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Essential Oil (*Citrus Aurantium L.*) Induced Changes in Metabolic Function of Mitochondria Isolated from Yeast Cells *(Saccharomyces Cerevisiae)*.

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ABSTRACT

Essential oils (EOs) play some important roles as antimicrobial and antioxidant compounds. EOs act at several cell sites and target numerous subcellular organelles such as mitochondria because of its importance in a triggering of many pathologies. However, their subcellular toxic mechanism is still under investigation. This study is carried out in order to examine the effects of *Citrus aurantium L* essential oilon the morphology and function of mitochondria isolated from yeast cells *S. Cerevisiae.* Treatment with EOs extracted from *C. aurantium* affects the oxygen consumption of mitochondria respiration chain by a decrease in the rate of ADP phosphorylation (inhibition of State 3). Strong Stimulation of oxygen consumption in state 4 is observed. A decrease in the respiratory control ratio (CR) obtained is due to the disturbances observed in oxidations rates of states 3 and 4. At the same time, exposition to EOs extracted from *C. aurantium*depressed the mitochondrial membrane potential ($\Delta\Psi$) by the same manner as protonophore CCCP. In parallel, EOsinduce a reduction about 80% in ATP activity. Moreover, EOs treatment generated an important oxidative stress observed with all stimulation of bioindicators stress parameters (CAT, GSH and MDA). These results suggest that mitochondrial damage observed, resulting in the uncoupling of the oxidative phosphorylation, ATP synthesis, CR and ($\Delta\Psi$) which is the fundamental factor for the oxidative phosphorylation via the mitochondrial complex V, probably due to ROS produced during oxidative stress localized on the mitochondrial respiratory chain and which could be involved in the cytotoxic activity mechanism of EOs . Finally, EOs extracted from *C. aurantium*seem to exert their cytotoxic effects on the mitochondrial respiratory chain, probably by ROS generated disrupting the

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functioning of the mitochondrial complex V responsible for the energetic interactions, which are essential for physiological cell function.

Keywords: Essential oils, *C. aurantium*, Mitochondria, Respiration chain, Uncoupling.

Introduction

Essential oils have long occupied an important place in the lives of men and women who used them as perfume, aroma, food or treatment of several pathologies, (Farhat *et al.*, 2010). Actually, Essential oils arouse a totally renewed therapeutic interest. Essential oils enter the body using all types of administration and they can be easily absorbed by the tissues and reach all organs.

Currently, the therapeutic properties of essential oils make them a very important source of interest in various sectors due to the variety of their chemical and volatile components (Bakkali *et al.*, 2008, Ojo et al., 2016). All these properties allow essential oils to play a key role in antimicrobial control (Lahlou, 2004; Chassaing and Gewirtz, 2014). In Algeria, the use of essential oils is a growing field due to the importance and diversity of aromatic and medicinal plants, including bitter orange.

Studies show that bitter orange essential oils are composed of two mono terpenes: limonene as the majority constituent at 97.83% and myrcene at 1.43% in addition to an aldehyde: octanal at 0,45%. (Pultrini *et al*, 2006). These compounds give *Citrus aurantium* EOs an inhibitory action on many bacterial strains (Frassinetti *et al*, 2011).

Previously, EOs have been shown to exert their potential antimicrobial action through interference with the lipid bilayer of the bacterial membrane by disrupting its hydrophobic properties, leading to increased permeability, loss of cellular constituents, and acidification of the intracellular medium of the bacterium thus blocking the production of cellular energy, the synthesis of structural components which leads to the death of the bacterium (Caillet and Lacroix, 2007).

The disruption of membrane integrity affects its functioning both as a barrier but also as an enzymatic support for enzymes, particularly involved in energy metabolism (Gill *et al.*, 2006). Some studies (Dipasqua *et al*., 2006) show significant variations observed in the fatty acid

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composition of bacterial cell membranes (increase in unsaturated fatty acids) linked to changes in membrane fluidity. Other studies report that these compounds are effective against Leishmania (Monzote *et al*., 2007), and that the action mechanism observed would be linked to their interference in mitochondrial bioenergetic processes.

