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Msc thesis in
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Work supervised by
Professor Doutor Alberto Carlos Pires Dias

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Declaração

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PUBLICATIONS

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Produção de metabólitos secundários em culturas de células em suspensão de *Ocimum sanctum* L.

RESUMO

A espécie *Ocimum sanctum* pertence à família Labiatae. Conhecido pelo seu potencial medicinal e terapêutico, é tradicionalmente utilizado no tratamento do reumatismo, paralisia, epilepsia, constipações, gripe, febres altas, dores abdominais, tosse e outras sintomatologias. *O. sanctum* é descrita como uma "Rasayana", isto é, uma planta com propriedades adaptogénicas, e é conhecida pela medicina tradicional Ayurveda como sendo o "elixir da vida" e por aumentar a longevidade. Vários investigadores demonstraram que *O. sanctum* possui várias atividades biológicas, maioritariamente relacionadas com mecanismos antioxidantes enzimáticos e não enzimáticos e, também, a capacidade de manter a homeostasia e a resistência do corpo a diferentes tipos de stresse devido ao conteúdo em compostos fenólicos, em particular o ácido rosmarínico.

Assim, este estudo teve como principais objetivos: 1) o aumento da produção de ácido rosmarínico e compostos fenólicos bioactivos através da eliciação de culturas celulares em suspensão de *O. sanctum*; 2) a determinação *in vitro* das propriedades antiradicalares dos extratos respetivos; e, 3) a incorporação dos extratos num creme e avaliação *in vitro* das suas propriedades antiradicalares.

Culturas celulares em suspensão de *O. sanctum* foram tratadas com vários eliciadores de forma a aumentar a produção e acumulação de compostos fenólicos, nomeadamente ácido rosmarínico. O conteúdo em compostos fenólicos dos extratos celulares e dos extratos de folhas *in vivo* foi determinado por HPLC-DAD. As culturas celulares em suspensão de *O. sanctum* demostraram uma produção de ácido rosmarínico 93% superior à das plantas *in vivo*.

As propriedades antioxidantes dos extratos das culturas celulares de *O. sanctum* foram avaliadas através do método DPPH, atividade quelante do ferro, inibição da produção de óxido nítrico e remoção do anião superóxido. Os extratos das culturas celulares de *O. sanctum* demonstraram elevada atividade antioxidante, mostrando um elevado potencial na inibição da produção de óxido nítrico e de superóxido evitando, desta forma, a interação destes e prevenindo a formação de $ONOO^-$. Contrariamente ao ácido rosmarínico puro, os extratos celulares têm também a capacidade de quelar iões de ferro diminuindo a formação de grupos hidroxilo produzidos

na reação de Fenton. Estes extratos demonstram, ainda, um elevado potencial de *scavenging* de DPPH, podendo atuar como antioxidantes devido ao alto conteúdo em compostos fenólicos.

Depois de ter realizado testes sobre as actividades antioxidades dos extractos de *O. sanctum*, uma porção dele foi incluído com sucesso numa base de creme, com objetivo avaliar as capacidades antioxidantes do creme obtido para executar próximo teste em ratos.

Production of Secondary Metabolites in Cell Suspension Cultures of *Ocimum sanctum* L.

ABSTRACT

The *Ocimum sanctum* belongs to the Labiatae family. Commonly known for their medicinal and therapeutic potential, traditionally used to treat rheumatism, paralysis, epilepsy, colds, flu, high fever, influenza, abdominal pains, coughs and others symptomologies. *O. sanctum* is often described as a "Rasayana" (plant with adaptogenic properties), it is regarded in Ayurveda as a "elixir of life" and believed to promote longevity. Several investigators have reported that *O. sanctum* possess numerous biological activities mostly related with enzymatic and non-enzymatic antioxidant mechanisms and the capability of maintain body homeostasis and resistance to different stress due their phenolic compounds constituents, namely rosmarinic acid.

The objectives of this study were: 1) increase rosmarinic acid and phenolics production trough elicitation techniques in cell suspension cultures; 2) evaluate the *in vitro* antiradical properties of these extracts; 3) Incorporate the extract into a cream base and evaluate its *in vitro* antioxidant properties.

O. sanctum cell suspension cultures were treated with several elicitors in order to increase phenolic compounds production and accumulation (mainly rosmarinic acid); phenolic quantification of cell extracts and *in vivo* leaf extracts were analyzed by (HPLC-DAD). *O. sanctum* cell suspension cultures were able to produce 93% more rosmarinic acid than *in vivo* plants;

In vitro radical properties of the extracts were analyzed trough DPPH, iron chelating activity, inhibition of nitric oxide production and scavenging of superoxide anion. *O. sanctum* cell suspension cultures extracts have shown antiradical scavenging activity for the non-enzymatic assays tested in the present study displaying high potential to inhibit the nitric oxide production and superoxide anion, avoiding their interaction and preventing the formation of $ONOO^-$; contrary to pure rosmarinic acid, cell extracts can also chelate iron ions decreasing the formation of hydroxyl groups produced by the Fenton reaction; cell suspension extracts also displayed high DPPH scavenging potential and may act as chain breaking antioxidants.

After having performed tests on the antioxidant activities *O. sanctum* in extracts, a portion of it was included successfully in a cream base in order to evaluate the antioxidant capacities in order to determine their ability to perform next test in rats.

INDEX

FIGURE INDEX

TABLE INDEX

Abbreviations

•NO - Nitric Oxide Radical

2,4-D - 2,4-Dichlorophenoxyacetic acid

ABA - Abscisic acid

ALT - Serum Alanine Aminotransferase

AST - Serum Aspartate Aminotransferase

ATP - Adenosine Triphosphate

CAT - Catalase

DHPL - Dihydroxyphenilacetic acid

DNA - Deoxyribonucleic acid

DNBC - Dinitrochlorobenzene

DPPH - 1,1-diphenyl-2-picrylhydrazyl

Dwb - Dry Weigh Biomass

Dwr - Dry weighted Residual

FAK - Focal Adhesion Kinase

FRAP - Ferric Reducing Antioxidant Power

GABAergic - Gamma Aminobutyric acid

GGA - Glacial Acetic acid

GSH - Glutathione

H_2O_2 - Hydrogen Peroxide

HDL - High density Lipoprotein

HOO• - Hydroperoxyl Radical

HPLC-DAD - High Performance Liquid Chromatography Diode Array

ICA - Iron Chelating Activity

IPP - Isopentyl Pyrophosphate

ISO - Isoprenol

JNK - C-Jun N-terminal Kinase

LCC - Lewis Lung Cancer

LDL - Low density Lipoprotein

Mej - Methyl Jasmonate

MI - Myocardial Infarction

Na2EDTA - Ethylenedinitrilotetraacetic acid disodium salt dihydrate

NAA - Naphthalene Acetic acid

NADH - Nicotine Adenine Dinucleotide (Reduced form)

NADPH - Nicotine Adenine Dinucleotide Phosphate Hydrogenated

NBT - Nitroblue Tetrazolium Chloride

NED - N-(1-naphthyl) ethylenediamine

NF-kB - Nuclear Factor kB

NO - Nitric Oxide

$O_2^{\bullet -}$ - Superoxide Anion

OH• - Hydroxyl Radical

ONOO– - Peroxynitrite Anion

ORAC - Oxygen Radical Absorbance Capacity

PAL - Phenylalanine Ammonia-lyase

PMS - Phenazine methosulfate

RA - Rosmarinic acid

RAS - Rosmarinic acid synthase

RNS - Reactive Nitrogen Species

ROS - Reactive Oxygen Species

RUBISCO - Ribulose-1,5-bisphosphate carboxylase/oxygenase

Sa - Salicylic acid

SNP - Sodium Nitroprusside

SOD - Superoxide Dismutase

TAT - Tyrosine Amino Transferase

TNFα - Tumor Necrosis Factor

Ye - Yeast Extract

CHAPTER 1

GENERAL INTRODUCTION

1.1 Medicinal plants and secondary metabolites

Plants are able to produce two major groups of compounds: primary and secondary metabolites. Primary metabolites include mostly sugars, amino acids and fatty acids, mainly involved in growth, tissue development and reproduction. Kossel (1891) introduced the concept of secondary metabolism and secondary metabolites to define compounds that apparently did not have a role in the maintenance of fundamental life processes in the organism. Later, it was found that secondary metabolites play an important role in the interaction of plants with their environment. Plants chemical adaptations to environmental stress range from production of secondary metabolites that protect them from herbivores to production of UV absorbing compounds to prevent sunlight (Hartmann, 2004; Mazid*, et al.*, 2011).

Plant secondary compounds are classified, according to molecule size and their biosynthetic pathways, in three families (Figure 1.1): i- alkaloids commonly derived from aromatic or aliphatic aminoacids (Crozier*, et al.*, 2006), ii- terpenes and steroids synthetized from mevalonic acid in the cytoplasm or pyruvate and 3-fosfoglycerate in the chloroplast (Harbonre, 1999; Crozier*, et al.*, 2006), iii- phenolics derived from shikimate pathway or mevalonic acid pathway (W. Vermerris & R. Nicholson, 2006). Some families of secondary metabolites are widespread and can be found all over on Earth, for example phenolics compounds which are involved in lignin synthesis, are common to all higher plants; while other compounds, such as alkaloids are sparsely distributed among higher plants. Alkaloids presence is used as a taxonomic trait and constitutes the basis of chemotaxonomy and chemical ecology (Bourgaud*, et al.*, 2001).

Since immemorial times the human kind has been using its knowledge of the surrounding environment and resources to survive. Especially plants have been used to solve different kinds of adversities depending of their properties, namely medicinal and nutritional. The world population has been rapidly increased in the last decades, so a high demand for plants derived compounds has rapidly increased as well.

Plant secondary metabolites have been used in medicine and food industry due to their large biological activities. They correspond to valuable compounds used as cosmetics, paper industry, flavors, insecticides, food additives, medicinal and pharmaceuticals, fine chemicals, or more

recently nutraceuticals (Bourgaud, *et al.*, 2001; Verpoorte, *et al.*, 1994). In fact in 2002, USA valued the prescription drugs containing phytochemicals in US$30 billion (Raskin, *et al.*, 2002).

For example phenolic compounds are very present in our lives. Thus, much of the flavor, odor and staining of various vegetables are generated by phenolic compounds. Some of these compounds, such as cinnamic acid (*Cinnamomum zeyllanicum)* and vanillin (*Vanilla planifolia)*, are employed in the food industry (Peres, 2004). On the other hand, alkaloids are synthetized in the endoplasmic reticulum and stored in the vacuole, usually characterized by the presence of nitrogen atoms and a strong action in the central nervous system. Terpenes are considered polymers; their basic unit is a 5 carbons molecule called isopentyl pyrophosphate or IPP, derived from mevalonic acid; the addition of one or more IPPs will define the characteristics of the compounds, therefore the addition of IPP will produce monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40) and polisoprene ($C40^{+}$) (Humphrey & Beale, 2006).

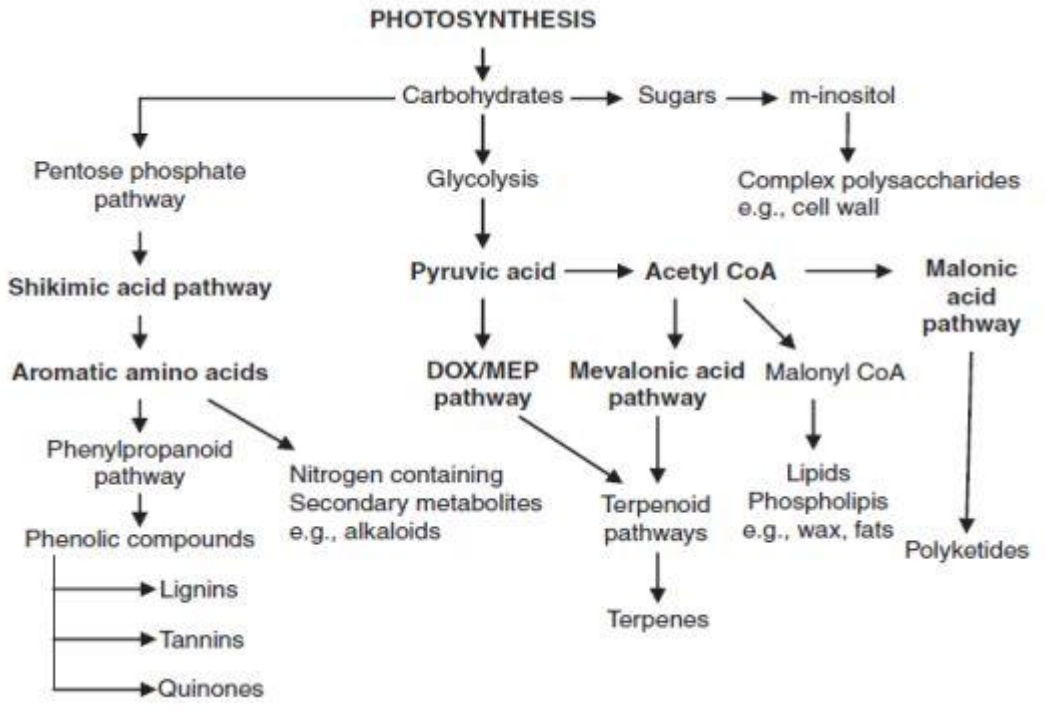

Figure 1. 1 - Secondary metabolites principal pathways (Ramawat 2010)

With human population reaching critical levels, the demand for plant derived products has increase. Field cultivation of selected plants is not enouhg to cover the high demand due several reasons: i- some species do not respond adequately to growth outside their natural habitat and become very susceptible to plagues and diseases, ii- species react differently depending on the

biotypes of origin and culture environment characteristics (Bourgaud*, et al.*, 2001). Other plants may grow very slowly, like quina trees that need about 10 years before be ready for harvesting (Verpoorte*, et al.*, 2002; Verpoorte*, et al.*, 1994) . Many of the plant derived products like cocaine, codeine, quinine, morphine, nicotine, caffeine, anti-cancer *catharanthus* alkaloids, belladonna alkaloids, phytostigminine, colchicines, pilocarpine, reserpine and steroids like diosgenin, digoxin and digitoxin have an high demand but not useful synthetic substitutes (Namdeo, 2007). Therefore many plants containing high-value compounds are becoming endangered because of overharvesting (Rates, 2001).

1.2 Secondary metabolites and plant defense

As a sessile organism, plants cannot escape from organism's attack trough locomotion; instead other strategies to defend themselves have appeared. Plants have the ability to synthetize approximately 200,000 compounds that evolved in response of particular changes in the environment (Pichersky & Lewinsohn, 2011), besides pathogens, herbivorous, arthropods and insects attacks.

Plant defenses can be distinguished in two broad categories: *1)* always present and *2)* inducible specifically by certain aggressor (Mithofer & Boland, 2012). Intracellular signals, membrane receptors, biochemical changes and cellular responses are mechanisms that help plants to discriminate the type of attack they faced and likewise determine responses. Plants respond differently to attack of pathogens, insects and herbivores or to other biotic and abiotic stresses by activating an array of defense mechanisms including induction of biosynthesis of secondary metabolites such as pigments, phytoalexins, hypersensitive responses and structural defensive barriers, for example lignin, deposition on cell wall, thorns, spikes, glandular hairs (Wink, 1988), among others (Vasconsuelo & Boland, 2007).

1.3 Reactive oxygen species (ROS)

Terms like oxidative stress, oxidative damage, free radical and antioxidants have become an integrated part of the scientific lexical. Molecular oxygen (O_2) is essential for the survival of all aerobic organisms, required by prokaryotic and eukaryotic cells for energy production, often via the electron transport chain in the mitochondria (Held, 2010). Oxidative stress occur when there is a serious imbalance in any cell compartment between production of reactive oxygen species

(ROS) and antioxidant defenses leading to damage at several levels. (Figure 1.2)(Agius, *et al.*, 1998; Apel & Hirt, 2004b; Maritim, *et al.*, 2003; V. J. Thannickal & Fanburg, 2000).

ROS production is an unavoidable consequence of aerobic metabolism. Oxidoreduction energy produced of the mitochondrial electron transport is converted into high energy phosphate bound adenosine triphosphate (ATP). O_2 perform as the final electron acceptor for the cytochrome-c oxidase complex, (Apel & Hirt, 2004b) the enzymatic terminal that catalyzes the reduction of O_2 to H_2O. However O_2 metabolites could also be formed like the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). When transition metals ions are involved more reactive species can be formed like hydroxyl radical (OH^-).

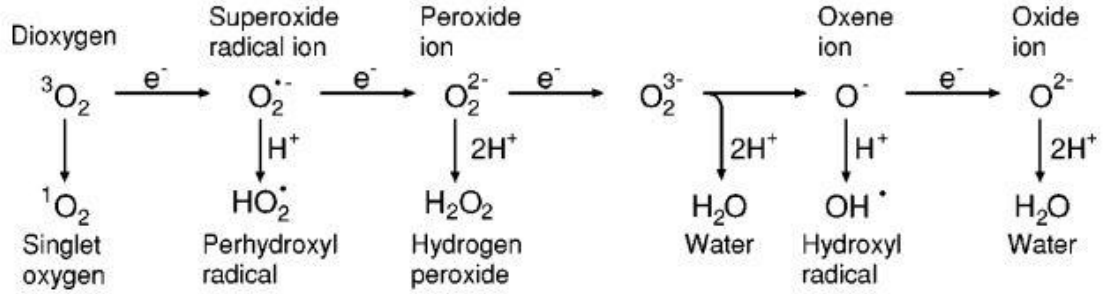

Figure 1. 2 - Generation of different ROS by energy transfer or sequential univalent reduction of ground state triplet oxygen (Apel and Hirt 2004).

ROS are produced mostly in mitochondria (Chance, *et al.*, 1979; Held, 2012), chloroplast, peroxisome and nitrogen fixing nodules (Becana, *et al.*, 2000) as unwanted products. ROS production also occurs in the course of major metabolic such as photosynthesis and respiration. External factors such as environmental stress, injuries and pathogen attacks organisms can also increase the concentration of free radicals to unsustainable limits for the proper functioning of the body. Imbalances in homeostasis could trigger a succession of events related to lipid peroxidation that can lead to the collapse of the cell membrane at several stages (Wilfred Vermerris & Ralph Nicholson, 2006b); reactive oxygen can cause oxidative damage to DNA bases, which can lead to mutagenesis and are finally implicated in cancer, peroxidation of unsaturated lipids can alter membrane structure, disrupting permeability properties and altering recognition of cellular components leading to compromise the immune system (Lambeth & Neish, 2014).

In the last years it has been attributed a dual leadership to ROS; oxygen radicals have a role in cellular regulation control and defense mechanisms, ROS production by phagocytes and by other cells in gastrointestinal and respiratory tracts act as defense against microorganisms (Nauseef,

2008). Oxygen radicals have also a role in cellular processes controlled by enzymes and transcriptional factors, vascular cells produce superoxide and other ROS, which in turn activate multiple intracellular signaling pathways such as ERK1, ERK2, JNK(c-Jun N-terminal kinase), NF-κB (nuclear factor κB), FAK (focal adhesion kinase), AP-1, Akt, Ras, Rac, and JAK-STAT (Buonocore*, et al.*, 2010; Lambeth & Neish, 2014; Simon*, et al.*, 1998).

1.4 The *Ocimum* genus

The genus *Ocimum* also known as Basil belongs to the Labiatae family. They are strong aromatic plants due to the presence of many essential oils, mainly monoterpenes, sesquiterpenes and phenylpropanoids (Raimo & Yvonne, 2005). *The Plant List* (Version 1.1) reports 327 species for the genus *Ocimum* recognizing 68 names as accepted and 241 as synonyms (Figure 1.3). (*List*, 2013). Still the exact number of species is not yet clear due to regular occurrence of interspecific hybridization within genus (Raseetha*, et al.*, 2009).

