CHAPTER 5

DISCUSSION

Plant extracts continue to play a major role in new drug discovery research as evidenced by the large number of promising new agents that are isolated, characterized, laboratory synthesized, efficacy tested and now in clinical developmental pipeline or already marketed as drug. Galantamine , Nitisinone, Apomorphine , Varenicline and Bevirimat are the few well know examples (Salim *et al*., 2008). Plant-derived natural products have been an important source of several clinically useful anti-cancer and anti-inflammatory agents. Shikonin and its derivatives, a red naphthoquinone pigment accumulating in the roots of Boraginaceae plants, such as *Arnebia hispidissima*, *Lithospermum erythrorhizon* and *Onosma paniculatum* have shown immense medicinal values and multiple pharmacological actions (Andujar *et al*., 2013). Shikonin, a biologically active compound shows wide spectrum of therapeutic effects in the broad area of oncology, inflammation, HIV infection, metabolic disorder and wound healing properties. Elaborate data accumulating from many literatures over the recent years has established the fact that shikonin and its closely active derivatives interferes through numerous signaling mechanisms to exert its effect in various human diseases.

In oncology, action of shikonin primarily is pro-apoptotic and anti-angiogenic that are activated either by induction of protein kinase, ROS and caspase pathways or by the inhibition of endothelial cell proliferation, migration and differentiation. Due to the huge unmet need for an efficacious drug in the area of oncology, we consider that shikonin has a great scope in the cancer chemotherapy research. This is further proven by the number of scientific articles published over the decades showing its *in-vitro* and *in-vivo* effects in cell based and preclinical studies. In addition to oncology, shikonin has also been found to be efficacious in inflammation where its primary targets are chemokine receptor (CCR1) and kinases. Shikonin subsequently down regulated the TNFα and NF-kB activation, leading to reversal of the autoimmune disease phenotype. A safe and efficacious small molecule anti-inflammatory agent against human RA and respiratory diseases is hugely sought after and shikonin may well play a vital role in

this area. Furthermore in metabolic disorder such as obesity and insulin resistance, shikonin interferes with the mechanism of adipogenesis and glucose sensitivity, indicating that its therapeutic implication cannot be overlooked in diabetes. Remarkably shikonin has also been shown to inhibit HIV replication and its entry in the immune cells via specific receptor. As evident from numerous scientific works published, shikonin has also been shown to exhibit disease reversing properties in the area of CNS disorders, cardiovascular disorders and wound healing.

During the last decade, extensive investigations have revealed distinctive biological and pharmacological effects of shikonin in diverse therapeutic areas. However, the precise mode of action of this extract has remained ambiguous. Hence, an in-depth study of the mechanism of signaling pathway modulation with shikonin holds a great potential in the basic biological field. Shikonin and its closely active ingredients have been widely accepted molecules in the academic research and are on the brink to be characterized and developed by global pharmaceuticals and biotech companies as a 'magic' drug towards diverse therapeutic areas. Scientific community worldwide is sincerely hoping for this molecule to move beyond bench and become a safe, efficacious drug for the common man in future.

In small molecule drug discovery paradigm, target-identification is a crucial step. Development of a new drug is a complex and very lengthy process involving 10-12 years of intense research starting from target identification to its launch in the market. The whole process of drug discovery includes several steps such as target identification and validation, synthesis, preclinical screen, clinical study (phase 1-3), approval, marketing and post marketing examination. In modern drug discovery research, selecting a specific disease target followed by the identification of a potent, druggable and safe compound is of prime interest. Thus a novel compound if shows a specific and selective effect on a target with a modulation of the signaling pathway, it becomes a differentiator among the same class of lead molecule.

Prominent key biological targets, which are currently sought aft identification of a new drug candidate are GPCRs, ion channels, proteins and enzymes. The target specific analysis of shikonin on these targets has not been explored completely. Therefore there remains an opportunity to identify novel disease relevant

targets that were not previously screened towards the effect of shikonin. In this study, our objective was to examine the therapeutic potential of shikonin on disease specific targets that are primarily involved in cancer, inflammation, pain, cardiovascular disorders. Efficacy of shikonin was evaluated in many established and validated targets that are of current interest in modern day drug discovery research in each therapeutic area.

