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„ Effects of beta-Thujone and *Salvia pomifera* Essential Oil on Cellular Stress Models for Neurodegeneration “

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1. Introduction

1.1. Thujone and Absinthe

In the second half of the nineteenth century, a wormwood (*Artemisia absinthium*) based liqueur, called absinthe was a common drink in the French bohemian society. Famous persons such as Edgar Allen Poe, Henri de Toulouse-Lautrec, Oskar Wilde or Vincent van Gogh praised this liqueur as a source of inspiration (Padosch et al., 2006). During this time, there was a discussion about a new disease. A change in the behaviour of persons diagnosed with an absinthe addiction was detected and a degenerative change in the central nervous system was suspected/ assumed (Magnan, 1864). Medical scientists called this absinthe abuse combined with the changes in mind "absinthism". It was defined as a general poisoning of the organism, a degeneration of the central nervous system, psychosis, convulsions that resemble epilepsy, and hallucination, resulting in a complete mental and physical deterioration (Magnan, 1874).

To clarify what substance of absinthe might be the reason for those changes, scientists focused on the elucidation of the composition of absinthe (Ossipow, 1914). In the early twentieth century it was postulated that the main ingredient in absinthe, the wormwood or more precise the main compounds, (α)- and (β)-thujone, is responsible for the previously described effects. Even though the amount of thujones in absinthe of the nineteenth century is largely unknown, it is estimated that the maximum amount of the thujones in absinthe was 260 mg/l (Bonkovsky et al., 1982). Based on observations by medical doctors and presumably due to politics, many European countries and the USA banned the production and the sale of absinthe (Olsen, 2000). In 1988, the Council of the European Union decided that the flavouring with thujone-containing-plants is allowed, while this is still prohibited in the USA (EEC, 1988). Based on this change, absinthe was again available on the market. Nonetheless, the need of regulation about the amount of wormwood and especially the concentration of the thujones in absinthe is still a matter of discussion (Padosch et al., 2006 and Radulovic et al., 2017).

The regulation of the European Union about the maximum level of thujone-containing-food and beverages is not restricted to absinthe alone, although wormwood was always in the focus of toxicological studies, but other plants like hyssop and sage contain thujone, too (Tisserand and Young, 2014).

1.2. Thujones

The thujones are unpolar, bicyclic monoterpenes which are widely found in the essential oil of common plants like wormwood (*Artemisia* spp.), sage (*Salvia* spp.) and conifers. The oil of the bark of the thuja tree contains a high amount of thujone (up to 60%) which this substance was named after (Tisserand and Young, 2014). In nature, the thujones exists as two different isomers, which is a result of the stereocenters of the bicyclo-hexanone core structure: (-)-α-thujone and (+)-β-thujone (Waidyanatha et al., 2013). The synthesis of thujone and thousands of other monoterpenes in the plant starts with the isoprenoid geranyl- pyrophosphate (GPP). GPP is converted to sabinene via sabinene synthase (SS), which is hydroxylated to sabinol. The next step to sabinone is catalysed by the sabinol dehydrogenase.A double-bond reductase converts sabinone into α-thujone and β-thujone (Figure 1). The content of thujone in the plant´s essential oil is based on the genetic variations.

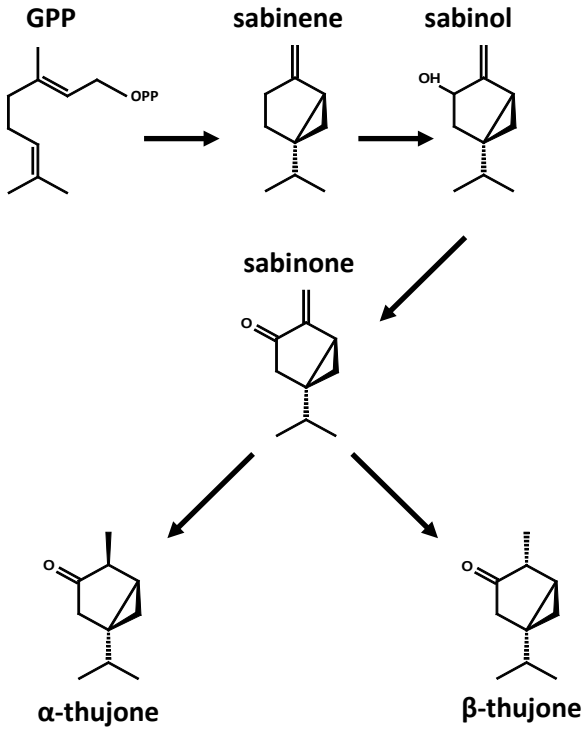


Figure 1: Synthesis of α-thujone and β-thujone in plants (modified from Za´mborine´ Ne´meth and Thi Nguyen, 2020)

Thuja occidentalis	**α-thujone** 48.7-51.5% **β-thujone** 7.9-9.9% **total 56.6-61.4% thujone in oil**
Artemisia absinthium	**α-thujone** 2.3-3.4% **β-thujone** 33.1-59.9% **total 35.4-63.3% thujone in oil**
Salvia officinalis	**α-thujone** 13.1-48.5% **β-thujone** 3.9-19.1% **total 17-67.6% thujone in oil**

Table 1: Different amounts of total thujone and variation of the composition (α-thujone and β-thujone) of the essential oil in different plant species (modified from: Tisserand and Young, 2014)

Therewith, the proportion of α- and β-thujone varies in different plant species (Table 1). As mentioned above, thujones are suspected to be neurotoxic. It is reported that α-thujone has a higher ability in binding to receptors, which are involved to neurotoxic effects (Höld et al., 2000). There are only very few reported cases about persons who ingested thujone containing essential oils. In the so-called "*thuja-case"*, a 50-year-old woman took 40 drops of the essential oil per day for 5 days by accident. After her last intake, she suffered a tonic seizure (approximately intake: 4.55 g of α-thujone and 0.9 g of β-thujone in total) (Millet et al., 2008). Another example is the "*wormwood case*". A 31 year old man had ingested approximately 14 ml of wormwood essential oil when he was hospitalized with tonic and clonic seizures (approximately intake: 0.27 g of α-thujone and 4.2 g of β-thujone) (Weisbord et al., 1997). In the "*sage case*", approximately 7 ml of sage essential oil induced several episodes of convulsions in a 44 year old woman (approximately intake: 2 g of α-thujone and 0.73 g of β-thujone) (Whitling, 1908). These three cases, with equally aged persons, but widely different intake of α- and β-thujone entailed comparable results. It can be assumed that the consequence of the intake should be different if α-thujone would be more neurotoxic than β-thujone, but the case reports do not support this assumption. Therefore, the thujone hypothesis of "absinthism" is questionable:

The concentration of thujone in absinthe was 0.3 g/l in total, which means that a person would need to drink approximately 15 litres of absinthe to reach toxic thujone levels comparable to the patients mentioned above.

1.3. Thujones in *Salvia officinalis*

Carl von Linné first described *Salvia officinalis* in 1753. The conventional name is sage, with the typical representatives common sage or garden sage. *Salvia* is a typical herb in the Mediterranean area. It is cultivated there since several hundreds of years and used for flavoring food and a fragrance in perfumes and other scents (Ghorbani and Esmaeilizadeh, 2017). The name *officinalis* points to its use in the traditional European medicine. An *officina* is the storage room of drug plants and medicine in a traditional monastery. It is also used as folk medicine mostly in form of teas against inflammation of the mucous membrane of the mouth and throat, dyspeptic complaints and increased sweat secretion (Ghorbani and Esmaeilizadeh, 2017, Gali-Muthabsi et al., 2000, Li et al., 2013, Hamidpour et al., 2014). The genus *Salvia* is widely spread but not exclusive around the Mediterranean Sea, with hundreds of species (Ghorbani and Esmaeilizadeh, 2017). This species differs in their composition of the essential oil of the plants. The differences are detectable in the aroma or odor of the plants. Thujone is one of the main contributors for the sage aroma. As shown in table 2, the content of thujones in the essential oil can be higher as in wormwood. Even with this high amount of thujone, *Salvia* is known and accepted as an herbal drug (HMPC, 2011). Interestingly, beside the use mentioned above, sage is also used as a drug with beneficial cognitive and memory enhancing effects. It could be shown that an intraperitoneal or an intracereboventricular administration of an ethanolic sage extract increased the memory retention in rats (Eidi, 2006).

Figure 2: Illustration of *Salvia officinalis* (Köhler, 1897)

S. officinalis	up to 65% thujone in oil
S. pomifera	up to 58-83% thujone in oil
S. lavandulifolia	up to 1.3% thujone in oil

Table 2: Different content of total thujone in different sage species (modified from: Tisserand and Young, 2014)

Furthermore, long time treatment with *S. officinalis* extracts show a beneficial effect in patients with Alzheimer´s disease. Akhondzadeh et al. (2003) could show that a treatment with 60 drops/day of an ethanolic *S. officinalis* extract for a duration of 4 months has beneficial effects in mild to moderate Alzheimer´s disease patients.

1.4. Thujones and the EU law

The current law in the European Union is a result of events occurring in Parisian Cafés in the 1860s. A green coloured liqueur called *absinthe* grew in popularity. One of the main reasons of the great success of absinthe was that it was very affordable. Even after the tax the spirit was cheaper than wine (Padosch et al., 2006). This popularity and the low price was the beginning of the success of and the opposition against this stimulant. Within a few years, the consumption of absinthe grew up to 222 million litres per year (Padosch et al., 2006). At the same time, the number of absinth opponents started to rise. Mostly wine farmers and anti-alcoholic organisations pointed to problems coming with absinthe. Medical doctors at this time, successfully described alcoholism as a severe disease a few years earlier supported the anti-absinth community with theories about illness connected to absinthe consumption like "*craziness*" or to the proposed capacity to cause tuberculosis and epilepsy (Padosch et al., 2006). Also, many crimes and acts of violence were related to the consumption of higher amounts of absinthe. Within a few years absinthe was banned in most of the European countries and in USA as a reaction to those crimes (Padosch et al., 2006). This law was not modified until the 1990ies. In 1988, a new novel of the law for aroma regulation used in groceries (88/388/EWG) was constituted in order to harmonize the market of the European Union (EEC, 1988). Henceforth, plants, part of plants and aroma extracts containing thujone were allowed to be used if the total maximum amount of thujone is not higher than:

0.5 mg/kg in food products and beverages with the exception of:

5 mg/kg in alcoholic beverages with not more than 25% volume of alcohol

10 mg/kg in alcoholic beverages with more than 25% volume of alcohol,

25 mg/kg in foods products containing preparations based on sage and 35 mg/kg in bitters (EEC, 1988).

In 1999, the Council of Europe allocated that the total daily intake (TDI) which is tolerable and not affecting the health status is 10 µg/kg bodyweight (Council of Europe, 1999). This determination was confirmed by the Scientific Committee on Food (SCF) in 2003 (SCF, 2003). This is based on a 14-week-study with rats, in which the rats were treated with 5 mg/kg bodyweight for 6 days/week (NTP, 2011). In a Public statement in 2010 of the Committee on Herbal Medicinal Products (HMPC) on *Salvia* it is written that the daily intake of sage leaf preparations is restricted to 5.0 mg per person for a maximum duration of two weeks (Public statement EMA/HMPC/41843/2009). Later on, in 2012, HPMC admitted having problems of determining the maximum limit of daily intake of thujone, which confirms that a dose-effect comparison is uncertain (Public statement EMA/HMPC/732886/2010).

1.5. Thujones and neurodegeneration

Since the 1940s, thujone is under suspicion to be neurotoxic. Several experiments were done to evaluate the maximum daily intake. Beside the neurotoxic effect in a single dose intake, long-term studies were performed in mice and rats (NTP, 2011). In the beginning of these studies the concentrations of thujone was quite high (2-8 g in dogs) while newer studies are performed with concentrations in a physiologically relevant range that were at most around of a tenth of the concentrations (up to 100 mg/kg in mice and rats) used in the 1940s (Eadie, 2009; NTP, 2011).

In 1939, Sampson and Fernandez could show that the highest non-convulsing intraperitoneal dose of thujone (mixture undefined) is 0.02 ml/kg and a non-lethal but convulsing subcutaneous dose is 36 mg/kg in rats. In mice it could be demonstrated that a subcutaneous dose of thujone (mixture undefined) of 590 mg/kg was convulsive but not lethal (Wenzel and Ross, 1957). On the other side, Millet et al. showed in 1981 that a dose above 0.2 ml/kg thujone is convulsive and lethal in rats.

Nonetheless, intraperitoneal (i.p.) administration of sage oil (thujone content 17-68%) at single doses of 300 mg/kg showed cortical effects, with convulsions at 500 mg/kg and a maximum non-lethal dose at 3.2 g/kg in rats (Millet et all., 2008). In 2000, Höld et al. determined the intraperitoneal LD_{50} of α-thujone in mice at 45 mg/kg.

At the highest dose of 60 mg/kg, the mice underwent tonic convulsion leading to death within one minute with 100% mortality. All those experiments that were conducted within the past 80 years reveal that the lethality (e.g. expressed as LD_{50}) cannot be precisely determined. However, it was clearly proven that thujone has an effect on biological systems. For instance, studies of Bonkovsky detected a porphyrinogenic activity in a primary cell culture of embryonic chick liver cells. This effect mimics an acute porphyria, which has a neurological effect via the high levels of hepatic ALA (5-aminolevulinate) and an overproduction of the heme precursor (Bonkovsky et al., 1992, Bonkovsky and Schady, 1982). All the experiments described above worked with a concentration range from 20 mg/kg to 3.2 g/kg in different model systems. Still, these studies do not confirm whether thujone is the trigger for the neurotoxic effects. They can be mediated by metabolites of thujone which are described in Figure 3. Höld showed that mice treated with 50 mg/kg i.p. of α-thujone metabolize thujone within 5 minutes. They also detected that the concentration of the metabolites is even higher (3-8 times higher) than the thujone itself (Höld et al., 2000). It can be assumed that not the thujone itself evokes the toxic effect within the central nervous system but rather one of thujone metabolites.

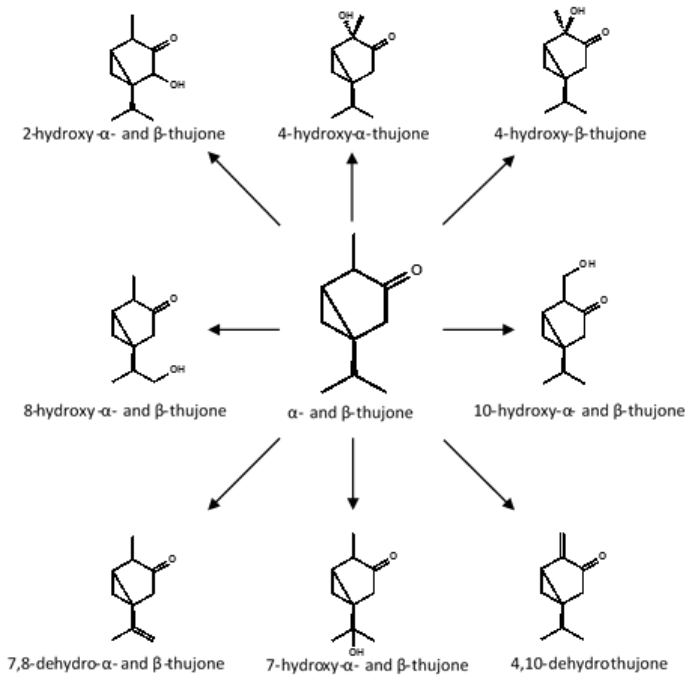


Figure 3: Metabolites of thujone (modified from Höld et al., 2000)

1.5.1. The GABA receptor hypothesis

To elucidate the mechanisms of thujone-induced neurotoxicity, several studies were conducted. In 1975, Del Castillo saw similarities between thujone and tetrahydrocannabinol and assumed that thujone acts via the cannabinoid system (Del Castillo, 1975). Nonetheless, 20 years later, Meschler and Howlett demonstrated a very low affinity of thujone to the cannabinoid receptors CB_1 and CB_2. They could show that thujone is unable to evoke a cannabimimetic response (Meschler & Howlett, 1999). A new hypothesis raised in 2001 when Höld presented data about the ability of thujone to bind to the γ-aminobutyric acid (GABA) type A receptor. In a competitive receptor binding assay with the GABA receptor antagonist [^{3}H]EBOB assay, α-thujone exhibited an IC_{50} of 13±4 µM and β-thujone an IC_{50} of 29±8 µM. For comparison, picrotoxin as a potent $GABA_A$ receptor antagonist has an IC_{50} of 0.6±0.1 µM. (Höld et al., 2001). Although the $GABA_A$ receptor affinity of thujone is comparably low, this study depicted for the first time that this compound is able to modulate the GABA system. GABA is the neurotransmitter of the widespread and most important inhibitory GABAergic system. The ionotropic $GABA_A$ receptor is a pentameric transmembrane chloride channel which is widely distributed on a variety of cell types in the central nervous system. The influx of chloride ions has a hyperpolarizing and therewith inhibitory effect on the postsynaptic neuron, which is crucial for the normal function of the brain. Therefore, the $GABA_A$ receptor has many binding sites for biological and pharmacological modulatory substances. Some of them, such as benzodiazepine or barbiturates, cause a higher opening frequency or a prolonged influx of chloride ions. In epilepsy and epilepsy research, the GABAergic system plays a prominent role. For example, bicuculline as an antagonist of the $GABA_A$ receptor is used as a model drug for investigating epileptiform activity (Albertson et al., 2013).

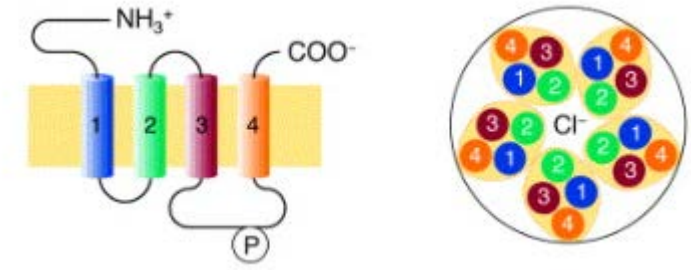


Figure 4: Architecture of the ionotropic $GABA_A$ receptor and its four transmembrane domains (Bormann, 2000)

1.5.2. Thujones and the GABAergic system

Like many other natural products, thujone is influencing the ionotropic $GABA_A$ receptor (Johnston et al., 2006). It is a negative allosteric modulator of this receptor, so the $GABA_A$ receptor seems to be the target of the postulated neurotoxicity of thujone. This was demonstrated in four individual experiments:

(i) the toxic effect of thujone is inhibited by $GABA_A$ blockers like benzodiazepine, barbiturate or picrotoxinin,

(ii) a strain of *Drosophila* flies, resistant to chloride channels blocker is resistant to the effects of thujone,

(iii) in a competitive $GABA_A$ receptor binding assay thujone inhibits the binding and,

(iv) thujone reversibly inhibited a $GABA_A$ receptor signal in an electrophysiological experimental set-up (Höld et al., 2000).

Following these findings, research focused on the influence of thujone on the $GABA_A$ receptor. To date, the site of action of thujone is still not clarified. As found in investigations on other lipophilic substances, the surrounding membrane of the receptors may be the target of thujones. But dihydroumbellulone, a similar lipophilic substance, does not alter the GABAergic miniature inhibitory postsynaptic currents (Szczot et al., 2012).

It could be shown that the action of thujone on $GABA_A$ receptor differs on the architecture and the composition of the receptor subunits, which points to the existence of a specific binding site. The composition of the subunits is relevant to the form of inhibition, because it determines whether a tonic or phase inhibition occurs. Czyzewska and Mozrzymas showed that the influence of α-thujone has a major impact on receptors involved in tonic currents (Czyzewska and Mozrzymas, 2013). In a study using neonatal chickens, Rivera could find a correlation of an anxiogenic-like effect and a modulation of the $GABA_A$ receptor when thujone was intracerebroventricularly administrated (Rivera et al., 2014).

It is known that there are receptors within the brain which are structure-related to the GABA receptors (Albertson et al., 2013). It can be assumed that if thujone acts via the GABA receptor system, a modulation of those related receptors may be possible.

