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Nano particle formulation of Phoenix dactylifera L. (ajwah and black date) seed and peel and investigating their antioxidant and antibacterial properties

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Nano particle formulation of *Phoenix dactylifera L.* (Ajwah and Black date) seed and peel and investigating their antioxidant and antibacterial properties

A Thesis Submitted to
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In partial fulfilment of the requirements for
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By:

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Under the supervision of:

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Thesis Committee Supervisor/Chair

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Abstract

Bacterial infection is one of the leading causes of death world-wide. Antibiotics have always been the first choice to be used against bacterial infections especially in post-surgery, diabetes mellitus due to their lack of immunity and in organ transplantation as well. On the other side the medical antibiotics have a major problem which is the resistance; meaning that after sometime the body begins to resist them and they became with no action on bacteria. That is why it is a global trend now to shift back to the natural resources from plant origin which exhibit potent antibacterial action. As an example is the date pit *Phoenix dactylifera L*. (Ajwah and Black date) which was proven to have many medical uses; antimicrobial, antifungal, anti-diabetes mellitus type 2, reduces obesity and hypercholesterolemia, protection against coronary heart diseases. This study is focused on using Black and Ajwah date pit and fruit, black date fruit to form nano formulation that are expected to have unique antioxidant and antibacterial activities. To the best of our knowledge No previous work was done to formulate a nano particle out of the pit. Date pit extract was prepared as aqueous and/or alcoholic. The chitosan nanoparticle was formulated to encapsulate the date pit extract inside it by the help of the sodium tri-poly phosphate (TPP) linker to enhance coiling and encapsulation by applying certain stirring criteria including the time, speed and temperature of stirring, centrifugation and sonication. Those separated nanoparticles will be measured to guarantee their size and potential. Spectroscopic and physicochemical assessments were done using X-ray diffractometer (XRD) and Transmittance Electron microscopy (TEM) to guarantee their homogenous spherical shape and size. Then measurements were done by Ultraviolet-Visible spectrophotometry (UV-Vis), Infra-red spectrophotometry (IR). They proved that AS OD NPs were of hydrodymanic size 152.3-254.93 ±60 nm and TEM size of 1.01-2.05 nm, poly dispersion index of 0.39±0.08 and potential of 32.49-35.21±0.4. the NPs encapsulation AS extract in ratio of chitosan : ASOD 1:7 (w/w) had encapsulation efficiency of 70.37% and loading capacity of 61%. Release rate of those NPs was 68.2%±0.04 at 48 hrs. They showed enhanced antioxidant and antibacterial actions where the NPS were 1.2 fold more antioxidant than the plant extract and 100 fold more antibacterial than the plant extract.

List of Abbreviations

AS: Ajwah Date Seed

AP: Ajwah Date Peel

AO%: % Antioxidant

BS: Black Date Seed

BP: Black Date Peel

BF: Black Date Fruit

BHT: Butylated Hydroxy Toluene

BHA: Butylated Hydroxy Anisole

CD: Cyclo Dextrin

DLS: Differential Light Scattering

Dpp: Dry Plant Powder

DW: Distilled Water

D: DMSO, Dimethyl Sulfoxide solvent

E: 52% Ethanol solvent

FTIR: Fourier Transform Infrared spectroscopy

FD: Freeze dried

GAE: Gallic Acid Equivalent

M: Quaternary mixture solvent system formed of DW: acetone: methanol: formic acid at ratio 20:40:40:0.1 (v/v)

NPs: Nano Particles

NE: Nano Emulsion

NC: Nano Capsule

NEMS: Nano Electro Mechanical System

OD: Oven dried

PS: Particle Size

PDI: Particle Dispersion Index

PEG: Poly Ethylene Glycol

PPG: Poly Propylene Glycol

PGA: Poly Glycolic Acid

PLLA: Poly L- Lactic Acid

PDLA: Poly D- Lactic Acid

PLCG: Poly Lactide Co- Glycolide

SEM: Scanning Electron Microscopy

TEM: Transmittance Electron Microscopy

TPP: Sodium Tri-Poly Phosphate

UV-Visible spectroscopy: UV-Vis Spectroscopy

WHO: World Health Organization

XRD: X-ray Diffractometer

TSB: Tryptone Soy Agar Broth Medium

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Thesis Scope and Objectives

Thesis Scope and Objectives

The date pit *Phoenix dactylifera L.* was proved to have many medical uses; antimicrobial, antifungal, anti-diabetes mellitus type 2, reduced obesity and hypercholesterolemia, protected against coronary heart diseases. No previous work was done to formulate a nanoparticle of it so as to be easily inoculated in drugs or food to do its promising action. Date pit extract would be prepared as aqueous and/or alcoholic and then formulated in a chitosan polymeric nano particle. The natural polysaccharide chitosan nano particle would be formulated to encapsulate the date pit extract inside it. The polymeric solution would be added to the extract solution by the help of the TPP linker to enhance coiling and encapsulation by applying certain stirring criteria including the time, speed and temperature of stirring, centrifugation and sonication to follow a protocol called ionic gelation technique. Those separated nano particles would be measured to guarantee their size and potential by Zeta-sizer Malvern. They would be imaged by X-ray diffractometer (XRD) and Transmittance Electron microscopy (TEM) to guarantee their homogenous spherical shape and size. Then measurements by Ultraviolet-Visible spectrophotometry (UV-Vis), Infra-red spectrophotometry (IR) would be done to guarantee that the extract got encapsulated inside the prepared nanoparticles and the time at which this extract would be released inside the body by encapsulation efficiency (EE), loading capacity (LC) and release tests and calculations. Finally, we would to test the efficacy of the NPs and see whether the total phenolic content, antioxidant activity and antibacterial action got enhanced or not compared to the plant extract itself. The presented thesis would be divided to 3 main chapters; chapter 1 is an introduction on anti-bacterial and nanotechnology and why the green synthesis is the followed approach nowadays to formulate NPs.

It includes as well a summary on the botanical origin of the date plant and why it attracted attention nowadays. Chapter 2 is the methodology, the materials used and the techniques and instruments used to monitor the prepared NPs. Finally, chapter 3 formed of 2 parts; part I shows the prepared NPs and the results of the measurements to reach the best preparation to get the minimum size and PDI, maximum zeta potential, EE, LC and release. Part II compares the prepared NPS with the plant extract itself in the total phenolic content, antioxidant activity and antibacterial action to see whether those parameters got enhanced, deteriorated or remained as such.

Chapter 1

General Introduction

&

Literature Review

Chapter 1: General Introduction & Literature review

1.1.Bacterial infections:

Bacterial infection is prevailing world- wide and is a high reason for mortality and morbidity [1]. The normal and traditional way of getting rid of bacteria is using antimicrobials either of narrow or wide spectra. Antibiotics are usually used in case of infection with high grade fever. Antibiotics are also used in post-surgery, organ transplantation and diabetes mellitus since diabetic patients lack immunity. On the other side, the chemical antibiotics have a major problem which is the resistance; meaning that after sometime the body begins to resist them and they have no effect on bacteria. That is why it is a global approach nowadays to shift back to the natural resources from plant origin. Fig.1.1 shows the main reasons of mortality world-wide

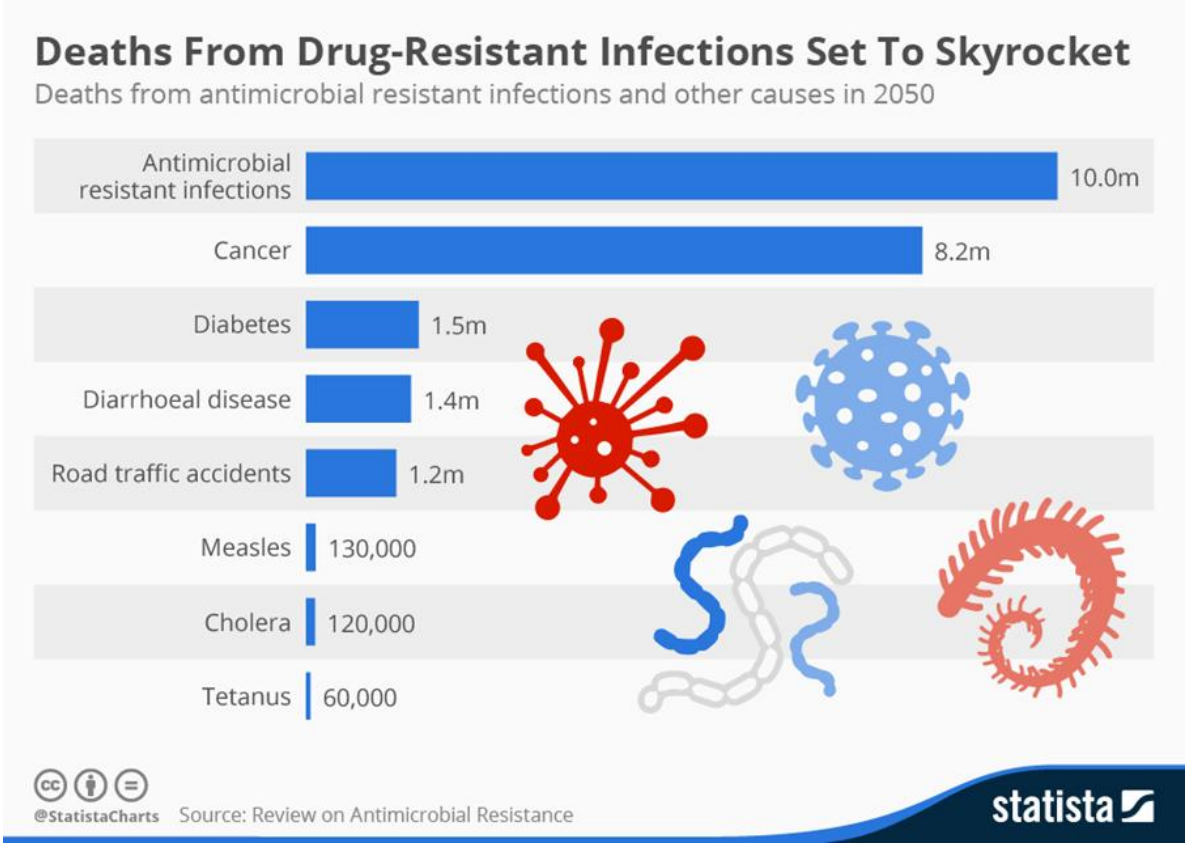

Fig1.1. Main reasons of mortality world-wide according to Statistica survey [1]

The pharmaceutical antibiotics target the bacteria by 3 mechanisms as shown in Fig. 1.2 ; either by inhibiting DNA replication of bacteria or inhibiting cell wall synthesis or affecting the translational machinery of the bacterial cells [2].

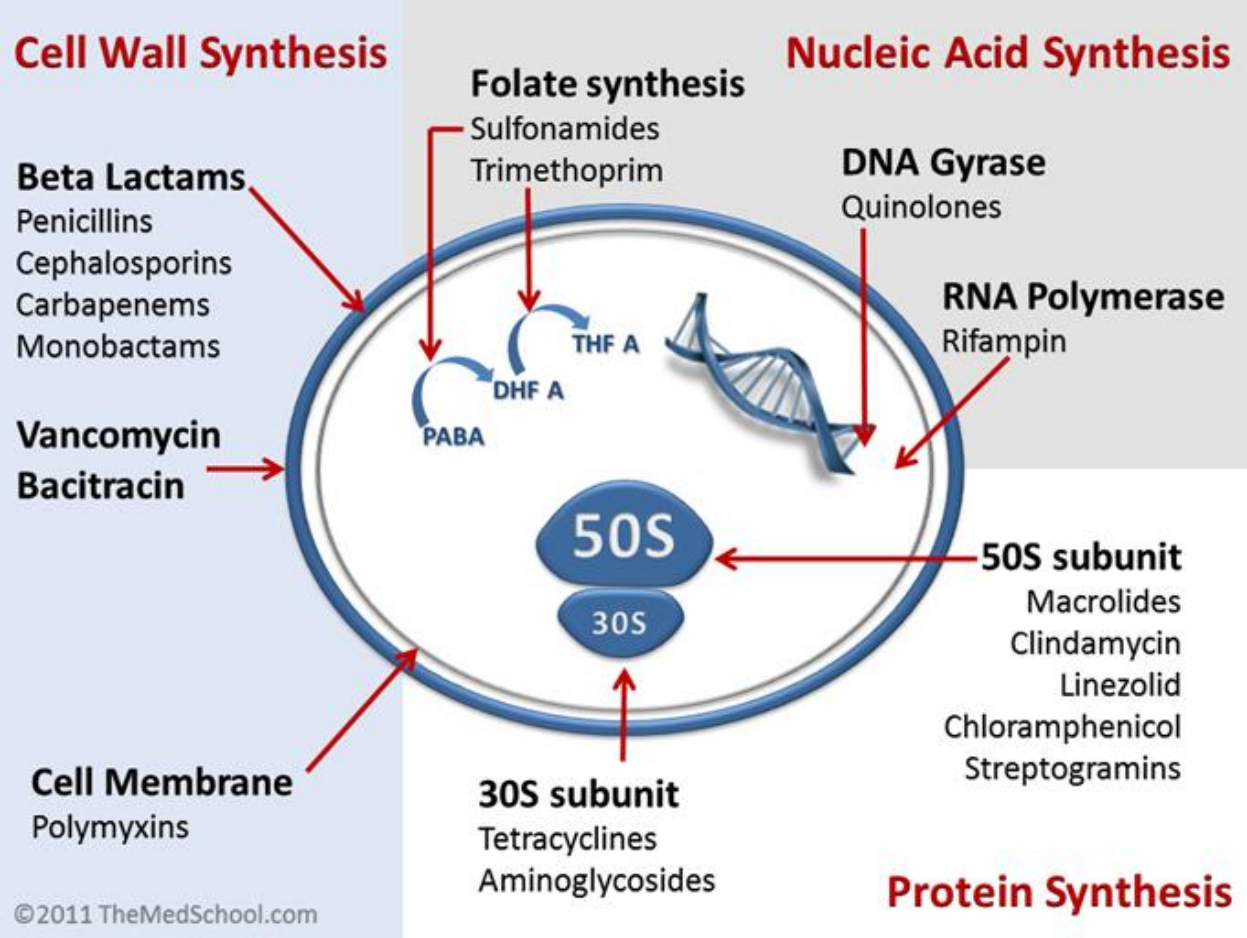

Fig1.2. Mechanism of actions of antibiotics to eradicate bacteria [8]

The main drawback of antibiotics rather than being a non-natural; bacteria can form a type of resistance against them if those antibiotics are misused like in case of common cold or overused with no accurate reason [3]. Thus, they will become ineffective. The main drawback of this resistance is that the antibiotics will be less efficient in controlling life threatening conditions like in case of Diabetes Mellitus, organ transplantation and surgeries which will lead to prolonged stays at hospitals and negative effects on the health of the populations and economy of the countries [3].

The bacteria form resistance by: beta lactamases, aminoglycosides, cell wall modification or efflux pump as clarified in Fig. 1.3. They form biofilms which at first adhere reversibly then irreversibly to peptidoglycans forming highly resistant colonies

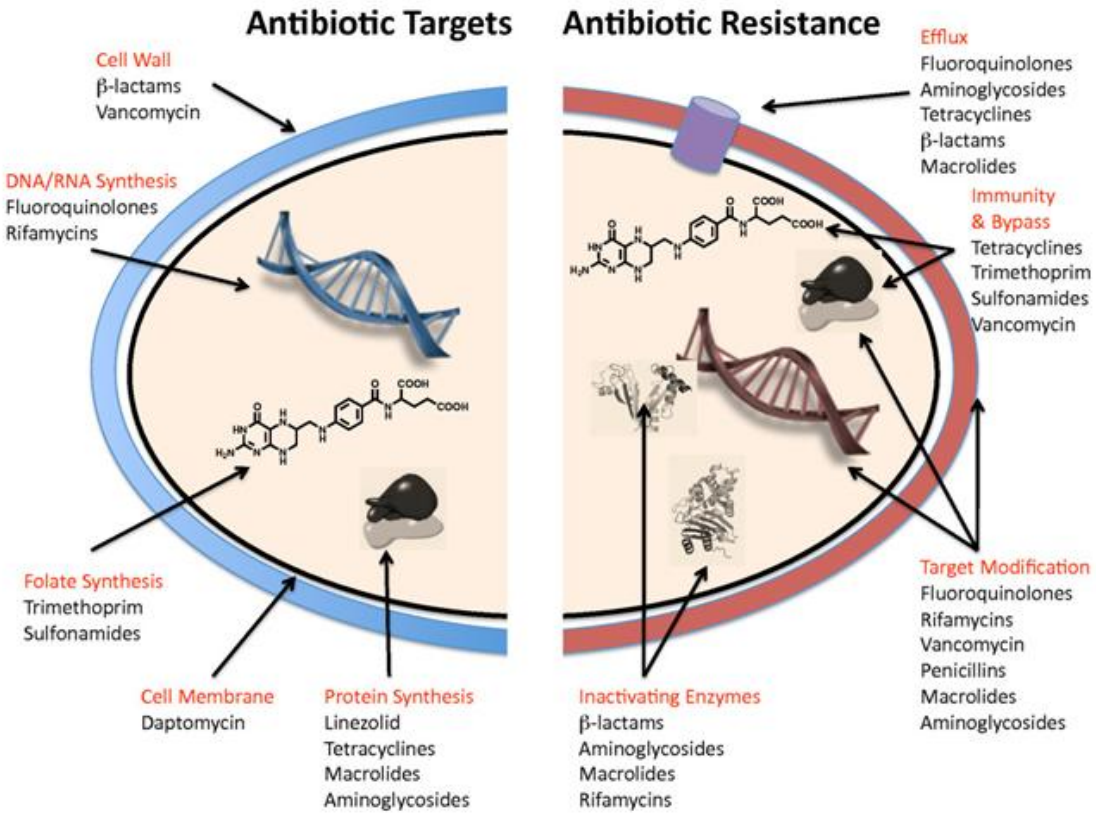

Fig1.3. Mechanism of bacterial resistance to antibiotics [9]

1. 2. Nanotechnology:

Recently, there was an increasing interest among the scientific community to shift to more effective natural antimicrobial treatments with advanced technologies such as nanotechnology [2] which assists in making antimicrobials to be more effective and less liable to resistance. Nanoparticles (NPs) are examples of nanomaterials which have been proven to be less prone to bacterial resistance since they enter the cell of the bacteria easily rather than normal antibiotics which remain on the bacterial walls. The NPs existed on Earth surface long ago before the evolution of nanoscience and nanotechnology. Particles that were released from combustion, food cooking, vehicle exhausts, photochemical volcanic eruption existed since 1974 and were found to be in the nano-size scale. The merge of nanoscience and nanotechnology just began 2 decades ago. NPs are metal atomic clusters which are extremely promising due to their commercial applications.

As mentioned in many nanotechnology publications; nano is a term that is dealing with a material of the size down below 100 nm and 1nm is equal to 10^{-9} m [1] [4]. NPs can be prepared from metal and a matrix of polymer or lipid nature and other forms as shown in Fig1.4. [6][7][10]

For example, the printed paper is about 75000 nm in thickness. The single strand of DNA is about 3 nm width. The various colors reflected by the peacock feathers and the soap bubbles are due to interaction between the light rays and their internal structures of dimensions $10^{-1}$of 1 nm in thickness [1]. Nanoscience is interdisciplinary science; it merges physics, chemistry, electronics, medicine, biology to get enhanced applications in our everyday life like quantum dots, nano fibers, nano crystals which could be fabricated from rare earth materials without the need for matrix or polymer and their characters depend on the surfactant used. Nano capsules have oil core and a polymeric outer shell where the drug is added to the core so it is encapsulated inside or added to the shell so it is loaded on the capsule. Yet, one of the drawbacks that nano capsules have is low drug loading capacity which does not exceed 30%.

Nano emulsion is a dispersion prepared at the nano level of 2 immiscible liquids by surfactant(s). The aim of adding surfactant is to decrease the surface tension and increase repulsion. The oil/water (o/w) nano emulsion can be converted to w/o by high pressure atomization; however,

one of the main drawbacks of nano emulsions is that they have low control and low release rate kinetics of the drug.

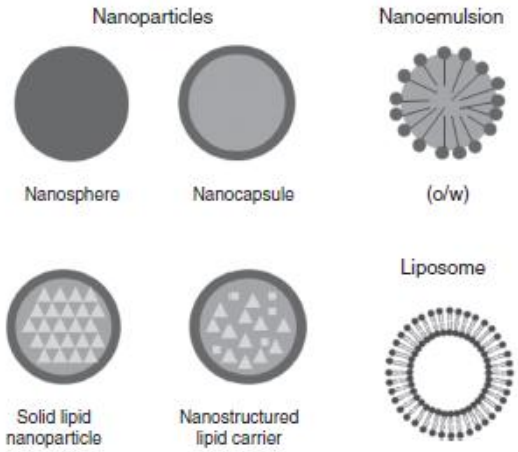

Fig.1.4. Different architectures of nano systems used to improve biopharmaceutical properties of plant extracts [6]

When those sciences intermingle for certain application like for example; Nano Electro Mechanical System (NEMS) which is focused on the fabrication of 3-D nanostructures could be applied in robotics or to move certain molecules it will turn to be Nanotechnology [1][7][10]. Nanotechnology combines new atoms and molecules and engineers them in a highly sophisticated manner so as to deal with the materials on the size level of organelles for example or the level of DNA for example so as to be able to formulate a customized drug for every patient targeting a certain nucleobase in the DNA. This will be highly beneficial in the inherited diseases and chromosomal mutagenic diseases. One branch of the applications of nanotechnology is nanomedicine [7]. In this branch nanotechnology fulfills many innovations in devices, sensors, theranostics, cardiology and dermatology [7].

Nanoparticles are produced by reducing the size to reach the nano level; either by the top down or bottom up approaches as shown in table 1.1 [11].

Table1.1 Top down versus Bottom up approaches [11]

Top down	Bottom up
Milling	Reactive precipitation
High pressure homogenization	Solvent displacement
Lithography	Molecular self-assembly
Photolithography	Atomic layer deposition
Electron beam lithography	Electrodeposition
Micro contact printing method	
Focused ion beam machinery	
Sonication	

Nanoparticles are either inorganic in nature like metal oxides such as titanium oxide, silver oxide, magnesium oxide or organic like lipids, polymers or carbon nanotubes [11].

Classification of polymers applied in nanotechnology:

Polymers are advantageous over other matrices since they are stable and easily tailored. They are classified into many classes but the most frequent three bases of classifications are:

1. Polymer degradation inside the body
2. Polymer type either hydrophilic or hydrophobic
3. Polymer nature (natural or synthetic) [6][11][12] [13]

According to the first base of classification; enzymatically degradable polymers are ether or amide polymers which need a catalyst for hydrolysis like synthetic ones; poly ethylene glycol (PEG), and Poly Propylene Glycol (PPG). Examples of natural polymers are collagen, elastin, albumin, fibrin, natural amino acids and polysaccharides (such as chitosan and poly-acids). The natural polymers are of high molecular weight thus easily targeted by the enzymes.

Hydrolytically degradable polymers are hydrolytic labile polymers with chemical bonds in their backbone that can be easily hydrolyzed. Those hydrolysable groups can be ester, cetal, anhydride, amide, carbonate, phosphate or urethane. Many examples of those polymers exist

such as poly glycolic acid (PGA), poly capro lactone (PCL), poly L-lactic acid (PLLA), poly D-lactic acid (PDLA), PLA and PGA form; poly lactide Co-glycolide (PLCG).

Any of those polymers get degraded by one of two mechanisms either by bulk erosion, where water diffuses at high rate and leads to mass shrinkage or by surface erosion where the drug release rate kinetics are controlled and it is favorable in sustained release drug preparations or both systems as shown in Fig1.5. [13]

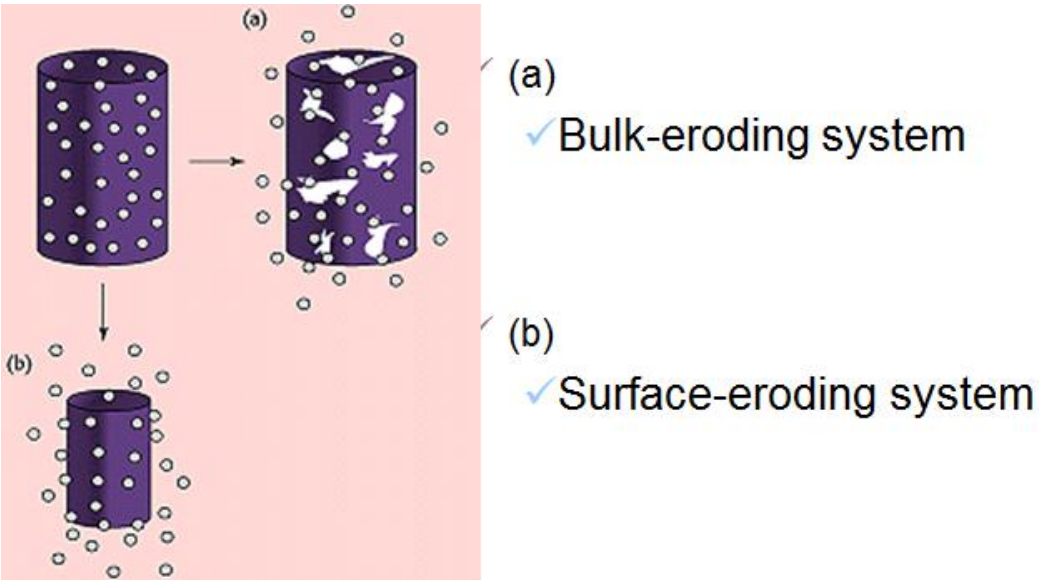

Fig.1.5. Erodible polymer matrices [13]

The second base for classification is according to polymer hydrophilicity and hydrophobicity [6]. Polymeric NPs are prepared either by hydrophilic polymer matrix which is prepared by ionic gelation or nanoprecipitation or by hydrophobic matrix which is prepared by solvent substitution or nano precipitation. The later form is suitable for oil derived drugs and plant constituents and in this case water miscible organic solvents like acetone or ethanol are used [6]. The hydrophobic NPs are classified into solid lipid NPs and nanostructured lipid carriers. The former need a surfactant or co-surfactant to be stable but they are of low holding capacity that is why the best preparation for lipid soluble drugs or extract is the nanostructured lipid carriers. The later uses liquid lipids to form disorganized matrix [6].

The third base of classification is according to the polymer being natural or synthetic [11]. The natural polymers have a large advantage over the synthetic ones as they are biodegradable,

biocompatible and non-toxic. They can be of agricultural origin like cellulose, starch or pectin. They can also be of marine origin like chitin and chitosan [11].

Those polymers are biodegradable and have marvelous applications in nanomedicine as shown in Fig1.6. [13]

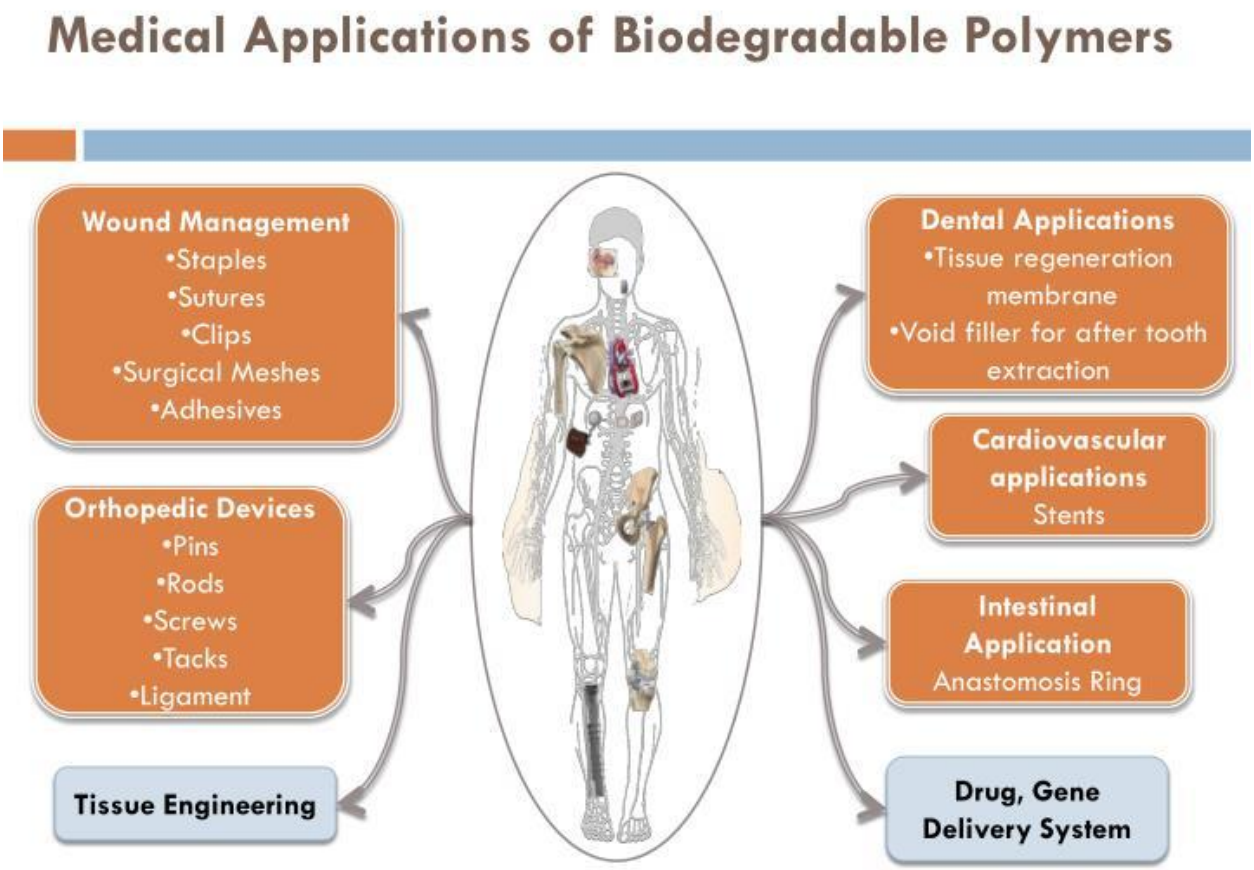

Fig1.6. Medical Applications of Biodegradable polymers [13]

Thus, preparing polymeric antimicrobial NPs to target the human body, it is preferred tha they would be prepared to be biodegradable and biocompatible to avoid being rejected by the body or form a foreign body to attack it. A good example for such polymer is to be derived from family Crustacea; which includes chitin and the deacetylated form from it which is chitosan.

1.3.Chitosan polymer:

In coastal and marine areas, shell fish meat is the edible part while the rest which is the head and the shell are discarded and form around 80,000 tons of waste annually. The rate of degradation of this waste is very slow forming a large source of pollution. The only solution for this huge source of pollution is to recycle this waste to a reusable natural polymer like chitin. Chitin was first discovered by Henri Barconnot who was performing a research on mushroom in 1811. Professor C. Rouget discovered a derivative of chitin upon applying alkaline hydrolysis which produced a chemical entity that was acid soluble in 1859. Hoppe Seiler discovered that this chemical entity was the N-deacteylated form of chitin and he called it chitosan. Nowadays chitosan is prepared from chitin either by chemical deacetylation or enzymatic deacetylation. Enzymatic deacetylation is either homogenous by dissolving in sodium hydroxide then cooling giving 45-52% DD. Heterogenous enzymatic deacetylation is performed by hot sodium hydroxide to give insoluble chitosan of 85-90% DD as shown in Fig.1.7. [11]

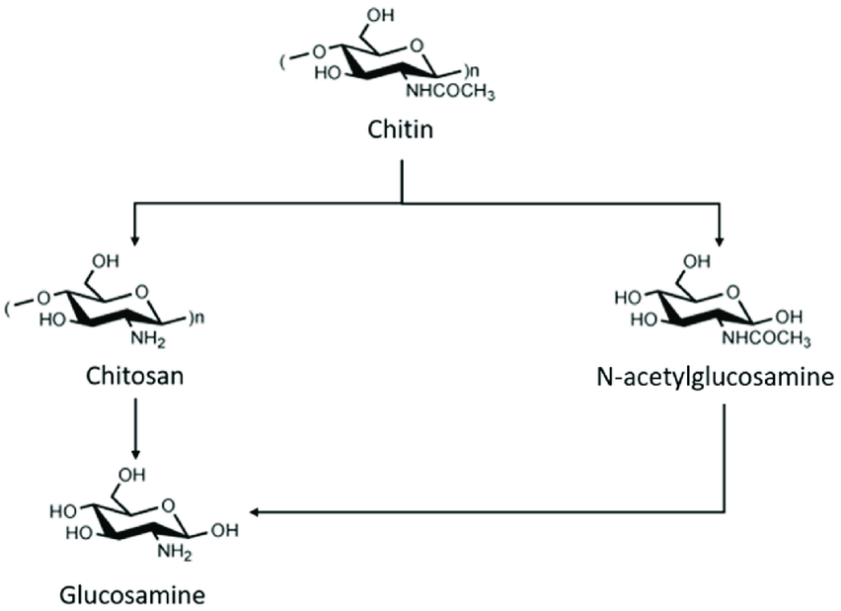

Fig.1.7. Structure of Chitin and Chitosan [11]

Chitin is the second most abundant natural polymer after cellulose. Its main origin is from the cuticle of insects, yeast, green algae, shrimps, crabs and fungus cell wall. As shown in Fig.1.8. Chitin is a homo polymer β 1,4-linked N-acetyl D glucosamine(Glc NAc, A unit).[11][14]

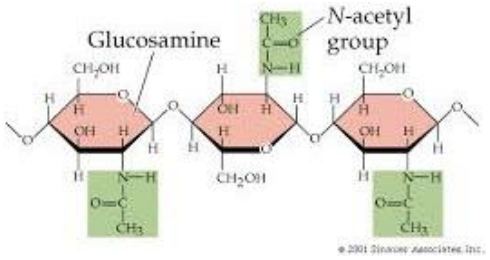

Fig.1.8. Chemical structure of chitin [14]

It is classified according to the bond orientation to 3 classes; α, β, and 8 where alpha is characterized by antiparallel chains, beta is characterized by intra-sheet hydrogen bonds by parallel chains and gamma is characterized by combination of alpha and beta chitin which means it acquires parallel and antiparallel chains.

Chitosan is a white inelastic hard nitrogenous polysaccharide material. Chitosan is N-deacteylated derivative of chitin. It is poly cationic in nature formed of alternate units of glucosamine and N-acetyl D-glucosamine linked by β1,4 glycosidic bonds. According to the molar fraction of D units the degree of deacetylation can be determined. This character is crucial to determine the molecular weight and the viscosity and whether or not the amino group remains free or will dissolve in acid to give acidic solution of chitosan. Those characters have great impact on the UV-Vis and IR spectra and the gel permeation properties. [14]

1.3.1. Advantages of using Chitosan as polymer for NPs fabrication:

Chitosan has many advantages since it is biocompatible, biodegradable, non-toxic, bio-adhesive, enhances permeability and is a good candidate as drug delivery system for many medical applications, genes, antigens and anticancer drugs. Chitosan by itself has high antibacterial and antitumor action since it inhibits the mutagenic cell growth and induces apoptosis according to Ding R.L.*etal.* [15]. Using it in the nano form is much more advantageous than using it in its bulk form[2] since they proved that the nano size preparation binds more irreversibly to the bacterial cell wall in a more or less electrostatic interaction. This electrostatic bond alters the membrane

polarity and permeability which will create reactive oxygen species (ROS) that will disrupt the respiratory mechanism of the bacterial cells [2]. So, the NPs in general can easily pass the physiological barriers and act as drug delivery carrier candidates [2]. One main challenge in preparation of the chitosan NPs with the sodium tri polyphosphate (TPP) linker is the high value of particle dispersion index which could be optimized by trial and error [14]. Many techniques are used to prepare chitosan NPs which have many pros and cons as shown in Fig.1.9. [11]

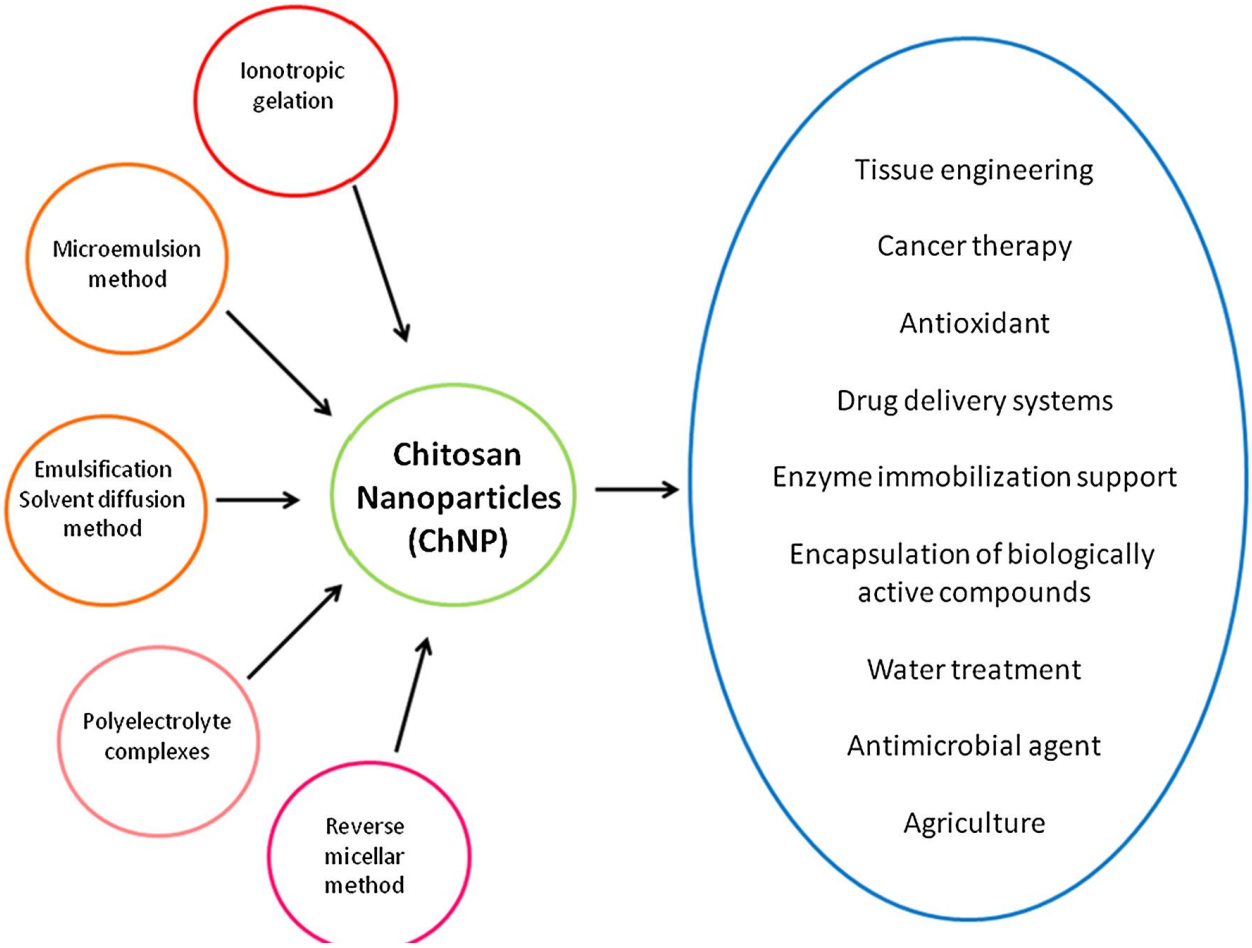

Fig1.9. Modes of Chitosan nanoparticles synthesis and their applications [11]

1.3.2. **History and methods of preparation for chitosan nanoparticles**:

Chitosan NPs were first prepared by emulsifier and cross-linker to deliver anticancer drug intra-venous route. Several techniques are adopted for their preparation as described below;

a. **Ionotropic gelation**; where an electrostatic bond occurs between the positive amino group and the negative group of TPP linker. First a stabilizer is added like poloxamer then the TPP linker is added to from NPs by stirring at room temperature. The size of the NPs can be controlled by controlling the ratio of the chitosan added or the ratio of chitosan to the TPP added. This is the best method to prepare chitosan NPs since it does not use high shear forces and uses organic solvents.
b. **Micro emulsion**; where chitosan is prepared and dissolved in acetic acid solution then the surfactant solution is prepared in N-hexane and glutaraldehyde is used as the cross linker is added to it. After crosslinking for overnight the excess surfactant with the solvent is evaporated under low pressure then precipitated using calcium chloride. The major drawback here is the usage of antigenic glutaraldehyde and this type of prepared NPs cannot incorporate proteins.
c. **Emulsification solvent diffusion Method;** where chitosan is prepared and the organic phase with the poloxamer stabilizer is injected in it and stirred under high pressure homogenization. After that the prepared solution is diluted with high amount of water so the organic solvent will diffuse in the water therefore the polymer precipitate and form NPs.
d. **Poly Electrolyte Complex**; where chitosan NPs are formed when the chitosan solution is added to the DNA solution and high stirring conditions are applied.
e. **Reverse Micelle Method**; where chitosan dissolved in water is added to surfactant solution in organic solvent to form reverse micelle of ultrafine NPs [7] [11] [15] [16] [17] [18] [19][20][21][22].

1.3.2.1. **Biological versus non- biological routes of chitosan nanoparticles synthesis**:

Any of the above procedures is performed using chemicals yet applying green synthesis is much more advantageous than the chemical one. The chemical and physical methods for NPs preparation involve many sophisticated steps and procedures. Those methods require high temperature and inert atmosphere and large amount of many additives like organic solvents, surfactants, stabilizers and reducing agents to avoid spontaneous oxidation upon storage.

Therefore, a system of preparation which is ecofriendly and safe is preferred to avoid any toxic byproducts. Table1.2. shows the biological and the non-biological routes for NPs synthesis and Table1.3. shows one shows a comparison of the different routes for NPs green synthesis. [4] [23]

Chitosan can either be used as a polymeric matrix for NPs or as a substrate to prepare NPs made of another matrix or metal as will be discussed below.

Table1.2. biological and non-biological routes for nanoparticles synthesis [4]

Route of NPs synthesis	**Method**	**Examples**
Non biological	**Top to bottom**	Mechanical, Ball milling, diffusion flame, chemical etching, chemical or laser ablation, sputtering, microwave, ultra film, plasma arching, molecular beam epistaxis and lithography
	Bottom to top	Chemical or electrochemical; precipitation, vapor deposition, atomic or molecular condensation, sol-gel process, spray pyrolysis, laser pyrolysis, aerosol pyrolysis .
Biological	**Bottom to top**	Green synthesis; microscopic: Bacteria, actinomycetes, Fungi Macroscopic; Algae, sea weeds, plant extracts (leaves, bark, stem, shoot, seed, latex, secondary metabolites, root, twigs, peel, fruit, seedling, essential oils, tissue cultures and gum)

Table1.3. Different Types of NPs green synthesis [22]

Plant route	Microorganism route	Green chemical route
Use the plant part like leaf, stem, seed, shoot, root, petal, fruit, extract either fresh or dried; the extract of any of those plant parts in distilled water will carry the reducing and capping agent needed for fabricating the NPs. Many factors will affect the final morphology of the nanoparticles like the metal ion concentration, the temperature, the pH and the extract concentration	Microorganism, either fungi or algae or bacteria grows on culture media where it will release the reducing agent or the capping agent needed to reduce the metal ion	eco-friendly pure chemicals like chitosan, starch, sucrose, calcium alginate are used as capping agent to form spherical NPs.

The mostly used polymer in green chemical synthesis is chitosan with its cationic amino group chelated with the poly-anionic group of TPP. All the pervious reported work were to prepare metallic NPs using chitosan biodegradable polymer and plant part extract. [4] [23] The outcome of the green approach was just metallic NPs like silver and copper NPs. Using this system did not only avoid costly and hazardous byproducts but produced better and smaller size NPS. It is a colloidal stable method to prepare copper nanoparticles in chitosan dissolved in aqueous medium. The protocol of chitosan to chelate metals like copper and silver produced NPs with stable uniform size. In this case, chitosan was used as a capping and reducing agent for silver and copper to get Ag^0 and Cu^0 metal NPs. Other reducing agents as ascorbic acid and other stabilizing agents and polymers like PEG or cyclo-dextrin in aqueous medium were used as a good source for hydroxyl ions which then reacted electrostatically with the protonated amino group NH_3^+ in chitosan. The time of stirring conditions and the chemical conformation of the

stabilizer and the plant extract used control effectively the size and morphology of the formed NPs [22][23][24]. The use of α cyclodextrine (CD) is better than β and ϑ as the first one gives the smallest NP size as shown in Fig.1.10.

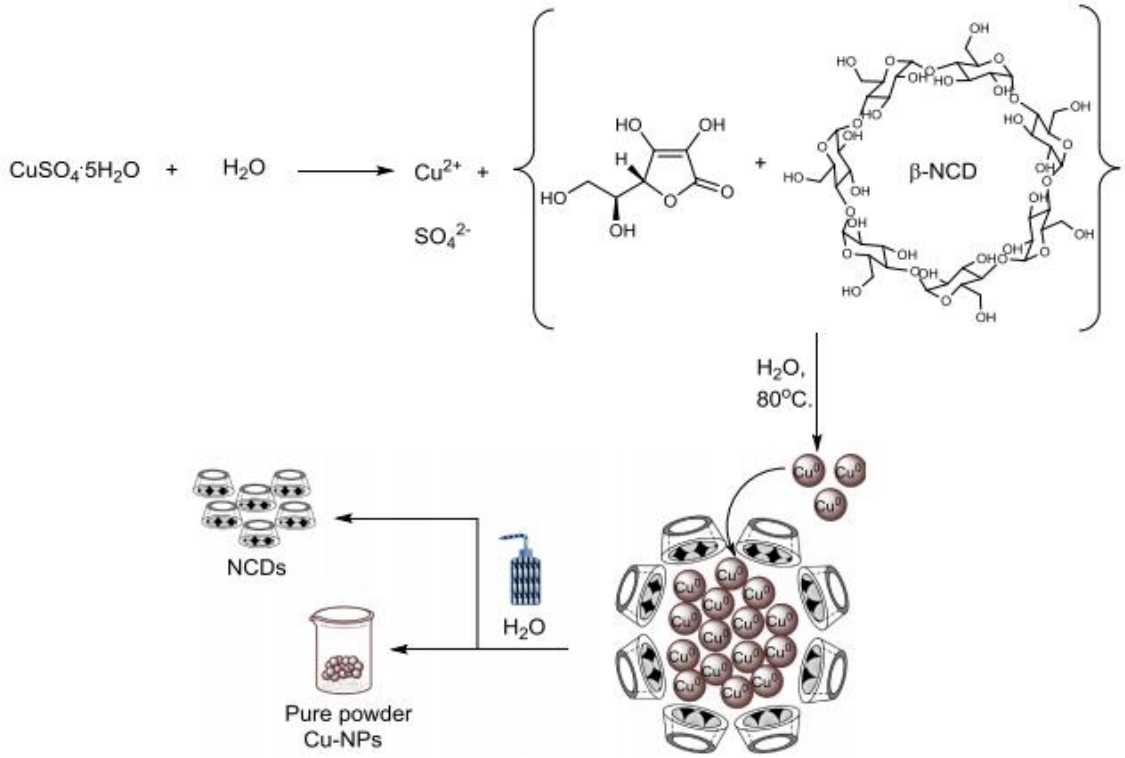

Fig1.10.General scheme for the synthesis of the copper NPs using CD [24]

For the preparation of silver NPs by green synthesis routs, the stirring conditions for too long time reaching 48 hours would make the stabilizer loses its control on the dispersion and the stability of the NPs. Using these vigorous conditions would produce large particle size. Thus, the optimum stirring time was just overnight not for 2 days otherwise oversized NPs would be produced as shown in Fig.1.11.

