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Development of date fruit nanoparticles and investigating its effect on *Pseudomonas aeruginosa* biofilm

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جامعة الاسراء

نموذج تفويض

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Dedication

I dedicate this work to **my wonderful parents** who have supported me to do the best and their best wishes to me all time.

I dedicate this thesis for **my lovely sister** and **lovely brothers**.

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I would like to express my gratitude to **Dr. Eman Zmaily** for accepting my research idea and supporting it as much as possible and for unlimited support and encouragement. In addition to her valuable comments and advices, her optimism was the most supportive of me.

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List of Abbreviations

ASM	Artificial sputum medium
COPD	Chronic obstructive pulmonary disease
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
COX	Cyclooxygenase
CDN	Chitosan date nanoparticle
DPI	Dry powder inhalers
DNase	Deoxyribonuclease
DTPA	Diethylenetriaminepentaacetic acid
Equiv	Equivalent
ED	Emitted dose
EE	Encapsulation efficiency
E. coli	*Escherichia coli*
FPF	Fine particle fraction
FEV_1	Forced Expiratory Volume in 1 Second
FDA	Food and Drug Administration
FTIR	Fourier-transform infrared spectroscopy
FPF-Theo	Fine particle fraction theoretical
Intravenous	IV
MCC	Mucociliary clearance
MIC	Minimum inhibitory concentration

MBC	Minimum biocidal concentration
NGI	Next generation impactor
P. aeruginosa	*Pseudomonas aeruginosa*
PMDIs	pressurized metered dose inhalers
PSA	Particle size analysis
RPM	Round per minuets
S. aureus	*Staphylococcus aureus*
WHO	World Health Organization

Development of date fruit nanoparticles and investigating its effect on *Pseudomonas aeruginosa* biofilm

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Abstract

Cystic fibrosis (CF) patients suffer from recurrent lung infections mainly bacterial infection such as *staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). Although prophylactic antibiotics are usually prescribed, bacterial resistance is a key challenge. Therefore, there are attempts to identify new antibiotics, particularly those from natural sources, that may enhance the prognosis of the disease. Therefore, the aim of this project was to investigate the use of date fruit extracts as a potential candidate for the development of dry powder inhaler (DPI) with potential biological activity against *P. aeruginosa*. Date fruit (*Phoenix dactylifera L.*) consists of flavonoids, polyphenols, minerals and vitamins, and in many studies, it was reported that it had an effect on gram-positive and gram-negative bacteria. When talking about lung diseases, the pulmonary route for drug delivery provides special advantages when compared with other routes. The drugs can reach the site of action more efficiently. Dry powder inhaler (DPI) was employed to deliver the powder formulations. Aqueous date fruit extract: chitosan-based nanoparticles with increasing concentration of chitosan were prepared. Chitosan 0.05% w/v nanoparticles demonstrated the highest encapsulation efficiency (55.91%) and delivered the highest emitted dose (98.92%) and Fine particle fraction of nominated dose (42.62%). Chitosan date nanoparticles (CDN) showed MIC of 4.5 mg/ml and demonstrated a significant inhibition of biofilm formation. CDN is suggested as a potential candidate for the management of cystic fibrosis as an DPI.

Chapter One
Introduction

1. Introduction

Many people are suffering from respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and lung cancer. Almost four million people die each year from chronic respiratory diseases. According to the World Health Organization (WHO), 235 million people suffer from asthma causing 180,000 deaths worldwide each year. Furthermore, the number of people suffering from COPD is 200 million people worldwide, where COPD is the fourth leading cause of death worldwide (Ferkol and Schraufnagel, 2014). The prevalence of CF is (1 in 2500) with carrier frequency of CF is 1 in 28 in North American white populations, and 1 in 84 in African Americans (Rohlfs *et al.*, 2011).

1.1 Cystic Fibrosis

Cystic fibrosis is an inherited disorder in white populations due to mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene that causes severe damage to the lungs, digestive system, and the reproductive organs. The importance of CFTR gene is the regulation of chloride transport across the cell membrane. The defect in this gene causes unnatural movement of salt and water across the cell, and affects the cells that produce mucus, sweat and digestive juices resulting in the production of sticky and thick secretion that block passageways, ducts and tubes, especially in the lungs and pancreas (Sockrider and Ferkol, 2017).

Cystic fibrosis affects mostly the lungs, but also the pancreas, liver, kidneys, and intestines. Long-term issues pertinent to CF include difficulty breathing, coughing up mucus and the accumulation of mucus in the lungs which allows bacteria to grow more easily causing infections. Other signs and symptoms may include sinus infections, poor growth, fatty stool,

clubbing of the fingers and toes, and infertility in most males. It is caused by the presence of mutations in both copies of the gene for the CFTR. Those with a single working copy of CFTR, are carriers, and otherwise mostly normal. CFTR is involved in the production of sweat, digestive fluids, and mucus. When the CFTR is not functional, secretions which are usually thin, become thick. The condition is diagnosed by a sweat test, and genetic testing. In parts of the world, where the defect in the gene is very common, screening of infants at birth with a sweat test may take place (Wright and Vera, 2017).

There is no known cure for CF, and the most common complications come from lung infections. Acute lung infections are treated with antibiotics, which may be given intravenously, inhaled, or by mouth. Airway clearance techniques, such as chest physiotherapy have some short-term benefits, but the long-term effects are unclear. Sometimes, when someone has repeated infections, the antibiotic azithromycin is used for long term. Inhaled hypertonic saline, and salbutamol, may also be useful and lung transplantation may be an option, when lung function becomes very poor. Aside from treatment of the lungs, pancreatic enzyme replacement, and fat-soluble vitamin supplementation are also important, especially in the young (Wright and Vera, 2017).

The average life expectancy for CF patients is between 42 - 50 years. Lung problems are responsible for around 80% of related deaths. For people of Northern European ancestry, about one out of every 3,000 newborns. In addition, about one in 25 people in Northern Europe is a CF gene carrier. The disease is least common in Africans, and Asians (Bannon *et al.*, 2015).

Additionally, CF patients usually suffer from osteoporosis, sinus disease, reproductive health issues and mal absorption of food, leading to nutritional problems and unhealthy low weight. Also enzyme and fat-soluble vitamins (A, D, E, and K) supplement should be taken (Sockrider and Ferkol, 2017).

Management of CF is done by taking many supplements and antibiotics. The supplements are used to compensate the shortage in many digestive enzymes (e.g., Lipase, Protease and Amylase), and vitamins particularly fat-soluble vitamins. Excessive thick mucus that is present in airways causes complications such as chronic bacterial and fungal infections and this will accompany the formation of biofilm, which is the biggest challenge in CF disease. Managing CF entails using airway clearance therapies, anti-inflammatory medications, and antibiotic for treatment and prophylaxis. Oral, intravenous **(**IV), and aerosolized antibiotic formulations are indicated and are used in CF patients. A major disadvantages in CF patients is that pathogens are not completely destroyed from the airways and the resistance will often develop (Wright and Vera, 2017). Drugs prescribed for the management of CF are summarized in Tables 1.1 to 1.4.

Long-term use of prophylactic antibiotic can improve clinical status, improve lung function, and improve survival. On the other hand, it causes adverse effects such as diarrhea, skin rash, candidiasis, and it leads to the emergence of antibiotic resistance and earlier isolation of *P. aeruginosa* from respiratory cultures (de Boer *et al.*, 2017).

Dry powder inhalers (DPI) is an ideal choice to reduce adverse effect because it acts locally by delivering antibiotics or other drugs directly to the lungs and reach infected airways,

therefore, allows effective treatment of lung infections. In addition to that, it enhances the patient adherence to the medication (de Boer *et al.*, 2017).

Table1. 1: Antimicrobial Agents used to manage Cystic Fibrosis (H: hour, IV intravenous, Q: every) (adapted from (Wright and Vera, 2017)).

Drug	**Route of administration / Dosage form**	**Adult Dose**	**Frequency Range**	**Pathogens**	**NOTE**
Ciprofloxacin	Oral	750 mg	Q 12 H	*Pseudomonas, Alcaligenes*	
	IV	400mg	Q 8 H		
Doxycycline	Oral	Max 200mg/day	Q 12-24 H	*Stenotrophom-nas, Staphylococcus*	
	IV				
Amikacin (Arikayce®)	IV	15 mg/kg/day	Q 8 H	*Pseudomonas*	
	Oral inhalation	590 mg/8.4 mL	Q24H	*Pseudomonas*	Liposome suspension for oral inhalation, administered by Lamira Nebulizer System.
Aztreonam (Cayston®)	IV	2 g	Q 6-8 H	*Pseudomonas*	
	Inhalation solution	Lyophilized aztreonam (75mg/vial)	Q 8H	*Pseudomonas*	Patients should use a bronchodilator before administration of Cayston.
Cefepime	IV	2 g	Q8 H	*Pseudomonas*	
Ceftazidime	IV	2 g	Q8 H	*Pseudomonas, Burkholderia, Alcaligenes*	
Colistimethate	IV	5-8 mg/kg/day	Q8 H	*Pseudomonas*	
Colistimethate (Colobreathe®)	Inhaled dry powder	125 mg	Q 12 H	*Pseudomonas*	Each capsule contains 1,662,500 IU, which is approximately equal to 125 mg of colistimethate sodium.
Tobramycin	IV	7.5-10mg/kg/day	Q 6-8H	*Pseudomonas*	
Tobramycin (Tobi	Inhaled dry powder	224 mg/day	Q 12 H	*Pseudomonas*	28 mg/ capsule 4 capsules each

Drug	Route of administration / Dosage form	Adult Dose	Frequency Range	Pathogens	NOTE
Podhaler®)					dose.
Tobramycin (Tobi®, Bethkis®)	Inhalation Solution	60-600mg/day	Q 12 H	*Pseudomonas*	
Gentamicin	IV	7.5-10mg/kg/day	Q 6-8 H	*Pseudomonas*	
Vancomycin	IV	15 mg/kg	Q 6-12 H	*Staphylococcus (*MRSA, MSSA)	

Table1. 2: Airway Clearance Therapies used to manage Cystic Fibrosis (adapted from (Wright and Vera, 2017)).

Drug	Dose
Albuterol	2 puffs prior to therapy 2-4 times a day
HyperSal® (hypertonic saline)	4 mL delivered via a nebulizer 2-4 times a day
Pulmozyme® (dornasealfa)	2.5 mg delivered via a nebulizer 1-2 times a day
Mannitol (Bronchitol)	400 mg twice a day; ten capsules via the inhaler device twice a day.

Table1.3: Anti-inflammatory Therapies used to manage Cystic Fibrosis (adapted from (Wright and Vera, 2017))

Drug	Dose
Ibuprofen	(20-30 mg/kg) of body weight twice daily
Azithromycin	(250-500 mg) given three times weekly

Table1. 4: Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein as a Therapy used to manage Cystic Fibrosis (adapted from (Wright and Vera, 2017)).

Drugs	Dose	Note
Ivacaftor (Kalydeco®)	150mg Q12H given orally with fatty food	For patients 6 years or older with the G551D mutation.
Lumacaftor and Ivacaftor (Orkambi®)	800mg/500mg Q24H given orally with fatty food	For patients 12 years or older with the ΔF508 mutation.

1.2 Dry Powder Inhalers

DPIs are good option for the effective delivery of drugs to the lung with reduced side effects and dose when compared to systemic drug delivery. The success of DPIs formulations is dependent on several factors such as the particle size distribution of the drug, inhalation flow rate, fine-lactose content, lactose (or carrier) source, and the dispersion capacity of the respective DPIs device. The most used excipient in DPI formulations is Lactose monohydrate, which is used as a carrier. To ensure effective delivery of the drug to the lower parts of the lungs, the drug particles size should be between 1– 5 μm, but the carrier particles size should be between 60 μm – 160 μm. During inhalation, the drug particles detach from the lactose and the larger carrier particles impact the oropharyngeal region. Figure 1.1 demonstrates the two main methodologies used in developing DPI and their dispensing mechanisms. When the dose is low (50-500 µg), carrier-based formulation is employed. The major advantages of using a carrier are enhancement the flowability, handling small doses, dispensing, actuation of the micronized drug and prevent aggregation. However, large doses, require alternative techniques such as loose agglomerates (Figure 1.1 A) (Mehta, 2016).

Physical and chemical characteristics of powder, stability, biocompatibility, biodegradation without any adverse effect and aerosolization properties of the formulation these are the key requisites that should be taken into consideration when preparing DPIs (Mehta, 2016).