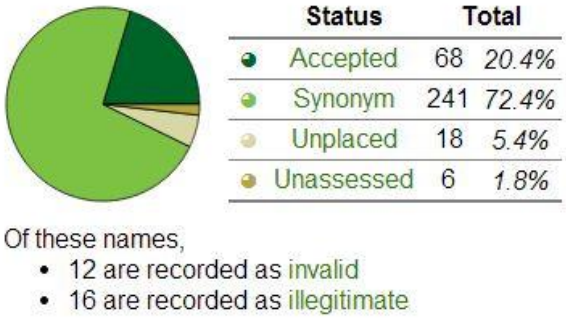

Figure 1. 3 - The Plant List status for the 333 species names for *Ocimum* genus

The genus has been known in many cultures and associated to history and folklore (Khosla, 1995); characterized for the production essential oils that confers strong flavors, *O. basilicum* (sweet basil) is used intensely in culinary, *O. americanum* and *O. minimum* for fragrances and pot herbs (Raimo & Yvonne, 2005). Some species like *O. kilimandscharicum, O. forskolei, O. gratissimum, O. canum* and *O. sanctum* are used across the world in traditional medicine for treatments against pains, diabetes, diarrhea, poisons, gonorrhea, epilepsy, anthelmintic, etc. (Demissew & N.; Saha*, et al.*, 2013). Some of the most important *Ocimum* species are summarized in table 1.1. The genus involves the most important economically medicinal and aromatic herbs, undershrubs or shrubs in the world, being rich in biological activities due their variable chemical composition (Saha*, et al.*, 2013). *Ocimum* metabolites are very variable and production and accumulation depends of several biotic and abiotic factors; glycosides flavonoids are accu-

mulated in the budding stage and increase during the flowering period; flavone aglycones, tannins and polyphenols began after sowing and increase during the vegetative phase (Lemberkovics*, et al.*, 1996).

Table 1. 1- Biological activities of some species of the *Ocimum* genus modified from (Raimo and Yvonne 2005)

Species	Common name	Pharmacological activity
O. americanun	American basil	Antimicrobial
O. basilicum	Sweet basil	Antimicrobial, Antinociceptive, Antiviral, Larvicidal
O. campechianum	Least basil	Antidiabetic
O. canum	Kali tulasi	Acaricidal
O. citriodorum	Lemon basil	Antioxidant
O. forskolei	Basilikum	Antimosquito
O. gratissimun	African basil	Anticancer ,Antimicrobial, Antifungal, Antinociceptive, Antiviral, Antitrypanosoma
O. kenyense	Kenya basil	Insecticidal
O. kilimandscharicum	Camphor basil	Antimosquito, Insecticidal, Wound healing
O.lamiifolium	Basilikum	Anti-inflammatory
O. micranthum	Peru basil	Antibacterial, Antiradical activity
O.minimum	Bush basil	Phytoremediation
O. sanctum	Holy basil	Anthelmintic, Anticandidal, Antiaflatoxine, Antifungal, Hepatoprotector, wound healing
O. selloi	Pepper basil	Antidiarrheic, Anti-inflammatory, Antispasmodic
O. suave	Scented basil	Antimicrobial, Antipyretic

Several flavonoids have been found in basil species. Three flavones: eriodictyol, eriodictyol -7-glucoside and Vicenin-2 (Apigenin di-C- glycoside) were isolated from *O. basilicum* from Greece; xanthomicrol (5,4'-dyhydroxy-6,7,8-trimethoxyflavone) was isolated from the same species collected in Nigeria. In average basil species present 0.5 – 1.5% of glycosides flavonoids and 0,6 - 1,5% of flavone aglycones (Viorica, 1987). In *Ocimum* species phenolic acids are described as the most active compounds, especially in *O. sanctum*; caffeic acid and rosmarinic acid were reported to be present in all growing stages (Raimo & Yvonne, 2005). In minor proportion gallic acid, ethyl ester, protocatechuic acid, vanillic acid, 4- hydroxybenzoic acid, vanillin and chlorogenic acid, are also compounds present (Raimo H., 2005).

1.5 *Ocimum sanctum* L.

Also known as Holy basil or *Tulsi* is an important symbol of the Hindu religion and tradition (Govind & Madhuri, 2010). Marked by its strong aroma and astringent taste it has been used as flavoring in Asian cuisine, many Hindus ingest small quantities of the leaves as food additive or after divine worship in temples (F. L. Hakkim*, et al.*, 2007). It is regarded in Ayurveda as a kind of "elixir of life" and believed to promote longevity (Priyabrata*, et al.*, 2010). Native from tropical Asia and best likely originated in India is also well distributed in tropical areas (Pandey & Madhuri, 2010).

O. Sanctum (Figure 1.4) grows between 30-60 cm, leaves are simple, opposite, elliptic, oblong, obtuse or acute with entire or subserrate or dentate margins, growing up to 5 cm long. The Tulsi flowers are small, purplish in elongate racemes in close whorls. The fruits are small and the seeds are reddish-yellow in color (Govind & Madhuri, 2010).

Figure 1. 4 *Ocimum sanctum* plant

1.6 Chemical composition of *O. sanctum* species

Geographical location and several biotic and abiotic factors have strong repercussions in the chemical composition of *O. sanctum;* even plants in the same space could present major differences between them (Pattanayak*, et al.*, 2010). Some of the major chemical constituents are represented in Figure 1.5. Several studies report the presence of alkaloids, saponin, tannins trace of citric and tartaric acid (Nipum*, et al.*, 2013). The major chemical constituents found in

the essential oils are eugenol 60-71%, 20% of methyl-eugenol and, 3% of carvacol (Nipum*, et al.*, 2013; Saha*, et al.*, 2013). Extraction of leaves and different parts of the plant yield some compounds as caffeic acid, ursolic acid, apigenin, luteolin, rosmarinic acid cholorgenic acid, cirsilineol, isothymusin, apigenin, and eugenol (Govind & Madhuri, 2010; Nipum*, et al.*, 2013). Fixed oil contain 11-12% of palmitic acid, 52-53% of linoleic acid, 16-7% of linolenic acid, 13-14% of oleic acid and 3-4% of stearic acid (Nipum*, et al.*, 2013).

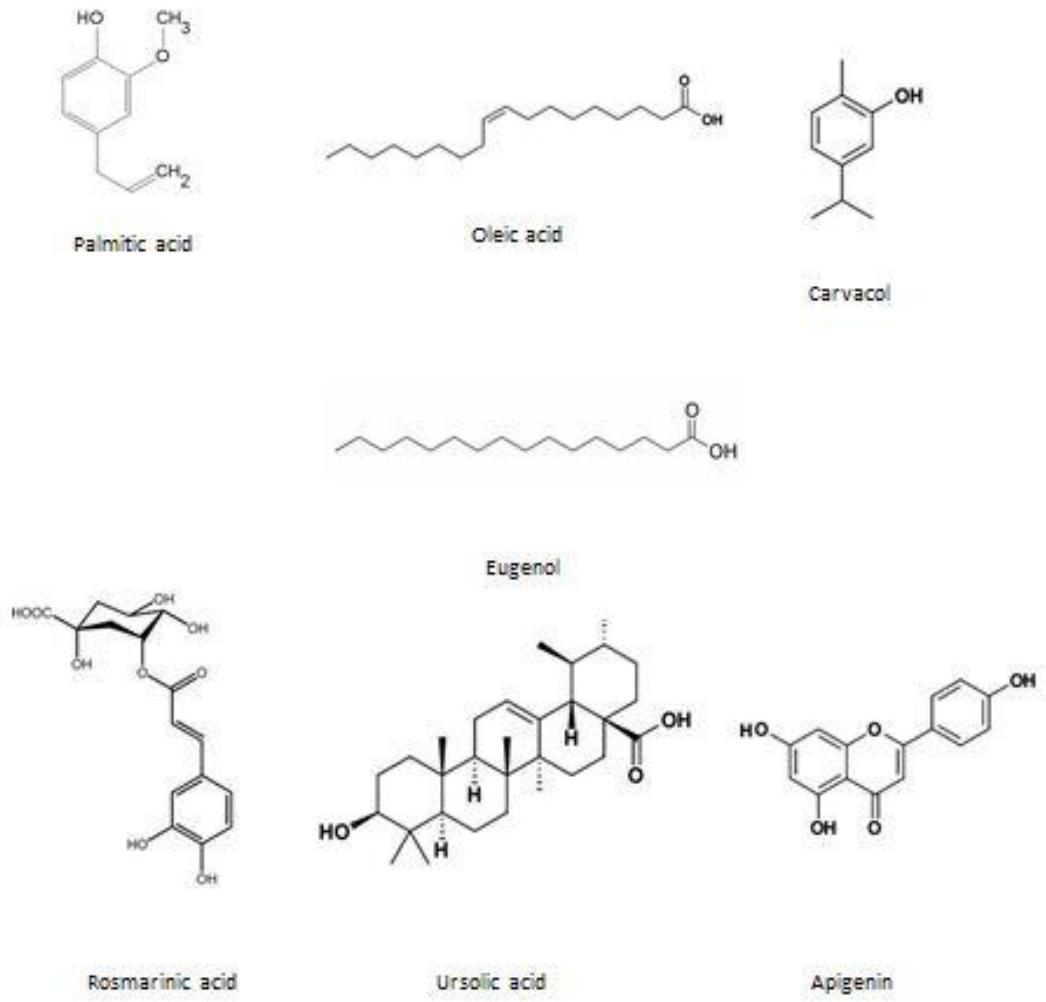

Figure 1. 5 - Chemical structures of active constituents in various *O. sanctum* species.

1.6.1 Biological activities

Due the complex chemical composition of *O. sanctum* species several biological activities mostly related with enzymatic and none-enzymatic mechanisms antioxidant activity and the capability of maintain homeostasis and resistance to different stress factors (Giraldo & Dias, 2014).

1.7 Plant Cell cultures

Plant cells are totipotent, which means that, in principle, every cell contains all genetic information and is able to originate all the tissues to grow a new plant (Mustafa*, et al.*, 2011). Each cell in culture retains complete genetic information and under the right condition is viable to produce the range of chemicals found in the parent plant (S. R. Rao & Ravishankar, 2002). So, plant cell cultures appeared as an alternative source for the production of secondary metabolites. Several advantages are appointed comparing to plant *in vivo* production of secondary metabolites, like the production of novel compounds, free of influence of microorganisms and diseases, independent of seasonal and environmental variations, reduction of the growth period and possibility of using rare or nom crop plants (Kieran*, et al.*, 1997).

Several alternatives have been developed and investigate to increase secondary metabolites production, like plant cells suspensions, organ and tissue cultures combined with media modification, screening of high yield cell lines, elicitation and precursor feeding, immobilization, biotransformation, bioreactor system (Namdeo, 2007; Rao & Ravishankar, 2002; Vasconsuelo & Boland, 2007). Shooty teratomas, hairy roots, cell suspension, and immobilization are other alternatives (Hellwig*, et al.*, 2004). Cell suspensions constitute a good biological material for studying biosynthetic pathways. Indeed, compared to callus cultures they allow the recovery of large quantities of cells from which enzymes can be easily isolated (Dougall, 1981).

However, there are a few successful examples of using plant cells as factories to produce high valuable secondary metabolites (Namdeo, 2007). Some cases can be pointed-out for large scale production of secondary metabolites, namely after the pioneer work with cell suspension cultures of *Morinda citriflora*m where the production of 2.5 g of anthraquinone per liter of medium was achieved (Zenk, 1991). Other examples of industrial production could be indicated, like the rosmarinic acid production of *Coleus blumei* (M. Petersen, Hausler*, et al.*, 1993; Szabo*, et al.*, 1999; Zenk*, et al.*, 1977), and paclitaxel from *Taxus* sp (Dicosmo & Misawa, 1995; Kajani*, et al.*, 2012; Naill*, et al.*, 2012; Namdeo, 2007). Some more examples of species used to produce secondary metabolites are indicated in the table 1.2.

During the early stages of cell suspension cultures carbon allocation is mainly distributed for primary metabolism when growth is very active. So, in many cases secondary metabolites are

produced during the plateau (stationary) growth phase, when growth is stopped and carbon is no longer needed for the primary metabolism and secondary compounds are synthetized (Bourgaud, *et al.*, 2001). These results have conducted researchers to find new strategies to increase successful cases of plant derivate compounds obtained from cell suspension cultures like medium and culture conditions optimization, elicitation, cell immobilization and genetic transformation (Figure 1.6).

Table 1. 2 - Secondary metabolites by cell plant cultures modified from (Karuppusamy, 2009)

Compound	Plant species	Uses
Anthraquinone	*Rubia akane;Rubia tinctorium;Morinda citrifolia*	Dye, paper production, medicine, insecticide
Berberine	*Berberies sp;Coptis japonica*	Anti-microbial, Histological dye, Medicine
Capsaicin	*Capsicum sp*	Anti-fungal, Food flavor, Medicine, Non-lethal forces
Crocetin	*Crocus sativa*	Dye, Saffron color, Food additive
Digoxin	*Digitalis lanata*	Medicine, Pharmaceuticals
Diosgenin	*Dioscorea roseus*	Medicinal, Pharmaceuticals
Gingkolides	*Gingko biloba*	Medicinal, Pharmaceuticals
Ginsenoside	*Panax ginsen*	Medicine, Pharmaceuticals
Imperatorin	*Angelica dahurica*	Medicine, Pharmaceuticals
Morphine & Codeine	*Papaver somniferum*	Analgesic, Medicine, Pharmaceuticals
Rosmarinic acid	*Rosmarinus officinalis; Coleus blumei, Ocimum sp*	Antioxidant, Medicine, Pharmaceuticals
Taxol	*Taxus sp*	Medicine, Pharmaceuticals
Vitriscine	*Catharanthus roseus*	Medicine, Pharmaceuticals

1.7.1 Strain improvement

Selection of a parent plant with high contents of the desired product is the first step for starting cell suspension cultures. Once the *in vitro* culture process has begun, is necessary to make a screening of the clones or seedlings to check their biochemical heterogeneity. After, selection of the best parental tissue, the callus induction is started from several explants, to select and obtain high production cell lines (S. R. Rao & Ravishankar, 2002). For example in case of morphine, vinblastine and vincristine, the production is virtually zero in the cell cultures. Appar-

ently the biosynthetic pathway is not expressed under culture conditions and it is necessary a very selective process to obtain cells suspension with an high content in these secondary metabolites (Verpoorte*, et al.*, 2002).

Growth is considered a strong polygenic character and is necessary to stabilized the cell lines to avoid somaclonal variation (Bouque*, et al.*, 1988), a process that leads reduction in productivity, mutations, and changes in biochemical production due the subculture process (Dornenburg & Knorr, 1995). A cell line could not be considerate as a stable culture until growth parameters are established. A cell line is considered stable when it stops erratic growth parameters or abnormal production of secondary metabolites (Bourgaud*, et al.*, 2001).

Mutation strategies have been also used to improve cell lines (S. R. Rao & Ravishankar, 2002). Using mutants or transgenic plants, after transformation whit *Agrobacterium tumefaciens* or particle bombardment, overexpressing the metabolism of interest of parental tissue, callus induction will provide high production cell lines (Hellwig*, et al.*, 2004).

Some successful cases can be mentioned, like the production of shikonin from *Lithospermum erythrorhizon* (Deno*, et al.*, 1987), ginsenoides from *Panax ginseng* (Kieran*, et al.*, 1997), berberine from *Coptis japonica* (Fujita*, et al.*, 1988), diosgenin from *Dioscorea* (Dicosmo & Misawa, 1995), transformed cell lines of *Nicotiana tabacum* cv BY-2 for the production of the human interferon α2b and human growth hormone (Xu*, et al.*, 2010; Xu*, et al.*, 2007), *Oriza sativa* L. cv. Donjin for human growth hormone (T. G. Kim*, et al.*, 2008), *Glycine max* cv Williams 82 for Hepatitis B antigen (Smith*, et al.*, 2002).

1.7.2 Medium optimization and culture conditions

Several chemical and physical factors can influence secondary metabolites production (Dornenburg & Knorr, 1995). Many of the constituents of plant cell culture media are important determinants of growth and accumulation of secondary metabolites and may affect the expression of the metabolic pathways (S. R. Rao & Ravishankar, 2002).

Changes of nutrients, sugar levels, hormones, pH, temperature, light, precursor feeding may lead to the improvement of the culture production, like the production of shikonin by *L.*

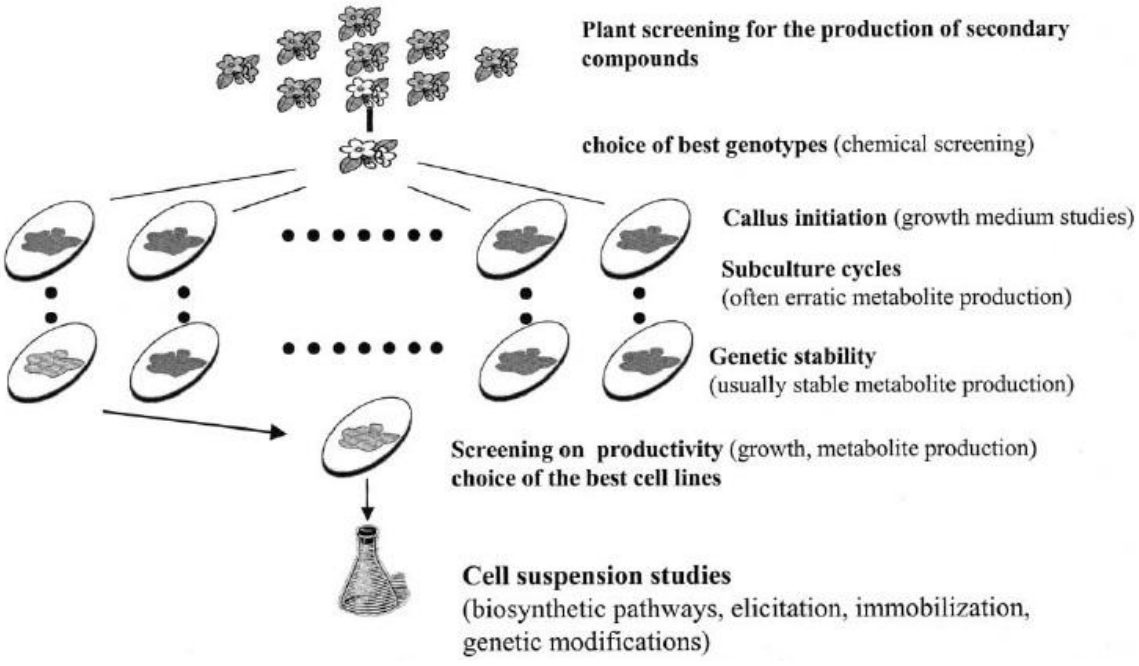

Figure 1. 6- Standard cell suspension cultures procedure (Bourgaud et al. 2001)

erythrorhizon cell cultures induced by avoiding ammonium ions in medium (Dornenburg & Knorr, 1995).

