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ORIGINAL ARTICLE

Effect of tramadol dependence on male sexual dysfunction

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ABSTRACT

Tramadol dependence became an increasing and alarming problem in the Egyptian community. Wide availability of tramadol as a pain killer and its role in the treatment of premature ejaculation may be the most apparent causes of increased magnitude of the problem among youth who believe that tramadol has a positive impact on their sexual functions. This study aimed to explore the real impact of chronic tramadol administration on sexual functions in males dependent on tramadol. The study was carried on 80 subjects (50 subjects were tramadol dependent group and 30 subjects represented the control group). Personal, family and past histories were obtained from all the participants in addition to the toxicological history from tramadol dependent group. Urine screening for tramadol was done for all cases of history of tramadol dependence then confirmation by HPLC technique to measure tramadol blood level was done. Both groups were investigated for serum testosterone and prolactin level. Curiosity (22%) and treatment of premature ejaculation (20%) were the main motives for dependence. Erectile dysfunction and decreased libido occurred in 44% and 48% of tramadol dependent group respectively. Significant increase in erectile dysfunction and decreased libido was noted as the duration of dependence increased. Additionally, significant decrease in serum testosterone level and increase in serum prolactin level as tramadol daily dose and duration increased was found. In conclusion, men who take tramadol for premature ejaculation or any other purpose must know that they are very susceptible to many sexual dysfunctions.

KEY WORDS: tramadol; dependence; sexual dysfunction; testosterone; prolactin

Introduction

Opioids are the most potent and effective analgesics available and they are accepted as appropriate treatment for cancer and non-cancerous pain (Atici *et al.,* 2005). Tramadol is the most widely sold opioid analgesic in the world (Shipton, 2000). It was the second most frequent opioid reported for abuse among physicians (Adams *et al.,* 2006). Tramadol was considered as a controlled substance in some United States of America and Canada and requires a prescription. However, it is readily available by remote prescription including internet pharmacies with relative ease (Solarino *et al.,* 2010).

Tramadol dependence became an increasing and at the same time alarming problem in the Egyptian community. This could be noted through its popularity and massive use especially among Egyptian youth (Shipton, 2000; Eassa & El-Shazly, 2012). Easy and wide availability of tramadol as a pain killer for intermediate pain and many other forms of chronic pains could be basic factors of its wide spread and abuse (Marquardt *et al.* 2005; Tashakori & Afshari, 2010). Additionally, its role in treatment of premature ejaculation could be one of the most apparent causes of increased magnitude of the problem among youth who believe that tramadol has a positive impact on their sexual functions. (Vorsanger *et al.* 2008).

Disorders of sexual interest/desire, erectile dysfunction and ejaculation disorders are the most common sexual dysfunctions detected in men. It could be attributed to different factors – psychological, physical and iatrogenic (Hatzimouratidis, 2007; Kaminetsky, 2008). In 29 countries, 28% of men aged 40–80 years had one or more sexual dysfunctions (Nicolosi *et al.,* 2004). Worldwide, erectile dysfunction is the most prevalent dysfunction whereas limited information are available about other sexual dysfunctions (Hatzimouratidis, 2007).

The aim of this study was to explore the real impact of chronic tramadol administration on sexual functions in males dependent on tramadol.

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Subjects and methods

Patients

This study was carried out on 80 male subjects who were divided into 50 patients (tramadol dependent group (TDG) who fulfilled the DSM V criteria of dependence (American Psychiatric Association, 2013) and 30 healthy volunteers (control group). The protocol of this work was approved by the ethical committee of faculty of medicine, Tanta University. A written informed consent was obtained from each participant prior to participation. Subjects coding was done to maintain records confidentiality.

Inclusion criteria

Tramadol dependent group: adult male aged below 40 years dependent on tramadol only, with fulfillment of DSM V criteria of dependence**.** Control group: apparently healthy adult male of the same age range.

Exclusion criteria

Patients with chronic disease such as diabetes, bronchial asthma, hepatic, renal, cardiovascular diseases, cancer and those under radio or chemotherapy. Dependents on mixed drugs were also excluded.

Methods

For all subjects the followings were done:

History

Personal history: name**,** age**,** occupation**,** marital status and residence. Past history: chronic diseases and long term medications. The motive for tramadol dependence; initiation and urge by friends, curiosity, financial and/or social troubles, pain killer or for premature ejaculation. Smoking: (Mild: <10 cigarette/day – Moderate smokers: 10–19 cigarette/day – Heavy smokers: ≥20 cigarette/day) (Rebecca et al., 2010). Toxicological history: amount and form of tramadol received per day and duration of tramadol dependence (lifetime use of tramadol) and diseases complicating dependence. Family history: smoking, substance or drug abuse by parents or other siblings, family troubles (broken or united family).

Laboratory investigations:

Quick view tramadol test card: (rapid one step drug of abuse test): it is an *in vitro* immunochromatographic assay for the rapid visual qualitative detection of tramadol in human urine (Marquardt *et al.,* 2005).

Positive cases were investigated for tramadol blood level: a high-performance liquid chromatographic method using UV detection was used for confirmation of diagnosis and for determination of tramadol concentration in human plasma according to Geng-Chang *et al.* (1999).

Both positive cases and the control group were investigated for: Serum testosterone level: Normal level: 270–1070 ng/dl)(Travison *et al.,* 2007) and serum prolactin level: Normal level: 2–18 ng/ml (Frantz *et al.,* 1997).

Statistics

Statistical presentation and analysis of the present study was conducted using the mean standard deviation, Student (T) test (for estimation of the difference between two means), chi-square (χ^2) (for comparison between two groups as regards qualitative data), Analysis of variance [ANOVA] tests (F) (for comparison among different times in the same group in quantitative data) and Linear Correlation Coefficient [r] test by SPSS Version 20. *Post-hoc* test (Tukey test) was used when F is significant. A *p*-value <0.05 was considered significant.

Results

The age of control group was 31.07±5.79 and that of tramadol dependent group was 30.90±5.06 with no significant difference between both groups (t=0.018 and *p*=0.893). It was found that 45% of TDG were skilled workers, 32% were non skilled workers, 14% were employees, and 8% had no job. Regarding the control group, 53.3% were non skilled workers, 23.3% were skilled workers, 16.7% were employees, whereas only 6.7% has no work. Concerning education, about 56% and 86.7% of the patients were educated, in TDG and control group respectively. More than half of the patients in TDG (56%) and control group (56.7%) were from rural residence.

All cases were married with no history of chronic diseases, long term medications or abuse of any psychotropic substances (other than tramadol) and received tramadol in tablet forms.

The present study showed significant difference between tramadol dependent group and control group regarding the family history of drug dependence which was positive in 38% of tramadol dependent group and in 10% of the control group. It also revealed non-significant difference between tramadol dependent group and control group regarding smoking, non-smokers represented 38% of cases in tramadol dependent group, 18% of cases were mild smokers, 12% were moderate smokers while, 32% of cases were heavy smokers.

Table 1 shows the different motives that pushed the cases for dependence, curiosity was the most common motive (22%) followed by tramadol taken as a treatment of premature ejaculation (20%) and the least found motive was the social and/or financial troubles (10%).

Table 1. Types of motives for dependence in tramadol dependent group (TDG)

		Tramadol dependent group (n =50)	
		N	%
Motives for dependence	Social/financial troubles	5	10
	Friends	7	14
	Pain killer	8	16
	Combined motives	9	18
	Premature ejaculation	10	20
	Curiosity	11	22

The majority of cases (62%) received tramadol in a dosage ranged from 400 to 1000 mg/d, 24% in a dosage more than 1000 mg/d and 14% in a dosage less than 400 mg/d (the maximum therapeutic dose = 400 mg/d). Furthermore, 52% of the cases took tramadol for more than 5 years, 38% of them took it for 2–5 years and 10% took it for less than 2 years.

As shown in Table 2 significant difference was detected regarding erectile dysfunction and decreased libido when comparing cases in tramadol dependent group and control group. Erectile dysfunction and decreased libido occurred in 44% and 48% of tramadol dependent group respectively.

A statistically significant decrease in serum testosterone and significant increase in serum prolactin level was noticed in cases of tramadol dependent group compared to control group (Table 3).

Table 2. Prevalence of erectile dysfunction and decreased libido among tramadol dependent group (TDG) and control group

			TDG n=50	Control group n=30	χ^2	*p*-value
Erectile dysfunction	Present	N	22	3	10.089	0.001*
		%	44%	10%		
	Absent	N	28	27		
		%	56%	90%		
Decreased libido	Present	N	24	5	7.966	0.005*
		%	48%	16.7%		
	Absent	N	26	25		
		%	52%	83.3%		

χ^2: Chi-square test.* Significant at *p*-value <0.05. TDG: Tramadol dependent group.

Table 3. Serum testosterone and prolactin hormonal levels in tramadol dependent group (TDG) and control group

Hormonal levels (Mean±SD)	TDG n=50	Control group n=30	T-test	*p*-value
Serum testosterone (ng/dl)	400.66±163.48	674.20±221.97	22.654	0.001*
Serum prolactin (ng/ml)	22.79±8.37	9.45±4.04	21.907	0.001*

T-test: Student t-test.* Significant at *p*-value <0.05. TDG: Tramadol dependent group.

Table 4 showed a significant relation between tramadol daily dose and both testosterone and prolactin serum levels. Significant decrease in serum testosterone level and significant increase in serum prolactin level occurred as tramadol dose increased. On the other hand, increasing the dose from 400–1000 to >1000 mg/d resulted in statistically non-significant decrease in the level of serum testosterone.

There was no significant relation between tramadol dose and both erectile dysfunction and decreased libido as shown in Table 5.

Table 6 illustrates significant decrease in testosterone and significant increase in prolactin serum levels as the duration of tramadol dependence prolonged till 5 years, while the change in hormonal levels became non-significant as the duration of dependence increased from 2–5 years to >5 years.

As the duration of dependence prolonged, significant increase in erectile dysfunction and decreased libido was noticed (Table 7).

Figures 1 and 2 show that there was a significant negative correlation between tramadol blood level and serum testosterone where r=–0.450 and *p* value=0.0010. However, a significant positive correlation was evident between tramadol blood level and serum prolactin (r=0.553 and *p*-value=0.001).

Discussion

The choice of tramadol in this study was based on its wide prevalence among drug dependent patients in Egypt. This was in agreement with International Narcotics Board Report International Narcotics Control Board Report (2013) which found that in the first six months in 2013 tramadol was at the top of narcotic substances in Egypt followed by poly drugs abuse and then by cannabis and other materials. On the other hand, Abou Eleinen *et al.* (2014) studied the prevalence of drug dependence among toxicology unit patients in Mansoura Emergency Hospital and Hamdi *et al.* (2013), whose study included eight different governorates in Egypt, found that cannabis (marijuana) was the most common substance of abuse.

Table 4. Comparison between tramadol daily dose and both testosterone and prolactin serum levels

Hormonal Levels (Mean ±SD)	Tramadol dose (mg/d) ≤400	400–1000	>1000	F-test	*p*-value	Tukey test	
Serum testosterone (ng/dl)	706.29±273.40	374.13±246.16	290.92±169.27	7.467	0.002*	P1	0.001*
						P2	0.001*
						P3	0.302
Serum prolactin (ng/ml)	18.84±4.71	22.38±11.35	26.05±11.36	6.997	0.012*	P1	0.043*
						P2	0.008*
						P3	0.032*

F. test: Analysis of variance [ANOVA]. * Significant at *p* value <0.05. *Post hoc* test (tukey test): P1: comparison between cases received tramadol doses ≤400 mg/d and cases received tramadol doses 400–1000 mg/d, P2: comparison between cases received tramadol doses ≤400 mg/d and cases received tramadol doses >1000 mg/d, P3: comparison between cases received tramadol doses 400–1000 mg/d and cases received tramadol doses >1000 mg/d.

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The age of participants in this study was selected to be below 40 years to exclude the effect of aging on sexual dysfunction. The prevalence of erectile dysfunction increases as the age increase. It increases from 40% to 70% as the age increase from 40 to 70 years (Feldman *et al.*, 1994).

Curiosity was the most prevailed motive of dependence among cases of tramadol dependent group in this study (22% of cases). This is similar to De Micheli *et al.* (2002) who found that curiosity and pleasure seeking were the most prominent reasons for initiation of drug abuse, and he suggested that drug use prevention should not simply focus on reducing drug availability but also on helping young people to develop good family/peer relationships and find healthy ways to enjoy themselves. Administering tramadol as a pain killer is another motive for its dependence that was found in 16% of tramadol dependent cases in this study. Similarly, Deering *et al.* (2008) clarified that prolonged self-administration of tramadol for the purpose of pain relief was one of the most important motives for its dependence among the studied cases.

