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Cinnamon plant extracts: a comprehensive physico-chemical and biological study for its potential use as a biopesticide

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Abstract

Cinnamon is a widely used herbal remedy and has many applications in perfumery, flavoring and pharmaceutical industries. The aim of the study is to examine the biological activity of different cinnamon extracts and major active constituent against a series of fungal strains and weeds. The extracts were obtained from two types of cinnamon quillings and essential leaf oil supplied from the local markets. Four different extracts were obtained: i) oleoresins, ii) essential oils, iii) water and iv) ethanol. Chemical composition, total phenolics and antioxidant activities of extracts were examined.

The extracts were tested by *in vitro* bioassays. Antifungal activity was evaluated against *Botrytis cinerea* and *Phytophtora capsici* and phytotoxic on seeds of *Lolium perenne*, *Licopersicum esculentum* and *Lepidium sativum*. Extracts exhibited significant inhibitory effect on mycelial growth, conidia and zoospore germination and root elongation for plant seeds. Both cinnamaldehyde and eugenol significantly contribute to biological activities, beside several other components.

Keywords: cinnamon extracts, eugenol, cinnamaldehyde, phenolic compound, biological activities.

Extraits de plante de cannelle: a compréhension physico-chimique et l'étude biologique pour son potentiel usage comme biopesticide

Résumé

La cannelle est un remède à base de plantes largement utilisé et à de nombreuses applications en parfumerie, assaisonnement et les industries pharmaceutiques. L'objectif de l'étude est d'examiner l'activité biologique de différents extraits de cannelle et de principal constituant actif contre une série de souches de champignons et de mauvaises herbes. Les extraits ont été obtenus à partir de deux types de quillings de cannelle et de l'huile essentielle des feuilles fournie a partir des marchés locaux. Quatre différents extraits ont été obtenus : i) oléorésine, ii) les huiles essentielles, iii) l'eau et iv) l'éthanol. Composition chimique, composés phénoliques totaux et les activités antioxydantes des extraits ont été examinés. Les extraits ont été testés par des essais biologiques *in vitro*. L'activité antifongique a été évaluée contre *Botrytis cinerea* et *Phytophtora capsici et phytotoxique sur les semences de Lolium perenne*, *Licopersicum esculentum* et *Lepidium sativum. Les extraits ont montré un effet inhibiteur significatif sur la croissance mycélienne, la germination des conidies et zoospore et sur l'allongement des racines pour les semences des plantes. Les deux, cinnamaldehyde et eugenol contribuent de manière significative à l'activité biologique, a coté de plusieurs autres composants.*

Mots-clés**:** *extraits de cannelle, eugenol, cinnamaldehyde, composés phénoliques, activités biologiques.*

Dedication

I praise Almighty Allah for giving me strength, passions, courage and guidance agency to achieve this work, despite all difficulties

I would like to express my gratitude to thanks my family for their continuous guidance, advice, support, inspiration and love

In memory of my brothers Djamel and Abdelaaziz

To my dearest

My parents;

My brother;

Nieces and nephew;

And for all

My friends whenever and wherever they are

To Algeria *Hakima*

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Abbreviation	
B1	Bark 1
B2	Bark 2
CE	Cinnamon Extract
CAD	Cinnamaldehyde
DPPH	Diphenylpicryl- hydrazyl
EOL	Essential Oil Leaf
EE	Ethanol Extract
EUG	Eugenol
ED_{50}	Effective Dose 50
EO	Essential Oil
EOs	Essential Oils
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
GRAS	Generally Recognized as Safe
GC	Gas Chromatography
GI	Germination Index
GC-MS	Gas chromatography-Mass Spectrometry
GAE	Gallic Acid Equivalents
RC_{50}	Half inhibition Concentration
HPLC	High Performance Liquid Chromatography
LOD	Limit of Detection
LC/ESI-MS/MS	Liquid Chromatography/Electron Spray Ionization coupled Mass Spectrometry
MICs	Minimum Inhibitory Concentrations
OECD	Organisation for Economic Co-operation and Development
OR	Oleoresin
PAD	Photodiode Array Detector
PDA	Potato Dextrose Agar
RSG	Relative Seed Germination
RRG	Relative Root Growth
TEAC	Trolox Equivalent Antioxidant Capacity
UV	Ultrat Violet
WE	Water Extract
WHO	World Health Organization

Introduction

Plants contain a multitude of chemical substances and the same is true for plant extracts (e.g. water and ethanol extracts), one or several substances or a cluster of very similar substances may be active against the target pest, the extract is therefore to be considered as the active ingredient and also as the plant protection product. Indeed, some natural substances of plant origin have good antimicrobial properties and have been used as seasonings for centuries (Sherman and Billing, 1999). Natural antimicrobials have been identified in herbs and spices and several studies have reported on the preservative action of spices or their essential oils.

Organic pesticides or biopesticides represent an important option for the management of plant diseases and are usually considered those pesticides that come from natural sources: plant, animal, microbial or mineral origin (EEC, 2007). In the USA, there is a list of substances that can be used as pesticides without any registration. These substances are called Minimal Risk Pesticides; the list is known as «25b list» and includes cinnamon and cinnamon oil (EPA, 2000).

In the Lauraceae family, the *Cinnamomum* genus (cinnamon) is a very popular spice throughout the world. The species *Cinnamomum zeylanicum* originates from Ceylon, being also native to South-East India, are a source of cinnamon bark and leaf and their essential oils. Its sensorial qualities are flavour, slightly sweet, pleasant, warm and bitter, besides being strongly aromatic (WHO, 1999). This cinnamon species are one of the world's finest spices, mainly exported as ''cinnamon quills''.

Cinnamon are recognized for their flavor and aroma in addition to their antimicrobial medicinal applications and are generally recognized as safe (GRAS) natural products by the U.S. Food and Drug Administration (FDA) and it is generally accepted that their volatile compounds are the main reason for their antimicrobial properties (Ayala-Zavala *et al*., 2008; Tzortzakis ,2008).

Cinnamon provides various kinds of oils, it has been established that the oils and extracts from cinnamon possess a distinct antioxidant activity, which is especially attributed to the presence of phenolic and polyphenolic substances (Schmidt E, 2006; Muchuweti *et al*., 2007). Recently, natural antioxidants are in high demand because of their potential in health promotion and disease prevention, and their improved safety and consumer acceptability.

The main constituent of cinnamon bark oil is cinnamaldehyde, whereas eugenol is the main constituent of cinnamon leaf oil. Several authors have reported various important biological effects associated with cinnamon. Extracts and essential oils or some of their constituents are indeed effective

against large variety of organisms including bacteria, fungi, mites and nematodes.

Essential oils also contain allelochemicals that inhibit seed germination. The inhibitory activity against seed varied with the species from which the essential oil was extracted (Dubai *et al*., 1999). Cinnamon sources of essential oils and aromatic compounds may be used as a viable weed control technology in organic farming systems but basic information on phytotoxicity is required before performing field experiments.

However, the action mechanism related with cinnamon is not fully understood. The use of different assays could allow for evaluating and obtaining more information about the possible action mechanism of this herbal medicine and spice which would be very important in the nutrition field.

Considering the requirements of effectiveness and convenience of the application of natural antimicrobial products, there has been a constant increase in the search of alternative and efficient compounds for food preservation aimed at partial or total replacement of antimicrobial chemical additives. In order to explore the potential usefulness of cinnamon extracts, it is important to know their chemical constituents and understand the effect of chemical structure on phytotoxic and antifungal activity.

Therefore, the aims of the present work, was to test the activities of different types of cinnamon extracts obtained from different extraction methods and to identify and quantify major bioactive components contributing to the biological activities.

To achieve these objectives further studies were performed:

- Application of different extraction methods on two types of cinnamon quillings.

- Determination of antioxidant activity and total phenolic compounds of cinnamon extracts.

- Identification and quantification of main compounds by means of HPLC/PAD/ESI-MS/MS and GC/MS analyses.

- Assessment of biological activities of extracts by *in vitro* studies:

    1. Phytotoxicity assay on *Lolium perenne, Licopersicum esculentum* and *Lepidium sativum.*

    2. Antifungal activity against *Botrytis cinerea* and *Phytophtora capsici*.

Chapter 1

Literature Review

1.1. Botanical pesticides

Plants, herbs, and spices as well as their derived essential oils and isolated compounds contain a large number of substances that are known to inhibit various metabolic activities of bacteria, yeast, and molds, although many of them are yet incompletely exploited. The antimicrobial compounds in plant materials are commonly contained in the essential oil fraction of leaves (rosemary, sage), flower buds (clove), fruit (pepper, cardamom), bark (cinnamon), or other parts of plant (Malo-Vigil *et al*., 2005).

Biopesticides is a term that encompasses many aspects of pest control such as microbial (viral, bacterial and fungal) organisms, entomophagous nematodes, plant-derived pesticides (botanicals), secondary metabolites from micro-organisms (antibiotics), insect pheromones applied for mating disruption, monitoring or lure and kill strategies and genes used to transform crops to express resistance to insect, fungal and viral attacks or to render them tolerant of herbicide application (Copping and Menn, 2000).

The botanical materials include crude extracts and isolated or purified compounds from various plants species and commercial products (Liu *et al*., 2006). Not unlike pyrethrum, rotenone and neem, plant essential oils or the plants from which they are obtained have been used for centuries to protect stored commodities or to repel pests from human habitations and use as fragrances, flavourings, condiments or spices, as well as medicinal uses (Isman and Machial, 2006). Pesticides based on plant essential oils could be used in a variety of ways to control a large number of pests, due to the rapid volatilization of these products; there is a much lower level of risk to the environment than with current synthetic pesticides (Isman and Machial, 2006).

Quantitatively, the most important botanical is pyrethrum, followed by neem, rotenone and essential oils, typical uses are as insecticides (e.g. pyrethrum, rotenone, rape seed oil, quassia extract, neem oil, nicotine), repellents (e.g. citronella), fungicides (e.g. laminarine, fennel oil, lecithine), herbicides (e.g. pine oil), sprouting inhibitors (e.g. caravay seed oil) and adjuvant such as stickers and spreaders (e.g. pine oil) (Isman, 2006).

1.2. Plant secondary metabolites

1.2.1. Introduction

Plants are capable of synthesizing an overwhelming variety of small organic molecules called secondary metabolites, usually with very complex and unique carbon skeleton structures (Sarker *et al*., 2005). By definition, secondary metabolites are not essential for the growth and development of a plant but rather are required for the interaction of plants with their environment (Kutchan and Dixon, 2005).The biosynthesis of several secondary metabolites is constitutive, whereas in many plants it can be induced and enhanced by biological stress conditions, such as wounding or infection (Wink, 2006). They represent a large reservoir of chemical structures with biological activity. This diversity is largely the result of co-evolution of hundreds of thousands of plant species with each other and with an even greater number of species of microorganisms and animals. Thus, unlike compounds synthesized in the laboratory, secondary compounds from plants are virtually guaranteed to have biological activity and that activity is highly likely to function in protecting the producing plant from a pathogen, herbivore, or competitor. It has been estimated that 14 - 28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up on ethnomedicinal use of the plants (Ncube *et al*., 2008).

1.2.2. Function of secondary metabolites

In order to be effective, secondary metabolites need to be present at the right site, time and concentration in plant (Wink, 2006). Many secondary compounds have signalling functions influence the activities of other cells, control their metabolic activities and co-ordinate the development of the whole plant. Other substances such as flower colours serve to communicate with pollinators or protect the plants from feeding by animals or infections by producing specific phytoalexines after fungi infections that inhibit the spreading of the fungi mycelia within the plant (Mansfield, 2000). Plants use secondary metabolites (such as volatile essential oils and colored flavonoids or tetraterpenes) also to attract insects for pollination or other animals for seed dispersion, in this case secondary metabolites serve as signal compounds (Figure 1).

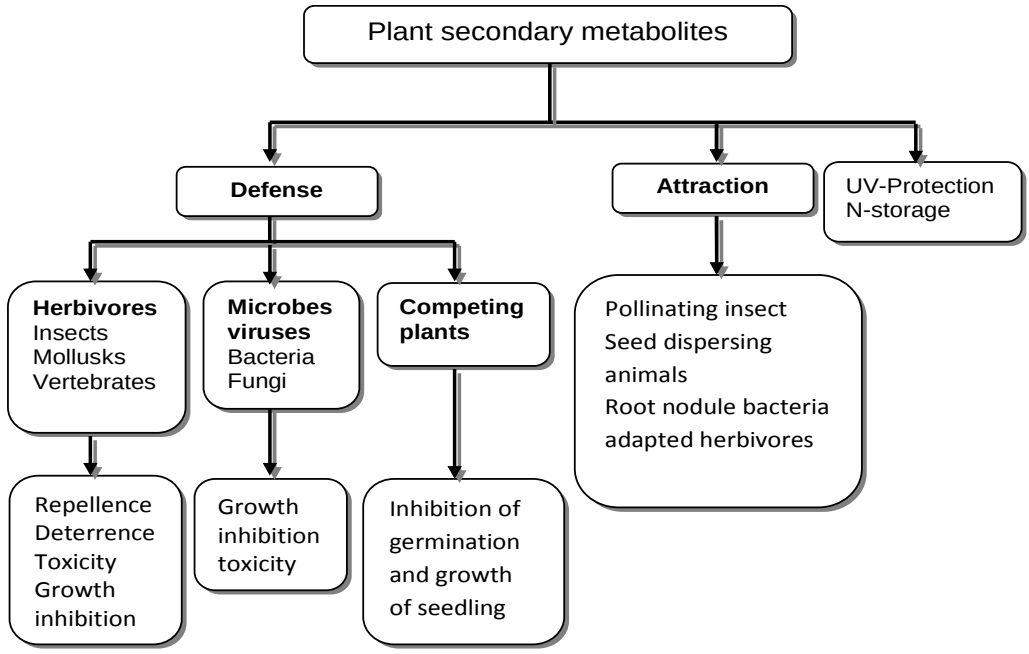

Figure 1. Function of secondary metabolites in plants (Wink, 2006)

Plant primary products refer to the chemical groups of carbohydrates, proteins, nucleic acids, fats and lipids. Their functions are related to structure, physiology and genetics, which imply their crucial role in plant development. In contrast, secondary metabolites normally occur as minor compounds in low concentrations. Although many of these metabolites show structural similarities to primary products, one can divide secondary metabolites into the main chemical groups: terpenoids, alkaloids, phenolic, rare amino acids, plant amines and glycosides (Rohloff, 2003).

A wide range of secondary compounds have been implicated as anti-nutritional components of food and animal feed with several types of phenolic compounds directly affecting the digestibility of plant tissues. These include phenolic components of the cell wall, lignification of cells and the presence of polyphenols such as condensed tannins. Plant terpenoids have dominated the subject of chemical ecology since they have been studied for their activities against a variety of insect models (Langenheim, 1994; Gutierrez *et al*., 1997). Presence of volatile monoterpenes or essential oils in the plants provides an important defense strategy to the plants, particularly against herbivorous insect pests and pathogenic fungi (Langenheim, 1994). These volatile terpenoids also play a vital role in plant–plant interactions and serve as attractants for pollinators (Tholl, 2006). They act as signaling molecules and depict evolutionary relationship with their functional roles (Theis and Lerdau, 2003). Soluble secondary compounds such as cyanogenic glycosides isoflavonoids and alkaloids can also be toxic to animals (Morris and Robbins, 1997) (Figure 2).

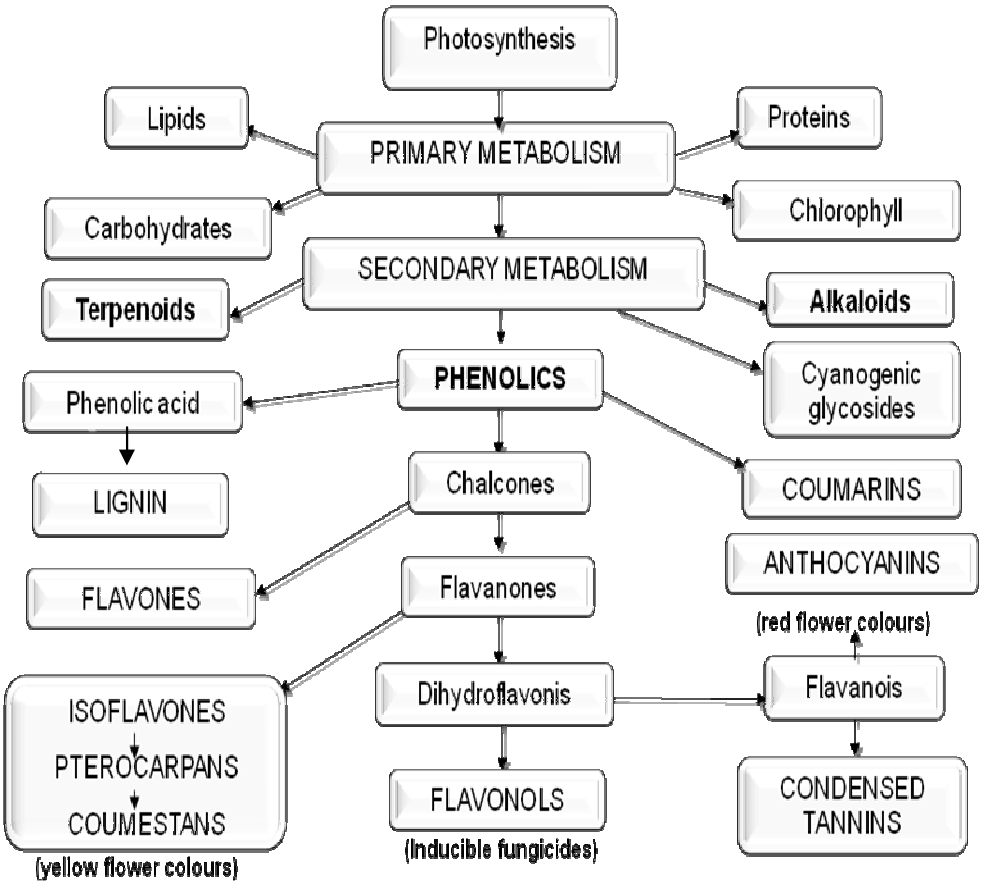

Figure 2: Interrelations between primary and secondary metabolism in plant (Morris and Robbins, 1997).

