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Research Article

Formulation, Characterization and Evaluation of Herbal Extracts Loaded Microspheres against Ovarian Cancer

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2. Key words

Ovarian cancer; Microspheres; Ellagic acid; Phytoconstituents; Drug loading

1. Abstract

1.1. Background: Ovarian cancer is the most life threatening disorder prevailing at a higher rate among the females in India. The drug regimen available is not sufficient enough to combat the disease. Traditionally, herbal drugs have shown promising effects in treating severe complications of this disease.

1.2. Objective: Therefore, the objective of the present study was to formulate and characterize microspheres of selected herbal extracts and investigate their potential against ovarian cancer.

1.3. Method: To achieve this ethanolic extract of *Embilica officinalis* and *Glycine max Merrill* were formulated in the form of microspheres using solvent evaporation method and conjugated with the folate as ligand for easy cellular uptake by ovarian cells. The prepared microspheres were characterize for particle size, zeta potential, surface analysis (SEM), FTIR,XRD and drug loading , in-vitro drug release and stability studies. The prepared microspheres were evaluated for MTT assay using against (SKVO3) Human ovarian cancer cells.

1.4. **Result:** The result showed that *E.Officinalis* extract loaded microspheres exhibited particle size of average 191.1±2.34µm and good stability, *Glycine max merill* extract loaded microsphere exhibited particle size at range of 467.2±1.28µm, whereas folate conjugated *E.Officinalis* extract loaded microspheres exhibited particle size of 195.09±1.45µm , FA-*Glycine max*extract loaded microsphere exhibited particle size 468.3µm±0.72µm. Comparative study showed that *E.Officinalis*

3. Introduction

Ovarian cancer is the fourth most commonly diagnosed and may be cause deadly gynaecologic malignancies in worldwide [1,2]. The main causes of ovarian cancer are not well understood. Tumour of ovarian benign nature may be develop at any point of life but mostly occur during child bearing age and it's constitute about 90% of ovarian cancers [3]. Histologically, ovarian cancer can be divided into three broad groups: Epithelial tumor, Sex-cord stromal tumor and Germ cell [4]. Ovarian cancer is difficult to diagnose in early stage due to the lack of original initial symptoms, therefore cancer in patients are usually diagnosed at advanced stage [5-7]. Mostly ovarian cancer can be treated by surgically, chemotherapy and radiation therapy. Surgical is main treatment for ovarian cancer, through surgery tumor cell are operated from the ovaries, and because of that it has been increasing the cell survival. Chemotherapy is a therapy in which anti-cancer drugs are used to kill the cancer or shrink tumor in ovarian cancer. Due to the increased adverse effect caused by the chemotherapy in treatment of cancer with the common drugs like alkylating agents, antibiotic, steroid analogue, herbs and drug obtained from these such as vincristine, vinblastine, taxols, etoposide shows lesser toxicity and better effectiveness in ,numbers of oncological conditions [6-8]. Such phytoconstituents which are having antioxidant properties and strong anticancer activity against ovarian cancer can be used as an alternative to conventional drug regimen [9-10]. These phytoconstituents plays an important role in the development of several clinically useful drugs. The present research aims in developing folate conjugated microspheres of phytoactives for ovarian cancer & to study their stability profile [11-13].

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4. Material and Method

4.1. Material

Dried *Embilica officinalis* fruit and *Glycine max Merrill* seeds was purchased from authentic supplier of herbal products of Raipur District. The plants were authenticated by Department of Botany, Govt. K.L. Arts, and Science & Commerce Collage of Mahasamund. 2-2-Diphenyl-1-Picrylhydrazyl (DPPH) was purchased from allied scientific laboratories, Nagpur and petroleum ether 60-80ºC, Ethanol was purchased from supplier. L-ascorbic acid was from institution laboratory. Ellagic acid was obtained as gift sample from our institute (Shri Rawatpura Sarkar institute of Pharmacy,Kumhari) and other materials was given from our institution.

4.1.1. Cell Culture: All the SKOV3 human ovarian cancer cells were obtained from the National Centre for Cell Science, Pune, India.3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased fromSigma Aldrich Ltd.(India) and cultured accordingly.