Peer influence play a crucial role in the involvement in drug dependence; this motive was found in 14% of the studied cases in the present study. A study done in Saudi Arabia by Bassiony (2013) reported that peer pressure was a risk factor for initiation as well as relapse of drug and substance abuse. Being accepted by peers is of critical importance to youth. Many of them are worried about how well they are liked and accepted by their peers and accordingly they adjust their behaviors, attitudes and beliefs (McElhaney *et al.*, 2008; Monahan *et al.*, 2011). Social and financial troubles represent 10% of the motives for tramadol dependence in the present study and this goes with Oshodi *et al.* (2010) in Nigeria who stated that social troubles are among the main causes of substance dependence.

Twenty percent of tramadol dependent cases in the current study received tramadol as a treatment for premature ejaculation and then prolonged self-administration that led them to become tramadol dependent. In accordance with that, Goda (2013) in Cairo University found that

Table 5. Association between tramadol dose and presence of erectile dysfunction and decreased libido in tramadol dependent group.

			Tramadol Dose (mg/d)			χ^2	*p*-value
			≤400	400–1000	>1000		
Erectile dysfunction	Present	N	1	15	6	2.926	0.232
		%	14.3%	48.4%	50%		
	Absent	N	6	16	6		
		%	85.7%	51.6%	50%		
Decreased libido	Present	N	1	15	8	4.865	0.088
		%	14.3%	48.4%	66.7%		
	Absent	N	6	16	4		
		%	85.7%	51.6%	33.3%		

χ^2: Chi-square test.

Table 6. Comparison between the duration of tramadol dependence and both testosterone and prolactin serum levels.

Hormonal levels (Mean ±SD)	Duration of dependence			F-test	*p*-value	Tukey test	
	1-2 years	2-5 years	>5 years				
Serum testosterone (ng/dl)	583.60±172.71	404.00±198.70	360.35±113.75	4.695	0.021*	P1	0.035*
						P2	0.018*
						P3	0.052
Serum prolactin (ng/ml)	17.08±3.43	23.33±3.56	26.03±4.70	5.589	0.017*	P1	0.021*
						P2	0.008*
						P3	0.687

F test: Analysis of variance [ANOVA]. * Significant at *p* value <0.05. *Post hoc* test (tukey test): P1: comparison between cases with 1–2 years duration and cases with 2–5 years duration. P2: comparison between cases with 1–2 years duration and cases with >5 years duration. P3: comparison between cases with 2–5 years duration and cases with >5 years duration.

Table 7. Association between the duration of tramadol dependence and presence of erectile dysfunction and decreased libido.

			Duration of dependence			χ^2	*p*-value
			1–2 years	2–5 years	>5 years		
Erectile dysfunction	Present	N	1	7	14	6.327	0.042*
		%	20%	30.4%	63.6%		
	Absent	N	4	16	8		
		%	80%	69.6%	36.4%		
Decreased libido	Present	N	1	6	17	10.859	0.004*
		%	20%	26.1%	77.2%		
	Absent	N	4	17	5		
		%	80%	73.9%	22.8%		

χ^2: Chi-square test. * Significant at *p*-value <0.05

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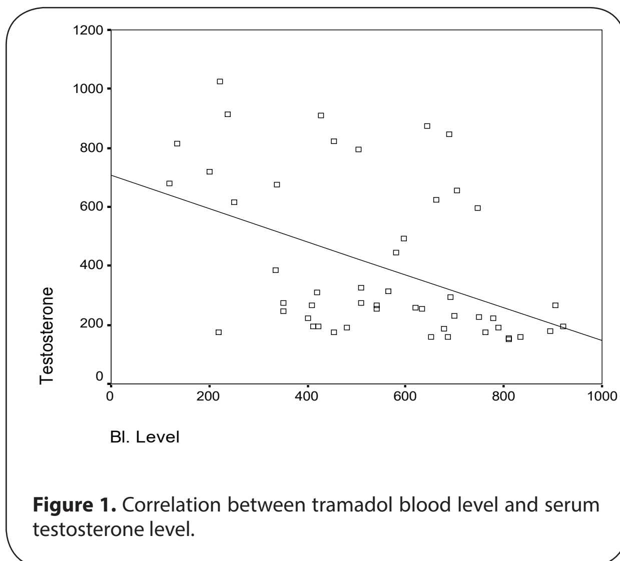

Figure 1. Correlation between tramadol blood level and serum testosterone level.

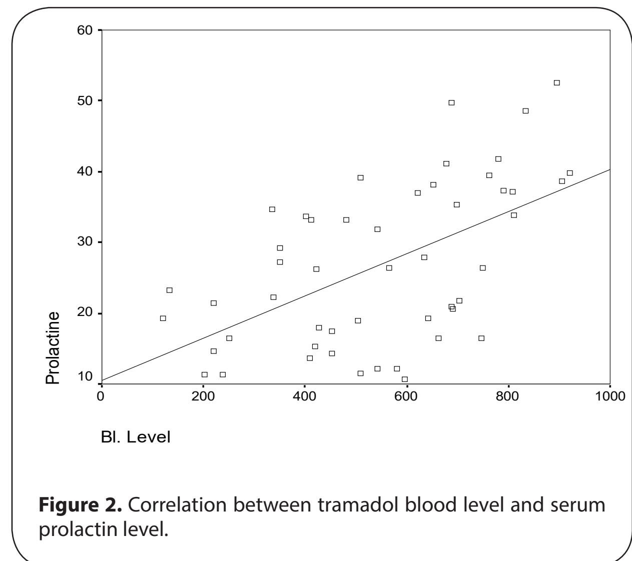

Figure 2. Correlation between tramadol blood level and serum prolactin level.

30% of his tramadol dependent cases, who sought medical advice at National Egyptian Center for Toxicological Research (NECTR), used tramadol with the same motive. Salem *et al.* (2008) used treatment with tramadol on demand (2 hours before sexual intercourse) for cases of premature ejaculation. They stated that tramadol showed good results in improving the condition and increased the intravaginal ejaculation latency time but they also reported that a potential risk of tramadol dependence should be kept in mind during long term therapy.

Significant differences in the serum testosterone and prolactin levels were found between tramadol cases and controls in this study. This is compatible with the study done by Daniell (2002) who measured the hormonal profile of 54 outpatient men consuming oral form of opioids several times daily and he reported significantly decreased levels of serum testosterone with increased prolactin levels referring this to the disturbance of hypothalamic pituitary gonadal axis. Auernhammer *et al.* (1993) stated that opioids bind to specific receptors in the hypothalamus and pituitary gland, disrupt the pulsatile release of corticotrophin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH), and interfere with the production of cortisol and androgen precursors. Testosterone is also reduced due to direct inhibition of testicular testosterone synthesis (Daniell, 2002). Chan *et al.* (2011) also explain the lowering of serum testosterone level by that tramadol might lead to adrenal insufficiency secondary to chronic use.

In the present study significant difference was detected regarding erectile dysfunction and decreased libido when comparing tramadol dependent group and control group. This is in accordance with other studies that reported hypogonadism as serious sequelae of long-term tramadol administration. Symptoms of tramadol-induced hypogonadism include loss of libido, infertility, fatigue, depression, anxiety, loss of muscle strength and impotence in men (Daniell, 2002; Katz *et al.,* 2009). Daniell (2002) contributed the erectile dysfunction and decreased libido to the subnormal sex hormone levels induced by chronic tramadol administration that predisposed his studied cases to a diminished quality of sexual life.

Das (2014) had implicated that chronic use of tramadol, fentanyl, and hydromorphone can lead to adrenocortical insufficiency. Patients with dependence usually have chronically low levels of ACTH, cortisol and testosterone concentrations which impair an individual's ability to respond to physical, emotional and metabolic stresses. This impaired ability leads to abnormal mental health, metabolic alterations and sexual troubles in the form of diminished sexual motivation, experiences and sexual preference.

In the present study, it was observed that the effect of tramadol on hormonal levels (testosterone and prolactin) was dose and duration dependent. This goes with the result of Mckim (2003) who reported that changes in sex hormones levels are dependent on the administered dose of tramadol. Additionally, Gowing *et al.* (2000); Herzog *et al.* (2004) found that the influence of tramadol on testosterone and prolactin serum levels was significantly related to its dose. Large doses of tramadol administration lead to more pronounced effects. Moreover, Mckim (2003) stated that tramadol is known to decrease the levels of sex hormones and this lowered hormonal level is thought to be a chronic outcome. Furthermore, an animal study done by El-Gaafarawi (2006) revealed a slight influence on testosterone and prolactin levels in rats receiving 40 mg/kg of tramadol after 30 days but 80 mg/kg tramadol exerted moderate effects at 20 and 30 days.

There was no significant relation between tramadol dose and both erectile dysfunction and decreased libido in this study. On contrary Daniell (2002) revealed that erectile dysfunction and decreased libido had a dose-related pattern in his study. Besides that, Goodyear-Smith *et al.* (2008) stated that reduced libido and erectile dysfunction were dose dependent effects and both were improved by lowering the administered dose.

In the present study, increased duration of dependence led to significant increase in both erectile dysfunction

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and decreased libido. This is in agreement with Daniell (2002) who declared that tramadol induced sexual dysfunction should be kept in mind during long term therapy especially in large doses. Additionally El-Ghawet (2015) concluded that chronic tramadol administration led to reproductive dysfunction and increased average of infertility. Also, Katz *et al.* (2009) reported that long-term tramadol administration for either dependence or chronic pain treatment often induces hypogonadism.

Significant negative correlation between tramadol blood level and serum testosterone level and significant positive correlation between tramadol and serum prolactin was noted among tramadol dependents in this study. Deer & Gunn (2015) reported low testosterone level and high prolactin level with increasing tramadol blood level among cases of chronic tramadol administration. They also recommended measuring serum testosterone level in patient on long-term therapy by tramadol to avoid hypogonadism.

Conclusion

From this work we can conclude that the majority of studied cases were smokers and curiosity played a crucial role as a strong motive in tramadol dependence. Men who take tramadol for premature ejaculation or any other purpose must know that they are very susceptible to many sexual dysfunctions.

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ORIGINAL ARTICLE

Melamine migration measurement through spectrophotometry device and the effect of time and tableware type on it

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ABSTRACT

Melamine is an organic-based chemical material widely used in the production of tableware. Given the adverse effects of melamine on human health, melamine tableware can be a source for its introduction into the human body. The aim of this study was to use a simple method for monitoring the rate of melamine migration from the tableware to food and the effect of time and tableware on this migration. To measure the migration, spectrophotometry was used. The limit of detection (LOD) of the method was 0.2 (µg/ml), which is functional for measuring the rate of migration. The investigation of sample migration of melamine tableware revealed that migration has occurred across all samples. The rate of migration in all samples was less than the standard level of the European Union (30 µg/ml). Statistical analysis indicated that time is an important factor in melamine migration, which significantly increased ($p<0.05$) in 93% of cases with lengthening the contact time from 30 minutes to 90 minutes. The type of tableware (new or old) and production conditions (standard or non-standard) were found to significantly affect ($p<0.001$) the rate of migration. Statistical analysis of the results suggested that old tableware increased melamine migration in 41% of cases ($p<0.05$). Non-standard tableware significantly ($p<0.001$) increased the rate of migration and thus the effect of non-standard production on melamine tableware was more significant than the age of the tableware.

KEY WORDS: melamine; migration; spectrophotometry; tableware type

Introduction

Melamine is an organic-based chemical with 67% of its mass being composed of nitrogen. This material has a strong framework of 1,3,5 triazine connected to six hydrogen atoms, which can be replaced with other groups, and produces various compounds of melamine. One of these derivations are melamine thermoset resins widely used in plastic industries, chemical fertilizers, floor and wall coverings, textiles, adhesives, dyeing, pharmaceutical products, and food-related tableware (JR, 2008; Rima, 2013). The use of melamine and its material is very common in producing plastic tableware due to its good heat resistance, high durability, cheap cost, and coming in various and attractive types. Melamine tableware has turned to one of the most important and common ways of human contact with melamine (Richardson, 2004; Bradley, 2010).

Longtime contact with melamine through digestive, respiratory or dermal absorption can threaten human health causing kidney failure and probably cancer (Lynch, 2015). In low concentrations, this substance causes acute oral toxicity and in high concentrations it results in kidney diseases and even death, especially in newborns and children. The entry of melamine into the body by different food may lead to the formation of a complex with cyanuric acid, which is in the form of an insoluble crystal for urine pH and leads to kidney stones, urinary tract tissue pathologies, and ultimately urinary retention due to blockage of the urinary tract (Chansuvarn, 2013).

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There is no particular law for using melamine in non-plastic substances in Europe. Nevertheless, since melamine is one of the raw materials for producing plastic products, used for serving food, a special limit of migration has been considered as a standard. Specific Migration Limit (SML) for melamine has been determined as 30 µg/ml by plastic unit in the European Union. The value of SML is derived from daily tolerable amount for humans per kilogram of body weight (Commission, 2009; Lynch, 2015). Since digestive contact is an important way of human contact with melamine and melamine is used in the synthesis of food-related utensils, there is some concern about melamine migration from tableware to food, where the availability of this tableware for children is of particular concern (Chien, 2011). The primary migration of melamine from melamine tableware to food may be due to remaining monomers. However, secondary reasons might be due to the breakdown and decomposition of polymers. Notably, the major concern is related to this second type migration (Hsu, 2010).

