



## ANTI CANCEROUS EFFECT OF NANO-ENGINEERED ENCAPSULATING CHEMOTHERAPEUTIC DRUG ON HUMAN COLORECTAL HT-29 CELL LINE.

\*SOURABH TIWARI<sup>1,2</sup>, RAVI UPADHYAYA<sup>2</sup>, RUCHI SHROTI<sup>3</sup>, SHARAD TRIVEDI UPADHYAYA<sup>3</sup>

1. Department of Research, Bhopal Memorial Hospital & Research centre, Bhopal India.
2. Department of Biotechnology, Govt. P.G. College, Pipariya, India.
3. Department of Biotechnology, Govt. P.G. College, Hoshangabad, India.

### Abstract

#### Accepted Date:

13/09/2012

#### Publish Date:

27/10/2012

#### Keywords

Solid lipid Nanoparticle

5-Fluorouracil

Ca-Leuovorin

Irinotecan

#### Corresponding Author

Ms. Sourabh Tiwari

Department of Research,  
Bhopal Memorial Hospital  
& Research centre,  
Bhopal India.

Colorectal cancer is fourth most prevalent cancer worldwide. In recent years, there have been important advances in the management of colorectal cancer. Several obstacles frequently encountered with anticancer compounds, such as toxicity of drugs to normal tissues, poor specificity and stability and a high incidence of drug-resistant tumor cells, are at least partially overcome by delivering them using solid lipid nanoparticles (SLN). Therefore, a need is felt to incorporate chemotherapeutic drugs into better drug delivery carriers such as SLN to minimize systemic side effects and toxicity. Measures of anti-carcinogenic potential of the nano-engineered formulations were investigated using cultured adenocarcinoma cells. Evaluation of anti-carcinogenic potential by Annexin-V-FITC/PI apoptosis assay following 48 h treatment with SLN and native drugs delineated significant differences, establishing better potential efficacy of nano-engineered drugs. Higher internalizing ability, better antitumor efficacy and lesser cytotoxicity of SLNs was attributed to increased accumulation of drugs in cancerous cells. The results of our study implied that these nano-carriers could possibly enhance antitumor effect in vivo with low systemic toxicity for the treatment of malignant cells.

## INTRODUCTION

Colorectal cancer remains one of the most feared gastrointestinal cancer worldwide with an annual incidence of approximately 1 million cases and an annual mortality of more than 500,000<sup>1</sup>. Incidence of colorectal cancer in male: female is 6.7:5.5 per 100,000 population in India<sup>2</sup>. Approximately 25% of patients with colorectal cancer present with overt metastatic disease, and metastatic disease develops in 40 to 50% of newly diagnosed patients. About 72% of cases arise in the colon and about 28% in the rectum. The majority of colon cancer is preceded by benign adenomas that gradually transform into malignant, invasive tumors. This gradual process allows for effective screening for colorectal cancer and a greater rate of potentially curative section<sup>3</sup>. The risk for CRC varies from country to country and even within countries. The risk also varies among individual people based on diet, lifestyle, inflammatory bowel disease and hereditary factors<sup>4</sup>.

Chemotherapy is a systemic therapy, which means it affects the whole body by going

through the bloodstream. Cytotoxic drugs are used as single agents or as combinations of several antitumor agents with different mechanisms of action<sup>5</sup>. A standard chemotherapy regimen widely used in colorectal cancer is composed of 5-fluorouracil (5-FU), irinotecan, and leucovorin (LV)<sup>6-8</sup>. In contrast to the modest benefits accruing from addition of LV to 5-FU, the addition of irinotecan to 5-FU and LV dramatically has improved the survival benefit of chemotherapy, with numerous studies confirming the superiority of this regimen based on objective response, survival, and time to progression, as well as demonstrating different toxicities as the dosage of each individual component is varied<sup>9-11</sup>.

