Republic of Iraq

Ministry of Higher Education and Scientific Research

University of Babylon / College Sciences

Department of Chemistry

The Cowpea Mosaic Virus modification as acarrier for some new Chloroquine derivatives and Its examinad as anti Breast cancer agent

A thesis
Submitted to the Council of the College of Science,
The University of Babylon as a Partial Fulfillment of the Requirements for a
Master's Degree in Sciences / Chemistry

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1444 .A.H **2022 A.D**

بِسْمِ اللهِ الرَّحْمَنِ الرَّحِيمِ

فتعالى الله الملك الحق ولَا تَعْجَلْ بِالْقُرْآنِ مِن قَبْلِ أَن يُقْضَىٰ إِلَيْكَ وَحْيُهُ ۖ وَقُل رَّبِّ زِدْنِي عِلْمًا

صَدَقَ اللهُ العليُّ العظيمُ

((سوره طه/ الايه١١٤))

CERTIFICATION

I certify that this thesis entitled **(The modification of cowpea mosaic virus with some of new chloroquine derivatives and its application as anti- cancer drug)** was prepared under my supervision at the Department of chemistry/ College of Science / University of Babylon , in partial requirements for the Degree of Master of Science in Biochemistry and this work has never been published anywhere.

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DEDICATIONS

To my dear mother's soul....may Allah have mercy on her.

To my dear father's soul....may Allah have mercy on him.

I dedicate a special appreciation to my dear husband , and also

For my dear brothers and sisters.

Khalida Mohee Hbeeb Ali AL Kafage

Acknowledgements

"In The Name of God Most Gracious Most Merciful"

Thanks and praise be to Allah prayers and peace be upon His Prophet Muhammad and his pure good family to complete this stage

From my heart, I would like to thank my supervisor, Assistant Prof. Dr. Rana Abd Al-Aly Al Refai'a for her guidance and support for the project and for providing the necessary facilities

Many thanks to the head and members of the Chemistry Department, College of Sciences, University of Babylon, for their assistance in this work. Thanks were also extended to Prof. Dr. Ahmed Ali Abdulsahib Alkarimi and Dr. Saba. AL-Obaidy, the rapporteur for higher studies, for her efforts to help postgraduate students.

I also thank Assist. Prof. Dr. Noor Salman Kadhim, for helping me complete the research requirements, I would like to express my gratitude to anyone who directly or indirectly contacted this project

Finally, I would like to thank all my friends who supported me in finishing my research.

The work was done in the advanced biochemistry laboratory in the Department of Chemistry,College of Science, University of Babylon which is under the Management of **Dr. Oda M. Yasser Al -Zamely.**

Khalida Mohee Hbeeb Ali AL Kafage

Summary

Multifunctional plant virus nanoparticles offer significant benefits for the field of nanotechnology. In this research, a plant virus called cowpea mosaic virus (CPMV) was utilized because it is easily isolated in gram quantities, its structure is known to atomic resolution, and it is very stable. It could be used as a molecular entity in synthesis, especially as a nanochemical building block. Each asymmetric unit of CPMV has an enhanced-reactivity lysine residue, for a total of 60 per virus particle.

In this work, (CPMV) was used as a potential carrier vehicle for some new chloroquine derivatives as chemotherapeutic medicines. The external capsid surface has been chemically modified with new chloroquinoline derivatives, addressable carboxyl groups have been modified with 4,7dichloroquine-Doxorubicine (CQ-DOX) and4,7 dichloroquine-Docetaxel (CQ-DOC). While, addressable amine on the exterior surface has been attached to 4,7dichloroquine-Salcylic acid (CQ-SAL) with a hope to produce promising anticancer drugs. The ability to bind each derivative to CPMV has been confirmed.

The capacity to alter exterior amine and carboxyl of cowpea mosaic virus (CPMV) with synthesized chloroquinoline derivatives has been investigated. Additionally, the conjugation was confirmed using UV-Vis spectrophotometer , electrophoresis and transmission electron microscopy (TEM) while the number of each conjugated derivative was calculated using Alexa Flour Dye 488 and CF-488 A to be 87±**2** ,79±**1** and 89± **1** respectively for (CQ-DOX,CQ-DOC and CQ-SAL).

All conjugated and unconjugated CQ-derivatives have tested as anticancer activator using MTT assay and ADPI.

Summary

In this study, new chloroquine derivatives derived from the reaction of 4,7-dichloroquine with (doxorubicin ,docetaxel and salicylic acid) were synthesized first and fully characterize using FTIR, CHN and ^{1}HNMR to produce (CQ-DOX,CQ-DOC and CQ-SAL).

The presented results were evaluated to be more than 50% reduction in cell viability (IC_{50}) for each CQ- derivative.

The affectivity of attached and unattached CQ-compounds to the CPMV's surface, was examined by MTT assay and ADPI, and the IC_{50} for each CQ-derivative with and without conjugation with CPMV was evaluated to be (70.395µg/ml for CQ-DOX, 14.384µg/ml for CQ-DOC and 74.065µg/ml for CQ-SAL) before modification then after modification o.o15 nM for CQ- DOX,0.038 nM for CQ-DOC and 0.042 nM for CQ-SAL.

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LIST OF ABBREVIATION

Abbreviation	Details
AKT	A serine/threonine protein kinase
BMV	Brome Mosaic Virus
CPMV	Cowpea Mosaic Virus
CPMVB	bottom CPMV
CPMV-M	central CPMV
CPMVT	top CPMV
CQ	Chloroquine
Ds	double-stranded
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DOX	Doxorubicin
DNA	Deoxyribonucleic acid
DOC	Docetaxel
DMSO	Dimethyl sulfoxide
DAPI	4,6-Diamidino-2-phenylindole dihydrochloride
EDC	1-ethyl-3- (3-dimethyl aminopropyl) carbodiimide hydrochloride
EMP	Ethyl mercury phosphate
FTIR	Fourier transform infrared spectroscopy
Gd(DOTA)	Gadoteric acid
Gd	Gadolinium
HCRSV	Hibiscus Chlorotic Ring Spot Virus
HCQ	Hydroxychloroquine
JgCSMV	Johnson grass chlorotic stripe mosaic virus
L and S	Large and Small coat proteins
L-DOX-CQ	doxorubicin and chloroquine loaded liposomes
MTT	3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide
NPs	Nanoparticles
NP	Nanoparticle
NHS	N- hydroxy succinimide
Ph MV	Physalis Mottle Virus
PEG	poly ethylene glycol

PH	Negatives logarithm to the base ten of the concentration of $[H]^+$ ions
PBS	Phosphate-buffered -saline
PEP	Polyphosphazene
P18/TPGS	poly (ethylene oxide) -block - poly (propylene oxide) -block - poly (e - caprolactone) (PEO68 – PPO34 – PCL18) and o - a - tocopheryl poly (ethylene glycol) (TPGS
P36/TPGS	poly (ethylene oxide) -block - poly (propylene oxide) -block - poly (e - caprolactone) (PEO68 – PPO34 – PCL36) and o - a - tocopheryl poly (ethylene glycol) (TPGS).
PEG-b-PLGA	poly(dl-lactide-co-glycolide-b-ethylene glycol-b-dl-lactide-co-glycolide)
RCMNV	Red clover necrotic mosaic virus
RNA	Ribonucleic acid
SAL	Salicylic acid
TMV	Tobacco mosaic virus
TME	Tumor microenvironment
TEM	Transmission- electron- microscopy
TPGS	Tocopherol Polyethylene Glycol Succinate
VLPs	Virus like particles
VLP	Virus like particle
VNPs	Virus *nanoparticles*

Chapter One

Indrodaction

1- Introduction

1.1- Nanoparticles

Nanotechnology has grown significantly over recent years and has been applied to medical applications, focusing on the diagnosis of diseases and their treatment. Diagnostics, nutraceuticals, and the development of biocompatible materials are all examples of *in vitro* and *in vivo* drug delivery applications. Nanoparticles are minuscule entities that, in terms of mobility and attributes, behave like whole units. Nanoparticles have been investigated for their possible applications in biotechnology, pharmacology, and pure technology. They function as a link between bulk materials and atomic species. [1,2] This is where a series of atoms will generate a cluster; a collection of clusters will form a nanoparticle. Large quantities of nanoparticles will aggregate to form a bulk particle, often on the micron scale or above. the physical and chemical properties of most nanoparticles are frequently dictated by their size.[3,4] Both science and technology are referred to with the term nano; nanoscience is found in everyday life sciences, physical sciences and materials science. Nanotechnology, on the other hand, has a broader scope because it is used in a variety of fields for example medicine, energy, textiles, electronics and transportation. In general, when a nanoparticle's size range is between 1 and 100 nm, the properties are much different and it depends on the application at hand. If a nanoparticle is very small, the surface area will far exceed its volume, resulting in higher surface–to–volume ratios than bigger nanoparticles or bulk materials.[5,6] The greater surface area for nanomaterials can mean much higher reactivity, magnetic properties or conductivity. [7]

Nanomedicine is a new discipline that blends nanotechnology with pharmaceutical and biomedical sciences in the hopes of creating more effective medications and imaging agents with enhanced safety, higher efficacy and toxicity characteristics. Because of their sub-micrometer size and high surface area to volume ratio, these materials exhibit significant differences in biochemical, magnetic, optical, and electronic properties as compared to bulk materials. [8-11]

1.1.1- Medicine and nanoparticles

Nanoparticles (NPs) have a wide range of applications in modern medicine. In recent years, these materials had significant roles in this field, with therapeutic applications ranging from contrast agents in imaging to drug and gene delivery carriers into tumors. There are specific cases where nanoparticles are a must-have for conducting analyses and carry out therapies that would not be possible. In the last ten years, NPs had been received significant attention due to their use in drug delivery systems. [12,13]Some of biological and medicinal applications of nanoparticle (NP) are included :

- Research and illness diagnostics, biological labels for major biological markers.
- Biological recognition of diseases
- Investigating the structure of DNA
- Eradication of tumors with medicines or heat

- Protein identification
- Genetic and tissue engineering
- Genetic therapy as gene delivery systems
- Purification and separation of cells and biological molecules
- MRI investigations
- Drug delivery systems
- Pharmacokinetics

Many compounds have been investigated for the drug delivery of anti-malarial therapy and cancer treatment. Nanoparticles are utilized to drastically minimize toxicity and negative effects of pharmaceuticals, they are used as a vehicle to deliver small and large molecules by changing their pharmacodynamics and pharmacokinetic properties. [14,15]

Furthermore, NPs have the ability to prevent the medication from being released throughout its storage and delivery, until they reach an area at which they are activated. Thus, there's a possibility for the development of targeted drugs.[16]

1.1.2- Methods of preparing nanomaterials

1- Gas Condensation

2- Vacuum Deposition and Vaporization

3- Chemical Vapor Deposition (CVD)

4- Chemical Vapor Condensation (CVC)

5-Mechanical Attrition

6- Chemical Precipitation

7- Sol-Gel Techniques

8- Electrodeposition[17]

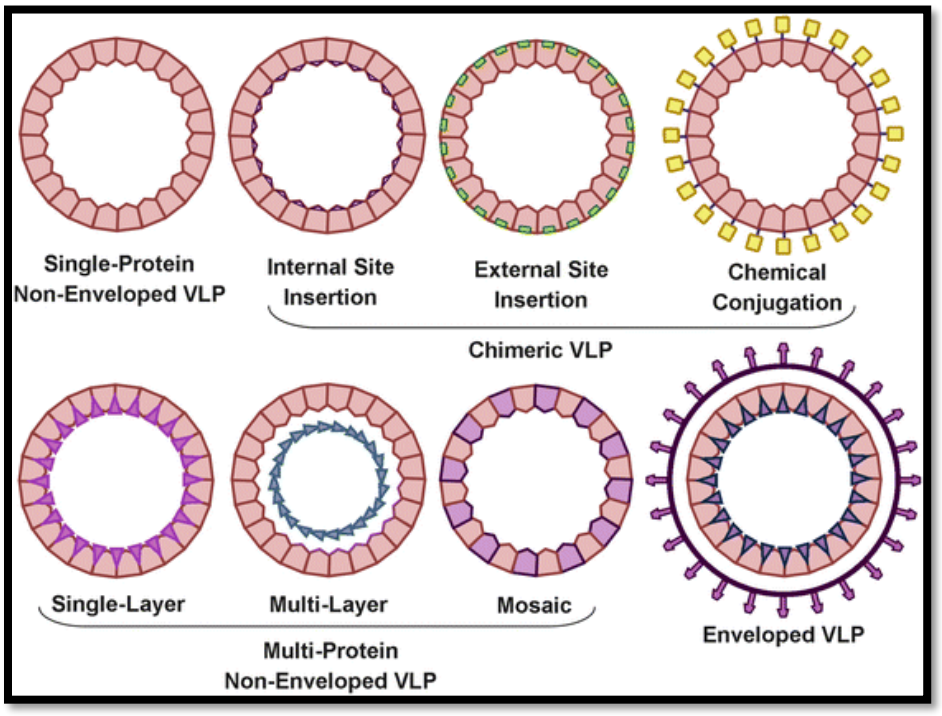
1.1.3-Virus like particles (VLPs)

Virus like particles (VLPs) are self-assembling capsid protein complexes that have the same general structure as their parent virus. These noninfectious particles, which lack viral genetic material, have biologically beneficial characteristics that are attributed to the particulate viral structure **(Figure 1.1)** . [18,19]

VLPs are existed nanoparticles in different living system that can be made by humans, plants, insects, and microorganisms. Because of the cavity within their structure, VLPs can be utilized to transport bio- and nanomaterials for instance, medicines,vaccinations,quantumdots,and imaging. [20-22]

The structures of VLP is icosahedral or rodshaped, formed by viral structural proteins selfassembling.[23] In 1968, researchers observed these nanoparticle forms in the serum of people with Down syndrome, hepatitis, as well as leukaemia patients. Despite the discovery of antigenic surface functional groups of these particles, their biological nature stayed unidentified.[24] Following that, VLP structures have been identified in virus capsids, envelopes, and, in certain cases, core viral proteins. Recombinanting virus-related proteins expressed in various expression organisms, comprising mammals cell lines, yeast, insects cell cultures, plants, and prokaryotic cells, can be used to make VLPs in the lab. [24,25] While

most VLPs are produced from the protein(s) of asingle virus, chimeric VLPs can produce via linking a structure of proteins from many distinct viruses.[26] The potential of VLPs in vaccination, which may possibly provide a major benefit from over traditional vaccine techniques, has been underutilized.[27] Because of their size and shape, which matches that of genuine virusess, the structure, can effectively provoke immuneresponses, and VLP lacks viral genomes with no possibility for duplication inside target cells; this improves vaccine's safety, particularly for immuncompromised or old vaccines.[24,28] Both pathophysiologic and cellular immunological responses can be activated by VLPs. [29]



Figu 1.1VLP Structure. VLPs can be categorised based on characteristic structural features such as capsid protein composition, encapsulation inside a lipid bilayer envelope, and incorporation of antigens by recombinant insertion or chemical conjugation. Additional combinations other than those illustrated also exist, such as multi-protein chimeric VLPs and enveloped mosaic or chimeric VLPs [30]

1.1. 4 -Virus nanoparticles (VNPs)

Plant viruses are generally identified according to their morphologies to icosahedral or helical, which provide alots of properties that enable them to be used in a variety of medical and technical applications. [31,32] Both morphologies

are possible transport medications or reagents for imaging that are attached to the outer surface of the protein's capsid, resulting remarkably regular repeating for attached materials on the virus particle's surface.[33] Several plant viruses' have helical shape that seems to contribute to their capability to target solid tumours.[34] Icosahedrall virusess, in contrast, have the ability to conjugate with active compounds, for example, medicines in their interior cores and discharge them when their physiologic condition are appropriate.[35,36]The use of icosahedrall and helicall viruses as immunotherapeutic(Immunotherapy is a type of cancer treatment. It uses substances made by the body or in a laboratory to boost the immune system and help the body find and destroy cancer cells)[37]agents toward help fighting chronic diseases like cancer .[38]

Icosahedral virus nanoparticles

Plantviruses that have manufactured as a nanoparticle and have an icosahedral morphology involve, Cowpea MosaicVirus (CPMV), Hibiscus Chlorotic Ring SpotVirus (HCRSV), Red clover necrotic mosaic virus (RCMNV) and Physalis Mottle Virus (Ph MV) **Table 1.1**. The multivalent scaffold for eptiope display is provided by icosahedral viruses' highly ordered symmetry. [39,40]Empty virus-like particles based on icosahedral plant viruses, on the other hand, it can be used as amolecular frame platform to transport medications, imaging agents, and supplementary resources.[41] Covalent binding to particular active moieties on the capsid protein allows drugs to load hooked on icosahedral viruses. A regulated sensitivity mechanism to PH ,and metallic ion concentration can be used to load

the interior cavity of a VLP. Some VLPs, for example, are tempted toward swelling and opening pores at high pH, allowing drug cargo to enter. The swelling is reversed when the PH is reduced again, and then the drug is locked inside. Outside of virus particles can be conjugated with a number of tissue-specific ligands to target these icosahedral nanoparticles. [42,43] The following are some examples of how icosahedral plant virus nanoparticles are currently being used.**Table1.1 .**

Table 1.1 Icosahedral plant virus and its application.

Plant virus nanoparticle	Application tested	Reference
Cowpea mosaic virus(CPMV)	Solid tumors	[44-46]
Red clover necrotic mosaic virus (RCNMV) Incapsulating DOX	Ovarian cancer, melanoma	[47]
Hibiscus Chlorotic ringspot virus(HCRSV) Encapsulating DOX ,PSA and PAA Johnson grass Chlorotic stripe mosaic virus	Ovarian cancer	[34]
Physalis mottle virus (PhMV) encasiulating DOX and MTS	Untested	[34]

1.1.5- The lifestyles of plant virus

There are four different lifestyles of plant viruses; these are, determined, acute, chronic and endogenous. Several viruses have the ability to switch between lifestyles, mostly between acute and chronic; further changes are possible but their occurrence is very uncommon. Plant viruses that are persistent have a number of characteristics: They are asymptomatic, though paucity of un-infected.plants makes diagnosis difficult in most circumstances; and they are spread vertically by gametes, they cannot transfer between cells in plants. Finally, they can be found in all cells, including those in the meristem.[48,49] All viruses in this classification have double-stranded (ds) RNA genomes so far. Fungus and particularly endophytic fungi, are infected by members of all of the virus families that contain persistent plant viruses. Persistent viruses constitute the majority of all plant viruses, according to ecogenomic examinations of wild plants. According to unpublished statistics obtained by M. J. Roossinck, incidence rates in some plant groups can reach 70%. This explanation of a persistent plant virus is worlds apart from that of the persistent mode of vector transmission. Vector transmission refers to the dispersion of a pathogen via both air borne and land based arthropods. Thus this is not relatable with the lifestyle of the plant virus itself.[50,51]

Plant viruses that are acute, as opposed to persistent viruses have an alternative life cycle. Their transmission is horizontal, some hosts allow the virus to be transmitted vertically, where vertical transmission can be through embryos or gametes. In this area, special proteins are designed and encoded to allow cell-to-cell

Acute viruses are flourishing in monoculture, this is comparable with acute animal viruses and it has been suggested that the lifestyle of an acute human virus is typically associated with large built up human areas, as well as domesticated animals.[50,52]

Another group of plant viruses are called Endogenous, this species integrates with the genome of the plant directly. A sizeable amount of these viruses are actually remnants of a benign ancient virus infection. There's a high possibility of their activation under specialized conditions. Discovering new plant viruses is imperative due to a matter of plant biosecurity. The motivation for identifying new plant viruses is impacted by; the causes of viral diseases in crops, specific virus screening and the ability to sequence viruses regardless of initial symptoms in an ecosystem The main motivation for this is that new sequencing technologies have paved the way to discover new viruses and a range of other microorganisms.[50,53]

Plant virus classification is affected and depends on aspects such as size, shape, type, organisation of genome and chemical structure of proteins and replication. Nucleotide sequences of viral genomes have been determined. However, virus classification will remain important for the future as the majority of phenotypic characters used at the present time will still be relevant **(Figure 1.2)** . [54,50]

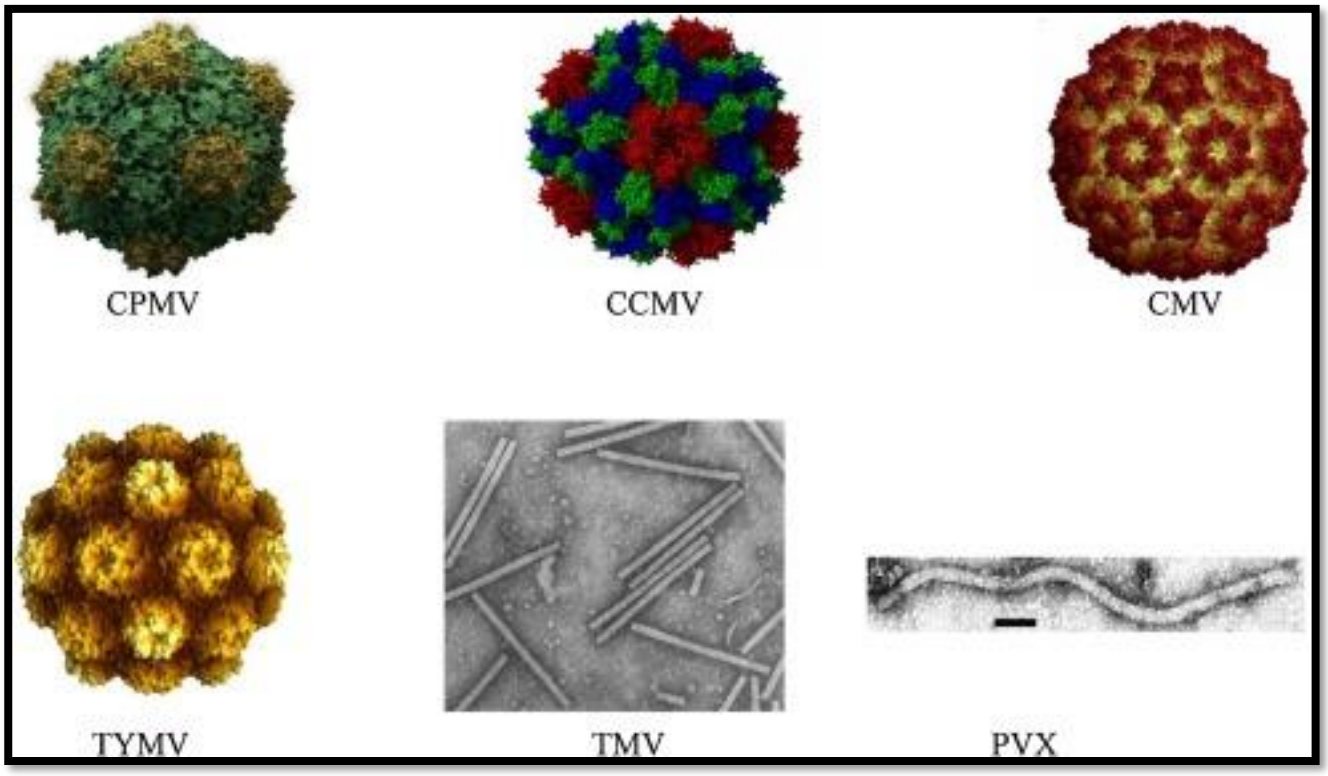


Figure 1.2 Types of plant viruses CPMV- Cowpea mosaic virus,CCMV- Copea chlorotic virus,CMV- Cytomega virus,TYMV-Turnip yellow mosaic virus,TMV-Tobacco mosaic virus,PVX-Patato virus X[50]

1.1.6- Cowpea mosaic virus (CPMV)

Cowpea mosaic virus (CPMV) is a plant-infecting virus with nonenveloped capsids that has been intensively considered, it contains eight families plus hundreds distinct viruses that include members that infect vertebrates (for example, viral diseases of feet and mouth and also polio virus, insects (like acute bee paralysis virus). [55,56]

Cowpea mosic virus is a member of the Comoviridaesub family, and contains a single stranded, and positive sense RNA genome. The genome of CPMV has two parts of RNA, RNA1 (6kb) and RNA2 (3.5kb) encapsidated independently **(Figure. 1.3a)**. The icosahedral capsid structure of CPMV has a diameter of 30 nm and is made up of 60 copies of the Large (L) and Small (S) coat proteins.[50,57]

With the activity of 24K viral proteinase, which encodes using RNA1, the two coat proteins are produced by a single RNA2 encoded as precursor polyprotein (VP60). As a result, both genomic regions in an infected plant cell are required for capsid construction and viral infection. [58] Density gradient ultracentrifugation can be used for separating the two different genomic RNAs **(Figure 1.3b)**, particles having RNA1 are known as the bottom CPMV-B part, RNA2 as central CPMV-M, and the top part of the CPMV particle with no packaged genome are known as CPMV-T. [59]

Cowpea mosic virus capsid's structure is well understood, and three Comoviruses have crystallographic structures: CPMV,BPM, also red clover mottle viruses.[60] Large and Small Subunits together form three β barrel domaines (2 from L, 1 from S; Figure. 1.3c), with a jely roll arrangement. The jelly roll, found in various viruses structures, is made up of two twisted antiparallel β sheets, each one with 4 strands.[50,57,61,62] **(Figure. 1.3c)**.The S subunit's C terminal 24 amino acids are required for viral assembly and genome encapsidation; nevertheless, these amino acids cleave during typical virus maturation and hence absent from Xray structures of Comovirus, with Lys189 being shown as the amino acid in the S subunit of CPMV. As a result, the three jelly-roll domains correspond to the three quasi-equivalent conformers of a $T = 3$ icosahedral lattice, and Como virus capsids adopt $pT = 3$ quasi symmetry **(Figure. 1.3d)**. At the particle's fivefold axes, there are

noticeable turrets created by the S subunit. Indeed, for all Picornavirales capsids, the penton of a set of coat protein subunits appears to represent the basic building block: for example, a penton of L and S for CPMV or a penton of VP1/2/3/4 for poliovirus. [57,63]

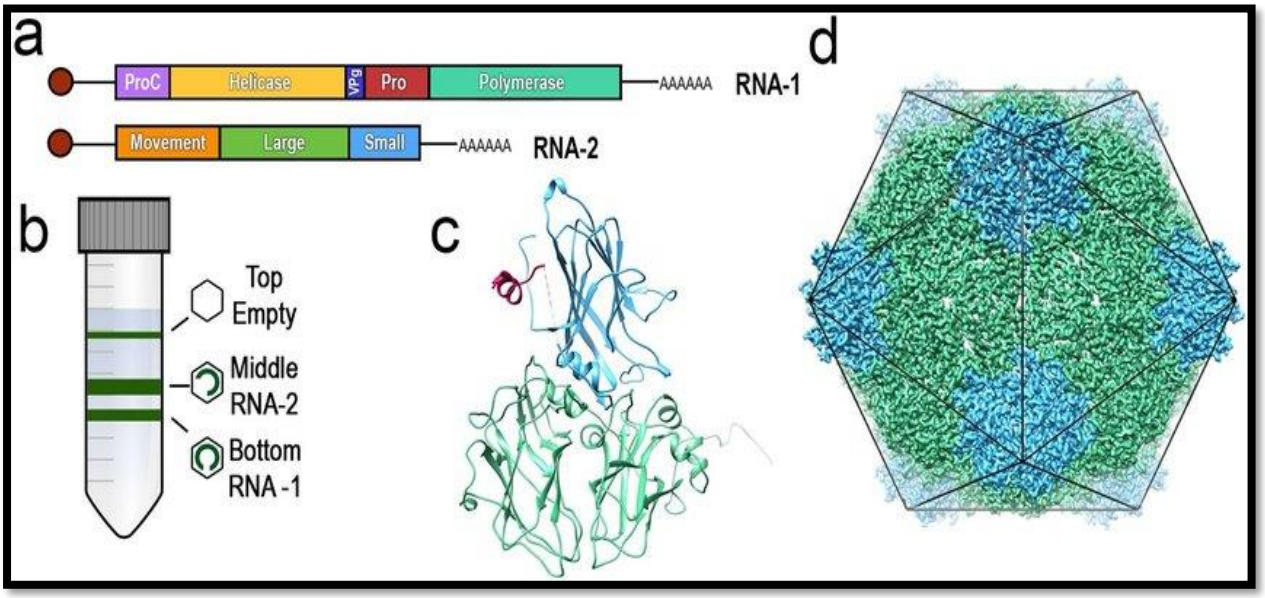


Figure 1.3. a, A single-stranded CPMV bipartite RNA genome. b, Centrifugation of CPMV. c, an asymmetrical unit of empty CPMV and d, EM a derived map of empty particles.[57]

CPMV has the benefit of being a nanoparticle since it can be genetically and chemically changed. It is possible to create multilayer arrays and it has the ability to conjugate with inorganics, organics, and biological molecules made into electroactive nanoparticles and used as a template for metallization .[50]

1.1.6.1 -CPMV exploitable surface

CPMV's structure is known to be near atomic resolution. According to X–ray diffraction examination, the particles have an average diameter of 28-30 nm, and the capsid protein is 3.9 nm thick. [64] The L and S coat proteins are duplicated 60 times in the CPMV capsid. As a result, functional groups like glutamate, aspartate, arginine, and lysine are highly symmetrical and repeated on the outside of a virus capsid. These functional groups have been used as a template for the synthesis of nanoparticles. Since 2002, when CPMV was originally explored as an accessible nano building block, surface–exposed amino acids have been used as targets for bioconjugation chemistry . [65,66,67]

The function groups on the external surface (–NH2 or –COOH) are the most typically used for chemical modification because they provide a specific addressable site. Various covalent coupling techniques including "click" chemistry have been employed to achieve this, on the other hand, the genetic variations of CPMV have been exploited as scaffolds for chemical modification; Early studies of CPMV's chemical reactivity focused on lysine- and cysteine-selective derivatives.[56,68] Each asymmetric unit contains five solvent–exposed Lys, equating to three hundred exposed Lys sidechains per CPMV particle according to the Xray structure and coordinates of CPMV (VIPER database).[69] **(Figure 1.4)** Different moieties have been added to four of the five solvent-exposed Lys.[56,69]

Single, double, triple, and quadruple Lys–minus mutants, in which the addressable lysines were sequentially replaced with arginine groups, were created and the ability of chemical labeling was assessed in a proof–of–concept study on CPMV. According to the investigations, all five Lys have availability for functionalization;

however, the degree of labeling efficiency differs between sites, and only four per subunit are generally changed. Lys (38) on the S protein and Lys (99) on the L protein were revealed to be the most reactive groups.[56,70]

Furthermore, carboxylates formed from Asp and Glu have been chemically attached to amine–containing compounds using EDC/NHS, as coupling reagents. Interparticle linkage is a major weakness of this method, which can be overcome by utilizing short linkers. Different moieties have been added to address 180 carboxylates per virion on the outer surface. [71,72,73]

Structural data from the CPMV capsid shows that five outer lysines as well three outers are solvent-exposed , as observed in **(Figure 1.4) [50]**

