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SYNERGISTIC HEPATOCARDIOPROTECTIVE AND ANTIOXIDANT EFFECTS OF MYRRH AND ASCORBIC ACID AGAINST DIAZINON-INDUCED TOXICITY ON RABBITS

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Abstract:

Diazinon (DZN) is a widely used organophosphrus synthetic and acaricide widely used for agricultural and veterinary purposes. However, its human and animal exposure leads to hepatocardiotoxicity. Our experimental objective was to evaluate protective effects of Myrrh; *Commiphora molmol* or/ and ascorbic acid; vitamin C against DZN-induced hepatocardiotoxicity in healthy male white New Zealand rabbits (*Oryctolagus cuniculus*). DZN-treated animals revealed significant alterations in serum biochemical parameters related to hepatic and cardiac injuries. There was a significant increase in hepatic and cardiac lipid peroxidation and significant inhibition in tissue antioxidant biomarkers due to DZN intoxication. Both myrrh and vitamin C protect against DZN-induced serum as well as hepatic and cardiac tissue biochemical parameters when used alone or in combination along with DZN-intoxication. Furthermore, both myrrh and vitamin C produced synergetic hepatocardioprotective and antioxidant effects. Therefore, it could be concluded that myrrh and/or vitamin C administration able to minimize the toxic effects of DZN by its free radical-scavenging and potent antioxidant activity.

Introduction

Organophosphorous pesticides have fully replaced the chlorinated pesticides in the 4 decades ago. The main advantage of the organophosphorous compounds was their low cumulative effect and short-term persistence in the ecosystem (Zavon, 1971). Although the organophosphates have been replaced by pyrethroids within the last 10–15 years, there is still a very intensive use of organophosphorus insecticides (Salem and Olajos, 1988). They are used throughout the world for control of agricultural, veterinary and domestic insect pests. Diazinon [phosphoric acid, O, O-diethyl O (2-isopropyl-6-methyl-4-pyridinyl)] phosphorothioate is an organophosphorus insecticide widely used in agricultural, veterinary and domestic practice throughout the world to control ticks, flies, lice, fleas and other insect pests of ornamental plants, food crops and domestic animals (Larkin and Tjeerdema, 2000). Toxic effects of Diazinon on in target and non-target organisms are due to the inhibition of acetyl cholinesterase (AChE). Diazinon exerts its toxicity through binding its oxygen analog to the neuronal enzyme AChE, resulting in the accumulation of the endogenous neurotransmitter; acetylcholine in neurons and effectors organ (Larkin and Tjeerdema, 2000) . Furthermore, it affects mitochondrial membrane transport in rat liver and disrupts cytochrome P450 system in human liver (Sams et al., 2004). The The toxic effects of diazinon on mammalian cells were studied by some researchers (Al-Attar and Abu Zeid, 2013; ElMazoudy and Attia, 2012; Elmazoudy et al., 2011; Larkin and Tjeerdema, 2000; Razavi et al., 2013). Diazinon toxicity induced oxidative stress resulting in hematological changes, neurotoxicity, hepatotoxicity, nephrotoxicity, cardiotoxicity and both male and female reproductive toxicity (Al-Attar and Abu Zeid, 2013; ElMazoudy and Attia, 2012; Elmazoudy et al., 2011; Razavi et al., 2013).

The cells combat oxidative stress by either removing the damaged nucleotides and lipid peroxidation products or directly scavenging oxygen radicals via endogenous enzymatic and non-enzymatic antioxidants (Abdel-Daim et al., 2013; Azab et al., 2013; Madkour and Abdel-Daim, 2013).