4.2. Method

4.2.1. Preparation of extract: All dried materials of *Embilica officinalis*fruit and *Glycine max Merrill* seedswere cleaned and crushed for powder. Exactly 60gm of each herb was weighed and were deffating with 200 ml of petroleum ether 60-80ºC and dried in air. After that herb were extracted with ethanolic solvent for 24hr by a continual hot extraction method, until complete exhaustion of the drug using a soxhlet apparatus [14].

4.2.2. Preparation of Blank Microspheres: Pure Ellagic acid microspheres were taken as standard and were prepared based on o/w emulsion solvent evaporation technique by using ethyl cellulose as a polymer. Different formulations were carrying varied concentration of the polymer and the drug in ethyl acetate and acetone as specified in (Table 2). This solution was poured drop wise means one ml in one minute in the 250 ml of distilled water (aqueous phase) containing tween 80 as the emulsifying agent with continuous stirring on mechanical stirrer. The resultant mixture was emulsified at speed constant for 4 h. The dispersed drug and polymer solution was immediately transformed into fine droplets, which return into solid form rigid microspheres due to the solvent evaporation. The particles were collected by filtration, washed to remove excess oil by distilled water and dried in hot air oven at 60˚C and characterized.[15] *E. officinalis*extract loaded microspheres [EMS] ,*Glycine max Merrill* (Soyabean) extract loaded microspheres [SMS], Ellagic acid loaded microsphere [EAMS] were prepared by same procedure.

4.2.3. Preparation of Folate Conjugated Microspheres: The method was similar to that of adopatated by Gupta at.al, 2015 with slight modifications. The conjugation of folate (FA) with the*E. officinalis* extract loaded MS, Ellagic acid loaded MS, and *Glycine max Merrill* extract loaded MS was done by activating the carboxylic end of the *E. officinalis* extract loaded MS, Ellagic acid loaded MS, and *Glycine max Merrill* extract loaded MS by EDC–NHS conjugation chemistry. *E. officinalis* extract loaded MS, Ellagic acid loaded MS, and *Glycine max Merrill* extract loaded MS (1 mg in ml) were suspended in PBS buffer (pH 7.4) and incubated in the dark with EDC for 30 min, at room temperature. Thereafter the sample was mixed with NHS for 6 h, under similar conditions. The sample was then washed several times with Millipore water and filtered. To the filtrate, FA was added at a concentration of 100 mg/mLin PBS, at a pH of 7.4, under the condition of overnight stirring. The filtrate was again centrifuged for 10 min at 11,000 rpm, twice. Excess linking reagent and soluble by products were removed by washing thrice with 1 ml of PBS, at a pH of 7.4. Finally, the Folate conjugated MS of ellagic acid. *E. officinalis* extract and *Glycine max Merrill* extract was lyophilized further to get the dry sample [16].

4.2.4. DPPH Scavenging Activity: In order to estimate the antioxidant potential of the extracts the DPPH activity was carried out.0.004% DPPH solution was prepared by using 20 mg DPPH with 500ml of ethanol [17-19]. A stock solution was prepared by using 10mg ethanolic extract within 10 ml of ethanol and different dilution 0.25µg/ml, 0.50µg/ml, 0.75µ g/ml, 1.00µg/ml, 1.25µg/ml of working solution were done and made up to 10 ml with help of 0.004% of DPPH solution in volumetric flask. The solution was kept aside for 30 minutes, in an incubator for 20ºC .and then the absorbance at 517 nm in a UV Shimadzu spectrophotometer was taken. The %DPPH Scavenging activity was calculated and compared with Ascorbic acid as standard. Where $A_{CONTROl}$ is the absorbance of the control reaction and A_{TEST} is the absorbance in the presence of the sample of the extracts [20,21].

$$\% \text{ DPPH Scavenged} = \frac{A_{CONTROL} - A_{TEST}}{A_{CONTROL}} \times 100$$

4.2.5. Physico-chemical characterization of prepared Microspheres

4.2.5.1. Particle Size Analysis and Zeta Potential: Particles having size range between 50 and 1000µm were estimated by using Transmission Electron microscope [22]. Mean particle size of microspheres was calculated by using the following formula [23,24]. The zeta potential is a measure of the stability of colloidal suspensions, was obtained by measuring the mobility distribution of a dispersion of charged particles as they were subjected to an electric field. The zeta potential was also measured at the same dilution and temperature, to examine the surface charge as well as stability of the MSs [16].