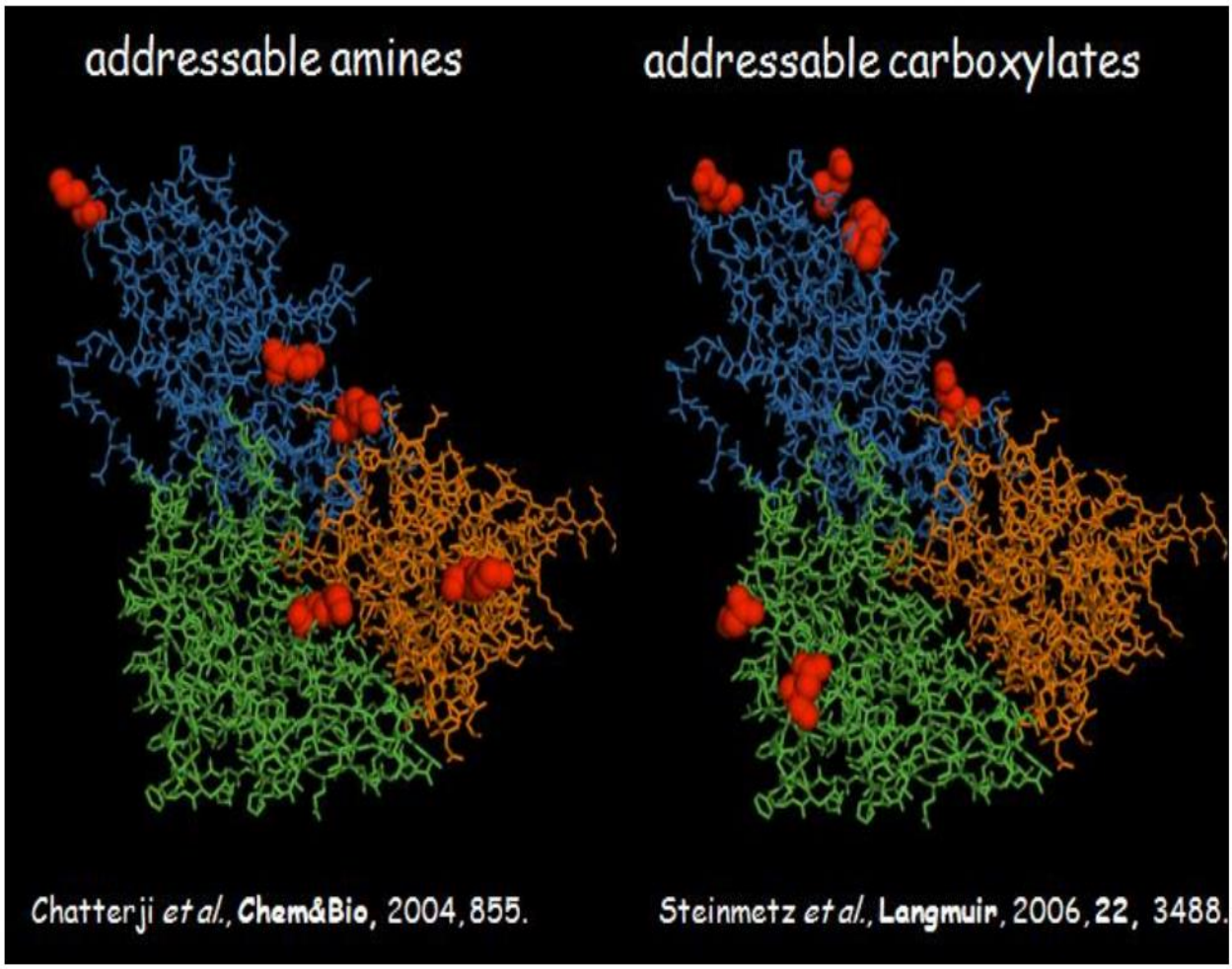


Figure 1.4 The surface exposed addressable amines and carboxylates of the CPMVcapsid . [50]

Tyr side chains have an aromatic group that could be modified chemically. Nevertheless, since the aromatic group is only moderately reactive, the tyrosine phenol must be oxidised by one electron to achieve bioconjugation. [74,75] Diazonium coupling is the most frequent reaction involving Tyr side chains on viruses, and it can be widely employed on MS2 and TMV.[76,77] In the S subunit of CPMV particles, two accessible Tyr side chains can be chemically modified, and fluorescein was covalently linked to CPMV Tyr side chains as described in demonstration studies.[56,78]

On the solvent.exposed surface of viruses, thiols produced from Cys side chains is rarely found in the free active form, although Cys has been detected on the interior solvent expose surface of CPMV.[56,59,79]

Previously, CPMV has also been labelled with Gd ions as a coordinatively bind well; as clinical approval is acknowledged for Gd(DOTA), Gd(DOTA) complexes can be attached by copper-mediated azide-alkyne cycloaddition to the linker that has been NHS/EDC conjugated to the CPMV surface exposed amine groups. Gadolinium attached to CPMV has exhibited a three-fold enhancement in relaxivity compared to free Gd(DOTA). Indicates that modified CPMV has the potential to be a superior contrast agent. [50]

Additionally, the Evans group demonstrated that CPMV could be covalently decorated with 240 redox-active ferrocene molecules, which has led to ferrocene's application as a CPMV decoration.[80]

Evans and colleagues have targeted carboxylate groups connected to the outer surface of decorated CPMV particles to demonstrate the adaptability of these particles. The amount and variety of addressable surface group options increased

as a result. Using the carboxylate-selective fluorescent dye can improve that carboxylates are addressable, CPMV particles can be decorated with about 180 redox-active, methyl (aminopropyl) viologen moieties by attaching to carboxylate groups. The presence of several redox sites on the surface of virus particles may facilitate the synthesis of unique electrontransfer mediators for homogeneous redox catalysis, as well as biosensor and nanoelectronic device. [81,50]

Furthermore, CPMV has been approved as a doxorubicin-related chemotherapy medication (DOX) Delivery, 80 DOX molecules attached the outer surface carboxylates of viral nanoparticle, has been used for a variety of cancer therapies, however, due to serious adverse side effects such as congestive heart failure, limited clinical effectiveness, low solubility, and heart problems are drawbacks. It

becomes essential to have a carrier system to decrease the harmful effects and boost overall effectiveness.[82]

The development of tailored medication delivery systems could benefit from the employment of viruses; for instance, they are highly effective in delivering the required encapsulated material to host cells.[83]

CPMV can be utilised to create viral particles with enzyme functionality. Multiple conjugation sites, controlled shape, and the ability to create multi-layer arrays are benefits of coupling enzymes to VNPs.[84]

Addressable groups have also been discovered on the interior of the capsid in addition to the reactive outside. Although CPMV possesses at least two reactive cysteine residues inside, they are not present on the surface that is visible to the outside world. It has been demonstrated that internal cysteines efficiently react

with tiny thiol-selective chemical molecules, including ethyl mercury phosphate (EMP), 5-maleimidofluorescein, and thiol-selective stilbene derivatives. [50] Chemical modifications were made to the wild-type CPMV to create a carrier for anti-malarial drugs. Carboxylates and phenolic groups,

previously used to attach entities such as metallic and semiconducting nanoparticles, are among the functions exposed on the surface of CPMV particles.[85, 50] It has also used organic materials like DNA, fluorescent dyes, carbohydrates, proteins, and antibodies. According to structural evidence, five outside lysines on the CPMV capsid are solvent-exposed. [86,50]

1.2- cancer

Now adays, cancer assumes the position as one of the major threats to survival on a global basis, It is the second leading cause of death in high-income nations, with roughly 21 million people expected to be affected by 2030.[87,88] Cancer is caused by a variety of endogenous and exogenous variables, including genetics, lifestyle, and living situations. To demonstrate their impacts, these risk factors can act in concert or in sequence.[89] Signals that control cell proliferation, differentiation, and survival are no longer perceived by cancerous cells. Angiogenesis and apoptosis inhibition in these aberrant cells can lead to metastasis into distant tissues and potentially death of the organism. Despite the medications' unfavorable side effects, chemotherapy is presently the most widely accepted traditional cancer treatment method. [90,91]

1.3- Breast cancer

Breast cancer is the leading reason of cancer death in women worldwide. It is the main reason of cancer-related death in women in developing countries (where many have advanced disease at presentation), and it is the secondleading cause in women in developed countries . Breast cancer has one of the highest potential years of life lost: almost 137,000 years, reflecting the burden of Breast cancer in younger women. Screening mammography is the method most commonly utilized worldwide for the detection of early Breast cancer in asymptomatic women, and it is the only imaging modality proven to significantly lower Breast cancer mortality.[92]

1.4- Anticancer

Anticancer immunotherapies are classed as passive or active depending on their ability to re-activate the host immune system against cancerous cells**(Figure 1.5)**. [93]Tumour-targeting monoclonal antibodies (mAbs) and adoptively transplanted T cells are both considered as passive forms of immunotherapy because they have inherent anticancer efficacy.[94,95,96] Anticancer vaccines and checkpoint inhibitors, on the other hand, have anticancer effects only when the host immune system is engaged, making them unambiguous, examples of active immunotherapy are shown in **(Figure 1.5)** . [97-100]

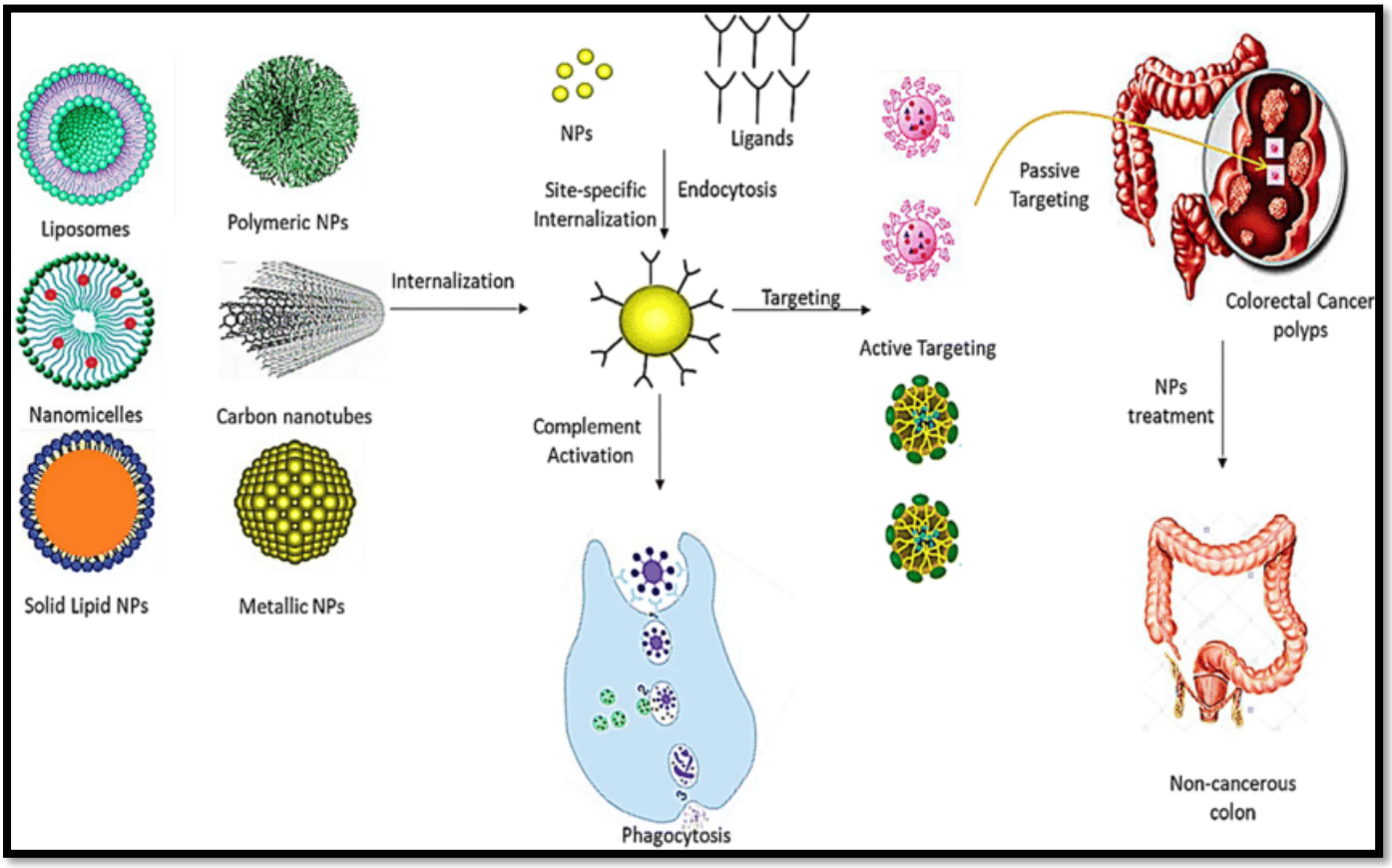


Figure1.5 Active and passive targeting of anticancer therapeutics using various nanoparticulate systems for targeting colorectal cancer.[101]

Cancer immunotherapies make it easier for the immune system to eliminate altered cells to be effective, however, such a therapy regimen must conquer the immunosuppressive tumor microenvironment (TME), which reduces the antitumor immune response quantitatively and qualitatively via various mechanisms [102,103] A lots of anticancer have been used in the field of cancer treatment such as doxorubicin (DOX) and docetaxel **(**DOC**).**

Doxorubicin, which has shown remarkable therapy potential and is one of the most potent of the FDA-approved chemotherapeutic drugs **(Figure1.6)**. [104] For decades, the ability to fight rapidly dividing cells and delay disease progression has been well recognised,. The compound is an anticancer antibiotic that belongs to the anthracycline class of drugs with aglyconic and sugar moieties. A tetracyclic ring containing adjacent quinine-hydroquinone groups, a methoxy substituent short side chain, and a carbonyl group make up the aglycone.[105,106] Doxorubicin (DOX) is a highly effective cancer medicine, but its medical efficacy is restricted via its low solubility and side effect, like heart failure. To maximise efficiency while, limiting offtarget effect, a delivery vehicle becomes clearly essential.[107] The ideal drug delivery system combines targeted distribution with monitoring discard inorder to increase therapeutic efficacy while reducing systemic toxicity. Because viruses have developed transport cargos toward host cells efficiently, they are an excellent starting point for creating specialized drug delivery vehicles. [108] Several nanomedicines based on viral nanoparticles (VNPs) are in the works, because plant viruses and bacteriophages are non-infectious to mammals, VNPs from these platforms are desirable.[109,110] Plant viruses such as (RCNMV) and Hibiscus chlorotic ringspot virus, as well as animal VLP_S to rotavirus's VP_6 main protein structure, have been employed as drug carriers for DOX distribution. [111-113]

(DOX) is the most frequent chemotherapy can be used to treat different metastatic cancer types; nevertheless, to lessen its toxicity, modify numerous signalling pathways in cancer cells, and improve anti-tumour efficiency, it must be paired with another highly effective non-toxic drug. [114] AKT (a serine/threonine

protein kinase) has previously been shown to be an essential regulator of cell survival and proliferation by Fabi et al. work's. [115]

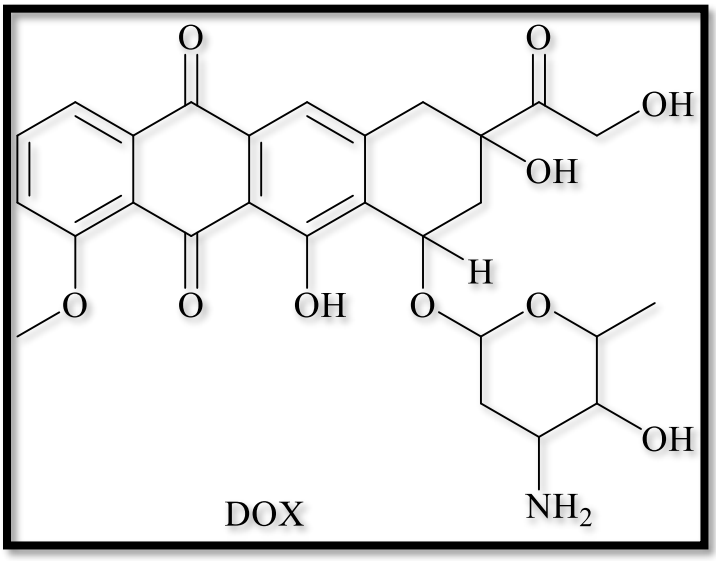


Figure 1.6 The structural formula of doxorubicine[113]

Docetaxel (DOC) is another anti-cancer drug**,** it is a taxoid-family antineoplastic agent. Its semi-synthetic preparation begins with a precursor derived from the regenerative needle biomass of yew plants.[116] It has a high lipophilicity and is almost water insoluble.**(Figure 1.7)** shows the structural formula for docetaxel. (DOC) known as a taxoid antineoplastic drug which used to treat tumors.[117,118] It interferes with the microtubule growth of the natural function. In opposite to treatments like colchicine, which cause the depolymerization of microtubules in *vivo*, DOC can inhibit their function by hyper-stabilising their structure; as a result, cell's capacity to employ its cytoskeleton is flexibly destroyed. [117]

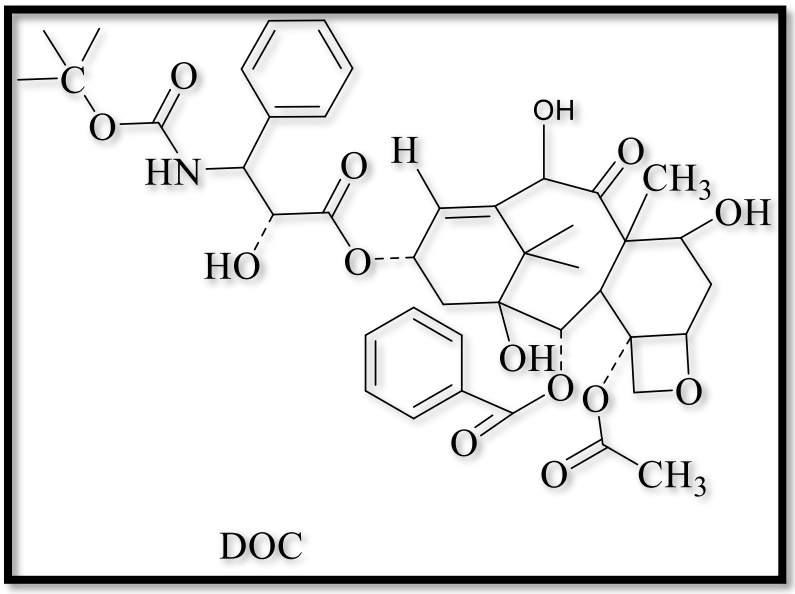


Figure 1.7 The structural formula of docetaxel[116]

1.5- Chloroquine derivatives

Chloroquine (CQ) and hydroxychloroquine (HCQ) are two drugs that have shown promise in the treatment of cancer **(Figure 1.8)**. [119] Several clinical investigations have indicated that chloroquine has beneficial effects as a novel antitumor drug.[120] The mechanism action of these drugs is still unknown, but the anticancer effects of CQ may be partially due to its inhibitory action on macroautophagy. The term autophagy shows how protein, organellee, and metabolitee are to break down and turn over regularly as response to hunger or as a protector of the cell from damage. Autophagy pathway come from macro autophagy, micro autophagy, and chaperonemediated autophagy. [121]

Macroautophagy is the best characterised and well understood; it was initially studied in yeast and involved the formation of lipid vesicles, known as autophagosomes, that surround cargo to be degraded. After forming, the autophagosome is transported to a lysosome, where a fusion process occurs, degrading the cargo inside the autophagosome. [122]

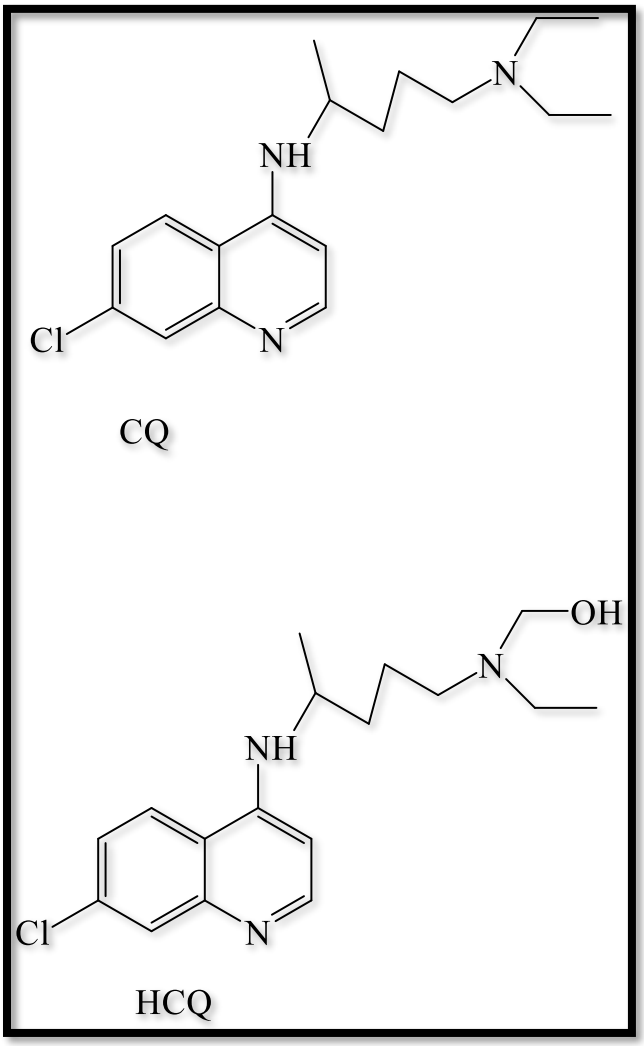


Figure 1.8 chemical structure of chloroquine(CQ) and hydroxychloroquine(HCQ) .[123]

Autophagy degrades and recycles a wide variety of cellular components, including long-living proteins, drips of fat, protein aggregatess, matuere ribosomes, glycogens, and even whole organelles like the endoplasmic reticulum, mitochondria, and Golgi apparatus. [124] The autophagy process is shown in **(Figure 1.9)**. [125]An autophagosome, a double-membraned vesicle, initially swallows the cytoplasm and cellular organelles. The autolysosome allows various lysosomal hydrolytic enzymes to degrade the sequestered materials. Following degradation producing, amino acids, sugars, fatty acids, and nucleosides, which are then recycled for macromolecular synthesis and energy production.[126,127] During starvation, this recycling system is very critical. Autophagy takes place at alow basale levels to maintaining cellulars homeostasise via eliminatings damaged proteines and organelles. Under hunger, autophagys are similarly activated to provide amino acids by degrading proteins and it is activated when cells need to get rid of damaging cytoplasmic components.[128]

Additionally, autophagy can be induced in cells as a survival mechanism in acidic environments. This deficiency has encouraged research into chloroquine derivatives that potentially increase autophagy suppression in acidic tumor microenvironments.[129,130]

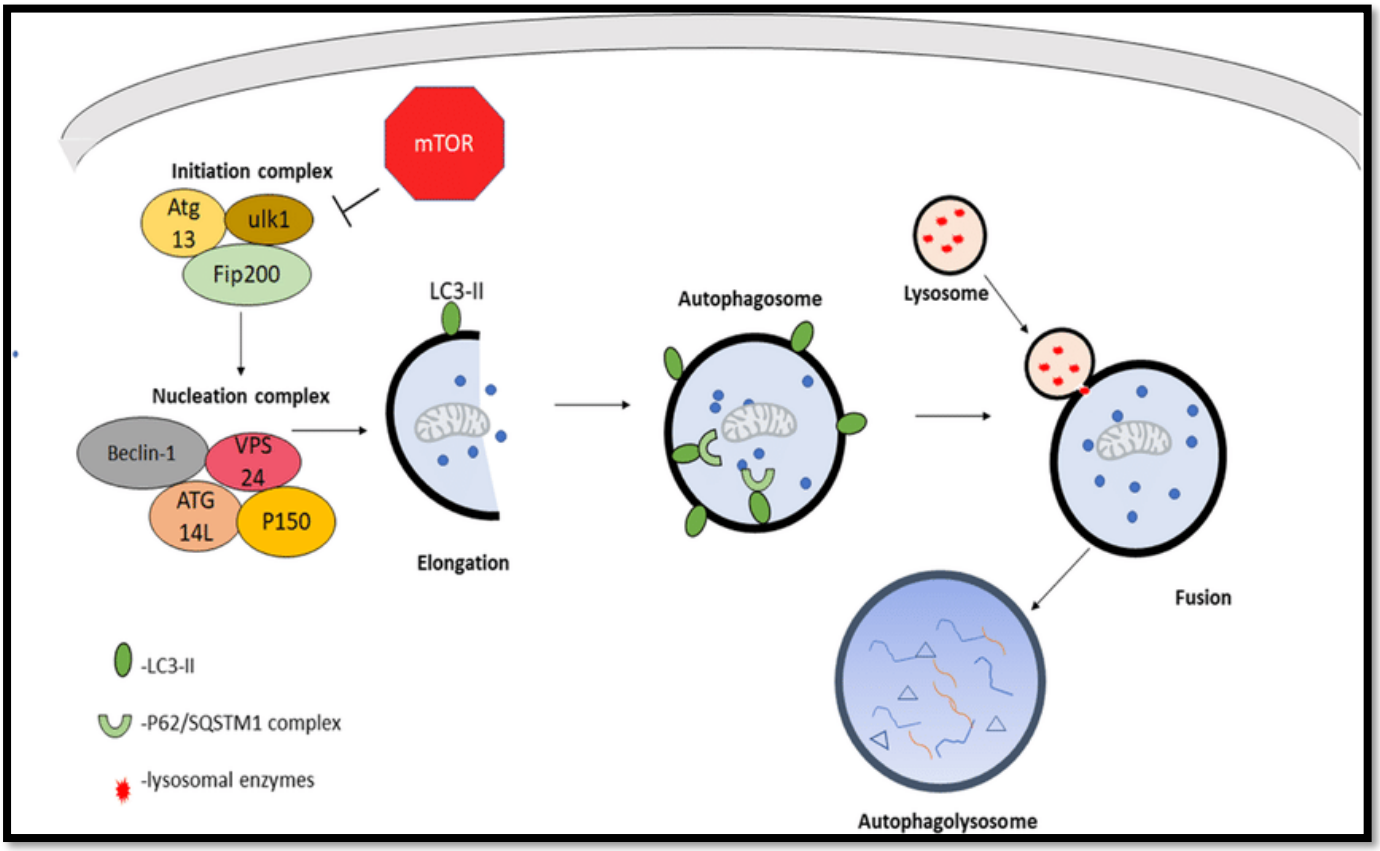


Figure 1.9 The mechanism of autophagy[125]

(CQ) as an antimalarial medication and a lysosomotropic agent, shown cytotoxicity effects against a variety of humanity's cancers by inducing cell cycle arrest, inhibiting autophagy, plus ultimately inducing death in tumour cells. It also has the ability to increase sensitivity to numerous therapeutic types used in chemotherapy for cancer. [114] Moreover, CQ has a powerful cancer-specific chemosensitizer when it used with other chemotherapies as it has described to enhancement the intracellular marks of the medications through inhibitory degradation of autophagic cargo.[131,114]

Previous reports indicated that MCF-7 might be successfully sens itized to DOX by the combination of CQ and DOX. To prevent DOX from efluxing outside of cancer cells, coencapsulated DOX ,and CQ liposome were identified as viable formulations for considering DOX resistant to MCF7 breaste cancer. In recent times, Guo et al. displayed that autophagy activation may be linked to the development of DOX resistance in breast cancer because DOX sensitivity was restored when autophagy was blocked. [114,132]

Combination therapy indicated the promise in targeting -to-treat tumours and making cancer cells more susceptible to commonly used anti-cancer medications like doxorubicin. [114]

1.6 -Combination treatment

A key component of cancer therapy is combination therapy, a mode of care that combines two or more therapeutic drugs. The combinations of antii-cancers medicationes improve efficacity in comparing too monotherapys because it targets critical pathways in a manner that is typically additive or synergistic. This approach may also lessen medication resistance and therapeuticc antii-cancers effectt, including reducing tumouring growth and metastating potential, stopping mitotically activy of cells, lowerings cancer stems to cell populaetions, and inducing apoptosis. Most metastatic tumours still have low 5-year survival rates, and creating a new anti-cancer medicine is expensive and time-consuming. New tactics are therefore being proposed that focus on the survival pathways that deliver efficient and effective outcomes at a reasonable cost. Repurposing prescription drugs eventually prescribed that will someday be used as a strategy to treat other illnesses than cancer.[133,134,135]

1.7-The mechanism action of chloroquine as anti-cancer drug

The term of autophagy means a complex, multi-faceted process. It has both pro and anti-tumour functions, depending on the particular stage and tissue type.[136]

Autophagy has protected cells by isolating and removing faulty cellular components, for instance damaged mitochondria, and also preserving cellular homeostasis, which plays a tumour-suppressing role in early carcinogenesis. [137,136]. Additionally, it has been demonstrated that autophagy may digest proteins that promote the growth of tumors, such as p62/SQSTM1, and that a number of autophagic proteins, such as Beclin-1, UVRAG, and Bif-1, can directly inhibit the development of tumors. [138] Deregulation of autophagy has also frequently been linked to human malignancies. [136]

In contrast, during more advanced stages of cancer, autophagy might encourage the growth of tumours, and several anticancer medications can also be induced as prosurvival autophagy. Nutrient recycling and other autophagic abilities can help cancer cells to survive without limitation to starvation, proteostasis loss, organelle damage and hypoxia. Furthermore, by increasing autophagy, important growth-controlling factors are destroyed, and the DNA damage response can also be inhibited. [139,136] The inhibition of autophagy is an interesting anticancer strategy while cancer cells start dependent on autophagy to survive, which is named autophagic switching.[140]

With the combination of autophagosomes within lysosomes and consequent degradation to the autolysosome, which are late-stage autophagic flux, both CQ and HCQ can be inhibited at the latest stage. **(Figure 1.10)**. When CQ and HCQ enter the lysosomes, they become protonated, which causes them to become trapped in acidic lysosomes and raises the pH of the lysosomes, inhibiting lysosomal degradation enzyme.[136] According to Loehberg et al., CQ may affect autophagy via altering the PI3K/Akt/mTOR pathway.

In conclusion, depending on both timing and context, autophagy has a double function in cancer treatment with crucial inhibition of the autophagy suppression utilizing latestage inhibition of CQ and HCQ.

