Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
SCIENZE FARMACEUTICHE

CO-TUTELA CON

THE UNIVERSITY OF
SYDNEY

Ph.D. in
Discipline of Pharmacology
Sydney Medical School
The University of Sydney

CICLO *XXVIII*

COORDINATORE Prof. Stefano Manfredini

INHALABLE LIPID MICROPARTICLE AND SPRAY DRIED FORMULATIONS BASED ON POLYPHENOLIC COMPOUNDS

Settore Scientifico Disciplinare CHIM/09

Dottorando
Dott.ssa Trotta Valentina

Tutore Unife
Prof. Scalia Santo

Tutore Sydney
Prof.ssa Traini Daniela

Co-Tutore Sydney
Prof. Young Paul Michael

Anni 2013/2015

TABLE OF CONTENTS

ACKNOWLEDGMENTS

At the end of my PhD I would I would like to thank all those people who made this thesis possible with their endless support, enthusiasm and encouragements.

Firstly, I would like to express my sincere gratitude to Professor Santo Scalia and Professor Daniela Traini, my research supervisors, and to Professor Paul Michael Young, my research co-supervisor, for their guidance, enthusiastic encouragements and support. Thanks for your excellent guidance during these years.

My sincere thanks goes to Wing-Hin Lee, Ching-Yee Loo and Mhera Haghi for your help, teaching, support and your friendship.

Moreover I would like to thank all rest of the people of the RespiTech group of the Woolcock Institute of Medical Research for the time that we spent together. I will never forget our conferences, coffee, dinners and all the special moments that we shared.

My thanks and appreciation go to Dott. Anna Bianchi for your good advices.

In addition, a thank you to the students of the Prof. Scalia, for the happy times that we spent together.

Many thanks to my very special friends Maria, Margherita, Antonio, Domenico, Alessandro, Flavio, Luca e Michele for encouraged me with their best wishes.

Special thanks go to my love Angelo, for your endless love, patience and support every time.

Finally and most importantly, I warmly thank my parents and sisters for their unconditional love.
Thanks to my grandmother Lina, for your endless love! You will live in my heart for ever!

I never could have done this work without the love, guidance and support of my grandmother Lina, my parents, my supervisors and Angelo. I dedicate this thesis to you!

Valentina

LIST OF PUBLICATIONS

Trotta, V., Lee, W. H., Loo, C. Y., Haghi, M., Young, P. M., Scalia, S., and Traini, D. (2015). In vitro biological activity of resveratrol using a novel inhalable resveratrol spray-dried formulation. *International journal of pharmaceutics*, *49*(1), 190-197.

Scalia, S., **Trotta, V.,** Iannuccelli, V., Bianchi, A. (2015). Enhancement of in vivo human skin penetration of resveratrol by chitosan-coated lipid microparticles. *Colloids and Surfaces B: Biointerfaces*, *135*, 42-49.

Scalia, S., **Trotta, V**., Bianchi, A. (2014). In vivo human skin penetration of (–)-epigallocatechin-3-gallate from topical formulations. *Acta Pharmaceutica*,*64*(2), 257-265.

Trotta, V., Goios, F., Monteiro, H., Almeida, I. F., Scalia, S. (2014). Influence of lipid microparticle encapsulation on in vitro efficacy, photostability and water resistance of the sunscreen agents, octyl methoxycinnamate and butyl methoxydibenzoylmethane. *Drug development and industrial pharmacy*,*40*(9), 1233-1239.

Scalia S., Haghi M., Losi V., **Trotta V.,** Young P. M.,Traini D. (2013). Quercetin solid lipid microparticles: A flavonoid for inhalation lung delivery. *European Journal of Pharmaceutical Sciences* Vol. 49 Issue: 2 278–285.

Scalia S., **Trotta V.,** Traini D., Young P. M., Sticozzi C., Cervellati F., Valacchi G. (2013) Incorporation of quercetin in respirable lipid microparticles: Effect on stability and cellular uptake on A_{549} pulmonary alveolar epithelial cells. *Colloids and Surfaces B: Biointerfaces* Vol.112, 322– 329.

CONFERENCE POSTER CONTRIBUTION

3rd international tb-meeting inhaled therapies for tuberculosis and other infectious diseases. Parma, Italy, October 14-16, 2015.

"Resveratrol: dry powder inhaler (DPI) formulations for pulmonary and nasal delivery".

Trotta V., Lee WH, Loo CY, Martignoni I., Haghi M, Young P.M., Traini D, Scalia S.

Respiratory Drug Delivery (RDD), Europe 2015. Antibes, France, May 5-8, 2015.

"Novel co-spray dried resveratrol and budesonide inhalable formulation for the treatment of chronic obstructive pulmonary diseases".

Trotta V., Lee WH., Loo CY., Scalia S., Young P. M., Traini D.

Drug Delivery to the lungs (DDL) 25. Edinburgh, Scotland, December 10-12, 2014.

"Resveratrol, a novel spray dried inhalation powder for the treatment of COPD and other inflammatory lung diseases".

Trotta V., Haghi M, Scalia S., Young P.M., Traini D.

ISAM (international society for aerosols in medicine) and Woolcock 2014, conference and workshop. Sydney, Australia, September 25-26, 2014.

"Resveratrol, a novel spray dried inhalation powder for complementary therapy in the treatment of COPD."

Trotta V., Haghi M, Lee WH., Scalia S., Young P. M., Traini D.

1 INTRODUCTION

1.1 Chronic inflammatory lung diseases: asthma and COPD

Acute and chronic alveolar and/or bronchial inflammation is thought to be central to the pathogenesis of many lung disorders such as asthma and chronic obstructive pulmonary disease (COPD) [1].

Asthma is a common chronic inflammatory disease of the airways characterise by variable and recurring symptoms, such as reversible airflow obstruction and bronchospasm (figure 1) [2]. Moreover, these effects are promoted by underlying inflammation, which increases mucus production and contraction of muscles around the airways or bronchospasm. The mucus not only obstructs airways but also contributes to an increasing of airway hyperresponsiveness [3]. The pathogenesis of this disease is correlate to different genetic factors such as innate immunity, genetic sex and age that coupled to the environmental factors like allergens and respiratory bacterial/viral infections are responsible to the susceptibility, pathogenesis and development of asthma [4-6].
The airflow limitation in asthma is recurrent and caused by a the presence of increased numbers of eosinophils, neutrophils, lymphocytes, mast cells, dendrites cells and macrophages in the bronchial tissues, specifically in the airways, and by a release of inflammatory mediators (e.g. chemokines, cytokines, leukotrienes and nitric oxide) [7].

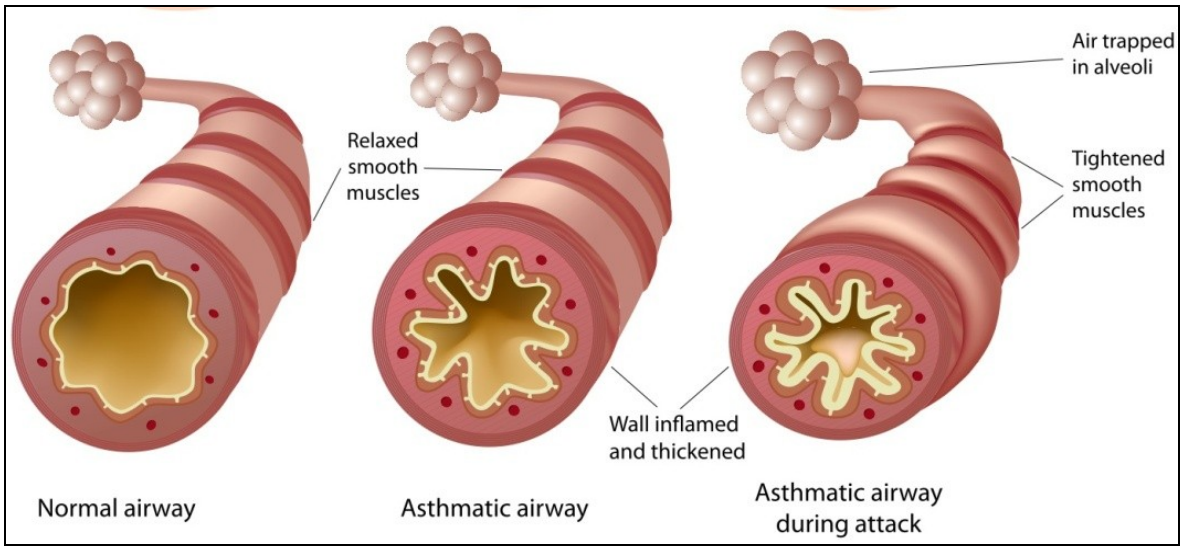


Figure 1: Airway in physiologic condition compare to asthmatic airways [8].

Chronic obstructive pulmonary diseases is one of the leading causes of morbidity and mortality worldwide [9,10], characterised by chronic inflammation, airflow limitation, hyper mucous production, emphysema, bronchoconstriction, a decline of respiratory activity and eventual death. COPD could be consider as a mix of two diseases: chronic bronchitis and emphysema (figure 2) [11].

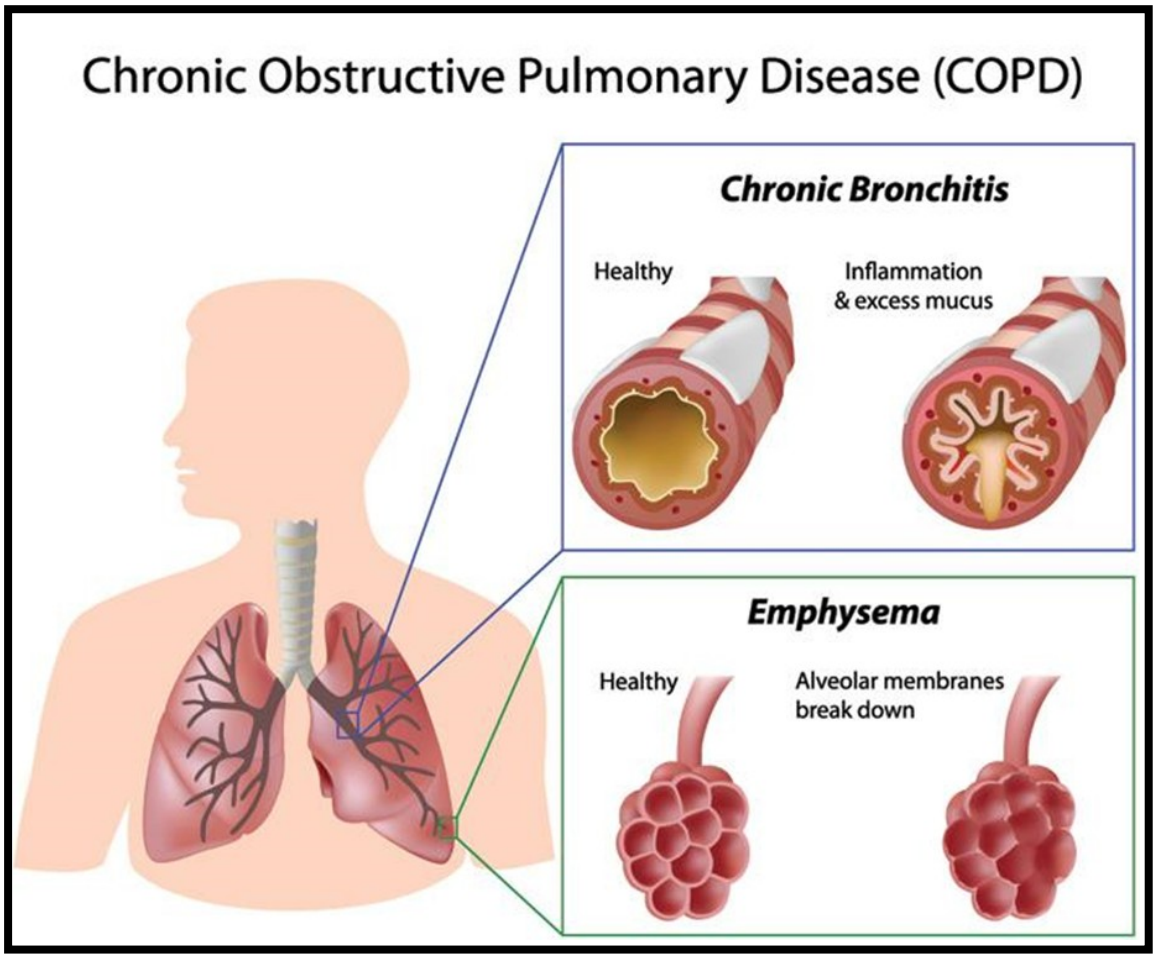


Figure 2: Airways and alveolus during COPD [12].

- ***Bronchitis*** is a diseases characterise by inflammation to the bronchi of the lungs [13]. Symptoms of bronchitis are the coughing mucus wheezing, the shortness of breath and chest discomfort. Bronchitis is divided into two types: acute and chronic.

  Acute bronchitis, is a short term inflammation of the bronchi of the lungs.

  The most common symptom is a cough. Other symptoms include coughing up mucus, wheezing, shortness of breath and fever. The infection may last from a few to ten days. The cough may persist for several weeks afterwards with the total duration of symptoms usually around three weeks, and in some cases until up to six weeks.

Chronic bronchitis is defined as a productive cough that lasts for three months or more per year for at least two years. Cigarette smoke is the most common cause, with a number of other factors such as air pollution and genetic playing a smaller role.

- ***Emphysema*** is a condition that it involves enlargement of the air sacs in the lung, that consequently make progressively short of breath [14].
  The site and specific characteristics of the inflammatory responses may be different in each of these diseases, but all are characterised by the recruitment to the lungs and activation of immune and inflammatory cells, that consequently produce cytokines, oxidants and many other mediators which are involved in inflammation [15].

1.2 Pharmacotherapy for asthma and COPD

Pharmacotherapy for asthma and COPD could have different target and also different types of drugs and administration routes.

Asthma is thought to be caused by a combination of genetic and environmental factors and the pharmacotherapy treatment is determined to a large extent after an initial assessment of severity and subsequent establishment of control, both of which can be variable over time and assessed in 2 domains: impairment (current) and risk (long-term consequences) [16].
The treatment includes different classes of drug in order to control the symptoms and a list of the common medications are listed below and are shown in Table 1:

- ✓ **Beta-2 agonist**, is the oldest classes of drugs used in the treatment of asthma to relieve bronchoconstriction and depending their time of action could be classified in short- and long- acting beta 2 agonist [17].
  Short-acting beta-2 agonists such as salbutamol and terbutaline are the drugs for acute relief of asthma symptoms, and the prevention of exercise induced bronchoconstriction. This class of drugs, provides quick relief of symptoms during asthma attacks and also is used in order to prevent bronchoconstriction induced by asthma symptoms.
  Short-acting beta-2-agonists are available different pharmaceutical formulations: inhaled, pill, liquid, and injectable forms. The inhaled form is preferred for asthma treatment.

Long- acting beta-2 agonists provide prolonged bronchodilation, indeed salmeterol and formoterol are potent and selective beta-2-adrenoceptor agonists with durations of action >12 h. These drugs are usually used to reduce in day- and night-time symptoms and improved quality of sleep.

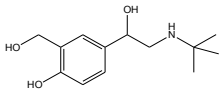
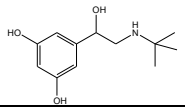
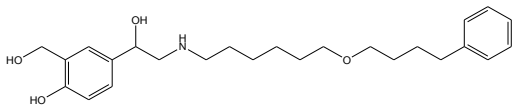
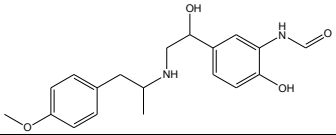
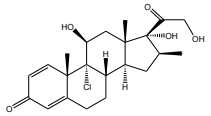
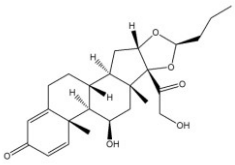
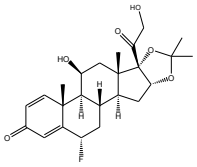
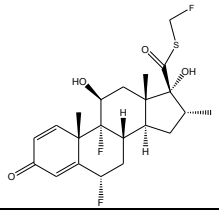
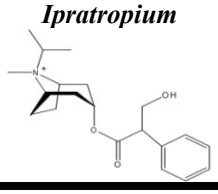
- ✓ **Inhaled corticosteroids,** such as beclomethasone, budesonide, flunisolide, fluticasone, are drugs used to treat inflammation in the airway [18].

  This class of drugs, represents the most effective anti-inflammatory medications for the treatment of persistent asthma and in general, they are used every day as a part of daily asthma treatment.

  Inhaled corticosteroids are the preferred treatment for long-term control of persistent asthma symptoms in children, teens, and adults by helping control of the narrowing and inflammation in the bronchial tubes. In general, they are part of daily asthma treatment and are used every day. Their clinical benefits include decreased asthma symptoms, improved lung function and a reduction of exacerbations;
- ✓ **Leukotriene receptor antagonists** may also be referred to as anti-inflammatory, to prevent bronchoconstriction and they may be an alternative treatment for mild persistent asthma. This class of drugs may be used in addition to inhaled corticosteroids if asthma symptoms remain uncontrolled [19,20];
- ✓ **Inhaled anticholinergics** (e.g. ipratropium) are usually used for severe asthma attacks while is limited their use in the day-to-day management of asthma [21].

The treatment of acute asthma symptoms is usually with an oral corticosteroids but there are more likely to cause side effects than inhaled corticosteroids, because with the inhaled therapy the medication is deliver directly to the lungs [22]. In very severe cases, intravenous corticosteroids, magnesium sulfate, and hospitalization may be required [23].

Table 1: Classes of drugs using for asthma treatment with same molecular samples.

CLASSES OF DRUG using for asthma treatment	Chemical structures
Beta-2 agonist	**Short-acting** ***Salbutamol***  ***Terbutaline*** 
	Long-acting ***Salmeterol***  ***Formoterol*** 
Corticosteroids	***Beclomethasone***  ***Budesonide***  ***Flunisolide***  ***Fluticasone*** 
Anticholinergics	***Ipratropium*** 

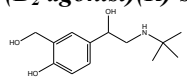
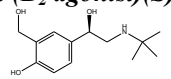
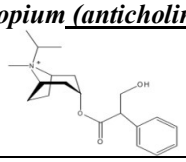
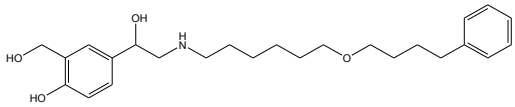
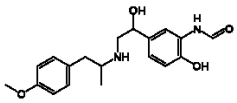
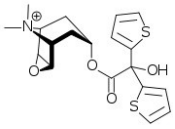
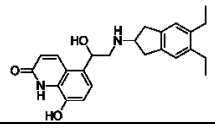
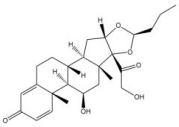
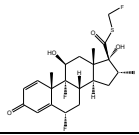
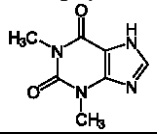
The pathogenesis of COPD is multifactorial which includes genetic predisposition, age, inhaled pollution and cigarette smoke.

There is no cure for COPD but the treatment can help slow the progression of the condition and reduce the symptoms [24]. Indeed the pharmacological therapies are focus on control symptoms, reduce risk of complications and exacerbations in order to improve the life quality of the patients.

The list of the different type of medication are listed below and are shown in Table 2:

- ✓ ***Bronchodilators*** are different drugs that are able to relax the muscles around your airways. The action of bronchodilators can help relieve coughing and shortness of breath and make breathing easier by relaxing the muscles around your airways. Usually are administrated by inhalation and depending on the severity of the disease and the duration of the actives. Short-acting bronchodilators include albuterol, levalbuterol and ipratropium [25]. The long-acting bronchodilators include tiotropium, salmeterol, formoterol, arformoterol, indacaterol and aclidinium [26];
- ✓ ***Inhaled steroids*** are medications that can reduce airway inflammation and help prevent exacerbations and are useful for people with frequent exacerbations of COPD. Fluticasone and budesonide are examples of inhaled steroids [27];
- ✓ ***Combination inhalers,*** some medications combine bronchodilators and inhaled steroids. Salmeterol/fluticasone and formoterol/budesonide are examples of combination inhalers [28,29];
- ✓ ***Phosphodiesterase-4 inhibitors***, is a new type of medication that is able to decreases airway inflammation and relaxes the airways in people with severe COPD and symptoms of chronic bronchitis [30];
- ✓ ***Theophylline*** is a very inexpensive medication that helps to the improve breathing and prevents exacerbations [31];
- ✓ ***Antibiotics*** are usually used for respiratory infections, such as acute bronchitis, pneumonia and influenza, that can aggravate COPD symptoms. Antibiotics help treat acute exacerbations, but are not generally recommended for prevention [32].

Table 2: Classes of drugs using for COPD treatment with same molecular samples.

CLASSES OF DRUG **Using for COPD treatment**	**Chemical structures**
Bronchodilators	**Short-acting** ***Albuterol (B_2 agonist)(R)-salbutamol***  ***Levalbuterol (B_2 agonist)(S)-salbutamol***  ***Ipratropium (anticholinergic)*** 
	Long-acting ***Salmeterol (B_2 agonist)***  ***Formoterol (B_2 agonist)***  ***Tiotropium (anticholinergic)***  ***Indacaterol (B_2 agonist)*** 
Corticosteroids	***Budesonide***  ***Fluticasone*** 
Xantine	***Theophylline*** 

The pharmacotherapy of these inflammatory lung diseases need to enhance in order to improve the life quality of the patients. In literature is show that inflammation and oxidative stress are the principle markers in the pathogenesis of these disease [33,34].

Previous studies have shown that oxidative stress plays an important role in the pathogenesis of the inflammatory lungs diseases and also that cigarette smoke is the main risk factor for the development and progression of COPD. This is because cigarette smoke causes a production of reactive oxygen species (ROS) that increase the oxidative stress and for this reason it is implicated in the pathogenesis and in irreversible airway inflammation [35]. Indeed the oxidative stress causes airway inflammation by stimulating the release of inflammatory mediators such as interleukin (IL)-6, IL-8 and TNF-α [36]. Moreover these inflammatory mediators result in an increase of ROS and hence an increase in oxidative stress in the lungs and consequently the inflammation (figure 3).

Identification of various anti-oxidant agents such as polyphenols have made it possible to modulate various biochemical aspects of inflammatory lungs diseases such as COPD and asthma. Moreover the therapy based on polyphenolic compounds could improve the treatment of inflammatory lung diseases, because these molecule have shown to have a good anti-oxidant and anti-inflammatory activities [37].

For this reason the polyphenols are suitable candidates to add to the pharmacotherapy of these lung diseases.

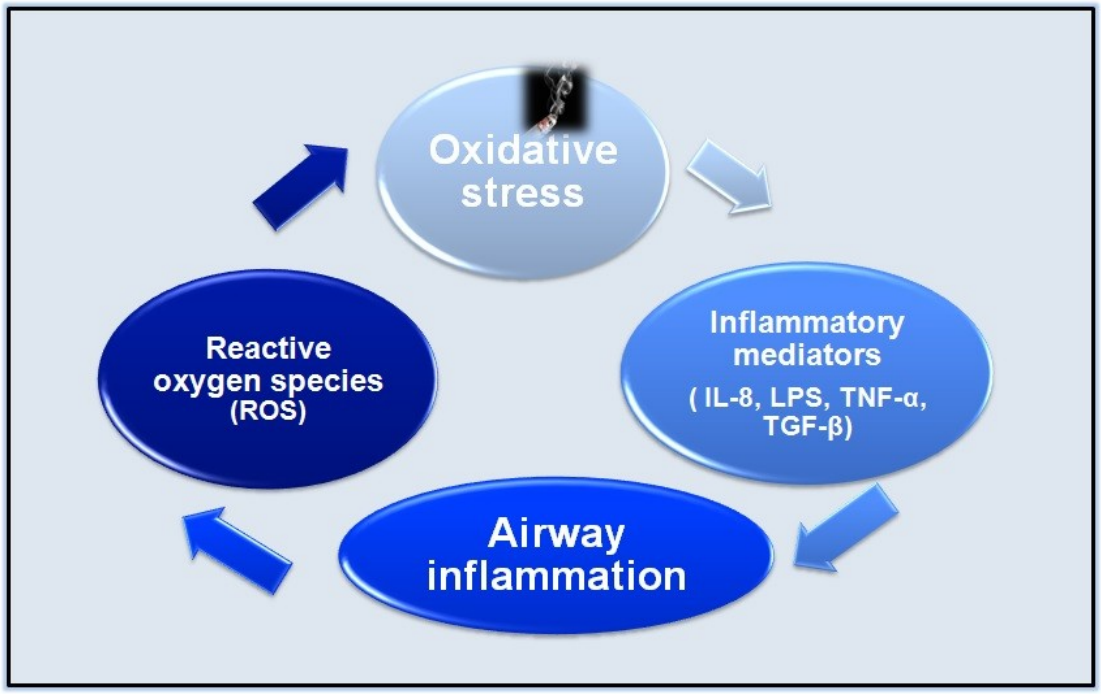


Figure 3: Oxidative stress play an important role in the pathogenesis of the inflammatory lungs diseases.

1.3 Polyphenols

Polyphenols refer to diverse groups of naturally occurring compounds which are secondary metabolites of plants [39,40].

The chemical structure of these molecules is a C6-C3-C6 carbon skeleton. Indeed their chemical structure is characterise by presence of multiple aromatic rings, characterized by presence of one or more hydroxyl groups. Depending to the number of phenolic rings and the structure elements that link together the rings, the polyphenols can be classified into different classes [39].

Polyphonic compounds can be classified in two main groups:

- Flavonoids;
- Non-flavonoids.

1.3.1 Flavonoids: chemical structures and biological activities

The flavonoids have a chemical structure formed by two aromatics rings, named as A and B, linked each other through an oxygenated heterocycle, indicated as C ring (figure 4) [41].

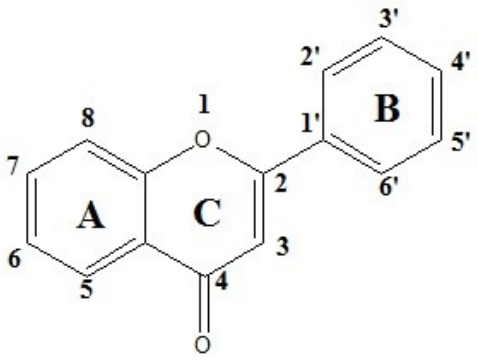


Figure 4: Chemical structure of flavonoids.

The aromatic rings A and B could have hydroxyl groups in different positions. Specifically, in the ring A, the hydroxyl groups are usually present in position 5 and 7, while in position 3' and 4', or 3', 4' and 5' for the B ring. Moreover the C ring could have different oxidation states.

The flavonoids can be further subdivided into six main subclasses, depending on the degree of oxidation of the central pyran ring (C ring) which are shown in figure 5 and are listed below [41]:

- Flavonols;
- Flavones;
- Isoflavones;
- Flavonones;
- Flavanols;
- Anthocyanidins.

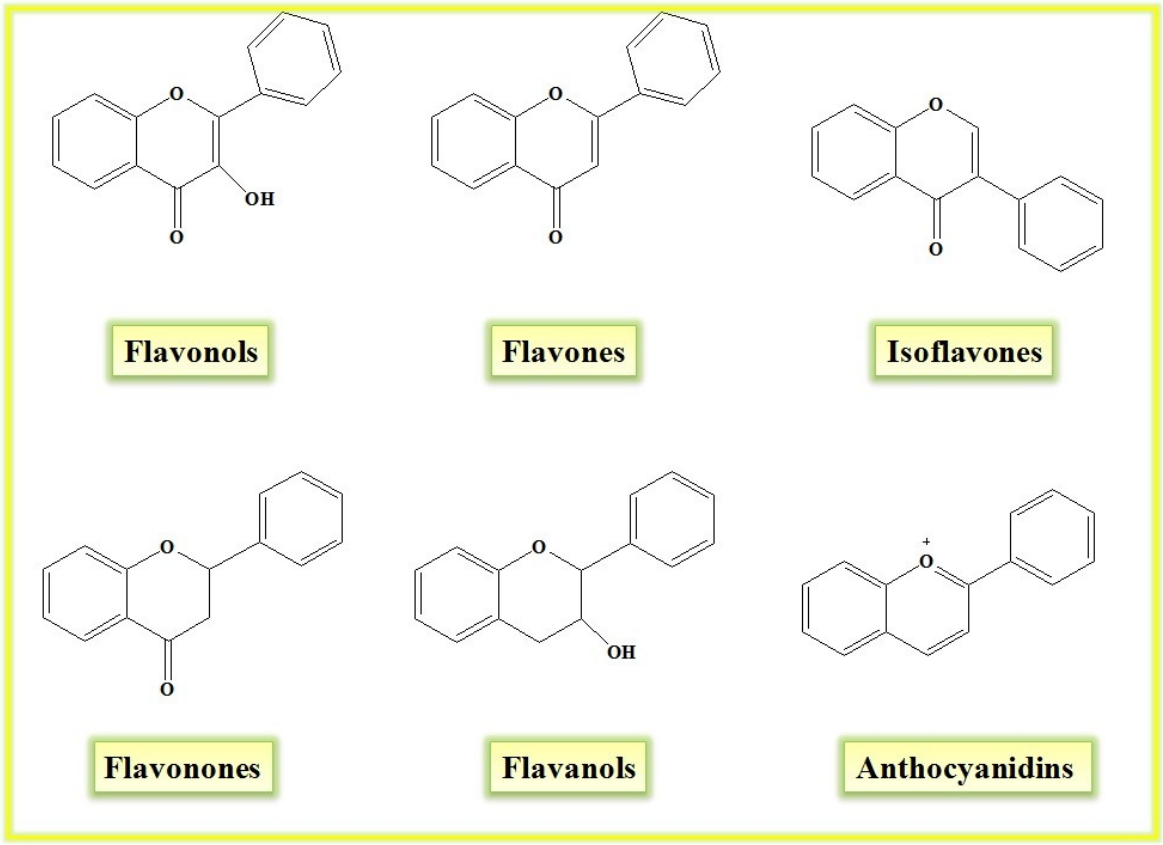


Figure 5: Classification of flavonoids depending on the degree of oxidation of the central ring.

There has been an increasing interest in the research on flavonoids due to their versatile health benefits with respect to different diseases as reported in a previously studies [42]. Indeed, flavonoids present different activities [42] such as anti-oxidant, anti-inflammatory, hepatoprotective, coronary heart diseases prevention, effect on blood and antimicrobial; however, the anti-oxidant [41] and the anti-inflammatory [43] activities are the most relevant for these compounds.

- ✓ The ***anti-oxidant*** capability of polyphenols represents the most important activity for these compounds and depends on the arrangement of functional groups connected in the chemical structure. Specifically, the anti-oxidant activity is influenced by the configuration, the substitution and number of hydroxyl groups [41].

  Flavonoids employed the anti-oxidant activity by two mechanisms: radical scavenging (I) and metal ion chelation (II) [44,45].