Mitochondria play a key role in basic cellular metabolism, in ATP synthesis, in calcium signaling, in cellular processes (apoptosis and necrosis) and especially in triggering many pathological processes (Goudjil *et al.,* 2022, Wallace, 2005; Gazotti *et al*, 1979). They can thus represent a privileged biological target for essential oils (Stani *et al*., 2007; Lambert *et al*., 2001; Gill and Holley, 2006; Moreira *et al*., 2006; Moreira *et al*., 2007).

The aim of this work is to study the effects of essential oils extracted from *Citrus aurantum L*. on the oxidative and phosphorylative properties of mitochondria isolated from yeast cells of *S. cerevisiae* in suspension. This research explores the action mechanism of these compounds, in particular at the level of the energy metabolism of the target organelles (mitochondria) which constitute the main sites of production of ATP, which is an essential compound for the physiological functioning of cells, and the disturbance of which could have serious effects on human health.

Material and Methods

Extraction of essential oils

The essential oil of *Citrus aurantium L*. is used for carrying out the experiments. The extraction is carried out from dried leaves and the essential oil content is determined by hydro-distillation using a Clevenger type apparatus . The device comprises a 2-liter flask containing a quantity of dry leaves and sufficient water (2/3), the whole is placed above a heat source for 3 hours. The oil-laden vapors are condensed in a refrigerant and the essential oils separate by density difference. The oil was collected in a glass tube and was kept at 4 ° C until further use. (Khadir *et al*., 2013). The essential oil yield is expressed by the amount of oil obtained per 100g of dry plant matter (Atailia *et al*., 2015).

Isolation of mitochondria

Yeast mitochondria were isolated from yeast cells suspension by the protocol described by Pan *et al.,* 2018. Mitochondria were isolated using a LC-MS compatible KPBS isolation buffer (180 mM KCl, 10 mM KH_2PO_4 (pH 7.2)) (Chen *et al*., 2016). Briefly, the

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harvested cells were first incubated in dithiothreitol buffer before being treated with zymolyase. The cells were washed and pottered in ice-cold KPBS buffer. Mitochondria were separated by differential centrifugation. After protein concentration determination using Bradford assay, mitochondria were aliquoted into 1 mg aliquots.

Proteins Assay

The mitochondrial proteins are determined according to the method described by Bradford (1976). The absorbance is measured by spectrophotometer (Jenway 6505) at the wavelength 596 nm. BSA is used as a standard protein.

Treatment of mitochondria by essential oil (EO)

Different concentrations of the essential oil of *C. aurantium L.*, (2µl/ml, 4µl/ml and 6µl/ml) are diluted in a solution of methanol, up to 0.2% of the final volume of the experiment and then incubated with mitochondria for 5 minutes at 25°C before the addition of succinate. Controls are carried out at the same time in the presence of 0.2% methanol.

Mitochondrial respiration and membrane potential activities

Determinations of oxidation rates in phosphorylation state 3 (Vox_3) and non-phosphorylation state 4 (Vox_4) are measured in parallel and simultaneously with potential membrane $\Delta\psi$ at 25°C using Clark electrode oxygen (Hansatech unit, UK), connected to a computer and a TPP^+ (TetraPhenylPhosphonium) sensitive electrode (Duthie *et al*., 1991; Davy de Virville *et al.*, 1994. The reaction mixture with a volume of 2ml contains 0.3M of mannitol, 10mM of phosphate buffer (KH_2PO_4/K_2HPO_4) and (pH7.2); 5mM of $MgCl_2$, 10mM of KCl and 1 $g.L^{-1}$ of BSA. Initiation of respiratory state 3 is obtained by adding 5 mM of succinate and a small quantity of ADP (30 mM). Respiratory state 4 is obtained after total consumption of the added ADP (ADP phosphorylated into ATP). The mitochondrial membrane potential ($\Delta\psi$) was monitored by evaluating transmembrane distribution of the (TPP^+) TPP^+ uptake was measured from the decreased TPP^+ concentration in the medium. At the end of each trace, the membrane potential is completely reduced by adding valinomycin (100 ng/mg of Mito. Protein). Carbonyl cyanide-m-chlorophenylhydrazone (CCCP), valinomycin and Tetraphenylphosphonium (TPP^+) were purchased from Sigma Chemical Corporation.

