"SYNTHESIS OF CURCUMIN-QUERCETIN LOADED CHITOSAN NANOPARTICLES FOR ANTIMICROBIAL AND ANTICANCER ACTIVITY"

Project report submitted in partial fulfillment of the requirement for the degree of Master of Science

in

Biotechnology

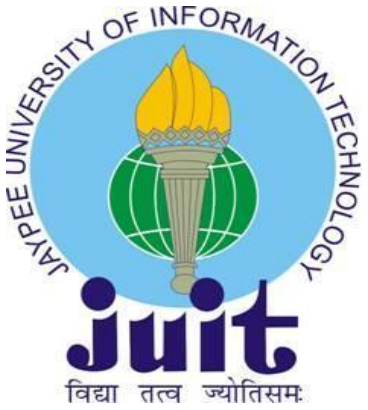
By

Tulika Goswami (217817)

Under the Supervision of

Dr. Abhishek Chaudhary

to



Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology Waknaghat, Solan-173234, Himachal Pradesh

DECLARATION

I hereby declare that the work presented in this report entitled **"Synthesis of curcumin-quercetin loaded chitosan nanoparticles for antimicrobial and anticancer activity"** in partial fulfillment of the requirements for the award of the degree of "Masters in Biotechnology" submitted in the Department of Biotechnology & Bioinformatics, "Jaypee University of Information Technology Waknaghat", is an authentic record of my own work carried out over a period from January 2023 to May 2023 under the supervision of **Dr. Abhishek Chaudhary** (Assistant Professor). The matter embodied in the report has not been submitted for the award of any other degree or diploma.

Tulika Goswami

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SUPERVISOR'S CERTIFICATE

This is to certify that the project work titled **"Synthesis of curcumin-quercetin loaded chitosan nanoparticles for antimicrobial and anticancer activity"** by **Tulika Goswami** during her end semester in fulfillment for the award of the degree of Master's in Biotechnology of Jaypee University of Information Technology, Solan, has been carried out under my supervision. This work has not been submitted partially to any other University or Institute for the award of any degree or appreciation.

Signature of Supervisor- _____

Name of Supervisor- **Dr. Abhishek Chaudhary**

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TABLE OF CONTENT

TABLE OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

CUR	Curcumin
TNBC	Triple-negative breast cancer
HER-2	Human Epidermal Growth Factor Receptor-2
MIC	Minimum Inhibitory Concentration
AST	Antibiotic Sensitivity Test
TME	Tumor Microenvironment
DMSO	Dimethyl sulfoxide
Q	Quercetin
PLGA	Poly (lactic-co-glycolic acid)
TPP	Sodium Tripolyphosphate
PBS	Phosphate Buffer Saline
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
DPPH	2,2-diphenylpicrylhydrazyl
ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
CSQNP	Chitosan-Quercetin Nanoparticle
CSCURNP	Chitosan-Curcumin Nanoparticle
CSCURQNP	Chitosan-Curcumin-Quercetin Nanoparticle
CDK	Cyclin-dependent kinases
ROS	Reactive Oxygen Species

ABSTRACT

Chitosan nanoparticles are frequently utilized in the pharmaceutical industry as a medication delivery system or as an antibacterial agent. This work used the ionotropic gelation process with acetic acid to create chitosan nanoparticles that were loaded with curcumin and quercetin. Utilizing sodium tripolyphosphate as a chelator, polymeric nanoparticles were synthesized using chitosan. For in vitro drug delivery testing, the nanoparticles were coated with hydrophobic curcumin and quercetin in a nanocarrier. As well as being subjected to controlled drug delivery investigations in vitro against bacteria and fungus, the synthesized nanoparticles were subjected to UV-VISIBLE spectroscopy that confirmed the formation of NPs an at absorption peak of 250nm. DLS, SEM, and Zeta potential analyses, as well as drug encapsulation efficiency, invitro drug release kinetics, and antimicrobial assays like the Agar Disc Diffusion method (AST) and Minimum Inhibitory Concentration (MIC), confirmed antimicrobial activity against different bacterial and fungal strains, Antioxidant Assays like DPPH radical scavenging test and ABTS method tests were also performed under lab conditions to confirm desired properties in synthesized nanoformulations. The synthesis of nano-drugs using various bioassays was investigated in the second section.

In order to achieve this, the McCoy cell line's cytotoxicity was assessed using the methyl-thiazol-tetrazolium (MTT) assay, Additionally, a variety of drug release techniques will be used to examine the drug release profile of manufactured medications. Quercetin-curcumin nanoformulations have demonstrated encouraging outcomes in terms of their absorption by the epithelial system as well as improved transport to the target location. Therefore, the primary goal of the current work was to synthesize a natural polymer-based nanomedicine that fights microbial attack and cancer with synergistic effects, improved drug loading efficiency, and bioavailability of the natural compounds.

KEYWORDS: Curcumin, Quercetin, Chitosan, Hydrophobic, Biopolymer, Encapsulation, synergistic, bioavailability, Nanocurcumin.

CHAPTER 1
INTRODUCTION

Curcumin, Nano curcumin, Quercetin, and Nano quercetin, and also their nanocomposites created using biodegradable polymer chitosan with enhanced aqueous solubility and bioavailability, are very promising, dependable, safe, and long-lasting antibacterial molecules against clinically significant species of bacteria that use multitarget mechanisms such as antioxidant enzyme inactivation, reactive oxygen species(ROS)-mediated cellular damage, and inhibition of acyl-homoserine-lactone synthase necessary for quorum sensing and biofilm formation, therefore, passing all the mechanisms of bacterial antibiotic resistance. Curcumin nanoformulations might thus be viewed as a promising and long-term antibacterial therapeutic alternative for dealing with the current problem of antibiotic resistance. These nanoformulations can be used as anticancer as well as antimicrobial agents due to their anticancer properties.

According to Cancer Statistics 2022, a research study that appeared in the American Cancer Society journal CA: A Cancer Journal for Clinicians, has a companion educational resource called Cancer Facts & Figures 2022. The yearly Facts & Figures report offers that around 10 million people are going to die from cancer worldwide in 2022, making it the top reason for death. In terms of new cases of cancer in 2022, these types are the most predominant: Breast cancer cases are 2.26 million, lung cancer cases are 2.21 million, and colorectal cancer is 1.93 million cases, prostate cancer includes 1.41 million cases worldwide. [1] Major cancer-related deaths in males are due to lung cancer while the majority of death related to females is due to breast cancer and cervical cancer. Cancer begins when body cells begin to proliferate uncontrollably and normally spreads gradually over several years. Consequently, cells in virtually any portion of the body have the potential to become cancerous and spread to other regions of the body. Cancer therapeutic interventions and survival are largely influenced by the cancer's stage, the likelihood of tumor progression, as well as the patient's general condition. Surgery, chemotherapy, radiation, and targeted therapy are the four methods of cancer treatment that are most frequently used. [2] Chemotherapy is still a very effective method for treating cancer, but it frequently comes with

serious risks and side effects. Scientists are looking into herbal remedies, nutrients, and natural medicine marks the common primary challenges in conventional cancer treatment.[3]

Nanoscale drug carriers may be able to minimize the severe adverse effects associated with specific molecular anti-cancer therapy selective absorption. The required ligand is typically chemically conjugated to the nanocarrier's constituent components before synthesis or conjugated to nanocarriers that have already been created utilizing reactive modifiers or coupling reagents.[4]

A better understanding of the molecular alterations that lead to the onset and spread of cancer is essential for its prevention and treatment. Targeting certain cancer cells allows for the prevention of tumor growth, spread, and advancement despite having any negative side effects. In addition to the anticancer medications that are chemically created, numerous anticancer compounds that are being isolated from different plants, especially Curcuma longa (C. longa) from the Turmeric plant and Quercetin compound which is a powerful flavonoid present in onions, grapes, citrus fruits, etc. [5]

Finding novel treatments for breast cancer has recently been the focus of numerous investigations and also a major objective of this study. Some plant-based substances function as pro-oxidants, causing cancer cells to produce reactive oxygen species (ROS). The polyphenolic compound is taken out of rhizomes of the turmeric plant *Curcuma longa.* contains the substance **Curcumin.** Two researchers, Pelletier & Vogel, initially identified this substance in 1815. With this finding, there was an increase in research activity on CUR, which resulted in the identification of CUR's many health advantages. A wide range of biological and pharmacological actions like antioxidant, antiseptic, analgesic, and anticancerous, is exhibited by Curcumin.[6] A flavonoid known as **quercetin** (3,3',4',5,7-pentahydroxyflavone) is found in many plants, especially raspberry, red grapes, and onions have anticancer activity. The key ingredients in many plants, including berries, red grapes, and onion particularly in cases of breast, pancreatic, prostate, hepatic, and thyroid cancers, quercetin can trigger apoptotic cell death and cell cycle, inhibiting tumor growth. It should be mentioned that, in addition to treatment, nanomedicine can perform a variety of other roles for cancer patients, such as tissue regeneration, detection of diseases, cancer scanning, and theranostic applications. This study will concentrate solely on treatment and to check the synergistic effect of the desired formulations, and their antimicrobial and antioxidant use, with a focus on the advanced usage of nanomaterials to transport anticancer drugs (i.e., as nanocarriers).[7]

CHAPTER 2

REVIEW OF LITERATURE

In both industrialized and developing nations, chemotherapy is considered the most accepted type of therapy for many human illnesses. Poor adherence and several negative effects have been linked to this strategy. As a result, there has been a lot of work done recently to find a better therapeutic method that makes use of natural substances or extracts. Curcumin, one of the other commonly occurring phenolic chemicals that exhibit a yellow color molecule that superficially benefits as an ingredient for artificial coloring in the food industry as well as household kitchens, also has therapeutic potential for several pathological disorders. It is also quite safe. The biological activities of anti-microbial, anti-tumor, and anti-diabetic curcumin make it solely responsible for its usage in several disease detections.

BRIEF DESCRIPTION OF THE PROPOSED ANTICANCER/ANTIMICROBIAL AGENTS:

Curcumin has also been shown to be a compound with hypoglycemia, hepato-, nephron-, cardio-, and neuroprotective properties. Furthermore, this chemical also prevents heart attack and reduces thrombosis. It is the most noticeable polyphenol and is frequently ingested daily with food (in countries such as India, Eastern Asia, and some of Africa), improving cooperation. The use of curcumin dates back to ancient times, but it wasn't until 1949 that the journal "Nature" published the very first experimental proof of curcumin’s antimicrobial effectiveness at concentrations of very minute levels. Only then did major research on the compound start. There have been over 8000-9000 peer-reviewed studies, reports, inventions, and views of preclinical and clinical trials during the past 20 years that demonstrate curcumin's potential as a medicinal chemical. Furthermore, the Food and Drug Administration of the US (USFDA) considers this chemical as GRAS i.e., generally recognized as safe and curcumin molecule has multitargeting capacity in diverse pathological situations, allowing the development of nutraceuticals as well as a medicinal drug.

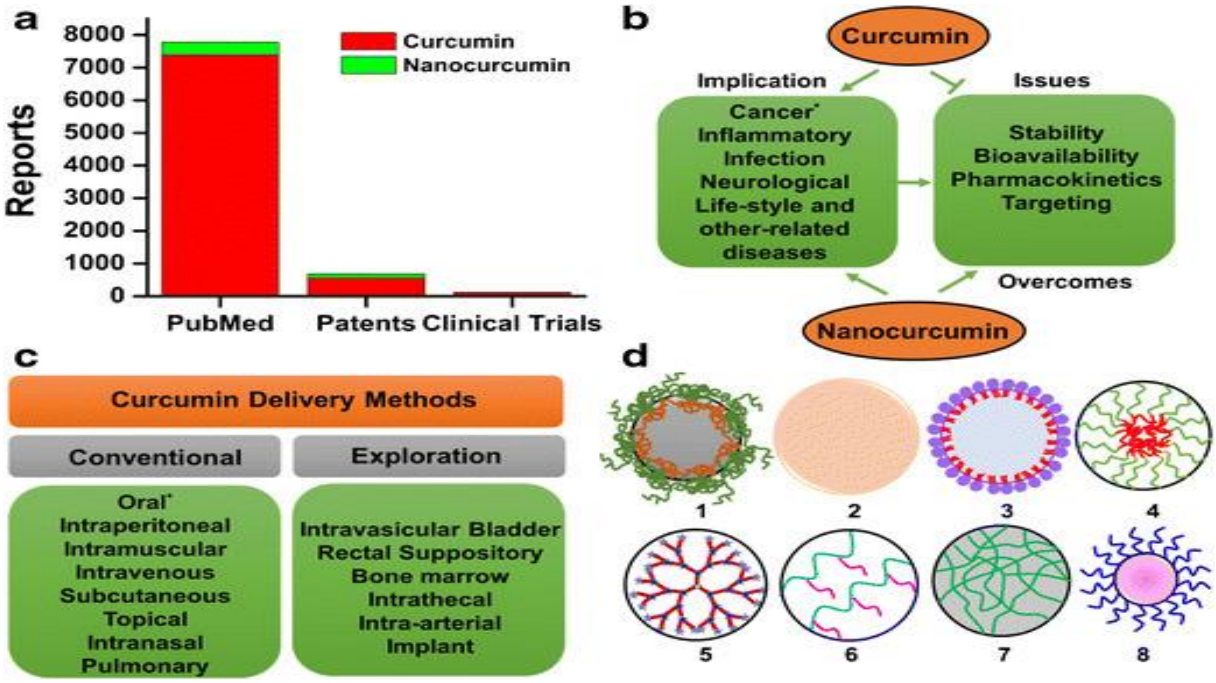


Fig: 1 Schematic illustration of curcumin nanoformulations. Source: (PubMed)

Curcumin, like numerous other lipid-soluble therapeutic small pharmacological compounds, has constraints for its effective application in analytical schemes to handle disease states. There are some examples: Minimal hydrophilic nature and inherent dissolution rate(s), low physicochemical instability, quick metabolization, absorption with low bioactive content, minimal pharmacokinetic and related bioavailability, and limited diffusion and targeted potency. These parameters have a substantial impact on the efficacy of curcumin as a medicinal chemical. Thus, various formulations of curcumin, comprising native, modified, and micro/nanoforms, are manufactured as emulsions, creams, solutions, tablets, gel, band-aids, and so on have been intended for conventional or exploratory administration to acquire the best results in a variety of clinical circumstances. For efficient local, circulation, affinities, or active targeting uses, both noninvasive and invasive methods of administration have been created.

To deliver a proper drug concentration of curcumin to the illness site, personalized and customized ways of administration may be required. Despite a few crucial claims which should be taken into consideration, curcumin has many advantages, including its centuries-long use, exceptional pre-clinical and biological activity, and diagnostic, human, and animal trials boost the fast growth of curcumin-based preparations for medicinal purposes.

All these encouraging results have prompted scientists in creating and refining effective nano curcumin formulations for enhanced soluble, stable, intracellular efficiency, specificity, tolerance, and high therapeutic index. Some of these novel ingredients and formulations, first and foremost, improve the cellular targets' sustained release of drugs and boost the efficiency of therapeutics.

Several nanoformulation-based techniques have been developed over the past years to improve all the use using curcumin *in vitro and in vivo*, and preclinical setup, including adjuvants, polymers, stabilizers, and other chemical moieties, hydrogels, lipids, and nanoparticles. Nanoparticles-based efficient curcumin helps in difficulties with solubility, rapid metabolism of the drug, hydrolysis, and stability of drugs and should also disseminate specific cells at the same time while reducing inadvertent damage at adjacent healthy tissues. [8]

According to numerous studies, polyphenols, flavones, and flavonoids have significant anti-tumor characteristics that can be used to treat various types of cancer. In this respect, quercetin is a common phenolic compound that may be found in a variety of foods such as nuts, beverages, vegetables, other plants, and, in general, people's regular dietary programs.

