Universidad de Valladolid

ESCUELA DE INGENIERÍAS INDUSTRIALES

DEPARTAMENTO DE INGENIERÍA QUÍMICA Y TECNOLOGÍA DEL MEDIO AMBIENTE

TESIS DOCTORAL:

INNOVATIVE PRODUCT DEVELOPMENT FROM THE VALORIZATION OF CLINACANTHUS NUTANS LINDAU MEDICINAL PLANT

Presentada por Ana Najwa Mustapa para optar al grado de doctor por la Universidad de Valladolid

Dirigida por:

Dr.- Ing. Ángel Martín Martínez
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Memoria para optar al grado de Doctor,

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Certifica que:

ANA NAJWA MUSTAPA ha realizado bajo su dirección el trabajo *"Innovative Product Development from the Valorization of Clinacanthus Nutans Lindau Medicinal Plant"*, en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

Reunido el tribunal que ha juzgado la Tesis Doctoral titulada *"Innovative Product Development from the Valorization of Clinacanthus Nutans Lindau Medicinal Plant"* presentada por el Ingeniero Químico Ana Najwa Mustapa y en cumplimiento con lo establecido por el Real Decreto 99/2011 de 28 de enero de 2011 acuerda conceder por_____ la calificación de _____.

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PRESIDENTE SECRETARIO

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Summary

The increased public awareness to have safer and healthier therapies has led to a wide use of natural products and drugs derived from plants and, particularly, medicinal herbs. Medicinal plants are a rich source of bioactive compounds that possess significant therapeutic activity such as antioxidant, anti-viral and anti-inflammatory effects. However, the application of the herbal medicines in pharmaceutical industry still faces huge challenges, particularly in terms of the efficiency of the delivery system. Therefore, an innovative study on the enhancement of the extraction and formulation of the herbal medicine is needed.

In this thesis, the valorization of the medicinal plant Clinacanthus nutans (*C. nutans*) by the extraction of high-value compounds and the evaluation of their potential for pharmaceutical applications is studied. The investigation involved the extraction of phytochemical compounds, the formulation of the herbal extracts in dosage form by loading on a mesoporous aerogel matrix, and the determination of their bioavailability.

In the first part, the *C. nutans* plant was extracted by Soxhlet, supercritical fluid extraction (SFE) and microwave-assisted extraction (MAE) method with the aim of determining the most efficient technique for the extraction of the bioactive compounds present in the plant. The phytochemical compounds were screened to determine the major compounds obtained with each method and other significant marker compounds. Results showed that the *C. nutans* extract consist of phytol as the major compound, and important amounts of other high value constituents such as phytosterols (2.36 ± 0.15 mg BS/g DM obtained by direct saponification in MAE) and polyphenols. The highest total phenols and flavonoid content (i.e. 11.30 ± 0.39 mg GAE/g DM and 4.66 ± 0.22 mg QE/g DM, respectively) was obtained with MAE using 50%vol ethanol/water in comparison to other extraction methods. MAE was demonstrated as the most efficient method for extracting the phytochemical compounds from the *C. nutans* with reasonable high yield in comparison to SFE and Soxhlet method, yielding valuable compounds and, particularly, polyphenols.

In the second part, the performance of MAE in enriching polyphenols was investigated by studying its kinetic modelling and optimizing the effect of specific energy absorbed, ethanol/water concentration as the solvent and the solvent-to-feed ratio (S/F) on the polyphenols content of extracts obtained from *C. nutans*. The effectiveness of the MAE pre-

treatment was compared to the conventional method, and it was found that the MAE technique increased the concentration of polyphenols by 2–5 times compared to the classical solvent extraction method. Polyphenols yield was found to be the maximum using a 50%vol ethanol-water solvent mixture at 14 mL/g of the S/F best ratio whilst the Patricelli's model gives excellent profiling kinetics behavior and accurate predictions of polyphenols yield and extraction rate. The microwave pre-treatment has markedly improved the extractability of polyphenols from the medicinal plant and identified as a promising approach to enrich compounds of interest in shorter time.

In the third part, the *C. nutans* extracts derived from the MAE by different ethanol/water concentrations were impregnated into silica and alginate aerogels. In addition, the major compound identified in the previous studies, phytol, was also employed as a model compound to be impregnated in the aerogels and its loading content and bioavailability were studied. The impregnation was carried out with two methods. In the first method, the drugs and *C. nutans* extracts were loaded into the gels by liquid absorption between the solutions of ethanol/compounds into gels that immersed in the solution for certain time. The wet impregnated gels were then dried by CO_2 under supercritical conditions. With this, supercritical CO_2 ($SCCO_2$) extract the ethanol, leaving the compounds deposited in the aerogels during the CO_2 depressurization. Poor solubility of the compounds in the CO_2 results on a high impregnation yield. On the other hand, the drugs and *C. nutans* also were impregnated by method called supercritical impregnation (SCI). In this technique, the compounds were loaded into dried aerogels by the saturated solution of supercritical CO_2/compounds. High diffusivity and low viscosity of the $SCCO_2$ enabled the saturated solution to penetrate into porous structure of the aerogels. Upon depressurization, the compounds entrapped in the aerogels either by deposition or molecular dispersion mechanism. The different mechanisms governing the two methods results in different behavior of the compounds interaction with host matrix as well as their dissolution efficacy.

Results demonstrated that by impregnation via liquid absorption the *C. nutans* and phytol have higher loading content in the alginate (11.5 ± 0.4 and 23.9 ± 1.0 wt% of the extracts obtained with 50% ethanol/water and pure ethanol solvents, respectively) than in silica aerogels

(5.2 ± 1.0 and 13.1 ± 0.9 wt% of the extracts obtained with 50% ethanol/water and pure ethanol solvents, respectively). On the other hand, by SCI, the compounds showed higher loading in the silica (11.5 ± 0.4 and 23.9 ± 1.0 wt% for extracts obtained with 50% ethanol/water and pure ethanol solvents, respectively) than in alginate aerogels. The differences were attributed to the higher specific surface are of the silica compared to alginate aerogels in the SCI, meanwhile the effect of the presence of ethanol in the liquid absorption impregnation stimulate the interaction between the compounds and alginate matrix thus increased the compounds loading. This finding was evidenced from the scanning electron microscopy (SEM). Furthermore, the dissolution tests revealed that the *C. nutans* in the alginate demonstrated sixteen times faster compounds release in 6h compared to the case of the silica aerogel, thereby suggesting that the *C. nutans* extract can have a good bioavailability when loaded in the alginate material. On the other hand, the phytol loaded in the alginate and silica by both impregnation methods showed poor dissolutions from the matrix. This demonstrates that this compound has extremely poor-water soluble regardless any impregnation method or host matrix used. These results motivated to the next task to develop a host matrix that can improve the solubility of the poorly soluble compounds in water.

In the last part, hybrid of β-cyclodextrin/alginate aerogels were synthesized. In this study, the synthesis was only applied to the alginate material due its simple gelation mechanism and because it was compatible with the β-cyclodextrin (βCD) characteristics. The alginate/β-cyclodextrin was prepared as aerogels beads through physical cross-linking, that is an ionotropic gelation mechanism. Two types of the hybrid aerogels i.e. core and floating beads were produced. In the preparation of the gels, based on the solubility data of the β-cyclodextrin in water, a saturated solution of βCD/alginate and βCD/alginate/$CaCO_3$ mixture was prepared and extruded into two different gelation bath solutions yielding the core and floating beads. The aerogels beads were dried by $SCCO_2$ and subsequently undergone supercritical impregnation with phytol. Results demonstrated that in the presence of βCD the loading capacity of phytol was slightly improved from 54.4 ± 0.5 wt% to 57.3 ± 1.2 wt% in the core beads whereas in the floating beads the loading increased from 56.7 ± 1.5 wt% to 60.5 ± 0.9 wt% of phytol. The release of phytol from the core and floating hybrid aerogels were

significantly improved three-fold and six times higher, respectively, in comparison to the release from non-hybrid aerogels. This indicated that the developed new host matrix successfully improved the solubility of the compounds with poor aqueous solubility.

In overall, this thesis presents the research works on the valorization of the *C. nutans* plant as herbal drug in order to investigate its applicability in drug delivery systems for pharmaceutical industry. The findings revealed that the medicinal plant has a great potential in pharmaceuticals industry as alternative medicine drugs, promoted by the intensification of extraction provided by microwave technology, the exploitation of supercritical fluids technology, and the use of aerogels as matrix for drug delivery.

Introduction

1.0 Overview of herbal medicinal plants

Herbal medicine derived from the plants has been used since ancient times to cure a variety diseases as well as to prevent disease to promote good health, as it provides low toxicity and good therapeutic effects. Medicinal plants are a rich source of numerous therapeutic compounds that have tremendous applications in pharmaceutical industry. The use of herbal medicine and natural products in medical and pharmaceuticals has received growing attention worldwide due to increasing public health concern on safety and quality of the drug therapies, and as alternative to modern drugs treatments. It is estimated that more than 50% of all drugs in modern therapy nowadays are derived from natural products or medicinal plants [1]. According to World Health Organization (WHO), about 80% of population in Africa or in other developing countries exploits traditional plants as their main source of medicine [2-4]. Furthermore, herbal remedies has also been widely accepted in developed countries [5-8].

Medicinal plants contain various organic compounds called secondary metabolites that are derived from secondary metabolism. This includes terpenoids, alkaloids, steroids, glycosides, flavonoids and phenols with attractive therapeutic activities. Herbal medicines can treat many health conditions such as asthma, allergies, migraine, diabetes, cardiovascular, skin irritation such as eczema, rheumatoid arthritis, Alzheimer's disease, Parkinson’s, premenstrual symptoms, fertility care, chronic fatigue, and for cancer therapies such as for lung, colon and kidney which taken as complementary and alternative to conventional treatment [9-15].

There is a wide range of herbal extracts including grape seed, green tea, *Ginkgo Biloba*, hawthorn, chamomile, ginger, garlic, curcumin, St. John's wort and many more. The herbal plants may different to each other’s by different climatic, geographical and ethnological properties [16] and can be classified as Western herbs, South Central America herbs, Native America herbs, South Africa herbs, Ayurveda herbs and Chinese herbs [17]. In Southeast Asia, more than 20 medicinal plant species have been cultivated in Thailand, Vietnam, Indonesia and Malaysia for research and development purposes. In particular, in Malaysia about 22 herbal species have been extensively planted for research activities, including *Eurycoma longifolia* (Tongkat Ali), *Labisia pumila* (Kacip Fatimah), *Orthosiphon aristatus* (Misai Kucing), *Ficus deltoidea* (Mas Cotek) and *Clinacanthus nutans* (Belalai Gajah). Some of these herbal plant

have reached commercialization stage. In fact, these crops have been highlighted as primary objective for product development under National Key Economic Areas (NKEA) plan with the focus of producing high-value product and commercialization [18, 19].

1.1 Clinacanthus nutans Lindau (*C. nutans*)

Clinacanthus nutans Lindau (*C. nutans*) (Figure 1) is a medicinal plant belonging to the family of Acanthaceae, commonly called as Sabah Snake Grass or Belalai Gajah. It is grown in the Asia tropical region, mainly in Malaysia, Thailand and Indonesia. At present, the plant has been widely used in different regions in Asia as alternative remedies of traditional medicine to treat hypertension, diabetes, fever, skin rashes, gout and diabetes mellitus. The plant leaves usually are boiled in water or blended with other ingredients and consumed as herbal tea or juice drink. In previous works, it has been demonstrated that plant extracts were capable of treatment of herpes infection, allergic responses and snake bites [20-22]. Scientific reports have claimed that that *C. nutans* extracts possess antimicrobial, anti-viral activity and anti-inflammatory effect against herpes simplex virus (HSV) and varicella-zoster virus (VZV) lesions [20, 23-25]. Recently, the plant has attracted numerous researchers due to some alleged activities for cancer treatment [26-28].

Figure 1 Clinacanthus nutans Lindau (*C. nutans*)

Studies on the phytochemicals revealed that the medicinal plant contains several important bioactive constituents including β-sitosterols and lupeol [29], stigmasteols [23], belutin [30], flavonoids such as vitexin, isovitexin, isoorientin, orientin, six known *C*-glycosyl flavones, isomollupentin 7-*O*-b-glucopyranoside and isoshaftoside in different extraction solvent used such as n-butanol, methanol and water [31, 32].

In addition, isolation of the acetate-soluble fraction of plant extract ethyl allowied discovering a mixture of cerebrosides and a monoacylmonogalactosyl glycerol (2S)-1-*O*-linolenoyl-3-*O*-b-dgalactopyranosylglycerol [22]. Meanwhile, by isolation of hexane and chloroform extract compounds of 13-hydroxy-(13-*S*)-phaeophytin b, pupurin-18-phytyl ester and phaeophorbide-a have been identified[33], whereas trigalactosyl and digalactosyl diglycerides that showed the highest inhibitory activity against herpex simplex virus (HSV) derived from the extracts [25].

Furthermore, the chemistry of primary metabolites of chlorophyll has also been investigated. Phaeophytin, i.e. chlorophyll-derivatives compounds that has the structures related to chlorophyll a and chlorophyll b, was identified as 13^2-hydroxy-(13^2-R)-phaeophytin b, 13^2-hydroxy-(13^2-S)-phaeophytin a and 13^2-hydroxy-(13^2-R)-phaeophytin a in the isolated chloroform extract [20]. These compounds have shown anti-viral activity against herpex simplex infection.

Cyctoxicity tests of ethanolic extracts of *C. nutans* leaves on mice at 1.3 g/kg of body weight (bw) did not showed any abnormalities on the internal organs of the mice [34]. In other analysis, mice that received orally administrated extracts of *C. nutans* leaves obtained with methanol with concentration ranging from 300 to 900 mg/kg bw did not show any adverse effects and damage on liver and kidney of mice. It was indicated that 900 mg/kg consumed to mice is equivalent to 9 mg/kg in human [35]. Furthermore, analysis of the *C. nutans* extracts on nervous system demonstrated that *C. nutans* was be able to modulate cholinergic neurotransmission by stimulating the nerves enzymes activity i.e. acetylcholinesterase in mice kidney, liver and heart without any changes or adverse effect on organ behavioural. In these tests, the extracts were orally administrated to mice at various amounts ranging from 250 to 1000 mg/kg bw [36].

It has been reported that the *C. nutas* extracts was extracted in traditional way i.e. using organic solvent such as ethanol, methanol, chloroform, hexane, dichloromethane, petroleum ether, ethyl acetate by Soxhlet extraction or maceration method [20, 27, 35, 37]. Nevertheless, this conventional extraction technique often use large amount of organic solvent, it is time-consuming, tedious and restricted by the selectivity of the solvent used [38]. In addition, degradation of thermal-sensitive phytocompounds also may occur since the samples are extracted at the boiling point of the solvent over long periods of extraction time. Furthermore, further purification of the extracts could lead to products contamination as well as waste disposal problems.

1.2 Extraction methods

Over the past decades, established modern extraction method such as supercritical fluid extraction (SFE) have been used to extract vegetable oils [39, 40], essential oils [41, 42], microalgae [43, 44] or to recover high valuable compounds from solid [45-47]. Carbon dioxide is the most common solvent employed in the SFE process and it is technically approved as non-toxic, non-flammable, inert and safe for food and pharmaceuticals-based applications. Low critical temperature at 31.1^{o}C allow its use for thermolabile and easily oxidized compounds. Furthermore, low boiling point of CO_2 at atmosphere condition is an advantage for the SFE method as the CO_2 can easily be removed and leaves no residues in the products. Another advantages of the SFE is that the dissolving power of the CO_2 and its selectivity can be controlled by proper selection on the temperature and pressure. Carbon dioxide is non-polar solvent, thus co-solvent or modifier such as ethanol can be used to increase its dissolving power over polar samples compounds [48]. All these advantages of the SFE coupling with the unique properties of CO_2 brought this method to be widely applied in diverse fields as well as commercialized in industry.

Another promising extraction process is microwave-assisted extraction method (MAE). This method has been recognized to offer many advantages over traditional technique such as less solvent consumption, less tedious laboratory work and special heating mechanism lead to high products yield in shorter extraction time. In principle, the MAE exploits microwave

energy, dissipation factor and dielectric constant of the solvent and materials to extract target compounds from various matrix. In classical method, energy transfer from the heating process involves convection and conduction to the materials, which are driven by the thermal gradients [49]. In contrast, the microwave heating process is governed by the energy conversion from microwave as source to thermal energy [50]. The microwave energy directly acts on the materials through molecular interaction with the electromagnetic field. This leads to localised heating in solid and sample caused enormous expansion as well as rupture of cell walls. Hence, the solutes desorb and diffuse out from the matrix towards bulk organic solvent.

The heating efficiency is dependent on the dielectric constant (ε') and dissipation factor (δ) to absorb and dissipate energy to surrounding molecules [51]. High values of ε' and δ result in better conversion from microwave to thermal energy. Nevertheless, other factors also important roles such as solvent, moisture content, solvent-to-feed ratio, power and temperature [51]. Optimization of these factors can maximize the extraction yield.

1.3 Formulation of herbal medicine in drug delivery

Until today, although herbal medicine has been widely recognized and has been used over years, however, systematic approach to measure their safety [52] and the efficacy of its absorption are still a challenge in pharmaceutical industry [53]. One of the reasons is due to the complexity of the chemical medicinal extracts. Most of the biologically medicinal extracts constituents such as flavonoids, glycosides, alkaloids, tannins and terpenoids have good water-solubility, but also poor ability to cross lipid membrane of intestine because of low lipid-solubility or having large molecules size, resulting in low absorption and hence poor bioavailability and efficacy [54, 55]. On the other hand, several phytochemical compounds are pH sensitive and can easily metabolized by the liver before reach stomach. This results to the low level of drug dosage in the blood than the required by the body and indicated as no therapeutic effect [53].

Nowadays, many scientific research effort on designing novel drug delivery system for herbal constituents in order to increase the therapeutics properties with reduced toxicity and improved bioavailability [56, 57]. The bioavailability of the low absorption natural products

can be improved with the use of novel drug delivery systems such as micelles, liposomes, nanoparticles, solid dispersion, microemulsions, solid-lipid nanoparticles and matrix systems including polymer or biopolymer [58-62]. The purpose of the development of drug delivery system for the herbal extracts over conventional method consumption are to enhance pharmacological activity and to improve bioactive compounds stability. In addition, drug vehicles play important roles to improve the solubility of the drugs, to minimize chemical and physical degradation of the bioactive compounds, and to be able to control the absorption of the active ingredients to the target sites with appropriate release while maintaining their therapeutic effect properties.

Some developments on the uses of herbal extracts combined with drug delivery system have been reported at laboratory level and some have reached market industry. For example, a US patent by Blatt, Kimmelman, Cohen and Rotman [63] described formulations for the controlled release or stable storage of granulated herbs and comprising microencapsulated granulated herbs with at least one carrier or excipient in order to improve the herbal preparation in oral dosage comprising granulated herbs (powder). The formulation showed released up to 75% of the active ingredients in 4 to 18h of the dissolution test. Another patent by Marechal, Yang and Yuzhang [64] claimed the sustained release of microgranules containing Ginkgo Biloba extract homogenized with pharmaceutical excipients and coated by cellulosic polymer via extrusion-spheronization, fluid air bed process or a coating-pan method. Application of the herbal delivery via transdermal also has been reported. Verma, Gupta, Varsha and Purohit [65] formulated a transdermal film incorporating herbal drug components i.e. boswellic acid (*Boswellia serrata)* and curcumin (*Curcuma longa)*.

There are several known ways to increase drugs bioavailability and release rate, for example delivery by lipids system, soft-emulsifying system, particle size reduction, solids dispersion with fillers and modification of drugs crystalline structure [66, 67]. Other alternative strategy to improve bioavailability of bioactive compounds is the use of aerogels as polymer or biopolymer matrix and deliver the compounds by oral administration to the target sites. One example is a patent by Attia [68] described drug delivery mechanism by using polyethylene glycol (PEG) as aerogels particles for cancer therapy. The aerogels containing drugs were

functionalized to release the drugs on the disease cells, thus reducing side effect while permitting high local drug concentration [69].

1.4 Aerogels as drug delivery system

Aerogels are porous structure materials derived from either synthetic inorganic precursor for example, silica or from organic substance such as polymer/biopolymer materials by method of sol-gel to produce wet gels (alcogels or hydrogels) and dried by supercritical drying. The wet gels are containing large portion of pore liquid content and are require to undergone solvent exchange procedure during the aging process to remove excess of reactants, replace water content and strengthen the gels structure prior to the supercritical CO_2 drying (SC drying) at pressure near 90 bar and temperature 40^{o}C in certain time [70]. Under the SC drying, the pore-filling liquid are extracted to produce three-dimensional network with highly porous structure gels. Low viscosity and high diffusivity of the supercritical CO_2 preserve the porous structure from damage and produce aerogels with attractive properties such as low-density, high porosity (~98%) and high surface as high as 1200 m^2/g. This interesting characteristics allows its use in wide range of application such as thermal insulation [71], catalyst [72], chemical sensor [73] and biomedical [74] and pharmaceuticals particularly for drug delivery system (DDS) [75, 76]. In the DDS application, aerogels have demonstrated great potential due to the large surface area and porosity that lead to high drugs loading.

Many works have reported on the use of silica aerogels to enhance the solubility of drugs for oral [76-78] or transdermal drug delivery [75, 79]. Bioavailability rate and release rate of drugs are prospective to increase through impregnation of the active pharmaceutical ingredients (APIs) or drugs into aerogels [80]. The impregnation of drugs into aerogels can done via three methods: 1) during the sol-gel i.e. before the formation of gel, 2) during aging process i.e. at the end of solvent exchange of alcogels and 3) post treatment of the dried aerogel called as supercritical impregnation. In the first method, the drug is dissolved in precursor solution and expected to be trapped within the gel network after the addition of chemical cross-linker or changing the physical condition of the sol-gel solution such as pH or temperature. It is important to take into account of any possibility on the physical or chemical modification

which may occur to the drugs or bioactive compounds that sensitive to pH or temperature effect. The synthesized gels are proceeded to solvent exchange prior to the SC drying to produce aerogels.

In the second method, alcogels are immersed into contact with organic solvent containing drugs prior to the SC drying. The drug will diffuse into the pores of alcogels from the organic solution due to the concentration gradients until reach equal concentration in the pores and in the solution. The diffusion rate depends on the size of the drug molecules, pore sizes and initial concentration of the drug solution [81]. Subsequently, the impregnated alcogels are dried by SC drying to extract the solvent and leaves the drug deposited in the pores of the aerogels. For successful of this method, the organic solvent must have good miscibility with the drug and the $SCCO_2$ whereas the drug must be insoluble with the $SCCO_2$ to avoid drug leaching from the aerogels matrix during the SC drying.

Supercritical impregnation (or adsorption) is a method where the drug is incorporated into aerogels under supercritical conditions. In this technique, the drug is solubilized into the $SCCO_2$ at specified temperature and pressure and contacting the result mixture with the aerogels matrix materials for certain time. During the contact, the supercritical CO_2-drug mixture is diffuses into the pores of the aerogels and the drug is adsorbed into the surface of the matrix. Upon the depressurization, the drug precipitated and trapped within the aerogels matrix pores. [82, 83]. Drugs impregnated by this technique resulted in increase the specific surface area of the adsorbed drug [84] and modification on the drugs structure from crystalline to amorphous in the aerogels [85]. This was found to improve the release of poorly soluble drugs at a significant level in comparison to the conventional drugs [69, 86].

Apart from the use of silica aerogels, polysaccharides also have been widely applied for drug delivery, including aerogels made of alginate [87, 88], starch [89], pectin [90], β-glucan [91], cellulose [92, 93] and others. These biomaterials has gained increasing attention due to demands from pharmaceuticals for biodegradable, biocompatibility and safer carrier materials. In addition, simple gelation formation of the polysaccharides allow its use extensively in many other fields.

Numerous studies have demonstrated the impregnation of model drugs such as ibuprofen, paracetamol [89], ketoprofen and benzoic acid [87] nicotinic acid [94], vitamin D [95]. The drug loading and its release rate are highly dependent on the material of the matrix, structural properties such as surface area, pore size and pore volume, its stability in water and drugs loaded characteristics in the aerogels [89]. The incorporation of the drugs into polysaccharides aerogels had improved the bioavailability rate and release kinetics either for fast or sustained release purposes. In addition, impregnation of bioactive compounds such as thymol [96] or flax oil [97] also are anticipated open its potential as carrier for food packaging and nutraceuticals delivery. In overall, performance of the conventional drugs has been proven enhanced by incorporating with aerogels as vehicles in the development of promising and novel drug delivery system.

2.0 Outlook of *C. nutans* potential as herbal drugs

It has been highlighted that the *C. nutans* plant has a great potential as alternative or complementary medicine to modern therapy in pharmaceutical industry. Nevertheless, the application of *C. nutans* as high-value natural product are not extensively explored. In fact, data on the best extraction method of the key bioactive compounds are never reported. In addition, knowledge on the bioavailability of the *C. nutans* extracts are also essential in order to understand the efficacy of the herbal delivery. From the information available in the literature, it is anticipated this medicinal plant will benefit people healthcare. However, the challenge of the medicinal plants in the pharmaceutical application must be taken into account. Herbal formulation for drug delivery might enhance the extracts stability, protect from toxicity, improve the therapeutic efficacy and provide sustained release behaviour. A wise strategy and thorough scientific work must be planned to open chances of the plant to be fully commercialized in herbal market industry.

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Objectives

Clinacanthus nutans (*C.nutans*) medicinal plant has been known as a high value herbal product, source of complementary natural drugs in modern therapies, and it is foreseen that it could contribute to developments in public's healthcare and in the pharmaceutical industry. Nevertheless, the potential applications of this plant still are debatable due to the lack of fundamental knowledge of aspects such as efficient extraction methods and the bioavailability of the extracted compounds. Therefore, the main objective of this work is to establish the bioactive compounds of interest that can be extracted from *Clinacanthus nutans* Lindau (*C.nutans*), to develop appropriate methods to extract these compounds, and to formulate the extracts in a suitable drug delivery system for pharmaceuticals industry. This study involves the following key steps:

I. Characterization of the *C. nutans* plant extracts, identifying and quantifying the phytochemical compounds available in the plant extracts and selecting key compounds of interest for pharmaceutical applications.

II. Investigation on the intensification of the extraction technique in comparison to the conventional solvent extraction method, aiming on high extraction yield with optimum conditions, investigating alternative extraction techniques including supercritical fluids extraction (SFE) and microwave-assisted extraction (MAE) at different process conditions.

III. Development of a suitable formulation of the medicinal plant extracts into solid dosage form with different carrier materials for oral administration, evaluated through *in-vitro* dissolution study in order to determine the behaviour of the extracts release, applying conventional and novel, supercritical CO2 assisted technologies.

IV. Study on the possible limitations of the applicability of the developed formulations and proposals for improvement, designing new strategies of drug delivery system to enhance the oral bioavailability of poorly soluble drugs, with the objective of synthesizing an innovative drug carrier with suitable excipients to improve the solubility and

bioavailability of the drugs. As the herbal medicine extract may contain insoluble water compounds due to its complex phytochemical composition, thus this research is very important for future development.

Chapter 1

Extraction of phytocompounds from the medicinal plant *Clinacanthus nutans* Lindau

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Abstract

The composition and bioactivity of natural plant extracts strongly depends on the extraction technique employed. Clinacanthus nutans Lindau (C. nutans) is a well-known medicinal plant in South-East Asia that has been traditionally used for treatment of hepatitis, skin-rashes and snake venom poisoning, and recently has attracted attention for its applications for treatment and prevention of cancer diseases. In previous studies, the extraction of bioactive compounds from C. nutans by conventional Soxhlet solvent extraction has been described, but this method shows limitations in terms of selectivity, extraction yield and toxicity of the solvents employed. In this study, phytochemical compounds were extracted from leaves and stems of C. nutans by microwave-assisted extraction (MAE), pressurised microwave-assisted extraction (PMAE), supercritical carbon dioxide extraction (SFE) and Soxhlet method to investigate the best technique in terms of yield, extraction time and recovery of bioactive compounds: phenols, flavonoids, phytosterols and β-sitosterol. The extracted phytocompounds and phenolics were characterized by Gas Chromatography Mass Spectrometry (GC/MS) and Ultra Performance Liquid Chromatography (UPLC). The results showed that MAE was the best technique to achieve a high yield and a maximal total polyphenol content (11.30 ± 0.39 mg GAE/g DM) and flavonoids content (and 4.66 ± 0.20 mg GAE/g DM), whereas SFE was the best method for phytosterols and β-Sitosterol extraction. P-MAE merely enhanced the polyphenol and flavonoids yield to 14.56 ± 0.77 mg GAE/g DM and 5.29 ± 0.30 mg QE/g DM respectively, without significant variations on the type of compounds obtained. MAE appears as the most efficient technique for the extraction of phytochemical compounds from C. nutans in a short time with a reasonable yield and a good selectivity towards bioactive nutraceutical compounds, with high concentrations of antioxidants, anti-inflammatory and antimicrobial compounds.

1 Introduction

Clinacanthus nutans Lindau (*C. nutans*), commonly known as snake grass, is a medicinal herb belonging to the family of Acanthaceae widely grown in the tropical region, mainly in Southeast Asia. It has been traditionally used as herbal medicine for treatment of herpes infection, insect and snake bites and allergic responses [1-3]. Its uses in traditional medicine have been scientifically supported by numerous studies that demonstrate that *C. nutans* extracts show anti-inflammatory, antimicrobial and anti-viral activity against herpes simplex virus (HSV) and varicella-zoster virus (VZV) lesions [1, 4-6]. In Thailand, the plant has been accepted as an essential medicinal herb for primary healthcare by the Ministry of Public Health of the country after extensive research on the *C. nutans* medicinal properties [2]. Moreover, in recent years, *C. nutans* has attracted considerable research interest due to its alleged properties for cancer treatment [7-9].

Various factors must be examined in order to determine the effectiveness of medicinal herb extracts. Among them, the extraction technique employed to obtain the bioactive compounds from the plant is a key factor. This is because the efficiency of extraction of different bioactive compounds from plant materials is influenced by several factors such as solvent polarity and concentration, solvent-to-feed ratio, extraction time and thermal degradation. In addition, a reasonable sample preparation is also very important to prevent the deterioration of the plant extract. For example, the use of activated charcoal to remove interferences such as chlorophyll in plant extracts, reported in some previous works, should be avoided. We have found in preliminary experiments of this work that treatment with activated charcoal eliminates many phytocompounds from the medicinal plant, thus reducing the potential biological activity of the extract.

In previous studies, C. *nutans* was treated by Soxhlet technique using methanol, chloroform, ethanol and hexane as solvents. Depending on the solvent employed, the bioactive constituents of *C. nutans* extracts were discovered to comprise stigmasterol, β-sitosterol , lupeol [10], betulin [11], six known C-glycosyl flavones, vitexin, isovitexin, shaftoside, isomollupentin, 7-O-β-glucopyranoside, orientin, isoorientin [12], sulphur containing

glucosides [13], glycoglycerolipids, a mixture of nine cerebrosides, monoacylmonogalactosylglycerol [3].

From these previous studies it can be concluded that to date, only simple maceration and Soxhlet extraction techniques have been reported for extraction of bioactive compounds from *C. nutans* medicinal herbs. In this work, it is hypothesised that the composition and properties of the extract are highly dependent on extraction method as well as on the solvent employed. The application of enhanced extraction techniques with non-toxic solvents (supercritical carbon dioxide extraction and microwave-assisted extraction MAE with ethanol-water solvent mixtures) on *C. nutans* is reported for the first time and compared with the conventional Soxhlet extraction method. Supercritical fluid extraction is a well-known method for producing high quality plant extracts in a safe and clean way. On the other hand, MAE extraction has been developed as a simple, faster and less solvent consumption method for the production of high valuable extracts from plant materials. In addition, we introduced the use of pressure in microwave-assisted extraction (P-MAE) to further enhance the extraction of phytocompounds from the medicinal plant. Theoretically, increases of pressure and the corresponding increments in extraction temperature could increase the solubility of the bioactive compounds in the extracting solvent.

The aim of this study is to determine the best technique for the extraction of phytocompounds from *C. nutans*, considering the extraction yield and the content on significant phytocompounds: phenols, flavonoids, phytosterols and β-sitosterol. Chlorophyll content in the *C. nutans* also was determined as complement to the phytocompounds characterization regardless the effect of extraction technique. In addition, the phenolic compounds in *C. nutans* extracts were characterized by Ultra-Performance Liquid Chromatography (UPLC) coupled to electrospray ionization and quadrupole-time of flight-mass spectrometry (ESI-QTOF/MS). This study is the first work reporting on the effect of modern extraction techniques on the phytocompounds and the characterization of phenolic compounds present in *C.nutans* extract based on the UPLC-ESI-QTOF/MS analysis.

2. Experimental

2.1 Materials

β-sitosterol (analytical chromatography grade), gallic acid, quercetin (analytical grade) and ethanol (96%) were purchased from Sigma-Aldrich Co. (Spain). Water was purified by a Milli-Q water purifier system from Millipore (Milford, MA, USA). The Folin-Ciocalteu reagent was obtained from Merck (Darmstadt, Germany). Dried Clinacanthus nutans (Burm.f.) Lindau samples were purchased from Mr Lee Huat Lye, Kuala Lumpur, Malaysia.

2.2 Sample Preparation

The total moisture content of leaves and stems was determined gravimetrically by air drying in an oven set at 105 ^{o}C for 24 hours. Prior to extraction, samples were dried in the oven at 60 ^{o}C for 2 hours in order to reduce the moisture content, ground and sieved to a particle size in the range of 500 to 100 µm. The prepared samples were then stored in airtight bags, swept with nitrogen gas and kept in a refrigerator (-8 ^{o}C) until used in extraction experiments.

2.3 Microwave-assisted extraction (MAE) and Pressurised-MAE (P-MAE)

MAE and P-MAE were carried out in a laboratory CEM Discover® microwave oven (300 W maximum power) operating at a frequency of 2.45 GHz, using ethanol-water solvent mixtures. The microwave oven was equipped with an optical fibre probe to measure the real temperature profile during the extraction process. MAE was operated as an open system whereas P-MAE was performed in closed system. Microwave irradiation in P-MAE was terminated when 2.7 bar of pressure build-up in the system was achieved. The parameters studied were the concentration of the ethanol-water solution (44 – 86 %vol) with a solvent-to-feed ratio of 14 g/g, at a constant power of 300 W. The extraction time (5, 10, 15, and 20 s) was determined in preliminary experiments to find an appropriate relation of temperature-time at a fixed irradiation power and to identify a sufficient time to achieve a temperature close to the boiling point of the ethanol-sample system at a constant irradiation power of 300W. From

the preliminary study, it was found that 15 s was sufficient to achieve ethanol's boiling point at 300W based on solvent-to-feed ratio of 14.

In extraction experiments, about 1.0000±0.0002g of *C. nutans* sample was mixed with 14 mL of ethanol-water solution (44 – 86%vol) in a 100 mL round-bottom flask. The mixed ethanol-sample material system was kept under magnetic stirring for 3 minutes to allow the samples to be soaked by the solvent. This promotes the diffusion of the solvent into the sample matrix and improves the mass transfer of active compounds into the solvent. During the microwave irradiation, the ethanol-sample was constantly stirred using a magnetic stirrer to avoid the formation of hot spots within the sample, thus homogenizing the temperature of the mixture during the extraction process. After irradiated by MAE, the sample was rapidly cooled down to 40^{o}C in an ice bath, 6 mL of cold fresh ethanol was added to the sample and the experiment proceeded with a conventional extraction in warm water (40^{o}C) for 80 minutes. Prior to the sample collection, nitrogen gas was flowed into the amber vials used to store the sample to remove air. Extracts were subjected to filtration using 0.20 µm PTFE to remove solid residues before storage.

2.4 Supercritical fluid extraction (SFE)

The supercritical fluid extraction of *C. nutans* was carried out over 80 g of *C. nutans* (7 – 8 %*wt* of moisture) at 350 bar and 60^{o}C in a custom-made SFE setup (Fig. 1). The system consisted of an extraction vessel made of stainless steel with a volume of 4 L, height of 49.5 cm and inner diameter of 9.7 cm. Pressure in the vessel was regulated by means of a back-pressure GO valve installed in the line between the extraction vessel and the separator. The reduction of pressure with this valve causes the transition from a supercritical to a gaseous CO_2 state, and therefore the separation of the compounds extracted from CO_2 by condensation and precipitation. The separator was a vessel of 2.5 L, designed to generate a cyclonic flow pattern and equipped with a heating/cooling jacket that can be used to adjust the temperature desired for an optimum separation of the extracted compounds from CO_2. CO_2 was pressurized by means of a diaphragm pump. The system operated in a closed CO_2 circuit, and gaseous CO_2 leaving the separator was condensed in a cooler operating at -25^{o}C and recompressed with the

pump into the extractor. Temperature and pressure conditions in the extraction apparatus were monitored through probes connected to a Picolog data acquisition software, and the amount of CO_2 consumed was measured by a Coriolis gas flowmeter.

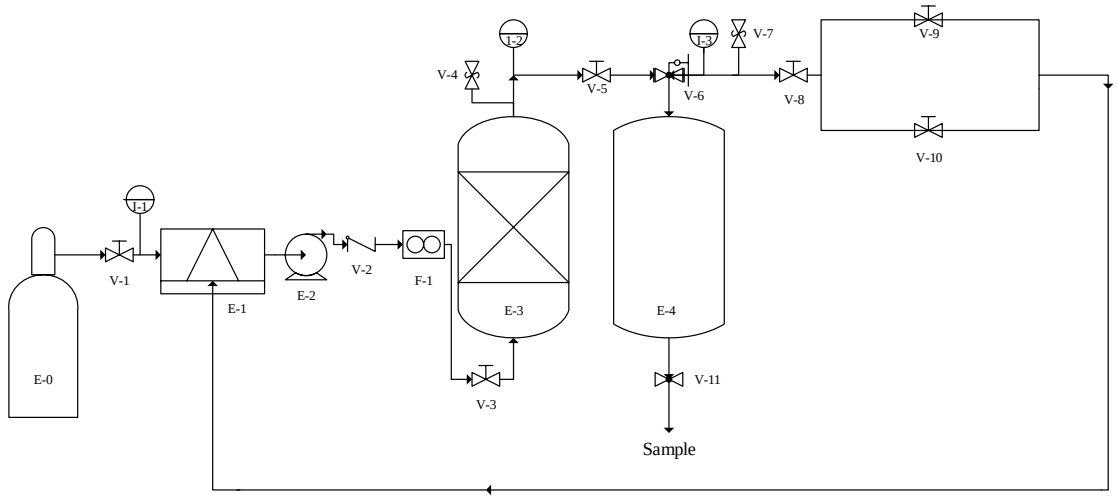

Figure 1 Supercritical fluid extraction plant. E-0: CO_2 tank, E-1: refrigerator, E-2: pump, E-3: extractor, E-4: separator, F-1: gas flowmeter.

In a SFE experiment, the extraction vessel was filled with the dried sample of *C. nutans,* covered at top and bottom with a mesh filter. Before the extraction started, the apparatus was flushed with CO_2 to remove air from the system, and afterwards the extraction vessel was filled with CO_2 up to the desired operating pressure and temperature (350 bar and 60ºC). After reaching these conditions, the back pressure valve between extractor and separator was regulated in order to maintain the conditions at the separator at 50 bar and 40ºC to achieve an efficient extract-CO_2 separation. The extraction proceeded for a total time of 120 min with a CO_2 flowrate of 9 kg/h. Finally, the system was depressurized and a sample of the extract was taken from the separator. The sample was collected in an amber vial to protect it from light, weighed and stored in a refrigerator (-8ºC) until analysis.

2.5 Soxhlet extraction

Solvent extraction was carried out in a Soxhlet apparatus for 8 hours using 80 mL of absolute ethanol over about 3.0 g of dry plant. Afterwards, extracts were subjected to rotary vacuum evaporation (Heidolph) at 40ºC to remove the solvent from the oil. The extracts was weighed and stored in an amber vials and kept in a refrigerator (-8ºC) until analysis.

2.6 Gas Chromatography Mass Spectrometry (GC/MS) Analysis

The phytochemical composition of *C. nutans* extract was analysed on an Agilent Gas Chromatograph Model 6890 (Agilent Technologies, J&W Scientific Products, Palo Alto, CA, USA) equipped with an Agilent 122-7032 capillary column, DB-WAX (30m x 0.25mm x 0.25µm) and coupled to a mass selective detector (MSD5973) working at 70 eV of ionization voltage. Helium was used as a carrier gas at 1.0 mL/min with injection in splitless mode. The oven temperature was programmed as follows: 110ºC held during 3 min, then increased to 200ºC at rate of 5ºC/min, then increased to 250ºC at rate of 10ºC/min and finally maintained at 250ºC for 10 min. Components identification was made based on comparison of their mass spectra with those in Wiley Registry of Mass Spectral Data, 7th edition (Agilent Technologies, Inc.) and National Institute of Standards and Technology 05 MS (NIST) mass spectral library data.