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Adenosine Triphosphatase Activity

ATPase activity determination is based on the assay of ATP by bioluminescence using the reaction catalyzed by the luciferin-luciferase association (Bomsel, 1973). The reaction mixture with contains 0.3M of mannitol, 10mM of phosphate buffer (KH_2PO_4/K_2HPO_4) and (pH7.2); 5mM of $MgCl_2$, 10mM of KCl, 1 $g.L^{-1}$ of BSA and 200 to 300 µg of membrane proteins. The reaction is triggered by addition of 1 mM ATP. Small aliquots of 10 µl are taken over time and then injected into 300 µl of mixture. The assay is carried out using a luminometer (Pico-ATP), a pneumatic injection system allowing the rapid introduction of 50 µl of luciferin-luciferase extract. The light energy is transformed into an electrical signal proportional to the quantity of ATP present in the tube. Standard solutions of determined concentrations of ATP are used.

Assay of Catalase Activity (CAT)

The method used for determination of catalase activity (CAT) in yeast is the method of Regoli and Principato, (1995).

Measurement of malondialdehyde

The proportionnig of malondialdehyde is carried out according to method of Draper and Hadley, (1990) by using the colorimetric method, based on the reaction of thiobarbituric acid with MDA.

Determination of GSH level

The level of total SH compound (GSH) was determined with Ellman's reagent according to Tukendorf and Rauser (1990). GSH was assayed by adding 2 ml of 0.5 Mm 5, 5' –dithio-bis - 2-nitro benzoic acid, (DTNB) prepared in 0.2 M phosphate buffer, pH 8.0 to appropriate volume of treated cell lines solution. The GSH reacts with DTNB and forms a yellow colored complex with DTNB. The absorbance at 412 mm was read after 2 min (Σ=13.6 $mM^{-1}cm^{-1}$).

Results

In this study we investigate the effects of Essential Oil "EOs" on the oxidative and phosphorylative properties of mitochondria isolated from yeast cellsand to understand the action mechanism of these EOs.

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1. Extraction of essential oils (EOs)

The weight yield in EO of *Citrus aurantium* is expressed in g per 100 g of plant material. It is 0.7%.

2. Effect of EOs on oxidative rate «state 3» in isolated yeast cellsmitochondria

Mitochondria isolated from yeast cells (*S. cerevisiae*) show good functional properties. The addition of succinate followed by ADP induces a high consumption of oxygen and consequently the oxidation rates in state 3. In the control mitochondria these rates vary from 97nmol.O_2/min/mg Prot. at 0 minutes up to 68nmol.O_2/min/mg Prot. at 8 minutes (Fig.1). The treatment of the mitochondria by the EOs causes an inhibition of the respiratory rate which reaches at 8 minutes 64nmol.O_2/min/mg Prot. at a concentration of 2µl/ml EOs, then 38nmol.O_2/min/mg Prot. at a concentration of 4µl/ml and finally 28nmol.O2/min/mg Prot. at the highest concentration of EOs (6µl/ml). It is observed that the 4 µl/ml and 6 µl/ml concentrations cause a very strong reduction in the respiratory rate (approximately 70% compared to the values obtained in the control mitochondria and nearly 50% compared to those obtained in the mitochondria treated with the 2 µl/ml and 4 µl/ml concentrations). These results show that EOs affect mitochondrial respiration through the values of oxidation rates obtained in respiratory rate state 3.

