Original Article

Formulation and Characterization of Chrysin Loaded Phytosomes and its Cytotoxic Effect against Colorectal Cancer Cells

Namit Kudatarkar, Sunil Jalalpure*, Bhaskar Kurangi

KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi, Karnataka, INDIA.

ABSTRACT

Background: Chrysin is a phytoconstituent which has anticancer activity. The study aims to formulate, characterize and evaluate the cytotoxic effect of chrysin loaded phytosomes against HT29 cells. **Materials and Methods:** Antisolvent precipitation technique was employed to prepare phytosomes. Particle size, polydispersity index, zeta potential, entrapment efficiency, scanning electron microscope and Fourier transform infrared spectroscopic analysis were carried out for the characterization of chrysin loaded phytosomes. Cell viability was done to evaluate the cytotoxic effect of developed phytosomes comparing with plain chrysin. **Results:** The developed chrysin loaded phytosomes showed the particle size of 94.40nm, polydispersity index of 0.31, and zeta potential -1.33 mV. The entrapment efficiency was 74.28 %. Chrysin loaded phytosomes showed increased cytotoxic effect on HT-29 cells. **Conclusion:** This research work produces confirmative indication for the use of Chrysin loaded phytosomes in experimental animals to further gain in depth analysis for anticancer activity of chrysin loaded phytosomes against colon cancer.

Keywords: Chrysin, Phytoconstituent, Nanoformulation, Colorectal cancer, Cytotoxicity.

INTRODUCTION

As per the data provided by GLOBOCAN, it is found that colorectal cancer (CRC) is being the 3rd most fatal cancer and also 4th most diagnosed cancer throughout the world.[1] An elevation in the incidence rate of CRC has being reported in both the developed and developing countries due to the consumption of artificial foods which contain low fiber diet and also due to sedentary lifestyle.[2] Treatment for CRC is available using chemotherapy if diagnosed with CRC at an earlier stage. But it has been found that these chemotherapy drugs possess many adverse effects due to which the risk is more than the benefits. To avoid the adverse effects of the chemotherapeutic drugs, it is recommended to use alternative medicines which include the herbal drugs either in the form of extracted crude drugs or pure phytoconstituents. Chrysin also known as 5'7 dihydroflavone is one phytoconstituent which falls under flavonoid category and is also known to possess anticancer properties against various types of cancer. Chrysin is a phytoconstituent mainly found in honey, propolis and passion flower.[3] Reviews from scientific literature which were previously published have revealed that chrysin exhibited enhanced cytotoxic effect against various colorectal cancer cell lines,[4] and also *in vivo* studies.[5] One study was done in Wistar rats and it revealed that the goblet cells in the crypts of colon had deteriorated due to cis-diamminedichloroplatinum which was protected by phytoconstituent chrysin through breakdown of oxidative stress and apoptosis.[6] However, phytoconstituent Chrysin is found to be beneficial in the treatment of colorectal cancer.[7] Studies revealed that Chrysin had

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Correspondence:
Dr. Sunil Jalalpure
Professor and Principal, Department of Pharmacognosy, KLE College of Pharmacy, Belagavi, KAHER, Belagavi-590010, Karnataka, INDIA.
E-mail: jalalpuresunil@rediffmail.com

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very low bioavailability due to which the dose required to treat the colorectal cancer was more.[8] Phytosomes is a novel nanoformulation which aids in reducing the size of the phytoconstituent which in turn improves the surface area and thus better permeability.[9] Studies also suggest that the stability of the drug is enhanced by incorporating into phytosomal form.[10] Since there are no studies that are being carried out to check the anticancer potential of chrysin in phytosomal form, the primary objective of this study is to enhance the pharmacokinetic and pharmacodynamics profile of chrysin by incorporating it into phytosomal form and to characterize and pharmacologically evaluate anticancer activity of chrysin loaded phytosomes for the treatment of colorectal cancer in Wistar rats.

MATERIALS AND METHODS

Materials

Soya lecithin and Chrysin (95%) was obtained from Tokyo chemical industries limited (Japan). The other chemicals and reagents used were of analytical grade.

