Preventive effect of *Xanthoria parietina* polyphenols on the complications of diabetes in white rat

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Abstract: Polyphenols have shown antioxidant activity and an ability to prevent the toxic effects of oxidative stress in diabetes. The objective of this study was to evaluate the hypoglycemic and antioxidant effect of the methanolic extract of *Xanthoria parietina* on rats diabetes induced by streptozotocin (40 mg/g). The results obtained show that streptozotocin induces diabetes in the animal characterized by hyperglycemia, elevation of oxidative stress markers and a decrease of enzymatic and non-enzymatic antioxidant defense system. However, the methanolic extract results in a marked improvement in the antioxidant state in the liver. Indeed our results show a decrease in the malonyldialdehyde concentration of 25.91% and an increase in the reduced glutathione rate of 23.62%, an increase in the superoxide dismutase activity of 23.53% and catalase activity of 49.10%. The effect of the polyphenolic extract on the blood glucose level is tested on rats rendered hyperglycemic. The feeding with the extract showed a significant hypoglycaemic effect during 120 minutes of treatment. In conclusion, the present study suggests that *Xanthoria parietina* has a beneficial effect on the control of blood glucose, lipid profile and oxidative status, activating antioxidant enzymes and decreasing lipid peroxidation in the liver. Such treatments may help reduce the development of complications associated with diabetes.

Keywords: Diabetes, *Xanthoria parietina*, polyphenols, oxidative stress, antioxidant enzymes.

INTRODUCTION

It is recognized that high concentrations of glucose in extra and intracellular media induce an oxidative stress defined as an imbalance between the pro-oxidant and the antioxidants (Guerci *et al.,* 2001). A study of human endothelial cells shows that high concentrations of glucose increase antioxidant enzyme activities and the expression of the RNA of these enzymes. The cellular over expression of these antioxidant enzymes is a response to oxidative stress caused by high levels of glucose. Several mechanisms appear to be involved in the development of oxidative stress in the presence of high glucose concentration: auto-oxidation of glucose, protein glycation and polyol pathway auto-oxidation of glucose (Auberval, 2010). The body has developed very effective defense systems against the production of free radicals. The molecules controlling this production are referred to as "antioxidant". In addition to the specific substances of the body, food and plants ar*et al*so important sources of antioxidants (Pham-Huy *et al.*, 2008; Kalem *et al.*, 2012). Thus the extracts from some plants: the butanolique extract of *Ranunculus repens* L. (Kebiéche *et al.*, 2011), the ethanolic extract of *Anacyclus pyrethrum* L. (Sujith *et al., 2011*), the methanolic extract of *Nigella Sativa* L. (Houcher *et al.*, 2007) and others, are tested for their antidiabetic activity. Recently, studies have been focuses to methanol extracts of lichens for their antidiabetic activity (Fraser and Currier, 2007). Lichen is the result of symbiosis between a fungus, mycobionte and algae, phytobionte prokaryote (cyanobacteria in 10% of cases) or eukaryotic (green algae chlorophyceae) in 80% of cases, and in 5% of cases the three partners are associated (tripartite symbiosis) (Amirouche *et al*., 2008). Symbiosis as also produces secondary lichenous substances having interesting active properties in pharmaceuticals, bio - indicator and cosmetic (Hans, 2011). Thus, lichens are anti vomiting, stomachic, febrifuge and tonic, anti-anemic and anti-inflammatory (Bugni *et al.*, 2009). The objective of this study consists to evaluate the *in vivo* antioxidant and hypoglycemia effect of the lichen *Xanthoria parietina.*

MATERIAL AND METHODS

Samples were collected from locations in the region of Boumerdes from trunks of oak and olive trees. Harvests were conducted during March until June at the level of the forests of Zemmouri and Corso. The identification of the *Xanthoria parietina* (species) is carried out based on the general morphological characteristics, such as shape, color, height, orientation of the ends and the type of

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branching by using Bauwens's key of identification (Bauwens, 2003). The identification is also based on a colorimetric characterization of the thallus and the apothecia, according to the method described by Rashmi and Rajkumar (2014). Once harvested, the species *Xanthoria parietina* is dried in the open air in the absence of light for 72 hours (fig. 1) and then ground into a fine powder.