In this regard, a study on $GABA_A$ receptors and glycine receptors could show that thujone has an effect on glycine receptors, too, but not at such a high extend (Hall et al., 2004). Another closely related receptor, the serotonin receptor, can also be modulated by thujone. The response of the receptor is concentration-dependently reduced when thujone is administrated (Deiml et al., 2004). Contrarily, when human α_7-nACH (nicotinic acetylcholine) receptor cRNA was injected in *Xenopus* oocytes and a functional acetylcholine receptor subunit is expressed, thujone is able to inhibit acetylcholine induced currents of the α_7-subunit of the receptor. The inhibitory effects of thujone were recorded by electrophysiology (Sultan et al., 2017). All those findings substantiate the suspicion that thujone acts via the $GABA_A$ receptor. Contrarily, thujone is also acting on a whole set of neuronal receptors, even if they are all closely related, the neurological effect is not related to only one receptor. Furthermore, there is a difference in $GABA_A$ receptor binding between the α-and the β-form of thujone. Höld demonstrated that α-thujone is 2.3 times more active than β-thujone in binding on the $GABA_A$ receptor. Furthermore, as mentioned above, the 7-hydroxy-α-thujone levels in the brain are higher than the α-thujone itself, even if it is not as toxic as α-thujone (Höld et al., 2000). So far, no receptor binding studies for 7-hydroxy-α-thujone or other thujone metabolites were conducted.

1.6. Investigating thujones in neural cell culture systems

In this thesis, I focused on working with cell culture models instead of animal experiments. The major advantage of using cell cultures is the possibility to mimic the conditions in the living body but being an *in vivo* replacement model at the same time, rising less ethical concerns. Another important advantage is that there is no biological modification of the testing substances, which makes it possible to focus on the effects of the substance not on the metabolites. Additionally, toxins and drugs have immediate access to the cells when administered.

1.6.1. Neural cell cultures

Different kinds of cell culture models are common for investigating the effects of potential neurotoxic or neuroprotective substances. In general, they differ in their source and in the grade of sharing *in vivo*-like properties and of standardisation. As shown in Figure 5, it can be stated that the higher the standardisation is, the lower is the comparability to the in vivo situation.

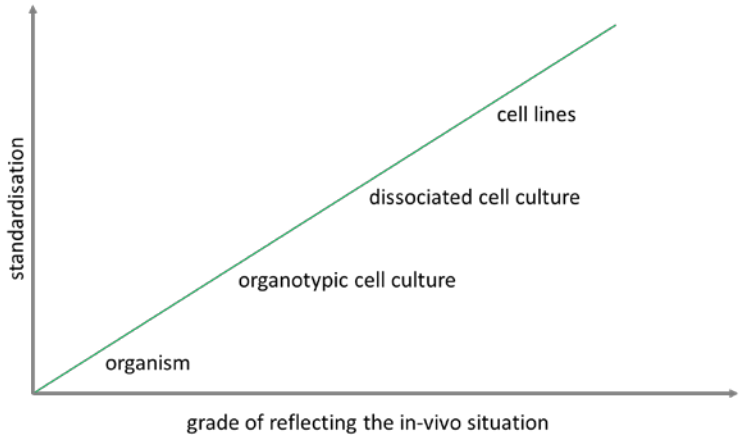


Figure 5: Relationship of in-vivo like situation and standardisation in different types of cell culture

The primary mesencephalic cell culture is directly prepared from embryonic brains. Because of its origin, mesencephalic cell culture consists of different cell types. These cell types build up a network, which closely resembles the brain network *in vivo*. This cell culture type is cultivated as a monolayer, providing a two-dimensional culture system. These properties of the primary mesencephalic cell culture make them suitable for a wide field of applications. The broad spectrum of applicable screening assays for evaluating the cell culture and the single cell fitness is a benefit of this cell culture model. Not to the latest, these cultures can be observed easily by microscopy.

1.6.2. Stressors in mesencephalic cell culture

In an optimal in-vivo situation, cells have unlimited access to nutrition they need, and the environmental conditions are perfectly balanced. But during stress situations this balance is disturbed. To mimic pathologic conditions in cell culture, three model compounds for different pathologic settings were used to maximize the effect of the administered sage-derived substances.

Stress in cell culture can be performed in different ways. Reduction of the levels of essential nutrients like glutamine or glucose causes starvation and leads to biochemical change of the cell metabolism. Another way to manipulate the biochemistry of the cell is the administration of drugs. The mitochondrion is a common target to induce stress in a more or less specific way. Therefore, the mitochondrial respiratory chain is a target which is widely used. Another approach to evoke stress in culture is via overload cells with compounds in non-physiological concentrations. For instance, excitatory neurotransmitters can be used to elicit stress in neurons and surrounding cells.

1.6.2.1. Glutamate

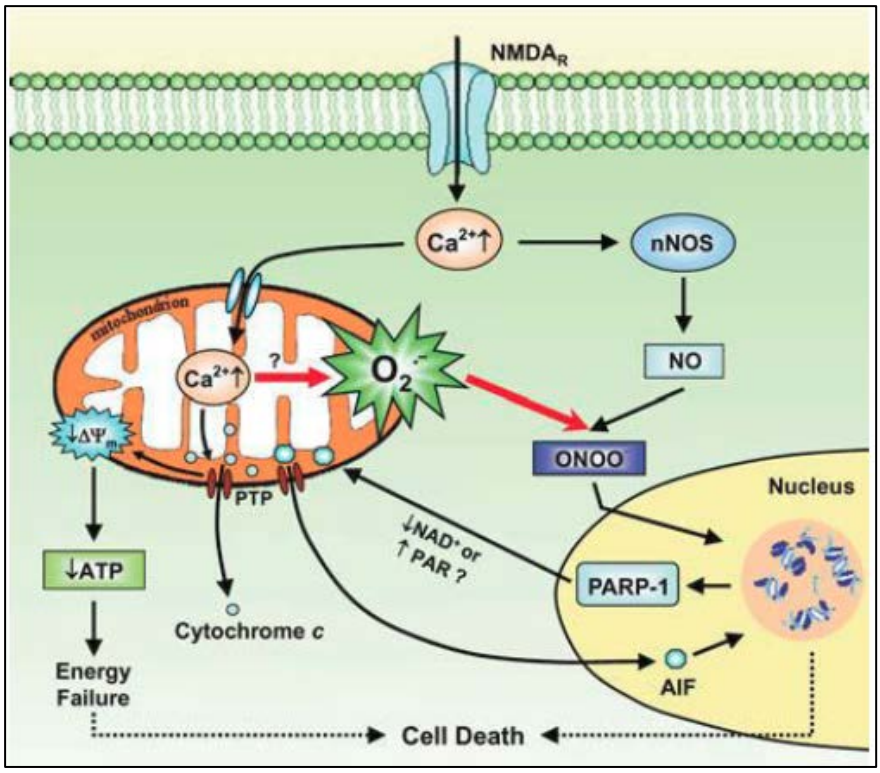


Figure 6: Mechanism of neuronal death induced by excitotoxicity
AIF: apoptosis inducing factor, **PAR**: poly (ADP-ribose), **PARP-1**: poly (ADP-ribose) polymerase 1, **PTP**: permeability transitions pore, **$\Delta\psi_m$**: membrane potential (Duan et al., 2007)

Glutamate is beside its use as a proteinogenic amino acid one of the most important neurotransmitters in the mammalian central nervous system. It acts on two major groups of receptors, the ionotropic and the metabotropic glutamate receptors. The ionotropic glutamate receptors can be divided in different subtypes including AMPA (2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate), NMDA (methyl-D-aspartate) receptor and kainate receptors.

The glutamatergic system and the influx of cations in the cell play an important role in neuronal signal transduction and the regulation of the brain function (Swanson et al., 2005). Beside its important role of the physiological function like learning and memory, synaptic plasticity and synaptic consolidation, excess concentrations of glutamate can also lead to neurodegeneration (Moldzio et al., 2006). An over-activation of AMPA and NMDA receptors can trigger pathologic pathways in the cell. The activation of the AMPA receptor increases the intracellular levels of positive ions like sodium and potassium. Depolarisation of the postsynaptic neuron by glutamate leads to the abolishment of the magnesium block in the NMDA receptor. This coincidental activation of the NMDA receptor increases the intracellular calcium levels. While calcium ions are important intracellular messengers, excessive levels of intracellular calcium cause severe changes within the cell, such as activation of phospholipases, calcium dependent proteases and an impairment of the mitochondria. Consecutively, nitric oxide, superoxide anion and hydrogen peroxide can increase to a harmful level. Since reactive oxygen species (ROS) are produced through the impairment of the mitochondria, glutamate overload can lead to cell death (Duan et al, 2007) (Fig.6). This mitochondrial stress is just one characteristic of cellular changes, when high levels of calcium lead to a pathologic mechanism called glutamate excitotoxicity. Other changes are activation of caspase-3, an enzyme involved in apoptosis, and in the regulation of proteins which are involved in the replication of mitochondrial DNA (Brecht et al., 2001). Therefore, providing the culture medium with high concentrations of glutamate is a widely used model for excitotoxicity.

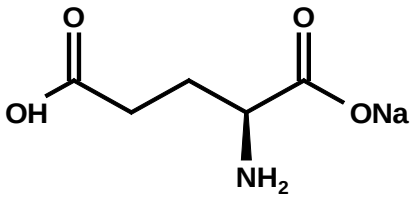


Figure 7: Chemical structure of glutamate

1.6.2.2. Rotenone

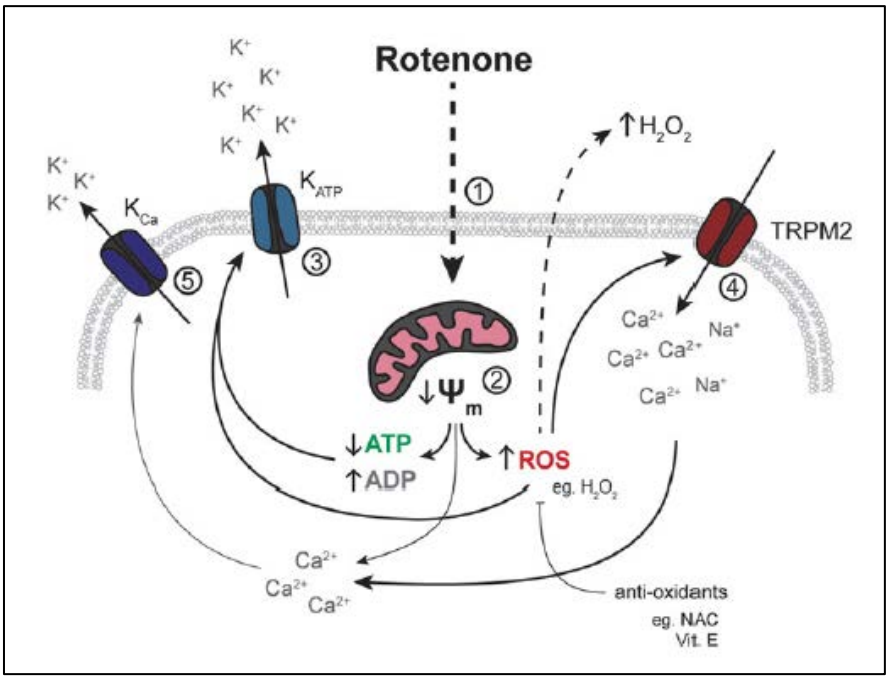


Figure 8: Schematic cellular consequences of rotenone treatment (Yee et al., 2017)

K_{Ca}: Ca^{2+} activated potassium channel, **K_{ATP}:** ATP sensitive potassium channel, **TRPM2:** transient receptor potential ion channels, **Ψ_m:** mitochondrial membrane potential, **ATP:** adenosine triphosphate, **ADP:** adenosine diphosphate, **ROS:** reactive oxygen species, **NAC**: N-acetylcysteine, **Vit.E.:** vitamin E

In the past, native Peruvians used plant extracts for catching fish. In south East Asia, extracts of plants were commonly used as an insecticide since hundreds of years. Botanists showed that all those plants are part of the legume family (species like *Mundulea*, *Derris* or *Thephrosia*) and a naturally occurring source of rotenone. In the nineteenth century, chemists could isolate the pure crystal form of rotenone. Since the 30s of the 20th century, the lipophilic compound rotenone is an intensively used compound in fishery management and in the use as an insecticide (Isman, 2006 and Ling, 2003). Rotenone has a highly differential toxicity in different kind of species, and the way of intake is important for the toxicity. In fishes, the LD_{50} of rotenone is 1-5 mg/L water (Isman, 2006). In mammals, the oral LD_{50} is a 100fold higher with 300-500 mg/kg body weight (b.w.). This remarkable higher toxicity in fishes based on the intake through the gills and the direct exposure of the body to the toxin. The way of exposure is also important in mammals: In mice, the oral LD_{50} is 350 mg/kg b.w. while the estimated intraperitoneal and intravenous LD_{50} is 2.8 mg/kg b.w. (Gupta and Mitatovic, 2014).

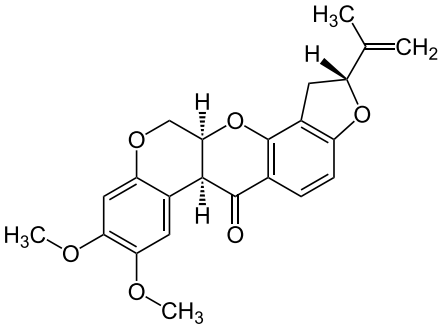


Figure 9: Chemical structure of rotenone

The general toxic characteristic is based on the fundamental mode of action of rotenone. In mitochondria, ADP is converted to ATP through the mitochondrial oxidative phosphorylation system. The components of this system are NADH-coenzyme Q oxidoreductase (complex I), Succinate-Q oxidoreductase (complex II), Q-cytochrome c oxidoreductase (complex III), Cytochrome c oxidase (complex IV), ATP synthase (complex V). The complex I possess two binding sites to rotenone, and this pesticide is thereby inhibiting the start of the respiratory chain. This inhibition leads to a decrease in the ATP level within the cell influencing all energy consuming cell processes (Heinz et al., 2017, Yee et al., 2017). Beside this mode of action, rotenone influences the leakage of electrons at the complex I. This leads to a higher production of reactive oxygen species (ROS) like hydrogen peroxide and superoxide (Sherer et al., 2001). Beside this, in neuronal cells, inflammation is an important mechanism to response to the effect of rotenone. Liang et al. (2017) could show that pro-inflammatory interleukins are upregulated if cells are exposed to rotenone. Gao et al. (2019) supported these findings by demonstrating that rotenone is able to activate the microglia, which likewise leads to a release of cytotoxic pro-inflammatory cytokines. Rotenone induced neuroinflammation ends up in the activation of intracellular death signaling pathways (Hirsch and Hunot, 2009). Another important regulatory mechanism beside inflammation is apoptosis which is induced by rotenone. In our neurochemistry group at the University of Veterinary Medicine, Vienna, we could show that the exposure of dopaminergic neurons in a primary cell culture to rotenone lead to a lower number of tyrosine hydroxylase positive cells and apoptotic changes (Moldzio et al., 2010).

1.6.2.3. Glucose deprivation

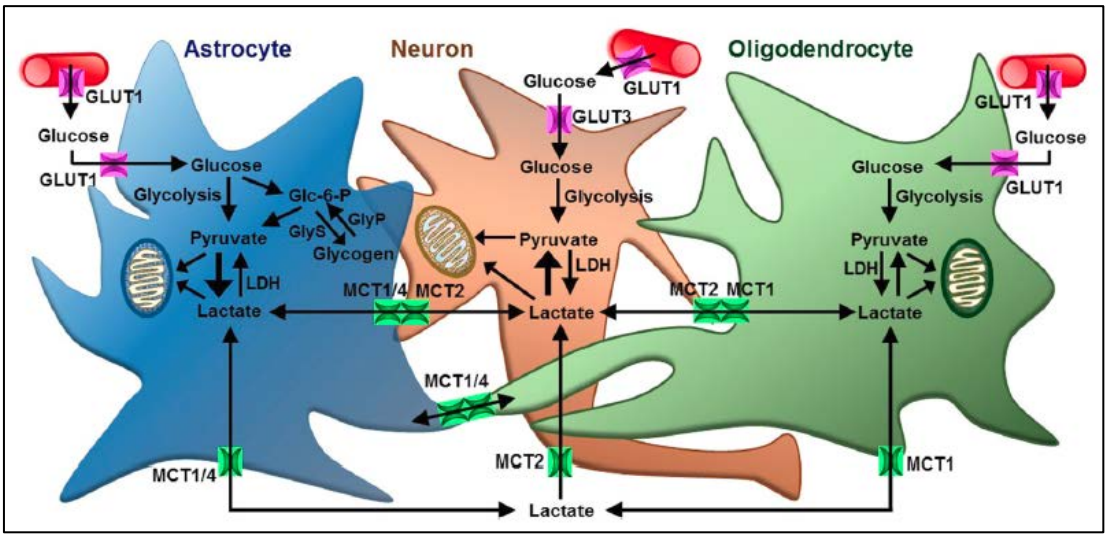


Figure 10: Use of glucose under physiological conditions (Jha and Morrison, 2018)

GLUTs: glucose transporters, **MCTs:** monocarboxylate transporters, **Glc-6-P:** glucose-6-phosphate, **GlyS:** glycogen synthase, **GlyP:** glycogen phosphorylase, **LDH:** lactate dehydrogenase

The physiological function of the cells in the brain depends on the adequate access to and the subtle regulation of nutrition. Sokoloff showed in 1960 that in the brain, the main source of energy is glucose (Sokoloff, 1960). Later studies suggested that glucose is not mostly consumed by neurons. Astrocytes and oligodendrocytes mostly incorporate the glucose through the blood-brain-barrier and release metabolites of glucose, like lactate to neurons depending on the energy requirements (Conquet et al., 2010). Beside the important role of astrocytes and oligodendrocytes, Simpson and colleagues could demonstrate that neurons express a specific transporter for glucose (GLUT3), which allows glucose to directly enter the cell (Simpson et al., 2007). The distribution of energy in form of glucose is maintained by the network of neurons glial cells connected with gap junctions (Froes et al., 1999). The physiological function of the brain is provided for when an adequate ATP level is generated. This is ensured via the sufficient access to glucose and the potential conversion to lactate. The homeostasis of the glucose level in the brain but also in the cell culture is crucial as shown in figure 10.

2. Aims of the study

This study is based on preliminary data from a bachelor thesis done in the neurochemistry group of the University of Veterinary Medicine, Vienna (Schefzig, 2017). α -thujone and some other sage-derived components were tested to investigate neurotoxic effects (Supplement 4). Based on the previous data in this study, the hypothesis is that thujones are not toxic in our cell cultures, even if co-cultivated with stressors. First, it should be elucidated if α-thujone, β-thujone and *Salvia pomifera* essential oil has a neurodegenerative effect on a primary cell culture model. Furthermore, I tested if this effect elicited by the thujones or the essential oil is enhanced in cell culture with cellular stress models for neurodegeneration. β-Thujone is commercially not available and has to be synthesized or isolated. Therefore, the following four questions should be answered:

1) Is it possible to synthesise β-thujone from α-thujone?
2) Do β-thujone and essential sage oil exert a toxic effect in the whole cell culture or to dopaminergic neurons in particular?
3) Could stress in the cell culture be elicited by the following different model?
   - High levels of glutamate
   - Complex I inhibition by rotenone
   - Low levels of glucose
4) Do stress in the cell culture enhance the hypothesized effect of α-thujone, β-thujone or essential sage oil?

Answering these questions may help for understanding possible damaging effects of Salvia derived compounds in neural cells. Further, this study may contribute to the discussion of thujones hazardousness in food and absinthe.

3. Materials and methods

3.1. Materials

3.1.1. Primary mesencephalic cell culture

Pregnant OF1/SPF mice, Himberg, Austria

3.1.2. Chemicals

2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1*H*-benzimidazole trihydrochloride (Hoechst 33342), Sigma-Aldrich, Germany

3,3´-diaminobenzidine (DAB), Sigma-Aldrich, Germany

5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1), Sigma-Aldrich, Germany

α- & β-thujone, Sigma-Aldrich, Germany

α-thujone, Sigma-Aldrich, Germany

Anti-tyrosine hydroxylase mouse antibody, Szabo, Austria

B27 supplement minus AO, Invitrogen, UK

Colourless Dulbecco´s modified Eagle´s medium (DMEM), Sigma-Aldrich, Germany

D-Glucose, Sigma-Aldrich, Germany

Dimethyl sulfoxide (DMSO), Sigma-Aldrich, Germany

DNase, Roche Germany

Dulbecco´s modified Eagle´s medium (DMEM), Sigma-Aldrich, Germany

Dulbecco´s modified phosphate buffered saline (D-PBS), Invitrogen, UK

Ethanol (96%), Sigma-Aldrich, Germany

Foetal bovine serum, Sigma-Aldrich, Germany

Glutamate, Sigma-Aldrich, Germany

Hexane Sigma-Aldrich, Germany

Histochoice, Sigma-Aldrich, Germany

Horse serum, Sigma-Aldrich, Germany

Hydrogen peroxide 30%, Sigma-Aldrich, Germany

Kaisers glycerol gelatine, Sigma-Aldrich, Germany

LDH, Roche, Germany

L-Glutamine, Sigma-Aldrich, Germany

N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), Sigma-Aldrich, Germany

Na_2SO_4, Sigma-Aldrich, Germany

Peroxidase mouse IgG ABC-Kit Vectastain, Vector Laboratories, USA

Poly-D- Lysine, Sigma-Aldrich, Germany

Propidium iodide, Sigma-Aldrich, Germany

Resazurin sodium salt, Sigma-Aldrich, Germany

Rotenone, Sigma-Aldrich, Germany

Sodium bisulfite, Sigma-Aldrich, Germany

Sodium carbonate, Sigma-Aldrich, Germany

Sodium hydroxide, Sigma-Aldrich, Germany

Sodium pyruvate, Sigma-Aldrich, Germany

Triton X, Sigma-Aldrich, Germany

Trypan blue, Sigma-Aldrich, Germany

β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate, Sigma-Aldrich, Germany

Salvia pomifera essential oil, Institute for Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine, Austria

3.2. Methods

3.2.1. Synthesis of β–Thujone

As mentioned above, different plant species differ in the relation of α-thujone and β-thujone in the essential oil. To distinguish the effects of either of the thujones, it was necessary to synthesise the β form, since it was not commercially available. The synthesis was performed according to the protocol of French with minor modifications (French, 2011).

