Indian Squid Ink Nano-Suspension: Formulation, Characterization, *In vitro* Anti-tumor Activity against Human Breast Cancer and Human Kidney Epithelial Cell Lines

Jismi Jose[1], Dineshkumar B.[1*], Krishnakumar K.[1], Rajeshkumar R.[2] and Tamilselvan N.[3]
1. Department of Pharmaceutics, St James College of Pharmaceutical Sciences, St. James Hospital Trust Pharmaceutical Research Centre, (DSIR Recognized) Chalakudy, Kerala, INDIA
2. Department of Pharmaceutical Biotechnology, JSS College of Pharmacy, Ooty, Tamilnadu, INDIA
3. Department of Pharmaceutics, KMCH College of Pharmacy, Coimbatore -641048, Tamilnadu, INDIA
*dinsbiotech@gmail.com

Abstract

Cancer is one of the leading causes of adult deaths worldwide. In India, the International Agency for Research on Cancer (IARC) estimated indirectly the death of people from cancer. Almost 60% of drugs approved for cancer treatment are of natural origin. In this study we have developed different formulations of Indian squid ink extract nanosuspension from squid fish (namely F1, F2 and F3) and characterized for its various physiochemical evaluations and investigated its anti-tumour effect in human breast cancer cell lines (MCF- 7) and kidney epithelial (Vero) cell lines.

The result revealed that the formulation F3 showed the better nano-scale particle size in the range of 87-125nm, better zeta potential of -21.2mV and in vitro drug release of 98.18±0.2 for 6 hours. The in vitro antitumor study indicated that the F3 formulation of Indian squid ink nanosuspension showed potential anti-tumour activity in a dose dependent manner against human breast cancer (MCF-7) cell line and human kidney epithelial (Vero) cell line compared with standard Indian squid ink extract. This study concluded that high pressure homogenization method could be suitable to formulate Indian squid ink nanosuspension (ISI-NS) with anti-tumour potential against MCF-7 and vero cell lines.

Keywords: Marine products, nanosuspensions, anticancer activity, cell lines.

Introduction

Non-communicable diseases (NCDs) are now responsible for the majority of global deaths and cancer is expected to rank as the leading cause of death and it is the single most important barrier to increasing life expectancy in every country of the world in the 21st century.

The drugs approved for cancer treatment are being obtained from natural origin. Moreover, research studies showed that the currently available drugs for cancer treatments are not specific for the cancer cells, resulting in toxicity to normal cells. Hence, natural formulations has been used widely for the treatment of cancer due to their easy availability, low cost and lesser side effects. The demand for marine natural formulations is increasing day by day in the world market[1]. Natural products continue to play a major role in anticancer medicine, and marine metabolites have emerged as important antitumor drugs. Most of the top selling pharmaceutical agents are obtained from natural products.

So, it is anticipated that the aquatic environment will become an invaluable source of novel compounds in the future. Attempts are nowadays being made to utilize these waste materials to produce bioactive substances such as antibacterial, anticancer and antiviral agents.[2]

One such product is Indian squid ink obtained from Indian squid fish present in mediterranean and atlantic ocean. It has a very special place in homeopathic medicine and in traditional Chinese medicine along with its therapeutical uses. The contents of Indian squid fish are large quantities of proteins, lipids and minerals. The squid ink has spectacular activities in many ailments like uterine dysfunction, anticancer, antimicrobial, anti-inflammatory activity along with hypertensive effects.[3]

Nanosuspensions are formulations that are widely used as drug delivery systems for better therapeutic efficacy. Among different methods, high pressure homogenization technique is the simplest and most common technique with less contamination.[4]

Due to the cell injury cause in chemotherapy, the search for cell protective effective drugs from marine sources has become an important and necessary activity.[5,6] This inspired to develop a nano suspension formulation of Indian squid ink extract and investigation of its *in vitro* anti-tumor activity against different tumor cell lines such as human breast cancer (MCF- 7) cell line and kidney epithelial (Vero) cell line.

Material and Methods

The Indian squid (*Loligo duvauceli*) which are commercially used for processing and export were collected and identified from the fishing harbor, Kochi, Kerala and all other chemicals used were of analytical grade purchased from Himedia.

