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Potentiation of antifibrotic activity of Atacand using grape seed extract (Gervital) in male albino rats

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Abstract

Carbon tetrachloride (CCl_4) is a known potent hepatotoxic agent. The present investigation clarified the ameliorative effects of Atacand and Gervital on CCl_4 –induced hepatotoxicity. 24 male albino rats were divided into 4 groups, 6 rats each. Group I, served as normal control. Group II, animals were injected subcutaneously with CCl_4 (1ml/kg b.w.) twice a week for 90 days. Group III, rats were injected with CCl_4, as Group II, then treated orally with Atacand (8mg/kg b.w.), daily, for 30 days. Group IV, rats were injected with the same dose of CCl_4 for 90 days, then treated with Atacand (8mg/kg b.w.) along with Gervital (100mg/kg b.w.), orally, daily for 30 days. CCl_4 treatment showed a marked deterioration of liver function as a high increase in the levels of serum AST and ALT (153.8% and 157.8%, respectively). Also a significant decrease in liver antioxidant machinery was represented by reduced glutathione (GSH, 68.1%), glutathione-s-transferase (GST, 69.9%) and glutathione reductase (GR, 65.65%). On the other side, the levels of liver protein carbonyl groups (PCO) and lipid peroxidation (LPO) were elevated 439.6% and 258.7%, respectively, compared to their control groups. These results indicated the oxidative damage as a result of CCl_4-induced liver fibrosis. Both treatments with Atacand or Atacand + Gervital showed significant ameliorative effect for both liver and serum CCl_4-induced alterations. The current study recorded that the treatment with Gervital along with Atacand scored more antifibrotic effectiveness than that of Atacand alone. So, Gervital acted as a potentiator for antifibrotic activity of Atacand.

Keywords: *Atacand; Carbon tetrachloride; Gervital; Liver function;Oxidative stress.*

1 Introduction

Hepatitis C virus is considered the most common etiology of chronic liver disease in Egypt - 20% of those progress to cirrhosis, and possibly 2-3% per year die as a result of its complications or hepato-cellular carcinoma.

Liver is the main detoxifying organ in the body, and as such it possesses a high metabolic rate and it is subjected to many insults potentially causative of oxidative stress. Consequently, a correct status of the hepatic antioxidant defense system is of major importance for the maintenance of health (Ahmed and Fatani, 2007).

Hepatic fibrosis is a reversible wound-healing response to liver injury, which has the potential to progress to cirrhosis. Several studies have shown that hepatic fibrosis is a reversible disease; therefore an effective treatment would probably prevent or reverse the fibrotic process in the liver (Farci et al., 2004; Satapathy et al., 2007). CCl_4-intoxication, give a suitable animal model, similar to the human fibrosis. Carbon tetrachloride (CCl_4) is one of the chlorinated hydrocarbons that have a widespread use in various industries as a solvent. On the other hand, exposure to CCl_4 takes place by inhalation, ingestion or absorption through the skin.

Potent hepatotoxin in a variety of experimental animal models (Weber et al., 2003), and induces necrosis (Sivikova et al., 2001) and apoptosis in the liver. Prolonged administration of CCl_4 leads to fibrosis, cirrhosis and hepatic carcinoma (Wernke and Schnell, 2004).

Atacand (Candesartan cilexetil) is a selective angiotensin II

(AT-II) type 1 receptor (AT1-R) blocker widely used as an antihypertensive in clinical practice and safe when administered for a long time (Weinberg et al., 2004; Rosei et al., 2005). Paizis et al. (2002) reported that Angiotensin II (AT-II) is the principal effector molecule of the renin angiotensin system (RAS). It is synthesized in chronically damaged tissues by resident myofibroblasts (Katwa et al., 1997). Locally produced AT-II binds to angiotensin type 1 (AT1) receptors to stimulate angiogenesis, recruitment of inflammatory cells, growth of myofibroblasts and synthesis of extracellular matrix proteins. Due to its biological properties, the RAS is a target to prevent fibrosis in chronic inflammatory (Ruiz-Ortega et al., 2003). The blockade of the RAS, either with angiotensin- converting enzyme (ACE) inhibitors or AT1 antagonists, attenuates fibrosis development in experimental cardiac and renal fibrosis and are widely used as antifibrotic therapy in patients with chronic cardiac and renal diseases (Lijnen and Petrov, 2003).