Myrrh is a yellow aromatic oleo-gum resin obtained from the stems of a number of small, thorny tree species of the genus Commiphora, particularly *C. molmol* and *C. myrrha* (Burseraceae family). It consists of approximately 30–60% water-soluble gum, 20–40% alcohol soluble resin and 3–8% volatile oil (al-Harbi et al., 1997). It also contains eugenol, cuminic aldehyde, terpenes and sesquiterpenes (al-Harbi et al., 1997). It has been demonstrated that myrrh has many pharmacological activities, including: local anesthetic, analgesic, anti-inflammatory and anti-ulcer, immunomodulatory, antimicrobial and antiparasitic (al-Harbi et al., 1997; Ashry et al., 2010; Dolara et al., 2000; Shen et al., 2012). It has a potent anticancer activity against Ehrlich ascites carcinoma(Qureshi et al., 1993). Myrrh protected liver against diethylnitrosamine-induced injury and hepatocarcinogenesis (El-Shahat et al., 2012). It reduced Pb-induced oxidative damage in hepatic mice tissue by reducing lipid peroxidation and enhancing GST activity (Ashry et al., 2010; El-Ashmawy et al., 2006). Myrrh active constituent; guggulsterone induced cardioprotective effects against doxorubicin cardiomyocyte injury (Wang et al., 2012). Its antioxidant properties might be through its free radical-scavenging activities (Ashry et al., 2010; El-Ashmawy et al., 2006). The free radical-scavenging effect of myrrh essential oils also provide a protection against lipid peroxidation induced by lipophilic pharmaceuticals and cosmetic preparations (Auffray, 2007).

Ascorbic acid; vitamin C is probably the most commonly used vitamin and perhaps the most important antioxidant in extracellular fluids. It is an essential component in the diet of some mammals, including human and rabbits. It is highly water soluble and acts as an effective reductant. It is the most effective antioxidant in inhibiting lipid peroxidation initiated by peroxyl radicals, and considered as an effective radical scavenger of ROS (El-Demerdash et al., 2005; Kojo, 2004). It may also regenerate other antioxidants such as vitamin E (Carr and Frei, 1999). To our knowledge, the role of myrrh and vitamin C against DZN-induced alterations in serum biochemical parameters as well as lipid peroxidation and antioxidant status in rabbits has not been studied yet. Therefore, in the present study, was designed to investigate the alterations in serum biochemical parameters related to liver and heart injuries as well as hepatic and cardiac lipid peroxidation and oxidative stress induced by DZN in rabbits. Moreover, the role of myrrh or/and vitamin C supplementation in alleviating these DZN-induced hazard effects could be evaluated.

MATERIAL AND METHODS

Chemicals:

Diazinon® and ascorbic acid were purchased from Adwia Pharmaceuticals, Cairo, Egypt. Diazinon® 60 was applied as a commercial emulsifiable formulation containing 60% active ingredient. It was diluted in deionized water for the final required concentration. Myrrh oleo-resin emulsion from was prepared by Pharco Pharmaceuticals, Alexandria, Egypt. Briefly, known weight of myrrh powder was extracted with ethanol by percolation at room temperature. The extracts were evaporated under vacuum at 40°C to give a yellowish-brown semisolid residue. The yield of myrrh ethanol extract was stored in a refrigerator. The used emulsion was prepared according to (Massoud and Labib, 2000). The concentration used in the experiment was based on the dry weight of the extract. All kits used for evaluation of serum biochemical parameters, tissue lipid peroxidation and antioxidant biomarkers were purchased from Biodiagnostics Co., Cairo, Egypt except that of LDH was purchased from Randox Laboratories Ltd, U.K. and CK was purchased from Stanbio™ CK-NAC (UV-Rate) kit (Texas, USA). Other chemicals used in this study were analytical grade.

