UNIVERSITÀ DEGLI STUDI DI SALERNO

UNIVERSITÀ DEGLI STUDI DI SALERNO
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PhD Thesis in

Chemical and Biological study of natural substances active on the Central Nervous System

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To all who have always believed in me

Abstract

The research project "Chemical and biological study of natural substances active on the Central Nervous System" has been focused on the possible effects of essential oils, their main constituents (in particular monoterpenes), plant extracts and/or their fractions and/or their constituents on the expression of some proteins involved in the adenylate cyclase 1 pathway, on cell electrophysiology and their potential effect on *in vivo* models.

Eight species were selected including three aromatic plants (*Lavandula angustifolia*, *Coriandrum sativum*, *Laurus nobilis*); two *Citrus medica* cultivars (cv '*liscia*' and cv '*rugosa*'); two species of *Ipomea* genus known because also *Ipomea violacea*, a famous 'smart drugs', belongs to this genus; and *Hypericum hircinum* belonging to the same genus of *Hypericum perforatum* known for its antidepressive properties. Essential oils have been obtained from the aerial parts of *L. angustifolia* and *L. nobilis*, from *C. sativum* fruits and from the two cultivars of *C. medica* flavedo, while from the aerial parts of the two *Ipomea* species and *H. hircinum* we obtained different extracts by using solvents with increasing polarity.

We analyzed the chemical composition of essential oils and the extracts by GC-MS for the first one, and by thin-layer chromatography (TLC), adsorption chromatography and HPLC for the second one. With this procedure it was possible to identify the main constituents of essential oils and the fractions with a chemical profile of interest.

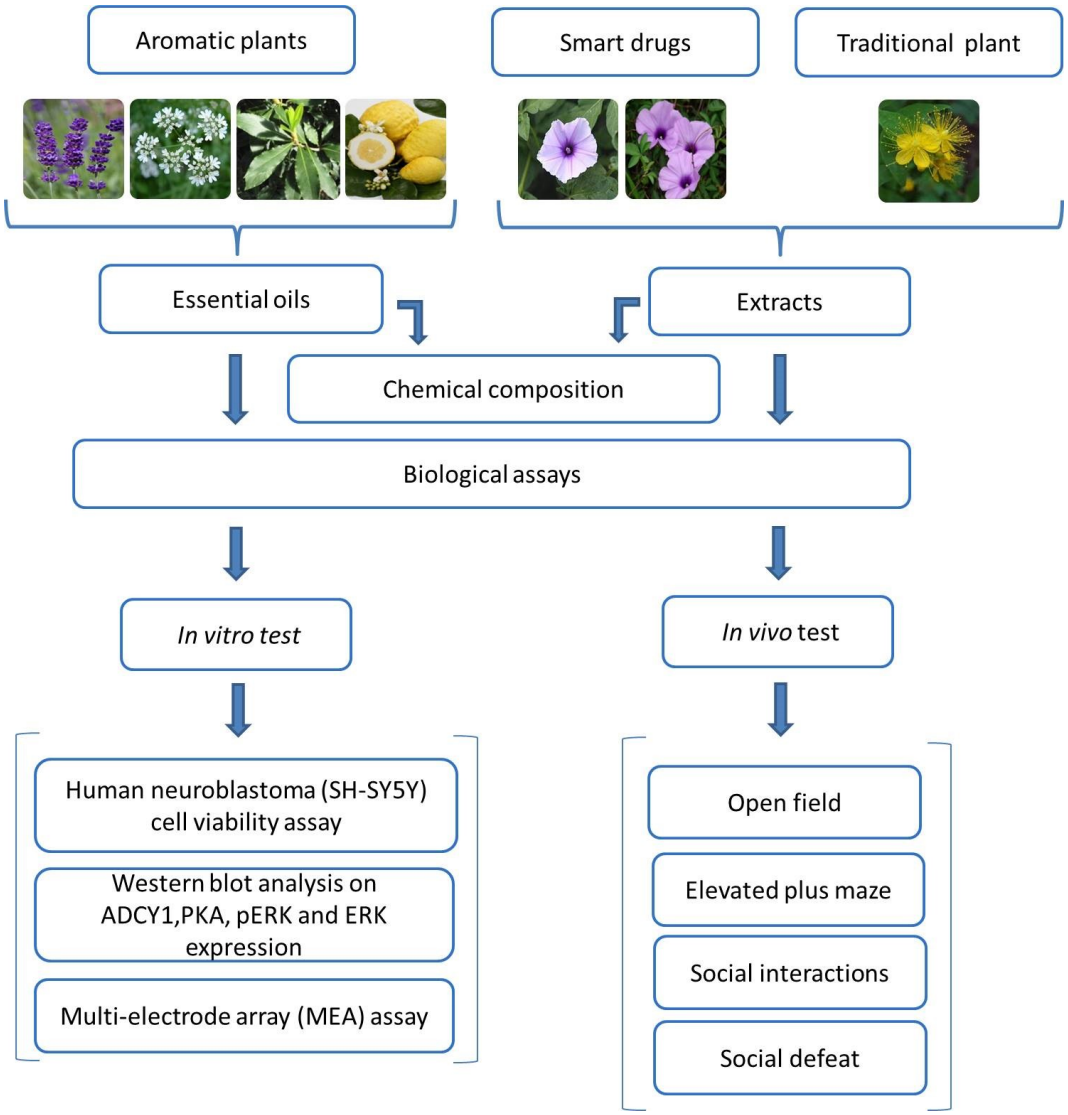
Subsequently, were performed several *in vitro* and *in vivo* assays following a bioassay guided fractionation.

We evaluated the cytotoxicity of the substances on human neuroblastoma cells (SH-SY5Y) in order to determine the most appropriate concentrations to treat the cells to study the effect on adenylate cyclase 1, protein kinase A, pERK and ERK protein expression.

In collaboration with the University of Genoa, we evaluated the possible effects on cellular electrophysiology of *L. angustifolia* essential oil, its main constituent and of *H. hircinum* methanolic extract.

Finally, in the period of research carried out at the Department of Psychobiology of the University of Valencia in Spain, we evaluated the effects of the essential oil of *L. angustifolia* and of the linalool on stress and social interactions with different experimental procedures *in vivo*.

Graphical abstract



1. Introduction

1.1 Plants used in traditional medicine for their effects on Central Nervous System

Traditional medicine is an important source for the study of medicinal plants. Nowadays, this science is revalued by an extensive activity of research on different plant species and their therapeutic principles (Nencini et al. 2006).

Often, in the course of the story, the humans have utilised plants not only as food sources or dietary supplements but also as part of their ritual and healing practices. In most preliterate cultures, a central role in therapeutic rites is played by "magical plants", most of which are represented by hallucinogenic species. The use of these plants is socially accepted and often there is a close relationship between the supernatural and the alteration of the habitual state of consciousness produced by hallucinogenic plants. This fundamental property has led these plants to be considered divine or sacred, and appropriate for use in religious and curative ceremonies. In fact, these species are seen as intermediaries between the human world and that of supernatural forces (Furst 1972). For these reasons, the knowledge and the practice of using plants for healing rituals assumed a special characteristics: often it is secretly kept and conveyed by shamans, priests and other religious figures, who are very knowledgeable about herbs and who combine their botanical, phytotherapeutical and toxicological knowledge with religious elements and rituals based on magic, superstition and ancestral beliefs (Diaz et al. 1979, De Feo et al. 1992).

Phytochemical and pharmacological investigations on some of these species revealed that they really act on Central Nervous System. The rational study of "magical plants" appears now to be a useful and chartered way to study psychoactive drugs. Moreover, data collected clearly show a deep knowledge of these hallucinogenic species.

Some of the “magical” plants belong to the solanaceous genus *Brugmansia* known with vernacular names “floripondio”, “campanchu” or “yerba del diablo”, and are used in the traditional Peruvian medicine to reach altered states of consciousness (De Feo 2003).

Figure 1.1 *Brugmansia arborea* (A); *Iresine herbstii* (B).

Brugmansia sanguinea (R. et P.) D. Don leaves tincture, for example, is claimed to have hallucinogenic effects when absorbed through the nasal mucous and is also used during the ritualistic ceremonies to help in divination (De Feo 2008). *B. arborea* extracts showed the property to reduce morphine withdrawal *in vitro* (Capasso and De Feo, 2002). *Iresine herbstii* exerts important psychotropic effects on CNS (Capasso and De Feo, 2002).

Also *Claviceps purpurea*, a pathogenic fungus that infects rye and cause a disease known as ‘ergot’, has been used in traditional medicine for a long time. A small dose can be used for migraine or blood pressure regulation but their effects on the Central Nervous System (CNS) are deleterious, in fact consumption of rye bread contaminated was responsible for epidemic ergotism that occurred in the past (Douhan et al. 2008).

Figure 1.2 *Claviceps purpurea* sclerotium (A); rye contaminated by *Claviceps purpurea* (B).

1.2 Plant secondary metabolites

The psychoactive properties of plants are attributable to the presence of their secondary metabolites. These chemical compounds are not required for the basic photosynthetic or respiratory metabolism such as primary metabolites, but increase the plant ability to survive and overcome local challenges allowing them to interact with their environment, including pathogens and herbivorous and symbiotic insects (Harborne 2014). One of their fundamental roles is to be allelopathic defenders of plants, against competitor plants (Wink 2003). Moreover, some secondary metabolites are toxic to herbivores acting with their Central and Peripheral Nervous System as agonist or antagonist of neurotransmitter system or forming structural analogs of endogenous hormones (Wink 2003, Miller and Heyland 2010). In some plants, secondary metabolites can give also resistance to salt or drought (Trossat et al. 1998, Nuccio et al. 1999).

Plant secondary compounds are usually classified on basis of their chemical structure and synthetic pathways in three large molecule families: alkaloids, terpenes and phenolic compounds (Bourgaud et al. 2001, Kennedy and Wightman 2011). Alkaloids are a group of nitrogen-containing compounds present in about 20% of plant species; they are specific to define plant genus and species and sparsely distributed in the plant kingdom (Zulak et al. 2006, Bourgaud et al. 2001).

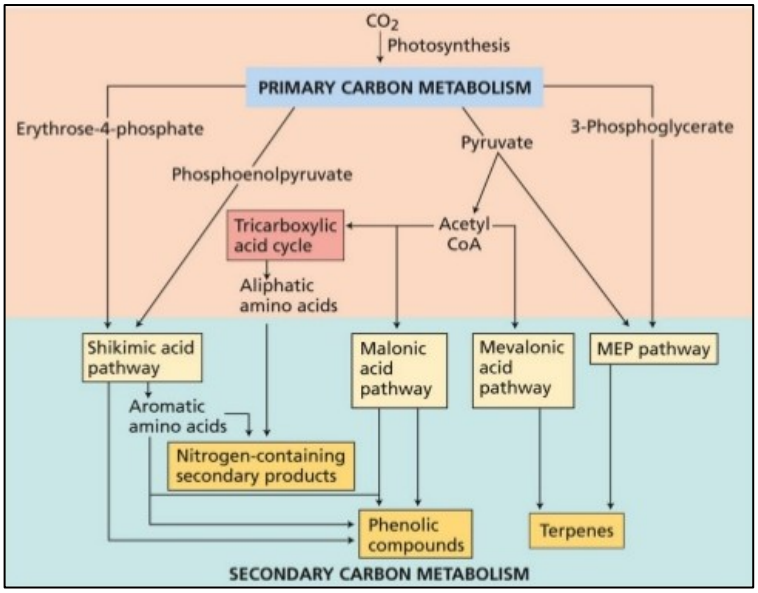


Figure 1.3 Major pathways of secondary metabolite biosynthesis and their relationship with primary metabolism (Taiz and Zeiger 2002).

As secondary metabolites, they play a defensive role against herbivores and pathogens interfering with signal transduction or binding to neuroreceptors (Wink 2000). Often alkaloids are classified on the basis of their structural similarity or their common precursor; some of them are used as hallucinogens, social drugs or psychotropic medicine.

Terpenes are a group of more than 30,000 lipid soluble compounds and are classified according to the number of isoprene units that they contain.

They have different functions: herbivore deterrence, pollinator symbiotes attraction, antigerminative and phytotoxic actions, and insect toxicity, in fact they can affect their CNS (De Almeida et al. 2010, De Martino et al. 2010, Rattan 2010). Moreover, they can be also solvents for mass compounds that would solidify and clog transport systems in plants (Zulak et al. 2006).

Phenolic compounds are synthesized from the phenylpropanoid pathway. Structurally, they have at least one aromatic hydrocarbon ring with one or more

hydroxyl groups attached. These secondary metabolites are common to all higher plants because they are involved in lignin synthesis. Moreover, they attract symbiotic insects and deter herbivores (Bourgaud et al. 2001). The ecological roles of phenolic compounds include functions as phagostimulants, allelopathic agents in intra-plant relationships; they can act also in antioxidant defenses and the absorption of UV light (Treutter 2006).

1.3 Essential oils and aromatherapy

Essential oils (EOs) are natural complex mixtures of volatile compounds isolated usually by hydro-distillation, and characterized by a strong odour. They can be synthesized by all plant organs and possess various biological activities on humans, animals, and other plants.

In nature, essential oils play an important role in the plant care as antibacterial, antivirals, antifungals, insecticides and also against herbivores by reducing their appetite for such plants.

They also may attract or repel insects to favour interplant communication (Baser and Buchbauer 2015). Essential oils are considered as multifunctional agents thanks to their strong stimulation of the human smell because olfactory information reaches a number of cortical areas without being relayed in the thalamus (Wiesmann et al. 2001) (fig. 1.5).

Studies *in vitro* with bacteria and mammalian cells demonstrated that EOs seem to have no specific cellular targets but as lipophilic compound they pass through the cytoplasmic membrane and disrupt its typical structure; this evidence could explain their cytotoxicity (Bakkali et al. 2008).

Moreover, the available literature reports antinociceptive, anticancer, antiviral, antioxidant and anticancer effects of essential oils, among others (Adorjan and Buchbauer 2010, Nunes et al. 2015).

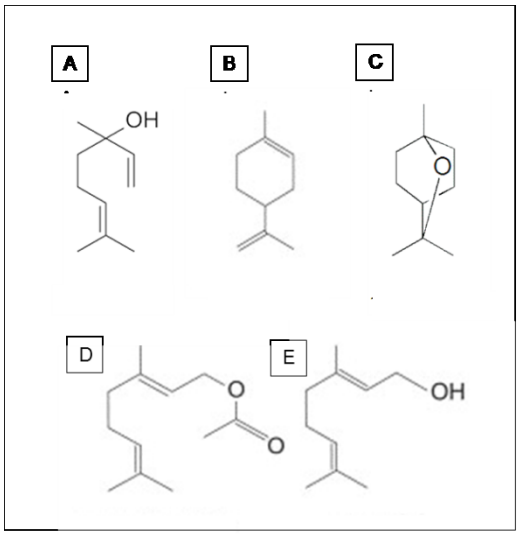


Figure 1.4 Structural formulae of selected components of essential oils: Linalool (A); Limonene (B); 1,8-Cineole (C); Geranyl-acetate (D); Geraniol (E).

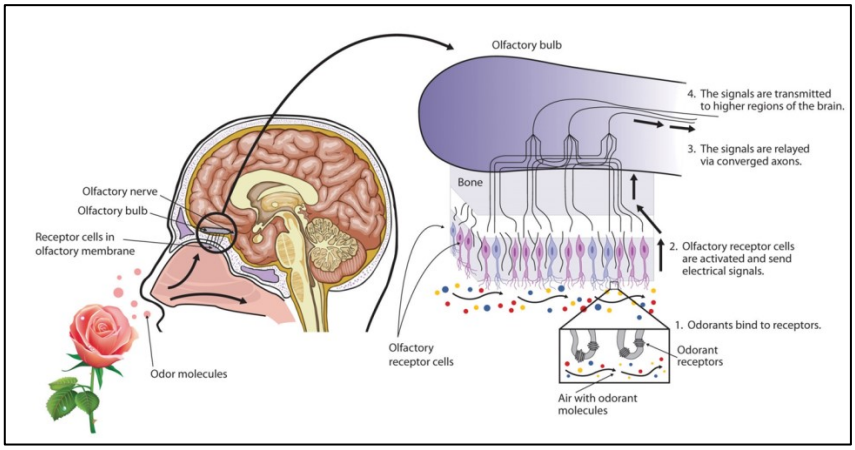


Figure 1.5 Limbic system: how odor molecules act on brain.

Different studies have been carried out to unravel the effects of essential oils and aromatic species on CNS have been reported effects on learning, memory, attention, and effects on the treatment of stress (Dobetsberger and Buchbauer 2011).

Aromatherapy is the therapeutic use of essential oils extracted from leaves, flowers, and other plant parts combined with massage and the olfactory system involvement to treat various physical or psychological conditions (Lee et al. 2011). Its relevance is growing, due to the anxiolytic properties showed by some aromatic plants such as *Lavandula angustifolia* Mill., *Salvia sclarea* L., *Citrus limon* L., *Anthemis nobilis* L. (Setzer 2009, Baser and Buchbauer 2015).

Anxiety is among the most common forms of psychopathology worldwide; the symptoms are shortness of breath, heart palpitations and pale skin. In recent years, its prevalence as a medical condition has increased because both animals and humans are continually exposed to various anxiety-promoting situations in their environment. Usually the treatment of persistent anxiety required the use of benzodiazepines but they have many side-effects so the alternative management of anxiety and social relationships has become salient in contemporary life (Linck et al. 2010, Woelk and Schläfke 2010).

Essential oils are used in aromatherapy also for the effects of their constituents in the treatment and prevention of some diseases related to the Central Nervous System such as epilepsy, Alzheimer's disease and Parkinson's disease (Dobetsberger and Buchbauer 2011, Babar et al. 2015).

1.4 Smart drugs

Nowadays, there is a growing interest in recreational drugs also called 'smart drugs' derived from natural materials, natural products or their simple derivatives. These agents have become popular for personal use to enhance performance in exams or at work, mental energy, concentration and alertness acting upon the synthesis of neurotransmitters, such as acetylcholine (Appendino et al. 2014, Canterbury and Lloyd 1994). It is possible to compare smart drugs with anabolic steroids; in fact, the first enhance mental performance, the second ones improve physical performance.

Figure 1.6 Common smart drugs.

The fundamental difference is that steroids have shown serious negative side-effects, instead there is no sufficient information about smart drugs negative effects (Canterbury and Lloyd 1994, Scheske and Schnall 2012).

Internet have increased the diffusion of smart drugs, that now are available on line in different formulations such as pills, tablets, powders, liquids, chewing gum and single plant material or extracts (Schmindt et al. 2011).

These products represent the legal alternative to cannabis, in fact they can be plants rich in alkaloids or stimulant and psychotropic substances, or plant mixtures that act as a 'shuttle' hiding illegal compounds and allowing them to avoid legal restriction and to be marked (Cornara et al. 2013).

The main problem is the identification of these herbal mixtures because often the material is very fragmented. Cornara et al. demonstrated, with combination of micromorphological, molecular and chemical technique, that it is necessary a multidisciplinary approach to identify plant material and to understand if plants contain alkaloids or play a 'green shuttle' role (Cornara et al. 2013).

1.5 Plant selected

1.5.1 Lavandula angustifolia

Figure 1.7 *Lavandula angustifolia*.

Order: Lamiales

Family: Labiatae (Lamiaceae)

Species: *Lavandula angustifolia* Mill.

Plant morphology: Lavender is a perennial herbs or small shrubs with purple-blue aromatic flowers. The leaves are linear or lanceolate with herbaceous branches. The flowers are small with blue caliber; the blue or violet corolla has 4 stamens.

Linalool, camphor, terpinen-4-ol, linalyl acetate, β-ocymene and 1,8-cineole are reported to be the main components of lavender essential oil (Price 1993, Koulivand et al. 2013). This essential oil possesses different biological activities and only few studies have investigated the effects of its major constituent, linalool, on brain activity or specific receptor populations (Elisabetsky et al. 1995; Elisabetsky et al. 1999).

Re and coworkers showed that linalool inhibits acetylcholine release at the neuromuscular junction modifying ion channel function (Re et al. 2000). Linalool has also antidepressant like effects in fact decrease the immobility time in tail suspension test (Coelho et al. 2013).

Studies *in vivo* have demonstrated that an intraperitoneal injection of lavender essential oil enhanced rotarod activity and increased dopamine receptors subtype D3 expression in the olfactory bulbs of mice (Kim et al. 2009). Moreover, this essential oil improves the cognitive performance of scopolamine induced mice, showing a neuroprotective effect in Alzheimer disease model *in vivo* (Xu et al. 2016).

1.5.2 Coriandrum sativum

Figure 1.8 *Coriandrum sativum*.

Order: Apiales

Family: Apiaceae (Umbelliferae)

Species: *Coriandrum sativum* L.

Plant morphology: Coriander is an erect herbaceous plant up to 80 cm tall with isolated and pinnate leaves, the upper leaves instead are more irregularly divided into narrowly linear segments. The inflorescences are umbels with peduncles up to 8 cm long and flowers are small white to pinkish. Fruits are subglobose.

Coriander is originating from the Mediterranean region and cultivated in different parts the world. All parts of the plant are edible, the plant and its fruits are used as a spice in different countries. In folk medicine, the fruits of coriander are recommended for the treatment of anxiety, insomnia or for relief of

nervousness (Mandal and Mandal 2015). Moreover it is useful in the treatment of inflammation, indigestion, vomiting, dysentery, diarrhoea (Varier 1994). Leaf preparations have been used to treat coughs and abdominal discomforts (Bruneton 1995). Linalool, geranyl acetate, nerol and neral are the main components of *C. sativum* essential oils (Ebrahimi et al. 2010).

This oil showed inhibitory activity against Gram-positive and Gram-negative bacteria, indicating that it is adapt for food preservation (Matasyoh et al. 2009).

Studies *in vivo* have reported coriander extracts and its essential oil for potential hypnotic sedative activities, anxiolytic, muscle relaxant and anticonvulsant effects (Emamghorashi and Heidari-Hamedani 2004, Hosseinzadeh and Madanifard 2005, Emamghorashi and Heidari-Hamedani 2006, Mahendra and Bisht 2011).