Extraction of polyphenols

The extraction was carried out by the method of Merghem *et al.* (1995). In order to proceed with depigmentation, 20 g of finely crushed dry vegetable matter are macerated in 60 ml of oil ether from 3 days. After 24 hour, filtered and kept the sediment once again in 60ml of oil ether. In the end, the filtrate was eliminated and the sediment recovered for the extraction of the polyphenols. The residues are macerated in 60ml of a mixture of methanol/water (60/40%) during 3 days. The filtration was carried out 3 times interrupted by a time interval of 24 hours where the filtrate is recovered each time and the sediment is undergoing another extraction by the same volume of the mixture of methanol/water during 24 hours. The three filtrates were combined and subjected to evaporation at 40°C and the Polyphenolic extract was stored for subsequent use. The yield of polyphenols was calculated using the following formula:

$$R(\%) = \frac{M - M0}{MT} \times 100$$

R (%): rate of the extracted material.
M: mass of the balloon with the extract (g).
M0: the mass of the empty balloon (g).
MT: total plant mass (g) used in extraction.

Determination of total polyphenols

Determination of the total polyphenols is carried out by a spectrophotometric method (Wong *et al.*, 2006), using the colorimetric reagent of Folin-ciocalteu. The concentration of the total polyphenols was determined as a function of a reference of a calibration curve made by the gallic acid standard extract at different concentrations under the same conditions as the sample. The results are expressed in mg of gallic acid equivalent of per gram of the powder.

Study of the biological activities of polyphenols of lichens

Study of antioxidant activity in vivo of polyphenols of lichens

For this study, 18 Wistar male rats with an average weight of 130 g are used and divided into 4 lots, of which three consist of 5 rats each and one lot of 3 rats as controls, according to the experimental protocol of Kebiéche *et al.* (2011). A first batch treated by Lichen extract at the rate of 10 mg/Kg, the second by Gallic acid (positive controls) at 200 mg/kg, the third batch control receives serum at 0.9 per cent. Animals received all these substances an hour and two hours after injection of Streptozotocin at a dose of 40 mg/Kg of body weight. Blood sugar was measured before experimentation and during the five days of treatment. At the end of the experiment, rats were killed and the livers were taken out in order to measure the activity of liver antioxidant enzymes.

Measurement of enzymatic activities in a Cytosolic fraction of liver

Hepatic reduced glutathione (GSH)

The dosage of the GSH was based on colorimetric method of Ellman (1959). For that, 1g of liver was homogenized in three volumes of trichloracetique (TCA) at 5% in a mortar and then centrifuged at 2000 rpm. The supernatant (50µl) was diluted in 10ml of phosphate buffer (0.1M; pH 8). To 3ml of mixture of dilution, added 20µl of DTNB (0.01M). The absorbance was read at a wave length of 412 nm against a white prepared under the same conditions with the TCA at 5%. The concentrations are expressed in nmole/g of liver.

Dosage of the malondialdehyde (MDA) in a Cytosolic fraction at 10% of liver

Hepatic MDA levels were evaluated according to the method of Ohkawa *et al.* (1979). To this end, 1 g of liver was added to 3ml of KCl solution (1.15M) and then ground. 0.5ml of 20% trichloroacetic acid and 1ml of 0.67% thiobarbituric acid (TBA) are added to 0.5ml of the homogenate. The mixture is heated at 100°C for 15 minutes. It is cooled and then 4 ml of n-butanol are added. After centrifugation at 3000 rpm for 15 minutes, the optical density is determined on the supernatant at the spectrophotometer at 530 nm. The amount of MDA in the sample is expressed in nmole / gram of liver tissue

Evaluation of the enzymatic activity of CAT

The enzymatic activity of CAT is determined by the method of Claiborne (1985). To perform this test, an enzymatic fraction is prepared according to the method of Iqbal *et al*. (2003). 2g of liver are cut in three volumes of phosphate buffer (0.1M, pH 7.4) containing KCl (1.17%). The homogenate is centrifuged at 2000 rpm for 15 minutes at 4°C. The supernatant obtained is centrifuged at 9600 rpm for 30 minutes at 4°C. The supernatant obtained is used for the evaluation of the enzyme activity (CAT and SOD). To this end, 1ml of phosphate buffer (KH_2PO_4, 0.1M, pH 7.2), 0.975ml of freshly prepared H_2O_2 (0.091 M) and 0.025ml of the enzyme source (cytosol) were mixed. The absorbance is read at 240nm every 30 seconds for two minutes, and enzymatic activity is calculated in terms of international unit per minute and per gram of protein (µl / min per gram of protein). The activity of the enzyme is expressed in units / mg of tissue protein (liver).

$$K = \frac{2.3033}{T} X \log \frac{A1}{A2}$$

K: Rate constant of the reaction.
A1: Absorbance in the first minute.

