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# *Diospyros cuneata* Inhibition of *Fusarium oxysporum*: Aqueous Extract and its Encapsulation by Ionic Gelation

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## Abstract

The application of plant extracts to control fungal crop pathogens is an ecological strategy that could potentially be useful in agriculture. Aqueous extracts of some species of the genus *Diospyros* spp have been tested against fungal pathogens of crops. Nevertheless, there is no information about the inhibitory effect of aqueous extracts of *Diospyros cuneata* on the micelial growth of *Fusarium oxysporum*, a pathogen responsible for "Panama disease" and "vanilla stem rot disease". Therefore, the antifungal activity of aqueous extracts of *Diospyros cuneata* leaves collected during the dry and rainy seasons was tested *in-vitro* against spores of *Fusarium oxysporum*. Only the aqueous extract from leaves collected during the dry season had an inhibitory effect on the micelial growth of asexual spores (2.5% minimum inhibitory concentration). Phytochemical analysis showed that both aqueous extracts contained mainly flavonoids and tannins; the chromatographic profile showed a larger abundance of polar compounds in the dry season extract. Furthermore, the antifungal activity observed is probably correlated with the abundance of some secondary metabolites produced by water stress and dry season conditions. The bioactivity of aqueous extracts of *Diospyros cuneata* leaves could be stored and released through encapsulation; an effective example of this was tested using alginate-inulin to prepare microbeads by ionic gelation.

**Keywords:** Antifungal activity; Bioassays; Phytochemical analysis; Alginate-inulin; Liberation studies

## Introduction

Over the last eighty years, synthetic fungicides have been the most useful strategy for eliminating crop pathogens. However, the problems of environmental contamination and fungal resistance generated by the inadequate application of synthetic fungicides (i.e., high concentration doses) and the high cost needed to produce new synthetic fungicides have made it so that the use of natural compounds is being considered as a potentially useful strategy to control fungal pathogens in agriculture [1,2]. Although the fungicidal effect of plant extracts obtained using different solvents is variable; aqueous extracts from plants have been more effective than methanolic and ethanolic extracts at combating fungal crop pathogens [3-6]. On the other hand, the pharmacological applications and the chemical compounds of the genus *Diospyros* (*Ebenaceae* family) have been well documented [7]. For example, aqueous extracts of *Diospyros ebenaster* leaves have been shown to inhibit 100% of the sporulation of *Colletotrichum gloeosporioides* in *in vitro* conditions, and the extracts have further reduced *Colletotrichum gloeosporioides* infections in mango, *Mangifera indica*, by 80% [8]. *Diospyros cuneata* and *D. anisandra* have been reported ethnobotanically to have medicinal properties that can be used to treat dermatological problems. Organic extracts of these plants have been tested on human fungal and bacterial pathogens [9]. Conversely, there is currently no information regarding the antifungal activity of aqueous extracts of *Diospyros cuneata* for combating crop pathogens such as *Fusarium oxysporum*. *Fusarium oxysporum* is a soilborne plant pathogen responsible of many crop diseases in tropical and subtropical regions; examples of diseases caused by *Fusarium oxysporum* include "Panamá disease" in banana and plantain [10] and "basal stem rot" in *Vanilla planifolia* [11]. On the other hand, the genus *Diospyros* is one of the most important sources of bioactive compounds mainly for anticancer and antibacterial applications [12].

Plants have an unlimited ability to synthesize phytochemicals such as secondary metabolites. Secondary metabolites have antimicrobial properties and serve as the plant's defence mechanism against pathogenic microorganisms. Phytochemicals with pro-oxidant properties may be able to cause damage to pathogenic microorganisms similar to how they have been shown to act against highly mitotic cells, i.e., cancer [13]. It is thought that bioactive polyphenols are toxic to microorganisms depending on the sites and number of hydroxyl groups that the compounds have [14]. It is thought that flavonoids are able to inhibit cytoplasmic membrane function and are also able to change cell morphology by damaging the formation of filamentous cells [15]. Moreover, flavonoids may inhibit the activity of DNA gyrase and β-hydroxyacyl-acyl carrier protein dehydrates; thus the synthesis of DNA and RNA can be inhibited by flavonoids [16]. Other compounds have also been reported to affect microbial activity. For example, tannins have been shown to bind to polysaccharides or enzymes on the surface of cells thereby blocking the membranes of microorganisms. Additionally, terpenes are known to directly cause membrane disruption, and coumarins are capable of reducing cell respiration in microorganisms [17].