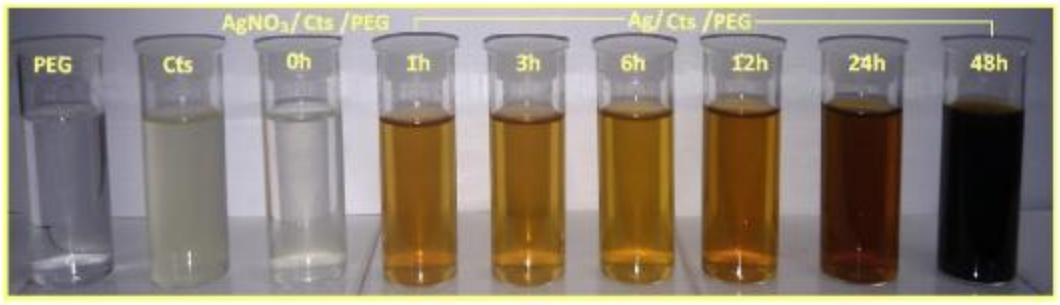

Fig.1.11. Silver NPs prepared by green synthesis using PEG and chitosan polymers at various stirring conditions and times 1, 3, 6, 12, 24 and 48 hours [22]

1.4.Different plant seeds extracts used in the fabrication of polymeric or metallic NPs:

For the plant extract used,they used Neem or Geranium leaf extract to produce gold NPs of decahedral or icosahedral morphology which showed the optimum optical and catalytic properties depending on the terpenoidal and flavonoidal content in the extract. Core and shell gold and silver bimetallic nano particles were formed by this sequence by adding persimmon leaf and mushroom extract in ratios 1:1. Other examples of plant seed extracts used are shown in table 1.4. [23][25]

Table.1.4.Different seed extracts used to prepare metal NPs[4]

Plant part used	Plant name	Metal prepared	significance	References
Seed	Elaeocarpus granitrus	Silver	antimicrobial activity	[55]
	Jatropha curcas	Silver		[56]
	Macrotyloma uniflorum	Silver		[57]
	Trigonella foenumgraecum	gold		[58]
	Artocarpus heterophyllus	silver	antibacterial	[59]
	Abelmoschus esculentus	gold	antifungal	[60]

As mentioned above; all work reported on seed extract was for the synthesis of metal NPs using chitosan polymer. Only Madadlou reported using the date pit extract to prepare micro emulsion suspended in it nano capsules made from starch polymers. Table1.5. shows a summary for all previous work using done on seed extracts in metal NPs preparation [4] [23] [33].

Table1.5. Seed extract used to prepare metal nanoparticles and their significance [4] [23] [33]

Seed extract used	Nano preparation	Polymer if used	Significance
Elaeocarpus granitrus	Silver NPs	chitosan	Antimicrobial
Jatropha curcas	Silver NPs		Spherical 15-50nm
Macrotyloma uniflorum	Silver NPs		Anisotropic NPs with 12 nm size
Trigonella foenumgraceum	Gold NPs		Reducing and protective size 15-25 nm
Artocarpus heterophyllus	Silver NPs	Alkaline Solid Polymer electrolyte (ASPE) seed powder extract	Antibacterial against gram positive bacteria *Staphylococcus aureus, Bacillus subtilis* and *Bacillus cereus* and gram negative bacteria *Pseudomonas aeruginosa*
Abelmoschus esculentus	Gold NPs		Antifungal against *Puccinia graminis trictci, Aspergillus flavus, Aspergillus niger* and *Candida albicans*
Phoenix dactylifera	Starch nano capsules	Micro emulsion	Antibacterial anti-fungal action was not investigated (no numbers were reported)

So the plant extract and the green chemical approach merge where the natural polymers with the plant extract are used simultaneously is the most optimum condition, suitable for large scale production of NPs and the most ecofriendly method. It is more advantageous than the microorganism based method according to the pros and cons listed in Table1.6. and Fig.1.12. which shows a general scheme for the plant route[18][22][23][224 [25][52][53].

Table1.6. Plant route versus Microorganism route [23]

Point of comparison	Plant	Micro organism
Rate of the reaction	Minutes to hours	2-3 days for the culture
Safety	Safe and benign	Some bacteria like *Pseudomonas, Escherichia coli, Fuzarium*can produce toxic NPs to human
Temperature needed	Room temperature	Heat is needed for the culture medium and the reaction mixture

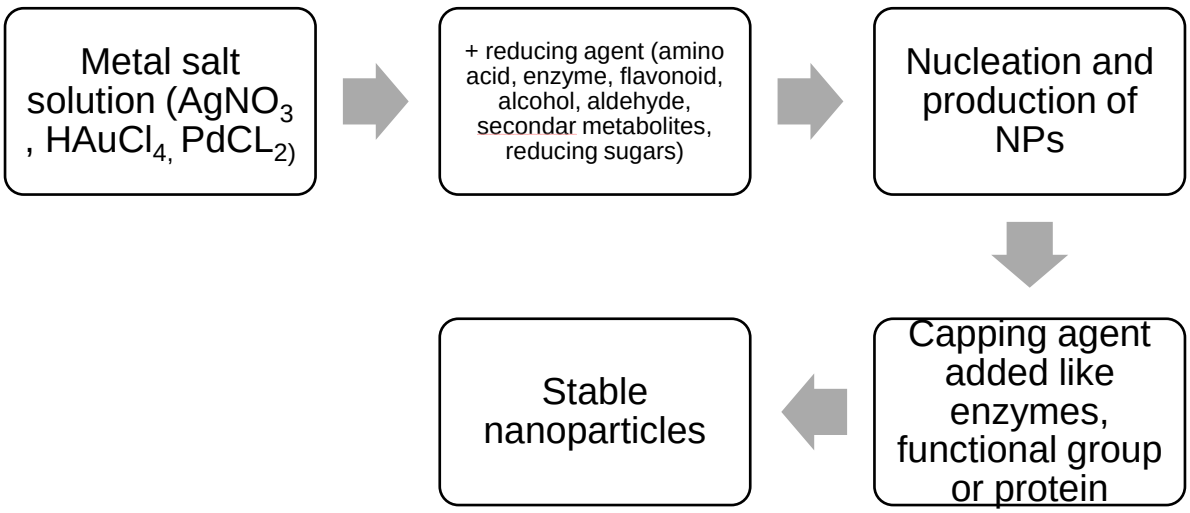

Fig 1.12. Scheme of the biogenic or plant route alone for the synthesis of the metal NPs [25]

There are some cons of using the plant like the parameters of the process as temperature, pH and incubation time which can be easily tackled and controlled since any variations in them affects the size and the morphology of the NPs as shown in Table1.7. [25]

Table1.7. Factors affect the biogenic plant route of synthetizing the nanoparticles [25]

Factor	Effect
Temperature	In case of gold NPs manufacture; it turns spherical at high temperature and triangular at low temperature
pH	As the pH is low from 2-4 the NPs get larger
Incubation time of the metal ion with the plant extract	As the time increases from 5 minutes to for example 2 hours, it gives sharper and better peaks at UV

In addition it is a slow process yet the best plant part extract which shows the fastest route is the seed extract compared to the other plant parts. *T.chebula* seed extract in which is rich in tannins was used to act as a capping agent to reduce gold and form spherical gold NPs of the least possible size 8-10 nm and of the best antibacterial action against both Gram positive *Staphylococcus aureus* and Gram negative *Escherichia coli.* This reaction just took 20 seconds to form the desired NPs [26] .

1.4.1. **Seed, definition and classification** :

The seed is the center part in the plant responsible for the sexual reproduction. Seeds are the main storage factory of food in the form of carbohydrates and fixed oil on which the embryo feed on. They are the main reason for the differentiation of the plant to different varieties and species which is a good consequence for evolution of mature fertilized ovule. The difference between seed, fruit and nut is illustrated in table1. 8 and the classification of seed is described in table1. 9.

The typical seed consists of 3 parts; the embryo which is the young sporophyte, endosperm and seed coat. In each stage of the seed development, the minerals derived for the embryo nutrition

either from the male or female; the pollen grain or the ovule; are either used in the growth process or stored as reserve material for late use. Those nutrients to feed the embryo accumulate in the nucellus or the pericarp they then get stored in the seed and the seed coat after finishing their role. They are then used by the quiescent embryo after abscission from parent plant for further germination.

Table1.8. The difference between seed, nut and fruit [27]

Name	Definition	Example
True seed	Mature fertilized ovule	Beans and cashew nuts
False seed	Any structure that works as a propagule	Cereal grains, potatoes and other tubers
True fruit	A mature ovary	Apples, legume pods, cereal grains and chestnuts
False fruit	Any fleshy sweet tasting structure; structures involved in zoochorous seed dispersal	Cashew apples (maranon), arils, ovuliferous scales and other appendages of gymnosperm seed
True nut	A dry one-seeded fruit with an extremely hard pericarp	Acorns, hazels and chestnuts
False nut	Any oily edible seed including those derived from true nuts	Peanuts, cashew nuts

Table1.9. Seed classification [27]

According to:	Types of seed	Description
Ovule morphology	Anotropous	Ovules and seeds with a raphe; a continuation of the funicle that ends at the chalaza
	orthotropous	Ovules in which funicle chalaza nucellus and micropyle are in a direct line
Embryo morphology	Campylotropous basal	Ovules with curved embryo sacs small embryo restricted to the lower half of the seed
	Peripheral	Large elongated curved embryo contiguous to the testa
	Axile or axial	Small to total embryo central straight curved coiled bent or folded
Origin of the mechanical layer of the seed coat	Exotegmic or exotestal	Inner tegmen or outer testa derived from outer epidermis
	Mesotegmic or mesotestal	Inner tegmen or outer testa derived from middle layer
	Endotegmic or endotestal	Inner tegmen or outer testa derived from inner epidermis
endosperm	Exalbuminous	Seeds with little or no endosperm
	albuminous	Seeds with a well-developed endosperm or perisperm

1.4.2. The Advantages of the seed over other plant parts:

As was reported in the above section, the biogenic route for NP synthesis is very tedious yet using the seed extract makes the process requiring few seconds to be done. [23]

Seeds are mainly rich in polyphenolics and antioxidants (Carlsen *etal.* 2010). For example; almonds are rich in flavonoids, catechins, flavonols and flavanones. Peanuts and pistachios are rich in flavonoids like Resveratrol. Walnuts contain polyphenols and tocopherols and non-flavonoid Ellagitannins. Cashews contain Alkyl phenols. As shown in table1. 10 antioxidants exist in many food types around 3100 yet nuts and seeds show the most antioxidant rich food category. Plant based foods include herbs, spices and plant medicines are folds more rich in antioxidants than animal based ones with median antioxidant (AO) value of 0.88,0.1, 0.31 m mol/ 100 gm [27].

Table1.10. Antioxidant rich food categories [27]

Product	**Country of origin**	**Product in antioxidant content (mmol/100 gm)**
Almond with pellicle	Norway	0.37
Chestnuts with pellicle	Italy	4.67
Ground flax seed	USA	1.13
Roasted kernel from water melon	Iran	3.27
Pecan with pellicle	Norway, Den Lille	8.24
Pecan with pellicle	Mexico	10.62
Pecan with pellicle	Norway, the green valley	7.31
Walnuts with pellicle	USA	15.16
Walnut with pellicle	Norway	25.41
Walnut with pellicle and shell	Norway	33.29

It has been reported that 20% of the seeds are thrown as waste products and not reused again. Date pits are just reused as food for cattle, sheep, camel and poultry. Around 9 million tons of dates are produced annually and 960 thousands of those 9 million tons are date seeds (FAO 2010). Simply, it is reported that the seeds when got soaked in water overnight and then this water is drunk; this water gives protection against tumors and cancer. Seeds and to a lesser extent the peels waste byproducts of the edible plants are rich in secondary metabolites mainly tocopherol, carotenoids, phenolic acids and flavonoids. [30] [34] Seeds as those of *Nux vomica, Colchicum* are rich in essential oils and terpenoids. Other seeds as *Cardamom and Nutmeg* are rich in carcinogenic glycosides. Fenugreek seed is rich in saponins, Guragum seed is rich in gum and Jojoba seed is rich in wax [27]. As shown in table1.11, table1. 12 and table1.13 .*Malus domestica*; the apple seeds show the least values of total phenolic and antioxidant contents. On the contrary, *Vitis vinifera* seeds; the grapes seeds show the maximum values of total phenolic and antioxidants contents [30].

Table1.11. The TPC and antioxidants in random seeds of plants [30]

Fruit seeds	Total phenol (mg GAE/100g)	DPPH%
Vitis Vinifera (Var. Feteasca Neagra)	21.19±0.04	95.18±0.00
Malus domestica (var. Golden)	2.02±0.08	6.19±0.04
Citrullus lanatus (var.Dabuleni)	3.75±0.02	25.79±0.03

Table1.12. Aqueous extracts of different plant parts showed polyphenolics [61] [62] [63] [64] [65]

Plant	Part used	Active compound	Activity	System
White Tea	Leaves	Phenolic compounds catechins	Antioxidants	NPs of PECL, alginate, Pluronic®F127
Phoenix dactylifera	Pit	Phenolic compounds	-	Nano capsule whey protein
Centella asiatica	Dry leaves	Asiaticoside Asiatic acid Madecassoside	Antioxidant	NPs of gelatin
Camellia sinesis	Leaves	Phenolic compounds; Epigallocatechin Epigallocatechin-3-gallate Epicatechin Epicatechin-3-gallate	Antioxidant	Nano emulsion of Lecithin palm or sunflower oil, Tween® 20 or 80
Hibiscus sabdariffa	flowers	Phenolic compounds Anthocyanins	Antioxidant	Liposome Lecithin

*PECL= poly(epsilon-caprolactone)

PLuronic F127= poly oxy ethylene-poly oxy propylene block copolymer

Tween®20= polyoxy ethylene sorbitan monolaurate

Tween ®80= poly oxy ethylene sorbitan monooleate

Table1.13. A comparison of the plant parts which are rich in antioxidant and polyphenolics and used as a good candidate for food, drug and cosmetics preservation[32][33][34][35][36][37][38][39][40][41][42].

Plant used	The AO effect on minced beef meat	Any comments added
Grape seed	55% ethanol extract showed high polyphenolic content 5000-8000 mg/kg TPC	Rich in catechin, epicatechin, procyanidin B1 and B2,gallic acid, epicatechin gallate
Kronaki cultivarL. Olive leaf	215 ppp caffeic acid	When was compared to synthetic BHT after 1 week administration it increased the levels of Alkaline Phosphatase AP, Alanine Amino transferase ALT, Aspartate Amino Transferase AST and increase serum total lipids.
Sage and rosmary	Inhibits oxidative changes for 100 days at -18 degrees	
Punica granatum L. peel Pomegranate peel	Punicalagin 503.7 and 762.85 mg/L and anthocyanins 62.43 -276 mg/L and ellagic acid 268.67-389.64 mg/L	Punicalagin PG is hydrolyzed to punicallin PL and Ellagic acid EA
Phoenix dactylifera L. Date palm pit	Rich in procyanidin, luteolin, oleic acid 41.4-58.8%, quercetin, apigenin	The AO activity ranges from 13.13-37.42mmol of Fe^{+2}/ 100mg dry seed The phenolic content ranges from 1260-3541 mg GAE/100gm dry plant seed

Another important example of the seeds to be used is the Avocado seeds, *Persea Americana mill* [28]. The peel and the seed are rich in flavonols monomers, proanthocyanidins, catechins, flavonols glycosides, hydroxy cinnamic acids, procyanidin A, 3-o-p-coumaroyl quinic acid and 3-o-caffeoyl quinic acid. Table1.14.shows the seed and peel are having higher percent of polyphenolics and antioxidants than the mesocarp and the pulp.

Table1.14. Trolox Equivalent Antioxidant Capacity (TEAC) and Oxygen Radical Absorbance Capacity (ORAC) tests to measure TPC and Antioxidants in the different plant parts of Avocado [28]

	Variety	**TPC (mgCE/DW)**	**TEAC (mmol Trolox /gDW)**	**EC_{50}(mgDW)**	**ORAC (Mmol Trolox/gDW)**
peel	Hass	25.32±0.242a	0.161±0.0024a	0.358	0.47±0.036a
	Shepard	15.61±0.241b	0.112±0.0034b	0.927	0.29±0.020c
	Hass	9.51±0.161d	0.094±0.0007c	0.920	0.21±0.014d
	Shepard	13.04±0.211c	0.091±0.0047c	0.776	0.35±0.021b

1.5.**Antioxidants and Polyphenolics role in treatments**:

Antioxidants adopt certain mechanism to protect us from many diseases. When body faces oxidative stress which is the accumulation of non-enzymatic oxidative damage to molecules caused due to inflammatory diseases, pollution, tobacco, alcoholics or certain drug abuse; this will affect normal cell functions and metabolism negatively (Blomhoff, 2005). This condition will create many free radicals spread through all the body as reactive oxygen species (ROS) and reactive nitrogen species (RNS). If those free radicals were not chelated or attacked by antioxidants, they will react with the human body cells negatively and with the cell components like the cell membrane, lipoprotein, protein, carbohydrate, DNA and RNA which will lead to seemingly unrelated diseases and inflammatory diseases like lupus erythromatosis, respiratory distress syndrome (RDS); vasculitis, glomerulonephritis, Ischaemic heart disease (IHD) and many other diseases with variable degree of severity it can cause Acquired Immune Deficiency Syndrome (AIDS) and cancer diseases as well. The oxidative damage for cells occurs when the balance between the free radicals and the antioxidants gets distorted. That is why it is very

crucial to acquire antioxidants in our daily routine in the form of diethyl for example which exists in chocolate, fruits, vegetables, tea, wine and marine organisms. PPH or polyphenolics is a large ubiquitous family of secondary hydroxy aromatic metabolites of all vascular plants. They have major role in plant life; in growth, reproduction and pathogenic and disease resistance due to acting as powerful ROS; reactive oxygen scavenger thus they turn the food and plants unpalatable for herbivorous animal. They have the ability to interact with protein, enzymes and cell receptors to modify their action in a certain manner [29]. They are synthetized within the plant via shikimate and phenyl propanoid pathways [33]. The following scheme shows the pathway for polyphenolic synthesis. Figure1. 13 shows different examples of polyphenolics exist in the plant and Fig.1.14 illustrates the different chemical structures for polyphenolics. [29] [33] [76] [34]

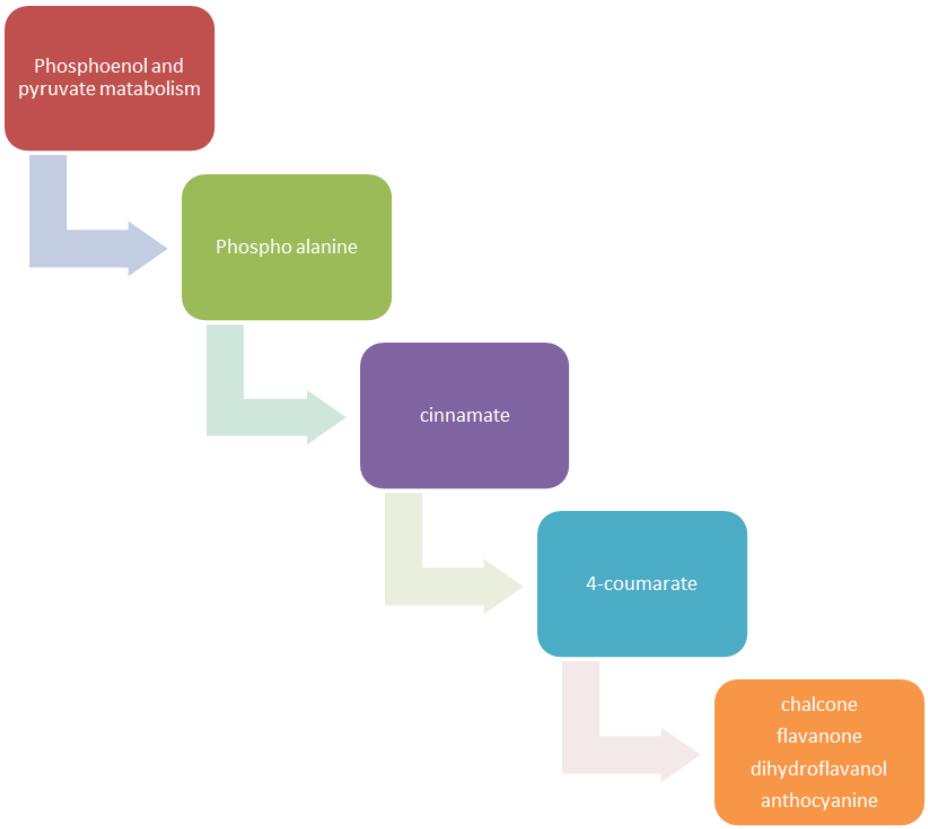

Fig.1.13. The general scheme for Polyphenolics synthesis [33]

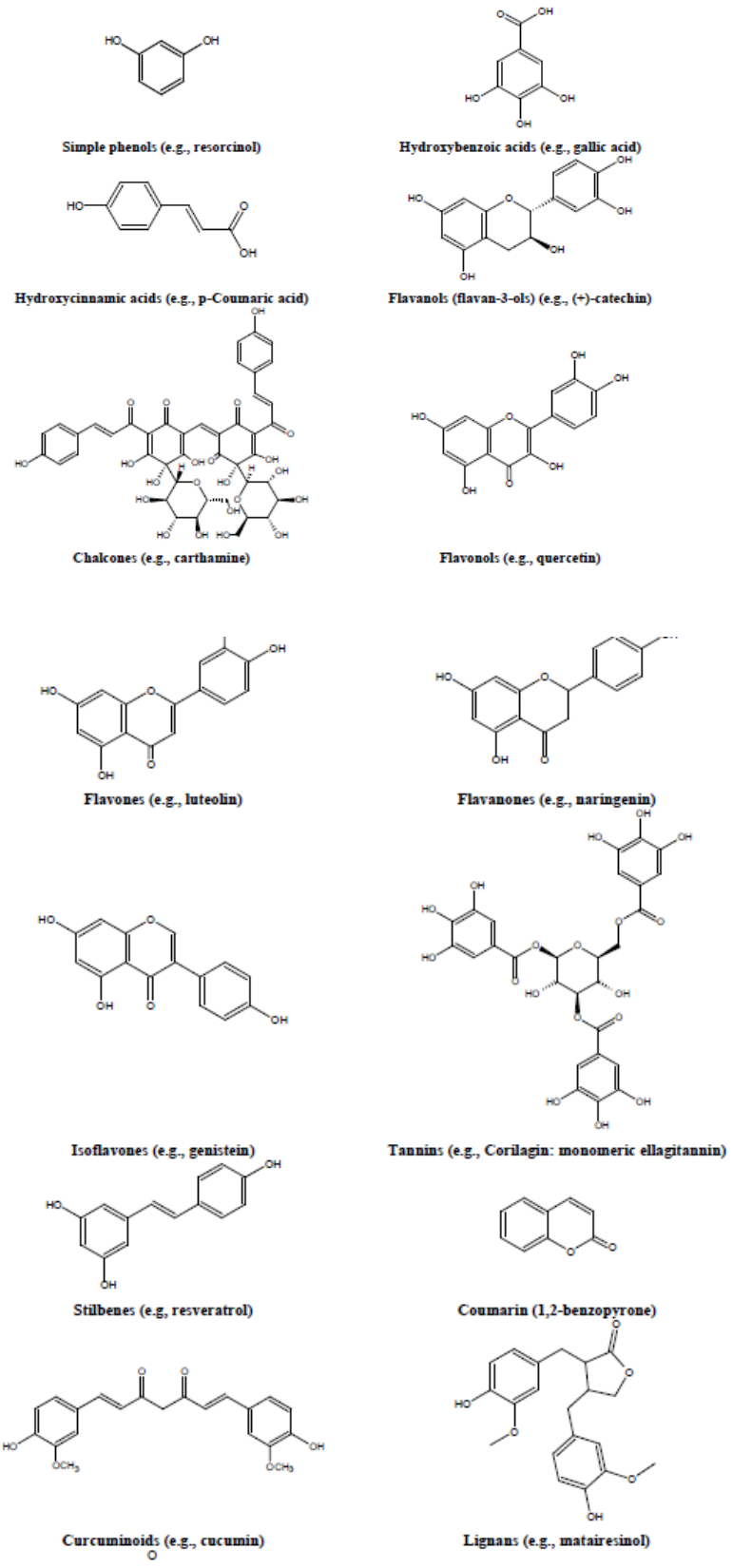

Fig1.14. Different chemical structure for polyphenolics [33]

Antioxidants and polyphenolics have the ability of free radical scavenging since they are proton donors. They can chelate metal ions, and form protein complex with pro oxidant protein. The importance of those polyphenolics and antioxidants is being used as functional food and antioxidant additive which act much better and safer than the chemical food additives like Butylated hydroxy toluene (BHT) and Butylated hydroxy anisole (BHA). BHT and BHA keep frozen pork patties intact for longer time as they inhibit oxidation [26] yet they cause cancer and other diseases. One disadvantage of the natural polyphenolics and antioxidants is that they are highly unstable suffering from low bioavailability and low water solubility and highly sensitive to light. That is why protocols shifted to formulate them in nano forms to increase their efficacy, bioavailability and stability. [27] [28] [29][30][31][32][34].

That is why the pit or seed extracts in the nano form were chosen to be studied and to synthetize chitosan NPs since they are one of the richest sources of active constituents and yet considered as a big source of waste of plants as shown in Fig 1.15. [45]

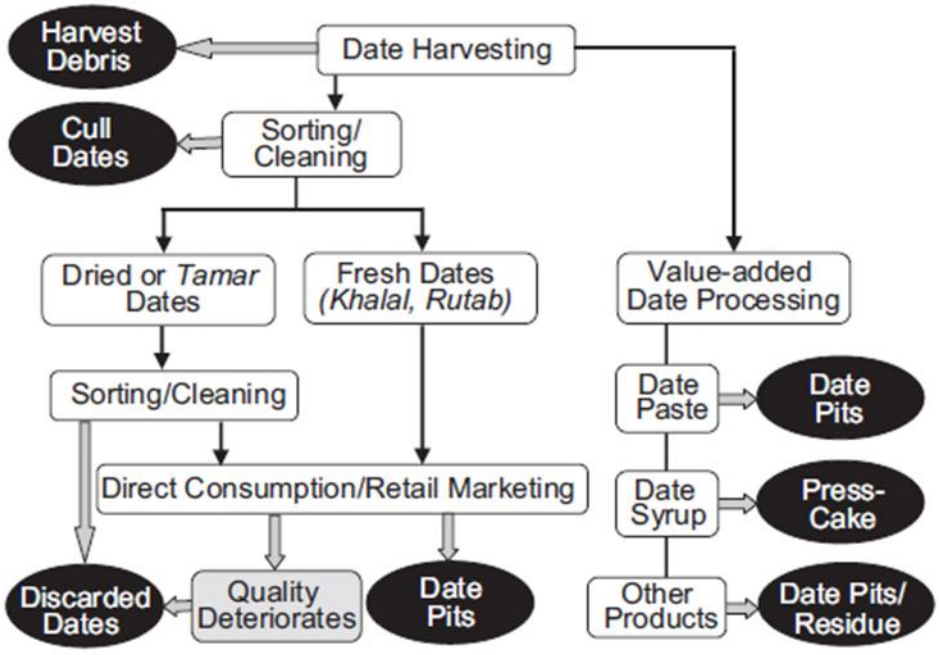

Fig.1.15. Waste and byproducts generation from date plant [45]

1.6.Date pit application:

Table1.15.shows the most important active constituents in the date pit which made it the best candidate to be used as a source of antioxidants and polyphenolics despite other seeds [2] [31] [32] [44] [45].

Table1.15. Chemical composition of the date from 2 different varieties [31]

Percentage content of the polyphenolics and flavonoids								
	Ethyl acetate		**acetone**		**ethanol**		**Aqueous**	
A}Phenolic acids:	DPPT	DPPK	DPPT	DPPK	DPPT	DPPK	DPPT	DPPK
Gallic acid	9.33	13.72	2.73	11.57	5.16	23.17	27.43	00.92
Caffeic acid	00.10	12.00	41.98	25.15	-	09.64	10.36	84.35
Vanillic acid	3.67	3.69	4.92	-	5.08	56.23	5.91	2.10
Coumarin	30.50	13.96	42.08	23.57	55.76	-	12.25	1.24
Total	43.60	43.37	91.71	60.29	66.10	89.04	55.65	88.61
B}Falvonoids: uercetin	00.11	01.21	-	05.35	00.11	-	00.17	00.55
Rutin	-	00.9	-	1.2	00.6	2.02	00.03	00.03
Catechin	03.63	03.46	-	-	04.04	03.06	04.76	-
Epicatechin	52.57	53.16	8.28	39.71	29.95	07.90	39.29	11.40
Total	56.31	57.92	8.28	46.26	34.16	12.98	44.25	11.98

According to what was reported by Amany M.M.B.*etal.* 2012, on comparing the antioxidant action of date pit aqueous extract versus the chemical synthetic antioxidants BHT and BHA [34], synthetic antioxidants are highly health threatening. They cause hepatocellular degeneration, cloudly swelling, vacuolar degeneration and fatty degeneration which appeared in rats after they ingested 200 ppm BHT after 6 weeks. That is why it is a future trend to use safer and cheaper antioxidants. ThioBarbituric Acid (TBA) in the ground beef; is a compound that evoked on meat deterioration. On meat storage for long time the phospholipids on the cell wall and the cell membrane are negatively charged and ionically bonded to iron ions. Those iron ions interact with

the unsaturated fatty acids and lead to their peroxidation and rancidity. On adding 1% date pit extract, it decreased the TBA value to 0.69 mg/kg meat versus no action was done on adding 1% BHT. This indicated the high antioxidant action of the date extract which reduced the formation of the hydrogen peroxides and kept the meet intact for longer storage period of time which complies with what was reported by Abdelhamied *etal.*2009 and ElRayes *etal.*2009 as shown in the following chart in Fig1.16 [34].

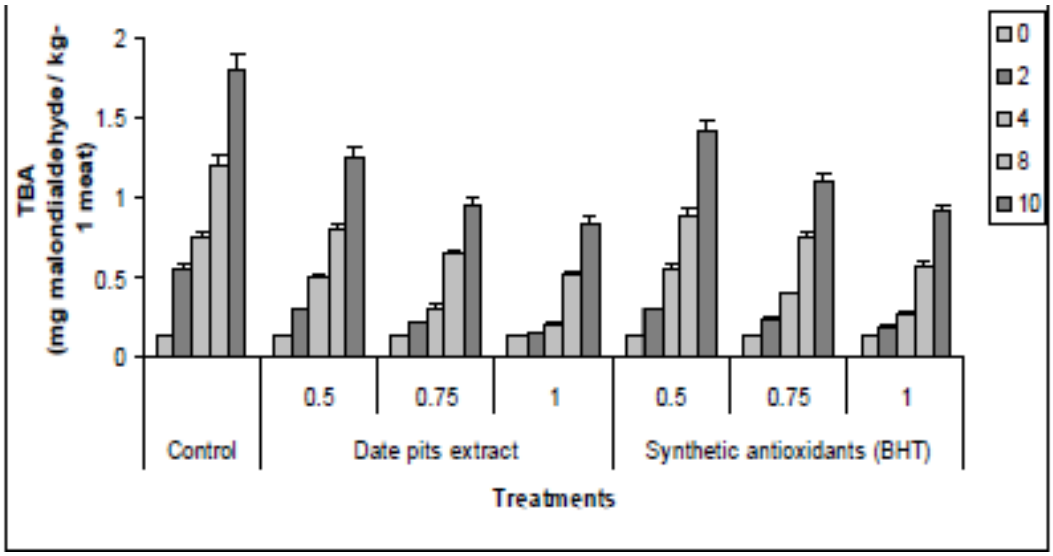

Fig1.16. Chart shows the antioxidant and polyphenolic content of date pit extract versus the BHT 1% extract expressed as mg malondialdehyde /kg meat [34]

Date palm is a dioecious monoctotyledonary plant which belongs to family Arecaceae. Date or *Phoenix dactylifera Bo*tanical classification is as follows; kingdom Plantae, Angiosperms, Monocotyledonaea, Order Arecales, Family Arecaceae, Sub family Caryophoideae, Genus Phoenix; this family Arecaceae has 183 genera and 2364 species. It includes as well 5 strong sub families; Arecoideae, Calamoideae, Ceroxyloideae, Coryphoideae and Nypoideae. The fruit is formed of the fleshy pericarp and the pit or the seed which forms 10-15% of the date fruit by weight (Hussein *etal.* 1998) [43].

Male and female flowers are on 2 separate trees. This plant is widely spread in the Middle East and North Africa. This plant was chosen due to its tremendous benefits and endless active constituents. Flower was reported to have anti-bacterial, anti-viral, anti-fungal and anti-parasitic actions. The date seeds are highly rich in secondary metabolites polyphenolics ranging from 2058-2983 mg GAE/ 100gm and has antioxidant activity equivalent to IC_{50}= 0.112 g/L in DPPH assay where the free radical gets reduced in this test . It is one class of antioxidants which works by prevention of hydrogen peroxide conversion to oxyradical by inactivation of lipoxygenase. The percentage of flavonoids and TPC differ from one variety to the other depending on many factors such as; salinity, environment, genotype, maturation and temperature according to table 1.16. [31] Antioxidant and anti-inflammatory content were prepared in acetone extract 50% i.e. solvent: solid ratio is 6:1 at 45°C. Their main use is to delay the onset of the Cardio Vascular Diseases (CVS), cancer diseases and aging [2] [46]. Its anti-oxidant content is 10 fold more than that in grape seeds, tea extract and date fruit itself [2].

Table1.16. Phenolic content and flavonoids in date palm plant with variable solvents [29]

Extracts	DPPT				DPP K			
	Total phenolic (mgGAE /g)	Total flavonoid (mg QE/g)	IC_{50}µg/mL		Total phenolic(mg GAE/g)	Total flavonoids (mg QE/g)	IC_{50}	
			DPPH	βcarotene			DPPH	βcarotene
Hexane	5.40±0.87	4.67±0.96	ND	170.86 ±0.02	48.29± 2.81	8.85±0.83	ND	233.40 ±0.07
Chloroform	133.14± 6.53	7.69±1.13	ND	43.25± 0.07	53.53± 5.30	3.79±0.26	ND	46.30± 0.03
Ethyl acetate	100.36± 4.69	69.72± 3.76	67.50± 0.95	34.50± 0.03	31.93± 1.62	9.48±1.51	ND	41.23± 0.04
Acetone	213.36± 5.72	75.1±4.37	46.56± 0.28	28.18± 0.04	197.62 ±7.41	30.85± 1.98	ND	36.75± 0.03
Ethanol	211.11± 10.02	22.25± 2.86	144.86 ±0.54	ND	13.42± 0.95	4.29±0.31	ND	ND
Aqueous	237.74± 9.58	73.59± 5.62	534.37 ±0.05	60.75± 0.06	180.04 ±6.72	27.05± 1.84	ND	64.52± 0.05
Vitamin E	-	-	21.86± 0.57		-	-	21.86± 0.57	-
BHT	-	-	32.17± 0.42	36.82± 0.53	-	-	32.17± 0.40	36.82± 0.5

DPPT and DPPK, date palm pollen extract was collected from two regions in Tunisia (Tozeur and Kerkennah)

The data expressed as mean ±SD n=3 - Not tested

mg GAE/g= mg gallic acid equivalent per g dry plant extract ND; not detected

mg QE/g= mg of Quercetin equivalent per g of dry plant extract IC_{50}µg/mL= values corresponding to the amount of extract required to scavenge 50% of the radicals present in the reaction

Date was reported as well to have a large spectrum against virus and bacteria. Date was tested against 10 bacterial strains a. Gram positive bacteria like; *Bacillus cereus, Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus, Micrococcus luteus, Listeria monocytogenes,* Gram negative bacteria like *Salmonella enteritidis, Salmonella typhimiurium, Escherichia coli, Klebsiellla pneumoniae* against chloramphenicol as control and fungi like *Aspergillus niger* and *Penicillium* against Cyclodextrines as control. The results were reported in table1.17 to show large spectra and large inhibition zones against bacteria [2] [31]. This property is used to formulate

osmotic dehydration process to give osmotic dehydrated fruit which is stable and does not get rotten at 48°C for 3 months. The antimicrobial effect is due to the antioxidant content which affects the membrane permeability of the bacterial cell and hinders the enzyme entry or excretion by changing the chemical composition of the active constituents [47]

Table1.17. The inhibition zone sizes of gram positive and negative bacteria and fungi on adding date palm seed different extracts on [33]

strains	**Inhibition zone diameter in mm**						
extracts	Hexane	Chloroform,	Ethyl acetate	Acetone	Ethanol	Aqueous	Chloramphenicol control
DPPT Gram + *Staphylococcus aureus*	9.5±05	9±0	15.5±0.5	14.5±0.5	12±0.0	8±0.0	16.5±0.5
Gram – *Escherichia coli*	10±0.1	-	13.5±0.5	12±1.0	10±1.0	8±0.0	23.5±0.5
Fungi *Aspergillus niger*	-	-	-	-	-	-	17±1.0
DPPK Gram + *Staphylococcus aureus*	11.5±0.5	8.5±0.5	20±1.0	13±1.0	14±2.0	8±1.0	16.5±0.5
Gram – *Escherichia coli*	-	-	16±1.0	8±0.0	10.5±0.5	8±0.0	23.5±0.5
Fungi *Aspergillus niger*	-	-	-	-	-	-	17±1.0

The data are expressed as mean± S.D. (n=3).
(a)Diameter of inhibition zones of extract including diameter of well 6 mm. (b)No inhibition.

(c) Chloramphenicol was used as a standard antibiotic at a concentration of 15 lg/well. (D) Cycloheximide was used as a standard antibiotic at a concentration of 20 lg/well.

Other active constituents are also present like; fatty acids as oleic, lauric, myristic, palmitic, linoleic and stearic. Minerals are as potassium, magnesium, calcium and sodium. The recommended daily allowance (RDA); the recommended daily allowance of date daily is around 100 grams which would provide the body with its daily needs of 14% magnesium, iron, copper

and manganese, 7% of zinc and 4% of calcium. That is why the date seed powder can be used as additive in food to fortify the food [30].Date can be used as adsorbent to remove pesticides from aqueous solution (EL Bakouri *etal.* 2009) [45].Date seed fibers are used as diet supplement and important source to prevent certain disease conditions like; diabetes mellitus, obesity, cholesterolaemia, coronary heart diseases, hyperlipidemia, intestine disorder, prostate cancer and colorectal cancer (Johnson and Southgate, 1994, Kritchev sky 1988 and Tariq *etal.* 2000) [34] [48]. Date seeds increase spermatogenesis, testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Date pollen grains increase female fertility and reduce the side effects of some neurotransmitter brain drugs like gamma-Aminobutyric acid (GABA). It can be administered with a dose of 21.0-62.0 mg/gm.

Date pits are light to dark brown in color with slight bitterness [45]. Table1. 18 shows that pits are rich in fats, carbohydrates, minerals, protein, steroids, vitamins, phenols, crude fibers, they are source of activated charcoal and caffeine substitute. It is used as a metal chelator since it has the capability to chelate copper, cadmium and zinc ions by batch absorption process. It also has a hormonal effect which induces uterus contractions that was tested on rats which induces labor. Around 720,000 tons of date oils are produced per year. Its life span is one year. [46] [66]

Table1.18. Chemical composition of date seeds on dry matter basis (% db) [66]

Component	Content %db; dry matter basis
Ash	1.0±0.1
Crude protein	5.95±0.01
Hot water extract	10.5±0.1
Fat	10.3±0.01
Cellulose	23.9±0.1
Hemicellulose	26.8±0.1
Legnin	21.6±0.1
Minerals: potassium	26.68
Phosphorus	11.96
magnesium	7.25
calcium	2.54
chloride	1.86

Date pits can be used as a coffee alternative yet it is caffeine free and it is a good source for biofuel by hydro deoxygenation and carbonization as shown in Fig.1.17. [50]

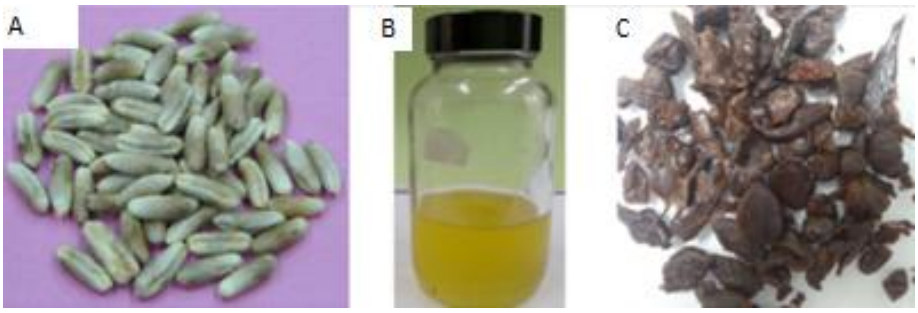

Fig.1.17. (a) dry date pits (b) pit oil extract (c) freeze dried water extract [48]

All the above listed health applications are at their very early stage and were applied using crude extracts. Seed or pit extract is usually prepared as aqueous or alcoholic extract [49]. From the pharmacological point of view it is better to use the crude extract rather than the single active compound due to: multi-target effect of the extract, synergistic effect and lack of knowledge of the extract active constituent of the plant [49]. Yet the hydrophilic extracts have very little antimicrobial action and organic extracts are much better [47]. Acetone, methanol, butanone or butanol extracts are the best choices to be prepared [47] [48]. Butanol and butanone are the best solvents to be used since they have high affinity for the molecules which contain hydroxyl group and highly economic (Peschel *etal*, 2006) [48]. However, the drawback of those organic solvents is that they cannot be applied to the human body which is why either aqueous or alcoholic extracts are always preferred.

Chapter 2

Materials & Methods

Chapter 2: Materials & Methods

2.1 Materials:

The plants used in this study are Ajwah and black date which belong to *Phoenix dactylifera* L. family Arecaceae. Ajwah and black date were purchased from the Egyptian local market. Chitosan of low molecular weight (89.9% degree of dealkylation and its viscosity is 65 cp) was purchased from (Primex ehf, Chitoclear, Iceland). Solvents used were; methanol 99.9% (Sphinx, Egypt) , acetic acid 100% (Burdick and Jackson, Germany) , acetone (Medical Pure Life, Europe) , absolute ethanol 99.9% (Alamia,Egypt) formic acid 85% (Adwic, Egypt) , tert. butanol 99+% (Sigma-Aldrich, USA), Dimethyl Sulfoxide(DMSO)99% (Chemajet,Egypt) , Folin-Ciocalteu reagent (Alpha Chemika, India) , Sodium tripolyphosphate (TPP) (Central Drug House CDH, India) , gallic acid anhydrous EMD (Millipore corporation,MS, USA) and 2,2-diphenyl-1-picryl hydrazyl (DPPH) (Aldrich, USA). Distilled water (DW) was used throughout the experiments.

2.2.1 Synthesis of Phoenix dactylifera L. Chitosan nanoparticles (NPs):

Date parts surface sterilization:

The Ajwah date/ black date were incised to the three main parts of the plant which are the seed, the fruit and the peel. The seeds were surface sterilized by maceration in DW for 15 minutes. Water was discarded then macerate in a mixture of DW: methanol in a ratio of 1:1 (v/v) for another 15 minutes. Finally the seeds were rewashed with DW for another 15 minutes. DW was discarded and the seeds were placed under microwaves for complete surface sterilization for few minutes. The previous work showed complete sterilization of microorganisms after exposure to microwave for 20 sec. Those seeds must be tightly sealed to avoid any further contaminations [67]

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Drying of the date plant parts:

According to previous published protocols [44][68][69] , it was reported that date plant parts were dried by oven, though most of the other plants when studied for their active constituents were dried by lyophilizer, unlike the work done by Madadlou *etal.* where the date palm seeds were sundried. [62]

The oven drying (OD) method was applied for the date parts for 72 hours at 60°C after full investigation resulting into similar results by using both oven drying and freeze drying (FD) which was done for 72 hrs at -60°C [68][69]. The following Fig. 2.1 and Fig .2.2 show the Ajwah and black date plant parts that were oven dried. In Madadlou *etal* protocol Kabkab variety from Tahran was manipulated as the plant of choice. [62]

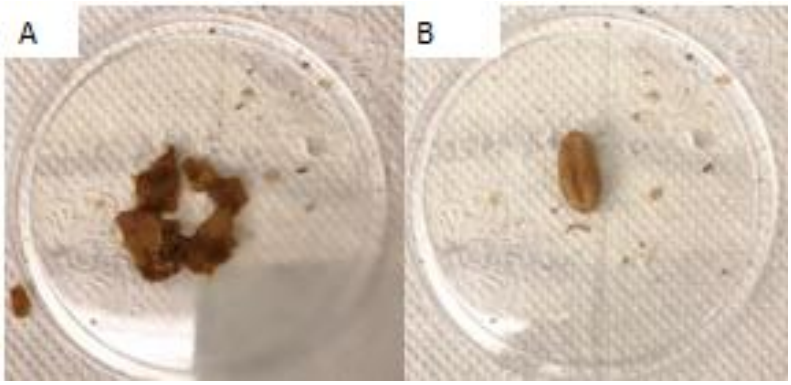

Fig.2.1 Phoenix dactylifera L. Variety Ajwah date plant parts oven dried (a) peel (b) seed

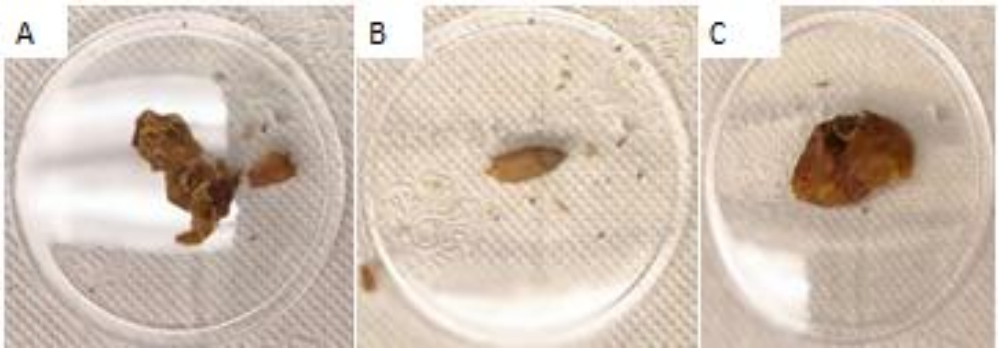

Fig.2.2 Phoenix dactylifera L. variety Ajwah date plant parts freeze dried (a) peel (b) seed (c) fruit

Preparation of the date extract:

The different parts of the date were extracted by various methods as described below,

Peel extract: it was prepared in hot water bath. 0.2 grams of the crushed peel was weighed and dissolved in 5 mL 52% ethanol and then left in rotating hot water bath at 42°C for 30 min. Similar experiment was done on grape peel [70].