2.7 High Performance Liquid Chromatography (HPLC) Analysis

A reversed-phase and isocratic HPLC method was set up to analyze the β-sitosterol content in extracts. The HPLC apparatus consisted of pump (515 HPLC Pump Waters), Waters 717 Plus Autosampler, Waters 432 Conductivity Detector, Waters 2487 Dual λ Absorbance Detector and column (Symmetry C18 5µm, 4.6 x 150 mm). The mobile phase was methanol/2-propanol (4:1 v/v) at a flowrate of 0.7 mL/min and the injection volume was 20 µL in each analysis. β-sitosterol was detected by an Evaporating Light Scattering Detector (ELSD) and the chromatogram was monitored at a UV wavelength of 210 nm. In order to quantify the amount of β-sitosterol in the sample, a linear calibration curve was developed based on standard solutions of β-sitosterol at six different concentrations, in the range 50 – 500 mg/L. About

0.005 g of oil extracts from SFE experiments was diluted with methanol (4 mL). For MAE sample, the extracts were subjected to saponification technique prior to the HPLC analysis.

2.8 Chlorophyll Analysis

Clinacanthus nutans (*C. nutans*) has been reported to contain chlorophyll a and b (Sakdarat et al., 2009). To determine the total chlorophyll content in *C. nutans*, ethanol (abs) and acetone (abs) solvents were used in the MAE at 300 W for 15 s. The sample extracts were filtered and subjected to UV-Vis spectrophotometer detection. Total concentrations of chlorophyll a and b were determined by measuring the absorbance using a UV-Vis Spectrophotometer. The equations proposed by Wintermans and de Mots [14] for ethanol and by Wellburn [15] for acetone were used to evaluate the chlorophyll concentration.

2.9 Total phenol content (TPC) analysis

The total phenolic content in the SSG extracts was determined by the Folin–Ciocalteu colorimetric method and expressed as Gallic Acid Equivalents (GAE) per gram of dry material (mg GAE/g dried material, DM) [16]. For this, 40 µL of sample extracts were mixed with 3 mL of distilled water followed by addition of 200 µL of Folin-Ciocalteu reagent under gentle stirring. After 5 minutes, 600 µL of saturated Na_2CO_3 solution was added, shaken gently and incubated in a warm water bath (40^{o}C) for 30 minutes. Absorbance was measured at 765 nm (UV- 2550 Shidmadzu UV-Vis Spectrometer). Standard solutions of known concentration of gallic acid (50–900 ppm) were used for calibrtion.

2.10 Total flavonoids content

Prior to the flavonoids determination, it is necessary to eliminate chlorophyll from ethanolic extracts as it causes interference. Some authors suggest to use activated charcoal to eliminate the chlorophyll colour. However, from our preliminary trials we found that the use of charcoal for more than 2 h to remove any interference traces including chlorophyll in the *C. nutans* extract significantly reduced the phenols content from 5.54 to 1.51 mg GAE/g dried material (DM). Thus, use of charcoal may cause deficiency on the therapeutic strength as some

bioactive compounds from medicinal plant extract could be absorbed. In fact, it has been established that charcoal has been used as antidote for substance poisoning including alkaloid [17].

Thus, to remove chlorophyll in this work the ethanolic extracts were subjected to liquid-liquid extraction by adding hexane in the ratio of 5:6 (v/v) for extract:hexane. The mixture was shaken vigorously and the phases were allowed to separate. The bottom layer that contained flavonoids was recovered to proceed with the flavonoids analysis. A method performed by Sólyom et al. [16] was referred where 1 mL of *C.nutans* extract was taken into a test tube and mixed with 300 µL of 5% sodium nitrite solution ($NaNO_2$). After 5 min, 500 µL of 2% aluminium chloride solution ($AlCl_3$) was added into the test tube followed by the addition of sodium hydroxide (NaOH) after 6 min of equilibration. The mixture was thoroughly mixed during 10 min and diluted with 10 mL with MilliQ water. The absorbance of the reaction mixture was then measured at 510 nm by UV-VIS Spectrophotometer against a blank which was prepared with the same procedure as samples. The total flavonoids content was calculated from a standard calibration curve (100 – 500 mg/L) and the results were expressed as quercetin equivalent, mg quercetin (QE) /g dried material (DM).

2.11 Total phytosterols content

The total phytosterols content in *C.nutans* extracts was determined according to the method described by Araújo et al. [18] with slight modifications. Prior to the analysis, the extracts were subjected to alkaline saponification to cleave the acetal bond between the phytosterol and the carbohydrate moiety [19]. In this work, a direct saponification was performed as done by Xiao et al. [20] with slight modification, under microwave irradiation of 20 ml of 1.5 mol/L ethanolic of KOH at 300 W within 15 s. Unsaponifiable matter was removed by two consecutive liquid-liquid extractions with hexane (5 mL) and washed with 0.5 M KOH (2×5 mL) and 2×5 mL of MilliQ water. Hexane was vaporized and the extract residue was dissolved in 5 mL of chloroform prior to the analysis. For SFE extract, the oil extracts (3 mL) were saponified with 3 mL of 2 mol/L KOH ethanolic solution at 80^{o}C for 1 hour. After the saponification, the solution was cooled down to room temperature and 2 mL of MilliQ water

were added to homogenize the solution. The unsaponifiable matter was separated from the extract by liquid-liquid extraction as previously described and dissolved in 5 mL of chloroform. Prior to the phytosterols detection, about 2 mL of the Liebermann-Burchard (LB) reagent was added to 5 mL of chloroform sample extracts and volume was increased with chloroform to 10 mL. After 5 min of reaction time, the absorbance of the samples was measured by UV-VIS Spectrophotometer at 625 nm against a blank sample. The Liebermann-Burchard (LB) reagent was prepared by adding about 50 mL of acetic anhydride into an amber glass vial and kept in an ice bath. After 30 min, 5 mL of sulphuric acid was added to the acetic anhydride. Results of the total phytosterols measurements were determined by referring to a standard calibration curve of β-sitosterol (20 – 100 mg/L) and expressed as mg of β-sitosterol (BS)/g dried material (DM).

2.12 Ultra-Performance Liquid Chromatography (UPLC) analysis

Ultra-Performance Liquid Chromatography (UPLC) analyses were carried out using a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA). Samples were separated on reverse phase in Acquity UPLC BEH C18 (1.7 µm x 2.1 x 50mm) column with mobile phases of 0 0.1% formic acid in MilliQ water and 0.1% formic acid in acetonitrile in a gradient elution mode. The addition of formic acid significantly enhances the efficiency of ionization [21]. The injection volume was 1 µL and the oven temperature was maintained at 25ºC. For the ESI/QTOF/MS, UPLC was coupled with to a Quadrupole-Time-of-Flight micro-TOF-Q (Bruker Daltonik GmbH, Bremen, Germany), an orthogonal accelerated QTOF mass spectrometer, equipped with an ESI. A negative ion mode was set with spectra acquired over a mass range from m/z 50 to 1300. The optimum parameters of the ESI/MS were found to be: capillary voltage, +2.5 kV; drying gas temperature, 200°C; drying gas flow, 8.0 l/min; nebulising gas pressure, 2.0; collision RF, 300 Vpp; transfer time 120.0 µs; and pre-pulse storage, 8.0 µs. An automation of MS was set at collision energy as -10 eV. The mass data was processed by Data Analysis 4.1 software (Bruker Daltonics, Bermen, Germany), that provided a list of possible elemental formulae using the Generate Molecular Formula editor.

2.13 Statistical analysis

All analyses were done in triplicate. Results are reported as mean values and standard deviations. One-way analysis of variance (ANOVA) tests were performed in Microsoft Excel 2012 to analyze the effect of solvent concentration, determining the cases where the difference between individual means was statistically significant, with $p<0.05$.

3.0 Results and Discussion

The extraction yields obtained by MAE, SFE and Soxhlet methods were compared to each other by considering solvent consumption and extraction time as presented in Fig. 2. In this Figure, the extraction yield is expressed as g/g dry material while the moisture content was 11.77%*wt* in dry weight basis. As it can be seen in Fig. 2, the lowest extraction yield was obtained by SFE method, with 3.19%*wt* on dry basis after 120 min of extraction at 350 bar and 60^{o}C, while the highest yield of 21.28%*wt* was obtained by Soxhlet extraction, and an intermediate yield of 17.39 %*wt* was achieved by MAE method. The low yield obtained by SFE is probably due to the non-polar nature of the carbon dioxide solvent that is unfavourable for extracting the abundant polar compounds present in *C. nutans* oil, such as chlorophyll and polyphenols (see Section 3.3). Besides, a slower extraction rate in the course of extraction (shown in Fig. 5 in Section 3.6) indicated that the oil extraction was controlled by mass transfer resistance as the solutes were strongly bound with the plant matrix. As consequences, the fluid-phase concentration became much lower than the solubility [22] and prolonged the extraction time needed to achieve asymptotic yield. The solubility of *C. nutans* oil was as low as 0.242 mg oil/g CO_2. A relatively high amount of carbon dioxide of 315 g solvent/g feed compressed for 120 min needed in order to achieve an extraction yield of only 3.19%*wt*, indicates that the supercritical extraction for the *C. nutans* was an unfavourable method considering the economic feasibility of the process.

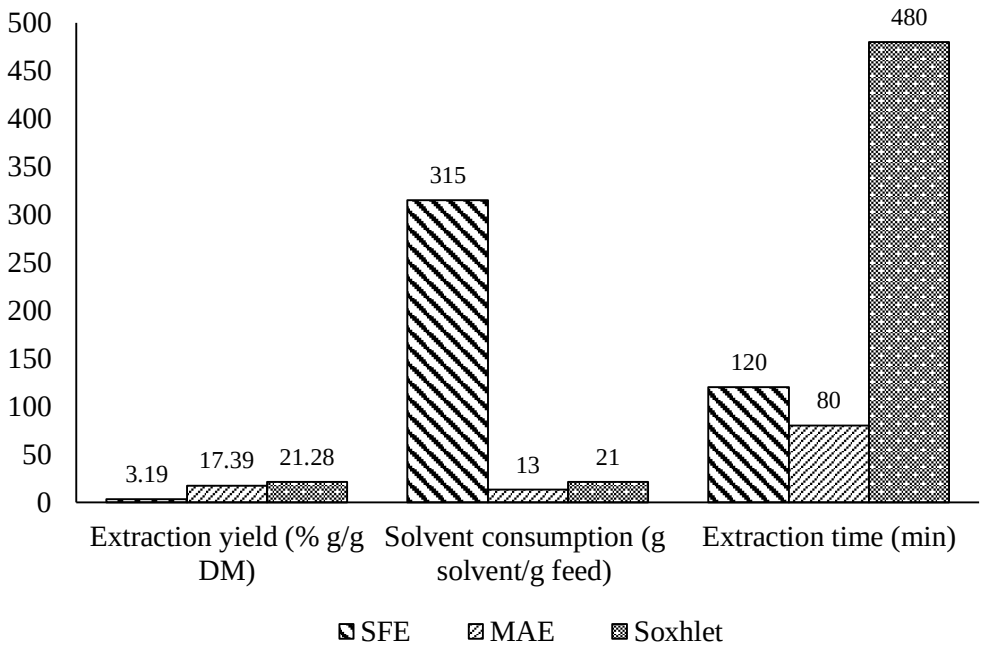

Figure 2. Comparison of extraction techniques based on different factors

On the other hand, the highest yield achieved by Soxhlet extraction can be explained by application of the high temperature (82^{o}C) during a longer period (480 min) compared to MAE which the temperature was 70^{o}C at 300W during 15 s and completed with conventional extraction at 40^{o}C in 80 min. The elevated temperature contributed to the process efficiency as the saturation concentration was increased improving the compounds extractability [23]. Besides, the different concentration of the ethanol solutions used for the extraction by Soxhlet (100% ethanol) and MAE (86%v/v) may also contribute to the lesser yield achieved by MAE. However, considering the factor of solvent consumption where MAE requires only 13 g solvent/g feed instead of 21 g solvent/g feed in Soxhlet the extraction time which is significantly shorter for MAE (15s) than for Soxhlet (480 min) and the energy consumption, microwave treatment appears as a favourable method for the *C. nutans* extraction.

3.1 Phytocompounds identification

GC/MS analyses were performed on the ethanolic extracts from MAE and Soxhlet as well as on the SFE extract. The various phytochemical compounds identified that can contribute to the bioactivity of *C. nutans* are presented in Table 1. The analysis on the extracts

revealed that *C. nutans* extract comprises diterpene, triterpene, fatty acids and other bioactive compounds. Most of the compounds identified can exhibit several biological activities such as antibacterial, anti-inflammatory and antioxidants, as for example phytol, squalene and lupel. Phytol was found as the major compound in the MAE and Soxhlet whilst palmitic acid was the major component in the SFE extract. These compounds have been claimed to have antiradical, anti-microbial, anti-inflammatory, antitumor and chemopreventive properties [24, 25]. Other bioactive compounds such as neophytadiene have been identified as strong bactericidal and anti fungal terpenoids compounds [26, 27]. Moreover, fatty acids such as palmitic acid, linoleic acid and linolenic acid have also been identified to act as antibacterial and antifungal agents against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* [28].

In addition, SFE extract included interesting compounds based on the triterpene group, as squalene and several long fatty acids that were not found in MAE or Soxhlet extracts. Squalene has been reported to exhibit anti-cancer, antioxidant, chemopreventive, anti-tumor [29], anti-cancerogenic and reducing serum cholesterol levels [30]. These results show that the type of compounds present in extracts is highly dependent on the nature of solvent and on the technique of extraction employed. Due to the characteristics of solvents employed, MAE extraction enabled obtaining several minority polar compounds, while non-polar fatty compounds mainly were obtained by SFE. In contrast, Soxhlet extracts did not include any of these minority compounds. This can be due to a partial degradation of these compounds during the long processing times at high temperature required for Soxhlet extraction.

Table 1 Phytocomponents identified by GC/MS in *C.nutans* extracts obtained by microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and Soxhlet extraction.

Compounds	Peak Area (%)			Nature of compound	Biological activity
	MAE (86%v/v)	SFE (350 bar, 60ºC)	Soxhlet (abs ethanol)		
Neophytadiene	13.44	2.73	-	Diterpene	Anti-microbial, anti-flammatory
Iron	3.00	-	-	Metal ions	Haemoglobin formation agent, maintain immune function
7,9-dodecadien-1-ol	4.25	-	-	Alcohol	NA
Myristic acid	-	2.12	-	Fatty acid	Flavouring, skin care
Palmitic acid acid, methyl ester	3.09	-	5.91	Fatty acid	Antibacterial, antifungal
Palmitic acid	29.23	43.49	12.13	Fatty acid	Antibacterial, antifungal
Benzenethanol	3.74	-	-	Alcohol	NA
Phytol	34.99	11.34	75.42	Diterpene	Antiradical, antimicrobial, anti-cancer
Squalene	-	5.58	-	Triterpene	Anticancer, antimicrobial, antioxidant, chemo preventive, pesticide, anti-tumour, sunscreen
Stearic acid, methyl ester	4.53	17.43	-	Fatty acid	Antibacterial, antifungal
Margaric acid, ethyl ester	-	0.50	6.54	Fatty acid	NA
Lupeol	3.73	-	-	Triterpene	Antiprotozoal, antimicrobial, anti-inflammatory, antitumor and chemopreventive properties
Linoleic acid, ethyl ester	-	15.77	-	Fatty acid	Antibacterial, antifungal
Linolenic acid, methyl ester	-	1.04	-	Fatty acid	Anti-microbial, anti-inflammatory

3.2 Chlorophyll content

Sakdarat et al. (2009) identified three chlorophyl derivatives related to the structures of chlorophyll a and b, namely 13(2)-hydroxy-(13(2)-R)-phaeophytin b, 13(2)-hydroxy-(13(2)-S)-phaeophytin a and 13(2)-hydroxy-(13(2)-R)-phaeophytin a. These compounds were investigated by these authors and exhibited anti-herpes simplex activity. It has been demonstrated by several researchers that the chlorophyll pigments show a diverse range of biological properties such as antioxidant, antimutagenic activities, chemopreventive action and induction of apoptosis activity in cancer cells [31, 32]. Therefore, it is considered that the the presence of chlorophyll in the extract also contribute to the medicinal properties of *C. nutans.*

Total amounts of chlorophyll a and b determined in this work are shown in Table 2. Results showed a high concentration of chlorophyll in the *C. nutans* leaves and stems. On the other hand, as it can be observed from Table 2, ethanol extractions attained higher chlorophyll content compared to acetone extractions. Even though acetone has been used for many decades for chlorophyll determination as it could give a very sharp chlorophyll absorption peak, it is a poor extracting solvent of chlorophyll from many vascular plants [33]. In addition, very low chlorophyll contents were obtained by extraction with a 50%v/v ethanol/water mixture, and the highest amounts of chlorophyll were obtained by extraction with a 86%v/v ethanol-acidified water mixture.

Table 2 Chlorophyll content in *C.nutans* extracts

Solvent	Chlorophyll a (g/g sample)	Chlorophyll b (g/g sample)	Total chlorophyll (g/g DM)
Ethanol	0.38	0.77	1.30
Acetone	0.48	0.45	1.06

It is interesting to note that different colour of extracts was obtained at different ethanol concentration (see Fig. 3). It was observed that the higher ethanol concentration the greener the colour of extract. This is possibly due to the higher solubility of chlorophyll at high ethanol concentration.

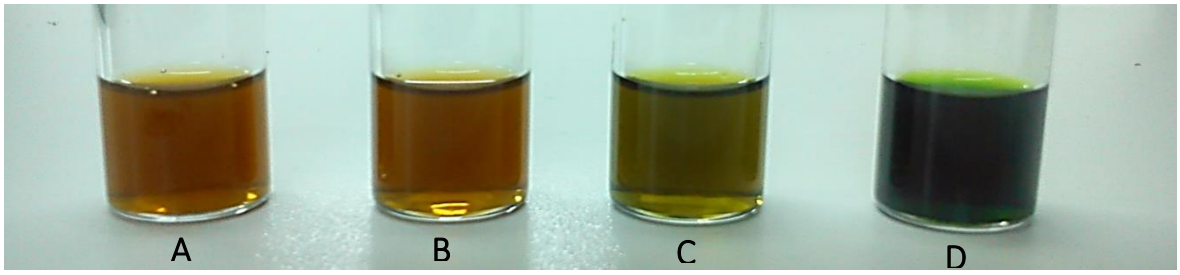

Figure 3. Colour of extracts at different concentration of ethanol.
A: 44%v/v, B: 50%v/v C: 65% and D: 86%v/v

3.3 Total phenols content (TPC)

The total phenols content of sample extracts are presented in Table 3. In general, a higher TPC was obtained by ethanol extraction assisted by microwave irridiation (MAE) than by supercritical fluid extraction (SFE). This phenomenon can be explained by the nature of the solvent used in the extraction, as the non-polar nature of supercritical carbon dioxide makes it less suitable than ethanol-water mixtures for the extraction of polar polyphenol compounds [34, 35]. Phenolic compounds comprise one or more hydroxyl groups (polar fragments) and phenyl groups or aromatic rings (non-polar fragments). These compounds are often found in plants as esters or glycosides rather than as free molecules [36]. With this structure, they can form a hydrogen bond with water molecules as well as with an electronegative oxygen of ethanol and vice versa [37], making them soluble in polar solvents.

In our method, an additional amount of solvent (6 mL) was added after the MAE irridation to increase the compounds solubility into the solvent and minimize the mass transfer limitation of compounds. The solvent amount was determined by the solvent-to-feed-ratio and the desired final volume of solvent. Indeed, from preliminary experiments, it was found that by adding the solvent after the MAE enhanced the total phenols content from 9.19 to 11.09 mg GAE/g DM at 44%v/v ethanol (not shown in this work). Hence, this method improved the extractability of polyphenols from sample matrix into extracting solvent.

The proportion between solvents in water/ethanol mixtures must be set depending on the specific compounds targeted. At low concentration of ethanol (i.e. 50%v/v), a higher amount of polyphenols can be extracted, whereas less polyphenols can obtained at high ethanol

concentration due to the solubility competition between phenolics compounds and chlorophyll that is governed by polarity factor and also might be a thermodynamic property i.e. activity coefficient. Galanakis et al. [37] explained that a tedency of phenols to be transferred, solubilized and diffused was dependent on activity coefficient, as with smaller activity coefficients the solubility was higher and vice versa.

Table 3 Total phenols content (TPC) and total flavonoids (TF) obtained with different methods of extraction

Method	Ethanol concentration (%v/v)	TPC (mg GAE/g DM)	TF (mg quercetin/g DM)
MAE	44	11.09 ± 0.28	4.24 ± 0.12
	50	11.30 ± 0.39	4.66 ± 0.20
	65	9.31 ± 0.18	4.54 ± 0.20
	86	5.74 ± 0.29	3.41 ± 0.76
*P-MAE	44	13.23 ± 0.40	5.23 ± 0.40
	50	14.56 ± 0.77	5.29 ± 0.30
	65	12.89 ± 0.90	5.07 ± 0.56
	86	8.88 ± 0.85	2.71 ± 0.47
Control (MAE)	44	8.89 ± 0.46	4.27 ± 0.22
Soxhlet	Absolute	7.95 ± 0.21	3.04 ± 0.02
SFE	No Ethanol CO_2 350 bar, 60°C	7.01 ± 0.15	5.88 ± 0.22

* At pressure = 2.7 bar
Values are mean ± S.D of triplicate experiments

On the other hand, the maximum contents of polyphenols were obtained with ethanol/water mixtures in a 50 %v/v proportion, with lower contents (with a statistically significant difference with $p<0.05$) when the proportion of ethanol was either increased or decreased. This result agrees with the findings by Spigno et al. [38] who found that the phenols concentration decreased when the proportion of ethanol in the mixture was increased beyond 50 %v/v. This behavior is due to the molecular structure of phenols which comprises both polar and non-polar fragments, limiting its solubility in ethanol-water mixture depending on the proportions between the two solvents. Moreover, the lower phenols content observed at 44%v/v of ethanol can also be due to the co-extraction of other compounds which decrease the phenols content in the extract. Spigno et al. [38] mentioned that the addition of water to ethanol

improved the extraction rate, but excessive water in solvent mixture could reduce the phenols content as other contaminants were also extracted.

As previously indicated, the low TPC content in SFE extracts can be expected due to the non-polar nature of supercritical carbon dioxide. This result is in agreement with the observations of Rombaut et al. [39] who performed an extraction of polyphenols from grape seed by supercritical cabon dioxide without the aid of any co-solvent also found a very low extracion yield of polyphenols i.e. between 0.061 to 0.067 g extract/g dry material after 120 min at 53.8 MPa and 104^{o}C. A higher TPC content is expected if a co-solvent such as ethanol is employed in the SFE process due to the increasing solubilities of high molecular weight phenolics [40], as reported by several researchers [41]. However, the use of organic co-solvents in SFE processes consideraby complicates the purification and recycling of carbon dioxide, increasing costs, and it is rarely used in commercial SFE plants.

Furthermore, the TPC of Soxhlet extract was also lower compared to the contents in MAE and P-MAE extracts, even though the total extraction yield obtained by Soxhlet was higher. This is probably due to the absence of water in the ethanol solvent used in Soxhlet, leading to a lower solubility of the polyphenols in the solvent.

3.4 Flavonoids content

Flavonoids are secondary metabolites of plants with polyphenolic structure and have several pharmocological activities such as anti-cancer, antioxidant, anti-viral and anti-inflammatory. In this work, the trend of total flavonoids content in extracts was similar as the trend of polyophenol content described in the previous setion. The total flavonoids content obtained by MAE showed a maximum value of. 4.66 ± 0.20 mg quercetin/g DM at 50%v/v when the concentration of ethanol was increased from 44%v/v to 50%v/v. However, a further increase in the ethanol concentration to 86%v/v insignificantly (p>0.05) reduced the total flavonoids content to 3.41 ± 0.76 mg quercetin/g DM. The lowest flavanoids content obtained at 86%v/v of ethanol was probably due to the extraction of non-polar aglycone flavonoids by hexane during the pre-treatment of liquid-liquid extraction (LLE) to remove chlorophyll thereby leaves merely polar flavonoids (glycosides flavonoids) in the extracts [42], thus

reduced the total amount of flavonoids content. Furthermore, it also might be due to the extraction competition between chlorophyll and flavonoids from the plant leaves as a function of ethanol concentration. Previously, flavonoids content in *C.nutans* has been reported by several reseachers, for example Ho et al. [43] found 0.04 ± 0.001 mg QE/g of total flavonoids content after extracted *C. nutans* using methanol maceration for 3 days whilst Tiew et al. [44] discovered 0.21 ± 0.005 mg QE/g dry extract by using the same extraction procedure. Differences values of the flavonoids might be due to different extraction method and solvent employed. In addition, a microwave pre-treatment could signficantly enhance the extractability of the polyphenols from the *C. nutans* plant. Moreover, the degradation of flavonoids during the extraction procedure can be an important factor, as flavonoids are labile compunds that can easily undergo degradation reactions in aqueous media. With respect to this, it is remarkable the high flavonoid content achieved by SFE (Table 3), even though flavonoids are polar compounds that are not easily extractable by SC-CO_2. This result is probably a consequence of a lower degradation of the compounds during SFE, as with this procedure a dry extract is obtained along the course of the extraction thus avoiding possible degradation reactions in aqueous media.

3.5 MAE vs Pressurised-MAE (P-MAE)

In this work, an extraction assissted by microwave under pressure (P-MAE) at 2.7 bar was also carried out with same parameters as MAE experimens. The extracts were analysed to determine the TPC and flavonoids, phytosterols content as well as polyphenols characterization by UPLC/MS. The results are shown in Tables 3, 4 and 5, respectively. In general, the TPC and flavonoids content in the P-MAE extracts were similar to the values obtained by MAE. The TPC value decreased from 13.23 to 8.88 mg GAE/g DM significantly (p<0.05) when ethanol concentration was reduced from 44 to 86%v/v. Pressurised-MAE (P-MAE) enhanced the extractability of the polyphenols about 2 to 4 mg GAE/g DM of the TPC in MAE extracts at different ethanol concentrations. The temperature increase produced by a higher pressure could have caused damage to the cell walls of the plant, stimulating compounds releasing and enhancing its dissolution into the solvent. The high temperature achieved in P-MAE that is up

to 108ºC also contributed to the high extractability of the compounds from plant sample. This factor has further affected the flavonoids extraction when the its content was found to be much higher compared to the flavonoids content obtained by conventional MAE. The maximum value of flavonoids, i.e. 5.29 ± 0.33 mg quercetin/g DM, was observed at 50%v/v and the effect of a variation in ethanol concentration from 44 to 86%v/v on this amount was significant. Furthermore, as the ethanol concentration was increased to 86%v/v the flavonoids content decreased to the lowest amount of 2.71 ± 0.50 mg quercetin/g DM. It is important to note that the application of P-MAE did not improve any major difference in the variety of compounds extracted compared to MAE. As it can be seen from Fig. 4 and Table 4, both extracts of MAE50 and P-MAE50 comprised similar phytochemical compounds. However, the extraction yields obtained by P-MAE were slightly higher than the yields obtained by MAE. This result suggested that MAE treatment is sufficient to improve the extractability of compounds from the plant material. Morevover, the P-MAE solely enhanced the extraction yield rather than the variety of compounds extracted.

In MAE and P-MAE process, 50%v/v of ethanol was found as the optimum concentration for the *C. nutans* extraction in producing the highest TPC and TF. Even though the flavonoids content was increased with ethanol, small differences were found. Moreover, ethanol with more than 50% v/v can caused local mucosal lesions through dehydration and albumin precipitation [45] meanwhile ethanol concentration in excess of 60% v/v could be toxic for short-term and prolonged use of ethanol-containing herbal medicinal products [46]. There is also another study by Chung et al. [47] concerning the ethanol concentration for human consumption, who studied the antioxidant activity and the safety of 50% ethanolic extract from red bean fermented. They claimed that the 50% ethanolic extract was found to be safe in genotoxicity as no mutagenicity or toxicity effect was found on the tester strains. Genotoxicity is a phenomenon describing the property of chemical solvent can cause a mutation of cells which lead to cancer. Thus, from the economic, safety and polyphenols extractability, point of view, it is suggested to use 50% v/v of ethanol as an optimum concentration to extract total phenols compounds from *C. nutans* sample.

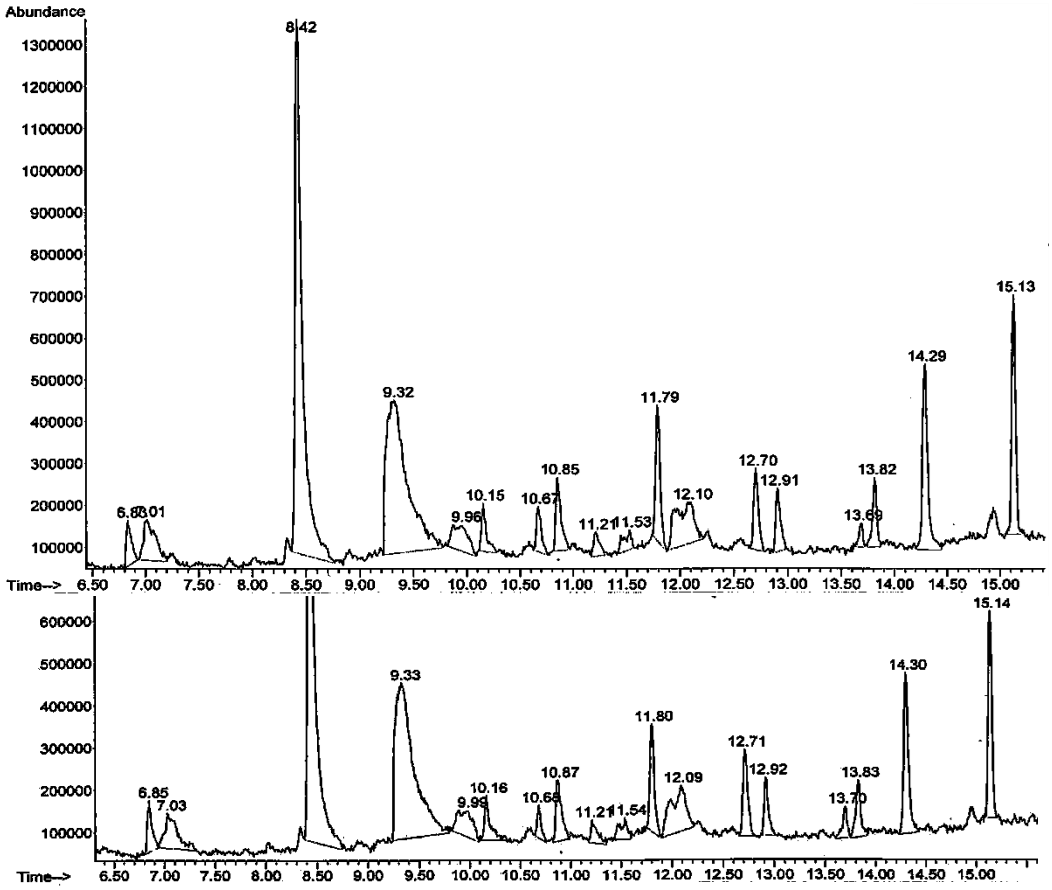

Figure 4. GCMS chromatograms for extracts at MAE50 and P-MAE50

Table 4 GC/MS phytochemical compounds identified in extracts obtained by MAE and P-MAE with 50% v/v ethanol-water mixtures

No.	Compound	RT (min)	Peak area (%)		Nature of compound	Activity/Uses
			MAE50	P-MAE50		
1	2-Butanol	6.85	1.97	1.98	Carboxylic acid	Antimicrobial
2	Butanamide	7.02	3.63	3.66	Amide	Antimicrobial
3	2-cyclopenten-1-one, 2-hydroxy	8.43	27.64	28.00	Cyclic Ketone	Fragrance
4	Glycine	9.33	22.05	22.24	Amino acid	NA
5	Pentanal	9.99	3.18	3.21	Aldehyde	Antimicrobial
6	Isoveraldehyde	10.16	2.15	2.11	Aldehyde	Food flavouring
7	Dimethyl trisulfide	10.68	1.05	1.09	Organosulfur	Antimicrobial
8	Thiophene	10.87	2.94	2.82	Heterocyclic	Antimicrobial
9	Succinic acid	11.21	1.17	1.25	Organic acid	Antimicrobial
10	Glycolic acid	11.54	1.30	1.44	α-hydroxy acid	Food preservative, skin care agent
11	Oxazolidine	11.80	3.87	3.99	Nitrogen compound	Antimicrobial
12	Thiophene	12.09	5.56	5.74	Heterocyclic	Antimicrobial
13	9-Azabicyclo (6.1.0) non-4-4en-9-amine	12.71	3.51	2.77	Azo compound	NA
14	4-vinyl-2-methoxy-phenol	12.92	2.03	2.20	Phenol	Antioxidant
15	Phenol,2,6-dimethoxy	13.70	1.20	1.31	Phenol	Antioxidant
16	4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	13.83	2.33	2.51	Flavonoid fraction	Antimicrobial, anti-inflammatory
17	Glycerine	14.30	6.46	6.95	Alcohol	Antimicrobial, preservative
18	4-vinylphenol	15.14	6.36	6.73	Phenol	Antioxidant

3.6 Total phytosterols and β-sitosterol

Phytosterols are plant sterols members of the 'terpene' family of natural products and classified as low polarity or non-polar compounds. Their nutraceutical activities include anti-inflammatory, anti-bacterial, anti-ulcerative and anti-tumor properties [48, 49]. The most abundant phytosterol components found in plant oil are β-sitosterol, stigmasterols and campesterol. In this work, analyses of total phytosterol (TP) and β-sitosterol by UV-Vis spectrophotometry and HPLC respectively were done to quantify the compounds in *C. nutans* extract.

Results are shown in Table 5. In general, by MAE and PMA extraction, phytosterols content was found to increase with a decreasing proportion of water in the solvent mixture, in agreement with the non-polar nature of these compounds. Results showed that MAE pretreatment yielded higher TP and β-sitosterol content than Soxhlet extraction when the proportion of ethanol in the solvent mixture was higher than 65%v/v. It is also remarkable that in the control experiment performed by conventional hot water extraction without MAE treatment, no phytosterols were observed in the extract. As shown in Table 5, certain phytosterol content was observed in the control hot water extraction without microwave irradiation performed with addition of KOH as saponification reagent, using 20 mL of 1.5 mol/L ethanolic of KOH at 40^{o}C for 80 min, but the TP and β-sitosterol content obtained by MAE were much higher. Therefore, irradiation of microwave towards the sample materials improved the extraction efficiency and reduced the extraction time. Furthermore, by applying a direct saponification with the aid of MAE the time consuming sample preparation can be simplified, minimizing the steps of the experimental procedure.

Analyzing the results obtained by SFE, as presented in Table 5 a remarkable content of TP and β-sitosterol, of 1.35 and 0.83 mg BS/g DM, respectively was obtained that was higher than the concentrations showed by MAE or Soxhlet methods. This behavior can be explained by the similar polarity between phytosterol and carbon dioxide that enhanced the extraction efficiency by SFE. Moreover, the higher temperature applied in Soxhlet extraction probably caused the degradation of β-sitosterol and resulted in a lower phytosterols concentration.

Sajfrtová et al. [50] stated that it was also possible that atmospheric oxygen degraded and reduced the β-sitosterol content in the oil extracted by Soxhlet method.

Table 5 Total phytosterol (TP) content and β-sitosterol (BS) yield obtained with methods of extraction

Method	Ethanol concentration (%v/v)	TP (mg BS/g DM)	BS (mg/g DM)
MAE	50	0.19 ± 0.13	-
	65	0.41 ± 0.20	0.16 ± 0.22
	86	0.70 ± 0.10	0.52 ± 0.10
*P-MAE	50	0.35 ± 0.12	-
	65	1.04 ± 0.15	0.45 ± 0.20
	86	1.19 ± 0.22	0.65 ± 0.14
Control	44	-	-
Soxhlet	Absolute	0.47 ± 0.20	0.23 ± 0.18
SFE	350 bar, 60°C	1.35 ± 0.12	0.83 ± 0.10
MAE-KOH	1.5 mol/L ethanolic KOH	2.36 ± 0.15	0.64 ± 0.13
Control-ethanolic KOH		0.88 ± 0.14	0.40 ± 0.10

* At pressure = 2.7 bar

The effect of solvent-to-feed ratio on the extraction yield of β-sitosterol by SFE at 350 bar and 60°C is shown in Fig. 5. As the solvent-to-feed ratio was increased, the yield of β-sitosterol increased and passed through a maximum before dropping as the consumption of CO_2 per mass of raw material was further increased. Initially, the concentration of β-sitosterol was low and then slowly increased as the time of extraction prolonged, and the maximum of β-sitosterol was obtained at the third fraction of the extraction course. High amount of β-sitosterol in the middle of the extraction fractions is probably due to the low solubility of the other compounds (triglycerides or fatty acids) in CO_2 during that time then in turn offer an advantage to the β-sitosterol to dissolve in SC-CO_2. However, this behaviour is in contradiction with some previous works. For example, Arevalo [51], reported that the highest concentration of β-sitosterol from soybean distillate waste was found at the first fraction of the extraction at pressure 200 and 300 bar, 80°C, claiming that the highest concentration of the compound obtained was due to the low solubility of other compounds in CO_2 at the initial part of the extraction course. Nevertheless, differences in the extraction β-sitosterol might be due to different structure and chemical composition of plant materials [52].

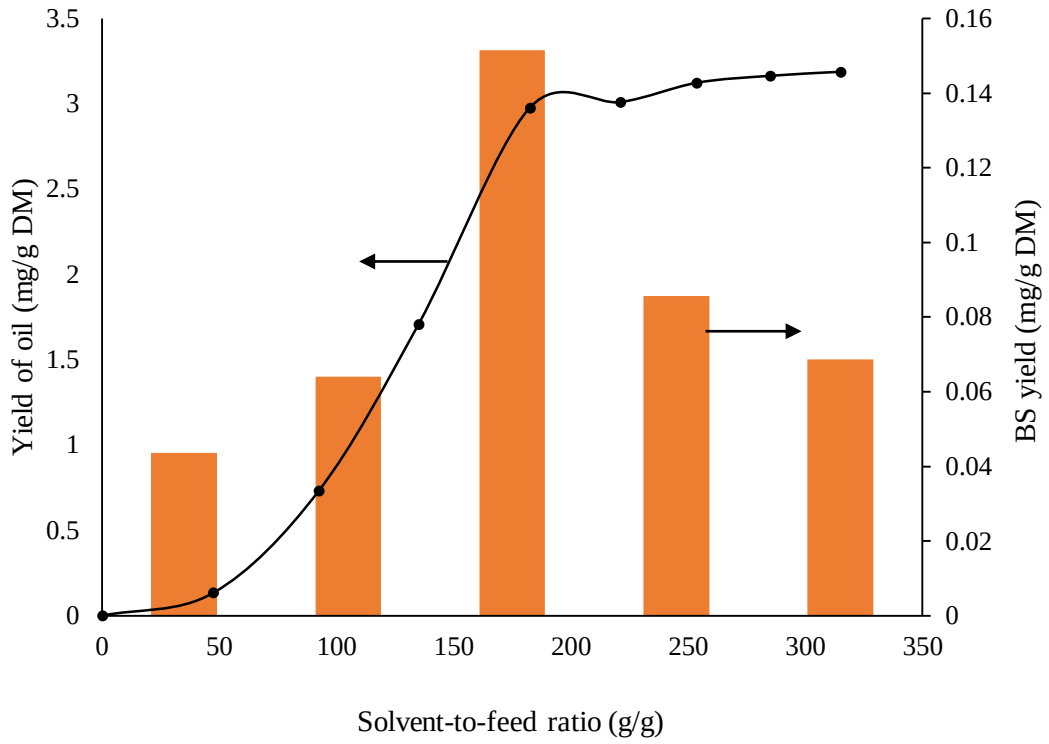

Figure 5. Oil and β-Sitosterol yield as a function on S/F

3.7 Characterization of metabolites in *C.nutans* extract by UPLC-QTOF/MS

Plant extracts generally contain primary metabolites such as lipids, protein and carbohydrates whereas the secondary metabolites comprises alkaloids, terpenoids and phenolics. Phenolic compounds play an important role in cancer preventation and treatment by exhibiting diverse physiological properties such as antioxidant, anti-mutagenic, anti-imflammatory [53]. The characterization of phenolic compounds from *C.nutans* extracts were done by UPLC-QTOF/MS as this technique provides high selectivity of detection and wide range of determination [21]. A negative ionization mode of MS data was used in this work since polyphenols contain one or more hydroxyl and/or carboxylic acid groups [54].