A major hurdle to successful cancer treatment is tumour resistance to chemotherapy. Cancer chemotherapy is limited by intrinsic or acquired multidrug resistance of tumor cells and toxicity to normal cells<sup>12</sup>. Chemotherapeutic agents do not specifically target tumor cells, but rather interfere with cell division or inhibit enzymes involved DNA replication or

metabolism. These drugs therefore also damage the normal dividing cells of rapidly regenerating tissues, such as those of the bone marrow, gut mucosa and hair follicles. Cancer chemotherapy is limited by a lack of specificity, resulting in damage to not only cancer cells but also normal cells. This creates a narrow therapeutic index. Chemotherapy is associated with a high rate of adverse reactions, although in general toxicities were mild and manageable. The principle adverse effects are leucopenia, nausea, vomiting, anaemia, diarrhoea and elevation of hepatic enzymes. However major challenges exist in conventional chemotherapy that relate to toxicity on healthy proliferating cells and multi-drug resistance (MDR) against anticancer agents<sup>13</sup>. The selective increase in tumor tissue uptake of anticancer agents would be of great interest in cancer chemotherapy since anticancer drugs are not specific to cancer cells.

The development of engineered nanoparticles with substantial biomedical significance has portrayed new opportunities and challenges for pharmacology and therapeutics. Nanoparticles and their use in drug delivery

is a far more effective antitumor strategy than conventional chemotherapy, which is typically limited by the toxicity of drugs to normal tissues, short circulation half-life in plasma, limited aqueous solubility, and non-selectivity restricting therapeutic efficacy<sup>14</sup>. Nanoparticulate drug delivery systems are being developed to deliver smaller doses of chemotherapeutic agents in an effective form and control drug distribution within the body<sup>15</sup>. Indeed, anticancer drugs, which usually have large volume of distribution, are toxic to both normal and cancer cells. Therefore, precise drug release into highly specified target involves miniaturizing the delivery systems to become much smaller than their targets. With the use of nanotechnology, targeting drug molecules to the site of action is becoming a reality resulting in a personalized medicine, which reduces the effect of the drug on other sites while maximizing the therapeutic effect. This goal is mainly achieved by the small size of these particles, which can penetrate across different barriers through small capillaries into individual cells. In addition, nanoparticles can be prepared to entrap, encapsulate or bind molecules improving the solubility, stability and absorption of

several drugs, as well as avoiding the reticuloendothelial system, thus protecting the drug from premature inactivation during its transport. The development of engineered nanoparticles with substantial biomedical significance has posed new opportunities and challenges for pharmacology and therapeutics<sup>16</sup>. Targeting an anticancer drug to the disease location is a distinctive feature of most studies, the aim being to convey a sufficient dose of drug to the tumor.

Solid Lipid Nanoparticles (SLN) consists of a solid lipid matrix, where the drug is normally incorporated, with an average diameter below 1  $\mu\text{m}$ . Solid lipid nanoparticles have been proposed as alternative drug carriers<sup>17</sup>. Nanoparticles made from solid lipids are gaining increasing attentions as colloidal drug carriers for intravenous application. At room temperature the particles are in the solid state. Therefore, the mobility of encapsulated drugs is reduced, which is an essential for controlled drug release<sup>18</sup>. The drug can either be directly incorporated during polymerization or by adsorption onto preformed nanoparticles. The ability to incorporate drugs into nanocarriers

offers a new prototype in drug delivery that could be used for secondary and tertiary levels of drug targeting. Another benefit of drug localization in the targeted tissue could be an improvement in its pharmacokinetic profile. Hence, solid lipid nanoparticles hold great promise for reaching the goal of controlled and site-specific drug delivery and hence have attracted wide attention of researchers.

The main aim of the present study was to evaluate the cytotoxic effect of incorporated SLN drugs, versus free drugs, on the human colorectal cancer cell line, HT-29. We evaluated apoptosis index through annexin-V/PI assay, DNA cell cycle and ploidy assay through BD<sup>TM</sup> Cycle TEST PLUS DNA.

## ***MATERIALS & METHODS***

### **Reagents**

The culture petri-dishes were procured from BD Falcon (Rockville, MD, USA). Fetal calf serum was obtained from HyClone Labs (Logan, Utah, USA). Dulbecco's Modified Eagle's Medium (DMEM) growth medium was procured from Gibco/BRL Life Technologies, Inc. (NY, USA). Antibiotic-antimycotic solution was obtained from Hi-

Media Labs (Mumbai, India). The cell growth supplements sodium pyruvate, nonessential amino acids and sodium bicarbonate were obtained from MP Biomedicals, Solon, USA. To quantify apoptosis, the Annexin-V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay kit from Roche Applied Sciences, Mannheim, Germany, was used. SLN encapsulated drug sample was kindly gifted by Mahakal Institute of Pharmacy, Ujjain. DNA cell cycle and ploidy were investigated using a BD<sup>TM</sup> Cycle TEST PLUS DNA Reagent Kit, BD Biosciences, USA.