  I. The configuration of the hydroxyl groups present in the B ring (figure 4) is the most significant determinant of scavenging the ROS and reactive nitrogen species (RNS) [46-49]. Indeed these hydroxyl groups are able to donate an hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to relatively stable and less-reactive flavonoids radicals, according to equation:

  $$\textbf{Flavonoid (OH)} + \mathbf{R^{\bullet}} \rightarrow \textbf{Flavonoid } (\mathbf{O^{\bullet}}) + \textbf{RH} \qquad \textbf{(1)}$$

  II. The metal ions chelation [45] plays an important role for the prevention of radical generation which damage target biomolecules. Flavonoids, with their multiple hydroxyl groups on C ring have several sites for metal complexation, and in literature is shown that these compounds are able to chelate transition metal ions, especially iron and copper, giving a stable complex that entrapping metals, prevent the generation of free radicals. Indeed, base on the Fenton reaction, hydroxyl radicals are produced from hydrogen peroxide in the presence of metal, according to following equation 2:

  $$\mathbf{H_2O_2} + \mathbf{M^{n+}} \rightarrow \mathbf{OH^{-}} + \mathbf{HO^{\bullet}} + \mathbf{M^{(n+1)+}} \qquad \textbf{(2)}$$

- ✓ The ***anti-inflammatory*** properties of flavonoids have been intensely studied because these compounds have shown to have anti-inflammatory activity in vitro and in vivo but their action mechanism is not full understood [43]. Indeed the flavonoids are able to decrease the transcription and also the release of inflammatory mediators [50].

Before to consider the anti-inflammatory activity of flavonoids, the arachidonic acid (AA) and its role during the inflammation need to be explained. This is because the AA plays a role very important for the release of different inflammatory mediators being involved in cellular signalling as a lipid second messenger involved in the regulation of signalling enzymes [51].

Arachidonic acid is present in the phospholipids of cellular membranes and is freed by the enzyme phospholipase A_2 (PLA_2) and represents the precursor of a wide range of biologically and clinically important signalling molecules during the inflammatory processes, that are named eicosanoids.

Indeed the AA is metabolized to eicosanoids and metabolites of these eicosanoids by various enzymes (figure 6). The cyclooxygenase and 5-lipoxygenase are the enzymes involved in the metabolization of the AA. Specifically, enzymes cyclooxygenase-1 and -2 (COX-1 and COX-2) metabolize AA to prostaglandins, while the enzyme 5-lipoxygenase (LOX) metabolizes AA to 5- hydroperoxyeicosatetraenoic acid (5-HPETE), which in turn is metabolized to various leukotrienes.

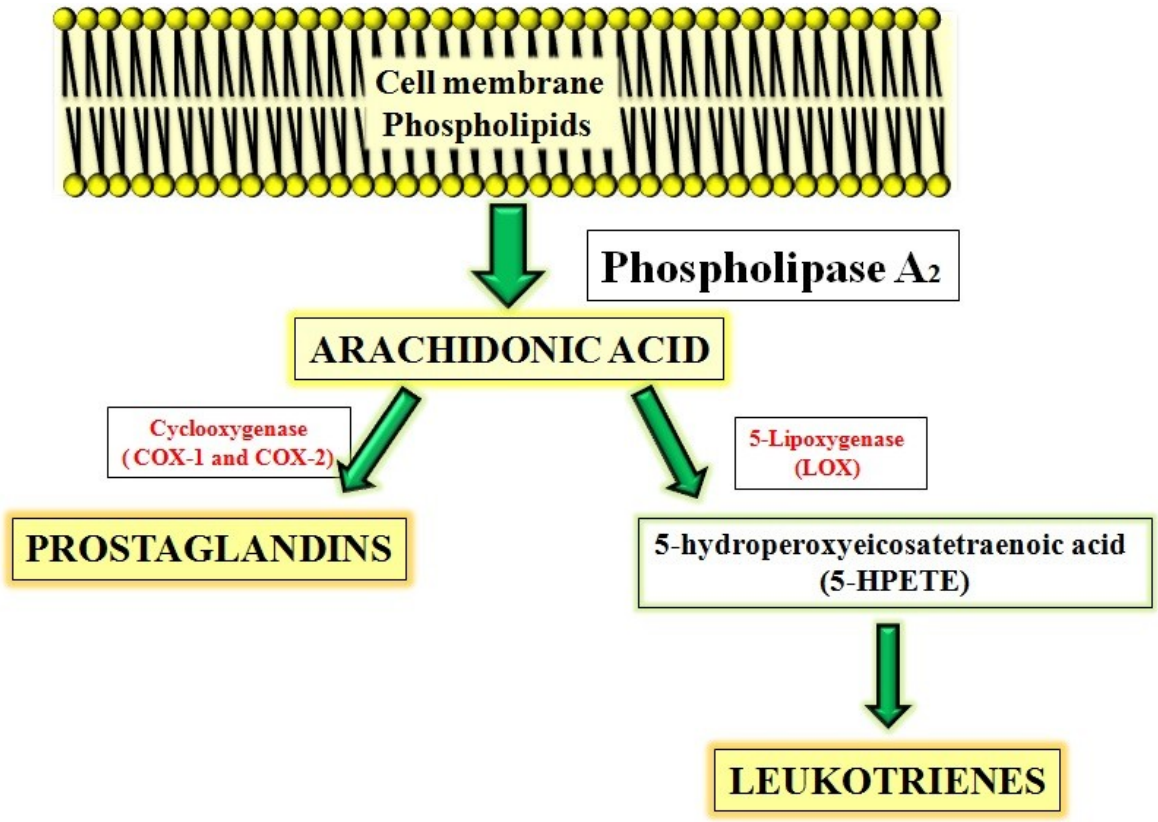


Figure 6: Role of arachidonic acid in inflammation.

Different studies have shown that flavonoids exert their anti-inflammatory activity by inhibition of AA and by the inhibition of the enzymes such as PLA_2, COX and LOX that results in the reduction of the production of AA, prostaglandins, leukotrienes (LT) and nitric oxide (NO) that are the crucial mediators of inflammation [43,52]. Moreover, flavonoids exert the anti-inflammatory activity by inhibition of two important enzymes such as the inducible nitric oxide syntetase (iNOS) and the COX-2, and also of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) [43,52,53].

1.3.1.1. Quercetin and biological properties

Quercetin, chemically named 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (figure 7), is a flavonoid compound that is part of the flavonols group and is found in the form of glycoside in many vegetables and fruits.

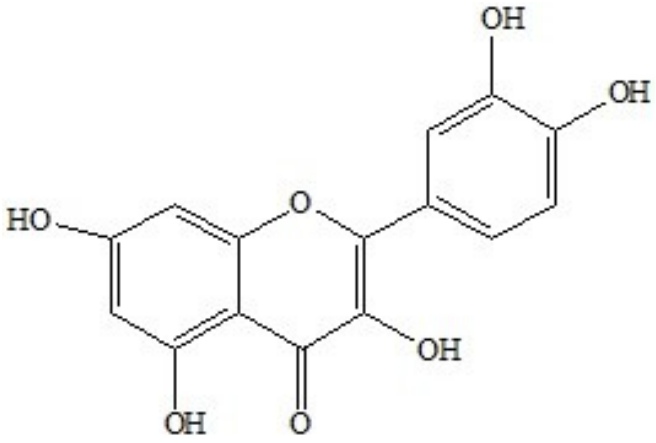


Figure 7: Chemical structure of quercetin.

Quercetin is the aglycone form of a number of other flavonoid glycosides, such as rutin and quercitrin (figure 8) [54,55].

This flavonoid is a molecule that possess potential anti-inflammatory, anti-allergic, anti-carcinogenic and anti-asthmatic properties, but the most important activity is the anti-oxidant action that it is exerted by scavenging of free radicals and binding transition metal ions.

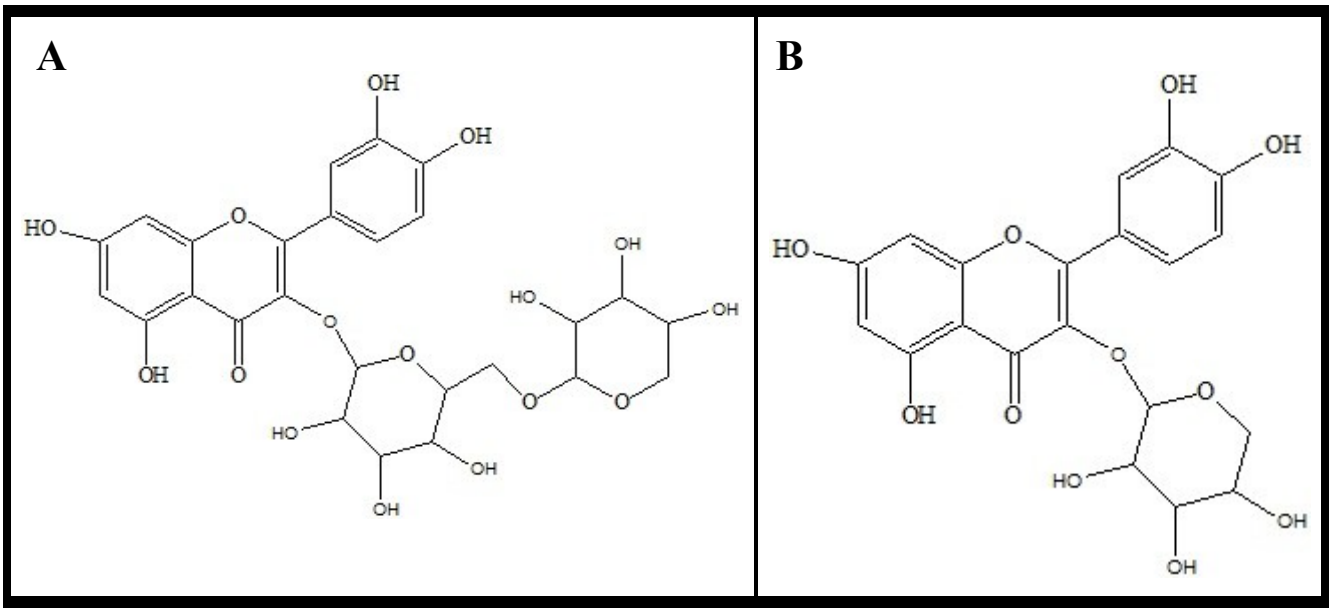


Figure 8: Chemical structures of A) rutin and B) quercitrin.

Previous studies have shown its anti-inflammatory activities *in vivo* and *in vitro.* In inflammation, bacterial products and proinflammatory cytokines induce the formation of large amounts of NO by inducible nitric oxide synthase (iNOS), consequently the compounds that inhibit NO production have anti-inflammatory effects. Quercetin, is able to inhibited iNOS protein and mRNA expression and also NO production in macrophages, exposed to an inflammatory stimulus (lipopolysaccharide, LPS), in a dose-dependent manner. Specifically, quercetin inhibits the activation of NF-κB and also the activation of the signal transducer and activator of transcription 1 (STAT-1), which are a significant transcription factors for iNOS [53]. Moreover quercetin exert anti-inflammatory activity by modulation of iNOS, COX-2 and reactive C-protein (CRP) expression, and variation in the NF-κB in liver cells, via mechanisms likely to involve blockade of NF-κB activation and the resultant up-regulation of the pro-inflammatory genes [56]. Add to this quercetin produced a decrease of iNOS, COX-2 and CRP protein level at different concentrations (from 5 to 200 μmol/l), but the percentage of inhibition induce by quercetin was reduced at high concentrations [56].

In vivo studies have shown that quercetin is able to reduce acute and chronic inflammation in rat, after intraperitoneal administration given at daily doses equivalent to 80 mg/kg [57] and also could be used for treating allergies because is an effective eosinophilic inflammation suppressors in a murine model of asthma [58].

In addition to these effects, chronic inflammation is being shown to be increasingly involved in the onset and development of several pathological disturbances such as arteriosclerosis, obesity, diabetes, neurodegenerative diseases and even cancer [59]. Treatment for chronic inflammatory disorders has not been solved, and there is an urgent need to find new and safe anti-inflammatory compounds [59].

Quercetin is able to inhibit cytokine and iNOS expression through inhibition of the NF-κB pathway (both *in vitro* and in *vivo*) [60,61] and also it is a potent inhibitor of leukotriene B4 formation in leukocytes (IC_{50} 2 mM), and its activity was dependent on specific structural features, particularly the 2,3-double bond of the C-ring. For this reason, the structural modifications of quercetin due to metabolic transformation had a profound effect on bioactivity. Moreover, the structural features required for antioxidant activity of quercetin and related flavonoids were unrelated to those required for inhibition of inflammatory eicosanoids [62]. Quercetin also reduced the gene expression of specific factors implicated in local vascular inflammation. Moreover this flavonoid reduces the expression of human CRP and cardiovascular risk factors (SAA, fibrinogen) in mice in vivo [63].

Specifically to pulmonary pathologies, quercetin inhibits the production of reactive oxygen species and also and inhibit the intercellular adhesion molecule-1 (ICAM-1) that is one of the important pro-inflammatory factors, in human alveolar epithelial A_{549} cells [64,65]. Furthermore, it reduces the induction of pro-inflammatory cytokines, and chemokines in human bronchial and alveolar A_{549} epithelial cells. Moreover, in vivo administration of quercetin has been reported to decrease allergic airways inflammation and asthmatic reactions including airway resistance, mucus production in the lung, recruitment of leukocytes and eosinophils to the broncho-alveolar lavage fluid in murine and guinea-pig models of asthma. In addition, inhibition of lung cancer cell proliferation (including the A_{549} human cell line) by quercetin has also been demonstrated [66,67].

However, the therapeutic application of quercetin is hampered by its extensive metabolism, poor cell permeability and high chemical instability in physiological fluids, which prevent adequate flavonoid concentrations to be reached and maintained during in vitro and in vivo investigations [68-71].

1.3.2. Non – flavonoids chemical structure and biological activities

The non-flavonoids compounds can be further subdivided into four main subclasses [72] and are shown in figure 9 and are listed below:

- ***Phenolic acids*** are types of aromatic acid compounds. Included in that class are substances containing a ring and an organic carboxylic function and constitute about one-third of the dietary phenols, which may be present in plants in free and bound forms [73]. Phenolic acids consist of two subgroups: hydroxybenzoic and hydroxycinnamic acids, which have the C6–C1 and C6–C3 structures, respectively.
- ***Tannins*** have diverse effects on biological systems since they are potential metal ion chelators, protein precipitating agents and biological antioxidants. Because of the varied biological roles that tannins can play and because of the enormous structural variation, it has been difficult to develop models that would allow an accurate prediction of their effects in any system [74].
- ***Lignans*** are produced by oxidative dimerisation of two phenylpropane units; they are mostly present in nature in the free form, while their glycoside derivatives are only a minor form [74].
- ***Stilbenes*** are a class of compound which have in common the C6–C2-C6 structure. Chemically, stilbene is a hydrocarbon consisting of a trans ethene double bond substituted with a phenyl group on both carbon atoms of the double bond. Stilbene exists as two possible isomers, *trans*- and *cis*-1,2-diphenylethylene. The *cis*-stilbene isomer is less stable because the steric interactions force the aromatic rings out-of-plane and prevent conjugation.

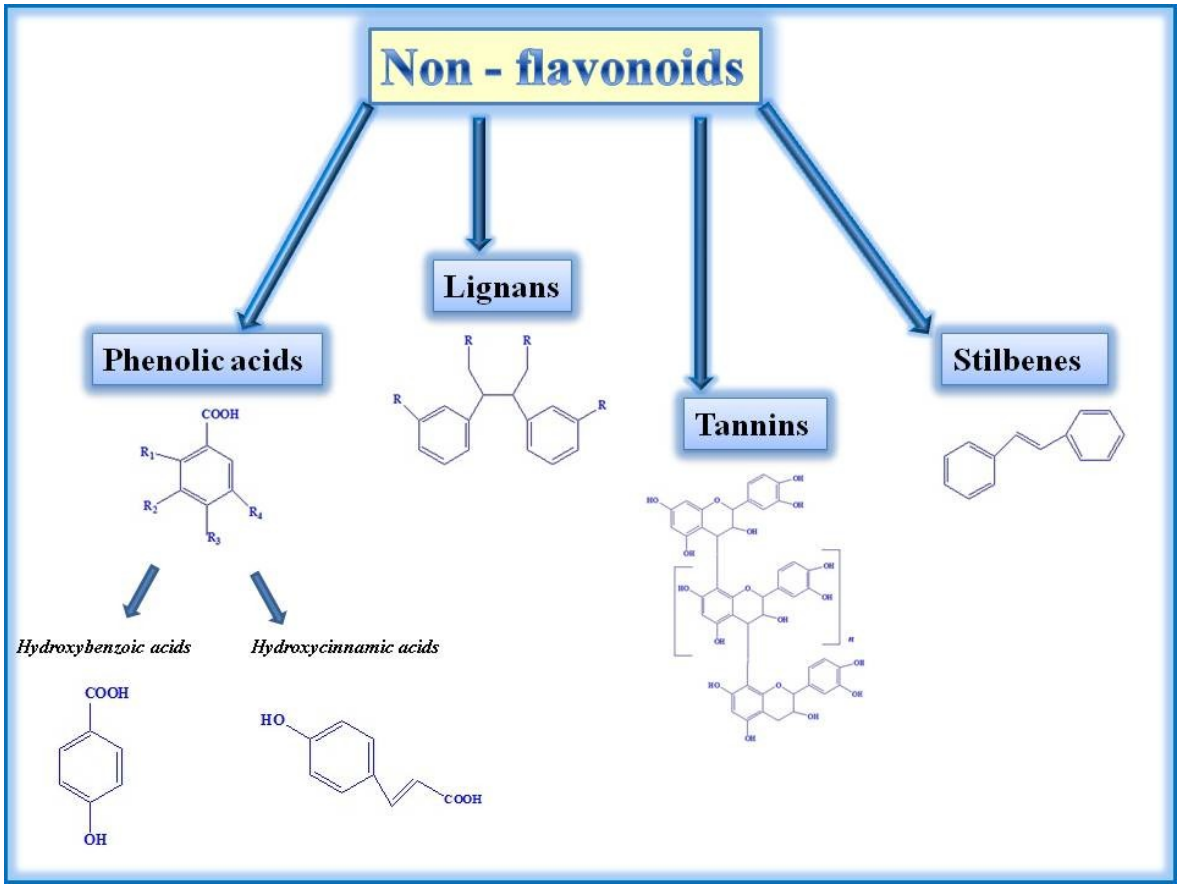


Figure 9: Classification of non-flavonoid compounds.

1.3.2.1. Resveratrol

Resveratrol, chemically named 3,5,4'-trihydroxystilbene (two phenol rings linked by a styrene double bond) (figure 10), is a secondary metabolite compound produced by plants, especially abundant in grapes and in red wine [75]. This polyphenol is produced from plants against exogenous substances such as fungi, UV irradiations and ozone exposure.

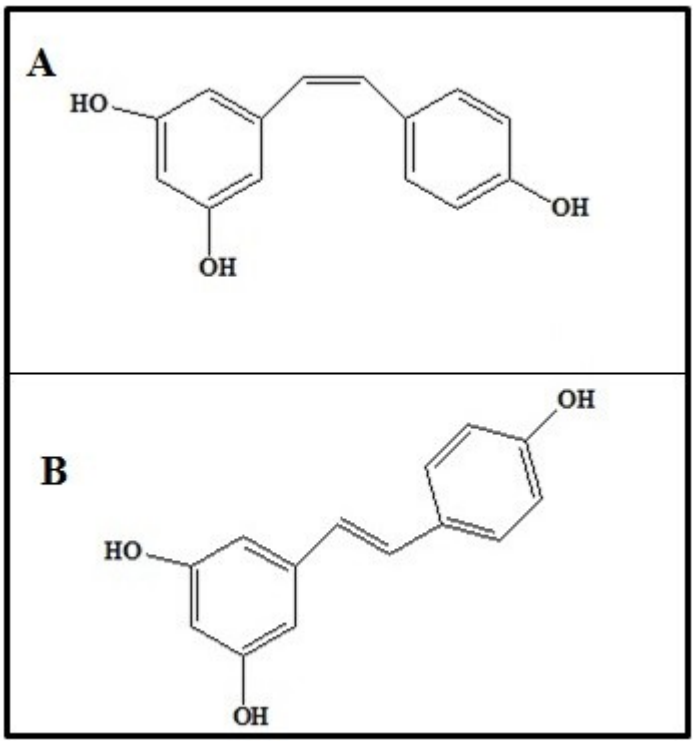


Figure 10: Chemical structure of (A) *cis*- and (B) *trans*—resveratrol.

Regarding its chemical structure resveratrol shows two conformations: *cis*- (Z) and *trans*- (E) (figure 10). The *trans*- isomer is more stable and more biologically active than the *cis*- one [76]. Even though *trans*-resveratrol is the more stable natural form, it has also a high level of photosensitivity. It has been studied that one hour of natural sunlight converts about 80-90% of *trans*-form into the less stable *cis*-form when in solution. It has also been estimated that UV exposure for 120 minutes may isomerise around of 90.6% of the *trans*-resveratrol into the *cis*-resveratrol [76]. Regarding metabolization, it has been shown that resveratrol, when administered orally, is absorbed and metabolized within 30 minutes [75]. Resveratrol also shows a broad range of beneficial health effects, with the anti-inflammatory and anti–oxidant being the most relevant.

- **Anti-inflammatory activity**

Resveratrol suppresses the expression of the genes of the NF- κB and the AP-1. These two molecules cause the expression of some enzymes that catalyse the production of pro-inflammatory molecules like prostaglandins and NO. Moreover through the same pathway resveratrol also blocks the expression of pro-inflammatorycytokines/chemokines such as interferon gamma (IFN-γ), IL-1, IL-6 IL-8 and TNF-α, all involved in inflammation [77-

79]. Specifically for the lung, during the inflammation process, multiple inflammation markers, such as IL-8, IL-6 and TNF-α, are expressed [80]. There is also further evidence showing resveratrol to have the potential to inhibit the release of different types of inflammatory cytokines, such us granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-8 from human bronchial smooth muscle cells and macrophages in COPD patients after exposure to TNF-α and LPS, respectively [79]. More recently, resveratrol has shown the potential to inhibit the release of inflammatory mediators from human airway epithelial cells, which has been proven to be involved in inflammatory lung disease, such as COPD and asthma [81]. Donnelly *et al*., reported that the reduction of IL-8 expression from human primary epithelial cells was due to the inhibition of NF-κB activity by resveratrol [81].

- **Anti-oxidant activity**

Resveratrol has been found to have an *in vitro* anti-oxidant activity by radical scavenging activity. Data in the literature have demonstrated that resveratrol scavenged free radical activity through the interaction of the *para* and m*eta* hydroxyl groups with free radicals [82]. This molecule is capable of scavenging lipid hydroperoxyl free radicals, as well as hydroxyl and superoxide anion radicals. Resveratrol had the highest anti-oxidant activity, but further increase in the concentrations of resveratrol resulted in reduced anti-oxidant activity. In addition, Acquaviva et al. have conducted a detailed study on the anti-oxidant activity of resveratrol, whereby resveratrol showed significant inhibition on xanthine oxidase, membrane lipid oxidation and DNA cleavage activities [83]. At a molecular level, it has been shown that resveratrol could reduce ROS production in lung epithelial cells induced by smoking via stimulation of glutamate-cysteine ligase (GCL) and glutathione (GSH) production [84]. Additionally, it has been shown that the level of GCL in smoker's airway and COPD patients was considerably lower than healthy and non-smokers, which further suggests that this protein is involved in progression of lung injury via oxidative stress. Nitrosative stress (contributed by NO production) and nitration of protein in airway epithelial cells have been hypothesized to be the culprit for steroid resistances in COPD. A study has previously demonstrated that resveratrol was able to reduce the expression of NO on epithelial cells in the presence of different inducers (cigarette smoke and cytomix). Furthermore, a study by Bi *et al*., showed that the inhibition of NO release by resveratrol in microglia cells after stimulation with LPS, was concentration dependent [85]. Resveratrol (100 μM) has also been reported to reduce by about 90% the NO production induced in

macrophages with LPS, although the concentration of NO was evaluated with a different method [85].

For the above reasons, polyphenolic compounds are potential candidate to improve the treatment of inflammatory lungs diseases. However, these molecules have a very low solubility in water, chemical stability, rapid metabolization and consequently they exhibit low bioavailability [86].
To overcome these problems and enhance the beneficial properties of quercetin and resveratrol, multiparticulate forms, have been developed in the course of this thesis, specifically inhalation powder using intelligent drug carriers and site-specific delivery in order to protect and enhance the bioavailability of the studied polyphenols.
Solid lipid microparticles (SLM) were produced for quercetin, while resveratrol formulations were based on powder production using the spray dried technique.

1.4 Formulation strategy for lung delivery using inhalable dry powder

1.4.1 Spray dried microparticles

One of the most known instrument which uses the spray drying technology the Mini Spray Dryer (figure 11) can be used with a small quantity of materials.
Three basic operations can be recognized in the process of spray drying: atomization, drying and separation.
The product to be dried, prepared in liquid form, is sucked from a solution and is transported to a pneumatic atomizing nozzle by means of a peristaltic pump. Subsequently, the feed solution is nebulized by atomizers in small droplets in a drying chamber in which hot air circulates. The thermal contact between the spray droplets and the hot air causes the rapid evaporation of the solvent with the formation of solid particles dried or partially wet, generally spherical shaped, which are then separated from the gas through a cyclone, an electrostatic precipitator or a filter.

Figure 11: Mini Spray Dryer B-290[87].

The chemical composition of the solid particulates depends on the content within the feed solution, whereas particle size and morphology are strongly dependent on process parameters such as liquid and gas feed rate, inlet temperature, gas pressure and aspiration.

1.4.2 Solid lipid microparticles (SLM)

Solid lipid microparticles were introduced since the early 1990s as an effective alternative drug carrier systems to the traditional colloidal carrier system such as emulsions, liposomes and polymeric microparticles. These particulate systems have gained great interest for the modulation and the targeting of active ingredients in the pharmaceutical, cosmetic and food fields and also for different route of administration like parenteral, topic and oral. A further advantage in using SLM for pulmonary targeting for local diseases (e.g., asthma); because they can be prepared in a size range suitable for lodging within bronchioles.

Solid lipid microparticles carriers consist of hydrophobic core, solid at ambient and body temperature, stabilized by a layer of surfactant in their surface (figure 12) [88,89].

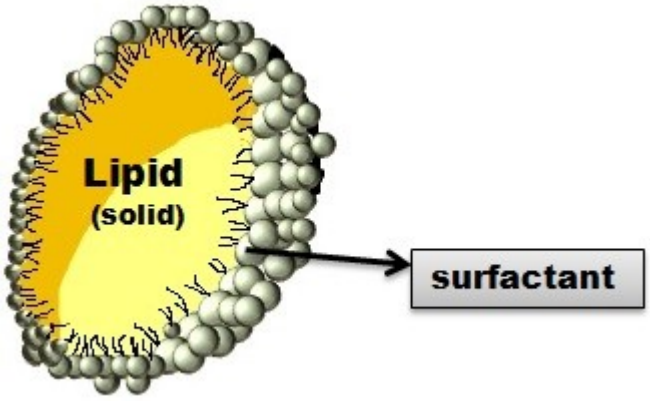


Figure 12: Solid lipid microparticles (SLM).

Moreover the nature of the ingredients ensure that SLM are compatible and biodegradable, providing good *in vivo* tolerability and optimal biodegradability [88,90,91].

Advantages of SLM include:

I. Modified release of the drug;
II. Drug targeting;
III. Increasing bioavailability of sparingly water-soluble substances;
IV. Protection of the active component incorporated in the particles from degradation;
V. Chemical and physical storage stability (for both drug and carrier system);
VI. Use of biodegradable and biocompatible ingredients;
VII. Ease of production on a large industrial scale;
VIII. Low cost of production due to their low manufacturing cost and raw materials cost.

One of disadvantages is that the drug loaded in SLM needs to be lipophilic enough to achieve a proper entrapment efficacy.

There are other disadvantages of SLM:

1. Possibility of particle growth;
2. SLM dispersions have high water content:
3. Safety profile may be uncertain;
4. Drug loading may be limited due to the solubility and miscibility of the drug in the melted lipid, chemical and physical structure of lipid materials, and their polymorphic state.

1.4.2.1 Methods to produce SLM

There are several techniques for the production of SLM:

- **Melt emulsification method**

For the melt emulsification technique, the drug is dissolved or dispersed into the melt lipid (figure 13). This melt phase is subsequently mixed into a preheated polar phase (mainly water) containing the surfactant. The mixture is then homogenised with a high-shear apparatus at a temperature above the lipid melting point and the obtained emulsion is cooled at room temperature to form a microparticles suspension [92].

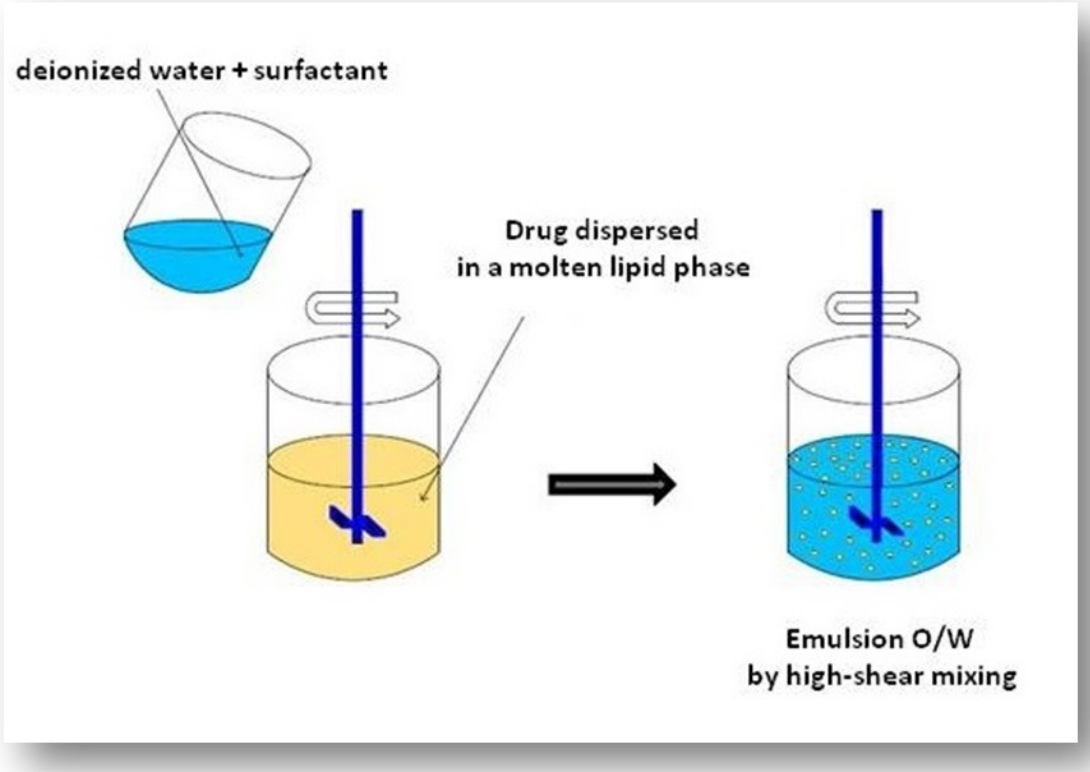


Figure 13: Preparation of SLM by melt emulsification technique.

The water-free SLM are then recovered by filtration, centrifugation and /or lyophilization. Solvent removal enhances the stability of the LM preparation and facilities characterization studies and formulation of the particles into a dosage form. This method is the most commonly used in order to prepare the SLM [93-95].

- **Solvent emulsification-evaporation method**

This technique differentiates from the melt emulsification method, since the lipid matrix instead of being melted is dissolved with the drug in a water-immiscible organic solvent, which is then emulsified in aqueous phase containing the surfactant by high-speed homogenization. Thereafter, the organic solvent is evaporated from the emulsion by mechanical stirring at room temperature or under reduce pressure (rotary evaporator), resulting in the precipitation of the lipid as SLM [92].

The main advantages for this procedure is the limited exposure of thermolabile substance to high temperature [88,89]. However the use of organic solvents represents a disadvantage for pharmaceutical applications.