After the autophagic change, autophagy can present a fascinating therapeutic target and sensitive inhibition. Nevertheless, it can vary depending on the tumour cells' dependence on autophagy and any combination therapy.[136,141]

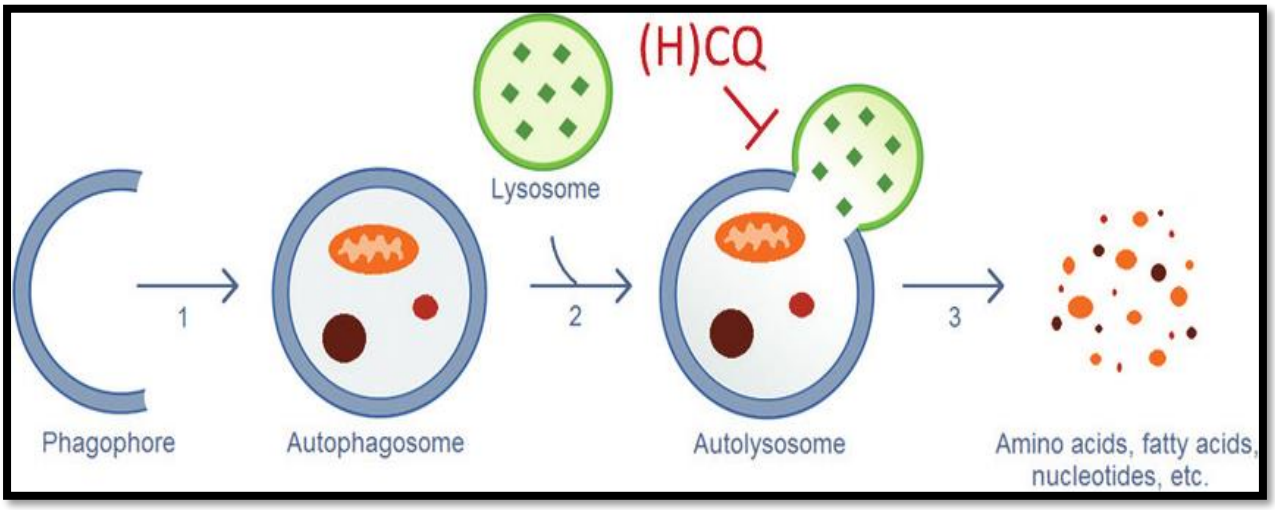


Figure1.10 The mechanism action of chloroquine as anti-cancer drug.[136]

In this chapter many distinct protein-based nanomaterials have been described in this chapter. The role of organic nanoparticles such as plant viruses and their applications in medicinal research has been described, and the ability of the exterior surface of CPMV with organic components has been reported. The favourable properties of viral nanoparticles for applications in medication, including their ability to carry payloads of drugs far more considerable than traditional drug delivery systems, have been discussed.

1.8-The aims of this study

1-Synthesis some of new chloroquine derivatives.

2-Using Cowpea Mosiac Virus(CPMV) as a carrier for each CQ-derivative by targating the external carboxyls and amines of CPMV.

3- Culculat the number of each conjugated CQ-derivative on the external surface of CPMV.

4- Conjugated and unconjugated CQ-derivatives with CPMV would be tested as anticancer drugs.

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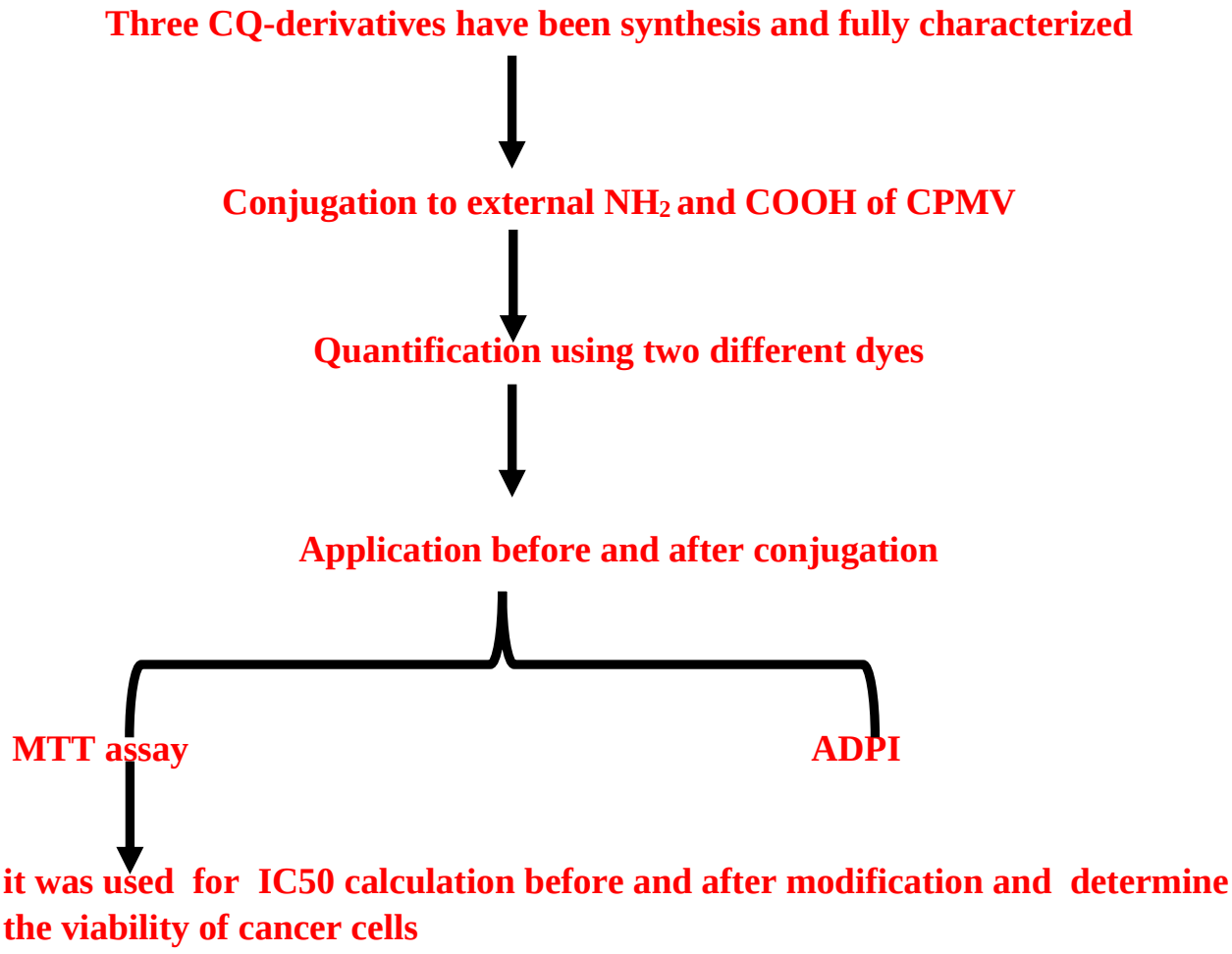
Chapter Two

Experimental part

Design of study



2-Experimental part

2.1-Materials and regents: The chemicals and supplied companies which used in the present study are listed in **Table 2.1 .**

Table2.1: Chemicals andReagents.

Chemicals	Symbol	Purity%	Supplied company
4,7-dichloroquinoline	$C_9H_5C_{l2}$	98%	Sigma-Aldrich
doxorubicin	$C_{27}H_{29}NO_{11}$	98%	Johnson and Johnson
docetaxel	$C_{43}H_{53}NO_{14}$	≥ 99%	Sanofi
salicylic acid	$C_7H_6O_3$	99%	Tomas Baker
Dimethyl sulfoxide (DMSO)	C_2H_6OS	99.9%	Sigma-Aldrich
disodium hydrogen phosphate	Na_2HPO_4	99.9%	Sigma-Aldrich
Sodium dihydrogen phosphate	NaH_2PO_4	99%	Sigma-Aldrich
1-ethyl-3- (3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC)	$C_8H_{17}N_3 \cdot HCl$	99%	Sigma-Aldrich
N- hydroxy succinimide (NHS)	$C_4H_5NO_3$	98%	Sigma-Aldrich
1-Butanol	$C4H_9OH$	99.9%	Laboratory chemicals
Chloroform	CHCl3	99%	Laboratory chemicals
poly ethylene glycol(PEG)	$C_{2n}H_{4n+2}O_{n+1}$	99.8%	Sigma-Aldrich
Ethidium bromide	$C_21H_{20}BrN_3$	~95%	Sigma-Aldrich
Coomassie stain	$C_{45}H_{44}N_3NaO_7S_2$		Sigma-Aldrich
CF-488A dye	$C_{35}H_{41}N_2N_8S_2$	90%	Sigma-Aldrich
Alexa Fluor dye	$C_{21}H_{15}N_4O_{10}S_2$	90%	Sigma-Aldrich

2.2 Instruments: The following table includes the equipment and instruments that have been utilised in this study**.**

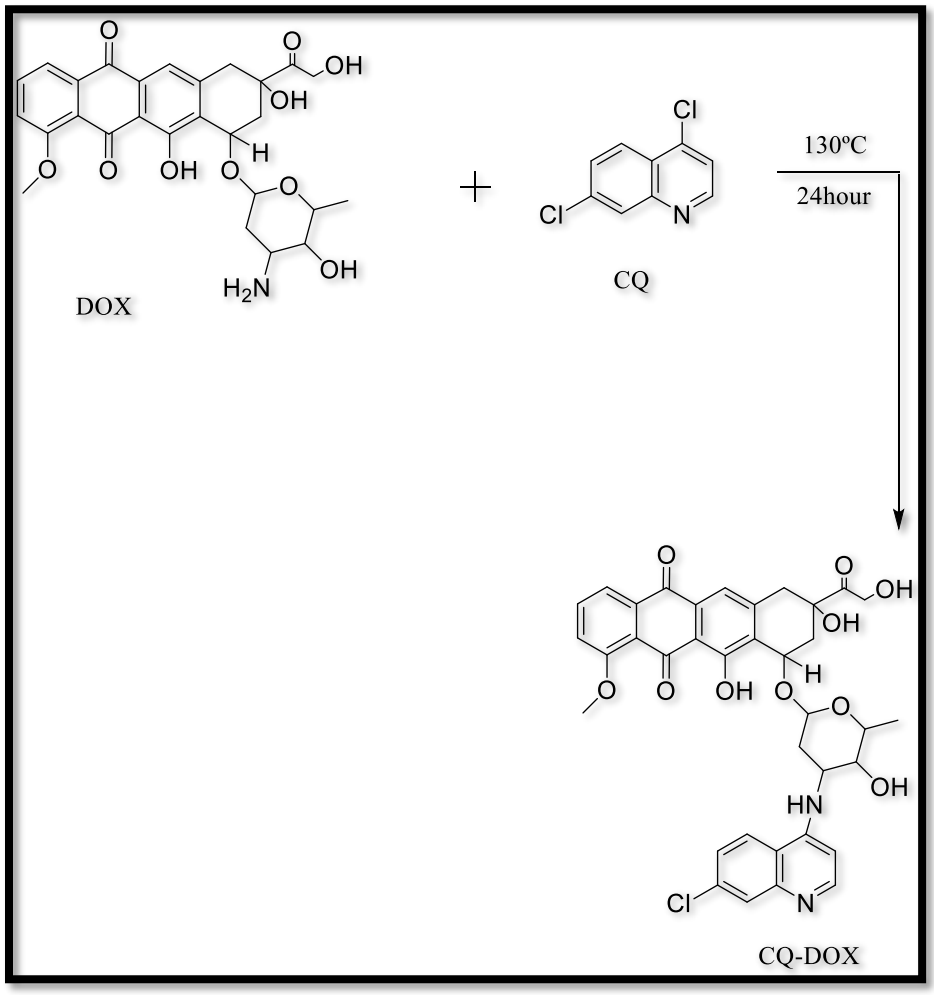
Table 2.2 Instruments and equipment

Instruments	Supplied company
Balance sartorlus	**Welghing Technology-German**
Centerifuge,Laboratory Retirezle	**Gordon-Germany**
Incubator	**Memert Edelsta(Germany)**
Magnatic stirrer VF2	**Funkentstort-Germany**
Oven	**Memmert854 schwabach(Germany)**
pH meter 7110 WTW 82362	**Wellheim-Germany**
Water Bath	**Memmert WNB(Germany)**
UV-visible spectrophotometer	**Biotehengireer ingmanagement CO. LTD.UK.**
Nano Drop Spectrometer ND	**Marshall scientific**

2.3- Chemical synthesis

2.3.1.The synthesis of CQ-DOX

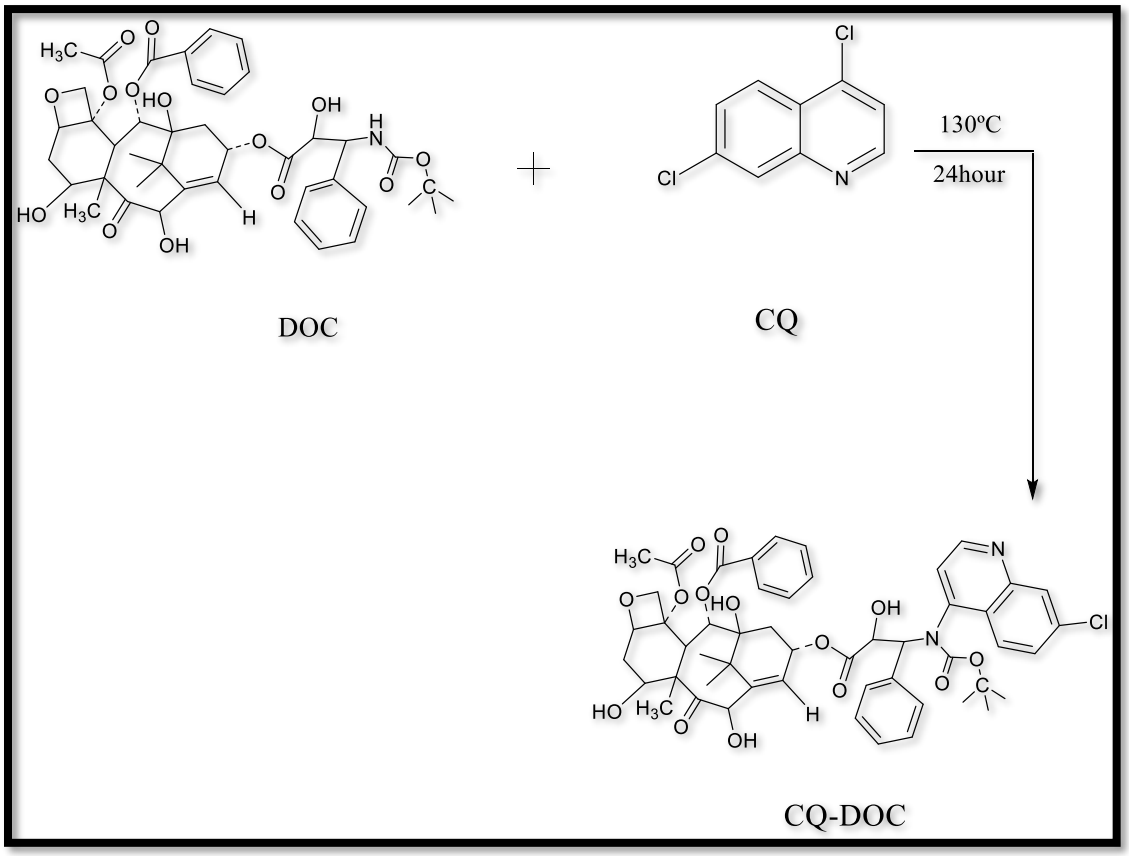
4,7-dichloroquinoline (0.5 g, 2.5 µmol) and doxorubicin (0.0460g, 84 µmol) with normal saline were mixed before being heated at 120–130°C for 22 hours with stirring. The reactionn wase emptied into 100 mL of water ,and filtered after cooling, and the solid was recrystallized from 100 mL ethyl acetate before being heated the precipitate was washed with chloroform and the crude was purified by TLC (DCM/MeOH, 5/2) to give the chloroquinoline amino acid compound as a reddish solid to give yield (o.6181 g, 81 %). Elemental analysis expected C: 62.75%, H: 4.83%, N: 4.07%: found C: 62. 09 %, H: 7.45 %, N: 4.12% for a chemical formula $C_{36}H_{33}ClN_2O_{10}$ with a molecular weight of 689.11. [142] the reaction was shown in **scheme** 2.1. The melting point for CQ-DOX was 354 C.



Scheme 2.1 Reaction CQ with DOX to CQ-DOX

2.3.2- The synthesis of CQ-DOC

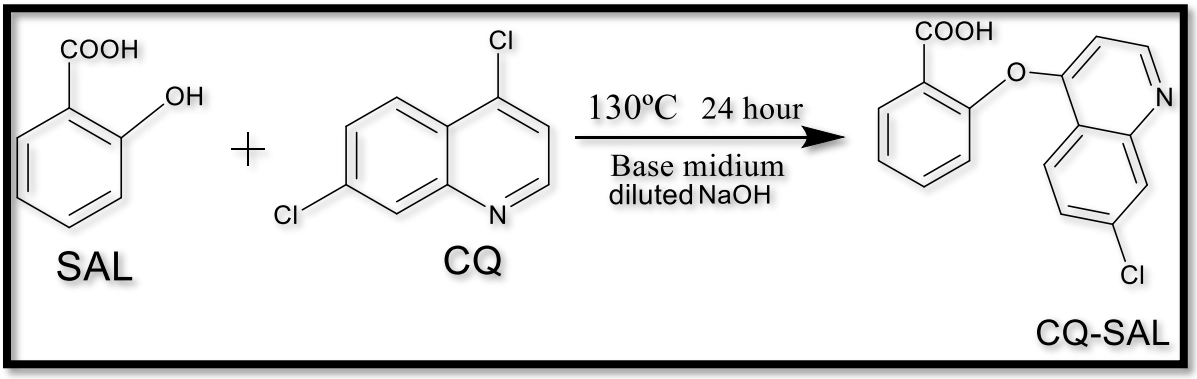
4,7-dichloroquinoline (0.5 g , 2.5 µmol) and docetaxel (0.08 g , 98 µmol) were mixed before heating at 120–130 °Cfor 23 hourse with stirring. The reaction wass emptied into 100 mL of water ,and filtered after cooling, then the solid recrystallised was in 100 mL of ethy acetate before being heated the precipitate was washed with chloroform and the crude was purified by TLC (DCM/MeOH, 5/2) to give the chloroquinoline amino acid compound as dark brown oil to give yield (o.78g, 64%). Elemental analysis expected C:, 64.11%, H:; 5.80%, N:; 2.93%: found C:; 64. 46 %, H:; 7.28 %, N:; 2.90% for a chemical formula $C_{51}H_{55}ClN_2O_{14}$ with a molecular weight of 955.44.[142] the reaction was presented in **scheme 2.2**.The melting point for CQ-DOC was 380 C.



Scheme 2.2 the Reaction of CQ with DOC to CQ-DOC

2.3.3 -The synthesis of CQ-SAL

4,7-dichloroquinoline (0.5 g, 2.5 µmol) and docetaxel (1.3g, 9.4 µmol) mixed then heated att 120–130 °Cfor 24, hours with stirring. The mixture was emptied in.to 100 mL of water, and filtered after cooling, and the remains solid was recrystallized from 100 mL of ethyl Acetate before being heated the precipitate was washed with chloroform and the crude was purified by TLC (DCM/MeOH, 5/2) to give the chloroquinoline amino acid compound as brown solid to give yield (0.8535 g ,59%) as brown solid. Elemental analysis expected C :; 64.12%, H :; 3.36%, N :; 4.67%: found C :; 64. 4 %, H :; 12.33 %, N :; 5.12% for a chemical formula $C_{16}H_{10}ClNO_3$ with a molecular weight of 299.71. [142] the reaction was shown in **scheme 2.3**.The melting point of CQ-SAL was 270 C.



Scheme 2.3 The Rreactionof CQ with SAL to CQ-SAL

2.4-Fourier transform infrared spectroscopy

For characterization of the functional groups on the surface of the prepared materials under study Fourier transform infrared spectroscopy (FTIR) spectra were documented on a Spactra IR-2, Perkin Elmer Instrument and spectrometer by utilising the potassium bromide KBr pellets method at room temperature. The FTIR spectrum was collected between the waves number of (400 - 4000 cm-1)

2.5- Sodium phosphate buffer

Na_2HPO4 (6.75 g, 47.5 µmol) and NaH_2PO_4 (7.1 g, 59 µmol) solutions in double distilled water (250 mL) for each were produced, then autoclaved before mixing. In order to make a buffer solution 0.1 M and PH7, 152.5 mL from Na_2HPO4 solution , and 97.5 mL from NaH_2PO4 solution with 250 mL of double distilled water to make a buffer solution 0.01 M and PH7.[50]

2.6- Cultivation and virus infection of plants

Cucumber plants were cultivated in February when low temperatures and a brief lighting period prevailed, so the glass houses were used for cucumber plant growth. The plants were infected by mechanical inoculation. In order to enforce the mechanical lesions, the leaves were treated with carborundum(silicon carbide).The primary leaves' inoculation was carried out using 0.5µg/mLof purifiied CPMV particle diluted with 500 µL of 0.01 M sodium phosphate buffer at pH 7.0 per leaf. [143]

2.7- Diseas symptoms which appeared on plant because of CPMV and extracttion of CPMV from the cucumber plant

CPMV causes lesions on inoculated leaves after 10-12 days with no distinction between the phenotype of CPMV wt **(Figure 2.1)**.

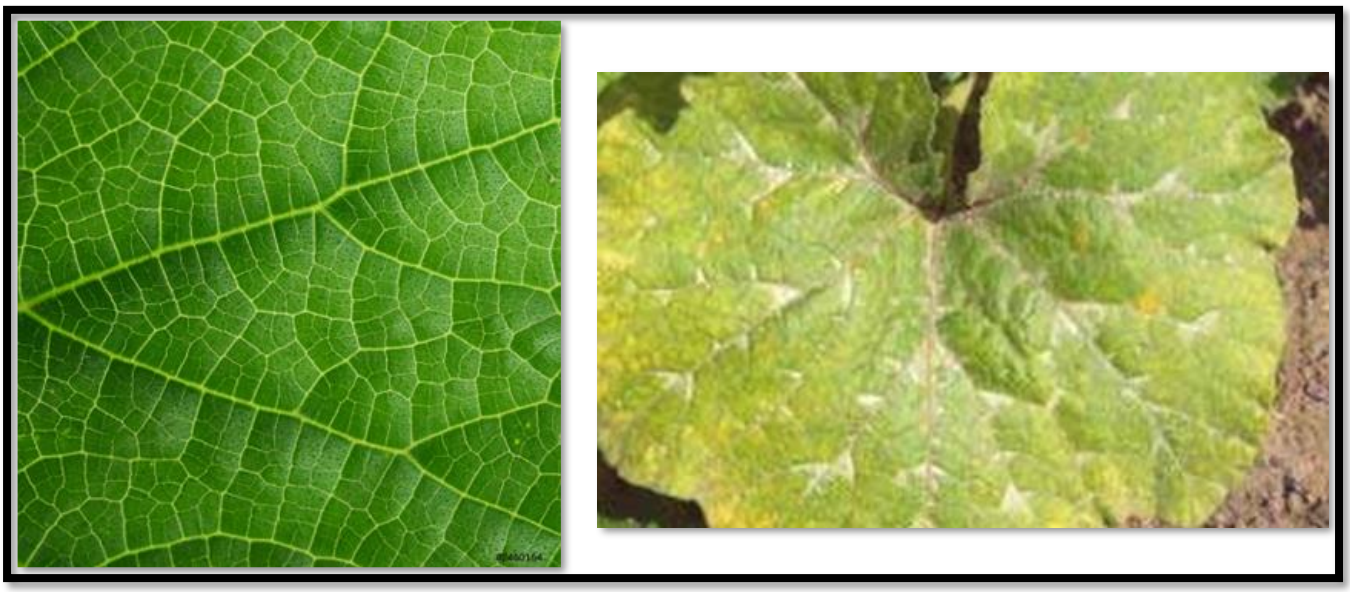


Figure 2.1 leaves of Cucumber before and after infected with CPMV

Each infected primary leaf was stored at -20 °C after being harvested. (The frozen CPMV wt infected plant tissue (. 100 g) was homogenised using a mechanical blender (Moulinex) in 150 mL to 200 mL of cooled 0.1 M sodium phosphate buffer PH 7.0. all solutions) and extracts were kept on ice. The extract was subsequently clarified through 2 muslin layers; this muslin was squeezed to extract as much sap as possible. Centrifugation at 15000 g for 20 min. at four °C, the plant sap was performed, and the supernatant was collected while, the pellet was re-suspended within 0.1 M sodum phosphate buffer PH 7.0 ,and reseparated using a centerfuge 15000 gx aimed in20 minu At 4.0 °C. The supernatantse werecollected and

extracted with a 0.7 vol. 1:1 (v/v) combination of n-butanol and chloroform. To separate two phases, the mixture was centrifuged at 8000 g for 5 min. at 4 °C after being stirred for 20 to 30 min. At that temperature. The upper aqueous phase, which contains the virus, was collected and 0.25 vol. of a solution containing 1 M NaCl/20% (v/v) PEG6000 is added and, the resultant solutions wasstirred at 4 °C for at least 2 h, followed by centrifugation at 15000 g for 15 min. at 4 °C. For every gram of plant tissue, the pellet was resuspended in 10 mM sodum phosphate buffer with PH7 using the volume of, 0.50 mL. Then re-suspended particle was centrifuged for 15 minutes at 27000 g at 4 °C. Supernatant containing the virus, was retained ,and pellet re-extracted in the same buffer solution before re-centrifuging at 27000 g, for 15 minu.at the temperature 4 °C. Both supernatants were mixed, and then ultracentrifuged at 13000 g, at 4 °C within three hours. The virus pellett was re-suspended in a buffer solution (e.g. 0.05 mL for every gram of plant tissue). After giving a clearing spin (13000 g for 10 minu), cleared supernatantwas together ,and stored at 4 °C. Photometrical measurement has been used to evaluate virus concentration using Bradford Assay. The yields obtained were about 0.5 to 1 g of virions per of infected leaf tissue . [143] Virus purification steps shown in **Figure2.2**.

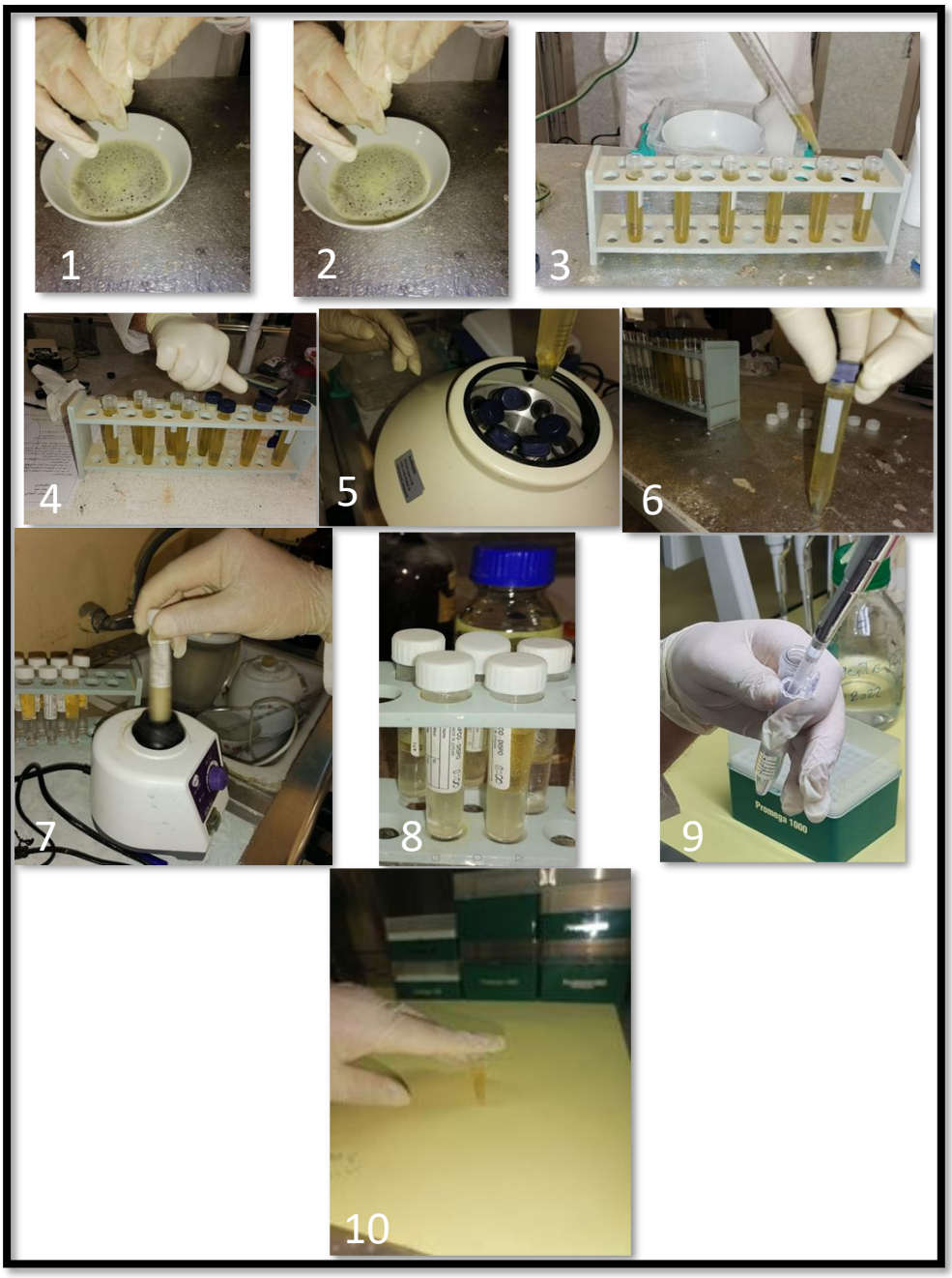


Figure 2.2 Virus purification steps

2.8- Determination of the Concentration of virus.

Both photometrical measurement and NanoDrop® ND-1000 spectrophotometer were used to determine the purified virus concentration**(Figure 2.3)**.

The maximum absorbance of CPMV particles has been recorded at λ = 262 nm (mainly resulting from encapsulated RNA). Concerning molar extinction coefficient of CPMV with ε_{CPMV}= 8.1 mL $mg^{-1}cm^{-1}$ the concentration can be calculated by the law of Beer Lambert: [50]

$$A = \varepsilon dc$$

Where **A**, is absorbance.

ε is the molar extinction coefficient in (mL $mg.^{-1}cm^{-1}$).

d for length ofthe lightpath in a cm .

c for particles concentration (mg. mL^{-1}),

.