Collection and Extraction of Indian Squid Ink (*Loligo Duvauceli)*

Collection of Indian squid ink: The Indian squids (*Loligo duvauceli*) commercially used for processing and export were collected and identified from the fishing harbor, Kochi, Kerala and brought to the laboratory in ice cold condition. It was authenticated by Mr. K.K Joshy, Principal Scientist and Head of marine scientific division, Cochin as urotheuthis (*photololigo)* duvauceli (old name: *loligo duvauceli).* The squid is carefully dissected to remove the ink sac. The ink was used for extraction.

Extraction of Indian squid ink: Known weight of the ink was delipidated with 4 volumes of acetone at -20ºC for 96 hr (4 days). The acetone was changed every 24 h after vacuum filtration. The delipidated residue was vacuum dried to give a black powder. This powder was extracted with 40 volumes of 0.1 M Tris-HCl buffer (pH 6.8) at 4ºC for 48 h followed by centrifugation at 13,000 x g at 40C for 30 min. The supernatant was dialyzed against distilled water at 4ºC for 48 h and then lyophilized to obtain an off-white powder.[7]

Pre-formulation Studies

Fourier Transform Infrared Spectroscopy (FTIR): FTIR spectra are obtained by placing the samples on discs containing a mixture of 0.2 mg lyophilized squid ink extract and about 10 mg potassium bromide (KBr) ground under drying conditions. The spectra are recorded using infrared spectrophotometer (e.g. Nicolet 200SXV) from 4000 to 500 cm^{-1} at a data acquisition rate of 2 cm^{-1} per point. The resulting spectra were analyzed using the software[8].

Formulation of Indian Squid Ink Nanosuspension (ISI-NS) by High Pressure Homogenization Technique: The sophisticated high pressure homogenization technique was applied for the formulation of nanosuspension. Different formulations of Indian squid ink extract nanosuspension (ISI-NS) namely F1, F2 and F3 were prepared by changing the concentration of polyvinyl alcohol as dispersion medium (Table 1). Briefly, 100mg of lyophilized powder of Squid ink extract was dispersed in 1.0%w/v PVA (Polyvinyl alcohol) solution which was prepared with continuous magnetic stirring at 1000rpm for 15 min in 100ml of water to form a pre-suspension.

This pre-suspension was homogenized in high pressure homogenizer at low pressure for pre-milling. This forms a nanosuspension of large particle size. It was then homogenized at high pressure homogenizer for 10-25 cycles at high pressure of 1500 bar until the squid ink nanosuspension of desired particle size (nano-scale) was obtained[9]. The resulting F1 of ISI-NS was formulated and stored in refrigerator. The same procedure was used to formulate F2 and F3 of ISI-NS by changing the concentration of polyvinyl alcohol as dispersion medium using high pressure homogenization technique and stored in refrigerator.

Characterisation of Indian squid ink nanosuspension

Physical Appearance of ISI-NS: ISI-NS Formulations F1, F2, F3 were subjected to physical parameters like colour, homogeneity and consistency measured by visual examination.

Viscosity of ISI-NS: Viscosity of the prepared formulation (F1 formulation of ISI-NS) was measured at room temperature using Ostwald viscometer at laboratory scale. Sample (10ml) was immersed in the sample holder before testing. The S62 spindle was used at 10 rpm and the viscosity was measured. The same procedure was followed in order to determine the viscosities of F2, F3 formulations of ISI-NS.

pH of ISI-NS: Prepared formulation (F1 formulation of ISI-NS) was taken in 10ml beaker and pH was measured using pH meter (Digital pH meter). The pH meter was calibrated before each use with standard pH 4 and pH 7 buffer solutions. 20ml of formulation was taken in suitable beaker and pH was measured.[10] The same procedure was followed in order to determine the pH of F2, F3 formulations of ISI-NS.

Scanning Electron Microscopy (SEM) Analysis of ISI-NS: SEM analysis was used to examine the microstructure of prepared F1, F2, F3 formulations of ISI-NS. The particle surface morphology can be determined by SEM analysis. The prepared F1, F2, F3 formulations of ISI-NS were placed on a carbon film coated copper grid for SEM. Studies were performed using JEOL model JSM-6390 LV Japan equipped with selected area electron diffraction pattern(SAED). The copper grid was fixed in to the sample holder and placed in a vacuum chamber of the Scanning microscopy and observed under low vacuum and SEM images of F1, F2, F3 formulations of ISI-NS were recorded [11].

