



Kaunas University of Technology

Faculty of Chemical Technology



Lithuanian University of Health Sciences

Faculty of Pharmacy

Design and Development of Liposomal Astaxanthin and Curcumin as a Nanocarrier with Anti-cancer Potential

Master's Final Degree Project

Hamidreza Sahaleh

Project author

Prof. Dr. Jurga Bernatoniene

Supervisor

Kaunas, 2026



Kaunas University of Technology
Faculty of Chemical Technology



Lithuanian University of Health Sciences
Faculty of Pharmacy

Liposominio astaksantino ir kurkumino nanonešiklio, pasižyminčio priešvėžiniu veikimu, kūrimas ir vystymas

Master's Final Degree Project
Medicinal Chemistry (6281CX001)

Hamidreza Sahaleh

Project author

Prof. dr. Jurga Bernatoniene

Supervisor

Prof. dr. Valdas Jakštas

Reviewer

Kaunas, 2026



Kaunas University of Technology

Faculty of Chemical Technology

Lithuanian University of Health Sciences

Faculty of Pharmacy

Hamidreza Sahaleh

Design and Development of Liposomal Astaxanthin and Curcumin as a Nanocarrier with Anti-cancer Potential

Declaration of Academic Integrity

I confirm the following:

1. I have prepared the final degree project independently and honestly without any violations of the copyrights or other rights of others, following the provisions of the Law on Copyrights and Related Rights of the Republic of Lithuania, the Regulations on the Management and Transfer of Intellectual Property of Kaunas University of Technology (hereinafter – KTU) and Lithuanian University of Health Sciences (hereinafter – LSMU) and the ethical requirements stipulated by the Code of Academic Ethics of KTU and LSMU;
2. All the data and research results provided in the final degree project are correct and obtained legally; none of the parts of this project are plagiarised from any printed or electronic sources; all the quotations and references provided in the text of the final degree project are indicated in the list of references;
3. I have not paid anyone any monetary funds for the final degree project or the parts thereof unless required by the law;
4. I understand that in the case of any discovery of the fact of dishonesty or violation of any rights of others, the academic penalties will be imposed on me under the procedure applied at KTU or LSMU; I will be expelled from the KTU or LSMU and my final degree project can be submitted to the Office of the Ombudsperson for Academic Ethics and Procedures in the examination of a possible violation of academic ethics.

Hamidreza Sahaleh

Confirmed electronically

Sahaleh, Hamidreza. Design and Development of Liposomal Astaxanthin and Curcumin as a Nanocarrier with Anti-cancer Potential. Master's Final Degree Project /supervisor Head of the Department of Pharmaceutical Technology, Prof. dr. Jurga Bernatoniene; Faculty of Chemical Technology, Kaunas University of Technology; Lithuanian University of Health Sciences, Faculty of Pharmacy

Study field and area (study field group): Chemistry, Physical Sciences.

Keywords: liposome, astaxanthin, curcumin, anticancer, drug delivery, particle size, encapsulation efficiency, stability

Kaunas, 2026. 71 pages.

Summary

Astaxanthin and Curcumin have a strong antioxidant capacity and exhibit various biological activities, such as anti-cancer potential, anti-inflammatory and antitumor activities, improving immunity. However, due to poor solubility and low bioavailability, their clinical use in drug treatment is limited. To address these limitations, liposomal astaxanthin and curcumin have been offered. Liposomes which are similar in structure to cell membranes and are commonly utilized as drug delivery systems capable of encapsulating hydrophobic bioactive compounds. The aim of this study is develop and characterize a liposomal drug delivery system containing astaxanthin and curcumin. Successful design and optimised formulation of liposomal astaxanthin and curcumin, enhanced drug delivery performance, optimized formulation techniques, and enhanced outcomes in quality and control assessments are expected results of this study. In addition, the assessment of scalability and stability under different conditions of the final formulation is another expected outcome to ensure its possible potential in the pharmaceutical market. According to previous studies, liposomal formulations of curcumin and astaxanthin have been studied individually and single liposomal system has not been explored. By formulating them together despite their different characteristics, and converting the liposome dispersion, this study presents a unique and innovative strategy and offers a novel approach with potential benefits in drug delivery systems.

Sahaleh, Hamidreza. Liposominio astaksantino ir kurkumino nanonešiklio, pasižyminčio priešvėžiniu veikimu, kūrimas ir vystymas. Baigiamasis magistro projektas/ Farmacinės technologijos departamento vadovas, Prof. dr. Jurga Bernatienė; Kauno technologijos universitetas, Cheminės technologijos fakultetas; Lietuvos sveikatos mokslų universitetas, Farmacijos fakultetas.

Studijų kryptis ir sritis (studijų krypčių grupė): chemija, fiziniai mokslai.

Reikšminiai žodžiai: liposoma, astaksantinas, kurkuminas, priešvėžinis, vaistų pernaša, dalelių dydis, inkapsuliacijos efektyvumas, stabilumas.

Kaunas, 2026. 71 p.

Santrauka

Astaksantinas ir kurkuminas pasižymi stipriu antioksidaciniu poveikiu ir įvairiomis biologinėmis savybėmis, tokiomis kaip priešvėžinis, priešuždegiminis, priešnavikinis aktyvumas bei imuniteto stiprinimas. Dėl prasto tirpumo ir mažo biologinio prieinamumo jų klinikinis naudojimas vaistų terapijoje yra ribotas. Siekiant išspręsti šiuos trūkumus, buvo pasiūlyti liposominiai astaksantino ir kurkumino preparatai. Liposomos, kurios savo struktūra yra panašios į ląstelių membranas, dažnai naudojamos kaip vaistų pernašos sistemos, galinčios inkapsuliuoti hidrofobinius bioaktyvius junginius. Šio darbo tikslas yra sukurti ir charakterizuoti liposominę vaistų pernašos sistemą, turinčią kurkumino ir astaksantino, bei optimizuotą liposominę formulę paversti mikrokapsulėmis ir tabletėmis. Tikėtini projekto rezultatai yra sėkmingas mikrokapsulių ir tablečių su liposominiu astaksantinu ir kurkuminu sukūrimas bei formulavimas, pagerintas vaistų pernašos efektyvumas, optimizuoti formulavimo metodai ir puikūs kokybės bei kontrolės vertinimo rezultatai. Be to, galutinės formulės stabilumo didinimas įvairiomis sąlygomis yra dar vienas tikėtinas rezultatas, siekiant užtikrinti galimą jos potencialą farmacijos rinkoje. Remiantis literatūra, liposominės kurkumino ir astaksantino formulės buvo tiriamos atskirai, tačiau bendra liposominė sistema nebuvo nagrinėta. Formuluojuojant juos kartu, nepaisant jų skirtingų savybių, ir paverčiant liposomų tirpalą stabiliomis mikrokapsulėmis, šis projektas pristato unikalią ir inovatyvią strategiją bei siūlo naują požiūrį su potencialiais privalumais vaistų pernašos sistemose.

Table of contents

List of figures	8
List of tables	9
List of abbreviations and terms.....	10
Introduction	12
1. Literature review	14
1.1. Astaxanthin.....	14
1.1.2. Physicochemical and biological properties of astaxanthin.....	14
1.1.3. Oral Bioavailability and Absorption Mechanisms of Astaxanthin.....	15
1.1.4. Chemical structure of Astaxanthin	16
1.1.5. Astaxanthin sources and biosynthetic production	17
1.1.5.1. Astaxanthin biosynthesis pathways	17
1.2. Curcumin	19
1.2.1. Biological activities and pharmaceutical potential of curcumin	19
1.2.2. Targeted Molecular Actions of Curcumin in Oncology	21
1.2.3. Chemical structure of Curcumin.....	21
1.2.4. Synthetic Approaches of Curcumin.....	23
1.3. Liposomes.....	25
1.3.1. Drug Delivery Systems (DDSs)	25
1.3.2. Liposomes as nanocarriers for targeted drug delivery.....	25
1.3.3. Liposome structure	27
1.3.4. Composition and Physicochemical Properties of Liposomes	27
1.3.5. Liposome preparation methods	28
1.3.5.1. Thin film hydration method.....	28
1.3.6. Challenges of liposomes formulation	29
1.3.7. Research Gap and Justification of the Study	30
2. Materials and methods	32
2.1. Materials	32
2.3. Experiment.....	32
2.3.1. Pre-formulation studies.....	32
2.3.2. Physical appearance.....	32
2.3.3. Solubility analysis	33
2.3.4. λ max determination	33
2.3.5. Calibration curve	33
2.3.6. Identification test by FT-IR spectrophotometer	33
2.2.3. Preparation of astaxanthin and curcumin loaded liposomes by solvent injection method.....	33
2.2.4. Preparation of astaxanthin and curcumin loaded liposomes by thin film hydration method ..	34
2.2.5. Freeze-Drying (Lyophilization) of astaxanthin and curcumin loaded liposomes	36
2.3. Evaluation and characterization of formulated liposome	37
2.3.1. Average size and Polydispersity index (PDI) evaluation:	37
2.3.2. Zeta potential evaluation:	37
2.3.3. Drug encapsulation efficiency	37
2.3.3.1. Encapsulation efficiency of Astaxanthin	37

2.3.3.2. Encapsulation efficiency of Curcumin	37
2.3.4. Structural analysis of the liposomal astaxanthin and curcumin	38
2.3.4.1. Identification test by FT-IR spectrophotometer	38
2.3.4.2. Scanning Electron Microscopy (SEM) analysis	38
2.3.5. Yield	38
2.3.6. Moisture content	38
2.3.7. Bulk density	39
2.3.8. Stability studies.....	39
3. Results and discussion	40
3.1. Pre-formulation studies.....	40
3.1.1. Physical appearance.....	40
3.1.2. Solubility analysis.....	40
3.1.3. λ max determination	40
3.1.4. Calibration curve	42
3.2. Evaluation of liposomal formulations prepared by the ethanol injection method.....	42
3.2.1. Average size and Polydispersity index (PDI) evaluation	42
3.2.2. Justification for changing method	45
3.3. Evaluation of liposomal formulations prepared by the thin-film hydration method.....	45
3.3.1. Average size and Polydispersity index (PDI) evaluation	46
3.3.2. Influence of hydration media on particle size, PDI and zeta potential.....	46
3.3.3. Characterisation of liposomal formulations	47
3.3.4. Identification test by FT-IR spectrophotometer	50
3.3.5. Scanning Electron Microscopy (SEM) analysis.....	53
3.3.5.1. SEM Morphological Analysis for formulation F9	53
3.3.5.2. SEM Morphological Analysis for formulation F10	54
3.3.5.3. Comparative discussion between two different formulation	55
3.3.6. Structural analysis based on SEM and FTIR:.....	55
3.3.7. Encapsulation efficiency (%).....	55
3.3.8. Yield	56
3.3.9. Moisture content	56
3.3.10. Bulk density.....	56
3.3.11. Stability studies.....	56
3.3.11.1. Appearance	57
3.3.11.2. Particle size.....	58
3.3.11.3. Encapsulation efficiency (EE%).....	58
Recommendations.....	62
Conclusions	63
List of references.....	64
List of publications	71

List of figures

Fig. 1. Chemical structures of astaxanthin	16
Fig. 2 . Biosynthetic pathway of β -carotene from IPP and DMAPP in <i>H.pluvialis</i>	18
Fig. 3 . Astaxanthin biosynthesis from β -carotene in <i>H.pluvialis</i>	19
Fig. 4. Schematic of the biological activities	20
Fig. 5. Chemical structure of curcumin	22
Fig. 6. Molecular structure of curcuminoids	23
Fig. 7. Structural keto-enol tautomerism of curcumin	23
Fig. 8. Molecular structure of curcumin with key functional groups	24
Fig. 9. Schematic of natural curcumin synthesis	24
Fig. 10. Functionalised liposomes as nanocarriers for targeted delivery of anticancer compounds .	26
Fig. 11. General structure of liposomes.....	27
Fig. 12. Schematic of solvent injection method	34
Fig. 13. Sonification and particle size analysis of liposomal formulations.....	35
Fig. 14. λ max of astaxanthin	41
Fig. 15. λ max of curcumin.....	41
Fig. 16. Particle size distribution of liposomal formulation F4.....	43
Fig. 17. Particle size distribution of liposomal formulation F1 and F2.....	43
Fig. 18. Zeta potential distribution of liposomal formulation F4	44
Fig. 19. Zeta potential distribution of liposomal formulation F1	45
Fig. 20. Preparation of lyophilised liposomal astaxanthin and curcumin formulations.....	46
Fig. 21. Particle size distribution of liposomal formulation F6.....	47
Fig. 22. Particle size distribution of liposomal formulation F9.....	49
Fig. 23. Zeta potential of liposomal formulation F9.....	49
Fig. 24. IR spectrum of astaxanthin.....	50
Fig. 25. IR spectrum of curcumin.....	51
Fig. 26. IR spectrum of sample formulation F9	51
Fig. 27. IR spectrum of formulation F10.....	51
Fig. 28. SEM micrographs of formulation F9 at different magnifications.....	53
Fig. 29. SEM micrographs of formulation F10 at different magnifications.....	54
Fig. 30. Appearance stability of liposomal astaxanthin and curcumin liposomes after six weeks ...	58
Fig. 31. PDI changes during storage at 4 °C and 25°C	59
Fig. 32. Z-average changes during storage at 4 °C and 25 °C.....	59
Fig. 33. Zeta potential changes during storage 4 °C and 25 °C.....	60
Fig. 34. Curcumin encapsulation efficiency during storage at 4°C and 25°C	61
Fig. 35. Astaxanthin encapsulation efficiency during storage at 4 °C and 25 °C	61

List of tables

Table 1. Composition of liposomal formulations by solvent injection method	34
Table 2. Composition of liposomal formulations by thin-film hydration method.....	36
Table 3. Solubility of astaxanthin and curcumin in different solvents	40
Table 4. λ max of determination of astaxanthin	41
Table 5. λ max of determination of curcumin	41
Table 6. Z-average and PDI of solvent injection liposomal dispersion.....	42
Table 7. Zeta potential of solvent injection liposomal dispersion.....	43
Table 8. Z-average and PDI of liposomal formulations F6 and F7	47
Table 9. Zeta potential of liposomal formulations F6 and F7	47
Table 10. Z-average and PDI of thin-film hydration liposomal dispersion.....	48
Table 11. Zeta potential of thin-film hydration liposomal dispersion.....	48
Table 12. The main Characteristic FTIR peaks of curcumin	52
Table 13. The main Characteristic FTIR peaks of astaxanthin	52
Table 14. Characteristic FTIR peaks of the lyophilized formulation F9.....	52
Table 15. Characteristic FTIR peaks of the lyophilized formulation F10.....	52

List of abbreviations and terms

Abbreviations:

ABC:	ATP-binding cassette
ASES:	aerosol solvent extraction systems
AST:	Astaxanthin
BKT:	β -carotene ketolase
CMC:	critical micelle concentration
CO ₂ :	Carbon dioxide
CUR:	Curcumin
DDS:	Drug delivery system
DMAPP:	Dimethylallyl pyrophosphate
DMSO:	Dimethyl sulfoxide
DOPC:	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DOPE:	1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine
DPPC:	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
DPPG:	1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol
DRV:	Dried reconstituted vesicle
DSPC:	1,2-Distearoyl-sn-glycero-3-phosphocholine
DSPE:	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine
DSPG:	1,2-Distearoyl-sn-glycero-3-phosphoglycerol
EP:	European Pharmacopoeia
FCC:	Food Chemicals Codex
GAS:	Gas anti-solvent
GGPP:	Geranylgeranyl pyrophosphate
GGPPS:	GGPP synthase
GMP:	Good Manufacturing Practice
GUV:	Giant unilamellar vesicles
H. pluvialis:	<i>Haematococcus pluvialis</i>
HMGR:	Hydroxymethylglutaryl-CoA reductase
IPP:	Isopentenyl pyrophosphate
LUV:	Large unilamellar vesicles

MEP: Methylerythritol phosphate
MLV: Multilamellar vesicle
MTX: Methotrexate
MVA: Mevalonate
MVD: Mevalonate diphosphate decarboxylase
MVK: Mevalonate kinase
MVV: Multivesicular vesicle
NLC: Nanostructured lipid carrier
PA: Phosphatidic acid
PAINS: Pan-assay interference compounds
PC: L- α -phosphatidylcholine
PDI: Polydispersity index
PDS: Phytoene desaturase
PE: Phosphatidylethanolamine
PEG: Polyethylene glycol
PG: Phosphatidylglycerol
PI: Phosphatidylinositol
PMK: Phosphomevalonate kinase
PS: Phosphatidylserine
PSY: Phytoene synthase
SAS: Supercritical anti-solvent
SCF: Supercritical fluid
SCRPE: Supercritical reverse-phase evaporation
SLN: Solid lipid nanoparticle
SR-B1: Scavenger receptor class B type 1
SUV: Small unilamellar vesicles
ULV: Unilamellar vesicle
UV: Ultraviolet spectrophotometer
ZDS: ζ -carotene desaturase

Introduction

Cancer remains one of the major global health challenges, and despite advances in treatment, many therapies are still limited by poor efficacy, systemic toxicity, and lack of targeted delivery. A key issue in cancer treatment is the effective delivery of therapeutic agents to the desired site of action while maintaining their stability and biological activity. There are many bioactive compounds in the nature which have various and unique biological activities. For instance, some of them have anticancer effects in laboratory studies; however, they are limited in clinical application due to poor solubility, low bioavailability, rapid degradation and limited stability over time.

Astaxanthin and Curcumin are two natural bioactive compounds that have attracted considerable attention particularly because of their antioxidant, anti-inflammatory, and anticancer properties. Curcumin is a polyphenolic compound from *Curcuma longa*, and astaxanthin is a lipid-soluble xanthophyll carotenoid which mostly known for their antioxidant and anticancer properties. According to previous studies, natural astaxanthin is highly potent antioxidant with singlet oxygen quenching activity approximately 6000 times greater than Vitamin C and 100 times higher than Vitamin E [1].

Also, curcumin interacts with multiple cellular signalling pathways involved in tumour growth and progression, while astaxanthin is known for its strong ability to reduce oxidative stress. Despite these biological benefits, both compounds have significant limitations. For instance, the clinical application of curcumin is limited due to poor oral bioavailability because of its low aqueous solubility, poor absorption and rapid metabolism in the body. Same issue for astaxanthin which is hydrophobic and chemically unstable and some factors such as oxygen, temperature and light can cause its degradation and as a result, it restricts its oral bioavailability. These factors reduce their therapeutic effectiveness and highlight the need for improved delivery strategies.

Liposomal drug delivery systems are considered an effective approach to address these challenges. They are vesicular structures composed of phospholipid bilayers that can encapsulate both hydrophilic and lipophilic compounds which this feature make them practical and unique in terms of drug delivery. Moreover, this allows them to protect sensitive compounds from degradation, improve their stability, and modify their pharmacokinetic behaviour. In addition, liposomes can improve the delivery of bioactive compounds to target cells and tissues, which improve their therapeutic. With microencapsulation techniques liposomal structures can be protected. Liposomal astaxanthin and curcumin have been studied individually in previous research; however, in this project, they are formulated together. This combination of both bioactive compounds in a one formulation shows a unique way and innovative strategy and offers a novel approach with potential benefits for drug delivery. In addition, this study explores the optimisation of formulation parameters and evaluates key physicochemical characteristics of final formulation to ensure its effectiveness.

The main aim of this study was to design and develop liposomal formulations which contain curcumin and astaxanthin in order to overcome their limitations and improve their solubility and bioavailability. To achieve this aim, liposomal formulations were prepared by using the thin-film hydration method and ultrasonication. The prepared liposomes were optimised and then characterised based on particle size, polydispersity index (PDI), zeta potential, and encapsulation efficiency and etc.

In addition, the physicochemical properties and stability of the lyophilised liposomal formulations were evaluated under different conditions (4 °C and 25 °C) in a dark place to investigate their potential production in large scale such as pharmaceuticals.

This study is expected to contribute to the development in the effective way to deliver natural bioactive compounds and provide a foundation for future research in advanced pharmaceutical formulations.

1. Literature review

1.1. Astaxanthin

1.1.2 Physicochemical and biological properties of astaxanthin

Astaxanthin ($C_{40}H_{52}O_4$) is hydrophobic bioactive compound and marine carotenoid that widely found in marine crustaceans and microorganisms such as shrimp, salmon, crabs and etc. beside the natural sources, synthetic astaxanthin has attracted attentions particularly in industry scale due to low production costs as well as its manufacturing process. However, during the project, synthetic astaxanthin proved that synthetic form can't be always good choice to formulate liposomal astaxanthin and even can affect on biological activity and as a result can make some limitation to use them [2].

Astaxanthin has many health benefits due to its antioxidant effects, anti-inflammatory activity, anti-diabetic activity, anticancer activity, cardiovascular disease prevention but nowadays, astaxanthin mostly known by its anticancer properties and antioxidant activity. It attracted widespread attention in pharmaceutical and biopharmaceutical science, cosmetic as well as food industry. Recent studies highlight its widespread use in a variety of commercially available products, which are formulated as powders, biomass, lotions, capsules, soft gels, tablets, energy drinks, oils, and extracts. In some formulations same as this project, astaxanthin is combined with additional ingredients such as omega-3 and omega-6 fatty acids, multivitamins, carotenoids, and herbal extracts. Astaxanthin is considered one of the most powerful naturally antioxidants, with activity reported to exceed that of α -tocopherol (Vitamin E), Vitamin C and many other carotenoids. Similar to other carotenoids, astaxanthin is biosynthesised from isoprenoid units and shows a 40 carbon polyene backbone [3]. Its strong antioxidant performance which mostly comes from the terminal ring groups that help to neutralise and stabilise reactive molecules. Because of these properties, astaxanthin was approved as a nutritional supplement in 1991[4]. Moreover, it has since been applied in human health and livestock systems [5]. Beyond antioxidant action, it has been linked to anticancer potential, activation of the Nrf2/Keap1 pathway, inhibition of NF- κ B signalling, and a range of biological effects which include anti-inflammatory, antihypertensive, anti-obesity, antimicrobial, neuroprotective, and gastroprotective activities [5]. Moreover, astaxanthin proved that it can modulate mitochondrial function and cellular signalling pathways associated with oxidative stress [6]. The red colour in salmon, shrimp, and flamingos is obtained from dietary astaxanthin, which they acquire from microalgae in the food chain [7]. Industrial production currently depends on chemical synthesis, microbial fermentation or extraction from crustacean by-products, with synthetic routes remaining the lowest-cost option. However, by increasing consumer awareness and regulatory support for natural colourants, demand for microalgal astaxanthin has risen. It is frequently reported to show stronger antioxidant activity than synthetic counterparts, due to its predominantly esterified form. Natural astaxanthin is produced by several microorganisms. They include *Haematococcus pluvialis* (*H. lacustris*), *Chromochloris zofingiensis*, *Chlorella vulgaris*, and the yeasts *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous*. Recent studies suggest that the biological functions of astaxanthin are more extensive than previously recognised. Although it is a non-provitamin A carotenoid and cannot be converted into retinoids, evidence suggests that astaxanthin may nevertheless interact with cellular signalling pathways involving retinoic acid receptors and other nuclear receptors [8] This raises the possibility that astaxanthin contributes to the regulation of gene expression through mechanisms functionally comparable to those of retinoic acid. Despite all benefits of astaxanthin, it

has some limitations that make it challenging to use this bioactive compounds in pharmaceuticals. For instance, astaxanthin shows poor stability and very sensitive to oxygen, heat, temperature, light and pH changes; in other words, it chemically unstable and reduce its biological effectiveness even before absorption takes place [9]. This can be explained by its molecular structure, which contains a long chain of conjugated double bonds, makes it vulnerable to oxidative degradation. Other limitation of astaxanthin can be its poor bioavailability. The poor bioavailability of astaxanthin is connected to its physicochemical characteristics as well as its sensitivity to environmental conditions. One of the main issues is its strong lipophilic nature, which results in very low solubility in aqueous phase. Because of this, astaxanthin does not dissolve efficiently in gastrointestinal fluids, and this directly limits the amount that can be absorbed through the intestinal tract [10, 11]. In practical terms, its absorption depends heavily on the presence of dietary fats, since the compound needs to be incorporated into mixed micelles before it can be taken up by intestinal cells [11]. Several recent studies have pointed out that the absorption of astaxanthin is not only low but also inconsistent. This can be explained by its large molecular size and its limited ability to pass through intestinal membranes [11]. After absorption, metabolic processes in the body can quickly transform the compound into other forms, which reduces the amount of active astaxanthin available in systemic circulation [6].

1.1.3 Oral Bioavailability and Absorption Mechanisms of Astaxanthin

After oral administration, astaxanthin (AST) does not directly enter systemic circulation and it first undergoes several stages within the gastrointestinal tract. Firstly, it is released from the food or formulation matrix, and then undergoes stages such as dissolution in digestive fluids, absorption through the intestinal epithelium, distribution via the bloodstream, and finally metabolism and excretion. The term bioavailability refers to the fraction of AST that successfully reaches the circulation and becomes available for biological activity in tissues [12]. This is not a fixed value, as it can depending on multiple factors, such as the individual's metabolic condition, the chemical nature of AST, the composition of the ingested food, and the involvement of transport proteins. The structure of AST also plays an important role in how it is absorbed. The molecule contains a system of conjugated double bonds along with chiral centres, which results in the presence of different stereoisomeric forms. During digestion, these forms can change to some extent. Evidence suggests that cis-isomers are absorbed more efficiently than the all-trans form, although the mechanism behind this difference still remains unclear [13]. The absorption of AST in the intestine contains membrane-associated proteins, but the process cannot be explained by a single pathway. Proteins such as scavenger receptor class B type 1 (SR-B1) and cluster of differentiation 36 (CD36) are believed to contribute to its uptake into enterocytes [14]. At the same time, efflux transporters, particularly those which belonging to the ATP-binding cassette (ABC) family, can transport AST in the opposite direction. As a result in the compound being returned to the intestinal lumen or redirected towards lymphatic transport. Their activity is not constant and can be influenced by other compounds present in the system. For instance, phytochemicals such as curcumin, piperine, quercetin, and resveratrol have been introduced to modify transporter behaviour, which, in turn, can increase or reduce AST absorption depending on the conditions [15]. The physiological condition of the individual can influence AST absorption. One factor is the gut microbiota, which is connected to metabolic health [16].