- **Solvent emulsification-diffusion method**

In this technique, the organic solvent used is partially water-miscible. The lipid and active ingredient are dissolved in organic solvent and emulsified with the external aqueous phase, containing the surfactant under high-speed mixing [92]. Water is then added to the obtained emulsion in order to favour the organic solvent diffusion from the droplets to the external phase, producing the solidification of the dissolved lipophilic material [96,97]. The SLM are isolated by centrifugation or filtration.

This technique has the advantages of satisfactory reproducibility and reduced exposure to high temperature. The major drawback is low drug entrapment due its diffusion into the aqueous phase.

- **Double emulsion (w/o/w) method**

This technique is used for polar drug in order to overcome their poor entrapment in the hydrophobic matrix of the SLM [92]. A hot aqueous solution of the hydrophilic drug is added to the molten lipid containing a lipophilic surfactant under mixing. This primary w/o emulsion is then dispersed in aqueous phase maintained at the temperature above the lipid melting point and containing a hydrophilic surfactant. The mixture is stirred and the resulting double emulsion is cooled at room temperature. The obtained SLM are isolated by centrifugation or freeze-drying [98-100]. Particles formed from such technique are by nature, poorly controlled in both size and structure, thus limiting their use.

- **Sonication method**

The dispersion of the emulsion obtained according to one of the methods reported in previous section can be performed by sonication alone or in combination with stirring. Sonication of the sample using an ultrasonic probe leads to a reduction of the particle size to few microns, suitable for specific administration route such as pulmonary delivery [100].

- **Spray congealing method**

Spray congealing represents a relative novel and attractive technique for the preparation of SLM, which is not based on emulsification method. The fluid mixture, consisting of the drug dissolved or dispersed in the molten lipid carrier is atomised through an atomisation device. The atomisation leads to the formation of multiple droplets, which then solidify into a vessel maintained at ambient temperature or in dry ice. The obtained SLM are then collected and in some cases dried in a desiccators [89,92]. The performance of this method depends primarily on the atomisation efficiency of the molten mixture. The different types of atomisation device available include: a) centrifugal atomisers, b) airless atomiser and c) the air nozzle or pneumatic nozzle. The latter can be in the internal (the molten fluid and atomisation air come in contact in the mixing chamber) or external (the molten fluid and the atomisation air come in contact outside the nozzle) mixing configuration. The inner diameter of pneumatic nozzle, the air temperature and pressure represent the main operating parameters influencing the performance of the obtained. This technique has been shown to be more efficient than the melt emulsification for the incorporation of polar drug into the matrix lipid of the SLM. The main disadvantages is that rather larger particles are produced suitable for oral or topical delivery.

- **Spray Drying**

Using the spray drying technique the lipid and the active are dissolved or dispersed in an organic solvent. The obtained mixture is then atomized into a current of warm filtered air, which removes the solvent from the droplets yielding solid microparticles. Due to the heat supplied by the air for solvent evaporation, the use of lipids with high melting point (e.g. >70°C) is required to avoid the melting of the SLM during the spray process, this aspect being probably the reason of the limited application of this method to LM preparation [92].

1.4.3. Microparticles characterization

- ***Optical microscopy and scamming electron microscopy***

Optical microscopy and scanning electron microscopy (SEM) are both used to measure particles size and evaluate particles morphology and also the presence of agglomerates [92]. The OM is less accurate in the dimensional range of few microns. The main disadvantage of microscopy technique is the rather small number of particle examined (300-500) that limits the statistical significance of the results.

- ***Laser diffraction***

The technique is based on the correlation between the angle of the laser light scattered by particle and their size (the angle increases with decreasing particle size).

The dimension of the particles are calculated from light scattering angle through complex algorithms based on Frauhofer and /or Mye calculations. Laser diffraction covers a broad size range from about 0.1 to 1000 μm and can by use with dry powder or particle suspension [92]. It is a simple and fast sizing method and represents the most commonly used technique for the analysis of particle size distributions.

- ***Crystallinity and polymorphism***

The characterization of degree of drug and /or lipid crystallinity and the modification during particle preparation and storage are very important parameters. Differential scanning calorimetry and powder X-ray diffraction are the most commonly used techniques for microparticles and LM solid state studies [92].

Differential scanning calorimetry (DSC) is a thermal method which measure the thermal response of a sample to a temperature gradient, thus enabling the measurement of transition enthalpies and related transition temperature. Differential scanning calorimetry analysis is based on the fact that different polymorphic forms exhibit different melting temperatures and enthalpies.

Powder X-ray diffraction analysis exploits the diffraction of X-rays by crystals, due to their wavelengths being comparable to the distance between atoms and molecules of crystals. A powder can be also investigated by X-ray diffraction by comparing the position and intensity of the diffraction peaks with those of a known substance and polymorphic form. Polymorphic modification can be detected by the presence of diffraction signals at different positions and with varying intensities. Moreover, since amorphous forms produce

broad and diffuse maxima in diffraction patterns, it is generally recognized that the disappearance of the crystalline peaks of substance indicates its amporphisation.

1.4.3.1 SLM characterization: drug loading, encapsulation and in vitro release.

Once the SLM are manufactured, it becomes important to estimate the drug loading and the encapsulation efficiency, in order to assess the quality of the drug carrier system [92]. There are several factors that influence the drug loading and the encapsulation efficiency of SLM. These are:

- The solubility of the drug in the melted lipid. If the solubility of the drug is high, in turn, the encapsulation efficiency and the drug loading should be high as well;
- The chemical and physical state of the lipids and of the active substance;
- The size of the particles; large SLM can load a higher amount of drug.

The selection of a drug carrier system and its effectiveness in the encapsulation of a drug depends on the loading capacity of the carrier system. Drug loading indicated the amount of drug incorporated into the matrix lipid matrix and is determined as the percentage ratio between the amount of effective ingredient assayed in the SLM and the total weight of the particle sample according to this equation:

$$\text{Drug loading (\%)} = \frac{Mass\ of\ drug\ in\ microparticles}{Mass\ of\ microparticles\ recovered} \text{ x } 100 \qquad \textbf{(3)}$$

The encapsulation efficiency is calculated as the percentage ratio between the quantify of active entrapped in the microparticles and added to the melted lipid phase during particle production according to the following equation:

$$\text{Encapsulation efficiency (\%)} = \frac{Mass\ of\ drug\ in\ microparticles}{Starting\ mass\ of\ drug} \text{ x } 100 \qquad \textbf{(4)}$$

Some of the factors which affect the loading capacity of a drug in a lipid are the solubility of the drug in the lipid melt and the miscibility of the drug and the lipid melt.

Since the general aim of microparticles system is protection and controlled release of the incorporated drug, in vitro release studies have to be carried out on the obtained particles to ascertain the release profiles. These studied are performed according to the Pharmacopeia

dissolution test such as apparatus I, II and IV, although non-official apparatus are also often used. The method measures the diffusion of the incorporated substance through the particle matrix into a suitable dissolution fluid. In order to ensure the sink conditions, surfactants, water miscible organic solvents have to been added to the release medium. The release fluid should ensure adequate solubility of the incorporated drug, whereas it should not affect the integrity of SLM.

1.5 Pulmonary drug delivery

Drugs delivery to the lungs is an attractive target in the health care research area due to the lung capability of absorbing pharmaceuticals either for local deposition or for systemic delivery. In order to delivery the drugs to the lungs by inhalation is important understand the anatomy of the respiratory tract, the physical-chemical characteristics and the aerosol performance of the formulations and finally the specific device that is require for different types of formulations.

1.5.1 Respiratory system: anatomy

The respiratory system is a biological system involved in the respiration that is the exchange of oxygen and carbon dioxide between the organism and the environment through two phases: inhalation and exhalation.

The inhalation is the passage of air into the lungs to supply the body with oxygen while the exhalation is the passage of air out of the lungs to expel carbon dioxide. This biological system is composed of specific organs and structure and the respiration takes place in the respiratory organs called lungs.

In addition to air distribution and gas exchange, the respiratory system carry out different activities such as filter, warm and humidifies the air breath. For this reason the organs of the respiratory tract play a specific role to guarantee these passages.

The respiratory system is divided into two main components: upper and lower respiratory tract.

The organs that compose the **upper respiratory tract** (figure 14) are located outside the chest cavity and are the nose and nasal cavity, the pharynx, the larynx. Below there is a list of the specific activities for each organs.

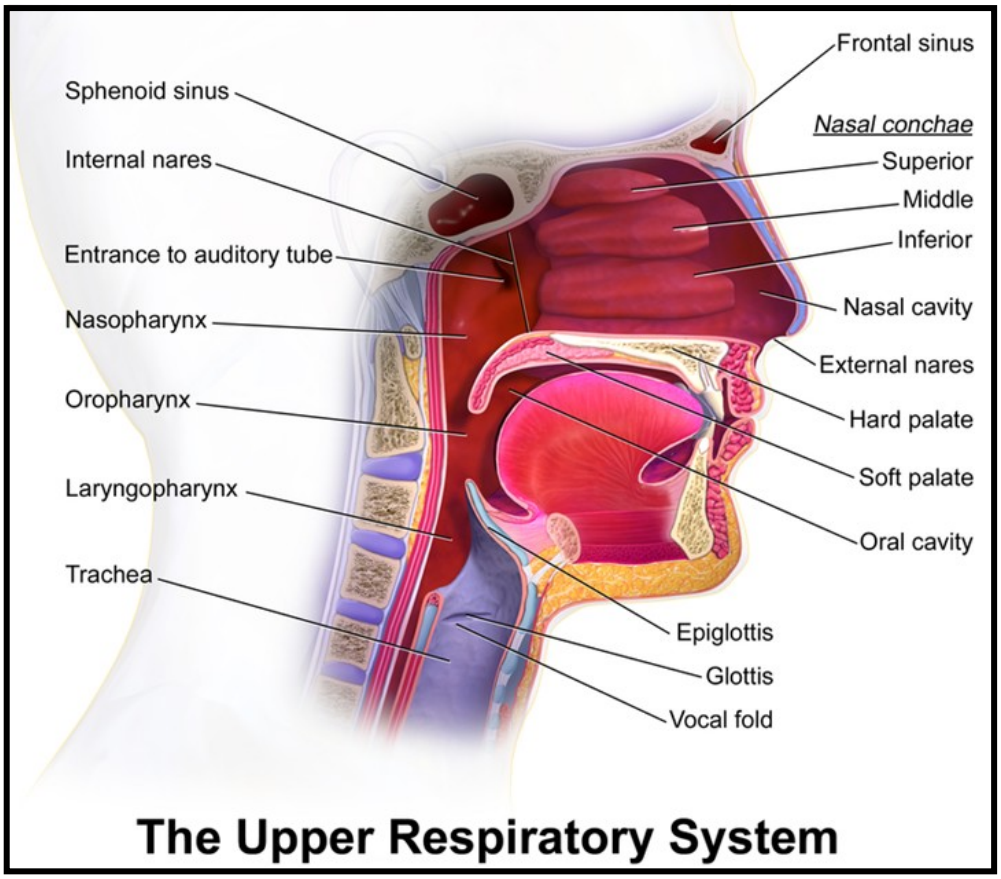


Figure 14: The upper respiratory tract [101].

- The **nasal cavity** is the internal side of the **nose** and it consists in two chambers separated by the nasal septum. It extends from the nasal vestibule, which opens to the face through the nostrils, to the nasopharynx: the muscular-membranous tube that regulates the right passage of food and air. Between the nasal vestibule and the nasopharynx, is located the atrium, a transition and wider compartment, and the respiratory region.

  Therefore the nasal district, apart the olfactory function, is geared to fulfil these two roles before conditioning the processed inhaled air into the lungs:

  1. Humidify and warm the air

  The air needs to be heated and moisturized before travelling to the lungs. The transition through the nasal cavity allows air to reach over 80% of humidity and to be warmed really efficiently because of the opposite direction between blood and air flow. Therefore the heat exchange from the warm blood to the cold air is very powerful.

2. Filtration

All particles larger than 30 μm are completely filtered from entering the lung and most of the particles over 10 μm are also cleared.

The removal of the particles after their deposition is mostly achieved by the mucociliary clearance.

- The **pharynx** is part of the digestive system and also of the conducting zone of the respiratory system. after the inspiration the air pass through the pharynx before reaching the lower respiratory tract.

- **Larynx**: The larynx is essential to human speech.

In the **lower respiratory tract** (figure 15) all the organs are located inside the chest cavity and are trachea, the lungs, bronchi and alveoli.

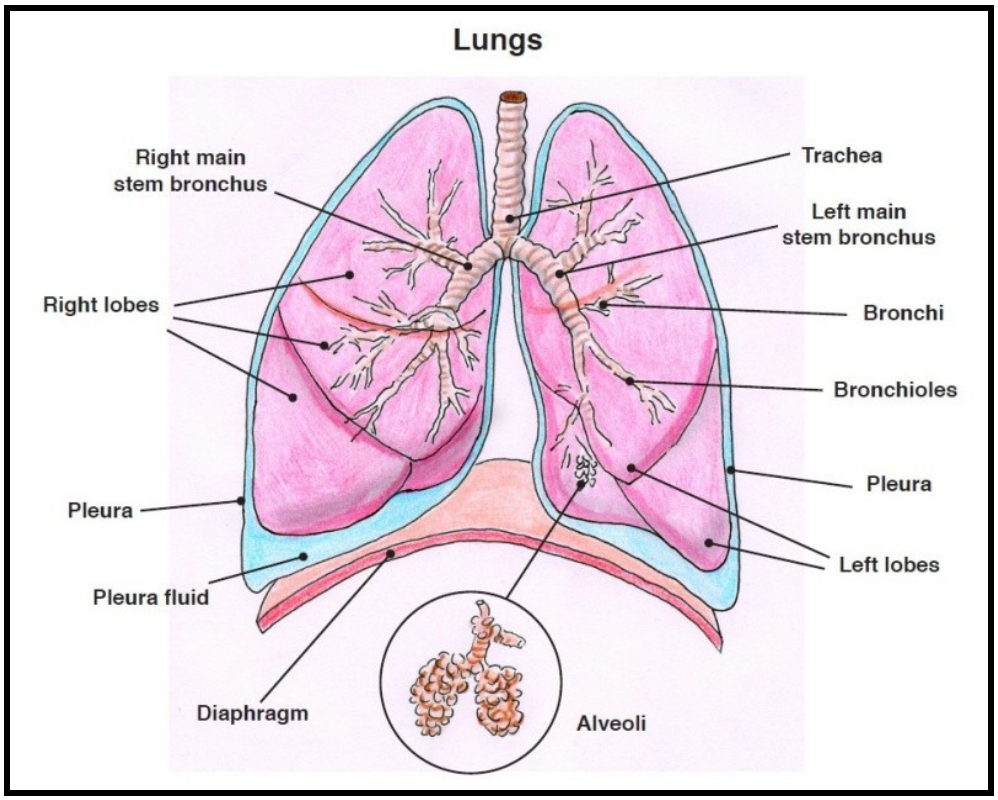


Figure 15: Lower respiratory tract [102].

- **Trachea:** Located just below the larynx, the trachea is the main airway to the lungs. The tube is made of muscles and elastic fibres with rings of cartilage, has a role of connecting the throat to the bronchi.
- **Bronchi:** The bronchi branch from the trachea into each lung and the bronchi branches off into smaller tubes called broncheoli which end in the pulmonary alveolus.
- **Pulmonary alveoli** – tiny sacs (air sacs) delineated by a single-layer membrane with blood capillaries at the other end. The exchange of gases takes place through the membrane of the pulmonary alveolus, which always contains air: oxygen (O_2) is absorbed from the air into the blood capillaries and the action of the heart circulates it through all the tissues in the body. At the same time, carbon dioxide (CO_2) is transmitted from the blood capillaries into the alveoli and then expelled through the bronchi and the upper respiratory tract. The inner surface of the lungs where the exchange of gases takes place is very large, due to the structure of the air sacs of the alveoli.

1.5.2 Drug deposition in the lungs

In order to have an appropriate and efficient process of drug administration by inhalation, **aerosolization** design the inhalable formulation (aerosol) and choose selection of the appropriate device that can permit an efficiency delivery of formulations are required [102]. After aerosolization the particles is required an efficient **deposition** at the appropriate site of the respiratory tract is required, since failure in depositing the formulation will result in poor drug absorption [104,105**]**. Finally, the last step is the drug **absorption** (after dissolution and permeation) [105,106].

For this reason is important to recognize the mechanisms of particle deposition in the airways and how they may affect the performance of formulations for drugs delivery to the airways of the lungs.

The most important mechanisms of particle deposition are impaction, sedimentation, diffusion, interception and electrostatic precipitation [107-110].

- ***Inertial impaction*** is the dominant deposition mechanism for particles larger than 1 μm in the upper tracheobronchial regions. A particle with large momentum (large size or velocity or both) may be unable to change direction with the inspired air as it passes the bifurcations of the airways, and instead it will collide with the airway walls. Because

impaction depends on the momentum of the particle, large or dense particles moving at a high velocity will show greater impaction [111].

- Gravitational ***sedimentation*** is an important mechanism for deposition of particles bigger than 0.5 μm and smaller than 5 μm in the small conducting airways where the air velocity is very low. Deposition due to gravity increases with enlarging particle size and longer residence times but decreases as the breathing rate increases [112]**.**
- Submicron-sized particles (especially smaller than 0.5 μm) acquire a random motion caused by the impact of surrounding air molecules. This Brownian motion may then result in particle deposition by ***diffusion***, especially in small airways and alveoli, where bulk airflow is very low. Therefore, to reach the lower respiratory tract and optimize pulmonary drug deposition, aerosols need to have aerodynamic diameters between 1 and 5μm [113].
- ***Interception*** is likely to be the most effective deposition mechanism for aggregates. For such particles, deposition may occur when a particle contacts an airway wall, even though its centre of mass might remain on a fluid streamline [114,115]**.**
- ***Electrostatic charges*** enhance deposition by increasing attractive forces to airway surfaces, in particular for fresh generated particles**.**

1.5.3 Factors affecting aerosol deposition

The bioavailability of pulmonary formulations, such as an aerosol, depends of the dose of drug that is able to deposit in the lower respiratory tract. For this reason drug formulation plays an important role in producing an effective inhalable medication because a failure in deposition may result in lack of efficacy. Since particles geometry appears so relevant to the behaviour of the inhaled particle, the concept of aerodynamic diameter has been introduced to measure the size of the inhaled particle.

Hence, when the inhaled particle is not spherical, the aerodynamic diameter d_{ae} is defined as the diameter of a sphere of unitary density (ρ_0) that has the same sedimentation velocity as the particle in consideration [116], and can be calculated using this equation:

$$Dae = dv\sqrt{\rho/X\rho 0} \qquad \textbf{(5)}$$

With:

- Dv is the volume-equivalent diameter,
- ρ is the particle density and
- X is the dynamic shape factor.

The dynamic shape factor is a correction factor that takes into account the non-sphericity of the particle, equal to 1 for a sphere and greater than 1 for irregular particles.
Hence, particle geometry, density and volume diameter are the main characteristics that affect inhalation performance.
Particles with an aerodynamic diameter of 1-5 μm (referred to as the "respirable range") tend to deposit in the lungs, while particles larger than 5 μm are trapped in the upper respiratory tract and smaller particles (<1 μm) are generally exhaled during normal breathing [110].

1.5.4 Device used for lungs delivery

In order to have local or systemic effect of drugs by inhalation is important that the inhaled medications are able to reach the lung region and for this reason a suitable device need to be use by the patients.
The inhaled medication can be delivered into the lung region in the form of aerosol using different types of devices in according to the *European Pharmacopoei*a.
The device could be classified depending to the formulations that need to be delivered and the different design and three main types of inhaler devices are available and are classified:

- **Nebulizers;** aerosolize aqueous solutions or suspensions containing the drug by means of a source of energy (figure16);

Figure 16: Nebulizer [117].

- **Pressurized metered dose inhalers (pMDI);** the drug is dissolved or suspended with a propellant in a pressurized dispenser and is aerosolized through an atomization nozzle using a metering valve and an actuator (figure 17);

Figure 17: Pressurized metered dose inhaler [118].

- **Dry powder inhalers (DPI);** deliver the drug to the pulmonary system as a dry powder aerosol (figure 18).

Figure 18: Dry powder inhaler [119].

The **nebulizer** has been the first device developed for inhalation therapy market used to administer medication in the form of a mist inhaled into the lungs and is commonly used for the treatment of inflammatory lung diseases such as asthma, COPD and cystic fibrosis. This device consists of a reservoir containing a solution or suspension of drug connected to a facemask or mouthpiece through which the patient breathes normally. Moreover, the nebulizer emit aerosol microparticles from solutions or suspensions formulations using

oxygen, compressing air or ultrasonic power. Nebulizers have some disadvantages such as delivery inefficiency, drug wastage, poor reproducibility, great variability and high cost.

Pressurised metered dose inhaler (pMDI) are inhalation devices were the drug is dissolved or suspended with a propellant in a pressurized dispenser and is aerosolized through an atomization nozzle using a metering valve and an actuator [120].

The medication is placed into the chamber and a propellant that turns the medication into a fine mist. Then the patients have to push the button onto de device button to force the medication out through the mouthpiece.

The propellant formerly used because of their non-toxicity is chlorofluorocarbon (CFC), but the medical application of CFC as propellants has been phased out because is implicated in atmospheric ozone depletion and for this reason the CFC was substituted by hydrofluoroalkanes, given their safety profile [121]. One of disadvantages of this device is that the patients have to co-ordinate device actuation with their inhalation because of the high exit velocity of the aerosol cloud. To improve the efficacy of the device and consequently helping the patients in the co-ordination the Breath-actuated pMDIs and the use of spacer devices could be use (figure 19).

Figure 19: Pressurised metered dose inhaler with spacer device [122].

Dry powder inhaler (DPI) is a device that delivers medication to the lungs in the form of a dry powder (figure 18).

The advantages of delivery dry powder are due to the possibility to deliver high doses of formulation and also the greater stability of the powder compared to liquid formulations. These advantages coupled with the absence of propellant (not require for this device) that

reduce the cost associated with the generation, transport, and storage of the propellant and their unwanted environmental impact, cause that makes these devices an advantageous alternative to MDI.

Currently there are essentially four types of DPI:

- Single-unit dose (capsule); this inhaler requires the patient to load a single hard gelatin capsule containing the powder formulation into the device before each use.
- Single-unit dose (disposable); It is a device containing a pre-metered amount of a single dose that is discarded after use.
- Multi-unit dose (pre-metered unit replaceable set); Multi-unit devices deliver individual doses from pre-metered replaceable blisters, disks, dimples or tubes.
- Multiple dose (reservoir); Multiple dose reservoir inhalers contain a bulk amount of drug powder in the device with a built in mechanism to meter a single dose from the bulk and individual doses are delivered with each actuation.

2 AIM OF WORK

Since that inflammation and oxidative stress are the principle markers in the pathogenesis and development of chronic inflammatory lungs diseases, such asthma and COPD, the identification potential use of various anti-oxidant agents such as polyphenols (quercetin and resveratrol) could be a potential candidate to improve the treatment of inflammatory lungs diseases.

However, these molecules exhibit very low solubility in water, chemical instability, rapid metabolization and consequently they have low bioavailability.

To overcome these problems and enhance the beneficial properties of quercetin and resveratrol, multiparticulate forms, have been developed in the course of this thesis, specifically inhalation powder using drug carriers and site-specific delivery in order to protect and enhance the bioavailability of the studied polyphenols.

Solid lipid microparticles (SLM) were produced for quercetin, while resveratrol formulations were based on powder production using the spray dried technique, in a single spray dried formulation and in co-spray dried with budesonide (corticosteroid drug).

The experimental work was divided in three sections as listed below:

I. **SECTION A:** Inhalable dry powder based on quercetin-loaded lipid microparticles for pulmonary delivery
II. **SECTION B:** Inhalable resveratrol spray dried formulation to improve the treatment of chronic inflammatory lung diseases
III. **SECTION C:** *In vitro* activities of co-spray dried resveratrol and budesonide inhalable formulations in alveolar macrophages

3 MATERIALS

Quercetin, resveratrol (*trans*-3,4',5-trihydroxystilbene) and budesonide were purchased from Sigma (Steinheim,Germany), Fagron Italia (Bologna, Italy) and Yicheng Chemical Corp (Jiangsu, China), respectively.

3.1 Ingredients to manufacturing the solid lipid microparticles

- Lipids: tristearin was supplied by Sigma (Steinheim,Germany)
- Surfactants: phosphatidylcholine was received as a free sample from Cargill (Hamburg, Germany).

3.2 Cell lines

- A549 cells were purchased from ATCC(Rockville, MD). Ham's F-12, fetal bovine serum, RPMI-1640, penicillin/streptomycin and l-glutamine were obtained from Lonza (Milan, Italy).
- Calu-3 cell line (HTB-55) was purchased from the American Type Cell Culture Collection (ATTC, Rockville, USA) and Dulbecco's Modified Eagle's medium (DMEM) from Sigma–Aldrich (Sydney, Australia).
- Rat alveolar macrophage NR8383 cell line (HTB-55) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

3.3 Ingredients for the in vitro studies

CelLytic™ M reagent, α-Lipoic acid, Nitro-L-arginine methyl ester (L-NAME), L-ascorbic acid, non-essential amino acids, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,3-diaminonaphthalene (DAN) and LPS from Escherichia coli 0111:B4 were purchased from Sigma-Aldrich (Sydney, Australia). L-glutamine, Hank's balanced salt solution and TNF-α were purchased from Invitrogen, (Sydney, Australia). Transforming growth factor beta (TGF-β1) was from Sapphire Bioscience (Sydney, Australia) and Human IL-8 ELISA Kit II BD OptEIATM were purchased from Becton Dickinson (Sydney, Australia). Analytical grade solvents were purchased from Sigma (Sydney, Australia).

Other cell culture reagents including were purchased from Invitrogen, (Sydney, Australia). Elisa kit for determination of inflammation markers such as IL-6 and TNF-α were supplied from BD Bioscience (Sydney, Australia).

3.4 Solvents

All solvents used were analytical grade were purchased from Biolab (Victoria, Australia) and from Sigma.

4 METHODS

4.1 Manufacturing of powder

4.1.1 Preparation of the solid lipid microparticles (SLM)

For the development of SLM loaded with quercetin, melt o/w emulsification technique was employed (figure 20), since it circumvents the use of organic solvents [89, 90, 92-94]. This approach eliminates the potential drawback for residual solvent in the final dosage form.

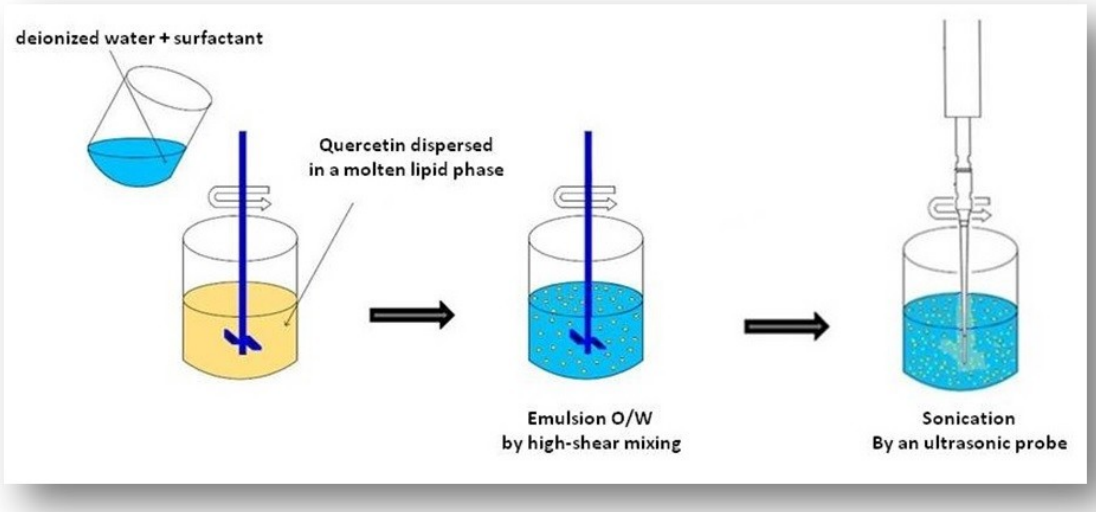


Figure 20: Preparation of SLM using melt emulsification technique with a step of sonication.

Briefly, SLM were prepared by adding preheated (75–85°C) deionised water (50 ml) containing the surfactant (1.4%, w/v) to the molten lipid phase (3.6 g), in which quercetin (0.7 g) was dispersed. When, both aqueous and lipid phases, were at the same temperature (~75°C), the hot aqueous phase was poured into the molten lipid (phase-inversion process) to avoid loss of lipid and drug during the preparation process. The mixture was then subjected to high-shear mixing (17500–21500 rpm for 2–3 min) using an Ultra-Turrax T25mixer (IKA-Werk, Staufen, Germany) at 75–85°C. For the optimized preparation, the sample was also sonicated at 22 kHz for 2 min (power output, 40 W), using an ultrasonic probe (Microson XL2000Ultrasonic Cell Disruptor; Misonix, Farmingdale, NY).

The obtained emulsion was rapidly cooled at room temperature under magnetic stirring. The formed suspension was subjected to centrifugation (6000 rpm for 15 min) and lyophilizatrion to obtain water-free microparticles.
Unloaded particles were also prepared with the same procedure, by omitting quercetin.

4.1.2 Spray-dried microparticles

Respiratory size microparticles of resveratrol, alone or in combination with budesonide were produced by spray drying using a Buchi spray dryer (Buchi B-290 Mini Spray Dryer, Buchi, Switzerland).

- ✓ Resveratrol (20 mg/ml) was dissolved in ethanol–water (50:50% v/v) and spray-dried at a feed rate of 40%, flow rate of 12.5 ml/min, aspiration rate 100%, inlet temperature 100 °C and a measured outlet temperature of 40 °C in a closed loop configuration.
- ✓ In order to evaluate the effects of resveratrol and budesonide in combination powder, single and combination microparticles were produced by spray drying. Both resveratrol and budesonide, either alone or in combination, were dissolved in ethanol-water (80:20% v/v) and spray dried using a nozzle of 1.4 mm at feed rate of 40% and aspiration of 100% in a close loop configuration. Single or combinations of resveratrol(RES) and budesonide (BD) with final dry weight percentages (%w/w) were labeled as follow: 100% RES, 75%:25% RES-BD, 50%:50% RES-BD, 25%:75% RES-BD and 100% BD.

4.2. Particle characterisation of powder

4.2.1 Scanning electron microscopy (SEM)

Scanning electron microscopy was used to study the morphology of the SLM, spray dried and co-spray dried formulations.

- ➢ The morphological features of the SLM were observed by variable-pressure (ca. 90 Pa) scanning electron microscopy (VP-SEM; Zeiss EVO40XVP, Arese, Milan, Italy). The images were taken at random locations.

- Spray dried resveratrol, spray dried budesonide and all co-spray dried resveratrol and budesonide formulations were dispersed casually on carbon tapes, placed onto aluminum stubs and coated two times with gold using a Smart Coater (JEOL USA Smart Coater). A bench top SEM (JMC, 6000 JEOL, Japan) operating at 15KV was used for imaging samples.

4.2.2 Optical microscopy

Preliminary information of SLM was observed using an optical microscope (B-500 TPL microscope, Optika Microscopes,Bergamo, Italy) (figure 21). Moreover the estimation of particle dimensions were performed by computerized image analysis (MicrometricsTMcamera 122CUand software vision 2.02) on a minimum of 100 particles.

