in Fig. 5.7. It was observed that concomitant administration of morin and atorvastatin calcium decreased significantly the bioavailability of atorvastatin calcium. Fig. 5.7 clearly suggests that there was no alteration in the T_{max}. In other words, the onset of action of the drug, atorvastatin calcium remains unaltered but, the maximum concentration C_{max} decreased significantly.

The mean plasma concentration–time profiles of atorvastatin calcium in the presence and absence of morin were characterized in rats and illustrated in Fig. 5.7. The mean pharmacokinetic parameters of atorvastatin calcium were also summarized in Tab. 5.31.

Fig. 5.7 displays the atorvastatin plasma concentration–time profiles in the animals after orally administration of the atorvastatin suspension with morin solution. Morin decrease the C_{max} of atorvastatin by approximately -14.23 % from 3.921 to 3.363 µg/ml while there was no significant change in T_{max} of atorvastatin in presence of morin. These results indicate that co-administration of atorvastatin with morin seems to decrease the C_{max}.

From the experimental analysis the results indicates that the silymarin and naringin significantly increase the concentration of drug in rat blood plasma while morin decrease the concentration as compare to control (Atorvastatin calcium).

6.2.2 Carvedilol

6.2.2.1 Effect of silymarin as absorption enhancer

The method was applied to a bioavailability study for examining the influence of the co-administration of silymarin on the absorption of carvedilol. Findings of the pharmacokinetic studies were shown in Tab. 5.32 and the plasma concentration profile at different time intervals was depicted in Fig. 5.9. It was observed that concomitant administration of silymarin and carvedilol increased significantly the bioavailability of carvedilol. Fig. 5.9 clearly suggests that there was no alteration in the T_{max}. In other words, the onset of action of the drug, carvedilol remains unaltered but, the maximum concentration C_{max} increased significantly.

The mean plasma concentration–time profiles of carvedilol in the presence and absence of silymarin were characterized in rats and illustrated in Fig. 5.9. The mean pharmacokinetic parameters of carvedilol were also summarized in Tab. 5.32.

Fig. 5.9 displays the carvedilol plasma concentration–time profiles in the animals after orally administration of the carvedilol suspension with silymarin solution. Silymarin increased the C_{max} of carvedilol by approximately 31.95 % from 9.517 - 12.558 µg/ml, while there was no significant change in T_{max} of carvedilol in the presence of silymarin. These results indicate that co-administration of carvedilol with silymarin seems to increase the C_{max}.

6.2.2.2 Effect of naringin as absorption enhancer

Same as above the method was applied to a bioavailability study for examining the influence of the co-administration of naringin on the absorption of carvedilol. Findings of the pharmacokinetic studies were shown in Tab. 5.33 and the plasma concentration profile at different time intervals was depicted in Fig. 5.10. It was observed that concomitant administration of naringin and carvedilol increased significantly the bioavailability of carvedilol. Fig. 5.10 clearly suggests that there

was no alteration in the T_{max}. In other words the onset of action of the carvedilol remains unaltered but, the maximum concentration C_{max} increased significantly. The mean plasma concentration–time profiles of carvedilol in the presence and absence of naringin were characterized in rats and illustrated in Fig. 5.10. The mean pharmacokinetic parameters of carvedilol were also summarized in Tab. 5.33.

Fig. 5.10 displays the carvedilol plasma concentration–time profiles in the animals after orally administration of the carvedilol suspension with naringin solution. naringin increased the C_{max} of carvedilol by approximately 28.01 % from 9.517 - 12.183 µg/ml, while there was no significant change in T_{max} of carvedilol in the presence of naringin. These results indicate that co-administration of carvedilol with naringin seems to increase the C_{max}.

6.2.2.3 Effect of morin as absorption enhancer

Same as above the method was applied to a bioavailability study for examining the influence of the co-administration of morin on the absorption of carvedilol. Findings of the pharmacokinetic studies were shown in Tab. 5.34 and the plasma concentration profile at different time intervals was depicted in Fig. 5.11. It was observed that concomitant administration of morin and carvedilol increased significantly the bioavailability of carvedilol. Fig. 5.11 clearly suggests that there was no alteration in the T_{max}. In other words the onset of action of the carvedilol remains unaltered but, the maximum concentration C_{max} increased significantly.

The mean plasma concentration–time profiles of carvedilol in the presence and absence of morin were characterized in rats and illustrated in Fig. 6.9. The mean pharmacokinetic parameters of carvedilol were also summarized in Tab. 6.10. Fig. 5.11 displays the carvedilol plasma concentration–time profiles in the animals after orally administration of the carvedilol suspension with morin solution. Morin increase the C_{max} of carvedilol by approximately 5.67 % from 9.517 - 10.057 µg/ml, while there was no significant change in T_{max} of carvedilol

in the presence of morin. These results indicate that co-administration of carvedilol with morin seems to increase the C_{max}.

From the experimental analysis the results indicates that the silymarin, naringin and morin significantly increase the concentration of drug in rat blood plasma, as compare to control (Carvedilol).

6.2.3 Sildenafil citrate

6.2.3.1 Effect of silymarin as absorption enhancer

The method was applied to a bioavailability study for examining the influence of the co-administration of silymarin on the absorption of sildenafil citrate. Findings of the pharmacokinetic studies were shown in to the Tab. 5.35 and the plasma concentration profile at different time intervals was depicted in Fig. 5.13. It was observed that concomitant administration of silymarin and sildenafil citrate increased significantly the bioavailability of sildenafil citrate. Fig. 5.13 clearly suggests that there was no alteration in the T_{max}. In other words, the onset of action of sildenafil citrate remains unaltered but, the maximum concentration C_{max} increased significantly.

