

Protective effect of oregano water extract against Carbon tetrachloride induced Hepatotoxicity in female rats

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ABSTRACT

Carbon tetrachloride (CCL₄) is one of the most toxic chemicals causes oxidative stress in the living organisms. Our experiment started with twenty female rats that were divided into 4 groups; The 1st group G1 served as control group, 2nd group G2 injected twice a week with CCL₄ diluted in olive oil (1:1 v/v), 3rd group G3 were fed daily for 14 days with 1ml of Oregano water extract and the 4th group G4 were injected with CCL₄ plus daily feeding with 1ml of Oregano water extract. The biochemical results indicated a significant elevation in the liver and kidney functions including ALT, AST, creatinine, uric acid and urea for the 2nd group due to the toxicity induced by the CCL₄. The levels of ALT, AST, creatinine, urea and uric acid were significantly decreased after the administration of oregano water extract in the 4th group. Lipid profile was evaluated in the four groups by measuring the levels of total cholesterol, HDL-C and triglycerides. The variation noticed by the over and down expression of the liver genes due to CCL₄ was explored. These up and down regulated genes were tracked using differential display technique.

Keywords: Oregano water extract, carbon tetrachloride, hepatotoxicity, rats

INTRODUCTION

In the recent days, the rates of human being diseases and organs toxicity have been increased as a result of exposing to multi environmental factors. These factors include xenobiotics, heavy metals, and toxic chemicals in addition to different microbial infections.

The liver is almost involved with all the biochemical pathways related to growth, fight against disease, nutrient supply and energy provision (Palanivel *et al.*, 2008). It is also responsible for the process of detoxification of toxic chemicals. There are some factors that can easily cause liver injury including non-adequate nutrition, alcohol and drug

abuse, viral infections and incidental poisoning (Domitrovic' *et al.*, 2013).

Carbon tetrachloride (CCl₄) intoxication is a frequently used model of liver injury (Weber *et al.*, 2003). CCl₄ is activated by hepatic microsomal cytochromes to form the trichloromethyl radical, CCl₃. This radical induces oxidative stress by binding to cellular molecules, thus impairing numerous cellular processes that lead to necrotic cell damage, inflammation and apoptosis (Sun *et al.*, 2001). Non lethal intoxication triggers liver tissue remodeling and healing through the activation of hepatic stellate cells (HSCs), leading to liver fibrosis (Friedman, 2000). Carbon tetrachloride (CCl₄) is extensively used

hepatotoxic agent in preclinical animal studies and considered the best-characterized chemical to study xenobiotic induced free radical mediated acute liver injury in rats (Recknagel and Glende, 1973). In addition, CCl₄ can cause pathological lesions in treated animals that closely resemble the symptoms of cirrhosis in human, and so considered an excellent model to evaluate the efficacy of hepatoprotectants (Desai *et al.*, 2012).

A large variety of methods are used for the treatment of liver diseases include, pharmacotherapy, surgery as well as liver transplantation, all of which have shown limited therapeutic benefits and are associated with serious complications (Mihailovic *et al.*, 2013). Consequently, there is a great demand to figure out an alternative approaches for the treatment of liver diseases (Bishayee *et al.*, 2010). The medicinal plants are considered the most recommended approaches for the treatment of liver disorders (Jaishree and Badami, 2010).

Oregano or Marjoram (*Majorana hortensis*) is the gray green leaf of the mint family (Lamiaceae or Labiatae). It is widely distributed in Egypt and was known to the ancient Egypt for its healing properties (Badee *et al.*, 2013). It has been used for treatment of arthritis, rheumatism, muscle and nerve pain, headaches, circulatory disorders, respiratory infections (Rashwan, 2011) in addition to its medicinal effect as gastrointestinal tract stimulant, tonic, carminative, diaphoretic, hypoglycemic, diuretic as well as antibacterial (Leeja and Thoppil, 2007) and as antioxidant (Handl *et al.*, 2008).

Our study was targeted to use carbon tetrachloride (CCl₄) as hepatotoxic agent with subsequent treatment with marjoram. The efficacy of marjoram was tracked using physiological, biochemical and advanced molecular methods.

MATERIALS AND METHODS

Chemicals:

Carbon tetrachloride used in the experiment was purchased from Sigma-Aldrich Co., Germany. All the diagnostic kits assaying liver, kidney function tests and the lipid profile were obtained from Biodiagnostic Company, Egypt. Olive oil used for dissolving CCl₄ was purchased from the local market.