5.1 IDENTIFICATION OF SIGNALING MECHANISM OF INVOLVED IN PAIN PATHWAYS

Although shikonin has shown multifaceted pharmaceutical properties in many therapeutic areas including oncology, diabetes, inflammation, wound healing and many more, its anti-nociceptive efficacy remained unexplored. In this study, shikonin was analyzed in several *in vitro* and *in vivo* pain assay read-outs and our results demonstrated that it has substantial anti-nociception property. Shikonin inhibited pain in *in vivo* rodent models of pinch pain, inflammatory hyperalgesia and induced pain possibly via sodium channel modulation (Table 4.1).

There are strong evidences suggesting a predominant association of sodium channels in pain. Voltage-gated sodium channels (NaVs) are well validated targets for treating pain based both on human genetics and clinical experience (Cohen, 2011) and developing selective and potent sodium channel blocker might be favourable to patients. Sodium channels are responsible for generation of action potential in excitable cells. Newer class of sodium channel blockers includes drugs like benzazepinone series compounds, which are selective towards NaV1.7 channels (Hoyt *et al*., 2013). A-803467 is reported to be a selective NaV1.8 blocker (Jarvis *et al*., 2007) and CDA54 is a dual NaV1.7/NaV1.8 blocker (Brochu *et al*., 2006). In this study shikonin was found to bind the sodium channels, block the voltage gated dependent channel opening and further inhibits generation and propagation of action potential that eventually modulates the transduction of pain signal.

For analyzing anti-nociception efficacy of shikonin: multiple receptors, ion channels and enzymes that are implicated in pain pathophysiology were evaluated in *in vitro* pharmacological screens. Significant inhibitory effect of shikonin was found

specifically on pan sodium channels and NaV1.7 channels along with moderate inhibition on B1 receptor.

To study the effect of shikonin on sodium channels, a FLIPR based membrane potential *in vitro* assay was performed. FLIPR membrane potential assay has been a choice of *in vitro* assay for high throughput screening of ion channels including sodium channels in recent years (Trivedi *et al*., 2008). In this assay, shikonin blocked 100uM Veratridine (channel opener) in a dose dependent manner with IC_{50} of 7.6µM on N1E115 cells (Figure 4. 4). Rat neuroblastoma cell line, N1E115, natively expresses pool of sodium channels and provides an excellent platform for *in vitro* screening of sodium channel modulation (Kondratiev *et al*., 2003; Errington *et al*., 2008). Tetracaine is a gold standard sodium channel blocker (Clare, 2010) and showed IC_{50} of 4.5uM on N1E115 cell line. In our study, we found strikingly comparable potency of shikonin with Tetracaine that gave the opportunity to evaluate shikonin in other assays. We also tested a local anaesthetic drug Lidocaine in the same assay that showed IC_{50} value of 671µM (Figure 4. 4). Interestingly, IC_{50} of Lidocaine is ~ 100 fold higher than shikonin. To examine a specific NaV channel effect of shikonin, we then tested it in NaV1.7 channel inhibition assay. Inhibitory effect of shikonin was tested on NaV1.7 over expressed cells (NaV1.7/HEK-293) along with standard inhibitor Tetracaine in FLIPR membrane potential assay. Shikonin distinctly blocked the binding of 75µM Veratridine (EC_{80} conc.) to NaV1.7 channels with an IC_{50} of 6.4µM, comparable to Tetracaine (IC_{50} : 4.2µM) (Figure 4.5). It ceased the Na+ influx, further activation of the channels and finally concomitant transient change in membrane potential.

These *in vitro* results which are novel findings in shikonin research, its vote as a sodium channel blocker encouraged us to evaluate shikonin in preclinical models of pain for analyzing its translation from *in vitro* potency to *in vivo* efficacy. Hence, three acute pain models; pinch pain, mechanical hyperalgesia and formalin induced pain were used in our study to determine the analgesic property of shikonin. The pinch pain model is an acute and quick model of nociception and very well accepted to analyze the local analgesic property of drug like molecules (Kau *et al*., 2006). This model is routinely used in our facility and optimized using the standard drug Lidocaine, a known sodium channel blocker. In order to do a parallel comparison of nociceptive efficacy,

shikonin was evaluated in this model along with Lidocaine. When rat paw was pinched to elicit pain, in 0.02% w/v shikonin treated rats, we found a marked and significant anti-nociception at 30 min, which came down at 2 h post dosing. Lidocaine showed similar trend of efficacy but at very high dose (2% w/v), almost 100 fold higher than that of shikonin. At higher doses (0.05% and 0.08% w/v), Shikonin showed a significant and long lasting anti-nociception for more than 48 h (Figure 4.7). The present study showed that shikonin possesses an anti-nociception efficacy approximately 100 times more potent than that of Lidocaine. This *in vivo* data is comparable to the *in vitro* potency of shikonin and Lidocaine (IC_{50}: 7.6µM VS 671µM) in membrane potential assay.