1.7.2.1 Sugar levels

Plant cell cultures usually grown heterotrophically using simple sugars, as a carbon source, and inorganic supply of other nutrients. The level of sucrose has been shown to affect the productivity of secondary metabolites in cultures (S. R. Rao & Ravishankar, 2002). Sugar metabolism is a very dynamic process, and metabolic fluxes and sugar concentrations are altered dramatically during development and in response to environmental signals (Rolland*, et al.*, 2006). When cells are exposed to concentrated aqueous solutions of low molecular compounds (sugar, salts), an osmotic pressure appear changing water fluxes between sink organs, cell, and medium. Under osmotic stress abscisic acid (ABA) is accumulated and consequently prevent cell death. ABA plays important roles under some stress conditions, such as osmotic, cold and drought stresses. It acts as a signal molecule, participating in the regulation of stress responsive genes and also mediating the biosynthesis of certain secondary metabolites in plant cell cultures (Zhao*, et al.*, 2005). The osmotic stress created by sucrose was found to regulate anthocyanin production in *Vitis vinifera* cell suspension cultures (Do and Cormier, 1990), indole alkaloids in *Catharanthus roseus* (Zhao*, et al.*, 2000), taxol in *Taxsus chinensis* (Luo*, et al.*, 2001) and puerarin in *Pueraria tuberoses* (Karwasara & Dixit, 2012).

1.7.2.2 Nitrogen levels

Nitrogen concentration affects the level of proteinaceous or amino acid products in cell suspension cultures, and the ratio of the ammonia, nitrate, nitrogen and overall levels of total nitrogen markedly affect the production of secondary plant products (Nagella & Murthy, 2011).

Nitrogen is incorporated into amino acids and may also serve as a reprogramming signal for the metabolism of nitrogen and carbon, resource allocation, and root development (Wang*, et al.*, 2000). Also regulates the expression of specific proteins through mechanisms affecting transcription and or mRNA stability (Sugiharto & Sugiyama, 1992). Nitrogen sources are important for secondary product synthesis of compounds such as alkaloids, anthocyanins, and shikonin (Zhong, 2001). Interestingly, the NH_4^+ to NO_3^- ratio in the medium affects not only the growth of plant cell cultures but also the production of secondary compounds (Smetanska, 2008). For example, the production of betacyanin in *Phytolacca americana* has been shown to be increased in a high NO_3^-/NH_4^+ ratio medium (Sakuta*, et al.*, 1987).

1.7.2.3 Phosphorus levels

Higher levels of phosphate were found to enhance the cell growth, whereas it had negative influence on secondary product accumulation (S. R. Rao & Ravishankar, 2002). Phosphate levels are very important for cell cultures. It is an essential element in DNA and RNA biosynthesis, connecting individual ribonucleic acids to form macromolecules; it is also involved in the formation of high energy molecules (ATP), very important for anabolism and catabolism. Also, it has influence in many metabolic processes through enzymatic regulation (Duchefa, 1996). Reduced phosphate levels induced the production of ajmalicine and phenolics in *Catharanthus roseus*, caffeoyl and putrescines in *Nicotiana tabacum,* and of harman alkaloids in *Peganum harmala* (S. R. Rao & Ravishankar, 2002).

1.7.3 Growth regulators

Also called hormones, growth regulators are defined as an organic compound synthesized in one part of the plant and transfer to another part; where at low concentration, causes a physiological response (Feitosa de Lacerda*, et al.*, 2007). Auxins, abscisic acid, cytokines, ethylene, and gibberellins are commonly recognized as the five main classes of naturally occurring plant hormones (Gaspar*, et al.*, 1996). Auxins, cytokines, and auxin-cytokine interactions are

usually considered to be the most important for regulating growth and organized development in plant tissue and organ cultures (S. R. Rao & Ravishankar, 2002).

Several experiments have proved that auxins have effects on secondary metabolites production profile; 2,4 dichlorophenoxyacetic acid (2,4-D) strongly inhibits the production of some metabolites on *Nicotiana tabacum* and shikonin derivatives in *L. erythrorhizon* (Nakagawa*, et al.*, 1986). Anthocyanin production has been enhance trough the replacement of 2,4-D by indole acetic acid (IAA) or naphthalene acetic acid (NAA) in cell suspension of *Daucus carota* (S. R. Rao & Ravishankar, 2002). Sometimes the effects of hormones depend of the type of metabolite and the species, for example kinetin stimulates the production of anthocyanin in *Haplipappusgracilus* and inhibit their formation in *Populus* cell cultures (Seitz & Hinderer, 1988). Ethephon has been shown to promote alkaloid production in both *Coffea arabica* and *Thalictrum rugosum* cell suspension cultures (Cho*, et al.*, 1988; Schulthess & Baumann, 1995); ethylene may be useful for stimulating secondary product biosynthesis in other systems (Dong & Zhong, 2002; Songstad*, et al.*, 1989).

1.7.4 Temperature

Stress caused by temperature can affect many physiological, biochemical and molecular processes such as lipid liquefaction and protein denaturation. Many of these changes can induce and enhance secondary metabolism production that maintain the homeostasis inside the cell (Zobayed*, et al.*, 2005). Plants exposed to low temperatures usually experience damage in proteins, nucleic acids, polysaccharides, lipid oxidation and photosynthetic rates due ROS production, mostly generated by mitochondrial electron transport (Nacif de Abreu & Mazzafera, 2005). Detoxification of reactive oxygen species is paramount to the survival of all aerobic life forms. At high temperature dissipative process like photorespiration is increased, reflected in the decline of carbon metabolism, decreasing growth and secondary metabolite anabolism; CO_2 uptake could be also limited due the imbalance of RUBISCO enzyme (Weis & Berry, 1988).

1.7.5 Illumination

Light is an important physical factor, which influences the growth and the formation of primary and secondary metabolites by regulating the CO_2 incorporation (Yu*, et al.*, 2005). In the photosynthetic apparatus, light is absorbed by the antenna pigments, and the excitation energy is

transferred to the reaction centers of the two photosystems. Plant responses to red, blue, and UV radiation, evoking different responses via signal transudation pathways (Kendrick & Kronenberg, 1993), have stimulatory effect on the formation of anthocyanins (Zhong*, et al.*, 1991), betalains (Shin*, et al.*, 2003), and flavonoids (Kendrick & Kronenberg, 1993). It was reported that simultaneous irradiation with blue and UV-B light stimulates the phenylpropanoid pathway (Ebisawa*, et al.*, 2008).

1.7.6 Precursor feeding

Precursor feeding is based in the idea that increasing a compound that acts as intermediate, at the end or beginning of metabolic pathway, that could increase the production yield of determined metabolite. Some cases have been successful, achieving higher yields of metabolite production (S. R. Rao & Ravishankar, 2002). Phenylpropanoid (coniferin) precursors increased 12,8 fold podophyllotoxin in *Podophyllum hexandrum* (van Uden*, et al.*, 1990). Phenylalanine addition has increased rosmarinic acid production in *C. blumei* (Ibrahim, 1987) and *Salvia officinalis* (Ibrahim, 1987). Leucine led to enhancement of volatile monoterpenes and α- and β-pinene in cultures of *Perilla frutescens* (S. R. Rao & Ravishankar, 2002). *Taxus cuspidate* supplemented with phenylalanine increased taxol yields (Fett-Neto*, et al.*, 1992).

1.7.7 Elicitation

Secondary metabolites are produced by plants as defense mechanisms against biotic or abiotic factors. Some compounds called "elicitors" are signals capable of elicit secondary metabolites synthesis by the activation of secondary pathways. Elicitors could be defined as small concentration molecules introduced in a living system, that initiate or improve biosynthesis of specific compounds and can trigger morphological and physiological responses and phytoalexins accumulation (Namdeo, 2007). Microorganism invasion of plants prompt the synthesis of secondary metabolites in the same way as stress factors like UV-irradiation, osmotic imbalance, fatty acids, inorganic salts and heavy metal ions.

Elicitors can be classified by their nature as abiotic or biotic elicitors, or by their origin (Table 1.3). Biotic elicitors are compounds or substances with biological origins, they include glycoproteins, intracellular proteins coupled to cell receptors, and cell wall derived polysaccharides. Abiotic elicitors are non-biological substances, mostly inorganic salts and environmental factors that

could lead to stimulation of Cd^{+2}, Cu^{+2}, Ca^{+2} influx ions and pH (Namdeo, 2007). Substances originated outside the cell such as polyamines, fatty acids and polysaccharides are called exogenous elicitors, on the other hand substances originated inside the cell like galacturonidases or hepta-β-glycosides, methyl jasmonate.

Table 1. 3 - Elicitor's classification by their origin

Endogenous	Exogenous
Via secondary reactions induced by biotic or abiotic signals: Alginate oligomers, galacturonidases, Hepta-β-glycosides, Methyl jasmonate	Originated outside the cell, or endogenous mediators; Polysaccharides, Peptides and Poly-cations, Polyamines, Fatty acids

Biologic system responses and elicitation mechanisms are not very clear yet; the primary step is the recognition of the elicitor and its binding site to a specific receptor, then ATPase plasma membrane is inhibited and the proton electromechanical gradient across the membrane is increased or decreased followed by at least one signal cascade which usually result in the accumulation of secondary compounds (Dornenburg & Knorr, 1995).

Chapter 2

OCIMUM SANCTUM L.: A SYSTEMATIC REVIEW OF ITS PHARMACOLOGICAL ACTIVITIES

2.1Introduction

Explosion in professional healthcare information in the latter half of the 20th century makes primary research evidence a very difficult feat. Systematic reviews became crucial tools for summarizing accurately and reliably information(Liberati*, et al.*, 2009). They facilitate investigators to keep updated; it provide summarized information of previous research; starting point for clinical and health practices, punctual clinical trials and help editors judge the merits of publishing reports of new studies. A systematic review attempts to collate all empirical evidence that fits pre-specified eligibility criteria to answer a specific research question (Liberati*, et al.*, 2009). It use explicit, systematic protocols (Urrutia & Bonfill, 2010) to reduce slant in inclusion criteria, thus providing reliable findings from which conclusions can be drawn.

Ocimum sanctum L.: a systematic review of its pharmacological activities

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Abstract

Ocimum sanctum L. has been known and used in Ayurvedic medicine for centuries. It presents wide spectrum of biological activities and is considered a versatile medicinal plant. The main goal of this study was to systematically review the scientific literature on the biological activities and chemical composition of plant extracts, providing a summary of the potential benefits of use of this species. A systematic review analysis was performed using several databases: ScienceDirect, PubMed, Mednar, and Scielo. The words (*O. sanctum*) and (extract) were used as relevant terms for the incoming data. Essential oils and biological activities of pure compounds present in in the species were excluded of the incoming data. 115 articles were included in this review: 91% specifying extracts, 4% fixed oils, 3% seed oil and 2% dermal applications. Among the several bioactivities found most papers dealt with both non-enzymatic and enzymatic antioxidant mechanisms. Several studies concern the potential effect of *O. sanctum* on human health and relate it with the capability of maintaining ROS homeostasis and resistance to different stress factors. Other relevant studies include anticancer, radio-protector and inmonumodulator, neuroprotection and protection against cardio diseases.

Introduction

The genus *Ocimum* belongs to the Labiatae family. It is known for their medicinal and therapeutic potential (Jamal, 2011), traditionally used to treat rheumatism, paralysis, epilepsy, colds, flu, high fever, influenza, abdominal pains, coughs and others symptomologies (Raimo H., 2005; Saha*, et al.*, 2013; Vani*, et al.*, 2009). Commonly known as basil, plants of this genus are

strongly aromatic due to the presence of essential oils, mainly monoterpenes, sesquiterpenes and phenylpropanoids (Raimo & Yvonne, 2005). *Ocimum* plants are native of warm temperate and tropical regions of Europe, Africa, America and Asia (Raimo & Yvonne, 2005). *The Plant List* (Version 1.1) reports 327 species for the genus *Ocimum* recognizing 66 names as accepted and 243 as synonyms (*List*, 2013). Still the exact number of species is not yet clear due to regular occurrence of interspecific hybridization within genus (Raseetha*, et al.*, 2009).

The genus *Ocimum* involves important economically medicinal and aromatic herbs, undershrub's or shrubs in the world; being rich in biological activities due their variable chemical composition (Saha*, et al.*, 2013). In Table 1 is summarized some relevant *Ocimum* species and their activities.

O. sanctum is regarded in Ayurveda as a "elixir of life" and believed to promote longevity (Priyabrata*, et al.*, 2010). It is often described as a "Rasayana" (plant with adaptogenic properties) (S. U. Yanpallewar*, et al.*, 2004). This plant, popularly known as "Holy basil" or "*Tulsi"*, is an important symbol of the Hindu religion and tradition (Govind & Madhuri, 2010). Marked by its strong aroma and astringent taste it has been used as flavoring in Asian cuisine, and many Hindus ingest small quantities of the leaves as food additive or after divine worship in temples (F. L. Hakkim*, et al.*, 2007). Antique poems describe the plant as highly sacred that protect from adversities and misfortunes. Also, who carefully takes care of it will be allow to go to heaven (Khosla, 1995). There are two types of *"tulsi"* venerated in Hinduism: *"Rama Tulsi"* with light green leaves and larger in size, and *"Shyama Tulsi"* a variety with dark green leaves, important for Hanuman's worship (G. Chatterjee*, et al.*, 2001). Traditionally, tulsi is planted in the center of the central courtyard of Hindu houses. It is also frequently grown next to Hanuman temples, especially in Varanasi (de Sousa*, et al.*, 2005). The plant is considered so sacred that during solar or lunar eclipses their leaves are stored to protect them from bad (Khosla, 1995).

Several ayurveda websites have information about health benefits and home remedies of this plant. *O. sanctum* contains several chemicals that synergisticaly interact to elicit their pharmacodynamics (S. K. Gupta*, et al.*, 2002). In the last 15 years several biological activities such as anti-inflammatory (Kalabharathi*, et al.*, 2011), antifungal (Balakumar*, et al.*, 2011), antioxidant (Govind & Madhuri, 2010; Kumar Mundey*, et al.*, 2013; Samson*, et al.*, 2007; Subramanian*, et al.*, 2005), inmonumodulator (Jadhav*, et al.*, 2005; R. Mukherjee*, et al.*, 2005) radioprotector (Arora*, et al.*, 2005; Kumarchandra*, et al.*, 2010; S. Rao*, et al.*, 2014; Reshma*, et al.*, 2008), among others, have been confirmed by investigators. Also, it has been employed as repellent of insects and several extracts have been used against numerous diseases vectors like *Aedes aegypti, Anopheles subpictus, Aphis gossypii, Culex quinquefasciatus, Culex tritaeniorhynchus* (A. M. Anees, 2008; A. Bagavan*, et al.*, 2009; Bagavan*, et al.*, 2008; Das & Chandra, 2012; Jain & Suneetha, 2013; C. Kamaraj*, et al.*, 2008).

Geographical location and several biotic and abiotic factors have strong impacts in the chemical composition of the species; even plants in the same space could present major differences among them (Pattanayak*, et al.*, 2010). Some studies reported the presence of alkaloids,

saponin, tannins, traces of citric and tartaric acid (Nipum*, et al.*, 2013). The major chemical constituents found in the essential oils are eugenol (60-71%), and methyl-eugenol (20%) (Nipum*, et al.*, 2013; Saha*, et al.*, 2013). Several phenolic compounds were isolated from leaves (E. Singh*, et al.*, 2012). Leaf extracts yield compounds like caffeic acid, luteolin, apigenin, ursolic acid, rosmarinic acid, cirsilineol, circimaritin, isothymusin and small quantities of eugenol (Awasthi & Dixit, 2007; Govind & Madhuri, 2010; Nipum*, et al.*, 2013; E. Singh*, et al.*, 2012; S. U. Yanpallewar*, et al.*, 2004). Fixed oil contains palmitic acid (15-12%), linoleic acid (52-53%), linolenic acid (16-17%), oleic acid (13-14%), and of stearic acid (3-4%) (Nipum*, et al.*, 2013).

Table 2.1 - Biological activities of some species of the *Ocimum* genus

Species	Common name	Pharmacological activity	Reference
O. americanun	American basil	Anti-microbial	[33, 34]
O. basilicum	Sweet basil	Antimicrobial, antinociceptive, antiviral, larvicidal, antioxidant	[33, 35-38]
O. campechianum	Least basil	Anti-diabetic, anti-bacterial	[39, 40]
O. canum	Kali tulasi	Insecticidal, anti-diabetic	[41-43]
O. citriodorum	Lemon basil	Anti-bacterial, antioxidant	[40, 44]
O. forskolei	Basilikum	Antimosquito	
O. gratissimum	African basil	Anti-cancer, antimicrobial, antinociceptive, antiviral, antitrypanosoma.	[43, 45-51]
O. kenyense	Kenya basil	Insecticidal	[52-54]
O. kilimandscharicum	Camphor basil	Insecticidal, wound healing	[55, 56]
O. lamiifolium	Basilikum	Anti-inflammatory, hematological	[57, 58]
O. micranthum	Peru basil	Antinociceptive, antiradical activity	[59, 60]
O.minimum	Bush basil	Phytoremediation	[61-63]
O. selloi	Pepper basil	Anti-diarrheic, anti-inflammatory	[64-66]
O. suave	Scented basil	Anti-pyretic, repellent, wound healing	[67-70]

The main goal of this study was to systematically review the scientific literature concerning the biological activities of *O. sanctum* extracts, seed oil, providing a summary of the potential benefits of use of this species.

Methodology

A systematic review analysis of published studies reporting biological activities of *O. sanctum* was performed using PRISMA statement guidelines (Preferred Reporting Items for Systematic reviews and Meta-Analyses) (Urrutia & Bonfill, 2010). A wide-ranging search of the literature was conducted using the following databases: ScienceDirect® (Elsevier B.V, USA), PubMed® (National Center for Biotechnology Information, National Library of Medicine, USA), Mednar® (Deep Web Technologies, USA), and Scielo® (Scientific Electronic Library Online, BRA), for studies published before October 2014. The words "*O. sanctum*" and "extract" were used as relevant terms for the incoming data (inclusion criteria). Essential oils and biological activities of

pure compounds present in *O. sanctum*, as well as in other plants, were excluded from the review (excluding criteria).

In a second stage, the total hits obtained from searching the databases using the above search criteria were pooled together and duplicate articles were removed. The remaining articles were initially screened by reading the 'title' and thereafter the 'abstracts'. Papers not satisfying the inclusion criteria were excluded at these stages. The remaining articles were screened in the final stage by reading the full-text and those not meeting inclusion criteria were excluded. This screening process was conducted independently by two reviewers (LG and AD) and the final group of articles to be included in the review was determined after an iterative consensus process.

Results and Discussion

Literature search

In this review it was excluded the literature that reported about *O. sanctum* essential oils and their pharmacological effects, since this topic was already focus of several reviews (Devi, 2001; Ashok Kumar*, et al.*, 2010; Miguel, 2010; Thamolwan Suanarunsawat*, et al.*, 2010; Zheljazkov*, et al.*, 2007). The literature search using the above search criteria identified the following number of articles in the respective databases: ScienceDirect® (n= 187), PubMed® (n= 433), Mednar® (n= 530) and Scielo® (n= 11). After removing duplicates and exclude papers accordingly the methodology used, a total of 115 articles were included in this review. The search strategy is summarized in Figure 1.

Of the 115 articles, 105 specifying extracts, 5 fixed oils, 4 seed oil and 1 dermal applications; 6% of the total information correspond to extraction and chemical characterization of *O. sanctum* compounds. Of the incoming data of all extracts, the majority correspond to hydroalcoholic extracts (47%) and aqueous preparations (46%)

Antinociceptive activity

O. sanctum alcoholic extracts have shown positive effects against pain. The administration of 50 to 200 mg/kg body weight (orally), and 50, 100 mg/kg (intraperitoneally) of *O. sanctum* extract showed an analgesic effect in albino mice models [42]. This action is exerted both centrally as well as peripherally involving an interplay between several neurotransmitter systems (Khanna & Bhatia, 2003). Additionally, root methanolic extract showed influence in general CNS behavior profiles of Wistar rats and mice, including analgesic effects (J. Mukherjee*, et al.*, 2009).