Mean particle size = $\sum$ (mean particle size of the fraction X weight fraction) / $\sum$ (weight fraction)

4.2.5.2 Surface Morphology: The samples for the scanning electron microscope (SEM) analysis were prepared by sprinkling the microspheres on one side of an adhesive stub. Then the microspheres were coated with diameter 2.5um thin layer of gold-platinum by using sputter coater before microscopy. Finally the morphology and size of the microspheres were observed with the scanning electron microscope (Zeiss, EVO 18 Special Edition) [23].

4.2.5.3 Fourier Transform Infrared Spectroscopy (FTIR) Studies: The infrared (IR) spectra was recorded using an FTIR spectrophotometer (Perkin Elmer Spectrum GX) by the KBr pellet method in the wavelength region between 400 and 4000 cm-1. The spectra obtained for Ellagicacid, Amla extract and Soya bean extract of physical mixtures with polymers were compared to check compatibility of drug with polymers and to confirm the conjugation [24- 29].

4.2.5.4 XRD: The crystalline state of different samples wasevaluated with X-ray powder diffraction. Thediffraction patterns were obtained at roomtemperature using a Philips Analytical X-ray BV1710 with cobalt as anode material and graphitemonochromatic, operated at a voltage of 30 kV. The samples were analyzed in the 2θ angle range of 10^{o} 500 and process parameters were set as scan rate of 0.020 sec[23,24].

4.2.5.5 Drug Loading: The amount of ellagicacid, *E. officinalis* extract and *Glycine max Merrill* extract present in the ethylcellulose microspheres was determined by taking the known amount of microspheres in which 1 gm of drug should be present theoretically [27,28]. Then the microspheres ware crushed and the powdered microspheres was taken and dissolved in 10 ml of phosphate buffer (pH-7.4) solution and stirred for 15 minutes with an interval of 5 minutes and allowed to keep for 24 hours. The solution was filtered through filter paper. 0.1ml of this solution was diluted up to 10ml with phosphate buffer (pH-7.4) solution and the absorbance was measured spectrophotometrically at 267 nm against phosphate buffer (pH-7.4) solution as blank with the help of UV Shimadzu 1800 spectrophotometer [24,29-30].

4.2.5.6 Percentage of Folate Content Attached: The amount of folate attached on the surface of MSs prepared was determined by the dialysis method as stated by Gupta et.al,2015 with slight modifications. The content was analyzed under UV spectrophotometer by measuring absorbance at 358 nm. The analysis was carried out in CH_2C_{l2}/DMSO (1/4) solvent. All the measurements were done in triplicate [16].

4.2.5.7 *In-vitro* Drug Release Studies: About 10 mg of microspheres were taken in dialysis bag (10000 M.W. cut off). This dialysis bag was transferred to a vial containing 5 ml of phosphate buffer (pH 7.4; 100 mM) and placed in reciprocal shaking water bath maintained at 37ºC. An aliquot (1 ml) of the release medium was withdrawn at preset time points and volume withdrawn was replaced with fresh release medium for 6 hr. Drug content in samples was analyzed by UV Spectrophotometer at 267 nm wavelength of ellagic acid as standard drug [25,29-30].

4.2.5.8 MTT Assay: Cells were seeded in 96 well plates at a density of $1x10^5$ cells/ well. There after cells of the cell lines (SKOV3 human ovarian cancer cells) were subjected to DMSO and DMSO containing folate (FA) with the amla extract loaded MS , Ellagic acid loaded MS , and soyabean extract loaded MS at concentrations from 0-75 µm, taking as control and test sample respectively. The cells were treated for 48 hours. At the end of each treatment time, 20µl of MTT stock solution (5mg/ml) was added to each cell and again left under incubation for 5 hours. Following this, all media was aspirated out and 200 µl of fresh DMSO was added to each well. The reading was taken at 550 nm (Absorbance) against an appropriate blank [16].