A2: Absorbance in the second minute.
T: Time-to-minute interval.
The activity of the enzyme is calculated according to the following equation:

$$U/mg = \frac{k}{n}$$

n: mass of proteins present in the volume of the sample used.

Evaluation of the enzyme activity of SOD

The evaluation of SOD was carried out on the cytosol by the method of Beauchamp and Fridovich (1971). A mixture consists of 300 µl of each of these solutions: NBT (nitroblue of tetrazolium), methionin, riboflavin (pH 7.8) and 5 µl of cytosol. This mixture is exposed to the light of a 15 W lamp for ten minutes to induce the photo-reaction of riboflavin and O_2. The reading is performed on a spectrophotometer at 560 nm. The enzymatic activity is calculated in terms of IU / mg of proteins. The activity of the enzyme is expressed in units /mg of tissue protein (liver). One unit of SOD activity is defined as the enzyme that would cause the inhibition of 50% of H_2O_2 autoxidation. It is calculated according to the following equation:

$$Total\ inhibition = \frac{\text{Optical density of white} - \text{optical density of sample}}{\text{White optical density}} \times 100$$

$$\text{SOD Unit} = \frac{\text{The total Inhibition}}{\text{n X 50}}$$

n: mass of protein in mg present in the volume of the sample used.

Study of the hypoglycaemic activity of lichen polyphenols

Glucose tolerance is the body's ability to metabolize glucose. The first objective of the oral glucose tolerance test (TTOG) is to test the sensitivity of cells to endogenous insulin (Wright *et al.*, 1980). In order to determine the hypoglycemic power of polyphenols extracted from lichen, 11 rats are divided into four groups. The principle consists in administering glucose (control batch), glucophage (standard batch) and lichenic extract (test batches) at the rate of 4g/kg, 2.5mg/Kg and 10g/kg, the control animals received the same volume in physiological water one hour before gavage of a glucose solution. The blood glucose evolution is monitored before and at 30 minutes over a period of 2 hours.

STATISTICAL ANALYSIS

Results are expressed as averages and standard error at mean (mean ± ESM). Statistical evaluation is carried out using Student's t-test using statistica software 6.

RESULTS

Identification by binocular lens

The morphological and phytochemicals characteristics of the study species are shown in tables 1 and 2.

Yield of the methanolic extract of Xanthoria parietina

The polyphenol yield of the methanolic extract calculated from the *Xanthoria parietina* powder revealed a high value of 51%.

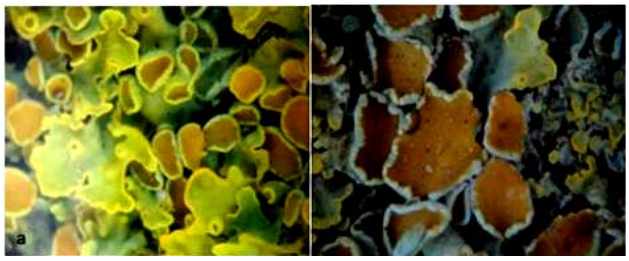


Fig. 1: Lichen *Xanthoria parietina* before (a) and after (b) drying.

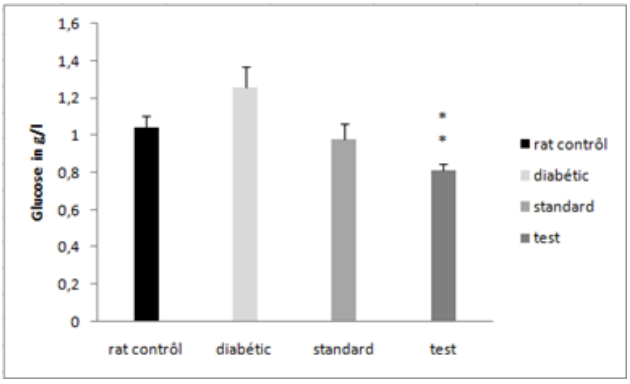


Fig. 2: Evolution of blood glucose in diabetic rats, treated with gallic acid and lichen extract after five days of experimentation (p□ 0.05).

