“FORMULATION, CHARACTERIZATION AND PHARMACOLOGICAL EVALUATION OF CHRYSIN LOADED PHYTOSOMES IN COLORECTAL CANCER IN WISTAR RATS”

Thesis Submitted to

The KLE Academy of Higher Education and Research, Belagavi

(KLE DEEMED UNIVERSITY)
[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide

Govt. of India Notification No.F.9-19/2000-U.3 (A)]

[Accredited ‘A+’ Grade by NAAC (3rd Cycle)]
[Placed in category ‘A’ by MoE (GoI)]

For the award of the degree of

DOCTOR OF PHILOSOPHY

IN

THE FACULTY OF PHARMACY

By

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ACKNOWLEDGEMENT

I would like to thank great almighty and all those numerous individuals whose blessing, continuous support and enthusiasm kept my vision alive.

I would like to sincerely thank my research guide, **Prof** .(**Dr). Sunil Jalalpure** for his expertise, support and helping me develop my knowledge of research strategies and provide encouragement leading to completion of this thesis.

I wish to express my deep sense of gratitude to entire staffs at Dr. Prabhakar Kore Basic Science Research Centre and KLE College of Pharmacy, Belgaum for providing all necessary support required during the course of study.

My sincere thanks to Dr Bhaskar Kurangi for his constant support and encouragement during various stages of research work.

I whole heartedly thank my colleagues and batch mates who have directly or indirectly helped me in my project.

Most importantly, I would like to express my huge gratitude to my parents who were always supportive and encouraged me through all difficulties and challenges.

Thanking you, one and all.

Date: ***Mr. Namit Kudatarkar***

Place:

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LIST OF ABBREVATIONS

ACF	Abberant crypt foci
AOM	Azoxymethane
APC	Adenomatous polyposis coli
CLP	Chrysin loaded phytosomes
CMC	Carboxymethyl cellulose
CRC	Colorectal cancer
DC	Diseased control
DMEM	Dulbecco modified eagle medium
DMH	Dimethyl hydrazine
DMSO	Dimethylsulfoxide
5-FU	5-Flurouracil
MMR	Mismatch repair
NC	Normal control
NCCS	National centre for cell science
PBS	Phosphate buffer solution
STD	Standard

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ABSTRACT

Background

Chrysin is a phytoconstituent belonging to flavonoid category which exhibits various pharmacological activities including anti-cancer. However, the oral bioavailability of chrysin is severely low due to its poor aqueous solubility, extensive first pass metabolism and stability problems.

Objectives

The main objective of the present study is to formulate and characterize chrysin loaded phytosomes and pharmacologically evaluate its anticancer activity for the treatment of colorectal cancer in Wistar rats.

Methodology

Chrysin loaded phytosomes were prepared using antisolvent precipitation technique. respectively. The prepared formulations were characterized for particle size, polydispersity index, zeta potential, entrapment efficiency, surface morphology, transition temperature, polymorphic state, compatibility between chrysin, soya lecithin and chrysin loaded phytosomes. In *in vitro* studies, MTT assay and DPPH assay was carried out. Toxicity study was carried out according to OECD guideline 423. Finally, the colorectal cancer was induced in male Wistar rats using Dimethyl hydrazine and various parameter like polyps count, colon weight by length ratio, hematological parameters like red blood cells, white blood cells, platelets, hemoglobin, monocytes and lymphocytes and liver enzyme estimation like AST, ALT and ALP were carried out. At last the samples of colon were screened for histopathological changes.

Results

Chrysin loaded phytosomes were prepared and the particle size was found to be 91.40nm with polydispersity index of 0.29 and zeta potential value of -1.33mv. The amount of pure chrysin entrapped within the phytosome was found to be 74.28%. Pure chrysin showed an irregular arrangement of bright particles with ill defined morphology and a wide particle size distribution, wheras chrysin loaded phytosomes displayed a more defined structural arrangement in which bright particles that is chrysin were engulfed by phospholipids. The results of differential scanning calorimetry (DSC) and X-ray diffraction (XRD) study indicated that chrysin was in amorphous state when encapsulated into phytosomes. FTIR analysis concluded that there was no difference between the internal structures and confirmation of these samples at the molecular level.

In vitro cytotoxicity and anti oxidant study showed that chrysin loaded phytosomes had significantly higher antioxidant activity and cytotoxicity in HT29 cells, compared to pure chrysin. There was no toxicity seen in the dose of 2 gram per kilogram and hence one $1/5^{th}$, $1/10^{th}$ and $1/20^{th}$ dose was considered for the study. Invivo studies showed that chrysin loaded phytosomes was able to bring back the malignancy condition to normal condition in a dose dependent manner.

Conclusion

The present research concludes that chrysin loaded phytosomes can be a better option for increasing the bioavailability of the phytoconstituents which leads to a better therapeutic anticancer agent for the treatment of colorectal cancer.

Keywords: Chrysin; colorectal cancer; phytosomes; nanoformulation.

1. INTRODUCTION:

1.1 BACKGROUND OF RESEARCH:

According to data published by GLOBOCAN, the third most fatal and fourth-most diagnosed cancer in the world is colorectal cancer (CRC) [1]. Because of sedentary ways of living in addition to consuming processed foods with little fiber, the incidence rate of CRC is increasing in emerging nations [2]. If CRC is discovered at an early stage, chemotherapy can be used as a treatment. However, it has been discovered that these chemotherapy medications have a number of side effects, making the danger outweigh the advantages. It is advised to use alternative medications, such as herbal remedies in the form of pure phytoconstituents or extracted crude drugs, to avoid the side effects of chemotherapy treatments. One phytoconstituent, chysin, belongs to the flavonoid class and is known to have anticancer activities in numerous forms of cancer. It is also known as 5'7 dihydroflavone. Passion flower, propolis, and honey are the primary phytoconstituents that contain chysin [3]. Reviews of previously published scientific research have demonstrated that chysin has a superior cytotoxic impact on a variety of colorectal cancer cell lines [4] and in in vivo tests [5]. Wistar rats were used in one study, which found that "cis-diamminedichloroplatinum-induced deterioration of the goblet cells in the crypts of the colon was protected by phytoconstituent Chrysin which was induced by cisplatin through amelioration of oxidative stress and apoptosis" [6]. The phytoconstituent chyrsin has been shown to be beneficial in the treatment of colorectal cancer [7].

However, investigations have shown that because chysin has a very low bioavailability, a higher dose is needed to treat colorectal cancer [8]. A new nanoformulation called phytosomes helps reduce the size of the phytoconstituent, which increases surface area and, as a result, enhances permeability. [9]. Studies contend that putting a medication into a phytosomal form improves its stability [10]. This work's primary goal is to develop, define and pharmacologically assess the anticancer activity of chrysin-loaded phytosomes in Wistar rats because there are currently no research being done to investigate the phytosomal version of chrysin's possible anticancer properties.

1.2 REVIEW OF LITERATURE:

Colorectal cancer

Large intestine and rectum cancer is referred to as colorectal cancer. According to research, colorectal cancer begins as a tiny polyp. Adenomatous polyps and inflammatory polyps, which operate as risk factors, are the two types of polyps. Some of these polyps eventually develop into cancer. (11).

Types of tumor depending on the origin

Table 1

Type	Origin
Adenocarcinoma of colon	Cancer of gland cells present in the lining of colon wall
Carcinoid tumor of intestine	an intestinal cancer that affects the specialized hormone-producing cells.
Stromal tumour of the digestive system	Cancer of intestinal cells
Lymphoma of intestine	Cancer of lymphatic system cells
Sarcoma of colon	Cancer of blood vessels, muscles and connective tissues from the wall of colon and rectum
Squamous cell colon cancer	Cancer of the squamous cells of colon lining

Depending on cancer risk and hereditary effect

1) Sporadic CRC: Hereditary influence is absent

2) Familial CRC: A family with at least one case of CRC without a germ-line mutation or distinctive pattern.

3) Hereditary CRC: Inherited mutation of the germ line

2.2. Colorectal cancer warning signs and symptoms include:

- A change in bowel habit
- constipation
- Rectal bleeding
- diarrhea
- Persistent abdominal discomfort
- A feeling of unemptied bowel
- Weakness or fatigue
- Weight loss

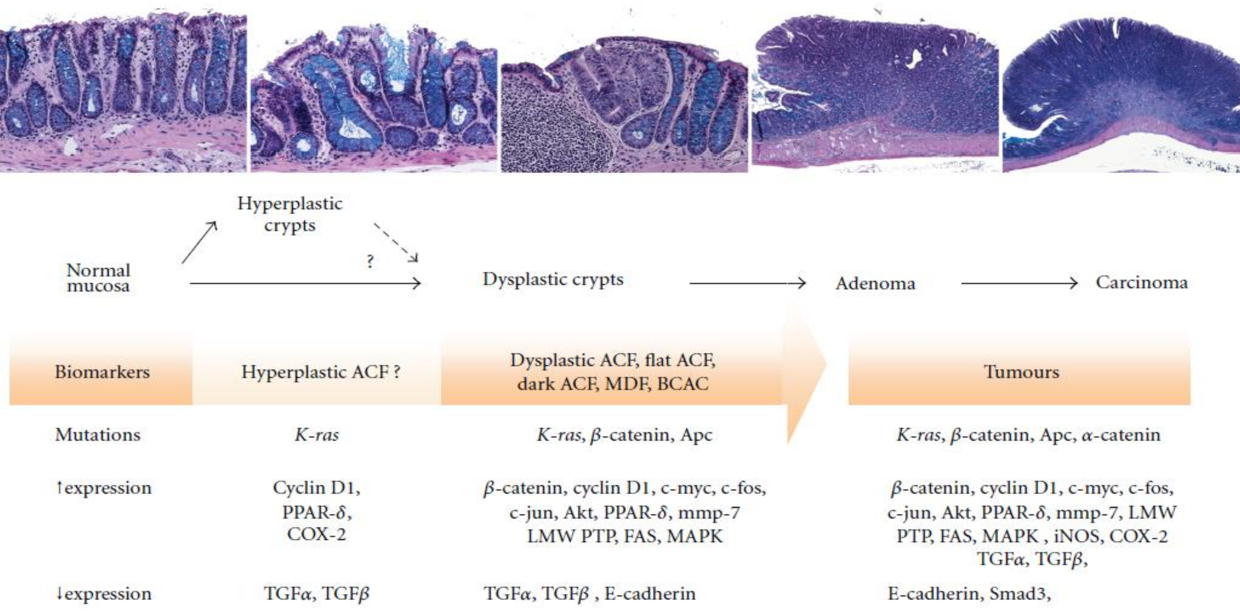
Risk factor for colorectal cancer

Among the elements that increase the risk of colorectal cancer are the following ones:

- A person having a family history of colorectal cancer.
- Diseases such as Crohn's disease and ulcerative colitis.
- Inherited genetic conditions like hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis.
- A diet with little fiber and lots of calories.
- A sedentary way of life.
- People with diabetes and insulin resistance have an increased chance of developing colorectal cancer.
- Obesity.
- Smoking.
- Alcohol.

Pathogenesis of colorectal cancer

Figure 1



Perše & Cerar, 2011

2.5. Diagnosis of colorectal cancer

Following tests are performed to diagnose colorectal cancer.

FOBT (fecal occult blood test)

Sigmoidoscopy

Colonoscopy

Computerized tomographic colonography.

Stages of colorectal cancer

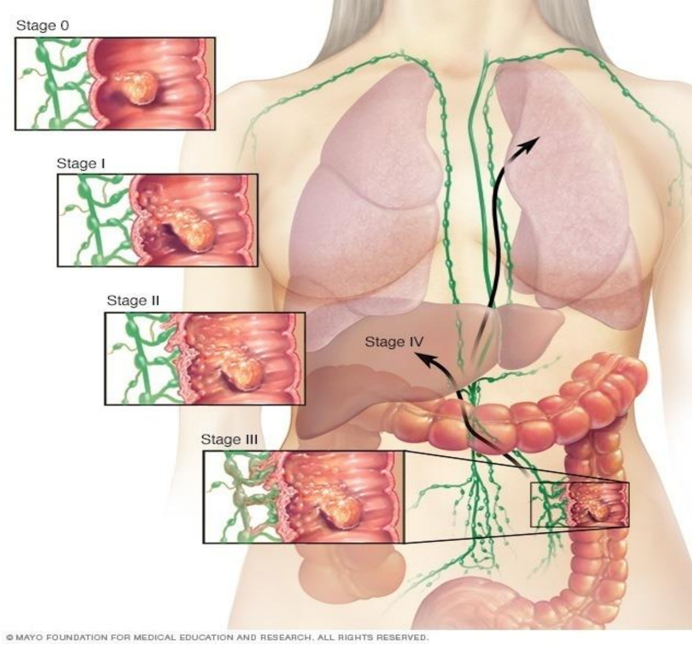


Figure 2

Stage 0.- The Stage 0: The illness has become ingrained in the rectum or colon lining. Therefore, colonoscopy-based polypectomy or surgery (if the lesion is too large) are the two most prevalent forms of treatment.

Stage 1: In this stage, the illness has penetrated the intestinal wall but not spread outside of it. The usual course of action in this situation is a colectomy alone.

Stage 2: At this point, the disease has gone into the tissue around the large intestine's muscular layers (stage 2B) and into nearby tissue (stage 2C), but it hasn't yet spread to the lymph nodes.

Stage 3: Lymph nodes have been affected by the cancer's spread at this point.

Stage 4: The liver, ovaries or lungs are among the distant organs to which the cancer has metastasized or spread at this time. In this situation, the tumor may be treated with chemotherapy, cryotherapy, or radiofrequency ablation (heat destruction).

Management of colorectal cancer

Various treatments are employed depending on the stage of colorectal cancer, including

Surgical removal of colorectal cancer

It is the typical course of treatment for colorectal cancer at all stages. There are two forms of surgery: colon resection with anastomosis and local incision. By maintaining a tube with a cutting instrument through the rectum and into the colon, a local incision describes the removal of the cancer. The procedure is referred to as a polypectomy if malignancy is found within the polyps. The healthy portion of the colon is sewed back together after the cancer and a small quantity of surrounding healthy tissue have been removed during resection of the colon with anastomosis. A partial colectomy is another name for it. Only if the cancer is large is this type of treatment performed.

Radiofrequency ablation of colorectal cancer

This method entails inserting a unique probe with small electrodes designed to destroy cancer cells. The probe is put straight into the skin after receiving local anesthesia. In a different scenario, the probe is introduced through an abdominal incision while the patient is under general anesthesia.

Cryosurgery of colorectal cancer

During this procedure, a device that freezes and kills aberrant tissues is used.

Radiation therapy for colorectal cancer

In order to eradicate cancer cells, this treatment uses radiation, such as x-rays. Radiation treatment includes internal and exterior radiation therapies. During external radiation therapy, a machine emits radiation outside the body. A radioactive chemical is directly administered into the malignancy during internal radiation therapy using wires, seeds, needles, or catheters.

Colorectal cancer chemotherapy

It includes

- 5-Fluorouracil (5-FU)
- Capecitabine, As soon as it touches the tumor, it transforms into 5-FU.
- Irinotecan
- Oxaliplatin
- Tipiracil and Trifluridine.

2.8. Preclinical models to assess the anticancer activity in colorectal cancer

Animal models for colorectal cancer

The most ideal animals for inducing colorectal cancer are rodents (rats and mice). For chemically induced models, rats are recommended, whereas mice are favored for genetically modified models.

Mouse colorectal cancer models

In gene related models mice are widely used. Some of the gene related models include Adenomatous polyposis coli (APC), TGF-B models, mismatch repair (MMR) deficient models, inflammation mediated models, carcinogen-induced models and immune system related models.

APC (Adenomatous polyposis coli) gene related models: Small intestinal polyps and sporadic cancer appeared in APC Min/+ mouse models. The colon practically has no tumors in the C57BL/6J mice background (12). Even though this results in familial adenomatous polyposis in humans, the majority of colon adenomas progress to aggressive adenocarcinomas. Human intestinal neoplasms and the APC - Min/+ models

are equivalent in terms of phenotype and histological. In order to investigate therapeutic and chemo preventive approaches, these models are employed. They aid in comprehending the function of the APC gene in colorectal cancer. (13).

Mismatch repair (MMR) deficient models: DNA mismatch repair (MMR) genes such as MLH1, MSH2, MSH6, and PMS2 are inactivated in hereditary non-polyposis colorectal cancer, an inherited condition that affects a range of malignancies, including colon cancer.

TGF-B models: Mice lacking RAG2/TGF-B1 showed a significant increase in colon and cecum cancer incidence. Thus, this model was used to investigate how inflammation in CRC relates to TGF-B1 signaling (15). After exposure to the carcinogen, it was discovered that mice lacking TGF-B1 in the colonic epithelium developed more adenomas and adenocarcinomas.

Inflammation mediated models: When mice were given dextran sulphate sodium (DSS), it caused persistent colitis and the development of high-grade dysplasia (16). Crohn's disease and ulcerative colitis increase the risk of CRC (17). These provided evidence that continuous inflammation had a part in the emergence of colorectal cancer. Intestinal tumorigenesis was brought on by the injection of AOM and DSS together. Furthermore, the JAK/STAT and NFκB pathways in inflammation induced CRC can also be defined by combined AOM/DSS model (18).

Immune system related models: To better understand how the immune system contributes to the development of colorectal cancer, models have been created.

Major contributors to the inflammatory response include immune cells. CRC and inflammation are closely connected. Therefore, IL-2 and IL-10 knockout animals were created in order to research the function of dietary factors and inflammation in the development and beginning of colorectal cancer. (19).

Colon cancer xenograft models: Xenograft models of CRC are frequently used to analyze the pathogenesis of human illness as well as to evaluate more recent treatments. They are produced by implanting the cells under the skin, inside the spleen or inside the renal capsule. The xenograft are implanted into the immunosuppressed mice. The NOD-SCID mice or t-cell deficient nude mice are frequently employed (20). The fact that subcutaneous xenografts don't require anesthesia and the tumors are simple to access for external monitoring makes this
model advantageous for CRC research. The subcutaneous model has the drawbacks of lacking a tumor microenvironment typical of CRC and not showing metastases in subcutaneous xenograft animals. In contrast, it has been discovered that intrasplenic or intrarenal metastasis in xenograft models has metastasis that is identical to human CRC.

Orthotopic model: Tumor tissue or colorectal cancer cells are injected into the intestinal submucosa and are linked to all angiogenic, proliferation, and cytokine factors, as well as every component of the tumor microenvironment. The subcutaneous model and this paradigm are similar. (21).

Additionally in terms of metastasis and microenvironments, it is similar to human CRC. The impact of alterations in the microenvironment on the emergence and development of tumors can also be assessed using the orthotropic model. This model has the disadvantage of requiring more specialized knowledge and time than subcutaneous models.

Rat models for screening new chemical entity or substance to treat colorectal cancer: Common carcinogens like azoxymethane (AOM), 1,2-dimethylhydrazine are used for developing the rat model of colon cancer. In addition to AOM and methyl azoxy methanol, various carcinogens such nitroso methyl urea (MNU) and 7- hydroxy-7 - methoxy -4,4 -bis(1,3 - benzodioxole) -5,5 -dicarboxylic acid dimethyl ester (DMBDD) are frequently utilized in the development of tumors in rat models.

Pathophysiology of dimethyl hydrazine (DMH) induced colorectal cancer in animals

Figure 3, The process of DMH's biotransformation is described in Figure 3. DMH is specialized for the colon, and AOM has been helpful in the development of malignancies. DMH is utilized to comprehend the molecular pathways driving sporadic colorectal carcinogenesis (22). The Wnt/-catenin pathway is altered by carcinogen-mediated cancers (23). Mutations observed in the Ctnnb gene was found to be the primary cause of AOM-induced colorectal carcinogenesis which generates

catenin and promotes the growth of colorectal adenomas as well as the overexpression of proliferation markers such cMyc and cyclin D1 (24).

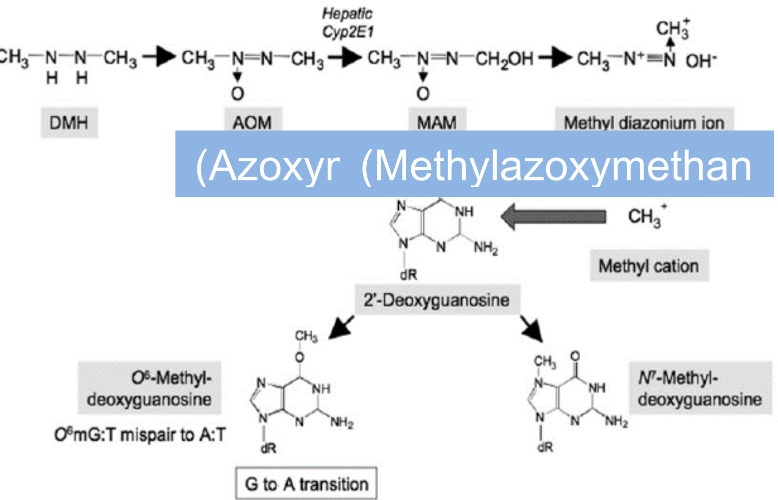


Figure 3 **Rosenberg et al, 2009**

Chrysin- its availability, uses and problem of chrysin when given orally

Chysin (5,7-dihydroxyflavone) is a naturally occurring flavone found in several plant extracts, such as propolis, honey, and blue passion flower (Passiflora caerulea), all of which have important medicinal and commercial value. (25). Numerous biological effects of chysin have been demonstrated, including antioxidant and anti-inflammatory effects (26), antibacterial (27), antihypertensive (28), anti-allergic (29), vasodilator (30), antidiabetic (31) anxiolytic (32), antiviral (33), anti-estrogenic (34), hepatoprotective (35), anti-aging (36), anticonvulsant (37), and anticancer effects (38), according to literature reports. Pharmaceutical investigations revealed that the largest difficulty in in vivo efficacy tests is the oral bioavailability of chrysin. Chrysin's absolute oral bioavailability was estimated to be less than 1% (39). Chrysin has poor aqueous solubility and a quick metabolism in the GI tract and liver because it is a good substrate for the phase II metabolic enzymes UGTs and SULTs that mediate glucuronidation and sulfonation, respectively.