Formulation of Chrysin Loaded Phytosomes

Chrysin loaded phytosomes (CLP) were prepared by antisolvent precipitation technique.[11] The required quantity of soy lecithin and chrysin were taken in 250ml round bottomed flask and refluxed in the presence of dichloromethane in the temperature 60°C for 3 hr. The resultant solution was concentrated to about 5-10ml and hexane was administered to the concentrated solution with continuous stirring till a precipitate (ppt) was formed. Then the ppt was filtered out. It was kept under desicator for complete drying. The dried ppt was sieved in 100 mesh and stored in ambor coloured bottles.

Formulation and Structural Characterization of Chrysin Loaded Phytosomes

Visual Observation, Particle Size Distribution, Polydispersity Index and Zeta Potential

10mg of chrysin loaded phytosomes was added in 100ml phosphate buffer saline along with rapid shaking and it was observed in the presence of white light for the presence of particle aggregates and also for a characteristic opalescence. Particle size of chrysin loaded phytosomes was measured with the help of Nanotrac. Here 1mg of the formulation were added with 10ml of water and this was ultrasonicated for an hour. After sonication, the solution was administered in nanotrac for determining particle size and polydispersity index. Zetasizer (Malvern Instruments, United Kingdom) was utilized for determining the zeta potential. The prepared formulation (1mg) was added in 10ml of water by shaking rapidly. 0.5 ml of chrysin loaded phytosome was administered in disposable zeta sizing cuvette. It was analyzed at 25°C at a measurement angle of 90°.

Entrapment Efficiency

The amount of phytoconstituent Chrysin present in the phytosomal form was determined using High performance thin layer chromatography (CAMAG, Switzerland).

Scanning Electron Microscopy (SEM)

Scanning electron microscope (Hitachi S3400 Japan) was utilized to check the surface morphology of chrysin and chrysin loaded phytosomes. Photomicrographs were obtained by placing the dried samples which was coated with 5 nm gold.

FTIR Analysis

FTIR spectroscopy was utilized to determine molecular states of CLP, pure chrysin and soya lecithin (Shimadzu, Japan). After collecting the background, sufficient quantity of samples was administered above the crystal surface and gripper was set down on above the sample by turning it until a 'click' sound was heard to verify the gripper is in constant contact with the sample. Scanning was performed and the process was repeated for every individual sample respectively.

In vitro activity on HT-29 Cells

Cell Culture

Cell line of human colorectal cancer that is HT-29 was procured from NCCS Pune and grown in McCoys 5A+2mM medium at 37°C and a pH of 7.4 supplemented with 0.25% trypsin under humidified atmosphere of 5% carbon dioxide. These cells were then subcultured in McCoys 5A+2mM medium with an average density of 1-3x10,000 cells/cm^2.

Cell viability

MTT [3- (4,5-dimethylthiazol-2-yl) - 2,5- diphenyltetrazolium bromide] assay were carried out according to previously published method. A 96-well plate was taken and 200 µl of HT-29 cells was seeded. It was then incubated at a temperature of 37°C for 48 hr under 5% CO_2. Solution of pure chrysin and chrysin loaded phytosome solution were administered into the wells in the concentrations of 20,40,60,80,100 and 200µM. MTT solution (2ml) that is 5 mg/ml was added to individual well. It was incubated at temperature of 37°C under 5% CO_2. The medium which was present in the well was replaced with 2 ml of dimethyl sulfoxide after incubating it for 4 hr and the optical density was measured at

570 nm. Calculation of percentage of cell viability with pure chrysin and CLP was carried out.

RESULTS

Formulation and Structural Characterization

The composition of phospholipid: phytoconstituent that soya lecithin and pure chrysin in the ratio of 2:1 showed better formulation.

Visual Observation, Particle Size Distribution, Polydispersity Index and Zeta Potential

The dispersion of CLP in water appeared to be yellow coloured with a slight turbidity. Suspended particulate matter was absent in the dispersion. Chrysin loaded phytosomes exhibited a particle size of 94.40nm. The polydispersity index showed uniformity in the particle size distribution that is 0.31. Zeta potential of CLP was found to be -1.33 mv.