Figure 2.3 NanoDrop image for Cowpea mosaic virus particles

Depicted is the absorbance (A) virsus the wavelength in nm. The value of the absorption maximum can calculate the concentration (c) of the sample at 260 nm by making use of the molar extinction coefficient of $\varepsilon_{CPMV} = 8.1$ mL. $mg.^{-1}$ cm^{-1}; $c = (A/\varepsilon) * F$; where by F is the dilution factor. [143]

Dilution Factor is the factor by which the stock solution is diluted. It may be expressed as the ratio of the **volume of the final diluted solution (V_2) to the initial volume removed from the stock solution (V_1)**. [144]

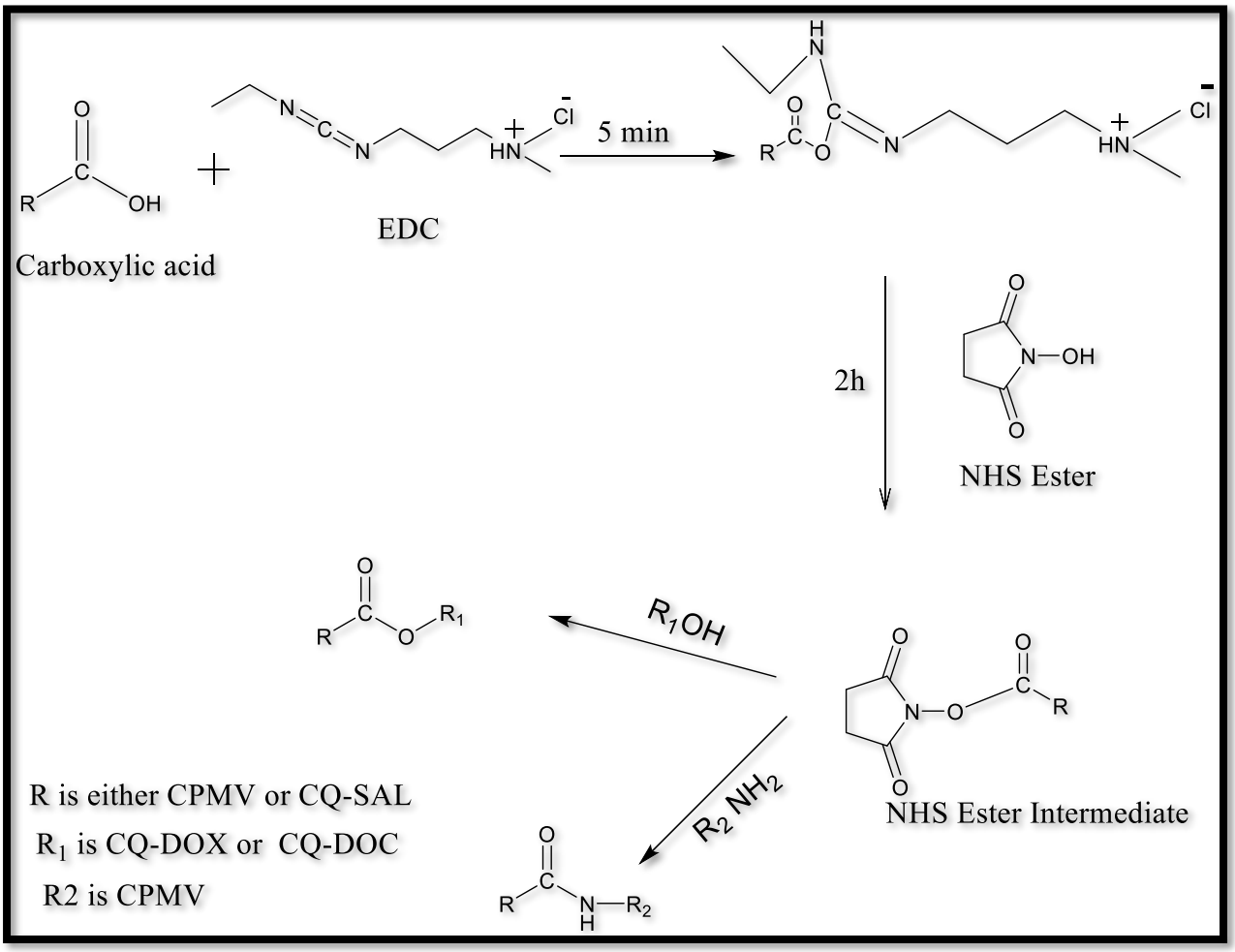
2.9 -The modification of CPMV

2.9.1- Addressability of CPMV's external carboxyl

Freshly prepared (EDC) (0.1 g , 580 µmol) in 10.0 mL sodium phosphate buffer PH7.1 which was used in a1000, molar excess, with NHS (0.1g, 860 µmol) in 5Ml DMSO in 4000 molar excess were added to 200 µL CPMV (3 mg/mL), the reaction mixture was then allowable run for two hours with gentley stirring .Then 250 µL of CQ-DOX (0.01 g, 14.5 µmol) in 5 mL DMSO was added in 6000 molar excess and 306 µL of CQ-DOC (0.01 g, 10.4 µmol) in 5 mL DMSO in the same molar excess were added separately. The reaction mixture's final volumetric concentration of DMSO was used into 20.%. After stirring, for 20h at room- temperature each sample was then centrifuged (14000 r.p.m) for 10 min . At the same temperature; using 100 kDa cut off columns to separate virus particles , they were washeed three- times with sodium phosphate buffer (80 µL). The modified virions were re-suspended inside (100 µL) of 10 mM sodium phosphat PH 7.1.Recovery of the virus was 76.6% for CQ-DOX and 72.2% for CQ-DOC, according to the initial concentration of CPMV.[`145]

2.9.2- Addressability of CPMV's external amine

In addition prepared 79.6 µL in 6000 molar excess of CQ-SAL (0.01 g, 3.3 µmol) in 5 mL DMSO was added to 1000 molar excess of (EDC) (0.1 g, 850 µmol) in 10 mL sodium phosphate buffer PH 7.1 for five min. and then 4000 molar excess of NHS (0.1g, 860 µmol) in 5 mL DMSO, the reaction was then allowed process for, 2 h using gentley stirring .After that, 200 µL CPMV (3 mg./mL) was to add for the mixture. Final concentration, of DMSO in this reaction was attuned to be 20 % by volume. After 22 h. stirring at room- temperature, virus was purified by centrifugation using (14000 r.p.m, 10 minu.,and room temperature); using 100 kDa, cutoff columns, then they washed for three times with in (100 µL) sodium phosphate buffer. The conjugated particle was re-suspended again inside 10 mM from the same buffer. Recovery of virus was 72.8%. The mechanism action of EDC and NHS as conjugation reagents is shown in **scheme 2.4.** EDC(1-ethyl-3-(3-dimethyle aminopropyl) Carbodimide) generally med as carboxyl agent activating agent for the coupling of primary amine to yield amide bond.



Scheme 2.4 The mechanism action of EDC and NHS as conjugation reagents.

2.10- practical purification

2.10.1-Ultrafiltration

Ultrafiltration was employed to purify and concentrate virus particles coated with chloroquine derivatives. Samples were purified using (100 μL – 1 mL) of 100 kDa cut off columns (Microcon, Amicon Millipore) and centrifuged. While, buffersolution ,and tiny impurities can be passed through membrane, and particles are put on the filter. [50]

2.10.2-Dialysis

This approach of nanoparticle purification is quite successful. The semipermeable membrane keeps particles inside while allowing tiny molecules to pass through. If the protein concentration is too low for further processing or analysis, centrifugal concentrators can immediately concentrate the sample. Float–A–Lyze tubing 100KD has been used throughout the rese. [50]

2.11- UV–Vis determines wavelengths of CPMV and CQ- derivatives

The purification of CPMV particles and CQ- derivatives wavelengths were determined using a 1 cm quartz cuvette and photometric measurement at ambient temperature .

2.12 Agarose gel electrophoresis

Modfied and unmodified CPMV samples were taken in (17μL) from each sample and added seperetly to 3 μL of loading dye (Coomassiee Staining Solu.) and loaded or going on 1.2% agarose gel in 50mL TBE buffer by spending an electric field between 1to5 Vcm^{-2} for 1.5 hour. For the staining of ethidiumbromide(Et.Br) (nucleic Acid staining) 0.10 μg per mL (Et.Br) (5.0 μL) in 1.0 x TBEbuffer was

mixed with a gel before running as shown in**(Figure 2.4)**. Each particle was visualised on a UV-transilluminator at 302nm using E-GRAPH device . Gel was stained with Coomassie staining solution overnight for coat protein visualisation. A camera or scanner was used to capture gel images. [50]

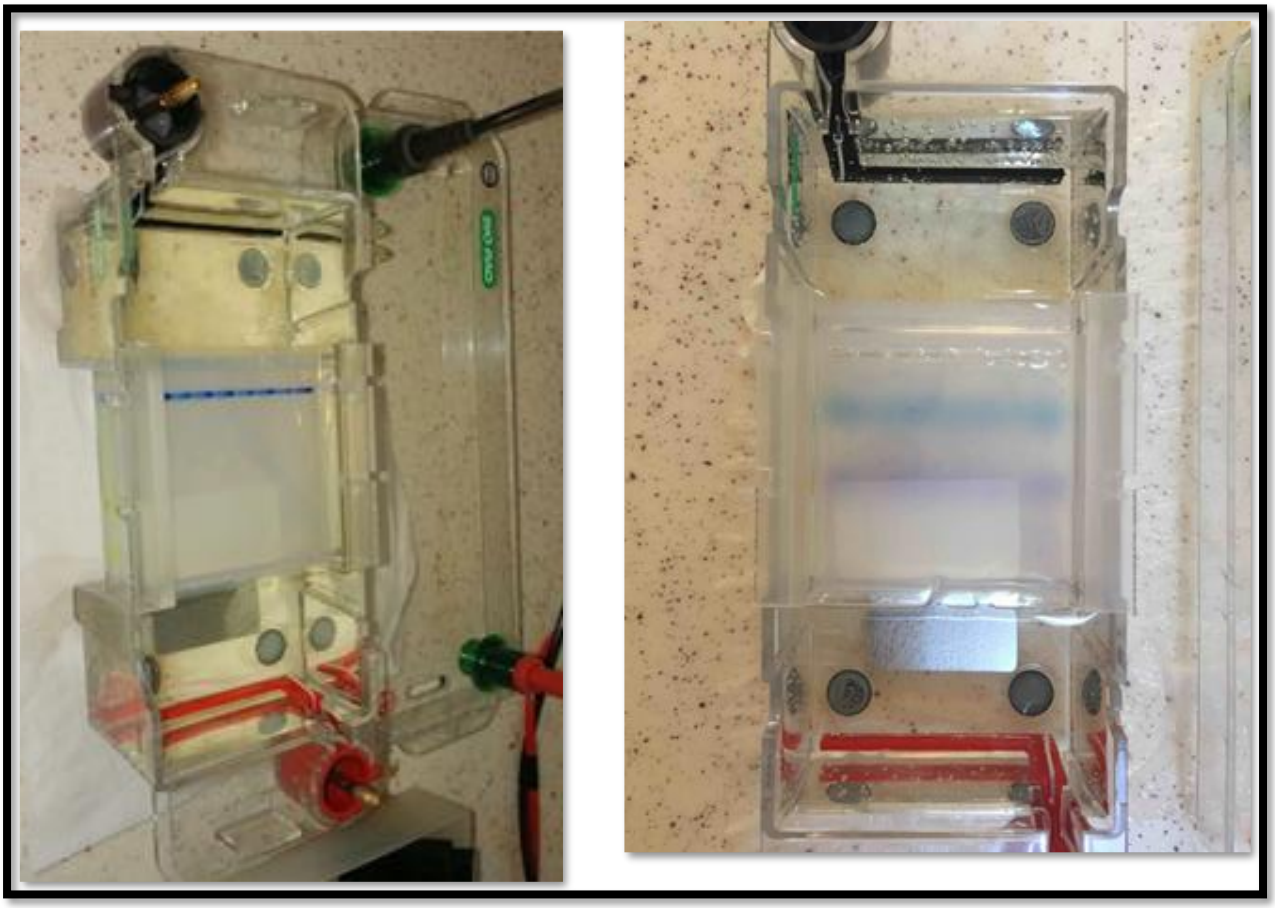


Figure 2.4 Agarose-gel electrophoresis of CPMV-CQ derivatives

2.13 Labelling of CPMV-CQ -derivatives with fluorescent dye

molar excess of 2000 of Alexa Flour 488 dye (0.028g , 43.5 µmol), was to liquefied within dry DMSO (2 mL) ,then mixed with 100.0 µL of CPMV, CPMV-CQ-DOX and CPMV-CQ-DOC particles in 0.01 M sodium phosphate buffer (PH 7.1). The final DMSO content was reduced to be 20 precent (vol/vol). Each mixture incubated for 2 h at room temperature before being allowed to continue for 22 hours at a temperature of 4.0°C for 22 hours while, stirring delicately . then the products were purified by dialysis against 500 mL sodium phosphate buffer for 8 hours. The final products were purified three times with 100 µL sodium phosphate buffer PH 7.1 and centrifuged each time at 14000 rpm for 10 minutes. [50]

While CF-488A (succinimidylester dye) (0.028 gm, 43.5 µmol) within molar excess of 2000 ws used for labeling of CPMV-CQ-SAL particles, it was added to (100 µL) in 0.01 M sodium phosphate buffer PH 7.1 of modified and unmodified particles, the dye was first dissolved in dry DMSO (2 mL) and the DMSO concentration was setto 20 precent (vol/vol). The reaction later incubated for 2 hours at room temperature before being allowed to continue for 22 hours at a temperature of 4°C while stirring carefully. then the products were purified by dialysis against 500 mLsodium phosphate buffer for 8 hours. The final products were purified three times with 100 µL sodium phosphate buffer PH 7.1 and centrifuged each time at 14000 rpm for 10 minutes [50]

2.14- Transmission- electron- microscopy (TEM)

The particles were dyed within Silver nitrate for visibility. For 3-5 minutes, 10 µL of 3 mg.mL^{-1} of virions with 10 µL of 2% (w/v). Silver nitrate was incubated on carbon film-coated nickel or copper grids. The filter paper was used to remove the solution, and the grid was left to dry by air.[50]

2.15 Cytotoxicity of synthesized compounds

The Pasteur Institute (located in Tehran, Iran) provided the MCF-7 cell line. Cells were grown and maintained at 37°C in a humidified incubator with 5% CO_2 in air in Dulbecco's Modified Eagle Medium (D.M.E.M; Gibco;, Life. Technologies,; Waltham, M.A, U.S.A) were supplemented within 10% fetal- bovine-serum (F.B.S; Bio-West S.A.S, Nuaille,; France) and 1% P.S.F (antibiotic- antimycotic solution, Sigma-Aldrich, St.. Louis;, M.O, U.S.A). Cells were detached at 37°C using, 0.250% trypsin (Gibco,; Invitrogen,; Waltham;, M.A, U.S.A) and 0.10% ethylene diamine tetraacetic acid (Merck,; Darmstadtt, Germany) in phosphate-buffered - saline (PBS) after reaching ~75% confluency . The cells were then re-suspended in DMEM with 10% FBS and 1% PSF. Prior to the trials. At a densitty of, 5000 cells per-well, cells were then seeded onto 96-well.

plates ,and incubated for 24 hours. Then they were rinsed with PBS pH 7.4 (phosphate- buffered- saline) before incubating for 72 hours in a freshly medium having samples at various concentrations (20, 40, 80, 160, and 320 times dilutions). 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide The assay of (MTT) dye was used to evaluate cell viability and materials cytotoxicity effect at varying concentrations. After 72 hours incubation (37C°, and5 per cent CO2 in a steamy atmosphere), MTT in (0.50 mg..mL^{-1} in PBS) was added to each well, while, each

plate was thenincubated for anadditional 4 hours at the same temperature. At 37°C, theformazan was liquefied with 100 µL of DMSO with shaking gently. An ELISA reader was used to determine the absorbance at 570 nm. The average of three separate experiments was used to present the results. Then, the concentrations of substances (i.e., IC_{50}values) that caused a 50% reduction in cell viability were determined.

2.16- 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) nuclear staining

After being given the treatment as mentioned earlier for 72 hours, MCF-7 cells were fixed with methanol and acetic acid in (3:1, v/v) and then rinsed with PBS buffer.

Cells were stained for 20 minutes in the dark with 1 $mg.mL^{-1}$ of DAPI stain after washing. A fluorescent microscope with a suitable excitation filter was used to record the stained pictures.[146]

Chapter Three
Results and Discussion

3.1 Chemical Synthesis

The most important role in this study is to increase the effectiveness of small molecule chemotherapeutic drug (SMCD) and moderate its toxic and side effects. Before the beginning, two selected models of chemotherapy drugs doxorubicin (DOX) and docetaxel (DOC) were conjugated with chloroquine (CQ) to produce new chloroquine derivatives. Additionally, salicylic acid was used as well with(CQ) to give another CQ-derivative (sections 2.3.1 to 2.3.3) , and then all these compounds were fully characterized **(Figure 3.1)** represents the structures of these derivatives.

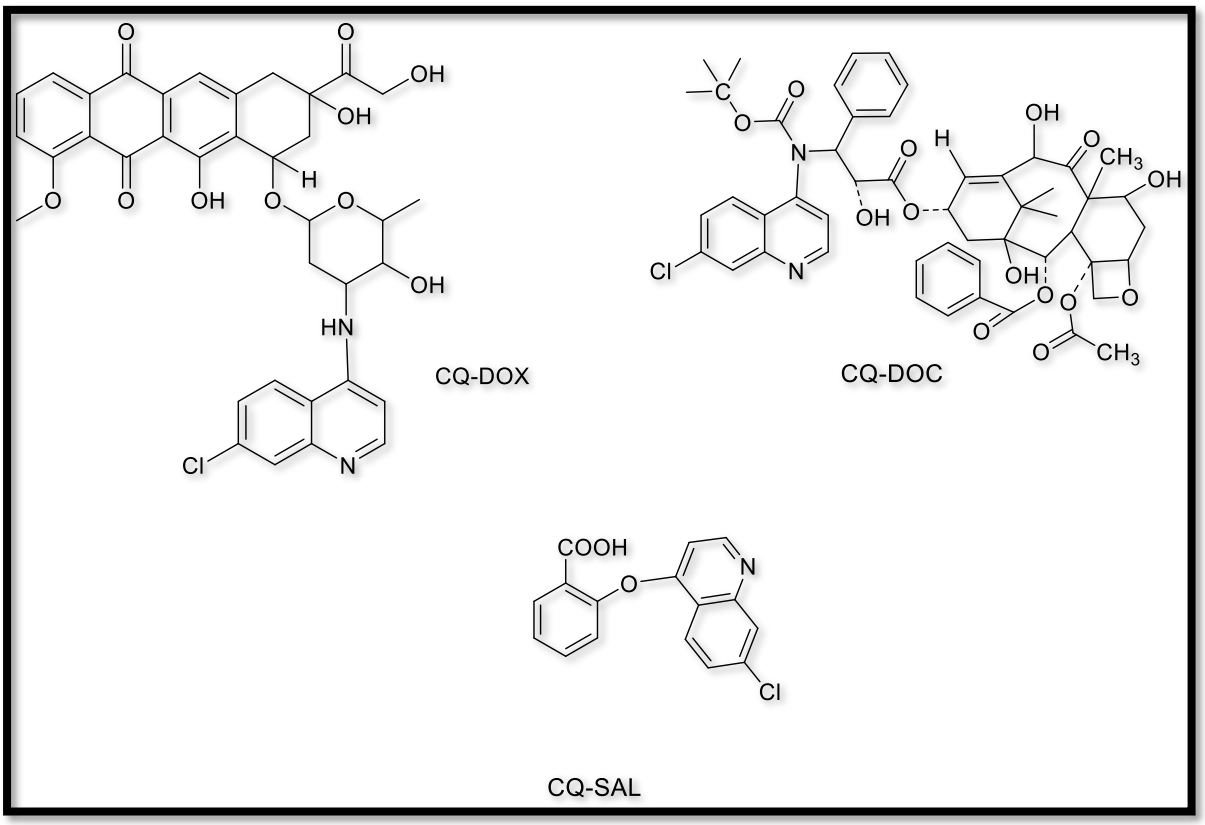


Figure 3.1: The suggested structure of CQ-derivatives

3.1.1.1 UV-Vis analysis of CQ-DOX

the derivative CQ-DOX was created by the reaction between dichloroquine (CQ) and doxorubicin (DOX) , compound that are more active than the original compound. One way to identify each conjugated compound is by using UV-Vis spectroscopy. The maximum wavelengths of 316 , 325 nm for CQ-DOX . As shown in **(Figures 3.2)**.

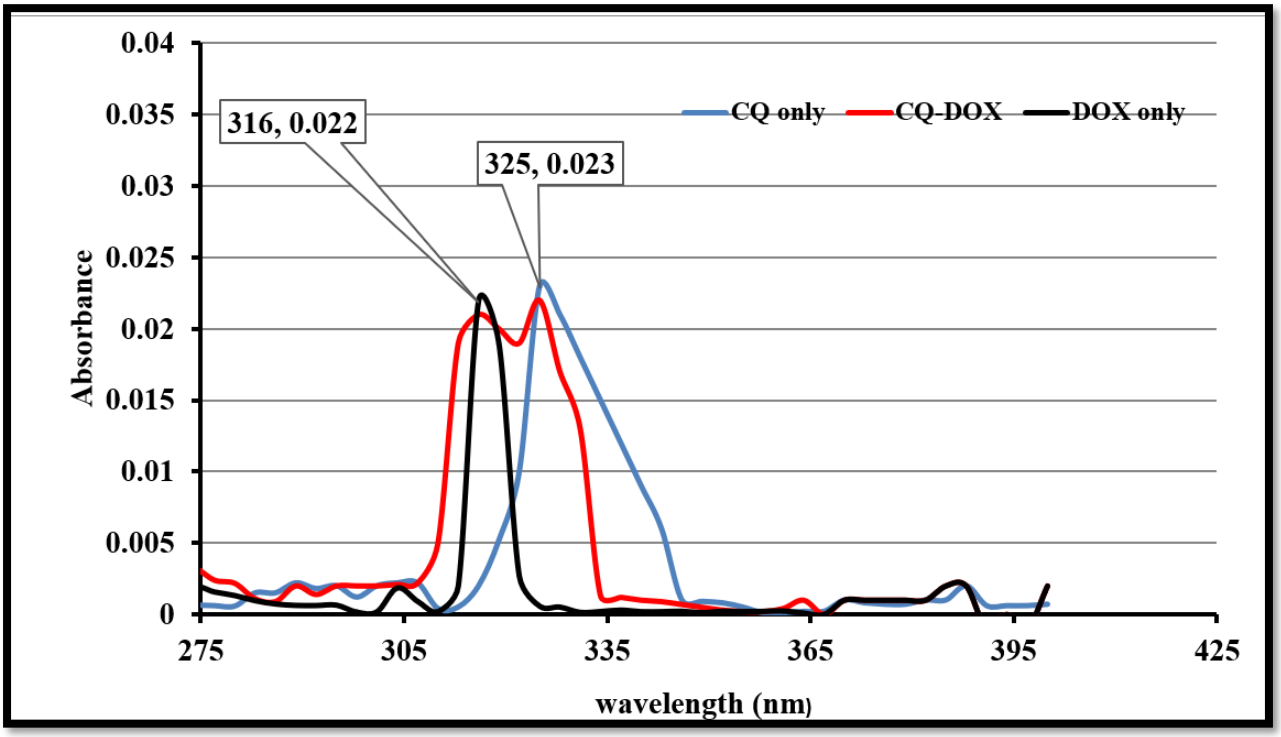


Figure 3.2: UV-Visible spectra for CQ-DOX

3.1.1.2 FTIR Analysis of Chloroquinoline Derivative of CQ- DOX

FTIR spectra for CQ-DOX compound provided good evidence for the production compound, and data displayed an intense band of stretching vibrations of N-H at 3350 cm^{-1} with no shoulder band, which is indicated the conjugation between CQ and DOX as illustrated in **(Figure 3.3)**. Moreover, the rest characteristic absorption peaks corresponding to vibrations of different functional groups of the drug molecule have been listed in **Table 3.1**.

Table 3.1: IR spectrum of CQ-DOX

3086, 2862	C–H stretch
1635	C=O stretch
1593, 1558, 1491	C=C ring stretch
1197, 1087	C–O–C stretch
805, 688	C=H bend, C=C ring bend
1352	C-N-C

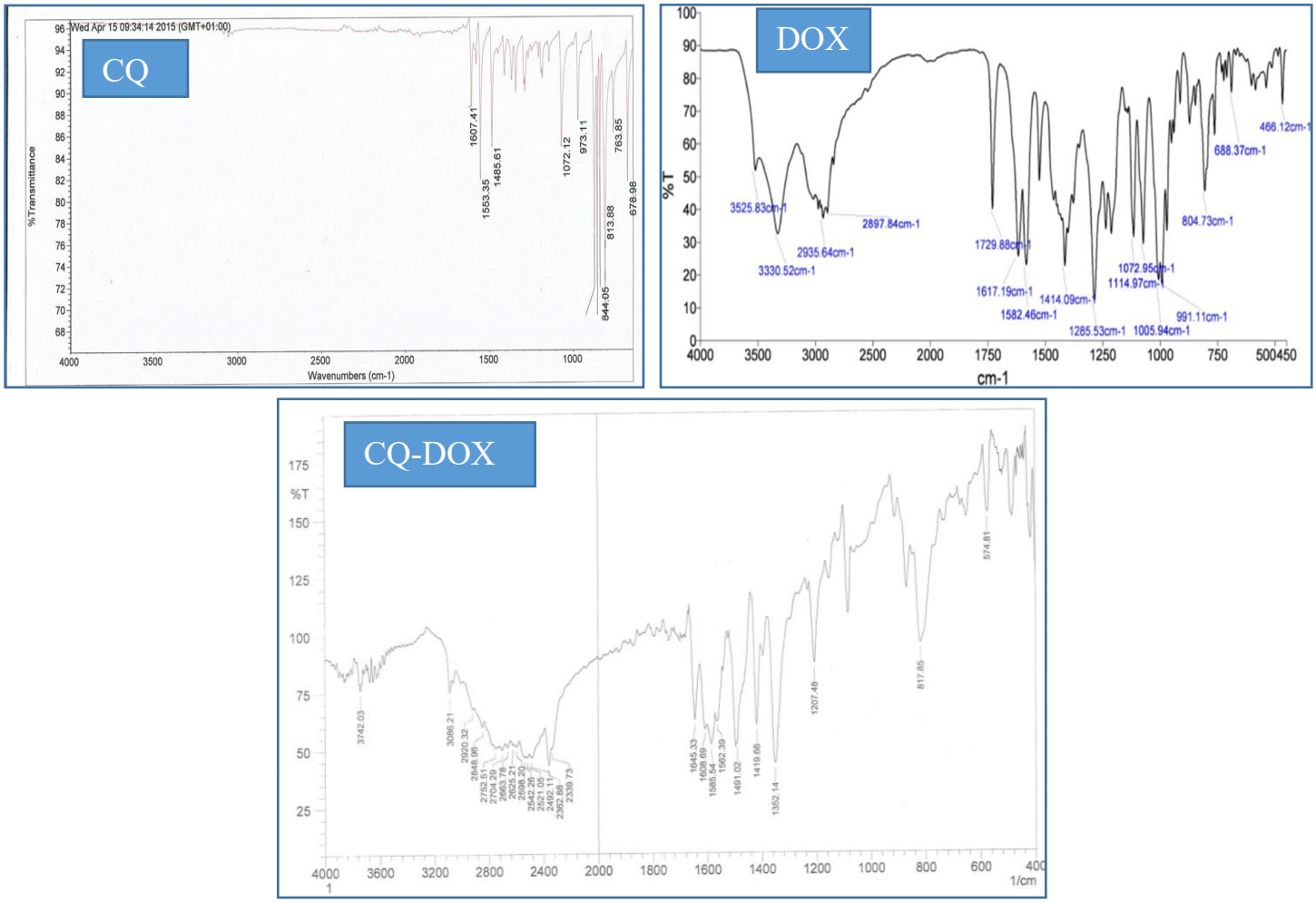


Figure3.3 FTIR spectra of CQ , DOX and CQ-DOX

3.1.1.3 Proton-NMR of CQ-DOX

The proton-NMR is the most important indication for identifying any compound; therefore, it was used to further improvement CQ-DOX production. 1HNMR spectra has been illustrated in**(Figure 3.4)** . The peak at 6.8 ppm due to secondary amine provided evidence for conjugation CQ with DOX

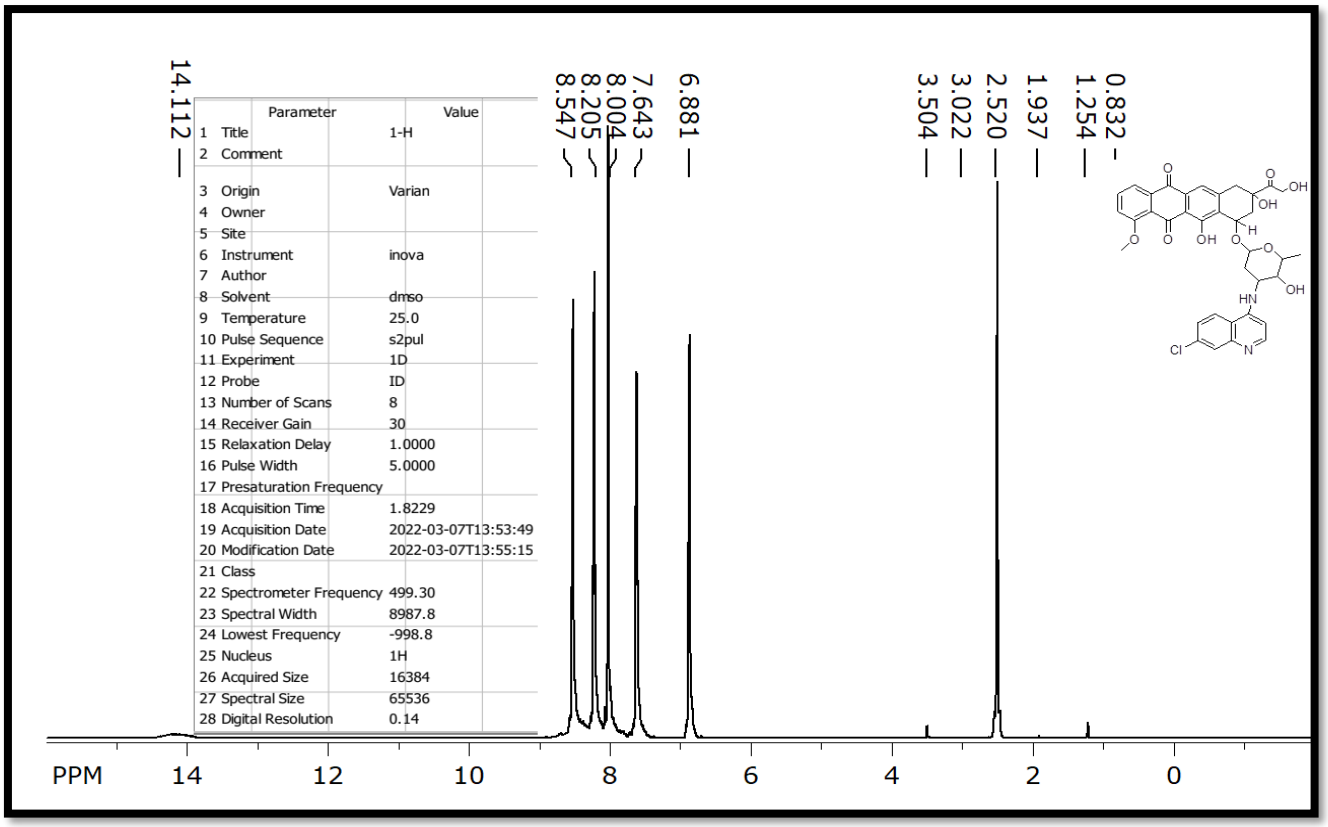


Figure 3.4^{1}HNMR spectrum for CQ-DOX

3.1.2.1- UV-Vis analysis of CQ-DOC

CQ -DOC derivative was created by the reaction between dichloroquine (CQ) and docetaxel (DOC) compounds which is more active than the original compound. One way to identify each conjugated compound is by using UV-Vis spectroscopy. The maximum wavelengths of 319 , 325 nm for CQ-DOC , As shown in **(Figure 3.5)**.