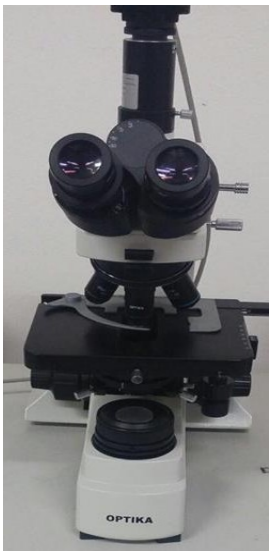


Figure 21: B-500 TPL microscope.

4.2.3 Laser diffraction

Particle size distribution of spray dried and co-spray dried microparticles was analysed using laser diffraction (Mastersizer, Malvern, United Kingdom) (figure 22). Specific experimental conditions were applied to each formulation.

Approximately 10 mg of spray dried microparticles were dispersed using the Scirocco dry dispersion unit with a feed pressure of 4 bars. Specific condition, for spray dried resveratrol, were a feed rate of 75% with an obscuration value between 0% and 15% and a reference refractive index of 1.762. The particle size distribution of co-spray dried

microparticles were determinate using feed rate of 35% with an obscuration value between 0% and 15%. Moreover, a refractive index of 1.67 was used for all measurements and was calculated by the average of the refractive index of budesonide and resveratrol.
All samples were analyzed in triplicate and the geometric diameter was expressed as volume median diameter (Dv50), namely the particle size below which 50% of the sample (in terms of volume) lies.

Figure 22: Mastersizer, AERO [123].

4.2.4 Thermal analysis

The thermal responses of single and co-spray dried microparticles were investigated using DSC (DSC823e, Mettler-Tolledo, Switzerland). Samples (3–5 mg) were crimp-sealed in DSC aluminium pans with the lid pierced to ensure constant pressure, and heated at 10°C/min over a temperature range of 25–320°C. Exothermic and endothermic peaks were determined using STARe software V.11.0x (Mettler Toledo, Greifensee, Switzerland).
In addition, the temperature stability and solvent evaporation of each formulation was assessed using the thermal gravimetric analysis (TGA; Mettler-Toledo, Switzerland). Approximately 13 mg of spray dried powders were placed onto aluminum cubicle pans. The weight loss of the samples was evaluated by heating the samples from 25–200°C with scanning rate of 10ºC/min under constant nitrogen gas at flow 110 ml/min. Data were analysed using STARe software V.11.0x (MettlerToledo, Switzerland) and expressed as the percentage of weight loss with respect to initial sample weight.

4.3 High-performance liquid chromatography (HPLC)

4.3.1 Chemical quantification of quercetin

The HPLC was used in order to quantify the quercetin and the system used is composed of a Model LabFlow 3000 pump (Lab-Service Analytica, Bologna, Italy), a Model 7125 injection valve with a 20 µl sample loop (Rheodyne, Cotati, CA, USA) and a Model 975-UV variable wavelength UV–vis detector (Jasco, Tokyo, Japan). Data acquisition and processing were performed with a personal computer using Borwin software (JBMSD eveloppements, Le Fontanil, France). Sample injections were performed with Model 701 and Model 710 syringes (Hamilton,Bonaduz, Switzerland).

Separations were accomplished using a 5 µm Zorbax SB-C18 column (150 mm × 4.6 mm i.d.; Agilent Technologies,Waldbronn, Germany) fitted with a guard column (5 µm particles,4 mm × 2 mm i.d.; Phenomenex, Torrance, CA, USA) and eluted at a flow-rate of 0.8 ml/min, with sodium acetate (pH 4.0;0.02 M)-acetonitrile (60:40, % v/v) containing EDTA (0.5 mM). The identity of quercetin peak was assigned by co-chromatography with the authentic standard at 370 nm and the quantification was carried out by integration of the peak areas using the external standardization method. Calibration curves were linear over the range 0.05–3 µg/ml, with correlation coefficients greater than 0.998. The precision of the method, evaluated by repeated analyses (n = 6) of the same sample solution containing quercetin at levels of 0.05 and 3 µg/ml, was demonstrated by relative standard deviation values lower than 5.0%.

4.3.2 Chemical quantification of resveratrol and budesonide

Chemical quantification of resveratrol and budesonide were performed using a Shimadzu Prominence UFLC system equipped with a DGU-20 A5R Prominent degasser unit, LC-20 AD Liquid chromatography, SIL-20A HT Autosampler and SPD-20A UV-Vis detector was used (all Shimadzu Corporation, Japan).

Specifically for resveratrol the conditions were XbridgeTM C18 column (5 mm, 4.6 x 150 mm) (Waters, Massachusetts, USA), mobile phase consisted of methanol-water (60:40% v/v) with 0.5% v/v of acetic acid and run at the flow rate 0.7 ml/min. The volume of samples injection was 100 µl and the content of resveratrol was quantified at 306 nm from

the peak area correlated with the predetermined standard curve between 0.2 and 50 μg/ml (R^2 =0.999).

Quantification of budesonide was conducted using methanol: water 80:20 (%v/v) as the mobile phase at flow rate of 1 ml/min in isocratic mode with a Luna C18 column (3 μm, 4.6 × 150 mm) (Phenomenex, Sydney, Australia). The volume of sample injection was 100 μl and the detection wavelength was set up at 243 nm. The linearity was confirmed between 0.2 and 50 μg/ml with correlation coefficients (R^2 = 0.999).

4.4 Solid lipid microparticles characterisation

4.4.1 Quercetin loading of SLM

The weighed of microparticles (25 mg) loaded with quercetin were dissolved in ethanol (20 ml) under heating (80 °C for 5 min) and were sonicated (10 min), in sealed glass vials. The obtained sample was diluted to volume (50 ml) with methanol. After that, the samples were filtered through 0.45 μm membrane filters and assayed by HPLC in order to quantify the amount of quercetin entrapped in the SLM. The encapsulation efficiency was calculated as the percentage ratio between the quantity of quercetin entrapped in the microparticles and the amount of flavonoid initially added to them melted lipid phase. Data were calculated from the average of at least three determinations.

4.4.2 *In vitro* release

For the purpose to study the efficiency of solid lipid microparticles the in vitro studies were performed. In brief, quercetin (2.0 mg) or SLM, containing an equivalent amount of flavonoid, were added to 100 ml of phosphate buffer (0.05 M, pH 7.4), containing polysorbate 20 (0.5%, w/w) as solubilizer to ensure sink conditions. After that, the samples were kept under mechanical stirring at 50 rpm and 37°C. At different time points, aliquots of the medium (1ml) were collected and replaced with an equal volume of fresh fluid. The samples were filtered (0.45 μm) and then assayed for quercetin by HPLC. The quercetin release was calculated as a percentage of the amount of quercetin release in each time point respect the total quercetin content of each LM preparation, that was determined by extraction of the particles, after the release experiment, using the method described above, a minimum of 5 replicates were conducted for each formulation.

4.5 Aerosol performance

In order to evaluate the aerosol performance and the aerodynamic particles size distributions of the formulations, in vitro studies were performed using the anderson cascade impactor (ACI) and the multi stage liquid impinger (MSLI).

- *Experimental procedure for spray dried resveratrol*

Spray dried powder (5 ± 0.1 mg) was located into a size 3 gelatin capsule (Capsugel®, Sydney, Australia) and then placed into the sample compartment of a low resistance RS01 dry powder inhalation device (Plastiape®, Osnago, Italy). The device was attached to the USP throat of the ACI (figure 23) and the flow was adjusted to 60 l/min using a calibrated pump (Westech Scientific Instrument, UK) and flowmeter (Serie 4000, TSS Inc., MN, USA). After actuation, at 60 l/min for 4 seconds, the capsule, device, adaptor, throat and all ACI stages were washed separately with 50:50% v/v ethanol–water and further mixed with mobile phase (methanol:water 60:40% v/v with 0.5% acetic acid) at ratio 1:1 to improve the HPLC peak resolution.

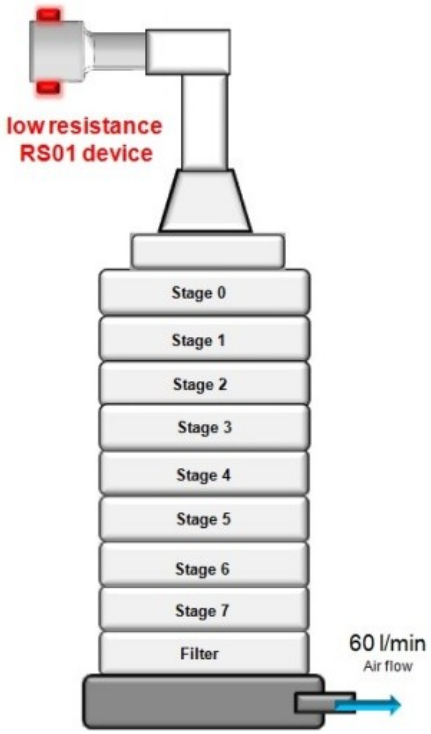


Figure 23: Anderson cascade impactor (ACI) with low resistance RS01 dry powder inhalation device.

The fine particle dose (FPD) (drug recovered from stages 3 to filter, <4.7 μm), the fine particle fraction (FPF) (FPD/Total dose x 100) and total mass recovery were calculated. Experiments were conducted in quadruplicate and samples were analysed using a validated HPLC method.

- *Experimental procedures for single and co-spray dried resveratrol and budesonide formulations*

Approximately 10 mg of spray dried powders was loaded in a size 3 gelatin capsules (Capsugel®, Sydney, Australia) and placed into the dosage chamber of standard-resistance RS01 dry powder inhalation device (Plastiape®, Osnago, Italy). The device was connect to a custom-built mouthpiece adaptor connected to a US Pharmacopeia induction port (throat), connected to the MSLI. The aerosol performance was assessed at two different flow rates, 60 l/min and 90 l/min (calibrated using a suitable flow meter). After actuation, the device, capsule, adaptor, throat, all MSLI stages and filter were washed separately with methanol: water (80:20 %v/v) into suitable volumetric flasks.

All samples, after filtration, were analysed using a validated HPLC method. All experiments were conducted in triplicate and the aerosolization efficiency was evaluated in terms of FPD and FPF.

The FPD was calculated by linear regression analysis of cumulative percentage deposition vs. logarithmic stage cut-off diameter (amount of compounds recovered from stage 3 to filter corresponding to the amount of microparticles with a diameter <6.8μm), while the FPF was defined as the percentage ratio between the FPD and the total quantity of drug collected from the adapter, throat and MSLI stages. Data were calculated from the average of at least three experiments.

4.6 DPPH assay

The chemical compound named 2,2-diphenyl-1-picrylhydrazyl (DPPH) (figure 24) is a stable free-radical molecule and it is used in a common anti-oxidant assay called DPPH assay.

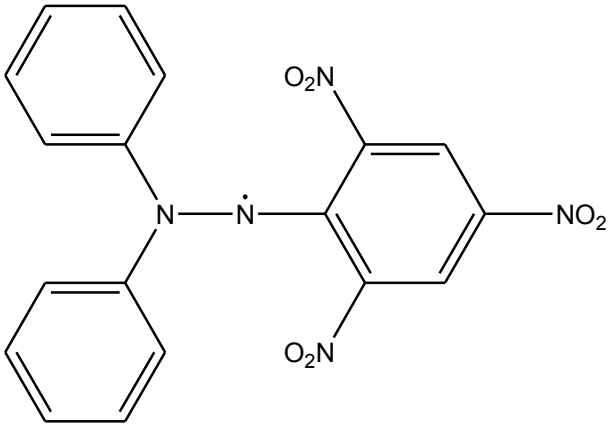


Figure 24: Chemical structure of DPPH.

It is a dark-color powder that due to the strong absorption at 520 nm, the DPPH free radicals has violet color in solution and became colorless or yellow (figure 25) when is neutralize by compound with radical scavenging activity, with a reaction t that is shown in figure 26.

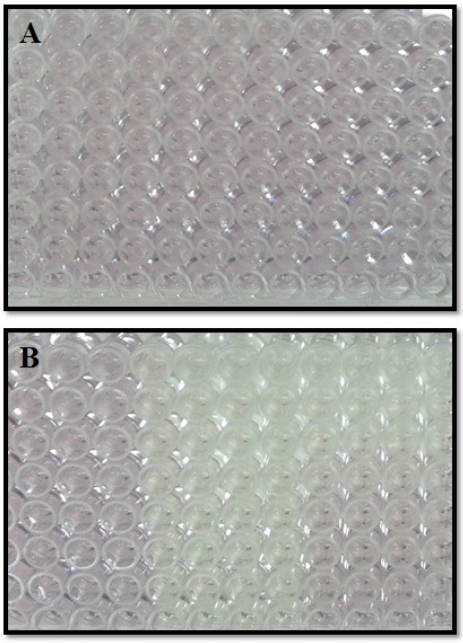


Figure 25: (A) DPPH solution became (B) colorless or yellow in presence of compound with radical scavenging activity.

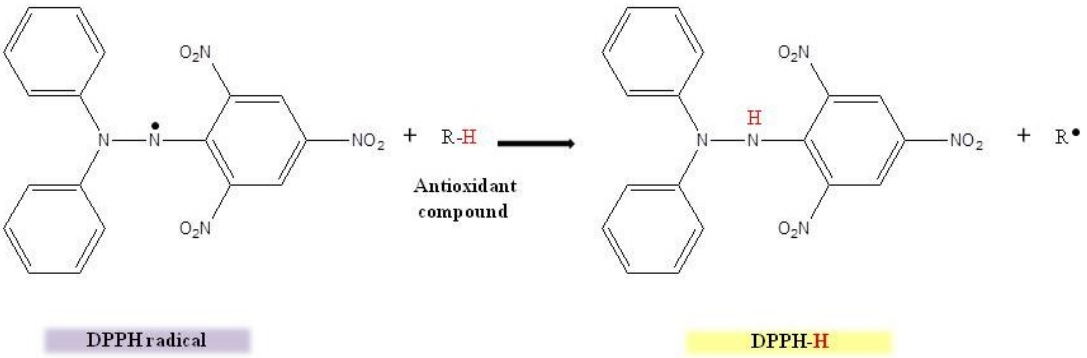


Figure 26: DPPH reaction with an antioxidant compound.

In brief, different concentrations of resveratrol and budesonide either in single or combination formulation were added to the same volume of DPPH ethanolic solution (60 mM) [124]. Samples were stored in the dark at room temperature for 30 min and the intensity of DPPH radical's absorbance was measured at 520 nm. The same concentrations of α-Lipoic acid and L-NAME were used as negative controls, while ascorbic acid was used as the positive control.

4.7 Stability of polyphenolic compound in cell culture media

Before starting the in vitro experiment, the chemical stability of resveratrol was evaluated in completed DMEM:NMF-12 media.

Briefly, resveratrol solution was prepared by dissolving in ethanol and diluting in the media with a final concentration of resveratrol of 100 μM. Ethanol concentration was kept to less than 1%, in order to maintain cell viability. The samples were incubated in culture condition (37 °C, humidified atmosphere and 5% CO_2) for 72 h. At different set time points (0, 6, 24, 48 and 72 h) samples were collected and the resveratrol content was quantified by HPLC. The stability of resveratrol was expressed as the remaining of resveratrol after incubation at different time points.

4.8 Cytotoxicity in pulmonary cell lines

4.8.1 Cytotoxicity of resveratrol on Calu-3

The cytotoxicity of resveratrol was assessed by measuring the viability of Calu-3 cells after exposure with increasing resveratrol concentrations (from 1.25 nM to 160 μM). Following

incubation with different concentrations of resveratrol and the addition of CellTiter 961 Aqueous assay (MTS reagent, Promega, USA) the absorbance was measured at 490 nm using a plate reader (Wallac 1420 VICTOR2TM, Multilaber Counter, Massachusetts, USA). Cell viability was calculated with reference to the untreated cells and the absorbance values were directly proportional to cell viability.

4.8.2 Single and co-spray dried resveratrol and budesonide formulations cytotoxicity using alveolar macrophages

To evaluate the suitability of delivering resveratrol and budesonide into lungs, a *in vitro* MTS cytotoxicity assay was conducted. Alveolar NR8383 macrophages were seeded onto 96-well plates at a density of 50,000 cells/well and incubated at 37°C in a humidified atmosphere at 5% CO_2 overnight. This was followed with addition of drugs (resveratrol and budesonide) with increasing concentrations, from 0.612 µM to 50 µM, either as single or combined formulations. After 72 h of incubation, 20 µl of CellTiter 96® Aqueous assay (MTS reagent) was added into each well and incubated for 4 h. The absorbance of the samples were measured at 490 nm with microplate reader (Wallac 1420 VICTOR^{2TM}, Multilaber Counter, Massachusetts, USA).

4.9 Cellular uptake

After reaching 80–90% confluency, the A_{549} cells were subcultured into 6-well plates and treated with serum-free Ham's F-12medium containing plain or microencapsulated quercetin (5 µM), at 37 °C in a humidified atmosphere of 5% CO_2. Free quercetin was added as a 250 µl aliquot of a freshly prepared stock solution in ethanol (final ethanol concentration <0.5%). The flavonoid-loaded microparticles were dispersed in the medium. After incubation for specified times up to 4 h, the medium was removed and the cells monolayer washed with 2 ml of ice-cold phosphate buffer and lysed with RIPA buffer. Cells lysates and samples from the extracellular medium were filtered and analyzed by HPLC. The results were the average of at least six experiments.

4.10 Deposition and transport of spray dried resveratrol on Calu- 3 cell lines

Calu-3 cells were seeded on Snapwells polyester membrane (0.4 mm pore size, 1.12 cm^2 surface area) (Corning Costar, Lowell, MA, USA) and maintained in air-interface configuration for 17–19 days. Spray dried resveratrol was deposited on Calu-3 cells grown on Snapwells using a modified ACI according to the method described by Haghi et al. [125]. Briefly, a capsule containing the spray dried powder was placed in the RS01 device. The cells were placed on stage 3 of the ACI (cut off diameter 2.1–3.3 μm) and spray dried resveratrol was deposited at flow rate of 60 l/min for 4 seconds. The Snapwells were transferred to a 6-well plate containing Hank's balanced salt solution (HBSS). Sampling of the basal chamber was conducted at set time points (30, 60, 120, 180, 240 min).
At the end of the experiment, the surface of the Calu-3 cells was washed to quantify the residual apical drug and intracellular content of resveratrol was analysed according the cell lysis method using CelLyticTM reagent.
The experiments were conducted in triplicate and all samples analysed using HPLC.

4.11 Resveratrol *in vitro* activities using Calu-3pulmonary cell lines

4.11.1 Anti-inflammatory activity

Anti-inflammatory effects of spray dried resveratrol using modified ACI plates The anti-inflammatory activity of resveratrol was studied after deposition of spray-dried resveratrol on Calu-3 cells, using the modified ACI as described by Haghi et al. [125]. The Snapwells were transferred to a 6-well plate and incubated at 37 °C, 5% CO_2 for 24 h. Tumor necrosis factor alpha, TGF-β1 and LPS were added at a concentration of 5 ng/ml and plates incubated for further 48 h to allow for the production of inflammatory cytokine, IL-8. Samples of the culture medium were analysed for IL-8 using Human IL-8 ELISA Kit II BD OptEIA^{TM} according to the manufacturer's instructions.

4.11.2 Anti-oxidant activity of spray dried resveratrol: DAN assay

The cells were treated with TGFβ-1 and LPS 100 ng/ml for 48 h and then with resveratrol 100 μM (final concentration) for further 24 h. The amount of NO produced from Calu-3 cells was measured with 2,3-diaminonaphthalene (DAN). Ascorbic acid and L-NAME at

100 μM (final concentration) were used as positive and negative control, respectively, according to the method described by Choi et al. [126].

Serial concentrations of nitrite (0.19–25 μM) were prepared as standard. Based on the fluorescence intensity (excitation = 360 nm and emission = 430 nm), the amount of NO detected from Calu-3 after treatment with either resveratrol, ascorbic acid, lipoic acid or L-NAME was calculated against the standard curve.

4.12 *In vitro* activities using alveolar macrophages cell lines

4.12.1 Anti-oxidant activity of single and co-spray dried formulations

The ability of single and co-spray dried formulations to reduce the production of NO in LPS-induced alveolar macrophages were evaluated using DAN assay in accordance to the method described previously. Briefly, alveolar macrophage cells were treated with 5 ng/ml LPS for 24 h. This was followed with treatment of single and co-spray dried formulations with different concentrations. Samples were collected at different time points (12 h to 72 h).

4.12.2 Anti-inflammatory activity of single and co-spray dried formulations against LPS-induced alveolar macrophages

The anti-inflammatory effects of spray dried formulations were evaluated using a LPS-induced alveolar macrophages cell study. Approximately 500,000 cells/well were seeded into 6 well plates with 5 ng/ml of LPS before incubation for 24 h at 37°C, with 5% CO_2. The cells were subsequently treated with the drugs concentration of 50 μM, at different incubation time points. At different time points, the samples were harvested by centrifugation at 13,000 rpm at 4 °C for 5 min. A clear supernatant was kept in –80 °C. Cytokine expression (IL-6 and TNF-α) was measured using Rat IL-6 and TNF-α ELISA kits according to the manufacturer instructions.

4.13 Statistical analysis

One-way ANOVA or unpaired 2-tailed t-tests were performed to determine the significance (which was quoted at the level of $p < 0.05$) between treatment groups and control.

5. RESULTS AND DISCUSSIONS

SECTION A

INHALABLE DRY POWDER BASED ON QUERCETIN-LOADED SOLID LIPID MICROPARTICLES FOR PULMONARY DELIVERY

5.1. Scientific background and research aim

Quercetin has been shown to have several beneficial pharmacological properties against different diseases due its ant-oxidant activity and specifically to pulmonary pathologies, published *in vitro* and *in vivo* studies have demonstrated that this flavonoid had a good properties as potential compound that could be potentially useful for asthma treatment [127,128]. Quercetin is able to inhibits the production of reactive oxygen species and decrease the induction of pro-inflammatory cytokines and chemokines in human alveolar epithelial A_{549} cells [129,130]. Moreover, previously studies reported that *in vivo* administration of quercetin decrease the allergic airways inflammation and asthmatic reactions including airway resistance, mucus production in the lung, recruitment of leukocytes and eosinophils to the bronchoalveolar lavage fluid in murine and guinea-pig models of asthma [131,132]. However, the therapeutic application of quercetin is hampered by its low biovaibility due to the extensive metabolism, poor cell permeability and high chemical instability in physiological fluids [127,133,134].
For this reason, the encapsulation of quercetin into micro- and nano-particles has been reported [68,128,135]. Specifically, SLM represent a suitable carrier to delivery the flavonoid directly to the lung thereby increasing its concentration at the site of action [99]. Lipid microparticles, consisting of a lipid core stabilized by surfactants, represent an optimal delivery system for inhalation, since they are based mainly on physiologically compatible and biodegradable constituents and are characterized by adequate physico-chemical stability. Furthermore, *in vitro* and *in vivo* published studies have demonstrated that lipid particles do not induce inflammatory responses in the lung tissue [136].

This study was aimed on the preparation, characterization and in vitro activity of the inhalable dry powder based on quercetin loaded-SLM for pulmonary delivery.
The morphology and physico-chemical characterization of the SLM coupled with the entrapment efficiency of quercetin in SLM were evaluated. Moreover, the effect of the flavonoid-loaded SLM on the uptake in A_{549} lung alveolar epithelial cells was examined.

5.2 Results and discussion

5.2.1 Preparation of the solid lipid microparticles

A melt emulsification technique was employed to prepare the quercetin loaded SLM, since it circumvents the use of organic solvents and also because has been previously utilized to prepare SLM for pulmonary drug delivery. Based on optimization studies previously performed in the laboratory of Prof. Scalia, the components that achieved the more suitable condition for SLM preparation were tristearin and phosphatidylcholine, as lipid and emulsifier, respectively. The optimized conditions for unloaded and loaded particles are shown below (table 3).

Table 3: Name and amount of components used for produce unloaded SLM and loaded SLM.

		Unloaded SLM	**Loaded SLM**
Component	***Name***	***Amount (g)***	***Amount (g)***
Lipid	Tristearin	4.3	3.6
Active	Quercetin	-	0.7
Surfactant	Phosphatidylcholine	0.7	0.7

Briefly, SLM were prepared by adding preheated (75–85°C) deionized water (50 ml) containing the surfactant (1.4%, w/v) to the molten lipid phase (3.6 g), in which quercetin (0.7 g) was dispersed. The hot aqueous phase was poured into the molten lipid (phase-inversion process) to avoid loss of excipient and drug during the preparation process. The mixture was then subjected to high-shear mixing at 21500 rpm for 2 min using an Ultra-Turrax T25mixer at 75–85°C. The sample was also sonicated at 22 kHz for 2 min using an ultrasonic probe. The obtained emulsion was rapidly cooled at room temperature under magnetic stirring.

The formed suspension was subjected to centrifugation (6000 rpm for 15 min) and lyophilization to obtain water-free microparticles. Unloaded particles were also prepared with the same procedure, by omitting quercetin.

5.2.2 Solid lipid microparticles characterisation

The quercetin content of the microparticles and the encapsulation efficiency were 11.8 ± 0.3% (w/w) and 72.4%, respectively.

In order to charactherise the microparticles in term of both, morphology and release, different studies were performed.

5.2.2.1 Optical microscopy and scanning electron microscopy

Preliminary analysis of the microparticles by optical microscopy indicated a particle size between ca.1 and 30 µm, with the majority of the population (more than 70%) in the 1–5 µm range (figures 27 and 28) that indicate an appropriate size to be delivery to the lungs.

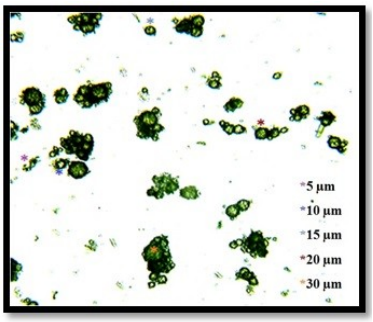


Figure 27: Optical microscopy image of SLM loaded with quercetin.

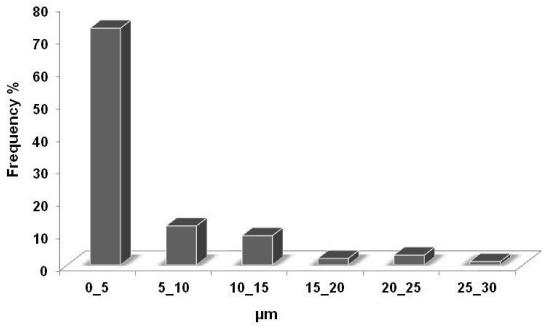


Figure 28: Percentage of SLM loaded with quercetin respect to the size diameter of microparticles.

Moreover the morphology of microparticles was observed using the SEM (figure 29) which indicated that the microparticles had a rather spherical shape and a size suitable to inhalation. However, some quercetin crystal not encapsulated were also observed.

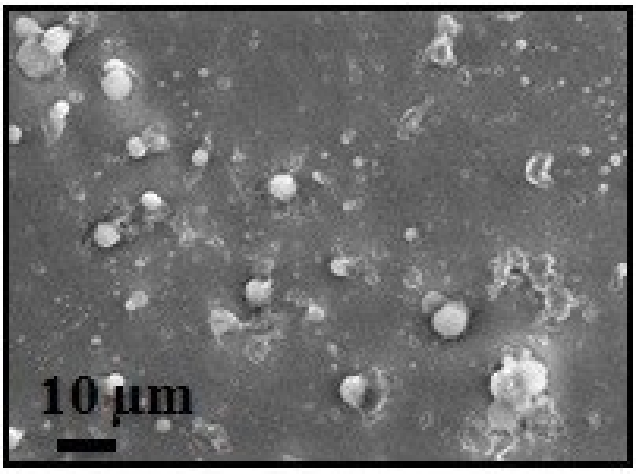


Figure 29: Scanning electron microscopy image of SLM loaded with quercetin.

5.2.2.2 *In vitro* release

To study the entrapment effectiveness of SLM, the *in vitro* release studies were performed. The quercetin release was calculated as a percentage of the amount of quercetin release in each time point with respect to the total quercetin content of SLM samples and the data are shown in figure 30.

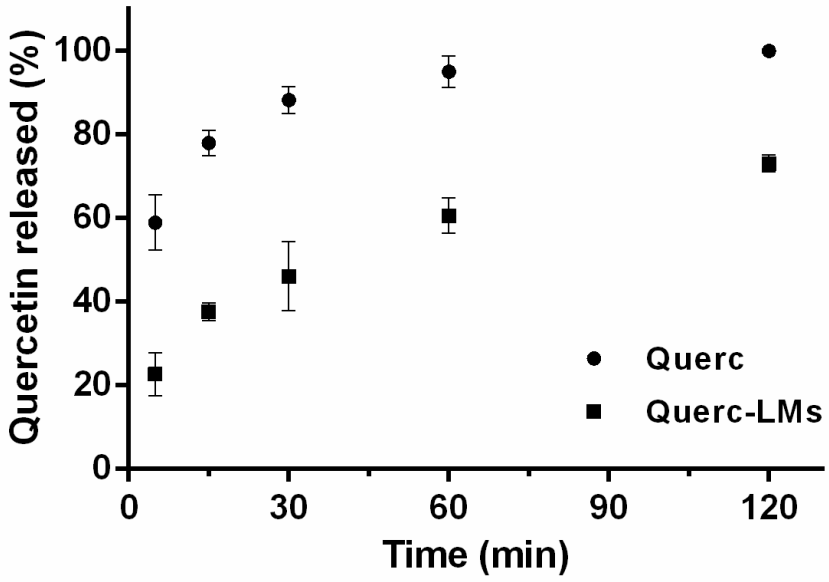


Figure 30: Quercetin dissolution and release profiles from SLM. Data represent–mean ± SD (n = 5).

The flavonoid release profiles show that the SLM produced a quercetin release significantly lower ($p < 0.05$; ANOVA and Tukey's post test) than the dissolution of the pure flavonoid drug (figure 30). This indicated that quercetin was encapsulated in the lipid matrix, the diffusion through this matrix producing an efficient modulation of quercetin release.

5.2.3 Cellular uptake

In order to investigate if the SLM are able to influence the in vitro activity of quercetin, alveolar epithelial cells A_{549} were selected for the study and the uptake of free and microencapsulated quercetin by lung alveolar A_{549} cells was investigated.

Solid lipid microparticles loaded with quercetin were administered to cultured lung alveolar A_{549} cells and compared to a flavonoid solution of identical drug level (5 μM). The quercetin concentration used was selected based on that needed for anti-inflammatory activity in vitro. Moreover, the A_{549} cells were incubated under conditions that did not affect cell viability as reported in a study of our group [71].

In brief, free or microencapsulated quercetin was added to the cell culture system and the concentrations of flavonoid present in the Ham's F-12 medium and taken up by the A_{549} cells were determined at different time points, up to 4 h and the results are shown in figures 31 and 32.