To 0.5 ml of a mixture of α- and β-thujone, 0.36 ml of an ethanolic NaOH solution (5% NaOH in 95% ethanol) was added. The mixture was shaken for 30 minutes at room temperature. 0.8 ml of a freshly prepared saturated $NaHSO_3$ solution was added afterwards. The mixture was allowed to stand at room temperature until the next day. Because of the small volumes, the following filtration was adapted to a centrifugation (1 min at 12,000 RCF) to collect the precipitated bisulfite product. To remove eventually clinging oil, the precipitate was washed twice with ice cold 95% ethanol. 3.6 ml of a 10% aqueous Na_2CO_3 was added to separate free ketone as oil. Therefore, the solution was stirred for 10 minutes at room temperature. To extract the oil from the aqueous phase, 1 ml of hexane was added three times to the solution. To remove the remaining water, the organic phase was dried over Na_2SO_4 overnight. A separation of β-thujone was performed with a rotary evaporation system (showed in Figure 11). The efficiency of the synthesis and the purity of the end-product were evaluated with a HP6890 gas chromatograph coupled with a HP5972 mass spectrometer (GC/MS). For the analysis, the thujone mixture, the oil and the end-product of the synthesis were diluted 1:10 with hexane. The gas chromatography, which is the separating step, was performed with 5 µl sample, an injector temperature of 250 °C and a 80 °C-105 °C temperature program with a heating speed of 6 °C per minute. Subsequently the main components where identified with a quadrupole mass spectrometer.

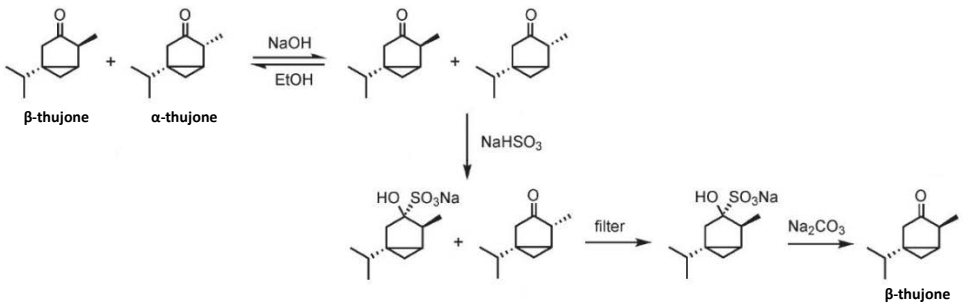


Figure 11: Reaction equation of ß-thujone synthesis (modified from French, 2011)

3.2.2. Primary mesencephalic cell culture

Experiments were discussed and approved by the institutional ethics and animal welfare committee in accordance with GSP guidelines and national legislation: Pregnant OF1/SPF mice were cared and handled in accordance with the guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals. The protocol is slightly modified of Radad et al. (2004). Mice of the Oncins France 1 strain (specific-pathogen-free) (OF1/SPF) at the gestation day 14 were sacrificed with CO_2 followed by a cervical dislocation. The abdomen was disinfected with a solution of 70% ethanol in water. The abdomen was opened and the embryos containing uterus horns transferred into a petri dish filled with D-PBS. All further steps of the preparation were performed under sterile condition under a laminar flow (biosafety cabinet). Furthermore, to avoid contaminations, each step of the preparation was performed in a new petri dish filled with sterile fresh D-PBS. The tissue of the uterus was opened and separated from the amnion coated embryos. Subsequently, the placenta and the amnion were removed, and the embryos were cut in half. The further steps were performed with a binocular microscope placed in the laminar flow. The brain was cut out and the mesencephalon separated from the prosencephalon and rhombencephalon. The meninges were cautiously removed from the mesencephalon and the obtained brain tissue were dissected into small pieces and transferred in a centrifuge tube. The tissue was treated with trypsin (0.1% in D-PBS, 2 ml) and DNase (0.02% in D-PBS, 2 ml) for 7 minutes at 37 °C. After the incubation time, the supernatant was discarded, 3 ml of basic medium and 60 µl of DNase were added. To obtain the cell suspension, the tissue was carefully triturated using a fire-polished Pasteur pipette.

After 10 minutes at room temperature the remaining tissue separated from the single cell suspension. 3 ml of the supernatant was transferred into an Erlenmeyer flask containing 5 ml of pre-warmed basic medium. With the remaining tissue, the trituration and sedimentation step was done two more times. To determine the cell density, the cell suspension was diluted 1:5 with trypan blue and counted using a Neubauer counting chamber. The cell suspension was diluted with basic medium to a final concentration of 750,000 cells/ml and seeded onto 96- or 48- well cell culture plates (pre-coated with poly-D-lysine (50 µg/ml in D-PBS, overnight) using 150 µl or 340 µl. The cell cultures were incubated at 37 °C and 5% CO_2. In Table 3, the composition of the medium is listed, and the medium changing timetable is shown in Table 4.

basic medium		**N4 medium**	
DMEM with phenol red	50 ml	DMEM w/o phenol red	50 ml
fetal bovine serum	5 ml	B-27 supplement (50x)	1 ml
L-glutamine (200 mM)	1 ml	L-glutamine (200 mM)	1 ml
HEPES-buffer (1 M)	0.5 ml	HEPES-buffer (1 M)	0.5 ml
glucose solution (20%)	0.37 ml	glucose solution (20%)	0.37 ml

Table 3: Primary mesencephalic cell culture media composition

day in vitro (DIV)	**used medium**	**procedure**
0	BM	**preparation**
1	BM	
3	BM	
5	BM&N4 (50:50)	
6	N4	
8	N4	
10	N4	
12	N4	**treatment**
14	-	**analysis**

Table 4: Experimental timeline

3.2.2.1. Treatment of the primary mesencephalic cell culture

At DIV (day *in vitro*) 12, the maintaining medium was removed and the cells were treated with different concentrations of α-thujone, β-thujone and the essential oil of sage. As the stocks of the thujones and the essential oil were dissolved in dimethyl sulfoxide (DMSO), wells used as references contained the same amount of the vehicle DMSO. To evaluate the neurotoxicity of the thujones and the essential oil alone cells were treated with different final concentrations (f.c.) of the substance (100, 10, 1, 0.1, 0.01 µM). Furthermore, the cells were stressed with different model substances, like rotenone and glutamate or with a low concentration of glucose in the medium. Rotenone was used in a f.c. of 80 nM respectively 10 nM (for the staining of the dopaminergic neurons). The final concentration of the treatment with glutamate was 30 mM. To provoke a stress reaction mediated by a low level of glucose, the concentration was lowered to 25.5 mM f.c.. The co-administration of thujone and the essential oil of sage at a high concentration of 100 µM with the stressors was meant to show if the effect of thujone is even higher in the stress model. Treated cells were incubated at 37 °C and 5% CO_2 for 48 h.

3.2.3. Tyrosin hydroxylase staining

In the central nervous system tyrosin hydroxylase (TH) is mainly expressed in dopaminergic neurons. The enzyme TH catalyses the conversion of the amino acid tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA), which is a precursor of the catechol amines, like dopamine. Therefore, TH can be used to specifically determine the number of dopaminergic neurons, while other types of neurons will stay unstained. The TH-staining was performed in the 48-well plate because of the larger surface of each well (1 cm^2, versus 0.32 cm^2 of a 96 well). After the treatment on DIV (day *in vitro*) 14 before removing the whole medium 100 µl of the medium was transferred in a 96-well plate for the measurement of the lactate dehydrogenase levels. Cells were fixed with the tissue-fixative-solution Histochoice for 15 min at 4 °C.

The volume which was used for each step during the staining process was 200 µl per well.

Afterwards the fixative was removed. The cells were washed with D-PBS and, to permeabilize the cell membrane, a Triton-X solution (0.4% in D-PBS) was applied for 30 min at room temperature. The cells were washed three times with D-PBS and for blocking the unspecific binding sides of the tissue a horse serum solution (5% in D-PBS) was used. After the incubation of 1.5 h the anti-tyrosine hydroxylase antibody (diluted 1:1000 in horse serum solution) was added. To make sure that the first antibody binds to the epitope, the incubation took place overnight at 4 °C. The following day the cells were washed 3 times with D-PBS to remove the unbound primary antibody. Following, the secondary biotinylated antibody (horse anti-mouse, diluted 1:200 in D-PBS) was incubated for 1.5 h at room temperature. 30 minutes before the incubation time ended, an equal mixture of an avidin and biotin solution (ABC peroxidase staining kit) was prepared. Three washing steps at the end of the incubation time removed unbound secondary antibodies. The avidin-biotin mixture was diluted 1:500 with D-PBS and applied for 1.5 h at room temperature. After this incubation, cells were again washed three times with D-PBS. A 1.4 mM solution of 3,3'-diaminobenzidine (DAB) was prepared and mixed with a 3.3 mM hydrogen peroxide solution (1:0.025) and applied to the cells. The staining procedure was observed under optical control and stopped by removing the DAB-solution followed by a washing step with D-PBS. To preserve the staining, the culture was mounted with Kaiser´s glycerol gelatine.

3.2.4. Measurement of LDH activity

The disintegration of the cytoplasm membrane of cells is a morphological marker for cell death. This process leads to a release of the components of the cytoplasm like lactate dehydrogenase (LDH), which is an enzyme as stabile in the extracellular space as intracellularly. The measurement is based on the oxidation of β-nicotinamide adenine dinucleotide (NADH) via lactate dehydrogenase. Therefore, in a LDH-buffer (D-PBS containing 0.02 M HEPES), sodium pyruvate (0.6 mM) and NADH (0.2 mM) was solved.

As mentioned above the measurement of the lactate dehydrogenase activity started with the transfer of 100 µl medium into a 96-well plate after treatment. Before the measurement, a few wells were filled with 100 µl D-PBS to establish a starting point of the reaction.

In each well of the 96-well plate a volume of 170 µl of the LDH-buffer was added. After one minute of incubation the measurement was performed with a plate reader at a wavelength of 340 nm. The measurement was repeated after another minute and after two minutes. The mean of the slopes between t_1-t_2 and t_2-t_3 was calculated to evaluate the activity.

3.2.5. Resazurin cell viability assay

To determine the overall cell metabolism, a resazurin-resorufin assay was performed. The non-toxic compound resazurin (7-hydroxy-10-oxido-phenoxacin-10-ium-3-on) is

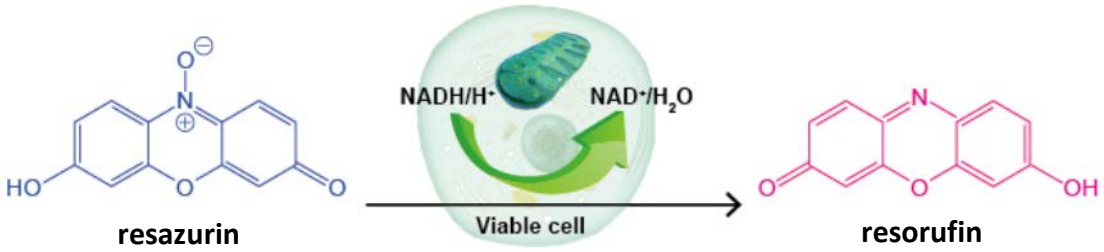


Figure 12: Resazurin cell viability assay overview, reduction of resazurin to resorufin (adapted from: http://file.biotool.com/pic_img/Vita-Blue-Cell-Viability-Reagent-2015042001.png)

able to cross the cell membrane. In viable mitochondria, the bluish coloured and non-fluorescent resazurin is reduced to the pink-coloured fluorescent resorufin (Fig: 12). The rate of conversion of resazurin to resorufin is directly proportional to the mitochondrial activity. Therefore, the assay can be used as an adequate indicator of an eventual metabolic impairment as a result of the toxicity of the tested substances.

A 10-fold stock solution of resazurin (500 µM in D-PBS) was used for the measurement. 15 µl of the stock solution was added to each well of a 96-well plate (final concentration of resazurin: 50 µM). Immediately after adding the resazurin solution the colorimetric absorbance was measured at 570 nm (resorufin) and at 600 nm (resazurin) as a reference wavelength (t_0).

After an incubation of two hours at 37 °C and 5% CO_2, a second measurement was done (t_1). The decrease of resazurin level compared to the increase of resorufin level during the incubation time is reflecting the mitochondrial activity of the cells.

3.2.6. Propidium iodide and Hoechst 33342 fluorescence staining

3,8-diamino-5-(3-(dieethylammonio)propyl)-6-phenyl-phenanthridinium-diiodide (propidium iodide, PI (Fig.: 13)) is a stabile fluorescence dye. The excitation wavelength is in the blue-greenish (493 nm) area of the light spectrum while the emission wavelength is at 630 nm (red). The PI staining is used for the visualisation of degenerated or damaged cells. PI has the ability to penetrate the cell membrane of those cells, because of the ongoing disintegration processes. Within the cell, propidium iodide produce the full fluorescence characteristics if it´s bound to DNA or RNA.

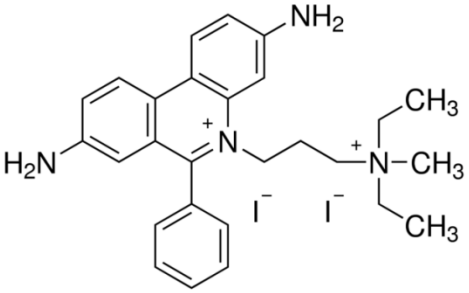


Figure 13: Propidium iodide molecule

Hoechst 33342 (2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloride) (Fig.: 14) on the other hand has the ability to penetrate both, the cell membrane of intact living cells and the cell membrane of damaged cells. Therefore, Hoechst 33342 is widely used in fluorescence microscopy as a nuclear counterstaining dye. However, in immunohistochemistry and in fluorescence measurements it can be used to determine the overall cell number and as a normalisation marker. Hoechst 33342 intercalates in dsDNA and in its bounded state it fluoresces with an excitation wavelength of 346 nm (purple) and an emission of dark blue light with a wavelength of 460 nm. Therefore, PI staining combined with Hoechst 33342 staining is a good parameter to determine the overall toxicity of compounds in cell culture.

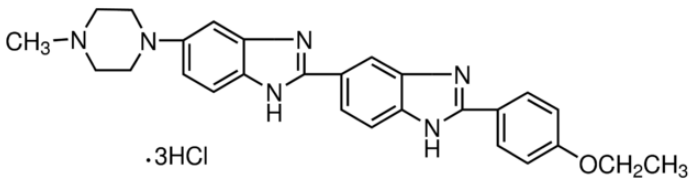


Figure 14: Hoechst 33342 molecule

After the incubation time the supernatant was removed. A solution of pre-warmed DMEM without phenol red containing 5 µg/ml Hoechst 33342 and 600 ng/ml PI was added and incubated for 10 minutes at 37 °C. Before the measurement with a fluorescence plate reader, cells were washed once with D-PBS and each well filled with 100 µl D-PBS. The intercalation and the fluorescence is quite stable.

Therefore, there is no need for a defined sequence of measurement, but for the comparability the Hoechst 33342 measurement was always done before the PI measurement.

3.2.7. Mitochondrial integrity detection (JC-1)

The status of the mitochondrial membrane potential is a potent marker for cellular health. Changes in the mitochondrial membrane potential are an initial sign for early

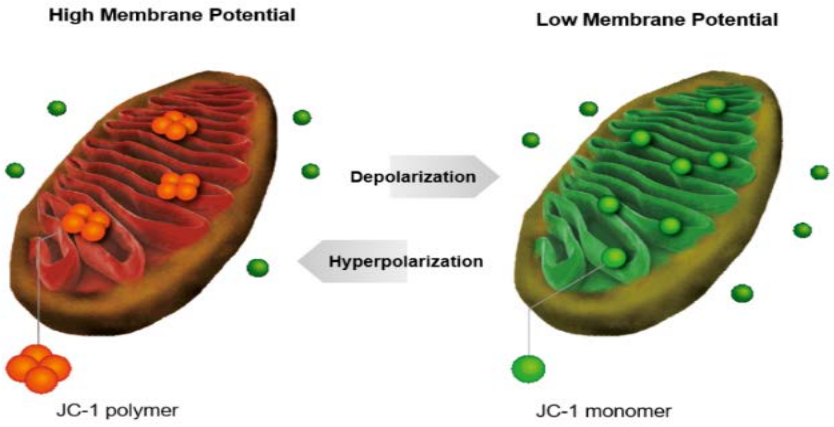


Figure 15: Change in fluorescence of JC-1 dependent on mitochondrial membrane potential (adapted from: https://www.dojindo.eu.com/JC-1-MitoMP-Detection-Kit.aspx

stages of apoptosis and other ongoing mechanisms of cell death. Those changes are a result of an uncontrolled influx of ions in the mitochondria, which has its origin in the disruption of the mitochondrial membrane or in a decoupling process of the respiratory chain. This influx of ions is often connected with oxidative stress. Therefore, the JC-1 dye (Fig: 15) can be used to evaluate a broad spectrum of influence, which all condensate in the change of mitochondrial membrane potential changes. For the measurement of JC-1, a stock solution of 10 µg/ml in DMSO was prepared. 100 µl of the stock solution was mixed with 900 µl pre-warmed DMEM without phenol red and mixed very well. In each well of a 96 well plate 15 µl of the JC-1 solution were added.

After the incubation time of 30 minutes at 37 °C the fluorescence was measured. Because of the higher sensitivity for bleaching the green monomer was measured first with an excitation wavelength at 540 nm and an emission wavelength at 570 nm. Afterwards the red fluorescence polymer was measured at 590 nm excitation wavelength and an emission wavelength at 610 nm with a fluorescence plate reader.

3.3. Statistics

Data were obtained from independent experiments in different quantities, mentioned in each figure description. All experiments were performed in duplicates. The obtained data are presented as mean ± SEM and the statistically analysed with the non-parametric Kruskal-Wallis (H)-test followed by the Chi square (χ^2) test.

The non-parametric Mann-Whitney U-test was used to determine statistical significances between two independent groups.

4. Results

4.1. Synthesis **of β**- Thujone

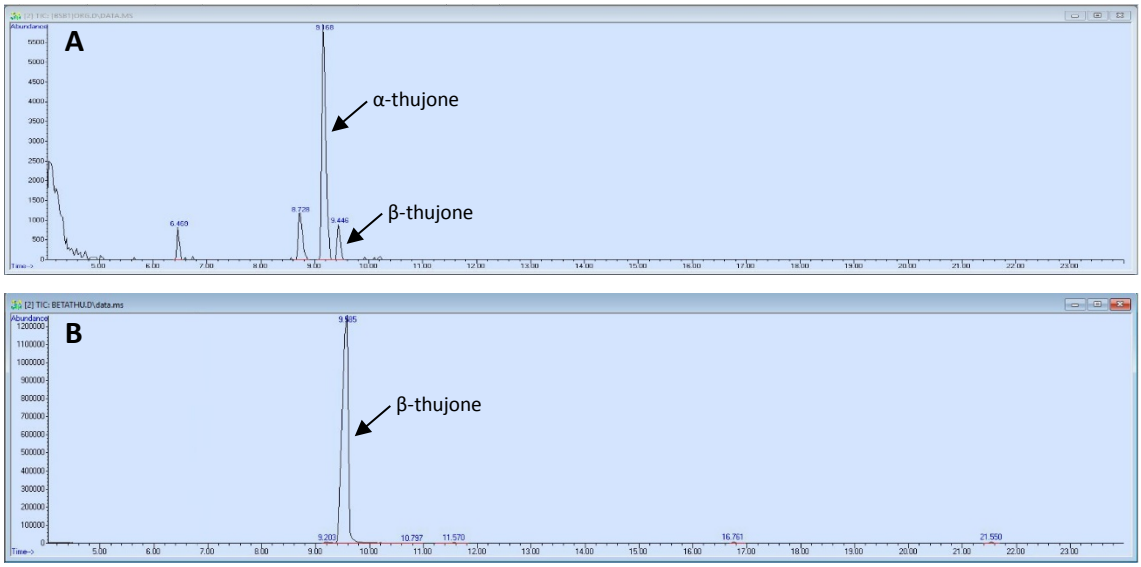


Figure 16: GC/MS analysis of the thujones in the oil before and after synthesis.