The mean plasma concentration–time profiles of sildenafil citrate in the presence and absence of silymarin were characterized in rats and illustrated in Fig. 5.13. The mean pharmacokinetic parameters of sildenafil citrate were also summarized in Tab. 5.35.

Fig. 5.13 displays the sildenafil citrate plasma concentration–time profiles in the animals after orally administration of the sildenafil citrate suspension with silymarin solution. Silymarin increase the C_{max} of sildenafil citrate by approximately 24.88 % from 1.049 to 1.310 µg/ml while there was no significant change in T_{max} of sildenafil citrate in the presence of silymarin. These results indicate that co-administration of sildenafil citrate with silymarin seems to increase the C_{max}.

6.2.3.2 Effect of morin as absorption enhancer

Same as above method, the method was applied to a bioavailability study for examining the influence of the co-administration of morin on the absorption of sildenafil citrate. Findings of the pharmacokinetic studies were shown in Tab. 5.36 and the plasma concentration profile at different time intervals was depicted in Fig. 5.14. It was observed that concomitant administration of morin and sildenafil citrate increased significantly the bioavailability of sildenafil citrate but it was some less as compare to silymarin. Fig. 5.14 clearly suggests that there was no alteration in the T_{max}. In other words, the onset of action of sildenafil citrate remains unaltered but, the maximum concentration C_{max} increased significantly.
The mean plasma concentration–time profiles of sildenafil citrate in the presence and absence of morin were characterized in rats and illustrated in Fig. 5.14. The mean pharmacokinetic parameters of sildenafil citrate were also summarized in Tab. 5.36.

Fig. 5.14 displays the sildenafil citrate plasma concentration–time profiles in the animals after orally administration of the sildenafil citrate suspension with morin solution. Morin increase the C_{max} of Sildenafil citrate by approximately 11.09% from 1.049 to 1.154 µg/ml while there was no significant change in to the T_{max} of sildenafil citrate in presence morin. These results indicate that co-administration of sildenafil citrate with morin seems to increase the C_{max}.

6.2.3.3 Effect of naringin as absorption enhancer

Same as above methods, the method was also applied to a bioavailability study for examining the influence of the co-administration of naringin on the absorption of sildenafil citrate. Findings of the pharmacokinetic studies and the plasma concentration profile at different time intervals was depicted in Tab. 5.37 and Fig. 5.15 respectively. It was observed that concomitant administration of naringin and sildenafil citrate increased significantly the bioavailability of sildenafil citrate but it is some less as compare to silymarin and morin. Fig. 5.15 clearly suggests that there was no alteration in the T_{max} as like as silymarin and morin. In other

words the onset of action of sildenafil citrate remains unaltered but, the maximum concentration C_{max} increased significantly.

The mean plasma concentration–time profiles of sildenafil citrate in the presence and absence of naringin were characterized in rats and illustrated in Fig. 5.15. The mean pharmacokinetic parameters of sildenafil citrate were also summarized in Tab. 5.37.

Fig. 5.15 displays the sildenafil citrate plasma concentration–time profiles in the animals after orally administration of the sildenafil citrate suspension with naringin solution. Naringin having not any significant effect on to the C_{max} of sildenafil citrate by approximately -1.90 % from 1.049 to 1.029 µg/ml, while there was no significant change in T_{max} of sildenafil citrate in the presence of naringin.

From the experimental analysis the results indicates that the silymarin and morin significantly increase the concentration of drug in rat blood plasma, as compare to control (Sildenafil citrate) while naringin having not any significant effect on rat blog plasma concentration.

6.2.4 Zidovudine

6.2.4.1 Effect of silymarin as absorption enhancer

Same as above methods, for examining the influence of the co-administration of silymarin on the absorption of zidovudine in bioavailability study this method was used. Findings of the pharmacokinetic studies and the plasma concentration profile at different time intervals was depicted in to the Tab. 5.39 and Fig. 5.18 respectively. Fig. 5.18 clearly suggests that the concomitant administration of silymarin and zidovudine increased significantly the bioavailability of zidovudine. And there was no alteration in to the $T_{max.}$ In other words, the onset of action of zidovudine remains unaltered but, the maximum concentration C_{max} increased significantly.

The mean plasma concentration–time profiles of zidovudine in the presence and absence of silymarin were characterized in rats and illustrated in Fig. 5.18. The mean pharmacokinetic parameters of zidovudine were also summarized in Tab. 5.39.

Fig. 5.18 displays the zidovudine plasma concentration–time profiles in the animals after orally administration of the zidovudine suspension with silymarin solution. Silymarin having significant effect on the C_{max} of zidovudine by increasing approximately 28.31 % from 5.407 - 6.938 µg/ml, while there was no significant change in T_{max} of zidovudine in the presence of silymarin. These results indicate that co-administration of zidovudine with the silymarin seems to increase the C_{max}.

6.2.4.2 Effect of morin as absorption enhancer

As like above methods, this method also applied for examining the influence of the co-administration of naringin on the absorption of zidovudine in bioavailability study. Findings of the pharmacokinetic studies and the plasma concentration profile at different time intervals was depicted in to the Tab. 5.40 and Fig. 5.19 respectively. It was observed that concomitant administration of morin and zidovudine increased significantly the bioavailability of zidovudine. Fig. 5.19 clearly suggests that there was no alteration in the T_{max}. In other words, the onset of action of the drug, zidovudine remains unaltered but the maximum concentration C_{max} increased significantly.