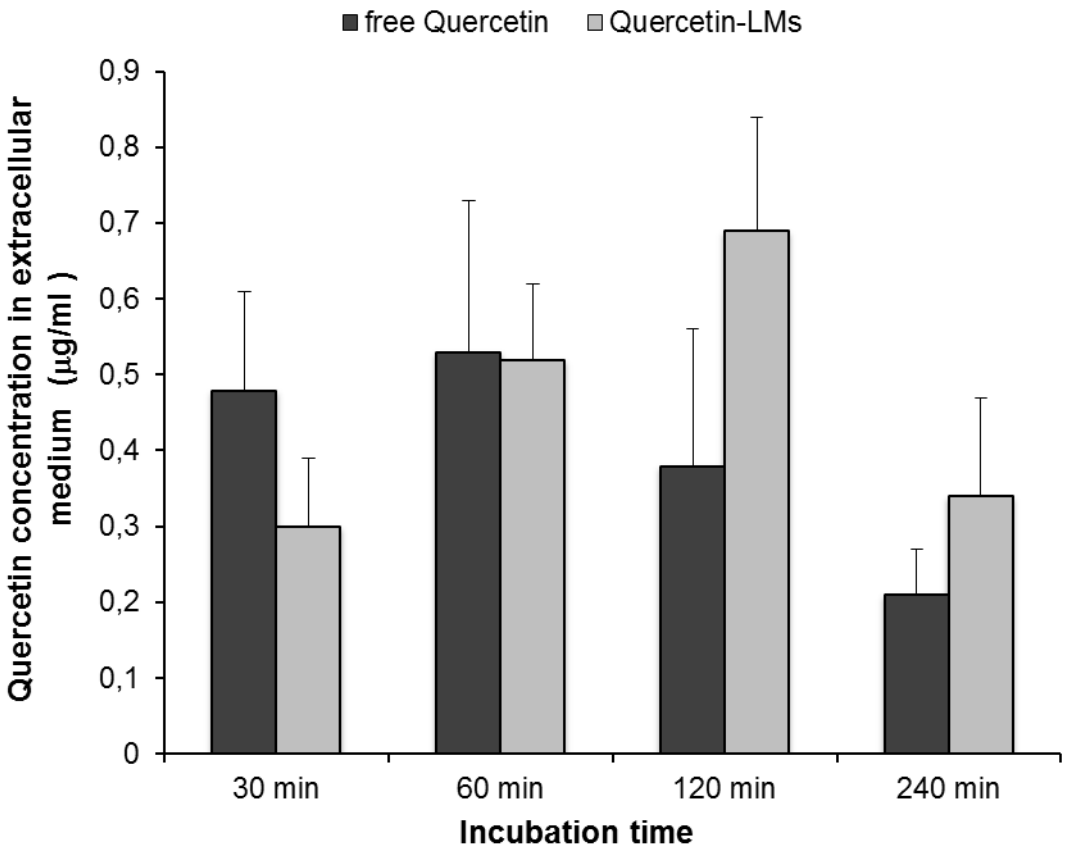


Figure 31: Time dependent quercetin concentration in extracellular medium of A_{549} lung alveolar cell culture. Data represent-mean ± SD (n = 6) [71].

The flavonoids concentrations remaining in the extracellular culture medium during incubation are depicted in figure 31. Following addition of plain quercetin to the cell culture, the flavonoid level in the medium decreased to less than 50% of the initial quercetin content after 4 h (figure 31). When the A_{549} cells were exposed to the medium containing quercetin entrapped in the SLM, the flavonoid level increased up to 2-h incubation and then decreased to about the same concentration measured at the beginning of the experiment (figure 31). During incubation with the culture medium containing the flavonoid-loaded SLM, the amount of quercetin accumulated into the cells increased over time (figure 32). Initially, the level of flavonoid diffused in the cells was lower for the microencapsulated quercetin (although the difference was not significant; ANOVA and Tukey's post test), demonstrating the gradual release nature of the LM system. However, after 4-h incubation, the quercetin levels found into the A_{549} cells were significantly higher ($p < 0.01$; ANOVA and Tukey's post test) for the microparticle entrapped flavonoid, the

cellular uptake of quercetin being more than 2.3 times greater than that of the non-encapsulated flavonoid (figure 32).

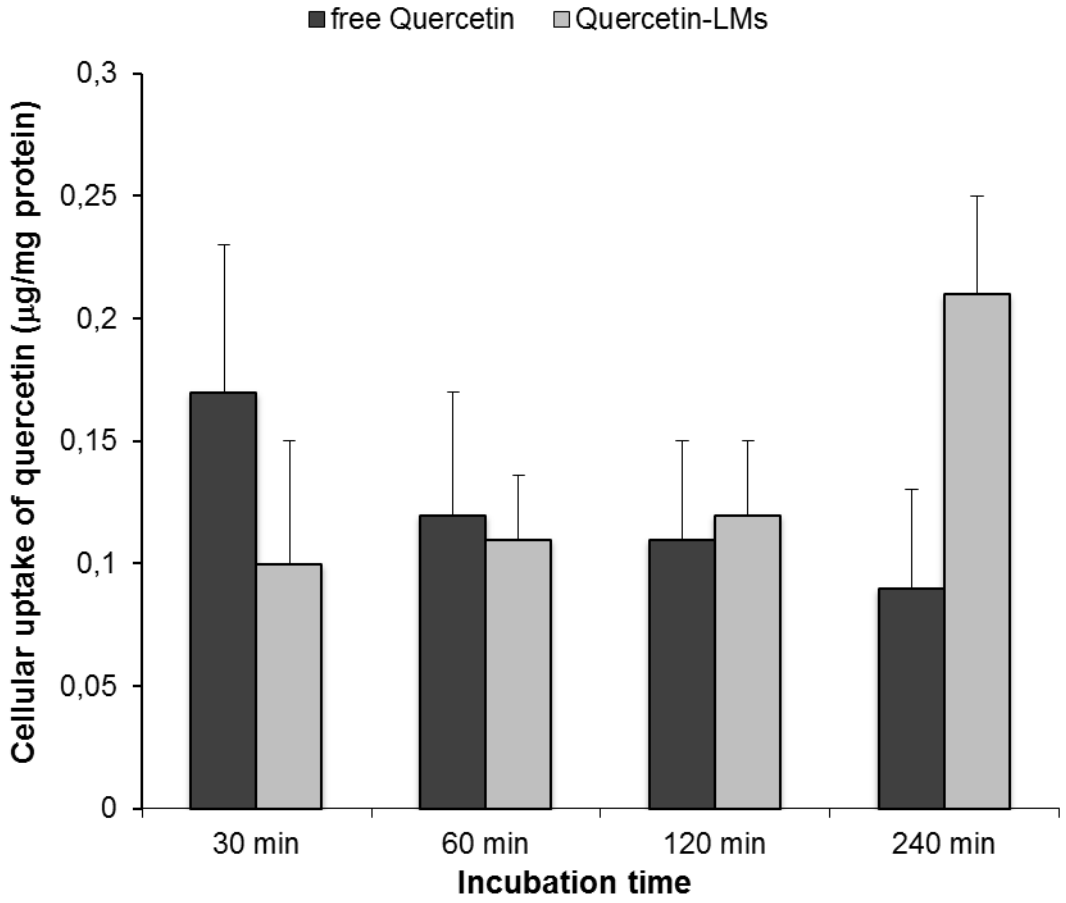


Figure 32: Time dependent uptake of quercetin by A_{549} lung alveolar cells. Data represent mean ± SD (n = 6) [71].

The data from the cell permeability experiments demonstrated that quercetin-loaded SLM influenced the rate and efficiency of the uptake of the flavonoid by the A_{549} lung alveolar cells (figures 31 and 32). More specifically, the intra-cellular delivery of quercetin was significantly increased when the flavonoid was encapsulated in the SLM (figure 32). This effect can be explained on the basis of the sustained-release properties of the SLM, which reduced the exposure of the drug to the oxidative environment compared to the free flavonoid that is immediately available. Therefore, the enhanced accumulation in the A_{549} cells of encapsulated quercetin compared to its free form, is likely to be due to differences in cell culture medium concentrations over time (figure 32), resulting from the protective effect of the SLM. Another possible mechanism for the observed greater intracellular

delivery of quercetin by the SLM could have been attributed to a more effective interaction between the encapsulated flavonoid and the cell surface.

The surfactant (phosphatidylcholine) used for the preparation of the SLM may contribute to increased cell permeability, via partition with the membrane bilayer and destabilization of its ordered structure[64,71]. However, this hypothesis was ruled out, since unloaded SLM did not affect the uptake of quercetin by the A_{549} cells [64,71].

5.3 Conclusions

Solid lipid microparticles obtained by melt emulsification technique represent an efficacy strategy to delivery quercetin directly to the lungs. Indeed, SLM exhibit both, particles morphology and size suitable to lung delivery. The protective activity of the SLM formulation on the flavonoid stability in the A_{549} cellular medium under atmospheric oxygen was reflected in the enhanced uptake of microencapsulated quercetin by the alveolar epithelial A_{549} cells. This effect should improve the flavonoid cellular activity.

The data obtained in this study suggest that SLM could represents a promising system for inhalation delivery of quercetin as a new potential therapeutic approach for the treatment of airways inflammatory diseases.

6 RESULTS AND DISCUSSIONS

SECTION B

INHALABLE RESVERATROL SPRAY DRIED FORMULATION TO IMPROVE THE TREATMENT OF CHRONIC INFLAMMATORY LUNG DISEASES

6.1 Scientific background and aim

Chronic obstructive pulmonary disease is characterised by chronic inflammation, bronchoconstriction, airflow limitation and mucus hyper secretion. Current treatment for COPD is symptomatic and does not inhibit the progression of the disease or restore normal lung function [37]. The pharmacotherapy of COPD includes inhaled β_2-agonists, inhaled anticholinergics and inhaled corticosteroids (e.g., beclometasone, budesonide) [10, 138-142]. In several cases, COPD airway inflammation becomes refractory to corticosteroids and the therapy fails [143]. Therefore, development of novel efficient therapies for the treatment of COPD is essential to improve patients' quality of life.

Oxidative stress is one of the components involved in the pathogenesis of airways inflammatory diseases, such as COPD [144]. Furthermore, oxidative stress can change normal cell signalling pathways. One instance is the reduction in expression and activity of histone deacetylases ($HDAC_2$) in COPD. These conditions are worsened in smokers, since several studies in literature have demonstrated that oxidative stress and cigarette smoke increase histone acetylation, which leads to increased expression of inflammatory genes [143,144]. The inflammation caused by these agents results in the release of reactive oxygen species. Hence, oxidative stress can be an important pharmaceutical target for the treatment of COPD and antioxidant compounds could have the potential to restore the responsiveness to corticosteroids [144].

Resveratrol exhibits potent anti-oxidant and anti-inflammatory properties and has shown potential against different diseases [145-150]. Specifically, in lungs it has been shown that this compound is capable of scavenging oxygen-derived free radicals and therefore has the potential to be used as a adjunct therapy in the treatment of COPD [79, 81, 151-153].

It has also been shown that polyphenolic compounds, such as resveratrol, are candidate molecules for the development of novel anti-inflammatory therapies for airway diseases, especially when patients become non-responsive to corticosteroids [81,152].

The delivery of resveratrol as solution is not suitable due to its instability, propensity for rapid oxidative degradation in water and to its low solubility in water [154], for this reason the dry powder could be a suitable formulation to delivery the polyphenol directly to the lung.

The aim of this study was to investigate the potential of resveratrol as inhalable powder formulation and its anti-inflammatory and anti-oxidant activity in Calu-3 bronchial cell

lines. The inhalable microparticles were produced using the spray drying technique. The physicochemical characteristics of spray dried resveratrol formulation were investigated and the aerosol performance evaluated using ACI. After that, the deposition, transport and cell uptake of DPI resveratrol were investigated using the Calu-3 cell line incorporated onto a modified ACI. Finally, the anti-inflammatory and anti-oxidant activities of resveratrol on Calu-3cell were studied.

6.2 Results and discussion

6.2.1 Physicochemical characterisation of raw and spray dried resveratrol

In this study, resveratrol was manufactured as dry powder for inhalation to be delivered directly to the lung for the reduction of inflammation and oxidative stress. Particle size distributions of both, raw and spray dried resveratrol, was analysed using laser diffraction and the results are shown in figure 33.

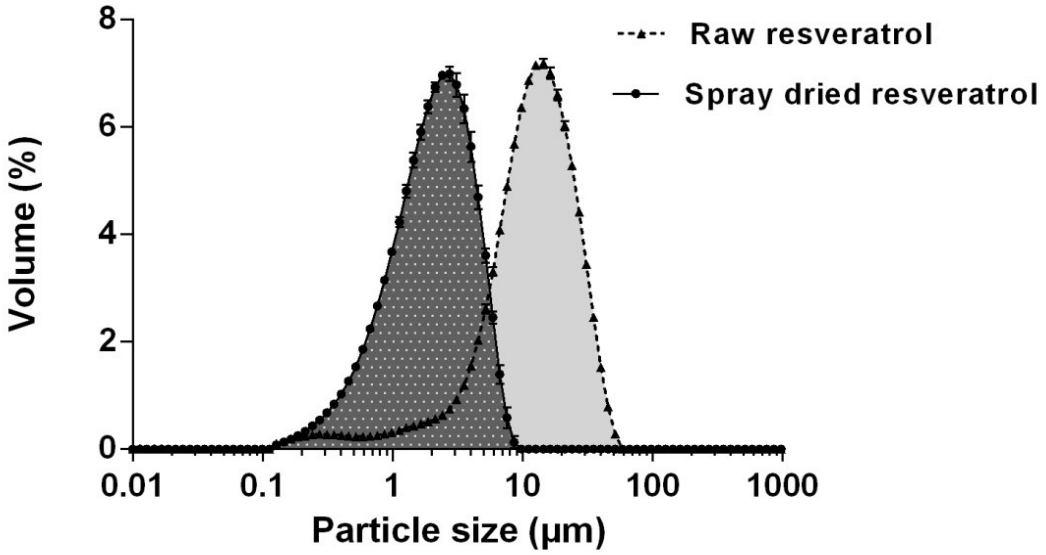


Figure 33: Particle size distribution of raw and spray-dried resveratrol. Data represent mean ± SD (n=3).

Analysis of the data showed that the size distributions for both samples varied significantly. Indeed, values of median volume diameters of 13.3 ± 0.1 µm and 3.9 ± 1.0 µm were observed for raw resveratrol and spray dried resveratrol, respectively. These results showed that raw resveratrol had a size that did not fall within the respirable range while spray dried resveratrol is suitable for pulmonary administration [155].

Add to this, the SEM images of raw resveratrol presented a columnar shape with a volume size above 120 µm (figure 34), while, the spray dried resveratrol particles showed a corrugated plate-like morphology (figure 35), with a suitable size for lung deposition (≤ 5 µm).

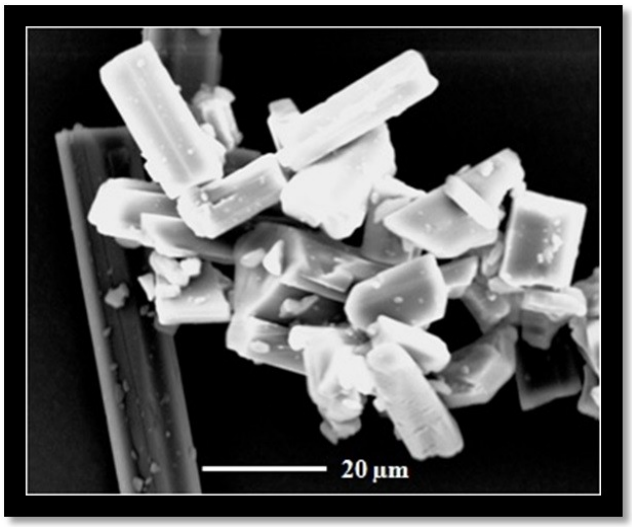


Figure 34: Scanning electron microscopy image of raw resveratrol.

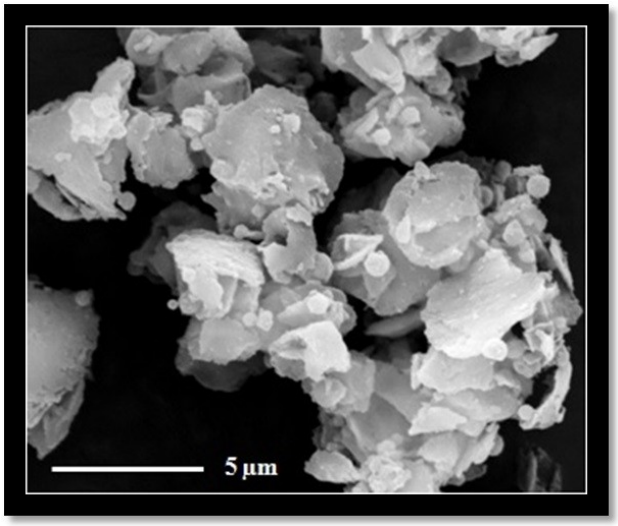


Figure 35: Scanning electron microscopy image of spray dried resveratrol.

Io order to study the influence of heat flow on the changes in the dry particle system, the thermal response of both raw and spray dried resveratrol was investigates using DSC and the results are shown in figure 36.

A single sharp endothermic peak at 270.0°C was presented and was attributed to the melting of raw resveratrol [156]. The absence of any exothermic peaks prior to the melting peak suggested that no phase transition from amorphous to crystalline did occur; thus indicating that the raw resveratrol exists as a crystalline material.

Similarly, spray dried resveratrol showed a melting peak at 267.3°C, with no exothermic events in the low temperature region prior to this peak, suggestive the spray dried sample was crystalline.

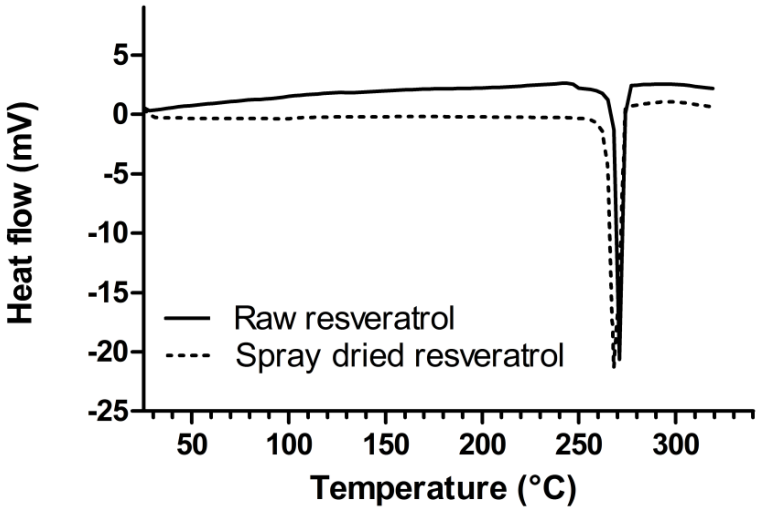


Figure 36: Differential scanning calorimetric thermographs of raw and spray dried resveratrol.

6.2.2 *In vitro* aerosol performance

The aerosol performance of spray dried resveratrol dry powder was studied using the ACI. Data presented are the percentage of the total drug deposited in device, throat and each stage of ACI over the emitted dose and are shown in figure 37.

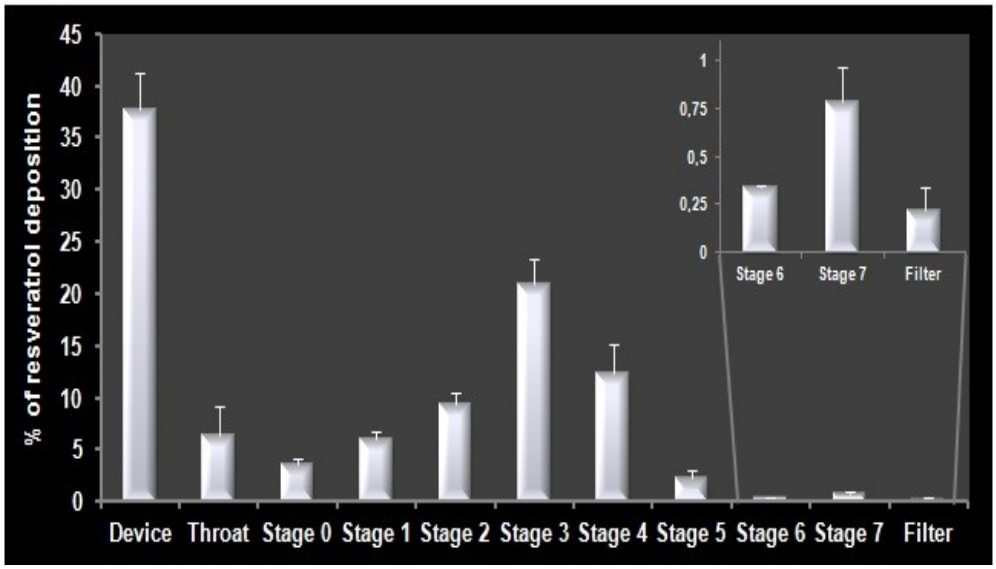


Figure 37: *In vitro* aerosol deposition of spray dried resveratrol using the ACI and RS01 dry powder inhalation device at 60 l/min for 4 seconds. Data represents mean ± SD (n=3).

The mass median aerodynamic diameter (MMAD) of the particle was 3.7 ± 0.1 μm, with a geometric standard deviation (GSD) of 1.3. The total recovery of resveratrol was 105.9 ± 4.7% of the loaded dose, falling within the acceptable pharmacopeia range of 100 ± 25% [157]. The FPD and FPF were calculated to be 2054.6 ± 191.0 μg and 39.9 ± 1.1%, respectively. These data demonstrated that spray dried resveratrol powder had efficient aerosol performance, most likely due to the particles' corrugated surface which reduces the cohesive forces between particles, thus facilitating aerosolization of DPI.

6.2.3 Biological responses using Calu-3 cell lines

6.2.3.1 Stability of resveratrol in cell culture media

Before performing biological tests, resveratrol was studied with respect to its chemical stability in cell culture media in order to eliminate the possibility of resveratrol inactivation or degradation during cell experiments.

Data obtained for resveratrol stability in cell culture media are shown in figure 38. The data indicated that resveratrol was stable during early incubation, in which the amount of resveratrol present at 24 h was 86% of the initial level. Following incubation to 48 h and 72 h, the amount of remaining resveratrol was 77.19% and 57.15% of the initial concentration, respectively (figure 38).

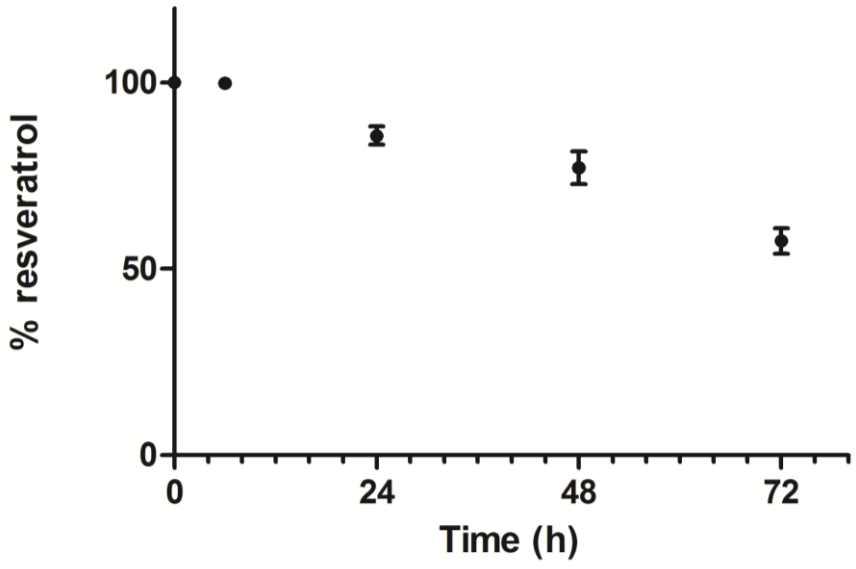


Figure 38: Time course of resveratrol concentration during incubation in completed DMEM:NMF-12 media. Data represents mean ± SD (n=3).

These results demonstrated that the stability of resveratrol was adequate in culture medium containing serum, with 50% of degradation after three days of incubation.

6.2.3.2 Cytotoxicity profiles of resveratrol

The dose response viability profile of resveratrol for Calu-3 cells is shown in figure 39. The MTS assay demonstrated that the wide range of resveratrol concentrations used, from 1.25 nM to 160 μM, were well-tolerated and non-toxic to Calu-3 cells. The viability of Calu-3 cells was maintained above 95% for this concentration range (figure 39).

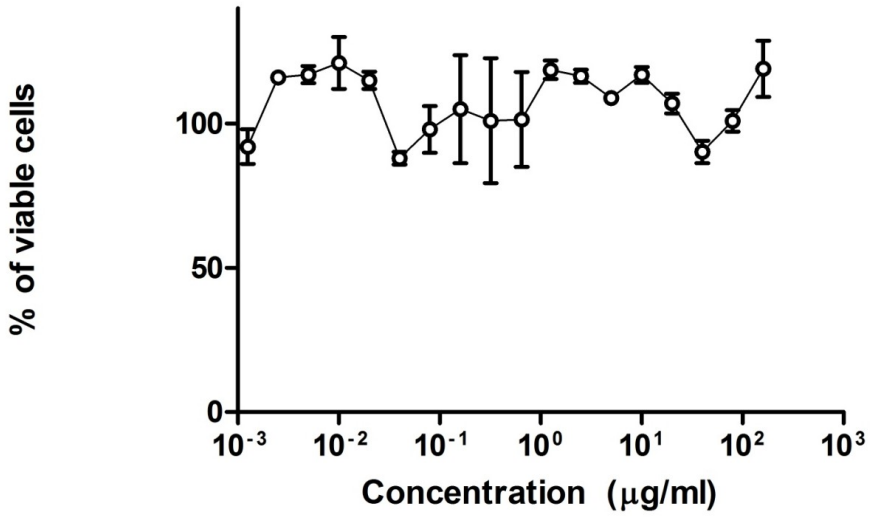


Figure 39: Calu-3 cell viability profile after 72h resveratrol treatment. Data represents mean ± SD (n=3).

6.2.3.3. Resveratrol transport across Calu-3 cell line using spray dried formulation

The in vitro transport of resveratrol across Calu-3 cell monolayer was investigated by deposition of spray-dried resveratrol directly onto Calu-3 cells using a in vitro lung model (modified ACI) and the data are shown in table 4. The values are presented as the percentage of total drug deposited (sum of resveratrol transported from the apical to the basal chamber, remaining on the cells surface and retained within the cells).

Results shown that more than 80% of resveratrol was transported across Calu-3 cells within 4 h of deposition, and the rate of transport was independent of the initial deposited amount.

The high resveratrol transport rate across cell monolayer could be correlated to the presence of fatty acids on the cell membrane that provide a lipophilic environment to improve the binding efficiency towards resveratrol.

Table 4: *In vitro* deposition and transport of resveratrol across Calu-3 cell monolayer. Data represents means ± SD (n=3).

% of resveratrol	Dose of spray dried resveratrol	
	0.5 mg	**1 mg**
Transported across Calu-3 monolayer	82.31 ± 15.49	87.18 ± 7.81
On the cell monolayer	1.86 ± 1.47	2.97 ± 1.94
Retained inside the cells	15.83 ± 14.47	9.85 ± 6.49

In addition, the data on the transport indicate that resveratrol could also exert its anti-oxidant activity in the blood to scavenge the free radicals and reactive oxygen species present. In a previous study, it was noted that unusually high levels of RNS and radicals were released by peripheral blood neutrophils in smokers' patients that subsequently lead to pathogenesis and development of COPD [144].

6.2.3.4 Anti-inflammatory effects of resveratrol

The anti-inflammatory activity of resveratrol was determinate as its ability to decrease the amount of IL-8 in Calu-3 cells after stimulation of the cells with TGF-β1, TNF-α and LPS. IL-8 levels were measured at 48 h and the results are shown in figure 40. Statistically significant differences were observed between samples from the culture media after 48 h for resveratrol pre-treated cells (p<0.05).

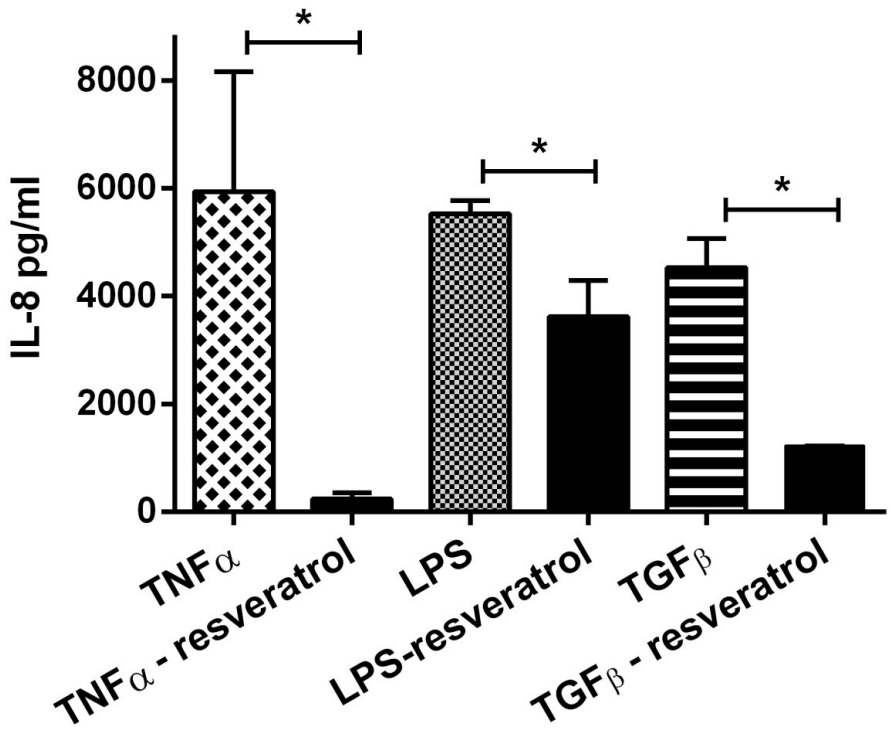


Figure 40: Concentration (pg/ml) of the inflammatory cytokine, IL-8, in culture media after stimulation of Calu-3 cells with TNF-α, LPS and TGF-β1 in presence and absence of resveratrol treatment. Data represents mean ± SD (n=4). * $p < 0.05$.

IL-8 levels were measured to be 4526.2 ± 534.95 pg/ml, 5935 ± 2219.74 pg/ml and 5525.75 ± 250.06 pg/ml after 48 h of stimulation with TGF-β1, TNF-α and LPS, respectively; while the level of IL-8 after 48 h of exposure to TFG-β1, TNF-α and LPS for the cells pre-treated with resveratrol was 1229.67 ± 15.56 pg/ml, 236.33 ± 117.96 pg/ml and 3615.67 ± 676.18 pg/ml, respectively. The results showed that resveratrol exhibited strong anti-inflammatory activity towards TGF-β1, TNF-α and LPS induced Calu-3 cells. More recently, resveratrol has shown the potential to inhibit the release of inflammatory mediators from human airway epithelial cells, which has been proven to be involved in inflammatory lung disease, such as COPD and asthma [81]. Nuclear factor kappa is a key factor for activation of inflammatory proteins expression such as GM-CSF, IL-8, COX-2 and iNOS [158]. It is known that NF-κB activity is regulated by different pathways such as IκB kinase and modification of p65 subunit of NF-κB complex [159].

The reduction of IL-8 obtained in this study indicated that resveratrol could potentially inhibit the activation of NF-κB. Donnelly *et al.*, reported that the reduction of IL-8 expression from human primary epithelial cells was due to the inhibition of NF-κB activity by resveratrol [81].

Moreover, data obtained during in this study, showed that resveratrol is more potent at suppressing the expression of IL-8 in cells induced with TNF-α, compared to LPS or TGF-β1(figure 40). It has been reported that small amount of resveratrol (5 μM) could inhibit the activation of AP-1 involved in inflammation [160].