A: content of α- and β- thujone in the mixture oil before synthesis.

B: the analysis after the synthesis revealed an almost pure β thujone oil.

The graphs in figure 16 show the relative content of thujones in the mixture oil before and after synthesis. On the x-axis time is shown in minutes, which the substances need to pass through the GC separation column (retention time). The y-axis shows the relative abundance of the separated substance on the quadrupole mass spectrometer detector, which is dimensionless. GC/MS analyses revealed that the α- and β- thujone in the mixture oil has a high content of α-thujone while the β-thujone content is quite small when compared to each other (Figure 16 A). After the synthesis, the GC/MS analysis shows an almost pure β- thujone oil with a purity of 99.58% (Figure 16 B). The small differences in the retention time in the two analysis is due to the amount of substances that was analysed. The β- thujone peak in B is much higher and wider than the peak in A. Therefore, the retention time in figure B is slightly shifted to the right.

4.2. β- Thujone

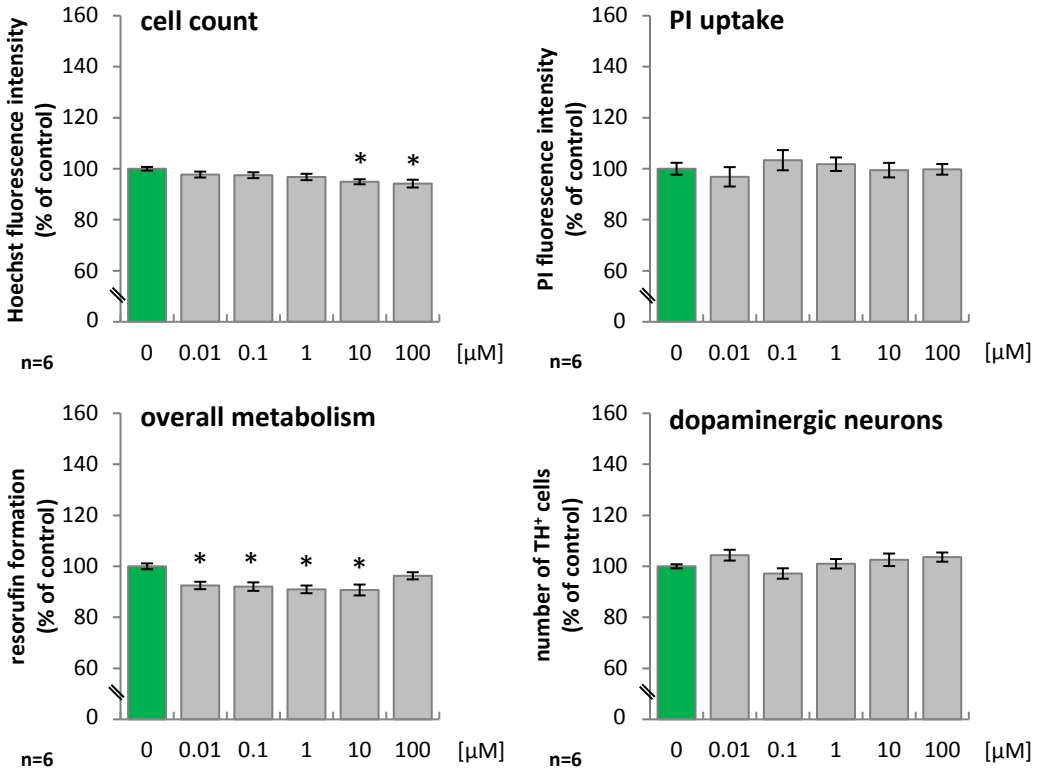


Figure 17: Primary mesencephalic cell culture treated with β-thujone. Cells were treated on DIV 12 for 48 h with different concentration of β-thujone and the status of the culture was determined using four different assays. 100% correspond to the value of the untreated controls. Data are expressed as means ± SEM of six independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$) and displayed with (*).

The treatment of primary culture on DIV 12 for 48 h with β-thujone in final concentrations of 0.01, 0.1, 1, 10, and 100 µM shows marginal dose-dependent influences (Figure 17). A decreased cell count could be observed with a significant decrease at 10 and 100 µM to 94.9 and 94.2% compared to control. The uptake of PI was not influenced by the administration of β-thujone. The overall cell metabolism was slightly affected by the treatment with 0.01, 0.1, 1 and 10 µM of β-thujone with a decrease to 92.5, 92.0, 91.0 and 90.7% compared to the untreated control. Comparable to the observations regarding the PI uptake, there was no observable influence of β-thujone in the number of TH immunopositive cells.

4.3. Essential oil

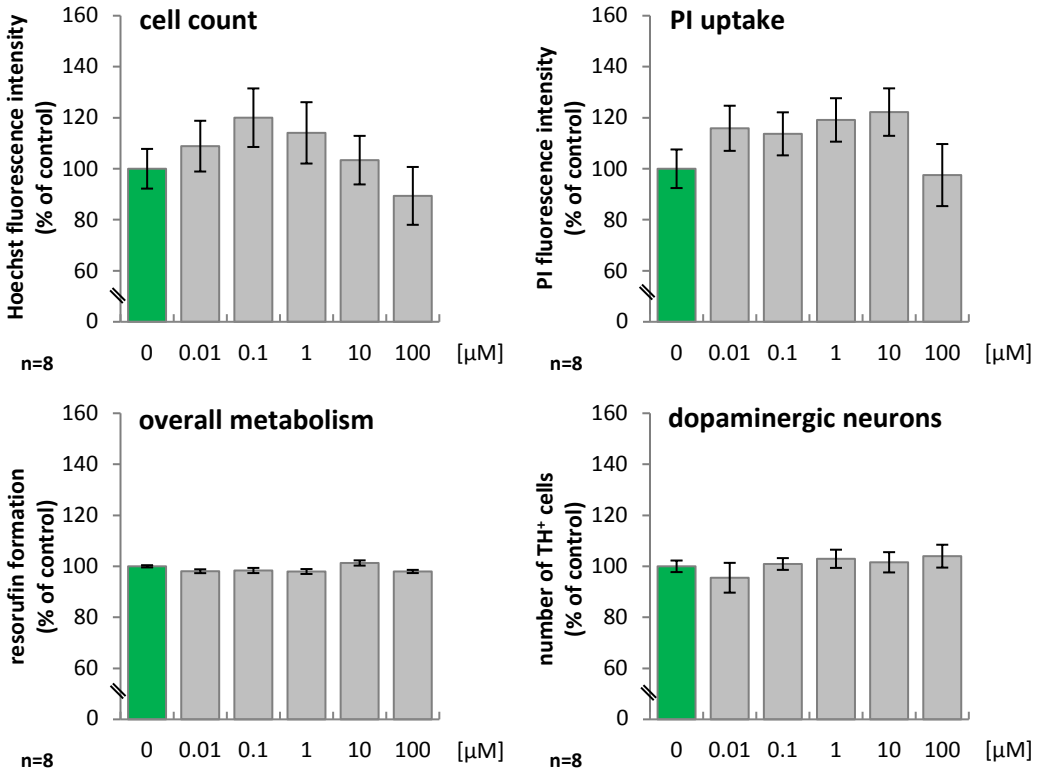


Figure 18: Effects of sage essential oil on primary cell cultures. Cells were treated on DIV 12 for 48 h with different concentration of sage oil, and the status of the culture was determined using four different assays. 100% correspond to the value of the untreated controls. Data are expressed as means ± SEM of eight independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the $\chi 2$-test ($p < 0.05$) and marked with (*).

The sage essential oil used for treatment of primary cell cultures at concentrations of 0.01, 0.1, 1, 10 and 100 µM showed almost no effect (Figure 18). The Hoechst 33342 fluorescence intensity was insignificantly, but prominently higher at 0.1 and 1 µM compared to control. Interestingly, also the PI uptake was higher at the concentrations 0.1 - 10 µM, but also not significantly. The overall metabolism as well as the number of dopaminergic neurons was not influenced by sage essential oil in all used concentrations.

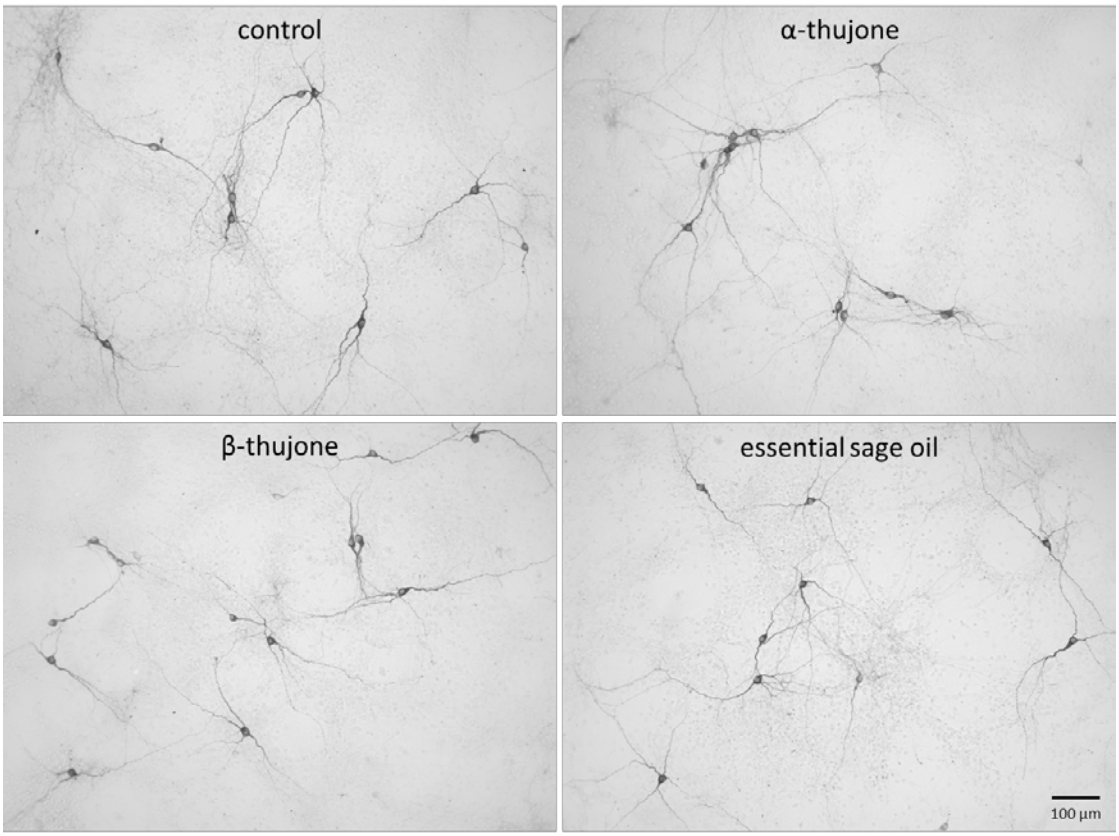


Figure 19: Representative photographs of dopaminergic neurons. Cells were treated on DIV 12 for 48 h with 100 µM α-thujone, β-thujone and essential sage oil.

4.4. Stressors

4.4.1. Controls

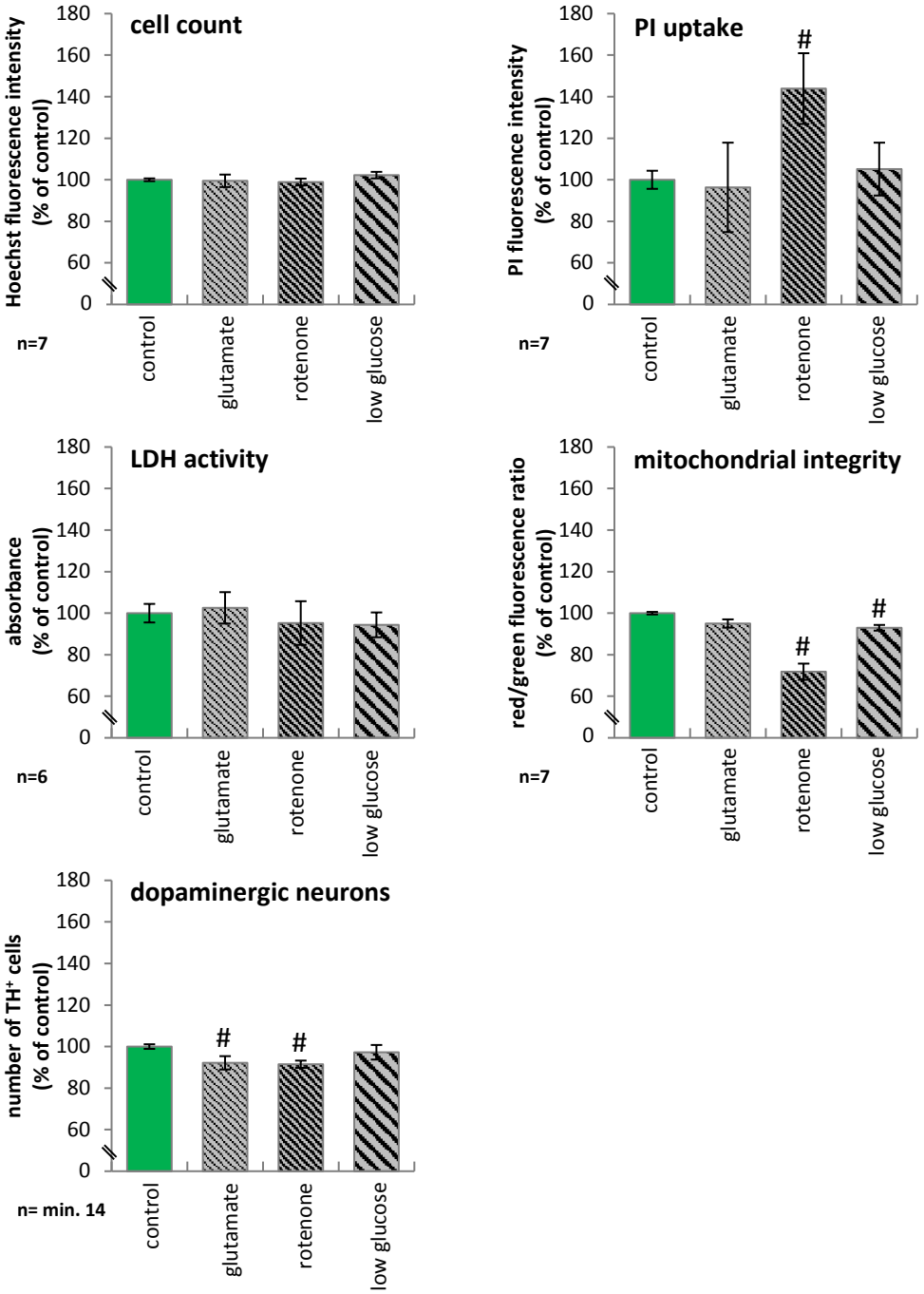


Figure 20: Controls of primary mesencephalic cell culture under stressed conditions. Stressful conditions were established on DIV 12 for 48 h by the treatment with glutamate (30 mM), rotenone (80 nM or 10 nM (dopaminergic neurons) or a low level of glucose (25.5 mM). 100% correspond to the value of the untreated controls. Data are expressed as means ± SEM of **n** independent experiments (shown in the figure), whereas each condition was carried out as duplicates. Pair comparison of the stressor to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$) and marked with (#).

The following parameters, namely cell count of the whole culture via the Hoechst 33342 dye for measuring the total dead cells in the population, and detection of LDH activity show no significant change in these conditions. Propidium iodide uptake was increased to 143.9% when treated with 80 nM rotenone. Treatment on DIV 12 with 80 nM rotenone and low glucose environment (f.c. was 25.5 mM) for 48 h decreased the mitochondrial integrity to 71.8 and 93.0% compared to the control. The number of dopaminergic neurons was slightly decreased when treated with 30 mM glutamate (92.2%) and to 91,5% when treated with 10 nM rotenone for 48 h, both significantly (Figure 20).

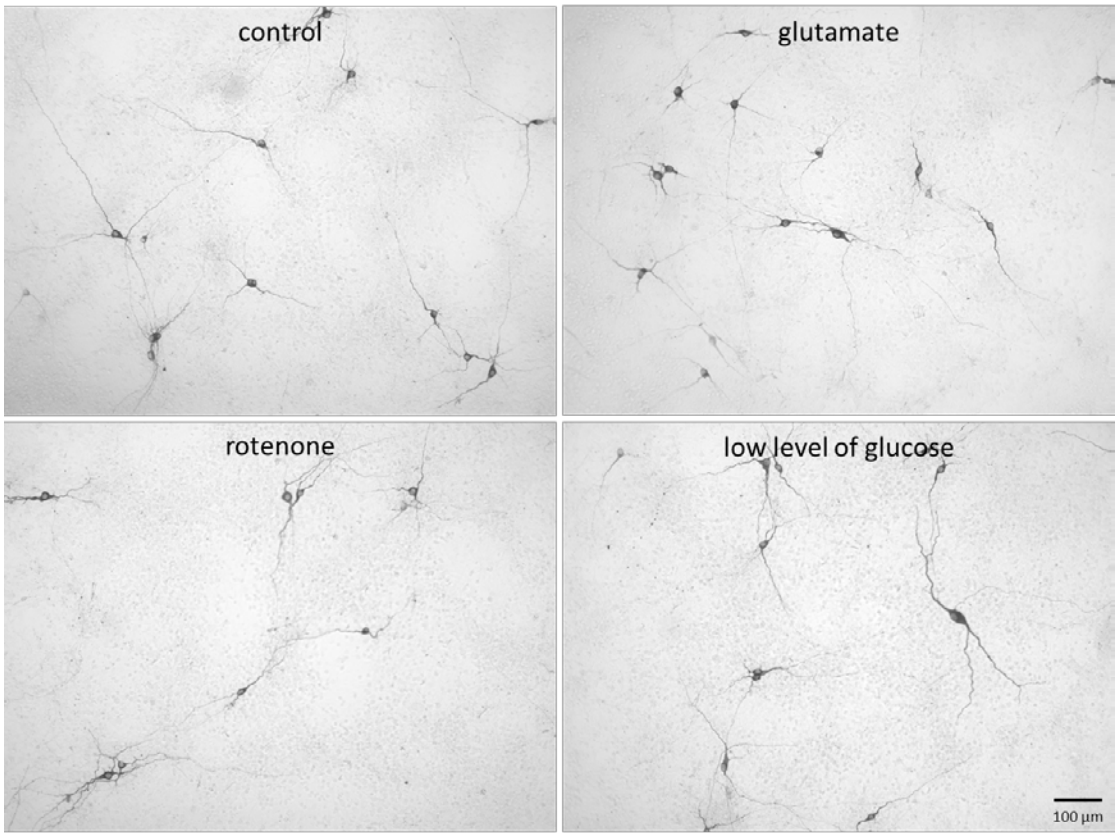


Figure 21: Representative photographs of dopaminergic neurons. Cells were treated on DIV 12 for 48 h with glutamate [30 mM], rotenone [10 nM] and low level of glucose [25.5 mM].

4.4.2. Glutamate

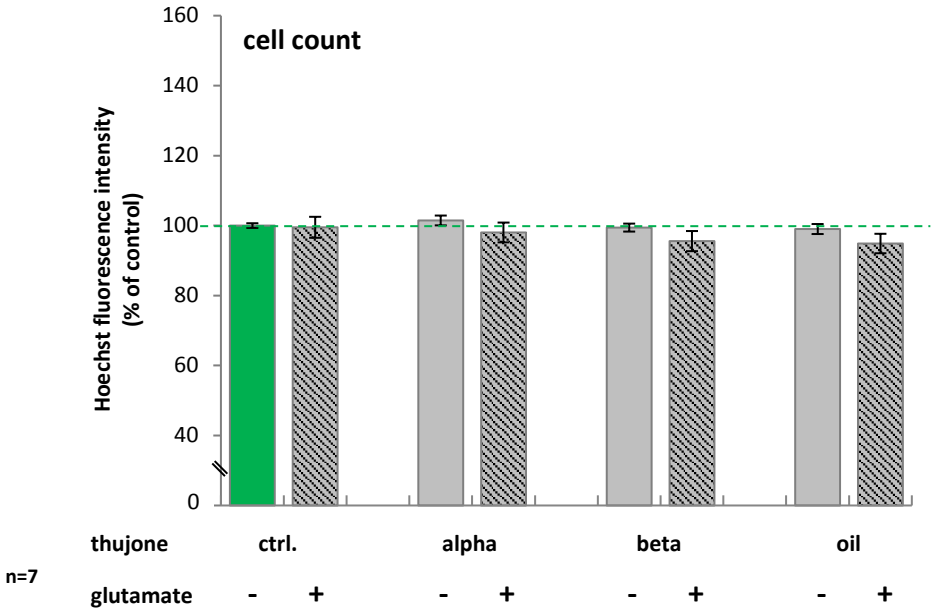


Figure 22: Total cell count of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil under normal treatment and stressed condition due to glutamate overload. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with 30 mM glutamate. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 7 independent experiments, whereas each condition was carried out as duplicates. No statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$).