The mean plasma concentration–time profiles of zidovudine in the presence and absence of morin were characterized in rats and illustrated in Fig. 5.19. The mean pharmacokinetic parameters of zidovudine were also summarized in Tab. 5.40.

Fig. 5.19 displays the zidovudine plasma concentration–time profiles in the animals after orally administration of the zidovudine suspension with morin

solution. morin having significant effect on the C_{max} of zidovudine by increasing approximately 16.17 % from 5.407 to 6.219 µg/ml while there was no significant change in T_{max} of zidovudine in the presence of morin. These results indicate that co-administration of zidovudine with morin seems to increase the C_{max}.

6.2.4.3 Effect of naringin as absorption enhancer

Same as above methods, the method was applied to a bioavailability study for examining the influence of the co-administration of naringin on the absorption of zidovudine. Findings of pharmacokinetic studies and the plasma concentration profile at different time intervals was depicted in to the Tab. 5.38 and Fig. 5.17 respectively. It was observed that concomitant administration of naringin and zidovudine increased significantly the bioavailability of zidovudine. Fig. 5.17 clearly suggests that there was no alteration in the T_{max}. In other words the onset of action of drug, zidovudine remains unaltered but the maximum concentration C_{max} increased significantly.

The mean plasma concentration–time profiles of zidovudine in the presence and absence of naringin were characterized in rats and illustrated in Fig. 5.17. The mean pharmacokinetic parameters of zidovudine were also summarized in Tab. 5.38.

Fig. 5.17 displays the zidovudine plasma concentration–time profiles in the animals after orally administration of the zidovudine suspension with naringin solution. Naringin having significant effect on the C_{max} of zidovudine by increasing approximately 60.49 % from 5.407 - 8.678 µg/ml, while there was no significant change in T_{max} of zidovudine in the presence of naringin. These results indicate that co-administration of zidovudine with naringin seems to increase the C_{max}.

From the experimental analysis the results indicates that the silymarin and morin significantly increase the concentration of drug in rat blood plasma, as compare

to control (zidovudine) while naringin having very significant effect on rat blood plasma concentration.

6.3. *In vitro* cytotoxicity studies by SRB assay

The three flavanoids named silymarin, morin & naringin were selected for the study of its cytotoxicity against colon cancer cell line (HCT-15) and breast cancer cell line (MCF-7) by SRB assay method.

6.3.1 *In Vitro* Cytotoxicity of HAPs against human cancer cell lines

In vitro cytotoxicity against two human cancer cell lines was determined using 96-well tissue culture plates. The 100 µl of cell suspension was added to each well of the 96-well tissue culture plate. The cells were allowed to grow in carbon dioxide incubator (37 °C, 5 % CO_2, 90 % RH) for 24 hr.

The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in presence of test material was calculated considering the growth in absence of any test material as 100 % and in % growth inhibition in presence of test material was calculated.

From the experimental analysis the results indicates that the silymarin and morin significantly having not any considerable growth inhibition of selected cell lines i.e. HCT-15 and MCF-7 as compare to positive control 5-furouracil and temoxifen.

6.4 *In Vitro* enzyme activity

6.4.1 *In Vitro* P-gp ATPase enzyme activity

P-gp ATPase enzyme activity has been found in the present investigation, it indicated that silymarin possessed affinity for P-gp and it could be a putative modulator of this protein: an inhibition has implications in bioavailability enhancement. Tab. 5.42 shows that at the dose level of 10 µmol and 30 µmol the

silymarin significantly inhibit the P-gp ATPase enzyme activity as compared to control with $P < 0.05$ when analyzed statically by Dunnett's test. At the dose level of 100 µmol of silymarin significantly inhibited the P-gp ATPase enzyme activity as compared to control with $P < 0.01$ when analyzed statically by Dunnett's test. The P-gp ATPase enzyme inhibition activity of silymarin was also shown in Fig. 5.21 which was graphical representation as % change vs control. From above results for silymarin it can be concluded that silymarin having a significant inhibition effect on P-gp ATPase enzyme activity by this *in vitro* study.

P-gp ATPase enzyme activity has been found in the present investigation, it indicated that naringin possessed affinity for P-gp and it could be a putative modulator of this protein: an inhibition has implications in bioavailability enhancement. Tab. 5.43 shows that at the dose level of 10 µmol, 30 µmol and 100 µmol naringin highly significantly inhibit the P-gp ATPase enzyme activity as compared to control with $P < 0.0001$ when analyzed statically by Dunnett's test. The P-gp ATPase enzyme inhibition activity of naringin was also shown in Fig. 5.22 which was graphical representation as % change vs control. From above results for naringin it can be concluded that Naringin having a highly significant inhibition effect on P-gp ATPase enzyme activity by this *in vitro* study.

P-gp ATPase enzyme activity has been found in the present investigation, it indicated that morin possessed affinity for P-gp and it could be a putative modulator of this protein: an inhibition has implications in bioavailability enhancement. Tab. 5.44 shows that at the dose level of 10 µmol, 30 µmol and 100 µmol morin highly significantly inhibit the P-gp ATPase enzyme activity as compared to control with $P < 0.001$ when analyzed statically by Dunnett's test. The P-gp ATPase enzyme inhibition activity of morin was also shown in Fig. 5.23 which was graphical representation as % change vs control. From above results for morin it can be concluded that morin having a highly significant inhibition effect on P-gp ATPase enzyme activity by this *in vitro* study.