Anti-microbial activity

Several *O. sanctum* extracts of leaves showed antibacterial activity against: *Streptococcus mutans* (Nipum*, et al.*, 2013), *Aeromona hydrophila* (Balasundaram*, et al.*, 2013), *Salmonella typhi* and *Salmonella typhimurium* (Rani & Khullar, 2004) *Klebsiella sp., E. coli,* and *Staphylococcus aureus* (Govind & Madhuri, 2010)*, Neisseria gonorrhea* (Shokeen*, et al.*, 2005), *Myco-*

bacterium tuberculosis (Farivar, *et al.*, 2006). It also showed strong antifungal activity against some *Aspergillus* species (Geeta, *et al.*, 2001), *Colletotrichum lindemuthianum* (Amadioha, 2003), and dermatophytic fungi as *Trichophyton mentagrophytes, Trichophyton rubrum, Microsporum canis, Microsporum gypseum* and *Epidermophytom floccosum* (Balakumar, *et al.*, 2011).

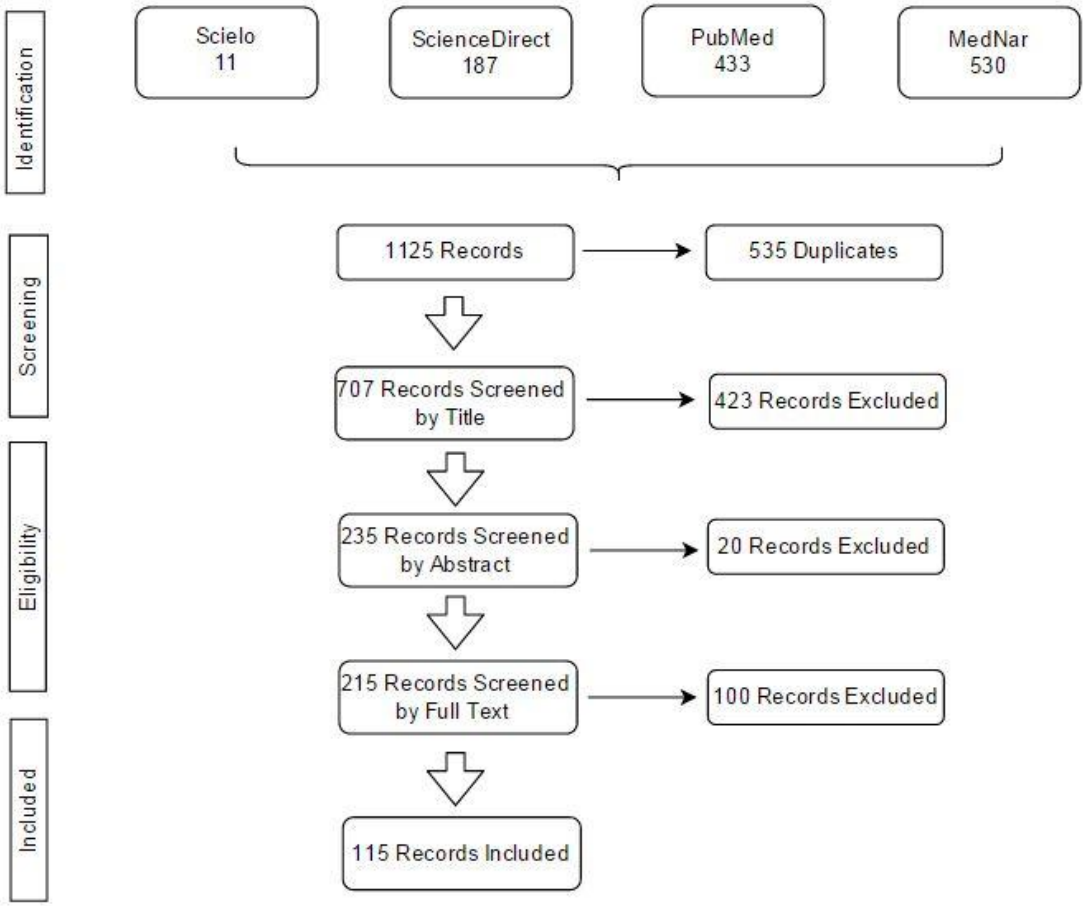

Figure 2.1 - Flow diagram: Search strategy and analyzed data

Anticancer activity

Oral and topically administration of ethanolic and aqueous extracts of *O. sanctum* delayed the incidence of papilloma and increased the survival rate of animals (hamster) induced with DMBA for buccal pouch carcinogenesis (K. Karthikeyan, *et al.*, 1999). Ethanolic leafs extracts and essential oils significantly inhibit the aggressiveness, motility, invasion and inhibit the growth of pancreatic cancer (PC) cells as well as orthotopically implanted PC cells (Shimizu, *et al.*, 2013). Ethanolic extracts also inhibited the invasion of murine Lewis lung cancer (LLC) changing caspase-3 levels, a key executioner of apoptosis (Magesh, *et al.*, 2009). Aqueous extract possess significant free radical scavenging and combined with WR-2721 (an effective radioprotector) results in higher bone marrow cell protection, shielding DNA, and decreasing toxicity of

high doses of radiation or chemoprotective treatments (U. S. Bhartiya*, et al.*, 2010; A. Ganasoundari*, et al.*, 1998; Niture*, et al.*, 2006).

Antidiabetic

Several reports appointed positive effects of *O. sanctum* in diabetic pathology. Its aqueous extract increased the activity of antioxidant enzymes, like superoxide dismutase (SOD) and catalase (CAT), and acted against free radical damage induced by the diabetic condition (Chandra*, et al.*, 2007; G. Muralikrishnan*, et al.*, 2012). Combination of *O. sanctum* and Vitamin E (α-tocopherol) reverse the changes of diabetic retinopathy (Eshrat M & Mukhopadhyay, 2006). *O. sanctum* produces a marked decrease in blood glucose levels in normal as well as streptozotocin induced diabetic rats (Grover*, et al.*, 2002; Mahdi*, et al.*, 2003). *O. sanctum* acts as a natural drug that regress and mitigate dyslipidemia and oxidative stress in diabetes (Husain*, et al.*, 2014). Dietary intake of *O. sanctum* either before or after induction of diabetes in Wistar rats, significantly reduced the elevated serum lipid profile, serum enzyme serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and creatinine (T. Suanarunsawat & Songsak, 2005).

Anticonvulsant

Chloroform-ethanol extracts (95%) of *O. sanctum* stem *calli*, stem and leaf were tested in albino mice to evaluate its anticonvulsant activity. All extracts exhibited significant decrease in the time spent in the extensor phase of protective effect against MES-induced convulsion (Jaggi*, et al.*, 2003). Stem calli at 400 and 800 mg/kg body weight exhibited a decrease in mortality of albino mice. Leaf extract was as potent as phenytoin at 400 and 800 mg/kg body weight doses. The anticonvulsant activity of the extracts may be related with the presence of saponins, tannins and proteins (Anca*, et al.*, 1990; De Lucia*, et al.*, 1997; Nguyen*, et al.*, 1993).

Antifertility

Benzene leaf extracts of *O. sanctum* (250 mg/kg body weight) induced a decrease of fertile parameters (sperm count, sperm motility and forward velocity) in albino rats (Ahmed*, et al.*, 2002). This effect seems related with disturbance in plasma membrane as well as acrosomal membrane of spermatozoa (Ahmed & Nazeer Ahamed, 2009).

Anti-inflammatory activity

Aqueous and methanolic extracts of *O. sanctum* have shown to inhibit acute and chronic inflammation induced by carrageenan pedal edema, croton oil granuloma and exudate in rat models (Godhwani*, et al.*, 1987). Fresh leaves 500 mg/kg body weight significantly reduce inflammation induced by carrageenan (Kalabharathi*, et al.*, 2011). *O. sanctum* fixed oil inhibited cyclooxygenase and lypoxygenase pathways of arachidonic acid metabolism, as well as inhibited leukotriene-induced paw edema (S. Singh*, et al.*, 1996). *O. sanctum* fixed oil also showed an inhibitory effect in 5-lipoxygenase and leukotriene pathways, in rats submitted to stress induced ulceration (S. Singh & Majumdar, 1999).

Antioxidant activity

Intensive stress could result in an increase of reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and superoxide anion radical (O_2^-) that cause lipid peroxidation, especially in membranes, and can play an important role in tissue injury (Samson*, et al.*, 2007). Lipid peroxidation and oxidative stress play critical role in several physiologic responses such as inflammation, cancer and cardiac diseases, gastric ulceration, and diabetes. Antioxidant supplementation constitutes important defense against various oxidative stress-related diseases (Nipum*, et al.*, 2013).

Due to an high polyphenolic content, *O. sanctum* showed effective activity preventing lipid peroxidation and imbalance of ROS, modifying SOD, CAT and glutathione (GSH) levels or non-enzymatic activities, like free radical scavenging or iron chelation (K. Reshma*, et al.*, 2005).

Oral administration of aqueous extracts of *O. sanctum* (200 mg/kg body weight) attenuated cadmium-induced oxidative stress in rats, mitigating the elevation of serum enzymes; it also conferred protection against tissue damage and protects the glutathione complex of misbalances (E. Mitra*, et al.*, 2014). Treatments with hydro-alcoholic extracts (100 mg/kg body weight) showed a significantly reduction in lipid peroxidation and increase levels of GSH, CAT and SOD in blood and brain tissue caused by oxidative stress induced by arsenic (Kumar Mundey*, et al.*, 2013).

Administration of *O. sanctum* aqueous extracts significantly decrease the levels of TBARS and antioxidant enzymes (CAT, SOD, GPx) in both plasma as well as in in vital organs such as the liver and kidney of streptozocin-induced diabetic rats (G Muralikrishnan*, et al.*, 2012). Aqueous extract (50 mg/kg body weight) also protected mice against radiation induced lipid peroxidation (Uma Devi*, et al.*, 1999). It also increased erythrocyte number, SOD, and CAT activities in albino rats (Naresh Khanna*, et al.*, 2010). *O. sanctum* aqueous extracts also suppressed the high level of lipid peroxidation in the serum, liver, and cardiac tissues in rats feed with an high cholesterol diet for seven weeks (Thamolwan Suanarunsawat*, et al.*, 2011). Additionally, aqueous leaves extracts (100 mg/kg body weight) promote reduction of blood rat lipid levels, decrease low density lipoproteins (LDL), cholesterol, triglycerides and increase high density lipoproteins (HDL), and enzymatic and non-enzymatic antioxidant defenses (N. Khanna*, et al.*, 2010).

Ethanolic extracts exhibited a protective effect against lipid oxidation in cooked ground pork during storage at 5 °C for 14 days in a dose-dependent manner (Juntachote*, et al.*, 2007).

Adaptogenic Activity

O. sanctum is considered as an adaptogenic plant, increasing the body capability to deal with challenging environments and maintain the homeostasis (Mohan*, et al.*, 2011). Preliminary studies have revealed that methanolic extracts of leaves have anti-stress activity in acute stress and chronic unpredictable stress models (P. Gupta*, et al.*, 2007). Doses of 50-100 mg/kg body weight decrease the transfer latency in young and aged mice, dosages of 200 mg/kg enhance the learning and memory of aged mice as reflect of marked decreasing in transfer latency (Joshi

& Parle, 2006; S. R. Kumar*, et al.*, 2007). Administration of *O. sanctum* extracts to rats reduces the corticosterone levels caused by chronic exposure to noise stress (P. Gupta*, et al.*, 2007; Sembulingam*, et al.*, 1997, 2005), and showed a significant enhancement in the neutrophil functions and plasma corticosterone (R. Archana & A. Namasivayam, 2000).

Antiulcer activity

Aqueous extract of *O. sanctum* (100 mg/kg body weight) showed protection against piroxicam-induced gastric ulceration in rats (Basu*, et al.*, 2013). Namely, lipid peroxidation levels were reduced and gastric GSH levels increased. Additionally, it also improved the levels of gastric peroxidase, Cu-Zn SOD, CAT, xanthone oxidase and xanthine dehydrogenase. Ethanolic extracts (50-100 mg/kg body weight) also prevented ulcerogenic effects in rats submitted to several ulcer gastric models and improved healing process due to a cytoprotective effect (Dharmani*, et al.*, 2004). Standardized methanolic extracts given orally, twice daily at 50-200 mg/kg body weight, showed dose-dependent ulcer protective effect and significantly inhibited offensive acid-pepsin secretion, lipid peroxidation levels, and increase gastric defensive factors like mucin secretion, cellular mucus and life span of mucosal cells (R. Goel*, et al.*, 2005).

Anxiolytic activity

An *O. sanctum* hydro alcoholic extract proved to attenuate anxiety disorders in rat model, being its efficacy similar to diazepam (R. R. Chattopadhyay, 1994). Ethanolic leaf extract intake at dose of 50 mg/kg body weight protected stressed-mice against anxiety and depression effects and also improved motor coordination (M. Chatterjee*, et al.*, 2011). Similar effects have been observed also in humans. In a clinical study, patients suffering of anxiety and depression problems significantly attenuated these neurological disorders after the ingestion of 500 mg of leaves, twice a daily, for a 60 days period (Bhattacharyya*, et al.*, 2008).

Antitussive activity

O. sanctum has been used traditionally as an expectorant; crude form is used in combination with other herbs or alone as a remedy. Aqueous extracts show 73 % of cough inhibition, whereas methanolic extracts displays less activity (only 35% of cough inhibition) (Nadig & Laxmi, 2005). It was suggested that the antitussive effect is due to a central action probably mediated by both opioid and GABAergic systems (Nipum*, et al.*, 2013).

Cardioprotective activity

O. sanctum hydro-alcoholic extracts protected rat heart from chronic restraint stress-induced changes, decreasing myocardial glutathione levels and rising myocardial SOD and CAT activities (Sood*, et al.*, 2006). A combined formulation containing *Ginkgo biloba* and *O. sanctum* administrated orally to rats submitted to isoproterenol (ISO)-induced myocardial necrosis resulted in significant cardiac protection, decreased lipid peroxidation, and restored antioxidant activities (Panda & Naik, 2009). Pre and co-treatments of animals with ethanolic extracts (25-100 mg/kg body weight) offered a significant protection against ISO induced myocardial infarction

(MI) (M. Sharma*, et al.*, 2001). Aqueous dosage of 50 mg/kg resulted in an increasing production of endogenous myocardial antioxidants like SOD and CAT and reducing of loss of muscle fiber and myocardial necrosis (Sood*, et al.*, 2005).

Chemoprotector

Ethanolic and aqueous extracts of *O. sanctum* induced significant and reproducible increases in O^6-methylguanine- DNA methyltransferase (MGMT) activity in human lymphocytes and human tumor cells (Niture*, et al.*, 2006). MGMT is the first-line of defense against alkylation DNA damage being highly relevant for avoiding carcinogenesis. Seed oil ingestion (100 microl/kg body weight) also displayed a significant delay and reduction in 20-methylcholanthrene (MCA) induced-fibrosarcoma tumors in mice (Prakash & Gupta, 2000). Several liver enzymes (SOD, CAT, glutathione-S-transferase), non-enzymatic antioxidants (GSH) and lipid peroxidation levels were significantly modulated, resulting in a protective effect against MCA. Fresh leaf paste, aqueous and ethanolic extracts were topically applied and orally administered to buccal pouch mucosa of animals exposed to 0,5% of 7,12-dimethylbenz anthracene (DMBA). Incidence of papillomas and squamous cell carcinomas were significantly reduced, and increased the survival rate of hamsters (K Karthikeyan*, et al.*, 1999).

Hepatic-protector

O. sanctum leaves have displayed hepatic-protective effect in several models of hepatotoxicity like paracetamol and carbon tetrachloride induced liver damage in rats (Bhargava & Singh, 1981; R. Chattopadhyay*, et al.*, 1992). Ethanolic leaf extract also decreased induced hepatotoxicity of anti-tuberculosis treatments modulating enzymatic and non-enzymatic mechanisms in rats liver (Ocimum, 2003).

Immunomodulatory activity

O. sanctum extracts seems to modulate both humoral and cellular responses that may be mediated by GABAergic pathways (Lee*, et al.*, 1985). Alcoholic and aqueous extracts improved immunity of rats submitted to immunotoxicity with endosulfan (Bharath*, et al.*, 2011). Extracts also show biphasic responses in a dose dependent manner. At lower concentration (5-20 μg/ml), it stimulated nitric oxide production by monocytes and macrophages, helpful in case of pathogen attack. However, at higher doses (25-250 μg/ml) it inhibits the nitric oxide production, which is usually required for inflammatory processes (Jadhav*, et al.*, 2005). Intra-mammary infusion of leaf extracts showed antimicrobial and immunomodulatory activities. The effect appears to be related with the enhancement of the activity of the polymorphonuclear cells in the bovine mammary gland (R. Mukherjee*, et al.*, 2005). Aqueous leaf extract administered intraperitoneally to Mozambique tilapia stimulated antibody responses and neutrophil activity (Logambal*, et al.*, 2000).

Larvicidal Effect

Several reports indicate that *O. sanctum* is used as a larvicidal. Crude extracts of this plant displayed repellent activity against *Helicoverpa armigera* and *Sylepta derogata* larvae, with 100% of mortality observed for acetone extract at 1,000 ppm (C Kamaraj*, et al.*, 2008). Similar effects have been shown for *Spodoptera litura* and *Culex quinquefasciatus* (C. Kamaraj*, et al.*, 2008), *Anopheles subpictus*, *Culex tritaeniorchyncus* and *Aedes aegypti* (A Mohamed Anees, 2008; A Bagavan*, et al.*, 2009; Bagavan*, et al.*, 2008). Nematicidal effect was also observed against *Meloifogyne incognita* (A. Chatterjee*, et al.*, 1982).

Metal Toxicity

O. sanctum aqueous extracts reduce mercury toxicity in mice via increasing GSH content and decrease lipid peroxidation (Mukesh Kumar Sharma*, et al.*, 2005), and lowering the levels of serum glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SPT), and an increase in alkaline phosphatase activity (M Kumar Sharma*, et al.*, 2002). It has also shown protective effect against cadmium induced hepatic(MITRA*, et al.*, 2013), and heart (ELINA MITRA*, et al.*, 2014) tissue damage in rats.

Neuroprotection

Pre-treatments with hydro-alcoholic extracts of *O. sanctum* significantly attenuate vincristine-induced alterations in neuropathic pain perception in response to noxious and non-noxious stimuli in rats (Kaur*, et al.*, 2010). It also inhibits H_2O_2 induced neuronal death, ROS generation, lipid peroxidation and DNA damage in neuronal cells (Venuprasad*, et al.*, 2013). *O. sanctum* pretreatment (200 mg/kg body weight/day for 7 days) significantly prevented the rise in malondialdehyde (MDA) levels and up-regulation of SOD activity, suggesting that it attenuates the excessive formation of ROS during transient cerebral ischemia and long-term cerebral hypoperfusion in Charles-Foster rats (S. Yanpallewar*, et al.*, 2004). Hydro-alcoholic extracts showed to reduce serotonin levels and reverse changes in central cholinergic parameters in noise stress induced Wistar strain albino rats (R Archana & A Namasivayam, 2000; Ravindran*, et al.*, 2005). Other studies shows that *O. sanctum* extratcs attenuates oxidative damage and neurological deficits following focal cerebral ischemia/reperfusion injury in rats (Ahmad*, et al.*, 2012).