1.2.3. Shikimate pathway

Cinnamaldehyde and eugenol are the mains constituents of cinnamon bark and leaf oil, respectively, and are components of the shikimik acid pathway leading to the lignin formation. Cinnamic aldehyde is directly formed by the reduction of cinnamic acid, the latter being formed from phenylalanine (PA) via shikimik acid pathway (Figure 3). It was shown that cinnamic aldehyde gets further reduced to cinnamyl alcohol, which is precursor of lignin. Eugenol, due to the absence of a hydroxyl group in the allyl side-chain, cannot readily contribute to the lignin formation.

Since lignification occurs in the xylem tissues, it has to be established whether synthesis of cinnamic aldehyde and eugenol occurs in the xylem tissues or whether they are with regard to the sequence (Sennayake and Wijesekera, 2003): Cinnamic acid ⟶ Cinnamyl alcohol ⟶ allyl derivative. The favoured pathway for eugenol biosynthesis is accepted to be:
L-phenylalanine ⟶ Cinnamic acid ⟶ p-coumaric acid ⟶ caffeic acid.

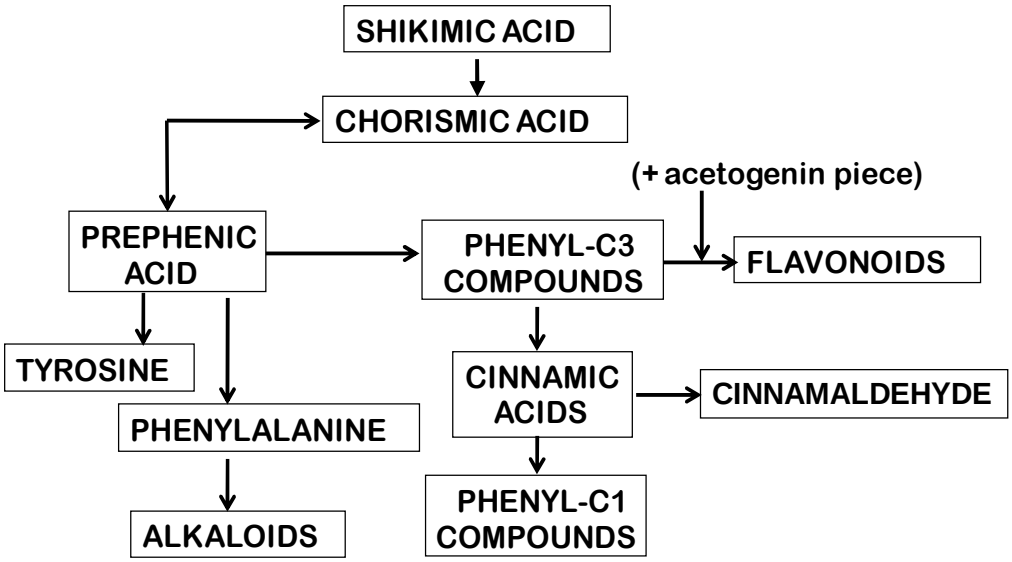

Figure 3. Cinnamaldehyde biosynthesis (Sennayake and Wijesekera, 2003)

1.3. Cinnamon species

1.3.1. Historical overview, geographical sources and major economic species of Cinnamomum

The name cinnamon refers to the tropical evergreen tree as well as the bark that is extracted from the plant. Cinnamon is known as *cannelle* in French; *ceylonzeimt/kaneel* in German; *cannella* in Italian; *canela* in Spanish, *yook gway* in Chinese, *dal-chini* in Hindi and *kurunda* in Sinhalese (Peter, 2001).

Cinnamon is classified in the botanical division Magnoliophyta, class Magnoliopsida, order Magnoliales and family Lauraceae. The tree grows to a height of 7 to 10 m in its wild state and has deeply veined ovate leaves that are dark green on top and lighter green underneath, both bark and leaves are aromatic, it has small yellowish-white flowers with a disagreeable odour and bears dark purple berries (Figure 4).

Figure 4. Cinnamon plant (Lee and Balick, 2005)

The genus *Cinnamomum* has 250 species and many of them are aromatic and flavouring (Lee and Balick, 2005).In many instances, very little distinction is made between the bark of *Cinnamomum verum* (syn. *Cinnamomum zeylanicum*, true cinnamon) and *Cinnamomum cassia* (Chinese cinnamon). *Cinnamomum verum* provides cinnamon bark of the finest quality and oil of cinnamon whereas *Cinnamomum cassia* provides cassia bark and oil of cassia (also known as oil of cinnamon).Cassia was used in China long before the introduction of true cinnamon but is now considered an inferior substitute. There are still other species of *Cinnamomum* which are often traded as cinnamon or cassia (Peter, 2001) (Table 1).

Cinnamon spice is obtained by drying the central part of the bark and is marketed as quills or powder. The production of cinnamon is mostly limited to the wettest lowland areas of Southeast Asia, cultivated up to an altitude of 500 meters above mean sea level where the mean temperature is 27ºC and annual rainfall is 2000–2400 mm, it prefers sandy soil enriched with organic matter (Peter, 2001).

Table 1. Major economic species of *Cinnamomum sp.*

Botanical name	Common name	Origin	Part used	Major use
Cinnamomum verum Presl. Syn *Cinnamomum zeylanicum* Blume	True cinnamon/Ceylon cinnamon	Sri Lanka, Malabar Coast, Seychelles	bark, leaves	Flavouring, perfumery, medicinal
Cinnamomum cassia Presl.	Cassia, Chinese cinnamon	Southeast China	bark, leaves, buds	Flavouring, medicinal, Chewing pan
Cinnamomum camphora	Camphor	Southern China/ Indonesia	Wood/ leaves	Medicinal/perfumery
Cinnamomum loureirii Nees	Saigon cinnamon, Vietnam cassia	Vietnam	bark, bark oil	Flavouring
Cinnamomum burmanii Blume	Cassia vera, Korinjii cassia	Indonesia	bark (Massoi bark)	Spice and oleoresin in flavouring
Cinnamomum tamala	Indian cassia I	India	bark, leaves	Medicinal, leaves as bay leaves for flavouring
Cinnamomum ineris	Wild cinnamon of Japan	Japan, Southern India	bark	Mosquito repellent
Cinnamomum sintok	Java cassia	Java and Sumatra	bark	Flavouring
Cinnamomum obtusifolium		Northeast India, Myanmar	bark	Substitute for true cinnamon
Cinnamomum culilawan Cinnamomum rubrum		Moluccas and Amboyana	bark, bud	Flavouring, substitute for clove bud
Cinnamomum olivera	Australian cinnamon	Australia	bark	Flavouring
Cinnamomum glaucascens	Sugandha kokila	Nepal	bark/leaves	Perfumery

1.3.2. Cultivation, collection, preparation and quality issues

Sri Lanka contributes 80 to 90% of the world trade of cinnamon production (Sial, 1995). As in the case of other cassias, the true cinnamon is obtained from the bark of small stems of cultivated cinnamon trees which have been cut down after an initial establishment period and the bushy regrowth stems are harvested at regular intervals thereafter. A first harvest may be obtained after 3-4 years although both quality and yields improve with subsequent cuttings.

The shoots are ringed at the nodes, about 30 cm apart, with a sharp copper or brass knife, longitudinal incision are made to connect the rings and the bark is removed in strips (Figure 5). Brass or copper knives are used to avoid the discoloration that steel would cause by the reaction with tannin of the bark, the pieces of bark are made into bundles, which are wrapped in matting and allowed to remain for about 24 hrs, when a slight fermentation occurs, which loosen the outer layers. Each strip is stretched on a wood stick and the epidermis, cork and green cortex are removed by scraping with curved knife. After slightly drying for 24hrs the pieces are sorted and packed one inside the other. These compound quills are first dried in the shade of a shed for a day and then for a second day in the sun (Lee and Balick, 2005).

Figure 5. Cultivation, collection and preparation (A) leaves, (B) flowers, (C) bark, (D) bark with different colours of *Cinnamomum zeylanicum* Blume (Lee and Balick, 2005)

The sweet taste of cinnamon is due to the presence of cinnamaldehyde. It is reported that, when combined with sweet food, the sweet sensation of the food is enhanced because of the synergetic effect between the sweet taste of sugar and sweet aroma of cinnamon (Leela, 2008). Sweetish bark with pungent taste and low mucilage (about 3%) is preferred by the food industry. The deodouring/masking property of cinnamon bark is due to the presence of trimethyl amine.

The quality of cinnamon is assessed primarily on the basis of its appearance and on the content and aroma/ flavour characteristics of the volatile oil. Good quality cinnamon should be light brown with wavy lines and produce a sound of fracture when broken. When chewed it should become soft, melt in the mouth and sweeten the breath. Freshly ground cinnamon bark of good quality contains 0.9 to 2.3% essential oil depending on the variety (Thomas and Duethi, 2001). The quality of bark is greatly influenced by the soil and ecological factors. The bark obtained from the central branches is superior to that from the outer shoots and that from either the base or the top (Benini, 2007).

1.3.3. Cinnamon oil

1.3.3.1. Origin, uses and factors affecting quality

Essential oils are a rich source of biologically active compounds. Cinnamon produces two different oils, cinnamon bark oil obtained from the dried inner bark, whereas cinnamon leaf oil is obtained from the leaves and twigs. It is important to distinguish between the two variations of cinnamon oil, cinnamon bark oil has a spicy smell whereas cinnamon leaf oil is said to smell like cloves; leaf oil is considered to be considerably safer to use in aromatherapy (Jayaprakasha *et al*., 2000). The bark oil, bark oleoresin and leaf oil are important value added products from cinnamon. Bark oil is used in the food and pharmaceutical industries while, oleoresin is used mainly for flavouring food product such as cakes and confectionary.

The most important cinnamon oils in world trade are those from *Cinnamomum* zeylanicum, *Cinnamomum* cassia and *Cinnamomum* camphora (Guddadarangavvanahally *et al*., 2002). The other species provide oils, which are utilized as sources for chemical isolates.

In order to obtain essential oils of constant composition, they have to be extracted under the same conditions from the same organ of the plant which has been growing on the same soil, under the same climate and has been picked in the same season (Rohloff, 2004). Most of the commercialized essential oils are chemotyped by gas chromatography and mass spectrometry analysis. Temperature, humidity, duration of daylight (radiation), and wind patterns all have a direct influence on volatile oil content, especially in those herbs that have superficial histological storage structures (e.g. glandular trichomes). When the localization is deeper, the oil quality is more constant .Genetic, physiological and environmental factors as well as processing conditions may play an important role (Lawrence, 2002; Rohloff, 2004).

1.3.3.2. Chemical composition

From different parts of the plant of *Cinnamomum zeylanicum* Blume syn. C. *verum* J. Presl, Laurus *Cinnamomum* L. (bark, roots and leaves) essential oils with variations in the composition by especially geographic and technical reasons can be obtained (Schmidt, 2006). Major compounds present in stem-bark oil and rootbark oil are cinnamaldehyde (75%) and camphor (56%), respectively (Senanayake *et al.*, 1977).

Singh and coauthors (2007) identified by GC and GC–MS analysis of cinnamon leaf volatile oil the presence of 19 components accounting for 99.4% of the total amount. The major component was eugenol (87.3%) followed by bicyclogermacrene (3.6%), a-phellanderene (1.9%), b-carryophyllene (1.9%), aromadendrene (1.1%), p-cymene (0.7%) and 1,8-cineole (0.7%).The analysis of cinnamon bark volatile oil showed the presence of 13 components accounting for 100% of the total amount . (E)-cinnamaldehyde was found as the major component along with d-cadinene (0.9%), α-copaene (0.8%) and α-amorphene (0.5%).

Twenty-six compounds constitutes 97% of the volatile oil from cinnamon flowers were characterized with *(E)*-cinnamyl acetate (42%), *trans*-α-bergamotene (8%) and caryophyllene oxide (7%) as the major compounds (Jayaprakasha *et al.*, 2000). Thirty-four compounds representing 98% of the volatile oil from the buds of *Cinnamomum zeylanicum* were characterized using GC and GC-MS. It consists of terpene hydrocarbons (78%) and oxygenated terpenoids (9%). α-Bergamotene (27.38%) and α-copaene (23.05%) are found to be the major compounds (Guddadarangavvanahally *et al*., 2002). The important chemical constituents of *Cinnamomum zeylanicum* oil are cinnamaldehyde and eugenol (Senanayake *et al*., 1977).

1.3.4. Biological activities

1.3.4.1. Antioxidant activity

For many centuries, cinnamon and its essential oil have been used as preservatives in food, due to the antioxidant property of cinnamon. Deterioration of food is due to lipid peroxidation. *In vivo* lipid peroxidation can cause tissue damage, which can lead to inflammatory diseases. Phenolic compounds, such as hydroxyl cinnamaldehyde and hydroxycinnamic acid present in the cinnamon extract, act as scavengers of peroxide radicals and prevent oxidative damage (Mathew and Abraham, 2006; Leela, 2008).

The presence of oligomeric proanthocyanidins (OPC), a class of bioflavonoid, opened a new area of research on its antioxidative effect. Through agriculture research, type A and type B oligomeric proanthocyanidins were identified in cinnamon spice via mass spectrometer analysis. Antioxidants are essential to the human body to neutralize free-reactive oxygen species, also known as free radicals to maintain functional cellular membrane and structure (Maxwell and Tran, 2007).

Polyphenolic compounds in plant, including catechins exert anticarcinogenic, antimutagenic and cardioprotective effects linked to their free radical scavenging (Parr and Bolwell, 2000). Cinnamon is a good source of antioxidant and antimutagenic phenolics, reported by Jayaprakasha *et al*., (2007), that the total phenolics content of the extracts were found to be the highest in water extract from dried fruit of cinnamon and showed strong antimutagenicity. In addition, Su *et al*., (2007) indicated that cinnamon may serve as potential dietary sources of natural antioxidants for improving human nutrition and health.

1.3.4.2. Anti-inflammatory activity

Cinnamon is reported to possess anti-inflammatory activity (Lee *et al*., 2007). The ethanolic extract (70%) of cinnamon was effective on ocute inflammation in mice. An herbal ophthalmic preparation, called ophthacare containing 0.5% cinnamon was found to be effective as anti-inflammatory agent on ocular inflammation in rabbits (Leela, 2008).

1.3.4.3. Antidiabetic activity

Cinnamon is reported to reduce the blood glucose level in non-insulin-dependent diabetics. Therapeutic studies have proved the potential of cinnamaldehyde as antidiabetic agent. Anderson *et al*., (2004), reported that water soluble polymeric compounds isolated from cinnamon have *in vitro* insulin enhancing biological activity in the *in vitro* assay measuring the insulin dependent effects on glucose metabolism and also function as antioxidants, these results suggest that compounds present in cinnamon may have beneficial in the treatment of diabetes.

1.3.4.4. Antibacterial activity

Essential oils of cinnamon were found to possess antimicrobial properties *in vitro* and shown to inhibit the growth of *Bacillus cereus* (Valero and Salmeron, 2003). Alcoholic extracts of cinnamon were found most effective against *Helicobacter pylori* in reducing its growth (Tabak *et al*., 1996). In addition, Azumi *et al*., (1997), showed that 67% ethanol/water extract of cinnamon bark inhibited the activity of bacterial endotoxin. This was the first report, which states that an inhibitor of bacterial endotoxin exists in a plant.

It was found that a combination of cinnamon and nisin accelerated the death of *Salmonella Typhimurium* and *Escherichia coli* O157:H7 in apple juice, and hence enhanced the safety of the product (Yuste and Fung, 2004). A study by Mau and co authors., (2001) on the antibacterial activity of extracts of chive (*Allium tuberosum*), cinnamon and *corni fructus* (*Cornus officinalis*) against common seven foodborne microorganisms, alone and in combination, showed that the mixed extract, consisting of three extracts in equal volumes possessed an antimicrobial spectrum and had excellent stability to heat, pH, and storage on growth of *Escherichia coli* at 2-5 mg/ml.

The mixed extract also inhibited the growth of *Pichia membranaefaciens* at 2 mg/ml. When the mixed extract was used in foods, an expected antimicrobial effect in orange juice, pork, and milk was observed.