In general, the UPLC analysis revealed that the MA extraction of *C. nutans* at different concentrations of ethanol yielded the same compounds, only with differences in their amounts when extracted with pressurised MAE. Similarly, the application of pressure in P-MAE

experiments merely enhanced the extraction yield of phenolic compounds instead of improving the extractability of different compounds, as indicated also by GC/MS assays (Table 4). The UPLC-MS chromatograms of MAE and P-MAE extracts are shown in Fig. 6 whilst the characterized phenolic compounds are shown in Table 6 and 7 for 86% and 50%v/v ethanol, respectively. All the compounds are characterized by interpretation of their mass spectra from TOF-MS and based on the data provided in literature databases. The identification of the phenolic compounds by QTOF/MS was based on the deprotonated ions $[M-H]^-$ and the MS/MS fragmentation released.

From ethanolic/water extracts of 86% and 50%v/v, a wide range of metabolites groups were detected comprising phenolic compounds, terpene glycosides and oxygenated fatty acids. The identified phenolics belong to several classess of flavonoids, mainly phenolic acids, flavones, flavonol, flavanone, isoflavones, flavan-3-ol, C-glycosylated flavone and anthocynidin. In both extracts of 50 and 86%v/v, compounds of shaftoside, vitexin, isoorientin and 3',7-Dimethoxy-3-hydroxyflavone has been identified. The identification of vitexin fragment at *m/z* 311 was also observed by Zhang et al. [55] after the elimination of ions of $[M-H-120]^-$ and $[M-H-C_4H_8O_4]^-$ and consistent with the characteristics ion of a C-glycosidic flavonoid [56]. The presence of vitexin in *C.nutans* extract has been reported by Teshima et al. [12] from the *n*-Butanol extract of the stems and leaves of *C.nutans*. The medicinal properties of vitexin have been reported in many works that claimed that the compound possess antioxidant, anti-tumor, anti-inflammatory, anti-viral and anti-depressant activities [57-60]. Meanwhile the ion fragments pattern obtained for isoschaftoside was similar to that reported by Simirgiotis et al. [61] at m/z 353, 383 and 443. For the isoorientin that has molecular anion at m/z 448 shown ion fragments at m/z 327 and 357.

Table 6 Characterization of phenolic compounds from ethanolic extracts (86%v/v)

Peak	*Rt	Compound	Class	$[M-H]^-$	Major fragments (*m/z*)
1, 3	1.10	Kaempferol-7-neohesperidoside	Flavones	594	593
4	6.02	Isoschaftoside	C-glycosylated flavone	563	353, 383, 443
10	9.90	Unknown flavones	Flavones	613	225, 243
12	12.7	Isoorientin	Flavones (Luteolin glucoside)	448	327, 357
17, 20	16.6	Vitexin	C-glycosylated flavone	432	283, 311
24, 26	19.3	3',7-Dimethoxy-3-hydroxyflavone	Flavones	298	297

*Rt = retention time

Table 7 Characterization of phenolic compounds from ethanolic extracts (50%v/v)

Peak	*Rt	Compound	Class	$[M-H]^-$	Major fragments (*m/z*)
1	1.10	2',6-Dihydroxyflavone	Flavone	209	147, 191, 209
2	2.4	(+)-Catechin	Flavan-3-ol	290	175, 227, 229, 231
10	6.2-6.8	Isoschaftoside	C-glycosylated flavone	563	353, 383, 443
13	7.0	7-Hydroxyflavone	Flavones	193	-
16	8.2	Gallic acid	Phenolic acid	169	125
23-25	12.5-13.3	Isoorientin	Flavones (Luteolin glucoside)	448	327, 357
27	15.8	Vitexin	C-glycosylated flavone	432	283, 311
31	18.8	Flavanone-7-O-glycoside	Flavones	580	295, 459
32	19.1	Unknown flavonoid	Flavonoid	415	-
34	19.7	3',7-Dimethoxy-3-hydroxyflavone	Flavones	298	297

*Rt = retention time

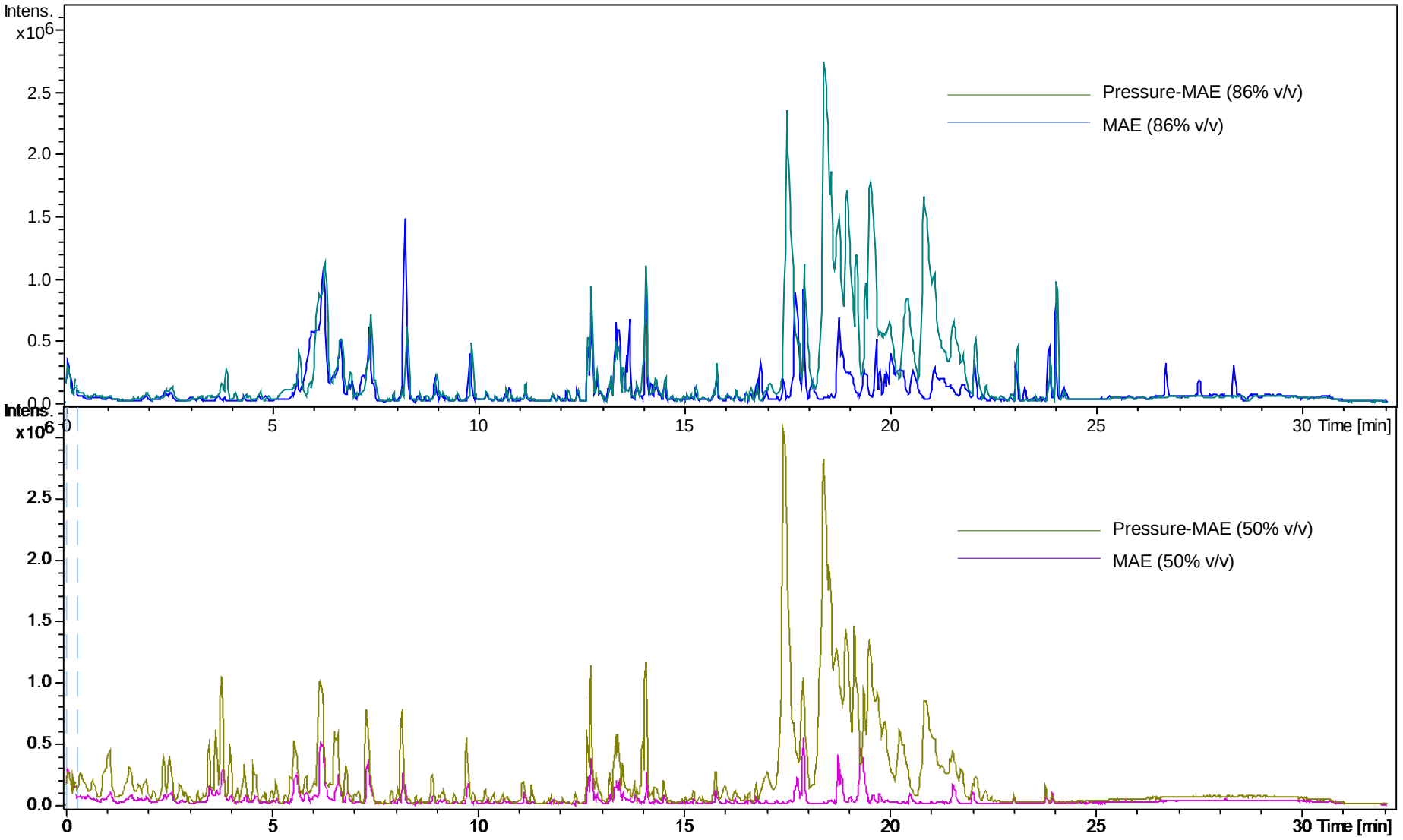

Figure 6. UPLC-QTOF ESI/MS chromatogram of MAE, PMAE at 86%v/v and 50%v/v of ethanol

On the other hand, a group of flavanone glycosides was identified such as 2,6-dihydroxyflavanone and 3-hydroxy-3',4',5'-trimethoxyflavone that identified at *m/z* 255 and *m/z* 297, respectively that have been claimed to possess anti-inflammatory effect [62]. In addition, flavan-3-ol a type of natural phenols and antioxidant i.e. catechin and gallic acid were identified in 50%v/v extract at m/z 245 and 193, respectively. Nevertheless, these compounds were not found in 86%v/v but the presence of kaempferol-7-neohesperidoside might increase the value-added of the extract. The variation of compounds found in both ethanolic extracts were probably due to their different solubility properties in ethanol/water.

4. Conclusions

Extraction of compounds of interest from *C. nutans* is highly dependent on the nature of the solvent and the extraction method. The highest total phenols and flavonoid content (i.e. 11.30 ± 0.39 mg GAE/g DM and 4.66 ± 0.22 mg QE/g DM, respectively) was obtained with MAE. These values were increased to 14.56 ± 0.77 mg GAE/g DM for total phenols and 5.29 ± 0.30 mg QE/g DM for flavonoids when P-MAE was applied. Extraction of phytosterols including β-sitosterol was successful when using SFE with maximal yields as high as 1.35 ± 0.12 mg of phytosterols/g DM and 0.83 ± 0.10 mg of β-sitosterol/g DM. In contrast, extracts obtained with the conventional Soxhlet method were very poor in minority bioactive compounds.

Microwave pre-treatment enhanced the extraction of phenols and flavonoids from *C. nutans* medicinal plant whereas for the extraction of phytosterols, SFE was found to be superior to microwave and Soxhlet. MAE produced a comparable total extraction yield to Soxhlet whilst SFE yielded the lowest yield among the extraction methods studied. Pressurised microwave-assisted extraction (P-MAE) merely enhanced the total extraction yield and polyphenols content of ambient pressure MAE without variations in the range of compounds extracted. Therefore, the comparison of MAE, SFE and Soxhlet extraction showed that MAE technique was more efficient than Soxhlet methods for the extraction of *C .nutans* in a shorter time, with reasonable yield and high content of compounds interest, particularly of polar polyphenols.

C. nutans extracts is rich of chlorophyll and contained abundant nutraceutical compounds, mainly phytol, palmitic acid, polyphenols, flavonoids and phytosterols. Thus, *C. nutans* extract has a great potential to be used as a source of natural bioactive, nutraceutical compounds. High phenolic compounds qualitatively screened by UPLC-ESI-QTOF/MS in the 50%v/v ethanolic extract rather than 86%v/v showed that 50-50 is the best ethanol-water composition to extract polyphenols from *C .nutans* medicinal plant. Nevertheless, further investigation on the antioxidant and anti-microbial activity of the recovered phytocompounds obtained by microwave pre-treatment is essential to determine the bioactive activity of the extracts.

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Chapter 2

Microwave-assisted extraction of polyphenols from *Clinacanthus nutans* Lindau medicinal plant: Energy perspective and kinetics modelling

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Abstract

Clinacanthus nutans Lindau (C.nutans, "snake grass") is a well-known medicinal plant in South-East Asia that recently has attracted attention for its high polyphenol content and its medical and nutraceutical applications. In this work, polyphenols have been recovered from C. nutans by Microwave-Assisted Extraction (MAE). The influence of the main process parameters has been analysed: ethanol concentration in the extraction solvent mixture, microwave energy applied and solvent-to-feed ratio. Model results have been correlated with mathematical models of the extraction process, that allow linking the observed variations in extraction curves with modifications of mass transfer and equilibrium parameters. Moreover, in order to assess the energy efficiency of the process, results were studied and explained in view of the specific energy absorbed during the microwave treatment (J/g). Results indicate that microwave pre-treatment improves extraction rate by a factor of 2-5, yielding the best results when a water-ethanol solvent mixture with 50%vol of ethanol is used at a solvent-to-feed ratio (S/F) of 14 mL/g.

1. Introduction

Polyphenols are secondary metabolites synthesized in plants that play important roles in the protection against ultraviolet light or against plant pathogens and animal aggression. In human nutrition, polyphenol antioxidants have been long used for treatment and prevention of different diseases [1, 2]. Due to their scavenging activities, these compounds principally protect cells and body from being damaged by oxidation by free radicals. Numerous research works have investigated and proved the activity of polyphenols as antioxidant, antimicrobial and anti-cancer agents. Polyphenols can be abundantly found in dietary products such as fruits, vegetables, cereals, beverages and herbs.

Clinacanthus nutans (*C.nutans*) is a medicinal plant that has recently received much attention by numerous researchers due to its therapeutic characteristics for treatment and prevention of diseases. These activities can be correlated with their high polyphenol content. Several reported works have shown that polyphenol contents as high as 23.5 mg GAE (Gallic Acid Equivalents)/g dry material can be obtained by Soxhlet extraction of *C.nutans* with methanol [3, 4]. However, these previous studies only report the application of simple maceration and solvent extraction methods, although the extract composition and yield in general is highly dependent on the extraction technique and solvent employed.

In a previous work [5], authors reported an exploratory study of the extraction of bioactive compounds from *C.nutans* employing different techniques: supercritical fluid extraction, microwave-assisted extraction (MAE) pressurized microwave-assisted extraction (PMAE) and Soxhlet solvent extraction. With that previous work, it was concluded that MAE was the most efficient technique for the extraction of polyphenols from *C.nutans* in terms of extraction yield, use of solvents and extraction time. Therefore, this work presents a detailed study of the extraction of polyphenols from *C.nutans* by MAE.

MAE has been reported by many researchers as a fast extraction technique with several advantages over conventional extraction methods such as high extraction rates, shorter extraction times and low solvent consumption [6]. Pare, Belanger and Punt [7] first highlighted that the absorbed energy, rather than the power of microwave irradiated, is a significant factor

on MAE, influencing the extraction efficiency. Later on, numerous researchers have reported similar conclusions [8-10]. In addition, the time of irradiation, temperature of solution and solvent–to-feed ratio (S/F) also are important parameters in the extraction of solutes by MAE [11]. In this work, specific energy absorbed is introduced to understand the quantity of energy absorbed per gram unit of solvent-sample system. The influence of ethanol concentration (44 – 86%vol) and solvent-to-feed ratio (S/F) (7 – 20 mL/g) at constant irradiation power (300 W) and irradiation time (15 s, energy emitted 4500 J) on total phenols content (TPC) yield were investigated in view of the absorbed energy. In addition, the extraction curves of polyphenols were analysed by the kinetic models of Patricelli, Assogna, Casalaina, Emmi and Sodini [12] and Peleg [13] in order to study the kinetics behaviour of polyphenols extraction initiated by microwave pre-treatment. By doing this the extraction rates were estimated and the predicted extraction yield was determined.

2. Materials and Methods

2.1 Materials

Clinacanthus nutans (Burm.f.) Lindau dried leaves and stems were obtained from Mr Lee Huat Lye, Kuala Lumpur, Malaysia. For the extraction, ethanol (96%) was purchased from Sigma-Aldrich Co. (Spain), whilst for the determination of phenols content, gallic acid and Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany) and 20%wt Na_2CO_3 solution was prepared by dissolving anhydrous sodium carbonate in Milli-Q deionized water. Water was purified by a Milli-Q water purifier system from Millipore (Milford, MA, USA).

2.2 Sample preparation

The moisture content in dried *C.nutans* was gravimetrically determined by treatment in an oven at 105ºC for 24 h. Prior to the extraction, the dried samples were grounded and sieved into particles sizes ranging from 100 to 500 µm. The prepared samples were stored in air tight bags, swept with nitrogen to remove air and kept in a refrigerator (-8ºC) until used in the extraction. Ethanol (96%) was diluted by acidified water (pH 1 by sulphuric acid) into several

proportions, i.e. 44, 50, 65 and 86%vol. Distilled water type 2 was boiled prior to the dilution to remove dissolved gasses.

2.3 Extraction procedures

2.3.1 Microwave-assisted extraction (MAE)

An open-system MAE was carried out in a laboratory CEM Discover® microwave oven of 300 W of maximum power, operating at a frequency of 2.45 GHz and equipped with Synergy software to set the program used for the extraction process. *C.nutans* samples (1.0000±0.0002g) were placed in 100 mL round bottom Duran flasks, mixed with water-ethanol solvent and kept under stirring for 3 min before irradiating them with microwaves, to homogenize the sample-solvent system as well as to ensure that the sample particles were fully immersed in the solvent. A magnetic stirrer was used to achieve a uniform temperature distribution in the sample-solvent system during microwave irradiation. System temperatures before and after the irradiations were recorded by an optical fibre probe, to determine the energy absorbed by the system. After the exposure to microwaves, the sample was rapidly placed in an ice-bath to reduce the temperature to 40^{o}C, 6 mL of fresh solvent were added and the sample was kept under magnetic stirring, thus proceeding with a conventional infusion.

The extract was sampled in different steps of the process: i) before microwave irradiation, ii) after the irradiation (i.e. after the addition of 6 mL of fresh ethanol solvent), and iii) during the infusion every 20 min until a total of 80 min of extraction time. For the kinetics study on the influence of energy emitted, an additional sampling before the addition of the 6 mL of fresh ethanol solvent was also done to observe the effect of energy absorbed on the phenols extracted by microwave irradiation. For study on the effect of solvent-to-feed ratio (S/F), the weight of *C.nutans* was kept constant whilst the solvent volume was varied according to the targeted S/F value. Prior to the sample collection, nitrogen gas was flowed into the 5 mL amber vials to remove air. The extract samples were filtered through 0.20 µm PTFE syringe filters to eliminate any suspended solids and kept in the refrigerator (-8^{o}C) until further used for analysis. All extractions were carried out in triplicate.

2.3.2 Conventional extraction

About 1.0000±0.0002g of *C.nutans* sample was placed in a 100 mL round-bottom flask and mixed with ethanol (44%vol). The system was magnetically stirred to keep it homogenized throughout the extraction at 40°C. Sampling was done according to the same schedule used in MAE experiments. The extracts were filtered through 0.20 µm PTFE syringe filters into amber vials that had been previously swept with nitrogen, and they were stored in refrigerator (-8°C) until further analysis. Extraction experiments were carried out in triplicate.

2.4 Determination of total phenols content (TPC)

Total phenols content (TPC) in the *C.nutans* extract were determined by Folin–Ciocalteu colorimetric method and expressed as mg Gallic Acid Equivalents (GAE) per gram of extract (mg GAE/g extract). Briefly, 40 µmL of sample extract was mixed with 3 mL of deionized water and 200 µmL of Folin–Ciocalteu reagent. After 5 min, 400 µmL of $NaCO_3$ was added to the mixture and left in water bath (40°C) for 30 min before proceeded with UV-Vis Spectrophotometer analysis at 765nm. The phenols yields of samples were calculated as in the Eq. (1) [14]. The moisture contents were determined in oven method at 105°C until reached a constant weight.

$$\text{Phenols yield (mg GAE/g DS)} = \frac{\text{concentration (mg/L)} \times \text{volume of extract (ml)}}{\text{Weight of dried sample (DS) (g)} \times 1000} \quad \text{Eq. (1)}$$

2.5 Determination of energy absorbed density

For the evaluation of the energy efficiency of the process, the energy absorbed by the solvent-sample system is a more relevant parameter than the total energy emitted by the microwave system. This is because the microwave energy emitted is not completely absorbed by the system and converted into thermal energy because several factors must be considered in the heat determination, such as heat loss and dielectric constant (ε) of solvent and sample, as well as geometrical and design parameters of the laboratory microwave system that are not intrinsic of the microwave extraction process and would be different employing different equipment or scaled-up processes. Furthermore, the maximum nominal power reported by the

microwave manufacturer in general does not correspond with the real power emitted by the microwave, as this value of nominal power is estimated from the amount of energy absorbed by a water sample under a specific set of conditions (amount of sample, geometrical properties of container, heating time). Therefore, although microwave powers are presented in this work as a convenient way of indicating the experimental conditions employed, for the discussion of results an estimation of the amount of energy actually absorbed by the sample is needed.

The amount of energy absorbed by the solvent-sample system can be measured by taking into account the mass of material exposed to the source of heat and the temperature difference between initial and final state. In other reported works, that type of energy is named as sensible heat [15]. Since the dielectric constant of *C.nutans* is not measured in this work, the moisture content of the *C.nutans* is taken into account as the mass of the material that absorbed the microwave energy, as shown in Eq. (2) and Eq. (3):

$$Q_{sensible} = m_s C_{p,s}\left(T_f - T_i\right) + m_w C_{p,w}(T_f - T_i) \quad \text{Eq. (2)}$$

$$= \left(m_s C_{p,s} + m_w C_{p,w}\right)\left(T_f - T_i\right) \quad \text{Eq. (3)}$$

Where m_s is the weight of solvent, $C_{p,s}$ is heat capacity of solvent, m_w is weight of water in *C.nutans*, $C_{p,w}$ is heat capacity of water ($C_{p,w}$ = 4.184 kJ/mol·K), T_i is initial temperature and T_f is final temperature. In addition, latent heat of evaporation of the solvent must be considered since the solvents have reached their normal boiling point in this work. Thus, the enthalpy of the solvent due to the vaporization can be calculated as in Eq. (3):

$$Q_{latent} = m_{vap} H_{vap} \quad \text{Eq. (4)}$$

Where m_{vap} is the weight of solvent evaporated, that was experimentally determined by weighing, and H_{vap} is the latent heat of vaporization of the solvent. Furthermore, the heat of vaporization H_{vap} was calculated as the molar average of the heats of vaporization of ethanol and water according to the molar composition of the solvent used in each experiment, and without considering the variations in the solvent composition due to the preferential evaporation of ethanol over water that in reality cause small variations in the hat of vaporization along the experiment.

Therefore, the total heat absorbed by the solvent-sample system can be determined as shown in the Eq. (5) and Eq. (6):

$$Q_{absorbed} = Q_{sensible} + Q_{latent} \quad \text{Eq. (5)}$$

$$Q_{absorbed} = (m_s C_{p,s} + m_w C_{p,w})(T_f - T_i) + m_{vap} H_{vap} \quad \text{Eq. (6)}$$

To further understand the heat absorption, Chan et al. [8] introduced the concept of absorbed energy density (AED), inspired from Alfaro et al. [10] due to the effect of solvent loading in the absorption of microwave energy. In this work, specific absorbed energy (J/g) is used to determine the amount of heat absorbed from microwave per unit mass of solvent-sample system.

3. Modelling of polyphenols kinetics profile

The extraction curves obtained with the experiments of microwave assisted extraction were fitted to the models derived by Patricelli, Assogna, Casalaina, Emmi and Sodini [12] and Peleg [13]. The basis of the Patricelli's model is to consider the extraction of active compounds as controlled by two phase boundaries. The first boundary explains the washing step where the compounds are dissolved into bulk solvent whose its temperature has been increased by energy obtained from the environment (in this case, the microwave irradiation), thus reducing the mass transfer limitations and enhancing the solvent penetration into ruptured wall cells. On the other hand, the second phase considers a diffusion step where the solutes from the internal wall cells diffuse into the solvent. This step typically is slower than the first extraction step due to mass transfer limitations.

The extraction yield for the Patricelli's model is given by the following equation that expresses concentration (C_t) as a function of time (t):

$$C_t = C_1(1 - \exp(-k_1 t)) + C_2(1 - \exp(-k_2 t)) \quad \text{Eq. (7)}$$

where C_1 (%*w*/*w*) is the solutes equilibrium yield at the washing phase, C_2 (%*w*/w) is the solutes equilibrium yield at the diffusion step, k_1 (min^{-1}) is the mass transfer coefficient at the washing

step and k_2 (min^{-1}) is the mass transfer coefficient at the diffusion step. Derivation of Eq. (7) yields an extraction rate estimation:

$$v = \left(\frac{dC}{dt}\right) = k_1 C_1 \exp(-k_1 t) + k_2 C_2 \exp(-k_2 t) \qquad \text{Eq. (8)}$$

Values of k_1 usually are higher than values of k_2 due to the fast extraction rate at the beginning of the course. Thus, to determine the extraction rate of the process i.e. at the washing phase is when $t = 0$ and yielding extraction rate, v (Eq. 9), whereby total equilibrium yield, C_e can be determined from the summation of the equilibrium yield from both regions, $C_1 + C_2$.

$$v_0 = \left(\frac{dC}{dt}\right) = k_1 C_1 + k_2 C_2 \qquad \text{Eq. (9)}$$

In contrast, Peleg [13] proposed a non-exponential empirical model to fit data of moisture content *vs* time where absorption of solutes into the solvent was established as the basis of the model development. This model has been successfully applied by several researchers to describe adsorption of solutes from water [16, 17]. However, since the sorption shapes exhibited by Peleg's model was found to be similar to a few of extraction kinetics of natural products, the model has been applied to profiling the extraction behaviour of active compounds from solid matrix. For example, Bucic-Kojic Bucić-Kojić, Planinić, Tomas, Bilić and Velić [18] employed the model to described kinetic behaviour of polyphenols extraction from grape seeds by 50%vol ethanol in a conventional method. They found that the Peleg's model fitted well the experimental data and became a remarkable tool for the prediction of the yield of extraction of polyphenols.

The Peleg's model is described by the following equation:

$$C(t) = C_0 + \frac{t}{K_1 + K_2 \bullet t} \qquad \text{Eq. (10)}$$

where, C_t is the concentration of polyphenols at t time, C_o is the concentration of polyphenols at initial of extraction i.e. when $t = 0$, t is the extraction time, K_1 is Peleg's rate constant (min g extract/g GAE)and K_2 is Peleg's capacity constant (g extract/mg GAE). In the context of

experiments performed in this work, the initial concentration C_0 is zero at all conditions, as described by Bucić-Kojić, Planinić, Tomas, Bilić and Velić [18], giving rise to Eq. (11) , that was the working equation used to fit the experimental data:

$$C(t) = \frac{t}{K_1 + K_2 \bullet t} \qquad \text{Eq. (11)}$$

The Peleg's constant rate, K_1 and capacity constant, K_2, are related to extraction rates at the very beginning course (t = 0) and equilibrium yield (t = ∞), respectively. At the equilibrium yield the polyphenols content is considered as maximum at the condition. Their relationships can be described as below:

$$B_o = \frac{1}{K_1}\left(mg\ GAE\ /\ g\ dried\ sample,\ DS\right) \qquad \text{Eq. (12)}$$

$$C\big|_{t\to\infty} = C_e = \frac{1}{K_2}\left(mg\ GAE\ /\ g\ dried\ \ sample,\ DS\right) \qquad \text{Eq. (13)}$$

All data fittings and analysis were done by Solver program package implemented in Microsoft Office. The parameters for Patricelli's and Peleg's models were estimated based on the experimental data by using non-linear regression, using the percentage average absolute relative deviation (%AARD) between experimental and predicted TPC yield as the objective function for the minimization procedure:

$$\%AARD = \frac{100}{N}\sum_{i}^{N}\frac{\left|C_{\exp} - C_{pred}\right|}{C_{\exp}} \qquad \text{Eq. (14)}$$

where N is number of experimental data. The coefficient of determination R^2 was calculated to determine the quality of the fit. Residuals between experimental and calculated data from both model fittings were also determined and compared.

4. Results and Discussion

4.1 Conventional infusion *versus* microwave-assisted extraction (MAE)

A conventional extraction (S/F = 14 mL/g) without microwave pre-treatment was carried out using out a solvent mixture with 44 %vol of ethanol at 40 ^{o}C. The polyphenols extraction curve was compared to the results obtained when sample was pre-treated with a microwave power of 300 W during 15 s and a solvent/fluid S/F ratio of 14 mL/g. The extraction curves obtained with both extraction approaches and fitted with Patricelli and Peleg's model are presented in Figure 1. It is observed that the pre-treatment with microwave irradiation resulted in a considerable increase in the polyphenol extraction yield. Moreover, a significantly faster polyphenols extraction was observed with MAE pre-treated samples. Indeed, as shown in Table 1, according to Patricelli's model the extraction rates were improved from 1.69 to 3.17 mg/g/min, whereas by Peleg's the estimated increase was from 1.16 to 5.55 mg GAE/ min·g DS. Additionally, the conventional method needs more than 80 min to reach equilibrium yield whereby with MAE pre-treated samples the equilibrium yield is attained in less than 40 min. As a conclusion, MAE pre-treatment clearly increased the extraction yield and extraction rate of polyphenols from *C.nutans.*

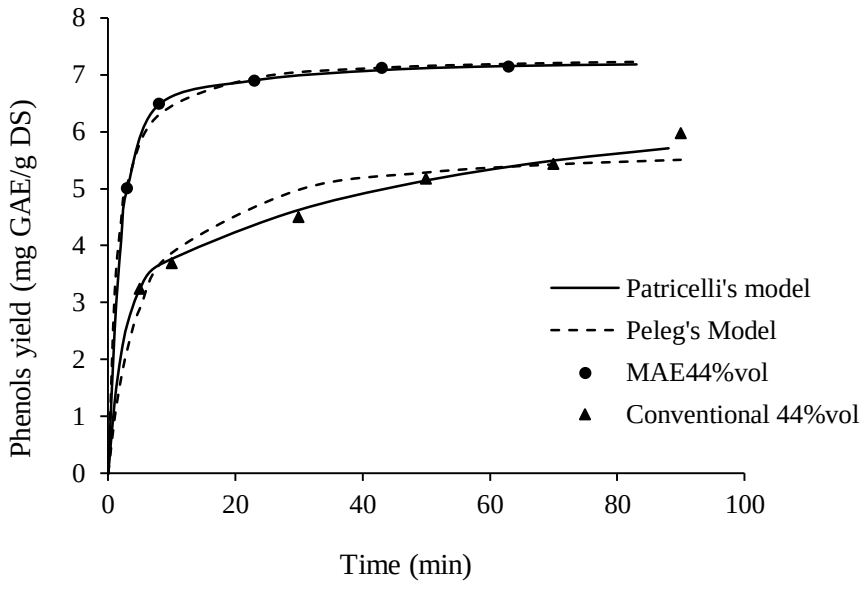


Figure 1 Kinetics profile of phenols yield by MAE44%vol and conventional 44%vol

Table 1 Mass transfer coefficient, yield and extraction rate of polyphenols by MAE (i) and conventional (ii) fitted by Patricelli and Peleg's model

Method	*Patricelli's model*						
	Mass transfer coefficient (min^{-1})		TPC concentration (mg GAE/g DS)		Extraction rate (mg/g/min)	Equilibrium yield, C_e (mg GAE/g DS)	R^2
	k_1	k_2	C_1	C_2			
MAE44%vol	0.49	0.05	6.33	0.87	3.17	7.20	0.999
Conventional 44%vol	0.50	0.01	3.26	3.89	1.69	7.14	0.995
Method	*Peleg's model*						
	K_1 (min·g DS/mg GAE)		K_2 (g/mg GAE)	Extraction rate, B_o (mg GAE/ min·g DS)		Equilibrium yield, C_e (mg GAE/g DS)	R^2
MAE44%vol	0.18		0.14	5.55		7.34	0.985
Conventional 44%vol	0.86		0.17	1.16		5.81	0.918

One clear reason might explained the significant different between the conventional and MAE process is the energy absorption by the sample mixture. Absorbed energy by the sample mixture that irradiated by the microwave energy apparently was higher than the energy gained by the system without the microwave pre-treatment (Table 2). The conventional method was solely supplied by the normal heating at 40ºC and resulted very low specific energy absorbed for the polyphenols extraction i.e. 59.01 J/g in comparison to 203.71 J/g by MAE pre-treatment. By microwave pre-treatment the extraction rate was improved 2-5 times from the conventional extraction with shorter time achieve an equilibrium yield. Therefore, it is clearly showed that with MAE pre-treatment the extraction of solutes has been improved in terms of extraction rate, equilibrium yield and extraction time.

Table 2 Energy absorption in different extraction method

Method	Temperature (ºC)	Energy emitted (J)	Energy Absorbed (J)	Specific energy absorbed (J/g)
MAE	80.7 ± 0.8	4500	2627.08	203.71
Conventional	40.0 ± 0.2	-	761.06	59.01

4.2 Effect of absorbed energy on TPC

In order to analyse the influence of the absorbed energy, at constant ethanol proportions in the solvent of 50%vol and S/F ratios of 14 mL/g, the solvent-sample was irradiated by i) 200W during 15s, ii) 300W during 15s and iii) 300W during 30s. These combinations of power and time resulted in emitted energies of 3000, 4500 and 9000 J, respectively. Thus, as a result different amounts of energy absorbed were attained, as presented in the Table 3. The corresponding kinetic curves of polyphenols extraction are shown in Figure 2. As it can be seen, with a higher absorbed energy, higher TPC extraction yield was attained. This result is due to solvent power increases at high temperature of solvent-sample system as a results of solvent's viscosity and surface tension, hence enhance the solutes release from plant cells and increase its solubility in the solvent. This explanation is consistent with the mass transfer coefficient, k_1 estimated by Patricelli's model, showed in Table 4, which increased as the

energy absorbed increased as a result of the enhanced accessibility of the solute due to cellular rupture.

Table 3 Energy absorbed by 50%vol of ethanol-*C.nutans* at different energy emitted (J)

Conditions	Final temperature (°C)	Energy emitted (J)	Energy absorbed (J)	Specific absorbed energy (J/g)
200W+15s	62.3 ± 0.3	3000	2094.90	145.77
300W+15s	75.2 ± 0.4	4500	2453.46	191.15
300W+30s	80.8 ± 0.2	9000	2688.02	230.56

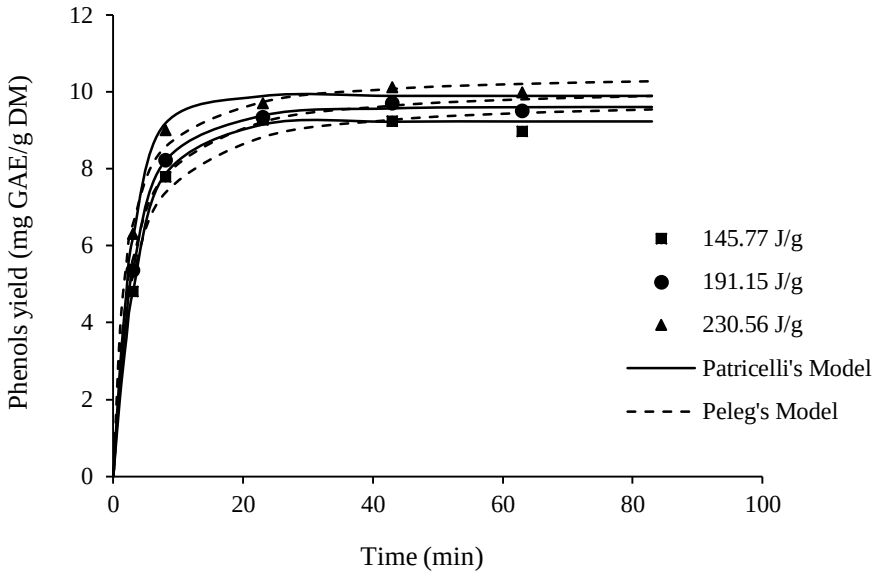


Figure 2 Kinetics profile of phenols yield at 50%vol ethanol concentration, S/F: 14 at different specific energy absorbed; ■145.77 J/g, ●191.15 J/g, ▲230.56 J/g

.

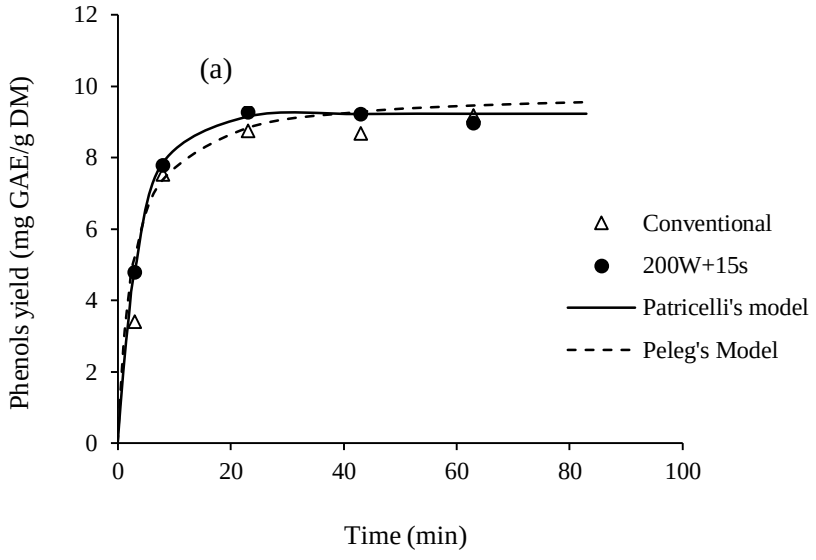
Table 4 Mass transfer coefficient, yield and extraction rate of polyphenols at different specific absorbed energy by Patricelli's and Peleg's model

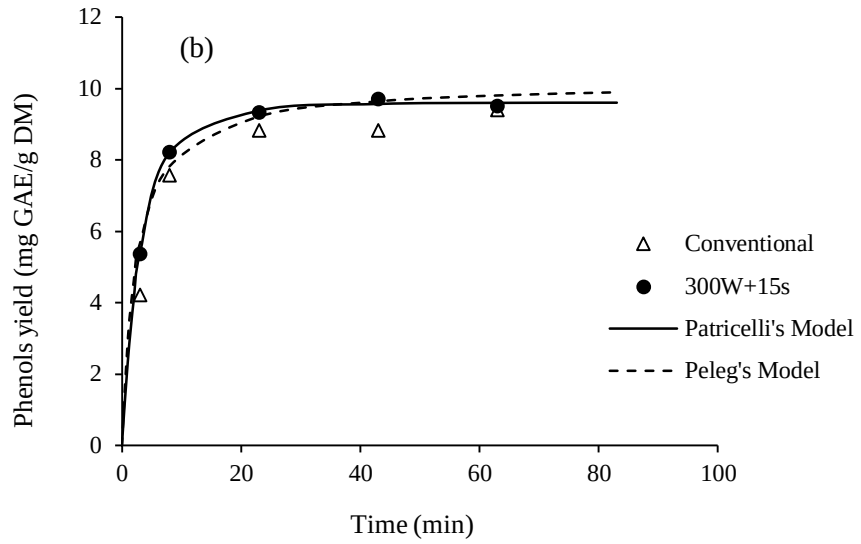
Specific absorbed energy (J/g)	*Patricelli's model*						
	Mass transfer coefficient (min^{-1})		TPC (mg GAE/g DS)		Extraction rate (mg/g/min)	Equilibrium yield C_e (mg GAE/g DS)	R^2
	k_1	k_2	C_1	C_2			
145.77	0.26	0.10	8.61	0.63	2.28	9.24	0.994
191.15	0.33	0.09	7.90	1.71	2.76	9.61	0.998
230.56	0.33	0.26	9.90	0.002	3.25	9.90	1.000
Specific absorbed energy (J/g)	*Peleg's model*						
	K_1 (min·g DS/mg GAE)		K_2 (g/mg GAE)		Extraction rate, B_o (mg GAE/ min·g DS)	Equilibrium yield, C_e (mg GAE/g DS)	R^2
145.77	0.27		0.101		3.66	9.86	0.947
191.15	0.24		0.098		4.22	10.19	0.974
230.56	0.17		0.095		5.81	10.50	0.999

However, although experiments performed with 300W microwave power during 30s showed the highest TPC yield and extraction rates, this condition was not chosen for the further investigation due to instability of sample mixture during the microwave pre-treatment such as severe hurdling of materials that caused important losses of sample extracts.

To further determine the effect of microwave power pre-treatment on the extraction of polyphenols from *C.nutans,* and ensure that the differences observed in the results Figure 3 were related to the different powers applied and not only to the final temperatures achieved after microwave irradiation, a series of conventional method extractions (without microwave treatment) were carried out at a similar temperature as that found in MAE experiments with different powers, i.e. 62.3, 75.2 and 80.8°C in 15s followed by infusion at 40°C using 50%vol ethanol and S/F = 14ml/g

Results of these experiments are presented in Figure 3. It is observed that the yield of phenols obtained by the conventional method was slightly lower and kept slowly evolving during a long time until the end of extraction. However, opposite situation was observed in the MAE where the yield was higher at much shorter times and tend to decrease towards the end, probably due to a slow degradation of the dissolved polyphenols. This shows that the increase in microwave power has a more pronounced effect on the extraction than a simple increase in the extraction temperature.