4.2.5.9. Stability Studies: The prepared microspheres were packed in glass container and subjected to stability studies at 40 ±2°C/75±5% RH as per ICH guidelines for a period of 4 months. Samples were withdrawn at 1 month time intervals and evaluated for physical appearance, pH, and rheological properties [26].

4.2.5.10. Statistical Analysis: Data are shown as means ± standard deviation (n = 5). Statistical data were analyzed by the Student's *t*-test at the level of P=0.05.

5. Result

5.1.Percentage of Free Radical Inhibition

The value of % DPPH scavenging activity of standard L-Ascorbic acid and test samples (extract of *Embilica officinalis* and extract of *Glycine max Merrill*) were done and the comparison of standard and test samples was plotted with help of plotted graph (**Table 1**) (**Figure 1**).

5.2. Particle Size and Zeta Potential : The average size of *Embilica officinalis* extract microsphere, ellagic acid and *Glycine max Merrill* extract microsphere was 194.100 ± 2.34 µm , 884.418± 1.32 µm and 467.232 ± 28µm. The average particle size offolate conjugated *Embilica officinalis* extract microsphere, ellagic acid and *Glycine max Merrill* extract microsphere of 195.09 ± 1.45 µm, 885.425 ±0.92 µm and 468.3 ± 0.72 µm. The average particle size of microspheres increased with increasing the concentration of the polymer (**Table 2**). *Embilica officinalis* extract microsphere, Ellagic acid and *Glycine max Merrill* extract microsphere have an anionic surface charge of -30.98 ± 2.2 mV, -27.67±0.3

mV and 17.99±0.23mV. The physical adsorption folateleads to significant increase in the zeta potential of folate conjugated *E. officinalis* extract microsphere, ellagic acid and *Glycine max Merrill* extract microsphere 29.43 ± 1.65 mV, -26.57±0.3 Mv, and 16.56± 0.56Mv. The average zeta potential of microspheres is represented in (**Table 2**) (**Figure 2**) (**Figure 3**).

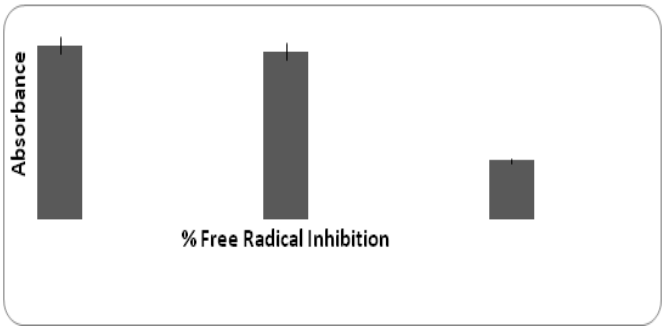

Figure 1: Percentage free radical inhibition of *E. officinalis*and ***Glycine max Merrill*** **of extracts when compared with standard.**

**The results are expressed as mean ± S.E.M. The statistical analysis values Statistical tests as well as mean and S.E.M calculations and graphical representation of result were performed.

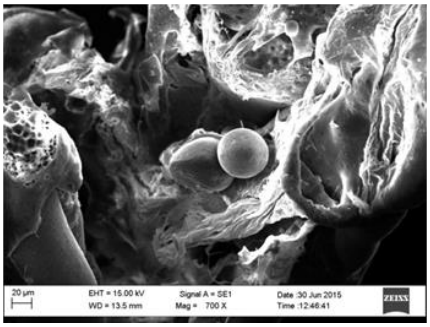

Figure 2: SEM image of Formulation of folate conjugated amla extract microsphere.