1.5.3 Citrus medica

Figure 1.9 *Citrus medica*.

Order: Geraniales

Family: Rutaceae

Species: *Citrus medica* L.

Plant morphology: Citron is a woody plant with simple leaves, has white flowers with 5 petals, 5 sepals and stamens stacked in bunches. The carpels are welded throughout their length.

Citron, native to Southeast Asia, was imported to the Mediterranean around 300 B.C. probably, it arrived in Italy through the Hebrews who introduced the cultivation of citron on the Calabrian coasts, Amalfi Coast, and Garda Lake (Gabriele et al. 2009; Savo et al. 2011). Two local cultivars of *Citrus medica* L. are grown on the Amalfi Coast: *C. medica* cv. '*liscia*', known by the vernacular name of 'cedro', and *C. medica* cv. '*rugosa*', known as 'ponsino'. These two cultivars contributed to the agricultural biodiversity of this area, as well as other Citrus species. However, their diffusion is decreasing, due to the technical difficulties for their cultivation and to the competition of lemon cultivations.

The taxonomy of Citrus species is complex. In fact, recent genetic analyses have shown that only three species belong to the genus Citrus: *C. maxima* (Burm.) Merr., *C. medica* L., and *C. reticulata* Blanco (Uzun and Yesiloglu 2012). Moreover, the *Citrus* species are able to crossbreed, producing fruits with a wide range of morphological and organoleptic characteristics. Today, the fruits of both cultivars are used locally only for fresh alimentary consumption. In past times, both citrons have also been employed in traditional medicine as an anti-infective, an anti-inflammatory, and to treat digestive disorders. Fruits and leaves are used in different countries in the treatment of allergic inflammation, for treating colds, as a decongestant, an expectorant, and a carminative, in the treatment of pathologies of the intestinal tract and rectum, as well as a stomachic, an antispasmodic, a diuretic and a digestive (Yeung 1985, Uzun and Yesiloglu 2012).

The citron essential oils are used for flavouring, for perfuming, in fruit beverages, in soft drinks, in cosmetics, and in household products (Yeung 1985). Different studies reported evidence that *Citrus* consumption is associated with a reduced cancer incidence (Li et al. 2010). Menichini and coworkers (2010) reported the chemical profile and the photo-induced cytotoxic activity of *Citrus bergamia* Risso and Poit. and *Citrus medica* cv. 'Diamante'. Both oils exhibited a

selective inhibition of the A375 tumoral cell line. Russo and coworkers (2014) studied the cytotoxic effect of the Bergamot (*Citrus x bergamia* Risso & A. Poit.) essential oil on SH-SY5Y neuroblastoma cells and its components, limonene and linalyl acetate, were able to induce cell death.

There are no studies on *Citrus medica* essential oil effects *in vivo*. Nevertheless, CNS effects of other *Citrus* species are reported: *C. limon* essential oil has showed a sedative and anxiolytic effect, probably involving the $GABA_A$ receptor complex (Lopes Campelo et al. 2011); *C. aurantium* enhances the time spent in the open arms of elevated plus maze, suggesting an anxiolytic effect (Carvalho-Freitas and Costa 2002).

1.5.4 Laurus nobilis

Figure 1.10 *Laurus nobilis.*

Order: Laurales

Family: Lauraceae

Species: *Laurus nobilis*

Plant morphology: Laurel is an evergreen dioecious tree or shrub. The leaves are alternate leathery with slightly wavy margins. The leaves have an aromatic smell and taste a little bitter. The flowers have a white perianth and they are grouped in small umbels. The fruit is a black ovoid drupe containing a single seed.

The laurel is native to the southern parts of Europe and the Mediterranean area; is widely cultivated in many countries. Its dried leaves and the essential oil deriving from leaves are used as a valuable spice and a flavouring agent in culinary and food industry. The leaves have been used, in Iranian folk medicine, to treat epilepsy, neuralgia, and parkinsonism (Zargari 1990, Aqili 1992). Leaves and fruits have been reported to possess aromatic, stimulant, and narcotic properties (Abu-Dahab et al. 2014). Several studies reported the antimicrobial and the antioxidant properties of laurel essential oil and/or extracts (Santoyo et al. 2006, Derwich et al. 2009, Ozcan et al. 2010).

The leaves of *L. nobilis* are traditionally used orally to treat the symptoms of gastrointestinal problems, such as epigastric bloating and flatulence (Qnais et al. 2012). The essential oil of laurel leaves is widely used in the perfume and soap industries (Kosar et al. 2005). Moreover, it has been used for relieving haemorrhoid and rheumatic pains (Zargari 1990). It also has diuretic and antifungal activities (Zargari 1990, Aqili 1992, Patrakar et al. 2012). Sayyah and coworkers (2002) showed that *Laurus nobilis* essential oil showed a sedative effect protecting NMRI mice against seizure and reduced time spent on rotarod.

1.5.5 Ipomea transvaalensis

Figure 1.11 *Ipomoea transvaalensis*

Order: Solanales

Family: Convolvulaceae

Species: *Ipomea transvaalensis* A. Meeuse

Plant morphology: Perennial plant with long fusiform tuberous root-stock. Stems are annual, herbaceous, and suberect or prostrate, up to 1 m long. Leaves are narrowly deltoid-cordate to broadly cordate-suborbicular, up to 45 mm long. Corolla is funnel-shaped, 20-40 mm long, pink to magenta or white with purple centre. The most peculiar aspect is the bright orange fuzzy seeds.

There are no studies in literature on *Ipomea transvaalensis* extracts and their biological activities and composition.

1.5.6 Ipomea cairica

Figure 1.12 *Ipomoea cairica* flowers.

Order: Solanales

Family: Convolvulaceae

Species: *Ipomea cairica* (L.) Sweet

Plant morphology: A rampant perennial climber reaching up to 5 m or more in height, or creeping along the ground. The alternately arranged leaves are divided into five or seven narrow lobes. The tubular flowers are purple to pinkish-purple with a darker purple centre. The fruit capsules are more or less globular and turn from green to brown in colour. The seeds have smooth surfaces interspersed with dense tufts of long silky hairs.

An *I. cairica* ethanolic extract presents an antinociceptive effect instead aqueous extract showed anti-RSV (respiratory syncytial virus) activity *in vitro*

(Ma et al. 2002, Ferreira et al. 2006). Lima and Braz-Filho (1997) demonstrated that the main constituents of the *I. cairica* methanol extract were scopoletin, umbelliferone and arctigenin. Arctigenin presents antioxidant, anti-inflammatory and cytotoxic activities (Cho et al. 2004). Moreover, *I. cairica* essential oil has showed larvicidal properties (Thomas et al. 2004).

Only few studies have been carried out to explore the *in vivo* effects of *Ipomea* species on Central Nervous System. Sivaraman and Muralidaran (2010) demonstrated that *Ipomea aquatica* methanol extract had a CNS depressant activity in Swiss albino mice; instead Herrera-Ruiz and co-workers (2007) studied the biological activities of *Ipomea stans* ethyl acetate extract highlighting its anxiolytic, anticonvulsant and potential sedative effects.

1.5.7 Hypericum hircinum

Figure 1.13 *Hypericum hircinum.*

Order: Malpighiales

Family: Hypericaceae

Species: *Hypericum hircinum*

Plant morphology: An herbaceous perennial growing freely wild in uncultivated round, woods, hedges, roadsides, and meadows. Leaves pale green, sessile and oblong. Flowers have five sepals and petals and are yellow. Calyx and corolla are marked with black dots and lines.

Some plants belonging to the genus *Hypericum* are used in different part of the world as traditional plants (Yazaki and Okada 1994). Unlike *H. perforatum*, known as St. John's wort, which has been the subject of many phytochemical and pharmacological studies in the treatment of moderate depression (Greeson et al. 2001), no many studies have been carried out on *H. hircinum* biological activities. This plant is traditionally used in Lucanian folk medicine for the treatment of cough (Pieroni et al. 2004) and in Sardinian medicine for its antiseptic properties and in form of decoction to treat bronchitis (Ballero et al. 1997).

H. hircinum essential oil shows antioxidant, and antiproliferative activities (Quassinti et al. 2013). Pistelli and coworkers (2000) demonstrated that methanolic extract have a stronger activity against *Staphylococcus aureus* than the isolated constituents (quercetin, quercitrin, biapigenin). *Hypericum hircinum* leaves methanol extract contain shikimic acid, chlorogenic acid, rutin, quercetin, quercetin-7-O-glucoside (Mandrone et al. 2017). Quercetin inhibits the viral enzyme that catalyzes the HIV dsDNA integration into the cell genome (HIV-1 Integrase) and has MAO-inhibitory properties (Lee et al. 2001, Vandegraaff et al. 2001).

Studies *in vivo* on *Hypericum* species reported controversial results: *H. hircinum* and *H. perfoliatum* extracts reduced CD1 mice locomotor activity in open field test highlighting an anxiogenic activity (Diana et al. 2007). Instead Beijamini and Andreatini (2003) reported that the administration of *H. perforatum* produces anxiolytic effects in an elevated plus maze.

1.6 SH-SY5Y cells as a model for *in vitro* studies on Central Nervous System

SH-SY5Y cells, derived from human neuroblastoma, have been often utilized as a cellular model for *in vitro* experiments in neuroscience because of their many

biochemical and functional characteristic of neuronal cells (Xie et al. 2010, Kovalevich and Langford 2013).

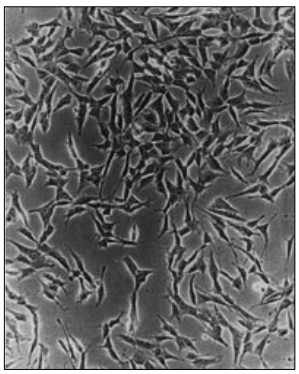


Figure 1.14 Neuroblastoma human cells (SH-SY5Y) (Raguenez et al. 1999).

In fact, this cell line have showed catecholaminergic neuronal properties such as moderate activity of dopamine-β-hydroxylase and tyrosine hydroxylase (Biedler et al. 1978, Ross and Biedler 1985), and basal noradrenaline release (Påhlman 1984).

These properties have made SH-SY5Y cells adapt as a model to study neurotoxicity, neuroprotection and Parkinson's disease for their dopaminergic characteristics (Xie et al. 2010). Moreover, they are used to investigate secondary messengers response associated with delta opioid and muscarinic receptors (M_1, M_2 and M_3) (Lambert and Nahorski 1990, Vaughan et al. 1995).

1.7 Role of adenylyl cyclase 1, Protein kinase A and Extracellular Signal Regulated Kinase in Central Nervous System

Cyclic adenosine-3',5'-monophosphate (cAMP) is involved in different physiological cell functions such as differentiation, development and cellular

death; in particular in neurons can regulated synaptic plasticity, learning and memory process and chronic pain (Willoughby and Cooper 2007, Zhuo 2012).

Adenylyl cyclase (ADCY) is an integral membrane protein composed of two repeated domains, each containing six hydrophobic transmembrane segments, short loops that linked them and two cytoplasmic regions (C1 and C2) responsible for forskolin- and G-protein stimulated catalysis and catalyses the conversion of ATP into a key intracellular second messenger cAMP (Zhang et al. 1997) (fig. 1.15).

Nine isoforms of membrane-bound adenylyl cyclases (ADCY1-9) modulated by G proteins and one cytoplasmic isoform of soluble ADCY have been reported with their own tissue distribution and distinct biochemical properties (Sunahara and Taussig 2002, Pavan et al. 2009).

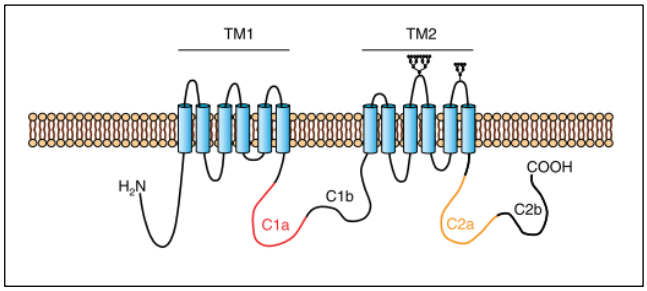


Figure 1.15 Adenylyl cyclase (ADCY) structure. The protein can be divided into 2 transmembrane domains (TM1 and TM2 with extracellular N-glycosylation sites) and 2 cluster cytoplasmic loops (C1 and C2). C1a and C2a are highly conserved catalytic ATP-binding regions, which dimerize to form the catalytic site. C1b and C2b domains are less conserved (Willoughby and Cooper 2007).

ADCY1 is neurospecific and is expressed downstream from the glutamate N-methyl-D-aspartate (NMDA) receptors in the areas of the brain associated with neuronal plasticity (Abdel-Majid et al. 1998, Zhuo 2012).

Intracellular levels of cAMP regulate Protein kinase A (PKA) (fig. 1.16), which begins an enzymatic cascade of phosphorylation reactions in the cell activating the Rap1 B-Raf complex.

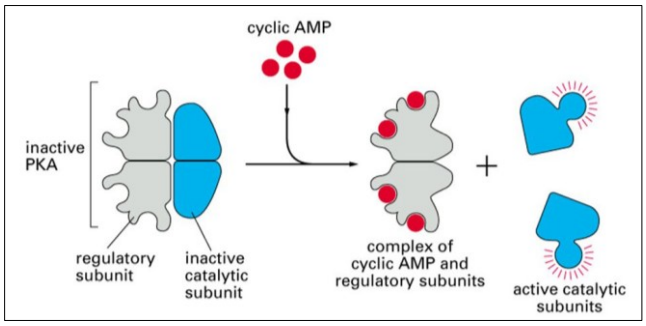


Figure 1.16 cAMP dependent protein kinase A (PKA) (Alberts et al. 2017).

This cytoplasmatic pathway ends with the phosphorylation of Extracellular Signal Regulated Kinase (ERK) that allows this protein to translocate into the nucleus of neurons where activate CREB-dependent gene transcription (Impey et al. 1998, Duhan et al. 1999, Kawasaki et al. 1999, Sweatt 2000, Zanassi et al. 2001) (fig.1.17).

In the nervous system, ERK pathway is implicated in a number of different forms of plasticity, including activation of gene transcription, structural modification at the synapse, receptor insertion and regulation of dendritic protein synthesis (Davis and Laroche 2006). The activation of this pathway occurs in a variety of locations and situations, some of which contribute to painful conditions (Cruz and Cruz 2007). ERK is involved in the learning process, acquisition and maintenance of long-term memory in mammals (Martin et al. 2000). However, PKA stimulation of ERK activity may regulate neuronal survival and synaptic plasticity (Grewal et al. 1999).

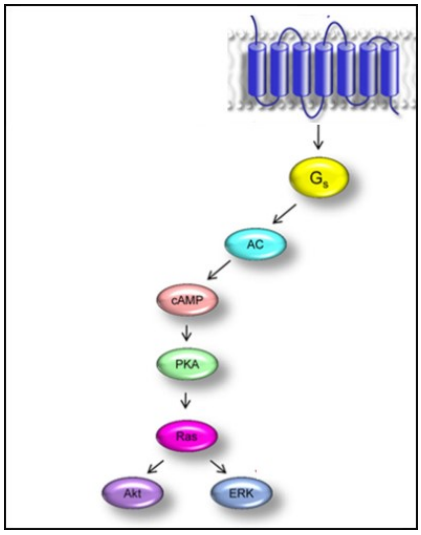


Figure 1.17 Schematic representation of signalling pathway studied (Guseva et al. 2014).

2. Aim of the study

The aims of this study are:

- to select vegetal species with a possible activity on CNS;

- to characterize the chemical composition of essential oils derived from *Lavandula angustifolia* and *Laurus nobilis* aerial part, from *Coriandrum sativum* fruits and from the peel of the fruits of the two cultivars of *Citrus medica*;

- to characterize the chemical composition of extracts derived from *Ipomea transvaalensis*, *Ipomea cairica* and *Hypericum hircinum* aerial parts;

- to evaluate the cytotoxicity of the EOs, their main constituent and the extracts against SH-SY5Y cell line;

- to study the role of EOs, their main constituent and the extracts on expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cell line;

- to study the possible influence of *Lavandula angustifolia* and *Coriandrum sativum* essential oils and their principal component in cellular electrophysiology;

- to study *in vivo* the possible effects on central nervous system of *Lavandula angustifolia* essential oil and its main constituent, linalool.

3. Results

3.1 Essential oils composition

Tables 3.1-3.4 show the percent chemical composition of the essential oils; compounds are listed according to their elution order on a HP-5MS column

3.1.1 Lavandula angustifolia

Hydrodistillation of the aerial parts of *L. angustifolia* furnished a pale yellow oil in 5% on a dry mass basis. Altogether, 59 compounds were identified, accounting for 97.3% of the total oil. Linalool (33.1%), linalyl acetate (10.4%), 1,8-cineole (8.0%) and borneol (4.5%) are the main components.

Table 3.1 Chemical composition of the essential oils of *Lavandula angustifolia* (LA).

No.	Compound	LA	Ki[a]	Ki[b]	Identification[c]
1	α-Pinene	0.8	922	939	1,2
2	Camphene	0.6	935	954	1,2
3	Thuja-2,4(10)-diene	0.1	957	960	1,2
4	β-Pinene	0.9	980	979	1,3
5	Myrcene	1.9	985	990	1,2
6	α-Phellandrene	0.2	991	1002	1,2
7	δ-3-Carene	0.9	1000	1008	1,2
8	α-Terpinene	0.2	1000	1017	1,3
9	p-Cymene	0.4	1009	1024	1,2
10	Limonene	2.1	1014	1029	1,2,3
11	1,8-Cineole	8	1017	1031	1,2
12	(Z)-β-Ocimene	0.6	1025	1037	1,2
13	(E)-β-Ocimene	1.4	1036	1050	1,2
14	γ-Terpinene	0.3	1046	1059	1,2
15	cis-sabinene Hydrate	0.2	1057	1070	1,2

16	cis-Linalool oxide	0.1	1063	1072	1,2,3
17	Terpinolene	0.7	1076	1088	1,2,3
18	Linalool	33.1	1099	1096	1,2,3
19	(2E)-Heptenyl acetate	0.2	1100	1113	1,2
20	Menth-en-2-ele-1-ol	t	1109	1121	1,2,3
21	Allo-Ocymene	1.3	1115	1132	1,2
22	trans-pinocarveol	0.1	1125	1135	1,2
23	Camphor	11.0	1135	1146	1,2,3
24	Lavandulol	0.1	1153	1169	1,2
25	Borneol	4.5	1155	1160	1,2
26	Pinocarvone	0.1	1165	1164	1,2
27	Neo-iso-Isopulegol	2.3	1166	1171	1,2
28	cis-Linalool oxide	t	1172	1170	1,2,3
29	Menthol	0.2	1177	1171	1,2
30	α-terpineol	1.6	1182	1188	1,2
31	Hexyl butanoate	0.5	1183	1192	1,2
32	Nerol	0.2	1215	1229	1,2
33	Hexyl-(2E)- butanoate	0.3	1228	1242	1,2
34	Linalyl acetate	10.4	1247	1257	1,2
35	Iso-3-Thujanol acetate	0.1	1275	1270	1,2
36	Neo-3-Thujanol acetate	0.1	1281	1276	1,2
37	α-Terpinen-7-ale	0.1	1283	1285	1,2
38	ρ-Cymen-7-ol	t	1297	1290	1,2
39	Terpinyl acetate	0.1	1315	1317	1,2
40	Mirtenyl acetate	t	1315	1326	1,2,3
41	α-Terpinyl acetate	0.8	1355	1349	1,2
42	α-Cubebene	0.2	1368	1348	1,2
43	α-Copaene	1.4	1374	1376	1,2
44	β-Cubebene	0.2	1378	1388	1,2
45	Longifolene	0.2	1394	1407	1,2
46	(Z)-Caryophyllene	3.3	1408	1408	1,2
47	(E)-Caryophyllene	0.4	1413	1419	1,2,3
48	cis- Thujopsene	0.3	1424	1431	1,2
49	β-copaene	0.3	1433	1432	1,2

50	α-Guaiene	0.3	1442	1439	1,2
51	6,9-guaiadiene	0.9	1445	1444	1,2
52	alloaromadendrene	0.3	1458	1466	1,2
53	cis-muurola-4(14,5)diene	0.2	1468	1468	1,2
54	β-selinene	0.3	1486	1490	1,2
55	δ-selinene	0.5	1490	1492	1,2
56	γ-cadinene	0.2	1511	1513	1,2
57	Caryophyllene oxide	0.4	1572	1583	1,2,3
58	epi-α-cadinol	0.2	1629	1640	1,2
59	β-bisabolol	1.2	1672	1675	1,2
	Total compounds	97.3			
	Oxygenated Monoterpene	72.9			
	Monoterpene	12.6			
	Sesquiterpenes	9.2			
	Oxygenated sesquiterpenes	1.6			

[a]Kovats retention index on HP-5 MS column; [b]Kovats retention index on HP Innowax; [c]1 = Kovats retention index, 2 = mass spectrum, 3 = coinjection with authentic compound; t = trace, less than 0.05 %

3.1.2 Coriandrum sativum

Hydrodistillation of fruits of *C. sativum* furnished a pale yellow oil in 2.1% yield on a dry mass basis. Thirty-six compounds were identified accounting for 99.3% of the total oil. The main compounds are linalool (67.8%), α-pinene (5.0%) and camphor (5.0%). Other compounds, in a lesser amount are *p*-cymene (2.8%), γ-terpinene (2.7%) and limonene (2.6%).

Table 3.2 Chemical composition of the essential oils of *Coriandrum sativum* (CS).