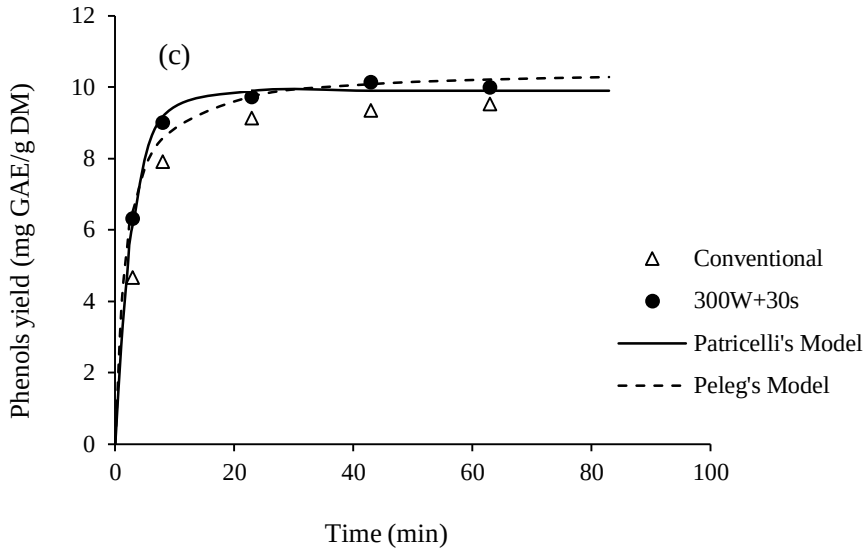


Figure 3 Kinetic of phenols yield by MAE and conventional at different power and temperature: 200W+15s, 62.3°C (a), 300W+15s, 75.2°C (b), 300W+30s, 80.8°C (c)

A different mechanism of heating in both methods can explain the results. Unlike the conventional method that proceeds through a conductive heating, the extraction of solutes in MAE is driven by electromagnetic waves that heat the whole sample simultaneously. As a result, localised heating occur leads to expansion and rupture of cell walls and improve the release of solutes from the material [7, 19]. To illustrate the effect of these two heating

mechanisms on samples, scanning electron microscopy (SEM) of *C.nutans* residuals from MAE and conventional extraction (without microwave pre-treatment) were compared and presented in Figure 4. No clear damaged of cells can be observed in the residual of samples extracted by the conventional method. Nevertheless, the SEM of residual from MAE exhibits damaged cell structure because of sudden rise of temperature during MAE heating followed by a drop of temperature through fast cooling technique in ice bath. This leads to a generation of internal pressure inside the cells which ultimately accelerates the release of solutes from the materials. Dahmoune, Nayak, Moussi, Remini and Madani [20] also confirmed in their work on the microwave extraction of polyphenolic compounds from medicinal plant that the electromagnetic waves caused cell damage and rupture due to sudden temperature rise during microwave irradiation. In addition, heat and mass transfer occur in the same direction from inside to outside in MAE, accelerating the solubilisation of solutes. Meanwhile the heat that transferred from the outside to the inside of samples in the conventional method prolongs the extraction period needed for the solutes to diffuse out and become solubilised in the solvent [6].

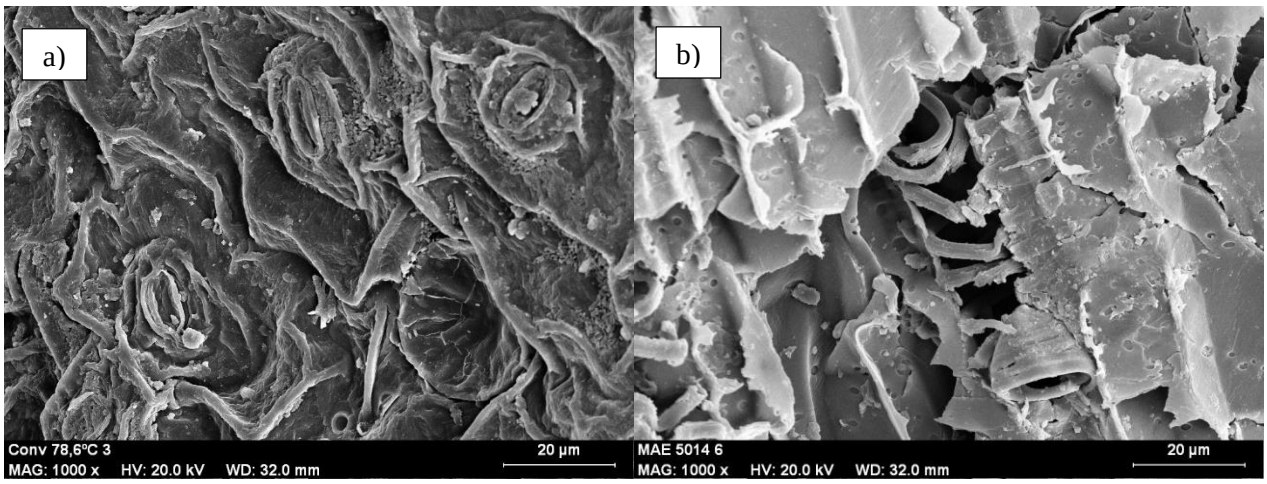


Figure 4 Scanning electron microscopy images of *C.nutans* residuals from conventional extraction-without microwave pre-treatment (a) and MAE

4.3 Influence of ethanol concentration

MAE experiments with ethanol-water mixtures in proportions ranging from 44% to 86%EtOHvol were performed at constant S/F of 14 mL/g, with a microwave power of 300 W applied during 15 s. With these conditions, the ethanol-water solvent mixtures reached their normal boiling point, as verified by calculations of ethanol-water boiling points performed with the Aspen Hysys thermodynamic package. Results of the extracted polyphenols and absorbed energy at different ethanol concentration are presented in Figure 5.

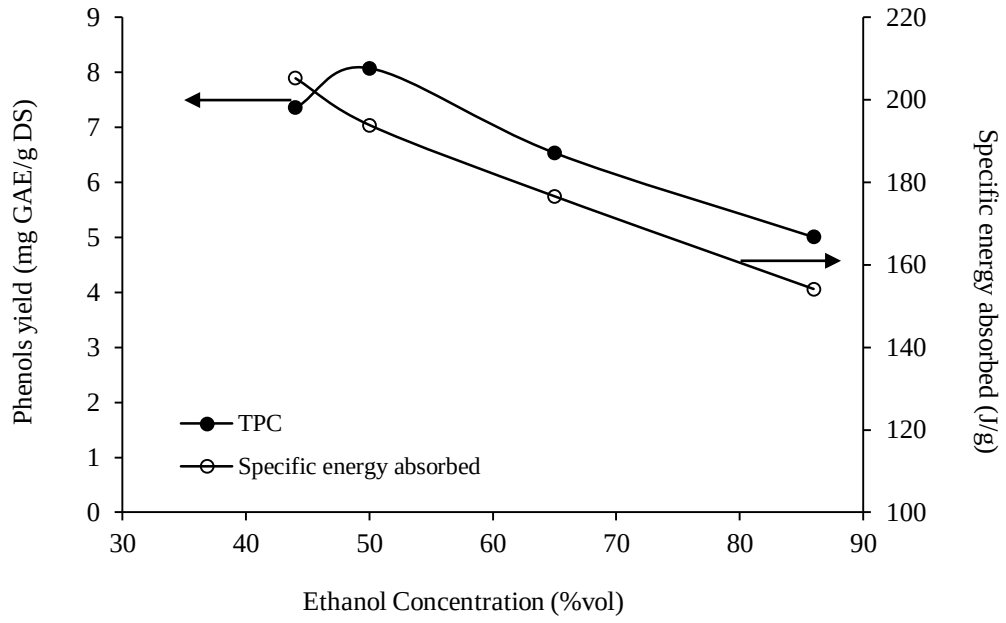


Figure 5 Phenols yield and specific energy absorbed as a function of ethanol proportion (%vol)

It is observed that decreasing ethanol ratio from 86% to 50%vol significantly increased the TPC concentration. However further decreasing the ratio to 44%vol slightly reduced the TPC value (Figure 5). The highest phenols yield (8.08 mg GAE/g DS) was obtained with 50%vol of ethanol as an optimum extracting solvent whereas the lowest (only 5.01 mg GAE/g DS) was observed using 86%vol ethanol-water mixtures. An improved performance of conventional or microwave-assisted extractions when a mixture of ethanol and water in certain, application-specific proportions is used rather than pure ethanol or pure water has been

frequently observed and reported. In particular, several works have also reported a 50%vol proportion as the optimum ethanol concentration for the extraction of polyphenols and/or antioxidants from plant materials [21, 22]. The reasons for this general behaviour are not entirely clear, but it is considered that some important factors are the possibility to modify the polarity of the solvent mixture according to the proportion of ethanol, that can increase the selectivity of the extraction towards the desired compounds [23], and the swelling of the plant material by the addition of water that can improve the contact between solvent and solutes [24]. For example, several authors also have reported that higher water concentration reduced the extraction yield since higher water content caused the solvent mixture to reach a degree of polarity that is no longer favourable for the extraction [6, 25, 26]. In addition, Galanakis, Goulas and Gekas [27] also reported that a balance of high and moderate polar structure in the phenols compounds contribute to a remarkable effect in the extraction when equal ratio of ethanol-water concentration is employed as extracting solvent.

The energy absorbed under a constant value of energy emitted of 4500 J shows a decreasing value as the proportion of ethanol in the solvent mixture increased (Figure 5). The energy absorption is dependent on the dielectric loss factor of the system [28]. At ambient temperature, the dielectric loss factor decreases as the proportion of ethanol in the solvent mixture is increased [29], thus justifying the experimental trends, although at higher temperatures this trend may be reversed as the dielectric loss factor of ethanol increases at higher temperatures [28]. As shown in Figure 5, it is particularly remarkable that a reduction of ethanol proportion from 50%vol to 44%vol implies an increase in the specific energy absorbed that, as previously described, is not accompanied by an improvement in the extraction yield, indicating that the influence of this parameter is associated with the variations in the properties of the solvent mixture and not with the energy input provided.

The kinetics profile of polyphenols extraction at different ethanol proportions were correlated with Peleg's and Patricelli's models, as presented in Figure 6. In general, the experimental data were well-fitted by both models, that gave comparable curve shapes. The extraction of polyphenols from *C.nutans* using 50%vol as extracting solvent gave the fastest extraction rate with 3.60 mg/g/min and 5.97 mg/g·min (in Table 5) estimated by Patricelli's

and Peleg's model, respectively. Accordingly, the time needed to reach equilibrium decreased from about 40 min in experiments with 86%vol solvent mixture, to about 25 min in experiments with 44%vol or 50%vol solvent mixtures. The highest equilibrium TPC was obtained at 50%vol ethanol and were estimated by Patricelli and Peleg's models as 7.68 and 7.82 mg GAE/g DS, respectively, in good agreement with the experimental results.

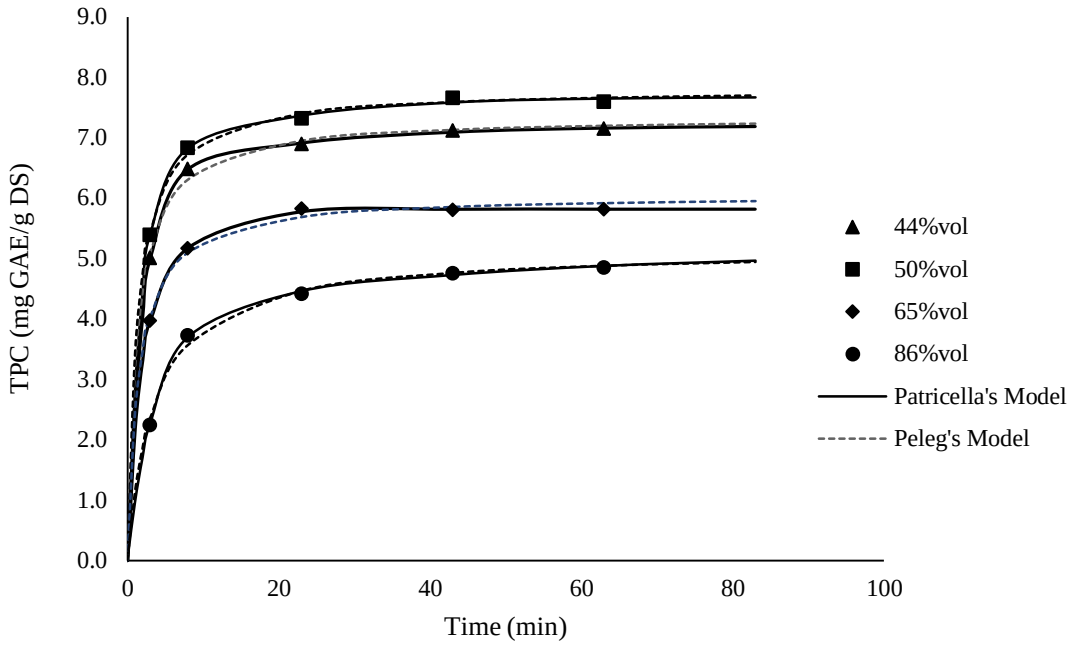


Figure 6 Kinetics profile of phenols yield at different ethanol concentration fitted by Patricelli's and Peleg's Model

As can be observed from the Patricelli's parameters in Table 5, the yield of TPC at the washing step was higher than at the diffusion step ($C_1 > C_2$). This can be explained by the rapid dissolution of solutes from the broken cell walls due to the microwave irradiation into the solvent during the washing period. Towards the end, the extraction rate decreased and became slower as the extraction was prolonged until reaching the plateau yield. This behaviour can also be observed in the mass transfer coefficient that was much higher in the washing step rather than in the diffusion step ($k_1 > k_2$). On the other hand, Peleg's model described the extraction process globally and thus those characteristics were not explicitly described.

Table 5 Mass transfer coefficient, yield and extraction rate of polyphenols at different ethanol proportions fitted by Patricelli and Peleg's model

Proportion (%vol)	*Patricelli's model*						
	Mass transfer coefficient (min^{-1})		TPC (mg GAE/g DS)		Extraction rate (mg/g/min)	Equilibrium yield C_e (mg GAE/g DS)	R^2
	k_1	k_2	C_1	C_2			
44	0.50	0.05	6.31	0.89	3.17	7.20	1.000
50	0.55	0.06	6.42	1.25	3.60	7.68	0.997
65	0.81	0.18	3.10	2.71	3.02	5.81	0.999
86	0.27	0.03	3.92	1.15	1.09	5.08	0.999
Proportion (%vol)	*Peleg's model*						
	K_1 (min·g DS/mg GAE)	K_2 (g/mg GAE)	Extraction rate, B_o (mg GAE/ min·g DS)		Equilibrium yield, C_e (mg GAE/g DS)	R^2	
44	0.18	0.14	5.55		7.34	0.985	
50	0.17	0.13	5.97		7.82	0.990	
65	0.25	0.17	4.01		6.06	0.983	
86	0.69	0.19	1.44		5.15	0.991	

The residuals of calculations of both models are presented in Figure 7. Patricelli's model showed better agreement with the experimental data compared to the Peleg's model, with $R^2 > 0.995$ (Table 5) at all ethanol proportions studied. In particular, as shown in Figure 3 fittings with Peleg's model showed higher residuals at intermediate extraction times (10-20 min). This result indicates that this model, based on desorption/sorption phenomenon, is not able to provide a good fit of results when the extraction process is controlled by two processes, i.e. washing and diffusion step. A similar observation was reported by Sant'Anna, Brandelli, Marczak and Tessaro [30] who investigated the kinetics of aqueous extraction of solutes from Fennel. The high value of the correlation coefficient R^2 and reasonable model characteristics of the Patricelli's model compared to Peleg's model indicate that the Patricelli's model is a useful tool to profiling the microwave extraction of *C.nutans* for the conditions studied.

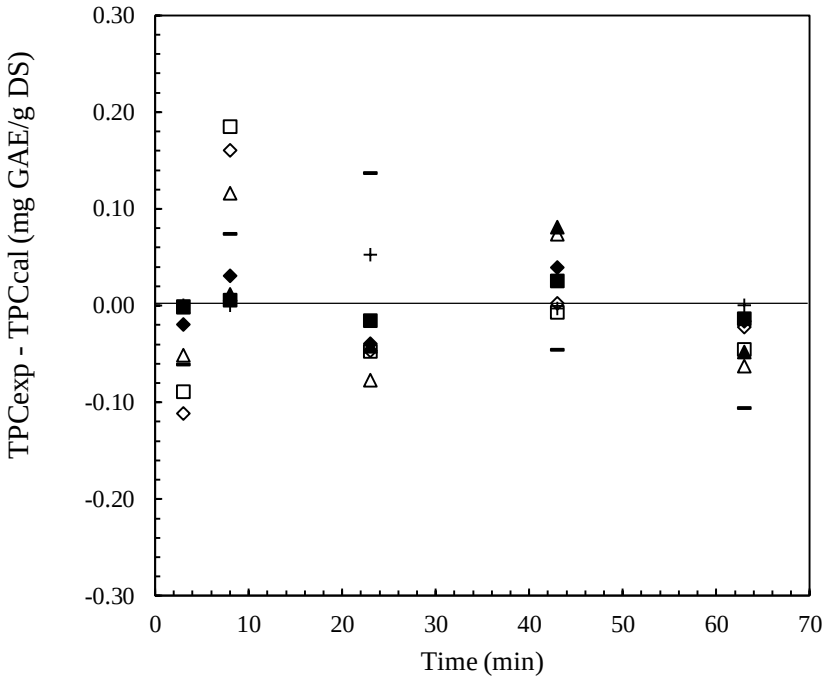


Figure 7 Residual plot between experiments and calculated of phenols yield at different ethanol concentration, Peleg's Model: □44%vol, Δ50%vol, ─65%vol, ◊86%vol; Patricelli's Model: ■44%vol, ▲50%vol, +65%vol, ♦86%vol

4.4 Influence of solvent-to-feed ratio (S/F)

In order to study the influence of the solvent-to-feed ratio (S/F) on the extraction, the power and time were fixed at 300 W and 15 s. According to the results obtained in the previous section, a constant ethanol proportion in the solvent mixture of 50%vol was used.

The S/F ratio is important in MAE to ensure that sample materials are fully immersed in the extracting solvent and experience a uniform sample heating rate. Solvent-to-feed ratio (S/F) can be varied either at constant solvent volume (varying sample weight) or a constant sample weight (varying solvent volume). The latter technique is more interesting since the sample heating rate is subjected to the energy absorption by solvent-sample system which determined by the dielectric properties and sample size [31]. Thus, the amount of solvent was varied with a fixed amount of solid sample, with S/F ratios of 7, 14 and 20 mL/g.

The specific energy absorbed by the solvent-sample system and the phenols yield after irradiation over a constant microwave energy total power at different S/F are shown in Figure 8. As it can be seen in this Figure, the phenols yield was enhanced when solvent volume was increased from 7 to 14 mL over 1 g of sample material, but when it was further increased to 20 mL/g the yield dropped to 5.32 mg GAE/g DS. This happened because the large amount of solvent incorporated resulted in a low specific energy absorbed by the S/F 20 mL/g sample mixture. On the other hand, despite the experiment with S/F = 7 mL/g resulted in a higher specific energy absorbed than in S/F = 14 mL/g due to the smaller solvent volume, the phenols yield was slightly lower in comparison to the yield obtained with S/F= 14 mL/g. One possible reason is the mass transfer limitation due to small solvent volume that hindered the diffusion of solutes from intracellular to the solvent bulk phase.

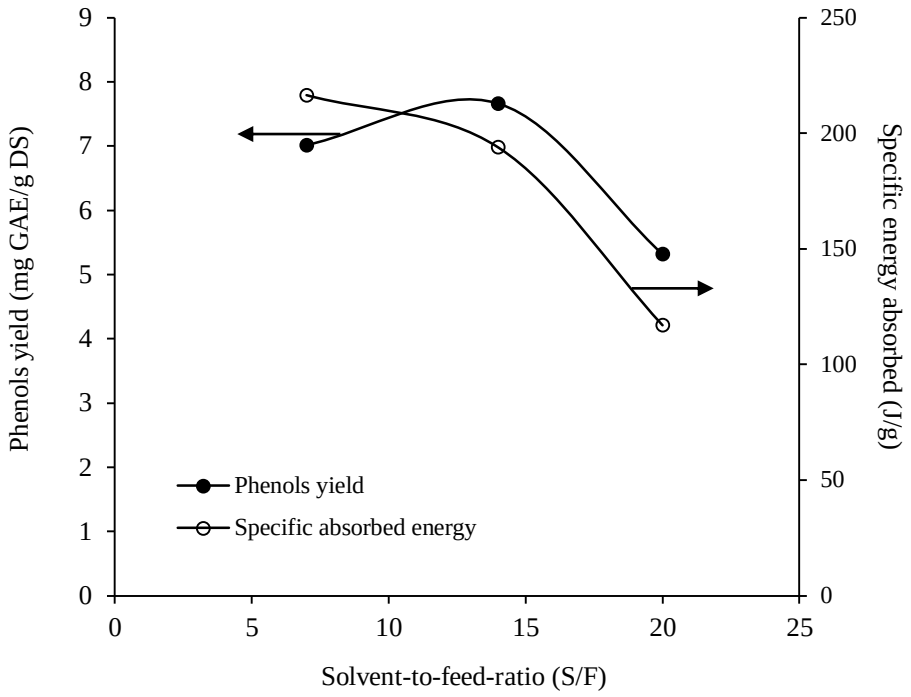


Figure 8 Phenols yield and specific energy absorbed as a function of solvent-to-feed ratio (S/F)

Kinetics modelling of the TPC yield at different S/F ratios using Patricelli's and Peleg's models is presented in Figure 9 meanwhile the estimated parameters for both models are presented in Table 6. According to Patricelli's model (in Table 6), it is observed that the mass transfer coefficient at the washing step k_1 was higher when the S/F ratio was 7 mL/g (k_1 = 0.73 min^{-1}) than when it was 14 mL/g (k_1 = 0.55 min^{-1}). In contrast, the TPC yield at the washing step was higher when the S/F ratio was higher, and as the extraction time was prolonged the extraction rate of the experiment with S/F=7 mL/g became slower until trace amounts of TPC was extracted in the diffusion step i.e. C_2 = 1.11E-05 mg GAE/g DS. These results clearly indicate that with S/F ratio of 7 mL/g the solvent quickly became saturated during the first minutes of the extraction process.

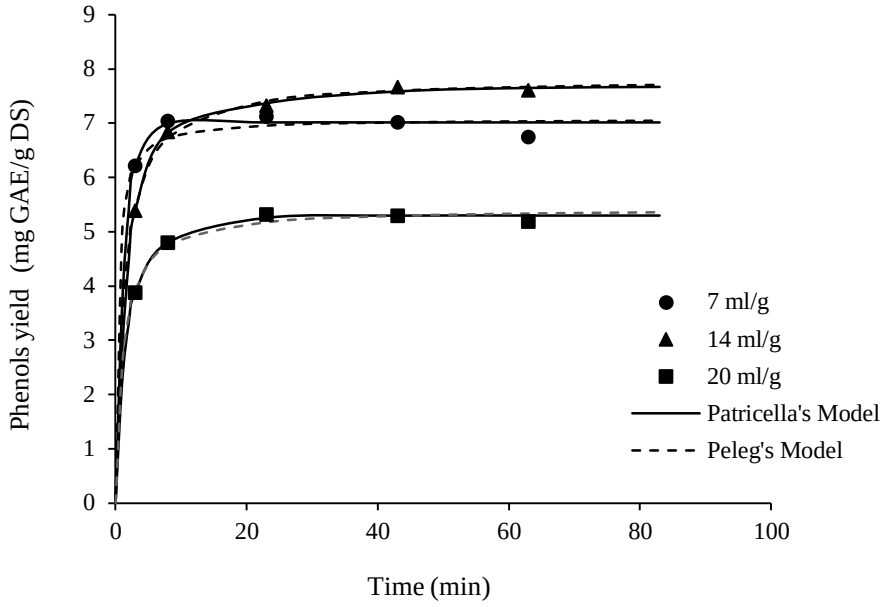


Figure 9 Kinetics profile of phenols yield at different solvent-to-feed ratio (S/F) fitted by Patricelli's and Peleg's Model

On the other hand, with S/F = 20 mL/g the slowest extraction rate was observed: 3.13 mg/g·min^{-1} and 4.71 mg GAE/min·g DS calculated by Patricelli and Peleg's model, respectively. From the Patricelli's model, a low TPC yield at the washing step, C_1, was found. Thus, a large solvent volume did not improve the extractability of solutes from *C.nutans* sample material. The low specific energy absorbed by the sample mixture, as reported in Figure 8, might retarded the extraction of polyphenols. In contrast, the experiment with S/F = 14 ml/g showed a reasonable yield in both extraction regions and resulted in a higher equilibrium TPC yield than in experiments with S/F = 7 and 20 mL/g with an equilibrium TPC yield as high as 7.68 mg GAE/g DS within 43 min after 15 s of irradiation by 4500 J of microwave energy.

Table 6 Mass transfer coefficient, yield and extraction rate of polyphenols at S/F fitted by Patricelli and Peleg's model at 50%vol, 300W and 15s

S/F (ml/g)	*Patricelli's model*						
	Mass transfer coefficient (min^{-1})		TPC (mg GAE/g DS)		Extraction rate (mg/g/min)	Equilibrium yield C_e (mg GAE/g DS)	R^2
	k_1	k_2	C_1	C_2			
7	0.73	0.002	7.01	1.11E-05	5.09	7.01	0.846
14	0.55	0.06	6.42	1.25	3.60	7.68	0.997
20	0.16	0.81	1.83	3.47	3.13	5.30	0.986
S/F (ml/g)	*Peleg's model*						
	K_1 (min·g DS/mg GAE)	K_2 (g/mg GAE)	Extraction rate, B_o (mg GAE/ min·g DS)			Equilibrium yield, C_e (mg GAE/g DS)	R^2
7	0.06	0.14	17.00			7.08	0.678
14	0.17	0.13	5.97			7.82	0.988
20	0.21	0.18	4.71			5.44	0.959

5. Conclusions

Extraction kinetics of ethanol-*C.nutans* system irradiated over microwave energy were reported. Influencing parameters on the extraction yield of polyphenols such as ethanol concentration, solvent-to-feed ratio (S/F) and energy absorbed were studied. The variations in the effect of ethanol concentration and S/F were also explained in view of energy absorption, and the specific energy absorbed (J/g) was introduced in this work in order to have better understanding on effect of energy absorbed towards extractability of compounds interest. Polyphenols yield was found to be the maximum using a 50%vol ethanol-water solvent mixture. The yield of polyphenols energy absorbed-dependent at short extraction times, but the solvent composition was more dominant enhancing the final extraction yield. S/F at 14 mL/g was the best ratio to produce the highest polyphenols yield. In terms of modelling, Patricelli's model gives excellent profiling kinetics behaviour and accurate predictions of polyphenols yield and extraction rate. Microwave pre-treatment has substantially improved the extractability of polyphenols from the medicinal plant and could be as a promising approach to enrich compounds of interest in shorter time.

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Chapter 3

Impregnation of medicinal plant phytochemical compounds into silica and alginate aerogels

The Journal of Supercritical Fluids (2016), 116, 251-263

Abstract

Herbal medicinal plants are important sources of bioactive, medicinal compounds. In this work, impregnation of phytol as a model compound and multicomponent Clinacanthus nutans (C.nutans) plant extracts into alginate and silica aerogels was investigated, applying two different methods: wet impregnation and supercritical impregnation. Two types of C.nutans extracts were prepared by microwave-assisted extraction (MAE) using either ethanol-water solvent mixtures or pure ethanol as solvent. The impregnated compounds were analyzed by Infrared spectroscopy (FTIR), thermogravimetric analysis (TGA) and chromatography (HPLC and UPLC-MS/MS). Results showed that supercritical impregnation method yielded the highest loading content with silica aerogels, with a content of 30.1 ± 0.6 wt% of the model compound phytol, and 11.5 ± 0.4 and 23.9 ± 1.0 wt% of the extracts obtained with ethanol/water and pure ethanol solvents, respectively. In the wet impregnation method, alginate aerogels exhibited higher loading than the silica aerogels regardless of their surface area properties, indicating that in this case other properties of carrier materials and the nature of compounds have a stronger influence on the compounds loading than the surface properties of the carrier. Impregnation in alginate aerogels yielded higher total phenols and flavonoids contents than in silica aerogels. Plasticized alginate biopolymer was formed when the supercritical impregnation was performed with excess of phytol at 200 bar and 40°C, indicating that the swelling and/or plasticizing effect of $SCCO_2$ was remarkable combined with the effect of excess solute. Release kinetics results indicate that the alginate aerogel impregnated with the extract obtained with pure ethanol is suitable for controlled drug release whereas the alginate aerogel impregnated with the ethanol/water extract is more appropriate for fast release purposes. The release kinetics results were fitted into several mathematical models: zero order, first order and Higuchi model, to evaluate the mechanism of the release. Results showed that diffusion and erosion and/or swelling control the release of extract from the aerogels.

1.0 Introduction

Medicinal herbs have received much attention for many years as sources of natural drugs for therapeutic purposes. Herbs with high content of phytochemicals with antioxidant, anti-cancer, anti-inflammatory compounds have applications for diseases treatment and prevention, in chemotherapy and to form good health. Clinacanthus nutans Lindau (*C.nutans*) is a popular medicinal plant in South-East Asia. Recently, it has received much attention due to its therapeutic applications related to its antioxidant, anti-inflammatory, antimicrobial and anti-viral properties. In our previous study, *C.nutans* was extracted by Soxhlet extraction, supercritical CO_2 extraction and microwave-assisted extraction (MAE), and it was found that the MAE method provided the highest extraction yield of bioactive compounds in the shorter extraction time. Among various phytochemical compounds identified, phytol was found as the major bioactive compound in the *C.nutans* extracts [1].

Phytol is an acyclic diterpene alcohol. It is an integral part of chlorophyll and it is abundantly found in green plants and planktonic algae. Phytol is commonly used as aromatic ingredient in cosmetic and in food additives. In the medicinal field, it has been claimed that phytol has antibacterial and anti-inflammatory properties, and acts as an excellent adjuvant in vaccine formulation to stimulate humoral immunity response [1-3]. Recent studies have revealed that phytol has several unique therapeutic activities such as antioxidant and antinociceptive action on the central nervous system (CNS) [4]. This study claimed that phytol showed *in-vitro* antioxidant activity at dosages of 25, 50, 100 and 200 mg/kg. Furthermore, the antibacterial and antiradical properties of phytol could complement new therapies for heart disease, and its antischistosomal effect make it active in the treatment of major endemic diseases [5].

However, these compounds are prone to degradation by ambient conditions. One way to protect the bioactive compounds is by incorporating them into a polymer or biopolymer matrix. For application for oral drug delivery of pharmaceutical compounds, it is necessary to use biodegradable materials with suitable release kinetic characteristics. Alginate is a natural polysaccharide biopolymer mainly derived from brown algae and consists of α-L-guluronic acid and β-D-mannuronic acid (M) residues, linearly linked by 1,4-glycosidic linkage [6]. This

natural biomaterial has been accepted in several areas of application as it is biodegradable, low cost, non-toxic and stable. Over many years, alginate has been extensively investigated for food applications, controlled drug delivery, encapsulation and environmental applications [7-10]. Several attempts have been reported on the application of alginate aerogels with desirable therapeutic features for drug delivery either in monolith forms [11] or microsphere beads [9, 12]. Hydrogel alginate can be synthesized by inducing the gelation of alginate solution based on two fundamentals methods: i) diffusion method or ii) internal setting method. Typically, they can be prepared as hydrogels and dried under ambient condition or by supercritical fluids to produce xerogels and aerogels, respectively. By supercritical drying high specific surface area, large porosity and low density of aerogels can be produced while maintaining the network structure of the polymer or biopolymer. These attractive characteristics make aerogels suitable for delivery of bioactive compounds or drugs for pharmaceutical applications.

Incorporation of active substances into carrier matrices (particularly, polymeric) by impregnation has been applied to protect and preserve valuable compounds from free radicals, oxygen or UV, and for controlled drug release purposes [13, 14] or bioavailability enhancement [15]. Among other methods, processes based on supercritical fluids are promising ways to impregnate active substances in a matrix without using organic solvents. Carbon dioxide is the most commonly employed supercritical fluid due to its non-toxic and non-flammable character and because it can be easily separated at ambient conditions leaving negligible residues in the sample [16]. The resulting technology of Supercritical Impregnation (SCI) has been recognized as a promising alternative method to conventional process due to excellent properties of the fluids under supercritical conditions. High density of the fluids encourages the solubilisation of the compounds and thus increases the rate of the impregnation. In addition, low viscosity and high diffusivity enables the fluid mixture to penetrate and impregnate the compounds into the inner parts of the materials without damaging the matrix structure. Another interesting point is that the supercritical fluids could act as a swelling and/or plasticizer agent and cause the polymer or biopolymer carrier matrix to swell, thus facilitating the penetration of the fluid-compounds mixture into the matrix [13, 17]. These advantages of the SC fluids properties have

been applied in impregnation techniques for drug delivery [18, 19], food packaging [20] and dyeing [21, 22].

In general, two basic mechanisms of impregnation have been described: 1) physical deposition and, 2) molecular dispersion [17, 23]. In the first, the key driving force for the impregnation is the solubility of the substance in the $SCCO_2$. During the process, the $SCCO_2$ solubilize the substance in a fixed time. The saturated $SCCO_2$-substance mixture swells the carriers without dissolving them. The swollen carrier enables the saturated $SCCO_2$-substance to diffuse into the internal part of the carrier, and it is then physically deposited by precipitation upon depressurization. Other factors that influence this type of impregnation include the physical-chemical properties of the carrier materials such as surface area, porosity and chemical functional groups. On the other hand, the second mechanism, called as molecular dispersion impregnation, consists on the chemical adsorption of the solute on the carrier surface at the molecular level, and depends on: interaction of $SCCO_2$-carrier, interaction of solute-carrier (that controls the solubility of solute in the carrier) and solubility of the solute in $SCCO_2$, which determine the corresponding partition coefficients of solute between supercritical fluid and carrier matrix. The saturated $SCCO_2$- solute fluid mixture acts as swelling and/or plasticizing agent and dissolves into the carrier material. The presence of other compounds such as co-solvents and additives may influence the impregnation performance [18, 24].

Apart from the SCI method, liquid absorption has also been extensively studied for the impregnation of drugs or active pharmaceutical ingredients. In the conventional method, the drug is dissolved in organic solvent and brought into contact with the carrier material by soaking for a certain time followed by solvent removal. The drawback of this technique is the use of organic solvent and it is only applicable for drugs that have high solubility in the organic solvent [24].

In the case of impregnation of aerogels, impregnation of bioactive compounds or drugs via liquid absorption integrated with $SCCO_2$ drying of the aerogels could eliminate the impregnation step in post-SC drying. Low viscosity, high diffusivity and null surface tension of $SCCO_2$ allows the gel drying while maintaining their porosity without damaging the surface structure of the material. During the SC drying, the $SCCO_2$ extracts the organic solvent, causing

solute precipitation by an antisolvent mechanism [25, 26]. Upon depressurization, the $SCCO_2$ is released, leaving the solute precipitated and trapped in the matrix pores. Nevertheless, some issues must be taken into account such as undesired reactions, drugs degradation and residual levels of toxic organic solvent in the final product that may lead to low processing efficiency [24, 27].

The objective of this study is to impregnate a phytochemical compound in silica and alginate aerogels and to investigate the interaction behavior of the encapsulated compounds with the silica and alginate matrix. The silica aerogels were synthesized via sol-gel methods whilst the alginate hydrogels were prepared via internal setting method. In both cases, aerogels were dried by supercritical CO_2. Pure phytol was chosen as a model herbal compound for this impregnation study, impregnating it into the aerogels and investigating its thermal release characteristics. On the other hand, additional experiments were carried out on the impregnation of multicomponent extracts of the medicinal plant Clinacanthus nutans Lindau (*C.nutans*) into silica and alginate aerogels. Prior to the impregnation, the *C.nutans* extract was obtained by microwave-assisted extraction (MAE) as reported in our previous work (Mustapa et al. [1]). Two impregnation methods were employed: wet impregnation (WI) during the aging process of the alcogel and supercritical impregnation (SC) over preformed, dried aerogel monoliths. Effect of different impregnation methods was discussed and dissolution tests of the phytochemical compounds from the aerogels were also carried out to determine their release kinetics.

2.0 Methodology

2.1 Materials

Alginic acid sodium salt from brown algae (low viscosity, 4-15 cP), calcium carbonate ($CaCO_3$) and glucono-δ-lactone (GDL), tetramethoxysilane (TMOS), methanol, ammonium hydroxide (NH_4OH) and phytol standard were purchased from Sigma Aldrich, Spain. Distilled water (Type II) was used in the preparation of silica and alginate alcogels.

2.2 Preparation of alcogels

2.2.1 Silica alcogels synthesis

Hydrophilic silica alcogels were produced based on a sol-gel technique with the molar ratio TMOS:methanol:H_2O:ammonium hydroxide; 1:3:4:5x10^{-3} [28]. TMOS was mixed with methanol and the solution of ammonium hydroxide-water was added drop by drop under stirring conditions. A specific volume of the mixture was then pipetted into molds and covered with parafilm for gelation to take place. After several minutes, the gels formed were immersed into methanol for aging process, which was maintained at least 7 days. The solvent was changed at least twice during aging to completely remove excess water and traces of excess of reactants from the sol-gel reactions from the alcogels.

2.2.2 Alginate alcogels synthesis

Alginate hydrogel was prepared by a method of internal gelation based on lowering of pH as described elsewhere [6, 11]. Sodium alginate was dissolved in distilled water (Type II) under stirring conditions of 400 rpm overnight to allow complete dispersion to obtain a 2% (w/v). In this work, calcium carbonate was used as Ca^{2+} divalent cations source to create cross-linking of alginate-Ca^{2+} and form alginate gels. The $CaCO_3$ was added into the solution and stirred for 1h. This was followed by the addition of GDL to liberate the Ca^{2+}, reduce the pH of the solution and to allow the solution to become a gel. The resulting mixture was poured into molds, covered with parafilm and stored in refrigerator (4^oC) for 18 h, period during which it solidified. The monoliths of alginate hydrogels were then immersed in ethanol-water solution (stepwise from 30, 50, 70, 90% v/v and twice with pure ethanol, every 24h) to substitute the water content from the hydrogel with alcohol prior to the supercritical drying.

2.3 Impregnation methods

Two different impregnation methods were tested: Wet Impregnation (WI) and Supercritical Impregnation (SC) into silica and alginate aerogels for two bioactive compounds i.e. phytol and *C.nutans* extracts. For the latter substance, two types of extracts were prepared

by microwave-assisted extraction (MAE) as described previously in Mustapa et al. [1] with slight modification, depending on the different concentration of ethanol-water solution as extraction solvent. i.e. 50vol% (thereafter referred to as CN50 extract) and pure ethanol (CN100). The target compounds for the CN50 and CN100 extracts are identified as polyphenols and phytol, respectively which has been previously demonstrated by Mustapa et al. [1] as the major bioactive compounds when different ethanol concentration as the extracting solvent.

2.3.1 Wet Impregnation (WI)

In the WI method, the absorption of substance into the alcogels was carried out at the end of the solvent exchange period. First, the compounds of interest or the plant extracts were dissolved in an organic solvent to produce a saturated compounds-ethanol solution. The solution was then put into contact with the alcogels that had been aged for at least a week, and kept in contact to allow compounds diffusion into the pores of alcogels until equilibration was reached. Subsequently, the solvent was extracted by supercritical CO_2 drying, leading to the precipitation of the compounds on the surface and pores of aerogels due to their insolubility in CO_2. It is important to note that the appropriate diffusion of the compounds into the pores of alcogels depends on the time of diffusion, concentration of the compounds solution and the porosimetry properties of the alcogels.