Based on our promising *in vitro* results of shikonin as pan sodium channel blocker, we hypothesized that this anti-nociceptive effect of shikonin could be mediated via sodium channel modulation. Our results showed a pronounced anti-nociceptive effect of shikonin in pain in *in vivo* model, although the precise mechanism responsible for long lasting effect of shikonin at higher doses in pinch pain model remains to be elucidated.

These exciting results inspired us to evaluate the analgesic efficacy of shikonin through systemic delivery. CFA induced mechanical hyperalgesia rat model, which is a well-established and preferred model for studying drug efficacy in inflammatory pain (Larson *et al*., 1986) was chosen for analyzing the effect of shikonin subsequently. CFA produces hyperalgesia when injected directly in foot pad. We studied the effect of shikonin in rat CFA model with both intraperitoneal (i.p.) and oral routes of administration and in both the cases we found very encouraging results with marked analgesia. Due to limited data on bioavailability, effect of shikonin was first evaluated by i.p. route of administration. In i.p. study, shikonin notably attenuated the CFA induced hyperalgesia in dose dependent manner at 10 mg kg^{-1} and 3 mg kg^{-1} and exhibited significant reversal (67% and 35% respectively) at 0.5 h. The effect was found to be quite comparable to that of a standard analgesic, Diclofenac sodium salt, which showed 64% reversal at 10 mg kg^{-1}. The analgesic effect at 10 mg kg^{-1} dose lasted for 1 h in both shikonin and Diclofenac treated animals with significant reversal of 45% and 37% respectively (Figure 4.8). Based on the remarkable results in study

with i.p. dosing, we assessed efficacy of shikonin through oral route of administration.

When administered orally, shikonin showed a distinct analgesic effect with 39% reversal at 30 mg kg^{-1} dose at 1 h post dosing compared to 55% reversal with Diclofenac at same dose (Figure 4.9). Efficacy of shikonin through oral administration was found to be little less than Diclofenac sodium salt, might be due to poor bioavailability of shikonin.

Our data on the effect of shikonin in both the rodent pain models implies that the anti-nociception phenotype exerted by this compound mediated possibly via sodium channels modulation, however some effect on B1 receptor could not be completely ruled out. B1 is also a target for inflammatory pain and has been shown to be involved in CFA induced hyperalgesia signaling (Petho and Reeh, 2012). There are few B1 receptor antagonists reported in literature with very high *in vitro* potency (~1nM) that have shown significant and specific effect in rat CFA pain model (Pal *et al*., 2010). In *in vitro* assay, shikonin showed IC_{50} of 1.84nM against B1 receptor. As shikonin displayed moderate potency on B1 receptor in cell-based pharmacology assay, we presume that exhibited a nalgesic effect in CFA model might not be predominantly via B1 receptor but more due to sodium channel inhibition. Interestingly, shikonin seems as a promising anti-nociceptive agent gains confidence from the fact that it is 100 times more efficacious than Lidocaine through local application. Moreover it showed similar efficacy with Diclofenac sodium salt through systemic administration. Both Lidocaine and Diclofenac sodium salt are known drugs for current pain therapy and hence leaves shikonin as a very compelling molecule to be used in future pain therapeutics.

The present study also showed efficacy of shikonin in animal model of inflammatory pain. In order to differentiate the analgesic mechanism of shikonin from NSAIDs, its effect was evaluated in FIP model. FIP is a robust and well established animal model for pain and consists of two distinct phases of pain. First phase persisting for 10 min post formalin injection is acute nociceptive pain mediated via C fibres involving CNS neurons whereas second phase involves both peripheral and central components (Yaksh *et al*., 2001). In current study, we compared effect of shikonin with that of Diclofenac sodium, a known NSAID, marketed analgesic in first phase of FIP. In general, NSAIDs show little or no effect in first phase of FIP model where only

centrally acting drugs are found effective (Hunskaar and Hole, 1987). As reported in our investigation, Diclofenac sodium failed to show any effect in first phase of FIP whereas shikonin showed very pronounced (~ 71%) reduction of flinch count. The effect of shikonin was found even higher than that of Gabapentin, a centrally acting drug which showed ~ 36% reduction in paw flinching (Figure 4.10). Shikonin showing significant effect in first phase of FIP and that too higher than Gabapentin clearly indicates that the analgesic effect of shikonin is through CNS component, possibly mediated via sodium channels but not through cyclooxygenase pathway.