No.	Compound	CS	Ki[a]	Ki[b]	Identification[c]
1	α-Thujene	0.1	909	929	1,3
2	α-Pinene	5.0	922	939	1,2
3	Camphene	1.0	935	954	1,2
4	Sabinene	0.7	961	975	1,2
5	β-Pinene	0.8	980	979	1,3
6	Myrcene	0.1	985	990	1,2
7	δ-3-Carene	0.1	1000	1008	1,2
8	p-Cymene	2.8	1009	1024	1,2
9	Limonene	2.6	1014	1029	1,2,3
10	1,8-Cineole	0.1	1017	1031	1,2
11	(Z)-β-Ocimene	1.1	1025	1037	1,2
12	(E)-β-Ocimene	0.1	1036	1050	1,2
13	γ-Terpinene	2.7	1046	1059	1,2
14	*cis*-sabinene Hydrate	t	1057	1070	1,2
15	*cis*-Linalool oxide	0.7	1063	1072	1,2,3
16	Terpinolene	0.5	1076	1088	1,2,3
17	Linalool	67.8	1099	1096	1,2,3
18	Camphor	5.0	1135	1146	1,2,3
19	Borneol	0.3	1155	1160	1,2
20	Terpinen-4-ol	0.3	1167	1177	1,2
21	*p*-cimen-8-ol	0.1	1176	1182	1,2
22	α-terpineol	0.6	1182	1188	1,2
23	Methyl Chavicol	0.1	1188	1196	1,2
24	Safranal	t	1197	1196	1,2
25	*n*-decanal	t	1207	1201	1,2
26	Citronellol	0.3	1217	1225	1,2
27	Neral	0.1	1230	1238	1,2
28	Geraniol	2.0	1248	1252	1,2
29	Geranial	0.1	1268	1267	1,2
30	Thymol	0.1	1296	1290	1,2
31	10-undecenal	t	1294	1299	1,2

32	Mirtenyl acetate	0.2	1315	1326	1,2,3
33	Neryl acetate	t	1346	1361	1,2
34	(E)-2-undecenal	0.1	1359	1360	1,2
35	Geranyl acetate	3.7	1382	1381	1,2
36	(E)-Caryophyllene	0.1	1413	1419	1,2,3
	Total compounds	99.3			
	Oxygenated Monoterpene	77.8			
	Monoterpene	17.6			
	Sesquiterpenes	3.7			

[a]Kovats retention index on HP-5 MS column; [b]Kovats retention index on HP Innowax; [c]1 = Kovats retention index, 2 = mass spectrum, 3 = coinjection with authentic compound; t = trace, less than 0.05 %

3.1.3 Citrus medica

Hydrodistillation of the peel from fruits of *C. medica* cv. '*liscia*' and *C. medica* cv. '*rugosa*' gave yellow essential oils characterized by a typical citrusy and floral odor, with yields of 0.9% and 0.75%, respectively.

In all, 100 compounds were identified, 82 for *C. medica* cv. '*liscia*', accounting for 91.4% of the total oil, and 88 for *C. medica* cv. '*rugosa*' accounting for 92.0% of the total oil. Monoterpene hydrocarbons are the main constituents in both oils, 79.1% for cv. '*liscia*' and 80.2% for cv. '*rugosa*'. In both oils, limonene (67.2%–62.8%), camphene (8.5%–10.9%), and β-pinene (1.4%–1.7%) were other main components.

In the oil from *C. medica* cv. '*liscia*' other components in a lesser amount are geranyl acetate (0.9%), and α-trans-bergamotene (0.5%); in the oil from cv. '*rugosa*' geraniol (0.7%), geranial (0.7%), neral (0.5%), isopulegol (0.7%), and α-bisabolol (0.5%) are present.

Table 3.3 Chemical composition of the essential oils isolated from the peels of *Citrus medica* cv. '*liscia*' (CL) and *C. medica* cv. '*rugosa*' (CR).

No.	Compound	CL	CR	Ki[a]	Ki[b]	Identification[c]
1	α-Thujene	-	0.1	915	930	1,2
2	α-Pinene	0.8	1.2	921	939	1,2
3	α- Fenchene	0.1	0.1	934	952	1,2
4	Camphene	8.5	10.9	964	954	1,2
5	β-Pinene	1.4	1.7	980	979	1,3
6	α-Phellandrene	0.5	0.6	991	1002	1,2
7	δ-2-Carene	0.1	0.3	1004	1002	1,2
8	*p*-Cymene	-	0.1	1012	1024	1,2
9	Limonene	67.2	62.8	1022	1029	1,2,3
10	(Z)-β-Ocimene	t	0.1	1028	1037	1,2
11	(E)-β-Ocimene	0.1	0.3	1038	1050	1,2
12	γ-Terpinene	0.3	0.7	1047	1059	1,2
13	Linalool oxide furanoid	0.3	t	1064	1072	1,2
14	*trans*-Linalool oxide	-	t	1086	1086	1,2
15	Terpinolene	0.1	0.3	1077	1088	1,2,3
16	Linalool	0.3	1.3	1091	1096	1,2,3
17	α-Pinene oxide	t	0.1	1095	1099	1,2
18	Perillene	t	t	1103	1103	1,2
19	1,3,8-*p*-Menthatriene	-	t	1100	1110	1,2
20	*trans*-Thujone	t	0.1	1106	1114	1,2,3
21	Dehydro Sabina ketone	0.1	0.1	1111	1120	1,2
22	*allo*-Ocymene	t	0.1	1119	1132	1,2
23	*cis*-*p*-Mentha-2,8-dien-1-ol	-	t	1126	1137	1,2
24	*cis*-Limonene oxide	-	0.5	1133	1136	1,2
25	*trans*-Limonene oxide	-	t	1140	1142	1,2,3
26	neo *allo*-Ocimene	-	t	1152	1144	1,2
27	Isopulegol	-	0.1	1144	1149	1,2
28	Citronellal	t	0.2	1155	1153	1,2
29	Isoborneolo	-	t	1163	1160	1,2,3
30	neo-*iso*-Isopulegol	0.8	0.7	1167	1171	1,2

31	α-Terpineol	0.7	0.6	1180	1188	1,2
32	Hexyl butanoate	-	t	1183	1192	1,2
33	Dihydrocarveol	t	t	1185	1193	1,2
34	Methyl chavicol	-	t	1190	1196	1,2
35	*trans*-4-Caranone	0.3	0.1	1195	1196	1,2,3
36	Decenal	-	t	1198	1196	1,2
37	2-Decanol	0.3	0.1	1202	1199	1,2
38	*cis*-4-Caranone	0.1	0.3	1209	1200	1,2
39	*endo*-Fenchyl acetate	0.9	0.4	1219	1220	1,2
40	Tymol methyl-ether	-	t	1223	1235	1,2
41	Neral	0.1	0.5	1231	1238	1,2
42	Geraniol	0.9	0.7	1246	1252	1,2,3
43	Geranial	0.1	0.7	1261	1267	1,2
44	*n*-Decanol	0.3	-	1263	1269	1,2
45	*trans*-Carvone oxide	0.1	0.1	1276	1276	1,2,3
46	Thymol	-	0.4	1283	1290	1,2,3
47	*p*-Cymene-7-ol	-	t	1292	1290	1,2
48	10-Undecenal	0.1	t	1294	1299	1,2
49	*n*-Nonanyl acetate	0.1	t	1301	1312	1,2
50	Citronellic acid	t	t	1314	1313	1,2
51	δ-Elemene	0.4	0.2	1326	1338	1,2,3
52	α-Terpinyl acetate	t	0.1	1355	1349	1,2,3
53	Citronellyl acetate	0.1	0.1	1343	1352	1,2,3
54	Eugenol	-	t	1348	1359	1,2
55	Neryl acetate	0.7	0.6	1354	1361	1,2,3
56	α-Ylangene	t	t	1364	1375	1,2,3
57	α-Copaene	-	t	1368	1376	1,2,3
58	Geranyl acetate	0.9	0.5	1373	1381	1,2,3
59	β-Patchoulene	0.1	0.1	1380	1382	1,2
60	Methyl eugenol	0.1	0.1	1396	1403	1,2,3
61	Italicene	0.1	t	1399	1405	1,2
62	Sesquithujiene	0.1	t	1403	1405	1,2
63	Longifolene	0.5	0.6	1407	1407	1,2
64	β-Duprezianene	0.1	0.1	1417	1422	1,2

65	γ-Elemene	0.1	0.1	1422	1436	1,2,3
66	α-*trans*-Bergamotene	0.5	0.4	1424	1434	1,2
67	α-Guaiene	t	t	1432	1439	1,2,3
68	Aromadendrene	0.1	0.1	1441	1441	1,2,3
69	(Z)-β-Farnesene	0.1	0.1	1445	1442	1,2,3
70	(E)-β-Farnesene	t	t	1449	1456	1,2
71	*cis*-Cadin-1(6),4-diene	-	t	1457	1463	1,2
72	9-*epi*-(E)-Caryophyllene	t	0.1	1469	1466	1,2,3
73	β-Acoradiene	t	t	1473	1470	1,2
74	γ-Gurjenene	t	-	1478	1477	1,2
75	α-Amorphene	0.1	t	1482	1484	1,2,3
76	Aristolochene	t	t	1486	1488	1,2
77	β-Selinene	0.1	0.1	1490	1490	1,2
78	α-Selinene	1.0	0.6	1496	1498	1,2
79	α-Cuprenene	0.1	t	1502	1505	1,2
80	δ-Amorphene	-	0.1	1511	1512	1,2
81	γ-Cadinene	0.1	-	1523	1523	1,2
82	(Z)-Nerolidol	t	-	1526	1532	1,2
83	γ-Cuprenene	t	t	1530	1533	1,2
84	(E)-Nerolidol	0.3	t	1552	1563	1,2
85	Caryophyllene oxide	-	0.1	1572	1583	1,2,3
86	Globulol	t	t	1580	1590	1,2
87	β-Oplopenone	t	t	1597	1607	1,2
88	Guaiol	0.1	t	1599	1600	1,2
89	1-*epi*-Cubenol	t	t	1618	1628	1,2
90	Eremoligenol	t	-	1629	1631	1,2
91	α-Muurolol	t	-	1631	1646	1,2,3
92	epi-α-Muurolol	0.1	0.1	1644	1642	1,2
93	Pogostol	0.3	t	1647	1653	1,2
94	Cedranol	0.1	0.1	1658	1673	1,2
95	α-Bisabolol	0.1	-	1674	1685	1,2,3
96	Eudesm-7(11)-en-4-ol	t	0.5	1682	1700	1,2
97	(Z)-α-trans-Bergamotol	0.1	-	1688	1690	1,2
98	Nootkatol	0.3	-	1703	1715	1,2

99	(2Z,6E)-Farnesol	t	-	1711	1723	1,2
100	Oplopanone	t	-	1717	1740	1,2
	Total compounds	91.4	92.0			
	Monoterpene hydrocarbons	79.1	80.2			
	Oxygenated Monoterpene	4.8	6.9			
	Sesquiterpenes hydrocarbons	4.2	3.2			
	Oxygenated sesquiterpenes	2.5	1.6			
	Non terpenes	0.8	0.1			

[a]Kovats retention index on HP-5 MS column; [b]Kovats retention index on HP Innowax; [c]1 = Kovats retention index, 2 = mass spectrum, 3 = coinjection with authentic compound; t = trace, less than 0.05 %

3.1.4 Laurus nobilis

The hydrodistillation of the leaves of *L. nobilis*, harvested in Montecorice (Campania, Southern Italy) provided an essential oil characterized by a typical odor, in a yield of 0.57% on the fresh weight. In all, 55 compounds were identified, accounting for 91.6% of the total oil. Oxygenated monoterpenes represent 48.6% of the EO, with 1,8-cineole (31.9%), sabinene (12.2%), and linalool (10.2%) as the main components. Other components were α-terpinyl acetate (5.9%), α-pinene (5.8%), α-terpineol (3.3%), methyl-eugenol (3.3%), neoiso-isopulegol (2.5%), eugenol (1.6%), β-pinene (1.4%), and γ-terpinene (1.0%). Sesquiterpenes represent 3.4% of the oil, the hydrocarbons 3.2% (β-funebrene 0.5%, β-elemene 0.4%, spathulenol 0.4%), and the oxygenated compounds 0.2%.

Table 3.4 Chemical composition of the essential oils of *Laurus nobilis* (LN).

No.	Compound	LN	Ki[a]	Ki[b]	Identification[c]
1	Methyl pentanoate	0.1	850	828	1,2
2	Ethyl isovalerate	0.1	853	858	1,2
3	α-Thujene	0.7	916	930	1,2
4	α-Pinene	5.8	922	939	1,2,3
5	Camphene	0.8	935	954	1,2
6	Sabinene	12.2	962	975	1,2
7	β-Pinene	1.4	980	979	1,2,3
8	α-Phellandrene	0.5	991	1002	1,2,3
9	δ-2-Carene	0.4	997	1002	1,2
10	α-Terpinene	0.6	1004	1017	1,2,3
11	*o*-Cymene	0.3	1013	1026	1,2
12	1,8-Cineole	31.9	1016	1031	1,2,3
13	(Z)-β-Ocimene	0.2	1028	1037	1,2
14	(E)-β-Ocimene	0.2	1038	1050	1,2
15	γ-Terpinene	1.0	1048	1059	1,2,3
16	*cis*-Sabinene hydrate	0.3	1057	1070	1,2
17	o-Mentha-3,8-diene	0.5	1077	1072	1,2
18	*trans*-Sabinene hydrate	0.1	1093	1098	1,2
19	Linalool	10.2	1096	1096	1,2,3
20	*exo*-Fenchol	0.1	1111	1121	1,2
21	*allo*-Ocymene	0.2	1118	1132	1,2
22	*trans*-Sabinol	0.2	1128	1142	1,2
23	Camphor	0.2	1133	1146	1,2,3
24	β-Pinene oxide	0.1	1147	1159	1,2
25	Isoborneolo	0.5	1155	1160	1,2
26	*iso*-Isopulegol	0.6	1157	1159	1,2
27	neo-*iso*-Isopulegol	2.5	1165	1171	1,2
28	α-Terpineol	3.3	1180	1188	1,2,3
29	*cis*-Carveol	0.2	1219	1229	1,2
30	*cis*-*p*-Mentha-1(7),8-dien-2-ol	0.1	1232	1230	1,2

31	*trans*-Sabinene hydrate acetate	0.7	1246	1256	1,2
32	2-(1E)-Propenyl-phenol	0.1	1265	1267	1,2
33	*neo*-3-Thujanol acetate	0.4	1275	1276	1,2
34	α-Terpinen-7-al	0.3	1284	1285	1,2
35	*iso*-Verbanol acetate	0.3	1306	1309	1,2
36	α-Terpinyl acetate	5.9	1340	1349	1,2
37	Eugenol	1.6	1347	1359	1,2,3
38	Cyclosativene	0.1	1360	1371	1,2
39	Longicyclene	0.2	1373	1374	1,2
40	β-Elemene	0.4	1381	1390	1,2
41	Methyl-eugenol	3.3	1394	1403	1,2,3
42	β-Funebrene	0.5	1408	1414	1,2
43	*cis*-Thujopsene	0.2	1427	1431	1,2
44	Spirolepechinene	0.1	1445	1451	1,2
45	*allo*-Aromadendrene	0.1	1449	1460	1,2,3
46	γ-Himalachene	0.1	1474	1482	1,2
47	α-Amorphene	0.1	1483	1484	1,2
48	δ-Amorphene	0.1	1502	1512	1,2
49	δ-Cadinene	0.2	1512	1523	1,2
50	Elemicin	0.5	1546	1557	1,2
51	Spathulenol	0.4	1563	1578	1,2,3
52	Caryophyllene oxide	0.3	1572	1583	1,2,3
53	Thujopsan-2-α-ol	0.1	1580	1587	1,2
54	Viridiflorol	0.2	1591	1592	1,2
55	Eremoligenol	0.1	1630	1631	1,2
	Total compounds	91.6			
	Monoterpene hydrocarbons	34.0			
	Oxygenated monoterpene	48.6			
	Sesquiterpenes hydrocarbons	3.2			
	Oxygenated sesquiterpenes	0.2			
	Phenolic compounds	5.6			

[a]Kovats retention index on HP-5 MS column; [b]Kovats retention index on HP Innowax; [c]1 = Kovats retention index, 2 = mass spectrum, 3 = coinjection with authentic compound; t = trace, less than 0.05 %

3.2 Extracts composition

3.2.1 Hypericum hircinum

Thin layer chromatography (TLC) highlighted the presence of flavonoids in the *Hypericum hircinum* methanol extract that was purified by size-exclusion chromatography on Sephadex LH-20. We obtained 139 fractions; their homogeneity was evaluated by TLC which allowed them to be combined into 15 major fractions (fig. 3.1). Analytic HPLC chromatograms revealed in flavonoid fraction the presence of three flavonoids: isoquercetin, rutin and quercetin (fig.3.2-3.3).

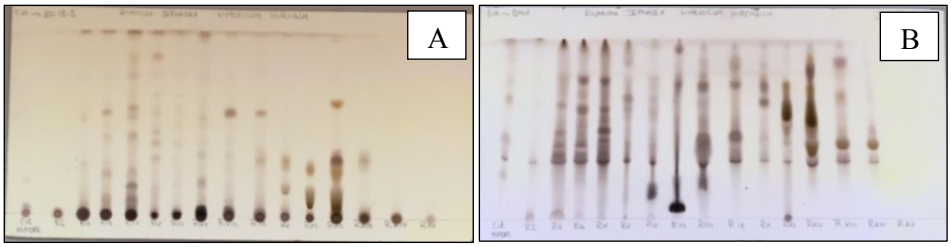


Figure 3.1 TLC profiles of the 15 major fractions (10 µl) developed with mobile phase A ($CHCl_3$: CH_3OH: H_2O; 80:18:2 v/v/v) and B (BAW, $C_4H_{10}O$: CH_3COOH: H_2O; 60:25:15 v/v/v) and revealed with cerium sulfate.

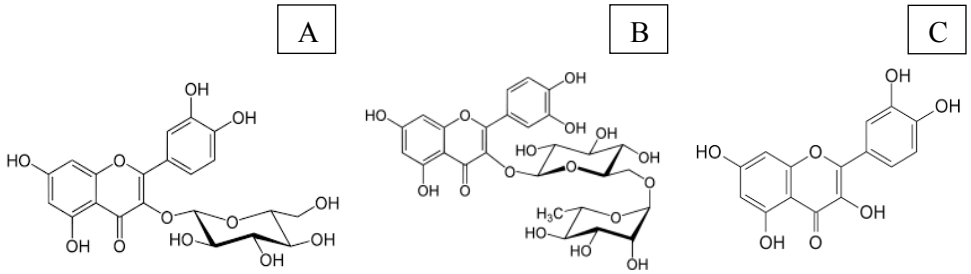


Figure 3.2 Flavonoids identified: Isoquercetin (A), Rutin (B), Quercetin (D).

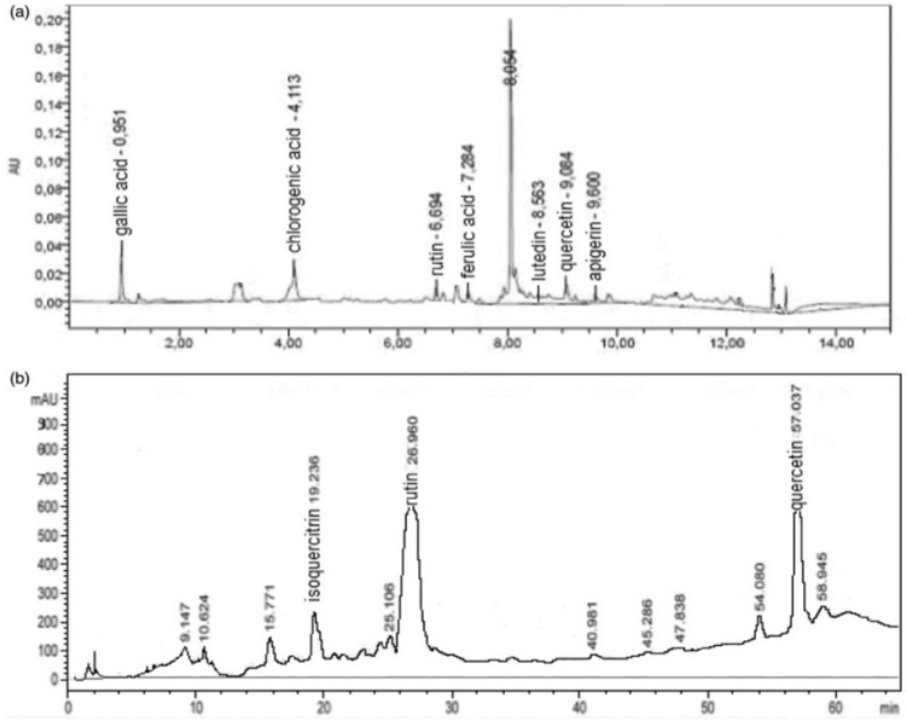


Figure 3.3 Representative HPLC analytic chromatograms of flavonoid fraction.

3.2.2 Ipomea cairica

Thin layer chromatography (TLC) highlighted the presence of alkaloids in the *Ipomea cairica* methanol extract (fig. 3.4) that were purified by size-exclusion chromatography on Sephadex LH-20.

We obtained 138 fractions; their homogeneity was evaluated by TLC which allowed them to be combined into 10 major fractions.

However, no biological activities were found for the extract and its fraction, so the chemical characterization of these extracts was no performed.

Figure 3.4 TLC profiles of the chloroform: methanol (9:1) and methanol extract of *Ipomea cairica* (10 µl) developed with mobile phase BAW ($C_4H_{10}O$: CH_3COOH: H_2O; 60:25:15 v/v/v) showing the alkaloids (orange spots) after revealing them with the Dragendorff's reagent.

3.2.3 Ipomea transvaalensis

Thin layer chromatography (TLC) revealed the presence of alkaloids in the *Ipomea transvaalensis* chloroform: methanol (9:1) extract (fig. 3.5).

We separated the methanolic fraction containing the alkaloids from the chloroform one, by solvent extraction. Then, one gram of the methanol fraction was purified by adsorption chromatography.