Additionally, several efflux transporters, including as MRP2 and BCRP, are good substrates for chrysin glucuronide and sulphate, facilitating their removal through urine and faeces. Pharmaceutical studies showed that chrysin's oral bioavailability is very low (1%), as will be explained in more depth below, due to its poor water solubility and significant metabolism (such as glucuronidation and sulfonation). Accordingly, in vivo efficacy trials without pharmacological analysis could lead to incorrect conclusions about chrysin. Future clinical investigations should define the maximum chrysin absorption dose and in vivo chrysin exposure in order to optimize the dosing regimen based on in vivo exposure. For a more precise evaluation of chrysin's in vivo effectiveness, it should also be taken into account to give chrysin in certain formulations (such as nanoparticles) that can boost its oral bioavailability. It's interesting to see how

chrysin works to reduce the GI toxicity caused by irinotecan. Despite having very little systemic exposure, chysin undergoes remarkable enterohepatic recycling, resulting in recurrent exposure in the GI tract. Chrysin's poor oral bioavailability but frequent GI tract exposure may be useful in treating illnesses or drug-induced toxicity in the GI tract since it could achieve local efficacy with no or few off-target side effects. For the lower GI tract, chrysin can be compared to a drug with a local delivery method (40).

Nanoparticles as cancer therapy medication delivery systems:

In comparison to traditional herbal extracts, phytosomes are more advanced herbal formulations that are more absorbed, producing improved bioavailability and effects. (41).

When plant extracts and phosphatidylcholine (or any other hydrophilic polar head group) combine in an aprotic solvent, a complex of phospholipids and naturally occurring active phytochemicals are bound in their structures (42). The existence of a chemical link between phospholipids and molecules of flavonoid vegetal derivatives was initially acknowledged by Bombardelli et al (43). The stoichiometry of the supramolecular adducts that phospholipids and polyphenols generate can be ascertained using thermal analysis. This attribute was investigated by Semalty et al. They found that hydrophobic interactions, or hydrogen bond formation, resulted from interactions between the two molecules (44). Between the polar head and the active ingredient's polar functions, the phospholipid-active ingredient is in charge of forming a hydrogen connection (45).

What are the drugs which are available in phytosomal form?

Silybin Phytosome TM, Ginkgo Phytosome TM, Panax Ginseng Phytosome TM, Green Tea Phytosome TM, Super Milk thistle Extract Grape seed (PCO) phytosomes, Hawthorn Phytosomes, Centella Phytosome. (46)

What are the advantages of phytosomes over other types of nanoparticles? (47)

The advantages of phytosomes over typical herbal formulations include:

I. It shows enhanced bioavailability, enhancing lipid insoluble polar phytoconstituent absorption when used topically or orally, leading to significantly higher therapeutic benefit.
II. When an active constituent's absorption is improved, less of the substance is needed.
III. The phosphatidylcholine molecule and phytoconstituent form chemical bonds, which leads to the phytosomes' better stability profile.
IV. Phospholipids have additional nutritional benefits.
V. Profound drug entrapment.

Advantages of nanoparticles for which they are used in cancer therapy (48) (49):

1. Reduced drug concentration in healthy tissues which reduces chemotherapeutic medication’s negative effects.
2. The drug concentration in the tumor is increased by the selective aggregation of nanoparticles at tumor sites due to the EPR effect.
3. High pay load of drugs in nanoparticles.
4. High organ drug concentration.
5. Prolonged and site-specific drug delivery.
6. Reduces the required dose with maximum therapeutic effect.
7. Non-toxic degradability, better patient compliance and relief.

1.3 JUSTIFICATION FOR THE STUDY:-

Due to the westernization of lifestyle and eating habits, colorectal cancer incidence rates are rising in India. Although there are many chemotherapies for treatment of colorectal cancer, it has been seen that severe adverse effects and multi-organ toxicity are the major problem due to which the success rate of drugs as an anticancer agent is low. To overcome these limitations, more preferences are given to herbal formulation as these formulations are found to have a minimal adverse effect when compared to the chemotherapeutic drugs. Chrysin is a phytoconstituent belonging to flavonoid category. Treatment for cancer, AIDS, diabetes, inflammation, and aging of the skin has shown it to be therapeutically effective. There are both invitro and in vivo studies which shows that chrysin possess anticancer activity. The major problem of administrating chrysin orally was that the oral bioavailability of chrysin in pure form was low due to which administration of chrysin in pure form showed less therapeutic effect as an anticancer agent. This highlights the requirement for creating a unique medication delivery mechanism with the aim of enhancing oral bioavailability and targeting the tumor in the affected region resulting in decrease in adverse effects and multiorgan toxicities. The rational use of nanoparticles for targeting tumors is mainly dependent on the capability of nanoparticles to deliver the therapeutically effective dose of drug in the region of the tumor targets and also minimal exposure of healthy tissues to drug is limited by nanoparticles which decreases the damage caused by the drug to healthy cells. Ideally, a delivery system of drug should be such that it should quickly respond to physiological needs, sense the changes and accordingly alter the drug- release from the system.

As a carrier, ideal nanoparticles are required to possess high percent drug loading as well as delivery of drug specifically to the pathological region without any leakage of drug on the way, while quickly unload the drug at the target site and also increased oral

bioavailability. In this context, phytosomes are found to have better qualities compared to other forms of nanoformulations to achieve increased oral bioavailability and improved medication release at the target location. Therefore, adding a phytoconstituent to phytosomal form is advised to boost the drug's oral bioavailability. With the aim to further increase the oral bioavailability of the drug, phytoconstituent chrysin were incorporated into phytosomal form. There are no research being conducted to determine the phytosomal form of chrysin's anticancer efficacy against colorectal cancer.

To overcome the pharmacokinetic and pharmacodynamic limitations, phytosomes, a novel nanoformulation, incorporates chysin. Determining the anticancer effects of Chrysin loaded phytosomes in Dimethyl hydrazine caused colorectal cancer in Wistar rats was the purpose of the current investigation. A delivery system of drug should be such that it should quickly respond to physiological needs, sense the changes and accordingly alter the drug- release from the system. As a carrier, ideal nanoparticles are required to possess high percent drug loading as well as delivery of drug specifically to the pathological region without any leakage of drug on the way, while quickly unload the drug at the target site and also increased oral

bioavailability. In this context, phytosomes are found to have better qualities compared to other forms of nanoformulations to achieve increased oral bioavailability and enhanced release of drug at the target site.

Therefore, adding a phytoconstituent to the phytosomal form is advised to boost the medication's oral bioavailability. With the intention of further boosting the medication's oral bioavailability, phytoconstituent chrysin were incorporated into phytosomal form. Studies to determine the phytosomal version of chrysin's anticancer efficacy against colorectal cancer are not currently being conducted.

A novel nanoformulation called phytosomes contains chysin to circumvent pharmacokinetic and pharmacodynamic limitations. Determining the anticancer effects of Chrysin loaded phytosomes in Dimethyl hydrazine caused colorectal cancer in Wistar rats was the purpose of the current investigation.

1.4 AIM, OBJECTIVES AND PLAN OF WORK:

Aim of the study:

Formulation, characterization and pharmacological evaluation of chrysin loaded phytosomes against colorectal cancer in Wistar rats.

Objectives of the study:

1. To formulate and characterize chrysin loaded phytosomes
2. To evaluate the anticancer activity of chrysin loaded phytosomes using HT29 cells.
3. To perform oral acute toxicity study.
4. To evaluate the anticancer activity of chrysin-loaded phytosomes in the treatment of colorectal cancer in Wistar rats.

Plan of work:

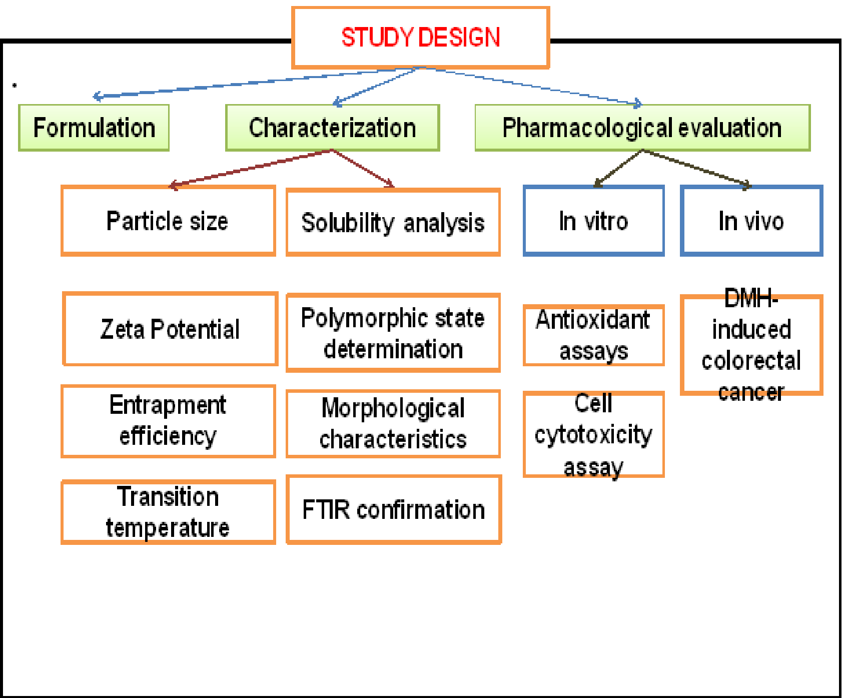


Figure 4

2. MATERIALS AND METHODS :

MATERIALS :

The materials listed below were either of the best grade available or were of the AR/LR grade as given by the manufacturer.

Table 2. List of chemicals used and supplier.

Sr No	Chemicals	Company
1	1,2-Dimethyl hydrazine	Tokyo chemical industries,Japan
2	Dimethyl Sulfoxide	Merk Inc, India
3	Disodium hydrogen phosphate	Sigma Aldrich, USA
4	Ethanol	Merk Inc, India
5	Potassium hydroxide	Sigma Aldrich, USA
6	Sodium dihydrogen phosphate	Sisco lab private limited, India
7	Dulbecco's Modified Eagle Medium	Himedia, India
8	5-Fluorouracil	Zydus, India
9	MTT	NCCI, India
10	EDTA	Gibco-invitrogen, USA

Table 3. List of instruments used.

Sr No	Equipment	Company
1	Dynamic Light Scattering (DLS)	NanotracR-150, Germany
2	Differential Scanning Calorimeter	Perkin Elmer, USA
3	HPTLC	Camag, Switzerland
4	Electronic balance	Sartorius, Germany
5	X ray diffractometer	Siemens D5000, Germany
6	Fourier transform infrared spectrophotometer	Shimadzu Scientific, Japan
7	Micro pipettes	Thermo scientific , USA
8	Laminar air flow	Labline , India
9	MilliQ	Millipore
10	pH meter	Mifa systems, India
12	Sonicator	Biolinx labsystem, India
13	Tissue homogenizer	Chief Scientifich, India
14	96 well plates	Acrobiosystems, USA
15	Aluminium foil	Diamond, India
16	Centrifuge tubes	Tarsons, India
18	Elisa plate reader	Biotek, USA
19	Deep freezer	Electrolux, India
20	Automated blood cell counter	ERMA Inc, Tokyo
21	Scanning electron microscope	Hitachi-3400, Japan

METHODS:

2.1. PREFORMULATION STUDIES:

During this initial stage of dosage form formulation for drug material, a pure drug's physical and molecular characteristics alone and a pure drug coupled with excipients are investigated.

2.1.1. Identification & Characterization of Pure Drug

2.1.1.1. Identification of pure drug:

Chrysin (drug) was identified by Fourier-transform infrared spectroscopy (FTIR). The pure chrysin supplied by Tokyo chemical industry, Tokyo Japan was further confirmed by FTIR.

2.1.1.2. Standard calibration curve of chrysin (HPTLC Method) (50)

To obtain accurate results, HPTLC method was used for chrysin quantification and standard calibration curve of chrysin was plotted by using HPTLC method mentioned in USP Bulletin.

Preparation of standard stock solution and sample solution:

Five milligram of chrysin was used as standard and chrysin loaded phytosomes as sample. Both were accurately weighed and then transferred in two different volumetric flasks of 10ml and methanol was used as a solvent.

Chrysin solution with 500 microgram per ml was obtained by making up the volumes to the mark with methanol. The working solutions of stock solution were prepared by diluting them with methanol to get a working standard of 50microgram per ml of chrysin and chrysin loaded phytosomes.

Chromatographic Conditions:

Standard solution of reference chrysin of different concentrations was prepared and it was spotted as bands which were obtained on the pre-coated silica gel aluminum Plate 60 F254, using Camag Linomat 5 sample applicator having a bandwidth of 6mm using micro syringe. A twin trough glass chamber was employed for the development of plates. The mobile phase was allowed to saturate in the twin trough chamber (20 min) before the development of the plate. The Plate was allowed to run for a distance of about 80mm for the solvent system. After the plate reaches the 80mm distance, the plate is allowed to dry with the help of an air dryer. Once the plate is completely dry it is been used to analyze by the TLC scanner 4 using the wavelength and the results were interpreted from the chromatograms. The spectra of the standard are identified by scanning them under the UV-Range. The wavelength selected after scanning the plates under ultraviolet light was found to be 268 nm. The mobile phase was selected after many trials and finalized to n-hexane: ethyl acetate: methanol: formic acid in the ratio 8:8:1:0.2 v/v which showed better separation for the reference and the sample and the spectra was obtained.

2.1.1.3. Solubility determination (51):

One mg of chrysin and chrysin loaded phytosomes was taken in a test tube containing 10ml of methanol. It was ultrasonicated for 3hours.The sample was filtered through 0.45μ membrane filter and diluted suitably using distilled water and analyzed by UV spectrophotometer at 268 nm wavelength.

2.1.1.4. Determination of melting point (52):

By using a DSC analysis, the melting point of pure chrysin was determined.

2.1.1.5 Drug-Excipient Compatibility Study:

The drug with other excipient like soya lecithin (in 1:2 ratio) was subjected to storage at normal room temperature for one month. The mixtures of drug and excipients were then evaluated by IR spectra by using FTIR spectroscopy.

2.1.1.6 Differential scanning calorimetric study:

The thermal behavior of phytoconstituent Chrysin and CLP was analyzed using Differential scanning calorimeter (Perkien Elmer, USA). One aluminium pans was empty and was used as reference and in another pan, 10mg of plain chrysin,soya lecithin and chrysin loaded phytosomes sample were administered. Under inert nitrogen at a rate of 10°C/min, the pans were heated at a temperature ranging from 20–200°C. The peaks obtained were intepreted for drug excipient compatibility.

2.1.1.7 FT-IR spectroscopic studies:

Determination of the molecular states of pure chrysin, soya lecithin and CLP was done using Fourier transform infrared (FTIR) analysis (Shimadzu Scientific Instruments, Japan). “After caliberating the background, sufficient quantity of sample was administered above the clear crystal surface. The gripper was set down on top of the sample by turning it until a ‘click’ sound was heard to verify the gripper is in constant contact with the sample. Scanning was done and it was repeated for every indivisual sample respectively”.

2.2 PRELIMINARY STUDY:

Preliminary study was performed to study the effect of concentration of pure chrysin and soya lecithin on various properties like particle size, poly dispersity index (PDI) and zeta potential. To study the effect of concentration on nanoformulation, the concentration of pure chrysin and soya lecithin were altered. At first the concentration of both pure chrysin and soya lecithin was kept constant. Further the concentration of pure chrysin was doubled while keeping the concentration of soya lecithin constant. At last the concentration of soya lecithin was doubled keeping the concentration of pure chrysin constant. The compositions were as shown in the following Table 4.

Table 4. Composition for chrysin and soya lecithin for preliminary study

Ratio	Soya lecithin (mg)	Chrysin (mg)
1:1	100	100
1:2	100	200
2:1	200	100

2.3 Preparation of Chrysin loaded phytosomes :

Antisolvent precipitation technology was used to create CLP. (53). The phytoconstituent was incorporated into phytosomal form by reacting with phospholipid that is soya lecithin in ratios of 1:1, 2:1 and 1:2. Dichloromethane (30 ml) was refluxed with the medication and phospholipids in varied ratios for 3 hours at 60 degrees Celsius. The resultant solution, containing 5–10 ml, was combined with 20 ml of n-hexane while being continuously stirred to produce a precipitate. This precipitate was filtered through a Whatmann filter, dried at room temperature, and then put into amber-colored vials for storage.

2.4. Characterization of Chrysin loaded phytosomes:

2.4.1 Determination of particle size :

The size distribution of phytosomes was determined using Dynamic Light Scattering (DLS) (Nanotrac R-150 ULTRA Microtrac, Inc).

The cell of the Nanotrac was cleaned, followed by background was taken with Millipore water. 1mg of chrysin loaded phytosomes was diluted in 10ml of Millipore water. A cell containing 3ml of the material was filled, and after six measurements, the mean diameter (SD) was determined.

2.4.2. Polydispersity index (54):

The polydispersity index (PDI) was calculated using dynamic light scattering equipment. The PDI is a metric for particle size distribution width. Lower PDI values suggest monodispersed samples, while greater PDI values indicate samples with a polydisperse nature and a larger size dispersion of particles.

The following equation can be used to determine PDI.

Polydispersity = [D90 – D10]/ D50

The usual value of PDI <0.5 indicates nearly homogenous dispersion ofparticles and PDI value > 0.5 indicates heterogeneous dispersion of particles

2.4.3. Zeta Potential (55):

Using Zetasizer Ver. 7.11 (Malvern), the zeta potential of phytosomes that had been loaded with chrysin was calculated. After inserting the electrodes and placing

the cell under a microscope, the sample was added to the cell. As soon as the electrodes are attached to the Zetasizer Ver. 7.11 unit and powered up, colloids are observed in the microscope's eyepiece as they move over a grid. Simply hold down the "track" button as the colloid moves through the grid to track one. The Zetasizer Ver. 7.11 instantaneously calculates and shows the colloid's zeta potential (or electrophoretic mobility) as soon as the "track" button is released.

2.4.4. Determination of Percentage Yield.

The percentage yield of chrysin loaded phytosomes was calculated

Using the formula:

$$\% \text{ yield} = \frac{\text{Practical yield} \times 100}{\text{Theoretical yield}}$$

2.4.5. Determination of Entrapment efficiency (EE)

Ultracentrifugation was used to calculate how much chrysin was trapped inside the nanovesicular structures (45). A centrifuge tube containing an aliquot of the vesicular suspension was placed into a SorvallTM MTX 150 Micro-Ultracentrifuge (Thermo Scientific, India) and centrifuged for three hours at 4 °C at 40,000 rpm. After centrifugation, the free chrysin in the supernatant was separated from the pellet, suitably diluted, and its concentration was measured using HPTLC. To ascertain the entire chrysin content of the created nanovesicular formulations, the vesicular dispersion was lysed with methanol and sonicated afterwards. After being diluted and passing through 0.45 m filters, the solution was analyzed using HPTLC.

The entrapment efficiency percentage (% EE) was calculated as follows: EE % = (T−S)/T x 100, where T is the overall concentration of chrysin, S is the concentration found solely in the supernatant, and T-S is the concentration found inside the nanovesicles.

2.4.6 Analysis of thermal behavior (56) (57):

The thermal behavior of phytoconstituent Chrysin and CLP was analyzed using Differential scanning calorimeter (Perkien Elmer, USA). One aluminium pans was empty and was used as reference and in another pan, 10mg of plain chrysin and CLP sample were administered. Under inert nitrogen at a rate of 10°C/min, the pans were heated at a temperature ranging from 20–200°C.

2.4.7 Determination of polymorphic states (58):

X ray diffractometer with Ni filtered Cu Kα radiations (Siemens D5000, Germany) was utilized to understand the patterns of pure phytoconstituent, its lipid component and their physical mixtures. "The measurements was carried out at a voltage of 40 kV and 25 mA. The scanned angle was set from $2° \leq 2\theta \geq 50°$ with scanned rate of 1°min−1"

2.4.8 Examination of surface morphology of pure chrysin and CLP (59):

The surface morphology of chrysin loaded phytosomes was observed using scanning electron microscope (Hitachi S3400 Japan). Dried sample of pure chrysin and CLP was coated using 5 nm gold and the photomicrographs were collected using scanning electron microscope.