For determining the whole cell number within in the culture, the cells were stained with a Hoechst 33342 dye, which is intercalating in the nucleus of all cells. The stressor glutamate at a concentration of 30 mM has no significant influence on the total cell population compared to the control (Figure 22). α-thujone, β-thujone and the essential sage oil at a concentration of 100 µM did not affect the total cell number compared to the control. When co-treated with the stressor and the thujones or the essential sage oil, changes in the total cell population due to adding effects were also not detected.

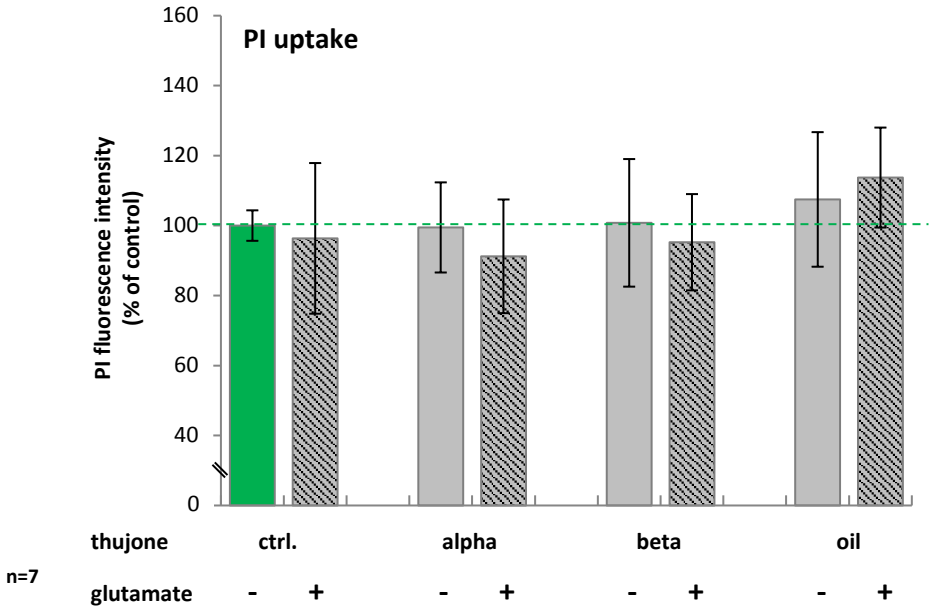


Figure 23: PI uptake of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil under normal treatment and under glutamate induced stress. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with 30 mM glutamate. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 7 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$).

Propidium iodide uptake as a marker for detecting acute cell death in the culture through intercalation in the degrading cell nucleus, was not influenced when cultures were treated with 30 mM glutamate (Figure 23). Additionally, the treatment with α-thujone, β-thujone and the essential sage oil at a concentration of 100 µM do not show a significant change compared to control. Co-treatment of glutamate (30 mM) and the thujones or the essential oil did not result in an aggravating effect.

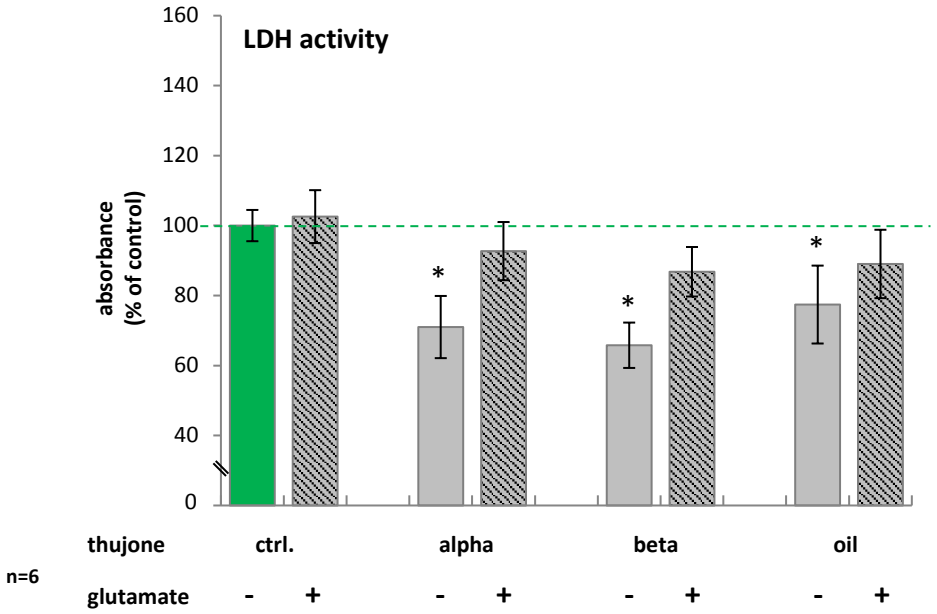


Figure 24: LDH activity of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil under normal treatment and stressed condition by glutamate administration. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with 30 mM glutamate. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 6 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$) and displayed with (*). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$).

LDH as a very stable intracellular enzyme, it can be used to determine cell dead during the treatment period of the culture. Glutamate itself at a concentration of 30 mM has almost no effect to the culture when compared to the untreated control (Figure 24). In culture treated with α-thujone, β-thujone, or with essential sage oil with a concentration of 100 µM, a significant decrease of the LDH activity was measured by 29.0, 34.2 and 22.6% compared to the untreated control. However, co-treatment of the thujones or the essential sage oil with the stressor did not show significant effect, since the LDH activity was almost at the control levels with 92.7, 86.8 and 89.0% compared to untreated control.

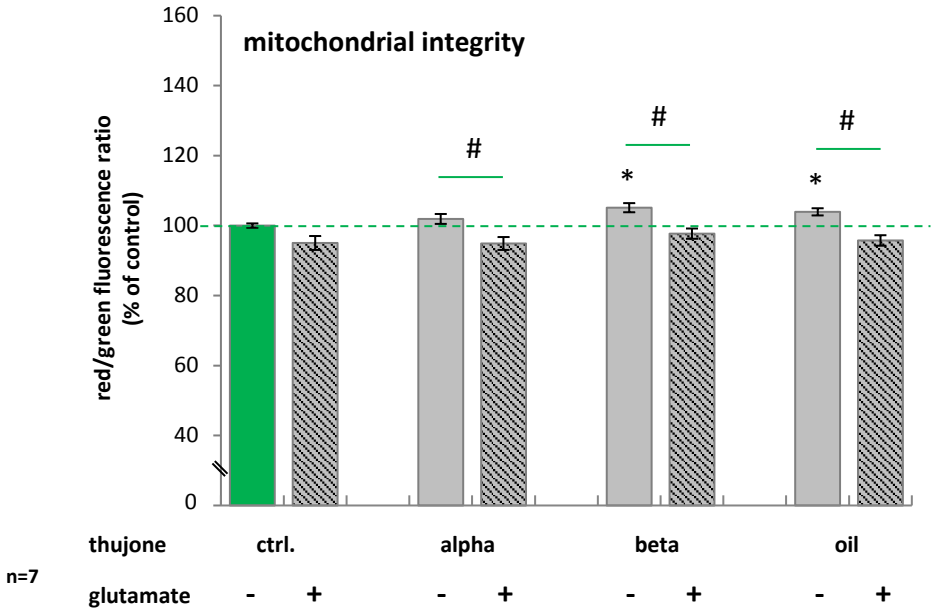


Figure 25: Mitochondrial membrane integrity of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil under normal and glutamate overload condition. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with 30 mM glutamate. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 7 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$) and displayed with (*). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$) and marked with (#).

The integrity of the mitochondrial membrane is a suitable marker for stress in the cell. The stressor glutamate at the concentration of 30 mM was slightly but not significantly reducing the mitochondrial activity (Figure 25). Treatment with 100 µM β-thujone and 100 µM of the essential sage oil has a significant positive influence on the mitochondrial membrane integrity with 105.1 and 103.2% of the red/green ratio compared to the untreated control, while α-thujone has no significant effect (101.9%). Co-treatment of α-thujone, β-thujone and the essential oil with glutamate lowers the red/green fluorescence ratio to 94.9, 97.7 and 95.7% compared to the untreated control, which is significant if compared to the respective controls.

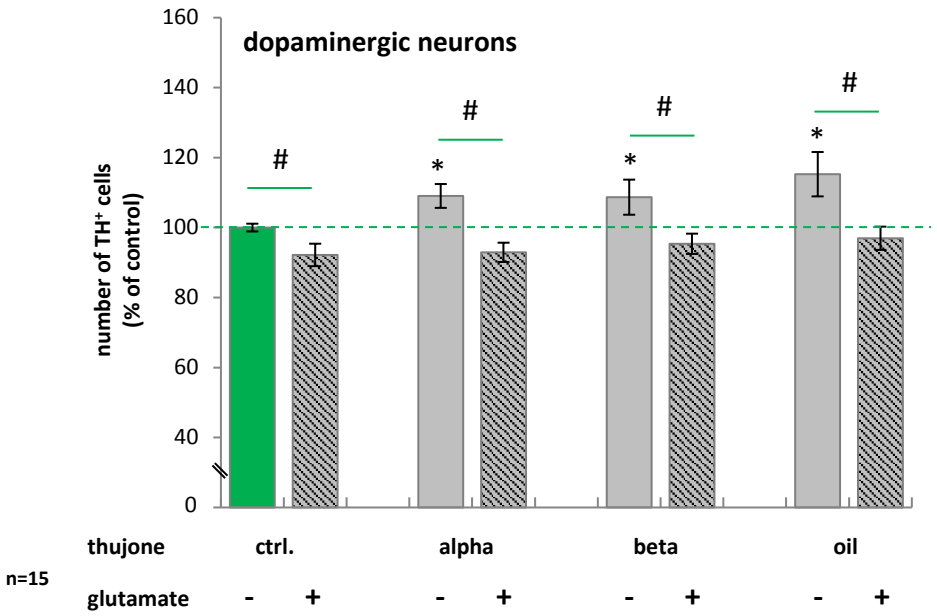


Figure 26: Number of dopaminergic neurons treated with alpha thujone, beta thujone and sage essential oil under normal treatment conditions and under stress condition with excess concentrations of glutamate. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with 30 mM glutamate. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 15 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$) and displayed with (*). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$) and marked with (#).

The number of dopaminergic neurons is a sensitive marker for neurotoxic or neuroprotective abilities of substances. The stressor glutamate at the concentration of 30 mM significantly reduced the total count of dopaminergic neurons to 92.2% compared to the untreated control (Figure 26). The thujones and the essential sage oil increased the number of dopaminergic neurons by 9.0, 8.7 and 15.3% compared to the untreated control. Co-treatment of the thujones or the essential sage oil with the stressor abrogated the positive effect and lowers the number of dopaminergic neurons to a level like the stressor itself with 92.9, 95.4 and 96.9%, which is significant when compared to the respective thujone or essential oil controls.

4.4.3. Rotenone

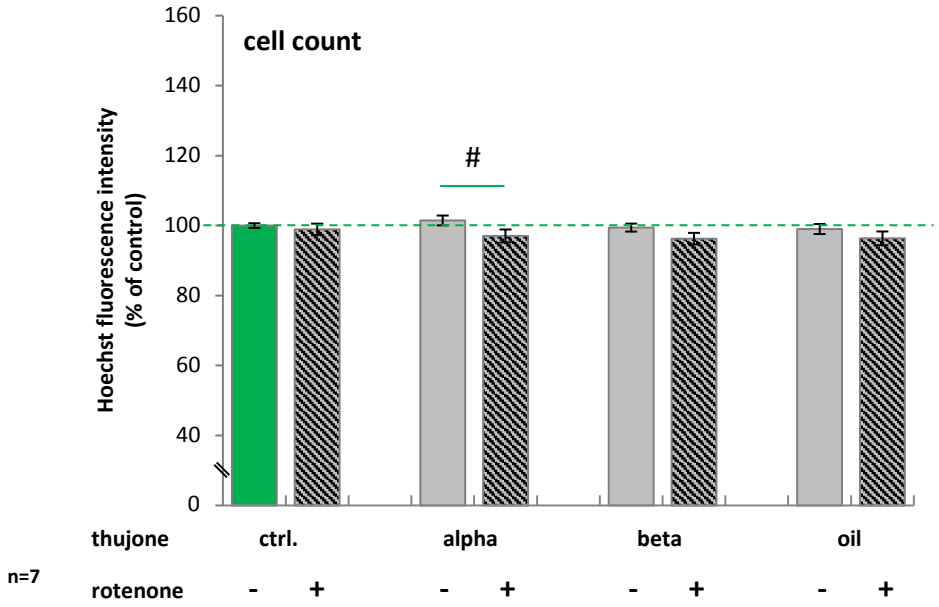


Figure 27: Total cell count of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil under normal treatment and stressed condition due to rotenone administration. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with 80 nM rotenone. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 7 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$) and marked with (#).

The stressor rotenone has no significant influence on the total cell amount in the cell culture at the concentration of 80 nM compared to the untreated control (Figure 27). The thujones and the essential oil also did not affect the cell number at the concentration of 100 µM. When rotenone was co-treated with α-thujone, it slightly increased the total cell count from 97.0 to 101.5%, which is significant when compared to the particular control, while the other co-treatment conditions showed no change.

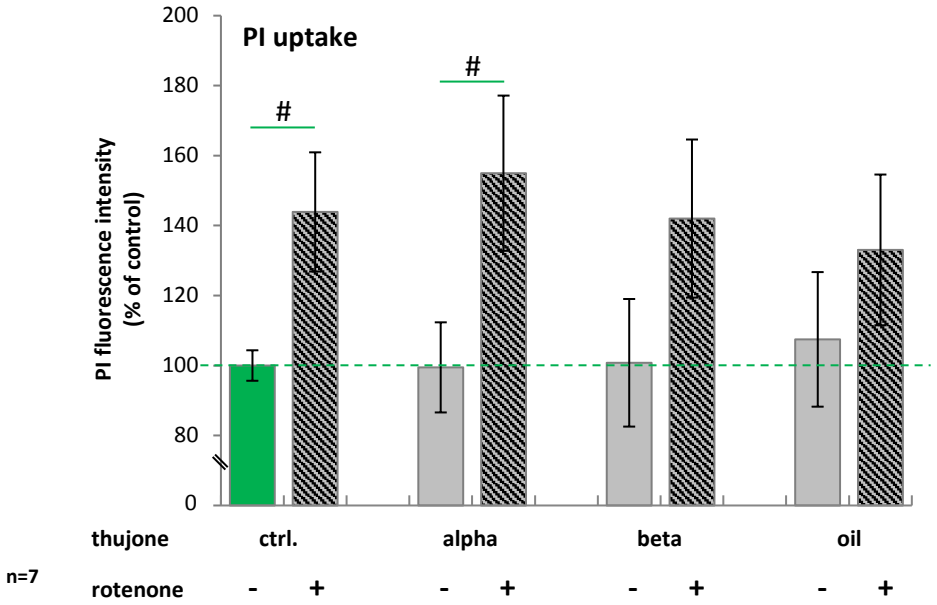


Figure 28: PI uptake of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil under normal treatment and under rotenone induced stress. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with 80 nM rotenone. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 7 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$) and marked with (#).

Treatment with 80 nM of rotenone significantly increased the uptake of propidium iodide by 43.9% compared to the untreated control (Figure 28). α-thujone, β-thujone and the essential sage oil did not influence the PI uptake if 100 µM was used for the treatment. The co-treatment of rotenone with α-thujone increased the propidium iodide uptake by 55.5% compared to the specific control (99.5%), while β-thujone and the essential sage oil led to a slight decreasing effect, which was nonetheless not significant when compared to the specific controls.

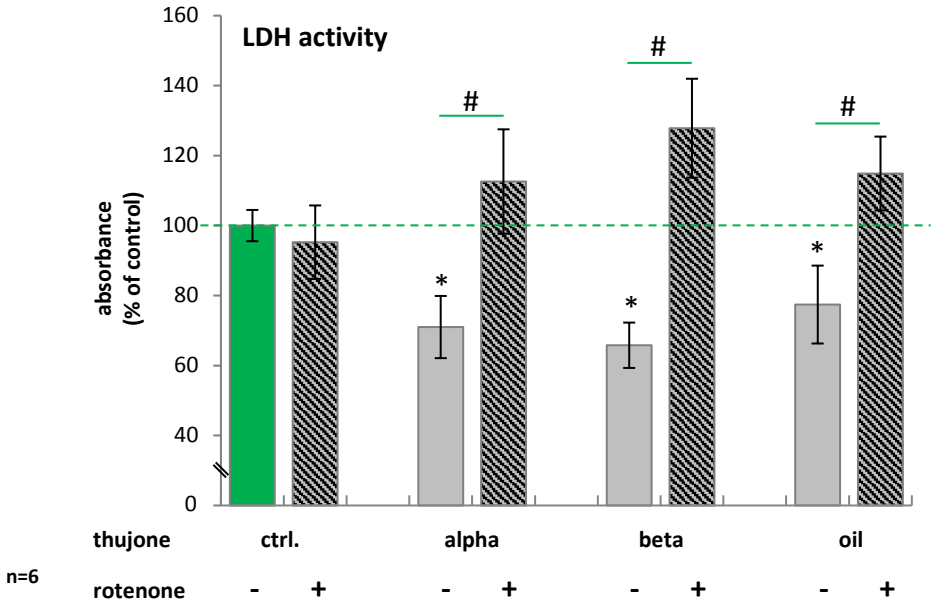


Figure 29: LDH activity of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil under normal treatment conditions and stressed condition by rotenone administration. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with 80 nM rotenone. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 6 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$) and tagged with (*). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$) and marked with (#).

Measurement of the LDH activity did not lead to significant changes in the 80 nM treated condition compared to the untreated control (Figure 29). The treatment with 100 µM of α-thujone, β-thujone or essential sage oil decreased the LDH activity significantly to 71.0, 65.8, 77.4% respectively compared to the untreated control. When the thujones and the essential sage oil were co-treated with rotenone, the diminished LDH activity was counteracted. The co-treatment of rotenone with α-thujone, β-thujone and essential sage oil significantly increased the LDH activity by 58.6, 94.2, and 48.4% compared to the specific control.

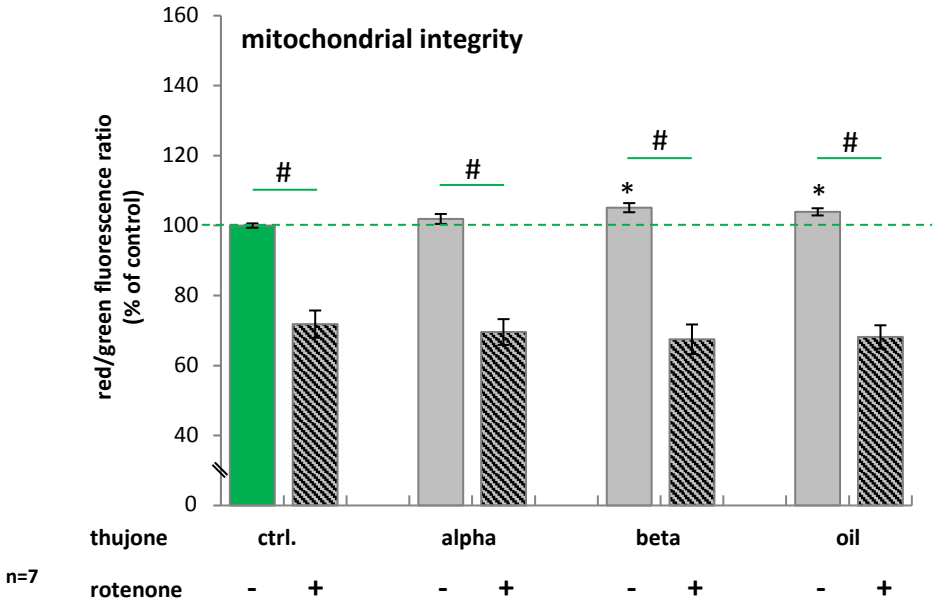


Figure 30: Mitochondrial membrane integrity of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil under control and rotenone conditions. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with 80 nM rotenone. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 7 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$) and indicated with (*). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$) and marked with (#).

