# Development and evaluation of various pharmacological activities of sustained release formulation of phytochemical PITC-2, isolated from the tissue cultured medicinal plant *Pluchea indica* (L.)Less.

Thesis submitted by

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# CERTIFICATE FROM THE SUPERVISOR

This is to certify that the Ph.D thesis entitled **"Development and evaluation of various pharmacological activities of sustained release formulation of phytochemical PITC-2, isolated from the tissue cultured medicinal plant *Pluchea indica* (L.) Less."** submitted by **Smt Soumita Goswami**, who got her name registered on **29th March, 2016** for the award of Ph.D (Pharmacy) degree of Jadavpur University is absolutely based upon her own work under the supervision of **Prof. (Dr.) Tapan Kumar Chatterjee** and that neither her thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.

…….

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Date: ----------

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# CONTENTS

# PREFACE

The major treatment for cancer patients are chemotherapies which have various toxic side effects. To overcome these problems various traditional medicines are used. PITC-2 was isolated from the methanolic root extract of tissue cultured medicinal plant *Pluchea indica*(L.) Less. PITC-2 is a Thiophen derivative which is [2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene]. First objective of the study is to evaluate the in-vivo antitumor activity of PITC 2 against sarcoma- 180 cancer cell and EAC cell line in Swiss albino mice. The antitumor activity was evaluated by treatment with PITC-2 at different dose for 14 days and 21 days on EAC and Sarcoma-180 mice model. The histopathological and immunohistopathological examination indicates that PITC-2 induces apoptosis and suppresses tumor cell proliferation along with G1 cell cycle arrest through the down–regulation of the intratumoral expression of Bcl-2, cyclic D1 and Ki-67 and thus highlighting anti-proliferative and apoptotic properties against sarcoma-180 solid tumor model. Furthermore treatment with PITC-2 decreases body weight, tumor weight, tumor volume and increases lifespan and survival time of tumor bearing mice. Another major objective of the study is to formulate PITC-2 in a sustained release formulation and evaluation of the prepared formulation. So here we have performed designing, preparation and characterization of PITC-2 loaded Solid Lipid Nanoparticle for i.v. administration. Solid Lipid Nanoparticles were prepared by emulsion evaporation technique. PITC-2 SLNs formed are smooth spherical particles observed in cryo-FESEM with less than 200nm in size. 52% encapsulation efficacy is found with a stable zeta potential of -35nV. DSC and PXRD studies indicates complete encapsulation of drug within the nanoparticle matrix in amorphous form. The drug release study demonstrated a sustained and prolonged drug release from the SLNs. A comparison on therapeutic effectiveness is presented between PITC-2 SLNs and free phytochemical PITC-2 on EAC cell in Swiss albino mice. Treatment with PITC-2 SLNs decreases tumor volume and increases lifespan of cancer bearing mice in comparison to phytochemical PITC-2. The histopathological examination also indicates that PITC-2 SLNs have promising apoptotic activity on tumor cells. Along with this SLNs show no significant manifestation of toxic symptoms on liver and kidney of mice. Hence, the developed PITC-2 loaded SLNs can be used as drug carrier for sustained and prolonged drug release with improve therapeutic activity and bioavailability.

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## Abbreviations:

EAC- Ehrlich Ascites Carcinoma

SLN- Solid Lipid Nanoparticle

DSC- Differential scanning calorimetry

PXRD- Powder X-ray diffractometry

FESEM- Field emission scanning electron microscopy

5FU- 5 Fluoro Uracil

DMSO- Dimethyl Sulfoxide

PE- Petroleum Ether

EA- Ethyl Acetate

NMR- Nuclear Magnetic Resonance

NCCS- National Centre for Cell Science

CNCRI- Chittaranjan National Cancer Research Institute

DMEM- Dulbecco's modified Eagle's medium

PBS- Phosphate Buffer Saline

S-180- Sarcoma-180

MST- Mean Survival Time

RBC- Red Blood Cell

WBC- White Blood Cell

SGOT- serum glutamate oxaloacetate transaminase

SGPT- serum glutamate pyruvate transaminase

H&E- Hematoxylin and eosin

BSA- Bovine Serum Albumin

ILS- increase in life span

AST- Aspertate Aminotransferase

ALT- Alanine Aminotransferase

ALP- Alkaline Phosphatase

EE- Entrapment Efficacy

DL- Drug Loading

PI- Polidispersity Index

ZP- Zeta Potential

# INTRODUCTION

# Introduction:

Cancer and tumors are considered as the most killer disease all over the world. Cancer is caused by abnormalities of the genetic material of the affected cells. Tumorigenesis is a multistep process that involves the accumulation of successive mutations in oncogenes and suppressor genes that deregulates the cell cycle. It is not only a cell disease but also a titular disease in which the normal relationship between epithelial cells and their underlying stomach cells are altered (Bissel MJ 2001). The growth division takes place beyond limit and this uncontrolled growth finally leads to the disastrous and fatal disease of cancer. This abnormal cell growth starts in a part and with the help of blood and lymph it spreads over other areas of as well called as metastasis. Tumor metastasis is responsible for approximately 90% of all cancer related death (Abd-El Fattah AA 2017). Although struggling efforts against cancer have grown tremendously in last few years but still it is the second leading cause of death in economically developed countries. Factors such as obesity, poor diet, tobacco, radiation, environmental pollutants, lack of physical activity and age increases cancer risk. An increase in worldwide death rate was found on cancer patients which is 7.1 million and 8.2 million on 2007 and 2012 respectively (Jemal A 2007), (Hussein BHM 2012). By 2030 the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer death (Global cancer facts and figures, 3rd Edition 2015).

## Pathology of malignant disease:

Cancer possess a complex Pathophysiology. Pathologists who are concerned primarily with the study of disease in all its aspects. This includes cause of the disease, diagnosis, how the disease develops, mechanism and natural course of the disease. This disease also deal with biochemical features, progression, and prognosis or outcome of the disease. Cancer severity and staging refers to the site of the original (primary) tumor and spreading of cancer cells in the body. Cancer stage is based on factors such as the location of the primary tumor, tumor size, regional lymph node involvement, and the number of tumors present. Cancer cell occurs by the transformation of normal cell occurs through three distinct phases, initiation, promotion, and progression. Due to exposure of carcinogenic and mutagenic agents initiation of cancer occurs in the normal cells. These initiated cells are irreversibly altered and are at greater risk of neoplastic transformation. However tumor formation is not sufficient by this initiation alone (Rajesh E 2015).

Tumor grading is and pathophysiology is slightly different from cancer. Tumor grade is the description based on how abnormal the tumor cells and the tumor tissue look under a microscope. This indicators how quickly a tumor cells can grow and spread. When the cells of the tumor and the organization of the tumor's tissue are close to the normal cells and tissue, the tumor is called well-differentiated . These tumors tend to grow and spread at a slower rate than tumors that are "undifferentiated" or "poorly differentiated," which have abnormal-looking cells and may lack normal tissue structures. The nature of the tumor is determined by biopsy. Generally a doctor removes a small part of the suspected tumor tissue for biopsy. Then pathologists examines the biopsied tissue to determine whether the tumor is benign or malignant. They also identifies other characteristics of the tumor and grades the tumors. There are four grades of malignant tumors depending on the amount of abnormality. Grade one are when organization of the cancer cells appears close to normal orientation of cells. These type of tumor cells tends to grow and spreads slowly and have low degree of malignancy. A huge difference in cell orientation was found in tumors of grade three and four. They grow rapidly and usually spread at a faster rate in the body.

## Therapies of cancer:

Cancer therapy is based on surgery, radiotherapy and on systemic chemotherapy. Approximately half of cancer patients are not cured by this type of treatments. This only prolongs the survival span or even not beneficial for some cases. Chemotherapeutic drugs mainly kills malignant tumor cells by inhibiting some of the mechanisms implied in cellular division. Chemotherapy induces apoptosis which initiates tumor cell death. Apoptosis is an evolutionarily conserved form of death of cell in order to maintain tissue homeostasis which allows removal of damaged or superfluous cells. This can be mediated either by death receptor pathway or by mitochondrial pathway. First indication of apoptosis is activation of several caspases within the same cell and second evidence from different timing of caspase activation after addition of apoptotic stimuli are consistent with a hierarchy of caspase activation. (Skommer J 2010)(Swanton E 1999). It is obvious that cancer chemotherapy is a very difficult task(Nygren P 2003). Antitumor activity of these drugs approach are cytostatic or cytotoxic. However in past cast decades tumor biology explored a lot and this pave the way for more active targeting of anticancer drugs. A highly effective drug in chronic myeloid leukemia and gastrointestinal stromal tumors was

introducesnamed tyrosine kinase inhibitor Imatinib which was a proof of the effective development in drug based on knowledge of tumor biology (Capdeville R 2002)(Alkain JH 2002). But targeted therapies may be suitable and effective for small subgroups of patients..

One of its main associated problems in the nonspecific toxicity of most anticancer drugs due to their bio distribution throughout the body. This needs the administration of a large total dose to achieve high local concentrations of the drug in a tumor. The aim of drug targeting is to accumulate in the target cells independently on the method and route of drug administration. Another problem in cancer chemotherapy is drug resistance. It may be due to cancer cell mutation or by repairing the DNA breaks caused by anticancer drugs or by adopting some other mechanism that inactivates the drugs. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents. Recent interest in these secondary metabolites has been focused upon their medicinal properties (Harborne 2000).

## Natural products in cancer treatment:

For over 40 years, natural products have served us well in fighting against cancer. Microbes and plants from terrestrial and marine environment are the main sources of these successful compounds (Bulter 2005). A major source of natural products with anti-tumor activity are microbes. The first discovered of these products were as antibiotics. Plant alkaloids, flavonoides, taxoids and podophyllotoxins are other major contributors.

For example, flavonoides are a large group of aromatic plant secondary metabolites that are produced in the plant for the purpose of protection from photosynthetic stress, reactive oxygen species, wound and herbivores. Studies on flavonoid have produced the most compelling data for the antitumor activities of plant secondary metabolites in various types of cancer and severalflavonoides have been shown to inhibit cancer development which exhibiting antioxidant activities in various animal models (Kuo 1997). Vast studies and research on these products especially phytochemicals have exhibited anti-carcinogenic activities by interfering with the initiation, development and progression of cancer through the modulation of various mechanisms including cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis (Rajesh E 2015). Marine world also is a vast array of biological metabolites which can be used for effective

cancer treatment. Due to the rapid development of resistance to chemotherapeutic drugs, the search of a novel drugs is still a priority aim for cancer therapy.

Form 20th century use of plant and microbial secondary metabolites has doubled our life. They have reduced our sufferings and pains. Transplantation of organs is possible by this revolutionized medicine. The entire globe is explored for bio prospecting by researchers as the chemical diversity is based on biological and geographical diversity. Most of the pharmaceutically successful natural products originates due to easier access to terrestrial life. However, the ocean hosts a vast repertoire of life forms brimming with natural products of potential pharmaceutical importance. Marine life is a relatively unexplored area of opportunity as marine bio prospecting is a relatively new phenomenon. From both the soil and the sea new methods are being developed to grow the so-called 'un culturable' microbes. Most biologically active natural products are secondary metabolites which holds complex structures. In some cases, the natural product itself can be used, but in others, derivatives made chemically or biologically are the molecules used in medicine. To yield the desired product biosynthetic pathways manipulation is often needed. Thousands of new derivatives can now be made by this biological technique, which is complementary to combinatorial chemistry. Natural products holds more structural diversity than standard combinatorial chemistry so they often merge noble opportunities for finding low molecular weight structures that are active against a wide range of assay targets. In addition they are capable of being absorbed and metabolized in the body easily. Experimental agents which are derived from natural source offer great opportunities to evaluate not only new chemical class if anticancer agents but also novel and potentially relevant mechanisms of action. An evolutionary process going on in which producers of secondary metabolites evolved according to their local environments. The biosynthetic genes were retained if the metabolites were useful to the producing species and genetic modifications further improved the process. Combinatorial chemistry practiced by nature is much more sophisticated than combinatorial chemistry in the laboratory, which results a yield of exotic structures rich in stereochemistry, concatenated rings and reactive functional groups (Verdine 1996). This results are found to be of amazing variety and number of products have been found in nature. Combination of complementary technologies such as natural product discovery, high-throughput screening, genomics, proteomics, metabolomics and combinatorial biosynthesis are the main success of the pharmaceutical industry.

Taxol is the most important natural product derived from a medicinal plant which possess a high antitumor activity in recent years (Lahiri-Chatterjee M 1999) (Indram D 1997). Taxol is a powerful antitumor drug which is first isolated from the bark of *Taxusbrevifolia Nutt* and specially used in ovarian and breast cancers (Wain MC 1971). From the medicinal plant *Camptotheca acuminate* an alkaloid was found named Camptothecan (Wall ME 1971). A significant anti neoplastic activity was shown by this compound in various experimental tumor model and is too insoluble for drug use, but its analogues topotecan and irinotecan are used to treat gastric, rectal, colon and bladder cancer (Wall ME 1971) (Arun B 2001). Antineoplastic compounds of Vinca alkaloids named Vincristine and Vinblastine were isolated plant *Catharanthus roseus.* This alkaloids are used for the treatment of breast, bladder, lung, lymphomas, leukemia and various neoplastic diseases. Some other naturally derived anticancer products are Navelbine, Etoposide, Teniposide, Topotecan and Irinotecan. These proves the herbal remedies of cancer and tumor have very effective antitumor features which effectively controls tumor growth and uncontrolled cell divisions. All these are without any serious side effects and hence these can be used very safely with many assured benefit and least expensive.

In this project we are using a tissue cultured plant *Pluchea indica*. The plant was tissue cultured with the purpose to obtain more secondary metabolites (Pramanik 2007). From the methanolic root extract of tissue cultured medicinal plant *Pluchea indica* PITC 2 was isolated. It is a Thiophen derivative which is [2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene].

## Drug delivery system:

Cancer, a life threatening disease all over the world. Therapy of cancer is based on surgery, radiotherapy and on systemic chemotherapy. Cytotoxic drugs plays a major role in chemotherapy for cancer except for a few cancer types where hormonal therapy and immunotherapy is used. These drugs acts primarily by being toxic to the cancer cells that are rapidly growing and dividing. Cytotoxic drugs are earlier administered typically by free drug solutions as intravenous bolus or infusion (Ho Lun Wong 2007). Drug carrier system plays a great role in therapeutic effectiveness and safety profile for this conventional forms of this cancer therapies. Different type of drug carrier systems was used previously like colloidal drug delivery system which includes lipid emulsion, liposome and polymeric nanoparticles. But from a long time trials are done to utilize solid lipid nanoparticle (SLN) as an alternative drug carrier system. SLNs are

advantageous to use as drug carrier by many ways. It combines the advantages of lipid emulsion and polymeric nanoparticle systems by overcoming the *in vivo* stability issues as well as polymeric approach of drug delivery system (Mehnert W 2001). SLNs also provide advantage over other colloidal drug delivery system by improving the bioavailability of drugs (Olbrich C 2002) and obtains sustained release of lipophilic drugs (Yang S 1999). Other advantages of SLNs includes low biotoxicity of carrier, avoidance of organic solvents, possibility of controlled drug release, drug targeting and increased drug stability. Moreover production of SLN are simple and in large scale with low toxicity (Muller RH 2000).

## Properties of Solid Lipid Nanoparticle:

- High drug loading capacity.
- Large surface area.
- Small in size.
- More stable than liposomes.
- Readily biodegradable.
- Incorporation of both hydrophilic and hydrophobic substances.
- Less toxic than ceramic or polymeric nanoparticles.
- Feasible for drug delivery through various routes of administration(oral, inhalation, iv,etc)

**Solid lipid nanoparticles** are generally spherical in shape with an average diameter of 10 – 1000 nanometer. SLN consists of an amphiphilic outer core of surfactants and a biocompatible lipid inner core. The lipid core of these SLNs are stabilized by surfactants. Lipid which can be used in SLNs formulation can be triglycerides, diglycerides, monoglycerides, fatty acids, steroids and waxes. In many cases a combination of emulsifiers are used to avoid particle agglomeration more effectively (Mehnert W 2001). The physically stable and biocompatible lipid core can incorporate lipophilic drug by emulsification of lipid molten matrix and subsequent recrystallization of the dispersed phase. SLN also provide a good protection of drugs against chemical degradation, a good drug release profile modulation avoidance of organic solvents and combination of a wide range of lipid/surfactant which results in high drug loading (Kim BD 2005). There are few examples of use of SLN as drug delivery systems for anticancer drugs. 5FU

a commonly used anticancer drug to treat lung carcinoma was incorporated into a SLN formulation for better and effective drug delivery system in a study by Du et al (Du B 2010). Like many problems of anticancer drugs, 5FU has its limit in use due to its high toxicity, short half-life and low bioavailability. But after formulating it in SLNs, It allows to protect it from chemical degradation, increase drug bioavailability and decreasing the dosing frequency (Du B 2010).

Although different type of carrier systems had developed for the delivery of cytotoxic drugs but one main problem is its nonspecific toxicity due to their bio distribution throughout the body, which requires the administration of a large total dose to achieve high local concentrations in a tumor. Another problem in cancer chemotherapy is drug resistance. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents. All these points are already discussed earlier elaborately. Recent interest focused on medicinal properties of the secondary metabolites of these medicinal plants (Harborne JB 2000.) In the present study, isolation, purification and characterization of pharmacologically active tissue cultured medicinal plant was *Pluchea indica* used.

## Medicinal Plant *Pluchea indica:*

In this project tissue cultured medicinal plant *Pluchea indica* was used. The plant was tissue cultured with the purpose to obtain more secondary metabolites (B. R. Pramanik KC 2007). *Pluchea indica* (L.) Less is a flowering plant in the family Asteraceae. Its common named as Indian camphorweed, Indian fleabane, and Indian pluchea. This is a branching shrub and grows up to 2 meters in length. The leaves have toothed oval blades and are papery but not thin, and often have a fine coating of hairs. They have pinkish purple florets. Florets along the edges of the head produce fruits. Fruits are a millimeter in length which have a white pappus about 5 millimeters long. It is an evergreen large shrub found abundantly in salt marshes and mangrove swamps in Sunderbans (India), Bangladesh, Myanmar, China, Philippines, Malaysia, Tropical Asia and Australia (Chakraborty and Chatterjee 2017). *Pluchea indica* is already familiar for its multiple pharmacological activities. Many a compounds are already isolated from *Pluchea* species, which includes *Pluchea chingoyo, Phuchea foetido, Pluchea odorata, Pluchea rosea, and Pluchea suaveolens.* Many a compounds were also isolated from different parts of the

*Pluchea indica* which includes 3-(2',3;-Diacetoxy-2'-methyl butyryl)-cuauhtemone a eudesmane derivative from the leaf part of plant (Mukhopadhyay S 1983), some terpenic glycosides identified includes linaloyl glucoside, linaloyl apiosyl glycoside, 9-hydroxylinaloyl glycoside, plucheosides A, B and C from plants areal part . Other lignin glycosides are plucheosides $D_1$, $D_2$, and $D_3$ from the root of the plant. Three sesquiterpenes isolated which are plucheols A and B and plucheoside E. in our study we had isolated PITC-2 from the methanolic root extract of tissue cultured plant *Pluchea indica*. It is a Thiophen derivative which is [2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene]. This plant is used for various range of disorders. It has antimicrobial activity, a dose-related diuretic effect, anti-inflammatory effect, protective action against ulcers, activity against experimentally induced liver damage, has wound healing property, anti-oxidant effect, anti-hyperglycemic activity and anti-bacterial activities (C. T. Pramanik KC 2008) (Sen T 2002) (B. P. Pramanik KC 2006 ). A decoction of the roots or leaves is recommended for treating fever, headache, rheumatism, sprains, dysentery and dyspepsia also. Our study focuses on the anticancer activity of PITC-2 isolated from this plant. For this I had prepared various cancer cell mice models to establish its anticancer activity and also to identify the active principal and mechanisms involved in this anticancer activity. I had also prepared a suitable formulation to deliver this compound in system. A solid lipid nanoparticle was formulated for PITC-2 whose mode of administration is intravenous. Then characterization of this prepared formulation and in-vivo pharmacological activities were analyzed.

## Aims and Objectives:

1. To develop and evaluate a suitable sustained release formulation of PITC-2 obtained from the plant *Pluchea indica* Less.
2. To characterize the prepared sustained release formulation.
3. Pharmacological evaluation of prepared formulation.

## References:

Abd-El Fattah AA, Darwish HA, Fathy N, Shouman SA. 2017. "Carbition anhydrase inhibition boost the antitumor effect of Imatinib mesylate via potentiating the antiangiogenic and antimetastatic machineries,." *Toxicology and applied pharmacology* 316:123-138.

Alkain JH, Gershell LJ. 2002. "Selective anticancer drugs." *Nat. Rev. Drug Discov 1* 491.

Arun B, Frenkel EP. 2001. "Topoisomerase I inhibition with topotecan: pharmacologic and clinical issues." *Expert Opin Pharmacother.* 2(3):491-505.

Bissel MJ, Radisky D. 2001. "Putting Tumours in Context." *Nature Review Cancer* 1: 46-54.

Bulter, MS. 2005. "Natural products to drugs: natural product derived compounds in clinical trials." *Nat Prod Rep.* 22(2), 162-95.

Capdeville R, Buchdunger E, Zimmermann J, Matter A. 2002. "Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug." *Nat. Rev. Drug. Discov* 1(7),493-502.

Chakraborty S, Chatterjee TK. 2017. "Targeting viseral leishmaniasis by mannosylated liposome incorporated PITC-2 isolated from the plant *Pluchea indica* (L) less." *International Journal of Pharma and Bio Sciences.* 8: 396-408.

Chakraborty S, and T. K. Chatterjee. 2017. "Targeting viseral leishmaniasis by mannosylated liposome incorporated PITC-2 isolated from the plant *Pluchea indica* (L) Less." *Int J Pharm Bio Sci* 8(2), 396-408.

Du B, Yan Y, Li Y, Wang S, Zhang Z. 2010. "Preparation and passive target of % - fluorouracil Solid Lipid Nanoparticle." *Pharm Dev Technol* 15(4), 346 - 353.

2015. *Global cancer facts and figures, 3rd Edition.* American Cancer Society, 1.

Harborne, JB. 2000. "Arsenal for Survival: Secondary Plant Products." *Taxon* 49: 435-449.

Ho Lun Wong, Reina Bendayan, Andrew. M. Rauth, Yongyiang Li, Xiao Yu Wu. 2007. "Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles." *Advanced drug delivery reviews* 59 , 491 - 504.

Hussein BHM, Azab HA. 2012. "A novel antitumor agent Ln(lll) 2-thioacetate benzothiazole induces anti-angiogenic effect and cell death in cancer cell lines." *European journal of medicinal chemistry* 51: 99-109.

Indram D, Sanders K, Kolybaba M, Lopez D. 1997. "Case control study of phyto - oestrogens and breast cancer." *Lancet* 350:990 - 994.

Jemal A, Siegel R, Murray T, Xu J, Thun MJ. 2007. "Cancer Statistics." *CA: A cancer journal for clinicians* 57: 43-66.

Kim BD, Na K, Chio HK. 2005. "Preparation and characterization of solid lipid nanoparticles (SLN) made of cocoa butter and curdlan." *European journal of pharmaceutical scienses* 24, 199-205.

Kuo, SM. 1997. "Dietary flavonoid and cancer prevention: evidence and potential mechanism." *Crit Rev Oncogenesis* 8:47 - 69.

Lahiri-Chatterjee M, Katiyar SK, Mohan RR, Agarwal R. 1999. "A flavonoid antioxidant, silymarin, affords exceptionally high protention against tumor promotion in the SENCAR mouse skin tumorigesis model." *Cancer Res* 59: 622 - 632.

Mehnert W, Mader K. 2001. "Solid lipid nanoparticles: production, characterization and application." *Adv Drug Deliv Rev* 47(2-3), 165-196.

Mukhopadhyay S, Cordell GA, Ruangrungsi N, Rodkird S, Tantivatana P and Hylands PJ. 1983. "Traditional medicinal plants of Thailand. IV. 3-(2',3'-Diacetoxy-2'-methyl butyryl)-uauhtemone from *Pluchea indica.* " *J Nat. Prod.* (46) 671-674.

Muller RH, Madar K, Gohla S. 2000. "Solid lipid nanoparticles (SLN) for controlled drug delivery-a revier of the stste of the art." *Eur, J. Pharm. Biopharm* 50, 161-177.

Nygren P, Larsson R. 2003. "Overview of the clinical efficacy of investigational anticancer drugs." *Journal of Internal Medicine* 253: 46-75.

Olbrich C, Kayser O, Muller RH. 2002. "Lipase degradation of Dynasa 114 and 116 solid lipid nanoparticles (SLN) - effect of surfactents, storage time and crystallinity." *Int.J. Pharm* 237, 119-128.

Pramanik KC, Bhattacharya P, Biswas R, Bandyopadhyay D, Mishra M, Chatterjee TK. 2006 . "Hypoglycemic and antihyperglycemic activity of leaf extract of *Pluchea indica* Less." *Orient Pharm Exp Med.* (6)232-6.

Pramanik KC, Biswas R, Mitra A, Bandyopadhyay D, Mishra M, Chatterjee TK. 2007. "Tissue culture of the plant *Pluchea indica* (L.) Less. and evaluation of diuretic potential of its leaves." *Orient Pharm Exp Med* . 7(2), 197-204.

Pramanik KC, Chatterjee TK. 2008. " *In vitro* and *in vivo* antibacterial activities of root extract of tissue cultured *Pluchea indica* (L.) Less." *Orient Pharm Exp Med.* 8(3):295-301.

Pramanik, KC, Biswas R, Mitra A, Bandyopadhyay D, Mishra M, Chatterjee TK. 2007. "Tissue culture of the plant *Pluchea indica* (L.) Less. and evaluation of diuretic potential of its leaves." *Oriental Pharmacy and Experimental Medicine* 7: 197-204.

Rajesh E, Sankari LS, Malathi L, Krupaa JR. 2015. "Naturally occurring products in cancer therapy." *J Pharm Bioallied Sci* 181 - 183.

Sen T, Dhara AK, Bhattacharjee S, Pal S, Nag Chaudhuri AK. 2002. "Antioxidant activity of the methanol fraction of *Pluchea indica* root extract. ." *Phytother Res.* 16(4):331-5.

Skommer J, Brittain T, Raichoudhuri S. 2010. "Bcl-2 inhibits apoptosis by increasing time-to-death and intrinsic cell-to-cell variations in the mitochondrial pathway of cell death." *Apoptosis* 15: 1223-1233.

Swanton E, Savory P, Cosulich S, Woodman P. 1999. "Bcl-2 regulates caspase 3/caspase 2 apoptotic cascade in cytosolic extracts." *Oncogene* 18: 1781-1787.

Uchiyama T, Miyase T, Ueno A, Khan U. 1989. "Terpenic glycosides from *Pluchea indica*." *Phytochemistry* (28)3369-3372.

Verdine, GL. 1996. "The combinatorial chemistry of nature." *Nature.* 384,11–13.

Wain MC, Taylor HL, Wall ME, Coggon P, Mc Phail AT. 1971. "Phant antitumor agents VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus Vrevifolia ." *Taxus brevifolia J Am Chem Soc* 93, 2325 - 2327.

Wall ME, Taylor HL, Wall ME, Coggon P, Mc Phail AT. 1971. "Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia." *J Am Chem Soc* 93(9):2325-7.

Yang S, Zhu J, Lu Y, Liang B, Yang C. 1999. "Body distribution of camptothecin solid lipid nanoparticle after administration." *Pharm. Res* 16(5), 751-757.

# LITERATURE REVIEW

# Literature Review

**S.K. Sahoo** *et al.* described Nanotechnology is a multidisciplinary field which comprises of a vast and diverse devices derived from engineering, physics, chemistry, and biology. The prevalent new field of nanotechnology, opted up by rapid advances in science and technology, which creates new opportunities for advancement of medical science for treatment of various diseases in human health care. Applications of nanotechnology to medicine and physiology imply materials and devices designed to interact with the body at subcellular (i.e., molecular) scales with a high degree of specificity. This can be targeted into cellular and tissue-specific sights and can be designed to achieve maximal therapeutic efficacy with a minimum side effects. In this review they have discussed some of the technical aspects of Nano technology to achieve potential clinical applications (S.K. Sahoo 2007).

**A. Madhusudhan** *et al.* in 2012 designed a Controlled release solid lipid nanoparticles of Efavirenz by solvent emulsification followed by evaporation. Efavirenz is an oral nucleoside reverse transcriptase inhibitor (NRTI). Particle size, zeta potential, drug entrapment efficiency (EE%), *in vitro* drug release of the SLNs are the parameters investigated in this study. Solid lipid nanoparticles prepared by this technique had particle size of 85.55±0.8 nm, zeta potential of -24.44±(0.4) mV, EE of 92±9.7% and cumulative drug release of 83.75±2.54% in 48 hours. Studies for comparison of formulation were done by using FTIR Spectroscopy and DSC. FTIR spectra and DSC thermograph of Efavirenz, lipids, indicates no chemical interaction between them and confirmed the stability of the drug. Solid lipid nanoparticles shape are confirmed by SEM study which were non-spherical in shape. The solid state of the drug in the Solid lipid nanoparticles and lipid modification was characterized. No significant change in the mean particle size was found from accelerated stability studies of the formulation after stored for a period of three months. These Efavirenz-Solid lipid nano particles are can be considered to be promising vehicles for oral drug delivery due to its enhanced bioavailability (A. Madhusudhan 2012).

**Surajit Das** *et al*. aimed to prepare solid lipid nanoparticles (SLNs) of a hydrophobic drug, tretinoin, by the method of emulsification ultra sonication. Solubility of tretinoin in the solid lipids was examined. Particle size, polydispersity index (PI), zeta potential (ZP), drug

encapsulation efficiency (EE), and drug loading (L) of the SLNs were investigated for process variables examination. Shape and surface morphology of the SLNs were investigated by cryogenic field emission scanning electron microscopy (cryo-FESEM). Cross-polarized light microscopy and differential scanning calorimetry (DSC) studies were conducted to identify encapsulation of drug in the nanoparticles. DSC and powder X-ray diffraction (PXRD) were analyzed for crystallinity of the formulation. Drug release and stability studies were also performed for formulation optimization. From the results it was found that 10 mg tretinoin was soluble in 0.45 ± 0.07 g Precirol® ATO5 and 0.36 ± 0.06 g Compritol® 888ATO, respectively. Process variables indicates a significant influence in the production of SLNs. SLNs with <120 nm size, <0.2 PI, >I30I mV ZP, >75% EE, and ∼0.8% L can be produced followed by appropriate formulation conditions. Spherical and smooth surface particles were shown by Cryo-FESEM study. Cross-polarized light microscopy was also performed which shows that drug crystals in the external aqueous phase were absent when the SLNs were prepared at ≤0.05% drug concentration. Indication of complete drug encapsulation within the nanoparticle matrix as amorphous form was analyzed by DSC and PXRD studies. The drug release study performed shows a sustained and prolonged drug release pattern from the SLNs. Furthermore, stability study of tretinoin-loaded SLNs shows a stable formulation 3 months at 4 ◦C. After investigating these all parameters it is concluded that the developed SLNs can be used as drug carrier for sustained drug release and to improve oral absorption and bioavailability (Surajit Das 2011).

**Carla Vitorino** *et al.* in 2010 investigate the role of different factors what can affecting the size of solid lipid nanoparticles (SLN), prepared by the emulsification–solvent evaporation method. A double factorial design was conducted which is to cover a wide range of sizes, highlighting zones with different behavior with respect to changes in the controlled variables like lipid concentration, solvent: lipid ratio and emulsifier concentration. The main factor influencing particle size was solvent: lipid ratio constitutively. A decrease in the size was found with increasing the amount of solvent induction. This was a general trend which is independent from the solvent and lipid type. A non-trivial impact on size was found on amount of emulsifier and it depends on whether systems were located below, above or close to the optimal surface coverage. A limited influence upon particle size was observed on amount of lipid, which is more relevant for lower lipid concentrations. An optimal formulation was selected for intermediate levels of the

three variables. Particle size and polydispersity both are reduced by sonication. As a model of lipophilic drug these particles were also tested for drug carriers using simvastatin. In conclusion SLN were able to entrap a high amount of simvastatin, with a little effect on the size and zeta potential, constituting a promising carrier for lipophilic drugs (Carla Vitorino 2011).

**YiFan Luo** *et al.* designed a technique ultrasonic-solvent emulsification which was adopted to prepare vinpocetine loaded Glyceryl monostearate (GMS) nano dispersions with narrow size distribution. The process was performed at 50 °C to increase the lipid load in the formulation, using an ultrasonic solvent emulsification technique. To characterize the prepared formulation a detailed investigation was done on mean particle size and droplet size distribution, drug loading capacity, drug entrapment efficiency (EE%), zeta potential, and long-term physical stability of the SLNs. Drug release was studied by dialysis bag method for VIN-SLNs. Male rats were used for pharmacokinetic study. Formulation was administered orally at 10 mg kg−1 VIN and different formulations. It was found that the relative bioavailability of VIN in SLNs was significantly increased compared with that of the VIN solution. Oral absorption of VIN with SLN formulations had a marked effect depending on the amount of surfactant. The absorption mechanism of the SLN formulations was also discussed. These results concludes that absorption of VIN is enhanced significantly by giving it in this SLN formulations. SLNs offer a new approach for improving the oral bioavailability of poorly soluble drugs (YiFan Luo 2006).

**Shengpeng Wang** *et al*. on 2012 prepared and characterize emodin (EMO)-loaded solid lipid nanoparticles (E-SLNs) and evaluate their *in vitro* antitumor activity. EMO and pharmaceutical lipid material were used to prepare E-SLNs in high pressure homogenization (HPH). Surfactant used in this process was Poloxamer 188 and Tween 80. E-SLNs were investigated for particle size analysis, zeta potential measurement, drug entrapment efficiency (EE), stability and *in vitro* drug release behavior for formulation qualification and quantification. It was reported that E-SLNs showed stable particle size at 28.6 ± 3.1 nm, ideal drug EE and relative long-term physical stability after a storage of 4 months. The drug release from E-SLNs lasts for 72 h and exhibited a sustained profile, which made it a promising vehicle for administering the drug in oral route. *In vitro* cytotoxicity assay (MTT assay) showed that E-SLNs could significantly cytotoxic effect against human breast cancer cell line MCF-7 and MDA-MB-231 cells compared to the EMO

solution, while free EMO, blank SLNs (B-SLNs) and E-SLNs all showed no significant toxicity to human mammary epithelial line MCF-10A cells. E-SLNs also showed more significant cell cycle arrest effect in MCF-7 cells compared to bulk EMO solution found by Flow cytometric analysis. Hoechst 33342 staining and Annexing V-FITC/PI double staining further confirmed that higher apoptotic rates in MCF-7 cells induced by E-SLNs, which clearly indicating that cell cycle arrest and apoptosis maybe the underlying mechanism of the enhanced cytotoxicity. Taken these results together, it seems that HPH was a simple, available and effective method for preparing high quality E-SLNs to enhance its aqueous solubility. Moreover, these results suggest that the EMO delivery in the form of lipid nanoparticles maybe a promising approach for the therapy of cancer (Shengpeng Wang 2012).

**Mustafa Cengiz** *et al.* in 2015 prepared nanostructure-mediated drug delivery to obtain a potential to improve drug bioavailability. Apart from this it is also designed to obtain a release deviation of drug molecules and enabling precision drug targeting. Scientists found a solution to minimize pharmaceutics limitations of the drug's use by the designing of Solid lipid nanoparticles (SLNs). Silymarin (Sm) is a drug used for treating diverse liver and gallbladder disorders, such as cirrhosis, hepatitis, and jaundice, and for protecting the liver against poisoning from chemical and environmental toxins by its antihepatotoxic and antioxidative properties. The present study aims in the development of a novel silymarin-loaded solid lipid nanoparticle (Smloaded SLN) system which will enhance its bioavailability and ability to provide an excellent hepatic protection for poorly water-soluble drugs. A significantly reduced of D-GaIN/TNF- α-induced hepatotoxicity was found by treatment with Sm-loaded SLN based upon our investigation results with apoptotic markers, PCNA and light microscopic findings. Results also suggested improved bioactivity compared to Sm. In conclusion, it may be said that Sm loaded SLN could be a useful system for the delivery of poorly water-soluble Sm, apart from providing favorable hepatic protection (Mustafa Cengiz 2015).

**Kopparam Manjunath** *et al.* in 2005 prepared solid lipid nanoparticle of Clozapine which is a lipophilic effective atypical antipsychotic drug. Due to the effect of first pass it have very poor oral bioavailability (<27%). Using various triglycerides (trimyristin, tripalmitin and tristearin), soylecithin 95%, poloxamer 188 and stearyl amine as a positive charge inducer Clozapine solid

lipid nanoparticles have been developed by the technique of hot homogenization followed by ultrasonication method. Malvern Zeta sizer were used for particle size and charge measurements. Pharmacokinetics study was conducted by intravenous (i.v.) administration of clozapine solid lipid nanoparticles (SLNs) to male Wistar rats. The main objective of this research was to find out whether the bioavailability of clozapine be improved by administering clozapine SLN duodenally to rats. In Swiss albino mice tissue distribution studies of clozapine SLN and suspension were carried out. Average size and zeta potential of SLNs of different lipids with stearyl amine ranged from 96.7F3.8 to 163.3F0.7 nm and 21.3F1.3 to 33.2F0.6 mV, respectively. It is found that by administering clozapine entrapped in SLNs with stearyl amine intravenously $AUC_{(0–l)}$ was increased (up to 2.91-fold) and clearance was decreased (up to 2.93-fold). After intra duodenal administration compared with that of clozapine suspension bioavailability of clozapine SLNs were 2.45- to 4.51-fold. In tested organs, the AUC and MRT of clozapine SLNs were higher than those of clozapine suspension especially in brain and reticulo endothelial cell-containing organs. These results helps to conclude thar that SLN are suitable drug delivery system and increases bioavailability of lipophilic drugs such as clozapine (Kopparam Manjunath 2005).

**Rohan Shah** *et al*. in 2014 Optimizes and study Stability Assessment of Solid Lipid Nanoparticles using Particle Size and Zeta Potential. Due to physiological and biodegradable lipid matrix Solid lipid nanoparticles (SLNs) act as a well-tolerated lipid carrier system. Physicochemical properties to identify of SLNs are particle size, polydispersity index (PI) and zeta potential. A good indicator of in-vitro instability is the increased particle size. Importance in selecting a suitable lipid matrices and excipients is the main focus of this work that can achieve the particle size and stability required if such formulations are to be utilized in the pharmaceutical market. A Taguchi model of experimental design was applied to understanding the influence of variation in SLN composition (lipid and emulsifier concentration). Tested factors included the concentration of lipid (stearic acid) and the concentration of surfactant Tween®20. Here SLNs were successfully prepared by the method of micro emulsion. Based on the hydrophilic lipophilic balance (HLB), different combinations of emulsifiers/co-emulsifiers (Tween®20/Span®20, Tween®20/Span®80, Tween®20/n-butanol, and Tween®20/iso-propanol) were also used to control the physicochemical properties of SLNs. During and after

the preparation of SLNs the influence of pH (addition of HCl or NaOH) and electrolyte (addition of NaCl), were also investigated for the selected SLN formulations. It is found that polydispersity (PI < 0.3) nanoparticles with a particle size < 450 nm and zeta potential range of +5 to –50 mV were developed. After a 2 month storage period physical stability of optimized stearic-acid based SLNs were assessed by particle size measurement. SLNs were found to be stable when refrigerated. Selection of lipid and lipid excipients is very essential for successful preparation and physical stability of SLNs which can be confirmed by the above results. The preliminary physicochemical characterization facilitates favorable encapsulation of lipophilic and hydrophilic drugs (Rohan Shah 2014).

**Neha Yadav** have written a review on solid lipid nanoparticle. In early 1990s as an alternative to other traditional colloidal carriers systems like liposomes, polymeric nanoparticles and emulsions solid lipid nanoparticles were developed as they have advantages like controlled drug release and targeted drug delivery with increased stability. This study was done to understand and to give an overview about the potential advantages and also the disadvantages of solid lipid nanoparticles. Membrane contractor method is used for the preparation of this SLN formulation. Stability of SLN and the effect of various excipients (used in SLN production) on stability with other secondary steps involved in their stabilization like freeze drying, spray drying etc. A thoroughly discussion was done in this paper about the problems associated with SLN production and instrumental techniques followed in production. To develop the models of drug loading in SLN and the pattern of drug release from SLN a special attention was given to design it. For formulation optimization various analytical methods involved in SLN evaluations are discussed in detail and the major approach for targeting drugs delivery by SLNs preparation are discussed here (Neha Yadav 2013).

**Dianrui Zhang** *et al*. in 2006 designed Solid lipid nanoparticle (SLN) of Oridonin, which is a lipophilic Chinese medicine. It has a very low oral bioavailability due to its poor solubility. To overcome this problem solid lipid nanoparticle delivery systems of oridonin have been formed where ingredients used are stearic acid, soybean lecithin and plutonic F68x`. Technique used in the preparation of SLN is Emulsion evaporation method and then solidification at low temperature. To analyze the particle size and morphology of SLN transmission electron

microscopy (TEM) study was performed, and the zeta potential was measured by a television micro-electrophoresis apparatus to analyze stability of formulation. On the basis of drug loading and entrapment efficacy process and formulation variables have been studied and optimized. Differential scanning calorimetry (DSC) and powder x-ray diffraction (PXRD) studies were performed to characterize the crystalline state of the drug in the formulation. At pH 7.4 in phosphate-buffer solution (PBS) *in vitro* release studies were performed. To evaluate the tissue targeted property of SLNs the tissue distribution in mice and the pharmacokinetics in rabbits were studied. It is found that stable SLN formulations of oridonin having a mean size range of 15–35 nm and mean zeta potential −45.07 mV were developed. Entrapment of drug in SLN was found to be more than 40%. Dispersed of oridonin in SLNs in an amorphous state was confirmed DSC and PXRD result analysis. The release pattern of the drug was found to follow the Higuchi equations. Observation and results based on *in vivo* studies demonstrated that oridonin-loaded SLNs increased the concentration of oridonin in liver, lung and spleen, while the distribution of drug is found to be decreased in heart and kidney (Dianrui Zhang 2006).

**Rahul Nair** *et al*. on 2012 aims at preparing aqueous suspension of Solid lipid Nanoparticles containing Chitosan (CT). This is a biopolymer that can exhibits a number of interesting properties for controlled drug delivery. Carbamezapine (CBZ) is a lipophilic drug which by inactivating sodium channels shows it antiepileptic activity. Solvent injection method is used for preparation of this solid lipid Nanoparticles (SLN) of Chitosan-CBZ, using ethanol as organic solvent. The SLN formulations prepared by this technique exhibited high encapsulation efficiency and high physical stability. A controlled release pattern of drug was studies from this SLNs for prolonged period of time. The prepared SLNs were characterized by SEM analysis for surface morphology, entrapment efficiency, zeta potential for stability, FTIR and DSC for excipient interaction and crystalline nature of drug in formulation. In-vitro diffusion studies was performed for release study. The results shows mean diameter and zeta potential were 168.7±1.8 nm and −28.9 ±2.0 mV for SLN-chitosan-CBZ respectively. Therefore prepared chitosan-SLN may be claimed to be a good candidates to encapsulate CBZ and to increase its therapeutic efficacy for the treatment of Epilepsy (Rahul Nair 2012).

**Senthil Kumar** *et al*. prepared solid lipid nanoparticles of enrofloxacin. The objective of this study was to formulate a solid lipid nanoparticles (SLNs) of enrofloxacin by the technique of hot homogenization which is coupled with ultra-sonication method to achieve a sustained oral delivery and then evaluation of the formulation. Ingredients used for preparation of SLNs were tripalmitin as lipid carrier, tween 80 and span 80 as surfactants and polyvinyl alcohol (PVA) as a stabilizer. To optimize the formulations investigation was done on the factors such as composition and concentration of lipid carrier and surfactant on the particle size. For the optimization of SLNs formulations characterizations done are characterized for particle size, polydispersity index (PDI), zeta potential (using dynamic light scattering), shape (using atomic force microscopy (AFM) and transmission electron microscopy [TEM]), drug encapsulation efficiency (EE) , loading capacity (LC) (using by dialysis and ultracentrifugation methods), and *in vitro* drug release (using by dialysis). To confirm the cross-linking reaction between drug, lipid and surfactants the prepared SLNs were analyzed by Fourier transform infrared (FT-IR) spectroscopy. The results obtained demonstrates that the particle size, PDI, zeta potential, EE and LC of the enrofloxacin SLNs were 154.72±6.11 nm, 0.42±0.11, −28.83±0.60 mV, 59.66±3.22% and 6.13±0.32%, respectively. Spherical to circular particles with well-defined periphery was shown by TEM and AFM images. A biphasic pattern of drug release was exhibited by *in vitro* drug release study with an initial burst release of 18% within 2 hrs, followed by sustained release over 96 hrs. From the FT-IR study it can be suggested that during the process of formulations, lipid and surfactants have not reacted with the drug to give rise to reactant products and it was only physical mixture. All these results indicated that prepared SLNs might be a promising delivery system which may prolong and enhance the pharmacological activity of enrofloxacin (Senthil Kumar P 2015).

**Laith Hamza Samein** in 2014 planned to prepare SLN to incorporating an antifungal drug Nystatin and study its effects on skin localization of drug when administered through a suitable semisolid vehicle such as gel. So by preparing solid lipid nanoparticles of Nystatin and theoptimize formulation was selected by using Box Behnken Design and then the formulation was characterized for particle size, percent entrapment efficiency, XRD, FTIR and DSC to confirm the formation of SLN and entrapment of Nystatin in SLN which was formulated as a gel containing solid lipid nanoparticles. While for the selection of SLN delivery system was the

maximum solubility of Nystatin in different lipids and also on melting point of lipid as the type of drug-lipid matrix and drug release pattern would depend on it. To solubilize Nystatin in lipid various co-solvents were also used. Nystatin showed maximum solubility out of different lipids used in mixture of glyceryl monostearate (GMS) and propylene glycol (PG). GMS, Span 60 and tween 80 which were used as formulation ingredients determined by pre-optimization study. The optimized formulation by using different concentration of Carbopol 940 with 0.4 % gel which was finalized, and pseudo plastic behavior was observed by rheology study. Nystatin in the Ny-SLN was found to be effective against *Candida albicans* after going through various process conditions. The result supports for a successful new developed pharmaceutical formulation which is able to deliver the active substance to the target organ at therapeutically relevant levels, with negligible amount of discomfort and side effects. In addition the stability study indicates that there is no significant difference between the parameters tested before and after the stability studies (Samein 2014).

**Ghada Abdelbary** *et al.* aim in the present study to investigate the feasibility of the inclusion of a water insoluble drug (diazepam, DZ) into solid lipid nanoparticles (SLNs). This may offer various advantages of rapid onset and prolonged release of the drug. Preparation of DZ-loaded SLN dispersions may describe a new approach as a potential drug carrier for the rectal route. Techniques involved in the preparation of SLNs were modified high-shear homogenization and ultrasound. Investigation was done to analyze the effect of incorporation of different concentrations of Compritol® ATO 888 or Imwitor® 900K and Poloxamer 188 or Tween 80. From the result it was found that by varying the type or concentration of lipid matrix or surfactant there was a noticeable influence on the entrapment efficiencies, particle size, and release profiles of prepared SLNs. From the results of differential scanning calorimetry and X-ray diffraction it showed that the majority of SLNs possessed less ordered arrangements of crystals than the corresponding bulk lipids. This effect was favorable for increasing the drug loading capacity. Transmission electron microscopy and laser diffractometry studies revealed the shape and morphology of the particles. It is found that the prepared nanoparticles were round and homogeneous and 60% of the formulations were less than 500 nm. Additionally release study of SLN formulations showed significant ($P<0.05$) prolonged release than DZ solution. The subsequent step encompassed the preparation and evaluation of SLN-based suppositories

utilizing SLN formulations that illustrate doptimal release profiles. From the *in vitro* release of DZ it is observed that suppositories prepared using DZ-loaded SLN dispersions (equivalent to 2 mg DZ) was significantly ($P<0.05$) extended compared to suppositories containing 2 mg DZ free drug (Fahmy 2009).

**Nehal M. Elsherbiny** *et al*. aimed to investigate the anti-cancer activities of vanillin against breast cancer and possible synergistic potentiating effect of doxorubicin DOX chemotherapeutic effects by vanillin. Study was carried out for 21 days by i.p. administration of Vanillin (100 mg/kg), DOX (2 mg/kg) and their combination to solid Ehrlich tumor-bearing mice. MCF-7 human breast cancer cell line was treated with vanillin (1 and 2 mM), DOX (100 M) or their combination. Study was carried out in rats to confer the protection against DOX-induced nephrotoxicity that received vanillin (100 mg/kg, ip) for 10 days with a single dose of DOX (15 mg/kg) on day 6. Vanillin shows good anticancer effects in comparison to DOX and synergistically potentiated DOX anticancer effects observed both in-vivo and in-vitro. The apoptosis and antioxidant capacity of vanillin in-vivo was mediated. It also shows a good an *in-vitro* growth inhibitory effect and cytotoxicity mediated by apoptosis by increased caspase-9 and Bax:Bcl-2 ratio, along with anti-metastasis effect. Vanillin also give protection against DOX–induced nephrotoxicity in rats. In conclusion, it can be said that vanillin can be used as a potential lead molecule for the development of non-toxic agents for the treatment of breast cancer either alone or a combination with DOX (Nehal M. Elsherbiny 2016).

**A.J. Ruby** researched on Natural curcuminoids, curcumin, I, II and III isolated from turmeric *Cunruma longa* and they were compared for their cytotoxic effect, tumor reducing and antioxidant activities. It is found that isolated compound curcumin III was found to be more active than the other two as a cytotoxic agent in the inhibition of Ehrlich ascites tumor in mice (ILS 74.1%). Antioxidant activity were also checked which indicates the potentiality in using it as an anti-promoters. For 50% inhibition of lipid peroxidation the amount of curcuminoids (I, II and III) needed was 20, 14 and 11 g/ml. 50% inhibition of superoxide's were found in concentrations are 6.25, 4.25 and 1.9µg/ml and those for hydroxyl radical were 2.3, 1.8 and 1.8 µg/ml, respectively. The ability of these compounds to suppress the superoxide production by macrophages activated with phorbol-12-myristate-13- acetate (PMA) indicated that all the three

curcuminoids inhibited superoxide production and curcumin III produced maximum effect. From these results it can be said that curcumin III is the most active amongst the other curcuminoids present in turmeric. Synthetic curcumin I and III had similar activity to natural curcumins (A.J. Ruby 1995).

**Adrita Chakrabarti** *et al*. researched on Methyl glyoxal (MG). This is a potent anticancer agent which has been conjugated to a nontoxic, biocompatible polymer, chitosan, to protect it from degradation by *in vivo* enzymatic. On treatment with elicits macrophage-mediated immunity in tumor bearing mice, this polymeric complex, 'Nano-MG' shows remarkable antitumor property on intravenous (0.4 mg/kg body wt/day) treatment more efficiently than MG (20 mg/kg body wt/day). These activated macrophages appear more in numbers in the peritoneum and produce more superoxide and nitrite. Moreover it is noted that immunomodulatory cytokines and surface receptors of these macrophages like iNOS, IFN-c, TNF-a, IL-1b, IL-6, M-CSF, TLR-4 and TLR-9 exhibit a marked up-regulation in Sarcoma-180 tumor bearing mice after treatment with Nano-MG compared to that of untreated tumor bearing counterpart. Hence, from this study it can be suggested that, treatment with Nano-MG is capable of stimulating the immune system in immunosuppressed tumor bearing mice models at a much lower dose than treatment with MG alone can execute. From these all studies it can be concluded that Nano-MG may be considered as a promising futuristic potential drug in cancer immunotherapy (Adrita Chakrabarti 2014).

**Belal H.M. Hussein** *et al.* synthesize, characterize and experimented on new complexes which has a potent DNA-binding anti-tumor agent, europium(III)- and terbium(III)-2-thioacetate benzothiazole. Using fluorometric and electronic absorption spectroscopy, it was found that these complexes showed strong binding affinity to calf thymus DNA. An inhibition of proliferation of EAC cells and ascites formation was resulted by the synthesized complexes. By the reduction in the micro vessel density and by down regulation of VEGF receptor type-2 (Flk-1) the anti-tumor effect was found to be through anti-angiogenic activity. From the result analysis of capillary electrophoresis it was found that EAC cells had distinct DNA fragmentation patterns in the treated animals. Moreover, significant cytotoxic activity was exhibited by synthesized complexes against HepG2 and MCF7 cell lines. Furthermore, complexes synthesized showed a potent anti-bacterial activity against Escherichia coli and Salmonella which are two

pathogenic bacteria's. In addition, an antiangiogenic effect was exerted by our novel compounds as it was evident by the reduction of micro vessel density. From the apoptotic study it is evident that the two complexes can effectively induce the apoptosis of EAC cells by inter nucleosomal DNA ladder formation. Moreover when compared to cisplatin as a reference drug, Eu (III)- and Tb(III)-2-thioacetic benzothiazole acid complexes exhibited more significant anticancer activity. From these results it can be concluded that these synthetic compounds have a strong therapeutic potential as anti-tumor and anti-bacterial agents (Belal H.M. Hussein 2012).

