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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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 1H-NMR analyzes.

2.6.1. 1H-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...
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 tubules 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 1H-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)

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 NOESYRESAT 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

Reduced levels of citrate (δ2.57, δ2.69), α-ketoglutarate (δ2.44, δ3.01), and allantoin (δ 5.34)
Table 1: Means of variations in food/water consumption and body weight.

<table>
<thead>
<tr>
<th></th>
<th>Food consumption (g)</th>
<th>Water consumption (ml)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dcf</td>
<td>H2-Dcf</td>
<td>Dcf</td>
</tr>
<tr>
<td>pre-test</td>
<td>27±01</td>
<td>26±00</td>
<td>27±02</td>
</tr>
<tr>
<td>Day 4</td>
<td>27±03</td>
<td>30±03</td>
<td>33±12</td>
</tr>
<tr>
<td>Day 8</td>
<td>25±02</td>
<td>27±01</td>
<td>29±04</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Dcf group</th>
<th>H2-Dcf group</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Pro (g/dl)</td>
<td>3.80±0.28</td>
<td>4,8±0,26</td>
<td>4,5±0,10</td>
</tr>
<tr>
<td>Alb (g/dl)</td>
<td>4,3±0,14</td>
<td>3,60±0,10</td>
<td>3,40±0,10</td>
</tr>
<tr>
<td>t-bil (mg/dl)</td>
<td>0.7±0.28</td>
<td>0,57±0,06</td>
<td>0,55±0,05</td>
</tr>
<tr>
<td>GOT (UI/L)</td>
<td>46,50±19,09</td>
<td>73,33±12,34</td>
<td>66,50±3,50</td>
</tr>
<tr>
<td>GPT (UI/L)</td>
<td>49,00±11,31</td>
<td>122,00±10,58**</td>
<td>79,00±9,00</td>
</tr>
<tr>
<td>HDL (UI/L)</td>
<td>3468.50±571</td>
<td>499,00±138,11</td>
<td>437,50±8,50</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>08.50±0.71</td>
<td>9,67±0,58</td>
<td>12,50±0,50*</td>
</tr>
<tr>
<td>UA (mg/dl)</td>
<td>1.70±0.42</td>
<td>6,20±0,26</td>
<td>2,95±0,45</td>
</tr>
<tr>
<td>Cre (mg/dl)</td>
<td>0.45±0.07</td>
<td>2,90±0,35**</td>
<td>2,55±0,05</td>
</tr>
</tbody>
</table>

Values: Means ± SD; * P<0.05; ** p<0.001.

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).
Increased levels of descriptors corresponding to sugars (δ 3.61, δ 3.77, δ 3.81, δ 3.85), hippurate (δ 3.97) and dimethylamine (δ 2.73).

4.4.2. Urine ¹H-NMR spectra

¹H-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 (δ 2.44, δ 3.01), oxo-glutarate (δ 3.00), betaine (δ 3.25), dimethylamine (δ 2.73), creatinine (δ 3.05 and δ 4.06), fumarate (δ6, δ 53 and δ 8.46), β-glucose (δ 5.23), trans-aconitate (δ 6.58), allantoin (δ 5.38), as well as in the region corresponding to sugars (δ 2.61, δ 3.77, δ 3.81, δ 3.85). In the same group, differences in the levels of acetate, alanine and an unidentified metabolite (δ 2.15, S) were also noticed.

On the other hand, except for a very important increase in succinate (δ 2.40), these alterations...
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 Officinalis 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 polyphne-
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 metabolomic 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.

References


Slotkin TA (2011) Does early-life exposure to organophosphate insecticides lead to prediabetes and obesity?. Reproductive Toxicology 31(3):297-301.


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