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FORMULATION AND EVALUATION OF BIODEGRADABLE POLYPHENOLIC MICROSPHERES FOR CANCER

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Abstract

Objective: The purpose of this research was to formulate and evaluate biodegradable polyphenolic microspheres for cancer.

Materials and methods: Polyphenolic compounds like catechin, gallic acid and tannic acid were prepared as microspheres using polycaprolactone and polyvinyl alcohol as polymers (PCL). Microspheres were prepared using o/w solvent evaporation technique. All formulations were evaluated for particle size, surface morphology, drug loading and encapsulation efficiency, *in-vitro* release and *in-vitro* anti cancer activity using Human epidermoid cancer cells (HEP2).

Result: Formulations CF 4(Catechin), GF 4(Gallic acid) and TF 4(Tannic acid) showed a better drug loading as well as good sustained drug release. *In-vitro* release study was carried out using phosphate buffered saline (pH 7.4) as the release medium. Regardless of drug loading, the release profile exhibited a biphasic release, i.e., small initial burst release followed by a slow release. Effect of formulations CF4, GF4, TF4 on HEP2 cells are determined by MTT assay. At the concentration of 100µg/ml, formulations showed a relative cell viability of 19.09, 22.72 and 4.54 with the cell control 100. Cells shrinkage and cells rounding were the cytotoxic changes observed.

Discussion: It is observed that the drug release from the microspheres depends on the PCL concentration. When PCL concentration was 5% the rate of drug release was the fastest, as the PCL concentration increased, the drug release behaviour showed a more sustained pattern. The formulations CF4, GF4 and TF4 were found to be efficient to possess *in-vitro* anti cancer activity in HEP2 cells.

Key words: Polyphenol, catechin, gallic acid, tannic acid, o/w solvent evaporation.

Introduction

Polyphenols are a group of chemical substances found in plants, characterized by the presence of more than one phenol unit or building block per molecule. Poly phenols are present in fruits, vegetables, drinks etc. They may reduce the risk of cardiovascular disease and cancer.[1] The growing amount of evidence from studies in epidemiology, cell cultures and animal tumour models demonstrates that a large number of natural compounds from the diet could lower cancer risk and some of them could sensitize tumour cells in anticancer therapies.[2,3]

Microsphere is a term used for small spherical particles, with diameters in the micrometer range (typically 1µm to 1000 µm)(1mm). They are characteristically free flowing powders consisting of proteins or synthetic polymers which are biodegradable in nature and ideally having a particles size less than 200 µm. These carriers received much attention not only for prolong release but also for targeting of the anticancer drugs to the tumour. These materials are mainly used for early detection of malignancy and precise localisation of cancer therapeutics without or with minimal adverse effect to the somatic tissues.[4,5]

Phenolic compounds can trigger apoptosis in cancer cells through the modulation of a number of key elements in cellular signal transduction pathways linked to apoptosis. This programmed cell death may be considered as one of the important targets in preventive approach against cancer at the moment.[6,7]

Natural compounds have been extensively studied and have shown 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. The cancer chemo preventive effects of polyphenolic anti oxidants is specifically important since environmental pollutants, radiation and physical stress exhibit the ability to produce enormous amount of free radicals which cause many diseases, including tumour promotion and cancer. For cancer prevention and chemotherapy, plant derived natural compound such as Catechin, Gallic acid, and Tannic acid are an invaluable treasure and worthy to be further explored[8,9].

So, our aim is to formulate microspheres containing Catechin, Gallic acid and Tannic acid and to evaluate its characters. Also the potency against the selected HEP2 cell line using MTT assay is established.

Materials

Catechin, gallic acid, tannic acid and Polycaprolactone were procured from Sigma Aldrich Chemicals, Bangalore, India. Dichloromethane was obtained from CIFT, Cochin, India. Polycaprolactone and polyvinyl alcohol

was purchased from S.D.Fine-Chem Limited, Mumbai, India. Sodium di hydrogen phosphate and disodium hydrogen phosphate were purchased from Merk specialities Private Limited, Mumbai, India. Sodium chloride and ethanol were purchased from Spectrum reagents and chemicals.