The administration of phenolic compounds requires the formulation of a finished product with a protective casing that has

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specific properties. The protective casing or exterior must be able to maintain the structural integrity of the polyphenol until consumption or administration, increase its water solubility and bioavailability, and convey the polyphenol precisely towards a target [18]. In the last thirty years, alginate has become the material of choice for encapsulation. It is widely used in bioengineering and in the biotechnology industry owing to its biocompatibility [19]. The ionic gelation process consists of extruding an aqueous solution of polymer through a syringe-needle or a nozzle in which the active material is dissolved or dispersed. Droplets are received in a dispersant phase and are transformed, after a reaction occurs, into spherical gel particles [19]; as is the case, for example, when sodium alginate is combined with a dispersant phase of calcium chloride. The hydrophilic property of the polymer enables entrapment of water-soluble products. By varying the cross-linking of the polymer and the bead concentration in the bioactive compound, one can control the rate and duration of bioactive compound release *in vivo*. Alginate beads can protect materials, such as proteins [20], amino acids/peptides [21], hormones or enzymes [22], polyphenols [23], antioxidant plant extracts [24], and antimicrobial plant extracts [25], so that they can be delivered to the target choosen [22]. An effective alternative to protect and scatter the bioactivity of aqueous *Diospyros cuneata* leaf extracts is through encapsulation. The main objectives of the present study are to test the antifungal activity of aqueous *Diospyros cuneata* leaf extracts against spores of *Fusarium oxysporum* and to produce the aqueous extract in alginate-inulin microbeads to control the antifungal compound release. Furthermore, the effect of the production parameters on the aqueous extract entrapment efficiency was also investigated. Specifically, the effect of the following parameters was studied: the concentration of alginate, the concentration of calcium chloride, the contact time with the hardening agent, and the addition of inulin to alginate. Finally, the effect of different encapsulation variables on the *in vitro* release behaviour of phenol compounds (efficiency measured indirectly) from alginate-inulin microbeads was examined.

## Materials and Methods

### Fungal material

The *Fusarium oxysporum* isolate VF11 was taken in 2012 from a naturally infected vanilla stem (*Vanilla planifolia* Jacks. ex Andrews) growing at a vanilla plantation in Veracruz State, Mexico. The fungus was isolated and cultured on potato dextrose agar (PDA) at 28°C.

### Isolation of DNA and molecular identification

For DNA extraction, 150 ml of nutrient broth was inoculated with 2 plugs of 5 mm diameter isolate growths, and the culture was incubated in an orbital shaker at 100 rpm at 28°C. The isolate mycelial mass was harvested with a glass hook and deposited in Corning sterile conical tubes. Total genomic DNA was extracted according to a method developed in the GeMBio laboratory [26]. The DNA concentration was determined spectrophotometrically, and DNA quality was determined according to published protocols [27].

The *Fusarium* isolate was identified by nucleotide sequence analysis. For this, the 5.8S-ITS regions were amplified with the universal primers ITS1 and ITS4 [28]. PCR reactions were performed in reaction volumes of 50 µl containing 25 ng of genomic DNA, 1x PCR buffer (Invitrogen, Carlsbad, California, USA), 0.20 mM of each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 1 µM primers and 1U Taq polymerase (Invitrogen). DNA amplification was performed in a GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer, Waltham, Massachusetts, USA), and the program consisted of an initial denaturing step at 94°C for 1 min, followed by 30 cycles of 60 s at 94°C, 1 min at 58°C and 60 s at 72°C; and a final extension step of 5 min at 72°C. PCR products (550 bp) were separated by electrophoresis in 1.5% (wt/vol) agarose gels and visualized by ethidium bromide staining.