For the WI with standard phytol, an amount of phytol was weighed and mixed with ethanol to achieve a 17wt% solution. From preliminary trials, concentration of phytol in ethanol higher than 18wt% resulted in severely damaged silica and alginate aerogels by shrinkage and cracking. About 6 alcogel monoliths (aged for 7 days) were added and maintained into the solution for 3 days. On the other hand, for the WI with *C.nutans* extracts, two types of the plant extracts were prepared, depending on the concentration of the water/ethanol solvent employed in the extraction. The plant was firstly extracted by MAE at 300W in 15 s using 100% ethanol (CN100) and 50%vol ethanol solution (CN50) with 14 ml/g of solvent-to-feed ratio as described in [1]. The *C.nutans* extracts extracted by pure ethanol (CN100) was diluted by a dilution factor of 2.50 using pure ethanol and the alcogels were then immersed in the CN100

solution for 3 days in refrigerator (4ºC). On the other hand, for the *C.nutans* extracted by 50%vol ethanol (CN50), it is necessary to remove the water content prior to the WI and SC drying. Thus, the ethanol content was first eliminated by rotary evaporator until 99.5wt% of ethanol was evaporated and subsequently 100wt% water content was removed by freeze drying. An amount of the dried extract was then dissolved in pure ethanol to achieve a 9000 ppm solution of CN50-ethanol, sonicated and the alcogels were then immersed in the solution for 3 days in refrigerator (4ºC).

After 3 days, the impregnated alcogels were subjected to supercritical drying at 120 ± 5 bar and 40ºC to extract the alcohol. It is important to note that phytol is nearly insoluble in water (0.00327 mg/l at 25ºC) [29] and highly soluble in ethanol. Hence, phytol has good diffusivity into the alcogels during the impregnation. Meanwhile its solubility in supercritical CO_2 at 200 bar and 40ºC is in the range of 10^{-2} to 10^{-3} in molar fraction [30].

2.3.2 Supercritical Impregnation (SCI)

Supercritical Impregnation consisted on the addition of the active compounds into previously dried aerogels, by immersing these aerogels into a supercritical CO_2 fluid saturated with the active compounds. Supercritical CO_2 impregnation process was performed by a static method (batch mode) as shown in Figure 1. The impregnation cell has 15 mL of volume capacity, and it is closed with stainless steel clamps on the bottom and on the top of the cell. Prior to the impregnation, the alginate and silica aerogels were left in oven at 60 and 105ºC, respectively to remove any traces moisture remain after the SC drying. Then, a known weight (w_a) of dried aerogels were placed in the impregnation cell meanwhile pure phytol was placed in a glass container at the bottom of the vessel and separated by metal filters to avoid direct contact with the aerogels. The cell was heated to 40ºC and slowly pressurized with CO_2 to 200 bar at rate of 2 bar/min. The conditions were chosen considering the relatively high solubility of phytol in the $SCCO_2$ under these conditions [30], as previously indicated. For the case of SCI of *C.nutans* extracts, due to its low solubility in $SCCO_2$, 10*wt%* ethanol as co-solvent was used to increase the solubility of the extracts into $SCCO_2$. The experiments were performed at 150 bar and 40ºC in 24h. An equivalent amount of the CN50 and CN100 extracts were used

for the silica and alginate aerogels impregnation. After the pressurization, all the valves were closed and the system was kept at constant pressure and temperature for 24h to ensure the phytol and *C.nutans* extracts were completely dissolved and to achieve saturation of $SCCO_2$-compounds thus maximizing the adsorption of the compounds in the aerogels. After 24 h, the system was slowly depressurized at a rate of 2 bar/min to avoid shrinkage or collapse of the mesopores structure of the alginate aerogels. Finally, the impregnated aerogels were taken out of the cell and the loading of compounds was determined as later shown in section 2.5.

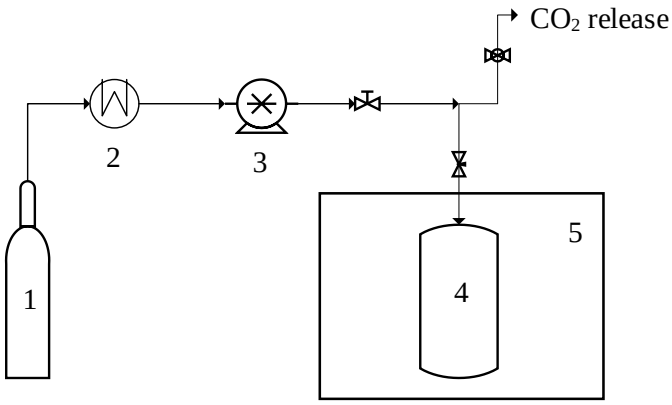


Figure 1: Supercritical CO_2 impregnation setup; 1) CO_2 tank, 2) pump, 3) cooler, 4) impregnation vessel and 5) oven

2.4 Supercritical drying of alcogels

The alcogels (silica and alginate), either neat or resulting from WI experiments, were subjected to supercritical drying by CO_2 at 120 ± 5 bar and 40ºC, employing the procedure and equipment described in a previous work (Figure 2) [28]. For this, about 8 alcogel monoliths were placed in the drying chamber, which was then filled with alcohol to prevent damage of alcogel structure during the pressurization of the system. The system was slowly pressurized to the desired pressure at a rate of 4 bar/min. After the operating conditions of drying were reached, the alcohol in the chamber was slowly removed by opening the lower valve. By doing this, a continuous circulation of CO_2 began to extract alcohol from the alcogels. The drying process was conducted in three cycles of 60 minutes minimum in each cycle. After the three

cycles of CO_2 circulation were completed, the system was slowly depressurized at a rate of 2 bar/min in order to avoid shrinkage or damage of aerogels.

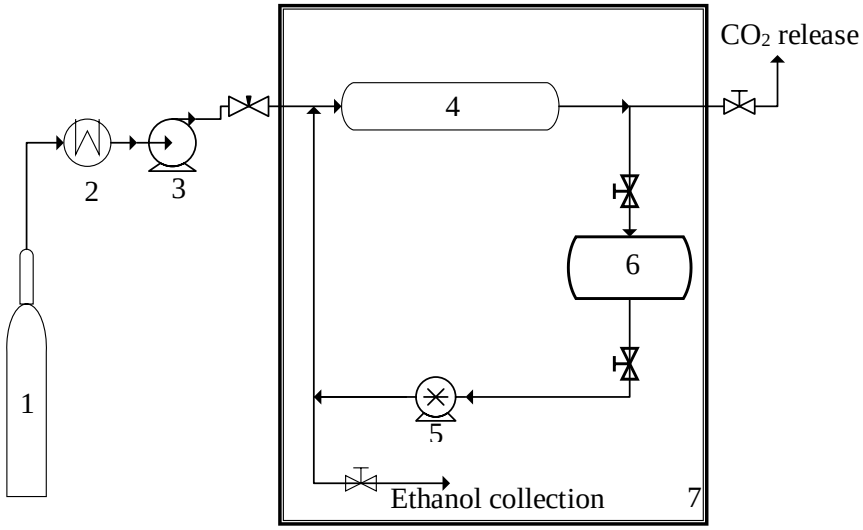


Figure 2: Supercritical CO_2 drying setup; 1) CO_2 tank, 2) cooler, 3) pump, 4) CO_2 buffer, 5) pump, 6) view cell chamber and 7) oven

2.5 Determination of compounds loading

Experiments were carried out in triplicate to measure the uncertainties of the compounds loading by SC method. The final weight of the impregnated alginate was measured (w_I) and the compounds loading were calculated as in Eq. 1:

$$\%L = \frac{w_I - w_a}{w_I} \times 100\% \qquad \text{Eq. 1}$$

where w_I is the weight of impregnated aerogels and w_a is the weight of the aerogels before the impregnation.

To determine the amounts of phytol and *C.nutans* extracts loaded via WI method, the aerogels were powdered (w_I) and dispersed in absolute ethanol and stirred at 250 rpm for 4 h. The extract was then filtered through 0.45 μm PTFE filters prior to the determination. To recover all the extracts, the powdered aerogels were washed several times with fresh pure

ethanol, filtered and combined with the first filtrate followed by drying under N_2 flow. The weight of the dried extract thus obtained corresponded to the loaded compounds and recorded as w_c (g). Thus the loading was calculated as in Eq. 2. The measurements were replicated at least three times, including in the replications the loading of samples with CN50- and CN100-ethanol solution and the assays of determination of solute content.

$$\%L = \frac{w_c}{w_I} \times 100\% \qquad \text{Eq. 2}$$

2.6 Characterization analysis

Textural characteristics of aerogels, mainly specific surface area, mean pore diameter and pore size distribution, were determined by N_2 adsorption-desorption analysis at low temperature (NOVA 3000e). Prior to the determination, the alginate and silica aerogels were degassed at 70 and 200^{o}C, respectively, under vacuum (<1mPa) for 20 h. The specific area was calculated by the method of Brunauer, Emmett, Teller (BET) and the pore size distribution was calculated from the desorption isotherm. The surface morphology of the alginate aerogels was determined using SEM JEOL JSM 820 and microanalysis (Bruker Quantax 2000). The samples were splattered-coated with gold layer prior to the scanning at voltage of 2-4kV. The impregnated aerogels (exterior and interior surfaces) were qualitatively characterized by FTIR (Bruker Platinum-ATR) equipped with software of OPUS Optik GmBH in the range from 400 to 4400 cm^{-1} of wavelength. To determine the thermal characteristics of the encapsulated phytol, thermal gravimetric analysis (TGA) was carried out from 20 to 600^{o}C at rate of 10^{o}C/min using a TGA Mettler Toledo SAE system. The density of the aerogels was determined by weighing and measuring their weight and volume using a Vernier caliper.

2.7 Analysis of impregnated *C.nutans* extracts

Determination of flavonoids content impregnated into the aerogels and extracts was carried out by ultra-performance liquid chromatography mass-spectrometry (UPLC-MS/MS). To obtain the liquid samples for this analysis, the impregnated aerogels were ground and immersed in pure ethanol to dissolve the *C.nutans* extracts. The same volume of pure ethanol

was applied to dissolve CN50 and CN100 from silica and alginate aerogels. An aliquot was then filtered through 0.45µm PTFE filters prior to the sample injection. The UPLCMS is consists of Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) with Acquity UPLC BEH C18 column (i.d., 1.7 µm x 2.1 x 50mm). Analysis was carried out with elution gradient of Acetonitrile (A) and water (B) (both containing 0.1% formic acid) at a flowrate of 0.35 ml/min and injection of 5µL, as follows: 0-0.5 min (98% A), 11 min (2% A) and 13 min (98%A). The column and auto-injector temperature were maintained at 25^{o}C.

Phytol content in the *C.nutans* extracts impregnated in silica and alginate aerogels was determined by HPLC apparatus that mainly consisted of a pump (515 HPLC Pump Waters), Waters 717 Plus Autosampler, Waters 432 Conductivity Detector, and Waters 2487 Dual λ Absorbance Detector. The column used was Symmetry C18 (5µm, 4.6 x 150 mm) and was maintained at 25^{o}C during the analysis. The mobile phase consisted of HPLC-grade methanol/acetonitrile (30:70 v/v) whilst 20 µL of the sample volume was injected at a flowrate of 1.0 mL/min. Phytol was detected by an Evaporating Light Scattering Detector (ELSD) at a UV wavelength of 210 nm. To quantify the phytol content, a calibration curve was prepared with standard phytol at a concentration range 10 – 100 mg/L. The method of analysis for total phenols content (TPC), total flavonoids (TF) and chlorophylls were applied as reported in previous work [1].

2.8 Aerogels-*C.nutans* dissolution test

C.nutans extracts release study was conducted at 37^{o}C in saline phosphate buffer (pH 6.8) as simulated intestinal fluid using an USP-certified Copley NE4-COPD dissolution tester equipment. Aerogel samples were weighed, placed in a basket metal filter and immersed into a vessel containing 500 mL of the simulated fluid. The vessel was covered and kept under 100 rpm of stirring condition. Aliquots of 2 mL were taken at predefined times and replaced with 2 mL of fresh fluid. The samples were centrifuged and subjected to UV-Vis spectrophotometer at 275nm and 665nm for CN50 and CN100, respectively. The wavelength was determined based on the maximum absorption of the *C.nutans* extracts in the phosphate buffer pH 6.8 at wavelength ranges from 700 to 250 nm.

2.9 Modeling of *C.nutans* release kinetics

The *C.nutans* extracts release kinetics from silica and alginate aerogels were fitted into several established mathematical models, i.e. zero order, first order and Higuchi model (Eq. 3, Eq.4 and Eq.5, respectively), to evaluate the mechanism of the release. The zero order model describes the drug release rate as independent on its concentration. The model is plotted as the cumulative of *C.nutans* extracts released against time in hours.

$$Q_t = Q_o + K_o t \qquad \text{Eq. 3}$$

where Q_o is the initial amount of *C.nutans* extract, Q_t is the amount of *C.nutans* released at *t* time, *K* is the zero order release constant and *t* is the time in h. For the first order kinetic model, the plot is constructed as a logarithm of the cumulative amount of *C.nutans* extract released *vs* time in hours (Eq.4). The model describes the release rate of the drugs from the system as concentration dependent.

$$Log\ Q_t = Log\ Q_o + K_o t \qquad \text{Eq. 4}$$

Higuchi model (Eq.5) describes the drug release behavior from matrix system by a diffusion model based on Fick's law. The model is plotted as cumulative of drug release *vs* square root of time.

$$f_t = Q = K_H \bullet t^{1/2} \qquad \text{Eq. 5}$$

where K_H is the Higuchi dissolution constant *t* is dissolution time in h. The best fitting model was determined based on the highest correlation coefficient (R^2) which also may explain the release kinetics mechanism whereas the release constants (*K*) were calculated as the slope of the linear plot of the models.

3.0 Results and Discussions

3.1 Characterization of the aerogels

Hydrophilic silica and acidic alginate were produced with average density of 0.23 and 0.18 g/cm^3, respectively. It was observed that the volume reduction by shrinkage during

preparation and drying of alginate aerogels was as high as up to 60% after SC drying. Other works have also reported the strong shrinkage behavior of alginate or other polysaccharides aerogels, with up to 64% volume reduction [11], meanwhile with β-glucan aerogels a shrinkage of 68% was reported [31], in comparison with typical shrinkages of less than 5% in the case of silica aerogels [32]. This behavior is consequence of the relatively large proportion of water in polysaccharides gels. The first shrinkage occurrence can be observed during the solvent exchange where the water in the alginate alcogels is displaced with the ethanol. For this reason, the exchange must be done in several steps with gradually increasing alcohol concentration in order to control the shrinkage phenomenon, as if the hydrogels are directly dehydrated in pure ethanol in the solvent exchange a large shrinkage of the alginate aerogels occurs. From our observation, four exchange steps with increasing ethanol concentrations (30, 50, 70 and 100%vol) and 24h of residence time in each concentration results in a shrinkage of the hydrogels as high as 30-45% of the initial volume. The residence time after each solvent exchange is also important to ensure that all water has been displaced by the ethanol, because the hydrogels that are not well-dehydrated can suffer extensive shrinkage during the SC drying. Typically, the additional shrinkage of the polysaccharides aerogels after the SC drying can reach up to 5-15%. Polysaccharide aerogels are lightweight materials and consist of fragile open-porous network, thus particularly sensitive to expanded CO_2 organic solvent within the gels at high pressure condition. Other possible reasons for shrinkage are residual of solvent or traces water in the gels which caused capillary stress, insufficient drying time and inappropriate depressurization rates [33].

Table 1 shows the porosimetric properties of the blank and impregnated alginate and silica aerogels. As it can be seen in this table, the specific surface area and pore volume of the prepared blank silica was higher in comparison to the alginate aerogels. On the other hand, the reduction of the specific surface areas of the impregnated aerogels indicates that the phytol has filled and partially blocked the pores of the aerogels. The alginate+phytol aerogels impregnated by SCI show smaller specific surface area and pore volume compared to the alginate+phytol impregnated by WI. A similar behavior of the porosimetric properties was also observed for

the silica+phytol aerogels impregnated by different methods. The final specific surface and pore volume were smaller by SCI than by the WI method.

Table 1 Porosimetric of blank and impregnated alginate and silica aerogels by different methods

Aerogels	Specific surface area (m^2/g)	Average Pore volume (cm^3/g)	Average pore diameter (nm)
Silica blank	881.5 ± 0.5	2.9 ± 0.1	12.9 ± 1.5
Silica+phytol (WI)[a]	400.4 ± 0.3	2.0 ± 0.2	20.4 ± 0.8
Silica+phytol (SC)[b]	219.3 ± 0.7	1.0 ± 0.1	17.1 ± 0.2
Alginate blank	125.9 ± 0.2	0.8 ± 0.4	25.5 ± 0.3
Alginate+phytol (DS)	97.4 ± 0.1	0.7 ± 0.3	27.8 ± 0.2
Alginate+phytol (WI)[a]	53.0 ± 1.2	0.3 ± 0.8	24.9 ± 0.5
Alginate+phytol (SC)[b]	28.1 ± 0.9	0.2 ± 0.1	33.3 ± 0.4

As a complement to the results reported in Table 1, Figure S1 provided as Supplementary Information shows the N_2 adsorption-desorption isotherms of the blank silica and alginate aerogels and the impregnated aerogels by SC method. The blank alginate aerogels and alginate aerogels impregnated with phytol exhibit adsorption-desorption isotherms of type IV, indicating the presence of mesopores in the alginate aerogels. It is observed that adsorption of nitrogen into the impregnated alginate aerogels has been decreased in comparison to the blank alginate aerogels. This is due to the pore filling with phytol that reduces the adsorption of nitrogen into the inner part of the aerogels. Additionally, Figure S2 shows impregnated alginate and silica+phytol aerogels produced by WI method.

3.2 Impregnation of phytol into silica and alginate aerogels

Two impregnation methods were studied: wet impregnation and supercritical impregnation. The results were discussed in terms of compounds loading, physical characterization of the impregnated aerogels, impregnation mechanisms and the compounds impregnated.

Table 2 presents the phytol loading capacities corresponding to the different types of aerogels and preparation methods. It is found that under SCI method, higher loading was obtained in silica aerogels with 30.1 ± 0.6*wt%*, compared with 20.1 ± 0.2 *wt%* attained by

alginate aerogels. The difference of the phytol loading can be explained by the high surface area of the silica aerogels, which is 881.5 ± 0.5 m^2/g compared to the alginate aerogels that have 125.9 ± 0.2 m^2/g of surface area. Higher specific surface area of the aerogels provides larger capacity for the compounds to be impregnated in the mesopores of the aerogels. In addition, the surface chemistry of the aerogels and substance-aerogels chemical interactions also influence the loading of the compounds [9]. Phytol has –OH groups on its structure, and tends to form hydrogen bonds with surface –OH groups on the silica aerogels. The presence of –OH groups increases the interaction with the solute, enhancing the adsorption of phytol onto the silica aerogel surfaces. As a consequence, high affinity of phytol to the silica matrix surface interaction increased the partition coefficient of the solutes towards the silica matrix [34]. In contrast, the presence of carboxyl acid groups in the molecule structure of alginate aerogels can lead to electrostatic repulsions between phytol and alginate, hence resulting in lower phytol loading in comparison to the silica aerogels.

Table 2 Loading of phytol in silica and alginate aerogels by different impregnation methods

Aerogels	Phytol loading, *L* (*wt*%)
Silica+phytol (WI)[a]	17.4 ± 0.5
Silica+phytol (SCI)[b]	30.1 ± 0.6
Alginate+phytol (WI)[a]	18.9 ± 0.8
Alginate+phytol (SCI)[b]	22.1 ± 0.2

[a]17wt% of phytol in ethanol

[b]Phytol pure: 0.3370 ± 0.0002 g

Among the impregnation methods studied, SCI technique exhibited higher phytol loading than the WI method. It is observed that the alginate aerogels impregnated by SC yielded a phytol content of 22.1 ± 0.2 *wt*%, compared to 18.9 ± 0.8 *wt*% attained by WI. In addition, silica aerogels also showed similar results of the loading capacity, with higher value obtained by the SCI method (30.1 ± 0.6 *wt*%) than by WI (17.4 ± 0.5 *wt*%). High solubility of phytol in the $SCCO_2$ in the pressure conditions employed and the favorable transport properties such as high diffusivity and low viscosity contributed to the higher loading obtained by SCI method. Those properties enabled the $SCCO_2$ to dissolve the phytol, promoting the diffusion of $SCCO_2$-phytol into the pores of the aerogels, which were then impregnated in the aerogels by the

precipitation and physical entrapment method during the depressurization. Furthermore, another reason that could also explain the lower compounds loading attained by WI was to the presence of ethanol during the SC drying of alcogels, which enhances the solvent power of the $SCCO_2$ and can cause desorption of the phytol from matrix sites into the $SCCO_2$-ethanol phase. This result indicates that the supercritical method is more feasible to impregnate phytol with higher loading capacity and without the use of organic solvent.

Nevertheless, different results were observed for the silica and alginate aerogels impregnated by the WI method, where it was found that the content of phytol in the alginate was slightly higher (18.9 ± 0.8 *wt*%) in comparison to the silica aerogels (17.4 ± 0.5 *wt*% of phytol). It has been mentioned that the $SCCO_2$-solutes mixture can dissolve into the polymer, promoting its inner plasticization and/or swelling [17, 24]. In fact, the presence of co-solvent, additive and other substances could influence the CO_2 sorption and polymer swelling and/or plasticization, thus increasing the performance of the impregnation process [35]. Alginate is a hydrophilic biopolymer network which may increase its volume by absorbing liquid solution. Thus, the ethanol used in the WI play roles as a media to swell the alginate biopolymer and act as a carrier for the absorption of the solutes into the matrix. The swelling of alginate was enhanced during the SC drying due to the increased amount of $SCCO_2$-solutes inside the alginate pores which promotes the contact between $SCCO_2$-ethanol-solutes with the biopolymer matrix. Upon depressurization, the solutes nucleate, grow and precipitate inside the pores becoming trapped within the swollen alginate biopolymer matrix. In contrast, no changes on the silica aerogels matrix after the impregnation process were observed, as SiO_2 matrix does not interact with $SCCO_2$ and plasticization and swelling effects do not take place [36]. In this case, although the compounds loading in silica and alginate involved a small difference, it was found that in the WI method alginate matrix is more appropriate than silica to impregnate phytol compounds. This result indicates that not only the surface properties, but also the type of polymer and its chemical properties are important factors to take into account in an impregnation process since it could influence the loading performance.

In addition to the experiments with the WI and SCI methods, some additional preliminary experiments were carried out according to a direct synthesis (DS) impregnation

method, by introducing the bioactive compounds i.e. phytol into alginate solution prior to its gelation. It was found that with this method, only traces of phytol remained impregnated in the alginate aerogels due to diffusion of the compounds during solvent exchange procedure and SC drying. This result made this technique inappropriate for impregnation of compounds that have high solubility in organic solvent.

3.3 Effect of impregnation methods on surface morphology

To visualize the changes of surface morphology of the alginate aerogels by the impregnation procedures by different methods, scanning analysis microscope (SEM) were carried out. As previously described, no changes on the surface morphology of silica aerogels after the SCI and WI method were observed. Therefore, the discussions on the effect of impregnation on surface morphology will be focused on the alginate aerogels.

The SEM micrographs structure of the blank alginate aerogels obtained after the SC drying at 120 ± 5 bar and 40^{o}C (Fig.3a) show the open pores and cross-linked structure which is the common surface morphology of alginate aerogels. On the other hand, the impregnated alginate aerogels with phytol by SCI method (Fig.3b) exhibits linkages of granular material, indicating the deposition of phytol on the surface structure of alginate aerogel. Nevertheless, no clear swelling effect is observed in the material.

To investigate the effect of amount of phytol impregnated on the surface morphology of the alginate aerogels, an additional experiment of SCI with excess of phytol (while maintaining the weight of the alginate aerogels) to attain a maximal loading was carried out. The loading of phytol increased to 38.1 ± 0.6 *wt%*. The increase of the value was due to the increase of phytol solubilization in the $SCCO_2$ which consequently enhances the affinity of the compounds to the alginate matrix in the presence of $SCCO_2$.

It is observed in the Fig.3c that in this experiment with higher phytol content the morphology of the material was obviously changed, appearing melted-like or plasticized. The apparent modification on the alginate biopolymer is due to the plasticizing effect of $SCCO_2$ occurred during the impregnation process. In comparison to the Fig.3b, it is observed that the

alginate structure materials became plasticized when the SCI was done with excess amount of phytol. Previously, Milovanovic et al. [37] found apparent changes that went beyond the swelling effect on the cellulose acetate polymer when excess of thymol was applied. It has been mentioned that solutes can significantly swell the polymer and lower the polymer glass transition temperature T_g [38]. The reduction of T_g of the polymer is due to the $SCCO_2$ and solutes dissolved into the polymer materials, which then exhibit a rubbery or plasticized aspect [23, 38, 39]. However, the behavior is dependent on the solubility of the solutes in $SCCO_2$, polymer-$SCCO_2$ interaction and partition coefficient between the solutes and polymer [13, 17, 23].

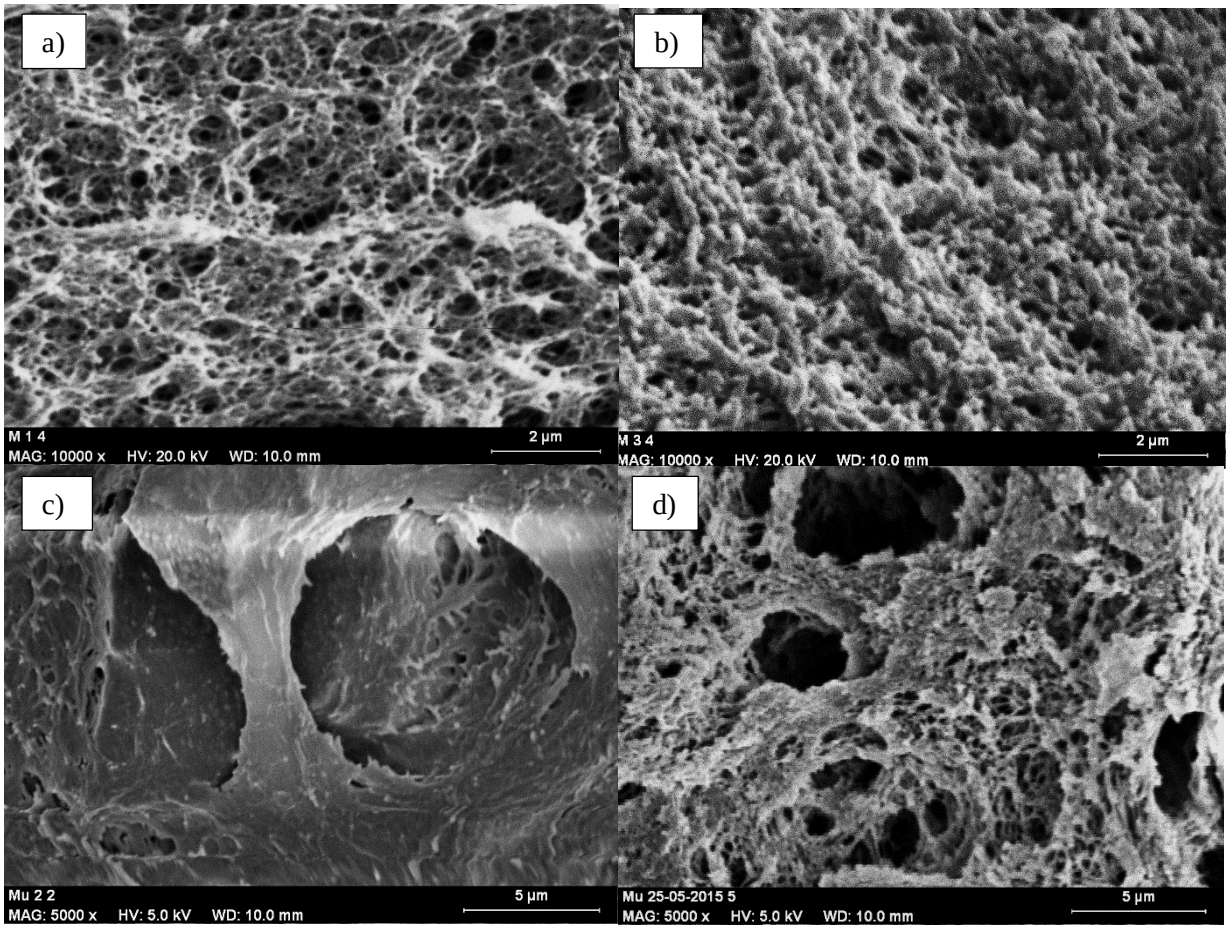


Figure 3: SEM images blank and impregnated alginate aerogels by several methods: a) blank alginate, b) supercritical impregnation (SCI), c) supercritical impregnation with excess of phytol and d) wet impregnation (WI)

Although in this experiment the loading of the compounds was increased, the severe alteration on the surface morphology might be a disadvantage if fast release is the main concern. This is because the compounds impregnated possibly have strong interaction with the materials and thus longer time needed for compounds dissolution. Nevertheless, other factors such as solubility and hydrophobicity also might influence the release rate of the substance.

On the other hand, it has been indicated that alcohol plays a role SCI in promoting the diffusion of the solutes into the insides pores of the polymer matrix [17]. In this work it is speculated that by WI method, the $SCCO_2$ and ethanol swelled the alginate biopolymer. And as a result, alginate matrix became stretched and coarser, losing their fibril linkages structures as shown in Fig.3d. This suggests that absorption of phytol from the ethanol medium may also change the pore structure of alginate aerogels whilst enhancing the penetration of the solutes.

3.4 IR spectra analysis

The effect of different impregnation methods on the phytol loading in the silica and alginate aerogels has also been evaluated by FTIR analysis. The results of FTIR assays of the impregnated alginate with phytol by wet impregnation (WI) and supercritical impregnation (SCI) methods are shown in Figure 4, compared to the results with blank alginate aerogel and pure phytol. For the blank alginate aerogel (Figure 4a), several characteristic absorption bands can be observed at 3450 cm^{-1} (O-H stretching), 2500 cm^{-1} (C-H stretching), and alginic acid bands in between 1800-700 cm^{-1} which can specifically classified as 1464 cm^{-1} for COO^- symmetric stretching and 1000-1200 cm^{-1} for C-O-C stretching vibrations [7, 40].

Clear absorption bands of pure phytol can be seen in the IR spectra at 2800 to 2960 cm^{-1} and 3360 cm^{-1} due to the stretching of –C-H and –OH bonds, respectively. These functional groups are attributed to the presence of acyclic diterpene alcohol structure of the phytol compound. The spectra were compared to the impregnated aerogels (Figure 4c-d). It is observed that the -C-H and –OH stretching also appeared in the spectra of the alginate aerogels impregnated by wet and supercritical method. This indicates that the phytocompounds were successfully impregnated in the alginate aerogels by those methods.

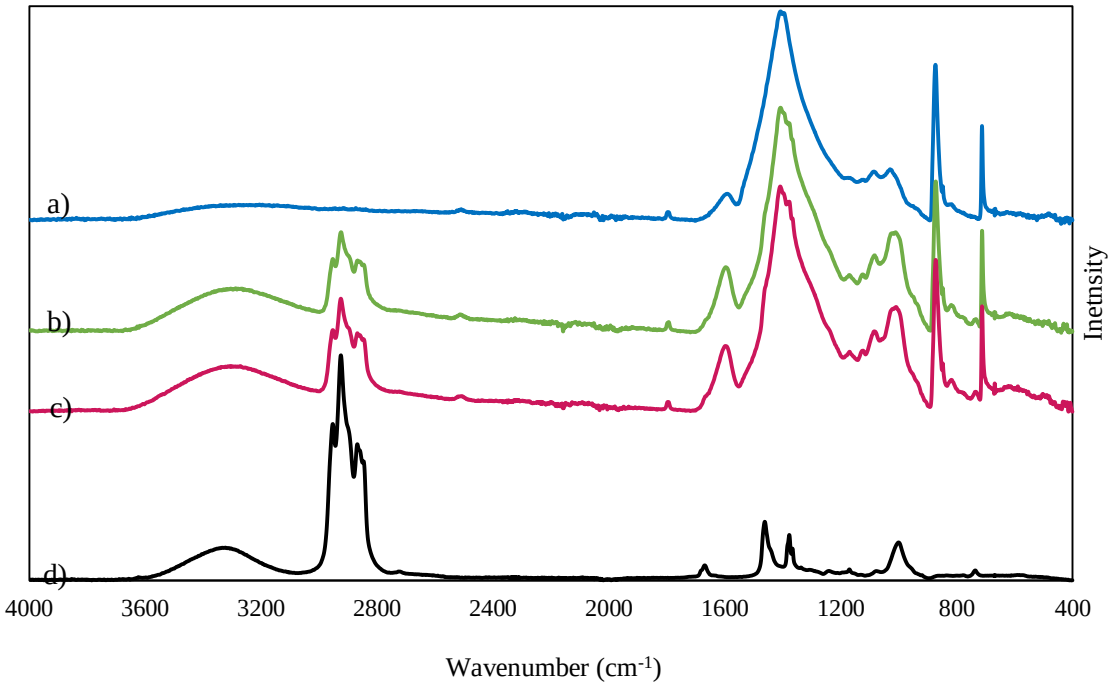


Figure 4: IR spectra of alginate encapsulated by different methods: a) blank alginate, b) wet impregnation, c) supercritical impregnation and d) pure phytol

On the other hand, a similar performance of the phytol impregnation in the silica aerogels was also observed, as shown in Figure 5. Strong absorption bands at 2800 to 2960 cm^{-1} (-C-H stretch) and 3360 cm^{-1} (-OH stretch) were found by the SCI method. In comparison, the WI of phytol into silica yielded weaker absorption bands, indicating that a fraction of phytol was co-extracted with ethanol by supercritical CO_2 during the drying process, resulting in lower phytol loading. However, Belhadj-Ahmed et al. [41] reported a different observation where the drug loading obtained by supercritical media was similar to that impregnated by liquid media, but in their case they used hexane as solvent. This can be due to the apolar and aprotic characteristics of both solvents (CO_2 and hexane) used by these authors.

From the IR features of the alginate and silica aerogels shown in Fig 4 and 5, it is found that there is no chemical reaction between the phytol compound and the aerogels materials. Further analysis by FTIR was carried out to verify the possibility of compound oxidation after the encapsulation. For this, after the initial FTIR analysis, the impregnated silica and alginate aerogels were kept in glass vials at room temperature for 3 months. After this, no new, shifted

or missing peaks were observed in the IR spectrum in comparison to the spectrum of pure phytol. This indicates that phytol encapsulated in the aerogels via WI and SCI has not undergone oxidation process after the encapsulation during this period.

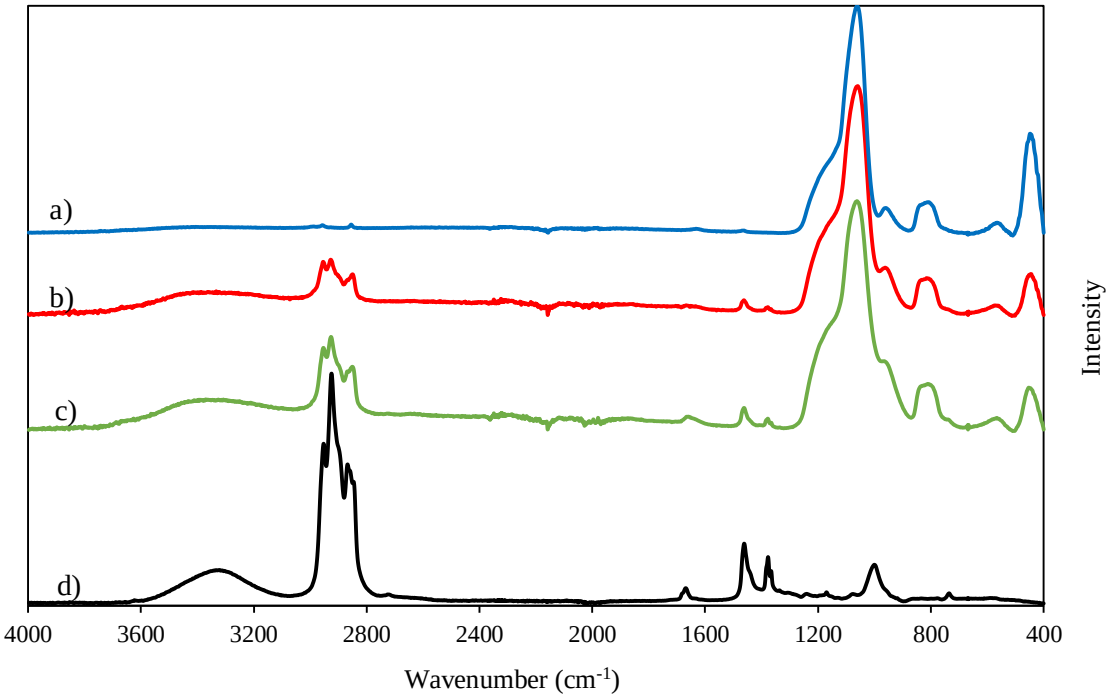


Figure 5: FTIR spectrum of silica aerogels impregnated with phytol using different methods: a) blank silica, b) wet impregnation, c) supercritical impregnation, d) pure phytol

3.5 Thermal release of alginate+phytol aerogels

TGA assays were carried out to characterize the strength of interactions between the substances impregnated and the aerogel carrier material. Besides, information from TGA could also explain any chemical interaction (if present) between the substances impregnated and the backbone of the aerogel material. Figure 6 shows the TGA curve of blank, impregnated alginate aerogels and pure phytol over the temperature range from 20 to 600°C at a heating rate of 10°C/min. From the figure, it is observed an initial mass loss from the alginate aerogels of about 5% occurring in the temperature interval from 30 to 180°C that corresponds to water desorption. Furthermore, for the blank alginate aerogels, the thermal degradation of the biopolymer occurred from 180 to 250°C, and it was followed by the decomposition of carbonaceous material until 500°C. The same decomposition behavior of alginate aerogels was

also reported by several works [12, 42]. As it can be observed from the thermograms, the curves of the impregnated alginate-phytol dropped at 220°C i.e. the same point where the pure phytol begins to decompose regardless to the decomposition of the alginate material. This indicates that the impregnated phytol has no strong interaction with the biopolymer backbone and thus it has a similar decomposition point as pure phytol. It is also observed that the weight loss of the alginate+phytol aerogels was much higher than the loss from the blank alginate aerogels. This was attributed to the release and decomposition of impregnated phytol in between 220 and 280°C.

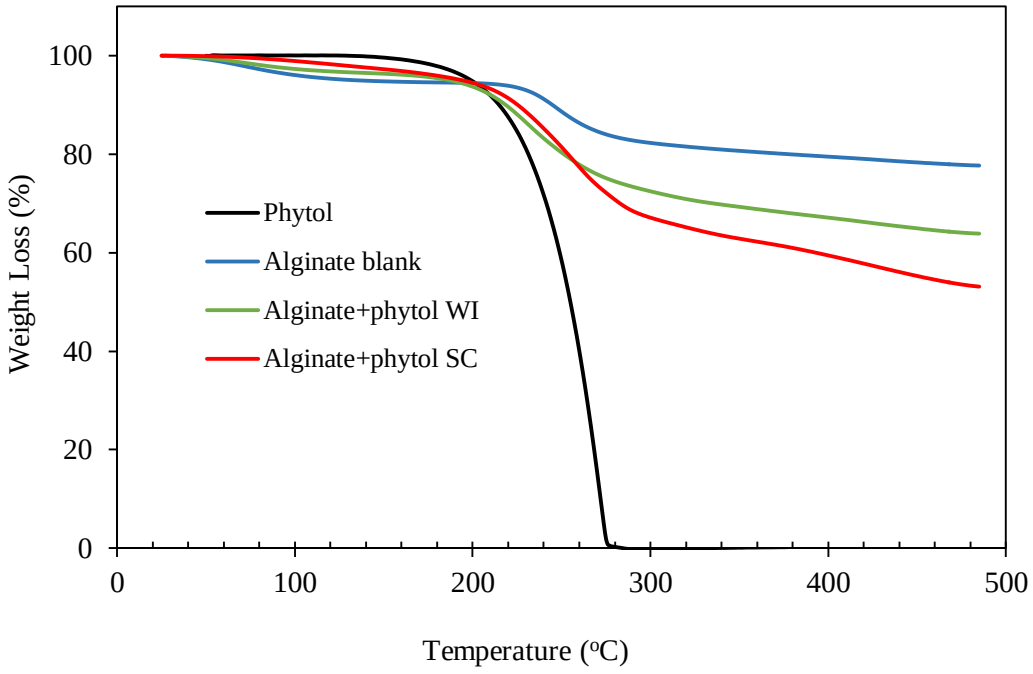


Figure 6: Comparison of TGA curve for impregnated phytol in alginate aerogels by few methods

3.6 Impregnation of *C.nutans* extract into silica and alginate aerogels

In previous work Mustapa et al. [1], authors showed that the supercritical CO_2 extraction of the *C.nutans* from its plant exhibited a very low yield of about 3.19 *wt%* on dry basis by $SCCO_2$ 350 bar 60°C. This indicates that the *C.nutans* extracts has very low solubility in $SCCO_2$. Thus, the most feasible method to impregnate the plant extracts is by adding a co-

solvent in the impregnation system to increase the solvent power of the $SCCO_2$ thus enhancing partition coefficient between the plant extracts and the $SCCO_2$ phase.

