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REGULAR ARTICLE

Protective potential of the essential oil of *Thymus vulgaris* L. against Dicofol-induced poisoning in rats as established through clinical chemistry, histopathology and 1H-NMR-based metabonomics

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ABSTRACT

Pesticides, organochlorines, analogues and derivatives of DDT, those are all terms often associated with health risks, intoxications, nephropathies and other hepatotoxicities. These are phytosanitary products that interfere with the internal metabolism of sensitive organs, such as the kidney or the liver, which orchestrate the metabolism and elimination of xenobiotics. In this study, a group of Wistar rats was intraperitoneally exposed to Dicofol, a pesticide analogue of DDT. A second group received preventive injections of a diluted thyme essential oil solution for four days before exposure to Dicofol. The evaluation of the toxic effects possibly induced by Dicofol in the two experimental groups was carried out using a multidisciplinary approach including clinical chemistry, histopathology and ^{1}H-NMR-based metabonomics. Histopathological examination showed pulmonary inflammation and kidney damage in the group exposed to Dicofol. The metabonomic study revealed metabolic disturbances in the liver and kidney. The protective role of the essential oil of thyme was clearly demonstrated from the metabonomic profiles and was confirmed by histological examination of organs.

1. Introduction

Pesticides are chemical substances voluntarily introduced into the environment. These products are used in chemical pest control, which remains the most effective solution to fight against plant diseases. However, the health risks associated with the use and intensive spreading of these chemicals have given rise in recent years to growing concern about ecotoxicological studies on human health in general and the health of the farmer in particular. Among these products, Dicofol [2,2,2-trichloro-1,1-bis (4-chlorophenyl) ethanol], a non-systemic organochlorine pesticide, has been widely used to control phytophagous mites on fruits, vines, ornamentals, vegetables, teas, and field crops (Chan et al., 2009). And yet, numerous studies confirm the high toxicity of this product (Bayomy et al., 2008;; Kavitha et al., 2016; XP Xu 2014). A recent study shows in particular a hypoactivity of the thyroid gland due to the degeneration of follicular cells in rats exposed to Dicofol. This degeneration is due to oxidative lesions resulting from the generation of free radicals in the target cells (Kavitha et al., 2016).

Faced with this challenge, scientists are increasingly looking for alternatives that are less dangerous

and more respectful of human health and the environment, such as the use of natural extracts through processes based on green chemistry and innovative technology.

In this respect, Thyme, a medicinal and aromatic plant belonging to the labiate family, shows various beneficial effects: antiseptic, antimicrobial, bactericidal, deworming, as well as antioxidant properties. Many *in vitro* and *in vivo* studies point out the fascinating antimicrobial and antioxidant properties of Thyme extracts (Kačániová et al., 2012, Proestos et al., 2013), but also its anti-tumor and hepatoprotective potential (Ait M'barek et al., 2007; El-Banna et al., 2013; Özkan and Erdoğan, 2011). Based on those promising effects of thyme, we investigated in the present *in vivo* study the protective potential of the essential oil of thyme, extracted through green chemistry procedures, against the Dicofol-induced toxicity in Wistar Han rats.

2. Materials and Methods

2.1. Animals

Male Wistar Han rats weighing 250 ± 50 g (Elevage Janvier, Le Genest Saint Isle, France) were housed in individual metabolic cages with free access to food (Carfil Quality, Oud-Turnhout, Belgium) and water. The environmental conditions were set at a temperature of 25 ± 1 ° C with a relative humidity of 50% and a light cycle of 12h (day/night). All animal experiments were conducted at the University of Mons (Mons, Belgium), in agreement with the local ethics committee of the Institution.