Given the extensive use of melamine in food tableware for maintaining, transporting, and serving food materials, melamine migration to food has changed into a topic of discussion as an important problem in the health and safety of food materials. The necessity of measuring and monitoring the magnitude of melamine migration from tableware to food is felt in order to control this hazard in food materials. Spectrophotometric method, which is a simple, cheap, and available method for most laboratories, was used in this study for measuring the rate of melamine migration. To this end, the method was first validated. Then, the rate of migration was investigated along with the effect of time and type of tableware on this migration in terms of newness or oldness as well as the conditions of tableware production in terms of being standard and non-standard.

Materials and methods

To determine the rate of melamine migration from melamine tableware in this study, three standard brands were selected. They are prepared based on standard number 612 of Iranian national standards organization with the strongest sales in the market and as a result with the greatest consumption. A sample of melamine dish with no label and sign of production site was selected as the non-standard sample, while a sample of melamine tableware, which had been used for two years, was selected as the old sample for investigating the effect of oldness on melamine migration.

As food stimulants, distilled water and acid acetic 3% were selected to get into contact with melamine tableware. Simulants were contacted to melamine tableware at 90 °C for 30 and 90 minutes to determine the effect of time on melamine migration. The rates of melamine migration from melamine tableware to simulants were measured by spectrophotometry.

Migration measurement by spectrophotometry method

The method was based on the complexation of melamine with a mixture of formaldehyde and chemicals, including a ketone group. This complex was the result of Mannich reaction. Uranin was used as a ketone compound; By UV/VIS (200–400 nm) spectrophotometry device the quantitative value of melamine was measured (Rima, 2009 ; Rima, 2013).

Mannich reaction consists of amino alkylation of an acidic proton located next to a carbonyl functional group by formaldehyde and a primary or secondary amine or ammonia. The final product is a β-amino-carbonyl compound (Figure 1).

Materials and device

Melamine (Sigma, Germany, Lot number: 1422105v), uranin (C20H10Na2O5) purity 99.0% (Sigma,Germany), formaldehyde (Merck,Germany), deionized distilled water, acetic acid 3% (Merck, Germany), spectrophotometer UV/VIS (Perkinelmer, America), scale with microgram detection limit (Sartorius,Germany), ultrasonic device (Elma, Germany), sampler (Eppendorf, Germany).

Preparation of the solutions and drawing the calibration curve

Melamine stock solution with a concentration of 6.3 µg/ml was prepared along with one solution of uranin with a concentration of 6.3 µg/ml. This was followed by preparing pure formaldehyde, which was another component of the intended complex.

To draw the calibration curve, 10 solutions were mixed with different volumes of stock melamine (0.05–2 ml) with 0.5 ml of uranin solution as ketone and 1 ml of pure formaldehyde. Next, different volumes of deionized distilled water were added to each of the solutions to reach a final volume of 5 ml (Table 1). In the final solution,

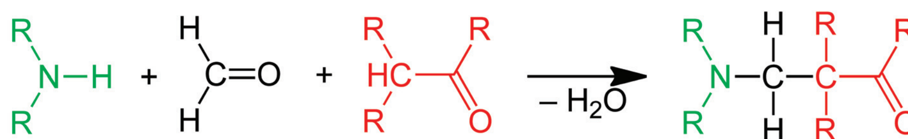

Figure 1. Mannich Reaction

Table 1. Concentration of different sections of melamine-uranin-formaldehyde complex for plotting the calibration curve.

Volume of Water (ml)	Volume of formaldehyde (ml)	Volume of Uranin [6.3 µg/mL] (ml)	Melamine Con (µg/ml)	Volume of melamine [6.3 µg/mL] (ml)
3.45	1	0.5	0.063	0.05
3.3	1	0.5	2.52	0.2
3.25	1	0.5	3.15	0.25
3	1	0.5	0.630	0.5
2.75	1	0.5	0.945	0.75
2.5	1	0.5	1.260	1
2.25	1	0.5	1.575	1.25
2	1	0.5	1.890	1.5
1.75	1	0.5	2.205	1.75
1.5	1	0.5	2.520	2

the concentration of uranin and formaldehyde was fixed, while the concentration of melamine varied between 0. 63 and 2.52 µg/ml. Then the solution was shaken and absorption of the solutions was measured by the device and the calibration curve was drawn. Three replications were considered for each concentration.

Validating the spectrophotometry method

To validate this method, the protocol proposed in the international conference of coordinated instructions was used for validating analytical methods including LOQ, LOD, linearity, and accuracy (Rima, 2009).

To obtain the value of LOD and LOQ, the value of signal was calculated by noise. To check the linearity, the relationship between the concentration and peak area was investigated. Ten different concentrations of melamine standard were prepared and read by the device and the correlation coefficient was determined.

To check the accuracy, iteration in one day (three iterations) and iteration in three consecutive days were used. At first volumes of 0.5, 1 and 2 ml of melamine standard solution with a concentration of 6.3 µg/ml were selected (the concentrations of .63, 1.26 and 2.52 µg/ml) and transferred to three test tubes. Then, 1 ml of formaldehyde and 0.5 ml of uranin were transferred to each of the test tubes and different volumes of deionized distilled water were added to each solution to reach the ultimate volume of 5 ml, shaken for 2 min. Their absorption was then measured at the wavelength of 210 nm. This protocol was repeated on three consecutive days. The accuracy of the method was ultimately calculated based on statistical analysis results. Note that to have an acceptable accuracy for a method, the percentage of standard deviation should be less than 20%.

Preparing and measuring the sample absorption

The sample bowls were first washed with distilled water and completely dried using hot air oven with 30 °C and were filled up to 1 cm off the edge with the solutions of simulants, previously reaching 90 °C. To maintain the intended temperature of the simulants during contact with the tableware, the samples were put in an oven with the desired temperature. Once the contact time was finished, the simulants were withdrawn and maintained in glass tubes and refrigerator until measurement time.

In the next phase, to measure each sample, 3.5 ml of it was transferred to the test tube and 0.5 ml of uranin plus 1 ml of formaldehyde were added to it. After mixing, they were placed in a spectrophotometry device to measure their absorption.

In order to measure the absorption of the samples, the absorption of melamine-uranin-formaldehyde complex, formed from Mannich reaction, was first scanned within the range of UV (200–400 nm). Then, a wavelength at which the complex had the maximum absorption was specified. The device became zero with a solution, containing all available materials in the sample except for melamine. The absorption of the samples was measured at the wavelength with the maximum absorption of M-F-U complex.

Results

The results indicate that melamine standard solution has no absorption within the UV range (200–400 nm). Similarly did formaldehyde and uranin have no absorption spectrum within the UV range. However, when 0.5 ml of uranin was mixed with 1 ml of formaldehyde, it had an absorption at 205 nm (Figure 2). The scan of M-U-F complex, obtained from Mannich reaction within the UV range, indicated that the greatest absorption occurred at the wavelength of 210 nm (Figure 3). Accordingly, the wavelength of 210 nm was considered for monitoring the samples and measuring melamine quantitatively.

Calibration curve and validating the spectrophotometry method

The absorption of 10 solutions of M-U-F complex was measured with different concentrations of melamine (0.063–2.52 µg/ml) at the wavelength of 210 nm. The calibration curve was drawn using these results (Figure 4). Table 2 reports the results related to the method validation.

Measuring the migration rate of the samples

As stated previously, we had five types of melamine dish, two types of stimulant, and two times of 30 and 90 minutes for absorption to be measured individually in three iterations. The measured absorptions for each sample were incorporated in the formula of calibration line to determine the rate of melamine migrated from tableware to the simulants based on ppb. Tables 3 and 4 present the results as the mean of the three iterations for each sample. (Also see Tables 5 and 6)

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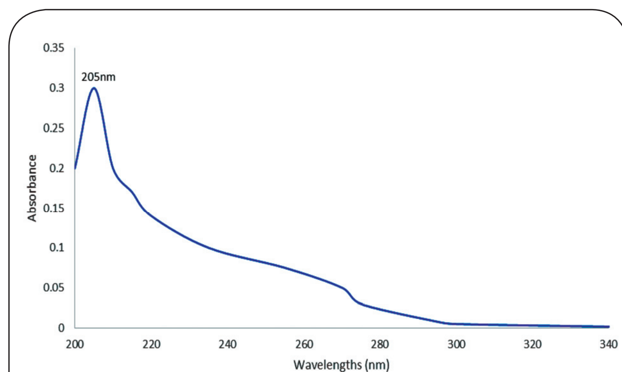

Figure 2. Scan of absorption spectrum of uranin-formaldehyde solution within the UV range

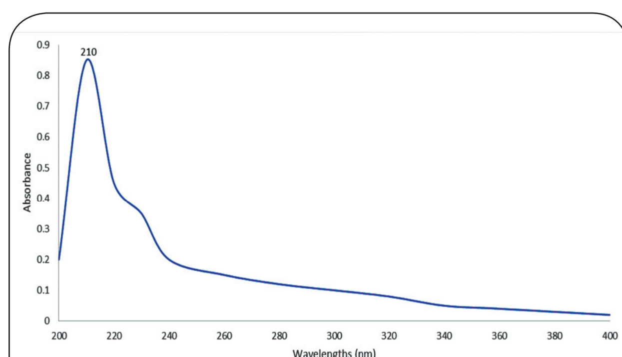

Figure 3. Scan of absorption spectrum of melamine-uranin-formaldehyde complex within the UV range

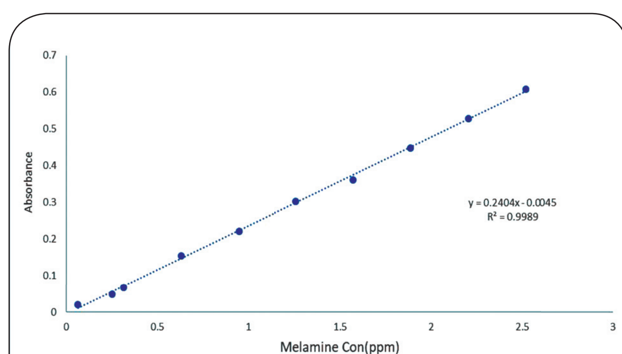

Figure 4. calibration curve of melamine-uranin- formaldehyde complex

Table 2. Results of validating the spectrophotometry method.

Parameter	Result
Maximum absorption	210 nm
Accuracy (Recovery%)	%95.3
Precision	96.2%
Slope	0.0002
Intercept	−0.0045
Linearity range (μg/ml)	0.063–2.520
Standard equation regression	y = 0. 240x – 0.0045
Correlation coefficient	R^2 = 0.9989
SE of intercept	0.00404
SD of intercept	0.01211
LOD (μg/ml)	0.19990
LOQ (μg/ml)	0.60578

Table 3. Results of migration rate of the samples through spectrophotometry method after 30 minutes of contact.

Mean ± SD (ppm)	Test	Sample
1.545+0.07	Water 90 °C - 30 min	Old dish
5.309+0.07	Acetic acid (3%) 90 °C - 30 min	
3.729+0.05	Water 90 °C - 30 min	Non standard dish
5.768+0.07	Acetic acid (3%) 90 °C - 30 min	
2.015+0.01	Water 90 °C- 30 min	Standard dish A
2.452 +0.02	Acetic acid (3%) 90 °C - 30 min	
2.127+0.01	Water 90 °C - 30 min	Standard dish B
3.111+0.009	Acetic acid (3%) 90 °C - 30 min	
2.625+0.02	Water 90 °C - 30 min	Standard dish C
5.956+ 0.01	Acetic acid (3%) 90 °C - 30 min	

Table 4. Results of migration rate of the samples through spectrophotometry method after 90 minutes of contact

Mean ± SD (ppm)	Test	Sample
1.678+0.06	Water 90 °C- 90 min	Old dish
7.604 +0.06	Acetic acid (3%) 90 °C- 90 min	
3.982+0.02	Water 90 °C- 90 min	Non standard dish
8.737+0.10	Acetic acid (3%) 90 °C- 90 min	
1.342+0.03	Water 90 °C– 90 min	Standard dish A
3.242+0.04	Acetic acid (3%) 90 °C- 90 min	
2.855+0.03	Water 90 °C- 90 min	Standard dish B
5.628+ 0.01	Acetic acid (3%) 90 °C- 90 min	
2.738+0.04	Water 90 °C- 90 min	Standard dish C
6.750+0.05	Acetic acid (3%) 90 °C- 90 min	

Discussion

In this study, a method was first designed and then validated using a spectrophotometry device for measuring the rate of melamine migration from tableware as there was no routine method of using spectrophotometry for checking the rate of melamine migration from food-related tableware and since the migration rate of melamine in food materials has not been examined in food quality control laboratories in Iran. The results indicated that this method is reliable with an acceptable accuracy and standard deviation. Given the acceptable values of limit of detection (LOD) and limit of quantitation (LOQ), this method can be functional for checking the rate of melamine migration from tableware to food. Further, statistical analysis of the results of measuring the melamine

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Table 5. Summary of statistical comparisons of tableware types and simulants.