5.2 IDENTIFICATION OF SIGNALING MECHANISM OF INVOLVED IN DIABETES PATHWAY

The increasing prevalence of type 2 diabetes mellitus (T2D) leading to tremendous interest in identifying compounds that can improve glucose tolerance. T2D is a heterogeneous disorder characterized by chronic hyperglycemia due to impaired insulin secretion and insulin sensitivity. Controlling blood sugar (glucose) level is the major goal of diabetes treatment, in order to prevent complications of the disease.

Anti-obesity and anti-diabetic effect of shikonin has been elucidated in few reports. Research studies including clinical trials show evidences that shikonin has a potential role in the treatment of diabetes through its role in glucose sensitivity and inhibition of adipocyte signaling. Shikonin was found to inhibit fat droplet formation and triglyceride accumulation in 3T3-L1 adipocytes by down regulating PPARγ and C/EBPa (Lee *et al*., 2010). Other possible mechanisms of shikonin intervention are activation of protein kinase B (PKB) (Nigorikawa *et al*., 2006) and stimulation of the insulin release from pancreatic beta cells by inhibiting KATP ion channel of beta cells (Park *et al*., 2010). Shikonin has also been shown to inhibit adipogenesis by novel modulation of the WNT/β-catenin pathway (Lee *et al*., 2011).

To analyze the therapeutic efficacy in shikonin in diabetes and metabolic disorders, 11 targets including GPCRs and enzymes of diverse signaling mechanism were investigated *in vitro* assays (Table 4.2). As per our knowledge and literature precedence, this is the first study which shows effect of shikonin on diabetes and obesity targets such as Orexin1, Orexin2, Neuropeptide Y1 (NPY1), Neuropeptide Y2

(NPY2), CB1, GLP-1 and melanocortin4. Few enzymes, for example alpha-glucosidase, DPPIV, FAAH and PTP1b, were also assayed in this study (Table 4.2).

In this study, shikonin showed feeble effect on CB1, NPY1, NPY2 and MC4 receptors in their respective assay with IC_{50} > 10µM. Shikonin was tested on GLP-1 receptor for its agonistic activity as GLP-1 activation leads to insulin secretion. There was no specific effect of shikonin was observed on GLP-1 receptor. When tested in enzyme inhibition assays, shikonin did not show notable inhibitory effect on alpha-glucosidase, DPPIV, FAAH and PTP1b enzymes. However, it showed substantial inhibition of orexin-1 and orexin-2 receptor with IC_{50} of 7.5 and 3.2µM (Figure 4.11).

Orexins (hypocretins) are novel Neuropeptides that appear to play a role in the management of energy balances. Orexin receptors are involved in appetite stimulation and regulation of glucose metabolism and are well known targets for diabetes. As shikonin showed substantial effect on Orexin2 receptor, there remains a possibility to develop shikonin analogues in terms of better potency to combat with diabetes.

5.3 IDENTIFICATION OF SIGNALING MECHANISM OF S INVOLVED IN INFLAMMATION PATHWAY

Inhibitory role of shikonin on inflammatory disorders is not yet well studied. Few scientific groups have shown broad involvement of shikonin in cellular signaling pathways like inhibition of CCR1 (Chen *et al*., 2001), TNFα (Chiu and Yang, 2007), MMP-1 (Kim *et al*., 2010), COX2 (Prasad *et al*., 2015), NOS (Cheng *et al*., 2008), Syk kinase and histamine release (Takano-Ohmuro *et al*., 2008). However, specific mechanism of action is not well defined and the limited knowledge in this field provides scope to screen shikonin against many novel anti-inflammatory targets. In this study, we have examined some of these new and relevant targets such as bradykinin1, CXCR2, Cannabinoid 2, Prostaglandins: PGE2 and PGE4, Adenosine 2b, Histamine 1, CRTh2, Autotaxin, Pantetheinase 1, TACE and NRF2 for analyzing the shikonin efficacy in inflammation. Our results demonstrated the promising effect of shikonin in inflammation through Bradykinin 1, CXCR2 and CRTh2 receptors and NRF2 protein (Table 4.3).