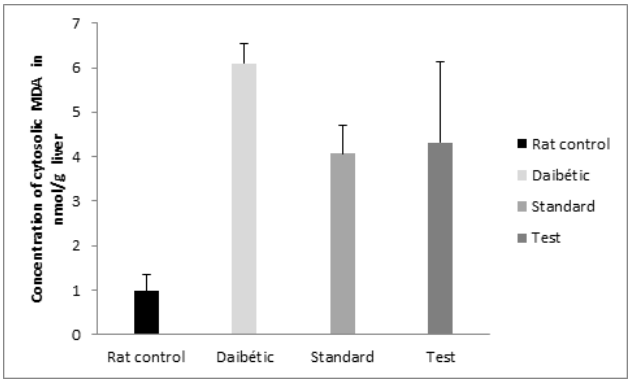


Fig. 3: Evolution of cytosolic MDA levels in diabetic rats treated with gallic acid and lichen extract after five days of experimentation.

Determination of polyphenols

The results obtained show that the extract of *Xanthoria parietina* is very rich in polyphenols with a value of 858.18µg gallic acid equivalents /g of dry plant material, referring to the calibration curve of gallic acid.

Evaluation of antioxidant activity in vivo

Evolution of blood glucose

The results obtained indicate a decrease in blood glucose levels in the batch treated with lichen extract (0.81g/l± 0.03) from the first day after treatment. This decrease was

statistically very significant (p℟0.01) at the end of the experiment compared to diabetic control rats (1.26g/l± 0.1). However, in the batch treated with gallic acid (0.97g/ l ± 0.08) this difference is not significant (fig. 2). This efficiency of the lichenic extract is demonstrated by the calculation of the percent reduction in blood glucose which is equal to 30% and that of the gallic acid to 24% relative to the diabetic control rats.

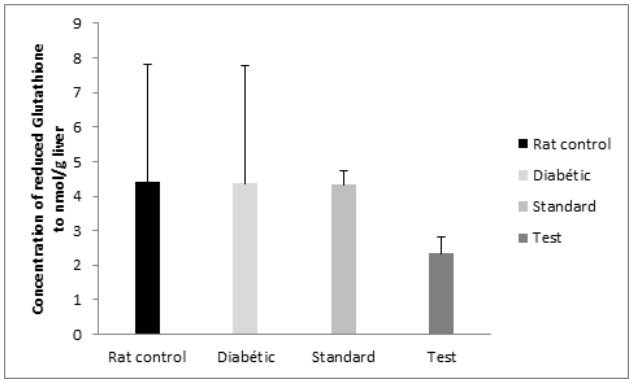


Fig. 4: Evolution of cytosolic reduced Glutathione levels in diabetic rats treated with gallic acid and lichen extract after five days of experimentation.

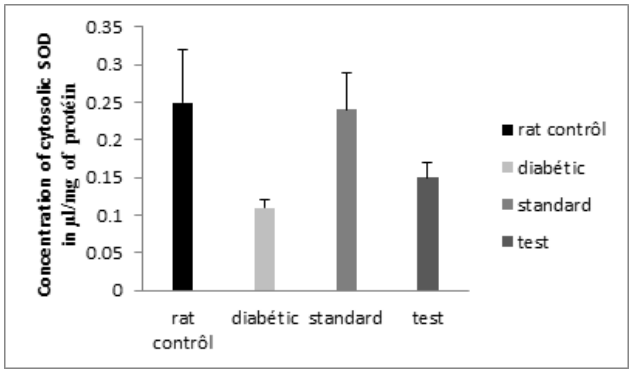


Fig. 5: Evolution of cytosolic superoxide dismutase (SOD) levels in diabetic rats treated with gallic acid and lichen extract after five days of experimentation.