Drug Entrapment

The quantity of chrysin embedded within the 1ml of chrysin loaded phytosome was found to be 164.9 microgram respectively and the percentage of entrapment efficiency was found to be 74.28%.

Scanning Electron Microscopic Examination

The morphology of chrysin (Figure 1a) and chrysin loaded phytosomes (Figure 1b) was revealed by scanning electron microscopy.

Fourier Transformed Infrared Spectroscopy

Evaluation of the molecular states of pure chrysin, soya lecithin and chrysin loaded phytosomes was done using FTIR analysis. The bands at 1649.19, 1579.75, 3529.85-3287.11 in chrysin is assigned to C=O stretch, C=C and OH group. Similar peaks were seen in CLP and is shown in Figure 2.

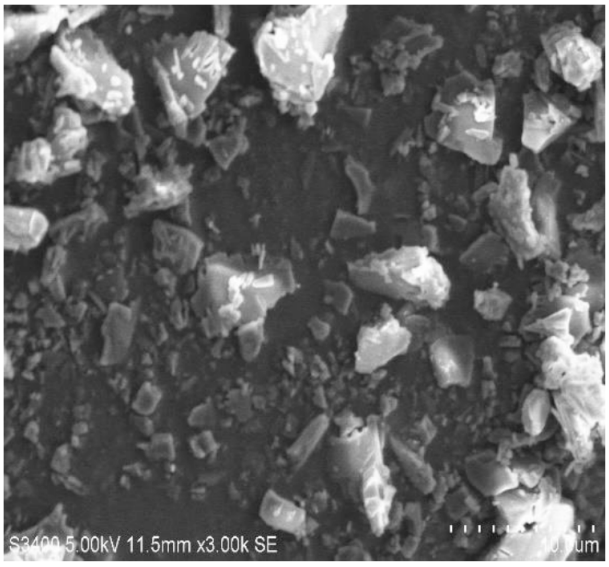

Figure 1a: SEM image of chrysin.

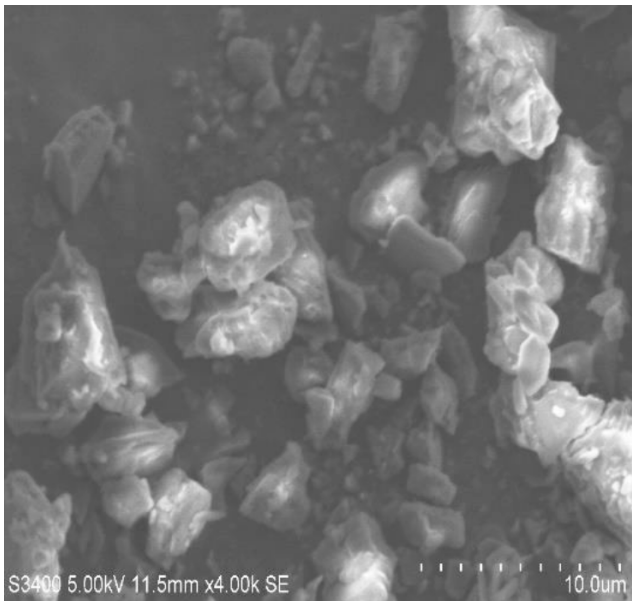

Figure 1b: SEM image of chrysin loaded phytosome.

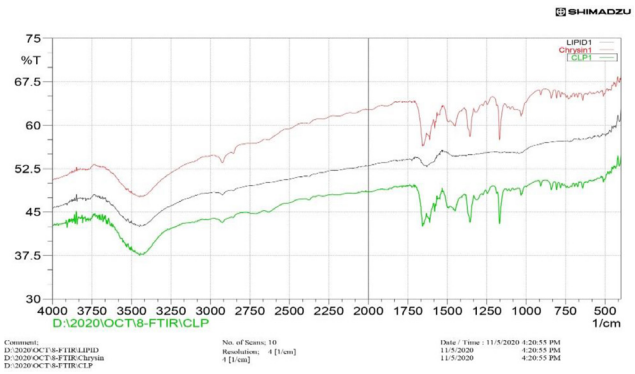

Figure 2: FTIR Image of chrysin, soya lecithin and chrysin loaded phytosomes.