Plant material:

Marjoram leaves were obtained as a gift from a farmer in Minia province, Egypt.

Extraction of oregano (marjoram):

15 gm of dry leaves of the plant were grinded and extracted by warming in 300 ml distilled water for 30 min at 80°C and finally cooled. The extract was filtered using small diameter mesh, and the remaining fine particles were filtered using small pores filter paper and stored in 4°C until use.

Experimental design:

A total of twenty female rats were used for the study; divided into four groups with total of five rats for each. The first group served as control group which fed orally on normal diet with distilled water, the second group served as treated group which injected interperitoneally with CCl₄ dissolved in olive oil (1:1 V/V), the third group served as positive control group which only fed orally on oregano extract and the last group served as treated group injected interperotinely with CCl₄ and orally fed on oregano extract.

The study was run for two weeks with injection of 0.25 ml of CCl₄ twice a week and daily feeding of 1 ml oregano/ rat.

At the end of the study, the rats were sacrificed under ether anesthesia and serum samples were collected in test tubes and preserved at 4° C, while, liver samples were kept in phosphate buffer and preserved in – 80° C for the molecular analysis.

Biochemical analysis:**Measurement of liver function markers:**

Total proteins (TP) were determined by means of the Biuret reaction as described by Gornall *et al.* (1949). In the presence of an alkaline cupric sulfate, the protein produces a violet color, the intensity of which is proportional to their concentration. Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were determined following the method of Reitman and Frankel (1957). The catalytic activity was measured by spectrophotometry at 505nm.

Measurement of kidney function markers:

Creatinine level was determined by colorimetric kinetic method as described by Larsen (1972), where Creatinine in alkaline solution reacts with picric acid to form a colored complex. Urea levels in the serum were measured by means of the coupled reactions described by Fawcett and Soctt (1960). The blue dye indophenol product reaction absorbs light between 530 nm and 560 nm proportional to initial urea concentration. Uric acid level was determined as described by Barham and Trinder (1972), where the uric acid was hydrolyzed enzymatically to release hydrogen peroxide which reacts with 4-aminoantipyrine in the presence of 3,5 Dichloro-2- hydroxybenzen sulphonate to form a quinoneimine which has color measured at 510 nm.

Lipid profile:

The total cholesterol was determined after enzymatic hydrolysis and oxidation according to Allain *et al.* (1974). The quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. The HDL-cholesterol was determined by enzymatic colorimetric method as described by Lopez-Virella *et al.* (1977) where phosphotungstic acid and magnesium

ions selectively precipitating all lipoproteins except the HDL fraction, cholesterol present in the supernatant can be determined by the same method used for total cholesterol.

Measurement of glutathione peroxidase (GPx):

The assay is an indirect measure of the activity of c-GPx according to Paglia and Valentine (1967). Oxidized glutathione (GSSG), produced upon reduction of organic peroxide by c-GPx, and is recycled to its reduced state by the enzyme reductase (GR).

Statistical analysis:

The data were given as individual values and as mean \pm standard error. Comparisons between the means of various treatment groups were analyzed using one way ANOVA. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using the statistical software SPSS, version 10.

Molecular methods:**RNA extraction:**

The frozen liver tissues were thawed, grinded and submitted to total RNA extraction according to the manual instructions of the used RNA extraction kit (Qiagen, Germany). The RNA was separately extracted from all the rats groups whether the controls or the treated ones.

The successful extraction of RNA was ensured through agarose gel electrophoresis. Ten micro liters of the extracted RNA was injected into the wells of 1% agarose gel, submitted to electrical migration for 30 min at 120V and finally photographed using gel documentation system (Bio Rad, GelDoc, 2000).

Reverse transcriptase PCR (cDNA Synthesis)

At first, five micro liters of total RNA was treated with DNaseI enzyme to emphasize complete absence of the genomic DNA. The RNA was eluted with 100 μ l of an elution buffer followed

by incubation with 70 units of DNaseI at 37° C for 1 h. The enzyme was then inactivated at 75° C for 10 min and the RNA was precipitated using 100 µl of isopropanol followed by centrifugation at maximum speed (13.000 rpm) for 30 min at 4° C. Two 70% ethanol washes were followed, using the same centrifuge settings. The ethanol was removed and the RNA pellets were allowed to air dry for 15 min, at which point 32 µL RNase-Free water were added. The mRNA was then transcribed to cDNA using a TaqMan Reverse Transcriptase kit (Applied Biosystems) at the presence of oligo(dT) 16-mers primers according to the manufacturer's instructions. The mixture was incubated at 37° C for 1 h followed by enzyme inactivation step at 75° C for 10 min.