Results of SCI of alginate and silica aerogels of CN50 and CN100 extracts are presented in Table 3. The impregnations were performed in 24h with the addition of 10*wt%* pure ethanol as co-solvent for $SCCO_2$. It was found that the loading of the extracts in alginate aerogels were extremely low with 4.7 ± 0.6 *wt%* and 12.7 ± 0.8 *wt%* for CN50 and CN100 extracts, respectively. On the other hand, a significantly higher loading of CN50 and CN100 extracts were obtained in silica aerogels: 11.5 ± 0.4 *wt%* and 23.9 ± 1.0 *wt%*, respectively. This result can be related to the higher surface area of silica aerogels, and to the hydrophilic surfaces of these aerogels that lead to higher affinity for CN50 and CN100 than in alginate aerogels. On the other hand, the impregnation of CN100 was found to be much higher compared to the CN50 in both aerogels silica and alginate aerogels. It has been reported that CN50 extracts are rich of phenols and flavonoids which may have higher polarity [1]. In addition, these extracts also contain high molecular weight compounds such as tannins, saponins and terpenoids that have very poor solubility in $SCCO_2$, therefore resulting low CN50 extract solubilized in $SCCO_2$ thus lower its loading in the aerogels.

Moreover, in contrast with the results obtained with pure phytol, no swelling or plasticized alginate material was observed for the case of impregnation of *C.nutans* extract in aerogels. This could be due to the low solubility of the compounds in $SCCO_2$, as solutes influence polymer swelling, but this effect is essentially relying on the solubility of the solutes in polymer-$SCCO_2$ mixture [38].

For the impregnation of the plant extracts through liquid absorption or WI method, the same behavior was observed for the loading as described in the experiments with pure phytol compounds in previous section. The transfer of the compounds were enabled through diffusion by means of ethanol. The plant extract was carried into the pore structures, absorbed and dispersed into the alginate material matrix. Upon the depressurization, solubility of the extracts in the $SCCO_2$ was reduced and caused to its precipitation and trapped inside the mesopores. Results presented in Table 3 shows that the alginate aerogels have higher *C.nutans* extract loading capacity compared to the silica aerogels. In the case of silica, competition between the

compounds' solubility in organic solvent and surface chemistry might take place. High polarity of the solvent and affinity of compounds towards the organic solvent reduces their interaction with the surface hydroxyl groups on the silica matrix.

On the other hand, compatibility between the polysaccharides of alginate organic material with the phytochemical compounds probable encourage the biopolymer-compounds interaction. It is then suggested that the materials of the carrier and nature of compounds may influence the impregnation of compounds by WI method. It is then suggested that the WI is a feasible method to impregnate liphophilic drugs or compounds that have high solubility in organic solvent but low solubility in $SCCO_2$ without damaging the porous structure of aerogels. Braga et al. [43] also had demonstrated that different polymer structure chemically and physically can influenced the impregnation rate and drug releases.

Table 3 Loading of *C.nutans* extract; CN50 and CN100 by wet impregnation (WI) and supercritical impregnation (SCI) method into silica and alginate aerogels

Method	Aerogels	Extracts loading, *L*(*wt*%)
Wet Impregnation (WI)	Silica+CN50	5.2 ± 1.0
	Alginate+CN50	9.6 ± 2.1
	Silica+CN100	13.1 ± 0.9
	Alginate+CN100	18.5 ± 0.5
Supercritical Impregnation (SCI)	Silica+CN50	11.5 ± 0.4
	Alginate+CN50	4.7 ± 0.6
	Silica+CN100	23.9 ± 1.0
	Alginate+CN100	12.7 ± 0.8

The images of the impregnated silica and alginate aerogels by WI method are presented in Figure 7. It is observed that both aerogels have green and yellow colors indicating the presence of *C.nutans* extracts in the aerogels whilst the same characteristics were observed for the exterior and interior of the impregnated alginate aerogels, indicating a homogeneous loading of the whole aerogel monolith. In addition, analysis of FTIR showed the appearance of small peaks at 2800cm^{-1} in silica and alginate aerogels (see Figure 8). The peak absorbance is due to the vibrations of –OH groups that are present in *C.nutans* extracts. Nevertheless, no

peaks corresponding to –OH groups can be observed in the spectra of the aerogels impregnated with CN50. This is probably due to the low loading of the CN50 extract into the aerogels.

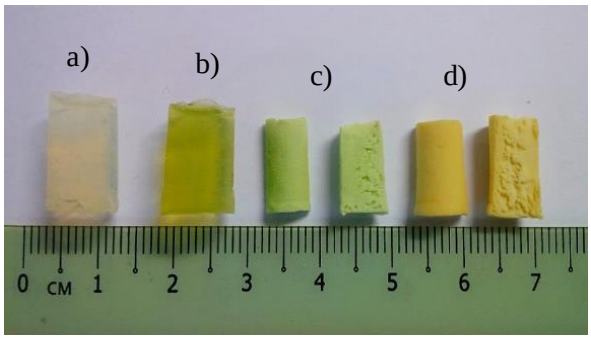
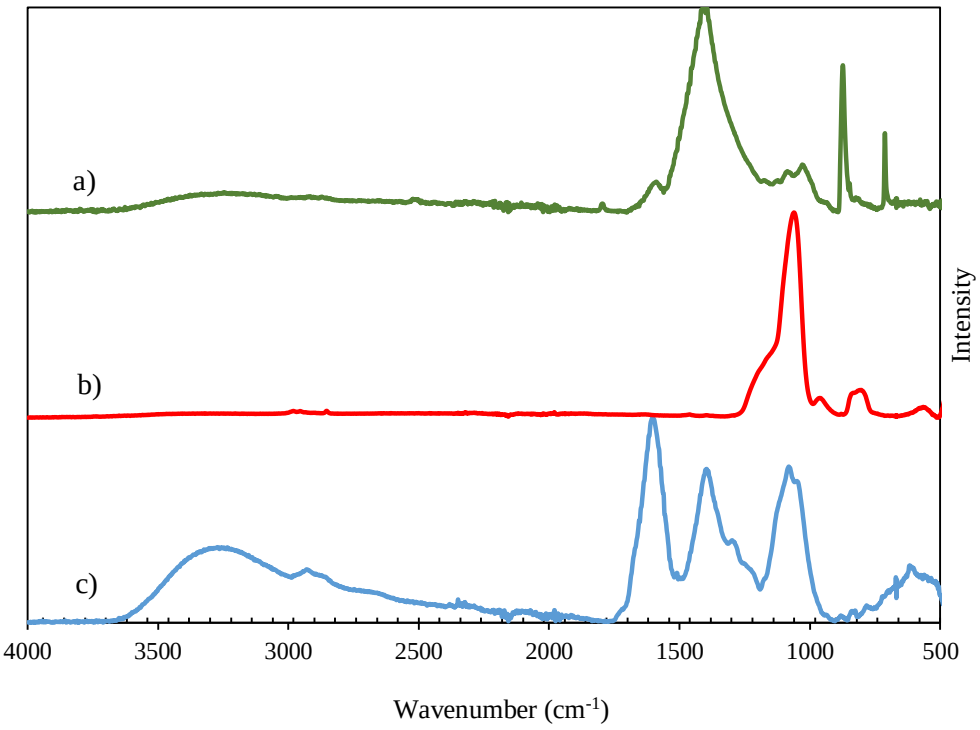


Figure 7: Impregnated silica and alginate aerogels with CN50 and CN100 extract by WI method; a) CN50+silica aerogel, b) CN100+silica aerogel c) CN100+alginate aerogel interior and exterior and d) CN50+alginate aerogel interior and exterior



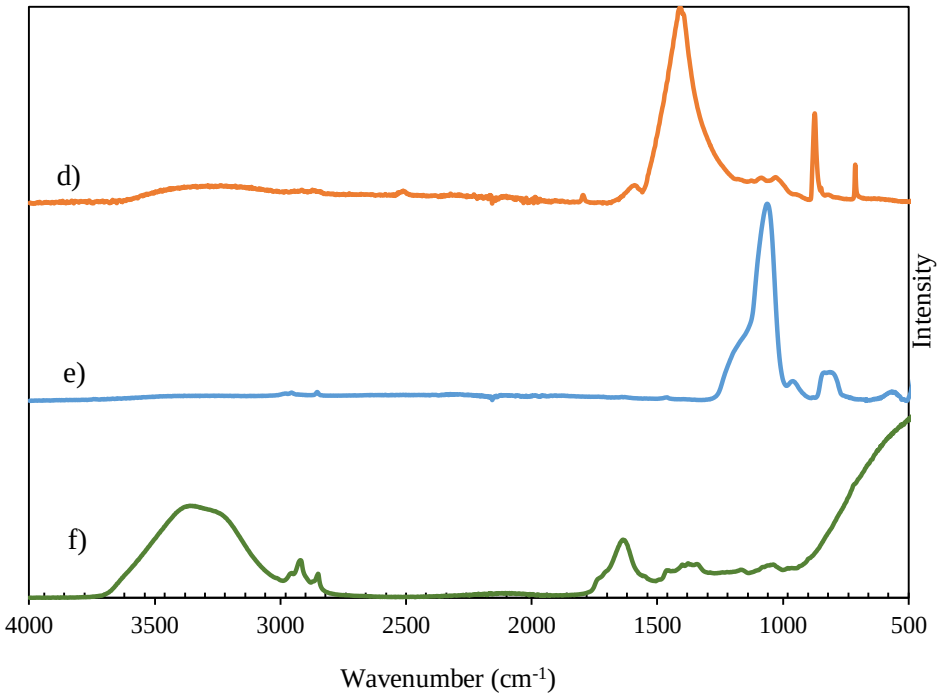


Figure 8: FTIR spectrum of silica and alginate impregnated with medicinal plant *C.nutans* using wet impregnation: a) alginate+CN50, b) silica+CN50, c) CN50, d) alginate+CN100, e) silica+CN100 and f) CN100

To evaluate any differences between the compounds in the CN50- and CN100-ethanol solution with respect to the compounds impregnated due to a preferential impregnation of certain extract compounds over the others, UPLCMS analysis of their composition was carried out. The results are presented in Figure 9. The analysis of UPLCMS was employed due to its high sensitivity to identify compounds at low concentration. It is found that the impregnated alginate aerogels comprises almost all main compounds from the CN50 extracts such as vitexin, isoshaftosides, 6,7-dihydroxycoumarin, chlorophyll b, vitexin-2”-o-rhamnoside, glucocerebrosides, (-)-epicatechin and few unknown compounds whereas compounds such as myricetin and some unidentified compounds from extract were not observed in the impregnated aerogels. Furthermore, it is observed that only some compounds: vitexin, L-Trytophane, 6,7-dihydroxycoumarin, chlorophyll b and vitexin-2-“-o-rhamnoside were identified in the silica aerogels and showed smaller intensity in comparison to the CN50 extracts in the alginate aerogels. One possible cause it the high solubility of phytocompounds in the CN50 extracts

into the alginate aerogels instead of the silica aerogel, as the organic aerogels has more compatibility with the plant extracts than the inorganic silica aerogels.

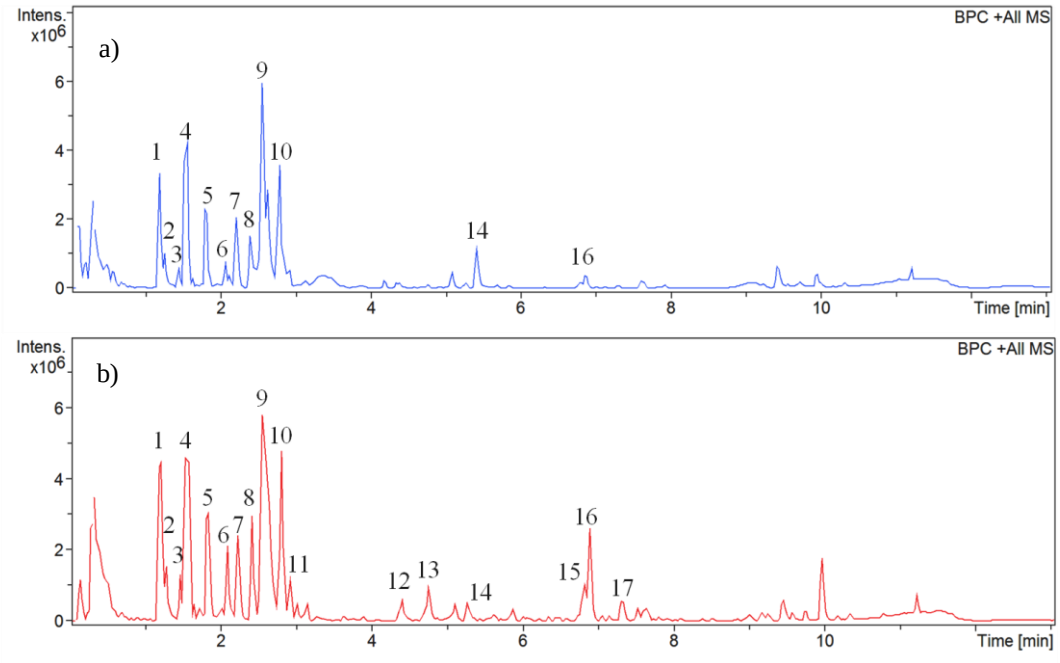


Figure 9: UPLCMS chromatograms; impregnated CN50 in alginate aerogels a) and CN50 extract b):
Vitexin (1 and 4), L-Trytophane (5), 6,7-dihydroxycoumarin (6), chlorophyll b (7), Vitexin-2-“-o-rhamnoside (8), Isoshaftosides (9), glucocerebrosides (10), myricetin (11), epicatechin (14), unknown compounds (2, 3, 12, 13, 15-17)

On the other hand, the same results were observed for the CN100 impregnation into alginate aerogels. The CN100 extracts comprises isoshaftosides, epicatechin and terpenoids that were also identified also in impregnated alginate aerogels meanwhile only isoshaftosides were found in the silica aerogels. Some compounds contained in the initial CN50 and CN100 extracts did not appear in the aerogels silica and alginate aerogels, and they probably were co-extracted with ethanol by $SCCO_2$ during the supercritical drying, and thus they did not remain impregnated in the aerogels. Nevertheless, wet impregnation of the phytocompounds into alginate aerogels was more promising in comparison to the silica aerogels.

Determination of the total chlorophyll content was carried out by extracting the CN100 impregnated in the silica and alginate aerogels using pure methanol. The results are presented in the Table 4 as mg/g sample with the standard deviation for the TPC and TF are ±0.040 mg/g and ±0.003 mg/g, respectively meanwhile ±0.002 mg/g for chlorophyll and phytol. As it can be seen from the table, the chlorophyll content impregnated in the silica and alginate were comparable, i.e. 0.170 mg/g and 0.168 mg/g, respectively. On the other hand, it is observed that the total phenols and total flavonoids content impregnated in the alginate for both CN50 and CN100 of plant extracts were higher in comparison to the content in silica aerogels. Results shows that CN50 impregnated in the alginate aerogels comprises 1.889 mg/g of total phenols and 0.142 mg/g of total flavonoids, whereas the CN50 impregnated in the silica aerogels presents significantly lower total phenols and total flavonoids contents, i.e. 0.242 mg/g and 0.023 mg/g, respectively.

Similar characteristics were also observed for the aerogels impregnated with CN100 plant extracts. The alginate aerogels loaded with CN100 showed higher content of phenols and flavonoids than silica aerogels loaded with the same extract. These results were consistent with the loading percentage of the CN50 and CN100 in the aerogels. In addition, this result may also suggest that the impregnation of the CN100 extract by means of WI method was independent on the specific surface area of the aerogels. Furthermore, the analysis of phytol content by HPLC revealed that the CN100 impregnated in a silica and an alginate aerogels comprises 0.015 and 0.034 mg/g of phytol, respectively. These results indicate that the target compounds were successfully impregnated by wet impregnation method.

Table 4 Total chlorophyll, phenols and flavonoids content impregnated in silica and alginate aerogels by WI method

Aerogels	Total chlorophyll (mg/g)	Phytol (mg/g)	Total Phenols (mg/g)	Total Flavonoids (mg/g)
Silica+CN50	-	-	0.242	0.023
Alginate+CN50	-	-	1.889	0.142
Silica+CN100	0.170	0.015	0.262	0.059
Alginate +CN100	0.168	0.034	0.796	0.067

3.5 Kinetics dissolution of *C.nutans* extract

Release kinetics studies were only carried out for the aerogels impregnated with the *C.nutans* extracts and not applied to the aerogels impregnated with the pure phytol in this current work due to its extremely low solubility in the water and strong interaction of the phytol with the material carrier, as it was found that impregnation of phytol into aerogels by supercritical fluid was unable to improve the bioavailability of the compounds. Nevertheless, it is anticipated that this could be improved by applying additives such as β-cyclodextrin or preparing aerogels template-surfactant prior to the SCI, which are in the scope of ongoing work.

The dissolution kinetics of the *C.nutans* extract from alginate aerogels are presented in Figure 10. It is found that about 50*wt*% of the CN100 was released after 6h in phosphate buffer solution (pH 6.8) and the rest of the CN100 remained in the alginate material at the end of the test. It was assumed that some of the CN100 extracts were dissolved within the alginate gel network during the wet impregnation and thus had strong bonding with the gels. Possible hydrogen bonding between the carboxyl groups of the alginate aerogels and the hydroxyl groups that may be attributed to some of the components constituting the multicomponent CN100 extracts have taken place. In addition, some of the compounds in the CN100 extract that was obtained with pure alcohol may have low solubility in water and thus show a slow release from the alginate aerogels.

In contrast, release kinetics of the CN50 extract from the alginate aerogels showed a faster dissolution. The high solubility of the CN50 extract in water encouraged good dissolution of the extract from the alginate into the phosphate buffer solution. It was found that about 50*wt*% of the CN50 was released within 1.5 h, reaching equilibrium after 6 h of dissolution time with 80*wt*% of the extract released. In addition, it was also observed that the alginate material was completely dissolved in the buffer after 7 h, which is also observed for the alginate+CN100 extracts. Thus, the biodegradable characteristics of the alginate contribute to the complete release of the CN extract from the matrix carrier. Furthermore, the results also may suggest that the alginate+CN100 compound is more suitable as controlled drugs release system whereas the alginate+CN50 can be appropriate for fast release purposes.

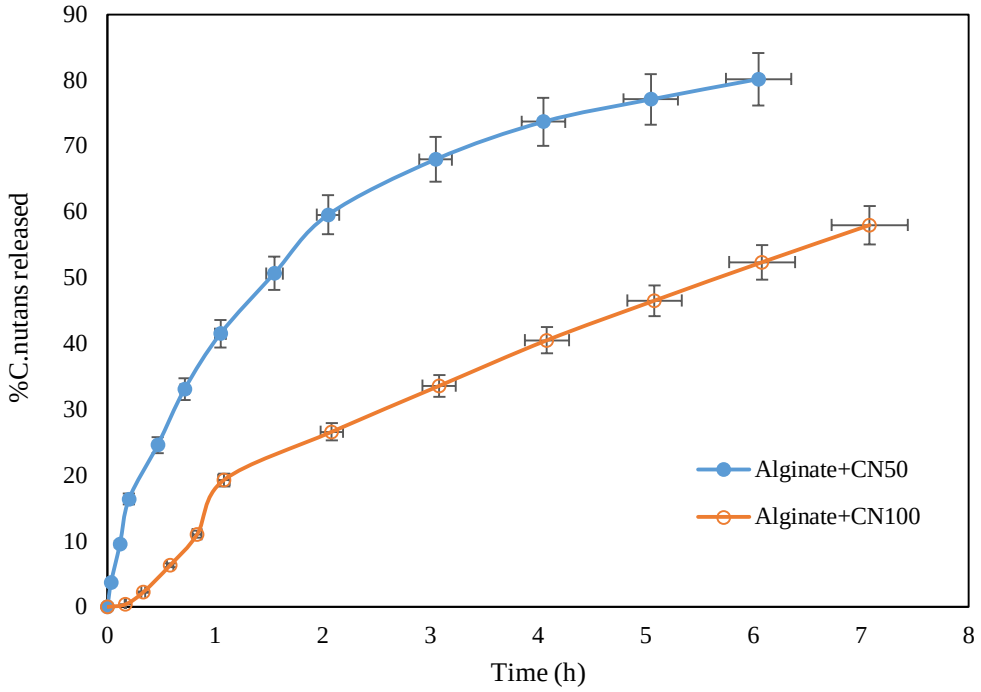


Figure 10 *C.nutans* extract release kinetics from alginate aerogels in phosphate buffer pH 6.8 at 37°C

On the other hand, silica aerogels showed a very poor *C.nutans* extract dissolution rate into phosphate buffer for both cases of CN50 and CN100 extracts. It was found that after 6 h of dissolution time, the release of the *C.nutans* was as low as 5*wt*%. Strong interaction between the *C.nutans* extract with the silica matrix could be the reason for the slow release kinetics of the phytocompounds from the silica aerogels. A strong interaction can be formed between the –OH groups on the silica aerogels surface with certain molecules from phytocompounds of the *C.nutans* extract. The FTIR results (from Figure 8) may confirm this finding as the reduction of the band at 3750 cm^{-1} that can attributed to the disappearance of free hydroxyl groups of the aerogel after the impregnation, indicating the presence of hydrogen bonding between the molecules of adsorbate and silica aerogels surfaces [44]. On the other hand, the ethanol that was used as solvent in the wet impregnation may play an important role due to its "solvent effect" that can enhance the bonding by improving the dielectric properties. This possible interaction includes ion-dipole, dipole-dipole and dipole-quadrupole electrostatic interaction [45]. From the previous report [1], the *C.nutans* extract comprises a wide variety of compounds, mainly chlorophyll, phytol and significant level of polyphenols. Thus, it is difficult

to determine if specific interactions have been formed with the free hydroxyl groups on the silica aerogel surface.

The kinetics data of both cases were fitted into several mathematical models to understand the mechanism of release of *C.nutans* from the aerogels. Table 5 shows the correlation coefficients fitted to zero order, first order and Higuchi release kinetics models. As it can be seen from the table, the correlation coefficient of the Higuchi model was the highest in comparison to the zero and first order models, i.e. 0.971 and 0.994 for alginate+CN50 and alginate+CN100, respectively. Nevertheless, the sample of alginate+CN100 showed a better fitting with Higuchi compared to alginate+CN50 confirmed that the release kinetics of the CN100 was diffusion controlled and depended on its initial concentration. Comparing the other two models, the first order model gave higher correlation coefficient than the zero order model. The less precise fitting to the of zero order model in comparison to the other models shows that the release kinetics of the alginate+CN50 and alginate+CN100 depends on the extract concentration in the aerogels.

As previously indicated, the alginate+CN100 system appears to be appropriate for controlled drug release, as confirmed by the correlation with the Higuchi method. To further understand the mechanism of the dissolution profile, the release data was fitted to the Korsmeyer-Peppas model:

$$\left(\frac{M_t}{M_\infty} = kt^n \right) \qquad \text{Eq. 6}$$

that is a simple relationship to describe drug release from polymeric material, with M_t/M_∞ is the cumulative drug release at time *t* and infinite time (which the $M\infty$ is the drug in the polymer at time *t* is 0), K_{KP} is the rate constant and *n* is diffusional exponent, indicative of release mechanism depending on geometric shape of the polymer carrier. For this work, cylindrical shape was consider since we the aerogels were produced in monoliths form. The value $n < 0.45$ indicates Fickian diffusion, $0.45 < n < 0.89$ shows anomalous (non-Fickian) diffusion, $n = 0.89$ specifies the Case-II transport and $n > 0.89$ exhibits as super Case-II transport. Note that the

model only be applied to the first 60% fraction of release curve or $M_t/M_\infty < 0.6$ regardless of geometric shape [46, 47].

Results of correlation to the Korsmeyer-Peppas model shown in Table 5 yield $n = 0.61$ indicating that the release of the CN100 from the alginate aerogels is anomalous transport, suggesting that the release is controlled by two phenomena i.e. drug diffusion and polymer erosion. These phenomena happen simultaneously as polymer undergoes relaxation process in aqueous media. This result was coincident with the curve of the dissolution profile where slower release was found within the first hour possibly due to the erosion and/or swelling that was also observed during the test. The dissolution was then primarily controlled by the drug diffusion. Nevertheless, swelling effect also could be a factor influencing the dissolution rate which occurred simultaneously with the erosion, causing a moving boundary and changing the effective diffusivity of the drug. Polymer erosion increases the drug dissolution rate thus compensating the swelling which consequently reduce the drug diffusion due to the increasing of diffusional path length [48]. It is suggested to investigate the contribution of these two mechanisms by performing swelling and erosion analysis in future works.

Table 5 Release kinetics model parameters of alginate impregnated with CN50 and CN100 extracts

Sample	Zero Order		First Order		Higuchi		Korsmeyer-Peppas		
	R^2	K_o	R^2	K_1	R^2	K_H	R^2	K_{KP}	n
Alginate+CN50	0.849	12.21	0.956	0.115	0.971	35.15	-	-	-
Alginate+CN100	0.961	8.18	0.990	0.053	0.994	25.92	0.925	0.23	0.61

4.0 Conclusions

Supercritical CO_2 impregnation of phytol has produced higher loading content of phytol and *C.nutans* extracts than the wet impregnation. In the SCI method the main factor influencing the compounds loading is the specific surface area of the aerogels. In contrast, in the wet impregnation method, alginate aerogels yielded higher phytol and *C.nutans* extracts content than silica aerogels regardless of their surface area properties, suggesting that nature of compounds and carrier material influenced the compounds loading. For both types of plant

extracts (CN50 and CN100), the total phenols and flavonoids contents attained were higher in alginate than in silica aerogels, which is consistent with the compounds loading pattern. For both cases of compounds, alginate aerogels yielded higher loading content of the compounds of interest through the wet impregnation method whilst by $SCCO_2$ impregnation method silica aerogel yielded higher loading content, but with poor dissolution kinetics. Due to high solubility of CN50 extracts in water, the release kinetics behavior of these extracts was much faster than the kinetics of CN100 extracts which on the other hand showed a sustained drug release behavior. Data fitting with release kinetics models indicated that the dissolution mechanism of CN100 extracts was controlled by diffusion and erosion and/or swelling of the alginate aerogels.

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Chapter 4

Synthesis of hybrid alginate/β-cyclodextrin aerogels for drug delivery of compounds with low water solubility

Abstract

The application of some natural bioactive compounds such as phytol is limited by the low water solubility of these compounds and the correspondingly low bioavailability. Incorporation of these compounds into high surface area carriers, or inclusion into β-cyclodextrin (βCD), can enhance the solubilization. In this work, both approaches were combined, synthetizing hybrid alginate/βCD aerogels beads via an ionotropic gelation method. Two types of hybrid beads: core and floating beads, were obtained by extruding a mixture of alginate/βCD and alginate/$CaCO_3$/βCD into cross-linking baths. The beads were characterized by nitrogen adsorption-desorption analysis, scanning electron microscopy (SEM) and infrared spectroscopy (IR) whereas its thermal stability was examined by differential calorimetry (DSC) analysis. Their drug loading capacity was assessed by impregnating phytol using a supercritical CO_2-assisted method at 200 bar and 40ºC in 24 h. Results of DSC analysis confirmed the presence of βCD, and the loaded phytol in the hybrid alginate/βCD was stable over temperature. The specific surface area of core and floating beads was 447 and 545 m^2/g, respectively. The surface area of hybrid beads with addition of βCD was smaller, 424 and 495 m^2/g, correspondingly. Phytol loading increased when βCD was added into the alginate aerogels beads despite this reduction of surface area, suggesting that the interaction between βCD/phytol is responsible of this enhanced the phytol loading. The dissolution test showed an increased phytol release in hybrid aerogel beads containing βCD, with a total release as high as 90% in 8 h. The release from core beads was faster than from floating beads. High compounds loading and improved release kinetics demonstrated that the development of highly porous hybrid aerogels with βCD is a simple and promising way to improve the solubility of poorly soluble compounds for drug delivery.

1.0 Introduction

Phytol is a natural compound with pharmaceutical applications that is abundantly available from chlorophyll-based plants. Many studies have revealed that phytol shows a wide range of medicinal properties. It is an excellent immunostimulant, superior to a number of commercial adjuvants in terms of long-term memory induction and activation of both innate and acquired immunity [1]. Furthermore, phytol exhibits antimicrobial activity against *Mycobacterium tuberculosis* [2, 3], *Staphylococcus aureus* [4] and possess several therapeutic properties such as anti-inflammatory and antiallergic effects [5], antinociceptive and antioxidant activities [6]. It has also been claimed that this compound is a cholesterol-lowering agent and it can be administered to healthy individuals to maintain normal levels of serum cholesterol [7]. Costa et al. [8] demonstrated in tests on mice that phytol has anticonvulsant activity and reduces the mortality rate by seizures. A more recent study by de Moraes et al. [9] revealed that phytol is a promising drug for chemotherapy of human schistosomiasis.

Nevertheless, the applicability of phytol is often restricted by its limited bioavailability due to its low aqueous solubility, that is 0.00327 mg/l at 25^{o}C [10]. According to the United States Pharmacopeia (USP) [11, 12] and European Pharmacopoeia [13], drugs that have solubility <0.01 mg/ml are regarded as insoluble and their bioavailability is considered as unacceptable when the aqueous solubility is lower than 1µg/ml [14]. This is because poor solubility of drugs cause them to be eliminated from the body before being fully dissolved and absorbed into bloodstream. In a previous work [15], the dissolution rate of phytol impregnated into silica and alginate aerogels could not even be assessed due to its extremely low solubility in water.

One way to improve the solubility of poorly soluble drugs is by incorporating the compounds into β-cyclodextrin (βCD). The βCD are cyclic osoligosaccharides produced by the transformation of starch by certain bacteria such as *Bacillus macerans, Bacillus sutilis* and *Bacillus circulan* [16, 17]. The molecular structure of βCD comprises a hydrophilic surface at the exterior whereas the central cavity is built of hydrophobic sites. Generally, the

special characteristics of the βCD are due to its ability to form solid inclusion complexes through host-guest interactions at the central hydrophobic cavity. In an aqueous solution containing βCD and a hydrophobic guest, the formation of host-guest complexes takes place when water molecules in the cavity are displaced by the more hydrophobic molecules of guest [18]. The formation of these complexes increases the accessibility of the hydrophobic guest molecules in aqueous environment.

Due to these properties, βCD has been used as excipient in pharmaceutical industry to enhance the solubility of water-insoluble drugs or active pharmaceutical ingredients (APIs) [19-21]. Numerous works have been carried out on the encapsulation of βCD into hydrogels in order to improve the dissolution of poor solubility compounds in water [22-24], with various techniques of polymer and/or biopolymer preparation, either by physical or chemical crosslinking method. In the later technique, βCD is modified to form long chains of polymer prior to the encapsulation of poorly soluble compounds into their network. This technique involves either grafting of monomers on the backbone of polymer or utilizing chemical cross-liner agents to link two different polymers [25]. For example, Li et al. [26] prepared drug inclusion complexes with water-soluble cationic β-cyclodextrin polymers (CPβCDs). The CPβCDs polymer was produced through a one-step polymerization procedure, mixing β-cyclodextrin (βCD) and reagents of epichlorohydrin (EP) and choline chloride (CC).

In addition to synthetic polymers, natural polymers have also been widely studied for pharmaceutical uses due to their non-toxic, biodegradable and biocompatible properties. Alginates are anionic linear polysaccharides consisting of (1-4) linked *β*-D-mannuronic acid (M block) and α-L-guluronic acid (G block) in sequential arrangements, that are biodegradable and inert, and therefore safe for biomedical applications. In the work of Izawa et al. (2013), βCD was cross-linked with alginate by chemical reaction, producing Alg-βCD hydrogel as a controlled released system for drug delivery. For this, βCD was grafted onto the sodium alginate backbone via amino link condensation in order to form continuous linkage chains of βCD-alginate network, thus synthesizing β-cyclodextrin-conjugated alginate (Alg-βCD). Then, the hydrogel produced was dried at ambient conditions or in an oven at a

specific temperature. This material enhanced the aqueous solubility and controlled release of poorly water-soluble compounds.[27, 28].

Alternatively, βCD-based alginate hydrogels can also be prepared by a simple and fast method consisting of physical crosslinking gelation in absence of toxic chemicals. In this technique, gelation is initiated by the presence of various divalent cations that participate in ionic interaction with the G-block of alginate molecules and create a three-dimensional network. Ionotropic gelation is an attractive gel formation method in which the alginate solution is extruded into a solution containing cross-linker cations, by which alginate hydrogel beads are formed. This method has also been applied to encapsulate bioactive compounds in a single-step preparation method. For example, Moses et al. [29] performed the encapsulation of βCD+insulin in chitosan/calcium alginate in form of beads dried at 10^{o}C to investigate the effect of pH and insulin concentration on the loading capacity. On the other hand, Goindi et al. [30] formulated floating alginate beads containing an inclusion complex of βCD+curcumin dried by freeze drying, in an attempt to improve the solubility and bioavailability of curcumin for sustained drug release. In the recent study by Nguyen et al. [31], the synthesis of novel βCD/alginate nanoparticles loaded with ketoprofen by ionotropic method was studied to determine its potential in drug associations and delivery systems. The formation of βCD/alginate nanoparticle was achieved by dropwise addition of $CaCl_2$ solution into sodium alginate solution followed by the addition of βCD solution to the pre-gel Ca-alginate. The concentration of βCD was varied and the effect of the amount of βCD on the loading efficiency and association was studied. To load ketoprofen into the nanoparticles, the drug was dissolved in an alcohol/water mixture (1:1) prior to its incorporation into calcium chloride. The synthesized nanoparticles were centrifuged and subsequently freeze-dried.

In some of these applications, drug loading and drug release properties of the complex are limited by the porosimetric properties of the polysaccharide support: surface area and pore volume. Compared with conventional polysaccharide materials, polysaccharides aerogels are highly porous solid materials with densities as low as 0.01 g/cm^3, high specific surface area (~400 m^2/g) and high porosity (~ 90%). The aerogel is derived from hydrogels

after the drying by supercritical CO_2 ($SCCO_2$), in a procedure that is able to maintain the three-dimensional network of the gel, thus leading to the high pore volumes previously indicated. These attractive porosimetric properties can enhance the drug loading capacity, making these materials suitable candidates for drug delivery.

To date, no reported work on the production of alginate/βCD hybrid aerogel beads has been reported. Hybrid alginate/βCD aerogels dried under supercritical conditions show highly porous structure that can increase drugs loading capacity. Thus, investigation on the porosimetric characteristics of the aerogels/βCD such as specific surface area, pore volume and pore size and the corresponding drug release kinetics is significant for the drug delivery field. In addition, it is important to determine the interaction of βCD with the aerogels matrix and with the compounds interest, as this fundamental information can assist in the enhancement of the inclusion complexes. Therefore, it is envisioned that the development on the production of aerogels based on the βCD concept is a novel contribution for controlled release and insoluble-water drugs or APIs.

In this work, two types of hybrid alginate/βCD aerogels beads were synthesized via ionotropic gelation method. Core beads were prepared by physically crosslinking the alginate/saturated βCD mixture in ethanol $CaCl_2$ solution while floating beads were obtained by dropping a mixture of alginate/saturated βCD containing insoluble calcium salt into ethanol acidified with acetic acid. In the later case, calcium carbonate is acting as gas-forming agent, forming floating beads of Ca-alginate in acidic medium. The dried aerogels beads were characterized by Scanning Electron Microscopy (SEM) to investigate the cross-section of the beads and analyzed by nitrogen adsorption-desorption to determine their porosimetric characteristics. The synthesized aerogels were loaded with phytol by supercritical impregnation to determine their drug or APIs loading capacity. Differential scanning calorimetry (DSC) was employed to assess the presence of βCD in the aerogels as well as to investigate the thermal stability of phytol loaded in the hybrid aerogels. Finally, the dissolution of phytol was performed to determine the effect of βCD added in alginate aerogels on the solubility of phytol.

2.0 Materials

Alginic acid sodium salt from brown algae, β-cyclodextrin, calcium carbonate and phytol were purchased from Sigma Aldrich. Calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), acetic acid was purchased from Carl Roth GmbH.

2.1 Supercritical inclusion

The purpose of these experiments is to determine the effect of $SCCO_2$ in the formation of inclusion complex of βCD+phytol. Formation of inclusion complex of βCD and phytol by $SCCO_2$ was conducted in a static mode using a high pressure autoclave of 70 mL, as shown in Figure 1. The inclusion was performed at pressures of 100 and 150 bar while tested temperatures were 40 and 50°C. Initially, a mixture of βCD and phytol with molar ratio of 1:1 was prepared by physically mixing the compounds in a ceramic mortar for 10 min. The mixture was then placed in the high pressure autoclave and heated to the desired temperature. The system was then slowly pressurized with CO_2 at a rate of 5 bar/min up to the desired pressure and held at these conditions for 2 h, in a static operation mode. According to Bounaceur et al. [32], the effect of contact time over $SCCO_2$ condition on the formation of the complexes was very significant at contact times up to 2 h, and became weaken at longer complexation times. Thus, after 2 h, the system was depressurized at a rate of 7-10 bar/min and the product was homogenized in a mortar [33] and stored in a vial. After each run, the setup was flushed with ethanol and CO_2 meanwhile the autoclave was cleaned with acetone to remove leftover traces of the inclusion complex.

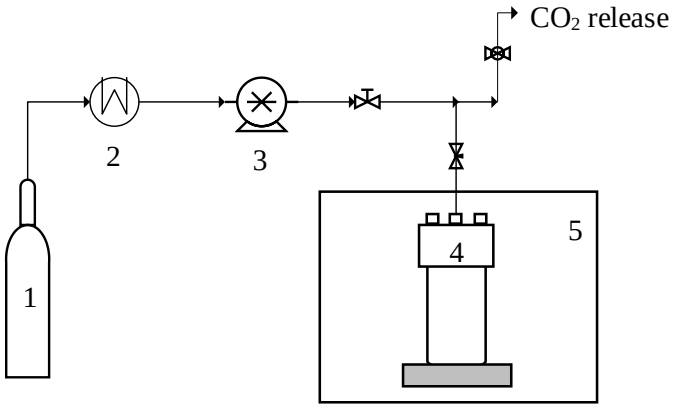

Figure 1: Supercritical CO_2 impregnation setup; 1) CO_2 tank, 2) cooler, 3) pump, 4) impregnation autoclave and 5) oven

2.2 Synthesis of alginate beads

Two types of hybrid alginate/βCD hydrogels beads were prepared by ionic interaction using different preparation methods. In the first method, a mixture of sodium alginate solution concentrated with βCD was extruded into an ethanol solution containing $CaCl_2$ as a cross-linker. The gel was formed through interaction between ionic groups on alginate (COO^-) with divalent cations, Ca^{2+}, creating a three-dimensional network [25]. In the second method, a mixture of sodium alginate+$CaCO_3$ saturated with βCD was dripped into acidified pure ethanol, where the carbonate salt was dissociated due to reaction with the acid forming a gas. The Ca^{2+} ions delivered interacted with the carboxyl groups of alginate, creating gels. The amount of βCD added was based on the maximum solubility of the βCD in water i.e. 1.6g/100 mL [34] to attain a saturated βCD in alginate solution.

Method I / Core beads: A known weight of sodium alginate was dissolved in distilled water under stirring to produce a 3.0 wt% alginate solution. β-cyclodextrin powder was then dissolved in the solution and was maintained under stirring until a uniform dispersion was obtained. The mixture was allowed to stand still to remove the bubbles from the solution prior to the dripping into the gelation bath.

Method II / Floating beads: In distilled water sodium alginate was dissolved under stirring condition to produce 3.0 wt% of alginate solution. Calcium carbonate, with a constant 1:1 $CaCO_3$/alginate (w/w) weight ratio, was added when the sodium alginate powder was completely dissolved, followed by the addition of β-cyclodextrin. To ensure a complete reaction and to produce homogeneous Ca-alginate beads the acetic acid was prepared in excess of 3.5 mole ratio in relation to the number of moles of calcium carbonate. The $CaCO_3$/alginate ratio was chosen after preliminary trials that showed that a ratio of $CaCO_3$ lower than 1 resulted in the formation of non-floating beads whereas a ratio higher than 1 lead to the rupture of beads due to blast gas released during the gel formation. Nevertheless, this finding is not general as the behavior may vary with the concentration of acetic acid in the ethanol solution.