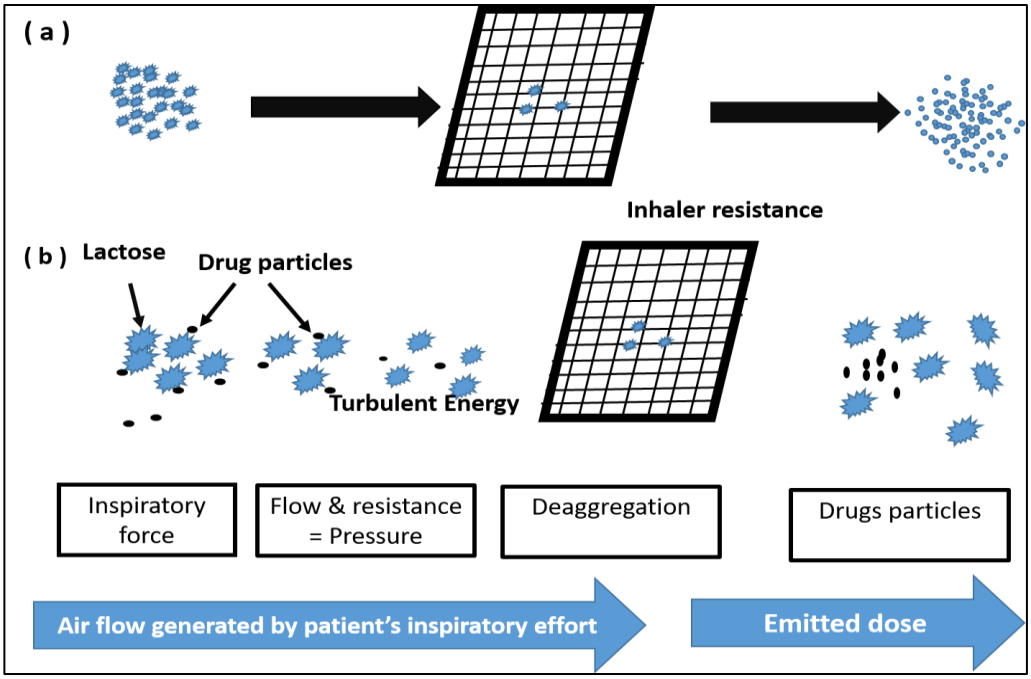


Figure1. 1: Schematic diagram of dry powder inhaler formulation strategies and dispensing mechanisms. (A) Drug-only formulation (drug agglomerates); (B) Carrier-based formulation. Adopted from (Lavorini, Pistolesi and Usmani, 2017).

Various novel drug delivery systems were developed, for example, solid lipid nanoparticles, liposome, nano-aggregates, polymeric nanoparticles, microspheres, nano-composites and polymeric microparticles, have obtained a number of advantages for traditional DPI, including chemical stability and physical flow properties, distribution, dispersion, and bioavailability (Mehta, 2016).

DPIs are designed for low drug doses in the management of COPD, asthma and CF. Research regarding the development of inhalers design for future applications of DPIs is to make it cost-effective and safe. However, with the increasing interest in systemic delivery of drugs via the lungs, high dose drug delivery is under extensive research employing particle engineering and other innovative technologies. Safety is served by minimizing the use of unnecessary excipients, designing simple inhalers and increasing patient adherence to the

therapy. For some applications, like delivery of hygroscopic formulations and vaccines; disposable inhalers may be preferred (de Boer *et al.*, 2017). Development of inhalation technology for treating various extra pulmonary and intrapulmonary diseases is supported by the lungs' unique geometry such as thin alveolar epithelial lining, large surface area, avoidance of first pass metabolism and high vascularization (Mehta, 2016).

DPIs were introduced to overcome some of the challenges and drawbacks associated with the nebulizers and pressurized metered-dose inhalers (pMDIs) (Al-Hallak *et al.* 2011). Nebulizers require a source of compressed air also pMDIs have limitations such as crystal growth, sedimentation, synchronize actuation inhalation and build deposition in the oropharynx due to high velocity therefore, increase the systemic absorption risk (Winkler, Hochhaus and Derendorf, 2004). Whereas for DPIs such limitations were not reported, and it has several advantages as stated above.

Therefore, the use of DPIs is the focus of this project owing to its multiple merits and to enable better deposition of drug to the lung as well as enabling enhanced control of the prognosis of the disease.

1.3 Mechanisms of drug deposition within the lung

The mechanisms of drug deposition in the lung are impaction, sedimentation, and diffusion, as depicted in (Figure 1.2). The particle size of drug plays a significant role in determining the area of settling within the lung. The larger particles of more than 8 μm settle in the upper respiratory system, oropharyngeal region by impaction and sedimentation, small particles (1-5 μm) reach to lungs through diffusion, while particles with size between 1–2.8 μm reach

the alveoli region. However, in some cases submicron particles are exhaled and fail to deposit in the lungs (Lavorini, Pistolesi and Usmani, 2017).

To improve inhalation products performance, the nanotechnology and particle engineering techniques can be employed to introduce a progress in developing these products and also improve particle dispersion and lung deposition of the inhalation formulation (Cheng, 2014).

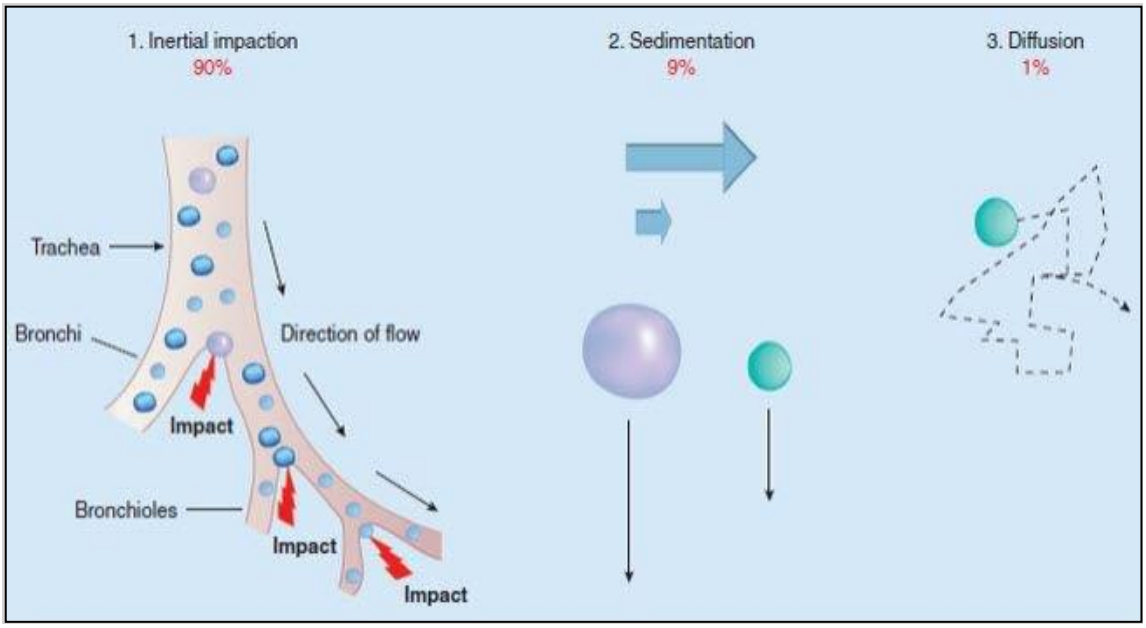


Figure1. 2: Schematic diagram highlighting particle deposition process in the respiratory tract. Adopted from (medescape, 2019).

1.4 Natural biodegradable polymer and DPI excipient

Many types of polymers are used in drug targeting, drug delivery, biotechnology and other pharmaceutical applications. For pulmonary delivery, the natural biodegradable polymers are usually selected, such as albumin, collagen, alginate, gelatin, chitosan and cyclodextrin. Also, poly(lactide-co-glycolide) is an example of synthetic polymer that could be used for pulmonary applications (Rytting *et al.*, 2008). The main properties of these polymers are

biocompatibility, biodegradability, non-toxic and their structure could be chemically modified (Jana and Jana, 2016).

1.4.1 Chitosan

Chitosan is a natural polycationic linear polysaccharide derived by partial deacetylation of chitin. It contains a primary amine group. Chitosan has many biological effects such as antimicrobial activity and antioxidant effect, and it has many uses, for example: encapsulation of biologically active compounds, carrier for sustained drug release and drug delivery systems such as pulmonary, nasal, brain and oral drug delivery. Also, it is employed in anticancer delivery, gene delivery and insulin delivery. Also it is mucoadhesive, biodegradable, biocompatible, and permeability enhancer (Divya and Jisha, 2018; Jana *et al.*, 2013). It has a pKa value of 6.2, therefore, it is soluble in acidic media but not alkaline media or neutral pH (Adolfo *et al.*, 2017). In a recent study, chitosan nanoparticles were used to encapsulate date (*Phoenix dactylifera L.*) seed extract to enhance the biological activity, antioxidant and antibacterial effects. Indeed, there is a significant difference in biological activity between the date seeds extract and date seeds extract nanoparticles that enhance antioxidant and antibacterial activities of about 1.2 fold more antioxidant than the plant extract and 100 fold more antibacterial than the plant extract (Badawi, 2018).

1.4.2 Magnesium stearate

Magnesium stearate is a fatty acid salt, very fine white powder, and commonly used as lubricant and force control agent in pharmaceutical industry. It is widely used in formulation of tablets and DPIs. Micro and nano scale particles are more cohesive and demonstrate poor

flowability. To improve these properties, force control agents can be used such as licithin, leucine and magnesium stearate. The exact principles for improving the powder flowability by coating or surface layering are not well understood. The modification of the surface properties of the particles, reduction of interparticle cohesive forces improve powder flowability (Begat *et al.*, 2005).

1.5 Effect of date on the prognosis of CF

1.5.1 Date composition

Date fruits have many health benefits because of its nutrients content such as carbohydrates, minerals, lipids, proteins and phenolic compounds (Gu *et al.*, 2003). In addition, date fruits have biological activities, such as anti-mutagenic and antioxidant (Vayalil, 2002), anti-carcinogenic (Rahmani *et al.*, 2014), anti-inflammatory (Tang, Shi and Aleid, 2013) and antimicrobial activity (Taleb *et al.*, 2016).

Date fruits have high composition of carbohydrates, salts and minerals, dietary fibers, vitamins, fatty acids and amino acid which gives a unique value in human nutrition (Al-Shahib and Marshall, 2003). The amino acid content in fresh and dried dates are shown in (Table 1.5). A 100 g serving of dates provides almost 75 g of carbohydrates, 85% of total carbohydrate is present in the form of simple sugars (glucose and fructose).

Dates fruits are rich in ascorbic acid, vitamin K and B-complex vitamins (thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), folate (B9)) (Ahmed, Al-Jasass and Siddiq, 2014). In addition, Dates fruits contain essential minerals, where 100 g of date contains 696 mg of potassium, 362 mg of copper, 90 mg of magnesium, and 90 mg

of iron, that are essential for bone growth. The significantly high potassium in dates are favorable for people who have hypertension (Appel *et al.*, 1997). Potassium helps to control blood pressure and muscle contractions (Al-Shahib and Marshall, 2002). Also, copper is essential for red blood cells production. Dates are good source of antioxidant constituents including, carotenoids (0.92–2.91 mg/100 g), total antioxidant selenium (0.356 to 0.528 mg/100 g), and phenolics (217–343 mg of ferulic acid equiv/100 g) (Baron, 2006).

Owing to high content of nutrients, this project will investigate its use for the management of cystic fibrosis, this project will build on such results and investigate the use of inhalable date powder for the treatment of cystic fibrosis.

Table1. 5: Summary of the range of amino acids content in fresh and dried dates (Ahmed, Al-Jasass and Siddiq, 2014)

Amino acid	Content (mg/100g) Fresh date fruit	Content (mg/100g) dried date fruit
Alanine	30	133
Arginine	34	148
Aspartic acid	59	309
Cysteine	13	67
Glutamic acid	100	382
Glycine	42	268
Histidine	0.1	46
Isoleucine	4	55
Leucine	41	242
Lysine	42	154
Methionine	4	62
Phenylalanine	25	67
Proline	36	148
Serine	29	128
Threonine	23	95
Tryptophan	7	92
Tyrosine	15	156

1.6 Study Aim and Objectives

The aim of this research project is to develop DPI formulation containing dried dates fruit powder or extract. The formulation will be investigated to deliver high fine particle fraction to the lung and assessed the antibacterial and anti-biofilm effect. The project will investigate the possibility of developing DPIs from date's powder using carrier-based technology and or nanoparticles then testing the developed formulation. Specific challenges that will be tackled in this project are pertinent to optimization of effective DPI formulation to delivers high fine particle fraction (FPF) (>20%) and emitted dose (>60%) and enables the targeting into designated area within the lung. The specific objectives are as follows:

- Preparation of dates powder DPI formulation using carrier-based technology (carrier lactose or coarse dates powder) or nanoparticles using biocompatible polymer such as chitosan.
- Evaluating biofilm inhibition of *P. aeruginosa* grown in artificial sputum medium by date extract and the DPI formulations.

Chapter Two
Literature Review

2. Literature Review

This chapter will highlight the research that was conducted for the development and evaluation of various drugs and /or dosage forms for the management of CF and hence the motive of this research.

2.1 Inhaled antimicrobial agents

2.1.1 Gentamicin

In a study comparing gentamicin in three different dosage forms; DPIs versus intravenous and nebulized solution, results revealed that there was no significant difference observed in pulmonary function, no bronchospasm detected after gentamicin administration after each of the three types of dosage forms. However, a significant reduction was noted in the bacterial count for both inhalation treatments when compared to intravenous. Further, a significant difference was found in drug distribution, sputum concentrations of gentamicin after nebulizer administration which was approximately 20-fold greater than intravenous administration. However, the gentamicin sputum concentration after DPI administration was 7-fold lower than with a current standard “wet” nebulization device. DPI delivery achieved minimal systemic absorption of gentamicin, no apparent bronchial irritation, and eliminate the risk of nephrotoxicity and ototoxicity. All patients in this study stated that they favored to take antimicrobial therapy with a DPI (Renée Crowther Labiris *et al.*, 1999).