2.4.9 Determination of molecular states of chrysin in pure form, soya lecithin and chrysin loaded phytosomes (60):

Fourier transform infrared (FTIR) analysis was used to determine the molecular states of pure chrysin, soy lecithin and CLP (Shimadzu, Japan). "After caliberating the background, sufficient quantity of sample was administered above the clear crystal surface. The gripper was set down on top of the sample by turning it until a 'click' sound was heard to verify the gripper is in constant contact with the sample. Scanning was done and it was repeated for every indivisual sample respectively".

2.5 Anti-oxidant activity:-

DPPH radical scavenging activity (61):-

It is one of the most widely used models for assessing the anti-oxidant activity of radical scavengers. In 80% methanol, 0.1mM DPPH was produced by dissolving. The samples were made with a stock of 1 mg/ml, and they were then dissolved in methanol. Different concentrations ranging from 10 to 40 g/ml were generated from the stock. In 96 well plates, 100 µL of DPPH was applied after the samples were diluted with Tris-HCl. 30 minutes are spent keeping the samples in the dark. The absorbance was determined at 517 nanometers. Each experiment was carried out three times. For radical scavenging, the results are shown as an activity in %.

DPPH free radical scavenging capacity. (%) = (A0 –A1) /A0 × 100

(A0 = Control group absorbance, A1 = Experiment group absorbance).

2.6 *In-vitro* cytotoxicity study:

2.6.1 Cell culture (62)

Human colon cancer (HT-29) cell lines were cultured in 96-well micro titer plates using DMEM media supplemented with 10% heat-inactivated fetal calf serum (FCS), containing 5% of penicillin (100 Units/mL), gentamycin (10 Ug), and streptomycin (100 g/L) and in the presence of 5% CO2 at 37 °C for 48 hours.

2.6.2. Maintenance of cell lines

HT29 cells were obtained from NCCS Pune. The cells were allowed to grow

in 25 cm^2tissue culture flasks which contained suitable media. In order to prevent microbial growth, 10% FBS and 4 mg/L of streptomycin were added to DMEM media. The cells were kept in a 95% air and 5% CO2 environment at 37°C in a CO incubator.

2.6.3. Sub-culturing of cells

1) The degree of confluence and the absence of bacterial and fungal contamination of the cells cultivated in T-25 flasks were assessed using an inverted microscope.
2) After discarding the used media, 3ml of PBS was used to wash the cell monolayer.
3) To cover the monolayer, 0.5 ml of trypsin-EDTA salt was applied. For 1-3 minutes, the flask was kept in the incubator.
4) The cell detachment was confirmed after removing the flask from the incubator by observing under inverted microscope.
5) 3 ml of DMEM containing 10% FBS was added to the flask and thoroughly mixed after the cells had been fully detached from the flask.
6) Trypan blue dye exclusion testing was used to measure cell viability in a tiny sample of the suspension.
7) 1X 10^4 viable cells/ml were seeded from the stock cell suspension into a 25 cm2 tissue culture flask with approximately 4 ml of new medium, and the flasks were incubated until they reached 80–90% confluency.

8) The method was repeated as necessary to meet the cell line's growth requirements.

2.6.4. Freezing of cells

1) Cells were harvested by trypsinization when they were 80-90% confluent. Re-suspending the collected cells in full media, they underwent a 5-minute centrifugation at 1000 rpm.
2) The cell pellets thus obtained were then suspended in freezing mixture (complete medium + FBS+DMSO in the ratio 7:2:1.
3) Each cryogenic vial received 1ml of cell suspension, which was also marked.
4) The cryogenic vials were put in a polystyrene box and kept overnight at -80C.
5) The following day, vials are placed in a container with liquid nitrogen.

2.6.5. Thawing of cells

1) Cryogenic vial containing the cells was taken from liquid nitrogen storage and brought immediately to room temperature by rolling between the hands.
2) The vials was wiped with 70% alcohol before placing inside the laminar hood.
3) The entire contents of the vial are transferred to a microcentrifuge tube, where they are centrifuged for five minutes at 1000 rpm.
4) The resulting cell pellets were suspended in ml of complete media after the supernatant was discarded.
5) The flask was placed in incubator.
6) After 24 hrs the culture was checked to ensure the attachment of cells to the flask the medium was changed after 2 days or when it changed from pink to yellow.

2.6.6. MTT Assay

1) The developing cells were removed from T-25 tissue culture flasks, and a 1X 10^6 stock cell suspension was made using the appropriate medium.

2) A sterile, flat-bottomed 96-well tissue culture plate has 5000 seeded cells in each well. that it might remain for a day.

3) Just prior to the experiment, the test substance was produced and serially diluted with the appropriate medium to get various concentrations of (0.05-50) g/ml. DMSO ultimate concentration should not exceed 0.2%.

4) Following a 24-hour incubation period, cells were exposed to 100 µl of test substance from corresponding top stocks for 48 hours.

5) 20 µl of MTT reagent was added to a 96-well plate, and it was then incubated for three hours at 37 C using a stock solution containing five milligrams per milliliter PBS with a 7.4 pH.

6) Cells in the control group only received medium and vehicle control media containing 0.2% DMSO

7) Each procedure was carried out three times.

8) 200 µl of 100% FMSO were added to each well after the incubation period of three hours to dissolve the crystals of formazan.

9) The optical density was measured at 540 nm using a 96-well plate reader and a 540 nm reference wavelength.

10. The vitality of the cells was determined.

2.7. *In-vivo* studies

2.7.1 Utilization of animals.

Wistar rats (male) were bought from Invivo Bioscience in Bangalore. Rats were acclimated in an experiment room with a regulated humidity level of 75%, a light cycle of 12:12 hours and temperature 23°C. In sterile polypropylene cages with bedding made of sterile rice husk, rats were housed. The animals were given water and autoclaved rat food as food. The study employed male rats that were 8 to 10 weeks old and weighed 190±10 grams. The KLE College of Pharmacy KAHER, Karnataka, India, institutional animal ethics committee developed guidelines that were adhered to when handling and caring for animals.

2.7.2. Oral Acute Toxicity Studies

According to OECD 423 recommendations, research on acute oral toxicity are conducted. Wistar rats that were 8 weeks old were used. According to OECD 423 standards, the limit test was carried out by giving a 2 g/kg p.o. dosage to each animal at a time, followed by three animals following a 24 hour pause. For 72 hours, animals were monitored cage side for signs of fatality or any other acute toxicity.

2.7.3. DMH-induced colorectal cancer and its therapy with chrysin and chrysin-loaded phytosomes.

Rats were distributed randomly based on their body weight. According to the literature, In order to induce colorectal cancer in rats, DMH was given to them in doses of 20 mg /kg i.p. for the first 10 weeks and 30 mg /kg i.p for the following 10 weeks (63)

as shown in table 5.

After the 20th week of 1,2 dimethylhydrazine induction, rats in the standard group received 5-Fluorouracil (weekly once 10 mg/kg i.p.) . After 20 weeks of receiving DMH, the rats in the fourth group were given 500 mg/kg of pure chrysin orally. The 5th, 6th and 7th group received chrysin loaded phytosomes with an oral dose of 100, 200 and 400mg/kg after 20th week of DMH administration for 21 days. A diagrammatic illustration of the treatment planner is shown in Table 5. The colorectal tissue was examined for polyps, colon weight to length ratio, hematological estimation, biochemical estimation, and histological inspection after the rats had been sacrificed.

Figure 5

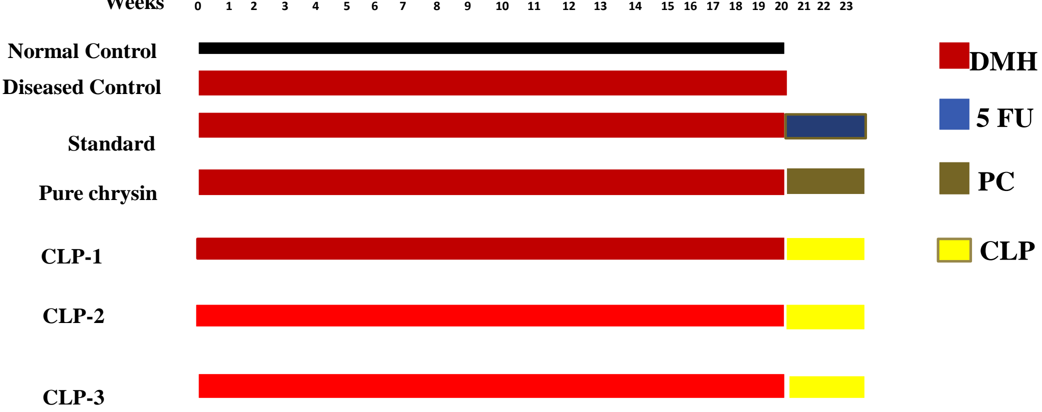


Table 5. Planner for the dosage regime for DMH-induced colon cancer treatment

Group no	Group name	Dosage
1	Normal control	Normal food and water
2	Diseased control	DMH 20 mg/kg i.p, for first 10 week and 30 mg/kg i.p, for next 10 weeks
3	Standard control	DMH + 5-FU (10 mg/kg i.p)
4	Pure Chrysin	DMH + Pure chrysin (500 mg/kg p.o)
5	Chrysin loaded phytosomes -I (CLP-I)	DMH + CLP-I (100 mg/kg p.o)
6	Chrysin loaded phytosomes -II (CLP-II)	DMH-induced + CLP-II (200 mg/kg p.o)
7	Chrysin loaded phytosomes -III (CLP-III)	DMH-induced + CLP-III (400 mg/kg p.o)

DMH: 1,2-Dimethyl hydrazine; 5-FU: 5-Flurouracil; CLP: Chrysin loaded phytosome; (n=6)

2.7.4. Parameters monitored in DMH model

1) Number of polyps present

2) Colon length/weight ratio

3) Hematological parameters

4) Biochemical estimation

5) Histopathological examination

Number of polyps present

The incidence of ACF was measured in each group of rats and expressed as a percentage = [No of rats with aberant crypt foci / No of rats examined] x100

Determination of colon length by weight ratio

Colon weight / length ratio= weight of colon in gram / length of colon in cm

Hematological parameters

A retro-orbital puncture was used to remove the blood right before the rats were sacrificed. Blood was drawn into a 2 ml eppendorf tube with 0.5% EDTA anticoagulant. The animal-cell count analyzer determines the hematological parameter.

Between the treatment groups, the following variables were compared and a summary was provided

Total RBC count

Total WBC count

Total platelet count

Hemoglobin content

Percentage of lymphocytes and monocytes

Biochemical estimation

Estimation of liver enzymes like AST, ALT and ALP was carried out as per standard procedure.

Histopathological examination

For histopathological examination, the samples of colorectum was fixed using neutral formalin solution (10%) using normal saline after which the washing was done in sterilized water. It was washed with alcohol for dehydration of the specimen.

Cleaning of the specimen was done with xylene. It was embedded in paraffin at an temperature of 56^0C using hot air oven for a period of 24 hours.

The A sledge microtome was used to slice paraffin bees wax tissue blocks into 4 m-thick sections. Following deparaffinization and hematoxylin and eosin staining, the obtained tissue sections were mounted on glass slides. The material was analyzed for histopathological alterations using an electric light microscope.

2.8 Statistical analysis:

To examine the data, one-way analysis of variance (ANOVA) was employed. To assess if there had been any noteworthy variations between the treatment groups, the Post Hoc Tukey's test was employed. The results were regarded as significant at p 0.05. For all statistical analyses, the Prism 5.03 Demo Version (Graph Pad Software Inc., La Jolla, CA, USA) was utilized.

RESULTS & DISCUSSION

3.1. PRE-FORMULATION STUDIES :

3.1.1. Identification and Characterization of Chrysin in pure form

The FTIR spectrum of chrysin obtained (TCI) was recorded (Fig 6) and interpreted for its structure. All the characteristic peaks corresponding to the functional groups of drug were found in the spectrum and were matching with the structure of drug. Table 6 shows the interpretation of FTIR spectrum of drug along with its functional groups and corresponding peaks obtainedin the spectrum.

Fig 6. FTIR Spectrum of Chrysin

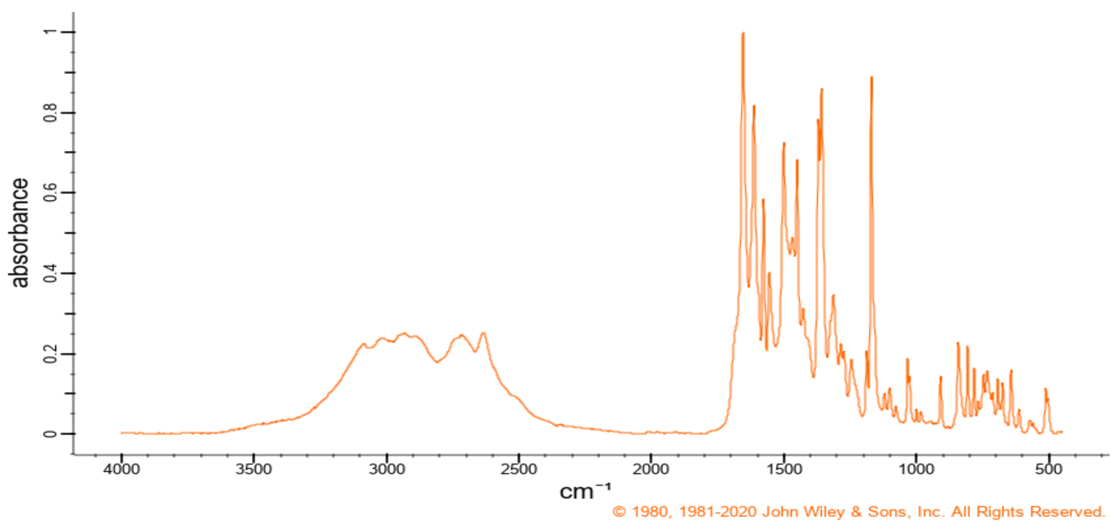
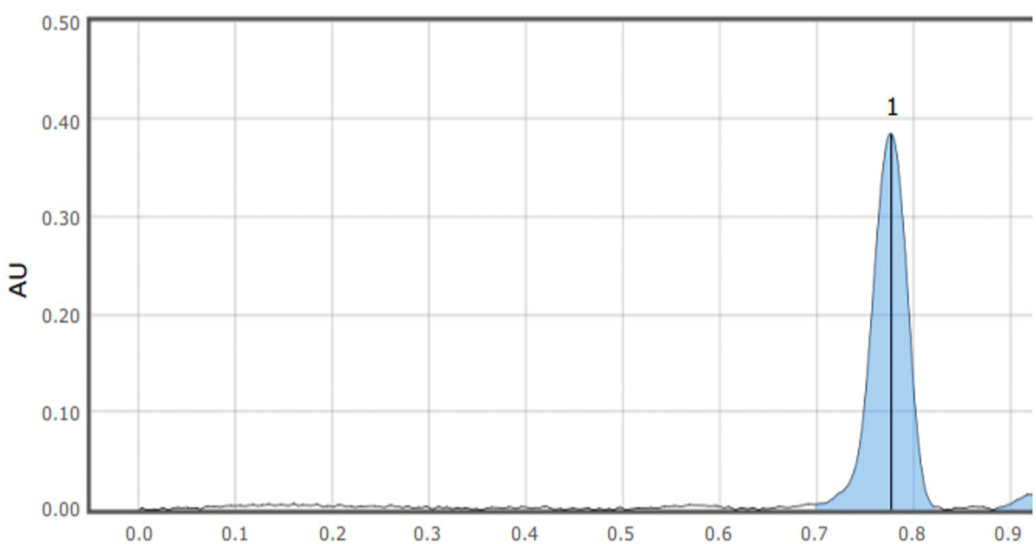


Table 6. Functional groups of chrysin and their corresponding peaksobtained in FTIR spectrum of drug.

Functional Group	Wave number (cm^{-1})
C=O (Stretch)	1649.19
C=C	1579.75
OH	3529.85-3287.11

3.1.2. Standard calibration curve of chrysin:

Chrysin exhibited maximum absorbance with a retention time of 8.2 min at 268nm, which was selected as wavelength for further analysis**.** The standard calibration curve to quantify chrysin was found to be linear having 0.99 of correlation coefficient (over the range of 50-250 ng/ml). Fig 7. Shows the chromatogram of chrysin at 50 µg/ml.



AU-Area under the curve

Fig 7. Chromatogram of chrysin (50µg/ml)

3.1.3. Solubility study of drug:

Chrysin was insoluble in water. The solubility of drug was found to be 200 µg/ml in methanol.

3.1.4. Determination of melting point of drug:

Differential scanning calorimetry (DSC) measurements revealed that the chrysin's melting point is 294.08 0C.

Fig 8. shows the Endothermic peak of chrysin. using DSC thermogram

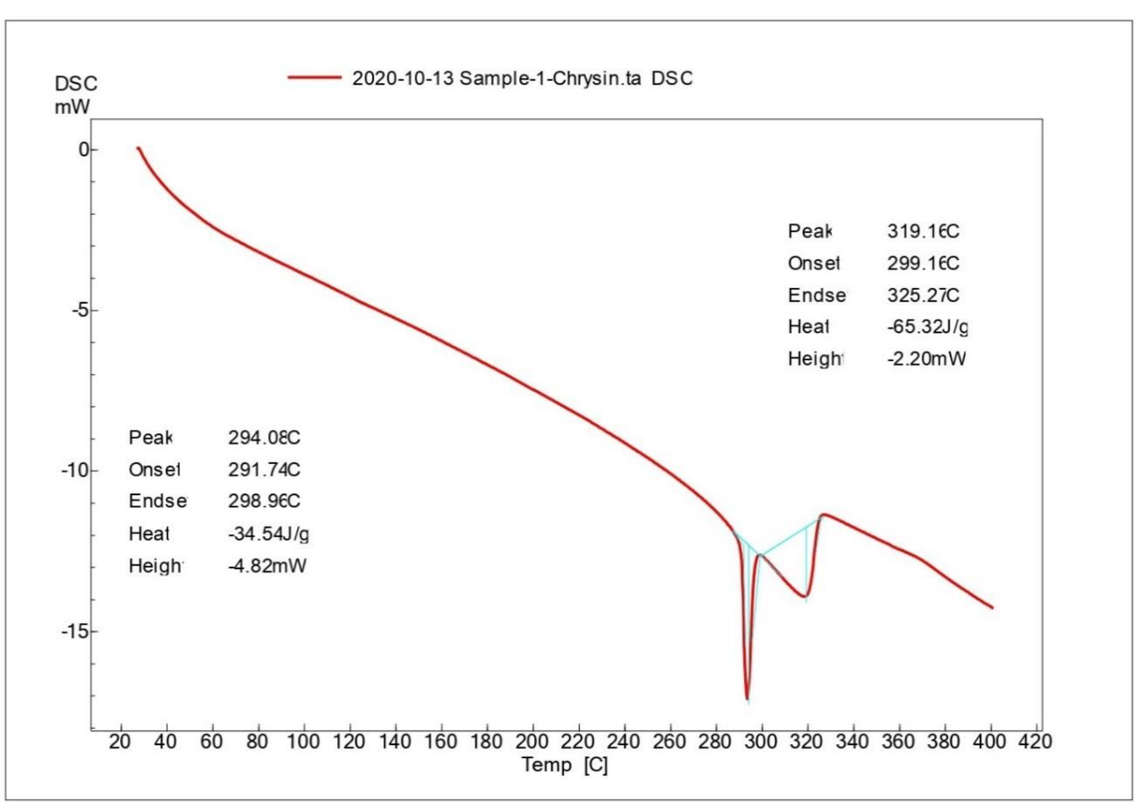


Fig 8. DSC Thermograms of chrysin

3.2. Characterization of chrysin loaded phytosomes.

3.2.1 Particle size and Polydispersity index

Formulation of Chrysin loaded phytosomes was done in the ratio of 1:1, 1:2 and 2:1. Better results were obtained in the molar ratio of 2:1 (phospholipid:Chrysin)

Molar ratio of phospholipid:chrysin	Particle size	Polydispersity index
2:1	91.40	0.29

3.2.2. Zeta potential

Using Zetasizer Ver. 7.11 (Malvern), the charge of chrysin-loaded phytosomes was calculated and discovered to be -1.33 mv.

3.2.3 Percentage Yield

The % yield was found to be 74.28%.

3.2.4. Determination of Entrapment efficiency

- Using high performance thin layer chromatography (CAMAG, Switzerland), the amount of chrysin in the phytosome was determined.
- For the quantification of phytosomes that are loaded with chyrsin, a new method was created and validated.
- It was found that one milliliter of chrysin loaded phytosome contain 164.9 µg of chrysin.

3.2.5. Determination of polymorphic state

It was discovered that the XRD patterns of CLP were extremely similar to those of the pure medication chrysin, proving that the incorporation of nanoparticles into the formulation had no impact on chrysin's physical properties. Both the pure drug and the CLP included distinct peaks at diffraction angles of 2 ϴ: 7.43, 17.70, 14.87, 17.73, 22.37, 24.63, 25.61, and 27.64. In contrast to those of CLP, pure chrysin's XRD patterns showed higher and more strong peaks, indicating a decrease in crystallinity.