Both alginate mixtures were dripped using a syringe pump KD Scientific Model 100 through a syringe (BD Plastipak diameter 16 mm) at 0.2 mL/min through a 25G x 0.625 inch syringe needle. The mixture from Method I was dropped into a 90% v/v ethanol/water mixture containing 20 wt% calcium chloride while the mixture from Method II was dropped into 20wt% of acetic acid in ethanol under gentle stirring. In addition, the volume ratio of gelation bath (V_b) to alginate solution (V_a) must be optimized to ensure complete reaction and gelation for the beads via Method II as well as gelation of alginate beads for Method I. After several trials, it was found that the optimum volume ratio of gelation bath (V_b) to sample dripped (V_d) for Method I was 7:1 (V_b/V_d) meanwhile for Method II the optimum volume ratio was 4:1 (V_b/V_d).

Beads were left in the gelation bath for 20 min (hardening time) to ensure internal gelification and to allow complete reaction of carbonate salt-acid for core and floating beads, respectively. The beads were then recovered, washed several times with the gelation bath to remove the calcium acetate salt (for the beads in Method II), and immersed in pure ethanol. For the case of core beads preparation (MI), it has been reported that the solubility of βCD in 90% v/v ethanol solution is as low as 0.05g/mL [34]. Thus it was assumed that the diffusion of βCD into the gelation was negligible in the 20 min of hardening time.

2.3 Supercritical CO_2 drying

The alginate alcogels beads obtained after the solvent exchange with ethanol were subjected to supercritical drying by CO_2 at 125 ± 5 bar and 45°C. The alcogels beads were packed in a bag of filter paper and placed in a 250 mL autoclave at 45°C, which was then filled with alcohol to prevent the premature solvent evaporation from the alcogels. Liquefied CO_2 was slowly pumped into the system at a rate of 4 bar/min, up to the desired pressure. After the operating conditions of drying were reached, a continuous circulation of CO_2 through the autoclave was initiated to extract the alcohol from the alcogels. The drying process was completed in 6 h and the system was slowly depressurized at a rate of 2 bar/min in order to avoid shrinkage or damage of aerogels.

2.4 Supercritical impregnation

The impregnation process was conducted in a batch system consisting of a 500 mL stainless steel high pressure autoclave with a maximum working pressure of 300 bar. A known weight of pure phytol was placed at the bottom of the autoclave meanwhile a known weight (w_a) of dried hybrid alginate/βCD aerogels beads was wrapped in filter paper and loaded in the autoclave separated from the phytol with a metal support. The autoclave was heated to 40°C and pressurized with CO_2 to 200 bar. The impregnation process was maintained for 24 h under stirring to ensure maximum solubilization of phytol in supercritical CO_2. After 24 h, the system was slowly depressurized at a rate of 2 bar/min to avoid shrinkage or collapse of the mesopore structure of the aerogels. The impregnated aerogels were taken out of the autoclave and the loading of compounds was calculated as follows:

$$\frac{w_a\,(g) - w_b\,(g)}{w_a\,(g)} \times 100\% \qquad \text{(Equation 1)}$$

where w_a is the weight of alginate beads after impregnation, w_b is the weight of alginate beads before impregnation (g).

2.5 Alginate aerogels beads characterization

2.5.1 Physicochemical characterization

The average diameter of 100 dried beads was determined by Venier caliper whereas the surface morphology of the hybrid alginate aerogels beads was examined by Scanning Electron Microscopy (SEM) Model JEOL JSM 820. The beads were coated with gold prior to the SEM imaging. FTIR analysis to investigate the interaction βCD/alginate and βCD/ Fourier-transform infrared (FT-IR) spectroscopy analysis were conducted with a Bruker Platinum-ATR system equipped with OPUS Optik GmBH software in the wave range from 400 to 4400 cm^{-1}.To determine the alginate aerogel beads porosimetric characteristics, the beads were subjected to nitrogen adsorption-desorption isotherms analysis using ASAP 2420 V2.09 instrument. Samples were degassed at 60°C under vacuum condition (<1mPa) for 12 h prior to this analysis. Specific surface areas were calculated from the Brunauer-Emmett-Teller (BET) equation, average pore volume was determined by single point adsorption at P/Po 0.998 and average mesopores diameter distributions were determined from adsorption isotherms by the Barrett-Joyner-Halenda (BJH) method.

2.6 Thermal characterization

2.6.1 Differential scanning calorimetry (DSC)

DSC analyses were conducted for pure βCD, physical mixtures and SC inclusions samples, using a Mettler Toledo DSC 822e SAE. For each sample, about 5-7 mg of sample was placed in an aluminum pan, sealed and heated from 25 to 400°C at a rate of 10°C/min under a dry nitrogen purge.

2.7 Dissolution studies

Dissolution test of phytol loaded in non-hybrid and hybrid alginate/βCD aerogels beads were performed using a USP-certified dissolution test apparatus Copley NE4-COPD with phosphate buffer solution (PBS) at pH of 6.80 and at a constant temperature of 37°C.

For this, a known weight of sample was dispersed into the buffer solution (350 mL) and was kept stirring during the test using an impeller speed of 100 rpm. At specific time intervals, an aliquot of 2 mL was withdrawn, filtered through 0.45μm PTFE microfilter and subsequently replaced with 2 mL of fresh PBS that had been maintained at 37^{o}C. The aliquots were then analyzed by UV-Vis Spectrophotometry at 363 nm against blank PBS. In preliminary studies, absorbance of βCD in PBS was analyzed, founding that these compounds did not interfere during the samples measurement.

3.0 Results and Discussion

3.1 Preparation of core and floating beads

The first step in the development of the aerogel beds is the optimization of the gelation conditions. With respect to the composition of the gelation solvent mixture (gelation bath), it has been reported that βCD has a relatively high solubility in water of 1.6 g/100 mL. Thus, another solvent such as ethanol solvent must be used as the gelation bath to avoid premature βCD release from the alginate hydrogels beads. However, in preliminary experiments, it was observed that the use of a $CaCl_2$ solution in pure ethanol as the gelation bath resulted in severe shrinkage and flattened the sphere shape of the alginate beads. This is due to the fast release of water from the alginate hydrogels beads during the gelation in the bath. The formation of alginate polymeric network causes water displacement when divalent cations interacts ionically with carboxyl groups of the alginate [35].

This problem can be minimized by adding a small amount of water in the gelation solution to decelerate the water exchange during gel formation. For this reason, 90%v/v of ethanol/water containing 10%w/w of $CaCl_2$/ethanol solution was found as the optimum composition of the gelation bath solution, for completing the formation of the internal pore structure of alginate/βCD hybrid hydrogels while maintaining the sphere shape of the beads. In addition, hardening time is also important in order to maintain the spherical shape of the beads and to decrease their shrinkage rate. Excessively prolonged hardening time results in the formation of beads of small diameter and low porosity due to shrinkage whereas shorter

time leads to incomplete internal gelation. It was observed that 20 min of hardening time allowed maintaining the sphere shapes of the core beads with complete internal gelation. This result agrees with the conclusions of Smrdel et al. [36], who demonstrated that alginate beads size decreased from 4.47 to 3.61 mm after 30 min of gelation, while the cross-linking of beads was completed after 10 min of hardening time.

The formation of the core hybrid alginate/βCD gels proceeds through two mechanisms, as illustrated in Figure 2. This involves 1) alginate gelation via Ca^{2+} ion interaction with the alginate carboxyl groups and 2) electrostatic interaction between negatively charged βCD and Ca^{2+} that caused the entrapment of the βCD molecules in the Ca-alginate polymeric network. These mechanisms occur simultaneously as they are initiated by the same divalent cations. In the second mechanism, the Ca^{2+} acts as bridge ion to interact electrostatically with the negative charge on the oligosaccharides polymer and the βCD [37-39]. A similar mechanism of βCD entrapment takes place in the formation of the floating hybrid alginate beads, however the $CaCO_3$ has to be dissociated by the reaction with acid to liberate the Ca^{2+}, inducing the gelation of alginate ionically and with βCD in electrostatic manner.

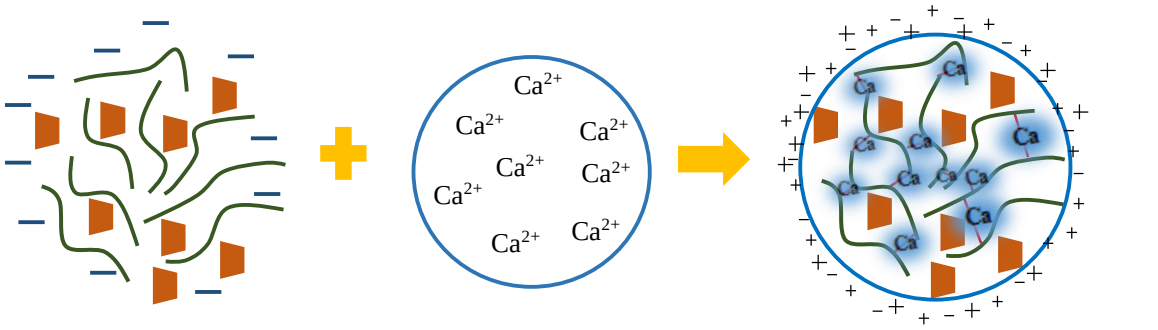

Figure 2 General mechanism for the formation of hybrid alginate/βCD beads

3.2 Physical characterization

All the hybrid core and floating alginate beads produced were spherical and transparent. The mean diameter of dried aerogels was 2.64 ± 0.01 and 2.72 ± 0.01 mm for

core and floating beads, respectively. Surface and cross-sectional SEM images of the alginate/βCD hybrid beads are presented in Figure 3. The presence of hollows or large pores in the interior of the floating beads, shown in Fig.3a, is attributed to the release of carbon dioxide produced in the reaction of carbonate salt with acid during the gel formation. The emergent gas permeates through the alginate matrix, leading to the formation of pores. The morphology of the internal cross-section of the beads is similar to that of the floating beads prepared by Choi et al. [40] with different ratios of $CaCO_3$/alginate (w/w) from 0.5 to 1:1, extruded into 1% (w/v) $CaCl_2$ containing 10% (v/v) and subsequently dried by freeze drying. These authors observed that the amount of internal large pores is related to the amount of $CaCO_3$ used. In Fig. 3b, βCD distributed on the surface of alginate matrix is observed meanwhile the curved marks observed on the alginate morphology could be attributed to the release of gas from inside the beads. On the other hand, in Fig. 3b, it is observed that the interior surface of the core beads is rougher compared to the surface of floating beads (shown in Fig. 3c). Furthermore, βCD particles in the core beads are also visible in the Fig. 3d which confirm the presence of the excipient in the hybrid alginate/βCD aerogels beads.

The porosimetric characteristics of the non-hybrid and hybrid alginate/βCD aerogels beads are shown in Table 1. As presented in the table, the specific surface area of both core and floating hybrid alginate aerogels was comparable to the surface area of the non-hybrid alginate/βCD aerogels. This indicates that the addition of βCD did not significantly affect the specific surface area, pore volume and pore size of the original alginate aerogels matrix. On the other hand, floating beads exhibited lower surface area than core beads.

Table 1 Porosimetric characteristics of non-hybrid and hybrid alginate/βCD aerogels beads

Aerogels beads	Specific surface area (m^2/g)	Average pore volume (cm^3/g)	Average pore size (nm)
Non-hybrid core	447.26	4.44	39.7
Hybrid core	424.73	4.04	38.0
Non-hybrid floating	545.59	6.03	44.2
Hybrid floating	495.37	5.29	42.7

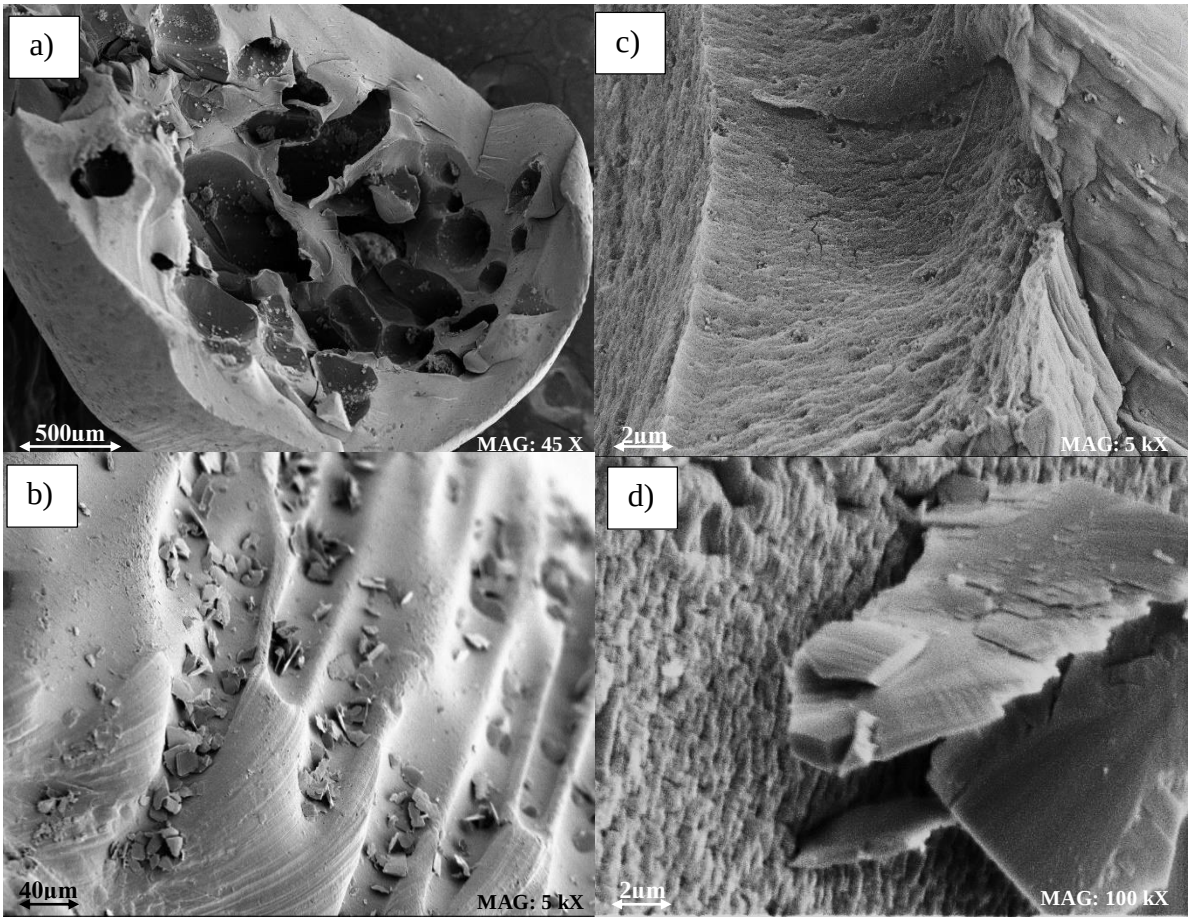

Figure 3 SEM images of cross-sectional: floating beads (a, b) and core beads (c, d)

3.3 Thermal analysis

3.3.1 Inclusion complex β-cyclodextrin+phytol

A βCD-guest inclusion complex can be identified from the thermal behavior observed in the DSC thermogram. Disappearance or shift of melting, boiling and sublimation points to a different temperature indicates the incorporation of the guest molecules in the βCD host [41]. In Figure 4, DSC curves of βCD+phytol inclusion complexes formed by $SCCO_2$ and physical mixing are compared with the curves corresponding to pure phytol, untreated βCD and $SCCO_2$-treated βCD. Pure βCD shows a large peak at 105ºC corresponding to the release of "surface" and "bound" water molecules. Variations in this important peak can be examined to indicate the formation of inclusion complex between the host and guest, because appearance of the peak at temperatures similar or higher than 105ºC suggests a

significant bound water content in the complex, thus corresponding to the presence of non-complexed or not completely complexed βCD [42].

As shown in the Fig 4, inclusion complexes formed by $SCCO_2$ at 100 bar, 40°C and 150 bar, 50°C exhibited a slightly attenuated and displaced peak (shifted from 100°C to 110°C), indicating the incomplete formation of inclusion complexes βCD+phytol. Moreover, the appearance of an endothermic peak at 220°C could be due to phytol+βCD interaction, indicating that phytol has been embedded on the βCD host molecules. This peak can be observed also in the physical mixture complex.

On the other hand, it is observed that only the SC inclusion at 100 bar and 50°C exhibited a complete formation of inclusion complex: the endothermic peak was broadened and shifted from 100°C to 80°C with reduced intensity compared to the peak of pure βCD. This indicates the complete formation of βCD+phytol complex due to the replacement of the bound water content inside the host cavity. In other words, in this case the water molecules in the βCD were displaced by the lipophilic guest molecules to attain apolar-apolar association, resulting in a lower peak intensity with a peak shifted from 100°C to 70°C. Moreover, the small endothermic event observed at 220°C in other samples disappeared in this case, also indicating a change of structure that could be associated to a successful incorporation of phytol inside the βCD cavities. This significant effect of temperature on the formation of the inclusion complex could be due to the faster kinetics of formation of the complexes reported at higher temperatures [32]. In contrast, increasing pressure to 150 bar at constant temperature did not improve the formation of the βCD+phytol inclusion complex.

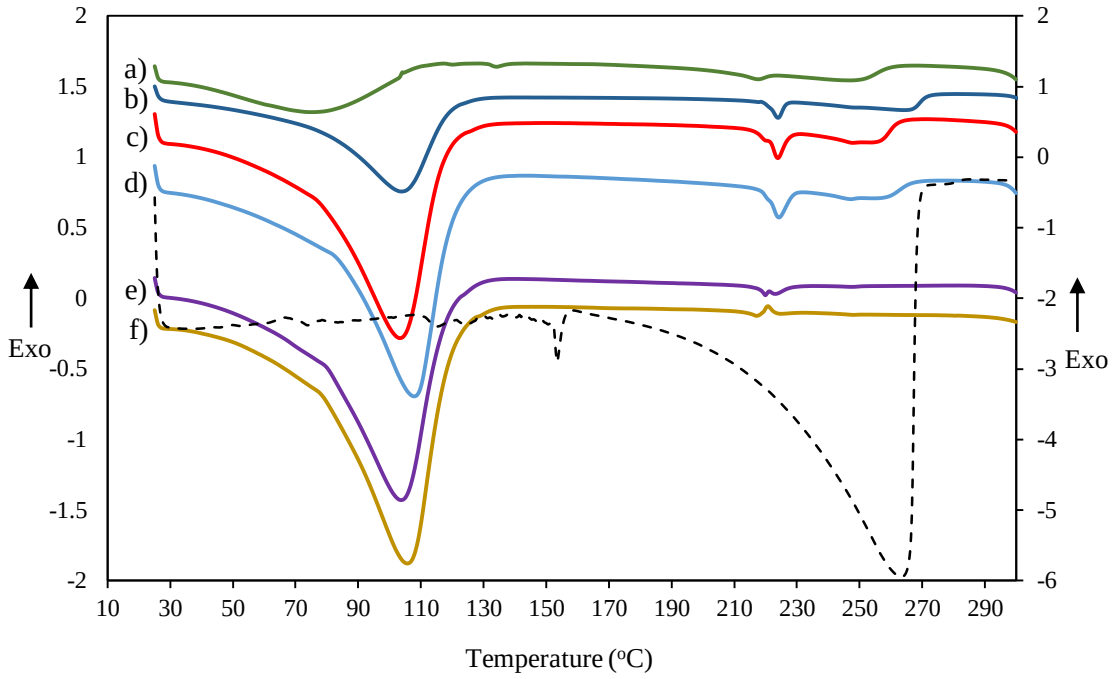

Figure 4 DSC curves comparison for βCD inclusion complex formed by $SCCO_2$ at several operating conditions: 100 bar, 50°C a), physical mixture (PM) b), 100 bar, 40°C c), 150 bar, 50°C d), β-cyclodextrin treated with $SCCO_2$ at 100 bar 40°C e) untreated β-cyclodextrin f) and untreated phytol g)

3.3.2 Alginate/β-cyclodextrin hybrid aerogels

The results of DSC analyses of the hybrid alginate/βCD aerogels and its comparison with the results of blank alginate (without βCD) and pure βCD are depicted in Figure 5. As shown in the figure, pure βCD (Fig. 5a) exhibited two important peaks, i.e. the endothermic peak at 110°C and a small exothermic peak at 220°C, which is similar as the peak observed at the same temperature in Figure 4. Furthermore, peaks at 310 and 330°C correspond to phase transition and fusion of βCD followed by the decomposition of pure βCD [43, 44].

On the other hand, in the blank alginate (Fig. 5b) the endothermic peak observed at 90°C was due to water dehydration whereas the exothermic peak at 240°C indicates the decomposition of the alginate. The profiles corresponding to the hybrid alginate/βCD aerogels in Fig. 5c) demonstrate the combination peaks of pure βCD and blank alginate with

the presence of dehydration peak at 80ºC, decomposition peak of alginate at 240ºC and decomposition of βCD at 300ºC. These peaks demonstrate the presence of βCD in the alginate aerogels.

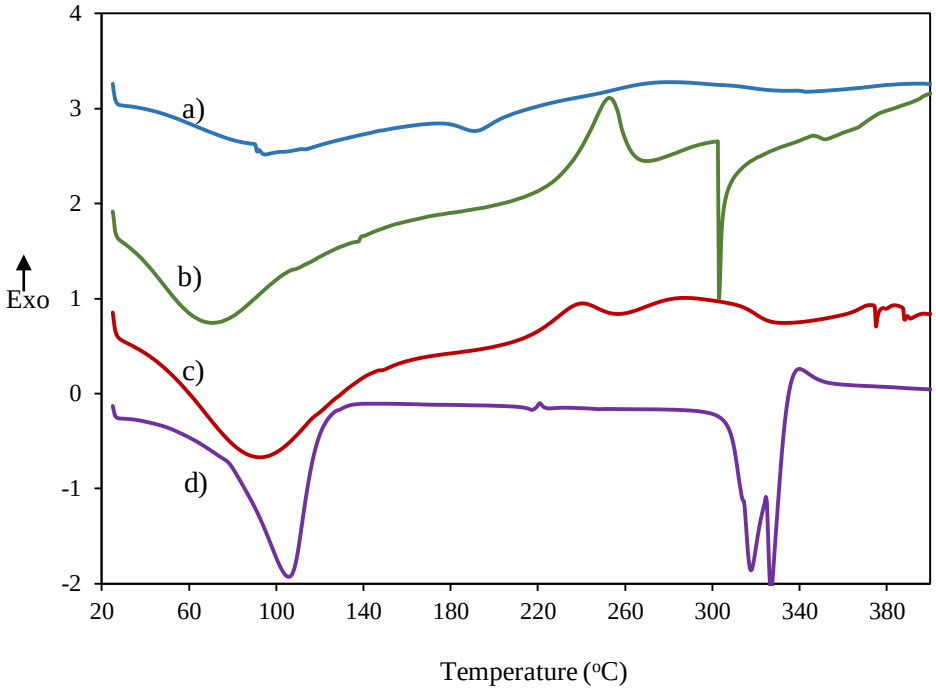

Figure 5 DSC curves comparison for: core hybrid aerogels impregnated with phytol (a), non-impregnated core hybrid aerogels (b), alginate aerogels (c) and β-cyclodextrin (d)

Furthermore, in the DSC thermogram of the impregnated hybrid alginate/βCD aerogels with phytol showed in Fig.5d, it is observed that the main peaks from the pure βCD and the blank alginate are not present. The disappearance of the peak corresponding to water dehydration from the βCD molecules between 80 to 110ºC indicates that some phytol has formed an inclusion complex with βCD, meanwhile the appearance of a small endothermic peak at 190ºC could be the pre-decomposition peak of phytol that probably was impregnated on the alginate rather than embedded in the βCD. Indeed, a competing effect between the tendency to form apolar-apolar association of βCD+phytol and the affinity of the phytol towards the alginate material takes place between these two impregnation sites for phytol. Therefore, the impregnation of phytol into the hybrid alginate/βCD aerogels beads is

probably governed by the formation of βCD+phytol inclusion complex and the interaction of phytol-alginate. In overall, disappearance of phase transition and fusion thermal events indicates that the complexation of βCD/phytol formed in the hybrid alginate beads was stable over a wide temperature range.

3.4 Loading capacity

Impregnation of phytol into the non-hybrid alginate aerogel beads and hybrid alginate/βCD aerogel beads were performed to determine the effect of βCD addition on the loading capacity. Results of the loading capacity of phytol in both core and floating hybrid alginate/βCD aerogel beads are presented in Table 2. It was found that with both types of alginate hybrid aerogels, i.e. core and floating beads, the phytol loading slightly increased with the presence of βCD in the alginate aerogels. The loading capacity of phytol was slightly improved from 54.4 ± 0.5 wt% to 57.3 ± 1.2 wt% in the core beads whereas in the floating beads the loading increased from 56.7 ± 1.5 wt% to 60.5 ± 0.9 wt% of phytol. Although the hybrid alginate/βCD aerogels have lower surface area compared to the non-hybrid alginate beads, the higher phytol loading found could be due to the presence of βCD, that tend to create apolar-apolar association through inclusion complexation between the lipophilic phytol with the hydrophobic cavity of the βCD. This result is in agreement with the observations from the DSC analysis in Section 3.3.2 (Figure 5d) that showed the disappearance of βCD peaks, indicating the formation of inclusion complex βCD/phytol in the hybrid alginate beads. Nguyen et al. [31] also reported increasing ketoprofen loading efficiency due to the incorporation of the drug into the central cavity of βCD to form βCD-ketoprofen complexes. They found that increasing the amount of βCD increased the loading efficiency and association value of ketoprofen.

Phytol loading in floating beads was slightly higher than the loading in core beads. The higher loading of phytol in floating beads was due to the void space of the beads that provide a larger capacity for the compounds to be impregnated into the aerogel beads. Higher specific surface area of the floating beads may have also contributed to the higher

phytol loading in comparison to the core beads (see Table 1). Results of an analysis of variance (ANOVA) on the effect of hybrid alginate/βCD on the phytol loading at a significant confidence level of 95% indicates that the effect of βCD on the loading capacity is significant with a *p*-value below 0.05. The increase of phytol loading in the hybrid alginate/βCD relative to the non-hybrid alginate/βCD was found significant both for core (*p*-value = 0.018) and floating (*p*-value = 0.011) beads.

Table 2 Comparison of loading capacity of non-hybrid alginate beads aerogels and alginate/β-cyclodextrin hybrid aerogels

Alginate hybrid beads	Loading capacity, %*L*	
	Without βCD	With βCD
Core beads – MI	54.4 ± 0.5	57.3 ± 1.2
Floating beads – MII	56.7 ± 1.5	60.5 ± 0.9

The hybrid alginate/βCD aerogels impregnated with phytol were characterized by FTIR to investigate the possible interactions of impregnated pythol with βCD and the alginate matrix. The results or the IR spectrum are presented in Figure 6 for the core system. As indicated in Figure 6a, the main bands in the IR spectrum of βCD were as follows: 1155 cm^{-1} (C–O vibrations), 1640 cm^{-1} (O–H bending vibrations), 2930 cm^{-1} (C–H stretching vibrations) and 3400 cm^{-1} (O–H stretching vibrations) [45]. On the other hand, for the blank alginate beads (without βCD) in Fig. 6b, the vibration bands exhibited at 3400, 1596 and 1404 cm^{-1} correspond to O–H stretching vibrations, COO^- symmetrical and COO^- asymmetrical stretching vibration of carboxyl groups, respectively. The IR spectrum of hybrid alginate/βCD aerogels showed in Fig.6c, did not reveal any different peaks in comparison to the spectrum of pure βCD (Fig. 6a) and blank alginate aerogels beads (Fig. 6b). On the other hand, the impregnated hybrid alginate/βCD aerogels exhibited absorption bands at 2940 cm^{-1}, corresponding to C–H stretching vibrations due to the presence of phytol in the hybrid alginate aerogels. A similar observation was found in the floating hybrid alginate/βCD beads.

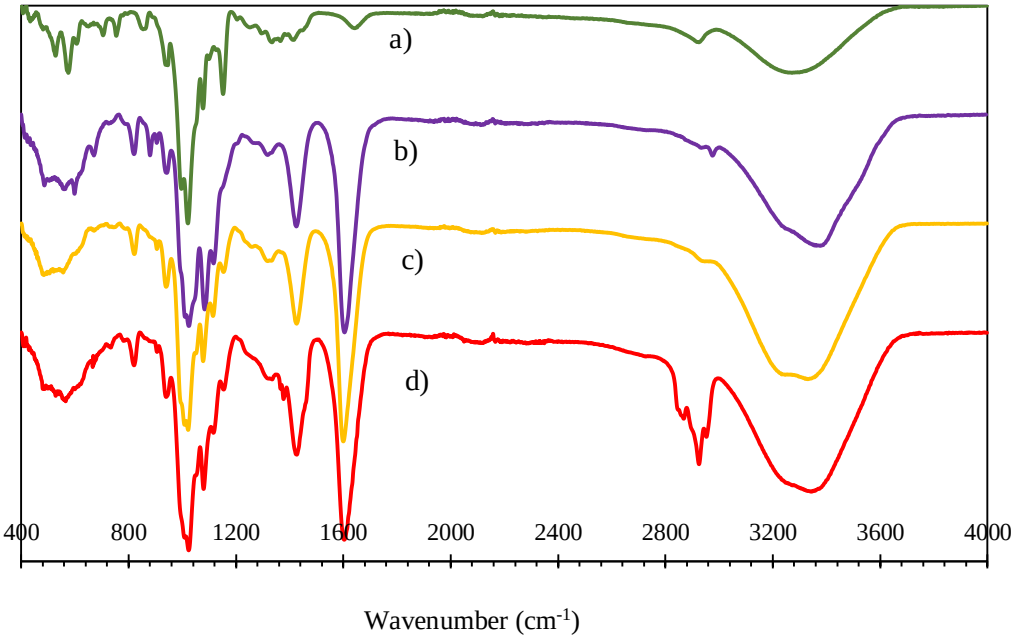

Figure 6 IR spectra of pure βCD (a), blank alginate beads MI (b), Hybrid alginate/βCD aerogels beads MI (c) and impregnated Hybrid alginate/βCD aerogels beads MI with phytol (d)

3.5 Phytol dissolution

The comparative dissolution tests of core and floating hybrid and non-hybrid alginate aerogels beads are presented in Figure 7. It is observed that within the first 2 h, the release of phytol from the core and floating hybrid beads reached 45% and 28%, respectively. The total phytol release was improved three-fold for the core hybrid beads while it was six times higher for the floating hybrid beads, compared to the non-hybrid alginate beads. For both non-hybrid beads, the phytol release was significantly poorer, as 30% was released from core beads and only 12% of phytol was released from floating beads after 6 h of dissolution test.

It is also observed that the release of phytol from floating beads was much slower in comparison to the release of compounds from the core hybrid beads. Almost 95% of the phytol was released from the core hybrid beads at the end of the dissolution experiment whereas for the floating beads a slower dissolution was observed with 70% phytol released

in 8 h. This result could be expected as floating or hollow beads based on alginate are some of the systems applied as floating drug dosage system (FDDS) in order to prolong gastric retention time (GRT) for 13 to 24h and maintain an effective concentration of the drug in serum for longer period of time [40, 46-49]. In addition, the release of phytol from the floating beads is anticipated to be much slower in simulated gastric fluid as the drug release from the floating beads in alkali media is more rapid compared to the release in acidic media [49].

The dissolution profile obtained in the present work was significantly improved in comparison to the results reported in a previous work by Mustapa et al. [15]. In the previous work, the release test of impregnated phytol could not be even carried out due to the extremely low solubility of phytol in water and the strong interaction of phytol with silica and alginate used as carriers in that work. The improvement of the phytol release reported here is due to the presence of βCD in the alginate aerogels that promotes the solubilization of phytol in water. In addition, the geometry shape of the beads accelerated the rate of release (flux) of the compounds, compared to the release from larger cylindrical monoliths tested in the previous work.

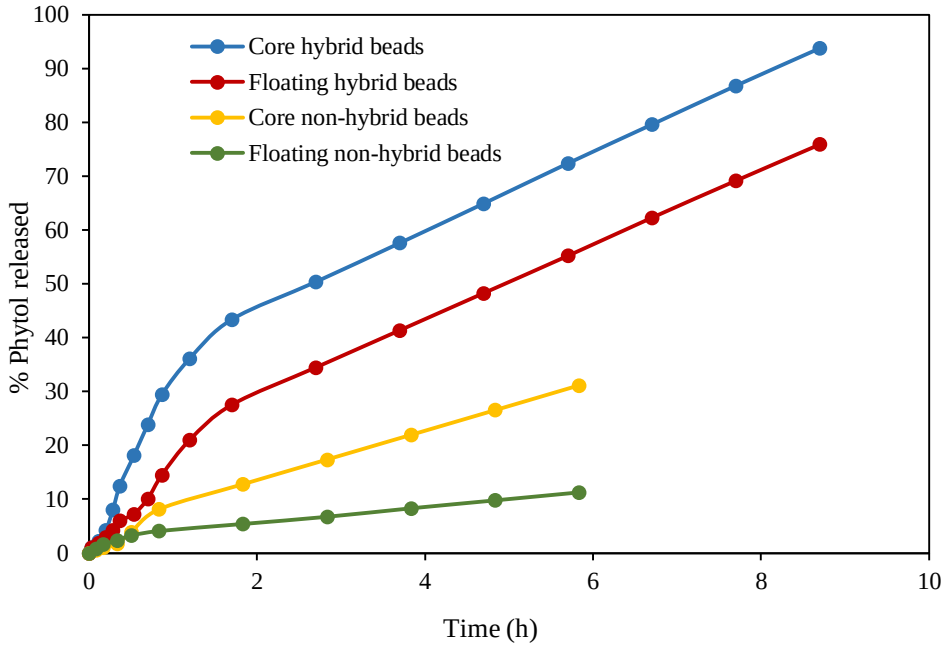

Figure 7 Dissolution profile of non-impregnated and impregnated alginate/β-cyclodextrin hybrid aerogels with phytol and comparison with non-hybrid alginate beads aerogels

4.0 Conclusion

In this work, the synthesis of hybrid alginate/βCD aerogels beads to improve the solubility of insoluble drugs or APIs was reported for the first time. In the formation of inclusion complex by supercritical CO_2, temperature is more influencing factor than pressure to form an inclusion complex of βCD+phytol. SEM and DSC analysis have confirmed the presence of the βCD excipient in the hybrid beads. The loading capacity of the hybrid alginate/βCD aerogels beads with phytol was only slightly increased in comparison to the non-hybrid aerogels beads, and floating beads attained slightly higher loading than core beads. However, the dissolution of phytol was remarkably improved three-fold from the case of core and six times higher from the floating beads type. The floating beads showed slower release in contrast to the core beads due to its buoyancy characteristics that make them suitable for sustained drug release. These results indicate that hybrid alginate/βCD aerogels can have a great potential in pharmaceuticals industry for the production of dosage form in simple, safe and efficient way with significantly high loading capacity as well as enhanced dissolution of poorly soluble drugs.

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Conclusions & Future Works

In this chapter, general conclusions are discussed for each of the objectives that have been addressed. In addition, the outlook for future development are also pointed out particularly to enhance the potential of the medicinal plant or generally for other high value products.

Conclusion I

Phytochemical compounds from *C. nutans* medicinal plant extracts have been identified and quantified. The plant is containing valuable bioactive compounds such as phytol, β-sitosterols, and it is rich in polyphenols content. The major compounds in supercritical fluid extraction (SFE) is a palmitic acid (29.23%) whereas phytol has been identified as major in Soxhlet and microwave-assisted extraction (MAE) with 75.42% and 34.99%, respectively. In contrast to the method of extractions, microwave extraction produced the *C. nutans* extracts were enriched of polyphenols from 5.74 ± 0.29 to 14.56 ± 0.77 mg GAE/g DM and flavonoids from 3.41 ± 0.76 to 5.29 ± 0.30 mg quercetin/g DM. Substantially high of major compounds i.e. phytol and rich of polyphenols content obtained from MAE allow this method to be recognized as an efficient for extracting the bioactive compounds from the *C. nutans* plant. In addition, these compounds have been selected as the key and high valuable compounds for further investigation.

For future development, isolation of the valuable marker compounds such as β-sitosterols or flavonoids are necessary to be carried out to enrich these compounds at higher purity. This enable for the determination of their biological activity and better formulation in absence of any toxic compounds that might attribute to the impotent of the plant extracts.

Conclusion II

Extraction of phytochemical compounds from *C. nutans* medicinal plant has been enhanced. Microwave-assisted extraction (MAE) appears as the most efficient technique over conventional method (Soxhlet extraction) and supercritical fluid extraction (SFE) method in a shorter time and reasonable yield. Although SFE has been recognized as a safer and cleaner extraction method, the poor solubility of key *C. nutans* extract compounds in supercritical limits the application of this technology in this case.

The kinetics process of MAE pre-treatment were studied by considering the specific energy absorbed (J/g), the effect of solvent concentration and solvent-to-feed ratio (S/F) on the polyphenols content. Higher specific energy absorbed (230.56 J/g) led to higher polyphenols content and 50%v/v of ethanol/water with S/F of 14 were found as the optimum conditions for extracting *C. nutans* in faster rate with reasonable equilibrium concentration of polyphenols. The special heating mechanism in the MAE method successfully enhanced the extraction of phytochemical compounds by rupturing cell walls due the microwave energy absorbed by the *C. nutans* and solvent. This facilitated the release of polyphenols into the extraction solvent. Due to the effect of this extraction pre-treatment, MAE is more efficient than the conventional extraction in maximizing equilibrium yield with rich of compounds of interest in shorter time.

Nevertheless, further thorough investigation on the other factors that influence the MAE such as effect of temperature and dielectric properties of the medicinal herbal are essential in order to further optimize the method.

Conclusion III

Incorporation of *C. nutans* extracts into silica and alginate aerogels is a novel approach in drug delivery system. Loading of the extracts can be done with two methods: 1) impregnation before supercritical CO_2 drying by liquid absorption of extracts solution into aerogels and 2) supercritical impregnation (SCI) of $SCCO_2$-extracts solution on dried aerogels. The successfulness of the loading of phytochemical compounds by SCI is highly dependent on the solubility of the compounds in $SCCO_2$, surface area of the aerogels and interaction of compounds with carrier materials and with CO_2. Meanwhile, the impregnation by liquid absorption is governed by the solubility of extracts in organic solvent and with the concept of either molecular or physical dispersion. In the SCI method, silica gave higher loading (30.1 ± 0.6 *wt%* of phytol, 11.5 ± 0.4 *wt%* CN50 extract and 23.9 ± 1.0 *wt%* of CN100 extract), whereas via liquid absorption technique, alginate is superior to the silica aerogels (18.9 ± 0.8 *wt%* of phytol, 9.6 ± 2.1 *wt%* of CN50 extract and 18.5 ± 0.5 *wt%* of CN100 extract). The *C. nutans* showed faster release when loaded in alginate with 80*wt%* CN50 extract released in 6h whilst 55*wt%* of CN100 extract release in 7h, than in silica aerogels, indicating that the extracts has good bioavailability with alginate and thus appear as more appropriate carrier materials for the *C. nutans*.

Based on these results, it is suggested to extend the use of biomaterials to other polysaccharides such as pectin, starch, cellulose and amylose to determine the best association of herbal drugs with the materials in considering the drugs loading and its bioavailability. In addition, a combination of these polysaccharides with silica or other biopolymer also would be interesting to be explored in the development of herbal drugs delivery system. Furthermore, association of herbal drugs in micro- or nanoparticles aerogels by emulsion and supercritical impregnation is also another interesting innovation of novel drug delivery to improve applicability and the efficacy of natural drugs in pharmaceuticals field.

Conclusion IV

The development on the new dosage form based on aerogels integrated with β-cyclodextrin as excipient is a promising method to improve the bioavailability of poorly soluble compounds for oral drug delivery system. The solubility of poor-water soluble drugs has been increased three-fold and six-times higher with the presence of β-cyclodextrin in the core and floating alginate beads aerogels, respectively in comparison to the drugs in the aerogels without β-cyclodextrin. Slight increase of phytol loading in the hybrid aerogels was found to be 3 – 4 *wt%* compared to the loading in the non-hybrid aerogels.