The mitochondrial integrity measured with the JC-1 dye was significantly reduced by the stressor rotenone when used at 80 nM to 71.8% compared to the untreated control (Figure 30). While 100 µM α-thujone did not influence the mitochondrial integrity, the treatment with 100 µM β-thujone and essential sage oil slightly but significantly increased the red/green ratio by 5.1 and 3.9% compared to the untreated control. Thujones and the essential sage oil cannot compensate for the negative influence of the stressor in the co-treatment conditions. Rotenone decreased the mitochondrial integrity by 68.3, 64.2 and 65.6% when it is co-treated with α-thujone, β-thujone and the essential sage oil.

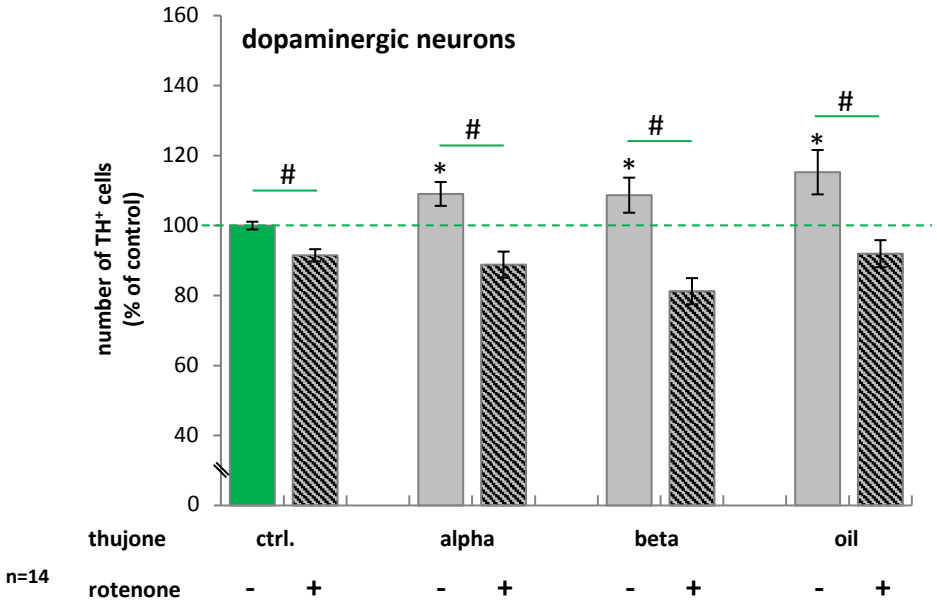


Figure 31: Number of dopaminergic neurons treated with alpha thujone, beta thujone and sage essential oil under normal treatment conditions and under rotenone-induced complex I inhibition. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with 10 nM rotenone. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 14 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$) and marked with (*). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$) and indicated with (#).

The number of dopaminergic neurons was significantly reduced to 91.5% by the stressor rotenone (10 nM) (Figure 31). Treatment with 100 µM α-thujone, β-thujone and the essential sage oil slightly but significantly increased the number of dopaminergic neurons to 109.0, 108.7 and 115.3%. The negative effect of rotenone could not be compensated by the positive effect of the thujone or the essential oil. Co-treatment of rotenone and α-thujone, β-thujone and the essential oil significantly decreased the number of dopaminergic neurons by 81.5, 74.8, and 79.8% compared to the specific control.

4.4.4. Low level of Glucose

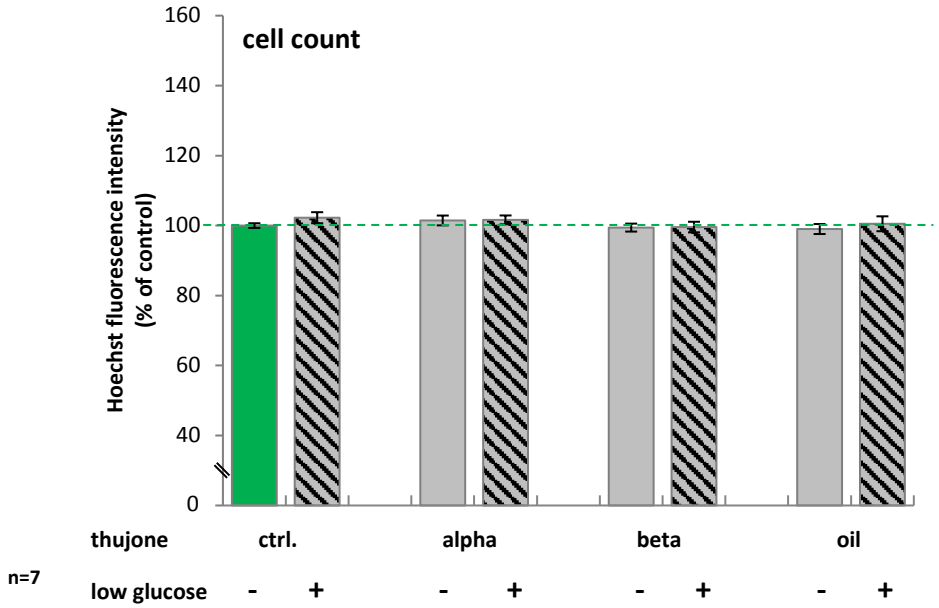


Figure 32: Total cell count of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil in controls and cultures treated with low levels of glucose. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with medium containing a final concentration of 25.5 mM glucose. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 7 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the $\chi 2$-test ($p < 0.05$). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$).

Under low glucose concentration (25.5 mM) in the treatment medium, the whole cell number was not affected (Figure 32). Also, the treatment with α-thujone, β-thujone and the essential sage oil (100 µM) had no effect on the total cell number in the culture. Those two conditions also have no influence on the cell count when co-treated.

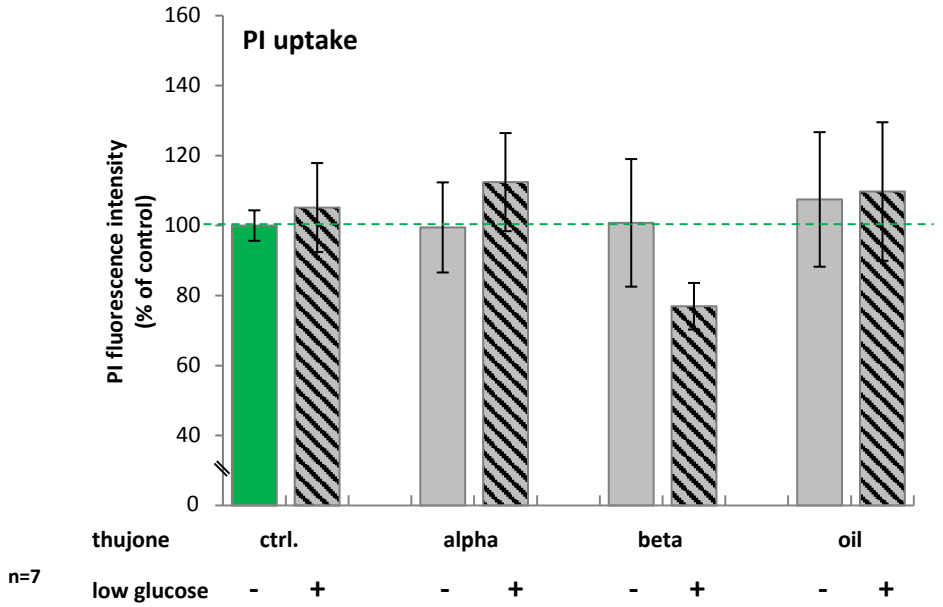


Figure 33: PI uptake of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil under normal treatment conditions and glucose deprivation. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with medium containing a final concentration of 25.5 mM glucose. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 7 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the $\chi 2$-test ($p < 0.05$) Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$.

The propidium iodide uptake was not significantly increased when media with a low concentration of glucose was added (f.c. in the culture was 25.5 mM) (Figure 33). The co-treatment in of sage derived compounds with low glucose containing media, like the treatment with 100 µM α-thujone, β-thujone and the essential sage oil alone, did not affect the uptake of propidium iodide.

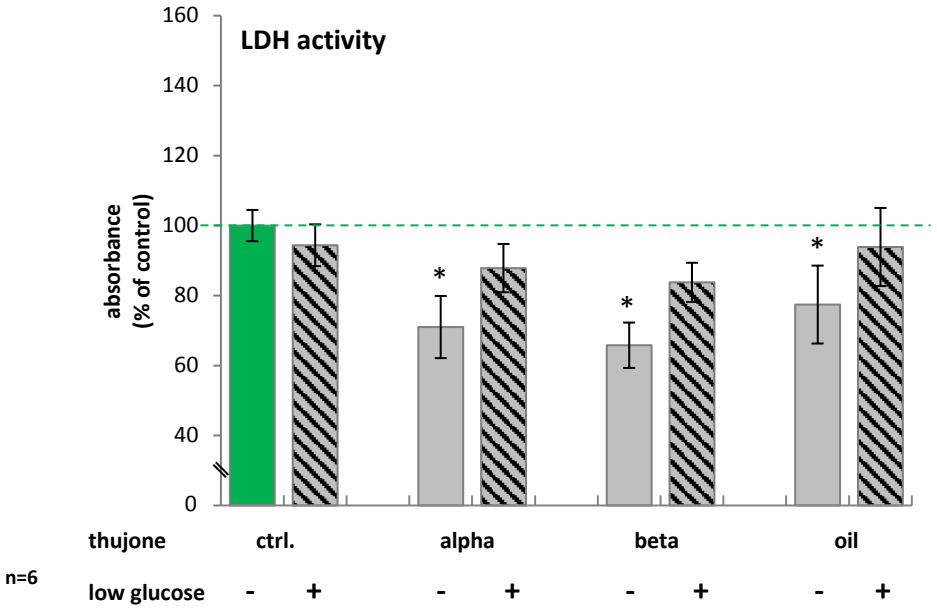


Figure 34: LDH activity of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil under normal treatment conditions and stress condition induced by low concentration of glucose. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with medium containing a final concentration of 25.5 mM glucose. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 6 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the $\chi 2$-test ($p < 0.05$) and indicated with (*).Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$).

Under lowered levels of glucose (25.5 mM), a moderate but not significant decrease of LDH activity was measured (Figure 34). Treatment with α-thujone, β-thujone and the essential sage oil, used at 100 µM, decreased the LDH activity in all three conditions by 29.0, 34.2 and 22.6% compared to the untreated control. The co-treatment of the thujones and the essential sage oil increased the LDH activity slightly but not significantly when compared to the specific control.

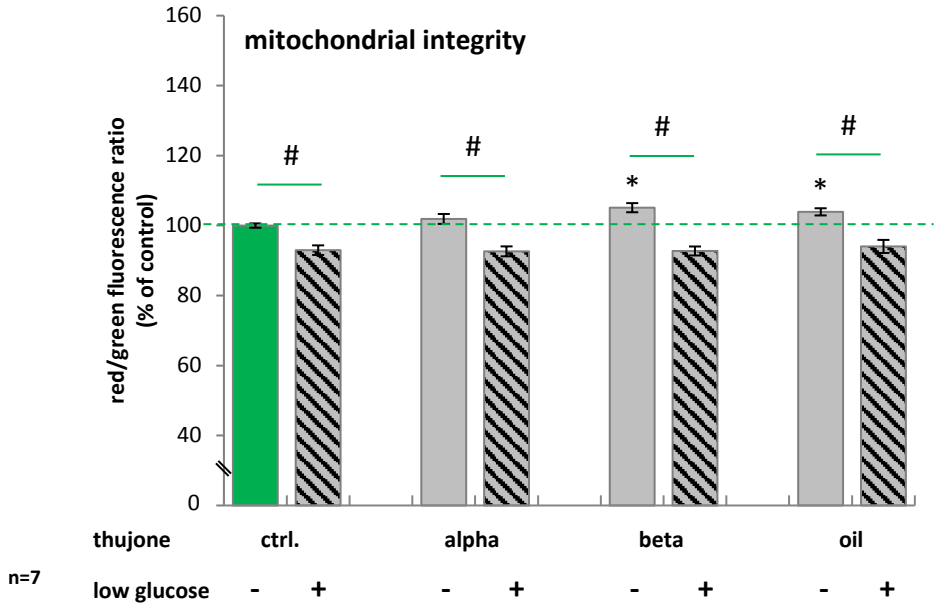


Figure 35: Mitochondrial membrane integrity of primary mesencephalic cells treated with alpha thujone, beta thujone and sage essential oil in cultures with control or low glucose media. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with medium containing a final concentration of 25.5 mM glucose. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 7 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$) and marked with (*). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$) and indicated with (#).

Glucose deprivation significantly decreased the red/green fluorescence ratio to 93.0% compared to the untreated control, therewith indicating damaged mitochondrial membrane integrity (Figure 35). β-thujone and the essential sage oil significantly increased the membrane integrity to 105.1 and 103.9% if 100 µM was added to the media. In cultures treated with low glucose and sage derived compounds, neither the thujones nor the essential oil could restore the integrity of the mitochondrial membrane. The red/green ratio was significantly reduced by 90.9, 88.2, and 90.5% compared to the respective control.

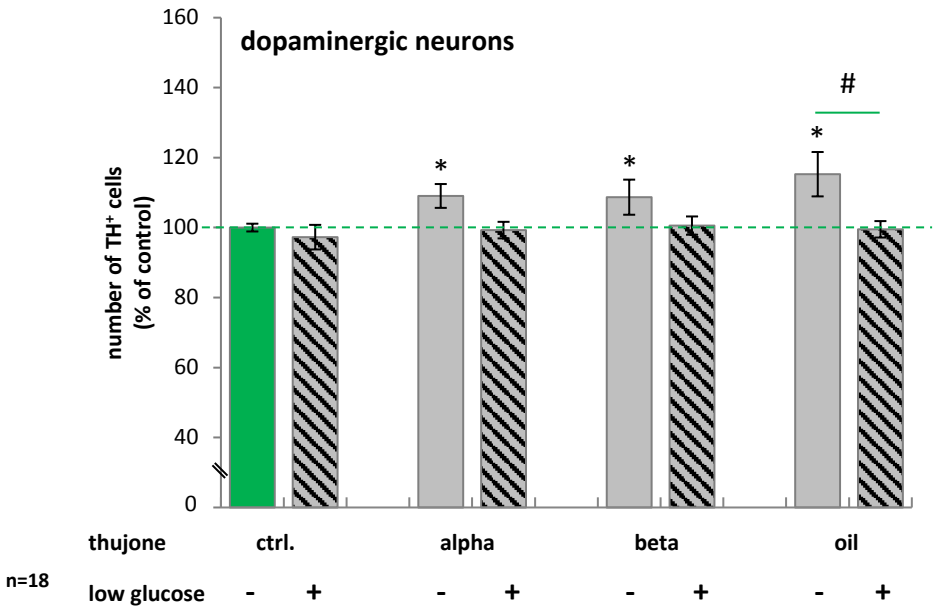


Figure 36: Number of dopaminergic neurons treated with alpha thujone, beta thujone and sage essential oil under normal treatment conditions and under a stress condition due to low glucose administration glucose. Cells were treated on DIV 12 for 48 h with 100 µM of α-thujone, β-thujone or with essential sage oil co-treated with medium containing a final concentration of 25.5 mM glucose. 100% correspond to the value of the untreated control. Data are expressed as means ± SEM of 18 independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the $\chi 2$-test ($p < 0.05$) and displayed with (*). Pair comparison of α-thujone, β-thujone or with essential sage oil to the untreated control was performed using the Mann-Whitney *U* test ($p < 0.05$) and marked with (#).

The number of dopaminergic neurons was not significantly affected by the lowered level of 25.5 mM glucose (Figure 36). Administration of 100 µM α-thujone, β-thujone and the essential sage oil significantly increased the number of dopaminergic neurons in the cell culture by 9.0, 8.7 and 15.3%% compared to the untreated control. The beneficial effect of the thujones and the essential sage oil could not be observed in the co-treatment cultures. Co-treatment of essential sage oil and lowered level of glucose, so the TH^+ cell count was significantly reduced by 86.3% compared to the specific control treated with sage oil alone.

5. Discussion

5.1. Synthesis of β-thujone with α-thujone as an educt

The content of α-thujone and β-thujone in the essential oil of different plant species differ, as mentioned above (Table 1). To elucidate differences between the effects of the specific thujones, it was necessary to synthesise the β-form, since it is to date not commercially available. The synthesis was performed according to the protocol of French with minor modifications (2011). One of those modification was a downscaling of the used volume. In the protocol of French, 7 g of western red cedar essential oil was used. Because of the limited α-/β-thujone mixture stock, I used 0.46 g of the oil as an educt of the reaction. A lower purity of the oil was expected as a result of the downscaling of the method, since washing steps performed with filters and high volumes of washing fluid could not be used. Instead of this, all steps including washing steps, were performed in reaction tubes using a centrifuge to collect the precipitate. Surprisingly the evaluation of the obtained oil revealed a high amount of the β-form of thujone which was 99.58% (Figure 16).

5.2. Effects of β-thujone or *Salvia pomifera* essential oil on primary dissociated neuronal cell culture

To evaluate if β-thujone and sage essential oil influences the primary mesencephalic cell culture itself, a concentration range was chosen corresponding to previous studies (Meschler and Howlett, 1998, Höld et al., 2000, Sultan et al., 2017, Deiml et al., 2003). A maximum concentration of 100 µM with a dilution range of five magnitudes to 0.01 µM was used. The highest concentration of 100 µM, which is not likely to be of pharmacological relevance at the first glance, was chosen since it could be shown that peak concentrations of around 60 µM of thujone are detectable in the brain (Höld et al., 2000).

In this study, possible effects of β-thujone and essential sage oil were evaluated using four assays. Cell counting via Hoechst 33342 staining in combination with propidium iodide staining gives a good outline of the living to dead cell ratio and, therefore, of the toxicity of a chemical compound.

Both, β-thujone and sage essential oil do not show a remarkable influence in either on the cell count nor on the PI staining (Figure 17, 18). Due to the fact that neurons no longer proliferate in the cell culture and proliferation of the glial cells is inhibited by choosing a specific supplemented serum-free medium (N4, Table 3), resazurin reduction assay could be used as a cell metabolism marker rather than a cell count parameter (Rampersad, 2012). Even the observed significant lowering of the metabolism in cultures with β-thujone treatment (0.01-10 µM) was not dose dependent and very moderate (Figure 17, 18). The number of dopaminergic neurons is a sensitive marker for neurotoxic characteristics of a substance. Dopaminergic neurons are highly vulnerable to excitotoxicity and to oxidative stress (Moldzio et al., 2006). In contrast to the proposed effect of thujones on the GABAergic system (Höld et al., 2000), inhibition of $GABA_A$ receptors would inevitably lead to a failure in inhibition of excitatory signals. This would lead to excessive activation of the neuronal network, and a possible excitotoxicity effect, which could be detected by changes in dopaminergic cell count and morphology, when these cells were stained with anti-TH immunocytochemistry (Tepper and Lee, 2007). In my study both, β-thujone and sage essential oil do not show a decrease in the number of dopaminergic neurons in the cell culture to a remarkable extent (Figure 17-19).

5.3. Primary mesencephalic cell culture under stress conditions

β-thujone and the essential sage oil show no highly toxic effect under the chosen parameters. Like under normal physiological condition in the body, cells in culture are treated with unlimited access to nutrition they need. To mimic pathologic conditions in cell culture, three models for different pathologic settings were used to maximize the effect of the co-administered sage-derived substances. In the further experiments, LDH activity and mitochondrial membrane integrity was measured instead of resazurin cell viability assay to assess possible effects more efficiently. Concentrations of the stressors where chosen to influence the cellular behaviour, but not completely to disturbing the cellular network (Figure 21).

