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The Protective Effect of Grape Seed and Skin Extract and *Ulva rigida* Against Oxidative Stress Induced by Cisplatin on The Testis of Rats

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Abstract

Cisplatin, an anticancer drug used in chemotherapy, has made considerable progress recently; particularly in the treatment of testicular cancer. However, the side effects of this treatment, limit its use. Grape Seed and Skin Extract (GSSE) and *Ulva rigida* (*U. rigida*) marine seaweeds are giving compounds with high antioxidant capacity. This work aims to develop a model in vivo to evaluate the protective effect of extracts of GSSE or *U. rigida* against the oxidative stress induced by cisplatin. This stress occurring in healthy cells is one of the major causes of the adverse effects of chemotherapy. Healthy male rats received an intraperitoneal injection of cisplatin only or cisplatin and GSSE or *U. rigida* extract. At the end of the treatment, the rats were sacrificed and from the crushed testicles; the supernatant was recovered for biochemical assays. The data showed that the dose of cisplatin induces testicular toxicity, and a pro-oxidant state characterized by increased levels of malondialdehyde, carbonyl proteins, superoxide anion, calcium and iron. Besides, treatment with cisplatin increases the activity of superoxide dismutase and inhibits the activity of catalase. GSSE or *U. rigida* extract exerts a protective effect on the testes of cisplatin-treated rats, or they protect against the adverse effects of oxidative stress induced by cisplatin to restore levels near control. This study demonstrated that GSSE and *U. rigida* can protect against cisplatin-induced cytotoxicity.

Keywords: Cisplatin, GSSE, *U. rigida*, Oxidative Stress, Pro-Oxidant, Antioxidant.

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1. Introduction

Cancer presents a global public health challenge that affects countries around the world becoming one of the leading causes of death. According to the World Health Organization (WHO), the number of cancers could reach 15 million by new cases per year from the beginning of 2020. Nowadays, medicine has several weapons against this scourge thanks to hormone therapy, radiotherapy and chemotherapy. Chemotherapy has significantly evolved in the last few decades thanks to the discovery of new chemotherapeutic molecules. However, this progress remains as every evolution a double-edged sword, because most anti-cancer treatments are often with side effects such as hair loss, nausea, vomiting and fatigue, caused mainly by inequality of the balance between pro and antioxidants generating oxidative stress in healthy cells. Some side effects may be limited or even avoided by appropriate care and medication. In chemotherapy, in particular, a lot of progress has been made in recent years to improve the quality of life of patients treated with chemotherapy. Synthesis chemical's molecules are used as pretreatment or post-treatment to reduce damage from chemotherapy. Nevertheless, most of these molecules did not give a satisfactory result because of the toxicity associated with certain chemical molecules or because of their intolerance. It is thus important to continue to develop new molecules with greater tolerance and less toxicity. Currently, searches are increasingly moving towards the medicinal plants and herbal medicine known by their beneficial effects on health. In this context, our work aims to search in extracts of *Ulva rigida* (*U.*

rigida) and seeds and grape skin (GSSE: Grape Seed and Skin Extract) an antioxidant effect. This effect will be evaluated against the oxidative stress induced by cisplatin, a molecule used in chemotherapy on the testes of healthy rats, to minimize the side effects related to this molecule during chemotherapeutic treatment.

2. Materials and Methods

2.1 Animals

Thirty male Wistar rats weighed between 110 and 290 g (8 to 10 weeks old) from the Pasteur Institute of Tunis were used for these experiments in accordance with the local ethics committee of Tunis University, and care of animals was in conformity with NIH recommendations (National Research Council 1985). They are raised and grown in the laboratory in a pet shop at a constant temperature (21 + 1°C) and submitted to a photoperiodic regime (12h dark/12h artificial light). Food and drink are provided an ad libitum.