In vitro study on HT-29 Cells

Cell Viability

MTT assay showed that pure chrysin solution and CLP ($p < 0.05$) showed the cytotoxic effect on HT-29 cells when compared with normal control group. The inhibition of cell viability significantly increased in CLP when compared to chrysin solution under all concentrations that is 20–200 µM with $p < 0.05$. Both the treatment group showed a raise in HT-29 cell viability in a dose dependent manner. IC_{50} value for chrysin and CLP was found to be 53.21 and 17.92 respectively and is shown in Table 1.

Table 1: Result of MTT assay.

Compound	Concentration	% Viability	IC_{50} value
Chrysin	200	46.4264082	53.21
	100	57.3894609	
	80	64.3549364	
	60	79.0732889	
	40	81.5263477	
	20	82.1017565	
Chrysin loaded phytosomes	200	38.5466617	17.92
	100	43.7845813	
	80	54.9612689	
	60	57.1007008	
	40	63.1132423	
	20	74.6587975	

DISCUSSION

Nowadays the prevalence rate of colorectal cancer is increasing vigorously.[12] This might be because of various factors like consumption of processed food,[13] which contains low fiber diet and also contains preservatives which is also known to be a cause of colon cancer,[14] and sedentary lifestyle.[15] Oxidative stress is another factor known to produce colon cancer.[16] Thus it important to come up with a treatment for colon cancer. Now there are number of drugs which are being used for the treatment of colorectal cancer such as 5-flurouracil, capacitabine etc.[17] But there are multiple adverse effects observed when using such drugs like hepatotoxicity.[18] Thus it is of utmost importance to come up with a treatment for colon cancer which does not cause any adverse or toxic effect. Through literature review,[19] we found that herbal constituents play a crucial role for the treatment of cancer,[20] and therefore we selected a phytoconstituent that is chrysin which is an flavonoid and known to have anticancer effect on colon cancer. The major problem seen with respect to chrysin administration was that it had poor bioavailability due to its low lipid soluble property.[21] Thus it is of utmost importance to increase the bioavailability of chrysin so that a better therapeutics effect can be achieved. To encounter this low bioavailability problem, we incorporated chrysin into the phytosomal form. In the current study, the chrysin loaded phytosomes were prepared by anti-solvent precipitation technique,[22] by incorporating soya lecithin and pure chrysin in the ratio of 2:1. Characterization of the chrysin loaded phytosomes was done. However, in the present work, we prepared phytosomes by incorporating pure chrysin and soya lecithin. Formulation of chrysin loaded phytosomes was done in the molar ratio of 1:1, 1:2 and 2:1. Better results were obtained in the molar ratio of 2:1 (phospholipid: chrysin). Nanotrac was utilized to determine the size of the formulation and polydispersity index and it was found to be 94.40 nm and 0.31 which revealed that the prepared formulation was within the nano range and also there was reduced prevalence rate of flocculation. Zeta potential of Chrysin loaded phytosomes was determined using Zetasizer Ver. 7.11 (Malvern) and it was found to be -1.33mv which determines better stability in acidic medium.

As seen in the present research study, smaller particle size were observed for chrysin loaded phytosomes than chrysin alone.

In scanning electron microscope, an irregular arrangement of bright particles were observed in pure chrysin (Figure 1a) with an less defined morphology and scattered distribution of particle size. In case of chrysin loaded phytosomes (Figure 1b), a well- defined arrangement in the structure was seen in which bright particles that is chrysin were engulfed by phospholipids. FTIR analysis was carried out to determine molecular states of CLP and pure chrysin. Bands at 1649.19, 1579.75, 3529.85-3287.11 in chrysin was assigned to C=O stretch, C=C and OH group. Similar peaks were observed in CLP. From the given spectra, it can be concluded that pure chrysin and CLP had no difference in the internal structures and the confirmation of these samples at the molecular level.