Shan *et al*.,(2007) demonstrated that the proanthocyanidins-(epi)catechins from cinnamon bark exhibited strong antibacterial properties and that procyanidin B2 had similar antibacterial properties to the proanthocyanidins-(epi)catechins, but (+)-catechin did not have any antibacterial properties against any of the tested bacteria. This suggested that the antibacterial properties of the proanthocyanidin-(epi) catechin fractions were fully from contribution of the proanthocyanidin components. This indirectly indicates that the proanthocyanidins were also important bioactive components contributing to its antibacterial properties.

1.3.4.5. Antifungal activity

The antifungal properties of cinnamon have also drawn great attention from many researchers. The effect of cinnamon extract on mycelial growth inhibition of *phytophtora capsici* was observed by Nguyen *et al*., (2009). They evaluated the effects of medicinal plant extracts on the development of mycelium of *Phytophthora capsici*, *Rhizoctonia solani, Fusarium solani, Colletotrichum gloeosprorioides, and Botrytis cinerea*. Cinnamon extract showed inhibitory activity against mycelial growth of *phytophtora capsici* and the highest fungicidal activity against *Rhizoctonia solani.* Similar effect was found by Tzortzakis (2008), against *Botrytis cinerea* affected by essential oil. In addition, Amiri *et al*., (2008), found that mixture of eugenol and soy lecithin reduced the disease incidence caused by *Botrytis cinerea.*

Singh *et al*., (1995), have showed that *Cinnamomum zeylanicum* bark oil has fungitoxic properties against fungi involved in respiratory tract mycoses, such as *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus nidulans*, and *Aspergillus flavus*). Cinnamaldehyde and eugenol have also been demonstrated to have inhibitory properties against *Aspergillus flavus, Aspergillus ochraceus, Aspergillus niger, Aspergillus terreus, Aspergillus citrinum, Penicillium viridicatum* (Singh *et al*., 2007). In addition, *Cinnamomum zeylanicum* oil has been tested for maize kernel protection against *Aspergillus flavus* (Montes-Belmont and Carvajal, 1998). The results have revealed that it can effectively inhibit the growth of *Aspergillus flavus* and has no phytotoxic effect on germination and corn growth.

1.3.4.6. Insecticidal activity

Cinnamaldehyde obtained from an extract of *Cinnamomum cassia*, is a potent insecticide against adults of *Sitophilus orycae* and *Callosobruches chinensis* (Kim *et al*., 2003a). Repellent and insecticidal activities of essential oils extracted from leaves of *Artemisia princeps* and seeds of *Cinnamomum camphora* (L.) Presl against storage pests *Sitophillus oryzae* L. and *Bruchus rugimanus* Bohem were investigated. Results showed that the two individual oils displayed good, but their mixture exhibited much better repellent activities (Liu *et al*., 2005).

1.3.4.7. Nematicidal activity

Cinnamon oil possessed strong nematicidal activity against the male, female and juveniles of pinewood nematode *Bursaphelenchus xylophilus* (Park *et al*., 2005). Cinnamyl acetate, the active ingredient in the oil, at a concentration of 32.81µg/l resulted in 50% mortality of nematodes. Kim *et al*., (2003b), reported that at the rate of 0.2 %(weight by volume of soil) of stem bark of *Cinnamomum cassia* powder used for soil amendment significantly reduced by 91.1% gall number of *Meloidogyne incognita* infection (root gall formation) of tomato seedling compared with control.

Chapter 2

Materials and method .2

2.1. Cinnamon quillings

Barks or quillings of *Cinnamomum zeylanicum* were purchased from the local market (*Prada, Erbe e Specie*, Milano, Italy). Two kind of cinnamon quillings were utilized: 50 mm long (use in cosmetic and perfumery industries) **B1**; and 250 mm long (use in food industry) **B2** (Figure 6). Before applying extraction procedure quillings were grounded in order to obtain a fine powder.

Figure 6 : Cinnamon bark (1) B1 and (2) B2

2.2. Chemicals and materials

Standards of trans-cinnamaldehyde (CAD), eugenol (EUG), gallic acid, trolox, linalool and α-terpineol and essential oil leaf extract (EOL) were purchased from Sigma-Aldrich (Steinheim-Germany).

Solvents like ethanol and methanol (HPLC grade) from Sigma-Aldrich (Steinheim-Germany) and hexane from Carlo Erba (Rodano-Milan-Italy). Ultra pure water was obtained from the Millipore (Billerica, MA) Milli-Q system.

Nonionic surfactant Tween80, diphenylpicrylhydrazyl (DPPH), Folin-Ciocalteu reagent (1N), and sodium carbonate were purchased from Sigma-Aldrich (Steinheim –Germany).

2.3. Extraction procedures

Cinnamon barks B1 and B2 were used as starting material and four different extractions were applied (Annexe 1):

- Water extract (**WE**)
- Ethanol extract (**EE**)
- Oleoresin (**OR**)

- Essential oils (**EO**)

At the end of each extraction, the extraction yields were calculated and corrected according to humidity.

2.3.1. Water and Ethanol extracts

Water and ethanol extracts were prepared by soaking 1 g of the ground cinnamon barks in 10 ml of water and ethanol, respectively. The suspensions were mixed for four days at room temperature of 25°C on a rotatory shaker (IKA KS 130 basic) at 120 rpm. During the extraction procedure, seven different samples corresponding to 0.4, 1, 3, 6, 24, 48 and 96 hours of extraction, were taken for analysis. Samples were centrifuged at 6500 rpm for 20 min and finally filtered through Whatman No 1 filter paper before analysis. The experiments were carried out in triplicate. The aqueous extracts were daily prepared for phytotoxicity and *in vitro* assays.

2.3.2. Oleoresins extraction

Oleoresins (OR) were obtained by extraction in the *Soxhlet* apparatus. 12 g of cinnamon bark powder were extracted with 120 ml n-hexane (1:10, w:v) for 6 hours at 68℃. At the end of the extraction proce ss, solvent in the flask was separate by evaporation at 40℃ over night.

2.3.3. Essential oils distillation

Essential oils (EO) were obtained by water steam distillation for 6 hours in accordance with European Pharmacopoeia 5^{th} (2005). Fifty grams of cinnamon bark powder with 0.5 l of distilled water (1:10 w:v) were extracted in the clevenger apparatus at 100°C for 6 hours.

Both essential oils and oleoresins were stored at 4^0C until further use for phytotoxicity and *in vitro* assays and analysis.

2.4. Instrumental analysis

2.4.1. HPLC-PAD conditions and analysis

The HPLC system Ultimate 3000 (Dionex, Germering, Germany) was equipped with an photodiode array detector (PAD 3000), low pressure pump Ultimate 3000 pump, injector loop Rheodyne (Rheodyne, USA) of 20 µl, the column Acclaim C18 reverse (150 x 4.6 mm; 3 µm) and precolumn Acclaim C18 reverse (10 x 4.6 mm; 5 µm) and column oven. The HPLC was controlled and data were elaborated using Chromeleon Software *vs* 6.8 (Dionex, Germering, Germany). The gradient profile for the separations of cinnamon extracts was as follows: starting with methanol/water (45/55, v/v) for 5 min, then linear gradient from 55% to 15% water in 40 min, maintained for 5 min at methanol/water (85/15, v/v) and equilibration for 5 min by methanol/water (45/55, v/v) mobile phase and flow rate of 1 ml/min The analysis were performed at UV wavelengths of 280, 287, 290 nm for extracts,

cinnamaldehyde and eugenol, respectively and scan mode range was 190-400 nm.

2.4.2. HPLC/ESI-MS/MS conditions and analysis

A Varian tandem mass spectrometer (Palo Alto, CA, USA) consisting of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200 L triple quadrupole mass spectrometer equipped with an electrospray ionization source was used. Varian MS workstation, version 6.7 software was used for data acquisition and processing. Chromatographic separation was performed on an Phenomenex Synegri 4 u Max-RP80℃olumn (4.6 × 180 mm I.D., particle size 5 *μ*m, Milford, MA). The mobile phase consisted of (A) methanol and (B) bidistilled water with 0.1% of formic acid. Elution started with A-B (10:90, v/v) reaching A-B (100:0, v/v) for 20 min maintained for 5 min and then equilibration time for 5 min till A-B (10:90, v/v). The mobile phase, previously degassed with high-purity helium, was pumped at a flow rate of 0.4 ml/min, and the injection volume was 10 *μ*l. The electrospray ionization-mass spectrometer was operated in the switching mode. The electrospray capillary potential was set to 70 V, while the shield was at 300 V. Nitrogen at 57 psi was used as a drying gas for solvent evaporation. The atmospheric pressure ionization (API) housing and drying gas temperatures were kept at 250 ℃, respectively. Protonated analyte molecules of the parent compounds were subjected to collision induced dissociation using argon at 3.80 mTorr in the multiple reaction monitoring (MRM) mode. The scan time was 1 s, and the detector multiplier voltage was set to 1450 V, with an isolation width of *m*/*z* 1.2 for quadrupole 1 and *m*/*z* 2.0 for quadrupole 3. ESI mass spectra were acquired by scanning over the 100-1000 mass range.

2.4.3. GC-MS conditions and analysis

The DSQ II, configured with the proven Thermo Scientific TRACE GC UltraTM gas chromatograph and single quadrupole GC/MS was used. The capillary column was TR CP-Wax 52 CB fused silica WCOT, 10 m x 0.1 mm, dF=0.2 µm. The GC–MS transfer line temperature was maintained at 200℃. The following temperature program was employed: initial temperature 50℃ held for 1 min; ramped at 3° C/min up to 220℃, hel d for 13 min. Helium was used as a carrier gas. Helium was the carrier gas at 0.9 ml/min; the sample (1µl) was injected in the split mode. The ionization was used in the electron ionization (EI) mode, scanning the 40–300 *m*/*z* range used to determine the appropriate mass for quantitative analysis in the selected ion monitoring (SIM) mode.

2.4.4. Quantitative and qualitative analysis

The cinnamon extracts components were identified by comparison of relative retention times with those of authentic standards of cinnamaldehyde and eugenol. Calibration curve were constructed with the external standard method, correlating the area of the peaks with the concentration. The correlation values were 0.99 for cinnamaldehyde and eugenol.

Other components were identified by computer matching against commercial (NIST library) as well as MS literature data. Quantitative analysis of each extracts components (expressed in percentages) was carried out by peak area normalization measurements.

2.5. Total phenolic content

Total phenolic contents (TPC) of the extracts were assayed according to Folin-Ciocalteu method. Briefly, stock solutions were prepared by dissolving 10 mg of CAD, EUG, OR, EO and 10 µl of WE and EE in 10 ml of deionized water. 300 µl of stock solutions, calibration solutions and blank were pipetted into separate test tubes and 300 µl of Folin-Ciocalteu reagent were added to each. The mixture was mixed well and allowed to equilibrate. After 2 min, 2.4 ml of a 5% (w/v) sodium carbonate solution was added. The mixture was swirled and put in a temperature bath at 40℃ for 2 0 min. Then, the tubes were rapidly cooled and the maximum adsorption was measured at 740 nm using spectrophotometer (UNICAM BS DISC PD 2000-1). Data were expressed as Gallic Acid equivalent (GAE) using gallic acid calibration curve.

2.6. Antioxidant activities

A specrophotometric analysis that used DPPH was performed. This assay is based on the ability of the antioxidant to scavenge the radical cation DPPH. Data were expressed as Trolox equivalent antioxidant capacity (TEAC) using Trolox calibration curve. The *in vitro* antioxidant activities of cinnamon extracts were performed in the following way: 10 µl of cinnamon extracts were added to 3 ml of 0.04 mM DPPH ethyl acetate solution and mixed with glass baquet. The samples were kept in the dark for 60 min at room temperature and then decrease in absorbance at 517 nm was measured using spectrophotometer (UNICAM BS DISC PD 2000-1). Calibration curve in the range of 0.2/0.4/0.6/1.0/2.0/4.0/6.0 mmol/l has been prepared for Trolox.

2.7. *In vitro* study

2.7.1. Phytotoxicity assay

Phytotoxicity assay was conducted according to the Organisation for Economic Co-operation and Development (OECD, 2003) guideline for testing the chemical which is designed to assess potential effects of substances on seedling emergence and growth.

Phytotoxicity assay was performed on the seeds of *Lolium perenne, Licopersicum esculentum* and *Lepidium sativum* by using different cinnamon extracts solutions prepared in 0.1% ethanol and 0,1% Tween80 deionized water solution. Ten seeds were placed in 100 mm disposable polyethylene containers with filter paper in the bottom as support; afterwards, 2 ml of increase range of CAD and EUG concentrations (5, 10, 25, 50, 75, 100, 175 and 250 mg/l) in testing extracts were applied. 0.1% ethanol and 0.1% Tween80 deionized water solution were used as control treatment. All

treatments were performed in five replicates. Assay was performed in the growing chamber at 23 ± 1ºC in dark condition.

Germinated seeds are defined as a primary root of ≥ 3mm and the test was terminated when seedlings in control for all tested species were developed roots at least 20 mm long. The data were recorded at 3, 5 and 6th days for *Lepidium sativum, Licopersicum esculentum* and *Lolium perenne*, respectively.

- **RC_{50} and GI determination**

Phytotoxicity was assessed using the following relative seed germination (RSG), relative root growth (RRG) and germination index (GI) tests (Tiquia *et al*., 1996). After incubation, the parameters were determined using the following formulas:

$$\text{RSG}\,(\%) = \frac{\text{Number of seeds germinated in the treatment}}{\text{Number of seeds germinated in control}} \times 100$$

$$\text{RRG}\,(\%) = \frac{\text{Mean root length in the treatment}}{\text{Mean root length in control}} \times 100$$

$$\text{GI}\,(\%) = \frac{\text{RSG x RRG}}{100}$$

Half inhibitory concentration (RC_{50}) is the concentration which inhibits 50% of germination index and was calculated according to linear dose-response relationship graphs.

2.7.2. Antifungal activities

In order to determine the antifungal efficacy of the cinnamon extracts the pathogenic fungi: *Botrytis cinerea,* isolated from artichoke and *Phytophtora capsici* isolated from pepper were undertaken. The fungi isolates were procured by Prof Franco Nigro from Plant protection and applied microbiology department, University of Bari.

2.7.2.1. Mycelial growth assay

The mycelia disc (5 mm diameter) was taken from the periphery of an actively growing agar culture and placed at the centre of a Petri dish. Petri dish containing 13 ml of potato dextrose agar (PDA) and different amounts of cinnamon extracts were added in growing media in the case of direct contact assays or on filter paper placed on the cover inside the dish in the case of volatile phase assays. The final concentrations used were 10, 50, 100, 250 and 500 mg/l of CAD or EUG, respectively, in the extracts.

For each extract five concentrations per five replicates were tested. Control treatments consisted of 13 ml of PDA inoculated with the fungi. Test was terminated when fungal micelle in control for each fungi reached the edges of

the control dishes. During the incubation at 23˚C in the dark, diameter of the fungal micelle was measured with ruler every day. The experiment was repeated twice. The data were recorded during the seven and fifteen days of incubation for *Botrytis cinerea* and *Phytophtora capsici*, respectively.

To investigate the effectiveness of various treatments, at the end of experiment MIC (minimum inhibitory concentration) and ED_{50} (effective dose 50) was determined.

MICs are considered the "gold standard" for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. The MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of an organism after incubation (Messele, 2004). ED_{50} is the concentration which inhibits 50% of mycelial growth.

2.7.2.2. Conidial, zoospores germination and vitality assays

Conidia and zoospores from 10 days old colonies of *Botrytis cinerea* and *Phytophtora capsici* inoculated on PDA and water agar, respectively were collected by adding 5 ml of distilled sterile water to each Petri dish by rubbing the surface with a sterile spatula. The suspension was collected and then centrifuged at room temperature at 1000 rpm for 2 minutes. Then supernatant was discarded and the number of conidia and zoospores were obtained by Thoma cell counting method.

For liquid phase, aliquot of 100 µl of conidia and zoospores suspension containing $1x10^7$ conidia and zoospores per ml were added to 5 ml of cinnamon extracts previously diluted in sterile distilled water to reach the concentration of 10, 50, 100, 250 and 500 mg/l of CAD or EUG. The controls consisted on conidia or zoospores suspensions in 0.1% Tween80 and 0.1% ethanol solution.

In volatile phase, Petri dishes containing PDA were inoculated with 100 µl of conidia and zoospores suspensions dispersed on the surface with a sterile L-shaped spreader. Aliquots of each extracts were added to sterile filter paper placed on the cover inside the dish. The Petri dishes were then sealed using adhesive tape then incubated at 23˚C in the dark for 3-5 days.

After the incubation, fungistatic or fungicidal effects were examined by vitality test. 100 µl of conidia or zoospores suspensions, prior exposed to cinnamon extracts, were transferred on PDA medium and in 5 ml of sterilized distilled water. The treatments were incubated at 23˚C in the dark for 3-5 days.

Experiments were carried out with three replicates per treatment. The percentage of conidia and zoospores germination was determined microscopically by looking for the presence of germ tubes and was calculated in comparison with the control assay. The data was recorded after 24, 48 and 72 hours of incubation to establish the effect of cinnamon extracts on

germination. Germinated conidia or zoospores are defined as a germinated tube two times longer than their diameters.

- **Percentage of inhibition determination**

Percentages of mycelial growth and conidia / zoospores germination inhibitions were calculated according to the formula:

$$\text{Inhibition (\%)} = \frac{(\text{Control - Treatment})}{\text{Control}} \times 100$$

- **Growing rates (mm/day) determination**

Growing rates (mm/day) were calculated according to the linear equation $y = \acute{\alpha}x - \beta$ where $\acute{\alpha}$ is growth rate mm/day.