2.2 Treatments

Rats were divided according to their weights and the type of injection into six lots into 6 groups of 5 animals each. Group 1 received ethanol 10% (control), group 2 ethanolic extract of garlic (2.5 g/kg bw), group 3 ethanolic extract of *U.rigida* (2.5 g/kg bw), group 4 cisplatin (5mg/kg/bw) as a dose that may induce a toxicity according to literature [1, 2], group 5 cisplatin plus GSSE and group 6 cisplatin plus *U.rigida*. Animals were daily administered IP with one of these treatments. The treatment was performed at fixed times for 7 days according to the following protocol:

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Table 1 : Protocol of follow-up of the different injections during 7 days

Group 1	D1		Ethanol 10%		D7
Group 2	D1		GSSE (2.5g/kg/bw)		D7
Group 3	D1		*U.rigida* (2.5g/kg/bw)		D7
Group 4	D1		Cisplatin (5mg/kg/bw)		D7
Group 5	D1	GSSE	Cisplatin (day 4)	GSSE	D7
Group 6	D1	*U.rigida*	Cisplatin (day 4)	*U.rigida*	D7

2.3 Preparation of the extract of *U. rigida*

The green seaweed *U. rigida* was harvested in spring in a rocky habitat on the sea coasts of the Ras Djebel region (Bizerte, Tunis) and transported in the ice to the laboratory. The samples were cleaned of the epiphytes, washed successively with distilled water, dried overnight at 30°C and then grounded with an electronic mortar to obtain a powder. The mixture is dissolved at the rate of 5g of powder in 30 ml of 10% ethanol and centrifuged at 5000 rpm for 30 minutes [3]. The supernatant is recovered and then injected to the animals intraperitoneally with a dose of 2.5mg/kg/bw after calculation and

2.4 Preparation of the extract of GSSE

We used the vinification waste of the *Carignan Vitis Vinifera* variety which is available in the north of Tunisia. GSSE extract is produced from residues of winemaking that were obtained from the wine cooperative Ain Ghelal, Tunis. It's an extract consisting of 50% seeds and 50% pulp. The mixture is dissolved at the rate of 5 g of powder in 10 ml of 10% ethanol, centrifuged at 3000 rpm for 10 min and thus the supernatant is recovered then injected to the animals intraperitoneally with a dose of 2.5g/kg/bw knowing that GSSE was used at

a dosage 500 mg/kg/bw and it was safe and near the optimal concentration recognized. Also, GSSE was tested at wide-ranging doses, reaching 4 g/kg/bw with no sign of toxicity [4].

2.5 Sacrifice of animals

The rats were sacrificed and the testes taken were weighed, crushed and then homogenized using an ULTRA-TURRAX in a Tris Base buffer solution (TBS, 50mM, pH=7.4) at a rate of 1g $2ml^{-1}$. The homogenates were centrifuged (10 000 rpm, 10 min, 4°C) and the supernatant is gently removed and placed in eppendorfs and stored at -20°C for subsequent biochemical assays.

2.6 Biochemical assays

2.6.1 Determination of superoxide anion $O_2^{°-}$:

DHE is a non-fluorescent compound that is rapidly oxidized to fluorescent ethidium (λ excitation=488 nm and λ emission=575 nm) under the action of $O_2^{°-}$. The samples are incubated with the dihydroethidine probe (DHE, 2μM) for 15 min in the dark. The stock solution of DHE was prepared in dimethyl sulfoxide (DMSO, 1.6mM). This solution is later used at the final

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concentration of 2µM. After incubation for 15min at 37°C, the analysis is performed by a fluorescence microplate reader (Bio-Tek FL800TBI).

2.6.2 Determination of malondialdehyde (MDA):

This colorimetric assay consists of reacting the thiobarbituric acid (TBA) reactive substance on one of the end products of lipid peroxidation (MDA) [5]. The reaction is carried out in acid and hot medium to form a pink coloring pigment whose intensity is proportional to the concentration of MDA in the sample. A 25µl of each sample is mixed with 125 µl TCA-BHT (trichloroacetic acid, Butylhydroxytoluene) and centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant was mixed with 0.5N HCl, 50 µl Tris-TBA (thiobarbituric acid) and then heated at 80°C for 10 minutes. After incubation at 80°C for 10 minutes, the absorbance measured at 530 nm is directly proportional to the amount of MDA present in the reaction medium.