In this study, HT-29 cell lines were selected as colorectal cancer cells and in order to examine the efficiency of pure chrysin and chrysin loaded phytosome, the cytotoxicity test that is MTT assay was done. In HT-29 cells, the chrysin loaded phytosomes showed IC_{50} value of 17.92 µg/ml whereas pure chrysin showed IC_{50} value of 53.21 µg/ml. Similar result was observed in the study.[23] They also found that cytotoxicity of phytoconstituent loaded in phytosomal form was more when compared to the free drug[24] and it may be because of the reduced particle size of chrysin loaded phytosomes which enhanced the penentration of chrysin loaded phytosomes through the cell wall because of which there was maximum inhibibitory cytotoxicity was observed in the solution of chrysin loaded phytosomes when compared with pure chrysin alone.

CONCLUSION

The purpose of this research was to formulate, characterize and evaluate the cytotoxic effect of chrysin loaded phytosomes against HT29 cells. Looking into the results, we can say that formulation of chrysin loaded phytosomes in the ratio of 2:1 showed better cytotoxic

effect against HT29 cells and this provides indication for the use of Chrysin loaded phytosomes in experimental animals to further gain in depth analysis for anticancer activity of chrysin loaded phytosomes against colorectal cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

ABBREVIATIONS

CRC: Colorectal cancer; **CLP:** Chrysin loaded phytosomes; **HT-29:** Human Colorectal Adenocarcinoma Cell Line; **FTIR:** Fourier Transform Infrared; **NCCS:** National Centre for Cell Science.

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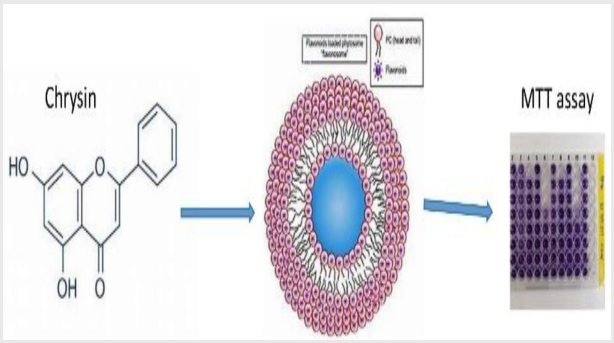
PICTORIAL ABSTRACT



SUMMARY

Chrysin loaded phytosomes were formulated by antisolvent precipitation technique. Particle size distribution, polydispersity index and zeta potential were determined. The amount of phytoconstituent entrapped within phytosomes was determined using High performance thin layer chromatography. Photomicrographs of chrysin and chrysin loaded phytosomes were obtained by scanning electron microscopy. FTIR spectroscopy was utilized to determine molecular states of Chrysin loaded phytosomes, pure chrysin and soya lecithin. Cytotoxicity of chrysin and chrysin loaded phytosomes were determined using MTT assay on colorectal cancer cell

About Authors

Mr. Namit Kudatarkar is presently working as an Assistant Professor, Department of Pharmacology, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi. His areas of research include screening of phytoconstituents and drugs for cancer using *in vitro* and *in vivo* studies.

Prof. (Dr.) Sunil Jalalpure is presently working as a Professor and Principal, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi. His areas of research include isolation/ characterization of active principles from medicinal plants and their pharmacological screening for various biological activities and training the research students in Pharmacognosy, Phytochemistry and Biotechnological aspects with modern tools and techniques. He is actively involved in nanoparticles drug delivery system of herbal phytoconstituents and green nanotechnology.

Dr. Bhaskar K. Kurangi is presently working as an Assistant professor, Department of Pharmaceutics, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi. His research areas include the design and evaluation of novel drug delivery systems, development of analytical and bioanalytical techniques using HPLC, HPTLC and the application of nanoparticulate drug delivery in prophylactic and therapeutic treatment of cancer.

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