Fruit extract: 0.2 grams powder of the dried fruit after crushing with the mortar and pestle was dissolved in either 100% ethanol or methanol. [71][72]

Pit or seed extract: according to previous work on date seed [73] [74] 0.1 grams powder was weighed in 10 mL mixed solvent (DW: 50% acetone in a ratio of 1:1(v/v)). Then the extract solution was placed on a magnetic stirrer for 1 hour at 450 rpm and 45°C followed by centrifugation at 1000 rpm for 10 min. Finally fractioning occurred at 60-70 °C to collect the acetone layer followed by the addition of butanol and then the butanol layer rich in polyphenolics was collected and the UV-Vis spectroscopy measurements for every layer collected was carried out[73][74].

Another method was suggested and was much better since it decreased the loss in the extract and was carried out by weighing 0.2 grams of the powder and dissolving it in 5 mL in any of the two solvents that were used; dimethyl sulfoxide (DMSO) [75] and a solvent mixture composed of DW : acetone: methanol: formic acid in a ratio of 20:40:40:0.1(v/v) [34]. DPPH and Folin Ciocalteu tests were applied to all of the prepared extracts to investigate the antioxidant and polyphenolic content in each extract [34][69][72][75][76].Fig.2.3 shows the shape and color of the extract after the date plant part was macerated in a solvent like DW.

Fig.2.3. Ajwah date seed extract in DW

2.2. **Date Characterization:**

Assay of polyphenolics:

A stock solution for gallic acid was prepared with concentration of 1 mg/mL followed by serial dilutions of 0.01, 0.03, 0.05, 0.07, 0.09 and 0.11 and then 2.5 mL of the Folin-Ciocalteu reagent was added and the solutions were left for 5 min followed by the addition of 2 mL of 7.5% sodium carbonate. The samples were kept in the dark for 120 min and then the absorbance at 765 nm was measured and plotted on the regression line of the stock gallic acid to get the equivalent concentration of every sample. After that the histogram which indicated each sample contained the amount in milligrams of gallic acid in its net weight was plotted [34][69][75][77].

Assay of antioxidant activity:

DPPH is an easy and rapid method for assaying the free radical scavenging activity. In this test, the ability of the plant extract to inhibit the oxidative cell damage attack of lipoproteins, poly unsaturated fatty acids was measured. The IC_{50} (which is the concentration required to scavenge the DPPH free radical by 50%) was calculated according to equation 2.1. The lower the IC_{50} values the greater is the antioxidant action.

Equation2.1. [34]

$IC_{50}\% = (1 - As/Ac) \times 100$

Where As is the absorbance of the sample and Ac is the absorbance of the control. The percentage of the antioxidant activity in every sample was calculated and then the histogram was plotted.

The antioxidant action of the polyphenols is pertained to the OH group which donates hydrogen to the free radicals and scavenges them. That is why the aglycones possess higher antioxidant property than the glycosides derivatives.

First, the DPPH was prepared as 0.01 M of the reagent which is equivalent to 0.00394 grams in 100 mL methanol. Then the control sample which is the reagent alone was prepared which contains 4 mL . Then, 0.05 gram of the powder was weighed followed by the addition of 2 mL of the reagent and left on a shaker for one hour. The blank used was methanol. Then the absorbance at wave length 517 nm was measured. The antioxidant activity was plotted and estimated from a graph where x-axis represents the sample and y-axis represents the IC_{50} values [77] .Table2.1 shows the samples prepared from different plant parts and varieties and the scheme of work is illustrated in Fig.2.4.

Table2.1. the Ajwah and Black date plant parts oven and freeze dries from three different solvents

Plant part	*Samples*	*Oven drying (OD)*			*Freeze drying (FD)*		
		Methanol (M)	*Ethanol 52%(E)*	*DMSO*	*Methanol (M)*	*Ethanol 52% (E)*	*DMSO*
Ajwah seed (AS)	*ASOD M*	√					
	ASOD E		√				
	ASOD D			√			
	ASFD M				√		
	ASFD E					√	
	ASFD D						√
Black date seed (BS)	*BSOD M*	√					
	BSOD E		√				
	BSOD D			√			
	BSFD M				√		
	BSFD E					√	
	BSFD D						√
Black date peel (P)	*POD M*	√					
	POD E		√				
	POD D			√			
	PFD M				√		
	PFD E					√	
	PFD D						√
Black date Fruit	*Fruit FDM*				√		
	Fruit FD E					√	
	Fruit FD D						√

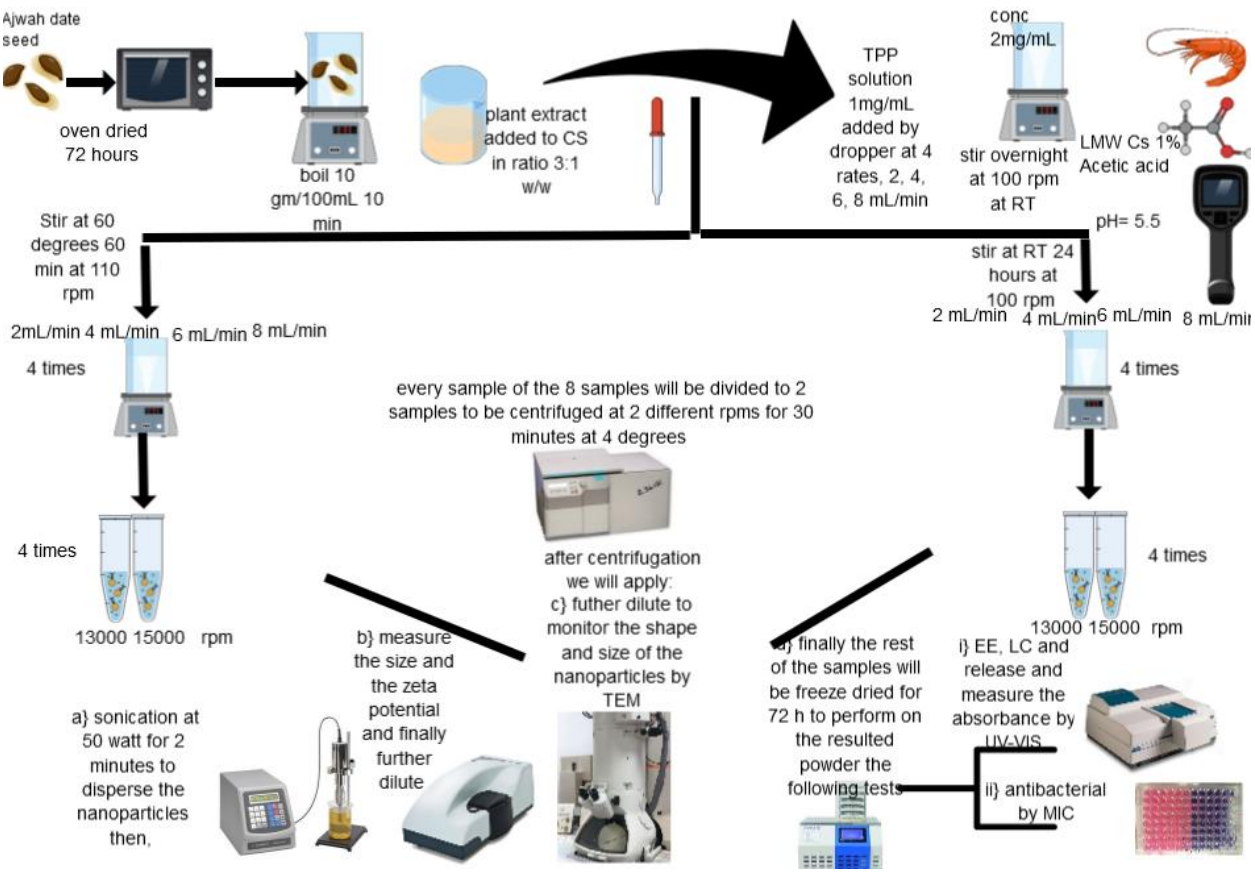

Fig.2.4. the Scheme of work

Nano particle formulation:

Chitosan NPs were prepared by ionotropic gelation technique. This technique is the optimum for the encapsulation of the extract [17][18][19][33][91]. The extract was encapsulated in chitosan polymeric NPs which were prepared first as follows; chitosan was dissolved in 1% (v/v) acetic acid with concentration 2 mg/mL. The chitosan prepared solution was placed on the magnetic stirrer for 24 hours at room temperature and at 100 rpm and the pH of the solution was adjusted at 5 by adding 1N Na OH or 1N HCl solution. The two plant extracts which were chosen to complete the protocol since they proved to be most successful were; ASOD and BSOD were prepared in DW (but not in 52% ethanol) as stock as reported by previous work[18] [137][140]. Samples were prepared with concentration 10 gm in 100 mL DW that was either left till boiling for 10 min or leave the plant parts to be macerated in DW for 72 hours. The prepared plant

extract was added to the chitosan solution in a ratio of 3:1 (w/w). The same preparation was done for ASOD and BSOD in the same ratios. Each of the two samples followed 2 different stirring cases; 2 preparations were done for each concentration; case one was to leave either the ASOD or the BSOD dissolved with the chitosan solution on the stirrer at 60 °C for 60 min at 110 rpm while case two was to be left at room temperature for 24 hrs at 100 rpm. After the elapsed time; either 60 min in case 1 or 24 hrs in case2, the sodium tri polyphosphate TPP, the linker or precipitating salt was added dropwise with 4 different volumes 2, 4, 6 and 8 mL respectively for the 4 sets of prepared NPs. Each one of those 4 beakers with variable TPP volumes were divided in to 2 centrifugation tubes for centrifugation at 2 speeds 13000 and 15000 rpm for 30 min at 4°C.The precipitates and pellets from our 32 samples were collected and dispersed in DW then sonicated at 50% watt, for 2 min and then the samples were diluted at concentration of 0.5 mL to 1 mL DW. Size, poly dispersion index (PDI) and the zeta potential were measured using the zeta sizer. The rest of the samples were lyophilized at -60°C for 72 hrs to perform the rest of the characterization tests. The following table 2.2 showed the samples prepared. This was different from the way nano preparation was formulated according to Madadlou *etal.* and Manal Awad *etal.*[62] [105] where in Madadlou *etal.* protocol the starch polymer which was dissolved in 37% HCl and cross-linker used was sodium hexa metaphosphate to prepare nanocrystals. Then, nano crystals (NCs) first prepared then loaded with the date pit extract which was dissolved in glacial acetic acid which causes tissue irritation in ingested and then suspended in the micro emulsion prepared from sunflower oil using surfactant Span 80. For separation of NCs prepared, centrifugation was applied at 5000 rpm for 5 min [62] In Manal Awad *etal.* protocol, no polymer was used on the contrary the date palm seed was crushed and turned to powder then dissolved in 20-50 mL of 38% hydrochloric acid (HCl). After that, the solution was stirred at 1000 rpm at temperature 30°C ±2. Finally the formed NPs were separated from the main solution by filtration using Millipore filter of size 220 nm and then centrifugation at 9000 rpm for 15min.[105]

Table2.2. Summary of Ajwah and black date samples prepared with the designation (protocol 1)

Sample	TPP rate ml/min 60°C 60min				TPP rate ml/min 24 hrs @ RT				Cent. RPM		plant variety	
	2	4	6	8	2	4	6	8	13000	15000	BSOD	ASOD
sample1												
sample2												
sample3												
sample4												
sample5												
sample6												
sample7												
sample8												
sample9												
sample10												
sample11												
sample12												
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sample27												
sample28												
sample29												
sample30												
sample31												
sample32												

2.3. **Characterization techniques of the prepared chitosan/extract NPs**

After applying the first protocol to choose the optimum conditions for NPs preparation, the effect of variation of the plant extract concentration was studied by the following characterization techniques as shown in table 2.3

Table2.3 Summary of the samples after chitosan/extract NPs preparation optimization (following protocol 2)

Sample (Chitosan: ASOD) (w/w)	Chitosan 2 (mg/mL)	Ajwah seed extract (mg)	TPP added (1 mg/mL)
1:0	**2 mg**	**0**	**6 mL**
1:1		**2**	
1:3		**6**	
1:5		**10**	
1:7		**14**	

2.3.1. Oven drying: TITANOX (Italy), was applied for 72 hrs on the dessicated plant parts; peel, seed and fruit in the oven at temperature 60°C.

2.3.2. Freeze drying*:* BIOBASE (Jinan, China), Bio-medical samples and heat labile samples were completely dried and remove the whole solvents. It has a CD touch screen, it can show data and export them on the display panel, it has a huge ice condenser without a coil inside. It has shelves for pre-freezing [95]. Freeze drying is a process where the water content of the substance is completely withdrawn from the material to turn it to a completely dry material. It is useful for those materials need to be stored for long period of time and yet they are thermos labile.

2.3.3. UV-VIS: CARY 500 SCAN Varian (Hi-tech, NJ, USA), It scans the sample at wave length range 200-400 nm for the ultraviolet range and 400-800 nm for the visible range. Samples would absorb the UV-VIS spectra depending on the chromophores they carried. Then the instrument examined spectra of photo initiators that started the reaction upon light absorption [99]. It performed qualitative and quantitative analyses for the sample. It was used to measure the intensity of the Ultra violet or visible light beam absorbed by the sample.

2.3.4. Probe sonicator Polytron (Thermo-fisher, USA): Ultrasonic apparatus is used to produce sub-micron or nano particulate matter. It consists of generator, ultrasonic converter (transducer), metal horn and probe. Generator produces electrical waves of certain frequency (normally 20–24 kHz) while ultrasonic transducers consist of piezoelectric (electrostrictive) material as quartz and ceramics such as barium titanate, lead titanate and lead zirconate titanate; which converts electrical oscillations into mechanical vibrations called acoustic waves of the similar frequency which transfer to the liquid carrying the particles need to be homogenized through direct immersion of a probe into liquid

2.3.5. Scannig electron microscope (SEM); Gemini Sigma USA, microscopic morphological analysis was done by model Gemini drops of nano particles. Aqueous solution was put on aluminium stub double sided tape and dried in oven for 2 or 3 minutes and then analyzed at excitation voltage (EHT) of 6.00 KV, signal A= in lens, magnification ranging from 20.00 to 40.00 K x, WD= 3.5 mm and aperture size= 60 µm after gold sputtering approximately 10 nm under argon atmosphere [33].

2.3.6. Transmission electron microscope (TEM); for size and morphology determination ; Using the apparatus TEM JEOL, USA, the samples were prepared after dilution to 0.02 mg/mL and then one drop was placed on a filter paper. The drop was left to dry after which the phosphotungesten dye was added which turned the chitosan more electrically conductive. The sample was prepared on copper holder and coated with carbon plate and then held by metal handle to be adjusted in the apparatus and begin measurement [91].

Measurement of Particle size and poly dispersion index (PDI): use photon correlation spectroscopy , Malvern UK nano ZS at 25 degrees and scattering angle 90 degrees after sample dilution to the concentration of 1 mg/mL DW and sonication at 50% watt. The sample was prepared 5 min before measurement [33].

2.3.7. Zeta-sizer Measurement Malvern UK zetasizer nano ZS, particle size and particle dispersion index (PDI): use photon correlation spectroscopy , at 25 degrees and scattering angle 90 degrees after sample dilution to the concentration of 1 mg/mL DW and sonication at 50% watt. The sample was prepared 5 min before measurement [33].

2.3.8. Fourier-Transform Infrared Spectroscopy (FTIR) Thermo Fisher Scientific Nicolet 380 Spectrophotometer (USA): the chemical composition of the date extract, chitosan and chitosan loaded plant extract NPs were mixed with KBr to form pellets, and the wave number ranged from 400-4000 cm^{-1} and resolution of $4cm^{-1}$ [33].

2.3.9. X-ray Diffraction (XRD): Bruker D8 (MS, USA), XRD patterns for Ajwah date seed powder, the chitosan powder, the unloaded cross linked chitosan NPs and the cross linked chitosan NPs loaded with the Ajwah seed extract were performed on the X ray diffractometer. The samples were irradiated with mono chromatized Cu Kα radiation 1.542°A. They were analyzed between 2 and 50 degrees angles; 2θ. The voltage and the current used were 30 KV and 30 mA respectively. The range and the chart speed were 2×10^3 cps and 10 mm/° 2θ respectively. [92] [93]

2.3.10. Centrifugator *HERMLE* Labotechnik 36 HK (Germany) [103][104]

2.4. Encapsulation efficiency (EE%) and Loading Capacity (LC%):

Encapsulation efficiency and loading capacity were studied in terms of total phenolics and antioxidant content; flavones content in the sample [4][15][17][18][19][77][78][79][80][81][82].

First the reference regression line was plotted for our plant extract. Serial dilution for our extract in DW was prepared from 50-350mg/mL using DW as the blank. Regression line was plotted where the X axis represented the concentration mg/mL and the Y axis represented the absorbance and calculate the coefficient of determination (r^2) factor was calculated. In total, 5 different samples of the prepared chitosan NPs, were under investigation; one carried no plant extract in it; pure chitosan NPs 100 mg of concentration (2 mg/mL in 50 mL 1% v/v acetic acid) . The remaining four samples were loaded with the plant extract with ratio of plant extract: chitosan 1:1, 3:1, 5:1 and 7:1 w/w. The freeze dried samples were weighed to reach a concentration of 20 mg dissolved in 10 mL DW [45] [46]. Trials were applied on the samples at variable rpms; 20000 and 15000 for 30 min, 60 min and 90 min at either room temperature or at 40°C. Finally samples were stirred on the magnetic stirrer for 24 hours at 500 rpm at room temperature or 40°C or avoid stirring and directly were measured by UV-Vis spectroscopy [44][45][46][47] . The absorbance of the extract in the supernatant was measured by UV-Vis

spectroscopy at the wavelength which was concluded from the graph of the serial dilutions and then was substituted in the following equations (2.2 and 2.3) to calculate the EE% and LC%.

Equation2.2. [15][19][77]

LC%= [(total amount of extract loaded – free amount of the drug extract) × 100] / (extract – nanoparticles dry weight)

Equation2.3. [15][19][77]

EE%= [(total amount of extract added – free amount of extract) ×100] / total extract added or

= TPC encapsulated in the NPs / TPC in the plant extract

2.5. *In-vitro* release kinetics:

After encapsulation of the plant extract within the chitosan NPs, the activity of the plant extract should remain the same or get enhanced. That is why the following tests were done [17][19][20][76][78]. A known weight of the NPs of the selected 4 samples; Chitosan:AS; 1:1, 1:3, 1:5, and 1:7 (w/w) was weighed , 40 mg in 5000 µL= 20mL of the media under test; either PBS (pH=7.4) or HCL (pH=1.2) . Those 2 experiments of 2 different human body simulation media were performed in dialysis membrane of dimensions 16 mm diameter and 5 mm length MW 12000-14000 and pore diameter ca 25 °A and suspended in 2 different media; (a.) 20 mL of 0.1 N HCl at PH= 1.2 for gastric media simulation and the other media was (b.) 20 mL of PBS buffer solution at pH 7.4 for intestinal fluid simulation. Aliquot samples were incubated at body temperature 37°C ± 0.5 at 400-600 rpm in Statim shaker bath and at certain time intervals aliquot samples of 1mL were withdrawn and analyzed which were replaced by fresh media to attain constant volume every time. The TPC released was analyzed and measured in the supernatant by UV-Vis spectroscopy measurement. [94]

2.6. Stability test of total phenolic content (TPC) in the prepared chitosan NPs:

According to what was reported by previous protocols [6][17][20][77][80] stability of the plant extract alone was examined and compared to the chitosan NPs encapsulating the Ajwah date plant extract. It was concluded that the lyophilized NPs were stable at 4°C and RH of 32%. Depending on the results of the EE% , LC% and release profile (R) , the previous mentioned 5 NPs (1;0, 1:1, 1:3, 1:5, 1:7 (w/w)) were prepared for the chitosan NPs loaded with the plant extract and tested at concentration of 2 mg/mL; similar to the concentration manipulated in the EE% , LC% and R with the Folin-Ciocalteau reagent and the time at which the maximum poly phenolic content was released was observed. [6][17][20][77][80]

2.7. Antioxidant activity:

The concentration of each prepared NPs sample which gives the highest antioxidant activity (AO) compared to gallic acid serial dilutions was required. Various concentrations of each of the 5 samples (1:0, 1:1, 1:3, 1:5 1:7 (w/w)) mentioned in Table2.4 were prepared to show the accurate weight at which AO was the maximum possible value. [3][14][15][25][26]

Table2.4. Summary of the samples of prepared NPs for antioxidant assay

S serial	Chitosan : plant: TPP (w/w/w)	Total weight of NP (mg)
1:0/ 5	2: 0 : 6	5
1:0/10		10
1:0/20		20
1:0/40		40
1:0/50		50
1:1/5	2 :2 :6	5
1:1/10		10
1:1/20		20
1:1/40		40
1:1/50		50
1:3/5	2: 6: 6	5
1:3/10		10
1:3/20		20
1:3/40		40
1:3/50		50
1:5/5	2: 10 :6	5
1:5/10		10
1:5/20		20
1:5/40		40
1:5/50		50
1:7/5	2 :14 : 6	5
1:7/10		10
1:7/20		20
1:7/40		40
1:7/50		50

2.8. Antibacterial assay:

Bacterial assay was determined by two techniques, (a) MIC minimum inhibitory concentration or minimum bactericidal concentration (MBC) to determine at which concentration the NPS had potent antibacterial effect ; the blank used in all experiments was the broth medium only. Reference sample for the bactericidal test was the broth medium inoculated with potent antibacterial Doxycycline®. Positive control samples were just the media with the bacteria strains under test. The samples were prepared in concentration 2 mg/mL as mentioned in table

2.3. Gram positive bacteria *Staphylococcus aureus*, Gram negative bacteria *Escherichia coli* were the two bacterial strains under test.

All tubes were prepared then left for 24 hours for incubation at 37°C. The tubes which showed the least amount of turbidity and visible growth were considered bactericidal at this concentration. [115][121]

(b) the Log reduction method was used in which , at first stock cultures were prepared by; inoculating the two strains under test as mentioned above at 37°C for 48 hours on Oxoid basing stoke media UK; Tryptone Soy Agar Broth Media (TSB) media till reaching the mid log phase. Then after stock preparation they were kept frozen at temperature under 80°C for further use. An aliquot was taken from those frozen samples of the two bacterial strains and inoculated on the phosphate buffer saline solution (PBS) at pH 7.4 at concentration 1×10^6 CFU/ mL. Samples were prepared by inoculating the bacteria under test at volume of 10µL (concentration of 1×10^6CFU) per 90 µL of the PBS for every strain, then addition the chitosan NPs loaded with the AS extract under test with concentration of 200/100 µg/µL of the media prepared. Control samples were prepared at concentration of 100µL of the bacterial stain in the buffer media without adding any antibacterial drugs. After all samples were prepared, they were incubated for 4 days at 37°C. At 24 hour time intervals samples were withdrawn and inoculated on plates of TSB media to count t(he colony forming unit (CFU) and expressed as Log_{10} CFU/mL . [6][17][18][26][68][84][85] [116]

2.9. Statistical study:

All experiments were done in triplicates. The statistical analysis was performed by ANOVA method of analysis and student T test and results were expressed as mean ±SD. $P<0.05$ was considered as a significant difference between results. [14][21][29] [87] [88] [89] [90].

2.10. Theoretical back ground:

a. Lyophilizer or freeze dryer:

Freeze drying or Lyophilization is a process where the water content of the substance is completely withdrawn from the material to turn it to a completely dry material. It is useful for those materials need to be stored for long period of time and yet they are thermos labile. It is accomplished in two processes starting with sublimation where water in the ice solid form turns

directly to vapor or gas state under vacuum and high temperature not to be below the triple point of water , at 4.579 mm of Hg and 0.0099°C. Fig.2.5 shows the phase diagram illustrating the triple point graph of water. The concentration gradient of water vapor between the drying front and condenser is the driving force for removal of water during lyophilization process. The vapor pressure of water increases with an increase in the temperature and turns to ice under conduction or radiation but yet not above the critical temperature otherwise a collapse in the main substance solid structure would occur. After the first drying step a second drying step is performed where severe cooling occurs which let the ice pieces separate apart from the solute or the thermos labile material that need to be dried. [96]

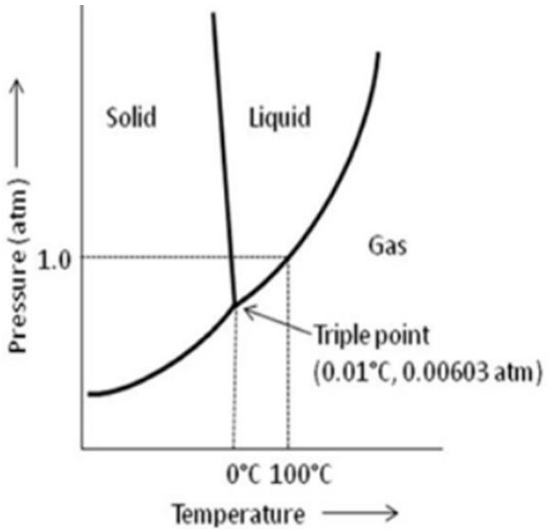

Fig.2.5. Phase diagram showing the triple point of water at 0.01°C, 0.00603 atmosphere [96]

Annealing is the second part which is an optional step in the freeze drying process. Annealing is a process targeting the solute and not the water. It is a process to crystallize the outcome solute which depends on the nature of the solute. If it is crystalline so this temperature is called the eutectic temperature and if it is amorphous then it is called the glass transition temperature. Fig. 2.6 showed the summary of the lyophilization process with an image of the apparatus itself used in this study. [96]

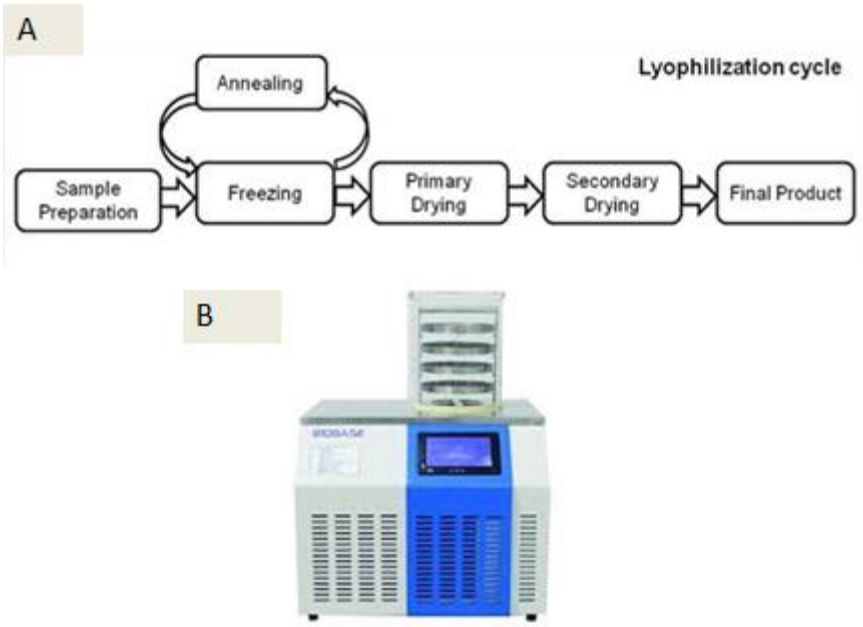

Fig2.6. (a) Lyophilization process [96] (b) lyophilizer [98]

b. UV-Vis Spectroscopy:

In order to characterize the chemical characteristics of the samples, UV-Vis spectroscopy is used. It scans the sample absorption of radiation at wave length range 200-400 nm for the UV range and 400-800 nm for the visible range. Samples absorb the UV-Vis spectra depend on the chromophores they carry. Then the instrument examines spectra of photo initiators that start the reaction upon light absorption [99]. Moreover, it performs qualitative and quantitative analyses of the absorption behavior of the sample. It is used to measure the intensity of the UV or visible light beam absorbed by the sample by applying Beer Lambert's law in the measurements and calculations as shown in equations 2.4, 2.5 and 2.6. When an absorbing substance is put in a transparent cell and a beam of light is passing through it then the intensity of light emerging out will be less than the incident one.

Beer–Lambert's Law:

Equation2.4. [98]

$$A=abc$$

Equation2.5. [98]

A= A 1%/1cm bc

A= absorbance or optical density

a= absorptivity or extinction coefficient

b= path length of radiation through sample (cm)

c= concentration of solute in solution.

Both a and b are constants so (A) is directly proportional to the concentration (c) gm/100mL

When a substance is measured quantitatively a calibration curve will be plotted for the absorbance on the y-axis and concentration on the x-axis. The absorbance at certain wave length is measured. The concentration of the substances in the sample is calculated from the proportional relationship that exists between absorbance and concentration as mentioned in equation2.6.

Equation.2.6. **[98]**

$C_{test} = (A_{test} \times C_{standard})/A_{standard}$

where C_{test} and C_{std} are the concentration of the sample and standard blank solutions respectively and A_{test} and A_{std} are the absorbance of the sample and standard blank solutions respectively.[98] Fig.2.7. illustrates the mechanism of action of the UV-Vis spectroscopy and an image of the apparatus itself used in this study.

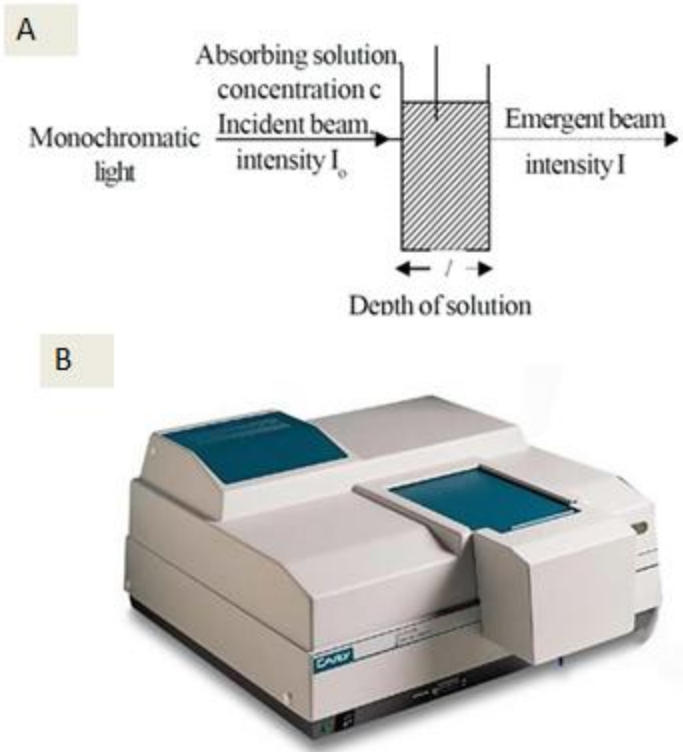

Fig.2.7. (a) mechanism of action of the UV-VIS spectrophotometer [98] (b) UV-VIS: CARY 500 SCAN Varian spectrophotometer [99]

c. FTIR Spectroscopy

In order to determine the chemical composition of the samples, FTIR spectroscopy is the most commonly used technique. Samples were prepared by mixing with lyophilized KBr pellets to attain the fingerprint spectrum for every material for precise identification [99].

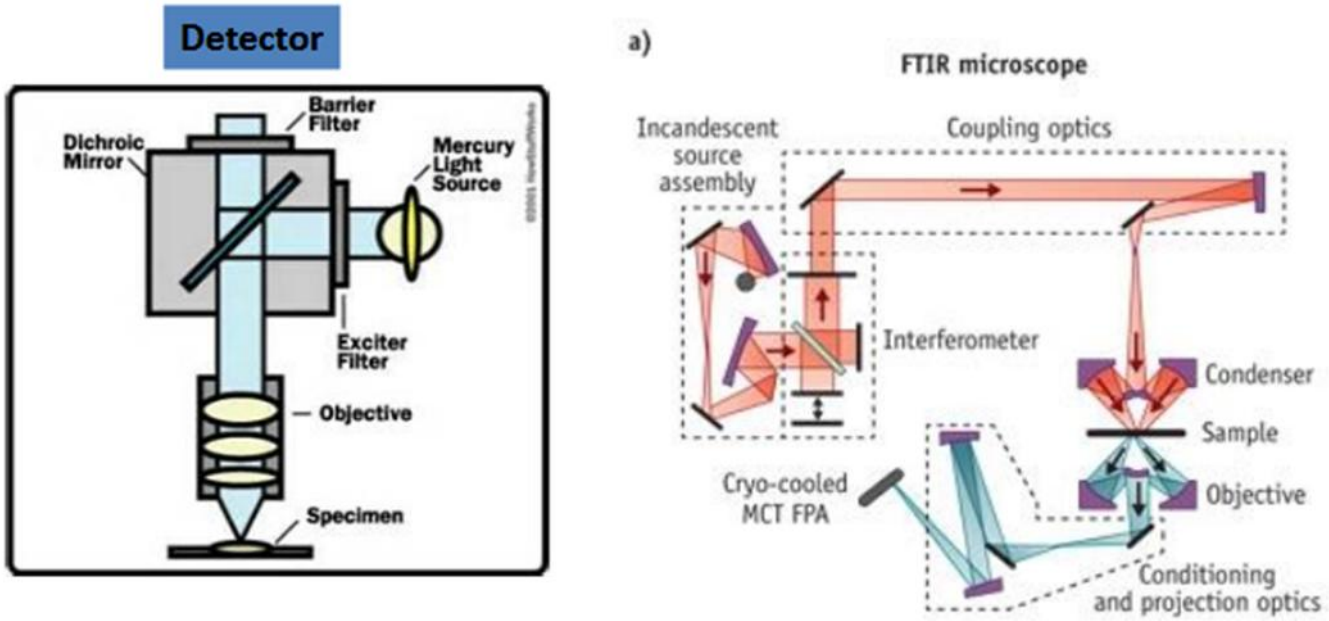

Fig.2.8. FTIR spectrophotometer [100]

It is a crucial apparatus to scan molecules especially organic compounds and it is used to search for certain functional group or to make a complete scan of the compound to detect the presence of any foreign compounds. Fig.2.8. and Fig.2.9. show a basic design of the FTIR principle.

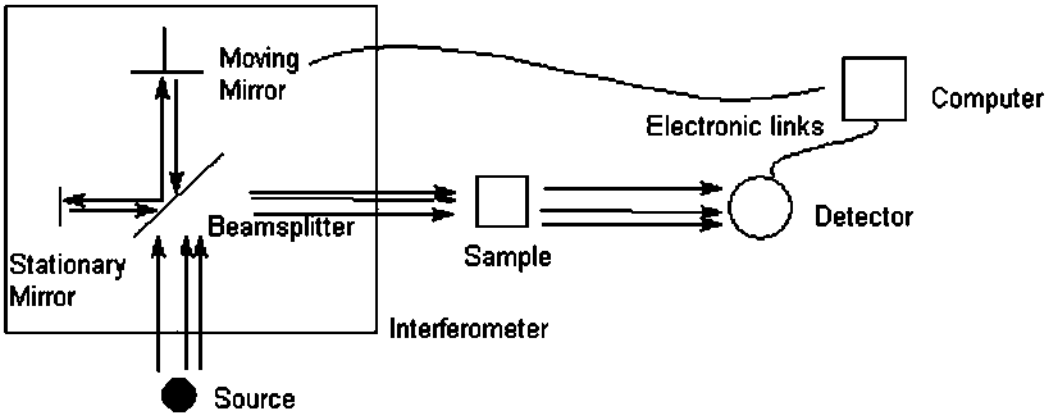

Fig.2.9. Principle of FTIR [101]

As shown in both Fig.2.8. and Fig.2.9., there is a source of multi-chromatic beam, a sample and a detector similar to the UV-Vis spectrophotometer. All the source energy is sent through a Michelson interferometer and onto the sample. The interferometer differs from the mono-chromator where the light passes through a beam splitter, which sends the light in two directions

at right angles. One beam goes to a stationary mirror and then back to the beam splitter while the other goes to a moving mirror. The motion of the mirror makes the total path length variable versus that taken by the stationary-mirror beam. When the two beams meet up again at the beam splitter, they recombine, but the difference in path lengths creates constructive and destructive interferences which make the measurement by the FTIR apparatus to be more precise and with less noise ratio compared to the conventional IR apparatus. The recombined beam passes through the sample and the sample absorbs all the different wavelengths characteristic or finger print of its spectrum, and this subtracts specific wavelengths from the interferogram. The detector reports the variation in energy versus time for all wavelengths simultaneously. Then a graph of wave number as x-axis versus the % Transmittance as the y-axis is plotted as shown in figure2.10 and according to the equation2.7:

Equation2.7. [100]

$$A(r) = X(k) \exp(-2\pi\, trK/N)$$

Where A (r) and X (k) are the frequency domain and time domain points, respectively, for a spectrum of N points. Fig.2.11. shows an image of the entire apparatus used in this study. [100]

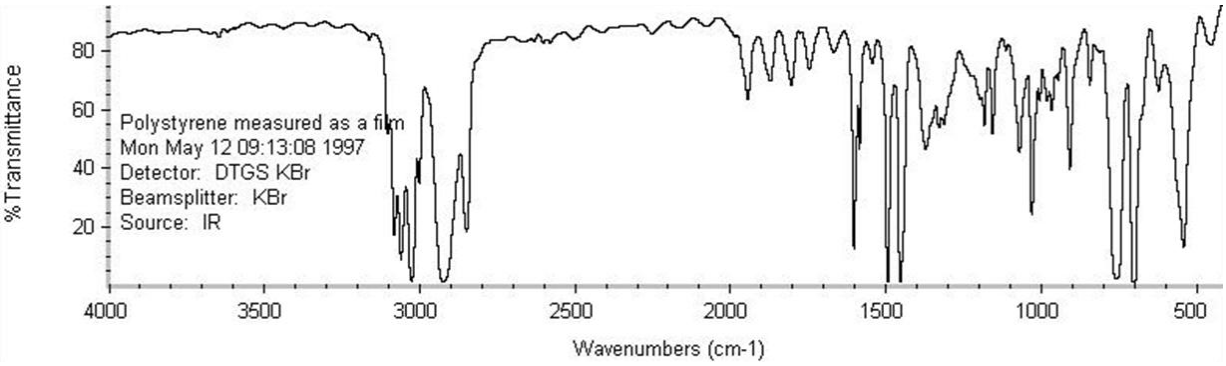

Fig.2.10. FTIR graph sample[101]

Fig.2.11. FTIR apparatus[102]

d. Zeta sizer and zeta potential Malvern;

In order to measure the size and surface potential of colloidal mono and poly-dispersed NPs and molecules without agitation to make the sample suitable for analysis, zeta-sizer is the most world-wide used apparatus for that purpose. It measures NPs size range between 0.3 nm to 10µm [106]. It can be connected to a chromatographic detector and is operated by applying the principle of dynamic light scattering (DLS) as shown in Fig.2.12.

The principle of DLS is that fine, micro and NPs suspensions are in constant random Brownian motion, diffuse at a speed related to their size, in which smaller particles diffuse faster than larger ones. The speed of Brownian motion is also affected by the surrounding temperature, therefore precision temperature control is essential for accurate size measurement. To measure the diffusion speed, the scattering intensity of light was used to illuminate the particles at a specific angle will fluctuate with time, and this is detected using a sensitive avalanche photodiode detector (APD). The intensity changes are analyzed to give the size and the size distribution.