Sequences were obtained from Macrogen Inc, Korea. Alignment and edition were carried out with the BioEdit Program v 7.0.5 [29]. The VF11 strain sequence was then compared against those available in the GenBank database. The sequence obtained showed 100% homology with DNA sequences from other *Fusarium oxysporum* strains deposited in GenBank (Banklt 1826294).

### Plant material

Leaves of *Diospyros cuneata* were collected from the coastal dunes of Yucatán, México (21°19'22" N 89°25'55" W) during the dry (i.e. 0.8 mm daily mean rainfall and 76% RH, [30]) and rainy (i.e., 3.9 mm daily mean rainfall and 83% RH; [30]) seasons (2013). The identity of the plant was confirmed with one sample that was deposited at CICY's herbarium.

### Aqueous extract preparation

Fresh leaves were collected, washed, and dried in an oven at 50°C for about 2-3 days. Ten grams of dried leaves were blended (1.5 min) in a laboratory blender (Waring, commercial, USA), and 100 mL of sterilized distilled water were added and blended again. The mixture was placed in 50 mL falcon tubes and centrifuged in a bench refrigerated centrifuge (Eppendorf, Hamburg, Germany) at 3220 g, 20°C, and for 30 min. The supernatant was recuperated and centrifuged (Eppendorf) again at 20817 g, room temperature (RT), and for 30 min. The supernatant was recuperated and successively filtered through 0.65 µm, and 0.45 µm membrane filters (Merck Millipore, Darmstadt, Germany) to obtain an aqueous solution free of particle waste. To finish the supernatant was sterilized with 0.22 µm membrane filter and the aqueous extract (AE) was then considered ready to use for bioassays or for the encapsulation assay.

### Antifungal susceptibility

In order to determine the minimum inhibitory concentration (MIC) of the aqueous extract needed to use against *Fusarium oxysporum*, the agar dilution method was used to assess *in vitro* antifungal susceptibility. Four concentrations, 0.5, 1, 2.5 and 5%, of the extract were assayed in PDA (potato dextrose agar from Difco, Leeuwarden, The Netherlands) medium poured in 3 cm diameter Petri dishes; three replicates were made per concentration. Twenty-five microliters of a spore suspension of 200 conidia/µL were spread on the differently diluted mediums. A commercial fungicide (Miconazole nitrate 20 mg/ml) was added to the positive control at a final concentration of 0.2 ng/µL. The negative control included only PDA medium. After the inoculation, Petri dishes were incubated at 26 ± 2°C and with a 12/12 h photoperiod. The MIC was determined as the lowest concentration of aqueous extract preventing growth of macroscopically visible mycelium. The dishes were evaluated 4 and 18 days after inoculation. To verify if the AE had a fungicidal or a fungistatic effect, we incubated 50 µl (200 conidia/µl) of *Fusarium oxysporum* spores with 450 µl of AE; the spores were incubated in microtubes with each of the AE concentrations (0.5, 1, 2.5 and 5%) separately. The inoculations were incubated for four days after which an aliquot of each inoculation (AE and spores) was spread on PDA medium (three replicates per concentration).

### Phytochemical analysis

In order to know the profile of secondary metabolites present in the samples, the aqueous extracts were tested according to the methods

described by Harborne [31], Trease and Evans [32]. The samples were lyophilised and 10 mg of the dry samples were used for the analysis. The compounds tested were: alkaloids, flavonoids, saponins, tannins, and terpenoids. For testing for flavonoids, 2-aminoethyl diphenylborinate and Polyethylenglicol 4000 (PEG 4000) were used as the derivatizing agents in the Thin Layer Chromatography (TLC). Ferric chloride was the agent used when testing for tannins.

### Chromatographic profile

An Agilent 1100 Series Liquid Chromatograph with a diode array detector and a 1200 automatic injector was used. The column used was a Hibar 250-4 Lichrosorb RP-18 with a length of 250 mm, an internal diameter of 4 mm, and with a 5 µm particle size bond phase. The mobile phase was on isocratic mode utilizing a solution of pH 2.6 including 86% water with trifluoroacetic acid (TFA) and 14% of acetonitrile; the flow rate was 1 mL/min. The detection was run at: 254, 350, 450 and 505 nm. The samples were dissolved in HPLC-grade methanol and were filtered with 0.45 µm pore diameter acrodiscs; the injection volume used was 20 µL.