Animals and experimental design

The design of the experiments conforms to the guidelines of the National Institutes of Health (NIH) and was approved by a local committee at Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt and Faculty of Agriculture, University, Moshtohor, Egypt. The present work was carried out at Rabbit Farm, Animal Production Department, Faculty of Agriculture, University, Moshtohor, Egypt. Total forty healthy male white New Zealand rabbits (*Oryctolagus cuniculus*) (weighing 1500± 200 g) were used in the current study. All animals were housed in metal batteries and supplemented with the basal diet and water ad libitum through the experiment. After one weeks of acclimatization, all rabbits were randomly divided into five experimental groups of nine rabbits each. Groups I (control) received saline, and II (DZN) orally received the emulsifying agent at a dose of 5.28 mg/kg body weight (Tsitsimpikou et al., 2013) and served as positive controls, respectively. Groups III (DZN-CME) and IV (DZN-AA) were administered with CME in a dose rate of 50 mg/kg body weight(Ashry et al., 2010), and AA at a dose of 20 mg/kg body weight (Ozdem et al., 2011) respectively, while rabbits at group V (DZN-CME-AA) were administered both CME and AA at the same dose and regimen used for the group III and IV. DZN, CME and AA were orally administered using intra-gastric intubation every 48 hours for successive 30 days **(Table 1).** At the end of the experiment, blood samples were collected at room temperature, left to clot and centrifuged at 3000 rpm for 15 minutes to obtain clear sera, and then all animals were slaughtered and sacrificed, and livers and hearts were excised for further preparation for estimation of lipid proxidation and antioxidant biomarkers.

Serum biochemical analysis

Freshly separated sera were used for estimation of serum hepatic and cardiac injury biomarkers according to manufacturer protocol. The appropriate kits were used for the determination of the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) according to (Reitman and Frankel, 1957). Alkaline phosphatase (ALP) was determined according to (Tietz et al., 1983). The enzyme activity was expressed as units/liter computed directly from the absorbance values. The level of total proteins were evaluated according to (Lowry et al., 1951), While total bilirubin was determined according to (Schattmann, 1952). Nevertheless, cholesterol was measured according to (Allain et al., 1974; Richmond, 1973). Serum LDH activity was determined enzymatically the according to the manufacturer's protocol using kits from Randox Laboratories Ltd, U.K. (Buhl and Jackson, 1978). CK activity was estimated according to the method developed by (Szasz et al., 1979) using Stanbio™ CK-NAC (UV-Rate) kit (Texas, USA).

Evaluation of lipid peroxidation and antioxidant biomarkers

Hepatic and cardiac tissues of the collected from each rabbit group were rapidly removed, cleaned from any extraneous materials and immediately perfused with cold saline. The tissues were homogenised in cold phosphate buffer saline (0.1 M pH 7.4) then the homogenate was filtered and centrifuged at 1500 rpm for 20 min .The supernatant was stored in -80°C until use for biochemical analysis of lipid peroxidation and antioxidant biomarkers.

Lipid peroxidation was evaluated using measurement of MDA content in the tissues according to (Mihara and Uchiyama, 1978). Oxidative stress was assessed by evaluation of the enzymatic antioxidant biomarker; superoxide dismutase (SOD) according to (Nishikimi et al., 1972), and the non-enzymatic antioxidant marker; reduced glutathione (GSH) according to (Beutler et al., 1963).

Statistical analysis

All data were expressed as means ± S.E.M. and statistically analyzed using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL). Statistical significance of differences among different study groups were evaluated using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test for post-hoc analysis. Statistical significance was acceptable to a level of P≤0.05.

Result and Discussion

Reactive oxygen species (ROS) are continuously generated inside the body as a result of exposure to many exogenous drugs and xenobiotics in our environment and/or a plenty of endogenous metabolic events involving redox enzymes and electron transport mechanism (Abdel-Daim et al., 2013; Azab et al., 2013; Madkour and Abdel-Daim, 2013). Under normal conditions, there is equilibrium between the ROS generated and the antioxidants present as the ROS generated are neutralized by the endogenous antioxidants (Abdel-Daim et al., 2013; Sun, 1990). Deleterious effects caused by ROS occur as a consequence of an imbalance between the formation and inactivation of these species leading to irregularities in cellular physiology and different pathological conditions (Abdel-Daim et al., 2013; Sun, 1990). Free radicals have been implicated in the aetiology of many degenerative diseases such as cancer, cataract, coronary heart disease, stroke, rheumatoid arthritis, diabetes, Alzheimer's disease and ageing process (Abdel-Daim et al., 2010a, b; Funasaka et al., 2012; Willcox et al., 2004).