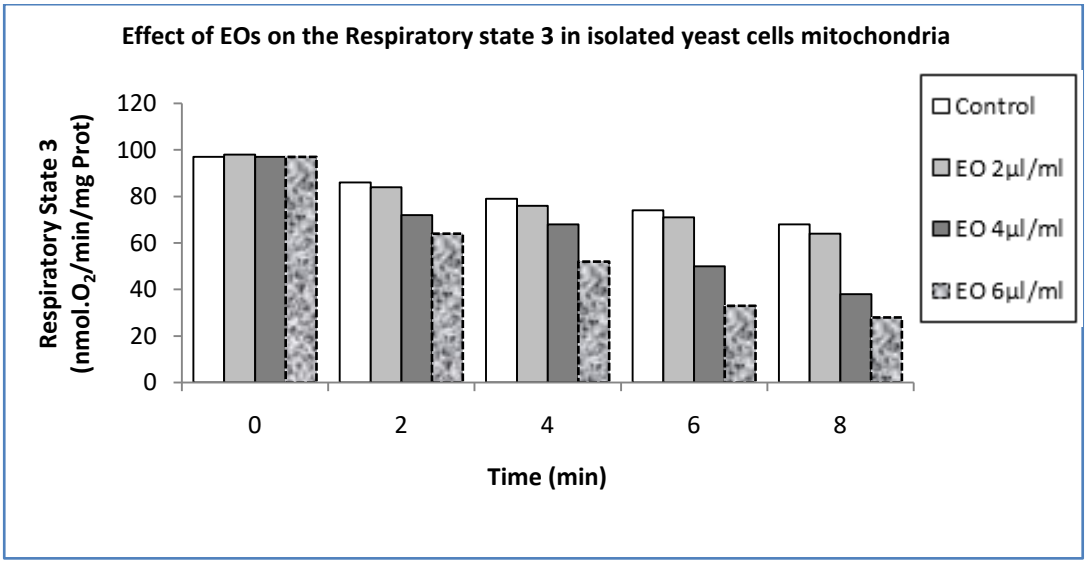

Figure 1. Effect of EOs on oxidative rate, state 3 in isolated yeast cells mitochondria.

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3. Effect of EOs on oxidative rate « state 4 » in isolated yeast cells mitochondria

The oxidation rates obtained in state 4 after total consumption of ADP (phosphorylation of ADP into ATP) show an oxygen consumption of the control mitochondria (13 to 14nmol.O_2/min/mg Prot. at the start of the kinetics) which will reach (20 nmol.O_2/min/mg Prot. at 8 minutes) (Fig.3). At the same time, treatment with the lowest concentration of EOs does not significantly modify the rate of oxidation in state 4. However, at 4µl/ml and 6µl/ml concentrations of EOs, a strong stimulation of respiratory state 4 is observed after treatment with EOs (28 and 30nmol.O_2/min/mg Prot., this value is 50% higher than that obtained in control mitochondria treated with 2µl/ml EOs). At the same time, we observe a stimulation of the oxidation rate in state 4 at 4µl/ml and 6µl/ml concentrations of EOs. This effect could reflect a movement of facilitated exchange of ions capable of affecting mitochondrial membrane permeability and consequently causing a dysfunction of the mitochondrial respiratory chain which could be the basis of a cellular energy disturbance.