Radioprotector

Aqueous leaf extracts proved to reduce the gamma-irradiation toxicity and increased the activity of the well-known radioprotector WR-2721 in rats (A Ganasoundari*, et al.*, 1998). Extracts reduce salivary gland weight and maintain micro-nucleic index in the bone marrow polychromatic (PECs) and normochromatic erythrocytes (NCEs) in exposed radioiodine exposed-mice (Uma S Bhartiya*, et al.*, 2010). It also prevented γ-radiation-mediated cell death in mouse splenocytes (Subramanian*, et al.*, 2005). A clinical study has been reported that *O. sanctum* flavonoids extract act as radioprotector on the erythrocyte antioxidants in oral cancer patients (K Reshma*, et al.*, 2005).

Wound healing

O. sanctum is widely used in Ayurveda massage and in topical applications. Aqueous extract of *O. sanctum* increased collagen synthesis and cellular proliferation at the wound site in rats (Shetty, *et al.*, 2008). Ethanolic extracts have been shown to promote epithelialization of wounds and to counteract the healing suppressant effect of dexamethasone in albino rats topical applications (A. Goel, *et al.*, 2010). *O. sanctum* extract in petroleum jelly accelerate the healing process in rats *via* TNF-α modulation (A. Goel, *et al.*, 2010).

Conclusion

The use of medicinal plants for the treatment of various ailments has being reported since ancient times in several cultures around the world. *Ocimum* genus includes important economically medicinal and aromatic plants. *O. sanctum* is well known in Hindu religion, rituals and ayurvedic medicine, being considered a sacred plant. This plant has been used in several Rasayanas (formulas) for the treatment of several illnesses or just as a flavoring agent in culinary. Several websites offers information about traditional uses, homemade treatments, and are commercialized in several presentations. However, such claims need to be scientifically proved and reliable information is required. In the present review 115 scientific documents were selected trough a systematic selection of biological activities of the plant extracts. The occurrence (in percentage) of bioactivities found for *O. sanctum* in the literature selected in this review is presented in Figure 2. Most of the information, 21% of the incoming data, was directly related with important antioxidant activities of this plant *via* enzymatic and non-enzymatic mechanisms. We can conclude that *O. sanctum* presents important anti-oxidant properties reflected in a wide spectrum of the biological activities with potential use for health care. We also disclosed that *O. sanctum* is involved in possible treatments against related oxidative stress diseases. Other relevant activities were recorded, including antinociceptive activity (10% of the information), followed by radioprotection and antimicrobial activities (9 and 8% respectively), anticancer (7%), and anti-diabetic (5%).

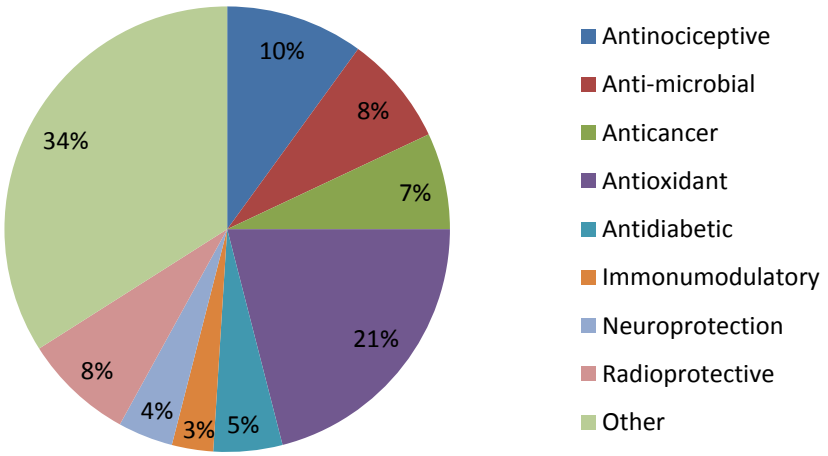

Figure 2.2 - Percentage of categorized information found in literature data of bioactivities of *Ocimum sanctum*

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Chapter 3

PRODUCTION OF PHENOLICS BY CELL SUSPENSION CULTURES OF *O. SANCTUM*

3.1 Introduction

Rosmarinic acid (RA) is an ester of caffeic acid (3,4-dihydroxyxinnamic acid) and 3,4-dihydroxyphenyllacic acid (DHPL), isolated for first time from rosemary (*Rosmarinus officinalis*) (Scarpati & Oriente, 1958). The structure was clarified in 1970 when *Mentha arvense* and *Mentha piperita* were fed with C^{14} labeled aminoacids, showing that the caffeic acid part incorporated phenylalanine and the DHPL part incorporated tyrosine (Ellis & Towers, 1970). This shows that RA is not a derivative of two caffeic acid units as often falsely described in the literature (Maike Petersen, 2013). It mostly occurs throughout Boraginaceae and Lamiaceae families, restricted to Neotoideae subfamily (Maike Petersen, 2013; M. Petersen & M. S. Simmonds, 2003). However it also occurs in archaic groups and other families as is showed in table 3.1.

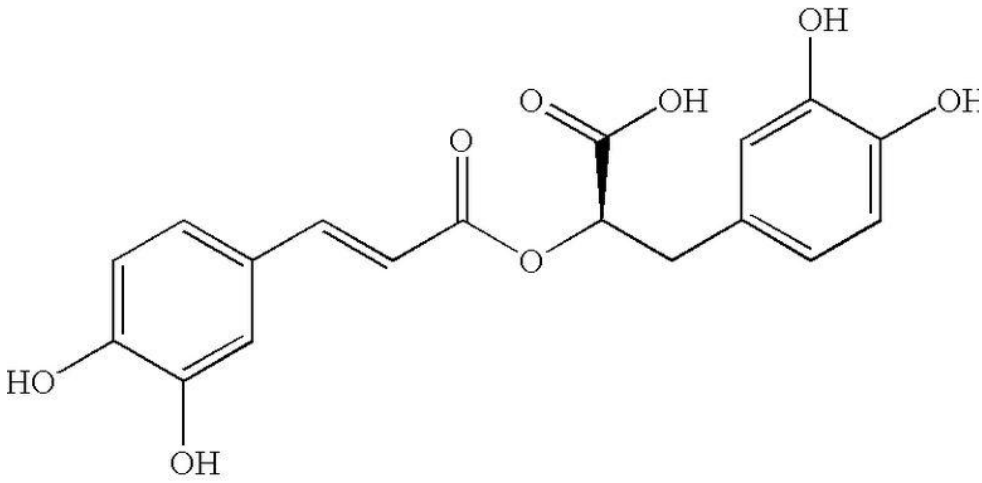

Figure 3. 1 - Rosmarinic acid structure

RA has received attention because it displays some interesting biological activities such as anti-inflammatory (Osakabe, Yasuda*, et al.*, 2004), anti-allergic (Osakabe, Takano*, et al.*, 2004), anti-oxidant (Chen & Ho, 1997; Frankel*, et al.*, 1996). A number of related derivatives can be isolated. Isorinic acid (caffeoyl-4'-hydroxyphenyllactate), 4-O-glucosylated, isomers of RA, lithospermic acid, salvionic acid, melitric acid, sagerinic acid are compounds often described as caffeic acid dimmers, trimmers or tetramers (Maike Petersen, 2013).

The aromatic amino acids L-phenylalanine and L-tyrosine are the precursors for RA synthesis. Phenylalanine is only incorporated into the caffeic acid part and tyrosine only into the DHPL moiety (Maike Petersen, 2013).

Table 3. 1 - Rosmarinic acid and its occurrence in plant kingdom

Dicotyledon		
Apiaceae	Araliaceae	Boraginaceae
Cucurbitaceae	Hydrophyllaceae	Lamiaceae
Plantaginaceae	Rubiaceae	
Sterculiaceae	Tiliaceae	
Monocotyledon		
Cannaceae	Potamogetonaceae	Zosteraceae
Pteridophyta		
Blechnaceae		

Phenylalanine is transformed to *t*-cinnamic acid by phenylalanine ammonia-lyase (PAL). The resulting molecule is hydrolyzed to 4-coumaric acid by cinnamic acid 4-hydroxylase (C4H), and finally 4-coumaric acid CoA-ligase (4CL) produce 4-coumaroyl-CoA. In the other pathway, tyrosine is transformed into 4-hydroxyphenylpyruvic acid by the pyridoxalphosphate dependent tyrosine aminotransferase (TAT). The hydroxyphenylpyruvate dioxygenase transform the product into 4-hydroxyphenylacetic acid (DHPL). Once 4-coumaryl-CoA and DHPL are formed the specific hydroxycinnamoyltransferase (rosmarinic acid synthase, RAS) transfer the 4-coumaryl moiety to the aliphatic OH group of DHPL releasing the CoA. Final biosynthetic stages correspond to the introduction of hydroxyl (OH) groups in position 3 and 3' of the aromatic rings mediated by the cytochrome p450 (M. Petersen, Häusler*, et al.*, 1993). The biosynthesis of rosmarinic acid is described in Figure 3.2.

The research on the biosynthesis of RA has shown that *in vitro* cultures of some plant species usually synthesize and accumulate the compound in vacuoles (Maike Petersen, 2013). In some cases, *in vitro* production of RA is higher than that observed in the original plant (M. Petersen & M. S. Simmonds, 2003). The first report of plant cell cultures that accumulate RA was *C. blumei* (8-11% of dry weigh) (Razzaque & Ellis, 1977) and 13-15% of dry weigh (Zenk*, et al.*, 1977). Suspension cultures from these species were the first ones used for biotechnological production of RA. Cell cultures of *C. blumei* supplemented with 4% of sucrose accumulate maximum RA contents of 20% of dry weigh (M. Petersen, Häusler*, et al.*, 1993). Fungal preparations and methyl jasmonate (Mej) have been used as elicitors in effort to enhance RA accumulation in cell suspension cultures of *C. blumei* (Szabo*, et al.*, 1999), *L. erythrorhizon* (Mizukami*, et al.*,

1993), *O. basilicum* (H. J. Kim*, et al.*, 2006), *S. officinalis* (Hippolyte*, et al.*, 1992). *Ocimum* species have been also object of biotechnological approaches in order to increase the accumulation of RA *in vivo* and *in vitro*. *O. basilicum* cell line T2b rich in RA were treated with physical stress in order to increase RA and anthocyanin (AC) production (Strazzer*, et al.*, 2011), Mej was also used as an elicitor to evaluate total phenolic compounds (TPC) in *in vivo O. basilicum* plants. Increase in RA content was reported (H. J. Kim*, et al.*, 2006). Due the high content of RA and TPC *O. sanctum* is receiving special attention. Calli cultures of *O, sanctum* shows several levels of AR accumulation according with the type of explant used (F. L. Hakkim*, et al.*, 2007), cell suspension cultures treated with several growth regulators and Mej were also tested in order to increase RA amounts (L. Hakkim*, et al.*, 2011a, 2011b). In order to find new sources of biological compounds at low cost *O. sanctum* cell suspension cultures supplemented with 2,4-D and 2% of sucrose were treated with several elicitors to enhance RA production and accumulation.

3.2 Materials & Methodos

3.2.1 Elicitor preparation

Metil jasmonate (Mej) (Aldrich, Spain) and Salicylic acid (Sa) (Merck, Germany) were dissolved in ethanol to reach 63 mg/ml and 9.3 mg/ml respectively. Yeast extract (Cultimed, Uk), was dissolved in deionized water (Millipore, MA, USA) to afford a stock concentration 3 mg/ml. *Agrobacterium tumefaciens* and *Escherichia coli* were obtained from the Centro de Biologia Funcional (CBF) Biology department (University of Minho, Braga, Portugal) and incubated in LB medium, during 24h in a rotary shaker at 200 rpm, at 30 +/- 0.1°C. Biomass was collected by centrifugation (Eppendorf centrifuge 5804R) at (5.000 rpm from 10 min) frozen at -80°C and lyophilized (B. Braun. Christ Alpha 2-4 Christ LDC-1m) for 48h. The biomass was re-suspended in deionized water to afford stock concentration, 20 mg/ml and stored at -4°C.

3.2.2 Cell suspension cultures

Ocimum sanctum cell suspension cultures were obtained from the Biology department of the University of Minho. Cultures were incubated in 16h light photoperiod at 25 +/- 0.1°C, illuminated by fluorescent light bulbs (Osram-Fluora) providing a photon flux of 30 μMol/s and kept in a rotatory shaker at 100 rpm. The growth was done in MS medium (Murashige & Skoog, 1962) supplemented with 2 mg/l of 2,4-D (SIGMA), 20% of sucrose, and sub culture each 8 days, by pipetting 10 ml of old cultures and transferring it for 60 ml of fresh medium.

3.2.3 Culture elicitation

O. sanctum suspension cultures were divided in groups for a preliminary test. One group of flasks was kept as control. Sets were treated at the exponential phase (6th day) of culture with several elicitors as follows: *i)* Sa 100 µM; *ii) Sa* 50 µM; *iii)* Yeast extract 2.27 µg/ml (Ye); *iv)* Ye 1.13 µg/ml; *v) A. tumefaciens* 2.27 µg/ml; *vi) A. tumefaciens* 1.13 µg/ml; *vii) E. coli* 2.27 µg/ml; *viii) E. coli* 1.13 µg/ml.

A new set of elicitors and combinations was chosen for final experiment on base in the preliminary results and bibliography research. The first group of flasks was kept as control. Second group was treated with Mej (100 µM), third group was treated with Ye (1.13 µg/ml), a fourth group was primed in the 5ht day of culture with Mej (100 µM) and 24h hours later elicited with Ye (1.13 µg/ml). Cells suspension culture samples were harvested on the 7th day of growth by centrifugation at (5.000 rpm from 10 min) frozen at -80°C, and lyophilized for 72 h and stored for future analysis.

3.2.4 Cell viability test

Cell cultures were checked for their viability throughout the experiment using the trypan blue (Sigma-Aldrich, Spain). Briefly, 0.1 ml cells suspension samples were mixed with 0.1 ml of 0.4% trypan blue and incubated for 10 min. A part of the mixture after incubation was transferred onto a glass slide and covered with a cover slip. Cell viability was checked at light microscope. The percentage of cell viability for each sample was calculated by counting the number of non-stained cells in relation to the total cells at four different locations of the glass slide.

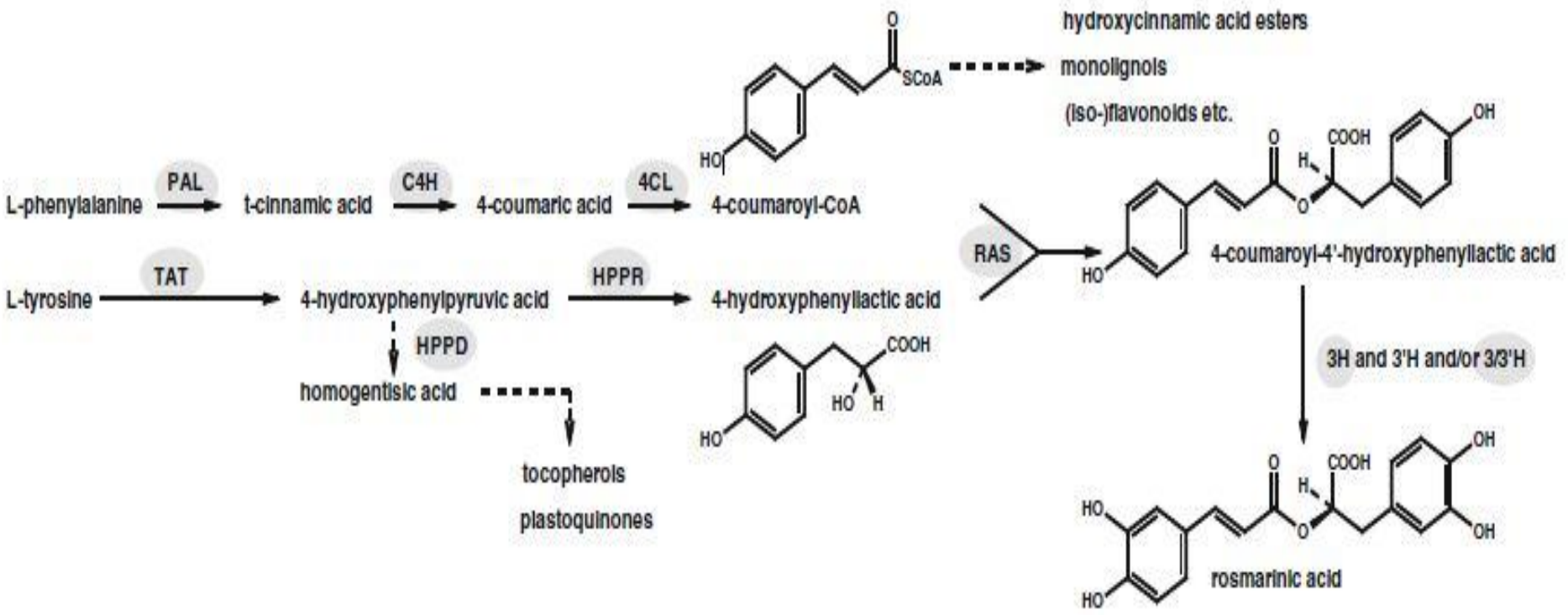

Figure 3. 2 - Biosynthetic pathway of rosmarinic acid in Coleus blumei. PAL phenylalanine ammonia-lyase, CAH cinnamic acid 4-hydroxylyase, 4CL 4-hydroxycinnamic acid CoA-ligase, TAT tyrosine aminotransferase, HPPR hydroxyphenilpyruvate reductase, HPPD hydroxyphenylpyruvate dioxygenase, RAS rosmarinic acid synthase, 3-H, 3-H and 3'-hydroxylase.

3.2.5 High Performance Liquid Chromatography Diode Array (HPLC-DAD) analysis

O. sanctum lyophilized biomass was used for HPLC analysis of phenolics content. Ethanolic extracts were prepared using 100mg dry weighted biomass (dwb) of cells mixed with 5ml of ethanol-water solution (80:20). This mixture was sonicated in a Ultrasonicator (Brandson 2510) for 30 min, and 1 ml of each extract was filtered using a nylon filter (Tracer, 0.45 µm, Spain), and stored in dark at 4°C till further analysis for HPLC. *O. sanctum* leaves collected in New Delhi (India) were also used as biomass to prepare ethanolic extracts as was described above. A pure standard sample of RA (Aldrich, Spain) was also run in order to determine and quantify its production on biomass.

Samples were run in a HPLC-DAD apparatus (HITACHI, LabChrom Elite, Japan) controlled by the computer software EZChrome elite. The detection was performed in a 250-600 nm rage, and chromatograms were recorded at 260, 280 and 350 nm, for quantification. The separation was executed on a LichroCART column (Lichrospher 100. Rp-18e. 5µm, Merck, Germany). Mobile phase used as solvent A, ultra-pure water containing formic acid (0.2%), and B, methanol for liquid chromatography (Merck, Germany). Elution was executed using the gradient described on table 3.2.

Table 3. 2 - Elution specifications for HPLC-DAD analysis

Time (Min)	Solvent A %	Solvent B%
0	70	30
2	70	30
5	65	35
10	30	70
15	10	90
20	10	90
25	70	30
30	70	30

3.2.7 Statistical analysis

All data were calculated using the software GraphPad 5 (Prism, USA). Data were expressed as the mean ± S.E.M., of three independent experiments. The significance of the differ-

ences between the means obtained was evaluated using the unpaired two-tailed Student's t-test. A difference of $p \leq 0.05$ was considered significant.

3.3 Results and Discussion

O. sanctum cell suspension cultures were treated with several elicitors with the purpose of increasing phenolics production, namely RA accumulation. A first set of elicitors was tested to evaluate cell suspension behavior. Most of the treatments showed very low or no significant toxicity (Figure 3.3). Treatments with Ye and *E. coli* with a concentration of 2.72 µg/ml significantly reduced the viability of the cultures. Due to this fact, treatmens with Ye and *E. coli* at 2.72 µg/ml were excluded from elicitation experiments. Moreover, SA significantly reduced the accumulation of RA in cells suspension (results not shown) and it was also removed from potential elicitors to be tested.