We obtained 180 fractions; their homogeneity was evaluated by TLC which allowed them to be combined into 10 major fractions. The fraction X contains alkaloids and had a weight of 100 mg. The yield was very low so we decided to purify alkaloids by extraction with solvents. In this way we obtained 415 mg of alkaloids from 1 g of the methanol fraction of the $CHCl_3$: CH_3OH extract. The alkaloid fraction was purified by RP-HPLC; then structural determination of two isolated compounds (ergine and ergometrine) was performed by ^1H NMR (fig. 3.6-3.7).

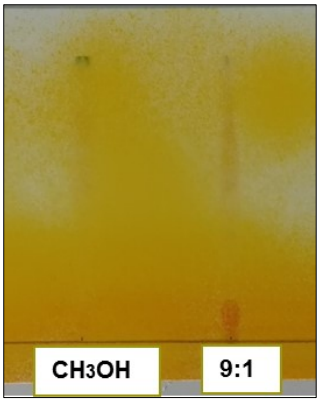


Figure 3.5 TLC profiles of the chloroform: methanol (9:1) and methanol extract of *Ipomea transvaalensis* (10 µl) developed with mobile phase BAW ($C_4H_{10}O$: CH_3COOH: H_2O; 60:25:15 v/v/v) showing the alkaloids (orange spots) after revealing them with the Dragendorff's reagent.

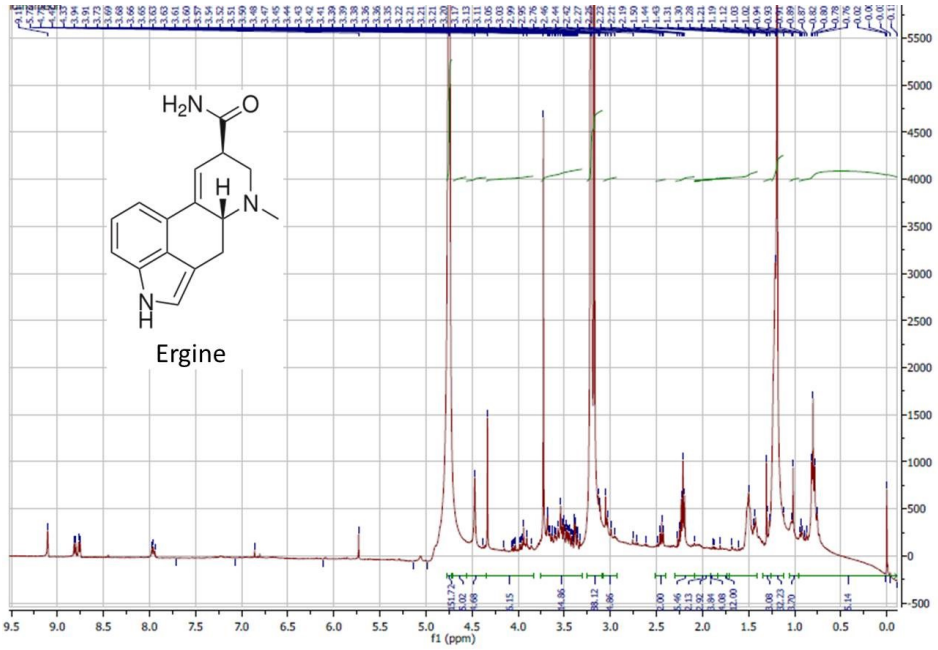


Figure 3.6 ^{1}H NMR Spectrum (600 MHZ, CD_3OD) and molecular structure of Ergine.

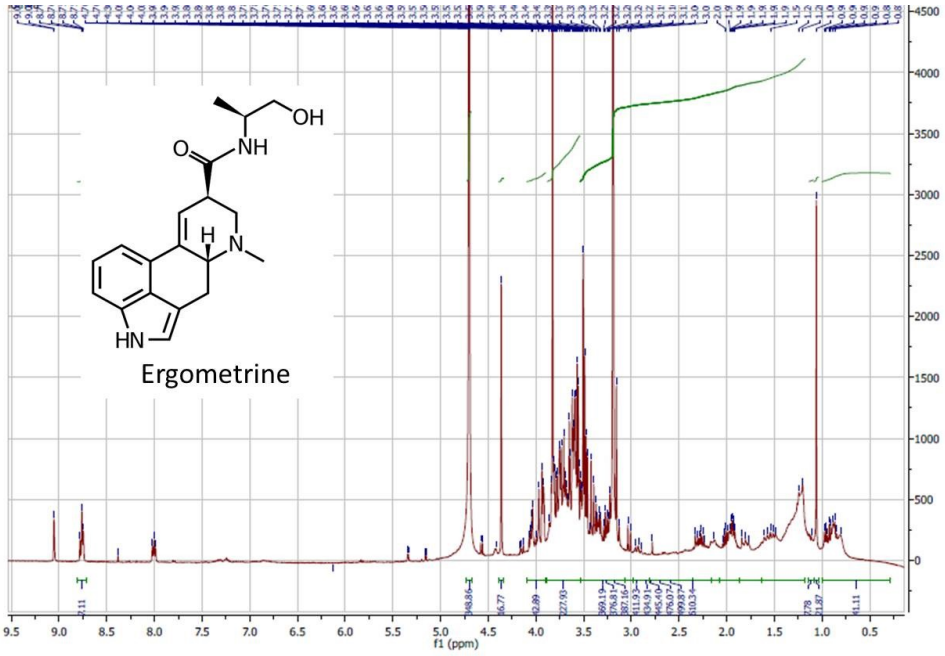


Figure 3.7 ^{1}H NMR Spectrum (600 MHZ, CD_3O) and molecular structure of Ergometrine.

3.3 Cytotoxicity on SH-SY5Y cells

Cytotoxicity of essential oils, their main components and extracts was evaluated using an MTT assay performed on the human neuroblastoma cell line (SH-SY5Y).

3.3.1 Linalool, Lavandula angustifolia and Coriandrum sativum essential oils

After 24 h of treatment, linalool, *L. angustifolia* and *C. sativum* essential oils revealed different cytotoxic activities, with IC_{50} values of 334.5, 591.8 and 663.2 µg/ml, respectively. Treatment of SH-SY5Y neuroblastoma cells with 800 µg/ml of linalool for 24 h resulted in a strong cytotoxic activity with 92% cell death.

However, treatment with 800 µg/ml of *L. angustifolia* and *C. sativum* essential oils resulted in 78% and 63% cell death, respectively (fig. 3.8).

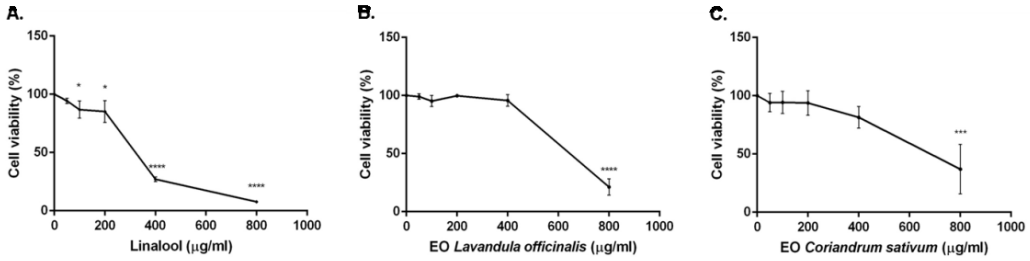


Figure 3.8 Cell viability calculated as a percentage after MTT assay. Cells were treated with different concentrations (50-800 µg/ml) of linalool (A); *L. officinalis* (B); and *C. sativum* (C) essential oils, for 24 h and solvent (DMSO, 0.1%) alone. Data are the mean ± SD of three experiments ($^{*}p< 0.05$, $^{***}p< 0.001$, $^{****}p< 0.0001$ vs. DMSO).

3.3.2 Limonene, Citrus medica cv 'liscia' and Citrus medica cv 'rugosa' essential oils

Limonene, *C. medica* cv. '*liscia*', and *C. medica* cv. '*rugosa*' essential oils revealed different cytotoxic activities. Limonene and *C. medica* cv. '*rugosa*' EO showed an IC_{50} > 2000 µg/ml, instead *C. medica* cv. '*liscia*' EO showed an IC_{50} of 718.2 µg/ml. Treatment of SH-SY5Y neuroblastoma cells with 800 µg/ml of limonene for 24 h resulted in a low cytotoxic activity. However, treatment with 800 µg/ml of *C. medica* cv. '*liscia*' EO resulted in a stronger cytotoxicity than *C. medica* cv. '*rugosa*' EO with 38% cell death (fig. 3.9).

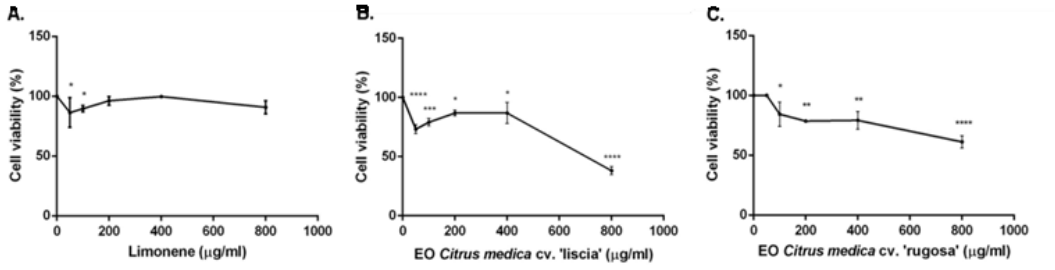


Figure 3.9 Percentage of cell viability after MTT assay. Cells were treated with different concentrations (50–800 µg/ml) of limonene (A); *C. medica* cv. 'liscia' (B) and *C. medica* cv. 'rugosa'(C) essential oils, for 24 h and solvent (DMSO, 0.1%) alone. Data are the mean ± SD of three experiments $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. DMSO.

3.3.3 1,8 Cineole and Laurus nobilis essential oil

The treatment of SH-SY5Y neuroblastoma cells with of 1,8-cineole (800 – 50 µg/ml) and *Laurus nobilis* essential oil (800 – 50 µg/ml) for 24 h resulted in a low cytotoxic activity.

1,8-Cineole and essential oil showed an $IC_{50} > 2000$ µg/ml and $IC_{50} = 471.1$ µg/ml, respectively. However, the treatment with essential oil resulted in a stronger cytotoxicity ($IC_{50} < 500$ µg/ml) (fig. 3.10).

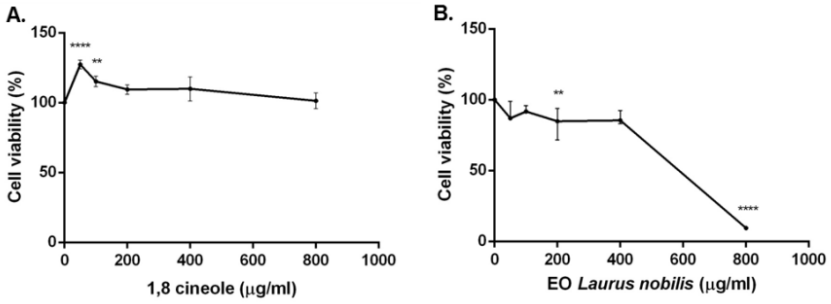


Figure 3.10 Cell viability calculated as percentage after MTT assay. Cells are treated with different concentrations (800-50 µg/ml) of 1,8 cineole (A) and *L. nobilis* essential oil (B), for 24 h and solvent (DMSO, 0.1%) alone. Data are the mean ± SD of three experiments $^{**}p< 0.01$, $^{****}p<0.0001$ vs. DMSO.

3.3.4 Ipomea transvaalensis extracts

Ipomea transvaalensis cloroform: methanol (9:1) extract and its metanolic fraction revealed different cytotoxic activities. *I. transvaalensis* 9:1 extract showed an IC_{50} = 1338.9 µg/ml, instead its methanolic fraction, that contained some alkaloids, showed an IC_{50} of 258.4 µg/ml (fig. 3.11).

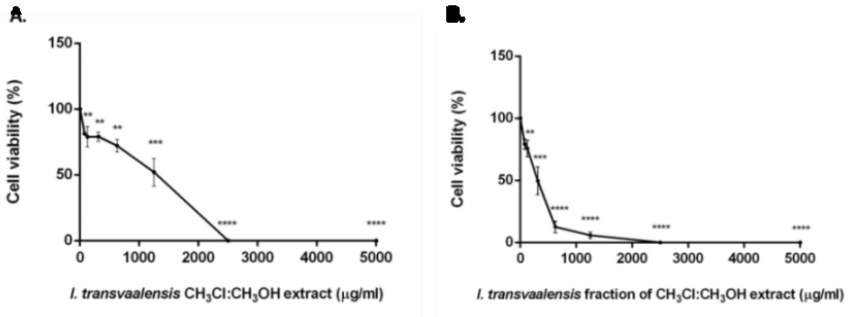


Figure 3.11 Cell viability calculated as percentage after MTT assay. Cells are treated with different concentrations (5000-78 µg/ml) of *I. transvaalensis* 9:1 extract (A), and *I. transvaalensis* fraction of 9:1 extract (B) for 24 h and solvent (DMSO, 0.1%) alone. Data are the mean ± SD of three experiments $^{**}p< 0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ vs. DMSO.

3.3.5 Ipomea cairica extract

After a treatment for 24 h of SH-SY5Y neuroblastoma cells with *I. cairica* methanol extract (5000 – 78 µg/ml), the results showed an IC_{50} = 353.5 µg/ml; instead after a treatment with *I. cairica* fraction of methanol extract (5000 – 78 µg/ml) we have an IC_{50} > 5000 µg/ml (fig. 3.12).

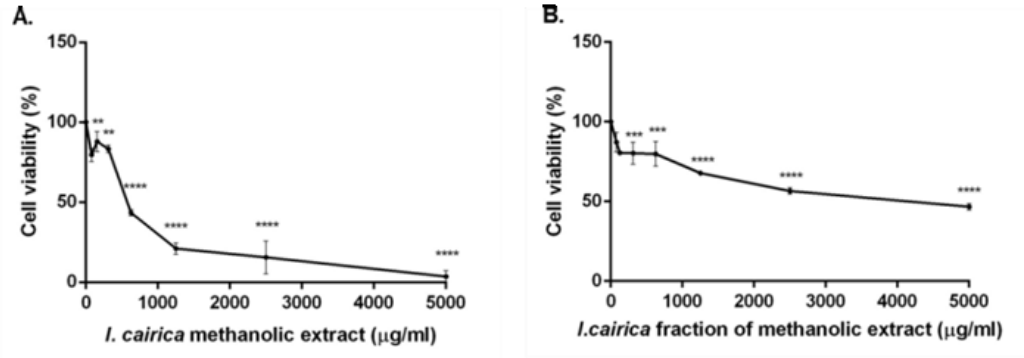


Figure 3.12 Cell viability calculated as percentage after MTT assay. Cells are treated with different concentrations (5000-78 μg/ml) of *I. cairica* methanol extract (A) and *I. cairica* fraction of methanol extract (B) for 24 h and solvent (DMSO, 0.1%) alone. Data are the mean ± SD of three experiments $^{**}p< 0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ vs. DMSO.

3.3.6 Hypericum hircinum extract

The treatment of SH-SY5Y neuroblastoma cells with of (5000–78 μg/ml) *Hypericum hircinum* methanol extract for 24 h resulted in a low cytotoxic activity, with an IC_{50} = 451.5 μg/ml (fig. 3.13).

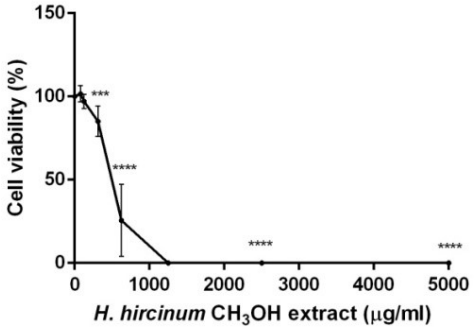


Figure 3.13 Cell viability calculated as percentage after MTT assay. Cells are treated with different concentrations (5000-78 μg/ml) of *H. hircinum* extract for 24 h and solvent (DMSO, 0.1%) alone. Data are the mean ± SD of three experiments $^{***}p<0.001$, $^{****}p<0.0001$ vs. DMSO.

3.4 ADCY1, pERK, ERK, PKA: Western Blot Analysis

We investigated the effects of essential oils, their main components and extracts in an SH-SY5Y cell line. More representative Western blots and quantitative densitometric analysis for protein expression in SH-SY5Y human neuroblastoma cells are shown in Figures 3.14-3.36.

3.4.1 Linalool

Treatments of SH-SY5Y neuroblastoma cells with 200 and 100 µg/ml of linalool for 24 h significantly reduced ADCY1 and PKA protein expression. Only the concentration of 200 µg/ml inhibited pERK and ERK expression (fig. 3.14-3.15).

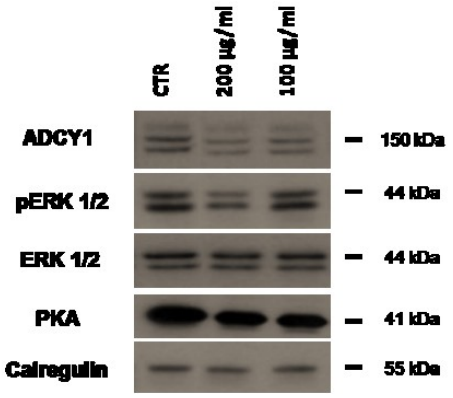


Figure 3.14 Representative Western blot of ADCY1, pERK 1/2, ERK 1/2, PKA proteins in SH-SY5Y cells treated with linalool.

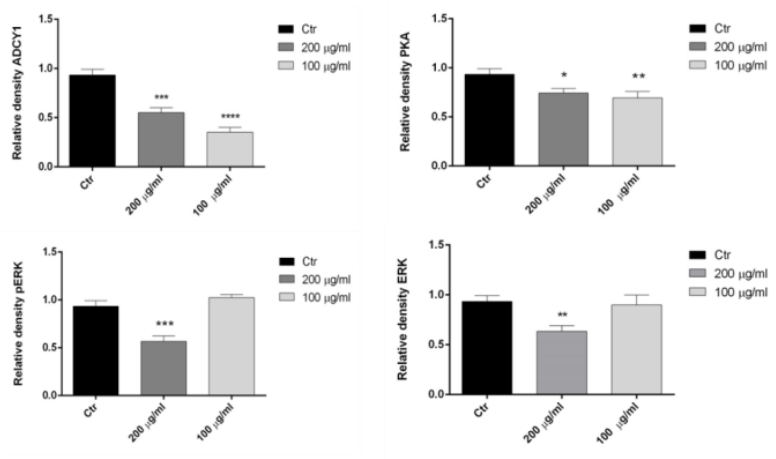


Figure 3.15 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cells treated with linalool. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, compared to control (ANOVA followed by Dunnett's multiple comparison test).

3.4.2 Lavandula angustifolia essential oil

Treatment for 24 h with 200 µg/ml of *L. angustifolia* appears to increase ADCY1 and ERK expression, but there are no significant variations in the expression of pERK and PKA (fig. 3.16 - 3.17).

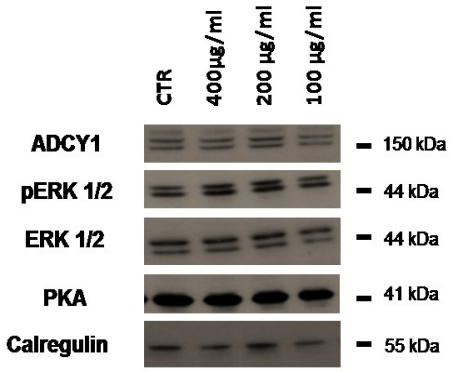


Figure 3.16 Representative Western blot of ADCY1, pERK 1/2, ERK 1/2, PKA proteins in SH-SY5Y cells treated with *L.angustifolia* essential oil.

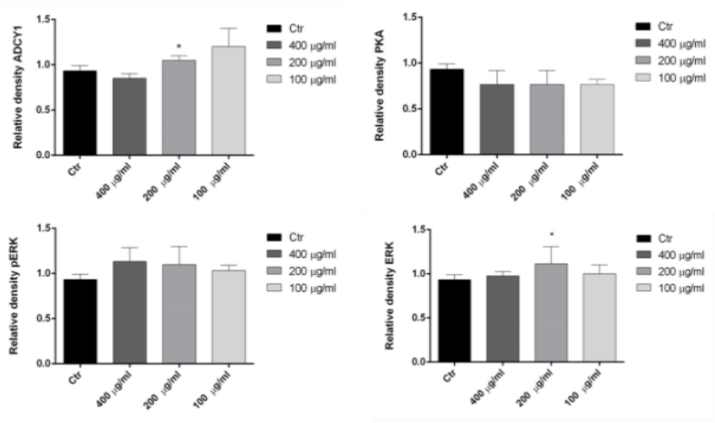


Figure 3.17 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cells treated with *L. angustifolia* essential oil. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{*}p < 0.05$, compared to control (ANOVA followed by Dunnett's multiple comparison test).

3.4.3 Coriandrum sativum essential oil

C. sativum essential oil increased both pERK and PKA expression at concentration of 100 µg/ml, instead concentration of 50 µg/ml increased only pERK expression. Moreover, no concentrations of *C. sativum* essential oil had significant effects on ADCY1 and ERK expression (fig. 3.18-3.19).

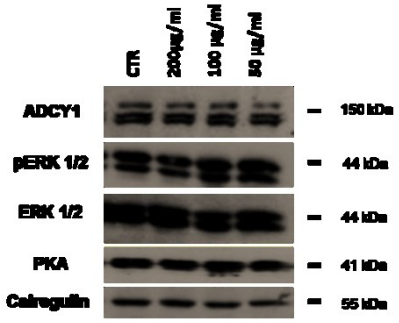


Figure 3.18 Representative Western blot of ADCY1, pERK 1/2, ERK 1/2, PKA proteins in SH-SY5Y cells treated with *C. sativum* essential oil.