Methods

1. Preparation of microspheres[4,5]:

O/w solvent evaporation technique was selected to prepare polyphenolic microspheres. Polycaprolactone (polymer) at different concentration was dissolved in 3 ml of dichloromethane. Micronized drug was dispersed in the polymer solution. This drug polymer solution was added drop wise into 40 ml of 0.1% polyvinyl alcohol aqueous solution, and the resultant mixture was stirred with a three blade propeller 6000 rpm for 2 hours. After complete evaporation of dichloromethane, the microspheres were collected by filtration, washed with deionised water (three times 10ml per time) and dried in vacuum desiccators at room temperature. The different formulations and their codes were given in Table no.1

Table No.1: Formulation codes for microspheres.

Formulation Codes	Concentration of PCL	Volume of (0.1%) PVA added	Amount of drug added	Volume of DCM added
CF 1	5%	40 ml	100 mg	3 ml
CF 2	10%	40 ml	100 mg	3 ml
CF 3	15%	40 ml	100 mg	3 ml
CF 4	20%	40 ml	100 mg	3 ml
GF 1	5%	40 ml	100 mg	3 ml
GF 2	10%	40 ml	100 mg	3 ml
GF 3	15%	40 ml	100 mg	3 ml
GF 4	20%	40 ml	100 mg	3 ml
TF 1	5%	40 ml	100 mg	3 ml
TF 2	10%	40 ml	100 mg	3 ml
TF 3	15%	40 ml	100 mg	3 ml
TF 4	20%	40 ml	100 mg	3 ml

PCL- polycaprolactone, PVA- polyvinyl alcohol, DCM- dichloromethane

2. Analysis of particle size, shape and surface morphology[10]:

2.1 Optical microscopy[5]:

Optical microscopy involves the use of transmitted light, reflected light, polarised light and fluorescence. All the formulations were viewed through optical microscope for the confirmation of microspheres and its sphericity.

2.2 Particle size determination[5]:

The particle size and size distribution of the prepared microspheres were measured by a laser light scattering analyser (Microtrac Inc.). A suitable amount of dried microspheres, from each formulation was suspended in water and sonicated for one minute before measurement. The resulting homogenised suspension was then analysed for the volume mean diameter and particle size distribution using software Microtrac flex (Table no. 2).

2.3 Polydispersity index[5]:

Polydispersity (microspheres of non uniform size) was calculated from the formula,

$$\text{Polydispersity} = (D_{0.9} - D_{0.1}) \div D_{0.5}$$

Where, $D_{0.9}$, $D_{0.1}$ and $D_{0.5}$ particle diameters determined at 90^{th}, 50^{th}, and 10^{th} percentile of undesired particles respectively. Higher the polydispersity index values indicate high level of non-uniformity and its value is used to characterize the microsphere as monodisperse, homogenous and heterogeneous systems (Table no. 2).

2.4 Scanning electron microscopy[5]:

The prepared microspheres were sprinkled on one side of the adhesive stub. The stub was then coated with conductive gold with JOEL-JFC 1600 auto coater and was examined under JOEL-JFC 6360 SEM for qualitative assessment of microsphere morphology (Figure no.4, Figure no.5 and Figure no.6)

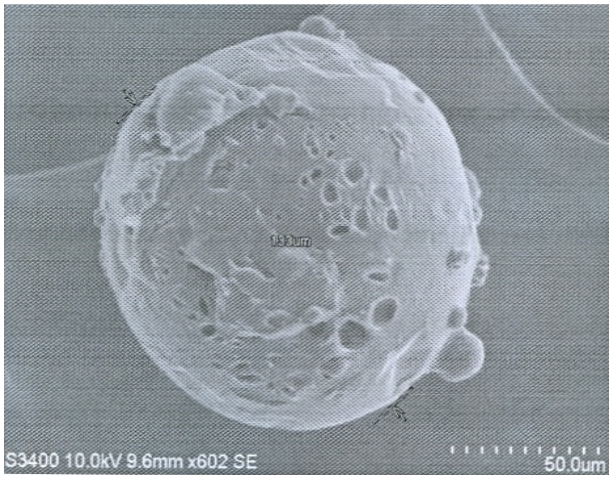

Figure no.4: Scanning Electron Microscopy of Catechin microspheres.