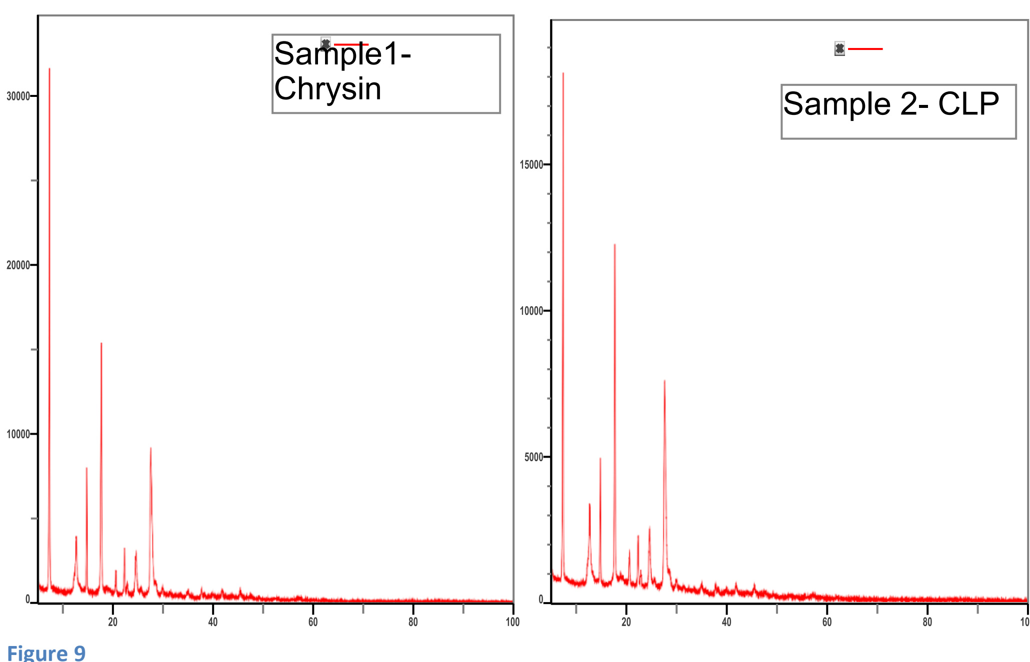


Figure 9

3.2.7. Surface Morphology

While chrysin in its pure form (Fig. a) showed an erratic arrangement of brilliant particles with a wide particle size variation and poorly defined shape. CLP (Fig. b) revealed a more defined structural arrangement in which brilliant particles, which are chrysin, were engulfed by phospholipids.

Fig a Fig b

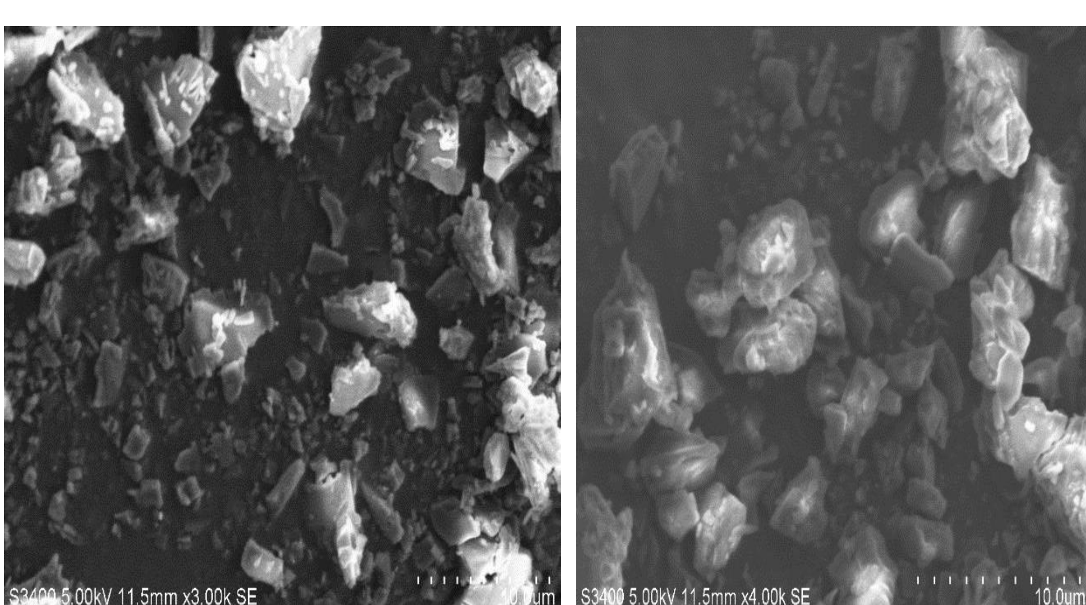


Figure 10

3.2.8. Compatibility study

FT-IR analysis was performed to evaluate the molecular states of pure chrysin and CLP. The bands at 1649.19, 1579.75, 3529.85-3287.11 in chrysin can be assigned to C=O stretch, C=C and OH group. Similar peaks were observed in CLP. From the spectra, it can be concluded that there was no difference between the internal structures and confirmation of these samples at the molecular level.

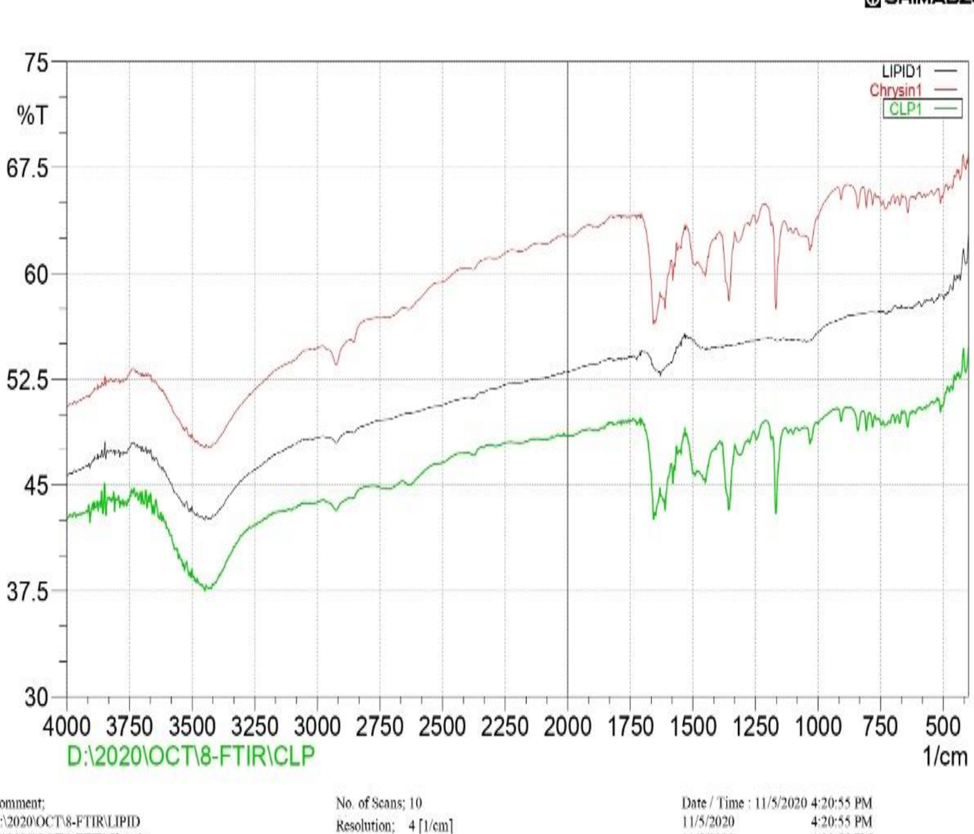


Figure 11

3.3. MTT assay

Preliminary cytotoxicity screening was carried on HT29 colon cancer cell line. Concentration of the Chrysin and chrysin loaded phytosomes (CLP) used for MTT-assay was within the range 20 to 200 μg/ml. The 50% inhibitory concentration (IC_{50}) of the chrysin and CLP calculated is given in table. The pure chrysin had low anticancer activity on HT29 colon cancer cell line compared to CLP. The CLP showed a dose dependent cytotoxicity and it might be due to cytotoxic activity of soy lecithin which might synergize the cytotoxic effect of chrysin. Hence, the MTT-assay is validated by the method followed. (Figure 12, Table 7).

Table 7

Compound	Concentration	%viability	IC50 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	

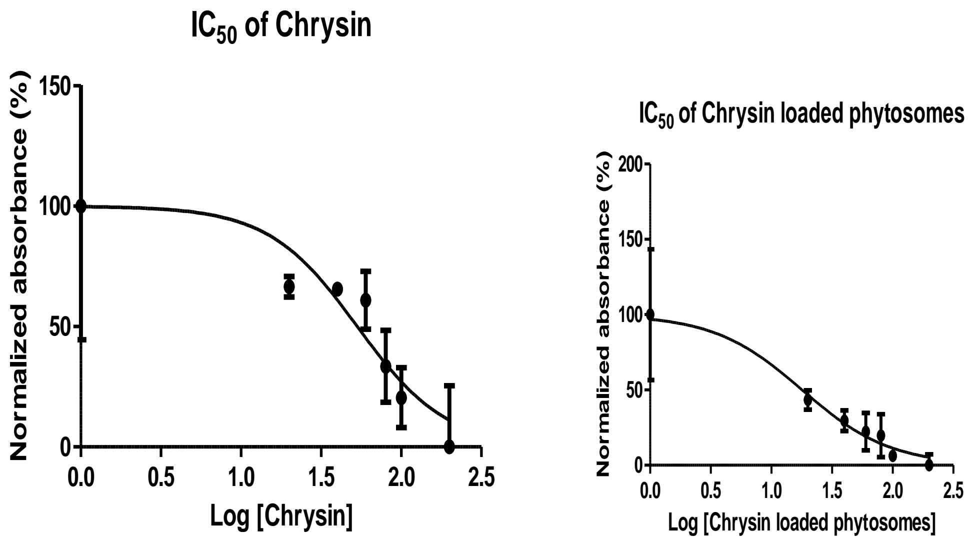


Figure 12 (Graph of IC50 value)

3.4. Antioxidant activity

Antioxidant activity of reference chrysin and chrysin loaded phytosomes was carried out using DPPH assay and % inhibition was determined.

Table 8

Group	Absorbance	% inhibition
Blank	0.385	-
Ascorbic acid	0.146	62.07
Chrysin	0.206	46.49
Chrysin loaded phytosomes	0.156	59.48

3.5 Oral acute toxicity studies:

Toxicity study was done in accordance with OECD 423 regulations. Chrysin loaded phytosomes (CLP) at a dose of 2 g/kg were given orally to rats; no mortality was noted, and the animals exhibited no adverse effects. After additional 14-day observation, it was concluded that there was no toxicity because the animals displayed no adverse effects on their immune systems, central neurological systems, or peripheral nervous systems. We have chosen 1/5, 1/10, and 1/20 as the therapeutic doses p.o. for animal models based on prior research and non-lethal effects in our acute toxicity investigations.

3.6 Effect of CLP in the treatment of DMH induced colorectal cancer model.

3.6.1. Effect of CLP treatment on Polyp count as ACF incidence

The polyps were counted from the indivisual animals of all the groups and are given in Table below. The results shows that there was more number of polyps in Diseased control group. The number of polyps were reduced in rats administered with chrysin loaded phytosomes.

Table 9

Group	Number of polyps in each animal						
	1	2	3	4	5	6	Total
Normal control	0	0	0	0	0	0	0
Diseased control	8	6	5	7	8	5	39
Standard control	3	4	4	3	3	4	21
Chrysin	6	5	7	6	4	6	34
CLP-I	6	6	6	4	6	3	31
CLP-II	4	4	5	5	6	4	28
CLP-III	3	5	4	4	5	4	25

3.6.2. Effect of chrysin loaded phytosomes treatment on Colon weight/length ratio

In the present study it was found that Colon weight/length ratio was increased in DMH group and improvement was seen in standard and CLP group in a dose dependent manner.

The Colon weight/length was determined and is given in Table below

Table 10

Treatment group	Colon weight/length ratio
Normal	179.67±2.92
Diseased	636.13±24.39$^{\#}$
Standard	231.65±9.82$^{\#*}$
Chrysin	357.59±5.84$^{\#*\wedge}$
Chrysin-I	316.60±24.12$^{\#*\wedge\$}$
Chrysin-II	274.01±10.37$^{\#*\wedge\$a}$
Chrysin-III	266.06±12.17$^{\#*\wedge\$a}$

#p<0.001 compared to Normal control, *p<0.05 compared to disease control, ^p<0.05 compared to standard control, $p < 0.05 compared to pure chrysin, ^{a}p< 0.05 compared to CLP1, ^{b}p<0.05 compared to CLP2.

3.6.3. Effect of chrysin loaded phytosomes treatment on Hematological parameters.

Comparing the groups hematological data revealed substantial differences, with the DMH control group's RBC level falling below that of normal control group. There was also significant difference seen that is increased level in standard, chrysin, CLP-I, CLP-III and CLP-III groups when compared with disease control group. It indicates that 5-FU, pure chrysin and chrysin loaded phytosomes did not have any destructive effect on Red blood cells.

When comparing the DMH control group with normal control group, there was rise in WBC level having significant difference in the case of WBC. There was significant difference seen that is the standard control, pure chrysin group and the three groups of chrysin loaded phytosomes had a reduction in the WBC count when compared with disease control group. Comparing to the control group, the WBC level of the pure chrysin and chrysin-loaded phytosomes differed significantly. CLP-II and CLP-III group showed significant difference with $p < 0.05$ compared to pure chrysin group. There was also significant difference seen in CLP-II and CLP-III group with $p < 0.05$ when compared to CLP-I group.

When compared to the normal control, the platelets of standard, pure chrysin, CLP-1, II, and III exhibited a significant difference ($p < 0.05$). Significant increase was observed in the platelet level of standard, pure chrysin, CLP-1, II and III with $p < 0.05$ when compared with diseased control. Pure chrysin and CLP-I showed significant difference with $p < 0.05$ when compared to standard control. CLP-I,II,III showed significant elevation in the platelet level compared to pure chrysin group. CLP-III showed significant elevation in the platelet level compared with CLP-I group.

Hemoglobin level showed significant decrease in disease control group when compared to normal control with $p < 0.05$. The hemoglobin level elevated significantly in standard

control, pure chrysin, CLP-I, CLP-II and CLP-III groups with $p<0.05$ when compared to disease control group. This showed DMH administration reduced the hemoglobin level which might be because of methylation effect of DMH on cells. The hemoglobin level of CLP-III showed significant increase in hemoglobin level compared to pure chrysin group. This may be because of increase permeability of CLP-III in the systemic circulation and protective effect on hemoglobin. The hemoglobin level of CLP-III were increased significantly when compared with CLP-I and CLP-II groups with $p<0.05$.

There was significant decrease of monocyte level in disease control group compared to normal control with $p < 0.05$. Monocyte level of standard, pure chrysin, CLP-I, CLP-II and CLP-III group increased significantly when compared to disease control group. CLP-III group showed an significant elevation in the monocyte level compared to pure chrysin group and CLP-II group. In case of lymphocyte, there was no significant changes seen between the groups.

Table 11

Effect of Chrysin loaded phytosomes on Hematological variables						
Groups	**Hematological Parameters**					
	RBC	**WBC**	**Platelets**	**Hemoglobin**	**Monocyte**	**Lymphocyte**
Normal control	7.77±0.25	7.58±1.95	78.66±3.43	14.43±0.54	08.66±1.36	85.66±1.36
DMH control	6.2±0.86$^{\#}$	12.18±0.05$^{\#}$	28.73±4.86	11.66±0.68$^{\#}$	04.00±0.89$^{\#}$	84.83±1.47
5-FU control	7.35±0.36*	7.8±0.55*	73.33±0.50$^{\#*}$	13.93±0.76*	8.66±3.14*	83.00±4.47
Pure chrysin	7.56±0.34*	12.06±1.72$^{\#\wedge}$	54.93±2.77$^{\#*\wedge}$	13.86±0.88*	8.33±1.03*	81.66±1.36
CLP-I	7.52±0.22*	10.59±0.37$^{\#\wedge}$	54.23±0.85$^{\#*\wedge\$}$	13.96±0.93*	9.66±0.51*	80.66±3.72
CLP-II	8.03±0.24*	8.32±0.95$^{*\$a}$	58.81±2.45$^{\#*\$}$	14.08±0.14*	7.66±1.36*	84.33±2.25
CLP-III	7.96±0.28*	7.95±0.61$^{*\$a}$	73.43±2.52$^{\#*\$a}$	15.66±1.15$^{*\$ab}$	11.50±2.25$^{*\$b}$	84.00±0.89

#p< 0.05 compared to NC, *p < 0.05 compared to DC, ^p < 0.05 compared to Std, \$p < 0.05 compared to pure chrysin, ^{a}p < 0.05 compared to CLP1, ^{b}p < 0.05 compared to CLP2

3.6.4. Effect of chrysin loaded phytosomes treatment on Biochemical parameters.

The AST levels in the disease control, standard, pure chrysin, CLP-I, II, and III groups showed significant differences (p <0.05) from the normal control. When compared to the diseased control, the standard, pure chrysin, CLP-I, II, and III group demonstrated a substantial decrease in the AST level (p<0.05). Pure chrysin, CLP-I,II,III group displayed

significant changes in the AST level when compared with Standard control with $p<0.05$.There was significant decrease seen in CLP-I,CLP-II and CLP-III group when compared with pure chrysin group. There was also significant decrease seen in CLP-II and CLP-III group when compared to CLP-I group. CLP-III group had significant decrease when compared with CLP-II group.

The ALT level showed significant increase in the Diseased control group. The ALT level displayed that standard, pure chrysin, CLP-I,II,III group had significant differences when compared with normal control group with $p<0.05$. The standard group, pure chrysin and CLP-I,II,III group showed significant decrease in the ALT level when compared with disease control group. Pure chrysin group showed significant decrease when compared to standard control group. CLP-I, CLP-II and CLP-III group had significant decrease in ALT level when compared with pure chrysin group with $p<0.05$. CLP-II and CLP-III group had significant decrease in ALT level when compared with pure CLP-I group with $p <0.05$.

When compared to the normal control, the ALP level in diseased control, pure chrysin, CLP-I, CLP-II, and CLP-III groups revealed significant variations ($p<0.05$).Standard, pure chrysin, CLP-I, CLP-II and CLP-III group had significant differences when compared with diseased control group with $p <0.05$. When compared to the standard control group, the pure chrysin group, CLP-I, CLP-II and CLP-III groups showed significant changes ($p <0.05$).When compared to the pure chrysin group, the ALP levels in the CLP-I, CLP-II, and CLP-III groups decreased significantly ($p<0.05$).

Table 12

Effect of Chrysin loaded phytosomes on Biochemical variables			
Groups	**Biochemical estimation**		
	AST	**ALT**	**ALP**
Normal control	75.26±3.73	27.33±1.36	106±2.94
DMH control	304.66±5.75$^{\#}$	127±8.04$^{\#}$	415.33±6.59$^{\#}$
5-FU control	260.33±3.14$^{\#*}$	98±7.79$^{\#*}$	117.33±9.42*
Pure chrysin	289.33±4.50$^{\#*\wedge}$	115±4.09$^{\#*\wedge}$	311.66±8.99$^{\#*\wedge}$
CLP-I	246.66±1.86$^{\#*\wedge\$}$	97±11.62$^{\#*\$}$	205.33±5.73$^{\#*\wedge\$}$
CLP-II	198.16±2.92$^{\#*\wedge\$a}$	78.66±1.36$^{\#*\wedge\$a}$	186.66±7.58$^{\#*\wedge\$}$
CLP-III	178.33±3.61$^{\#*\wedge\$ab}$	71.66±3.14$^{\#*\wedge\$a}$	132.66±5.73$^{\#*\wedge\$}$

#p<0.001 compared to normal control, *p<0.001 compared to DC, ^p<.001 compared to Std, $p < 0.001 compared to pure chrysin, ^{a}p < 0.001 compared to CLP1, ^{b}p < 0.001 compared to CLP2.

3.6.5. Effect of chrysin loaded phytosomes treatment on histopathological findings of colorectum tissue in DMH treated rats:

The findings of the histological examination revealed that there were inflammatory cells infiltrating the wounded area, which is a sign that the rats had developed cancer. After DMH injection in the current investigation, there was an infiltration of mild to moderately inflammatory cells in the mucosal area. Chronic inflammatory cells, such as eosinophilic cell infiltration and proliferating lymphoid follicles, were scarcely present in the colon mucosa of DMH-induced rats. In rats exposed to DMH, the entire colon was edematous, and fibrin deposition was seen. In the colon mucosa and submucosa of the DMH-induced rats, there were haemorrhages, mucosal congestion, desquamation, epithelial necrosis, and epithelial ulcers. Furthermore, the mucosal epithelium had hyperplasia and dysplastic alterations. These serve as the physical indicators of the development of adenocarcinoma. As a result, rats were given DMH to produce symptoms of cancer. A 500 mg/kg oral dose of pure chrysin produced findings that were similar to those of rats who had been given DMH. Chrysin-loaded phytosome treatment at oral dosages of 100 mg/kg and 200 mg/kg avoided most of the parameters, including general malignancy condition signs, haemorrhages, and eosinophil infiltration, although only mild dysplasia was produced. Treatment with 400mg/kg of chrysin-loaded phytosomes produced effects that are comparable to those of normal 5-FU.