Analysis of oxidative stress parameters

Effect of polyphenols on lipid peroxidation

According to the results obtained, the treatment of diabetic rats with gallic acid and the lichenic extract led to a 33% and 29% reduction in the levels of the oxidative stress marker, malondialdehyde (MDA) in Liver cells compared to the diabetic controls. The respective values are: 4.07mmol/g±0.62; 4.32mmol/g±1.8; 6.09mmol/ g±0.45. However, these values remain statistically insignificant (fig. 3).

Effect of polyphenols on the cytosolic level of Glutathion peroxidase

The results obtained for the cytosolic levels in the liver cells of reduced glutathione show a reduction of 49.5% in the diabetic rats treated with the lichenic extract. No change was observed in diabetic rats treated with gallic acid (0.09%) compared to diabetic controls. The respective average values were 2.34nmol/g ±0.48; 4.33 nmol / g±0.41 and 4.37nmol / g±3.4 (fig. 4).

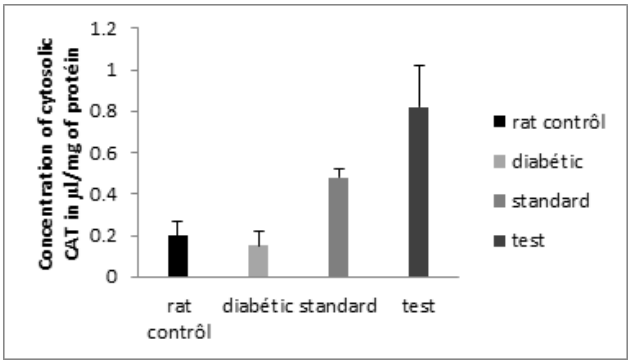


Fig. 6: Evolution of cytosolic catalase (CAT) levels in diabetic rats treated with gallic acid and lichen extract after five days of experimentation.

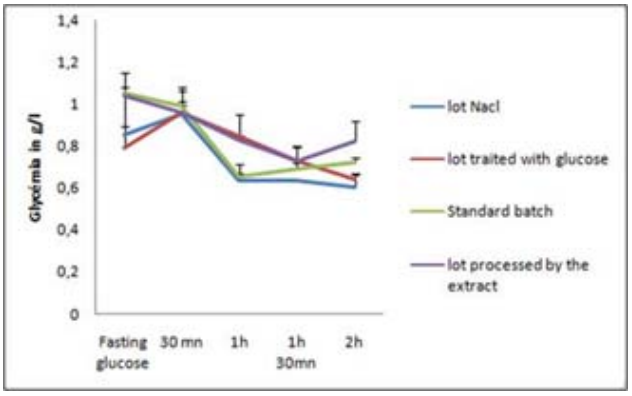


Fig. 7: Blood glucose evolution in two hours in rats treated with a hypoglycaemic glucophage and lichenic extract.

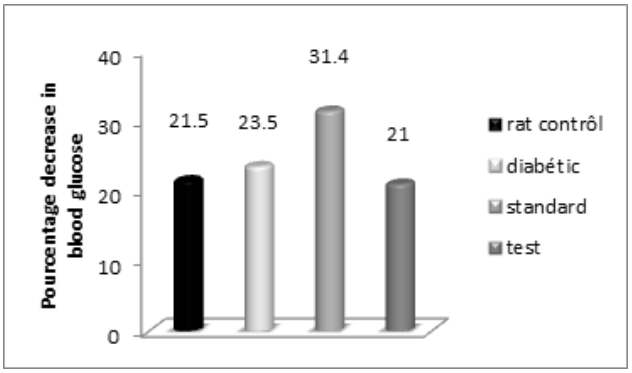


Fig. 8: Percentage of blood glucose reduction in rats treated with glucophage and lichen extract.

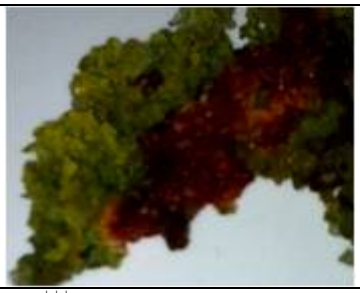
Effect of polyphenols on superoxide dismutase (SOD)

The results indicate a marked increase (54%) in cytosolic SOD levels in the liver with treatment of diabetic gallic rats than those treated with lichen extract (26.6%) compared to diabetic controls. The respective mean values were 0.24µl / mg protein ±0.05; 0.15µl/mg protein ±0.02 and 0.11µl/mg protein ±0.01 (fig. 5).