Grapes and grape products are good sources of dietary flavonoids, which are powerful antioxidant compounds. Furthermore, the inedible contain some compounds that are able to scavenge superoxide radicals in living cells (Yilmaz and Toledo, 2004). Gervital which is grape seed extract contains proanthocyanidins as naturally occurring plant metabolites widely available in fruits, vegetables, nuts, flowers, wine, bilberry, ginko, black and green tea (Delnunay et al., 2002). A variety of proanthocyanidins have been shown to be anti-bacteria, anti-viral, anti-carcinogenic (Carnésecchi et al., 2002), anti-inflammatory (Li et al., 2000), anti-allergic and consequently reduce the concentration of reactive oxygen species (Bagchi et al., 1997) and low density lipoprotein oxidation (Rein et al., 2000).

The present study was designed to evaluate the effect of the co-treatment with Atacand (Atac), as an antifibrotic drug, and Gervital (Gerv), as a grape seed natural extract rich with proanthocyanidin, against CCl_4- induced liver fibrosis.

2 Materials and Methods

Chemicals:

Carbontetrachloride was obtained from Sigma-Aldrich company (St. Louis, MO, USA). Atacand (Candesartan Cilexetil) was obtained from Astrazenca for Pharmaceutical, Cairo, Egypt. Gervital (Proanthocyanidins) was obtained from Minapharm for pharmaceuticals, Cairo, Egypt. Thiobarbituric acid was obtained from Fluka (Berlin, Germany). The fine chemicals; Sodium nitrite, ammonium molybidate, 1-chloro-2,4-dinitrobenzene(CDNB), ascorbic acid, ammonium acetate, riboflavin, Nitroblue Tetrazolium (NBT), sulfanilamide and trichloroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Thiobarbituric acid was obtained from Fluka (Berlin, Germany).

Animals:

Male albino rats weighing 100-120 g were obtained from National Research Institute, Cairo, Egypt and acclimatized for two weeks prior to the experiment. They were maintained under standard laboratory conditions of room temperature (22-25 $^{\circ}$C) and a relative humidity (55±5 %) with a light period of 12 h light/dark cycle/day. Animals received standard laboratory balanced commercial diet and water *ad libitum*.

Expirmental design:

Twenty four rats were separated randomly into 4 groups, 6 rats each.

Group I: served as normal control, animals received saline solution.

Group II: animals were injected subcutaneously with CCl_4 (1ml/kg b.w.) twice a week for 90 days (El-Elaimy and Abdel-Ghaffar, 1997).

Group III: rats were injected with the same dose of CCl_4 for 90 days, then treated, orally, with Atacand (8mg/kg b.w.), daily, for 30 days (Yoshiji et al., 2001).

Group IV: rats were injected with the same dose of CCl_4 for 90 days, then treated with Atacand (8mg/kg b.w.) along with Gervital (100mg/kg b.w) (Bagchi et al., 2001), orally and daily, for 30 days

After 24 hours from the last treatment, the animals of each group were sacrificed by cervical dislocation then blood samples from hepato-portal vein were withdrawn. Serum was separated by centrifugation at 4000 rpm for 15 min, at 4 $^{\circ}$C. Liver was discarded, blotted dry, weighed and homogenized in cold, 0.15 M, KCl to give a final concentration of 10% (w/v) homogenate. All samples were stored at -20°C till biochemical analyses.

Biochemical analysis:

The Biochemical analyses were determined according to the corresponding methods. Serum AST and ALT activities (Reitman and Frankel, 1957). Liver reduced glutathione (GSH) content (Beutler and Kelley, 1963) and activities of liver antioxidant enzymes; glutathione- S- transferase (GST) (Habig et al., 1974), glutathione reductase (GR) (Carlberg and Mannervik, 1975). Also, liver lipid peroxidation (LPO) (Ruiz- Larrea et al., 1994) and protein carbonyl (PCO) (Levine et al., 1990) contents were assayed.