This substance is a pental-hydroxyl-flavanol with 5 hydroxyls functional group on the flavanol molecule at positions 3,5,7,30,40. The substitution based on different groups with functional moieties results in distinct chemical and biological and pharmacologic properties of quercetin. According to certain studies, quercetin is present in two conditions Aglycone/1free and 2 coupled alongside other moieties. Quercetin interacts with moieties such as biomolecules like carbohydrates, sulfate, and alcoholic groups for the formation of quercetin byproducts including Quercetin ethers, glycoside, prenylated quercetin, and sulfate.

It is detrimental to active cells, but it has a cytotoxicity effect on tumor cells via a variety of pathways, making it a promising choice for breast cancer treatment or as a supplement to other anti-cancer drugs.[9]

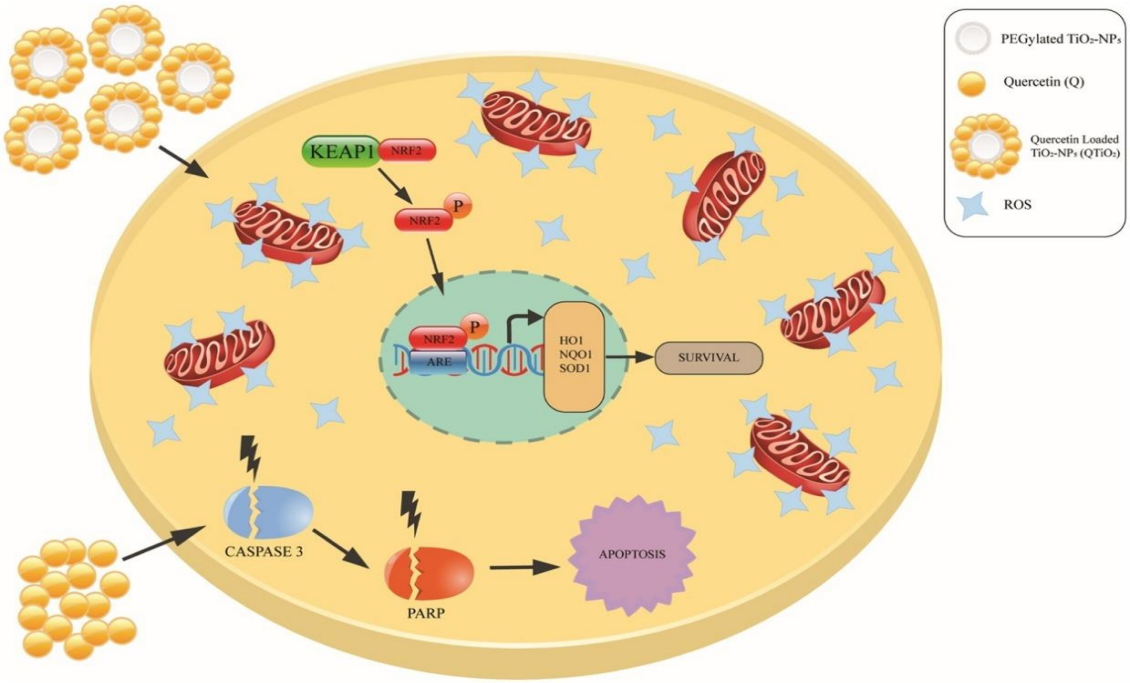


Figure: 2 Nano quercetin. Source: (Enzyme and Microbial Technology, Volume 138, August 2020, 109559)

[2.1] NANOMEDICINES AS THERAPEUTIC AGENTS FOR BREAST CANCER

Breast cancer is a very diverse disease. As a result, breast cancer is classified in a variety of ways. Breast cancer is now frequently categorized based on clinical and pathological classification. Invasive ductal carcinoma is one of the major types of breast cancer however fewer common types continue to gain interest because of their aggressive nature and incidence in distinct patient subpopulations like younger patients are more prone to breast cancer. This tumor stage is usually the next most serious worry. The actual tumor at stage 1 in the breast rapidly spreads into neighboring lymphatic systems and tissues or the other distant organs as the disease progresses (distant metastasis, i.e., stage 4). The most common locations of metastasis from breast cancer are the lung, bone, liver, and brain. Breast cancer is further classified based on the presence or overexpression of progesterone, estrogen, and HER-2 receptors like IDC, TNBC, and IBC, MBC.

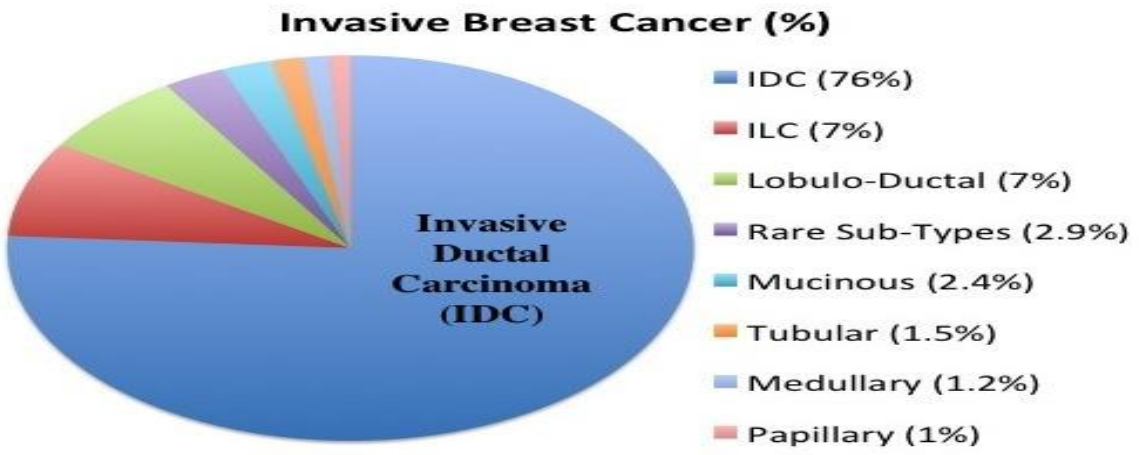


Fig: 3 Types of breast cancer. Source: (SEER, 2001)

Nanomedicines have small size and flexible characteristics which make them a promising candidate in the field of therapeutics and diagnostics. Nanomedicine is defined as the biomedical field of material having a minimum one dimension less than 100 nm, while apparatus with dimensions of 100-200 nm are constantly used in nanomedicine applications. Lipid membranes, nanoparticles, micelles, dendrimers, nanotubes, and other nanomedicine examples can be constructed of a variety of components, including lipids and their derivatives like glycolipids, polymers, proteins, or their combinations.

Doxil which is a liposome and nanomaterials like Abraxane already are generally utilized in clinical therapy of breast cancer having effectiveness. However, all these compounds are first created as common drug vehicles for anticancer. Having greater knowledge in breast cancer molecular biology, various deliverable nano-delivery techniques for breast cancer are being highly studied in recent times. This makes it appropriate that can provide an update on the present state of affairs along with the most current developments in this subject.

[2.2] Applications of Nanomedicines

S. No.	Objections in breast cancer chemotherapy	Functions of Nanomedicines
1.	Lack of specificity in receptors of breast cancer	Increased tumor drug levels can be achieved by active and passive targeting
2.	Insufficient reach of drugs at the metastatic locations like brain/bone	Some nanoformulations intrinsically boost the brain and bone perforation
3.	Unwanted pharmacological conditions like a short lifetime and rapid clearance	The PEGlyation method can be used to enhance the circulation duration.
4.	Usage of organic solvent/ surfactants limits the dose toxicity of anticancer drugs	Increased tumor specificity as above, controlled nanocarrier drug release from surfactant-free nanoformulations
5.	At the cellular /micro level drug resistance occurs. Insufficient drug loading effluxes.	Improving endocytosis via passive and active targeting nanoformulations inhibits the efflux and transport of drugs and the delivery of chemotherapeutic agents together for drug-target resistance approaches.
6.	At the tumor microenvironment level drug resistance occurs like hypoxic conditions, and low ph.	By catalytic receptive-based nanoformulations like ph/temp-controlled devices, one can target the tumor microenvironment (TME)

Table: 1 Challenge in Nanomedicine and its Solutions

At a minimum, some of these restrictions may be overcome via nanomedicine.

Because nanocarriers have an exceptional property of a high surface area-to-volume ratio, it is possible that the surface characteristics can be altered for better treatment such as tumor attacking, extensive circulation, and enhanced transcytosis that helps in achieving better entrance into the tumor sites, cancer sites and metastatic locations of tumor cells.

Furthermore, medicinal compounds can obtain greater stability, higher solubility, and regulated release kinetics by enclosing in or attaching to nanocarriers. Drug mixtures may also be administered concurrently for enhanced additive or synergistic anticancer effects. Solid-lipid nanoparticles, liposomes, and polymers are examples of nanomaterials that are commonly used.

These nanocarriers aid in improving anticancer drug water solubility, increasing drug carrier efficiency to tumor locations, and enabling anticancer agents' site-targeted delivery. Only a few nanomedicine products have received FDA approval as of today, with Abraxane and Doxil being the most effective nano-based drug globally utilized in clinical settings for the treatment of breast cancer. Nanomedicine development and incorporation into routine medication for cancer-related therapy also give valuable findings for scientists working in the nanomedicine area and doctors. Moreover, a few potential nano-based formulations in the clinical phase are already described [10].

[2.3] IMPORTANT FINDINGS

Due to an increment in the cases of side effects of synthetic drugs, there is an enormous growth in the utilization of natural products in the past few years, and near about 80% of FDA-approved drugs used for cancer treatment are natural products or their byproducts.

A few of the substances, including resveratrol, and sulforaphane have demonstrated favorable efficacy against cells of breast cancer. Green tea catechin i.e., EGCG has been demonstrated to increase cell death in breast tumor cells via causing upregulation of genes like caspase 3, 8, 9 inducing proapoptotic pathways. Furthermore, the human telomerase reverse transcriptase i.e., hTERT gets inhibited by sulforaphane in a dosage and time-related manner. The mitochondrial membrane potential is raised by resveratrol and breaks it down to release cytochrome c, which then promotes cell death via multiple cascade enzymes. Curcumin, an active component in the culinary spice used as turmeric, has received special importance because of its numerous pharmacological effects like anticancer, and antioxidant activities.

Curcumin's advantages, although, are hampered by its rapid first-pass metabolism from the liver and poor water solubility due to which system circulation is restricted. Due to this several nano-based formulations are being developed to overcome such problems.[11]

[2.3.1] Curcumin as a Phytochemical with Hydrophobic Properties in Medicine

Curcumin Composition:

(1E,6E)-1,7-bis(4-hydroxy-methoxyphenyl)-1,6-heptadiene-3,5-dione, is the chemical moiety of curcumin which consists of 2 aromatic ring structures having methoxy and hydroxy groups linked by a 7-carbon-containing chain of the molecule containing unsaturated diketone. Generally, available commercial curcumin consists of 3 curcuminoids: bisdemethoxycurcumin desmethoxycurcumin, and di-feruloyl methane. Curcumin exhibits a mechanism of tautomerism with keto and enol form, having keto form prevailing in acidic and neutral settings and the constant enol type prevailing in alkaline environments. Due to its ionization capabilities, at neutral or acidic ph curcumin is less water soluble. It absorbs alkaline ethanol, methanol, ketone, CH3COOH, DMSO, etc. Curcuminoids like diferuloylmethane which is responsible for the yellow color of curcumin show different pharmacological and biological behavior patterns of the substances.

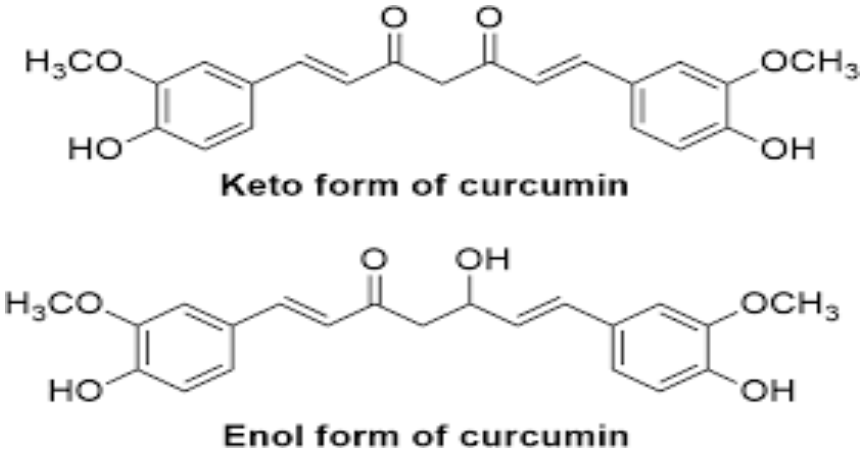


Figure: 4 Keto-Enol form of curcumin. Source: (Bio nanocomposites, Green Synthesis and Applications Micro and Nano Technologies,2020, Pages 233-257)

Regarding the United States Food and Drug Association (FDA), various studies have been conducted in-depth on the inherent well-tolerance, safety, and biocompatible properties of pure curcumin.

[2.3.2] Curcumin as a therapeutic agent and Its Disadvantages

However, CUR exhibits limited bioavailability, poor absorption, and quick excretion from the body. It is also not soluble in water and unstable in solutions. With these factors, CUR has been solubilized using solvents that are organically based like methanol, DMSO, ethanol, and acetone). These limitations make it difficult to employ CUR as a medicinal agent in vivo. Native curcumin has been shown to exhibit an unexpected potential in vitro, but due to its limited solubility in lipids of 0.6 g/ml, it exhibits very little to no action in vivo. Its non-specific circulation and insufficient aggregation after intravenous injection restrict its therapeutic effectiveness.

Curcumin must distribute its active ingredient to the most prominent location of injury at a steady state and amount in order to have the desired therapeutic effects. Depending on each drug's specific therapeutic goal, the amount and dose may range from quick and complete absorption when a rapid commencement of an action is necessary, as in the case of acute diseases like asthma or heart attacks, to slow and maintained when a longer circulation is required.

Therefore, more study on curcumin is needed to discover potential solutions to these constraints. By far, a number of biochemical developments have been shown as promising candidates for enhancing its bioavailability, including an adjuvant fusion alongside additional dietary variables, metals hybridization, curcumin-based lipid nanoformulations, its phospholipid structures and conjugation with polymeric compositions and artificially made analogs. Yet, all of the above research mentioned uses curcumin in its natural form.[27]

As a result, many methods have been employed to address the issues with CUR, including the application of adjuvants in drug delivery devices. Chitosan, a natural polymer, which is found in crustaceans' shell and in chitin found in the cell wall of fungi, have been linked to CUR to increase its bioavailability.

[2.3.3] Antimicrobial Properties

CUR has been shown to have antibacterial activity against Gram-positive and Gram-negative bacteria, including those responsible for human illnesses and antibiotic resistance. CUR also inhibits the formation of microbes like biofilms out of bacteria, which are colonies of cells encased within the created matrix of polymers that are resistant to therapies associated with antimicrobial activity. CUR's antibacterial mechanism of action includes membrane or cell wall damage, disturbance with a cells-related cascade of processes via protein and DNA targeting, and restricts bacterial quorum sensing.[22] Curcumin is effective in helping treat bacterial infections since it possesses antibacterial activity against bacterial pathogens. Curcumin has been shown to possess a variety of antimicrobial actions, including bacterial cell membrane rupture, inhibition of DNA replication, motility impairment, and changes in bacterial gene expression. Curcumin is regarded as a wide-spectrum antibacterial agent since it is effective against a variety of Gram-positive and Gram-negative bacteria. Curcumin's amphipathic characteristics enable it to pass across bacterial cell membranes and render them permeable to antibiotic absorption.