There are compelling evidences linking Bradykinin 1 receptor (B1) with the pathophysiological processes that accompany tissue damage and inflammation (Dray and Perkins, 1993). Bradykinin 1 is a GPCR, involve in many chronic inflammatory responses including neuroinflammation. Bradykinin antagonists might be beneficial in the treatment of inflammation, asthma and endotoxic shock. In this investigation, shikonin displayed very strong inhibitory effect on Bradykinin 1 receptor and showed IC_{50} of 484nM on B1 over expressed cells in intracellular calcium mobilization assay (Figure 4.1).

CXCR2 is a chemokine GPCR coupled to Gi/o, binds to proinflammatory chemokine (hIL-8) with high affinity. This signaling event induces leukocyte recruitment and their activation at the site of inflammation. Chemokine mediated cell migration is an important event in the process of inflammation and CXCR2 is best known for its ability to control leukocyte migration. CXCR2 receptor has critical role in various cellular processes, such as angiogenesis, proliferation and invasion (Role of CXCR2 has also been well established in psoriasis, atherosclerosis (Murdoch and Finn, 2000), asthma, arthritis, chronic obstructive pulmonary disease (COPD) (Dwyer and Yu, 2014), pulmonary fibrosis (Russo *et al*., 2009) and pancreatitis (Steele *et al*., 2015). Few CXCR2 receptor antagonists have also been reported in clinical trial (Donnelly and Barnes, 2011). Our data demonstrated a notable effect of shikonin on CXCR2 receptor, both on over expressed cells as well as on human neutrophils. Neutrophils are the primary cells which express CXCR2 receptor and upon binding to IL-8, migrate from blood to the site of injury during inflammation. In current study, shikonin displayed strong inhibitory effect on recombinant CXCR2 cells and neutrophils in intracellular calcium mobilization assay with IC_{50} of 1.3µM and 1.4µM (Figure 4.13b, 4.13c) respectively. Shikonin was also screened in IL-8 driven neutrophil migration assay, where it showed specific effect on CXCR2 receptor and exhibited noticeable effect with IC_{50} of ~1µM (Figure 4.14).

CRTh2 a GPCR is expressed on Th2 cells and a current drug target for inflammation associated diseases. Role of CRTh2 is validated in many inflammatory diseases like asthma, allergic rhinitis (Birkinshaw *et al*., 2006) and colitis (Iwanaga *et*

al., 2014). The re are many CRTh2 antagonists in clinical development for asthma, such as AMG-853, OC000459 and MK-2746 (Barnes *et al*., 2012). In this study shikonin displayed notable efficacy on CRTh2 over expressed cells with IC_{50} of ~ 1µM (Fig ure 4.12b) in a functional assay. When tested in radioligand binding assay using cell membrane, shikonin could not displace radiolabelled hPGD2. T here might be few possible reasons for the difference in potency between functional and binding assay; a) shikonin might be acting as allosteric inhibitor in functional assay and affecting PGD2 signaling from a distant site, but in binding assay, it is un able to displace the bound radioligand. b) There could be a possibility of partial displacement of PGD2 upon binding of shikonin, which is more visible in function assay.

NRF2 is a transcription factor and has emerged as a regulator of cellular homeostasis. It protects the cells from oxidative stress through activation of a pathway. NRF2 is a high value therapeutic target for chronic obstructive pulmonary disease (COPD) (Boutten *et al*., 2011), oncology (Leinonen *et al*., 2014) and kidney diseases (Leal *et al*., 2015). Shikonin was found to have a marked effect on NRF2 in ARE bla gene blazer assay with EC_{50} of 7µM (Fig ure 4.15) in current study. Moreover this effect was better than the standard compound tBHQ, which showed EC_{50} of ~8µM in same assay (data not shown). This data corroborated with the work of a few scientific groups who evaluated the effect of shikonin on NRF2 protein and found similar results (Zhang *et al*., 2012).