#### Cell line & culture conditions

The human colorectal adenocarcinoma cell line, HT-29, was obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were seeded at  $2 \times 10^5$  cells/60 mm culture dishes in DMEM supplemented with 10% fetal calf serum, 1.5 g/L sodium bicarbonate at 37°C in the humidified atmosphere of 5% CO<sub>2</sub> in air according to NCCS catalogue instructions. After optimum confluency, the cells were treated with the experimental agent, free drugs and encapsulated drugs and harvested with trypsin-EDTA for use in the following experiments.

#### Study design

Cells were treated with a fixed 0.005 µM concentration of free drugs (5-Fluorouracil, Ca-leucovorin and Irinotecan) and SLN loaded 5-FU, leucovorin and irinotecan at different sampling intervals (n=5) for time course studies ranging from 0 to 96 h, dosage gradient of 0.1 to 100 µg following 6 h of treatment and passage levels ranging from passage 1 to passage 5.

#### Assessment of apoptotic index

Measurement of apoptotic index of cultured cells was performed using the Annexin-V-FITC/PI assay kit according to manufacturer recommendations. From each cell, forward light scatter (FSC), orthogonal light scatter (SSC), and annexin-V-FITC and PI fluorescence were measured using the Cell-Quest software (BD-IS, USA). The gate was applied in the FSC/SSC dot plot to restrict the analysis to cultured cells only. For the gated cells, the percentages of annexin-V-FITC-positive or -negative or PI-positive or -negative cells were evaluated. In each case total 10,000 events were recorded in HI mode with 10/10 log quadrant gate.

#### Analysis of DNA cell cycle arrest

Analysis of nuclear DNA cell cycle was performed by staining the cultured cells with Propidium Iodide (PI) using Cycle TEST PLUS DNA Reagent kit as per the manufacturer's protocol. PI fluorescence was measured through BD™ FACS Calibur (USA) using Cell-Quest Software (BD-IS, USA). A sum total of 30, 000 events were acquired in LO mode and were subjected for analysis by Cell-Quest software (BD-IS, USA). Histogram displays were overlaid with graphical representations of the modeled G0/G1, S and G2/M populations and data were expressed as percentages of cells for any given phase of the cell cycle.

## RESULTS AND DISCUSSION

### **Apoptotic index**

Annexin V, a Ca<sup>2+</sup> dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS), is used as a sensitive probe for PS exposure upon the outer leaflet of the cell membrane, for detecting apoptotic cells. The number of apoptotic FHC cells increased with the length of their exposure to drugs. The maximum number of such cells was 49% at 24 h (Fig 1).

### **Analysis of DNA Cell Cycle Arrest**

Nuclear DNA cell cycle analysis performed through flow cytometry revealed the arrest of HT-29 cells at different phases of cell cycle during time course treatment starting from 6 hr till 96 hrs. Interestingly, there was significant arrest of cells in G0/ G1 phase after 24 hrs of treatment and in G2/M phase after 96 hrs of treatment followed by apoptosis, as observed in all the treated cells after 96 hrs (fig 2). These results are probably the response of check-point proteins, and the consequent of blockage of DNA replication and inactivation of cell cycle progression.

### **Discussion**

The SLNs overcome all the above disadvantages of the chemotherapeutic drugs with their various properties especially their site specificity and less toxicity. The experimental set up for the evaluation of cytotoxic potential of 5-fluorouracil, calcium leucovorin, and irinotecan loaded in solid lipid nanoparticles targeted to colorectal malignancies comprised of the concentration and time course gradients. Fig 1 shows that the number of apoptotic cells was observed in an increment with increase in the

treatment is evidence for increased susceptibility to apoptosis on exposure.