6.4.2 *In Vitro* CYP 450 enzyme studies

6.4.2.1 CYP1A1 Enzyme: Benzopyrene Hydroxylase Activity

CYP1A1 enzyme activity has been found in the present investigation, it indicated that silymarin possessed not having significant affinity for CYP1A1 and it could be a putative modulator of this enzyme. Tab. 5.45 shows that at the dose level of 10 µmol, 30 µmol and 100 µmol silymarin not significantly inhibit the CYP1A1 enzyme activity as compared to control with $P > 0.05$ when analyzed statically by Dunnett's test. The CYP1A1 enzyme inhibition activity of silymarin was also shown in Fig. 5.24 which was graphical representation as % change vs. control. From above results for silymarin it can be concluded that silymarin having a not significant inhibition effect on CYP1A1 enzyme activity by this *in vitro* study.

CYP1A1 enzyme activity has been found in the present investigation, it indicated that naringin possessed having significant affinity for CYP1A1 at higher dose level and it could be a putative modulator of this enzyme. Tab. 5.46 shows that at the dose level of 10 µmol naringin not significantly inhibit the CYP1A1 enzyme activity as compared to control with $P > 0.05$ when analyzed statically by Dunnett's test. At the dose level of 30 µmol and 100 µmol naringin significantly inhibit the CYP1A1 enzyme activity as compared to control with $P < 0.05$ when analyzed statically by Dunnett's test. The CYP1A1 enzyme inhibition activity of naringin was also shown in Fig. 5.25 which was graphical representation as % change vs control. From above results for naringin it can be concluded that naringin having a significant inhibition effect on CYP1A1 enzyme activity at higher dose level by this *in vitro* study.

CYP1A1 enzyme activity has been found in the present investigation, it indicated that morin possessed not having significant affinity for CYP1A1 and it could be a putative modulator of this enzyme. Tab. 5.47 shows that at the dose level of 10 µmol, 30 µmol and 100 µmol morin not significantly inhibit the CYP1A1 enzyme activity as compared to control with $P > 0.05$ when analyzed statically by Dunnett's test. The CYP1A1 enzyme inhibition activity of morin was also shown

in Fig. 5.26 which was graphical representation as % change vs control. From above results for morin it can be concluded that silymarin having a not significant inhibition effect on CYP1A1 enzyme activity by this *in vitro* study.

6.4.2.2 CYP3A4 enzyme: Erythromycin demethylase

CYP3A4 enzyme activity has been found in the present investigation, it indicated that silymarin possessed having significant affinity at higher dose level for CYP3A4 enzyme and it could be a putative modulator of this enzyme. Tab. 5.45 shows that at the dose level of 10 µmol silymarin not significantly inhibit the CYP3A4 enzyme activity as compared to control with $P > 0.05$ when analyzed statically by Dunnett's test. At the dose level of 30 µmol and 100 µmol silymarin significantly inhibit the CYP3A4 enzyme activity as compared to control with $P < 0.05$ when analyzed statically by Dunnett's test. The CYP3A4 enzyme inhibition activity of silymarin was also shown in Fig. 5.24 which was graphical representation as % change vs control. From above results for silymarin it can be concluded that silymarin having significant inhibition effect at higher dose level on CYP3A4 enzyme activity by this *in vitro* study.

CYP3A4 enzyme activity has been found in the present investigation, it indicated that naringin possessed not having significant affinity for CYP3A4 enzyme and it could be a putative modulator of this enzyme. Tab. 5.45 shows that at the dose level of 10 µmol, 30 µmol and 100 µmol naringin not significantly inhibit the CYP3A4 enzyme activity as compared to control with $P > 0.05$ when analyzed statically by Dunnett's test. The CYP3A4 enzyme inhibition activity of naringin was also shown in Fig. 5.24 which was graphical representation as % change vs control. From above results for naringin it can be concluded that naringin having a not significant inhibition effect on CYP3A4 enzyme activity by this *in vitro* study.

CYP3A4 enzyme activity has been found in the present investigation, it indicated that morin possessed not having significant affinity for CYP3A4 enzyme and it could be a putative modulator of this enzyme. Tab. 5.45 shows that at the dose

level of 10 µmol, 30 µmol and 100 µmol morin not significantly inhibit the CYP3A4 enzyme activity as compared to control with P > 0.05 when analyzed statically by Dunnett's test. The CYP3A4 enzyme inhibition activity of morin was also shown in Fig. 5.24 which was graphical representation as % change vs control. From above results for morin it can be concluded that Morin having a not significant inhibition effect on CYP3A4 enzyme activity by this *in vitro* study.

6.5 Primary compatibility study of HAPs with drugs

In this study the IR spectra and DSC spectra were taken of selected drugs i.e. atorvastatin calcium, carvedilol phosphate, sildenafil citrate and zidovudine with each of the selected HAPs i.e. silymarin, naringin and morin along with individual spectra of each drug and HAPs. This will give a conformation about the compatibility of selected HAPs with the selected drugs and about the physical and chemical change occur due to interaction between them.

6.5.1 Infrared spectroscopic study

6.5.1.1 Atorvastatin calcium

The FT-IR spectra of atorvastatin with HAPs were shown in Fig. 5.30. Mixture of Atorvastatin with HAPs i.e. silymarin, saringin and morin were also found to be identical. The principal IR absorption peaks of atorvastatin were all observed in the spectra of atorvastatin and solid mixture with HAPs. From these spectral observations it was indicated that no significant interaction between the atorvastatin and HAPs. So it can be concluded that this herbal ingredients were compatible with selected drug.

6.5.1.2 Carvedilol phosphate

The FT-IR spectra of carvedilol phosphate with HAPs were shown in Fig. 5.31. Mixture of carvidilol with HAPs i.e. silymarin, naringin and morin were also found to be identical. The principal IR absorption peaks of carvedilol were all observed in the spectra of carvedilol and solid mixture with HAPs. From these spectral observations it was indicated that no significant interaction between the carvedilol

and HAPs. So it can be concluded that this herbal ingredients were compatible with selected drug.