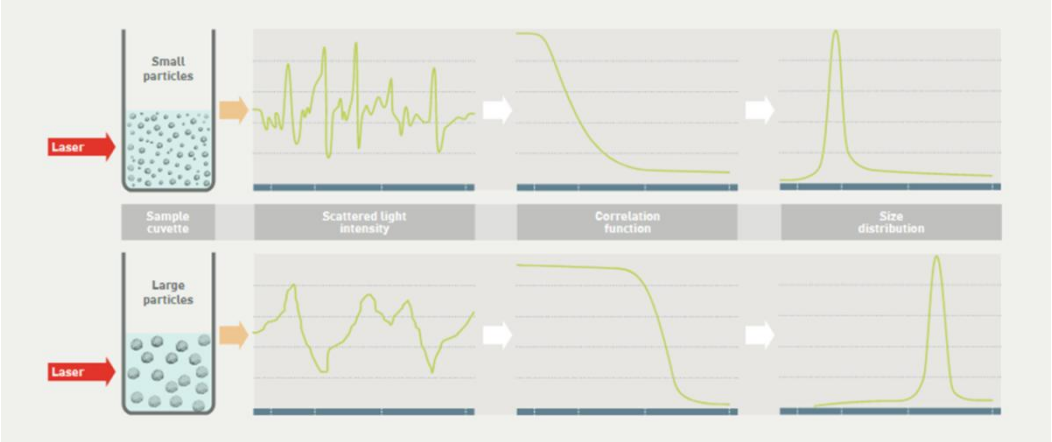

Fig.2.12. The mechanism of DLS differential light scattering to determine the size of the particles [106]

In addition to measuring the particle size, the charge acquired by a particle or molecule in a given medium is known as its zeta potential and arises from the surface charge and the concentration and types of ions in the solution. Since particles of similar charge will repel each other, resist flocculation and aggregation for longer periods making such samples more stable. This means that the stability can be modified by altering the pH of the solution, the ionic concentration, the type of ions and by using additives such as salt linkers, surfactants and polyelectrolytes. Knowledge of the zeta potential of particles in a formulation can be used to make logical choices to select the most appropriate materials to guarantee stability and prolonged shelf life. Particle surfaces in contact with a liquid contains ions can have a zeta potential in the same way as dispersed particles and molecules. The charge or zeta potential of particles and molecules is determined by measuring their velocity while they are moving by electrophoresis. Particles and molecules that have a zeta potential will migrate towards an electrode if a field is applied. The velocity by which they move is proportional to the field strength and their zeta potential. If the field strength value is known, the speed of movement, using laser Doppler electrophoresis will be measured, and then application of established theories to calculate the zeta potential is performed. The patented M3-PALS multi-frequency phase analysis light scattering measurement determines the mean and distribution during the same measurement. The

whole measurement procedure is automated to simplify the measurement process as shown in Fig.2.13. and an image of the apparatus used in this study is shown in Fig.2.14. [107]

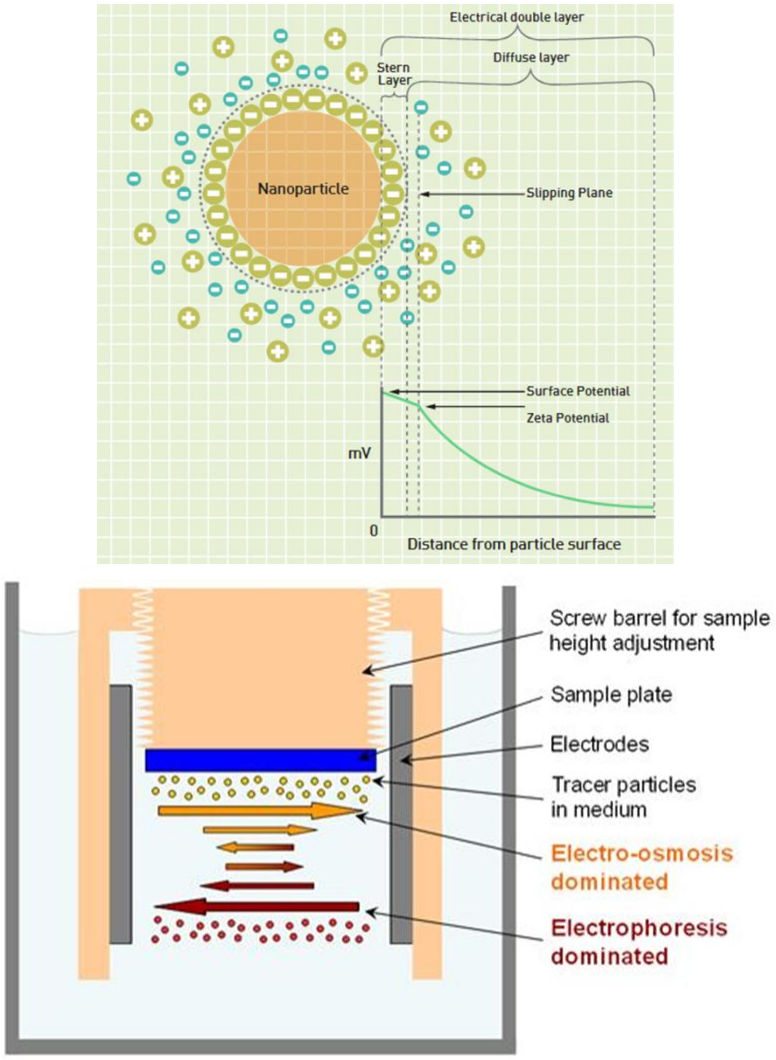

Fig2.13. the mechanism of measurement of zeta potential and surface charge and plotting the curve [107]

Fig2.14. (a) Zeta sizer and zeta potential Malvern apparatus (b) potential cuvette [108][109]

e. Probe sonicator:

Ultrasonic apparatus is used to produce sub-micron or nano particulate matter. It consists of generator, ultrasonic converter (transducer), metal horn and a probe. The generator produces electrical waves of certain frequency (normally 20–24 kHz) while the ultrasonic transducers consist of piezoelectric (electrostrictive) material as quartz and ceramics such as barium titanate, lead titanate and lead zirconate titanate; which converts electrical oscillations into mechanical vibrations called acoustic waves of the similar frequency which are transferred to the liquid carring the particles that need to be homogenized through direct immersion of a probe into the liquid. Ultrasound waves are in the range of 20–100 kHz of frequency. When those frequencies interact with matter in the form of a smooth planar surface vibrates with certain frequency and amplitude, longitudinal waves are generated and propagate into the surrounding liquid medium. These waves induce series of compressions and rarefactions amplified with the help of metal horn leading to acoustic cavitation phenomena. The generated sound waves dissipate part of the acoustical energy into heat energy. The apparatus work according to the equation.2.9.;

Equation2.9 [110]

$P_a = P_{amax} (2\pi ft)$

Where these sinusoidal waves exert a pressure (P_a) which is known as acoustic pressure, on the liquid medium and are dependent on the time (t), frequency (f) and the maximum pressure amplitude (P_{amax}) of the wave. P_{amax} of the wave in equation 2.9.is directly proportional to the power input of the transducer, acoustic streaming is the predominant phenomena at lower amplitude while acoustic cavitation is initiated at higher amplitudes. Summary of the process is shown in Fig.2. 15. and an image of the apparatus used in this study in Fig.2.16. [110]

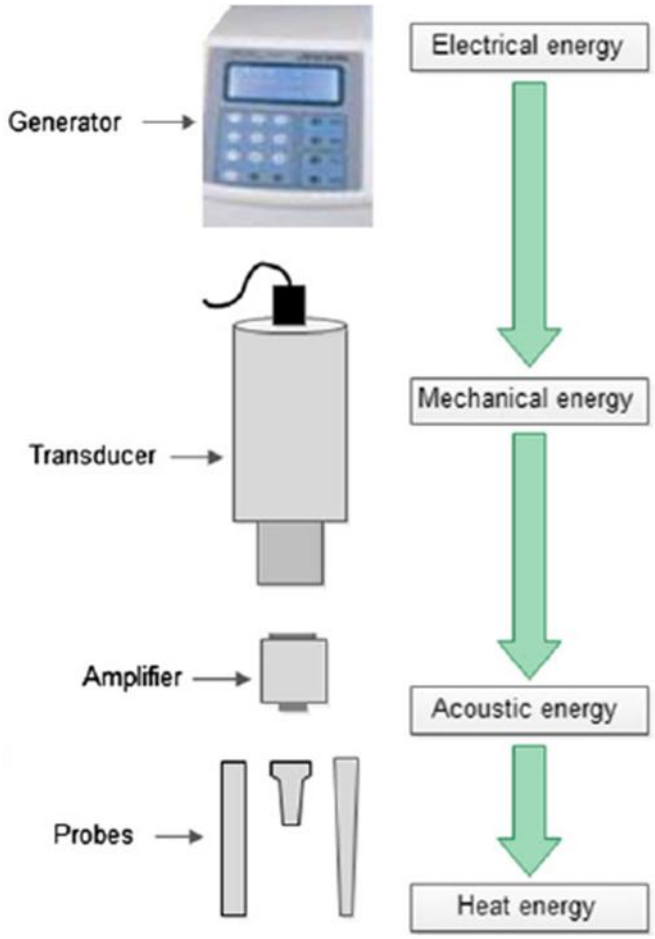

Fig2.15. the summary of the principle of probe sonicator [110]

Fig2.16. Probe sonicator [111]

f. SEM:

A scheme of the SEM principle of work and a diagram showing the path of the primary and secondary electrons inside the SEM for 3D image formation are shown in Fig.2.17 and Fig.2.18. The accelerated primary electrons under effect of grid and anode activations from the ejector gun are emitted. Those primary electrons strike the sample using the condensing lens and coil, it produces lower energy secondary electrons known as Auger electrons. Typical Auger electron energies are in the range of a few hundred eV to a few thousand eV. The secondary electrons or Auger electrons are collected by a positive charged electron detector which in turn gives a 3-D image of the sample. Those secondary electrons when emitted from the sample leave holes in their places when those holes and electrons bind they will emit light and form cathode luminescence. The collection of secondary electrons is very difficult and hence a high voltage is applied to the collector. These collected electrons produce scintillations on to the photo multiplier tube which are then converted into electrical signals. These signals scan the sample from left to right and the whole picture of the sample is obtained in the cathode ray oscilloscope (CRO) screen. [112]

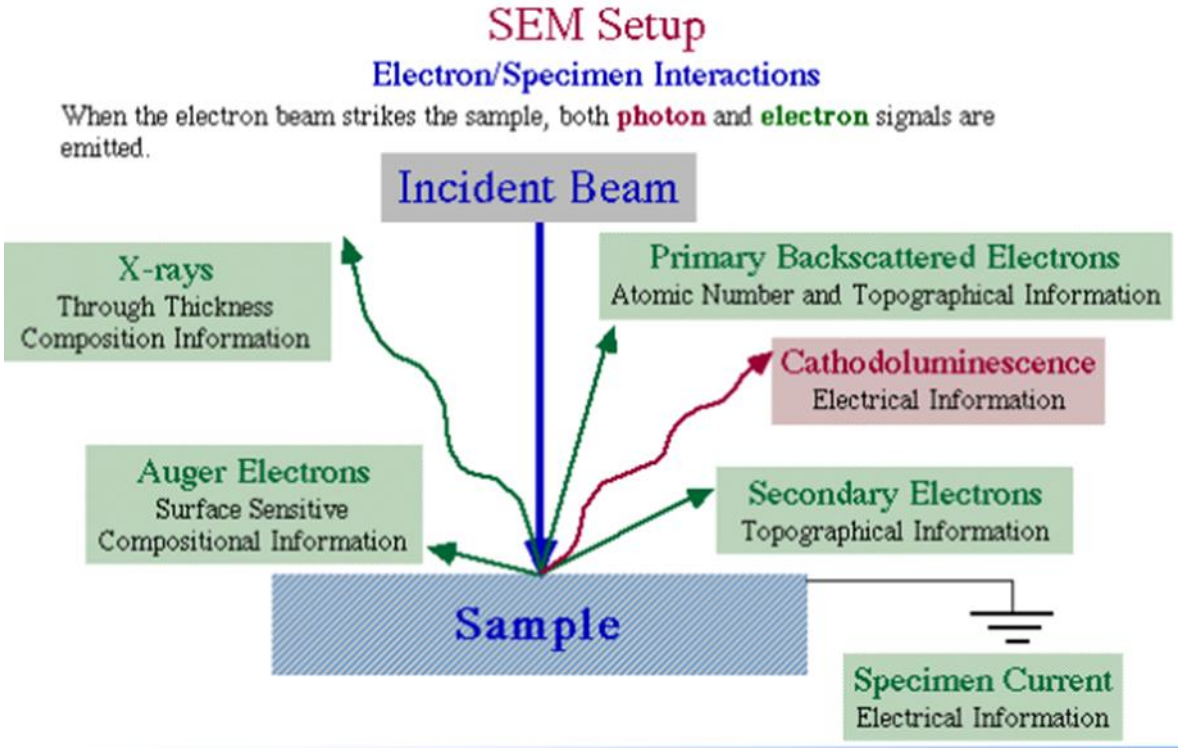

Fig.2.17. A scheme of the SEM principle of work [113]

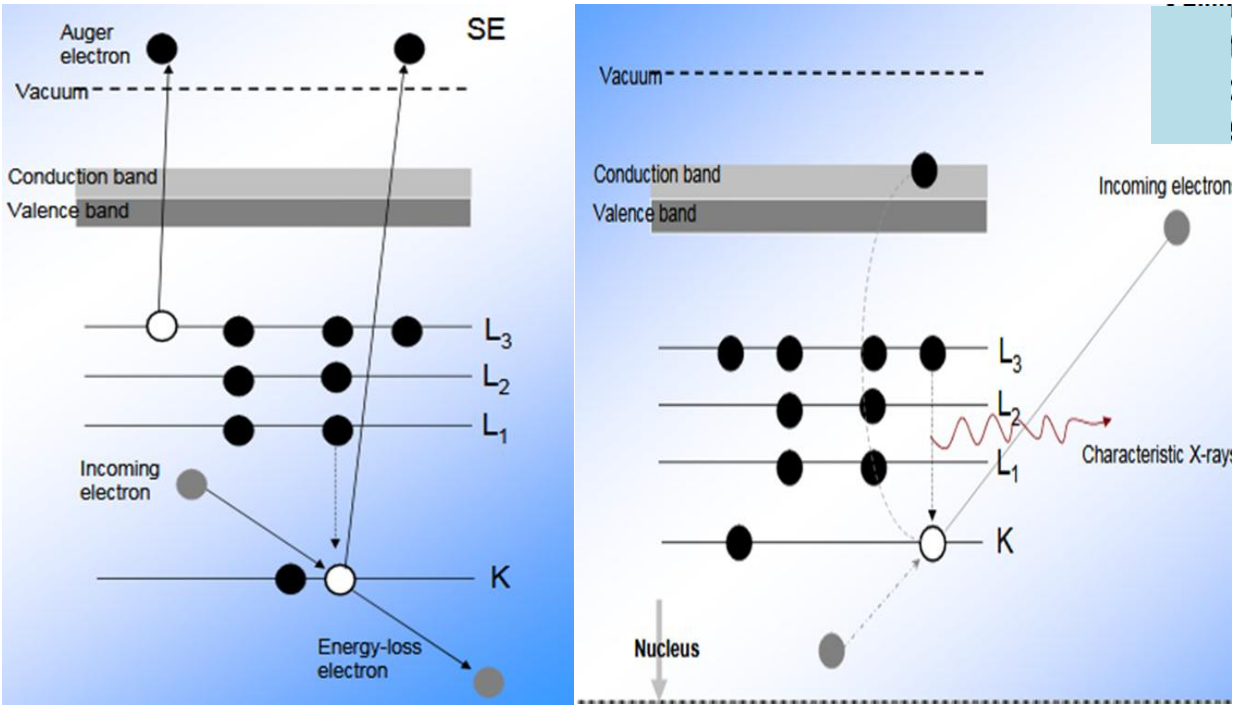

Fig.2.18. A diagram showing the path of the primary and secondary electrons inside the SEM for 3D image formation [113]

g. TEM:

The development of TEM is based on the quantum mechanical behavior of electrons. The interaction of electrons with the material was obtained due to the inherent nature of electron which is a quantum mechanical object; meaning that electrons have both wave and particle dual nature according to de Broglie wavelength principle.

De Broglie stated that nature did not single out light as being the only matter which exhibits a wave-particle duality. He proposed that any ordinary ``particles" such as electrons, protons, or bowling balls could also exhibit wave characteristics in certain circumstances according to equation 2.10. [106]

Equation2.10. [112]

$\lambda = h/mU$

where λ is the wavelength, m is the mass, U is the velocity and h is Plank's constant

TEM forms a major analysis method in a range of scientific fields, it scans particles in size range of tens of thousands nm. Fig.2.19. shows a schematic diagram of a TEM apparatus. TEMs works by shining a beam of electrons (like the light in a slide projector) through the specimen (like the slide). The transmission of electron beam is highly dependent on the properties of material being examined. For example, porous material allows more electrons to pass through while dense material allows less electrons passage. As a result, a specimen with a non-uniform density can be examined by this technique. The X-rays emitted are detected by a semi-conductor and processed on a phosphorescent screen by a detector protected by an ultrathin window and cooled at liquid nitrogen temperature to avoid the thermal noise and the diffusion of the dopant in the semi-conductor. An energy dispersive X-ray spectrum (EDS) is constituted by a background produced by the Bremsstrahlung X-rays and by peaks characteristic to the chemical elements of the material. [112]

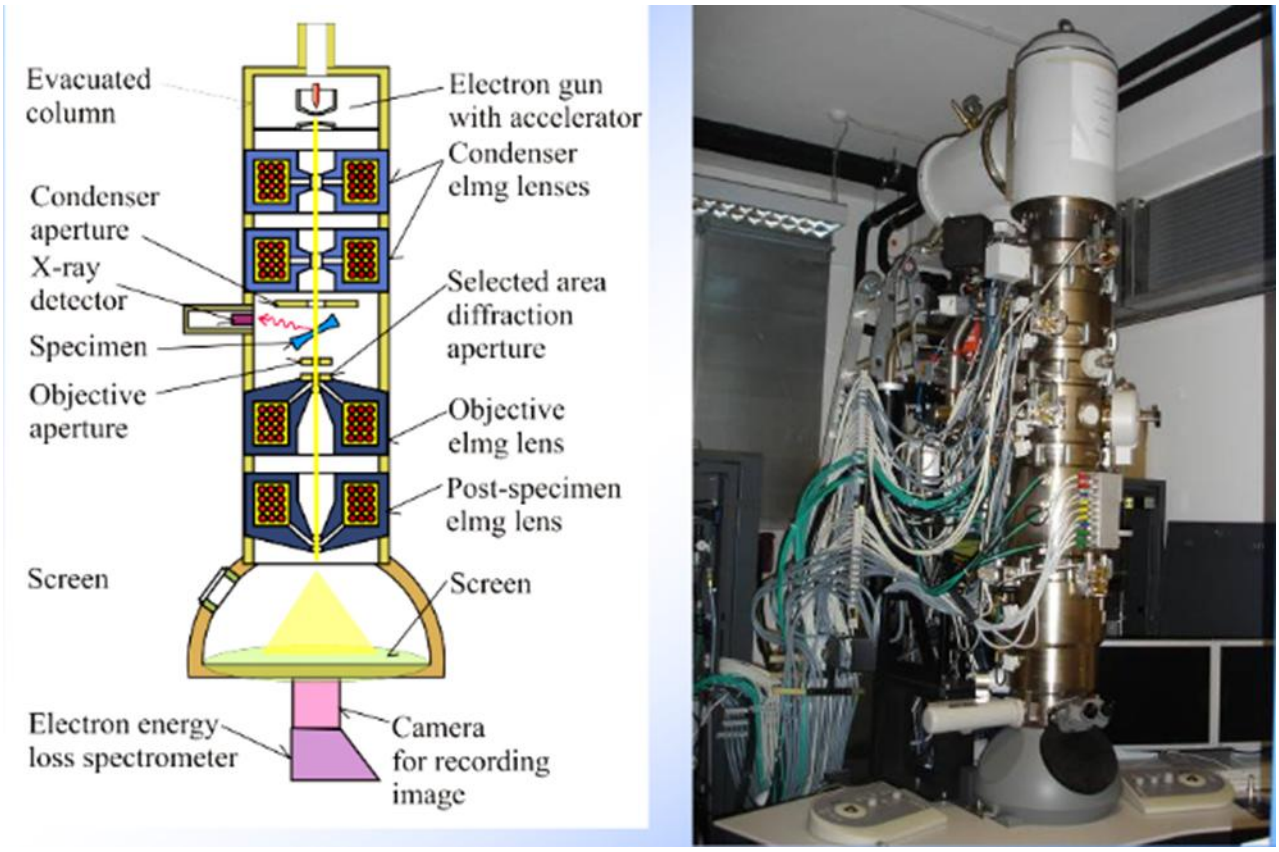

Fig.2.19. Schematic diagram of TEM apparatus [113]

h. XRD:

When the X rays fall on the atom and get emitted due to the atomic planes the x rays get out interfere with one another and lead to X ray diffraction. Crystals should be homogenous and their size does not exceed 10 mm otherwise results will be of variable peaks intensity. Examples of diffraction peaks are shown in Fig.2.20 where air has no peaks at all, mono crystalline material has one set of peaks and if sample composed of more than one component, many diffraction peaks will be monitored. [115]

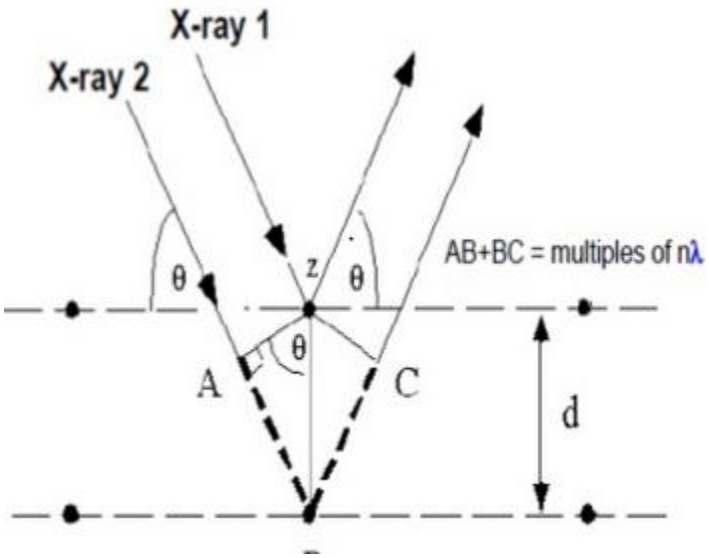

Fig.2.20.The X ray interferences between the incident rays and the reflected ones [115]

Bragg's law explains the X ray interferences when scattered by the crystal material according to equation.2.11.

Equation2.11. [115]

$n\lambda = 2d_{hkl} \sin\theta$

where n is an integer, ƛ is the wave length of the incident x-ray and d is the spacing distance between the planes. Fig.2.21 shows XRD pattern of air and an example of XRD pattern of ZrO2 NPs [115] and Fig.2.22 shows an image of the X-ray Diffractometer used in this study.

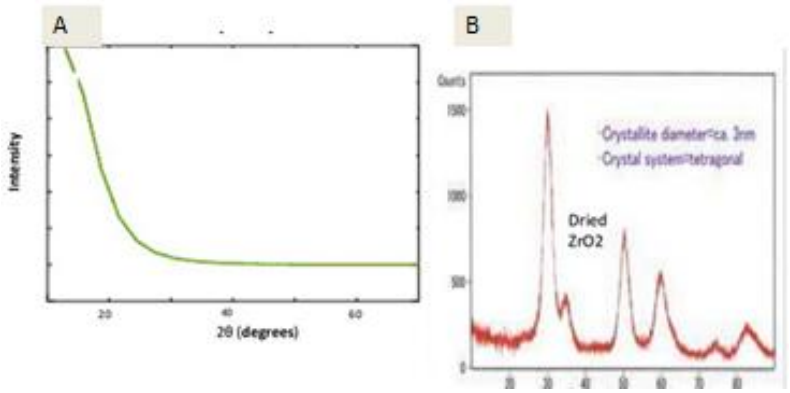

Fig.2.21. (a) XRD pattern of air (b) XRD pattern of ZrO_2 NPs[115]

Fig.2.22. X-ray Diffractometer [116]

i. Ionic gelation technique to prepare NPs:

Polyelectrolyte complexation is considered to be one of the best methods to prepare chitosan NPs. It requires the interaction of an ionic polymer with opposite charged ions to initiate cross-linking. In the simple monomeric ions, the interaction of poly-anions with poly-cations cannot be completely explained by the electro neutrality principle. Ionotropic gelation technique is mainly used to generate the 3-D hydrogels NPs and is divided into two methods, external and internal ionotropic gelation. In external ionotropic gelation method, the cross-linker ion is positioned externally. The advantages of external cross-linking is the production of thinner films with smoother surface, greater matrix strength , stiffness and permeability, thus produces micro or nano pellets capable of greater drug encapsulation efficiency and slower drug release rate. On

the other hand, in internal ionotropic gelation method, the cross-linker ion is incorporated within the polymer solution in active form. Fig.2.23 shows the different ionotropic gelation techniques.

There is a wide range of natural and synthetic polymeric systems that have been investigated for their controlled release of drugs. From which many hydrophilic poly-anionic polymers were used. Targeted in this era is the application of chitosan and sodium alginate as polymers to prepare NPs. One of their advantages is that they are prepared by using DW as a solvent and not organic solvents thus, decreasing the negative impact on the environment. As compared to other natural polymers, sodium alginate and chitosan show no variations in viscosity and hence produce more uniform gel structure which forms stronger cross linked structure and more loading of entrapped material.[117]

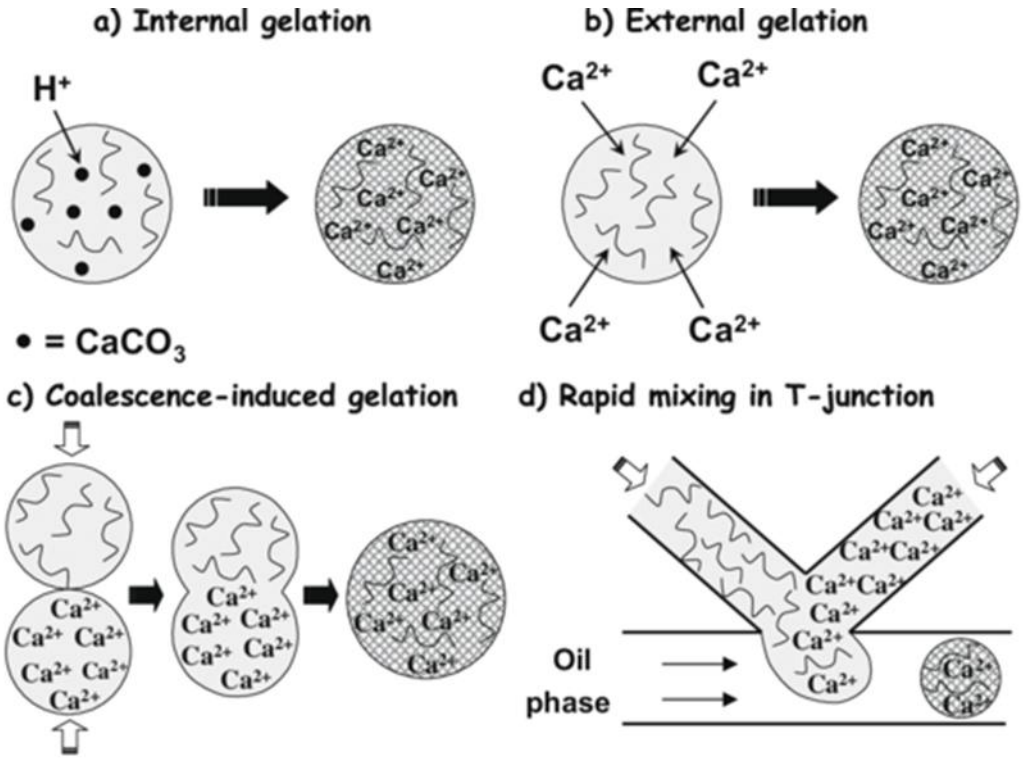

Fig.2.23 (a) external ionotropic gelation (b) internal ionotropic gelation [117]

k. DPPH test for antioxidant assay:

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in methanol. This free radical in the DPPH reagent is stable at room temperature and in the dark, is reduced in the presence of an

antioxidant molecule if exists inside the material under test, giving rise to colorless methanol solution.

The percentage of antioxidant activity (AA%) of each substance was assessed by DPPH free radical assay according to Brand-Williams *et al.*[119]. The reaction mixture consists of 0.05 mg of sample, 2 mL of the reagent formed of 0.01 M of the reagent (which is equivalent to 0.00394 grams in 100 mL methanol) and is left on the shaker for one hour. When DPPH reagent which acts as oxidizing agent reacts with an antioxidant compound, which can donate hydrogen, it gets reduced. The absorbance of the changes in color (from deep violet to light yellow) are measured at 517 nm using a UV-Vis spectrophotometer (du 800; Beckman coulter, Fullerton, ca,USA). The mixture of methanol (2 mL) and reagent (0.01M DPPH) acts as control. The blank solution is just the methanol solvent. The scavenging activity percentage (AA%) is determined according to Mensor et al. [120]

Equation2.12. [120][121]

$AA\% = (1-A_s/A_C) \times 100\%$

Where A_s is the absorbance of the sample, A_c is the absorbance of the control at 517 nm [121]

l. Folin-Ciocalteu test for polyphenolic content:

Folin-Ciocalteu phenol reagent consists of a mixture of the hetero poly acids, phosphor molybdic. Molybdenum and Tungsten have oxidation number +6. On reaction with a reductant, the molybdenum blue and the tungsten blue are formed and the mean oxidation state of the metals is between 5 and 6. According to the following equation 2.13;

Equation 2.13. [122]

$Na_2 WO_4 / Na_2 MO$ yellow $\Rightarrow$ $(Phenol\text{-}MoW_{11} O_{40})^{-4}$ blue

Mo^{+6} (yellow) + e^{-1} $\Rightarrow$ Mo^{+5} (blue)

$Mo^{+5} + e^{-1} \Rightarrow Mo^{+4}$ (blue)

$ØOH \Rightarrow ØO\bullet + H^{+1} + e^{-1}$

$ØO^{-1} \Rightarrow ØO\bullet + e^-$

A stock solution for Gallic acid is prepared of concentration; then serial dilutions are prepared from it and then on every prepared 0.5 mL of the serial dilution add 2.5 mL Folin reagent and wait 5 min then add 2 mL 7.5% sodium carbonate. Blank is prepared by Folin reagent and sodium carbonate only. After that the samples are left in the dark for 120 min. Samples are then centrifuged for 3 min. Absorbance of the samples is measured at 765 nm. Phenolic content is calculated as gallic acid equivalents, gm extract/mg GAE. [122]

m. Anti- bacterial assay:

The efficacy of the prepared NPs is investigated as potent wide spectrum antibacterial materials. Many tests and requirements are manipulated to test the potency and efficacy of antibiotics in the market. Antibiotic potency testing is a biological assay whereby the efficacy of the antibiotic at a range of prepared concentrations is tested against a live microorganism; the resulting biological response is then measured and evaluated against a median standard and five-point (minimum) standard curve. The biological response is referred to as antibiotic activity or potency.

Antibiotic potency testing is a multi-variable test which depends on: 1) test microorganism growth requirements 2) test antibiotic concentration and 3) preparation and use of growth media, reagent, test organism and antibiotic standards. Antibiotic potency testing is performed either by the plate (cylinder-plate or diffusion or Log reduction) or tube (turbidimetric, colorimetric or MIC) method. Both plate and tube methods demonstrate measurable levels of growth inhibition. Growth inhibition measurements are tabulated and integrated into a linear regression curve, resulting in extrapolated antibiotic potency values.

Potency is denoted in units (U) or μg of activity and may or may not be exact in equivalence to the μg (weight) of the active compound and related to the amount of remaining living bacteria expressed in units of colony forming unit (CFU) or colony forming unit. In any of the two techniques, MIC or Log reduction a serial dilution of the bacterial strain under test is prepared. The tested organism must be pure and robust and further portioned into a primary and working culture. From the working culture an inoculum is prepared. Inoculum preparation and standardization is required prior to antibiotic potency testing. A working inoculum stock should

also be prepared to prevent possible cross contamination of the primary stock. After that, serial dilution of which is prepared to just work on a range of 50µL.

a. **MIC; Minimum inhibitory concentration technique**: The antibacterial activity of natural products was studied by employing a microdilution method using Luria Bertania (LB).

1. Inoculum preparation and dilution: The inoculum was prepared by selecting three to five well-isolated colonies of the same morphological type from the agar plate on which the bacterial strain was replicated. The top of each colony is touched with a wire loop and the growth is transferred to a tube containing 4–5 mL of suitable LB broth medium. The broth culture is incubated at 35˚C until it achieves the turbidity of a 0.5 McFarland standard (usually 2–6 hours). Fig.2.24. shows the serial dilutions of the inocula of the organisms to be tested:

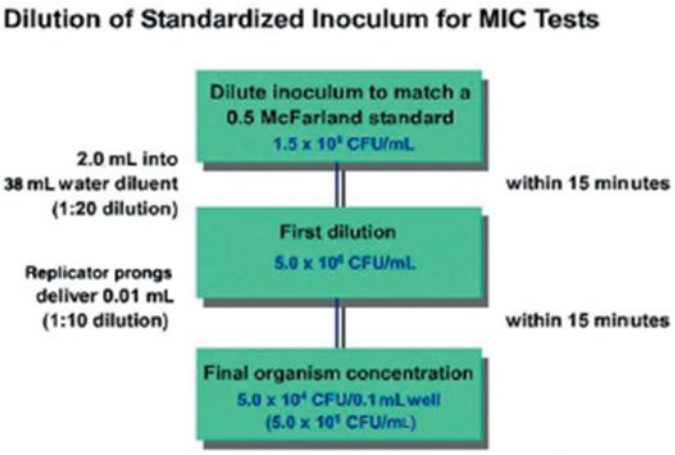

Fig.2.24. Serial dilutions of inoculum to reach standardized concentration to perform MIC test [123]

2. Natural products were dissolved in DW (50µL of the final volume) and diluted with culture broth around 200-230 µL. 100 µL of each dilution were distributed in 96-well plates.
3. Sterility negative control and a growth control (containing culture broth plus inocula of living bacteria, without antimicrobial or antifungal substance). Four control carriers should be used in each test. Each test and growth control well was inoculated with 5 µL of a bacterial suspension. All experiments were performed in triplicate and the

microdilution trays were incubated at 36ºC for 18 h. Bacterial growth was detected former by optical density (ELISA reader or measured by UV,VIS spectrophotometer). The trays were again incubated at 36ºC for 30min, and in those wells, where bacterial growth occurred, Iodonitrotetrazolium chloride; p-Iodonitrotetrazolium Violet (INT) changed from pink to purple. MIC values were defined as the lowest concentration of each natural product, which completely inhibited microbial growth. The results were expressed in mg/mL. [123][125][126][127][129]

b. Log reduction method:

1. Stock culture preparation: the bacterial strain under test was inoculated on Tryptone Soy Agar media (TSB), till reach the mid log phase, the stage at which bacteria reachs the maximum growth rate. After that it was sub-divided to aliqoutes and incubated at -80°C. Diluted suspensions were inoculated on agar plates Trypticase Soy Agar (TSA) and incubated for 48 hours at 37°C.
2. Antibacterial assay: 10 µL of the bacterial suspension which was equivalent to 10^6 CFU were added to 90µL of the buffer assay phosphate buffer saline (PBS), those samples were used as negative control samples. Results obtained were compared with those of the natural plant and the plain nanoparticles. Samples were incubated at 37°C for 4 days under rotation. Aliquots were withdrawn at 24 hrs intervals from each sample and plated on TSA media plate. Results were plotted as Log_{10} CFU/ mL. [124][120]

c. Calculating Log10 Reductions

A method for determining log10 in the viability titer of the test organism by the test substance in quantitative carrier tests such as this one has been described by the following equations 2.14, 2.15, 2.16. (DeVries and Hamilton 1999) [128]

Equation2.14. [128]

$$\% \text{ reduction rate} = (A-B)\times 100/A$$

Where A is number of viable microorganisms before treatment and B is number of viable microorganisms after treatment

Equation2.15. [118]

Inhibition rate= $1-[OD_{ttt}/OD_{control}] \times 100$

OD is the optical density recorded by the plate reader

Equation2.16. [128]

Log reduction= Log_{10} A/B

1 log reduction = 90% reduction

2 log reduction = 99% reduction

3 log reduction = 99.9% reduction

4 log reduction = 99.99% reduction

5 log reduction = 99.999% reduction

6 log reduction = 99.9999% reduction

Chapter 3

Results and Discussion:

I. Synthesis and Characterization of chitosan NPs loaded with the Date seed extract

Chapter 3: Results & Discussion:

I. Synthesis & Characterization of Chitosan NPs loaded with the AS/BS plant extract:

3.1.1. Phytochemical analysis:

Standardization of plant parts active constituents for the pharmaceutical industry plays an important role in product outcomes. Therefore, the quality of the botanical material, as well as the adequate processing of the fresh material, including drying, transportation, storage, and the use of appropriate and reproducible extraction techniques have a straight outcome on health benefits and economic issues. Previous studies were conducted to determine the best solvent and plant part for extraction and preparation of antioxidants and polyphenolics which are the main outcome searched for to act as antibacterial and radical scavengers [17] [18] [19] [33][68][69][70][130] .Tests and trials were done on 3 plant parts; seed, peel and fruit. First we applied fractional distillation technique using distilled water: 50% acetone in ratio 1:1. The dried sample was placed on magnetic stirrer for 1 hr at 450 rpm and 45°C and then centrifuged at 1000 rpm for 10 min. Fractionation occured at 60-70°C to collect acetone layer then add butanol and the butanol layer was collected which was rich in polyphenolics and then UV-VIS spectrophotometry for every layer collected was applied [73] . It was actually a failure and separation was not 100% that is why ordinary extraction was the method of choice of extraction which was accomplished either by; maceration in solvent for 72 hrs or by boiling in hot water bath for 30 min. We used 4 different solvents; methanol100%, ethanol 52%, dimethyl sulfoxide (DMSO) and finally a quaternary system formed of water: Acetone: methanol: formic acid in ratio: 20:40:40:0.1. Methanol is indeed the most common and effective solvent for extracting anthocyanins; however, it is an environmental pollutant and it is also more toxic than other alcohols. Thus, 52% ethanol was preferred over methanol for the extraction of polyphenolics from plant material.

The physicochemical properties of the prepared chitosan nanoparticle and the nanoparticle loaded with the Date seed extract were investigated. From the trials were done, it was concluded that the prepared nanoparticles size and charge got affected by group of factors like; the variety of the plant used, the stirring speed rpm and temperature, the pH of the prepared chitosan solution, the rate of Sodium tri polyphosphate added mL/min, the speed in rpm the time and the temperature of centrifugation and finally the time and pulse power of sonication .[17] [18] [19] [33][68][69][70][130]

And that is why all the previous factors got settled to certain set to be followed to be able to study other parameters effect on the prepared nanoparticles to get the most delayed release rate, antioxidant and antibacterial action such as; the variation in the Date seed extract concentration, the media used to evaluate the drug release, the presence or absence of stirring, the temperature and time of homogenization to monitor the Encapsulation efficiency (EE),loading capacity(LC) and drug release (RR).

3.1.1.1. Comparison of the oven dried versus the freeze dried plant parts using IR spectra:

Fourier Transfer Infra-red Spectrophotometer (FTIR) was performed on all the crushed dried plant parts; the peel, seed and fruit parts of the Date plant either were dried by oven at 60°C for 72 hrs or lyophilized at -60°C for 72 hrs. [42] The dried plant part after drying and crushing was mixed with Potassium bromide (KBr) at ratio 2:500 then they got pressed to form a disc .The plant part under examination was scanned at wave number range from 4000-400 cm^{-1} and resolution 4 cm^{-1} .

It was reported that plant parts either oven or freeze dried got the same pattern on IR as shown in Fig. 3.1 and referring to Appendix [4.1]. Table3.1. shows the wave numbers that appeared in the chart indicated a number of polyphenolics and antioxidants that probably are present in the plant parts. IR is not highly indicative since mostly all parts have the same active constituents but with variable percentages and this actually was reported in other plants as well like *Phylanthus* in Brazil. 13 authentic samples were prepared from 3 sets; stem only, leaf only and aerial parts only. Leaves and stem had more or less the same active constituents. They showed mostly the same IR pattern according to what was reported by Santos M.S.*etal.* [4][15][17][18][19][22][77][79][80][137]

That is why oven dried samples would be used as was reported in most previous work on extraction from date plant parts.

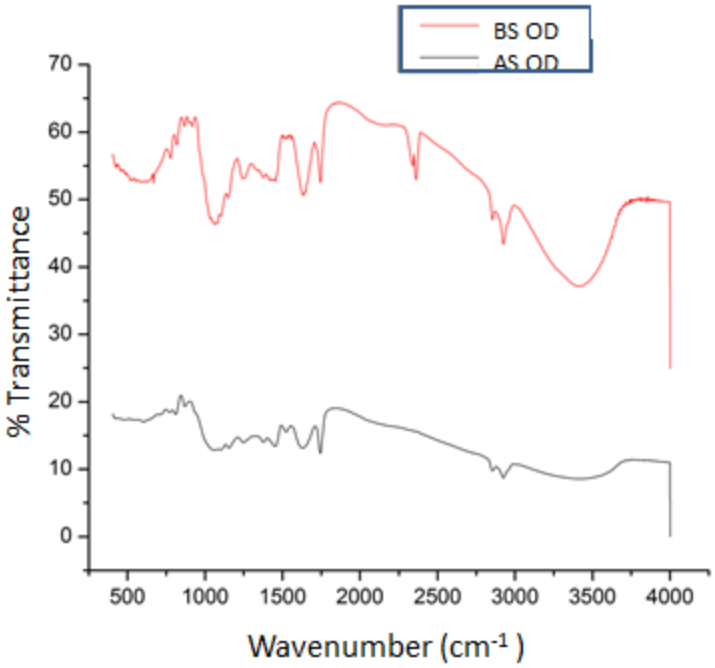

Fig.3.1. Ajwah and Black date seed plant parts oven dried IR charts

Table3.2 and table3.3 show the probability of the reference of the appearing peaks in IR chart to which compounds they may refer to.

Table3.1. The wave numbers and the probable polyphenolics present in Date seed. . [4][15][17][18][19][22][77] [79] [80] [137]

Absorption peak (wavenumber cm^{-1})	Active constituent
3418.8	Apigenin-8-C-glucoside (CO stretching)
3399.9-321	Ferulic acid (COOH stretching)
2924.7	Protocatechuic acid (COOH and OH stretching)
2853.8-270	Gallic acid (COOH bending and OH stretching)
2853.7	Catechin , epicatechin (OH stretching)
2348.4	(those two peaks disappear in Ajwah plant parts)
2283.7	

Table3.2. The reported absorption maxima in nm for variable polyphenolics, flavonoids and anti-oxidants [138]

Phenolic compounds	**Absorption peaks (nm)**					
Flavonoids:	**1**	**2**	**3**	**4**	**5**	**6**
Catechin	328	279				
Rutin	299	295	256			
Quercetin	326	282	274	253		
Fisten	301	256				
Non flavonoids:						
cinnamic acid	340	333	328	270		
caffeic acid	312	287	285			
Benzoic acid derivatives:						
gallic acid	344	340	333	261		
Salicylic acid	299	295	256			
p-Hydroxy benzoic acid	271					
2,3-dihydroxy benzoic acid	306					
2,4-dihydroxy benzoic acid	342	292				
2,5-dihydroxy benzoic acid	321					
3,4- dihydroxy benzoic acid	341	290	253			
2,4,6- trihydroxy benzoic acid	341	293	255			
2,3,4- trihydroxy benzoic acid	347	295	260			
Others:						
Caffeine	340	333	328	324	314	272
Catechol	346	335	328	273		
4-hydroxy phenylacetic acid	343	325	317	306	275	
Resorcinol	335	328	314	309	302	273
Tannic acid	348	340	338	277		
Vanillin	311					

Table3.3. Polyphenolics and their A max nm (Cont.) [139]

Polyphenolic	ƛmax (nm) (reported)
Protocatechuic acid hexoside	257, 291
Caffeoyl hexoside	234, 88sh*, 297
Caffeoyl-D-glucose	-
p-hydroxy benzoic acid	278, 310 sh*
Cis- caftaric acid	232, 277,321
Caffeoyl hexoside	233, 291
Quinic acid derivative	230, 266
Chlorogenic acid	236, 303sh*, 326
Caffeic acid	241, 305sh*, 323
Hydroxy cinnamic acid derivative	241, 291, 319
Quercetin-pentoside-hexoside	231, 260, 358
Quercetin acid derivative	231, 260, 358
Caffeoyl dihydroxy phenyl lactoyl tartaric acid	246, 300sh*, 332
Quercetin 7-o-hexoside-3-o-malonyl hexoside	-
Luteolin hexoside hexoside	255, 266sh*, 345
Caffeic acid derivative	240, 310sh*, 325
Luteolin 7-o-rutinoside	242, 305sh*, 328
Quercetin pentoside	255, 266sh*, 348
Quercetin hexoside	256, 300sh*, 354
Luteolin-7-o-glucoside	255, 266sh*, 347
Quercetin pentoside	-
3,5-di-o-caffeoylquinic acid	243, 303sh*, 327
Chicoric acid	242, 305sh*, 328
Chicoric acid derivative	242, 305sh*, 328
Caffeoyl hexose-deoxyhexoside	290, 320

N.B. * Sh, shoulder or tailing for the peak appeared at this wave length

Antioxidants appeared in the range of 2000 to 800 cm^{-1}. They represented high degree of unsaturation and that is why they could be used as radical scavengers and thus as natural food preservatives or as antimicrobials. The wave numbers from 2000 to 1500 cm^{-1}; referred to groups like; CO, CN, NH, NH2, CC, CN. The wave numbers from 1500 to 1000 cm^{-1}; referred to groups like COC, COH. The summary of the wave numbers of different groups was shown in table3.4.

Table 3.4.Wave numbers of different functional groups [61] [79] [81] [106] [107] [108]

Absorption peaks (wavenumber (cm^{-1}))	Active constituents
1746.5 (in all plant parts)	Ester carbonyl of glyceride
1700-1650	Ester carbonyl may shift when gets oxidized
1633.6	C=C stretching of olefins
1519.8-1455.4	Aliphatic bending CH2 and CH3
1378.5	CH2=CH2 bending
1246.3	Asymmetric stretching –C-O-C
1156	OH bending

3.1.1.2.<u>The preparation of the different plant parts extracts and assay of polyphenolics and antioxidants contents</u>*:*

Extract from the peel and seed of Ajwah date and Black date plant; oven and freeze dried and added to it extract of the black date fruit freeze dried were prepared to examine which plant part and with which solvent the maximum polyphenolic and antioxidant contents could be reached. Folin-Ciocalteau test was applied on all plant part extracts with 3 different solvents as mentioned before; ethanol 52% (E), dimethyl sulfoxide (DMSO) (D) and finally a quaternary system formed of water: Acetone: methanol: formic acid in ratio: 20:40:40:0.1. (M). 2,2, diphenyl -1-picryl hydrazyl (DPPH) test was applied on the dry plant powder (dpp). Results of the TPC and AO% for the freeze dried plant parts were mentioned in Appendix [4.2]. It was reported that the best plant plant showed the maximum polyphenolic and optimum antioxidant contents was the

seed either of Ajwah date or Black date plant using 52% ethanol as solvent for extraction which complied with previous work as shown in Fig.3.2. and Table3.5

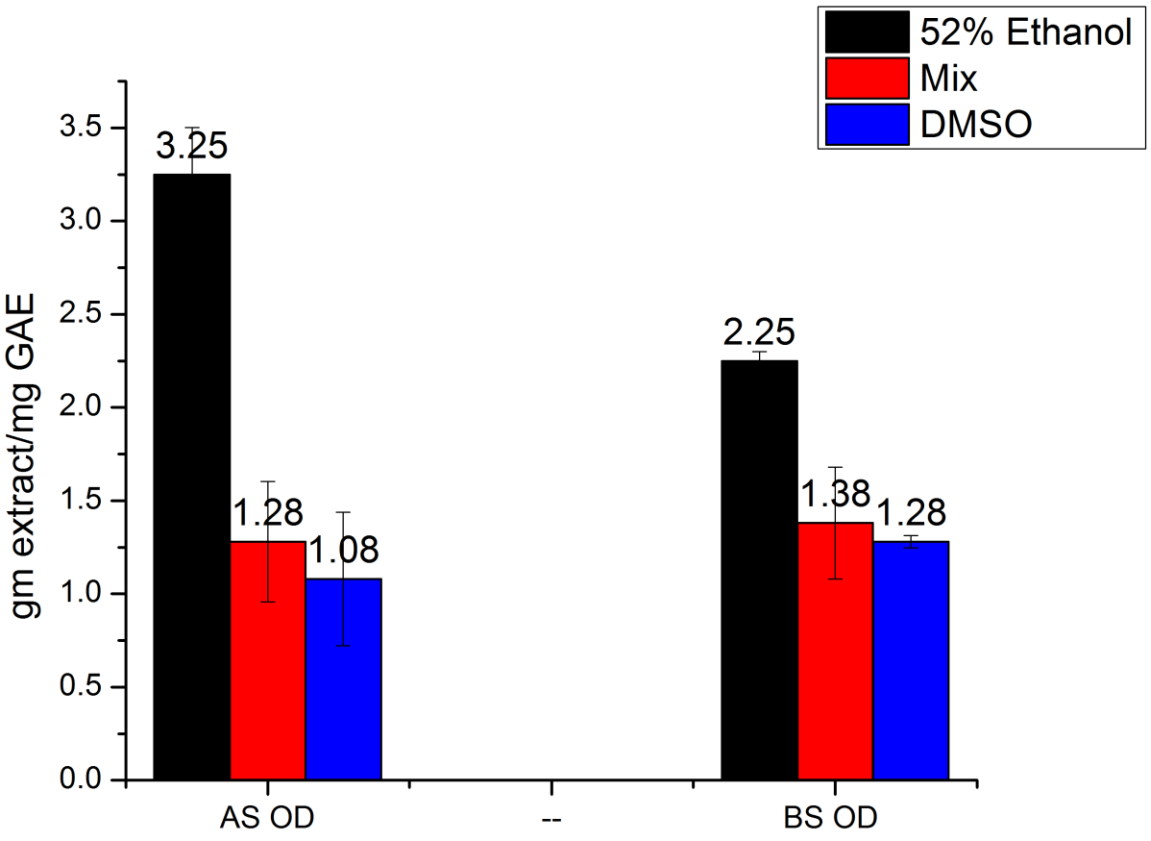

Fig.3.2. Folin-ciocalteau test of Ajwah seed oven dried (AS OD) and black date seed oven dried (BS OD) in 3 solvents 52% ethanol (E), Dimethyl Sulfoxide DMSO (D) and mixture of water:methanol:acetone:formic acid (M)

Table3.5. the Total phenolic content in the Ajwah peel (APOD) and Ajwah seed (ASOD) oven dried ; Black date peel(BPOD), black date seed (BSOD) oven dried in 3 different solvents; 52% ethanol (E), Dimethyl Sulfoxide DMSO (D) and mixture of water :methanol :acetone :formic acid (M)

Solvent / Plant part	E	D	M
ASOD	3.25±0.25	1.08±0.36	1.28±0.32
APOD	2.65±0.15	1.02±0.23	1.42±0.22
BSOD	2.52±0.05	1.28±0.03	1.38±0.30
BPOD	1.36±0.66	0.24±0.06	0.41±0.36

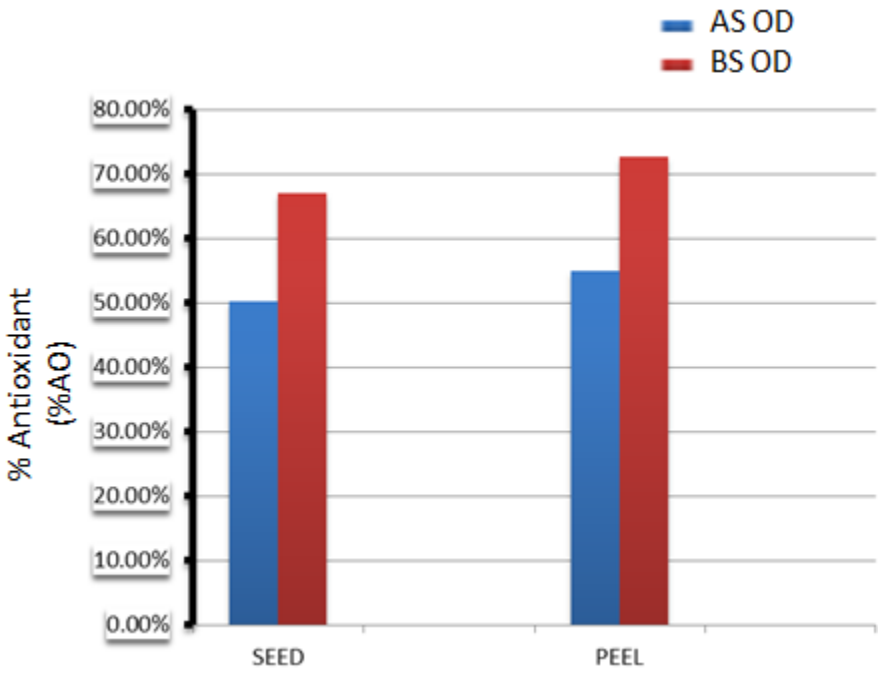

Fig3.3. DPPH test for Ajwah seed oven dried (ASOD), black date seed oven dried (BSOD), Ajwah date peel oven dried (APOD) and black date peel oven dried (BPOD) on dry plant powder (dpp) and measure the antioxidant activity % (AO)

Table3.6. Antioxidant activity (AO%) of dry plant part powder (dpp) of Ajwah peel (APOD) and Ajwah seed (ASOD) oven dried ; Black date peel (BPOD), black date seed (BSOD) oven dried

Plant part	AO%
ASOD	50.17%
APOD	55%
BSOD	67%
BPOD	72.7%

According to what was reported by Raseae Iman *etal.,* Ali.K. *etal.*and Santos M. *etal.,* The variable solvent or variable ratios could alter the yield and the degree of active constituents extraction. That is why for example in the egg- plant peel on using methanol or formic acid showed the highest yield for polyphenolic compounds especially anthocyanins thus it showed the highest absorbance in the Folin test using those solvents, on the other hand on using 70% ethanol for extraction of the same plant part it showed maximum extraction for carotenoids. Aside from the usage of variable solvents the level of antioxidants are not the same in all plant parts as an example in egg -plant, the order of percentage of antioxidants and polyphenolics was reported to be as follows; anthocyanins > other phenolics> flavonoids> tannins> carotenoids. On the basis of active constituents and biological applications; polyphenolics and antioxidants were considered to be the same class of compounds yet on applying chemical tests basis and assays they were considered not identical depending on the solvent used in the extraction as what was reported on *Limnophila aromatic L.* it is a medicinal herb and spice used in South Asia. On applying extraction using 52% ethanol it got the maximum yield of polyphenolics and antioxidant contents. Though this fact, this was not the solvent of choice to prepare the NPs. The same in NPs prepared in the following protocol, though the 52% ethanol showed the maximum polyphenolic yield; DW was used as the solvent to extract the polyphenolics and to prepare the nanoparticles [18] [137][140] . From the above results, due to the difficulty of separating the peel from the fruit and the difficulty to dry them due to the high stickiness of the fruit, and added to this the difference in the %AO between the peel and the seed of either the Ajwah date or the Black date was not of high significance compared to the difference in TPC contents which was

higher in seed than the peel of either of the 2 date varieties by 1 or 2 folds. This made the seed the plant part of choice it was concluded that Ajwah date seed and black date seed oven dried were both selected for the preparation of the chitosan NPs.