Diazinon; an organophosphrus insecticide has been widely used in industrial agriculture and veterinary practice worldwide that would be potentially an exposure risk to human and animals, including rabbits (Larkin and Tjeerdema, 2000). Although several reports about the toxicity of DZN have been published, little study has been performed about the use of natural products for prevention of such toxicity and mechanism of their ameliorative action (Al-Attar and Abu Zeid, 2013; ElMazoudy and Attia, 2012; Elmazoudy et al., 2011; Larkin and Tjeerdema, 2000; Razavi et al., 2013).

In the present study, hepatic and cardiac injuries caused by DZN may be attributed to the oxidative stress resulted from free radical production. DZN intoxication significantly (P≤0.05) increased serum liver and heart injury biomarkers; AST, ALT, ALP, cholesterol and total bilirubin, LDH and CK. Moreover, it significantly (P≤0.05) reduced serum total protein level (Table 2). DZN treatment significantly (P≤0.05) increased lipid peroxidation through elevated hepatic and cardiac MDA level, significantly (P≤0.05) decreased hepatic and cardiac enzymatic; SOD and CAT as well as non-enzymatic; GSH antioxidant level (Tables 3&4).

All these effects are involved in the cascade of events leading to DZN-mediated hepatocardiac oxidative stress and toxicity. This indicates that hepatocardiac injuries induced by DZN is the result from oxidative stress that arises as a result of excessive generation of ROS, which have been reported to attack various biological molecules, including lipids and causing lipid peroxidation. The activities of antioxidant enzymes, including the enzymes involved in glutathione metabolism were also perturbed in DZN treated group (Tables 3 and 4) indicating the involvement of oxidative stress in DZN-mediated hepatocardiac injuries. These results are consistent with the literature (Al-Attar and Abu Zeid, 2013; Razavi et al., 2013) and point towards the role of ROS in DZN-mediated injury and toxicity (Al-Attar and Abu Zeid, 2013; Larkin and Tjeerdema, 2000; Razavi et al., 2013; Salem and Olajos, 1988)

The organophosphorus insecticide; DZN caused an increase in AST, ALT, ALP, LDH, CK, CK-MB enzymes activity, cholesterol total protein, albumin and total billirubin as well as hepatic and cardiac MDA levels and reduced GSH, CAT, SOD, glutathione peroxidase (GPx) and glutathione S-transferase (GST) in mice and rats (Al-Attar and Abu Zeid, 2013; El-Demerdash and Nasr, 2013; Razavi et al., 2013; Tsitsimpikou et al., 2013). These alterations might differ dependent on exposure dose and duration.

In the current study, the pre-administration of CME (50 mg/kg) as well as AA (20 mg/kg) reduced the serum hepatic and cardiac injury biomarkers. Moreover, they reduced the lipid peroxidation in hepatic and cardiac tissues. In addition, there were elevations of hepatocardiac antioxidant enzymes and glutathione levels due to both preventive agents administration. Both myrrh and vitamin C induced synergistic protective effects against DZN-induced serum and tissues biochemical alterations (Tables 2, 3 &4)

The antioxidant and protective effects of myrrh are owed to their content of antioxidant active constituents such as eugenol, cuminic aldehyde and sesquiterpenes (al-Harbi et al., 1997; Shen et al., 2012) . Many previous literatures showed the hepatoprotective, cardioprotective and antooxiant effects of myrrh and its active constituents against drugs, chemicals and xenobiotics (Ashry et al., 2010; Auffray, 2007; El-Ashmawy et al., 2006; El-Shahat et al., 2012; Shen et al., 2012; Wang et al., 2012)

Pre-treatment with vitamin C might play a role in reducing the toxic effect of DZN, and its antioxidant properties seem to mediate such a protective effect, indicated by the reduction of MDA as well as the elevation of GSH and SOD, CAT levels in hepatic and tissue (Devrim et al., 2008; El-Demerdash et al., 2005; Kojo, 2004).