6.2.3.5 DPPH radical scavenging activity

The anti-oxidant activity of resveratrol was determined using the *in vitro* DPPH assay by measuring the amount of DDPH radical after incubation for 30 min. In this assay, the free radical scavenging potential of resveratrol was measured in terms of its potential to reduce the concentrations of stable free radical DPPH.

In the presence of hydrogen donating-antioxidant molecules, the odd electron of free radical DDPH was reduced and paired with hydrogen which resulting in a colour change from purple to yellow (a decrease in absorbance). The anti-oxidant activity results were shown in figure 41. Lipoic acid, L-NAME and ascorbic acid were used as a negative and positive control, respectively. The scavenging effect was investigated in the range of concentrations from 0.39 to 100 μM.

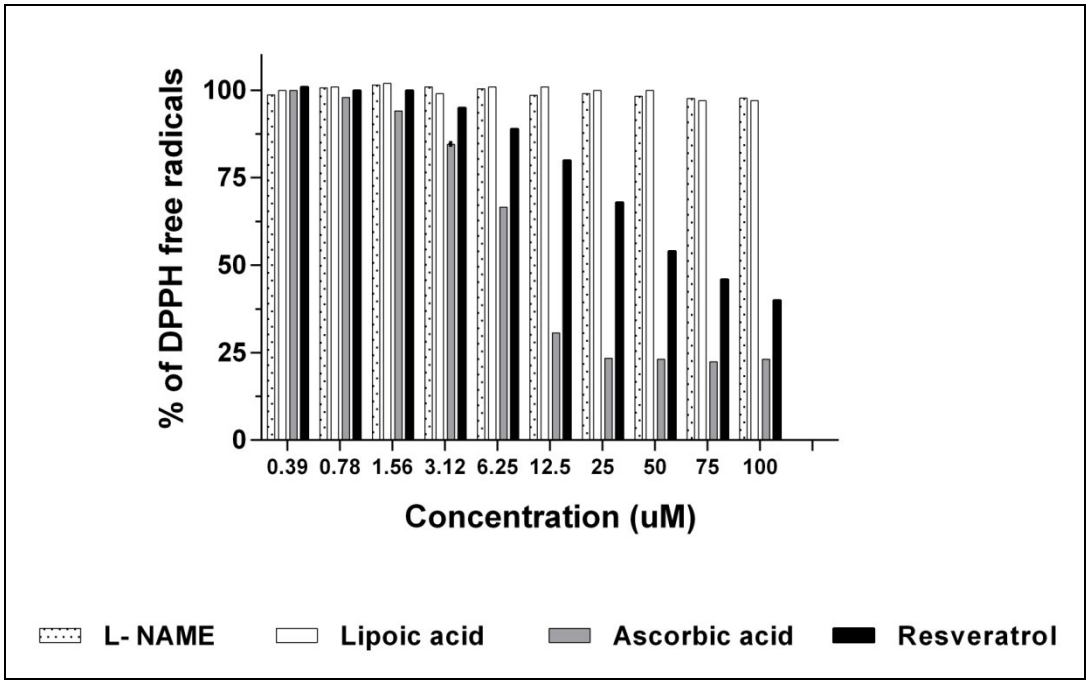


Figure 41: Antioxidant activity of resveratrol compare to L-NAME, lipoic acid and ascorbic acid. Absorbance values are directly proportional to % of DPPH free radicals. Data is represents mean ± SD (n=4).

The obtained results demonstrated that the anti-oxidant activity of ascorbic acid and resveratrol were concentration dependent. No scavenging activity was observed when up to 1.56 μM of these compounds reacted with the DPPH radicals.

Both NAME and lipoic acid compounds did not show any significant anti-oxidant effect as the DPPH level was still maintained above 80% even at high concentrations. The reduction of DPPH radical was determined with resveratrol at relatively low concentration. Resveratrol has been known for its potential role in preventing oxidation damage by cigarette smoke in human lung epithelial cells.

Data demonstrated that this polyphenol molecule efficiently scavenges free radicals (about 50%) at the concentration of 100 μM, and the anti-oxidant activity was dose dependent (figure 41). These results are in good agreement with Soares *et al.,* who reported that 100 μM of resveratrol had the highest anti-oxidant activity [161]. In addition, Acquaviva *et al.* have conducted a detailed anti-oxidant activity of resveratrol whereby resveratrol showed significant inhibition on xanthine oxidase, membrane lipid oxidation and DNA cleavage activities [162]. Additionally, it has been shown that the level of GCL in smoker's airway and COPD patients was considerably lower than healthy and non-smokers, which further suggests that this protein is involved in progression of lung injury via oxidative stress [163,164].

6.2.3.6 DAN assay

Nitrosative stress (contributed by NO production) and nitration of protein in airway epithelial cells have been hypothesized to be the culprit for steroid resistances in COPD [143]. In addition, NO is also involved in vasodilatation, inhibiting platelet aggregation and smooth muscle cell proliferation, which could contribute to pulmonary hypertension. Here, the effectiveness of resveratrol to inhibit the production of NO in Calu-3 induced with LPS and TGF-β1 was studied (figures 42 and 43). In this study resveratrol was shown to effectively inhibit the expression of NO in Calu-3 cells induced by LPS and TGF-β1.

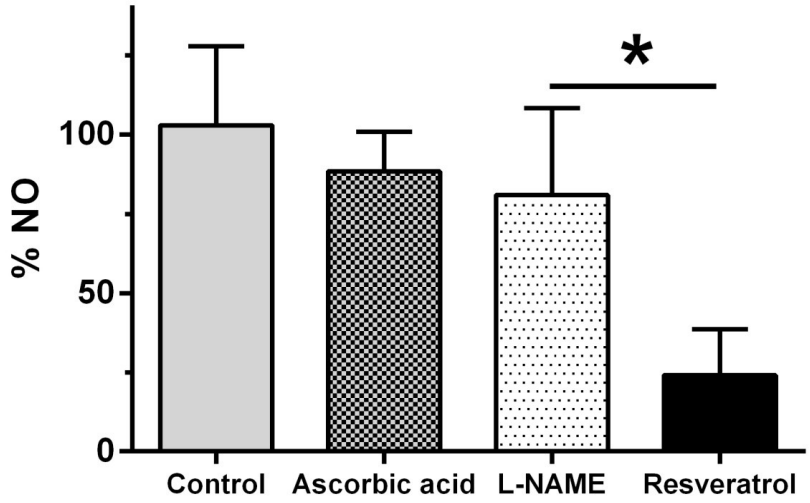


Figure 42: Percentage of NO production in Calu-3 cell after induction inflammation with LPS. Data represents mean ± SD (n=9). * $p < 0.05$.

As observed in the reduction of NO production, NAME and ascorbic acid showed comparatively weak inhibitory effect towards NO production, either in LPS or TNF-α induced Calu-3 cells. However, following resveratrol treatment, significant reductions (p<0.001) in NO production by Calu-3-induced with LPS was observed.

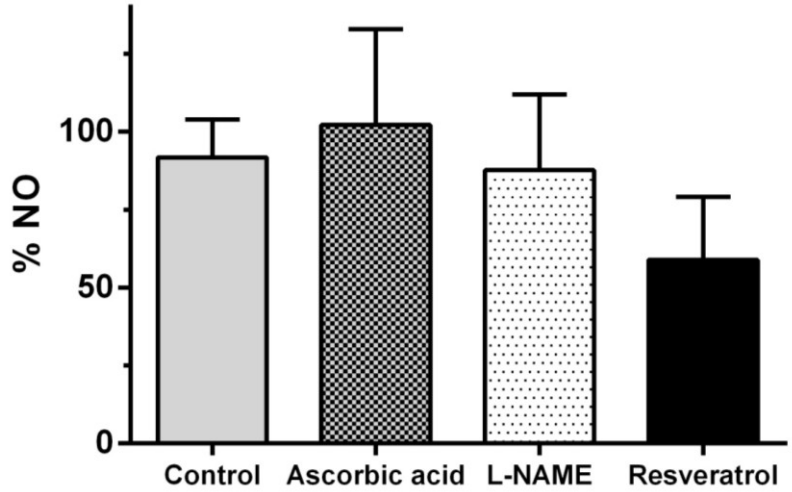


Figure 43: Percentage of NO production in Calu-3 cell after induction inflammation with TFG-β1. Data represents mean ± SD (n=9).

NO was reduced by more than 75.96% and 41.09% when 100 μM of resveratrol was used to treat Calu-3 cell after induction with LPS and TGFβ-1 (final concentration of 100

ng/ml), respectively. A study has previously demonstrated that resveratrol was able to reduce the expression of NO on epithelial cells in the presence of different inducers (cigarette smoke and cytomix) [180]. Furthermore, a study by Bi *et al.*, showed that the inhibition of NO release by resveratrol in microglia cells after stimulation with LPS, was concentration dependent) [85].

6.3 Conclusions

In this study, a inhalable resveratrol dry powder formulation, to be used as alternative or add on-therapy chronic inflammatory lung diseases, was prepared and characterised. Resveratrol was non toxic for the Calu-3 cells in the range investigated and the spray dried microparticles had physico-chemical characteristics and aerosol performance suitable for lung delivery. Moreover, resveratrol was found to have good anti-oxidant and anti-inflammatory properties on Calu-3.

Hence, resveratrol could be of high therapeutic value in diseases like asthma and COPD where inflammation and oxidation is present and the spray dried formulation could be an appropriate formulation to delivery this polyphenol directly to the lungs.

SECTION C

IN VITRO ACTIVITIES OF CO-SPRAY DRIED RESVERATROL AND BUDESONIDE INHALABLE FORMULATIONS IN ALVEOLAR MACROPHAGES

7.1 Scientific background and aim

The pathogenesis of COPD is multifactorial which includes genetic predisposition, age, inhaled pollution and cigarette smoke. Previous studies have shown that cigarette smoke is the main risk factor for the development and progression of COPD [165]. This is because cigarette smoke causes a production of ROS that increase the oxidative stress and for this reason it is implicated in the pathogenesis and in irreversible airway inflammation). Oxidative stress causes airway inflammation by stimulating the release of inflammatory mediators such as IL-6, IL-8 and TNF-α. These inflammatory mediators result in an increase of ROS and hence an increase in oxidative stress in the lungs) [144]. Alveolar macrophages are one of the first lines of defence of the respiratory tract against inhaled noxious agents. In COPD patients, alveolar macrophages play an important role in regulating the release of inflammatory mediators that attract neutrophils into the airway) [166,167]. Corticosteroid molecules are able to suppress the release of these inflammatory mediators in alveolar macrophages but these drugs are relatively ineffective in COPD patients due to the inhibition of the histone deacetylases ($HDAC_2$) activities [143]. For this reason a novel therapeutic strategy is needed. Previous studies have shown that ROS have been implicated in initiating inflammatory responses in the lungs through the activation of transcription factors such as NF-κB [15]. This results in vicious cycle of oxidative stress by ROS and airway inflammation [143,168]. Histone deacetylases activities are required for NF-κB blockade by corticosteroid receptors [144,168]. In several cases COPD patients became non-responsive to corticosteroid treatment as $HDAC_2$ activities can become inhibited in presence of oxidative stress [144].

For these reasons, the use of anti-oxidant compounds in association with one of the corticosteroids drugs could provide a new therapeutic approach for the treatment and management of COPD [169]. Polyphenolic compounds are potential candidate molecules since these compounds naturally exhibit potent anti-oxidant and anti-inflammatory activities.

Specifically in the lungs, *in vitro* and *in vivo* experiments have shown that resveratrol can reduce inflammation in lung cells, scavenging oxygen-derived free radicals; subsequently, resveratrol maybe a potential adjunct therapy in the treatment of COPD [170]. In addition, resveratrol has been shown to inhibit the release of inflammatory cytokines from alveolar macrophages in COPD and therefore can be considered a suitable candidate for pharmacotherapy of macrophages) [171].

For these reasons, the aim of this study was to develop inhalable microparticles containing resveratrol and budesonide, a common anti-inflammatory corticosteroid in order to delivery a combination formulation containing anti-oxidant and anti-inflammatory compounds for improvement of COPD. Different series of co-spray dried formulations were prepared and the physico-chemical characteristics and *in vitro* aerosol performance were investigated. Importantly, the biological responses of alveolar macrophages cell lines in terms of cell viability, anti-inflammatory and anti-oxidant activities were evaluated with the prepared spray dried formulations.

7.2 Results and discussion

7.2.1 Physical-chemical characterization of spray dried formulations

7.2.1.1 Particles size and morphology

The microparticles containing anti-oxidant resveratrol and anti-inflammatory budesonide compounds were formulated using spray drying technique. The inlet temperature range was chosen so that the outlet temperature would be below the glass transition temperature (Tg) of budesonide and resveratrol, which are reported as 90°C and >340°C, respectively. Through the optimization of spray-drying conditions, five formulations with resveratrol (RES) and budesonide (BD) concentration were successfully prepared. These included: 100% BD, 75%BD/25%RES, 50%BD/50%RES, 25%BD/75%RES and 100% RES. Throughout the manuscript, these formulations were denoted as formulation 0%, 25%, 50%, 75% and 100% respectively, in accordance to the percentage w/w resveratrol in the final SD particles.

Table 5: Component, ratio (% w/w), feed concentration, inlet temperature, outlet temperature and flow rate used to produce inhalable microparticles.
RES, resveratrol; BD, budesonide.

Component	Ratio	Feed	Inlet	Outlet	Flow rate
	(% w/w)	concentration (mg/ml)	temperature (ºC)	temperature (ºC)	(ml/min)
RES	100	50	100	52	12.5
RES : BD	75 : 25	50	100	49	12.5
RES : BD	50 : 50	48	100	48	12.5
RES : BD	25 : 75	48	100	46	12.5
BD	100	30	100	46	12.5

As seen from the SEM images presented in figure 45, the microparticles of single spray dried and co-spray dried combinations at different concentrations showed spherical geometries; morphological characteristic typical of a spray-drying process. Uniform spherical microparticles with smooth surface morphology were characteristics of spray dried budesonide alone (figure 44 A). In comparison, spray dried resveratrol alone (100%) exhibited spherical particles with rough surfaces (figure 44E). When co-spray dried, the particles exhibited a change in morphology between the two single drug formulations based on the ratio of budesonide to resveratrol.

Interestingly, overall co-spray dried microparticles exhibited increase in smoothness of particles' surface proportional to the increase of budesonide concentration (figure 44 B, C and D). It is speculated that, as budesonide is more hydrophobic and with higher molecular mass, in the binary system it would preferentially accumulate on the air-liquid interface of the droplet during the spray drying process [172].

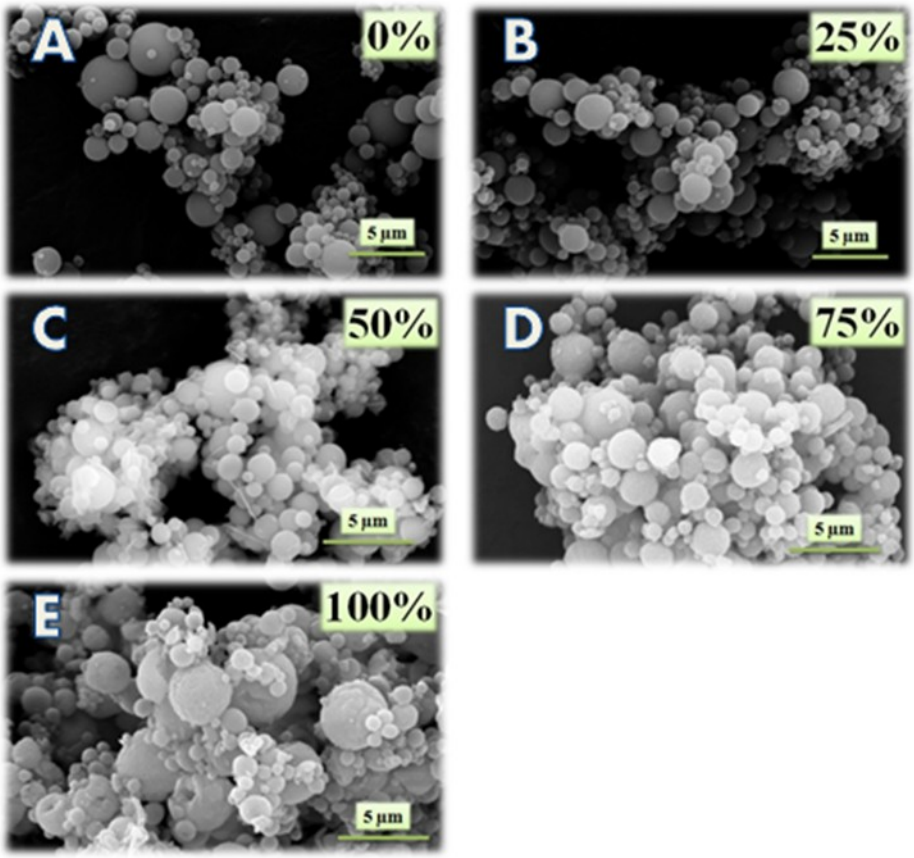


Figure 44: Scanning electron microscopy images of single and co-spray dried formulations containing resveratrol and budesonide at different ratios. The percentages referred to the resveratrol concentration present in spray dried formulations.

Particle size analysis of co-spray dried microparticles were measured using laser diffraction. The volume weighted median diameter (D_{50}) of spray dried budesonide was 1.0 ± 0.01 µm, significantly smaller ($p < 0.05$) than spray dried resveratrol (7.74 ± 1.66 µm). A gradual increase in particle size for co-spray dried formulations with increasing resveratrol concentration was observed. As such, the diameter for co-spray dried containing 25%, 50% and 75% resveratrol were 1.2 ± 0.02, 2.32 ± 1.01 and 6.23 ± 1.32 µm, respectively. Moreover the presence of spray dried budesonide on the surface reduced the cohesiveness between particles of resveratrol, thus resulting in decreased particles' agglomeration and increased aerosol performance[173].

7.2.1.2 Thermal characteristics of spray dried formulations

The thermal response of spray dried formulations containing either resveratrol and/or budesonide is shown in figure 45. Both thermograms of crystalline raw spray dried budesonide and resveratrol demonstrated only a single endothermic peak indicative of melting at 260°C and 270°C, respectively [170,174].

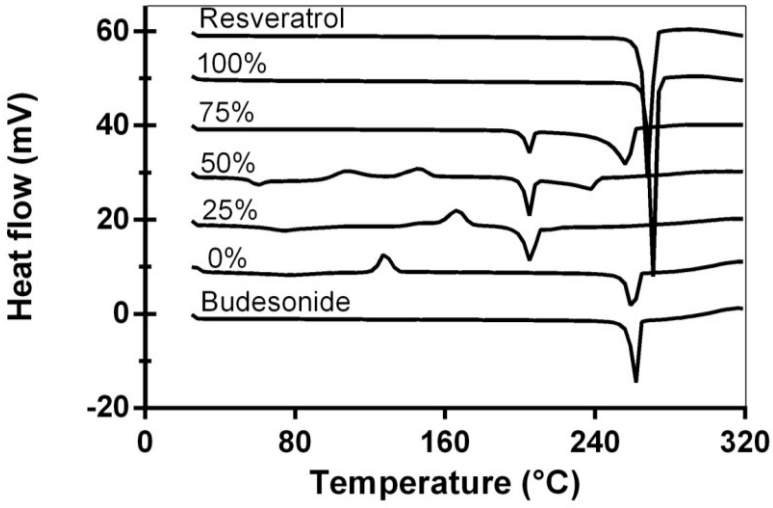


Figure 45: Differential scanning calorimetric thermograms of raw budesonide, raw resveratrol and co-spray dried formulations. The percentages referred to the resveratrol concentration present in spray dried formulations.

Spray drying processes did not change resveratrol crystallinity, as seen in the presence of single endothermic melting at 269°C for spray dried resveratrol alone [170]. The DSC thermogram for spray dried budesonide alone however showed a broad exothermic peak at

130 °C, followed by a sharp endothermic peak at 260°C. The presence of exothermic peak prior to melting suggests that spray dried budesonide particles were amorphous and underwent phase transition from amorphous to crystalline when heated to 130°C. In general, the melting peaks of co-spray dried formulations were shifted to lower temperature. In addition, irrespective of the concentrations of resveratrol and budesonide used, the co-spray dried formulations exhibited a 'new' endothermic peak at 190°C, which was not thermodynamically related to raw resveratrol or budesonide compounds (figure 45). The presence of this peak suggested the chemical interaction between the two compounds which resulted in the shifting of the melting peak proportional to the strong solid–solid interaction) [175]. For instance, for co-spray dried containing 75% resveratrol, two endothermic peaks were observed at 190°C and 257 °C, respectively. In the co-spray dried formulations containing 75% budesonide, an exothermic peak at 167°C and one endothermic peak at 206 °C were also observed.

Further, spray dried formulations were stable up to 200 °C with TGA data reporting a weigh lost ≤0.1% w/w.

7.2.2 Aerosolization efficiency of spray dried formulations

The evaluation of aerosol efficiency for dry powder inhalers is an important tool for predicting the amount of the inhalable microparticles that could reach the lungs. The aerosol performance of single spray dried and the co-spray dried formulations were tested using the MSLI at two different flow rates (60 l/min and 90 l/min). Data are expressed as the percentage deposition of budesonide and resveratrol recovered in each stage of MSLI, throat, and device over the total mass calculated (figure 46).

The FPF values of spray dried budesonide were significantly higher than for spray dried resveratrol at both flow rates (p<0.001). At 60 l/min, the FPF for spray dried budesonide and spray dried resveratrol were 39.4 ± 2.8% and 26.3 ± 1.5 %, respectively. Meanwhile, the FPF of spray dried budesonide (45.7 ± 1.4%) was 2-fold higher than spray dried resveratrol (25.8 ± 4.1%) when the flow rate was set at 90 l/min. Furthermore, spray dried resveratrol had a higher throat deposition (11.5 ± 1.6% and 17.8 ± 0.9%) compared to spray dried budesonide with values of deposition of 3.6 ± 0.9% and 5.0 ± 0.3 % at 60 l/min and 90 l/min, respectively. This could be attributed to the adhesive/cohesive nature of resveratrol and high surface area of the microparticles, which promoted agglomerates; hence the poor dispersion at set flow rates (figure 44 E).

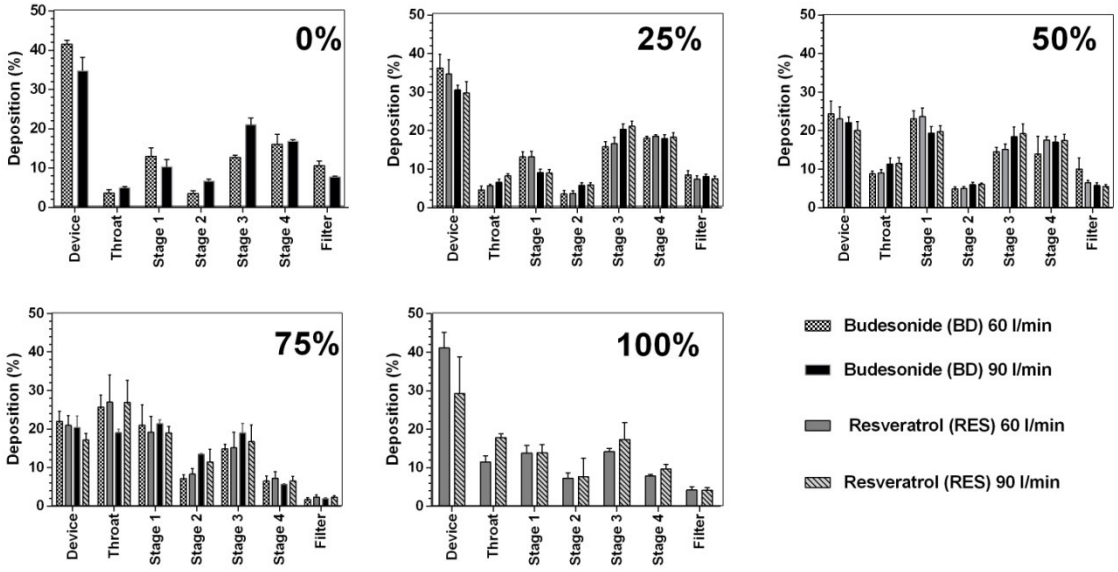


Figure 46: *In vitro* aerosol deposition of the single spray dried and co-spray dried powders at various concentrations (% w/w) of resveratrol using MSLI at flow rate of 60 l/min and 90 l/min. Data represents mean ±SD (n=3).

It was also observed that deposited doses of each component (resveratrol and budesonide) in the throat, devices and all stages of the MSLI had no significant differences, irrespective of the co-spray dried combinations. These results indicate that the co-spray dried particles were homogeneous in composition, rather than binary system containing individual components. Analysis of the deposition profiles of co-spray dried formulations showed an increase in aerosol performance proportional to resveratrol decreasing concentration. The FPF of resveratrol for co-spray dried formulations containing 25%, 50% and 75% of resveratrol were 42.5 ± 1.7%, 38.8 ± 2.9%, and 23.8 ± 3.7 %, respectively at 60 l/min (figure 46). Similarly, the FPF calculated for budesonide were 42.5 ± 1.6%, 38.5 ± 3.1%, and 23.0 ± 1.2%, respectively (figure 46). At 90 l/min, the FPF of resveratrol for co-spray dried formulations containing 25%, 50% and 75% resveratrol were 46.7 ± 2.8%, 41.7 ± 4.4%, and 25.9 ± 4.2 %, respectively (figure 46). Moreover the FPF of budesonide for co-spray dried formulations containing 25%, 50% and 75% RES were 46.5 ± 2.8%, 41.2 ± 4.3% and 26.4 ± 2.7% at 90 l/min.

7.2.3 In vitro activity of resveratrol and budesonide using alveolar macrophages

7.2.3.1 Viability of alveolar macrophages cells to resveratrol and budesonide

It is widely accepted that inflammation and oxidative stress is the central pathogenesis contributing to the development of chronic lung diseases such as asthma and COPD [176]. Budesonide is the treatment of choice to control inflammation but some COPD patients are unresponsive towards corticosteroids. Therefore, combining budesonide with resveratrol, which possesses strong anti-oxidant and anti-inflammatory properties, could be beneficial for COPD treatment. Prior to anti-inflammatory and anti-oxidant studies, any potential cytotoxicity effects of resveratrol and budesonide towards alveolar macrophage cells need to be evaluated. Different spray dried formulations, both as combination or single compounds, were added to alveolar macrophages with increasing concentrations (0.612 to 50 μM) and evaluated using the MTS cytotoxicity assays.

As shown in figure 47, alveolar macrophage cells demonstrated higher tolerance towards budesonide compared to resveratrol.

These data implied that resveratrol induced higher cytotoxicity effect compared to budesonide. Therefore, it was noted that the decrease of budesonide concentration in co-spray dried formulations resulted in enhanced cytotoxicity effect towards alveolar macrophages (figure 47).

Many studies have reported that budesonide is relatively safe for lung cells even at micromolar concentration [177-178]. Cell viabilities of healthy alveolar macrophage remained approximately 90% after receiving 100 μM budesonide [179]. Although budesonide imparts negligible cytotoxicity to alveolar macrophages at concentrations up to 100 μM, it was demonstrated that the phagocytosis functions of alveolar macrophages was impaired [179].

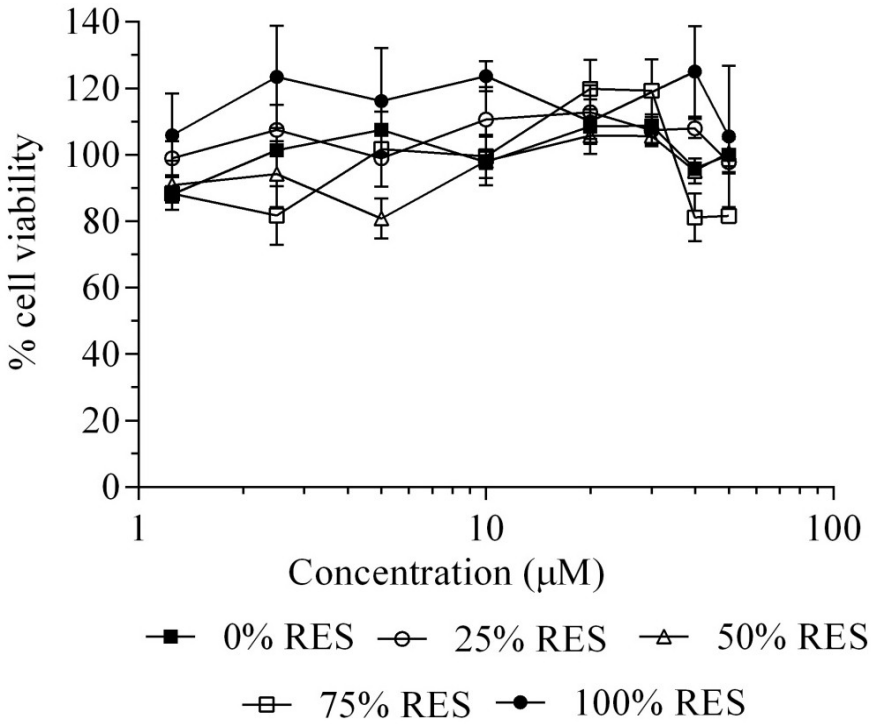


Figure 47: Viabilities of alveolar macrophages cells evaluated using MTS cytotoxicity assay after 72 h of treatment with spray dried formulations. The percentages referred to the resveratrol concentration present in spray dried formulations. Data represents mean ±SD (n=3).

This could indirectly explain the unresponsiveness of COPD patients towards corticosteroids during exacerbation as the inflammatory mediators (alveolar macrophages) are partially inhibited. Resveratrol was well-tolerated by airway lung cells, such as Calu-3 and A_{549} cells, as 95% were viable at 160 µM and 100 µM, respectively.

7.2.3.2 Anti-oxidant activities of co-spray dried formulations

The anti-oxidant activity of resveratrol and budesonide were investigated by measuring their ability to scavenge free reactive radicals, using the DPPH assay. Lipoic acid and L-NAME were used as negative controls while ascorbic acid, a potent free radical scavenger, was used as positive control in this study (figure 48). As expected, no scavenging activities were observed for lipoic acid and L-NAME even at high concentration (500 µM). Ascorbic acid demonstrated the highest anti-oxidant potential by reducing up to 80% DPPH free radicals at 40 µM.

As for budesonide, no detectable radical scavenging activities were found in the ranges of concentrations studied (25 to 500 µM). Meanwhile, it was observed that the anti-oxidant activities of resveratrol were dose-dependent. The increase of resveratrol from 2 to 500 µM led to significant reduction of free DPPH radicals, whereby only 87.8% and 25.2% of DPPH remained, respectively (figure 48). The dose-response curve of resveratrol showed a gradual increase in DPPH scavenging activity while dramatic elevation of anti-oxidant activity was observed for ascorbic acid. For instance, approximately 70% and 31% of DPPH free radicals remained after treatment with 3.9 and 7.8 µM ascorbic acid, respectively (figure 48).

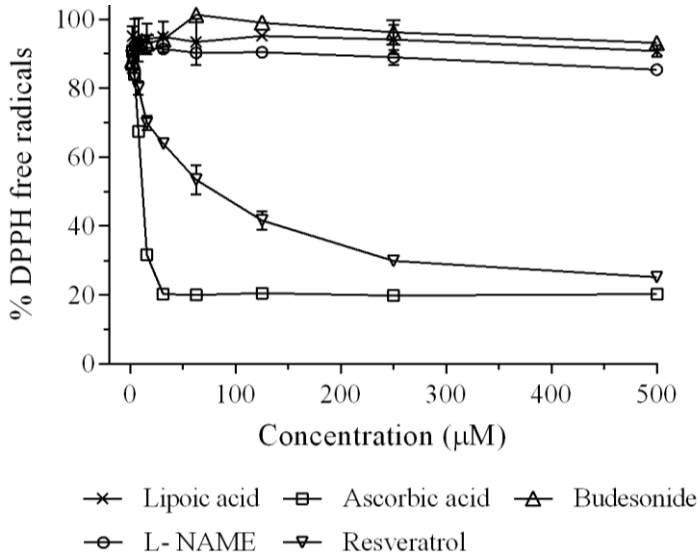


Figure 48: The DPPH free radical scavenging activities in the presence of lipoic acid, ascorbic acid, L-NAME, budesonide and resveratrol. Data represents mean ±SD (n=3).