3.1.2. Chitosan NP preparation and encapsulation of the date pit extract in it:

3.1.2.1. Physicochemical and spectroscopic measurements of the prepared plant seed/ chitosan NPs:

Preparation of the ASOD or BSOD /chitosan nanoparticle was done according to the following concentrations; first chitosan powder was dissolved in acetic acid 1%v/v at concentration of 2mg/mL at pH=5. Then, plant extract in DW was added to the chitosan solution at ratio 3:1 (w/w) and 1:1 (v/v) to reach total volume of 50 mL. Sodium tri-polyphosphate (TPP) was added at concentration of 1mg/mL with variable volumes 2,4,6 and 8 mL as shown in Table 3.7. Applying different centrifugation and stirring conditions for every prepared sample to get out the best protocol to be followed for the preparation of the chitosan NPs.

Table3.7. Samples of the Chitosan NP loaded with AS and BS at variable mLs of TPP

	Set A			**Set B**		
	Plant part AS/DW	**Chitosan (1% acet.ac.)**	**TPP/DW**	**Plant part BS/DW**	**Chitosan (1% acet.ac.)**	**TPP/DW**
Conc.	6 mg/mL	2 mg/mL	1mg/mL	6 mg/mL	2mg/mL	1mg/mL
Samples	**Volumetric ratios (v/v, mL)**					
Sample1	25	25	2			
Sample 2	25	25	4			
Sample 3	25	25	6			
Sample 4	25	25	8			
Sample 5				25	25	2
Sample 6				25	25	4
Sample 7				25	25	6
Sample 8				25	25	8

According to Rasaee Iman *etal.* and Swarnalatha. Y *etal.* the prepared seed extract was added to the chitosan acidic solution before adding the cross-linker to guarantee that the NPs prepared would encapsulate the plant extract. There was no significant difference between the plant extract prepared in DW either by to leaving it till boiling for 10 min or the plant seed to be macerated in DW for 72 hrs. [16][139] Chitosan NP was prepared by applying ionic gelation technique [17][18][19][33][129]. It is a conventional method to prepare chitosan NPs. The poly anionic groups like Phosphate ($PO4^{3-}$) in Sodium Tri polyphosphate (TPP) would bind ionically with the amino group (NH^{+}_{3}). This would lead to cross linkage between monomers to form chitosan NPs as shown in Fig.3.4.

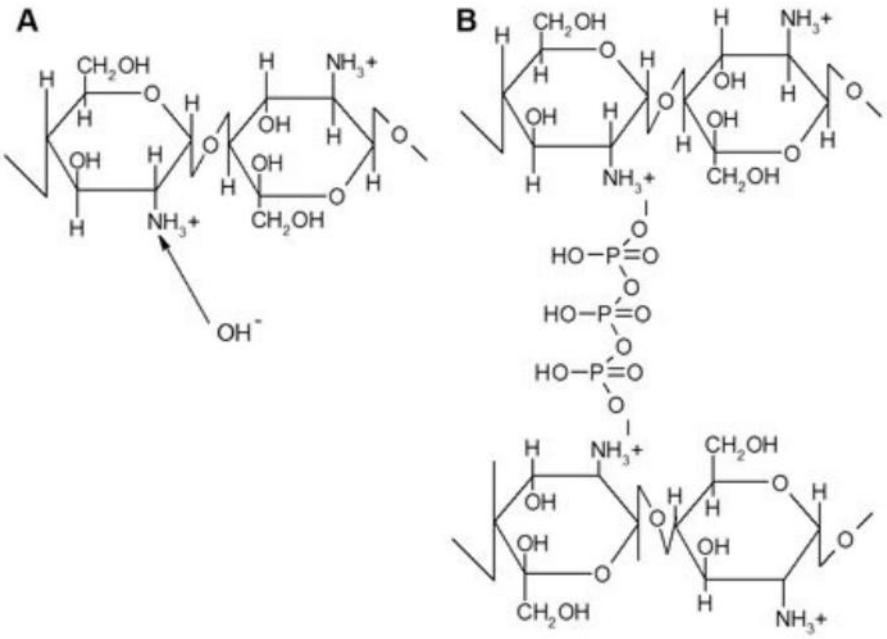

Fig3.4. Interaction of chitosan with TPP (a) deprotonation, (b) ionic cross-linking[135]

Many factors controlled the prepared nanoparticles shape and size and charge like;

* concentration of chitosan

* concentration of chitosan :TPP,

* pH of the prepared chitosan solution

* speed of sonication in correlation to the power and time of sonication.

* plant variety used

* stirring conditions, time, temperature and rpm

* centrifugation conditions, rpm, temperature and time

To isolate the effect of centrifugation, stirring, homogenization sodium tri-polyphosphate was used as precipitation salt and all other preparation parameters were fixed according to Shi W.*etal.* and Koppolu B.P.*etal.*[83][142] The size, poly dispersion index and zeta potential were measured by Zeta-sizer Nano (Malvern Instruments).

According to Koppolu B.P.*etal.*, when anions of TPP got highly hydrated in case of using high concentration of TPP linker, this would lead to very low poly dispersive index (PDI) which was required. On the other side, the high sonication power would not reduce the PDI since it had minimum effect on the PDI and mean particle size. The sonication aim was just to re-disperse the particles and make them distributed homogenously through the system used [83]. The specific size needed whether to the minimum border line approaching the 10nm or to the maximum border line reaching the 100 nm is dependent on the target of the nanoparticle inside the human body. According to Koppolu *etal.* the large particle size approaching the 100 nm; approximately in the size range of bacteria or virus thus it would be easily engulfed by the immune system ; the antigen progenitor cells (APC), lead to increased immune response and the drug would be released at fast rate. If sustained or controlled drug release was required as in the case of the NPs prepared in this protocol so very small size would be needed which do not exceed 50 nm [83].

For the volume of added TPP; when the volume of TPP added ranged from 8-10 ml, this would decrease the particle size and lead to uniform PDI. Depending on Hofmeister series; the chitosan had pKa 6.3, it is poly cationic in nature thus when dissolved in acid it would release the protonated NH_3^+ ions that would cross-link with the OH^- and the TPP^{---}ions that released from the sodium tri-poly phosphate (TPP) when got hydrated. The sodium would act as a cationic constant and TPP^- would act as anionic constant. They would get high degree of hydration and thus the smallest possible particle size. The minimum particle size and PDI are required but the drawback of this approach was the decrease in the drug load. Therefore, as in Table 3.8., an

optimization done between the sonication or homogenization power and the rate TPP should be fulfilled to get the maximum drug loading with the least possible particle size and PDI [83] [135]. In the Chitosan NPs that were prepared encapsulating (AS) or (BS) extract; on applying sonication it was observed that sonication affected the Particle dispersion index PDI decreased from 0.9 to 0.4 at 50% watt .

Table.3.8. The relation between the TPP rate and volume versus the sonication power [82]

Factor	**PS and PDI**		**Drug loading**	
	High	**Low**	**High**	Low
Rate and volume of TPP	Low	High	**Optimum**	Very high loading
Sonication power	Low or high	**Low or high but more applicable in low power**	Optimum sonication	Very high

N.B. the bolded text referred to the optimum conditions needed

A very crucial parameter was the chitosan itself; concentration, viscosity, molecular weight , temperature of preparation and pH. The NPs were prepared with Low molecular weight chitosan (LMW) and with minimum concentration 2 mg/mL in acetic acid 1%v/v. According to Jarudilokkul S.*etal.*, on using high concentration or high molecular weight of chitosan this would lead to the formation of a barrier against the swelling mechanism and consequently against the release of the drug, since mostly NPs released the drug from the matrix by swelling and erosion mechanism [20] [142]. On increasing chitosan concentration or molecular weight; the particle size would increase dramatically reaching micron level without increasing the drug loading efficiency [83]. There should be an optimum ratio of chitosan to TPP to be added as was reported by Calvo *etal.*to guarantee a PDI which does not exceed 0.05, thus we should work with chitosan concentration which would not exceed 2.5 mg/mL and TPP concentration which would not exceed 1mg/mL [16]. According to Bhumkar *etal.* when the mL of the TPP added increase they would not only increase the efficiency of cross linkage and get the NP size to the minimum value

but at the same time would increase conductivity. Yet the degree of conduction depended more or less on the pH as would be discussed later. Fig.3.5. showed the IR chart with the sharpest peaks referred to the maximum cross-linkage and the minimum particle size at the optimum TPP volume of 6 mL. Fig.3.6. showed the difference in the IR patterns before and after cross linkage of chitosan and the change in the IR pattern after date pit extract encapsulation. Appendix [4.3] showed the same charts for black date seed in chitosan NPs and Ajwah date seed in chitosan NPs with varied stirring, centrifugation conditions and TPP rates. [135]

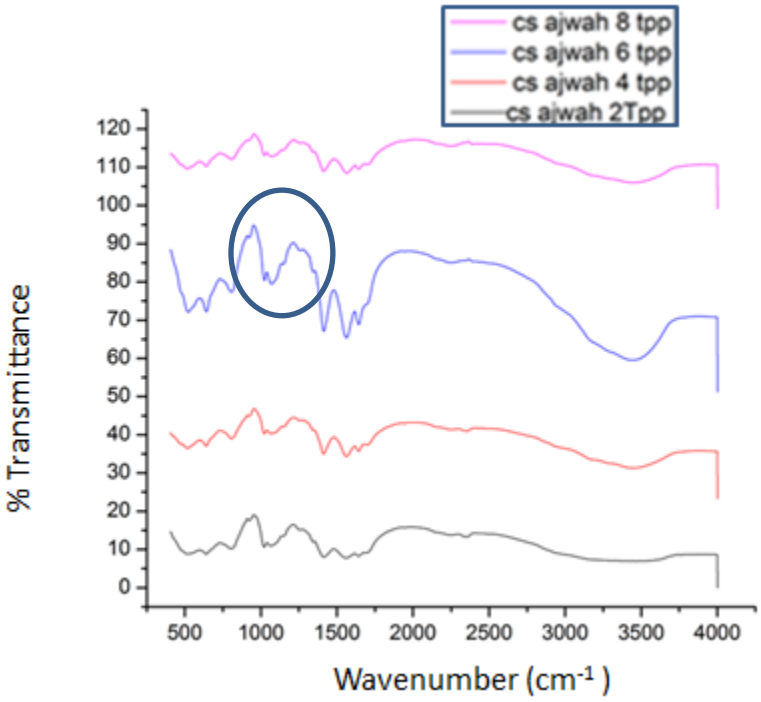

Fig.3.5. IR chart of the Ajwah date seed extract in chitosan NP and study the impact of TPP volume added variation

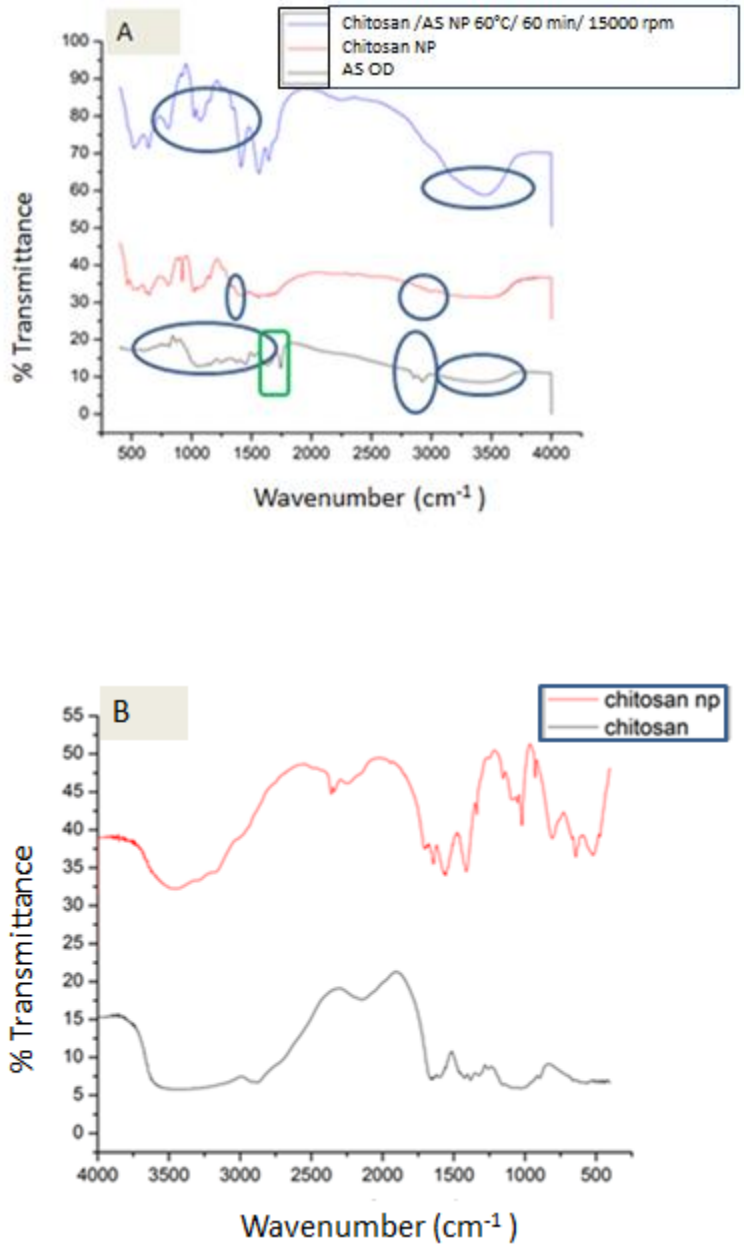

Fig.3.6. FTIR charts of (a) the Chitosan loaded with date seed extract using TPP at volume of 6 mL and compare it with vacant chitosan NPs and pure Ajwah date seed powder (b) the chitosan powder and chitosan NP

Fig.3.6. showed that date seed extract was proved to be rich in polyphenolics and antioxidants. Peaks at 3418 cm^{-1} referred to the apigenin glucoside, peaks at 2924 and 2853 cm^{-1} referred to Protocatechuic and gallic acids respectively. Other peaks appeared between 2000 and 1000 cm^{-1}

referred to the ester group, C=C, C=O, CH_2 and CH_3 groups of the glucoside parts of the polyphenolics and antioxidants. For the chitosan NPs, all the peaks were shifted to smaller wave number, larger wave length, smaller frequency and energy since the free ionic groups became more stable and free charge got neutralized after cross linkage with the TPP salt; for example 3240.56 cm^{-1} referred to the OH group stretching and 2869 referred to the CH group stretching due to the CO effect. On adding the Ajwah seed (AS) extract, OH groups peak got broadened due to the flavonoids and polyphenolics rich in OH groups and glycosides as well and that is why the CO group peak disappeared since mostly all the CH converted to COH, thus the peak at 3437 cm^{-1} got shifted to 3452 cm^{-1} due to increased hydrogen labile bonds with the acidic medium. The peak at 1641 cm^{-1}got shifted to 1620 cm^{-1} due to the cross linkage occurred between the amino group of chitosan with the phosphate anions of TPP^{--}. The chitosan NPs loaded with the plant seed extract acquired the same peaks but with the addition of more peaks. Those results were more promising unlike those IR results reported by Madadlou *etal.*, where the IR chart of the free and the loaded starch NCs did not show any significant differences as shown in Appendix [4.3]. [33] Due to the interaction between the free amino group 1 and 2 of the chitosan with the free OH , CC and CO groups of the polyphenolic glycosides, this led to the formation of 2 distinctive peaks; when the CO group of the polyphenolics that already appeared at range of 1750 cm^{-1} interacted with the NH_3^+ group would form amide groups which would shift the wave number of the amino group to 3500-3180 cm^{-1} and the CO shifted from 1750 cm^{-1} to 1680 cm^{-1}; to smaller wave number since the vibrational stretching increased and it became more stable due to the sharing of the lone electron pair between N and O anions. In chart (a) the highlighted green squared peak which appeared between 2000 and 1500 cm^{-1} disappeared in the NPs formulated indicating that the plant got encapsulated inside the NP and that is why the peak no more appeared. All the shifting of the peaks that took place to smaller wave number or larger wave length was pertained to the interaction between the chitosan matrix and the date seed extract. On the other side, when the free amino group of the chitosan NH_3^+ interacted with the alkene groups in the aromatic ring it formed imide bond where the wave number shifted from 1600 to 1690 cm^{-1}; it shifted to larger wave number since the degree of un stability increased due to the presence of the free lone pair of electrons over the aromatic ring which increased the electron density. And as was mentioned above TPP should be added in an optimum rate in correlation with the stirring and centrifugation rpm so as not to get the minimum size and the

minimum drug loading that is why it was ideal to decrease the rpm of centrifugation and increase the TPP not to a very high level to get the maximum cross-linkage and the maximum drug load with the least possible particle. [17] [18] [66] [135] [137] [143][144]

For the stirring conditions; many protocols were tried as shown in Appendix [4.4] in preparing the NPs. According to Fen w.*etal.* it was reported that when temperature applied in the preparation ranged from ambient temperature to 60°C, the PDI decreased from 1 or 2 to reach 0.05; which was highly favorable as clarified in Fig.3.7. Since as the temperature increased, the intrinsic viscosity decreased, the radius of chitosan gyration would decrease, the hydration of the H bonds with the water molecules would decrease and this would increase the chitosan flexibility. [16]

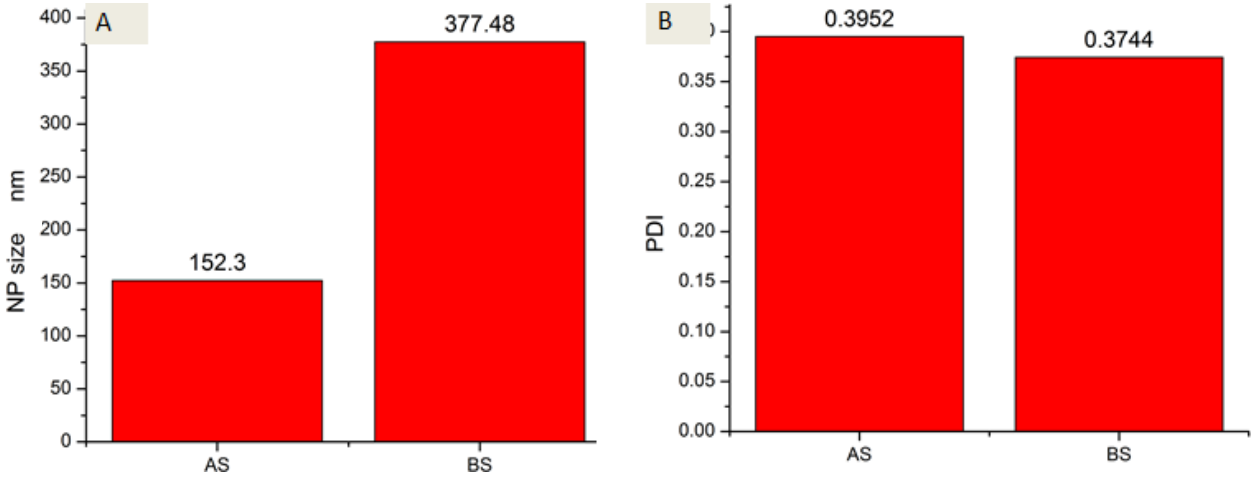

Fig.3.7. (a) the NP size and (b) PDI for Ajwah date seed (AS) and Black date seed (BS) at stirring condition of 60°C and TPP volume 6mL at 15000 rpm centrifugation speed

According to Bhumkar *etal.* and Wen F.*etal.* pH of the prepared chitosan solution was very important factor in chitosan nanoparticle preparation. Acidic media was more favorable for cross-linkage and interactions rather than neutral or alkaline media. In low pH there were repulsion forces between the protonated chitosan and the positive charged amino group which existed in equilibrium with the inter-chain interaction between the chitosan molecules. [16] [135] it was expected that the pH of the TPP and chitosan had crucial role in the degree and type of cross-linkage that would occur. In acidic pH using acetic acid which does not exceed concentration 1% (v/v), the NH_3^+ group released from chitosan would get hydrated. After that the TPP also would get hydrated and release the TPP^- anions ($P_2O_5^{--}$). This ionic bond would

increase the conductivity and zeta potential till reach equivalence point. After reaching the equivalence point, the conductivity would continue to increase due to the ionic interaction between the H^+ ions and the phosphate of TPP. Unlike the alkaline medium, at high pH the chitosan structure got more porous and more degradable led to fast release of the drug [20].In alkaline medium, OH^-ions and phosphate ions of TPP would compete to interact with the NH_3^+group of chitosan. Finally OH^- would interact with the NH3+group by deprotonation. This latter mechanism would decrease the conductivity and zeta potential [135]. Fig.3.8. showed the variations of the conductivity and zeta potential of the prepared chitosan nanoparticles between the 2 varieties of date seeds.

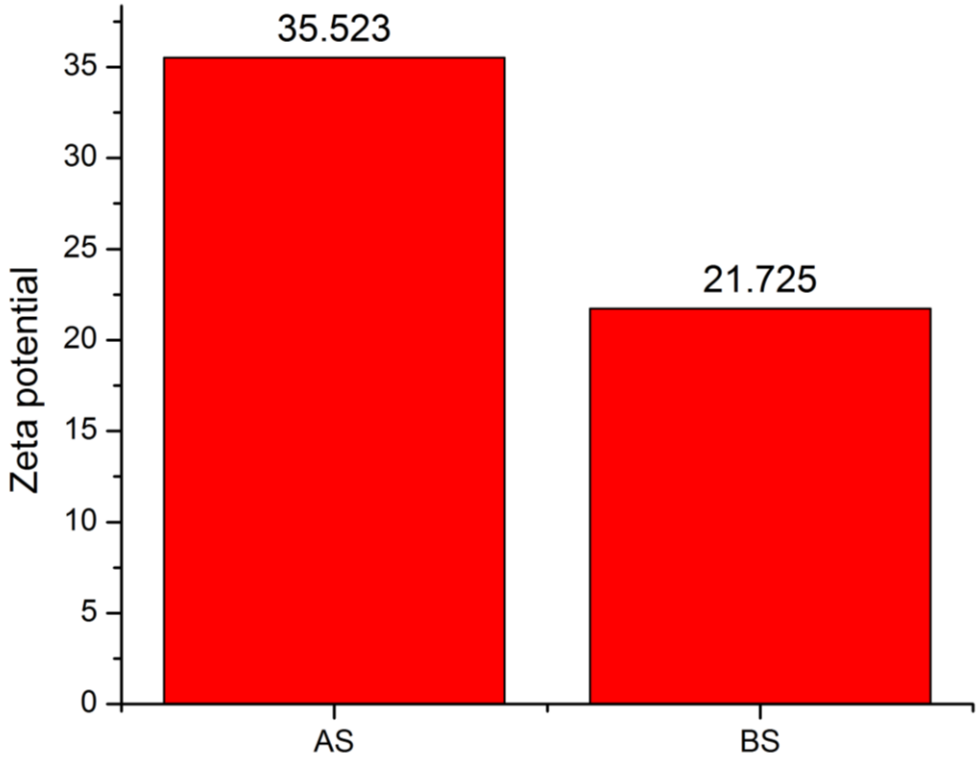

Fig3.8. The zeta potential values for the prepared NPs of AS and BS at stirring conditions of 60°C for 60min, centrifugation 15000 rpm at 4°C for 30 minutes and TPP of volume 6ml

Table3.9. Summary of the average particle size, PDI and zeta potential for the prepared NPs

Plant/ centrifugation rpms at 60 min 60°C	Particle size in nm	PDI	Zeta potential
Ajwa 13000	531.3±302.1	-	41.2±1.28
Ajwah 15000	152.3±43.7	0.39525±0.06	35.52±1.61
Black 13000	337.5±161.7	-	22.67±1.24
Black 15000	377.48±156.6	0.3744±0.082	21.72±0.454

N.B. A is Ajwah date seed, B is Black date seed, 13000 and 15000 are the rpms of the centrifugation and both at the same stirring conditions. _ means that the samples were out of range Values are shown as mean ±standard deviation and n=5

It was concluded that; the least hydrodynamic size was the Ajwah date seed/black date seed at 15000 rpm centrifugation and stirring at 60°C for 60 min at 110 rpm and at TPP volume of 6/8 mL. They showed a high degree of stability of potential above +35 as shown. After settling on the protocol regarding the amount of chitosan, the stirring and centrifugation conditions, further imaging techniques and morphological characterization would be done to determine the plant variety used and the TPP rate to be added at which rate.

1.6.1.1.Morphological characterization of the prepared plant seed/chitosan NPs:

Scan Electron Microscope (SEM):

Microscopic morphological analysis was done by model Gemini drops of NPs aqueous solution was put on aluminium stub double sided tape and dried in oven for 2 or 3 min and then analyzed. Gold sputtering is needed for chitosan NPs to look clearer and to increase their conductivity. Sputtering in general is coating the sample with a thin layer of thickness 2-20 nm of a metal like gold (Au), gold palladium (Au/Pd), Platinum (Pt), Silver (Ag), Chromium (Cr), Iridium (Ir). This turned the sample more conductive and inhibited the charging of the specimen which could occur due to static electric field. [118] Sputtering was a crucial step in SEM for the following advantages:

- Reduced microscope beam damage.
- Increased thermal conduction.
- Reduced sample charging (increased conduction).
- Improved secondary electron emission
- Reduced beam penetration with improved edge resolution
- Protects beam sensitive specimens [150]

We would find that for example the chitosan/Ajwah date seed NP SEM image approximate size at TPP volume 6 mL was 129.935 nm without gold sputtering versus 37.899 nm with gold sputtering.

Appendix [4.6] and Fig.3.9. showed the different crystalline patterns of the prepared NPs and Table3.10. and Table3.11. showed the partice size of the NPs as measured by SEM.

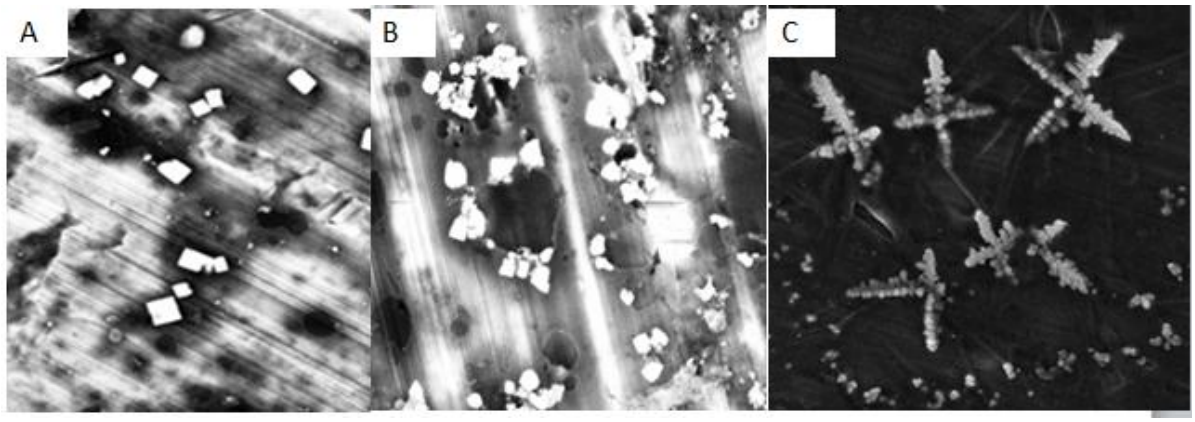

Fig.3.9. Scanning Electron Microscope of (a) aggregated plain vacant chitosan NPs showed square shaped crystals (b) aggregated chitosan NPs loaded with the black date seed extract showed square shaped crystals (c) aggregated chitosan NPs loaded with the Ajwah date seed extract showed star shaped crystals

Fig.3.9. showed that NPs were spherical,yet when they aggregated and coalesced they turned to form clustered pattern either square shaped as in black date seed or star shaped as in Ajwah date

seed. According to Pranami G. *etal.* This aggregation pattern was dependant half on the biodegradable polymer and half on the plant itself. The shape and pattern of the aggregation was dependent on the morphology of the NPs as in Fig.3.10. and Fig.3.11.[145]

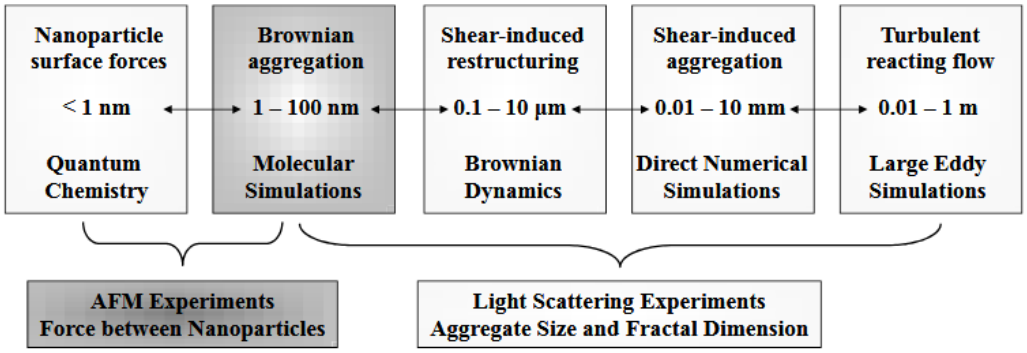

Fig.3.10. Organization of the inter-disciplinary research framework for modeling NP aggregation was at vastly varying multiple length-scales. The experimental contributions are shown at the bottom [145]

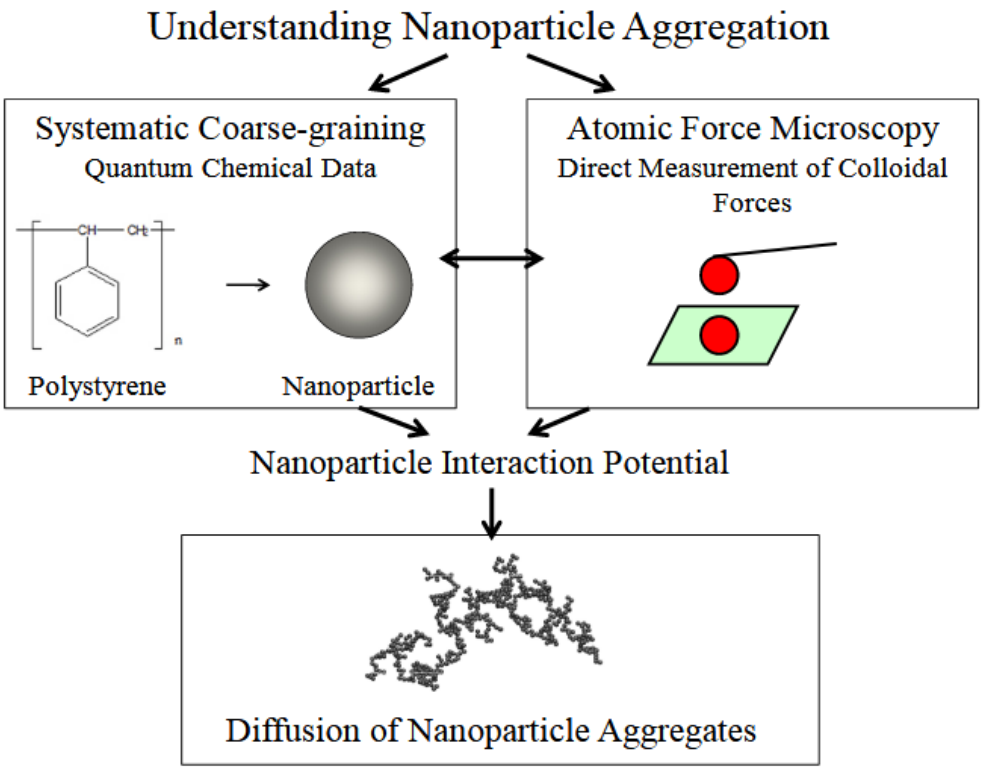

Fig.3.11. Mechanism of nanoparticle aggregation [145]

A second factor which highly affected the aggregation pattern of the NPs was the pH. According to R.K. Saini *etal.* the chitosan NPs were stable at pH from 3 to 6.5. In this pH, kinetic stability of the colloidal suspension was dependent on the repulsive forces of electric double layers of the surface of NPs and attractive Vander-wall forces. When the charge on the surface of NPs decreased, the amino group would be neutralized, the repulsive forces would decrease so the attractive Vander-wall forces would pre-dominate the repulsive forces and superimpose them, thus aggregation would occur at pH above 6. [146]

On the other side, the plant type itself has a very crucial role in the shape and morphology of the crystals. According to, Nabili A.*etal* 2016 and 2017, the first reason for the date crystallinity is the cellulose of the cotyledons and the seed. Cellulose and the acetylated form of the cellulose as well were responsible of the crystallinity of the plant as would be discussed in a clearer way in the X ray diffraction patterns [113] [116]. Another reason was the structural composition of the cotyledon itself. According to Darleen A.*etal.*, the cotyledons were formed of 3 teguments, protoderm, procambium and storage parenchyma. The protoderm was formed of multilayered sympodia which later on bifurcates by mitosis to multiply in number. Storage parenchyma contained crystalline bodies as globoids or phytin. Phytin was a group of insoluble mixture of potassium, magnesium, calcium salts of phytic acids and myoinositol hexaphosphoric acid. They were membrane bound and irregularly shaped as shown by the Electron micrograph. [148]

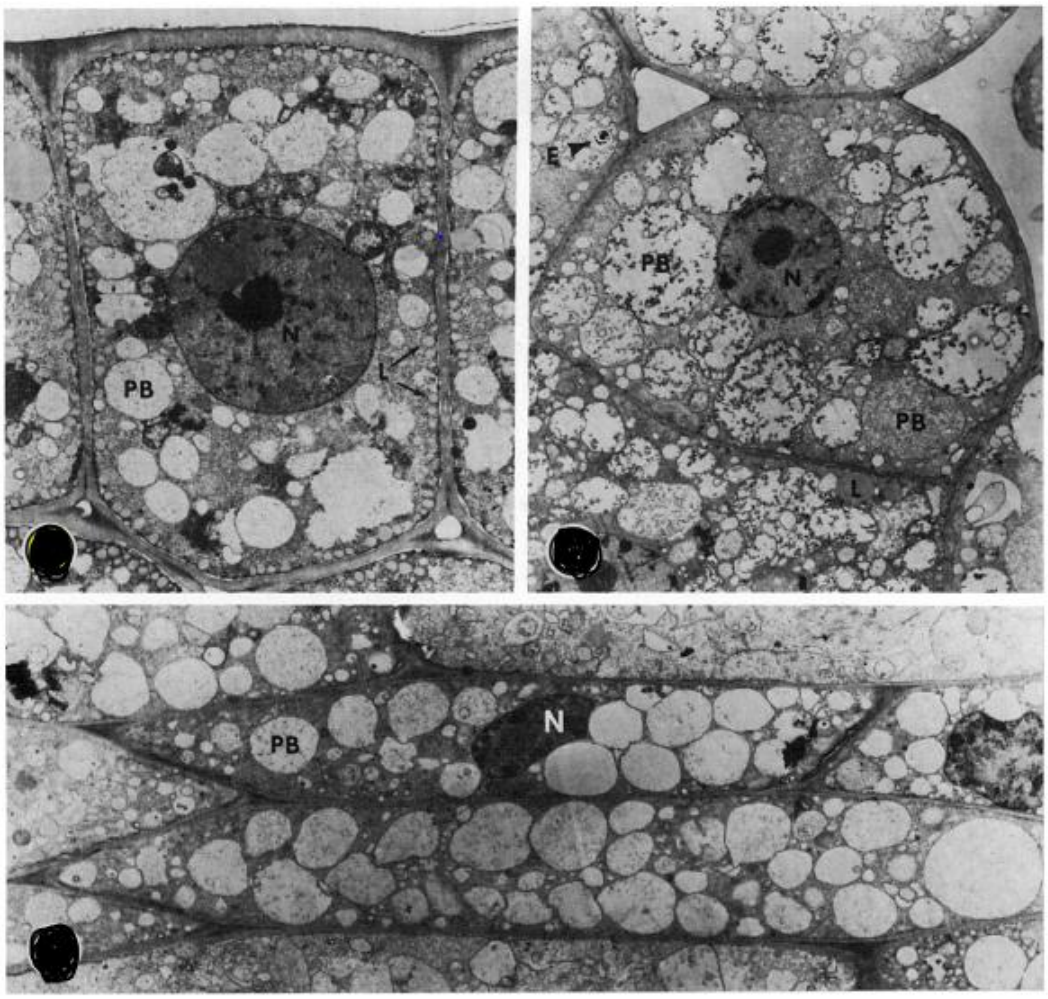

Fig.3.12. the electron micrograph of the protoderm and the parenchyma cells to show the inclusion dark colored crystals [148]

Table3.10. SEM average sizes for. Chitosan / AS or BS plant extract NPs at centrifugation power 13000 and 15000 rpm

Plant variety and centrifugation speed	Particle size in nm by SEM
AS 13000	177.53±3.7
AS 15000	24.07±0
BS 13000,	36.48±3.8
BS 15000	51.93±23.52

Table3.11. SEM average sizes for Phoenix dactylifera L. chitosan nanoparticles at centrifugation power 15000 rpm and stirring conditions 60 minutes at 60 degrees; at various TPP volumes, 6 and 8 mL.

Plant variety	TPP 6 mL	TPP 8 mL
AS	37.89±23	24.07±0
BS	53.11±3.07	51.96±23.52

Table 3.10. and Table 3.11. showed that the size by the SEM was much smaller than that by the zeta sizer since the focused beam of electrons beam made the particles more or less far away from each other; that is why for example in case of the Ajwah plant seed extract NP we would find that the size by zeta sizer was approximately 152.37±43 nm whereas the same sample by the SEM appeared to have size of 24.070±0 nm. Yet we would observe the minimum size was achieved at TPP volume of 8 ml. In which the size of the NPs reached a range of 2-5nm as would be shown in Table 3.14. in which they proved to have much better size range than that reported by Madadlou *etal.*, where the NCs prepared were around 198 nm but the SEM images showed high aggregation as shown in Appendix [4.6]. [33] For those NPs prepared by Manal Awad *etal.*, where they had wide range of size from 1-220 nm. This wide range could be due to the absence of cross-linker that kept the size range narrower and stable for long time period avoiding aggregation or agglomeration. [105]

From all the above physicochemical and morphological characterization, though the black date seed (BSOD) showed better TPP and AO% than Ajwah seed extract (ASOD), ASOD was the plant extract of choice to prepare the NPs from since it gave the smallest PDI, the smallest NPs size and the highest zeta potential. It was concluded as well that the best protocol for the preparation of the NP was of plant variety Ajwah date seed/ chitosan NP in v/v at stirring conditions of 60°C, 60min at 110 rpm and centrifugation at 15000 rpm at 4°C for 30 min using 6mLs cross linker TPP of concentration 1mg/mL. one crucial factor was needed to be investigated was the effect of variation of the Ajwah date seed extract to reach the maximum loading capacity (LC), encapsulation efficiency (EE) and most prolonged drug release rate (RR) .

3.1.3. Effect of variation of the concentration of the Ajwah seed extract loaded in the prepared chitosan NP:

3.1.3.1. Physicochemical and spectroscopic measurements of the prepared plant seed/ chitosan NPs:

Concentration of the plant seed extract under test would be varied to reach the optimum ASOD concentration which could be encapsulated in the chitosan NPs. Chitosan : ASOD was added with equal v/v but variation was done w/w. Applying the same procedures as above and measure the size of the produced NPs, PDI and the zeta potential the following results were obtained.