2.1.2 Tobramycin

In other study, a single dose administration of tobramycin inhalation powder resulted in three times more efficient and rapid delivery of tobramycin compared to tobramycin solution for inhalation in CF patient, but the pharmacokinetic properties were still the same. Systemic

exposure to tobramycin achieved after administration of the 112 mg of tobramycin inhalation powder (4 capsules x 28 mg) were very similar to that seen after 300 mg dose of tobramycin solution for inhalation (Geller *et al.*, 2007).

2.1.3 Ciprofloxacin

Dry powder ciprofloxacin may be more successful in patients with non-cystic fibrosis bronchiectasis because of the less lifetime exposure to ciprofloxacin and lower microbial load (Wilson *et al.*, 2013). After 30 days of ciprofloxacin powder inhalation, the antimicrobial effect of ciprofloxacin was attenuated because ciprofloxacin rapidly induced resistance and contributed to the lack of improvement in Forced Expiratory Volume in 1 Second (FEV_1) (Dasenbrook, Konstan and VanDevanter, 2015).

In a research conducted by Yang and co-workers, where they compared between the inhaled ciprofloxacin powder and the combination of two inhaled powder; ciprofloxacin with recombinant deoxyribonuclease (DNase) which is a mucolytic agent. The higher activity of the combination is attributable to the mucolytic activity of DNase, which promotes the penetration and diffusion of ciprofloxacin. This strategy for local antipseudomonal therapy can be achieved efficiently using ciprofloxacin delivery to the CF airway (Yang *et al.*, 2010).

2.2 Prevention of Biofilm Formation

Biofilm formation is the biggest challenge in CF disease. It is the main cause of chronic infection specially the opportunistic pathogen *P. aeruginosa*. The chemistry of infected host environments should be considered at different stages of the disease to design effective treatment strategies. Iron detected in sputum samples collected from patient in different stages of the disease. The iron concentration increases with increasing in disease severity. In

a study revealed that the presence of iron in airways is an important factor in the formation of biofilm and the use of ferric iron chelation therapy may be a novel therapeutic strategy for CF lung infections (Hunter *et al.*, 2013).

2.2.1 Cysteamine

Cysteamine is a novel mucoactive antimicrobial and anti-biofilm agent for the treatment of cystic fibrosis. Cysteamine prevent the formation of biofilms, and it disrupt established *P. aeruginosa* biofilms. Also, Cysteamine acts as mucolytic activity and rapidly bactericidal against both persistent cells and metabolically active of *P. aeruginosa*. It has a synergistic effect with conventional CF antibiotics, this synergistic effect related to its ability to reverse antibiotic resistance or insensitivity in CF bacterial pathogens. Cysteamine can be used to synthesize glutathione, which is the most potent intracellular antioxidants (Charrier *et al.*, 2014).

2.2.2 D-amino acid

Biofilms are communities of cells held together by a self-produced extracellular matrix typically consisting of exopolysaccharide, protein, and often DNA. The mixture of D-leucine, D-methionine, D-tyrosine, and D-tryptophan prevent biofilm formation and can break down existing biofilms. D-amino acids may be a widespread signal for biofilm disassembly, they also prevent biofilm formation by *Staphylococcus aureus* and *P. aeruginosa*. The presence of D-amino acids cause alteration in a protein (YqxM) that is required for the formation and anchoring of the fibers to the cell (Kolodkin-Gal *et al.*, 2010; Hochbaum *et al.*, 2011). Therefore, the merits of dates as it is rich in amino acids could be of potential effect on CF in our study.

2.3 Airway Clearance Therapies

2.3.1 Mannitol

Inhaled mannitol powder has beneficial effects on lung function, sputum properties, quality of life and mucociliary clearance (MCC). Mannitol dry powder inhaler increases the MMC. The mechanism of action of mannitol is indirect effects of increased osmolarity on the rheology of the mucus and on the ciliary activity. The increase in osmolarity of the airway lumen causes depolarization of the epithelial cell membrane which could cause an increase in ciliary activity. Further, the increase in osmolarity stimulate water movement out of the cells, this water movement lead to shrinkage of the epithelial cells and increase intracellular calcium level. Increasing calcium within the cell has been shown to increase ciliary activity (Daviskas *et al.*, 1997). Therefore, it is proposed that dates high sugar content could support the change in rheology of sputum and hence enhance the prognosis of CF.

A comparative study between the response of asthmatic and healthy subjects to administered mannitol DPI was made by Daviskas and colleagues. The results revealed that there was a significant increase in MCC in all lung regions for both asthmatic and healthy subjects. The increase in MCC was of a similar degree in both asthmatic and healthy subjects although it tend to be greater in asthmatic subjects because asthma patients were more sensitive to change in osmolarity than healthy individuals (Daviskas *et al.*, 1997).

Inhaled mannitol 400 mg twice a day was prescribed for CF patients for long-term. In a randomized controlled trial, that evaluated the efficacy and safety in the long-term use of inhaled mannitol, the conclusions in this study confirmed its potential role for the chronic therapy of CF and the safety profile was also acceptable (Aitken *et al.*, 2012).

2.3.2 Hypertonic saline

Hypertonic saline enhances MCC, it has multiple mechanisms of action (e.g., mucolytic, expectorant, mucokinetic, ion-transport modifier). The mucolytic effect of hypertonic saline is not the primary mode of action. Hypertonic saline can reduce cross-linking within the mucus gel by breaking down the ionic bonds. Also, hypertonic saline has another mucolytic mechanism which is the ability to dissociate DNA from the mucoprotein. This facilitates digestion of the mucoprotein by natural proteolytic enzymes (Elkins and Bye, 2011). Hypertonic saline acts also as expectorant. It prevents chloride secretion and allows sodium absorption from the epithelial cells in the airway lumen that maintain the usual hydration of the epithelial surface, thus decrease mucus secretion (Elkins and Bye, 2011).

In several randomized controlled trials that compared hypertonic saline with mucolytic agents. DNase led to a greater increase in FEV1 % than hypertonic saline (5 mL twice daily) at 12 weeks. Hypertonic saline and mannitol showed no differences in sputum clearance, but mannitol was reported to be more 'irritating' (Wark and McDonald, 2018).

2.3.3 Dornase alfa

Dornase alfa cleaves extracellular DNA and actin, resulting in reduced DNA length and concentration, therefore it reduces the viscosity of mucus in the lungs, improves clearance of secretions and improves pulmonary function (JS Wagener, 2012). There is no serious side effects, with only a change in voice and rash seen frequently in those people taking dornase alfa (Yang *et al.*, 2016). Dornase alfa has an additive effect to treatments such as inhaled hypertonic saline, inhaled dry powder mannitol and inhaled antibiotics.

2.4 Cystic Fibrosis Transmembrane Conductance Regulator protein

2.4.1 Ivacaftor and Lumacaftor

Food and Drug Administration (FDA) approved Ivacaftor on January, 2012 and also Approved Lumacaftor on July, 2015. Almost 4-5% of CF patients worldwide demonstrate a defect in G551D and ivacaftor is used for mutation in G551D. It is available as oral granules or tablets taken two times a day with fat-containing food (FDA, 2017). Its mechanism of action is by acting as a potentiator of the CFTR protein, an ion channel involved in the transport of chloride and sodium ions across cell membranes of the pancreas, lungs, and other organs (Saint-Criq and Gray, 2017) .

Combination of Lumacaftor and Ivacaftor was given to manage of CF with F508 del-CFTR mutations which 70% of patients with CF worldwide have this mutation type, is known to improve CF symptoms and underlying disease pathology by helping the conformational stability of F508del-mutated CFTR, producing an enhancement in processing and trafficking of mature protein to the cell surface. Also, Lumacaftor prevents misfolding of CFTR ion channels and consequent destruction during processing in the endoplasmic reticulum (Mayer, 2016).

2.5 Anti-inflammatory Therapies

In CF airways and chronic inflammation are closely linked. The inflammation process in the epithelial cells and immune cells lead to secretion of proinflammatory mediators that promote overly exuberant of neutrophil influx into the airways, then neutrophils release proteases, such as neutrophil elastase, anti-inflammatory agents should be used to reduces the exacerbation and to improve lung function (Elizur, Cannon and Ferkol, 2008). It is proposed

that the natural contents of dates that promote anti-inflammatory effect with no reported side effects provides an additional benefit in this project.

2.5.1 Ibuprofen

Ibuprofen is a drug used in high dose to manage inflammation and give apparent benefit. However, high dose ibuprofen therapy causes renal and gastrointestinal toxicities for that infrequently applied to CF patients. Corticosteroids and ibuprofen have demonstrated benefits, but their use have been limited by adverse effects (Elizur, Cannon and Ferkol, 2008).

2.5.2 Azithromycin

Recently, azithromycin was employed as a potential anti-inflammatory therapy for CF lung disease. Its mechanism of action remains unclear, although it is increasingly used as an immunomodulatory agent. Fewer pulmonary exacerbations and better lung function are the outcomes of azithromycin use (Elizur, Cannon and Ferkol, 2008).

2.6 Therapeutic uses of date *(Phoenix dactylifera)*

Antioxidant activity of date is related to the wide range of phenolic compounds present in dates including ferulic, p-coumaric, and, flavonoids, sinapic acids, and procyanidins (Mansouri *et al.*, 2005; Gu *et al.*, 2003). Another study indicated that date fruit consisted of 13 flavonoid glycosides of quercetin, apigenin, and luteolin. Despite the antitumor activity of date that was established, the exact date composition mechanism of action in the prevention of tumor was not clearly elaborated. Previous studies reported that β-D-glucan, a content in dates, had shown antitumor activity. The date's constituents such as flavonoid and phenol have an important and significant role in cancer control through the regulation of genetic

pathways without any side effect (Ishurd *et al.*, 2002). In a recent study, that show preparation of nanoparticle of date seed and investigate their antioxidant and antibacterial properties, the result of this study indicated that date seed nanoparticles multiplicate the antibacterial activity around 100 fold more than the date seed extract and 1.2 fold more antioxidant effect than the date seed extract (Badawi, 2018).

The transcription factors LOX and NF-kB play a critical role in cancer, diabetes, inflammation and other diseases. Regulation of these factors is significant step in the prevention of different disease, flavonoids and phenols act as great anti-inflammatory agents dates inhibit the lipid peroxidation cyclooxygenase enzymes COX-1 and COX2 (Zhang *et al.*, 2013).

Previous studies presented the efficacy of date sap for the treatment of lung injury. After the application of a murine model of pulmonary fibrosis, the result demonstrated that date sap can attenuate bleomycin induced lung fibrosis due to its richness in phenolic compounds, vitamins, anti-oxidative and anti-fibrotic effects (Bahri *et al.*, 2018; Bahri *et al.*, 2019).

For all such benefits, this research project will investigate the benefits of date powder in inhalation dosage forms on CF.

2.6.1 Microbiological effect of date

A study tested the effect of date syrup on gram negative *Escherichia coli* (*E. coli*) and Gram positive *Staphylococcus aureus* (*S. aureus*), the result indicated that there is an antibacterial effect, the minimum inhibitory concentration (MIC) for E. coli and *S. aureus* was 30 mg /ml and the minimum bactericidal concentration (MBC) is 40 mg/ml, 35 mg/ml respectively (Taleb *et al*., 2016).

The leaf, fruit and seeds of date palm have a significant antibacterial activity on gram positive (*S. aureus, Streptococcus pyogenes*) and gram negative (*E. coli* and P *aeruginosa*) (Al-Daihan and Bhat, 2012). Moreover, it is preferable to identify the active ingredients present in the extracts that can be exploited for pharmaceutical use (Al-Daihan and Bhat, 2012).

In a recent study, novel formulation of nanoparticle of date seed extract with chitosan was done by using ionotropic gelation technique. Furthermore, this formulation was tested on *E. coli and S. aureus* and the result suggested that date seed nanoparticle enhanced the antioxidant and antibacterial actions for 100 fold more antibacterial than the plant extract and 1.2 fold more antioxidant effect than the plant extract (Badawi, 2018).

Chapter Three Materials and Method

3. Materials and methods

3.1 Materials

Zahidi date powder was donated by Wood spur Farms (CA, USA). α-Lactose monohydrate was purchased from Central Drug House (New Delhi, India). Magnesium stearate and magnesium citrate were purchased from Sinopharm Chemical Reagent Co., Ltd (Mainland, China), Glycerin, Glycine and L-cysteine were obtained from Guangdong Guanghua Chemical Factory Co., Ltd. (Guangdong, China). DNA from fish sperm, cellulase, chitosan, and mucin from porcine stomach (type II) were purchased from Sigma Aldrich (Steinheim, Germany). L-tyrosine, L- Alanine, L-Arginine, L-Asparagine, L- Aspartic acid, L-Glutamine, L-Glutamic acid, L-Histidine, L-Isoleucine, L- Leucine, L-Lysine, L-Methionine, L-phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L- - Valine were purchased (Sigma Aldrich, USA). Tris (hydroxymethyl) aminomethane and diethylenetriamine Penta acetic acid were purchased from SD Fine-Chem Limited (Mumbai, India). Potassium hydroxide, Potassium chloride, Sodium chloride were obtained from AZ Chemicals, Inc. (ON, Canada). Egg yolk emulsion, nutrient agar and nutrient broth were purchased from Biolab (Budapest, Hungary). Hard gelatin capsules size 3 were donated from Pharmacare (Amman, Jordan). Acetic acid was purchased from Labchem (NJ, USA). Acetone was obtained from Alpha Chemika (India).