Oxidative damage in lung tissues caused by stimuli such as cigarette smoke is well known. The introduction of an anti-oxidant molecule such as resveratrol to existing therapies to potentially treat oxidative-stress related lung injury (i.e. COPD) could consequently be advantageous. In both smokers and COPD patients it was shown that the expression of GCL and GSH was reduced compared to healthy subjects, hence an indication of lung injury caused by oxidative stress [163,164], while resveratrol can stimulate lung repair by enhancing GCL and GSH productions [84]. Steroid resistance in COPD patients has been linked to nitrosative stress which is contributed by NO production and protein nitration in airway cells) [143].

In this study, the inhibitory effect of spray dried formulations on NO production was investigated using LPS-induced alveolar macrophage cells (figure 49).

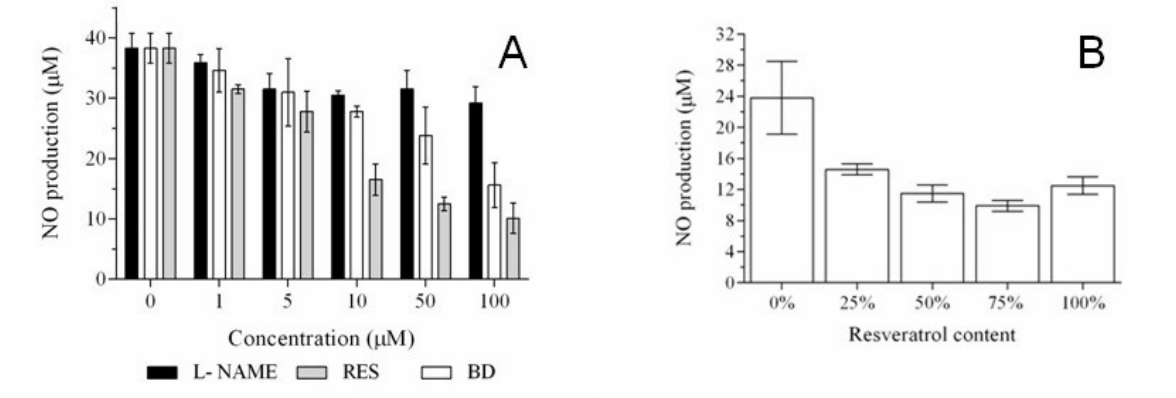


Figure 49: The effect on NO productions in alveolar macrophages. NO production (μM) in the presence (A) of L-NAME, budesonide and resveratrol and (B) of combination spray dried formulations. The percentages referred to the resveratrol concentration present in spray dried formulations. Data represents mean ±SD (n=3).

It has been demonstrated that LPS stimulated iNOS expression in macrophages cells, which in turn led to enhanced production of NO via conversion of L-arginine to L-citrulline [163]. When high-output of NO levels is non-attenuated, it could facilitate synthesis of peroxynitrite intermediates, which react to form 3-nitrotyrosine that causes severe lung epithelial damage [180,181]. In a study by Peng et al, the increase of NO production was correlated to 3-nitrotyrosine concentration in lungs [180]. It is therefore likely that during COPD exacerbations caused by bacterial infection, activated alveolar macrophages result in elevated NO levels and inflammatory cytokines, which lead to lung injury and fibrosis. These unstable NO radicals are rapidly converted to NO_2^- or NO_3^-.
Therefore the amount of NO_2^- measured using Griess reagent could indirectly determine NO production by alveolar macrophages. Results demonstrated that the inhibition of NO was dose-dependent, irrespective to the drugs used (figure 49 B). The concentration of NO decreased from 38.3 ± 2.5 μM (untreated control) to 23.8 ± 4.7 μM, 12.5 ± 1.1 μM and 31.5 ± 3.1 μM when treated with 50 μM BD, RES and L-NAME, respectively. Resveratrol demonstrated the strongest activity, while NAME showed weak inhibitory effect across all concentrations assayed (p<0.05). Only 25% inhibition of NO production could be observed even at high concentration (100 μM) of NAME used. Comparatively, at the same concentration, RES and BD inhibited 75% and 60% of NO production from alveolar

macrophages, respectively (figure 49). The combination of resveratrol and budesonide was also studied to evaluate possible additive/synergistic inhibitory effects of combination therapies towards NO productions in alveolar macrophage cells (figure 49). Interestingly, the reduction of NO was more pronounced when resveratrol and budesonide were used in combination. The reduction was directly correlated to the increase of resveratrol in the formulation. The amount of NO produced showed the following trend: 23.8 ± 4.7 μM (0% RES or 100% BD) > 14.6 ± 0.7 μM (25% RES + 75% BD) > 12.5 ± 1.1 μM (100% RES or 0% BD) > 11.5 ± 1.1 μM (50% RES + 50% BD) > 9.9 ± 0.7 μM (75% RES + 25% BD) (figure 49).

As mentioned, NO synthesis in alveolar macrophages is regulated by iNOS expression, which in turn is controlled by NF-κB. Inhibiting high-output of NO by down-regulating the iNOS expression at the transcription level, through inactivation of the NF-kB signalling pathway, could be a treatment strategy [182,183]. In work by Li et al, the inhibition of NO and iNOS was achieved in LPS-stimulated alveolar macrophages by pre-treating the cells with low concentrations of budesonide (10^{-10} M) [183]. In contrast, higher concentrations of budesonide or resveratrol were needed in order to inhibit iNOS expression, as well as translocating NF-kB compared to NO production in LPS-induced alveolar macrophages, consistent with our results [166]. In addition, resveratrol was not effective to totally inhibit NO productions once cells were stimulated with LPS. This suggests that both inhibition of iNOS protein transcription and NF-kB inactivation is not the primary resveratrol target towards NO regulation. Cho et al. hypothesized that resveratrol interferes with LPS-induced expression of genes responsible for NO expression, independent of the NF-kB signalling pathway [182].

7.2.3.3 Anti-inflammatory activity

The anti-inflammatory properties of resveratrol and budesonide have been widely documented. Results showed that resveratrol and budesonide alone inhibited cytokine expressions (IL-6 and TNF-α) in LPS-stimulated alveolar macrophages as shown in figure 50. These data are consistent with other findings using either resveratrol or budesonide to inhibit cytokine expressions in different pulmonary cells line such as RAW 264.7, A549 and Calu-3 [81,170,184]. Based on these results, resveratrol is more effective than budesonide to reduce IL-6 and TNF-α (p<0.05). Approximately 60% and 20% of IL-6 was

inhibited after 72 h of treatment with resveratrol and budesonide alone, respectively (figure 50).

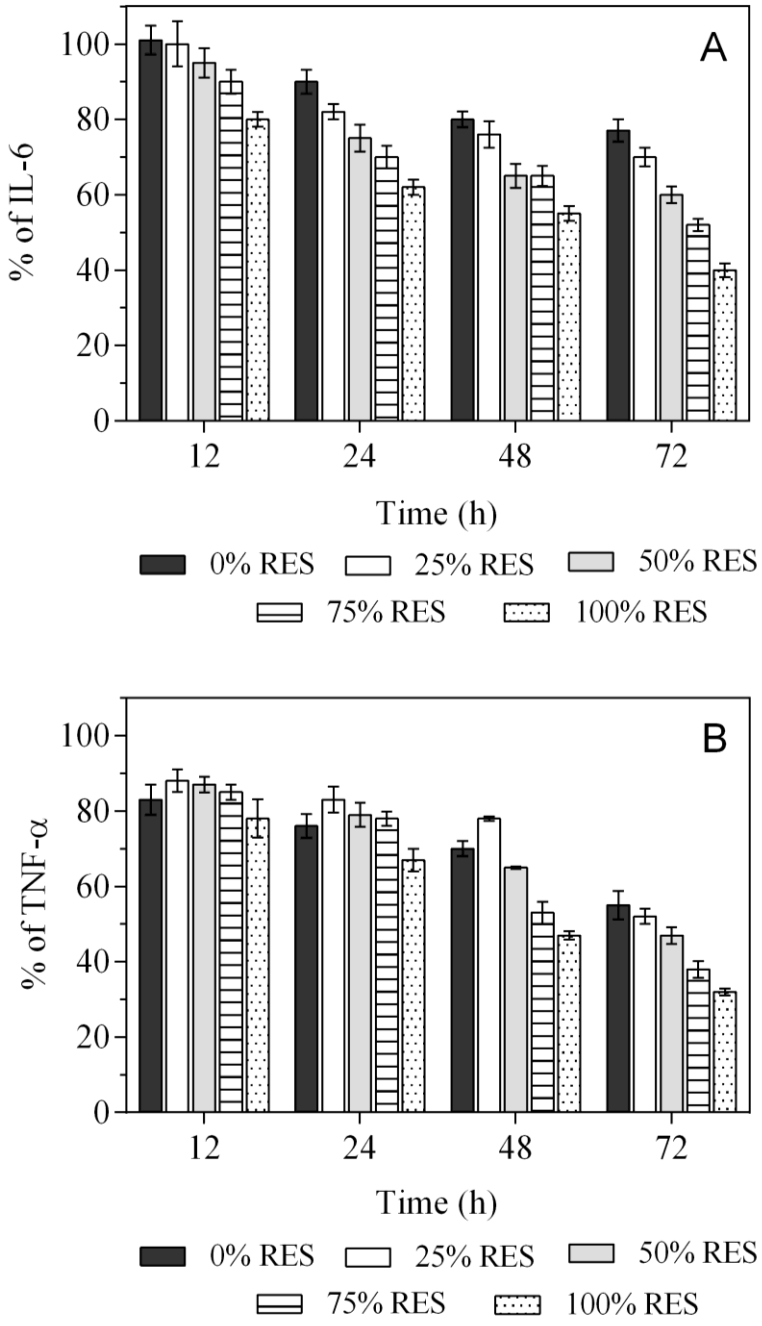


Figure 50: Cytokines expression in culture media of LPS pre-stimulated alveolar macrophage cells after treatment with spray dried formulations at different time points. (A) IL-6 and (B) TNF-α. The percentages referred to the resveratrol concentration present in spray dried formulations. Data represents mean ±SD (n=3).

A similar inhibitory profile was observed for TNF-α production in alveolar macrophages. After 72 h treatment with resveratrol and budesonide, only 45% and 32% of TNF-α markers remained, respectively (figure 50 B). Our data also demonstrated that budesonide preferentially inhibited the expression of TNF-α compared to IL-6 in LPS-stimulated alveolar macrophage cells. This was supported by earlier findings whereby budesonide almost completely inhibited TNF-α release, while only partially inhibited IL-6 expression from macrophages. These results clearly demonstrated the time-dependent response for both resveratrol and budesondie in inhibiting cytokines (figure 50). For budesonide, the percentages of TNF-α remained after 12 h, 24 h, 48 h and 72 h were 83 ± 4%, 76 ± 3%, 70 ± 2%, and 55 ± 4%, respectively. As for resveratrol, the amount of IL-6 remained after 12 h, 24 h, 48 h and 72 h were 80.0 ± 1.9%, 62.0 ± 2.0%, 55.0 ± 2.2%, and 40.0 ± 1.8%, respectively (figure 50). Consistent with previous studies, TNF-α release was significantly inhibited irrespective to the sequence of steroid treatment addition (either prior, during and after stimuli incubation) [185,186].

These results thus suggest the cytoplasm, but not nucleus, is the major site of action for budesonide and resveratrol via interaction with transcription factors, including NF-κB and AP-1, hence the rapid onset of action [186].

The effectiveness of corticosteroids in bacterial-induced COPD exacerbations remains debatable. In a study, IL-8, MMP-9 and MCP-1 expressions from LPS-stimulated alveolar macrophages were resistant to corticosteroids while IL-6 releases were effectively inhibited) [153].

The authors also demonstrated that the provision of resveratrol alone reduced IL-8, MMP-9, IL-6 and MCP-1 expressions down to baseline readings in alveolar macrophage isolated from COPD patients. Therefore it was hypothesized that treatment of alveolar macrophages with inhalable formulations containing budesonide and resveratrol might enhance the anti-inflammatory effect in COPD. As shown in figure 50, cytokine releases from alveolar macrophages were significantly inhibited with the presence of resveratrol in the co-spray dried formulations. By increasing the ratio of resveratrol from 0% to 75%, a reduction of IL-6 expression from 75% to 52% after 72h of treatment was observed. Similarly, significant reductions of TNF-α production were observed by increasing resveratrol (figure 50). The exact reason underlying stronger attenuation effect of resveratrol towards cytokine compared to budesonide is still unclear, but could be due to the antioxidant properties of resveratrol in addition to its anti-inflammatory effect.

In addition, the lack of corticosteroid efficacy could be the result of increased steroid-resistance in pulmonary macrophages. The disparities in steroid sensitivity towards inflammatory genes have been postulated to be attributable to impacts of corticosteroid-sensitive and corticosteroid-resistant transcription mechanisms) [152,187]. In other words, the corticosteroids' efficacy towards specific cytokine is highly dependent on the ratio of corticosteroid-sensitive and corticosteroid-resistant signalling genes for transcriptions of cytokine proteins. For instance, LPS-induced cytokine genes transcription in alveolar macrophages are dependent on NF-κB signalling and mitogen-activated protein kinases (MAPK)/AP-1 pathway, whereby the former is sensitive to corticosteroids while the latter is non-responsive to corticosteroids) [187]. In contrast, resveratrol is known to be effective against both NF-κB and MAPK signalling, which could explain the higher efficacy of resveratrol to reduce IL-6 from alveolar macrophages) [188].

Mechanistically, corticosteroid-resistance in COPD is also augmented by reduced HDAC activities in alveolar macrophages caused by oxidative and nitrosative stress. As resveratrol scavenges ROS and RNS radicals readily, resveratrol could restore HDAC functions. This could also be a contributing factor for the enhanced anti-inflammatory activity showed when combination of budesonide and resveratrol was administered.

7.3 Conclusions

In this study novel co-spray dried formulations of an anti-oxidant (resveratrol) and anti-inflammatory (budesonide) compounds were produced. The spray dried powders showed appropriate morphologies and suitable aerosol properties for inhalation drug delivery. *In vitro* studies showed that alveolar macrophages could tolerate resveratrol and budesonide in the range of concentrations investigated. Moreover, resveratrol and budesonide showed to have anti-inflammatory activities due to their ability to reduce the levels of TNF-α and IL-6. The data presented provide preliminary evidence that these compounds if delivered in combination could be suitable for the treatment of chronic inflammatory lung diseases such asthma and COPD, where both inflammation and oxidative stress are present.

REFERENCES

1. Oudijk, E. D., Lammers, J. J., and Koenderman, L. (2003). Systemic inflammation in chronic obstructive pulmonary disease. *European Respiratory Journal*, *22*(46 suppl), 5s-13s.

2. Lemanske, R. F., and Busse, W. W. (2010). Asthma: clinical expression and molecular mechanisms. *Journal of Allergy and Clinical Immunology*, *125*(2), S95-S102.

3. Rogers, D. F. (2004). Airway mucus hypersecretion in asthma: an undervalued pathology?. *Current opinion in pharmacology*, *4*(3), 241-250.

4. Busse, W. W., and Rosenwasser, L. J. (2003). Mechanisms of asthma. *Journal of Allergy and Clinical Immunology*, *111*(3), S799-S804.

5. Gern, J. E. (2000). Viral and bacterial infections in the development and progression of asthma. *Journal of allergy and clinical immunology*, *105*(2), S497-S502.

6. Eagan, T. M. L., Bakke, P. S., Eide, G. E., and Gulsvik, A. (2002). Incidence of asthma and respiratory symptoms by sex, age and smoking in a community study. *European Respiratory Journal*, *19*(4), 599-605.

7. Bousquet, J., Jeffery, P. K., Busse, W. W., Johnson, M., and Vignola, A. M. (2000). Asthma: from bronchoconstriction to airways inflammation and remodeling. *American journal of respiratory and critical care medicine*, *161*(5), 1720-1745.

8. http://simplebiologyy.blogspot.it/2015/10/what-is-asthma-what-are-asthma-symptoms-causes-treatments-and-prevention.html

9. Pauwels, R. A., Buist, A. S., Calverley, P. M., Jenkins, C. R., and Hurd, S. S. (2012). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine*.

10. Vestbo, J., Hurd, S. S., Agustí, A. G., Jones, P. W., Vogelmeier, C., Anzueto, A., and Stockley, R. A. (2013). Global strategy for the diagnosis, management, and

prevention of chronic obstructive pulmonary disease: GOLD executive summary. *American journal of respiratory and critical care medicine*,*187*(4), 347-365.

11. Barnes, P. J. (2007). Chronic obstructive pulmonary disease: a growing but neglected global epidemic. *PLoS Med*, *4*(5), e112.

12. http://www.devilbisshc.com/patient_information/copd/

13. Lacoste, J. Y., Bousquet, J., Chanez, P., Van Vyve, T., Simony-Lafontaine, J., Lequeu, N., and Godard, P. (1993). Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease. *Journal of Allergy and Clinical Immunology*, *92*(4), 537-548.

14. Mattison, S., and Christensen, M. (2006). The pathophysiology of emphysema: considerations for critical care nursing practice. *Intensive and Critical Care Nursing*, *22*(6), 329-337.

15. Rahman, I., and MacNee, W. (1998). Role of transcription factors in inflammatory lung diseases. *Thorax*, *53*(7), 601-612.

16. Busse, W. W., and Lemanske, R. F. (2007). Expert Panel Report 3: Moving forward to improve asthma care. *Journal of Allergy and Clinical Immunology*,*120*(5), 1012.

17. Chowdhury, B. A., and Dal Pan, G. (2010). The FDA and safe use of long-acting beta-agonists in the treatment of asthma. *New England Journal of Medicine*,*362*(13), 1169-1171.

18. Suissa, S., Ernst, P., Benayoun, S., Baltzan, M., and Cai, B. (2000). Low-dose inhaled corticosteroids and the prevention of death from asthma. *New England Journal of Medicine*, *343*(5), 332-336.

19. Ducharme, F. M., Lasserson, T. J., and Cates, C. J. (2006). Long-acting beta2-agonists versus anti-leukotrienes as add-on therapy to inhaled corticosteroids for chronic asthma. *Cochrane Database Syst Rev*, *4*.

20. Peters, S. P. (2003). Leukotriene receptor antagonists in asthma therapy.*Journal of allergy and clinical immunology*, *111*(1), S62-S70.

21. Rodrigo, G. J., and Rodrigo, C. (2002). The role of anticholinergics in acute asthma treatment: an evidence-based evaluation. *CHEST Journal*, *121*(6), 1977-1987.

22. Cochrane, M. G., Bala, M. V., Downs, K. E., Mauskopf, J., and Ben-Joseph, R. H. (2000). Inhaled corticosteroids for asthma therapy: patient compliance, devices, and inhalation technique. *CHEST Journal*, *117*(2), 542-550.

23. Rowe, B. H., Bretzlaff, J. A., Bourdon, C., Bota, G. W., and Camargo, C. A. (2000). Intravenous magnesium sulfate treatment for acute asthma in the emergency department: a systematic review of the literature. *Annals of emergency medicine*, *36*(3), 181-190.

24. Siafakas, N. M., Vermeire, P., Pride, N. A., Paoletti, P., Gibson, J., Howard, P., and Rees, J. (1995). Optimal assessment and management of chronic obstructive pulmonary disease (COPD). *European Respiratory Journal*, *8*(8), 1398-1420.

25. Donohue, J. F. (2004). Therapeutic responses in asthma and COPD: bronchodilators. *CHEST Journal*, *126*(2_suppl_1), 125S-137S.

26. Cazzola, M., and Molimard, M. (2010). The scientific rationale for combining long-acting β 2-agonists and muscarinic antagonists in COPD. *Pulmonary pharmacology and therapeutics*, *23*(4), 257-267.

27. Keatings, V. M., Jatakanon, A., Worsdell, Y. M., and Barnes, P. J. (1997). Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *American journal of respiratory and critical care medicine*, *155*(2), 542-548.

28. Palmqvist, M., Arvidsson, P., Beckman, O., Peterson, S., and Lötvall, J. (2001). Onset of bronchodilation of budesonide/formoterol vs. salmeterol/fluticasone in single inhalers. *Pulmonary pharmacology and therapeutics*, *14*(1), 29-34.

29. Calverley, P. M., Boonsawat, W., Cseke, Z., Zhong, N., Peterson, S., and Olsson, H. (2003). Maintenance therapy with budesonide and formoterol in chronicobstructive pulmonary disease. *European Respiratory Journal*, *22*(6), 912-919.

30. Lipworth, B. J. (2005). Phosphodiesterase-4 inhibitors for asthma and chronic obstructive pulmonary disease. *The Lancet*, *365*(9454), 167-175.

31. Barnes, P. J. (2006). Theophylline for COPD. *Thorax*, *61*(9), 742-743.

32. Adams, S. G., Melo, J., Luther, M., and Anzueto, A. (2009). Antibiotics are associated with lower relapse rates in outpatients with acute exacerbations of COPD. 2000. *Chest*, *136*(5 Suppl), e30.

33. Kirkham, P., and Rahman, I. (2006). Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. *Pharmacology and therapeutics*, *111*(2), 476-494.

34. Corrigan, C. J., and Kay, A. B. (1991). The roles of inflammatory cells in the pathogenesis of asthma and of chronic obstructive pulmonary disease. *Am Rev Respir Dis*, *143*(5 Pt 1), 1165-1168.

35. Rahman, I., Biswas, S. K., and Kode, A. (2006). Oxidant and antioxidant balance in the airways and airway diseases. *European journal of pharmacology*, *533*(1), 222-239.

36. Ito, K., Lim, S., Caramori, G., Chung, K. F., Barnes, P. J., and Adcock, I. M. (2001). Cigarette smoking reduces histone deacetylase 2 expression, enhances cytokine expression, and inhibits glucocorticoid actions in alveolar macrophages. *The FASEB Journal*, *15*(6), 1110-1112.

37. Rahman, I., Biswas, S. K., and Kirkham, P. A. (2006). Regulation of inflammation and redox signaling by dietary polyphenols. *Biochemical pharmacology*, *72*(11), 1439-1452.

38. Tomás-Barberán, F. A., Ferreres, F., and Gil, M. I. (2000). Antioxidant phenolic metabolites from fruit and vegetables and changes during postharvest storage and processing. *Studies in natural products chemistry*, *23*, 739-795.

39. Ignat, I., Volf, I., and Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry*, *126*(4), 1821-1835.

40. Ferrazzano, G. F., Amato, I., Ingenito, A., Zarrelli, A., Pinto, G., and Pollio, A. (2011). Plant polyphenols and their anti-cariogenic properties: a review.*Molecules*, *16*(2), 1486-1507.

41. Heim, K. E., Tagliaferro, A. R., and Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *The Journal of nutritional biochemistry*, *13*(10), 572-584.

42. Xiao, Z. P., Peng, Z. Y., Peng, M. J., Yan, W. B., Ouyang, Y. Z., and Zhu, H. L. (2011). Flavonoids health benefits and their molecular mechanism. *Mini reviews in medicinal chemistry*, *11*(2), 169-177.

43. Kim, H. P., Son, K. H., Chang, H. W., and Kang, S. S. (2004). Anti-inflammatory plant flavonoids and cellular action mechanisms. *Journal of pharmacological sciences*, *96*(3), 229-245.

44. Seyoum, A., Asres, K., and El-Fiky, F. K. (2006). Structure–radical scavenging activity relationships of flavonoids. *Phytochemistry*, *67*(18), 2058-2070.

45. Mira, L., Tereza Fernandez, M., Santos, M., Rocha, R., Helena Florêncio, M., and Jennings, K. R. (2002). Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free radical research*, *36*(11), 1199-1208.

46. Pannala, A. S., Chan, T. S., O'Brien, P. J., and Rice-Evans, C. A. (2001). Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics.*Biochemical and Biophysical Research Communications*, *282*(5), 1161-1168.

47. Haenen, G. R., Paquay, J. B., Korthouwer, R. E., and Bast, A. (1997). Peroxynitrite scavenging by flavonoids. *Biochemical and biophysical research communications*, *236*(3), 591-593.

48. Burda, S., and Oleszek, W. (2001). Antioxidant and antiradical activities of flavonoids. *Journal of Agricultural and Food Chemistry*, *49*(6), 2774-2779.

49. Kerry, N., and Rice-Evans, C. (1999). Inhibition of peroxynitrite-mediated oxidation of dopamine by flavonoid and phenolic antioxidants and their structural relationships. *Journal of neurochemistry*, *73*(1), 247-253.

50. Rathee, P., Chaudhary, H., Rathee, S., Rathee, D., Kumar, V., and Kohli, K. (2009). Mechanism of action of flavonoids as anti-inflammatory agents: a review. *Inflammation and Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation and Allergy)*, *8*(3), 229-235.

51. Kuehl, F. A., and Egan, R. W. (1980). Prostaglandins, arachidonic acid, and inflammation. *Science*, *210*(4473), 978-984.

52. Raso, G. M., Meli, R., Di Carlo, G., Pacilio, M., and Di Carlo, R. (2001). Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A. 1. *Life sciences*, *68*(8), 921-931.

53. Hämäläinen, M., Nieminen, R., Vuorela, P., Heinonen, M., and Moilanen, E. (2007). Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. *Mediators of inflammation*, *2007*.

54. Sheu, J. R., Hsiao, G., Chou, P. H., Shen, M. Y., and Chou, D. S. (2004). Mechanisms involved in the antiplatelet activity of rutin, a glycoside of the flavonol quercetin, in human platelets. *Journal of agricultural and food chemistry*, *52*(14), 4414-4418.

55. Wagner, C., Fachinetto, R., Dalla Corte, C. L., Brito, V. B., Severo, D., Dias, G. D. O. C., and Rocha, J. B. (2006). Quercitrin, a glycoside form of quercetin, prevents lipid peroxidation in vitro. *Brain research*, *1107*(1), 192-198.

56. García-Mediavilla, V., Crespo, I., Collado, P. S., Esteller, A., Sánchez-Campos, S., Tuñón, M. J., and González-Gallego, J. (2007). The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells. *European journal of pharmacology*, *557*(2), 221-229.

57. Guardia, T., Rotelli, A. E., Juarez, A. O., and Pelzer, L. E. (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Il farmaco*, *56*(9), 683-687.

58. Rogerio, A. P., Kanashiro, A., Fontanari, C., Da Silva, E. V. G., Lucisano-Valim, Y. M., Soares, E. G., and Faccioli, L. H. (2007). Anti-inflammatory activity of quercetin and isoquercitrin in experimental murine allergic asthma. *Inflammation Research*, *56*(10), 402-408.

59. García-Lafuente, A., Guillamón, E., Villares, A., Rostagno, M. A., and Martínez, J. A. (2009). Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflammation Research*, *58*(9), 537-552.

60. Chen, J. C., Ho, F. M., Chao, P. D. L., Chen, C. P., Jeng, K. C. G., Hsu, H. B., ... and Lin, W. W. (2005). Inhibition of iNOS gene expression by quercetin is mediated by the inhibition of IκB kinase, nuclear factor-kappa B and STAT1, and depends on heme oxygenase-1 induction in mouse BV-2 microglia. *European journal of pharmacology*, *521*(1), 9-20.

61. Comalada, M., Camuesco, D., Sierra, S., Ballester, I., Xaus, J., Gálvez, J., and Zarzuelo, A. (2005). In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-κB pathway. *European journal of immunology*, *35*(2), 584-592.

62. Loke, W. M., Proudfoot, J. M., Stewart, S., McKinley, A. J., Needs, P. W., Kroon, P. A., and Croft, K. D. (2008). Metabolic transformation has a profound effect on anti-inflammatory activity of flavonoids such as quercetin: lack of association between antioxidant and lipoxygenase inhibitory activity. *Biochemical pharmacology*, *75*(5), 1045-1053.

63. Kleemann, R., Verschuren, L., Morrison, M., Zadelaar, S., van Erk, M. J., Wielinga, P. Y., and Kooistra, T. (2011). Anti-inflammatory, anti-proliferative and anti-atherosclerotic effects of quercetin in human in vitro and in vivo models. *Atherosclerosis*, *218*(1), 44-52.

64. Robaszkiewicz, A., Balcerczyk, A., and Bartosz, G. (2007). Antioxidative and prooxidative effects of quercetin on A549 cells. *Cell biology international*, *31*(10), 1245-1250.

65. Ying, B., Yang, T., Song, X., Hu, X., Fan, H., Lu, X., and Xu, D. (2009). Quercetin inhibits IL-1 beta-induced ICAM-1 expression in pulmonary epithelial cell line A549 through the MAPK pathways. *Molecular biology reports*, *36*(7), 1825-1832.

66. Yang, J. H., Hsia, T. C., Kuo, H. M., Chao, P. D. L., Chou, C. C., Wei, Y. H., and Chung, J. G. (2006). Inhibition of lung cancer cell growth by quercetin glucuronides via G2/M arrest and induction of apoptosis. *Drug Metabolism and Disposition*, *34*(2), 296-304.

67. Nguyen, T. T. T., Tran, E., Nguyen, T. H., Do, P. T., Huynh, T. H., and Huynh, H. (2004). The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. *Carcinogenesis*, *25*(5), 647-659.

68. Li, H., Zhao, X., Ma, Y., Zhai, G., Li, L., and Lou, H. (2009). Enhancement of gastrointestinal absorption of quercetin by solid lipid nanoparticles. *Journal of Controlled Release*, *133*(3), 238-244.

69. BOULTON, D. W., WALLE, U. K., and WALLE, T. (1999). Fate of the flavonoid quercetin in human cell lines: chemical instability and metabolism. *Journal of pharmacy and pharmacology*, *51*(3), 353-359.

70. Haenen, G. R., Arts, M. J., Bast, A., and Coleman, M. D. (2006). Structure and activity in assessing antioxidant activity in vitro and in vivo: A critical appraisal illustrated with the flavonoids. *Environmental toxicology and pharmacology*,*21*(2), 191-198.

71. Scalia, S., Trotta, V., Traini, D., Young, P. M., Sticozzi, C., Cervellati, F., and Valacchi, G. (2013). Incorporation of quercetin in respirable lipid microparticles: Effect on stability and cellular uptake on A549 pulmonary alveolar epithelial cells. *Colloids and Surfaces B: Biointerfaces*, *112*, 322-329.

72. Ignat, I., Volf, I., and Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry*, *126*(4), 1821-1835.

73. Robbins, R. J. (2003). Phenolic acids in foods: an overview of analytical methodology. *Journal of agricultural and food chemistry*, *51*(10), 2866-2887.

74. Ignat, I., Volf, I., and Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry*, *126*(4), 1821-1835.

75. R Neves, A., Lucio, M., LC Lima, J., and Reis, S. (2012). Resveratrol in medicinal chemistry: a critical review of its pharmacokinetics, drug-delivery, and membrane interactions. *Current medicinal chemistry*, *19*(11), 1663-1681.

76. Amri, A., Chaumeil, J. C., Sfar, S., and Charrueau, C. (2012). Administration of resveratrol: what formulation solutions to bioavailability limitations?. *Journal of Controlled Release*, *158*(2), 182-193.