### Preparation of microbeads

A $2^3$ factorial design with four central points was used (Table S1) to establish the best conditions of encapsulation. The tested factors and levels were Na-alginate concentration (3-5% w/v), calcium chloride concentration (1-3% w/v), and hardening time (10-30 min). The response variable was the encapsulation efficiency. The Na-alginate was dispersed in 10 mL of the *Dyospiros cuneata* aqueous extract that showed major antifungal activity (dry season) at the concentrations established in the experimental design; inulin (20% mass) was also added. Once dispersed and using a 2 mm diameter hose, the solutions were passed through a peristaltic pump at 0.17 mL/s using a blunt stainless plastic needle. As drops exited the hose, they fell a distance of 10 cm into a calcium chloride solution to form into microbeads; they were then left to harden in the cationic solution for the time determined in the experimental design. Hardened microcapsules were recovered by decanting the solution. Following recovery, they were washed with deionized water. All reagents used were of analytical grade (Sigma-Aldrich, USA).

### Determination of total phenol content

Total phenol content (TPC) was determined using the Folin-Ciocalteu reagent (Sigma-Aldrich) according to Brat et al. [33]. Gallic acid was used as the standard and the results were expressed as mg/L of gallic acid equivalents (GAE).

### Encapsulation efficiency

Encapsulation efficiency (EE) was calculated as the amount of TPC encapsulated in microbeads (mb) divided by the TPC of the solution used for the preparation of the microbeads (ms); this is shown in the equation: Encapsulation efficiency=(mb/ms) × 100. Quantification of TPC in the microbeads (mb) was performed after dissolving the microbeads in 2% (w/v) Na-citrate (Sigma-Aldrich) solution (in a weight ratio of 1:5). A vortex mixer was used to chemically dissolve the microbeads at room temperature. TPC was determined using the Folin-Ciocalteu method according to the procedure described in the previous section.

### Release studies

The release studies of polyphenols from freshly prepared hydrogel microbeads were performed at laboratory conditions [34]. About 1.0 g of microbeads was suspended in 10.0 mL of distilled water. The samples were submitted to continuous agitation on an orbital shaker operating at 100 rpm. At defined time intervals (2, 5, 10, 20, 30, 45, 60 min), an aliquot (100 µL) was taken for TPC and chromatographic profile analyses.

### Analysis of release data

To analyse the mechanism of release from the microbeads, the *in vitro* release data were fitted to various mathematical models. The models tested included zero order, first order, Higuchi, and Korsmeyer-Peppas models [35].

Zero-order Model: $F=K_0 t$, where F represents the fraction of phenol compounds released in time t, and $K_0$ is the apparent release rate constant or zero-order release constant.

First-order Model: $\ln (100-F)=-K_1 t$ where F represents the fraction of phenol compounds released in time t, and $K_1$ is the first–order release constant.

Higuchi Model: $F=KH t^{½}$ where F represents the fraction of phenol compounds released in time t, and $K_H$ is the Higuchi dissolution constant.

Korsmeyer-Peppas Model: $\ln (F/100)=K_p t^n$ where F represents the fraction of phenol compounds released in time t, $K_p$ is the rate constant and n is the release exponent. $K_p$ is a constant incorporating structural and geometric characteristics of the release device, and n is the release exponent indicative of the mechanism of release.

### Statistical data analysis

All results were analysed using descriptive statistics taking into account measures of central tendency and dispersion. Regression analysis and response surface methods were conducted for the encapsulation process. One way ANOVAs were run to evaluate total phenol content and *in vitro* antioxidant activities. The least significant difference (LSD) multiple range test was used to determine differences among treatments. All analyses were done according to Montgomery [36] and processed with the Statgraphics Plus version 5.1 software.