2.6.3 Determination of carbonylated proteins:

The oxidation of the proteins by the ROS (reactive species of oxygen) leads to the formation of a carbonyl group (C=O) in the protein. The determination of the carbonylated proteins was carried out according to Levine et al., (1990) [6]. After precipitation of the proteins with 20% TCA and centrifugation at 11 000 rpm for 3 minutes at 4°C, the pellet then was on the edge in buffer with 10mM DNPH. Stirring was performed every 10min during the time of incubation. After 3 washes with ethanol-ethyl acetate, the pellet was dissolved in 20mM potassium phosphate containing 6M guanidine chloride. The density was then read at 366nm.

2.6.4 Determination of intracellular mediators

Calcium: By the use of a commercial kit (Biomaghreb, Tunis), we measured the intracellular calcium following Stern and Lewis, (1957)[7]. A purple colorful complex is formed after the interaction of calcium with cresolphtalein at basic pH, measurable at 570nm. Briefly, 50µL of testicular extract was added to 650µL of the mixture containing 2-amino-2-methyl-1-propanol (500 mmol L^{-1}), Cresolphthalein (0.62 mmol L^{-1}) and 8-hydroxyquinoline buffer (69 mmol L^{-1}). Assuming that the complex was stable for one hour, incubations were realized at room temperature for 5 min.

Iron: The level of free iron in the testes was determined by Leardi et al., (1998) [8] using a commercial kit (Biomaghreb, Tunis). At acidic pH 4.8, ferric iron Fe^{3+} is released from transferrin. Ascorbic acid reduces it to ferrous iron Fe^{2+}, which gives ferrozine a colored complex in violet measurable at 562 nm. A 50 µL of our samples was added to 250µL of reaction mixture containing ascorbic acid (5g L^{-1}) and ferrozine (40 mM) with incubation at 37°C for 10 min. The density is measured at a wavelength $\lambda=562$ nm.

2.6.5 Assaying the activity of antioxidant enzymes

The superoxide dismutase (SOD): The assay of SOD activity is based on the method described by Misra and Fridovich, 1972 [9] which relies on the use of epinephrine and bovine catalase to generate superoxide radicals. It relies on the ability of SOD to inhibit the oxidation of epinephrine to a pink-coloured compound, adenochrome, by trapping the superoxide anion flux. A 10µl of the sample is mixed in a solution of Na_2CO_3/$NaHCO_3$ buffer (62.5mM, pH 10.2) with 10µl of bovine catalase (0.4U $µl^{-1}$), 20

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μl of epinephrine (5mg ml^{-1}). The density is measured per minute for 1 to 4 minutes at λ=480 nm.

Catalase (CAT): The principle of the assay is based on the decomposition of H_2O_2 into H_2O and O_2 in the presence of catalase activity in the solution. The decomposition of H_2O_2 is determined by the fall in absorbance at λ = 480 nm. A 20 μl of the tissue extract is mixed in a solution of phosphate buffer (50 mM, pH 7) containing 33 mM H_2O_2. The catalase activity, in the samples, is evaluated using a spectrophotometer (Bio-Rad) followed by the kinetics of the disappearance of H_2O_2 at the wavelength of 240 nm [10].

2.6.6 Determination of total protein

It is a colorimetric assay using the Biuret method [11], which consists in quantifying the number of proteins contained in the sample. In fact, the proteins form blue-violet complexes in the presence of Cu^{2+} in an alkaline medium, so the quantity of the proteins is proportional to the intensity of the coloration. The density is determined at λ=546nm. The amount of protein contained in each sample was directly determined from a calibration curve. The proteins were measured using a commercial kit (Bio-Maghreb). A 20μl of the sample was mixed with 1ml of the working reagent. The reading of the density is carried out after mixing and incubation of the solution for 5min at room temperature.