Simulant	Temperature	Tableware type (I)	Tableware type (J)	Mean Difference (I-J)	*p* value	95% Confidence Interval for Difference	
						Lower Bound	Upper Bound
Distilled water	90 °C	Old dish	Non standard dish	−0.868*	0.000	−1.086	−0.650
			Standard dish A	−0.022	1.000	−0.239	0.196
		Non standard dish	Old dish	0.868*	0.000	0.650	1.086
			Standard dish A	0.846*	0.000	0.629	1.064
		Standard dish A	Old dish	0.022	1.000	−0.196	0.239
			Non standard dish	−0.846*	0.000	−1.064	−0.629
Acetic acid (3%)	90 °C	Old dish	Non standard dish	−0.111	1.000	−0.329	0.107
			Standard dish A	0.813*	0.000	0.595	1.030
		Non standard dish	Old dish	0.111	1.000	−0.107	0.329
			Standard dish A	0.923*	0.000	0.706	1.141
		Standard dish A	Old dish	−0.813*	0.000	−1.030	−0.595
			Non standard dish	−0.923*	0.000	−1.141	−0.706

Table 6. Summary of statistical comparisons of tableware types, stimulants and temperatures.

Brand	Stimulant	Temperature (I)	Temperature (J)	Mean Difference (I-J)	*p* value	95% Confidence Interval for Difference	
						Lower Bound	Upper Bound
Old dish	Distilled water	30 °C	90 °C	−0.151*	0.040	−0.295	−0.007
		90 °C	30 °C	0.151*	0.040	0.007	0.295
	Acetic acid (3%)	30 °C	90 °C	−0.142	0.054	−0.286	0.002
		90 °C	30 °C	0.142	0.054	−0.002	0.286
Non standard dish	Distilled water	30 °C	90 °C	−0.181*	0.014	−0.325	−0.037
		90 °C	30 °C	0.181*	0.014	0.037	0.325
	Acetic acid (3%)	30 °C	90 °C	−0.235*	0.002	−0.379	−0.090
		90 °C	30 °C	0.235*	0.002	0.090	0.379
Standard dish A	Distilled water	30 °C	90 °C	0.030	0.686	−0.115	0.174
		90 °C	30 °C	−0.030	0.686	−0.174	0.115
	Acetic acid (3%)	30 °C	90 °C	−0.479*	0.000	−0.623	−0.334
		90 °C	30 °C	0.479*	0.000	0.334	0.623

migration rate, based on spectrophotometry, indicated that the designed model was significant ($p<0.001$).

Measuring the rate of melamine migration from melamine tableware to the simulants under different conditions by spectrophotometry showed that the migration occurred in all samples. However, the rate of migration in all samples was less than the standard level of the European Union for melamine migration, which is 30 µg/ml (SML).

The results also suggested that time was an important factor in melamine migration. It was observed that lengthening the contact duration of tableware with the simulants, when other variables were fixed, increased the migration significantly ($p<0.05$) in 93% of samples. This high percentage of the significant role of time is noticeable in raising melamine migration.

The other factor tested on melamine migration was the type of tableware in terms of being standard or non-standard as well as old and new, whose effects were measured on the rate of migration. It was seen that the type of tableware significantly influences the rate of melamine migration to the simulants ($p<0.001$). The statistical analysis of the results revealed old melamine tableware increased melamine migration in 41% of cases ($p<0.05$). Also, non-standard tableware affected the rate of migration such that in 90% of cases, being non-standard significantly ($p<0.001$) increased the rate of migration, and thus the effect of non-standard production of melamine tableware was more significant than the age of the tableware.

In a similar study, Rima *et al.* measured melamine migration through spectrophotometry method based on melamine-formaldehyde and a group of ketone complex. They managed to separate the melamine migrated to fish with a recovery (accuracy) percentage of 97% within similar results (Rima, 2009).

In another study, Rima *et al.* used the spectrofluorometric method to measure melamine migration.

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Their method was based on Mannich reaction. With similar results to the current study, they could detect the melamine added to milk powder with a recovery (accuracy) percentage of 97% (Rima, 2009). Chansuvarn *et al.* also used spectrophotometry method in a similar study to measure contamination of milk and its products with melamine. Their method was also based on Mannich reaction. They stated that this is a reliable method for measuring melamine in food material samples (Chansuvarn, 2013).

Chik *et al.* measured melamine migration from 41 samples of food serving dishes to food simulants. In that study, samples were exposed to two types of food simulants (3% acetic acid and distilled water) under three test conditions (25, 70 and 100°C) for 30 min using LC-MS/MS device. The results of their study recommended that excessive heat and acidity may directly affect melamine migration from melamine-ware products (Chik, 2011)

Conclusion

Spectrophotometry method is a simple, cheap and available method for most food quality control laboratories. It is capable of measuring the acceptable accuracy of melamine and has the potential of being further researched and developed. Also, as melamine migration occurred across all samples though it was less than the standard level of the European Union in all of them, it should be noticed that long-term and continuous use of tableware, especially for long-term maintenance of hot food, may lead to bad effects, arising from toxicity with melamine. e, Developing methods for monitoring melamine in food materials is necessary to cope with its adverse effects, thus enhancing public health.

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ORIGINAL ARTICLE

Mechanism of protection of rat hepatocytes from acetaminophen-induced cellular damage by ethanol extract of *Aerva lanata*

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ABSTRACT

The aim of this study is to evaluate the protective effect of ethanol extract of *Aerva lanata* (EEAL) in preventing acetaminophen induced liver toxicity. EEAL was prepared and its hepatoprotective effect was studied in both isolated primary hepatocytes *in vitro* and in Sprague Dawley rats *in vivo*. For *in vivo* studies, the animals were grouped as Group I – Control; Group II – ACN (2 g/kg b.w.); Group III – EEAL (50 mg/kg b.w.) + ACN (2 g/kg b.w.), Group IV – EEAL (100 mg/kg b.w.) + ACN (2 g/kg b.w.). Extracellular activities of the enzymes liver aminotransferease (GOT, GPT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in isolated hepatocytes and rat plasma were studied colorimetrically. Expression of GST, Nrf2, COX 1 & COX2 genes in rat liver were evaluated by RT-PCR. The results showed that ACN induced down-regulation of Nrf2 and upregulation of GST gene expression, which were modulated by EEAL treatment. GOT, GPT, ALP and LDH levels were found to be lowered in both hepatocyte culture media and plasma following EEAL treatment. In addition, the medium GOT and GPT levels were diminished following EEAL treatment only. Moreover, only ALP and LDH in serum appeared to be at normal level following EEAL treatment, whereas GOT and GPT showed levels lower than control. ACN treatment increased the expression of pro-inflammatory COX 1 and COX 2 genes and the levels of these genes were reduced by EEAL treatment. EEAL pre-treated rats exposed to ACN were found to retain normal hepatic structure compared to ACN alone treated rats. From these results it can be concluded that ethanol extract of *A. lanata* possesses both anti-inflammatory and hepatoprotective activity.

KEY WORDS: *Aerva lanata*; hepatotoxicity; Nrf2; cyclooxygenases; acetaminophen

Introduction

Parenchyma and non-parenchyma cells of the liver are involved in several functions which regulate the homeostasis of our body. The presence of chemical and/or biological toxins causes severe damage to the liver leading to hepatitis and cirrhosis mediated through lipid peroxidation and other oxidative complex reactions (Kumar *et al.*, 2011). Severe lipid peroxidation induced by continuous oxidative stress triggered by oxidants is one of the major attributes to the initiation and progress of liver damage (Albano *et al.*, 1985). Hepatic injury and subsequent hepatic failure due to overdose of acetaminophen (ACN) is a serious health concern (Yoon *et al.*, 2016). Under conditions of ACN overdose, the glucuronidation and sulfation process become saturated and more extensive bioactivation of ACN to *N*-acetyl-*p*-benzoquinone imine (NAPQI) occurs, which covalently binds to produce cellular protein adducts, leading to liver failure (Jollow *et al.*, 1973). This evidence suggests that removal and/or deactivation of agents creating oxidative stress is a protective mechanism against the development of ACN hepatotoxicity. Thus, components of natural origin, i.e. detoxifying enzymes that contribute to enhance intracellular antioxidant potential are important in the protection or treatment of such injury. Though many synthetic drugs/natural preparations are now available in the market for treating liver damage, they all have been found to have some toxic side effects. Thus the development of effective drugs with lower toxicity is required. Plant derived components/products always have a potential role in the research of medicine and pharmacology. There are several plant species that are considered to have significant hepatoprotective effects in animal model (Kumar *et al.*, 2011).

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Aerva lanata (Amaranthaceae family) is a common plant found throughout the tropical region of India. Previous research on *A. lanata* showed that different parts of this plant have anti- cancer, anti-diabetic, anti-inflammatory, nephroprotective, hepatoprotective and antihelimintic properties (Ragavendran *et al.,* 2012; Nevin and Vijayammal, 2005; Anusha *et al.,* 2016). This study evaluates the hepatoprotective effect of the ethanol extract of *A. lanata* on primary hepatocytes and rat liver from toxicity induced by ACN.

Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic–antimycotic solution, EZcount^{TM} LDH cell assay kit, cell culture chamber slides and other cell culture reagents were procured from Hi-Media Laboratories, India. Taq PCR Smart Mix 2x was purchased from Orion-X, India. Verso cDNA Synthesis Kit was procured from Thermofisher Inc, USA. Oligos were synthesized by Xcelris Labs and Integrated DNA Technologies, USA.

Plant extraction

A. lanata was collected from different areas of Mahatma Gandhi University, Kottayam, Kerala, India. The plant was identified by the botanist of the Department of Botany, ST. Thomas College, Pala, Kerala, India and a voucher specimen was deposited at their herbarium (Voucher specimen No. 1503). *A. lanata* whole plant was washed and shade dried. The dried plant material was powdered and extracted with 300 ml of petroleum ether (PE; BP-60-80) using Soxhlet apparatus to remove all fatty materials. After PE extraction, the plant material was extracted with ethanol. The ethanol extract (EEAL) thus obtained was dried using a rotary evaporator, weighed and stored for further experiments.

Preliminary component identification in EEAL

The extract was analyzed for phytochemicals qualitatively for the presence of protein (xanthoproteic test) phenolic compounds (Lead acetate test), flavonoids (Alkaline reagent test), tannins (ferric chloride test), steroids, triterpenoids (Salkowski's test), saponins (Froth test), cardiac glycosides (Keller Killiani test) and alkaloids (Wagner's test) using standard procedures (Dyana and Kanchana, 2012).

In vitro anti- lipid peroxidation assay

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed, using liver homogenate as lipid rich medium (Ohkawa *et al.,* 1979; Shabbir *et al.,* 2013). The isolated liver lobes were washed with 0.9% sodium chloride solution and dried using blotting paper. One gram of tissue was then homogenized with 10 ml of cold phosphate buffer (pH 7.4) using glass-teflon homogenizing tubes, filtered and centrifuged at 3000 rpm at 4°C for 10 min. The supernatant was diluted with phosphate buffered saline (PBS) to obtain final concentration of protein equal to 8.0–15.0 mg/ml. Liver homogenates (3.0ml) were pre-treated with different concentrations of EEAL. Lipid peroxidation was initiated by adding 100 µl of 15 mM ferrous sulphate solution. After 30 min, 200 µl of this reaction mixture was mixed with 3.0 ml of 10% trichloroacetic acid (TCA). After 10 min, the tubes were centrifuged and the supernatant was separated and mixed with 3.0 ml of 0.67% thiobarbituric acid in acetic acid. The mixture was heated in a water bath at 85°C for 30 minutes, followed by heating in boiling water bath. The intensity of the pink-colored complex formed was measured at 535 nm. The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of control. The percentage of inhibition of lipid peroxidation was calculated by the formula: Inhibition (%) = [Abs (Control) – Abs(Sample)]/ Abs (Control) × 100.