2.2. Extraction

Extraction of the essential oil was carried out by Clevenger type hydrodistillation. A 150g sample of the flowering tops of *Thymus vulgaris* was put in a flask containing the distilled water and the mixture was boiled for about 90min. The passage of the condensed vapor through a refrigerating column allowed, due to the temperature change, to condense and liquefy the molecules driven by the vapor. The harvested distillate contained two phases, oily and aqueous. The pure oil was recovered in a brown bottle and stored at 4°C. The sample was mixed in corn oil to obtain a 2% dilution of essential oil (H2).

2.3. Experimental protocol

The animals were randomly distributed into three groups of three animals each. After two pretest days in the metabolic cages, animals in the first group received daily intraperitoneal injections of corn oil for eight days. Animals in the second group received daily IP injections of Dicofol solution at the dose of 25 mg/kg b.w. (Dcf) for eight consecutive days. As for the third group, the animals received the essential oil of thyme diluted to 2% during the first four days of treatment and then injections of Dicofol until the eighth day (H2Dcf). Blood samples were collected Before euthanasia. In addition, urine samples were taken every 24 hours in tubes cooled at 4°C, placed on automated mobile racks, and containing 1ml of 1% sodium azide for a 24h harvest. This procedure avoids bacterial contamination of the collected urine samples. The rats were euthanized at the end of the exposure period and lungs, kidney and liver were dissected for histopathology.

2.4. Clinical Chemistry

Serum samples were analyzed for clinical chemistry on an automatic biochemical analyzer (spotchem EZ SP 4430 Menarini Diagnostics) SPOTCHEM multi liver-1 and SPOTCHEM multi kidney-2.

SPOTCHEM multi Liver-1 was used to measure total bilirubin (T.bil), transaminases and lactate dehydrogenase (LDH) and SPOTCHEM II Kidney-2 kit was used to measure total protein (T.pro), albumin (Alb), blood urea nitrogen (BUN), creatinine (Cre) and uric acid (UA).

2.5. Histopathology

Lungs, kidneys and liver were removed at autopsy and immediately fixed in Bouin solution for 24 hours. Next, the fixed tissues were exposed to three successive ethanol baths for 24 hours followed by three baths in butanol for 24 hours as well. Then, the samples were embedded three times in paraffin baths. 4-5µm sections were obtained on a microtome and stained with hematoxylin-eosin before being analyzed by light microscopy.

2.6. Metabonomic assessment

After collection, the urine samples were immediately stored at -80°C for subsequent ^{1}H-NMR analyzes.

2.6.1. ^{1}H-NMR Spectroscopy of urine samples

Urine samples were centrifuged for 5 min at 5000 g (at 4 ° C) to remove solid debris. Aliquots (400 µl) of supernatant were mixed with 200 µl of 0.2M sodium phosphate buffer (0.04 M NaH2PO4 + 0.2M

Na2HPO4, pH 7.0) prepared in H2O/D2O solution (80/20). Deuterated trimethylsilyl propionic acid (TSP, lot number MBBB0475V, Sigma-Aldrich Chemie GmbH, Germany), 1 mM final concentration, was used as an external reference. 550 µl of this preparation were transferred to 5 mm NMR tubes and analyzed by 1H-NMR spectroscopy at 11.5T (500 MHz proton) using a Bruker Avance spectrometer (Bruker, Karlsruhe, Germany). Solvent suppression of residual water signal was achieved by using the NOESYPRESAT pulse sequences for urine samples.

2.6.2. Multivariate data analysis

The phases and baselines were manually corrected for all spectra, Chemical shifts were referenced to TSP (singlet resonance arbitrarily placed at 0.0 ppm) and spectra were normalized to TSP.

The data were reduced to 250 subregions by binning the global spectral width (from 0.08 to 10.0 ppm) by 0.04ppm steps. This procedure was performed using MestreNova 5.2.0 software (Mestre Research Lab, Santiago de Compostela, Spain). Segments ranging from 4.5 to 5.0 ppm and 5.5 to 6.0 ppm were next removed to eliminate the uncertainty of the residual water and urea signals, respectively. The area under the binned subregions (descriptors) curve were integrated and the numerical values obtained for each spectrum were imported into the SIMCAP +12 software (Umetrics AB, Sweden) for PCA analysis.