2.7 Statistical test

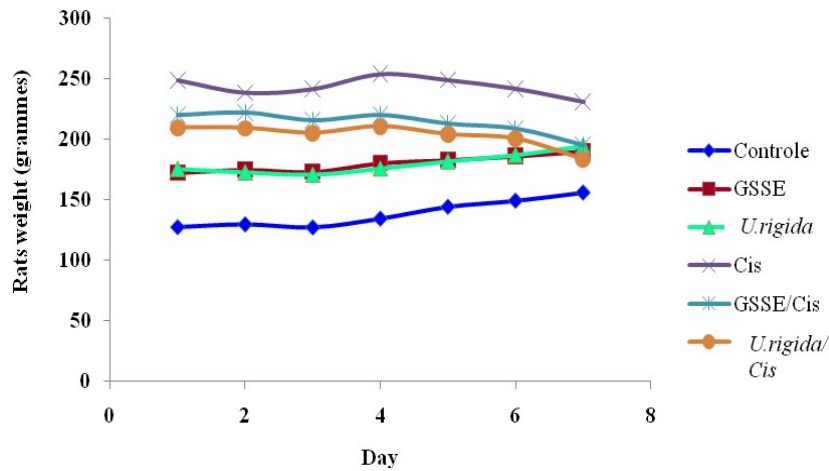Statistical analyses were performed using the GraphPadPrism software (GraphPad Software). The data are expressed on average (± SEM) of at least 4 independent animals. A two-way ANOVA test followed by a Bonferroni post-test is used for the intergroup comparison. The value found by the calculation can affirm that the groups are different with a risk of error p. The GSSE, *U.rigida* and Cisplatin groups are compared with the control group. The group treated with Cis and GSSE, Cis and *U.rigida* are compared with the Cisplatin group.

3. Results and Discussion

As a whole, our study consists at looking in the natural extracts of GSSE and *U.rigida*, a beneficial effect against the cytotoxicity induced by cisplatin, chemotherapeutical agent, in the cells of the testes of rats.

3.1 Effect of treatments on body weight

This study conducted to determine the variation of rats the weight in each batch. The rats are weighed each day at the same time; the variation is represented below at Figure 1.

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Figure1: Variation of the body weight of rats during 7 days of treatment. The results are expressed by the mean ± SEM (n = 5).

The variation in the body mass of the rats, during our study (Figure1), shows that the weight of the animals increases throughout the treatment period (7 days), except animals treated with cisplatin (cisplatin alone or in co-treatment with GSSE or *U. rigida*) where a non-significant reduction in the weight of the animals is observed.

In the present work, we observed an increase in the weight of the rats following treatment with an extract of GSSE or *U. rigida*. This corroborates the data showing that these extracts are used as nutritional supplements thanks to the plethora of minerals, proteins and vitamins [12, 13]. Treated rats by cisplatin only do not show a decrease in their weight which is not in correlation with other

cisplatin.

work which has shown that treatment with cisplatin induces a decreased body weight [14].

This could be explained by a difference between the duration of treatment used in both studies. In addition, the rats treated with extracts from GSSE or *U. rigida* with cisplatin revealed a decrease in their weight which could be explained by the toxicity of