**Flavia R.F. Nascimento** *et al.* researched on leaves of *Chenopodium ambrosioides* L. [Chenopodiaceae] (Fmastruz) which have been indicated for the treatment of several type of diseases, among which one is cancer. There are no results focusing the effect of *in vivo* treatment of C. ambrosioides on tumor development. This study was aim to investigate the effect of C. ambrosioides treatment on Ehrlich tumor development. Leave extract of C. ambrosioides were treated by intraperitoneal route (i.p.) with hydro alcoholic on swiss mice in the dose (5 mg/kg) or with PBS (control group) 48 h before or 48 h later the Ehrlich tumor implantation. The tumor cells were implanted either on the left footpad (solid tumor) or in the peritoneal cavity (ascites tumor). Footpad of mice was measured in a interval of 2 days until the fourteenth day, to determine the solid tumor growth when the feet were weighed. After 8 days ascites tumor development was evaluated for tumor implantation by quantification of the ascites fluid volume and tumor cell number. The treatment of C. ambrosioides extract administered i.v. before or after the tumor implantation shows a significant inhibition of the solid and ascites Ehrlich tumor. Similarly this inhibition was observed in ascites tumor cell number, in the ascites volume, in the tumor-bearing foot size and foot weight when compared to control mice. It is also found that treatments shows an increase in the survival of tumor-bearing mice. In conclusion, C. ambrosioides with a small dose has a evident potent anti-tumoral effect and even when the treatment was given two days after the tumor implantation. This effect is also related with anti-oxidant properties of C. ambrosioides (Flavia R.F. Nascimento 2006).

**Juanjuan Yi** *et al.* worked on a bioactive dietary constituents Pinecone polyphenols that enhance health and help prevent and treat cancer by the improvement of antioxidant and immune regulatory activities. This study was basically designed for investigating antitumor, antioxidant

and immunoregulatory activities of the 40% ethanol eluent of polyphenols from pinecone of *pinuskoraiensis* (PPP-40) on *in vivo* Sarcoma 180 (S180)-bearing mice models. A significant inhibitory effect on S180 tumor growth was identified from the results of antitumor activity indicated that PPP-40 and the dose exhibited the highest antitumor activity was 150 mg/kg. Moreover apoptosis of S180 tumor cells was further confirmed by TdT-mediated dUTPnick end labeling (TUNEL) assay results. In addition, PPP-40 could promote the expressions of Bax protein and inhibit the Bcl-2 protein, which will accordingly improve the expressions of activated Caspase-3 as well. This finally results in the activation of mitochondrial apoptotic pathway of tumor cells in S180 mice eventually. S180 mice treated with PPP-40 results antioxidant activity, higher superoxide dismutase (SOD) and glutathione per-oxidase (GSH-Px) activities, the more glutathione (GSH) content, and the lower malon di aldehyde (MDA) level in plasma compared to that of non-treated control group. Moreover, with PPP-40(150 mg/kg) administration a significantly accelerated the solenocytes proliferation ($p < 0.01$) and increased the mono-cyte phagocytosis activity *in vivo* simultaneously. From these results it can be revealed that PPP-40 exerts an effective antitumor activity by mitochondrial apoptotic pathway activation and improving the antioxidant and immunoregulatory activities (Juanjuan Yi 2017).

From the previous studies it was demonstrated that alginates from *Sargassum sp.* (Phaeophyta) showed a considerable anticancer activity against various murine tumors. **Alessandra Paula Alves de Sousa** *et al.* aimed in the present study to investigate brown seaweed *Sargassum vulgare* C at different viscosity for *in vivo* antitumor activity of two alginates (SVHV and SVLV). For this Sarcoma 180 tumor cells are transplanted in mice. Growth of sarcoma 180 was inhibited by both alginates. A more effective route of administration was oral route for both alginates, leading to an inhibition of 51.8 and 74.8% for SVLV at the doses of 50 and 100mg/m2/day, respectively, and of 66.2 and 88.8% for SVHV at the same doses. It is found that with doses at the dose of 100mg/m2/day, SVLV was 2.04 times more active after oral administration, while SVHV was 1.89. As observed by reduction of Ki67 staining in tumor for the treated-animals relation was found that alginates-antitumor activity was related to the tumor proliferation rate inhibition. Due to treatment with SVHV and SVLV, histopathological analysis for liver and kidney showed affected organs. However, acute tubular necrosis was only caused by SVLV. Alginates in treated animals causes the enlargement of the white pulp of the spleen,

which suggests that the antitumor activity observed could be related to alginates immunomodulatory properties (Alessandra Paula Alves de Sousa 2007).

**Meiling Zheng** *et al.* aimed to prepared and characterized Carboxy methyl chitosan (CM-chitosan) by FTIR and NMR spectroscopy, and its biological safety in tumor application was investigated both *in vitro* and *in vivo*. MTT assay was performed for cytotoxicity study, indicates that CM-chitosan was safe both on normal cell L02 and three tumor cell lines: Bel-7402, SGC-7901 and Hela in *vitro*. TGF-β secretion of L02 cell ($P < 0.05$) was improved by CM-chitosan, whereas decreased levels of TGF- β and VEGF secreted by Bel-7402 cell ($P < 0.05$), which are compatible with the observations at cell levels. *In vivo*, sarcoma 180 tumor model was established in mice and they are treated with CM-chitosan which is administered through intraperitoneal injection. Experimental data indicated that CM- chitosan can slightly inhibited growth of sarcoma 180, it is safe for *in vivo* and enhanced body immunity via elevation of serum IL-2 and TNF-β levels in treated mice ($P < 0.05$). These results suggest that in the field of biomedical materials like CM-chitosan is safe for treatment of tumor (Meiling Zheng 2011).

**T.A. Ajith** *et al.* in this work studies the cytotoxic and antitumor activities of ethyl acetate, methanol and aqueous extracts of a wood inhabiting polypore macro fungus, named *Phellinus rimosus* (Berk) Pilat. A good *in vitro* cytotoxic activity against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines was shown by Ethyl acetate and methanol extracts of *Phellinus rimosus* (Berk) Pilat. No cytotoxicity activity against the tested cell lines was observed with the aqueous extract. It is found that in case of solid tumor induced by DLA cell line all the three extracts were highly effective in inhibiting cell growth of in mice. However, a better antitumor activity was shown by ethyl acetate extract than that of methanol and aqueous extracts. The ethyl acetate extract was also effective in preventing the development of cell growth by EAC induced ascites tumor in mice. The antitumor activity was compared between all the three extracts against solid tumor at a dose of 50 mg/kg (i.p.) and clinically used standard reference drug, cisplatin (4 mg/kg, i.p.). Pre-treatment of the extracts was also effective in the inhibition of tumor growth induced by DLA cell lines. From these all experimental results it can be said that ethyl acetate extract of P. rimosus possessed a significant antitumor activity.

The findings helps us to conclude that this mushroom is potential as a antitumor agent (T.A. Ajith 2003).

**Paulo Michel Pinheiro Ferreira** *et al*. studied on *Casearia sylvestris* (Salicaceae) which is found in South America. This possesses antiulcerogenic, cytotoxic, antimicrobial, anti-inflammatory and anti hypertensive activities. In this study author is going to assess *in vivo* and ex vivo antitumor action of a fraction with case arias (FC) and its main component-CasearinX-isolated from C. Sylvestris leaves. Firstly, treatment is done with FC and CasX for 7 days on Sarcoma180 bearing Swiss mice. Secondly, treatment given with FC for 4 days on BALB/nude animals received hollow fibers with colon carcinoma (HCT-116) orglio- blastoma (SF-295)cells. On the $5^{th}$day, cell proliferation was detected by MTT assay. FC given in i.p. dose 10 and 25 mg/kg/day and oral dose 50 mg/kg/day and CasX given in i.p. dose 25 mg/kg/day and oral dose 50 mg/kg/ day revealed tumor growth inhibition rates of 35.8, 86.2, 53.7, 90.0 and 65.5 % and such tumors demonstrated rare mitoses and coagulation necrosis. Similarly in case of FC reduced multiplying of HCT-116 and SF-295 cells when evaluated by the Hollow Fiber Assay (2.5 and 5mg/kg/day i.p. and 25 and 50 mg/ kg/day oral), with cell growth inhibition rate ranging from 33.3 to 67.4 % ($p<0.05$). From the Flow cytometry experiment it was found that FC reduced membrane integrity and induced DNA fragmentation and mitochondrial depolarization ($p<0.05$). So from these results it can be concluded that FC and Cas X were efficient antitumor substances against human cancer cells. It causes reversible morphological changes in liver, kidneys and spleens, emphasizing clerodane di- terpenes as an effective emerging class of anti cancer molecules (Paulo Michel Pinheiro Ferreira 2016).

**Magdy A.H. Zahran** studied, designed and synthesized on a series of 16 novel thalidomide sulfur analogs containing one and two sulfur atoms 2 and 4–18, respectively. These compounds exhibited potent cytotoxic activity on screening with *in vitro* tumor cells of Ehrlich ascites carcinoma (EAC) cell line. On the bases of the results obtained from *in vitro* cytotoxic activity, *in vivo* EAC-induced solid tumor are treated with thalidomide sulfur analogs containing two sulfur atoms 8, 9, 13 and 14 in female mice. In compared to thalidomide 1 it is found that analog 2 exhibited a highly significant reduction in tumor volume (TV). As it is found that level of hepatic lipid peroxidation decreased and levels of antioxidant enzymes like superoxide dismutase

(SOD) and catalase were elevated so it illustrate the antioxidative activity of these compounds. From the histopathological studies against solid tumor it can be said that thalidomide sulfur analogs 2, 8, 9, 13 and 14 have antimitotic, apoptotic and necrotic activities. These compounds lead in the increase of Fas-L expression. From the immunohistochemical studies, results shows a good decrease in Ki67 and vascular endothelial growth factor (VEGF) staining in tumor cells from treated-animals in compared to that of the non-treated groups. This fact suggests an inhibition of tumor proliferation rate and antigenic process associated with tumor growth. Compounds 9 and 13 were the most potent compounds as it caused tumor necrosis but no significant liver necrosis where as on treatment with compound 9 resulted in liver degeneration (Magdy A.-H. Zahran 2008).

**References:**

A. Madhusudhan, G. Bhagavanth Reddy, M. Venkatesham, G. Veerabhadram. 2012. "Design and Evaluation of Efavirenz loaded Solid Lipid Nanoparticles to Improve the Oral Bioavailability." *International Journal of Pharmacy and Pharmaceutical Science Research* (International Journal of Pharmacy and Pharmaceutical Science Research) 2(4): 84-89: 2(4): 84-89.

A.J. Ruby, G. Kuttan, K. Dinesh Babu, K.N. Rajasekharan, R. Kutta. 1995. "Anti-tumour and antioxidant activity of natural curcuminoids." *Cancer Letters* 94: 79-83.

Adrita Chakrabarti, Dipa Talukdar, Aparajita Pal, Manju Ray. 2014. "Immunomodulation of macrophages by methylglyoxal conjugated with chitosan nanoparticles against Sarcoma-180 tumor in mice." *Cellular Immunology* 287: 27–35.

Alessandra Paula Alves de Sousa, Márcia Rocha Torres, Claudia Pessoa, Manoel Odorico de Moraes, Francisco Dario Rocha Filho, Ana Paula Negreiros Nunes Alves, Letícia Veras Costa-Lotufo. 2007. "*In vivo* growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed Sargassum vulgare." *Carbohydrate Polymers* 69: 7–13.

Belal H.M. Hussein, Hassan A. Azab, Mona F. El-Azab, Abdullah I. El-Falouji. 2012. "A novel anti-tumor agent, Ln(III) 2-thioacetate benzothiazole induces anti-angiogenic effect and cell death in cancer cell lines." *European Journal of Medicinal Chemistry* 51: 99-109.

Carla Vitorino, Filomena A. Carvalho, António J. Almeida, João J. Sousa, Alberto A.C.C. Pais. 2011. "The size of solid lipid nanoparticles: An interpretation from experimental design." *Colloids and Surfaces B: Biointerfaces* 84:117–130.

Dianrui Zhang, Tianwei Tan, Lei Gao. 17: 5821–5828. "Preparation of oridonin-loaded solid lipid nanoparticles and studies on them *in vitro* and in *vivo.*" *Nanotechnology* 2006.

Fahmy, Ghada Abdelbary and Rania H. 2009. "Diazepam-Loaded Solid Lipid Nanoparticles: Design and Characterization." *American Association of Pharmaceutical Scientists* 10(1): 211-219.

Flavia R.F. Nascimento, Gustavo V.B. Cruz, Paulo Vitor S. Pereira, Marcia C.G. Maciel. 2006. "Ascites and solid Ehrlich tumor inhibition by Chenopodium ambrosioides L. treatment." *Life Sciences* 78, 2650–2653.

Haining Tan, Shenglin Yang, Chunhui Liu, Jichao Cao, Guoying Mu, Fengshan Wang. 2012. "Enhanced anti-angiogenesis and anti-tumor activity of endostatin by chemical modification with polyethylene glycol and low molecular weight heparin." *Biomedicine & Pharmacotherapy* 66: 648–654.

Juanjuan Yi, Hang Qu, Yunzhou Wu, Zhenyu Wang, Lu Wang. 2017. "Study on antitumor, antioxidant and immunoregulatory activities of the purified polyphenols from pinecone of Pinus koraiensis on tumor-bearing S180 mice *in vivo*." *International Journal of Biological Macromolecules* 94: 735–744.

Kopparam Manjunath, Vobalaboina Venkateswarlu. 2005. "Pharmacokinetics, tissue distribution and bioavailability of clozapine solid lipid nanoparticles after intravenous and intraduodenal administration." *Journal of Controlled Release* 107: 215–228.

Magdy A.-H. Zahran, Tarek A.-R. Salem, Rehab M. Samaka, Hussein S. Agwa, Ayman R. Awad. 2008. "Design, synthesis and antitumor evaluation of novel thalidomide dithiocarbamate and dithioate analogs against Ehrlich ascites carcinoma-induced solid tumor in Swiss albino mice." *Bioorganic and Medicinal Chemistry* 16: 9708–9718.

Meiling Zheng, Baoqin Han, Yan Yang, Wanshun Liu. 2011. "Synthesis, characterization and biological safety of O-carboxymethyl chitosan used to treat Sarcoma 180 tumor." *Carbohydrate Polymers* 86: 231– 238.

Mustafa Cengiz, H. Mehtap Kutlu, Dilek D. Burukoglu, Adnan Ayhancı. 2015. "A comparative study on the therapeutic effects of Silymarin and Silymarin-Loaded Solid Lipid Nanoparticles on D-GaIN/TNF-α-induced Liver Damage in Balb/c Mice." *Food and Chemical Toxicology* 77: 93–100.

Neha Yadav, Sunil Khatun, Udai Vir Singh Saha. 2013. "Solid lipid nanoparticles- a review." *International Journal of Applied Pharmaceutics* 5(2): 8-18.

Nehal M. Elsherbiny, Nahla N. Younis, Mohamed A. Shaheen, Mohamed M. Elseweidy. 2016. "The synergistic effect between vanillin and doxorubicin in ehrlich ascites carcinoma solid tumor and MCF-7 human breast cancer cell line." *Pathology – Research and Practice* 212: 767–777.

Paulo Michel Pinheiro Ferreira, Daniel Pereira Bezerra, Jurandydo Nascimento Silva *et al.* 2016. "Preclinicalanticancereffectivenessofafractionfrom Casearia sylvestris and itscomponentCasearinX: *in vivo* and ex vivo methods and microscopyexaminations." *Journal ofEthnopharmacology* 186: 270–279.

Rahul Nair, Ashok CK Kumar, Vishnu K Priya, Chakrapani M Yadav and Prasanna Y Raju. 2012. "Formulation and evaluation of chitosan solid lipid nanoparticles of carbamazepine." *Lipids in Health and Disease* 11(72): 1-8.

Rohan Shah, Daniel Eldridge, Enzo Palombo, Ian Harding. 2014. "Optimisation and Stability Assessment of Solid Lipid Nanoparticles using Particle Size and Zeta Potential." *Journal of Physical Science* 25(1): 59–75.

S.K. Sahoo, S. Parveen, J.J. Panda. 2007. "The present and future of nanotechnology in human health care." *Nanomedicine* 3: 20– 31.

Samein, Laith Hamza. 2014. "PREPARATION AND EVALUATION OF NYSTATIN LOADED-SOLID-LIPID NANOPARTICLES FOR TOPICAL DELIVERY." *International Journal of Pharmacy and Pharmaceutical Sciences* 6(2): 592-597.

Senthil Kumar P, Arivuchelvan A, Jagadeeswaran A, Subramanian N, Senthil Kumar C, Mekala P. 2015. " Formulation, optimization and evaluation of enrofloxacin solid lipid nanoparticles for sustained oral delivery. ." *Asian journal of pharmaceutical and clinical research* 8(1): 231-236.

Shengpeng Wang, Tongkai Chen, Ruie Chen, Yangyang Hu, Meiwan Chen, Yitao Wang. 2012. "Emodin loaded solid lipid nanoparticles: Preparation, characterization and antitumor activity studies." *International Journal of Pharmaceutics* 430: 238– 246.

Surajit Das, Wai Kiong Ng, Parijat Kanaujia, Sanggu Kim, Reginald B.H. Tan,. 2011. "Formulation design, preparation and physicochemical characterizations of solid lipid nanoparticles containing a hydrophobic drug: Effects of process variables." *Colloids and Surfaces B: Biointerfaces* 88:483– 489.

T.A. Ajith, K.K. Janardhanan. 2003. "Cytotoxic and antitumor activities of a polypore macrofungus, Phellinus rimosus (Berk) Pilat." *Journal of Ethnopharmacology* 84: 157-162.

YiFan Luo, DaWei Chen, LiXiang Ren, XiuLi Zhao, Jing Qin. 2006. "Solid lipid nanoparticles for enhancing vinpocetine's oral bioavailability." *Journal of Controlled Release 114 (2006) 53–59* 114: 53–59.

# CHAPTER 1

# Extraction and Isolation of Phytochemical PITC-2 from Medicinal Plant *Pluchea indica*

## Plant Collection:

The plant *Pluchea indica* (L.) Less belongs to the family of Asteraceae. It is Tan evergreen large shrub found abundantly in salt marshes and mangrove swamps in Sunderbans (India), Bangladesh, Myanmar, China, Philippines, Malaysia, Tropical Asia and Australia (Chakraborty and Chatterjee 2017). The plant was collected in the month of August 2015, from the garden Agri-Horticultural Society of India, Alipur, Kolkata where they were planted after *in vitro* regeneration cultured in the laboratory. Originally this tissue cultured explant was collected from mangrove forest of Sunderban, West Bengal, India. The plant was then identified and authenticated from Botanical Survey of India, Central Natural Herberium, Howrah-711103, Ministry of Environment, Forest & Climate Change, Government of India. Its Specimen No. is JU/SG-001 and Reference No. is CNH/2017/Tech.II/37.

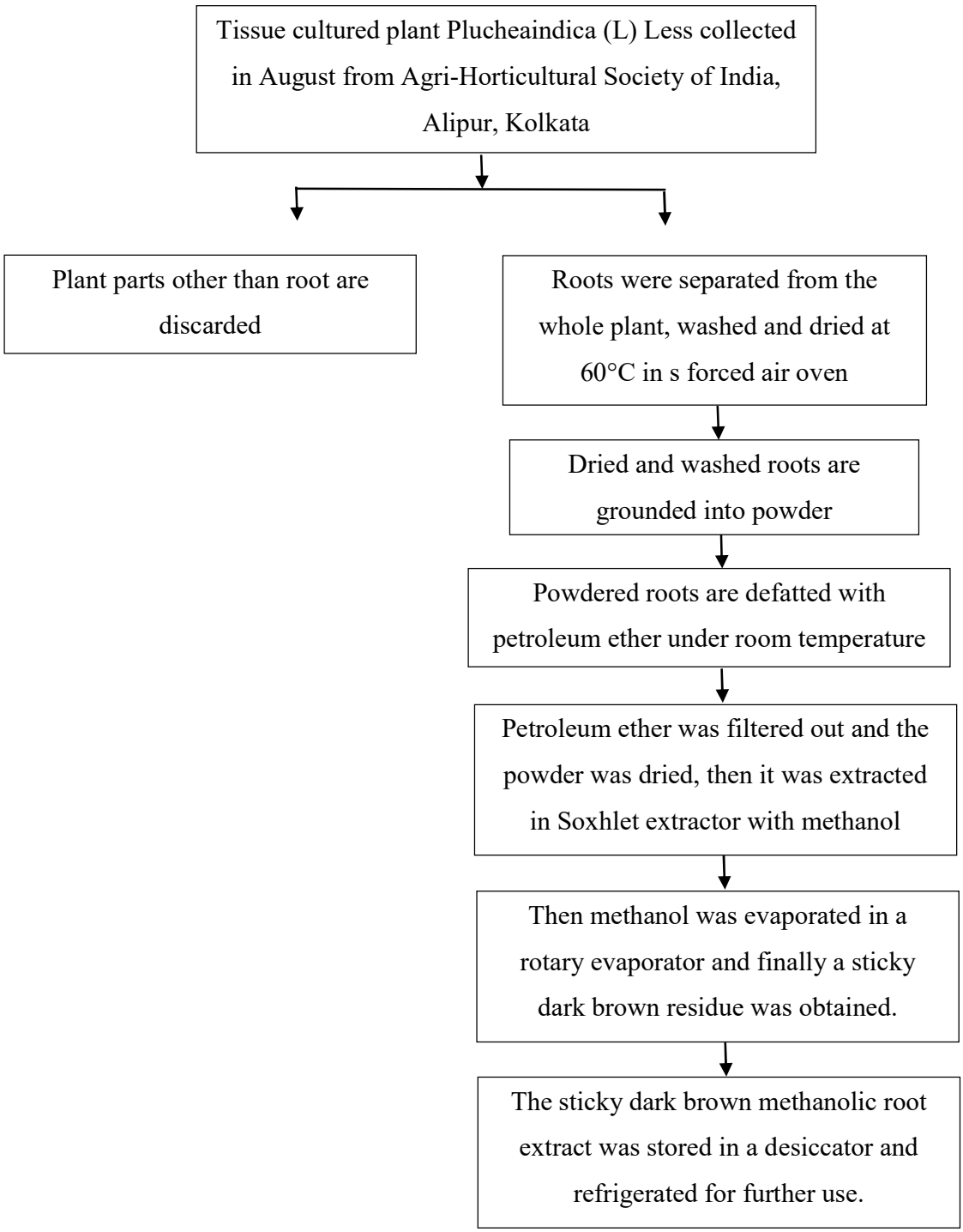
## Plant Material Preparation:

After collection of plants, roots are separated from the whole plant. The roots after being separated from the whole plant were washed with water and again re washed with distilled water to separate all unwanted discards and debris in it. Then it was dried at 60°C in a forced air oven. Dried roots were then grounded into powder. Finally the powders are preserved in airtight containers for further use.

## Extraction:

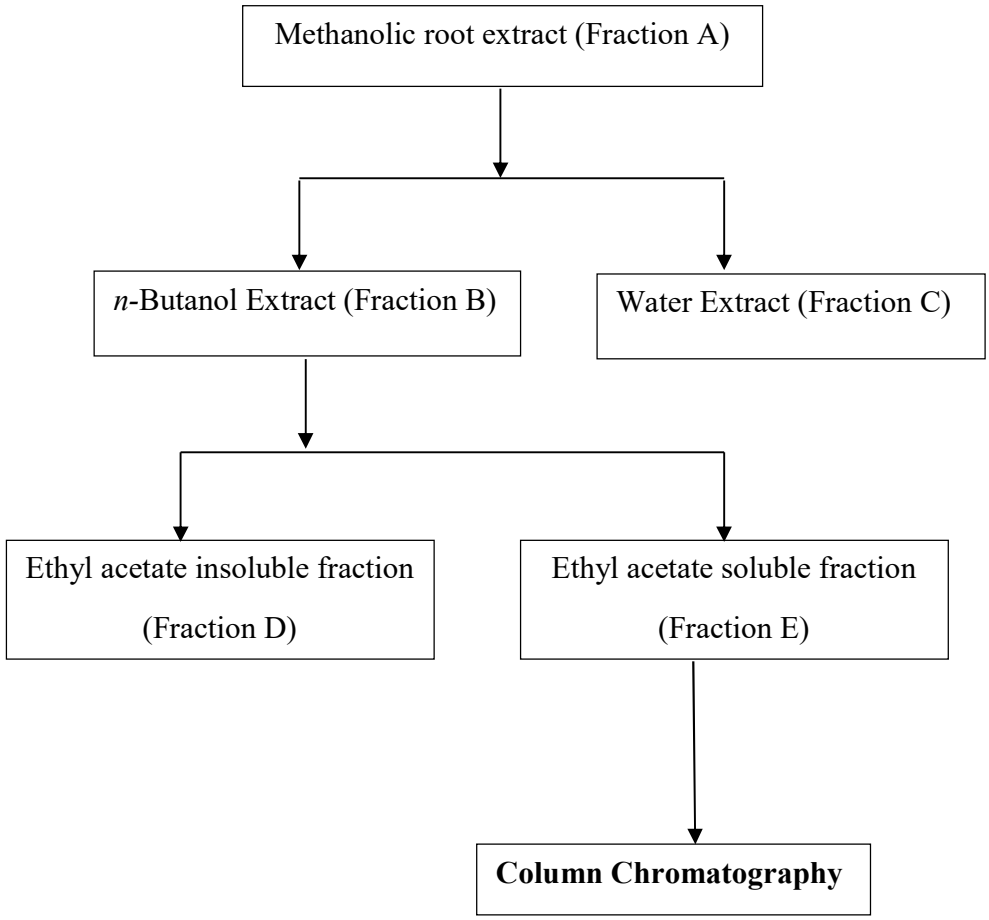
The powered root of *Pluchea indica* was first defatted. 500 g of powder was kept soaked in petroleum ether (for synthesis) under room temperature. The solvent was filtered out and the dried powder root was collected. Then it was extracted in a soxhlet extracting apparatus with methanol (AR Grade, 99.9% pure) to obtain methanolic root extract of plant *Pluchea indica.* Then the solvent was evaporated under reduced pressure using a rotary evaporator. After evaporation of the methanol a sticky dark brown residue was obtained. The yield of the extract was 9.1% (w/w)(43 gm). This was stored in a desiccators and refrigerated at 4°C for future use.

**Collection and extraction of *Pluchea indica*:**



## Isolation:

For isolation of PITC-2 from the methanolic root extract of the plant, it was first fractioned between water and n-butanol. The solvents are then separately evaporated and the butanol fraction is taken for further use. The butanol fraction was then shaken with ethyl acetate to get a ethyl acetate soluble and insoluble part. Ethyl acetate insoluble part was filtered out and soluble part is taken and solvent was evaporated. Then the ethyl acetate soluble residue (10g) was stored in refrigerator for further use in column chromatography to isolate desired compound PITC-2(Biswas R 2007).




Fractionation of methanolic root extract of tissue cultured medicinal plant *Pluchea indica* (L) Less.

For column chromatography silica gel used was 60-120 mesh. Eluents used in this chromatography was petroleum ether and ethyl acetate mixture with increasing polarity. Column was prepared and sample was loaded at the top of the column. First only Petroleum Ether was used as a eluent in the process of column chromatography. Then ethyl acetate (EA) and petroleum ether (PE) mixture was used in a ratio of PE:EA = 9.5 : 05. Then concentration of ethyl acetate was increased slowly. At a ratio 8:2 of pet ether and ethyl acetate from fraction 14 – 23 a prominent spot was observed in the TLC plate which was the desired compound PITC-2. Then the solvent was evaporated and PITC-2 was collected which is light yellowish in color (80mg, relative amount = 0.000015 mg w/w)(Biswas R 2007)(Kartick C. Pramanik 2009). The presence of light yellow solid PITC-2 was further confirmed by testing the isolated compounds on NMR spectroscopy and MASS spectroscopy.

***Table 1:*** Column chromatography fractions.

| Fractions | Solvent Ratio | Result |
|---|---|---|
| 1-4 | PE | No spot |
| 5-9 | PE : EA = 9.5 : 0.5 | No spot |
| 10-13 | PE : EA = 9 : 1 | Very light spot |
| **14-18** | **PE : EA = 8.5 : 1.5** | **A prominent spot** |
| **19-23** | **PE : EA = 8 : 2** | **A prominent spot** |
| 24-28 | PE : EA = 7.5 : 2.5 | A mixture of spots |
| 29-34 | PE : EA = 7 : 3 | A prominent spot |
| 35-37 | PE : EA = 6 : 4 | A mixture of spots |
| 38-41 | PE : EA = 6 : 4 | A prominent spot |

## PITC-2:

The melting point of the compound was recorded as 106 – 108°C which was recorded before as the melting point of PITC-2. In MASS spectroscopy a strong pick was observed at *m/z* 230. Molecular weight of compound PITC-2 is 230 shown in Fig 2. So the present peak in mass spectroscopy is may be for PITC-2 which will be confirmed by NMR shown in Fig 3. In $^{1}H$ NMR $\delta^{TMS}$ ($CDCl_3$, 300MHz): 1.64 ($D_2O$ exchangeable, OH merged with solvent $H_2O$),

2.04(3H, s), 3.78($^1$H, dd, J=11.4, 6.3Hz, $H_A$ of CH-$CH_2$OH), 3.82($^1$H, dd, J=11.4, 3.9 $H_Z$, $H_B$ of CH-$CH_2$OH), 4.69 ($^1$H, dd, J=6.3, 3.9Hz,CHOH--$CH_2$OH), 7.04, 7.18 ($^2$H, m, thiophene-H). The $^1$H NMR spectrum showed peaks for a methyl attached to unsaturation δ (2.04, s), a CHX-$CH_2$Y unit and an aromatic system. The compound finally identified is PITC-2 which is 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene shown in Fig 1.

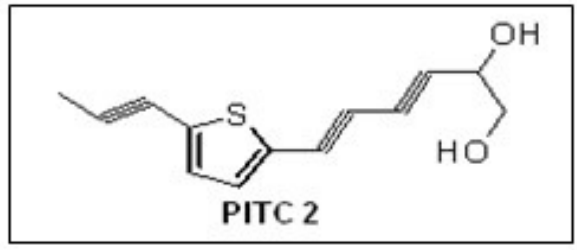



***Figure 1:*** 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene.

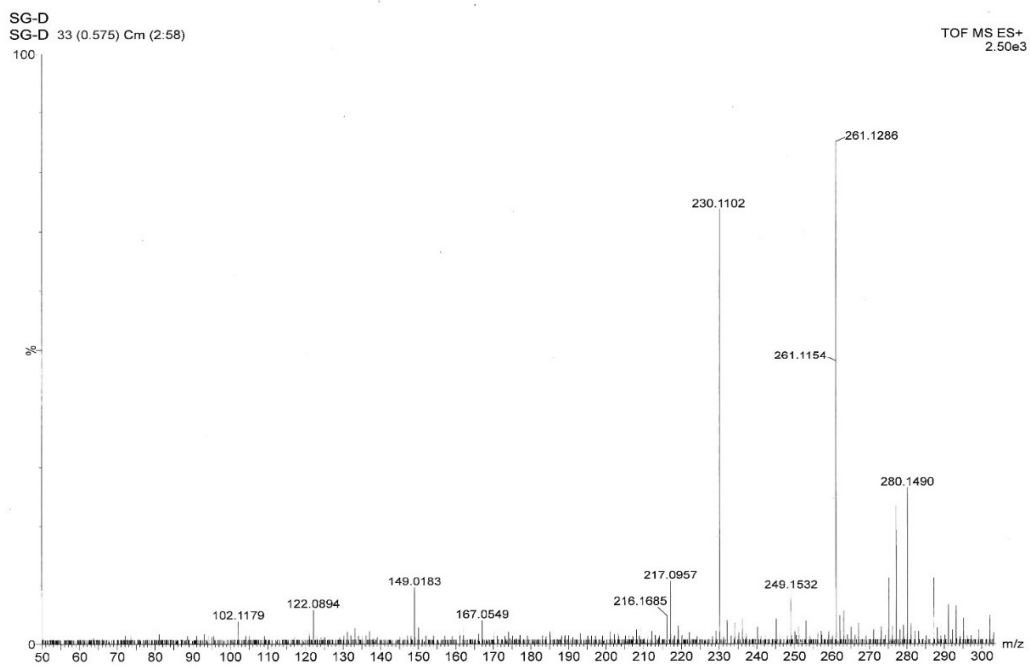



***Fig 2***: MASS spectroscopy of PITC-2.

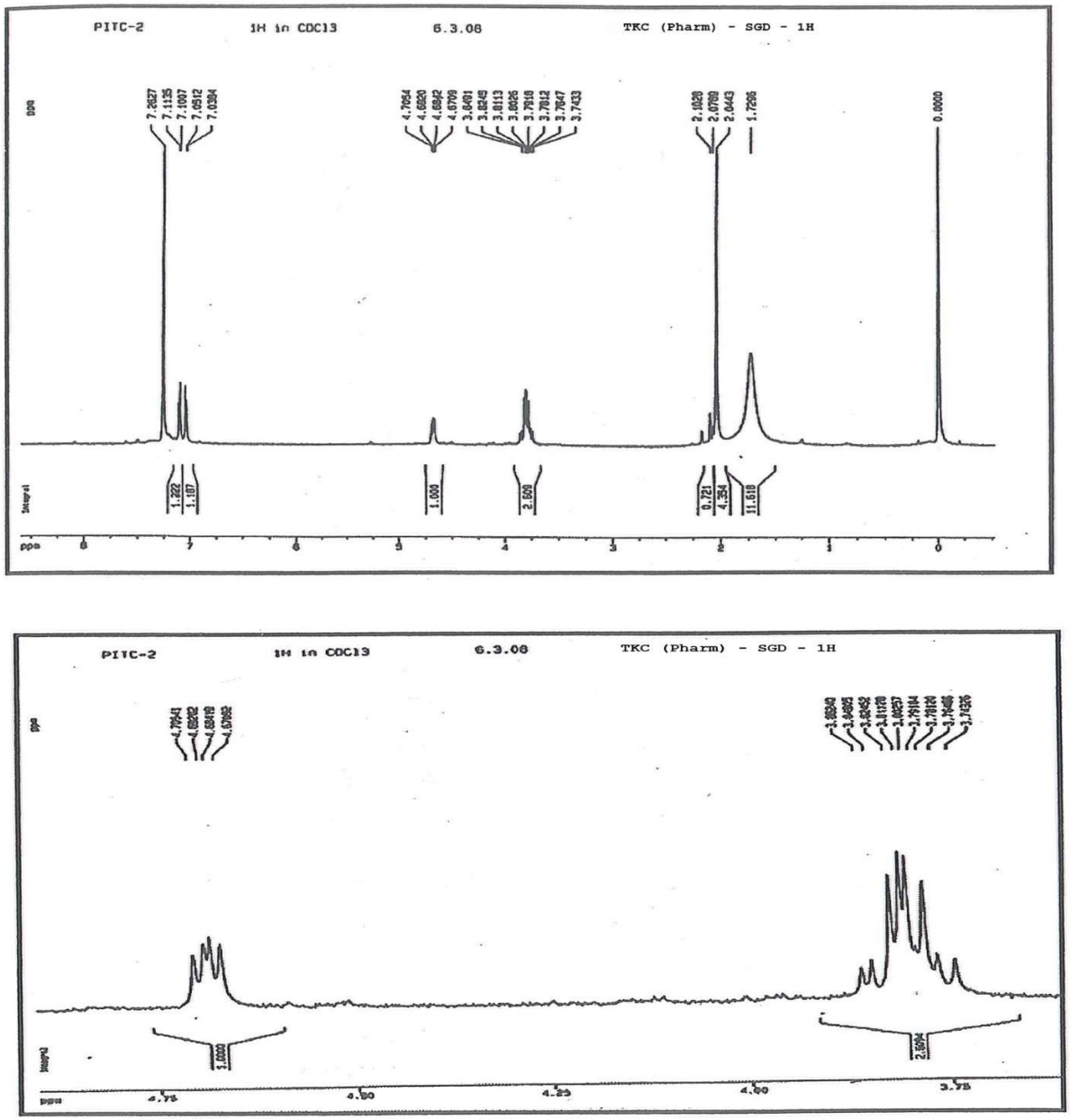



***Figure 3:*** $^{1}$H NMR spectrum of compound PITC-2 in $CDCL_3$ (300MHz)

# Reference:

R Biswas, D Bandyopadhyaya, M Mishraa , KC Pramanika, TK Chatterjee. "Isolation of pure compound R/J/3 from Pluchea indica (L.) Less. and its anti-amoebic activities against Entamoeba histolytica." Phytomedicine, 2007: 14: 534-537.

Sharmili Chakraborty and T. K. Chatterjee. "Targeting viseral leishmaniasis by mannosylated liposome incorporated PITC-2 isolated from the plant Pluchea indica (L) Less." Int J Pharm Bio Sci, 2017: 8(2): 396-408.

Kartick C. Pramanik, Tapan K. Chatterjee. "Isolation, characterization and sub-acute toxicity studies of a new compound PITC-2 isolated from tissue cultured medicinal plant *Pluchea indica*(L) Less ." *International Journal of Biomedicine and Pharmaceutical Sciences*, 2009: 3(1), 50-54.

# CHAPTER 2

# Anti –tumor Activity of phytochemical PITC-2 on Sarcoma-180 Solid Tumor Model in Mice

# Material and Methodology:

## Cell lines and cell culture:

*In-vitro* cancer cell lines were obtained from National Centre for Cell Science (NCCS), Pune, India and Sarcoma-180 cancer cells were collected from Chittaranjan National Cancer Research Institute (CNCRI), Kolkata, India. The *in-vitro* cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS at 37°C in $CO_2$ incubator in an atmosphere of humidified 5% $CO_2$ and 95% air. The cells were maintained by routine sub culturing in tissue culture flasks. Tumor cell counts were done using the trypan blue dye exclusion method. Cell viability was always found to be 95% or more.

## Cytotoxicity Assay (MTT assay):

This test is based on MTT (3-(4, 5- dimethylthiazol -2-yl)-2, 5-diphenyl tetrazolium bromide), which is reduced to a purple-blue soluble formazan by the living cells. Experiments were performed in 96-well flat bottomed culture plates (BD Biosciences, USA). MTT was dissolved in phosphate buffered saline (PBS) at 5mg/ml. Different concentrations of PITC-2 (0.1, 0.5, 01, 5,10,20,25, 30 µg/ml) were added and the plate was incubated for 24 hr. Following incubation with each compound for 24 h, 20 $\mu$L of MTT was added to each well. After incubation for 4 h at 37 °C, the culture medium was removed, and the formazan crystals were dissolved in 200 $\mu$L DMSO (Sinha A, 2014). Absorbance (A) of formazan dye was measured at 570 nm using a micro plate reader. The background absorbance was determined at 690 nm and subtracted from the 570 nm measurement. (Abd-El Fattah AA, 2017)

The percentage of viable cells was determined by the following equation: (Joseph MM, 2014)

$$Viablecells(\%) = \frac{A\ of\ treatedcells}{A\ of\ untreatedcells} \times 100$$

The cytotoxic activities of this compound PITC-2 were determined in human breast cancer cell MCF7, human lung cancer cell A549, human macrophage cell U937 and Sarcoma S-180.

## Animals and study groups:

*In vivo* studies were carried out on female Swiss albino mice weighing 22 ± 0.50g of either. The animals were kept under standard conditions of 12:12 hr light and dark cycle in polypropylene cages and fed with standard laboratory diet and water *ad libitum.* As the phytochemical PITC-2 was not freely soluble in water so 1% DMSO solution was prepared to make it solubilize. All *in vivo* experiments were conducted as per the guideline of Animal Ethics Committee of the Department of the Pharmaceutical Technology of Jadavpur University, India (Registration number: 147/1999/CPCSEA).

For experimental purpose animals are divided into 5 groups each containing 12 mice.

Group I: Normal animals (negative control).

Group II: Sarcoma cancer animals (positive control) + 1% DMSO 5ml/kg/day.

Group III: Sarcoma cancer animals (Test 1) + PITC 2 (2.5mg/kg/day given orally)

Group IV: Sarcoma cancer animals (Test 2) + PITC 2 (5mg/kg/day given orally)

Group V: Sarcoma cancer animals (Standard) + 5FU (20mg/kg in alternative day given i.p).

## Solid tumor induction by Sarcoma 180 (S – 180) cell line and experimental protocol:

Sarcoma 180 cells were preserved *in vivo* in the peritoneal cavity of mice by intra peritoneal inoculation of cells in sterile normal saline. (Pal A, 2009) Then solid tumors were inoculated in the left hind leg of mice by intra-muscular injection of S – 180 cells (0.2 ml of 2 x 106 cells/mouse) to all the groups intra peritonially except the normal group. (Nascimento FR, 2006). Treatment was given from day 8 after inoculation of S-180 tumor for 21 days. (Chakraborty A, 2014)From each group 6 mice were sacrificed and other 6 were kept for calculation of Mean survival time.

## Recording of survival rate:

Mean survival time and percentage increase in life span (%ILS) were calculated by the following formulas. (Bala A, 2010)

$$\text{Mean survival time (MST)} = \frac{\sum \text{Survival time(day) of each mice in a group}}{\text{total number of mice}}$$

$$\%\text{ILS} = \left[\frac{\text{MST of treated mice}}{\text{MST of control mice}} - 1\right] \times 100$$

## Tumor volume:

In case of solid tumor, tumor volumes were recorded using a Vernier Calipes and calculated as V(mm3) = (a2 x b)/2 where a (smaller diameter) and b (large diameter) are perpendicular. (Gaballah HH, 2017). Tumor growth inhibition ratio was calculated as follows: Inhibition ratio (%) = [(A - B) / A] x 100 where A is average tumor weight in negative control and B is average in each treatment group. (de Costa PM, 2015) But in case of ascetic tumor, peritoneal fluid was collected from the peritoneal cavity. Then the volume was measured by taking it in a graduated centrifuge tube.

## Hematological Parameters:

Hematological studies were performed by recording the hemoglobin content, red blood cell (RBC) and white blood cells (WBC) counts from freely flowing tail vein blood (Gupta M, 2004). Differential WBC leukocyte counts were carried out including neutrophils, lymphocytes and monocytes from leishaman stained blood smears of normal, Sarcoma 180 control and PITC 2 treated groups respectively.

## Biochemical Parameters:

After 24 hr of last dose and 18 hr of fasting the blood sample were collected by puncturing retro-orbital plexus. Blood was used for the assay of biochemical parameters serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, globulin, bilirubin, alkaline phosphatase and serum protein to investigate liver function alteration and creatinine was measured as renal function parameter.

## Histopathology:

The tumor, liver and kidney were excised and fixed in 10% buffered formalin and embedded in paraffin. +5-µm sectioned was stained with hematoxylin and eosin (H&E) (Elsherbiny NM, 2016). Histological analysis was performed under light microscope (40X). The morphological changes were photographed and then compared.

## Immunohistochemical analysis of Bcl-2, Ki-67, Cyclin-D1:

Tumor section were deparaffinized with xylene and dehydrated with ethanol. After blocking the slides with 5% bovine serum albumin (BSA) in Tris-buffered saline, the sections were then stained with primary antibody specific to Bcl-2, Ki-67 and Cyclin-D1, at a concentration of 1µg/ml at 4oC overnight, followed by mouse monoclonal antibody. After washing sections with PBS they were incubated with diaminobenzidine substrate and counter stained with Mayer's hematoxylin. Then they were visualized in a digital camera installed on a light microscope. (Abd-El Fattah AA, 2017) (Gaballah HH, 2017)

## Statistical analysis:

For cytotoxicity studies, the IC50 values and 95% confidence intervals were obtained by nonlinear regression. All *in vitro* studies were carried out in triplicate represented by independent biological evaluation. Data are extracted as mean ± standard error of the mean (SEM) and were compared by one way analysis of variance (ANOVA) followed by Kruskal Wallis Test using SPSS statistical software of 20.0 version. $P<0.05$ was considered to be statistically significant when compared with control.

# Result:

## *In-vitro* cell viability assay:

The phytochemical PITC 2 was screened for its cytotoxicity against human breast cancer cell MCF7, human lung cancer cell A549, human macrophage cell U937 and Sarcoma S-180at different concentrations (0.1, 0.5, 01, 5,10,20,25, 30 µg/ml) to determine the percentage of viable cells by MTT assay. MTT assay shows significant effect on Sarcoma S-180, MCF-7,A-549 and U-937 cancer cells (Figure 1). These results indicates anti-proliferative activity of PITC-2 in cancer cells. Among these cancer cells PITC-2 is most potent for U-937 cells.

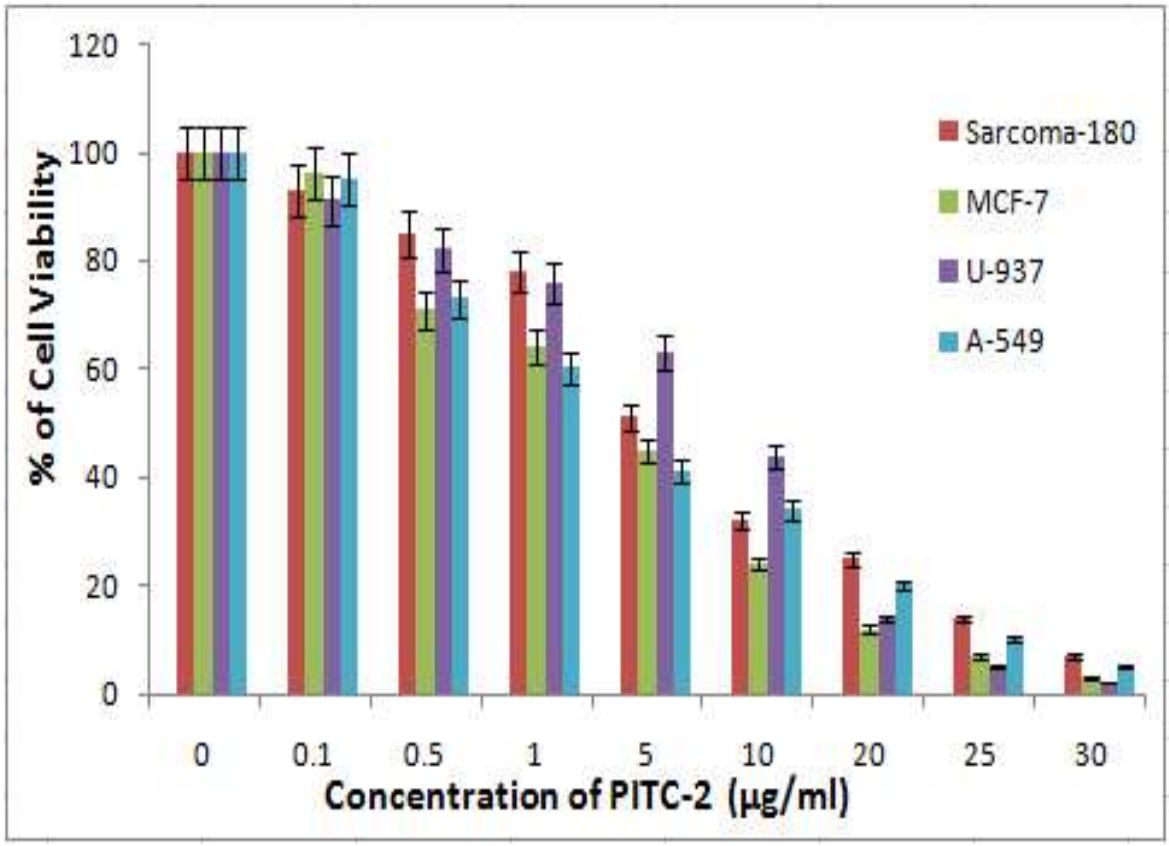



***Figure 1*****:** *In vitro* cytotoxic activity of PITC-2 against Sarcoma 180, MCF-7, U-937 and A-549. Values are expressed as mean ± standard deviation.

## Inhibition of Sarcoma-180 solid tumor growth by PITC-2:

PITC-2 at concentrations 2.5 mg/kg and 5 mg/kg shows good reduction of solid tumor volume and weight at the end of 21 days compared to that of control group mice. PITC-2 treated groups also shows significant reduction of body weight. (Figure 2)(Table 1)

***Table 1*** **:** *In-vivo* effect of PITC-2 on solid tumors and study groups

| | PITC-2 2.5mg/kg b.w | PITC-2 5mg/kg b.w | Std drug 5-FU 20mg/kg b.w | Sarcoma 180 Control |
|---|---|---|---|---|
| Tumor weight (gm) | 1.156 ± 0.03* | 0.649 ± 0.008* | 0.890 ± 0.03* | 3.90 ± 0.11* |
| % Tumor volume | 1900.75 | 1563.10 | 1823.64 | 6642.30 |
| Tumor inhibition ratio % | 71.38 ± 1.72* | 76.46 ± 0.27* | 72.54 ± 0.96* | 0 ± 0 |
| Body weight (gm) | 24.05 ± 0.43* | 22.71 ± 0.42* | 23.68 ± 0.55* | 27.10 ± 0.48* |
| MST (days) | 34.67 ± 0.98* | 52.83 ± 0.70* | 42.00 ± 0.85* | 19.50 ± 0.42* |
| %ILS | 77.79 | 170.92 | 115.38 | 0 ± 0 |

*<0.05 as compared to control group (n = 6 mice per group)

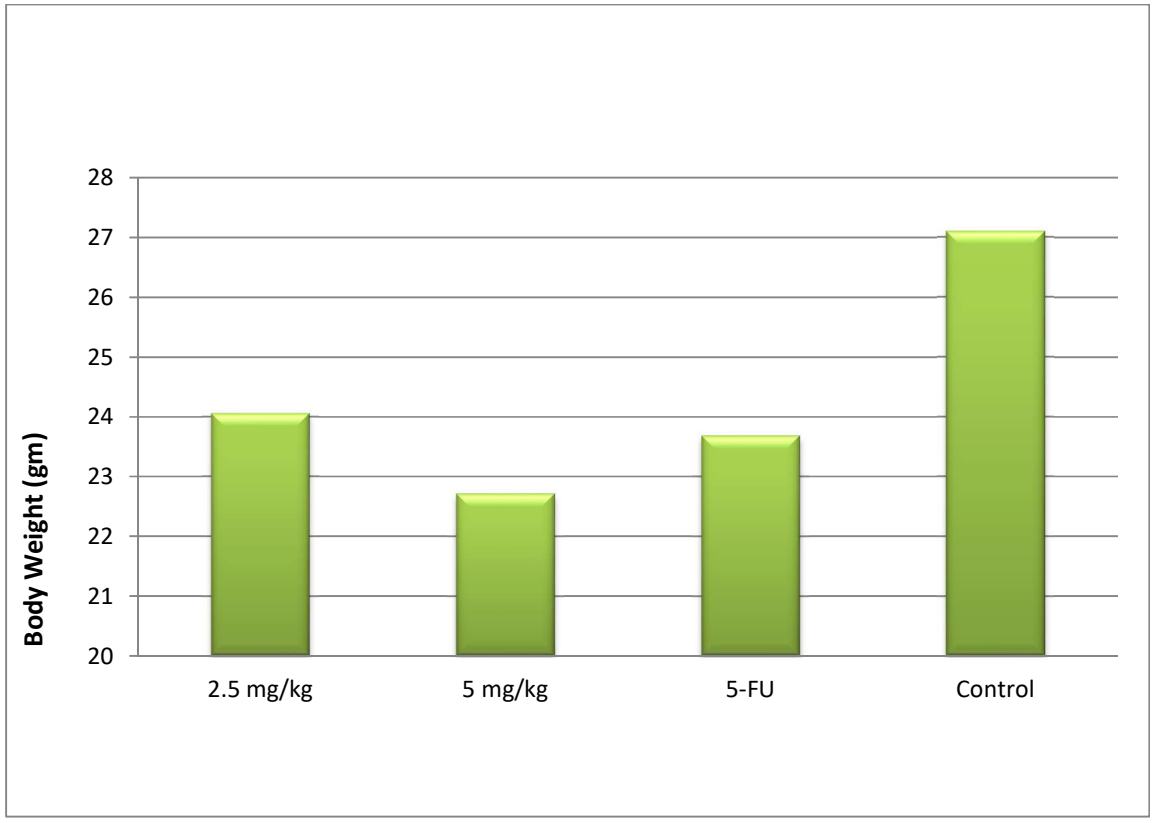



***Figure 2*** **:** Effect of PITC-2 treatment on body weight

## PITC-2 enhanced survival of sarcoma 180 bearing mice:

As shown in Table 1 and Kaplan Meier survival curve (Figure 3) a significant increase in survival time and life span of sarcoma 180 bearing mice treated with PITC-2 was found in comparison with sarcoma bearing control group. The %ILS was found to be dose dependent (Figure 4).