Rabbit is used for many researches as an important livestock for production of meat in most Mediterranean countries, offering more than 30% of world production. Besides, rabbits reproductive capacity, a feature that is attractive for its use as lab animal too.(Lazzaroni et al., 2009). The protective effect of myrrh and/or vitamin C against DZN-induced oxidative stress in our rabbit model could be either direct by inhibiting lipid peroxidation and scavenging free radicals or indirect through the enhancement of the activity superoxide dismutase and CAT; the enzymatic free radicals' scavengers in the cells. Therefore, myrrh and vitamin C could be used in combination to prevent and treat hepatic and cardiac diseases, especially those induced by oxidative damage.

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Table 1: Summary of different rabbit groups and their treatment

Group	DZN	CME	AA
Control	-	-	-
DZN	+	-	-
DZN-CME	+	+	-
DZN-AA	+	-	+
DZN-CME-AA	+	+	+

Diazinon 5.28 mg/kg body weight, every 48 hours for 4 weeks (DZN), Commiphora molmol; myrrh emulsion at 50 mg/kg body weight, every 48 hours for 4 weeks, 1 hour before DZN dose (CME), Ascorbic acid; vitamin C 20 mg/Kg body weight every 48 hours, 1 hour before DZN administration (AA)

Table 2: Serum enzymes activity and biochemical parameters in control and different treated groups

	AST U/L	ALT U/L	ALP U/L	Cholesterol	T. protein	T. billirubin	LDH U/L	CK U/L
Control	$49.72^{C}\pm2.06$	$54.42^{C}\pm3.08$	$15.31^{C}\pm0.49$	$60.80^{D}\pm1.38$	$8.23^{A}\pm0.14$	$1.442^{C}\pm0.02$	$43.90^{D}\pm1.89$	$119.48^{C}\pm3.41$
DZN	$123.4^{A}\pm7.95$	$151.6^{A}\pm8.18$	$29.84^{A}\pm2.61$	$87.39^{A}\pm2.06$	$6.26^{D}\pm0.11$	$1.916^{A}\pm0.02$	$72.19^{A}\pm2.75$	$337.02^{A}\pm13.21$
DZN-CME	$76.05^{B}\pm3.66$	$76.28^{B}\pm4.09$	$20.15^{B}\pm1.02$	$70.51^{B}\pm1.54$	$7.26^{C}\pm0.11$	$1.574^{B}\pm0.06$	$54.67^{B}\pm2.43$	$179.23^{B}\pm10.09$
DZN-AA	$60.26^{C}\pm1.46$	$69.26^{B}\pm0.88$	$18.91^{BC}\pm0.88$	$65.52^{C}\pm1.42$	$7.56^{BC}\pm0.12$	$1.421^{C}\pm0.03$	$50.62^{BC}\pm2.25$	$145.48^{C}\pm7.28$
DZN-CME-AA	$48.32^{C}\pm2.65$	$52.74^{C}\pm2.27$	$16.16^{BC}\pm0.45$	$61.35^{CD}\pm1.84$	$7.89^{AB}\pm0.17$	$1.405^{C}\pm0.03$	$45.42^{CD}\pm1.56$	$124.23^{C}\pm5.03$

Data are expressed as means ± SE (n=8).

Values having different superscripts within the same row are significantly different (P≤0.05).