Figure no.5. Scanning Electron Microscopy of Gallic acid microspheres.

Figure no.6. Scanning Electron Microscopy of Tannic acid microspheres.

3. Drug loading and encapsulation efficiency[11,12,13,14]:

The drug content of biodegradable microspheres were determined for all the formulations by dissolving 10 mg of the microspheres in 200µl of DCM taken in 2 ml eppendorf tubes and mixed in vortex for 10 minutes. Then 1800µl of ethanol were added to precipitate polycaprolactone (insoluble in ethanol) and again mixed in vortex mixer for 5 minutes to extract the drug in ethanol. The resulting solution was centrifuged for 10 minutes at 3000 rpm to settle down the precipitated polymer. The 200µl of supernatant was diluted to 3 ml using ethanol and the absorbance was measured by using UV spectrophotometer and equivalent concentration was determined by the calibration curve using the same proportion of solvents. The percentage drug loading and percentage encapsulation efficiency of the blend microspheres were calculated using the formula,

$$\%\ \boldsymbol{drug\ loading} = \frac{\boldsymbol{weight\ of\ the\ drug\ in\ microspheres}}{\boldsymbol{weight\ of\ the\ microspheres}} \times \mathbf{100}$$

$$\%\ \boldsymbol{Entrapement\ efficiency} = \frac{\%\ \boldsymbol{drug\ loading}}{\%\ \boldsymbol{theoretical\ drug\ loading}}$$

The drug loading and entrapment efficiency of the formulations were depicted in Table no. 2.

Table No-2: Effect of concentration of polycaprolactone, stabilizer on particle size and entrapment efficiency.

Formulation codes	Polycaprolactone concentration (% w/v)	Stabilizer concentration (40 ml)	Average particle size (µm)	Drug loading (µg)	Entrapment efficiency (EE %)	Polydispersity index (PDI)
CF1	5	0.1%	171.1	1.2105	26.13	2.13
CF2	10		126.3	2.0158	34.98	1.35
CF3	15		122.3	2.9185	49.55	1.25
CF4	20		61.13	3.5740	57.09	1.20
GF1	5	0.1%	202.9	1.0635	21.10	1.58
GF2	10		189.5	1.2640	38.90	1.26
GF3	15		150.5	2.3345	42.13	1.14
GF4	20		91.47	3.0300	53.99	1.05
TF1	5	0.1%	189.5	1.2635	19.16	1.54
TF2	10		100.5	1.7050	31.00	1.35
TF3	15		91.47	2.4627	45.03	1.17
TF4	20		79.79	3.0145	58.11	1.11

4. ***In-vitro*** **drug release[15,16,17]:**

Drug release from the microspheres was determined by using phosphate buffered saline (PBS pH 7.4) as the release medium. 10 mg of the prepared microsphere from various formulations are transferred into open ended cylinder whose one end was tied with a dialysis membrane (Hi media, Mumbai, India) with molecular weight cut off 12,000 to 14,000 Daltons. The tied end is dipped into the release medium and at different time intervals samples are withdrawn and it is replaced by the buffer to maintain the sink conditions and the absorbance was measured at suitable nm using double beam UV spectrometer.

5. Performance of drug toxicity assay[16,17,18,19]:

Cytotoxicity is the toxicity or damage caused to the cells on addition of the drug. After the addition of the drug, cell viability is estimated by staining technique, whereby cells are treated with trypan blue. Trypan blue is

excluded by live cells, but stains dead cells blue. The results are confirmed by additional metabolic intervention experiments[20] such as MTT assays. MTT assay is called as (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide. MTT assay was performed and the cytotoxic activity of prepared microspheres were determined. Cell shrinkage and cell rounding were the cytotoxic changes observed.

Results

Preparation of microspheres:

In this study, o/w solvent evaporation technique was selected to prepare the three different polyphenolic microspheres containing Catechin, Gallic acid and Tannic acid. The microspheres were evaluated for the particle shape, size, surface morphology, drug entrpement efficiency, *in-vitro* release studies and toxicity studies. In the formulations, CF3, CF4, GF4, TF2, TF4 microspheres were found to be uniform by the SEM analysis and particle size study.