5.4 IDENTIFICATION OF SIGNALING MECHANISM OF S INVOLVED IN CARDIOVASCULAR AREA

Cardiovascular disease (CVD) broadly includes heart or blood vessels such as coronary artery diseases (angina and myocardial infarction), stroke, hypertensive heart disease, cardiomyopathy, heart arrhythmia, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease and venous thrombosis. Several receptors, enzymes, proteins and ion channels are shown to be involved in the pathogenesis of cardiovascular diseases and are targeted for identification of the novel drugs.

Shikonin is a well known pleotropic medicinal agent. It also exhibited its therapeutic potential in cardiovascular diseases. Though there is very little data

available in this area; Shikonin showed dose dependent inhibition of nitric oxide synthases (NOS) in RAW 264.7 (macrophage) and neuroglial cells. Shikonin has not been tested for its effect in most of the disease relevant targets.

In our study, to understand the therapeutic potential of shikonin in cardiovascular disorders, few relevant targets such as angiotensin1 (AT1), Bradykinin 1, hERG, NaV1.5, P2X and HDAC were screened with and without shikonin (Table 4.4). AT1, Bradykinin 1, hERG, NaV1.5 and P2X receptors were over expressed and analyzed in *in vitro* assays. Shikonin did not displayed compelling effect on targets such as NaV1.5, P2X and HDAC. It showed moderate inhibition of hERG channels with IC_{50} of ~10µM. Shikonin displayed marked inhibitory effect on Bradykinin 1 receptor with IC_{50} of 484nM (Figure 4.1). It also exhibited strong promising inhibitory effect on AT1 receptor with IC_{50} of 2.1µM (Figure 4.16). Bradykinin 1 and angiotensin 1 are very crucial receptors, involved in various cellular signaling of cardiovascular system. Both receptor are the part of renin angiotensin trunk and are current targets for drug discovery and shikonin displayed notable inhibitory effect on these receptors. All these results lead to a new insight on shikonin as a regulator of cardiovascular signaling. NaV1.5 and hERG channels are the cardiac channels which maintain normal cardiac rhythm therefore these channels should not be affected by drugs. Shikonin showed very feeble effect on these targets.

5.5 IDENTIFICATION OF SIGNALING MECHANISM OF INVOLVED IN CANCER

Shikonin has been evaluated largely in anticancerous signaling pathways but its target specific effect is not well known . Inhibitory role of shikonin on GPCRs, ion channels, relevant enzyme targets and proteins has not been well established yet in the area of oncology.

To establish the target specific effect, we have chosen number of recent and disease relevant targets and evaluated shikonin for its activity as inhibitor or activator especially on GPCRs, enzymes and protein. Our initial effort was to determine the cytotoxic potency of shikonin on multiple cancerous cells from diverse origin followed by limited panel screening on few recent oncology targets.

Shikonin was tested on several cancerous cell lines of different origin such as liver cancer (HEPG2) breast cancer (MCF-7), cervical carcinoma (HeLa), prostate cancer (PC3), colon carcinoma (COLO205) and myelogenous leuke mia (K562) for its cytotoxic effects at varying incubation time from 30 min to 72 h. It displayed specific and differential cell killing effect at varying doses and incubations on these mammalian cells of diversified origin (Figure 4.17 a-f).

In our study, myelogenous leukemia cells were found to be most sensitive towards shikonin at longer incubation and exhibited 200nM and 12nM LD50 at 48 h and 72 h respectively. Myelogenous leukemia is a malignant cancer of white blood cells and is a huge burden for society with high mortality. So far, many drugs have been approved and tested for the treatment of myelogenous leukemia. These drugs are efficacious, but, have safety concerns and also induced drug resistance. Shikonin might be a new and interesting choice of lead compound in future considering its potent and lethal effect on these cells. Colon carcinoma cells also showed high sensitivity towards shikonin even at shorter incubation (~7µM LD50 at 30 min). Shikonin showed huge death of COLO205 cells at 48 h and 72 h with LD50 of 170nM and 40nM respectively. Liver carcinoma and prostate cancer cells showed moderate sensitivity for shikonin at early incubation but demonstrated substantial cell killing effect at 48 h and 72 h: HEPG2 cells showed LD50 of 250nM and 210 nM where as PC3 cells exhibited LD50 of 320nM and 180nM at 48 h and 72 h respectively. Breast cancer and cervical carcinoma cells displayed lesser sensitivity for shikonin and showed ~ 1µM LD5 h. Similar study was published in 2006, where scientists found LD50 of shikonin ~ 3.9µM on MCF-7 cells at 72 h incubation (Hou *et al*., 2006).