Thus, the parameters cell count, PI uptake and LDH activity do not show a significant change under stressed conditions with 30 mM glutamate, 80 nM rotenone and glucose deprivation with 25.5 mM glucose in the treatment media (Figure 20). This could be due to the parameters itself. Hoechst 33342 as a low toxic, in the DNA intercalating, marker detects all attached cells in the culture. PI uptake is referred to dead cells. Moreover, LDH activity is a marker for cell degeneration, too. It was the intention of the chosen stressor concentrations to harm, but not to detach the cells from the culture plate, what would make the cultures ineligible for experimental intervention. Due to the high sensitivity in changes in the mitochondrial membrane integrity, treatment with rotenone and glucose deprivation at the concentration of 80 nM and 25.5 mM are detectable significantly with the JC-1 dye (Figure 20). The onset of rotenone on mitochondrial integrity was expected, considering the fact that rotenone treatment results in mitochondrial impairment. Though, the change in the mitochondrial membrane integrity in low glucose cultures was unexpected. But during starvation, the interaction between endoplasmic reticulum (ER) and mitochondria at the mitochondria-associated membranes (MAMs) can be altered. This change could lead to a disturbed exchange of Ca^{2+} ions between ER and mitochondria and, therefore, evoke a mitochondrial membrane imbalance (Rieusset, 2018). For immunocytochemistry, rotenone was used at 10 nM final concentration to determine the dopaminergic neurons (Figure 20). Dopaminergic neurons are cells with a high energy consumption rate and vulnerability to oxidative stress (Alam and Schmidt, 2002). Influencing the metabolic rate of mitochondria with drugs like rotenone leads to misbalance in the ATP levels in the cell, and to cellular degeneration. Therefore, the number of dopaminergic neurons shows only in the rotenone and in the glutamate treatment condition a small but significant decrease, while in the glucose induced stress condition the chosen concentration wasn't influencing this specific parameter. Glutamate is used in a wide concentration range to investigate excitotoxicity and oxidative stress in neuronal cell cultures. Therefore, it is not clear if the used concentration was high enough to elicit excitotoxicity in mesencephalic cell culture. Nonetheless, the concentration is in a range to evoke oxidative glutamate toxicity or excitotoxicity in different cell lines and primary cell culture (Kritis et al., 2015)

5.3.1. Effects of α-thujone, β-thujone and sage essential oil under stress conditions induced with glutamate

Administration of sage-derived substances in combination with cellular stressors in culture should give a closer look on supposed cell damaging thujone action, since co-stressors might have additive or amplifying effects. In the single-dose treatments, thujones and sage oil did not influence the overall cell count (Figure 22) and the PI staining (Figure 23) even under excessive glutamate conditions. Data of PI staining show a high mean variation. This stained dead cells easily detach and could therefore not be detected. Due to these findings, PI staining wasn't used alone in my study to identifying disintegrating cells. LDH activity as a marker for lysed cells was measured as an additional and complementary parameter to PI (Figure 24). All used sage-derived substances show a significant decrease in the LDH activity while they were not able to retain this positive effect in the glutamate-induced stress condition. Glutamate, as mentioned above, can cause ROS formation in the cells and thereby damage them (Figure 6, Duan et al., 2007). Srinivasan and his colleagues (2020) could show that thujone has a moderate free radical scavenging activity. This antioxidative ability of thujone can be the reason for the moderate lower LDH activity in cultures co-treated with thujones and glutamate. Although treatment with the sage derived substances show a significant change in the LDH activity, this could be a result of a low level LDH activity in general. Therefore, small changes in the LDH activity can lead to a high value and percental changes. The mitochondrial membrane integrity was slightly but insignificantly positively influenced by β-thujone and the essential oil when treated alone (Figure 25). Nevertheless, the positive effect of the thujones does not lead to a counteraction of the small negative effect of glutamate. Nonetheless, an aggravating effect of thujone/glutamate co-treatment was not seen either. Interestingly in the co-treatment condition, the thujones and the essential sage oil show a significantly increased number of dopaminergic neurons (Figure 26). A significant increase was not seen in the foregoing experiments for detecting the suitable β-thujone and sage essential oil experiments, but also a small increment of TH^+ neurons by 3.6 and 4.0%, respectively.

In the stressor experiments, a higher number of experiments was performed, so to achieve statistical significances even to small percentage changes is more likely. Comparable to the other parameters, the beneficial effect of thujones wasn't high enough to counteract the reaction of cells to the exposition to excessive levels of glutamate. These positive effects could be a result of the above mentioned antioxidative ability of thujone, but to conclude, these small effects are possibly caused by a mean variation, so the data straggling might not represent a physiological effect.

5.3.2. Effects of α-thujone, β-thujone and sage essential oil under stress conditions by partial rotenone complex I inhibition

Rotenone is interacting with the complex I of the respiratory chain. This inhibition leads to a decrease in the ATP level within the cell influencing all energy consuming cell processes (Heinz et al., 2017, Yee et al., 2017). Beside this mode of action, rotenone influences the leakage of electrons at the complex I. This leads to a higher production of reactive oxygen species (ROS) like hydrogen peroxide and superoxide (Sherer et al., 2001). In my study, rotenone was used to stress the cells. The overall cell count was influenced neither by thujones nor by rotenone (Figure 27). This is not reflected by the rotenone-induced increase of propidium iodide uptake in all rotenone conditions on an equally percentage level. Sage derived substances do not counteract or aggravate the rotenone increased uptake of PI (Figure 28). Interestingly, the LDH activity wasn't significantly higher in the rotenone control condition (Figure 29). This indicates an impairment of the cell membrane and a binding of PI to the DNA but not a full lysis and excretion of LDH in the media. This may point to a very small population of cells affected by the treatment, as mentioned above, which was also found in the high glutamate experiments. Similar to the PI data (Figure 28), the combination of thujone or essential oil with the stressor has a higher LDH activity compared to the control (Figure 29). It is not obvious why thujones alone reduce the LDH activity at the first glance, but data may correspond to an increase in dopaminergic neuron numbers (Figure 31).

Nonetheless, the increase of THir neurons cannot explain the massive decrease in LDH activity, so I clarified whether the thujones might directly interact with the LDH detection system by performing experiments in a cell-free system (Supplement 2). Additionally, the possible radical scavenging ability of thujones wasn't able to counteract or fortify the disturbance of the mitochondrial membranes induced by rotenone (Figure 30). Metabolism of dopamine by monoamino oxidases leads to the production of peroxide, which can undergo Fenton reaction with Fe^{2+} to build even more harmful hydroxyl radicals and Fe^{3+}. Ferric iron can be reduced by neuromelanin to ferrous iron, to be available for another round of Fenton reaction. Neuromelanin is an oxidation product of catecholamines like dopamine. Therefore, dopaminergic neurons are highly susceptible to complex I inhibition. Consequently, the rotenone concentration was lowered to 10 nM. Thujones and the essential oil has no influence on the damaging effect of rotenone while rotenone reduced the number of dopaminergic neurons (Figure 31).

5.3.3. Effects of α-thujone, β-thujone and sage essential oil under stress caused by low level of glucose

Treatment with glutamate or with rotenone tends to result in an impairment of the mitochondria and consequently end up in oxidative stress. Lowering the glucose level in the media should not lead to oxidative stress, but disturbances of energy metabolism. Lowering the glucose concentration in the cell culture was not aiming to be lethal itself, which is verified by Hoechst 33342 cell count, PI uptake of dead cells, and LDH activity in the treatment media (Figure 32, 33, 34), but to elicit possible harmful effects of thujones when cultured in low glucose medium. Nonetheless, also in low glucose medium, thujones do not display any toxic effect. The sage derived substances itself had a mild but significant positive effect seen by the LDH activity and mitochondrial integrity, but also in this test models, the positive effect couldn't counteract the moderate negative effect of the lowered glucose level in the cell culture media (Figure 34, 35). The number of TH^+ cells wasn't influenced in cultures with low glucose media when co-treated with thujones (Figure 36).

Due to the fact that neurons are supported by glial cells *in vivo* but also in cell cultures, a decrease in the energy supply by lactate can be adapted. Glial cells compensate the direct carbohydrate deficit by lactate production and exocytosis (Zilberter et al., 2015). During the cultivation time in the high glucose environment, glial cells can build up a sufficient glycogen reservoir (Brown et al., 2003). In the hypoglycemia phase during the treatment, this glycogen is converted to lactate (Gallagher et al., 2009). Via monocarboxylate transporters, neurons ingest the lactate and convert it into pyruvate. Pyruvate is than used for ATP production and therefore for maintaining the ATP homeostasis (Demetrius et al., 2015). The glycogen reservoir of the glial cells is able to compensate a hypoglycemia treatment for 20 min in slice cultures (Brown et al., 2003). Therefore, a compensation for 48 hours is implausible but lowering the effect to an insignificant level is possible. Accordingly, a significant change in the number of dopaminergic neurons by lowering the glucose content in the treatment media wasn't seen.

6. Conclusion

In my thesis, the following questions could be answered:

-Is it possible to synthesise β*-thujone from* α*-thujone?*

The small availability of educts made it difficult to guarantee the purity necessary for the treatment of the cell culture. Nonetheless, the mixture of α/β-thujone was highly enriched in β-thujone (99.6%) and suitable for the treatment after following the protocol.

-Do β*-thujone and essential sage oil exert a toxic effect in the whole cell culture or to dopaminergic neurons in particular?*

In this study, neither β-thujone nor essential sage oil show a remarkable negative effect to the whole primary cell culture to dopaminergic neurons in particular.

-Could stress in the cell culture be elicited by 3 different models?

Glutamate:

A small effect could be seen in the number dopaminergic neurons. In all the other parameters the chosen concentrations show no effect. Therefore, the stressor glutamate only mildly elicited stress in the cell culture.

Rotenone:

The disturbance of the mitochondrial respiratory chain via rotenone is an adequate agent to evoke stress in the primary cell culture. The effects are from mild to moderate in the used parameters.

Low level of glucose:

Lowering the level of glucose in culture was delicate because of the thin line between stress and cell death in the whole cell population. This might be the reason for the very mild effects.

-Do stress in the cell culture enhance the possible effect elicited by α*-thujone,* β*-thujone or essential sage oil?*

Even under stress conditions the effect of the thujones and the essential sage oil was very mild. Most of the effects was by the stressor itself and wasn't increased by the sage derived substances.

To conclude, the hypothesis could be confirmed that thujones show low toxicity in the cell culture model used in my study. Nonetheless, it must be noted that further studies should be done to determine their stability in cell culture medium and to include investigations on thujone metabolites. Binding to $GABA_A$ receptors was proposed to be the main mode of action of thujones. Supporting the GABA hypothesis, I would expect to detect a toxic effect of thujones at least by co-cultivating high concentrations of glutamate, which was not the case in my experiments.

Finally, whether *Salvia* consumption is definitely unhazardous cannot be conclusively answered before the toxicity on other tissues is determined.

7. Summary

Since the nineteenth century, absinthe and later in the early twentieth century thujones were under suspicion to evoke neurotoxic effects. Until now, the manufacturing and trade of thujone containing products is still strictly regulated by the council of Europe. Thujones, especially the α- form, was previously demonstrated as a negative allosteric modulator of the $GABA_A$ receptor which might elicit epileptiform activity and thereby, the neurotoxic effect in the brain.

In this thesis, α- and β-thujone and an essential sage oil was used to investigate if the postulated neurotoxic effect could also be seen in murine a primary mesencephalic cell culture model. Data do not show an influence of the thujones and the essential oil on the whole cell population and the dopaminergic neurons in particular.

Furthermore, three stress model conditions should clarify if the postulated negative effect of the thujones was absent because conditions in the cell culture are optimized and the medium rich of nutrients. Concomitant treatment of glutamate, rotenone and a lower level of glucose and α-thujone, β-thujone and essential sage oil did also not show a high toxicity of the sage derived substances. So, the results of the study do not support the pro-epileptiform activity of thujones while further investigations are mandatory to finally confirm or falsify the $GABA_A$ hypothesis.

Further, this study may contribute to the discussion of the hazardousness related to the consumption of thujones by *Salvia*-containing food and absinthe intake.

8. Zusammenfassung

Seit dem neunzehnten Jahrhundert stand Absinth und später, im frühen zwanzigsten Jahrhundert, die Thujone unter dem Verdacht neurotoxisch zu sein. Bis jetzt ist die Herstellung und der Handel von Thujon-haltigen Produkten vom Rat der europäischen Union streng geregelt. Thujone, insbesondere die α-Form, wurden zuvor als negativer allosterischer Modulator des $GABA_A$-Rezeptors publiziert, dies könnte die epileptiforme Aktivität und damit die neurotoxische Wirkung im Gehirn hervorrufen.

In dieser Arbeit wurde untersucht, ob der postulierte neurotoxische Effekt von α- und β-Thujon, und einem ätherischen Salbeiöl auch in einem primären mesencephalen Zellkulturmodell der Maus gezeigt werden kann.

Die Ergebnisse zeigen keinen Einfluss der Thujone und des ätherischen Öls auf die Anzahl der Zellen in der gesamten Zellpopulation und der dopaminergen Neuronen im Speziellen.

Darüber hinaus sollten drei Modellbedingungen klären, ob der postulierte negative Effekt der Thujone auf die Zellviabilität zumindest unter Stresskonditionen detektierbar ist, da die Bedingungen in der Zellkultur optimiert sind und das Medium generell sehr nährstoffreich ist. Die Behandlung mit Medium, welches sehr hohe Konzentrationen von Glutamat, hier als Exzitotoxizitätsmodell, Rotenon oder einen niedrigen Glucose-Gehalt im Medium und den Thujonen zeigte ebenfalls keine hohe Toxizität der Salbei-Inhaltsstoffe oder dem Öl. Die Ergebnisse der Studie stützen daher nicht die Hypothese der pro-epileptiformen Aktivität von Thujonen, wobei weitere Untersuchungen erforderlich sind, um die $GABA_A$-Hypothese endgültig zu bestätigen oder zu falsifizieren.

Darüber hinaus kann diese Studie zur Diskussion der eventuellen Gesundheitsgefährdung beitragen, die mit dem Verzehr von Thujonen durch Salbei beinhaltende Lebensmittel und der Absinthaufnahme verbunden ist.

9. References

Akhondzadeh, Shahin et al. 2003. "Melissa Officinalis Extract in the Treatment of Patients with Mild to Moderate Alzheimer's Disease: A Double Blind, Randomised, Placebo Controlled Trial." *Journal of Neurology Neurosurgery and Psychiatry* 74(7): 863–66.

Alam, M., and W. J. Schmidt. 2002. "Rotenone Destroys Dopaminergic Neurons and Induces Parkinsonian Symptoms in Rats." *Behavioural Brain Research* 136(1): 317–24.

Amtsblatt der Europäischen Gemeinschaft (EEC), 1988, Richtlinie des Rates zur Angleichung der Rechtsvorschriften der Mitgliedstaaten über Aromen zur Verwendung in Lebensmitteln und über Ausgangsstoffe für ihre Herstellung (88/388/EWG)

Albertson, Asher J., Sidney B. Williams, and John J. Hablitz. 2013. "Regulation of Epileptiform Discharges in Rat Neocortex by HCN Channels." *Journal of Neurophysiology* 110(8): 1733–43.

Bonkovsky, Herbert L. et al. 1992. "Porphyrogenic Properties of the Terpenes Camphor, Pinene, and Thujone. (with a Note on Historic Implications for Absinthe and the Illness of Vincent van Gogh)." *Biochemical Pharmacology* 43(11): 2359–68.

Bonkovsky HL and Schady W, The neurological manifestation of acute porphyria. Semin Liver Dis 2: 108-124, 1982.

Bormann, Joachim. 2000. "The 'ABC' of GABA Receptors." *Trends in Pharmacological Sciences* 21(1): 16–19.

Brecht, Stephan et al. 2001. "Caspase-3 Activation and DNA Fragmentation in Primary Hippocampal Neurons Following Glutamate Excitotoxicity." *Molecular Brain Research* 94(1–2): 25–34.

Brown, Angus M., Selva Baltan Tekkök, and Bruce R. Ransom. 2003. "Glycogen Regulation and Functional Role in Mouse White Matter." *Journal of Physiology* 549(2): 501–12.

Chuquet, Julien, Pascale Quilichini, Esther A. Nimchinsky, and György Buzsáki. 2010. "Predominant Enhancement of Glucose Uptake in Astrocytes versus Neurons during Activation of the Somatosensory Cortex." *Journal of Neuroscience* 30(45): 15298–303.

Committee on Herbal Medicinal Products, 2011. "Public Statement on the Use of Herbal Medicinal Products Containing Thujone." *ReVision* 44(January): 1–9.

Committee on Herbal Medicinal Products, 2010. Public statement on *Salvia officinalis* (EMA/HMPC/41843/2009)

Council of Europe 1999, Document RD 4.2/14-44. Revised Detailed Datasheet on Thujone

Czyzewska, Marta M., and Jerzy W. Mozrzymas. 2013. “Monoterpene α-Thujone Exerts a Differential Inhibitory Action on GABAA Receptors Implicated in Phasic and Tonic GABAergic Inhibition.” *European Journal of Pharmacology* 702(1–3): 38–43. http://dx.doi.org/10.1016/j.ejphar.2013.01.032.

Deiml, T. et al. 2004. “α-Thujone Reduces 5-HT3 Receptor Activity by an Effect on the Agonist-Induced Desensitization.” *Neuropharmacology* 46(2): 192–201.

Del Castillo, J.; Anderson, M.; Rubottom, G. M.: Marijuana, absinthe and the central nervous system. Nature 253:365–366;1975.

Demetrius, Lloyd A., Pierre J. Magistretti, and Luc Pellerin. 2015. “Alzheimer’s Disease: The Amyloid Hypothesis and the Inverse Warburg Effect.” *Frontiers in Physiology* 6(JAN): 1–20.

Duan, Yuntao, Robert A. Gross, and Shey Shing Sheu. 2007. “Ca2+-Dependent Generation of Mitochondrial Reactive Oxygen Species Serves as a Signal for Poly(ADP-Ribose) Polymerase-1 Activation during Glutamate Excitotoxicity.” *Journal of Physiology* 585(3): 741–58.

Eadie, M. J. 2009. “Absinthe, Epileptic Seizures and Valentin Magnan.” *Journal of the Royal College of Physicians of Edinburgh* 39(1): 73–78.

Eidi, Maryam, Akram Eidi, and Massih Bahar. 2006. “Effects of Salvia Officinalis L. (Sage) Leaves on Memory Retention and Its Interaction with the Cholinergic System in Rats.” *Nutrition* 22(3): 321–26.

French, Larry G. 2011. “Isolation and Structure Elucidation of the Terpene β-Thujone from Cedar Leaf Oil.” *Journal of Chemical Education* 88(6): 829–31.

Fróes, Maira M. et al. 1999. “Gap-Junctional Coupling between Neurons and Astrocytes in Primary Central Nervous System Cultures.” *Proceedings of the National Academy of Sciences of the United States of America* 96(13): 7541–46.

Gali-Muhtasib, Hala, Christo Hilan, and Carla Khater. 2000. “Traditional Uses of Salvia Libanotica (East Mediterranean Sage) and the Effects of Its Essential Oils.” *Journal of Ethnopharmacology* 71(3): 513–20.

Gallagher, Clare N. et al. 2009. “The Human Brain Utilizes Lactate via the Tricarboxylic Acid Cycle: A 13C-Labelled Microdialysis and High-Resolution Nuclear Magnetic Resonance Study.” *Brain* 132(10): 2839–49.

Gao, Yurong, Sungwoo Kim, Yun Il Lee, and Jaemin Lee. 2019. “Cellular Stress-Modulating Drugs Can Potentially Be Identified by in Silico Screening with Connectivity Map (CMap).” *International Journal of Molecular Sciences* 20(22).

Ghorbani, Ahmad, and Mahdi Esmaeilizadeh. 2017. “Pharmacological Properties of Salvia Officinalis and Its Components.” *Journal of Traditional and Complementary Medicine* 7(4): 433–40.

Gupta RC, Mitatovic D. Insecticides. In: Biomarkers in toxicolo-gy. Elsevier Inc 2014; pp. 389-407.

Hall, Adam C. et al. 2004. “Modulation of Human GABA A and Glycine Receptor Currents by Menthol and Related Monoterpenoids.” *European Journal of Pharmacology* 506(1): 9–16.

Hamidpour, Mohsen, Rafie Hamidpour, Soheila Hamidpour, and Mina Shahlari. 2014. “Chemistry, Pharmacology, and Medicinal Property of Sage (Salvia) to Prevent and Cure Illnesses Such as Obesity, Diabetes, Depression, Dementia, Lupus, Autism, Heart Disease, and Cancer.” *Journal of Traditional and Complementary Medicine* 4(2): 82–88. http://dx.doi.org/10.4103/2225-4110.130373.

Heinz, Sabrina et al. 2017. “Mechanistic Investigations of the Mitochondrial Complex i Inhibitor Rotenone in the Context of Pharmacological and Safety Evaluation.” *Scientific Reports* 7(February): 1–13.

Hirsch, Etienne C., and Stéphane Hunot. 2009. “Neuroinflammation in Parkinson’s Disease: A Target for Neuroprotection?” *The Lancet Neurology* 8(4): 382–97.

Höld, Karin M. et al. 2000. “α-Thujone (the Active Component of Absinthe): γ-Aminobutyric Acid Type A Receptor Modulation and Metabolic Detoxification.” *Proceedings of the National Academy of Sciences of the United States of America* 97(8): 3826–31.