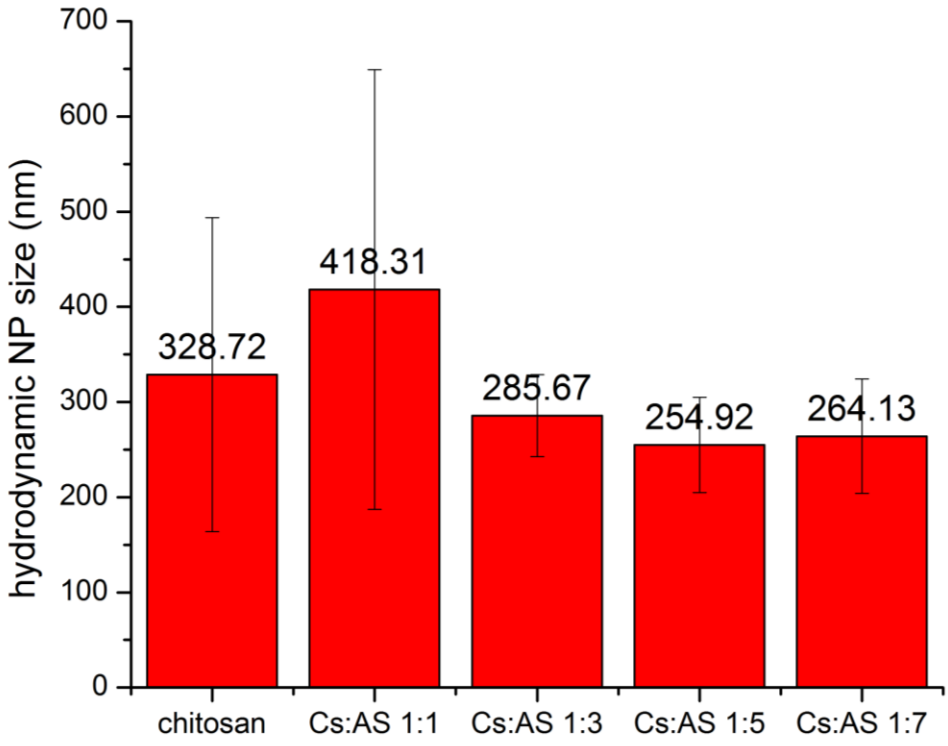

Fig.3.13. Impact of variation of the plant concentration on the hydrodynamic size

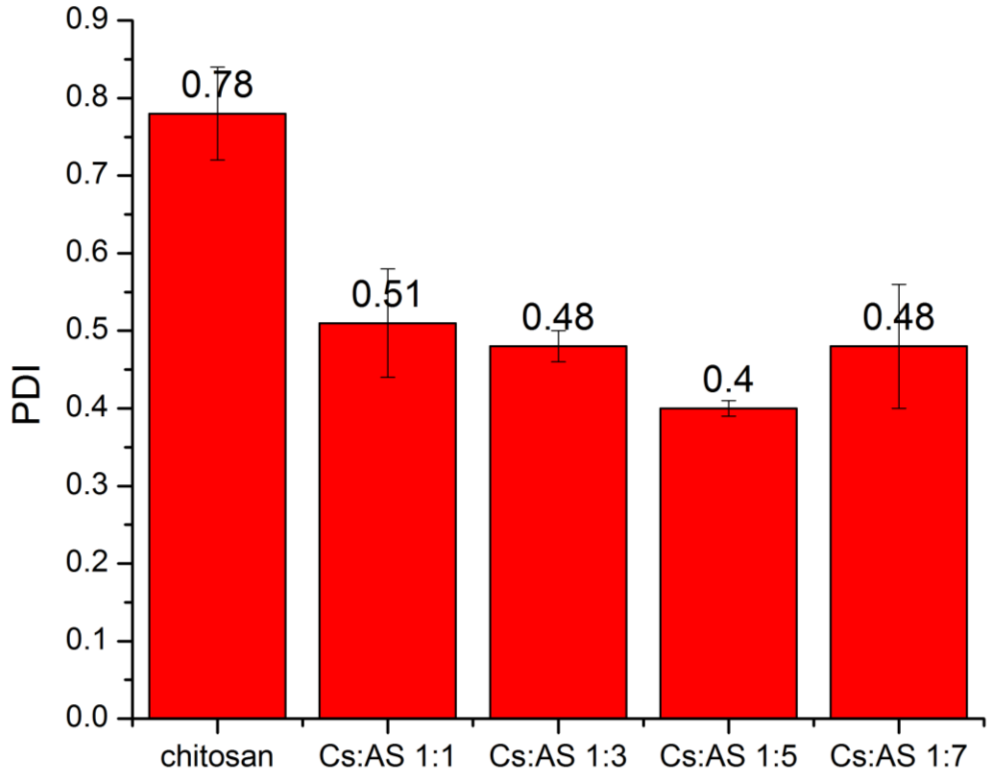

Fig.3.14. Impact of variation of the plant concentration on the particle dispersion index PDI

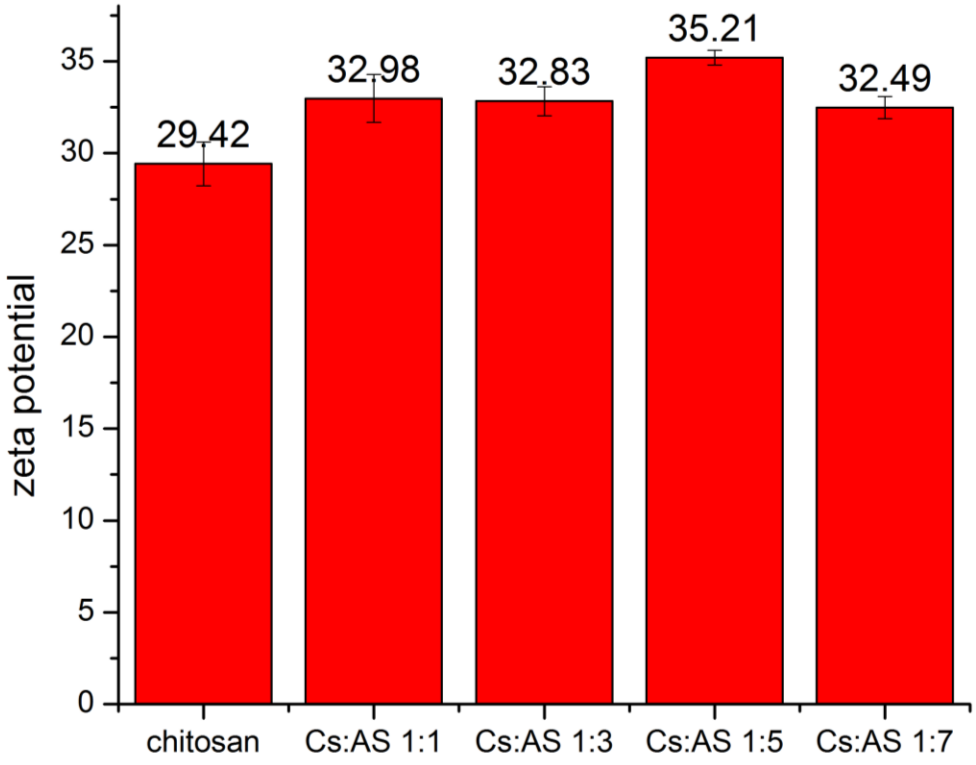

Fig.3.15. Impact of variation of the plant concentration on the zeta potential

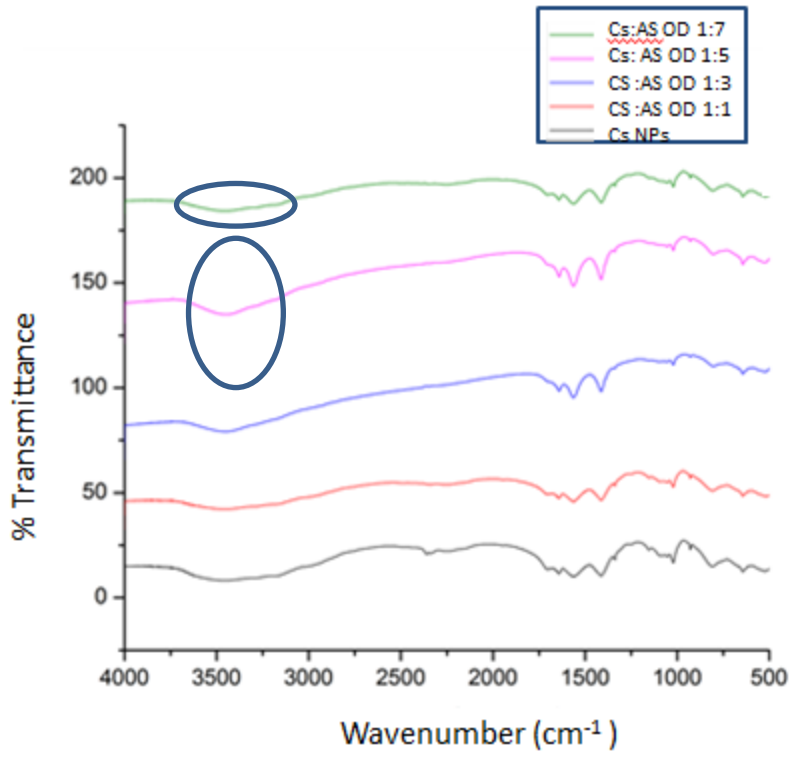

Fig.3.16. FTIR chart to show the impact of plant concentration variation over the encapsulation, shape and cross linkage degree

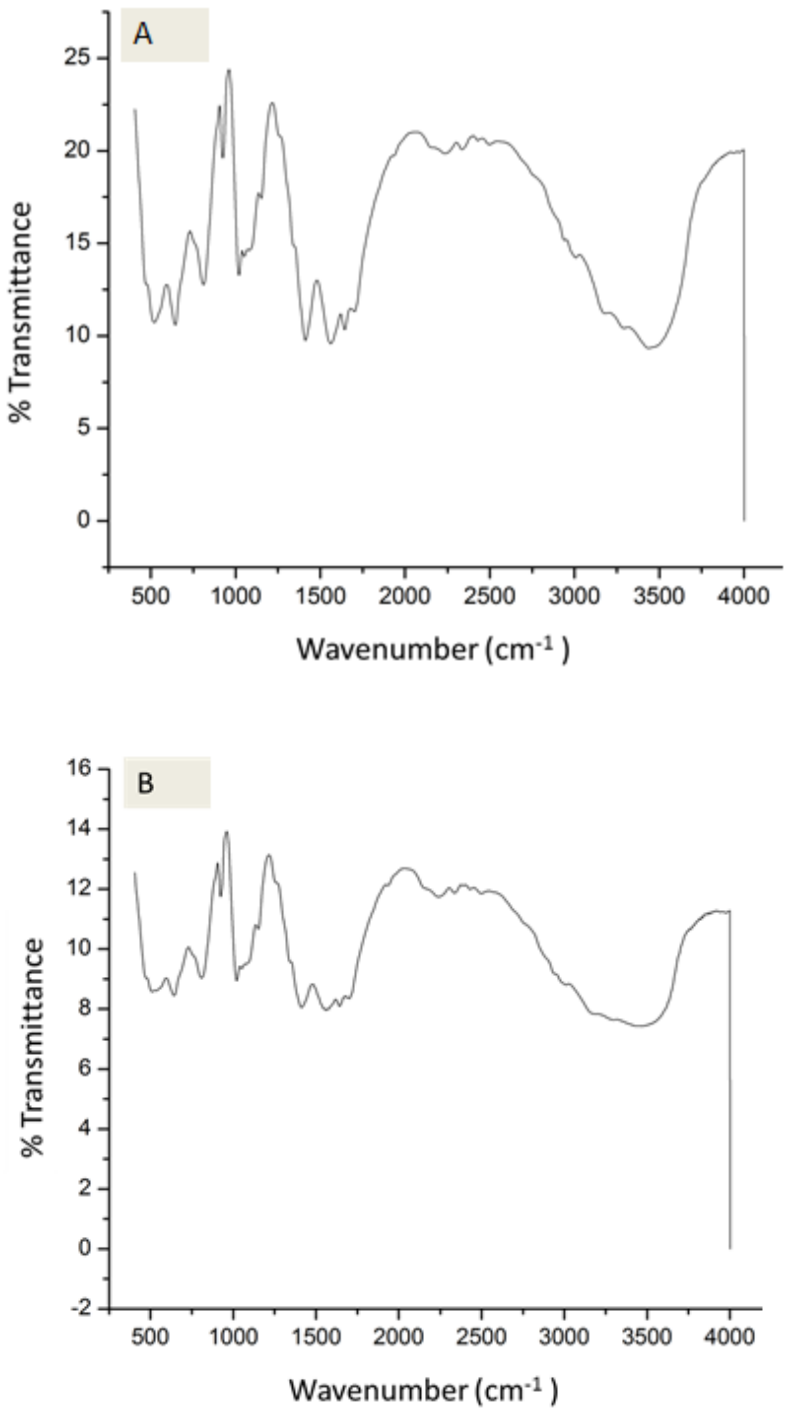

Fig.3.17. FTIR charts of the Chitosan: Ajwah NPs in ratios (a) 1:5 and (b) 1:7 (w/w)

In the IR chart Fig.3.16. and Fig. 3.17. The 4 main peaks mentioned before and encircled in the above graph which represented catechin, proto catechuic, Gallic acid and Ferulic and Apigenins

showed high enhancement when the Ajwah date seed was formulated in the nano form especially in the Cs:Ajwah concentrations in ratios; 1:3, 1:5 and 1:7.

Table3.12. The summary of the average particle size, PDI poly dispersion index and zeta potential using Zeta-sizer nano for second samples set trial 2

Sample name Chitosan : Ajwah ratio (w/w)	Particle size in nm	PDI	Zeta-potential
1:0	328.72±165	0.78±0.06	29.42±1.2
1:1	418.31±231	0.51±0.07	32.98±1.3
1:3	285.67±43	0.48±0.02	32.83±0.8
1:5	254.92±50	0.40±0.01	35.21±0.4
1:7	264.13±60	0.48±0.08	32.49±0.6

It was concluded that the best two preparations which gave the minimum size, PDI and maximum zeta potential were those of Chitosan: Ajwah in ratios of 1:5 and 1:7. Other investigations need to be made concerning the drug loading, encapsulation efficiency and morphological assay.

3.1.3.2. Morphological characterization of the prepared plant seed/chitosan NPs:

Transmission Electron microscope; TEM:

The prepared NPs were diluted to 0.02 mg/mL and then one drop was placed on a filter paper. The drop was left to dry then put the dyed using phospho-tungesten dye. The prepared chitosan NPs were hydrophilic and prepared in DW. Thus they required fixation so as the ultra-structure of the sample remained close to the biological cell. Formvar film supported the sample of ultra-thin section over the copper holder. Then this sample got coated with light carbon layer. The stain absorbed electrons and scattered part of the electron beam which was actually florescent. After that the sample was taken by a long metal handle to be adjusted in the apparatus TEM JEOL. [149]

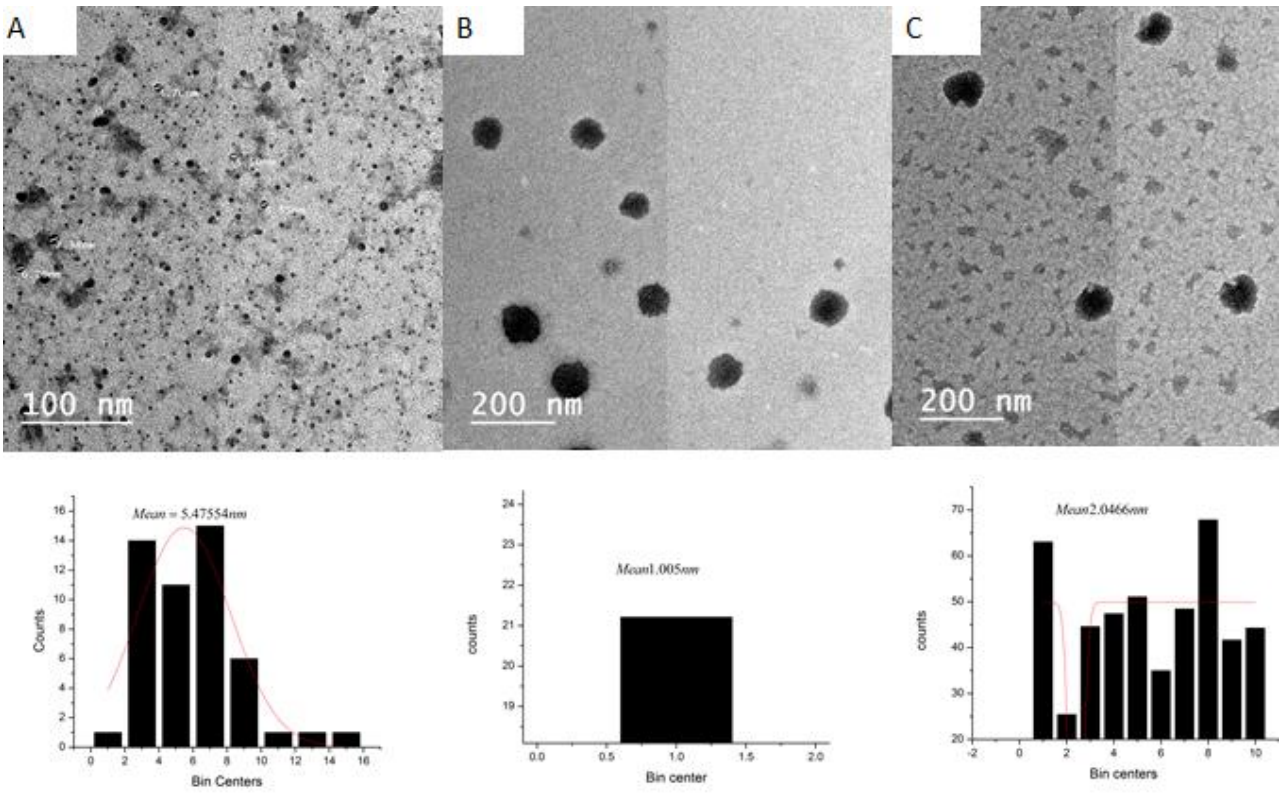

Fig.3.18. TEM image of chitosan NPs loaded with Ajwah date seed plant (ASOD) at ratios (a) 1:3 (b) 1:5 (c) 1:7 with mean particle size (nm)

As shown in Fig.3.18. The particle size by Transmittance electron microscope was much smaller than the scan electron microscope. It is a more accurate and sophisticated apparatus to monitor and measure the NP. The thermal evaporation of carbon is widely used for preparing specimens for electron microscopy. A carbon source – either in the form of a thread or rod was applied in a vacuum system between two high-current electrical terminals. When the carbon source was heated to its evaporation temperature, a fine stream of carbon was deposited onto specimens. [149]

Table3.13. showed a comparison between the imaging using the SEM versus the TEM

Table3.13.Compare between the SEM and TEM to show that the TEM is better in imaging [151]

SEM	TEM
Magnification power ×20000-40000	×1200000
200 nm index	10 nm index
Monitors macro size adhesion	Micron size adhesion
Monitor adhesion at scale of 200 nm	At scale of 70 nm or less thus it determines the adhesion and crystalline structure of the NPs

Table3.14. Compare the mean NP size for the samples of Chitosan : ASOD in ratios 1:3, 1:5 and 1:7 according to measurements by TEM

Sample name ; Chitosan: ASOD	**1:3**	**1:5**	**1:7**
Size nm	5.48	1.01	2.05

From Fig.3.18. and Table 3.14. it was clear that the best 2 preparations of NPs which showed the minimum particle size with uniform morphology and least aggregation were those of chitosan : ASOD in ratios 1:5 and 1:7 (w/w).

XRD; X- ray diffraction:

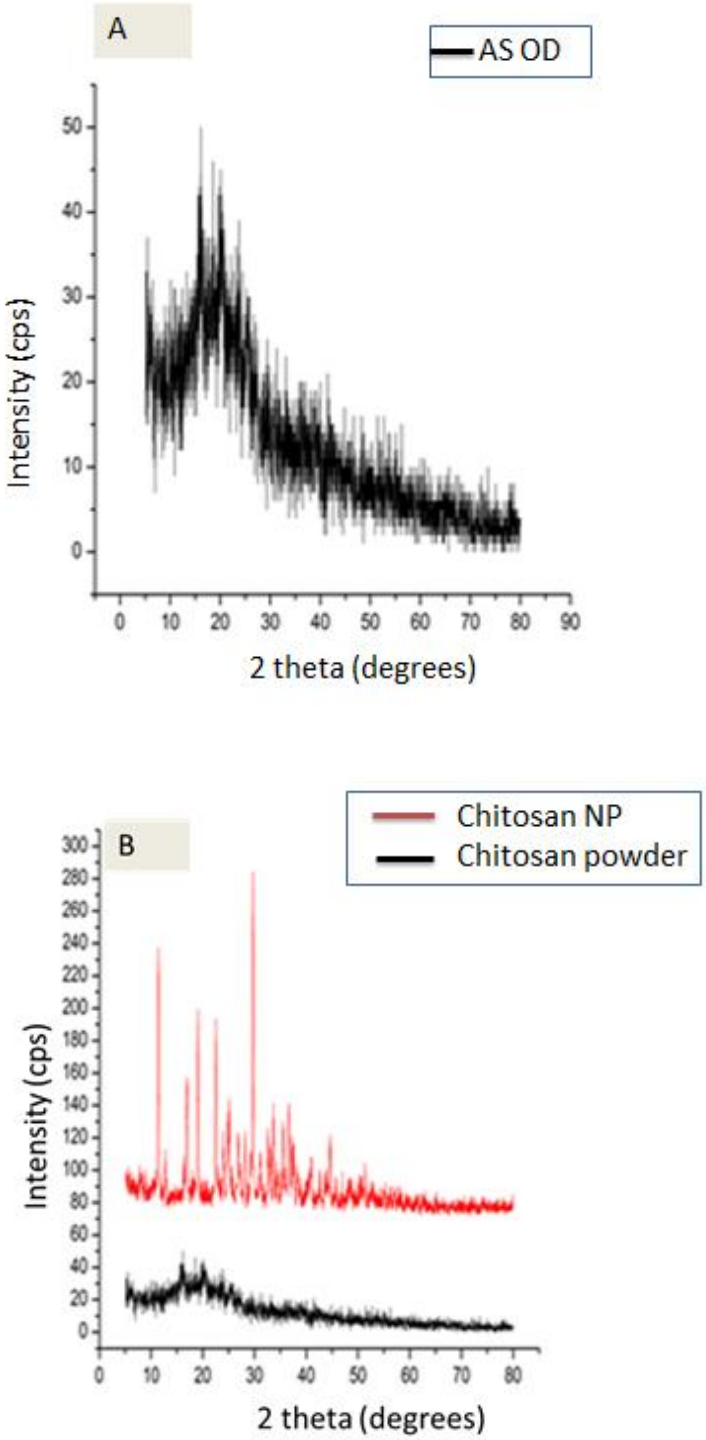

Fig.3.19. XRD of (a) Ajwah date seed (b) compare the Chitosan powder to Chitosan NP

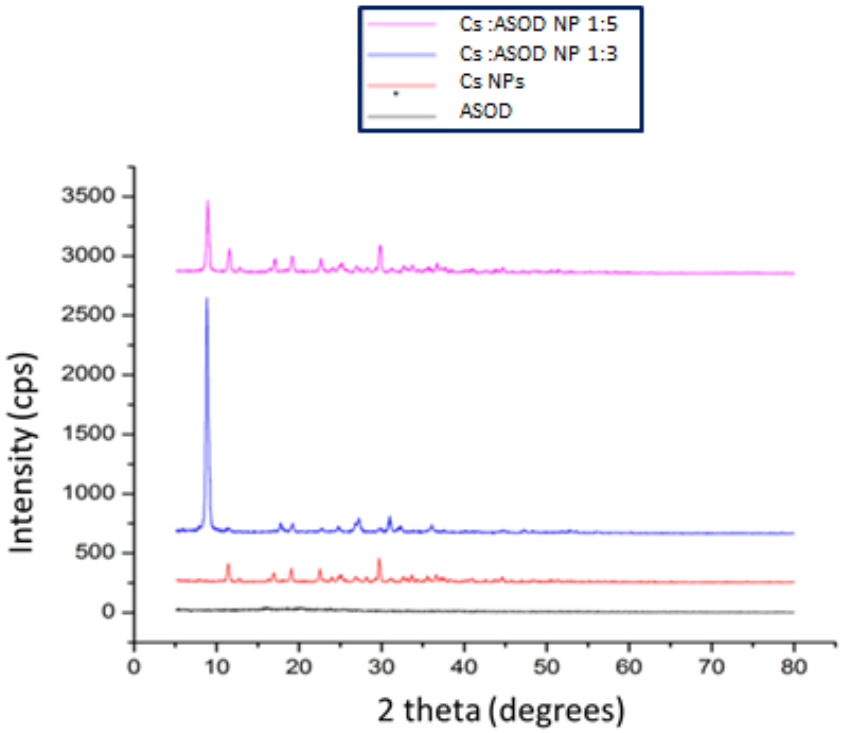

Fig.3.20. the XRD of Ajwah date seed and chitosan NP and compare them to the prepared Chitosan : Ajwah date seed in ratios 1:3 and 1:5 w/w to detect the occurrence of encapsulation

According to Wan *etal*. and Pokharkar *etal.,* chitosan was charachterized by 2 peaks at incidence angles 10° and 20°. Appearance of a peak at 30° was indicative of the encapsulation of the Ajwah date seed extract. The intensity of the peak changes due to the degree of cross linkage which altered the atoms arrangement in the crystal lattice. [135] [136]

The acidic medium made the peaks appeared sharper and stronger according to Long Mi F.*etal*. [151]

The morphology of the extract added affected the XRD pattern of the formed NPs for example as was reported by Awual S.M. *etal*. who used stone fish bio peptide as extract. It was highly amorphous that is why it made the NP looked less crystalline. The Ajwah date seed was used as such without neither mechanical, chemical treatment nor removal from the seed. According to B.Manoj.*etal*. and Nabili A.*etal.* 2014 keeping the cellulose increased the crystallinity of our structure and would acquire peaks at 16.1°, 20.2°, 39.2° and 25.4° for crystalline carbon. [66] [143] [144]

Using acetic acid 0.5% (v/v) in preparation of chitosan affected the cellulose of the Ajwah seed and produced acetylated cellulose. According to Nabili A.*etal*.2017 the acetic acid addition gave semi crystalline cellulose triacetate which had 2 sharp crystalline peaks at 22° and 7.9°. This was due to the increase in the inter-fibrillar distance on adding the acetyl group along the cellulose chains. [147]

3.1.4. Encapsulation efficiency and loading capacity*:*

According to Venna sanna *etal.* and Somnuk J.*etal.*, we first performed a calibration curve for serial concentrations of the Ajwah date seed extract to scan for the most prominent peak against which the absorbance would be measured of the extract encapsulated in the NP. It was realized from the measurement that it was at wave length= 285 nm which most possible referred to catechins and epi-catechins [4][15][17][18][19][22[]77] [79][80][137]

A plot of concentration at x-axis and absorbance at y-axis was drawn to calculate the regression line and regression equation with r^2 factor 0.99.

Y=ax+b where y is the absorbance and x is the concentration , b is the intercept and a is the slope.

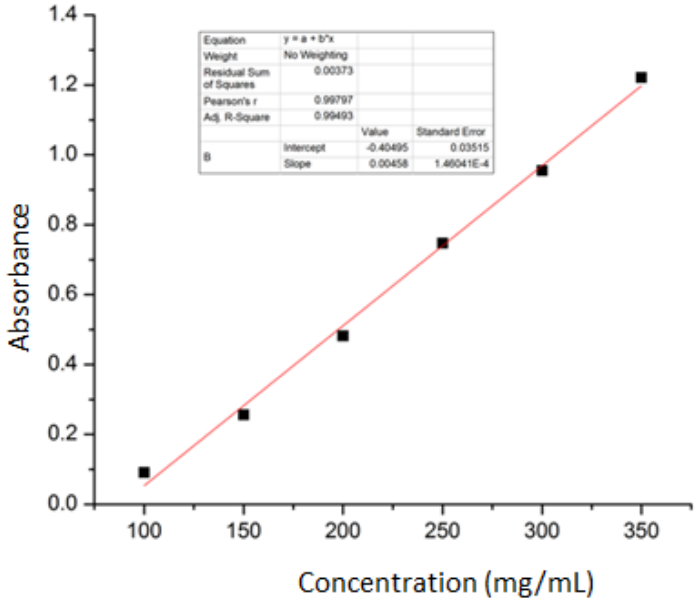

Fig.3.21. The regression line for the serial dilutions of Ajwah date seed at 285 nm

The straight line equation was; Y= 0.00458-0.40495X i.e. intercept= 0.00458, slope = -0.40495

Many conditions and parameters were followed by altering the stirring and centrifugation time, rpm and temperature to reach the optimum conditions which led to the best Encapsulation efficiency (EE)and loading capacity (LC) as shown in Table 3.15;

Table3.15. Trials of varying the conditions of stirring and centrifugation to study EE and LC

	Centrifugation			**Result**
Stirring	**RPM**	**Time**	**Temp °C**	Failure all the samples gave low absorbance or negative absorbance (negative results)
24 hrs	15000	30min	25	
No stir	20000	30min	40	
	20000	1 hr	40	
	20000	1.5 hrs	40	
Stir 24 hrs at 500rpm	20000	30 min	40	
	20000	1 hr	40	
No stir	20000	30min	25	
Stir 500 rpm 24 hrs at 25°C	20000	30min	25	(positive results)

From the above trials we studied 2 parameters in encapsulation efficiency and loading capacity study;

1. Stirring and centrifugation time, temperature; absence or presence of stirring, stirring at 500 rpm for 24 hrs, 12 hrs and centrifugation at 30, 60 and 90 min at 15000 and 20000 rpm and at 25 and 40°C. When stirring performed at low rpm or for 12 hrs only, when samples were centrifuged at 15000 rpm this led to lower moving rate of the drug. Increasing the centrifugation time to more than 30 min led to the return back of the drug inside the NPs. When temperature applied in release reached 40°C this led to increased

flocculation. That is why the optimum protocol was to prepare samples with concentration 2mg/mL in 10 mL DW, at stir at 500 rpm on magnetic stirrer at room temperature for 24 hrs then centrifuge at 2000 rpm for 30 min at room temperature. This was the only condition which gave positive absorbance values on the UV spectrophotometer. [20] [77] [152]

2. Effect of variation of Ajwah date seed concentration:

Table3.16. Study the impact of the plant concentration variation on encapsulation efficiency and loading capacity

Sample Chitosan :Ajwah NPs (w/w)	**Theoretical amount of the Ajwah date seed in the prepared NP in (mg)**	**EE%= drug mg/theoretical amount in mg ×100%**	**LC%= drug in mg/20 mg**
1:1	9.4	114.8	54
1:3	14.4	84.72	61
1:5	16.2	94.9	77
1:7	17.2	70.37	61

The encapsulation efficiency and loading capacity were 2 crucial factors to determine the efficiency of the nano carrier prepared. In order to increase the encapsulation efficiency and loading capacity percent one of the ways to be followed was to increase the concentration of the entrapped drug. In the above table it was clear that when the concentration of the plant increased from one fold to five-fold that of the chitosan the EE and LC increased till reach 94.9 and 76.9% respectively. Yet when the plant concentration was 7 fold that of the chitosan the preparation flocculated a little that is why the EE and LC percentages tended to retrieve back. [20] [77] [152] Though this smaller value of EE% and LC %of the NP of ratio 1:7 (w/w) yet it would be the NP of choice due to its antioxidant and antibacterial results as would be mentioned later.

In many cases the increase in EE% and loading capacity was pertained to the structure rather than the amount of the polymer or the drug added. For example when the pH suffered sharp decrease due to increase in the amount of carboxylic acid this led to increase of EE and loading capacity. [156]. When loading capacity reached high values it mean that minimum amount of the polymer was required to act as a drug delivery system. [158]

According to Stephanie J.W.*etal.*, when NPs were not well dispersed in the solution or the solution was not filtered by more sophisticated means of centrifugation like by ultrafiltration or pressure where the filtrate pass through tiny pores so as to have a filtrate completely free from any clogged, agglomerated or aggregated NPs, the filtrate would just carry the free plant extract. When the chitosan was equal in weight to the Ajwah date seed extract added, the NPs were aggregated which led to blurred UV readings due to inability to scatter light. The UV reading could explain the reason that EE% was above100%. [153]

3.1.5. ***In-vitro* release test*:***

According to Vanna Sanna *etal.* release rate was studied over 48 hrs in 2 different media, first; the gastric simulation media 0.1 M Hydrochloric acid (HCl) at pH=1.2 and the second was the intestinal simulation media 0.2M phosphate buffer saline (PBS) at pH=7.4. [75] Referring to Appendix [4.7] showed the graphs comparing The effect of the Ajwah date seed extract concentration variation and the pHs variations on the release profile.

1. Effect of the Ajwah date seed extract Concentration:

Fig.3.22. and Table3.17. showed the cumulative release profile in (a) HCl (b) PBS and (c) both media for the 4 samples of the chitosan NPs loaded with the Ajwah date seed extract to compare which one showed the maximum release rate

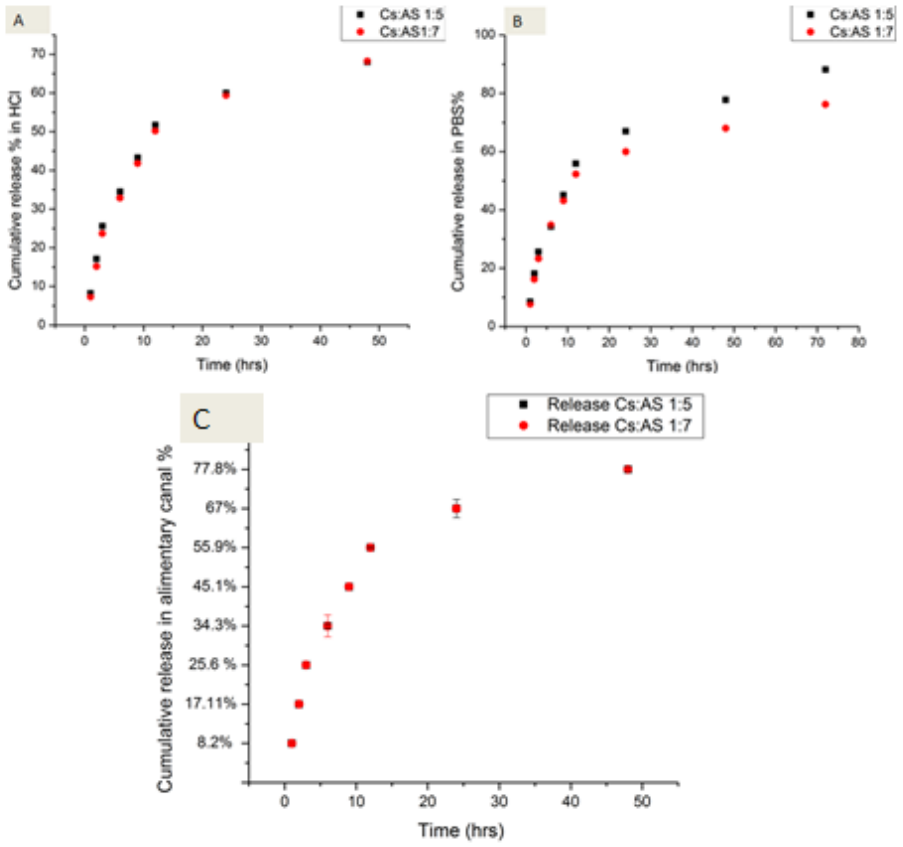

Fig. 3.22. Cumulative release profile in (a) HCl (b) PBS and for the chitosan NPs loaded with the Ajwah date seed in ratios of Chitosan:AS 1:5 and 1:7 (w/w) (c) Cumulative release profile in gastric simulation fluid (HCl) for first three hours followed by release profile in intestinal simulation fluid (PBS) for the chitosan NPs loaded with the Ajwah date seed in ratios of Chitosan:AS 1:5 and 1:7 (w/w)

Table3.17. Cumulative drug release for the 4 samples of Chitosan NPs loaded with the AS at certain time intervals in acidic and alkaline media

Release (Hrs)	Cs: Ajwah ratio 1:1		1:3		1:5		1:7	
Media	HCl	PBS	HCl	PBS	HCl	PBS	HCL	PBS
1	13% $\pm 7\times10^{-2}$	12% $\pm 2\times10^{-3}$	8.00% $\pm 2\times10^{-3}$	9.20% ±0.07	8.20% $\pm 6\times10^{-3}$	8.50% ±0.12	7.30% $\pm 4\times10^{-3}$	7.70% $\pm 4\times10^{-2}$
2	26% $\pm 3\times10^{-2}$		16.60%±1 $\times10^{-3}$	18.60% $\pm 7\times10^{-3}$	17.11% $\pm 6\times10^{-3}$	18.20% ±0.067	15.20%± 6×10^{-3}	16.20% $\pm 1\times10^{-3}$
3	39%± 4×10^{-2}	24% $\pm 4\times10^{-3}$	25.88%±1 $\times10^{-3}$	27.50% $\pm 3\times10^{-2}$	25.60% $\pm 3\times10^{-3}$	25.60% ±0.01	23.70%± 4×10^{-3}	23.30% $\pm 2\times10^{-3}$
6	53%± 4×10^{-2}		35.40%±5 $\times10^{-2}$	39.30% ±0.12	34.50% $\pm 3\times10^{-2}$	34.30% ±0.05	32.90%± 6×10^{-2}	34.80% ±0.28
9	68% $\pm 1\times10^{-2}$		44.60%±1 $\times10^{-2}$	49.60% ±0.11	43.30% $\pm 9\times10^{-2}$	45.10% ±0.06	41.81%± 5×10^{-2}	43.20% $\pm 6\times10^{-2}$
12	81% $\pm 7\times10^{-2}$		53.50%±5 $\times10^{-3}$	60.56% ±0.09	51.74% $\pm 2\times10^{-3}$	55.90% ±0.02	50.20%± 1×10^{-2}	52.30% $\pm 4\times10^{-3}$
24	95.40% $\pm 5\times10^{-2}$	36.30% $\pm 5\times10^{-3}$	62.80%±7 $\times10^{-3}$	71.10% ±0.05	60%±7 $\times10^{-3}$	67.00% ±0.23	59.40%± 1×10^{-3}	60% ±0.08
48	109%±2 .62	51.20%±2 $\times10^{-2}$	71.90%±5 $\times10^{-3}$	83.50% ±0.1	68%±5 $\times10^{-2}$	77.8% ±0.07	68.2%±. 2×10^{-2}	68%±0. 049

The NPs of Chitosan : Ajwah seed in ratios 1:5 and 1:3 w/w in HCl always showed zero order kinetics of drug release. The drug released independent on the initial concentration of the drug dissolved in the media. It was applicable in the non-disintegrative dosage forms as depot and topical applications according to the following equation

Equation.3.1. [154][155]

$$Q_t = Q_0 + K_0\, t$$

Where Q_t is the initial amount of the drug, Q_0 is the cumulative amount of the drug release at time t , T is time in hours and K_0 is the zero order kinetic constant.

Other preparations of NPs of chitosan: Ajwah in ratios is 1:5 and 1:7 in PBS were prepared and showed first order kinetics of drug release. In this model, the drug release was dependant on the initial drug concentration dissolved in the media. This model could explain the sustained release profile of the prepared NP sample 1:5 and 1:7 (w/w) to have prolonged antibacterial activity as shown in Equation 3.2.

Equation.3.2. [154][155]

$\text{Log } Q_t = \text{Log } Q_0 + Kt/2.30$

Where Q_t is the initial amount of the drug, Q_0 is the cumulative amount of the drug release at time t , T is time in hrs and K is the first order kinetic constant [154] [155]

The process of Drug release is the process by which the drug would leave the matrix to go to the outside medium. There existed many types of release, the zero order kinetics could be explained in the preparations of; immediate release, delayed release, targeted release. In those cases, the drug would not be released except once at certain time or certain place. Those preparations were characterized by a fast initial release of the drug followed by slower and continuous rate of release of the drug. The drug here released by either diffusion or polymer matrix erosion. Due to the hydrophilic nature of chitosan, the plant extract easily diffused through the surface pore matrix. [7]

The Chitosan : Ajwah seed NPs prepared which followed the first order kinetics were sustained or extended release preparations. The drug concentration in the plasma increased by time till reach a maximum value. That is why the rate of drug release here was mainly dependant on the increase in the drug or polymer concentration itself (first order kinetics). As the drug concentration increased, the polymer concentration to the drug concentration became relatively low so the polymer got highly diluted and highly dispersed which led to subsequent increase in Encapsulation efficiency, loading capacity and prolonged rate of release. [157]

According to Khaled Mohammed elsay *etal.* and Bahattarai N.*etal.*This protocol was more favorable with antibiotics so as there would be a maximum dose released at the desired time till another dose got administered. There would be a gradual increase in the drug released in the plasma. The release of the extract depended on the concentration gradient between the polymer

carring the extract and the surrounding media inside the body. It followed both 2 mechanisms, the sustained release was maintained by the diffusion through the pore wall of the polymer led to the diffusion of the outside release media in the polymer matrix that eventually led to its erosion as shown in Fig.3.23. [155] [156] [159] [160]

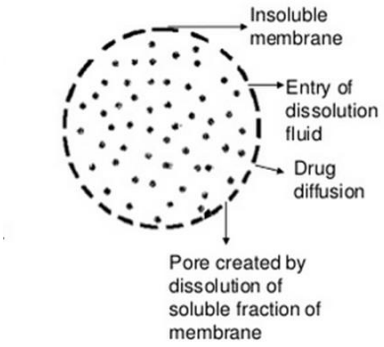

Fig.3.23. Dissolution or matrix erosion and diffusion binary system [155]

It was concluded that the NPs prepared by chitosan : plant ratio 1:7 showed the lower value of rate of cumulative release than 1:5 indicating that lower doses would be needed to be administered since the drug would be released at slower rate in case of the NP prepared at ratio of Chitosan :AS 1:7 (w/w).

2. Effect of pH of the media used:

According to Fig.3.23, Table 3.17 and Appendix [7], it was found that the NPs prepared at ratio of chitosan: plant ratio 1:5 and 1:7 w/w showed different release patterns depending on the media used. In Acidic gastric simulation media it showed smaller increase in rate of release kinetics versus the alkaline intestine simulation media showed bigger increase in release kinetics as time passed . NPs of Chitosan: Ajwah date seed extract at ratio 1:5 (w/w) showed release rate % of 51% in acidic pH versus 56% in alkaline medium at 12 hrs time . then at 48 hrs, they released 68% in acidic medium versus 78% in alkaline PBS medium. At 72 hrs time interval they released 88% drug in alkaline medium. For NPs of Chitosan: Ajwah date seed extract at ratio 1:7 (w/w) showed release rate % of 50.20% in acidic pH versus 52.3% in alkaline medium at 12 hrs time. then at 48 hrs, they released 68.2% in acidic medium versus 68% in alkaline PBS medium. At 72 hrs time interval they release 76% drug in alkaline medium. This meant that those NPs

would release the drug completely after around 4 or 5 days which means it could be administered once per week for example which would be highly reasonable for the patient. This sustained release profile was due to the strong bond between the polyphenolics and the polymer made them not easily dissolved in water or the outside plasma though their hydrophilicity. [77]

NPs released at continuous persistent profile in PBS rather than HCl because according to Ali shahi *etal*.in HCl the drug release occured only by diffusion and the bonds were highly strong and not easily broken. On the other side, in PBS the drug release occured by 2 mechanisms the diffusion and erosion due to the ion exchange between the polymer and release media. In alkaline and neutral pHs weakening of the electrostatic interactions between the poly-anion complexes and the nanoparticle polymer took place. And even the non-electrostatic bonds like hydrogen and hydrophobic bonds that formed in this media were highly fragile and that is why the release in PBS occured at higher rate. [157]

Chapter 3:

Results and Discussion:

II. Applications of chitosan NPs loaded with the Date seed extract

II. Applications:

Assay of the polyphenolic (PP) and antioxidant (AO) contents in the prepared NPs and comparing them with the date seed PP and AO contents is a very crucial step to study the efficacy of the prepared NPs. Those tests were not performed by Madadlou *etal.* nor by Manal Awad *etal.* Only the PP content was assayed by Madadlou and no results were reported concerning the PP content of the prepared NCs. [33] [105]

3.2.1. Polyphenolic content for the formulated NPs:

The 4 prepared NPs loaded with the Ajwah date seed plant extract, one sample of the vacant crosslinked chitosan NP and sample of Ajwah date seed macerated in DW were prepared at concentration of 2 mg/mL. 0.5 mL of each sample was added to 2.5 mL of Folin Cicalteau reagent 10%v/v and then 2 mL 7.5% sodium carbonate were added. All samples were left in dark for 48 hrs to reach the maximum release rate of the polyphenolic content and the results were shown in Fig.3.24.

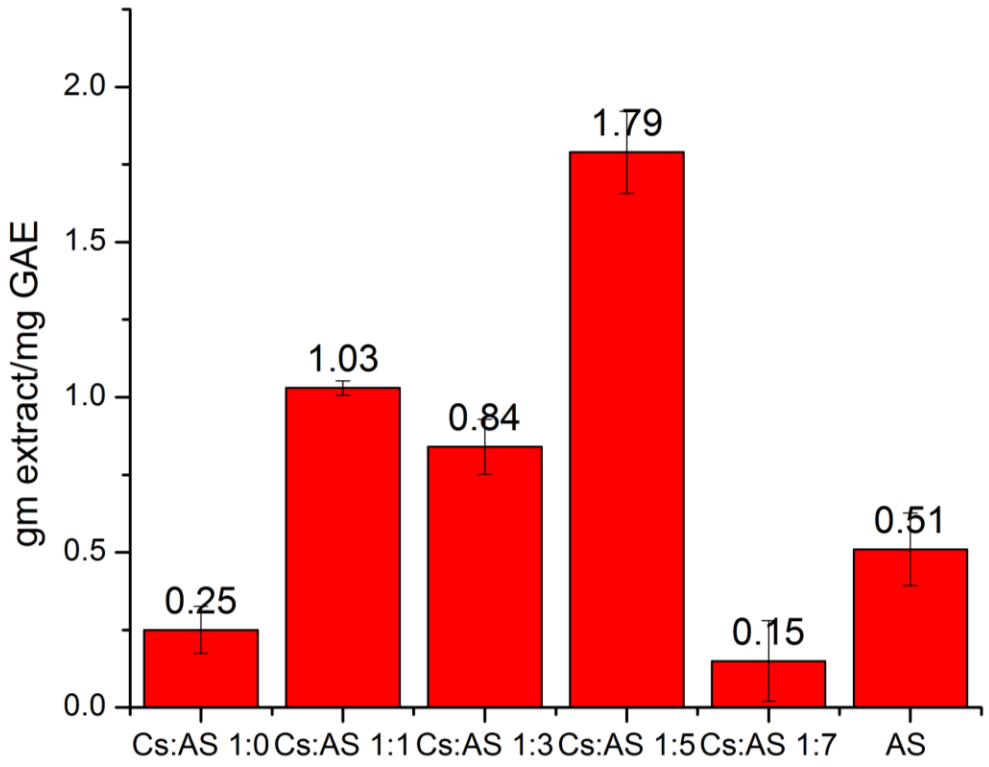

Fig.3.24. Polyphenolic content of the chitosan NPs loaded with the AS plant extract, Ajwah date seed extract alone and the chitosan NP not loaded with the plant extract expressed in gm extract/ mg GAE

It was clear that the best preparation showed the maximum polyphenolics content was the NP of chitosan : Ajwah seed prepared at ratio of 1:5 (w/w) that showed 1.7905 gm extract/mg GAE which was around 3.5 fold the amount of polyphenolics content in the same mass of the plant extract which was 0.50775 gm extract/mg GAE. It indicated what was reported about NPs that they enhanced the intrinsic characters of any original element or plant extract. [16] [135] [138] though those results the NP prepared at chitosan: Ajwah seed ratio of 1:7 (w/w) was the NP of choice due its Antioxidant and antibacterial activity.

3.2.2. Antioxidant activity measurement by DPPH reagent:

First the DPPH test was performed on variable concentrations of gallic acid to reach the optimum concentration which would give the maximum antioxidant effect. Gallic acid at concentration 0.07 mg/mL gave the maximum antioxidant activity at 88%.