Statistical analysis:

Results are expressed as mean ± S.E. Data were analyzed by SPSS software, version 19 (Chicago, IL, USA) followed

by Student's t- test. P value < 0.05 was considered statistically significant.

3 Results

CCl_4 caused highly significant (P< 0.001) elevation in activities of serum AST (153.8%) and ALT (157.8%) compared to control group. Atac-treatment ameliorated serum AST and ALT activities (31.95% and 32.24%), respectively. It reduced, significantly, the alterative CCl_4- induced effect, compared to CCl_4 treated groups. On the other side Gerv+Atac co-treatment produced additive amelioration, for serum AST and ALT activities (9.86% and 3.90%), respectively, compared to CCl_4 + Atac treated groups (Table 1).

On the other hand, CCl_4 caused a highly significant (P< 0.001) depletion (68.1%) in the level of liver GSH. Also, liver antioxidant enzymes GST and GR activities decreased with CCl_4 treatment (69.9% and65.65%), respectively, compared to their control values. Treatment with Atac significantly restored the level of liver GSH (70.33%) and liver antioxidant enzymes activities of GST and GR (109.19% and66.66%), respectively. But these levels still, significantly, lesser than their normal levels. Atac+Gerv co-treatment significantly resulted in more regulation for GSH level (11.94%) and liver antioxidant enzymes GST and GR activities (13.73% and 16.66%) respectively, compared with CCl_4 +Atac treatment groups (Table 2).

CCl_4 -intoxication significantly increased the levels of LPO and PCO (258.7% and 439.6%), respectively, when compared to their control groups. However, LPO and PCO levels have been restored with Atac-treatment (55.87% and 32.23%), respectively, compared with CCl_4 treated groups. On the other side, Atac + Gerv co-treatment showed more restoration for hepatic LPO and PCO levels (3.96% and13.18%), respectively, compared with CCl_4 + Atac treatment groups (Figs. 1& 2).

Table 1. Serum AST & ALT activities in different studied animal groups.

Expirmental groups	**AST** (U/L)	**ALT** (U/L)
Control	97.40± 1.12	61.60 ± 1.03
CCl_4	247.20 ±1.39 [a**] **(153.8%)**	158.80±1.74[a**] **(157.8%)**
CCl_4 + Atac	168.20 ± 1.66 [b**] **(31.95%)**	107.60 ± 1.21 [b**] **(32.24%)**
CCl_4 + Atac +Gerv	151.60 ± 1.86 [c**] **(9.86%)**	103.40 ± 1.21 [c*] **(3.90%)**

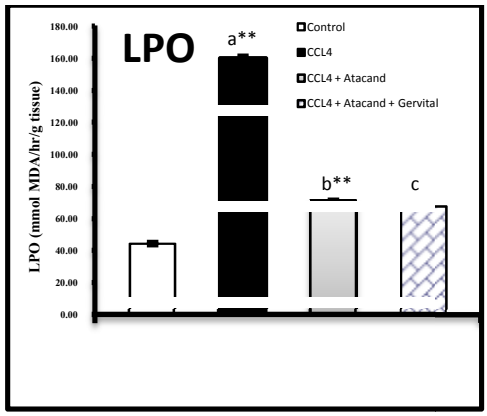
Each value represents mean ± S.E. (n = 6). *(%) value is compared to the corresponding group.* (a) significant compared to the control group, (b) significant compared to the CCl_4 treated group and (c) significant compared to the to the CCl_4 + Atac treated group. (*) P < 0.05 and (**) P <0.001.

Table 2. Liver GSH content, GST and GR activities in different studied animal groups.