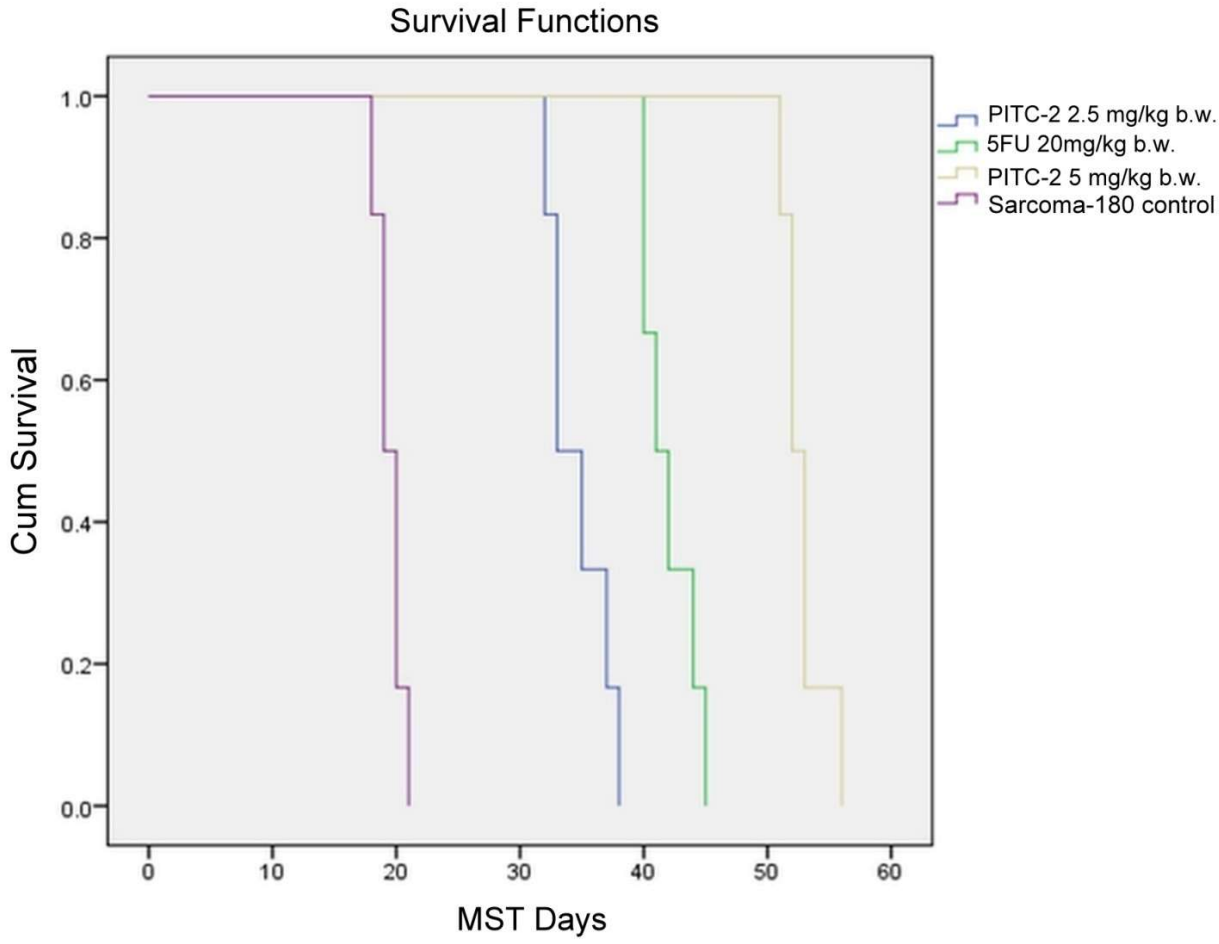



***Figure 3:*** Kaplan Meier survival curve showing survival of all groups

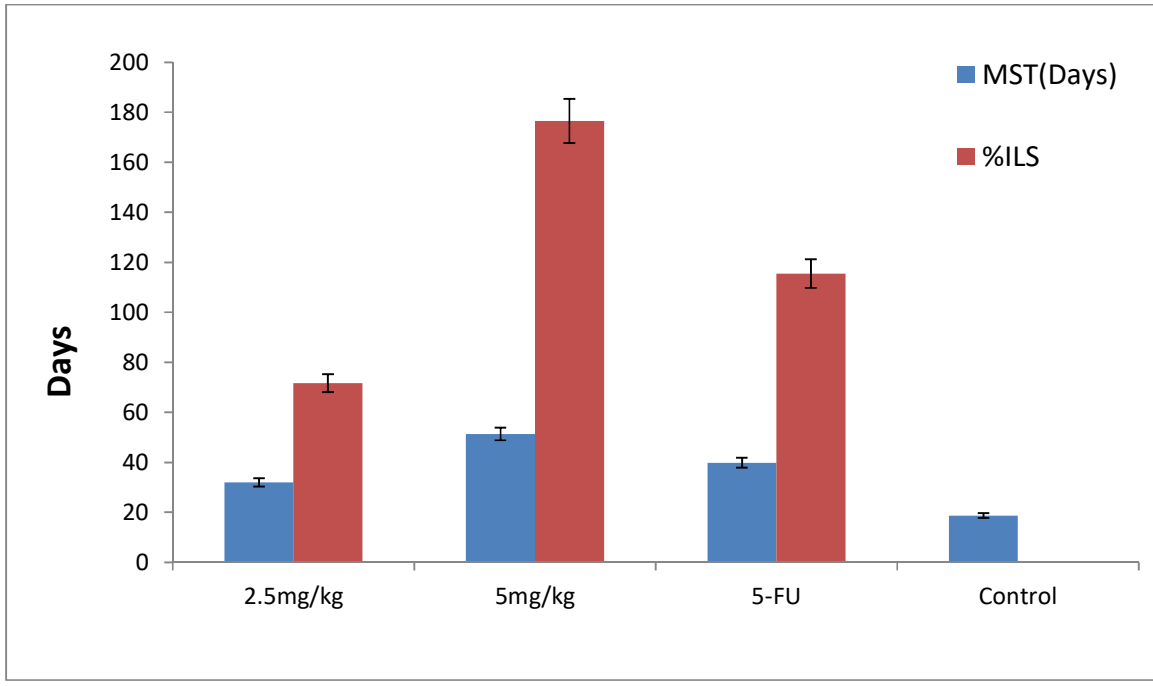



***Figure 4*** **:** Effect of PITC-2 treatment on mean survival time (MST), percentage of increase life span (%ILS)

## Effect of PITC-2 on hematological parameters:

An increase in hemoglobin level, RBC count, neutrophil level and decrease in WBC level, monocyte and lymphocyte count was found in PITC 2 treated groups in a dose dependent manner in compared to that of sarcoma 180 control group (Table 2).

***Table 2*** **:** Effect of PITC-2 on hematological parameters in Sarcoma-180 solid tumors bearing mice

| | PITC-2 2.5mg/kg b.w | PITC-2 5mg/kg b.w | Std 5-FU 20mg/kg b.w | Sarcoma 180 control | Normal Mice |
|---|---|---|---|---|---|
| Hemoglobin (gm %) | 7.5 ± 0.05* | 10.4 ± 0.03* | 8.5 ± 0.08* | 6.3 ± 0.01* | 12.8±0.12* |
| Erythrocyte(RBC) (cells x $10^6$/$mm^3$) | 4.34 ± 0.04* | 6.53 ± 0.02* | 5.6 ± 0.03* | 3.75 ± 0.04* | 7.90±0.02* |

| | | | | | |
|---|---|---|---|---|---|
| Leucocytes(WBC) (cells x $10^6$/$mm^3$) | 14.1±0.05* | 10.19± 0.05* | 4.29 ±0.05* | 16.6±0.02* | 6.40±0.03* |
| Neutrophil (%) | 42.3±0.03* | 39.46±0.03* | 30.1 ± 0.02* | 68.5 ±0.05* | 19.4±0.03* |
| Lymphocyte (%) | 48.0 ± 0.04* | 51.0 ± 0.04* | 63.0 ± 0.09* | 35.8 ± 0.11* | 64.1±0.23* |
| Monocyte (%) | 1.5 ± 0.03* | 1.4 ± 0.03* | 1.7 ± 0.03* | 1.3 ± 0.04* | 2.8 ± 0.01* |

*<0.05 as compared to control group (n = 6 mice per group)

## Effect of PITC-2 on biochemical parameters:

PITC-2 treated groups shows significant decrease in SGPT, SGOT and serum alkaline phosphatase parameters are found in a dose dependent manner (2.5 and 5 mg/kg b.w) in comparison with sarcoma 180 control group. A dose dependent increase in total protein was also found (Table 3).

***Table 3*** *:* Effect of PITC-2 on biochemical parameters in Sarcoma-180 solid tumors bearing mice

| | PITC-2 2.5mg/kg b.w | PITC-2 5mg/kg b.w | Std 5-FU 20mg/kg b.w | Sarcoma 180 control | Normal Mice |
|---|---|---|---|---|---|
| Bilirubin (total) mg/dl | 0.20±0.02* | 0.26 ± 0.02* | 0.29 ± 0.03* | 0.34 ± 0.01* | 0.4 ± 0.02* |
| Serum protein (total) g/dl | 8.12±0.09* | 9.69 ± 0.07* | 5.22 ± 0.12* | 15.4±0.08* | 8.19±0.08* |
| AST (SGOT) U/L | 0.71±0.01* | 0.60 ± 0.01* | 0.59 ± 0.01* | 0.6 ± 0.09* | 0.8 ± 0.01* |
| ALT(SGPT) U/L | 351 ± 0.04* | 175 ± 0.01* | 178 ± 0.06* | 199 ± 0.07* | 414 ± 0.04* |
| Serum alkaline | 73 ± 0.02* | 68 ± 0.02* | 18 ± 0.05* | 40 ± 0.03* | 74 ± 0.03* |

| | | | | | |
|---|---|---|---|---|---|
| phosphatase U/L | | | | | |
| Creatinine Mg/dl | 1 ± 0.01* | 1 ± 0.01* | 1 ± 0.01* | 1 ± 0.02* | 1 ± 0.01* |

*<0.05 as compared to control group (n = 6 mice per group)

## Histopathology of liver and kidney tissue:

Figure 5A shows H&E stained section of liver of healthy mice which bears all the normal features, including circular hepatic portal vein and of hepatic artery, as marked by arrow. It shows prominent nuclei and the tissue section comprises of hepatic sinusoid which are usual. But in case of Sarcoma-180 control group showed in Figure 5B none of the above mentioned regular features are observed, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Group receiving standard 5-FU shows lesser amount of hepatocellular lesion, which are close to normal as shown in Figure 5C. Although little changes are found like deformed hepatic artery and irregular bile duct. Groups receiving different dose of PITC-2 also shows a little hepatic deformation and altered hepatocyte population shown in Figure 5D and 5E. Healthy hepatocytes were observed, regular branch of bile duct found, although mild dilation of central vein observed.

Figure 6A shows H&E stained section of kidney of a healthy mice which bears all the normal features, while Sarcoma-180 control group showed in Figure 6B shows severe glomerular and tubular hemorrhage with increase swelling of tubular epithelium and presence of hyaline cylinders. Group treated with 5-FU and PITC-2 shows cellular features which have less deformation and close to normal in Figure 6C, 6D and 6E respectively with light glomerular and tubular hemorrhage.

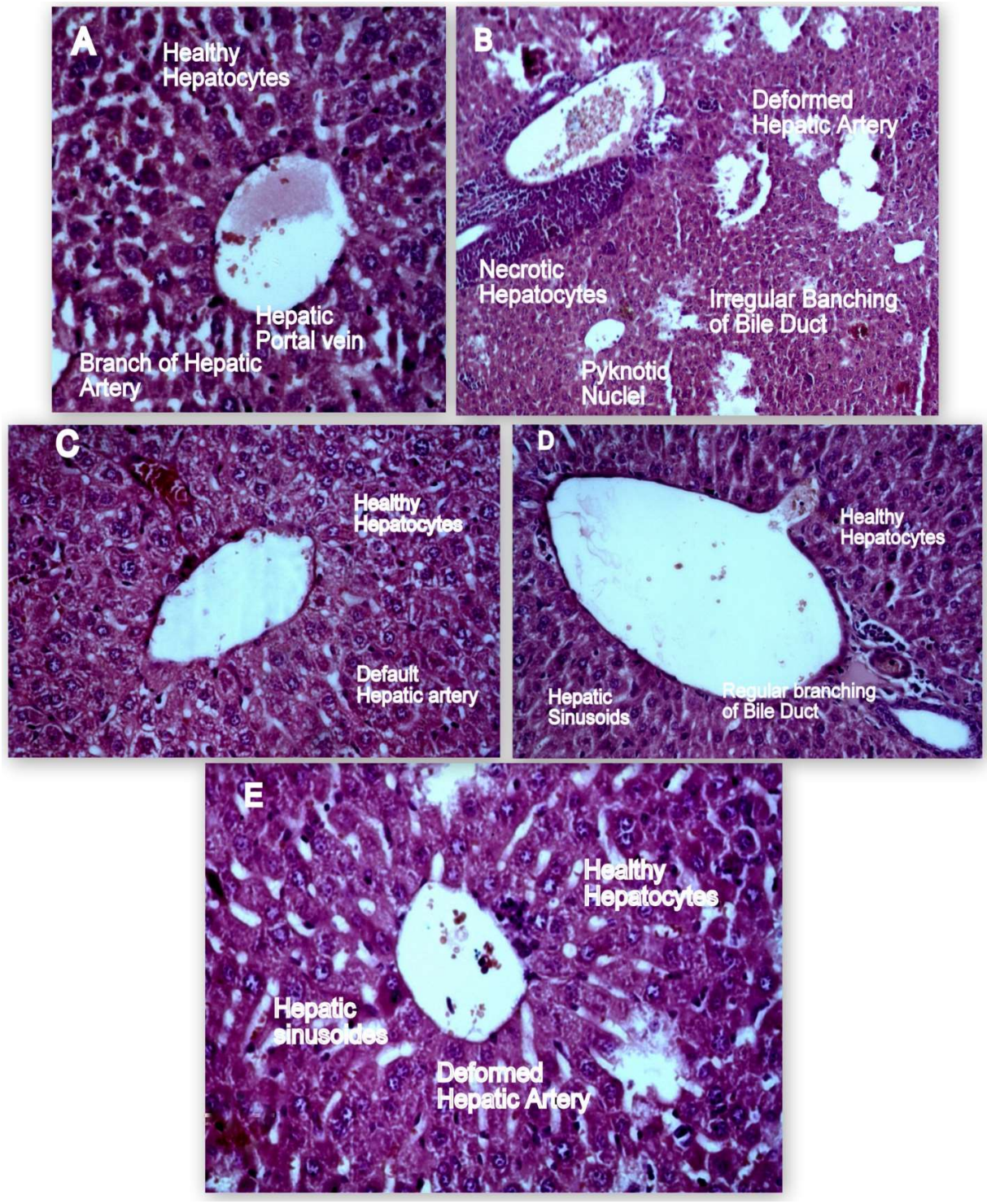



***Figure 1*** **:** H&E stained section of liver for A) normal healthy mice, B) Sarcoma-180 control group, C) Standard 5-FU treated group, D) 2.5 mg/kg of PITC-2, E) 5 mg/kg of PITC-2

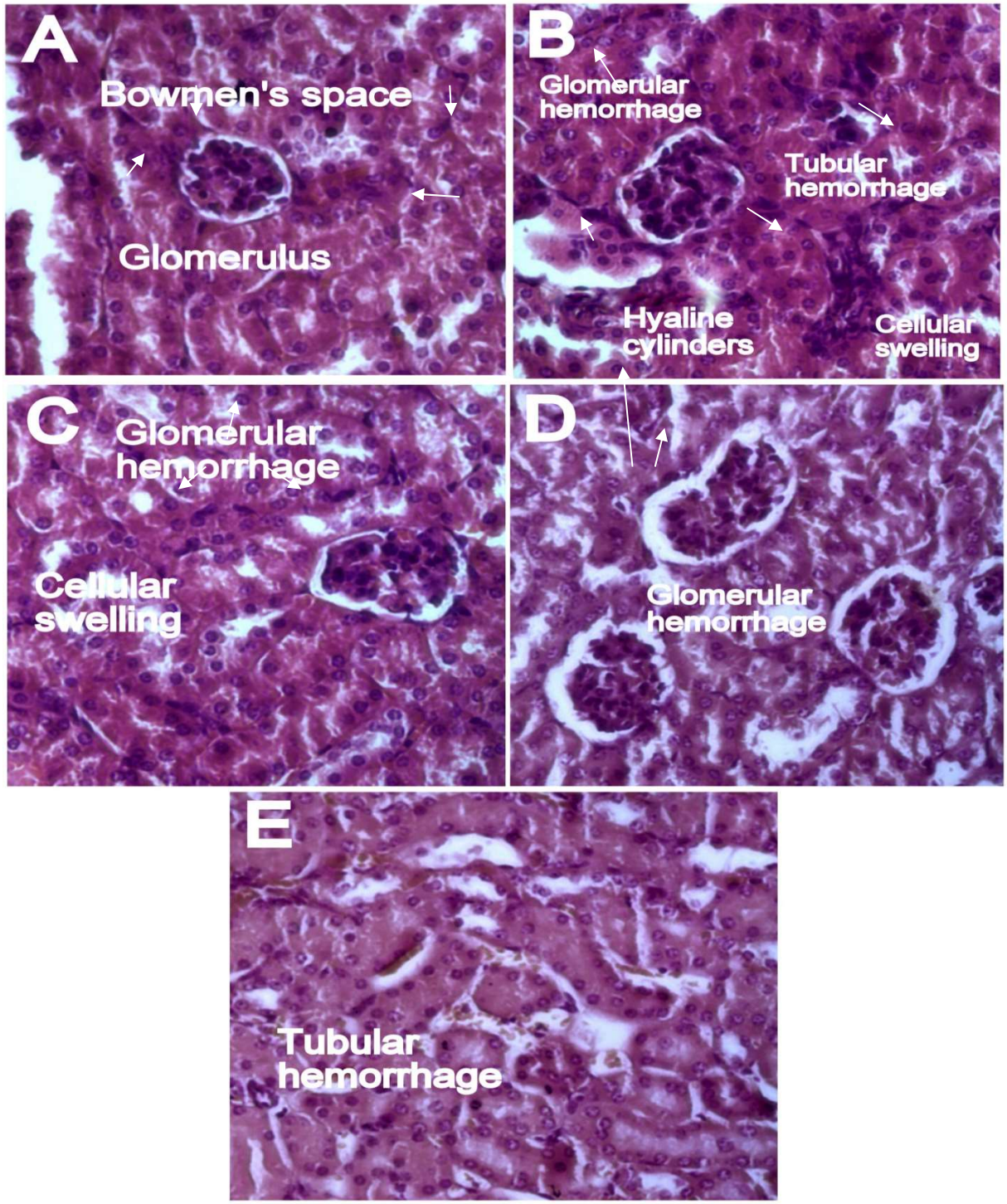



***Figure 2*** **:** H&E stained section of kidney for A) normal healthy mice, B) Sarcoma-180 control group, C) Standard 5-FU treated group, D) 2.5 mg/kg of PITC-2, E) 5 mg/kg of PITC-2

## Histopathological changes of solid sarcoma-180 tumor:

Histopathological examination of solid sarcoma-180 tumor of control group shows intact anaplastic cancer cells without any necrosis and focal hemorrhage reflecting viability and aggressiveness of tumor (Figure 8A). It shows sheets of large rounded and polygonal cells with hyper chromatic nuclear and binucleation. Whereas solid tumor of group treated with 5-FU (Figure 8B) and PITC-2 with different doses (2.5 mg/kg and 5 mg/kg) (Figure 8C and Figure 8D) shows areas of necrosis. Low cell proliferation and muscle invasion were found and with increase in dose tumor cells loose their details and architecture.

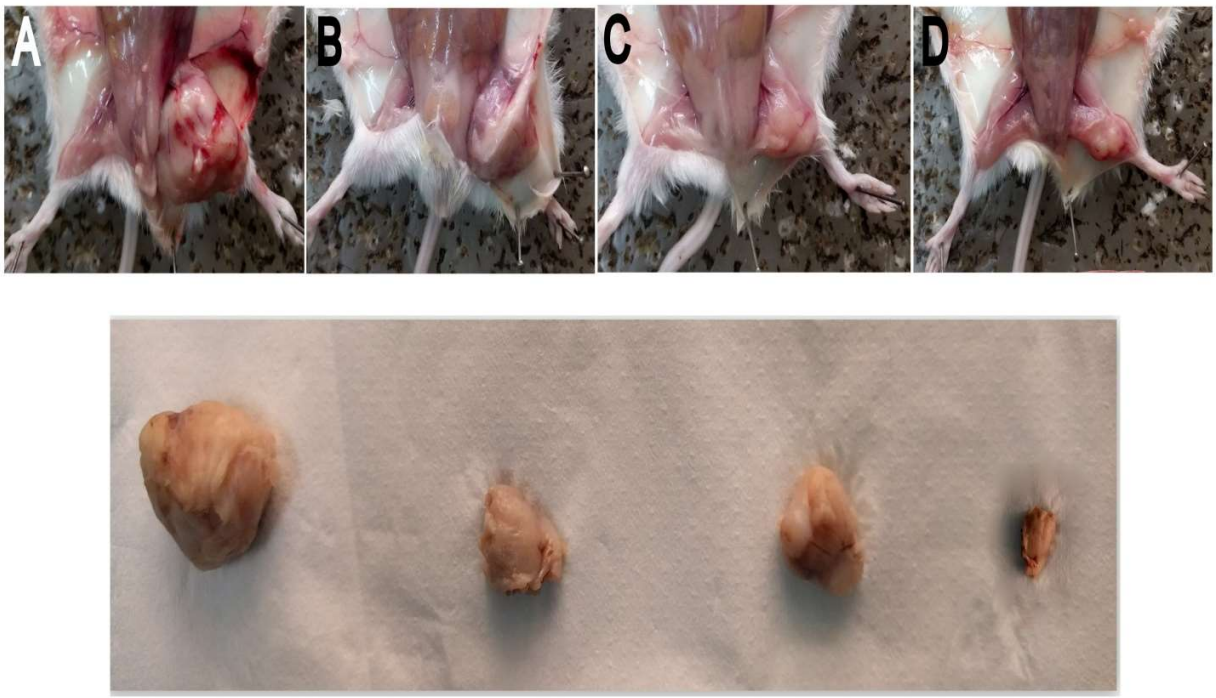



***Figure 7*** **:** Effect of PITC-2 treatment on Solid Sarcoma-180 tumor: A) Sarcoma-180 control group, B) Standard 5-FU treated group, C) 2.5 mg/kg of PITC-2, D) 5 mg/kg of PITC-2, panel 1) shows tumor growth on animals and panel 2) shows tumor isolated from animal body.

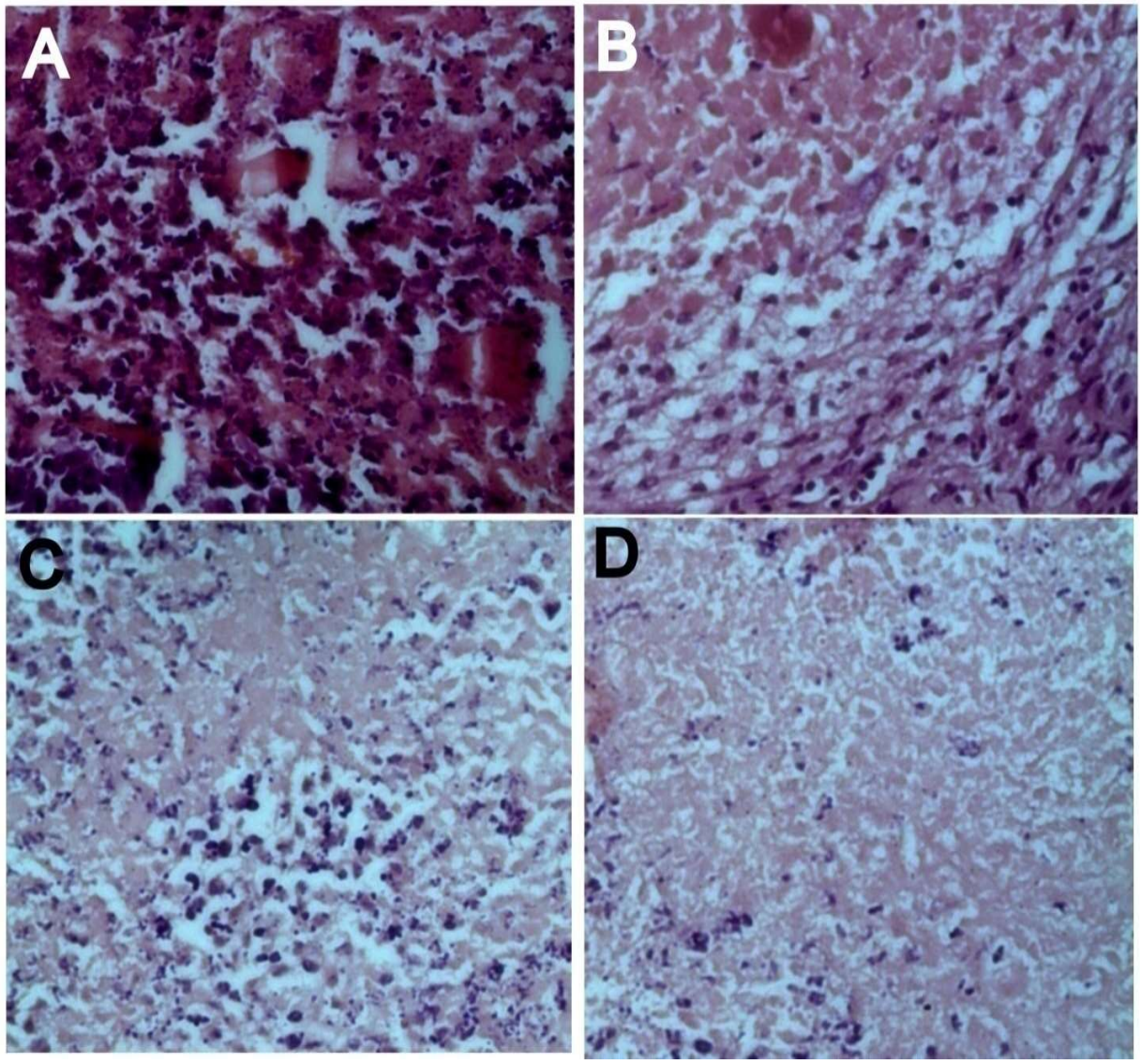



***Figure 8*** **:** H&E stained histological section of solid Sarcoma-180 tumor by treatment with PITC-2: A) Sarcoma-180 control group, B) Standard 5-FU treated group, C) 2.5 mg/kg of PITC-2, D) 5 mg/kg of PITC-2. The cellular features are marked by circle.

## Immunohistochemical analysis of Bcl-2, Ki-67, Cyclin-D1:

The tumor cells were considered positive Bcl-2, Ki-67 and Cyclic-D1 with presence of yellow brown nuclear staining in Ki-67 (Figure 9) and greenish brown nuclear staining in Bcl-2 (Figure 10) and Cyclic-D1 (Figure 11). Here we are studying the regression in tumor growth by PITC-2,

due to apoptosis that decreases expression of anti apoptotic protein Bcl-2, G1 cell cycle arrest by suppression of Cyclic-D1 cell cycle protein. Also PITC-2 reduces tumor cell proliferation by decreasing Ki-67 tumor proliferation protein. A significant decrease in positive Bcl-2, Ki-67 and Cyclic-D1 were found in PITC-2 treated group, compared to that of Sarcoma-180 control group was observed which shows effective suppression of tumor. Meanwhile it was found that the decrease in protein expression is dose dependent and is better in case of PITC-2 with dose 5mg/kg b.w.

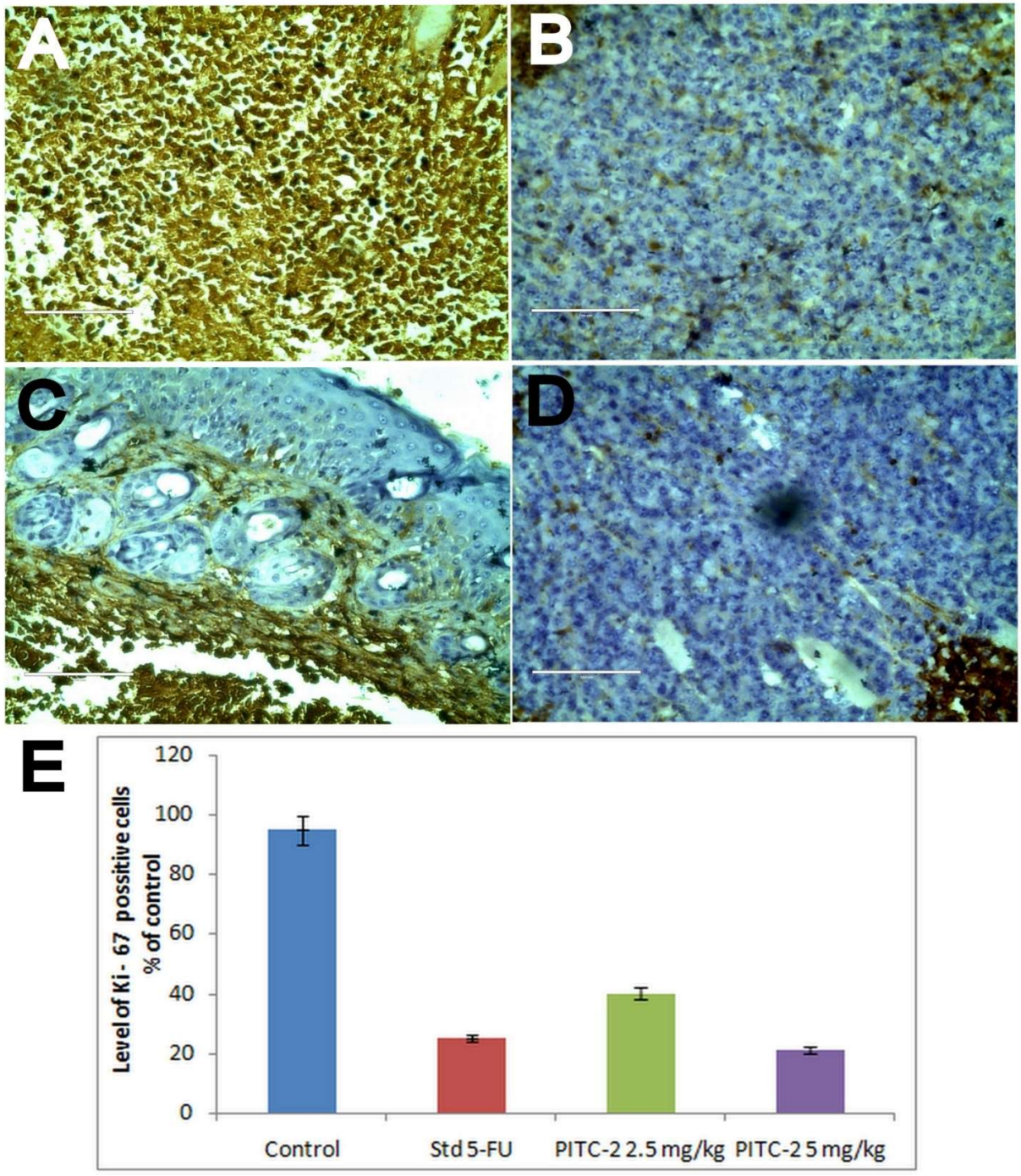



***Figure 9*** : A) Sarcoma-180 control, B) Std 5-FU, C) PITC-2 2.5 mg/kg, D) PITC-2 5 mg/kg shows Ki67 expression (Magnification 40 X). E shows level of immunopositive Ki67 cells, pvalues are given in mean ± SD.

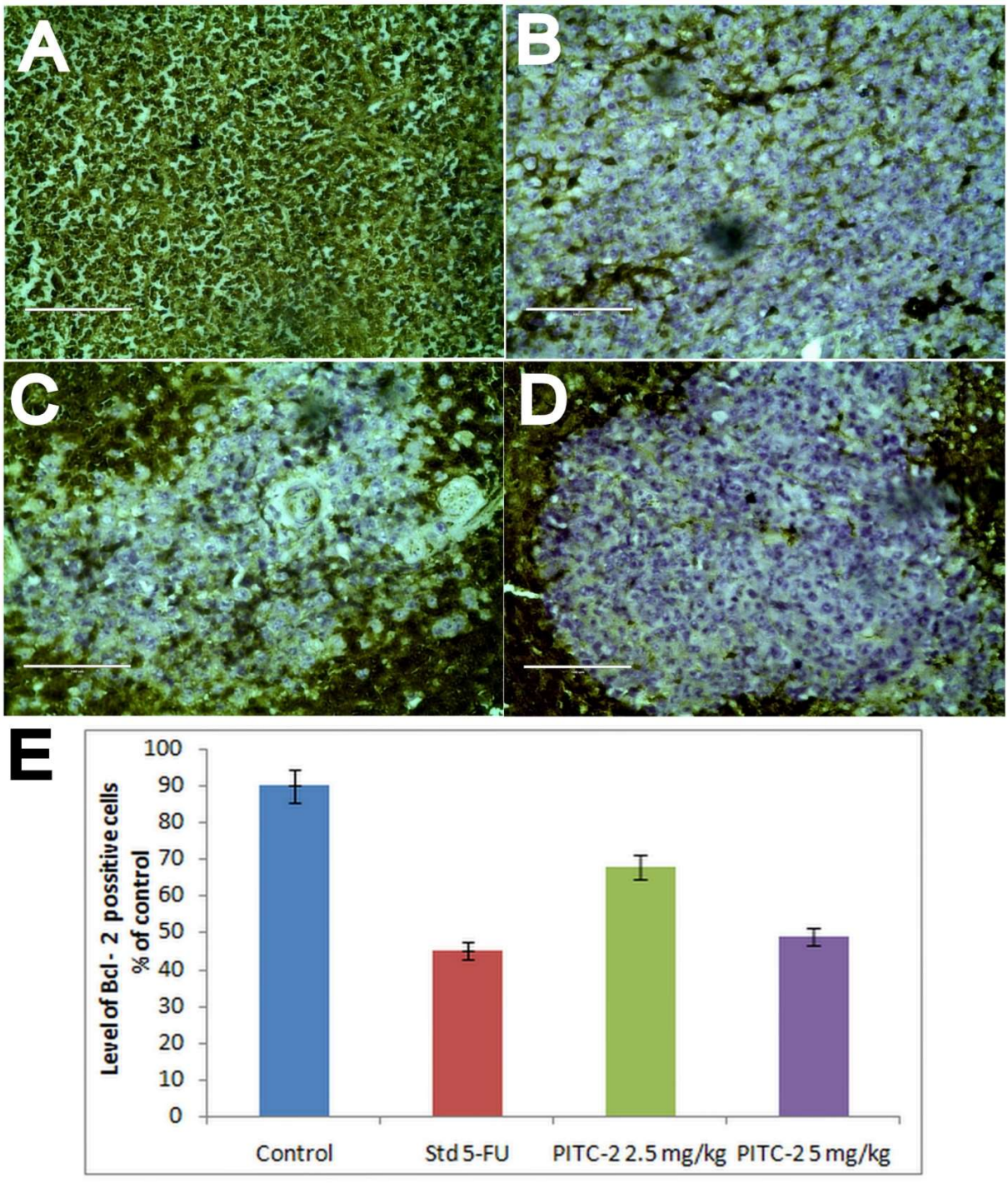



***Figure 10*** : A) Sarcoma-180 control, B) Std 5-FU, C) PITC-2 2.5 mg/kg, D) PITC-2 5 mg/kg shows Bcl-2 expression(Magnification 40 X). E shows level of immunopositive Bcl-2 cells, values are given in mean ± SD.

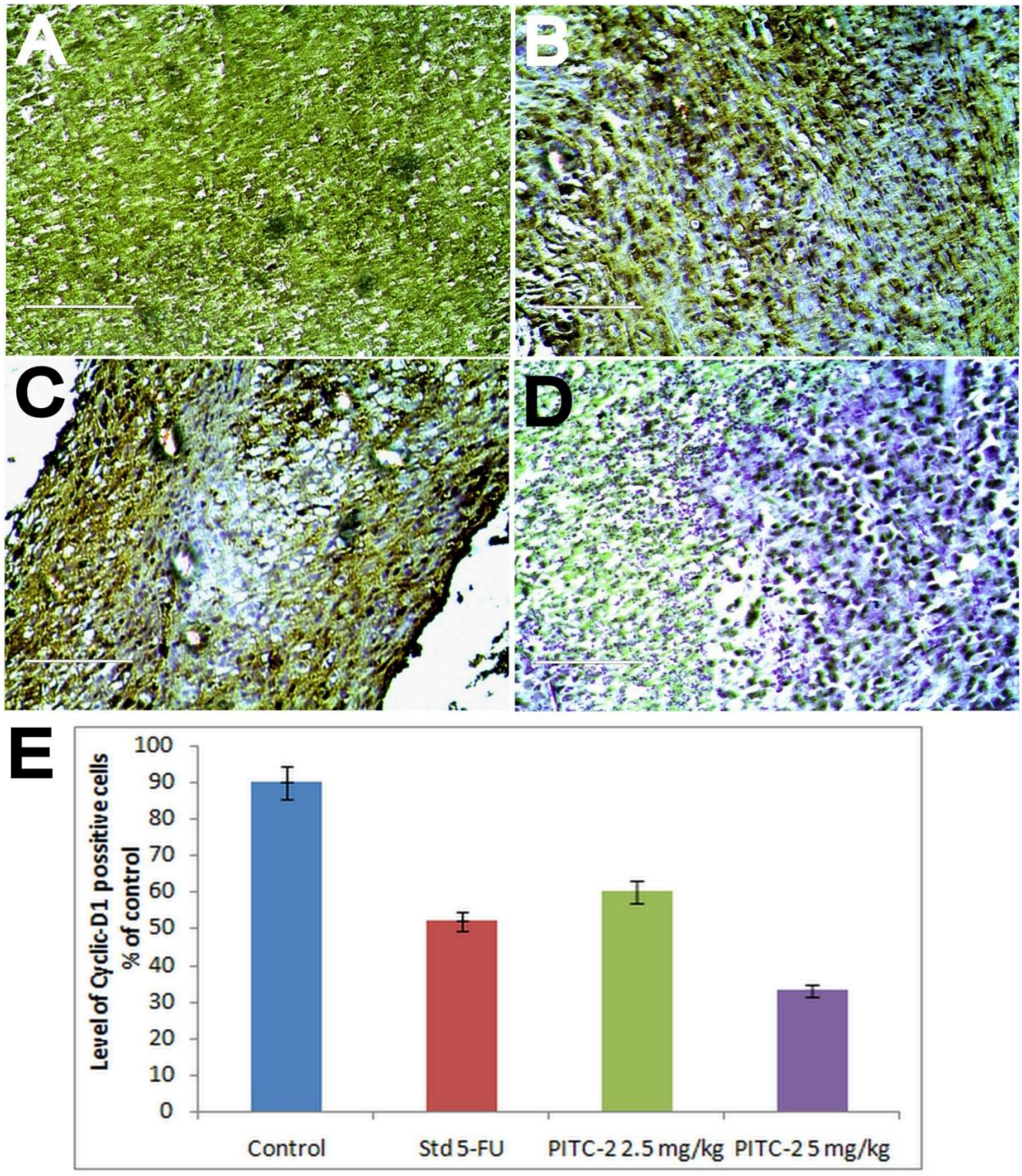



***Figure 11*** **:** A) Sarcoma-180 control, B) Std 5-FU, C) PITC-2 2.5 mg/kg, D) PITC-2 5 mg/kg shows Cyclic-D1 expression(Magnification 40 X). E shows level of immunopositive Cyclic-D1 cells, values are given in mean ± SD.

## Discussion:

Cancer is a group of disease which involves abnormal growth of cell which is rapid and uncontrolled with the potential to spread in other parts of the body. Anticancer drugs or antineoplastic drugs are drugs effective in the treatment of malignant disease which trigger signaling mechanism that initiate apoptosis or cell death. (Torre LA, 2015) Currently available synthetic drugs have many adverse reactions which can be minimized to a great extent by natural compound. The aim of the study was to evaluate the in-vivo antitumor activity of PITC-2 against sarcoma- 180 cancer cell in Swiss albino mice. Therefore we established a mouse model bearing sarcoma-180 in order to study the antitumor activity of the drug. The mice treated with PITC-2 at different dose inhibit body weight, tumor weight, tumor volume, tumor inhibition ratio and also brought hematological parameters to more or less normal level which is dose dependent. A same type of finding was observed by P.M de Costa et.al on *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives (de Costa PM, 2015). This study also shows inhibition of body weight, tumor volume, and tumor inhibition ratio. A good increase in lifespan and survival time was also noted in sarcoma-180 solid tumor bearing mice when treated with PITC-2. A major problem in cancer chemotherapy is myelo suppression and anemia. Tumor bearing mice have anemia mostly due to reduction in RBC or low hemoglobin percentage. This may occur due to iron deficiency or due to myelopathic condition (Gupta M, 2004). Treatment with PITC-2 stabilizes hemoglobin concentration, RBC and WBC cell count near to normal value. This reveals protective activity of PITC-2 on the hematopoietic system. Another problem of cancer chemotherapy is liver toxicity which is due to accumulation of drug metabolites in liver (Debnath S, 2017). In this study liver toxicity was analyzed by biochemical studies and histopathology of liver tissue. AST, ALT and ALP levels of control group mice, normal mice and drug treated mice were compared and it was found that PITC-2 treated mice had less toxic liver than others as the level of AST, ALT and ALP are close to normal value. These all features like decrease in body weight, tumor weight, tumor volume, increase in lifespan and survival time, stabilizing hematological and biochemical parameters of PITC-2 are dose dependent, that in stabilizing effects are better in animals treated with higher dose of PITC-2. These features helps in judging the drug activity.

Histopathological analysis of kidney for control, 5-FU and PITC-2 for different dose was done. It was found that several degree of hydropic changes of proximal tubular epithelium, glomerular and tubular hemorrhage. These finding were much more prominent in control group animal than drug treated groups where these effects are low but the glomeruli structures were essentially preserved. The necrosis is characterized by no nuclear staining and deeply eosinophilic cytoplasm (de Sousa AP, 2007). This show PITC-2 is less toxic to kidney than standard drug. Many drugs can cause lesion of liver found on biopsy. A large number of drugs with different pharmacological action and chemical structure give rise to liver lesion. Although liver shows a great adaptive and regenerative ability for example if anticonvulsant drugs are used for a long time it causes increase in endoplasmic reticulum which is regarded as adaptive phenomenon. Again regeneration of hepatic tissue occurs by the conjugative tissues after a hepatocellular necrosis due to treatment with alginates (de Sousa AP, 2007). In our study normal healthy mice shows prominent nuclei and the tissue section comprises of hepatic sinusoid which are usual. But in case of Sarcoma-180 control group none of the above mentioned regular features are observed, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Group receiving different dose of PITC-2 are close to normal although little changes are found like deformed hepatic artery and irregular bile duct which show PITC-2 is less toxic to liver.

From MTT assay it was found that PITC-2 have notable *in vitro* cytotoxic effect on S-180, MCF-7,A-549 and U-937 cancer cells. The mechanism of apoptosis is its diverse alteration of cells. So cell apoptosis are identified by anti-apoptotic protein Bcl-2. Bcl-2 plays an important role in apoptosis regulation in physiological and pathological conditions. Prevention of the loss of mitochondrial membrane potential is one of its notable works. It also prevents release of cytochrome c into the cytosol which activates caspases by the mitochondrial pathway. Over expression of Bcl-2 inhibits caspase-3 activation (de Silva Faria MC, 2015) (Debnath S, 2017). In our study it was found that after treatment with PITC-2 on sarcoma-180 cells a significant decrease in Bcl-2 was observed whereas an increase in level Bcl-2 was observed in control group animals. This result concludes that increase of Bcl-2 would be a cellular response to counteract the apoptosis which is already initiated. Therefore PITC-2 reduces the ability of Bcl-2 expression and initiates apoptosis.

To identify the tumor proliferation rate the cells tumor cells are stained with Ki67. Nuclear staining was observed in all tumor sections which are immunized with Ki67 antibody. Mouse monoclonal antibody of ki67 was used that identifies a nuclear antigen associated with G1, S, G2 and M phase. The expression of this molecule is found in all cell cycle except in G0 and early G1 phase (Gerdes J, 1984). In our study it was found that a decrease in positive Ki67 cells in animals treated with 5FU and PITC-2 when compared to control group. It was also noted that reduction in tumor proliferation rate is more for higher dose of PITC-2.

Cyclic D1 over expression is an indication of early cancer onset and tumor progression (Diehl, 2002). Cyclic D1 is a protein required for progression through G1 phase during cell cycle. It synthesizes rapidly during G1 phase and accumulates in the nucleus. Further it starts degrading when it enters S phase (Baldin V, 1993). Our study shows a marked decrease in expression of Cyclic D1 in animals treated with 5FU and PITC-2 when compared to control group. Even more effective result was shown by different dose of PITC-2 than 5FU which reveals anti-tumor activity PITC-2.

# References

Abd-El Fattah AA, D. H. (2017). Carbition anhydrase inhibition boost the antitumor effect of Imatinib mesylate via potentiating the antiangiogenic and antimetastatic machineries,. *Toxicology and applied pharmacology* , 316:123-138.

Alisa Bahar Beydogan, S. b. (2016). The effects of silibin adminiistration for different time periods on mouse liver with Ehrlich ascites carcinoma. *Pharmacological Reports* , 543-549.

Bala A, K. B. (2010). Evaluation of anticancer activity of Cleome gynandra on Ehrlich's Ascitec Carcenoma treated mice. *Journal of ethnopharmacology* , 129: 131-134.

Baldin V, L. J. (1993). Cyclic D1 is a nuclear protein requires for cell cycle progression in G1. *Genes and Development* , 5: 812-821.

Bissel MJ, R. D. (2001). Putting Tumours in Context. *Nature Review Cancer* , 1: 46-54.

Biswas R, B. D. (2007). Isolation of pure compound R/J/3 from *Pluchea indica* (L.) Less. and its anti-amoebic activities against Entamoeba histolytica. *Phytomedicine* , 14: 534-537.

Chakraborty A, T. D. (2014). Immunomodulation of macrophages by methyl glyoxal conjugated with chitosan nanoparticles against Sarcoma-180 tumor in mice. *Cellular Immunology* , 287: 27-35.

Chakraborty S, C. T. (2017). Targeting viseral leishmaniasis by mannosylated liposome incorporated PITC-2 isolated from the plant *Pluchea indica* (L) less. *International Journal of Pharma and Bio Sciences.* , 8: 396-408.

de Costa PM, d. C. (2015). Inprovement of *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives. *Chemico- Biological Interaction* , 239: 174-183.

de Silva Faria MC, d. S. (2015). Effect of diabetes on biodistribution, nephrotoxicity and antitumor activity of cicplatin in mice. *Chemico-Biological Interactions.* , 229: 119-131.

de Sousa AP, T. M. (2007). *In vivo* growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed Sargassum vulgare. *Carbohydrate Polymers* , 69: 7-13.

Debnath S, K. S. (2017). Poly-L-Lysine inhibits tumor angiogenesis and induces apoptosis in ehrlich ascites carcinoma and sarcoma S-180 tumor. *Asian pacific journal of cancer prevention* , 18: 2255 - 2268.

Diehl, J. (2002). Cycling to cancer with cyclic D1. *Cancer biology and therapy* , 3: 226-231.

Elsherbiny NM, Y. N. (2016). The synergistic effect between vanillin and doxorubicin in ehrlich ascites carcinoma solid tumor and MCF-7 human brest cancer cell line. *Pathology- Research and Practice* , 212: 767-777.

Gaballah HH, G. R. (2017). Apigenin potentiates the antitumor activity of 5-FU on solid Ehrlick carcinoma : Crosstalk between apoptotic and JNK mediated autophagic cell death platforms. *Toxicology and applied pharmacology* , 316: 27-35.

Gerdes J, L. H. (1984). Cell cycle analysis of a cell proliferation associated human nuclear antigen defined by the monoclonal antibody Ki67. *Journal of immunology* , 133: 1710-1715.

(2015). *Global cancer facts and figures, 3rd Edition.* American Cancer Society.

Gupta M, M. U. (2004). Antitumor activity and antioxidant role of Bauhinia racemosa against Ehrlich ascites carcinoma in swiss albino mice. *Acta Pharmacologica Sinica* , 38: 1070-1076.

Harborne, J. (2000). Arsenal for Survival: Secondary Plant Products. *Taxon* , 49: 435-449.

Hussein BHM, A. H. (2012). A novel antitumor agent Ln(lll) 2-thioacetate benzothiazole induces anti-angiogenic effect and cell death in cancer cell lines. *European journal of medicinal chemistry* , 51: 99-109.

JA, D. (2002). Cycling to cancer with cyclic D1. *Cancer Biology and Therapy* , 3, 226-231.

Jemal A, S. R. (2007). Cancer Statistics. *CA: A cancer journal for clinicians* , 57: 43-66.

Joseph MM, A. S. (2014). Antitumor activity of galactoxyloglucan gold nanoparticles against murine ascites and solid carcinoma. *Colloids and surfaces B: Biointerfaces* , 116: 219-227.

ML Salem, N. S. (2016). *In vitro* and *in vivo* antitumor effects of the Egyptian scorpion Androclonus amoreuxi venam in an Ehrlich ascitic tumor model. *Springer Plus* , 570.

Nascimento FR, C. G. (2006). Ascitic and solid Ehrlich Tumor ingibition by Chenopodium ambrosiodes L. Treatment. *Cellular Immunology* , 78: 2650-2653.

Nygren P, L. R. (2003, 253). Overview of the clinical efficacy of investigational anticancer drugs. *Journal of Internal Medicine* , 253: 46-75.

Pal A, B. I. (2009). Methylglyoxal induced activation of murine peritoneal macrophages and surface marker of T lymphocytes in sarcoma 180 bearing mice:involvement of MAP kinase NF-Kappa beta signal transduction pathway. *Molecular Immunology* , 46: 2039-2044.

Pramanik KC, B. R. (2007). Tissue culture of the plant Pluchea indica (L.) Less. and evaluation of diuretic potential of its leaves. *Oriental Pharmacy and Experimental Medicine* , 7: 197-204.

R.Perveen, F. J. (2012). Preventive effect of ethanolic extract of Alpinia calcarata Rose on ehrlichsascitic carcenoma cell induced malignant ascitic in mice. *Asian pecific journal of tropical medicine* , 121-125.

RH, B. (2007(1)). Topics in Medicinal Chemistry. *Springer - Verlog, Berlin Heidelberg* .

S.S.Agarwal, S. S. (2011). Cytotoxic and antitimor effect of brucine on ehrlich ascites tumor and human cancer cell line. *Life Sciences* , 147-158.

Sinha A, B. K. (2014). Synthesis characterisation and biological evaluation of a noble vanadium complex as a possible anticancer agent. *Journal of organometallic chemistry* , 772-773: 34-41.

Skommer J, B. T. (2010). Bcl-2 inhibits apoptosis by increasing time-to-death and intrinsic cell-to-cell variations in the mitochondrial pathway of cell death. *Apoptosis* , 15: 1223-1233.

Swanton E, S. P. (1999). Bcl-2 regulates caspase 3/caspase 2 apoptotic cascade in cytosolic extracts. *Oncogene* , 18: 1781-1787.

Torre LA, B. F. (2015). Global cancer Statistics. *CA: A cancer journal for clinicians* , 65: 87-108.

# CHAPTER 3

# Anti –tumor Activity of phytochemical PITC-2 on Ascites Tumor Model by EAC cell in Mice

# Material and Methodology:

## Ascites tumor induction by EAC cell line and experimental protocol:

EAC cells were preserved *iv vivo* by intraperitoneal inoculation of cells in the peritoneal cavity of mice with sterile normal saline.(Placeholder3)[10] .EAC cells were taken from those inoculated mice and injected (0.2 ml of 2 x $10^6$ cells/mouse) to all the groups intraperitonially except the normal group (A Sinha 2014). From day 1 after inoculation with EAC cells treatment was given for 14 days.(S.S.Agarwal 2011)(Alisa Bahar Beydogan 2016) From each group 6 mice were sacrificed and other 6 were kept for calculation of Mean survival time**.**

## Animals and study groups:

*In vivo* studies were carried out on Swiss albino mice weighing 22 ± 0.50g of either. The animals were kept under standard conditions of 12:12 hr light and dark cycle in polypropylene cages and fed with standard laboratory diet and water *al libitum.* All *in vivo* procedures described were reviewed and approved by the University Animal Ethical Committee.