Diazinon 5.28 mg/kg body weight, every 48 hours for 4 weeks (DZN), Commiphora molmol; myrrh emulsion at 50 mg/kg body weight, every 48 hours for 4 weeks, 1 hour before DZN dose (CME), Ascorbic acid; vitamin C 20mg/Kg body weight every 48 hours, 1 hour before DZN administration (AA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (T. protein), total billirubin (T. billirubin), lactatic dehydrogenase (LDH), creatine kinase (CK).

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Table 3: Hepatic oxidative stress markers and antioxidant parameters in control and different treated groups.

	MDA nmol/gm	GSH mg/g	SOD u/g	CAT u/g	TAC mmol/g
Control	$28.78^{D}\pm2.39$	$54.52^{A}\pm1.98$	$36.06^{A}\pm1.91$	$2.75^{A}\pm0.13$	$48.01^{A}\pm1.35$
DZN	$76.06^{A}\pm2.57$	$34.68^{C}\pm1.61$	$13.34^{D}\pm0.90$	$0.84^{E}\pm0.04$	$33.15^{C}\pm0.72$
DZN-CME	$52.54^{B}\pm1.92$	$43.44^{B}\pm1.49$	$24.14^{C}\pm1.78$	$1.49^{D}\pm0.09$	$42.36^{B}\pm1.29$
DZN-AA	$41.03^{C}\pm5.08$	$45.64^{B}\pm4.23$	$31.16^{B}\pm3.64$	$1.84^{C}\pm0.23$	$44.40^{B}\pm1.75$
DZN-CME-AA	$32.30^{D}\pm2.34$	$50.82^{A}\pm1.66$	$35.96^{A}\pm1.52$	$2.11^{B}\pm0.10$	$47.71^{A}\pm0.84$

Data are expressed as means ± SE (n=8).
Values having different superscripts within the same row are significantly different (P≤0.05).
Diazinon 5.28 mg/kg body weight, every 48 hours for 4 weeks (DZN), Commiphora molmol; myrrh emulsion at 50 mg/kg body weight, every 48 hours for 4 weeks, 1 hour before DZN dose (CME), Ascorbic acid; vitamin C 20mg/Kg body weight every 48 hours, 1 hour before DZN administration (AA), malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (TAC).

Table 4: Cardiac oxidative stress marker and antioxidant parameters in control and different treated groups.

	MDA nmol/gm	GSH mg/g	SOD u/g	CAT u/g	TAC mmol/g
Control	$43.17^{CD}\pm3.59$	$28.69^{A}\pm1.04$	$11.27^{A}\pm0.60$	$1.37^{A}\pm0.06$	$816.2^{A}\pm22.87$
DZN	$120.3^{A}\pm4.81$	$15.96^{C}\pm0.82$	$3.94^{D}\pm0.35$	$0.44^{D}\pm0.01$	$529.7^{D}\pm23.20$
DZN-CME	$68.79^{B}\pm4.30$	$22.86^{B}\pm0.79$	$7.55^{C}\pm0.56$	$0.79^{C}\pm0.05$	$720.1^{C}\pm21.98$
DZN-AA	$52.28^{C}\pm3.76$	$23.65^{B}\pm2.65$	$9.74^{B}\pm1.14$	$0.92^{C}\pm0.12$	$754.8^{BC}\pm29.82$
DZN-CME-AA	$39.92^{D}\pm2.99$	$29.06^{A}\pm1.85$	$11.37^{A}\pm0.47$	$1.07^{B}\pm0.05$	$803.0^{AB}\pm14.25$

Data are expressed as means ± SE (n=8).
Values having different superscripts within the same row are significantly different (P≤0.05).
Diazinon 5.28 mg/kg body weight, every 48 hours for 4 weeks (DZN), Commiphora molmol; myrrh emulsion at 50 mg/kg body weight, every 48 hours for 4 weeks, 1 hour before DZN dose (CME), Ascorbic acid; vitamin C 20mg/Kg body weight every 48 hours, 1 hour before DZN administration (AA), malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (TAC).