Entrapment efficiency:

Among several of the parameters of the formulation, the polymer concentration played an important role in the loading of the drug. The drug loading was increased with the increasing concentration of the polymer. The increase in entrapment efficiencywas mainly attributed to the increased viscosity of the drug polymer solution. The increased polymer concentration can accelerate the solidification of the phase seperated polymer domain. The reason for the increase in encapsulation efficiency is that the polymer is not overloaded with the amount of the drug. Formulations CF4,GF4, and TF4 showed entrapment efficiency of 57.09%, 53.99% and 58.11%

FTIR studies

IR spectrum of gallic acid showed sharp peaks at 3491 which is responsible for inter molecular hydrogen bonding. C-H strectching at 3377, C=O strecting at 1703, and peaks at 1617, 1539, 1453, 1254 cm^{-1} confirmed the presence of –OH bonds. Catechin and tannic acid showed a peak at 1600 cm^{-1} for aromatic ring. From the results, it is clear that there is no appreciable change in the positions of the characteristic bands of the drug along with the IR spectrum of the best formulation deriveed during the present investigation. Since there is no change in the nature and the positions of the bands in the formulation, it can be concluded that the drug maintains its identity without going chemical interactions with the polymer used. The IR spectrum of the 3 drugs were depicted in Figure no.1,2 and 3.