When this balance is disturbed, for example in hyperglycaemia or insulin resistance, the uptake of AST can decrease. The exact relationship is still not fully understood, but it is considered relevant. Diet also can affect AST absorption. The presence of lipids generally improves absorption. The reason is that AST dissolves better and mixed micelles can form more easily during digestion [17]. On the other hand, soluble dietary fibres can reduce absorption. They increase the viscosity of the intestinal contents, which may interfere with micelle formation. This effect has been observed in Caco-2 cell models. For this reason, AST bioavailability cannot be explained by a single factor. It depends on several factors which are acting together, such as molecular properties, physiological state, and diet. These factors should be considered when attempting to improve its delivery and potential therapeutic use.

1.1.4 Chemical structure of Astaxanthin

Astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) with chemical formula $C_{40}H_{52}O_4$ and a molecular weight of 596.8 g/mol is a naturally occurring keto-carotenoid widely distributed in marine organisms such as microalgae (*Haematococcus pluvialis*), yeast (*Phaffia rhodozyma*), salmon, krill and shrimp (Figure 1). It is responsible for the red to orange pigmentation observed in these species and is considered one of the most potent natural antioxidants among carotenoids [18].

From a structural perspective, it consists of a long polyene chain with 13 conjugated double bonds, flanked by two terminal β -ionone rings. Each ring contains both a hydroxyl ($-OH$) and a keto ($C=O$) functional group, which classifies astaxanthin as a xanthophyll carotenoid [18, 19]. This structural arrangement gives the molecule an amphiphilic nature, allowing it to cross lipid bilayers and effectively protect cell membranes against oxidative damage. The hydroxyl groups allow astaxanthin to form esters with fatty acids, which increases its solubility in cells and leads to the formation of monoesters and diesters. Astaxanthin can also be in non esterified form or bound to other cellular molecules. Compared to free astaxanthin, the esterified forms are usually more stable and often show stronger antioxidant behaviour. Their stability increases when the fatty-acid chains are longer, esterification is higher, and the fatty acids are less unsaturated [20]. Astaxanthin exists in different stereoisomeric and esterified forms in nature, with variations depending on its biological source. These structural features contribute to its strong radical-scavenging activity and high biological functionality, making it widely studied for nutraceutical, pharmaceutical, and cosmetic applications [19].

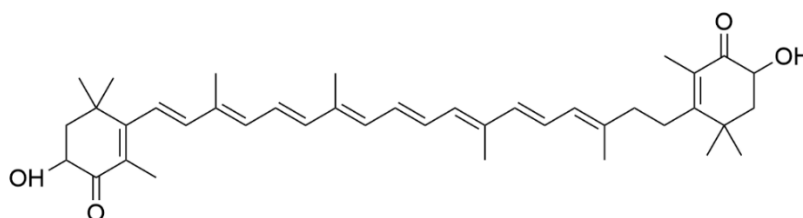


Fig. 1. Chemical structure of Astaxanthin

1.1.5 Astaxanthin sources and biosynthetic production

There are two main sources for astaxanthin: synthetic and natural sources. The synthetic form contains of a mixture of three stereoisomers (3S,3'S; 3R,3'S; 3R,3'R); however, the natural form is mainly present as the 3S,3'S isomer, which is considered more biologically active. In terms of safety and environmental effects, synthetic astaxanthin is largely restricted to non-human applications such as aquaculture [21]. Natural astaxanthin is produced by several microorganisms, such as bacteria, fungi, yeast, and microalgae. However, low yields and high production costs make a limitation to use them. Among the available sources, *Haematococcus pluvialis* is the most efficient producer, with reported astaxanthin content ranging from 1.5% to 10% of dry weight. In comparison, *Phaffia rhodozyma* contains approximately 0.40% (w/w), although improvements have been achieved through strain selection and optimisation of cultivation conditions [22]. As a result, microalgae are currently the main industrial source, although production capacity still falls short of global demand. Other potential sources, such as seafood processing waste and *Euglena sanguinea*, have been explored, but they are not yet widely used at an industrial scale.

1.1.5.1 Astaxanthin biosynthesis pathways

Astaxanthin biosynthesis proceeds in two main stages. The first stage involves the formation of β -carotene from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The second stage includes the conversion of β -carotene into astaxanthin through a sequence of ketolation and hydroxylation reactions.

IPP is generated through two metabolic pathways: the mevalonate (MVA) pathway in the cytosol and the methylerythritol phosphate (MEP) pathway in the chloroplast [21]. *Phaffia rhodozyma* utilises the MVA pathway, where acetyl-CoA serves as a precursor for IPP synthesis. In contrast, *Haematococcus pluvialis* relies on the MEP pathway due to the absence of key enzymes such as hydroxymethylglutaryl-CoA reductase (HMGR), mevalonate kinase (MVK), phosphomevalonate kinase (PMK), and mevalonate diphosphate decarboxylase (MVD). In this organism, IPP is formed from glyceraldehyde-3-phosphate and pyruvate through methylerythritol phosphate intermediates (Figure 2). After IPP is produced, it is converted into its isomer which is DMAPP. These two five-carbon compounds then take part in a series of enzyme-mediated condensation reactions that lead to the formation of geranylgeranyl pyrophosphate (GGPP). This step is catalysed by prenyltransferase enzymes with GGPP synthase (GGPS) [23]. In the next stage, two GGPP molecules are joined together by phytoene synthase (PSY) to form phytoene [23]. Then phytoene is gradually converted into lycopene by several dehydrogenation reactions. During this process, intermediates such as phytofluene, ζ -carotene, and neurosporene are formed through the activity of phytoene desaturase (PDS) and ζ -carotene desaturase (ZDS). Lycopene is cyclised to β -carotene by lycopene β -cyclase (LCYB/CrtL-b) located in the chloroplast membrane [24]. Although β -carotene synthesis occurs in the chloroplast, it is later accumulated in lipid droplets, and the transport mechanism remains unclear. Astaxanthin formation begins with the modification of β -carotene. The reactions are catalysed by β -carotene ketolase (BKT) and β -carotene hydroxylase (CrtR-b). Two main biosynthetic routes have been proposed (Figure 3). One pathway proceeds through intermediates such as echinenone, canthaxanthin, and adonirubin which involve sequential ketolation and hydroxylation reactions. The another pathway involves the formation of zeaxanthin through hydroxylation that it followed by ketolation to produce astaxanthin [25].

In *H. pluvialis*, astaxanthin is found in an esterified form. Some studies suggest that its synthesis does not occur only in the chloroplast, but can also take place in the endoplasmic reticulum, because the activity of the BKT enzyme in the chloroplast is limited [26].

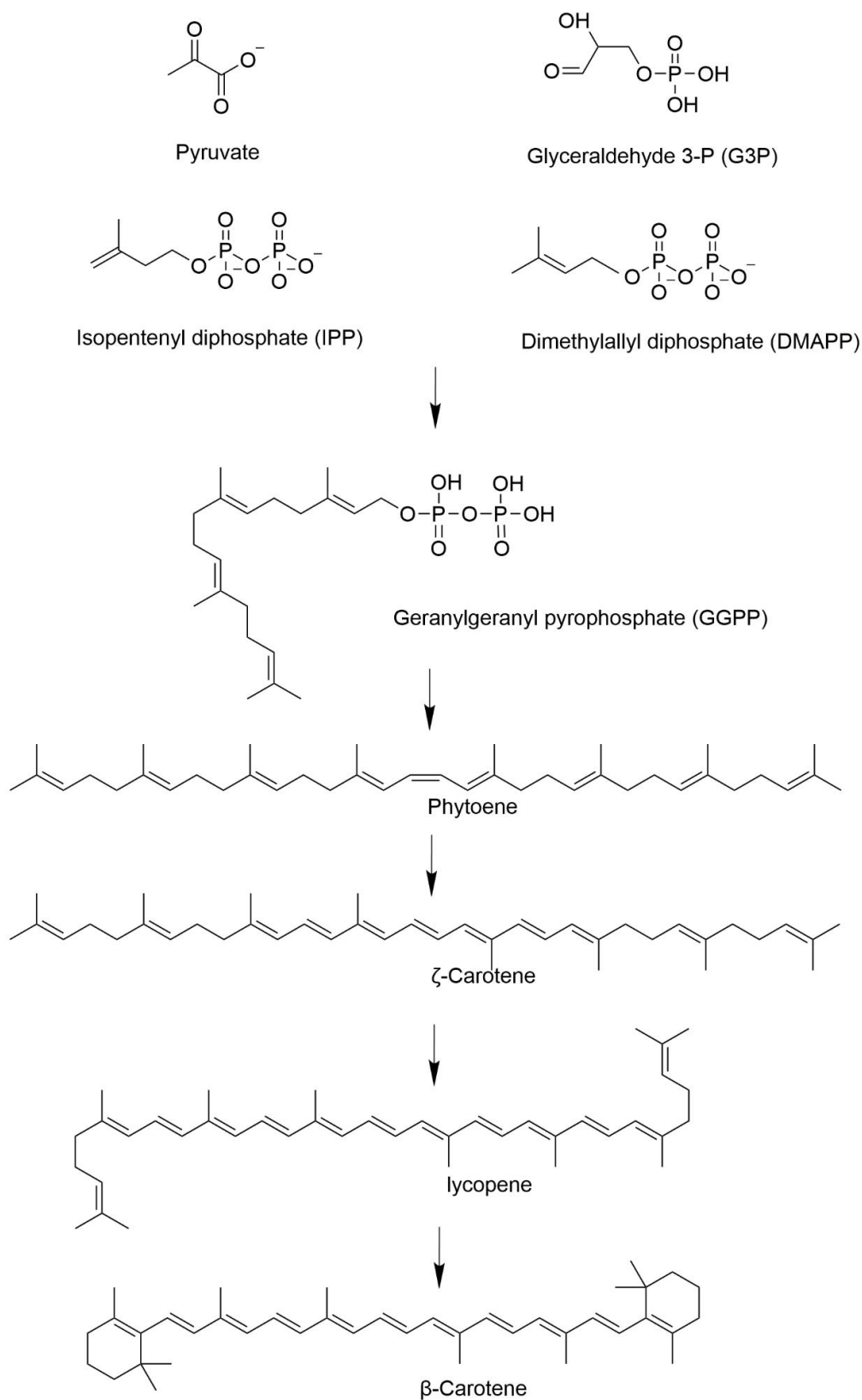


Fig. 2. Biosynthetic pathway of β -carotene from IPP and DMAPP in *Haematococcus pluvialis* [25]

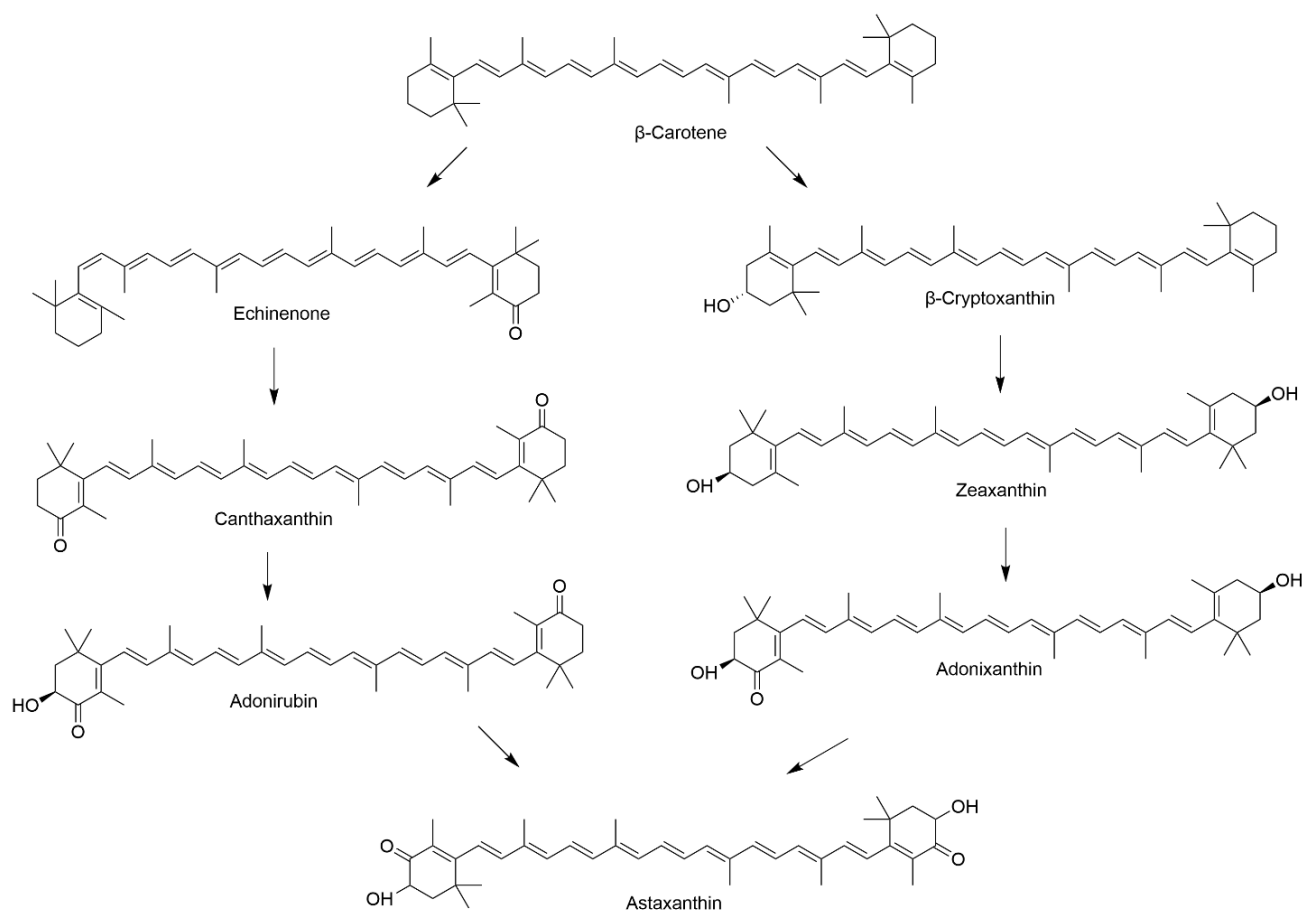


Fig. 3. Astaxanthin biosynthesis from β -carotene in *Haematococcus pluvialis* [26]

1.2. Curcumin

1.2.1 Biological activities and pharmaceutical potential of curcumin

Curcumin is polyphenol and a fat-soluble aromatic phytochemical responsible for the characteristic orange–yellow colour of turmeric, is derived from the rhizome of *Curcuma longa* L., a member of the Zingiberaceae (ginger) family. It mostly cultivated in India and other parts of Southeast Asia and was first isolated in 1870, while its chemical structure, known as diferuloylmethane, was elucidated in 1910. Early scientific interest in curcumin included reports of antibacterial activity published in *Nature* in 1949, whereas clinical observations had already appeared in *The Lancet* as early as 1937. Today, a substantial body of in vitro, in vivo, and clinical evidence shows that curcumin exhibits a wide range of biological activities, such as antioxidant, anti-inflammatory, anticancer, antidiabetic, and antiviral effects [27]. The biological and pharmacological activities of curcumin are presented in figure 4 [12].

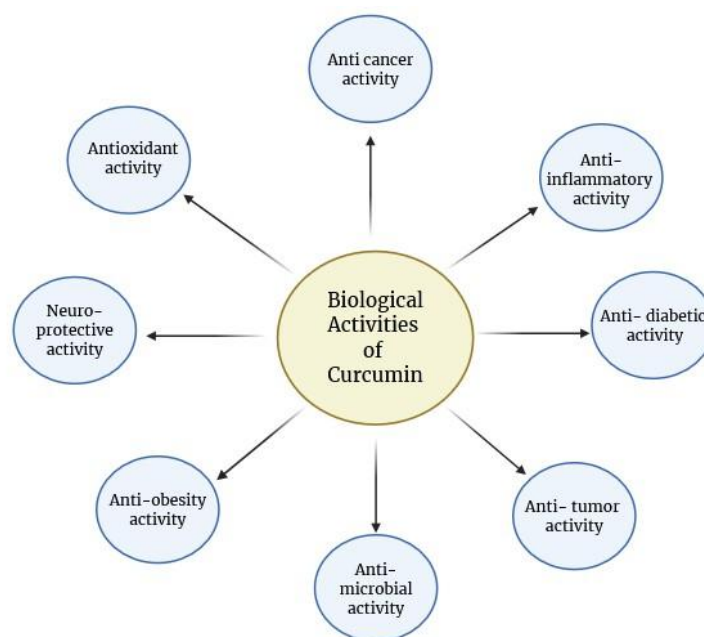


Fig. 4. Schematic of the biological and pharmacological activities of curcumin
(created by BioRender.com)

Curcuminoids are the main bioactive compound of turmeric and include several related compounds, such as curcumin, demethoxycurcumin, bis-demethoxycurcumin, dihydrocurcumin, as well as tetrahydrocurcumin which cause biological and pharmacological activities in turmeric. Among them, curcumin has been the most studied one and has shown potential roles in immune regulation, cardiovascular protection, and neuroprotection. However, curcumin has some limitations. It shows poor water solubility, low absorption, rapid metabolism, and quick elimination from the body and as a result cause low bioavailability. To overcome and address these drawbacks, many formulation strategies have been developed to improve its physicochemical properties and biological performance. In particular, nanotechnology-based delivery systems, such as liposomes, nanoparticle encapsulation, and surface-modified carriers, have demonstrated considerable potential in improving the stability, bioavailability, and biocompatibility of curcumin [28]. According to studies, liposomal formulations can increase stability and prolong systemic circulation [29]. Also, previous studies have investigated the co-administration of adjuvant compounds such as quercetin and piperine with aim of improving its therapeutic efficacy or minimise adverse effects [30]. In the present study, astaxanthin was selected as a co-administered compound due to its strong antioxidant and anti-inflammatory properties, with the aim of exploring its potential possibility in combination with curcumin. A wide range of experimental investigations has showed that turmeric and its main bioactive components indicate various biological effects. These include antimicrobial, insecticidal, larvicidal, antimutagenic, radioprotective, and anticancer activities. The obtained results from animal studies indicate that these compounds have beneficial effects in several pathological conditions, such as inflammatory disorders, obesity, diabetes, atherosclerosis, cancer, depression, and neurodegenerative diseases. In addition, clinical research has reported positive outcomes in humans, particularly in conditions such as lupus nephritis, irritable bowel syndrome, acne, fibrosis, diabetes, and various forms of cancer [30].

Curcumin has attracted considerable research interest over the years and remains one of the most frequently studied natural compounds, especially in studies related to chronic diseases. Although

many studies have described its molecular mechanisms of action, a detailed understanding of its interactions with biological targets is still incomplete. For this reason, recent research has increasingly focused on ligand–protein interactions, often using molecular docking techniques, to better explain the multi-target activity of curcumin in disease prevention and treatment [31].

1.2.2 Targeted Molecular Actions of Curcumin in Oncology

Cancer is still a major global health problem and remains one of the main causes of death worldwide. In most cases, its development is connected to a loss of balance between normal cell growth and programmed cell death (apoptosis) [32]. When apoptotic pathways do not function in the correct way, damaged or abnormal cells are not eliminated and can continue to divide, and as a result it leads to tumour formation and progression [32]. Although in some cases, improvements in early diagnosis and treatment have helped to reduce mortality, many therapies still face limitations, particularly due to the development of drug resistance. This situation proves the need for new treatment approaches that are both more effective and less toxic. Curcumin has been widely explored as a potential anticancer agent, mainly because it shows relatively low toxicity while being able to affect multiple cellular pathways at the same time. Some cancers, such as prostate, lung, liver, pancreatic, colorectal, and breast cancers, as well as haematological malignancies such as leukaemia and multiple myeloma has been studied. Among them, breast cancer is one of the most common diagnosed cancers in adults. According to previous studies, curcumin can make tumour development procedure slowly by influencing apoptotic mechanisms. It can activate the intrinsic pathway by disrupting mitochondrial function and decreasing the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, which then leads to caspase activation and cell death [33]. In addition, curcumin has been reported to interfere with different stages of the cell cycle, including G0/G1, G1/S, and G2/M phases, thereby limiting uncontrolled cell division [34].

Curcumin is known for its anticancer activity mainly because it can affect different cellular signalling pathways that are involved in tumour development. Studies have reported that it interacts with growth factor receptors such as EGFR and ERBB2, and also with transcription factors like NF- κ B and STATs. It can also influence inflammatory mediators and several protein kinases and as a result curcumin can disturb the normal processes that control cell growth and survival [34].

It has also been shown that curcumin can act on important pathways related to cancer progression, such as PI3K/Akt, MAPK, and Wnt/ β -catenin. When these pathways are affected, tumour growth tends to decrease and the risk of metastasis can be reduced and as a result curcumin can lower the viability of cancer cells [35].

In addition to these effects, curcumin influences the expression of genes that regulate apoptosis and cell cycle progression which include tumour suppressors such as p53 [33, 36]. Curcumin is able to influence many stages of cancer development and Because of that, it can interact with a wide range of biological targets, Researches have reported its activity against multiple cancer types, such as breast, prostate, colorectal, pancreatic, and lung cancers [34]. This multi-targeted behaviour suggests that curcumin may be useful not only as a single agent but also in combination with existing anticancer therapies.

1.2.3 Chemical structure of Curcumin

Curcumin is the main phenolic compound found in the rhizomes of *Curcuma longa* and because of its unique structure, it has attracted many attention in science such as organic chemistry,

pharmacology, and pharmaceutical sciences because of its ability to interact with multiple molecular targets and influence signalling pathways involved in disease progression, particularly in cancer [37]. Curcumin has been reported to show a wide range of biological activities such as antioxidant, antidiabetic, antimicrobial and antimalarial effects [38]. Among all, its anticancer activity that is the most important one, is related to specific structural features of the molecule, particularly the phenolic hydroxyl groups and the methylene group within the β -diketone system. Some studies have shown that curcumin can activate programmed cell death in different cancer cell lines, which proves its potential role as a chemotherapeutic agent [37].

From a chemical point of view, curcumin (diferuloylmethane) has the molecular formula $C_{21}H_{20}O_6$ and a molecular weight of 368.385 g/mol (Figure 5). Its structure as shown in the figure 5 can be described as 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione. The molecule consists of two substituted aromatic rings which are connected by a conjugated heptadiene chain that contains an α,β -unsaturated β -diketone moiety. Based on this structure, the 1,3-diketone group, the conjugated double bond system, and the ortho-methoxy phenolic groups can be identified: In turmeric, curcumin is not the only compound and it presents together with other curcuminoids. Curcumin (a) itself makes up approximately 77% of the total curcuminoid content. It also contains approximately 17% (b) and 3% of demethoxycurcumin (b) and bisdemethoxycurcumin (c) respectively as well as smaller amounts of cyclocurcumin (d) (Figure 6) [35].

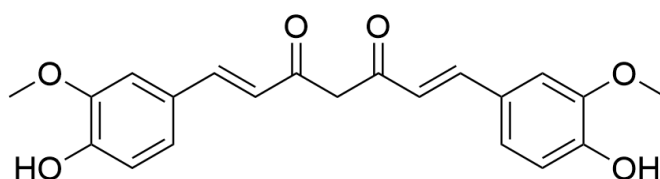


Fig. 5. Chemical structure of curcumin

A notable characteristic of curcumin is its ability to exist in keto–enol tautomeric forms due to the β -diketone group. These two forms are in equilibrium, although the enol form is generally more stable in solution because of intramolecular hydrogen bonding and extended conjugation [10–12]. On the other hand, the diketone form is more commonly observed in the solid state and under acidic or neutral conditions, while alkaline conditions tend to favour the enol form (Figure 7).