Both types of bacteria gram positive and gram negative are prevented from growing by curcumin. The MIC (Minimum Inhibitory Concentration) of curcumin required for the prevention in development of *Staphylococcus aureus* MRSA) is found to be 125-250µg/ml, data collected from the research *in vitro* studies. *In vitro*, all of the Helicobacter pylori species were inhibited from growing in individuals with gastroenteritis using curcumin, one of the primary components of turmeric. The pour plate method was used by Negi et al to assess curcumin's antibacterial properties. *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Pseudomonas aeruginosa* were among the bacteria against which various curcumin fractions were tested. They discovered that curcumin significantly inhibits all of the bacterial strains they had previously investigated. [33]

Curcumin also works well against 20 different kinds of Candida. According to Pandit et al.'s assessment of the antibacterial properties of curcumin nanoformulations alongside the synthetic antibiotics gentamycin and chloramphenicol that are available for purchase, curcumin nanoparticles rank amongst the remaining synthetic antibacterial agents.

As a result, lotions containing curcumin or nano curcumin might be created to treat illnesses brought on by different bacteria. The principal infectious bacterium S. aureus may be found in the inner elbow, on the bottom of heels and side of the groin area. It is a gram-positive microbe that can cause skin and soft tissue infections, bacteremia, infective endocarditis, osteoarticular infections, and pleuropulmonary infections, among other illnesses. Gram-negative E. coli bacteria are found mostly in the small intestine. Inguinal and perineal regions that have been exposed to urine and feces can contain E. coli. E. coli is generally resistant to antimicrobials due to its robust cell wall containing biological components like porins. This is because the bacterium's active efflux mechanism could eliminate the antimicrobials before they had a chance to do any damage. [31]

[2.3.4] Antioxidant Properties

Antioxidant defenses, such as enzymes that neutralize free radicals (antioxidants) and antioxidant dietary ingredients, are present in all aerobic organisms and are used to either eliminate or rebuild injured molecules. By delaying the process of peroxidation of lipids, which constitutes one of the main causes of degradation of items related to food and pharmaceuticals while their preparation and preservation, the chemicals have antioxidant properties like scavenging of free radicals that cause damage and extend their shelf life. And the effects of reactive oxygen species and free radicals can be prevented by antioxidants. Both the oxidation of lipids and the progression of many chronic illnesses are slowed down by them. As a result, an alternative, natural, and safe source of dietary antioxidants needs to be identified as having a plant origin, and in recent years, interest in natural antioxidants, particularly those with a plant origin, has grown significantly.

Antioxidants are frequently employed as food additives in order to protect foods from oxidative food deterioration. Currently, propyl gallate, tert-butyl hydroquinone, butylated hydroxy anisole (BHA), and butylated hydroxytoluene (BHT) are the antioxidants that are most often utilized. But inflammation of the liver and tumorigenesis have been linked to BHA and BHT respectively. As a result, demand for natural and nontoxic antioxidants is expanding and one such natural compound is curcumin. Due to its considerable medical potential, it has received a lot of attention recently. [32]

Curcumin's structure includes hydroxyl, methoxy, and carbonyl which are related to its antioxidant properties activity due to its ability to scavenge free radicals *in vivo*, especially peroxyl radicals (ROO).

However, curcumin consists of a diketone moiety that exists in a cis, Trans, or enol form in solutions. The asymmetrical di phenolic dienone series, which contains molecules that are kept or free of phenol-containing groups, is thought to be responsible for curcumin's antimicrobial characteristics.

According to studies, the formation of units containing 2 and 4 hydroxyphenyl as well as an alkoxy- in ortho form groups with enhanced antioxidant activity. Few studies have shown that the treatment of curcumin activated endogenous cellular antioxidant mechanisms, which in turn led to the activation of the cytoprotective Nrf2 gene activated the target genes, which helps in protection against free radicals or reactive oxygen species (ROS) by scavenging them and protecting cells from ROS-induced oxidative stress.[27] Therefore another aim of this study is to investigate the DPPH radical scavenging activity and ABTS scavenging activity of curcumin. Clarifying the antioxidant, radical-scavenging, and metal-chelating processes of curcumin was another significant major objective of this work.

[2.3.5] Anticancer Properties

Curcumin has an anti-cancer effect primarily by inhibiting the progression of cell cycles and inducing tumor cell death. Curcumin inhibits all 3 phases of oncogenesis, which include initiation, promotion, and progression. Curcumin also has an impact on several key targets, including growth regulators, cytokines, protein kinase C-associated receptors, and genes associated with cell growth and programmed cell death are all examples of proteins. Overexpression of proapoptotic proteins via curcumins effects is mediated by molecular pathways involving the Tumor suppressor gene p53. It might cause programmed cell death by stimulating p53 and decreasing the expression of other substrates, which play important roles in cancerous cell survival networks. Curcumin stimulates the p53 directly, which fixes the deoxyribonucleic acid (DNA)and reduces protein kinase B activity, which increases the activation of Bax genes and induces apoptosis.

Previous findings show that JAK2/STAT3 is an important signaling pathway that plays a function in several stages of carcinogenesis development. Curcumin suppressed JAK2 activation and decreased tumorspheres by blocking the JAK/STAT3 signaling pathway.

This is most likely related to nuclear factor B suppression, which regulates the expression of multiple genes associated with cell survival, death, carcinogenesis, and inflammation. Curcumin's effectiveness makes it a possible target for therapy. Curcumin also impacts a variety of cellular proteins and barriers like checkpoints, including the decreased expression of several cyclins and kinases that are cyclin-dependent (CDK), the increased expression of inhibitors related to CDK, and the suppression of synthesis of DNA. But the physicochemical reaction generated from curcumin is dependent on the kind of cell, the curcumin concentration (IC50: 2-40 g/ml), and the time of administration.

Curcumin's clinical application remains limited owing to its exceptionally low solubility in water (11 ng/ml) as well as poor bioavailability after oral treatment. Several investigations have shown that curcumin at 10-50 M (3.7-18.4 g/ml) increases cell death predominantly by apoptosis. The essential topic to be addressed, however, is how to deliver curcumin at such micromolar doses to tumour sites when curcumin has such a limited bioavailability. In order to address this issue, target specific and triggered medication delivery systems combined with nanotechnology have been recognized as key options.[26] Similarly the present study is based on the introduction of chitosan polymer as a nanocarrier system suitable for delivery of curcumin.

[2.3.5] Drug Release Study

The properties of nanocurcumin are governed by both their physical and chemical compositions. The primary physicochemical properties of nanocurcumin that enable it to be more powerful than conventional curcumin are its particle dimension, charge on the surface, its hydrophobicity and its surface area. Strong pharmacological qualities and effective target specificity, as well as other attributes, have been found to be associated with a better dissolution rate and greater bioavailability when administered orally. Its characteristics alter when curcumin's particle diameter varies on a nanoscale. It was shown that decreasing particle diameter enhanced nanocurcumin's efficacy and lifted it above natural curcumin. In comparison to conventional curcumin, nano curcumin is thought to be a better adjuvant for use as a medication because of its larger surface area. It was

shown that nano curcumin has a higher capacity for intracellular absorption than regular curcumin. This skill is necessary for identifying intracellular microorganisms in infectious diseases as well.

Nanocurcumin dissolves more quickly physiologically through tissues and plasma than free curcumin does. Ma et al. demonstrated in an in vivo investigation on rats that nano curcumin enhances biocompatibility and nanoparticle dispersion in tissues by offering a 60 times-fold increase in biologically related half-life in comparison with the administration of native curcumin.[30]

Numerous types of cancer cell lines have been used to examine the cytotoxicity using curcumin nanoformulations. Due to the long-term exposure of cells to high static levels of curcumin (whether it's in its free form or in its nanoformulation), which are not always correlated with the concentrations attained in vivo, makes it difficult to interpret relevant results. Yallapu et al. showed that the prolonged release of the active ingredient made the Nano-CUR6 formulation's intracellular retention of the drug superior to that of free curcumin (when dissolved in DMSO). Additionally, as compared to free curcumin, this formulation significantly boosted cellular absorption in MDAMB-231 metastatic breast cancer cells and A2780CP cisplatin-resistant ovarian cancer cells by 2 and 6 times, respectively.[29]

[2.3.6] Synthesis of Curcumin Nanoparticles

To generate nano curcumin, a variety of methods have been devised. Wet milling, spray pyrolysis, anti-solvent precipitation, ionic gelation, solvent evaporation, coacervation pro, and the Fessi technique are used. Curcumin nanostructures have higher stability and solubility, according to extensive literature evidence. The ionotropic gelation process is based upon the propensity of polymer composites to cross-link mostly when counter ions are present. This strategy has emerged as among the most effective. This approach has emerged as one of the most efficient ways of creating bioactive, nontoxic, biocompatible, and environment-friendly polymers like chitosan.

As a result, numerous research has been carried out to investigate the ability or usage of organic polymer nanoparticles (alginate/chitosan) for curcumin delivery via oral methods. Das *et al.* created a nanoformulation that is curcumin-based having chitosan or alginate composites and used an ionic gelation technique to deliver them to malignant cells. Antisolvent precipitation is another widespread and practical approach for producing curcumin nanoparticles, where success is

regulated via agitation temperature, rotation speed, and duration. Biopolymers also increase the stable nature and solubility of curcumin nanostructures. Because it is a simple procedure, it can be employed in the various industrial processes of medicinal nanomaterials.[12]

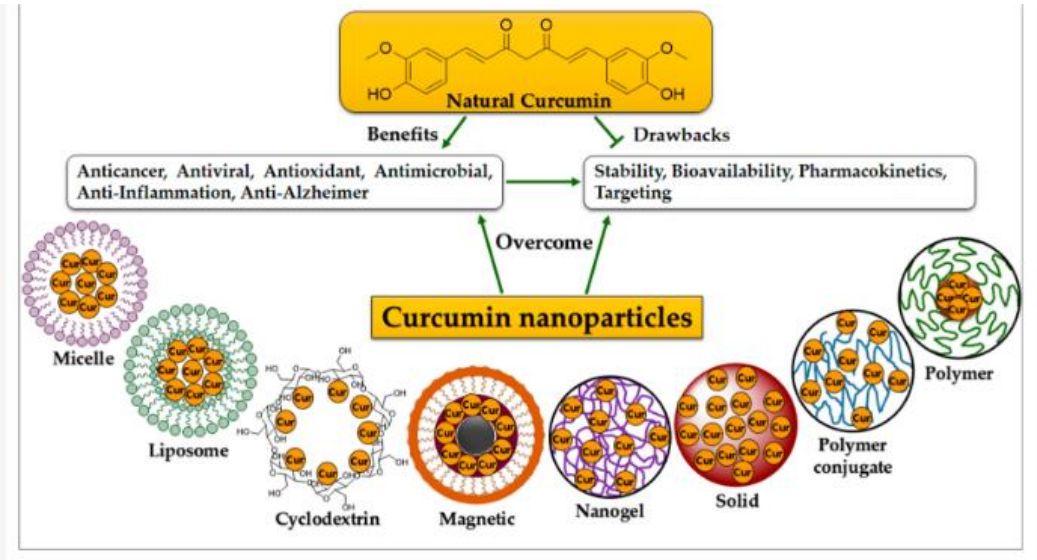


Fig: 5 Nano-based formulations of curcumin. Source: (Molecules 2022, 27, 5236)

[2.3.7] Flavonoid Nanoparticle: Quercetin

Flavonoids are by far the most ubiquitous and widely dispersed class of plant chemicals, appearing in almost every plant part. This family is further subdivided into flavones, flavonols, flavanones, flavanols, and isoflavones. Flavonoids' anticancer potential is receiving a lot of attention these days. Many studies have shown that flavonoids such as silibinin, genistein, and kaempferol are helpful against cancer. However, the use of flavonoid-based therapies is not satisfactory because of their limited solubility in solvents, low absorption, and quick metabolism. Hence, Modern nanotechnology could be useful in this regard.

Flavonoids' bioavailability can be improved by using nanocarriers. Flavonoid nanoparticles have been proven *in vitro* and *in vivo* and have anti-tumor efficacy against the breast cancer cell line MCF-7. One of the most prominent flavonoids found in plants is quercetin (QT). In vitro, it induces apoptosis in proliferative lymphoid tissues and prevents the proliferation and genesis among many tumor cell lines.

3, 3, 4, 5, 7-pentahydroxyflavone, the chemical structure of Quercetin is a bioflavonoid molecule that can remove reactive oxygen species. Quercetin can modulate lipid peroxidation because it chelates ion-related activities.

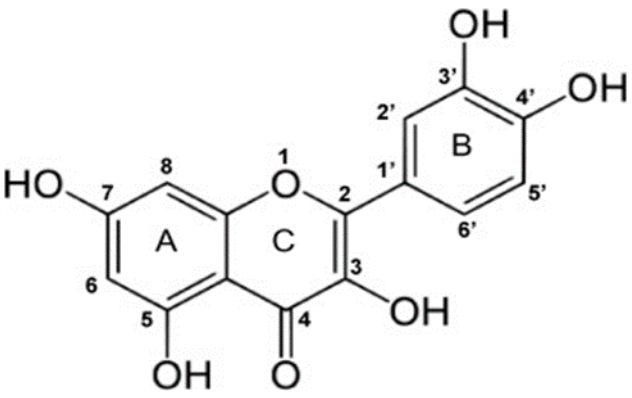


Figure: 6 Quercetin chemical structure. Source: Quercetin: (Fitoterapia Volume 106, October 2015)

[2.3.8] Antimicrobial Properties

Quercetin has antibacterial properties against a broad variety of bacterial species/strains, especially those that impact the digestive tract, the respiratory system, the urinary, and other systems. The antibacterial potential of quercetin has been associated with its solubility and interaction with the membrane of bacterial cells, which is primarily controlled when hydroxyl groups are present in quercetin. Infectious illnesses are mostly caused by bacteria and the metabolic byproducts of those bacteria. The process by which quercetin suppresses bacteria may be broken down into four parts: destroying and resisting the cell membrane, preventing bacterial adherence, preventing nucleic acid production, and suppressing biofilms associated with infections.

Peptidoglycan-based bacterial cytoderm is an essential component of the defense mechanism for germs. According to the leaky alkaline phosphatase enzyme, Wang et al. showed quercetin's capacity to damage Escherichia coli cytoderm. Additionally, quercetin therapy may directly result in the inhibition of the enzyme d-alanine-d-alanine ligase in bacteria responsible for producing the amino acid precursor to the peptidoglycan in bacteria, and hence the inhibition of cell wall production.[39]

Gram-negative bacteria are often resistant to quercetin's bacterial-related actions in comparison with Gram-positive bacteria. The variation within quercetin sensitivity among Gram-positive as well as Gram-negative bacteria is due to differences in the composition of cell wall between the

types of bacteria. Although, certain quercetin variants were more effective against bacteria that were Gram-negative.

The solubility of quercetin can be increased or decreased at different hydroxyl groups due to phosphorylation and sulfation at those sites' groups leading to the altered antibacterial potential for certain types of bacteria.[24] According to reports, quercetin works against bacteria by modifying their permeability, rupturing their cell wall, suppressing for the production of nucleic acids, which has an impact on proprotein expression and synthesis, and decreasing activity of enzymes.