3.2 Methods

3.2.1 Date powder, chitosan and CDN characterization studies

Initial attempts were made to characterize the date powder in terms of particle size analysis, moisture content, FTIR and UV Spectrophotometer analysis.

3.2.1.1 Particle size analysis (PSA)

PSA was conducted using sieving method. A nest of sieves were constructed with the following sizes: 1000, 500, 355, 250,180, 125 µm using vibration Erweka AR- 400 Esieve shaker Erweka, (Langen, GmbH). 50 g of date fruit powder was accurately weighted using analytical balance (Sartorius analytic AC 120 S),then placed on the upper sieve for PSA. Speed was set at 400 vibration min^{-1} to enable high vibration intensity for a duration of 5 min.. After that, the remaining powder in each sieve was collected and weighted. This experiment was repeated three times and particle size distribution curve was plotted.

3.2.1.2 Moisture content determination

Moisture content was measured using the mass weight loss upon heating. One g of date powder was placed in the oven (Gallenkamp, Fisons Erba Science (UK)) at 40°C, during three weeks the mass was measured until it was stabilized. The moisture content test was repeated three times, the moisture content was calculated using equation 3.1.

$$Moisture\ Content\ (\%) = \frac{w - d}{w} \times 100\% \ \ldots\ldots\ldots Eq.(3.1)$$

Where w: initial weight and d: weight after drying

3.2.1.3 Fourier transform infrared (FTIR) spectroscopy analysis

FTIR spectra of date powder, chitosan and CDN samples were recorded using Perkin Elmer FTIR spectrometer (OH, USA), coupled with Spectrum 10 software which was used to run and treat FTIR spectra. Few milligrams were loaded on the sample holder that is located above the laser lens, and then held in place by screwing down the relevant adaptor. For each

sample the FTIR spectra scans were obtained over the range of 450–4000 cm^{-1} with a resolution of 2 cm^{-1}.

3.2.1.4 UV- Spectrophotometric analysis of the crude date powder.

10 g of date fruit powder was soaked in 100 ml distilled water for 12 hours. Firstly, a sample of filtrated extract was taken to detect λ max measuring absorbance at different wavelengths from 190 nm to 375 nm using UV-Spectrophotometer Rayleigh UV-2601 (Beijing, China). Then serial dilutions of the filtrated extract was prepared to create a calibration curve, the process was repeated three times and results were presented as mean ± SD.

3.2.2 Preparation of date powder DPIs formulations.

DPI formulations with dates powder was prepared following carrier-based formulation. Initially, the date powder was milled using ball mill (Erweka AR-400, GmbH, Germany) and fine particles were obtained. The powder was sieved using sieve with aperture size of 32 μm (the smallest available sieve size). The milling process employed 16 ceramic balls in each batch with 50 g of date fruit powder. The ball mill run for cycles of 15 minutes at 40 rpm. The process was repeated until fine powder was obtained. The carrier powder was prepared by sieving lactose powder to obtain the required size range (60-160 μm) by sieving 50 g of lactose powder using 180 micron sieve and 125 micron sieve for 12 minutes at 400 vibration min^{-1} using Erweka vibration sieve shaker (GmbH).Then lactose powder with particle size ranging from 125 -180 μm was stored until use. The formula was based on fine dates powder, lactose, magnesium stearate and fine lactose powder. The different ratios of the fine date powder, magnesium stearate and lactose were mixed using high shear blending (employ either high shear blender (Philips, Amsterdam, Netherlands) or mortar and pestle using

trituration and geometric blending process).

3.2.2.1 Carrier-based formulation using lactose monohydrate

Many attempts were employed to develop carrier-based DPI formulation using lactose coarse carrier as a model carrier material. Table 3.1 summarizes the formulations used.

Table 3. 1: Described the method of carrier-based formulation using lactose.

Sample #	**Batch size (g)**	**Ball mill parameters for date powder**	**Date powder properties**	**Carrier properties and method**	**Lactose/ date ratio w/w g/g**	**Mixing method**
F1	2	Duration: 90 mins Speed:40 rpm	Sieved date powder (particles size less than 32µm)	Lactose sieved (particles size 125-180µm)	60: 40	Geometric dilution technique using mortar and pestle.
F2	2	Duration: 90 mins Speed:40 rpm	Sieved date powder (particles size less than 32µm)	Lactose sieved (particles size 125-180µm)	80:20	Geometric dilution technique using mortar and pestle.
F3	2	Duration: 90 mins Speed:40 rpm	Sieved date powder (particles size less than 32µm)	Lactose sieved (particles size 125-180µm)	80:20	Ball mill
F4	2	Duration: 90 mins Speed:40 rpm	Sieved date powder (particles size less than 32µm)	Lactose sieved (particles size 125-180µm)	20:80	Ball mill

3.2.2.2 Carrier-based formulation using lactose and force control agent

The use of force control agent such as magnesium stearate aimed at reducing the attraction force between the guest (fine date powder) and the carrier, hence enabling higher fine particle fraction. Also, owing to the enhancement of flowability upon its use in small percentage. Three formulations were used as can be seen in Table 3.2.

Table 3. 2: Summary of the carrier-based formulation of dates powder using lactose and force control agent

Sample #	Batch Size (g)	Ball mill parameters for date powder	Date powder properties	Force control agent	Carrier properties and method	Lactose/ date/ force control agent ratios w/w g/g	Mixing method
F5	2	Duration: 90 mins Speed:40 rpm	Sieved date powder (particles size less than 32µm)	Magnesium stearate	-	0: 95:5	Ball mill for 30 minutes
F6	2	Duration: 90 mins Speed:40 rpm Placed in the oven for 15 min at 25 °C before milling	Sieved date powder (particles size less than 32µm)	Magnesium stearate	Lactose sieved (particles size 125-180µm)	50: 47.5:2.5	Date and Magnesium in the mortar and pestle/ light shear blending Then put in the oven for 10 mins at 25°C to loss any humidity Next mixing the lactose
F7	2	Duration: 90 mins Speed:40 rpm Placed in the oven for 15 min at 25 °C before milling	Sieved date powder (particles size less than 32µm)	Magnesium stearate	-	0:95:5	Date and Magnesium Stearate in the ball mill alone for 20 minutes Sieved through 32 µm and use the mortar and pestle/ high shear blending

3.2.3 Assessment the carrier-based formulations using next generation impactor NGI.

The developed formulation was filled into size 3 hard gelatin capsules (Pharmacare, Jordan) and tested using NGI (Copley scientific limited, Nottingham, UK). Ten capsules were tested per run. The Aerolizer was used as the actuation device. Trays of NGI were treated with 1% glycerin in acetone solution and left to dry. The purpose of conditioning is to prevent particles to bounce back. Trays weight was measured before and after each actuation. Also,

using 10 ml of distilled water to collect the particle in each tray. Absorbance was measured using UV-spectrophotometer. The formulations were run on a flow rate of 60 L/min. The pre-separator was filled with 15 ml of distilled water. Each actuation was run over 4 seconds. Results were used to assess median mass aerodynamic diameter (MMAD), FPF and emitted dose.

3.2.4 CDN preparation

CDN were prepared using the method described by Badawi (Badawi, 2018) with modification. Ionotropic gelation technique was employed as an alternative to carrier-based formulation. This technique is the optimum for the encapsulation of the extract, using chitosan nanoparticle to encapsulate the date fruit extract (Badawi, 2018). Firstly, chitosan polymer was dissolved in acetic acid to produce various concentrations [(0.2g (F8), 0.1g (F9) and 0.05g (F10)) in 50 ml of 1% (v/v) acetic acid)], the solution was placed on the magnetic stirrer at 100 rpm for 24 hours at room temperature. After that, the pH was adjusted to 5 using 1N NaOH. Date extract was prepared by weighting 10 g of date fruit powder and marinating it in 100 ml distilled water for (48-72) hours. About 120 mg of cross linker sodium chloride was mixed with 50 ml of the filtrated extract then the chitosan solution was added dropwise. The solution was kept at room temperature for 24 hours with continuous stirring at 110 rpm. After the elapsed time, the solution was centrifuged (Hettich Universal 30 RF, Germany) at a speed 11,000 rpm for 30 min. The precipitate was placed at 30 °C in the oven to dry for 24 hours. Upon drying, it was crushed using the mortar and pestle, then the powder was sieved using the finest possible sieve (32 µm) to make sure it is well grinded. Several tests were done, NGI and biological effect evaluation **(**see Figure 3.1).

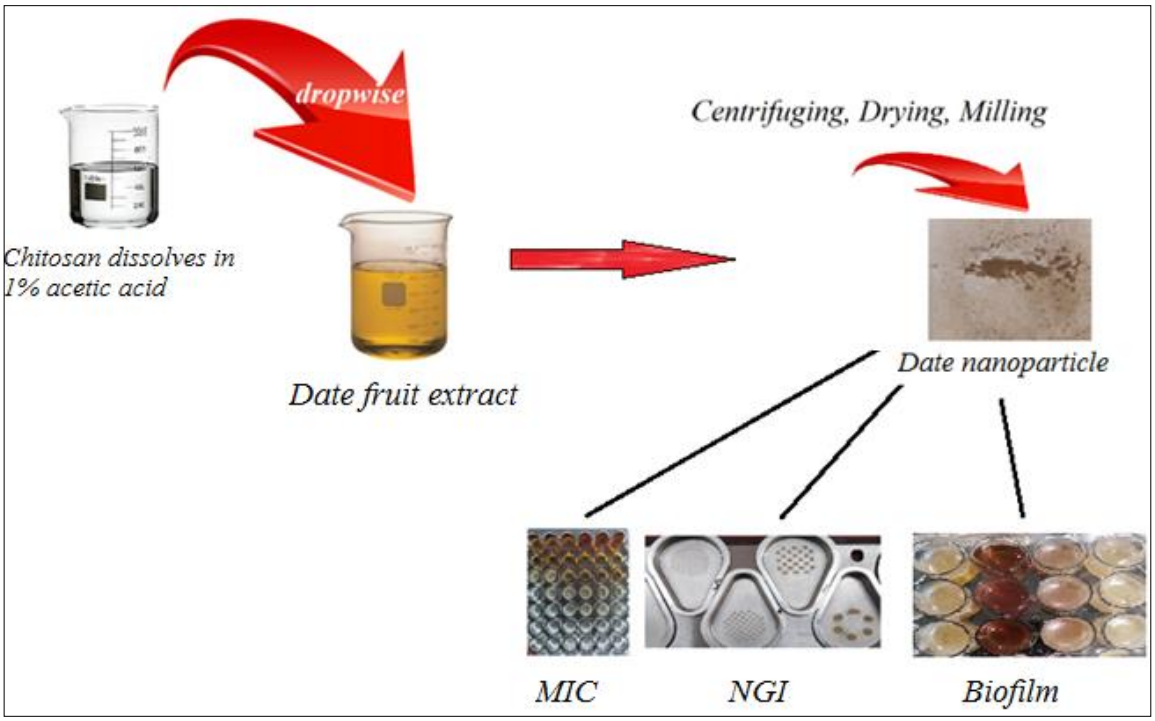


Figure 3. 1: A graphical presentation of the main steps involved in preparing and assessing the CDN.

3.2.5 Assessment of CDN using NGI.

The same procedure on section 3.2.3 was done for the assessment of CDN but for calculate FPF, ED and median mass aerodynamic diameter the trays weight was measured before and after each actuation. for each run 6 capsules of 20 mg each were used. The Aerolizer was employed as the actuation device.

3.2.5.1 Emitted dose and FPF

FPF was defined as the total mass of the particles that settled on the NGI trays from tray 2-7. The mass of particle was measured by weighting each tray before and after performing the NGI. The calculations were based on 6 capsules per batch each capsule contained 20 mg of the CDN. FPF of emitted dose was calculated by dividing the FPF on the emitted dose,

whereas the FPF of the nominal dose was calculated based on FPF divided by the theoretical (nominal dose). Respirable dose is the cumulative content that is collected from trays 2-7.

Emitted dose was calculated based on the cumulative content obtained from induction tube, pre-separator, and trays 1 to 8 based on equation 3.2. and also, the emitted dose was the different of empty capsules mass and the mass after performing the NGI.

$$\boldsymbol{Emitted\ dose}(\%) = \frac{\boldsymbol{Cumulative\ content\ x\ 100}}{\boldsymbol{X}} \ldots\ldots\ldots \boldsymbol{Eq\ (3.2)}$$

Where X is the theoretical drug content in each sample (~ 20 mg).