3. Statistical Analysis

Clinical chemistry data (blood parameters) and physical measurements (body weight, water and food consumptions) were expressed as means ± SD. Statistical comparisons were made using an analysis of variance (ANOVA), and the Newman-Keul test, the criterion of statistical significance was set at a value of $p < 0.05$.

4. Results

4.1. Effects of extracts on food/water consumptions and body weight

Increases in food and water consumption were observed in animals exposed to either thyme essential oil or Dicofol, accompanied by a body weight gain comparable to that of the control group (Table 1).

4.2. Histopathology

In the group of rats treated with Dicofol (Dcf), the lungs present a subnormal to normal microscopic appearance (Fig.1). The kidneys display tubulointerstitial lesions of minimal to discrete intensity, consisting of multifocal basophilic tubes in the renal cortex. In the liver, cytoplasmic vacuolation is observed in hepatocytes located in the subcapsular and centrilobular regions (Fig.2). Fibrosis of the liver capsule, of limited intensity, is also noticed as well as the occasional presence of lipogranulomas.

In rats receiving 2% essential oil before Dicofol treatment (H2-Dcf), the lungs and kidney have display normal microscopic appearance. In the liver, a minimal cytoplasmic vacuolation of hepatocytes is observed in the subcapsular region. This aspect appears slightly less intense than in the Dcf group.

4.3. Effects of extracts on clinical liver and kidney parameters

The results showed no difference between the groups for the values of certain liver parameters evaluated: T.pro, T.bil and GOT. In contrast, the GPT levels in the Dcf group (122.00 ± 10.58 IU) that received Dicofol for eight consecutive days increased significantly ($p < 0.0007$) relatively to the H2-Dcf group (79, 00 ± 9.00 IU).

With respect to serum creatinine levels, close values were observed between Dcf (2.90 ± 0.35 mg / dl) and H2-Dfc (2.55 ± 0.05 mg / dl) rats.

On the other hand, a very important increase is also observed for the serum uric acid level ($P < 0.0004$) in the group exposed to Dicofol (6.20 ± 0.26 mg / dl), whereas the rats of the H2- Dcf group had a uric acid level of the order of 2.95 ± 0.45 mg / dl.

4.4. Effects of extracts on urine metabonomic profiles

4.4.1. Principal component analysis of ^{1}H-NMR binned data

The scores plot (Fig. 3A) show that urine samples collected from animals exposed to Dicofol are metabolically different from the samples collected during the pre-treatment period as well as from their matching controls. The corresponding loading plot (Fig. 3B) displays the spectral regions that contribute the most to the separation of the groups observed in the scores plot:

Reduced levels of citrate (δ2.57, δ2.69), α-ketoglutarate (δ2.44, δ3.01), and allantoin (δ 5.34)

	Food consumption (g)		Water consumption (ml)		Body weight (g)	
	Dcf	H2-Dcf	Dcf	H2-Dcf	Dcf	H2-Dcf
pre-test	27±01	26±00	27±02	24±02	214±11	217±8
Day 4	27±03	30±03	33±12	32±11	236±12	240±12
Day 8	25±02	27±01	29±04	31±12	263±13	267±15

Table 1: Means of variations in food/water consumption and body weight.

	Control group	Dcf group	H2-Dcf group
T-Pro(g/dl)	3.80±0.28	4,8±0,26	4,5±0,10
Alb (g/dl)	4,3±0,14	3,60±0,10	3,40±0,10
t-bil (mg/dl)	0.7±0.28	0,57±0,06	0,55±0,05
GOT (UI/L)	46,50±19,09	73,33±12,34	66,50±3,50
GPT (UI/L)	49.00±11.31	122,00±10,58**	79,00±9,00
HDL (UI/L)	3468.50±571	499,00±138,11	437,50±8,50
BUN (mg/dl)	08.50±0.71	9,67±0,58	12,50±0,50*
UA (mg/dl)	1.70±0.42	6,20±0,26	2,95±0,45
Cre (mg/dl)	0.45±0.07	2,90±0,35**	2,55±0,05
Values: Means ± SD; * P<0.05; ** p<0.001.			