6.5.1.3 Sildenafil citrate

The FT-IR spectra of sildenafil citrate with HAPs were shown in Fig. 5.31. Mixture of sildenafil citrate with HAPs i.e. silymarin, naringin and morin were also found to be identical. The principal IR absorption peaks of sildenafil citrate were all observed in the spectra of sildenafil citrate and solid mixture with HAPs. From these spectral observations it was indicated that no significant interaction between the sildenafil citrate and HAPs. So it can be concluded that this herbal ingredients were compatible with selected drug.

6.5.1.4 Zidovudine

The FT-IR spectra of zidovudine with HAPs were shown in Fig. 5.31. Mixture of zidovudine with HAPs i.e. silymarin, naringin and morin were also found to be identical. The principal IR absorption peaks of zidovudine were all observed in the spectra of zidovudine and solid mixture with HAPs. From these spectral observations it was indicated that no significant interaction between the zidovudine and HAPs. So it can be concluded that this herbal ingredients were compatible with selected drug.

6.5.2 Differential scanning calorimetry

6.5.2.1 Atorvastatin calcium

The DSC thermograms of atorvastatin calcium and atorvastatin calcium with HAPs i.e. silymarin, naringin and morin were compared. The peak of atorvastatin calcium obtained in the DSC thermogram at 161 ^{o}C, while peak of atorvastatin calcium with HAPs were obtained at the same temperature and there were no any changes in thermogram patterns. On the basis of the temperature obtained in both cases it was observed that there was no any significant interaction between the atorvastatin calcium and HAPs.

6.5.2.2 Carvedilol phosphate

The DSC thermograms of carvedilol phosphate and carvedilol phosphate with HAPs i.e. silymarin, naringin and morin were compared. The peak of carvedilol phosphate obtained in the DSC thermogram at 158.8 ^{o}C while peak of carvedilol phosphate with HAPs were obtained at the same temperature and there were no any changes in thermogram patterns. On the basis of the temperature obtained in both cases it was observed that there was no any significant interaction between the carvedilol phosphate and HAPs.

6.5.2.3 Sildenafil citrate

The DSC thermograms of sildenafil citrate and sildenafil citrate with the HAPs i.e. silymarin, naringin and morin were compared. The peak of the sildenafil citrate obtained in the DSC thermogram at 192 ^{o}C while peak of sildenafil citrate with HAPs were obtained at the same temperature and there were no any changes in thermogram patterns. On the basis of the temperature obtained in both cases it was observed that there was no any significant interaction between the sildenafil citrate and HAPs.

6.5.2.4 Zidovudine

The DSC thermograms of zidovudine and zidovudine with HAPs i.e. silymarin, naringin and morin were compared. The peak of the zidovudine obtained in the DSC thermogram at 130 ^{o}C while peak of zidovudine with HAPs were obtained at the same temperature and there were no any changes in thermogram patterns. On the basis of the temperature obtained in both cases it was observed that there was no significant interaction between the zidovudine and HAPs.

7. SUMMARY AND CONCLUSION

For the current research on effect of bioavailability on the HAPs i.e. silymarin, naringin and morin selected with allopathic drugs i.e. atorvastatin calcium, carvedilol phosphate, sildenafil citrate and zidovudine. For analysis of these drugs as such as well from the plasma the sensitive methods was developed on HPLC. It was also validated and from results it was clear that the methods selected for the analysis of the drugs were selective, sensitive and accurate with high precision and accuracy and within the statistical limit.

In vitro cytotoxicity HRB assay shows that the selected HAPs silymarin, naringin and morin not shows any kind of the membrane damaging activity and cytotoxicity, while control group of 5-flurouracil and tamoxifen shows significant membrane damage and cytotoxicity after the 24 hr and in MCF-7 and HCT-15 cell lines. These results confirmed that the silymarin, naringine and morin were not having a membrane damaging cytotoxicity and safe to use.

Silymarin was significantly inhibiting the p-gp ATPase activity when compared with the control group at the concentration level of 10 µmol, 30 µmol and 100 µmol. The results indicate that all three HAPs having a potential inhibitory activity of p-gp ATPase enzymes. *In vitro* assay of CYP 450 1A1 enzyme activity of the silymarin and morin shows significant inhibition of enzyme at the concentration of 10 µmol, 30 µmol and 100 µmol. In naringin there was no significant inhibition of an enzyme activity of CYP 4501A1 at concentration of 10 µmol but shows significant inhibition at the concentration of 30 µmol and 100 µmol. *In vivo* assay CYP 450 3A4 enzyme for silymarin shows that it was not significantly inhibit the enzyme activity at the concentration 10 µmol and shows significant activity at 30 µmol and 100 µmol. Naringin and morin not shows significant inhibition activity at the concentration of 10 µmol, 30 µmol and 100 µmol.

The primary compatibility study of selected HAPs i. e. silymarin, naringin and morin not shows any significant interaction in FT-IR spectra and in the DSC

thermogram in primary compatibility with selected drugs i.e. atorvastatin calcium, carvedilol phosphate, sildenafil citrate and zidovudine. These studies conformed that selected HAPs may not interact potentially with the selected modern allopathic drug.

This studies shows that silymarin was increases bioavailability of atorvastatin by 32.31 %, carvedilol by 31.95 %, sildenafil by 24.88 % and the zidovudine by 28.31 %. It also potentially inhibit the p-gp ATPase activity in to the *in vitro* study at the concentration level of 10 µmol, 30 µmol and 100 µmol.