Figure 3. 2: A photograph of the next Generation Impactor highlighting its components. (1): induction tube, (2) pre-separator, (3) collection trays 1-8.

3.2.5.2 Mass median aerodynamic diameter (MMAD calculation)

MMAD was calculated based on the USP method <601>(USP-35, 2012), the flow rate equal to 60 L/min the base of the calculations of the cutoff diameter of each stage of the NGI. The amount of CDN that was collected from each stage from stage 1 to stage 8 was used to calculate the cumulative mass, then MMAD and geometric standard deviation (GSD) were calculated. All results were made in triplicates and reported as mean ±SD.

3.2.5.3 Encapsulation efficiency

To calculate the encapsulation efficiency (EE) of CDN the following procedure was done. Firstly, the mixture containing the CDN was centrifuged at 11,000 rpm for 30 minutes. Secondly, the amount of the free compounds in the supernatant was measured based on measuring the absorbance by UV-spectrophotometer at lambda max 350 nm. The encapsulation efficiency of date powder was calculated according to equation (3.3).

$$Encapsulation\ efficiency\ \% = \frac{Total\ amount\ of\ date\ extract - Total\ amount\ of\ free\ date\ extract}{Total\ amount\ of\ date\ extract} \times 100..\ Eq.\ (3.3)$$

3.2.6 Assessment the biological activity of CDN and date fruit extract.

For the assessment of the biological activity, the minimum inhibitory concentration (MIC) of the CDN, and ethanolic date extracts was tested. Effect on *P.aeruginosa* biofilm cultured in artificial sputum medium (ASM) that resembles cystic fibrosis sputum was evaluated.

3.2.6.1 Extract preparation

10 g of date powder was soaked in 100 ml of 100% and 90% ethanol for 48 hours at room temperature, then both of extracts were centrifuged at 11,000 rpm for 20 minutes (Hettich Universal 30 RF, Germany) and filtrated to obtain clear solution. The solutions were put to dry using water bath at 50 °C until ethanol completely evaporated. The residue was stored at -20 °C in amber bottle until it is used.

3.2.6.2 MIC and MBC test for CDN and date fruit extract

Overnight culture of *P. aeruginosa* (ATCC 15692) in nutrient agar plates at 37°C was adjusted in nutrient broth to an OD_{600} of (0.1±0.02). Firstly,100 µl of nutrient broth was added in all wells of 96 well plate. Solution of CDN, 100% ethanolic date extract, 90%

ethanolic date extract were added to the first wells of each raw at concentrations 36mg/ml, 732mg/ml, 740mg/ml respectively. Serial half dilutions of 100 µL were made using nutrient broth. Bacterial suspension of *P. aeruginosa* was added to each well in 10 µL volumes. Positive control with bacterial suspension and nutrient broth and negative control with only nutrient broth were included in each test. The plates were incubated overnight at 37°C. Minimum inhibitory concentration was considered as the lowest concentration with no visible *P. aeruginosa* growth or turbidity and minimum biocidal concentration as the lowest concentration that has no growth after subculture of the clear wells. Each test was repeated three times.

3.2.6.3 Preparation of Artificial Sputum Medium (ASM)

Artificial sputum medium was prepared according to the method described by Kirchner *et al*. 2012. To prepare the ASM, 4 g of DNA from fish sperm were added to 250 ml sterile water very slowly over a period of several hours then stirred overnight at room temperature. Next, 5 g of mucin from porcine stomach (type II) were added slowly to 250 ml sterile water until the mucin was completely dissolved. After that, 250 mg of each non-essential and essential L-amino acid, with the exception of L-cysteine and L-tyrosine, were dissolved in 100 mL sterile water. Then, dissolving 250 mg of L-cysteine was done in 25 mL of 0.5 M potassium hydroxide and 250 mg of L-tyrosine in 25 mL sterile water. In 100 mL of sterile water 5.9 mg diethylenetriaminepentaacetic acid (DTPA), 5 g sodium chloride and 2.2 g of potassium chloride were dissolved. Finally, in a 1 liter bottle the DNA, mucin, L-amino acids, DTPA, sodium chloride and potassium chloride were combined. Then 5 mL of egg yolk emulsion was added to the solution and the content within the bottle were filled to approximately 850 mL with sterile water. The pH was adjusted to 6.9 using 1 M Tris buffer

(pH 8.5) before completing the volume to 1 L with sterile water. Sterilization was done by filtration using a syringe filter with a pore size of 0.20 μm (Corning, USA). Unfiltered artificial sputum medium was stored at 4 °C in the dark for a valid shelf life of 4 weeks.

Table 3. 3: Summary of the materials used to prepare ASM including quantities and reconstitution media.

Material name	**Required amount**	**Dissolve in**
DNA from fish sperm	4 g	250 ml sterile water
Mucin from porcine stomach (type II)	5 g	250 ml sterile water
L- Alanine	250 mg	100 ml sterile water
L-Glutamic acid	250 mg	
L-Histidine	250 mg	
L-Arginine	250 mg	
L- Aspartic acid	250 mg	
L-Asparagine	250 mg	
L-Glutamine	250 mg	
L- Valine	250 mg	
L-Tryptophan	250 mg	
L-Threonine	250 mg	
L-Serine	250 mg	
L-Proline	250 mg	
L-phenylalanine	250 mg	
L-Methionine	250 mg	
L-Lysine	250 mg	
L- Leucine	250 mg	
L-Isoleucine	250 mg	
Glycine	250 mg	

L-tyrosine	250 mg	25 mL sterile water
L-cysteine	250 mg	25 mL of 0.5 M potassium hydroxide
Egg yolk emulsion	5 ml	-
Diethylenetriaminepentaacetic acid	5.9 mg	100 ml sterile water
Sodium chloride	5 g	
Potassium chloride	2.2 g	

3.2.6.4 Biofilm formation

Biofilm culture on ASM was prepared according to the model described by (Kirchner *et al.*, 2012). The day before experiment *P.aeruginosa* was cultured in nutrient agar plates and incubated overnight at 37°C. Six milliliter of sterile ASM was added on each of 80 mg of ethanolic date extract and 80 mg of CDN (F10) with final concentration of 13.3 mg/ml,13.3 mg/ml respectively in addition to chitosan 2 mg/ml of ASM was tested. Moreover, ASM with bacteria was considered as control. In a 24- well plate, 1.8 ml of sterile ASM was added to all wells. Overnight culture of *P.aeruginosa* was diluted in nutrient broth to an OD_{600} of (0.1±0.02). The ASM was inoculated with 18µl of the bacterial suspension in all wells except negative control that contained ASM only. Finally, the 24-well plate was secured with laboratory parafilm and incubated in shaker incubator for 48 h at 37 °C, while shaking at 75 rpm (Lab Shaker Incubator model:IN-666 (Gemmyco Taipei, Taiwan)). After incubation, 100 µL of 100 mg/mL cellulase (diluted in 0.05 M citrate buffer) was added to all wells to disrupt the bacterial biofilms and the plate was incubated at 37 °C for 1 h, while shaking at 150 rpm. Then manual pipetting of biofilms was done to ensure disruption. The ASM was serially diluted and 100 µL of each dilution was cultured on nutrient agar plates

and incubated overnight at 37°C. Colonies were counted from the plates to determine the CFU/ml of the ASM. Log transformed bacterial counts (CFU/mL) were evaluated.

3.2.7 Statistical analysis

All data was generated in replicates and analyzed statistically by One-Way or Two-way ANOVA from Minitab v. 18 statistical pack. Level of significance was quoted as $p<0.05$, with a confidence interval of 95%. For NGI experiments 6 capsules were used for each batch.

Chapter Four
Results and Discussion

4. Results and Discussion

4.1 Date powder characterization studies

Initial attempts were made to profile the date powder using several characterization techniques to enable the development of dry powder inhaler formulations containing date powder as a potential for use in cystic fibrosis.

4.1.1 Particle size analysis (PSA)

Particle size analysis of date powder revealed the particle size distribution of the material as shown in (Figure 4.1). The percentage of particles within each size range was plotted against the sieve size which demonstrated a monomodal particle size distribution pattern. Particle size distribution curve suggested that 92.68% of the date powder was less than 500 µm, but 46.20% was less than 250 µm, and just 7% was more than 1000 µm. The results also indicated that the particle size range is way beyond the targeted particle size range required for pulmonary drug delivery. Hence, the plan was to go for particle size reduction procedures.

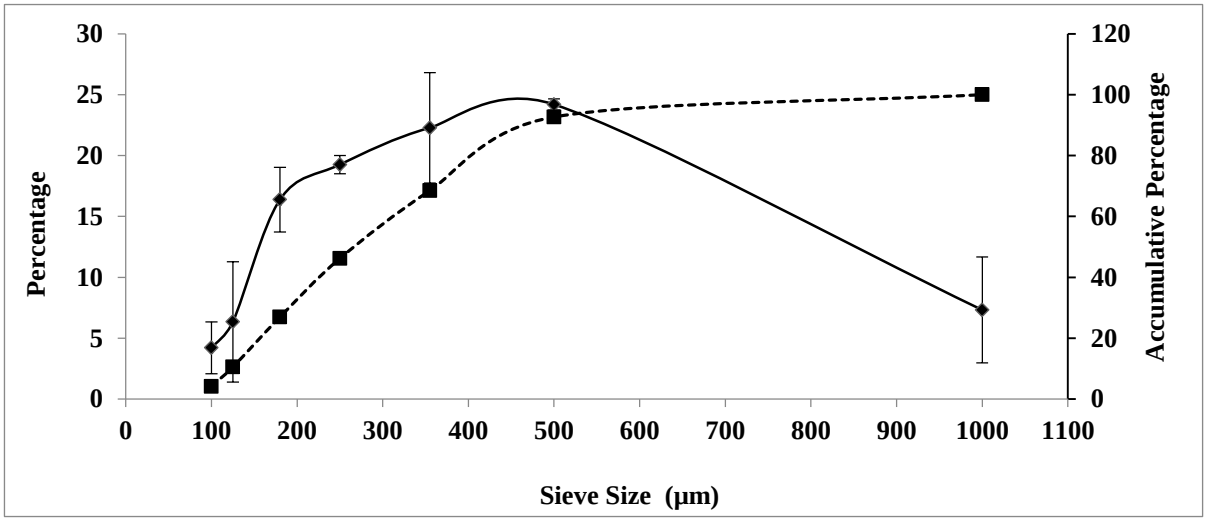


Figure 4. 1: Particle size distribution of date powder and cumulative percentage of particle obtained using sieve analysis (mean ± SD, n=3).

4.1.2 Moisture content determination

The next step in characterization process was to determine the moisture content of the powder. Moisture content of the date powder was 4.35% ± 1.8 (mean ± SD, n=3). Despite low humidity level, the date powder was cohesive which could be attributed to high percentage of simple sugars content. Hence, it is quite challenging to develop it as DPI.

4.1.3 FTIR analysis

Date is rich in many chemicals that includes carbohydrates, minerals, lipids, proteins, and phenolic compounds (Gu*et al.*, 2003). FTIR analysis of crude date powder showed troughs and peaks that represents either stretching, bending or vibration of specific bonds. (Figure 4.2) depicts a wide trough at around 3306 cm^{-1} which is assigned to hydroxyl groups O-H stretching. This was reported to be corresponding to cellulose and water content(George *et al.*, 2020). Additional characteristic trough was noted at 2913 cm^{-1} is attributed to C-H stretching vibrations, whereas troughs at 1612 and 1015 cm^{-1} are assigned to carbonyl

(C=O) and C-OH stretching respectively. Such results tally with results obtained from Farhadi and colleagues (Farhadi, Ajerloo and Mohammadi, 2017).

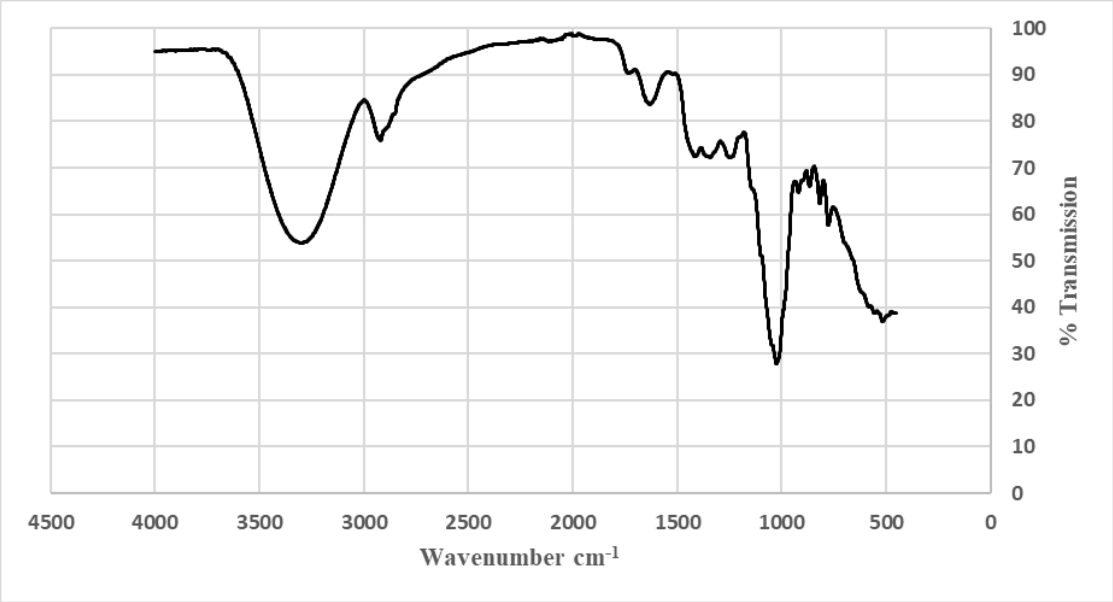


Figure 4. 2: FTIR spectra of Crude Date Fruit Powder scanned over the wavenumber range 450-4000 cm^{-1}.