Differential Display PCR (DD-PCR)

A total reaction volume of 25 µl containing 2.5 µl 10X Taq buffer, 2.5 µl MgCl₂, 2.5 µl dNTPs, 1 U Taq DNA polymerase, 3 µl of 10 pmol separately arbitrary primers OP1 5'-ACGGQACCTG-3' OR OP2 5'-GTCCCGAGGA-3' and 2 µl of each cDNA and finally 12 µl of sterile dH₂O. The amplification program was as follows; one cycle at 94° C for 5 min (hot start), followed by 40 cycles at 94° C for 1 min, 35° C for 1 min and 72° C for 1 min and final extension step at 72° C for 10 min. PCR products were visualized on 2% agarose gel and photographed using gel documentation system (Gel Doc, 2000).

The up and down expressed genes were excised from the gel and submitted to PCR-gel purification kit (Qiagene) and the purified bands were submitted to sequencing (Macrogen Inc., Korea).

RESULTS AND DISCUSSION

Biochemical analysis:

The serum biochemical parameters are considered the internal mirror that reflects the actual effect of both of the

CCl₄ toxin and marjoram as its counter. The measured biochemical parameters were as follows: ALT, AST, TP, creatinine, uric acid, urea, total cholesterol, HDL-C, TG and GPx. The results indicated that the injection of rats with CCl₄ was able to induce hepatotoxicity as shown in Figs. 1-7.

The activity levels of ALT and AST liver enzymes, in addition to the levels of total protein, creatinine, urea, total cholesterol, TG were significantly increased in CCl₄ group when compared with the control group ($p < 0.05$). On the other hand, the levels of uric acid and HDL-C were non significant ($p > 0.05$) in CCl₄ group when compared with the control group.

These results are in agreement with Leelaprakash, *et al.* (2011), who reported statistically significant increase in plasma activities of AST and ALT. They reported general statistically significant losses in activity of GPx which agree with our results that indicate a significant decrease in the activity levels of GPx in the CCl₄ group in comparison with that of control group. It was observed in our study that the feeding of rats with marjoram was able to reduce and sometimes completely remove the toxic effect of CCl₄. Some parameters including ALT, AST, creatinine, urea, total cholesterol and TG were significantly decreased ($p < 0.05$) in the group injected and fed with CCl₄ and marjoram compared with CCl₄ group. In contrast, total protein, uric acid and HDL-C showed no significance ($p > 0.05$) under the same conditions. Leelaprakash, *et al.* (2011), had also reported counteract of all the damage caused by CCl₄ using *Vernonia cinerea*. The slight difference between our results and Leelaprakash results may back to the type of the used medicinal plant or the sex of animal model.

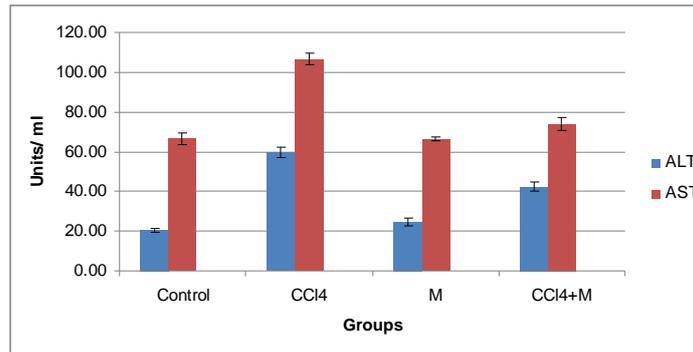


Fig. 1: Serum activity levels of AST and ALT liver enzymes in the different experimental rat groups.