Zeta Potential analysis of ISI-NS using photon correlation spectroscopy: The zeta potentials of prepared F1, F2, F3 formulations of ISI-NS were analyzed using Malvern zetasizer (Nano ZS90, Malvern instruments) at 25ºC.

Table 1
Formulation of Indian squid ink Nanosuspension (ISI-NS)

Formulation ingredients	F1	F2	F3
Squid ink powder	100mg	100mg	100mg
Polyvinyl alcohol (w/v)	1.0%	1.5%	2.0%
Millipore water	100ml	100ml	100ml

Zeta potential gives the particle charge of the formulated nanosuspension which determines the physical stability of nanosuspension. It is governed by both the stabilizer and the drug itself. An electrostatically stabilized nanosuspension shows a minimum zeta potential of -3 to -30mV.[12]

***In- vitro* release studies of ISI-NS:** *In vitro* drug diffusion studies were carried out using dialysis membrane. Prepared F1 formulation of ISI-NS was placed in the dialysis membrane (10kDa Molecular weight) and tied at both end. 200ml of 0.1M of phosphate buffer pH 7.4 was placed in receptor compartment and it should be under continuous stirring using magnetic stirrer.

Sample was withdrawn at several time intervals such as 1, 2, 3, 4, 5 and 6 hrs respectively and same volume was replaced with fresh phosphate buffer. Then the amount of drug released was estimated using UV/VIS spectrophotometer at 200nm.[13] The same procedure was followed in order to determine *in vitro* release studies of F2, F3 formulations of ISI-NS.

Stability studies of ISI-NS: Stability is an important factor for nanosuspension. Prepared F1, F2, F3 formulations of ISI-NS were subjected to stability study at room temperature (27^0C) and at cold temperature (4^0C) using stability chamber for 3 months under ambient conditions. A portion of all formulations were collected and finally evaluated for its physical appearance and particle size by SEM analysis[14].

***In vitro* anti-tumor study of ISI-NS against different tumors cell lines:** Prepared F1, F2, F3 formulations of ISI-NS were subjected to *in-vitro* antitumor activity against different cancer cell lines such as kidney cancer cell line (MCF-7) and breast cancer cell line (Vero). The cell viability was measured using MTT assay.

MTT assay of Indian squid ink (ISI) on human breast cancer (MCF 7) cell line and human kidney epithelial (Vero) cell line: Cyto-toxicity assay of ISI was studied on MCF-7 cell lines. MCF-7 cells were plated on to 48 wells plates 18 hrs before commencement of the test. Growth medium used was DMEM with 10 % foetal bovine serum. The plates were incubated in an animal cell culture incubator, maintained at 37ºC with 5% carbon dioxide. The wells achieved 70% confluency at the time of testing. The original growth medium in the 48 well plates was removed.

Different concentrations such as 10, 25, 50 and 100 µg/ml of Indian squid ink (ISI) were added to the appropriate wells. The plates were incubated for 96 hrs. At the end of 96 hrs the medium in the wells was carefully removed and fresh complete growth medium was added. To each well, MTT solution (5mg/ml of MTT dissolved in PBS, was added and replaced in the incubator for 3 hrs. After 3 hrs, the medium was carefully removed from the wells, and DMSO was added to each well and kept on a rocking platform for efficient mixing and extraction of formazen dye from the cells by DMSO. The optical density of the extracted dye was measured at 570 nm using a plate reader. The average optical density of the dye in the control wells was taken as 100% viability and the viability of the best wells was calculated. The same procedure was followed to evaluate cyto--toxicity assay of Indian squid ink (ISI) against human kidney epithelial (vero) cell line using MTT assay:

Percentage of viability of the test = (average optical density of the test wells/ average optical density of the control wells) ×100

MTT Assay of ISI-NS on Human breast cancer (MCF- 7) Cell Line: Based on the results of *in vitro* release study of ISI-NS, F3 formulation of ISI-NS was selected because of its better drug release due to better particle size in nano-scale for *in vitro* cyto-toxicity assay against MCF-7 cell line. MCF-7 cells were plated on to 48 wells plates 18 hrs before commencement of the test. Growth medium used was DMEM with 10 % foetal bovine serum. The plates were incubated in an animal cell culture incubator maintained at 37ºC with 5% carbon dioxide. The wells achieved 70% confluency at the time of testing. The original growth medium in the 48 well plates was removed. Different concentrations such as 10, 25, 50 and 100 µg/ml of F3 formulation of ISI-NS were added to the appropriate wells.