Naringin was significantly increases the bioavailability of atorvastatin calcium by 19.66 %, carvedilol phosphate by 28.01 % and Zidovudine by 60.49 % while it was not increases the bioavailability of sildenafil citrate significantly. Naringin having a potential inhibits the activity of p-gp ATPase activity when studied the *in vitro* assay. It was also shows that the significant inhibition of the *in vitro* activity of CYP 450 1A1 enzyme at the concentration of 30 µmol and 100 µmol and it was not significantly inhibits the activity of cyp 450 3A4 in the *in vitro* studies.

Morin was significantly increase the bioavailability of carvedilol phosphate by 5.07 %, sildenafil citrate by 11.09 % and zidovudine by 16.07 % and not having a significant increase in the bioavailability of atorvastatin calcium. Morin was significantly inhibits the activity of p-gp ATPase enzyme activity in the *in vitro* studies. At the concentration of 10 µmol, 30 µmol and 100 µmol. Marin not having a potential inhibitory activity of the CYP 450 1A1 enzyme and CYP 450 3A4 enzyme into the *in vitro* studies.

From above research work it can be concluded that the selected HAPs increases the bioavailability of the drugs which pose poor bioavailability by undergoing extensive first pass metabolism i.e. drug from the BCS class II-IV (poor stability and this may be due to the inhibition of brush border enzyme of intestinal wall i.e. CYP 450 and p-gp ATPase enzyme.

From this research we conclude that HAPs may exhibit high benefit to risk ratio to use as permission enhancers with modern drug which will help to reduce the dose of the drug and risk of few side effects associated with GI track and cost of the therapy may get reduced. This research may be extended for the exploration of clinical trials with modern drug which goes extensive first pass metabolism in human to establish the use of HAPs in to the formulation.

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9. ANNEXURES

Annexure – I

Internal Quality Assurance Cell
SAURASHTRA UNIVERSITY, RAJKOT

Phone : 0281 - 2576347
0281 - 2586417
Fax : 0281 - 2577633

Ref. No. : IQAC/
Date : 11 /2 /2008

Dr. Kamlesh Joshipura
Chairperson

Avadhesh Kumar Singh
Co-ordinator

To,
Dr. Ashwin Dudharejiya,
Department of
Saurashtra University,
Rajkot.

Sub: Award of the Seed Money Project-07

Dear Colleague,

On behalf of the IQAC team, I am glad to confratulate you for your selection for the Seed Money Project-2007. You are to complete your project by March, 2009. You have been sanctioned Rs. 50,000/-. The title of your project and the amount sanctioned are mentioned head-wise below. You are expected to spend money strictly as per the heads mentioned. You are also enclosed the cheque no. 104619 worth Rs. 50,000/-(Rs. Fifty Thousand only). Please send your acceptance to IQAC Office, Academic Centre Building, Saurashtra University Campus through your head of the Department by 1st March, 2008.

1.	Books & Journals	10,000/-
2.	Travel	10,000/-
3.	Chemicals	10,000/-
4.	Equipments (Testing & Analysis	10,000/-
5.	Contingency	5,000/-
6.	Any Other (Stationary + Printing)	5,000/-
	Total	50,000/-