Table 2: Comparison of Mean Values of Liver and Renal Parameters.

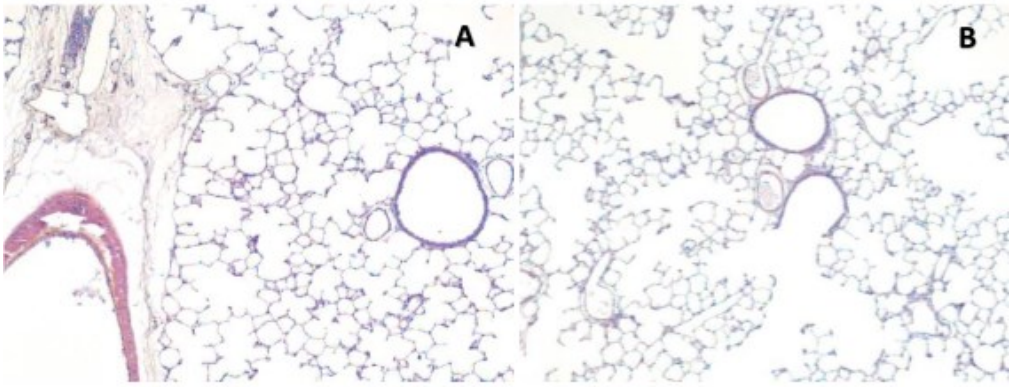

Figure 1 : Lung perivascular inflammation in the Dcf group (A) as compared to the H2-Dcf group (B) showing normal aspect (X40).

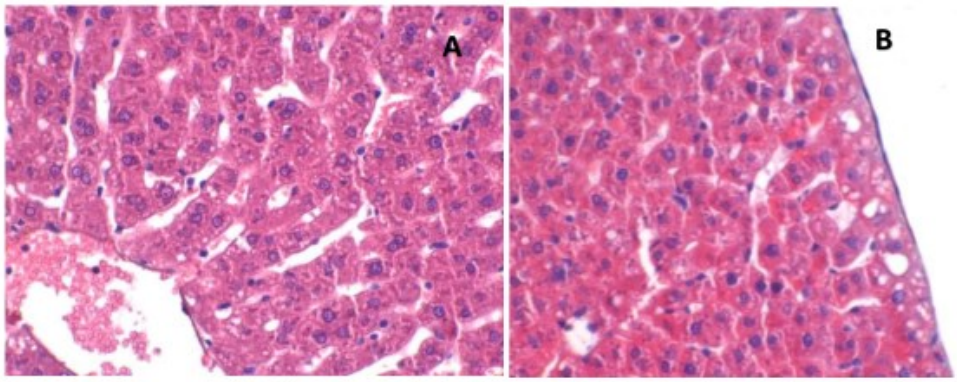

Figure 2: Hepatocellular vacuolisation in centrolobular (A) and subcapsular (B) regions in the Dcf group (X200).