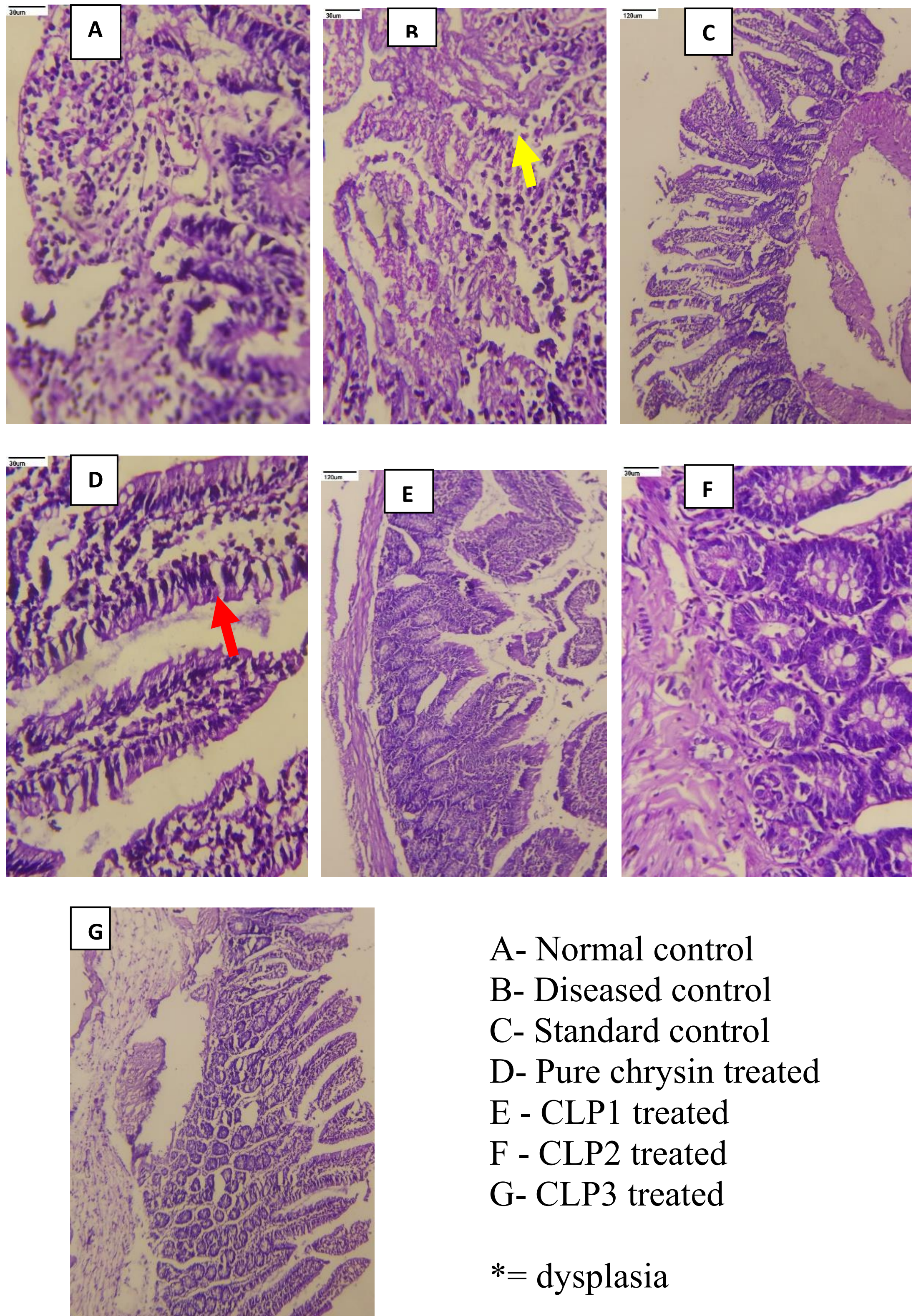


Figure 13: Effect of chrysin and chrysin loaded phytosomes treatment on histopathological findings of colorectum tissue in DMH treated rats

4. SUMMARY:

For the treatment of colon cancer, chysin, a member of the flavonoid class, has been employed. Its major drawback is its low bioavailability due to which the effectiveness of chrysin as an anticancer agent is reduced. Thus, in the current work, efforts were undertaken to create phytosomes that were loaded with chrysin and could target the tissues or cells of colorectal cancer.

In a preliminary investigation, three distinct formulations of varying combinations of pure chrysin and soya lecithin were created using an anti-solvent precipitation method, and their effects on the characteristics of the chrysin-loaded phytosomes were assessed. The data demonstrate the effective synthesis of chrysin-loaded phytosomes with a reduction in nanoparticle size, polydispersity index and zeta potential. On the basis of these preliminary results, combination of pure chrysin and soya lecithin in the ratio of 1:2 was considered as optimal. Finally, chrysin-loaded phytosomes were created utilizing an optimal ratio of pure chrysin and soya lecithin as the carrier, and they were evaluated for their physicochemical characteristics, cytotoxicity experiments on cancer cells (HT- 29), and invivo studies. By using Dynamic Light Scattering (Nanotrac) and scanning electron microscopy, the optimized chrysin-loaded phytosomes revealed a particle size of 91.40 nm, spherical morphology, and a zeta potential of around -1.33 mV, indicating increased electrostatic forces.stability of nanoparticles in the solution. In an MTT assay at pH 7.4 and 37 °C, chrysin-loaded phytosomes were more harmful to colon cancer cell lines HT-29 than pure chrysin (IC50-17.92 mg/ml).The increased rate of chrysin release from the nanoparticles at 37^0 C and 7.5 pH is responsible for the pronounced cytotoxicity of chrysin loaded under these conditions. When maintained in a glass container, chrysin in pure form and chrysin-loaded phytosomes remained stable at room temperatures for eight months.

According to OECD 423 recommendations, an oral toxicity research was conducted in-vivo, and the therapeutic dose for chrysin-loaded phytosomes was determined to be doses of 100, 200 and 400 mg/kg. Tumor was induced in rats by administering 1,2-Dimethyl hydrazine for 20 weeks and treated with reference drug 5-fluorouracil, pure chrysin and chrysin loaded phytosomes in 3 therapeutics doses. Polyps were counted in all the rats in the groups and was observed to be reduced in the groups administered with chrysin loaded phytosomes with a dose of 400mg/kg When compared to those receiving the reference medication and pure chrysin, groups receiving chrysin-loaded phytosomes experienced a dose-dependent decrease in colon weight/length.. Hematological and biochemical estimation was done .Chrysin loaded phytosomes with a dose of 400mg/kg was able to bring the various parameters to normal. Finally, a histological analysis was performed, and chrysin-loaded phytosomes demonstrated decreased dysplasia, a reduction in eosinophil infiltration, hemorrhages, and the overall symptoms of a malignancy state.

5. CONCLUSION:

Chrysin loaded phytosomes were proficiently generated by the antisolvent precipitation technique with the combination of chrysin and soy lecithin. Interesting property obtained from the nanoformulation is their particle size. Additionally, the MTT assay showed that the chrysin-loaded phytosomes might increase the anti-tumor activity in conditions of elevated temperature and pH in the extracellular space surrounding the tumor. DPPH assay confirmed that chrysin loaded phytosomes had better antioxidant activity then pure chrysin. The anticancer potential of Chrysin and phytosomes containing Chrysin was investigated in relation to treating DMH-induced colon cancer in male Wistar rats. Finally, an in vivo investigation showed a comparison of phytosomes loaded with chrysin and pure chrysin in a colorectal cancer model generated by DMH. Considering the findings in all the parameters with respect to polyps count, colon weight/length ratio, biochemical tests, hematological parameters and histological examination, when treated with chrysin loaded phytosomes in a dose dependent manner, a discernible improvement in the treatment of colorectal cancer was observed.

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ANNEXURE – I – POSTER PRESENTATIONS

KRUPACON PHARMA 2021
Innovation in healthcare - making a difference together

3rd GLOBAL ONLINE CONFERENCE & WORKSHOP ON
DRUG DEVELOPMENT 4.0:
EMERGING TECHNOLOGIES

CERTIFICATE OF PARTICIPATION

This is to certify that Namit Kudatarkar of KLE Academy of Higher Education and Research has presented a poster titled Analytical Method Development and Validation for Estimation of Chrysin using HPTLC in Chrysin loaded Phytosomes co-authored with Sunil Jalalpure In the

KrupaPharmaCon 2021 | 3rd GLOBAL ONLINE CONFERENCE & WORKSHOP ON
DRUG DEVELOPMENT 4.0: EMERGING TECHNOLOGIES
an event organized by Krupanidhi College of Pharmacy, Bengaluru, on 02-03 July 2021.

ORGANIZED BY
KRUPANIDHI COLLEGE OF PHARMACY

Dr. SV Rajendra
Convener & Principal
Krupanidhi College of Pharmacy

Prof. Dr. Suresh Nagpal
Chief Patron & Chairman
Krupanidhi Group of Institutions

International Congress of
Society for Ethnopharmacology, India
(Globalizing local knowledge and localizing global technologies)

SFEC 2021
27th to 29th, AUGUST 2021

THEME : Ethnopharmacology & Medicinal Plants - Approach towards product development

ORGANISED BY
SFE—INDIA PUNE CHAPTER
&
BHARATI VIDYAPEETH (DEEMED TO BE UNIVERSITY)
POONA COLLEGE OF PHARMACY & ALL PHARMACY COLLEGES OF PUNE REGION

This certificate is awarded to

Professor/Dr. /Ms. /Mrs. /Mr. **Namit Madan Kudatarkar**

from....................

for presenting Poster Presentation on the topic **System biology and chemoinformatics approach to decode the molecular mechanisms of Chrysin against colon cancer**

at International Congress of Society for Ethnopharmacology, India (SFEC 2021).

Dr. Sathiyanarayanan L,	Dr. K. R. Mahadik,	Mr. Birendra K. Sarkar
Organizing Secretary, SFE India	LOC Chairman, SFE India	SFE President

Graduate School of Pharmacy

Gujarat Technological University

&

Society of Pharmacognosy

Formerly Indian Society of Pharmacognosy

ESTD - 2007

CERTIFICATE NO.: GSPICON21PPR3

CERTIFICATE OF PARTICIPATION

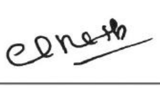
THIS CERTIFICATE IS BEING AWARDED TO

DR./MR./MS. **MR. NAMIT MADAN**

HAS SUCCESSFULLY PRESENTED A POSTER ✔ / ORAL ENTITLED

Analytical method development and validation for estimation of Chrysin in Chrysin loaded phytosomes using High performance thin layer chromatography

AND SECURED **THIRD RANK** DURING 25TH NATIONAL CONVENTION OF SOCIETY OF PHARMACOGNOSY & INTERNATIONAL CONFERENCE ON **"NEW HORIZONS OF NATURAL PRODUCTS** AND **AYUSH REMEDIES"** DURING NOVEMBER 27-28, 2021.



MS. JIGNA VADALIA	DR. SANJAY CHAUHAN	DR. UMESH PATIL	PROF. (DR.) NAVIN SHETH
Organizing Secretary, Loc & Asst. Prof., GSP- GTU	Chairman, Loc & Director GSP-GTU	General Secretory Society Of Pharmacognosy	Vice Chancellor Gtu & President Society Of Pharmacognosy

Department of Biochemistry, University of Kashmir
NAAC Accredited A+
and
Indian Society Of Cell Biology

44th All India Cell Biology Conference and International Symposium on Molecular & Cellular Insights of Human Diseases

CERTIFICATE OF PARTICIPATION

This is to certify that Dr/Mr/Ms Namit Kudatarkar from KLE University participated/presented a poster/~~delivered a talk~~ in the **44th All India Cell Biology Conference** held at the University of Kashmir, Srinagar from September 2-3, 2022.

Dr. Shajrul Amin
Convenor

Dr. Shaida Andrabi
Organizing Secretary

Prof. Bhupendra N Singh
Secretary ISCB

ANNEXURE-II: PAPER PUBLICATIONS

List of paper publication:

Sr. No	Title	Journal	I. F.
1.	System biology and chemoinformatic approaches to decode the molecular mechanisms of chrysin against colon cancer	Journal of applied pharmaceutical science	0.759
2.	Analytical method development and validation for estimation of chrysin in chrysin loaded phytosomes using high performance thin layer chromatography	Journal of Liquid Chromatography & Related Technologies .	1.467
3.	Formulation and Characterization of Chrysin Loaded Phytosomes and its Cytotoxic Effect against Colorectal Cancer Cells	Indian Journal of Pharmaceutical Education and Research	0.843
4	Development and validation of UV-Visible Spectrophotometric method for estimation of chrysin in bulk form	Journal of Pharmaceutical Negative Results	0.654

Journal of Applied Pharmaceutical Science Vol. 11(09), pp 057-065, September, 2021
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2021.110907
ISSN 2231-3354

System biology and chemoinformatics approaches to decode the molecular mechanisms of Chrysin against colon cancer

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ARTICLE INFO

Received on: 21/04/2021
Accepted on: 15/06/2021
Available online: 05/09/2021

Key words:
Chrysin, colon cancer, Erlotinib, network pharmacology, molecular docking, molecular dynamics.

ABSTRACT

Chrysin is a flavonoid possessing potential pharmacological activities against many diseases including cancer. Many studies have been reported on Chrysin showing anticancer activity against colon cancer. However, the mechanism with which Chrysin shows its anticancer activity is not known yet. Hence, the current study was framed to understand the molecular mechanism of Chrysin against colon cancer via gene set enrichment and network pharmacology analysis coupled with molecular docking study. Initially, Chrysin probable targets were identified by SwissTargetPrediction and their molecular pathway enrichment was analyzed by the STRING and KEGG pathway databases. The network among Chrysin, probable protein targets, and its pathways were constructed with the aid of Cytoscape 3.6.1v. Molecular docking was carried out with the aid of AutoDock Vina by PyRx 0.8v. Molecular dynamics was carried out by Schrodinger Desmond v6.1 software. Druggability, side effects, and ADMET analysis were determined using MolSoft, ADVERPred, and admetSAR2.0 web server, respectively. Chrysin potentially acts via metabolic pathways and Ras and PI3K–Akt signaling pathways associated with the progression of cancer. Among the probable targets, Chrysin exhibited the highest binding affinity against epidermal growth factor receptor comparable to standard molecule Erlotinib, and its root mean square deviation and interactions were found stable at 20 ns MD production run. Both Chrysin and Erlotinib shared common interactions with Asp831 active site residue and confirmed their potential antagonistic effect. In conclusion, Chrysin may serve as a potential anticancer small molecule in the future for the management and treatment of colon cancer.

INTRODUCTION

Among cancer, the third most common cancer worldwide is cancer of the colon with an incidence rate and mortality rate of 6.1% and 9.2% when combined for both sexes, respectively (Bray *et al*., 2018). Although there are several chemotherapies and surgeries for colon cancer patients, the rate of recurrence after surgery is high (Hellinger and Santiago, 2006). Thus, exploration of an effective clinical treatment for the treatment of colon cancer is essential. It is seen that herbal phytoconstituents have been broadly used for the treatment of various tumors such as colon carcinoma (Benarba and Pandiella, 2018). Chrysin is a herbal phytoconstituent that is found to have anti-inflammatory activity (Nunes *et al*., 2020) by effectively impairing cisplatin-influenced expression for iNOS and COX-2 (Rehman *et al*., 2014), neuroprotective activity by triggering nuclear factor-kB and inducing the expression of nitric oxide synthase (Zhang *et al*., 2015), and antidiabetic activity by significantly inhibiting advanced glycation end products-receptor for advanced glycation end products conciliated oxidative stress, activation of PPAR-g resulting into inflammation (Rani *et al*., 2016), and antidepressant activity by culmination in the uplift of nerve growth factor and BDNF levels (Filho *et al*., 2015). Reviews of previously published scientific literature have disclosed that the results of phytoconstituent

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Chrysin showed improved cytotoxic effects against colorectal cancer on various *in vitro* and *in vivo* studies (Bahadori *et al*., 2016). One study showed that in Wistar rats, Chrysin guarded against colon cancer which was induced by cisplatin through amelioration of oxidative stress and apoptosis which caused a significant decrease in cis-diamminedichloroplatinum-induced deterioration of the goblet cells in the crypts of the colon (Khan *et al*., 2012). However, Chrysin is found to be efficacious in the treatment of colon carcinoma (Lin *et al*., 2018). To date, there is no data available on Chrysin action on multiple protein targets and its pharmacological mechanisms on colon cancer. Thus, the drug targets and pharmacological mechanism can be predicted using network pharmacology analysis which provides insight for improving the discovery of various drugs for various diseases (Shi *et al*., 2020).

In the current study, the inherent mechanism of phytoconstituent Chrysin for the treatment of colon cancer was scrutinized with the help of a network-based systematic study. Briefly, the drug-target network was constructed considering the target sets of Chrysin and colon cancer. The genes which overlapped between Chrysin and colon cancer were inspected by comparative analysis with the aid of KEGG pathway enrichment analysis. Hence, the current study was designated to access and document the Chrysin probable protein targets and their mechanisms of action on colon cancer.

MATERIALS AND METHODS

Target identification

The canonical SMILE of Chrysin was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and its targets were predicted using the SwissTargetPrediction online server (http://www.swisstargetprediction.ch/). The server predicts molecular protein targets based on similarity measures depending on 2D and 3D combinations with known ligands. Furthermore, the protein molecules involved in colorectal cancer were identified using the Open Targets Platform (https://platform.opentargets.org/) and Therapeutic Target Database (http://db.idrblab.net/ttd/).

Gene set enrichment and network analysis

A set of gene IDs of probable protein target for Chrysin were submitted as input into the STRING database (https://string-db.org/) to study protein–protein interactions. Furthermore, molecular pathways modulated by a set of genes were identified using the KEGG pathway. The network among Chrysin, probable protein targets, and its pathways were constructed with the aid of Cytoscape 3.6.1 version. The constructed network was analyzed by edge count topological parameter by treating it as direct. The node size of the network was fixed to "low values to small size" with color to "low values to bright colors" (Patil *et al*., 2020, 2021).

Docking studies

Selection, preparation, and quality of protein target

Based on the network analysis, protein targets having larger node sizes and highest edge counts, and based on the clinical success full targets, we selected epidermal growth factor receptor (EGFR), a major potential therapeutic target of colon cancer. The 3D structure with X-ray crystallographic of EGFR having PDB ID: 1M17 was retrieved from Research Collaboratory for Structural Bioinformatics Protein Data Bank (https://www.rcsb.org). The distribution of amino acids and the quality of proteins were determined by PROCHECK and ERRAT (https://servicesn.mbi.ucla.edu/ERRAT) online servers, respectively. The active site residues that are involved in the ligand-binding were noted from the selected PDB file *via* Discovery Studio Visualizer 2019v (DSV v2019) and further, the listed amino acid residues were confirmed from P2RANK (https://prankweb.cz/analyze?database=v2-conservation&code=1M17) and Galaxy site web servers (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=SITE).

Preparation of ligand

The 3D structure of Chrysin and the reference drug molecule, i.e., Erlotinib, was taken from the PubChem chemical database. To avoid interference during docking simulation, the initial free energy of the ligand molecules was minimized by Marvin Sketch using the MMFF94 force field.

Docking validation

Before initiating the docking studies, the grid box and docking conformation are validated via the pre-docking step. The structure of Erlotinib from the crystal structure of EGFR (PDB ID 1M17) was retrieved in .pdb format using DSV v2019. Docking of EGFR with both Erlotinib extracted from the 1M17 and PubChem database was carried out using PyRx 0.8v software. The .pdb format protein molecule and the ligand were imported into the software and converted into AutoDock Vina molecules, i.e., .pdbqt files. The grid box was set to maximum for size and center [center x = 26.6909, y = 9.667, and z = 59.1236; size x = 58.124, y = 66.546, and z = 51.512] and the system exhaustiveness was set to 8. Intermolecular interactions and post-docking ligand orientation (conformation) root mean square deviation (RMSD) of both Erlotinib extracted from the 1M17 and PubChem was analyzed by DSV v2019 software and DockRMSD online server (https://zhanglab.ccmb.med.umich.edu/DockRMSD/).

Chrysin–EGFR docking

The affinity between Chrysin and EGFR protein target was determined using AutoDock Vina by using PyRx 0.8v software. The .pdb format protein molecule and the ligand were imported into the software and converted into AutoDock Vina molecule (i.e., Chrysin.pdbqt) file. As mentioned above, the grid box was set to maximum for both size and center [center x = 26.6909, y = 9.667, and z = 59.1236; size x = 58.124, y = 66.546, and z = 51.512] and the system exhaustiveness was set to eight. The ligand–protein interactions within the site-specific domains along with a transformation in the orientation of ligand after docking were envisioned with the aid of DSV v2019.

Molecular dynamics

Molecular dynamics of Chrysin with EGFR complex was carried out by Desmond software (version 6.1). The MD system was solvated by Simple Point Charge water model in a cubic box [dimension 10Å × 10Å × 10Å]. Chlorine ions (–5) were added to neutralize the system. Furthermore, the complex system was minimized for a 100ps production run. The Isothermal-Isobaric ensemble: moles (N), pressure (P) and temperature (T) was applied,

and 1.01325bar atmospheric pressure and temperature 300K were set. Furthermore, 2,000 frames were set for 20 ns MD production run at 10 ps sampling analysis time interval. Finally, the RMSD and residue-wise interaction fluctuations were analyzed.