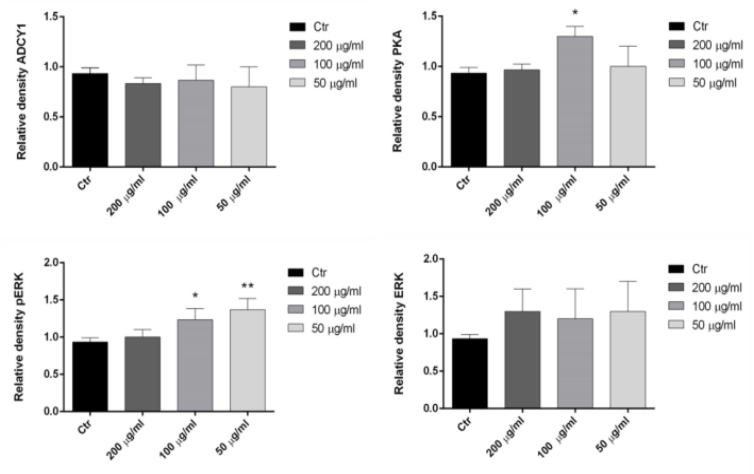


Figure 3.19 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cells treated with *C. sativum* essential oil. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{*}p < 0.05$, $^{**}p < 0.01$, compared to control (ANOVA followed by Dunnett's multiple comparison test).

3.4.4 1,8 Cineole

Treatments of SH-SY5Y neuroblastoma cells with 400 µg/ml of 1,8-cineole for 24 h increased pERK and PKA expression and no had influence on ADCY1 and ERK protein expression (fig. 3.20-3.21).

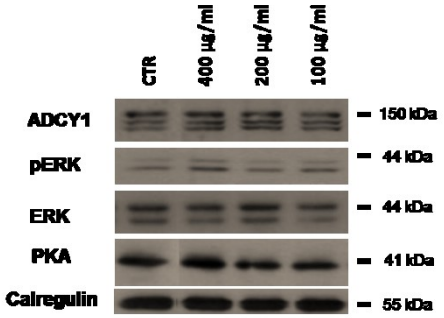


Figure 3.20 Representative Western blot of ADCY1, pERK 1/2, ERK 1/2, PKA proteins in SH-SY5Y cells treated with 1,8-Cineole.

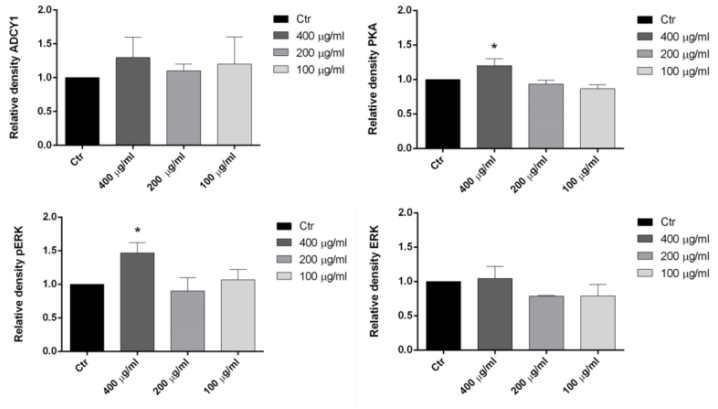


Figure 3.21 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cells treated with 1,8-Cineole. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{*}p < 0.05$, compared to control (ANOVA followed by Dunnett's multiple comparison test).

3.4.5 Laurus nobilis essential oil

L. nobilis essential oil reduced significantly ADCY1 expression in SH-SY5Y cells after a treatment with 200 and 100 µg/ml for 24 h, instead concentrations of 200-100 and 50 µg/ml inhibited pERK expression but none of used concentrations influenced PKA and ERK expression (fig. 3.22-3.23).

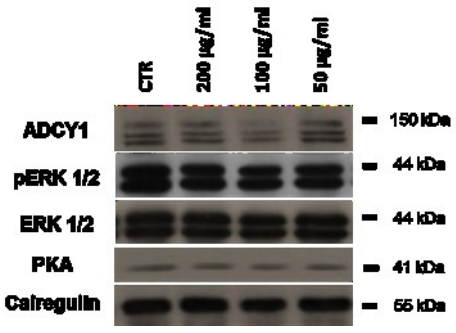


Figure 3.22 Representative Western blot of ADCY1, pERK 1/2, ERK 1/2, PKA proteins in SH-SY5Y cells treated with *L. nobilis* essential oil.

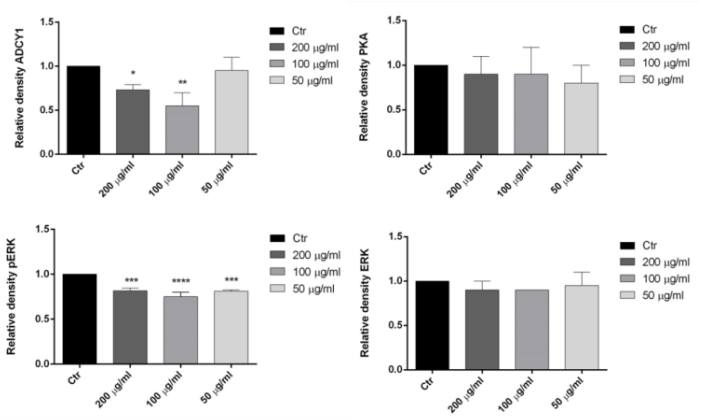


Figure 3.23 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y treated with *L. nobilis* essential oil. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared to control (ANOVA followed by Dunnett's multiple comparison test).

3.4.6 Limonene

Treatments of SH-SY5Y neuroblastoma cells with 800 µg/ml of limonene for 24 h significantly influenced ADCY1 expression in fact appear to increase ADCY1 expression. Instead, no significant effects on pERK, ERK and PKA expression have been registered (fig. 3.24-3.25).

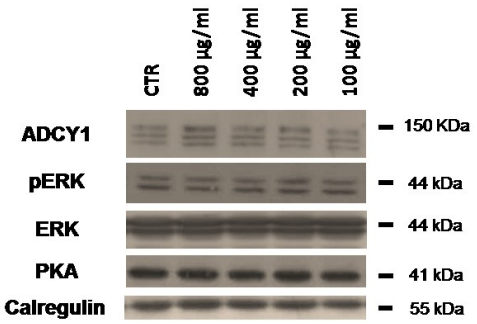


Figure 3.24 Representative Western blot of ADCY1, pERK 1/2, ERK 1/2, PKA proteins in SH-SY5Y cells treated with limonene.

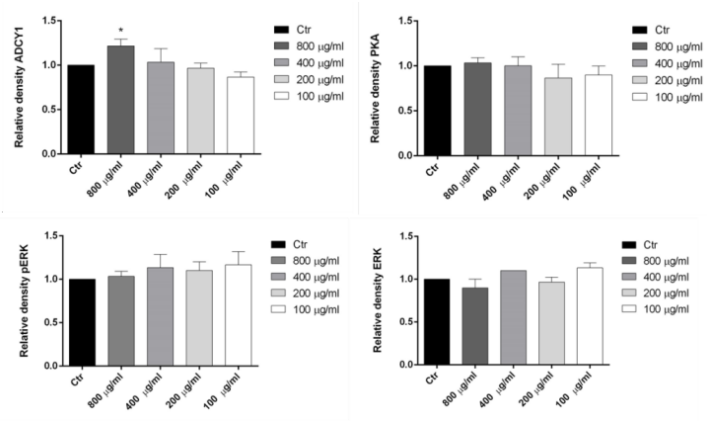


Figure 3.25 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cells treated with limonene. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{*}p < 0.05$, compared to control (ANOVA followed by Dunnett's multiple comparison test).

3.4.7 Citrus medica essential oils

Treatments with 400, 200, 100, 50 µg/ml of *C. medica* cv. '*liscia*' and *C. medica* cv. '*rugosa*' essential oils appear to influence significantly ADCY1 expression with an over expression and a down expression of ADCY1, respectively (fig. 3.26-3.28). Moreover, treatment with 400, 200 and 100 µg/ml of *C. medica* cv. '*liscia*' essential oil increased pERK and PKA expression (fig.3.26a-3.27), instead ERK expression only was affected by the concentration of 100 µg/ml. Treatments with 200 and 100 µg/ml of *C. medica* cv. '*rugosa*' essential oil decrease PKA and ERK expression, and treatment with 400 µg/ml increases pERK expression (fig. 3.26b-3.28).

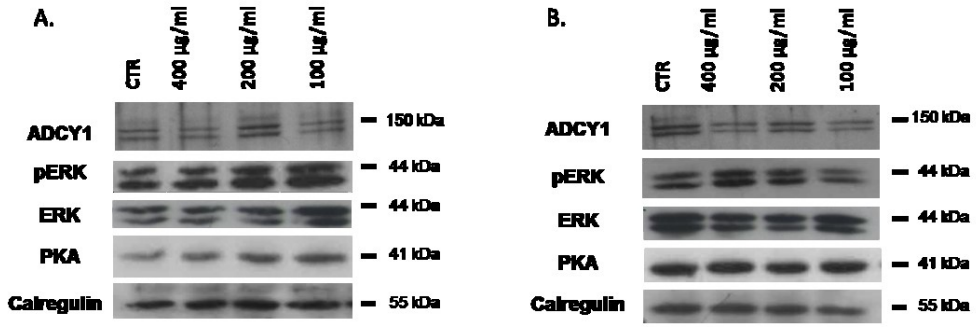


Figure 3.26 Representative Western blot of ADCY1, pERK 1/2, ERK 1/2, PKA proteins in SH-SY5Y cells treated with *C. medica* cv. '*liscia*' (A) and *C. medica* cv. '*rugosa*' (B) essential oils.

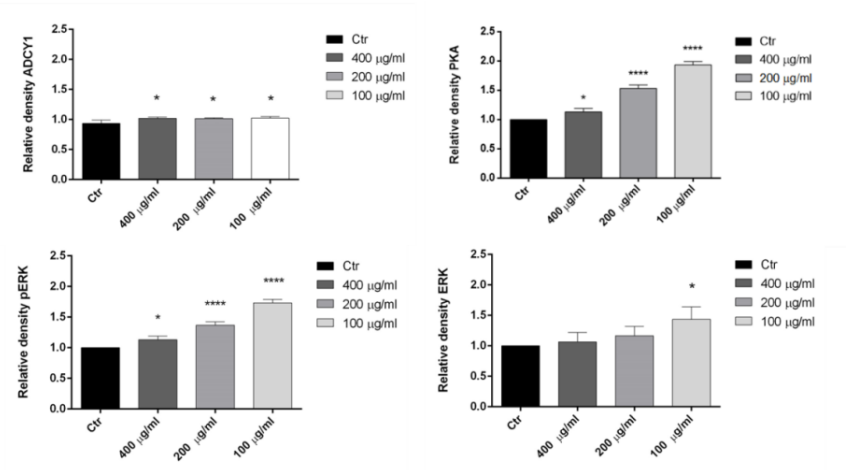


Figure 3.27 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cells treated with *C. medica* cv. '*liscia*' essential oil. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{*}p < 0.05$, $^{****}p < 0.0001$ compared to control (ANOVA followed by Dunnett's multiple comparison test).

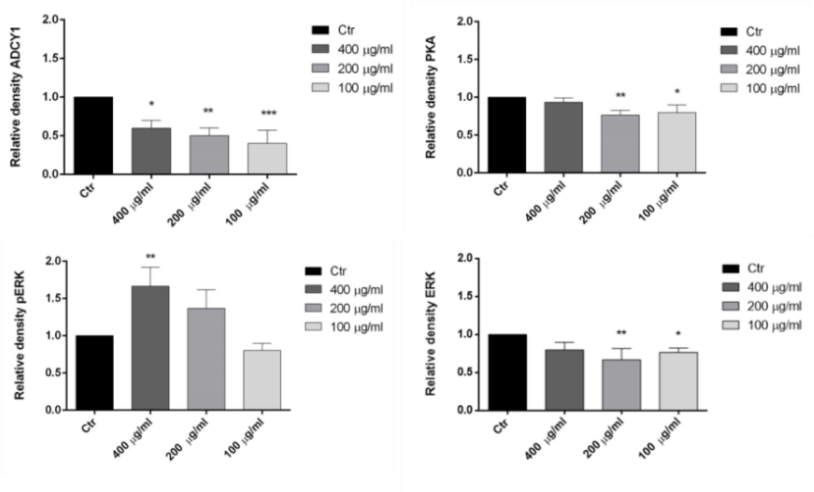


Figure 3.28 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cells treated with *C. medica* cv. '*rugosa*' essential oil. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control (ANOVA followed by Dunnett's multiple comparison test).

3.4.8 Ipomea transvaalensis extracts

Ipomea transvaalensis cloroform : methanol (9:1) extract no affects ADCY1, PKA and ERK expression. Moreover, treatment with 125 µg/ml increase pERK expression (fig. 3.29a-3.30). Treatment with each concentration (125 – 31.5 µg/ml) of *Ipomea transvaalensis* alkaloid fraction of cloroform: methanol (9:1) extract decreased ADCY1 expression. Instead, only treatments with 125 and 62.5 µg/ml decrease PKA expression, and concentration of 125 µg/ml increases pERK expression (fig. 3.29b-3.31).

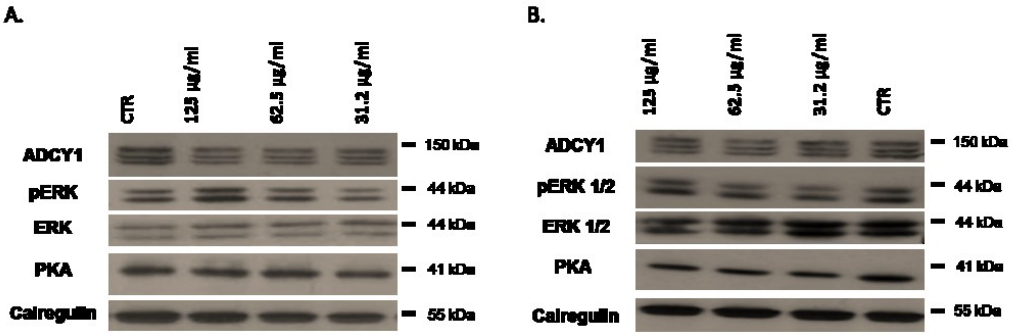


Figure 3.29 Representative Western blot of ADCY1, pERK 1/2, ERK 1/2, PKA proteins in SH-SY5Y cells treated with *Ipomoea transvaalensis* cloroform: methanol (9:1) extract (A) and *Ipomoea transvaalensis* alkaloid fraction of cloroform: methanol (9:1) extract (B).

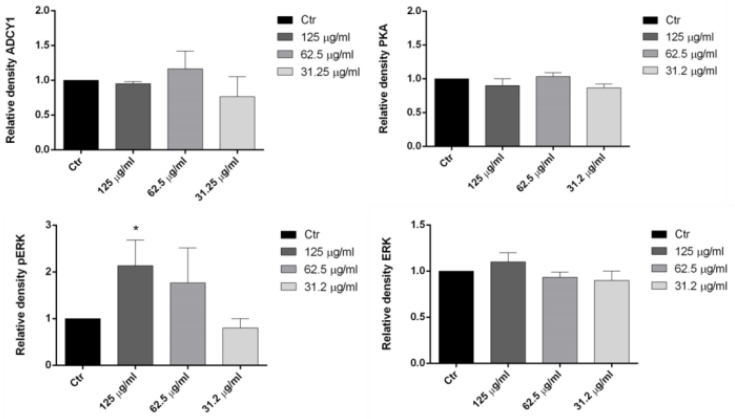


Figure 3.30 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cells treated with *Ipomoea transvaalensis* cloroform: methanol (9:1) extract. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{*}p < 0.05$ compared to control (ANOVA followed by Dunnett's multiple comparison test).

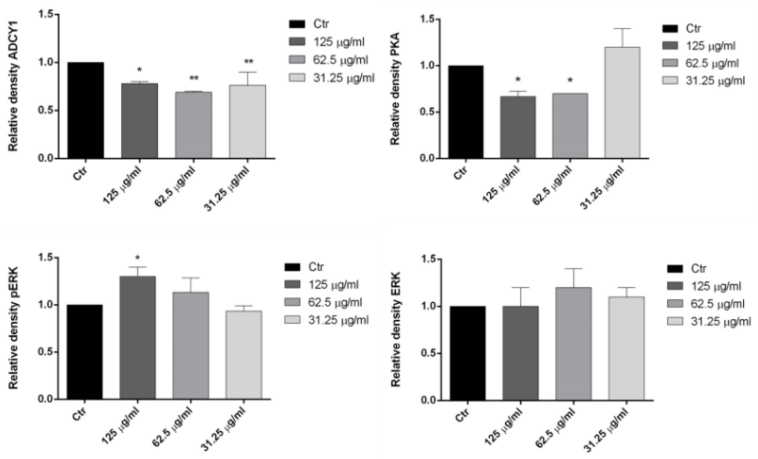


Figure 3.31 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cells treated with *Ipomoea transvaalensis* alkaloid fraction of cloroform: methanol (9:1) extract. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{*}p < 0.05$, $^{**}p < 0.01$, compared to control (ANOVA followed by Dunnett's multiple comparison test).

3.4.9 Ipomea cairica extract

Ipomea cairica methanol extract and its fraction no affect significantly the expression of proteins studied. However, results showed a tendency of *Ipomea cairica* fraction of metanol extract to increase expression of ADCY1 and ERK (fig. 3.32-3.34)

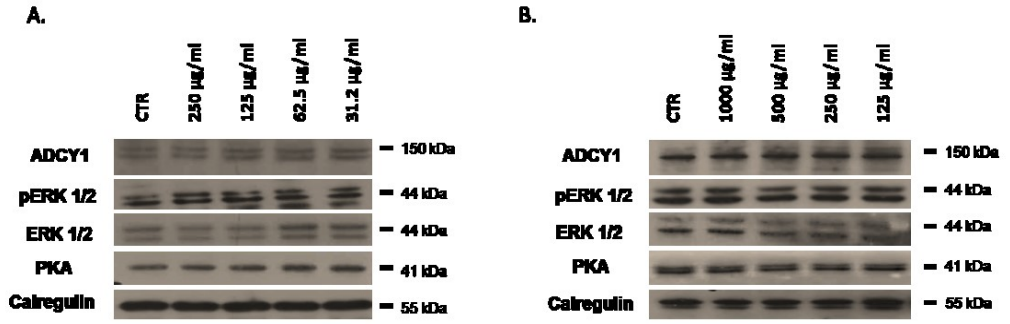


Figure 3.32 Representative Western blot of ADCY1, pERK 1/2, ERK 1/2, PKA proteins in SH-SY5Y cells treated with *Ipomoea cairica* methanol extract (A) and *Ipomoea cairica* fraction of methanol extract (B).

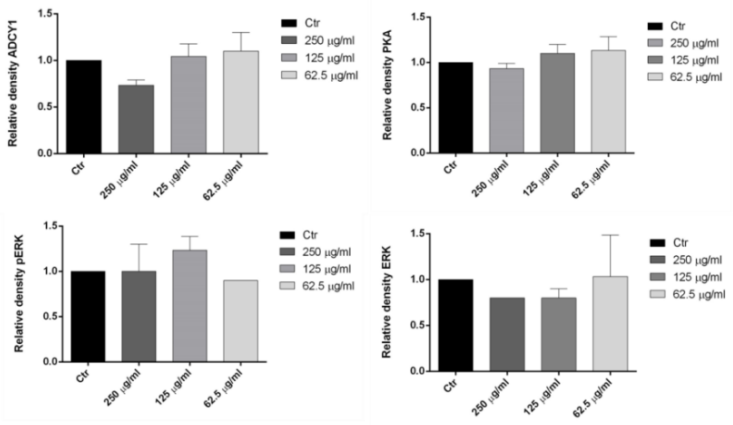


Figure 3.33 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y cells treated with *Ipomoea cairica* methanol extract. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3).

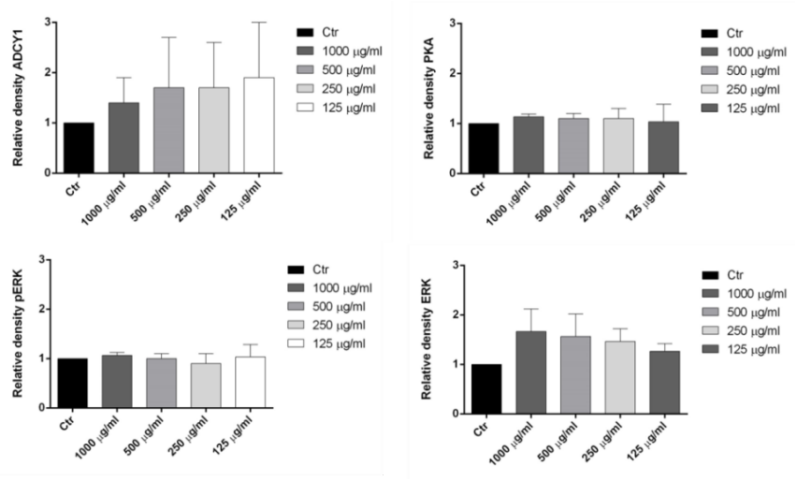


Figure 3.34 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y treated with *Ipomoea cairica* fraction of methanol extract. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3).

3.4.10 Hypericum hircinum extract

Treatment with 250 µg/ml of *Hypericum hircinum* methanol extract decreased ADCY1 expression. Instead, treatment with 250, 125 and 62.5 µg/ml increased pERK expression (fig. 3.35-3.36).

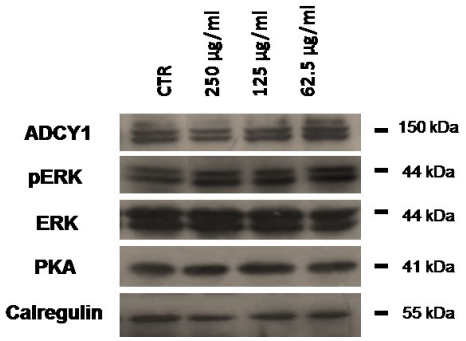


Figure 3.35 Representative Western blot of ADCY1, pERK 1/2, ERK 1/2, PKA proteins in SH-SY5Y cells treated with *H. hircinum* methanol extract.