## Results and Discussion

*Fusarium* wilt disease is one of the major plant diseases around the world, and currently there is no naturally derived commercial compound such as an aqueous plant extract to control this pathogen. We found that only the *Diospyros cuneata* aqueous leaf extract collected during the dry season inhibited the micelial growth of asexual spores of *F. oxysporum*. We observed micelial growth inhibition using 2.5% and 5% of the extract in agar, and the MIC value was found at 2.5% (Figure 1). Moreover, the spores that were re-incubated in PDA medium for 7 days after being in contact with the AE for 4 days did not grow. This indicates that the aqueous extracts of *D. cuneata* leaves had a fungicidal effect on the *F. oxysporum* spores in this *in vitro* assay. These results support the alternative use of aqueous *D. cuneata* leaf extracts for combating *F. oxysporum*. There are some other reports about the effect of aqueous plant extracts (AE) on *F. oxysporum*. For example, 10% Neem (*Azadirachta indica*) and willow (*Salix babylonica*) aqueous extracts reduced disease incidence by 25.5% and 27.8%, respectively, after 6 weeks of infection [37]. Among others, aqueous extracts of 8% onion (*Allium cepa*) and ≥ 4% marigold (*Tagetes sp*) inhibited 73% and 79% of *F. oxisporum* growth, respectively [38]. In addition, organic extracts of *Diospyros cuneata* have been tested on *Candida albicans*, a human pathogenic fungi, and results have shown that *Diospyros cuneata* can reduce fungal growth [9]. However, no information has

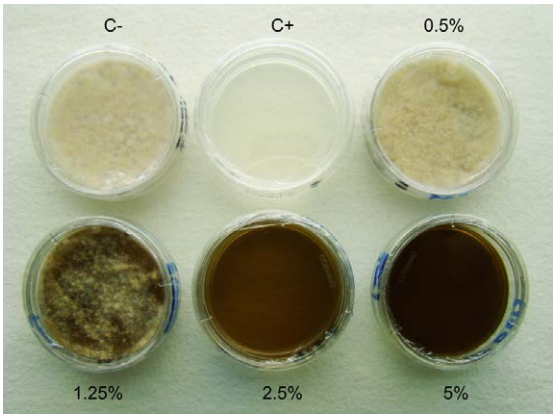


**Figure 1:** Inhibitory effect of *Diospyros cuneata* aqueous extract on the mycelia growth of *Fusarium oxysporum*. Negative control (C-) and Positive control (C+) miconazol nitrate 20 mg/ml.

yet been reported about the antifungal properties of aqueous extracts of *D. cuneata* as we have reported here.

The phytochemical analysis of the aqueous extracts of the *Diospyros cuneata* leaves collected during the dry and rainy seasons revealed that both extracts contained flavonoids, tannins, triterpenoids, and saponins. However, saponins were present in small amounts and also triterpenoids but only in the AE of rainy season (Table S2). The presence of all of these compounds with the exception of saponins and other metabolites like steroids and naphthoquinones has been reported in leaves of *Diospyros* species reviewed by Mallavadhani et al. [7]. In addition, the antifungal activity of flavonoids, triterpenoids, and saponins against crop pathogens has recently been reported [39-41]. Using TLC (data not shown), we found that the sample collected during the rainy season had a higher intensity of flavonoid compounds. This is interesting because it was expected that the sample collected during the dry season would show a higher intensity of these compounds as was demonstrated for *Labisia pumila* Benth growing with high water stress [42]. Though, in all accounts, the high antifungal activity observed for the dry season AE of *D. cuneata* could be correlated with some kind of flavonoids such as prenylated flavonoids [39].

The chromatographic profile at 254 nm showed the highest number of compounds in the AE of both seasons. For the rainy season extract, 36 compounds were detected and 31 compounds for the dry season extract. The retention time for the rainy season sample was 1.9-27.5 min, and for the dry season extract it was 1.9-22.7 min. Comparing the area (mAu*s) of each peak at 254 nm, we observed that the major compounds (>5000 mAu*s) of the dry season AE were polar (peaks 3, 13 and 15), and only one of the rainy season compounds was not polar (peak 35). Furthermore, the rainy season AE had a greater number of minor compounds (Figure 2). From this, it is noteworthy that with the humidity of the rainy season, the aqueous extract of the rainy season had more compounds overall compared to the dry season sample. However, the total area of the dry season sample (47460 mAu*s) was 1.2 fold greater than the area of the rainy season (40487 mAu*s). This indicates that some of the compounds of the dry season AE were abundant. Generally when plants are under water stress, they tend to increase their concentration of secondary metabolites [43], but seldom do they increase their total compound content as reviewed by Kleinwächter and Selmar [44].