Some findings showed that flavonoid like quercetin has broad-spectrum antimicrobial properties and inhibits growth of fungus as well as bacteria to a substantial degree. The development of dangerous bacteria such *Salmonella enterica, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Aspergillus* is also effectively inhibited by quercetin. Additionally, quercetin inhibits the development of E. coli through changing the way adenosine triphosphate functions.[36]

[2.3.9] Antioxidant Properties

Through its impact on glutathione (GSH), the activity of enzymes, pathways for signaling, and the formation of ROS i.e., the reactive oxygen species brought on due to ecological and toxic conditions, quercetin's antioxidant action is primarily demonstrated. By preserving the equilibrium of oxidative processes, quercetin has high antioxidant activity. By controlling GSH levels, quercetin boosts the body's potential for antioxidant defense. This is due to the fact that superoxide dismutase, also known as (SOD), quickly absorbs oxygen and converts it into hydrogen peroxide once free radicals from oxygen are produced inside the body. Furthermore, this enzyme catalysis leads to the conversion of hydrogen peroxide to nontoxic water. GSH is required as a donor of hydrogen in this process. Quercetin promotes GSH production, according to research done on animals and cells. [34] Free radicals are molecules that react with other substances very fast in order to grab their electron and become stable. When a molecule is attacked, it loses an electron and becomes a free radical, setting off a series of events that damage living cells.

It has been shown from the literature that the hydroxyl groups at positions 3, 5, 7, 3′, and 4′ of the rings A and B having double bond, that exists between the 2^{nd} and 3^{rd} carbons, and the group of carbonyls on the fourth carbon play a major part in the antioxidant capabilities of quercetin.[35]

As most oxidative injury in vivo is caused by ROS, which quercetin is able to scavenge, quercetin can protect against oxidative damage, including radiation-induced UVB wounds, pulmonary damage and its related oxidant damage illnesses. Skin is extremely resilient & capable of withstanding many environmental stresses, but UVB exposure causes an imbalance in the body's natural systemic antioxidants and a brief rise in Reactive oxygen species, that worsens inflammation and the production of free radicals while also having an impact on cellular functions.

According to studies, quercetin not only protects mitochondria from ROS-induced damage but also scavenges ROS, preventing the depolarization of the mitochondrial membrane as well as the movement of the cell membrane. It follows that by repressing this imbalance, quercetin appears to be able to prevent UVB-induced skin damage. [36]

[2.3.10] Anticancer Properties

Quercetin is present abundantly in fruits, and vegetables and can be comfortably extracted. Therapy related to any soft tissue damage with various quercetin preparations is an important application of quercetin. Furthermore, earlier research has revealed that quercetin has a promising role in cancer treatment. Natural chemicals, such as quercetin, are now recognized as important agents for cancer prevention and treatment because of their reliable performance, high therapeutic potential, and minimal toxicity of the chemicals. Quercetin appears to be a major anti-proliferative and anticancer drug, as well as a stimulator of apoptosis. Numerous research studied the effect of isolated Quercetin molecules on various cancer cell lines. It has been chemically synthesized and is commercially available because of its antiproliferative properties and importance in anti-hypertensive and neurotropic activity.

Its antioxidant and oxidative effects have been the subject of conflicting reports which call for additional investigation. Further research is also necessary to determine the mechanism through which quercetin induces cell cycle arrest, as inconsistent results have been reported.

For instance, it looks like quercetin administration may cause G0/G1 cell cycle arrest in leukemia, S stage in colorectal carcinoma, or the G2/M stage in breast carcinoma, leukemia, and adenocarcinoma of the gastrointestinal tract cell line.[28]

Clearly, quercetin inhibits the growth of malignancies that include breast cancer, gastric cancer, colorectal cancer, thyroid cancer, pancreatic cancer, and prostate cancer.[25] Because the contact area between both the particles and the surrounding region rises as particle size decreases, nanoparticles perform better in terms of absorbance and efficacy than larger particles. This study focuses on the synthesis of curcumin-quercetin-based nanoformulations for antimicrobial activity and anticancer activity.13][14]

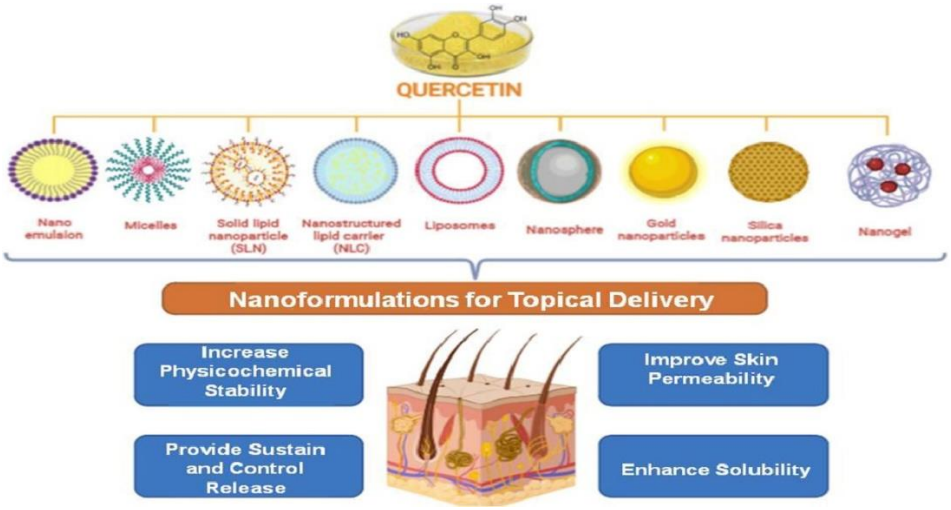


Fig:7 Quercetin Nanoformulations. Source: (Phytomedicine Plus, Volume 2, Issue 2, May 2022, 100257)

[2.3.11] Drug Release Study

Dialysis membranes are employed for separating NPs from the free drug that is released in several investigations assessing the drug release of kinetics in nanocarriers. Despite this issue has been highlighted in a number of recent works on drug release for nanoparticles and other researchers have chosen to overlook all the consequences of drug release kinetic of the nanoparticles using a dialysis bag. As a result, the amount of drugs that are released from the nanoparticles within the membrane is different from the amount of drugs outside the membrane. Washington mathematical formulations, which assume that the steady-state premise is true as well as that the amount at a certain rate of the release of drugs from formulated nanoparticles also the dialysis rate from the

membrane are comparable, were utilized to address additional diffusion barriers impacting the drug release kinetics from nanoparticles.

The coefficient of partition, or the amount of formulated drug distributed among the nanoparticles and their continuation phase, as well as the volumes within and outer surface the of membrane, the rate of transfer of membrane, also the rate of drug release are all factors in this model. Two scenarios are taken into consideration:(i) drug release is stimulated by diffusion from nanoparticles, and (ii) transport through the cell membrane maintains first-order kinetics.[40]

[2.4] PROPOSED SOLUTIONS

Curcumin-Quercetin nanoparticle administration has been found that alter its pharmacology profile, hence increasing its chemically induced therapeutics agent's potential. PLGA, chitosan, and alginate biopolymers, conjugated micelles, are some of the nano-drug delivery vehicles used for curcumin distribution. Aside from the composition of the medicine, the selection of material is heavily influenced by its physicochemical qualities and mode of administration.

Current findings from some papers have demonstrated that encapsulating curcumin-quercetin in Peg-PLGA NPs effectively enhances its liquid suspension and its bioavailable property, resulting in a significant increase in its medicinal and chemo-sensitizing efficiency. However, with PLGA being highly expensive it is not economically beneficial to use it as a drug delivery system for a long duration in chemoprevention. So, to tackle the above problem in this current work chitosan is being used as it is cost-effective and acts as a biocompatible nanocarrier, and can be given orally in a wide variety of populations Furthermore, chitosan is a mucoadhesive that behaves as a greater nano-based carrier of quercetin and curcumin for cancer prevention than another form of nano-based carriers.

Chitosan is a type of linear polysaccharide that is formed via the deacetylation of chitin and its structural components from crabs and shrimp shells or other crustaceans and is composed of β-(1,4)-linked N-acetyl glucosamine units. Because of their biodegradability and biocompatibility, as well as their ability to enclose hydrophobic medicines, chitosan, as well as its chemically induced versions, like chitosan carboxymethyl, is a very much studied nanocarrier.[15]

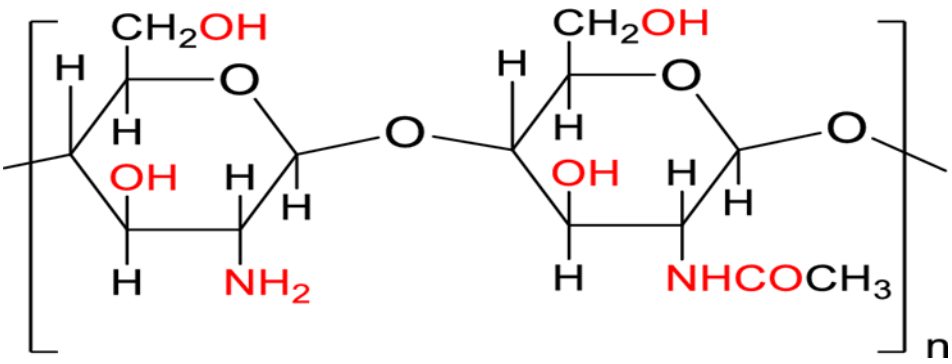


Figure: 7 Chitosan Structure. Source: (Carbohydrate Polymers, Volume 282, 15 April 2022, 119132)

Chitosan, which is available commercially, has a percentage of deacetylation ranging from 60-100% and MW (molecular weight) varying from 3500- 20,000 Da. In drug delivery systems, chitosan formulations with a higher degree of deacetylation are desirable since a higher level of deacetylation is associated with a faster disintegration rate. The FDA has approved chitosan as nontoxic for utilization in medical applications. The process of ionic gelation has distinct advantages over other forms of nanoparticle creation, such as emulsified extraction, spray drying, or micelle formation. TPP, the cross-linking agent, is non-toxic and has a high gelling capacity. The manufacturing process is straightforward; no harmful chemicals are required, and the zeta potential and particle size may be easily controlled by varying all the concentrations of chitosan and TPP.[16]

Numerous characteristics make CS an appropriate option to be used in the current investigation. For starters, it is regarded as biocompatible with living tissues, which means will not induce hypersensitive reactions within the body. Chitosan is biodegradable, producing non-hazardous and effortlessly removed breakdown products when fermented by bacterial colonies. Chitosan's distinctiveness stems from its multiple beneficial qualities, which allow it to be used in a wide range of industries.

This moderate-cost material is biodegradable, biocompatible, and has low toxicity, making it an FDA-approved substance that may be utilized effectively in the pharmaceuticals, bio-medical, food, or cosmetic sectors. The physiological and biological characteristics, such as antifungal, anti-inflammatory, antibacterial, antioxidants, antitumoral, or mucoadhesive actions, are also important.

They can be utilized effectively for medicinal purposes as well as in other industries such as agriculture, food production, and other domains (for example, the textile industry). Furthermore, chitosan's strong adsorption ability is employed in ecological uses to remove hazardous substances from water-based solutions.[23]

Toxic organic solvents don't require addressing during formulation given that it is quite soluble in an acidic aqueous medium. It is easily cross-linked with diverse anions because it is a polyamine linear form having lots of free amine groups. It can also be designed as a sustained release matrix.[17] Chitosan works as a concentration enhancer by releasing the epithelial tight junctions. As seen in Figure 8, chitosan enhances either paracellular or drug delivery via transcellular mode. Chitosan is involved in the formation of a compound with negatively charged mucus via noncovalent interactions like ionic bonding, hydrophobic interactions as well as hydrogen bonding. Also, the main amine in chitosan has a pKa of 6-6.5, because of the level of N deacetylation. Amine molecule helps in chitosan's solubility strength in acidic ph environments.[18]

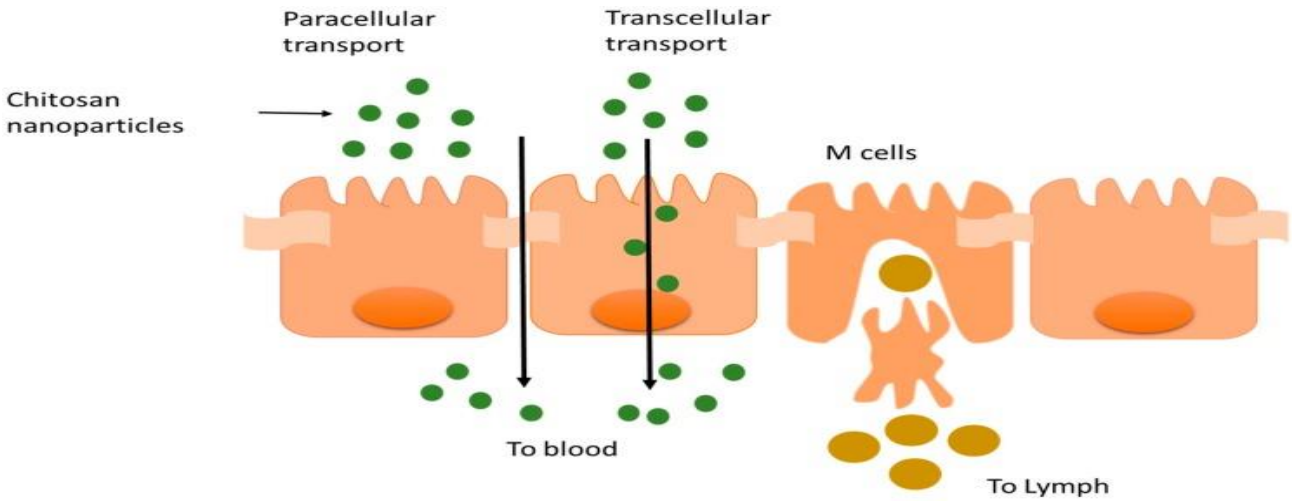


Figure: 8 Schematic illustrations of chitosan NPs transport. Source: (Pharmaceutics. 2017 Dec; 9(4): 53.)

When considering chitosan for use in pharmaceutical applications, it's also crucial to take its stability into account. Due to its high hygroscopy, chitosan is extremely sensitive to environmental factors, particularly humidity. Chitosan retains water by hydrogen bonding, which has been shown to alter its mechanical characteristics and partially reduce its mucoadhesive qualities. At both room temperature and 60oC, thermal deterioration of solutions containing chitosan was also seen.

These factors make low-temperature storage (2-8°C) in a dry environment preferable. Due to its mucoadhesive qualities and permeability-improving effects, nanoparticles of chitosan are also especially well-suited for local distribution at the skin's surface mainly dermis and mucosal region. Chitosan nanoparticles are a highly promising and adaptable approach to overcoming bioavailability and stability problems of the majority of active substances. They combine the inherent features of polymers with tunable dimensions and the potential for surface alteration and other modifications according to custom demands. The production of innovative therapeutic drug release mechanisms with higher bio-availability, greater sensitivity and specific target mechanism, and decreased toxicity from pharmaceutical perspective has made chitosan nanoparticles a hot topic in the field of nanomedicine, biomedical engineering, and these days.

The size and shape of CSNP may have an impact on certain activities. The unique chitosan nanoparticles, which are made up of aggregates of NP's diameters ranging from 10 to 100 nm, have prospective applications in the sectors of pharmaceuticals, medical engineering, industrial, and nanomedicine. The amazing physicochemical and biological properties of the chitosan-based nano systems, as well as their ability to change protein loading and adjust parameter values while manufacture process, make them suitable for application as high-tech drug delivery systems. Additionally, they may be manufactured as a frozen or lyophilized powder, have excellent protein packing efficiency, and are simple to store and transport. [37]

Chitosan and its analogs superficially perforate and exert non-proliferative effects in different ways like antioxidant-based defense, apoptosis, and enzyme modulation via modulating the signaling molecule pathways like nuclear factor k light chain enhancer of activated B cells [NF-kb] and chitosan controlling cell cycle markers, TGF and induce apoptosis.