The plates were incubated for 96 hrs. At the end of 96 hrs, the medium in the wells was carefully removed and fresh complete growth media was added. To each well MTT solution (5mg/ml of MTT dissolved in PBS, was added and replaced in the incubator for 3 hrs. After 3 hrs the medium was carefully removed from the wells and DMSO was added to each well and kept on a rocking platform for efficient mixing and extraction of formazen dye from the cells by DMSO. The optical density of the extracted dye was measured at 570nm using a plate reader. The average optical density if the dye in the control wells was taken as 100% viability and the viability of the best wells was calculated:

Percentage of viability of the test = (average optical density of the test wells/ average optical density of the control wells) ×100

MTT Assay of ISI-NS on Human Kidney Epithelial (Vero) cell line: Based on the results of *in vitro* release study of ISI-NS, F3 formulation of ISI-NS was selected because of its better drug release due to better particle size in nano-scale for *in vitro* cyto-toxicity assay against Vero cell line. Vero cells were plated on to 48 wells plates 18 hrs before commencement of the test. Growth medium used was DMEM with 10 % foetal bovine serum.

The plates were incubated in an animal cell culture incubator maintained at 37ºC with 5% carbon dioxide. The wells achieved 70% confluency at the time of testing. The original growth medium in the 48 well plates was removed. Different concentrations such as 10, 25, 50 and 100 µg/ml of

F3 formulation of ISI-NS were added to the appropriate wells.

The plates were incubated for 96 hrs. At the end of 96 hrs the medium in the wells was carefully removed and fresh complete growth media was added. To each well MTT solution (5mg/ml of MTT dissolved in PBS was added and replaced in the incubator for 3 hrs. After 3 hrs, the medium was carefully removed from the wells, and DMSO was added to each well and kept on a rocking platform for efficient mixing and extraction of formazen dye from the cells by DMSO. The optical density of the extracted dye was measured at 570nm using a plate reader. The average optical density of the dye in the control wells was taken as 100% viability and the viability of the best wells was calculated:

Percentage of viability of the test = (average optical density of the test wells/ average optical density of the control wells) ×100

Statistical analysis: All data were expressed in mean ± standard deviation. The student t-test statistical analysis was used in the study. $P < 0.05$ was considered as significance.

Results and Discussion

The Indian squid ink was extracted from Indian squid *(Loligo Duvauceli)* and percentage yield was found to be about 30% w/w. This lyophilized powder is further used for pre- formulation studies. The FTIR spectra of squid ink extracts, PVA and polymer were studied and interpreted. The results of interpretation have been discussed in table 2.

The FTIR analysis peaks give information about the components (amino acids, peptides and proteins) present in the squid ink extract. FTIR analysis showed that there was no drug incompatibility between poly vinyl alcohol and squid ink extract. Hence the PVA and squid ink extract could be used for the formulation of squid ink nanosuspension.

Nanosuspension of squid ink extract was prepared by using high pressure homogenizer. The high pressure homogenization is a simple, more facile, less complex method with less chance of contamination and suitable for large scale production. Prepared F1, F2, F3 formulations of ISI-NS (Figure 1) were brown turbid solution preparation with a smooth homogeneous texture. The physical properties of the prepared formulations are discussed in table 3.

Table 2
Interpretation of FTIR spectrum

S.N.	Functional groups	Name of the group	Frequency	Absorption peak of PVA	Absorption of peak of squid ink extract	Absorption peak of squid ink extract and PVA
1	OH stretch	Alcohol	3400-3700	3400	3400	3400
2	N-H Stretch	1º 2ºAmine, amides	3300-3350	3340	3379.29	3379.29
3	=C-H Stretch	Alkenes	1640-1680	-	1640.53	1650
4	=C=C-H Stretch	Alkynes	3300	-	3300	3300
5	C-=N Stretch	Nirile	2200-2250	-	2203.7	2205
6	C-H stretch	Alkyl	2840-3000	2840-3000	-	-
7	C=O	Ketone]-aectate	1720-1740	1730	-	-
8	C-O	Acetate group	1680	1680	-	-