There are some cases where water deficiency can decrease the production of secondary metabolites. For example, in *Salvia miltiorrhiza*, the content of furoquinones has been shown to be minor when plants are grown under water deficiency conditions compared to when grown with water [45]. However, in our case, the antifungal activity against *Fusarium oxysporum* observed for the dry season AE is possibly not directly correlated with the number of secondary metabolites produced during the season but is potentially correlated with the abundance of some secondary metabolites produced by water stress as has also been mentioned by Selmar and Kleinwächter [43]. Additionally, the antifungal activity could be related to metabolites produced only during dry conditions (Figure 2; peaks 15, 27, 30 and 31). As such, future studies should be focused towards isolating and studying the antifungal activity of metabolites produced during dry conditions.

The gelled microbeads were formed by the ionic interaction between the negatively charged carboxyl groups of sodium alginate and the positively charged ion $Ca^{2+}$. The addition of divalent ions such as $Ca^{2+}$ produced a partial neutralization of the carboxylate groups present on the alginate chain; this caused the formation of insoluble

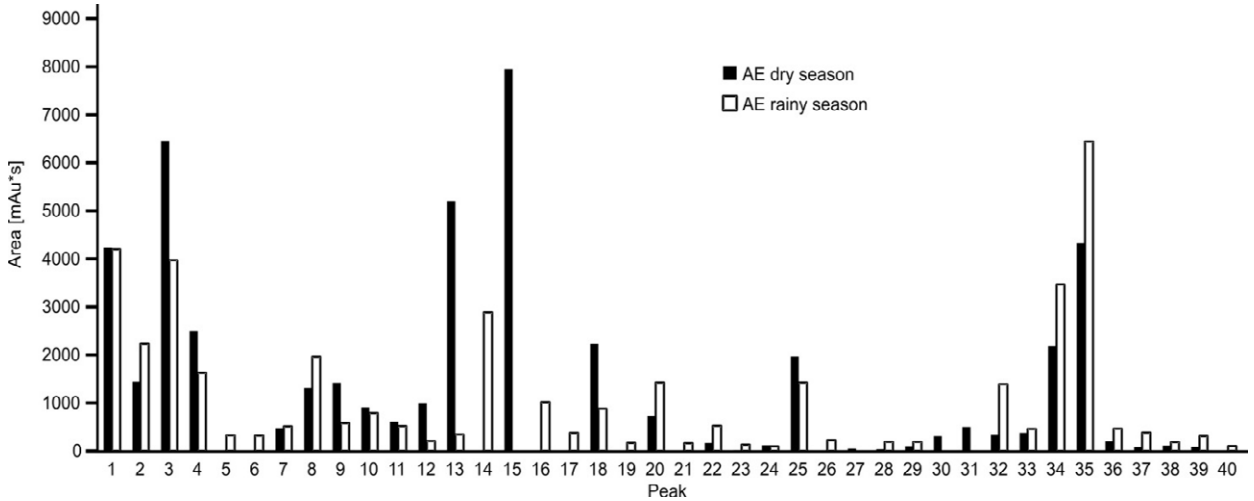


**Figure 2:** Schematic representation of the chromatographic profile at 254 nm considering the area (mAu*s) of the aqueous extract of *Diospyros cuneata* collected during the dry and rainy seasons.

gelatinous microbeads. Gelation occurred due to intermolecular cross-linking between the divalent calcium ions and the negatively charged carboxyl groups of the pectin and sodium alginate molecules. Also, inulin was investigated as a filler to improve the final properties of the alginate microbeads. Fillers are known to have the ability to induce structural changes in terms of influencing and increasing brittleness (i.e., creating a crunchier product) [46]. Specifically, alginate-inulin hydrogel has potential in food applications since inulin can be used as a functional food and as a source of dietary fibre.