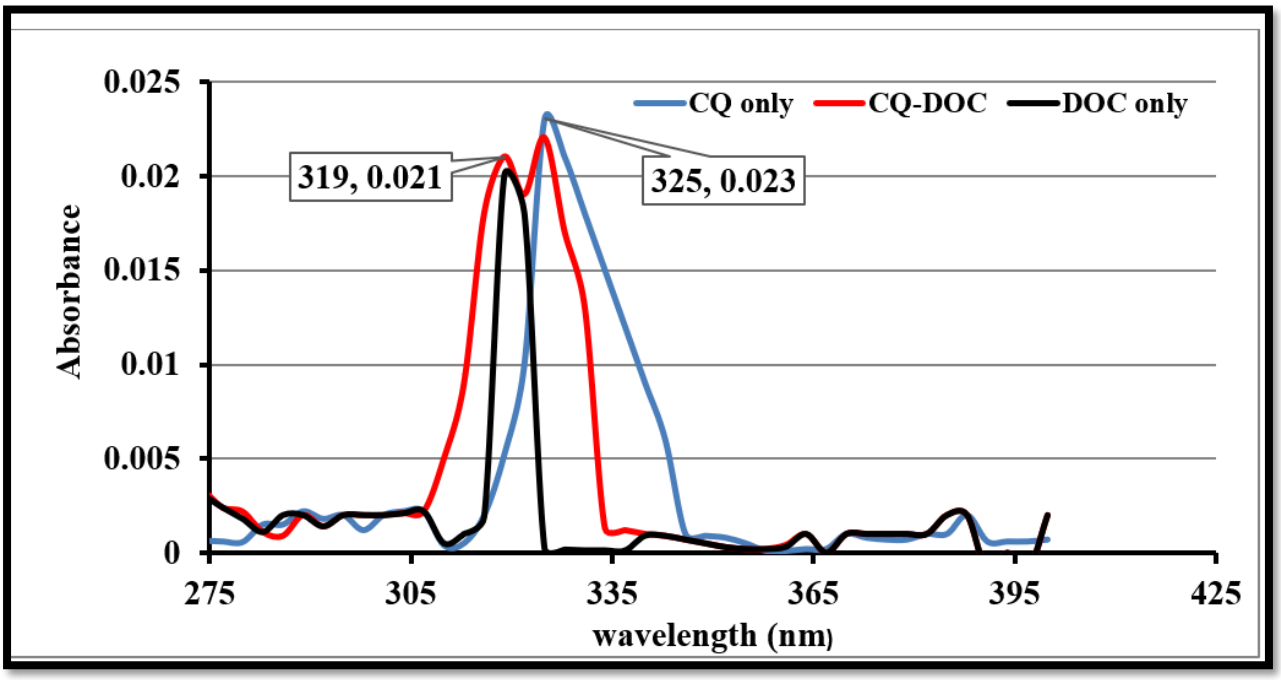


Figure 3.5: UV-Visible spectra for CQ-DOC

3.1.2.2- FTIR Analysis of Chloroquinoline Derivative of CQ- DOC

Similarly, FTIR spectra for CQ-DOC compound was indicated a clear extra band at 1350 cm-1 for stretching vibrations of C-N-C and disappearing a band at 3350 cm-1, which offers an indication for secondary amine of docetaxel, thus, providing good evidence for the conjugation between CQ and DOC as illustrated in **(Figure 3.6)** . From Table 3.2, the rest of characteristic absorption peaks correspond to different functional groups' vibrations.

Table 3.2: IR spectrum of CQ-DOC.

2924, 2860	C–H stretch
1739,1647	C=O stretch
1560, 1504, 1456	C=C ring stretch
1356, 1109	C–O–C stretch
1356	C-N-C

1

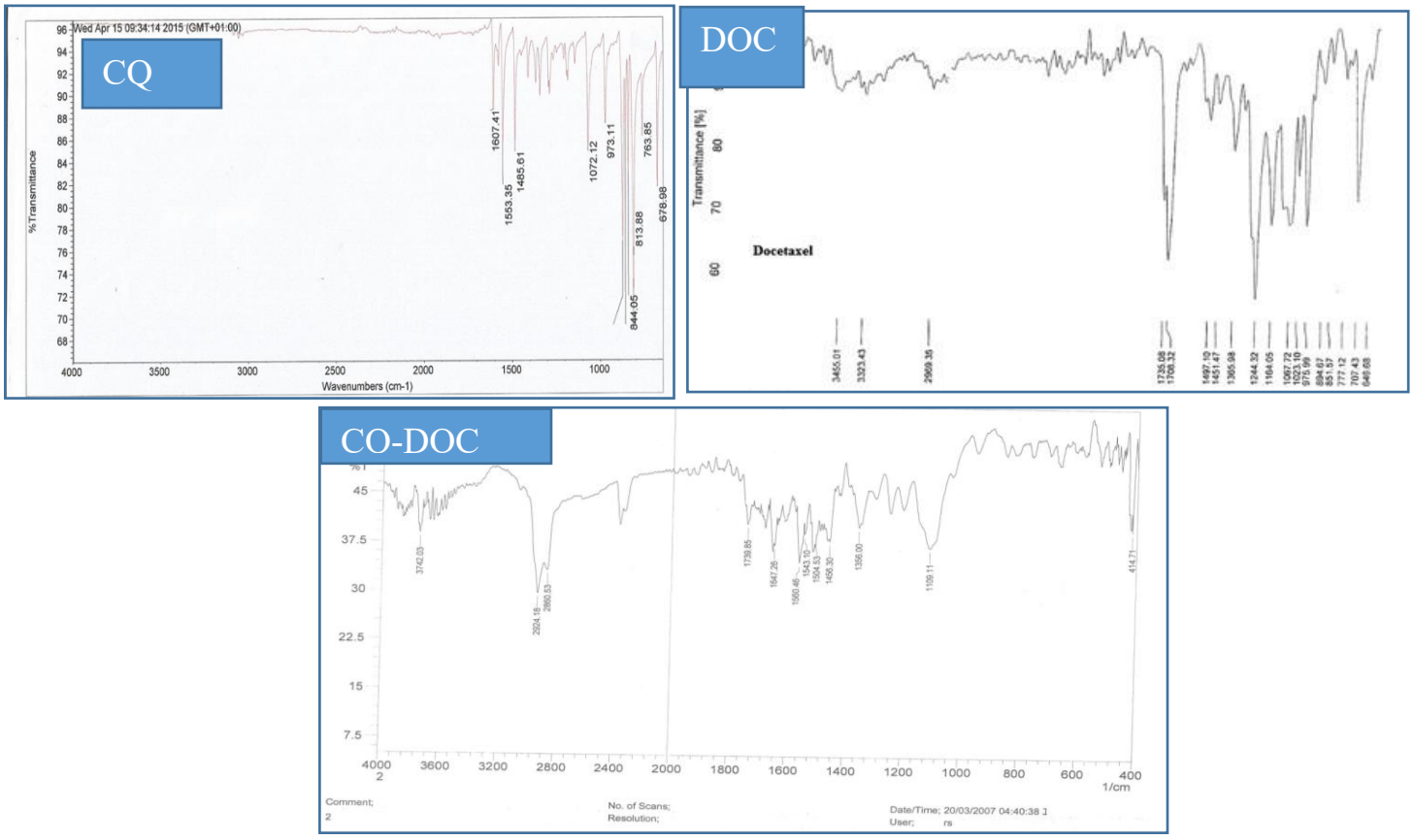


Figure 3.6 FTIR spectra of CQ,DOC andCQ-DOC

3.1.2.3 - Proton-NMR of CQ-DOC

The proton-NMR is the most important indication for identifying any compound; therefore, it was used to further improvement CQ-DOC production. 1HNMR spectra has been illustrated in**(Figure3.7) .** The peak at 6.8 ppm absent in the proton NMR for CQ-DOC due to the conjugation of CQ with DOC.

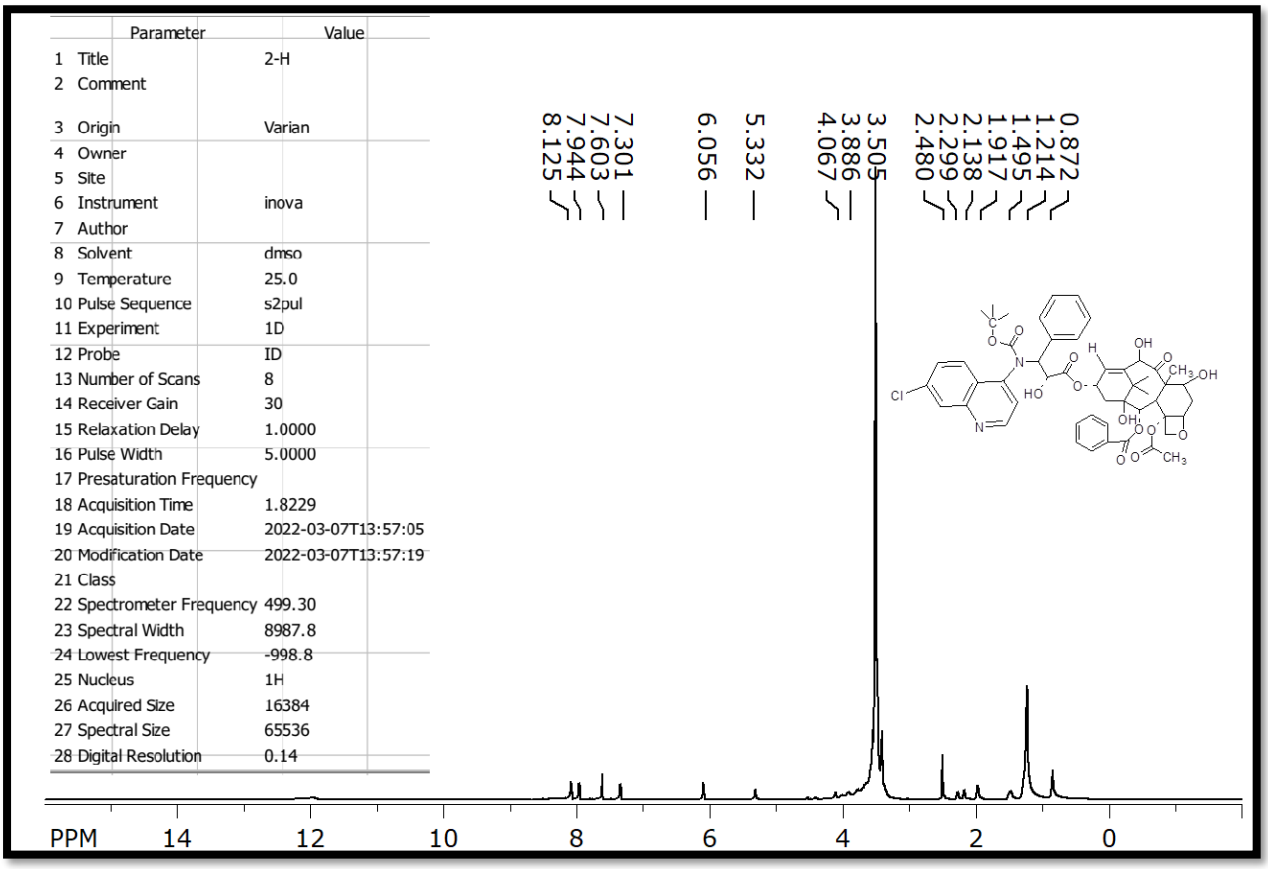


Figure 3.7 ^{1}HNMR spectrum for CQ-DOC

3.1.3.1- UV-Vis analysis of CQ-SAL

Chloroquine derivative CQ-SAL was created by the reaction between dichloroquine (CQ) and salicylic acid to create new CQ-SAL compound that are more active than the original compound.The maximum wavelengths for CQ-SALwere 340,325. As shown in **(Figure 3.8)** .

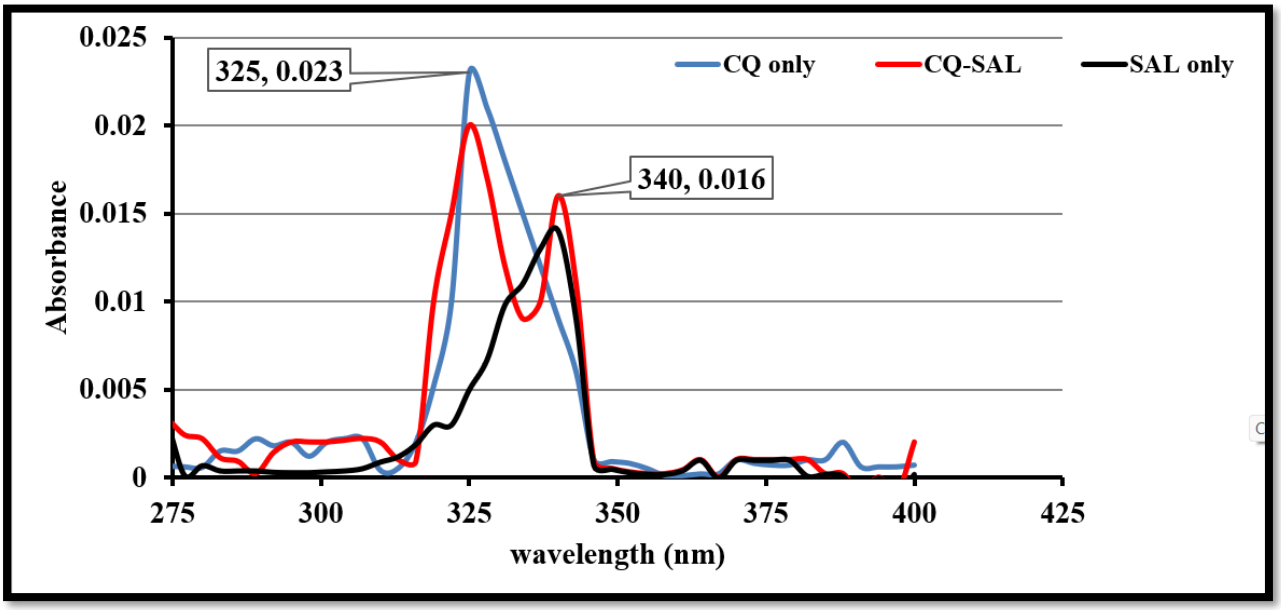


Figure 3.8: UV-Visible spectra for CQ-SAL

3.1.3.2- FTIR Analysis of Chloroquinoline Derivative of CQ- SAL

For the third derivative CQ-SAL, FTIR spectra was provided good evidence for the production compound, data displayed an intense band of stretching vibrations of C-O-C at 1100 cm^{-1}, which indicates the conjugation between initial compounds (CQ and SAL) as indicated in **(Figure 3.9)**. while, the rest characteristic spectrum of absorption peaks corresponding to vibrations of different functional groups is listed in **Table 3.3**

Table 3.3: IR spectrum of CQ-SAL

2926, 2856	CH stretch
1678	C=O stretch
1593, 1558, 1491	C=C ring stretch
1125,1175.1225	C-O-C

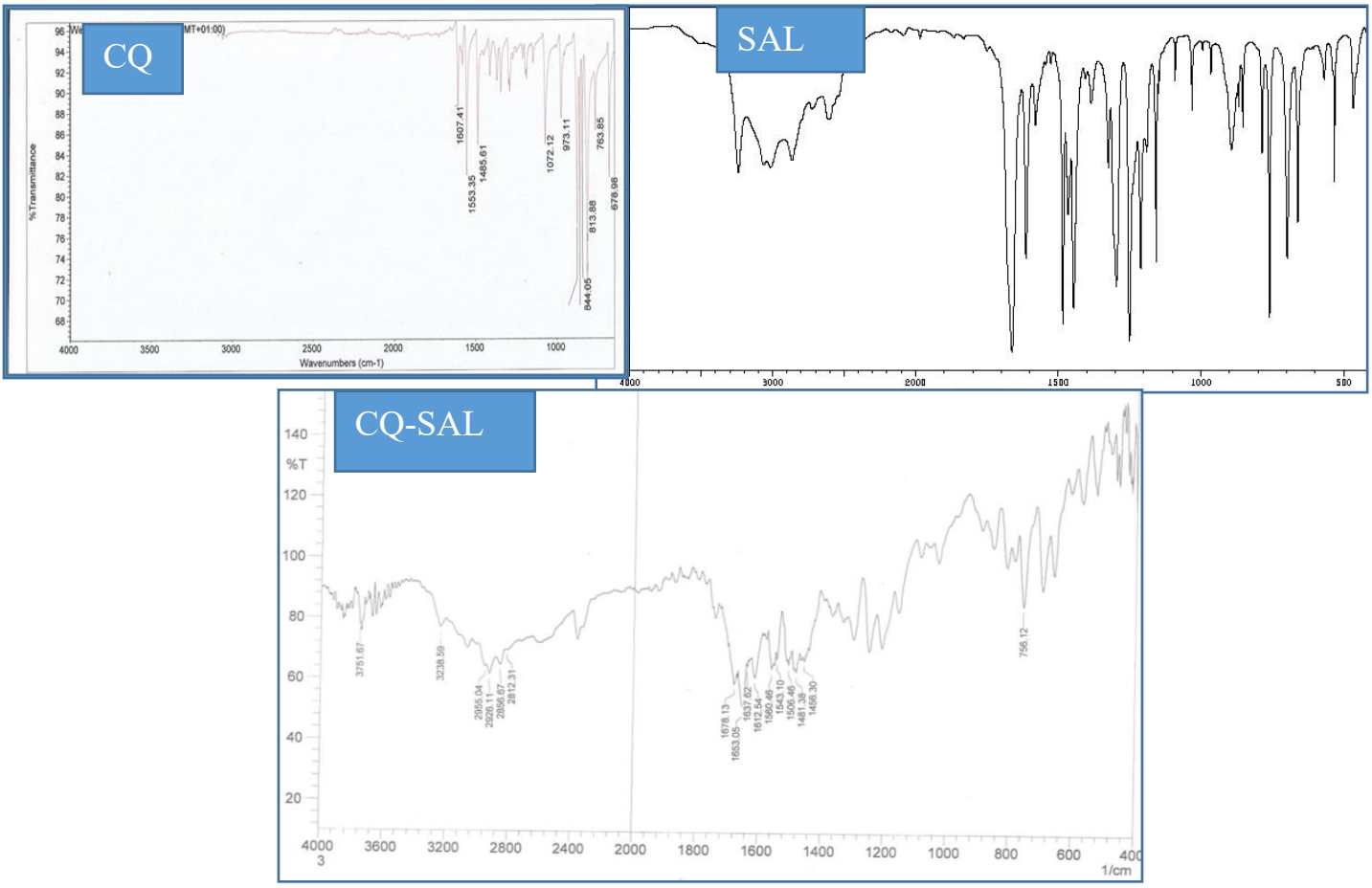


Figure 3.9 FTIR spectra of CQ,SAL and CQ-SAL

3.1.3.3 - Proton-NMR of CQ-SAL

the proton-NMR has been used as a farther evidence for producing CQ-SAL.. The peak at 9 ppm of phenolic OH driven from SAL disappeared.The fact that ether linkage was produced indicates the conjugation between CQ and SAL as illustrated in(**Figure3.10).**

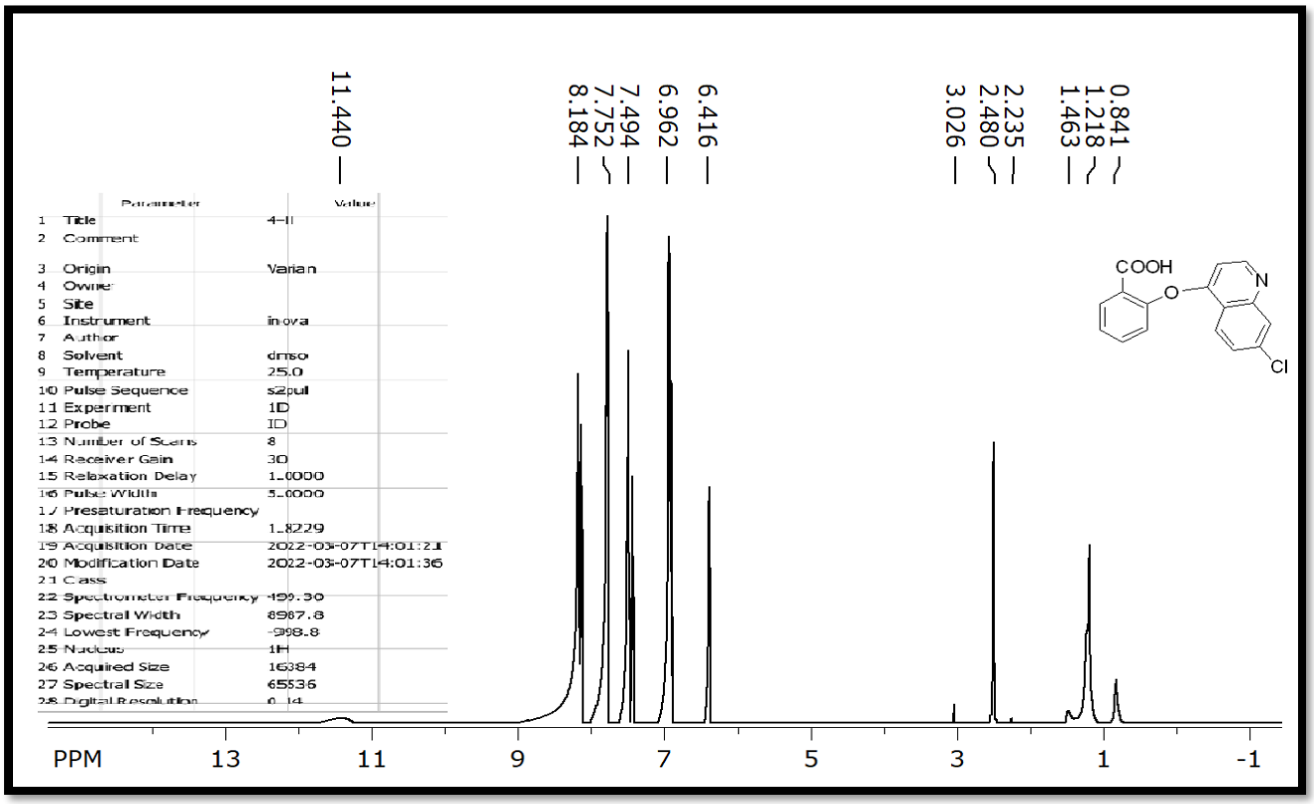


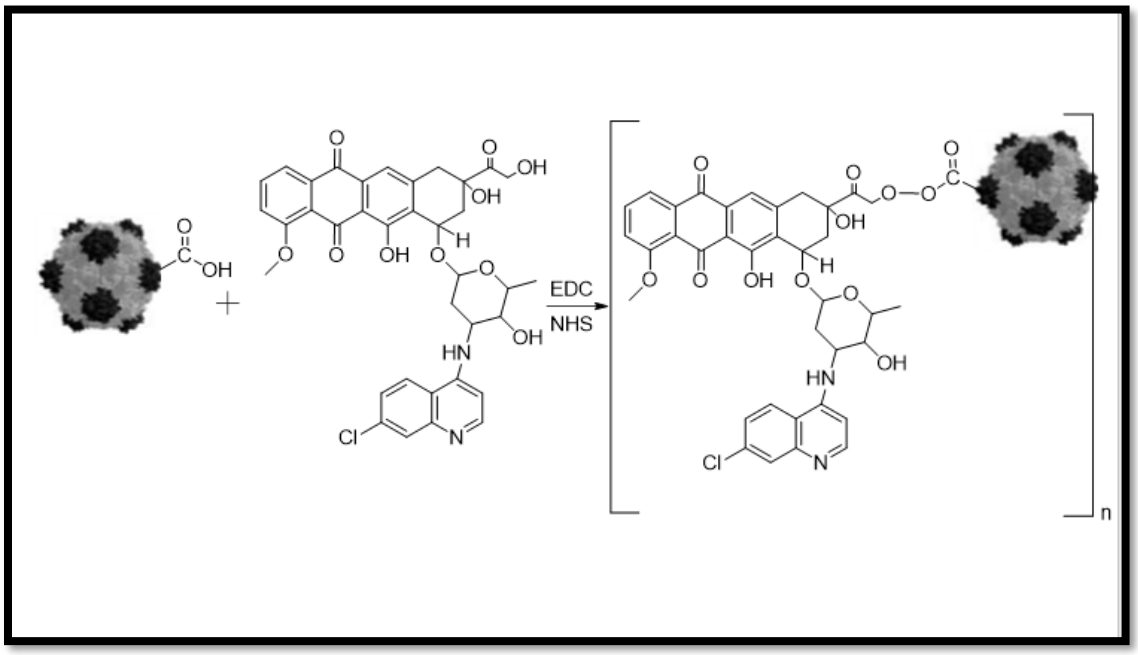
Figure 3.10 [1]HNMR spectrum for CQ-SAL

3.2 - Chemical modification of CPMV particles

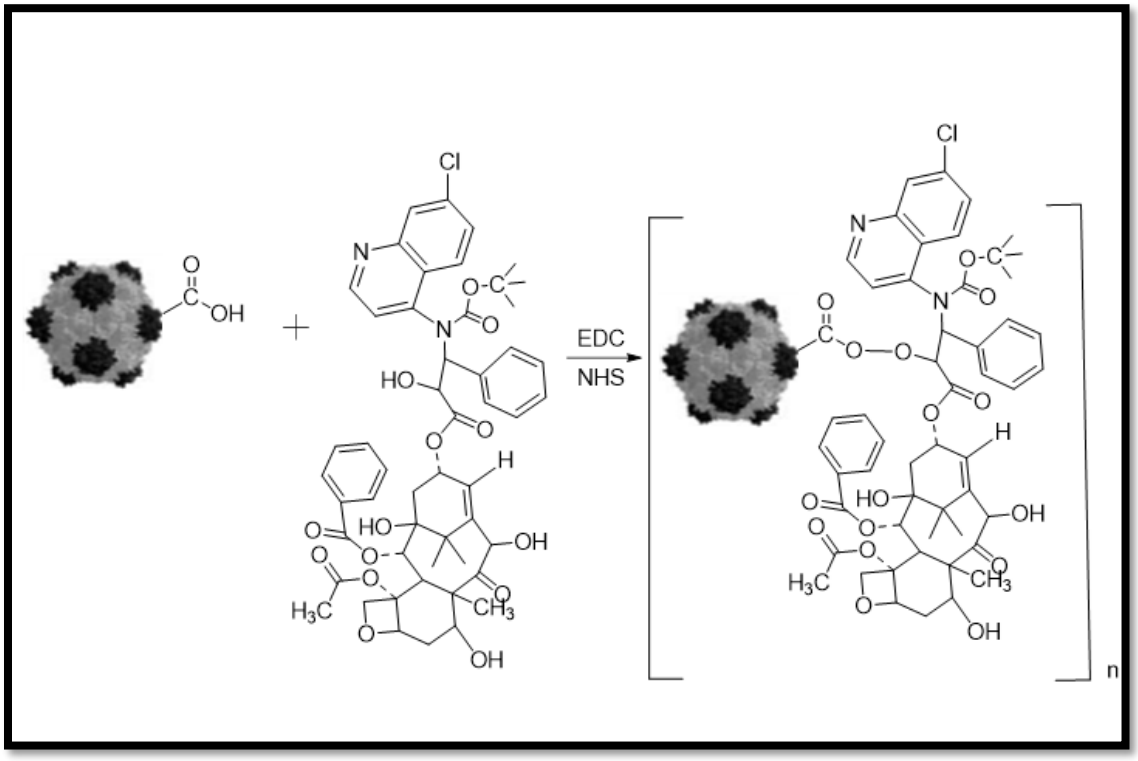
Viruses can be chemically modified to provide useful information on natural systems, new protein structures, and medicinal conjugates. There are several goals of chemical modification such as identifying which parts of the molecule are exposed to solvent and determining critical residues for a particular phenotype. [147] Additionally, it was reported that chemical modification can be a useful technique for enhancing the functional qualities of plant and yeast proteins and may enable the adaptation of proteins with certain functional properties.[50] This study uses a plant virus's chemical modification to create new drug can didates that may display potent anticancer activity.EDC/NHS activated external carboxyl groups on both CPMV and salicylic acid.

3.2.1- Addressability of carboxyle on the exterior surface of CPMV with CQ-DOX and CQ-DOC).

The addressable carboxylate on the outer surface of CPMV was modified with EDC and NHS together to form CPMV-NHS-ester, CQ-DOX and CQ-DOC were also conjugated to the external surface of CPMV to form CPMV-CQ-DOX and CPMV-CQ-DOC, as presented in **Schemes 3.1** and **3.2**. Addressable carboxyl is produced from acidic amino acides (aspartic and glutamic acids) on the exterior surface of wt CPMV, producing CPMV-CQ-DOX conjugates in 76.6% recovered yield depending on the virus's starting concentration (section 2.9.1) . Similarly, in order to produce CPMV-CQ-DOC in 77% recovery yield according to the initial virus concentration, the exterior surface of wt CPMV was modifed with CQ-DOC (section 2.9.1), **Schemes 3.2.**



Schemes 3.1 Chemical modification of CPMV with CQ-DOX



Scheme 3.2 The Chemically modification of CP MV with CQ-DOC

A verification of the changed particles' integrity was made by UV/Vis elctroscopy,Nano Drop spectrophotometer as shown in**(Figures 3.11,3.12,3.13,3.14)** and also agarose gel electrophoresis **(Figure 3.17)**.