Isolation of primary rat hepatocytes

Hepatocyte isolation was performed using the two-step enzymatic method (Seglen, 1976; Ateş *et al.,* 2012). The abdomens of Sprague Dawley rats were opened under general anesthesia, a cannula was inserted into the portal vein and the perfusion solution (0.9 g/100 mL NaCl; 0.04 g/100 mL KCl; 0.09 g/100 mL D-glucose; 0.21 g/100 mL $NaHCO_3$ and 2 mL HEPES – 25 mM in DMEM) was slowly infused at 37°C. After discoloration of the liver, 30 ml of the enzyme solution (0.24 mg type 4 collagenase/mL perfusion solutions) was infused via the portal vein. After the incubation period of 30 min, the liver was cut into small pieces and filtrated through 210 µm, 70 µm and 40 µm porous membranes. The samples thus obtained were centrifuged with washing solution (0.5 g/500 mLNaCl; 0.02 g/500 mL KCl; 0.48 g/500 mL $CaCl_2.H_2O$; 5 mL HEPES – 19.8 mM; 1 g/500 mL bovine serum albumin (BSA) dissolved in DMEM) at 150 g for 3 min. The supernatant was discarded and the pellet obtained was washed. Separation of the dead cells was performed with 10.8 mL percoll solution (100%) and 15 mL DMEM, the resulting cell suspension was centrifuged at 2000 rpm for 20 min. The medium layer was collected and percoll was removed by washing and centrifugation at 150 g for 3 min. The viability of isolated hepatocytes was monitored by Trypan blue method. The isolated hepatocytes were cultured in William's media at 37°C with 5% CO_2. Periodic acid – Schiff (PAS) staining was performed to identify glycogen synthesis and hepatocyte function. Isolated cells were washed with PBS three times and fixed within 4% formaldehyde for 30 min. The cells were oxidized with periodic acid for 5 min, processed with Schiff's reagent for 15 min, washed with dH_2O for 10–15 min and observed under the inverted microscope (Olympus 1×51) (Reintoft, 1978).

Analysis of nuclear changes by DAPI staining

Chromatin changes in EEAL treated cell lines were studied by DAPI.HCl. Isolated rat hepatocytes were pre-treated

with different concentrations of EEAL and exposed to 3 mM ACN for 24 h. After the treatment period, cells were washed with phosphate buffered saline (PBS) to completely remove the growth medium. Cells were fixed for 10 min in 3.7% formaldehyde and again rinsed three times in PBS before permeabilization in 0.2% TritonX 100 for 5 min. The cells were washed and incubated with DAPI labeling solution (stock solution: 10 mg/mL in water; dilution 1:5 000 in PBS) for 5 min in the dark. The labeling solution was aspirated and cells were rinsed thrice in PBS. Morphology of the cells and nuclei were observed using a fluorescence microscope (Olympus 1×51) with the DAPI filter (Chazotte, 2011).

Animal experiments

Male Sprague Dawley rats (4 weeks old, weighing between 100 and 150 g each) were purchased from the Government Veterinary College Mannuthy, Thrissur, India. After one week of acclimation at the departmental animal house, the rats were randomly assigned to 4 groups. Group I: Normal control (administered vehicle 10%DMSO), Group II: Acetaminophen control (2 g/kg b.w.), Group III: 50 mg/kg b.w. EEAL+ Acetaminophen(2 g/kg b.w.), Group IV: 100 mg/kg b.w. EEAL + Acetaminophen (2 g/kg b.w.); (n=6). EEAL was suspended in 10% DMSO (v/v) in PBS and was given intragastrically for 14 consecutive days. After 14 days, the rats were intragastrically given 2 g/kg acetaminophen. After 24 h post acetaminophen treatment, blood was collected in tubes containing heparin as an anticoagulant and plasma was prepared to evaluate the progress of hepatic damage. All animal experiments were approved by the University Animal Ethical Committee (CPCSEA No .732/03/ac/CPCSEA dated 17/07/2009).

RNA isolation and RT-PCR analysis

Total RNA was isolated from control, treated cells and rat liver by Trizol reagent (Himedia) as described by the manufacturer's instructions. cDNA was synthesized by adding 1ng of RNA to the 5X cDNA synthesis buffer (4 µl), dNTP Mix (2 µl) RNA primer (1 µl), RT Enhancer (1µl) Verso Enzyme Mix (1 µl) and nuclease free water (20 µl) using Thermo Scientific Verso cDNA Synthesis Kit. PCR was done by using GoTaq Green master Mix in thermal cycler using the following primer sequences. GAPDH was used as an endogenous control. PCR primers for COX 1 (F-5'-CTCACAGTGCGGTCCAAC-3', R-5'-CCAGCACCTGGTACTTAAG-3'), COX 2 (F-5'-TTCAAATGAGATTGTGGAAAAAT-3' R-5'-AGATCATCTCTGCCTGAGTATCTT-3'), GST (F-5'-GCCTTCTACCCGAAGACACCT-3' R-5'-GTCAGCCTGTTCCCTACA-3', and Nrf2 (F-5'-CCAGCTGAACTCCTTAGACTCA-3', R-5'-GCTCTGC TAGGAAAGCAGAGTAAAATT-3'), were designed and PCR for these genes were performed. The finished PCR product was run on 1.5% agarose (Kendell *et al.,* 2010).

Liver marker enzyme activities

To determine the effect of EEAL on liver marker enzyme activity in primary rat hepatocytes, cells were seeded on a 96 well plate and pre-treated with EEAL (10 µg/ml, 50 µg/ml, 150 µg/ml). Pure acetaminophen (final concentration 3mM) was added to the cells after 24 hours of pre-treatment with EEAL. The culture supernatants from the different experimental and control groups were collected after treatment with EEAL to estimate the levels of secreted proteins associated with normal development and functioning of the liver. Serum from rats treated with EEAL and ACN was used for biochemical analysis. To evaluate the hepatotoprotective activity of EEAL in both primary hepatocytes and in rats, we analyzed the activities of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), alkaline phosphate (ALP) and LDH using commercially available kits (Agapee, India) following the manufacturers´ instructions. LDH was assessed by the kit provided by HIMEDIA, India. Protein content was estimated colorimetrically (Bradford, 1976).

Histopathology of the liver

After the experimental period, the rats were anesthetized in the animal house and the livers were removed and preserved in 10% formaldehyde. Dehydration and clearing of the tissues were performed using different concentrations of ethanol. Five micrometer thick sections were prepared and stained with hematoxylin and eosin (H/E) (Mendez *et al.,* 2005). Stained sections were qualitatively evaluated using a digital microscope (Phase Contrast Fluorescence Microscope Olympus BX-43). The images were analyzed using software Q IMAGING, Micro Publisher 5.0 RTV.

Statistical analysis

Mean values ± SD were calculated for all parameters. The statistical difference was analyzed by one-way analysis of variance (ANOVA) using windows SPSS 19 and significance was calculated as *P* values. In all cases a difference was considered significant when *p*-value was <0.05. Duncan's *post-hoc* analysis was used for statistical analysis.

Results

EEAL prevented *in vitro* lipid peroxidation

Ethanol extraction of *A. lanata* plant gave a yield of about 5.69 g per 100 g of the plant material. Preliminary phytochemical screening was done on EEAL to identify the different classes of components present. The results indicated the presence of polyphenolic compounds, flavonoids and alkaloids in the preliminary compound identification (Table 1). Quantitative analysis showed that EEAL contained a higher amount of polyphenols (62.31±1.62 µg/100 mg) followed by flavonoids (25.12±0.75 µg/100 mg) and lower levels of alkaloids (15.20±0.43 µg/100 mg) (Table 1). The *in vitro* antioxidant activity of EEAL was studied using lipid peroxidation assays. The results showed an anti-lipid peroxidation effect of EEAL in *in vitro* conditions using rat liver extract. The IC_{50} value was found to be 281.25 µg/ml (Figure 1).

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Table 1. Preliminary component identification and their respective concentrations in EEAL.

Components	Presence	Concentration (µg/100mg extract)
Protein	-	NA
Phenolic Compounds	+	62.31±1.62
Flavonoids	+	25.12±0.75
Tannins	-	NA
Steroids	-	NA
Saponins	-	NA
Alkaloids	+	15.20±0.43

Results indicate the presence of polyphenols, compounds, flavonoids, cardiac glycosides and alkaloids Values are expressed as Mean ± SD of three independent experiments. NA: not applicable

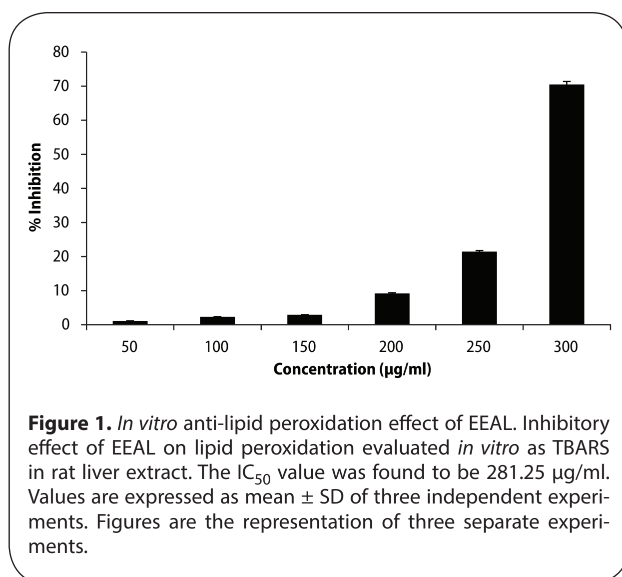

Figure 1. *In vitro* anti-lipid peroxidation effect of EEAL. Inhibitory effect of EEAL on lipid peroxidation evaluated *in vitro* as TBARS in rat liver extract. The IC_{50} value was found to be 281.25 µg/ml. Values are expressed as mean ± SD of three independent experiments. Figures are the representation of three separate experiments.

Previous studies showed that the extract of *Mucuna pruriens* significantly prevented *in vitro* lipid peroxidation with an IC_{50} value of 217.25 µg/ml (Rajeshwar *et al.*, 2005), while *Leucas plukenetii* extract showed an anti-lipid peroxidation activity at 536 µg/ml (Nandy *et al.*, 2012). In comparison with these results, *A. lanata* extract has a similar anti-lipid peroxidation activity.

EEAL prevents nuclear changes induced by ACN

Rat primary hepatocytes were isolated by collagenase perfusion method. The cells were found to be highly viable and were successfully propagated in William's media. PAS staining confirmed the viability of the hepatocytes (Figure 2). The nuclear changes induced by ACN and their prevention by EEAL were studied using DAPI staining. Acetaminophen (3 mM) treatment showed significantly higher decrease in hepatocyte viability (70%), while primary rat hepatocytes pre-treated with EEAL showed significant prevention of cell toxicity. The intensification of DAPI staining in ACN treated cells pointed to apoptotic chromatin condensation. EEAL treatment showed significant protection towards ACN exposed hepatocytes in dose dependent manner. The result indicated that EEAL had some mitogenic effects towards rat hepatocytes, since the cell number observed was much higher than that observed in control cells (Figure 3).

Liver marker enzyme activities were beneficially modulated by EEAL both *in vitro* and *in vivo*

Treatment of isolated hepatocytes with ACN showed a significant increase in the activity of LDH, ALP, GOT, GPT, while EEAL pre-treated hepatocytes when exposed to ACN showed reduced LDH ALP, GOT,GPT activity in the culture medium (Figures 4 and 5). Interestingly, the activities of GOT and GPT levels were found to be below the values of normal untreated control in hepatocytes treated with EEAL alone at a concentration of 50 and 150 µg/ml. Treatment with ACN (2 g/kg b.w.) in rats showed a significant increase in the activity of serum ALP, GPT, GOT and LDH. While pre-treatment of ACN-exposed rats with EEAL, at a concentration of 50 and 100 mg/kg b.w., resulted in reduction of upregulated ALP, GPT, GOT and LDH activities, with GOT and GPT activity below the levels of untreated control rats. Of the liver enzymes, the activity of LDH in serum of EEAL-treated rats was only mildly suppressed (Figure 6).

Gene expression analysis

PCR analysis was done to determine the expression of glutathione s transferase (GST), cyclooxygenases 1 and 2 (COX 1 and COX 2) and Nrf2 in the liver of EEAL and ACN treated rats. The inflammatory COX 1 gene was mildly overexpressed in ACN treated rats. Rat livers pre-treated with EEAL and exposed to ACN showed COX 1 gene expression downregulated compared to ACN treated animals. COX 2 gene was found to be upregulated in ACN treated rats, while EEAL pre-treatment showed a decrease in the COX 2 gene expression which was comparable with control animals. GST levels were found to be significantly upregulated in ACN treated rats. The expression levels of GST in EEAL pretreated rats were found to be normalized to those of control animals. Nrf2 levels were found to be mildly upregulated in EEAL pretreated rats exposed to ACN, while the expression levels of Nrf2 in ACN alone treated rats were found to be significantly lower (Figure 7).

Histopathological changes in the liver

Histopathological examination of the liver of ACN treated animals showed total loss of hepatic architecture, accumulation of fat globules, dilated sinusoids and centrilobular necrosis. EEAL pre-treated rats exposed to ACN were found to retain normal hepatic architecture with less damage to hepatocytes and sinusoids (Figure 8).