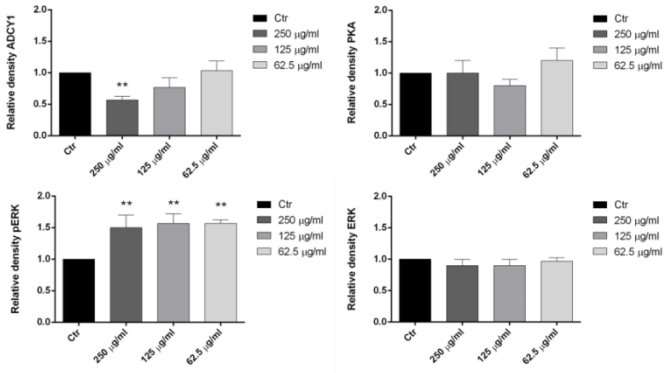


Figure 3.36 Relative expression of ADCY1, pERK, ERK and PKA in SH-SY5Y treated with *H. hircinum* methanol extract. The panel shows densitometric analysis of bands in the control and treated groups. Values are the mean ± SD in each group (n= 3). $^{**}p < 0.01$, compared to control (ANOVA followed by Dunnett's multiple comparison test).

3.5 Effects on Neuronal Activity

To evaluate if exposure to the selected essential oils affects neuronal spontaneous electrical activity, the mean firing rate (MFR) of primary cultures of rat cortical neurons was considered.

3.5.1 Linalool, Lavandula angustifolia and Coriandrum sativum essential oils

To evaluate the role of linalool, the major component of the two essential oils, in the reduction of neuronal networks functionality, we exposed neuronal cultures to increasing amounts of this compound. As illustrated in figure 3.37, linalool was considerably more potent than the two essential oils in reducing MFR, showing an IC_{50} of 25 µg/ml (fig. 3.37a). Neuronal networks subjected to *L. angustifolia*

essential oil induced a concentration-dependent inhibition of activity with an IC_{50} value for MFR of 100 µg/ml and a total block at 200 µg/ml (fig. 3.37 b).

Differently, *C. sativum* essential oil reduced electrical activity with an IC_{50} value for MFR of 88 µg/ml, while a dose of 200 µg/ml completely blocked the firing activity (fig. 3.37c).

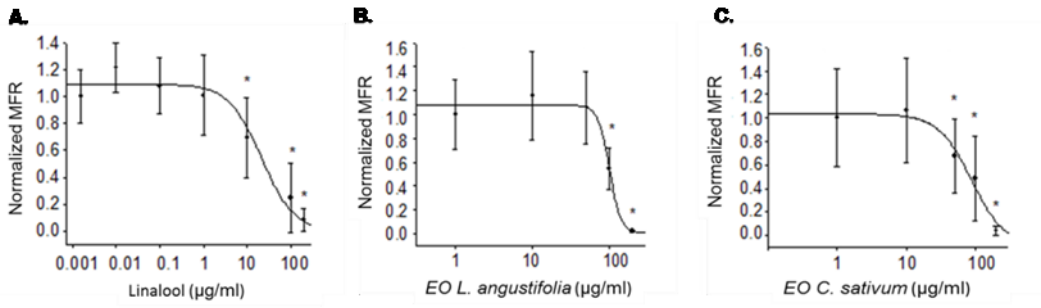


Figure 3.37 Effects of linalool (A); *L. angustifolia* (B); and *C. sativum* (C) essential oils on mean firing rate (MFR) of cortical cultures grown on microelectrode arrays. Each data point is the mean ± SEM of 3 independent experiments ($^{*}p$ <0.005 with respect to the normalised baseline values).

3.5.2 Hypericum hircinum

Hypericum hircinum methanol extract was considerably more potent than linalool and the two essential oils in reducing MFR, in fact, the treatment showed an IC_{50} of 0,01 µg/ml (fig 3.38).

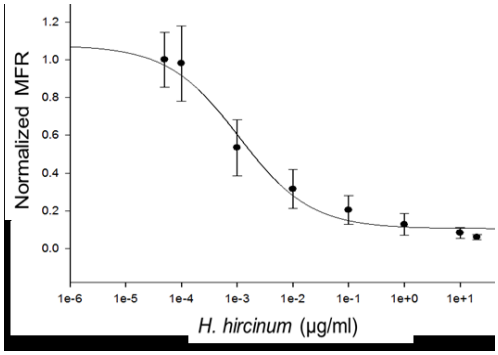


Figure 3.38 Effects of *Hypericum hircinum* methanol extract on mean firing rate (MFR) of cortical cultures grown on microelectrode arrays. Each data point is the mean ± SEM of 3 independent experiments ($^{*}p$ <0.005 with respect to the normalised baseline values).

3.6 Studies *in vivo*

3.6.1 Linalool and Lavandula angustifolia essential oil effects in OF1 mice

3.6.1.1 Open Field

The results of the basal activity of mice in the open field test (after saline administration) are shown in fig. 3.39. The ANOVA of the distance travelled showed that the administration of linalool [F (2,42) = 6.736; $p < 0.01$] essential oil had a significant effect. The ANOVA of the velocity showed that the administration of linalool [F (2,42) = 5.427; $p < 0.01$] essential oil had a significant effect. The results showed that that mice treated with linalool (100 mg/kg) travelled a lower distance with a lower velocity than mice treated with *L. angustifolia* essential oil (200 mg/kg) and their respective control group. The ANOVA of the time spent in the centre of the open field and the ANOVA of the frequency to enter in the central area of the open field did not reveal any significant effect (data not shown).

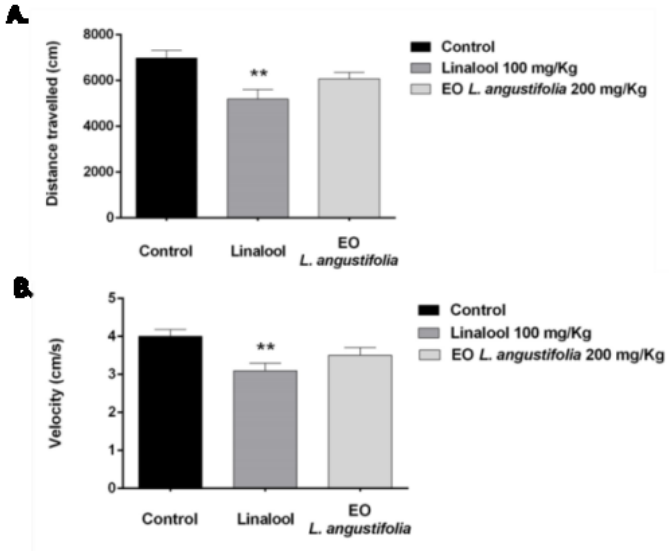


Figure 3.39 Effects of Linalool and *L. angustifolia* essential oil administration on the open-field test. A. Effects of linalool (100 mg/Kg) and *L. angustifolia* essential oil (200 mg/Kg) administration on the distance travelled by mice in the open field test. The bars represent the mean (± SEM) of the distance travelled (cm) by animals in the different treatments groups. B. Effects of linalool (100 mg/Kg) and *L. angustifolia* essential oil (200 mg/Kg) on the velocity of mice in the open field test. The bars represent the mean (± SEM) velocity (cm/s) of animals in the different treatment groups. $^{**}p < 0.01$ significant difference with respect to the CONTROL .

3.6.1.2 Elevated plus maze

EPM data (fig. 3.40) revealed that the number of entries in the closed arms was lower by animals treated with linalool and *L. angustifolia* essential oil than those in the control group ($p < 0.01$ and $p < 0.05$ respectively). The number of total entries [$F(2,42) = 2.964$; $p < 0.05$] was lower in mice treated with 100 mg/kg of linalool than in those receiving 200 mg/Kg of *L. angustifolia* essential oil.

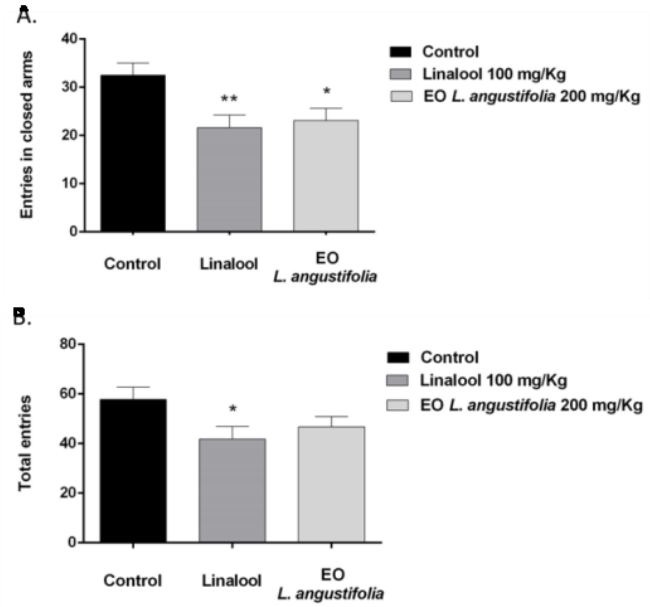


Figure 3.40 Effects of linalool and *L. angustifolia* essential oil administration on mice in the EPM. Animals were divided into the following three treatment groups: CONTROL (SALINE) (n=15), LINALOOL (n=15) and L. ANGUSTIFOLIA essential oil (n=15). Data are presented as mean values ± SEM. A. Entries in closed arms. $^{*}p<0.05$; $^{**}p<0.01$ differences with the control group. B. Total entries. $^{*}p< 0.05$ difference with the control group.

3.6.1.3 Tail suspension test

The ANOVA showed that administration of *L. angustifolia* essential oil [$F(2,42)= 7.806$; $p < 0.01$] had a significant effect. In fact, the results showed that the *L. angustifolia* group was immobile for longer than the control and the linalool group, indicating that the administration of *L. angustifolia* essential oil produces depressive-like behaviour (fig. 3.41).

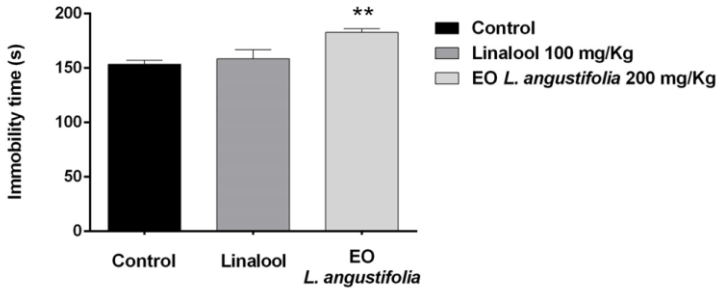


Figure 3.41 Effects of linalool and *L. angustifolia* essential oil administration on the time mice were immobile during the tail suspension test. Animals were divided into the following three groups: CONTROL (SALINE) (n=15), LINALOOL (n=15) and *L. ANGUSTIFOLIA* essential oil (n=15). The bars represent the mean time (± SEM) in seconds (s) that the animals were immobile. $^{**}p < 0.01$, significant difference with respect to the CONTROL group.

3.6.1.4 Social interaction test

The data for the different types of behaviour evaluated in the social interaction test are presented in Table 3.5. More time was spent in Social Investigation by mice receiving linalool (100 mg/Kg) and *L. angustifolia* essential oil (200 mg/Kg), showing a significant difference with respect to their saline control group ($p<0.05$). Similar results were obtained analysing the mean time spent in each contact (Unit of Social Investigation), which was higher among animals receiving linalool (100 mg/Kg) and *L. angustifolia* essential oil (200 mg/Kg) ($p < 0.0001$ and $p < 0.01$, respectively) than the control group.

Accordingly to this results, less time was spent in Non-Social Exploration by mice receiving linalool (100 mg/Kg) and *L. angustifolia* essential oil (200 mg/Kg) with respect to their saline control group ($p < 0.01$). Instead, values for Explore from a Distance and Latency of Social Investigation no showed significative differences with respect the control group.

Table 3.5 Means of accumulated times (in seconds) with SEM allocated to different categories of spontaneous behaviour from the social interaction test in adult mice divided into the following groups: CONTROL (SALINE) (n=15), 100 mg/Kg of LINALOOL (n=15) and 200 mg/Kg of *L. ANGUSTIFOLIA* essential oil (n=15).

	Control	**Linalool**	**EO *L. angustifolia***
Non-social exploration	534 ± 5	494 ± 9**	491 ± 12**
Explore from a distance	3 ± 0.3	2 ± 0.3	2 ± 0.4
Social investigation	50 ± 6	83 ± 8*	84 ± 10*
Unit of social investigation	1 ± 0.2	3 ± 0.2****	2 ± 0.3**
Latency of social investigation	20 ± 3	13 ± 2	12 ± 2

Data are the mean values ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, differences with the control groups.

3.6.2 Linalool and Lavandula angustifolia essential oil effects in OF1 mice with social stress

3.6.2.1 Elevated plus maze

EPM data (table 3.6) revealed that the number of entries in the closed and open arms were lower for animals treated with linalool 100 mg/kg after social defeat than those in the saline explora control group ($p < 0.001$ for the entries in closed arms, $p < 0.05$ for the entries in open arms). Moreover, mice treated with 100 mg/kg of linalool and 200 mg/Kg of *L. angustifolia* essential oil spent more time in closed arms than those in the saline explora control group ($p < 0.01$ and $p < 0.05$, respectively). Instead, the percentage of time spent in open arms was less for mice treated with linalool after social defeat than those of explora control group ($p < 0.05$).

The number of total entries [F(3,41) = 8.109; $p < 0.001$] was lower in mice treated with linalool and *L. angustifolia* essential oil after social defeat than those of saline explora control group ($p < 0.001$ and $p < 0.05$, respectively).

Table 3.6 Effects of linalool and *L. angustifolia* essential oil on stressed mice in the EPM.

	Control (explora)	**SD saline**	**SD Linalool**	**SD EO *L. angustifolia***
Time in open arms	50 ± 8	30 ± 6	18 ± 7	34 ± 9
% Time in open arms	22 ± 2	13 ± 3	8 ± 4*	14 ± 4
Time in central platform	38 ± 4	50 ± 9	37 ± 12	40 ± 10
Time in closed arms	169 ± 14	213 ± 13	244 ± 18**	225 ± 16*
Entries in open arms	24 ± 4	26 ± 6	9 ± 3*	15 ± 2
% Open entries	32 ± 3	35 ± 5	29 ± 7	31 ± 4
Entries in closed arms	49 ± 5	42 ± 3	22 ± 6***	33 ± 4
Total entries	73 ± 7	68 ± 8	28 ± 8***	48 ± 6*

Data are the mean values ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p<0.001$ differences with the explora control groups.

3.6.2.2 Social interaction test

The data for the different types of behaviour evaluated in the social interaction test are presented in Table 3.7. Lower time was spent in Social Investigation by mice receiving physiological solution after social defeat showing a significant difference with respect to their saline explora control group ($p < 0.01$). Accordingly to these results, more time was spent in Non-Social Exploration by mice receiving physiological solution after social defeat with respect to their saline explora control group ($p < 0.05$).

Moreover, mice treated with linalool (100 mg/Kg) and *L. angustifolia* essential oil (200 mg/Kg) after social defeat spent more time in Social Investigation and

less time in Non-Social Exploration respect to their control group treated with physiological solution after social defeat, showing significant differences (respectively $p < 0.01$ for linalool and $p < 0.01$, $p < 0.05$ for *L. angustifolia* essential oil).

Instead, the mean time spent in each contact (Unit of Social Investigation), values for Explore from a Distance and Latency of Social Investigation no showed significative differences with respect the control group.

Table 3.7 Means of accumulated times (in seconds) with SEM allocated to different categories of spontaneous behaviour from the social interaction test in adult mice divided into the following groups: CONTROL (EXPLORA SALINE) (n=11), SD SALINE (n=10); SD LINALOOL 100 mg/Kg (n=12) and SD *L. ANGUSTIFOLIA* essential oil 200 mg/Kg (n=12).

	Control (explora)	**SD saline**	**SD Linalool**	**SD EO *L. angustifolia***
Non-social exploration	448 ± 14	492 ± 8*	433 ± 13$^{\#\#}$	447 ± 12$^{\#}$
Explore from a distance	3 ± 0.5	3 ± 1	3 ± 0.4	3 ± 0.4
Social investigation	121 ± 12	77 ± 6**	132 ± 9$^{\#\#}$	129 ± 12$^{\#\#}$
Unit of social investigation	4 ± 1	3 ± 0.1	4 ± 0.4	4 ± 0.4
Latency of social investigation	12 ± 3	17 ± 3	15 ± 2	15 ± 2

$^{*}p < 0.05$; $^{**}p < 0.01$ differences with the explora saline control groups; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ differences with SD saline.

3.6.3 Linalool and Lavandula angustifolia essential oil acute or chronic administration effects on social stress and social interaction of OF1 mice

3.6.3.1 Acute administration

Elevated plus maze

EPM data (not shown) revealed no significant differences between the four groups of animals. No significant differences were detected between treated mice and their control with respect all the parameters considerated.

Social defeat

The ANOVA for resident mice revealed an effect of the time spent engaged in attack [$F(2, 33) = 12.39$; $p < 0.0001$], showing that mice displayed less attack behaviour in the social defeat against mice treated with linalool (100 mg/Kg) and *L. angustifolia* essential oil (200 mg/Kg) ($p < 0.01$ and $p < 0.0001$, respectively). In the case of intruder mice treated with *L. angustifolia* essential oil (200mg/Kg), the latency of defensive/submissive behaviour showed a significant effect [$F(2, 33) = 4.464$; $p < 0.05$]; mice displayed their first defensive/submissive behaviour significantly later than their control group ($p < 0.05$) (Table 3.8).

Table 3.8 Social interaction during the resident–intruder paradigm to induce social defeat (SD).

Social defeat	Control	Linalool	EO *L. angustifolia*
Intruder mice			
Avoidance	82 ± 9	66 ± 6	57 ± 13
Latency avoidance	12 ± 5	6 ± 2	19 ± 9
Defense/Submissive	48 ± 9	51 ± 11	34 ± 12
Latency defense/submissive	27 ± 9	11 ± 3	111 ± 43*
Resident mice			
Threat	3 ± 1	2 ± 0.3	1 ± 0.3
Latency threat	18 ± 6	17 ± 7	28 ± 9
Attack	33 ± 5	18 ± 2**	10 ± 2****
Latency attack	9 ± 5	15 ± 7	37 ± 26

Data presented as mean values ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p<0.0001$ differences with respect to the control.

Social interaction test

The data for the different types of behaviour evaluated in the social interaction test are presented in Table 3.9. Lower time was spent in Social Investigation by mice receiving physiological solution before social defeat showing a significant difference with respect to their saline explora control group ($p < 0.01$). According to these results, more time was spent in Non-Social Exploration by mice receiving physiological solution with respect to their saline explora control group ($p < 0.01$). The mean time spent in each contact (Unit of Social Investigation) was lower among animals receiving physiological solution before social defeat than the control group ($p < 0.05$).

Moreover, mice treated with linalool (100 mg/Kg) and *L. angustifolia* essential oil (200 mg/Kg) before social defeat spent more time in Social Investigation (respectively $p < 0.0001$ and $p < 0.05$) and less time in Non-Social Exploration (respectively $p < 0.001$ and $p < 0.05$) respect to their control group treated with physiological solution before social defeat, showing significant differences.

Instead, values for Explore from a Distance and Latency of Social Investigation no showed significative differences with respect the control group.

Table 3.9 Means of accumulated times (in seconds) with SEM allocated to different categories of spontaneous behaviour from the social interaction test in adult mice divided into the following groups: CONTROL (EXPLORA SALINE) (n=12), SD SALINE (n=12); SD LINALOOL 100 mg/Kg (n=12) and SD *L. ANGUSTIFOLIA* essential oil 200 mg/Kg (n=12).

	Control (explora)	**SD Saline**	**SD Linalool**	**SD EO *L. angustifolia***
Non-social exploration	454 ± 11	511 ± 8**	447 ± 12$^{\#\#\#}$	469 ± 13$^{\#}$
Explore from a distance	2 ± 0.3	3 ± 0.4	2 ± 0.3	2 ± 0.4
Social investigation	110 ± 11	63 ± 8**	126 ± 12$^{\#\#\#\#}$	96 ± 6$^{\#}$
Unit of social investigation	4 ± 0.4	2 ± 0.2**	3 ± 0.3	3 ± 0.6
Latency of social investigation	8 ± 2	9 ± 3	7 ± 2	8 ± 2

$^{**}p < 0.01$ differences with the explora saline control groups; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$, differences with SD saline.

3.6.3.2 Chronic administration

Elevated plus maze

EPM data (table 3.10) revealed that the number of entries in the closed arms were lower by animals treated with linalool 100 mg/kg before social defeat than those in the saline explora control group ($p < 0.05$). Moreover, mice treated with 200 mg/Kg of *L. angustifolia* essential oil spent more time in central platform than those in the saline explora control group ($p < 0.05$). The number of total entries was lower in mice treated with linalool before social defeat than those of saline explora control group ($p < 0.05$).

Table 3.10 Effects of linalool and *L. angustifolia* essential oil on stressed mice in the EPM.

	Control (explora)	**SD saline**	**SD Linalool**	**SD EO *L. angustifolia***
Time in open arms	43 ± 9	25 ± 4	25 ± 6	35 ± 5
% Time in open arms	16 ± 3	10 ± 2	12 ± 4	16 ± 2
Time in central platform	27 ± 6	23 ± 9	50 ± 13	74 ± 18*
Time in closed arms	229 ± 11	243 ± 14	237 ± 19	208 ± 20
Entries in open arms	22 ± 4	17 ± 2	13 ± 3	18 ± 3
% Open entries	36 ± 9	40 ± 4	35 ± 5	36 ± 4
Entries in closed arms	39 ± 6	31 ± 6	20 ± 4*	28 ± 3
Total entries	61 ± 9	49 ± 7	33 ± 7*	46 ± 5

Data are presented as mean values ± SEM. *$p < 0.05$ differences with the explora control groups.