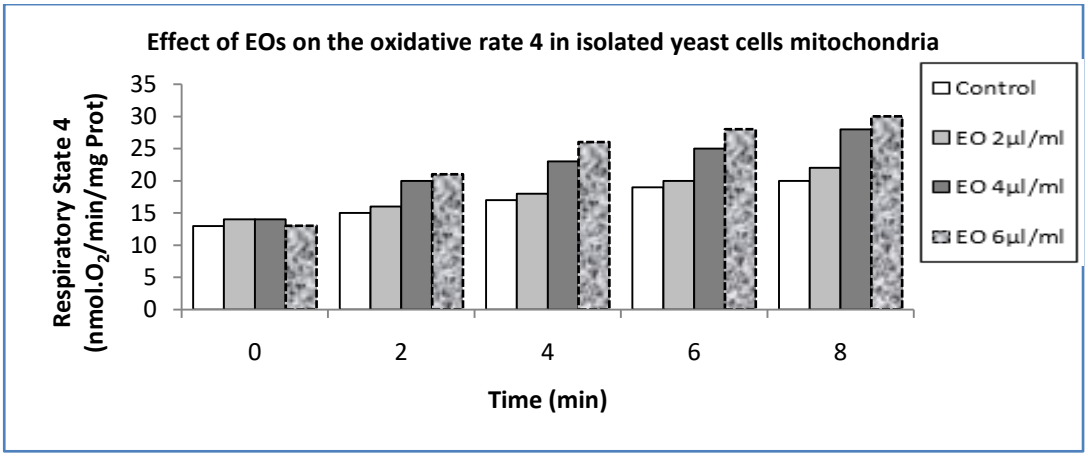

Figure 2. Effect of EOs on oxidative rate, state 4 in isolated yeast cells mitochondria.

4. Effect of EOs on Respiratory Control RC in isolated yeast cells mitochondria

In order to confirm the effects observed due to the EOs treatments on the oxidation rates in states 3 and 4, we determined the evolution of the RC respiratory ratios of mitochondria isolated from yeast cells (Fig. 4). It can be seen in this figure that the RC ratios decrease due to the decrease in oxidation rates in state 3 on one side and the increase in oxidation rates in state 4 on the other. A slight reduction in RC is thus observed at the lowest concentration of EOs (2µl/ml) followed by a strong reduction in RC after treatment with EOs at high concentrations.

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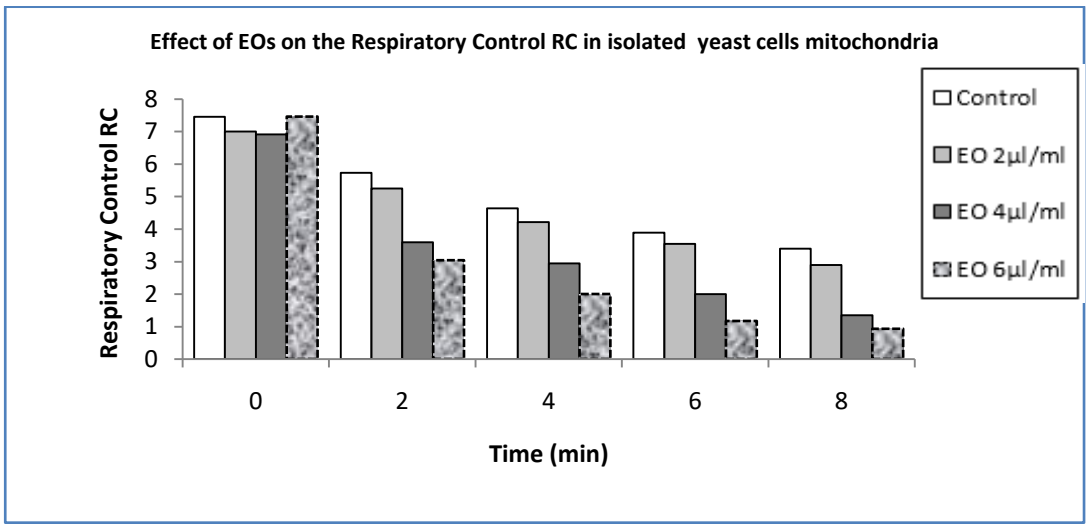

Figure 3. Effect of EOs on Respiratory Control «RC» in isolated yeast cells mitochondria.