Figure 1: Prepared F1, F2, F3 formulations of ISI-NS

Table 3
Physical appearance of Indian squid ink nanosuspension

Formulations	Colour	Consistency	Homogeneity
F1	Brown	Good	Good
F2	Brown	Excellent	Excellent
F3	Brown	Excellent	Excellent

Viscosity of prepared formulations (F1, F2, F3) was determined using Brookfield viscometer. Viscosity of the formulation directly indicates its consistency. The viscosity of F1, F2 and F3 was found to be 394±3.2, 336±2.8 and 457±2.1 respectively. The viscosity study indicated that F3-ISI-NS was found to be optimum viscous than F1 and F2 of ISI-NS. The pH of the three formulations F1, F2 and F3 was determined using digital pH meter. The pH of F1, F2 and F3 was found to be 6.75±0.2, 6.79±0.1 and 6.8±0.1 respectively. The PH of three formulations was found to be within the range between 6.75- 6.8.

The results of SEM analysis indicated that particle sizes of the prepared formulations (F1, F2, F3) were found to be 150-230nm, 175-210nm and 87-125nm respectively (Figure 2). Further, F3 formulation of ISI-NS was found to have better particle size at nano-scale (Table 4).

The zeta potentials of formulated nanosuspension of FI, F2 and F3 of ISI-NS were found to be -16.6 mv, - 17.7mv and -21.2 mv respectively. Zeta potential study indicated that F3 formulation was found to have better stability than F1 and F2 formulations. Then cumulative percentage drug releases of prepared F1, F2, F3 formulations of ISI-NS were calculated and the results are shown in figure 3. The cumulative percentage drug releases of prepared F1, F2, F3 formulations of ISI-NS were found to be 88.66±0.3%, 96.76±1%, 98.18±0.2% respectively. These results indicated that F3 formulation of ISI-NS was found to have better drug release due to better particle size in nano-scale with more surface area.

Stability study results indicated that particle sizes of the formulations F1, F2, and F3 were found to be within the range of 124 to 230nm. No significant color change is observed. A slight change in particle size is observed in all the formulations at room temperature. Therefore, stability study indicated that the F1, F2, F3 formulations of ISI-NS were stable in cold temperature only (Table 5).