- **Microscopic observation**

In order to investigate the effect of different extracts on the growth of mycelia, conidia and zoospores morphology of the fungal cells was observed under a binocular light microscope Leica DMR and Zeiss Photomicroscope, Germany, equipped with a digital camera.

2.8. Statistical analysis

Statistical analyses of all experimental data were done using the statistical General Linear Models Procedure (SAS Institute, Inc. 2001). Analysis of variance was followed by comparison of means for significant effect using Duncan Multiple Range Test. Differences were considered to be significant at $P \leq 0.05$. For RC_{50} and growth rates, correlation coefficient and standard errors were calculated according to the linear dose-responds relationship.

Chapter 3

Results and discussions

3.1. Water and ethanol extracts

Levels of CAD and EUG in ethanol and water extracts after 96 hours of contact time were determined. Results were reported in Table 2 and Table 3.

In EE, the highest amount of CAD was obtained after 6 hours of extraction with 2.12 mg/ml and 1.39 mg/ml in B1 and B2, respectively, remaining stable till the end of extraction time. Whereas in WE, the highest amount was obtained after 0.4 hour of extraction with 0.87 mg/ml and 0.52 mg/ml in B1 and B2, respectively, decreasing in time to 0.52 mg/ml and 0.48 mg/ml in B1 and B2, respectively.

On the other hand, some traces of EUG were found in B2, while in B1 it was < LOD (below limit of detection) (Table 3). Highest value was found after 6 hours in EE and 0.4 hour in WE. The amount of CAD varied in time and in extract, it was found that the highest amount of CAD was extracted from B1 in both ethanolic and aqueous extract. Significant differences of CAD and EUG contents have been found among the extracts type and materials (Table 2).

Table 2. Cinnamaldehyde concentration in WE and EE during extraction time

Time (hours)	B1		B2	
	WE	EE	WE	EE
	(mg/ml)			
0.4	0.87 ^{d}	1.82 ^{b}	0.52 ^{efg}	0.86 ^{d}
6	0.59 ^{e}	2.12 ^{a}	0.45 ^{a}	1.39 ^{c}
24	0.51 ^{efg}	2.07 ^{a}	0.46 ^{a}	1.31 ^{c}
48	0.58 ^{ef}	2.07 ^{a}	0.43 ^{a}	1.38 ^{c}
96	0.52 ^{efg}	2.07 ^{a}	0.48 ^{a}	1.36 ^{c}

Values reported represent the average of three replicates. Means within a row followed by the same letter are not significantly different at P<0.05 (Duncan test)

Table 3. Eugenol contents in WE and EE during extraction time

Time (hours)	B1		B2	
	WE	EE	WE	EE
	(mg/ml)			
0.4	< LOD	< LOD	0.03 ^{d}	0.04 ^{c}
6	< LOD	< LOD	0.02 ^{e}	0.04 ^{b}
24	< LOD	< LOD	0.02 ^{ef}	0.05 ^{a}
48	< LOD	< LOD	0.01 ^{f}	0.05 ^{a}
96	< LOD	< LOD	0.02 ^{ef}	0.05 ^{a}

Values reported represent the average of three replicates. Means within a row followed by the same letter are not significantly different at P<0.05 (Duncan test)

The differences in the extraction yield can be attributed to the different solvents used. Levels of CAD in aqueous extracts are due to the repartition of compounds in water as reported by Anderson *et al*., (2004). The majority of the lipid soluble components of cinnamon bark would remain in the insoluble fraction, so the relatively hydrophobic character of CAD and its chemical structure may explain the low extraction yield, while ethanol is organic solvent with high intermediate polarity, which can extract higher amount of CAD.

3.2. Yields and chemical composition of different extracts from cinnamon barks

In this study, two types of cinnamon quillings B1 and B2 were used in order to evaluate the influence of the diversity of materials subjected to different extraction methods on qualitative and quantitative characteristics of extracts.

3.2.1. Extraction Yields

The yields of extracts from B1 and B2 obtained by using different extraction methods are shown in Table 4. The results indicated that the highest and lowest amounts were obtained in EE and in EO, respectively. The yields were in this order EE>WE>OR>EO with different amounts depending to the materials used. From B1 extraction yields were 110.7 g/kg, 86.5 g/kg, 37.4 g/kg and 17.5 g/kg in EE, WE, OR and EO, respectively. The lowest amount were extracted from B2 with 50.5 g/kg, 45.0 g/kg, 29.8 g/kg, and 14.9 g/kg in EE, WE, OR and EO, respectively. Significant differences have been found among all extracts and materials.

In comparison with Singh *et al*., (2007), the yields of EO and OR found in two materials are lower. They have extracted 25 g/kg and 97 g/kg of EO and OR, respectively. On the other hand, Joy *et al*., (2005) reported that generally the oil yield varied from 5 to10 g/kg.

Table 4.Yields of extracts from cinnamon bark

Extracts	B1	B2
	(g/kg)	
OR	37.4 ^{e}	29.8 ^{f}
EO	17.5 ^{g}	14.9 ^{g}
WE	86.5 ^{b}	45.0 ^{d}
EE	110.7 ^{a}	50.5 ^{c}

Values reported represent the average of six replicates. Means within a row followed by the same letter are not significantly different at P<0.05 (Duncan test)

It is possible that the yield of EO will vary with the type and age of the plant as well as water content of the material and the method of distillation (Senanayake and Wijesekera, 2003). The EO normally contained higher levels of volatile components; therefore, it was reasonable to have lower yields. Rohloff (2004) reported that genetic, physiological and environmental factors as well as processing conditions have direct influence on volatile oil content.

In addition, the difference of extraction yield can be attributed to the solvents different polarity, which influences the extraction of cinnamon components. It was found that ethanol as a solvent extracts more efficiently proteinaceous compounds and made possible the extraction of some compounds of intermediate polarity (Tabak *et al*., 1999; Jones and Kinghorn, 2005), while as reported by Anderson *et al*., (2004), during aqueous extraction, the overwhelming majority of the lipid soluble components of cinnamon bark would remain in the insoluble fraction.

3.2.2. Chemical composition of cinnamon extracts

Chemical analysis of cinnamon extracts indicated the apparent difference in the compounds composition. Main components identified were CAD and EUG. Results are presented in Figure 7. The highest CAD contents were found in EOs with 81.2 % and 69.2 %, followed by ORs with 50.5 % and 42.3 % in B1 and B2, respectively. Chemical analysis of EOL extract, showed EUG as major constituent with 76.8 %. In addition some traces of EUG were found in EOs from B1 and B2 with 0.2 % and 6.9 %, respectively, while in OR was extracted 3.5 % from B2.

The major constituents identified in this work have been reported in some previous studies related to chemical analysis of cinnamon species. By GC-MS analysis, a total of 19 compounds were identified in the EOL with 87.3% EUG as the major compound. CAD was found major constituent in cinnamon bark with 97.7% of total volatile oil and 50% of oleoresin (Joy *et al*., 2005; Singh *et al*., 2007). Recently, Wang *et al*., (2008) identified 79.75% of EUG from EOL of *Cinnamomum zeylanicum*.

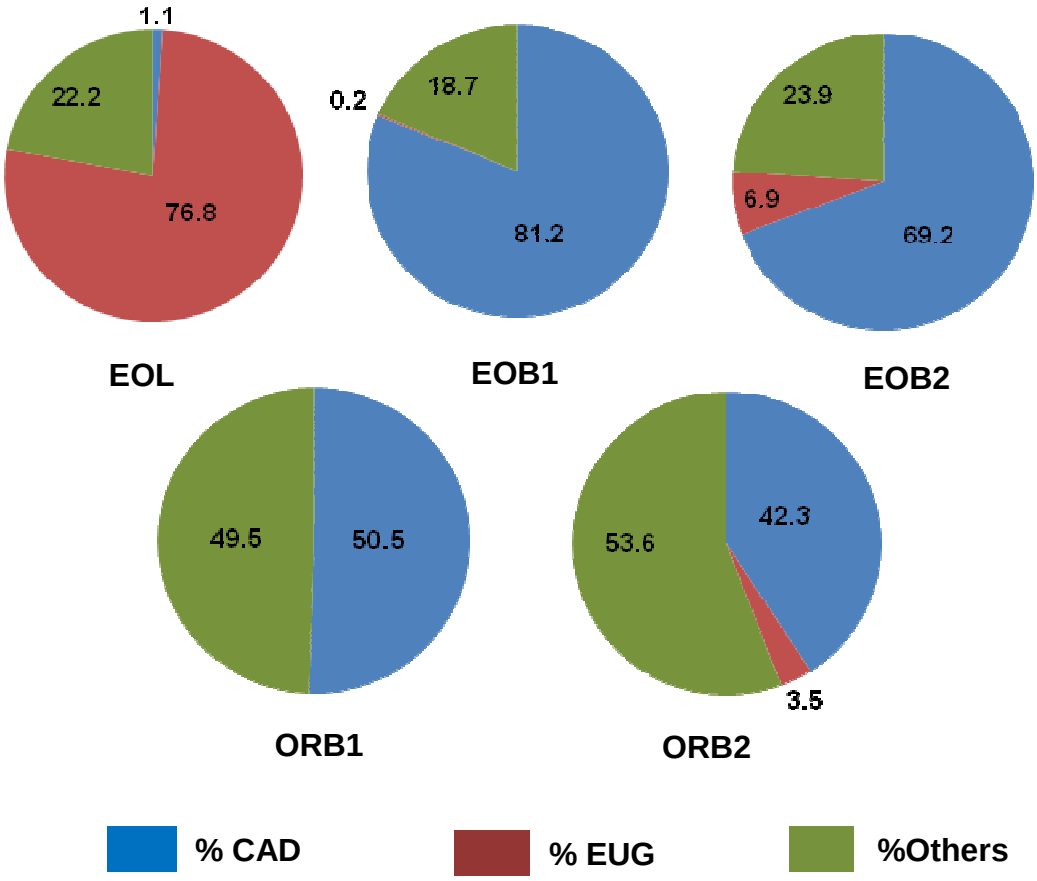

Figure 7. Chemical composition of cinnamon extracts

3.2.3. Total polyphenols and antioxidant activities

Total polyphenols and antioxidant activities of cinnamon extracts were determined and showed in Table 5. Indeed, EUG had the greatest capacity to react with DPPH and Folin-Ciocalteu reagents due to the its phenolic structure, whereas CAD had the least ability. Eugenol exhibited a higher reaction rate compared to cinnamaldehyde; similar results have been reported by Mathew and Abraham (2006).

It was found that EOs and ORs had the highest total phenolic contents in B2, while EEs and WEs showed the highest values in B1. In addition, results revealed that total polyphenols compounds contents were related to the antioxidant activities found in all extracts. Therefore, cinnamon bark extract showed good free radical scavenging capacity. The amount of polyphenols and antioxidant activities in the extracts were significantly different.

These results suggested the influence of extraction type on total phenolic content as reported by Zhou and Yu (2004). Previous study revealed that antioxidative potency of phenolic compound depends on their chemical structure, mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching oxygen or decomposing peroxides (Ogata *et al*., 2000; Karou *et al*., 2005).

Table 5. Total polyphenols and antioxidant activities of cinnamon extracts

Extracts	GAE (mmol /l) [a]		TEAC (mmol /l) [b]	
EUG	5.76 [f]		8.40 [c]	
EOL	4.67 [g]		8.64 [b]	
CAD	0.15 [n]		0.73 [m]	
	B1	**B2**	**B1**	**B2**
EO	0.22 [n]	2.59 [j]	0.65 [m]	8.54 [b]
OR	1.44 [l]	1.65 [k]	4.17 [h]	8.89 [a]
WE	1.60 [k]	0.88 [m]	6.42 [e]	3.39 [i]
EE	3.31 [i]	1.31 [l]	7.11 [d]	5.36 [f]

Significantly different at p<0.05

[a] GAE is the milimolar concentration of a Galic Acid Equivalents equivalent to that of 10µl of extracts or standards

[b] TEAC is the milimolar concentration of a Trolox solution having an antioxidant capacity equivalent to that of 10 µl of extracts and standards

3.2.4. Cinnamaldehyde and eugenol contents in cinnamon barks

CAD and EUG contents in cinnamon barks based on dry cinnamon materials were determined and showed in Figure 8. In EEs and WEs were extracted 20.7 g/kg and 8.7 g/kg of CAD, respectively from B1, while lower amounts were in B2 with 13.9 g/kg and 5.2 g/kg. EOs and ORs showed intermediate contents with 15.2 g/kg and 16.7 g/kg from B1 and 13 g/kg and 13.6 g/kg from B2. The amounts of CAD were in this order EE>OR>EO>WE.

On the other hand, lowest EUG content was found in B2 with 0.3 g/kg, 0.5 g/kg, 1.3 g/kg and 1.4 g/kg in WE, EE, EO and OR, respectively, while in B1 were below limit of detection.

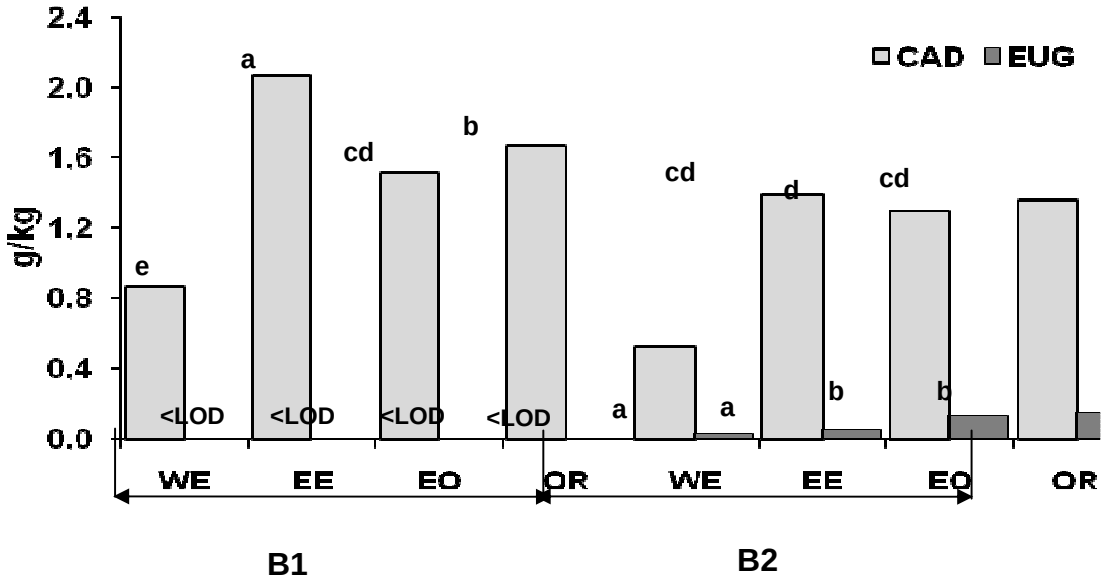

Figure 8. Cinnamaldehyde and eugenol contents in cinnamon barks from B1 and B2

3.2.5. Chemical composition of cinnamon extracts used in in vitro study

CAD contents in different extracts were higher in B1. Biological activities of cinnamon extracts are mostly related to CAD and EUG contents, cinnamon extracts from B1 were selected for *in vitro* study and subjected to others chemical analysis in order to identify major components.

GC-MS analysis of EO, OR and EOL resulted in the identification of 17 other components (Table 6). The analysis of EO and OR showed the presence of CAD as the major component. In EO were identified caryophyllene oxide (5.55%), borneolacetate (3.23%), α-terpineolo (1.94%), copaene (1.40%) and borneol (1.02%).

The analysis of OR showed the presence of copaene (4.27%), isocaryophyllene (1.31%), coumarins (2.51%) and others minor components. In EOL, major component present was EUG beside other components as caryophyllene (6.58%), linalolo (3.74%), phenol, 4-allyl-2-methoxy-, acetate (1.82%), m-cymene (1.75%), and α-phellandrene (1.69%).

Table 6. Chemical composition of EO, OR and EOL extracts

Compounds	**[x]EO**	**[x]OR**	**[y]EOL**
copaene[2]	1.40	4.27	1.25
benzaldehyde[2]	0.61		
linalolo[1]	0.29	0.11	3.74
borneolacetate[2]	3.23	1.02	
α-terpineolo[1]	1.94	0.68	
borneol[2]	1.02		
caryophyllene oxide[2]	5.55		
isocaryophyllene[2]		1.31	
unknown[2]		1.24	
coumarin[2]		2.51	
α-pinene[2]			1.36
α-phellandrene[2]			1.69
m-cymene[2]			1.75
caryophyllene[2]			6.58
phenol,4-allyl-2-methoxy-,acetate[2]			1.82
CAD[1]	100.00	100.00	1.01
EUG[1]	0.12	0.00	100.00

[x] % of the height in the respect to CAD
[y] % of the height in the respect to EUG
[1] Identification was based on standard compounds and [2] on composition of the compounds MS spectra with spectra in the NIST library

Results demonstrated that EO, OR and EOL are very heterogeneous mixture of a single substances, biological actions are primarily due to these components in a very complicated concert of synergistic or antagonistic effects reported by Singh *et al*., (2007). The major constituents identified have also been reported in some previous studies related to the chemical analyses by GC-MS of *Cinnamomum* species.