For experimental purpose animals are divided into 5 groups each containing 12 mice.

Group 1: Normal animals (negative control).

Group 2: EAC cancer animals + PITC 2 (2.5mg/kg/day given i.v)

Group 3: EAC cancer animals + PITC 2 (5mg/kg/day given i.v)

Group 4: EAC cancer animals (Standard) + 5FU (20mg/kg in alternative day given i.p).

## Collection of ascetic fluid:

After 14 days of drug treatment, on 15$^{th}$ day animals were sacrificed. 2ml of normal saline water was injected (i.p.) in the abdominal region and then tumor cells along with ascetic fluid was collected in 15ml centrifuge tubes. Then they are centrifuged for 10 min at 3000 rpm at 4°C. Ascites tumor volume was then calculated by subtracting injected saline volume from the collected whole ascetic fluid and packed cell volume was calculated.(Nicol B.M. 2006)

The viability and non-viability of the cells were checked by trypan blue assay. The cells were stained with trypan blue (0.4% in normal saline) dye. The cells that did not take the dye were viable and those who took the dye were non-viable. Viability percentage is calculated by (No. of viable/ No. of viable and non-viable cells) x 100.(M.L.Salem 2016)

Percentage cell growth inhibition was calculated by the formula = $1 - \frac{Tw}{Cw} \times 100$

Where Tw is mean of number of tumor cells of the treated group of mice and Cw is mean of number of tumor cells of the control group of mice.(R.Perveen 2012)

## Recording of survival rate:

Mean survival time and percentage increase in life span (%ILS) were calculated by the following formulas (S.S.Agarwal 2011).

$$\text{Mean survival time (MST)} = \frac{\sum \text{Survival time(day) of each mice in a group}}{\text{total number of mice}}$$

$$\%\text{ILS} = \frac{\text{MST of treated mice}}{\text{MST of control mice}} \times 100$$

## Hematological Parameters:

Hematological study includes hemoglobin content, red blood cell (RBC) and white blood cells (WBC) counts. After 14 days of treatment animals were sacrificed by cervical dislocation and blood samples were collected from heart. WBC leukocyte counts were also carried out including neutrophils, lymphocytes and monocytes from leishaman stained blood smears of all groups of animal.

## Biochemical Parameters:

After 24 hour of last dose and 18 hour of fasting the blood samples were collected from heart. Biochemical parameters includes serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, globulin, bilirubin, alkaline phosphatase and serum protein to identify alteration in liver function and creatinine was measured as renal function parameter.

## Histopathology:

After collecting ascetic fluid from treated and control group, they were centrifuged at 3000 rpm for 10 minutes and one part of it is fixed in glass slides with neutral buffer formation. Then they were stained with hematoxylin and eosin. Another part is fixed on glass slide using 95% ethanol and stained with papanicalaou stain. Then both stained slides were mounted with distend dibutyl phthalate xylene and were examined under light microscope (40X) (Debnath S 18(2017)). Liver and kidney tissues were isolated from the animals and washed with saline. Then it was fixed in 10% buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin(Elsherbiny NM 212(2016))Then the tissues were sectioned 5 to 6 μm, stained with hematoxylin and eosin (H&E) and examined under light microscope (40X).

## Statistical analysis:

All *in vitro* studies were carried out in triplicate represented by independent biological evaluation. Data are extracted as mean ± standard error of the mean (SEM) and were compared by one way analysis of variance (ANOVA) followed by Kruskal Wallis Test using SPSS statistical software of 20.0 version. $P<0.05$ was considered to be statistically significant when compared with control.

# Result:

## Inhibition of EAC cells growth by PITC-2:

A significant reduction of ascetic fluid volume, packed cell volume and viable tumor cell count was found with phytochemical PITC-2 at an increasing concentration that is 2.5mg/kg and 5mg/kg in compared to that of control group mice. Moreover the median survival time (MST) was also increased in PITC-2 treated groups of mice shown in Table 1.

***Table 1*****:** *In-vivo* effect of PITC-2 on EAC bearing Mice

| | PITC-2 2.5mg/kg b.w | PITC-2 5mg/kg b.w | Std drug 5-FU 20mg/kg b.w | Sarcoma 180 Control |
|---|---|---|---|---|
| Ascites fluid volume (ml) | 5.89 ± 0.28* | 4.64 ± 0.12* | 5.24 ± 0.23* | 11.90 ± 0.11* |
| Packed Cell volume (ml) | 1.45 ± 0.08* | 1.31 ± 0.04* | 1.22 ± 0.02* | 4.21 ± 0.08* |
| Body weight (gm) | 23.05 ± 0.43* | 21.71 ± 0.42* | 22.68 ± 0.55* | 30.10 ± 0.48* |
| MST (days) | 39.67 ± 0.98* | 44.83 ± 0.70* | 32.00 ± 0.85* | 18.40 ± 0.42* |
| %ILS | 215.59 | 243.64 | 173.91 | 0 ± 0 |
| Viable Cell($10^6$cells/ml) | 3.05 ± 0.13* | 2.79 ± 0.21* | 3.45 ± 0.43* | 10.65 ± 0.23* |

**<0.05 as compared to control group (n = 6 mice per group)*

## Effect of PITC-2 on hematological parameters:

An increase in hemoglobin level, RBC count, neutrophil level and decrease in WBC level, monocyte and lymphocyte count was found in phytochemical PITC 2 treated groups of different dose and standard 5FU treated group with compared to that of EAC control group shown in Table 2.

***Table 2*:** Effect of PITC-2 on hematological parameters in EAC bearing Mice

| | PITC-2 2.5mg/kg b.w | PITC-2 5mg/kg b.w | Std 5-FU 20mg/kg b.w | EAC control | Normal Mice |
|---|---|---|---|---|---|
| Hemoglobin (gm %) | 8.18± 0.05* | 9.6± 0.08* | 9.1 ± 0.06* | 6.3 ± 0.01* | 11.8±0.12* |
| Erythrocyte (RBC) (cells x$10^6$/$mm^3$) | 5.6± 0.04* | 6.53± 0.03* | 6.18± 0.08* | 3.75 ± 0.08* | 7.90±0.02* |
| Leucocytes (WBC) (cells x$10^6$/$mm^3$) | 10.2±0.05* | 9.14±0.05* | 8.29±0.05* | 16.69±0.02* | 6.40±0.03* |
| Neutrophil (%) | 32.3± 0.03* | 30.46±0.03* | 30.1±0.02* | 68.5 ± 0.05* | 19.4±0.03* |
| Lymphocyte (%) | 51.0± 0.04* | 54.0± 0.04* | 61.0±0.09* | 35.8 ± 0.11* | 64.1±0.23* |
| Monocyte (%) | 1.6 ± 0.03* | 2.2 ± 0.03* | 1.7 ± 0.03* | 1.3 ± 0.04* | 2.8±0.01* |

**<0.05 as compared to control group (n = 6 mice per group)*

## Effect of PITC-2 on biochemical parameters:

Phytochemical PITC-2 treated groups 2.5 mg/kg and 5 mg/kg, shows significant decrease in SGPT, SGOT and serum alkaline phosphatase parameters in comparison with EAC control group. An increase in total protein was also found in PITC-2 treated groups shown in Table 3.

***Table 3*:** Effect of PITC-2 on biochemical parameters in EAC bearing mice

| | PITC-2 2.5mg/kg b.w | PITC-2 5mg/kg b.w | Std 5-FU 20mg/kg b.w | Sarcoma 180 control | Normal Mice |
|---|---|---|---|---|---|
| Bilirubin (total) mg/dl | 0.25 ± 0.02* | 0.26 ± 0.02* | 0.27 ± 0.03* | 0.34± 0.01* | 0.45 ± 0.02* |

| | | | | | |
|---|---|---|---|---|---|
| Serum protein (total) g/dl | 8.22 ± 0.09* | 8.29 ± 0.07* | 8.52 ± 0.12* | 4.4± 0.08* | 8.19±0.08* |
| AST (SGOT) IU/L | 34.5 ± 0.01* | 37.3 ± 0.01* | 35.9± 0.01* | 74.9 ± 0.09* | 39.1±0.01* |
| ALT(SGPT) IU/L | 34.6 ± 0.04* | 35.4 ± 0.01* | 39.3 ± 0.06* | 71.4 ± 0.07* | 29.1±0.04* |
| Serum alkaline phosphatase IU/L | 71.1 ± 0.02* | 70.9 ± 0.02* | 72.8 ± 0.05* | 121.1±0.03* | 74.4 ± 0.03* |
| Creatinine Mg/dl | 0.78 ± 0.01* | 0.8 ± 0.02* | 0.81 ± 0.01* | 1.2 ± 0.02* | 0.62 ± 0.01* |

**<0.05 as compared to control group (n = 6 mice per group)*

## Histopathology of liver and kidney tissue:

H&E stained section of liver of a healthy mice was shown in Fig 1A, bearing all the normal features which includes circular hepatic portal vein and of hepatic artery, as marked by arrow. The tissue section comprises of hepatic sinusoid and nuclei which are usual. But none of the above mentioned regular features are observed in case of Sarcoma-180 control group showed in Fig 1B, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Although little changes are found in group receiving standard 5-FU like deformed hepatic artery and irregular bile duct but lesser amount of hepatocellular lesions observed which are close to normal as shown in Fig 1C. Groups receiving phytochemical PITC-2 2.5 mg/kg and 5 mg/kg also shows a little hepatic deformation and altered hepatocyte population shown in Fig 1D and 1E. Healthy hepatocytes were observed, regular branch of bile duct found, although mild dilation of central vein observed.

H&E stained section of kidney of a healthy mice was shown in fig no 2A, bearing all the normal features, while Sarcoma-180 control group shows severe glomerular and tubular hemorrhage with increase swelling of tubular epithelium and presence of hyaline cylinders showed in Fig 2B. Group treated with 5-FU and phytochemical PITC-2 shows light glomerular and tubular hemorrhage and cellular features are less deformation which are close

to normal shown in Fig 2C, 2D and 2E respectively.

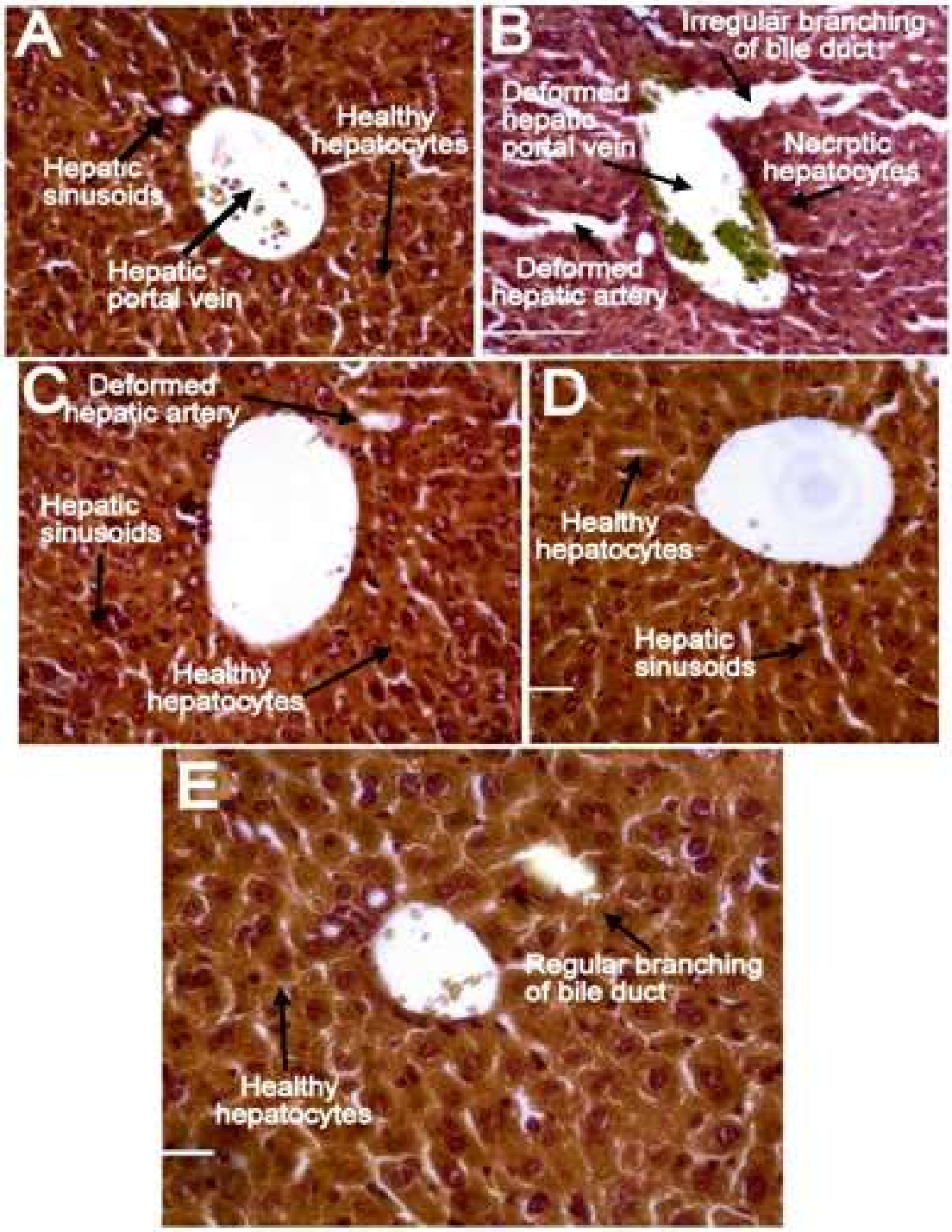



***Figure 1*** **:** H&E stained section of liver for A) normal healthy mice, B) EAC control group, C) Standard 5-FU treated group, D) 2.5 mg/kg of PITC-2, E) 5 mg/kg of PITC-2

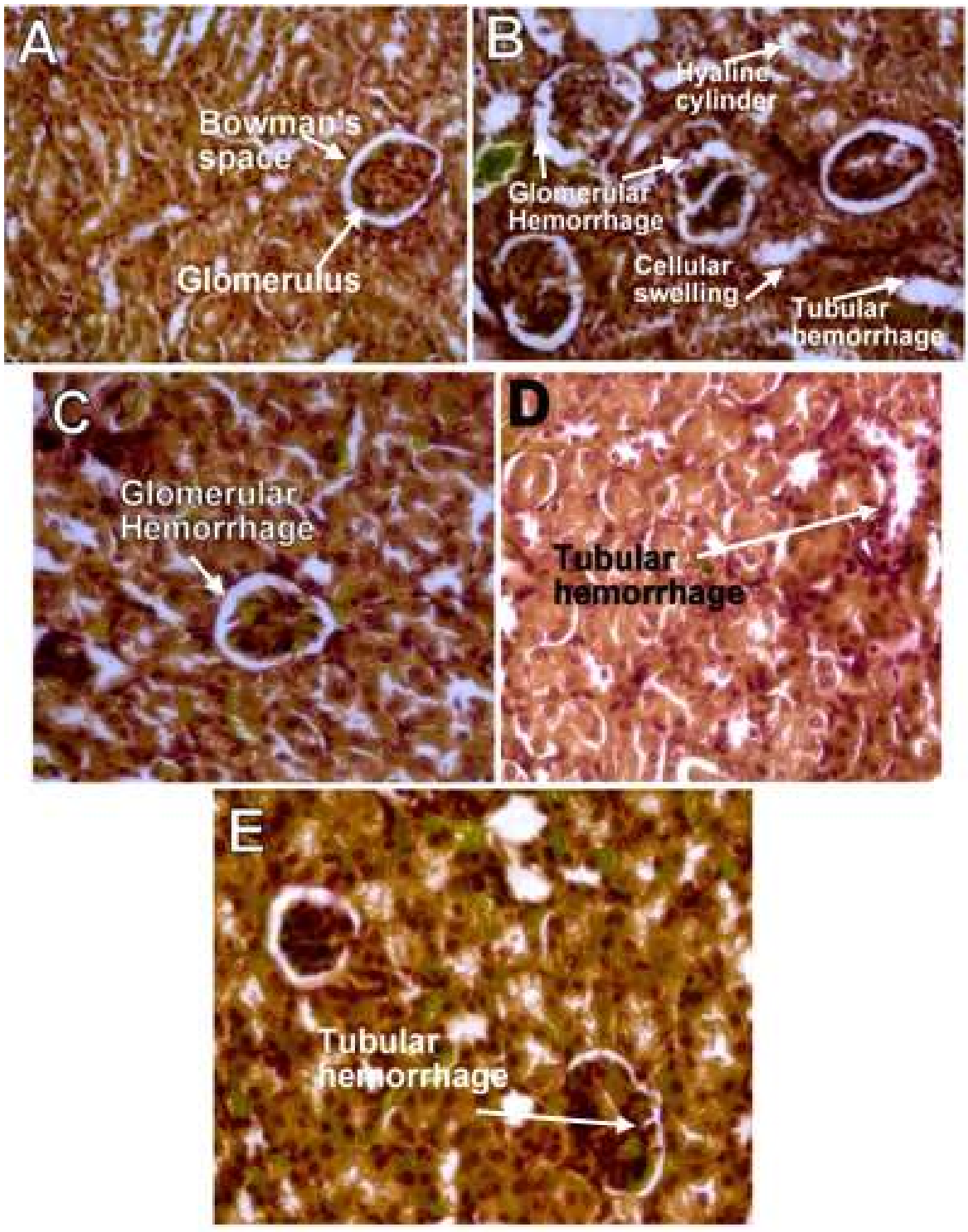



***Figure 2*** **:** H&E stained section of liver for A) normal healthy mice, B) EAC control group, C) Standard 5-FU treated group, D) 2.5 mg/kg of PITC-2, E) 5 mg/kg of PITC-2

## Morphological changes of EAC cells:

Morphological changes were observed in EAC cell with both Haematoxylin & Eosin (H&E) and Papanicolaou (Pap) staining. All morphological changes observed by H&E stain are shown in Fig 3. H&E staining cells of control group, a good circular morphology, intact plasma membrane and nucleus were found. But cell of groups treated with standard drug shows apoptotic bodies and nuclear contraction. Irregularity in cell morphology, blebbing of plasma membrane, chromatin condensation was also found in groups treated with phytochemical PTIC-2 at dose 2.5 mg/kg. In case of group treated with phytochemical PITC-2 at dose 5 mg/kg evident change in cell morphology was found which includes cell shrinkage, formation of apoptotic bodies, nuclear contraction and blebbing of plasma membrane.

Similarly in case of cells stained with Papanicolaou stain numerous features of cellular apoptosis was observed in standard and test drug groups which are shown in the figure by arrow which are characterized by change in cell morphology was found which includes cell shrinkage, formation of apoptotic bodies , nuclear contraction and blebbing of plasma membrane. It was also noticed that cellular apoptosis happens in a dose dependent manner of phytochemical PITC-2. More the dose of PITC-2, more is the rate of change in cellular morphologies. All morphological changes observed by Pap stain are shown in Fig 4.

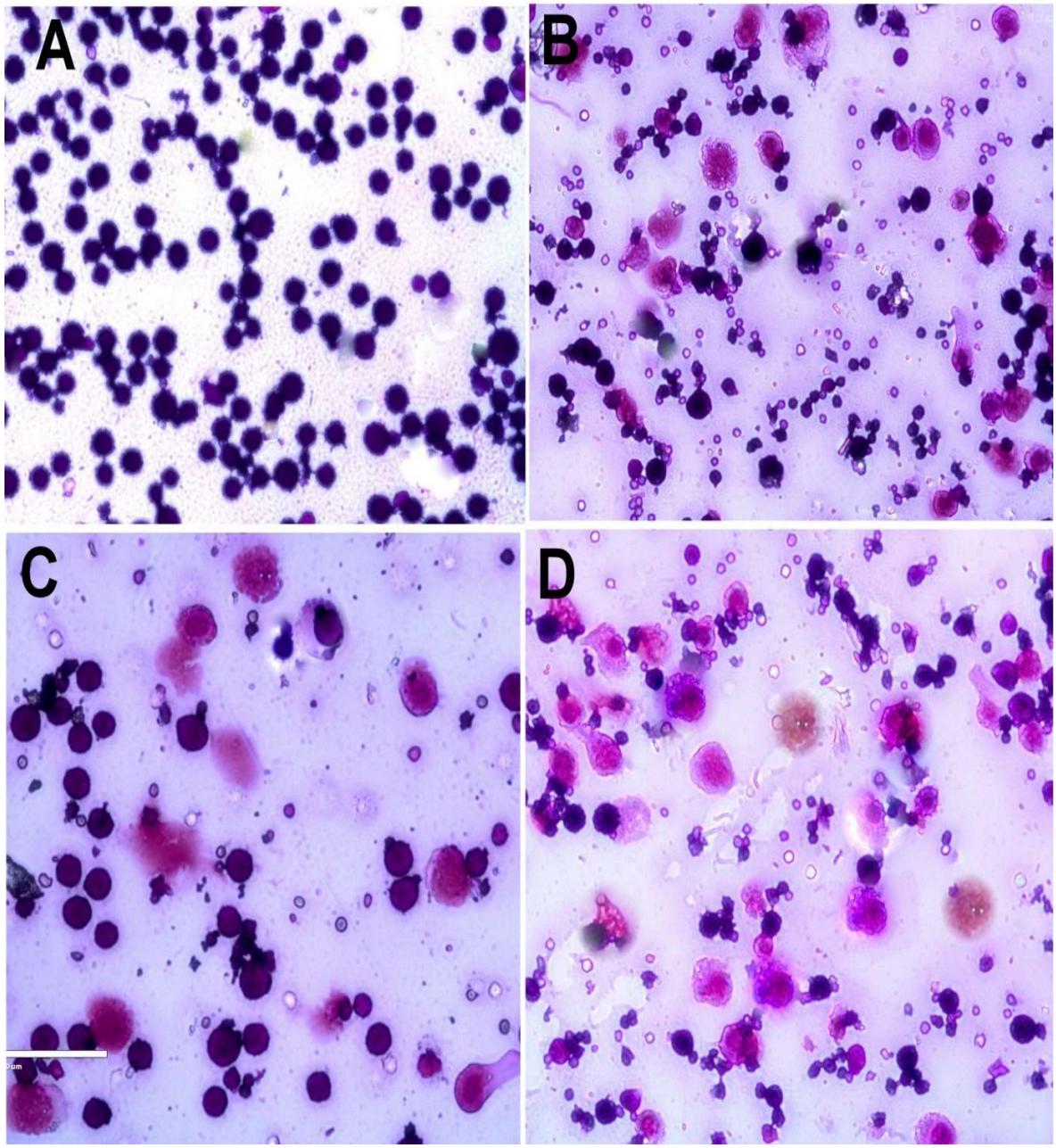



***Figure 3.*** Change in morphology of EAC cells by Haematoxylin & Eosin stain for groups A) Sarcoma-180 control group, B) Standard 5-FU treated group, C) 2.5 mg/kg of PITC-2 and D) 5 mg/kg of PITC-2. The cellular features are marked by arrow.

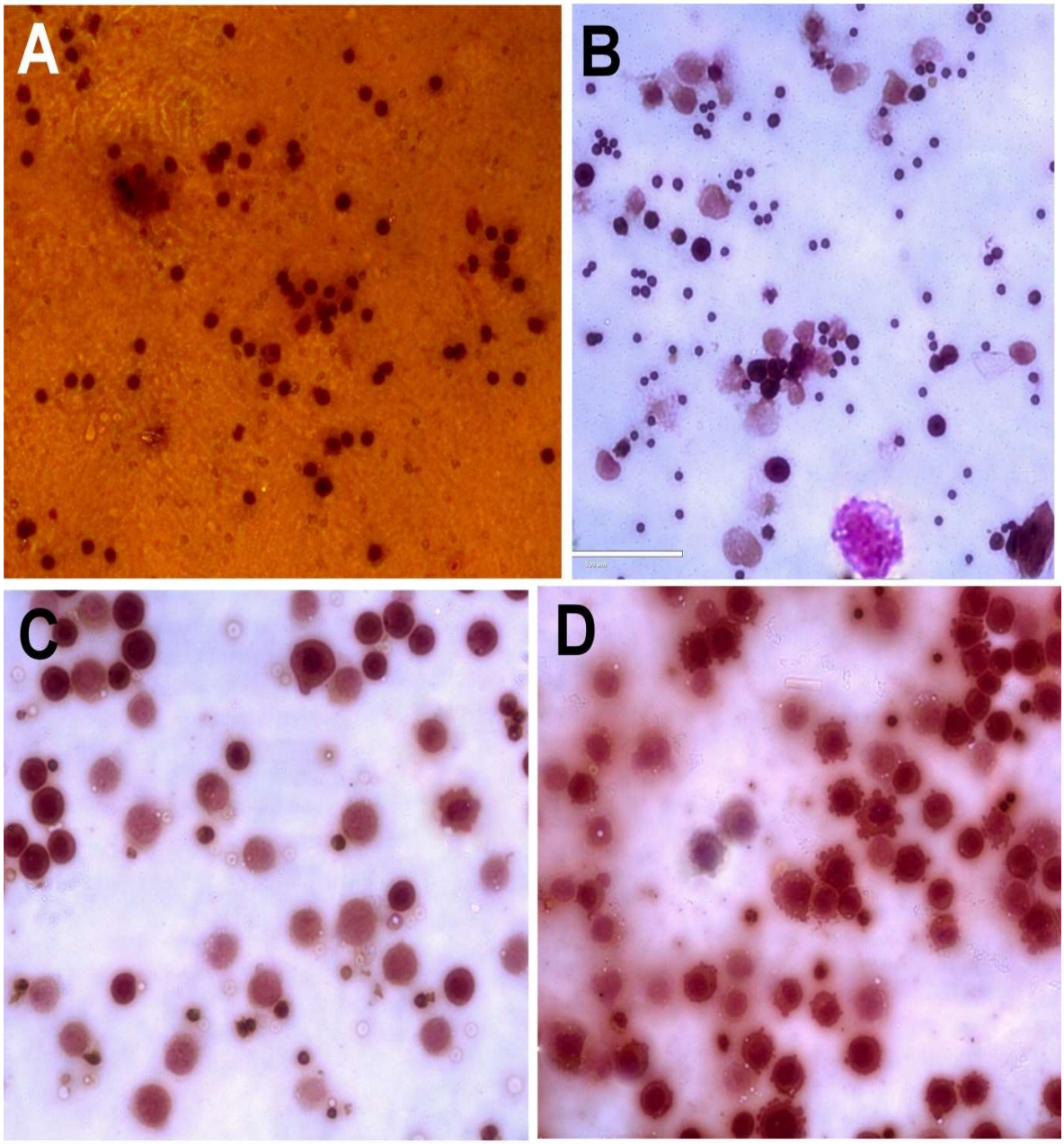



***Figure 4:*** Change in morphology of EAC cells by Papanicolaou stain for groups A) Sarcoma-180 control group, B) Standard 5-FU treated group, C) 2.5 mg/kg of PITC-2 and D) 5 mg/kg of PITC-2. The cellular features are marked by arrow.

# Discussion:

Cancer is considered as the most killer disease all over the world. Cancer therapies are remarkably consistent for last 50 years. Chemotherapeutic drugs plays a major role for its treatment but clinical success of these treatments reached a plateau (Isha Dhamija 65, 2013). Moreover **c**urrently available cytotoxic drugs have many adverse reactions. Another problem in cancer chemotherapy is drug resistance. These problems can be minimized to a great extent by natural compound. Plant alkaloids, flavonoids, taxoids and podophyllotoxins are major contributors of many diseases and abnormalities. Vast studies and research on these products especially phytochemicals have exhibited anti-carcinogenic activities by interfering with the initiation, development and progression of cancer through the modulation of various mechanisms including cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis(E. Rajesh 2015). Due to the rapid development of resistance to chemotherapeutic drugs, the search of a novel drugs is still a priority aim for cancer therapy. So in this study we are using phytochemical PITC-2, obtained from tissue cultured medicinal plant *Pluchea indica.* The aim of the study was to evaluate the in-vivo anticancer activity of PITC-2 against EAC cancer cell in Swiss albino mice.

In this study we also had established a mouse model which bears EAC cell in order to study the anticancer effect of the phytochemical PITC-2. The mice treated with PITC-2 inhibits body weight, ascetic fluid volume, viable tumor cell count and also brought hematological parameters close to the normal mice group. A same type of finding was observed by P.M de Costa *et al.* on *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives (de Costa PM 239 (2015)). A good increase in lifespan and survival time was also noted in EAC cell bearing mice when treated with phytochemical PITC-2. It was also seen that increase in life span of EAC cell bearing mice are phytochemical PITC-2 dose dependent. More good result was observed with a PITC-2 dose of 5 mg/kg than dose 2.5 mg/kg.

Myelosuppression and anemia are the major problems in cancer chemotherapy. Mostly due to reduction in RBC count, cancer cell bearing mice have anemia or due to low hemoglobin percentage. This may occur due to iron deficiency or due to myelopathic condition (Gupta M 25(8), 2004). Treatment with phytochemical PITC-2 stabilizes hemoglobin concentration, RBC

and WBC cell count near to normal value. This reveals protective activity of Phytochemical PITC-2 on the hematopoietic system. Another major problem of cancer chemotherapy is liver toxicity which is due to accumulation of drug metabolites in liver (Debnath S 2017). Here liver toxicity was analyzed in this experiment by biochemical studies and histopathology of liver tissue. AST, ALT and ALP levels of control group mice, normal mice and drug treated mice were compared. It was found that groups treated with phytochemical PITC-2 had less toxic liver and the level of AST, ALT and ALP are close to normal value. These all features like decrease in body weight, tumor weight, tumor volume, increase in lifespan and survival time, stabilizing hematological and biochemical parameters of PITC-2 at dose 5 mg/kg n are nearly close to normal and even better in comparison to the effect of std drug 5FU. These features helps in judging the anticancer property of phytochemical PITC-2.

Histopathological analysis of kidney was done for control, 5-FU and phytochemical PITC-2. Several degree of hydropic changes of proximal tubular epithelium, glomerular and tubular hemorrhage was observed which were much more prominent in control group animal than drug treated groups where these effects are low but the glomeruli structures were essentially preserved. The characterization of necrosis is done by no nuclear staining and deeply eosinophilia cytoplasm (de Sousa AP 2007).This show phytochemical PITC-2 are less toxic to kidney than standard drug and quite resembles to the normal mice. A large number of drugs with different pharmacological action and chemical structure give rise to liver lesion. Although liver has a unique regenerative ability and great adaptive efficiency. For example an adaptive phenomenon of liver is if an anticonvulsant drug was used for a long time then it causes increase in endoplasmic reticulum. Again regeneration of hepatic tissue occurs by the conjugative tissues after a hepatocellular necrosis due to treatment with alginates (de Sousa AP 2007) . In our study prominent nuclei and the tissue section comprises of hepatic sinusoid was observed in normal healthy mice which are usual. But in case of EAC cell bearing control group features observed are not normal as mentioned, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Group receiving phytochemical PITC-2 at different doses are more or less close to normal group although little changes like deformed hepatic artery and irregular bile duct are found which show they are less toxic to liver.

Now for analyzing the cytotoxic effect of the phytochemical PITC-2 histopathology of ascetic fluid was done. Cell apoptosis takes place and a significant change in cell morphology was observed. Apoptosis is a cell regulatory mechanism which is distinguished by diverse alteration in cell morphology including asymmetry in plasma membrane, cytoplasm shrinkage, chromatin condensation, formation of apoptotic bodies, etc. (Debnath S 2017). In our study control group shows good circular morphology, intact plasma membrane and nucleus. But cell of groups treated with standard drug shows apoptotic bodies and nuclear contraction. Irregularity in cell morphology, blebbing of plasma membrane, chromatin condensation was also found in groups treated with phytochemical PTIC-2 at dose 2.5 mg/kg. In case of groups treated with phytochemical PTIC-2 at dose 5 mg/kg evident change in cell morphology was found which includes cell shrinkage, formation of apoptotic bodies, nuclear contraction and blebbing of plasma membrane. A better cytotoxic activity was observed with greater dose of phytochemical PITC-2. So this study gives a clear idea about phytochemical PTIC-2 induced inhibition of EAC ascetic cell growth by cell apoptosis. This study also shows that phytochemical PTIC-2 is a good natural source of anticancer agent.

# Reference:

A Sinha, K. Banerjee, A Banerjee, S Das, SK choudhuri. "Synthesis characterisation and biological evaluation of a noble vanadium complex as a possible anticancer agent." *Journal of organometallic chemistry*, 2014: 34-41.

Alisa Bahar Beydogan, Sema bolkent. "The effects of silibin adminiistration for different time periods on mouse liver with Ehrlich ascites carcinoma." *Pharmacological Reports*, 2016: 543-549.

Biswas R, Bandyopadhyaya D, Mishraa M, Pramanika KC , Chatterjee TK. "Isolation of pure compound R/J/3 from *Pluchea indica* (L.) Less. and its anti-amoebic activities against Entamoeba histolytica." *Phytomedicine*, 2007, 14: 534-537.

de Costa PM, de Costa MP , Jamacaru VF, Ferreira PM. " Inprovement of *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives." *Chem Biol Interact* , 2015, 239: 174-83.

de Sousa AP, Torres MR, Pessoa C, Costa Lotufo LV. "*In vivo* growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed Sargassum vulgare." *Carbohydr Polym*, 2007, 69: 7-13.

Debnath S, Karan S, Debnath M, Chatterjee TK. " Poly-L-Lysine inhibits tumor angiogenesis and induces apoptosis in ehrlich ascites carcinoma and sarcoma S-180 tumor. ." *Asian Pac J Cancer Prev*, 2017: 18(8): 2255 - 68.

E. Rajesh, Leena S. Sankari, L. Malathi, Jayasri R. Krupaa. "Naturally occurring products in cancer therapy." *J Pharm Bioallied Sci*, 2015: 181 - 183.

Elsherbiny NM, Younis NN, Elseweids MM. " The synergistic effect between vanillin and doxorubicin in ehrlich ascites carcinoma solid tumor and MCF-7 human brest cancer cell line." *Pathol Res Pract*, 2016, 212: 767-77.

Gupta M, Mazumdar UK, Kumar SA, Kumar TS. "Antitumor activity and antioxidant role of Bauhinia racemosa against Ehrlich ascites carcinoma in swiss albino mice." *Acta Pharmacol Sin*, 2004, 25(8): 1070-6.

Isha Dhamija, Nitesh Kumar, S.N. Manjula, Vipan Parihar, M. MManjunath Setty, K.S.R. Pia. "Preliminary evaluation of *in vitro* cytotoxicity and *in vivo* antitumor activity of Premna herbacea Roxb. in ehrtich ascites carcinoma model and daltons lymphona ascites model." *Experimental and toxicologic pathology.*, 2013, 65: 235 - 242.

Jenning V., Gysler A., Schafer Korling M. Gohla SH. "Vitamine A loaded solid lipid nanoparticles for tropical use: Occlusive properties and drug targeting to the upper skin." *Eur J Pharm Biopharm*, 2000: 49: 211 - 21.

M.L.Salem, N.M.Shoukry, A.A.Rahman. "*In vitro* and *in vivo* antitumor effects of the Egyptian scorpion Androclonus amoreuxi venam in an Ehrlich ascitic tumor model." *Springer Plus*, 2016: 570-581.

Muller R.H., Madar K., Gohla S. "Solid lipid nanoparticles (SLN) for controlled drug delivery-a revier of the stste of the art." *Eur, J. Pharm. Biopharm*, 2000, 50: 161-177.

Nicol B.M., Prasad S.B. "The effects of cyclophosphamide alone and in combination with ascorbic acid against murine ascitesd Dalton's lymphoma." *India J Pharmacol*, 2006: 260-265.

P.K. Senthil, A. Arivuchelvin, A. Jagadeeswaran, N. Subramanian, C.K Senthil, P.Mekala. "Formulation, optimization and evaluation of enrofloxacin solid lipid nanoparticles for sustained oral delivery." *Asian journal of pharmaceutical and clinical research*, 2015, 8(1): 231 - 236.

R.Perveen, F.Islam, J.Khanum, T.Yeasmin. "Preventive effect of ethanolic extract of Alpinia calcarata Rose on ehrlichsascitic carcenoma cell induced malignant ascitic in mice." *Asian pecific journal of tropical medicine*, 2012: 121-125.

S.S.Agarwal, S. Saraswati, R. Mathur. "Cytotoxic and antitimor effect of brucine on ehrlich ascites tumor and human cancer cell line." *Life Sciences*, 2011: 147-158.

Surajit Das, Wai Kiong Ng, Parijat Kanaujia, Sanggu Kim,. "Formulation design, preparation and physicochemical characterizations of solid lipid nanoparticles containing a hydrophobic drug: Effects of process variables." *Colloida and Surfaces B: Biointerfaces*, 2011, 88: 483-489.

# CHAPTER 4

# Preparation of PITC-2 loaded Solid Lipid Nanoparticle Formulation

# Material and Methodology:

## Extraction and Isolation:

The roots after separating from the whole plant were dried, grounded and defatted with petroleum ether (60-80°C for synthesis). Then it was extracted in a soxhlet extractor with methanol (AR Grade, Purity 99.8%). After methanol distillation a sticky dark brown residue was obtained. Then methanolic root extract was partitioned in a mixture of n-Butanol and water and butanol fraction was taken. Then it was shacked with ethyl acetate to get an ethyl acetate soluble and insoluble part. Finally ethyl acetated soluble part is concentrated in rotary vacuum evaporator and dried to obtain crude residue which is further column chromatographed with a mixture of ethyl acetate and petroleum ether to get phytochemical PITC 2. A prominent spot was observed in the TLC plate when pet ether and ethyl acetate are in a ratio 8:2 from fraction 8 – 15 which is the desired phytochemical PITC-2 [2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene]. The presence of light yellow solid PITC-2 was further confirmed by testing the isolated compounds on NMR spectroscopy and MASS spectroscopy (Biswas R 2007).

## Preparation of calibration curve of PITC-2:

Stock solution of PITC-2 was prepared at 0.1 mg/ml in Methanol. Then serial dilution were made using methanol in 1, 2, 3, 4, 5 µg/ml. Then absorbance of the solution are taken at 325 nm and then absorbance is plotted against concentration. By this the calibration curve is prepared.

## Preparation of PITC-2 loaded solid lipid nanoparticles:

PITC-2 loaded solid lipid nanoparticles were prepared by emulsion evaporation method. Approximately PITC-2 (10 mg), soya lecithin (100 mg) and glycerol monostearate GMS (200 mg) were mixed in a beaker containing 10 ml of ethyl acetate. Then this mixture is sonicated which is the organic phase. In another beaker 40 mg of poloxamer was mixed with ultra-pure water and heated at around 75 °C and starred at 1000 r.p.m. Then slowly organic phase was injected into the aqueous one and the temperature was maintained at 75°C with 1000 r.p.m. By this the organic phase was evaporated and when the aqueous phase was also reduced add ice cold ultra pure water and maintain temperature at around 3°C with 650 r.p.m. Then it was sonicated

and centrifuged at 30,000 r.p.m. to remove free drug contains in it. After centrifugation the precipitate was separated and was lyophilized to obtain the final PITC-2 loaded SLN powdered formulation.

## Determination of drug content and Entrapment efficiency:

By measuring the concentration of free drug in the dispersion medium, entrapment efficiency of the drug in the formulation can be determined. The samples were subjected to centrifugation for 30 minutes at 4°C and 20,000 rpm using an ultracentrifuge. The unencapsulated amount of drug in the supernatant was analyzed using UV Spectrum. The amount of drug loading (DL) in the lipid phase and entrapment efficacy (EE) was also estimated by the given equation below.

$$\text{Entrapment efficiency (EE) \%} = \frac{\text{Total mass of PITC2} - \text{Mass of PITC2 in suspension}}{\text{Total mass of PITC2}} \times 100$$

$$\text{Drug loading (DL) \%} = \frac{\text{Total mass of PITC2} - \text{Mass of PITC2 in suspension}}{\text{Total mass of Lipid}} \times 100$$

## Particle side and polydispersity index:

Particle size (z-average diameter) and polydispersity index (PI) were measured using Malvern Zetasizer Nano ZS (Malvern 90) by photon correlation spectroscopy at 25°C. Before measurement nanoparticles are dispersed with ultra-purified water in a dilution between 100 and 1000$s^{-1}$ particle count. The measurement was conducted at 90° detection angle.

## Zeta Potential:

An electronic change on the particle size is identifies by Zeta potential (ZP). To determine the physical stability of a colloidal system, ZP is used. It measures the electrophoretic mobility Malvern Zetasizer Nano ZS with a field strength 20V $cm^{-1}$. Equation used for ZP conversion

$$\zeta = \frac{EM \times 4\pi\eta}{\varepsilon}$$

Where $\zeta$ is zeta potential, EM is electrophoretic mobility, $\eta$ is viscosity of the dispersion medium, and $\varepsilon$ is dielectric constant (Surajit Das 88(2011)). Before measurement nanoparticles are dispersed with ultra-purified water in appropriate dilution.

## Differential scanning calorimetry (DSC):

DSC was performed to investigate the melting point and crystalline behavior of crystalline materials. Samples were placed in aluminum pans and empty pans were used as reference. A heating rate of 10°C/ min was employed in the range of 30 – 300°C. Analysis was performed under nitrogen purge (50ml/min).

## Powder X-ray diffractometry (PXRD):

PXRD was performed on the samples by exposing them in CuKα radiation at 40Kv, 30mA. The results were scanned from 2° to 70° using diffraction angle $2\theta$ at a step size of 0.045° and step time 0.5 s.

## Cryogenic field emission scanning electron microscopy (Cryo-FESEM):

Cryo-FESEM is used to determine the surface morphology and shape of SLNs. 2-3 drops of dispersed SLNs were placed on a copper stub and freeze in liquid nitrogen at -196°C. Samples were then stored in liquid nitrogen and placed into cryopreservation chamber. Then it was fractured and sublimed at -95°C for 30 s. then the samples are coated with platinum and introduced into specimen stage of the FESEM at -140°C and examined at an excitation voltage of 5kV.

## Stability study:

Stability of the prepared SLNs were evaluated for 3 months. PITC-2 loaded SLNs optimized formulations were stored at 25°C and 4°Cand then average size, polydispersity index, zeta potential and entrapment efficacy were determined at an interval of 1 month for 3 months (Das S 2011).

## *In vitro* release kinetics of PITC-2 from SLN:

*In vitro* drug release studies of the selected optimized formulation were performed by using dialysis bag method. The molecular weight cut off of the used Dialysis membrane was 12,000-14,000. Before use of the membrane for drug release dialysis membrane was soaked for 12 hours. Each Dialysis bag was filled by 2 ml of formulation and the release media was 100 ml of

phosphate buffer consist of pH 7.4. At fixed intervals of 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 22, 24, 28, 32, 36, 44, 46 and 48 h, 1ml of sample was withdrawn and replaced with fresh buffer from release media and analyzed by UV at 325 nm

### Statistical analysis:

All *in vitro* studies were carried out in triplicate represented by independent biological evaluation. Data are extracted as mean ± standard error of the mean (SEM) and were compared by one way analysis of variance (ANOVA) followed by Kruskal Wallis Test using SPSS statistical software of 20.0 version. $P<0.05$ was considered to be statistically significant when compared with control.

## Result:

### Calibration Curve of PITC-2:

Standaed curve of PITC-2 is graphically shown in Fig 1. Here absorbance is plotted vercess drug concentration. Absorbance is taken at 325 nm.

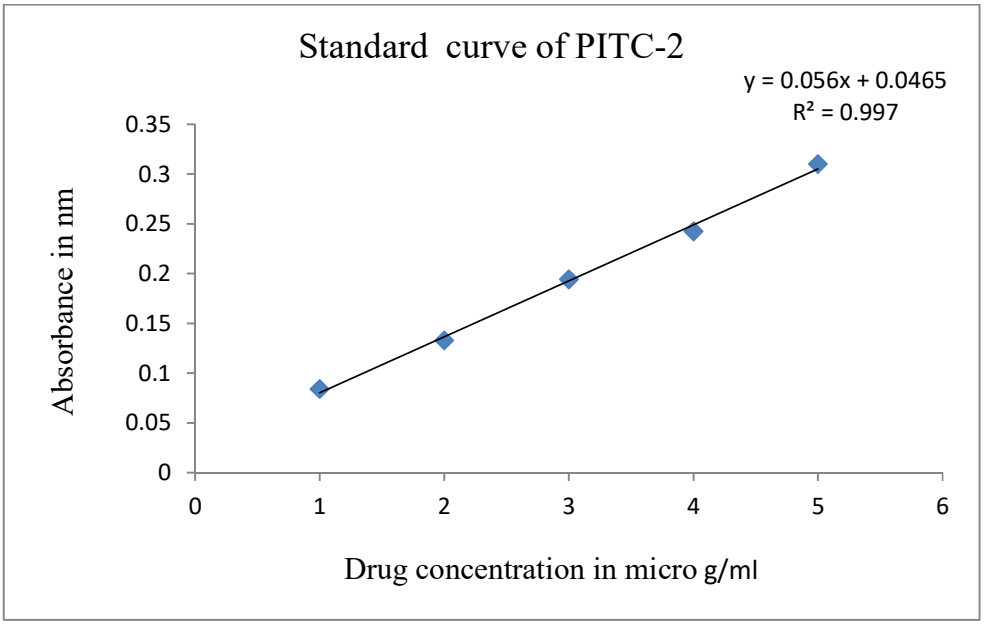



***Figure 1***: Standard calibration curve of PITC-2

## Particle size and Zeta Potential:

The size distribution of PITC-2 loaded SLNs were shown in Fig 2. In general PITC-2 loaded SLNs looks round, uniform sized and well dispersed. The mean size of our nanoparticles was 171.5d.nm.

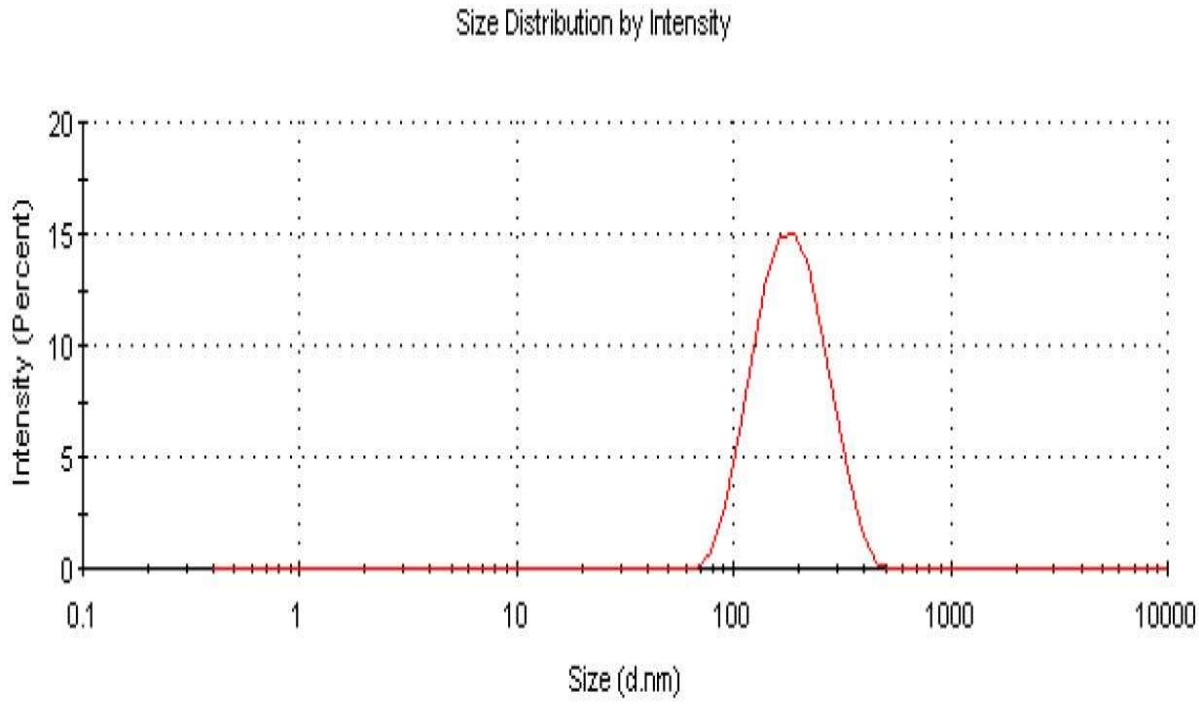



***Figure 2***: Particle size distribution curve of PITC-2 loaded nanoparticles.

Zeta potential and stability of nanoparticles are closely related to each other. By measuring zeta potential the stability of a nanoparticle system can be analyzed. In our study the zeta potential of all the prepared formulation were negatively charged and varied from -33.2 to -37.5 mV which ensured a system with good stability as it posse's sufficient repulsion. Zeta potential values were presented in Table 1 and shown in Fig 3.

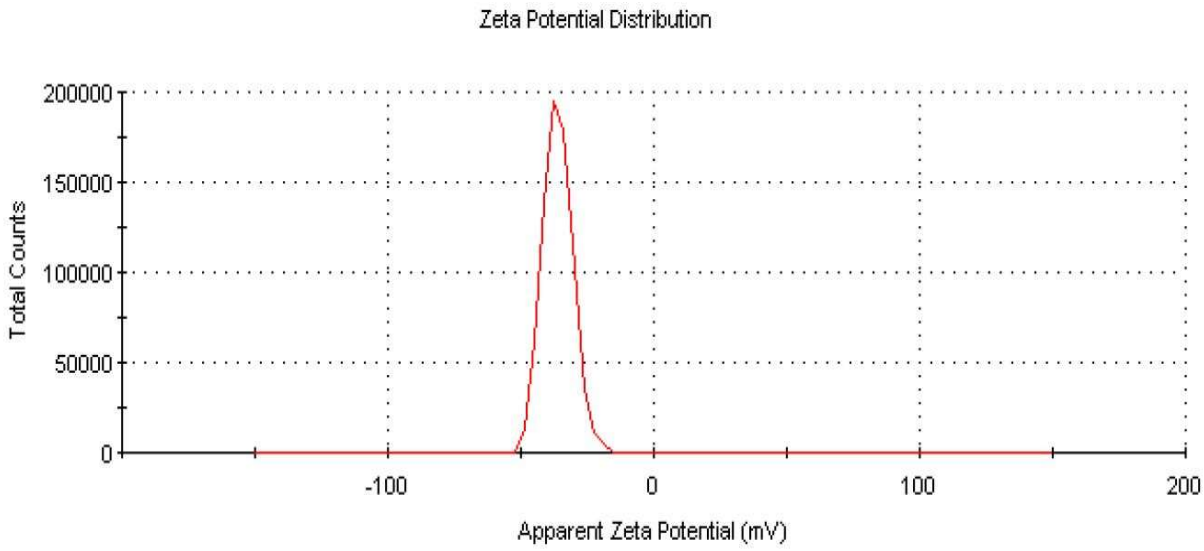



***Figure 3:*** Surface Zeta potential graph showing negative zeta potential value of PITC-2 SLNs.

***Table 1*:** Particle Size, Polydispersity index and Zeta Potential of drug free SLNs and PITC-2 loaded SLNs( data represents mean ±SD)

| Formulations | Particle size (d.nm) | | Polydispersity index | | Zeta Potential (mV) | |
|---|---|---|---|---|---|---|
| | Medium (n=3) | Standard deviation | Medium (n=3) | Standard deviation | Medium (n=3) | Standard deviation |
| Drug free SLNs | 290.5 | ±3.21 | 0.212 | ±1.32 | -35 | ±5.90 |
| PITC-2 loaded SLNs | 171.5 | ±4.87 | 0.101 | ±1.09 | -36.1 | ±5.57 |

## Entrapment Efficacy:

Total drug used in preparation of formulation was 10 mg.

Amount of drug present in the SLNs was found to be (10-4.8) 5.2 mg.

Entrapment Efficacy EE (%) = $\frac{\text{Total mass of PITC2} - \text{Mass of PITC2 in suspension}}{\text{Total mass of PITC2}}$ x 100

$$= \frac{5.2}{10} \text{ x } 100$$

$$= 52\ \%$$

So Entrapment efficacy percentage of the prepared formulation was 52%.

$$\text{Drug loading (DL) \%} = \frac{\text{Total mass of PITC2} - \text{Mass of PITC2 in suspension}}{\text{Total mass of Lipid}} \text{ x } 100$$

$$= \frac{5.2}{50} \text{ x } 100$$

$$= 10.4\ \%$$

So drug loading percentage of the prepared formulation was 10.4 %.