Social defeat

The ANOVA for resident mice revealed an effect of the time spent engaged in attack [$F(2, 33) = 15.18$; $p < 0.0001$], and an effect on latency of attack [$F(2,33) = 5.854$; $p < 0.005$] showing that mice displayed less attack behaviour in the social defeat against mice treated with *L. angustifolia* essential oil (200

mg/Kg) ($p < 0.0001$ for time spent in attack and $p < 0.05$ for latency of the attack) than those of control group.

In the case of intruder mice treated with *L. angustifolia* essential oil (200 mg/Kg), the time engaged in avoidance [$F(2, 33) = 7.721$; $p < 0.001$] and in defensive/submissive behaviour showed a significant effect [$F(2, 33) = 9.410$; $p < 0.001$]; these mice in fact displayed less defensive/submissive behaviour and avoidance than their control group ($p < 0.001$ and $p < 0.001$, respectively) (Table 3.11).

Table 3.11 Social interaction during the resident–intruder paradigm to induce social defeat (SD).

Social defeat	Control	Linalool	EO *L. angustifolia*
Intruder mice			
Avoidance	82 ± 9	57 ± 9	37 ± 4***
Latency avoidance	12 ± 5	7 ± 2	16 ± 4
Defense/Submissive	48 ± 9	43 ± 5	10 ± 4***
Latency defense/submissive	27 ± 9	20 ± 7	21 ± 6
Resident mice			
Threat	3 ± 1	3 ± 1	22 ± 1
Latency threat	18 ± 6	53 ± 30	88 ± 37
Attack	33 ± 5	25 ± 3	6 ± 2****
Latency attack	9 ± 5	5 ± 2	95 ± 36*

Data presented as mean values ± SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$; differences with respect to the control.

Social interaction test

The data for the different types of behaviour evaluated in the social interaction test are presented in Table 3.12. Less time was spent in Social Investigation by mice receiving physiological solution before social defeat showing a

significant difference with respect to their saline explora control group ($p < 0.01$). Accordingly to these results, more time was spent in Non-Social Exploration by mice receiving physiological solution with respect to their saline explora control group ($p < 0.01$). The mean time spent in each contact (Unit of Social Investigation) was lower among animals receiving physiological solution before social defeat than the control group ($p < 0.01$).

Moreover, mice treated with linalool (100 mg/Kg) and *L. angustifolia* essential oil (200 mg/Kg) before social defeat spent more time in Social Investigation ($p < 0.01$ and $p < 0.01$, respectively) and less time in Non-Social Exploration ($p < 0.05$ and $p < 0.001$, respectively) respect to their control group treated with physiological solution, showing significant differences.

Instead, values for Explore from a Distance and Latency of Social Investigation no showed significative differences with respect the control group.

Table 3.12 Means of accumulated times (in seconds) with SEM allocated to different categories of spontaneous behaviour from the social interaction test in adult mice divided into the following groups: CONTROL (EXPLORA SALINE) (n=12), SD SALINE (n=12); SD LINALOOL 100 mg/Kg (n=12) and SD *L. ANGUSTIFOLIA* essential oil 200 mg/Kg (n=12).

	Control (explora)	**SD saline**	**SD Linalool**	**SD EO *L. angustifolia***
Non-social exploration	454 ± 11	511 ± 8**	459 ± 11$^{\#\#}$	446 ± 12$^{\#\#\#}$
Explore from a distance	2 ± 0.3	3 ± 0.4	2 ± 0.3	2 ± 1
Social investigation	110 ± 11	63 ± 8**	115 ± 10$^{\#\#}$	114 ± 11$^{\#\#}$
Unit of social investigation	4 ± 0.4	2 ± 0.2**	4 ± 1	4 ± 0.4
Latency of social investigation	8 ± 2	9 ± 3	20 ± 5	15 ± 0.3

$^{**}p < 0.01$ differences with the explora saline control groups; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$; differences with SD saline.

4. Discussion

4.1 Essential oils composition

Oxygenated monoterpenes are highly predominant in *L. angustifolia*, *C. sativum* and *L. nobilis* essential oils, instead in both *C. medica* essential oils monoterpene hydrocarbons are predominant.

The major components of *L. angustifolia* oil were linalool (33.1%), camphor (11.0%), linalyl acetate (10.4%), and 1,8 cineole (8%). Our results agree with data by Koulivand and coworkers (2013) who reported linalool, linalyl acetate, 1,8-cineole, β-ocimene, terpinen-4-ol, and camphor as the main constituents of *L. angustifolia* essential oil. However, the percentage of single constituents varies in different samples (Cavanagh and Wilkinson 2002; Woronuk et al. 2011). In our essential oil borneol, (E)-ocimene, α-terpineol and (Z)-caryophyllene were found in moderate concentrations, comparable to the *L. angustifolia* essential oil from India (Verma et al. 2010).

The main constituents in our *C. sativum* essential oil were linalool (67.8%), camphor (5.0%), α-pinene (5.0%) and geranyl acetate (3.7%), in agreement with previous studies of Shahwar and coworkers (2012) and Mandal and Mandal (2015). The chemical composition of *C. sativum* essential oil may change depending on environmental conditions and is also affected by the duration and condition of storage (Ebrahimi et al. 2010). In our sample, the amount of linalool was 67.3%, higher than in the essential oils analysed by Khani and coworkers (2012), and by Mandal and Mandal (2015), who found percentages of linalool of 57.57% and 58%, respectively. The observed variations in the relative percentages of single constituents may be attributed mainly to environmental conditions, method of harvesting, and methods used to obtain the essential oil (Misharina 2001; Smallfield et al. 2001; Gil et al. 2002).

In the composition of the essential oil of *L. nobilis* 1,8 cineole (31.9%), sabinene (12.2%), and linalool (10.2%) are the main components, with other compounds in low percentages or even in traces. The comparison with most recent literature concerning the chemical composition of the essential oil of *L. nobilis* from other Mediterranean areas, showed substantial differences in fact, 1,8-cineole percentage found (31.9%) is lower than the values recorded in other studies: Turkey 44.97% (Ekren et al. 2013), Tunisia 56.0% (Snuossi et al. 2016), Cyprus 58.59% (Yalcin et al. 2007), Morocco 52.43% (Derwich et al. 2009), but similar to Algerian oil (34.62%) (Jemaa et al. 2012). In our sample, the amount of sabinene was 12.2%, higher than in the essential oils analysed by Derwich and coworkers (2009), Snuossi and co-workers (2016), and Yalcin (2007), who found percentages of sabinene of 6.13, 3.5, and 3.32%, respectively. Linalool was found in concentrations comparable to previous studies (Jemaa et al. 2012, Ekren et al. 2013) but in oil analysed by Derwick and coworkers (2009) linalool was not found.

In *C. medica* essential oils there were no relevant quali-quantitative differences in the chemical composition neither with regard to the percentages of the classes of compounds nor to the main components. In both oils, limonene and camphene are the main components, even if the composition of the EO of *C. medica* cv. '*rugosa*' is more complex, but many of its constituents are present in very low percentages or even in traces. The comparison with the available literature concerning the chemical composition of the EO of citron from other countries, had showed substantial differences. Limonene percentages found (67.2% and 62.8%) are significantly higher than the values recorded in different parts of the world or even in Italy: South Korea, 52.44% (Kim et al. 2013); China, 33.84% (Wu et al. 2013); Santa Maria del Cedro (Italy), 59% (Gabriele et al. 2009); and Iran, 56.6% (Monajemi et al. 2005).

Other notable differences relate to the content in γ-terpinene exceeding 20% and of α- and β-pinene, reported between 7.73% (Wu et al. 2013) and 16.3% (Monajemi et al. 2005). Camphene range is 8.5%–10.9% (cv. '*liscia*' and cv. '*rugosa*', respectively) in samples studies in this research, but this compound is practically absent or in traces in the oils reported in literature.

4.2 Extracts chemical composition

4.2.1 Hypericum hircinum

We identified in *Hypericum hircinum* methanol extract isoquercetin, rutin and quercetin. Our results corroborate with previous studies that reported that the *H. hircinum* leaves contain chlorogenic acid, rutin, isoquercitrin, quercetin and hyperforin (Pistelli et al. 2000; Sagratini et al 2008). Quercetin and rutin were found also in other *Hypericum* species (Stojanovic et al. 2013).

4.2.2 Ipomea transvaalensis

We identified from chloroform: methanol extract of *I. transvaalensis* two alkaloids, ergine and ergometrine.

Many species of genus *Ipomea* contain ergine such as *I. asarifolia* (Desr.) Roem. & Schult., *I. corymbosa* (L.) Roth ex Roem. & Schult; instead *I. tricolor* Cav., *I. muelleri* Benth. and *I. violacea* L. contains both ergine and ergometrine (Meira et al. 2012; Nowak et al. 2016).

4.3 Cytotoxicity on SH-SY5Y cells

4.3.1 Essential oils

MTT assay results showed that *L. nobilis* essential oil is more cytotoxic against SH-SY5Y cells than the other essential oils studied with an IC_{50} of 471

µg/ml. Our findings indicated that *L. nobilis* EO is also more cytotoxic than its main component: 1,8-cineole. Specific induction of apoptosis by 1,8-cineole was observed in human leukemia Molt 4B and HL-60 cells, but not in human stomach cancer KATO III cells (Hiroyukimoteki et al. 2002). Our results showed that the essential oil had less cytotoxicity than the one tested on ACHN and C32 cell lines (IC_{50} 202.6 and 209.7 µg/ml for ACHN and C32, respectively) (Loizzo et al. 2007) and the leaf extract tested on human neuroblastoma cell lines SK-N-BE(2)-C and SH-SY5Y (Pacifico et al. 2013).

Prashar and coworkers (2004) reported that *L. angustifolia* essential oil is cytotoxic to human skin cells in vitro (HMEC-1, HNDF, 153BR). Imelouane and coworkers (2010) studied the cytotoxicity of the essential oils of *Lavandula dentate* aerial parts and flower on five human cancer lines (P388D1, PC3, V79,U-373 MG, MCF7). They reported that the cytotoxicity of the flower oil is stronger than that of the oil from aerial parts. Conversely, to our knowledge no studies have been carried out to verify the cytotoxicity of *C. sativum* essential oil on neuroblastoma or other cell lines.

However, comparing the IC_{50} values, our findings indicated that *C. medica* cv. '*liscia*' EO is more cytotoxic than *C. medica* cv. '*rugosa*' EO and limonene. Monajemi and coworkers (2005) reported that the EO of *C. limon* with large amounts of limonene (98.4%) was less cytotoxic than *C. medica* with low content of limonene (56.6%) on MCF-7 and HeLa cell lines. Our results agree with previous studies reporting that low concentrations of limonene were ineffective in cell death in SH-SY5Y cells (Corasaniti et al. 2007, Russo et al. 2014).

Among the main components of the essential oil considered, linalool has stronger cytotoxic activity (IC_{50} = 334 µg/ml) than 1,8-cineole and limonene with IC_{50} > 2000 µg/ml. Ravizza and coworkers (2008) demonstrated that linalool possesses antiproliferative effects against two human breast

adenocarcinoma cell lines (MCF7 WT and MCF7 AdrR), and Sun and coworkers (2015) reported similar results in human prostate cancer cells (DU145), at concentrations of 50 and 80 μM, respectively.

However, in our experiments the IC_{50} values for all essential oils was > 20 μg/ml, indicating that they were not cytotoxic as judged by the criterion set by the National Cancer Institute (Geran et al. 1972), which states that only natural substances with IC_{50} < 20 μg/ml are considered cytotoxic against the treated cells.

The different cytotoxicity between each essential oil and their main component can probably be attributed to a synergistic activity of this and other minor components present in the essential oil.

4.3.2 Extracts and fractions

Ipomea cairica methanol extract is more cytotoxic against SH-SY5Y cells than the other extracts studied and its alkaloid fraction, with an IC_{50} of 353 μg/ml. However, fraction of chloroform: methanol *Ipomea transvaalensis* extract showed an IC_{50} of 258 μg/ml; probably the alkaloids present in this fraction are different or in different proportion respect to those present in fraction of *Ipomea cairica* methanolic extract.

Lin et al. reported that two components of *Ipomea cairica* methanol extract, arctigenin and trans-2,3-dibenzylbutyrolactone, have a strong cytotoxicity against A549 (human lung cancer cell) and human prostate cancer epithelial cell (LNCaP) (Lin et al. 2008).

No studies in literature reported data on *Ipomea transvaalensis* extracts cytotoxicity and its alkaloids.

The *Hypericum hircinum* methanol extract showed an IC_{50} = 451 μg/ml. Quassinti and coworkers (2013) demonstrated that the potential inhibitory effect

on tumor cell growth of *Hypericum hircinum* essential oil is higher than our extract for T98G human glioblastoma cell line, PC3 human prostatic adenocarcinoma cell line, A431 human squamous carcinoma cell line and B16-F1 mouse melanoma cell line.

However, in our experiments the IC_{50} value for all the extracts and their fractions was > 20 μg/ml, indicating that they were not cytotoxic as judged by the criterion set by the National Cancer Institute (Geran et al. 1972).

4.4 Western blot analysis

The available literature reports a well-established role for adenylyl cyclase in the regulation of multiple brain processes, such as synaptic plasticity, learning, and memory. Moreover, a cross-talk between the cAMP signal transduction system and other signalling pathways, such as the ERK/MAP kinase regulatory system, has been described (Impey et al. 1999; Davis et al. 2003).

There are no studies on the action of natural substances on expression of ADCY1, PKA, pERK and ERK in SH-SY5Y cells.

In this perspective, we carried out experiments to determine whether exposure to essential oils, their main components, different extracts and their alkaloid fraction can affect this or other pathways in SH-SY5Y cells.

4.4.1 Essential oils and their main components

Our results show that treatment with different concentrations of linalool inhibits ADCY1, PKA, pERK, and ERK expression in SH-SY5Y cell. The inhibition of these proteins could explain dose-dependent sedative effects in the Central Nervous System described by Elisabetsky and coworkers (1995; 1999). Furthermore, because high levels of ERK activation correlated with

allodynia and hyperalgesia in several pain models, inhibitors of ERK phosphorylation could be used in order to reverse those altered pain states (Cruz and Cruz 2007).

Differently to its principal component, *L. angustifolia* essential oil showed an ADCY1 and ERK increased expression. Probably, this essential oil influenced other intracellular pathway, respect to linalool, to determine a concentration-dependent inhibition of neuronal networks described by Caputo and coworkers (2016). However, our results agree with those of Impey and coworkers (1998) that showed that odorants generate transient increases in cAMP and Ca^{2+}, both of which stimulate ERK activity in CNS neurons and PC12 cells. *Coriandrum sativum* essential oil increase pERK and PKA expression.

These results could be explained by an action mediated by other components of *L. angustifolia* and *C. sativum* essential oil that can modify the linalool effect.

We also have studied the effect of 1,8-cineole and *Laurus nobilis* essential oil. Our results showed that 1,8 cineole increases pERK and PKA expression, instead *L. nobilis* essential oil reduced ADCY1 and pERK expression in SH-SY5Y cell. This result could explain at molecular level the use of *L. nobilis* leaves in Iranian folk medicine, to treat epilepsy, neuralgia and parkinsonism (Zargari 1990, Aqili 1992).

Limonene, the main component of two cultivars of *Citrus medica* increased ADCY1 expression at a concentration of 800 µg/ml in SH-SY5Y but have no effect on pERK, ERK and PKA expression. *Citrus medica* cv. '*liscia*' essential oil influenced ADCY1, PKA, pERK and ERK expression, in fact we have found an increase of these proteins expression.

Our results corroborate with previous studies on extracts of different species of *Citrus*. Extracts of *C. reticulata* peels facilitated PKA/ERK/CREB signaling in hippocampal neurons and potently enhanced CRE-mediated transcription (Kawahata et al. 2013). Also hexane extracts derived from the peels of *C. grandis*

had the ability to induce activation of ERK1/2 and CREB in cultured neurons (Furukawa et al. 2012).

Citrus medica cv. '*rugosa*' essential oil influenced in different way ADCY1, PKA and ERK expression; in fact, it decreases these proteins expression. Probably, in *L. nobilis* and *C. medica* essential oils there are other components that could affect proteins expression and the activities showed are not due to their principal components.

4.4.2 Extracts and their fractions

The genus *Hypericum* comprises a number of species with many different biological activities, such as antimicrobial (Pistelli et al. 2000) and antidepressant activities (Dar and Khatoon 2000). In this study we demonstrated that *Hypericum hircinum* methanol extract decreased ADCY1 and increased pERK expression. Different studies reported that some flavonoids can act on Central Nervous System in sedation, anxiolytic or anti-convulsive effects, and modulate protein and lipid kinase signalling pathways (Spencer 2008, Jäger and Saaby 2011). In particular, at molecular level, quercetin, a flavonoid present in our extract, increased cAMP response element-binding protein (CREB) expression (Suganthy et al. 2016).

However, hypericin, one of the main constituents of the genus *Hypericum,* inhibited the activating phosphorylation of extracellular signal-regulated MAP kinases (ERK1/2) in human retinal pigment epithelial cells and in EA.hy926 cells, an endothelial hybridoma expressing endothelial cell properties (Karioti and Bilia 2010). Our results corroborate with Pistelli and coworkers, who reported that *Hypericum hircinum* leaves contain chlorogenic acid, quercetin, quercetrin, and biapigenin, but no phloroglucinol compounds such as hyperforins or hypericins (Pistelli et al. 2000). *Ipomea cairica* methanol extract and its alkaloid fraction

showed no effects on protein expression. *Ipomea transvaleensis* $CHCl_3$: CH_3OH (9:1) extract only increase pERK expression at a concentration of 125 μg/ml instead its alkaloid fraction decrease ADCY1 and PKA expression.

There are no studies on biological activities of these *Ipomea* species extracts.

However, between alkaloids that we have identified in *I. transvaalensis* extract there is the most important ergoline derivative in Convolvulaceae*:* ergine (D-lysergic acid amide, LSA). This is a close analogue of best known hemisynthetic LSD (lysergic acid diethylamide) and presents hallucinogenic and psychotomimetic effects (Medeiros et al. 2003), but yet undiscovered mechanism of action (Paulke et al. 2013). Probably, ergine can increase oxytocin release and it can be used for treating or reducing the severity of psychotherapeutic or social disorders such as autism and in particular can act as an adjunct to behavioural therapy in obsessive-compulsive disorder, Tourette's syndrome, schizophrenia and depression (Young and Modi 2012).

Also, the other identified alkaloid, ergometrine, is known to be biologically active (Dawson and Moffatt 2012) and like other ergot alkaloids present in Convolvulaceae may have biological effect in humans, because of their structural similarity to LSD (Paulke et al. 2013).

The psychotropic effects of LSD may include indirect changes in the regulation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Marona-Lewicka et al. 2011). This compound interacts with specific dopaminergic and noradrenergic receptors (Minuzzi and Cumming 2010). These activities can explain the effects of its analogues, ergine and ergometrine, on the proteins involved in the signal transmission in CNS.

4.5 Effects on neuronal activity

4.5.1 Linalool, Lavandula angustifolia and Coriandrum sativum essential oils

In order to evaluate the potential effect of linalool and the essential oils of *L. angustifolia* and *C. sativum* on neuronal spontaneous electrical activity, we exposed rat neuronal networks grown on MEA to increasing concentrations of all products. The approach allowed, for the first time, an efficacy assessment of linalool and of the two essential oils. The results showed a concentration-dependent inhibition of neuronal networks firing activity for all the three agents, among which linalool is the most effective with an IC_{50} for MFR of 25 µg/ml. This result agrees with different studies *in vivo* that reported a sedative effect of linalool (Elisabesky et al. 1995, Linck et al. 2009).

Conversely, the essential oils of *L. angustifolia* and *C. sativum* showed a lower efficacy as demonstrated by IC_{50} values of 100 and 88 µg/ml for *L. angustifolia* and *C. sativum*, respectively.

Of note, the two essential oils revealed a similar efficacy, though the gas chromatography analysis demonstrated a considerable difference in their content of linalool. In fact, considering that *L. angustifolia* essential oil contains 33.1% linalool, the IC_{50} value corresponding to the pure compound is about 33 µg/ml, which is near that obtained with linalool alone. It could therefore be assumed that the inhibiting effect of *L. angustifolia* essential oil is principally mediated by linalool. Differently, *C. sativum* essential oil contains 67.3% of linalool and, consequently, the IC_{50} corresponding to the pure compound is about 59 µg/ml.

This result could be explained by a reductive effect mediated by other components of *C. sativum* essential oil on the linalool-induced inhibition of electrical activity and shows that the combination of chemical mixtures leads to completely different effects from those obtained with each component applied singularly.

However, the reduction of electrical activity can confirm the sedative effects observed in previous studies on *Lavandula angustifolia* essential oil and *Coriandrum sativum* hydro-alcoholic extract (Buchbauer et al. 1991, Rakhshandeh et al. 2012).

4.5.2 Hypericum hircinum extract

In our experiment *H. hircinum* methanol extract showed a decrease of the MFR with an IC_{50} of 0.01 µg/ml. Our result corroborate with different studies in literature that reported a sedative activity of some *Hypericum* species in relation to the CNS such as *H. perforatum, H. hircinum* (Gîrzu et al. 1997, Diana et al. 2007) and *H. montbretii* (Can and Özkay 2012).

4.6 Studies *in vivo*

In this part of study we focused our attention on pharmacological action on CNS of *L. angustifolia* essential oil and linalool, its main component.

We administrated these substances in different conditions: i) without stress, ii) after and iii) before a stress condition. The results showed that *L. angustifolia* essential oil and linalool affect motor activity in OF1 mice. In the experiment I, in fact, in open field mice treated with linalool travelled a lower distance with a less velocity than their respective control group. Shaw and coworkers (2007) demonstrated, instead, that in rats *L. angustifolia* essential oil reduced peripheral

movement in open field; in our experiment we observed a little reduction but it is not statistically significant.