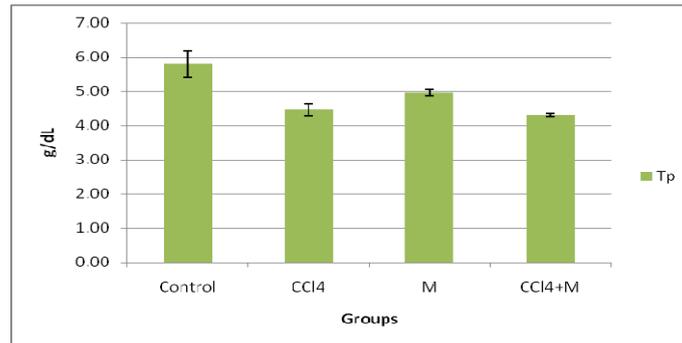


Fig. 2: Serum levels of Total Protein (TP) in the different experimental rat groups.

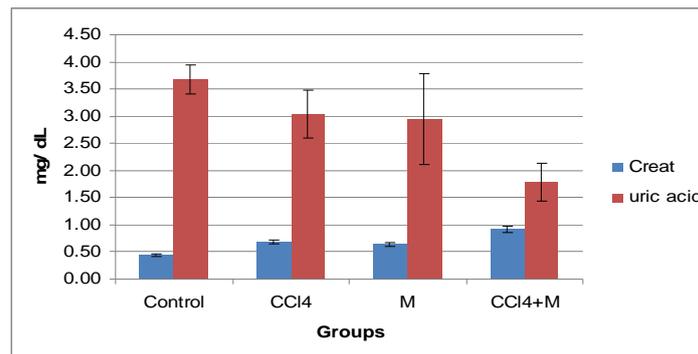


Fig. 3: Serum levels of Creatinine and uric acid in the different experimental rat groups.

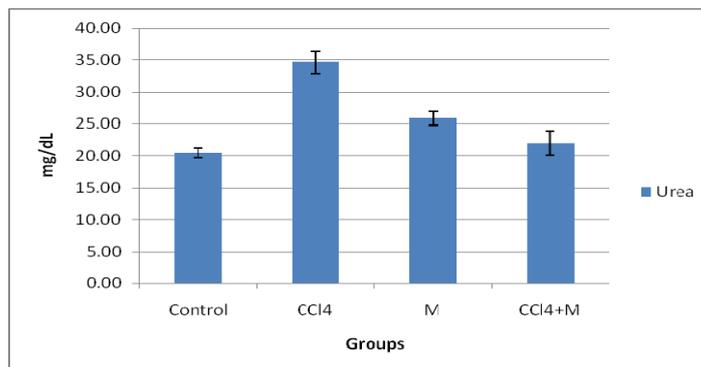


Fig. 4: Serum levels of urea in the different experimental rat groups.

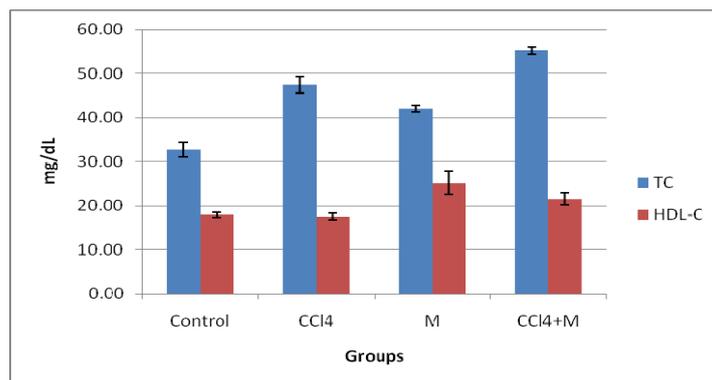


Fig. 5: Serum levels of total cholesterol and HDL-cholesterol in the different experimental rat groups.

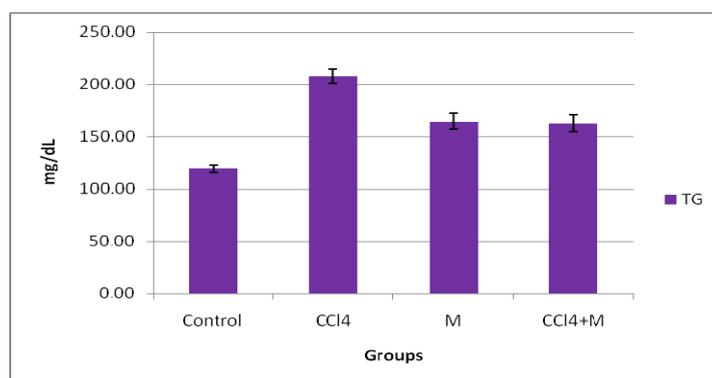


Fig. 6: Serum levels of triglycerides in the different experimental rat groups.