NPs prepared at Chitosan :Ajwah date seed extract were prepared at ratios 1:1, 1:3, 1:5 and 1:7 (w/w) at different masses to detect the mass to be used for maximum antioxidant effect and comparing them with the Ajwah date seed. It was concluded that the crosslinked chitosan alone gave negative results which was normal since the chitosan had no antioxidant actions. The NPs were measured for the antioxidant activity after incubation for 48 hrs as was previously studied in the release profiles and the polyphenolic content and the following results were obtained [163] ,

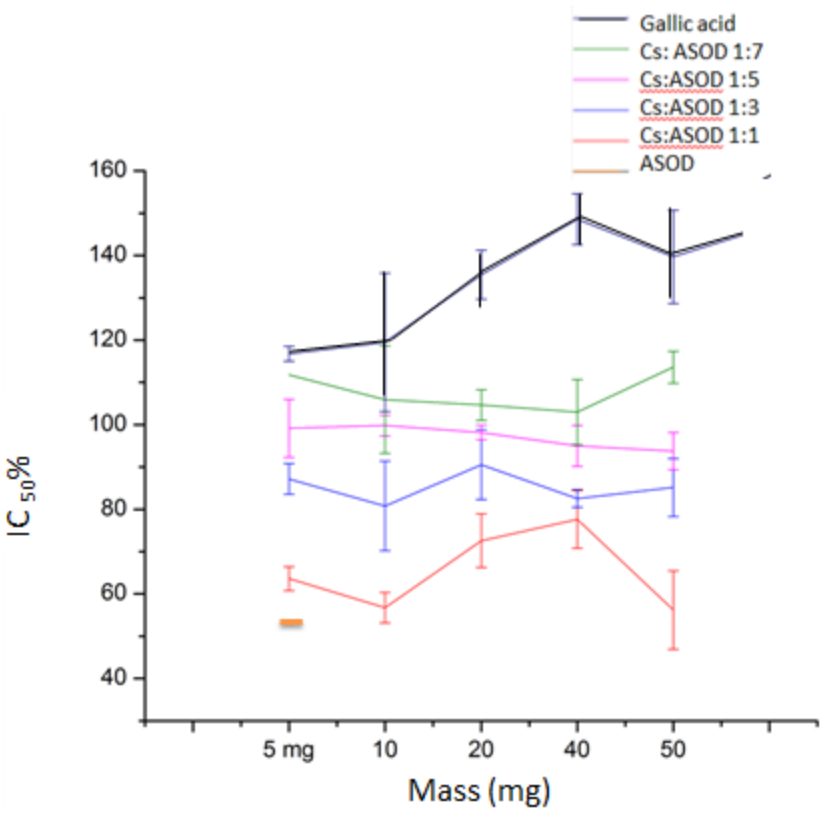

Fig3.25. The antioxidant activity of nanoparticles loaded with the Ajwah date seed extract chitosan: Ajwah seed .at different ratios, 1:1, 1:3, 1:5, 1:7 (w/w) and compare them with gallic acid serial dilutions and Ajwah date seed plant extract

Table3.18. The antioxidant activity of nanoparticles loaded with the Ajwah date seed extract chitosan: Ajwah seed at different ratios, 1:1, 1:3, 1:5, 1:7 (w/w) and compare them with gallic acid serial dilutions and Ajwah date seed plant extract

Chitosan:Ajwah w/w	Mass used mg	AO%
1:1	5	54.16
	10	47.30
	20	63.17
	40	68.2
	50	46.77
1:3	5	56.75
	10	50.38
	20	60.12
	40	52.14
	50	54.73
1:5	5	54.73
	10	55.37
	20	53.73
	40	50.57
	50	49.30
1:7	5	62.40
	10	56.50
	20	55.23
	40	53.57
	50	64.17

According to Brand-Williams *etal.* the DPPH reagent is an oxidizing agent which gets reduced to colorless methanol if there is a potent reducing agent in the media. [18] [111] [136] [140]

The polyphenolics existed in Ajwah date seed extract acted as potent reducing agent since they scavenged the molecular free radical species as super oxide radical $O_2^{\cdot}$, hydrogen peroxide H_2O_2,

hydroxyl radical $HO^{\cdot}$, singlet oxygen[1] O_2 or peroxyl radical $RO_2^{\cdot}$ [161].The polyphenolics would act as antioxidant or reducing agent by either of the 2 mechanisms,

a. Either they donated electrons to form a stable radical cation. In this case they act as singlet electron transferet (SET) and this mechanism was dependant on the ionization potential of the polyphenolic compound (ArOH) in order to give stable ($ArOH^{\cdot+}$) according to the equation 3.3, [161]

Equation3.3.[161]

$X^{\cdot} + ArOH \text{----------} > X^{-} + ArOH^{\cdot+}$

b. Or by hydrogen donation especially in neutral or acidic media, in this case they acted as hydrogen atom transferet (HAT). They transferred hydrogen by hemolytic rupture of OH bond. This mechanism was dependent on bonding and dissociation energy (BDE) . The BDE value depended on the position of the phenolic OH group and the conformation of the molecule itself to guarantee the easiness of electron delocatization according to equation 3.4. [161] [162]

Equation3.4 [161] [162]

$X^{\cdot} + ArOH \text{----------} > XH + ArO^{\cdot}$

The weaker the OH bond, the lower the IP value,the easier the Hydrogen and electron transfer would occur and thus the easier the reduction of ROS would take place.

From Fig.3.25. and Table3.18. It was concluded that the best preparation was the NP loaded with the Ajwah date seed extract at ratio of chitosan: AS extract 1:7 (w/w).

Another observation was that the NPs antioxidant activity did not vary greatly from the Ajwah seed extract which was 53.01%. This might lead to an interpretation of having the same antibacterial action, however this was completely the opposite as would be shown later in the antibacterial tests.

3.2.3. **Antibacterial test:**

Antibacterial assay was done on the 5 samples of Chitosan NPs loaded with the Ajwah date seed extract in ratios, 1:1, 1:3, 1:5, 1:7 (w/w) and compared to the Ajwah date seed extract plant. Bacterial rate of inhibition was studied against 2 strains; *Staphylococcus aureus L.* And *Escherichia coli L*. those 2 strains are considered the main 2 strains responsible of most of the bacterial infections. *Staphylococcus aureus* is more antibacterial resistant and more infectious than *Escherichia coli L*. according to Si Feng Shi *etal*., *Staphylococcus aureus L.*is stronger and more infectious than any other bacterial strain not only because of the infections it causes to man like wound infections, osteomyletis, foreign body device infections and orthopedics, but also due to the formation of highly resistant biofilm due to its production of high mucilaginous substance known as polysacharride intracellular adhesion (PIA) which is mainly formed of glucosaminoglycans made the bacteria very sticky and not easily detach from the surface on which it formed the biofilm. Nevertheless, its high resistance was pertained to its capability to withstand harsh conditions, like the presence of oxygen free radical, starvation, antibiotics and other planktonic counterpart. [171]

For the bactericidal action, the Log reduction technique was done unlike Manal Awad *etal*.who performed agar diffusion technique for antibacterial assay. [105] The following results were obtained according to Table3.19, Fig.3.26, Fig.3.27.and Appendix [4.8];

Table3.19. rate of reduction of the prepared NPs compared with Ajwah date seed against *Staphylococcus aureus L*. and *Escherichia coli L.* where, 1:1, chitosan NPs loaded with the Ajwah date seed in ratio 1:1 (w/w), 1:3; chitosan NPs loaded with the Ajwah date seed in ratio 1:3 (w/w), 1:5; chitosan NPs loaded with the Ajwah date seed in ratio 1:5 (w/w) and 1:7; chitosan NPs loaded with the Ajwah date seed extract in ratio 1:7(w/w).

Sample	**% Rate of inhibition**	
	Staphylococcus aureus L. **(Gram positive)**	***Escherichia coli L.*** **(Gram negative)**
Ajwah date seed extract	-400	-400
1:1	-400	-614
1:3	-490	-185
1:5	100	85.7
1:7	100	100

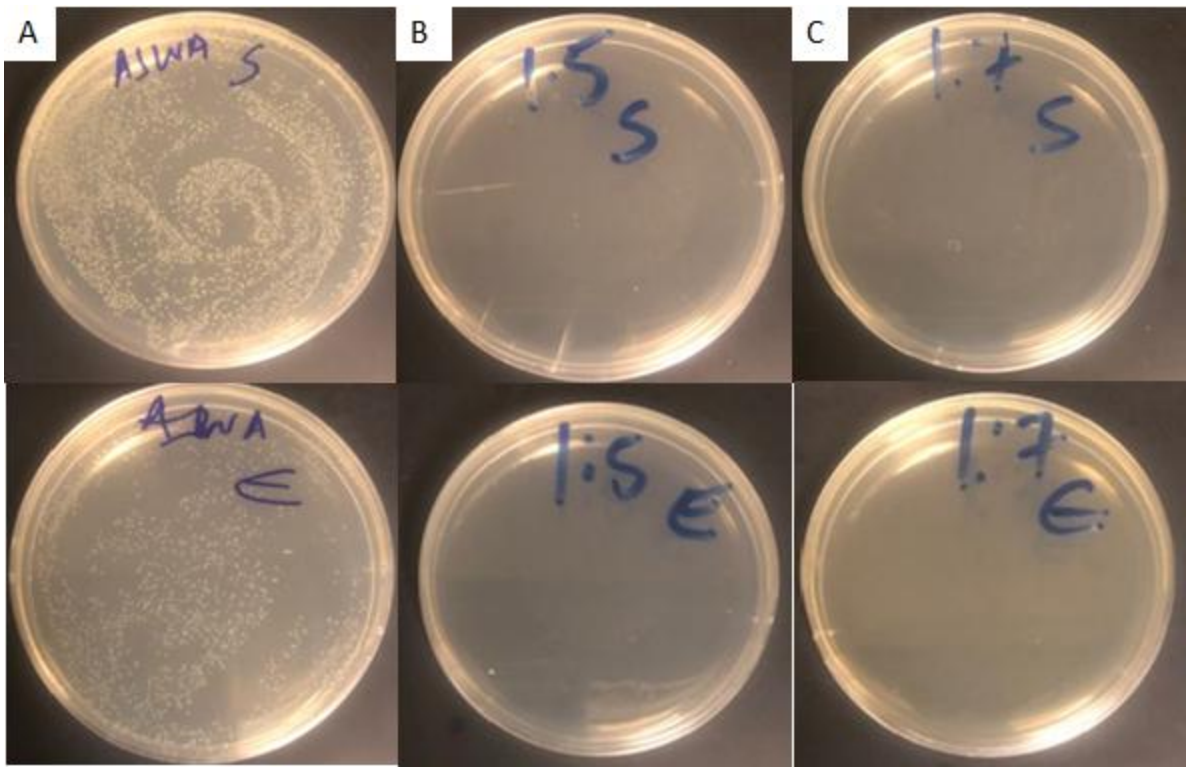

Fig.3.26. Rate of inhibition method for the chitosan NPs loaded with the plant at against S; *Staphylococcus aureus* and E; *Escheischia coli L.* (A) A S; Ajwah plant against *Staphylococcus aureus* L., AE Ajwah date seed against *Escherichia coli* L., (B) 1:5 S; chitosan NPs loaded with the Ajwah date seed extract in ratio 1:5 (w/w) against *Staphylococcus aureus* L., 1:5 E; chitosan NPs loaded with the plant extract in ratio 1:5 (w/w) against *Escherichia coli* L., just showing 2 or 3 colonies (C) 1:7 S; chitosan NPs loaded with the Ajwah date seed extract in ratio 1:7 (w/w) against *Staphylococcus aureus* L., 1:7 E; chitosan NPs loaded with the Ajwah date seed extract in ratio 1:7 (w/w) against *Escherichia coli* L.,

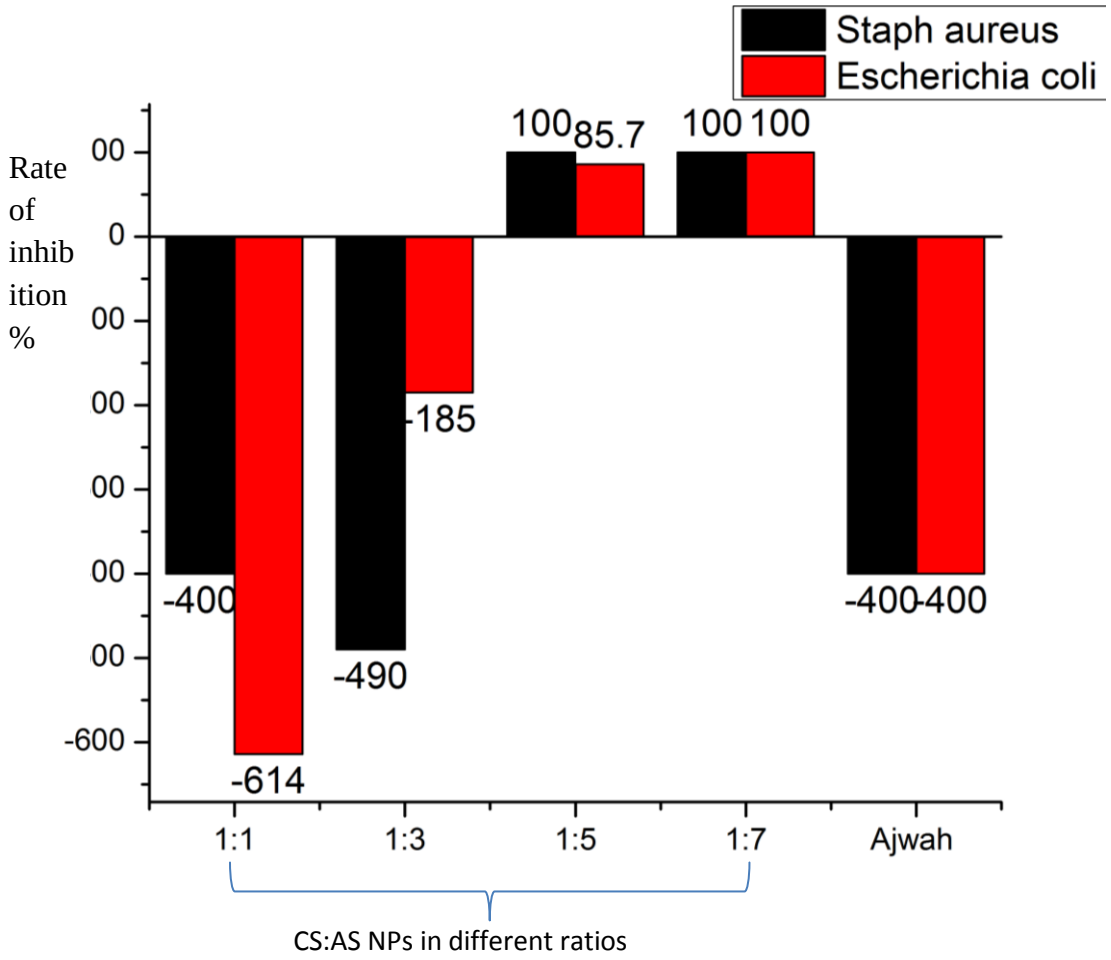

Fig3.27. Plot of rate of inhibition % of the chitosan NPs loaded with the Ajwah date seed extract in ratios 1:1, 1:3, 1:5 and 1:7 (w/w) against *Staphylococcus aureus L*. (S) and *Escherichia coli L*. (E) and compare them to the bactericidal action of the Ajwah date seed extract.

The polyphenolics, quinones, flavonols and falvonoids contained functional groups with low IP values which would easily dissociate to give hydroxyl (OH^-), carboxylate (COO^-), protons (H^+). Those free dissociable groups formed hydrogen bonds with the amino groups (NH_2) which were characterized by being highly electrophilic to give rise to the highly poly-cationic polyelectrolyte polysaccharide that formed from the bond between the plant polysaccharides and flavones with the cross-linked amino group of chitosan that mainly targeted the negative charged peptidoglycans of the cell membrane of the bacteria. The NP reacted more strongly than the chitosan powder to the bacterial cell membrane due to the larger surface area which led to the disruption of the cell membrane and leakage of the intracellular organelles [18]

From Table3.19, Fig.3.27.and Fig. 3.28.it was clear that the Ajwah date seed extract when got encapsulated in chitosan NP its bactericidal action was highly augmented compared to the Ajwah date seed extract alone. This was the same conclusion done by Manal awad *etal*.who was able to prove that the action of date palm NPs against bacteria was much better than the date palm seed not in the nano form. Yet they could not guarantee that their efficacy against gram positive or negative bacteria was 100% reduction rate as chitosan NPs were able to reach. [105] 1:7 (w/w) NPs were able to reduce bacteria by 100%. They acquired this bactericidal action against both Gram positive and Gram negative bacteria. The chitosan NP was very strong against the gram positive and gram negative bacteria though the structure of the cell membrane of the gram positive bacteria was different from that of gram negative bacteria [122]. Since gram positive bacteria cell membrane is composed of peptidoglycans associated to polysacharrides and teichoic acids, unlike gram negative bacteria where the cell membrane is formed of lipopolysacharrides, phosphates and pyrophosphates which made them acquire more negative charge as shown in Fig.3.28. [171] [172]

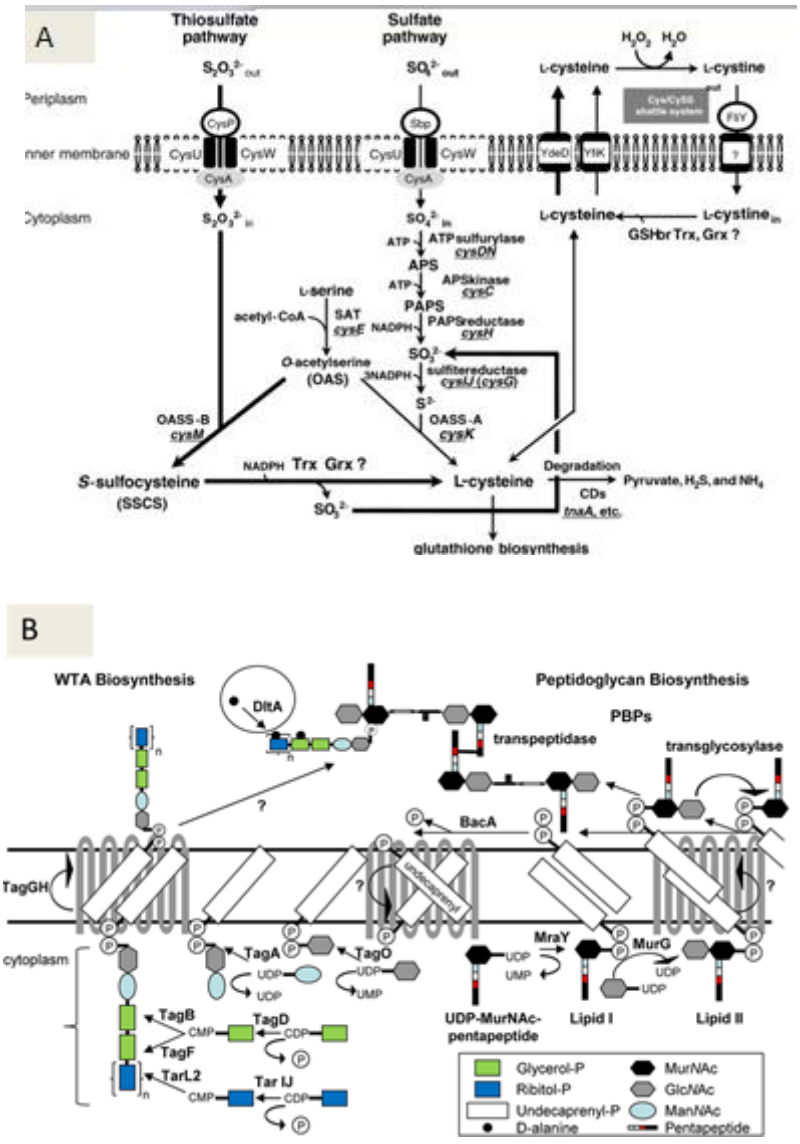

Fig3.28. Structure and charge of the cell membrane of (a) Gram negative versus (b) gram positive bacteria [172] [173]

Regarding the interaction with the gram positive bacteria as was mentioned above according to Si Feng Shi *etal* and Qi *etal.* ; the chitosan NPs could interact first through the teichoic acid which existed on the surface of the bacteria. They would bind to the membrane of the bacterial cell. Because of their nano size, they would be able to penetrate through the pores of the cell membrane or through the water channels of the biofilm to bind to the DNA of the bacteria which is formed of poly-anionic nucleobases and eventually would lead to the inhibition of the RNA replication and protein synthesis.[171] [174] The crosslinked chitosan because of its large positive

amino groups made the interaction with the gram negative bacteria cell membrane very strong and formed chito-oligosacharrides and consequently it would form its bactericidal action either by the cross-linkage with the cell membrane which would lead to cell wall damage and barrier dysfunction. Another suggested mechanism was that the diffused hydrolysis products due to the interaction between the chitosan and the gram negative bacteria would interact with the microbial DNA which would inhibit the transcription and translation processes. [175] [176] [177]

The chitosan NPs would not lose their antimicrobial action and they could be reused. Many factors as well would make this interaction stronger;

a. Effect of molecular weight of chitosan:

As the molecular weight decreased, the interaction with the cell membrane of bacteria would be stronger and from non-hydrolysable chito-oligosacharrides in gram negative bacteria or complexes with DNA and techoic acids in gram positive bacteria.

b. Degree of deacetylation and concentration:

As the degree of deacetylation and concentration increased, the antimicrobial action increased. [163] [164] [165] [166] [167] [168] [169] [170]

c. Cross-linkage:

When chitosan was prepared with cross-linker it would be more stable in bacterial solution and more potent. When the chitosan which is highly soluble in water crosslinked either by covalent, ionic, or vander-wall forces, it would be considered a more or less a type of composite formed, because an interaction occured and forms an interpenetrating polymer network(IPN) , which led to adding new properties to chitosan that made it more soluble in acidic medium [175]. An example was demonstrated in table 3.20. of the antibacterial action of chitosan versus crosslinked chitosan against *Borkholderia cepacia L. ; (*BCC) strainwhich is a potent aerobic gram negative bacteria that caused lethal acystic fibrosis according to BinLi *etal.*[176]

Table3.20. the chitosan versus the cross linked chitosan antibacterial action against BCC starin [173]

	Inhibition diameter in mm after incubation for 12 hrs	**24 hrs**	**48 hrs**
Chitosan	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Crosslinked chitosan NPs	20.0 ± 2.1	19.8 ± 2.1	19.6 ± 3.2

According to what was reported by; Qi *etal.*, La Thi Kim Ngan *etal.*, Du *etal.*, Xing *etal.*, Ngo *etal.*,K.Divya *etal.*where they tested the antimicrobial action of the chitosan NPs crosslinked and loaded with metal oxides that were reported to have more potent antibacterial and lesser nanosize range than cross linked chitosan NPs alone. Tests proved that the chitosan NPs had minimum inhibitory concentration (MIC) of 125-234 µg/mL . [178] [179][180] other work proved that chitosan NPs prepared by spray drying proved to have rate of inhibition of 76-98% against *staphylococcus aureus L.* at concentration of 100-500 mg/mL and rate of inhibition of 54-97% against *Escherichia coli L.* at concentration of 100-500 mg/mL. [118] [181] this proved that our results were more effective at lower concentration where the chitosan NPs loaded with the Ajwah date seed extract had % inhibition of 100% against *Staphylococcus aureus L.* and *Escherichia coli L.* at concentration of 20 mg/mL. [181]

d. Temperature:

As the temperature increased the antibacterial action increased [174].

e. Zeta potential:

As the zeta potential increased and the size of the nanoparticle decreased, the antibacterial action would increase [175] [181] .

f. Effect of pH:

In acidic pH, at pH 5.3, using the acetic acid, the uptake of the hydrophobic 1-N- phenyl naphthyl amine (NPN) by the gram negative bacteria which would lead to bacterial damage. In alkaline pH, no uptake had occurred. Another suggested mechanism was that on adding the acetic acid, the protonation will increase partially due to the H^+ ions from the acetic acid and the NH_3^+ on the C_2 of D- glucosamine repeated units in the chitosan backbone. The dense cloud of positive charge would interact with the negative charge on the gram negative bacteria, increasing the surface area by minimizing the particle size of the NPs would lead to easiness of penetration in the bacterial cell and would lead to the bacterial cell damage. Table3.21. showed a comparison between the presence and absence of acetic acid and its impact on the bacterial action according to W-L Du *etal.*[175][180]

Table3.21. MIC values of solid chitosan in bacterial broth, chitosan in acetic acid in the bacterial broth versus cross linked chitosan in acetic acid against *Staphylococcus aureus L., Salmonella choleraesuisL.., Escherichia coli L* .[175]

MIC against			
	Staphylococcus aureus L.	***Escherichia coli L.***	***Salmonella choleraesuisL***
Chitosan	-	-	-
Chitosan in acetic acid	656	468	468
Cross linked chitosan NPs in acetic acid	234	117	117

Chapter 4:

Appendix of chapter (3)

Appendix [1]:

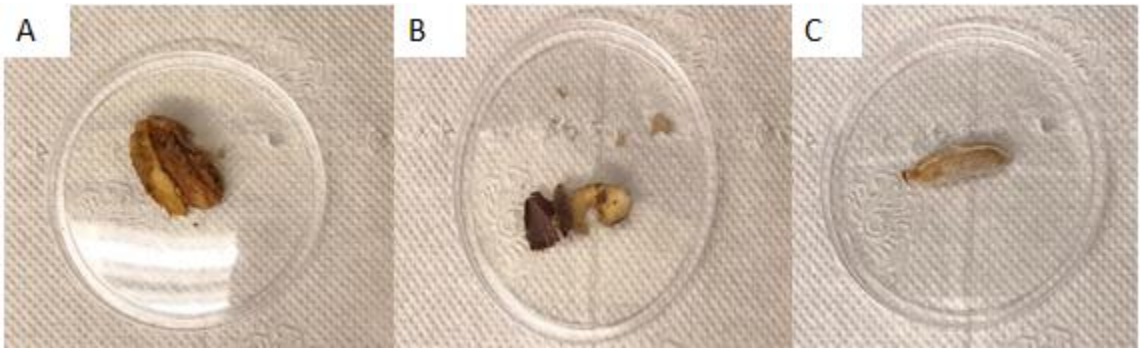

Fig4.1.1 (a) Black date fruit freeze dried, (b) black date peel freeze dried (c) black date seed freeze dried

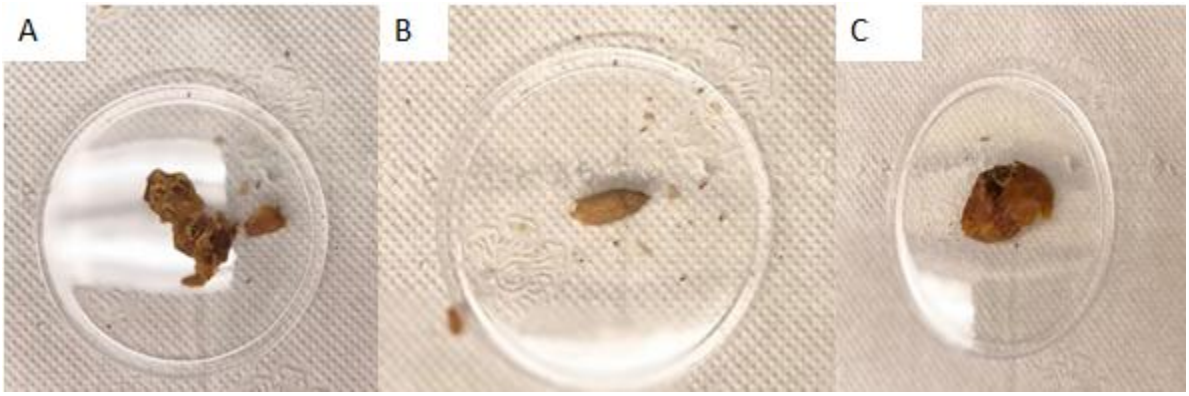

Fig4.1.2 (a) Ajwah date peel freeze dried (b) ajwah date seed Freeze dried (c) ajwah date fruit freeze dried

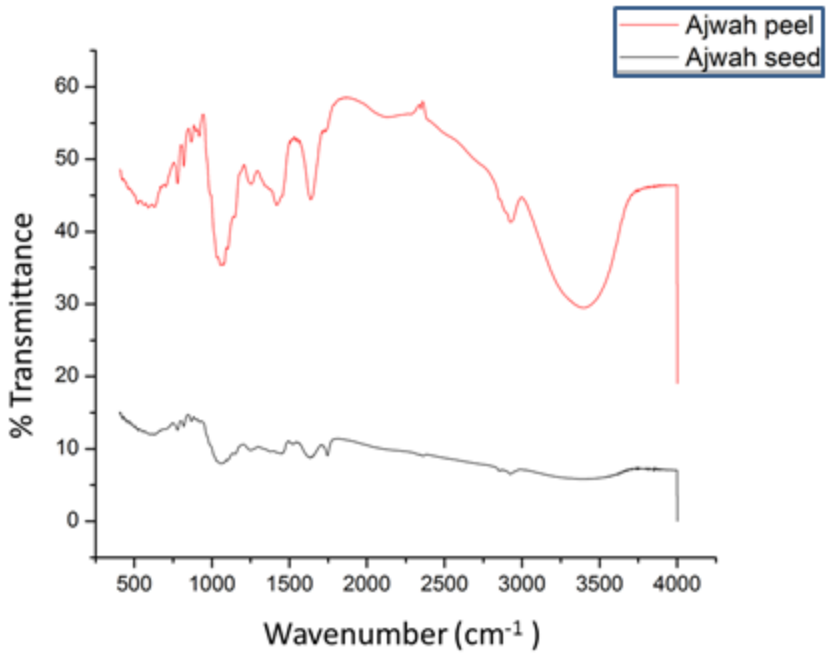

Fig4.1.3. IR charts of Ajwah date peel and seed freeze dried

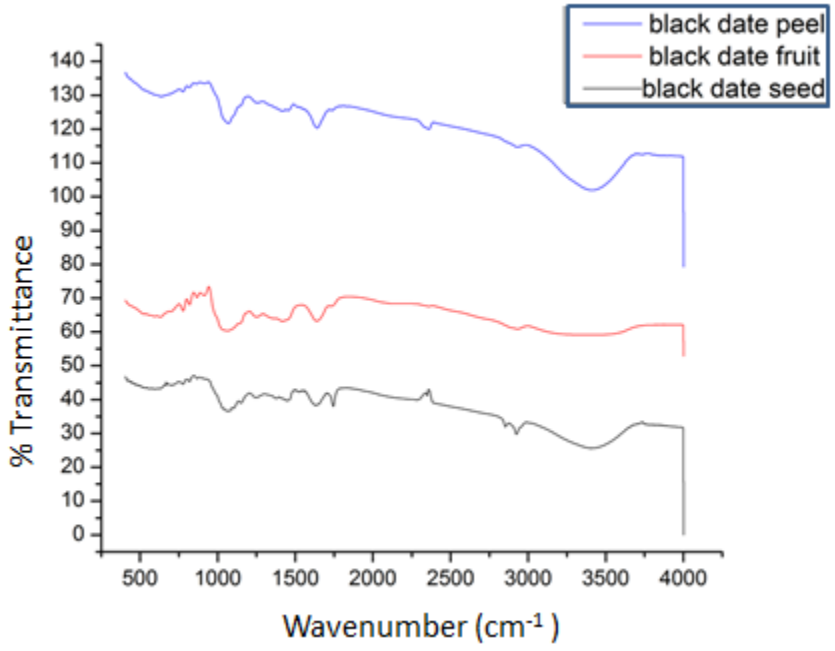

Fig4.1.4. IR charts of Black date seed, peel and fruit freeze dried

Appendix [2]:

Table4.2.1. the Total phenolic content in the Ajwah peel (APFD) and Ajwah seed (ASFD) freeze dried ; Black date peel(BPFD), black date seed (BSFD) freeze dried and black date fruit (BFFD) freeze dried in 3 different solvents; 52% ethanol (E), Dimethyl Sulfoxide DMSO (D) and mixture of water: methanol: acetone: formic acid (M)

Solvent / **Plant part**	**E**	**D**	**M**
ASFD	3.21	1.12	1.37
APFD	1.66	0.49	1.02
BSFD	2.01	0.85	1.56
BPFD	0.99	0.82	1.07
BFFD	0.39	-	1.48

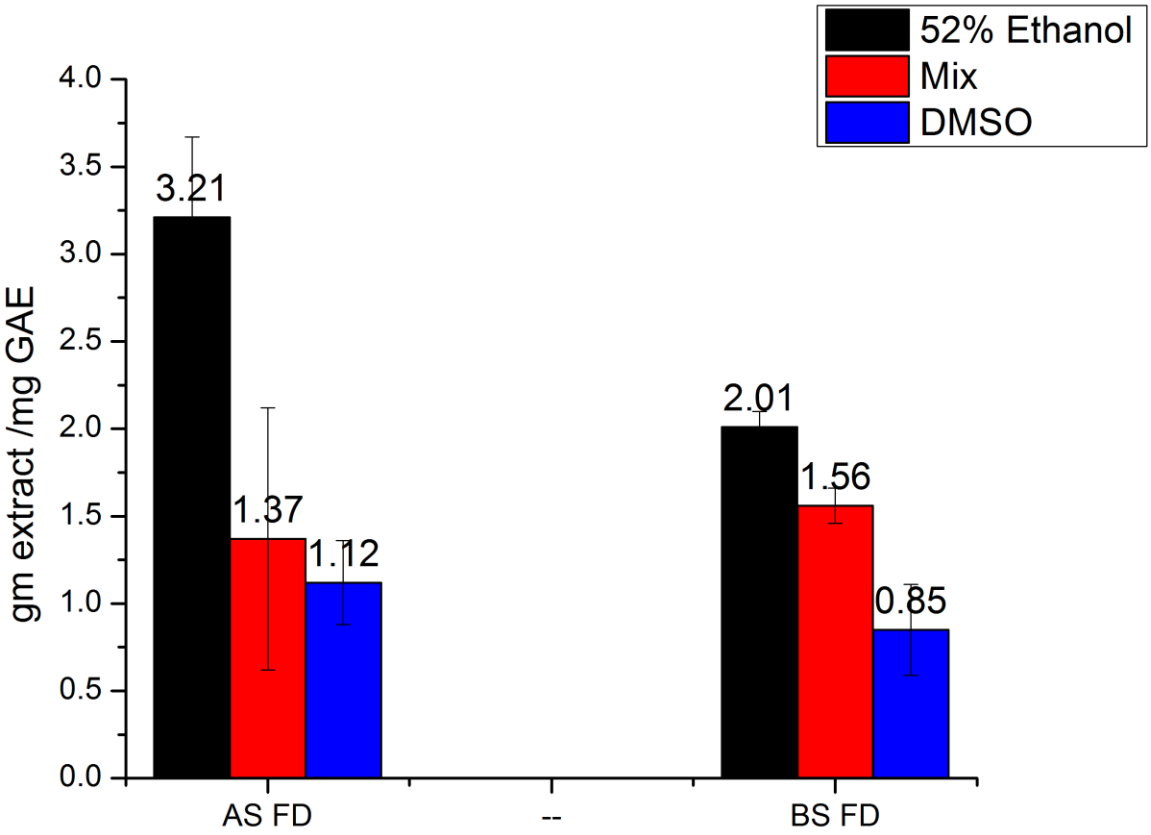

Fig4.2.1. Folin Ciocalteau chart for polyphenolic content gm extract /mg GAE of Ajwah seed freeze dried (ASFD) and black date seed freeze dried (BSFD) in different solvents; 52% ethanol (E), DMSO (D)and the mixture of water:methanol:acetone:formic acid (Mix)

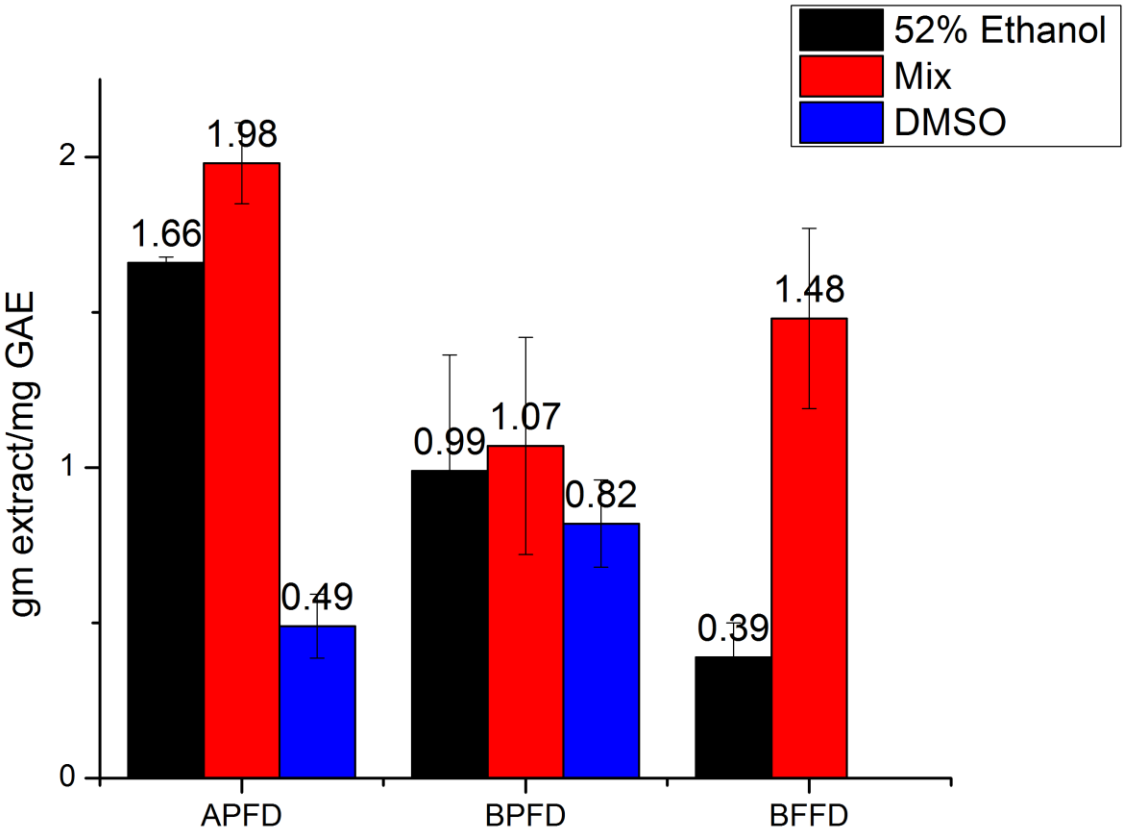

Fig4.2.2. Folin Ciocalteau chart for polyphenolic content gm extract /mg GAE of ajwah date peel freeze dried (APFD) and Black date peel freeze dried (BPFD) and black date fruit freeze dried (BFFD) in different solvents; 52% ethanol (E), DMSO (D)and the mixture of water:methanol:acetone:formic acid (Mix)

Table4.2.2. DPPH test for antioxidant activity (AO%) for Ajwah date seed freeze dried (ASFD), black date seed freeze dried (BSFD), Ajwah date peel Freeze dried (APFD), black date peel freeze dried (BPFD) and black date fruit freeze dried (BFFD)

Plant part	AO%
ASFD	51.27%
APFD	60%
BSFD	71.3%
BPFD	80%
BFFD	80%

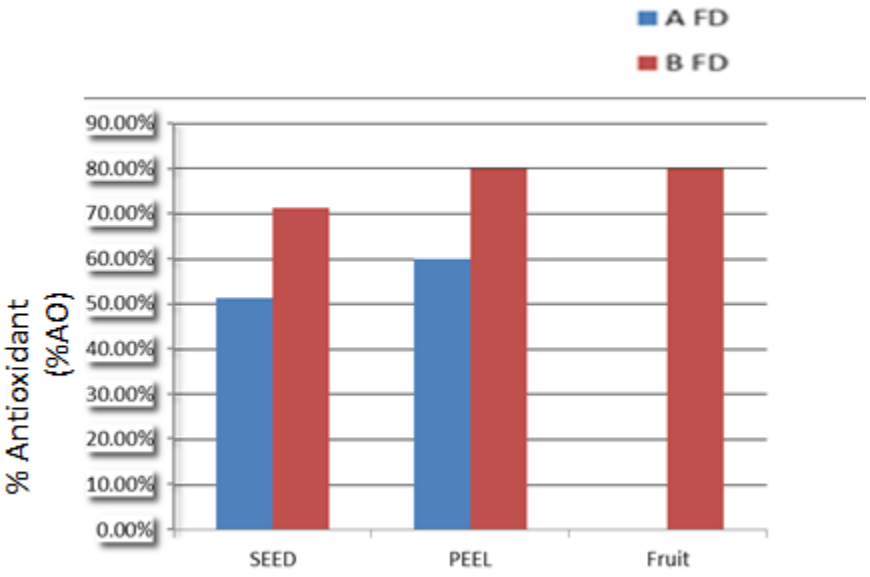

Fig4.2.3. DPPH test to measure the antioxidant activity (AO%) for dry plant powder (dpp) for Ajwah seed freeze dried (ASFD), Black date seed freeze dried (BSFD), Ajwah date peel freeze dried (APFD), black date peel freeze dried (BPFD), black date fruit freeze dried (BFFD)

Appendix [3]:

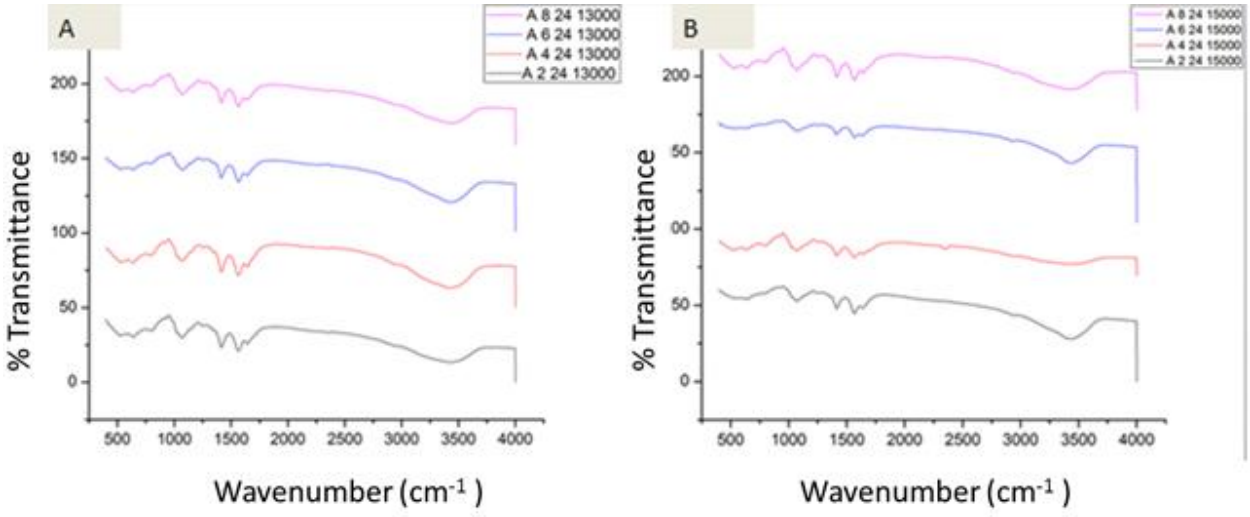

Fig4.3.1. the IR chart of the Ajwah date seed extract in chitosan NP and study the impact of TPP volume variation; 2, 4, 6 and 8 mL , at stirring conditions 100rpm at Room temperature(RT) for 24 hrs at centrifugation speed (a) 13000 rpm (b) 15000 rpm for 30 min at 4°C

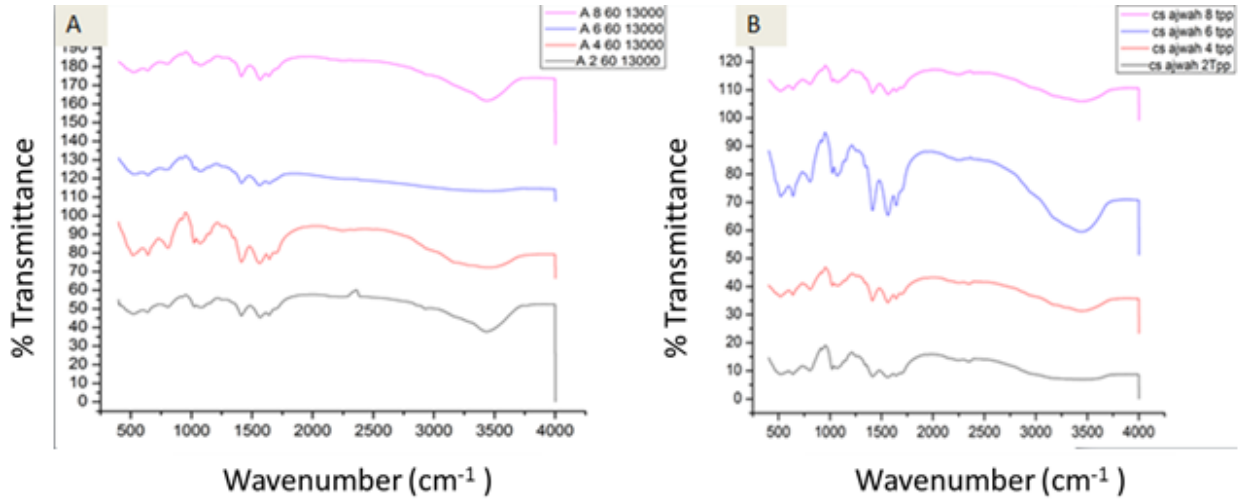

Fig4.3.2. the IR chart of the Ajwah date seed extract in chitosan NP and study the impact of TPP volume variation; 2, 4, 6 and 8 mL, at stirring conditions 110 rpm at 60°C for 60min at centrifugation speed (a) 13000 rpm (b)15000 rpm for 30 min at 4°C

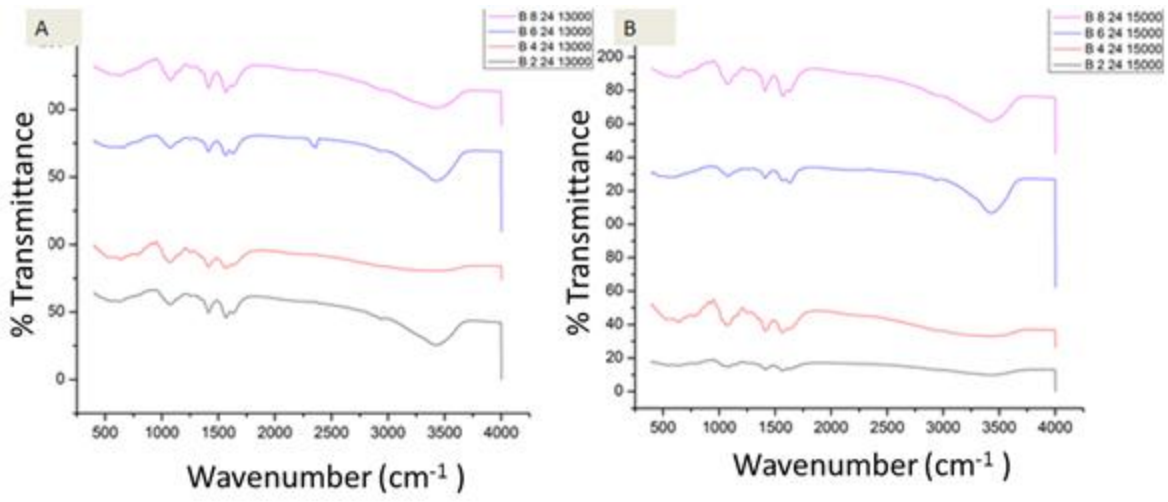

Fig4.3.3. the IR chart of the black date seed extract in chitosan NP and study the impact of TPP volume variation; 2, 4, 6 and 8 mL, at stirring conditions 100rpm at Room temperature(RT) for 24 hrs at centrifugation speed (a) 13000 rpm (b) 15000 rpm for 30 min at 4°C