Druglikeness characteristics and ADMET profile

Chrysin and Erlotinib canonical SMILES were retrieved to envisage the characteristics of druglikeness which includes the molecular weight of the drugs (MW), the total number of hydrogen bond donor (NHBD), the total number of hydrogen bond acceptor (NHBA), its lipid/water partition coefficient (Log P), and its druglikeness score (DLS) by employing MolSoft online server. With the aid of ADVERPred and ADMET SAR2.0 by the online server, numerous side effects along with the probable inactivity (Pi) and probable activity (Pa) and ADMET profile were anticipated, respectively.

RESULTS

Target prediction

A total of 40 protein targets were identified for the Chrysin from SwissTargetPrediction. Among them, 13 protein targets were significantly targeted by Chrysin and found to involve in colon cancer, namely calmodulin 1, EGFR, insulin-like growth factor 1, insulin receptor, myosin light chain kinase, glycogen synthase kinase 3 beta, neurotrophic receptor tyrosine kinase 2, cyclin-dependent kinase 6, nitric oxide synthase 2, monoamino oxidase A, arachidonate 15-lipoxygenase, arachidonate 12-lipoxygenase, and arachidonate 15-lipoxygenase.

Gene set enrichment and network analysis

The enrichment analysis identified 13 targets to play a major role in the 40 molecular pathways [false discovery rate (FDR) < 0.05]. Among them, pathways in cancer, metabolic pathways, Ras and PI3K–Akt signaling pathways, and nitrogen metabolism were significantly modulated via scoring the lowest FDR value. Table 1 summarizes the modulated pathways with their respected modulated genes by Chrysin and Figure 1 shows the network of interacted phytoconstituents with molecules of proteins and the pathways involved in colon cancer.

Active site residues of EGFR protein

The active site residues of EGFR present in the .pdb file 1M17 are Lys721, Val702, Ala719, Leu764, Met742, Leu694, Glu738, Asp831, Met769, and Asp831 (confirmed via P2RANK and GalaxySite web server).

Docking studies

Docking validation

The binding energy (BE) of Erlotinib extracted from the 1M17 and PubChem was –7.1 and –7.5 kcal/mol, respectively. Erlotinib extracted from the 1M17 formed one hydrogen bond interaction, i.e., Asp831, with NH group of Erlotinib and formed five non-hydrogen bond interactions with Gly695, Val702, Leu764, Ala719, and Met742. However, Erlotinib extracted from PubChem formed two hydrogen bond interactions, i.e., Met769…O- and Asp831…NH, and formed six non-hydrogen bond interactions with Leu694, Val702, Lys721 (2), Met742, and Leu764. Among the total interactions of Erlotinib extracted from 1M17 and PubChem with EGFR, Asp831, Val702, Met742, and Leu764 were found to be the common interactive residues. Furthermore, the Erlotinib docked orientation (conformation) was analyzed by the DockRMSD server and the RMSD was found to be 1.657 Å. Figure 2 shows the interactions and ligand orientation RMSD of both Erlotinib extracted from 1M17 and PubChem.

Chrysin and EGFR docking studies

Docking was carried out for Chrysin and compared its affinity and interactions with the docked conformation of Erlotinib extracted from PubChem due to its lowest BE and maximum intermolecular interactions. Chrysin scored the lowest BE of –8.8 kcal/mol with EGFR by forming two hydrogen bond interactions with site-specific residues that is Glu738…OH and Asp831…=O. However, Erlotinib scored the lowest BE of –7.5 kcal/mol with EGFR by forming two hydrogen bonds with active site residues, i.e., Met769…O- and Asp831…NH. Among these interactions, Asp831 amino acid residue was found to be common for Chrysin and Erlotinib. After docking, the BE and the swap in the orientation of ligand along with the hydrogen bond interactions are displayed in Table 2 and the interactivity of Chrysin and Erlotinib concerning EGFR is shown in Figure 3.

Molecular dynamics

Chrysin combined with EGFR exhibited a very stable RMSD (Å) at 20 ns production run. Initially, from 0 to 6 ns the ligand RMSD (Å) was found stable (0.3Å) and suddenly slight fluctuation was seen (0.3–0.4Å) at 6 ns and further from 6 to 20 ns again RMSD was found to be very stable at 0.4Å. Furthermore, Chrysin formed a very stable bond throughout the 20 ns production run. It formed stable interactions with Asp831 (56%), Thr766 (52%), Ala719 (17%), Glu738 (94%), Lys721 (32%), and Thr830 (12%). Figure 4 shows the Chrysin–EGFR RMSD and residue-wise interactions.

Druglikeness and side effects

Erlotinib, a clinically approved molecule was found to obey the rule of Lipinski via MW ≤500 g/mol (i.e., 393.17 g/mol), HBA ≤10 (i.e., 6), HBA ≤5 (i.e., 1), LogP ≤5 (i.e., 2.21), and scored positive DLS of 0.90. Similarly, Chrysin was also found to obey the rule of Lipinski via MW ≤500 g/mol (i.e., 254.06 g/mol), HBA ≤10 (i.e., 4), HBA ≤5 (i.e., 2), LogP ≤5 (i.e., 3.61), and scored negative DLS of –0.21. On looking at the side effects, Erlotinib was predicted to exert hepatotoxicity, myocardial infarction, nephrotoxicity, and cardiac failure via scoring Pa values of 0.932, 0.665, 0.594, and 0.362, respectively. Similarly, Chrysin was predicted to exert only hepatotoxicity via a scoring Pa value of 0.516. The results are shown in Table 3.

DISCUSSION

The present study was carried out to recognize the molecular mechanism of anticancer activity of phytoconstituent "Chrysin" which belongs to the flavonoid category with the analysis of gene set enrichment, network pharmacology, and *in silico* molecular docking analysis. Network pharmacology is a novel innovative method to forecast the association between the phytoconstituent and protein targets (Chandran *et al.*, 2017).

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Table 1. Gene set enrichment analysis of probable protein targets of Chrysin.

S. No.	KEGG ID	Pathway name	Observed gene count	False discovery rate
1.	hsa00910	Nitrogen metabolism	6	5.15E-09
2.	hsa05200	Pathways in cancer	12	1.11E-06
3.	hsa04014	Ras signaling pathway	8	1.31E-05
4.	hsa00590	Arachidonic acidmetabolism	5	4.26E-05
5.	hsa04151	PI3K–Akt signaling pathway	8	0.00014
6.	hsa04066	HIF-1 signaling pathway	5	0.00021
7.	hsa04010	MAPK signalingpathway	7	0.00031
8.	hsa01521	EGFR tyrosine-kinase inhibitor resistance	4	0.0011
9.	hsa04022	cGMP-PKG signaling pathway	5	0.0011
10.	hsa01100	Metabolic pathways	12	0.0013
11.	hsa01522	Endocrine resistance	4	0.0017
12.	hsa04510	Focal adhesion	5	0.0023
13.	hsa04015	Rap1 signaling pathway	5	0.0024
14.	hsa04726	Serotonergic synapse	4	0.0026
15.	hsa04722	Neurotrophin signaling pathway	4	0.0028
16.	hsa00330	Arginine and proline metabolism	3	0.0029
17.	hsa04068	FoxO signaling pathway	4	0.0036
18.	hsa04150	mTOR signaling pathway	4	0.005
19.	hsa05226	Gastric cancer	4	0.005
20.	hsa04115	p53 signaling pathway	3	0.0057
21.	hsa04971	Gastric acid secretion	3	0.0062
22.	hsa04020	Calcium signaling pathway	4	0.0078
23.	hsa00220	Arginine biosynthesis	2	0.0081
24.	hsa04964	Proximal tubule bicarbonate reclamation	2	0.0095
25.	hsa05205	Proteoglycans in cancer	4	0.0095
26.	hsa04750	Inflammatory mediator regulation of TRP channels	3	0.0096
27.	hsa00790	Folate biosynthesis	2	0.0109
28.	hsa00591	Linoleicacid metabolism	2	0.0128
29.	hsa04152	AMPK signaling pathway	3	0.016
30.	hsa04270	Vascular smooth muscle contraction	3	0.016
31.	hsa00350	Tyrosine metabolism	2	0.0166
32.	hsa04110	Cell cycle	3	0.0166
33.	hsa04960	Aldosterone-regulated sodiumreabsorption	2	0.0168
34.	hsa04728	Dopaminergic synapse	3	0.0177
35.	hsa04371	Apelin signaling pathway	3	0.0189
36.	hsa05206	MicroRNAs in cancer	3	0.0244
37.	hsa04218	Cellular senescence	3	0.0263
38.	hsa04923	Regulations of lipolysis inadipocytes	2	0.0276
39.	hsa04062	Chemokine signaling pathway	3	0.0358
40.	hsa04662	B-cell receptor signaling pathway	2	0.041

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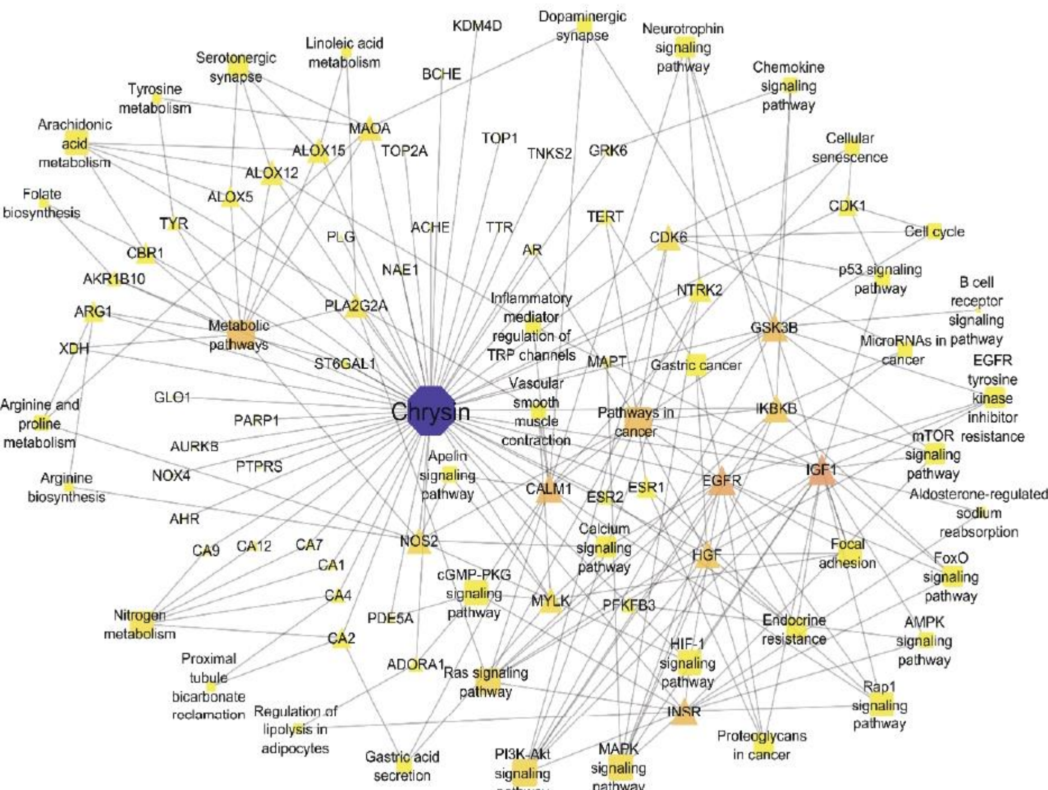

Figure 1. Network relationship between Chrysin (blue color node), probable protein targets, and pathways (yellow color nodes) involved in colon cancer.

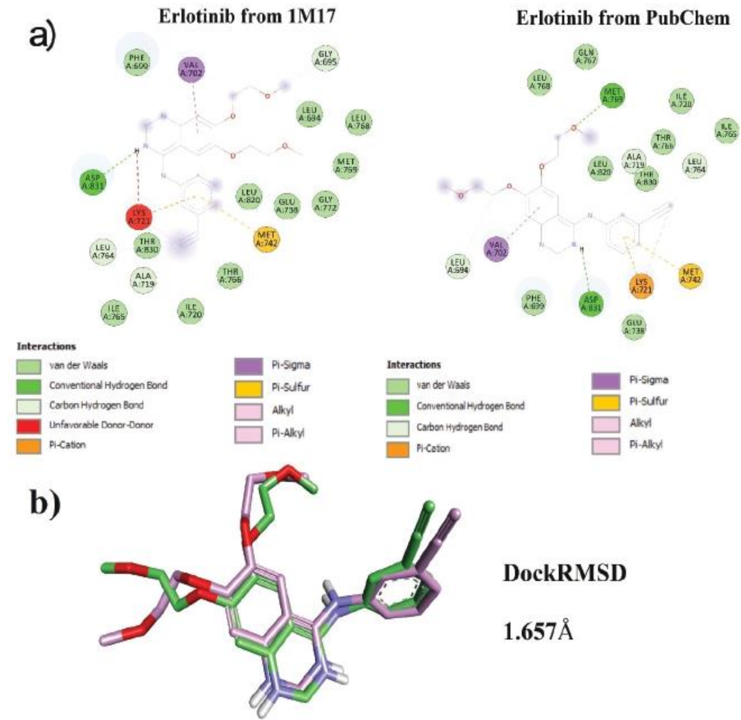

Figure 2. Docking validation. (a) Interactions of both Erlotinib extracted from 1M17 and PubChem. (b) Ligand orientation RMSD.

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Table 2. Affinity and interactions of Chrysin and Erlotinib with EGFR.

Compound name	BE (kcal/mol)	HBI (amino acid…ligand)	NHBI (No. of interactions)
Chrysin	−8.8	Glu738…OH, Asp831…=O	Lys721 (2), Val702 (2), Ala719 (2)
Erlotinib[a]	−7.5	Met769…O-, Asp831…NH	Ala719, Leu764, Lys721 (2), Met742, Leu694

BE = Binding energy; HBI = Hydrogen bond interactions; NHBI = Non-hydrogen bond interactions.
[a]Standard molecule (EGFR inhibitor);

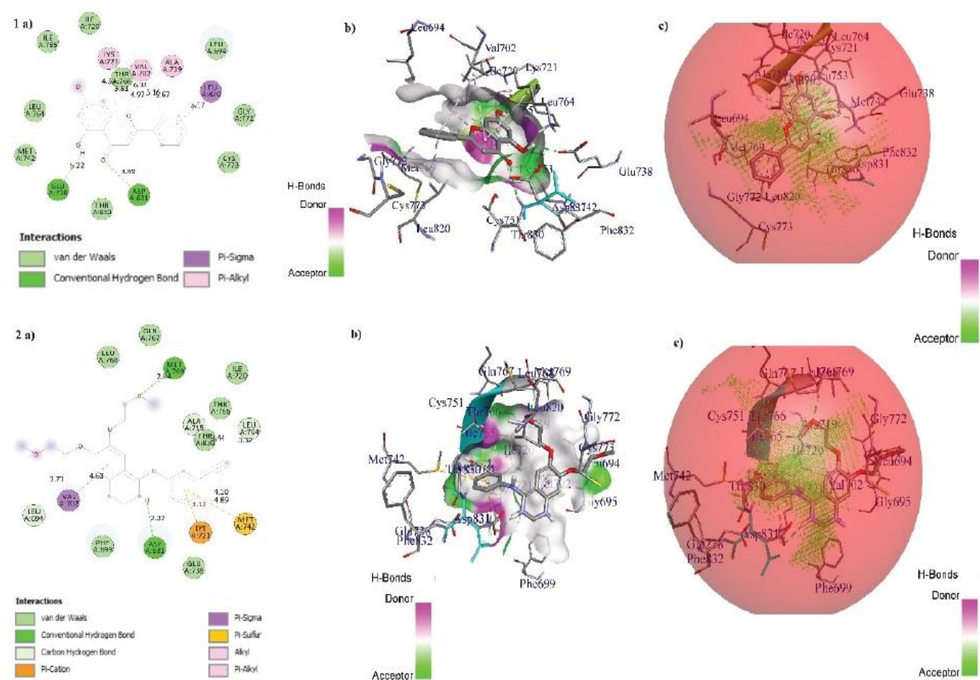

Figure 3. Interaction of (1). Chrysin and (2) Erlotinib with EGFR. (a) 2D representation, (b) Ligand within binding pockets, and (c) Ligands within active site domains, respectively.

Primarily, taking into account, the fundamentals of "similar compounds target similar proteins" forecasted the Chrysin targets with the help of SwissTargetPrediction and the protein molecules involved in targeting colon cancer concerning the approved targets which are available in the Therapeutic Target database. Later, we executed enrichment analysis of the compound gene set with the help of KEGG to recognize the biological pathways modulated by the Chrysin. Consequently, for colon cancer, 40 molecular pathways were notably clarified. The network was built displaying the interactivity and interconnection among Chrysin and its protein molecules, the protein molecules along with its associated pathways, and also with the phytoconstituents–targets pathways. Based on the polypharmacology perspective, it is important to assume that one pathway which consisted of several molecules of protein regulated by a single compound is commendable than the function of a single molecule of proteins that requires numerous pathways regulated by a single drug molecule (Patil *et al*., 2019). The influence of a single target in several routes can be fewer and the influence for that single route consisting of many protein targets regulated by that single compound could be vast. Our study disclosed that phytoconstituent Chrysin revealed the excessive gene count for various routes or pathways which are entangled in colon cancer and ultimately Chrysin falls under the polypharmacology therapy via multiple proteins and multiple pathways' mechanism. The constructed network linking phytoconstituents–proteins–pathways discloses the role of Chrysin (flavonoid) for the treatment of colon cancer by targeting significant molecules of protein. Flavonoids are polyphenolic compounds that are found drastically in plant sources. It contains two aromatic rings that are A and B which form a C ring by attaching a 3-carbon chain (Kumar and Pandey, 2013). Considering various *in vitro* and *in vivo* research studies, it can be seen that flavonoids had potential activity as an anticancer agent against innumerable types of cancer models which is progressed through modulation of key signaling pathways that are responsible for the transfer and conquering of cancer cells and also by arresting the metastatic progression like regulatory molecules like MMPs, TGF-β, uPA/uPAR, and other contributors of the complicated process which are involved in

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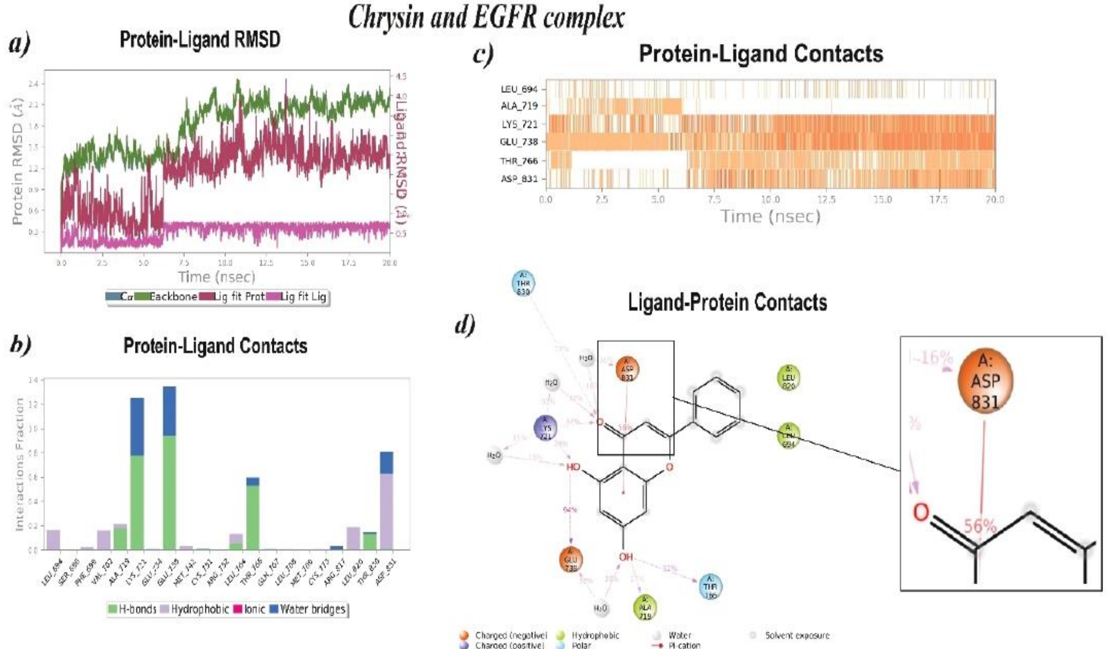

Figure 4. Stability of intramolecular interactions of Chrysin with EGFR. (a) RMSD fluctuations, (b–d) Residue-wise Chrysin–EGFR contacts.

Table 3. Druglikeness characteristics and its probable side effects of Erlotinib and Chrysin.