Expiremental groups	**(GSH)** (mg/gtissue)	**(GST)** (nmole CDNB conjugate formed/min/ ml)	**(GR)** (nmole/NADPH oxidized/min/ ml)
Control	74.00 ± 1.41	57.80 ± 1.16	31.40 ± 1.08
CCl_4	23.60 ±1.21 [a**] **(68.1%)**	17.40 ± 0.81 [a**] **(69.9%)**	10.80 ±1.16 [a**] **(65.6%)**
CCl_4 + Atac	40.20 ± 1.36 [b**] **(70.33%)**	36.40 ± 1.03 [b**] **(109.19%)**	18.00 ± 1.58 [b**] **(66.66%)**
CCl_4 + Atac + Gerv	45.00 ± 1.30 [c*] **(11.94%)**	41.40 ± 1.47 [c*] **(13.73%)**	21.00 ± 1.00 [c] **(16.66%)**

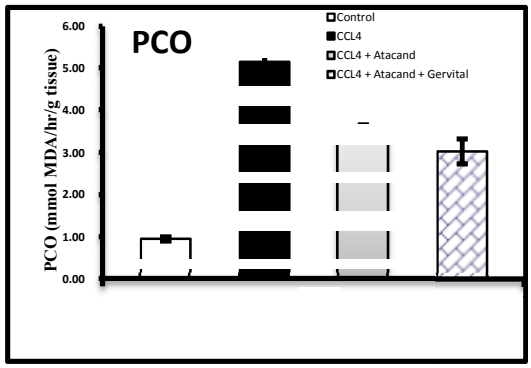
Each value represents mean ± S.E. (n = 6). *(%) value is compared to the corresponding group.* (a) significant compared to the control group, (b) significant compared to the CCl_4 treated group and (c) significant compared to the to the CCl_4 + Atac treated group. (*) P < 0.05 and (**) P <0.001.

Fig. 1. Liver LPO level in the different studied groups.



Each value represents mean ± S.E. (n = 6). (a) significant compared to the control group, (b) significant compared to the CCl_4 treated group and (c) significant compared to the CCl_4 + Atac treated group. (*) $P < 0.05$ and (**) $P < 0.001$.

Fig. 2. Liver PCO level in the different studied groups.



Each value represents mean ± S.E. (n = 6). (a) significant compared to the control group, (b) significant compared to the CCl_4 treated group and (c) significant compared to the CCl_4 + Atac treated group. (*) $P < 0.05$ and (**) $P < 0.001$.

4 Discussion

Oxidative stress is thought to play an important contributory role in the pathogenesis of numerous degenerative or chronic diseases (Ray and Husain, 2002). Human diseases, such as diabetes, neurodegenerative, cardiovascular diseases and specially carcinogenesis have been associated with oxidative stress. This condition occurs in the tissue when the concentration of the generated reactive oxygen species exceeds its antioxidant capability (Nakabeppu et al., 2006). AST and ALT are cytosolic enzymes of the hepatocytes. They are involved in the breakdown of amino acids into α-keto acids (Maiti et al., 2004).

In our study, CCl_4 provoked significant elevation in serum AST and ALT activities, reflecting impaired liver function. This result is in agreement with a previous study which reported that CCl_4 toxicity developed significant hepatic damage as manifested by a significant increase in activities of AST and ALT, which are indicators of hepatocyte damage and loss of functional integrity (Nabeshima et al., 2006).

It is well documented that the toxicity of CCl_4 is thought to involve two phases; first, CCl_4 metabolization by cytochrome P450 in the hepatocytes produces the highly reactive CCl_3- radical, which leads to lipid peroxidation and membrane damage. The second step is a Kupffer cell mainly related inflammatory response. Kupffer cells are activated by free radicals and secrete cytokines that attract and activate neutrophils. Neutrophils themselves release reactive oxygen intermediates (ROIs), thereby enhancing the liver injury (Louis et al., 1998). So, any increase in AST and ALT activities reflects a leakage in plasma membrane permeability, which in turn, is associated with cell death. Moreover, they are best indicators of liver necrosis (Hemida and Mohafez, 2008).

Recently, Sirage et al. (2011) found that CCl_4 – intoxication has a bad significant effect on the liver function, since the activities of serum AST and ALT were significantly higher than those of normal value.

All aerobic organisms have antioxidant defenses to remove or repair the damaged molecules. These antioxidants can protect the human body from free radicals and reactive oxygen species (ROS) effects. ROS related to the progress of many chronic diseases as well as lipid peroxidation (Lai et al., 2001; Gulcin et al., 2002).