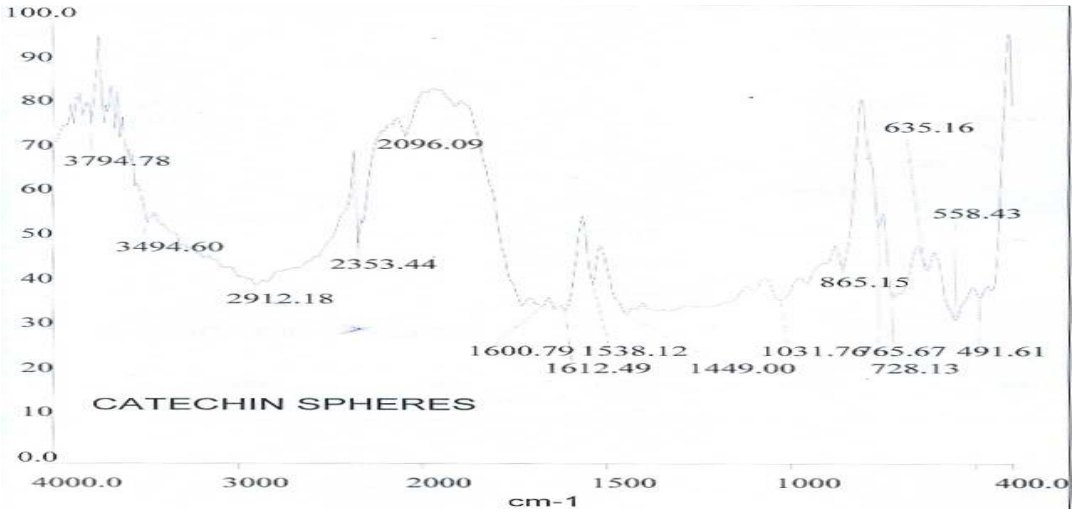

Figure no. 1. IR spectrum of Catechin microspheres

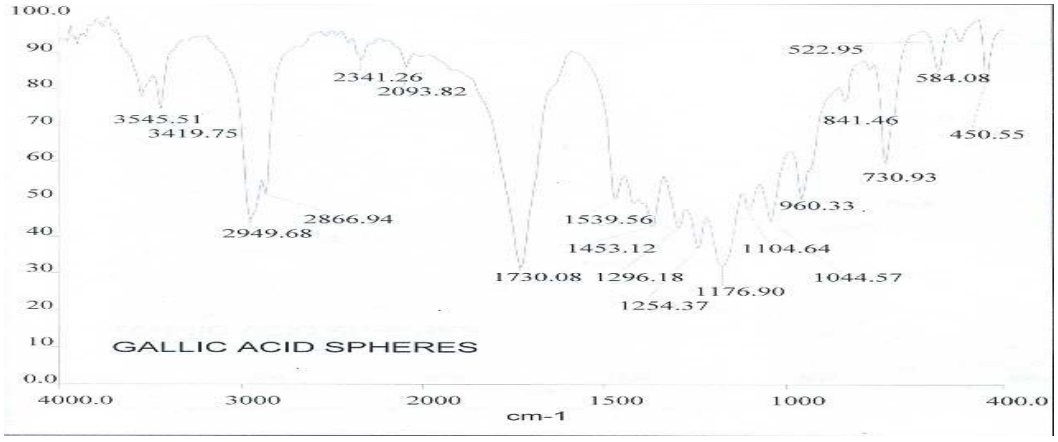

Figure no.2. IR spectrum of Gallic acid microspheres

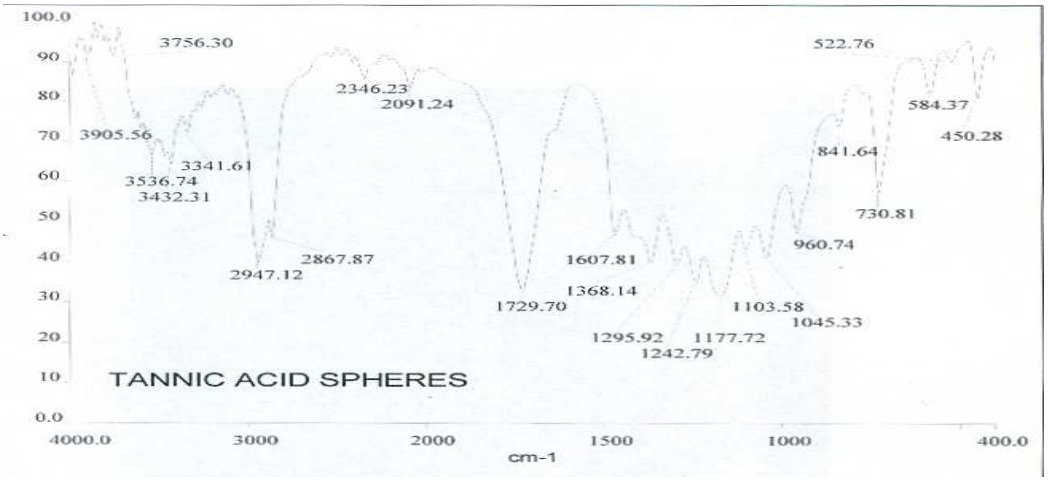

Figure no.3. IR spectrum of Tannic acid microspheres

Particle size

Microsphere particle size and particle size distribution were measured by a laser light scattering analyzer. A suitable amount of dried microspheres, from each formulation was suspended in water and sonicated for 1 minute before measurement. The resulting homogenised suspension was then analysed for the volume mean diameter and particle size distribution using software Microtrac flex. From the above results the particle size was observed in the range of 100 to 250µm.

Surface morphology of the microsphere

Scanning electron microscopy is used for the observation of surface morphology of microspheres. The catechin, gallic acid and tannic acid microspheres (CF4, GF4 and TF4), prepared by o/w solvent evaporation technique showed a good sphericity. Microspheres made with catechin shows the presence of pores in its surface (Figure no. 4) which indicates the faster release of drug, whereas the microspheres made with gallic acid and tannic acid shows the absence of pores in their surface (Figure no. 5 and 6).

***In-vitro* release studies**

In-vitro release profiles of drug loaded microspheres of Catechin, Gallic acid and Tannic acid in phospate buffered saline (pH 7.4) was shown in Graph no. 1, 2 and 3. The *in-vitro* release profile indicates the differences between polycaprolactone microparticle formulations caused by the polymer solution concentration. When the concentration of PCL was 5%, the rate of drug release was faster. As the PCL concentration increased, the drug release behavior showed a more sustained pattern.

Formulations CF4, GF4, and TF4 showed a better drug loading and as well as good sustained drug release. Regardless of drug loading, the release profiles exhibited a biphasic release.