To the best of our knowledge, this is the first comprehensive report capturing the cytotoxic effect of shikonin on several cancerous cell lines at six di incubations (30 min to 72 h) in a dose dependent manner. Our results confirm the substantial and significant cell killing ability of shikonin on broad range of cancerous cells. These results imply that shikonin could be developed as an anticancerous compound in future.

Numerous studies reported compelling effect of shikonin in cancer, which works via diverse signaling mechanism: inhibition of pyruvate kinase-M215, NF-κB16,

matrix metalloproteinase-9 (Jang *et al*., 2014), p-PI3K and p-Akt (Zhang *et al*., 2015), STAT3, IGF-IR phosphorylation (Kimura *et al*., 2005), exosome release (Wei *et al*., 2016), ERK1/2 signaling pathway (Wang *et al*., 2013) and modulation of the androgen receptor (Jang *et al*., 2014). In current scenario, considering the complex and intricate cellular signaling, a single drug might not be efficacious against cancer and it could also induce drug resistance. Taking this in account, combinatorial chemotherapeutic treatment for cancer could be a very effective treatment option (Kulhari *et al*., 2013).

In spite of significant research in this area, there is scope to identify novel oncology targets that were not previously screened towards the effect of shikonin. Current study has evaluated the potency of shikonin on a panel of recent oncology targets such as HDAC, MERTK, FLT3, TrkA, BRD4, Autotaxin, Adenosine 2b, CXCR2 and NRF2. All these enzymes, receptors and proteins are the current targets for oncology pipeline of major pharmaceutical and biotech companies. Few targets like HDAC, BRD4 and NRF2 are epigenetic targets that are currently drawing attention of scientific community. Epigenetic processes include histone modifications, DNA methylation, and chromatin remodelling and are affected by various environmental and genetic factors, contributing to disease progression. These epigenetic proteins are being targeted aggressively for novel drug development. MERTK, a member of the TAM receptor tyrosine kinases, has complex and diverse roles in cell biology. However, MERTK is primarily over expressed in a wide range of cancers, such as leukemia, lung cancer, glioblastoma, melanoma, prostate cancer, breast cancer, colon cancer, gastric cancer, pituitary adenomas, and rhabdomyosarcomas. It is suggested that MERTK inhibition by genetic or pharmacologic means can reverse pro-oncogenic phenotypes (Cummings *et al*., 2013). FMS-like tyrosine kinase-3 (FLT3), a receptor tyrosine kinase, is crucial for the hematopoietic and immune systems development. Mutation in the FLT3 gene are now considered as the most common molecular abnormality in acute myeloid leukemia and various small-molecule FLT3 inhibitors are currently in development (Levis and Small, 2003). TrkA over expression enhances growth and metastasis of breast cancer cells and there are few TrKA inhibitors are in clinical trial (Lagadec *et al*., 2009). Controlling cancer through the autotaxin–lysophosphatidic acid receptor axis is also a new strategy to combat cancer. A2b receptor is also an oncology target and found to be up regulated in various tumour cells. CXCR2 is a chemokine

GPCR and a target for pancreatic cancer treatment. Scientists are actively searching for CXCR2 inhibitor for cancer treatment. NRF2 activation is important in cancer chemoprevention and therefore NRF2 is being targeted for c of NRF2 agonist.

In this study, significant effect of shikonin was found on CXCR2 receptor and NRF2 protein. Shikonin did not show significant inhibitory effect on other targets (results have been discussed in inflammation target section). Shikonin displayed strong effect with IC_{50} of 1.3µM and 1.4µM on CXCR2 over expressed cells and on human neutrophils respectively. Chemotaxis or invasion is a key step in pathology of cancer. Chemotaxis of tumor cells and stromal cells in the surrounding microenvironment is an essential component of tumor dissemination during progression and metastasis (Roussos *et al*., 2011). Shikonin displayed very strong effect in neutrophil chemotaxis assay and exhibited marked inhibition of IL-8 with IC_{50} of 1µM. Shikonin also demonstrated promising stimulatory effect on NRF2 protein and showed ~ 7µM EC_{50}.

Shikonin has been reported to exert its effect by interfering multiple cellular signaling pathways rather than a single target, which has also been observed in the current study. Since cancer is a multifactorial disease, this feature of shikonin may be beneficial for the fight against cancer.