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3.2 Effects of the treatments on oxidation and the production of $O_2^{\circ-}$, MDA and carbonylated protein
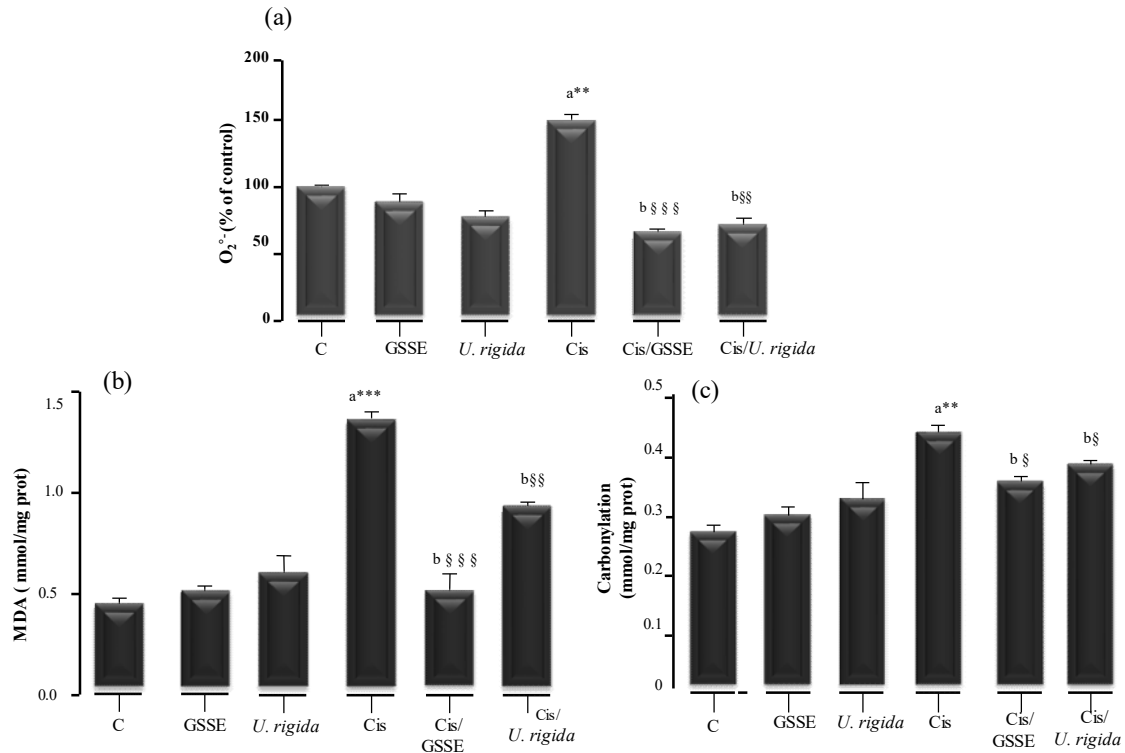
Figure 2. Effect of GSSE and *U.rigida* on cisplatin-induced changes in testicles lipoperoxidation, carbonylation and $O_2^{\circ-}$

Rats were on a daily basis administered with 10% ethanol (C), GSSE , *U.rigida* , cisplatin (Cis), *U.rigida* plus Cisplatin (Cis/*U.rigida*), or GSSE plus Cisplatin (Cis/GSSE) were administered to rats for 1 week (Fig. 2), $O_2^{\circ-}$ content (Fig. 2A), lipoperoxidation (Fig. 2B) and carbonylation (Fig. 2C) were determined. Results are expressed by means ±S.E.M. (n=5); **p < 0.01 where samples were compared with control (C). §§ p < 0.01where samples were compared with cisplatin (Cis).

We determined $O_2^{\circ-}$, MDA and carbonylated proteins as indexes of oxidative stress. Cisplatin significantly increased testis $O_2^{\circ-}$ (Fig 2A), MDA (Fig 2B) and carbonylated proteins (Fig 2C). Cisplatin has been described to induce testicular toxicity with stimulation of the production of free radical [15]. This has been confirmed in our study where we have detected in cisplatin-treated rats increased production of anion superoxide in the testicles. It is admitted that the superoxide radical crosses the biological membrane and its accumulation in cells causes damage irreversible oxidative effects on biomolecules including membrane lipids, proteins and nucleic acids [16]. This damage results in damaged cells that can lead to cell death. In agreement with these data, we have observed an increase in the level of MDA, a product of lipid peroxidation of acids polyunsaturated fat, an increase in the level

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of carbonylated proteins which is the consequence of the oxidation of proteins. Membrane lipid peroxidation at the level of

testicular has also been demonstrated in other studies [16, 17].

3.3 Effect of treatments on intracellular mediators of oxidative stress
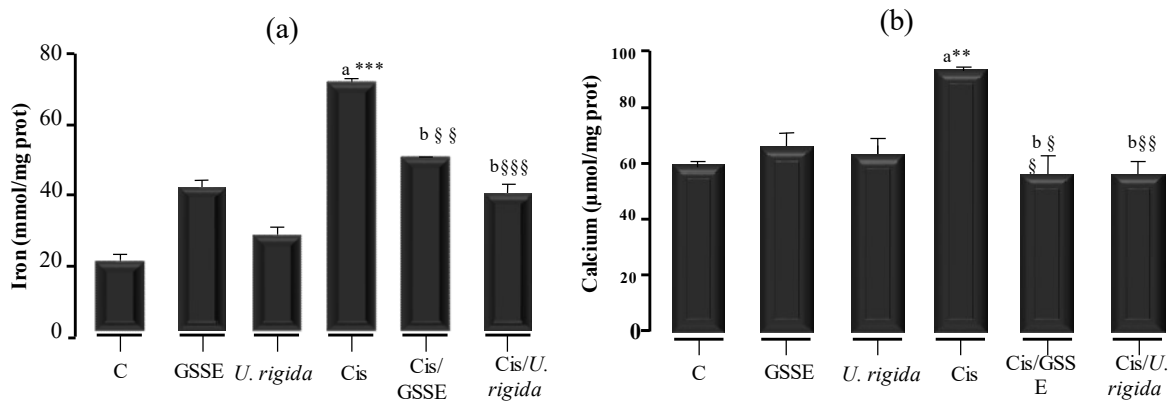
Figure.3: Effect of GSSE, *U.rigida* on cisplatin-induced intracellular mediators.