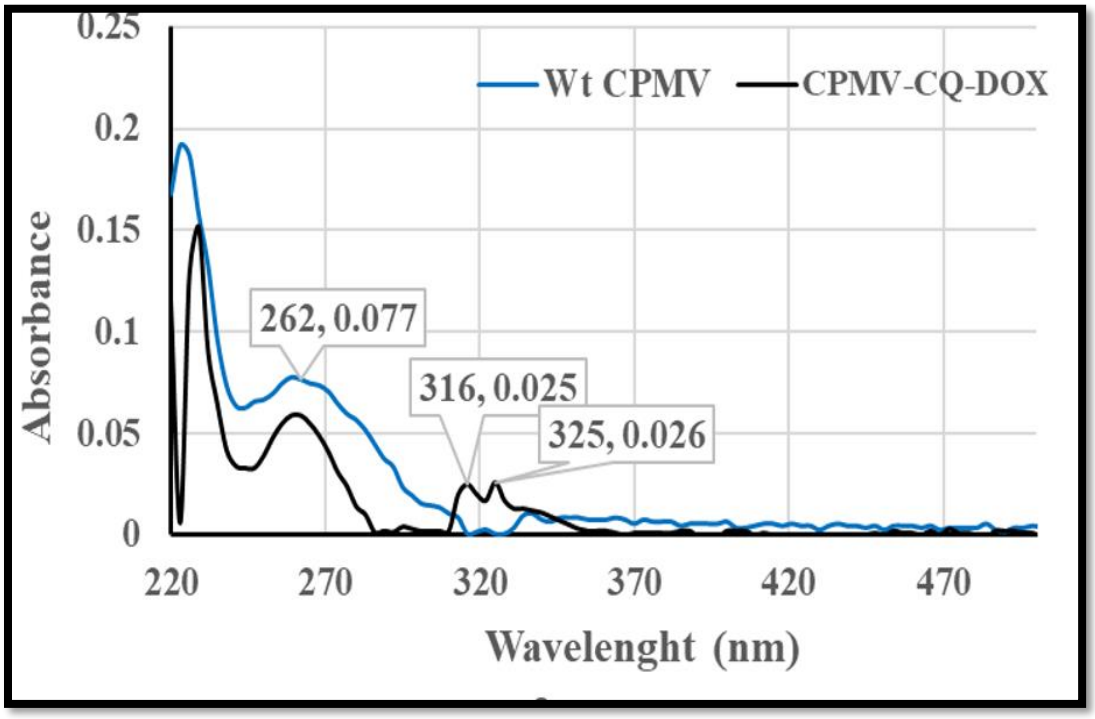
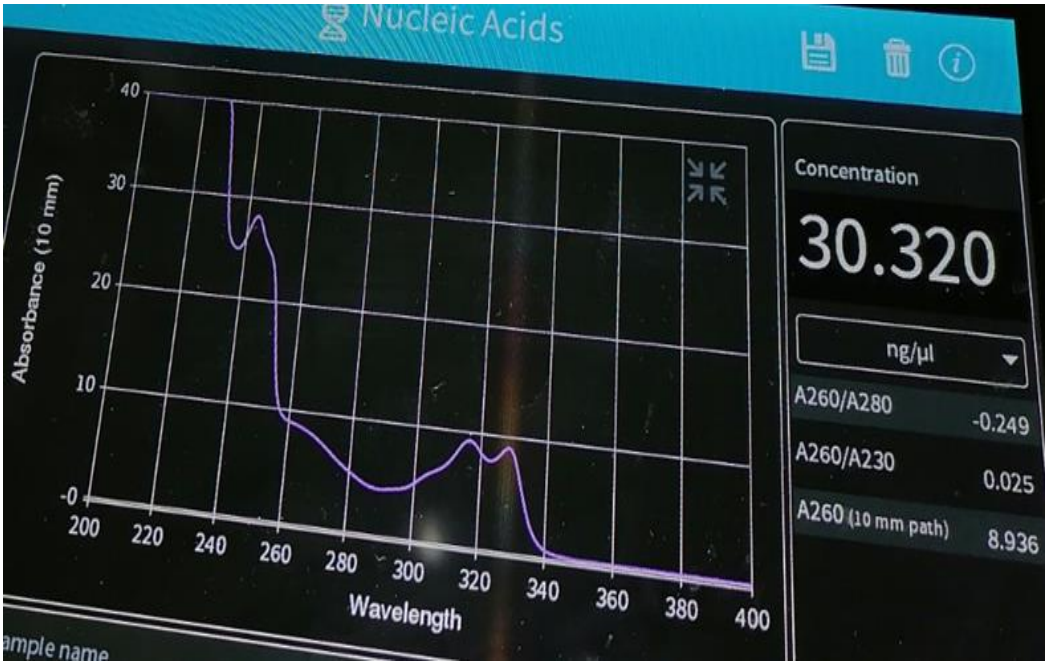


Figure 3.11 UV/ Vis spectrum of wt CPMV before and after modification with CQ-DOX.



3.12 Nanodrop image of modified wt CPMV with CQ-DOX.

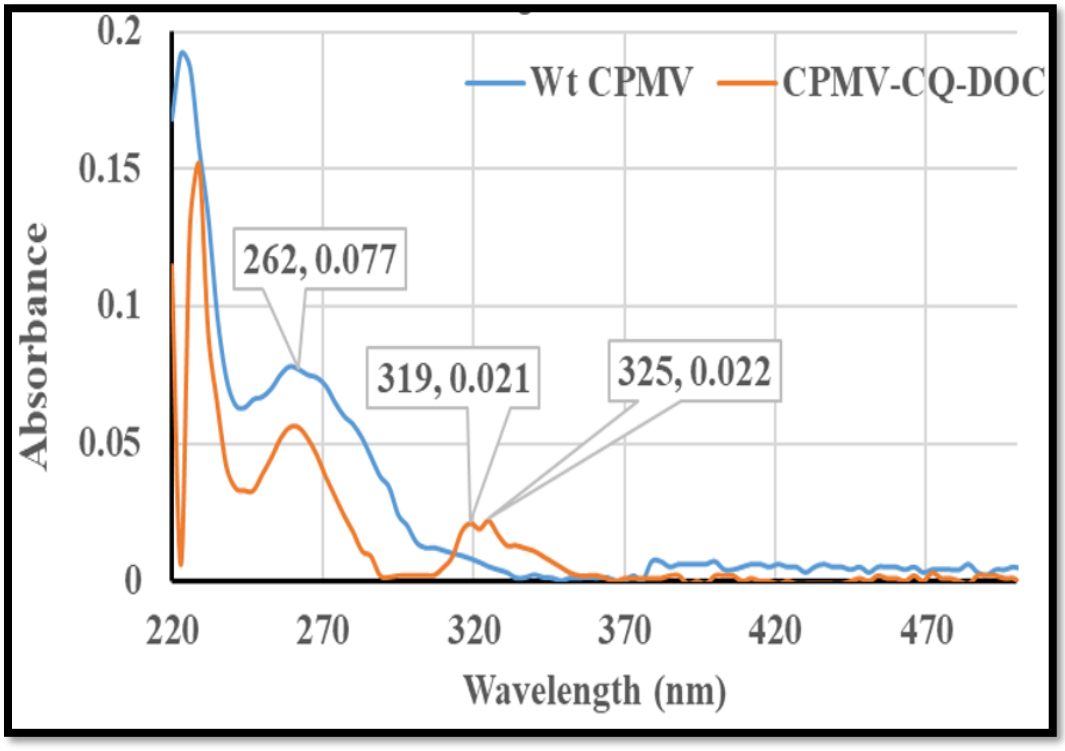
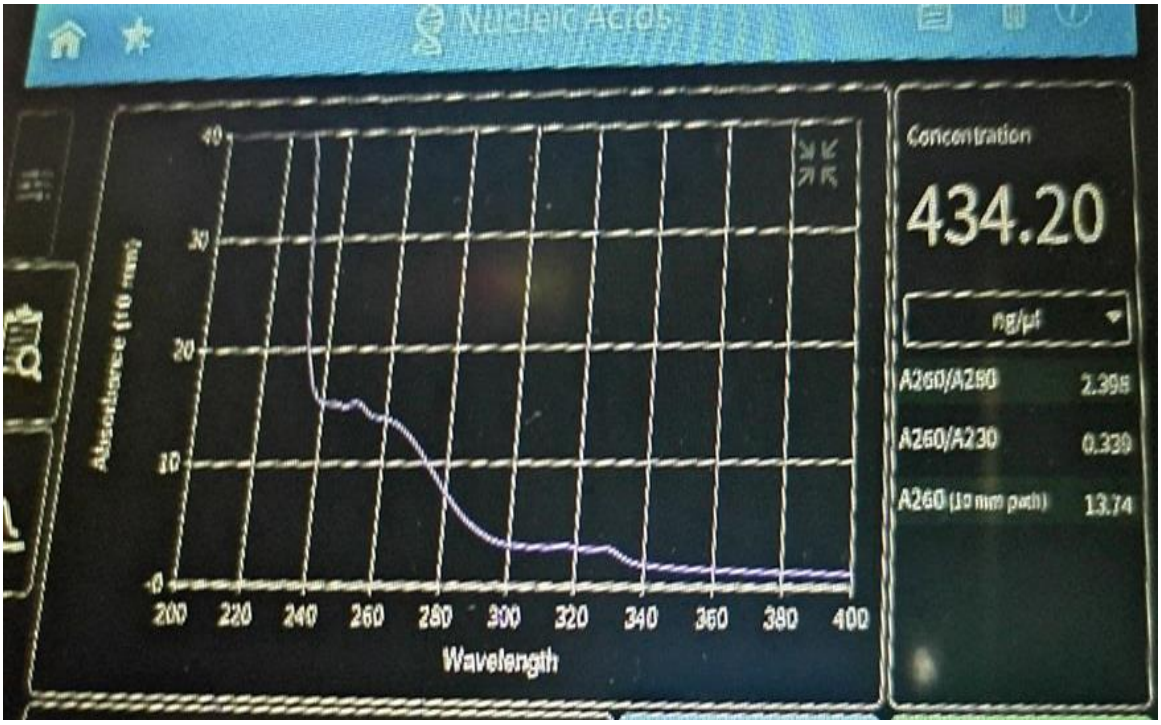


Figure 3.13UV/ Vis chart for modified and unmodified of wt CPMV with CQ-DOC.

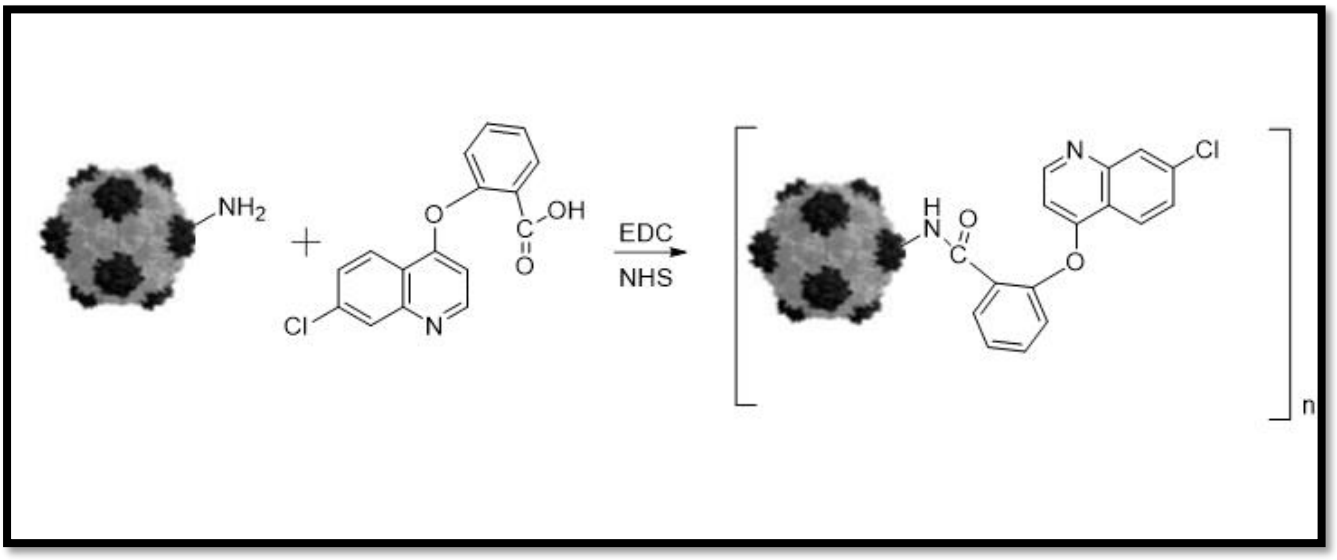


3.14 Nanodrop image of modified wt CPMV with CQ-DOC.

UV/Vis has been used to characterize CQ-DOX and CQ-DOC in aqueous 20% DMSO. Both CQ derivatives have two absorption maxima at 325, 316 and 325, 319 nm, respectively. In contrast, CPMV has absorption maxima at 262 nm, which makes identifying covalent bonding and successful coupling of ouet carboxylate of CPMV surface easy.

3.2.2- Addressability of amines on the external surface of CPMV with CQ-SAL

Addressable amines generated from lysines, which exposed on the exterior wt CPMVsurface was reacted with (CQ-SAL) producing CPMV-CQ-SAL (section 2.9.2) conjugates in 76.6% recovered yield depending on the started virus concentrations, as indicated in **Scheme 3.3**.



Scheme3.3 The Chemically modification of CPMV with CQ-SAL

The evidence for CQ-SAL conjugation onto the external surface of CPMV, UV–Vis spectrophotometer was used as shown in **(Figures 3.15 and 3.16)**, as well as agarose gel electrophoresis as displayed in **(Figure3.17).**

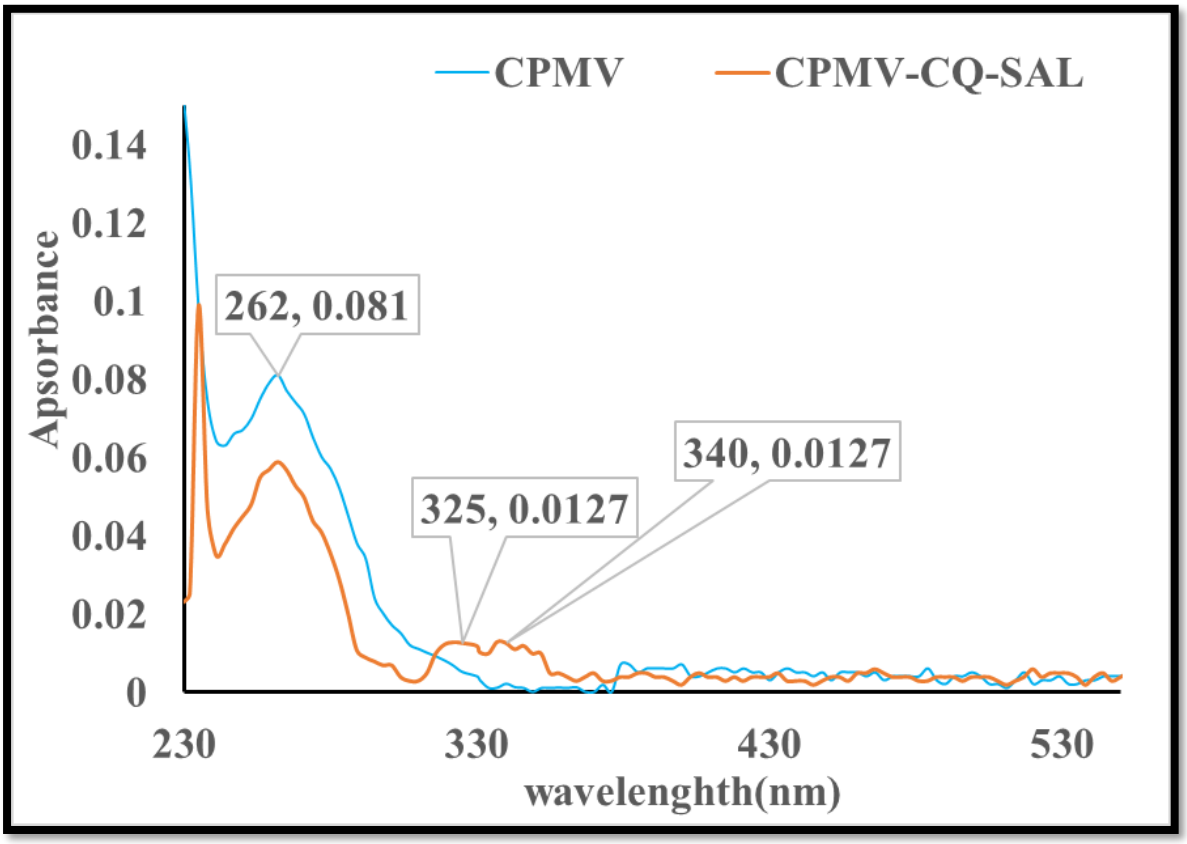


Figure 3.15 UV/ Vis spectrovotometer measurement for modified and unmodified CPMV with CQ-SAL

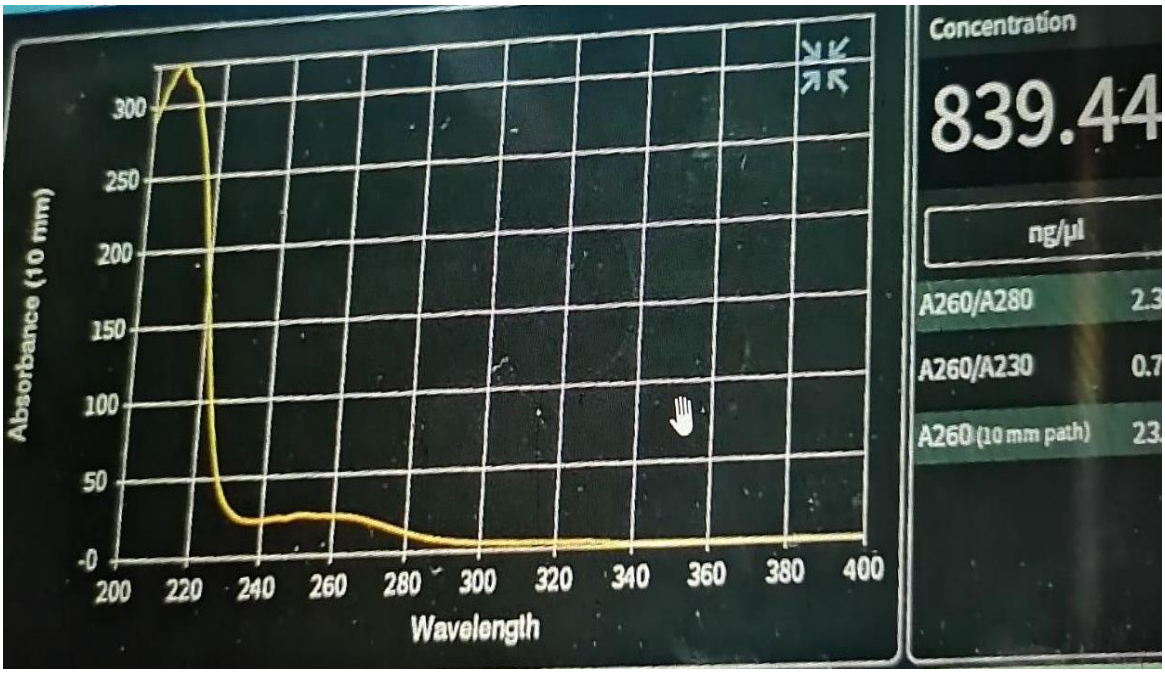
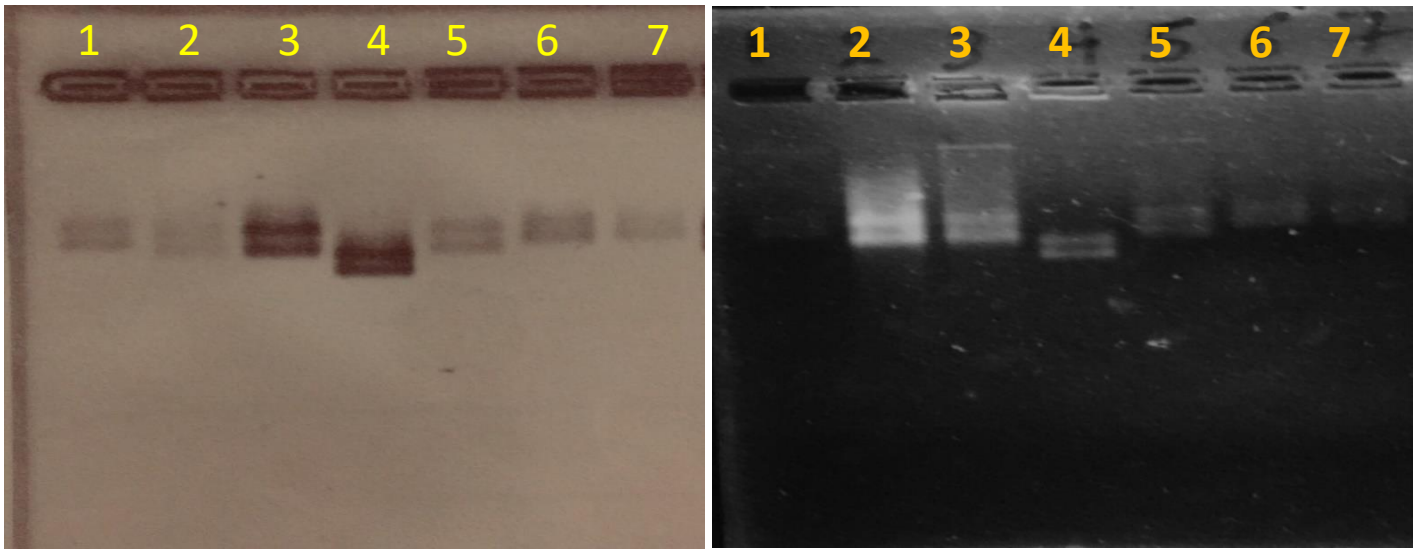


Figure3.16 Nanodrop image for modified CPMV with CQ-SAL

The conjugation of CQ derivatives to the external surface of wt CPMV was confirmed by running stained and unstained agarose gel electrophoresis with ethidium bromide; visible bands on agarose gel confirmed the movement of each CQ- derivative, which was loaded onto the surface of the CPMV.



A: (Coomassiee staine) **B: (ethidiumm-bromide staine)**

Figure 3.17 1.2% w/v agarose in agarose gel electrophoresis at 60 volts: Lan 1, 6 CPMV-CQ-DOX, lane 2 CPMV-DEC-NHS, lane3,5 CPMV CPMV-CQ-SAL, lane 4 WT CPMV, lane7 CPMV-CQ-DOC.

As evidenced from the above figure, the migration of RNA-containing un-modified CPMV particles is faster than that of modified CPMV, resulting in a slow movement of CPMV-CQ-DOX, CPMV-CQ-DOC and CPMV-CQ-SAL compared to wt CPMV. In contrast, the esterified CPMV particles were moved to the anode more slowly than the wild type, but they were faster than CPMV-CQ-DOX, CPMV-CQ-DOC and CPMV-CQ-SAL. Suggesting that it is linked to the molecular weight of each derivative and the number of negative charges, as shown in**(Figure 3.17)**.

There is an agreement with other researchers that have suggested ways to the successfully modification of CPMV with different compounds**.** [148]

3.3 - Quantification of CQ-derivatives binding on exterior CPMV surface

The number of each CQ-derivative has been calculated using two different dyes one for carboxyl (Alexa Fluor dye) and another for amine (CF-488A dye).

The absorption of CPMV and dye were considered to determine the quantity of unconjugated carboxylate and amine groups on the outer surface of CPMV. Besides, Beer's Lambert law was used to calculate the particle and dye concentration, while the number of CQ derivatives can be calculated according to Eq 1 and Eq 2 below:

C CPMV = A CPMV / ε CPMV , C dye = A dye/ ε dye **--- Eq 1**

The number of free carboxyls or amine = C dye / C CPMV---- **Eq 2**

Where A stands for absorption., C is the CPMV particle and dye concentration in mg mL^{-1} , d is the length of the light path in cm and ε, CPMV and dye molar extinction coefficients in mL mg^{-1} cm^{-1}. [50]

cpmv=8.1 mL mg^{-1} cm^{-1}

ε Alexa Fluor dye=73000 mL mg^{-1} cm^{-1}

ε CF-488 dye=70000 mL mg^{-1} cm^{-1}

extinction coefficients in mL mg^{-1} cm^{-1}.

3.3.1- Quantification of CQ-DOX and CQ-DOC

Each derivative has been calculated according to the most excellent absorbance of CPMV at a wavelength of 260 nm, with a molar extinction coefficient of 8.1 mL.gm^{-1} cm^{-1}.The highest absorbance of the Alexa Flour dye is 493-494 nm, with a molar extinction confusions of 73,000 mL mg^{-1} cm^{-1}. as shown in **(Figures 3.18 and 3.19).**

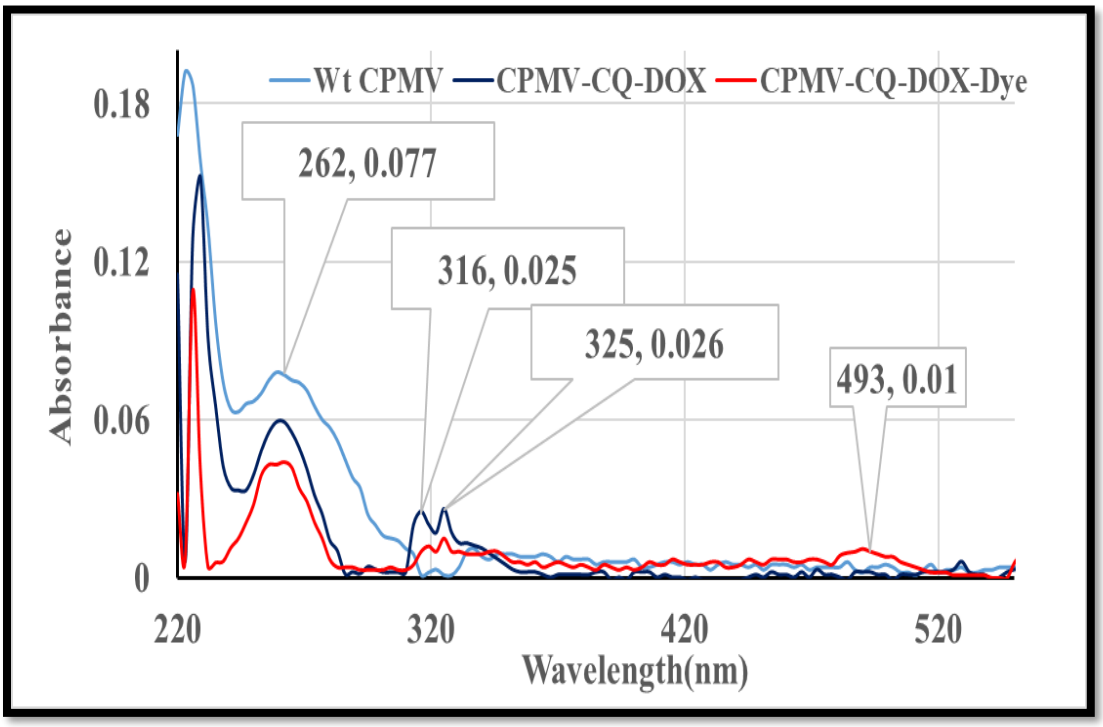


Figure3.18 UV–Vis spectrum of CPMV before and after modification with using Alexa Flour dye and CQ-DOX.

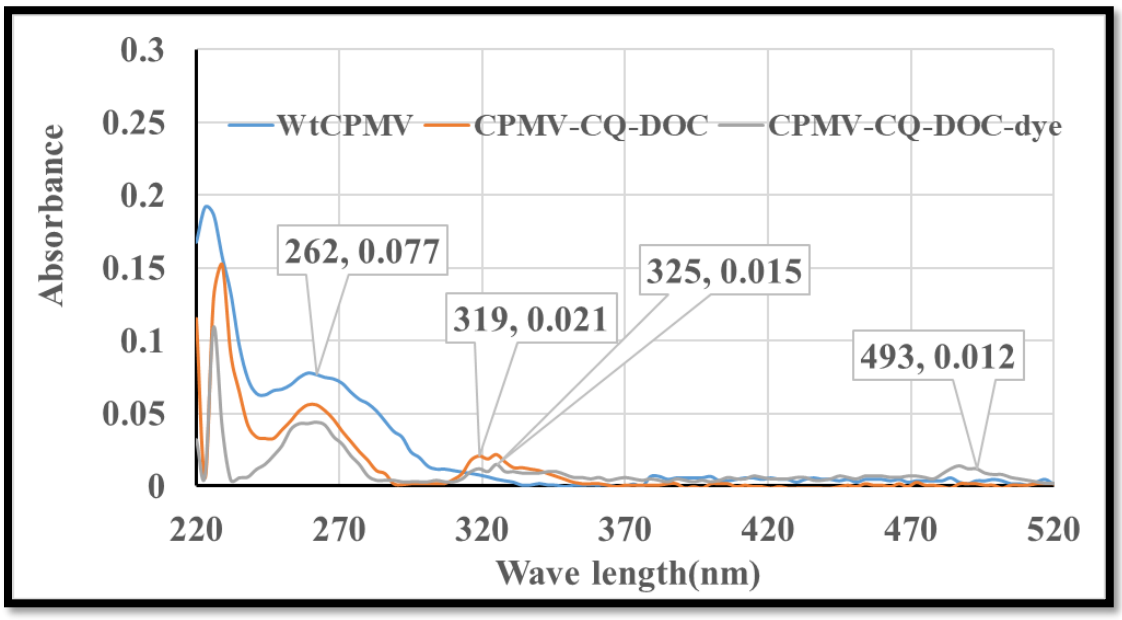


Figure 3.19 UV–Vis spectruphotomer measurement for modified and unmodified CPMV with CQ-DOC and Alexa Flour dye.

The number of each CQ-derivative (CQ-DOX, CQ-DOC) has been evaluated to be **87±1 and 79±1** per particle respectively . Results are given in **Table 3.4.**

Table 3.4 CPMV, CPMV- CQ-DOX, CPMV- CQ-DOC reactions with Alexae Flour-dye later 8h dialysis using 500 ml Sodium phosphate buffer.

React	Number of dye molecular/CPMV practical	CQ-DOX and CQ-DOC Numbers
CPMV dye	**180**	**…….**
CPMV-DOX-CQ - dye	**93**	**87±1**
CPMV-CQ-DOC dye	**101**	**79±1**

Number of dye molecular is yhe number of dye which conjugated with CPMV

3.3.2- Quantification of CQ-SAL

CF-488A dye was used to identify unpaired amines groups, which have a maximum absorption at 490 nm with a molar extinction coefficient ε = 70000 ml mg^{-1} cm^{-1}. as presented in **(Figure 3.20) .**

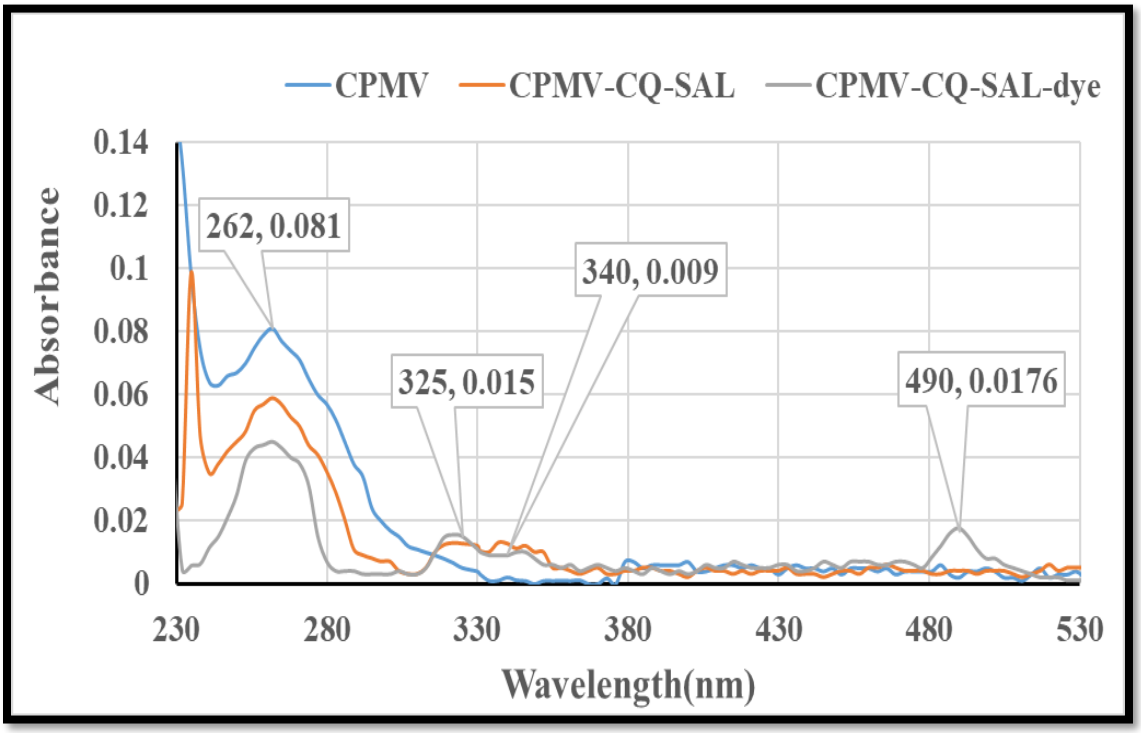


Figure3.20 UV–Vis spectrum of CPMV before and after modification with **CF-488A dye** and CQ-SAL.