### *CONCLUSION*

The treatment of metastatic colorectal cancer remains a challenge. Novel targeted drugs have received extensive attention in view of the relative insensitivity of colorectal cancer to conventional therapy. Promising new avenues of therapy are a reason for cautious optimism. In colorectal cancer, for instance new combinations have dramatically improved therapy although treatment continues to be palliative. In this context, it is absolutely necessary to explore new compounds in order to find much better treatments. Novel drugs that show broad activities, high efficacies, good

tolerability, and ease of use will rapidly find their place in the armamentarium of useful agents. Nanoparticulate systems had shown very promising results in the improvement of the pharmacokinetic profile of this antitumor drug and, simultaneously, in the enhancement of the anticancer action against experimental solid tumors. The research on nano-drug delivery system has become the hot spot in recent years. The use of nanoparticles as the drug-carrier system is a very attractive strategy to achieve controlled drug release.

### *ACKNOWLEDGEMENTS*

The authors are grateful to Bhopal Memorial Hospital Trust, India for providing financial support.

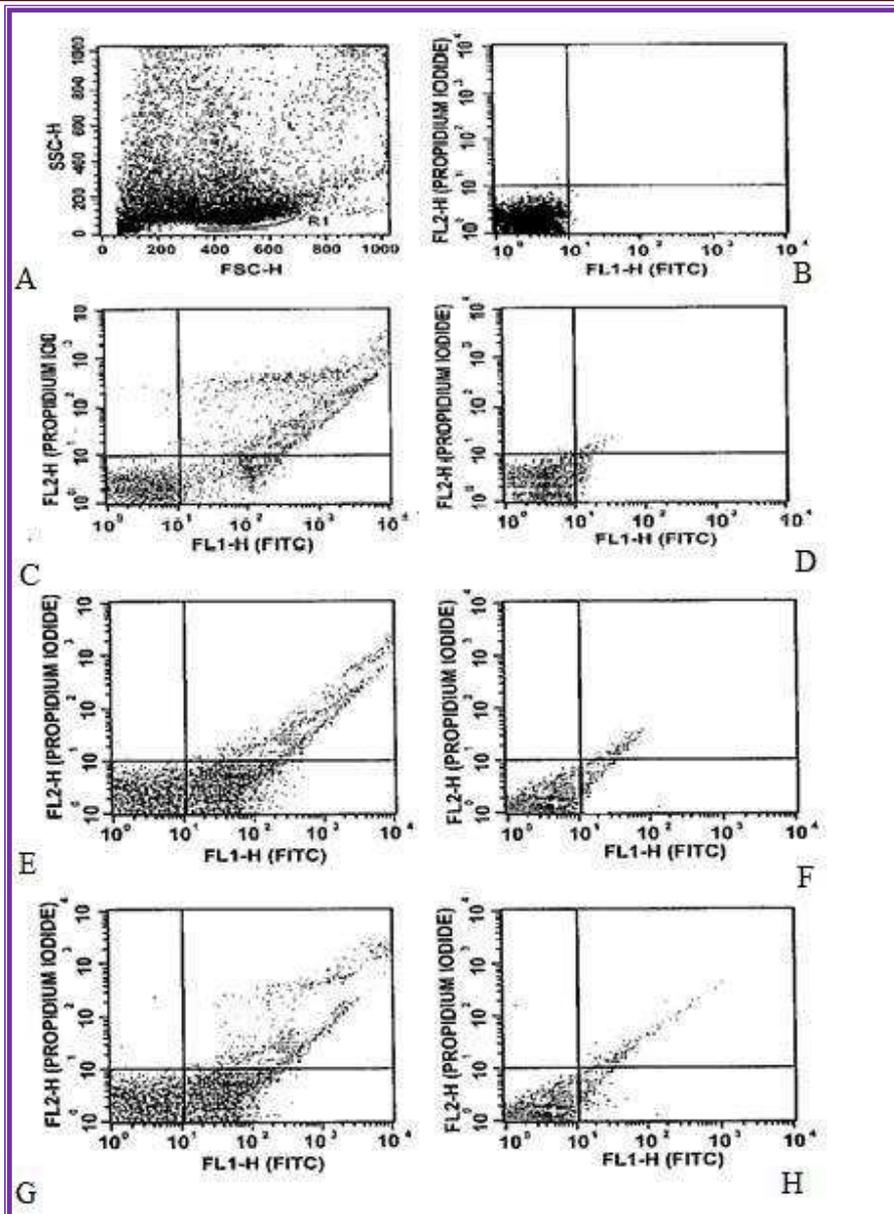


Figure 1 Flow cytometric analysis for apoptosis in cultured HT-29 cell lines following treatment with 5- Fluorouracil, Ca-leucovorin and irinotecan. (A) FSC/SSC dot plot showing the population of cells; (B) control cells; (C) cells treated with native 5-Fluorouracil (D) SLN Fluorouracil (E) Native Ca-leucovorin (F) SLN Leucovorin (G) Native Irinotecan and (H) SLN Irinotecan . These figures were observed at a concentration of 50 $\mu$ l at 24 hrs.