3.2.6. Chemical composition of water extract used in in vitro study

Results indicated that cinnamon bark not only possessed high levels of CAD, also contained many proanthocyanidins/ (epi) catechins. Chemical analysis of WE from B1 by LC-ESI-MS/MS showed beside CAD, the presence of polymeric polyphenols (mainly condensed tannins), that are proanthocyanidins and catechins (Table 7). Figure 9 clearly displays the predominant peaks isolated in the WE, that are proanthocyanidins and (epi) catechins (peaks 2-7) and (*E*)-CAD (peak 12). Proanthocyanidins and (epi) catechins, were tentatively identified as procyanidin (b1, b2), trimer and dimmers, and (+)-catechin and (-) epicatechin.

Proanthocyanidins and (epi) catechins have similar polarities; it is not easy to separate them well. Therefore, the mixed fractions of proanthocyanidins and (epi) catechins peaks 2-7 in Figure 9 (Shan *et al*., 2007).

Productions of polymeric polyphenols have antioxidant effects and may have significance in plant defense systems (Tanaka *et al*., 2008). The same authors reported that when the polyphenols were oxidized by the catalysis of enzymes, the oxygen molecules were simultaneously reduced to give reactive oxygen species, which act as antimicrobial agents.

Table 7. Tentative identification of major compounds (Proanthocyanidins /Catechins and CAD) in water extract of cinnamon bark B1 by LC/ESI-MS/MS

Compounds	**MS (m/z)**		**WEB1**		**Peak area (%)**	**Peak n. Figure 9.**
	$[M+H]^*$	$[M-H]^-$	$[M+H]^*$	$[M-H]^-$		
Procyanidin (b1)	579	577	+	+	0.41	2
Procyanidin (b2)	579	577	+	+	0.30	3
Procyanidin trimer	865	863	+	+	0.26	4
(+) catechin	290	289		+	2.43	5
Procyanidin dimer	577	575		+	3.41	6
(-) epicatechin	290	289		+	2.23	7
(E)-cinnamic acid	149	147	+	+	5.40	10
(E)- cinnamaldehyde	133	131			78.14	12
(S)- cinnamaldehyde	133	131			6.01	13

+ identified

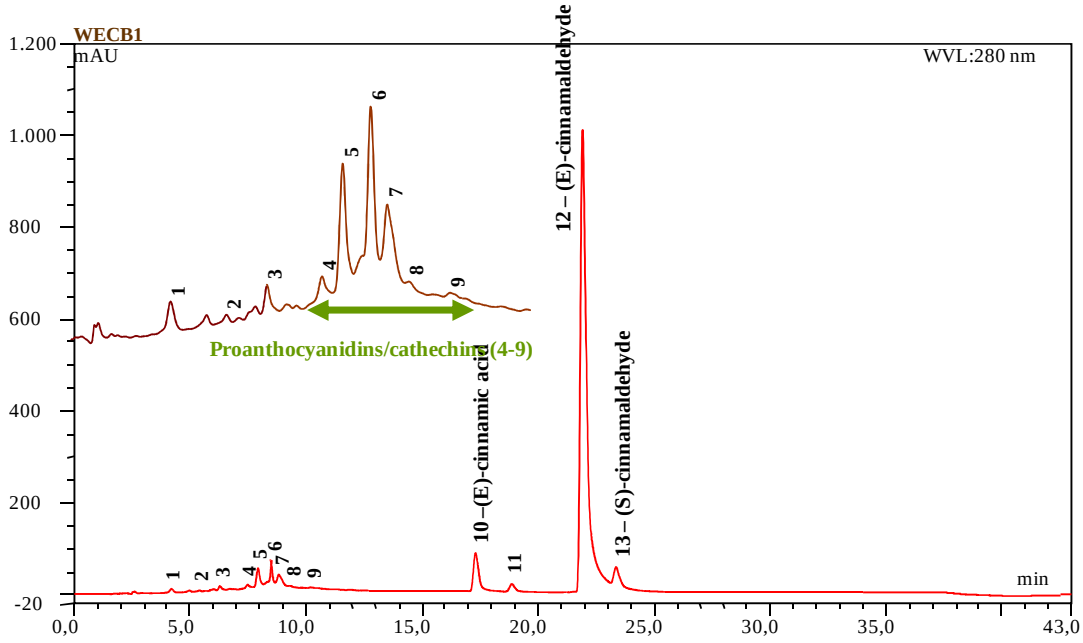

Figure 9. LC chromatogram of WEB1 (280 nm) major peaks were identified by ESI-MS/MS: 2-7 proanthocyanidins - (epi) cathecins; 12 CAD

3.3. *In vitro* study

In order to evaluate cinnamon extracts biological activity, CAD and EUG standards, EOL EO, OR and WE extracts from cinnamon bark B1 were used. EE due to ethanol effects on seeds germination and fungi were not taken in consideration. During the preliminary tests at ethanol concentration of 0.5% and 1%, results were markedly influenced.

3.3.1. Phytotoxicity assay

Results showed that germination indexes decreased with increases in concentrations of CAD or EUG (Figures 10, 11, 12, Annexe 2). At lower concentrations of cinnamon extracts ranging from 5 mg/l to 25 mg/l, highest values of GI was achieved in *Lepidium sativum* with above 85 % (Figure 12) in comparison to others species. *Licopersicum esculentum* showed the lowest value with above 60 % (Figure 11).

WE showed the lowest values of GI with above 80%, 70% and 40% for *Lepidium sativum, Lolium perenne* and *Licopersicum esculentum*, respectively (Figures 10, 11, 12). With increases concentrations of cinnamon extracts, GIs were significantly reduced and became more pronounced reaching 0% at 250 mg/l in all tested species.

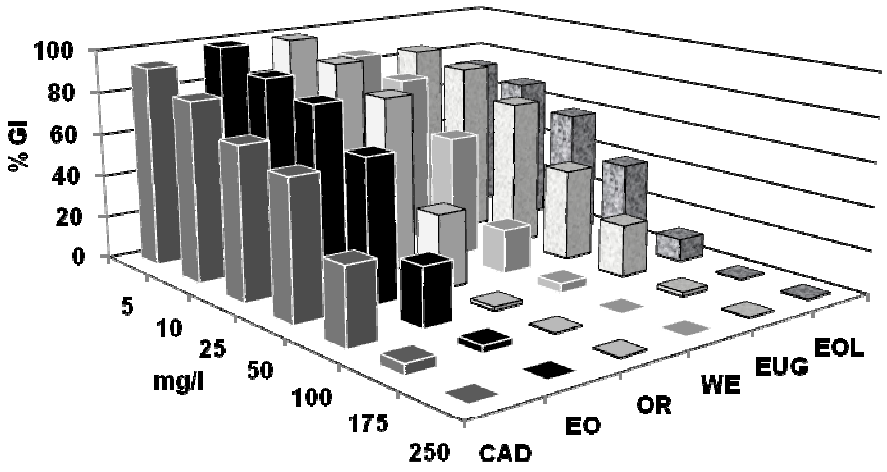

Figure 10. Germination index (%) of *Lolium perenne* in cinnamon extracts

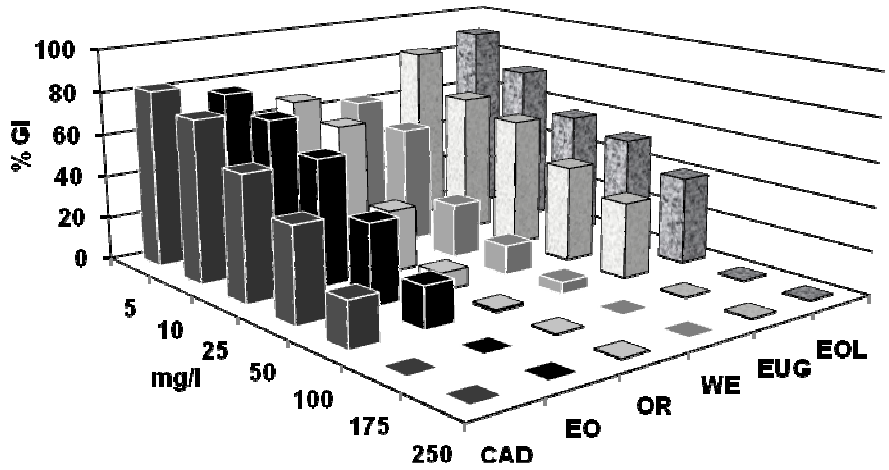

Figure 11. Germination index (%) of *Lycopesicum esculentum* in cinnamon extracts

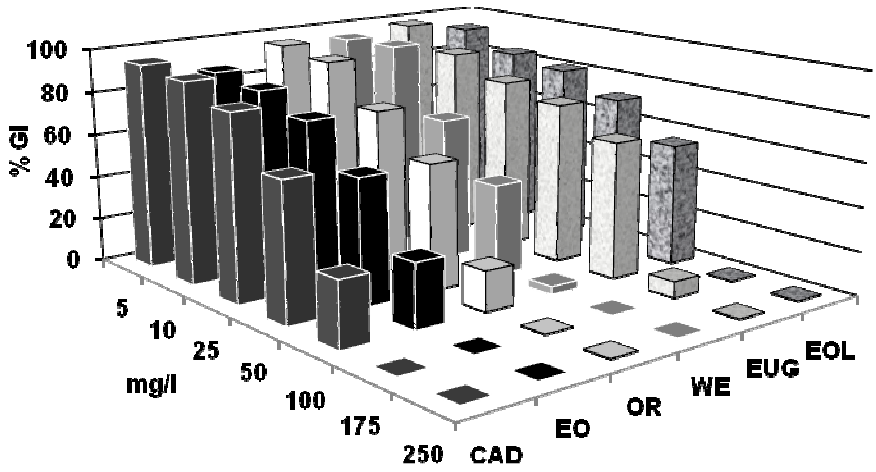

Figure 12. Germination index (%) of *Lepidium sativum* in cinnamon extracts

Phytotoxic effects of cinnamon extracts were indicated by decreases in number of germinated seeds and roots growth in a dose response relationship. From the dose response curve, RC_{50}s (amount of extracts required to cause 50% inhibition of germination) values were determined. Results presented in Table 8, showed lower RC_{50}s values in WE for all tested species with 30 mg/l, 12 mg/l and 49 mg/l in *Lolium perenne*, *Licopersicum esculentum* and *Lepidium sativum*, respectively. For others extracts, RC_{50} value varied from 12 mg/l to 108 mg/l depending of species and treatments. In comparison to CAD, lowest values of RC_{50} were found in EO, OR and WE in all species, CAD and EUG showed higher RC_{50}s values compared to the plant extracts. Significant differences were observed among the cinnamon extracts and tested species.

Table 8. Half inhibition concentration of cinnamon extracts on tested plant seeds

Treatments	***Lolium perenne***	***Licopersicum esculentum***	***Lepidium sativum***
		RC_{50} (mg/l)/SE	
CAD	78 / 5	48 / 4	79 / 6
EO	71 / 3	42 / 6	72 / 4
OR	49 / 10	16 / 8	60 /2
WE	30 / 1	12 /1	49 /8
EUG	52 / 7	78 / 4	108 /8
EOL	40 / 2	65 /6	89 /3

RC_{50} - Half inhibition concentration
SE- Standard error of estimate linear dose- response relations
Coefficients were $R^2 > 0.95$ and significantly different at $P<0.05$

WE showed strong phytotoxic effects, it can be due to the phenolic compounds catechins and proanthocyanidins. Several studies have demonstrated phytotoxic effect of catechins for many species at low concentration in petri dishes and in soils, able to induce stress responses in specific parts of root (Inderjit *et al*., 2008).

On the other hand, Weir *et al*., (2004), reported that (-) catechin from *Centaurea maculosa* (spotted knapweed) inhibits seed germination in plant species whose seedlings are generally tolerant of its phytotoxic effects. In addition, Orcutt *et al*., (2000), demonstrated that phenolic acids and coumarins show a range of activities such as suppressing mitochondrial metabolism stimulating hypocotyls elongation and rooting and affect the action of many enzymes.

Liu *et al*., (2005) reported that the effect of essential oils on seed germination and seedling growth is dependent on not only the individual effects of same main constituents but also their interaction because essential oils are a mixture of many compounds in different proportions. This can explain the higher phytotoxic effect of EO, OR compared to the CAD and similar for EOL compared to EUG. Other studies of Bainard and Isman (2006), on

phytotoxicity of clove oil and its primary constituent eugenol on broccoli, common lambsquarters and redroot pigweed, reported that higher phytotoxic effect found may be caused by others constituents (e.g., β-caryophyllene and ά-humulene) also identified in our extracts.

Tworkoski (2002) demonstrated that cinnamon oil was phytotoxic and caused electrolyte leakage resulting in cell death of leaves of dandelion by increasing membrane permeability. One suggested mechanism for the inhibition of seed germination is the disruption of mitochondrial respiration (Weir *et al*., 2004).

The tested plants had different responses to cinnamon extracts exhibiting differential species specificity. *Licopersicum esculentum* was the most affected, whereas *Lepidium sativum* showed the best response. Meyer *et al*., (2008) found that tomato seedlings were the most sensitive to clove oil than cucumber and muskmelon. It is reported that the sensitivity of plant species to toxicity depend on the quantity of its food reserves (Cheung *et al.,* 1989). Tomato seeds are quite small and have small quantity of food reserve.

3.3.2. Antifungal activities assay

Antifungal activity of cinnamon extracts was estimated by a growth inhibition assay on two different fungi *Botrytis cinerea* and *Phytophthora capsici*. They are an economically important phytopathogenic fungi caused the most destructive diseases in plants worldwide. The research for compounds active against those fungi is necessary which may have potential in disease control. *Botrytis cinerea belongs to the class of Ascomycetes,* while *Phytophthora capsici* belongs to the class of *Oomycetes*.

3.3.2.1. Mycelial growth assay

3.3.2.1.1. Botrytis cinerea

Results presented in the Figure 13 indicate that mycelial growth of *Botrytis cinerea* was partially or completely inhibited by all treatments. Indeed, at the highest extracts concentrations from 250 mg/l to 500 mg/l, results revealed complete inhibition of mycelial growth in volatile and solid phase, except for EUG which inhibited 50% and 91%, respectively in solid phase. EO, OR and WE exhibited the strongest activity compared to CAD, although EUG was less active in comparison to EOL. There were statistically significant ($P< 0.05$) reductions on the mycelial growth.

ED_{50} and MICs values of cinnamon extracts on mycelial growth were examined (Table 9). Mycelial growth of *Botrytis cinerea* was more affected by EO, OR, WE and EOL in solid phase. The ED_{50}s values varied between 50 mg/l and 100 mg/l and the MICs values was below 250 mg/l, except for EUG. In volatile phase, *Botrytis cinerea* was more sensitive to cinnamon extracts with ED_{50}s values varied between 10 mg/l and 50 mg/l in EO, OR, EUG and EOL while MICs values were below 50 mg/l, 100 mg/l, 250 mg/l and 250 mg/l ,respectively.