77. Santos, A. R. (2004). Anti-inflammatory compounds of plant origin. Part II. Modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. *Planta Med*, *70*, 93-103.

78. Venkatesan, B., Valente, A. J., Reddy, V. S., Siwik, D. A., and Chandrasekar, B. (2009). Resveratrol blocks interleukin-18-EMMPRIN cross-regulation and smooth muscle cell migration. *American Journal of Physiology-Heart and Circulatory Physiology*, *297*(2), H874-H886.

79. Knobloch, J., Wahl, C., Feldmann, M., Jungck, D., Strauch, J., Stoelben, E., and Koch, A. (2014). Resveratrol attenuates the release of inflammatory cytokines from human bronchial smooth muscle cells exposed to lipoteichoic acid in chronic obstructive pulmonary disease. *Basic and clinical pharmacology and toxicology*, *114*(2), 202-209.

80. Gan, W. Q., Man, S. F. P., Senthilselvan, A., and Sin, D. D. (2004). Association between chronic obstructive pulmonary disease and systemic inflammation: a systematic review and a meta-analysis. *Thorax*, *59*(7), 574-580.

81. Donnelly, L. E., Newton, R., Kennedy, G. E., Fenwick, P. S., Leung, R. H., Ito, K., and Barnes, P. J. (2004). Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *287*(4), L774-L783.

82. Shang, Y. J., Qian, Y. P., Liu, X. D., Dai, F., Shang, X. L., Jia, W. Q., and Zhou, B. (2009). Radical-scavenging activity and mechanism of resveratrol-oriented analogues: influence of the solvent, radical, and substitution. *The Journal of organic chemistry*, *74*(14), 5025-5031.

83. Acquaviva, R., Russo, A., Campisi, A., Sorrenti, V., Di Giacomo, C., Barcellona, M. L., and Vanella, A. (2002). Antioxidant Activity and Protective Effect on DNA Cleavage of Resveratrol. *Journal of food science*, *67*(1).

84. Kode, A., Rajendrasozhan, S., Caito, S., Yang, S. R., Megson, I. L., and Rahman, I. (2008). Resveratrol induces glutathione synthesis by activation of Nrf2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial cells. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *294*(3), L478-L488.

85. Bi, X. L., Yang, J. Y., Dong, Y. X., Wang, J. M., Cui, Y. H., Ikeshima, T., and Wu, C. F. (2005). Resveratrol inhibits nitric oxide and TNF-α production by lipopolysaccharide-activated microglia. *International immunopharmacology*,*5*(1), 185-193.

86. Manach, C., Scalbert, A., Morand, C., Rémésy, C., and Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American journal of clinical nutrition*, *79*(5), 727-747.

87. http://www.buchi.com/en/products/spray-drying-and-encapsulation/mini-spray-dryer-b-290

88. Mehnert, W., and Mäder, K. (2001). Solid lipid nanoparticles: production, characterization and applications. *Advanced drug delivery reviews*, *47*(2), 165-196.

89. Jaspart, S., Piel, G., Delattre, L., and Evrard, B. (2005). Solid lipid microparticles: formulation, preparation, characterisation, drug release and applications. *Expert opinion on drug delivery*, *2*(1), 75-87.

90. Elgart, A., Cherniakov, I., Aldouby, Y., Domb, A. J., and Hoffman, A. (2012). Lipospheres and pro-nano lipospheres for delivery of poorly water soluble compounds. *Chemistry and physics of lipids*, *165*(4), 438-453.

91. Comalada, M., Camuesco, D., Sierra, S., Ballester, I., Xaus, J., Gálvez, J., and Zarzuelo, A. (2005). In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-κB pathway. European journal of immunology, 35(2), 584-592.

92. Scalia, S., Young, P. M., and Traini, D. (2015). Solid lipid microparticles as an approach to drug delivery. *Expert opinion on drug delivery*, *12*(4), 583-599.

93. Dalpiaz, A., Mezzena, M., Scatturin, A., and Scalia, S. (2008). Solid lipid microparticles for the stability enhancement of the polar drug N 6-cyclopentyladenosine. *International journal of pharmaceutics*, *355*(1), 81-86.

94. Trotta, V., Goios, F., Monteiro, H., Almeida, I. F., and Scalia, S. (2014). Influence of lipid microparticle encapsulation on in vitro efficacy, photostability and water resistance of the sunscreen agents, octyl methoxycinnamate and butyl methoxydibenzoylmethane. *Drug development and industrial pharmacy*,*40*(9), 1233-1239.

95. Mezzena, M., Scalia, S., Young, P. M., and Traini, D. (2009). Solid lipid budesonide microparticles for controlled release inhalation therapy. *The AAPS journal*, *11*(4), 771-778.

96. Trotta, M., Debernardi, F., and Caputo, O. (2003). Preparation of solid lipid nanoparticles by a solvent emulsification–diffusion technique. *International journal of pharmaceutics*, *257*(1), 153-160.

97. Rawat Singh, M., Pradhan, K., and Singh, D. (2012). Lipid matrix systems with emphasis on lipid microspheres: potent carriers for transcutaneous delivery of bioactives. *Current drug delivery*, *9*(3), 243-254.

98. Cortesi, R., Esposito, E., Luca, G., and Nastruzzi, C. (2002). Production of lipospheres as carriers for bioactive compounds. *Biomaterials*, *23*(11), 2283-2294.

99. McCarron, P. A., Donnelly, R. F., and Al-Kassas, R. (2008). Comparison of a novel spray congealing procedure with emulsion-based methods for the micro-encapsulation of water-soluble drugs in low melting point triglycerides. *Journal of microencapsulation*, *25*(6), 365-378.

100. Scalia, S., Haghi, M., Losi, V., Trotta, V., Young, P. M., and Traini, D. (2013). Quercetin solid lipid microparticles: a flavonoid for inhalation lung delivery.*European Journal of Pharmaceutical Sciences*, *49*(2), 278-285.

101. https://en.wikipedia.org/wiki/Respiratory_tract

102. http://www.londoncancercentre.co.uk/cancer-types/lung/

103. Labiris, N. R., and Dolovich, M. B. (2003). Pulmonary drug delivery. Part I: physiological factors affecting therapeutic effectiveness of aerosolized medications. *British journal of clinical pharmacology*, *56*(6), 588-599.

104. Labiris, N. R., and Dolovich, M. B. (2003). Pulmonary drug delivery. Part II: the role of inhalant delivery devices and drug formulations in therapeutic effectiveness of aerosolized medications. *British journal of clinical pharmacology*, *56*(6), 600-612.

105. Nahar, K., Gupta, N., Gauvin, R., Absar, S., Patel, B., Gupta, V., and Ahsan, F. (2013). In vitro, in vivo and ex vivo models for studying particle deposition and drug absorption of inhaled pharmaceuticals. *European Journal of Pharmaceutical Sciences*, *49*(5), 805-818.

106. Stahlhofen, W., Gebhart, J., and Heyder, J. (1980). Experimental determination of the regional deposition of aerosol particles in the human respiratory tract. *The American Industrial Hygiene Association Journal*, *41*(6), 385-398a.

107. Haghi, M., Ong, H. X., Traini, D., and Young, P. (2014). Across the pulmonary epithelial barrier: Integration of physicochemical properties and human cell models to study pulmonary drug formulations. *Pharmacology and therapeutics*,*144*(3), 235-252.

108. Gonda, I. (1988). Drugs administered directly into the respiratory tract: modeling of the duration of effective drug levels. *Journal of pharmaceutical sciences*, *77*(4), 340-346.

109. Lippmann, M. (1977). Regional deposition of particles in the human respiratory tract. *Comprehensive Physiology*.

110. Heyder, J. J. G. C. F. W., Gebhart, J., Rudolf, G., Schiller, C. F., and Stahlhofen, W. (1986). Deposition of particles in the human respiratory tract in the size range 0.005–15 μm. *Journal of Aerosol Science*, *17*(5), 811-825.

111. Martonen, T., and Yang, Y. (1996). Deposition mechanics of pharmaceutical particles in human airways. *LUNG BIOLOGY IN HEALTH AND DISEASE*, *94*, 3-27.

112. Sbirlea-Apiou, G. A. B. R. I. E. L. A., Katz, I., Caillibotte, G. E. O. R. G. E. S., Martonen, T., and Yang, Y. A. D. O. N. G. (2007). Deposition mechanics of pharmaceutical particles in human airways. *Lung Biology in Health and Disease*, *221*, 1.

113. Zanen, P. L. T. G., Go, L. T., and Lammers, J. W. (1996). Optimal particle size for beta 2 agonist and anticholinergic aerosols in patients with severe airflow obstruction. *Thorax*, *51*(10), 977-980.

114. Darquenne, C., and Prisk, G. K. (2004). Aerosol deposition in the human respiratory tract breathing air and 80: 20 heliox. *Journal of aerosol medicine*,*17*(3), 278-285.

115. Gerrity, T. R., Garrard, C. S., and Yeates, D. B. (1983). A mathematical model of particle retention in the air-spaces of human lungs. *British journal of industrial medicine*, *40*(2), 121-130.

116. Lippmann, M. D. B. R. E., Yeates, D. B., and Albert, R. E. (1980). Deposition, retention, and clearance of inhaled particles. *British journal of industrial medicine*, *37*(4), 337-362.

117. http://www.rainbowcare.com.sg/image/cache/data/products/pumps/bd5002/bd5002-800x800.jpg

118. https://www.asthma.ie/sites/default/files/styles/latest_news_full/public/images/get-help/2013/Aug/reliever%20inhaler.jpg?itok=rqzpNOXd

119. http://www.webpackaging.com/en/portals/plastiape/assets/11023859/single-dose-inhalers-for-capsules-by-plastiape/

120. Newman, S. P. (2005). Principles of metered-dose inhaler design. *Respiratory care*, *50*(9), 1177-1190.

121. Emmen, H. H., Hoogendijk, E. M. G., Klöpping-Ketelaars, W. A. A., Muijser, H., Duistermaat, E., Ravensberg, J. C., ... and Schmit, B. (2000). Human safety and pharmacokinetics of the CFC alternative propellants HFC 134a (1, 1, 1, 2-tetrafluoroethane) and HFC 227 (1, 1, 1, 2, 3, 3, 3-heptafluoropropane) following whole-body exposure. *Regulatory Toxicology and Pharmacology*, *32*(1), 22-35.

122. http://www.personal.psu.edu/staff/r/r/rrz1/images/spacer_picture.jpg

123. http://www.malvern.com/Assets/600x400-aero-funnel-1.jpg

124. Basnet, P., Hussain, H., Tho, I., and Skalko-Basnet, N. (2012). Liposomal delivery system enhances anti-inflammatory properties of curcumin. *Journal of pharmaceutical sciences*, *101*(2), 598-609.

125. Haghi, M., Traini, D., and Young, P. (2014). In vitro cell integrated impactor deposition methodology for the study of aerodynamically relevant size fractions from commercial pressurised metered dose inhalers. *Pharmaceutical research*,*31*(7), 1779-1787.

126. Choi, J., Zhang, Q., Reipa, V., Wang, N. S., Stratmeyer, M. E., Hitchins, V. M., and Goering, P. L. (2009). Comparison of cytotoxic and inflammatory responses of photoluminescent silicon nanoparticles with silicon micron-sized particles in RAW 264.7 macrophages. *Journal of Applied Toxicology*, *29*(1), 52-60.

127. Teixeira, S., Siquet, C., Alves, C., Boal, I., Marques, M. P., Borges, F., and Reis, S. (2005). Structure–property studies on the antioxidant activity of flavonoids present in diet. *Free Radical Biology and Medicine*, *39*(8), 1099-1108.

128. Kumari, A., Yadav, S. K., Pakade, Y. B., Singh, B., and Yadav, S. C. (2010). Development of biodegradable nanoparticles for delivery of quercetin. *Colloids and Surfaces B: Biointerfaces*, *80*(2), 184-192.

129. Deiana, M., Dessi, M. A., Ke, B., Liang, Y. F., Higa, T., Gilmour, P. S., ... and Aruoma, O. I. (2002). The antioxidant cocktail effective microorganism X (EM-X) inhibits oxidant-induced interleukin-8 release and the peroxidation of phospholipids in vitro. *Biochemical and biophysical research communications*,*296*(5), 1148-1151.

130. Nanua, S., Zick, S. M., Andrade, J. E., Sajjan, U. S., Burgess, J. R., Lukacs, N. W., and Hershenson, M. B. (2006). Quercetin blocks airway epithelial cell chemokine expression. *American journal of respiratory cell and molecular biology*, *35*(5), 602-610.

131. Jung, C. H., Lee, J. Y., Cho, C. H., and Kim, C. J. (2007). Anti-asthmatic action of quercetin and rutin in conscious guinea-pigs challenged with aerosolized ovalbumin. *Archives of Pharmacal Research*, *30*(12), 1599-1607.

132. Rogerio, A. P., Dora, C. L., Andrade, E. L., Chaves, J. S., Silva, L. F., Lemos-Senna, E., and Calixto, J. B. (2010). Anti-inflammatory effect of quercetin-loaded microemulsion in the airways allergic inflammatory model in mice.*Pharmacological Research*, *61*(4), 288-297.

133. Tan, B. J., Liu, Y., Chang, K. L., Lim, B. K., and Chiu, G. N. (2012). Perorally active nanomicellar formulation of quercetin in the treatment of lung cancer. *Int J Nanomedicine*, *7*(6), 651-661.

134. Bischoff, S. C. (2008). Quercetin: potentials in the prevention and therapy of disease. *Current Opinion in Clinical Nutrition and Metabolic Care*, *11*(6), 733-740.

135. Ghosh, A., Mandal, A. K., Sarkar, S., Panda, S., and Das, N. (2009). Nanoencapsulation of quercetin enhances its dietary efficacy in combating arsenic-induced oxidative damage in liver and brain of rats. *Life sciences*,*84*(3), 75-80.

136. Nassimi, M., Schleh, C., Lauenstein, H. D., Hussein, R., Hoymann, H. G., Koch, W., and Braun, A. (2010). A toxicological evaluation of inhaled solid lipid nanoparticles used as a potential drug delivery system for the lung. *European Journal of Pharmaceutics and Biopharmaceutics*, *75*(2), 107-116.

137. Calverley, P. M., Anderson, J. A., Celli, B., Ferguson, G. T., Jenkins, C., Jones, P. W., and Vestbo, J. (2007). Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *New England Journal of Medicine*, *356*(8), 775-789.

138. Cazzola, M., Di Perna, F., Noschese, P., Vinciguerra, A., Calderaro, F., Girbino, G., and Matera, M. G. (1998). Effects of formoterol, salmeterol or oxitropium bromide

on airway responses to salbutamol in COPD. *European Respiratory Journal*, *11*(6), 1337-1341.

139. Celli, B. R., MacNee, W. A. T. S., Agusti, A. A. T. S., Anzueto, A., Berg, B., Buist, A. S., and Fein, A. (2004). Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper. *European Respiratory Journal*, *23*(6), 932-946.

140. Dahl, R., Chung, K. F., Buhl, R., Magnussen, H., Nonikov, V., Jack, D., and Kramer, B. (2010). Efficacy of a new once-daily long-acting inhaled β2-agonist indacaterol versus twice-daily formoterol in COPD. *Thorax*, *65*(6), 473-479.

141. Pauwels, R. A., Buist, A. S., Calverley, P. M., Jenkins, C. R., and Hurd, S. S. (2012). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine*.

142. Pauwels, R. A., Löfdahl, C. G., Laitinen, L. A., Schouten, J. P., Postma, D. S., Pride, N. B., and Ohlsson, S. V. (1999). Long-term treatment with inhaled budesonide in persons with mild chronic obstructive pulmonary disease who continue smoking. *New England Journal of Medicine*, *340*(25), 1948-1953.

143. Barnes, P. J., Ito, K., and Adcock, I. M. (2004). Corticosteroid resistance in chronic obstructive pulmonary disease: inactivation of histone deacetylase.*The Lancet*, *363*(9410), 731-733.

144. Rahman, I., and Adcock, I. M. (2006). Oxidative stress and redox regulation of lung inflammation in COPD. *European Respiratory Journal*, *28*(1), 219-242.

145. Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., and Moon, R. C. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, *275*(5297), 218-220.

146. Zhou, H. B., Chen, J. J., Wang, W. X., Cai, J. T., and Du, Q. (2005). Anticancer activity of resveratrol on implanted human primary gastric carcinoma cells in nude mice. *World J Gastroenterol*, *11*(2), 280-4.

147. Udenigwe, C. C., Ramprasath, V. R., Aluko, R. E., and Jones, P. J. (2008). Potential of resveratrol in anticancer and anti-inflammatory therapy. *Nutrition reviews*, *66*(8), 445-454.

148. Mokni, M., Elkahoui, S., Limam, F., Amri, M., and Aouani, E. (2007). Effect of resveratrol on antioxidant enzyme activities in the brain of healthy rat.*Neurochemical research*, *32*(6), 981-987.

149. Richard, T., Pawlus, A. D., Iglésias, M. L., Pedrot, E., Waffo-Teguo, P., Mérillon, J. M., and Monti, J. P. (2011). Neuroprotective properties of resveratrol and derivatives. *Annals of the New York Academy of Sciences*, *1215*(1), 103-108.

150. Turan, B., Tuncay, E., and Vassort, G. (2012). Resveratrol and diabetic cardiac function: focus on recent in vitro and in vivo studies. *Journal of bioenergetics and biomembranes*, *44*(2), 281-296.

151. Wood, L. G., Wark, P. A., and Garg, M. L. (2010). Antioxidant and anti-inflammatory effects of resveratrol in airway disease. *Antioxidants and redox signaling*, *13*(10), 1535-1548.

152. Knobloch, J., Sibbing, B., Jungck, D., Lin, Y., Urban, K., Stoelben, E., and Koch, A. (2010). Resveratrol impairs the release of steroid-resistant inflammatory cytokines from human airway smooth muscle cells in chronic obstructive pulmonary disease. *Journal of Pharmacology and Experimental Therapeutics*, *335*(3), 788-798.

153. Knobloch, J., Hag, H., Jungck, D., Urban, K., and Koch, A. (2011). Resveratrol Impairs the Release of Steroid-resistant Cytokines from Bacterial Endotoxin-Exposed Alveolar Macrophages in Chronic Obstructive Pulmonary Disease.*Basic and clinical pharmacology and toxicology*, *109*(2), 138-143.

154. Amri, A., Chaumeil, J. C., Sfar, S., and Charrueau, C. (2012). Administration of resveratrol: what formulation solutions to bioavailability limitations?. *Journal of Controlled Release*, *158*(2), 182-193.

155. Todoroff, J., and Vanbever, R. (2011). Fate of nanomedicines in the lungs.*Current Opinion in Colloid and Interface Science*, *16*(3), 246-254.

156. Ansari, K. A., Vavia, P. R., Trotta, F., and Cavalli, R. (2011). Cyclodextrin-based nanosponges for delivery of resveratrol: in vitro characterisation, stability, cytotoxicity and permeation study. *Aaps Pharmscitech*, *12*(1), 279-286.

157. Britain, G., and BP Commission. (2010). British Pharmacopoeia 2011.

158. Newton, R., Kuitert, L. M., Bergmann, M., Adcock, I. M., and Barnes, P. J. (1997). Evidence for involvement of NF-κB in the transcriptional control of COX-2 gene expression by IL-1β. *Biochemical and biophysical research communications*, *237*(1), 28-32.

159. Fiebich, B. L., Lieb, K., Engels, S., and Heinrich, M. (2002). Inhibition of LPS-induced p42/44 MAP kinase activation and iNOS/NO synthesis by parthenolide in rat primary microglial cells. *Journal of neuroimmunology*, *132*(1), 18-24.

160. Manna, S. K., Mukhopadhyay, A., and Aggarwal, B. B. (2000). Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-κB, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *The Journal of Immunology*, *164*(12), 6509-6519.

161. Soares, D. G., Andreazza, A. C., and Salvador, M. (2003). Sequestering ability of butylated hydroxytoluene, propyl gallate, resveratrol, and vitamins C and E against ABTS, DPPH, and hydroxyl free radicals in chemical and biological systems. *Journal of agricultural and food chemistry*, *51*(4), 1077-1080.

162. Acquaviva, R., Russo, A., Campisi, A., Sorrenti, V., Di Giacomo, C., Barcellona, M. L., and Vanella, A. (2002). Antioxidant Activity and Protective Effect on DNA Cleavage of Resveratrol. *Journal of food science*, *67*(1) 137–141.

163. Cerqueira, A. M., Khaper, N., Lees, S. J., and Ulanova, M. (2013). The antioxidant resveratrol down-regulates inflammation in an in-vitro model of Pseudomonas aeruginosa infection of lung epithelial cells 1. *Canadian journal of physiology and pharmacology*, *91*(3), 248-255.

164. Harju, T., Kaarteenaho-Wiik, R., Soini, Y., Sormunen, R., and Kinnula, V. L. (2002). Diminished immunoreactivity of γ-glutamylcysteine synthetase in the airways of

smokers' lung. *American journal of respiratory and critical care medicine*, *166*(5), 754-759.

165. Rabe, K. F., Hurd, S., Anzueto, A., Barnes, P. J., Buist, S. A., Calverley, P., ... and Zielinski, J. (2007). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *American journal of respiratory and critical care medicine*, *176*(6), 532-555.

166. Tetley, T. D. (2002). Macrophages and the pathogenesis of COPD. *CHEST Journal*, *121*(5_suppl), 156S-159S.

167. Kent, L., Smyth, L., Clayton, C., Scott, L., Cook, T., Stephens, R., ... and Singh, D. (2008). Cigarette smoke extract induced cytokine and chemokine gene expression changes in COPD macrophages. *Cytokine*, *42*(2), 205-216.

168. Barnes, P. J. (2009). Role of HDAC2 in the pathophysiology of COPD. *Annual review of physiology*, *71*, 451-464.

169. Biswas, S., W Hwang, J., A Kirkham, P., and Rahman, I. (2013). Pharmacological and dietary antioxidant therapies for chronic obstructive pulmonary disease. *Current medicinal chemistry*, *20*(12), 1496-1530.

170. Trotta, V., Lee, W. H., Loo, C. Y., Haghi, M., Young, P. M., Scalia, S., and Traini, D. (2015). In vitro biological activity of resveratrol using a novel inhalable resveratrol spray-dried formulation. *International journal of pharmaceutics*, *491*(1), 190-197.

171. Culpitt, S. V., Rogers, D. F., Fenwick, P. S., Shah, P., De Matos, C., Russell, R. E. K., and Donnelly, L. E. (2003). Inhibition by red wine extract, resveratrol, of cytokine release by alveolar macrophages in COPD. *Thorax*, *58*(11), 942-946.

172. Parlati, C., Colombo, P., Buttini, F., Young, P. M., Adi, H., Ammit, A. J., and Traini, D. (2009). Pulmonary spray dried powders of tobramycin containing sodium stearate to improve aerosolization efficiency. *Pharmaceutical research*,*26*(5), 1084-1092.

173. Adi, S., Adi, H., Tang, P., Traini, D., Chan, H. K., and Young, P. M. (2008). Micro-particle corrugation, adhesion and inhalation aerosol efficiency. *european journal of pharmaceutical sciences*, *35*(1), 12-18.

174. Tajber, L., Corrigan, D. O., Corrigan, O. I., and Healy, A. M. (2009). Spray drying of budesonide, formoterol fumarate and their composites—I. Physicochemical characterisation. *International journal of Pharmaceutics*, *367*(1), 79-85.

175. Budavári, Z. I. I. S., Zelko, R., Rácz, I., and Marton, S. (1999). Compatibility study between folic acid, vitamin B6 and tablet excipients using differential scanning calorimetry. *Pharmazie*, *54*(11), 861-862.

176. MacNee, W. (2001). Oxidative stress and lung inflammation in airways disease. *European journal of pharmacology*, *429*(1), 195-207.

177. Naikwade, S. R., Bajaj, A. N., Gurav, P., Gatne, M. M., and Soni, P. S. (2009). Development of budesonide microparticles using spray-drying technology for pulmonary administration: design, characterization, in vitro evaluation, and in vivo efficacy study. *AAPS PharmSciTech*, *10*(3), 993-1012.

178. Mali, A. J., Pawar, A. P., and Purohit, R. N. (2014). Development of Budesonide Loaded Biopolymer Based Dry Powder Inhaler: Optimization, In Vitro Deposition, and Cytotoxicity Study. *Journal of pharmaceutics*, *2014*.

179. Zetterlund, A., Larsson, P. H., Müller-Suur, C., Palmberg, L., and Larsson, K. (1998). Budesonide but not terbutaline decreases phagocytosis in alveolar macrophages. *Respiratory medicine*, *92*(2), 162-166.

180. Peng, X., Abdulnour, R. E. E., Sammani, S., Ma, S. F., Han, E. J., Hasan, E. J., ... and Hassoun, P. M. (2005). Inducible nitric oxide synthase contributes to ventilator-induced lung injury. *American journal of respiratory and critical care medicine*, *172*(4), 470-479.

181. Crosswhite, P., and Sun, Z. (2010). Nitric oxide, oxidative stress and inflammation in pulmonary arterial hypertension. *Journal of hypertension*, *28*(2), 201.

182. Cho, D. I., Koo, N. Y., Chung, W. J., Kim, T. S., Ryu, S. Y., Im, S. Y., and Kim, K. M. (2002). Effects of resveratrol-related hydroxystilbenes on the nitric oxide production in macrophage cells: structural requirements and mechanism of action. *Life sciences*, *71*(17), 2071-2082.

183. Li, Y. H., Yan, Z. Q., Brauner, A., and Tullus, K. (2002). Activation of macrophage nuclear factor-kappa B and induction of inducible nitric oxide synthase by LPS. *Respir Res*, *3*(1), 23.

184. Lilly, C. M., Nakamura, H., Kesselman, H., Nagler-Anderson, C., Asano, K., Garcia-Zepeda, E. A., and Luster, A. D. (1997). Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. *Journal of Clinical Investigation*, *99*(7), 1767.

185. Linden, M., and Brattsand, R. (1994). Effects of a corticosteroid, budesonide, on alveolar macrophage and blood monocyte secretion of cytokines: differential sensitivity of GM-CSF, IL-1β, and IL-6. *Pulmonary pharmacology*, *7*(1), 43-47.

186. Adcock, I. M., Brown, C. R., and Barnes, P. J. (1996). Tumour necrosis factor α causes retention of activated glucocorticoid receptor within the cytoplasm of A549 cells. *Biochemical and biophysical research communications*, *225*(2), 545-550.

187. Armstrong, J., Sargent, C., and Singh, D. (2009). Glucocorticoid sensitivity of lipopolysaccharide-stimulated chronic obstructive pulmonary disease alveolar macrophages. *Clinical and Experimental Immunology*, *158*(1), 74-83.

188. Cottart, C. H., Nivet-Antoine, V., Laguillier-Morizot, C., and Beaudeux, J. L. (2010). Resveratrol bioavailability and toxicity in humans. *Molecular nutrition and food research*, *54*(1), 7-16.

LIST OF FIGURES

Figure 35: Scanning electron microscopy image of spray dried resveratrol.

Figure 36: Differential scanning calorimetric thermographs of raw and spray dried resveratrol.

Figure 37: *In vitro* aerosol deposition of spray dried resveratrol using the ACI and RS01 dry powder inhalation device at 60 l/min for 4 seconds. Data represents mean ± SD (n=3).

Figure 38: Time course of resveratrol concentration during incubation in completed DMEM:NMF-12 media. Data represents mean ± SD (n=3).

Figure 39: Calu-3 cell viability profile after 72h resveratrol treatment. Data represents mean ± SD (n=3).

Figure 40: Concentration (pg/ml) of the inflammatory cytokine, IL-8, in culture media after stimulation of Calu-3 cells with TNF-α, LPS and TGF-β1 in presence and absence of resveratrol treatment. Data represents mean ± SD (n=4). * $p < 0.05$.

Figure 41: Antioxidant activity of resveratrol compare to L-NAME, lipoic acid and ascorbic acid. Absorbance values are directly proportional to % of DPPH free radicals. Data is represents mean ± SD (n=4).

Figure 42: Percentage of NO production in Calu-3 cell after induction inflammation with LPS. Data represents mean ± SD (n=9). * $p < 0.05$.

Figure 43: Percentage of NO production in Calu-3 cell after induction inflammation with TFG-β1. Data represents mean ± SD (n=9).

Figure 44: Scanning electron microscopy images of single and co-spray dried formulations containing resveratrol and budesonide at different ratios. The percentages referred to the resveratrol concentration present in spray dried formulations.

Figure 45: Differential scanning calorimetric thermograms of raw budesonide, raw resveratrol and co-spray dried formulations. The percentages referred to the resveratrol concentration present in spray dried formulations.

Figure 46: *In vitro* aerosol deposition of the single spray dried and co-spray dried powders at various concentrations (% w/w) of resveratrol using MSLI at flow rate of 60 l/min and 90 l/min. Data represents mean ±SD (n=3).

Figure 47: Viabilities of alveolar macrophages cells evaluated using MTS cytotoxicity assay after 72 h of treatment with spray dried formulations. The percentages referred to the resveratrol concentration present in spray dried formulations. Data represents mean ±SD (n=3).

Figure 48: The DPPH free radical scavenging activities in the presence of lipoic acid, ascorbic acid, L-NAME, budesonide and resveratrol. Data represents mean ±SD (n=3).

Figure 49: The effect on NO productions in alveolar macrophages. NO production (μM) in the presence (A) of L-NAME, budesonide and resveratrol and (B) of combination spray dried formulations. The percentages referred to the resveratrol concentration present in spray dried formulations. Data represents mean ±SD (n=3).

Figure 50: Cytokines expression in culture media of LPS pre-stimulated alveolar macrophage cells after treatment with spray dried formulations at different time points. (A) IL-6 and (B) TNF-α. The percentages referred to the resveratrol concentration present in spray dried formulations. Data represents mean ±SD (n=3).

LIST OF TABLES

LIST OF ABBREVIATIONS

5- HPETE: 5-hydroperoxyeicosatetraenoic acid

AA: Arachidonic acid

ACI: Anderson cascade impactor

AP-1: activator protein-1

BD: Budesonide

CFC: Chlorofluorocarbon

COPD: Chronic obstructive pulmonary diseases

COX: Cyclooxygenase

CRP: Reactive C-protein

DAN: 2,3-diaminonaphthalene

DMEM: Dulbecco's Modified Eagle's medium

DPI: Dry powder inhaler

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DSC: Differential scanning calorimetry

FPD: Fine particle dose

FPF: Fine particles fraction

GCL: Glutamate-cysteine

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GSD: Geometric standard deviation

GSH: Glutathione

HPLC: High performance liquid chromatography

ICAM-1: Intercellular adhesion molecule-1

IFN-γ : Interferon gamma

iNOS: Inducible nitric oxide synthase

LM: Lipid microparticles

L-NAME: Nitro-L-arginine methyl ester

LOX: 5-lipoxigenase

LT: Leukotrienes

MMAD: Mass median aerodynamic diameter

MSLI : Multi stage liquid impinger

NF-E2: Related factor 2

NF-κB: Nuclear factor kappa B

NO: Nitric oxide

PLA_2: Phospholipase A2

pMDI: Pressurised metered dose inhaler

RES: Resveratrol

RNS : Reactive nitrogen species

ROS: Reactive oxygen species

SEM: Scanning electron microscopy

SLM: Solid lipid microparticles

STAT-1: Activator of transcription 1

TGA : Thermal gravimetric analysis

TGF-β1: Transforming growth factor beta

TNF-α: Tumor necrosis factor alpha