The total phenol content was measured for two reasons. First, phenolic compounds are known to be present mainly in leaves. Secondly, phenolic compounds are known to have antimicrobial and antioxidant properties, often more so than has been found for other metabolites. The results of TPC and EE (%) analyses for all microbeads are given in Table 1. TPC in microbeads ranged from 9.8 to 16.9 mg GAE/g beads, and the highest amount of TPC was detected in beads obtained with 5%, w/v alginate, 3% w/v calcium chloride, and 10 min of hardening time. The results indicate that this type of microbead had the highest encapsulation capacity for this study. According to Rassis et al. [46], fillers like inulin prevent the leakage of the encapsulated compounds. In the present study, 20% (mass with respect to the content of alginate) of inulin mass was added for each treatment. An alternative to improve the EE% would be to increase the amount of inulin to reduce the loss of aqueous extract from the microbeads.

The chromatographic profiles of the release of AE compounds encapsulated using treatment 2 (T2) showed that from 2 min onwards, the compounds of high and middle polarity (849.70 mAu*s) were released in the medium, and this increased slightly (1625.97 mAu*s) until 20 min (Figure S1); the chromatographic profile of the aqueous extract before being encapsulated (control) showed compounds of high and middle polarity. These results suggest that the T2 encapsulated those compounds. On the other hand, the chromatographic profile of the AE and $CaCl_2$ showed compounds of low polarity, meaning that $CaCl_2$ encapsulated all of the compounds found in the aqueous extract. There are reports of the effective diffusion of polar and non-polar compounds through Ca-alginate capsules; examples of this include vitamin B12 or nicotinamide adenine dinucleotide [47]. Diffusion into Ca-alginate capsules has been reported to be a function of the residence time (for hardening) of capsules in the $CaCl_2$ solution. In this sense, diffusion can be optimized with long enough residence times in the solution or by using a high concentration of $CaCl_2$ [48]. In the present study the highest encapsulation efficiency was reached with a concentration of 3% (p/v) $CaCl_2$ in the hardening solution.

The release kinetics of encapsulated phenol compounds was determined by comparing their respective constants ($K$) and correlation coefficients ($r^2$) in different kinetic models. The results of the curve fitting for the above-mentioned mathematical models are given in Table 2. Phenols compounds release from the microbeads followed first-order kinetics. The plot of ln 100-F% vs. $T^{1/2}$ was found to be linear with correlation coefficients greater than that of zero order kinetics. Higuchi plots were found to be of highest linearity with correlation coefficients greater than that of zero order kinetics; the correlation coefficients corresponded to that of first order kinetics indicating that the drug release mechanism from these microbeads was diffusion controlled and follow first order kinetics (Table 2). Also, release of phenol compounds from microbeads was best explained by the Korsmeyer-Peppas equation indicating good linearity. The exponent, n, depends on the polymer swelling characteristics and the relaxation rate at the swelling front [49]. The values of release parameters, n and $K_p$ are inversely related. A higher value of $K$ may suggest burst compound release from the microbead. According to the criteria for release kinetics for swellable systems, a value of release exponent n=0.45, 0.45<n>0.89, and 0.89<n>1.0 indicates fickian (case-I) diffusion, non-fickian (anomalous) diffusion and zero order (case-II) transport, respectively [50]. The release exponent n ranged from 0.5317 to 0.9733. This indicates that the release of phenolic compounds from the capsules followed the case-II transport mechanism due to the rapid swelling and erosion of the capsules and diffusion of the aqueous extract compounds. The concentration of the cross-linking agent was found to have a significant effect on the release of the phenolic compounds from the capsules. The release was higher at low concentrations of $CaCl_2$. The results were influenced by the presence of calcium ions in the cross-linking solution. This kind of behaviour indicates that gel film formation occurs from outside the core of the capsule. All of these results can be easily explained by taking into consideration the gel formation, which is assumed to be controlled by the diffusion of the two components involved. In this regard, the fact that the metallic cation has a smaller size than the polymer molecules means that it is mainly the cation that diffuses between the polymer chains, binding to unoccupied binding sites on the polymers [51]. A lower concentration of $CaCl_2$ generates less cross-linked gels, thus