Discussion

ACN, a commonly used analgesics, is reported to have significant damaging effect on liver functions (Herndon

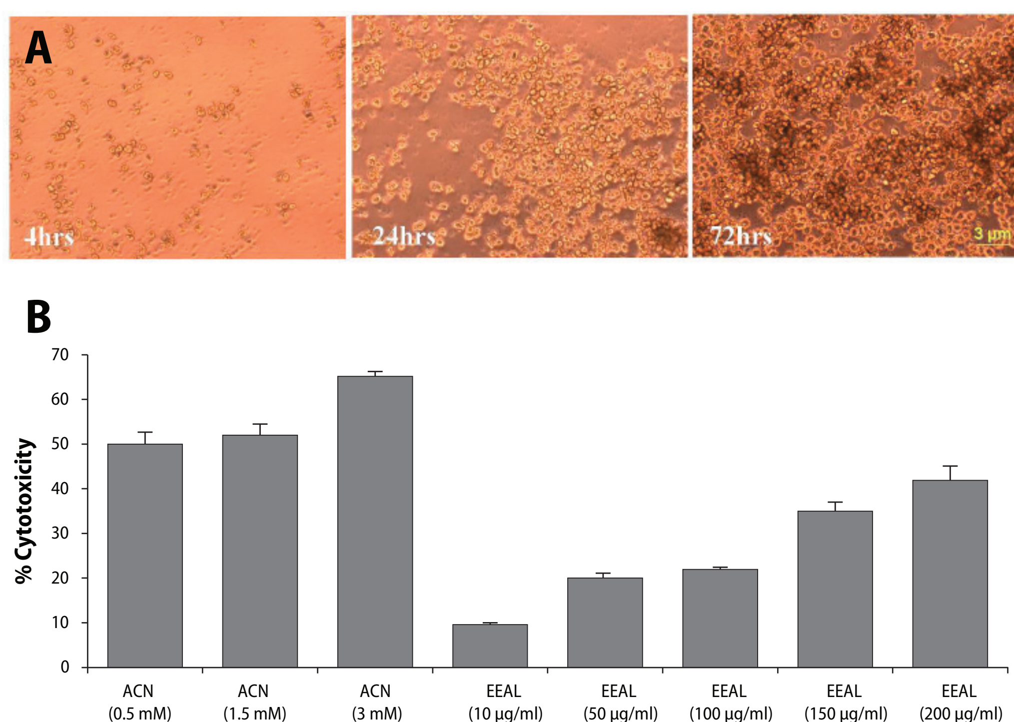

Figure 2. Isolation of rat hepatocytes and cytotoxic effect of different concentrations of EEAL and CAN. **A:** Growth progress of hepatocytes at different time intervals (10×), **B:** MTT assay of different concentrations of acetaminophen and EEAL on hepatocytes after 24 hrs. Values are expressed as Mean ± SD of three independent experiments. Figures are representation of three separate experiments. ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*.

et al., 2014). Treatment options for toxin-induced hepatic failure due to inconsistent efficacy and toxic adverse effect made us to look into the possibility of plant-derived compounds/extracts easily accessible and non synthetic (Fogden & Neuberger, 2003; Evans, 2002). In view of these observations, we selected *A. lanata* to study its hepatoprotective effect on ACN induced toxic liver and hepatocytes.

The *in vitro* antioxidant activity of EEAL was evaluated using lipid peroxidation assays. The results showed significant inhibitory efficacy of EEAL on Fe^{2+}-induced lipid peroxidation. Experiments showed that the metabolism of ACN triggers the oxidant stress due to the production of superoxide and hydrogen peroxide mediated by cytochrome P_{450} (Kunthan *et al.,* 1978; Wendel *et al.,* 1979). This was evidenced from the low lipid peroxides (LPO) in rats treated with cytochrome P450 inhibitors in ACN induced hepatotoxicity (Wendel & Feuerstein, 1981). There is a strong correlation between the oxidative damage to DNA and the formation of thiobarbituric acid reactive species. Malondialdehyde (MDA), the end product of lipid peroxidation, is a measure of membrane damage. High concentrations of substances like acetaminophen and CCl_4 cause tissue damage by initiating lipid peroxidation (Naskar *et al.,* 2009). EEAL may be interfering in the active metabolism of ACN and reduce its impact on hepatocyte damage by virtue of the antioxidant activity conferred by the biologically active components present in it.

Several phytomolecules including flavonoids, alkaloids, glycosides and saponins obtained from various plant sources have been reported as potent hepato-protective agents suppressing the initiation or propagation of the free radical chain reactions (Flora *et al.,* 1996; Hall & Cuppett, 1997). It was previously reported by several researchers that *A. lanata* is a rich source of flavonoids such as kaempferol, quercetin, isorhamnetin, etc (Saleh *et al.,* 1990). Apart from these compounds, *A. lanata* has also been reported to have β-cyanins (glycine betaine and trigonelline), sterols and carbohydrates (Zapesochnaya *et al.,* 1991; Zapesochnaya *et al.,* 1992a; Zapesochnaya *et al.,* 1992b). The results of our studies also confirmed that EEAL contains flavonoids, alkaloids and a polyphenolic class of compounds, which has to be isolated and evaluated individually for its biological effects. The results of our studies substantiate that the anti-lipid peroxidation effect of EEAL may probably be due to the presence of biologically active molecules in it.

ACN metabolism involves conversion to *N*-acetyl-*p*-benzoquinone imine (NAPQ1) and back to ACN or its conjugation with glutathione (GSH) which in turn initiate the hepatic injury leading to GSH depletion (Reid *et al.,* 2005). The expression of glutathione S transferase (GST) was found to be higher in rats treated with ACN, while

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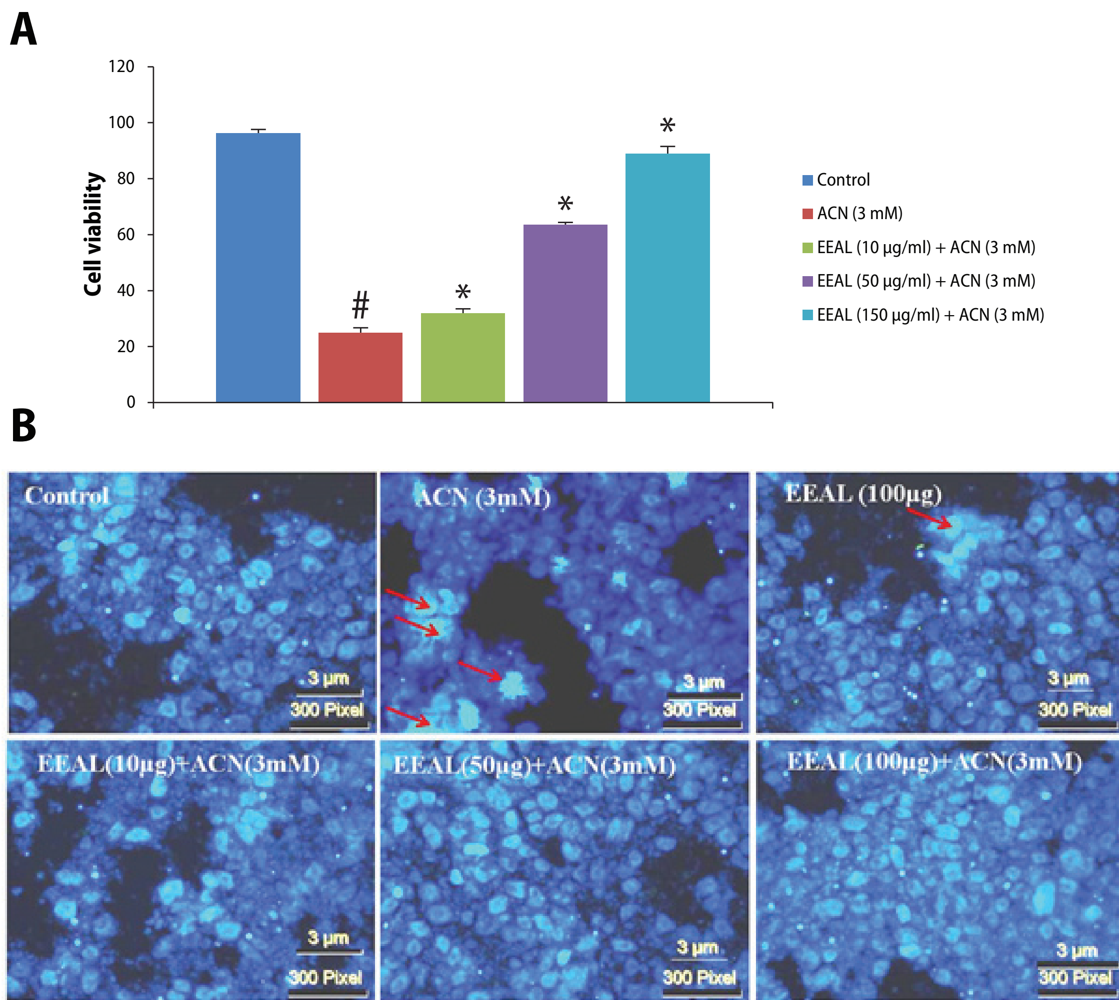

Figure 3. Prevention of ACN – induced cell death and nuclear changes in isolated rat hepatocytes by EEAL treatment. MTT assay of acetaminophen and EEAL after 24 hrs. Values are expressed as Mean ± SD of three independent experiments. (B) DAPI staining of control and treated cells (magnification 20×). Pictures are representation of three separate experiments. #Statistically significant compared to control cells (*p*<0.05), *Statistically significant compared to ACN treated cells (*p*<0.05). ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*. Red arrows indicate chromatin condensation.

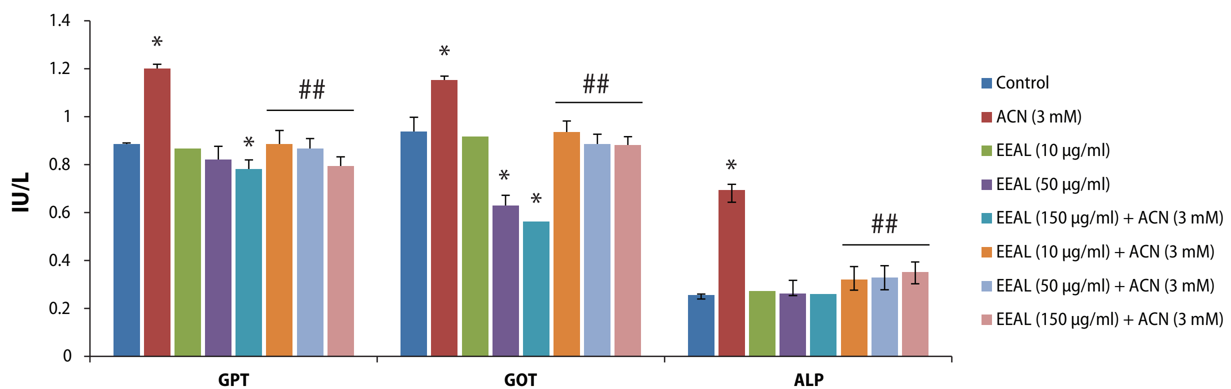

Figure 4. Effect of different concentrations of EEAL and ACN on GPT, GOT and ALP release from isolated rat hepatocytes. ACN-induced increase of GPT, GOT and ALP levels in hepatocytes media compared to the enzyme release by control and EEAL pre-treated cells. Values are expressed as Mean ± SD of three independent experiments. *Statistically significant compared to control cells (*p*<0.05), ##Statistically significant compared to ACN treated cells (*p*<0.05). GPT – Glutamine Pyruvate Transaminase; GOT – Glutamate Oxaloacetate Transaminase; ALP – Alkaline Phosphatase; ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*.

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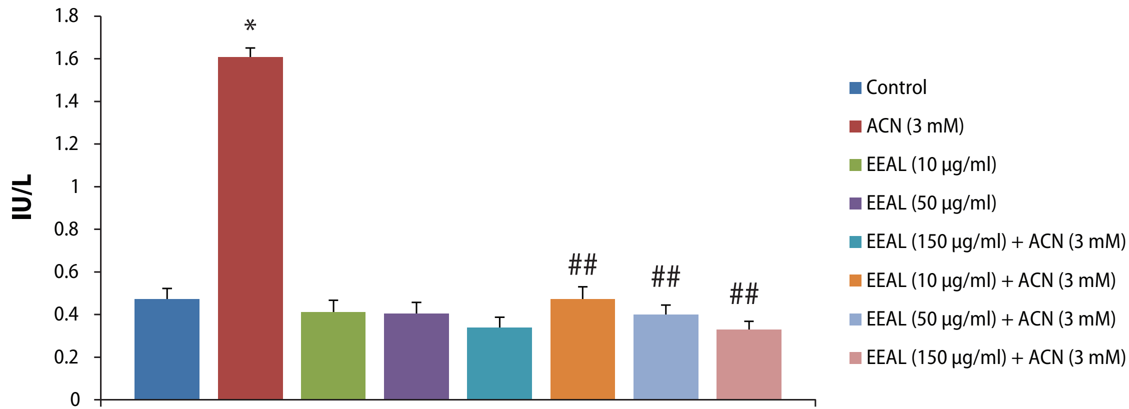

Figure 5. Effect of different concentrations of EEAL on LDH release from isolated rat hepatocytes. Values are expressed as Mean ± SD of three independent experiments. *Statistically significant compared to control cells ($p<0.05$), ##Statistically significant compared to ACN treated cells ($p<0.05$). ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*.