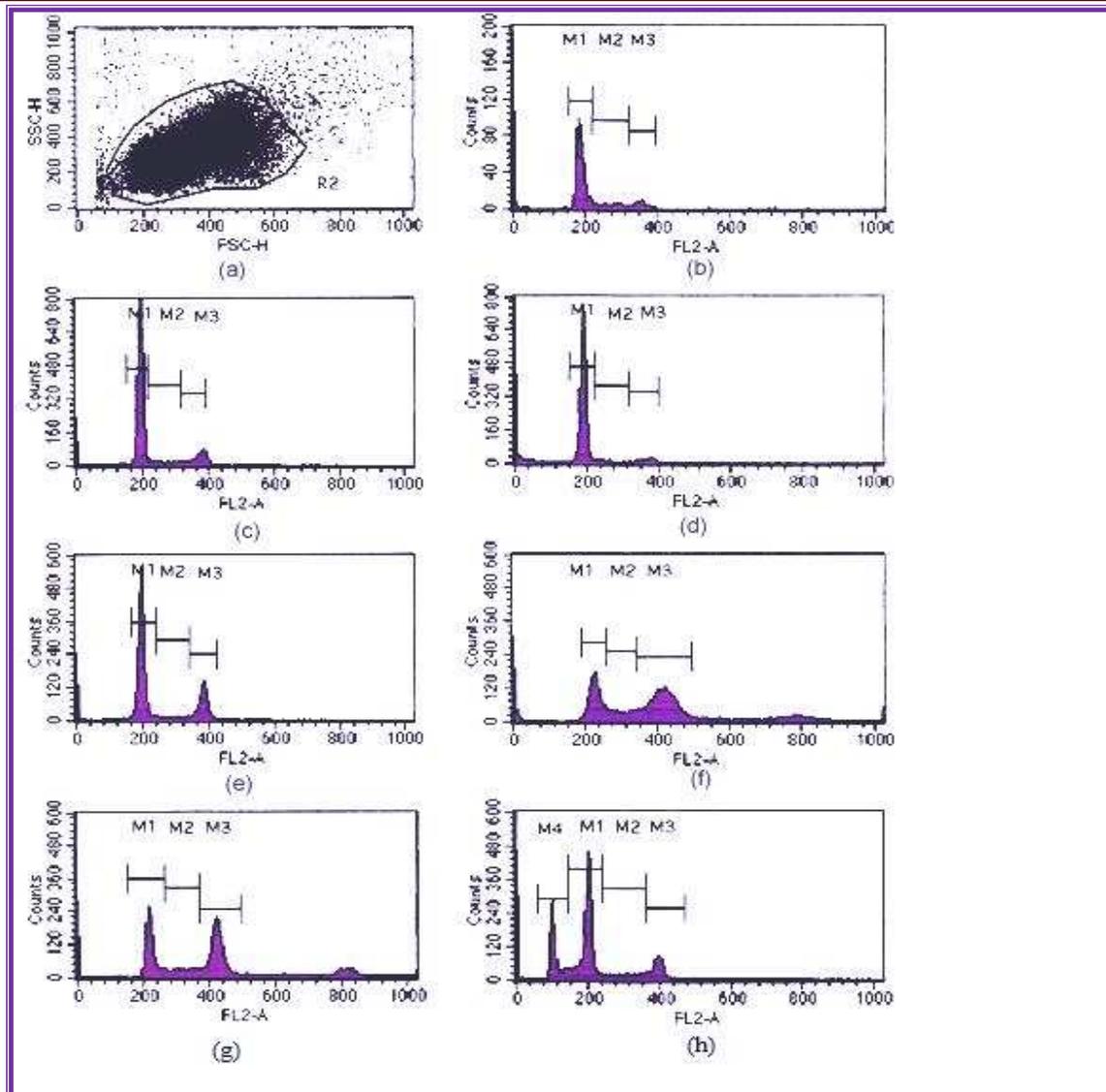


Figure 2 It shows DNA Cell Cycle Analysis of cells prior to and following treatment with chemotherapeutic drugs (a)FSC/SSC Dot Plot showing the population of gated cells, (b)Control cells showing G1, S and G2/M phases of the cell cycle denoted by M1, M2 and M3 respectively, (c)Treated cells after 12 hrs treatment showing onset of cell cycle arrest in G1 phase, (d)Significant arrest of cells as evident in G1 phase after 24 hrs of treatment, (e)Significant shift in peak from M1 to M2 depicting in cells after 48 hrs treatment, (f)Onset of M3 peak arrest after 72 hrs treatment, (g)Significant arrest in G2/M phase after 96 hrs, (h)Inclination of aneuploidy in treated cells observed at 2nd passage depicted by M4 peak.

REFERENCES

1. Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun M J: Cancer statistics, CA Cancer J Clin. 2007; 57: 43.
2. Adiga S M, Kumari M K, Bairy K L, Mohan Babu A, Vadiraja B M and Vidyasagar M S: The effect of using combination chemotherapy in colorectal cancer in India: A single institute survey, Cancer Res Exp Oncol, 2010; 2: 001.
3. Hill M J, Morsan B C and Bussey H J: Aetiology of adenoma-carcinoma sequence in large bowel, Lancet. 1978; 1: 245.
4. Albet D S: Reducing the risk of colorectal cancer by intervening in the process of carcinogenesis: a status report, Cancer J. 2002; 8: 208.
5. Frei E III and Antman K H: Combination chemotherapy, dose, and schedule, in Cancer Medicine. 1997: 817.
6. Douillard J Y, Cunningham D and Roth A D: Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial, Lancet. 2000; 355: 1041.