The primary goal of gene therapy is to input any type of genetic substance into the targeted cell paving the path for the control of genomic expression. The oppositely charged gene, on the other hand, is incapable of reaching the plasma and cell membrane via a passive diffusion mode. Furthermore, introducing DNA straight inside the cells results in fast nuclease breakdown.

The success of gene therapy is dependent on several factors, including the ability to target specific cells, protect nucleotides from degradation in the external medium of cells, and transport sufficient quantities of nucleic acid (NA)to produce a curative impact.[19]

Chitosan is a suitable polysaccharide with a low level of toxicity. It has been suggested that taking oligo chitosan/chitosan's may shield the body from oxidative stress brought on by cancer. While their increased penetration qualities are primarily responsible for their anti-metastatic action. A higher chitosan content inhibited the movement of MDA-MB-231 female breast cancer line (Nam and Shon, 2009). Another method by which chitosan exerts its anticancer properties is through improving the biodistribution of medicines. Due to chitosan's improvement of cell permeation and drug retention duration to low toxicity, the medication accumulates in tumor cells.[37]

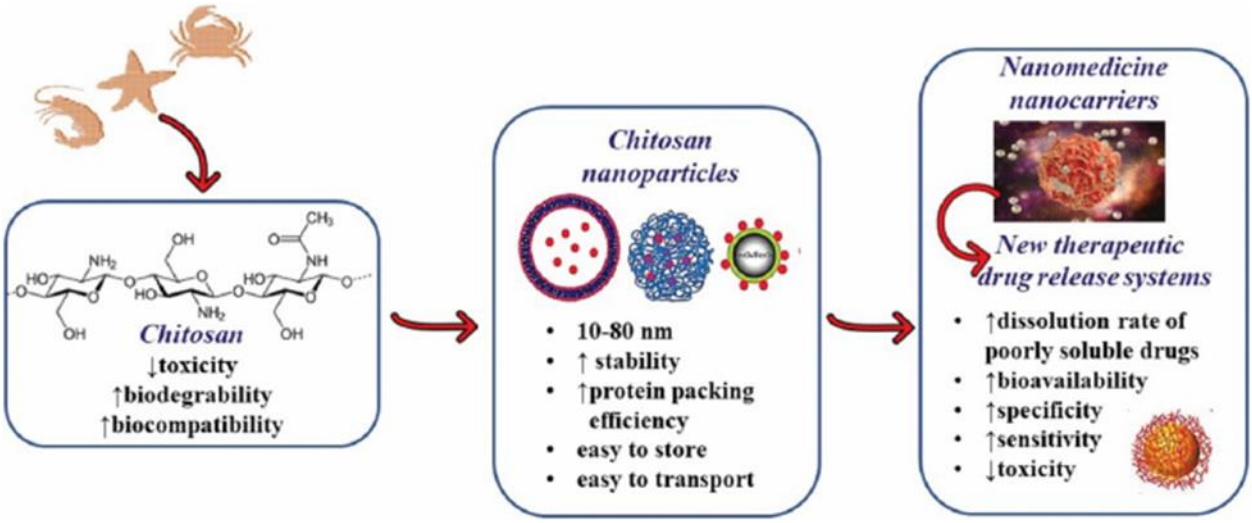


Figure:9 Advantages of chitosan encapsulation. Source: (Cancer Cell International volume 21, Article number: 318 (2021)

[2.4.1] Preparation of Chitosan Nanoparticles

Ionic gelation, which was initially described by Calvo et al., (1997), is one of the most popular production techniques for chitosan nanoparticles, which have been studied for more than 20 years. Its foundation is ionic crosslinking, which takes place in the presence of negatively charged polyanion groups like sodium tripolyphosphate (TPP) and positively charged amino chains of chitosan. Aqueous TPP solution is added while being vigorously stirred after chitosan has been dissolved in an acidic aqueous solution (often an acetic acid solution). Chitosan molecules that are positively charged diffuse into one another, causing crosslinking that results in the production of nanoparticles. demonstrates the electrostatic bond between TPP and chitosan, which results in the formation of spherical nanoparticles.

Chitosan NPs are produced by drying in the oven or freeze-drying after a few centrifugation and water-washing procedures. It uses a simple method without solvents or hazardous crosslinkers. Additionally, the procedure may be performed at constant regulated room temperature, the ultimate size of the nanoparticle can be modified by varying the Chitosan/TPP ratio, an important characteristic that directly influences the effectiveness of drug packaging and delivery.[38]

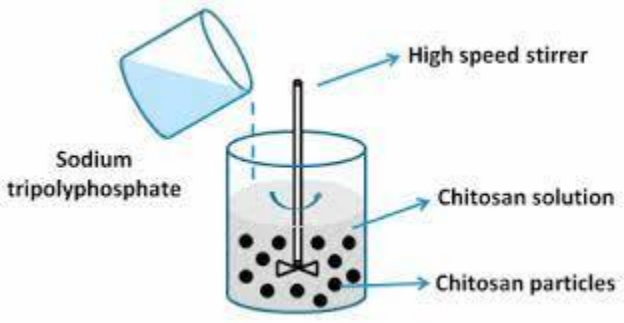


Figure 9: Schematic illustration of chitosan nanoparticle forming via ionic gelation. Source: (Nanomaterials, 6(2), p.26.)

CHAPTER 3

MATERIALS AND METHODS

[3.1] Materials: Chitosan (medium molecular weight), Curcumin, Quercetin powder, TPP (Sodium Tripolyphosphate) from Sigma Aldrich, Acetic Acid (Loba Chemie), NaOH, HCl, DMSO (Fisher Scientific), Ethanol, McCoy cell line (NCCS Pune), DMEM media, FBS for cell culture studies, PBS, MTT, DPPH, ABTS from (Himedia Lab.), Muller Hinton Agar, Muller Hinton Broth, Ampicillin (Himedia Lab.), *E.coli*.(ATCC 25922), *S. aureus*(ATCC 23235), Deionized water (Millipore Q.) All solvents used in this study are of analytical grade.

[3.2] Methodology

[3.2.1] Preparation of Chitosan Based Nanoformulations

A generalized method used for synthesizing nanoparticles using polymer is the Ion Gelation method, which was first described *by Calvo et al.*, (1997).

Chitosan medium molecular weight (100 mg) was dissolved in 100 mL of 3% v/v solution of acetic acid with continuous vigorous stirring in a magnetic stirrer at 600 rpm and 30°c for 1 hour to form a clear CS acidic solution. Drop by drop, while continual magnetic stirring, 14 ml of TPP solutions [0.1%, w/v] was added into the 20 ml CS acidic solution. The solutions were then agitated for another 30min. The pH of the solutions varied between 3-5.

CS-loaded Quercetin nanoparticles (CSQNP) were obtained when 10ml of Que dissolved in (30mg/ml) of 5% DMSO(v/v) solvent into 20ml CS solution and 14ml of (0.1%) TPP solution. The same process was followed for CS-loaded curcumin nanoparticles (CSCURNP) and CS-loaded curcumin-quercetin- nanoparticles. (CSCURQNP).

Above stated nanoformulations were also prepared using 10% ethanol(v/v) as a solvent which showed poor aqueous stability and solubility of curcumin and quercetin particles. In this study, the concentration of CS and solvent were optimized by preparing different ratios of mixing concentrations.

Cs Solution (Acetic Acid%)	TPP Solution %	Result
500mg /50ml 1% AA	-	Gel formation
250mg/100ml 1% AA	-	Not dissolved
175mg/100ml 1% AA	-	Not dissolved
100mg/ml 1% AA	1% 15ml	Ppt settled down(clear)
50/25mg/ml 1% AA	-	Solution clear, ppt settled down
25mg/ml 2% AA	1% 15ml	Ppt formed
50mg/ml (10ml) 2% AA	0.25% 15ml	Froth formed
12.5mg/ml 2% AA	1% 5ml-sonication	Solution clear –negative absorbance
12.5mg/50ml 2% AA	0.1% 5ml-overnight shaking	CSNP formed –negative absorbance
20mg/100ml 1% AA	0.1% 20 ml	CSNP- absorbance less
100mg/ml 2%AA (3:1) 20ml	0.1% 7ml	CSNP –negative absorbance
100mg/ml 3%AA (3:2) 20ml	**1% 14ml**	**CSNP formed-positive absorbance**

Table:2 Optimization of experimental factors

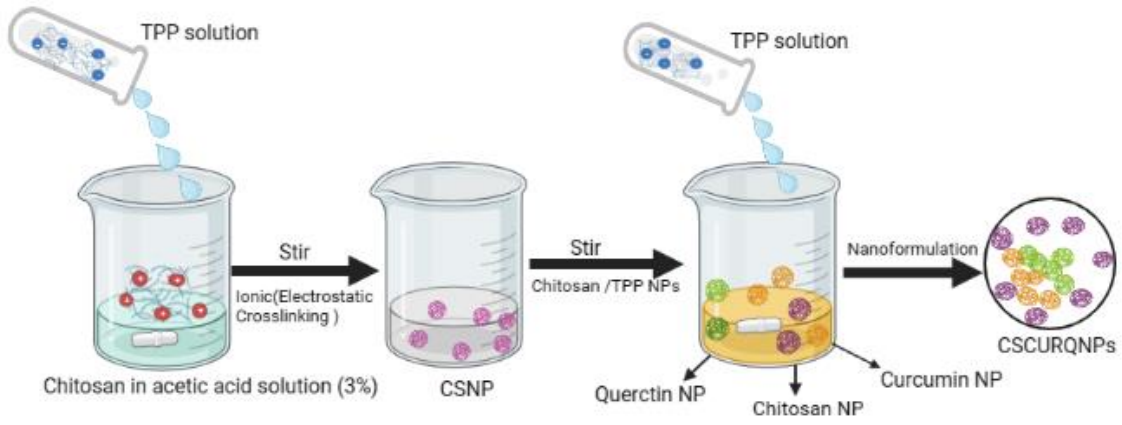


Figure: 10 Preparation of Chitosan-based Nanoformulation

[3.3] Physical and Chemical Characterization:

[3.3.1] UV-VISIBLE SPECTROSCOPY

Using a Thermo Fisher Scientific Spectrophotometer, the UV-visible absorption spectrum of the samples was produced in order to investigate the optical absorption characteristics of the photocatalyst. Understanding the spectral characteristics of chitosan nanoparticles was made possible by spectroscopy, that were recorded at constant room temperature within the wavelength of 200-700 nm (Janes et al. 2001).[41]

[3.3.2] FOURIER-TRANSFORMED INFRARED SPECTROSCOPY(FTIR)

Then, using a Nicolet IR100 FTIR Spectrometer (Thermo, USA), the Fourier transforms infrared (FTIR) spectra of curcumin, quercetin, chitosan, and curcumin-quercetin loaded chitosan-TPP nanoparticles were evaluated.[42]

[3.3.3] PARTICLE SIZE, POLYDISPERSITY INDEX AND ZETA POTENTIAL

Dynamic light scattering was used to measure the NP's hydrodynamic dimension, polydispersity index/average size at a particular length, and detection angles. To minimize multiple dispersions, all the samples were liquified/diluted from deionized water. NP comprising curcumin, quercetin, and both that have the proper size, and better zeta potential properties were named CSQNP, CSCURNP, and CSCURQNP and subjected to further testing.

[3.3.4] SCANNING ELECTRON MICROSCOPY(SEM)

SEM (Hitachi) was used to investigate the morphology of the particles. Centrifugation was used to separate the CNP for 30 minutes at 40,000 g. The pellet was freeze-dried using a lyophilizer then the supernatant was decanted before being analyzed by SEM at a 15.0 kV accelerating voltage. On a graphite surface, a single drop of nanoparticle was applied, and after the sample had dried, it was covered with gold utilizing ion sputter.[43]

[3.3.5] ENCAPSULATION EFFICIENCY

The ultrafiltration-centrifugation technique was used to test the entrapment efficiency of curcumin and quercetin in the nanoformulations.

All analyses were carried out in triplicate, as well as the encapsulation efficiency of curcumin-quercetin nanoformulation enclosed in CSCURQNP can be estimated via a formula:

> **Encapsulation efficiency (%)** = [**A-B/A *100]**, where A is the total quercetin weight in the pellet, and B is the free curcumin weight in the supernatant.

[3.3.6] EFFECT OF PH ON NANOPARTICLE

One of the most important factors that influence the size of nanoparticles is ph. Above nanoformulations were titrated and set using 0.1N NaOH and 0.1 N HCL in order to find the best isoelectric point of the samples ranging from ph 2-12.[43]

[3.3.7] EFFECT OF TEMPERATURE ON NANOPARTICLE

Different temperatures were set ranging from 20°c to 60°c using a hot plate for different nanoformulations in order to find out the stability of NPs.

[3.3.8] DRUG RELEASE STUDY

A measured quantity of CSQNP, CSCURNP, and CSCURQNP filled in dialysis membrane was kept in deionized water of a particular concentration pH 5, pH 7.4, and temperature 40°c and 50°c incubated at room temperature. In a time-lapse of 1,2,3,5,10,24 hrs., the solvent absorbance was measured using UV-VIS Spectro at around 420nm which determined the quantity of drug produced by nanoparticles. To maintain total volume, a constant concentration of D.W. was withdrawn at specific given time intervals and substituted with an equal number of fresh buffers. The quantity of curcumin produced by the nanoparticles will be determined using an ultraviolet spectrophotometer at around 420nm.[21]

Bioactive properties determined by:

[3.4] ANTIOXIDANT ASSAY

[3.4.1] The free radical scavenging test to check the capacity of chitosan-based nanoformulations was evaluated using the standard 2,2-diphenyl-1-Picrylhydrazyl DPPH assay. It is a free radical method based on electron transfer). The antioxidant's IC50 was determined. To determine the radical scavenging activity (RSA) formula RSA (%) = [(Acontrol Asample)/Acontrol] /100 is used

[3.4.2] The antioxidants are oxidized by the free radical ABTS. It is a cyan-colored (bluish-green) reagent that becomes colorless when an antioxidant is applied. Antioxidant activity is assessed as a function of color change intensity. The 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical scavenging test was used to test the antioxidant activity of curcumin and quercetin that had been encapsulated in chitosan. UV-Vis spectrometer was used to measure the absorbance at 734 nm (Shimadzu, Japan). The antioxidant's IC50 was determined. [43]. To determine the radical scavenging activity (RSA) formula RSA (%) = [(Acontrol Asample)/Acontrol] /100 is used

[3.5] ANTIBACTERIAL ACTIVITY

The antibacterial action of NP and NPC will be tested using different gram-positive or negative bacteria or fungi and the microdilution technique in a 96-well microtiter plate at concentrations ranging from zero to 400 g/mL via serial dilutions, by Clinical and Laboratory Standards Institute (CLSI, 2018). The Minimum concentration at which color change happens is the minimum inhibitory concentration (MIC).

Aliquots of materials from 96-well plates were utilized to determine the MIC of specimens that were transmitted to Petri plates for further microbial growth culture parameters and the Antibiotic Sensitivity Test (AST) was performed using Kirby Bauer Method at different concentrations and the zone of inhibition was calculated using diameter.

[3.6] CYTOTOXICITY ASSAY

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is a type of colorimetric method that will be carried out to determine cell viability after treatment with chitosan nano curcumin and quercetin. This colorimetric assay relies on cells with metabolic activity in which the reduction of yellow tetrazolium salt into purple formazan crystals takes place and measures mitochondrial activity.[20][44]

The cell viability can be calculated:

Cell Viability = [Treated cells absorbance/ Untreated cells absorbance*100]

CHAPTER 4

RESULTS AND DISCUSSION

[4.1] OPTIMIZATION OF THE CHITOSAN-BASED NANOFORMULATIONS

The shift in the color change of the chitosan solution from transparent to faint blue color determined the formation of chitosan nanoparticles (CSNP).