4.1.4 UV Spectrophotometric analysis of the crude date powder.

UV-spectrophotometric analysis of crude powder was assessed. A scan for the solution revealed a maximum absorbance at wavelength 350 nm as depicted in (Figure 4.3).

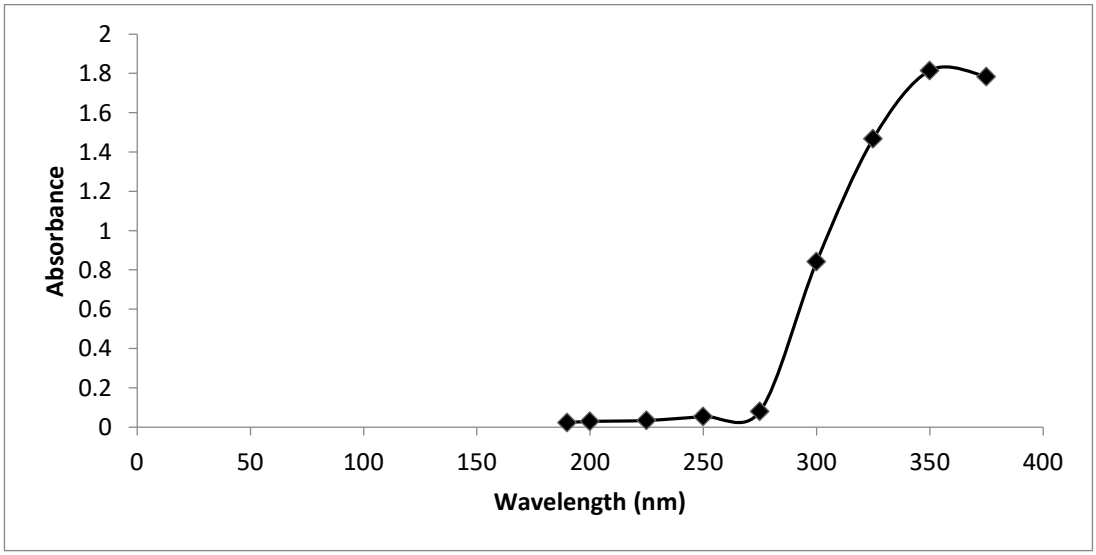


Figure 4. 3: The figure show lambda max after scanning the date solution on different wavelength.

A Beer-Lambert calibration curve was established by plotting absorbance against serial concentrations of the date fruit extract that ranged from 0.155- 9.96 mg\ml with coefficient of variation R^2= 0.999. The absorbance measuring at wavelength= 350 nm where it is the λ max (Figure 4.4)

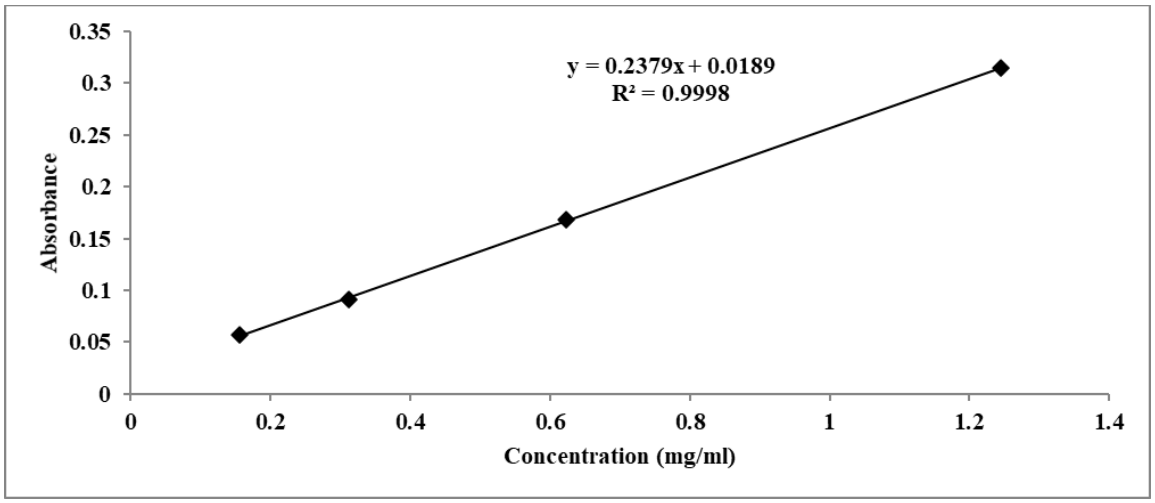


Figure 4. 4: Calibration curve of date fruit extract against concentration that ranged from 0.155- 1.45 mg/ml (mean, n=3).

4.2 Development of DPI containing crude date powder

4.2.1 Size reduction of date powder

The targeted size for respiratory drug delivery should be within the range of 1-5 µm, which is not obtained upon PSA of the powder. Therefore, the next step focused on reducing the particle size of the powder using ball milling technique. The date powder was milled using the ball mill and then sieved using sieve with apertures of 32µm. The powder that passed the sieve was very fine yet cohesive powder (Figure 4.5). However, the powder was showing aggregation, agglomeration, very cohesive and with poor flowability. To solve these challenges, the use of force control agent like magnesium stearate and carrier like lactose

were investigated. The grinding time and speed when using the ball mill were optimized as excessive grinding time resulted in production of cohesive solid pellets (Figure 4.6)

To avoid this problem, several attempts were made, for example: milling the date for several cycles, each cycle between 30 to 45 min. It was found that the obtained powder gets more moisture from surrounding environment. Another attempt was made to use a high shear blender instead of the ball mill was more effective.

Figure 4. 5:A photograph of the date fruit powder showing a very cohesive powder after sieving (particle size less than 32µm).

Figure 4. 6:A photograph of the date powder during ball milling highlighting the effect of excessive milling time formed solid cohesive pellets.

4.2.2 Carrier-based formulation

Carrier based formulation is a common formulation strategy in developing DPIs (Hamishehkar, Rahimpour and Javadzadeh, 2012) . Also, lactose monohydrate is a commonly used carrier and hence it was investigated in this study. Several ratios of lactose: fine date powder (L:D) blends were prepared employing different shear stress methods to prepare carrier-based formulation as shown in Table 4.1. The results revealed that although the blend produced emitted dose that exceeded 80%, fine particle fraction was negligible. This indicated that the blend was flowable. Increasing the date powder content did not result in any tangible increase in FPF. This is attributed to the cohesiveness of the blend and inability of the lactose to detach the fine dates powder. Or this could be attributed to the large size of the dates powder (i.e., above 5 µm). The lactose used in this study was lactose monohydrate, not the ones regularly employed for inhalation formulations and therefore, results were not favorable

Table 4. 1:Aerodynamic performance of DPI formulations of dates using NGI. The formulations were carrier-based using lactose (L: Lactose, D: Date). (mean ±SD, n=3)

Sample #	Shear stress	L:D ratio	FPF	Emitted dose
F1	mortar and pestle	60: 40	0	99.2% ±1.98%
F2		80:20	0	99.5%± 2.43%
F3	Ball mill	80:20	0	100%± 1.07%
F4		20:80	0. 04± 0.01	99.4%± 2.11%

4.2.3 Carrier-based formulation using lactose and force control agent

Force control agents are employed in developing DPI formulations as it tends to reduce the attraction force between the carrier (Lactose) and the active ingredient (see Figure 4.7) and hence should increase the FPF. Therefore, the formulation was composed of date powder, lactose, and magnesium stearate. The results in (Table 4.2) showed that regular lactose monohydrate was not a suitable option and the presence of force control agent did not enhance FPF. In addition, higher concentrations of magnesium stearate to reduce cohesiveness is not feasible. On the other hand, high sugar content in date powder made the task harder due to hygroscopic property of the powder. Drying of the blend was assessed, however, it did not result in better performance.

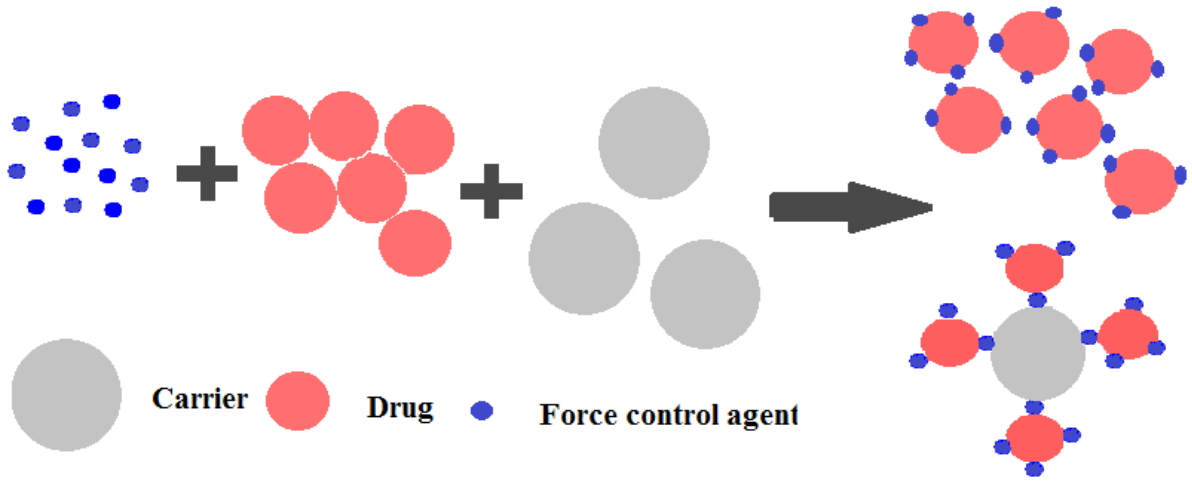


Figure 4. 7:A Graphical diagram highlighting the role of force control agent in DPI formulation.

Table 4. 2: Aerodynamic performance of DPI formulations of dates using NGI**.** Carrier-based formulation using lactose with force control agent magnesium stearate. (L: Lactose, D: Date, M: Magnesium stearate).(mean ±SD, n=3).

Sample #	**Shear stress**	**Oven**	**L:D:M ratio (w/w)**	**FPF**	**Emitted dose**
F5	Ball mill	Without	0: 95:5	2.4 ± 0.23	99.3% ± 2.43%
F6		With	50: 47.5 :2.5	0.74 ± 0.05	99.7% ± 5.12%
F7	Mortar and pestle	With	0: 95:5	0.8 ± 0.11	99.6% ± 1.65%

When the sieved date powder was collected from the sieve with aperture size of 32 µm, the powder was cohesive, hygroscopic and sticky. The addition of magnesium stearate within the 5% range did not enhance the results. Higher percentages might be needed, which will exceed the commonly used ratio of 5%. The FPF was 1.2%, however, the emitted dose (ED) was around 100%. Therefore, another method to prepare date nanoparticle was investigated.

4.3 Assessment of CDN

After several unsuccessful attempts, the best option was focused on developing nanoparticles as alternative option in DPI formulation preparation. Our method was based

on the study that was able to prepare date seed nanoparticle with modification (Badawi, 2018)**.** Three formulations were prepared, and the produced CDN resulted in fine and fluffy powder (Figure 4.8).

Figure 4. 8: A photograph of CDN after milling and sieving.

4.3.1 CDN encapsulation efficiency.

Encapsulation efficiency (EE) gives an indication on the nano carrier efficiency, and thus, ability to deliver higher doses to the respiratory system. Table 4.3 summarizes the results of EE, which increased with the reduction in chitosan concentration. F10 with chitosan concentration of 0.05% w/v produced the highest entrapment efficiency of 55.91%. One-way ANOVA analysis revealed that there is a significant difference between formulations (p= 0.000). Tukey post-test analysis demonstrated that the difference among all concentrations is significant. Several researchers reported an increase in entrapment efficiency upon the reduction of chitosan concentration (Zhang *et al.*, 2010; Lazaridou *et al.*, 2020). This could be attributed to the lower viscosity of the medium, which is associated with low chitosan concentration. This might enable a reduction in liquid phase resistance against dispersion and formation of nanoparticles (Wu *et al.*, 2005).

Table 4. 3: Encapsulation efficiency (EE) of CDN using various concentrations of chitosan (mean ± SD).