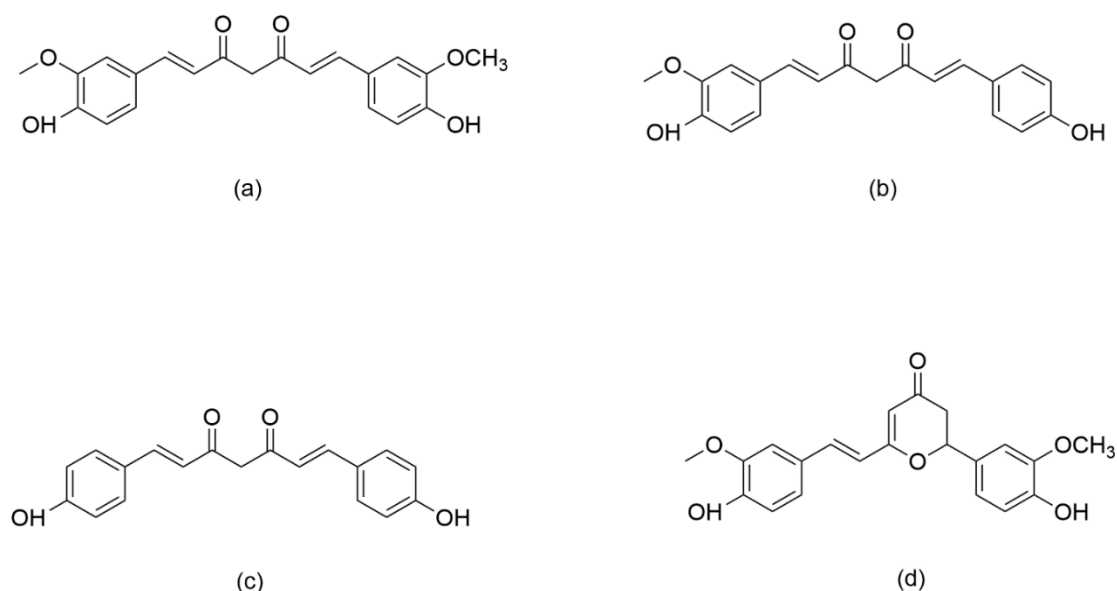


Fig.6. Molecular structures of curcuminoids. (a) Curcumin, (b) Demethoxycurcumin, (c) Bisdemethoxycurcumin, (d) Cyclocurcumin [35]

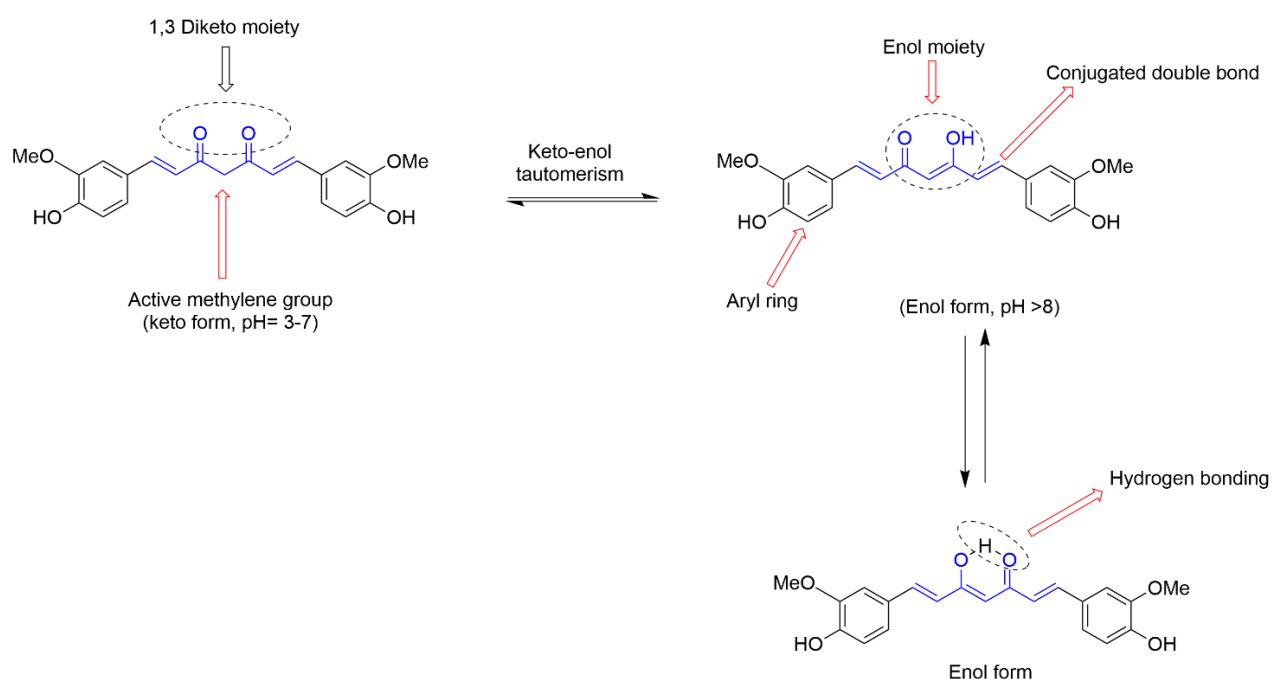


Fig. 7. Structural keto-enol tautomerism of curcumin [35]

1.2.4 Synthetic Approaches of Curcumin

Although curcumin shows valuable biological activity, it also has several well-known limitations, such as poor water solubility, low stability, and unfavourable pharmacokinetic properties [13]. In addition, the exact nature of its active pharmacophore is not yet fully understood. These factors caused that researchers start to explore different strategies for structural modification and optimisation.

The α,β -unsaturated 1,3-diketone moiety plays an important role in curcumin's biological activity, mainly because it can act as a Michael acceptor. At the same time, this structural feature is associated with pan-assay interference compound (PAINS) behaviour, which may lead to non-specific interactions in biological assays [10]. Other reactive parts of the molecule include the active methylene group, the conjugated linker, and the substituted aromatic rings. These regions are often used for chemical modification (Figure 8) [14].

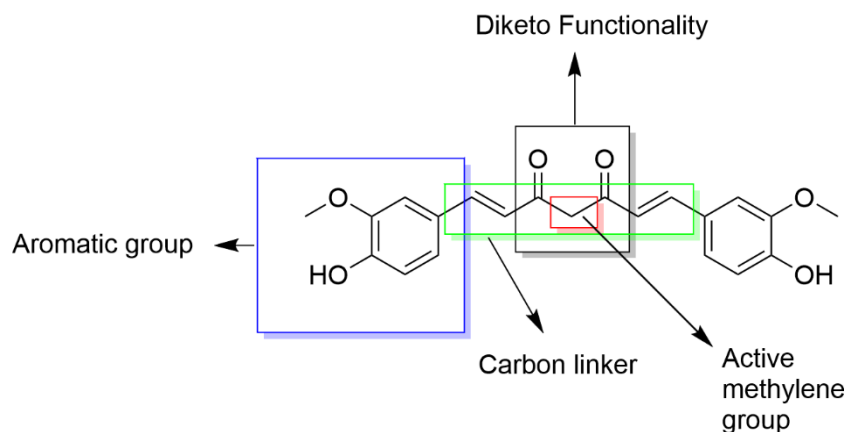


Fig. 8. Molecular structure of curcumin with key functional groups

Different methods have been investigated to improve the stability and biological performance of curcumin such as modifying or protecting phenolic and enolic hydroxyl groups, altering oxidation-sensitive sites, and introducing heterocyclic structures. The main idea of these modifications is to improve pharmacokinetic and pharmacodynamic properties, improve selectivity, and as a result, reduce problems such as resistance and recurrence in cancer treatment [16-18].

Extraction of curcumin from natural sources is often inefficient and causes low yields, which make difficult large-scale production. For this reason, synthetic methods have been developed.

One of the common methods was investigated by Babu and Rajasekharan (1994), which resulted in curcumin as an orange-yellow compound with a relatively high yield. The idea of this method is the condensation of substituted phenolic aldehydes with acetylacetone in the presence of boric acid in dry N,N-dimethylformamide. This is followed by treatment with 1,2,3,4-tetrahydroquinoline, tributyl borate, and glacial acetic acid. (Figure 9) [15].

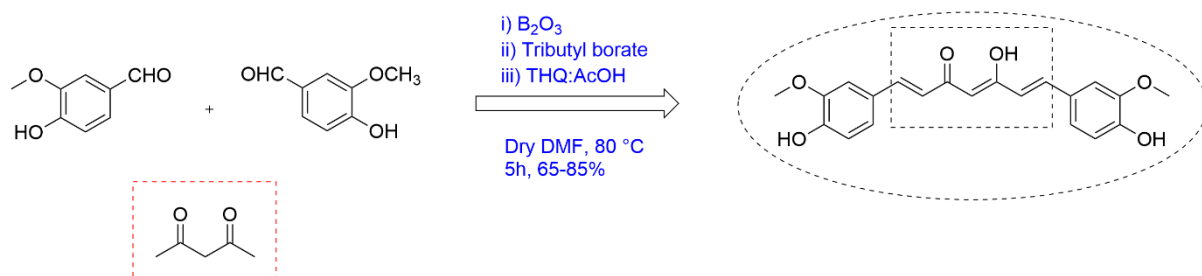


Fig. 9. Schematic of natural curcumin synthesis [35]

The 1,3-dicarbonyl moiety of curcumin has been known as an effective modification site for the introduction of heterocyclic groups, which can improve the stability and improve the anticancer activity of its derivatives. However, challenges such as first-pass metabolism and issues related to

PAINS behaviour are still significant, and more investigation is needed to develop improved curcumin-based systems.

1.3. Liposomes

1.3.1 Drug Delivery Systems (DDSs)

Nowadays, the role of drug delivery systems in pharmaceutical sciences is significantly important particularly in cancer study, as it directly influences the therapeutic efficacy and safety of drugs. Drug delivery systems (DDSs) are designed to transport active pharmaceutical compounds to their target site by using nano-scale carriers. These systems help improve the performance of free drugs by enhancing their pharmacokinetic behaviour, distribution within the body and at the same time also minimising their limitations.

There are many bioactive compounds in the nature with various pharmacological activities. Some of them have wide range of beneficial therapeutic effects such as anticancer activity, tumour-targeting potential, antioxidant, anti-inflammatory, and cardioprotective properties. However, their clinical application is often limited by their physicochemical and biological properties, such as poor water solubility, low stability, rapid degradation, limited bioavailability and their sensitive to different environmental degradation such as oxidation, enzymes, pH, temperature, etc. These challenges make it difficult to deliver sufficient drug concentrations to target tissues and as a result their therapeutic effectiveness significantly decreases.

To address these challenges, a variety of nanocarrier systems have been investigated in pharmaceutical research, such as liposomes, exosome, noisome, cubosome, phytosome polymeric nanoparticles, nano emulsions, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs). Each of these systems has different advantages depend on the nature of the drug and components and their application. For instance, lipid-based nanocarriers such as SLNs and NLCs have attracted many attention due to their biocompatibility, ability to encapsulate both hydrophilic and lipophilic compounds, as well as their potential to improve drug stability and bioavailability. The main advantage of nanocarrier-based delivery systems is to remove some limitations of formulations by protecting sensitive compounds from environmental degradation, and improve pharmacokinetic profiles, and finally facilitating targeted delivery to diseased tissues. However, these delivery systems can also have some limitations and challenges.

1.3.2 Liposomes as nanocarriers for targeted drug delivery

Liposomes were first discovered in the 1960s by the British haematologist Alec D. Bangham and his colleagues at the University of Cambridge [41]. Liposomes are spherical colloidal vesicles that consist of an aqueous core which enclosed within one or more biocompatible lipid bilayers. This structure allows to formulate both hydrophilic and lipophilic compounds, which makes them suitable and unique for delivering a majority of therapeutic agents. These include not only small drug molecules but also larger biomolecules such as proteins, DNA, microRNA, and siRNA. The internal aqueous phase has an important role in protecting sensitive molecules such as astaxanthin from enzymatic degradation and from rapid dilution in the bloodstream before reaching the target site. At the same time, the lipid bilayer interacts with biological membranes, which can improve cellular uptake and facilitate intracellular delivery. Another important role of liposomes is the possibility to adjust their physicochemical characteristics, such as particle size, surface charge, and lipid composition with different methods. This kind of modifications can influence their reaction in the body, particularly

their biodistribution. As a result, liposomal formulations often show lower systemic toxicity and, in some cases, improved therapeutic performance compared with conventional free drugs [41].

In figure 10, functionalised liposomes help to deliver bioactive compounds more directly and efficiently to cancer cells. In this type of system, ligands are added to the surface of the liposome so that it can interact with receptors found in higher amounts on cancer cell membranes. This binding can help the liposomes gather closer to tumour cells and can improve their entry into the cells through receptor-mediated endocytosis. Once the liposome reaches the cancer cell, the encapsulated compound is released close to or inside the cell, which increases the local concentration of the active substance at the target site. For hydrophobic compounds such as astaxanthin and curcumin which has some limitations to use, this type of delivery system is useful because it improves their dispersibility, protects them from degradation, and improves their potential anticancer effect while limiting unnecessary exposure to healthy tissues.

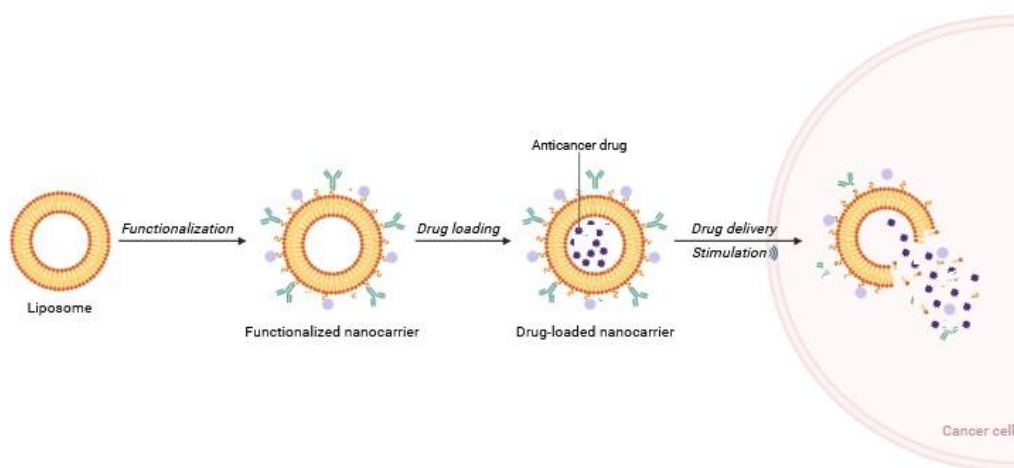


Fig. 10. Functionalised liposomes as nanocarriers for targeted delivery of anticancer compounds (created by BioRender.com)

The liposomal structure consists of one or more lipid bilayers (lamellae) arranged around an internal aqueous core, where the polar head groups face both the inner and outer aqueous phases. This structural organisation provides liposomes with the ability to encapsulate compounds with different physicochemical properties [42]. Hydrophilic compounds are retained within the aqueous core, whereas hydrophobic compounds are located within the lipid bilayer, and amphiphilic molecules position themselves at the interface between the aqueous phase and the lipid membrane (Figure 11).

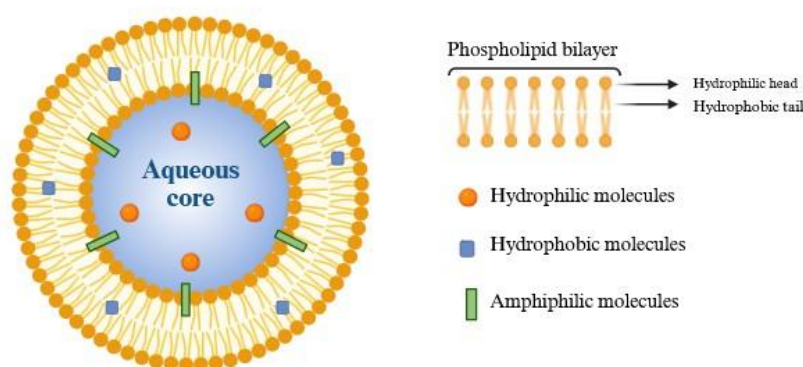


Fig. 11. General structure of liposomes (created by Biorender.com)

1.3.3 Liposome structure

According to lamellarity, they are divided into unilamellar vesicles (ULVs), multilamellar vesicles (MLVs), and multivesicular vesicles (MVVs). ULVs contain a single lipid bilayer and occur over a wide size range. MLVs are typically larger than 500 nm and consist of several concentric bilayers. However, MVVs usually larger than 1000 nm and surround multiple smaller vesicles within one outer membrane [43].

ULVs are also classified by size into small unilamellar vesicles (SUVs, 20-100 nm), large unilamellar vesicles (LUVs, >100 nm), and giant unilamellar vesicles (GUVs, >1000 nm). The single bilayer structure of ULVs allows efficient integration of hydrophilic substances. In contrast, MLVs have a layered structure that is more suitable for accommodating lipophilic compounds. MVVs differ structurally, as they contain several non-concentric internal compartments within a single bilayer, which enables them to hold relatively large amounts of hydrophilic material. The capacity of liposomes to encapsulate compounds depends not only on their size but also on the number of bilayers present, both of which influence loading efficiency [40].

1.3.4 Composition and Physicochemical Properties of Liposomes

Liposomes are mainly composed of glycerophospholipids, which are amphiphilic molecules made of a glycerol backbone, a phosphate group, and two fatty acid chains that can be saturated or unsaturated. The phosphate group may also be linked to different organic groups, which defines several types of natural phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidic acid (PA) [44].

These phospholipids can be of natural or synthetic origin. Natural phospholipids are commonly extracted from sources like egg yolk and soybean, with PC and PE being the most widely used due to their abundance. On the other hand, synthetic phospholipids are obtained through modifications of natural ones. Changes in head groups or fatty acid chains can improve stability. Examples can be DSPC, DPPC, DOPC, DSPG, DPPG, DOPE, and DSPE.

In aqueous systems, phospholipids tend to organise into bilayers because of their amphipathic nature. The hydrophilic heads interact with water, while the hydrophobic tails avoid it, which drives the

spontaneous formation of vesicles. Depending on their composition, liposomes contain one or multiple bilayers, and their surface charge can be neutral, positive, or negative. Their properties depend strongly on structural features such as chain length, saturation, and head group composition [45]. In general, longer and more saturated chains increase membrane rigidity, whereas shorter and unsaturated chains lead to more fluid and flexible membranes.

Cholesterol is often included in liposome formulations to improve membrane stability. It reduces permeability by inserting itself between phospholipid molecules and limiting their movement [45]. This also helps prevent interactions with plasma proteins that could destabilise the vesicles and reduce their effectiveness as drug delivery systems.

Polyethylene glycol (PEG) is another common additive used to extend circulation time. It forms a hydrophilic layer on the liposome surface, which reduces protein adsorption and recognition by the immune system. As a result, PEGylated liposomes remain in the bloodstream longer than conventional ones, which are quickly cleared by the liver and spleen [45, 46].

Liposomes can also be modified with targeting ligands to enhance their specificity, creating so-called targeted liposomes, which are designed for improved drug delivery. The delivery routes of liposomal drug carriers include intravenous, intramuscular, and subcutaneous injections, as well as oral, ocular, pulmonary, and dermal delivery.

1.3.5 Liposome preparation methods

Several conventional techniques are available for the preparation of phospholipid vesicles. Among the passive loading approaches, the thin-film hydration method is one of the most widely applied techniques at the laboratory scale. Despite its widespread use, the thin-film hydration method presents certain limitations, primarily related to the use of organic solvents. Trace amounts of these solvents may remain in the lipid or aqueous phase during preparation, which could potentially lead to toxicity after administration [47]. To overcome and address this issue, several alternative preparation methods such as direct sonication techniques, the detergent removal method, and heating-based preparation methods, have been developed that minimise or eliminate the use of organic solvents. It should also be noted that the method used for vesicle preparation can significantly influence the encapsulation efficiency of bioactive compounds. Some conventional techniques have showed higher loading capacities compared with others [48]. For instance, reverse-phase evaporation, the ether injection or vaporisation technique, and the freeze-thaw method [40] have been reported to achieve improved encapsulation efficiencies, particularly for hydrophilic molecules. Nevertheless, it is widely accepted that the selected preparation method should be capable of producing phospholipid vesicles with suitable physicochemical characteristics in a simple, reproducible manner and with the minimum number of processing steps. As the liposomal formulations of astaxanthin and curcumin were optimised by the thin-film hydration method in the present study, this preparation technique is discussed in more detail in the following section.

1.3.5.1 Thin film hydration method

The thin-film hydration method is one of the most widely used techniques for the preparation of multilamellar vesicles (MLVs) and remains popular because its simplicity to prepare without specialised equipment requirements. In this method, phospholipids are first dissolved in an organic solvent or combination of different organic solvents which methanol and chloroform in this project used and subsequently deposited as a thin lipid film on the inner surface of a round-bottom flask after

solvent removal by rotary evaporation (Heidolph) under reduced pressure. Then the prepared lipid film is hydrated with an aqueous medium under continuous agitation, which promotes the self-assembly of phospholipid molecules and the formation of vesicular structures [49].

This method typically produces multilamellar vesicles composed of several phospholipid bilayers separated by aqueous layers. These kind of structures enable the incorporation of both lipophilic and hydrophilic compounds. In most cases, the active substances are introduced through passive loading during the preparation process. Lipophilic molecules are usually dissolved in the organic phase together with the phospholipids and become incorporated into the lipid bilayer, whereas hydrophilic molecules are dissolved in the aqueous phase used during the hydration step and are entrapped within the aqueous compartments of the vesicles.

The hydration step is a critical and main stage in liposome formation. It is generally performed at a temperature above the phase transition temperature of the phospholipid used, which facilitates bilayer formation and improves the encapsulation of active compounds. The hydration time can be different and it depends on the phospholipid composition; however, sufficient mixing or agitation is required to ensure complete lipid hydration. The selection of the hydration medium is also important and depends on the intended biological application of the vesicular system. Distilled water, buffer solutions, saline, and sugar [50].

During the hydration process, phospholipid molecules can organise into different structural arrangements depending on the lipid composition and the characteristics of the hydration medium. The physicochemical properties of the phospholipids that can be charge and hydration capacity, significantly influence vesicle formation and stability. In some cases, specific phospholipids can form gel structures in solutions with low ionic strength or tend to aggregate during hydration. Consequently, the vesicular suspension which obtained after hydration is often heterogeneous with respect to particle size and lamellarity [51]. Therefore, additional processing steps are usually required to improve the physicochemical properties of the system. Commonly applied techniques for controlling vesicle size and distribution include sonication, membrane extrusion, and high-pressure homogenisation.

The thin-film hydration method has been used to encapsulate a wide range of bioactive compounds which include lipophilic molecules such as docetaxel, paclitaxel, quercetin, resveratrol, curcumin and astaxanthin as well as hydrophilic molecules such as proteins and siRNA. For instance, Jeon et al. developed a theranostic vesicular system by incorporating a gold layer onto the liposome surface to enable further functionalisation with polyethylene glycol (PEG). The results demonstrated good stability under both *in vitro* and *in vivo* conditions and allowed monitoring of vesicle biodistribution through imaging techniques [50].

Despite the simplicity of the thin-film hydration method, it shows several limitations such as relatively low encapsulation efficiency for hydrophilic compounds, limited control over particle size distribution, and difficulties in completely removing organic solvents which cause toxicity. In addition, these challenges make large-scale production difficult, particularly under Good Manufacturing Practice (GMP) requirements which must follow in pharmaceuticals, and it requires strict standardisation, monitoring, and validation of each manufacturing step.

1.3.6 Challenges of liposomes formulation

As discussed in the previous sections, liposomes play an important role in drug delivery systems, and can significantly remove some limitations to deliver the drug to the desired anatomical regions. Recent research has increasingly focused on the use of liposomes as carriers for antioxidant

compounds, with many studies reporting improved delivery performance. Encapsulation within liposomal structures has been associated with enhanced absorption and more stable release behaviour over time [52, 53]. In addition, modifying the surface of liposomes can help them reach specific target areas more effectively, which may lead to better therapeutic results [53].

Despite of the advantages of using liposomes, they have several challenges that limit the clinical efficiency of liposomal drug delivery systems. One of the main issue is its low proportion of the administered dose that reaches the target site, which is often reported to be less than 5% following systemic administration [54, 55]. Although surface modification with targeting ligands has shown potential in experimental models, these approaches have not consistently improved targeting in clinical applications. Immunological responses also is another challenge, as certain liposomal formulations can activate the complement system and cause adverse reactions [56]. Repeated administration, particularly of PEGylated liposomes, may lead to accelerated blood clearance and reducing their circulation time [57].

Stability is another critical limitation which explained in results and discussion. Liposomes are prone to physical and chemical instability, such as fusion and aggregation over time, particularly in aqueous environments. These issues become more noticeable when sensitive biomolecules, such as proteins or nucleic acids, are encapsulated. As a result, they must be stored at controlled temperatures, which make the whole process more complex and expensive [58]. According available studies, liposomal formulations have proved important clinical benefits in many applications; however their development and large-scale application in pharmaceutical industry are still challenging [55].

1.3.7 Research Gap and Justification of the Study

Recent research has started to study more at combining different bioactive compounds in one unique system to improve their overall effect. Nanocarrier systems such as liposomes have shown potential for delivering more than one active compound at the same time. These systems can improve overall effectiveness and provide a more controlled release compared to single-compound formulations [59, 60]. However, most of the available studies still focus either on traditional drug combinations or on single antioxidants and their possible combined effects are still not clearly understood. Curcumin, astaxanthin and liposomes have each been widely studied on their own. Even so, there is still a lack of research where all three are combined in one formulation. In particular, studies on co-encapsulation and optimisation of formulation conditions are limited. Information about important properties such as particle size, polydispersity index, zeta potential and encapsulation efficiency is also not sufficient.

Another issue that appears frequently in recent studies is related to stability. Although liposomes can improve the delivery of hydrophobic compounds, keeping them stable during storage is still difficult. Several laboratory stability studies conducted up to six months have proved that liposomes particularly those which contain sensitive bioactive compounds such as astaxanthin, still face considerable stability limitations.

According to Chang et al. [61], liposomal astaxanthin showed acceptable stability initially; however, their results proved that over time the stability of liposomal astaxanthin decreased and the reason explained as astaxanthin degradation and changes in the liposomal membrane. Similar observations, including leakage of encapsulated compounds and particle instability, have also been described in other studies [62]. This shows that stability remains one of the main challenges for these systems.