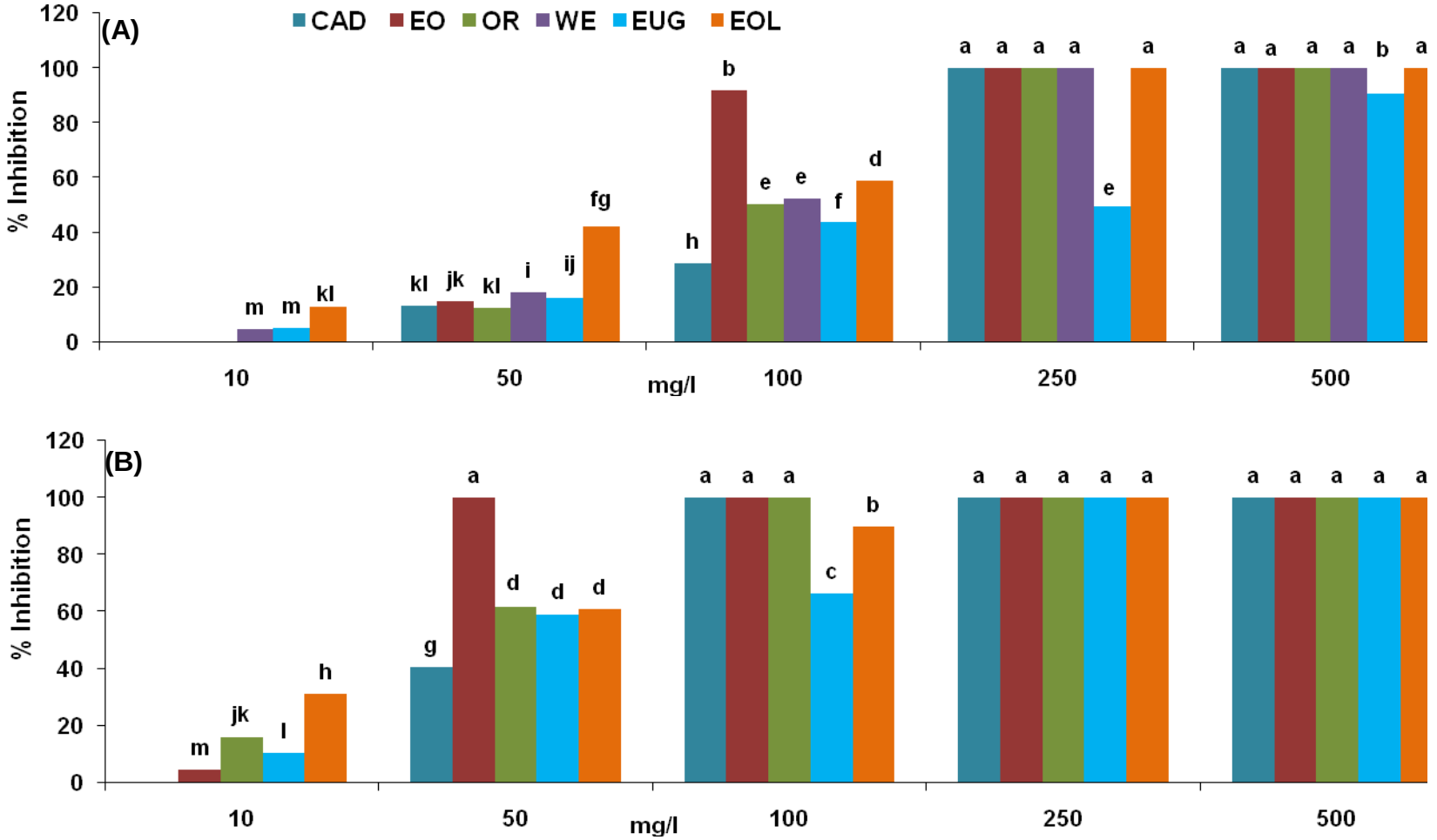

Figure 13. Inhibition of mycelial growth of *Botrytis cinerea* by extracts in solid (A) and volatile phase (B)

Table 9. The ranges ED_{50}s and MICs for mycelial growth of *Botrytis cinerea* in different treatments

Phase	Treatments	ED_{50}	MIC
		mg/l	
Solid	CAD	100-250	< 250
	EO	50-100	< 250
	OR	50-100	< 250
	WE	50-100	< 250
	EUG	100-250	> 500
	EOL	50-100	< 250
Volatile	CAD	50-100	< 100
	EO	10-50	< 50
	OR	10-50	< 100
	EUG	10-50	< 250
	EOL	10-50	< 250

On the other hand, fungi showed the presence of lag phases depending to treatments and concentrations (Figure 14, 15). In solid phase mycelia growth initiated after 2 days of incubation in OR extract at 100 mg/l and EUG at 500 mg/l (Figure 14). In volatile phase, after 1day of incubation, fungi mycelia began to increase at 50 mg/l in CAD, EO and EUG, and at 100 mg/l in EUG (Figure 15).

It is appeared that hyphal growth of the fungus was significantly retarded by cinnamon extracts. Microscopic observation of *Botrytis cinerea* hyphae exposed to treatment illustrated the effects of the extracts on the tested fungi. Alterations in the hyphal morphology when compared with the hyphae of the control were clearly visible. Shrinkage was observed in response to treatment EOL in solid and volatile phase. In addition, the distance between septa was shorter in the treatments than in control (Figure 16).

The results indicated strong inhibitory effect of cinnamon extracts toward *Botrytis cinerea* on suppression of fungi mycelia growth. Amoung the tested extracts, EO, OR and WE were the most active in all cases.

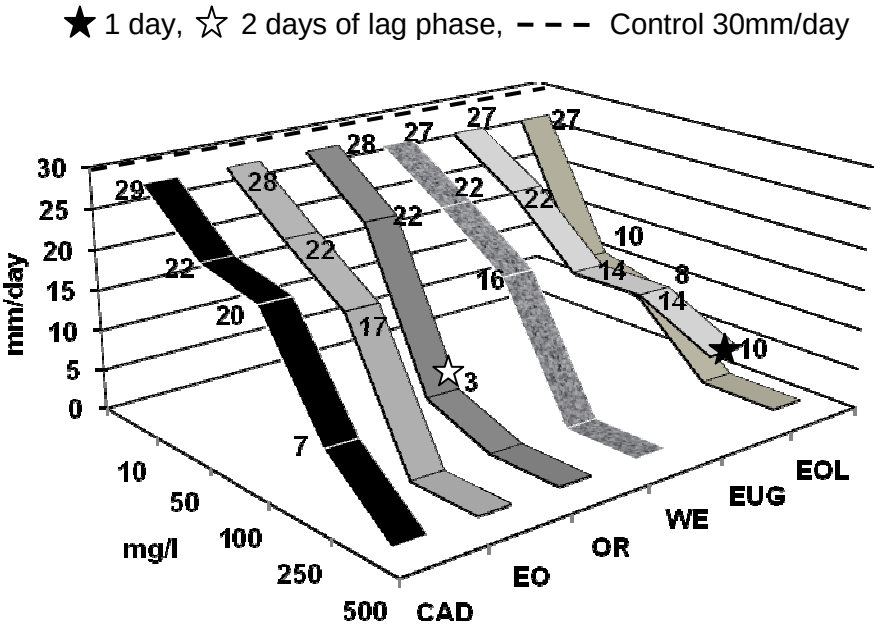

Figure 14. Growth rate (mm/day) of *Botrytis cinerea* affected by different treatments on solid phase

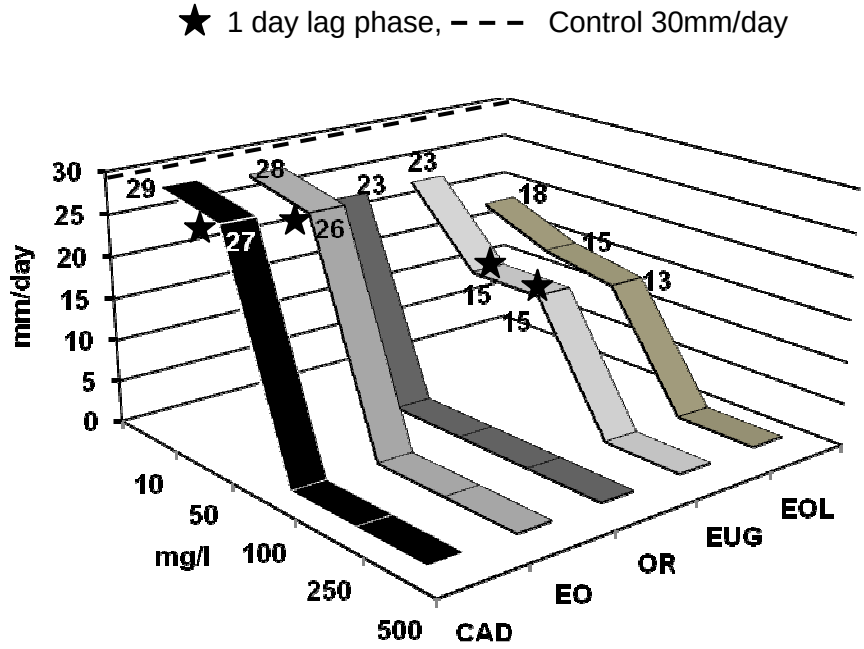

Figure 15. Growth rate (mm/day) *Botrytis cinerea* affected by different treatments in volatile phase

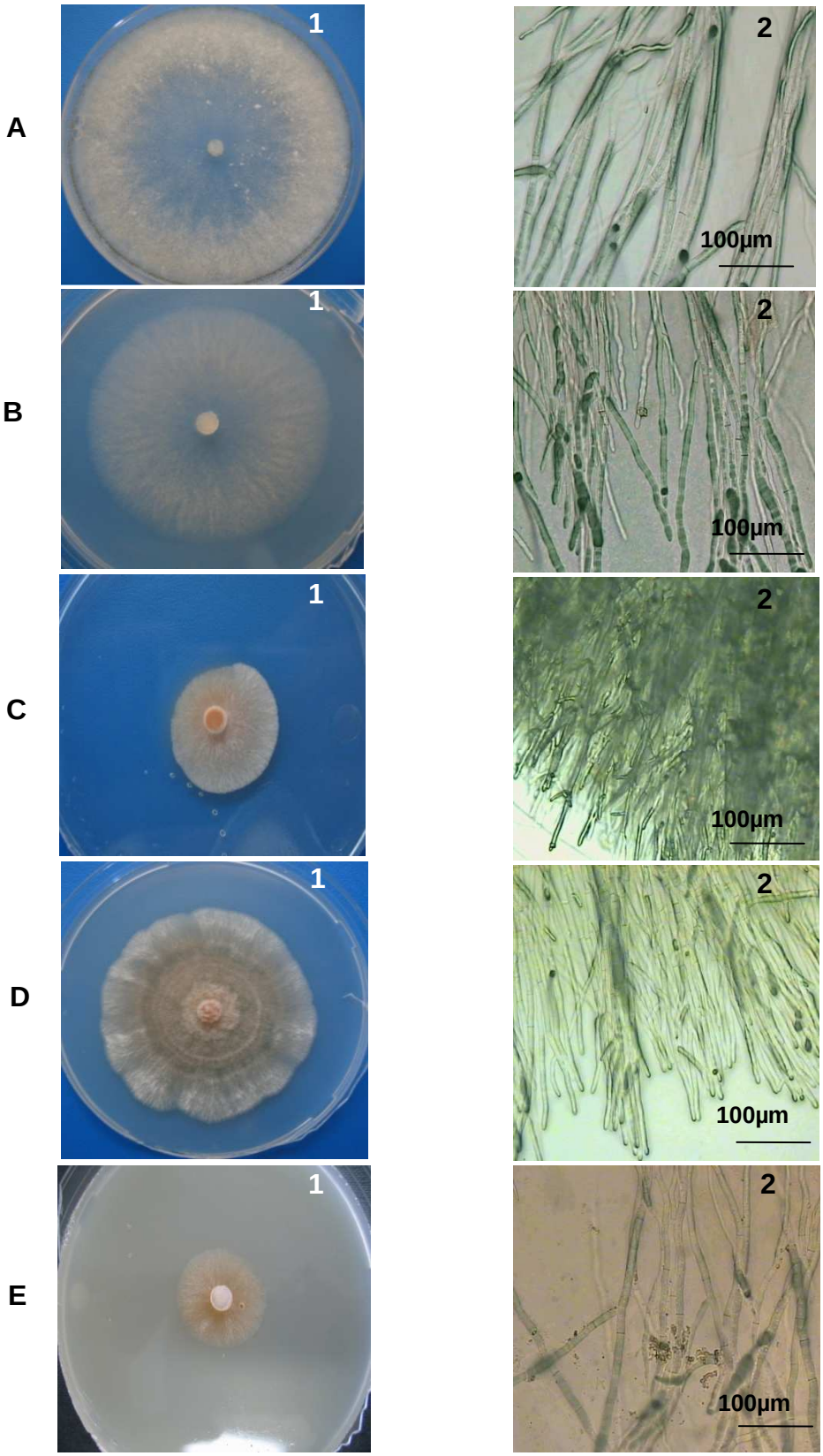

Figure16. Effects of cinnamon extracts on the mycelial (1) and hyphal (2) morphology of *Botrytis cinerea* observed with a light microscope (A. Control; B. CAD 100 mg/l solid phase; C.EOL 100 mg/l volatile phase; D. EOL 100 mg/l solid phase; E. WE at 100 mg/l)

3.3.2.1.2. Phytophtora capsici

The effects of cinnamon extracts on mycelial growth of *Phytophtora capsici* are showed in Figure 17. Results revealed that the mycelial inhibition exerted by the extracts occurred in a dose-dependent manner. At lower concentration of 10 mg/l, no relevant activities were observed in the opposite, cinnamon extract promoted mycelial growth. Cinnamon extracts stimulated the mycelial growth of *Phytophtora capsici* suggesting that it might have used extracts as energy supply, thus promoting mycelial growth.

It was stated that some plant pathogens use some organic materials as a source of energy which increases their inoculum potential (Linderman, 1989). On the other hand, Malo Vigil *et al*., (2005), mentioned that the effect of phenolic compounds is concentration dependent. At low concentration, phenols affected enzyme activity especially of those enzymes associated with energy production, whereas, at greater concentration phenols caused proteins denaturation. The absence of chitin in cell wall of *Phytophtora sp*. may explain the positive effect of extracts.

The effect on mycelial inhibition varied between treatments, EO, OR and WE showed strong inhibitory effect in comparison to CAD, while EOL was more effective than EUG. The differences of inhibitory activity were statistically significant ($P < 0.05$). The mycelial growth was significantly decreased with increases concentrations. Results showed that in the presence of cinnamon extracts, the growth of treated fungal cells was significantly inhibited. The cinnamon extract gave a stronger inhibition in volatile phase than in solid phase.

ED_{50} and MICs values of cinnamon extracts on mycelial growth were examined (Table 10). Mycelial growth of *Phytophtora capsici* was more sensitive to EO, OR and WE in solid phase. The ED_{50} values varied between 50 mg/l and 100 mg/l and MICs values were below 500 mg/l. Exposed to cinnamon extracts in volatile phase, *Phytophtora capsici* showed more sensitivity with ED_{50} varied between 10 mg/l and 50 mg/l in CAD, EO and EOL, respectively and MICs values below 250 mg/l.

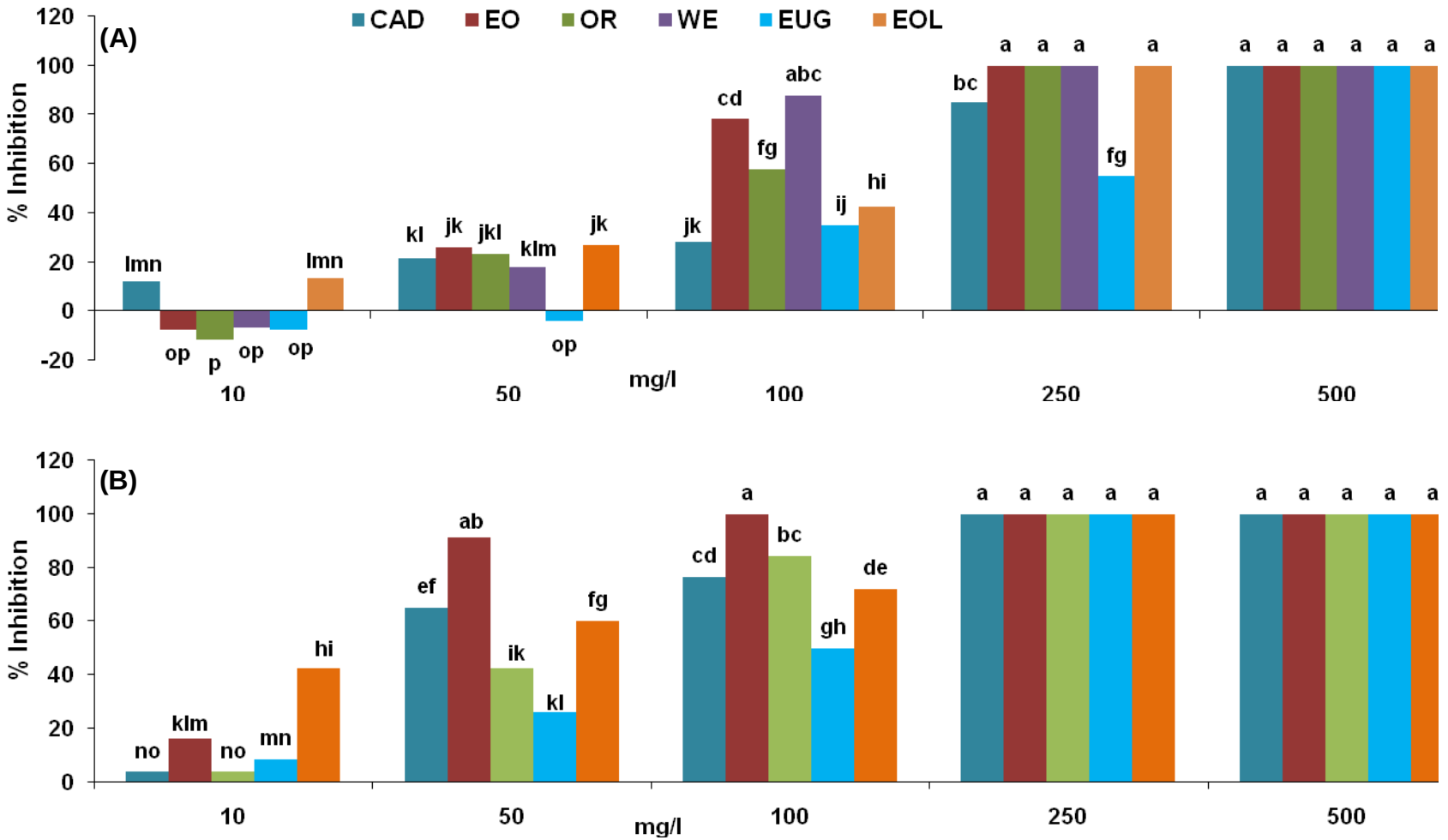

Figure 17. Inhibition of mycelial growth of *Phytophtora capsici* by extracts in solid (A) and volatile phase (B)

Table 10. The ranges ED_{50}s and MICs for mycelial growth of *Phytophtora capsici* in different treatments

Phase	Treatments	ED_{50}	MIC
		mg/l	
Solid	**CAD**	100-250	< 500
	EO	50-100	< 250
	OR	50-100	< 250
	WE	50-100	< 250
	EUG	250-500	< 500
	EOL	100-250	< 250
Volatile	**CAD**	10-50	< 250
	EO	10-50	< 100
	OR	50-100	< 250
	EUG	100-250	< 250
	EOL	10-50	< 250

Fungi had lag phases depending to treatments and concentrations (Figure 18, 19). In solid phase, after 2 days of incubation in WE and EO at 100 mg/l, fungi mycelia began to increase, while in EUG and EOL started after 1 day of incubation at 100 mg/l and 250 mg/l. In volatile phase, *Phytophtora capsici* showed an extended lag phase of 4 days in EUG and EOL at 100mg/l, and from 1 to 3 days at 50 mg/l in all extracts, except CAD.