7. Saltz L B, Cox J V and Blanke C: Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer, N Engl J Med. 2000: 905.
8. Kulig J, Popiela T, Richter P and Klek S: Evaluation adjuvant chemotherapy irinotecan + 5 fluorouracil + leucovorin in advanced colorectal cancer, Acta Cher Belg. 2007; 107: 297.
9. Tournigand C, Andre T, Achille E, Lledo G, Flesh M and Merymignard D: FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study, J Clin Oncol. 2004; 22: 229.
10. Hwang J J, Eisenberg S G and Marshall J L: Improving the toxicity of irinotecan/5-FU/leucovorin: a 21-day schedule, Oncology. 2003; 17: 37.
11. Douillard J Y: Irinotecan and high-dose fluorouracil/leucovorin for metastatic colorectal cancer, Oncology. 2000; 14: 51.
12. Magrath I T: Targeted approaches to cancer therapy, Int J Cancer. 1994; 56: 163.

13. Pal D K and Nayak A K: Nanotechnology For Targeted Delivery In Cancer Therapeutics, Int J Pharm Sci Rev Res. 2010; 1: 1.

14. Li C: Poly (L-glutamic acid)–anticancer drug conjugates, Adv Drug Deliv Rev. 2002; 54: 695.

15. Praetorius N P and Mandal T K: Engineered Nanoparticles in Cancer Therapy, Recent Pat Drug Deliv Formul. 2007; 1: 37.

16. Medina C, Santos-Martinez M J, Radomski A, Corrigan O I & Radomski M W, Nanoparticles: pharmacological and toxicological significance, Bri J Pharm, 150 (2007) 552.

17. Ravi Kumar M N V: Nano and microparticles as controlled drug delivery devices, J Pharm Pharmaceut Sci. 2000; 3: 234.

18. Jain P, Mishra A, Yadav S K, Patil U K and Baghel U S: Formulation Development and Characterization of Solid Lipid Nanoparticles Containing Nimesulide, Int J Drug Deliv Tech. 2009; 1: 24.