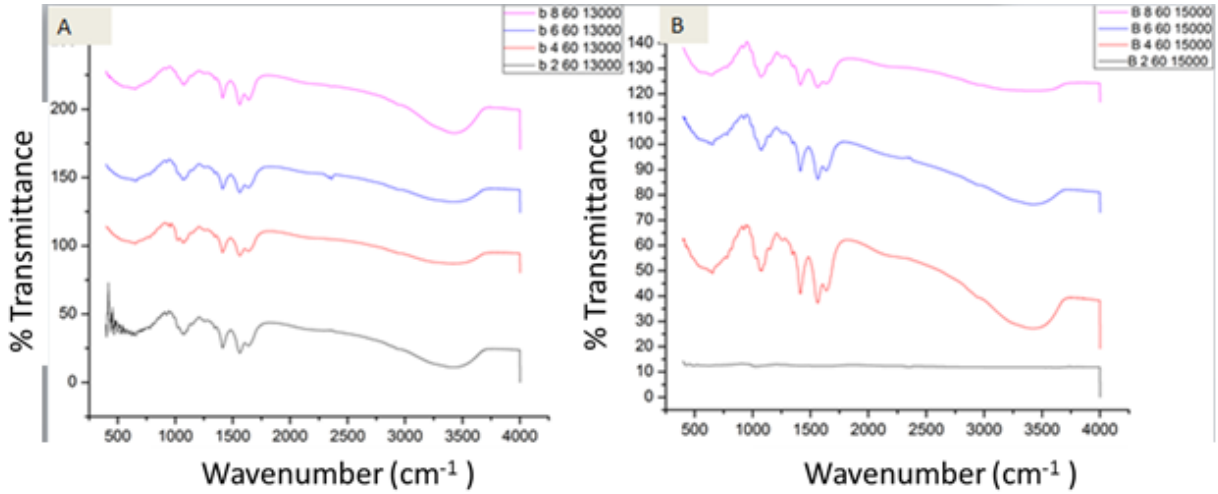

Fig4.3.4. the IR chart of the black date seed extract in chitosan NP and study the impact of TPP volume variation; 2, 4, 6 and 8 mL, at stirring conditions 110 rpm at 60°C for 60min at centrifugation speed (a) 13000 rpm (b)15000 rpm for 30 min at 4°C

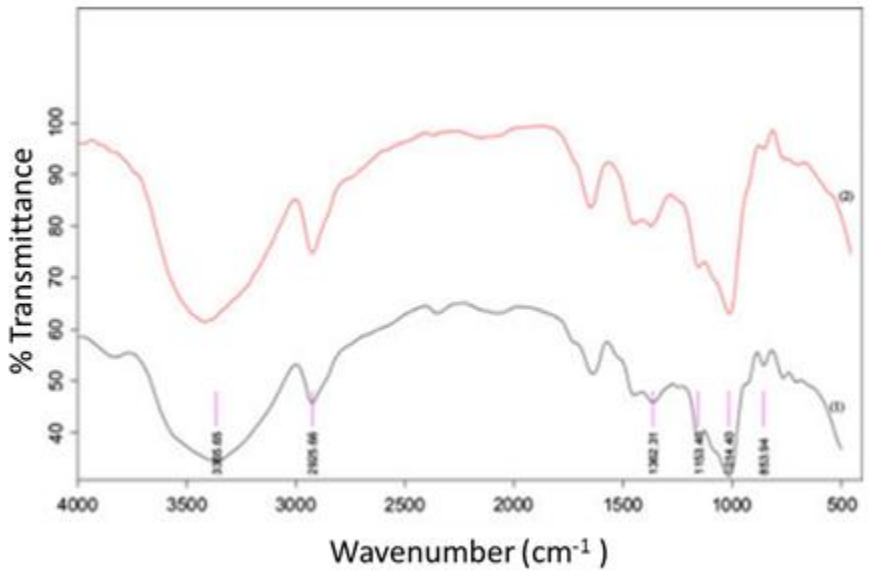

Fig4.3.5. the IR spectra of Madadlou *etal.* comparing the (1) free and (2) loaded starch NCs with the date seed extract [33]

Appendix [4]:

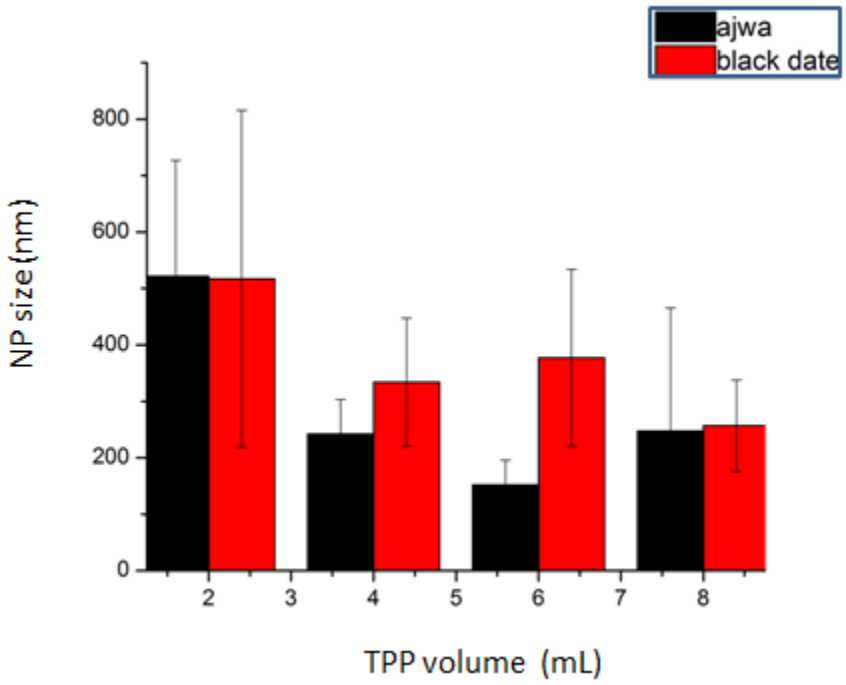

Fig4.4.1. comparison of the hydrodynamic size of the prepared NPs of chitosan loaded with the Ajwah date seed/Black date seed at different TPP volumes at stirring conditions of 60°C for 60 min at 110 rpm

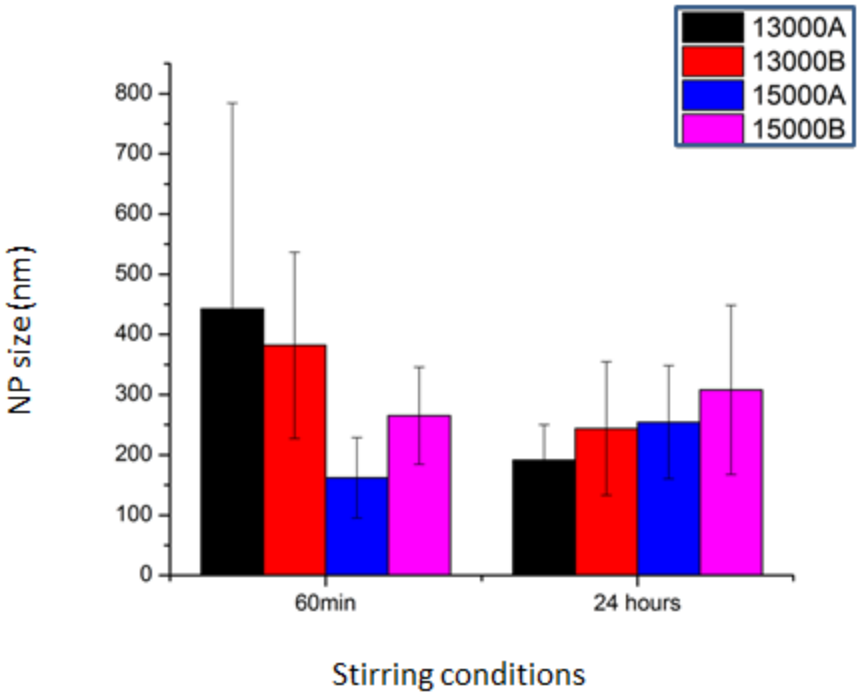

Fig4.4.2. comparison of the hydrodynamic size of the prepared NPs of Ajwah date seed (A) and Black date seed (B) at different stirring conditions; 60°C, 60 min at 110 rpm and RT, 24 hrs at 100 rpm and at different centrifugation conditions; 13000 and 15000 rpm at TPP volume 8 mL

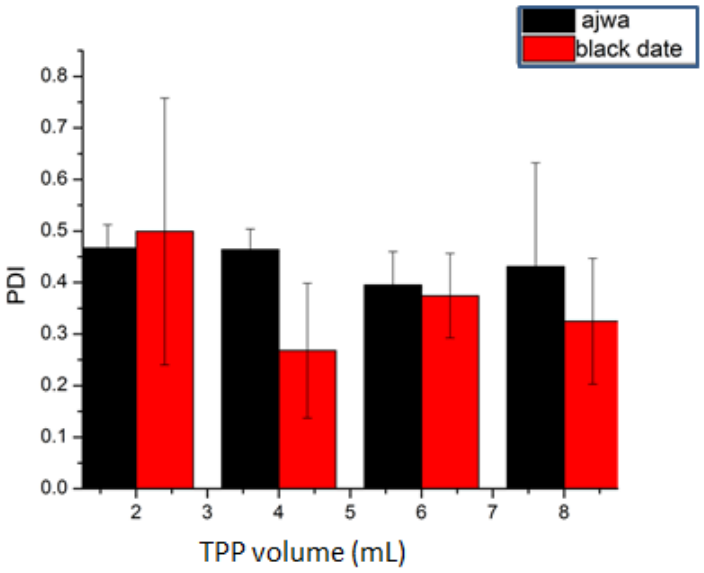

Fig4.4.3. comparison of the PDI of the prepared NPs of chitosan loaded with Ajwah date seed and black date seed extracts at different TPP volumes; 2, 4, 6, and 8 mL at stirring conditions of 60°C, 60 min at 110 rpm and centrifugation at 15000 rpm

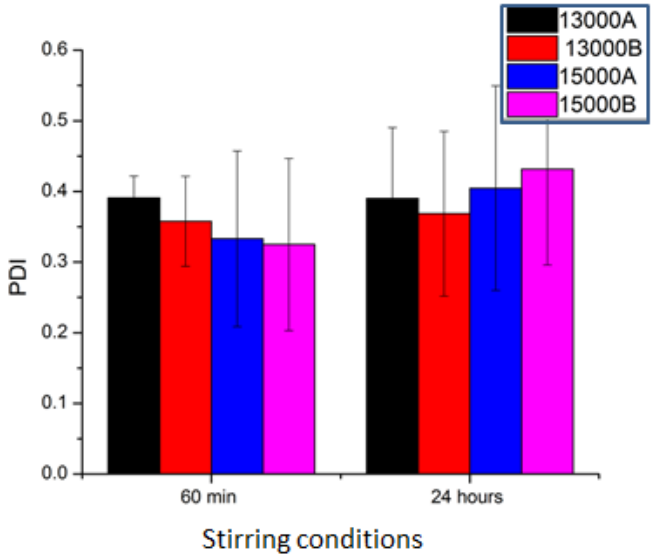

Fig4.4.4. comparison of the PDI of prepared NPS loaded with Ajwah date seed (A) and black date seed extract (B) at different centrifugation conditions; 13000 and 15000 rpm and at different stirring conditions; 60°C, 60 min at 110 rpm and at RT for 24hrs at 100 rpm at TPP volume 8 mL

Appendix [5]:

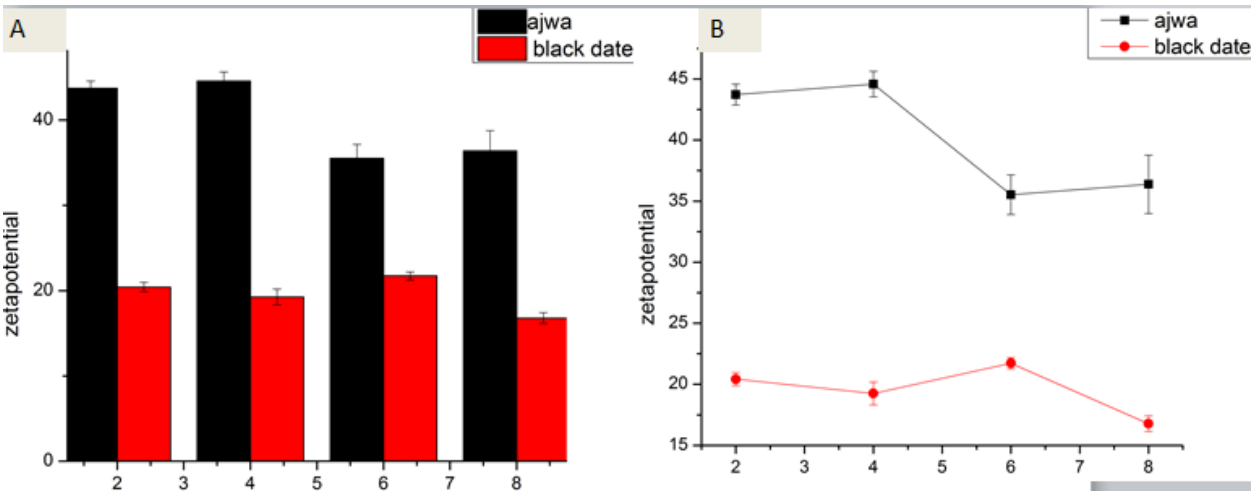

Fig4.5.1. comparison of the zeta potential of the prepared NPs of chitosan loaded with the Ajwah date seed and black date seed extracts at stirring conditions of 60°C, 60 min at 110 rpm and at centrifugation of 15000 rpm at different TPP volumes; 2, 4, 6 and 8 mL (a) bar chart (b) line chart

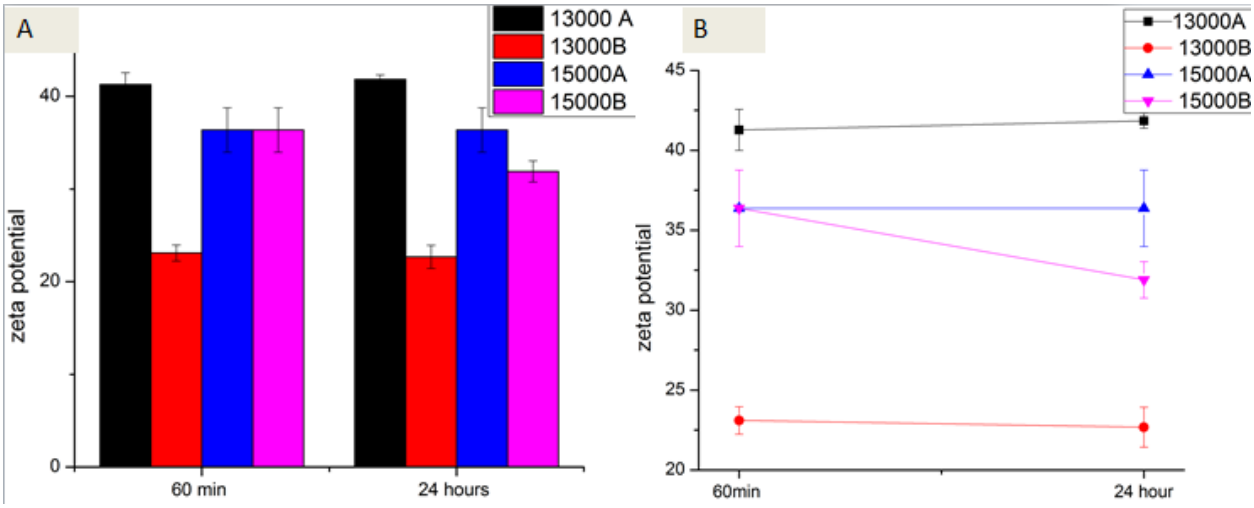

Fig4.5.2. comparison of the zeta potential of the prepared NPs of chitosan loaded with Ajwah date seed (A) and black date seed (B) at different stirring conditions; 60°C, 60 min at 110 rpm and at RT, 24 hrs at 100 rpm and at different centrifugation conditions; 13000 and 15000 rpm at TPP volume of 8 mL (a) bar chart (b) line chart

Appendix [6]:

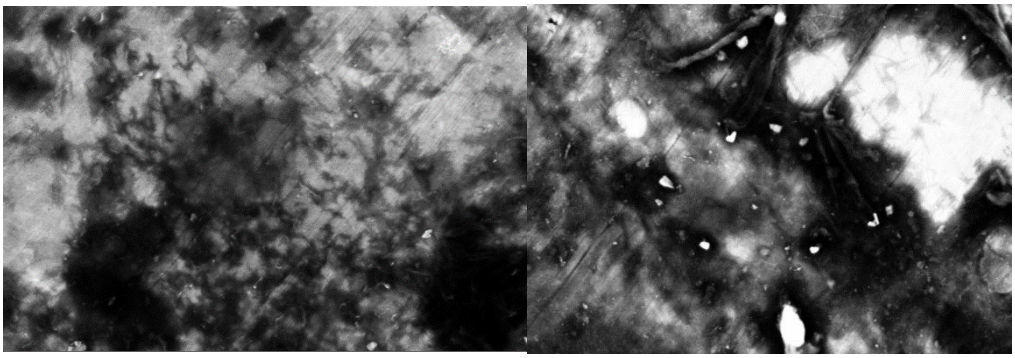

Fig4.6.1. SEM ; Scanning electron spectroscopy of freeze dried chitosan vacant NPs

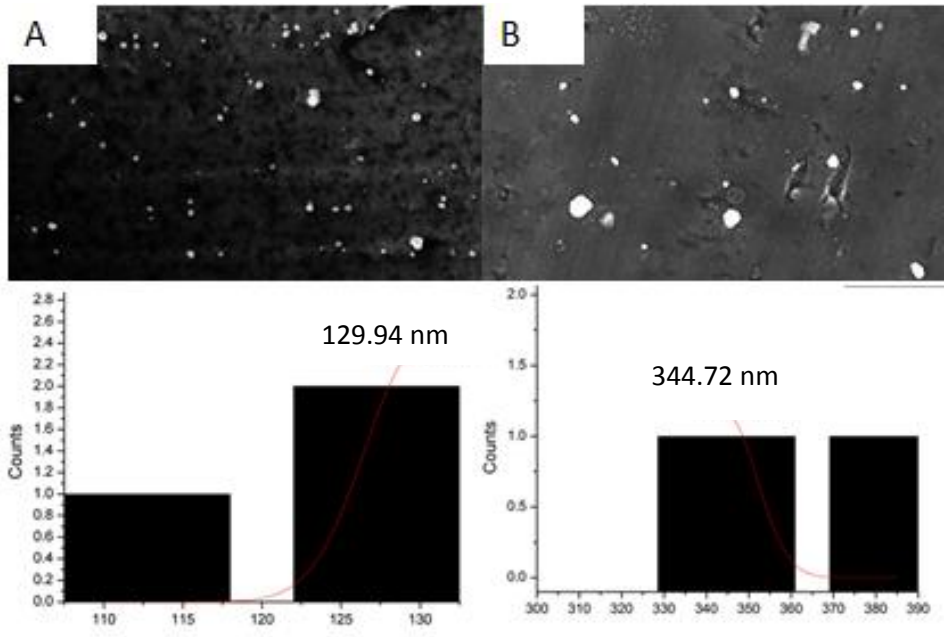

Fig4.6.2. Scanning Electron spectroscopy shows the chitosan NPs loaded with the Ajwah seed (AS) extract using cross linker TPP at volume (a) 6 TPP mL and (b) 8 mL and their image J charts to show the average particle size in nm. NB. Those samples were not gold sputtered

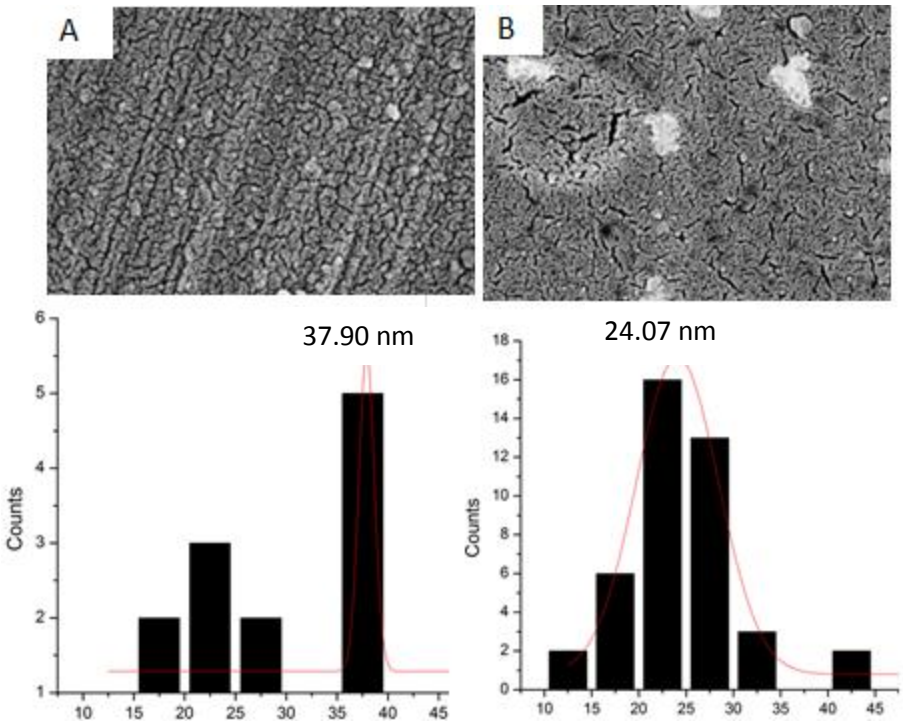

Fig4.6.3. Scanning Electron spectroscopy shows the chitosan NPs loaded with the Ajwah seed (AS) extract using cross linker at volume (a) 6 TPP mL and (b) 8 mL min and their image J charts to show the average particle size in nm. NB. Those samples were gold sputtered

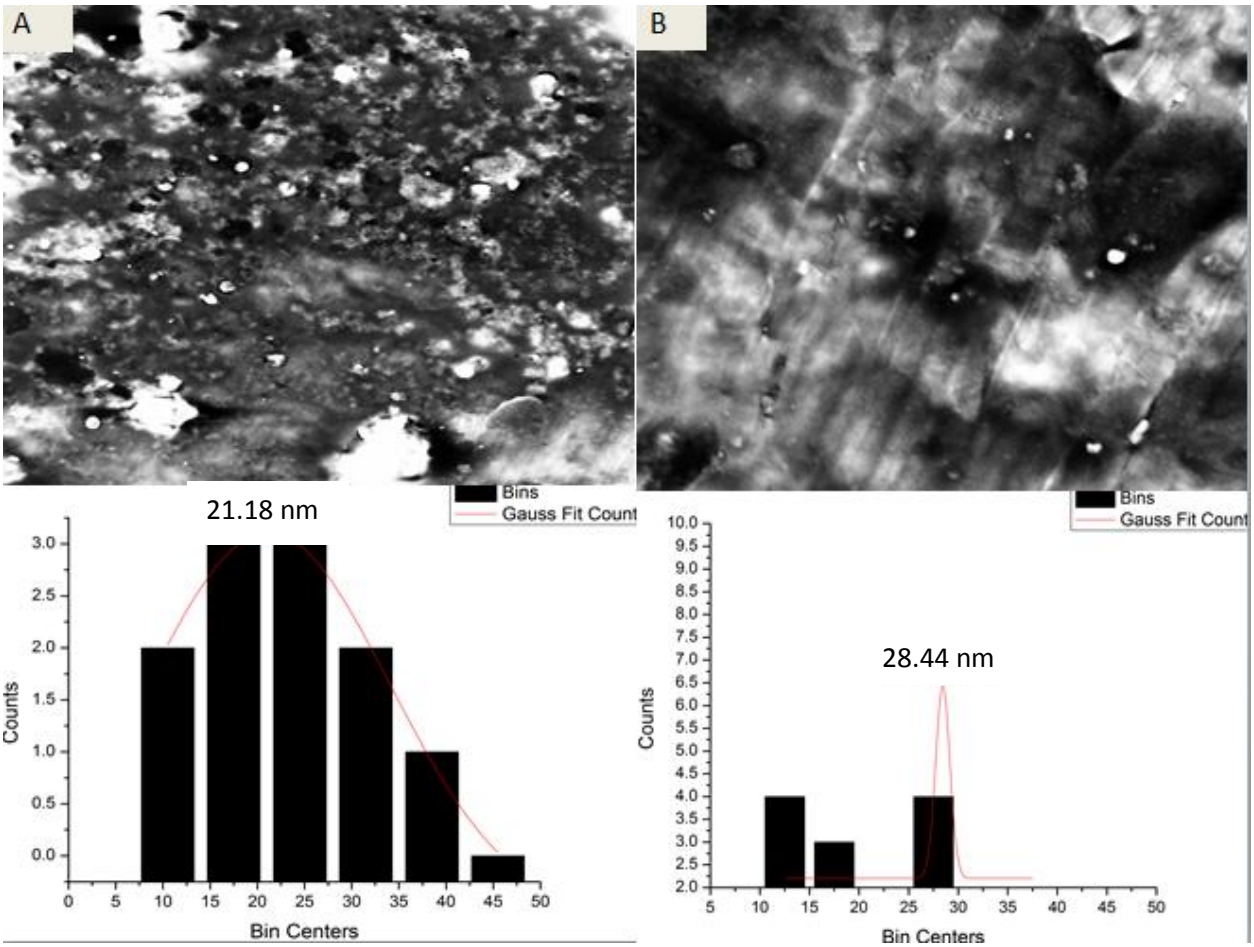

Fig4.6.4.Scanning Electron spectroscopy shows the chitosan NPs loaded with the Black date seed extract (BS) using cross linker TPP at volume (a) 6 TPP mL and (b) 8 mL and their image J charts to show the average particle size in nm. NB. Those samples were not gold sputtered

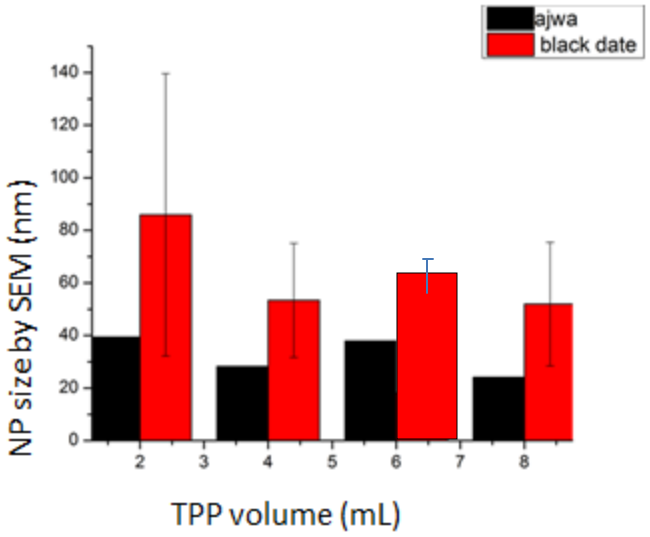

Fig4.6.5. Comparison of the particle size of chitosan NPs loaded with Ajwah (AS) and Black date seed extract (BS) at different TPP volumes, 2, 4, 6 and 8 mL respectively using Scanning electron microscope

Table4.6.1. SEM average sizes for chitosan NPs loaded with Ajwah date seed extract (AS) and Black date seed extract (BS) at centrifugation power 15000 rpm and stirring conditions 60 minutes at 60 degrees; at various TPP volumes; 2, 4, 6 and 8 mL respectively

Plant variety	**TPP volume**			
	2 mL	**4 mL**	**6 mL**	**8 mL**
Ajwah (AS)	39.41±3.2	28.22±2.3	37.90±23	24.10±0
Black date (BS)	85.93±53.75	53.41±21.7	53.41±3.07	51.96±23.52

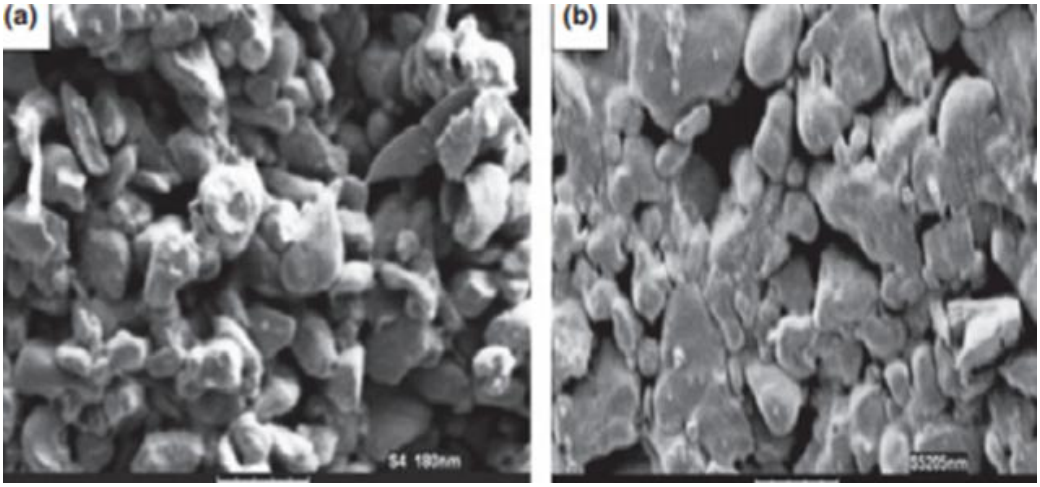

Fig4.6.6. the SEM images of the (a) free and (b) loaded starch NCs with the date seed extract [33]

Appendix [7]:

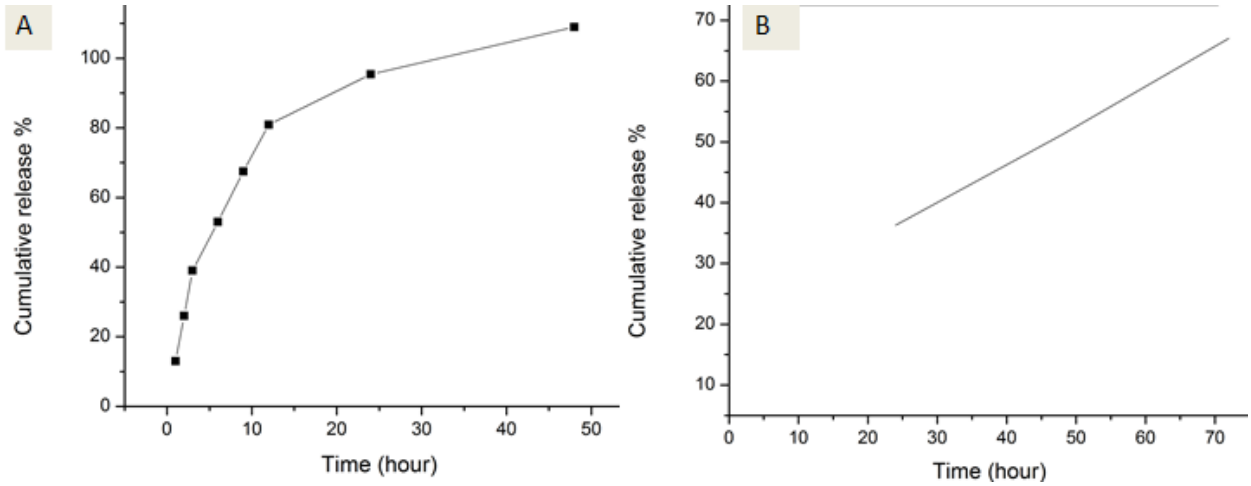

Fig4.7.1. Cumulative release profile in (a) HCl (b) PBS and for the chitosan NPs loaded with the Ajwah date seed in ratios of Chitosan : AS 1:1 (w/w)

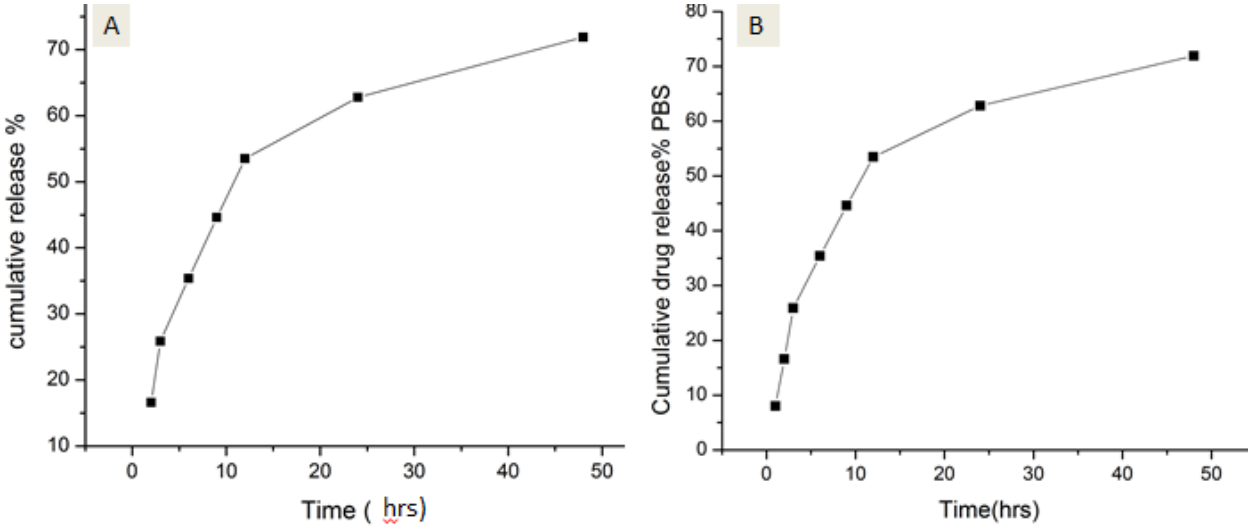

Fig4.7.2. Cumulative release profile in (a) HCl (b) PBS and for the chitosan NPs loaded with the Ajwah date seed in ratios of Chitosan : AS 1:3 (w/w)

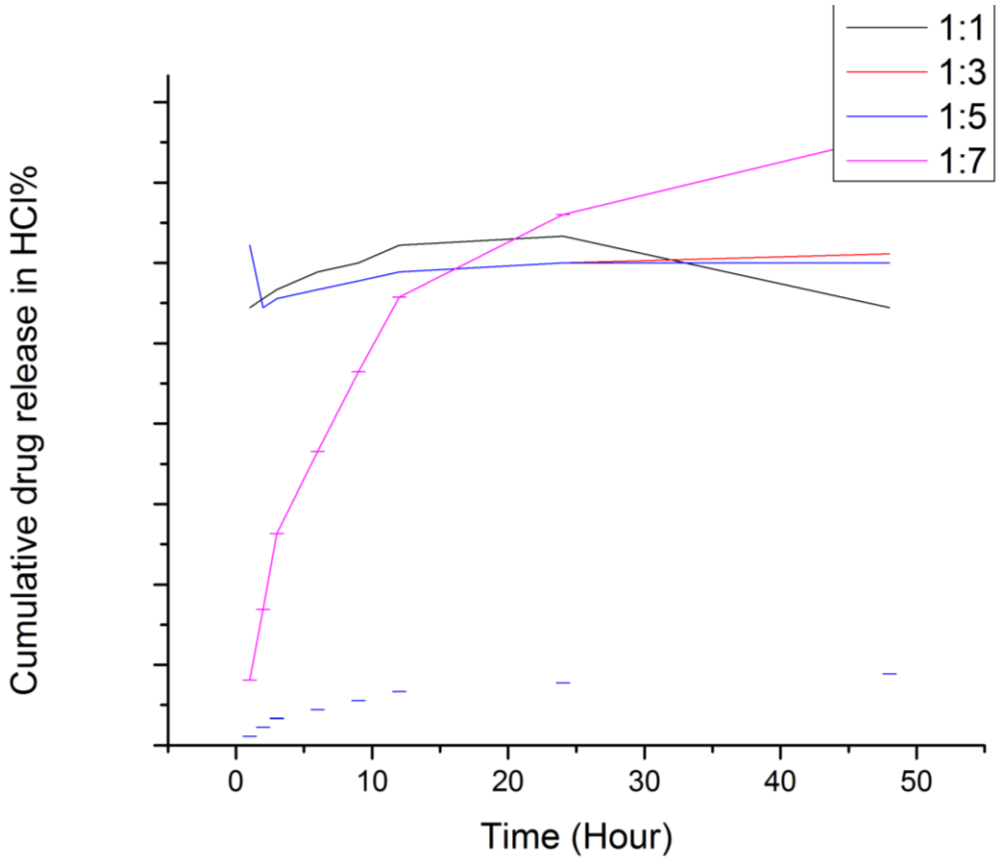

Fig4.7.3. Cumulative release profile in HCl for the chitosan NPs loaded with the Ajwah date seed in ratios of Chitosan: AS 1:1, 1:3, 1:5 and 1:7 (w/w)

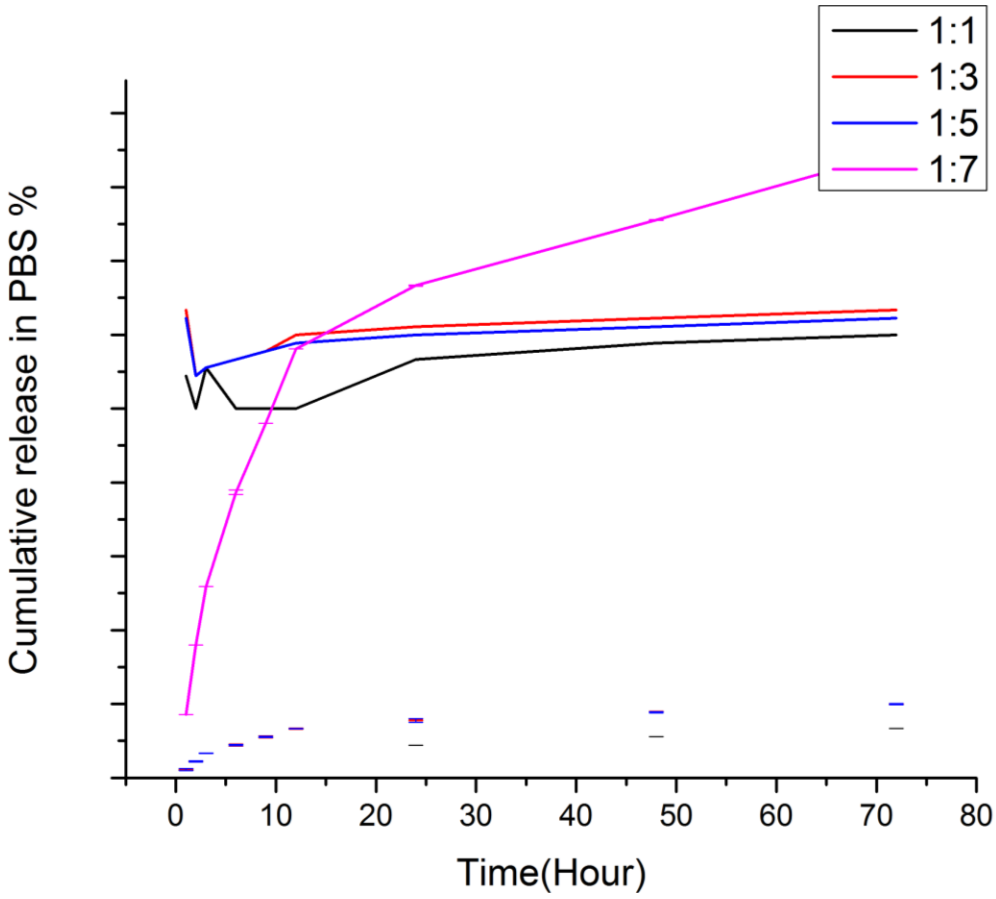

Fig4.7.4. Cumulative release profile in PBS for the chitosan NPs loaded with the Ajwah date seed in ratios of Chitosan : AS 1:1, 1:3, 1:5 and 1:7 (w/w)

Appendix [8]:

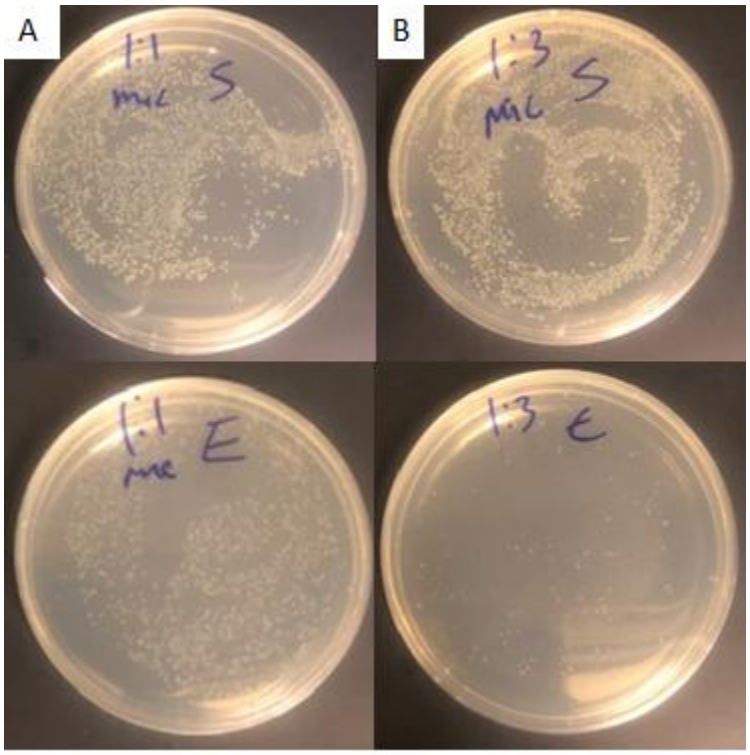

Fig4.8.1. Rate of inhibition method for the chitosan NPs loaded with the plant at against S; *Staphylococcus aureus* and E; *Escheischia coli L.* (A). 1:1 S; chitosan NPs loaded with the Ajwah date seed extract in ratio 1:1 w/w against Staphylococcus aureus L., 1:1 E; chitosan NPs loaded with the plant extract in ratio 1:1 w/w against Escherichia coli L.,, (B) 1:3 S; chitosan NPs loaded with the Ajwah date seed extract in ratio 1:3 w/w against *Staphylococcus aureus* L., 1:3 E; chitosan NPs loaded with the plant extract in ratio 1:3 w/w against *Escherichia coli* L.

Chapter 5:

Conclusion and Future prospects

Chapter 5: conclusion and future prospects

The date pit *Phoenix dactylifera L.* was proved to have many medical uses; antimicrobial, antifungal, anti-diabetes mellitus type 2, reduced obesity and hypercholesterolemia, protected against coronary heart diseases. The scope of this thesis was to test the capability of formulating NPs out of Ajwah date seed and peel, Black date seed, peel and fruit and monitor their antibacterial and antioxidant actions. No previous work was done to formulate a nanoparticle of it so as to be easily inoculated in drugs or food to do its promising action. Date pit extract would be prepared as aqueous and/or alcoholic and then formulated in a chitosan polymeric nano particle. The natural polysaccharide chitosan nano particle would be formulated to encapsulate the date pit extract inside it. The polymeric solution would be added to the extract solution by the help of the TPP linker to enhance coiling and encapsulation by applying certain stirring criteria including the time, speed and temperature of stirring, centrifugation and sonication to follow a protocol called ionic gelation technique. Those separated nano particles would be measured to guarantee their size and potential by Zeta-sizer Malvern. They would be imaged by X-ray diffractometer (XRD) and Transmittance Electron microscopy (TEM) to guarantee their homogenous spherical shape and size. Then measurements by Ultraviolet-Visible spectrophotometry (UV-Vis), Infra-red spectrophotometry (IR) would be done to guarantee that the extract got encapsulated inside the prepared nanoparticles and the time at which this extract would be released inside the body by encapsulation efficiency (EE), loading capacity (LC) and release tests and calculations. Finally, we would to test the efficacy of the NPs and see whether the total phenolic content, antioxidant activity and antibacterial action got enhanced or not compared to the plant extract itself.

From the above work and trials it was proved that natural food waste is a very rich source of medicinal compounds that had high impact on many diseases. Many scientists tackled using the seed, peel, fruit, rind and many other edible and non-edible plant parts to manufacture safer and less economic drugs, food preservatives, food packaging and metal chelators for purifying water.

From the above work it was proven that AS OD NPs were of hydro-dymanic size 152.3-254.93 ±60 nm and TEM size of 1.01-2.05 nm, poly dispersion index of 0.39±0.08 and potential of 32.49-35.21±0.4. the NPs encapsulation AS extract in ratio of chitosan : ASOD 1:7 (w/w) had encapsulation efficiency of 70.37% and loading capacity of 61%. Release rate of those NPs was

68.2%±0.04 at 48 hrs. They showed enhanced antioxidant and antibacterial actions where the NPS were 1.2 fold more antioxidant than the plant extract and 100 fold more antibacterial than the plant extract.

Future attention should be given to how to separate the peel of either Ajwah date or black date plant since they proved to be rich in TPC and AO. A cleaner and more sophisticated means will be required to separate them and make good use of them and here comes the role of the country and society to supply us with the tools, techniques or instruments needed to do so.

Many papers and protocols were recently published about the effect of the date plant on controlling the cardiac rhythm and protection against Coronary Heart diseases. Those trials were just tried on animals and still under in-vivo tests to prove their validity. Applying the pit extract as antibacterial in its nano-form will need further in-vivo tests on animals and man to prove its effectiveness against bacterial diseases in the human body.

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