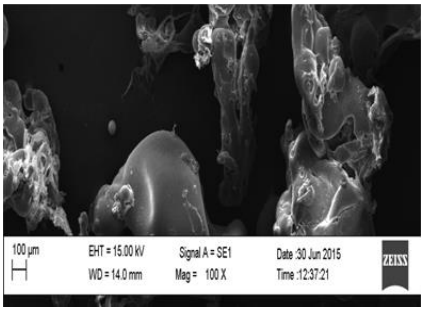

Figure 3: SEM image of Formulation of Soyabean extract loaded MS.

Table 1: Data of Percentage of free radical inhibition of standard L-Ascorbic acid extract of *Embilica officinalis* and extract *Glycine max Merrill*

Concentration of ascorbic acid	%free radical inhibition of ascorbic acid	%free radical inhibition *Embilica officinalis* fruit extract	%free radical inhibition *Glycine max Merrill* extract
0.25µg/ml	98.27 ± 0.68	95.57 ± 0.35	19.22 ± 0.76
0.50µg/ml	95.28 ± 0.13	94.91 ± 0.87	29.80 ± 0.22
0.75µg/ml	98.09 ± 0.20	91.72 ± 0.15	35.99 ± 0.48
1.00µg/ml	98.26 ± 0.35	94.36 ± 0.21	32.70 ± 0.83
1.25µg/ml	98.33 ± 0.97	93.81 ± 1.2	46.06 ± 1.03

@S.E.M: Standard error of Mean

Table 2: The average particle size, zeta potential, % drug loading and % folate content attached of prepared microspheres are represent in the table 3 below

Parameter	*E. officinalis* extract loaded MS	Ellagic acid loaded MS	G. max Merrill extract loaded MS	Folate conjugated AMS	Folate conjugated EMS	Folate conjugated SMS
Particle size	194.1 ± 2.34 µm	884.4 ± 1.32 µm	467.2± 1.28µm	195.09 ± 1.45µm	885.4 ± 0.92 µm	468.3 ± 0.72 µm
Zeta potential	30.98 ± 2.2 mV,	27.67±0.3 mV	17.99±0.23 mV.	29.43 ± 1.65 mV ,	26.57±0.3 mV	16.56± 0.56 mV
Drug loading %	90.78±0.2	62.77±0.5	82.98±0.4	89.45±0.3	61.88±0.2	81.89±0.2
Folate content attached	--	--	--	93.45±0.89	64.84±0.76	85.09±0.56

@Each batch was prepared by solvent evaporation method
@S.E.M: Standard error of Mean

5.3. Structural Analysis of Prepared Microspheres

Scanning electron microscopies was used to observe the surface morphology of all the prepared formulations and are shown in Figure. The SEM study reveals that the microspheres have been prepared are of uniform spherical shape and morphology.

5.4. FTIR Analysis

FTIR analysis was used to analysis of quantitative drug present in microsphere, the data was found shown in graph. IR peak of pure ellagic acid was observed in microspheres. As compare with standard IR spectrum of ellagic acid the confirmatory peaks were observed for both the extracts loaded microsphere showing characteristic peaks of O-H, C=O, C-OH, C-H in range 3600-850 cm^{-1}. The result of FTIR analysis showed that the Ellagic acid loaded microsphere, E. Officinalis extract loaded microsphere, G.max Merrill extract loaded microsphereshowed 3564.45, 3498.87 and 3483.44 that showed OH group was present. And 1701.22, 1739.79 and 1743.85 that showed carboxylic group was present. but in case of C-OH group EMS and AMS was showed peak in range of1354.03 and 1373.32 and C-H group only showed in extract loaded microspheres of *E. Officinalis* an *G.max Merrill* which showed peak in range of 817.72 and 655.80. The data obtained after FTIR analysis of standard and test samples are shown in the (**Table 3**) below.