## Thermal analysis by differential scanning calorimetry (DSC):

Fig 4 shows DSC curve of phytochemical PITC-2, glycerol monostearate GMS, drug free SLN, and PITC-2 loaded SLN formulation. A clear sharp pick observed at 106.26°C for phytochemical PITC-2 which corresponds to its melting temperature. PITC-2 loaded SLN shows pick at 65.14°C where as drug free SLN shows pick at 64.21°C. This slight shift of pick was due to loading of drug in the lipid matrix. Absence of drug pick at 106°C indicates either solubilization of PITC-2 in lipid matrix upon heating or due to amorphous dispersion of PITC-2 in lipid matrix.

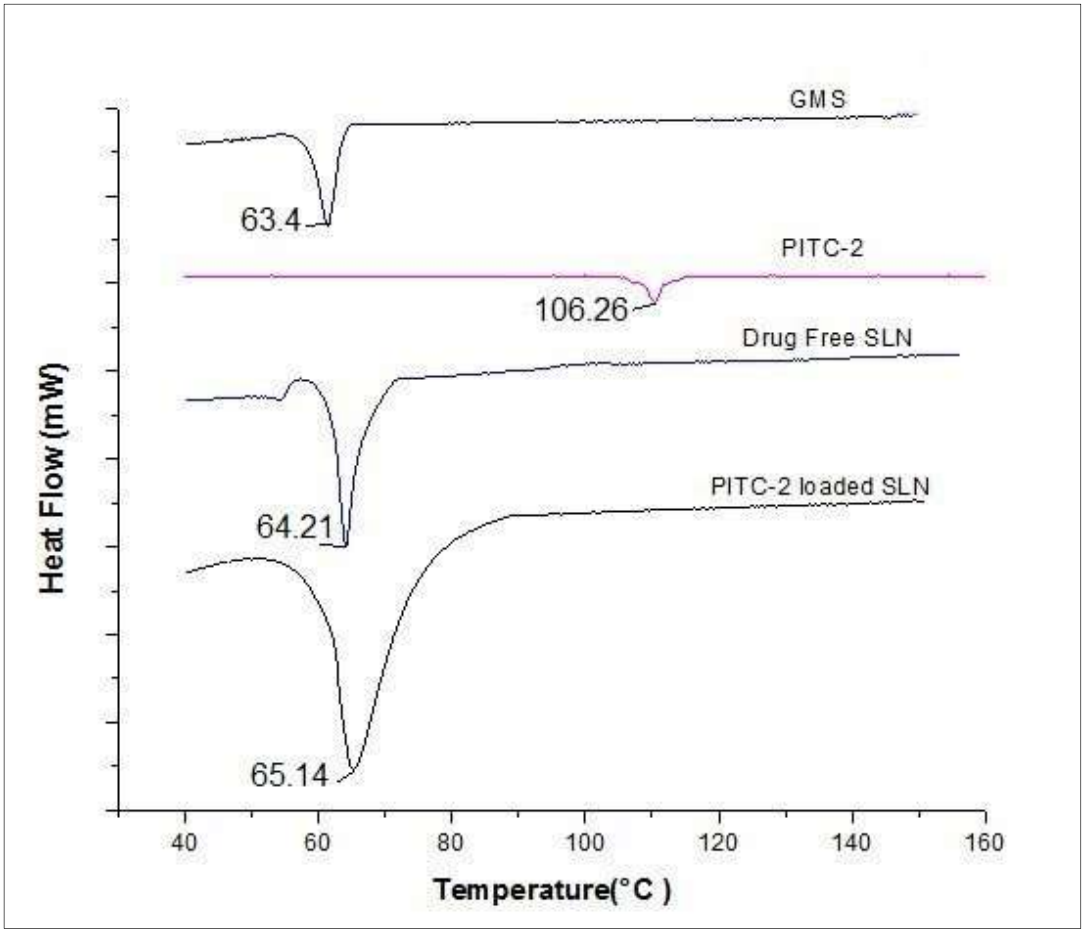



***Figure 4***: DSC Thermo gram of phytochemical PITC-2, glycerol monostearate GMS, drug free SLN, and PITC-2 loaded SLN formulation.

## Powder X-ray diffractometry (PXRD):

Fig 5 shows PXRD curve of phytochemical PITC-2, drug free SLN, and PITC-2 loaded SLN formulation. Phytochemical PITC-2 have picks at *2θ* scattered angle shown in fig no 4. Those picks are not found in the diffractogram of PITC-2 loaded SLNs which indicates PITC-2 was solubilized and stabilized in lipid matrix of formulation. If PITC-2 was present outside of lipid matrix then due to its poor solubility it would crystallized outside the lipid matrix and effect the diffraction curve. Now the diffractogram of drug free SLNs closely resembles to the diffractogram of PITC-2 loaded SLNs which indicates no change happens in the nature of SLNs due to addition of PITC-2.

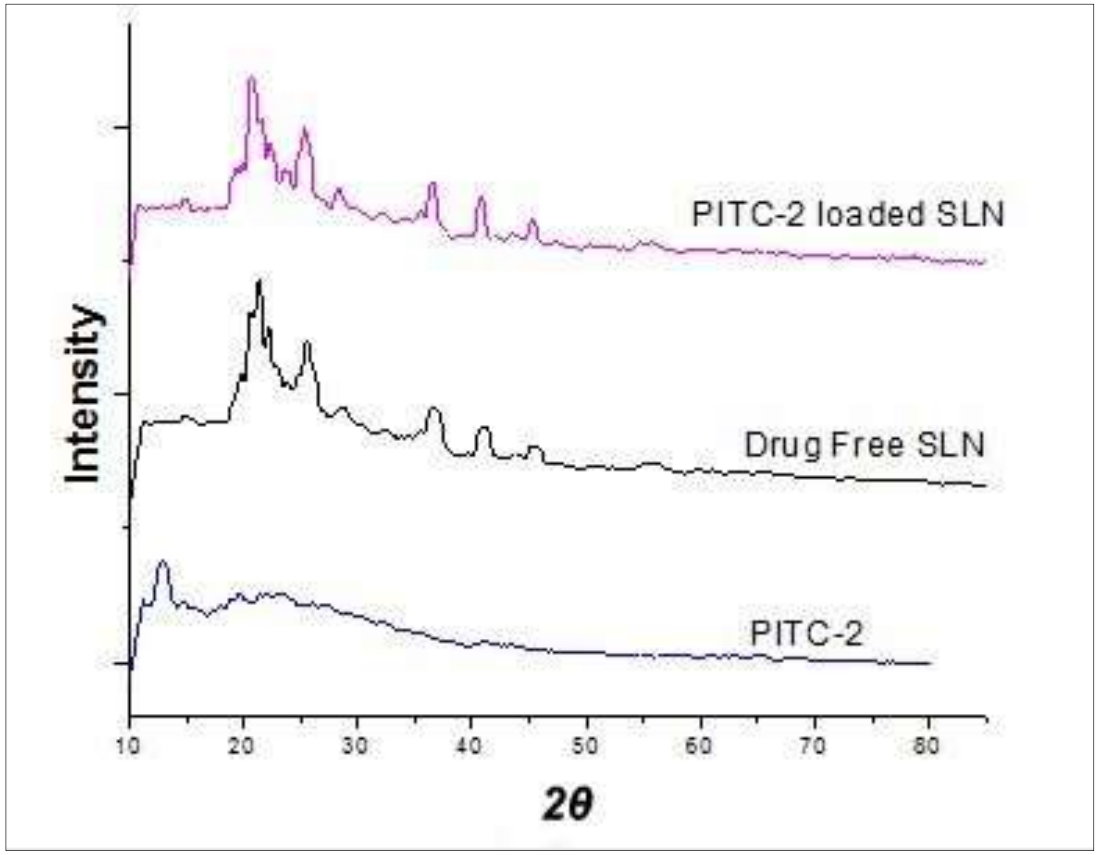



***Figure 5***: PXRD profile of phytochemical PITC-2, glycerol monostearate GMS, drug free SLN, and PITC-2 loaded SLN formulation.

## Cryogenic field emission scanning electron microscopy (Cryo-FESEM):

To check the surface morphology of nanoparticles Cryo-FESEM was done. Size and shape of the nanoparticles are identified by this method. In our study from Cryo-FESEM of PITC-2 loaded SLNs it was observed that they are almost spherical in shape, and have a smooth surface morphology shown in Fig 6A. Agglomeration of nanoparticles are also found which may be due to the lipid as a carrier system. On the other hand Fig 6B shows crystalline structure of free phytochemical PITC2. Absence of such crystalline structure of PITC-2 in SLNs formulation shows no unentrapped in the formulation.

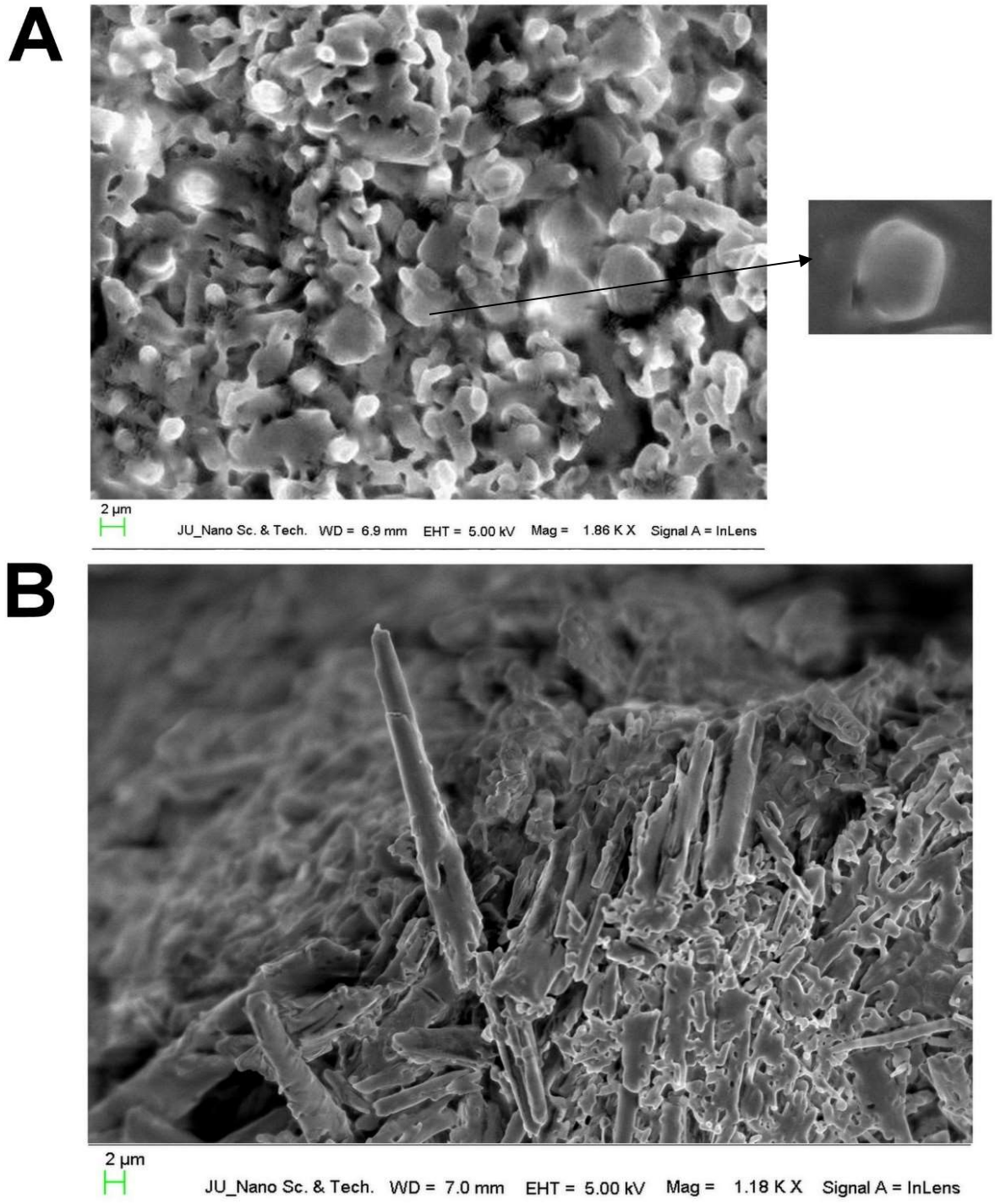



***Figure 6***: FESEM images of A.PITC-2 loaded SLNs, and B. phytochemical PITC-2.

## Stability of SLNs:

There are minor changes in particle size, PI, ZP and EE after 3 months of storage at 4°C shown in Table 2 but no changes at all were found in particles stored at 25°C shown in Table 3. However the changes are not that significant which indicates a good physical stability of PITC-2 SLNs for 3 months at 4°C and 25°C.

***Table 2*:** Particle Size, Polydispersity index, Zeta Potential and Entrapment Efficacy of PITC-2 loaded SLNs for 3 months at 4°C(data represents mean ±SD)

| Parameters | Fresh SLNs | SLNs after 1 month at 4°C | SLNs after 2 month at 4°C | SLNs after 3 month at 4°C |
|---|---|---|---|---|
| Particle size(d.nm) | 171.5 ± 4.87 | 173.45 ± 4.09 | 177.23 ± 3.78 | 182.43 ± 5.13 |
| Polydispersity index | 0.101 ± 1.09 | 0.103 ± 1.23 | 0.106 ± 0.98 | 0.116 ± 2.74 |
| Zeta Potential (mV) | -36.1 ± 5.57 | -35.87 ± 5.45 | -33.34 ± 4.87 | -31.87 ± 4.32 |
| Entrapment efficacy (%) | 52 ± 0.53 | 50.7 ± 0.98 | 50.35 ± 1.02 | 48 ± 1.23 |

***Table 3*:** Particle Size, Polydispersity index, Zeta Potential and Entrapment Efficacy of PITC-2 loaded SLNs for 3 months at 25°C (data represents mean ±SD)

| Parameters | Fresh SLNs | SLNs after 1 month at 25°C | SLNs after 2 month at 25°C | SLNs after 3 month at 25°C |
|---|---|---|---|---|
| Particle size (d.nm) | 171.5 ± 4.87 | 171.67 ± 4.45 | 171.86 ± 4.76 | 172.21 ± 3.08 |
| Polydispersity index | 0.101 ± 1.09 | 0.101 ± 0.89 | 0.102 ± 1.67 | 0.105 ± 1.65 |
| Zeta Potential (mV) | -36.1 ± 5.57 | -36.1 ± 5.86 | -35.54 ± 4.34 | -35.18 ± 4.68 |
| Entrapment efficacy (%) | 52 ± 0.53 | 51.89 ± 0.67 | 51.76 ± 0.61 | 51.24 ± 0.43 |

## *In vitro* release kinetics of SLNs:

Fig 7 and Table 4shows a sustained drug release of PITC-2 loaded SLNs at 7.4 pH phosphate buffer. At fixed intervals of 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 22, 24, 28, 32, 36, 44, 46 and 48 h, 1ml of sample was withdrawn and replaced with fresh buffer from release media and analyzed by UV at 325 nm. Percentage drug release verses time is plotted to determine the pattern of drug release. It was observed that 35 % of the drug was released in first 2 hr and then a sustained release of SLNs occurs. These burst release occurs may be due to presence of absorbed drug in the surface of the nanoparticles. After 48 hrs of percentage drug release was 79.02%. It was shown in Fig 7.

***Table 4*:** Release data of PITC-2 loaded SLNs:

| Time in hrs | Absorbance | Concentration | Amount drug release (mg) | % drug release |
|---|---|---|---|---|
| 0.5 | 0.093 | 0.830 | 0.830 | 16.60 |
| 1 | 0.108 | 1.098 | 1.098 | 21.96 |
| 1.5 | 0.128 | 1.455 | 1.455 | 29.10 |
| 2 | 0.145 | 1.758 | 1.758 | 35.16 |
| 3 | 0.158 | 1.991 | 1.991 | 39.82 |
| 4 | 0.170 | 2.205 | 2.205 | 44.10 |
| 6 | 0.179 | 2.366 | 2.366 | 47.32 |
| 8 | 0.192 | 2.60 | 2.60 | 52.00 |
| 10 | 0.207 | 2.866 | 2.866 | 57.32 |
| 12 | 0.219 | 3.080 | 3.080 | 61.60 |
| 22 | 0.242 | 3.491 | 3.491 | 69.82 |
| 24 | 0.244 | 3.526 | 3.526 | 70.52 |

| | | | | |
|---|---|---|---|---|
| 28 | 0.241 | 3.651 | 3.651 | 73.02 |
| 32 | 0.259 | 3.794 | 3.794 | 75.88 |
| 36 | 0.263 | 3.866 | 3.866 | 77.32 |
| 44 | 0.265 | 3.901 | 3.901 | 78.02 |
| 46 | 0.266 | 3.920 | 3.920 | 78.40 |
| 48 | 0.267 | 3.937 | 3.937 | 78.74 |

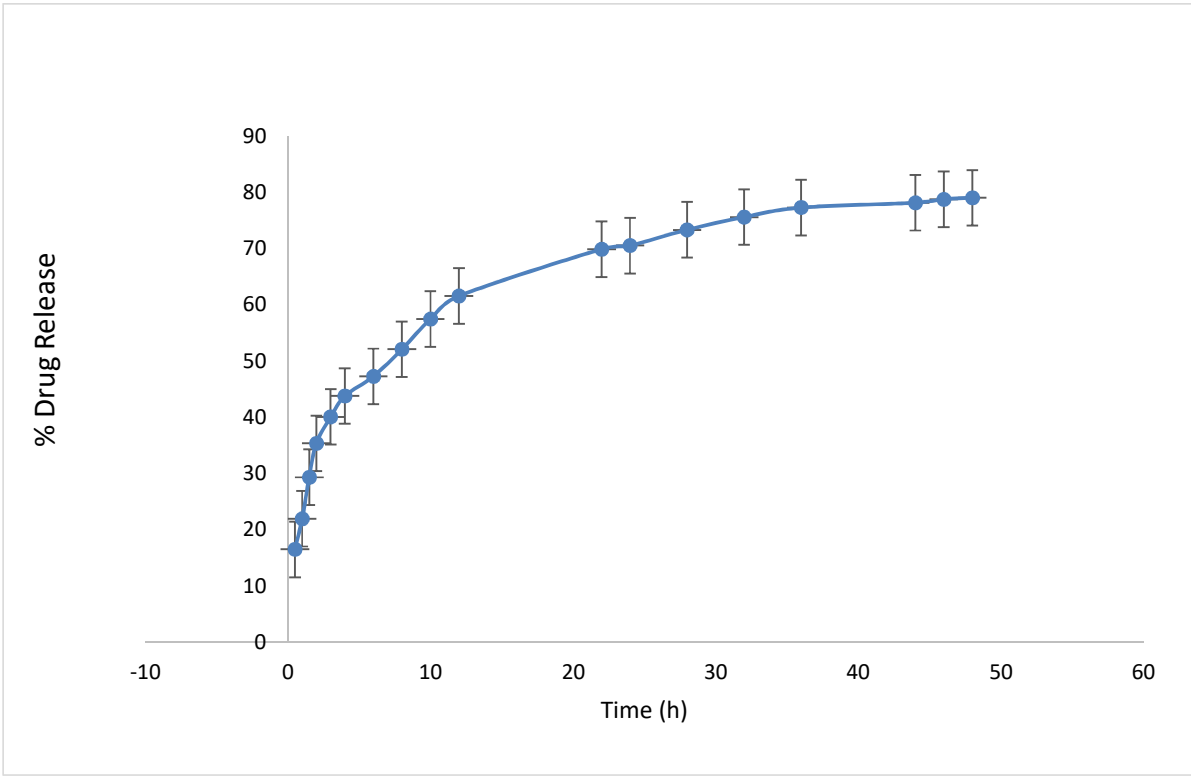



***Figure 7***: Drug release profile of PITC-2 loaded SLNs (data represents mean ±SD).

## Discussion:

Cancer is considered as the most killer disease all over the world. Cancer therapies are remarkably consistent for last 50 years. Chemotherapeutic drugs plays a major role for its treatment but clinical success of these treatments reached a plateau (Isha Dhamija 65, 2013).

Moreover **c**urrently available cytotoxic drugs have many adverse reactions. These problems can be minimized to a great extent by natural compound. So in this study we are using phytochemical PITC-2, obtained from tissue cultured medicinal plant *Pluchea indica* which has potent cytotoxic effect (Soumita Goswami 2018). Many techniques have been employed in the pharmaceutical industry to formulate a drug with good bioavailability and low toxicity. In this context SLN plays a major role and it also obtains good sustained release for lipophilic drugs. We had successfully prepared PITC-2 loaded SLNs for i.v. administration. In general SLNs obtained are round in shape, uniform particle size and are well dispersed. It is also observed that particle size of PITC – 2 loaded SLNs are smaller than drug free SLN formulation.

In the present study phytochemical PITC-2 have a melting point (106 – 108°C) more than the melting point of the lipid used (57 – 65 °C). Hence upon cooling the hot emulsion it is expected that the lipid will solidify faster than the drug and the drug will form a lipid core (Jenning V. 2000). And thus the prepared formulation is a PITC-2 enriched core SLNs formulation.

Zeta potential and stability of nanoparticles are closely related to each other. Due to Van Der Wall inter particle attraction if a dispersion have low zeta potential then particles will get aggregated (Muller R.H. 50(2000)). In our study the zeta potential of all the prepared formulation were negatively charged and varied from -33.2 to -37.5 mV which ensured a system with good stability as it possesses sufficient repulsion. The zeta potential with negative charge might not interfere with the absorption of the formulation (P.K. Senthil 8(1) 2015).

DSC was a tool which investigate the crystalline behavior and melting point. The thermogram of GMS and PITC-2 shows melting pick at 63.4°C and 106.26°C respectively. PITC – 2 also shows multiple sharp peaks at 2θ scattered angle which defines its crystalline nature. But in the DSC thermogram and XRD of PITC-2 loaded SLN nanoparticles there is no peak of phytochemical PITC-2. It shows PITC-2 is no more in a crystalline form in SLN formulations but in an amorphous form. This reveals a homogeneous dispersion of drug in the lipid. This phenomenon is also observed from the morphological study of the SLNs.

Cryo-FESEM of phytochemical PITC-2 shows crystalline structure of free phytochemical PITC2. But in PITC-2 loaded SLNs it was observed that they are almost spherical in shape, and

have a smooth surface morphology. Absence of such crystalline structure of PITC-2 in SLNs formulation shows no unentrapped in the formulation.

The release study shows a biphasic pattern. 35 % of the drug was released in first 2 hr and then a sustained release of SLNs occurs. These burst release occurs may be due to presence of absorbed drug in the surface of the nanoparticles. After 48 hrs of percentage drug release was 79.02%. This in important for the physiological requirements of human system. A similar type of pharmacokinetic study was observed by Shengpeng Wanga *et al.* (Shengpeng Wanga 2012)

Another major problem of cancer chemotherapy is liver toxicity which is due to accumulation of drug metabolites in liver (Debnath S 2017). Toxicity is a major problem of drug delivery system too. One can have a neat delivery system but it is more important to be a safe delivery system with low toxicity for its introduction in pharmaceutical and clinical market. Toxicity is due to the excipients in carrier system. Use of glycerides composed of fatty acid, contained in oils of parenteral fat emulsion may reduce toxicity (Muller R.H. 50(2000)). In our study we had used glyceryl monostearate which may not induce any toxicity for the SLNs. Furthermore surfactant used in our study was poloxomer which is recommended as an acceptable surfactant for use in parenteral formulations. (Muller R.H. 50(2000)). Here liver toxicity was analyzed in this experiment by biochemical studies and histopathology of liver tissue. AST, ALT and ALP levels of control group mice, normal mice and drug treated mice were compared. It was found that groups treated with PITC-2 loaded SLNs had less toxic liver as the level of AST, ALT and ALP are close to normal value. These all features like decrease in body weight, tumor volume, increase in lifespan and survival time, stabilizing hematological and biochemical parameters of PITC-2 loaded SLN formulation are nearly same and even better in comparison to the effect of phytochemical PITC-2. These features helps in judging the PITC-2 loaded SLNs activity.

# Reference

A Sinha, K. Banerjee, A Banerjee, S Das, SK choudhuri. "Synthesis characterisation and biological evaluation of a noble vanadium complex as a possible anticancer agent." *Journal of organometallic chemistry*, 2014: 34-41.

A. Pal, I. Bhattacharya, K. Bhattacharya, C. Mandal, M. Ray. "Methylglyoxal induced activation of murine peritoneal macrophages and surface marker of T lymphocytes in sarcoma 180 bearing mice:involvement of MAP kinase NF- Kappa beta signal transduction pathway." *Molecular Immunology*, 2009: 2039-2044.

Alisa Bahar Beydogan, Sema bolkent. "The effects of silibin adminiistration for different time periods on mouse liver with Ehrlich ascites carcinoma." *Pharmacological Reports*, 2016: 543-549.

Biswas R, Bandyopadhyaya D, Mishraa M, Pramanika KC , Chatterjee TK. "Isolation of pure compound R/J/3 from *Pluchea indica* (L.) Less. and its anti-amoebic activities against Entamoeba histolytica." *Phytomedicine*, 2007: 14: 534-537.

Byung Do Kim, Kun Na, Hoo Kyun Chio. "Preparation and characterization of solid lipid nanoparticles (SLN) made of cocoa butter and curdlan." *European journal of pharmaceutical scienses*, 24(2005): 199-205.

C. Olbrich, O. Kayser, R. H. Muller. "Lipase degradation of Dynasa 114 and 116 solid lipid nanoparticles (SLN) - effect of surfactents, storage time and crystallinity." *Int.J. Pharm*, 237(2002): 119-128.

Chakraborty S, Chatterjee TK. "Targeting viseral leishmaniasis by mannosylated liposome incorporated PITC-2 isolated from the plant *Pluchea indica* (L) Less." *Int J Pharm Bio Sci*, 8(2)2017: 396-408.

Das S, Ng WK, Kanaujia P, Kim S. "Formulation design, preparation and physicochemical characterizations of solid lipid nanoparticles containing a hydrophobic drug: Effects of process variables." *Colloids and Surf B: Biointerfaces*, 2011: 88, 483-489.

de Costa PM, de Costa MP , Jamacaru VF, Ferreira PM. " Inprovement of *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives." *Chem Biol Interact* , 239 (2015): 174-83.

de Sousa AP, Torres MR, Pessoa C, Costa Lotufo LV. "*In vivo* growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed Sargassum vulgare." *Carbohydr Polym*, 2007: 69: 7-13.

Debnath S, Karan S, Debnath M, Chatterjee TK. " Poly-L-Lysine inhibits tumor angiogenesis and induces apoptosis in ehrlich ascites carcinoma and sarcoma S-180 tumor. ." *Asian Pac J Cancer Prev*, 2017: 18(8): 2255 - 68.

Debnath S, Karan S, Debnath M, Chatterjee TK. "Poly-L-Lysine inhibits tumor angiogenesis and induces apoptosis in ehrlich ascites carcinoma and sarcoma S-180 tumor." *Asian Pac J Cancer Prev*, 18(2017): 2255 - 2268.

Elsherbiny NM, Younis NN, Elseweids MM. " The synergistic effect between vanillin and doxorubicin in ehrlich ascites carcinoma solid tumor and MCF-7 human brest cancer cell line." *Pathol Res Pract*, 212(2016): 767-77.

Gupta M, Mazumdar UK, Kumar SA, Kumar TS. "Antitumor activity and antioxidant role of Bauhinia racemosa against Ehrlich ascites carcinoma in swiss albino mice." *Acta Pharmacol Sin*, 25(8), 2004: 1070-6.

Harborne JB. "Arsenal for Survival: Secondary Plant Products." *Taxon*, 49 (2000).: 435-439.

Ho Lun Wong, Reina Bendayan, Andrew. M. Rauth, Yongyiang Li, Xiao Yu Wu. "Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles." *Advanced drug delivery reviews*, 59 (2007): 491 - 504.

Isha Dhamija, Nitesh Kumar, S.N. Manjula, Vipan Parihar, M. MManjunath Setty, K.S.R. Pia. "Preliminary evaluation of *in vitro* cytotoxicity and *in vivo* antitumor activity of Premna herbacea Roxb. in ehrtich ascites carcinoma model and daltons lymphona ascites model." *Experimental and toxicologic pathology.*, 65, 2013: 235 - 242.

Jenning V., Gysler A., Schafer Korling M. Gohla SH. "Vitamine A loaded solid lipid nanoparticles for tropical use: Occlusive properties and drug targeting to the upper skin." *Eur J Pharm Biopharm*, 2000: 49: 211 - 21.

M.L.Salem, N.M.Shoukry, A.A.Rahman. "*In vitro* and *in vivo* antitumor effects of the Egyptian scorpion Androclonus amoreuxi venam in an Ehrlich ascitic tumor model." *Springer Plus*, 2016: 570-581.

Muller R.H., Madar K., Gohla S. "Solid lipid nanoparticles (SLN) for controlled drug delivery-a revier of the stste of the art." *Eur, J. Pharm. Biopharm*, 50(2000): 161-177.

Nicol B.M., Prasad S.B. "The effects of cyclophosphamide alone and in combination with ascorbic acid against murine ascited Dalton's lymphoma." *India J Pharmacol*, 2006: 260-265.

P.K. Senthil, A. Arivuchelvin, A. Jagadeeswaran, N. Subramanian, C.K Senthil, P.Mekala. "Formulation, optimization and evaluation of enrofloxacin solid lipid nanoparticles for sustained oral delivery." *Asian journal of pharmaceutical and clinical research*, 8(1) 2015: 231 - 236.

Pal A, Bhattacharya I, Bhattacharya K, Mandal C, Ray M. "Methylglyoxal induced activation of murine peritoneal macrophages and surface marker of T lymphocytes in sarcoma 180 bearing mice:involvement of MAP kinase NF- Kappa beta signal transduction pathway." *Molecular Immunology*, 2009: 46: 2039-2044.

Pramanik KC, Biswas R, Mitra A, Bandyopadhyay D, Mishra M, Chatterjee TK. "Tissue culture of the plant *Pluchea indica* (L.) Less. and evaluation of diuretic potential of its leaves. ." *Orient Pharm Exp Med .*, 7(2)2007: 197-204.

R.Perveen, F.Islam, J.Khanum, T.Yeasmin. "Preventive effect of ethanolic extract of Alpinia calcarata Rose on ehrlichsascitic carcenoma cell induced malignant ascitic in mice." *Asian pecific journal of tropical medicine*, 2012: 121-125.

S. Yang, J. Zhu, Y. Lu, B. Liang, C. Yang. "Body distribution of camptothecin solid lipid nanoparticle after administration." *Pharm. Res*, 16(5) 1999: 751-757.

S.S.Agarwal, S. Saraswati, R. Mathur. "Cytotoxic and antitimor effect of brucine on ehrlich ascites tumor and human cancer cell line." *Life Sciences*, 2011: 147-158.

Shengpeng Wanga, Tongkai Chenb, Ruie Chena, Yangyang Hua, Meiwan Chena, Yitao Wanga. "Emodin loaded solid lipid nanoparticles: Preparation, characterization and." *International Journal of Pharmaceutics*, 2012: (460) 238-246 .

Soumita Goswami, Souvik Debnath, Saumen Karan, Tapan Kumar Chatterjee. "*In vivo* antitumor activity of phytochemical PITC-2 obtained from tissue cultured plant *Pluchea indica* on sarcoma-180 solid tumor mice model." *Asian journal of Pharmaceutical and Clinical research*, 2018: 4(11), 211 - 218.

Surajit Das, Wai Kiong Ng, Parijat Kanaujia, Sanggu Kim,. "Formulation design, preparation and physicochemical characterizations of solid lipid nanoparticles containing a hydrophobic drug: Effects of process variables." *Colloida and Surfaces B: Biointerfaces*, 88(2011): 483-489.

# CHAPTER 5

# Toxicity Study of PITC-2 loaded Solid Lipid Nanoparticle Formulation

# Acute toxicity study of PITC-2 loaded SLNs

## Animals:

*In vivo* studies were carried out on albino rats weighing 250 ± 10g of either. The animals were kept under standard conditions of 12:12 hr light and dark cycle in polypropylene cages and fed with standard laboratory diet and water *ad libitum.* They are provided with sawdust bedding which was changed twice a weak. Room temperature was maintained at 25 ± 2°C and relative humidity at 50 %. All *in vivo* experiments were conducted as per the guideline of Animal Ethics Committee of the Department of the Pharmaceutical Technology of Jadavpur University, India.

## Acute toxicity study:

Acute toxicity describes the adverse effects of a substance that result either from a single exposure, or from multiple exposures in a short period of time usually less than 24 hours ((CDER) 1996). Here we are using a single dose study for 24 hrs. For experimental purpose animals are divided groups each containing 10 mice (5 male and 5 female). The formulation is solubilized in water for injection and doses are prepared as 5mg/kg, 10mg/kg, 20mg/kg, 25mg/kg and 30mg/kg body weight. After administration of drug i.v., animals were closely observed for 2 hrs to identify any type of following symptoms includes i) Convulsion and tremors, ii) Righting reflex, iii) coordination of movement, iv) frequency of movement, v) size of pupil, vi) color of ear, vii)color of urine, viii) excretion. Number of dead rats within 24 hrs of drug administration was noted.

# Sub-acute toxicity study of PITC-2 loaded SLNs

## Animals:

*In vivo* studies were carried out on Swiss albino mice weighing 24 ± 1g of either. The animals were kept under standard conditions of 12:12 hr light and dark cycle in polypropylene cages and fed with standard laboratory diet and water *ad libitum.* They are provided with sawdust bedding which was changed twice a weak. Room temperature was maintained at 25 ± 2°C and relative humidity at 50 %. All *in vivo* experiments were conducted as per the guideline of Animal Ethics Committee of the Department of the Pharmaceutical Technology of Jadavpur University, India.

## Administration of sample:

The animals were divided into 3 groups 10 mice in each group (5 male and 5 female). Group 1 was not treated with drug. It was treated as normal group and administered with water for injection only. Group 2 receives 5mg/kg body weight dose of PITC-2 SLNs i.v. and group 3 receives 10 mg/kg body weight dose of PITC-2 SLNs i.v for 14 consecutive days.

## General parameters of observation:

During these whole experimental period some behavioral changes were monitored. Parameters which were monitored includes Diarrhea and Changes in food intake. Any sigh of mortality and changes in body weight were also monitored daily.

## Hematological Parameters:

Hematological studies were performed by recording the hemoglobin content, red blood cell (RBC) and white blood cells (WBC) counts. from freely flowing tail vein blood (Gupta M 2004). Differential WBC leukocyte counts were carried out including neutrophils, lymphocytes and monocytes from leishaman stained blood smears of normal and PITC 2 SLNs treated groups at different doses respectively. Blood were collected from freely flowing tail vein blood (Gupta M 2004) after $7^{th}$ day of treatment initiation and at $14^{th}$ day.

## Biochemical Parameters:

After 24 hr of last dose and 18 hr of fasting the blood sample were collected by puncturing retro-orbital plexus. Blood was used for the assay of biochemical parameters serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, globulin, bilirubin, alkaline phosphatase and serum protein to investigate liver function alteration and creatinine was measured as renal function parameter.

## Histopathology:

After 14 days of drug administration animals were sacrificed. Liver and kidney were excised from the mice body and fixed in 10% buffered formalin and embedded in paraffin. +5-µm sectioned was stained with hematoxylin and eosin (H&E) (Elsherbiny NM 2016). Histological

analysis was performed under light microscope (40X). The morphological changes were photographed and they are compared.

### Statistical analysis:

All *in vitro* studies were carried out in triplicate represented by independent biological evaluation. Data are extracted as mean ± standard error of the mean (SEM) and were compared by one way analysis of variance (ANOVA) followed by Kruskal Wallis Test using SPSS statistical software of 20.0 version. $P<0.05$ was considered to be statistically significant when compared with control.

## Result:

### Acute toxicity study:

It was found that PITC-2 loaded solid lipid nanoparticles are good tolerated up to a dose of 15mg/kg and no mortality was found in 24 hours after dosing. But at dose 20mg/kg 10% of the animal died. Mortality rate was shown in Table 1. Some behavioural changes were also observed in the period of 2 hours after dosing i.v. of PITC-2 SLNs at different doses which are shown in Table 2.

***Table 1***: Effect of PITC-2 SLNs on mortality in rats after 24 hours of dosing

| Groups | Dose (mg/kg) i.v | Dead | % Dead |
|---|---|---|---|
| 1 | 5 | 0 | 0 |
| 2 | 10 | 0 | 0 |
| 3 | 20 | 1 | 10 |
| 4 | 25 | 2 | 20 |
| 5 | 30 | 3 | 30 |

***Table 2***: Effect of PITC-2 SLNs on behavioural changes in rats after 2 hours of dosing

| Dose mg/kg | Convulsion or tremor | Coordination on of movement | Frequency of movement | Righting reflex | Size of pupil | Color of ear | Color of urine | Excretion |
|---|---|---|---|---|---|---|---|---|
| 1 | N | A | N | P | N | N | N | N |
| 2.5 | N | A | N | P | N | N | Y | N |
| 5 | N | A | N | P | N | N | Y | N |
| 10 | N | A | D | P | N | N | Y | N |
| 15 | NN | A | D | P | N | N | Y | NN |

N = Normal, NN = Not Normal, D = Decreased, Y = Yellow, A = Absent, P = Present

## Sub-acute toxicity study:

For sub-acute toxicity study we had divided the animals into 3 groups 10 animal each. After monitoring the body weight of test animals through 14 days it was found that 7.50 % gain in weight for group receiving only water for infection whereas groups receiving 5 mg/kg b.w. and 10 mg/kg b.w. of PITC-2 SLNs shows 6.42 % and 2.72 % gain in body weight respectively. Change in body weight after 14 days of treatment are not significant. This was shown in Table 3.During this period behavioural change in animals were also monitored which includes includes Diarrhea and Changes in food intake. No such type of behavioral changes are observed in both test and normal group of animals. The mortality rate in this groups are also monitored shown in Table 4. No mortality was found in group receiving 5 mg/kg b.w. of PITC-2 SLNs but in case of group receiving 10mg/kg bw of PITC-2 SLNs 10% of death case was recorded.

***Table 3***: Effect of PITC-2 SLNs at different dose on change of body weight in mice for 14 days of treatment

| Group | Body weight (gm) | | | | | | % of changes |
|---|---|---|---|---|---|---|---|
| | 0 days | 3 days | 6 days | 9 days | 12 days | 14 days | |
| PITC-2 SLNs 5mg/kg | 25.21 ± 0.76 | 25.49 ± 0.67 | 25.77 ± 0.81 | 26.18 ± 0.43 | 26.51 ± 0.92 | 26.83 ± 0.79 | 6.42 % gain in weight |
| PITC-2 SLNs 10mg/kg | 25.35 ± 0.82 | 25.57 ± 0.67 | 25.62 ± 0.73 | 25.71 ± 0.91 | 25.97 ± 0.32 | 26.04 ± 0.53 | 2.72 % gain in weight |
| Normal | 24.67 ± 1.21 | 24.89 ± 1.33 | 25.65 ± 1.15 | 26.06 ± 1.02 | 26.35 ± 0.98 | 26.52 ± 1.08 | 7.50 % gain in weight |

***Table 4***: Effect of PITC-2 SLNs at different dose on mortality in mice for 14 days of treatment

| Group | Mortality | | | | | | % of Mortality |
|---|---|---|---|---|---|---|---|
| | 0 days | 3 days | 6 days | 9 days | 12 days | 14 days | |
| PITC-2 SLNs 5mg/kg | 0 | 0 | 0 | 0 | 0 | 0 | 0 % |
| PITC-2 SLNs 10mg/kg | 0 | 0 | 0 | 0 | 0 | 1 | 10 % died |
| Normal | 0 | 0 | 0 | 0 | 0 | 0 | 0 % |

Table 5 shows the haematological parameters after a 14 day long treatment with PITC-2 SLNs at different dose. No significant change in haemoglobin level, RBC and WBC count, neutrophil, lymphocyte and monocyte are found in test groups in comparison to normal groups which are

not treated with PITC-2 SLNs. Like haematological parameters biochemical parameters were also analysed shown in Table 6. Serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, globulin, bilirubin, alkaline phosphatase and serum protein were analyzed. In case of groups treated with different doses of SLNs shows some changes in comparison with normal mice group but the changes are not that significant and remains within the normal range.

***Table 5***: Effect of PITC-2 SLNs at different dose on Haematological profile in mice after 14 days of treatment

| | PITC-2 SLNs 5mg/kg b.w | PITC-2 SLNs 10 mg/kg b.w | Normal Mice |
|---|---|---|---|
| Hemoglobin(gm %) | 10.89± 0.05* | 9.67± 0.08* | 11.8±0.12* |
| Erythrocyte(RBC) (cells x$10^6$/$mm^3$) | 7.90± 0.04* | 8.33± 0.03* | 7.28 ±0.02* |
| Leucocytes(WBC) (cells x$10^6$/$mm^3$) | 7.12±0.05* | 8.04±0.05* | 6.40±0.03* |
| Neutrophil (%) | 32.3± 0.03* | 35.46±0.03* | 30.4±0.03* |
| Lymphocyte (%) | 56.0± 0.04* | 50.23± 0.04* | 64.1±0.23* |
| Monocyte (%) | 2.3± 0.03* | 2.03± 0.03* | 2.8±0.01* |

***Table 6***: Effect of PITC-2 SLNs at different dose on Biochemical parameters in mice after 14 days of treatment

| | PITC-2 SLNs 5mg/kgb.w | PITC-2 SLNs 10 mg/kgb.w | Normal Mice |
|---|---|---|---|
| Bilirubin (total) mg/dl | 0.40 ± 0.02* | 0.36 ± 0.02* | 0.45 ± 0.02* |
| Serum protein (total) g/dl | 8.32 ± 0.09* | 8.49 ± 0.07* | 8.19±0.08* |

| | | | |
|---|---|---|---|
| AST (SGOT) IU/L | 45.5 ± 0.01* | 49.3 ± 0.01* | 39.1±0.01* |
| ALT(SGPT) IU/L | 39.6 ± 0.04* | 45.4 ± 0.01* | 29.1±0.04* |
| Serum alkaline phosphatase IU/L | 79.1 ± 0.02* | 88.9 ± 0.02* | 74.4 ± 0.03* |
| CreatinineMg/dl | 0.78 ± 0.01* | 0.8 ± 0.02* | 0.62 ± 0.01* |

## Histopathology of liver and kidney tissue:

H&E stained section of liver of a normal mice receiving water for injection was shown in Fig 1, bearing all the normal features which includes circular hepatic portal vein and of hepatic artery. The tissue section comprises of hepatic sinusoid and nuclei which are usual. Groups receiving PITC-2 SLNs 5 mg/kg and 10 mg/kg also shows liver tissues in a good condition and close to normal although little hepatic deformation and altered hepatocyte population shown in Fig 1B and 1C. Healthy hepatocytes were observed, regular branch of bile duct found, although mild dilation of central vein observed.

H&E stained section of kidney of a healthy mice was shown in Fig 2, bearing all the normal features. Group treated with PITC-2 SLNs shows a little deformation in cell structure though it is close to normal shown in Fig 2B and 2C.

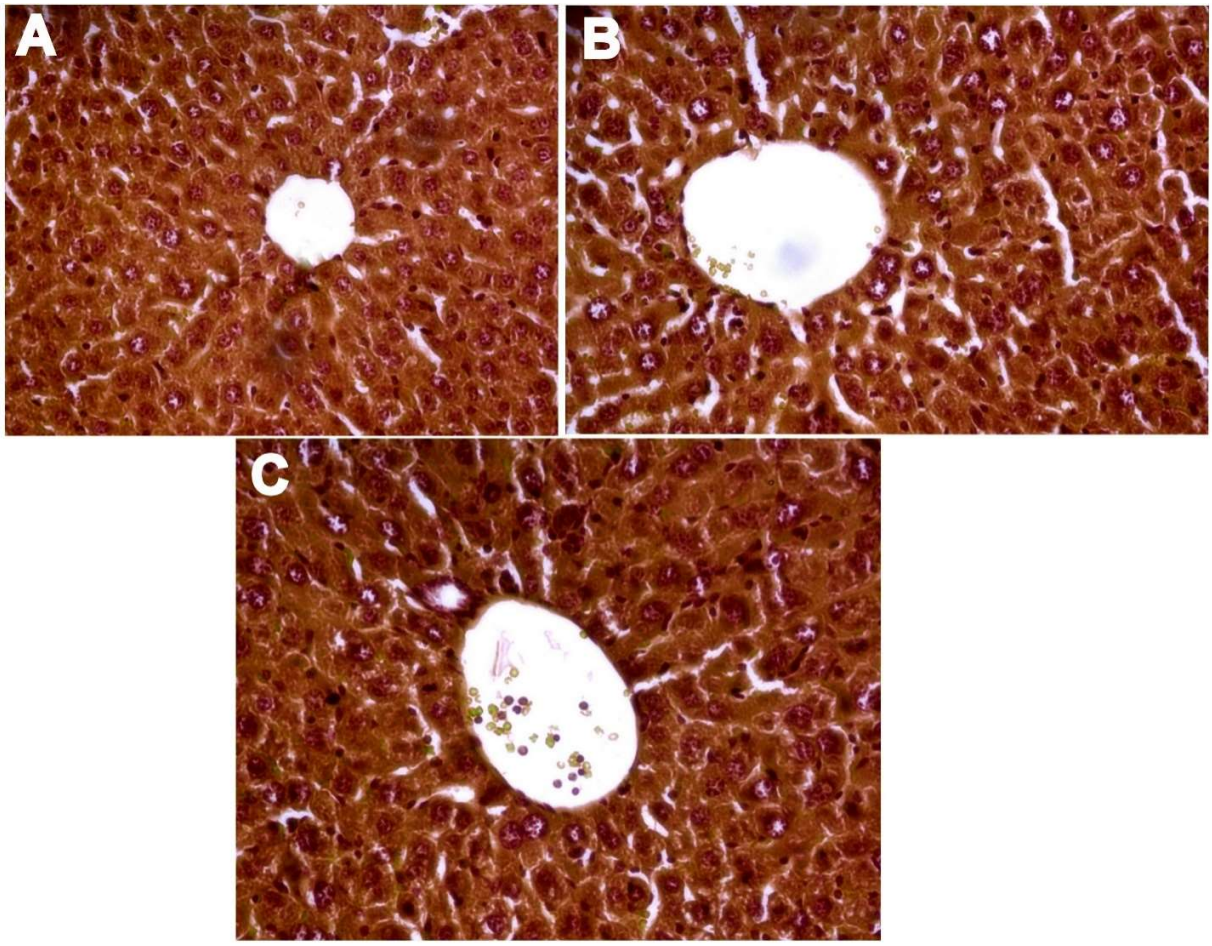



***Figure 1*** **:** H&E stained section of liver for A) normal healthy mice, B) 5 mg/kg of PITC-2 SLNs, C) 10 mg/kg of PITC-2 SLNs.

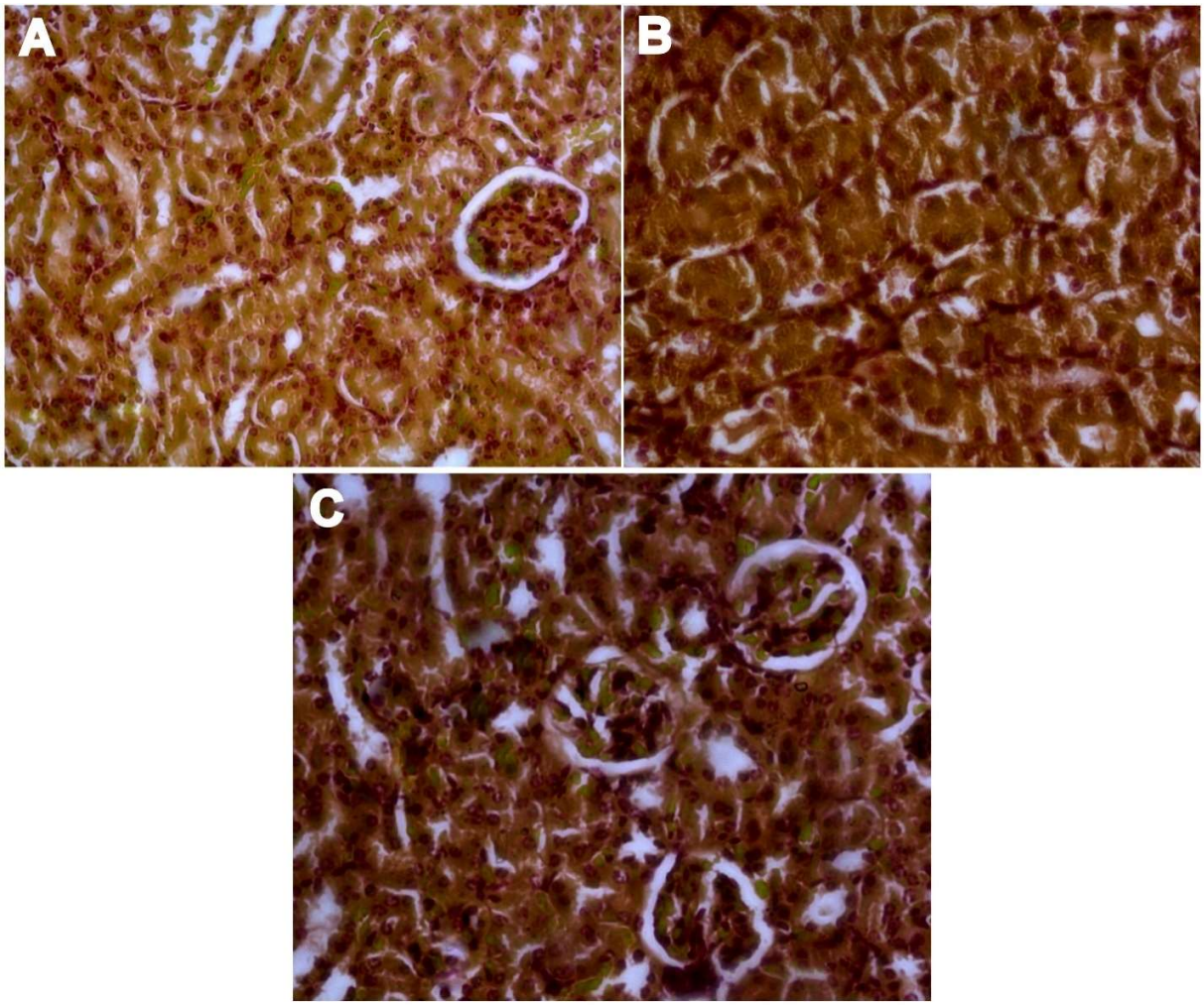



***Figure 2* :** H&E stained section of kidney for A) normal healthy mice, B) 5 mg/kg of PITC-2 SLNs, C) 10 mg/kg of PITC-2 SLNs.

# Discussion:

To observe the toxic manifestation of a drug or formulation toxicity study was performed. To increase the chances of toxic manifestation a dose is selected which is much higher than the therapeutic dose and was given for a long duration. The study was generally performed with a group of animals to get a statistically significant result. In this study we had taken 10 mice in each group to get a statistically sound result. For this study the animals are kept in identical laboratory condition for three weeks before starting of dosing to get acclimatized with the working environment. For acute toxicity study we are using a single dose of PITC-2 of different strength and study for 24 hrs. It was found that up to a dose of 20 mg/kg b.w. it was well tolerated but upon increasing of dose mortality rate increases. Sub acute toxicity was carried out for 14 days with repeated dose (D.L. Eaton 2010).It was found that 7.50 % gain in weight for group receiving only water for infection where as groups receiving 5 mg/kg b.w. and 10 mg/kg b.w. of PITC-2 SLNs shows 6.42 % and 2.72 % gain in body weight respectively. Change in body weight after 14 days of treatment are not significant. No behavioral changes like diarrhea or change in food intake were observed in both test and normal group of animals. The mortality rate in this groups are also monitored. No mortality was found in group receiving 5 mg/kg b.w. of PITC-2 SLNs but in case of group receiving 10mg/kg b.w of PITC-2 SLNs 10% of death case was recorded. Here liver toxicity was analyzed in this experiment by biochemical studies and histopathology of liver tissue. AST, ALT and ALP levels of normal mice and drug treated mice were compared. It was found that groups treated withPITC-2 SLNs had less toxic liver and the level of AST, ALT and ALP are close to normal value. A large number of drugs with different pharmacological action and chemical structure give rise to liver lesion although liver has a unique regenerative ability and great adaptive efficiency. For example an adaptive phenomenon of liver is if an anticonvulsant drug was used for a long time then it causes increase in endoplasmic reticulum. Again regeneration of hepatic tissue occurs by the conjugative tissues after a hepatocellular necrosis due to treatment with alginates (de Sousa AP 2007). In our study prominent nuclei and the tissue section comprises of hepatic sinusoid was observed in normal healthy mice which are usual. Group receiving PITC-2 SLNs at different doses are more or less close to normal group although little changes like deformed hepatic artery are found which show they are less toxic to liver.