Drug release kinetics

Since polycaprolactone possess semi crystallinity, the drug release from the PCL microspheres are commonly consiered as the result of diffusion.with regard to non swellable microspheres the kinetics of drug release indicates Fickian diffusion, non-fickian diffusion and zero order transport when n is 0.5 or less than 0.5, $0.5<n<1$ and n=1 respectively.the kinetic model showing highest correlation coefficient (r^2) was considerd as the most appropriate model. According to korsemeyer's Peppas equation n=0.4425 indicates Fickian release mechanism. In

the formulations, CF4; GF4; TF4 korsemeyer's Peppas n value lies between 0.48, 0.39 and 0.46 respectively. So it indicates the Fickian release mechanism.

***In-vitro* anticancer activity**

In-vitro anticancer activity of poly phenols have been extensively studiedin HEP2 cell line (Human Epidermoid cancer cells) at Life Teck Research Centre, vadapalani, Chennai and have shown anti cancerogenic activity. Effect of formulations CF4, GF4, TF4 on HEP2 cells are determined by MTT assay. At the concentration of 100µg/ml, formulations showed a relative cell viability of 19.09, 22.72 and 4.54 with the cell control 100. Cells shrinkage and cells rounding were the cytotoxic changes observed.

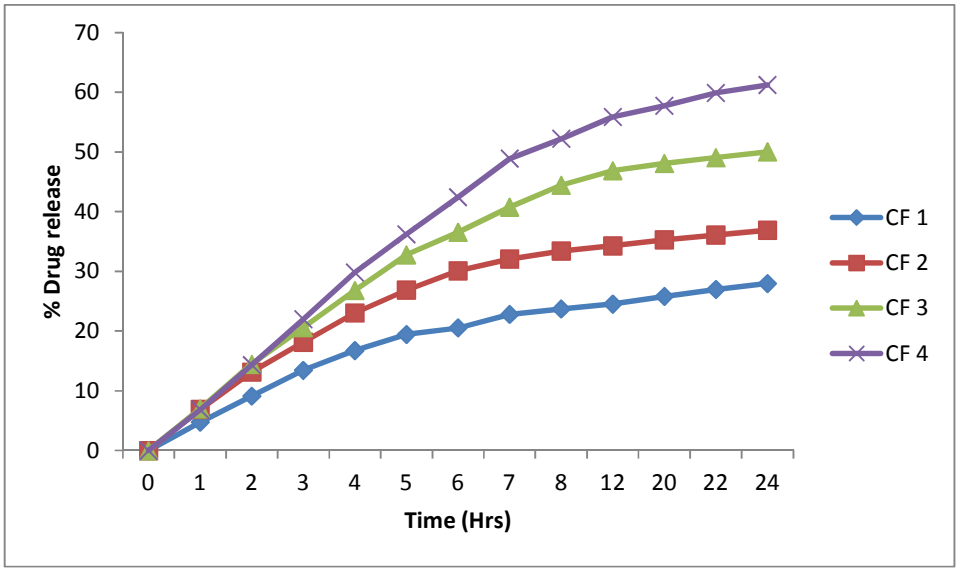

Figure no. 7: *In-vitro* release profile of CF 1- CF 4

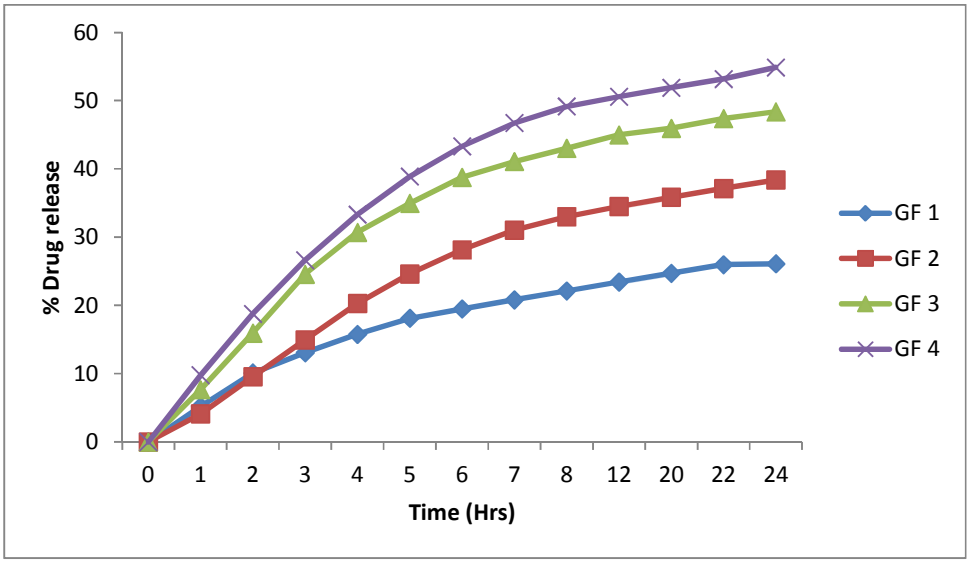