5.5. XRD Analysis

XRD Analysis of different prepared microspheres like Amla extract loaded microspheres Soyabean extract loaded microsphere and Ellagic acid loaded microsphere were done in order to analyse the nature of microspheres whether amorphous or crystalline and are shown in following (**Figure 4**), (**Figure 5**), (**Figure 6**). In figure 4, XRD spectrum data represent the graph of ellagicacid, which showed small peaks that denote ellagic acid present in microsphere of ethyl cellulose in crystal form. And figure 5, XRD spectrum data represent the graph of ellagic acid, which showed small peaks that denote Amla extract present in microsphere of ethyl cellulose in crystal form and it having amorphous nature and figure 6 data represent that the soyabean

extract present in microsphere of ethylcellulose, it also present in crystalline form with amorphous nature. In this XRD spectrum, the data represents characteristic peak of standard drug ellagic acid microspheres, but when compared with extract loaded microspheres, amla extract loaded microsphere showed small peak in same range of ellagic acid microsphere spectra while soyabean extract loaded microsphere also showed peak in region of ellagic acid confirming the crystalline nature of the drug.

5.6. *In-vitro* Drug Release Studies

Drug release was obtained from different formulation in phosphate buffer pH 7.4 medium. The data reported on (**Figure 7**) which shows as time increase drug release from polymer. The drug and extracts loaded in microspheres used as carrier for drug delivery system for treatment of ovarian cancer (**Figure 8**).

5.7. MTT Assay

The anticancer potential of folate conjugated with the *E. Officinalis* extract loaded microspheres (F-AE-MS), Ellagic acid loaded microspheres (F-EA-MS) and *G.max Merrill* extract loaded microspheres (F-SE-MS) was evaluated in SKOV3 human ovarian cancer cells. These cell lines express folate receptor on their surface. The results of our study potentiate the role of folate receptor mediate targeting of novel (natural) bioactive for the treatment of ovarian cancer. The results showed that cellular uptake by the cell lines were higher for conjugated microspheres as compared to the non-conjugated microspheres. Higher inhibition was observed with folate conjugated with the *E. Officinalis* extract loaded MS (IC50 =14.65 µg/ml), while Ellagic acid loaded MS gave a (IC50 =47.65 µg/ml) and *G.max Merrill* extract loaded MS gave least inhibition (IC50 = 52.65 µg/ml) (**Figure 9**).

Table 3: FTIR Analyze of prepared microspheres

Functional group	Standard (cm^{-1})	Ellagic acid loaded microsphere	E. Officinalis extract loaded microsphere	G.max Merrill extract loaded microsphere
O-H	3100-3600	3564.45	3498.87	3483.44
C=O	1650-1700	1701.22	1739.79	1743.85
C-OH	1350-1400	1354.03	1373.32	-
C-H	680-850	-	817.72	655.80

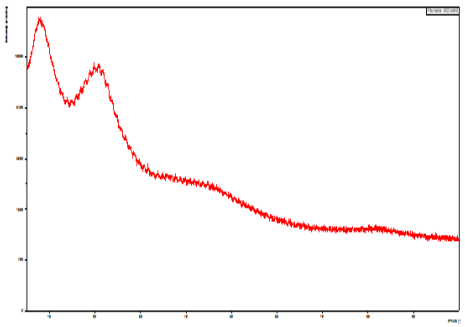

Figure 4: XRD Graph of Ellagic acid Microsphere.

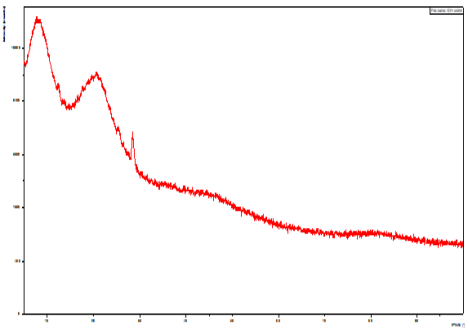

Figure 5: XRD Graph of Amlaextact loaded microsphere.

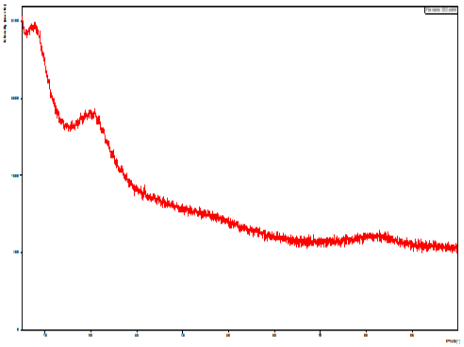

Figure 6: XRD Graph of soyabean extract loaded microsphere.