Table 4
SEM analysis of Indian squid ink nanosuspension

Formulations	Particle size (nm)
F1	150-230
F2	175-210
F3	87-125

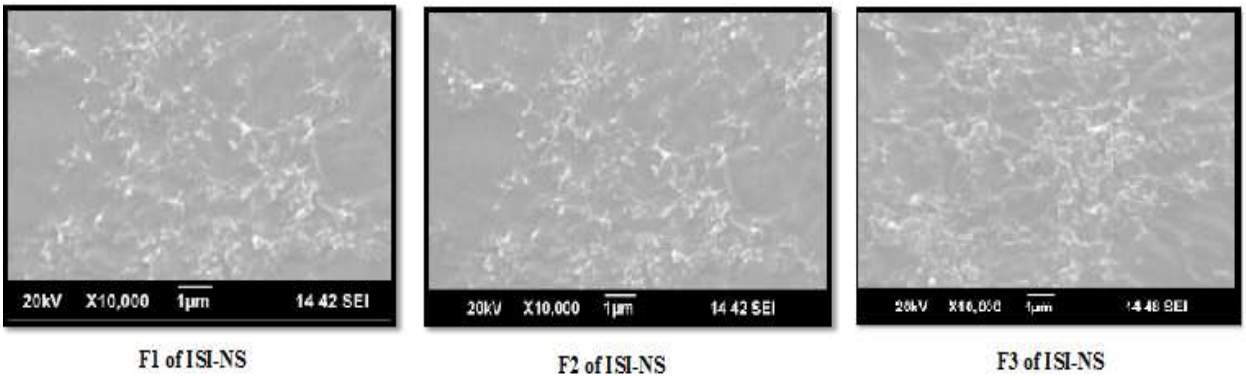

Figure 2: SEM Analysis of Prepared F1, F2, F3 formulations of ISI-NS

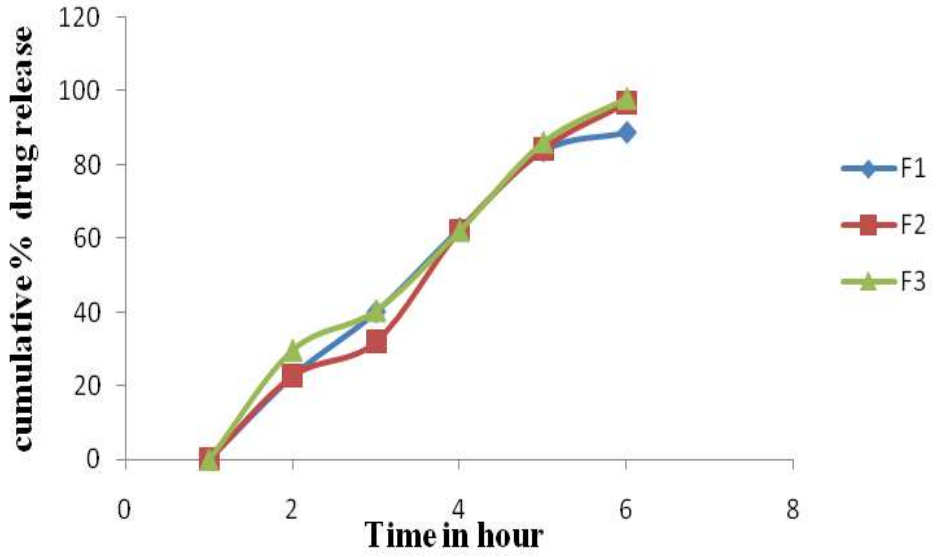

Figure 3: Cumulative percentage drug release of F1, F2, F3 formulations of ISI-NS

Table 5
Stability studies of Prepared F1, F2, F3 formulations of ISI-NS

S.N.	Formulation	Appearance	Particle size range	
			RT 27^{0}C	CT 4^{0}C
1	F1	Brown	230.25nm	226.12nm
2	F2	Brown	220.32nm	210.92nm
3	F3	Brown	126.32nm	120.25nm

* RT Room Temperature; *CT: Cold Temperature

In vitro antitumor activity of Indian squid ink extract results indicated that the Indian squid ink extract at different concentration (10, 25, 50 and 100 µg/ml) showed dose dependent cytotoxic activity against human breast cancer (MCF-7) cell line as well as Human Kidney Epithelial (Vero) cell line. The IC_{50} value of Indian squid ink extract was found to be 43.29 ± 0.50 (Table 6) for MCF-7 cell lines and 44.65 ± 0.53 µg/ml (Table 7) for Vero cell line respectively.

Table 6
% cell viability of Indian squid ink (ISI) extract on MCF-7 cell line

Concentration (µg/ml)	Percentage of cell Viability
100	29.03 ± 0.04
50	27.33 ± 0.46
25	62.12 ± 0.83
10	75.40 ± 0.43
IC_{50}	**43.29 ± 0.50 µg/ml**

Table 7
% viability of Indian squid ink (ISI) extract on Vero cell line

Concentration (µg/ml)	Percentage of cell Viability
100	21.10 ± 0.78
50	33.05 ± 0.74
25	56.35 ± 0.46
10	82.28 ± 0.90
IC_{50}	**44.65 ± 0.53 µg/ml**

In vitro antitumor activity of ISI-NS results indicated that F3 formulation of ISI-NS at different concentration (10, 25, 50 and 100 µg/ml) showed dose dependent cytotoxic activity against human breast cancer (MCF 7) cell line as well as human kidney epithelial (Vero) cell line. The IC_{50} value of ISI-NS was found to be 38.55 ± 0.41 µg/ml for MCF-7 cell line (Table 8) and 36.25 ± 0.75 µg/ml Vero cell line (Table 9) respectively.

Conclusion

In this present work we have extracted the Indian squid ink from the squid fish and different formulations (F1, F2, F3) ISI-NS were formulated by changing the concentration of polyvinyl alcohol as dispersion medium. The study indicated that F3 formulation of ISI-NS showed better particle size at nano-scale with highest zeta potential and considerable drug release due to better particle size at nano-size level with more surface area.

This study concluded that polyvinyl alcohol (2%) as dispersion medium and high pressure homogenization method could be suitable to formulate Indian squid ink nanosuspension (ISI-NS) with antitumor potential against human breast cancer (MCF- 7) cell line and human kidney epithelial (Vero) cell line compared with Indian squid ink extract (standard drug).