Table 1: Morphological characteristics of *Xanthoria parietina*

Type	Color	Apothecia / soralia	Species	Region collection	Support
Foliaceous Lichen	Yellow	The apothecia are not larger than the lobes of the lichen	*Xanthoria parietina*	Corso	*Olea europaea*
Form	The lichen is in the form of a "leaf" and is fixed in several places on the trunk				

Table 2: Colorimetric reactions (spot test) of the collected lichen.

Species	Sodium hypochlorite (C)	Potassium hydroxide (K)	C+k
Xanthoria parietina			
Results	C^-: orange	K^{+++}:red	CK^{+++}: red

(-) No staining ; (+) Clear color ; (++) Dark coloration ; (+++) Darker color. The colorings obtained for the thallus of Xanthoria parietina are red (K +++) indicates the presence of parietin, KC +++ indicates the presence of barbatic acid, and C is negative indicating the absence of thiophanic acid.

Effect of polyphenols on catalase (CAT)

The results show an increase in the cytosolic level of CAT in the liver, which is greater in the diabetic rats treated with the lichenic extract than with the gallic acid compared to the diabetic controls. The respective percentages are 446.6% and 220%. The respective mean values were 0.82µl /mg protein ±0.2; 0.48µl /mg protein ± 0.04 and 0.15µl / mg protein ±0.07 (fig. 6).

Evaluation of hypoglycaemic activity

The results obtained indicated a decrease in the glycemia of the rats treated with the lichen extract (0.96g/l±0.1) and those treated with the glucophage (0.99g/ l±0.09) from the first half hour compared to the glucose batch, where the glucose level is regulated from one hour (0.96g / l±0.05) (fig. 7).

The percentage of reduction in blood glucose shows a greater percentage of blood glucose reduction in rats treated with glucophage (31.4%) than those treated with lichen extract (21%) (fig. 8).

DISCUSSION

Lichens are a source of original compounds, and more particularly bioactive secondary metabolites such as polyphenols including thiophanic acid, strepsiline, parietin, ventosin, diffractic acid, and barbatic acid (Dieu, 2015). The results of the phytochemical characterization tests for the species *Xanthoria parietina* made it possible to demonstrate the presence of polyphenols represented by parietin, thiophanic acid and barbatic acid. The extraction method adopted is based on the solubility of the polyphenols in the methanolic solvent. The results obtained indicate a high yield of the methanolic extract (51%) of *Xanthoria parietina* which contains the most polar phenolic compounds. According to Pérez-Jiménez *et al.* (2008), the yield of phenolic compounds depends on several parameters: the nature of the solvent, its polarity, the pH of the medium, the temperature and the extraction time. The *in vivo* evaluation of antioxidant and hypoglycaemic activity is carried out on Wistar strain albino rats which serve as a reference for the study and understanding of the genesis and complications of diabetes (Fröde and Medeiros, 2008). STZ has bac widely used to induce experimental diabetes (Dhanabal *et al.*, 2007). It has the ability to induce excessive formation of reactive oxygen species that are highly toxic to cells, particularly cell membranes (Mazumder *et al.*, 2005). The β-cell have limited defenses against oxidative stress (Rigalleau *et al.*, 2007). Indeed, the reactive oxygen species (ROS) disrupts glucose-stimulated insulin secretion by decreasing the intracytosolic ATP / ADP ratio, abnormal hyperpolarization of the mitochondrial membrane and overexpression of the I chain complex Which leads to apoptosis of β-cells and could explain the reduction in β-cell mass observed in type 2 diabetes (Guillausseau *et al.*, 2008). ROS can interact with the lipid bilayer and cause the production of lipoperoxides (Sivajothi *et al.*, 2008). Monitoring of blood glucose levels in diabetic rats treated with the *Xanthoria parietina* methanol extract after five days of testing showed a decrease in blood glucose levels from the first day after treatment. This decrease was statistically very significant (p℟0.01) at the end of the experiment compared to the diabetic control rats with a 30% reduction percentage.