Höld, Karin M., Nilantha S. Sirisoma, and John E. Casida. 2001. “Detoxification of α- and β-Thujones (the Active Ingredients of Absinthe): Site Specificity and Species Differences in Cytochrome P450 Oxidation in Vitro and in Vivo.” *Chemical Research in Toxicology* 14(5): 589–95.

Isman, Murray B. 2006. “Botanical Insecticides, Deterrents, and Repellents in Modern Agriculture and an Increasingly Regulated World.” *Annual Review of Entomology* 51(1): 45–66.

Jha, Mithilesh Kumar, and Brett M. Morrison. 2018. “Glia-Neuron Energy Metabolism in Health and Diseases: New Insights into the Role of Nervous System Metabolic Transporters.” *Experimental Neurology* 309(May): 23–31. https://doi.org/10.1016/j.expneurol.2018.07.009.

Johnston, Graham A.R. et al. 2006. “Modulation of Ionotropic GABA Receptors by Natural Products of Plant Origin.” *Advances in Pharmacology* 54(06): 285–316.

Köhler, 1887, Köhler's Medizinal-Pflanzen in naturgetreuen Abbildungen mit kurz erläuterndem Texte. Atlas zur Pharmacopoea germanica, austriaca, belgica, danica, helvetica, hungarica, rossica, suecica, Neerlandica, British pharmacopoeia, zum Codex medicamentarius, sowie zur Pharmacopoeia of the United States of America, Gustav Pabst

Kritis, Aristeidis A., Eleni G. Stamoula, Krystallenia A. Paniskaki, and Theofanis D. Vavilis. 2015. “Researching Glutamate – Induced Cytotoxicity in Different Cell Lines: A Comparative/Collective Analysis/Study.” *Frontiers in Cellular Neuroscience* 9(March): 1–18.

Li, Minhui et al. 2013. “An Ethnopharmacological Investigation of Medicinal Salvia Plants (Lamiaceae) in China.” *Acta Pharmaceutica Sinica B* 3(4): 273–80. http://dx.doi.org/10.1016/j.apsb.2013.06.001.

Liang, Yanran et al. 2017. “Rifampicin Inhibits Rotenone-Induced Microglial Inflammation via Enhancement of Autophagy.” *NeuroToxicology* 63: 137–45. http://dx.doi.org/10.1016/j.neuro.2017.09.015.

Ling, Nicholas. 2003. “Rotenone—a Review of Its Toxicity and Use for Fisheries Management PART 1. A REVIEW OF THE USE AND TOXICITY OF ROTENONE FOR FISHERIES MANAGEMENT PURPOSES.” *Sciece for Conservation* 211: 5–40.

Magnan V. Accidents déterminés par l'abus de la liqueur d'absinthe. Union Med 1864; 92&94: 227-32&257-62. [11] Marcé LV. Sur l'action toxique de l'essence d'absinthe. C R Acad Sci 1864; 58: 628-9.

Magnan V. On the comparative action of alcohol and absinthe. Lancet 1874; 104: 410-2.

Meschler, Justin P., and Allyn C. Howlett. 1999. “Thujone Exhibits Low Affinity for Cannabinoid Receptors but Fails to Evoke Cannabimimetic Responses.” *Pharmacology Biochemistry and Behavior* 62(3): 473–80.

Millet, Y., Jouglard J., Steinmetz M. D., Tognetti P., Joanny P. & Arditti J.:Toxicity of Some Essential Plant Oils. Clinical and Experimental Study 1485-1498 | Published online: 25 Sep 2008

Moldzio, R. et al. 2006. “Glutamate-Induced Cell Death and Formation of Radicals Can Be Reduced by Lisuride in Mesencephalic Primary Cell Culture.” *Journal of Neural Transmission* 113(9): 1095–1105.

Moldzio, Rudolf et al. 2010. “Effects of Epigallocatechin Gallate on Rotenone-Injured Murine Brain Cultures.” *Journal of Neural Transmission* 117(1): 5–12.

National Toxicology Program, 2011, NTP technica report on the toxicology and carcinogenesis studies of α-, β-thujone (CAS No. 76231-76-0) in F344/N rats and B6C3F1 mice (Gavage studies)

Official Journal of the European Union, 2010, Directive 2010/63/EU of the european parliament and of the council on the protection of animals used for scientific purposes

Olsen, R. W. 2000. “Absinthe and γ-Aminobutyric Acid Receptors.” *Proceedings of the National Academy of Sciences of the United States of America* 97(9): 4417–18.

Ossipow WP: Über die Dosierung der Absinthessenz (essence d'absinthe cultivée) beim Hervorrufen von Anfällen experi- menteller Epilepsie bei Hunden. Monatsschr Psychiatr Neurol 1914, 85:516-525.

Padosch, Stephan A, Dirk W Lachenmeier, and Lars U Kröner. 2006. "Substance Abuse Treatment , Prevention , and Policy Absinthism : A Fictitious 19th Century Syndrome with Present Impact."*Substance abuse treatment prevention and policy*14(1):1–14. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1475830&tool=pmcentrez&rendertype=abstract.

Radad, Khaled et al. 2004. "Ginsenosides Rb 1 and Rg 1 Effects on Mesencephalic Dopaminergic Cells Stressed with Glutamate." *Brain Research* 1021(1): 41–53.

Radulović, Niko S. et al. 2017. "Toxic Essential Oils. Part V: Behaviour Modulating and Toxic Properties of Thujones and Thujone-Containing Essential Oils of Salvia Officinalis L., Artemisia Absinthium L., Thuja Occidentalis L. and Tanacetum Vulgare L." *Food and Chemical Toxicology* 105: 355–69.

Rampersad, Sephra N. 2012. "Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays." *Sensors (Switzerland)* 12(9): 12347–60.

Rieusset, Jennifer. 2018. "The Role of Endoplasmic Reticulum-Mitochondria Contact Sites in the Control of Glucose Homeostasis: An Update." *Cell Death and Disease* 9(3): 1–12.

Rivera, E. M. et al. 2014. "Central α- And β-Thujone: Similar Anxiogenic-like Effects and Differential Modulation on GABAA Receptors in Neonatal Chicks." *Brain Research* 1555: 28–35.

Schefzig, Marcus, 2017, Effekte von α-Thujon, Campher, Cineol und essentiellem Öl aus Salvia officinalis in neuralen Zellkulturen der Maus. bachelor thesis. Vienna: University of veterinary medicine

Scientific Committee on Food Commission, European. 2003. "Opinion of the Scientific Committee on Food on Carrageenan Opinion of the Scientific Committee on Food on Carrageenan." *Management* (March): 1–8.

Sherer, Todd B. et al. 2001. "Chronic Reduction in Complex I Function Alters Calcium Signaling in SH-SY5Y Neuroblastoma Cells." *Brain Research* 891(1–2): 94–105.

Simpson, Ian A., Anthony Carruthers, and Susan J. Vannucci. 2007. "Supply and Demand in Cerebral Energy Metabolism: The Role of Nutrient Transporters." *Journal of Cerebral Blood Flow and Metabolism* 27(11): 1766–91.

Sokoloff, L., 1960. Metabolism of the central nervous system in vivo. In: Field, J., Magoun, H.W., Hall, V.E. (Eds.), Handbook of Physiology -Neurophysiology. vol. III. American Physiological Society, Washington, D. C., pp. 1843–1864

Srinivasan, Ramalingam et al. 2020. “Antioxidant and Antiproliferative Potential of Bioactive Molecules Ursolic Acid and Thujone Isolated from Memecylon Edule and Elaeagnus Indica and Their Inhibitory Effect on Topoisomerase II by Molecular Docking Approach.” *BioMed Research International* 2020.

Sultan, Ahmed et al. 2017. “Thujone Inhibits the Function of A7-Nicotinic Acetylcholine Receptors and Impairs Nicotine-Induced Memory Enhancement in One-Trial Passive Avoidance Paradigm.” *Toxicology* 384(March): 23–32. http://dx.doi.org/10.1016/j.tox.2017.04.005.

Swanson, Chad J. et al. 2005. “Metabotropic Glutamate Receptors as Novel Targets for Anxiety and Stress Disorders.” *Nature Reviews Drug Discovery* 4(2): 131–44.

Szczot, Marcin, Marta Magdalena Czyzewska, Giovanni Appendino, and Jerzy Wladyslaw Mozrzymas. 2012. “Modulation of GABAergic Synaptic Currents and Current Responses by α-Thujone and Dihydroumbellulone.” *Journal of Natural Products* 75(4): 622–29.

Tepper, James M., and Christian R. Lee. 2007. “GABAergic Control of Substantia Nigra Dopaminergic Neurons.” *Progress in Brain Research* 160(06): 189–208.

Tisserand and Young, 2013, Essential Oil Safety: A Guide for Health Care Professionals, 2. Edition, Churchill Livingstone

Waidyanatha, Suramya et al. 2013. “Toxicokinetics of α-Thujone Following Intravenous and Gavage Administration of α-Thujone or α- and β-Thujone Mixture in Male and Female F344/N Rats and B6C3F1 Mice.” *Toxicology and Applied Pharmacology* 271(2): 216–28. http://dx.doi.org/10.1016/j.taap.2013.05.001.

Weisbord SD, Soule JB, Kimmel PL. Poison on line: acute renal failure caused by oil of wormwood purchased through the internet. N Engl J Med 1997;337:825-7

Wenzel, D.G., Ross, C.R., 1957. Central stimulating properties of some terpenones. J. Am. Pharm. Assoc. 46, 77–82.

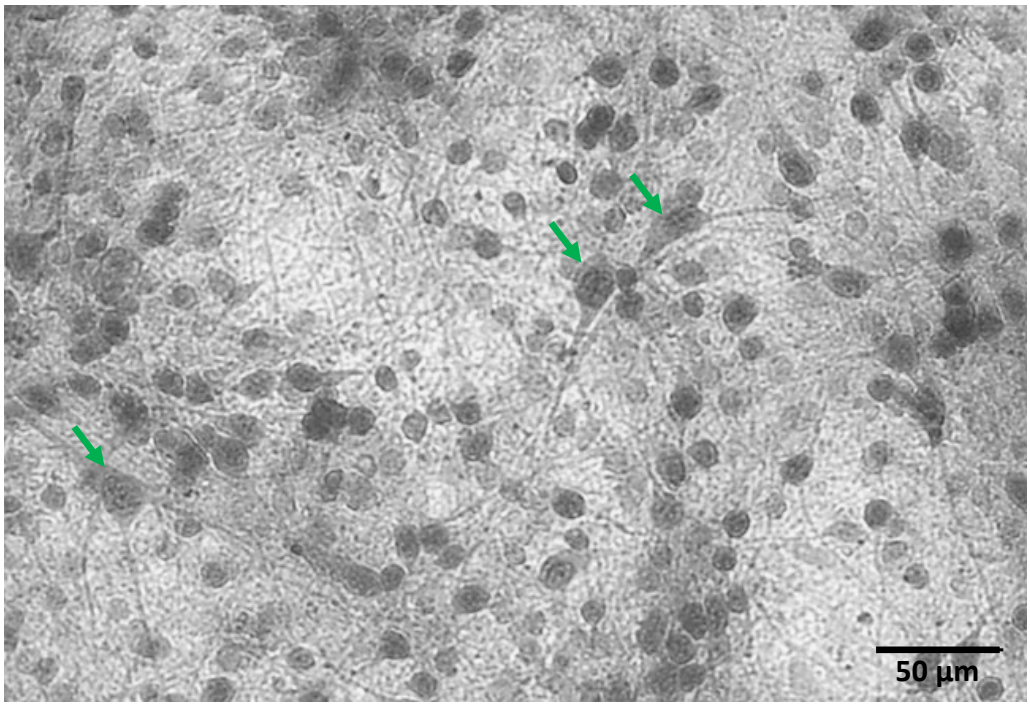
Whitling, H.T., 1908. “Cures” for asthma: fatal case from an overdose of oil of sage. Lancet 171 (4415), 1074–1075.

Yee, Andrew G., Peter S. Freestone, Ji Zhong Bai, and Janusz Lipski. 2017. “Paradoxical Lower Sensitivity of Locus Coeruleus than Substantia Nigra Pars Compacta Neurons to Acute Actions of Rotenone.” *Experimental Neurology* 287: 34–43. http://dx.doi.org/10.1016/j.expneurol.2016.10.010.

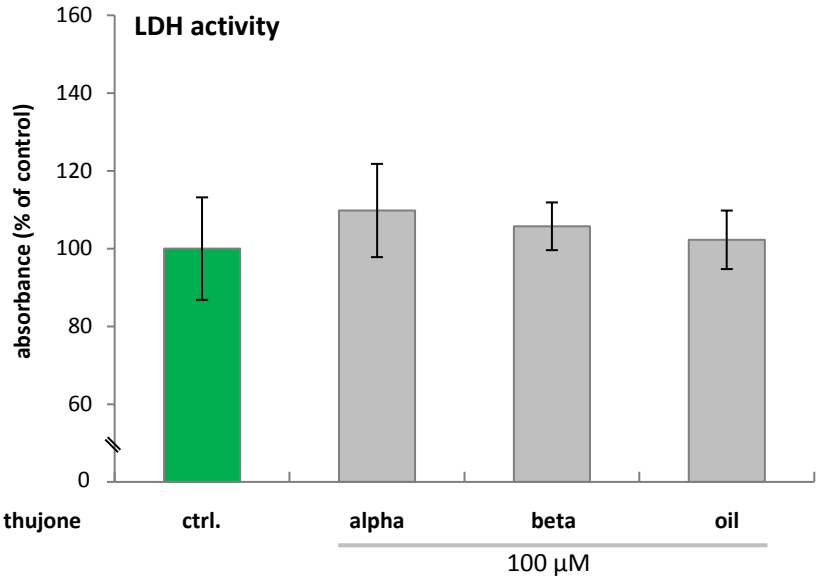
Zámboriné Németh, Éva, and Huong Thi Nguyen. 2020. “Thujone, a Widely Debated Volatile Compound: What Do We Know about It?” *Phytochemistry Reviews* 19(2): 405–23.

Zilberter, Yuri, Olena Gubkina, and Anton I. Ivanov. 2015. “A Unique Array of Neuroprotective Effects of Pyruvate in Neuropathology.” *Frontiers in Neuroscience* 9(FEB): 1–5.

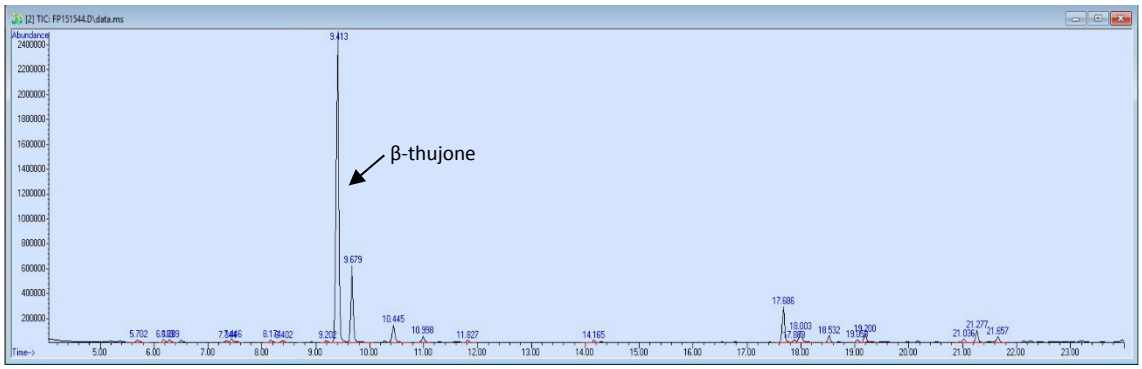
Supplement



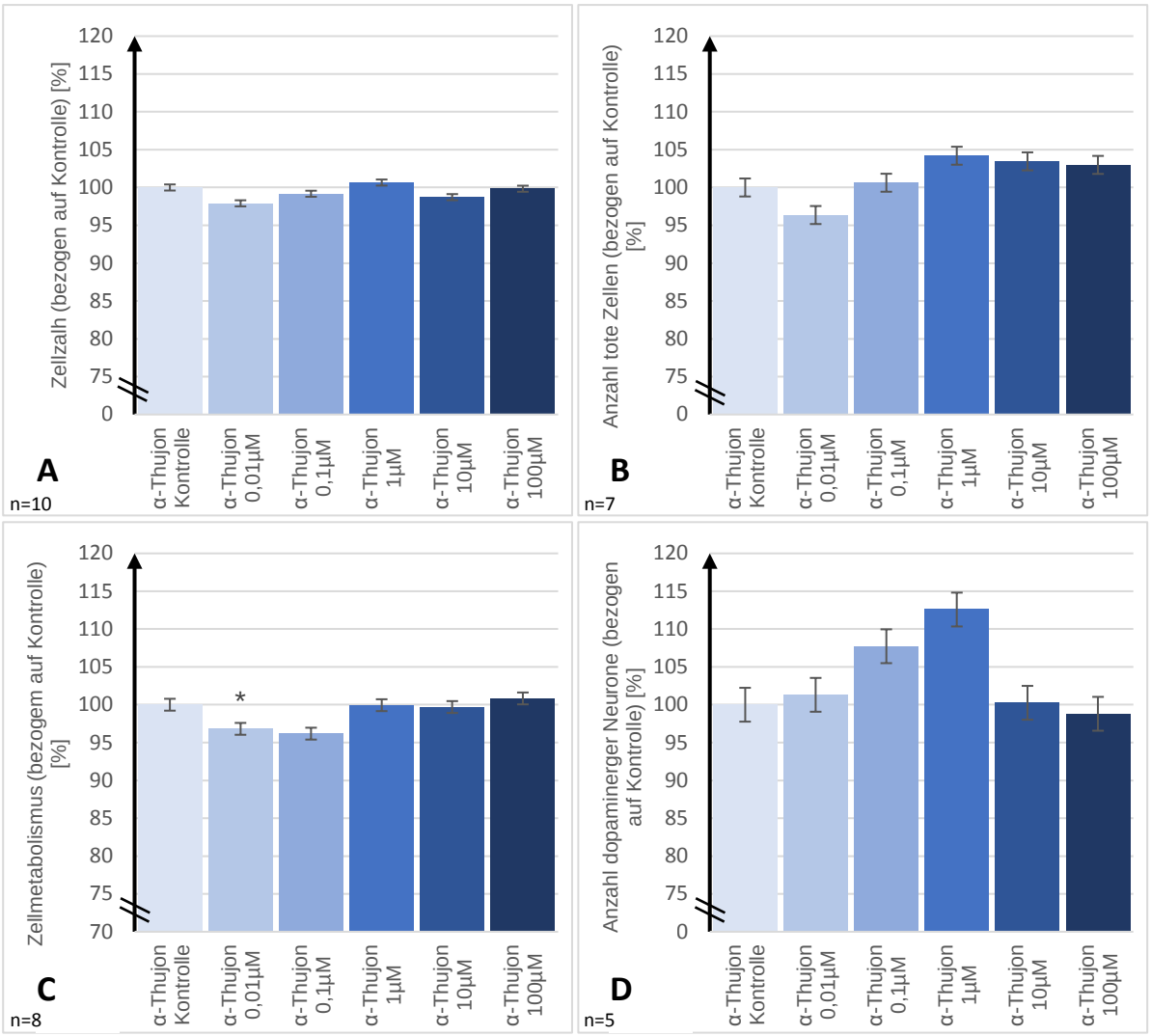
Supplement 1: Representative photographs of GABAergic neurons in the culture. To evaluate the contend and the presence of GABAergic neurons in the culture an immunohistochemical staining was performed with an anti-$GABA_A$ receptor antibody (anti-GABA A Receptor β2/3 Antibody, Sigma-Aldrich, Germany). Arrows indicate some of the $GABA_A$ positive neurons. The immunocytochemistry indicates rather the cytosolic subunit than the functional membrane-bond receptor.



Supplement 2: LDH activity in a cell-free system. Measurement was performed as described in material and methods. Instead of cell culture medium, a LDH solution of 0.5 µg/ml was used to exclude direct interaction between thujones and assay components.



Supplement 3: GC/MS analysis of the thujones containing *Salvia pomifera* essential oil. The GC/MS analysis revealed that the oil has a high amount of β-thujone and some other mono- and sesquiterpenes, which were not further evaluated substances.



Supplement 4: Preliminary data of α-thujone. Primary mesencephalic cell cultures were treated on DIV 12 for 48 h with different concentration of α-thujone and the status of the culture was determined using four different assays (**A**: Cell count with Hoechst 33342, **B**: PI uptake, **C**: overall metabolism, **D**: number of dopaminergic neurons). 100% correspond to the value of the untreated controls. Data are expressed as means ± SEM of **n** independent experiments, whereas each condition was carried out as duplicates. Statistical significance was determined with the Kruskal-Wallis (H)-test followed by the χ2-test ($p < 0.05$) and displayed with (*).