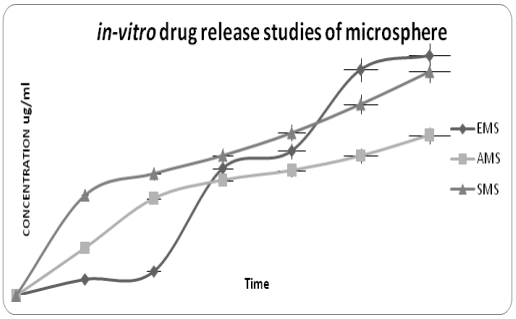

Figure 7: *In-vitro* drug release studies of microsphere Ellagic acid loaded microsphere, Amla extract loaded microsphere and *G.maxMerrill* extract loaded microsphere in phosphate buffer pH 7.4 at 267 nm. *In vitro* drug release of microspheres is expressed as mean ±S.D.

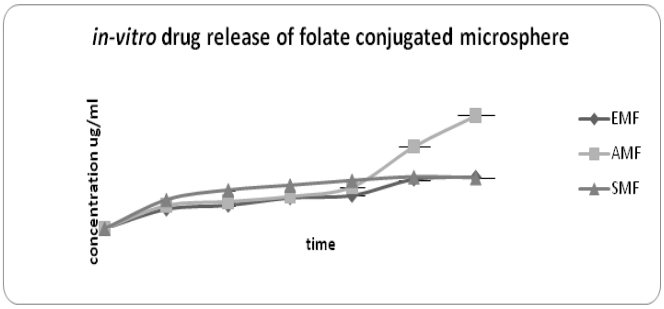

Figure 8: *In-vitro* drug release studies of microsphere Ellagic acid loaded microspheres, *E. Officinalis*ertract loaded microspheres and *G.maxMerrill* extract loaded microspheres in phosphate buffer pH 7.4 at 267 nm. *In vitro* drug release of folate conjugated microsphere is expressed as mean ± S.D.

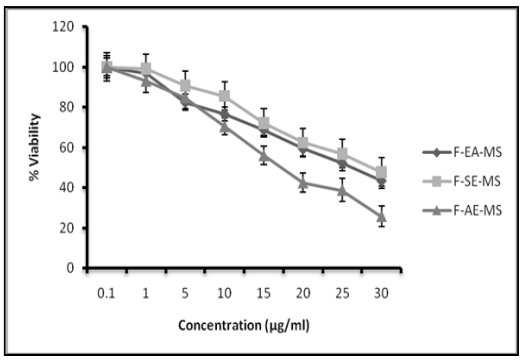

Figure 9: *In vitro* cytotoxicity of folate conjugated with the *E. Officinalis* extract loaded microspheres (F-AE-MS), Ellagic acid loaded microspheres (F-EA-MS), and *G.max Merrill* extract loaded microspheres (F-SE-MS) against SKOV3 cell line after 72 h measured with the MTT assay. Cell viability is expressed as mean± S.D.

6. Conclusion

Herbal drugs have always put forward an alternative to existing drug regimen, when they fail to provide a permanent solution to cure a disease. Female disorders find unbeatable curing remedies traditionally. Our ancient systems of medicines have various drugs underneath to reduce the complications of gynaecology. Satavari, Brahmi, Ashoka, Aloe, etc. are the herbs of known effects. Our objective in present study was to develop novel drug delivery system of herbal extracts in order to facilitate their entry inside the body by increasing their solubility and to target their effect by ligand binding to respective cells for maximum therapeutic activity. Herbal extracts suffers the problem of stability also. The prepared microspheres were competent enough to render a stable delivery system for developing them further into acceptable formulations. Our approach to develop a targeted moiety of herbal extract loaded system is in primary stage. Several studies are needed to groom it into a versatile carrier, but it can be hoped that future studies will promote it to a suitable form.

7. Acknowledgement

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