5. Effect of EOs on membrane potential «ΔΨ» in isolated yeast cells mitochondria

In mitochondria, the membrane potential (ΔΨ) plays a key role in maintaining the physiological function of oxidation of respiratory substrates and phosphorylation of ADP to ATP by ATP-Synthase) of the respiratory chain of mitochondria. To understand the action mechanism of EOs, we treated isolated yeast cells mitochondria with a protonophore, the CCCP. Figure 4 shows the relationship between ΔΨ and oxidation rate.

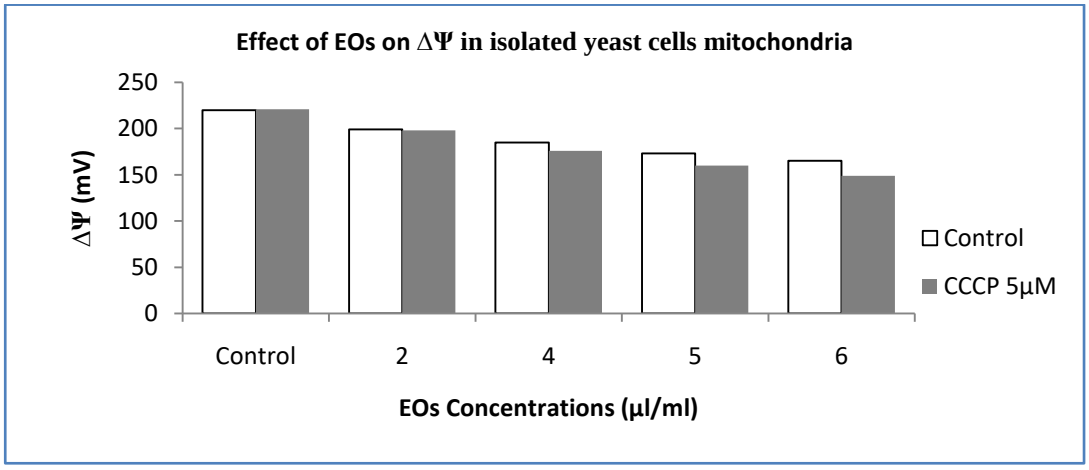

Figure 4. Effect of EOs and CCCP on membrane potential «ΔΨ» in isolated yeast cells mitochondria.

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We observe a similar evolution in mitochondria treated with either EOs concentrations or CCCP in the range of high of values of ΔΨ (between 221mv et 198mv). Below this value (EOs concentrations higher than 4µl/ml), the inhibiting effect of EOs on oxygen uptake was responsible for the loss of linearity in the relationships between oxidation rate and membrane potential. These results indicate that at concentrations between 2µl/ml and 4µl/ml, EOs exhibits uncoupling properties similar to those of the acidic protonophore CCCP. This could be explained by a similar mode of action between these two compounds at the level of the functioning of the mitochondrial respiratory chain of the isolated organelles.

6. Effect of EOs on ATP activity in isolated yeast cells mitochondria

To confirm the effects of EOs on oxidative phosphorylating coupling respiratory chain of mitochondria, we examine the effects of EOs on the ATPase activity.