The present study showed a highly significant reduction in GSH level after CCl_4 administration. Several studies suggested that CCl_4 administration cause depletion in GSH contents (Sirage, et al., 2011). On the same line Hayes et al. (2005) suggested that CCl_4 treatment depletes the hepatic GSH contents.

In general, GSH is one of the most common biologic non enzymatic antioxidant. Its function includes removel of free radicals such as H_2O_2 and superoxide anions, maintenance of membrane protein thiols and acting as a substrate for GPx and GR (Naik and Panda, 2007). GSH deficiency contributes to oxidative stress, which plays a key role in liver disease, cystic fibrosis, sickle cell anemia, cancer, heart attack, stroke and diabetes (Rana et al., 2002). On the other side Hayes et al. (2005) revealed that GSH conjugates play a major role in eliminating the CCl_4-induced toxic metabolites which are the main cause of liver

injuries. So, the maintenance of sufficient glutathione level is important for the prevention of CCl_4-induced damages.

Our data confirmed the concept that oxidative stress plays a role in CCl_4-induced tissue damage, whereas GSH reduction was accompanied by reduction in the antioxidant enzyme defense system represented as depletion in the levels of GST and GR. This is in agreement with the recent studies demonstrated that CCl_4 significantly lowered the levels of hepatic antioxidants coupled with high indices of lipid peroxidation (Jadeja et al., 2011; Desai et al., 2012).

Herein, a decrease in the activities of antioxidant enzymes can be explained either with their induction during the conversion of free radicals into inactive metabolites or secondary with the direct inhibitory effect of CCl_4 on enzymes activities.

The toxicity of CCl_4 and its reactive metabolites may result from covalent (primary) interactions with critical target molecules such as DNA, lipids, protein, or carbohydrates, or from the alteration of target molecules via secondary bond formation of lipid peroxidation (LPO), generation of reactive oxygen species and glutathione depletion (Boll et al., 2001; Das et al., 2004). Our results showed that CCl_4 administration increased the level of LPO. Surendran et al. (2011) showed that CCl_4 administration resulted in elevation in the level of LPO. It was reviewed also that CCl_4 treatment produced an elevation in the level of malonaldhyde (MDA) in the hepatic tissues (Bayraktar et al., 2012).

The increase in LPO levels suggests enhanced peroxidation leading to tissue damage and failure of the antioxidant defense mechanism to prevent the formation of excessive free radicals (Cetin et al., 2011).

Protein carbonyl groups (PCO), aldehydes and ketones, are produced by protein side chains when they are oxidized by almost all types of ROS (Beal, 2002).

Our data showed that CCl_4 administration increased PCO level in liver. Stadtman (2004) discussed that exposure of proteins to reactive oxygen species can alter the physical and chemical structure of the target protein causing consequent oxidation loading to oxidation of hydrophobic amino acyl residues to hydroxyl and hydroxyperoxy(P-OOH) derivatives, protein carbonylation (PCO), oxidation of total sulfhydryl groups (T- SH), and many others. Our results were in agreement with Chen et al. (2013) and Kamel et al. (2010) who found that CCl_4 toxicity caused a significant increase in protein carbonyl compared to the control group. The current study clarified that treatment with Atacand (candesartan cilexetil) markedly attenuated AST and ALT activities aganist CCl_4 toxicity. This is supported by the early study that serum levels of transaminases return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes (Thabrew et al., 1987). In other study D'Amico et al. (2001) showed that serum levels of transaminases return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes after candesartan cilexetil adminstration. On the same line Rajesh and Latha (2004) showed that administration of candesartan cilexetil to rats caused a decrease in the activity of the serum, hepatic enzymes which may be a consequence of plasma membrane regeneration as well as the repair of hepatic tissue damage caused by CCl_4. Sirag et al. (2011) reported that Atacand minimize the injuries and protect the liver from further side effect of CCl_4. This suggested that candesartan cilexetil may have direct effect on fibrosis rather than indirect effect mediated by hepatoprotection (Tuncer et al., 2003).