Both curcumin and astaxanthin have shown promising antioxidant and therapeutic effects, but their use is still limited because of low solubility, poor bioavailability and instability [19, 34]. Liposomes may help to overcome some of these problems by improving stability and absorption, as well as allowing a more controlled release. Combining both compounds in a single system may improve their overall effect. Based on this idea, the present study aims to prepare liposomal formulations containing curcumin and astaxanthin. These formulations will be optimised and then characterised by measuring parameters such as particle size, polydispersity index, zeta potential and encapsulation efficiency. The obtained results could provide useful information for developing more stable delivery systems for hydrophobic compounds.

2. Materials and methods

2.1. Materials

The following chemicals were used to prepare the liposome formulation:

Astaxanthin ($\geq 88\%$, Roth GmbH, Germany), curcumin (BLD Pharm, 98%, Germany), cholesterol (Roth GmbH, Germany), liopoid S100 (Lipoid GmbH, Germany), 1- α -phosphatidylcholine (egg yolk, $\sim 60\%$ (TLC), Sigma-Aldrich), tween 20 (Merck, Germany), tween 80 (Merck, Germany), methanol (Sigma-Aldrich), ethanol (96%, Vilniaus Degtinė, Lithuania), chloroform (Sigma-Aldrich), L-lysine monohydrochloride (CAS No: 657-27-2), olive oil (Roth GmbH, Germany), phosphate buffered saline (Sigma-Aldrich, USA), maltodextrin (Sigma-Aldrich, USA), trehalose (BLD Pharm, Germany), PEG (Sigma-Aldrich, USA), water (prepared by using a Millipore system, Rahway, Merck, USA), acetic acid (Sigma-Aldrich, USA), ammonium acetate (Sigma-Aldrich, USA).

1.4 Experiment

As discussed in the previous chapter, different methods can be used for the preparation of liposomal formulations. Based on previous published studies on liposomal curcumin and astaxanthin, the ethanol injection method and thin-film hydration method were selected for formulation development in this project. Initially, some main factors such as type of suitable phospholipids and determining their appropriate concentration and ratio were investigated. These factors can significantly influence vesicle formation, encapsulation efficiency, particle size, and formulation stability [63].

Preliminary formulations were first prepared by using ethanol injection method according to previously reported studies. However, based on the obtained experimental results and physicochemical characteristics of main compounds, the preparation method was later changed to the thin-film hydration method and the final liposomal formulation optimised and developed.

1.4.1 Pre-formulation studies

Pre-formulation studies are an important step in this project. In this stage, the main bioactive compounds which are astaxanthin and curcumin, are evaluated in terms of quality to better understand their physicochemical characteristics. This helps to build a clearer picture of how these compounds behave under different circumstances. This stage includes physical appearance, solubility, λ max determination, Calibration curve, FTIR Characterisation of Active Compounds. Among these parameters, solubility is one of the most important factors in liposomal formulations. Both compounds have low aqueous solubility, which can significantly affect drug loading, encapsulation efficiency, as well as stability of the liposomes. Therefore, a clear understanding of the behaviour of the main compounds is required.

1.4.2 Physical appearance

According to the European Pharmacopeia (EP) and Food Chemicals codex (FCC) the physical appearance of astaxanthin and curcumin were analysed by visually inspection of size, shape, colour and appearance and compared with standard.

1.4.3 Solubility analysis

A solubility study was conducted for both astaxanthin and curcumin to evaluate their behaviour in different solvents before liposomal formulation. This step was essential, as solubility directly influences drug loading capacity, encapsulation efficiency, and overall stability of liposomes. The solubility of astaxanthin and curcumin was determined by dissolving 10 mg of each in small amount of solvent in the test tube. The solubility of both compounds was tested in water, ethanol, methanol, Dimethyl sulfoxide (DMSO) and chloroform. Also, solubility of astaxanthin and curcumin was evaluated in olive oil.

1.4.4 λ max determination

According to previous studies, different wavelengths have been reported for astaxanthin and curcumin. To determine the maximum absorption wavelength in this study, solutions of astaxanthin and curcumin at a concentration of 4 ppm ($\mu\text{g/mL}$) were prepared in chloroform: methanol (2:1) and ethanol, respectively, and scanned using UV-Vis spectroscopy (Shimadzu, UV-3600 plus) over the range of 200-800 nm.

1.4.5 Calibration curve

Calibration curve of astaxanthin and curcumin was developed by UV-Spectrophotometer (Shimadzu, UV-3600 plus) in 429 nm for curcumin and 489 nm for astaxanthin. To development of the calibration curve, astaxanthin standard solutions at concentrations of 2, 4, 6, and 8 $\mu\text{g/mL}$ were prepared in chloroform: methanol at a ratio of (2:1), and curcumin standard solutions at the same concentrations were prepared in ethanol.

1.4.6 Identification test by FT-IR spectrophotometer

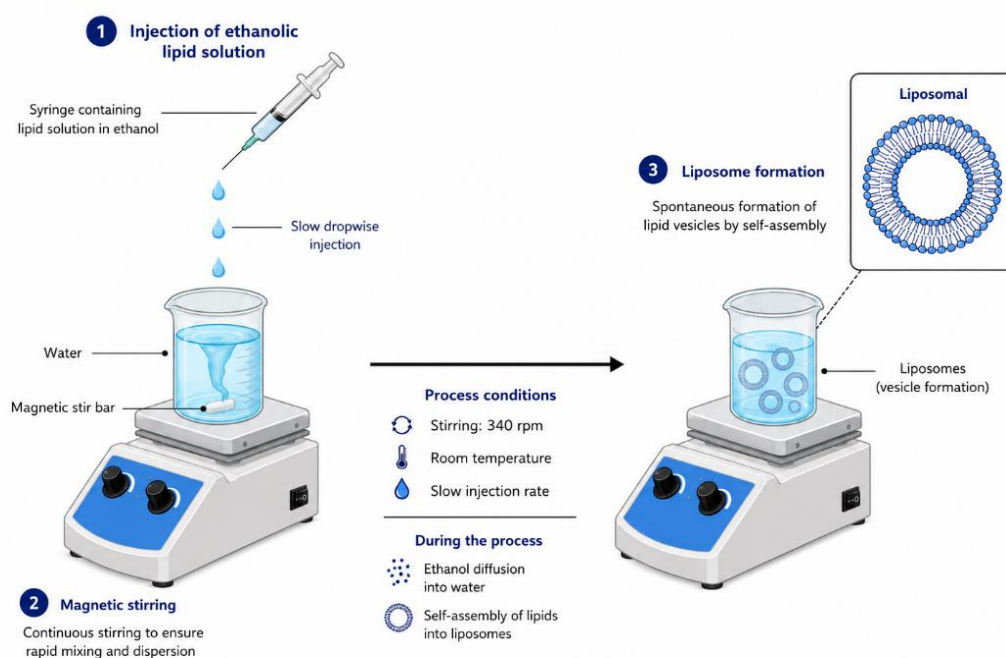
Fourier Transform Infrared (FTIR) spectroscopy was carried out as part of the pre-formulation studies to evaluate the chemical structure of the bioactive compounds. This technique can help to identify of characteristic functional groups present in astaxanthin and curcumin by analysing their specific absorption bands. The FT-IR attenuated total reflectance (ATR) spectra were recorded by using a Frontier spectrophotometer (Perkin Elmer, USA). A small amount of the sample was pressed against the diamond crystal plate, and the spectrum was recorded in the wavenumber from 4000 cm^{-1} to 560 cm^{-1} . The number of scans 6, resolution 4 cm^{-1} . The data was processed using the Spectrum software.

2.2.3 Preparation of astaxanthin and curcumin loaded liposomes by solvent injection method

Astaxanthin and curcumin loaded liposomes were prepared by solvent injection method as shown in figure 1 . Firstly lipids dissolved in ethanol then APIs added. The obtained solution is stirred for about 30 minutes with magnetic stirrer at a temperature of $45\text{ }^{\circ}\text{C}$ and a stirring speed of 340 rpm until all substances dissolve. 10 ml of resulting solution is drawn into the syringe and dropwise into the 50 ml of water and stirring with a magnetic stirrer speed of 560 rpm. The obtained solution is subjected to ultrasound homogenization using a ultrasonic homogenizer with a titanium probe at 32% power, performing 5 cycles which include (1 minute sonification, 1 minute rest). The composition of the liposomal formulations prepared by the ethanol injection method is presented in table 3. The overall procedure of liposome preparation by the ethanol injection method is illustrated in figure 12.

Table 1. Composition of liposomal formulations prepared by the ethanol injection method

Ingredient	Quantity				
	Formulation code				
	F1	F2	F3	F4	F5
Astaxanthin (mg)	2.6	2.2	2.5	2.6	2.3
Curcumin (mg)	2.8	2.6	2.4	2.6	2.7
Soy phospholipid (mg)	1651	-	408	-	-
L- α -Phosphatidylcholine (mg)	-	1682	-	445.3	416.6
Cholesterol (mg)	-	-	45	48	46
Tween 80 (mg)	25	37	-	-	-
Tween 20 (mg)	-	-	28	29	-
Ethanol 96% (ml)	25	25	25	25	25
Water (mL)	50	50	50	50	50

**Fig.12 .** Schematic representation of solvent (ethanol) injection method
(created by BioRender.com)

2.2.4 Preparation of astaxanthin and curcumin loaded liposomes by thin film hydration method

Liposomal containing astaxanthin and curcumin were prepared according to the thin film hydration method as shown in figure 2. In this method L- α -Phosphatidylcholine, cholesterol, astaxanthin, curcumin were dissolved in organic solvent chloroform: methanol mixture with ratio of 2:1. Polyethylene glycol (PEG) and tween 20 were added in separate formulations to the prepared solution and mixed for 1 hour. The organic solvents were evaporated by rotary evaporator (Heidolph) under

vacuum and 40 °C to form a thin film of liposome. The dried liposomal astaxanthin and curcumin hydrated with water or buffer and mixed for 45 minutes then vortexed for an hour. Acetate buffer (pH 4.2) was prepared by dissolving 7.7 g of ammonium acetate in water. 8 mL of acetic acid is added, and diluted to 100 mL with distilled water. Phosphate buffer saline (PBS, pH 7.3) was prepared by dissolving one PBS tablet in 200 mL of distilled water. To reduce particle size and improve homogeneity the liposomal dispersion was sonicated by with power at 32% and performing 10 cycles include 30 seconds sonification and 30 seconds rest (Figure 13). After this stage, to perform lyophilization, 2 g of cryoprotective excipient was added to the liposomal dispersion and mixed for 30 minutes by stirrer. Firstly, maltodextrin was selected as a cryoprotective excipient; however, based on the obtained results, trehalose was selected for the liposomal astaxanthin and curcumin. The composition of the liposomal formulations prepared by the thin film hydration method is presented in table 2.

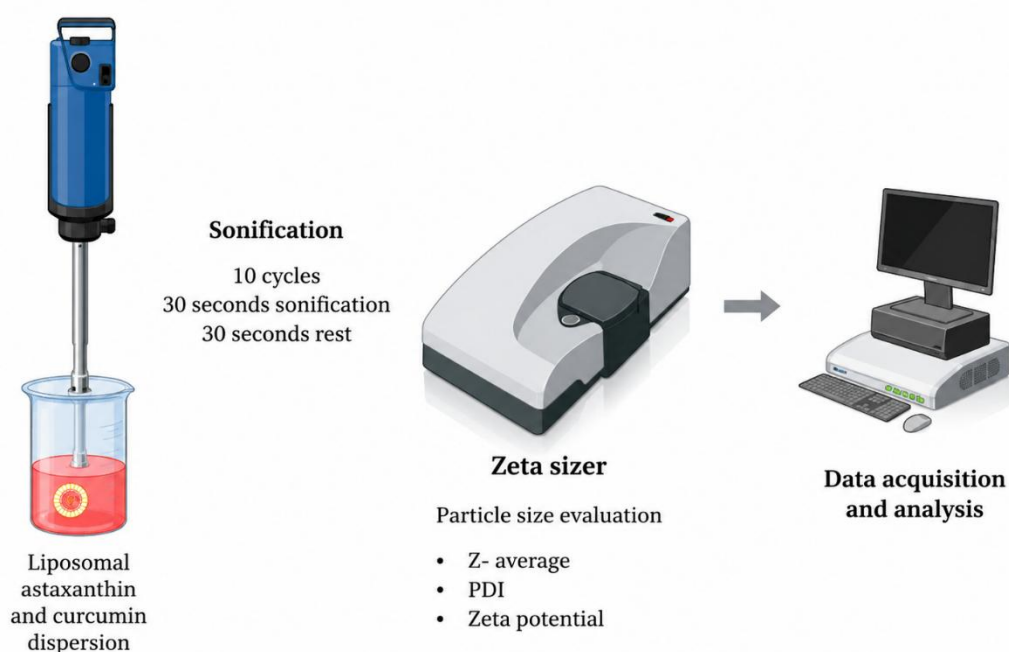


Fig. 13. Schematic of sonication and zeta potential analysis of liposomal formulations (created by BioRender.com)

Table 2. Composition of liposomal formulations prepared by thin-film hydration method

Ingredient	Quantity Formulation code				
	F6	F7	F8	F9	F10
Astaxanthin (mg)	2.24	2.11	2.78	9.47	9.32
Curcumin (mg)	2.06	2.43	2.54	8.84	9.48
L- α -Phosphatidylcholine (mg)	202.9	215.9	210.1	832.8	842.2
Cholesterol (mg)	20.7	20.9	22.6	89.8	93.4
Tween 20 (mg)	33.5	38.6	30.3	-	125.8
Polyethylene glycol (PEG) (mg)	-	-	29.8	118.8	-
Acetate buffer (mL)	-	40	-	-	-
Phosphate buffer (mL)	40	-	-	-	-
Water (mL)	-	-	20	80	80
Chloroform: methanol (2:1) (mL)	15	15	15	60	60

2.2.5 Freeze-Drying (Lyophilization) of astaxanthin and curcumin loaded liposomes

Spray-drying and freeze-drying (lyophilisation) are two common methods to convert liposomal dispersions into the dry powder [64-66]. Several studies have reported the use of spray-drying for the preparation of curcumin-loaded liposomal powders due to its simplicity and rapid processing [66, 67]. However, astaxanthin is highly sensitive to temperature which makes the use of spray-drying unsuitable for this experiment. As a result, freeze-drying was selected in the present study as a more suitable method for preserving the stability and bioactivity of astaxanthin and curcumin loaded liposomal formulations. In freeze-drying, excipients play an important role by maintaining liposome stability and particle size during freezing and drying. Amorphous excipients protect liposomes by forming a glassy structure, while crystalline excipients improve the strength and appearance of the lyophilised cake [68].

In this project, trehalose, maltodextrin, and sucrose were selected separately in individual formula as excipients for the freeze-drying of liposomal formulations because of their recognised stabilising properties and regulatory acceptance.

The prepared liposomal formulations which contain astaxanthin, curcumin and excipient were first transferred into freeze-drying plates and spread evenly to obtain a uniform layer. The samples were then placed in a lyophiliser (Telstar LyoQuest, Spain). Freeze-drying was carried out over three days. In the first step, the samples were frozen at $-60\text{ }^{\circ}\text{C}$ for 24 hours to ensure complete solidification. After freezing, the drying step was performed under reduced pressure (0.1 mbar) for 48 hours that during this stage, water was gradually removed.

2.3 Evaluation and characterization of formulated liposome

2.3.1 Average size and Polydispersity index (PDI) evaluation:

Z-average and polydispersity index (PDI) of liposomal astaxanthin and curcumin was analysed by a Malvern Zetasizer Nano ZS (Malvern Instruments) to determine the size distribution and homogeneity of the vesicles. The average particle size provides information about vesicle diameter and the PDI shows the uniformity of particle distribution [69]. A volume of 40 µL of the liposome solution is added to cuvette and diluted with 5% ethanol solution to a total volume of 2 mL. analysis was carried out at 25 °C.

2.3.2 Zeta potential evaluation:

The zeta potential of the liposomal formulations was determined by using a Malvern Zetasizer Nano ZS (Malvern Instruments) at 25 °C to evaluate the surface charge and stability of the vesicles. The zeta potential value provides the information about electrostatic repulsion between particles and has a important role in colloidal stability [70]. A volume of 40 µL of the liposome solution is added to cuvette and diluted with 5% ethanol solution to a total volume of 2 mL. analysis was carried out at 25 °C.

2.3.3 Drug encapsulation efficiency

The encapsulation efficiency of astaxanthin- and curcumin-loaded liposomes was determined by UV-spectroscopy (Shimadzu, UV-3600 plus) to evaluate the amount of bioactive compound entrapped within the vesicles. Encapsulation efficiency is an important parameter to determine the loading capacity and stability of liposomal formulation [71]. The amount of drug in liposomes was determined by multiplying the drug concentration of drug in liposomes by the total volume of liposomes. Encapsulation efficiency (%) was determined by using the equation below:

$$EE(\%) = \frac{\text{Amount of drug in liposome}}{\text{Amount of drug used to formulate liposomes}} \times 100$$

2.3.3.1 Encapsulation efficiency of Astaxanthin

The encapsulation efficiency (%) of astaxanthin was evaluated by UV-spectroscopy at 489 nm by centrifuging the formula at 4000 rpm for 30 minutes. This experiment conducted in triplicate. For stability study, 10 mg of lyophilized micro powder was dissolved in 2 ml of chloroform and methanol (2:1), then centrifuged the obtained solution at 4000 rpm for 30 minutes.

2.3.3.2 Encapsulation efficiency of Curcumin

The encapsulation efficiency (%) of curcumin was evaluated by UV-spectroscopy at 429 nm by centrifuging the formula at 4000 rpm for 30 minutes. This experiment conducted in triplicate. For stability study, 10 mg of lyophilized micro powder was dissolved in 2 ml of ethanol, then centrifuged the obtained solution at 4000 rpm for 30 minutes.

2.3.4 Structural analysis of the liposomal astaxanthin and curcumin

2.3.4.1 Identification test by FT-IR spectrophotometer

Fourier Transform Infrared (FTIR) spectroscopy was carried out as part of the pre-formulation studies to evaluate the chemical structure of samples after lyophilization and detect any possible interactions with excipients. This technique can help to identify of characteristic functional groups present in final formulation by analysing their specific absorption bands. The FT-IR attenuated total reflectance (ATR) spectra were recorded by using a Frontier spectrophotometer (Perkin Elmer, USA). A small amount of the sample was pressed against the diamond crystal plate, and the spectrum was recorded in the wavenumber range from 4000 to 560 cm^{-1} . The number of scans – 6, resolution – 4 cm^{-1} . The data was processed using the Spectrum software.

2.3.4.2 Scanning Electron Microscopy (SEM) analysis

Scanning electron microscopy (SEM) is one of important analytical technique used for the characterisation of liposomal formulations because it provides visualisation of particle morphology and surface structure. This technique provides a better understanding of external characteristics such as particle shape, surface texture, structural integrity, and aggregation behaviour which are important parameters in the assessment of liposomal systems [72]. SEM analysis is particularly useful for lyophilised formulations. It also helps to determine the liposomes maintain their structural stability after the drying process [72].

A small amount of each freeze-dried sample was carefully placed on an aluminium SEM stub. The samples were gently spread to form a thin layer and to reduce excessive particle overlap. The mounted samples were then analysed by using a scanning electron microscope (FEI Quanta 200 FEG) at Kaunas University of Technology.

SEM images were recorded at selected magnifications (1000 $\times$, 6000 $\times$, and 10,000 $\times$) to evaluate the morphology of the formulations. The analysis was performed using the whole-area electron probe scanning mode. Image resolutions of 512 $\times$ 442 and 2048 $\times$ 1768 pixels were applied during image acquisition. The obtained micrographs were used as supporting evidence for the physical characterisation of the liposomal formulations.

2.3.5 Yield

To evaluate the percentage of yield after freeze drying (lyophilization), the total mass of micro powder after freeze drying process weighted and calculated by using the following equation:

$$\%Yield = \frac{\text{Total mass of micro powder after lyophilization}}{\text{Total mass of raw materials in liposomal formulation}}$$

2.3.6 Moisture content

Approximately 1 g of micro powder was taken and the moisture content was determined by using (KERN DBS). This experiment conducted in triplicate and mean value was calculated.

2.3.7 Bulk density

Bulk density of the lyophilised liposomal powder was evaluated by measuring the mass of the obtained powder and recording its occupied volume in a graduated cylinder to assess the physical properties of the powder after freeze-drying [73]. This parameter is important because it can influence powder flowability, handling, and compressibility during further pharmaceutical processing and tablet formulation Bulk density was then calculated as the ratio of powder mass to bulk volume.

2.3.8 Stability studies

Stability studies are an important part of this study, since the stability of liposomes is always challenging. According to previous studies, encapsulation efficiency (EE%) and particle size were the main issues and challenging parts during the optimisation and stability studies. It becomes even harder when sensitive bioactive compounds such as astaxanthin exist in the formulation. In this part of the study, changes in appearance, particle size, and encapsulation were investigated.

3.1 Results and discussion

3.2 Pre-formulation studies

3.2.1 Physical appearance

According to the European Pharmacopeia (EP) and Food Chemicals codex (FCC) the physical appearance of astaxanthin and curcumin were analysed by visually inspection of size, shape, colour and appearance and compared with standard. According to the references, the physical appearance of both main compounds visually checked. Curcumin was found bright and yellow fined powder and astaxanthin was found dark red.

3.2.2 Solubility analysis

The solubility of curcumin and astaxanthin with different solvents was analysed and the result was found to be:

Table 3. Solubility of astaxanthin and curcumin in different solvents

S. No.	Solvent	Astaxanthin	Curcumin
1	Water	Insoluble	Insoluble
2	Ethanol	Soluble but need more time and high temperature	Soluble
3	Methanol	Soluble	Soluble
4	DMSO	Soluble	Soluble
5	Olive oil	Insoluble	Soluble
6	Chloroform	Soluble but not completely	Soluble

Based on the results of pre-formulation study on solubility of astaxanthin and curcumin, among the solvents, DMSO and combination of chloroform and methanol (2:1) were good choices for formulation; however, removing DMSO even with minimum amount of the solvent under vacuum condition and limited temperature was impossible and as a result Chloroform: methanol with ratio of 2:1 has selected for liposomal formulation. This solvent easily evaporated under vacuum condition with 40 °C temperature.

3.2.3 λ max determination

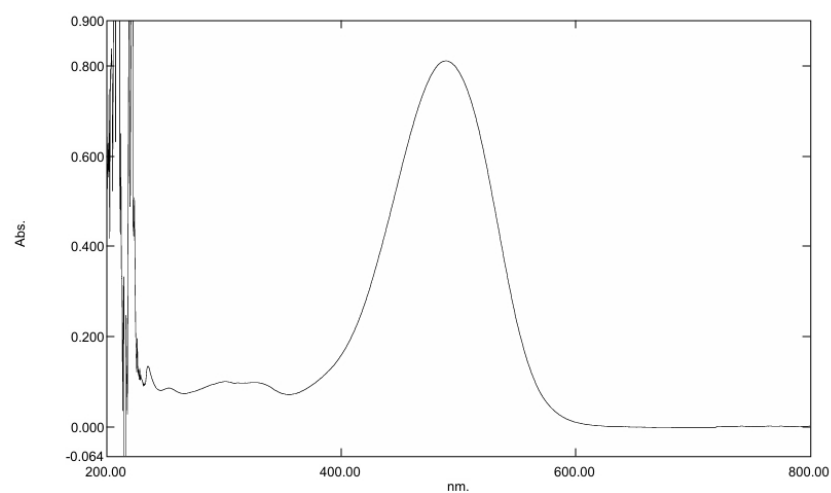
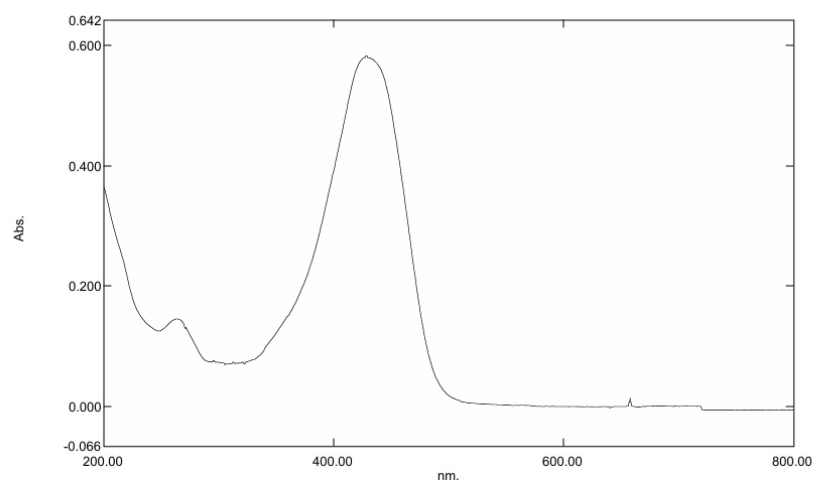
According to previous studies, different wavelengths have been reported for astaxanthin and curcumin. To determine the maximum absorption wavelength in this project, solutions of astaxanthin and curcumin at a concentration of 5 and 4 ppm ($\mu\text{g/mL}$) were prepared in chloroform: methanol (2:1) and ethanol, respectively, and scanned by using UV-Vis spectroscopy over the range of 200-800 nm. The λ max of astaxanthin was found to be 489 nm and curcumin was found to be 429 nm. The λ max analysis data are shown separately for both compounds in table 4 and 5. Moreover, corresponding graphs are presented in figures 14 and 15.