Histopathological analysis of kidney was done for identifying any degree of toxicity. In drug treated groups glomerular structures were essentially preserved. The characterization of necrosis is done by no nuclear staining and deeply eosinophilia cytoplasm (de Sousa AP 2007).This show phytochemical PITC-2 are not that toxic to kidney than standard drug and quite resembles to the normal mice. So from this study it was observed that it was safe to use PITC-2 SLNs upto a dose of 10mh/kg. No major acute or sub acute toxicity was found up to this dose.

## Reference:

(CDER), Center for Drug Evaluation and Research. *Guidance for Industry, Single Dose Acute Toxicity Testing for Pharmaceuticals.* Center for Drug Evaluation and Research (CDER), 1996.

D.L. Eaton, E.P. Gallagher. *Comprehensive Toxicology.* Second Edition: Elsevier Ltd, 2010.

de Sousa AP, Torres MR, Pessoa C, Costa Lotufo LV. "*In vivo* growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed Sargassum vulgare." *Carbohydr Polym*, 2007: 69: 7-13.

Ellman, GL. "Tissue sulfhydryl groups." *Archives in Biochemistry and Biophysics.*, 1959: 82, 70-77.

Elsherbiny NM, Younis NN, Elseweids MM. "The synergistic effect between vanillin and doxorubicin in ehrlich ascites carcinoma solid tumor and MCF-7 human brest cancer cell line." *Pathology- Research and Practice*, 2016: 212: 767-777.

Gupta M, Mazumdar UK, Kumar SA, Kumar TS. "Antitumor activity and antioxidant role of Bauhinia racemosa against Ehrlich ascites carcinoma in swiss albino mice." *Acta Pharmacologica Sinica*, 2004: 38: 1070-1076.

Jiang ZY, Hunt JV, Wolff SP. "Ferrous ion oxidation in the presence of xylenol orange for determination of lipid hydroperoxide in low - density lipoprotein. ." *Analytical Biochemistry*, 1992: 202, 384 - 387.

Nishius WG, Samuelsson D. "Formation of Malondialdehyde from phospholipid arachidonate during microsomal lipidperoxidation. ." *European Journal of Biochemistry*, 1968: 6, 126-130.

# CHAPTER 6

# Comparison of Anti-tumor Activity Between Phytochemical PITC-2 and PITC-2 Loaded SLNs Formulation both *in vitro and in vivo*

# Methodology:

## Cytotoxicity Assay (MTT assay):

This test is based on MTT (3-(4, 5- dimethylthiazol -2-yl)-2, 5-diphenyl tetrazolium bromide), which is reduced to a purple-blue soluble formazan by the living cells. Experiments were performed in 96-well flat bottomed culture plates (BD Biosciences, USA). MTT was dissolved in phosphate buffered saline (PBS) at 5mg/ml. Different concentrations of PITC-2 (0.1, 0.5, 01, 5,10,20,25, 30 µg/ml) were added and the plate was incubated for 24 hr. Following incubation with each compound for 24 h, 20 $\mu$L of MTT was added to each well. After incubation for 4 h at 37 °C, the culture medium was removed, and the formazan crystals were dissolved in 200 $\mu$L DMSO (A Sinha 2014). Absorbance (A) of formazan dye was measured at 570 nm using a micro plate reader. The background absorbance was determined at 690 nm and subtracted from the 570 nm measurement. (Abd-El Fattah AA 2017)

The percentage of viable cells was determined by the following equation (Joseph MM 2014):

$$Viablecells(\%) = \frac{A\ of\ treatedcells}{A\ of\ untreatedcells} \times 100$$

The cytotoxic activities of phytochemical PITC-2 and PITC-2 SLNs were determined in human breast cancer cell MCF7, human lung cancer cell A549 and human macrophage cell U937.

## Preparation of Phytochemical PITC-2 and PITC-2 Formulation:

As PITC-2 was not freely soluble in water so it was dissolved using 1% DMSO and prepared in concentration as required. SLNs formulation of PITC-2was dissolved in normal saline in required concentration.

## Ascites tumor induction by EAC cell line and experimental protocol:

EAC cells were preserved *in vivo* by intraperitoneal inoculation of cells in the peritoneal cavity of mice with sterile normal saline. (Placeholder3)[10] .EAC cells were taken from those inoculated mice and injected (0.2 ml of 2 x $10^6$ cells/mouse) to all the groups'intraperitoneally except the normal group (A Sinha 2014).From day 1 after inoculation with EAC cells treatment was given

for 14 days. (S.S.Agarwal 2011) (Alisa Bahar Beydogan 2016)From each group 6 mice were sacrificed and other 6 were kept for calculation of Mean survival time**.**

## Animals and study groups:

*In vivo* studies were carried out on Swiss albino mice weighing 22 ± 0.50g of either. The animals were kept under standard conditions of 12:12 hr light and dark cycle in polypropylene cages and fed with standard laboratory diet and water *al libitum.* All *in vivo* procedures described were reviewed and approved by the University Animal Ethical Committee.

For experimental purpose animals are divided into 4 groups each containing 12 mice.

Group 1: Normal animals (negative control).

Group 2: EAC cancer animals + PITC 2 (5mg/kg/day given i.p)

Group 3: EAC cancer animals + PITC2 formulation (5mg/kg/day given i.v.).

Group 4: EAC cancer animals (Standard) + 5FU (20mg/kg in alternative day given i.p).

## Collection of ascetic fluid:

After 14 days of drug treatment, on 15$^{th}$ day animals were sacrificed. 2ml of normal saline water was injected (i.p.) in the abdominal region and then tumor cells along with ascetic fluid was collected in 15ml centrifuge tubes. Then they are centrifuged for 10 min at 3000 rpm at 4°C. Ascites tumor volume was then calculated by subtracting injected saline volume from the collected whole ascetic fluid and packed cell volume was calculated. (Nicol B.M. 2006)

The viability and non viability of the cells were checked by trypan blue assay. The cells were stained with trypan blue (0.4% in normal saline) dye. The cells that did not take the dye were viable and those who took the dye were non viable. Viability percentage is calculated by (No. of viable/ No. of viable and non viable cells) x 100. (M.L.Salem 2016)

Percentage cell growth inhibition was calculated by the formula = $1 - \frac{Tw}{Cw} x100$

Where Tw is mean of number of tumor cells of the treated group of mice and Cw is mean of number of tumor cells of the control group of mice. (R.Perveen 2012)

## Recording of survival rate:

Mean survival time and percentage increase in life span (%ILS) were calculated by the following formulas (S.S.Agarwal 2011).

$$\text{Mean survival time (MST)} = \frac{\sum \text{Survival time(day) of each mice in a group}}{\text{total number of mice}}$$

$$\%\text{ILS} = \frac{\text{MST of treated mice}}{\text{MST of control mice}} \times 100$$

## Hematological Parameters:

Hematological study includes hemoglobin content, red blood cell (RBC) and white blood cells (WBC) counts. After 14 days of treatment animals were sacrificed by cervical dislocation and blood samples were collected from heart. WBC leukocyte counts were also carried out including neutrophils, lymphocytes and monocytes from leishaman stained blood smears of all groups of animal.

## Biochemical Parameters:

After 24 hour of last dose and 18 hour of fasting the blood samples were collected from heart. Biochemical parameters includes serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, globulin, bilirubin, alkaline phosphatase and serum protein to identify alteration in liver function and creatinine was measured as renal function parameter.

## Histopathology:

After collecting ascitec fluid from treated and control group, they were centrifuged at 3000 rpm for 10 minutes and one part of it is fixed in glass slides with neutral buffer formation. Then they were stained with hematoxylin and eosin. Another part is fixed on glass slide using 95% ethanol and stained with papanicalaou stain. Then both stained slides were mounted with Distrene Dibutyl Phthalate xylene and were examined under light microscope (40X) (Debnath S 18(2017)).

Liver and kidney tissues were isolated from the animals and washed with saline. Then it was fixed in 10% buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin (Elsherbiny NM 212(2016))Then the tissues were sectioned 5 to 6 µm, stained with hematoxylin and eosin (H&E) and examined under light microscope (40X).

### Statistical analysis:

All *in vitro* studies were carried out in triplicate represented by independent biological evaluation. Data are extracted as mean ± standard error of the mean (SEM) and were compared by one way analysis of variance (ANOVA) followed by Kruskal Wallis Test using SPSS statistical software of 20.0 version. $P<0.05$ was considered to be statistically significant when compared with control.

## Result:

### *In-vitro* cell viability assay:

The phytochemical PITC 2 and PITC-2 loaded SLNs were screened for its cytotoxicity against human breast cancer cell MCF7, human lung cancer cell A549 and human macrophage cell U937 at different concentrations (10,20,25, 30 µg/ml) to determine the percentage of viable cells by MTT assay. MTT assay shows significant effect on MCF-7,A-549 and U-937 cancer cells shown in Fig 1, 2 and 3.This result indicates anti-proliferative activity of PITC-2 SLNs in cancer cells is almost same and even much better than the anti-proliferative activity of phytochemical PITC-2 .So it is clear that prepared PITC-2 loaded solid lipid nanoparticles have good cytotoxic activity in compared to PITC-2 in free form. Results are represented graphically below.

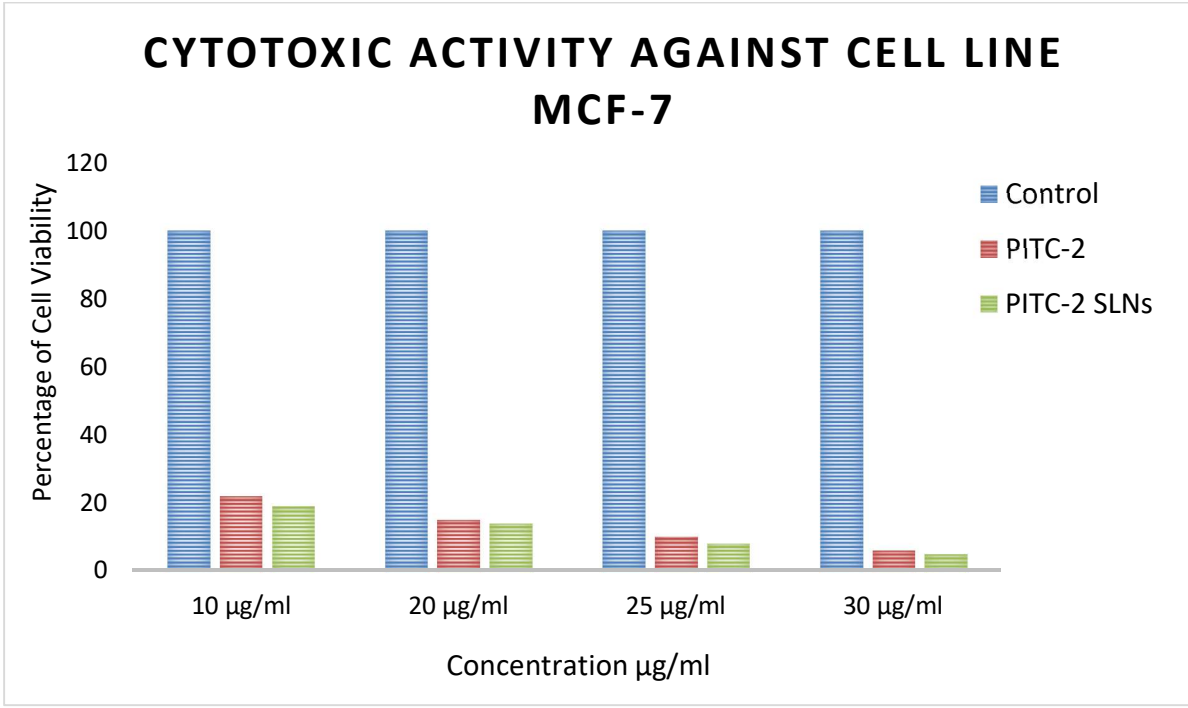



***Figure 1*** **:** *In-vitro* cytotoxic activity of PITC-2 and PITC-2 SLNs against MCF-7. Values are expressed as mean ± standard deviation

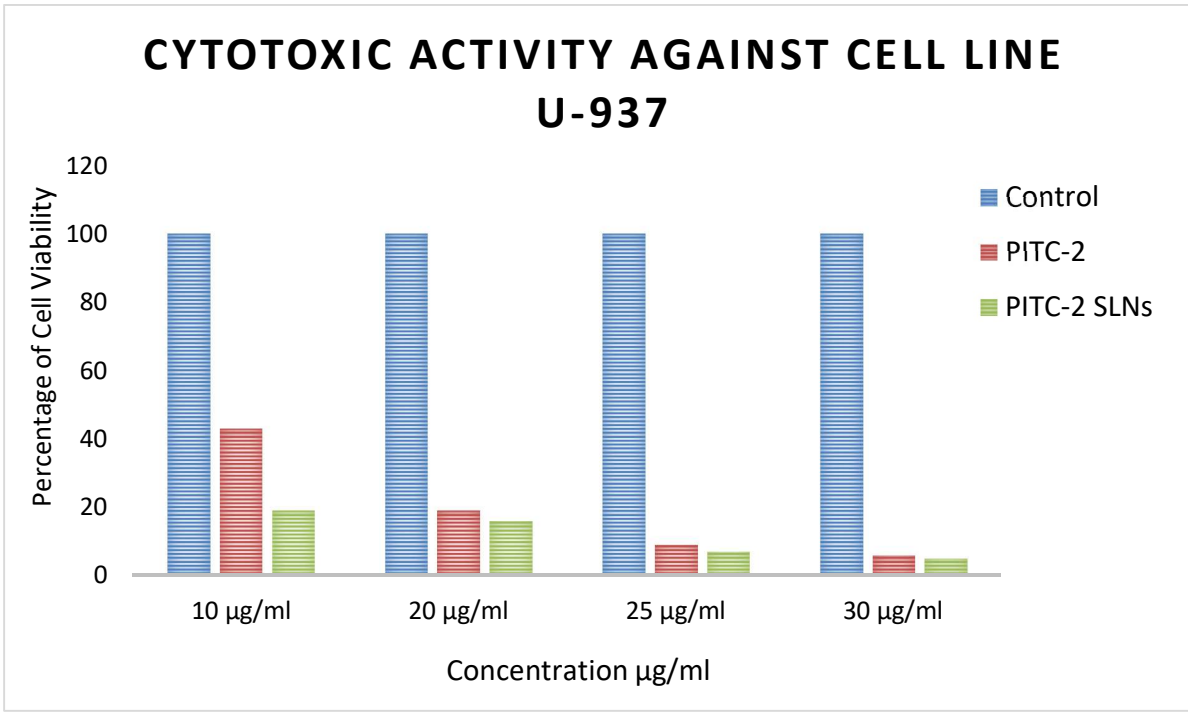



***Figure2*** **:** *In-vitro* cytotoxic activity of PITC-2 and PITC-2 SLNs against U-937. Values are expressed as mean ± standard deviation

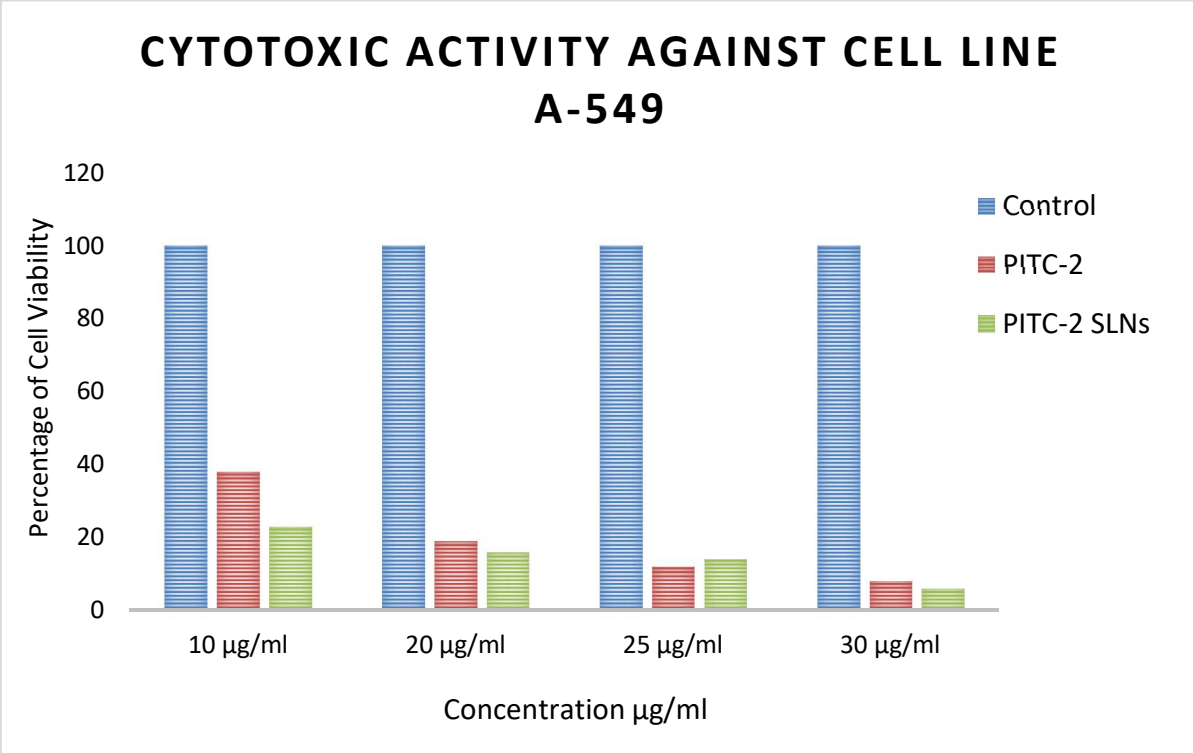



***Figure 3*** **:** *In-vitro* cytotoxic activity of PITC-2 and PITC-2 SLNs against A-549. Values are expressed as mean ± standard deviation

## Inhibition of EAC cells growth by PITC-2:

A significant reduction of ascetic fluid volume, packed cell volume and viable tumor cell count was found with PITC-2 SLNs formulation at dose 5mg/kg and phytochemical PITC-2 at concentration 5mg/kg b.w compared to that of control group mice. Moreover the median survival time (MST) was also increased in these above groups of mice shown in Table 1.

***Table 1:*** *In-vivo* effect of PITC-2 on EAC bearing Mice

| | PITC-2 5mg/kg b.w | PITC-2 loaded SLN Formulation 5mg/kg b.w | Std drug 5-FU 20mg/kg b.w | EAC Control |
|---|---|---|---|---|
| Ascites fluid volume (ml) | 5.64 ± 0.28* | 5.24 ± 0.12* | 5.89 ± 0.23* | 11.90 ± 0.11* |

| | | | | |
|---|---|---|---|---|
| Packed Cell volume (ml) | 1.45 ± 0.08* | 1.39 ± 0.04* | 1.32 ± 0.02* | 4.21 ± 0.08* |
| Body weight (gm) | 22.41 ± 0.43* | 22.13± 0.42* | 22.68 ± 0.55* | 30.10 ± 0.48* |
| MST (days) | 39.67 ± 0.98* | 41.83 ± 0.70* | 32.00 ± 0.85* | 18.40 ± 0.42* |
| %ILS | 215.59 | 224.89 | 173.91 | 0 ± 0 |
| Viable Cell($10^6$cells/ml) | 3.05± 0.13* | 2.92 ± 0.21* | 3.45 ± 0.43* | 10.65± 0.23* |

**<0.05 as compared to control group (n = 6 mice per group)*

## Effect of PITC-2 on hematological parameters:

An increase in hemoglobin level, RBC count, neutrophil level and decrease in WBC level, monocyte and lymphocyte count was found in phytochemical PITC 2 treated groups as well as in PITC-2 loaded SLNs with compared to that of EAC control group shown in Table 2.

***Table 2:*** Effect of PITC-2 on hematological parameters in EAC bearing Mice

| | PITC-2 5mg/kg b.w | PITC-2 loaded SLN Formulation 5mg/kg b.w | Std 5-FU 20mg/kg b.w | EAC control | Normal Mice |
|---|---|---|---|---|---|
| Hemoglobin (gm %) | 9.4± 0.05* | 9.6± 0.08* | 9.1± 0.06* | 6.3 ± 0.01* | 11.8±0.12* |
| Erythrocyte (RBC) (cells x$10^6$/$mm^3$) | 5.6± 0.04* | 6.53± 0.03* | 5.68± 0.08* | 3.75 ± 0.08* | 7.90±0.02* |
| Leucocytes (WBC) (cells x$10^6$/$mm^3$) | 9.1± 0.05* | 8.29±0.05* | 10.19 ± 0.05 * | 16.69±0.02* | 6.40±0.03* |

| | | | | | |
|---|---|---|---|---|---|
| Neutrophil (%) | 31.3± 0.03* | 30.46±0.03* | 30.1±0.02* | 68.5 ± 0.05* | 19.4±0.03* |
| Lymphocyte (%) | 53.0± 0.04* | 55.4± 0.04* | 61.0±0.09* | 35.8 ± 0.11* | 64.1±0.23* |
| Monocyte (%) | 1.7± 0.03* | 2.2± 0.03* | 1.6± 0.03* | 1.3 ± 0.04* | 2.8±0.01* |

**<0.05 as compared to control group (n = 6 mice per group)*

## Effect of PITC-2 on biochemical parameters:

Phytochemical PITC-2 treated groups and PITC-2 loaded SLNs groups shows significant decrease in SGPT, SGOT and serum alkaline phosphatase parameters in comparison with EAC control group. An increase in total protein was also found in PITC-2 treated groups shown in Table 3.

***Table 3:*** Effect of PITC-2 on biochemical parameters in EAC bearing mice

| | PITC-2 5mg/kg b.w | PITC-2 loaded SLN Formulation 5mg/kg b.w | Std 5-FU 20mg/kg b.w | EAC Control | Normal Mice |
|---|---|---|---|---|---|
| Bilirubin (total)mg/dl | 0.26± 0.02* | 0.27± 0.02* | 0.24± 0.03* | 0.34± 0.01* | 0.45± 0.02* |
| Serum protein (total)g/dl | 8.52 ± 0.09* | 8.29 ± 0.07* | 8.22 ± 0.12* | 4.4± 0.08* | 8.19±0.08* |
| AST (SGOT) IU/L | 34.5± 0.01* | 34.9± 0.01* | 35.9± 0.01* | 74.9± 0.09* | 39.1±0.01* |
| ALT(SGPT) IU/L | 35.6± 0.04* | 36.4± 0.01* | 39.3± 0.06* | 71.4± 0.07* | 29.1±0.04* |
| Serum alkaline phosphatase IU/L | 71.1± 0.02* | 69.9± 0.02* | 72.8± 0.05* | 121.1±0.03* | 74.4± 0.03* |

| | | | | | |
|---|---|---|---|---|---|
| Creatinine Mg/dl | 0.78± 0.01* | 0.8± 0.02* | 0.81± 0.01* | 1.2± 0.02* | 0.62± 0.01* |

**<0.05 as compared to control group (n = 6 mice per group)*

## Histopathology of liver and kidney tissue

H&E stained section of liver of a healthy mice was shown in fig no 4A, bearing all the normal features which includes circular hepatic portal vein and of hepatic artery, as marked by arrow. The tissue section comprises of hepatic sinusoid and nuclei which are usual. But none of the above mentioned regular features are observed in case of EAC control group showed in Fig. 4B, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Although little changes are found in group receiving standard 5-FU like deformed hepatic artery and irregular bile duct but lesser amount of hepatocellular lesions observed which are close to normal as shown in Fig. 4C. Groups receiving phytochemical PITC 2 and PITC-2 loaded SLNs also shows a little hepatic deformation and altered hepatocyte population shown in Fig. 4D and 4E. Healthy hepatocytes were observed, regular branch of bile duct found, although mild dilation of central vein observed.

H&E stained section of kidney of a healthy mice was shown in Fig 5A, bearing all the normal features, while EAC cell control group shows severe glomerular and tubular hemorrhage with increase swelling of tubular epithelium and presence of hyaline cylinders showed in Fig. 5B. Group treated with 5-FU and PITC-2 shows light glomerular and tubular hemorrhage and cellular features are less deformation which are close to normal shown in Fig. 5C, 5D and 5E respectively.

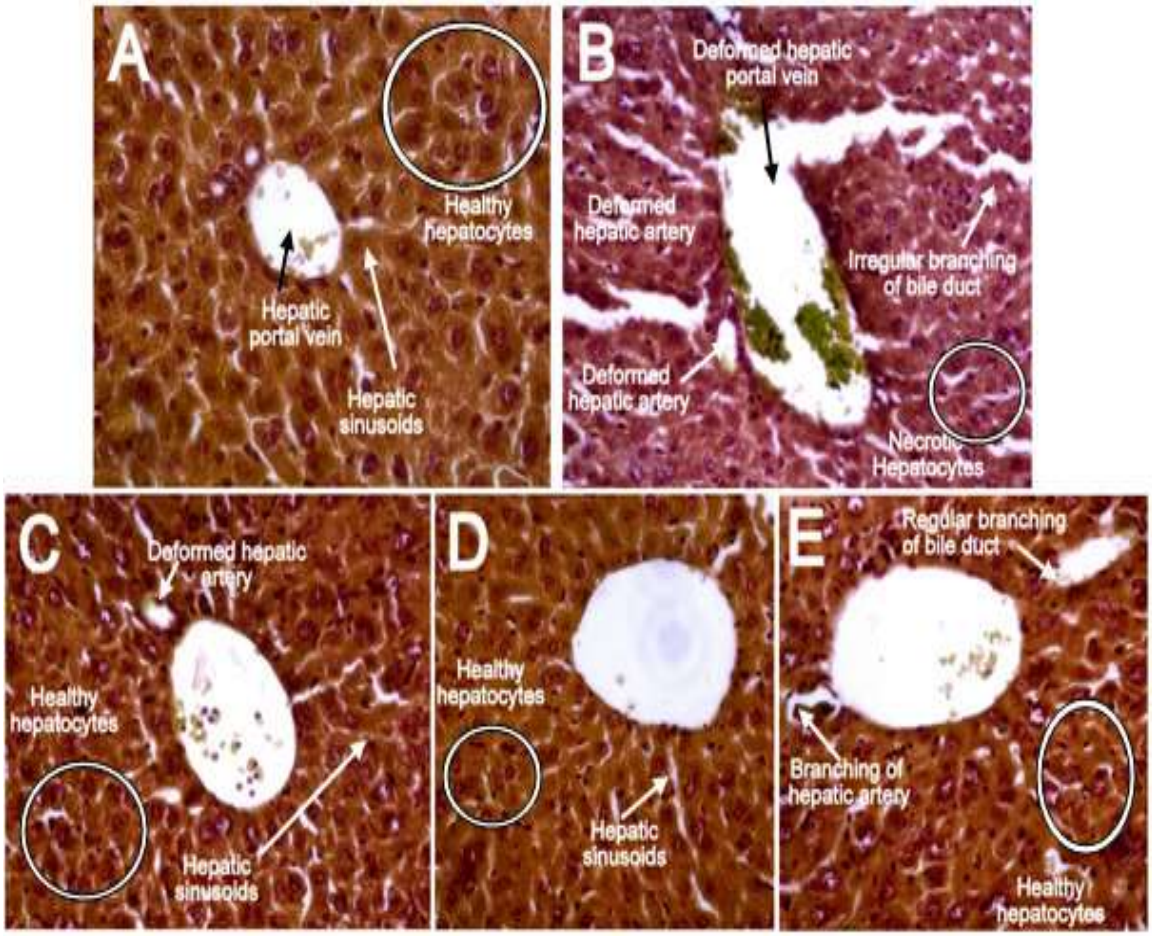



***Fig 4:*** H & E stained section of liver of mice showing cell morphology of groups A. Normal Mice, B. EAC control mice, C. 5 FU standard drug, D. 5 mg/kg PITC-2 and E. 5mg/kg PITC-2 SLNs.

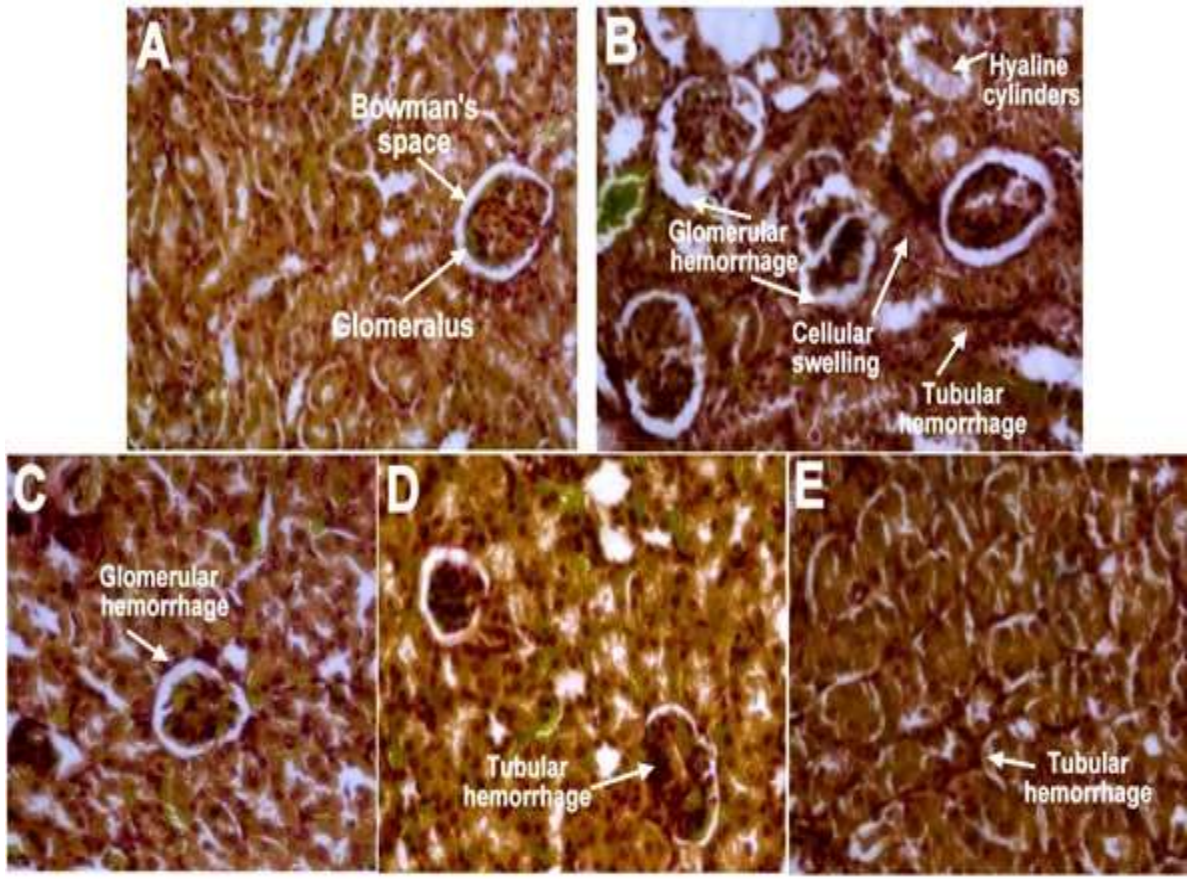



***Fig 5:*** H & E stained section of kidney of mice showing cell morphology of groups A. Normal Mice, B. EAC control mice, C. 5 FU standard drug, D. 5 mg/kg PITC-2 and E. 5mg/kg PITC-2 SLNs.

## Morphological changes of EAC cells

A morphological change was observed in EAC cell with both Haematoxylin& Eosin (H&E) and Papanicolaou (Pap) staining shown in Fig 6. In case of H&E staining cells of control group have good circular morphology, intact plasma membrane and nucleus. But cell of groups treated with standard drug shows apoptotic bodies and nuclear contraction. Irregularity in cell morphology, blebbing of plasma membrane, chromatin condensation was also found in groups treated with phytochemical PTIC-2. In case of groups treated with PITC-2 loaded SLNs evident change in cell morphology was found which includes cell shrinkage, formation of apoptotic bodies, nuclear contraction and blebbing of plasma membrane. Similarly in case of cells stained with Pap stain numerous features of cellular apoptosis was observed in

standard and test drug groups which are shown in the figure by arrow.

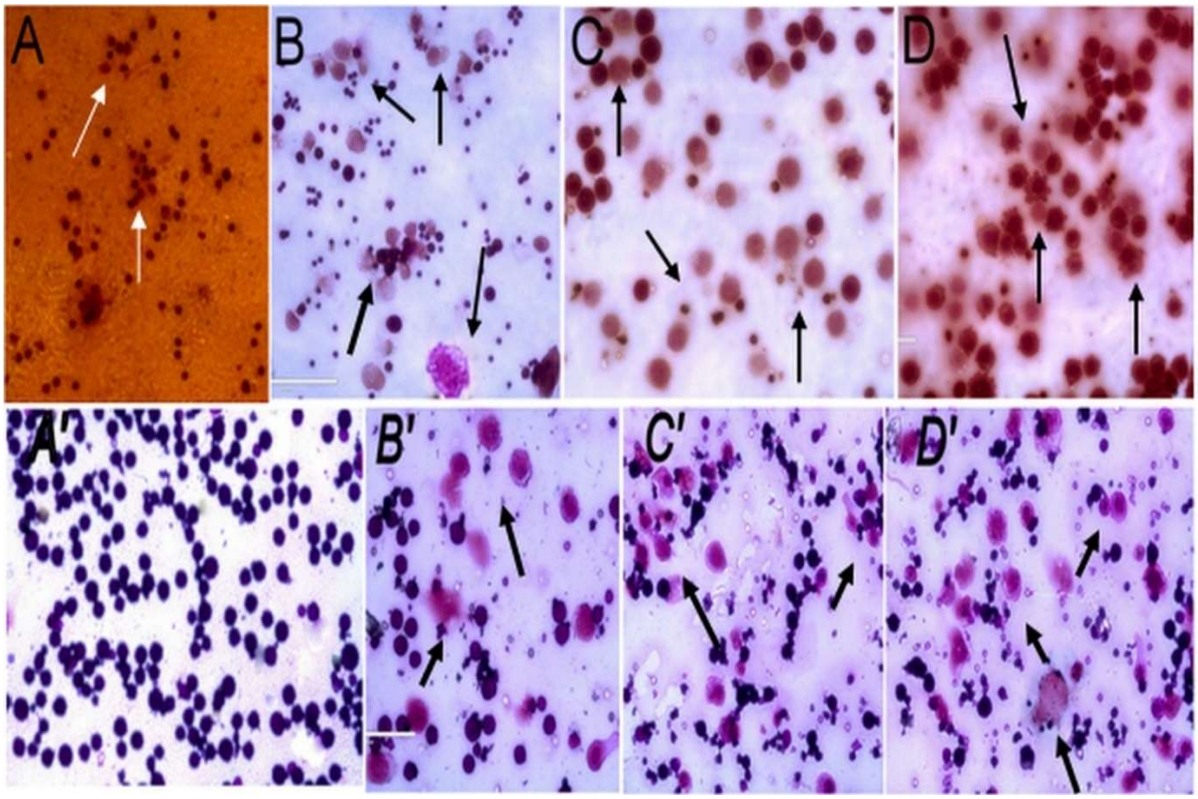



***Fig 6:*** Change in cell morphology of EAC cells by H&E stain of groups A. EAC control mice, B. 5 FU standard drug, C. 5 mg/kg PITC-2 and D. 5mg/kg PITC-2 SLNs and Papanicolaou stain of groups A'. EAC control mice, B'. 5 FU standard drug, C'. 5 mg/kg PITC-2 and D'. 5mg/kg PITC-2 SLNs

## Discussion:

Nanoparticles are a type of drug delivery system that lowers the toxicity level of a compounds and prolongs their half-life. In the present study the same thing is proved. In case if *in vitro* cytotoxicity study it was found that with same concentration of Phytochemical PITC-2 and PITC-2 in SLNs the formulation one is more effective. With same concentration PITC-2 SLNs shows more percentage of cell inhibition and more cytotoxic effect. In this study we also had also established a mouse model which bears EAC cell in order to study the anticancer effect of the prepared PITC-2 loaded SLN over phytochemical PITC-2.The mice treated with PITC-2 loaded SLN formulation inhibit body weight, tumor volume, tumor inhibition ratio and also

brought hematological parameters to more or less same as phytochemical PITC-2 does with same dose. A same type of finding was observed by P.M de Costa *et al.* on *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives (de Costa PM 239 (2015)). Inhibition of body weight, tumor volume, tumor inhibition ratio was also shown here. A good increase in lifespan and survival time was also noted in EAC cell bearing mice when treated with PITC-2 loaded SLNs. Myelosuppression and anemia are the major problems in cancer chemotherapy. Mostly due to reduction in RBC count, cancer cell bearing mice have anemia or due to low hemoglobin percentage. This may occur due to iron deficiency or due to myelopathic condition (Gupta M 25(8), 2004). Treatment with PITC-2 loaded SLNs and phytochemical PITC-2 stabilizes hemoglobin concentration, RBC and WBC cell count near to normal value. This reveals protective activity of PITC-2 SLNs on the hematopoietic system. Another major problem of cancer chemotherapy is liver toxicity which is due to accumulation of drug metabolites in liver (Debnath S 2017). Toxicity is a major problem of drug delivery system too. One can have a neat delivery system but it is more important to be a safe delivery system with low toxicity for its introduction in pharmaceutical and clinical market. Toxicity is due to the excipients in carrier system. Use of glycerides composed of fatty acid, contained in oils of parenteral fat emulsion may reduce toxicity (Muller R.H. 50(2000)). In our study we had used glyceryl monostearatewhich may not induce any toxicity for the SLNs. Furthermore surfactant used in our study was poloxomer which is recommended as an acceptable surfactant for use in parenteral formulations. (Muller R.H. 50(2000)). Here liver toxicity was analyzed in this experiment by biochemical studies and histopathology of liver tissue. AST, ALT and ALP levels of control group mice, normal mice and drug treated mice were compared. It was found that groups treated with PITC-2 loaded SLNs had less toxic liver as the level of AST, ALT and ALP are close to normal value. These all features like decrease in body weight, tumor volume, increase in lifespan and survival time, stabilizing hematological and biochemical parameters of PITC-2 loaded SLN formulation are nearly same and even better in comparison to the effect of phytochemical PITC-2. These features helps in judging the PITC-2 loaded SLNs activity.

Histopathological analysis of kidney was done for control, 5-FU,phytochemical PITC-2 and PITC-2 loaded SLNs. Several degree of hydropic changes of proximal tubular epithelium, glomerular and tubular hemorrhage was observed which were much more prominent in control group animal than drug treated groups where these effects are low but the glomeruli structures

were essentially preserved. The characterization of necrosis is done by no nuclear staining and deeply eosinophilic cytoplasm (de Sousa AP 2007).This show PITC-2 loaded SLNs are less toxic to kidney than standard drug and quite resembles to the action of phytochemical PITC-2. Lesion in liver was caused by many drugs as well as carrier systems which are analysed by biopsy. A large number of drugs with different pharmacological action and chemical structure give rise to liver lesion. Although liver has a unique regenerative ability and great adaptive efficiency. For example an adaptive phenomenon of liver is if an anticonvulsant drug was used for a long time then it causes increase in endoplasmic reticulum. Again regeneration of hepatic tissue occurs by the conjugative tissues after a hepatocellular necrosis due to treatment with alginates (de Sousa AP 2007). In our study prominent nuclei and the tissue section comprises of hepatic sinusoid was observed in normal healthy mice which are usual. But in case of EAC cell bearing control group features observed are not normal as mentioned, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Group receiving PITC-2 loaded SLN formulation are more or less close to normal group although little changes like deformed hepatic artery and irregular bile duct are found which show SLNs are less toxic to liver.

Now for analyzing the cytotoxic effect of the formulation histopathology of ascetic fluid was done. Cell apoptosis takes place and a significant change in cell morphology was observed. Apoptosis is a cell regulatory mechanism which is distinguished by diverse alteration in cell morphology including asymmetry in plasma membrane, cytoplasm shrinkage, chromatin condensation, formation of apoptotic bodies, etc. (Debnath S 2017). In our study control group shows good circular morphology, intact plasma membrane and nucleus. But cell of groups treated with standard drug shows apoptotic bodies and nuclear contraction. Irregularity in cell morphology, blebbing of plasma membrane, chromatin condensation was also found in groups treated with phytochemical PTIC-2. In case of groups treated with PITC-2 loaded SLNs evident change in cell morphology was found which includes cell shrinkage, formation of apoptotic bodies, nuclear contraction and blebbing of plasma membrane. Degree of change in cell morphology was more in SLN PITC-2 than phytochemical PITC-2.

# Reference

A Sinha, K. Banerjee, A Banerjee, S Das, SK choudhuri. "Synthesis characterisation and biological evaluation of a noble vanadium complex as a possible anticancer agent." *Journal of organometallic chemistry*, 2014: 34-41.

A. Pal, I. Bhattacharya, K. Bhattacharya, C. Mandal, M. Ray. "Methylglyoxal induced activation of murine peritoneal macrophages and surface marker of T lymphocytes in sarcoma 180 bearing mice:involvement of MAP kinase NF- Kappa beta signal transduction pathway." *Molecular Immunology*, 2009: 2039-2044.

Abd-El Fattah AA, Darwish HA, Fathy N, Shouman SA. "Carbition anhydrase inhibition boost the antitumor effect of Imatinib mesylate via potentiating the antiangiogenic and antimetastatic machineries,." *Toxicology and applied pharmacology*, 2017: 316:123-138.

Alisa Bahar Beydogan, Sema bolkent. "The effects of silibin adminiistration for different time periods on mouse liver with Ehrlich ascites carcinoma." *Pharmacological Reports*, 2016: 543-549.

Biswas R, Bandyopadhyaya D, Mishraa M, Pramanika KC , Chatterjee TK. "Isolation of pure compound R/J/3 from *Pluchea indica* (L.) Less. and its anti-amoebic activities against Entamoeba histolytica." *Phytomedicine*, 2007: 14: 534-537.

Byung Do Kim, Kun Na, Hoo Kyun Chio. "Preparation and characterization of solid lipid nanoparticles (SLN) made of cocoa butter and curdlan." *European journal of pharmaceutical scienses*, 24(2005): 199-205.

C. Olbrich, O. Kayser, R. H. Muller. "Lipase degradation of Dynasa 114 and 116 solid lipid nanoparticles (SLN) - effect of surfactents, storage time and crystallinity." *Int.J. Pharm*, 237(2002): 119-128.

Chakraborty S, Chatterjee TK. "Targeting viseral leishmaniasis by mannosylated liposome incorporated PITC-2 isolated from the plant *Pluchea indica* (L) Less." *Int J Pharm Bio Sci*, 8(2)2017: 396-408.

de Costa PM, de Costa MP , Jamacaru VF, Ferreira PM. " Inprovement of *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives." *Chem Biol Interact* , 239 (2015): 174-83.

de Sousa AP, Torres MR, Pessoa C, Costa Lotufo LV. "*In vivo* growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed Sargassum vulgare." *Carbohydr Polym*, 2007: 69: 7-13.

Debnath S, Karan S, Debnath M, Chatterjee TK. " Poly-L-Lysine inhibits tumor angiogenesis and induces apoptosis in ehrlich ascites carcinoma and sarcoma S-180 tumor. ." *Asian Pac J Cancer Prev*, 2017: 18(8): 2255 - 68.

Debnath S, Karan S, Debnath M, Chatterjee TK. "Poly-L-Lysine inhibits tumor angiogenesis and induces apoptosis in ehrlich ascites carcinoma and sarcoma S-180 tumor." *Asian Pac J Cancer Prev*, 18(2017): 2255 - 2268.

Elsherbiny NM, Younis NN, Elseweids MM. " The synergistic effect between vanillin and doxorubicin in ehrlich ascites carcinoma solid tumor and MCF-7 human brest cancer cell line." *Pathol Res Pract*, 212(2016): 767-77.

Gupta M, Mazumdar UK, Kumar SA, Kumar TS. "Antitumor activity and antioxidant role of Bauhinia racemosa against Ehrlich ascites carcinoma in swiss albino mice." *Acta Pharmacol Sin*, 25(8), 2004: 1070-6.

Harborne JB. "Arsenal for Survival: Secondary Plant Products." *Taxon*, 49 (2000).: 435-439.

Ho Lun Wong, Reina Bendayan, Andrew. M. Rauth, Yongyiang Li, Xiao Yu Wu. "Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles." *Advanced drug delivery reviews*, 59 (2007): 491 - 504.

Isha Dhamija, Nitesh Kumar, S.N. Manjula, Vipan Parihar, M. MManjunath Setty, K.S.R. Pia. "Preliminary evaluation of *in vitro* cytotoxicity and *in vivo* antitumor activity of Premna herbacea Roxb. in ehrtich ascites carcinoma model and daltons lymphona ascites model." *Experimental and toxicologic pathology.*, 65, 2013: 235 - 242.

Jenning V., Gysler A., Schafer Korling M. Gohla SH. "Vitamine A loaded solid lipid nanoparticles for tropical use: Occlusive properties and drug targeting to the upper skin." *Eur J Pharm Biopharm*, 2000: 49: 211 - 21.

Joseph MM, Aravind SR, Geroge SK, Sreelekha TT. "Antitumor activity of galactoxyloglucan gold nanoparticles against murine ascites and solid carcinoma." *Colloids and surfaces B: Biointerfaces*, 2014: 116: 219-227.

M.L.Salem, N.M.Shoukry, A.A.Rahman. "*In vitro* and *in Vivo* antitumor effects of the Egyptian scorpion Androclonus amoreuxi venam in an Ehrlich ascitic tumor model." *Springer Plus*, 2016: 570-581.

Muller R.H., Madar K., Gohla S. "Solid lipid nanoparticles (SLN) for controlled drug delivery-a revier of the stste of the art." *Eur, J. Pharm. Biopharm*, 50(2000): 161-177.

Nicol B.M., Prasad S.B. "The effects of cyclophosphamide alone and in combination with ascorbic acid against murine ascited Dalton's lymphoma." *India J Pharmacol*, 2006: 260-265.

P.K. Senthil, A. Arivuchelvin, A. Jagadeeswaran, N. Subramanian, C.K Senthil, P.Mekala. "Formulation, optimization and evaluation of enrofloxacin solid lipid nanoparticles for sustained oral delivery." *Asian journal of pharmaceutical and clinical research*, 8(1) 2015: 231 - 236.

Pal A, Bhattacharya I, Bhattacharya K, Mandal C, Ray M. "Methylglyoxal induced activation of murine peritoneal macrophages and surface marker of T lymphocytes in sarcoma 180 bearing mice:involvement of MAP kinase NF- Kappa beta signal transduction pathway." *Molecular Immunology*, 2009: 46: 2039-2044.

Pramanik KC, Biswas R, Mitra A, Bandyopadhyay D, Mishra M, Chatterjee TK. "Tissue culture of the plant *Pluchea indica* (L.) Less. and evaluation of diuretic potential of its leaves. ." *Orient Pharm Exp Med .*, 7(2)2007: 197-204.

R.Perveen, F.Islam, J.Khanum, T.Yeasmin. "Preventive effect of ethanolic extract of Alpinia calcarata Rose on ehrlichsascitic carcenoma cell induced malignant ascitic in mice." *Asian pecific journal of tropical medicine*, 2012: 121-125.

S. Yang, J. Zhu, Y. Lu, B. Liang, C. Yang. "Body distribution of camptothecin solid lipid nanoparticle after administration." *Pharm. Res*, 16(5) 1999: 751-757.

S.S.Agarwal, S. Saraswati, R. Mathur. "Cytotoxic and antitimor effect of brucine on ehrlich ascites tumor and human cancer cell line." *Life Sciences*, 2011: 147-158.

Shengpeng Wanga, Tongkai Chenb, Ruie Chena, Yangyang Hua, Meiwan Chena, Yitao Wanga. "Emodin loaded solid lipid nanoparticles: Preparation, characterization and." *International Journal of Pharmaceutics*, 2012: (460) 238-246 .

Soumita Goswami, Souvik Debnath, Saumen Karan, Tapan Kumar Chatterjee. "*In vivo* antitumor activity of phytochemical pitc-2 obtained from tissue cultured plant *Pluchea indica* on sarcoma-180 solid tumor mice model." *Asian journal of Pharmaceutical and Clinical research*, 2018: 4(11), 211 - 218.

Surajit Das, Wai Kiong Ng, Parijat Kanaujia, Sanggu Kim,. "Formulation design, preparation and physicochemical characterizations of solid lipid nanoparticles containing a hydrophobic drug: Effects of process variables." *Colloida and Surfaces B: Biointerfaces*, 88(2011): 483-489.

# CONCLUSION

# Conclusion:

In conclusion, the present results clearly demonstrate that PITC-2 significantly inhibits sarcoma-180 cell and EAC cell growth in a dose dependent manner in in-vivo mice model. Beside this, the study reveals a comprehensive perception of the possible mechanism behind the anti-tumor activity of PITC-2 by significant changes in the morphological, hematological, biochemical parameters in sarcoma-180 cells and EAC cells. Moreover the histopathological and immunohistopathological examination indicates that PITC-2 induces apoptosis and suppresses tumor cell proliferation along with G1 cell cycle arrest through the down–regulation of the intratumoral expression of Bcl-2, cyclic D1 and Ki-67 and thus highlighting anti-proliferative and apoptotic properties against tumor model. Along with this it shows no significant manifestation of toxic symptoms on liver and kidney of Swiss albino mice. Taking these results, we suggest that PITC-2 may be a potent prognostic factor and a potential target for tumor cells.

Again PITC-2 loaded SLNs were successfully prepared by emulsion evaporation method. Poorly water soluble phytochemical PITC-2 was effectively loaded in the nanoparticles. The method resulted in producing consistent smaller size nanoparticles in the range of 170- 180 nm with narrow size distribution. Most of the process variables shows significant effect on the formulation properties. SLNs prepared for systematic drug delivery obtains better therapeutic efficiency by sustained drug release, thus by improving bioavailability and will decrease dosing with lower side effects. Toxicity study also shown no such significant toxicity of the formulation. Moreover the formulation shown a good pharmacological activity against EAC liquid tumor cell in comparison to free phytochemical PITC-2. Finally this planning methodology has clearly shown its usefulness in this optimization process, and this research produces a framework for the understanding of effect of PITC-2 loaded SLN formation.

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Research Article

# *IN VIVO* ANTITUMOR ACTIVITY OF PHYTOCHEMICAL PITC-2 OBTAINED FROM TISSUE CULTURED PLANT *PLUCHEA INDICA* ON SARCOMA-180 SOLID TUMOR MICE MODEL

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**ABSTRACT**

**Objective:** PITC-2 was isolated from the methanolic root extract of tissue cultured medicinal plant *Pluchea indica* (L.) Less. PITC-2 is a thiophene derivative which is 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene. The main objective of the study is to evaluate the *in vivo* antitumor activity of PITC 2 against sarcoma-180 cancer cell in Swiss albino mice.

**Methods:** The antitumor activity was evaluated by treatment with PITC-2 at a dose of 2.5 and 5 mg/kg b.w for 21 days on sarcoma-180 mice model. Cell viability was studied using 3-(4, 5- dimethylthiazol -2-yl)-2, 5-diphenyl tetrazolium bromide assay and cell apoptosis, $G_1$ cell cycle arrest and reduction in tumor cell proliferation were evaluated by histopathological analysis and Bcl-2, cyclic-D1, and Ki-67 protein expression through immunohistochemistry study.

**Results:** Precisely, PITC-2 had a cytotoxic effect on various *in vitro* cancer cells. Significant decreases in solid tumor volume and weight along with increase lifespan also observed. The histopathological and immunohistopathological examination indicates that PITC-2 induces apoptosis, typical morphological changes and suppresses tumor cell proliferation along with $G_1$ cell cycle arrest through the downregulation of the intratumoral expression of Bcl-2, cyclic D1, and Ki-67 and thus highlighting antiproliferative and apoptotic properties against sarcoma-180 *in vivo* solid tumor model.

**Conclusion:** The present results clearly demonstrate that PITC-2 significantly inhibits sarcoma-180 cell growth in a dose-dependent manner in *in vivo* mice model. Besides this, the study reveals a comprehensive perception of the possible mechanism behind the antitumor activity of PITC-2 by significant changes in the morphological, hematological, biochemical parameters in sarcoma-180 cells.