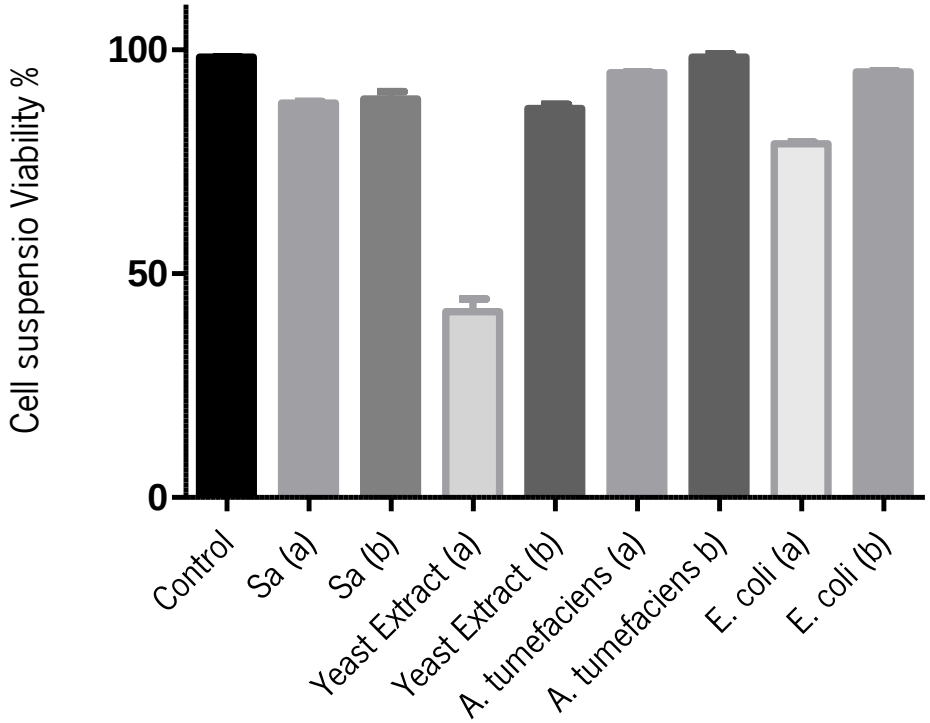

Figure 3. 3 - Cell viability assessed by Trypan blue test. Elicitors used were Sa (a) 100µM; Sa (b) 50 µM; Yeast extract, *A. tumefaciens* and *E. coli* (a) 2.27µg/ml; Yeast extract, *A. tumefaciens* and *E. coli* (b) 1.13µg/ml

A new set of experiments was done using only Ye 1.13 µg/ml, Mej 100 µM and combinations of priming cultures with Mej 100 µM 24h after Ye 1.13 µg/ml application. Viability test (Figure 3.6) and HPLC-DAD analysis of biomassa was done (Figure 3.7). RA determination

and quantification was done comparing the chromatograms, using the retention time, area and UV spectrum of a standard solution containing pure RA (Figure 3.4). A typical HPLC chromatogram of *O. sanctum* cells suspension phenolics is presented in figure 3.5.

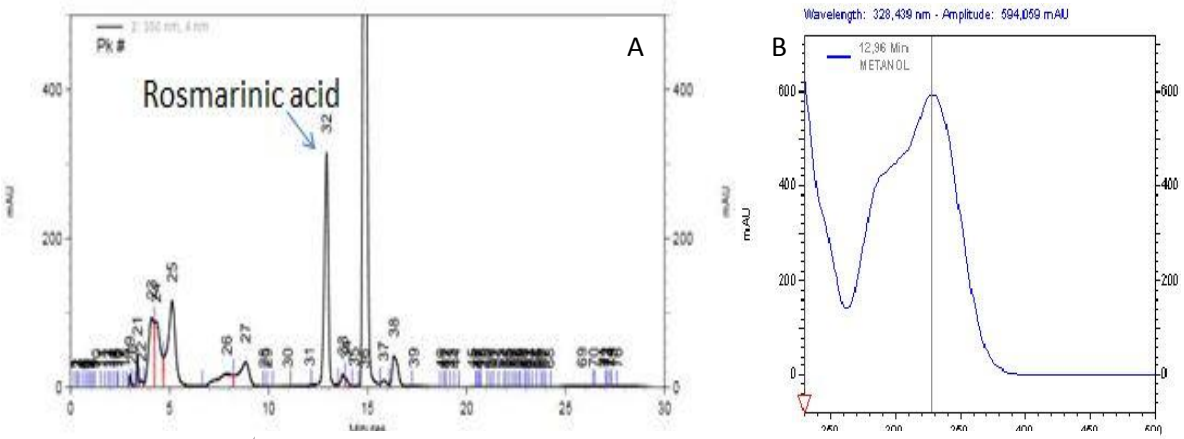

Figure 3. 4 - HPLC-DAD Chromatogram (350nm) of standard Rosmarinic acid solution (A). Rosmarinic acid UV spectrum detected by Diode array (B).

In some cultures, mostly cell suspensions, RA usually accounted approximately for 90% of total phenolics contents (Kintzios*, et al.*, 2004). In our experiment *O. sanctum* cell suspension produced approximately 92,7% more RA than *in vivo* plants with a production of 0.8 mg/gdwb of RA. This difference could be explained; cell suspension cultures grown in a nutrient rich medium, with a more carbon influx than field plants, leading to increasing metabolic flux biosynthesis and elevated amounts of phenolic compounds (F. L. Hakkim*, et al.*, 2007; Razzaque & Ellis, 1977), it was demonstrated that agitation and light intensity have a general increase in phenylpropanoid levels in *O. basilicum* by the stimulation of the 4-coumaroyl-CoA, PAL and cinnamic acid 4-hydroxylase (C4H) upstream regulation (Strazzer*, et al.*, 2011). Hormones, sugar and other signals can also interact as molecules that stimulate the phenylpropanoid synthesis (Davies, 1972; Westcott & Henshaw, 1976). Although Mej may have been related with PAL gene activation resulting in phenolic compounds accumulation, other factors like auxins, specially 2,4-D can act as a limiting factor of phenolic compounds accumulation in cell suspension (Phillips & Henshaw, 1977). In our experiment we were unable to increase RA concentration in cell suspension treated with different elicitors (Figure 3.7). One possible reason for these is the high concentration (2 mg/L) of 2,4-D used to growth and maintain the cultures.

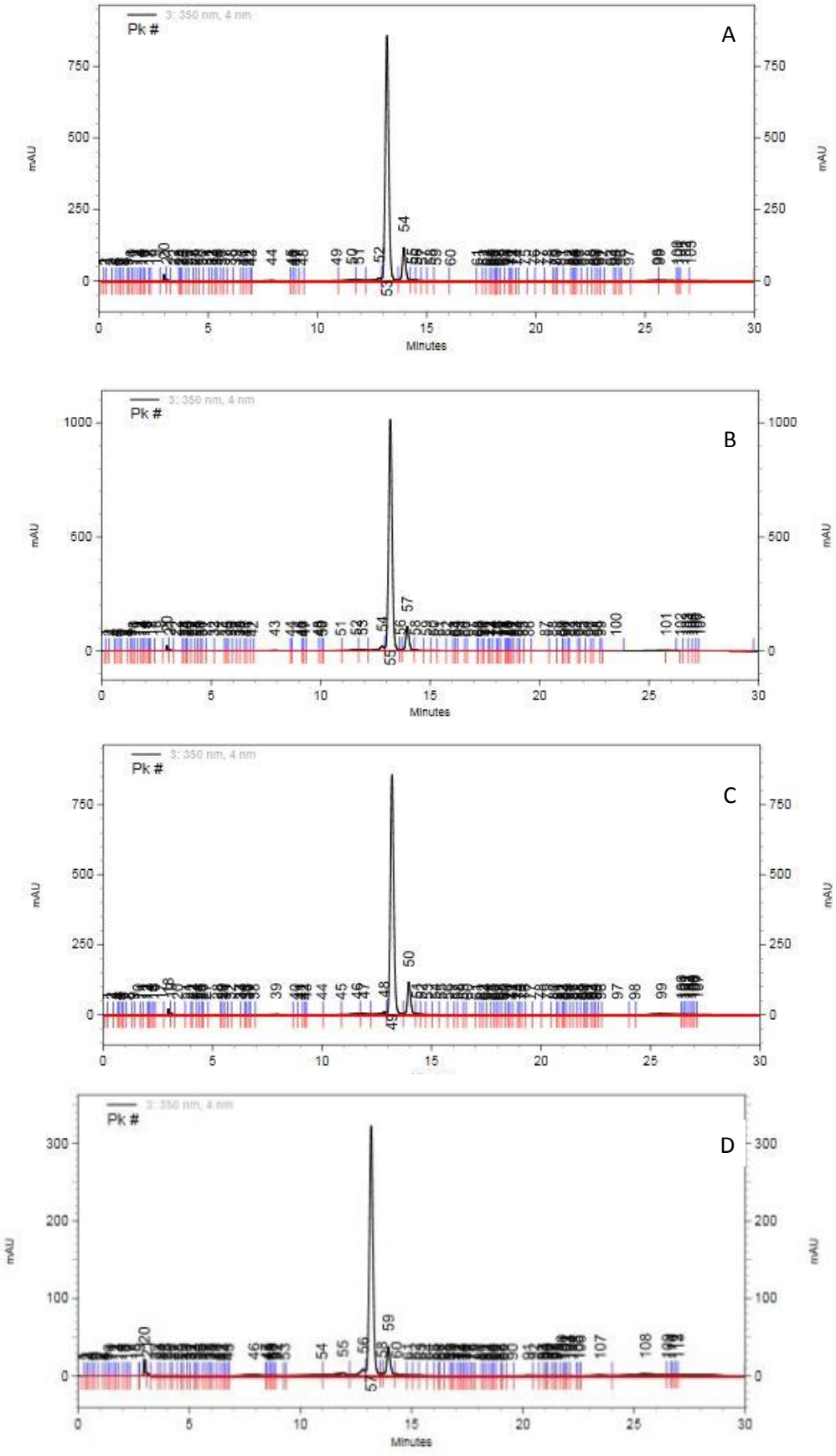

Figure 3. 5 - HPLC-DAD Chromatogram (350nm) of: (A) O. sanctum Control; (B) O. sanctum treated with Mej 100 µM; (C) O. sanctum treated with Ye 1,13 µg/ml; (D) O. sanctum treated with Mej 100 µM and 24h after Ye 1.13 µg/ml

RA production by cell suspension cultures of *L. erythrorhizon* was increased apparently due to the activation of the phenylpropanoid and tyrosine pathways confirmed by the incorporation of both radiolabelled phenylalanine and tyrosine into RA (Mizukami*, et al.*, 1993). Exogenous application of Mej induce *de novo* transcription of PAL gene, in *Glycine max* cell suspension cultures (Gundlach*, et al.*, 1992). Experiments with cell suspension cultures of *O. basilicum* treated with Mej suggest that PAL might be related with induction of phenolic compounds (H. J. Kim*, et al.*, 2006). Taking this account, medium supplementation with tyrosine and alanine could increase RA production serving as precursors in the biosynthetic pathway.

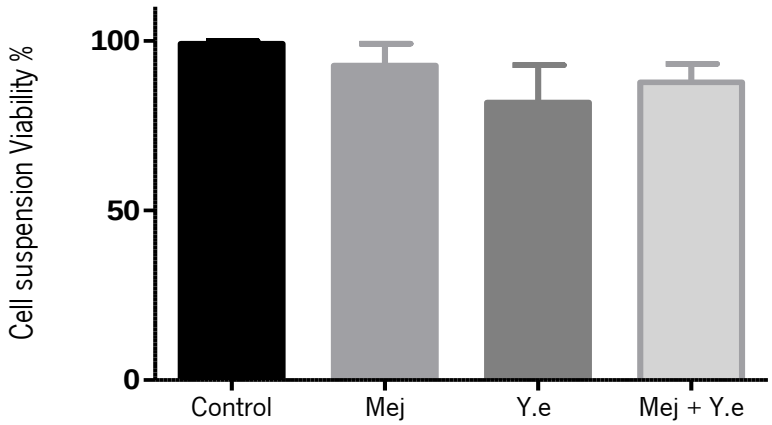

Figure 3.6 - Viability tes of *O. sanctum* cell cultures treated with Mej 100µM; Yeast extract 1,13µg/ml an combination of Mej 100µM and Yeast extract 1,13µg/ml. Each bar represents the mean ± S.E.M. considering the results obtained in at least three independent experiments.

Fungal elicitors are known to induce the *de novo* synthesis of compounds involved in defense responses. Cell suspension cultures of *Rauvolfia canescens* treated with a yeast cell wall preparation showed increment in the concentration of jasmonic acid. A peak concentration of the free acid appears after 45 min of elicitor treatment (Gundlach*, et al.*, 1992) Jasmonic acid and its derivatives have an integral role in the cascade of events that occur in the elicitation process, causing either directly or indirectly the activation of the genes of secondary metabolism, understating the eliciting process and transcription factors involved in RA biosynthesis is vital to increase total phenolic compounds in several species and particularly RA in *O. sanctum* cell suspension cultures.

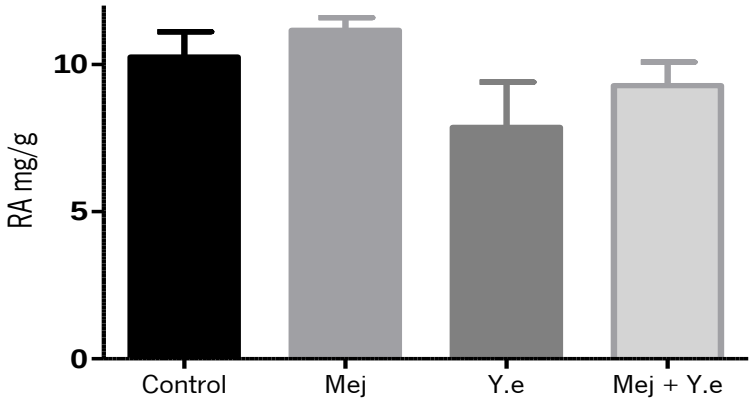

Figure 3.7 - Rosmarinic acid production on *O. sanctum* cell cultures treated with Mej 100µM; Yeast extract 1,13µg/ml an combination of Mej 100µM and Yeast extract 1,13µg/ml. Each bar represents the mean ± S.E.M. considering the results obtained in at least three independent experiments.

Chapter 4

ANTIOXIDANT ACTIVITIES OF CELL SUSPENSION CULTURES OF *O. SANCTUM*

4.1 Introduction

Oxygen have played a central role in the evolution of aerobic organisms on Earth (Buonocore, *et al.*, 2010), easily diffused across biological membranes and bounded to heme-proteins (cytochromes and hemoglobin) (Victor J Thannickal, 2009). Its reactivity allows its participation in high energy electron transference mostly to support the generation of ATP, through oxidative phosphorylation (Burton & Jauniaux, 2011). Molecular oxygen (O_2 bear four electron reduction when it is metabolized *in vivo*). Exited electrons can react with transition elements or other oxygen molecules to form high reactive species such as reactive oxygen species (ROS), nitrogen reactive species (RNS), carbon centered radicals and sulphur centered radicals (Halliwell, 2007). Several reactive species are presented at Table 4.1. The production of reactive species is inevitable, generated by different biological mechanisms, leading to both positive benefits and potentially damaging side effects for biological systems. At low or moderated concentrations, ROS and/or RNS acts as cellular signals, regulating several biological processes, on the other hand their excess can lead to harmful conditions known as "oxidative stress state". Oxidative stress can be defined as a "state in which oxidation exceeds the antioxidants systems in the body secondary to a loss of the balance between them" (Yoshikawa & Naito, 2002). This state results in several diseases mainly by the interaction and modification of lipids, proteins, aminoacids, DNA and transcription factors (Burton & Jauniaux, 2011; Giordano, 2005; Stadtman, 1992).

Table 4. 1 - High Reactive Species

	Major active oxygen species
$LO^{\cdot}$	Alkoxyl radical
LOOH	Alkylhydroperoxide
$LOO^{\cdot}$	Alkylperoxyl radical
$Fe^{+4}O$	Ferryl ion
H_2O_2	Hydrogen peroxide
$OH^{\cdot}$	Hydroxyl radical
ClO^{-}	Hypochorite ion
$NO^{\cdot}$	Nitric oxide
$Fe^{+5}O$	Periferryl ion
$_1O_2$	Singlet oxygen
$O2^{-}$	Superoxide radical

An electron addition to O_2 molecule produced superoxide anion ($O_2^{\cdot}$), considered as primary ROS. It occurs mainly in the mitochondrial respiration chain; it can be reduced to form molecular

oxygen and hydroxyl radical ($OH^{\cdot}$) or oxidized to produce hydrogen peroxide (H_2O_2), as follow the equation:

1) $O_2 + 1e^- + H^+ \longleftrightarrow HO_2^{\cdot} \longleftrightarrow H^+ + O_2^{\cdot-}$
2) $HO_2^{\cdot} + 1e^- + H^+ \longleftrightarrow H_2O_2$
3) $H_2O_2 + 1e^- + H^+ \longleftrightarrow H_3O_2 \longleftrightarrow H_2O + OH^{\cdot}$
4) $OH^{\cdot} + 1e^- + H^+ \longleftrightarrow H_2O$

Superoxide can also be generated by heme-oxidation; about 3% of the hemoglobin in erythrocytes is oxidized every day, and several enzymes (dihydrorotate dehydrogenase, aldehydeoxidase, xanthineoxidase) can produce superoxide and hydrogen peroxide: (Buonocore*, et al.*, 2010). Excessive generation of superoxide can also lead to interactions with nitric oxide to form peroxynitrite ($ONOO^-$), a powerful pro-oxidant capable of diffusing up to 5 mm. It may affect neighboring cells which can react directly with a variety of biomolecules to cause molecular damage (Burton & Jauniaux, 2011).

There are two mechanisms that maintain ROS/RNS homeostasis:

1) enzymatic defenses (transition metal core), are capable of take different valences during the electron transference in oxidation process; two isoforms of SOD are responsible for conversion of superoxide into hydrogen peroxide, which is also broken down into water by the action of CAT or glutathione peroxidase regulated by the presence of reduced glutathione (GSH) acting as substrate or co-factor in various enzyme reactions (Briviba & Sies, 1994).

Glutathione is the major cellular thiol redox buffer in cells, synthesized in the cytosol from L-glutamate, L-cysteine and glycine is involved in a large number of detoxifying reactions forming glutathione disulfide, which is converted back to GSH by the action of glutathione reductase at the expense of nicotinamide adenine dinucleotide phosphate hydrogenated (NADPH) (Burton & Jauniaux, 2011).

2) Non-enzymatic defenses, correspond to molecules synthetized in the body (glutathione & ubiquinol) or acquired by food ingestion (α-tocopherol, flavonoids, carotenoids & retinoids, ubiquinone and others), which may act at several levels as: a) radicals scavenging , b) binding to metal ions, c) scavenging of peroxyl radicals and d) removing oxidized damaged biomolecules (Briviba & Sies, 1994). Ascorbate and glutathione take part in detoxification enzyme reactions as

substrate and co-factor, and they also can deactivate free radicals and regenerate antioxidants from the hydrophobic phases, and plays a key role as antioxidant in lipid compartments. Aromatic and phenolic compounds are very effective as antioxidants due presence of conjugated bounds; given their aromatic nature, phenolic compounds are easily oxidized. The radical that is generated can subsequently react with other radicals to form a dimer. Since the radical electron is delocalized, several structures can be formed depending on the precise location of the radical electrons at the time of the reaction (Wilfred Vermerris & Ralph Nicholson, 2006a).