The hybrid aerogels were synthesized by a simple physical cross-linking at mild conditions resulted in the dispersion of β-cyclodextrin in the matrix without any association formed with the host polysaccharides polymer chains. With this, the inclusion complex between the β-cyclodextrin and drugs might not be fully attained in the whole host matrix. Therefore, it is worth to investigate the performance of hybrid of biopolymer aerogels/β-cyclodextrin prepared via chemical cross-linking that could graft the β-cyclodextrin molecules on the backbone of the biopolymer. Subsequently, the synthesized gels could be dried by supercritical CO_2 drying and producing aerogels with high porous structure. To-date, no reported on the production of the hybrid aerogels prepared by chemical and physical cross-linking is found in literature.

Resumen

1.0 Introducción y objetivos

El incremento del interés general por terapias más seguras y saludables ha llevado al aumento del uso de productos naturales y medicamentos derivados de plantas y, especialmente, hierbas medicinales. Las plantas medicinales constituyen una fuente rica en componentes bioactivos que posee importantes cualidades terapéuticas, como son efectos antioxidantes, antivirales y antiinflamatorios. Sin embargo, el uso de hierbas medicinales en la industria farmacéutica todavía presenta grandes desafíos, especialmente en términos de eficiencia del sistema de dosificación. Además, es necesario el desarrollo de procesos innovadores que mejoren tanto la extracción como la formulación de la fitoterapia.

En esta tesis se estudia la valorización de la planta medicinal *Clinacanthus nutans* (*C. nutans*) a través de la extracción de sus componentes de alto valor terapéutico y la evaluación de su potencial para aplicaciones farmacéuticas. La investigación comprende la extracción de componentes fitoquímicos, la formulación de los extractos herbarios en matrices de aerogel mesoporoso para facilitar su dosificación controlada, y la determinación de su biodisponibilidad. Los objetivos de esta tesis se pueden concretar en:

1. Caracterización de los extractos de *C. nutans*, identificando y cuantificando los componentes fitoquímicos disponibles en ellos y seleccionando los componentes de mayor interés para aplicaciones farmacéuticas.
2. Investigación de la intensificación de la técnica extractiva en comparación con el método tradicional de extracción con disolvente, con el fin de llegar a una alta extracción en óptimas condiciones, investigando técnicas alternativas de extracción que incluyen la extracción por fluido supercrítico (EFS, SFE en inglés) y la extracción asistida por microondas (EAM, MAE en inglés) en diferentes condiciones.
3. Desarrollo de una formulación adecuada de los extractos de la planta medicinal en forma de dosis sólidas de diferentes materiales para la administración oral, evaluados a través de estudios de disolución *in-vitro*, para determinar la conducta de la liberación de extractos, con tecnologías tradicionales y modernas asistidas con CO_2 supercrítico.
4. Estudio de las posibles limitaciones de aplicabilidad de las formulaciones desarrolladas y propuestas de mejora, a través del diseño de nuevas estrategias para el sistema de administración de medicamentos que realcen la biodisponibilidad oral de los medicamentos de baja solubilidad, con el objetivo de sintetizar un recipiente de

medicamentos novedoso con excipientes adecuados a la mejora de la solubilidad y la biodisponibilidad de los medicamentos.

2.0 Resultados y discusión

2.1 Extracción de fitocompuestos de la planta medicinal *Clinacanthus nutans* Lindau

En la primera parte de esta tesis (**Capítulo I**), la planta C. nutans ha sido extraída mediante tres procedimientos, extracción con Soxhlet, extracción con CO_2 supercrítico (ESC) y mediante extracción asistida por microondas (EAM). Estos procedimientos tienen como objetivo determinar que tecnología es la más eficiente para la obtención de los componentes bioactivos presentes en la planta. Los extractos han sido analizados para identificar los principales componentes fitoquimicos obtenidos con cada método y otros componentes significativos. Además, los resultados han demostrado que el método EAM produjo una extracción total comparable (17.39%) a la de Soxhlet en un tiempo más corto (80 min) mientras que EFS produjo el rendimiento más bajo entre los métodos de extracción estudiados (Figura 1).

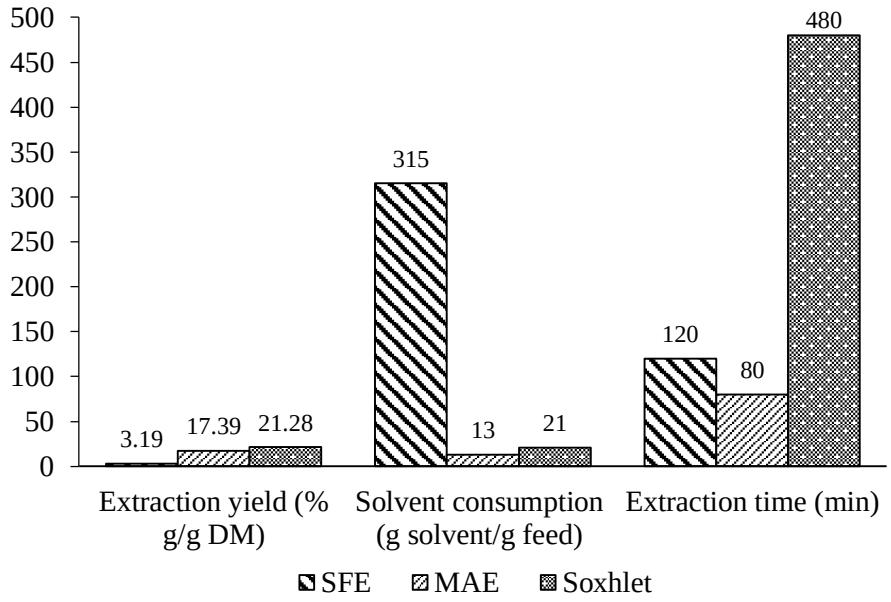

Figura 1 Comparación de las técnicas de extracción en base a diferentes factores

Los resultados han mostrado que el extracto de C. nutans tiene fitol como componente principal, y cantidades importantes de otros constituyentes de alto valor como fitoesteroles y

polifenoles (Tabla 1). Además, para la extracción de fitoesteroles, EFS aporta mejores resultados que la extracción con microondas y Soxhlet (Tabla 2).

Tabla 1 Fitocomponentes identificados por GC / MS en los extractos de C.nutans obtenidos mediante extracción asistida por microondas (EAM), extracción con fluidos supercríticos (EFS) y la extracción Soxhlet

Compounds	Peak Area (%)		
	MAE (86%v/v)	SFE (350 bar, 60ºC)	Soxhlet (abs ethanol)
Neophytadiene	13.44	2.73	-
Iron	3.00	-	-
7,9-dodecadien-1-ol	4.25	-	-
Myristic acid	-	2.12	-
Palmitic acid acid, methyl ester	3.09	-	5.91
Palmitic acid	29.23	43.49	12.13
Benzenethanol	3.74	-	-
Phytol	34.99	11.34	75.42
Squalene	-	5.58	-
Stearic acid, methyl ester	4.53	17.43	-
Margaric acid, ethyl ester	-	0.50	6.54
Lupeol	3.73	-	-
Linoleic acid, ethyl ester	-	15.77	-
Linolenic acid, methyl ester	-	1.04	-

Tabla 2 Contenido total de fitosteroles (TP) y de β-sitosteroles (BS) obtenidos con los diferentes métodos de extracción

Method	Ethanol concentration (%v/v)	TP (mg BS/g DM)	BS (mg/g DM)
MAE	50	0.19 ± 0.13	-
	65	0.41 ± 0.20	0.16 ± 0.22
	86	0.70 ± 0.10	0.52 ± 0.10
*P-MAE	50	0.35 ± 0.12	-
	65	1.04 ± 0.15	0.45 ± 0.20
	86	1.19 ± 0.22	0.65 ± 0.14
Control	44	-	-
Soxhlet	Absolute	0.47 ± 0.20	0.23 ± 0.18
SFE	350 bar, 60ºC	1.35 ± 0.12	0.83 ± 0.10
MAE-KOH	1.5 mol/L ethanolic KOH	2.36 ± 0.15	0.64 ± 0.13
Control-ethanolic KOH		0.88 ± 0.14	0.40 ± 0.10

En general, el contenido de fenoles totales (TPC) y flavonoides (TF) contenidos en los extractos mediante EAM presurizado (P-EAM) han sido similares a los valores obtenidos por EAM. El valor TPC disminuyó de 13,23 a 8,88 mg GAE / g DM significativamente ($p < 0,05$), cuando la concentración de etanol se redujo desde 44 hasta 86% v / v (Tabla 3). El P-MAE mejoró la capacidad de extracción de los polifenoles entre 2 y 4 mg GAE / g DM de TPC en extractos por EAM con diferentes concentraciones de etanol.

Tabla 3 Contenido de fenoles totales (TPC) y flavonoides totales (FT) obtenidos con diferentes métodos de extracción

Method	Ethanol concentration (%v/v)	TPC (mg GAE/g DM)	TF (mg quercetin/g DM)
MAE	44	11.09 ± 0.28	4.24 ± 0.12
	50	11.30 ± 0.39	4.66 ± 0.20
	65	9.31 ± 0.18	4.54 ± 0.20
	86	5.74 ± 0.29	3.41 ± 0.76
*P-MAE	44	13.23 ± 0.40	5.23 ± 0.40
	50	14.56 ± 0.77	5.29 ± 0.30
	65	12.89 ± 0.90	5.07 ± 0.56
	86	8.88 ± 0.85	2.71 ± 0.47
Control (MAE)	44	8.89 ± 0.46	4.27 ± 0.22
Soxhlet	Absolute	7.95 ± 0.21	3.04 ± 0.02
SFE	No Ethanol CO_2 350 bar, 60ºC	7.01 ± 0.15	5.88 ± 0.22

2.2 Extracción asistida por microondas de los polifenoles de *Clinacanthus Nutans* Lindau planta medicinal: Perspectiva de la energía y modelado cinético

En la segunda parte (**Capítulo II**), se ha estudiado el enriquecimiento de los extractos en polifenoles mediante la EAM. Se ha investigado la cinética de extracción y optimizando el efecto de la energía específica absorbida, la concentración de etanol/agua como disolvente y la ratio disolvente/alimento (D/A) en los polifenoles a partir de los extractos obtenidos de *C. nutans*. La efectividad del pretratamiento mediante EAM ha sido comparada con el método convencional, y se ha llegado a la conclusión de que la técnica EAM incrementa la concentración de polifenoles de dos a cinco veces en comparación con el método tradicional de extracción mediante disolvente (Figura 2). El barrido de electrones con microscopio (BEM) de los residuos de *C. nutans* por EAM y extracción convencional (sin microondas pre-tratamiento) indicó que no pueden observarse claros daños de las células en los residuos de las muestras extraídas por el método convencional. (Figura 3).El BEM de los residuos del

EAM muestra estructuras celulares dañadas por un repentino incremento de la temperatura durante el calentamiento de EAM seguido por una caída de la temperatura a través de la técnica de enfriamiento rápido en un baño de hielo.

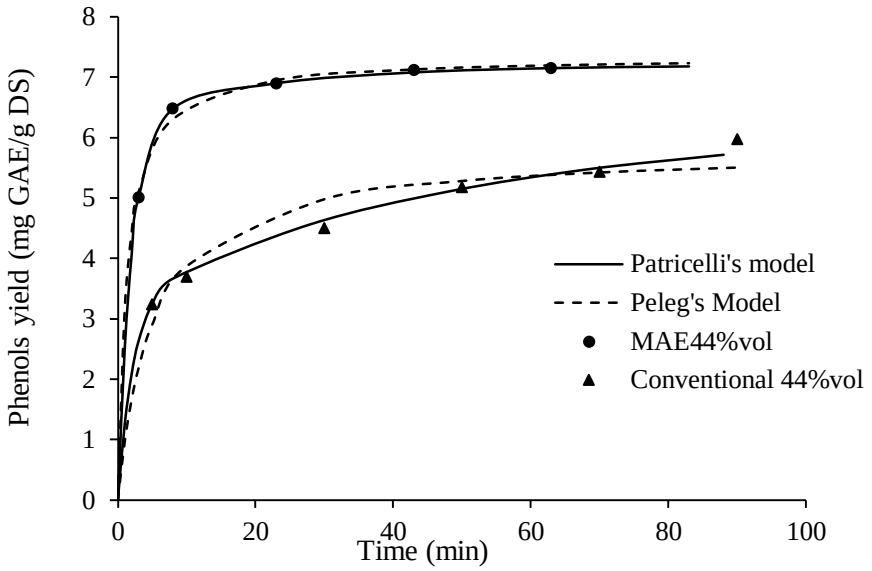

Figura 2 Perfil cinético de fenoles dado por EAM 44%vol y convencional 44%vol

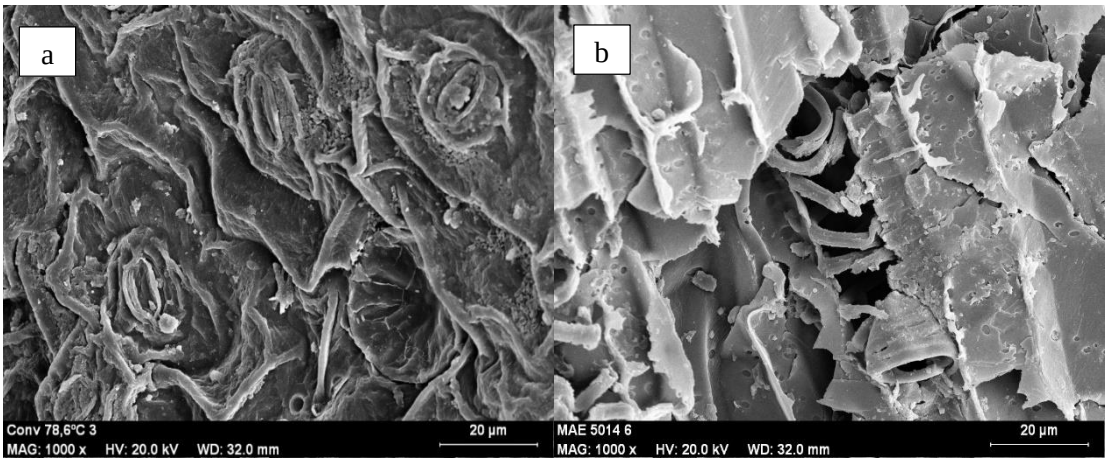

Figura 3 Imágenes del barrido de electrones con microscopio de los residuos de *C.nutans* a través de extracción convencional, sin microondas pre-tratamiento (a) y EAM (b)

Bajo la influencia de la concentración de etanol, se observa que la caída de la ratio de etanol de 86% a 50%vol incrementa significativamente la concentración de TPC. Sin embargo, la caída posterior de la ratio hasta 44%vol reduce ligeramente el valor de TPC (Figura 4). La energía absorbida en un valor constante de energía emitida de 4500 J muestra un valor descendente al tiempo que la proporción de etanol en el disolvente se incrementa. A pesar de ello, como se muestra en la Figura 4, la reducción de la proporción de etanol de 50%vol a 44%vol incrementa la energía específica absorbida, indicando que el TPC debe ser significativamente asociado con las variaciones de las propiedades del disolvente más que la energía de entrada suministrada.

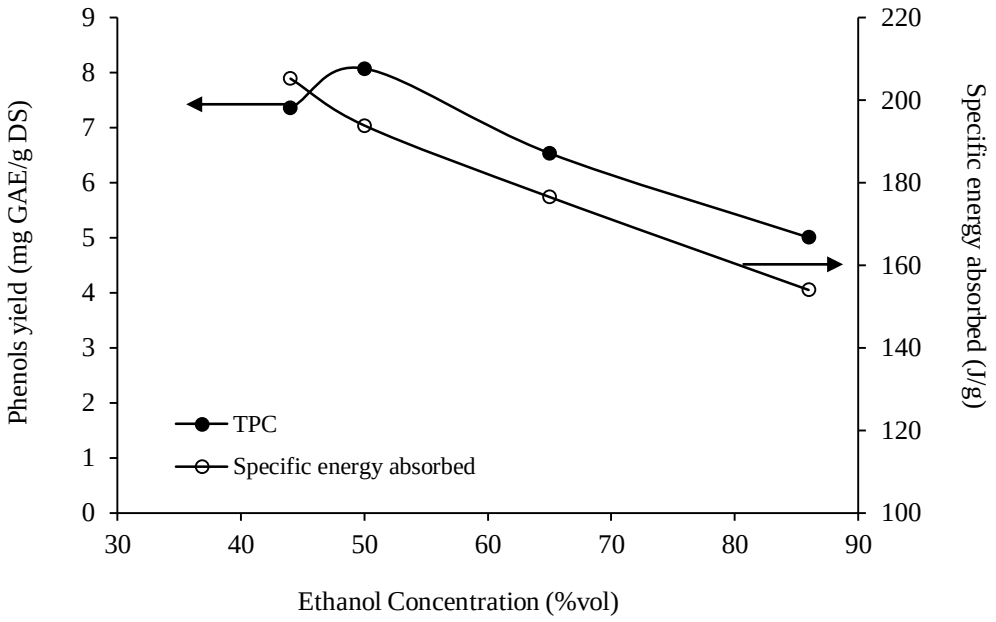

Figure 4 Phenols yield and specific energy absorbed as a function of ethanol proportion (%vol)

Por la influencia de la ratio disolvente/alimento (D/A), como se muestra en la Figura 5, la obtención de fenoles ha sido mejorada cuando el volumen de disolvente se incrementa de 7 a 14 mL para 1 g de material de muestra, pero cuando se incrementa a 20 mL/g la cantidad obtenida cae a 5.32 mg GAE/g DS. Esto sucede porque la gran cantidad de disolvente incorporado da lugar a una baja cantidad de energía específica absorbida por la

muestra de D/A 20 mL/g. por otro lado, a pesar de que el experimento con D/A = 7 mL/g ha dado lugar a una energía específica absorbida más alta que el que tenía una D/A de 14 mL/g debido al menor volumen del disolvente, la obtención de fenoles ha sido ligeramente más baja en comparación con la de la D/A de 14 mL/g. Una posible razón es la limitación de la transferencia de masa causada por el pequeño volumen del disolvente que impide la difusión solutos de la célula al disolvente.

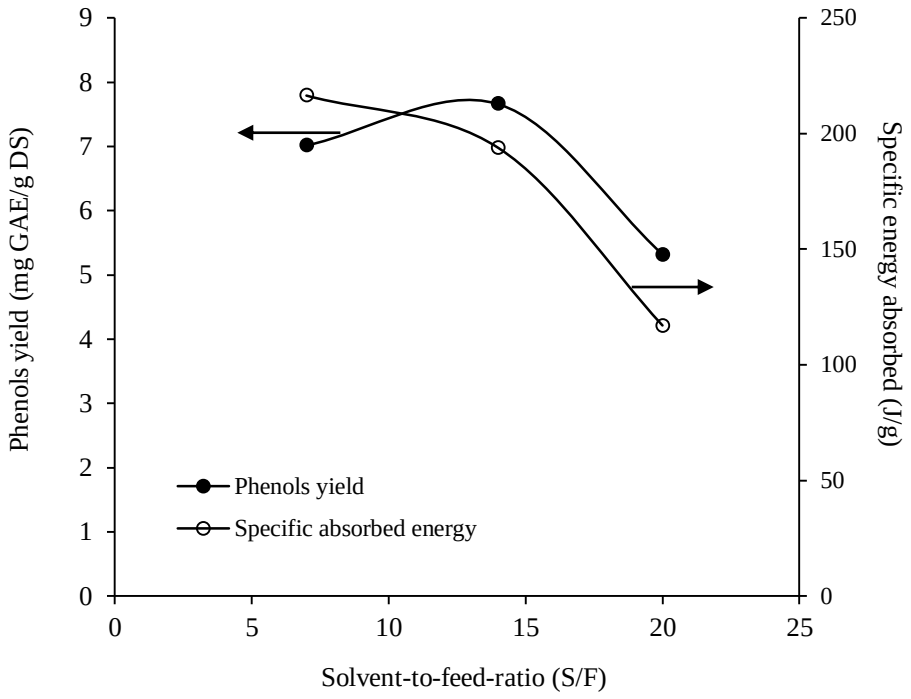

Figure 5 Fenoles obtenidos y energía específica absorbida en function de la ratio disolvente/alimento (D/A)

2.3 Impregnación de compuestos fitoquímicos de plantas medicinales en aerogeles de sílica y alginato

En la tercera parte (**Capítulo III)**, los extractos de *C. nutans* obtenidos con el método EAM con diferentes concentraciones etanol/agua han sido impregnados con aerogeles de sílice y alginato. Además, el principal componente identificado en los estudios previos, el fitol, también ha sido usado como componente modelo que impregnar los aerogeles y se han estudiado su concentración y su biodisponibilidad. La impregnación fue realizada con dos métodos.

En el primero, los compuestos activos y los extractos de *C. nutans* han sido cargados en los geles mediante absorción de líquidos, sumergiendo por un tiempo determinado los geles en las disoluciones de los compuestos activos en etanol. Los geles impregnados fueron secados con CO_2 en condiciones supercríticas. Con este procedimiento, el CO_2 supercrítico ($SCCO_2$) extrae el etanol, dejando los componentes depositados en los aerogeles durante la despresurización de CO_2. La baja solubilidad de los componentes activos en el CO_2 da lugar a una alta impregnación.

Por otra parte, los compuestos activos y los extractos de *C. nutans* han sido impregnados también con el llamado método de impregnación supercrítica (IS). Con esta técnica, los componentes han sido colocados en los aerogeles mediante el CO_2 saturado con la disolución de los compuestos activos. El elevado coeficiente de difusión y la baja viscosidad del $SCCO_2$ favorecen que la disolución penetre en la estructura porosa de los aerogeles. Mediante la despresurización los componentes quedan recogidos en los aerogeles por el mecanismo de deposición o por dispersión molecular. Los diferentes mecanismos que rigen estos métodos dan lugar a diferentes interacciones entre los componentes activos y la matriz receptora, además de una diferente eficacia de disolución.

Los resultados en la Tabla 4 han demostrado que con la impregnación mediante absorción de líquidos el extracto de la planta *C. nutans* y el fitol tienen mayor concentración en los geles de alginato (11,5 ± 0,4 y 23,9 ± 1,0% en peso de los extractos obtenidos con 50% de etanol/agua y disolventes de etanol puro, respectivamente) que en los de sílice (5,2 ± 1,0 y 13,1 ± 0,9% en peso de los extractos obtenidos con 50% de etanol/agua y disolventes de etanol puro, respectivamente), mientras que, con el método IS, los componentes mostraron una mayor concentración en los aerogeles de sílice (11,5 ± 0,4 y 23,9 ± 1,0% en peso para los extractos obtenidos con 50% de etanol/agua y disolventes de etanol puro, respectivamente) que en los de alginato. Las diferencias han sido atribuidas a la mayor superficie específica de la sílice respecto al alginato en el caso de la IS. En cambio, el efecto de la presencia de etanol en la impregnación por absorción de líquidos estimula la interacción entre los componentes y la matriz de alginato, lo que repercute en el incremento de su concentración. Este resultado ha sido validado mediante microscopia electrónica de barrido (MEB), en Figura 5.

La estructura de micrografías por BEM de los aerogeles de alginato vacíos obtenidas después del secado SC a 120 ± 5 bar y 40 °C (Figura 6a) muestra los poros abiertos y la estructura reticulada, que es la morfología de superficie común de los aerogeles de alginato.

En la Figura 6b, la matriz de alginato se estira y se hace más gruesa, perdiendo sus vínculos fibrilares estructurales. Esto indica que la absorción de fitol del etanol medio puede cambiar la estructura de poros de aerogeles de alginato mientras que mejora la penetración de los solutos.

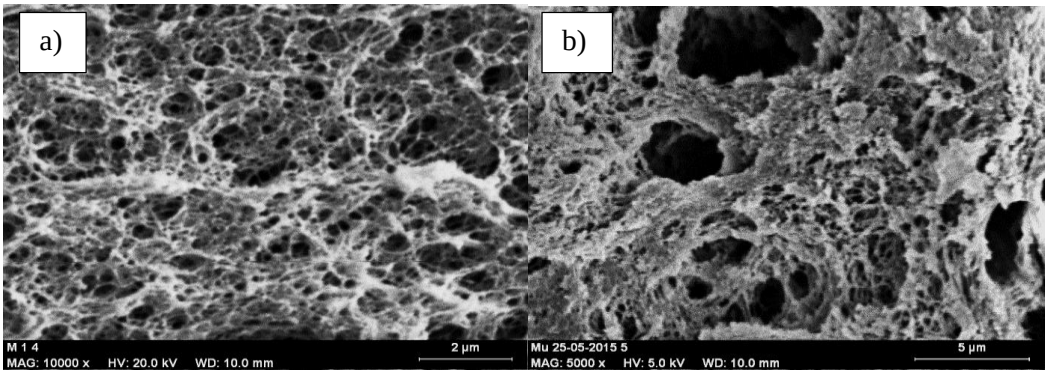

Figura 6 Imágenes de BEM vacío y con aerogeles impregnados con alginato mediante varios métodos: a) alginato vacío, b) impregnación húmeda (IH)

Tabla 4 Carga de extracto de *C.nutans*; CN50 y CN100 por impregnación húmeda (IH) y el método de impregnación supercrítica (ISC) en aerogeles de sílice y alginato.

Method	Aerogels	Extracts loading, *L*(*wt*%)
Wet Impregnation (WI)	Silica+CN50	5.2 ± 1.0
	Alginate+CN50	9.6 ± 2.1
	Silica+CN100	13.1 ± 0.9
	Alginate+CN100	18.5 ± 0.5
Supercritical Impregnation (SCI)	Silica+CN50	11.5 ± 0.4
	Alginate+CN50	4.7 ± 0.6
	Silica+CN100	23.9 ± 1.0
	Alginate+CN100	12.7 ± 0.8

A continuación, las pruebas de disolución (Figura 7) han revelado que los componentes del extracto de *C. nutans* cargados en alginato se liberan más rápido que los cargados en el aerogel de sílice, por lo que el extracto de *C. nutans* puede tener una mejor biodisponibilidad cuando está impregnado en alginato. Por otro lado, el fitol tanto impregnado en el alginato como en la sílice, mediante ambos métodos de impregnación, ha mostrado una baja velocidad de disolución, independientemente del método de impregnación o de la matriz receptora usada. Esto es debido a su extremadamente baja solubilidad en agua.

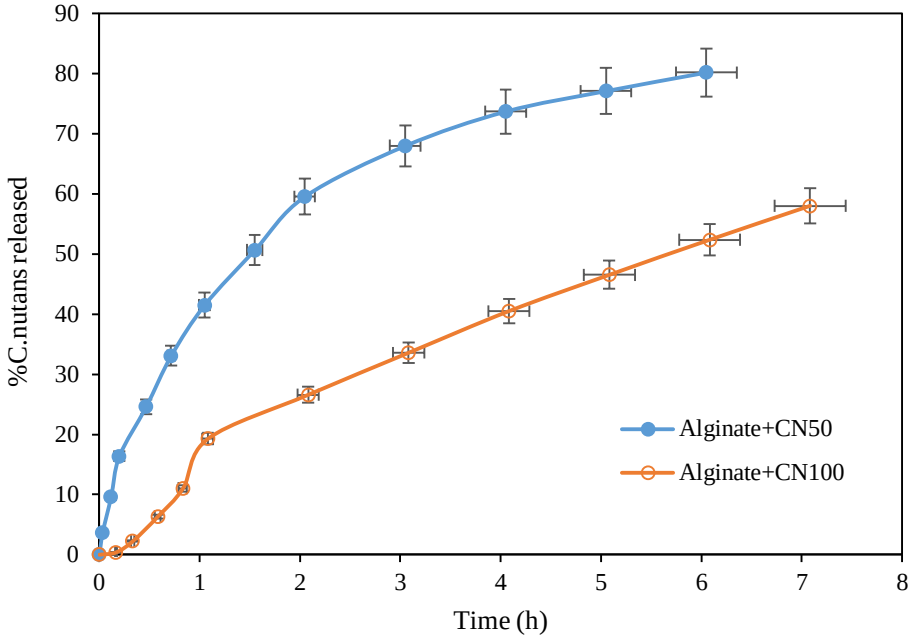

Figura 7 Liberación de extractos de *C.nutans* de los aerogeles de alginato en búfer de fosfato de pH 6,8 a 37ºC

2.4 Síntesis de aerogeles híbridos de alginato/β-ciclodextrina para la administración de fármacos de compuestos con baja solubilidad en agua

En la última parte (**Capítulo IV)**, se han sintetizado aerogeles híbridos de β-ciclodextrina/alginato. En este estudio, la síntesis ha sido realizada solamente con alginato debido a su simple mecanismo de congelación y a su compatibilidad con las características de la β-ciclodextrina (βCD). El alginato/β-ciclodextrina ha sido preparado con esferas de aerogel mediante reticulación física, con un mecanismo ionotrópico de congelación. Han sido producidos dos tipos de aerogeles híbridos: gotas de núcleo y de flotante. En la preparación de los geles, basados en los datos de solubilidad de la β-ciclodextrina en agua, una solución saturada combinación de βCD/alginato y βCD/alginato/$CaCO_3$ ha sido preparada y extrudida en dos diferentes soluciones de congelación obteniéndose esferas de núcleo y flotante. Las esferas de aerogel se han secado con $SCCO_2$ y posteriormente se han sometido a impregnación supercrítica con fitol. Los resultados obtenidos en la Tabla 5 demuestran que en presencia de βCD la capacidad de carga del fitol mejora ligeramente, de 54,4 ± 0,5 wt % a 57,3 ± 1,2 wt% en las gotas del núcleo, mientras que en las gotas de flotante el fitol aumenta de 56,7 ± 1,5 wt% a 60,5 ± 0,9 wt%.

Tabla 5 Comparación de la capacidad de carga de aerogeles no híbridos con gotas de alginato y aerogeles híbrodos de alginato/beta-ciclodextrina

Alginate hybrid beads	Loading capacity, %*L*	
	Without βCD	With βCD
Core beads – MI	54.4 ± 0.5	57.3 ± 1.2
Floating beads – MII	56.7 ± 1.5	60.5 ± 0.9

Además, la liberación de fitol (Figura 6) por parte de los aerogeles híbridos se ha incrementado significativamente tres y seis veces en comparación con la de los aerogeles no híbridos. Esto nos indica que la nueva matriz receptora desarrollada mejora con éxito la solubilidad de los componentes con baja solubilidad en agua.

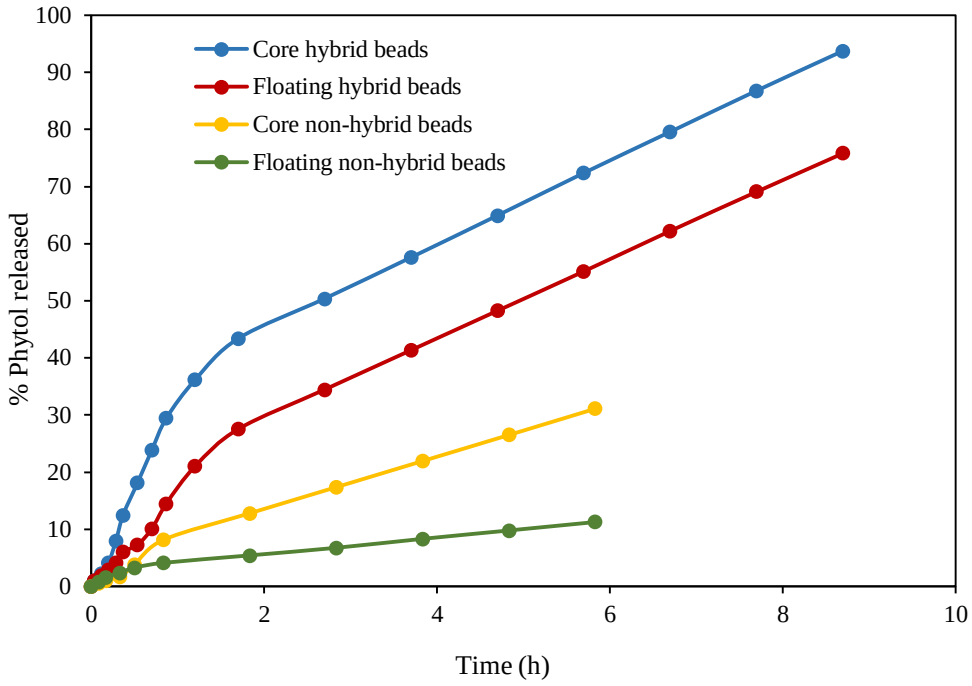

Figura 6 Perfil de disolución de los aerogeles no impregnados e impregnados híbridos alginato / β-ciclodextrina con fitol y comparación con aerogeles no híbridos con gotas de alginato

3.0 Concluciones y trabajo futuro

En resumen, esta tesis presenta los trabajos de investigación sobre la valorización de la planta *C. nutans* como planta medicinal con el objetivo de investigar su aplicabilidad en el

sistema de administración de medicamentos para la industria farmacéutica. Los resultados revelan que la planta medicinal tiene un gran potencial en la industria farmacéutica como medicina alternativa, promovida por la intensificación de la extracción llevada a cabo mediante la tecnología de microondas, la explotación de la tecnología de fluidos supercríticos y el uso de aerogeles como matriz para la administración de los compuestos activos.

Los resultados más significativos de este trabajo se recogen en las siguientes conclusiones:

1. El principal componente en la extracción por fluido supercrítico (EFS) es el ácido palmítico (29.23%), mientras que el fitol ha sido identificado como el más importante en la extracción mediante Soxhlet y la asistida por microondas (EAM) con 75.42% y 34.99%, respectivamente. La cantidad sustancialmente alta del principal componente, el fitol, y el alto nivel de polifenoles obtenidos con EAM, permite reconocer a este método como eficiente para la extracción de componentes bioactivos de *C. nutans*.

   Trabajo futuro:- Para futuras mejoras, es necesario llevar a cabo el aislamiento de valiosos componentes marcadores como β-sitosteroles o flavonoides para enriquecer estos componentes con una mayor pureza. Esto permite la determinación de su actividad biológica y una mejor formulación en ausencia de componentes tóxicos que puedan atribuirse a la baja calidad de los extractos de la planta.

2. La extracción asistida por microondas (EAM) se ha mostrado como la técnica más eficiente respecto al método tradicional (extracción con Soxhlet) y con fluido supercrítico (EFS) con menor tiempo y una razonable cantidad obtenida. Una energía específica absorbida más alta (230.56 J/g) lleva a un contenido más alto de polifenoles y 50%v/v de etanol/agua con S/F de 14, condiciones descubiertas óptimas para la extracción de *C. nutans* con una tasa más rápida y una razonablemente equilibrada concentración de polifenoles.

   Trabajo futuro:- Una investigación más a fondo de otros factores que influyen en la EAM, tales como el efecto de la temperatura y las propiedades dieléctricas de la planta medicinal, determina que estas son esenciales para una ulterior optimización del método.

3. La carga de los extractos puede llevarse a cabo de dos maneras: 1) secado con CO_2 supercrítico e impregnación mediante absorción de líquidos de una solución de extractos en aerogeles y 2) impregnación supercrítica (IS) de la solución de extractos de $SCCO_2$ en

aerogeles secados. El éxito de la carga de los componentes fitoquímicos con IS es depende en alto grado de la solubilidad de los componentes en $SCCO_2$, la superficie de los aerogeles y la interacción de los componentes con los materiales contenedores y con CO_2. Por otro lado, la impregnación mediante absorción de líquidos va en función de la solubilidad de los extractos en disolvente orgánico y la dispersión molecular o física. En el método IS, el sílice ofrece una mayor capacidad de carga (30.1 ± 0.6 *wt%* de fitol, 11.5 ± 0.4 *wt%* de extracto CN50 y 23.9 ± 1.0 *wt%* de extracto CN100), mientras que con la absorción de líquidos, los aerogeles son superiores a los de sílice (18.9 ± 0.8 *wt%* de fitol, 9.6 ± 2.1 *wt%* de extracto CN50 y 18.5 ± 0.5 *wt%* de extracto CN100). El *C. nutans* ha mostrado una liberación más rápida cuando está cargado en alginato, con 80*wt%* de extracto CN50 liberado en 6h por 55*wt%* de extracto CN100 liberado en 7h, que en aerogeles de sílice, lo que indica que los extractos tienen buena biodisponibilidad con alginato y por eso son contenedores más apropiados para la planta *C. nutans*.

Trabajo futuro:- Se sugiere extender el uso de biomateriales a otros polisacáridos como pectina, almidón, celulosa y amilosa para determinar la mejor asociación de medicamentos herbáceos con los materiales, considerando la carga de medicamentos y su biodisponibilidad. Además, también sería interesante explorar una combinación de estos polisacáridos con sílice u otros biopolímeros para la mejora del sistema de administración de los medicamentos herbáceos.

4. La solubilidad de medicamentos poco solubles en agua ha sido incrementada tres y seis veces con la presencia de β-ciclodextrina en los aerogeles de alginato con gotas de núcleo y flotante, respectivamente. Este incremento ha tenido lugar en comparación con los medicamentos en aerogeles sin β-ciclodextrina. Se ha descubierto un ligero aumento de la capacidad de carga de fitol en los aerogeles híbridos del 3 – 4 *wt%*, en comparación con la carga de los aerogeles no híbridos.

   Trabajo futuro:- Los aerogeles híbridos han sido sintetizados con una simple reticulación física en condiciones leves, resultando en la dispersión de β-ciclodextrina en una matriz sin ninguna asociación formada con las cadenas de polímeros receptoras de polisacáridos. Con esto, la integración compleja entre la β-ciclodextrina y los medicamentos puede no ser lograda completamente en toda la matriz receptora. Además, merece la pena investigar la actuación de los aerogeles híbridos de biopolímero y β-ciclodextrina preparados mediante reticulación química que puede injertar las moléculas

de β-ciclodextrina en la columna vertebral del biopolímero. En consecuencia, los geles sintetizados pueden ser secados con CO_2 supercrítico dando lugar a aerogeles con una estructura altamente porosa. Hasta el momento, no se han encontrado en la literatura sobre el tema menciones a la producción de aerogeles híbridos preparados con reticulación química y física.

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Ana Najwa Mustapa (Kuala Lumpur, Malaysia) completed her undergraduate studies (2005) and Master degree (2008) in Chemical Engineering at Universiti Teknologi Malaysia (UTM). Her Master study was about the utilization of R134a as an alternative solvent to supercritical CO_2 to extract palm oil and to recover its valuable minor compounds particularly the β-carotene. After completed the Master study, she was invited to join Centre of Lipids Engineering Applied Research (CLEAR), Universiti Teknologi Malaysia (UTM) as Research Fellow in the area of supercritical fluids extraction. Ana has been a member for the Asia-Pacific Chemical, Biological & Environmental Engineering Society since 2010 and was invited as a reviewer for the International Journal of Chemical Engineering and Application (IJCEA) from 2010 until 2013. She is also a member of The Institution of Engineers Malaysia (IEM) and Associate member of IChemE. She has been as a faculty member in the department of Chemical Engineering at the Universiti Teknologi MARA (UiTM) since 2009 before she pursued her PhD study in the Universidad de Valladolid, Spain in 2013.

In her PhD study, she has been working on the valorization of high potential medicinal plant *Clinacanthus nutans* Lindau to develop an innovative herbal drugs delivery in solid dosage form for pharmaceuticals field. Her research interest lie in the area of supercritical fluids, ranging from theory to process development with a focus on natural products, herbal and foods and pharmaceuticals applications.

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[3] Mustapa, A. N., Martín, A. and Cocero, M. J. Formulation of organic and inorganic aerogels-bioactive medicinal compounds for pharmaceuticals application, 11th International Symposium on Supercritical Fluids , Seoul Korea, October 11-14, 2015.

Courses / Workshops / Internship

[1] Socrates Course, *"Life-long Learning Intensive"*, University of Strathclyde, Glasgow, Scotland, 28th Jun – 18thJuly 2014.

[2] Autumn School on High Pressure Processes, Universidad de Valladolid, October 2014.

[3] Course with Professor Richard Smith Jr. *"Supercritical Fluid Science and Technology*", 3–5th November 2014.

[4] Course with Dr. Stephen Tallon, "*Near-critical solvents for processing of natural products*", Valladolid, 14–18 September, 2015.

[5] Workshop on emulsion gelation and supercritical drying in the frame of European project "Nanohybrids", TUHH, Eissendorferstr. 38 (Technikum) Hamburg University of Technology, 18 – 19 January 2016.

[6] Internship in Hamburg University of Technology (TUHH), 15 January – 15 April 2016.

[7] Winesense Spring School, *"Process Intensification and product development: a focus on grape polyphenols"*, Valladolid, 18 – 22 April 2016.

[8] Course with Prof. Rafa Luque, "*Bioproducts Engineering and Biorefineries"*, Valladolid, 14 – 17 Jun 2016.