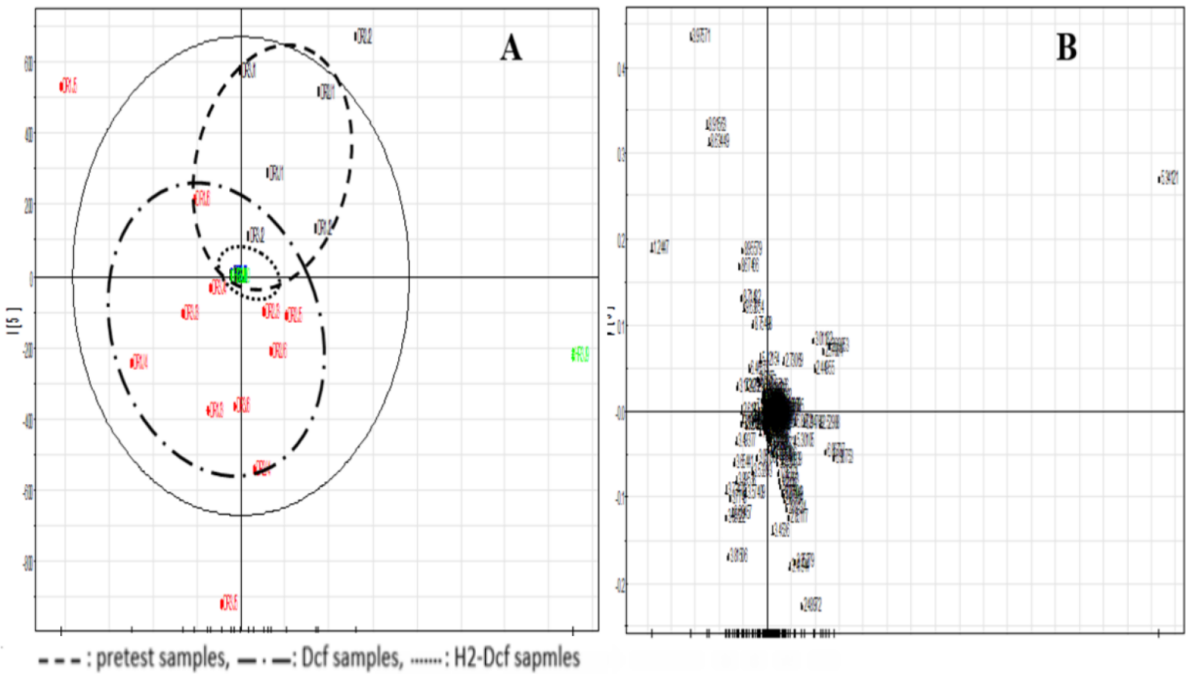

Figure 3: PCA scores (A) and loadings (B) plots performed on the numerical data obtained after integration of binned spectral data obtained form 1H-NMR analysis of urine samples from rats exposed to Dicofol without (Dcf) or with pre-treatment with Thyme essential oil 2% (H2-dcf).