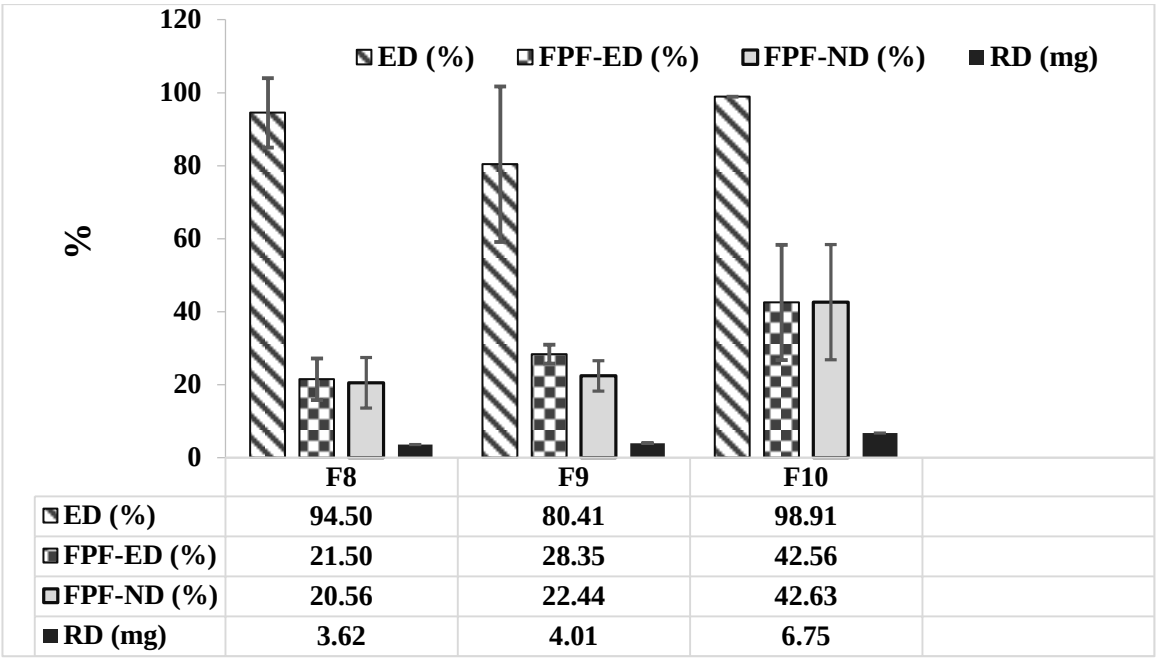
Sample #	Chitosan% (w/v)	EE%
F8	0.2	21.66 ± 1.04
F9	0.1	46.01 ± 1.23
F10	0.05	55.91 ± 0.70

4.3.2 *In-Vitro* assessment of the aerodynamic performance of the CDN using NGI

Date fruit has many biological activities, such as antioxidant, anti-inflammatory, and antimicrobial. In general, the amount of drug reaching the site of action plays a significant role in the biological response and pharmacodynamic effect. Therefore, CDN should reach the lower parts of the lungs to make the expected action. Therefore, this study aimed at determining the efficiency of the formulation employing FPF, which is the most commonly used parameter to assess *in-vitro* aerodynamic performance of the formulations. The targeted FPF was more than 20% and the desired ED was more than 60%.

Results for the pivot inhalation parameters [emitted dose (%ED), FPF of emitted dose (%FPF-ED), respirable dose (RD) and FPF from the nominated dose (FPF-ND)] were assessed (triplicate NGI readings from the same batch) (Figure 4.9). The results revealed that the CDN were able to successfully produce FPF-ED ranging from 21.50% to 42.56% which exceeded the targeted range for the three formulations.

From the (Figure 4.9**) ,**F10 produced the highest amount of CDN among the three prepared formulation, where the FPF-ND, FPF-ED and the respirable dose were significantly higher than F8 and F9 (one way ANOVA, $p<0.05$).



	F8	F9	F10	
ED (%)	94.50	80.41	98.91	
FPF-ED (%)	21.50	28.35	42.56	
FPF-ND (%)	20.56	22.44	42.63	
RD (mg)	3.62	4.01	6.75	

Figure 4. 9*:* Summary of *in-vitro* aerodynamic performance of CDN using NGI. Each capsulecontains20mg of the CDN. **F 8**: 0.2 % w/v chitosan, **F 9**: 0.1% w/v chitosan, **F 10:** 0.05% w/v chitosan. Results are presented as mean± SD, n=3. **ED**: %Emitted dose, **FPF-ED**: Fine Particle Fraction from Emitted Dose, **FPF-ND**: Fine Particle Fraction from Nominated Dose, **RD**: Respirable Dose.

The percentage of the nominal dose that is emitted from the capsules upon actuation is expressed in the emitted dose. From (Figure 4.9), the emitted dose was not significantly different between F8, F9 and F10 (94.5, 80.41, 98.91 respectively) (one-way ANOVA, p= 0.098). However, it was noted that F10 showed the lower variation between runs as can be seen from the small error bars. The second variable was FPF from emitted dose, which represented the percentage of the emitted dose that can reach the lower parts of the respiratory system and has aerodynamic particle size below 5 µm. F10 had the highest FPF-ED of 42.56% and the difference among formulation was statistically significant (one-way

ANOVA p=0.042). The higher the FPF the higher the chance of systemic absorption of CDN. Moreover, FPF from a nominal dose (FPF-ND) had a good representation of amount within the nominated dose that is delivered to the lower part of respiratory system. Results demonstrated a significant effect among formulations (one-way ANOVA p=0.040). All the three formulations produced FPF-ND that is above the anticipated target of 20%, however, F10 was superior (42.6%). When the results are presented in terms of respirable dose that presents the mass of the CDN which is in the micron size that would deliver to the lower parts of the respiratory system, F10 formulation showed the highest RD (6.75) mg per actuation followed by F9 (4.01 mg) followed by F8 (3.62 mg) per actuation. One-way ANOVA analysis demonstrated that there is a significant difference among formulations (p=0.040). The particle size of prepared CDN was varied and that is the result shown above, the concentration of the chitosan solution play a crucial role which effect on the particle size (Sreekumar *et al.*, 2018).

The mass median aerodynamic diameter (MMAD) values obtained for the three formulations using the NGI set at 60 l/min are depicted in Table 4.4. The results revealed that the MMAD values for the CDN ranged from 1.31 to 1.46 µm. However, there is a statistically significant difference among them (one-way ANOVA, p= 0.000) where reducing chitosan concentration resulted in lower particle size and such results are in line with the aerodynamic parameters of the three formulations. Lower chitosan concentration resulted in more EE, higher ED, FPF and RD. Furthermore, examination of the geometric standard deviation (GSD) revealed that the three formulations produced particles with similar spread of particle size (GSD) (one-way ANOVA, p= 0.427). Such results are expected as the method produces particles with narrow distribution of particle size. Also,

the results demonstrates a successful technique to deliver date powder extract to the lungs, where the CDN upon drying produced nanoaggregates within the particle size range 1-5 micron that enables dispersion through the respiratory system. It is expected that upon deposition on the alveoli, the nanoaggregates will further disintegrate to produce smaller nano sized particles hence avoid quick clearance with possible local effect.

Table 4. 4: MMAD and GSD of the three CDN formulation

Formulation	MMAD (µm)	GSD
F8	1.46 ± 0.09	0.073± 0.01
F9	1.39± 0.11	0.070± 0.009
F10	1.31± 0.13	0.068± 0.011

Table 4. 5: Cutoff aerodynamic diameter for stages of NGI apparatus set at 60L/min (adopted from(USP-31, 2008)

Stage	**Cut off diameter D_{50}**
1	8.06
2	4.46
3	2.82
4	1.66
5	0.94
6	0.55
7	0.34

The aerodynamic particle size distribution parameters obtained using the NGI where the three formulations of CDN with the cut-off diameter specifications (Table 4.5) of the NGI set at 60 l/min were compared (Figure 4.10). From the figure, the three formulations showed differences in the pattern of particle size distribution. Stages from 2-7 are the range that may produce deposition within the lower parts of the pulmonary system. The three formulations

had bi-modal aerodynamic particle size distribution within the particles deposited extra-thoracic with particles exceeding 8 µm (particles deposited at the mouthpiece, induction tube and stage 1). Then good percentage was deposited within the particle size range of 1.66-4.46 µm (stages 2-5) indicating that the produced aerosols particle size was low where the least proportion was produced between the range of 0.34- 1.66 µm (stages 6-8). Although, the percentage of produced particles of F10 is higher, the trend of aerodynamic particle size distribution is similar.

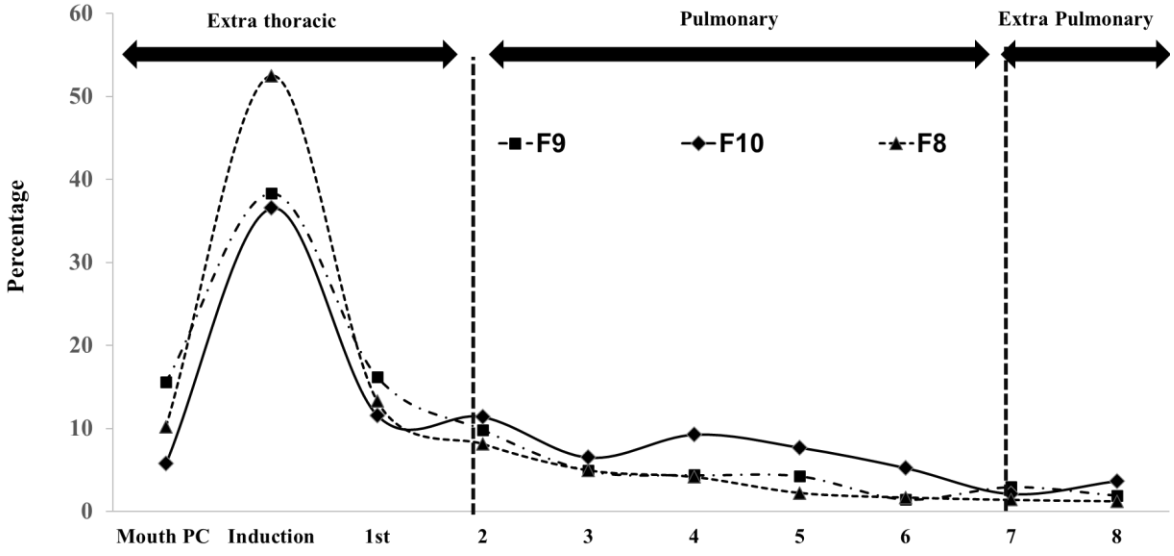


Figure 4. 10:*In vitro* comparison of aerodynamic particle size distribution of the three formulations demonstrating the lung deposition of CDNusing NGI set at flow rate of 60l/min.

(Figure 4.11) showed the deposition of CDN on the stages of the NGI device. As can be noted from the graph, deposition was mainly concentrated on trays 1-4.

Figure 4. 11: Deposition of the chitosan date nanoaparticles onto the NGI apparatus highlighting the brown powder from tray 1-7

4.3.3 FTIR results of CDN, chitosan and date fruit powder

FTIR spectra of the CDN demonstrated similar pattern to that of the date powder with slight reduction in the trough within the range of 2900 cm^{-1}. Overall no change in date composition upon inclusion in CDN.

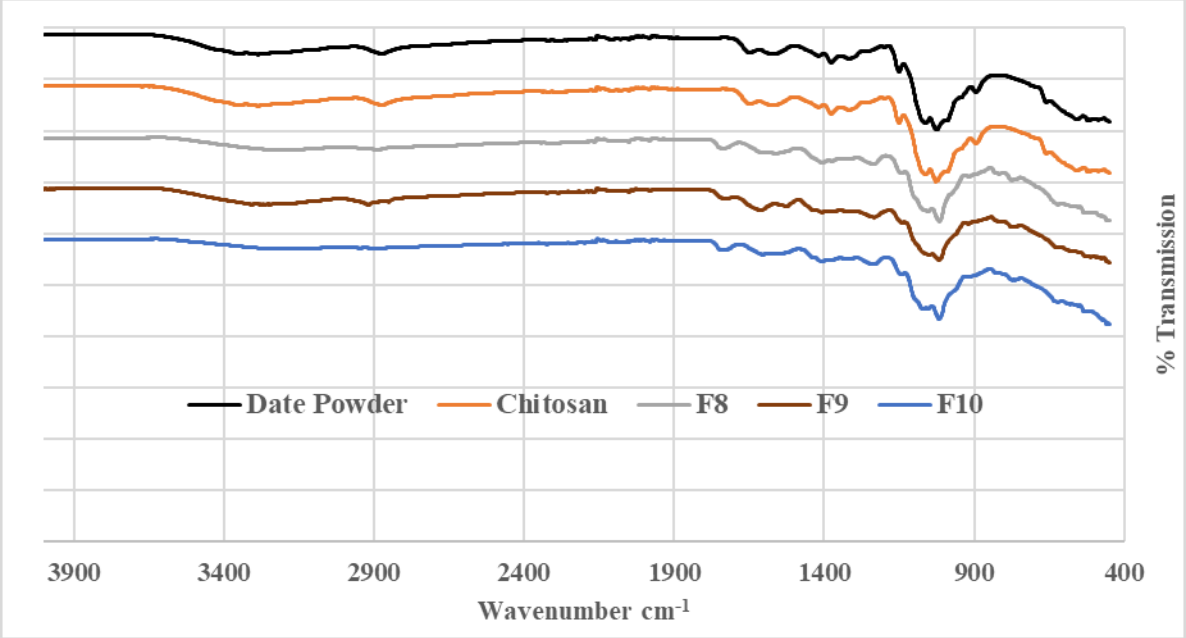


Figure 4. 12:FTIR spectra of date fruit powder, chitosan, F8, F9 and F10 over the wave number range from 450-4000 cm^{-1}.