In Elevated plus maze experiments, the number of entries in the closed arms was lower by animals treated with linalool and *L. angustifolia* essential oil than those in the control group. We obtained similar data in elevated plus maze of experiment II and III. The open field and elevated plus maze results showed an effect on motor activity of mice OF1 for both the substances studied.

In literature some contradictory effects on motor activity of *L. angustifolia* essential oil are reported. Chioca and collaborators (2013) observed that mice exposed to inhaled lavender essential oil increased the number of entries and time spent in the open arms, instead Kumar and coworkers (2013) reported that Silexan (a standardized essential oil produced from *L. angustifolia* flowers with 36% linalool and 34% linalyl acetate) decreased number of closed arm entries in the elevated plus-maze. However, our results agree with Linck and coworkers (2008) that demonstrated that linalool reduced locomotor activity.

Tail suspension test showed an increase of immobility time for mice treated with *L. angustifolia* essential oil; this could indicate that essential oil produces depressive-like effects but the data can also be affected by effects on motor activity.

Coelho and coworkers (2013) showed that linalool was able to reduce the immobility time; in our test there was this effect but the reduction was not significant.

Moreover, social interaction test in experiment I showed that in mice treated with linalool and essential oil of *L. angustifolia* the time spent in social investigation was increased respect to group control. This result agrees with Linck and coworkers (2010) that revealed a significant linalool effect in increasing the

social interaction time in comparison with controls. No data are present in literature about the effects of *L. angustifolia* essential oil on social interactions.

The social interactions test results of experiment II and III showed that our substances can cancel the stress effects of social defeat. In fact, both linalool and *L. angustifolia* essential oil increase time spent in social interactions respect to group control.

Moreover, social defeat data of experiment II and III highlighted that resident mice are less aggressive with mice treated with linalool and *L. angustifolia* essential oil than the group control, and the intruder mice treated with *L. angustifolia* essential oil spent less time in defense and submissive behaviour than the group control. The mechanism of this social behaviour is very complicate and there are no other studies in this field.

At this moment we can only hypothesize that probably the intruder mice produce pheromones that influenced residents or they reduced defensive behaviour for the effects on motor activity and consequently residents are less aggressive.

5. Conclusions

Different plant species are active on various organs and systems and the study of the scientific basis to support their use has grown and continues to grow by little more than a century.

Many vegetal extracts, essential oils and their main constituents are active on the Central Nervous System. In fact, they are used as sedatives, hypnotics, and tranquilizers and they may be useful in treating CNS disorders.

However, to date no many studies have been carried out to verify the subcellular mechanism of these effects.

Different studies reported an important role for adenylyl cyclase in the regulation of synaptic plasticity, memory, and other multiple brain processes.

Some of the substances that I have studied in this PhD program showed different effects on CNS. We studied the effects of secondary metabolites at different levels: i) effects on *in vitro* proteins involved in the transmission of the signal; ii) effects on the electrophysiology of neuronal cells; iii) effects on animals.

Our *in vitro* experiments demonstrated that even if the precise mechanism of the biochemical effects of essential oils and of secondary metabolites is still unclear, ADCY1 pathway might be involved (Caputo et al. 2016, Aliberti et al. 2016, Caputo et al. 2017). Moreover, linalool, *L. angustifolia* and *C. sativum* essential oils and *H. hircinum* methanolic extract affect, in a concentration-dependent manner, neural firing activity in micro-electrode array (MEA) (Caputo et al. 2016).

Our *in vivo* experiments showed that linalool and *L. angustifolia* essential oil had effects on the Central Nervous System. These substances affect mice OF1 motor activity, increase social interaction and reverse stress effects. These

results highlight that could exist a relation between CNS and the use of essential oil in the aromatherapy.

Furthermore, many studies correlate alterations of proteins studied in this doctoral project with genesis and development of different CNS diseases such as Parkinson and Alzheimer diseases (Du et al. 2014; Feld et al. 2014; Wang et al. 2014).

However, among the properties of many traditional medicinal plants, it has been reported their important role in alleviating or preventing symptoms of neurodegeneration (Howes et al. 2003). Some natural products, such as monoterpenoids have showed anti-Alzheimer and anti-Parkinsonian effects (Salakhutdinov et al. 2017) acting for example as acetylcholinesterase inhibitors (Chung et al. 2001).

In this perspective and on the light of the results obtained, we will continue this study in order to search for natural substances that, acting on ERK and or PKA expression, could ameliorate symptoms of Alzheimer and Parkinson deseases.

6. Materials and Methods

6.1 Plant material

Lavandula angustifolia aerial parts and *Coriandrum sativum* fruits were collected in the Garden of Medicinal—Aromatic Plants in the Campus of the University of Salerno in July and August 2015, respectively. Fruits of *Citrus medica* cv. '*liscia*' and *C. medica* cv. '*rugosa*' were collected in February 2016 from biological cultivations in the Coast of Amalfi. *L. nobilis* leaves were collected in February 2016, in Montecorice, Cilento area (Campania, Southern Italy) 90 m above sea level. Representative homogeneous samples of population were collected during the balsamic time. *H. hircinum* aerial parts were collected in June 2014, in Agerola (NA).

All plants were identified by Prof. Vincenzo De Feo. Voucher specimens (labeled as DF/367/2015 for *L. angustifolia* and DF/392/2015 for *C. sativum*; De Feo/134/2016 for cv. '*liscia*' and De Feo/133/2016 for cv. '*rugosa*'; DF/324/2016 for *L. nobilis* and De Feo/237/2014 for *H. hircinum*) were stored in the Herbarium of the Pharmaceutical Botany Chair at the University of Salerno on the basis of *Flora d'Italia* (Pignatti 1982).

Ipomea transvaalensis and *I. cairica* aerial parts were collected in September 2015 in the Hanbury Botanic Gardens in Ventimiglia (GE) and were identified by Prof. Laura Cornara of Dipartimento di Scienze della Terra, dell'Ambiente e della Vita, University of Genoa.

6.2 Chemicals

Linalool, 1,8 cineole, limonene, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased by Sigma Italia, Milano.

6.3 Isolation of the Volatile Oil

One kilogram of *L. angustifolia* and *L. nobilis* dried leaves*;* of *C. sativum* dried fruits of *C. medica* peels were ground in a Waring blender and then subjected to hydrodistillation for 3 h according to the standard procedure described in the European Pharmacopoeia (2004).

The oils were solubilized in *n*-hexane, filtered over anhydrous sodium sulphate and stored under N_2 at +4°C in the dark until tested and analysed.

6.4 Extraction Procedure

One kilogram of *Ipomea* species and *H. hircinum* leaves were air-dried and then extracted, at room temperature, successively with solvents of increasing polarity (petroleum ether, chloroform, and methanol). Finally, each extract was evaporated to dryness under reduced pressure.

6.5 Identification of the Extracts Components

6.5.1 Thin Layer Chromatography (TLC)

A 1 µl aliquot of the extracts or their fractions were spotted on silica-gel plates and developed with mobile phase A ($CHCl_3$: CH_3OH: H_2O; 80:18:2 v/v/v) and B (BAW, $C_4H_{10}O$: CH_3COOH: H_2O; 60:25:15 v/v/v). The developed TLC plate was dried and then sprayed with Dragendorff's reagent that is widly used to detect different groups of alkaloids, or with cerium sulfate reagent to detects the other extracts components.

6.5.2 HPLC Analysis

The methanolic extract of *H. hircinum* was fractionated by gel permeation chromatography on a Sephadex LH-20 column, eluiting with CH_3OH. One hundred thirty nine fractions of about 10 ml each were obtained and pooled in fifteen main fractions (I-XV) on basis of their TLC similarity in $CHCl_3$-CH_3OH-H_2O (80:18:2) and $C_4H_{10}O$: CH_3COOH: H_2O (60:25:15). Fraction B (fraction V+VI) and C (VII) were purified with RPHPLC on a C18 μ-Bondapack column (30 cm × 7.8 mm), eluting with CH_3OH-H_2O (70%:30%, 60 min, flow 1 ml/min). Structural determination of three isolated compounds (isoquercetin, quercetin and rutin) was performed of ^{1}H NMR, ^{13}C NMR, ^{13}C NMR DEPT data and their comparison with literature data (Agrawal 1989).

6.6 GC-FID Analysis

Analytical gas chromatography was carried out on a Perkin-Elmer Sigma-115 gas chromatograph equipped with FID and data handling processor. The separation was achieved using a HP-5 MS fused-silica capillary column (30 m 0.25 mm i.d., 0.25 m film thickness). Column temperature: 40°C, with 5 min initial hold, and then to 270°C at 2 C/min, 270°C (20 min); injection mode splitless (1 L of a 1:1000 n-hexane solution). Injector and detector temperatures were 250°C and 290°C, respectively. Analysis was also run by using a fused silica HP Innowax polyethylenglycol capillary column (50 m 0.20 mm i.d., 0.25 m film thickness). In both cases, helium was used as carrier gas ml/min.

6.7 GC/MS Analysis

Analyses were performed on an Agilent 6850 Ser. II apparatus, fitted with a fused silica DB-5 capillary column (30 m × 0.25 mm i.d., 0.33 μm film

thickness), coupled to an Agilent Mass Selective Detector MSD 5973; ionization energy voltage 70 eV; electron multiplier voltage energy 2000 V. Gas chromatographic conditions were as reported in the previous paragraph; transfer line temperature, 295 °C.

6.8 Identification of the Essential Oil Components

Most constituents were identified by gas chromatography by comparison of their Kovats retention indices (Ri) (determined relative to the tR of n-alkanes (C_{10}–C_{35})), with either those of the literature (Jennings and Shibamoto 1980; Davies 1990; Adams 2007; Goodner 2008) and mass spectra on both columns with those of authentic compounds available in our laboratories by means NIST 02 and Wiley 275 libraries (Wiley 1998). The components' relative concentrations were obtained by peak area normalization.

6.9 Human Neuroblastoma Cell Cultures

Human neuroblastoma (SH-SY5Y) cancer cells were cultured in in RPMI medium supplemented with 1% L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin (all from Sigma Aldrich, St. Louis, MO, USA) at 37°C in an atmosphere of 95% O_2 and 5% CO_2.

6.10 Primary Neuron Cultures

Cortical neurons derived from enzymatically and mechanically dissociated cortex of day 18 embryonic Wistar SPF rat brain, as previously described (Novellino et al. 2011). After counting, 50,000–60,000 cells were plated on each poly-D-lysine (100 g/ml) and Laminin (0.02 mg/ml) coated 60-electrode PEDOT-CNT MEA chip (Multi Channel Systems, Reutlingen, Germany). Neurons were

maintained in neurobasal medium (NB) supplemented with 2% B27 and 1% Glutamax-I, and maintained in a humidified incubator at 37°C in a 5% CO_2 enriched atmosphere. Half volume of the medium was exchanged three times a week. As previously reported, experiments were carried out from 4 to 6 weeks in vitro, when neuronal networks are mature and both neuronal and glial cells are present (Hogberg et al. 2011). Each preparation was tested in triplicate on neuronal networks derived from different isolations. All chemicals and reagents used for the preparation and maintenance of cultures were obtained from Invitrogen S.r.L. (Milan, Italy). All studies were performed according to the National Research Council's guide for the care and use of laboratory animals by following protocols approved by the Institutional Animal Care and Use Committee.

6.11 MTT Bioassay

Human neuroblastoma cancer cells (SH-SY5Y) were plated (5×10^3) in 96-well culture plates in 150 µl of culture medium and incubated at 37°C in humidified 5% CO_2. The day after, a 150 µl aliquot of serial dilutions of essential oils, their main component or extracts and their fractions were added to the cells and incubated for 24 h. DMSO alone was used as control. Cell viability was assessed through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Briefly, 30 µl of MTT (5 mg/ml) was added and the cells incubated for additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilized with 30 µl of a solution containing 50%, v/v, N,N-dimethylformamide, 20%, w/v, SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Thermo Scientific Multiskan GO, Monza, Italy) equipped with a 520 nm filter. Cell viability in response to treatment was calculated as a percentage of control

cells treated with DMSO at the final concentration 0.1% viable cells = (100 OD treated cells)/OD control cells (Picerno et al. 2005).

6.12 Extraction Proteins and Western Blotting

Cells were treated with different concentrations of essential oils, their main component or extracts and their fractions. The cells were collected after 24 h and lysed using the Laemmli buffer to extract total proteins. For Western Blot analysis, an aliquote of total protein was run on 8% SDS-PAGE gels and transferred to nitrocellulose. Nitrocellulose blots were blocked with 10% nonfat dry milk in Tris buffer saline 0.1% Tween-20 over night at 4°C and incubated with primary anti-ADCY1, anti-pERK, anti-ERK and anti-PKA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 h at room temperature. Immunoreactivity was detected through sequential incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Pittsburgh, PA, USA) and enhanced chemiluminescence reagents (ImmunoCruz, Santa Cruz Biotechnology, SantaCruz, CA, USA) (Petrella et al. 2006). The density of each band was measured by using ImageJ software (WS Rasband, ImageJ, NIH, Bethesda, MD).

6.13 Data Recordings, Signal Processing and Data Analysis

The spontaneous electrical activity was recorded by the USB MEA 120 INV 2 BC System from Multi Channel Systems (MCS GmbH, Reutlingen, Germany) as previously reported (Novellino et al. 2011). Briefly, the MEA chips were placed into the MEA Amplifier (Gain 1000) and data were recorded by the MC_Rack software (MCS GmbH, Version 4.4.1.0) at a sampling rate of 10 kHz. A band pass digital filter (60–4000 Hz) was applied to the raw signal in order to remove

electrical background noise. Only the electric signal overcomes the spike detection threshold of 5.5 times the standard deviation of the mean square root noise, was identified and recorded.Throughout the experiment, the cell cultures were maintained at 37°C by a temperature controller (TC02, MCS GmbH) and in a controlled and humidified atmosphere (9% CO_2, 19% O_2 and 72% N_2) to maintain the pH balance (pH was 7.0 ± 0.3). The data analysis was conducted by importing data (for MCS software, *.mcd files) into NeuroExplorer software (Littleton, MA, USA) and considering the network mean firing rate parameter (MFR; number of spikes/s). After exportation to Excel spreadsheets, data were averaged over several MEAs to create concentration curves for each treatment. The estimated IC_{50} values (half-maximal inhibitory concentration) were obtained by interpolating the normalised concentration–response curves of single treatments with the following four-parameter logistic function using SigmaPlot 8 software (Jandel Scientific, San Rafael, CA, USA):

$$f(x) = Max + (Min-Max)/(1+(\varepsilon /x)\ \beta)$$

where the variable x is the concentration of the compound; the parameter Min is the minimum effect; the parameter Max is the maximum effect; the parameter ε is the concentration at which the effect is reduced by 50% (IC_{50}); is a parameter related to the maximum slope of the curve, which occurs at concentration ε.

6.14 Animals

A total of 70 male mice of the OF1 strain (Charles River Barcelona, Spain) were employed in this study. The animals arrived at the laboratory at 21 days of age and were housed in groups of five in plastic cages (28 ×28×14.5 cm), under standard conditions: constant temperature (21 ± 2 °C), a reversed light schedule (white lights on: 19.30 – 07.30), and food and water available *ad libitum* (except during behavioral tests). Procedures involving mice and their

care were conducted in conformity with national, regional and local laws and regulations, which are in accordance with the European Communities Council Directives (86/609/EEC, 24 November 1986).

6.15 Drug treatment

Animals were injected intraperitoneally with 100 mg/kg of linalool (Sigma Aldrich, St. Louis, MO, USA) or 200 mg/kg of *Lavandula angustifolia* essential oil in a volume of 0.01 ml/g of mice body weight. Linalool and essential oil were solubilized in physiological saline solution with 2% Tween-80 and 1% DMSO. Control groups were injected with physiological saline (NaCl 0.9%), which was also used to dissolve the drug. The doses of linalool and *L. angustifolia* essential oil used to test the effects on behaviour of mice in different situations were selected on the basis of previous studies (Kim et al. 2009, Coelho et al. 2013, Xu et al. 2016).

6.16 Experimental design

An overall description of the experimental procedure is provided in Table 6.1.

Behavioral Tests began on PND 39. In experiment 1, 30 minutes after the administration of Linalool or *L. angustifolia* essential oil, OF1 mice (PND 39 to 47) performed the Open Field, Elevated Plus Maze (EPM) and Social interaction test. Three groups were employed in this experiment: Control, Linalool 100 mg/Kg, EO *L. angustifolia* 200 mg/Kg. In experiment 2, OF1 mice (PND 88) were exposed to social defeat (SD) followed, after 10 minutes, by intraperitoneal injection of linalool or *L. angustifolia* essential oil and after 30 minutes, they performed the Elevated Plus Maze (EPM) and Social

Interaction test. Four groups were employed in this experiment: Control (Explora Saline), SD Saline, SD Linalool 100 mg/Kg, SD EO *L. angustifolia* 200 mg/Kg.

In experiment 3, OF1 mice (PND 104 to 120) after 30 min of an acute or a cronic administration for 10 days of linalool or *L. angustifolia* essential oil were exposed to social defeat, and after 40 min they performed the Elevated Plus Maze (EPM) and Social interaction test. Again, four groups were employed in these experiments: Control (Explora Saline), SD Saline, SD Linalool 100 mg/Kg, SD EO *L. angustifolia* 200 mg/Kg.

Table 6.1 Experimental procedure.

PND	**39-47**	**88**	**104-120**	
	Experiment 1: effects in OF1 mice	Experiment 2: effects in social stressed OF1 mice	Experiment 3: effects on social stress and social interaction of OF1 mice	
	Acute administration	Social defeat	3a Acute administration	3b Cronic administration (10 days)
	Open Field Elevated plus maze Social interaction	Acute administration Elevated plus maze Social interaction	Social defeat Elevated plus maze Social interaction	

6.17 Open-field test

An open-field apparatus (32 × 30 × 32 cm) made of black Plexiglas was used to evaluate the motor behaviour after an intraperitoneal injection of physiological solution, linalool (100 mg/kg) or *L. angustifolia* essential oil administered 30 min before the test. The light in the room created an illumination of 150 lx at the centre of the open field. Animal behaviour was

tracked during a period of 30 min and analysed using EthoVision 3.1 software (Noldus Information Technology, Leesburg, VA). Total distance travelled (cm) and velocity (cm/s) were calculated. Additionally, we measured the time spent along the walls (periphery) and in the centre areas of the open field and the frequency to enter in the centre in order to evaluate if there were differences between the groups in the exploratory tendencies of mice. In this study, we used the open-field test to evaluate the possible sedative effects of linalool and *L. angustifolia* essential oil.

6.18 Elevated Plus Maze

The Elevated Plus Maze (EPM) consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm). The junction of the four arms formed a central platform (5 × 5 cm). The floor of the maze was made of black Plexiglas and the walls of the enclosed arms of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide the animals with additional grip. The entire apparatus was elevated 45 cm above floor level. In order to facilitate adaptation, mice were transported to the dimly illuminated laboratory 1 h prior to testing. At the beginning of each trial, subjects were placed on the central platform so that they were facing an open arm and were allowed to explore for 5 min. The maze was thoroughly cleaned with a damp cloth after each trial. The behaviour displayed by the mice was recorded automatically by an automated tracking control (EthoVision 3.1; Noldus Information Technology, Leesburg, VA). The measurements recorded during the test period were frequency of entries and time and percentage of time spent in each section of the apparatus (open arms, closed arms, central platform). An arm was considered to have been visited when the animal placed all four paws on it. Number of open arm entries, time spent in open

arms and percentage of open arm entries are generally used to characterize the anxiolytic effects of drugs (Pellow and File 1986; Rodgers et al. 1997).

6.19 Social defeat

Animals in the experimental group were exposed to one episodes of social defeat lasting 25 minutes each. An episode consisted of three phases, which began by placing the experimental animal or intruder in the home cage of the aggressive opponent or resident for 10 minutes. During this initial phase, the intruder was protected from attack by a wire mesh wall that permitted social interaction and species-typical threats from the male aggressive resident (Covington and Miczek 2001). In the second phase, the wire mesh was removed from the cage and a 5-minute period of confrontation began. In the third phase, the wire mesh was replaced for a further 10 minutes to allow social threats from the resident. The exploration group underwent the same protocol, but without the presence of a 'resident' mouse in the cage. The second phase of each social defeat protocol was video-recorded and ethologically analysed in the experiment 2 and 3. The following behaviors were scored for resident mice: threat and attack; and time needed to perform the first threat and attack (latencies). In the case of intruder mice, the following behaviours were analysed: avoidance/flee and defensive/submissive behaviors; and time needed to exhibit the first avoidance/flee or defense/submission (latencies).

6.20 Social interaction test

This test consisted of confronting an experimental animal and a standard opponent in a neutral cage (61 × 30.5 × 36 cm) for 10 minutes following a 1 minute adaptation period prior to the encounter. One day before testing,

standard opponents were rendered temporarily anosmic by intranasal lavage with a 4% zinc sulfate solution (Smoothy et al. 1986). This kind of mouse induces an attack reaction in its opponent, but does not outwardly provoke or defend itself since it cannot perceive a pheromone that is present in the urine of the experimental animals and functions as a cue for eliciting aggressive behavior in mice with a normal sense of smell (Brain et al. 1981; Mugford 1970). Behaviour was videotaped under white illumination. The videotapes were analysed using a custom-developed program that estimates the time devoted to different broad functional categories of behaviour (non-social exploration, social investigation, threat, attack and avoidance/flee,), each of which is characterized by a series of different postures and elements. A more detailed description can be found in Rodriguez-Arias et al. (Rodriguez-Arias et al. 1998).

6.21 Statistical Analysis

All experiments were carried out in triplicate.

The data for each experiment were statistically analysed using Graph Pad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA) followed by comparison of means (one-way ANOVA) using Dunnett's multiple comparisons test, at a significance level of $^{*}p < 0.05$.

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