Rats were on a daily basis administered with 10% ethanol (C), GSSE, *U.rigida*, Cisplatin (Cis), *U.rigida* plus Cisplatin (Cis/*U.rigida*), or Cisplatin plus GSSE (Cis/GSSE) for 7 days and we determined levels of testis free iron (Fig. 3a) and calcium (Fig. 3b).

We expressed Results as means ±S.E.M. (n=5); **$p < 0.01$ where lots were compared with control (C). §§ $p < 0.01$ where lots were compared with cisplatin (Cis).

We next required determining the putative involvement of intracellular mediators in cisplatin, GSSE and *U.rigida* mode of action (Fig.3). We have seen an increase in calcium levels on treated rats by cisplatin (Fig 3.B). This increase could be the cause of cell death caused by the oxidative stress generated by the treatment. Indeed, the Ca^{2+} is the primordial trigger of apoptosis, it accumulates in the mitochondria increasing its permeability leading to the release of apoptogenic factors such as cytochrome C which will activate caspases leading to irreversible death of cells by apoptosis [18, 19]. Moreover, the massive entry of Ca^{2+} into the Mitochondria blocks the electron transport chain which results in excessive production of ROS.

It is well established that oxidative stress leads to increased concentration of iron following hemolysis and attack of transport proteins by ROS [20]. This increase in iron concentration (Fig 3.A) allows the mobilization of intracellular Ca^{2+} [21].

In fact, iron stimulates the production of the hydroxyl radical, following the Fenton reaction, resulting to activation of xanthine oxidase which causes elevation Ca^{2+} levels [22]. This is consistent with our results showing an increased cellular concentration of iron and Ca^{2+} in the testes of rats subjected to oxidative stress caused by cisplatin.

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However, pretreatment of animals with GSSE or *U. rigida* followed by an injection of cisplatin and then a post-treatment with these extracts shows a very significant decrease in the Ca^{2+} and iron levels in the

two groups compared to the rats treated with cisplatin alone.

3.4 Effect of treatments on the activity of antioxidant enzymes

(a)   (b)
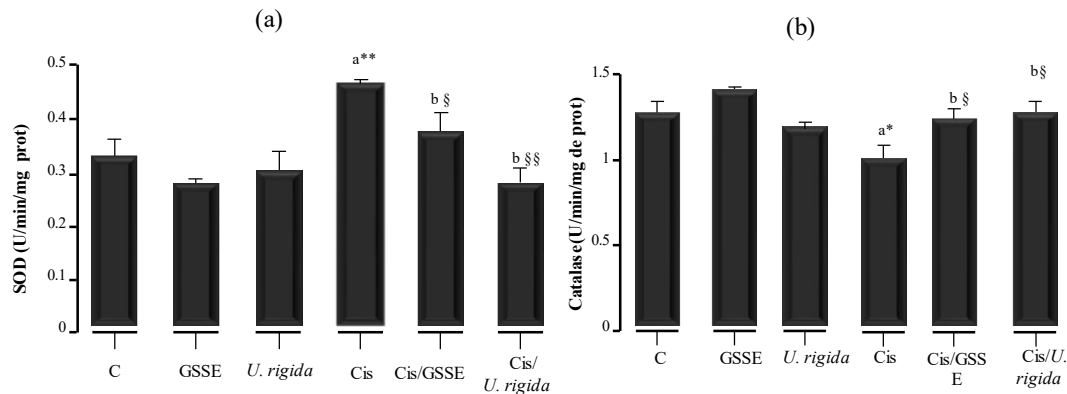
Figure.4: Effect of GSSE, *U.rigida* and cisplatin on testis antioxidant enzyme activities.