4.4 Assessment the biological activity of CDN and extract.

The next set of investigations focused on assessing the biological activity of the produced CDN. As discussed earlier, *P. aeruginosa* is the main bacteria in cystic fibrosis. Hence, this part of project will assess the antibacterial activity of our formulations using *P. aeruginosa* biofilm model cultured in ASM that mimics conditions in cystic fibrosis.

4.4.1 MIC and MBC of ethanolic date extract and CDN

To determine the MIC and MBC values of date extract, the concentration of 100% ethanolic extract from 366 mg/ml to 15 mg/ml were tested. The results as depicted in (Table 4.6) revealed that for the 100% ethanolic extract the MIC was 91.5 mg/ml and the MBC was 183 mg/ml.

Also, the concentration of 90% ethanolic extract from 370 mg/ml to 15.4 mg/ml were tested. The result of the 90% ethanolic extract (Table 4.6) showed that the MIC was 185 mg/ml and the MBC was 370 mg /ml. Then the concentration of prepared CDN from 18mg/ml to 0.75mg/ml was tested. Minimum inhibitory concentration for CDN was 4.5mg/ml. Overall, the 100% ethanolic extract produced better results in terms of MIC and MBC when compared with the 90% ethanolic extract. However, the CDN results suggested a superior effect with MIC and MBC values below those obtained from ethanolic extracts. Such results are in accordance with reports on the superiority of nanoparticles (Farhadi, Ajerloo and Mohammadi, 2017). Such enhancement of antimicrobial activity showed by the CDN have made them promising candidates as novel method for the management of cystic fibrosis.

Table 4. 6: MIC and MBC results of ethanolic extracts of date powder at 90% and 100% as well as F10 CDN.

Test	**90% ethanolic extract Date powder concentration (mg/ml)**	**100% ethanolic extract Date powder concentration (mg/ml)**	**CDN(mg/ml)**
MIC	185	91.5	4.5
MBC	370	183	18

4.4.2 Evaluationof biofilm inhibition.

Excessive thick mucus that is present in airways causes complications such as chronic bacterial and fungal infections and this is accompanied by the formation of biofilm, which is the biggest challenge in CF disease (Wright and Vera, 2017). Managing CF entails using airway clearance therapies, anti-inflammatory medications, and antibiotic for the treatment and prophylaxis (Wright and Vera, 2017). Therefore, this part of the project focused on assessing the ability of CDN and date extracts to inhibit biofilm formation in a model mimicking conditions in cystic fibrosis by utilizing artificial sputum medium as culture medium for biofilm formation.

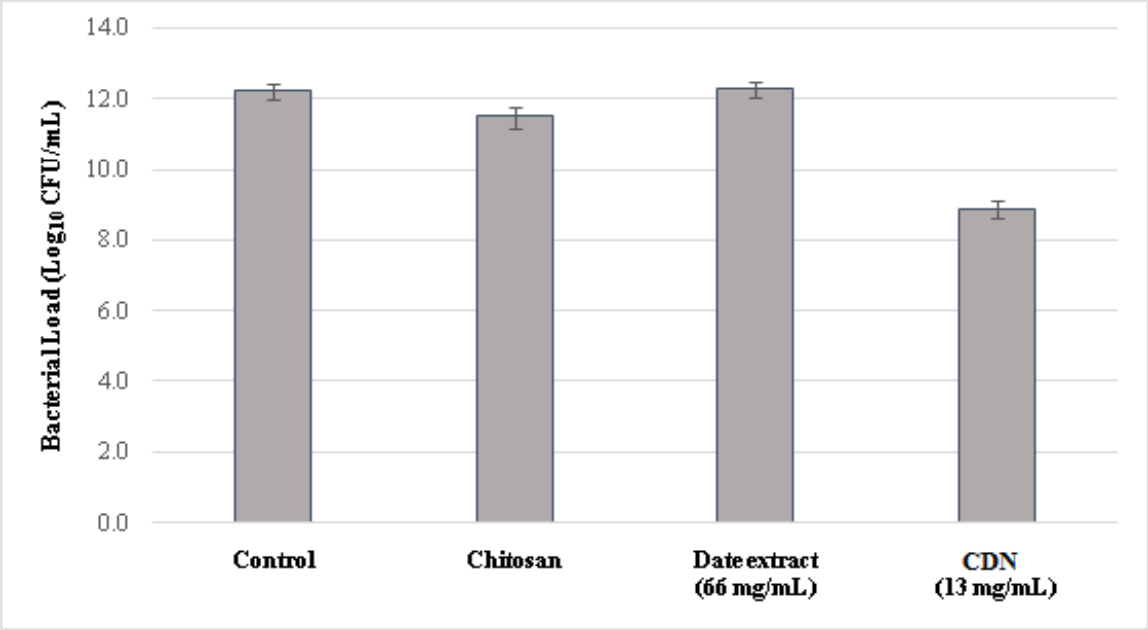


Figure 4. 13: Effect of CDN and date extract on *P. aeruginosa* biofilm after 48 hours (mean ± SD, n=3).

Date extract failed to inhibit biofilm formation (Figure 4.13). Chitosan showed biofilm inhibitory activity of almost one log cycle. Whereas CDN resulted in a significant 3.3 log cycle reduction of bacterial load. Knowing that cystic fibrosis patients will certainly have biofilm (Wright and Vera, 2017), this formulation could be introduced as prophylactic treatment to prevent biofilm formation in cystic fibrosis patients.

Several researchers reported about the anti-bacterial activity of date fruit, leaves and seeds on gram-positive and gram-negative bacteria. One example is the antimicrobial study of date fruit extract on *Bacillus subtilis*, *Salmonella typhi*, *Staphylococcus aureus*, and *P. aeruginosa* which revealed that date fruit extract induced around (80 - 99) % growth inhibition of all bacteria in nutrient broth cultures (El-Far *et al.*, 2016). Also, Sallal and Ashkenani reported that *Bacillus subtilis* was severely affected by date extract and based on the this result date fruit extract could be included in antimicrobial drugs and in topical

ointment manufacture (El-Far *et al.*, 2016).

In another study that isolated flavonoid glycosides such as quercetin, luteolin and apigenin from date fruit and tested them on *P. aeruginosa*, the cell morphology of *P. aeruginosa* was observed using scanning electron microscopy during 60 min after applying the extract. At 30 min, *P. aeruginosa* started to deform and at 60 min most cells were completely deformed, and pores were formed on the cell wall. Therefore, the strong antibacterial activity was related to how flavonoids work. Also, The possible antimicrobial mechanism of action based on disruption of hydrophobic structures within phospholipid bilayers of the bacterial cell (Selim *et al.*, 2012). The MIC of ethanolic date fruit extract when tested on *P. aeruginosa* ATCC 15442 was 4 mg/ml and MBC was 16 mg/ml (Selim *et al.*, 2012).

There are several reports that are addressing the benefits and medical uses of natural compound that are produced from plants as secondary metabolite. For example, a study that analyzed the phytochemicals of different parts of date palm, reported that the fruit consisted flavonoids, phenolic compounds, alkaloids, saponins, anthocyanins, sterols, carotenoids and tannins (Latrach and Verde Rodríguez, 2017). Therefore it is a good source of natural antioxidants that can be used against oxidative stress-related and infectious diseases (Al-Shwyeh, 2019). Zahidi date fruit that was cultivated in California consisted of total phenolics in fresh date fruit of about 2,546 mg Catechin/100 g and in dried date about 1,959 mg Catechin/100 g (Vinson *et al.*, 2005). Catechins have an activity against gram-positive and gram-negative bacteria. In a study by Cushnie and Lamb, Catechins were tested on liposomes as a model of bacterial membranes. Two observations were noted, the first, showed a leakage of small molecules from the intraliposomal space, and the second, induced aggregation in liposomes so the damage in bacterial membrane occurred. There are two

theories to explain this effect. First, catechins may disrupt and penetrate the lipid bilayers and damage the barrier function. Second, catechins may cause membrane fusion, a process that results in leakage of intramembranous materials and aggregation (Cushnie and Lamb, 2005).

Furthermore, date fruit is rich in acidic amino acids such as aspartic acid and glutamic acid and poor in sulfur containing amino acids. Hence, in a study of acidic amino acids with ciprofloxacin and their synergistic effect on *S. aureus* biofilm formation, there result showed that glutamic acid and aspartic acid can enhance the solubility of many poorly soluble drugs including ciprofloxacin also combination of D- acidic amino acids with ciprofloxacin was employed to overcome anti-microbial resistance in these biofilms and significantly gave synergistic effect on inhibition and dispersal *S. aureus* biofilm. Also, L- amino acids showed an effect on biofilm formation (Warraich *et al.*, 2020).

Owing to all the above, the ability to develop CDN with high loading efficiency, that was delivered into the lower parts of the respiratory system may be a potential candidate for the management of cystic fibrosis.

Conclusions

Cystic fibrosis is an inherited disorder that causes severe damage to the lungs, digestive system, and the reproductive organs. The disease causes excessive production of mucus, sweat and digestive juices resulting in the production of sticky and thick secretion that block passageways, ducts and tubes, especially in the lungs. Long-term concerns pertinent to cystic fibrosis include difficulty breathing, coughing up mucus and the accumulation of mucus in the lungs which allows bacteria to grow more easily causing infections particularly with P aeruginosa. Therefore, the aim of this research was to develop DPI formulation containing dried dates fruit extract as a potential candidate for the management of cystic fibrosis. There are many effective compomunds in date fruit that demonstrate an antibactrial effect.Chitosan based nanoparticles were prepared and assessed for their aeodynamic performance. Results revealed that low chitosan nanoparticles (0.05%w/v) had the hifghest EE of (56%) and delivered the highest FPF-ND (42.63%). The CDN were more effective than the extract,aginst *P. aeregnosa* biofilim formation. On the other hand, the propriate amount of prepared CDN that gave the inhibiton effect on biofilm could be successfully delivered to the lungs and act locally where broncial bacterial biofilm form which is the targeted area.

Futur work

Future work will focus on investigating the following:

- Incorporation of an antibiotic with CDN to investigate synergistic effect.
- Assessing the prepered formulations on *staphylococcus aureus.*
- Assessing the toxicty of CDN on cell culture
- Assessing the different conentrations of each of the three formulations aginst biofilm for 48 and 72 hour.

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Appendix 1

تطوير الجزيئات النانوية لثمار التمر ومعرفة تأثيرها على الأغشية الحيوية للبكتيريا الزائفة الزنجارية

ملخص البحث

يعاني مرضى التليف الكيسي من التهابات رئوية متكررة التي عادة ما تكون بكتيرية خاصة بكتيريا الزائفه الزنجاريه والمكورات العنقودية الذهبية. على الرغم من وصف المضادات الحيوية الوقائية عادة، إلا أن المقاومة البكتيرية تمثل تحديًا رئيسيًا. لذلك، هناك محاولات للتعرف على مضادات حيوية جديدة، خاصة تلك التي تأتي من مصادر طبيعية، والتي قد تعزز علاج المرض. لذلك، كان الهدف من هذا المشروع هو التحقيق في استخدام مستخلص فاكهة التمر كمرشح محتمل لتطوير جهاز استنشاق المسحوق الجاف (DPI) مع النشاط البيولوجي المحتمل ضد البكتيريا الزائفه الزنجاريه. تتكون فاكهة التمر (*Phoenix dactylifera L.*) من مركبات الفلافونويد والبوليفينول والمعادن والفيتامينات، وقد ورد في العديد من الدراسات أن لها تأثيرًا على البكتيريا موجبة الجرام وسالبة الجرام. عند الحديث عن أمراض الرئة، فإن الطريق الرئوي لتوصيل الدواء يوفر مزايا خاصة عند مقارنته بالطرق الأخرى، حيث يمكن للأدوية أن تصل إلى موقع التأثير بشكل أكثر كفاءة. تم استخدام جهاز الاستنشاق بالمسحوق الجاف (DPI) لتوصيل تركيبات المسحوق. حيث تم تحضير جسيمات نانوية من (مستخلص التمر المائي: الكيتوزان) وقام ذلك على زيادة نسبة الكيتوزان. أظهر تركيز الجسمات النانوية من الكيتوزان 0.05٪ (وزن / حجم) أعلى كفاءة تغليف (55.91٪) وأعطى أعلى جرعة انبعاث (98.92٪) ونسبة الجزيئات دقيقة من الجرعة المحددة (FPF) (42.62) ٪. أظهرت الجسيمات النانوية المنتجة أن التركيز الادنى لتثبيط نمو البكتيرياMIC يبلغ 4.5 مجم / مل وأظهر تتثبطا كبيرًا لتكوين الأغشية الحيوية. اقترحت الجسيمات النانوية لمستخلص التمر كمرشح محتمل لعلاج التليف الكيسي بشكل مسحوق جاف للاستنشاق (DPI).