Our results showed that, Atacand treatment impeded and consequently improved GSH level. Its treatment limited GSH reduction and restored the activity of GST and GR. Our results were in agreement with Sirag et al. (2011) who showed that the hepatic GSH level and GST activity were restored by treatment with candesartan cilexetil after CCl_4 toxicity. Darwish and Abdel-aziz, (2006) discussed that candesartan cilexetil have an efficient role in manipulating liver fibrosis by minimizing cytokine and extracellular matrix proteins production as well as affecting oxidative stress parameters.

In the present study, Atacand treatment significantly provides strong restoration against lipid peroxidation induced by CCl_4. As well as, ameliorative against PCO. Sirag et al. (2011) found that candesartan cilexetil treatment significantly decreased the serum markers of hepatic injury and lipid peroxidation against CCl_4 – induced liver injury in rat. The outhers suggested that this finding may prove that candesartan cilexetil has an important role in hepatocyte regeneration. Consequently, a remarkable depletion in the alterations of CCl_4 – induced hepatotoxicity was produced. Our results showed that, treatment with Gervital markedly attenuated AST and ALT activities aganist CCl_4 toxicity. These results were in agreement with the finding obtained by Ahmed and Fatani (2007) who showed that oral administration of grape seed proanthocyanidin (GSP) in rats, significantly protected the liver and attenuated activities of the estimated liver enzymes ALT and AST. In earlier study (Joshi et al., 2000) stated that GSP administration resulted in a significant decrease in acetaminophen-induced serum aminotransferases elevation, hepatic DNA damage, and mortality rate. This clarified that treatment with Gervital protected plasma membranes from the attacking of free radicals and prevented their damage.

Our results showed that, Gervital treatment improved GSH level and restored the activity of GST and GR. These results decoumented the role of proanthocyanidins in

protecting cells against oxidative stress caused by CCl_4 through modulation of metabolic functions, enhancement of detoxification pathways, and/or prevention of the interaction of xenobiotics with biological molecules (Bagchi et al., 2000). These results coincide with that of Shan et al. (2010) who stated that Grape seed proanthocyanidin extract (GSPE) can improve the activity of antioxidant GSH and GST in liver. This indicates that the antioxidant function of GSPE maybe work by increasing the activity of body's antioxidant enzymes.

The co-treatment of Gervital showed more restoration of hepatic LPO and PCO levels against CCl_4 induced oxidative stress. Early, investigators have demonstrated the efficacy of proanthocyanidin as an inhibitor of lipid peroxidation and as a powerful free radical scavenger *in vitro* as well as *in vivo* (Ray et al., 1999). Our results matched with Chis et al. (2009) who found that long-term administration of GSE offers enhanced antioxidant potential and protection of tissue lipid peroxidation and protein oxidation.

In general, grape seed proanthocyanidin extract (Gervital) can clear off free radicals, protect the over-oxidative damage caused by free radicals (Feng et al., 2005; Spranger et al., 2008), and prevent a range of diseases caused by free radicals, such as myocardial infarction, atherosclerosis, drug-induced liver and kidney injury; what's more, it has functions of anti-thrombotic, anti-tumor, anti-mutagenic, anti-radiation, and anti-fatigue (Qin et al., 2006; Engelbrecht et al., 2007).

Bagchi et al. (2000) concluded that GSPE act as a safe, novel, highly potent and bioavailable free radical scavenger and antioxidant possessing a broad spectrum of health benefits. GSPE functions at the genetic level and promotes therapeutic efficacy. Further mechanistic and clinical studies are in progress to unveil the mechanism of this novel natural antioxidant.

We concluded that Atacand and Gervital possessed modulatory mechanism against CCl_4- induced liver toxicity. Atacand (Candesartan cilexetil), an AT-II type 1 receptor (AT1-R) blocker, have hepatomodulative effects and effectively delayed the progression of hepatic fibrosis. Gervital (Proanthocyanidins from grape seed), having a very important function as antioxidant, by which it potentiated the antifibrotic activity of Atacand. Their co-treatment can clean off the free radicals, and reduce the membrane lipid peroxidation leading to hepatocyte regeneration.Our study showed that Gervital added more strength to the ameliorative effect of Atacand through their co-treatment against CCl_4-induced hepatic fibrosis.

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