Table 4. λ max of determination of astaxanthin

S. No.	Wavelength	Abs.
1	775.50	0.003
2	742.00	0.002
3	489.00	0.811
4	324.50	0.099
5	302.00	0.101
6	253.50	0.087
7	235.00	0.135

Table 5. λ max of determination of curcumin

S. No.	Wavelength	Abs.
1	658.00	0.013
2	428.50	0.583
3	264.00	0.145
4	721.00	-0.007
5	641.00	-0.003
6	306.00	0.069
7	247.50	0.125

**Fig. 14.** λ max of astaxanthin**Fig. 15.** λ max of curcumin

3.2.4 Calibration curve

Standard calibration curves of astaxanthin and curcumin were developed by UV-vis Spectrophotometer in 429 nm for curcumin and 489 nm for astaxanthin. Chloroform: methanol with a ratio of (2:1) for astaxanthin and ethanol for curcumin were used as a blank respectively. The absorbance for astaxanthin at concentration of 2,4,6,8 ppm were 0.273, 0.487, 0.790 and 0.989. The absorbance for curcumin at same concentration were 0.225, 0.459, 0.665 and 0.943. The linear equation for astaxanthin was $y = 0.1226x + 0.022$, with an R^2 value of 0.9937 and for curcumin $y = 0.118x - 0.017$ with an R^2 value of 0.9965 was obtained. In these equations, y represents the absorbance and x represents the concentration.

3.3 Evaluation of liposomal formulations prepared by the ethanol injection method

3.3.1 Average size and Polydispersity index (PDI) evaluation

Z-average, PDI, and zeta potential measurements were performed to evaluate the ethanol injection method. The particle size analysis showed significant differences between the prepared liposomal formulations which obtained by the ethanol injection method (Table 6). Formulations F4 and F5 indicated nanosized vesicles with a z-average of $86.0 \text{ nm} \pm 1.2\%$ and $87.0 \pm 1.1\%$, respectively. Also, both formulations had low PDI values $0.133 \pm 0.9\%$ for F4 and $0.125 \pm 0.8\%$ for F5, which indicate a homogeneous particle distribution and formation of stable nanoliposomes (Figure 16). The narrow size distribution which observed in these formulations, shows that the combination of phosphatidylcholine, cholesterol, and Tween 20 can have impact on the particle size to improved vesicle stability and reduced aggregation. Cholesterol can improve membrane rigidity and structural integrity, while Tween 20 provide steric stabilisation and improve dispersion uniformity.

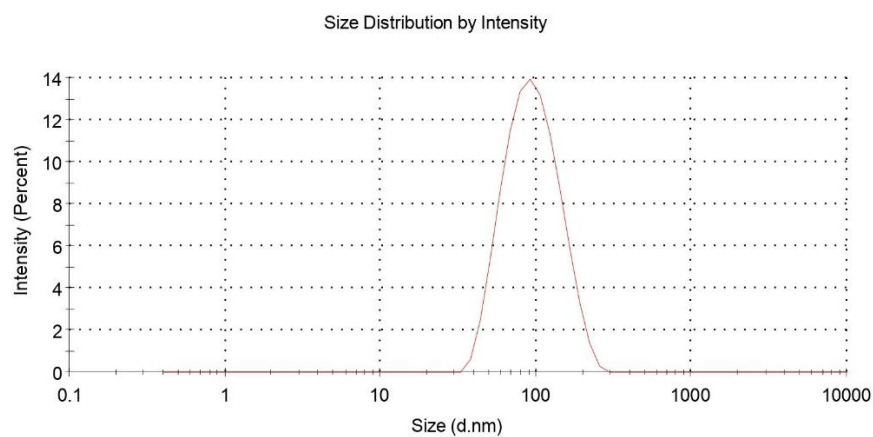
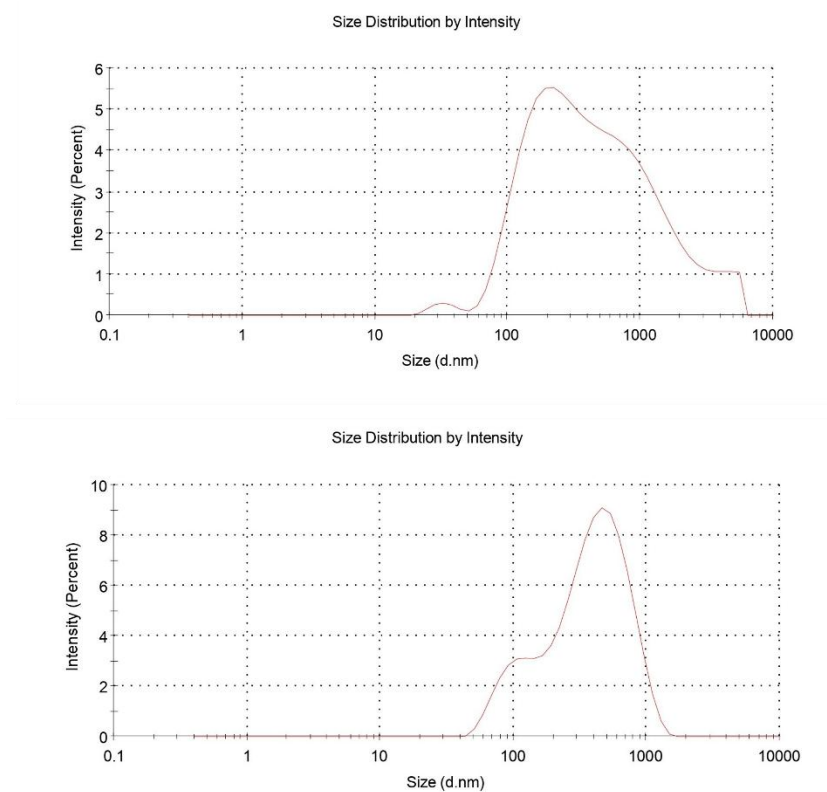
On the other side, formulations F1 and F2 showed larger particle sizes with a z-average of $280.1 \text{ nm} \pm 1.5\%$ and $273.5 \text{ nm} \pm 1.8\%$, respectively. In addition, both formulations had high PDI values $0.497 \pm 1.2\%$ for F1 and $0.433 \pm 0.9\%$ for F2, respectively. The DLS intensity distribution curves of these formulations showed broad and asymmetric peaks which extend toward larger particle sizes and indicate high heterogeneous particle population. The broad peak shape with long tailing showed the presence of vesicle aggregation, fusion phenomena, or coexistence of particles with different size populations that it is related to unstable liposomal systems (Figure 17). The poorer physical characteristics of F1 and F2 can be related to the absence of cholesterol and the high concentration of Tween 80 or different type of phospholipid, which could disturb proper bilayer formation and promote the formation of mixed micellar structures or aggregated vesicles.

Table 6. Particle size and polydispersity index (PDI)

S. No.	Formulation	Z-average (nm)	Polydispersity index (PDI)
1	F1	$280.1 \pm 1.5\%$	$0.497 \pm 1.2\%$
2	F2	$273.5 \pm 1.8\%$	$0.433 \pm 0.9\%$
3	F3	$145.1 \pm 2.0\%$	$0.279 \pm 0.5\%$
4	F4	$86.0 \pm 1.2\%$	$0.133 \pm 0.9\%$
5	F5	$87.0 \pm 1.1\%$	$0.125 \pm 0.8\%$

Table 7. Zeta potential of ethanol injection method

S. No.	Formulation	Zeta potential (mv)
1	F1	$2.0 \pm 0.9\%$
2	F2	$1.16 \pm 0.6\%$
3	F3	$-4.7 \pm 1.0\%$
4	F4	$-7.38 \pm 0.5\%$
5	F5	$-6.8 \pm 0.5\%$

**Fig. 16.** Particle size distribution of liposomal formulation F4**Fig. 17.** Particle size distribution of liposomal formulations F1 and F2

The zeta potential results supported the observations obtained from the z-average and PDI measurements (Table 7). Formulations with improved nanoscale properties demonstrated negative surface charge values -7.1 mV for formulation F4 (Figure 18). However, formulations with larger particle size and higher PDI showed near-neutral zeta potential values $+2.0$ for formulation F1 (Figure 19). Combination of phosphatidylcholine and cholesterol liposomal systems showed negative zeta potential values, and stable nano formulations containing steric stabilisers are reported the range of approximately -5 to -20 mV. The zeta potential value which observed for F4 and F5 is consistent with relatively stable nanosized liposomal dispersions; however, the positive value of zeta potential measured for F1 and F2 may have reduced electrostatic repulsion between particles, thereby increasing aggregation tendency and contributing to the higher particle size and PDI values. Although the measured zeta potential values were relatively low, the stability of F4 and F5 may still be explained by steric stabilisation provided by the non-ionic surfactants rather than strong electrostatic repulsion alone. The results indicate that lipid composition and surfactant type strongly influenced liposome formation, particle homogeneity, peak distribution characteristics, and colloidal stability in the ethanol injection method.

Based on obtained result, Tween 20 was a more suitable option for liposomal formulation compared to Tween 80. The reason may be related to the different physicochemical characteristics of the surfactants and their influence on liposomal bilayer formation and stability. Formulations containing Tween 20 showed smaller particle size, lower PDI values, narrower DLS peak distribution, and more negative value for zeta potential. On the other hand, formulations prepared with Tween 80 showed larger particle size, broader and asymmetric peak distribution, and higher PDI values which indicate heterogeneous particle populations and aggregation tendency and the reason can be related to the way that each surfactant interacted with the liposomal system.

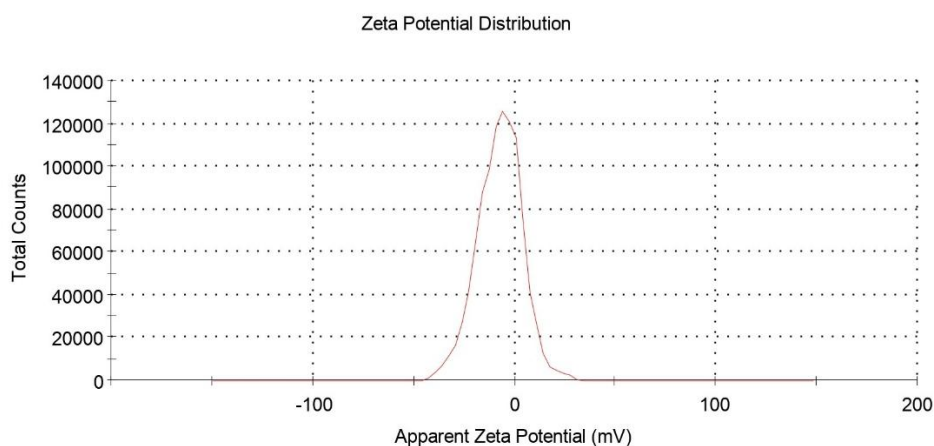


Fig. 18. Zeta potential distribution of liposomal formulation F4

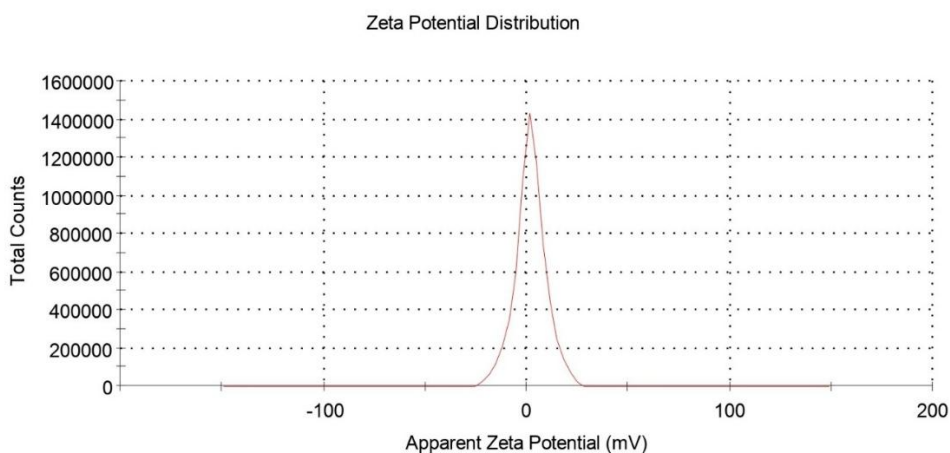


Fig. 19. Zeta potential distribution of liposomal formulation F1

3.3.2 Justification for changing method

The ethanol injection method was initially used as a preliminary formulation to evaluate proper method for formulation of liposomal astaxanthin and curcumin. Moreover, investigation of the proper lipid combination and their influence surfactant ratio on the physicochemical properties of liposome. Although several formulations, particularly F4 and F5, produced nanosized vesicles with almost acceptable particle size distribution, some limitations were still observed regarding colloidal stability and formulation homogeneity. The low zeta potential values indicated weak repulsive forces between the particles, while some formulations also showed wide particle size distribution and particle aggregation. In addition, astaxanthin is a highly hydrophobic compound with limited aqueous solubility, which can affect its uniform incorporation into liposomal bilayers during rapid ethanol injection. As a result, the formulation of liposomal astaxanthin and curcumin shifted to the thin-film hydration method since this method is the more suitable to prepare liposomal bioactive compounds. Thin film hydration method is widely reported to provide improved incorporation of hydrophobic compounds, better lipid film formation, enhanced bilayer organisation, and greater control over vesicle characteristics. After this stage, the thin-film hydration method was selected as a main formulation to improve liposomal stability, homogeneity, and drug incorporation efficiency.

3.4 Evaluation of liposomal formulations prepared by the thin-film hydration method

The liposomal formulations containing astaxanthin and curcumin were successfully prepared by the thin-film hydration method. During rotary evaporation, a thin orange-red lipid film was formed on the inner surface of the flask. After lyophilisation, the formulation formed a dry cake structure, which was then ground into a fine orange-red powder (Figure 20).

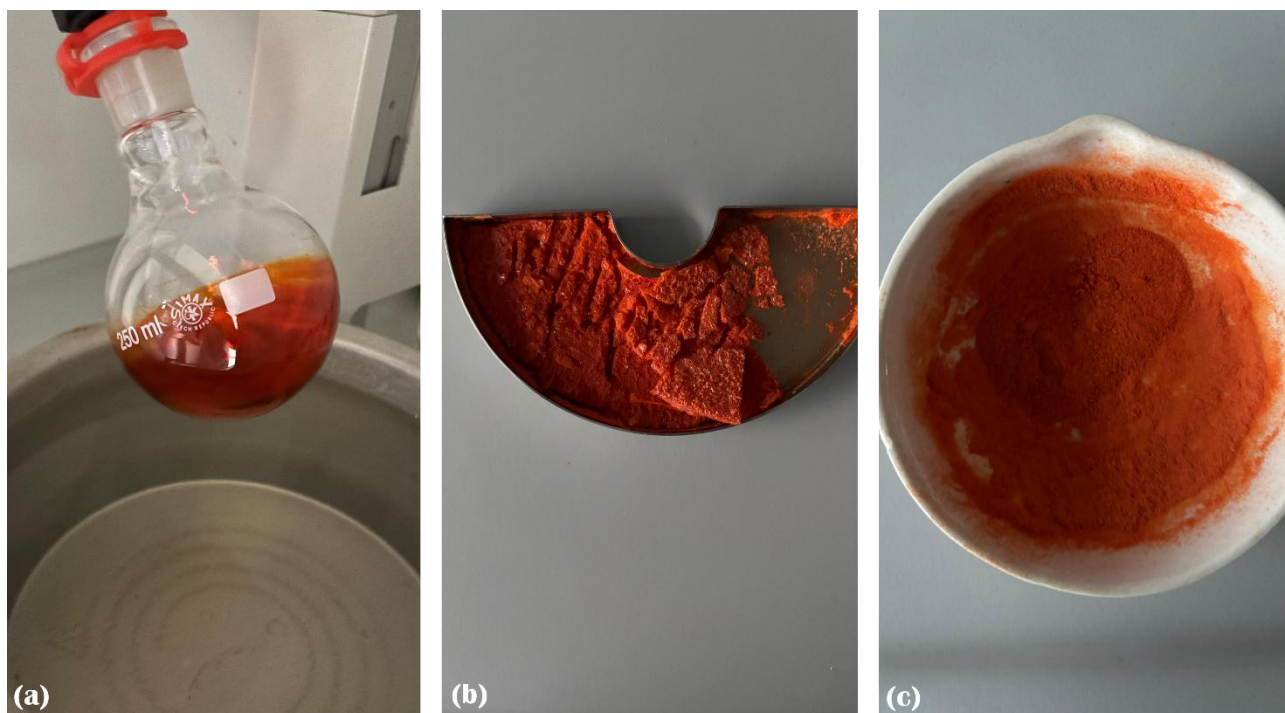


Fig. 20. Preparation of lyophilised liposomal astaxanthin and curcumin formulations: (a) thin lipid film formed after rotary evaporation, (b) freeze-dried liposomal cake, and (c) final powdered formulation after grinding

3.4.1 Average size and Polydispersity index (PDI) evaluation

3.4.2 Influence of hydration media on particle size, PDI and zeta potential

The effect of different hydration media on the liposomal formulations was evaluated firstly by using phosphate buffer and acetate buffer. Formulation F6, prepared with phosphate buffer, showed a large particle size of $358.7 \text{ nm} \pm 2.0\%$ together with a very high PDI value of $0.992 \text{ nm} \pm 1.3\%$, indicating poor homogeneity of the formulation (Table 8). The DLS graph of F1 showed broad and irregular peaks with multiple particle populations which showed aggregation and unstable vesicle formation (Figure 21). Although the zeta potential value was slightly negative at $-4.35 \text{ mV} \pm 0.9\%$, the low absolute value was not sufficient to prevent particle aggregation (Table 9).

The formulation F7 which prepared with acetate buffer showed improved results with a smaller particle size of $179.9 \text{ nm} \pm 0.9\%$ and a lower PDI value of $0.160 \text{ nm} \pm 1.4\%$, which indicate a more uniform particle distribution (Table 8). However, the zeta potential value of F2 remained relatively low at $2.11 \text{ mV} \pm 0.6\%$ that showed limited electrostatic stability of the dispersion (Table 9). The obtained results indicated that the hydration medium had a significant effect on particle size, homogeneity, and stability of the liposomal formulations.

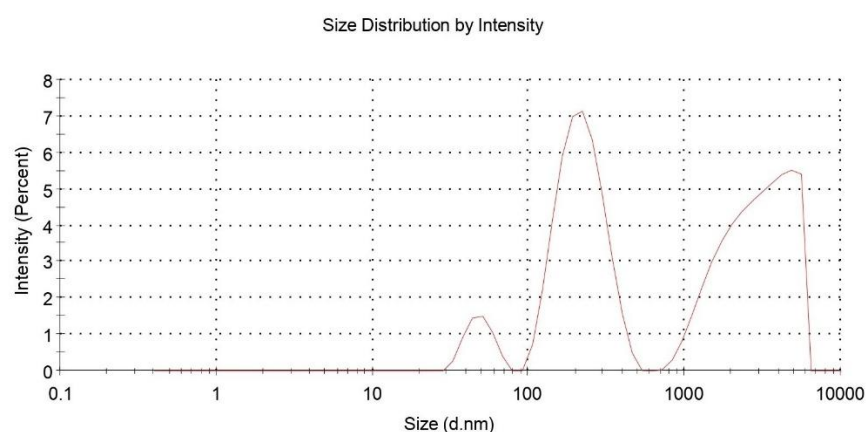
Although acetate buffer produced better results than phosphate buffer, distilled water was selected as the hydration medium for further studies due to its more suitable physicochemical properties and improved formulation behaviour during optimisation.

Table 8. Particle size and PDI of liposomal formulations F6 and F7

S. No.	Formulation	Z-average (nm)	Polydispersity index (PDI)
1	F6	$358.7 \pm 2.0\%$	$0.992 \pm 1.3\%$
2	F7	$179.9 \pm 0.9\%$	$0.160 \pm 1.4\%$

Table 9. Zeta potential of liposomal formulations F6 and F7

S. No.	Formulation	Zeta potential (mv)
1	F6	$-4.35 \pm 0.9\%$
2	F7	$2.11 \pm 0.6\%$

**Fig. 21.** Particle size distribution of liposomal formulation F6

3.4.3 Characterisation of liposomal formulations

The physicochemical properties of the prepared liposomal formulations were influenced by the composition of the formulation. The main differences were observed in particle size, polydispersity index (PDI), and zeta potential among the investigated formulations. These factors are significantly important to evaluate liposomal because it influences vesicle stability, drug encapsulation efficiency, cellular uptake, and the general effectiveness of the drug delivery system. As shown in table 10 and 11, F9 and F10 formulations indicated the most characteristics and improved homogeneity and colloidal stability.

F6 indicated the largest particle size of $360.5 \text{ nm} \pm 0.4\%$ with a very high PDI value of 0.991, which showed non-uniform particle distribution and possible vesicle aggregation. The particle size distribution graph of F6 also supported the obtained results, since it showed several peaks across a wide size range, which indicated the presence of non-uniform particles and aggregation within the formulation.

In addition to the main nanosized population, the presence of larger peaks in the micron range indicated particle aggregation and poor dispersion stability before sonication. According to studies, such broad and multimodal distribution patterns are commonly related to unstable liposomal systems and insufficient homogenisation. Although F7 showed a smaller particle size of $176.2 \text{ nm} \pm 1.9\%$, its surface charge remained relatively unstable. The poorer characteristics observed for F6 and F7 can

be related to the use of buffer during liposome preparation. The ionic components which is present in the buffer could have affected lipid hydration and vesicle stability, lead to aggregation and broader particle distribution and because of that, water was used instead of buffer in the subsequent formulations in order to improve dispersion stability and reduce particle aggregation.

The addition of Tween 20 and PEG had a significant effect on the physicochemical behaviour of the liposomes. Tween 20 has a non-ionic surfactant role in the system that reduces interfacial tension and improves particle dispersion. PEG also provides steric stabilisation and limits vesicle aggregation. Formulations which contain these stabilising agents produced smaller particle sizes and more acceptable PDI values compared to the earlier formulations. F9 indicated a sharp peak with the smallest particle size of $128.8 \text{ nm} \pm 0.28\%$ with a PDI of $0.355 \pm 1.4\%$ and a zeta potential of $-22.7 \text{ mV} \pm 0.4\%$ (Figures 22 and 23). Also, F10 was similar and showed a particle size of $150.4 \text{ nm} \pm 4.7\%$ and a zeta potential of $-25.8 \text{ mV} \pm 0.7\%$. These values indicated the successful formation of nanosized liposomal systems with acceptable colloidal stability.

According to the study which conducted by Gupta et al. (2024) [74], the results in terms of particle size was 150 nm and zeta potential values close to -30 mV . In this paper, they studies on formulations for the development of curcumin-loaded liposomes. The particle size and zeta potential values obtained for formulations F9 and F10 in the present study were similar to the results in their study which indicate acceptable physicochemical stability of the prepared formulations.

Almost same observations were reported by Chen et al. (2023) [75], a review which focused on astaxanthin-loaded lipid nanocarriers. Their results indicated particle sizes between 120 and 240 nm, while zeta potential values ranged from approximately -11 to -31 mV . The values obtained for F9 and F10 in the present study were within these reported ranges, indicating that the prepared liposomal formulations had physicochemical characteristics comparable to previously published lipid-based delivery systems particularly those which studied on astaxanthin and curcumin.

Table 10. Particle size and PDI of formulations prepared by the thin-film hydration method

S. No.	Formulation	Z-average (nm)	Polydispersity index (PDI)
1	F6	$360.5 \pm 0.4\%$	$0.991 \pm 1.2\%$
2	F7	$176.2 \pm 1.9\%$	$0.167 \pm 0.8\%$
3	F8	$240.4 \pm 1.6\%$	$0.426 \pm 1.0\%$
4	F9	$128.8 \pm 0.28\%$	$0.355 \pm 1.4\%$
5	F10	$150.4 \pm 1.7\%$	$0.361 \pm 0.3\%$

Table 11. Zeta potential of formulations prepared by the thin-film hydration method

S. No.	Formulation	Zeta potential (mv)
1	F6	$-4.35 \pm 0.4\%$
2	F7	$2.09 \pm 0.2\%$
3	F8	$-19.2 \pm 0.8\%$
4	F9	$-22.7 \pm 0.4\%$
5	F10	$-25.8 \pm 0.7\%$

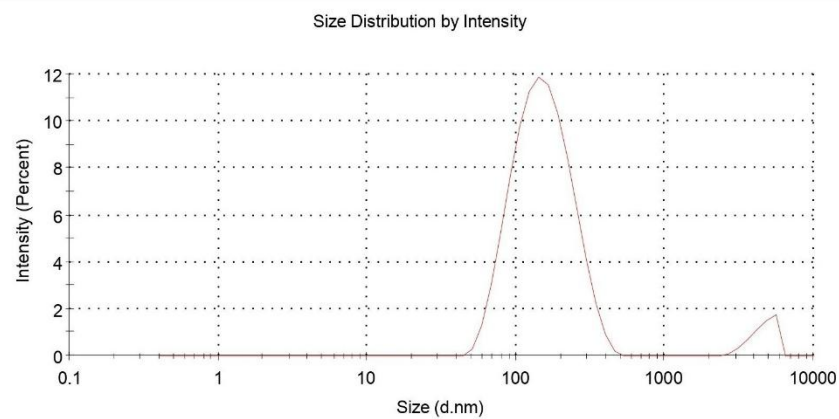


Fig. 22. Particle size distribution of liposomal formulation F9

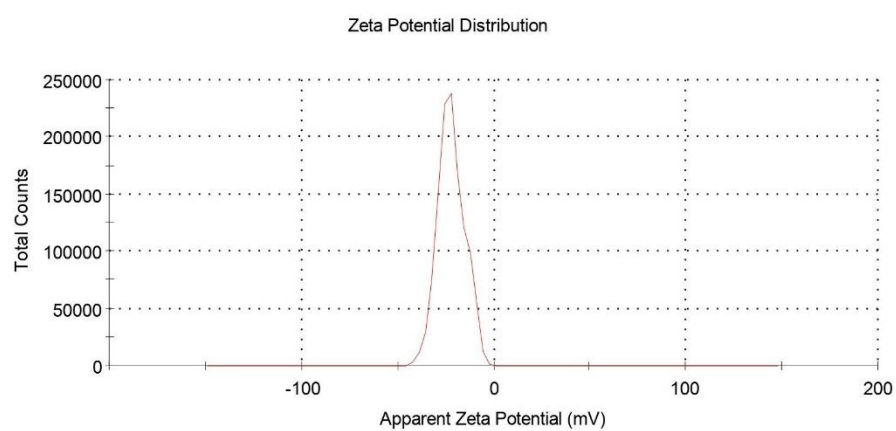


Fig. 23. Zeta potential of liposomal formulation F9

3.4.4 Identification test by FT-IR spectrophotometer

FTIR analysis was performed to identify the present functional groups in the main compounds as well as the lyophilised liposomal samples of formulations F9 and F10.