Microscopic observation of *Phytophtora capsici* hyphae exposed to cinnamon extracts revealed degenerative changes in the hyphal morphology when compared to the hyphae of the controls (Figure 20). Changes in the mycelial morphology were clearly visible. Differences in shape more pronounced in treatments OR and WE at concentration of 100 mg/l in solid phase and EO at concentration of 100 mg/l in volatile phase with coral and swollen forms.

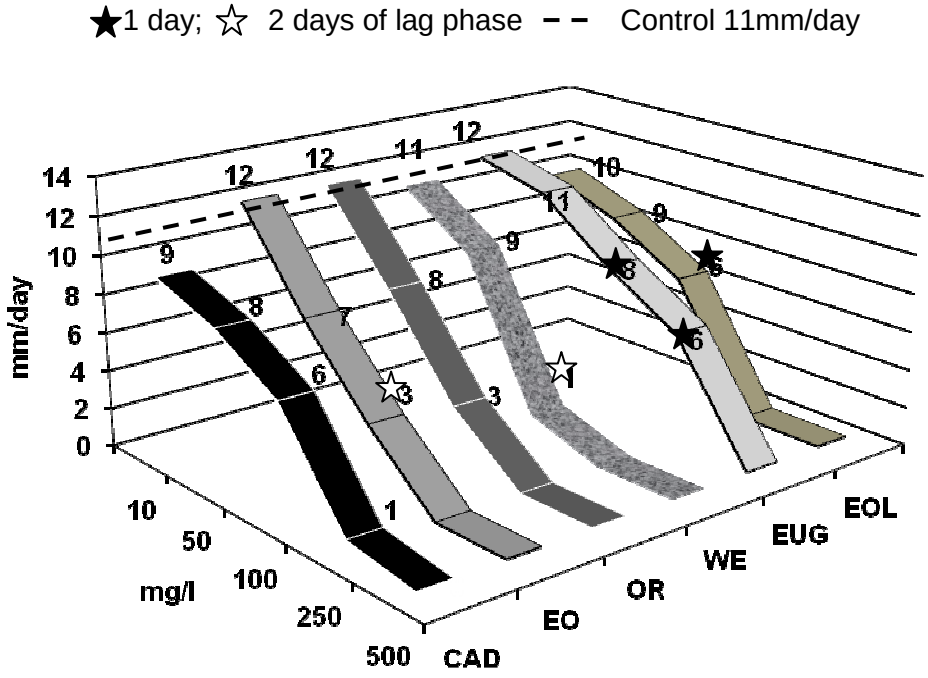

Figure 18. Growth rate (mm/day) of *Phytophtora capsici* affected by different treatments on solid phase

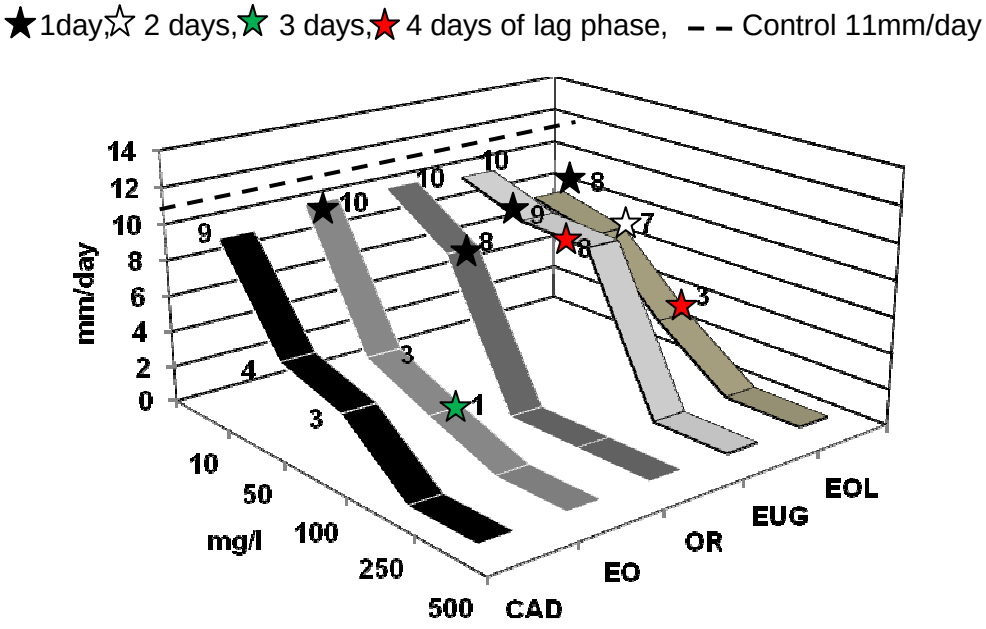

Figure 19. Growth rate (mm/day) of *Phytophtora capsici* affected by different treatments in volatile phase

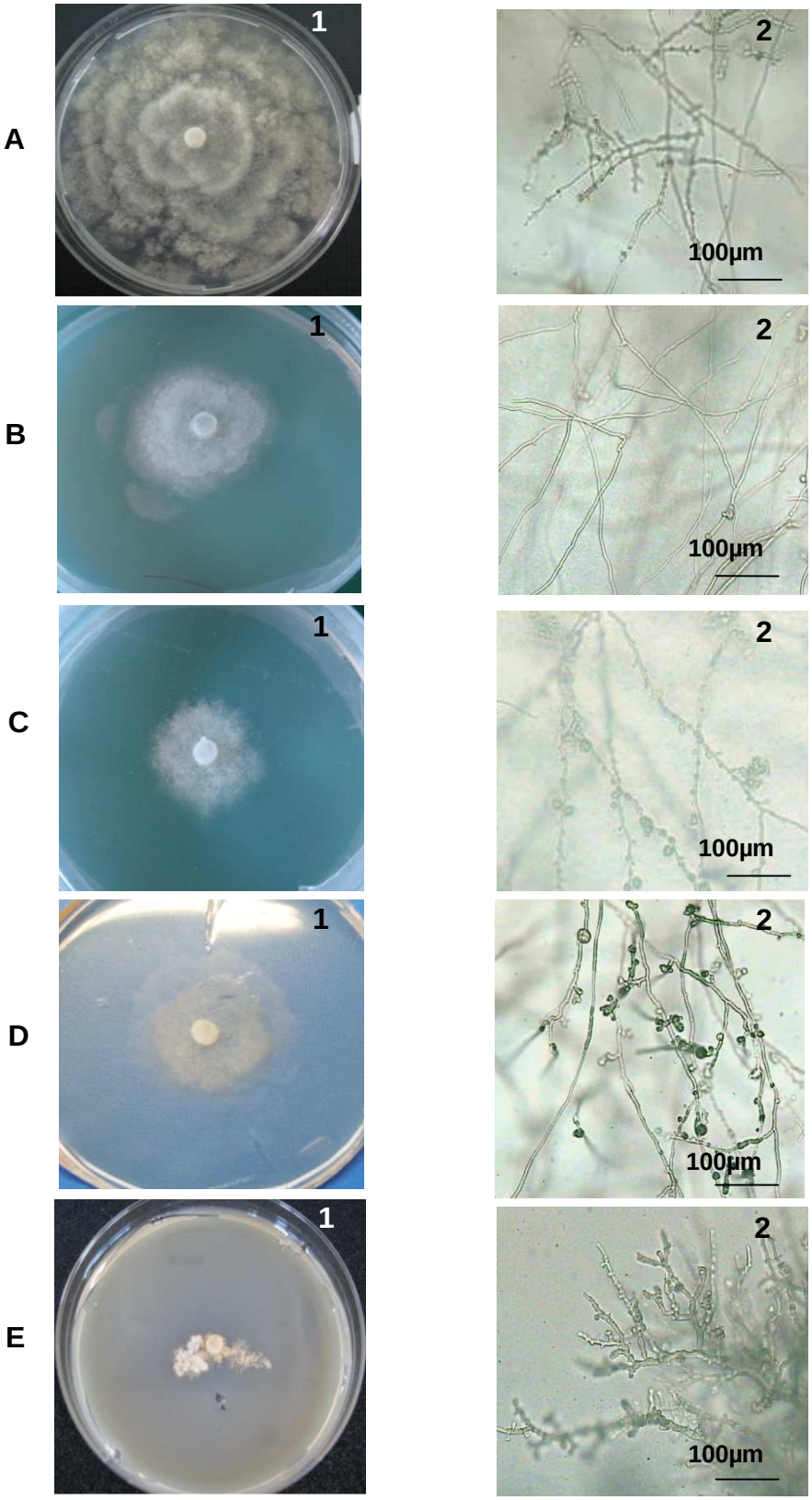

Figure 20. Effects of cinnamon extracts on the mycelial (1) and hyphal (2) morphology of *Phytophtora capsici* observed with a light microscope (A. Control; B.CAD solid phase at 100 mg/l; C.EOL solid phase at 100mg/l; D. EOL volatile phase at 100 mg/l; E. WE at 100mg/l).

3.3.2.2. Conidia and zoospores germination assay

3.3.2.2.1. Botrytis cinerea conidia germination

Results obtained from the effect of cinnamon extracts on conidia germination of *Botrytis cinerea* are showed in Table 11 and 12. After 24 hours of exposure there were no effects on conidial germination in all treatments, demonstrating the occurrence of lag phase of 1 day for all treatments. Due to this, results are not shown. The extracts at different concentrations caused an interesting inhibition. Results revealed that after 24, 48 and 72 hours, cinnamon extracts were effective and completely inhibited conidia germination (germ tube growth) in liquid phase. Although, EUG and EOL failed to inhibit germination at 10 mg/l.

However, after exposure in volatile phase, conidia initiated germination after 48 hours. The effect of cinnamon extracts at 10 mg/l reduced germination by 21%, 26%, 73%, 86% and 94% in OR, EUG, CAD, EOL and EO, respectively. At 50 mg/l inhibition was above 80%.

At the highest concentration, 250 mg/l and 500mg/l in liquid and volatile phase conidia germinations were completely inhibited. Compared to CAD, it was observed that EO, OR and WE showed greater effect during three days of exposure, whereas EUG was less effective than EOL.

Table 11. Percentage of conidia germination inhibition of *Botrytis cinerea* by different treatments in liquid and volatile phase after 48 hours of incubation

Extracts		Extracts concentration (mg/l)				
		10	**50**	**100**	**250**	**500**
Liquid phase	**CAD**	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EO	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	OR	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	WE	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EUG	0	100^{a}	100^{a}	100^{a}	100^{a}
	EOL	0	100^{a}	100^{a}	100^{a}	100^{a}
Volatile phase	**CAD**	94^{b}	100^{a}	100^{a}	100^{a}	100^{a}
	EO	73^{e}	100^{a}	100^{a}	100^{a}	100^{a}
	OR	21^{l}	96^{ab}	100^{a}	100^{a}	100^{a}
	EUG	26^{h}	81^{d}	100^{a}	100^{a}	100^{a}
	EOL	86^{cd}	92^{b}	97^{a}	100^{a}	100^{a}

Values reported represent the average of three replicates. Means within a row followed by the same letter are not significantly different at P<0.05 (Duncan test)

Table 12. Percentage of conidia germination inhibition of *Botrytis cinerea* by different treatments in liquid and volatile phase after 72 hours of incubation

Treatment		Extracts concentration (mg/l)				
		10	**50**	**100**	**250**	**500**
Liquid phase	**CAD**	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EO	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	OR	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	WE	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EUG	0	100^{a}	100^{a}	100^{a}	100^{a}
	EOL	0	100^{a}	100^{a}	100^{a}	100^{a}
Volatile phase	**CAD**	85.2^{cd}	87.1^{c}	98.1^{a}	100^{a}	100^{a}
	EO	77.7^{e}	97.1^{ab}	97.8^{a}	100^{a}	100^{a}
	OR	36.0^{g}	96.8^{ab}	98.8^{a}	100^{a}	100^{a}
	EUG	9.3^{h}	49^{f}	84.7^{cd}	97.3^{ab}	100^{a}
	EOL	75.7^{e}	82.9^{d}	94.1^{b}	100^{a}	100^{a}

Values reported represent the average of three replicates. Means within a row followed by the same letter are not significantly different at P<0.05 (Duncan test)

After 72 hours of incubation, above 80 % conidia germination inhibited at 50 mg/l except EUG (49%), while above 85 % of inhibition at 100 mg/l was observed in all treatments (Table 12).

The above results showed the activity of cinnamon extracts on complete suppression of conidia germination at highest concentrations. The fungitoxic potency of the essential oils might be due to the synergism between their components as reported by Amiri *et al*., (2008).

This is in agreement with previous results of Tzortzakis (2008). He showed high fungicidal activity of EO against *Botrytis cinerea.* In addition, Amiri *et al*., (2008), found that mixture of eugenol and soy lecithin suppressed the phytotoxic symptoms produced by eugenol on apples and reduced the disease incidence caused by *Botrytis cinerea* to less than 4 % after 6 months of storage at 2 °C.

3.3.2.2.2. Phytophtora capsici zoospores germination

The effect of cinnamon extracts on zoospores germination of *Phytophtora capsici* are showed in Table 13 and 14. After 24, 48 and 72 hours of incubation in liquid phase, cinnamon extracts completely inhibited zoospores germination in all concentrations. After 24 hours of exposure there were no effects on conidial germination in all treatments, demonstrating the occurrence of lag phase of 1 day for all treatments. Due to this, results are not shown.

In volatile phase, after 48 and 72 hours, CAD, EO and OR completely inhibited zoospores germination at 50 mg/l. EUG and EOL were less effective in comparison to CAD, EO and OR. Inhibition varied from 29 % to 43 % after 48 and 72 hours at lower concentration of 10 mg/l, while at 100 mg/l more than 80 % inhibition was observed.

Results indicated that zoospores started to germinate after 48 hours of incubation at concentrations ranged from 10 mg/l to 100 mg/l. Differences were observed between the treatments and concentrations. CAD, EO, OR and WE showed strong activity in both liquid and volatile phases.

Table 13. Percentage of zoospores germination inhibition *of Phytophtora capsici* by different treatments in liquid and volatile phase after 48 hours of incubation

Extracts		Extracts concentration (mg/l)				
		10	**50**	**100**	**250**	**500**
Liquid phase	**CAD**	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EO	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	OR	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	WE	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EUG	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EOL	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
Volatile phase	**CAD**	94^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EO	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	OR	97^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EUG	29^{h}	49^{f}	91^{b}	100^{a}	100^{a}
	EOL	43^{gh}	86^{cd}	91^{b}	100^{a}	100^{a}

Values reported represent the average of three replicates. Means within a row followed by the same letter are not significantly different at P<0.05 (Duncan test)

Table 14. Percentage of zoospores germination inhibition *of Phytophtora capsici* by different treatments in liquid and volatile phase after 72 hours of incubation

Treatment		Extracts concentration (mg/l)				
		10	**50**	**100**	**250**	**500**
Liquid phase	**CAD**	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EO	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	OR	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	WE	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EUG	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EOL	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
Volatile phase	**CAD**	65.4^{d}	100^{a}	100^{a}	100^{a}	100^{a}
	EO	98.1^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	OR	98.6^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	EUG	35.0^{gh}	67.3^{d}	89.1^{b}	100^{a}	100^{a}
	EOL	34.5^{gh}	73.6^{c}	92.7^{b}	100^{a}	100^{a}

Values reported represent the average of three replicates. Means within a row followed by the same letter are not significantly different at P<0.05 (Duncan test)

3.3.2.3. Vitality assay

Fungistatic or fungicidal effects of treatments were examined on conidia and zoospores vitality test. Results presented in Table 15. In fact, at concentration higher than 50 mg/l complete inhibition of conidia and zoospores germination were observed in all extracts. The effect on conidia and zoospores seems to be fungicidal rather than fungistatic. The effect of EUG and EOL seems to be fungistatic at concentration ranging between 50 mg/l and 100 mg/l.