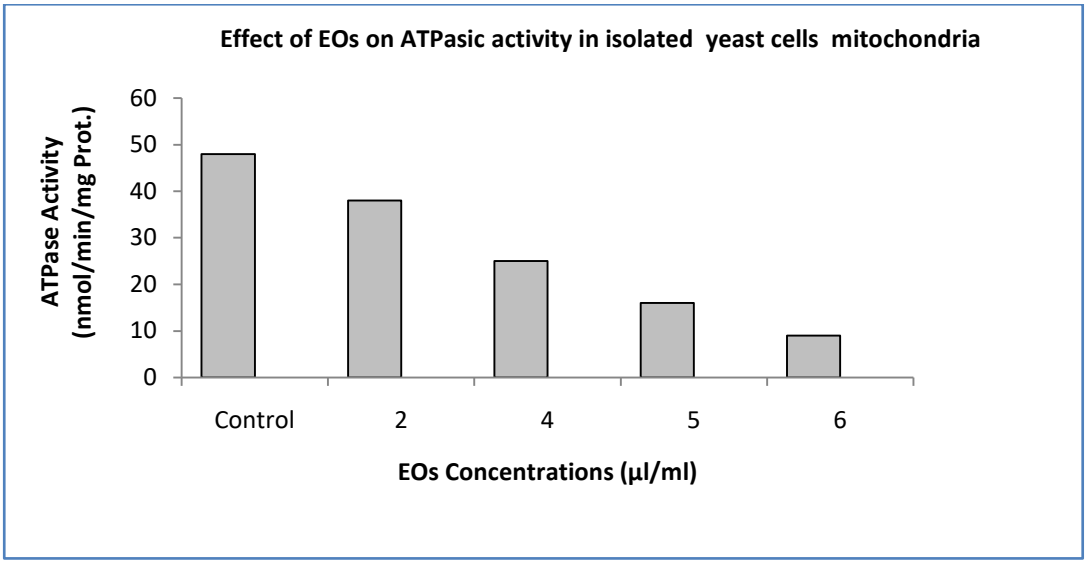

Figure 5. Effect of EOs on ATPase activity in isolated yeast cells mitochondria.

Figure 5 shows the effect of EOs concentration on the respiration of mitochondria energized by addition of substrate succinate. Following stimulation of the respiration rate, ATP activity fall approximately 80%.

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7. Effect of EOs on stress bio-indicators parameters in yeast cells mitochondria

The level of GSH obtained according to the different concentrations of EOs is presented in Table 1.

Table 1. Effect of EOs on stress bio-indicators parameters in yeast cells mitochondria.

EOs (µl/ml)	GSH (mg/l)	Catalase (nmol/min/mg Prot.)	MDA level (µM/mg Prot.)
0	30	5	0.08
2	33	7	0.12
4	52	13	0.18
5	74	27	0.31
6	12	3	0.02

The levels of GSH obtained in the samples after treatment with EOs were of the order of from 30 to 74 mg/l. The concentration of 4µl/ml EOs showed the highest GSH level compared to the other strains. The lowest production of glutathione (30mg/l) is obtained in the samples treated with the lowest concentration of EOs (2µl/ml). At the highest concentration of EOs, the GSH levels obtained rather reflect the beginning of a toxic effect of EOs

The measurements of catalase enzyme activity showed an increase in the isolated cells Yeast mitochondria treated with different concentrations of EOs. Indeed, after EOs treatment, the level of catalase increased from 5 nmol/min/mg Prot. in controls (4.96 to acetone controls, data not shown) to 27nmol/min/mg Prot. in isolated cells Yeast mitochondria treated by 5µl/ml concentration of EOs, this level is 5 times higher than that recorded in the control mitochondria and those treated with the lowest concentration of EOs. Treatment with 6 µl/ml EOs reflect a toxic effect of this concentration on the isolated organelles.

Finally, measurements of Malondialdehyde level show an important (0.08 to 0,031) elevation in the Malondialdehyde levels of the isolated cells Yeast mitochondria treated with different concentrations of EOs.

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All these characteristic parameters of oxidative stress clearly show that treatment with EOs generates stress in mitochondria isolated from yeast cells, more particularly in the respiratory chain, which constitutes one of the main sites of Reactive Oxygen Species (ROS) production in cells.

Discussion

EOs have been shown to display several beneficial effects on human health. They contain a variety of volatile molecules (terpenes, phenol-derived aromatic components and aliphatic components) which allow them to act as antioxidants. The molecular mechanisms responsible for most of the biological effects of EOs are not fully elucidated, and the toxicity targets have not been completely identified. Some authors seem to explain theses effects by an important perturbation of the lipid membrane structure of the microorganism (Gill *et al.,* 2006; Brul et Cotte, 1999).