The FTIR spectrum of curcumin showed several characteristic absorption bands that were consistent with its molecular structure. A broad band which observed between 3500 and 3200 cm^{-1} belonged to O–H stretching vibrations. Absorption bands around 1600–1625 cm^{-1} were related to conjugated C=O and C=C stretching vibrations, while bands in the 1500–1450 cm^{-1} region corresponded to aromatic C=C stretching. Additional bands observed between 1250 and 1150 cm^{-1} , as well as near 1025 cm^{-1} , were associated with C–O–C and C–O stretching vibrations, respectively.

The FTIR spectrum of astaxanthin also showed characteristic bands which associated with its molecular structure. A broad O–H stretching band was observed at 3497 cm^{-1} . A strong absorption band near 1731 cm^{-1} confirmed the presence of C=O stretching vibrations which belonged to the ketone group. Bands around 1650 cm^{-1} belonged to C=C stretching vibrations, while additional peaks in the range of 1200–1000 cm^{-1} indicated C–O stretching vibrations.

The FTIR spectra of the lyophilised liposomal formulation samples showed absorption bands of both curcumin and astaxanthin. The broad O–H stretching band was still present, although it showed a slight shift towards lower wavelength. The reason can be the formation of hydrogen bonding interactions within the formulation. The characteristic C=O and C=C stretching bands were also detected; however, some changes in their position and intensity were observed compared with results for the pure compounds.

These spectral changes may be related to molecular interactions between curcumin, astaxanthin, and the lipid components of the liposomal system. However, the main characteristic peaks of both active compounds remained visible in the lyophilised samples.

All of these results confirmed the presence of the characteristic functional groups of both active pharmaceutical ingredients in the liposomal formulations and prove the successful incorporation of curcumin and astaxanthin into the liposomal system without major structural changes or degradation. The FT-IR spectra of astaxanthin, curcumin, sample 1, and sample 2 were recorded in the range of 4000 cm^{-1} to 560 cm^{-1} and are presented in Figures 24-27. Moreover, the main characteristic peaks identified from the spectra are summarised in Tables 12-15.



Fig. 24. IR spectrum of astaxanthin



Fig. 25. IR spectrum of curcumin

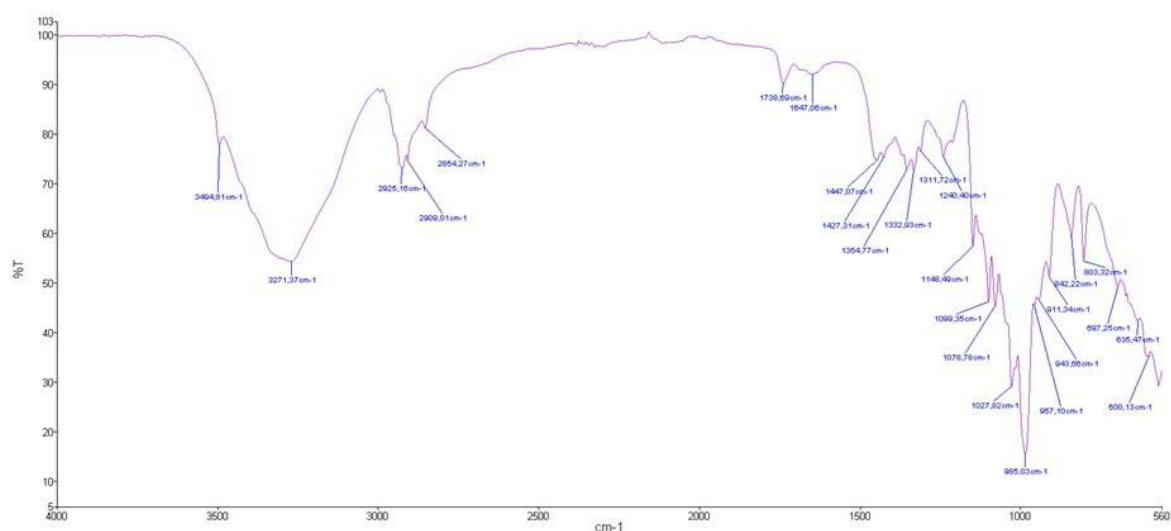


Fig. 26. IR spectrum of sample formulation F9

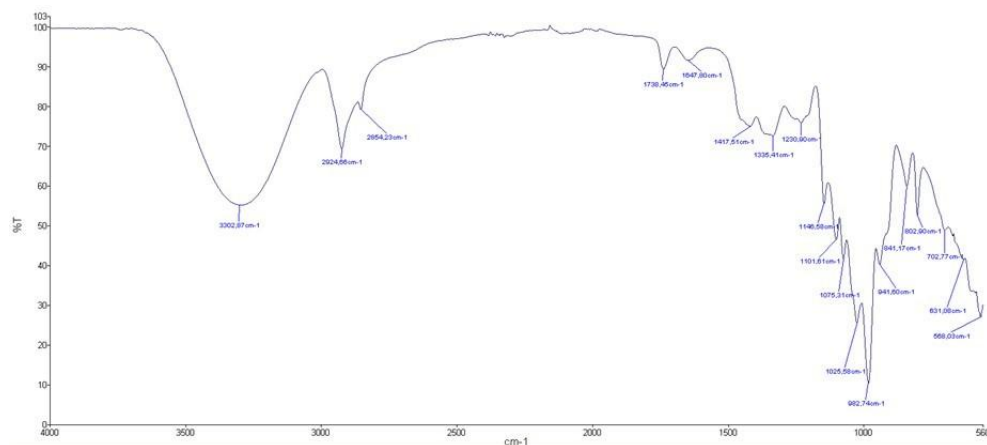


Fig. 27. IR spectrum of formulation F10

Table 12. The main Characteristic FTIR peaks of curcumin

S. No.	Functional group	Theoretical peak (cm ⁻¹)	Practical peak (cm ⁻¹)
1	Phenolic O–H stretching	3500-3200	3503,3356
2	Aromatic C–H stretching	3100-3000	3071
3	Aliphatic C–H stretching	2950-2850	2946, 2845
4	C=O / C=C stretching	1635-1600	1627, 1602
5	Aromatic C=C stretching	1510-1450	1505, 1456
6	C–O–C stretching	1275-1150	1261, 1181
7	C–O stretching	1150-1000	1026
8	Aromatic C–H bending	950-600	856-713

Table 13. The main Characteristic FTIR peaks of astaxanthin

S. No.	Functional group	Theoretical peak (cm ⁻¹)	Practical peak (cm ⁻¹)
1	O–H stretching	3500-3300	3497
2	Aliphatic C–H stretching	3000-2850	2969, 2921, 2864
3	C=O stretching	1740-1700	1731
4	C=C stretching	1650-1550	1650, 1604
5	CH ₂ /CH ₃ bending	1465-1360	1443
6	C–O stretching	1280-1000	1150-1035
7	=C–H bending	1000-600	878-782

Table 14. Characteristic FTIR peaks of the lyophilized liposomal astaxanthin and curcumin of F9

S. No.	Functional group	Theoretical peak (cm ⁻¹)	Practical peak (cm ⁻¹)
1	O–H stretching	3500-3200	3495
2	Aliphatic C–H stretching	2950-2850	2925, 2909, 2854
3	C=O stretching	1730-1700	1739
4	C=C stretching	1650-1600	1647
5	CH ₂ /CH ₃ bending	1450-1350	1447
6	C–O–C / C–O stretching	1250-1000	1150-1035
7	Fingerprint region	1000-600	985

Table 15. Characteristic FTIR peaks of the lyophilized liposomal astaxanthin and curcumin of F10

S. No.	Functional group	Theoretical peak (cm ⁻¹)	Practical peak (cm ⁻¹)
1	O–H stretching	3500-3200	3303
2	Aliphatic C–H stretching	2950-2850	2925, 2854
3	C=O stretching	1730	1738
4	C=C stretching	1650-1600	1648
5	CH ₂ /CH ₃ bending	1500-1300	1418
6	C–O–C / C–O stretching	1250-1000	1147-1026
7	Fingerprint region	1000-600	982

3.4.5 Scanning Electron Microscopy (SEM) analysis

Scanning electron microscopy (SEM) analysis was performed to evaluate the surface morphology and microstructural characteristics of the lyophilised liposomal formulations. The morphological properties of the F9 and F10 formulations, which had different formulation, were compared to investigate possible differences in their structural organisation after lyophilisation.

3.4.5.1 SEM Morphological Analysis for formulation F9

The scanning electron microscopy (SEM) micrographs of the F9 formulation show a highly porous, scaffold-like microstructure that is characteristic of a successful lyophilization process (Figure 28). At lower magnifications 1,000 ×, the sample consists of polydisperse and angular fragments which come from the mechanical breakdown of the lyophilized cake. High-resolution images 6,000× and 10,000× show an intricate, interconnected network of micro-pores, which are the physical remnants of ice crystals that have sublimated. This sponge-shape architecture shows that the trehalose cryoprotectant formed a robust matrix around the liposomes, leaving behind a high-surface-area framework. This kind of structure is suitable for pharmaceutical powders as it facilitates rapid rehydration and potentially protects the encapsulated astaxanthin and curcumin by maintaining their structural distribution across the protective scaffold.

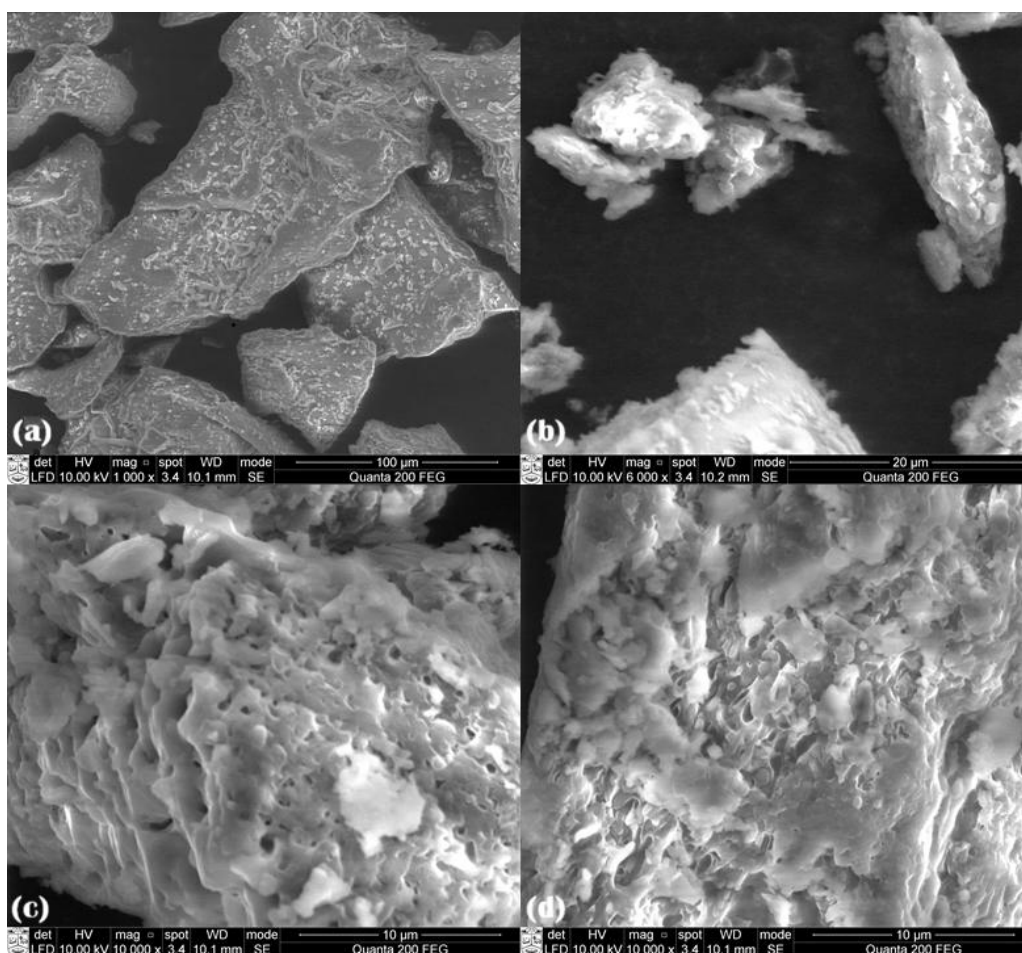


Fig. 28. SEM micrographs of formulation F9 at different magnifications: (a) 1,000×, (b) 6,000×, (c) 10,000×, and (d) 10,000×

3.4.5.2 SEM Morphological Analysis for formulation F10

The formulation F10 shows a different morphology in comparison of formulation F9 (Figure 29). It is characterized by dense, platelet-like aggregates and a smoother surface texture. At 1,000 $\times$, the particles appear as large, solid flakes with sharp, defined edges, suggesting a more brittle, "glassy" state of the trehalose matrix. Higher magnifications (6,000 $\times$ and 10,000 $\times$) show a significant reduction in visible micro-porosity compared to previous formulations. By looking closer to the results, the surfaces appear fairly smooth but are covered in small, dusty debris adhering to the main structure. This proves a very compact trehalose matrix. While a dense structure like this can sometimes slow down rehydration, it has the major advantage of acting as a solid physical barrier and acts as a better shield which protect the sensitive bioactive compounds such as astaxanthin from oxidation and moisture.

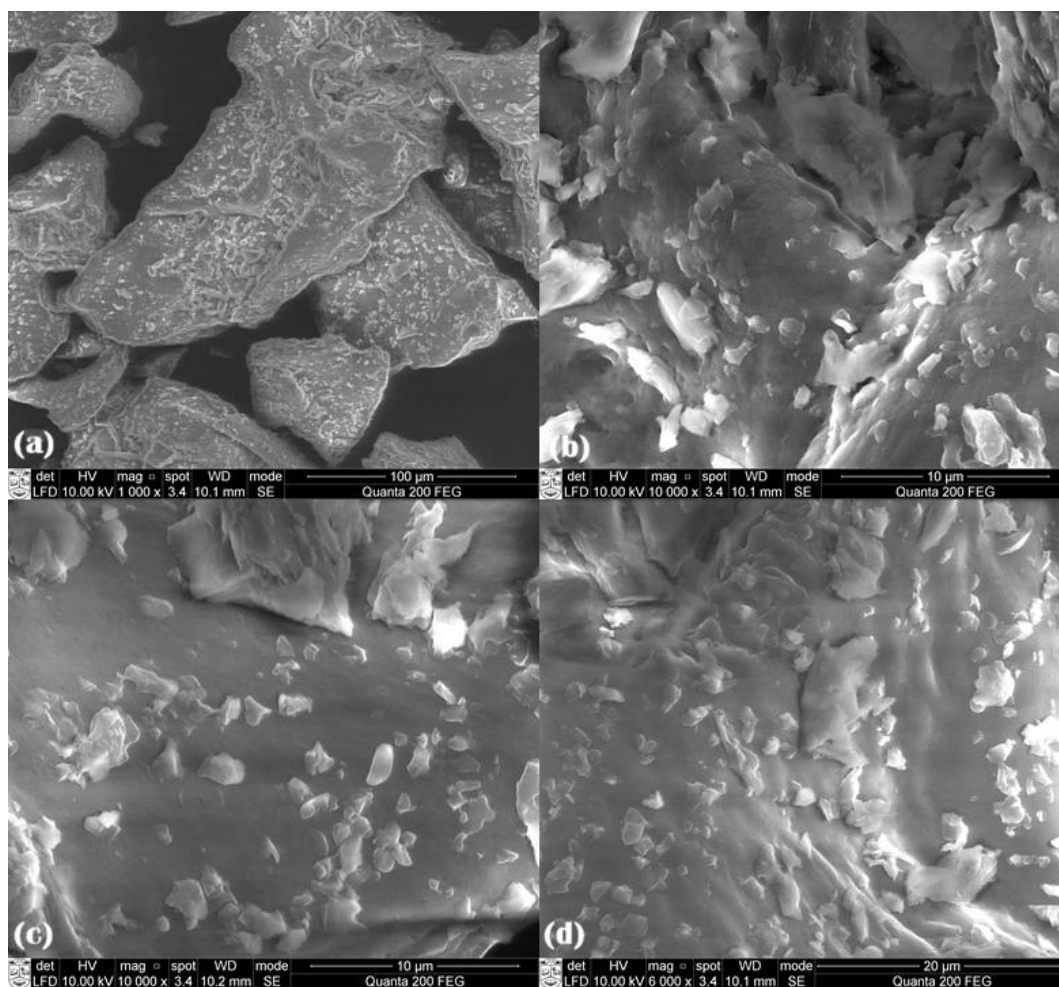


Fig. 29. SEM micrographs of formulation F10 at different magnifications: (a) 1,000 $\times$, (b) 6,000 $\times$, (c) 10,000 $\times$, and (d) 10,000 $\times$

3.4.5.3 Comparative discussion between two different formulation

The significant difference between two batches is the internal micro-structure and overall surface porosity. Formulation F9 has a much more open, aerated design, which is typically better for quick dissolution since water can penetrate the particles more easily. On the other hand, formulation F10 showed much more compact and solid. While this denser structure might slow down rehydration a bit, it actually serves as a better physical barrier and potentially protect the astaxanthin and curcumin particularly astaxanthin which is sensitive to environmental degradation such as oxidation by limiting air exposure. Ultimately, both batches show that trehalose is an effective stabilizer, but they highlight how even slight variations in lab conditions can significantly change the physical properties of the final powder.

3.4.6 Structural analysis based on SEM and FTIR:

Formulation F9 shows a highly porous, "sponge-like" architecture, a physical state largely attributed to the inclusion of PEG (Polyethylene Glycol). In this formulation, PEG acts as a plasticizer that disrupts the formation of a rigid, continuous glass which preserved the interconnected micro-channels created during ice crystal sublimation. This open design correlates directly with the sharper, more defined signals observed in the FTIR spectrum. Because the PEG-stabilized trehalose formed an airy scaffold rather than a solid mass, the infrared beam interacted more freely with the functional groups of the encapsulated curcumin and astaxanthin. This high-surface-area design is reflected in the sharp peaks within the 1000–1600 cm^{-1} fingerprint region and the lower intensity of the O-H stretching band 3271.37 cm^{-1} which indicate less trapped moisture within the pores. Ultimately, the use of PEG resulted in a high-accessibility formulation optimized for rapid rehydration and immediate release. On the other side, formulation F10 reflects a transition toward a dense, "glassy" state, by the addition of Tween 20 in liposomal formulation. Tween 20 as a surfactant, significantly reduces surface tension during the freezing phase, which promotes the formation of a more continuous and integrated matrix. This leads to the solid, platelet-like aggregates seen in the SEM micrographs, in other words, porosity is sacrificed for structural density. This physical transition is showed in the FTIR data by the significant broadening and increased intensity of the O-H band at 3302.871 cm^{-1} , a classic signature of extensive hydrogen bonding within a compact molecular environment. This Tween 20-mediated compaction can mask the individual vibration peaks of the curcumin and astaxanthin, as the bioactive molecules are more deeply shielded within the solid trehalose barrier. This structure provides superior molecular protection and acting as a robust physical shield against environmental oxidation; however, it leads to slower dissolution.

3.4.7 Encapsulation efficiency (%)

The encapsulation efficiency of the prepared liposomal formulation was found to be $87.5\% \pm 0.2\%$ for curcumin and $85.1\% \pm 0.8\%$ for astaxanthin. These results indicate successful incorporation of both hydrophobic compounds into the phospholipid bilayer of the liposomes. The high encapsulation efficiency can be related to the lipophilic nature of astaxanthin and curcumin, which improve strong interaction with the lipid membrane and improves their retention within the vesicles.

According to Luo et al., curcumin-loaded liposomes prepared by using the thin-film hydration method which showed an encapsulation efficiency of 84% [76]. In another study, Wang et al. reported encapsulation efficiencies higher than 90% for curcumin-loaded liposomes prepared with

phosphatidylcholine and cholesterol [77]. For astaxanthin, Chang et al. obtained high encapsulation efficiency for liposomal astaxanthin which prepared with soybean phosphatidylcholine [78]. In their studies, they obtained above 90% for encapsulation efficiencies, which indicate the suitability of liposomes for encapsulating hydrophobic carotenoids [78]. The differences between the present study and previous studies can be related to formulation composition, lipid-to-drug ratio, preparation conditions, and the simultaneous encapsulation of two hydrophobic compounds in the same liposomal system. According to previous studies and the value of encapsulation efficiency in this project, the obtained value in first step of evaluation shows good loading capacity and confirms the suitability of liposomes as carriers for hydrophobic bioactive compounds.

3.4.8 Yield

The freeze-dried formulations showed high production yield values. The % yield value for formulations F9 and F10 were 89% and 90%, respectively. These results indicate that only a small amount of material was lost during the preparation and freeze-drying steps. The high value of yield can be related to the presence of Tween 20 and PEG. They can significantly improve stability of the liposomal system and reduced particle aggregation during processing. Also, the high yield values indicate successful incorporation of curcumin and astaxanthin into the liposomal formulations which support the suitability of F9 and F10 for further analysis and formulation development. Previous studies on freeze-dried liposomal formulations have also reported almost same value which are usually between 80% and 95%. Differences in yield percentage are mainly influenced by the lipid composition and the stabilising agents used during freeze-drying.

3.4.9 Moisture content

The powder moisture content was found to be $4.65 \pm 0.61\%$ for F9 formulation and $4.89 \pm 0.99\%$, which are lower than the specified minimum moisture content of many powders used in food applications to inhibit microbiological spoilage and lipid oxidation and extend the product's shelf life.

3.4.10 Bulk density

The bulk density of the powder was $0.31 \pm 0.01 \text{ g/cm}^3$. A higher bulk density has several advantages, including more convenient storage conditions due to lower space requirements for storage and greater protection against oxidation during storage due to the presence of less air in powder. According to previous studies, bulk density values after the drying process were reported within the range of 0.20-0.40 g/cm^3 .

3.4.11 Stability studies

This part of study performed to investigate the stability of liposomal astaxanthin and curcumin. After the optimisation of formulation of F9 and F10, they stored in two different environments, cold and dark place and second in dark and 25 °C. They kept for six weeks and their physicochemical properties such as appearance, the value of z-average, poly dispersity (PDI), zeta potential as well as encapsulation efficiency (%EE).

3.4.11.1 Appearance

After six weeks of storage, significant differences were observed between the sample which stored at refrigerated condition and the sample which kept at 25°C. The samples stored at room temperature showed physical changes, such as loss of homogeneity, formation of dark red-orange aggregates, and development of an unpleasant odour (Figures 30b and 30c). On the other side, the sample in fridge, remained stable and homogeneous. These observations shows that storage temperature has a significant role in stability of the liposomal system (Figure 30a).

The instability observed at 25°C is related to changes in the lipid bilayer structure. The liposomal formulation was included L- α -phosphatidylcholine (PC), cholesterol, and PEG which help to improve liposomal stability. Cholesterol helps strengthen the phospholipid membrane and reduce permeability. Moreover, PEG provides steric stabilisation that limits vesicle aggregation. However, these components were not sufficient to keep the samples stable during storage. The higher storage temperature increased the movement and interaction of liposomes, which caused membrane fusion and destabilisation of the liposomal structure and as a result, encapsulated astaxanthin and curcumin were gradually released into the aqueous medium. Since both compounds are highly hydrophobic and poorly soluble in water, they tended to aggregate and crystallise after leakage from the vesicles. The reason for this can be the leakage of astaxanthin and curcumin from the liposomes, which resulted in their aggregation and crystallisation in the aqueous medium. Similar behaviour has been reported in previous studies involving carotenoid-loaded liposomal systems stored under non-refrigerated conditions.

The unpleasant odour which observed in the samples stored at 25°C also indicated chemical degradation during storage. The possible reason for this can be oxidation of the phospholipid membrane, since it contains PC which has unsaturated fatty acid chains and make it are highly sensitive to oxidation, particularly at higher temperatures. This oxidation process forms volatile compounds such as aldehydes and ketones, which are related to rancid odours. Although astaxanthin initially provides antioxidant protection, higher temperatures accelerate its degradation and gradually reduce its protective effect. In addition, microbial contamination can also have contributed to the unpleasant smell, particularly because no preservatives were used in the formulation.

On the other side, the sample which stored under refrigerated conditions in terms of appearance was stable during the six weeks. Lower temperatures helped maintain the rigidity of the liposomal membrane, reduced permeability, and prevented leakage of the encapsulated compounds. Based on the results, the best storage condition for the astaxanthin and curcumin liposomal dispersion was a dark environment at 4°C.