Compounds	MW (g /mol)	Molecular formula	NHBA	NHBD	DLS	Log P	Pa	Pi	Side effects
Erlotinib	393.17	$C_{22}H_{23}N_3O_4$	6	1	0.90	2.21	0.932	0.015	Hepatotoxicity
							0.665	0.017	Myocardial infarction
							0.594	0.027	Nephrotoxicity
							0.362	0.123	Cardiac failure
Chrysin	254.06	$\mathbf{C_{15}H_{10}O_4}$	4	2	−0.21	3.67	0.516	0.187	Hepatotoxicity

MW = Molecular weight; NHBA = Number of hydrogen bond acceptor; NHBD = Number of hydrogen bond donor; DLS = Druglikeness score; Log P = Partition coefficient; Pa = Probable activity; Pi = Probable inactivity.

metastatic spread (Liskova *et al*., 2020). In one study, the growth of epidermoid carcinoma A431 cells and upregulation of EGFR was inhibited significantly by tyrosine kinase inhibitors (Huang *et al*., 2009). The present study recognized Chrysin to potentially target EGFR as a vital protein molecule, which is inherently entangled in colon cancer, respectively. Previous studies reported that drugs like Erlotinib or Gefitinib produced a strong synergistic effect with serine/threonine-protein kinase B-Raf (BRAF) (V600E) inhibition which in turn leads to the inhibition of EGFR and thus such drugs are recommended as an inherent anticancer compound (Prahallad *et al*., 2012). In one study, it has been found that the therapeutic effect of anti-EGFR therapy is overruled by activated Kirsten rat sarcoma viral oncogene homolog protein mutations, which further lead to the transduction of the activation signal from EGFR. Apart from these, supplementary factors may be the portion for different signaling routes or pathways which are triggered by EGFR which include EGFR itself, the phosphatase, BRAF, and phosphatase and tensin homolog (PTEN). These are the genes that are the major targets of genetic modifications for colon cancer and they have been related to the effectiveness of therapies regulated against EGFR or the members of the EGFR-activated pathways (Laurent-Puig *et al*., 2009). Inhibition of EGFR is one of the restorative approaches in the management of colon cancer. In previous reports, it has been found that the subjects with KRAS wild type tumors and the administration of EGFR MAb drugs to standard treatment, there was progression of the disease which was reduced by 30%, and the rate of death was also shrunk by 12% and the tumor shrinkage rate was seen to be improved, i.e., from 31% to 46%. For subjects having both KRAS and neuroblastoma RAS viral oncogene homolog (extended Rat sarcoma) wild type, the risk of disease progression was found to be lowered, i.e., 40% and the risk of death was lowered by 23% and the rate of tumor shrinkage increased from 21% to 48% (Chan *et al*., 2017). In the present study, the phytoconstituent Chrysin is predicted for the inhibition of EGFR. It is suggested that Chrysin

reduced the proliferation of cancer cells by downregulation of the EGFR tyrosine kinase enzyme (Liu *et al.*, 2019). Additionally, this phytoconstituent is also envisaged to interconnect with numerous target proteins and regulate the pathways which are entangled in colon cancer. In the present study, we recognized Chrysin as a potent EGFR tyrosine kinase inhibitor. Considering, the small molecules function only on the active site domain on specific targets, according to the theory of the "lock and key" model. The present study employed molecular docking simulation to delineate the affinity of the standard drug and phytoconstituent toward envisaged target molecules. For the docking study, we selected the phytoconstituent Chrysin, which is predicted to target one inherent therapeutic protein target of colon cancer, i.e., EGFR. Furthermore, the binding affinity of the phytoconstituent Chrysin was compared with the therapeutically approved drug, i.e., Erlotinib. Among the selected drug, Chrysin expressed the highest binding affinity to EGFR. The results revealed that the phytoconstituent Chrysin, when coupled with long-chain, can obtain a better anticancer effect and those compounds with 16 carbon atoms chain were found to show inherent anti-proliferative activity (Lv *et al.*, 2010). Observing the hydrogen bond interactions, the selected standard drugs, i.e., Erlotinib and Chrysin, had hydrogen bond interactions with the predicted protein targets. The residues amino acids of EGFR having links with Chrysin were Lys721, Val702, and Ala719; and with Erlotinib are Ala719, Leu764, Lys721, Met742, and Leu69. The interactions of these residues were found within its active site domain. Likewise, Erlotinib and Chrysin shared Asp831 as the common residue of amino acids for EGFR. Furthermore, the interactions of Chrysin with EGFR were confirmed *via* a 20 ns MD production run. Chrysin was found to 56% stable with Asp831 and also it formed stable interactions with Thr766, Ala719, Glu738, Lys721, and Thr830. The study revealed that Chrysin was a better inhibitor of EGFR when compared to Erlotinib. Considering these findings from *in silico* molecular docking and MD analysis, Chrysin interacts with the active site domain and possibly plays a major role in the inhibition of EGFR as well as numerous protein molecules as observed in the Chrysin network-based mechanisms. Thus, employing phytoconstituents formulations for the management of complicated diseases could be the most favorable approach.

CONCLUSION

In this study, a gene set enrichment and network analysis of Chrysin possible protein targets coupled with its molecular interactions with EGFR via molecular docking and dynamics study were examined. We identified the mechanisms by which Chrysin modulates various signaling pathways which led to the inhibition of tumor progression. The study provided Chrysin as a major lead molecule as an anticancer agent against colon cancer. Chrysin majorly acts via pathways in cancer, metabolic pathways, and Ras and PI3K–Akt signaling pathways. However, it has major action on an EGFR as an inhibitor compared to standard molecule Erlotinib via molecular docking studies. The results which are obtained in the currents study will therefore narrow down the inherent protein targets of Chrysin which will direct a better pathway in future experiments. The major hurdle to deploy Chrysin as an anticancer agent for clinical use is its inadequate bioavailability and its reduced chemical stability under physiological conditions. However, we propound that novel drug delivery systems like nanoparticles might be intended to increase the bioavailability and stability of Chrysin. Thus, in the drug-development process, molecular docking analysis provides a rapid and cost-effective method to recognize the inherent protein targets for different anticancer compounds emerging from several natural sources along with the potential toxic side effects which occur through the interactions with proteins.

ACKNOWLEDGMENTS

The authors would like to thank KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research (KAHER) Belagavi, for providing support to carry out the research.

AUTHORS' CONTRIBUTIONS

All the authors have equally contributed to study design, collection, analysis, and interpretation of the data, writing, and drafting of the manuscript. All the authors read and approved the final version.

ETHICAL APPROVAL

This study does not involve any animals or human subjects.

CONFLICT OF INTEREST

The authors declare no conflict of interests for this manuscript.

FUNDING

None.

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How to cite this article:
Kudatarkar N, Jalalpure S, Patil VS, Kurangi B. System biology and chemoinformatics approaches to decode the molecular mechanisms of Chrysin against colon cancer. J Appl Pharm Sci, 2021; 11(09):057–065.

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES
https://doi.org/10.1080/10826076.2022.2038196

Analytical method development and validation for estimation of chrysin in chrysin loaded phytosomes using high performance thin layer chromatography

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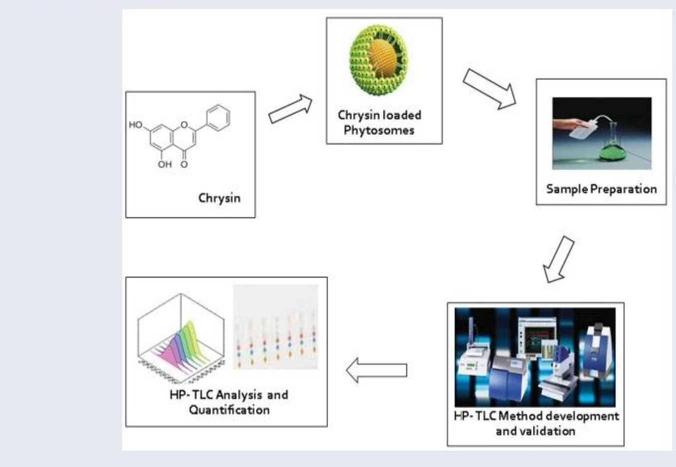
ABSTRACT

Chrysin is a potential flavonoid used in the treatment of various diseases and disorders, some of the major ones are inflammation, gout, HIV-AIDS, and cancer. In the present study, the Chrysin Phytosomes are prepared by applying the anti-solvent procedure and are quantified using an analytical method that is High Performance Thin Layer Chromatography (HP-TLC) which is developed and validated in accordance with International Council for Harmonization guidelines. In the method development for chrysin, silica gel aluminum plate 60 F254 is been used as stationary phase and the mobile phase used is *n*-hexane:ethyl acetate:methanol:formic acid in the ratio of 8:8:1:0.2 v/v/v/v is used. The method is validated for linearity, range, limit of detection, limit of quantification, precision, and robustness. The developed analytical method was found to be simple, reliable, precise, and robust. The validated High Performance Thin Layer Chromatography method was successfully applied for quantification of chrysin in chrysin-loaded phytosomes.

KEYWORDS
Chrysin; HP-TLC; ICH guidelines; phytosomes

GRAPHICAL ABSTRACT



Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide with an incidence rate and mortality rate of 6.1 and 9.2% when combined in both sexes.[1] It has been found that the incidence rate is more in developed countries with a western culture due to their low fiber diet and sedentary lifestyle. In recent years, the incidence rate of CRC was found to be increased with the age-standardized rate (ASR) for CRC to be 7.2 per 100,000 population in males and 5.1 per 100,000 population in women.[2] Though there are various chemotherapeutic drugs to treat specific cancer, multi-organ toxicity, targeted site-specificity, and low bioavailability are the major limitations. To overcome the limitations of current chemotherapeutic drugs, herbal compounds like flavanoids are playing a major role in prophylaxis and treatment of colorectal cancer.[3] Chrysin (5,7-dihydroxyflavone) is one known poor lipid-soluble phytoconstituent found in honey, propolis, and passion flower belonging to the flavonoid category.[4] It is found to possess chemopreventive and therapeutic effects in

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skin aging, atherosclerosis, inflammation, diabetes, AIDS, and cancer.[5] Reviews of previously published scientific literature have revealed that the effects of phytoconstituent Chrysin showed improved cytotoxic effects against colorectal cancer on various *in-vitro* and *in-vivo* studies.[6] Studies also revealed that decreased effect was seen in *in-vivo* studies due to reduced specificity and low permeability of Chrysin.[7] To overcome the pharmacokinetic and pharmacodynamic limitations, Chrysin is incorporated into phytosomes which is a novel nanoformulation.

The evolution of novel drug delivery systems like phytosomes would be useful in intensifying the therapeutic efficacy of the drug. Quantification of chrysin in the phytosomal form is of necessity for quality control and stability assessment.[8]

To confirm the suitability of pharmaceutical dosage forms for therapeutic use, quality control tests are the important aspect for identifying the predictability and consistency of the pharmaceutical dosage form. The quality control of the phyto-constituent would be possible only if an acceptable analytical method is available for the quantification of the phyto-constituent.[9] As the intended novel drug delivery system was phytosomes, the amount of chrysin would be in minute quantity. This intends for the development of a satisfactory analytical method that has an admissible limit of quantitation (QL) which is below the theoretical chrysin loading concentration in the final sample solution. From the multiple analytical methods which are available, high performance thin layer chromatography (HPTLC) is one method that offers numerous advantages due to its rigid tolerability about crude samples and also the possibility of separating samples in equivalent manner. HPTLC technique enables high samples throughput toward *in-situ* spectra recording and is also considered a cost-effective technique. Quantification of phyto-constituent in pharmaceutical dosage forms is simple and reliable using HPTLC. To date, no study has been done using HPTLC method for quantification of chrysin in phytosomal form. The mentioned HPTLC method for chrysin is for its determination in phyosomal form. The aim of the current study was to develop and validate an HPTLC densitometric method for the quantification of chrysin in chrysin-loaded phytosomes.

Materials and methods

Materials

Chrysin (Reference) (Purity—>98%) and Soy lecithin were purchased from TCI Hyderabad, India. Dichloromethane (99.8%), *n*-hexane (99%), ethyl acetate (95%), methanol (99%), formic acid (98%) were purchased from Molychem, Mumbai, India.

Instrumentation

Hamilton microliter syringe was used (Linomat syringe, Hamilton-Bonaduz Schweiz, Camag, Switzerland), precoated silica gel aluminum plate 60 F_{254} of 20×10 cm, with 1 mm thickness; (E. Merck, Darmstadt, Germany), sample applicator Linomat 5 (Camag, Switzerland), Twin trough chamber of 20×10 cm was used (Camag, Switzerland), UV chamber (Camag, Switzerland), TLC scanner 4 (Camag, Switzerland) was employed using vision CATS version 3 software (Camag, Switzerland) was used in the study.

Preparation of chrysin loaded phytosomes

Chrysin-loaded phytosomes were prepared by an anti-solvent precipitation technique. The required quantity of soy lecithin and chrysin was taken in a 250 ml round-bottomed flask and refluxed in the presence of dichloromethane at a temperature of 60 °C for 3 hr. The resultant solution was concentrated to 5–10 ml and *n*-hexane was added to the concentrated solution with continuous stirring until a precipitate was formed. The precipitate was filtered and then it was placed under a desiccator for complete drying. The dried precipitate was then sieved in 100 mesh and stored in amber color bottles. After 24 hr of equilibrium, the stored chrysin-loaded phytosomes were further characterized for particle size, polydispersity index, and zeta potential by DLS (Zetasizer Nano ZS, UK).

Preparation of standard solutions

Five milligrams of Chrysin was used as standard and Chrysin-loaded phytosomes as a sample. Both were accurately weighed and then transferred in two different volumetric flasks of 10 ml and methanol was used as the solvent. Chrysin solution with 500 µg/ml was obtained by making up the volumes to the mark with methanol. The working solutions of stock solutions were prepared by diluting them with methanol to get a working standard of 50 µg/ml of Chrysin and Chrysin-loaded phytosomes.

HP-TLC chromatographic procedure for method development

Standard solutions of reference Chrysin of different concentrations was prepared and it was spotted as bands which were obtained on the pre-coated silica gel aluminum Plate 60 F_{254}, using Camag Linomat 5 sample applicator having a bandwidth of 6 mm using micro syringe. A twin trough glass chamber was employed for the development of plates. The mobile phase was allowed to saturate in the twin trough chamber (20 min) before the development of the plate. The Plate was allowed to run for a distance of about 80 mm for the solvent system. After the plate reaches the 80 mm distance, the plate is allowed to dry with the help of an air dryer. Once the plate is completely dry it is been used to analyze by the TLC scanner 4 using the wavelength and the results were interpreted from the chromatograms. The spectra of the standard are identified by scanning them under the UV-Range.

HPTLC method validation

The developed analytical method is been validated using the ICH Q2 (R1) guidelines and the parameters performed are linearity, range, limit of detection(LOD), limit of quantification(LOQ), precision, specificity, and robustness.[10]

Linearity and range

The range of the standard is found out after applying many concentrations of the samples, the range that is selected depends on the linearity curve of the reference. The wavelength selected to perform the parameters at a selected wavelength.

Limit of detection

LOD is calculated derived on the slope obtained from linearity and standard deviation, the following formula is been used

$$\text{Limit of detection} = 3.3 \times \sigma/S$$

Where, σ = standard deviation; S = slope of calibration curve

Limit of quantification

The limit of quantification parameter is calculated similar to that of LOD depending on the slope and standard deviation, the formula used is

$$\text{Limit of quantitation} = 10 \times \sigma/S$$

Precision

The precision parameter is conducted in two parts namely intraday and interday precision. When the same procedure is repeated thrice on the same day then it is said as intraday precision, whereas when the repetition is done on three successive days then it is interday precision. The Center's most concentration of the calibration curve is selected for the precision study. The calculation of the precision study must have the %RSD < 2% according to the ICH guidelines.

Specificity

In specificity parameter, the phytosomes and standard are applied on the plate and allowed to develop. The developed plates are scanned for the chromatograms and are further compared for overlapping and the R_f value.

Robustness

In the robustness parameter, the deliberate changes are made in the parameters of the method like the slight modification in the ratio of mobile phase, time of chamber saturation, and mobile phase volume. The changes obtained must be in the criteria of the %RSD that is <2%.

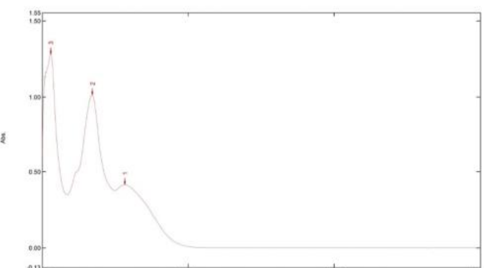


Figure 1. Spectra of chrysin.

Quantification

For the quantification of the Chrysin-loaded phytosomes, the stock solution was prepared with the concentration of 50 mcg/ml and the quantity applied was 3 µL. The procedure was carried out in triplicates for quantification of the chrysin-loaded phytosomes.

Results

Characterization of chrysin loaded phytosomes

Chrysin loaded phytosomes was prepared by anti-solvent precipitation technique the particle size, polydispersity index, and zeta potential for Chrysin loaded phytosomes was found to be 91.40 nm, 0.29, and −1.33 mV, respectively

Method development

The wavelength selected after scanning the plates under ultraviolet light was found to be 268 nm. The mobile phase was selected after many trials and finalized to *n*-hexane: ethyl acetate: methanol: formic acid in the ratio 8:8:1:0.2 v/v which showed better separation for the reference and the sample and the spectra was obtained[11–14] and is shown in Figure 1.

Method validation

The method was developed at 268 nm and the selected mobile phase was validated for the parameter below.

Linearity and range

The linearity was found to be in the range of 50–250 ng/band and the correlation coefficient was found to be 0.99 and coefficient of variation 0.08%, and the calibration curve is shown in Figure 2 and the data related to linear regression is shown in Table 1.

Limit of detection

The limit of detection was found to be 25.50 ng/band after calculation using the formula given in the method section.

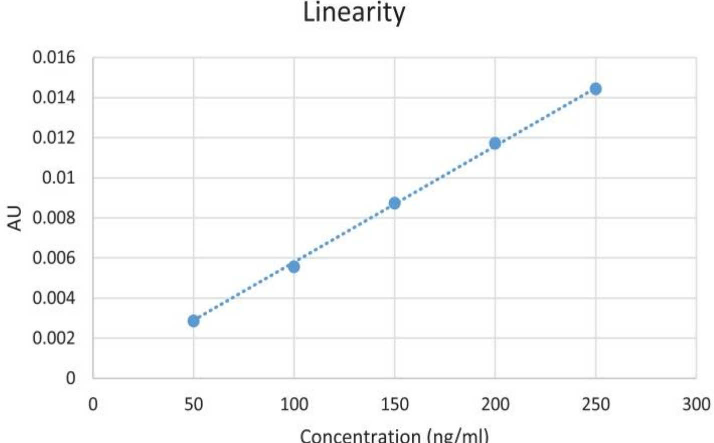


Figure 2. Linearity parameter.

Table 1. Linear regression data.

Parameters	Values
Linearity	50–250 ng/band
Correlation coefficient	0.99
Coefficient of variation	0.08%
Linear regression equation	$y = 0.00006x$

Table 2. Intraday precision.

	Peak area	R_f value
1	0.0161	0.7740
2	0.0162	0.7690
3	0.0161	0.7740
Mean	0.0161	0.7720
SD	0.00005	0.0028
%RSD	0.3578	0.3737

SD: standard deviation; RSD: relative standard deviation.

Table 3. Interday precision.

	Peak area	R_f value
1	0.0162	0.7770
2	0.0163	0.7850
3	0.0161	0.7970
Mean	0.0162	0.7863
SD	0.00014	0.0100
%RSD	0.8926	1.2801

SD: standard deviation; RSD: relative standard deviation.

Limit of quantification

The limit of quantification was found to be 77.29 ng/band after calculation using the formula mentioned in the method section.

Precision

The precision parameter was carried out for interday and intraday and the %RSD was observed to be within the criteria of <2% for the chrysin standard. The data obtained is presented in Tables 2, 3 for intraday and interday precision, respectively.

Specificity

After carrying out the specificity parameter it was found that chrysin standard and the phytosomes had a similar R_f value and overlapping position. The chromatograms for the standard and the chrysin-loaded phytosomes are obtained and are shown in Figures 3, 4.

Robustness

After carrying out the robustness parameter it was found that changes in mobile phase composition showed %RSD-1.3965%, Mobile phase volume changes showed % RSD-0.7748% and changes in the duration of chamber saturation showed %RSD-0.6025 this is within the criteria of <2%, which confirms that the developed method was robust and the data is shown in Table 4.

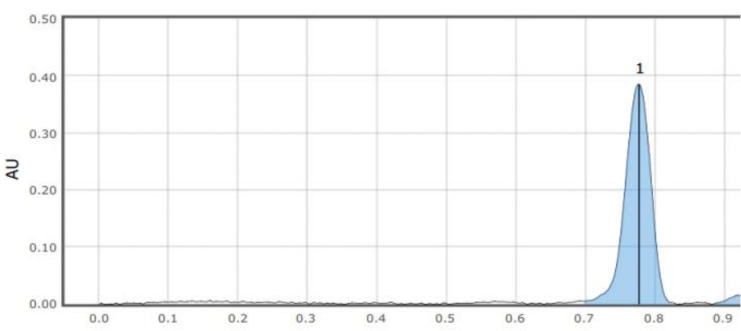
Quantification

The triplicate was carried out for the quantification of the Chrysin-loaded phytosomes. The data is presented in Table 5. And the mean and standard deviation of the results obtained were calculated.[15–19]

Discussions

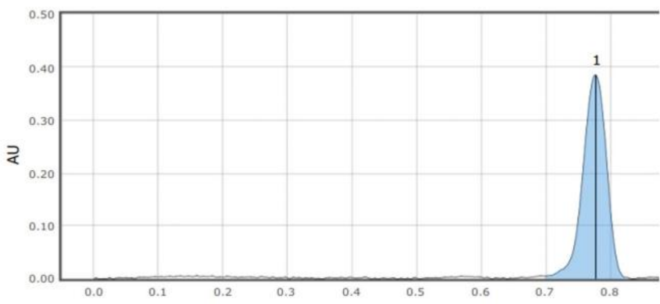
The phytosomes were prepared by using the anti-solvent precipitation method. The HP-TLC method was developed after the trial method for the solvent system several times. The mobile phase that was found to be more suitable is *n*-hexane:ethyl acetate:methanol:formic acid in the ratio 8:8:1:0.2 v/v/v/v which showed good separation on the stationary phase. The newly developed method was validated for the parameters as obeying the ICH Q2(R1) guidelines for the parameters which include linearity, range, limit of detection, limit of quantification, precision, specificity, and robustness.