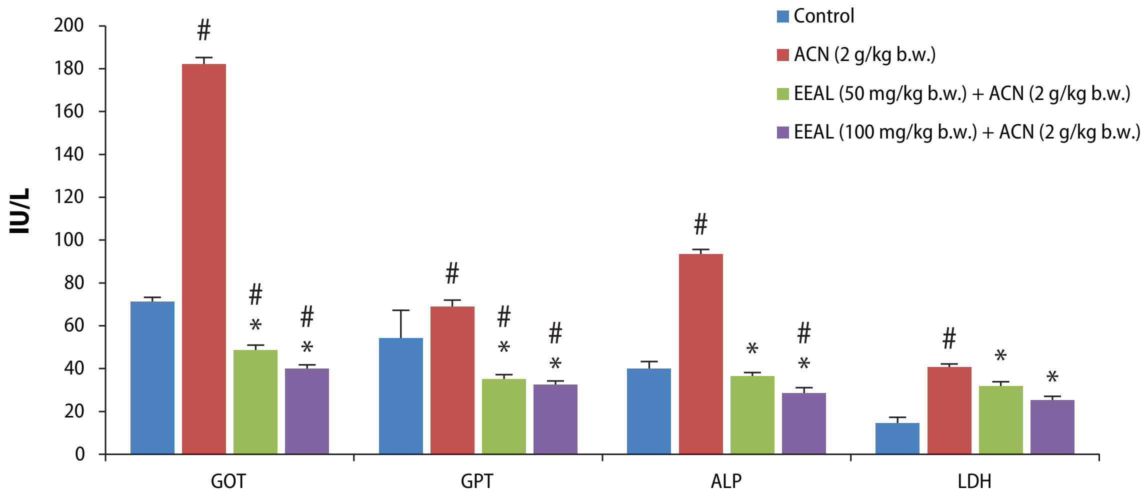

Figure 6. Levels of liver marker enzyme activities in serum of control, ACN and EEAL treated rats. ACN-induced increase of GPT, GOT and ALP serum levels compared to the serum levels of control and EEAL pre-treated rats .Values are expressed as Mean ±SD of three independent experiments. *Statistically significant compared to ACN treated rats ($p<0.05$), #Statistically significant compared to untreated control rats ($p<0.05$). ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*.

EEAL treated rats exposed to ACN showed a difference in the expression level with respect to the concentration of EEAL. This may be due the interference of EEAL on absorption of ACN or enhanced clearance from the circulation without affecting the normal homeostasis of the cells.

The rise in serum levels of GOT, GPT, ALT and LDH has been attributed to the damaged structural integrity of the liver, since they are cytoplasmic and released into circulation post cellular damage (Sallie *et al.*, 1991). In the present study we found that ACN elevated the activities of these marker enzymes, indicative of hepatocyte damage, while EEAL treatment restored the activity of these enzymes in rat liver. The results obtained *in vitro* using isolated hepatocytes showed the same pattern of activity.

The interesting observation we found *in vitro* was the reduction in the activities of GOT and GPT below the levels of normal cells in the culture medium of EEAL alone treated hepatocytes. This observation led us to the conclusion that EEAL may have some inhibitory effect on the production of these enzymes. Previous studies showed that it is possible that certain extracts/formulation containing pyrethroid and Neem have inhibitory effects on the activity of GOT and GPT in Khapra beetle larvae (Younes *et al.*, 2011). Studies by Tanani *et al.* (2009) also observed an inhibition of GPT activity by different solvent extracts of the wild plant *Fagonia bruguieri* and suggested that the inhibition may be due to its effect on synthesis or on functional levels of the enzyme directly or indirectly by altering the cytomorphology of the cells

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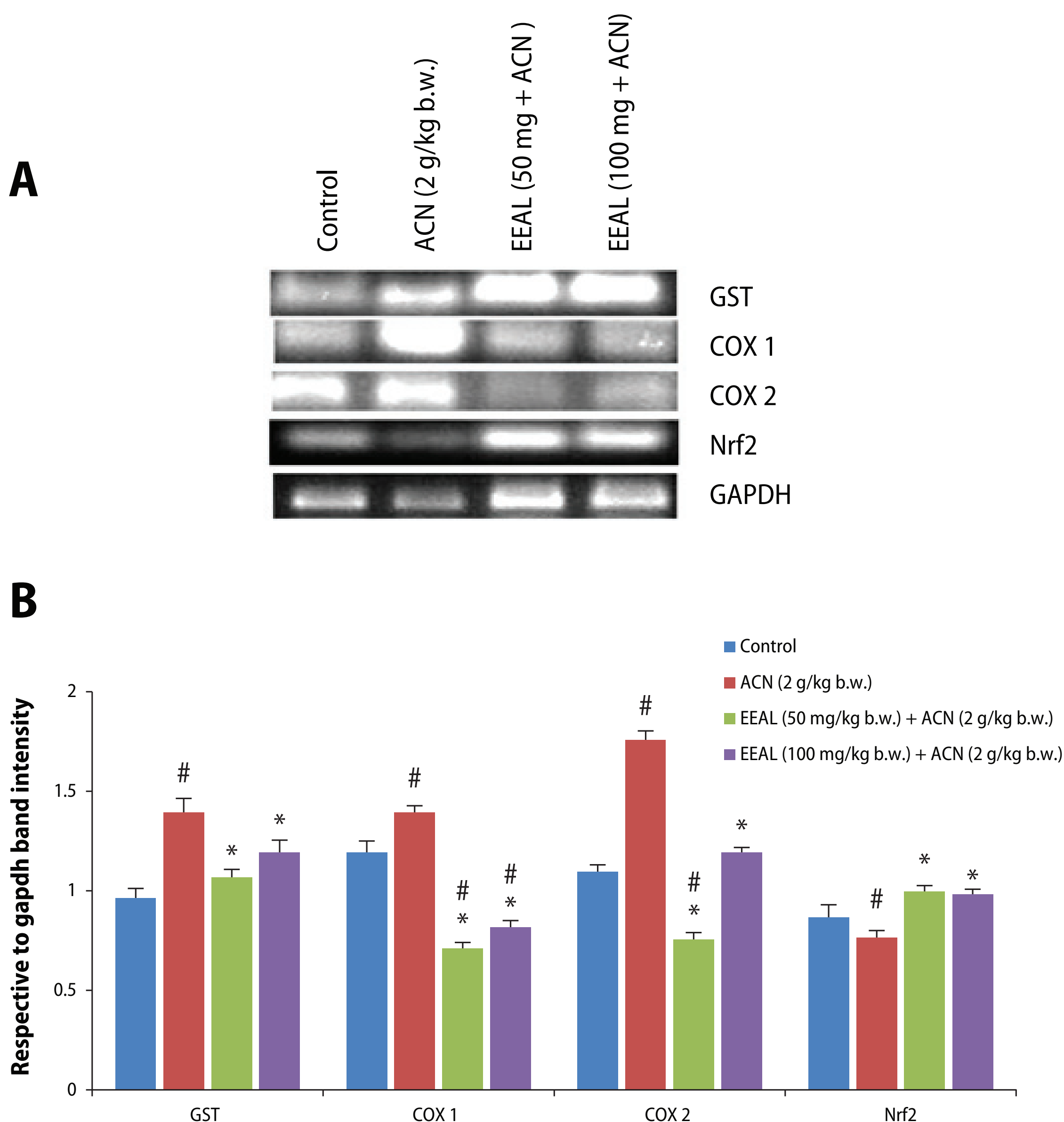

Figure 7. Expression of GST, COX1, COX 2 and Nrf2 genes in the liver of rats treated with EEAL and CAN. Expression of GST, COX 1, COX 2 and Nrf2 genes in EEAL treated rat liver. (B) Graph showing respective band intensity. EEAL dose is expressed in mg/kg body weight. Dose of ACN used was 2 g/kg body weight. ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*. The pictures are representation of three independent experiments. (B) Relative band intensity of gene products. Values are expressed as mean±SD of three independent experiments . #statistically significant compared to control (p<0.05). *statistically significant compared to ACN (p<0.05).

(Tananai *et al.*, 2009; Nath, 2000). We have found that EEAL did not affect the morphology of the hepatocytes, indicating the possibility of interfering in the synthesis of these marker enzymes.

Oxidative damage to biological macromolecules affects normal cellular functions and is implicated in cancer, inflammation, neurodegenerative diseases, cardiovascular diseases and aging. Eukaryotic cells have developed antioxidant defence mechanisms to neutralize reactive oxygen species (ROS) and prevent macromolecules from undergoing peroxidation. One of the most important cellular defence mechanisms against ROS and electrophilic intermediates are mediated through the antioxidant responsive element sequence (ARE), in the promoter regions of phase II and antioxidant enzyme genes. The ARE-dependent cellular defence system is controlled by the transcription factor, transcription factor nuclear factor-erythroid 2 related factor 2 (Nrf2). Nrf2 exerts its influence on hepato-protection of compounds through ARE by enhancing GSH synthesis, bio-conjugation and clearance of toxic metabolites (Gum & Cho, 2013). Results showed a mild decrease in expression of Nrf2 in rat hepatocytes treated with ACN which was restored by pre-treatment of EEAL. These results suggest that the modulation of the antioxidant master switch Nrf2 by EEAL can contribute to the restoration

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of redox homeostasis in ACN stressed hepatocytes. The Nrf2-ARE pathway is capable of inducing a phase-2 detoxification enzyme activity, promoting a disruption of (Nrf2)-Kelch-like ECH-associated protein interactions (Lau *et al.,* 2008). As a result Nrf2 translocates into the nucleus and modulates the expression of several target genes, especially HO-1 and GST, and protects the cells against oxidative stress-induced tissue. Here ACN treatment has significantly upregulated the GST expression and subsequently restored by EEAL treatment which may not be dependent on Nrf2, since the results showed down regulated levels of this transcription factor after ACN treatment. This prompts us to think about other alternative gene pathways affecting the regulation of GST expression. Such regulation can occur through another dimeric transcriptional factor as hepatic nuclear factor-1α (HNF1α), which is secreted by hepatocytes and mediates liver specific gene transcription (Park *et al.*, 2004).

ACN treatment induced a slight upregulation of COX 1 gene, while COX 2 gene expression was significantly higher in rat liver compared to untreated rats. COX 1 and COX 2 gene expression were found to be downregulated below normal levels by pre-treatment with EEAL (50 and 100 mg/kg b.w.). This effect of upregulation of COX 1 and 2 genes by ACN is uncharacteristic related to some of the previous research, which demonstrated a preferential COX-2 inhibition by ACN under different clinically relevant conditions (Hinz & Brune, 2012). Reports are also available suggesting that ACN is a potent antipyretic and analgesic drug with very weak anti-inflammatory effects, and activities of COX enzymes in homogenates of ACN treated rat tissues showed differential inhibitory action. The findings also suggested that ACN is a weak inhibitor of both COX 1 and COX 2 and there exists a possibility of inhibition of another unidentified variant of cyclooxygenase, COX 3 (Botting *et al.,* 2000). The inhibitory effect of EEAL on both COX 1 and 2 is very significant and the results can be related to previous reports suggesting that plant extracts may inhibit both COX 1 and COX 2 expressions, by interfering with the biosynthesis process (Shaikh *et al.,* 2016).