Figure no. 8: *In-vitro* release profile of GF 1- GF 4

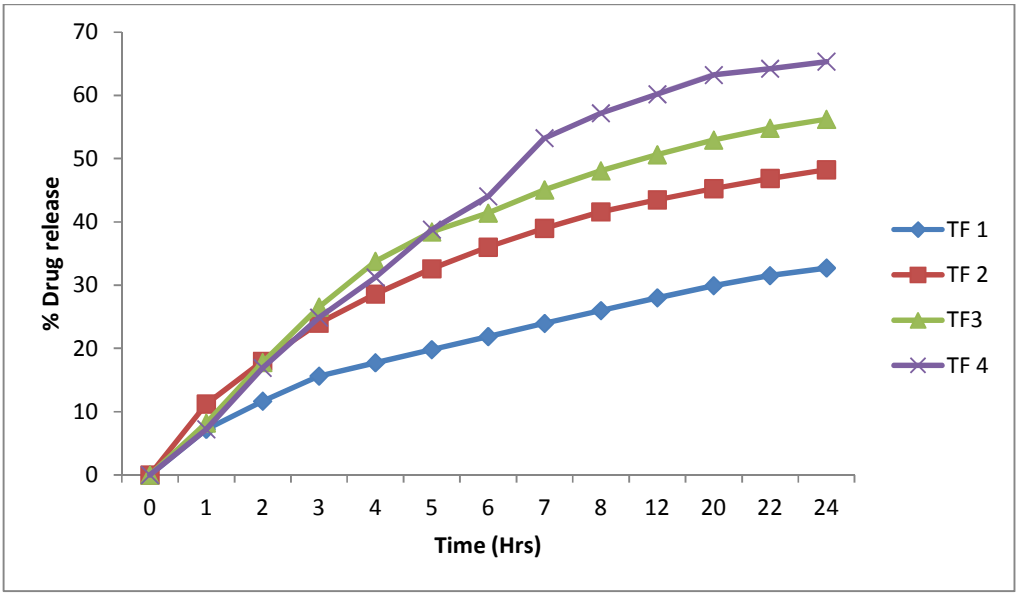

Figure no. 9: *In-vitro* release profile of TF 1- TF 4

Discussion

This study demonstrates that it is feasible to prepare biodegradable polyphenolic microspheres employing the o/w solvent evaporation technique. Among several parameters polymer concentration played an important role in the drug loading. From the above study, it was concluded that the entrapment efficiencey of the drug was increased with increasing concentration of polymer. The rate of the drug release from the microspheres depends upon difference in encapsulation efficiency, drug solubility, microsphere surface morphology, hydrophobic nature and crystallinity of the polymer. Invitro release studies showed an initial burst release followed by a rapid slow release. The drug release pattern showed zero order release indicating Fickian diffusion. Invitro cytotoxic studies of formulations were determined by MTT assay. At the concentration of 100µg/ml, formaulations CF4, GF4, TF4 showed a relative cell viability of 19.09, 22.72, and 4.54 with the control 100. Cells shrinkage and cells rounding were the cytotoxic changes observed. From the above results it was concluded that the formulations CF4,GF4 and TF4 were found to be efficient to possess *in-vitro* anti cancer activity in HEP2 cancer cell line.

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