With thanks,

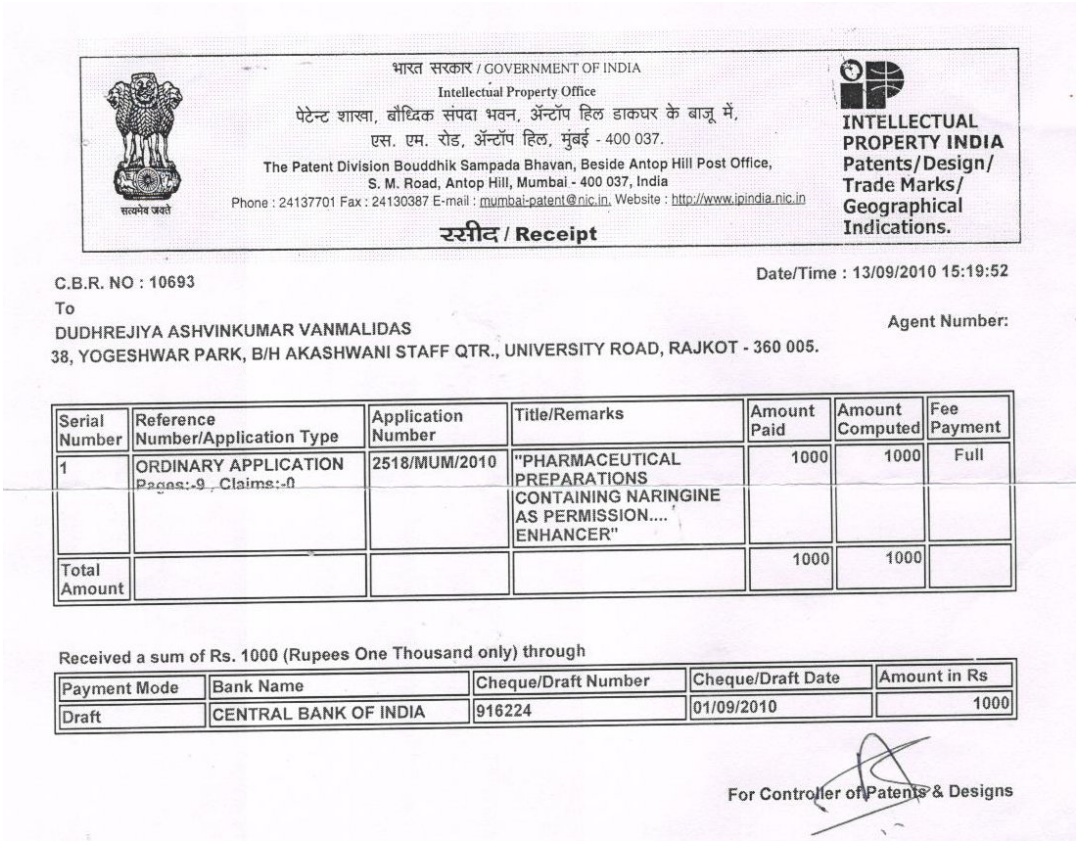
Sincerely yours,

Kamal Mehta
Prof. Kamal Mehta
Coordinator,
Internal Quality Assurance Cell,
Saurashtra University, Rajkot.

8

C/o. : DEPARTMENT OF ENGLISH & COMPARATIVE LITERARY STUDIES

Annexure - II



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Intellectual Property Office
पेटेन्ट शाखा, बौद्धिक संपदा भवन, अॅन्टॉप हिल डाकघर के बाजू में,
एस. एम. रोड, अॅन्टॉप हिल, मुंबई - 400 037.
The Patent Division Bouddhik Sampada Bhavan, Beside Antop Hill Post Office,
S. M. Road, Antop Hill, Mumbai - 400 037, India
Phone : 24137701 Fax : 24130387 E-mail : mumbai-patent@nic.in, Website : http://www.ipindia.nic.in

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INTELLECTUAL PROPERTY INDIA
Patents/Design/ Trade Marks/ Geographical Indications.

रसीद / Receipt

C.B.R. NO : 10693

Date/Time : 13/09/2010 15:19:52

To

DUDHREJIYA ASHVINKUMAR VANMALIDAS
38, YOGESHWAR PARK, B/H AKASHWANI STAFF QTR., UNIVERSITY ROAD, RAJKOT - 360 005.

Agent Number:

Serial Number	Reference Number/Application Type	Application Number	Title/Remarks	Amount Paid	Amount Computed	Fee Payment
1	ORDINARY APPLICATION Pages:-9 Claims:-0	2518/MUM/2010	"PHARMACEUTICAL PREPARATIONS CONTAINING NARINGINE AS PERMISSION.... ENHANCER"	1000	1000	Full
Total Amount				1000	1000	

Received a sum of Rs. 1000 (Rupees One Thousand only) through

Payment Mode	Bank Name	Cheque/Draft Number	Cheque/Draft Date	Amount in Rs
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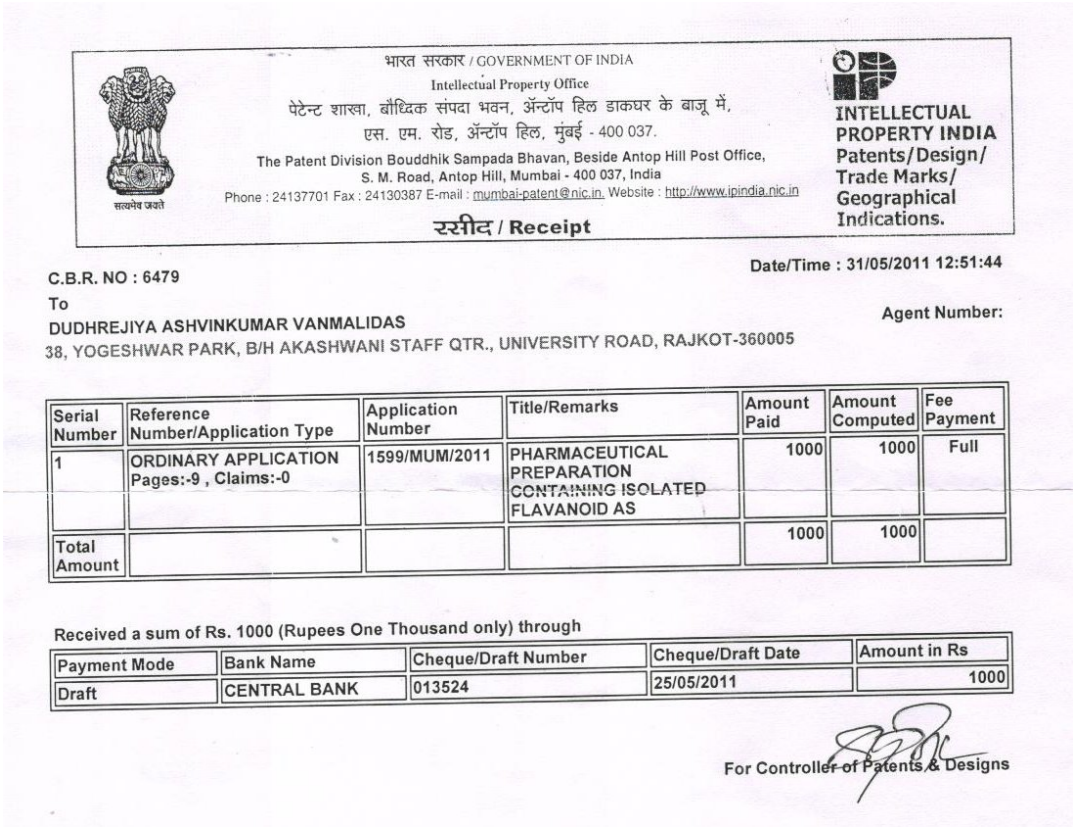
For Controller of Patents & Designs