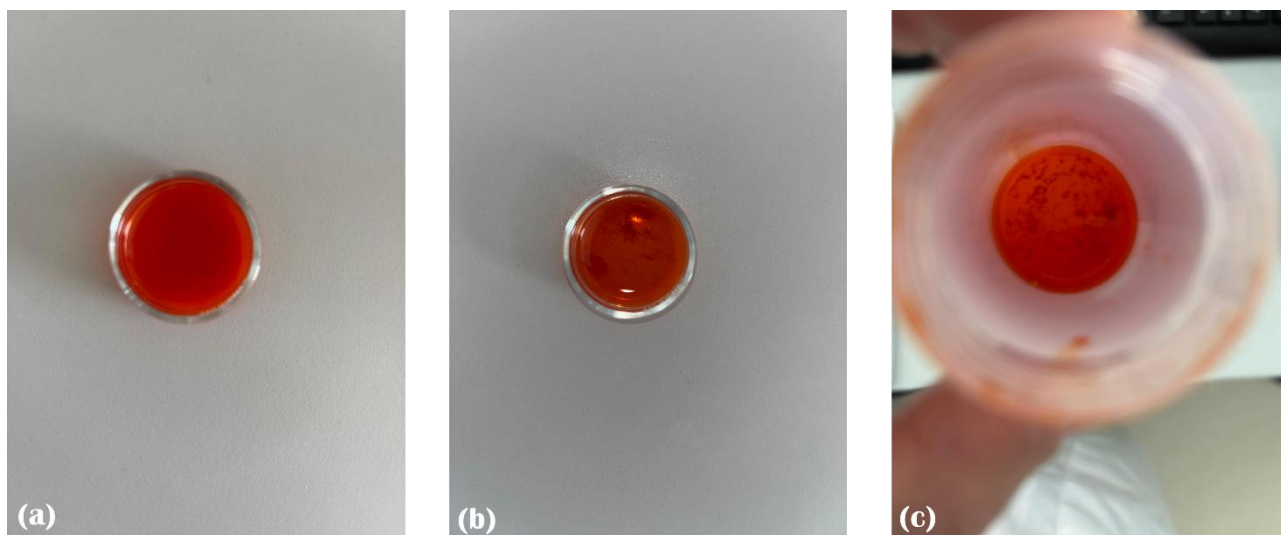


Fig. 30. Appearance stability of liposomal astaxanthin and curcumin liposomes after six weeks. (a) Refrigerated control (4°C). (b, c) Room temperature samples (25°C)

3.4.11.2 Particle size

This part of study is mainly important because particle size directly affects the physical stability, drug release behaviour, encapsulation efficiency, and therapeutic performance of the formulation. Changes in particle size during storage indicate vesicle aggregation, fusion, or degradation, which can reduce the stability and effectiveness of the liposomal system. In this study, the particle size of liposome dispersion over six weeks under different storage conditions (25 °C and 4 °C) in dark place monitored. Based on the obtained results storage temperature significantly affected the stability of the optimised formulation.

The results showed that storage temperature has a significant role in stability of the formulations. Based on the results, PDI values increased gradually at both temperatures at (4 °C and 25 °C) during storage (Figure 31). However, the increase was much higher at 25 °C. The sample at 4 °C remained lower PDI values, which indicate a more uniform particle distribution and better physical stability. On the other side, the higher value for PDI at 25 °C showed particle aggregation and reduced homogeneity of the liposomal astaxanthin and curcumin formulations.

The Z-average results also indicated better stability at 4 °C (Figure 32). Liposomes which stored at 4 °C remained stable during the first weeks, with only a moderate increase in particle size by the end of the study. However, the sample at 25 °C showed a rapid increase in particle size after week 2, which indicated aggregation or fusion of liposomal vesicles at higher temperature.

Zeta potential also showed the same trend (Figure 33). The sample which stored at 4 °C showed stable zeta potential values in six weeks and stayed close to -24 mV. This shows sufficient electrostatic repulsion between particles and good colloidal stability. In comparison, the zeta potential of samples stored at 25 °C became less negative. This value changed from -24 mV to -8.5 mV after 6 weeks. The reduction in surface charge likely increased particle aggregation and contributed to the increase in particle size and PDI.

This part of study indicated that in terms of particle size, refrigerated storage at 4 °C was more suitable too keep liposomal astaxanthin and curcumin stable

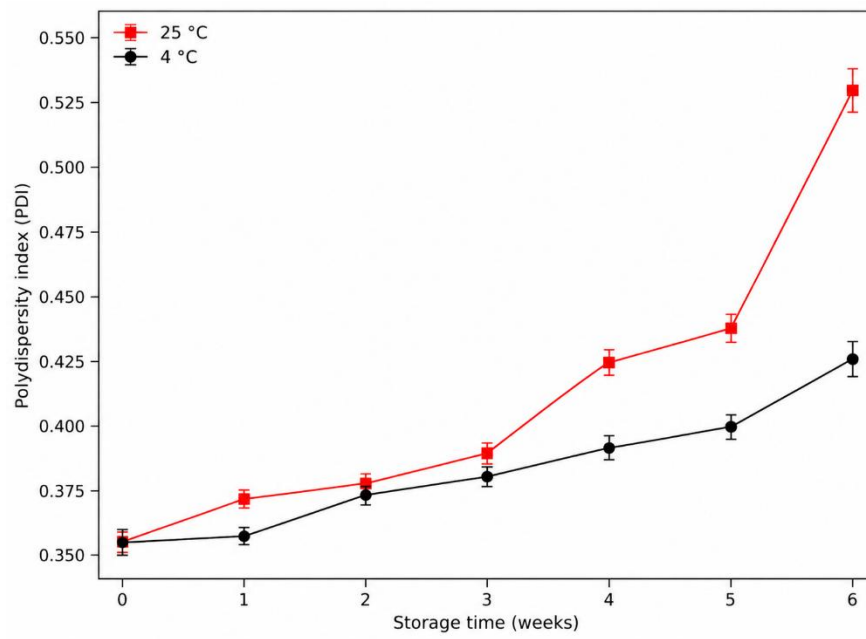


Fig. 31. PDI changes during storage at 4 °C and 25 °C

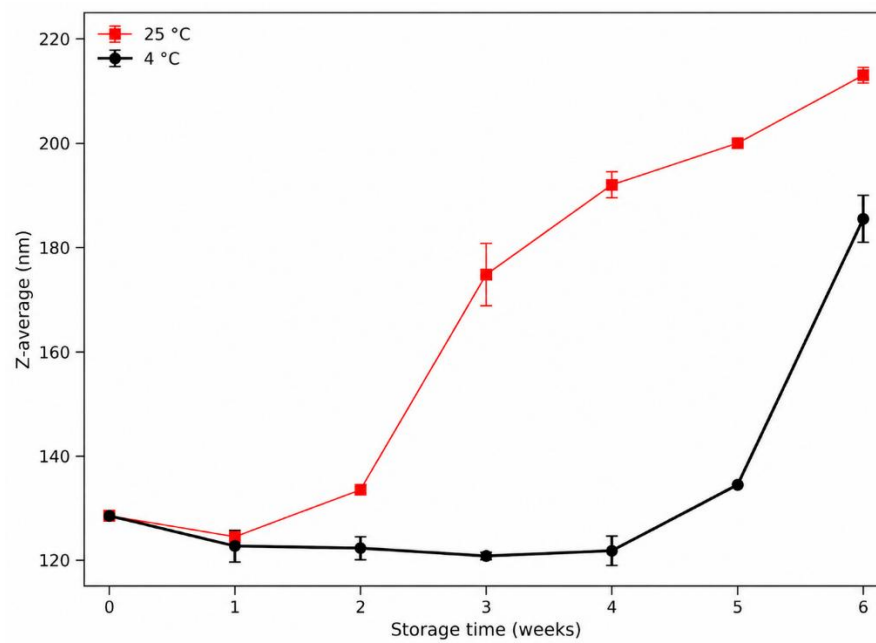


Fig. 32. Z-average changes during storage at 4 °C and 25 °C

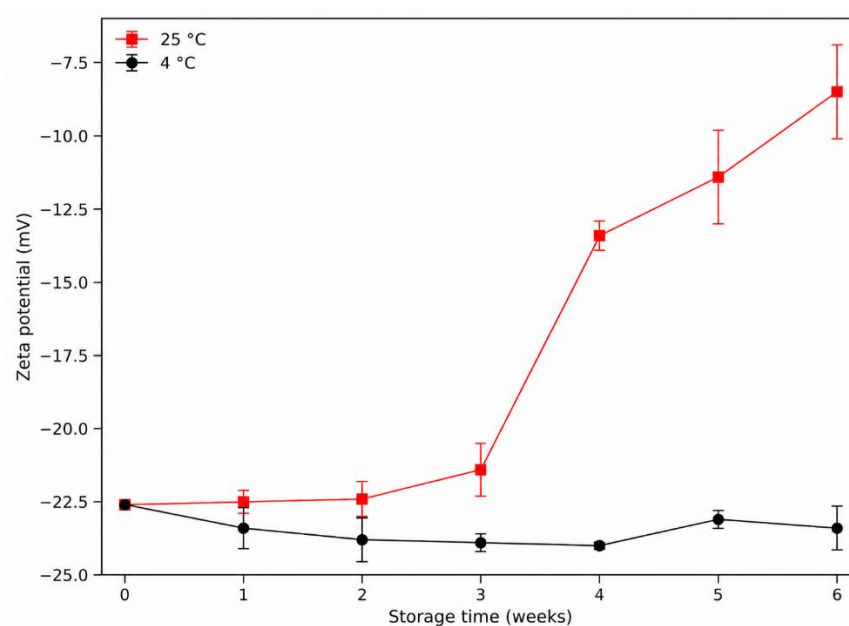


Fig. 33. Zeta potential changes during storage at 4 °C and 25 °C

3.4.11.3 Encapsulation efficiency (%)

Encapsulation efficiency is a key parameter because it indicates how effectively liposomes can preserve the encapsulated compounds during storage.. A decrease in encapsulation efficiency indicate leakage of bioactive compounds or destabilisation of the liposomal membrane. In this study, the encapsulation efficiency of liposomal astaxanthin and curcumin was evaluated over 6 weeks under different storage conditions at 4 °C and 25 °C in a dark place.

Both formulations showed a gradual decrease in encapsulation efficiency during storage. this reduction is greater at 25 °C condition. The encapsulation efficiency of curcumin liposomes decreased from 87% to 61% at 25 °C, however, sample with stored at 4 °C had better results and remained 71% after 6 weeks (Figure 34). A similar trend was observed for astaxanthin liposomes. The encapsulation efficiency of astaxanthin liposomes decreased significantly, from 84% to 44% at 25 °C and from 84% to 53% at 4 °C (Figure 35).

The main reason of greater reduction in astaxanthin liposomes is related to the high sensitivity of astaxanthin to heat, oxidation, and environmental conditions. As discussed in the literature review section of this study, astaxanthin is chemically less stable than curcumin and may degrade more rapidly during storage, particularly at higher temperatures. On the other side, curcumin liposomes showed better results of the encapsulated compound during the storage period. These results indicated that storage at 4 °C was more suitable in terms of the encapsulation efficiency and stability of both bioactive compounds.

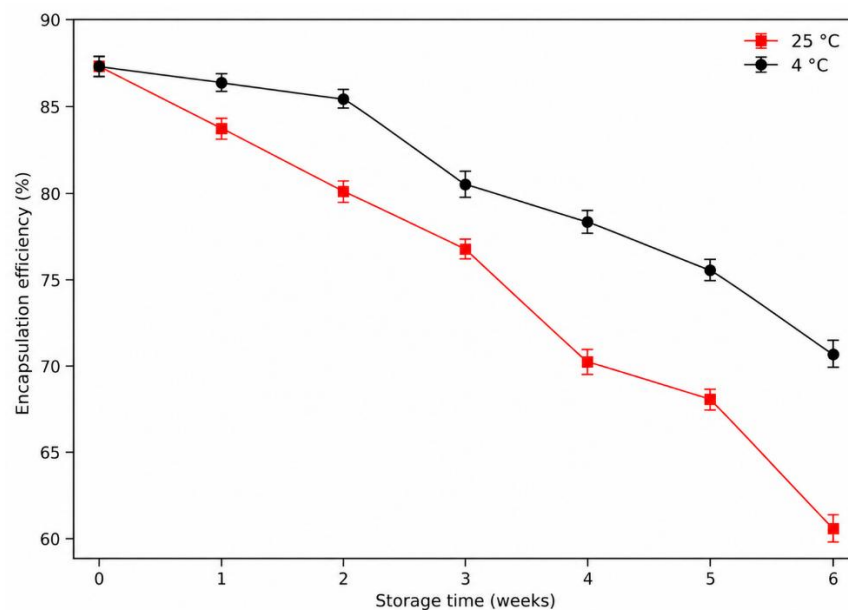


Fig. 34. Curcumin encapsulation efficiency during storage at 4 °C and 25 °C

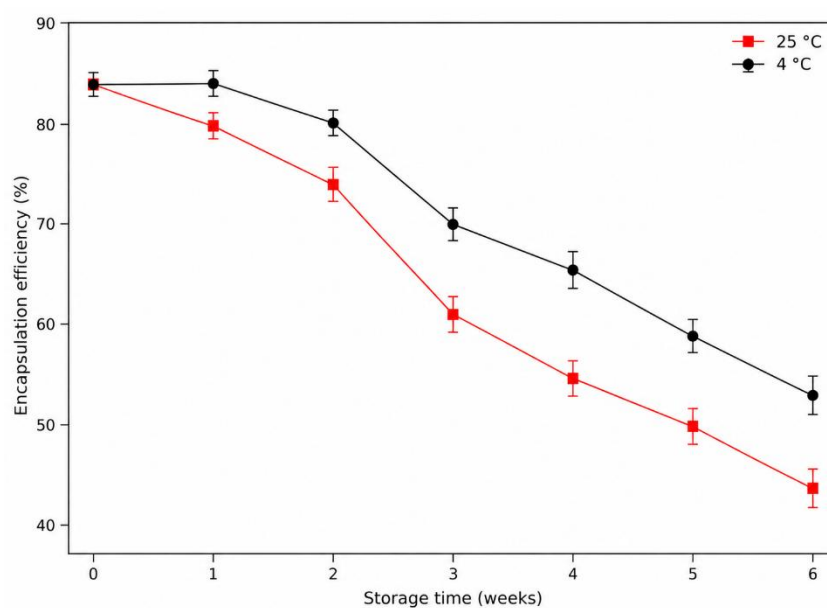


Fig. 35. Astaxanthin encapsulation efficiency during storage at 4 °C and 25 °C

The obtained stability results were similar to previous studies that showed better physicochemical stability of liposomal formulations at lower temperatures compared with room temperature. Previous studies reported increases in particle size and PDI together with decreases in zeta potential and encapsulation efficiency because of vesicle aggregation and leakage of encapsulated compounds at higher temperatures. The same trend appeared in the present study, as samples stored at 25 °C showed lower stability than samples stored at 4 °C. The better stability at refrigerated conditions is related to lower lipid oxidation and better preservation of the liposomal membrane structure during storage.

Recommendations

1. With molecular docking and computational drug design methods, liposomal formulations can be optimised in much better way. These methods can predict the interactions between lipids and bioactive compounds with target cell membranes. Computational modelling can also help to determine suitable active ingredient concentrations and optimise conditions such as pH, temperature, and sonication time.
2. Other recommendations for future studies can be the investigation of use of the amino acids in liposomal formulations to improve encapsulation efficiency, targeted delivery, with aim of improving formulation stability. According to previous studies, amino acids can effectively protect liposomes and prevent the degradation of sensitive compounds such as astaxanthin.

Conclusions

1. Based on the obtained results in this study, liposomal astaxanthin and curcumin were successfully developed and optimised by the thin-film hydration method. The final formulation showed acceptable physicochemical characteristics, such as particle size distribution, polydispersity index, zeta potential, and encapsulation efficiency, which the results are comparable to previous studies.
2. Structural and morphological analyses which performed by FT-IR spectroscopy and SEM, confirmed the successful incorporation of astaxanthin and curcumin into the liposomal systems and demonstrated the formation of vesicular structures with appropriate morphology.
3. The optimisation process of liposomal formulations was challenging, particularly in terms of liposome preparation method, phospholipid type and its ratio, sonication method, lyophilisation process, and analytical methods. Results showed that each factor during liposome formulation had a specific impact on the final formulation. Therefore, the use of molecular docking is recommended for future studies to make the formulation procedure shorter and more efficient.
4. Freeze-drying of liposomal formulations with cryoprotective excipients successfully formulated and dry powdered obtained. The obtained lyophilised formulations showed suitable physicochemical properties, which support the future development of stable pharmaceutical dosage forms such as microcapsules and tablets. However, the stability study results for lyophilised astaxanthin and curcumin, particularly astaxanthin, showed a significant decrease in encapsulation efficiency during storage, which make their production challenging particularly in industrial scale.
5. Based on results in stability studies, the most suitable storage condition for both the liposomal dispersion and lyophilized liposomal formulation was at 4 °C in a dark place.
6. According to the obtained results of the stability study, astaxanthin showed higher sensitivity to environmental conditions, as the encapsulation efficiency (%EE) decreased significantly during the six-week stability study. However, curcumin demonstrated comparatively better stability under the same conditions.

List of references

1. BJØRKLUND, Geir; GASMI, Amin; LENCHYK, Larysa; SHANAIDA, Mariia; ZAFAR, Saba, et al. The Role of Astaxanthin as a Nutraceutical in Health and Age-Related Conditions. *Molecules (Basel, Switzerland)*, vol. 27 (2022), no. 21, pp. 7167. doi: 10.3390/molecules27217167.
2. LI, Yifan; LI, Jiafeng; XU, Sheng; LI, Dongdong; ZHANG, Zhen, et al. Tetrahedral Framework Nucleic Acid-Based Delivery of Astaxanthin Suppresses Chondrocyte Pyroptosis and Modulates Oxidative Stress for the Treatment of Osteoarthritis. *Advanced healthcare materials*, vol. 13 (2024), no. 28, pp. e2401452. Available from: <https://pubmed.ncbi.nlm.nih.gov/38923865/>. [viewed 11-05-2026].
3. LI, Xin; WANG, Xiaoqian; DUAN, Chuanlan; YI, Shasha; GAO, Zhengquan, et al. Biotechnological production of astaxanthin from the microalga *Haematococcus pluvialis*. *Biotechnology Advances*, vol. 43 (2020), no. 1, pp. 107602. Available from: <https://pubmed.ncbi.nlm.nih.gov/32711005/>. [viewed 11/05/2026].
4. CHANG, Ming Xian and XIONG, Fan. Astaxanthin and its Effects in Inflammatory Responses and Inflammation-Associated Diseases: Recent Advances and Future Directions. *Molecules (Basel, Switzerland)*, vol. 25 (2020), no. 22, pp. 5342. doi: 10.3390/molecules25225342. Available from: <https://pubmed.ncbi.nlm.nih.gov/33207669/>. [viewed 11/05/2026].
5. LU, Qian; LI, Huankai; ZOU, Yao; LIU, Hui and YANG, Limin. Astaxanthin as a microalgal metabolite for aquaculture: A review on the synthetic mechanisms, production techniques, and practical application. *Algal Research*, vol. 54 (2021), no. 1, pp. 102178. Available from: <https://www.sciencedirect.com/science/article/pii/S2211926420310468>. [viewed 11/05/2026].
6. DONOSO, Andrea; GONZÁLEZ-DURÁN, Javiera; MUÑOZ, Andrés Agurto; GONZÁLEZ, Pablo A. and AGURTO-MUÑOZ, Cristian. “Therapeutic uses of natural astaxanthin: An evidence-based review focused on human clinical trials”. *Pharmacological Research*, vol. 166 (2021), no. 1, pp. 105479. Available from: <https://www.sciencedirect.com/science/article/pii/S1043661821000633>. [viewed 11/05/2026].
7. SUN, Tianhu. An Alternative Route for Astaxanthin Biosynthesis in Green Algae. *Plant Physiology*, vol. 183 (2020), no. 3, pp. 812–813. Available from: <https://pmc.ncbi.nlm.nih.gov/articles/PMC7333709/>. [viewed 11/05/2026].
8. KUMAR, Satish; KUMAR, Rakesh; Diksha; KUMARI, Anju and PANWAR, Anil. Astaxanthin: A super antioxidant from microalgae and its therapeutic potential. *Journal of Basic Microbiology*, vol. 62 (2022), no. 9, pp. 1064–1082. Available from: <https://doi.org/10.1002/jobm.202100391>. [viewed 11/05/2026].
9. SUN, Liwen; LI, Yang; YANG, Anquan; XIE, Min; XIONG, Ranhua, et al. Astaxanthin: A comprehensive review of synthesis, biological activities and applications. *Food Chemistry*, vol. 488 (2025), no. 9, pp. 144847. Available from: <https://www.sciencedirect.com/science/article/pii/S0308814625020989>. [viewed 11/05/2026].
10. AMBATI, Ranga Rao; PHANG, Siew Moi; RAVI, Sarada and ASWATHANARAYANA, Ravishankar Gokare. Astaxanthin: sources, extraction, stability, biological activities and its commercial applications--a review. *Marine drugs*, vol. 12 (2014), no. 1, pp. 128–152. Available from: <https://pubmed.ncbi.nlm.nih.gov/24402174/>. [viewed 11/05/2026].
11. ZHU, Yubo; HE, Linyang; QIN, Xuemin; XU, Mengmei and TAN, Chen. Recent advances in nanodelivery systems for astaxanthin: A review. *Food Bioscience*, vol. 67 (2025), no. 1, pp. 106305. Available from: <https://www.sciencedirect.com/science/article/pii/S221242922500481X>. [viewed 11/05/2026].
12. GENG, Tonghui; BAO, Shihan; SUN, Xiangyu; MA, Dexue; ZHANG, Hexin, et al. A clarification of concepts related to the digestion and absorption of carotenoids and a new standardized carotenoids

bioavailability evaluation system. *Food Chemistry*, vol. 400 (2023), no. 1, pp. 134060. Available from: <https://www.sciencedirect.com/science/article/pii/S0308814622020222>. [viewed 11/05/2026].

13. HONDA, Masaki; MURAKAMI, Kazuya; OSAWA, Yukiko; KAWASHIMA, Yuki; HIRASAWA, Kazuaki, et al. Z-Isomers of Astaxanthin Exhibit Greater Bioavailability and Tissue Accumulation Efficiency than the All-E-Isomer. *Journal of Agricultural and Food Chemistry*, vol. 69 (2021), no. 11, pp. 3489–3495. Available from: <https://doi.org/10.1021/acs.jafc.1c00087>. [viewed 11/05/2026].

14. LIU, Xiaojuan; ZHANG, Junlin; CHEN, Zhiqing; XIAO, Jie; ZHOU, Aimei, et al. Cluster-determinant 36 (CD36) mediates intestinal absorption of dietary astaxanthin and affects its secretion. *Food Research International*, vol. 173 (2023), no. 11, pp. 113328. Available from: <https://www.sciencedirect.com/science/article/pii/S0963996923008736>. [viewed 11/05/2026].

15. PENG, Xuan; LI, Dan and LIU, Yixiang. Construction of efficient carotenoid delivery vehicles based on the intestinal epithelial transport pathway: Current applications and future trends. *Trends in Food Science & Technology*, vol. 147 (2024), no. 11, pp. 104473. Available from: <https://www.sciencedirect.com/science/article/pii/S0924224424001493>. [viewed 11/05/2026].

16. YU, Yijing; RAKA, Fitore and ADELI, Khosrow. The Role of the Gut Microbiota in Lipid and Lipoprotein Metabolism. *Journal of Clinical Medicine*, vol. 8 (2019), no. 12, pp. 104473. Available from: <https://www.mdpi.com/2077-0383/8/12/2227>. [viewed 11/05/2026].

17. YAN, Xueyu; HUANG, Jinman; HUANG, Leiheng; LUO, Cong; LI, Zexin, et al. Effects of dietary lipids on bioaccessibility and bioavailability of natural carotenoids. *LWT*, vol. 200 (2024), no. 12, pp. 116171. Available from: <https://www.sciencedirect.com/science/article/pii/S002364382400450X>. [viewed 11/05/2026].

18. YANG, Shufang; LU, Xue; WANG, Jia; LIU, Ye; NIE, Man, et al. Astaxanthin from *Haematococcus pluvialis* and *Chromochloris zofingiensis*: Biosynthetic Pathways, Engineering Strategies, and Industrial Prospects. *Marine drugs*, vol. 23 (2025), no. 12, pp. 485. doi: 10.3390/md23120485. Available from: <https://pmc.ncbi.nlm.nih.gov/articles/PMC12734979/>. [viewed 11/05/2026].

19. SUN, Liwen; LI, Yang; YANG, Anquan; XIE, Min; XIONG, Ranhua, et al. Astaxanthin: A comprehensive review of synthesis, biological activities and applications. *Food Chemistry*, vol. 488 (2025), no. 12, pp. 144847. Available from: <https://www.sciencedirect.com/science/article/pii/S0308814625020989>. [viewed 11/05/2026].

20. HONDA, Masaki; MURAKAMI, Kazuya; ZHANG, Yelin and GOTO, Motonobu. Continuous production of Z-isomer-rich astaxanthin nanodispersions with stabilized Z-structures using selected ester-based nonionic emulsifiers. *Food Chemistry Advances*, vol. 1 (2022), no. 12, pp. 100028. Available from: <https://www.sciencedirect.com/science/article/pii/S2772753X22000168>. [viewed 11/05/2026].