**Keywords:** Apoptosis**,** Cell viability, PITC-2, Sarcoma-180, Antitumor activity, Cell proliferation.

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## INTRODUCTION

Cancer and tumors are considered as the most killer disease all over the world. Cancer is caused by abnormalities of the genetic material of the affected cells. Tumorigenesis is a multistep process that involves the accumulation of successive mutations in oncogenes and suppressor genes that deregulates the cell cycle. It is a cellular disease in which the normal relationship between epithelial cells and their underlying stomach cells are altered [1]. Tumor metastasis is responsible for approximately 90% of all cancer-related death [2]. Although struggling efforts against cancer have grown tremendously in past few years, still it is the second leading cause of death in economically developed countries. An increase in worldwide death rate was found on cancer patients which are 7.1 million and 8.2 million on 2007 and 2012, respectively [3,4]. By 2030 the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer death [5].

Cancer therapy is based on surgery, radiotherapy, and systemic chemotherapy. Chemotherapy induces apoptosis which initiates tumor cell death. Apoptosis is an evolutionarily conserved form of death of a cell to maintain tissue homeostasis which allows removal of damaged or superfluous cells. This can be mediated either by death receptor pathway or by mitochondrial pathway. First indication of apoptosis is activation of several caspases within the same cell and second evidence from different timing of caspase activation after addition of apoptotic stimuli are consistent with a hierarchy of caspase activation [6,7]. It is obvious that cancer chemotherapy is a very difficult task [8]. One of its main associated problems in the non-specific toxicity of most anticancer drugs due to their biodistribution throughout the body, which requires the administration of a large total dose to achieve high local concentrations in a tumor. Another problem in cancer chemotherapy is drug resistance. It may be due to cancer cell mutation or by repairing the DNA breaks caused by anticancer drugs or by adopting some other mechanism that inactivates the drugs. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents [9]. Recent interest in these secondary metabolites has been focused on their medicinal properties [10]. In this project, we are using a tissue cultured plant *Pluchea indica*. The plant was tissue cultured with the purpose to obtain more secondary metabolites [11]. *P. indica* (L.) Less. is a species of flowering plant in the family Asteraceae. The terminal flowers are rose purple and grow in rather loose few-flowered heads. It is an evergreen large shrub found abundantly in salt marshes and mangrove swamps in Sunderbans (India), Bangladesh, Myanmar, China, Philippines, Malaysia, Tropical Asia, and Australia [12]. From the methanolic root extract of tissue cultured medicinal plant, *P. indica* PITC 2 was isolated. It is a thiophene derivative which is 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene. The main objective of the study was to evaluate the *in vivo* antitumor activity of PITC 2 against sarcoma-180 cancer cell in Swiss albino mice.

## METHODS

### Extraction and isolation

The roots after being separated from the whole plant were washed and dried at 60°C in a forced air oven. Dried roots are grounded and extracted separately in petroleum ether (60–80°C for synthesis) and then dried and extracted in a Soxhlet extractor with methanol (AR grade, purity 99.8%). After distillation of methanol, a sticky dark brown residue was obtained. For isolation of the phytochemical PITC 2 from tissue cultured plant *Pluchea indica,* the methanolic root extract was partitioned in a mixture of n-Butanol and water. From there butanol fraction was taken and then shacked with ethyl acetate to get an ethyl acetate soluble and insoluble part. Finally, ethyl acetate soluble part is taken and concentrated in a rotary vacuum evaporator and dried to obtain a crude residue which is then column chromatographed with a mixture of ethyl acetate and Petroleum ether. At a ratio 8:2 of pet ether and ethyl acetate from fraction 8–15 a prominent spot was observed in the thin-layer chromatography plate which is the desired phytochemical PITC-2 [2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene] (Fig. 1). The presence of light yellow solid PITC-2 was further confirmed by testing the isolated compounds on nuclear magnetic resonance spectroscopy and MASS spectroscopy [13].

### Cell lines and cell culture

*In vitro* cancer cell lines were obtained from National Centre for Cell Science, Pune, India, and Sarcoma-180 cancer cells were collected from Chittaranjan National Cancer Research Institute, Kolkata, India. The *in vitro* cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% FBS at 37°C in $CO_2$ incubator in an atmosphere of humidified 5% $CO_2$ and 95% air. The cells were maintained by routine subculturing in tissue culture flasks. Tumor cell counts were done in a neubauer hemocytometer using the Trypan blue dye exclusion method. Cell viability was always found to be 95% or more.

### Cytotoxicity assay (3-(4, 5- dimethylthiazol -2-yl)-2, 5-diphenyl tetrazolium bromide [MTT] assay)

This test is based on MTT, which is reduced to a purple-blue soluble formazan by the living cells [14]. Experiments were performed in 96-well flat-bottomed culture plates (BD Biosciences, USA). MTT was dissolved in phosphate buffered saline (PBS) at 5 mg/ml. Different concentrations of PITC-2 (0.1, 0.5, 01, 5, 10, 20, 25, and 30 µg/ml) were added and the plate was incubated for 24 h. Following incubation with each compound for 24 h, 20 µL of MTT was added to each well. After incubation for 4 h at 37°C, the culture medium was removed, and the formazan crystals were dissolved in 200 µL dimethyl sulfoxide (DMSO) [15]. Absorbance (A) of formazan dye was measured at 570 nm using a microplate reader. The background absorbance was determined at 690 nm and subtracted from the 570 nm measurement [2].

The percentage of viable cells was determined by the following equation: [16]

$$\text{Viable cells}(\%) = \frac{\text{A of treated cells}}{\text{A of untreated cells}} \times 100$$

The cytotoxic activities of this compound PITC-2 were determined in human breast cancer cell MCF7, human lung cancer cell A549, human macrophage cell U937, and sarcoma S-180.

### Animals and study groups

*In vivo* studies were carried out on female Swiss albino mice weighing 22±0.50 g of either. The animals were kept under standard conditions of 12:12 h light and dark cycle in polypropylene cages and fed with standard laboratory diet and water *ad libitum.* As the phytochemical PITC-2 was not freely soluble in water so 1%, DMSO solution was prepared to make it solubilize. All *in vivo* experiments were conducted as per the guideline of Animal Ethics Committee of the Department of the Pharmaceutical Technology of Jadavpur University, India (Registration number: 147/1999/CPCSEA).

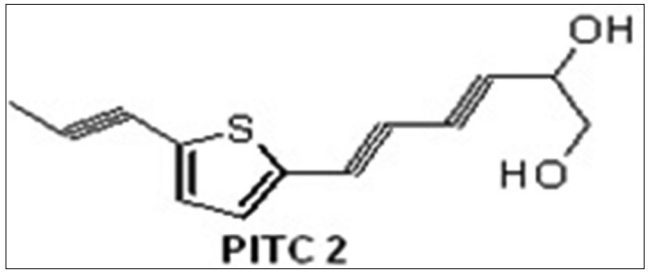


**Fig. 1: 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene**

For experimental purpose, animals are divided into five groups each containing 12 mice.

1. Group I: Normal animals (negative control).
2. Group II: Sarcoma cancer animals (positive control)+1% DMSO 5 ml/kg/day.
3. Group III: Sarcoma cancer animals (test 1)+PITC 2 (2.5 mg/kg/day given orally).
4. Group IV: Sarcoma cancer animals (test 2)+PITC 2 (5 mg/kg/day given orally).
5. Group V: Sarcoma cancer animals (standard)+5FU (20 mg/kg in alternative day given i.p).

### Solid tumor induction by sarcoma 180 (S-180) cell line and experimental protocol

Sarcoma-180 cells were preserved *in vivo* in the peritoneal cavity of mice by intraperitoneal inoculation of cells in sterile normal saline [17]. Then, solid tumors were inoculated in the left hind leg of mice by intramuscular injection of S-180 cells (0.2 ml of $2\times10^6$ cells/mouse) to all the groups' except the normal group [18]. Treatment was given from day 8 after inoculation of S-180 tumor for 21 days [19]. From each group, 6 mice were sacrificed, and other 6 were kept for calculation of mean survival time (MST).

### Recording of survival rate

MST and percentage increase in lifespan (%ILS) were calculated by the following formulas [20].

$$\text{MST} = \frac{\sum \text{Survival time(day) of each mice in a group}}{\text{total number of mice}}$$

$$\%\text{ILS} = [\frac{\text{MST of treated mice}}{\text{MST of control mice}} \times 100$$

### Tumor volume

In case of solid tumor, tumor volumes were recorded using a vernier calipes and calculated as V ($mm^3$) = ($a^2\times b$)/2 where a (smaller diameter) and b (large diameter) are perpendicular [21]. Tumor growth inhibition ratio was calculated as follows: Inhibition ratio (%) = ([A-B]/A)×100 where A is average tumor weight in negative control and B is average in each treatment group [22]. However, in case of an ascetic tumor, peritoneal fluid was collected from the peritoneal cavity. Then, the volume was measured by taking it in a graduated centrifuge tube.

### Hematological parameters

Hematological studies were performed by recording the hemoglobin content, red blood cell (RBC), and white blood cells (WBC) counts from freely flowing tail vein blood [23]. Differential WBC leukocyte counts were carried out including neutrophils, lymphocytes, and monocytes from Leishman stained blood smears of normal, sarcoma-180 control and PITC 2 treated groups, respectively.

### Biochemical parameters

After 24 h of last dose and 18 h of fasting the blood sample were collected by puncturing retro-orbital plexus. Blood was used for the assay of biochemical

parameters serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, globulin, bilirubin, alkaline phosphatase (ALP), and serum protein to investigate liver function alteration and creatinine was measured as a renal function parameter.

**Histopathology**

The tumor, liver and kidney were excised and fixed in 10% buffered formalin and embedded in paraffin. +5-µm sectioned was stained with hematoxylin and eosin (H and E) [24]. Histological analysis was performed under a light microscope (40×). The morphological changes were photographed and there compared.

**Immunohistochemical analysis of Bcl-2, Ki-67, cyclin-D1**

Tumor section was deparaffinized with xylene and dehydrated with ethanol. After blocking the slides with 5% bovine serum albumin in Tris-buffered saline, the sections were then stained with a primary antibody specific to Bcl-2, Ki-67, and cyclin-D1, at a concentration of 1 µg/ml at 4°C overnight, followed by mouse monoclonal antibody. After washing sections with PBS, they were incubated with diaminobenzidine substrate and counterstained with Mayer's hematoxylin. Then, they were visualized in a digital camera installed on a light microscope [2,21].

**Statistical analysis**

For cytotoxicity studies, the confidence interval ($IC_{50}$) values and 95% CI were obtained by nonlinear regression. All *in vitro* studies were carried out in triplicate represented by independent biological evaluation. Data are extracted as a mean ± standard error of the mean and were compared by one-way analysis of variance followed by Kruskal–Wallis test using SPSS statistical software of 20.0 version. $p<0.05$ was considered to be statistically significant when compared with control.

## RESULTS

***In vitro* cell viability assay**

The phytochemical PITC 2 was screened for its cytotoxicity against human breast cancer cell MCF7, human lung cancer cell A549, human macrophage cell U937, and sarcoma S-180 at different concentrations (0.1, 0.5, 01, 5, 10, 20, 25, and 30 µg/ml) to determine the percentage of viable cells by MTT assay. MTT assay shows a significant effect on sarcoma S-180, MCF-7, A-549, and U-937 cancer cells (Fig. 2). This result indicates antiproliferative activity of PITC-2 in cancer cells. Among these cancer cells, PITC-2 is most potent for U-937 cells.

**Inhibition of Sarcoma-180 solid tumor growth by PITC-2**

PITC-2 at concentrations 2.5 mg/kg and 5 mg/kg shows a good reduction of solid tumor volume and weight at the end of 21 days compared to that of control group mice. PITC-2 treated groups also shows significant reduction of body weight (Fig. 3 and Table 1).

**PITC-2 enhanced survival of sarcoma 180 bearing mice**

As shown in Table 1 and Kaplan–Meier survival curve (Fig. 4a) a significant increase in survival time and lifespan of sarcoma-180 bearing mice treated with PITC-2 was found in comparison with sarcoma bearing control group. The %ILS was found to be dose-dependent (Fig. 4b).

**Effect of PITC-2 on hematological parameters**

An increase in hemoglobin level, RBC count, neutrophil level and decrease in WBC level, monocyte, and lymphocyte count was found in PITC 2 treated groups in a dose-dependent manner in compared to that of sarcoma-180 control group (Table 2).

**Effect of PITC-2 on biochemical parameters**

PITC-2 treated group's shows a significant decrease in SGPT, SGOT, and serum ALP parameters are found in a dose-dependent manner (2.5 and 5 mg/kg b.w) in comparison with sarcoma-180 control group. A dose-dependent increase in total protein was also found (Table 3).

**Histopathology of liver and kidney tissue**

Fig. 5a shows H and E stained section of liver of healthy mice which bears all the normal features, including circular hepatic portal vein and

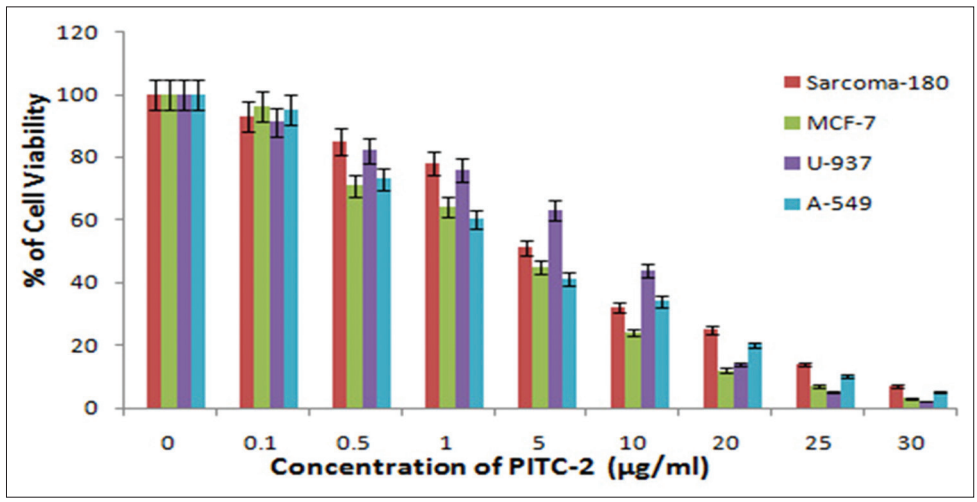


**Fig. 2: *In vitro* cytotoxic activity of PITC-2 against Sarcoma 180, MCF-7, U-937, and A-549. Values are expressed as mean ± standard deviation**

**Table 1: *In vivo* effect of PITC-2 on solid tumors and study groups**

| Parameters | PITC-2 2.5 mg/kg b.w | PITC-2 5 mg/kg b.w | Standard drug 5-FU 20 mg/kg b.w | Sarcoma-180 control |
|---|---|---|---|---|
| Tumor weight (g) | 1.156±0.03* | 0.649±0.008* | 0.890±0.03* | 3.90±0.11* |
| Tumor volume ($mm^3$) | 1900.75±45.30 | 1563.10±27.65 | 1823.64±33.73 | 6642.30±51.34 |
| Tumor inhibition ratio % | 71.38±1.72* | 76.46±0.27* | 72.54±0.96* | 0±0 |
| Body weight (g) | 24.05±0.43* | 22.71±0.42* | 23.68±0.55* | 27.10±0.48* |
| MST (days) | 34.67±0.98* | 52.83±0.70* | 42.00±0.85* | 19.50±0.42* |
| %ILS | 77.79 | 170.92 | 115.38 | 0±0 |

*<0.05 as compared to control group (n=6 mice per group). ILS: Increase in lifespan, MST: Mean survival time

**Table 2: Effect of PITC-2 on hematological parameters in sarcoma-180 solid tumors bearing mice**

| Parameters | PITC-2 2.5 mg/kg b.w | PITC-2 5 mg/kg b.w | Std 5-FU 20 mg/kg b.w | Sarcoma-180 control | Normal mice |
|---|---|---|---|---|---|
| Hemoglobin (g/dl) | 7.5±0.05* | 10.4±0.03* | 8.5±0.08* | 6.3±0.01* | 12.8±0.12* |
| Erythrocyte (RBC) (cells×$10^6$/$mm^3$) | 4.34±0.04* | 6.53±0.02* | 5.6±0.03* | 3.75±0.04* | 7.90±0.02* |
| Leukocytes (WBC) (cells×$10^6$/$mm^3$) | 14.1±0.05* | 10.19±0.05* | 4.29±0.05* | 16.69±0.02* | 6.40±0.03* |
| Neutrophil (%) | 42.3±0.03* | 39.46±0.03* | 30.1±0.02* | 68.5±0.05* | 19.4±0.03* |
| Lymphocyte (%) | 48.0±0.04* | 51.0±0.04* | 63.0±0.09* | 35.8±0.11* | 64.1±0.23* |
| Monocyte (%) | 1.5±0.03* | 1.4±0.03* | 1.7±0.03* | 1.3±0.04* | 2.8±0.01* |

*<0.05 as compared to control group (n=6 mice per group). RBC: Red blood cell, WBC: White blood cells

**Table 3: Effect of PITC-2 on biochemical parameters in sarcoma-180 solid tumors bearing mice**

| Parameters | PITC-2 2.5 mg/kg b.w | PITC-2 2.5 mg/kg b.w | Standard 5-FU 20 mg/kg b.w | Sarcoma 180 control | Normal mice |
|---|---|---|---|---|---|
| Bilirubin (total) mg/dl | 0.20±0.02* | 0.26±0.02* | 0.29±0.03* | 0.34±0.01* | 0.4±0.02* |
| Serum protein (total) g/dl | 8.12±0.09* | 9.69±0.07* | 5.22±0.12* | 15.4±0.08* | 8.19±0.08* |
| AST (SGOT) U/L | 0.71±0.01* | 0.60±0.01* | 0.59±0.01* | 0.6±0.09* | 0.8±0.01* |
| ALT (SGPT) U/L | 351±0.04* | 175±0.01* | 178±0.06* | 199±0.07* | 414±0.04* |
| Serum ALP U/L | 73±0.02* | 68±0.02* | 18±0.05* | 40±0.03* | 74±0.03* |
| Creatinine Mg/dl | 1±0.01* | 1±0.01* | 1±0.01* | 1±0.02* | 1±0.01* |

*<0.05 as compared to control group (n=6 mice per group). AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, SGOT: Serum glutamate oxaloacetate transaminase, SGPT: Serum glutamate pyruvate transaminase

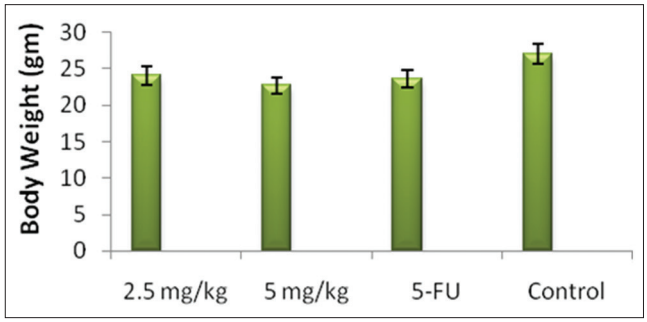


**Fig. 3: Effect of PITC-2 treatment on body weight**

of hepatic artery, as marked by arrow. It shows prominent nuclei, and the tissue section comprises hepatic sinusoid which is usual. However, in case of the Sarcoma-180 control group showed in Fig. 5b none of the above-mentioned regular features are observed, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Group receiving standard 5-FU shows a lesser amount of hepatocellular lesion, which is close to normal as shown in Fig. 5c. Although little changes are found such as deformed hepatic artery and irregular bile duct. Groups receiving a different dose of PITC-2 also show a little hepatic deformation and altered hepatocyte population shown in Fig. 5d and e. Healthy hepatocytes were observed, regular branch of bile duct found, although mild dilation of central vein observed.

Fig. 6a shows H and E stained section of kidney of a healthy mice which bears all the normal features, while sarcoma-180 control group showed in Fig. 6b show severe glomerular and tubular hemorrhage with increase swelling of tubular epithelium and presence of hyaline cylinders. Group treated with 5-FU and PITC-2 shows cellular features which have less deformation and close to normal in Fig. 6c-e, respectively, with light glomerular and tubular hemorrhage.

**Histopathological changes of solid sarcoma-180 tumor**

Histopathological examination of solid sarcoma-180 tumor control group shows intact anaplastic cancer cells with areas of necrosis and focal hemorrhage reflecting viability and aggressiveness of tumor (Fig. 7a). It shows sheets of large rounded and polygonal cells with hyperchromatic nuclear and binucleation. Whereas solid tumor of group treated with 5-FU (Fig. 7b) and PITC-2 with different doses (2.5 mg/kg and 5 mg/kg) (Fig. 7c and d) shows areas of necrosis. Low cell proliferation and muscle invasion were found, and with an increase in dose, tumor cells lose their details and architecture.

**Immunohistochemical analysis of Bcl-2, Ki-67, and cyclin-D1**

The tumor cells were considered positive Bcl-2, Ki-67, and cyclic-D1 with the presence of yellow-brown nuclear staining in Ki-67 (Fig. 8) and greenish brown nuclear staining in Bcl-2 (Fig. 9) and cyclic-D1 (Fig. 10). Here we are studying the regression in tumor growth by PITC-2, due to apoptosis that decreases expression of antiapoptotic protein Bcl-2, G1 cell cycle arrest by suppression of cyclic-D1 cell cycle protein. Futhermore, PITC-2 reduces tumor cell proliferation by decreasing Ki-67 tumor proliferation protein. A significant decrease in positive Bcl-2, Ki-67, and cyclic-D1 were found in PITC-2 treated group, compared to that of sarcoma-180 control group was observed which shows effective suppression of tumor. Meanwhile, it was found that the decrease in protein expression is dose-dependent and is better in case of PITC-2 with dose 5 mg/kg b.w.

## DISCUSSION

Cancer is a group of disease which involves abnormal growth of cell which is rapid and uncontrolled with the potential to spread in other parts of the body. Anticancer drugs or antineoplastic drugs are drugs effective in the treatment of a malignant disease which trigger signaling mechanism that initiates apoptosis or cell death [25]. At present, available synthetic drugs have many adverse reactions which can be minimized to a great extent by the natural compound. The aim of the study was to evaluate the *in vivo* antitumor activity of PITC-2 against sarcoma-180 cancer cell in Swiss albino mice. Therefore, we established a mouse model bearing sarcoma-180 to study the antitumor activity of the drug. The mice treated with PITC-2 at different dose inhibit body weight, tumor weight, tumor volume, tumor inhibition ratio and also brought hematological parameters to more or less normal level which is dose-dependent. A same type of finding was observed by de Costa *et al*. on *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives [22]. This study also shows inhibition of body weight, tumor volume, and tumor inhibition ratio. A good ILS and survival time was also noted in sarcoma-180 solid tumor-bearing mice when treated with PITC-2. A major problem in cancer chemotherapy is myelosuppression and anemia. Tumor-bearing mice have anemia mostly due to a reduction in RBC or low hemoglobin percentage. This may occur due to iron deficiency or due to myelopathy condition [23]. Treatment with

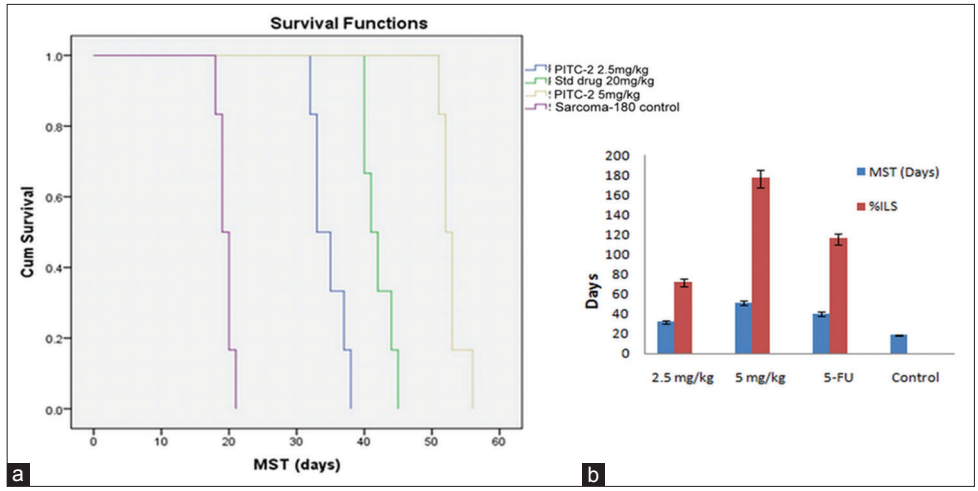


**Fig. 4: (a) Kaplan–Meier survival curve showing survival of all groups, (b) effect of PITC-2 treatment on mean survival time, and percentage of increase lifespan**

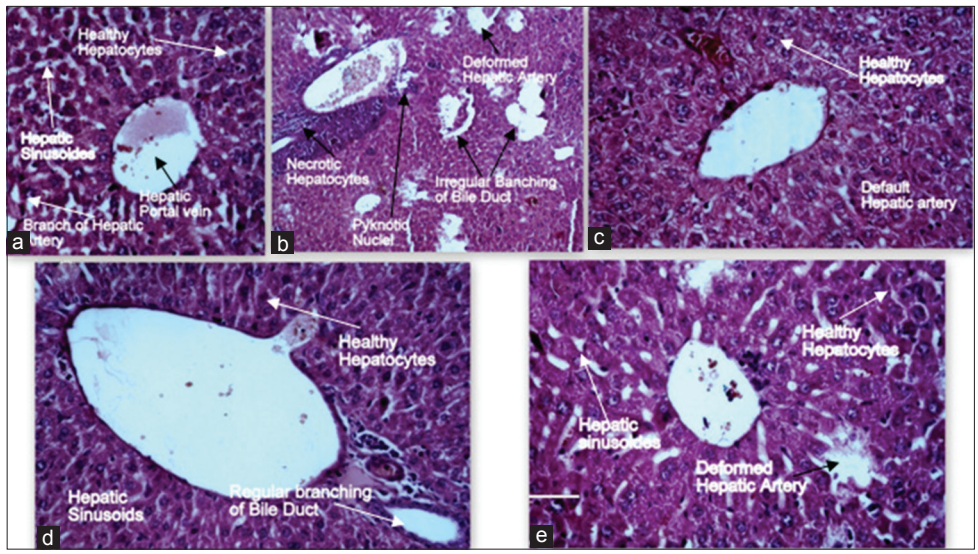


**Fig. 5: Hematoxylin and eosin stained section of mice liver showing cellular changes for (a) normal healthy mice, (b) sarcoma-180 control group, (c) standard 5-FU treated group, (d) 2.5 mg/kg of PITC-2, and (e) 5 mg/kg of PITC-2 (magnification 40×)**

PITC-2 stabilizes hemoglobin concentration, RBC and WBC cell count near to normal value. This reveals the protective activity of PITC-2 on the hematopoietic system. Another problem of cancer chemotherapy is liver toxicity which is due to accumulation of drug metabolites in liver [26]. In this study, liver toxicity was analyzed by biochemical studies and histopathology of liver tissue. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and ALP levels of control group mice, normal mice, and drug-treated mice were compared, and it was found that PITC-2 treated mice had less toxic liver than others as the level of AST, ALT, and ALP are close to normal value. These all features such as decrease in body weight, tumor weight, tumor volume, ILS and survival time, stabilizing hematological, and biochemical parameters of PITC-2 are dose-dependent, that in stabilizing effects are better in animals treated with higher dose of PITC-2. These features help in judging the drug activity.

Histopathological analysis of kidney for control, 5-FU, and PITC-2 for different dose was done. It was found that several degrees of hydropic changes of proximal tubular epithelium, glomerular, and tubular hemorrhage. These findings were much more prominent in control group animal than drug-treated groups where these effects are low, but the glomeruli structures were essentially preserved. The necrosis is characterized by no nuclear staining and deeply eosinophilic cytoplasm [27]. This show PITC-2 is less toxic to kidney than standard drug. Many drugs can cause lesion of liver found on biopsy. A large number of drugs with different pharmacological action and chemical structure give rise to liver lesion. Although liver shows a great adaptive and regenerative ability, for example, if anticonvulsant drugs are used for a long time, it causes an increase in endoplasmic reticulum which is regarded as an adaptive phenomenon. Again regeneration of hepatic tissue occurs by the conjugative tissues after hepatocellular necrosis due to treatment with alginates [27]. In our study, normal healthy mice show prominent nuclei and the tissue section comprises hepatic sinusoid which is usual. However, in case of sarcoma-180 control group none of the above mentioned regular features are observed, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Group receiving a different dose of PITC-2 are close to normal although little changes are found such as deformed hepatic artery and irregular bile duct which show PITC-2 is less toxic to liver.

From MTT assay it was found that PITC-2 has notable *in vitro* cytotoxic effect on S-180, MCF-7, A-549, and U-937 cancer cells. The mechanism of apoptosis is its diverse alteration of cells. Hence, cell apoptosis

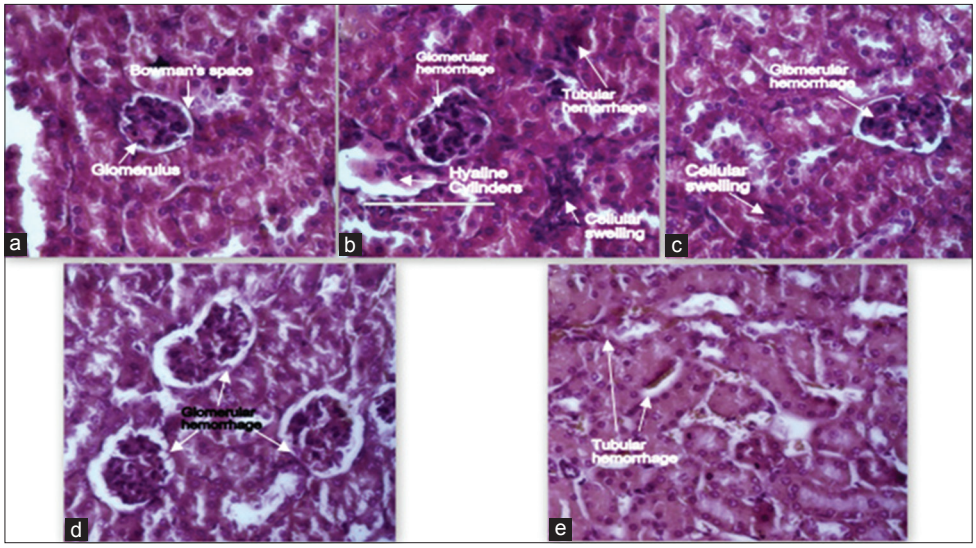


**Fig. 6: Hematoxylin and eosin stained section of mice kidney for (a) normal healthy mice, (b) sarcoma-180 control group, (c) standard 5-FU treated group, (d) 2.5 mg/kg of PITC-2, and (e) 5 mg/kg of PITC-2 (magnification 40×)**

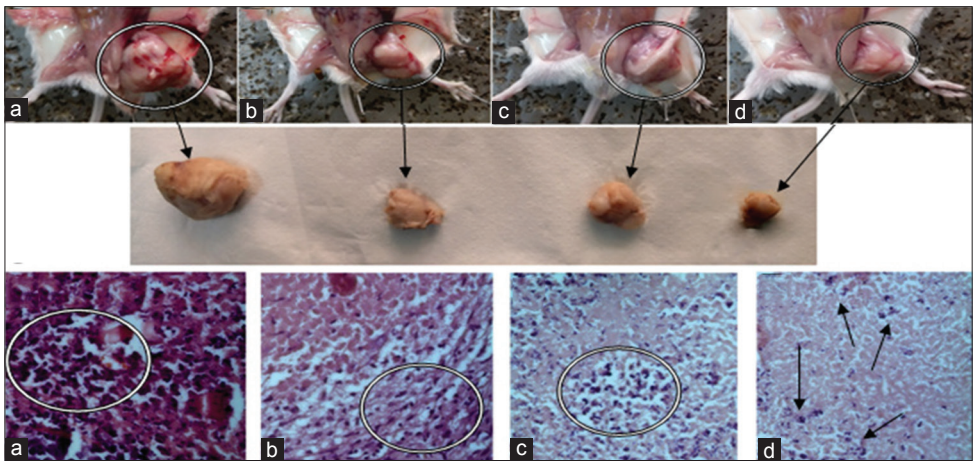


**Fig. 7: Effect of PITC-2 treatment on solid sarcoma-180 tumor: (a) Sarcoma-180 control group, (b) standard 5-FU treated group, (c) 2.5 mg/kg of PITC-2, (d) 5 mg/kg of PITC-2, panel (1) shows tumor growth on animals, panel (2) shows tumor isolated from animal body, panel (3) hematoxylin and eosin stained histological section of solid tumor (magnification 40×). The cellular features are marked by circle**

is identified by antiapoptotic protein Bcl-2 [28,29]. Bcl-2 plays an important role in apoptosis regulation in physiological and pathological conditions. Prevention of the loss of mitochondrial membrane potential is one of its notable works. It also prevents the release of cytochrome c into the cytosol which activates caspases by the mitochondrial pathway. Overexpression of Bcl-2 inhibits caspase-3 activation [30,26]. In our study, it was found that after treatment with PITC-2 on sarcoma-180 cells a significant decrease in Bcl-2 was observed whereas an increase in level Bcl-2 was observed in control group animals. This result concludes that increase of Bcl-2 would be a cellular response to counteract the apoptosis which is already initiated. Therefore, PITC-2 reduces the ability of Bcl-2 expression and initiates apoptosis.

To identify the tumor proliferation rate the cells, tumor cells are stained with Ki67. Nuclear staining was observed in all tumor sections which are immunized with Ki67 antibody. Mouse monoclonal antibody of Ki67 was used that identifies a nuclear antigen associated with G1, S, G2, and M phase. The expression of this molecule is found in all cell cycle except in G0 and early G1 phase [31]. In our study, it was found that a decrease in positive Ki67 cells in animals treated with 5FU and PITC-2 when compared to control group. It was also noted that reduction in tumor proliferation rate is more for a higher dose of PITC-2.

Cyclic D1 overexpression is an indication of early cancer onset and tumor progression [32]. Cyclic D1 is a protein required for progression through G1 phase during the cell cycle. It synthesizes rapidly during G1 phase and accumulates in the nucleus. Further, it starts degrading when it enters S phase [33]. Our study shows a marked decrease in expression of Cyclic D1 in animals treated with 5FU and PITC-2 when compared to control group. Even more effective result was shown by a different dose of PITC-2 than 5FU which reveals antitumor activity PITC-2.

## CONCLUSION

The present results clearly demonstrate that PITC-2 significantly inhibits sarcoma-180 cell growth in a dose-dependent manner in *in vivo* mice model. Besides this, the study reveals a comprehensive perception of the possible mechanism behind the antitumor activity of PITC-2 by significant changes in the morphological, hematological, and biochemical parameters in sarcoma-180 cells. Moreover, the histopathological and immunohistopathological examination indicates that PITC-2 induces apoptosis and suppresses tumor cell proliferation along with G1 cell

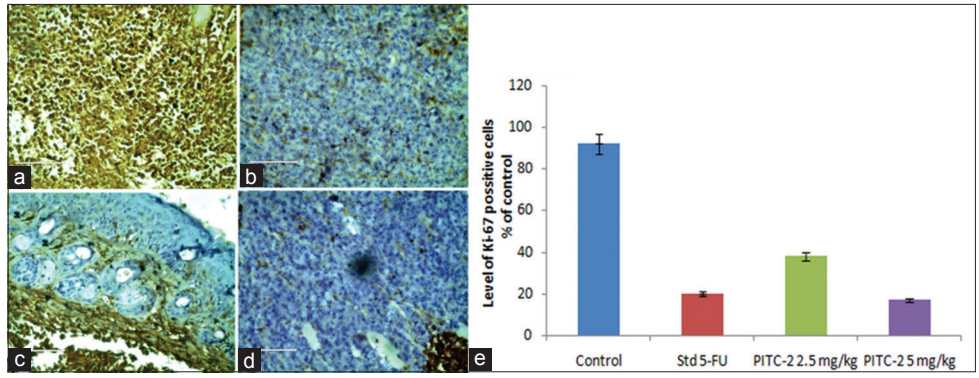


**Fig. 8: (a) Sarcoma-180 control, (b) standard 5-FU, (c) PITC-2 2.5 mg/kg, and (d) PITC-2 5 mg/kg shows Ki67 expression (magnification 40×). (e) Shows level of immunopositive Ki67 cells, values are given in mean ± standard deviation**

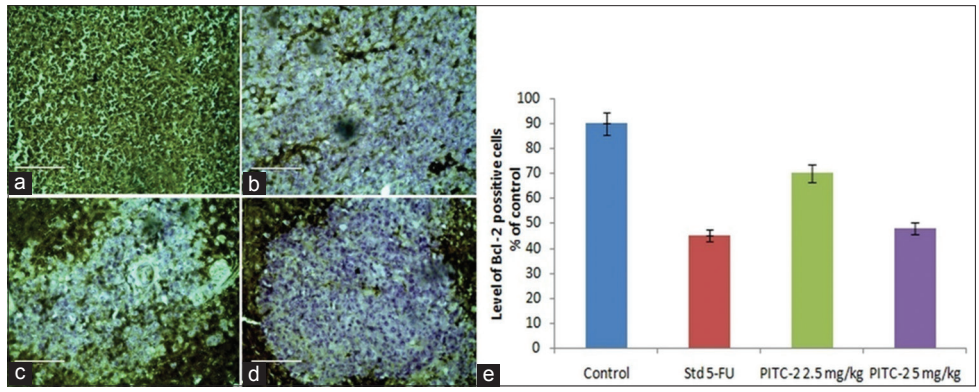


**Fig. 9: (a) Sarcoma-180 control, (b) standard 5-FU, (c) PITC-2 2.5 mg/kg, and (d) PITC-2 5 mg/kg shows Bcl-2 expression (magnification 40×). (e) Shows level of immunopositive Bcl-2 cells, values are given in mean ± standard deviation**

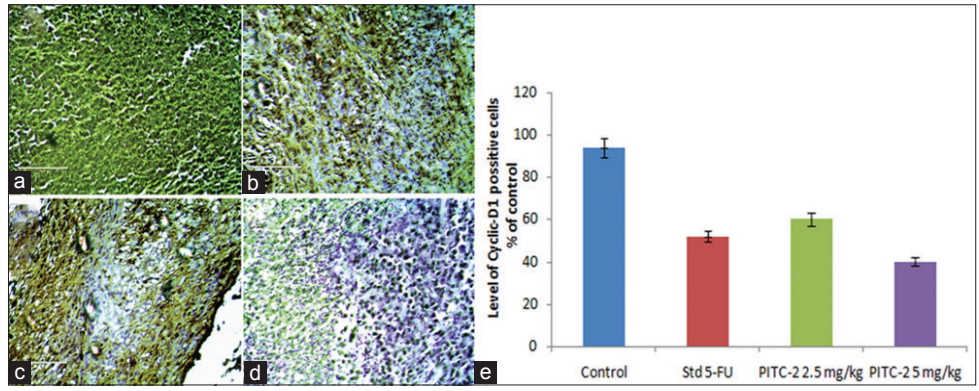


**Fig. 10: (a) Sarcoma-180 control, (b) standard 5-FU, (c) PITC-2 2.5 mg/kg, and (d) PITC-2 5 mg/kg shows cyclic-D1 expression (magnification 40×). E shows level of immunopositive cyclic-D1 cells, values are given in mean ± standard deviation**

cycle arrest through the downregulation of the intratumoral expression of Bcl-2, cyclic D1, and Ki-67 and thus highlighting antiproliferative and apoptotic properties against sarcoma-180 solid tumor model. Along with this, it shows no significant manifestation of toxic symptoms on liver and kidney of Swiss albino mice. Taking these results, we suggest that PITC-2 may be a potent prognostic factor and a potential target for tumor cells. Further investigations are in progress in our laboratory to identify the active principal and mechanisms involved in antitumor activity and to prepare a suitable formulation for modifying its way of administration.

## ACKNOWLEDGMENTS

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## AUTHORS CONTRIBUTION

Authors make substantial contributions in design, analysis and interpretation of data. Authors also participate in drafting the article. Authors give final approval of the version to be submitted and revised version

## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

## REFERENCES

1. Bissell MJ, Radisky D. Putting tumours in context. Nat Rev Cancer 2001;1:46-54.
2. Fattah AA, Darwish HA, Fathy N, Shouman SA. Carbition anhydrase

inhibition boost the antitumor effect of imatinib mesylate via potentiating the antiangiogenic and antimetastatic machineries. Toxicol Appl Pharmacol 2017;316:123-38.
3. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ, *et al*. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43-66.
4. Hussein BH, Azab HA. A novel antitumor agent Ln(lll) 2-thioacetate benzothiazole induces anti-angiogenic effect and cell death in cancer cell lines. Eur J Med Chem 2012;51:99-109.
5. American Cancer Society. Global Cancer Facts and Figure. 3rd ed. Atlanta: American Cancer Society; 2015. p. 1-61.
6. Skommer J, Brittain T, Raichoudhuri S. Bcl-2 inhibits apoptosis by increasing time-to-death and intrinsic cell-to-cell variations in the mitochondrial pathway of cell death. Apoptosis 2010;15:1223-33.
7. Swanton E, Savory P, Cosulich S, Woodman P. Bcl-2 regulates caspase 3/caspase 2 apoptotic cascade in cytosolic extracts. Oncogene 1999;18:1781-7.
8. Nygren P, Larsson R. Overview of the clinical efficacy of investigational anticancer drugs. J Intern Med 2003;253:46-75.
9. Nozlena SA, Ahmed AB, Rasedee A, Tengku IA, Rahman H, Keong SY. Zerumbone (ZER) induces apoptosis in HEPG2 cells via mitochondrial pathway. Int J Pharm Pharm Sci 2015;7:298-302.
10. Harborne JB. Arsenal for survival: Secondary plant products. Taxon 2000;49:435-49.
11. Pramanik KC, Biswas R, Mitra A, Bandyopadhyay D, Mishra M, Chatterjee TK. Tissue culture of the plant *Pluchea indica* (L.) Less. and evaluation of diuretic potential of its leaves. Orient Pharm Exp Med 2007;7:197-204.
12. Chakraborty S, Chatterjee TK. Targeting viseral leishmaniasis by mannosylated liposome incorporated PITC-2 isolated from the plant *Pluchea indica* (L) Less. Int J Pharm Bio Sci 2017;8:396-408.
13. Biswas R, Dutta PK, Achari B, Bandyopadhyay D, Mishra M, Pramanik KC, *et al*. Isolation of pure compound R/J/3 from *Pluchea indica* (L.) less. And its anti-amoebic activities against entamoeba histolytica. Phytomedicine 2007;14:534-7.
14. Mutiah R, Sukardiman, Widyawaruyanti A. Cytotoxic effect of crude exteact and fraction from Calotropis gigantea leaves on human colon cancer widr cell lines. Int J Pharm Pharm Sci 2017;9:83-6.
15. Sinha A, Banerjee K, Banerjee A, Das S, Choudhuri SK. Synthesis characterisation and biological evaluation of a noble vanadium complex as a possible anticancer agent. J Organi Chem 2014;772-773:34-41.
16. Joseph MM, Aravind SR, George SK, Pillai KR, Mini S, Sreelekha TT, *et al*. Antitumor activity of galactoxyloglucan-gold nanoparticles against murine ascites and solid carcinoma. Colloids Surf B Biointerfaces 2014;116:219-27.
17. Pal A, Bhattacharya I, Bhattacharya K, Mandal C, Ray M. Methylglyoxal induced activation of murine peritoneal macrophages and surface markers of T lymphocytes in sarcoma-180 bearing mice: Involvement of MAP kinase, NF-kappa beta signal transduction pathway. Mol Immunol 2009;46:2039-44.
18. Nascimento FR, Cruz GV, Pereira PV, Silva MC. Ascitic and solid Ehrlich tumor ingibition by Chenopodium ambrosiodes L. treatment. Cell Immunol 2006;78:2650-3.
19. Chakraborty A, Talukday D, Pal A, Ray M. Immunomodulation of macrophages by methyl glyoxal conjugated with chitosan nanoparticles against sarcoma-180 tumor in mice. Cell Immunol 2014;287:27-35.
20. Bala A, Kar B, Halder PK, Bera S. Evaluation of anticancer activity of Cleome gynandra on ehrlich's ascitec carcenoma treated mice. J Ethnopharmacol 2010;129:131-4.
21. Gaballah HH, Gaber RA, Mohamed DA. Apigenin potentiates the antitumor activity of 5-FU on solid ehrlick carcinoma: Crosstalk between apoptotic and JNK mediated autophagic cell death platforms. Toxicol Appl Pharmacol 2017;316:27-35.
22. de Costa PM, de Costa MP, Jamacaru VF, Ferreira PM. Inprovement of *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives. Chem Biol Interact 2015;239:174-83.
23. Gupta M, Mazumder UK, Kumar RS, Kumar TS. Antitumor activity and antioxidant role of bauhinia racemosa against ehrlich ascites carcinoma in swiss albino mice. Acta Pharmacol Sin 2004;25:1070-6.
24. Elsherbiny NM, Younis NN, Elseweids MM. The synergistic effect between vanillin and doxorubicin in ehrlich ascites carcinoma solid tumor and MCF-7 human brest cancer cell line. Pathol Res Pract 2016;212:767-77.
25. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A, *et al*. Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87-108.
26. Debnath S, Karan S, Debnath M, Chatterjee TK. Poly-L-Lysine inhibits tumor angiogenesis and induces apoptosis in ehrlich ascites carcinoma and sarcoma S-180 tumor. Asian Pac J Cancer Prev 2017;18:2255-68.
27. de Sousa AP, Torres MR, Pessoa C, Lotufo LV. *In vivo* growth-inhibition of sarcoma 180 tumor by alginates from brown seaweed Sargassum vulgare. Carbohydr Polym 2007;69:7-13.
28. Govindarasu M, Palani M, Vaiyapuri M. In ailico docking study on kaempferitrin with diverse inflammatory and apoptotic proteins functional approach towards the colon cancer. Int J Pharm Pharm Sci 201;9:199-204.
29. Paul S, Kundu R. Induction of apoptosis by fatty acid rich fraction of Solanum nigrum on carvical cancer cell lines. Int J Pharm Pharm Sci 2017;9:199-206.
30. de Silva Faria MC, dos Santos NAG, Rodrigues MA, dos Santos AC. Effect of diabetes on biodistribution, nephrotoxicity and antitumor activity of cicplatin in mice. Chem Biol Interact 2015;229:119-31.
31. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation associated human nuclear antigen defined by the monoclonal antibody Ki67. J Immunol 1984;133:1710-15.
32. Diehl JA. Cycling to cancer with cyclic D1. Cancer Biol Ther 2002;3(1):226-31.
33. Baldin V, Lukas J, Marcote MJ, Pagano M, Draette G. Cyclic D1 is a nuclear protein requires for cell cycle progression in G1. Genes Dev 1993;7:812-21.

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# PITC-2 Loaded Solid Lipid Nanoparticle: Design, Preparation, Characterization and Therapeutic Comparison with Free Phytochemical PITC-2 Isolated from Tissue Cultured Plant *Pluchea Indica*

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## Abstract

**Aim:** The objective of the study is to design, preparation and characterization of PITC-2 loaded Solid Lipid Nanoparticle for i.v. administration is performed.
**Methods:** Phytochemical PITC-2 is isolated from methanolic root extract of medicinal plant *Pluchea indica* which has a potent cytotoxic effect. PITC-2 loaded solid lipid nanoparticles were prepared by emulsion evaporation method. Formulation characterization was done using different technique and then pharmacological estimation of prepared SLNs was performed by comparing effects of PITC-2 SLNs with free phytochemical PITC-2 on ehrlich ascites carcinoma (EAC) cell in mice. **Results:** PITC-2 SLNs formed are smooth spherical particles observed in cryo-FESEM with less than 200nm in size. 52% encapsulation efficacy is found with a stable zeta potential of -35nV. DSC and PXRD studies indicate complete encapsulation of drug within the nanoparticle matrix in amorphous form. This formulation shows a sustained and prolonged drug release. Treatment with PITC-2 SLNs decreases tumor volume and increases lifespan of cancer bearing mice in comparison to phytochemical PITC-2. The histopathological examination also indicates that PITC-2 SLNs have promising apoptotic activity on tumor cells.
**Conclusion:** The developed PITC-2 loaded SLNs can be used as drug carrier for sustained and prolonged drug release with improve therapeutic activity and bioavailability.

**Keywords**
Solid Lipid Nanoparticle, Cytotoxicity, Apoptosis, Cell viability, PITC-2, Ehrlich Ascites Carcinoma (EAC).

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**Introduction:**
Cancers are considered to be the most killer disease all over the world. Therapy of cancer is based on surgery, radiotherapy and on systemic chemotherapy. Cancer therapies are remarkably consistent for last 50 years. Cytotoxic drugs play a

major role in chemotherapy for cancer except for a few cancer types where hormonal therapy and immunotherapy is used. Various categories of cytotoxic drugs are available which acts primarily by being toxic to the cancer cells that are rapidly growing and dividing. Cytotoxic drugs are earlier administered typically by free drug solutions as intravenous bolus or infusion (1).Drug carrier system plays a great role in therapeutic effectiveness and safety profile for this conventional form of this cancer therapies. Different type of drug carrier systems was used previously like colloidal drug delivery system which includes lipid emulsion, liposome and polymeric nanoparticles. But from a long time, trials are done to utilize solid lipid nanoparticle (SLN) as an alternative drug carrier system. SLN is advantageous over other colloidal drug delivery system as it improves the bioavailability of drugs (2) and obtains sustained release of lipophilic drugs (3).Moreover production of SLN are simple and in large scale with low toxicity (4). SLN consists of an amphiphilic outer core of surfactants and a biocompatible lipid inner core. The physically stable and biocompatible lipid core can incorporate lipophilic drug by emulsification of lipid molten matrix and subsequent recrystallization of the dispersed phase. SLN also provide a good protection of drugs against chemical degradation, a good drug release profile modulation avoidance of organic solvents and combination of a wide range of lipid/surfactant which results in high drug loading (5).

Although different type of carrier systems had developed for the delivery of cytotoxic drugs yet clinical success of these treatments reached a plateau (6).Moreover one main problem is its nonspecific toxicity due to their bio distribution throughout the body, which requires the administration of a large total dose to achieve high local concentrations in a tumor. Another problem in cancer chemotherapy is drug resistance. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents. Recent interest focused on medicinal properties of the secondary metabolites of these medicinal plants (7).

In this project tissue cultured medicinal plant *Pluchea indica* was used. The plant was tissue cultured with the purpose to obtain more secondary metabolites (8). *Pluchea indica* (L.) Less is a flowering plant in the family Asteraceae. The terminal flowers are rose-purple and grow in rather loose few-flowered heads. It is an evergreen large shrub found abundantly in salt marshes and mangrove swamps in Sunderbans (India), Bangladesh, Myanmar, China, Philippines, Malaysia, Tropical Asia and Australia (9). From the methanolic root extract of tissue cultured medicinal plant *Pluchea indica* PITC 2 was isolated. It is a Thiophen derivative which is [2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene].

The main objective of the study is to prepare SLN formulation of PITC-2 where lipid used is glycerol monostearate (GMS), poloxamer as a surfactant and soya lecithin as emulsifier. Then characterization of this prepared formulation and evaluation of in-vivo antitumor activity of the phytochemical PITC 2 against SLN PITC-2 formulation on ehrlich ascites carcinoma (EAC) cell in Swiss albino mice.

## MATERIAL AND METHODOLOGY:

### Materials:

Methanol (Sisco Research Laboratories Pvt. Ltd., boiling range 64 – 65°C), Petroleum ether (Marck Limited, boiling range 60-80°C), Ethyl acetate (Marck Limited), 1-Butanol (n-butyl alcohol for synthesis, Marck Limited), Silica gel (60-120 mesh, Sisco Research Laboratories Pvt. Ltd.), Lecithin from eggs, 30% from Himedia, Pluronic F 127 NF PrillPoloxamer 407 fromBASF**,** Glycerol monostearate (GMS) from Marck Limited,PLL hydrobromide (Mrs 30,000-70,000) and (4, 5-Dimethutthiazol-2-yl) -2, 5-dipheneyltetrazolim bromide(MTT) from Sigma Aldrich. Haematoxylin and Eosin stain, Papanicalaou stain from Qualigene (Mumbai, India). EDTA and Trypan blue from HiMedia. All chemicals are analytical grade (AR).

### Animals and their maintenance:

An anticancer study in Swiss albino mice of either sex weighing 20 to 30 gram was conducted according to the OECD 407 guideline (OECD, 2008). The maintenance of animal handling and care were performed under the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA). Swiss albino mice were selected for the anticancer evaluation animals of both sexes were designated within the same weight range. Precautions were taken to ensure that the selected female animals were nulliparous and non-pregnant. The animals were acclimatized under the standard controlled conditions, (temperature, 25 ± 5 C; relative humidity, 55± 10%) with light and dark cycle of 14 h and 10 h, respectively. A period of 7-10 days were provided to the animals to acclimatize under stipulated laboratory conditions prior to the initiation of experimentation. Throughout the experimentation period, animals were housed in

polypropylene cages provided with standard pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum* (10). All experiments were conducted as per guidelines cleared by the Animal Ethics Committee of the Department of the Pharmaceutical Technology of Jadavpur University, India (Registration number: 147/1999/CPCSEA).