Table 15. Vitality test: Percentages of germinated conidia and zoospores

Extracts	Extracts concentrations (mg/l)															
	10				**50**				**100**				**250**			
	SDW		**PDA**		**SDW**		**PDA**		**SDW**		**PDA**		**SDW**		**PDA**	
	BC	PH	BC	PH	BC	PH	BC	PH	BC	PH	BC	PH	BC	PH	BC	PH
CAD	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
EO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OR	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
WE	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
EUG	100	0	100	0	0	0	3	0	0	0	2.7	0	0	0	0	0
EOL	100	0	100	0	0	0	1.6	0	0	0	0	0	0	0	0	0

BC -*Botrytis cinerea,* PH-*Phytophtora capsici,* SDW- Sterilized Distilled Water, PDA- Potato Dextrose Agar

Botrytis cinerea* conidia** ***Phytophtora capsici zoospores

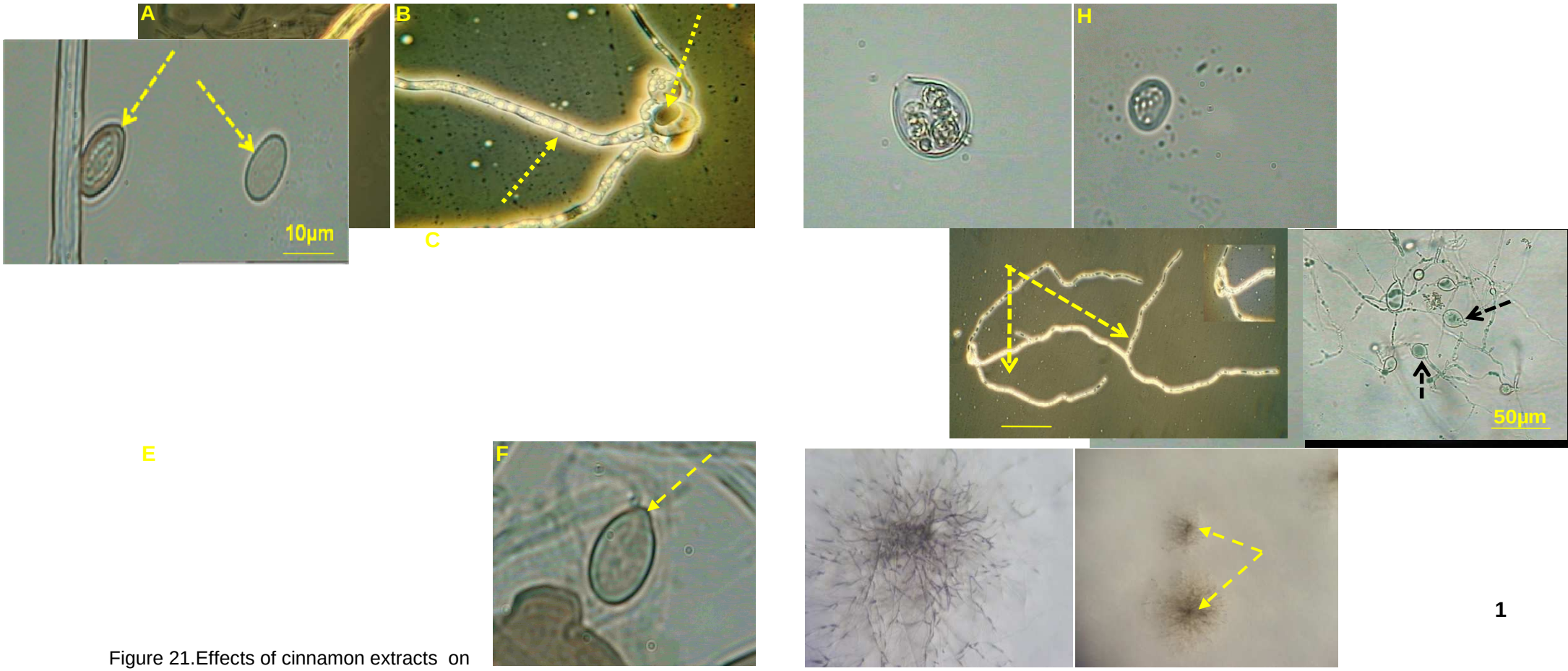

Figure 21.Effects of cinnamon extracts on conidia and zoospores of *Botrytis cinerea* and *Phytophtora capsici* (**A,B,C,D**. Vital conidia with presence of germ tube; **E1**.EUG solid phase 100 mg/l; **E2,F2**.CAD solid phase100 mg/l; **G,H**. zoospore in control ;**I,J**. zoospore in EUG 10 mg/l phase, K,L. zoospore in EUG and CAD 10 mg/l in volatile phase)

Microscopic observation of the control (untreated cell) showed a regular cell structure with homogenous cytoplasm and smooth surface, while fungi cell treated with CAD and EUG in liquid phase at 100 mg/l, showed considerable alteration with no cytoplasm or depleted level of cytoplasm (Figure 21).

The antifungal activities of cinnamon extracts against important phytopathogens *Botrytis cinerea* and *Phytophtora capsici* clearly showed strong inhibitory effect on fungi mycelia growth, conidia and zoospores germination. It is suggested that high efficacy of cinnamon extract is related to cinnamaldehyde and eugenol, as well as to various organic acids that have consistently been reported by different authors to show antifungal activities (Gill and Holly, 2004).

Amoung the extracts, EO, OR and WE were the most active in all cases. Nevertheless, it is importance note as identified by GC-MS analysis, essential oils and oleoresins are very heterogeneous mixtures of a single substance, biological actions are primarily due to these components in a very complicated concert of synergistic or antagonistic effects as reported by Singh *et al*., (2007). In addition, the antifungal properties of WE might be expected to be attributed to its major components that is many proanthocyanidins / (epi) catechins in addition CAD. As reported by Shan *et al.,* (2007), that proanthocyanidins-(epi) catechins from cinnamon stick exhibited strong antibacterial properties.

On the other hand, results showed that mycelial growth inhibition under solid phase was weaker than in volatile phase. Amiri *et al*., (2008), reported that is expected that the headspace volatile will increase in concentration following their release, which might be triggered by change in environment (e.g. increased temperature and humidity). In addition, due to the high lipohilic nature of mycelia coupled with large surface area relative to the volume of a fungus, volatile components may act mainly by accumulation on mycelia than in the agar (Inouye *et al*., 2000).

Phytophtora capsici tended to be more sensitive and more affected than *Botrytis cinerea.* This it can be due to the cell wall composition. Indeed, the cell walls of *Phytophtora sp.* are composed of cellulose and β-glucans and not chitin. Nevertheless, chitin appears to play a central structural role, conferring rigidity, physical strength and specific shape to the cell wall (Lipke and Ovalle, 1998), while *Botrytis cinerea* has β-glucans and chitin in its cell wall. On the other hand, *Phytophtora sp.* has short regeneration time because of the rapid production of flagellate zoospores (Erwin and Ribeiro, 2005).

There are many possible explanations for the antifungal activities. Some authors have suggested that the damage to the cell wall and cytoplasmic membrane was the loss of structural integrity and the ability of membrane to act as a permeability barrier (Carmo *et al*., 2008). In addition of inhibitory effects of CAD which is a cell wall active antifungal agents, inhibits the growth of fungal strains by inhibiting cell wall synthesizing enzymes, it may be useful lead compound for the development of antifungal agents through

the control of β-(1-3)-glucan and chitin synthesis in yeast and mold (Bang *et al*., 2000).

Velluti *et al.* (2003) report that eugenol presents its antimicrobial activity attributed to the presence of an aromatic nucleus and a phenolic OH group known to be reactive and to form hydrogen bonds with active sites of target enzymes, resulting in the deactivation of enzymes in fungi.

Some essential oils, plant extracts and oleoresins influence certain biochemical and/or metabolic functions such as respiration or production of toxins or acids, indicating that the active components in various oils and oleoresins may have different specificity with regard to target sites on or in microbial cells (Lopez-Malo *et al*., 2005).

Conclusions and recommendations

The main objectives of this work were to investigate the *in vitro* phytotoxicity and antifungal properties of cinnamon extracts and to identify and quantify active compounds.

A number of conclusions can be drawn from the work reported here:

Different extraction methods were applied on two types of cinnamon quillings. Results showed significant differences of extraction yields among extracts and materials, the yields were in this order EE> WE> OR> EO. Therefore, cinnamon extracts qualities and quantities are strongly correlated to the raw material and extraction types. Sample B1 showed the highest yield in all extracts.

Chemical analysis of the composition of different extracts allowed to detect a wide range of chemicals. CAD and EUG were major components present in bark and leaf oil, respectively. Results showed that extraction time affects significantly CAD and EUG contents, only 20 minutes of extraction gives the highest amounts in WE.

Free radical scavenging of phenolic compounds is an important property underlying their various biological activities. Determination of total polyphenols and antioxidant activities by DPPH method, showed a high radical scavenging capacity of cinnamon extracts. The antioxidant activity of polyphenols is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching oxygen, or decomposing peroxides. Total polyphenols compounds contents correlated with the antioxidant activities in all extract. Cinnamon bark extracts contain high amount of phenolic antioxidant activities.

Biological activities *in vitro* study revealed the effectiveness of cinnamon extract to induce phytotoxic and antifungal effects. Therefore, significant phytotoxic effect in all extract on seeds germination and root elongation were observed. The higher phytotoxicity was induced by water extract in all species probably due to the presence of polymeric polyphenols.

Cinnamon extract showed significant antifungal activity against *Botrytis cinerea* and *Phytophtora capsici* confirming the effects of cinnamon extracts on suppression of fungi mycelia growth, conidia and zoospores germination. *Phytophtora capsici* tended to be more sensitive to treatments. The effect seems to be fungicidal; this potency of cinnamon extract might be due to synergism between their components. Inhibition in volatile phase seems to be much more significant than direct contact. Thus, the volatile phase appears to be promising source in active packaging creating a protective atmosphere with minimum organoleptic alteration of the packaged foodstuffs.

In comparison among cinnamon extracts biological activities, EO, OR and WE extracts exhibited the strongest activities than CAD, while EUG was less active than EOL. CAD was more active than EUG. As seen in this study, the biological activity of cinnamon extracts might be due to CAD and EUG alone or in interaction with small amounts of identified components.

Results obtained in this study are only first step in seeking substances from natural source and biologically active. Based on the obtained results the following is strongly recommended:

1. Further studies are necessary in order to determine whether cinnamon could be applied in a manner that will be fungicidal but not phytotoxic. Accordingly, there is a need to develop a formulation to improve the biological effectiveness for agricultural applications and environmental performances.

2. Chemical compounds in extracts having significant positive or negative effects (phytotoxic or antifungal) should be studied in detail for their specific effects.

3. A large variety of fungal strains should be tested with cinnamon extract to validate the activities.

Such report provides a big impetus to evaluate these compounds alone or in combination to identify their potential in commercial formulations that can be used as bio-pesticides in organic and integrated pest management.

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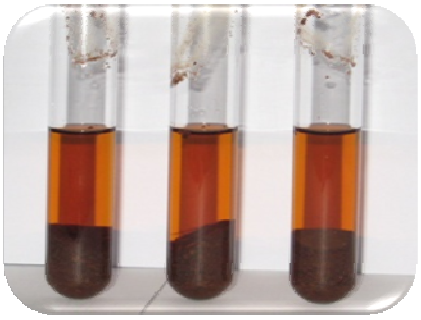
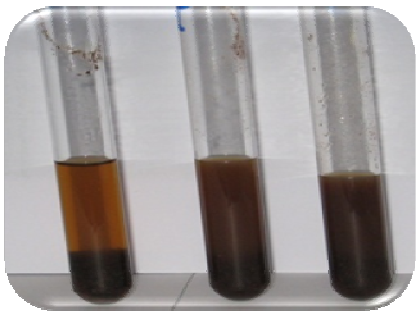
Annexes

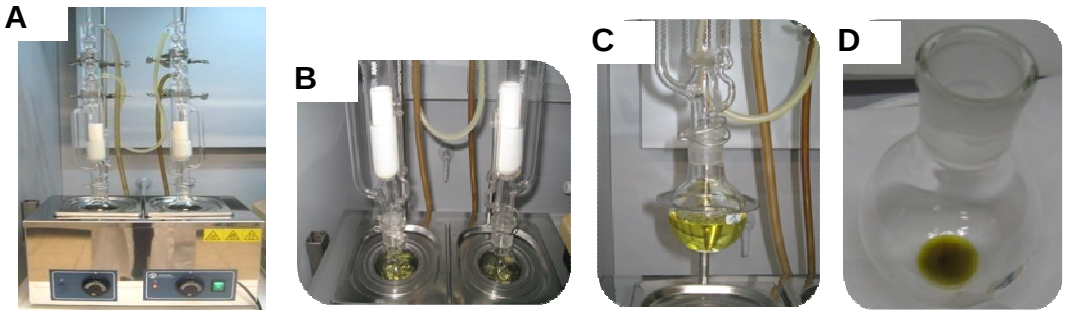
Annexe 1. Extraction procedures for compositional analysis of different cinnamon extracts



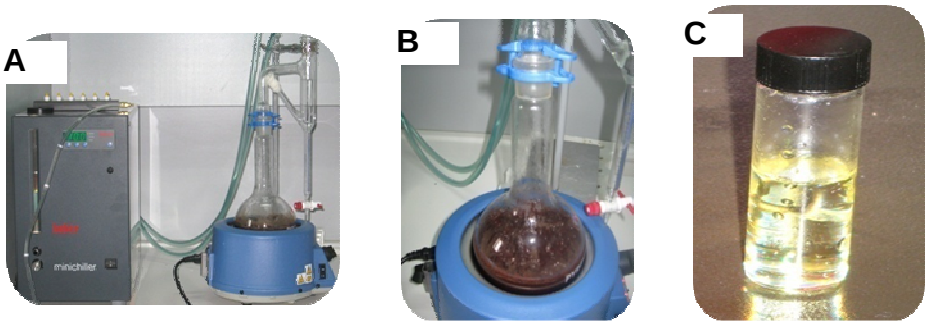
Water extract



Ethanol extract



Soxhlet apparatus (A) and oleoresin extraction (B, C and D)



Clevenger apparatus (A) and essential oils extraction (B and C)

Annexe 2. Effects of cinnamon extracts on root elongation of tested species in *in vitro* study

Root elongation (mm) of *Lolium perenne* seeds and their corresponding dose response relation

Extracts	Concentrations (mg/l)						
	5	10	25	50	100	175	250
CAD	45.3 ^{a}	41.6 ^{abcd}	40.9 ^{abcd}	37.1 ^{def}	21.0 ^{k}	12.8 ^{mn}	0
EO	33.2 ^{fghi}	30.0 ^{ghi}	29.5 ^{hi}	23.5 ^{jk}	15.2 ^{lm}	11.3 ^{mn}	0
OR	42.5 ^{abc}	39.6 ^{bcde}	35.1 ^{efg}	19.6 ^{kl}	8.5 ^{no}	0	0
WE	39.4 ^{bcde}	38.0 ^{cdef}	29.4 ^{hi}	12.2 ^{mn}	5.7 ^{o}	0	0
EUG	30.7^{ghi}	28.4 ^{ij}	24.1 ^{jk}	19.5 ^{kl}	12.3 ^{mn}	5.8 ^{o}	0
EOL	43.7^{ab}	36.7 ^{def}	34.5 ^{efgh}	28.1 ^{ij}	12.4 ^{mn}	0	0

Values reported represent the average of five replicates. Means within a row followed by the same letter are not significantly different at P<0.05 (Duncan test)

Root elongation (mm) of *Lycopersicum esculentum* seeds and their corresponding dose response relation

Extracts	Concentrations (mg/l)						
	5	10	25	50	100	175	250
CAD	31.0 ^{ab}	27.4 ^{bcd}	22.0 ^{ef}	17.5 ^{hij}	7.7 ^{lmn}	0	0
EO	27.4 ^{bcd}	24.2 ^{cde}	18.3 ^{gf}	12.4 ^{jkl}	6.1 ^{mno}	0	0
OR	24.4 ^{cde}	21.9 ^{ef}	14.1 ^{ijk}	7.7 ^{lmn}	5.6 ^{mno}	0	0
WE	22.9 ^{def}	20.2 ^{ef}	9.1 ^{klm}	4.8 ^{no}	2.0 ^{o}	0	0
EUG	28.2 ^{bc}	22.7 ^{def}	20.9 ^{ef}	15.0 ^{hij}	11.4 ^{jkl}	0	0
EOL	33.4 ^{a}	26.9 ^{bcd}	20.6 ^{ef}	19.7 ^{gf}	15.1 ^{hij}	0	0

Values reported represent the average of five replicates. Means within a row followed by the same letter are not significantly different at P<0.05 (Duncan test)

Root elongation (mm) of *Lepidium sativum* seeds and their corresponding dose response relation

Extracts	Concentrations (mg/l)						
	5	10	25	50	100	175	250
CAD	50.2 ^{a}	47.8 ^{ab}	43.9 ^{abcde}	33.3 ^{ghij}	16.8 ^{kl}	0	0
EO	44.1 ^{abcde}	44.4 ^{abcde}	42.1 ^{bcdef}	32.8 ^{ghij}	17.4 ^{kl}	0	0
OR	47.4 ^{abc}	45.8 ^{abcd}	41.5 ^{bcdef}	29.6 ^{j}	10.4 ^{l}	0	0
WE	39.8 ^{cdefg}	40.2 ^{bcdefg}	28.2 ^{j}	19.3 ^{k}	3.0 ^{m}	0	0
EUG	44.4 ^{abcde}	39.0 ^{defgh}	35.1 ^{fghij}	32.8 ^{ghij}	28.4 ^{j}	6.3 ^{ml}	0
EOL	44.9 ^{abcde}	39.2 ^{defgh}	38.2 ^{defgh}	37.4 ^{efghi}	30.2 ^{ij}	0	0

Values reported represent the average of five replicates. Means within a row followed by the same letter are not significantly different at P<0.05 (Duncan test)