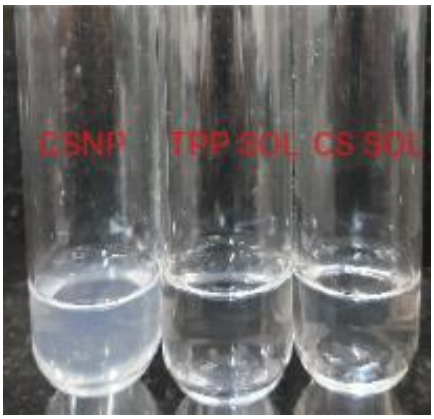


Figure 12: Formation Of CSNP

Similarly, color change in the solutions confirmed one of the parameters for the formation of CSQNP, CSCURNP, and CSCURQNP nanoformulations.

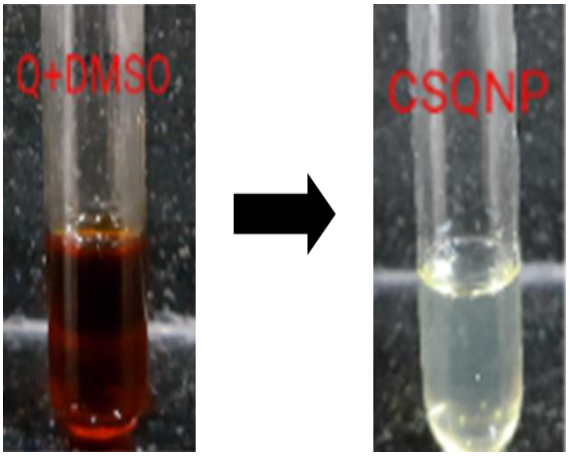


Figure 13: Formation of CSQNP

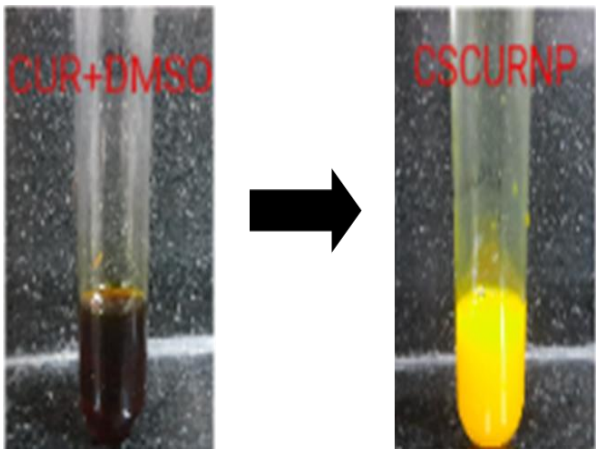
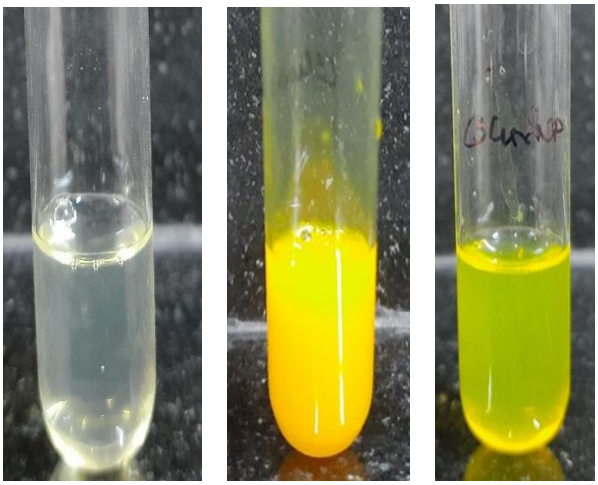


Figure 14: Formation of CSCURNP



A. CSQNP B. CSCURNP C. CSCURQNP

Figure 15: Formation of CSQNP, CSCURNP, CSCURQNP Nanoformulations

[4.2] CHARACTERIZATION OF CS-BASED NANOPARTICLES

[4.2.1] UV-VISIBLE SPECTROSCOPY

The UV spectra of CS nanoparticles, and quercetin-curcumin-assisted nanoparticles are shown in the following figures. The scanning of the solution takes place with CSNP using UV-VIS Spectroscopy provided an Absorption peak at around 290nm which preliminary confirmed the formation of CSNP. [45]

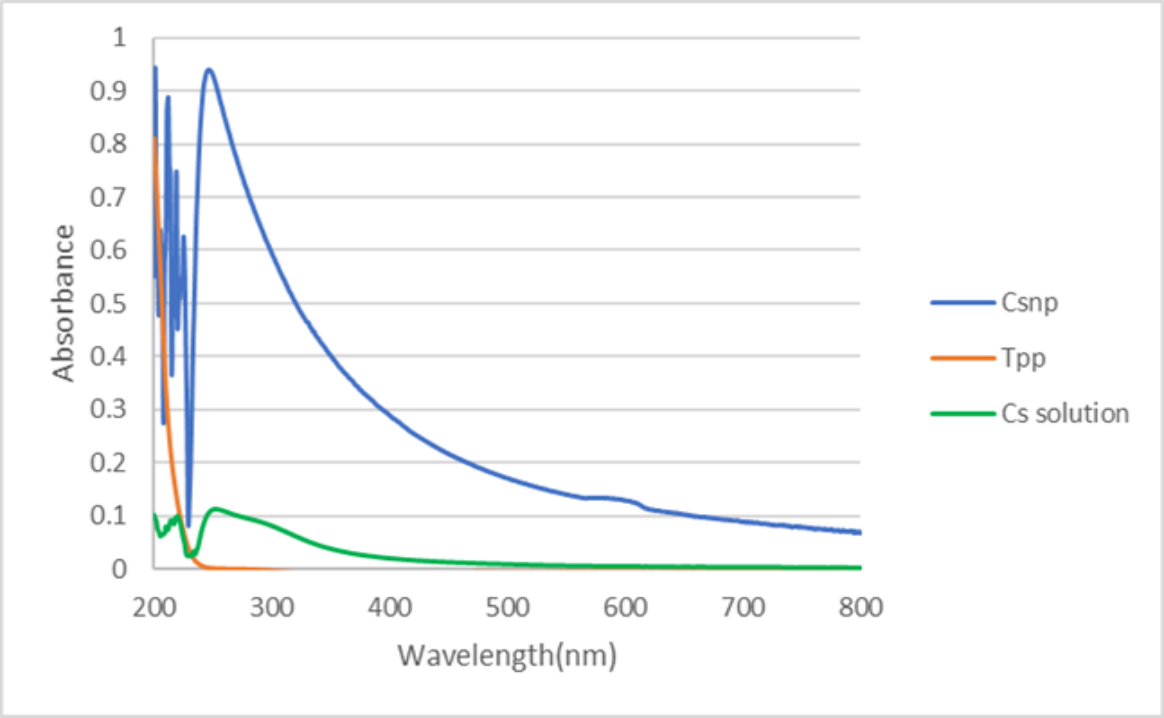


Figure: 16 UV-VIS spectra of CSNP

Similarly, the absorption peak of CS-QNP, CS-CURNP, and CS-CURQNP between 400-500 nm confirmed the formation of chitosan-based curcumin-quercetin nanoparticles. The peak intensity of curcumin and quercetin-encapsulated chitosan -nanoparticles was higher than that of native curcumin and quercetin.

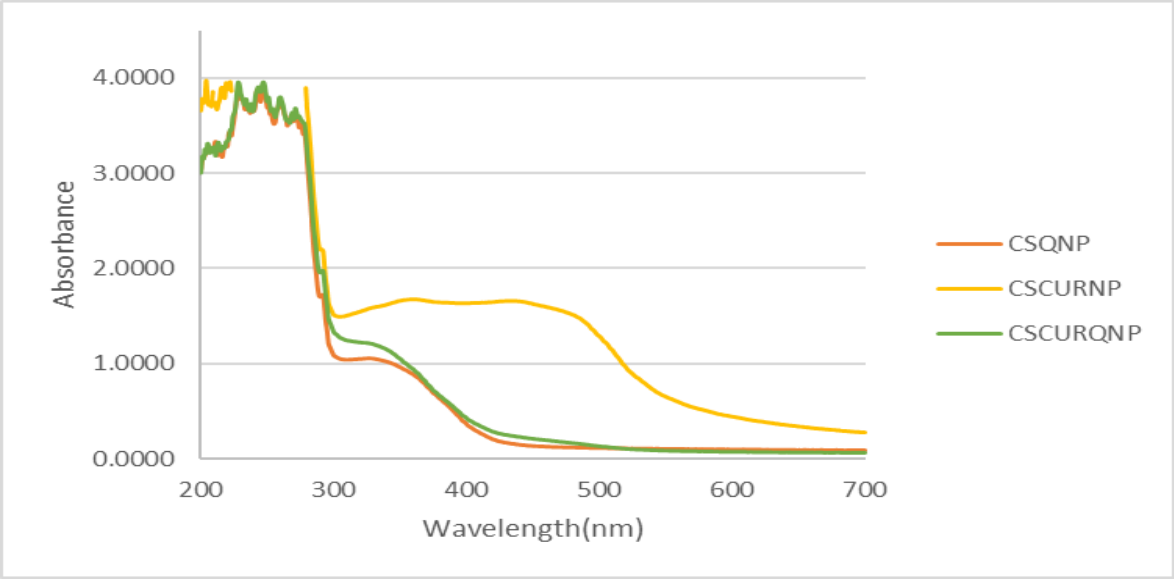


Figure:17 UV-VIS spectra of CSQNP, CSCURNP, CSCURQNP

[4.2.6] pH STABILITY STUDY USING UV-VIS SPECTROSCOPY

The following UV image depicts the most stable pH for CSQNP is around 5-6 and the absorbance increased with basic pH i.e. (8-12).

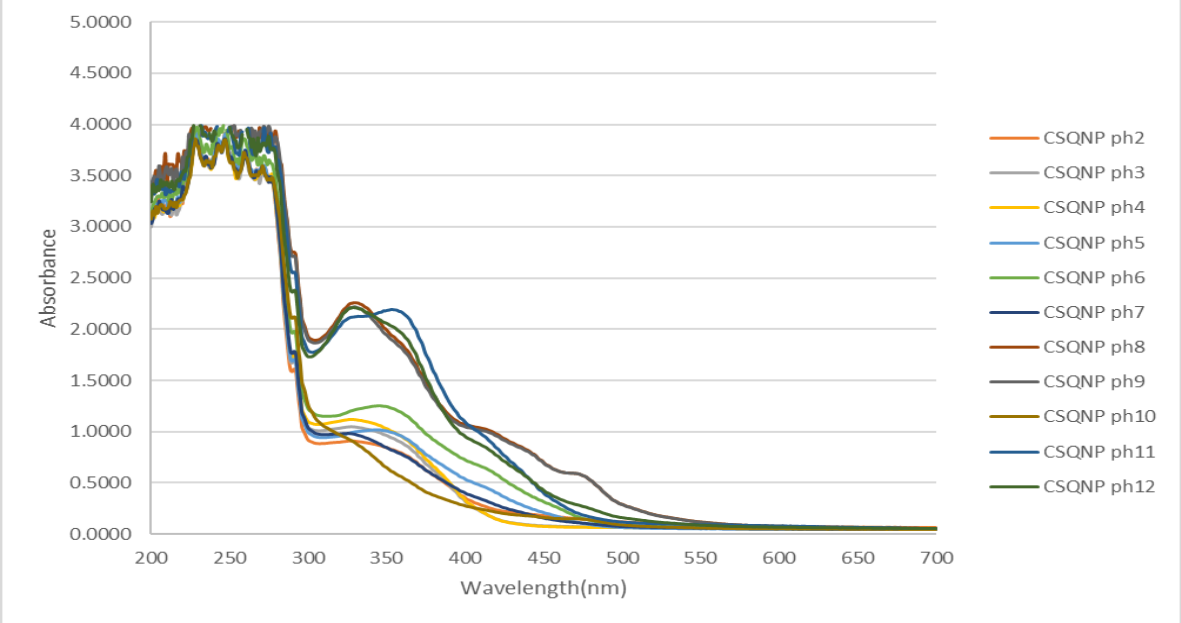


Figure: 18 UV-VIS spectra of CSQNP at different pH

Similarly, for CSCURNP, stability and increased absorbance were observed between 5-6 pH.

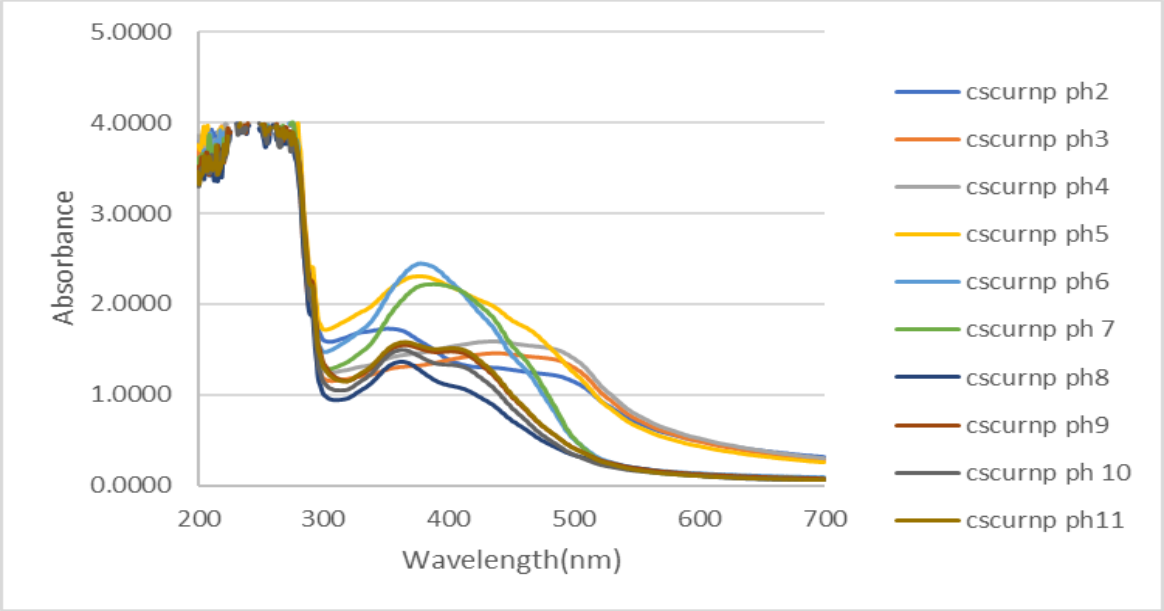


Figure 19: UV-VIS spectra of CSCURNP at different pH

And for CSCURQNP the most stability was found in the range of 5-7 pH and the absorbance increased around basic pH.