The aim of this study was to evaluate the antioxidant potential of cell suspension cultures extract of *O. sanctum*, comparing it with extracts of *in vivo O. sanctum* leafs and pure RA. Several assays were used to test this activity: DPPH assay (2,2-diphenyl-1-picrylhydrazyl), ICA (Iron chelating activity), inhibition of $NO^{\cdot}$ production and scavenging of superoxide assays. Additionally, it was tested the inclusion of *O. sanctum* cells extract in a cosmetic vehicle.

4.2 Material and Methods

4.2.1 Preparation of *O. sanctum* ethanolic extracts

O. sanctum lyophilized biomass was used for extract preparations. Ethanolic extracts were prepared using 100mg dry weighted biomass (dwb) to 5ml of ethanol-water solution (80:20), put it in Ultrasonicator (Brandson 2510) and stored in a dark and at ambient temperature for posterior use. *O. sanctum* leaves collected in New Delhi (India) were also used as biomass to prepare ethanolic extracts as was described above.

Extracts were concentrated under nitrogen flux, frozen at -80°C and lyophilized for 72 hours; residues were resuspended in DMSO (dimethyl sulfoxide) and stored at 4°C until further use.

4.2.2 Preparation of *O. sanctum* cream

O. sanctum extracts were incorporated into a cream. *O. sanctum* cell suspension extract was incorporated into 50g of a commercial crude base (Croda base, MG, Brasil), dissolved in 300 ml of water reaching a final concentration of 0.2% of extract in the base cream. The cream was stored at room temperature protected from light and humidity.

4.2.3 Antioxidant activities

Several methodologies: a) DPPH assay, b) chelating activity of Fe^{2+}, c) inhibition of nitric oxide and d) scavenging of superoxide radical were used to evaluation *in* vitro antioxidant activities of extracts of *O. sanctum* cell suspension culture, *in vivo* leafs and pure RA (Aldrich, Spain), and cream preparations.

4.2.3.1 DPPH Assay of *O. sanctum* extracts and cream

The ability to provide hydrogen to stable radicals was evaluated by the kinetic of DPPH (2,2-diphenyl-1-picrylhydrazyn, SIGMA, Spain) bleaching, or DPPH assay modified from (Silva*, et al.*, 2007). To evaluate the antiradical activity of *O. sanctum* cell suspension culture and wild plant extracts, several dilutions of the extracts were prepared as follows: 10 µl of extract (ethanol 100%) and 140 µl of a DPPH (400 µM), for a final range of extract between 1 mg dwr/ml to $6x10^{-4}$ mg dwr/ml.

The absorbance was monitored continuously for 60 min at 515nm with a SpectraMax-Plus microplate reader, until the reaction was completed. The free radical DPPH is reduced and the absorbance decreases in presence of radical scavenging substances. Ethanol was used as a blank and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, SIGMA, Spain) was used as a standard to perform a calibration curve and as a chemical reference in comparison to the antiradical capacities of the extracts. The DPPH solution was prepared daily, and stored in dark at 4°C. All measurements were performed in triplicate.

Results were expressed in terms of percentage of reduced DPPH at steady state, and were calculated as follows:

$$\%\ \text{DPPHr} = \frac{\text{Ai} - \text{Ar}}{\text{Ai}} \times 100$$

Ar – Absorbance registered at steady sate

Ai – Initial absorbance (time t= 0)

The EC_{50} was achieved through the percentage of reduced DPPH curve and the concentration needed to reduce DPPH color in 50%.

To evaluate the antiradical activity of *O. sanctum* cream we used the same methodology as described above but with some modifications. The dilution series were prepared as fol-

lows: 50 mg of cream and 700 µl of a DPPH (400 µM), and read in 1ml cuvette in a Thermo Scientific spectrophotometer. Final concentration 3.33 mg of cream. Cream without extract was used as control.

4.2.3.2 Iron Chelating Activity Fe^{+2} of *O. sanctum* extracts and cream

The iron chelating activity (ICA) test estimates the ability of extracts to chelate Fe^{+2}, (Iron II choride-4-hydrate, SIgma, Spain). In the ICA method, the Fe^{+2} is quantified by spectrophotometry using an iron colored complex which result by the conjugation of ferrozine (FZ) (3-(2-pyridyl)-5,6.diphenyl-1,2,4-triazine-4´,4'-disulphonic acid sodium salt, SIGMA, Spain) with iron (Fe2+-FZ) (Russo*, et al.*, 2005). The amount of iron chelated by the extract is calculated by absorbance difference (read at 562nm) between control (Fe2+-FZ) and sample (Fe2+-sample-FZ).

To evaluate the iron chelating activity (ICA) of *O. sanctum* cell suspension culture and *O. sanctum in vivo* leaves extracts, a dilution series were prepared as follows: 50 µl of extract (dissolved in ethanol 100%) at different concentrations (0,06 – 1 mg/ml), 50 µl of $FeSO_4$ stock solution (0.12 mM, ultrapure water) and 50 µl of Ferrozine stock solution (0.6 mM, ultrapure water). After 10 min of incubation in dark at room temperature, readings were taken at 515 nm with SpectraMaxPlus microplate reader. Disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA) (Merck, Germany) was used as a standard to perform a calibration curve and as a chemical reference in comparison to iron chelating activity capacities of the extracts. The different solutions was prepared daily, and kept protected from the light. All measurements were performed in triplicate.

Iron chelating activity is expressed in percentage of iron chelated and was calculated as follows:

$$\%\,\mathrm{ICA} = \frac{\mathrm{Abscontrol} - (\mathrm{Abssample} - \mathrm{Absblank})}{\mathrm{Abscontrol}} \times 100$$

$Abs_{control}$ - ferrozine absorbance with iron, without extract

Abs_{sample} - ferrozine absorbance with iron and extract

Abs_{blank} - absorbance of the extract without ferrozine

The EC_{50} was obtained from plotting the percentage of ICA against the concentration of the extract and represents the concentration needed to inhibit the formation of 50% of color complex Fe-Ferrozine.

To evaluate the antiradical activity of *O. sanctum* cream we used the same methodology as was described above but with some modifications. The dilution series were prepared as follows: 250 mg of cream, 250 µl of $FeSO_4$ stock solution (0.12 mM, ultrapure water) and 250 µl of Ferrozine stock solution (0.6 mM, ultrapure water). Read in 1ml cuvette in a Thermo Scientific spectrophotometer. Final concentration 83.33 mg of cream. Cream without extract was used as control.

4.2.3.3 Evaluation of Inhibition of Nitric Oxide Production by *O. Sanctum* extracts and cream

Nitric xide radical can be generated spontaneously *in vitro* from sodium nitroprusside (SNP) in aqueous solution at physiologic pH, and interact with oxygen to produce nitrite ions that can be estimated by the Gries's reagent. The nitrite ions diazotized with N-(1-naphthyl)ethylenediamide (NED) produce a pink chromophore that can be measured at 562 nm. A methodology modified from (Colle*, et al.*, 2012) was utilized for NO quantification.

To evaluate the NO scavenging activity of *O. sanctum* cell suspension culture and *O. sanctum in vivo* leaves extracts, a dilution series (0.06 – 1 mg/ml) were prepared as follows: 100 µl of extract (phosphate buffer pH 7.4) was added to 100 µl of SNP (Sigma, Spain) dissolved in ultrapure water 20 mM, and incubated for 1 hour. After that, 100 µl of Gries's reagent was added to mixture and readings were taken after 10 minutes of incubation, in the dark, at room temperature, at 562 nm with SpectraMaxPlus microplate reader. Gries's reagent contains sulfanilamide (1% sulphanilamide in 5% H_3PO_4) and same amount of NED (0.1%, water). The different solutions were prepared daily, and kept protected from the light. All measurements were performed in triplicate.

Inhibition of nitric oxide production was calculated as follows:

$$\%\ \text{Inhibition of nitric oxide production} = \frac{(\text{Abscontrol} - \text{AbsBlankcontrol}) - (\text{Abssample} - \text{AbsBlanksample})}{(\text{Abscontrol} - \text{AbsBlankcontrol})} \times 100$$

$Abs_{control}$ - SNP absorbance with Griess reagent, without extract

$Abs_{Blankcontrol}$ - SNP absorbance, without extract and Griess reagent

Abs_{sample} - SNP absorbance with Griess and extract

$Abs_{blanksample}$ - absorbance of the extract with SNP, without Griess

The EC_{50} was obtained from the percentage of inhibition of nitric oxide production plotted against the concentration of the extract, and represent the concentration of extract needed to inhibition 50% nitric oxide production

To evaluate the antiradical activity of *O. sanctum* cream we used the same methodology described above but with some modifications. The dilution series were prepared as follow: 300 mg of cream (1:1 diluted phosphate buffer pH 7.4) was added to 300 µl of SNP (SIGMA) dissolved in ultrapure water 20 mM and incubated for 1 hour, 300 µl of Gries's reagent was added and readings were taken after 10 minutes of incubation in the dark at room temperature. Final concentration 83,33 mg of cream. Cream without extract was used as control.

4.2.3.4 Evaluation of Superoxide Scavenging Activity of *O. sanctum* extracts and cream

Superoxide is generated *in vivo* by three key mechanisms: mitochondria respiratory chain, xanthine oxidase and NADPH oxidase activated phagocytes. It results in the reduction of oxygen by a single electron molecule (Apel & Hirt, 2004a).

A non-enzymatic system can be used to assess the potential to scavenge superoxide radical: phenazine methosulfate (PMS) is reduced by nicotinamide adenine dinucleotide (NADH) and react with oxygen to produce superoxide radicals. These radicals react with nitroblue tetrazolium chloride (NBT) reducing it to formazan, a blue compound that have maximum absorption at 560

nm. We used a methodology modified from (Valentao*, et al.*, 2002). In the presence of substances capable of scavenging superoxide radicals, the rate of NBT reduction decreases and consequently there is a decrease in color intensity.

To evaluate the superoxide scavenging activity of *O. sanctum* cell suspension culture and *O. sanctum in vivo* leaves extracts, dilution series (0,06 – 3 mg/ml) were prepared as follows: 50 µl of extract (phosphate buffer pH 7.4) added to 50 µl of NADH (166 µM in phosphate buffer pH 7.4), 150 µL of NBT (Roche, Switzerland) solution (43 µM in phosphate buffer pH 7.4) and 50 µl of PMS (Aacros, USA) solution (2,7 µM, phosphate buffer pH 7.4). Assays were conducted at room temperature and the readings were taken after 2 minutes at 562 nm with SpectraMaxPlus microplate reader, taken PMS addition as time 0. The different solutions were prepared daily, and kept protected from the light. All measurements were performed in triplicate.

The results of superoxide production are expressed as percentage of inhibition of the production of NBT relatively to the control, as follows:

$$\%\ \text{Inhibition of production of NBT} = \frac{(\text{Abscontrol} - (\text{Abssample} - \text{AbsBlanksample})}{\text{Abscontrol}} \times 100$$

$Abs_{control}$ – NBT absorvance, without extract

Abs_{sample} – NBT absorbance with extract

$Abs_{blanksample}$ - absorbance of the extract without PMS

The EC_{50} was obtained from the percentage of the production of NBT plotted against the concentration of the extract and represents the concentration of extract needed to inhibit 50% of iron superoxide

To evaluate the antiradical activity of *O. sanctum* cream we used the same methodology described above but with some modifications. A dilution series was prepared as follow: 150 mg of cream (1:1 dissolved in phosphate buffer pH 7.4) added to 150 µl of NADH (166 µM in phosphate buffer pH 7.4), 450 µL of NBT (ROCHE) solution (43 µM in phosphate buffer pH 7.4) and 150 µl of PMS (ACROS) solution (2,7 µM, phosphate buffer pH 7.4). Final concentration 83,33 mg of cream. Cream without extract was used as control.

4.2.3.5 Statistical Analysis

All parameters were calculated graphically using the software GraphPad 5 (Prism, USA). Data were expressed as the mean ± S.E.M., of three independent experiments. The significance of the differences between the means obtained was evaluated using the unpaired two-tailed Student's t-test. A difference of $p \leq 0.05$ was considered significant.

4.3 Results and Discussion

Antioxidants, including phenolic compounds, have diverse biological effects, such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic effects, as a result of their antioxidant activity (Chung*, et al.*, 1997). Compounds with more than one hydroxyl group in the phenolic rings can act as reducing agents, hydrogen donating antioxidant, chelate metal ions and single oxygen quencher's (C. A. Rice-Evans*, et al.*, 1996). RA has four phenolic hydroxyl groups separated into two pairs located in ortho-positions of the benzene rings, in a theoretical sense, rosmarinic acid can capture four radicals (Jayasinghe*, et al.*, 2003).

Our goal was to evaluate the antioxidant potential activity of *O. sanctum* cell suspension cultures, compare it to *in vivo* leafs extracts and cream formulation using DPPH assay, ICA, inhibition of nitric oxide production and superoxide scavenging activity as methodologies.

4.3.1 DPPH Assay

DPPH assay is one of the commonly techniques applied for screening vegetal extracts for the presence of antiradical compounds. The stable free radical DPPH in solution has intense purple color that changes into pale yellow when the radical is taken by a free radical scavenger (Cieśla*, et al.*, 2012). Solutions prepared with pure RA showed an higher scavenging activity (EC_{50} of 15 +/- 1,5 µg dwr /ml), followed by *O. sanctum* cell suspension cultures extract (EC_{50} of 30 +/- 3,4 µg dwr /ml), and finally less potent *O. sanctum in vivo* extracts (EC_{50} of 208 +/- 22.7 µg dwr /ml), as is showed in Figure 4.1. We also used Trolox as reference compound, the results were similar to the values showed for pure RA, so we decided use pure RA instead Trolox as reference. (Data not showed)

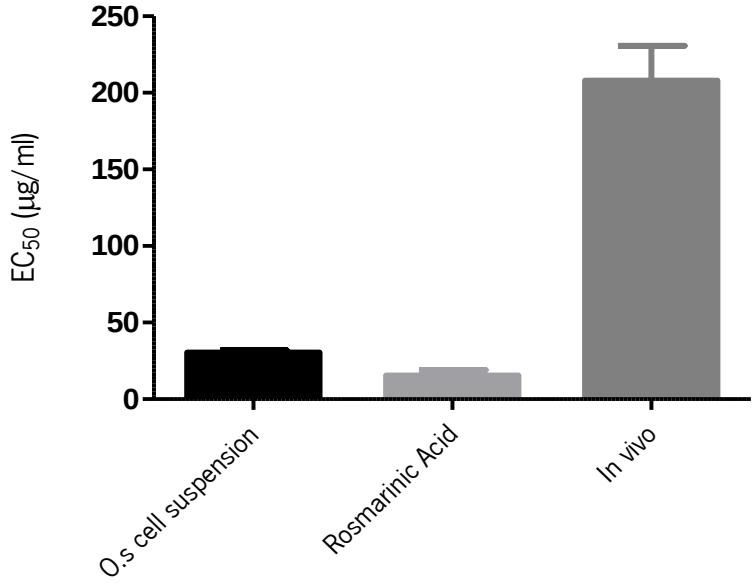

Figure 4.1 - EC50 values for DPPH assay of different treatments using *O. sanctum* extracts**.** Each bar represents the mean ± S.E.M. considering the results obtained at least three independent experiments. All the values are statistically different ($P \leq 0.05$)

The antiradical activity of phenolic compounds depends on their molecular structure, that is, on the availability of phenolic hydrogen and on the possibility for stabilization of the resulting pheoxyl radicals formed by hydrogen donation (Velioglu*, et al.*, 1998). *O. sanctum in vivo* extracts has low amount of RA, and a mix of compounds that their interactions may reduce the DPPH scavenging potential, when compared with pure RA and *O. sanctum* cell suspension cultures. The high content of RA in *O. sanctum* cell extracts is apparently the reason of the high DPPH scavenging potential showed.

Extracts with high DPPH scavenging potential may act as chain breaking antioxidants due the high content of phenolic compounds reducing alkoxyl or peroxyl radicals to alkoxiles or hydroperoxides (C. Rice-Evans*, et al.*, 1997). Peroxyl and alkoxyl are formed through the peroxidation of polyunsaturated fatty acids showing the high potential of *O. sanctum* cell suspension culture extracts to prevent lipid peroxidation *in vitro* systems.

4.3.2 Iron Chelating Activity

Iron is an essential element for life, due to its role in respiratory electron chain, and direct interaction with some enzymes, blood vascular cells, which may have important roles in human metabolism (Mladěnka*, et al.*, 2011). Excess iron is deposited as hemosiderin and ferritin

form in different parts of the body such liver, kidney, spleen, and myocardium. This situation can rise severe health complications such as heart failure, endocrine dysfunction, diabetes, hypothyroidism, liver failure and finally death (Amith Kumar*, et al.*, 2014). It was already mentioned that superoxide radical is produced in living systems by several mechanisms. Miss balances between transition metals and superoxide production may lead the formation of hydroxyl radicals trough Fenton reaction (Halliwell & Gutteridge, 1984; Amith Kumar*, et al.*, 2014) . Thus compounds with iron chelating capability may help to maintain body balances preventing a number of clinical disorders.

RA is an excellent reducing agent. As a reducing agent it is able to reduce catalytic metals such as Fe^{3+} and Cu^{2+} to Fe^{+2} and Cu^{1} (Buettner & Jurkiewicz, 1996). On the other hand, pure RA has pro-oxidant activity as well; in fact we could not find EC_{50} Values for any RA concentration tested (data not showed). these results may be related with the number of hydroxyl groups in the phenolic rings (Cao*, et al.*, 1997) Free ferrous iron is quite sensitive to oxygen and gives rise to ferric iron and superoxide, thereby generating hydrogen peroxide (Galey, 1997) while multiple hydroxyl especially in the B-ring, significantly increased production of hydroxyl radicals in a Fenton system (Heim*, et al.*, 2002).

Probably hydroxyl radical has the higher oxidant power and consequently is one of the most dangerous radical (Pastor*, et al.*, 2000). It has very short life and reacts in accelerated rates with almost every type of molecule found in living systems, attacking aminoacids, phospholipids, DNA, being involved in several diseases. *O. sanctum* cell suspension culture extracts showed high iron chelating activity with EC_{50} values of 47 +/- 1,8 µg dwr /ml, (Data not showed). *O. sanctum in vivo* extracts also has chelating activity, yet in much lower amounts comparing to cell suspension extracts (Figure 4.2). We cannot explain the difference in activity between extracts without elucidate the complete chemical profile and realize more experiments to complement the data. We can suppose that the presence of other phenolic compounds even at low concentration enhance iron chelating activity of both *O. sanctum* cell suspension cultures and *in vivo* leaf extracts when compared with RA.

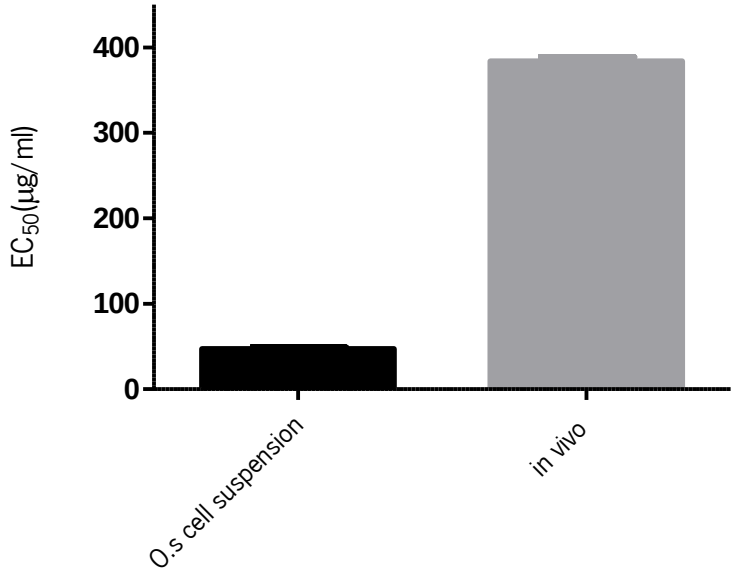

Figure 4.2 - EC50 values for ICA assay of different treatments using *O. sanctum* extracts. Each bar represents the mean ± S.E.M. considering the results obtained at least three independent experiments. All the values are statistically different ($P \leq 0.05$).