At the same time other results shown that EOs can affect the inner membrane of subcellular organists such as mitochondria (Helander *et al.,* 1998). Following all these observations, we focused our attention on mitochondria, which seem to represent the main intracellular targets of EOs, particular by its complexes forming the respiratory chain. (Orrenius *et al.,* 2007).

EOs are extracted from orange leaves. The results obtained show that the essential oil extracted from *Citrus aurantium* has a yield of around 0.7%. This value is close to that reported by Hamdani (2015) in the region of Chlef in Algeria, which is 0.73%. It is higher than the values revealed by Boussaada and Chemli (2007) obtained in Tunisia and which are 0.5%. On the other hand, higher values (1.2%) are obtained by EL-Akhal *et al.,* (2014) in Morocco. These differences in values can be explained by the influence of many factors on the yield such as the extraction method chosen, the geographical origin of the plant studied, the storage, drying and grinding conditions (Ghalem *et al.,* 2014) but also by genetic, climatic and edaphic factors of the harvesting region (Lagha-benamrouche *et al.,* 2017).

Exposure of isolated mitochondria to EOs elevated significant level of CAT, GSH and MDA profile, testes Higher production of theses parameters shows oxidative stress in isolated yeast mitochondria. [Cherait and Djebar, 2013; Kujumdzieva *et al*., 2002].

Secondly, we examined the effects of essential oils from *Citrus aurantium* on the oxido-phosphorylating properties of isolated mitochondria. We found that EOs cause Vox_3 reduction

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followed by Vox_4 stimulation. The stimulation observed in state 4 could be explained by a leak of H^+ protons. At the same time, the ratios of the respiratory controls (State3 /State4) obtained decrease due to the inhibition of Vox_3 and the stimulation of Vox4. This seems to indicate an effect of decoupling of the mitochondrial respiratory chain caused by the EOs of *Citrus aurantium*, (Wallace and Starkov, 2000).

The decrease in membrane potential observed in the presence of EOs is of the same type of magnitude as that observed following treatment with the protonophore CCCP. This result highlights a possible inhibition mechanism of the regenerative energy capacities of isolated mitochondria. (Diolez and Moreau, 1985).

The decrease in Vox_3 recorded associated with the decrease in membrane potential could be the cause of a disturbance in ATP synthesis (Wallace, 2005). In order to confirm this, we measured changes in the ATPase activity of mitochondria treated with different concentrations of EOs. The results obtained show a strong reduction in the ATPase activity of the mitochondria (about 80%). The reduction in membrane potential observed associated with the increase in Vox_4 would reflect a phenomenon of uncoupling of the mitochondrial respiratory chain. (Dorta 2003, Naseer 2017). This phenomenon would be induced by a disturbance of $\Delta\mu H^+$ (leakage of H^+ through the ATPase complex) following treatment with EOs, which would partly explain the stimulation of Vox_4 and the reduction of $\Delta\mu H^+$ leading to an uncoupling of the respiratory chain associated with an inhibition of ATPase activity, hence a significant loss of energy essential for cellular functioning. (Cardoso *et al.,* 2001; Vesga *et al.,* 2014; Abrahim *et al.,* 2003). Thus the EOs of *Citrus aurantium* exerts its cytotoxic action by a disturbance of the mitochondrial respiratory chain and probably by direct action on the ATPase complex (Mnatsakanyan and AnnJonas, 2020; Giovanna *et al.,* 2019).

Conclusion

It appears that EOs treatment can promote oxidative stress thus activating the detoxification system with an induction in the level of GSH, CAT and MDA. EOs provoked also a decrease in Vox_3 associated with the decrease in membrane potential showing an important reduction in the ATPase activity of the isolated mitochondria. These modifications are probably linked to the accumulation of ROS caused by the presence of EOs (oxidative stress). Finally, the present study demonstrates that EOs induced cytotoxicity by a disturbance of the

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mitochondrial respiratory chain and probably by direct action on the ATPase complex and uncoupling of the mitochondrial respiratory chain.

Conflict of interests

The authors declare no conflicts of interest.

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