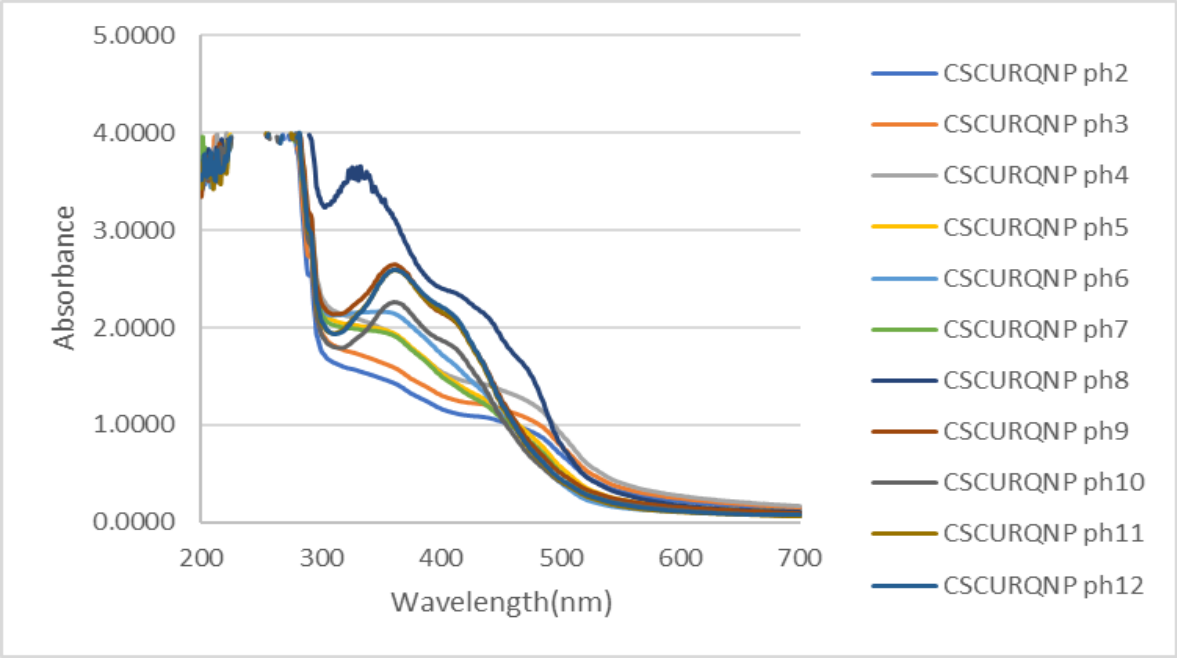


Figure 20: UV- VIS spectra of CSCURQNP at different pH

[4.2.7] THERMAL STABILITY STUDY USING UV-VIS SPECTROSCOPY

The thermal study of the chitosan-based nanoformulations was observed at different temperatures ranging from 20°c to 60°c using a hot plate. The UV-Vis data showed that with increasing temperature the absorbance of the formulation also increased.

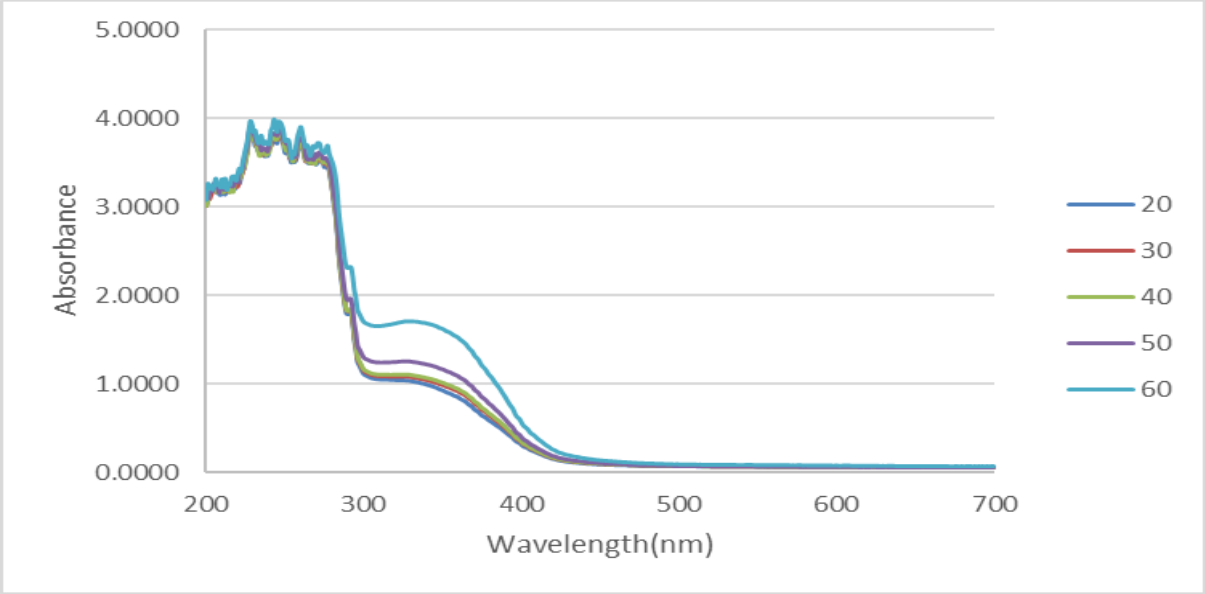


Figure:21 UV-VIS spectra of CSQNP at different temperaturesThe UV image of CSCURNP depicted that the most stable temperature for formation of curcumin nanoparticles is 20°c to 30°c

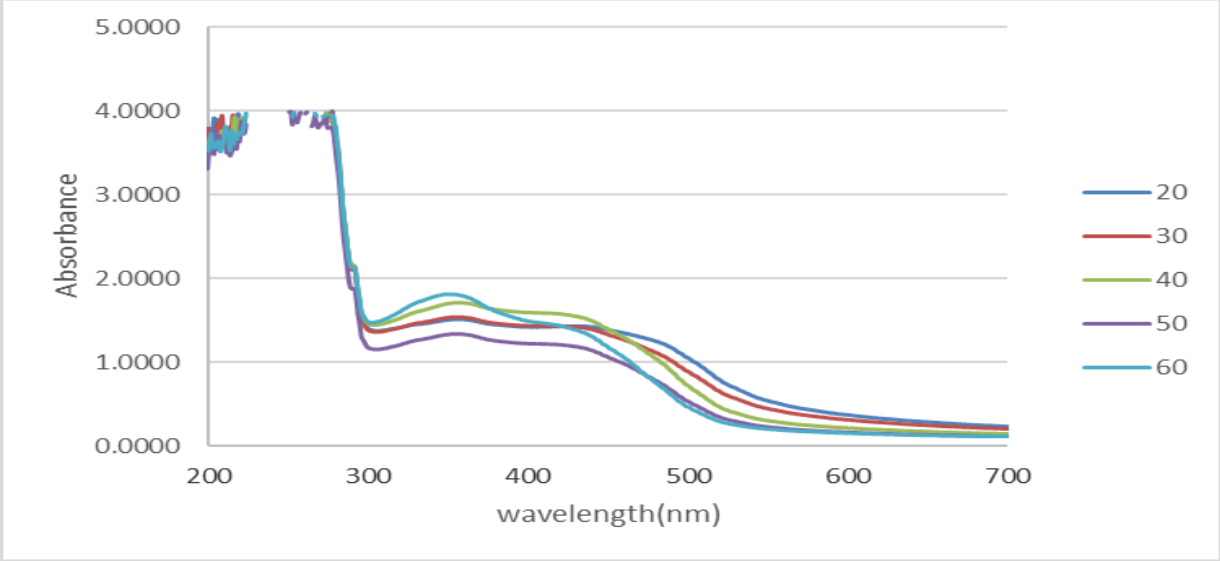


Figure 22: UV-VIS spectra of CSCURNP at different temperatures

The UV image of CSCURQNP determined that with increasing temperature absorbance also increases and the stable particle formation is between 40°c to 50°c.

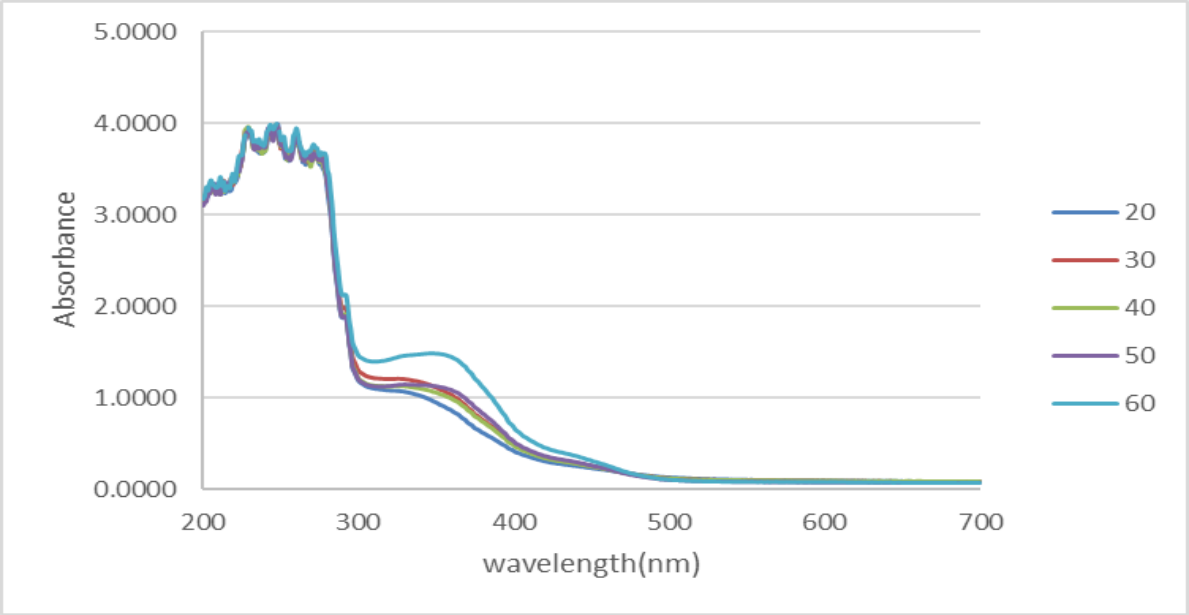


Figure 23: UV-VIS spectra of CSCURQNP at different temperatures

[4.3] ANTIMICROBIAL ASSAYS

The Antimicrobial activity of chitosan nanoparticles with the incorporation of curcumin and quercetin nanoparticles was investigated against *E. coli* (ATCC25922), *E. coli* DH5α (ATCC68322), and *Staphylococcus aureus* using disk well diffusion assay. The antimicrobial activity increased the most in CSNP with curcumin and quercetin nanoparticles in a (1:1) ratio showing the synergistic killing of bacteria and significant zone of inhibition (ZOI). Improved antibacterial activity of CSCURQNP was related to their large surface area that arranged to have more interactions on the surface with microorganisms. The negative DMSO control showed no inhibitory effect, having inhibition zones of 0 mm. [45]

S.No.	Nanoformulation	Concentration(µg/ml)	*E. coli* (25922) ZOI (mm)	DH5α ZOI (mm)	*S. aureus* ZOI (mm)
1.	CSNP	50	0	0	5±2
2.	Curcumin+DMSO	250	0	0	0
3.	Quercetin+DMSO	250	0	0	0
4.	CSQNP	250	10 ± 1	28 ± 0.5	4 ± 1
5.	CSCURNP	250	8± 0.85	30± 0.25	6 ± 0.95
6.	CSQCURNP	250	16 ± 1	35± 1	3 ± 0.80

Table: 3 ZOI of nanoformulations

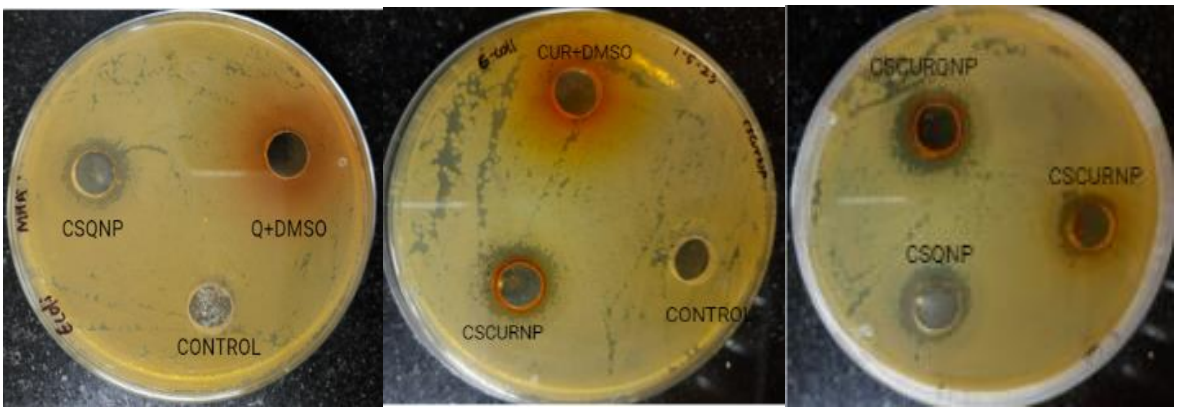


Figure: 24 AST of CSQNP, CSCURNP, CSCURQP against *E. coli* 25922

Figure: 25 AST of CSQNP, CSCURNP, CSCURQNP against *S. aureus*

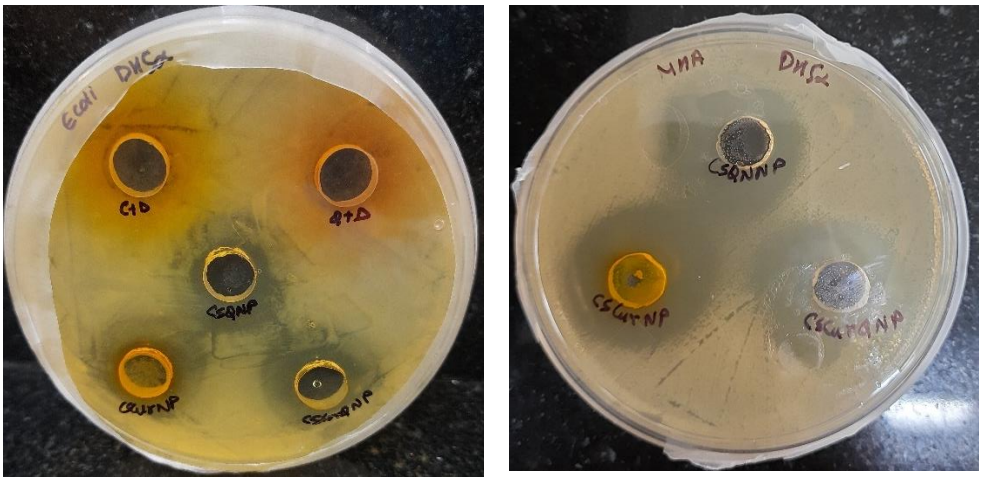


Figure: 26 AST of CSQNP, CSCURNP, CSCURQNP against DH5α

Another antimicrobial test used to determine the activity is the minimal inhibitory concentration (MIC) using 96 well plates taking *E. coli* (25922) as culture and Muller Hinton Broth (MHB) as culture medium. The MIC concentrations of the nanoformulations are depicted in the following table:

S.No.	Nanoformulation	Minimal Inhibitory Concentration (MIC)mg/ml (*E.coli* 25922)
1.	CSNP	0.29
2.	Curcumin+ DMSO	0.03125
3.	Quercetin+ DMSO	0.25
4.	CSQNP	0.0568
5.	CSCURNP	0.0285
6.	CSCURQNP	0.0231

Table:4 MIC of Nanoformulations

[4.4] ANTIOXIDANT ASSAY

DPPH assay:

The capacity of the suggested antioxidant to donate hydrogen to DPPH free radicals can be determined in part by the DPPH radical scavenging test. At 517.0 nm, the stable radical DPPH glows purple. In several in vitro investigations, curcumin and quercetin has previously demonstrated its potential to scavenge free radicals against the DPPH radical (Ak and Gulçin 2008). However, when curcumin is dissolved in an organic solvent, the majority of documented literature has demonstrated its efficiency in DPPH scavenging (Borra et al. 2013).[46] The sample's ability to scavenge free radicals or serve as an antioxidant is negatively correlated with the IC50 value.[48]