The linearity of the chrysin reference standard was found to be ranging from 50 to 250 ng/band after several trials had



AU-Area under the curve

Figure 3. Chromatogram for chrysin standard.



AU-Area under the curve

Figure 4. Chromatogram for chrysin loaded phytosomes.

Table 4. Robustness.

	Changes incorporated in chromatography	
Factors	Level	R_f value
Mobile phase composition (*n*-hexane:ethyl acetate:methanol:formic acid)	(±0.1 ml)	
8.1:8.1:1.1:0.2	+0.1	0.777
8:8:1:0.2	0	0.760
7.9:7.9:0.9:0.2	−0.1	0.780
%RSD	1.396%	
Mobile phase volume	(±1 ml)	
21	+1	0.789
20	0	0.788
19	−1	0.778
%RSD	0.774%	
Duration of chamber saturation	(±20%)	
16	+20%	0.788
20	0	0.779
24	−20%	0.786
%RSD	0.602%	

R_f: retention factor; RSD: relative standard deviation.

Table 5. Quantification of chrysin loaded phytosomes.

Trails ($n = 3$)	Quantification of chrysin loaded phytosomes (μg/ml)
1	106.2
2	106.8
3	106.4
Mean ± *SD*	106.46 ± 0.30

SD: standard deviation.

been conducted. So as the linearity was shown at 50–250 ng/band which can be selected for the range parameter.

The limit of detection and limit of quantification was determined by using the slope of the calibration curve obtained from the linearity parameter and the standard deviation and added up to the formula, and the LOD was found to be 25.50 ng/band and LOQ was found to be 77.29 ng/band.

The precision is carried out in two-part interday and intraday precision for standard chrysin at the concentration of 200 ng/band application. The results obtained after performing the precision data confirmed that the newly developed method was precise because the %RSD was found to be 0.3578% for the peak area in Intra-day and 0.8926% for the inter-day, which fits into the criteria of below 2%.

The phytosomes were quantified in triplicates to find out the concentration of the chrysin standard available in the phytosomes. The quantity of the phytosomes was found to be ranging between 106 ± 0.30 μg/ml.

Conclusion

The HP-TLC method is developed and validated per the ICH Q2 (R1) guidelines and was also found to be simple, easy, linear, precise, specific, and robust. The method which is developed can be used for the analysis of the chrysin-loaded drug delivery system.

Acknowledgments

The authors acknowledge the support of Dr. Prabhakar Kore Basic Science Research Centre for the support of the HP-TLC instrument facility and KLE College of Pharmacy, Belagavi for the timely support.

Disclosure statement

The authors declare no conflict of interest.

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Original Article

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

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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

Submission Date: 24-01-2022;
Revision Date: 17-04-2022;
Accepted Date: 06-06-2022.

DOI: 10.5530/ijper.56.3s.148

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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.

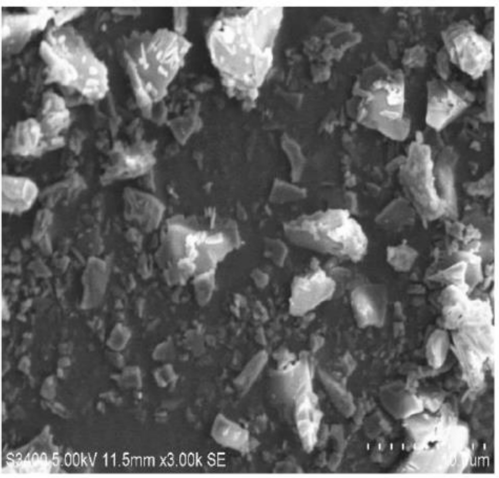

Figure 1a: SEM image of chrysin.

Figure 1b: SEM image of chrysin loaded phytosome.

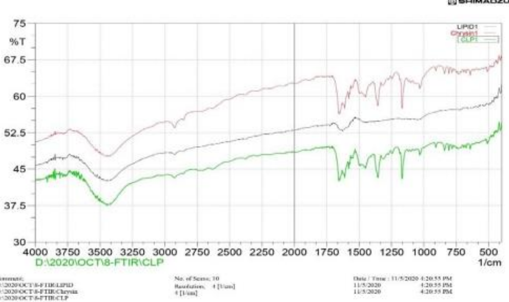

Figure 2: FTIR Image of chrysin, soya lecithin and chrysin loaded phytosomes.

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.

ACKNOWLEDGEMENT

The authors thank KLE College of Pharmacy-Belagavi for providing necessary support for conducting research activity.

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

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Cite this article: Kudatarkar N, Jalalpure S, Kurangi B. Formulation and Characterization of Chrysin Loaded Phytosomes and its Cytotoxic Effect against Colorectal Cancer Cells. Indian J of Pharmaceutical Education and Research. 2022;56(3s):s407-s412.

Original Article

Development And Validation Of UV-Visible Spectrophotometric Method For Estimation Of Chrysin In Bulk Form

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DOI:10.47750/pnr.2022.13.S08.621

Abstract

The goal of this work is to establish a straight forward UV-visible Spectrophotometric approach for determining chrysin in its pure form and to validate the method that has been devised. Using a UV-visible double beam spectrophotometer and a solvent solution containing dimethyl sulfoxide, a method was developed and validated for chysin. By following the International Conference on Harmonization (ICH) recommendations, analytical properties like linearity and precision were determined. According to Beer's law, the devised method demonstrated linear response for chrysin concentrations between 1 and 5 μg/ml, and a coefficient of correlation of 0.9987 was discovered. The method's sensitivity was determined and the limits of detection (LOD) and limit of quantification (LOQ) were discovered to be 0.2155 μg/ml and 0.6531 μg/ml respectively. The study found that the method for estimating chrysin is linear, consistent, precise and affordable and can be used to analyse pharmaceutical formulations.

Keywords: Chrysin, phytoconstituent, method development, validation.

INTRODUCTION

The validation procedure is a crucial step in the formulation and development of drugs because it shows if an instrument can consistently and effectively carry out its intended duties. Its primary goal is to verify that the processes have been properly established and managed. Additionally, it demonstrates that the devised approach can accurately determine the quantity of phytoconstituent contained in the sample.[1] Key components for the chemical analysis of the phytoconstituents are being revealed by the developed and validated method. Several techniques including spectrophotometry, thin layer chromatography and high pressure liquid chromatography can be used to identify and quantify phytoconstituents.[2] Among these techniques, spectrophotometry is a simple method to design and validate for flavonoid quantification. UV visible spectrophotometry assists in quantifying phytoconstituents based on the relative amounts of UV Visible radiation being absorbed by the substances in the solution using the Beer-Lambert equation. According to literature reviews, several phytoconstituents can be identified using UV spectro-photometric techniques either by themselves or in conjunction with other excipients.[3] There are numerous equipments utilized for quantitative determination of phytoconstituents including HPLC and HPTLC but their costs, complexity and lengthy procedures are their main drawbacks.

Also requirement of an highly trained personnel is required for operating the equipments. Thus UV visible spectroscopy is an better alternative when compared to other methods as it can overcome the above problems. Chrysin often referred to as 5'7 dihydroflavone is a phytoconstituent that belongs to the flavonoid class[4] and is also recognized for having anticancer activities against several types of cancer. Honey, propolis and passion flower are the main sources of the phytoconstituent chrysin.[5] Studies shows that chrysin has a potential as an anti-inflammatory, anti-diabetes, cardioprotective, hepatoprotective and anticancer agents.[6] Chrysin has the chemical formula $C_{15}H_{10}O_4$ and a molecular weight of 254.24g/mol. 285.50^{0}C is its melting point.[7] Chrysin's pharmacokinetic characteristics show that it is poorly absorbed after oral administration with only traces of the substances being seen in the blood, whilst the majority of it is excreted in the faceas.[8] Most critical evaluations pay attention to the biological activitiesof chrysin and comparable plant sources in vivo and in vitro as well as their their pharmacological effects on animal and human bodies rather than the analytical techniques for identifying chrysin. Chrysin has a limited solubility in buffers and fluids, however it is well soluble in dimethyl sulfoxide. Using HPLC and HPTLC, a number of analytical

techniques for chrysin estimation have been established.[9] Our study intends to develop a stable, repeatable, reliable, andtrustworthy method for determination of chrysin.

Figure 1: The chemical structure of chrysin

MATERIALS AND METHODS

Materials

Chrysin was purchased from Tokyo Chemical Industries in Japan. Spectral measurements were conducted using a double beam UV Spectrophotometric (Shimadzu 1900 Japan) and 10mm quartz curettes. The latter is less expensive andsimpler to use.[10] Only analytical-grade chemicals and reagents were used.

Method

Preparation of the standard solution

Chrysin was carefully weighed at 10 mg and then transferred into a 10 ml volumetric flask. A dimethylsulfoxide solution was added to the volumetric flask until it had a capacity of 10 ml. Bath sonicator was used to get a clearsolution.

Determining maximum wavelength

In a UV spectrophotometer, a 5 µg/ml solution of chrysin was scanned at 270 nm.11 As a control solution, dimethylsulfoxide was employed.

Creation of the reference calibration curve

By measuring the absorbance of a chrysin solution prepared from stock solutions in DMSO at 270 nm in triplicate, the standard calibration curve of chrysin was obtained. Chrysin concentration was plotted on the x-axis and absorbance on the y-axis to create the calibration curve.

Analytical method validation

A stated definition of validation is (ICH) providing recorded proof that offers a high level of assurance that a chosen activity can consistently deliver a desired outcome or product that complies with its predetermined requirements and quality attributes. For method validation, the following variables were assessed.[12]

Linearity and range

The analytical methodology's capacity to analyse data that are directly proportional to analyte concentration was referred to as its linearity. Chrysin standard solution ranging from 1 to 5 µg/ml was made from stock solution and examined to determine the linearity of the proposed methodology. Every measurement was made in triplicate.[13]

Precision

To assess the validity of the proposed methodical technique, precision studies were conducted. Through the use of three triplicates of the same sample at low concentrations of 1 µg/ml, medium concentrations of 3 µg/ml, and high concentrations of 5 µg/ml, repeatability was clearly confirmed. As a result, the absorbance was measured throughout the day and a precision research was conducted by making drug resolution at concentrations of low 1 µg/ml, medium 3 µg/ml, and high 5 µg/ml and analysing it three times throughout the day. Several completely separate days were treated the same way to produce work that was reportable as %RSD. The precision result demonstrated an honest reliability, buttwo intraday and interday precision study results were measured.

Ruggedness

By analysing low 1 µg/ml, medium 3 µg/ml, and high 5 µg/ml concentration solutions in DMSO solution three times bytwo different analyzers at 270 nm, ruggedness was assessed. The outcomes were reported as %RSD.[13]

Robustness

To assess the method's robustness, chrysin solution containing mild concentrations of 1 µg/ml, medium concentrations of 3 µg/ml, and high concentrations of 5 µg/ml were analyzed 2 times at two distinct wavelengths (265 and 275).[14]

LOD and LOQ

The lowest quantity of the sample's analyte which can be determined is known as the limit of detection (LOD). The lowest quantity of analyte in the sample that can be quantitatively determined with adequate precision and accuracy are termed as the limit of quantification (LOQ). The following equation was applied to determine LOD and LOQ: LOQ-10 s\m LOD-3 s\m where s is the response's standard deviation and m is the slope of the corresponding calibration curve. [15,16]

RESULTS AND DISCUSSION

The proposed methodology offers a simple, precise, cost effective and practical method for the UV Spectrophotometric analysis of chrysin. For both intraday and interday categories, it was discovered that the approach had an RSD of under two. The RSD values were found to be less than two, which further demonstrated the strategy's toughness and robustness. The predicted methodology's limit of detection and limit of quantification which were determined to be 0.2155 µg/ml and 0.6531 µg/ml respectively, show that the method devised is sensitive.

Determination of maximum wavelength.

The wavelength that has the greatest absorption in DMSO solution was found at 270nm (Figure 1). Figure 2: UV Spectrum of chrysin in DMSO.

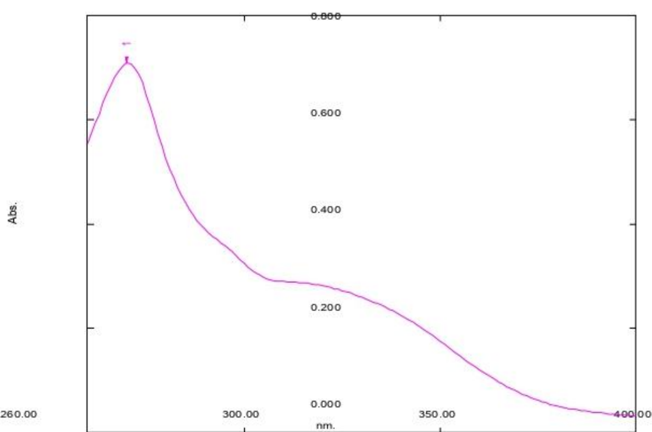

Figure 2: UV Spectrum of chrysin in Dimethyl sulfoxide

Preparation of standard calibration curve.

Chrysin's calibration plot was determined to be linear with a 0.9987 coefficient of correlation as shown in (Figure 3).

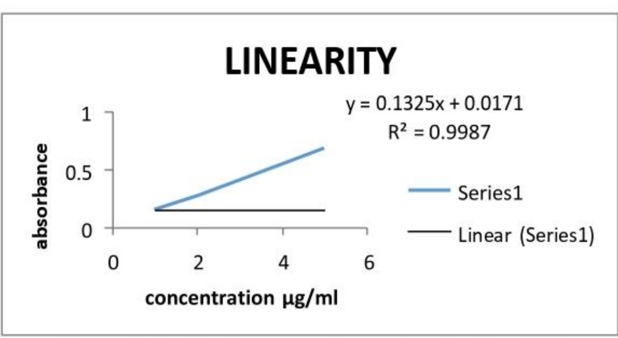

Figure 3: Calibration curve of chrysin in DMSO at 270nm

Analytical method validation

According to ICH guidelines, the method was evaluated for a set of parameters including precision, toughness, robustness, limit of detection (LOD) and limit of quantification (LOQ).[17]

Linearity and Range

The value of the correlation coefficients supported the linearity of the calibration curve (r2). According to Table 1, correlation coefficient for chrysin was determined to be 0.9987.

Table 1: Linearity table of chrysin

Concentration µg/ml	Absorbance	
1	**0.158**	**y=0.132x + 0.017** R^2 **=0.998**
2	**0.275**	
3	**0.406**	
4	**0.552**	
5	**0.682**	

Precision

There was no significant difference between the intraday and interday because they were performed on completely different days. The outcome demonstrates the consistency of the suggested methodology. The precision outcomes are shown in Table 2 and 3. Additionally, the relative variance proportion was estimated.

Table 2: Intraday Precision

Concentration µg/ml	Absorbance	Absorbance	Absorbance	Average %RSD
1	0.147	0.186	0.188	0.411286
1	0.148	0.185	0.187	
1	0.148	0.186	0.189	
%RSD	0.39098212	0.31096065	0.53191489	
3	0.406	0.445	0.452	0.26196
3	0.405	0.448	0.452	
3	0.405	0.445	0.454	
%RSD	0.14243839	0.3883522	0.25508848	
5	0.662	0.696	0.69	0.21579
5	0.659	0.696	0.693	
5	0.66	0.698	0.693	
%RSD	0.23132639	0.16574649	0.25029636	

Table 3: Intraday Precision.

Concentration µg/ml	Absorbance	Absorbance	Absorbance	Average %RSD
1	0.14	0.186	0.194	0.3404
1	0.139	0.185	0.195	
1	0.14	0.185	0.195	
%RSD	0.413377281	0.31151993	0.29658404	
3	0.388	0.445	0.457	0.2546
3	0.386	0.444	0.454	
3	0.386	0.444	0.455	
%RSD	0.29862945	0.12993629	0.33547406	
5	0.649	0.695	0.693	0.1330
5	0.649	0.696	0.695	
5	0.65	0.697	0.693	
%RSD	0.088914313	0.14367816	0.16646332	

Ruggedness

By performing the assay under identical conditions on completely different days by several analysts using various tools and at various times, ruggedness was determined. The test scores fell between 99 and 101%.

Table 4: Result of ruggedness

Concentration (µg/ml) Analyst 1	Absorbance	Statistical Analysis
1	0.145	Mean-0.146333333SD-
1	0.148	0.001527525
1	0.146	%RSD- 1.043866901
3	0.452	Mean-0.454333333SD-
3	0.456	0.002081666
3	0.455	%RSD-0.458180337
5	0.672	Mean-0.672666667SD-
5	0.672	0.001154701
5	0.674	%RSD-0.17166014

Analyst 2		
1	0.146	Mean-0.14466667SD-
1	0.144	0.0011547
1	0.144	%RSD-0.7981801
3	0.458	Mean-0.45766667SD-
3	0.456	0.00152753
3	0.459	%RSD-0.33376371
5	0.679	Mean-0.67733333SD-
5	0.676	0.00152753
5	0.677	%RSD-0.22552046

Robustness:

By securing the assay throughout the modification wavelength, robustness was firmly established. The sharp RSD was found to be less than 2%, which was within the permissible range as demonstrated in Table 5.

Table 5: Results showing robustness

Concentration (µg/ml) at wavelength 270nm	Absorbance	Statistical Analysis
1	0.152	Mean-0.1526667SD-
1	0.152	0.0011547
1	0.154	%RSD-0.7563541
3	0.502	Mean-0.5006667SD-
3	0.499	0.0015275
3	0.501	%RSD-0.3050982
5	0.675	Mean-0.6766667SD-
5	0.678	0.0015275
5	0.677	%RSD-0.2257426
Concentration (µg/ml) at wavelength 265nm		
1	0.234	Mean-0.23333333SD-
1	0.23	0.00305505
1	0.236	%RSD-1.30930734
3	0.518	Mean-0.51766667SD-
3	0.517	0.00057735
3	0.518	%RSD-0.11152935
5	0.696	Mean-0.69533333SD-
5	0.695	0.00057735
5	0.695	%RSD-0.08303216
Concentration (µg/ml) at wavelength 275nm		
1	0.135	Mean-0.1353333SD-
1	0.136	0.0005774
1	0.135	%RSD-0.4266135
3	0.486	Mean-0.4856667SD-
3	0.485	0.0005774
3	0.486	%RSD-0.1188779
5	0.662	Mean-0.663 SD-
5	0.665	0.0017321
5	0.662	%RSD-0.2612445

LOD AND LOQ:

LOD and LOQ values were found to be 0.2155 and 0.6531 µg/ml, respectively

Table 6: Optical characteristics.

Parameters	Result
Absorption maxima	270nm
Beers law range	1-5 µg/ml
Correlation coefficient	0.9987
Regression equation	0.132x + 0.017
Slope	0.1325
Intercept	0.0171
Precision	0.26196
LOD µg/ml	0.2155 µg/ml
LOQ µg/ml	0.6531 µg/ml

Chrysin at 270 nm was chosen as the phytoconstituent and the method was designed and validated in accordance with ICH requirements for linearity, accuracy, specificity, robustness, ruggedness, LOD and LOQ. Chrysin concentration was measured at three distinct concentrations: low (1 µg/ml), middle (3 µg/ml), and high (5 µg/ml). Beer's law is follow ed by all triplicates concentration of the phytoconstituent chrysin in the concentration range of 1 to 5 micrograms/ml. Chrysin was determined using the suggested approach which revealed linear regression with the equation y=0.1325x + 0.0171 and a coefficient correlation (r2) of 0.9987 (Figure 1). The relative standard deviation of the three test samples of chrysin was used to measure the precision. Each assay was calculated, and the resulting relative standard deviation of %assay was less than 1.5%. Limits of detection (LOD) and limit of quantification were discovered to be 0.2155 µg/ml and 0.6531 µg/ml respectively. Chrysin phytoconstituent can be analysed using the suggested method when it is taken in bulk dosage form.

CONCLUSION

The suggested UV Spectrophotometric approach which has been used in numerous research can be regarded as straightforward, quick and affordable. With outstanding accuracy, precision and linearity, the method is valid conformity with ICH criteria and appropriate for chrysin estimation.

ACKNOWLEDGEMENT:

The facilities for conducting this research were generously provided by KLE College of Pharmacy Belagavi for which the authors are appreciative.

CONFLICT OF INTEREST: Nil

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