| T | TPC (mg GAE/ g bead) | EE (%) |
|---|---|---|
| 1 | $12.4^b$ | $49.9^b$ |
| 2 | $16.9^e$ | $68.4^e$ |
| 3 | $12.9^b$ | $51.8^b$ |
| 4 | $14.7^c$ | $59.4^c$ |
| 5 | $15.3^d$ | $61.6^d$ |
| 6 | $15.1^d$ | $60.6^d$ |
| 7 | $9.8^a$ | $39.6^a$ |
| 8 | $12.9^b$ | $52.1^b$ |
| CP | $14.0^c$ | $57.2^c$ |

Values in columns with the same letter are not significantly different ($P$<0.05) as determined by Fisher's least significant difference procedure (LSD).

**Table 1:** Total phenol content (TPC) and encapsulation efficiency (EE). T=Treatment; CP=Central points.

| T | Zero-order | | First-order | | Higuchi | | Korsmeyer-Peppas | | |
|---|---|---|---|---|---|---|---|---|---|
| | $K_0$ | $r^2$ | $K_1$ | $r^2$ | $K_h$ | $r^2$ | $K_p$ | $r^2$ | n |
| 1 | 0.4057 | 0.8533 | 0.0199 | 0.9197 | 3.8935 | 0.9116 | 0.1119 | 0.9356 | 0.6868 |
| 2 | 0.3379 | 0.7144 | 0.0205 | 0.7843 | 3.4550 | 0.8664 | 0.0967 | 0.9672 | 0.5623 |
| 3 | 0.2667 | 0.7584 | 0.0105 | 0.8078 | 2.5785 | 0.8223 | 0.0940 | 0.8668 | 0.8519 |
| 4 | 0.3660 | 0.7892 | 0.0181 | 0.8772 | 3.6504 | 0.9105 | 0.1089 | 0.9755 | 0.6745 |
| 5 | 0.1784 | 0.8607 | 0.0120 | 0.8919 | 1.7391 | 0.9491 | 0.0498 | 0.9728 | 0.5317 |
| 6 | 0.1474 | 0.8258 | 0.0063 | 0.8428 | 1.4591 | 0.9390 | 0.0631 | 0.9923 | 0.9580 |
| 7 | 0.4918 | 0.8868 | 0.0169 | 0.9488 | 4.7587 | 0.9630 | 0.1604 | 0.9819 | 0.9714 |
| 8 | 0.4817 | 0.9143 | 0.0159 | 0.9376 | 4.5871 | 0.9617 | 0.1499 | 0.9396 | 0.9733 |
| CP | 0.3314 | 0.8291 | 0.0118 | 0.8669 | 3.2417 | 0.9201 | 0.1164 | 0.9575 | 0.9198 |

**Table 2:** Constant (K) and correlation coefficient ($r^2$) of different kinetic models for aqueous extract in microbeads. T=Treatment; CP=Central points.

resulting capsules lose their integrity faster. It is possible that a higher $CaCl_2$ concentration increased the cross-linking between the alginate and calcium ions at the droplet surface, resulting in the formation of tight Ca-alginate hydrogel wall barriers with a high internal content of *D. cuneata* aqueous extract.

## Conclusions

Crude aqueous extracts of leaves from *Diospyros cuneata* collected during the dry season, showed in *in vitro* antifungal activity against mycelial growth of asexual spores of *F. oxysporum* in *in vitro* conditions. Phytochemical analysis of the AE collected during the dry and rainy seasons revealed that both contained flavonoids, tannins, triterpenoids, and saponins. However, the chromatographic profile showed a larger abundance of compounds in the dry season sample. Further studies should be undertaken to isolate and to test the antifungal activity of major compounds observed in dry season aqueous extracts. Finally, alginate-inulin was efficient for encapsulating the compounds present in the aqueous extract of leaves collected during the dry season. Considering the rapid availability tested by release studies, we suggest that encapsulation is a good alternative for protecting and dosing aqueous extract antifungal compounds *in situ*.

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