Annexure - III



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एस. एम. रोड, ॲन्टॉप हिल, मुंबई - 400 037.
The Patent Division Bouddhik Sampada Bhavan, Beside Antop Hill Post Office,
S. M. Road, Antop Hill, Mumbai - 400 037, India
Phone : 24137701 Fax : 24130387 E-mail : mumbai-patent@nic.in Website : http://www.ipindia.nic.in

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C.B.R. NO : 6479

Date/Time : 31/05/2011 12:51:44

To

DUDHREJIYA ASHVINKUMAR VANMALIDAS

Agent Number:

38, YOGESHWAR PARK, B/H AKASHWANI STAFF QTR., UNIVERSITY ROAD, RAJKOT-360005

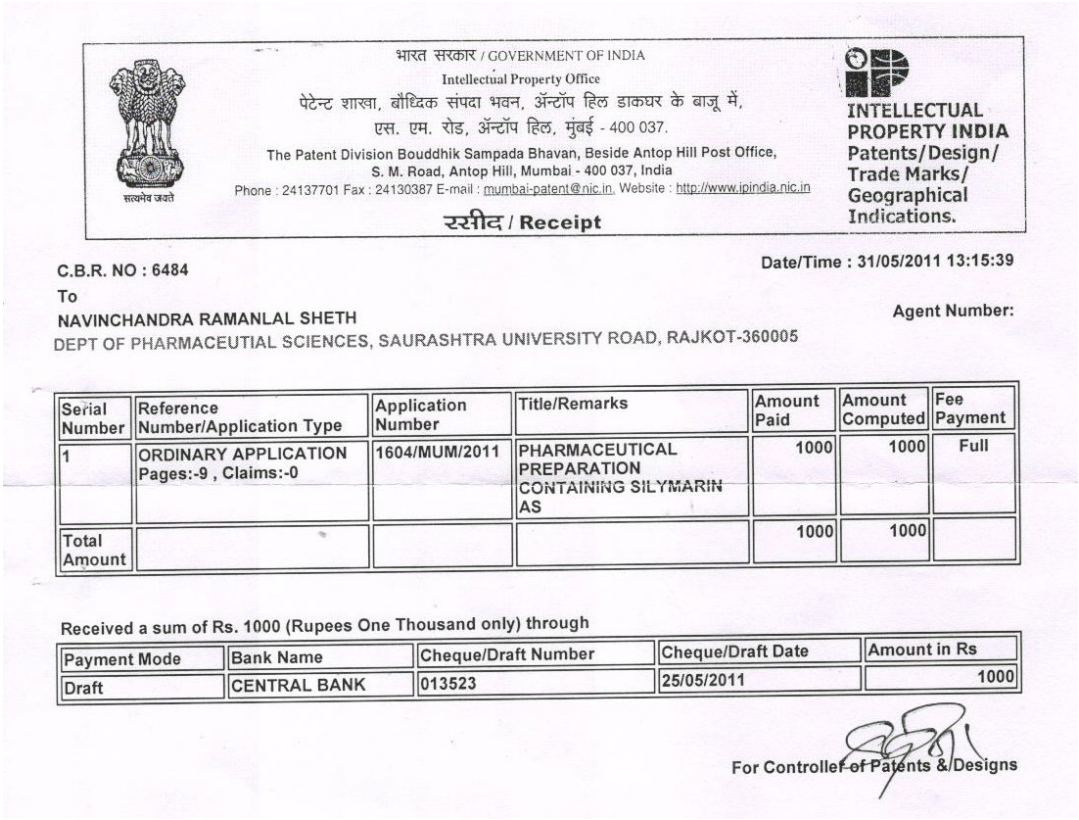
Serial Number	Reference Number/Application Type	Application Number	Title/Remarks	Amount Paid	Amount Computed	Fee Payment
1	ORDINARY APPLICATION Pages:-9 , Claims:-0	1599/MUM/2011	PHARMACEUTICAL PREPARATION CONTAINING ISOLATED FLAVANOID AS	1000	1000	Full
Total Amount				1000	1000	

Received a sum of Rs. 1000 (Rupees One Thousand only) through

Payment Mode	Bank Name	Cheque/Draft Number	Cheque/Draft Date	Amount in Rs
Draft	CENTRAL BANK	013524	25/05/2011	1000

For Controller of Patents & Designs

Annexure - IV



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एस. एम. रोड, अॅन्टॉप हिल, मुंबई - 400 037.
The Patent Division Bouddhik Sampada Bhavan, Beside Antop Hill Post Office,
S. M. Road, Antop Hill, Mumbai - 400 037, India
Phone : 24137701 Fax : 24130387 E-mail : mumbai-patent@nic.in, Website : http://www.ipindia.nic.in

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C.B.R. NO : 6484

Date/Time : 31/05/2011 13:15:39

To

NAVINCHANDRA RAMANLAL SHETH

Agent Number:

DEPT OF PHARMACEUTIAL SCIENCES, SAURASHTRA UNIVERSITY ROAD, RAJKOT-360005

Serial Number	Reference Number/Application Type	Application Number	Title/Remarks	Amount Paid	Amount Computed	Fee Payment
1	ORDINARY APPLICATION Pages:-9 , Claims:-0	1604/MUM/2011	PHARMACEUTICAL PREPARATION CONTAINING SILYMARIN AS	1000	1000	Full
Total Amount				1000	1000	

Received a sum of Rs. 1000 (Rupees One Thousand only) through

Payment Mode	Bank Name	Cheque/Draft Number	Cheque/Draft Date	Amount in Rs
Draft	CENTRAL BANK	013523	25/05/2011	1000

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