The number of CQ-SAL has been calculated according to **Table 3.5**

Table 3.5 The reaction of CPMV, CPMV- CQ-SAL with CF-488A dye after 8h dialysis against 500 ml sodium phosphate buffer.

React	Number of dye molecular/CPMV particle	CQ-SAL Number
CPMV dye	**240**	**……**
CPMV-CQ-SAL-dye	**115**	**89±2**

On the other hand, agarose gel electrophoresis was used to test whether the dye was linked to the external surface of CPMV or not. Ethidium-bromide-stained agarose gel showed excess fluorescent intensity in all lanes after 4h and 6 h dialysis, which means that all modified particles still have excess dye. Even though there was no possibility of calculating the correct number of conjugated CQ **(Figure 3.21)**, a solution was eventually brought into being continuous dialysis for extra two hours. Results are shown in **(Figure 3.22),** indicating clear cleavage of dye-modified CPMV and CPMV-CQ on agarose gel after 8h dialysis; this may be derived from the cleavage of 24 amino acids terminus on the small subunits**. [47]**

After six h of dialysis, surplus non-reacted dye was visible, but after an additional 2 hours, there is no excess dye (8 h dialysis). Thus demonstrating that 8 h of dialysis is the ideal amount of time to discontinue dialysis and determine the appropriate number of CQ-derivatives. The fluorescent intensity in all modified particle lanes compared to unmodified particles confirmed the dye's conjugation on the external surface of CPMV.

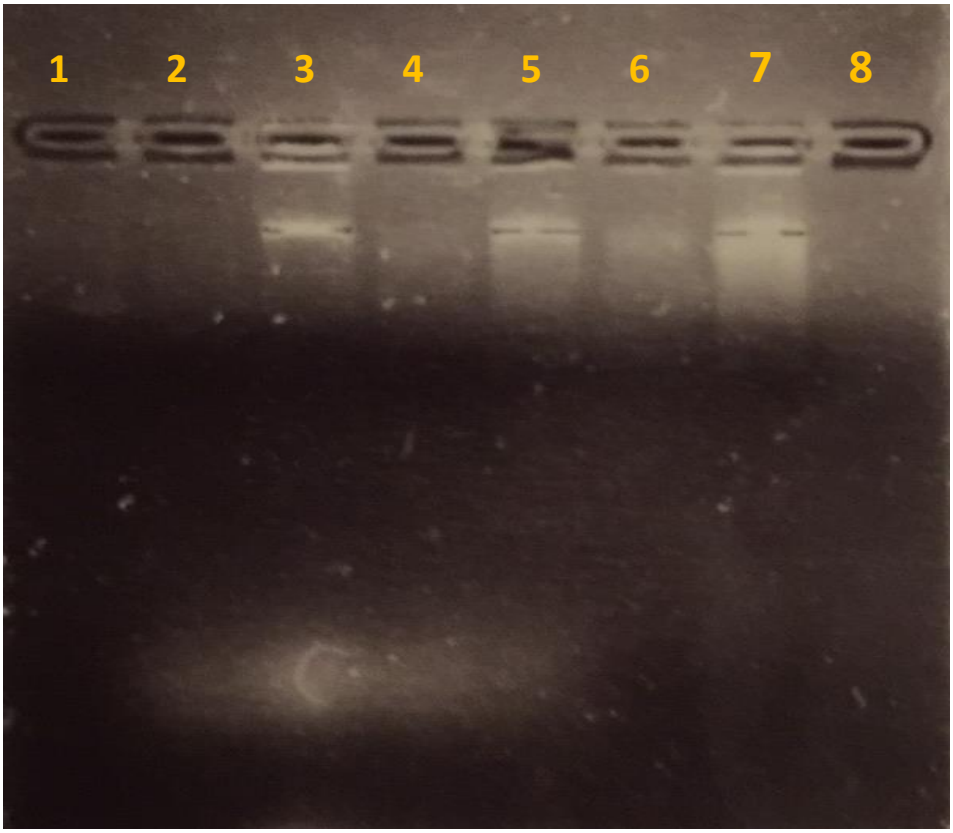


Figure 3.21 1.2% w/v agarose, 60 V agarose gel electrophoresis, stained with EtBr after 6h dialysis: lanee 1,2 CPMV-CQ-DOC, 3- CPMV -CQ-SAL-dye, 4-CPMV-CQ-DOX-dye, 5- CPMV-A lexa fluor dye ,6 - CPMV-CF-488 dye,7-CPMV, 8-empty.

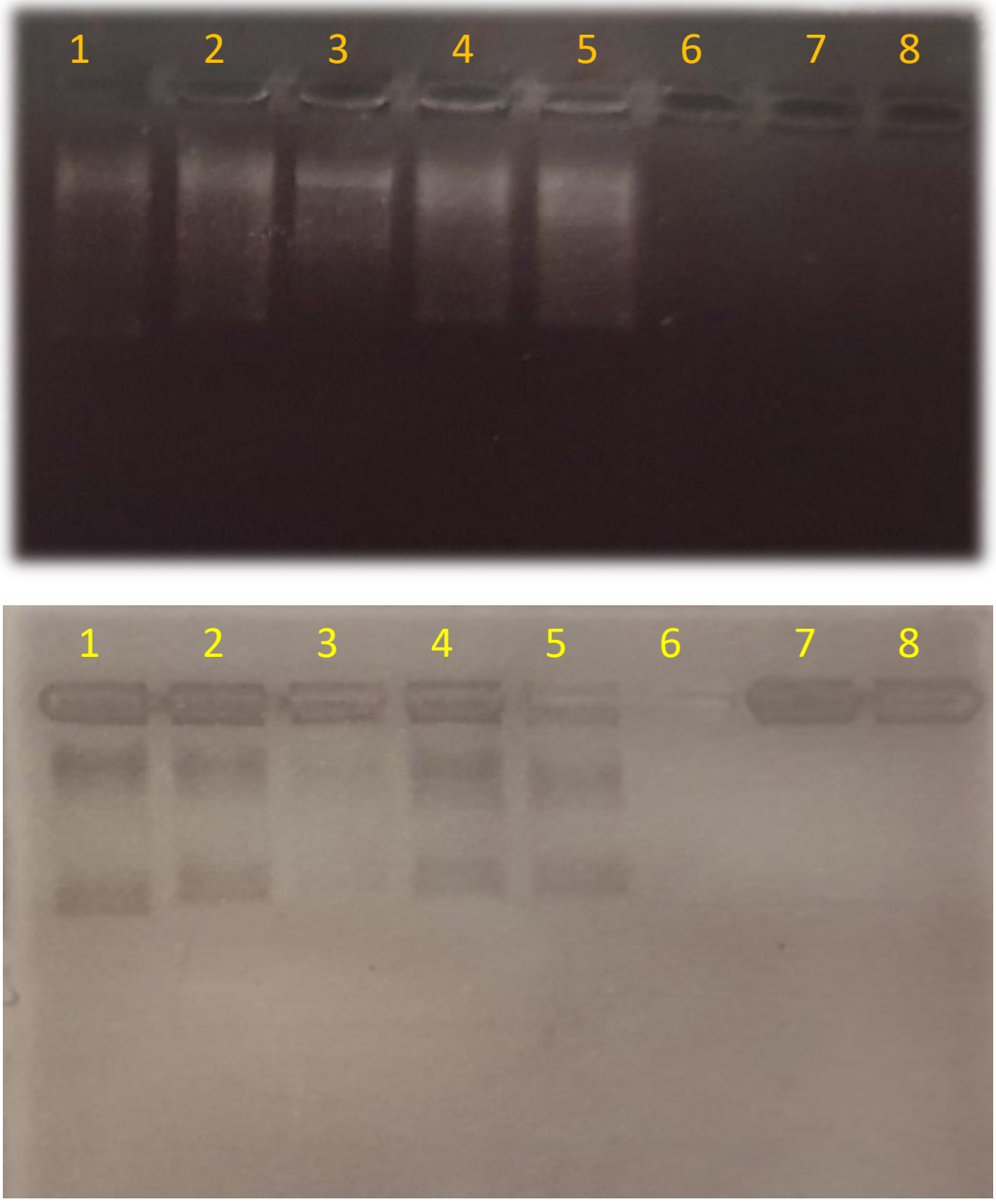


Figure3.22Agarose gel electrophoresis 1.2 % w/v agarose, 60 V: lane 1- CPMV-Alexa fluor dye, 2- CPMV -CF-488 dye , 3- CPMV-CQ-DOC-dye, 4- CPMV-CQ- SAL- dye , 5- CPMV-CQ-DOX dye, (6-7-8) empty

The conjugation of CQ derivatives to the outer surface of wt CPMV was confirmed by running stained Coomassie agarose gel after eight h dialysis, as shown in **(Figure 3.22)** .

3.4 - Transmission electron micrographs

A particle beam of electrons is used by transmission electron microscopes (TEM) to view specimens and produce a picture that is enormous quantity.[149]

In this study, the integrity of CPMV in CPMV-CQ-DOX , CPMV-CQ-DOC and CPMV-CQ-SAL were confirmed by negatively stained TEM (1% $AgNO_3$); the particles showed similar morphology to unmodified CPMV. No aggregation was noticeable in any of the TEM samples. As shown **(Figure 3.23,3.24,3.25)**, parts with a wide size distribution were obtained**.**

Negatively stained transmission electron micrographs showed a slight increase in

particle diameter, to 30.5 nm, after the formation of CPMV-CQ-derivatives compared with wild–type CPMV. In both cases, the CPMV remains intact with no aggregation.

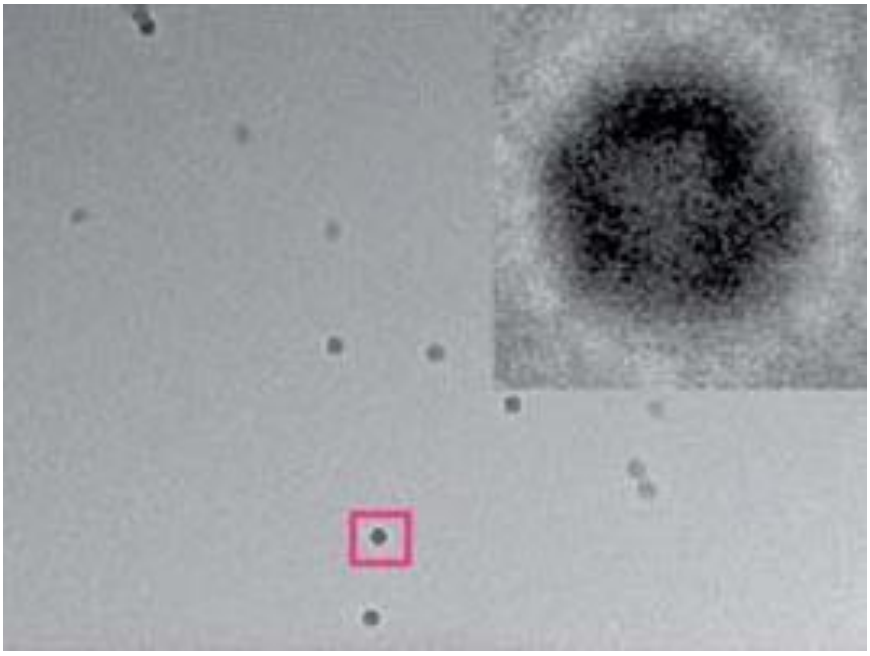


Figure 3.23TEM image of unmodified CPMV stained with 1% $AgNO_3$ solution[150]

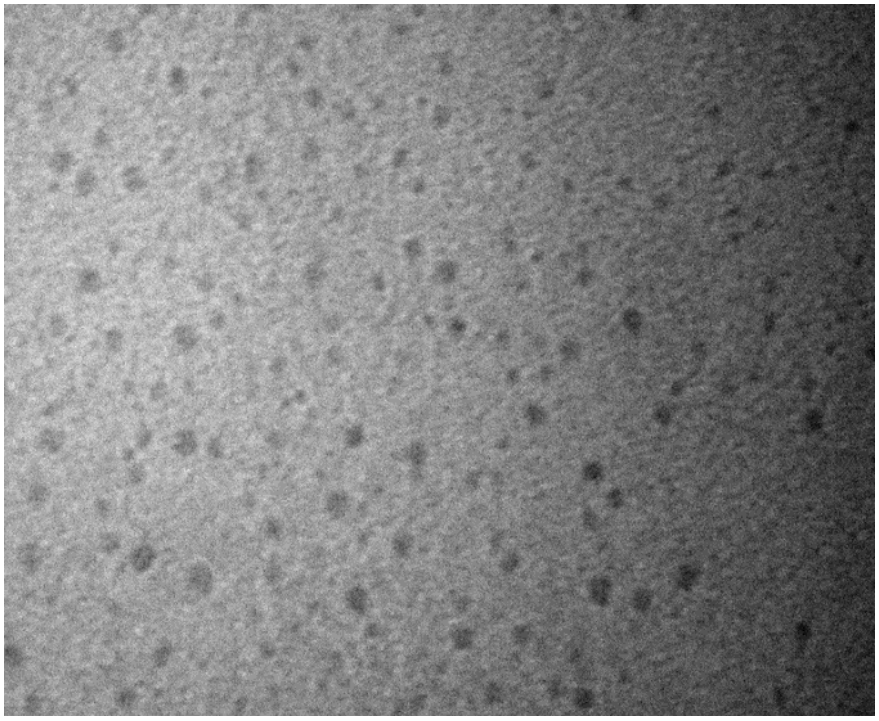


Figure 3.24 Stained TEM image of CPMV-CQ-DOX.

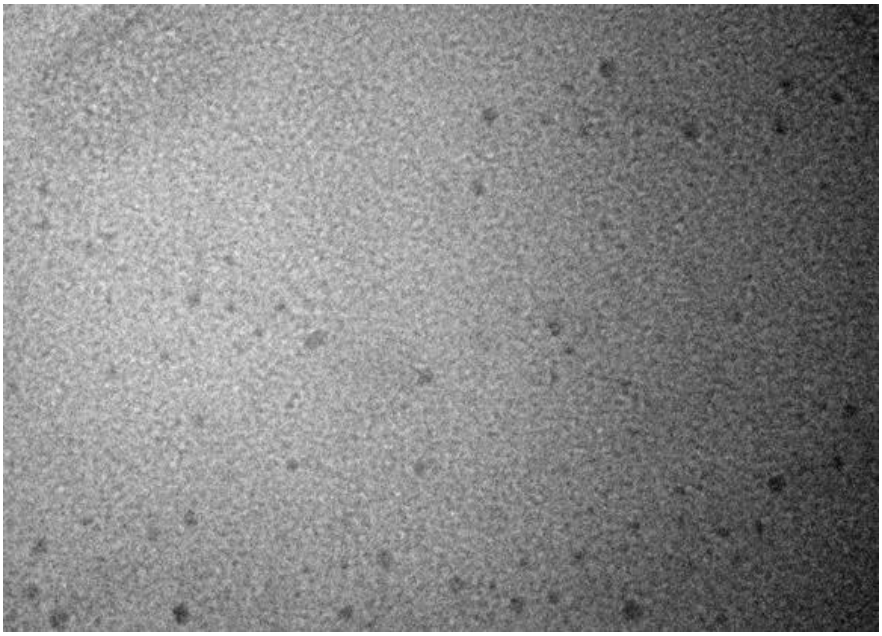


Figure 3.25 Stained TEM image of CPMV-CQ-DOC.

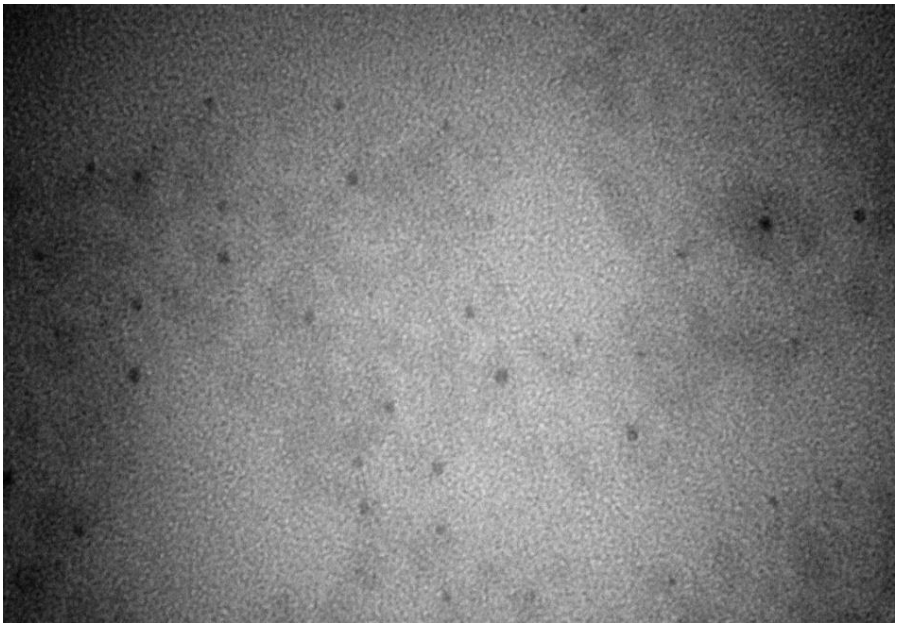


Figure 3.26 Stained TEM image of CPMV-CQ-SAL

3.5 -Applications

Recently, combination treatment is one way to reduce the toxicity and side effects of traditional anti-cancer drugs, chloroquine has been tried the most in this clinical example to suppress autophagy, and it may make healthy organs more sensitive to chemotherapy in addition to cancer cells.[151]

Repurposing pharmaceuticals by screening, modification, or identification of new combinations can minimize development costs and shorten drug development time, as an autophagy inhibitor, CQ makes tumour cells more sensitive to many different types of anticancer treatments.[152]

On the other hand, there are some disadvantages of this type of medication, such as gastrointestinal (nausea, vomiting and rarely diarrhoea). Even while side effects were minimal and toxicities were transient and reversible, it can still be harmful if one of the utilized medicines is a chemotherapeutic, making finding the best strategy to minimize these drawbacks imperative.[153]

However, three new CQ-derivatives have been synthesized to keep using CQ with chemotherapitic drugs DOX and DOC as traditional medicines of cancer and salysilic acid in order to enhance the activity of each one and provide protonated types of compounds which may be able to accumulate more inside tumour cells.

Next, each derivative's in vitro anti-cancer efficacy was tested. The MCF-7 breast cancer cell was used to study the intrecellular migration and anti-cancer mechanism of the CPMV particle as a carrier for CQ-derivatives.

3.6 - Cytotoxic Effect(MTT assay) for CQ-derivatives before and after modification

This study demonstrated that CQ-DOX, CQ-DOC and CQ-SAL might successfully stimulate death signalling pathways to inhibit the development of MCF-7 cells. The cytotoxic impact of CQ-derivatives on chosen cell viability was assessed using the MTT assay before and after conjugation with CPMV surface.

3.6.1- Cytotoxic effect (MTT assay) of CQ-DOX

To investigate the effect of using each CQ-derivative in breast cancer treatment, MCF-7cell cancer lines were treated with the first CQ-DOX using a range of concentrations (6.25-100 μg.ml) in two-fold dilutions as described in (section 2.15). Next, (IC_{50}) was calculated for CQ-DOX before conjugation with CPMV to be 70.375μg/ml and 0.051nM after conjugation according to the conjugation number of CQ-DOX on the CPMV surface. In contrast, (IC_{50}) was determined to be 4.885μg/ml with its carrier (CPMV-CQ-DOX) in a concentration range (0.34-5.5 885μg/ml) before releasing inside the autophagy. These results indicated a remarkable significant illumination of the live cells and improved the vital role of the delivery system in increasing the drug efficacy and activity. The IC_{50} values for CQ-DOX are shown **(Figures 3.27,3.28,3.29) .**

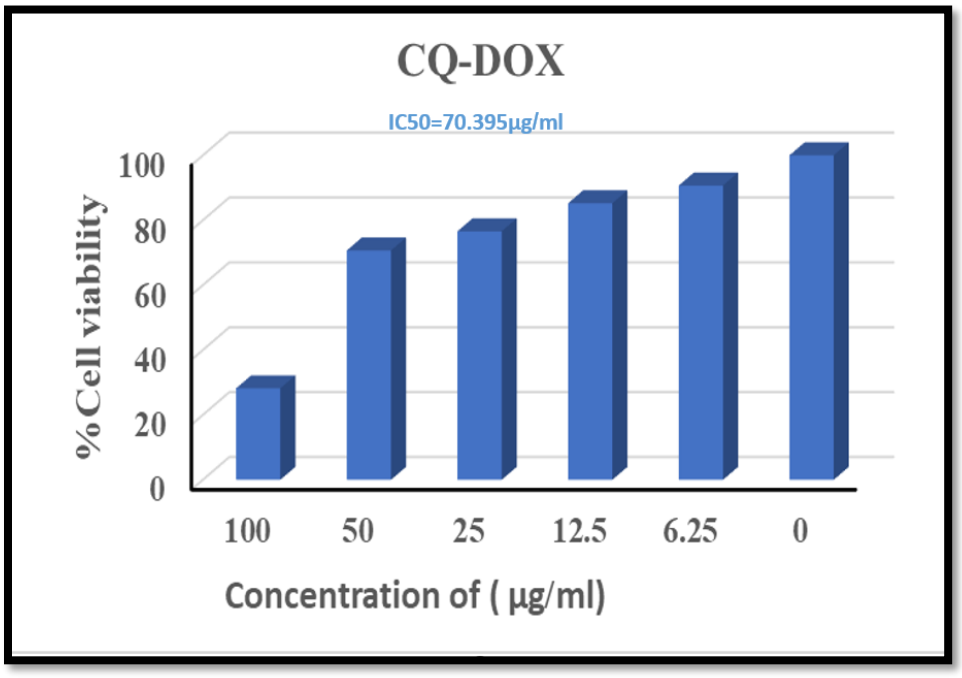


Figure 3.27 The effects of CQ-DOX on MCF-7cells vibilty before coupling to CPMV

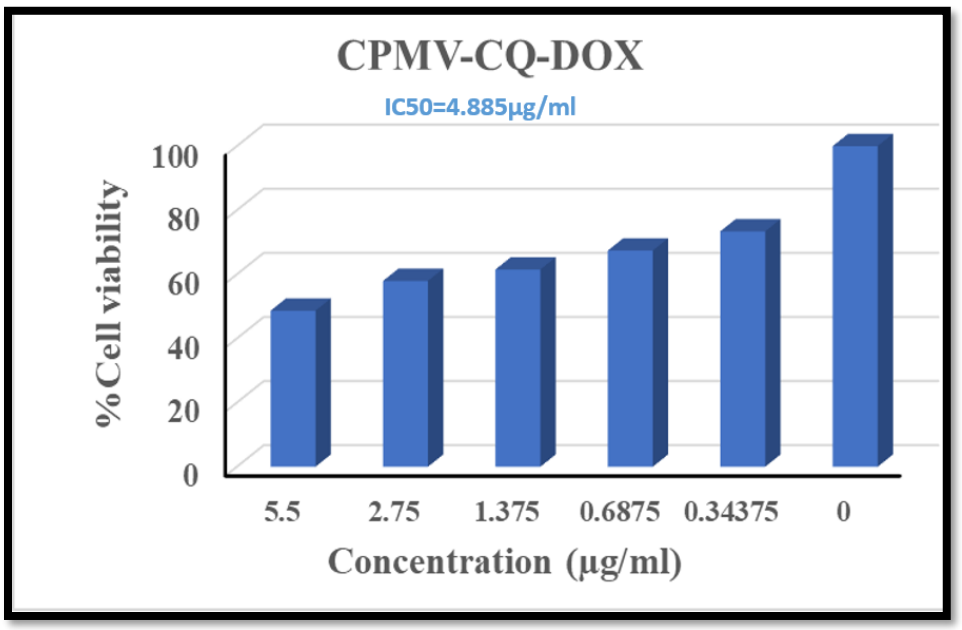


Figure 3.28 The effects of CQ-DOX on MCF-7 cells vibility when it is coupling with CPMV

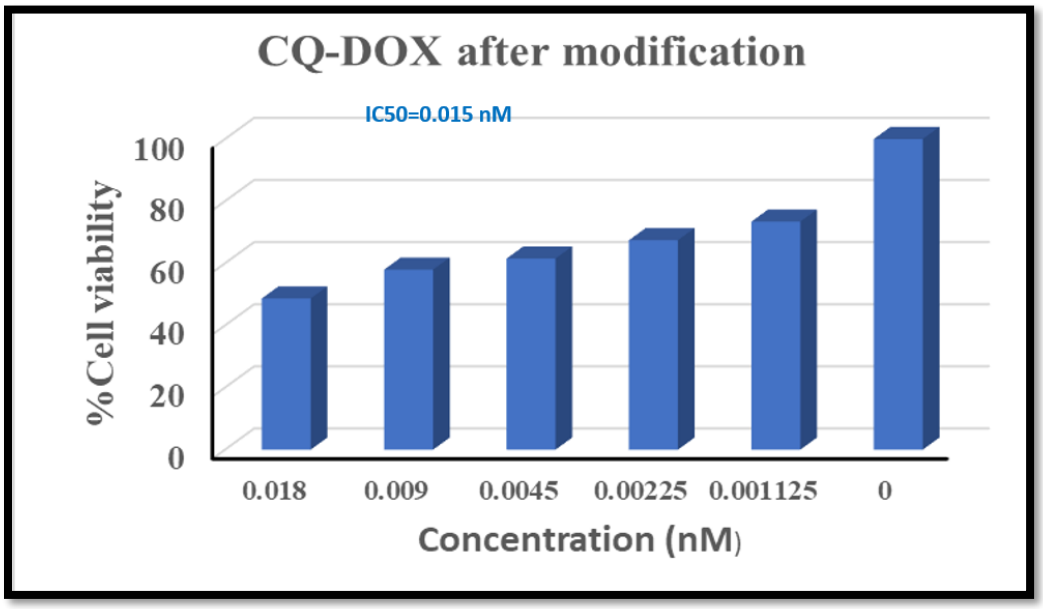


Figure 3.29 The effects of CQ-DOX on MCF-7 cells vibility after it is coupling with CPMV

3.6.2- Cytotoxic Effect(MTT assay) for CQ-DOC

Second tested derivative was CQ-DOC, which was tested before and after conjugation with CPMV particle. It was reported that DOC could be affected cancer cells (MCF7 cell lines) by preventing their depolymerization, thus disrupting normal cell division.

On the other hand, the combination of DOC plus CQ was more effective than DOCalone due to increasing the cytotoxic effect of DOC by CQ on MCF7 cell lines.[154]

While IC_{50} values were obtained at 14.384µg/ml, 13.686 µg/ml, and 0.038 nM for CQ- DOC before conjugation, when conjugated, and after conjugation with CPMV, respectively, the concentration of CQ-DOC after conjugation was

calculated depending on the number of CQ-DOC on the exterior surface, which was conjugated on CPMV.

It can easily recognize the inhibition in the viability of chosen cell after treated with loaded CQ-DOC on CPMV. The cell viability was decreased much more significan after using loaded derivative compared to CQ-DOC alone. The values are shown **(Figure 3.30,3.31,3.32)**

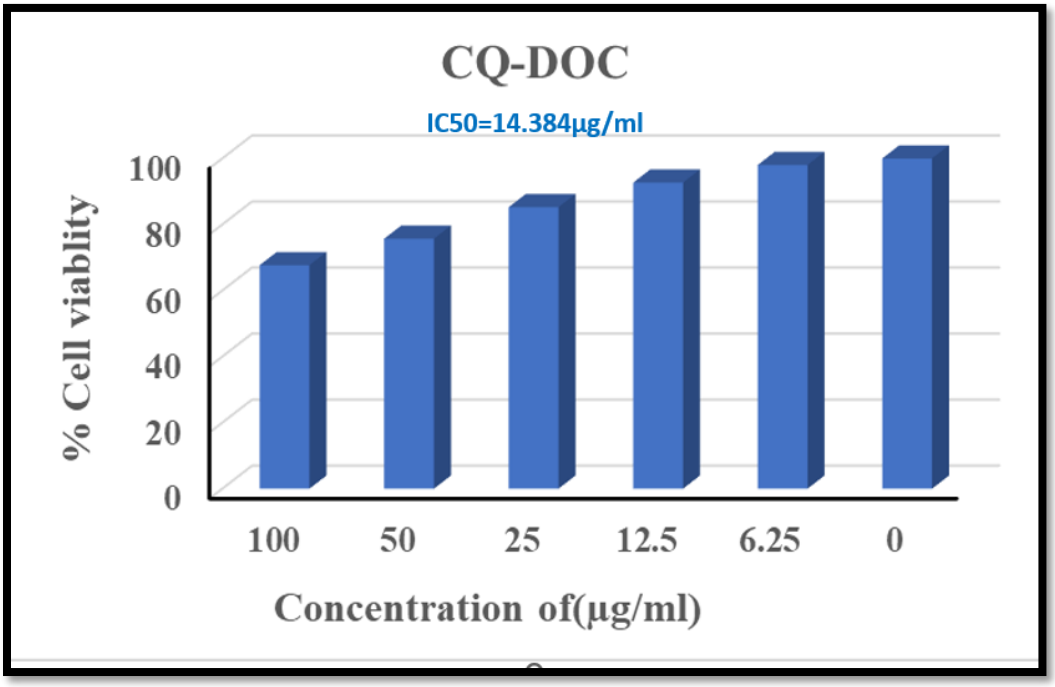


Figure 3.30The effects of CQ-DOC on MCF-7 cells vibility before it is coupled with CPMV

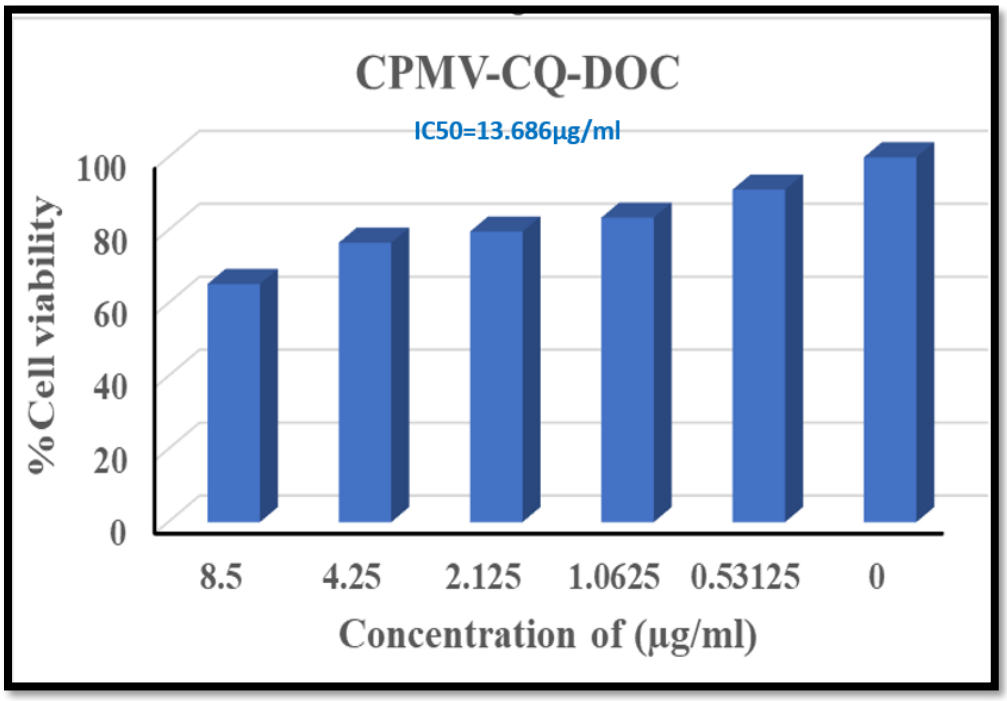


Figure 3.31 The effects of CQ-DOC on MCF-7 cells vibility when it is coupling with CPMV

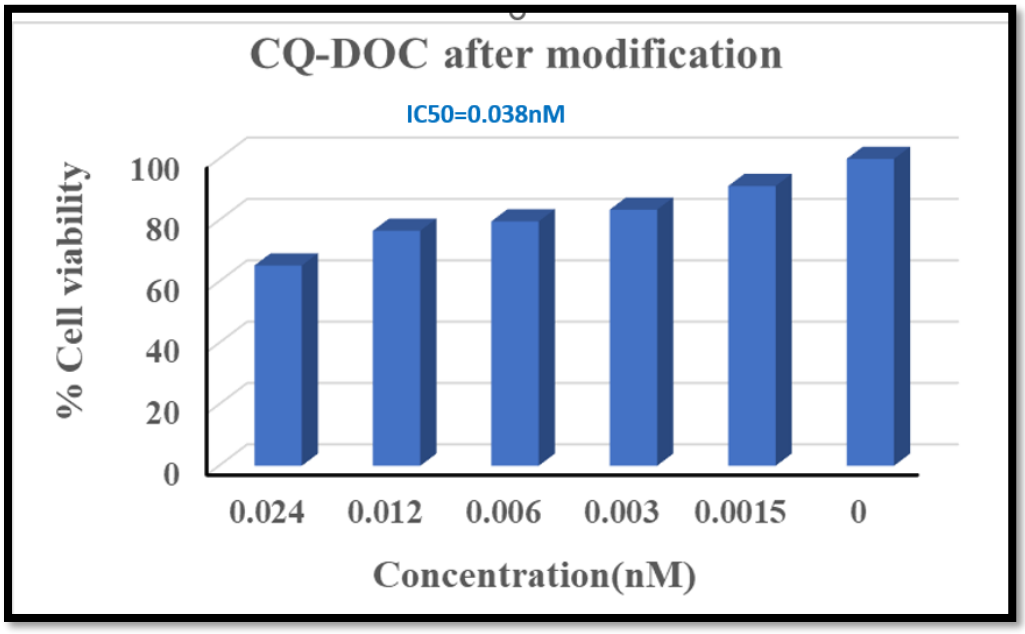


Figure 3.32 The effects of CQ-DOC on MCF-7 cells vibility after its coupling with CPMV

3.6.3- Cytotoxic Effect(MTT assay) for CQ-SAL

The last tested compound was CQ-SAL, in this study,SAL was used the first time as anticancer agent.