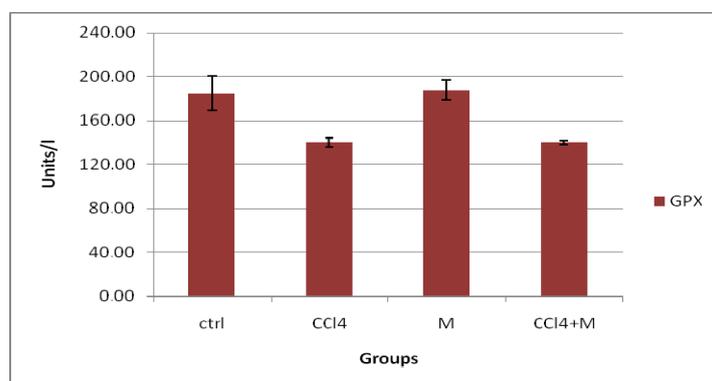


Fig. 7: Serum activity levels of glutathione peroxidase enzyme (GPx) in the different experimental rat groups.

The increased activities of liver marker enzymes such as AST and ALT in the serum of CCl₄ induced animals indicate damage to hepatic cells (Wolf, 1999). CCl₄-mediated acute toxicity increased permeability of the hepatocyte membrane and cellular leakage (Paduraru *et al.*, 1996; Huang *et al.*, 2011). The obtained results indicate the ability of marjoram to help liver cells to overcome the bad effect caused by CCl₄ toxicity. This may be achieved via stabilization of

plasma membrane as well as repair of hepatic tissue damages caused by CCl₄ (Ravikumar and Gnanadesigan, 2011). It had been reported that CCl₄ is metabolized by cytochrome P450 system and converted to trichloromethyl and trichloromethyl peroxy radicals (Zhou *et al.*, 2010).

These radicals initiate peroxidation of polyunsaturated fatty acid constituents of various membranes with secondary damage and severe enzymatic

disturbances (Stehbens, 2003). The body has a set of endogenous antioxidant enzymes which are able to detoxify free radicals by converting them back to more stable molecules within the cell, to be used or disposed accordingly (Saeed *et al.*, 2005). Marjoram is a medicinal plant that has antioxidant activity (Yasin and Abou-Taleb, 2007; Badee *et al.*, 2013). We attribute the ability of marjoram to help the liver to restore its activity to this antioxidant activity of marjoram.

Molecular part:

Differential display is considered one of the most recently valuable molecular techniques. It is able to provide researchers with general overview of the down and up expressed genes under specific treatments without previous knowledge about the extent of their expression or identity. In our study; we used differential display to compare between the genetic status of liver tissues in normal and treated conditions. The treated condition include exposure to specific dose of CCl₄ alone, exposure to specific dose of CCl₄ plus marjoram and

finally exposure to specific dose of marjoram alone. After two weeks of treatments, total RNA was extracted from all the mentioned tissues and mRNA was submitted to reverse transcription to cDNA using oligo dT primers. At the end, the obtained cDNA was randomly amplified through traditional PCR using OP1 and OP2 arbitrary primers.

As shown in figure 8, many genes with different sizes were amplified. Most of these genes were normally amplified in control samples (C) while same genes were down regulated in samples treated with toxin alone (T). On the other hand, the oral feeding of rats with marjoram was able to return the re-expression of these genes again wheather toxin is injected (T+M) or not (M). One of the down regulated genes in only toxin group (d) was excised from the gel and submitted to sequencing. The sequence result of this gene cleared that the gene is *Mus musculus* TBC1 domain family, member 30 (Tbc1d30), with somewhat similarity of (96%).

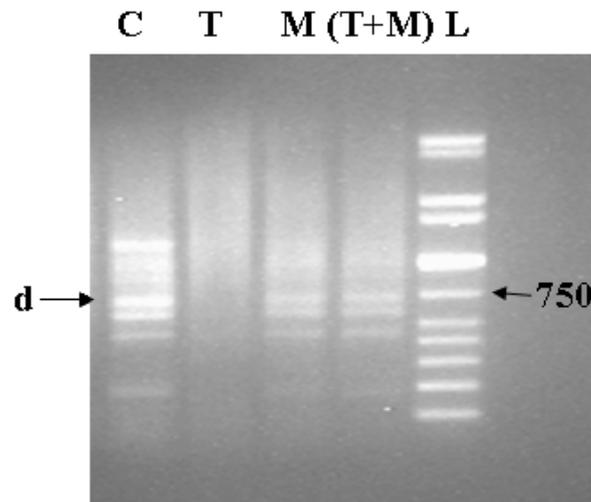


Fig. 8: DD-PCR of rat groups submitted to CCl₄ toxicity and treated with marjoram as medicinal plant. C: control group, T: CCl₄ injected group, M: marjoram feeding group, T+M: CCl₄ injected and marjoram feeding group, L: 1 kb DNA ladder, d: the down regulated gene.