4.3.3 Inhibition of Nitric Oxide Production

Nitric Oxide is a gas, containing an odd number of electrons that can form covalent links with other molecules by sharing a pair of electrons (Zade*, et al.*, 2013). Reactive oxygen species and RNS play an essential role as signaling intermediates; growth factors, environmental stress, and cytokines regulate specific cellular responses through redox-sensitive signaling pathways (Suzuki*, et al.*, 1997). Nitric oxide production can be enhance for macrophages and activated neutrophils in inflammatory events.

As is represented in Figure 4.3, *O. sanctum in* vivo leaf extracts showed low capacity to inhibit the nitric oxide production, with EC_{50} values of 277 +/- 19 µg dwr /ml. Pure RA and cell suspension extracts shows high capacity to inhibit the nitric oxide production, with EC_{50} values of 17 +/- 0,3 µg dwr /ml and 21 +/- 0,6 µg dwr /ml respectively.

It has been suggested that nitric oxide leads to an increase in the formation of $ONOO^-$ which is then used as part of the arsenal against invading pathogens (Ischiropoulos*, et al.*, 1992). However the oxidizing potential of $ONOO^-$ can directly initiate lipid peroxidation reactions by abstracting a hydrogen atom from a polyunsaturated fatty acid and also results in the formation of nitrated lipids. Nitric oxide inhibits oxidative modification of low density lipoprotein

LDL, model lipid systems and attenuates the consequent formation of secondary products of lipid oxidation.

4.3.4 Superoxide Scavenging Activity

O. sanctum cell suspension has high scavenging activity for superoxide radicals showing EC_{50} values of 22,1 +/- 3,1 µg dwr /ml been even higher than pure RA with EC_{50} values of 73,9 +/- 1,2 µg dwr /ml represented in figure 4.4. We could not determine EC_{50} for *O. sanctum in vivo* leaf extracts. We observed that the extract display some activity at low concentrations presenting maximum scavenging values for superoxide radicals of 27 % at 25 µg dwr /ml. We also saw that increasing the concentration of both extracts they start to act like pro-oxidant (Figure 4.6). However we cannot be sure that the pro-oxidant effect is real or could be miss interaction between the methodology and the intense color of the samples.

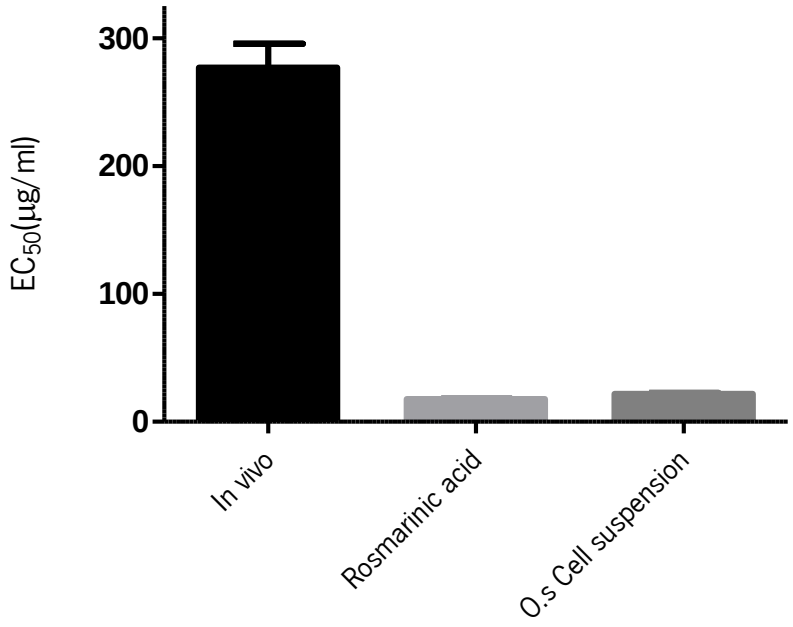

Figure 4.3 - EC50 values for Nitric oxide production inhibition assay of different treatments. Each bar represents the mean ± S.E.M. considering the results obtained at least three independent experiments. All the values are statistically different ($P \leq 0.05$).

Superoxide is the most produced reactive species in aerobic systems, and reacts with other molecules to form even more active species that could lead to harmful situations to living systems. It can acts as reducing or oxidizing agent according with the environment in which is involved (Halliwell & Gutteridge, 1984). Compounds that possess antiradical activity could have great relevance in health issues helping to maintain the homeostasis and preventing oxidative stress.

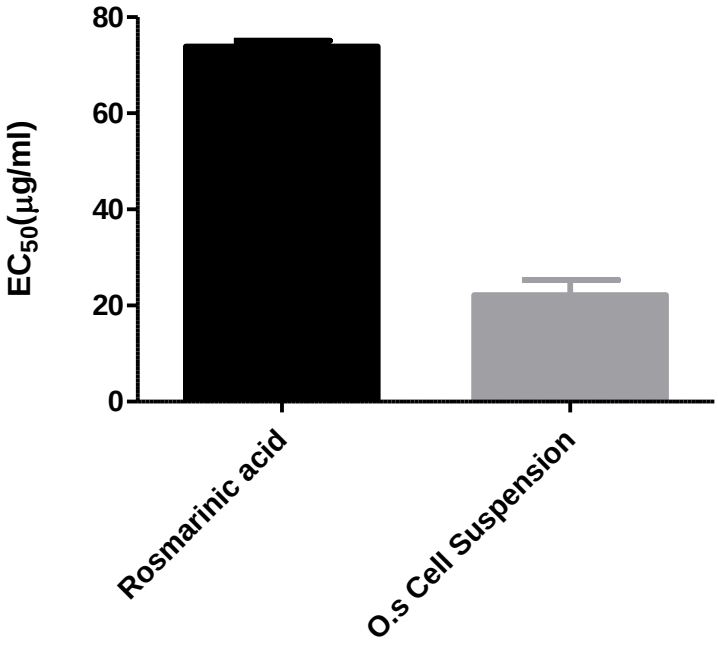

Figure 4. 4- EC50 values for superoxide assay of different treatments. Each bar represents the mean ± S.E.M. considering the results obtained at least three independent experiments. All the values are statistically different (P ≤ 0.05).

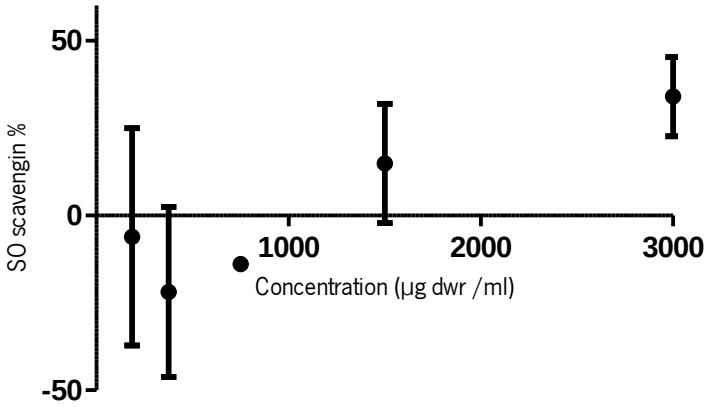

Figure 4.5 – Superoxide Scavenging activity of *O. sanctum* cell suspension cultures

4.3.5 Antiradical Activity for *O. sanctum* Cream

O. sanctum cream showed antiradical activity for all DPPH, Iron chelating activity, Nitric Oxide inhibition and Superoxide scavenging assay. Due the viscosity of the cream it was difficult to perform the serial dilutions and found EC_{50} values. Instead we decided to do punctual concentrations for each assay. At tested concentrations *O. sanctum* displayed potential in the antiradical activity, those properties could show an open window to develop treatments of skin diseases (Figure 4.6 – 4.9). The free antiradical activity of *O. sanctum* is a major mechanism to protect organisms against cellular damage (A Ganasoundari*, et al.*, 1998; Rasik & Shukla, 2000), it appear that several mechanisms like antiradical activity, changes in immune mechanism such as antibody production and release of mediators of hypersensitivity can increase the wounding healing effect of the species (Mediratta*, et al.*, 2002; Udupa*, et al.*, 2006).

4.4 Conclusions

Reactive species play important roles as second messengers in many intracellular signaling cascades aimed at maintaining the cell in homeostasis with its immediate environment .At higher levels, they can cause indiscriminate damage to biological molecules, leading to loss of function and even cell death. So is vital importance to find and evaluate new sources of potential compounds that may act as radical scavengers and finally as antioxidants that could lead to improvements in medicinal therapies and prevent oxidative stress damage.

O. sanctum cell suspension cultures extracts have shown antiradical scavenging activity for the non-enzymatic assays tested in the present study. This property could be due to more accumulation of active principles (phenolic compounds with one or more hydroxyl groups) in the cell suspension extract extracts (F. L. Hakkim*, et al.*, 2007). *O. sanctum* cell suspension extracts possess the ability of inhibit the nitric oxide production and superoxide avoiding their interaction and preventing the formation of $ONOO^-$ (Patel*, et al.*, 1999), nitric oxide is also involved in inflammatory responses mediated by macrophages and neutrophils, preventing acute inflammation. Contrary to RA, *O. sanctum* extracts possess the capability of chelate metal ions decreasing the formation of hydroxyl groups produced by the Fenton reaction. We can attribute the antiradical activity of the extracts to RA and its antioxidant activity extendedly documented (Khojasteh*, et*

al., 2014; Maike Petersen, 2013; M. Petersen & M. S. J. Simmonds, 2003). The presence of RA and other phenolic compounds in *O. sanctum* may be the reason why it has been attributed several biological activities to the specie (chapter 1).

However it should be remembered that to fully characterize a compound as an antioxidant other tests should also be performed oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC) assay, β-carotene bleaching test, lipid peroxidation, cytotoxicity and others (Cieśla*, et al.*, 2012) Thus it appears that different mechanisms like free radical scavenging, metal chelation as well as immune modulation may act at different levels individually or in combination increase healing effects of this medicinal plant (Shetty*, et al.*, 2008). For that reason several tests should be conducted to clarify the potential use of *O. sanctum* cell suspension cultures.

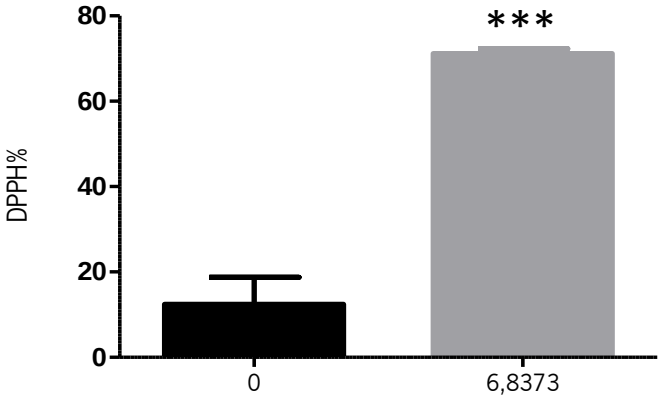

Figure 4. 6– % of DPPH Scavenging of *O. sanctum* cream 6,83 µg dwr in 3,33 mg of cream. Each bar represents the mean ± S.E.M. considering the results obtained at least three independent experiments.*** P < 0,001

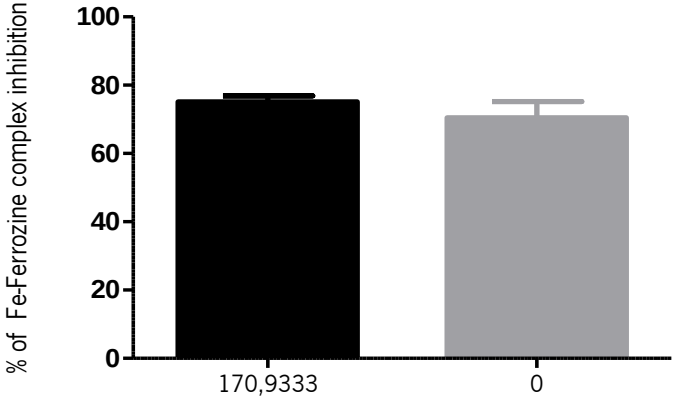

Figure 4. 7 - % of Fe-Ferrozine complex inhibition of *O. sanctum* cream 170,93 µg dwr in 83,22 mg of cream. Each bar represents the mean ± S.E.M. considering the results obtained at least three independent experiments.

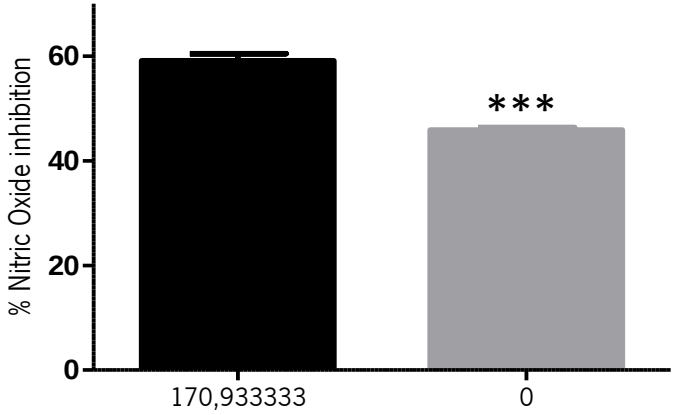

Figure 4. 8 - % of Nitric oxide production inhibition *of O. sanctum* cream 170,93 µg dwr in 83,3 mg of cream. Each bar represents the mean ± S.E.M. considering the results obtained at least three independent experiments.*** P < 0,001

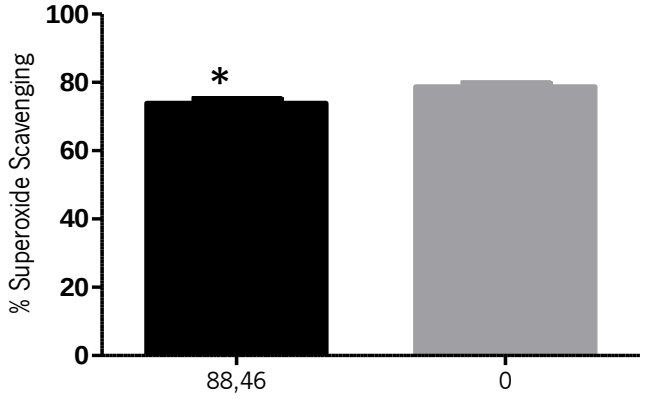

Figure 4. 9 - % of Superoxide Scavenging of *O. sanctum* cream 88,46 µg dwr in 83,3 mg of cream. Each bar represents the mean ± S.E.M. considering the results obtained at least three independent experiments.* P < 0,05

CHAPTER 5

FINAL REMARKS

5.1 General Discussion

Plants are one of the most important sources of medicines. Medicinal plants are rich in secondary metabolites of therapeutic importance. Different parts of *O. sanctum* have been reported in Ayurveda and Siddha Systems of Medicine for prevention and cure of many illnesses and everyday ailments like common cold, headache, cough, flu, earache, fever, colic pain, sore throat, bronchitis, asthma, hepatic diseases, malaria fever, as an antidote for snake bite and scorpion sting, flatulence, migraine headaches, fatigue, skin diseases, wound, insomnia, arthritis, digestive disorders, night blindness, diarrhea and influenza (Govind & Madhuri, 2010). As was described in chapter 1 *O. sanctum* has several chemical constituents that conduce to documented biological activities. RA is one of the major compounds found in *O. sanctum* extracts, usually described has a polyphenol with elevate antioxidant activity (Khojasteh*, et al.*, 2014; Maike Petersen, 2013; M. Petersen & M. S. Simmonds, 2003).

Plant cell cultures can be used has chemical factories for many reasons a) the desired product can be collected anywhere in the world maintaining strict production and quality control; b) herbicides and pesticides are out of question; c) climate and seasonal problems are avoided; d) growth cycles are of weeks rather than the years of an intact plant (Hussain*, et al.*, 2012). As has been reported in several experiments, production of secondary metabolites can be enhanced through metabolic engineering, transformation and elicitation (Deno*, et al.*, 1987; Fujita*, et al.*, 1988; Xu*, et al.*, 2010; Xu*, et al.*, 2007). Increase in the RA content in *O. sanctum* have been described (L. Hakkim*, et al.*, 2011a). In these study we were able to increase the production of RA in cell suspension cultures when is compared with *in vivo* plants, however as HPLC-DAD showed eliciting treatments did not result in the increasing of RA in the suspension cultures when are compared between elicited treatments. The increase in RA could be related with increased PAL and TAT activity (Kintzios*, et al.*, 2003; Mizukami*, et al.*, 1993; Szabo*, et al.*, 1999; Tada*, et al.*, 1996), as well the activity of the specific RA biosynthetic enzyme such as RAS (Szabo et al. 1999). We can suggest for incoming experiments with *O. sanctum* cell suspension cultures, aminoacids supplementation and new set of growth regulators and elicitors like taximin, which promotes secondary metabolism by interacting with Mej in *Taxsus sp*; coronatin or cyclodextrins in tobacco lines (Khojasteh*, et al.*, 2014), different concentrations of glucose may lead to increase the production of RA by the specie.

Chapter 4 was focused on the non-enzymatic antiradical activity of the cell suspension extracts evaluated by several methods. Free radicals have been implicated in several disease conditions in humans, including arthritis, hemorrhagic shock, atherosclerosis, advancing age, ischemia and reperfusion injury of many organs, Alzheimer and Parkinson's disease, gastrointestinal dysfunctions, tumor promotion and carcinogenesis (Bagchi*, et al.*, 2000). Supplementations of bioavailable and safe compounds that can act as antioxidants are essential for maintain ROS homeostasis preventing several illnesses. We confirm that *O. sanctum* cell suspension extracts display elevated *in vitro* antiradical activity for several radicals when is compared with some reference compounds and *in vivo* leaf extracts due the increment in phenolic compounds (Jayasinghe*, et al.*, 2003) mainly RA. However it seems that *O. sanctum* is involved also with enzymatic antioxidant defenses (SOD, GSH, CAT) activation of transcription factors and other mechanisms (R. R. Chattopadhyay, 1994; Eshrat M & Mukhopadhyay, 2006; R. S. Kumar*, et al.*, 2007; G. Muralikrishnan*, et al.*, 2012; Nipum*, et al.*, 2013; Ocimum, 2003). It is important standardize the chemical constituents profile in order to systematize the biological activities of the species and which compounds are responsible for the activity and elucidate the mechanisms of action.

We only evaluate the *in vitro* antiradical activity of *O. sanctum* cell suspension cultures, and it cannot be considered as antioxidant for disease treatments other aspects should be analyzed to fully characterize a compound as an antioxidant; oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC) assay, β-carotene bleaching test, lipid peroxidation, cytotoxicity and others (Cieśla*, et al.*, 2012)

Technologies for RA production have not yet been developed at an industrial level. Metabolic engineering can contribute to the establishment of RA of *O. sanctum* productive cell lines, new approaches to develop strategies for of RA biosynthesis, understanding of the regulation, potentially provided by proteomics and metabolomics technologies and new advances in eliciting technologies (Khojasteh*, et al.*, 2014).

CHAPTER 6

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