IC_{50} values for CQ-SAL before coupling ,when its conjugated ,after coupling with CPMV were 74.065 µg/ml,13.489 µg/ml and 0.042nM respectively.Those values are shown in **(Figures 3.33,3.34,3.35)**.

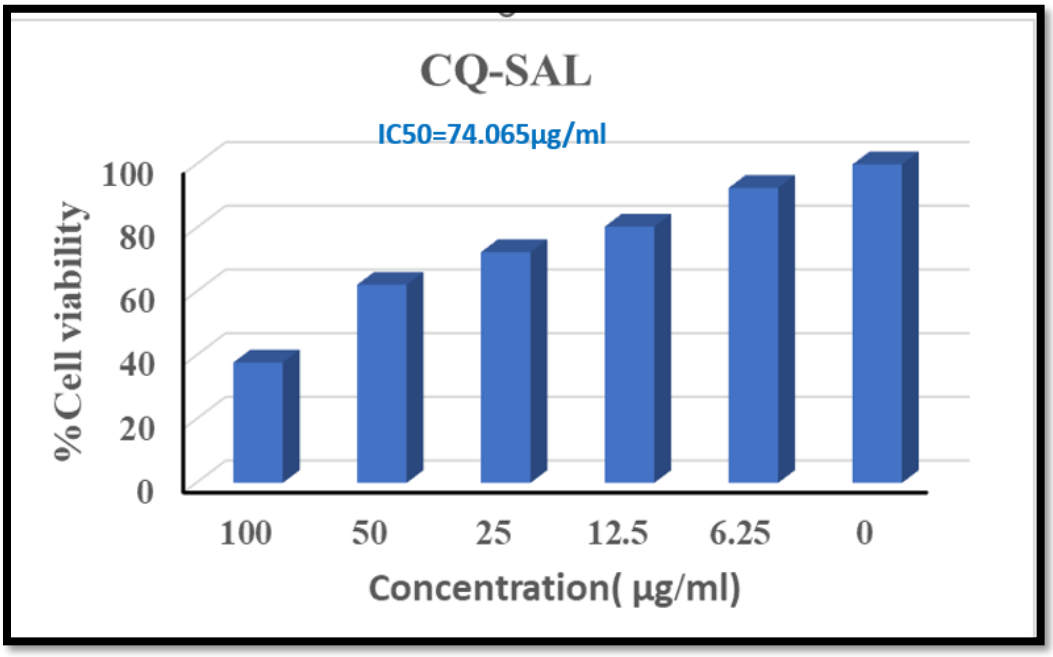


Figure 3.33 The effects of CQ-SAL on MCF-7 cells vibility before it is coupling with CPMV

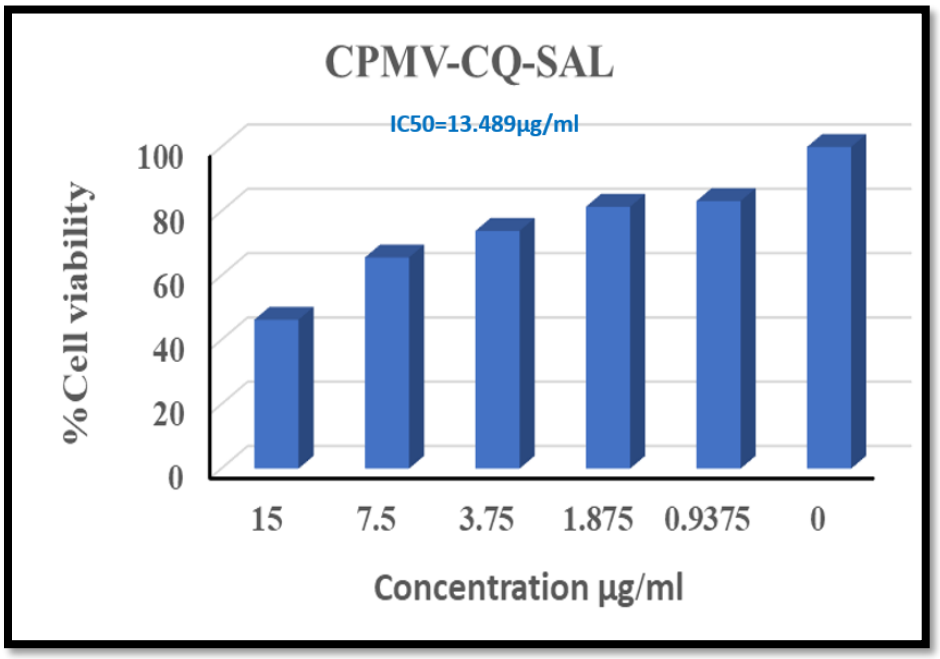


Figure 3.34 The effects of CQ-SAL on MCF-7 cells vibility when it is coupling with CPMV

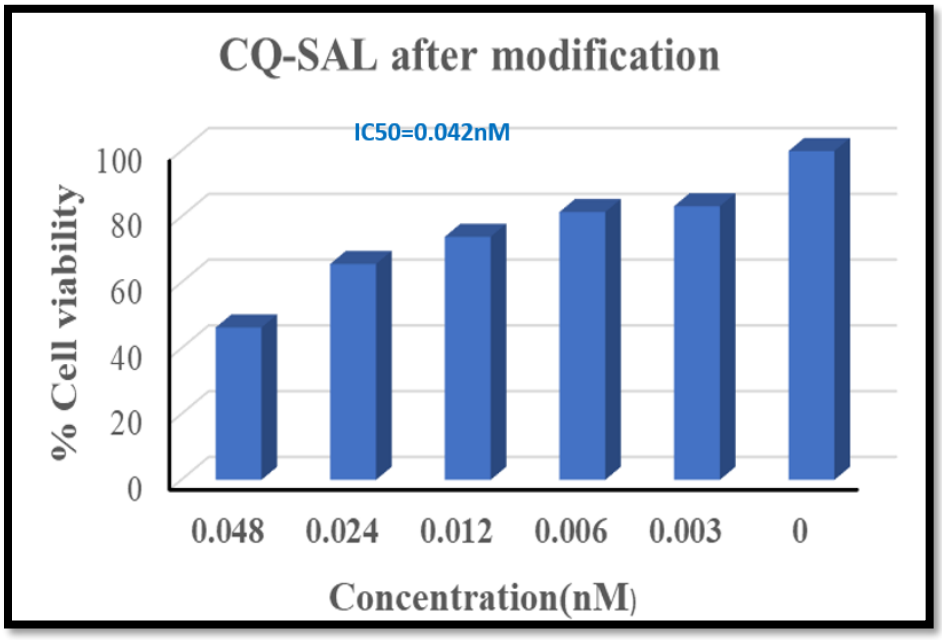


Figure 3.35The effects of CQ-SAL on MCF-7 cells vibility after it is coupled with CPMV

in this study CQ-SAL has the largest effect on MCF-7 cells compared to the CQ-DOX or CQ-DOC due to the short chain side.

Although, previous studies support the use of chloroquine sensitizes cancer cells especially in combination with conventional anticancer treatments and leads to anticancer effects through inhibiting autophagy and potentiating the therapeutic activity,the same effect of chloroquine to inhibit autophagy could also sensitize kidney cells to chemotherapy, leading to acute kidney injury.[155]

.

In this study, CQ has been used after linking with two traditional anti-cancer drugs separately (DOX ana DOC) and SAL as well, to produce CQ- derivatives that are more active and fewer drawbacks in one compound than using two.

According to several studies, inhibiting autophagy enhances chemotherapy's ability to kill tumour cells. [156]

In the above MTT assay results, IC_{50} values decreased significantly after loading each CQ derivate on the CPMV surface, which impmroved the activity of CPMV as a carrier compared with other carrires as represented in **Tables 3.4.**

The uptake of CPMV is mediated by specific interaction with a surface-displayed form of the cytoskeletal protein vimentin. Surface vimentin expression has been detected in endothelial cells *in vivo* as well as in activated macrophages, and the ability of cells to internalize CPMV is correlated with the presence of surface vimentin in these cell types. CPMV also accumulates within the tumour margin. . This feature was helped the drug reach the tumour areas without affecting other parts of the body.[157]

Moreover, the CPMV capsid's icosahedral structure allows precise therapeutic cargo loading to target tumor and cancer cells. Other features of CPMV include improved permeability

with a retention effect that enhances tumour penetration and intravital imaging, which allows for imaging living cells inside a multicellular organism.[158]

All CQ-derivatives had good conjugation effectiveness and loading capacity on CPMV particles , CPMN-CQ-DOC showed the highest *in vitro* cytotoxicity and eliminates against malignance cells.

According to the findings, CPMV as an NPs could shield medications from being recognized and rejected by P-glycoprotein (P-gp), giving them a chance to be transported by the autophagy process after being taken up by cells. So that, DOX, DOC, and SAL would not be affected by autophagy, CQ might exert its effectiveness and block autophagy during the migration of each loaded derivative.[159]

3.7- ADPI stain

The process of drug loading into CPMV and subsequent drug delivery to cancer cells was studied. *In vitro* experiments using DAPI-loaded stain established the efficacy of several CQ-derivatives in illuminating the number of living cells prior to and following conjugation with CPMV, proving the viability of these compounds.

In tissue culture, DAPI is used to color cell nuclei of MFC-7 cancer cell. Because its molecular structure allows it to pass through cell membranes, the dye is able to diffuse into the nucleus, where it intercalates with DNA. [160]

The apoptotic activity of conjugated and unconjugated CQ-derivatives on MCF-7 cells were examined by performing DAPI fluorescence staining assay. In general, the vital dye stains infected cells in blue. [161]

It was used to investigate whether or not the cytotoxicity of CQ-derivatives in MCF-7 cells was related to the activation of apoptosis. DAPI is a fluorescent dye that binds to DNA and is used to show DNA fragmentation. DAPI staining revealed that MCF-7 cells treated with CQ-derivatives had considerably more apoptosis than the control group, which revealed a significant percentage of cell death. DAPI staining results showed that CQ-derivatives could successfully trigger apoptosis in MCF-7 cells due to illimenation and morphology changing of maligante cells as highlighted in the **(Figures**3.36,3.37,3.38)

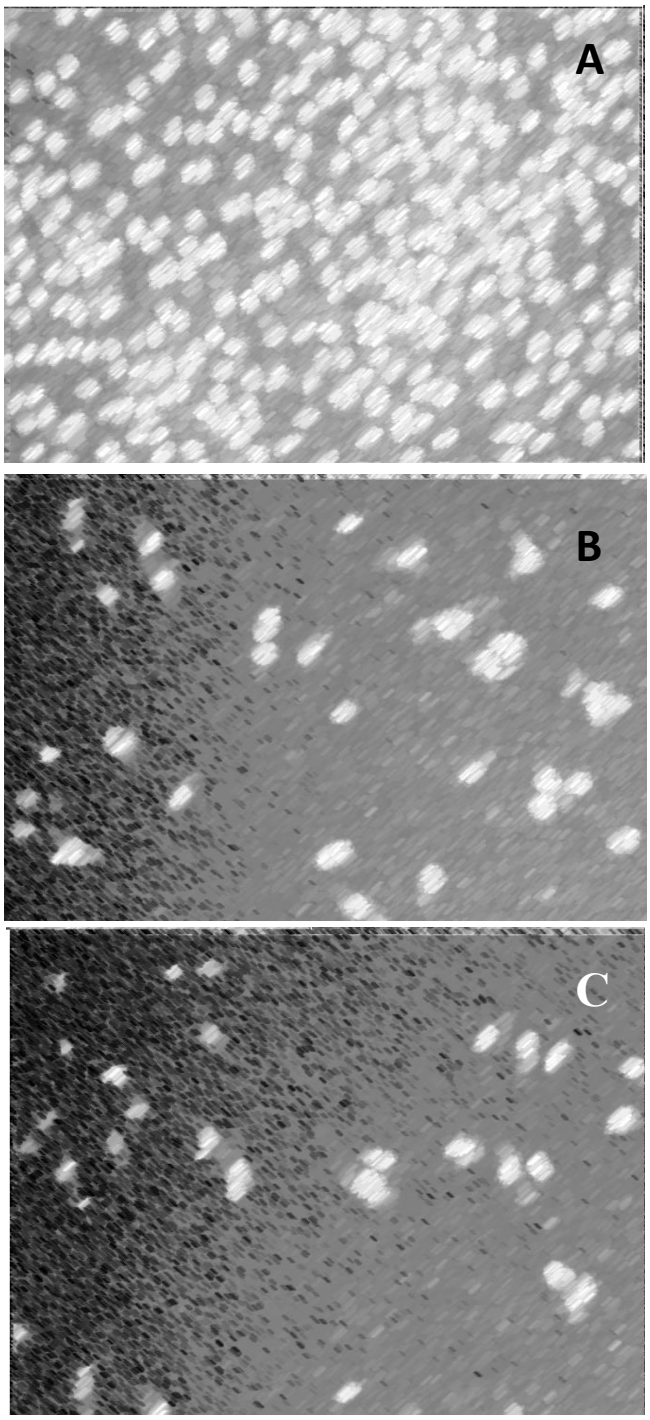


Figure 3.36. A- control of MCF-7cells ,B- ADPI assay for CQ-DOX before modification as anticancer drug C- ADPI assay for CQ-DOX after modification as anticancer drug

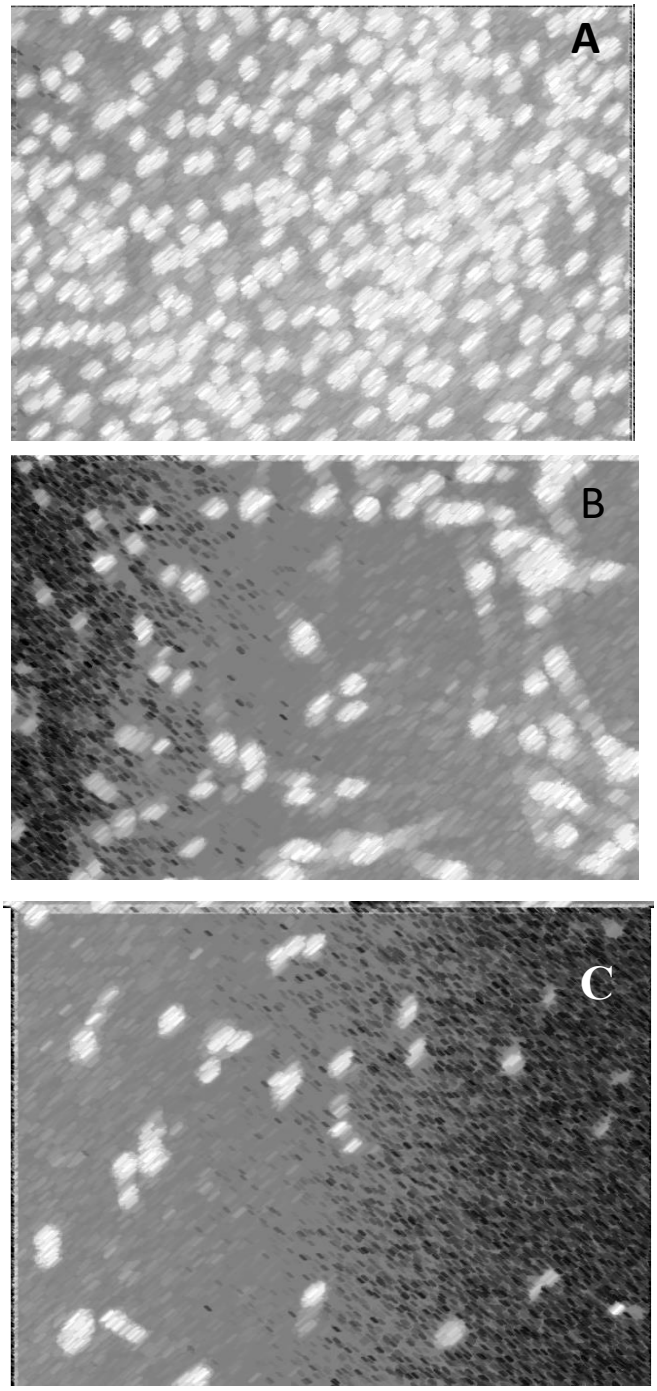


Figure 3.37 A- control of MCF-7cells ,B- ADPI assay for CQ-DOC before modification as anticancer drug C- ADPI assay for CQ-DOC after modification as anticancer drug

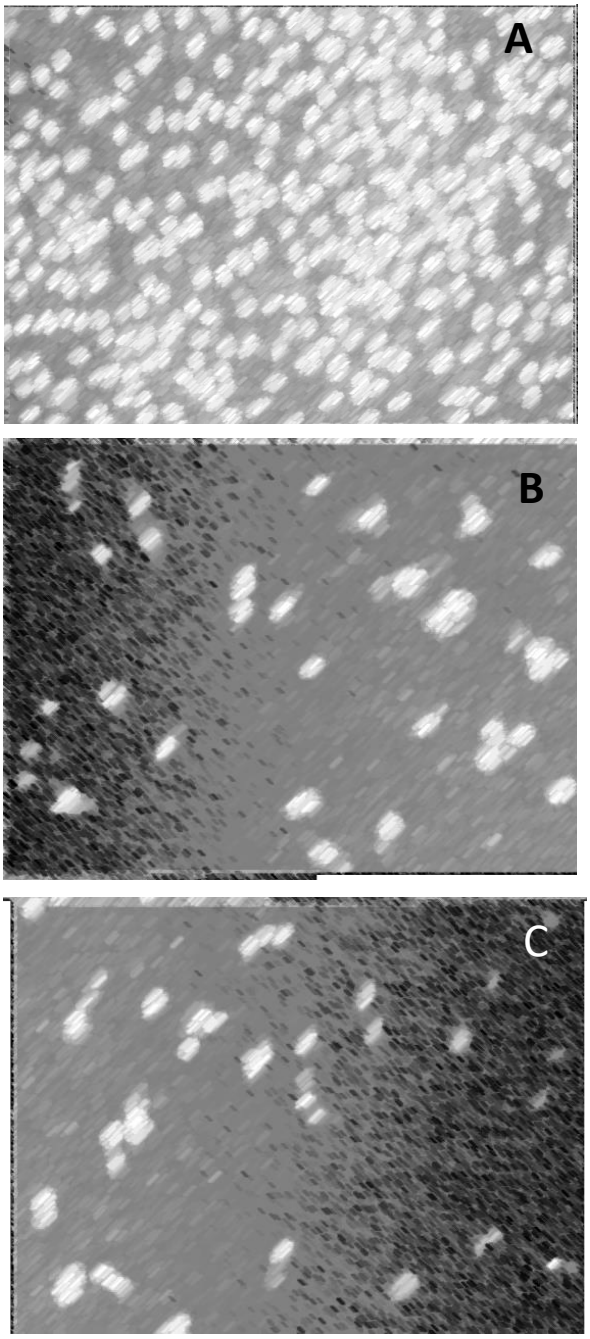


Figure 3.38 A- control of MCF-7cells ,B- ADPI assay for CQ-SAL before modification as anticancer drug C- ADPI assay for CQ-SAL after modification as anticancer drug.

Because there is no evidence that the CPMV carrier is toxic for mammalian as reported before. It was used rather than other carriers in the presented study.[162]

It has been documented before that CPMV nanoparticles interact with mammalian cells via binding to vimentin on their outer surfaces. This capability can be used to specifically target cancer cells like those found in cervical cancer, colon cancer, and prostate cancer.[163,164]

Each CQ treatment resulted in decrased blue fluorescence in treated cells, as compared to untreated onse (control cells). Additionally, morphologi\cal changes associated with apoptosis such as chromatin condensation, and nuclear fragmentation are evident in the cells upon treatment.

It could be concluded that creating more effective autophagy inhibitors is a very active and precise research topic, indicating that the anti-tumour impact can be attained through many mechanisms evoked when utilizing combination therapy in one compound instead of using two or more. As shown in **TableS 3.6,3.7**

The comparison between this study and previous studies is listed in **Table 3.6 and 3.7**.

Table 3.6 Combination of previous studies with presented ones. (Doxorubicin + Chloroquine).

Compound	IC_{50}	Cell type	Time	Doseg	Reference
(DOX+CQ) 1:2	2.830 mg/mL	MCF-7	48h	DOX-1 mg/mL CQ-2 mg/mL	[165]
DOX+CQ	5.200 mg/mL	MCF-7	72h	DOX-50 mg/mL CQ-250 mg/mL	[166]
DOX+CQ(1:2)	5.085 mg/mL	MCF-7 /ADR	24h	DOX-0.5 mg/ mL CQ-1 mg/ mL	[167]
CQ-DOX	0.0704 mg/mL	MCF-7	72h	(0.006-0.1) mg/mL	This study

Table 3.7 Combination of previous studies with presented ones. (Docetaxal + Chloroquine).

Compound	IC_{50}	cell Type	Time	The doseg	Reference
DOC+CQ (0.8:0.2)	4 µg/mL	MCF-7	48h	DOC-80 µg/mL CQ-20 µg / mL	[168]
CQ-DOC	14.384 µg/mL	MCF-7	72h	(6.5-100) µg/mL	This study

other carriers have been reported in the past for chemotherapeutic medicines, the results shown the remarkable activity of CPMV in comparision with others.

Table 3.8 IC_{50} values presented for CQ-DOX from this study compared to other carriers value.

Compound	IC_{50}	cell Type	Time	The doseg	Reference
PEP-DOX-CQ (1:1)	1790 nM	MCF7/Adr	72h	DOX 1839000 nM CQ 1938000 nM	[169]
PEP-DOX-CQ (2:1)	2000 nM	MCF7/Adr	72h	DOX-3679700 nM CQ1938000 nM	[169]
L-DOX-CQ (1:2)	4700 nM	MCF7/Adr	24h	DOX-4500 nM CQ-9700 nM	[167]
Loaded -CQ- DOX	0.015 nM	MCF-7	72h	(0-0.18) nM	this study

.

Table 3.9 IC_{50} values presented for CQ-DOC from this study compared to other carriers value.

Compound	IC_{50}	cell Type	Time	The doseg	Reference
DOC+CQ-P18/TPGS	260nM	MCF-7	24h	DOC100000nM CQ40000 nM	[168]
DOC+CQ-P36/TPGS	200 nM	MCF-7	24h	DOC100000nM CQ40000nM	[168]
DOC+ PEG-b-PLGA micelles then added CQ	IC_{50} DOC 27603nM IC_{50} CQ 3392nM	MCF-7	24h	DOC(3095-6189045)nM CQ-30000 nM	[170]
loaded-CQ-DOC	0.038Nm	MCF-7	72h in vitro	(0-0.024)nM	this study

3.8-Conclusion

1-Three different CQ derivatives have been synthesized successfully

2-Both external carboxyl and amine of CPMV have been successfully modified with each CQ-derivative, and the number of each derivative has been evaluated using two different dyes.

3- The activity of each CQ-derivative as an anticancer agent using DAPI and MTT assay before modification has shown more than a 50% reduction in cell viability, while the results have shown much more growth inhibition after modification with remarkable and unique IC_{50} values.

4- CQ-SAL has the largest effect on breast cancer cells compared to the other CQ-derivatives.

3.9- Future work :

1-Different CQ derivatives can be synthesized and tested as anticancer agents.

2- Same CQ- derivatives can be tested ***in vivo.***

3-Same derivatives can be used against other cancer cells.

4- Internal surface of CPMV can be modified.

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الخلاصه

أجزاء من بليون الجسيمات الصغيره الفايروسيه المزروعه متعدده الوظائف تقدم فوائد مهمه في مجال التكنولوجيا. في هذا البحث الذي يتناول داء الفسيفساء الفايروسي لنبات اللوبياCPMV كأحد أنواع الفايروسات النباتيه تم استخدامه بشكل نافع لانه يحتوي على شكل معروف في مجال التحلل الذري ولسهوله استخراجه بالغرام الذري وايضا كونه مستقر ويمكن ان يستخدم في تركيب الجزيئات أثناء عمليه تكوين مركب بطريقه الترابط لكيميائي لعناصر ليشكل كتله مبنيه من الجزيئات الكيمياويه. تحتوي كل وحده من CPMV الغير متماثله على بقايا اليسين تفاعليه معزز ليصبح المجموع ٦٠ لكل فيروس.

في هذا العمل تم اختبار الCPMV كحامل لبعض مشتقات الكلوروكوين الجديده كادويه علاج كيمياويه.

تم تعديل السطح الخرجي للقفيصه بمشتقات الكلوروكوين وتم تعديل مجموعات الكاربوكسيل القابله للعنونه باستخدام (CQ-DOCوCQ-DOX) في حين تم ربط الأمين القابل للتوجيه الموجود على السطح الخارجي ب(CQ-SAL) على أمل إنتاج ادويه واعده مضاده للسرطان. تم تأكيد القدره على ربط كل مشتق ب. CPMV

تم فحص القدره على تغيير الأمين الخارجي والكربوكسيل لفيروس فسيفساء اللوبيا CPMV بمشتقات الكلوروكوين.

بالاضافه إلى ذلك تم تأكيد الاقتران باستخدام مقياس الطيف الضوئي بالاشعه المرئي الفوق بنفسجيه والرحلان الكهربائي والمجهرالالكتروني النافذ

بينما تم حساب عدد كل مشتق مقترن باستخدام صبغه CF-488Aوصبغه Alexa Flour ليكون ٨٧ ± ١و ٧٩± ١و ٨٩ ± ٢ ل(CQ-DOXوCQ-DOCوCQ-SAL) على التوالي.

تم اختبار جميع مشتقات CQ المقترنه والغير مقترنه كمنشط مضاد للسرطان باستخدام فحص MTTوADPI وكمثبط للالتهاب الذاتي .

الالتهام الذاتي هو نظام إعادة تدوير خلوي يعمل على تحطيم العضيات الخلوية والبروتينات التالفة أو غير الضرورية بطريقة متوازنة . في البيئة المتنقله الغير ملائمه ، من المتوقع أن تستخدم الخلايا السرطانية الالتهام الذاتي كمصدر للطاقة ، وهناك عدد من التجارب السريرية تكشف الآن عن الدور المحتمل للكلوروكين (CQ) ، كمثبط للالتهام الذاتي. كثيرا ما يستخدم CQ في علاج السرطان بالاقتران مع أدوية العلاج الكيميائي والإشعاع. في هذه الدراسة ، تم تصنيع مشتقات الكلوروكين الجديدة المشتقة من تفاعل 4,7dichloroquineمع(DOXوDOCوSAL) تم تصنيعها أولاً وتوصيفها بالكامل باستخدامFTIRوCHNوHNMR لانتاج (CQ-DOXوCQ-DOCوCQ-SAL).

تم تقييم النتائج المقدمة في هذه الدراسة لتكون أكثر من 50 ٪ من انخفاض قابلية الخلية للحياة (IC_{50}) ثم تم حسابه لكل مشتق CQ.

إن تـأثير مركبـات CQ المرتبطـه وغيـر المرتبطـة بـسطح ال CPMV تـم فحصـه بواسـطه MTTوADPI وتـم تقيـيم IC_{50} لكـــل مشـــتق مـــن CQ مـــع وبـــدون اقتـــران مـــع CPMV ليكـــون(CQ-ل70.395µg/ml DOXو14.384µg/mlل CQ- DOCو74.065µg/mlل CQ-SAL) قبـــل التعـــديل ثـــم بعـــد التعـــديل 0.015 nMل CQ-DOXو0.038 nMل CQ-DOCو0.042 nMل CQ-SAL.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل/ كلية العلوم
قسم الكيمياء

تعديل فيروس فسيفساء اللوبيا كناقل لبعض مشتقات الكلوروكوين الجديدة واختبارها كعامل مضاد لسرطان الثدي

رسالة مقدمة إلى مجلس
كلية العلوم / جامعة بابل وهي جزء من متطلبات نيل درجة الماجستير
في علوم الكيمياء

من قبل
خالده محي حبيب علي الخفاجي
بكالوريوس علوم كيمياء /كلية العلوم /جامعة بابل ٢٠٠١

بإشراف

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1444هـ **2022م**