Table 8
Percentage viability of F3 formulation of ISI-NS on MCF- 7 cell line

Concentration (µg/ml)	Percentage of cell Viability
100	32.01 ± 0.81
50	39.22 ± 0.87
25	66.02 ± 0.81
10	78.08 ± 0.82
IC_{50}	**38.55 ± 0.41 µg/ml**

Table 9
Percentage of viability of F3 formulation of ISI-NS on Vero cell line

Concentration (µg/ml)	Percentage of cell Viability
100	24.95 ± 0.81
50	45.18 ± 0.85
25	72.35 ± 0.46
10	85.22 ± 0.87
IC_{50}	**36.25 ± 0.75 µg/ml**

Acknowledgement

The authors are thankful to management of St James College of Pharmaceutical Sciences for their valuable support for this research work. Authors are also thankful to Dept. of Pharmaceutical Biotechnology, JSS College of Pharmacy, Ooty, TN, for their support *in vitro* cancer cell line study.

References

1. Alvarez Roman R., Naik A., Kalia Y.N., Guy R.H. and Fessi H., Skin penetration and distribution of polymeric nanoparticles, *Journal of Controlled Release*, **99**, 53-62 **(2004)**

2. Sharamol A.R., Nair Smitha K., John Anish, Krishnakumar K. and Dineshkumar B., Nanosuspension of a drug and its biological activity: A review, *International Journal of Pharmacy Review and Research*, **6(2)**, 57-60 **(2016)**

3. Isabel Santacruz, Ketharm Anapoorni and John Benner, Preparation of high solid content nanozirconia suspension, *Journal of American Ceraminsociety*, **91(2)**, 398-405 **(2008)**

4. Meetali Mudgik and Pawar Pravin K., Preparation and invitro/ex vivo evaluation of moxifloxacin- loaded PLGA nanosuspension for ophthalmic application, *Sci Phrm*., **81**, 591-606 **(2013)**

5. Mohammad Ramjan Chisti, Raza Bahra Aasim, Singh Preet Amol and Niazi Junaid, Sepia ink: an untouched molecule from deep oceans, *International Journal of Research and Reviews in Pharmacy and Applied Sciences*, **6(1)**, 1303-1307 **(2016)**

6. Mossmann T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay, *J. Immunol. Methods*, **65(1-2)**, 55-61 **(1983)**

7. Nagavarma B.V.N., Yadav Hemant K.S., Ayaz A., Vasudha L.S. and Kumar H.G., Different techniques for preparation of polymeric nanoparticles- A review, *Asian Journal of Pharmaceutical and Clinical Research*, **5(3)**, 16-23 **(2012)**

8. Patravale V.B., Date A.A. and Kulkarni R.M., Nanosuspension: a promising drug delivery strategy, *Journal of Pharmacy and Pharmacology*, **56**, 827-40 **(2004)**

9. Dyshlovoy Sergey A. and Honecker Friedemann, Marine Compounds and Cancer: Where Do We Stand?, *Journal of Marine Drugs*, **13**, 5657-5665 **(2015)**

10. Tischleroval V. and Valencakova A., Use of nanotechnology in the diagnosis, prevention and therapy of cancer, *International Journal of Pharmaceutical Sciences and Drug Research*, **7(1)**, 1-7 **(2015)**

11. Senan V.P., Sherief P.M. and Nair J.R., Cytotoxic effect of ink extracts of Cuttlefish and Squid on Chick Embryo Fibroblasts, *Int J Pharma Sci Res*, **4(5)**, 1893-1896 **(2013)**

12. Sunil Rohilla, Aghamkar P., Shyam Sundar, Athul Kumar and Bhajanleal, Synthesis and characterization of Fe_4 $[Co\ (CN)_6]_3.16H_2O/SiO_2$ nanocomosites by coprecipitation method, *Advanced Material Letters*, **4(1)**, 53-57 **(2013)**

13. Saudagar R.B. and Deore Pradnya S., Formulation and Characterization of Fluorometholone Nanosuspension for Ophthalmic Drug Delivery by Precipitation Method, *Scholars Academic Journal of Pharmacy*, **5(7)**, 268-276 **(2016)**

14. Vanitha Pudata, Subramanyan V. and Jhansi K., A short note on cancer, *Journal of Carcinogenesis and Mutagenesis*, **2**, 128 **(2011)**.

(Received 17th January 2020, accepted 27th March 2020)