**Extraction and Isolation:**

The roots after separating from the whole plant were dried, grounded and defatted with petroleum ether (60-80°C for synthesis). Then it was extracted in a soxhlet extractor with methanol (AR Grade, Purity 99.8%). After methanol distillation a sticky dark brown residue was obtained which is 8.9% (w/w). Then the methanolic root extract was partitioned in a mixture of n-Butanol and water. From this the n-butanol fraction was taken. Then it was shacked with ethyl acetate to distinguish the ethyl acetate soluble and insoluble part. Finally ethyl acetated soluble part is concentrated by a rotary vacuum evaporator and dried to obtain crude residue which is further go through column chromatography technique with a mixture of ethyl acetate and petroleum ether to obtained pure photochemical PITC 2 (11). A prominent spot on TLC plate (Mobile Phase: pet ether and ethyl acetate are in a ratio 8:2) denoted the presence of PITC-2 and it was found from the fraction 8 to15 which is the desired phytochemical PITC-2 [2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene] of a yield of 0.016%(w/w). Then this compound is structurally elucidated by FT-IR, NMR and Mass spectroscopy.

**Preparation of PITC-2 loaded solid lipid nanoparticles:**

PITC-2 loaded solid lipid nanoparticles were prepared by emulsion evaporation method (12). Approximately PITC-2 (10 mg), soya lecithin (100 mg) and glycerol monostearate GMS (200 mg) were mixed in a beaker containing 10 ml of ethyl acetate. Then this mixture is sonicated which is the organic phase. In another beaker 40 mg of poloxamer was mixed with ultra-pure water (20 ml) and heated at around 75 °C and starred at 1000 r.p.m. (aqueous phase). Then slowly organic phase was injected into the aqueous one and the temperature was maintained at 75°C with 1000 r.p.m. By this way, the organic phase was evaporated and simultaneously the aqueous phase was also reduced. Then added ice cold ultra-pure water and maintained temperature at around 3°C with 650 r.p.m. Then it was sonicated and centrifuged at 30,000 r.p.m. to remove free drug contains in it. After centrifugation the precipitate was separated and was lyophilized to obtain the final PITC-2 loaded SLN powdered formulation. In the present study phytochemical PITC-2 have a melting point (106 – 108°C) more than the melting point of the lipid used (57 – 65 °C). Hence upon cooling the hot emulsion it is expected that the lipid will solidify faster than the drug and the drug will form a lipid core (13). And thus the formulation will be a PITC-2 enriched core SLNs formulation.

**Characterization of prepared SLNs**

**Determination of drug content and Entrapment efficiency:**

By measuring the concentration of free drug in the dispersion medium, entrapment efficiency of the drug in the formulation can be determined. The samples were subjected to centrifugation for 30 minutes at 4°C and 20,000 rpm using an ultracentrifuge. The encapsulated amount of drug in the supernatant was analyzed using UV Spectrum. The amount of drug loading (DL) in the lipid phase and entrapment efficacy (EE) was also estimated by the given equation below (14).

$$\text{Entrapment efficiency (EE) \%} = \frac{\text{Total mass of PITC2 - Mass of PITC2 in suspension}}{\text{Total mass of PITC2}} \times 100$$

$$\text{Drug loading (DL) \%} = \frac{\text{Total mass of PITC2 - Mass of PITC2 in suspension}}{\text{Total mass of Lipid}} \times 100$$

**Particle side and polydispersity index:**

Particle size (z-average diameter) and polydispersity index (PI) were measured using Malvern Zetasizer Nano ZS (Malvern 90) by photon correlation spectroscopy at 25°C. Before measurement, nanoparticles are dispersed in Milli-Q water (Milli-Q, Merck Millipore, Billerica, MA, USA). The measurement was conducted at 90° detection angle.

**Zeta Potential:**

An electronic change on the particle size was identifies by Zeta potential (ZP). To determine the physical stability of a colloidal system, ZP is used. It measures the electrophoretic mobility Malvern Zetasizer Nano ZS with a field strength 20V $cm^{-1}$. Equation used for ZP conversion

$$\zeta = \frac{EM \times 4\pi\eta}{\varepsilon}$$

Where ζ is zeta potential, EM is electrophoretic mobility, η is viscosity of the dispersion medium, and ε is dielectric constant (15). Before analysis weighed quantity of the experimental sample was dispersed in Milli-Q water (Milli-Q, Merck Millipore, Billerica,

MA, USA) by vortexing and sonication and then placed in a cuvette for zeta potential measurement.

**Differential scanning calorimetry (DSC):**

DSC was performed to investigate the melting point and crystalline behavior of crystalline materials. Samples were placed in aluminum pans and empty pans were used as reference. A heating rate of 10 °C / min was employed in the range of 30–300°C. Analysis was performed under nitrogen purge (50ml/min).

**Powder X-ray diffractometry (PXRD):**

PXRD was performed on the samples by exposing them in CuKα radiation at 40Kv, 30mA. The results were scanned from 2° to 70° using diffraction angle 2$\vartheta$ at a step size of 0.045° and step time 0.5 s.

**Cryogenic field emission scanning electron microscopy (Cryo-FESEM):**

Cryo-FESEM is used to determine the surface morphology and shape of SLNs. 2-3 drops of dispersed SLNs were placed on a copper stub and freeze in liquid nitrogen at -196°C. Samples were then stored in liquid nitrogen and placed into cryopreservation chamber. Then it was fractured and sublimed at -95°C for 30s. Then the samples are coated with platinum and introduced into specimen stage of the FESEM at -140°C and examined at an excitation voltage of 5kV.

**Stability study:**

Stability of the prepared SLNs were evaluated for 3 months. PITC-2 loaded SLNs optimized formulations were stored at 25°C and 4°C for 3 months and then average size, poly dispersity index, zeta potential and entrapment efficacy were determined (15).

**In vitro release kinetics of PITC-2 from SLN:**

*In vitro* drug release studies of the selected optimized formulation were performed by using dialysis bag method (16). The molecular weight cut off of the used Dialysis membrane was 12,000-14,000. Before use of the membrane for drug release dialysis membrane was soaked for 12 hours. Each Dialysis bag was filled by 2 ml of formulation and the release media was 100 ml of phosphate buffer consist of pH 7.4. At fixed intervals of 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 22, 24, 28, 32, 36, 44, 46 and 48 h, 1ml of sample was withdrawn and replaced with fresh buffer from release media and analyzed by UV at 325 nm.

**Anticancer activity of prepared SLN by liquid tumor model**

**Ascites tumor induction by EAC cell line:**

EAC cells were preserved *in vivo* by intraperitoneal inoculation of cells in the peritoneal cavity of mice with sterile normal saline. EAC cells were taken from those inoculated mice and injected (0.2 ml of 2 x $10^6$ cells/mouse) to all the group's intraperitoneally except the normal group (17). From day 1 after inoculation with EAC cells treatment was given for 14 days (18) (19). From each group 6 mice were sacrificed and other 6 were kept for calculation of Mean survival time**.**

**Experimental Design for anticancer study:**

SLNs formulation of PITC-2 was dissolved in water for injection before administration in required concentration. For experimental purpose animals were divided into 4 groups each containing 12 mice.

Group 1: Normal animals (negative control).

Group 2: EAC cancer animals + PITC 2 (5mg/kg/day given i.p)

Group 3: EAC cancer animals + PITC2 formulation (5mg/kg/day given i.v.).

Group 4: EAC cancer animals (Standard) + 5FU (20mg/kg in alternative day given i.p).

**Collection of ascetic fluid and determination of viable and non-viable cell count:**

After 14 days of drug treatment, on 15$^{th}$ day animals were sacrificed. 2ml of normal saline water was injected (i.p.) in the abdominal region and then tumor cells along with ascetic fluid was collected in 15ml centrifuge tubes. Then they are centrifuged for 10 min at 3000 r.p.m at 4°C. Ascites tumor volume was then calculated by subtracting injected saline volume from the collected whole ascetic fluid and packed cell volume was calculated (20). The viability and non-viability of the cells were checked by trypan blue assay. The cells were stained with trypan blue (0.4% in normal saline) dye. The cells that did not take the dye were viable and those who took the dye were non-viable. Viability percentage is calculated by (No. of viable/ No. of viable and non-viable cells) x 100 (21).

Percentage cell growth inhibition was calculated by the formula = $1 - \frac{Tw}{Cw} \times 100$

Where Tw is mean of number of tumor cells of the treated group of mice and Cw is mean of number of tumor cells of the control group of mice (22).

**Determination of mean survival time (MST) and percentage increase in lifespan(%ILS):**

Mean survival time and percentage increase in life span (%ILS) were calculated by the following formulas (18).

$$\textbf{Mean survival time (MST)} = \frac{\sum \textbf{Survival time(day) of each mice in a group}}{\textbf{total number of mice}}$$

$$\%ILS = \frac{\text{MST of treated mice}}{\text{MST of control mice}} \times 100$$

**Hematological Parameters:**

Hematological study includes hemoglobin content, red blood cell (RBC) and white blood cells (WBC) counts. After 14 days of treatment animals were sacrificed by cervical dislocation and blood samples were collected from heart. WBC leukocyte counts were also carried out including neutrophils, lymphocytes and monocytes from leishaman stained blood smears of all groups of animals.

**Biochemical Parameters:**

After 24 hours of last dose and 18 hours of fasting the blood samples were collected from heart. Biochemical parameters include serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, globulin, bilirubin, alkaline phosphatase and serum protein to identify alteration in liver function and creatinine was measured as renal function parameter.

**Study on change in morphology of EAC cells by staining method:**

After collecting ascetic fluid from treated and control group, they were centrifuged at 3000 rpm for 10 minutes and one part of it is fixed in glass slides with neutral buffer formation. Then they were stained with hematoxylin and eosin. Another part is fixed on glass slide using 95% ethanol and stained with papanicalaou stain. Then both stained slides were mounted with Distrene Dibutyl Phthalate xylene and were examined under light microscope (40X) (23).Liver and kidney tissues were isolated from the animals and washed with saline. Then it was fixed in 10% buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin (24). Then the tissues were sectioned 5 to 6 µm, stained with hematoxylin and eosin (H&E) and examined under light microscope (40X).

**Statistical analysis:**

All *in vitro* studies were carried out in triplicate represented by independent biological evaluation. Data are extracted as mean ± standard error of the mean (SEM) and were compared by one-way analysis of variance (ANOVA) followed by Kruskal Wallis Test using SPSS statistical software of 20.0 version. $P<0.05$ was considered to be statistically significant when compared with control.

**RESULT AND DISCUSSION:**

**Identification of PITC-2:**

The present light-yellow solid compound was further confirmed by NMR spectroscopy and MASS spectroscopy (25). In mass spectroscopy a strong pick was observed at *m/z* 230 shown in Fig. 1A. Molecular weight of compound PITC-2 is 230. So, the present peak in mass spectroscopy is may be for PITC-2 which will be confirmed by NMR shown in Fig. 1 B1 and B2. In $^1H$ NMR $\delta^{TMS}$ ($CDCl_3$, 300MHz): 1.64 ($D_2O$ exchangeable, OH merged with solvent $H_2O$), 2.04(3H, s), 3.78($^1H$, dd, J=11.4, 6.3Hz, $H_A$ of CH-$CH_2OH$), 3.82 ($^1H$, dd, J=11.4, 3.9 Hz, $H_B$ of CH-$CH_2OH$), 4.69 ($^1H$, dd, J=6.3, 3.9 Hz, CHOH--$CH_2OH$), 7.04, 7.18 ($^2H$, m, thiophene-H). The $^1H$ NMR spectrum showed peaks for a methyl attached to unsaturation δ (2.04, s), a CHX-$CH_2Y$ unit and an aromatic system. The compound finally identified is PITC-2 which is 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene.

**Characterizations of SLNs**

**Particle size and Zeta Potential:**

The size distribution of PITC-2 loaded SLNs were shown in Fig. 2A and Table 1. In general, PITC-2 loaded SLNs looks round, uniform sized and well dispersed. The mean size of our nanoparticles was 171.5d.nm. It is also observed that particle size of PITC – 2 loaded SLNs are smaller than drug free SLN formulation. Zeta potential and stability of nanoparticles are closely related to each other. By measuring zeta potential, the stability of a nanoparticle system can be analyzed. Due to Van Der Wall inter particle attraction if a dispersion has low zeta potential then particles will get aggregated (4). Intracellular distribution of nanoparticles depends upon its zeta potential (26). In our study the zeta potential of all the prepared formulation were negatively charged and varied from -33.2 to -37.5 mV which ensured a system with good stability as it posse's sufficient repulsion. The zeta potential with negative charge might not interfere with the absorption of the formulation (27). Zeta potential values were presented in Table1 and shown in Fig 2B.

**Table 1: Particle Size, Polydispersity index and Zeta Potential of drug free SLNs and PITC-2 loaded SLNs(data represents mean ±SD).**

| Formulations | Particle size (d.nm) | | Polydispersity index | | Zeta Potential (mV) | |
|---|---|---|---|---|---|---|
| | Medium (n=3) | Standard deviation | Medium (n=3) | Standard deviation | Medium (n=3) | Standard deviation |
| Drug free SLNs | 290.5 | ±3.21 | 0.212 | ±1.32 | -35 | ±5.90 |
| PITC-2 loaded SLNs | 171.5 | ±4.87 | 0.101 | ±1.09 | -36.1 | ±5.57 |

**Table 2: Particle Size, Polydispersity index, Zeta Potential and Entrapment Efficacy of PITC-2 loaded SLNs (data represents mean ±SD)**

| Storage | Particle size(d.nm) | Polydispersity index | Zeta Potential (mV) | Entrapment efficacy (%) |
|---|---|---|---|---|
| Fresh | 171.5 ± 4.87 | 0.101 ±1.09 | -36.1 ±5.57 | 52 ±0.53 |
| 4°C for 3 months | 182.43 ± 5.13 | 0.116 ± 2.74 | -31.87 ± 4.32 | 48 ± 1.23 |
| 25°C for 3 months | 172.21 ± 3.08 | 0.105 ±1.65 | -35.18 ± 4.68 | 51.24 ± 0.43 |

**Table 3: *In-vivo* effect of PITC-2 on EAC bearing Mice**

| | PITC-2 5mg/kg b.w | PITC-2 loaded SLN Formulation 5mg/kg b.w | Std drug 5-FU 20mg/kg b.w | Sarcoma 180 Control |
|---|---|---|---|---|
| Ascites fluid volume (ml) | 5.64 ± 0.28* | 5.24 ± 0.12* | 5.89 ± 0.23* | 11.90 ± 0.11* |
| Packed Cell volume (ml) | 1.45 ± 0.08* | 1.39 ± 0.04* | 1.32 ± 0.02* | 4.21 ± 0.08* |
| Body weight (gm) | 22.41 ± 0.43* | 22.13± 0.42* | 22.68 ± 0.55* | 30.10 ± 0.48* |
| MST (days) | 39.67 ± 0.98* | 41.83 ± 0.70* | 32.00 ± 0.85* | 18.40 ± 0.42* |
| %ILS | 215.59 | 224.89 | 173.91 | 0 ± 0 |
| Viable Cell($10^6$cells/ml) | 3.05± 0.13* | 2.92 ± 0.21* | 3.45 ± 0.43* | 10.65± 0.23* |

***<0.05 as compared to control group (n = 6 mice per group)***

**Table 4: Effect of PITC-2 on hematological parameters in EAC bearing Mice**

| | PITC-2 5mg/kg b.w | PITC-2 loaded SLN Formulation 5mg/kg b.w | Std 5-FU 20mg/kg b.w | EAC control | Normal Mice |
|---|---|---|---|---|---|
| Hemoglobin (gm %) | 9.4± 0.05* | 9.6± 0.08* | 9.1± 0.06* | 6.3 ± 0.01* | 11.8±0.12* |
| Erythrocyte (RBC) (cells x$10^6$/$mm^3$) | 5.6± 0.04* | 6.53± 0.03* | 5.68± 0.08* | 3.75 ± 0.08* | 7.90±0.02* |
| Leucocytes (WBC) (cells x$10^6$/$mm^3$) | 9.1± 0.05* | 8.29±0.05* | 10.19±0.05* | 16.69±0.02* | 6.40±0.03* |
| Neutrophil (%) | 31.3± 0.03* | 30.46±0.03* | 30.1±0.02* | 68.5 ± 0.05* | 19.4±0.03* |
| Lymphocyte (%) | 53.0± 0.04* | 55.4± 0.04* | 61.0±0.09* | 35.8 ± 0.11* | 64.1±0.23* |
| Monocyte (%) | 1.7± 0.03* | 2.2± 0.03* | 1.6± 0.03* | 1.3 ± 0.04* | 2.8±0.01* |

***<0.05 as compared to control group (n = 6 mice per group)***

**Table 5: Effect of PITC-2 on biochemical parameters in EAC bearing mice**

| | PITC-2 5mg/kg b.w | PITC-2 loaded SLN formulation 5mg/kg b.w | Std 5-FU 20mg/kg b.w | Sarcoma 180 control | Normal Mice |
|---|---|---|---|---|---|
| Bilirubin (total) mg/dl | 0.26± 0.02* | 0.27± 0.02* | 0.24± 0.03* | 0.34± 0.01* | 0.45± 0.02* |
| Serum protein (total)g/dl | 8.52 ± 0.09* | 8.29 ± 0.07* | 8.22 ± 0.12* | 4.4± 0.08* | 8.19±0.08* |
| AST (SGOT) IU/L | 34.5± 0.01* | 34.9± 0.01* | 35.9± 0.01* | 74.9± 0.09* | 39.1±0.01* |
| ALT(SGPT) IU/L | 35.6± 0.04* | 36.4± 0.01* | 39.3± 0.06* | 71.4± 0.07* | 29.1±0.04* |

| | | | | | |
|---|---|---|---|---|---|
| Serum alkaline phosphatase IU/L | 71.1± 0.02* | 69.9± 0.02* | 72.8± 0.05* | 121.1±0.03* | 74.4± 0.03* |
| Creatinine Mg/dl | 0.78± 0.01* | 0.8± 0.02* | 0.81± 0.01* | 1.2± 0.02* | 0.62± 0.01* |

***<0.05 as compared to control group (n = 6 mice per group)***

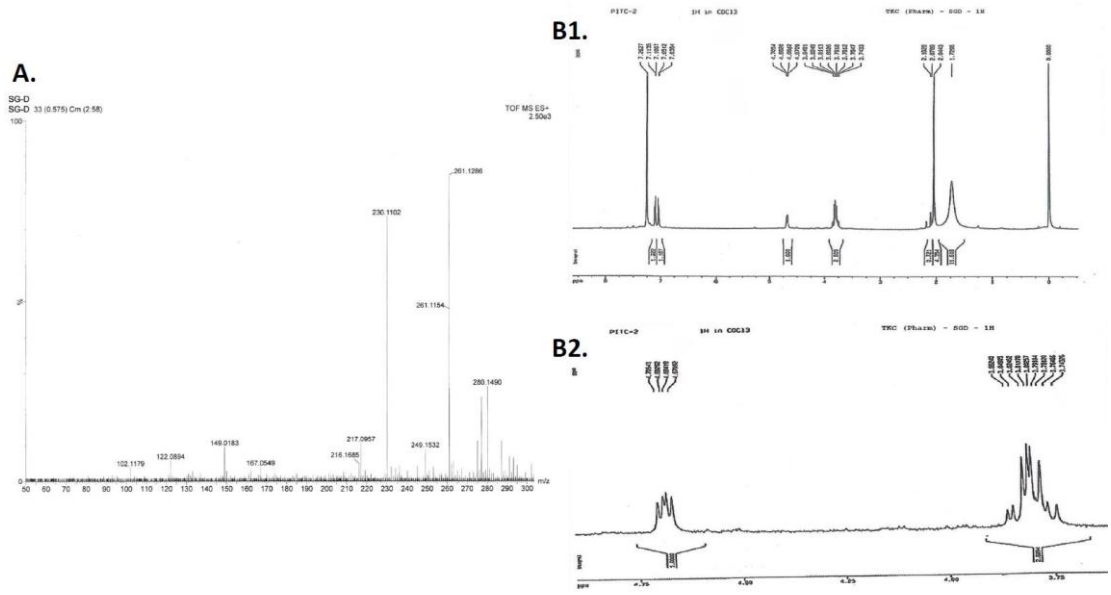


***Fig 1: A. Mass spectrum of compound PITC-2, B1 and B2. 1H NMR spectrum of compound PITC-2 in $CDCL_3$ (300MHz)***

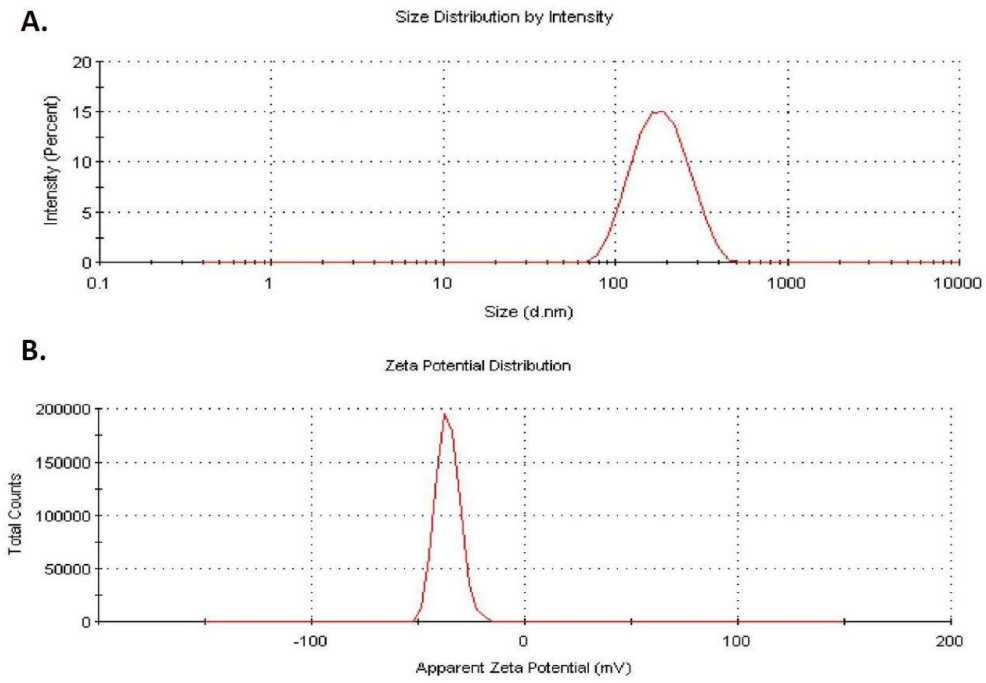


**Fig 2: A. Particle size distribution curve of PITC-2 loaded nanoparticles, B. Surface Zeta potential graph showing negative zeta potential value of PITC-2 SLNs.**

**Entrapment Efficacy:**

Total drug used in preparation of formulation was 10 mg. Amount of drug present in the SLNs was found to be (10-4.8) 5.2 mg. Entrapment efficacy of the prepared formulation was 52%.

**Thermal analysis by differential scanning calorimetry (DSC):**

DSC was a tool which investigate the crystalline behavior and melting point. Fig 3A shows DSC curve of phytochemical PITC-2, glycerol monostearate GMS, drug free SLN, and PITC-2 loaded SLN formulation. A clear sharp pick observed at 106.26°C for phytochemical PITC-2 which corresponds to its melting temperature. GMS shows its melting pick at 63.4°C. PITC-2 loaded SLN shows pick at 65.14°C whereas drug free SLN shows pick at 64.21°C. This slight shift of pick was due to loading of drug in the lipid matrix. Absence of drug pick at 106°C indicates either solubilization of PITC-2 in lipid matrix upon heating or due to amorphous dispersion of PITC-2 in lipid matrix. A similar type of phenomenon was reported by Das *et.al* for Tretinoin loaded SLNs (15). This reveals a homogeneous dispersion of drug in the lipid.

**Powder X-ray diffractometer (PXRD):**

Fig 3B shows XRD curve of phytochemical PITC-2, drug free SLN, and PITC-2 loaded SLN formulation. Phytochemical PITC-2 have picks at *2ϑ* scattered angle shown in the figure which defines its crystalline nature. Those picks are not found in the diffractogram of PITC-2 loaded SLNs which indicates PITC-2 was solubilized and stabilized in lipid matrix of formulation. If PITC-2 was present outside of lipid matrix then due to its poor solubility it would crystallize outside the lipid matrix and effect the diffraction curve. Now the diffractogram of drug free SLNs closely resembles to the diffractogram of PITC-2 loaded SLNs which indicates no change happens in the nature of SLNs due to addition of PITC-2. It shows PITC-2 is no more in a crystalline form in SLN formulations but in an amorphous form. This phenomenon is also observed from the morphological study of the SLNs.

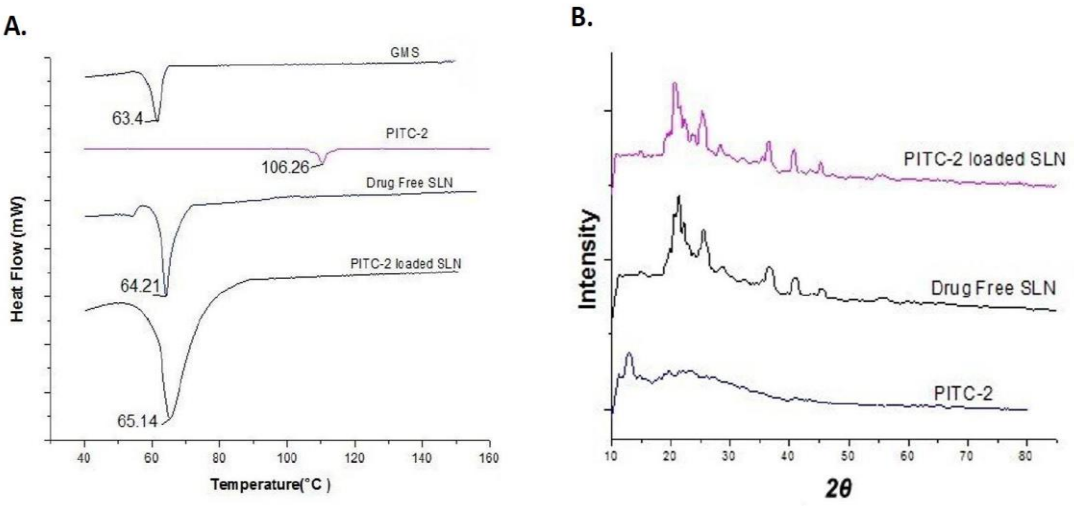


**Fig 3: A.DSC Thermogram of phytochemical PITC-2, glycerol monostearate GMS, drug free SLN, and PITC-2 loaded SLN formulation. B. PXRD profile of phytochemical PITC-2, glycerol monostearate GMS, drug free SLN, and PITC-2 loaded SLN formulation.**

**Stability of SLNs:**

There are minor changes in particle size, PI, ZP and EE after 3 months of storage at 4°C but no changes at all were found in particles stored at 25°C shown in Table 2. However, the changes are not that significant which indicates a good physical stability of PITC-2 SLNs for 3 months at 4°C and 25°C.

**Cryogenic field emission scanning electron microscopy (Cryo-FESEM):**

To check the surface morphology of nanoparticles Cryo-FESEM was done. Size and shape of the nanoparticles are identified by this method. In our study from Cryo-FESEM of PITC-2 loaded SLNs it was observed that they are almost spherical in shape and have a smooth surface morphology shown in Fig 4A. Agglomeration of nanoparticles are also found which may be due to the lipid as a carrier system. On the other hand, Fig 4B shows crystalline structure of free phytochemical PITC2. Absence of such crystalline structure of PITC-2 in SLNs formulation shows no unentrapped in the formulation (15).

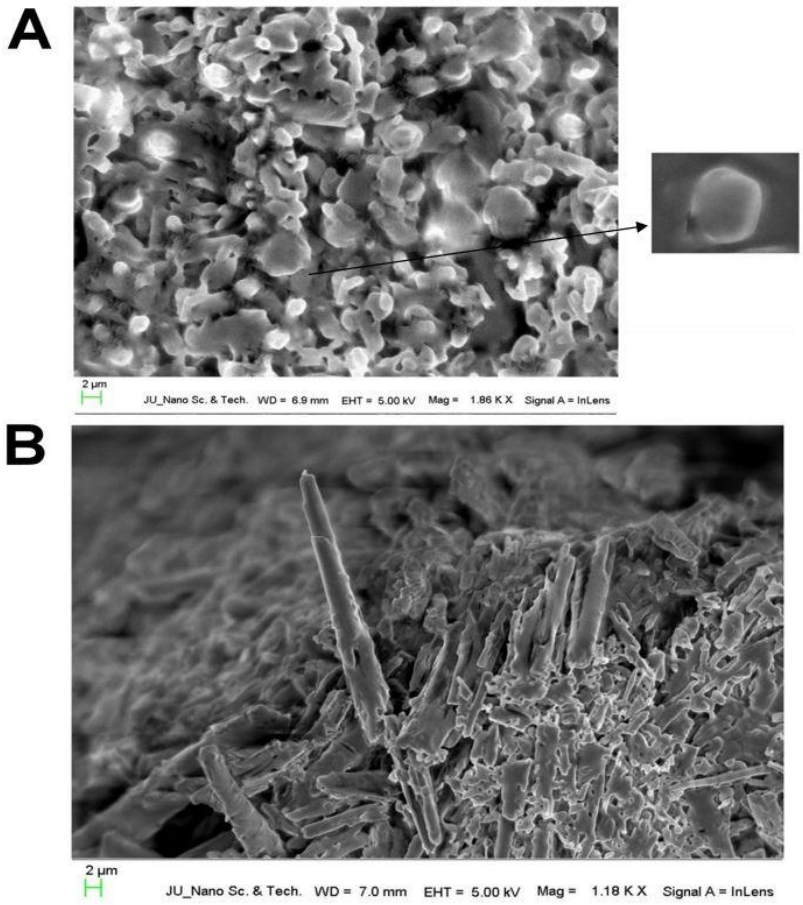


**Fig 4: FESEM images of A. PITC-2 loaded SLNs, and B. phytochemical PITC-2.**

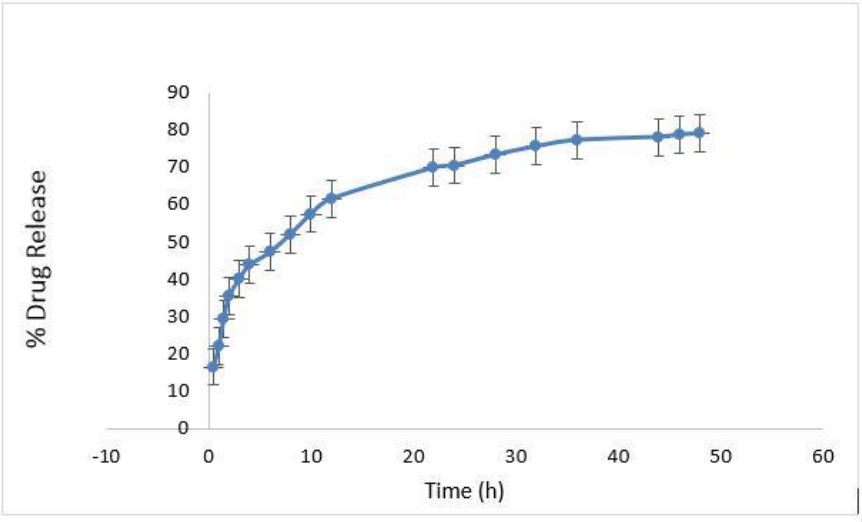


**Fig 5: Drug release profile of PITC-2 loaded SLNs (data represents mean ±SD).**

***In vitro* release kinetics of SLNs:**

Fig 5 shows a sustained drug release of PITC-2 loaded SLNs at 7.4 pH phosphate buffer. Percentage drug release verses time is plotted to determine the pattern of drug release. The release study shows biphasic pattern. It was observed that 35 % of the drug was released in first 2 hr and then a sustained release of SLNs occurs. These burst releases occur may be due to presence of absorbed drug in the surface of the nanoparticles. After 48 hrs of percentage drug release was 79.02%. This is important for the physiological requirements of human system. A similar type of pharmacokinetic study was observed by Shengpeng Wang et.al (28).

**Inhibition of EAC cells growth by PITC-2:**

A significant reduction of ascetic fluid volume, packed cell volume and viable tumor cell count was found with PITC-2 SLNs formulation at dose 5mg/kg and phytochemical PITC-2 at concentration 5mg/kg b.w compared to that of control group mice. A same type of finding was observed by de Costa *et al.* on *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives (29). Moreover, a good increase in lifespan and median survival time (MST) was also observed in EAC cell bearing mice when treated with PITC-2 loaded SLNs, shown in Table 3.

**Effect of PITC-2 on hematological parameters**

An increase in hemoglobin level, RBC count, neutrophil level and decrease in WBC level, monocyte and lymphocyte count was found in phytochemical PITC 2 treated groups as well as in PITC-2 loaded SLNs with compared to that of EAC control group (Table 4). Even SLNs shows comparative good result than free PITC-2. This reveals protective activity of PITC-2 SLNs on the hematopoietic system.

**Effect of PITC-2 on biochemical parameters**

One major problem of cancer chemotherapy is liver toxicity which is due to accumulation of drug metabolites in liver (30). Toxicity is a major problem of drug delivery system too. One can have a neat delivery system, but it is more important to be a safe delivery system with low toxicity for its introduction in pharmaceutical and clinical market. Toxicity is due to the excipients in carrier system. Use of glycerides composed of fatty acid, contained in oils of parenteral fat emulsion may reduce toxicity (4). In our study we had used glyceryl monostearate which may not induce any toxicity for the SLNs. Furthermore, surfactant used in our study was poloxomer which is recommended as an acceptable surfactant for use in parenteral formulations. (4). Here liver toxicity was analyzed in this experiment by biochemical studies and histopathology of liver tissue. Phytochemical PITC-2 treated group and PITC-2 loaded SLNs group shows significant decrease in SGPT, SGOT and serum alkaline phosphatase parameters in comparison with EAC control group shown in Table 5. It was found that groups treated with PITC-2 loaded SLNs had less toxic liver as the level of AST, ALT and ALP are close to normal value. An more increase in total protein was also found in PITC-2 formulation treated groups.

**Histopathology of liver and kidney tissue**

Lesion in liver was caused by many drugs as well as carrier systems which are analyzed by biopsy. A large number of drugs with different pharmacological action and chemical structure give rise to liver lesion. Although liver has a unique regenerative ability and great adaptive efficiency. For example, an adaptive phenomenon of liver is if an anticonvulsant drug was used for a long time then it causes increase in endoplasmic reticulum. Again, regeneration of hepatic tissue occurs by the conjugative tissues after a hepatocellular necrosis due to treatment with alginates (31). In our study H&E stained section of liver of a healthy mouse was shown in Fig. 6A, bearing all the normal features which includes circular hepatic portal vein and of hepatic artery, as marked by arrow. The tissue section comprises of hepatic sinusoid and nuclei which are usual. But none of the above-mentioned regular features are observed in case of Sarcoma-180 control group showed in Fig. 6B, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Although little changes are found in group receiving standard 5-FU like deformed hepatic artery and irregular bile duct yet lesser amount of hepatocellular lesions observed which are close to normal as shown in Fig. 6C. Groups receiving phytochemical PITC 2 and PITC-2 loaded SLNs also shows a little hepatic deformation and altered hepatocyte population shown in Fig. 6D and 6E. Healthy hepatocytes were observed, regular branch of bile duct found, although mild dilation of central vein observed found. This all features show SLNs are less toxic to liver.

Histopathological analysis of kidney was also done for control, 5-FU, phytochemical PITC-2 and PITC-2 loaded SLNs. H&E stained section of kidney of a healthy mice was shown in Fig. 7A, bearing all the normal features, while Sarcoma-180 control group shows severe glomerular and tubular hemorrhage with increase swelling of tubular epithelium and presence of hyaline cylinders showed in Fig. 7B. Group treated with 5-FU and PITC-2 shows light glomerular and tubular hemorrhage and cellular features are less deformation which are close to

normal shown in Fig. 7C, 7D and 7E respectively. The characterization of necrosis is done by no nuclear staining and deeply eosinophilic cytoplasm (31).This show PITC-2 loaded SLNs are less toxic to kidney than standard drug and quite resembles to the action of phytochemical PITC-2.

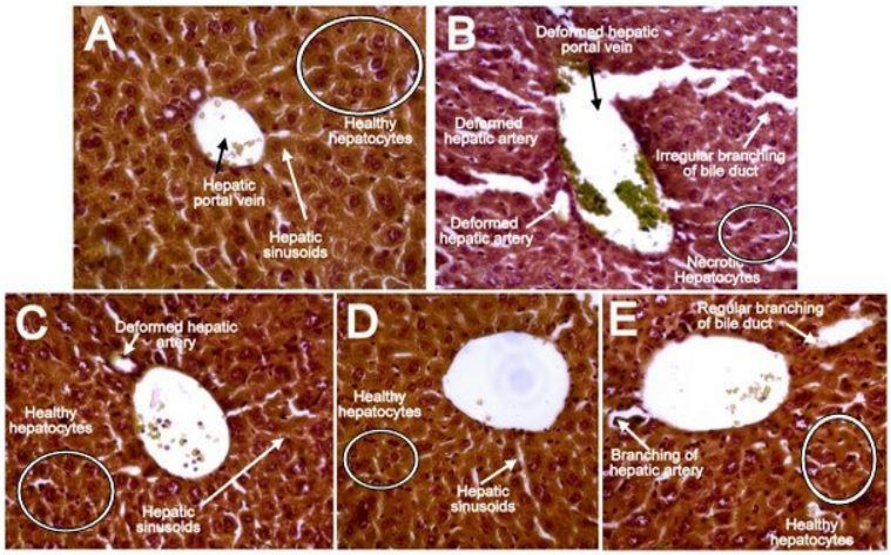


**Fig 6: H & E stained section of liver of mice showing cell morphology of groups A. Normal Mice, B. EAC control mice, C. 5 FU standard drug, D. 5 mg/kg PITC-2 and E. 5mg/kg PITC-2 SLNs.**

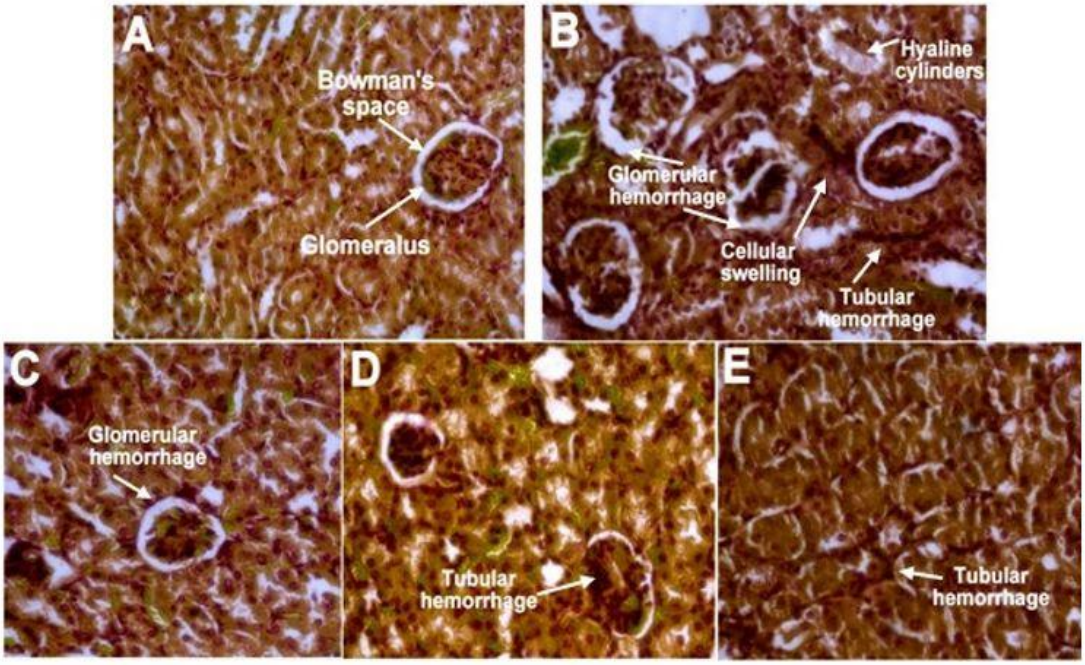


**Fig 7: H & E stained section of kidney of mice showing cell morphology of groups A. Normal Mice, B. EAC control mice, C. 5 FU standard drug, D. 5 mg/kg PITC-2 and E. 5mg/kg PITC-2 SLNs.**

**Morphological changes of EAC cells**

Now for analyzing the cytotoxic effect of the formulation histopathology of ascetic fluid was done. Cell apoptosis takes place and a significant morphological change was observed in EAC cell with both Haematoxylin & Eosin (H&E) and Papanicolaou (Pap) staining shown in Fig 8. Apoptosis is a cell regulatory mechanism which is distinguished by diverse alteration in cell morphology including asymmetry in plasma membrane, cytoplasm shrinkage, chromatin condensation, formation of apoptotic bodies, etc (32) (30). In our study, H&E

staining cells of control group have good circular morphology, intact plasma membrane and nucleus. But cell of groups treated with standard drug shows apoptotic bodies and nuclear contraction. Irregularity in cell morphology, blebbing of plasma membrane, chromatin condensation was also found in groups treated with phytochemical PTIC-2. In case of groups treated with PITC-2 loaded SLNs evident change in cell morphology was found which includes cell shrinkage, formation of apoptotic bodies, nuclear contraction and blebbing of plasma membrane. Similarly, in case of cells stained with Pap stain numerous features of cellular apoptosis was observed in standard and test drug groups which are shown in the figure by arrow. Degree of change in cell morphology was more in SLN PITC-2 than phytochemical PITC-2.

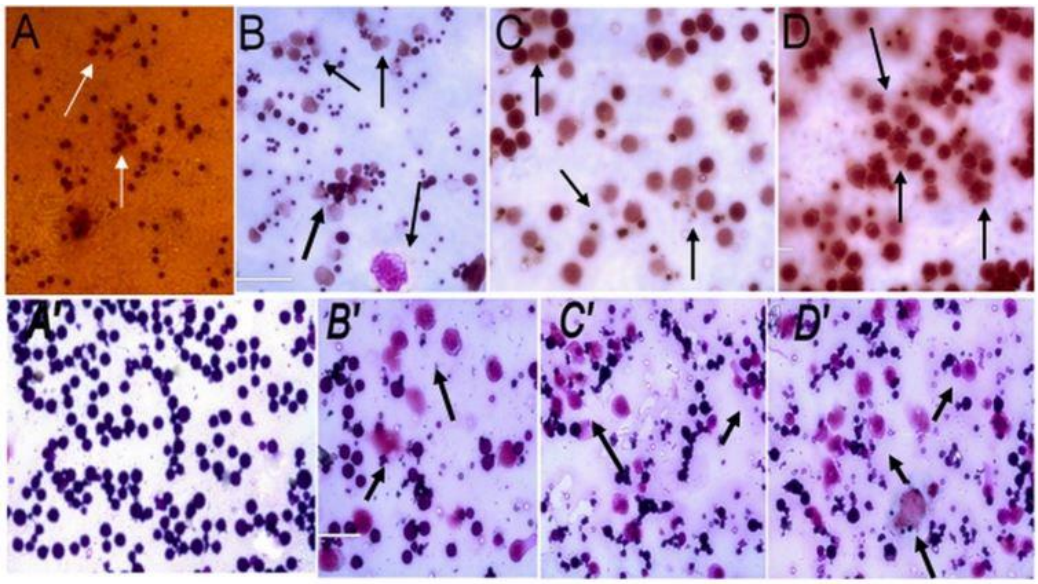


**Fig 8: Change in cell morphology of EAC cells by H&E stain of groups A. EAC control mice, B. 5 FU standard drug, C. 5 mg/kg PITC-2 and D. 5mg/kg PITC-2 SLNs and Papanicolaou stain of groups A'. EAC control mice, B'. 5 FU standard drug, C'. 5 mg/kg PITC-2 and D'. 5mg/kg PITC-2 SLNs**

## CONCLUSION:

In this study PITC-2 loaded SLNs were successfully prepared by emulsion evaporation method. Poorly water soluble phytochemical PITC-2 was effectively loaded in the nanoparticles. The method resulted in producing consistent smaller size nanoparticles in the range of 170- 180 nm with narrow size distribution. Most of the process variables shows significant effect on the formulation properties. SLNs prepared for systematic drug delivery obtains better therapeutic efficiency by sustained drug release, thus by improving bioavailability and will decrease dosing with lower side effects. Finally, this planning methodology has clearly shown its usefulness in this optimization process, and this research produces a framework for the understanding of SLN formation.

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## REFERENCES

1. Wong H.L., Bendayan R., Rauth A.M., Li Y., Wu X.Y., Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles. Adv Drug Deliv Rev, 59: 491-504, (2007)
2. Olbrich C.,Kayser O., Muller R.H., Lipase degradation of Dynasa 114 and 116 solid lipid nanoparticles (SLN) - effect of surfactents, storage time and crystallinity. Int.J. Pharm, 237: 119-128, (2002)
3. Yang S., Zhu J., Lu Y., Liang B., Yang C., Body distribution of camptothecin solid lipid nanoparticle after administration. Pharm. Res, 16(5): 751-757, (1999)
4. Muller R.H., Madar K., Gohla S., Solid lipid nanoparticles (SLN) for controlled drug delivery-a revier of the stste of the art. Eur, J. Pharm. Biopharm, 50: 161-177, (2000)
5. Kim B.D., Na K., Chio H.K., Preparation and characterization of solid lipid nanoparticles

(SLN) made of cocoa butter and curdlan. Eur J Pharm Sci, 24: 199-205, (2005)
6. Dhamija I., Kumar , Manjula S.N., Parihar V., Setty M.M., Pia K.S.R., Preliminary evaluation of in vitro cytotoxicity and in vivo antitumor activity of Premna herbacea Roxb. in ehrtich ascites carcinoma model and daltons lymphona ascites model. Exp Toxicol Pathol, 65: 235 - 242, (2013)
7. Harborne J.B., Arsenal for Survival: Secondary Plant Products. Taxon, 49: 435-439, (2000)
8. Pramanik K.C., Biswas R., Mitra A., Bandyopadhyay D., Mishra M., Chatterjee T.K., Tissue culture of the plant Pluchea indica (L.) Less. and evaluation of diuretic potential of its leaves. Orient Pharm Exp Med, 7(2): 197-204, (2007)
9. Chakraborty S., Chatterjee T.K., Targeting viseral leishmaniasis by mannosylated liposome incorporated PITC-2 isolated from the plant Pluchea indica (L) Less. Int J Pharm Bio Sci, 8(2): 396-408, (2017)
10. Gorain B., Choudhury H., Tekade R.K., Karan S., Jaisankar P. and Pal T.K., Comparative biodistribution and safety profiling of olmesartan medoxomil oil-in-water oral nanoemulsion. Regul Toxicol and Pharmacol, 82: 20-31, (2016)
11. Goswami S,. Dednath S., Karan S., Chatterjee T.K., In vivo antitumor activity of phytochemical PITC-2 obtained from tissue cultured plant Pluchea indica on sarcoma-180 solid tumor mice model. Asian J Pharm Clin Res 4(11): 211-218, (2018)
12. Chen J., Dai W.T., He Z.M., Gao L., Huang X., Gong J.M., Xing H.Y., Chen W.D., Fabrication and Evaluation of Curcumi nloaded Nanoparticles Based on Solid Lipid as a New Type of Colloidal Drug Delivery System. Indian J Pharm Sci, 75(2): 178–184, (2013)
13. Jenning V., Gysler A., Schafer Korling M., Gohla S.H., Vitamine A loaded solid lipid nanoparticles for tropical use: Occlusive properties and drug targeting to the upper skin. Eur J Pharm Biopharm, 49: 211 - 221, (2000)
14. Luo Y.F., Chen D.W., Ren L.X., Zhao X.L., Qin J., Solid lipid nanoparticles for enhancing vinpocetine's oral bioavailability. Journal of Controlled Release, 114: 53–59, (2006)
15. Das S., Ng W.K., Kanaujia P., Kim S., Formulation design, preparation and physicochemical characterizations of solid lipid nanoparticles containing a hydrophobic drug: Effects of process variables. Colloids and Surf B: Biointerfaces, 88: 483-489, (2011)
16. Madhusudhan A., Reddy G.B., Venkatesham M., Veerabhadram G., Design and Evaluation of Efavirenz loaded Solid Lipid Nanoparticles to Improve the Oral Bioavailability. International Journal of Pharmacy and Pharmaceutical Science Research, 2(4): 84-89, (2012)
17. Sinha A., Banerjee K., Banerjee A., Das S., Choudhuri S.K., Synthesis characterisation and biological evaluation of a noble vanadium complex as a possible anticancer agent. J Organomet Chem, 772-773: 34-41, (2014)
18. Agarwal S.S., Saraswati S., Mathur R., Cytotoxic and antitimor effect of brucine on ehrlich ascites tumor and human cancer cell line. Life Sci, 69(5-6): 147-158, (2011)
19. Beydogan A.B., Bolkent S., The effects of silibin adminiistration for different time periods on mouse liver with Ehrlich ascites carcinoma. Pharmacol Rep, 68(3): 543-549, (2016)
20. Nicol B.M., Prasad S.B., The effects of cyclophosphamide alone and in combination with ascorbic acid against murine ascited Dalton's lymphoma. India J Pharmacol, 38(4): 260-265, (2006)
21. Salem M.L., Shoukry N.M., Rahman A.A., In vitro and in Vivo antitumor effects of the Egyptian scorpion Androclonus amoreuxi venam in an Ehrlich ascitic tumor model. Springer Plus, 5: 570-581, (2016)
22. Perveen R., Islam F., Khanum J., Yeasmin T., Preventive effect of ethanolic extract of Alpinia calcarata Rose on ehrlichsascitic carcenoma cell induced malignant ascitic in mice. Asian Pac J Trop Med, 5(2): 121-125, (2012)
23. Debnath S., Karan S., Debnath M., Chatterjee T.K., Poly-L-Lysine inhibits tumor angiogenesis and induces apoptosis in ehrlich ascites carcinoma and sarcoma S-180 tumor. Asian Pac J Cancer Prev, 18: 2255 - 2268, (2017)
24. Elsherbiny N.M., Younis N.N., Elseweids M.M.,The synergistic effect between vanillin and doxorubicin in ehrlich ascites carcinoma solid tumor and MCF-7 human brest cancer cell line. Pathol Res Pract, 212: 767-777, (2016)
25. Biswas R., Bandyopadhyaya D., Mishraa M., Pramanika K.C. , Chatterjee T.K.,Isolation of pure compound R/J/3 from Pluchea indica (L.) Less. and its anti-amoebic activities against Entamoeba histolytica. Phytomedicine, 14: 534-537 (2007)
26. Chavanpatil M.D., Khdair A., Patil Y., Handa H., Mao G., Panyam J., Polymer-surfactant

nanoparticles for sustained release of water-soluble drugs. Journal of pharmaceutical sciences, 96(12): 3379–3389, (2007)
27. Senthil P.K., Arivuchelvin A., Jagadeeswaran A., Subramanian N., Senthil C.K., Mekala P., Formulation, optimization and evaluation of enrofloxacin solid lipid nanoparticles for sustained oral delivery. Asian J Pharm Clin Res, 8(1): 231 - 236, (2015)
28. Wang S., Chen T., Chen R., Hu Y., Chen M., Wang Y., Emodin loaded solid lipid nanoparticles: Preparation, characterization and antitumor activity studies. Int J Pharm, 460: 238-246, (2012)
29. de Costa P.M., de Costa M.P., Jamacaru V.F., Ferreira P.M., Inprovement of in vivo anticancer and antiangiogenic potential of thalidomide derivatives. Chem Biol Interact, 239: 174-183, (2015)
30. Debnath S., Karan S., Debnath M., Chatterjee T.K., Poly-L-Lysine inhibits tumor angiogenesis and induces apoptosis in ehrlich ascites carcinoma and sarcoma S-180 tumor. Asian Pac J Cancer Prev, 18(8): 2255 - 2268, (2017)
31. de Sousa A.P., Torres M.R., Pessoa C., Costa Lotufo L.V., In vivo growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed Sargassum vulgare. Carbohydr Polym, 69: 7-13, (2007)
32. Ahmed O.M., Ahmed R.R., Anti-proliferative and apoptotic effifi cacy of diallyl disulfifi de on Ehrlich ascites carcinoma. Hepatoma Research, 1(2): 67-74, (2015)