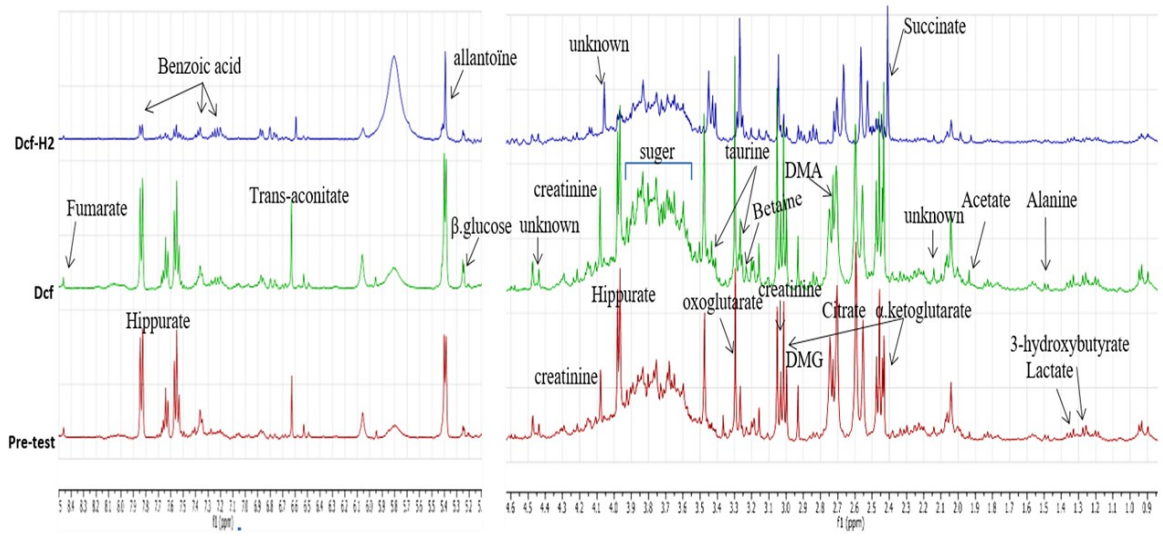

Figure 4: ^{1}H-NMR spectra of urine samples collected either from rats exposed to Dicofol (Dcf), rats pretreated with thyme and next with Dicofol (H2-Dcf), comparatively to samples collected during the pre-test period.

Increased levels of descriptors corresponding to sugars (δ3.61, δ3.77, δ3.81, δ3.85), hippurate (δ 3.97) and dimethylamine (δ 2.73).

4.4.2. Urine ^{1}H-NMR spectra

^{1}H-NMR spectra obtained from urine samples of rats exposed to Dicofol (Dcf) revealed some characteristic metabolic differences as compared to samples collected during the pre-test period in the same group (fig. 4). Very marked increases were observed in particular for α-ketoglutarate (δ2.44, δ3.01), oxo-glutarate (δ3.00), betaine (δ 3.25), dimethylamine (δ2.73), creatinine (δ3. 05 and δ4.06), fumarate (δ6, 53 and δ8.46), β-glucose (δ 5.23), trans-aconitate (δ 6.58), allantoin (δ 5.38), as well as in the region corresponding to sugars (δ 3.61, δ 3.77, δ 3.81, δ 3.85). In the same group, differences in the levels of acetate, alanine and an unidentified metabolite (δ2.15, S) were also noticed.

On the other hand, except for a very important increase in succinate (δ2.40), these alterations

(fumarate, trans-aconitate, allantoin, dimethylglycine (δ2.92), dimethylamine and acetate (δ1.94) are less or not detected when the animals were preventively exposed to Thyme essential oil 2% (H2 -Dcf).

5. Discussion

Under our experimental conditions, no mortality or suspicious physical signs was observed in rats from either experimental group (Dcf and H2-Dcf). According to a study by Chan et al. (2009), doses of 1, 10 and 25 mg/kg bw. Dicofol did not produce any significant changes in body weight of exposed rats. In contrast, according to these same authors, a relatively low dose of 1 mg / kg bw of Dicofol has the ability to induce many metabolic enzymes in the rat liver.

In our study, no effect on body weight was noticed in animals exposed to Dicofol. In addition, microscopic liver examinations showed mild to moderate hepatocyte swelling with small vacuoles in rats exposed to 25 mg / kg bw of Dicofol, as well as moderately severe lesions (Chan et al., 2009). Cytoplasmic vacuolization of hepatocytes was also observed in the subcapsular and centrolobular region, as well as limited liver fibrosis of the liver capsule and the occasional presence of lipogranulomas. This aspect is a reaction to the presence of a foreign body, probably consecutive to the intraperitoneal administration of oily compounds.

Several studies have shown the harmfulness of pesticides including organochlorines and DDT analogues (Ahlborg et al., 1995; Franco et al., 2010; Karami-Mohajeri and Abdollahi 2013; Slotkin, 2011; Shelton et al., 2012; Tag El-Din et al., 2003). And yet, even at the low dose tested in the present study, that approximately corresponds to about forty-six times lower than the LD_{50} reported for Diclofol, this pesticide that belongs to the organochlorine family is able to trigger obvious cellular and molecular perturbations.