As shown in Fig. 9, Tbc1d30 gene is highly expressed in some but not all the body tissues. Liver tissue is considered one of them (<http://www.genecards.org>

[/cgi-bin/carddisp.pl?gene=TBC1D30](http://cgi-bin/carddisp.pl?gene=TBC1D30)). This Tbc1d30 gene has the function of Rab GTPase activator activity. Rab GTPases are master regulators of

membrane trafficking (Stenmark, 2009). They exist in an active, GTP-bound state and an inactive, GDP-bound state (Ishibashi *et al.*, 2009). When active,

they bind a number of specific effector proteins that mediate their diverse roles in transport vesicle formation, motility, docking, and fusion (Goueli *et al.*, 2012).

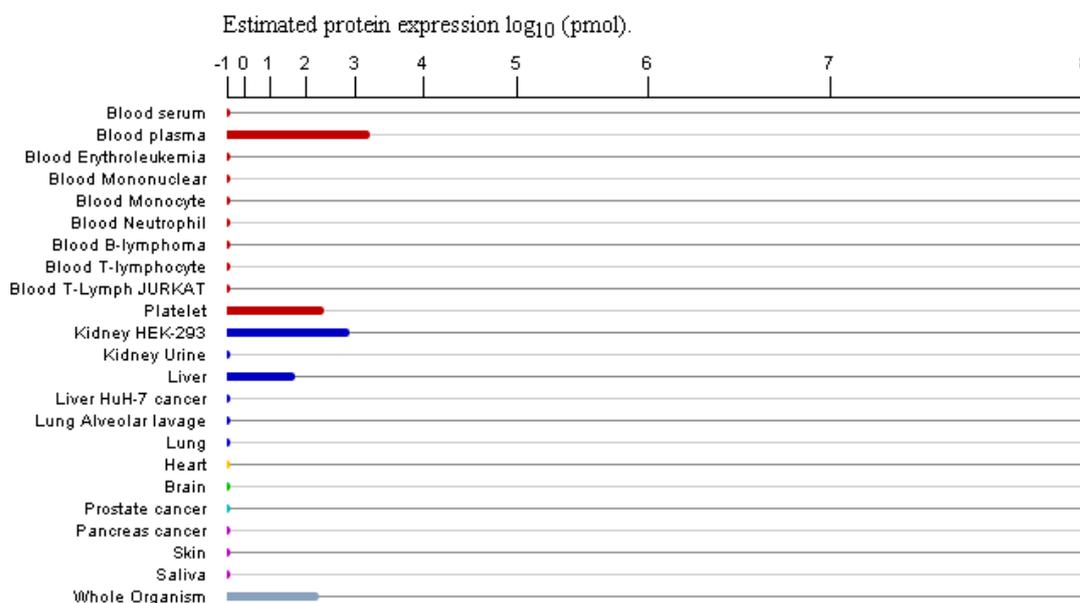


Fig. 9: Expression of Tbc1d30 gene in different body tissues.

According to Stenmark (2009); functional impairments of Rab signaling pathways could cause immunodeficiencies, cancer, and neurological disorders. From our point of view, down regulation of Tbc1d30 gene could cause serious inactivation of Rab GTPases that in turn might cause severe immunodeficiency where the liver cells became unable individually to resist CCl₄ toxicity. On the other hand, Rab GTPases inactivation could cause ceasing of vesicle trafficking. This may open the window to the importance of vesicles to resist and detoxify the toxic chemicals including CCl₄.

CONCLUSION

The use of natural products extracted from the medicinal plants found in Egypt is considered an alternative solution for the treatment of many diseases especially the liver diseases instead of the use of synthetic drugs. However, this needs further

studies to know the active ingredients of these natural products like those found in marjoram which affect the physiological and genetic status of the body.

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