21. SHAH, M. M. R.; LIANG, Y.; CHENG, J. J. and DAROCH, M. Astaxanthin-producing green microalga *Haematococcus pluvialis*: From single cell to high value commercial products. *Frontiers in Plant Science*, vol. 7 (2016), no. 12, pp. 100028. Available from: <https://www.scopus.com/inward/record.uri?eid=2-s2.0-84964838437&doi=10.3389%2ffpls.2016.00531&partnerID=40&md5=12e52ce85dbd1d4d8a2d92191a74c265>. [viewed 11/05/2026].

22. FANG, T. J. Batch cultivation and astaxanthin production by a mutant of the red yeast, *Phaffia rhodozyma* NCHU-FS501. *Journal of Industrial Microbiology and Biotechnology*, vol. 16 (1996), no. 3, pp. 175–181. Available from: <https://www.scopus.com/inward/record.uri?eid=2-s2.0-0029985556&doi=10.1007%2fbf01570001&partnerID=40&md5=275e7857dabdc0af8710b4f09637e675>. [viewed 11/05/2026].

23. LUO, Qiulan; BIAN, Chao; TAO, Ming; HUANG, Yu; ZHENG, Yihong, et al. Genome and Transcriptome Sequencing of the Astaxanthin-Producing Green Microalga, *Haematococcus pluvialis*.

Genome Biology and Evolution, vol. 11 (2019), no. 1, pp. 166–173. Available from: <https://doi.org/10.1093/gbe/evy263>. [viewed 11/05/2026].

24. LIANG, Ming-Hua; LU, Yan; CHEN, Hao-Hong and JIANG, Jian-Guo. The salt-regulated element in the promoter of lycopene β -cyclase gene confers a salt regulatory pattern in carotenogenesis of *Dunaliella bardawil*. *Environmental microbiology*, vol. 19 (2017), no. 3, pp. 982–989. Available from: <https://doi.org/10.1111/1462-2920.13539>. [viewed 11/05/2026].

25. LEMOINE, Yves and SCHOEFS, Benoît. Secondary ketocarotenoid astaxanthin biosynthesis in algae: a multifunctional response to stress. *Photosynthesis Research*, vol. 106 (2010), no. 1, pp. 155–177. Available from: <https://doi.org/10.1007/s11120-010-9583-3>. [viewed 11/05/2026].

26. GRÜNEWALD, Kay; HIRSCHBERG, Joseph and HAGEN, Christoph. Ketocarotenoid Biosynthesis Outside of Plastids in the Unicellular Green Alga *Haematococcus pluvialis* *. *Journal of Biological Chemistry*, vol. 276 (2001), no. 8, pp. 6023–6029. Available from: <https://www.sciencedirect.com/science/article/pii/S0021925819463564>. [viewed 11/05/2026].

27. ZIELIŃSKA, Aleksandra; ALVES, Henrique; MARQUES, Vânia; DURAZZO, Alessandra; LUCARINI, Massimo, et al. Properties, Extraction Methods, and Delivery Systems for Curcumin as a Natural Source of Beneficial Health Effects. *Medicina*, vol. 56 (2020), no. 7, pp. 6023–6029. Available from: <https://www.mdpi.com/1648-9144/56/7/336>. [viewed 12/05/2026].

28. VARAPRASAD, Kokkarachedu; SISUBALAN, Natarajan; JAYARAMUDU, Tippabattini and YALLAPU, Murali M. Nanocurcumin: A new and improved way to fight cancer and infections. *Nano-Structures & Nano-Objects*, vol. 40 (2024), no. 7, pp. 101352. Available from: <https://www.sciencedirect.com/science/article/pii/S2352507X24002646>. [viewed 12/05/2026].

29. CHEN, Wei-Ting; WU, Hung-Ta; CHANG, I. -Chia; CHEN, Hua-Wei and FANG, Wu-Po. Preparation of curcumin-loaded liposome with high bioavailability by a novel method of high pressure processing. *Chemistry and physics of lipids*, vol. 244 (2022), no. 7, pp. 105191. Available from: <https://www.sciencedirect.com/science/article/pii/S0009308422000196>. [viewed 12/05/2026].

30. FU, Yaw-Syan; CHEN, Ting-Hsu; WENG, Lebin; HUANG, Liyue; LAI, Dong, et al. Pharmacological properties and underlying mechanisms of curcumin and prospects in medicinal potential. *Biomedicine & Pharmacotherapy*, vol. 141 (2021), no. 7, pp. 111888. Available from: <https://www.sciencedirect.com/science/article/pii/S0753332221006703>. [viewed 12/05/2026].

31. AHMAD, R. S.; HUSSAIN, M. B.; SULTAN, M. T.; ARSHAD, M. S.; WAHEED, M., et al. Biochemistry, Safety, Pharmacological Activities, and Clinical Applications of Turmeric: A Mechanistic Review. *International Journal of Pharmaceutics*, vol. 2020 (2020), no. 7, pp. 111888. Available from: <https://www.scopus.com/inward/record.uri?eid=2-s2.0-85085574769&doi=10.1155%2F2020%2F7656919&partnerID=40&md5=369724cff35f038c6f08585a7713f315>. [viewed 12/05/2026].

32. BARONE, D.; CITO, L.; TOMMONARO, G.; ABATE, A. A.; PENON, D., et al. Antitumoral potential, antioxidant activity and carotenoid content of two Southern Italy tomato cultivars extracts: San Marzano and Corbarino. *Journal of cellular physiology*, vol. 233 (2018), no. 2, pp. 1266–1277. Available from: <https://www.scopus.com/inward/record.uri?eid=2-s2.0-85020406678&doi=10.1002%2Fjcp.25995&partnerID=40&md5=4df67bda482fe4ddf2d5882a4c14b3ba>. [viewed 12/05/2026].

33. GIORDANO, Antonio and TOMMONARO, Giuseppina. Curcumin and Cancer. *Nutrients*, vol. 11 (2019), no. 10, pp. 1266–1277. Available from: <https://www.mdpi.com/2072-6643/11/10/2376>. [viewed 12/05/2026].

34. TOMEH, Mhd A.; HADIANAMREI, Roja and ZHAO, Xiubo. A Review of Curcumin and Its Derivatives as Anticancer Agents. *International Journal of Molecular Sciences*, vol. 20 (2019), no. 5, pp. 111888. Available from: <https://www.mdpi.com/1422-0067/20/5/1033>. [viewed 12/05/2026].
35. ADHIKARI, Priyanka; SINGH, Meenakshi; PUNIA, Jyoti; RANJAN, Alok; PARTHIBAN, A., et al. 3 - Chemical structure and molecular targets of curcumin for cancer therapy In: *Curcumin-Based Nanomedicines as Cancer Therapeutics*, pp. 47–71 KESHARWANI, Prashant and SAHEBKAR, Amirhossein. 2nd ed. Elsevier: Academic Press, 2024. Available from: <https://www.sciencedirect.com/science/article/pii/B9780443154126000118>. [viewed 12/05/2026].
36. YAN, Yuxing; Kulsoom; SUN, Yanbo; LI, Yingjie; WANG, Zhenlong, et al. Advancing cancer therapy: Nanomaterial-based encapsulation strategies for enhanced delivery and efficacy of curcumin. *Materials Today Bio*, vol. 33 (2025), no. 10, pp. 101963. Available from: <https://www.sciencedirect.com/science/article/pii/S2590006425005332>. [viewed 12/05/2026].
37. PARTHIBAN, A.; SIVASANKAR, R.; RAJDEV, Bishal; ASHA, Radhakrishnan Nandini; JEYAKUMAR, Thayalaraj Christopher, et al. Synthesis, in vitro, in silico and DFT studies of indole curcumin derivatives as potential anticancer agents. *Journal of Molecular Structure*, vol. 1270 (2022), no. 10, pp. 133885. Available from: <https://www.sciencedirect.com/science/article/pii/S0022286022015393>. [viewed 12/05/2026].
38. SINGH, A.; SINGH, J. V.; RANA, A.; BHAGAT, K.; GULATI, H. K., et al. Monocarbonyl curcumin based molecular hybrids as potent antibacterial agents. *ACS Omega*, vol. 4 (2019), no. 7, pp. S1–S50. Available from: <https://www.scopus.com/inward/record.uri?eid=2-s2.0-85071521691&doi=10.1021%2facsomega.9b01109&partnerID=40&md5=ea1489fc950d92b5db8b5ea585e2b2b8>. [viewed 12/05/2026].
39. VERMA, Sant Kumar and THAREJA, Suresh. Chapter 11 - An overview on chemistry of natural aldose reductase inhibitors for the management of diabetic complications. *Studies in Natural Products Chemistry*, vol. 65 (2020), no. 9, pp. 381–429. Available from: <https://www.sciencedirect.com/science/article/pii/B9780128179055000111>. [viewed 12/05/2026].
40. LIU, Peng; CHEN, Guiliang and ZHANG, Jingchen. A Review of Liposomes as a Drug Delivery System: Current Status of Approved Products, Regulatory Environments, and Future Perspectives. *Molecules (Basel, Switzerland)*, vol. 27 (2022), no. 4, pp. 1372. doi: 10.3390/molecules27041372. Available from: <https://www.mdpi.com/1420-3049/27/4/1372>. [viewed 13/05/2026].
41. BAYDA, Samer; ADEEL, Muhammad; TUCCINARDI, Tiziano; CORDANI, Marco and RIZZOLIO, Flavio. The History of Nanoscience and Nanotechnology: From Chemical–Physical Applications to Nanomedicine. *Molecules*, vol. 25 (2020), no. 1. Available from: <https://www.mdpi.com/1420-3049/25/1/112>. [viewed 13/05/2026].
42. NSAIRAT, Hamdi; KHATER, Dima; SAYED, Usama; ODEH, Fadwa; AL BAWAB, Abeer, et al. Liposomes: structure, composition, types, and clinical applications. *Heliyon*, vol. 8 (2022), no. 5, pp. e09394. Available from: <https://pmc.ncbi.nlm.nih.gov/articles/PMC9118483/>. [viewed 13/05/2026].
43. CHELLIAH, Ramachandran; RUBAB, Momna; VIJAYALAKSHMI, Selvakumar; KARUVELAN, Murugan; BARATHIKANNAN, Kaliyan, et al. Liposomes for drug delivery: Classification, therapeutic applications, and limitations. *Next Nanotechnology*, vol. 8 (2025), pp. 100209. Available from: <https://www.sciencedirect.com/science/article/pii/S2949829525000786>. [viewed 13/05/2026].
44. NISINI, Roberto; POERIO, Noemi; MARIOTTI, Sabrina; DE SANTIS, Federica and FRAZIANO, Maurizio. The Multirole of Liposomes in Therapy and Prevention of Infectious Diseases. *Frontiers in Immunology*, vol. olume 9 - 2018 (2018). Available from: <https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2018.00155>. [viewed 13/05/2026].

45. MATHIYAZHAKAN, Malathi; WIRAJA, Christian and XU, Chenjie. A Concise Review of Gold Nanoparticles-Based Photo-Responsive Liposomes for Controlled Drug Delivery. *Nano-Micro Letters*, vol. 10 (2017), no. 1, pp. 10. Available from: <https://doi.org/10.1007/s40820-017-0166-0>. [viewed 13/05/2026].
46. HE, Haisheng; LU, Yi; QI, Jianping; ZHU, Quangang; CHEN, Zhongjian, et al. Adapting liposomes for oral drug delivery. *Acta Pharmaceutica Sinica B*, vol. 9 (2019), no. 1, pp. 36–48. Available from: <https://www.sciencedirect.com/science/article/pii/S2211383517307311>. [viewed 13/05/2026].
47. FILIPCZAK, Nina; PAN, Jiayi; YALAMARTY, Satya Siva Kishan and TORCHILIN, Vladimir P. Recent advancements in liposome technology. *Advanced Drug Delivery Reviews*, vol. 156 (2020), pp. 4–22. Available from: <https://www.sciencedirect.com/science/article/pii/S0169409X20300685>. [viewed 13/05/2026].
48. GÓMEZ, Saúl C.; QUEZADA, Valentina; QUIROZ, Isabella; MUÑOZ-CAMARGO, Carolina; OSMA, Johann F., et al. Design and Manufacture of a Low-Cost Microfluidic System for the Synthesis of Giant Liposomes for the Encapsulation of Yeast Homologues: Applications in the Screening of Membrane-Active Peptide Libraries. *Micromachines*, vol. 12 (2021), no. 11. Available from: <https://www.mdpi.com/2072-666X/12/11/1377>. [viewed 13/05/2026].
49. WILLIAM, Bigazzi; NOÉMIE, Penoy; BRIGITTE, Evrard and GÉRALDINE, Piel. Supercritical fluid methods: An alternative to conventional methods to prepare liposomes. *Chemical Engineering Journal*, vol. 383 (2020), pp. 123106. Available from: <https://www.sciencedirect.com/science/article/pii/S1385894719325185>. [viewed 13/05/2026].
50. SHAH, Sanket; DHAWAN, Vivek; HOLM, René; NAGARSENKER, Mangal S. and PERRIE, Yvonne. Liposomes: Advancements and innovation in the manufacturing process. *Advanced Drug Delivery Reviews*, vol. 154-155 (2020), pp. 102–122. Available from: <https://www.sciencedirect.com/science/article/pii/S0169409X20300788>. [viewed 13-05-2026].
51. KRAUSE, Rui; BAPOLISI, Alain; NKANGA, Christian and NNAMDI, Okafor. General Perception of Liposomes: Formation, Manufacturing and Applications In: *Liposomes - Advances and Perspectives*. CATALA, Angel ed., 1st ed. London: IntechOpen, 2019. Available from: <https://doi.org/10.5772/intechopen.84255>. [viewed 13/05/2026].
52. SUBRAMANIAN, Parthasarathi. Chapter 14 - Liposomes in food industries: Challenges and future scope In: *Liposomal Encapsulation in Food Science and Technology*, pp. 269–285 ANANDHARAMAKRISHNAN, C. and DUTTA, Sayantani. - ed. Academic Press, Cambridge, Massachusetts, United States: Academic Press, 2023. Available from: <https://www.sciencedirect.com/science/article/pii/B9780128239353000047>. [viewed 13/05/2026].
53. JENSEN, Gerard M. and HODGSON, Donald F. Opportunities and challenges in commercial pharmaceutical liposome applications. *Advanced Drug Delivery Reviews*, vol. 154-155 (2020), pp. 2–12. Available from: <https://www.sciencedirect.com/science/article/pii/S0169409X20301009>. [viewed 13/05/2026].
54. FULTON, Melody D. and NAJAH-MISSAOUI, Wided. Liposomes in Cancer Therapy: How Did We Start and Where Are We Now. *International Journal of Molecular Sciences*, vol. 24 (2023), no. 7. Available from: <https://www.mdpi.com/1422-0067/24/7/6615>. [viewed 13/05/2026].
55. AGRAWAL, Surendra S.; BALIGA, Vrinda and LONDHE, Vaishali Y. Liposomal Formulations: A Recent Update. *Pharmaceutics*, vol. 17 (2024), no. 1, pp. 36. doi: 10.3390/pharmaceutics17010036. Available from: <https://www.mdpi.com/1999-4923/17/1/36>. [viewed 13/05/2026].
56. CHEN, Jiayi; HU, Siyuan; SUN, Man; SHI, Jianan; ZHANG, Huan, et al. Recent advances and clinical translation of liposomal delivery systems in cancer therapy. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences*, vol. 193 (2024), pp. 106688.

Available from: <https://www.sciencedirect.com/science/article/pii/S0928098723003160>. [viewed 13/05/2026].

57. WANG, Huan; LIN, Shiqi; WU, Xiying; JIANG, Kuan; LU, Huiping, et al. Interplay between Liposomes and IgM: Principles, Challenges, and Opportunities. *Advanced Science*, vol. 10 (2023), no. 20, pp. 2301777. Available from: <https://doi.org/10.1002/adv.202301777>. [viewed 13-05-2026].

58. SAINAGA JYOTHI, Vaskuri G. S.; BULUSU, Raviteja; VENKATA KRISHNA RAO, Balaga; PRANOTHI, Mulinti; BANDA, Srikanth, et al. Stability characterization for pharmaceutical liposome product development with focus on regulatory considerations: An update. *International journal of pharmaceutics*, vol. 624 (2022), pp. 122022. Available from: <https://www.sciencedirect.com/science/article/pii/S0378517322005774>. [viewed 13-05-2026].

59. CHELLIAH, Ramachandran; RUBAB, Momna; VIJAYALAKSHMI, Selvakumar; KARUVELAN, Murugan; BARATHIKANNAN, Kaliyan, et al. Liposomes for drug delivery: Classification, therapeutic applications, and limitations. *Next Nanotechnology*, vol. 8 (2025), pp. 100209. Available from: <https://www.sciencedirect.com/science/article/pii/S2949829525000786>. [viewed 13-05-2026].

60. SUN, Liyan; LI, Zhe; LAN, Jinshuai; WU, Ya; ZHANG, Tong, et al. Better together: nanoscale co-delivery systems of therapeutic agents for high-performance cancer therapy. *Frontiers in Pharmacology*, vol. 15 - 2024 (2024). Available from: <https://www.frontiersin.org/journals/pharmacology/articles/10.3389/fphar.2024.1389922>. [viewed 13-05-2026].

61. CHANG, Hsin-I; SHAO, Chu-Wen; HUANG, Evelyn and HUANG, Kuo-Yuan. Development of Astaxanthin-Loaded Nanosized Liposomal Formulation to Improve Bone Health. *Pharmaceutics*, vol. 15 (2022), no. 4. Available from: <https://www.mdpi.com/1424-8247/15/4/490>. [viewed 13/05/2026].

62. PASARIN, Diana; GHIZDAREANU, Andra-Ionela; ENASCUTA, Cristina E.; MATEI, Catalin B.; BILBIE, Catalin, et al. Coating Materials to Increase the Stability of Liposomes. *Polymers*, vol. 15 (2023), no. 3. Available from: <https://www.mdpi.com/2073-4360/15/3/782>. [viewed 13/05/2026].

63. PANDE, Shantanu. Liposomes for drug delivery: review of vesicular composition, factors affecting drug release and drug loading in liposomes. *Artificial Cells, Nanomedicine, and Biotechnology*, vol. 51 (2023), no. 1, pp. 428–440. Available from: <https://doi.org/10.1080/21691401.2023.2247036>. [viewed 13/05/2026].

64. YU, Ji Y.; CHUESIANG, Piyanan; SHIN, Gye H. and PARK, Hyun J. Post-Processing Techniques for the Improvement of Liposome Stability. *Pharmaceutics*, vol. 13 (2021), no. 7. Available from: <https://www.mdpi.com/1999-4923/13/7/1023>. [viewed 13/05/2026].

65. ZHAO, Yingyuan; WANG, Zhaoxuan; LIU, Shuxian; XIE, Shiyang; XIE, Yinchun, et al. Lyoprotectant Formulation and Optimization of the J-Aggregates Astaxanthin/BSA/Chitosan Nanosuspension. *Biomolecules*, vol. 13 (2023), no. 3, pp. 106188. Available from: <https://www.sciencedirect.com/science/article/pii/S135041772200284X>. [viewed 13/05/2026].

66. DONG, Hualin; WANG, Peng; YANG, Zongyun; LI, Ru; XU, Xinglian, et al. Dual improvement in curcumin encapsulation efficiency and lyophilized complex dispersibility through ultrasound regulation of curcumin–protein assembly. *Ultrasonics sonochemistry*, vol. 90 (2022), no. 9, pp. 106188. Available from: <https://www.sciencedirect.com/science/article/pii/S135041772200284X>. [viewed 13/05/2026].

67. ADEL, Islam M.; ELMELIGY, Mohamed F.; ABDELRAHIM, Mohamed E. A.; MAGED, Amr; ABDELKHALEK, AbdelFattah A., et al. Design and Characterization of Spray-Dried Proliposomes for the Pulmonary Delivery of Curcumin. *International journal of nanomedicine*, vol. 16 (2021), pp. 2667–2687. Available from: <https://pubmed.ncbi.nlm.nih.gov/33854314/>. [viewed 13/05/2026].

68. WANG, Yuwei and GRAINGER, David W. Lyophilized liposome-based parenteral drug development: Reviewing complex product design strategies and current regulatory environments. *Advanced Drug Delivery Reviews*, vol. 151-152 (2019), pp. 56–71. Available from: <https://pubmed.ncbi.nlm.nih.gov/30898571/>. [viewed 13/05/2026].
69. ALI, Said A.; HELMY, Hagar I. and GABER, Mohamed H. Comparative in vitro cytotoxicity of free curcumin and a liposomal curcumin formulation on various human cancer cell lines. *Scientific Reports*, vol. 16 (2026), no. 1, pp. 6346. Available from: <https://doi.org/10.1038/s41598-026-36607-x>. [viewed 13/05/2026].
70. NÉMETH, Zsófia; CSÓKA, Ildikó; SEMNANI JAZANI, Reza; SIPOS, Bence; HASPEL, Henrik, et al. Quality by Design-Driven Zeta Potential Optimisation Study of Liposomes with Charge Imparting Membrane Additives. *Pharmaceutics*, vol. 14 (2022), no. 9, pp. 1798. doi: 10.3390/pharmaceutics14091798. Available from: <https://www.sciencedirect.com/science/article/pii/S0378517324013152>. [viewed 13/05/2026].
71. CHEN, Hua-Wei; CHEN, Su-Der; WU, Hung-Ta; CHENG, Chun-Hung; CHIOU, Chyow-San, et al. Improvement in Curcumin's Stability and Release by Formulation in Flexible Nano-Liposomes. *Nanomaterials (Basel, Switzerland)*, vol. 14 (2024), no. 22, pp. 1836. doi: 10.3390/nano14221836. Available from: <https://www.sciencedirect.com/science/article/pii/S0378517324013152>. [viewed 13/05/2026].
72. SINGH, Archu; NEUPANE, Yub Raj; SHAFI, Sadat; MANGLA, Bharti and KOHLI, Kanchan. PEGylated liposomes as an emerging therapeutic platform for oral nanomedicine in cancer therapy: in vitro and in vivo assessment. *Journal of Molecular Liquids*, vol. 303 (2020), pp. 112649. Available from: <https://www.sciencedirect.com/science/article/pii/S0167732219357113>. [viewed 15/05/2026].
73. WANG, Zijian; SONG, Sichen; ZHANG, Hongwei; LIU, Xiaohong; A. SIEGEL, Ronald, et al. Impact of solid content on the bulk properties of lyophilized powders. *International journal of pharmaceutics*, vol. 670 (2025), no. 6, pp. 125081. Available from: <https://www.sciencedirect.com/science/article/pii/S0378517324013152>. [viewed 13/05/2026].
74. JEETENDRA, Kumar Gupta; NAGABHOOSHANAM, N.; FALAH, Hassan Shari and HARIRAM, Venkatesan. Curcumin Nanostructured Drug Delivery Induces the Anti-cancer and Anti-inflammatory activity In Vitro and In Vivo. *Journal of Angiotherapy*, vol. 8 (2024), no. 10, pp. 1. Available from: <https://doi.org/10.25163/angiotherapy.8109997>. [viewed 13/05/2026].
75. CHEN, Siqian; WANG, Jiayi; FENG, Jiating and XUAN, Rongrong. Research progress of Astaxanthin nano-based drug delivery system: Applications, prospects and challenges?. *Frontiers in pharmacology*, vol. 14 (2023), no. 7, pp. 1102888. Available from: <https://pmc.ncbi.nlm.nih.gov/articles/PMC10034004/>. [viewed 13/05/2026].
76. ANDRIANI, Riza; SUBROTO, Toto; ISHMAYANA, Safri and KURNIA, Dikdik. Enhancement Methods of Antioxidant Capacity in Rice Bran: A Review. *Foods*, vol. 11 (2022), no. 19, pp. 1. Available from: <https://www.mdpi.com/2304-8158/11/19/2994>. [viewed 13/05/2026].
77. CARDOSO-DAODU, Ibilola; ILOMUANYA, Margaret Okonawan and AZUBUIKE, Chukwuemeka Paul. Development of curcumin-loaded liposomes in lysine–collagen hydrogel for surgical wound healing. *Beni-Suef University Journal of Basic and Applied Sciences*, vol. 11 (2022), no. 1, pp. 100. Available from: <https://doi.org/10.1186/s43088-022-00284-2>. [viewed 13/05/2026].
78. CHANG, Hsin-I; SHAO, Chu-Wen; HUANG, Evelyn and HUANG, Kuo-Yuan. Development of Astaxanthin-Loaded Nanosized Liposomal Formulation to Improve Bone Health. *Pharmaceutics*, vol. 15 (2022), no. 4, pp. 1. Available from: <https://www.mdpi.com/1424-8247/15/4/490>. [viewed 13/05/2026].

List of publications

Presentation of research results at conferences

1. Sahaleh H., Stabrauskienė J., Bernatoniene J. *Development and Bioinformatic Evaluation of Liposomal Astaxanthin and Curcumin for Targeted Cancer Therapy*. Poster presentation at the EUROPIN Summer School on Drug Design 2025, University of Vienna, Vienna, Austria, September 2025.
2. Sahaleh H., Stabrauskienė J., Bernatoniene J. *Design and Characterisation of Liposomal Curcumin and Astaxanthin Microcapsules for Oral Drug Delivery*. Presented at the 69th International Scientific Conference for Students of Physics and Natural Sciences “Open Readings 2026”, Vilnius, Lithuania, April 2026.
3. Sahaleh H., Stabrauskienė J., Bernatoniene J. *Addressing Drug Delivery Challenges of Astaxanthin Through Liposomal Techniques for Oral Anticancer Therapy*. Presented at the International Scientific Conference “Chemistry and Chemical Technology 2026” (CCT2026), Kaunas University of Technology, Kaunas, Lithuania, May 2026.