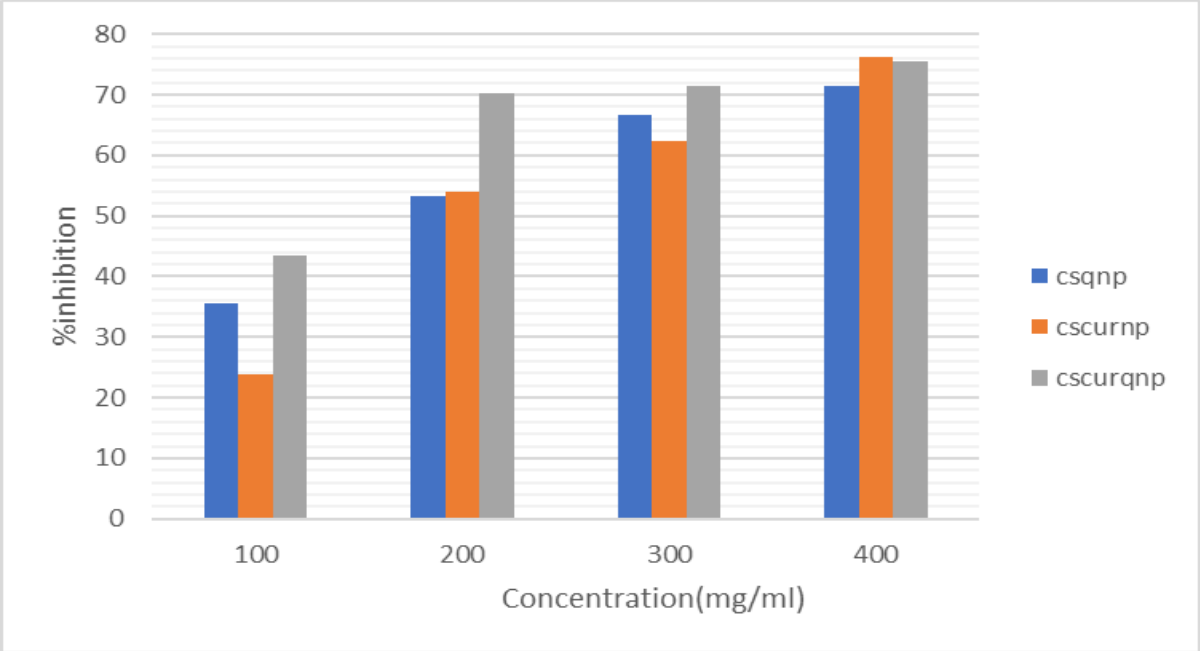


Figure:27 DPPH scavenging activity of CSQNP, CSCURNP, CSCURQNP

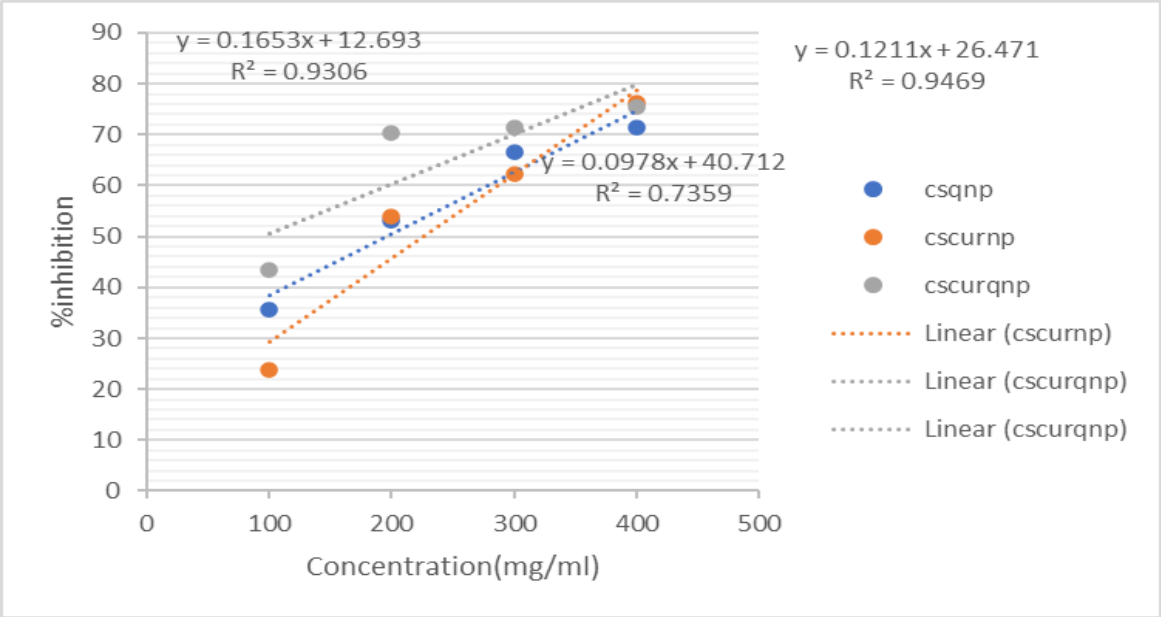


Figure: 28 Graphical representation of DPPH Assay

S.No.	Nanoformulation	IC50 value
1.	CSQNP	0.194
2.	CSCURNP	0.225
3.	CSCURQNP	0.094

Table 5 IC50 of nanoformulations

The IC50 value of the formulations CSQNP, CSCURNP, and CSCURQNP are **0.194,0.225,0.094 mg/ml.**

ABTS ASSAY:

Curcumin, Quercetin in both free and in encapsulated form, demonstrated substantial phenolic content (measured in gallic acid equivalents) and antioxidant activity, effectively scavenging ABTS.[47]

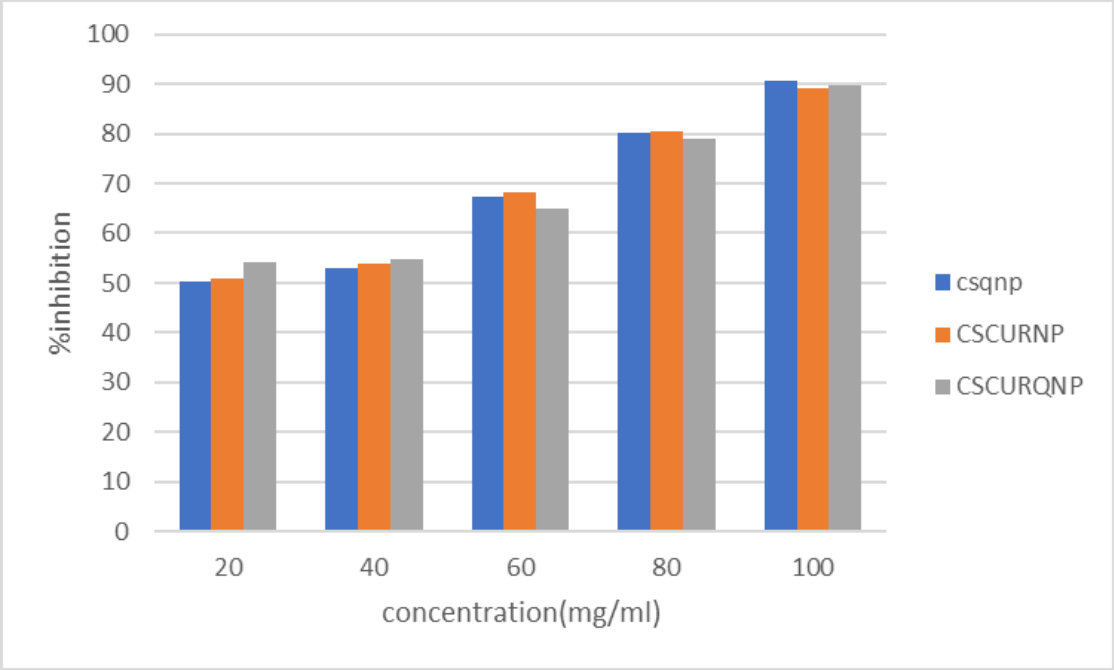


Figure:29 Graphical representation of ABTS Activity

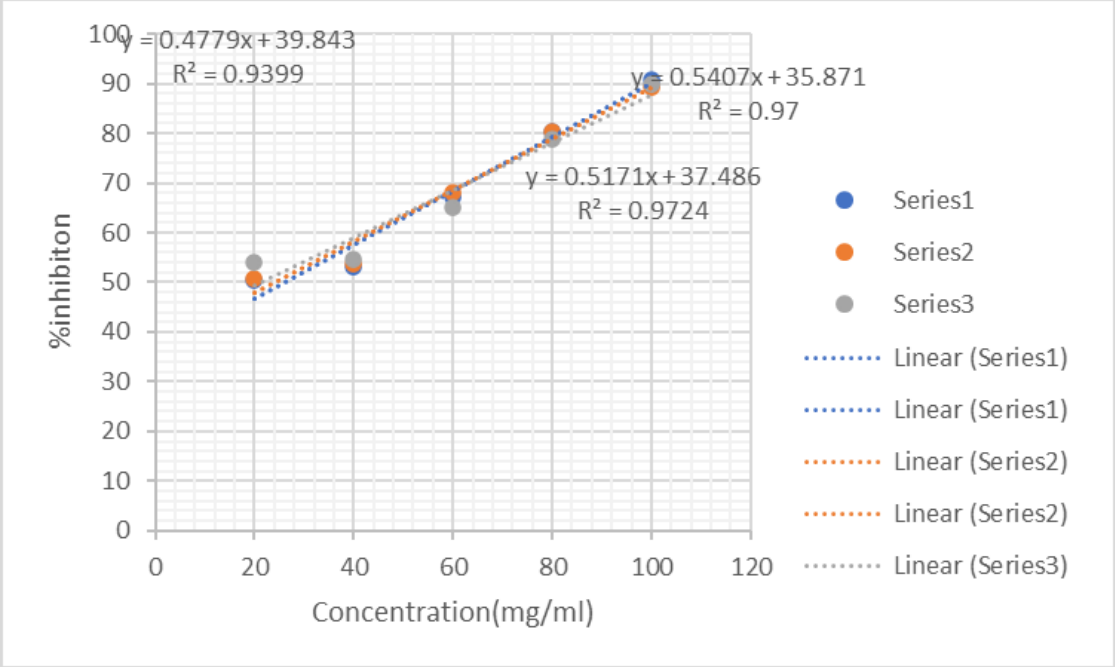


Figure:30 ABTS Scavenging activity of CSQNP, CSCURNP, CSCURQNP

The IC50 value of the nanoformulations CSQNP, CSCURNP, and CSCURQNP are **0.026,0.024,0.021 mg/ml.**

S.No.	Nanoformulation	IC50 value
1.	CSQNP	0.026
2.	CSCURNP	0.024
3.	CSCURQNP	0.021

Table 6: IC50 value of Nanoformulation (ABTS)

DISCUSSION

The goal of developing the curcumin-quercetin-loaded chitosan nanoparticles was to reduce the issues with curcumin administration, such as poor solubility, low bioavailability, and degradation. After the drug delivery system (chitosan nanoparticles loaded with curcumin and quercetin) was successfully produced, synthesized, and physiochemically characterized, it had an impact on the drug's physical stability, cellular absorption, biodistribution, and release.

Nanoparticles' small size, spherical shape, and zeta potential were useful in this regard for passive targeting of tumor tissue due to their improved permeability and retention impact. Furthermore, the curcumin's chemical makeup and its encapsulation inside the nanoparticles were clearly shown in the SEM, FTIR, Zeta potential, and DLS analysis.[49] In this paper, a new strategy to treat and target multi-resistant cancers and microorganisms utilizing a double combination of drug-loaded nanoparticulate is proposed according to previous literature reports. The Noyes-Whitney equation states that a reduction in the size of a nanometer can greatly use in the increase of interfacial surface area, the rate of dissolution is increased and solubility in water is increased, which results in an improvement in bioavailability of medication. However, an increase in the combined surface area also improves a drug's pharmacological activity and boosts its reactability to particular molecular targets. Because they are hydrophobic by nature, the majority of chemotherapeutic drugs, including quercetin, curcumin, piperine have limited bioavailability. Therefore, to counter the mentioned problem a nanoparticle drug delivery system is designed which not only increases reactability but also increases bioavailability.50]

Additionally, these organic bio-enhancers have a broad range of pharmacological actions, including anti-cancer potential. Therefore, the above stated bioenhancers are used to tackle multidrug resistance, to enhance the bioavailability of curcumin and quercetin by preventing intestinal and hepatic metabolism, and to produce a synergistic effect using curcumin and quercetin nanoformulation using chitosan as an encapsulating agent, all based on the aforementioned facts. Ionic gelation was used to create chitosan nanoparticles. The concentration of Chitosan: TPP was optimized using different concentration parameters for the proper distribution of chitosan with a nano-sized scale.

The biological efficacy of TPP/CS nanoparticles has been discovered to be influenced by their features. Because chitosan contains amino groups that may go through the addition of positively charged ions at low pH, and its solubility increases & gets soluble in acidic. TPP (Sodium Tripolyphosphate) acts as an agent for crosslinking and a negatively charged polyvalent anion. CSNPs are formed when positively charged chitosan and negatively charged TPP attracts each other. The concentration of chitosan and TPP solution has a significant impact on the size of the chitosan nanoparticles. Following the development of aggregation, it was shown that nanoparticle size rises when CS and TPP concentrations rise up to a certain concentration. The tight spatial spacing between chitosan molecules at a higher concentration led to the development of bigger particles, which might be the cause of the increase in particle size caused by the rise in CS concentration.[51] Positive results were found to be at concentration of 100mg/100ml in 3% acetic acid and TPP concentration should be around 0.1% for the proper cross linking of the nanoparticles having positive absorbance and the size ranged from 100-150 nm. After the preparation of CSNP, CSCURNP, CSQNP and CSCURQNP using the ionic gelation method the particles were characterized using various parameters like UV-VISIBLE spectroscopy that confirmed the formation of nanoparticles having absorption spectra at around 250nm and 400nm respectively.

According to an SEM investigation, CSNPs range in size from 80 to 100 nmWhile using SEM, we may estimate the projected area diameter, and DLS gives us the particle's hydrodynamic radius. In DLS, a tiny electric dipole layer from the solvent sticks to the surface of a dispersed particle as it travels through a liquid medium. This layer affects how the particle moves through the medium. Therefore, while a particle travels under the influence of Brownian motion, diameter of hydro dynamicity provides us with information about solvent layer, inorganic core, and coating material of the particle. DLS offers strong ensemble statistics at its core. Further FTIR analysis was performed to determine the possible biomolecules responsible for the bio reduction, capping, and stabilization of CSNP, and related nanoformulations and prominent peaks of the bonds were studied. The ph and thermal stability of the nanoformulations were also monitored using UV-VIS spectroscopy where the particles were found to be stable at ph 5-7 and temperature ranging between 30°c and 50°c. Encapsulation efficiency and drug loading capacity were also calculated which depicted the amount of free curcumin and quercetin is encapsulated in the system. Drug release study was studied using dialysis membrane and observed in time lapse of 1hr,2hr,3hr,5hr,10hr,24hr. Similarly, antimicrobial assays confirmed the synergistic effect of the

conjugated nanoformulation by giving prominently increased zones of inhibition and MIC when compared with control formulations. Strong antioxidant activity was observed using DPPH radical scavenging activity and ABTS method of the CSCURQNP showing maximum antioxidant potential of quercetin and curcumin as natural phenolic compounds and lower IC50 value indicated less toxicity of the compounds when administered in lower concentrations. MTT assay was performed to give the cytotoxic effect of formulations against McCoy cell line.

Future Perspective:

1. For characterization: FTIR, DLS, Zeta Analysis, and SEM are to be performed.
2. Drug Release Study of the nanoformulations.
3. Encapsulation Efficiency to be calculated.
4. MTT Assay repeat.

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