Rats were regulary administered with 10% ethanol (C), GSSE, *U.rigida*, Cisplatin (Cis), *U.rigida* plus Cisplatin (Cis/*U.rigida*) or Cisplatin plus GSSE (Cis/GSSE) for 7 days and testis free iron (Fig. 3a) and calcium (Fig. 3b) levels were outlined.

Results are described as means ±S.E.M. (n=5). ** $p < 0.01$ where lot where compared with control (C). §§ $p < 0.01$ where lots where compared with cisplatin (Cis).

In another part of our work, we followed the behaviour of the defensive enzymatic antioxidant in the presence of cisplatin and extracts of GSSE and *U. rigida*. The SOD and CAT are the first lines of defence against the toxic effects of ROS. Our results show that the activity of the SOD is largely stimulated at the level of testes of rats treated with cisplatin. It could be postulated that apart oxidative stress, the cells sought enzymatic defence. This issue does not agree with other results which showed a low activity of antioxidant enzymes including SOD [23]. Cisplatin upregulated SOD activity (Fig4.A) compared to control but decreased CAT (Fig4.B). Concerning CAT activities, cisplatin treatment appears to

disrupt its operation. This disturbance can be explained by the alteration of the structure of this enzyme. We noticed that the use of extracts from GSSE or *U. rigida* in pre-treatment by cisplatin and then post-treatment is not followed by the stimulation of the activity of the SOD. This suggests that these extracts can protect cells against stress oxidative so that the cells did not find the need to strongly stimulate the enzymatic defense to protect against oxidative stress-induced by cisplatin. In view of the activity of catalase, the treatment with the extracts makes it possible to recover an activity comparable to that measured in control rats. This proves the protective effect of the extracts against the alteration of catalase

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structure by the oxidative stress generated by cisplatin.

3.5 Overview of the effect of the extract of GSSE and *U.rigida* against oxidative stress product's

Treatment with the extract of GSSE or *U. rigida* protects against damage oxidative stress induced by cisplatin. It has been shown that the treatment of rats by these extracts decreases the production of the superoxide anion, one of the radicals responsible for the damage caused by oxidative stress. In addition, it has been shown that extracts of *U. rigida* or GSSE are able to protect lipids and cellular proteins from damage oxidative effects induced by oxidative stress. These results highlight the antioxidant potential of these extracts. In this context, work has revealed that the GSSE can protect against oxidative stress thanks to its power to trap ROS [4]. Even; Tian et al., (2018) [24] have shown that grape extracts have the ability to mitigate oxidative stress and to protect against inhibition of testosterone synthetase caused by cisplatin in the testes. Regarding *U. rigida*, previous work is done in our laboratory clearly demonstrate that this seaweeds possess a strong antioxidant power linked to the richness of its extracts in proteins, in compounds, phenolic and polysaccharides known for their remarkable antioxidant capacities [3, 12]. In addition, studies carried out in the laboratory showed that extracts of the seaweeds protect the cells against apoptosis induced by oxidative stress [25]. Moreover, our study reveals that extracts from the GSSE and *U. rigida* are devoid of any toxicity and has a high content of proteins, lipids, minerals and vitamins that have encouraged its extensive use as a dietary supplement for humans and animals [12, 26].

4.   Conclusion and Perspectives

Ultimately, all of this work contributes to the understanding of the molecular and cellular mechanisms involved in the toxicity of cisplatin on healthy cells during chemotherapy, and the beneficial antioxidant role of GSSE and *U.rigida's* extracts against this toxicity. These results strongly encourage the use of GSSE and *U.rigida* as an alternative to the use of molecules derived from chemical synthesis whose side effects are undesirable and often complicate.

In the perspective of this work, we will highlight the sterility and / or the decrease of fertility observed in rats treated with cisplatin. We will propose by biochemical and histological approaches to follow the effect of cisplatin on spermatogenesis and sperm mortality and to look for a protective effect in the extracts of GSSE and / or *U. rigida*.

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