According to the results of clinical chemistry, a significant increase in GPT was indeed observed in the diclofol-exposed animals, which most often reflects hepatocellular insufficiency. According to Chan et al. (2009), Diclofol induces changes in some liver parameters in rats including CYP1A1, CYP2B, CYP2E1, and CYP3A.

According to Karami-Mohajeri and Abdollahi (2013), organophosphorus compounds could cause some changes in the activity of enzymes involved in the Krebs cycle or the catabolism of ATP. Slotkin et al (2011) also reported that the exposure to organophosphorus compounds during critical periods of development could lead to a disruption of cyclic AMP signaling pathways and an increased response to gluconeogenesis stimuli. Also, according to Shelton.et al. (2012), these products are able to modify calcium signaling pathways, leading to direct effects on mitochondria and ROS production.

The work of Lim et al. (2009) and Lee et al. (2011) corroborated that exposure to atrazine and organochlorines may reduce the mitochondrial ability to beta-oxidize fatty acids.

According to our results, a recovery of serum levels of liver parameters in the group treated preventively with essential oil was noted. The work of Dadkhah et al. (2014) showed that acetaminophen-induced intoxication significantly modulated biochemical parameters by increasing the activity of CYP450, GOT and GPT, with a decrease in GSH and GST levels. According to these same authors, the levels of these parameters return to normal values under the effect of the essential oil of *Achillea wilhelmsii*.

Our results are in agreement with previous results by Fatemi et al., (2015), who orally pretreated rats with *Thymus Capitatus* and *Salvia Officinal* essential oils before exposing the animals to paracetamol. They showed a significant liver protection as demonstrated by the slowdown in serum and hepatic LDH activities, inhibition of reduced GSH levels, and increased SOD and GPx activity in the blood and liver.

According to Fatemi et al. (2015), the possible mechanism responsible for the protection of paracetamol-induced hepatic injury by such natural extracts would be an antioxidant action of compounds acting as a free radical scavenger by intercepting the radicals produced by microsomal enzymes.

Metabonomically, the reduced hippurate urinary level in the H2-Dcf group is very marked. This is explained by the presence of phenolic compounds and their metabolites in the body, the latter have a direct effect on the intestinal flora. According to Nicholls et al. (2003), a decrease in urinary hippurate excretion may be associated with disturbance of microbial colonies. Similarly for the levels of some intermediate metabolites of the Krebs cycle, a study in rats exposed to epicatechin, a polyphe-

nolic compound of the flavonoid family, showed a decrease in urinary excretion of certain TCA cycle metabolites, such as citrate and oxoglutarate (Solanky et al., 2003).

According to our results, particularly noticeable on the scores plot of the metabonomic study, the gathering of the individuals of the group treated preventively by the essential oil of thyme and those from the control group is quite clear, in contrast to the individuals of the group Dcf who are dispersed. In other words, the preventive low-dose of thyme essential oil obviously demonstrate a protective role against the poisoning induced by a synthetic pesticide, in this case Dicofol. The most discriminating metabolites are sugars, glucose, creatinine, as well as various certain amino acids and ketones. These results are consistent with our previous work (Benourad et al., 2014) where we showed that the loss of glucose in the urine would cause an energy imbalance in the tissues, which could be counteracted by the production of ketone bodies. During previous investigations, we have also found that the most remarkable metabolic changes following intraperitoneal injections of essential oil of thyme at two different doses (0.2% and 2%) were the increase in urinary glucose levels, lactate, and the appearance of ketone bodies. These changes were linked to a disruption in renal tubular reabsorption.

According to Domitrovic et al. (2013), phenolic compounds of natural origin have been shown to be effective in attenuating oxidative stress and preventing injury and liver failure.

In our study, the essential oil of thyme marked its effectiveness as a protective treatment against a poisoning induced by Dicofol. Some metabolic disturbances observed in H2-Dcf rats, probably induced by this 2% essential oil on energetic and hepatic metabolism, appear to be temporary, but this remains to be confirmed.

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