According to El ghoul *et al.* (2011), polyphenols have an activity similar to that of insulin, which is a hypoglycaemic hormone. The results for the activity of endogenous enzyme systems indicated a reduction in the products of lipid per oxidation (MDA) and GSH and an increase in the activity of the antioxidant enzymes (SOD and CAT). The evaluation of lipid per oxidation showed the generation of a high level of cytosolic MDA (521%) in the liver in diabetic rats and not protected by gallic acid or polyphenols. Several studies have reported an elevation of oxidative stress markers during STZ-induced diabetes (El ghoul *et al.*, 2011). This hyper peroxidation is due to the fact that STZ is a generator of free radicals responsible for the oxidation of DNA, lipid, carbohydrates, and leading to the degeneration of β cells (Huk *et al.*, 1998). The chronic accumulation of stress leads ultimately to the exhaustion of protective mechanisms, to the inability to adapt to the new cellular environment and thus to senescence, as well as to cell death (Thorin-Trescases *et al.*, 2010). However, in our experimental investigation, the previous administration of 10g / kg of the methanolic extract of *Xanthoria parietinaa* made it possible to effectively reduce the level of hepatic MDA (29%) compared to the control diabetics. On the other hand, the reduction of peroxidation in diabetic rats treated with polyphenols may also be due to the improvement of the glycemic control and thus to the improvement of the diabetic condition by inhibition of the lipid peroxidation.

Our results show a similar level in hepatic GSH for control, diabetic and gallic treated rats. According to (Thorin-Trescases *et al.*, 2010), treatment with STZ decreases the level of hepatic reduced glutathion which would probably be due, on the one hand, to an increase in its neutralizing action of the free radicals generated and, on the other hand, to a reduction in its synthesis or an increase in its degradation during oxidative stress caused by diabetes. Treatment of diabetic rats with polyphenols resulted in a decrease in the GSH concentration in the liver of 46.5% compared to control diabetic rats. Many studies have reported that the activity of SOD and CAT decreases during a diabetes mellitus (Taleb-Senouci *et al.,* 2009; Poongothai *et al.*, 2011). These results are in agreement with those obtained in our experiment. Indeed, there was a decrease in the activity of SOD (56%) and CAT (25%) in the liver. Pretreatment of the rats by the polyphenolic extract of *Xanthoria parietinaa* caused an increase in SOD (26.6%) and CAT (446.6%) compared to diabetic rats. According to Haleng *et al.* (2007), enzymatic endogenous antioxidants (SOD and CAT) and non-enzymatic such as reduced glutathione (GSH) are responsible for the detoxification of the organism of these deleterious free radicals and thus can improve hyperglycemia and prevent the development of complications Associated with diabetes. The results of the study of the antioxidant activity *in vivo* show that the polyphenols of *Xanthoria parietina* maintained the equilibrium of the antioxidant/prooxidant balance of the liver cells. Indeed, polyphenols possess a very effective free radical capture activity; this could exert a beneficial action against the pathological alterations caused by the presence of (O^{2-}) and (OH).

The study of the hypoglycemic power is carried out following a temporary induction by a solution of glucose (4g / kg). The results obtained show that the methanol extract possesses a hypoglycemic activity comparable to that of the oral hypoglycemic agent, glucophage. Indeed, the two substances had a hypoglycemic action from the first half hour of taking blood sugar. In addition, monitoring the progression of the latter before and every half hour over a period of 2 hours (30, 60, 90, 120 minutes) allowed us to note a decrease in blood glucose of 21% for the methanolic extract compared with that observed with the glucophage (31%). The hypoglycaemic effect of *Xanthoria parietina* may be induced by increased peripheral glucose metabolism and increased insulin secretion (Sathishsekar and Subramanian, 2005; Fernandes *et al.*, 2007; Abdollahi *et al.*, 2010; Dawei *et al.*, 2010). According to Hanhineva *et al.* (2010) and Bahadoran *et al.* (2013), polyphenols improve cell sensitivity to insulin, pancreas β-cell function, and glucose homeostasis in the liver by inhibiting gluconeogenesis and circulating glucose secretion, as well as increasing Glycogenesis.

CONCLUSION

All of our work has highlighted the beneficial effects of the administration of the methanolic extract of *Xanthoria parietina* either in lowering blood sugar levels or in preventing and limiting the toxic effects of free radicals.

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