Our experiments showed that treatment of primary rat hepatocytes with ACN induced significant cell death accompanied by changes in nuclear size and chromatin condensation, while pre-treatment with EEAL prior to ACN exposure protected hepatocytes from cell death and nuclear damage. ACN induced cellular damage resulted in elevated extracellular activity of GOT, GPT, ALP and LDH in both isolated hepatocytes *in vitro* and rat liver *in vivo.* Higher levels of ALP activity in both hepatocytes and rat serum showed the extent of cell membrane damage caused by ACN since ALP is a membrane bound glycoprotein enzyme, with high concentrations in sinusoids and endothelium and is excreted into the bile and its elevation in serum occurs in hepatobiliary diseases (Swamy *et al.,* 2012). Prophylatic treatment of

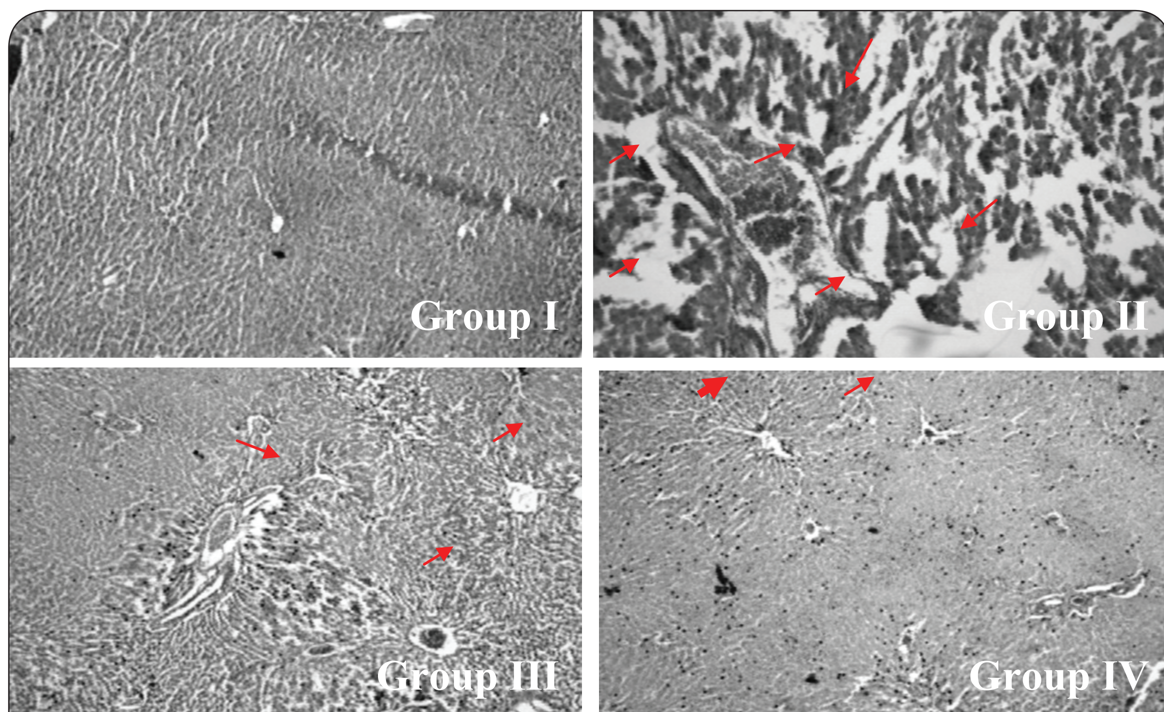

Figure 8. Histopathological changes in the liver of control and ACN exposed rats treated with EEAL. Representative microscopic photographs of histopathology of liver of Control, EEAL, EEAL and acetaminophen treated rats. Sections were routinely processed for hematoxylin and eosin staining, and were observed using a digital microscope at 10× magnification. Group I – Control; Group II – ACN (2 g/kg b.w.); Group III – EEAL (50 mg/kg b.w.)+ACN (2 g/kg BW), Group IV – EEAL (100 mg/kg b.w.) + ACN (2 g/kg b.w.).The arrows indicate loss of hepatic architecture, accumulation of fat globules, dilated sinusoids and centrilobular necrosis) ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*.

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rat liver and isolated hepatocytes with EEAL before ACN exposure reversed the increased extracellular activities of the hepatic marker enzymes possibly by preventing the leakage of intracellular enzymes by stabilizing the cell membrane from ACN action. These molecular effects of EEAL were substantiated by histopathological findings. ACN treatment induced significant structural changes to the individual hepatocytes with enlarged sinusoids, while EEAL pre-treated rats exposed to ACN showed a normal liver architecture.

In conclusion, it was observed that the ethanol extract of *A. lanata* has significant anti- inflammatory and hepatoprotective effects against ACN-induced liver injury, as evidenced by molecular and histopathological analysis. *A. lanata* contains polyphenols, flavonoid and alkaloids that contribute to the exhibited activity of *A. lanata*, which prompted us to conduct further studies to investigate the lead compound/s present in the plant and finally to carry out human clinical trials.

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ORIGINAL ARTICLE

Biochemical and histopathological effects of sub-acute exposure of albino rats to fumigants – dichlorvos and cypermethrin

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ABSTRACT

Cypermethrin (CYP) is one of the most common active ingredients in most insecticides, mosquito coils and powder used in Nigeria. dichlorvos (DDVP) is the most indiscriminately used fumigant in most rural and sub-urban areas in Nigeria. These fumigants can easily be accessed without proper method of usage thus exposing the population to their toxic effects. As a result, this study was initiated to determine the effects of sub-acute exposure of CYP and DDVP on some biochemical and histopathological parameters of albino rats. In this study, forty (40) albino rats of 10 groups of 4 rats per group, with one group serving as control, were exposed to these fumigants in a poorly ventilated area for 4hours per day over 2, 4 and 6 weeks. The results showed observable changes in liver enzyme activities ($p<0.05$) in groups exposed to DDVP for 2, 4 and 6 weeks. The groups exposed to CYP showed mild changes in liver enzyme activities when compared with the DDVP groups. Increase in activity of the liver enzymes was also observed in the groups exposed to a mixture of DDVP+CYP for 2, 4 and 6 weeks. The urea, creatinine and electrolytes levels in all the groups exposed to DDVP, CYP and DDVP+CYP for 2, 4 and 6weeks were significantly ($p<0.05$) increased. Also WBC and platelets in all the groups exposed to DDVP and CYP recorded significant changes. The histology report of the lungs and liver showed moderate lymphocytic infiltration and hepatocytic steatosis which progressed with duration of exposure to the fumigants, while the kidneys showed no remarkable changes. The results of this study suggest that DDVP and CYP have relative toxic effects in the exposed animals and should be used with caution to avoid human exposure to their visible toxicities.

KEY WORDS: dichlorovos; cypermethrin; fumigant; liver enzymes; sub-acute toxicity

Introduction

Fumigant is any volatile substance used to kill insects, nematodes, and other animals or plants that damage stored foods or seeds. Inadvertent exposure of humans and animals to fumigants is of great danger but the effects are not well quantified as many are not aware of toxic effects of these compounds. Many marketers and farmers through poor handling and ignorance are exposed to these substances. For example dichlorvos or 2,2-dichlorovinyl dimethyl phosphate (DDVP) is widely used worldwide in agriculture for controlling agricultural deleterious insects (Okamura *et al.,* 2005) and thus humans and animals are exposed to its toxicity. Organophosphate compounds such as DDVP are known to cause severe toxicity and death from acute poisoning in many parts of the world (Eddleston, 2000). The widespread use of pesticides produces a number of serious health hazards affecting both humans and animals. Poisoning by these compounds represents a serious public health problem especially in developing countries (Karki *et al.,* 2004). DDVP has been shown to endanger human health (Alavanja *et al.,* 2004; Forget, 1993). Thus ways to reduce human exposure is paramount.

DDVP also known as Ota-piapia in south-east Nigeria is indiscriminately used because of its accessibility and affordability (Nesheim *et al.,* 2002). In Nigeria, DDVP is commonly produced and used as an effective and potent insecticide (Owoeye *et al.,* 2012). The inactivation of acetylcholinesterase by dichlorvos seems to be the major mechanism of toxicity of this compound (Antonijevic *et al.,* 2016; Mostafalou *et al.,* 2017; Mileson *et al.,* 1998).

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Though, reactive oxygen species (ROS) production by dichlorvos has also been described as one of the mechanisms of its toxicity induction (Eraslan *et al.,* 2010; Eroglu *et al.,* 2013), causing irreversible damage to DNA and membrane lipid peroxidation (Ajiboye, 2010). Studies have also shown that chronic dichlorvos intoxication may result in increased brain intracellular Ca^{2+} levels and decline in Ca^{2+}-MgATPase activity (Raheja & Gill, 2002), which is deleterious to normal functioning of cells.

cypermethrin is extensively used not only as an ectoparasiticide in animals but also to control many agricultural pests. cypermethrin is classified by the World Health Organisation (WHO) as 'moderately hazardous' (WHO, 2009) which interacts with the sodium channels in nerve cells and interferes with other receptors in the nervous system.

Since most people use these fumigants in their houses against insects and pests, this study was initiated to determine the effects of sub-acute exposure of CYP and DDVP on aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), bilirubin (total and direct bilirubin), urea, creatinine and serum electrolytes in albino rats.

Materials and methods

Fumigants

Fumigants (dichlorvos and cypermethrin, 1000 mg/L each) were purchased from Molon Agrochemical company, GRA Enugu, Nigeria and then prepared in a dilution of 1:1 as recommended by the manufacturer (Hubei Samonda Co. Ltd, China), i.e. 50 ml of fumigant was mixed with 50 ml of distilled water.

Experimental animals

Forty (40) albino rats (*Rattus norvegicus*) of both sexes aged 6 to 8 weeks with an average weight range of 125.9 g to 135.6 g and mean body weight of 130.0±3.5 g were obtained from the animal house of the animal research unit of the College of Medicine, University of Nigeria, Enugu Campus, Enugu State, Nigeria. They were fed rat chow and water *ad libitum.* The animals were allowed to acclimatize for 2 weeks before being randomized into 10 groups of 4 rats per group as follows:

Group treatment

Group 0 served as control group and was not exposed to any fumigant; Group 1A was exposed to cypermethrin (50 ml CYP/50 ml distilled water – v/v) for 2 weeks of 4 hours per day at room temperature in a poorly ventilated compartment; Group 1B was exposed to dichlorvos (50 ml DDVP/50 ml distilled water – v/v) for 2 weeks of 4 hours per day at room temperature in a poorly ventilated compartment; Group 1C was exposed to a mixture of dichlorvos and cypermethrin (25 ml DDVP + 25 ml CYP/50 ml distilled water – v/v) for 2 weeks of 4 hours per day at room temperature in a poorly ventilated compartment; Group 2A was exposed to cypermethrin (50 ml CYP/50 ml distilled water – v/v) for 4weeks of 4hours per day at room temperature in a poorly ventilated compartment; Group 2B was exposed to dichlorvos (50 ml DDVP/50 ml distilled water – v/v) for 4 weeks of 4 hours per day at room temperature in a poorly ventilated compartment; Group 2C was exposed to mixture of dichlorvos and cypermethrin (25 ml DDVP + 25 ml CYP/50 ml distilled water – v/v) for 4 weeks of 4 hours per day at room temperature in a poorly ventilated compartment; Group 3A was exposed to cypermethrin (50 ml CYP/50 ml distilled water – v/v) for 6 weeks of 4 hours per day at room temperature in a poorly ventilated compartment; Group 3B was exposed to dichlorvos (50 ml DDVP/50 ml distilled water – v/v) for 6 weeks of 4 hours per day at room temperature in a poorly ventilated compartment; Group 3C was exposed to a mixture of dichlorvos and cypermethrin (25 ml DDVP + 25 ml CYP/50 ml distilled water – v/v) for 6 weeks of 4 hours per day at room temperature in a poorly ventilated compartment.

The doses were obtained using the simplified method of evaluating dose-effect experiments as described by Litchfield *et al.* (1949).

Exposure of animals to fumigants

Fumigants as described above were poured into an open container for easy diffusion of its odor in and out of the container and inhalation by the animals kept in a metal cage. The metal cage was placed inside a carton. The container was fixed in a corner inside the carton in such a way that the animals were unable to pour away the content of the container in order to prevent body contact.

Collection of blood samples and organs

At the end of 2 weeks, 4 weeks and 6 weeks, blood samples were collected from the rats through ocular puncture without the use of anesthetic agent. Blood samples of the animals were collected into plain tubes and into ethylenediaminetetraacetic acid (EDTA) tubes. The blood samples collected in plain tubes were allowed to clot and were centrifuged to get the serum and stored at 4 °C until used. The animals were sacrificed before the organs (livers, lungs and kidneys) were harvested and used for histopathology analysis.

Biochemical analysis

Aspartate transaminase (AST) and alanine transaminase (ALT) were determined according to the method of Reitman and Frankel (1957). Alkaline phosphatase (ALP) level was assessed using the method described by King (1965). The bilirubin (total and direct bilirubin) levels in serum was determined as described by Dangerfield and Finlayson (1953). Enzymatic method as described by Machado and Horizonte (1958) was used in urea determination. Creatinine was determined as described by Mitchell (1973). Electrolytes were determined using Ion-Selective Electrodes method as described by International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), (2000).

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Histological preparation and analyses

Kidney, liver and lungs were cut in sections at 5 µm and stained using hematoxylin and eosin method for demonstration of general tissue structure. Stained sections were examined using light microscopy and microscopically photographed using Nikon photomicroscope (Figures 1a–f).

Data analysis

The Statistical Package for Social Science (SPSS) computer software version 17 was used for data analysis. The results of the tests were analyzed using analysis of variance (ANOVA) and student's t-test at 95% confidence interval with p-value of ≤0.05 considered as significant.

Ethical permit

Ethical permit was sought and obtained from the research and ethics committee of the College of Medicine, University of Nigeria, Enugu Campus. The procedures followed in this study were in accordance with the guide for the care and use of laboratory animals by the National Research Council (National Research Council, 2011)

Results

The results of toxicity study of DDVP inhalation on liver enzymes of rats showed a significant increase in the activities of AST and ALT of rats exposed to DDVP for 2 weeks and 4 weeks and a significant decrease in the activity of AST and ALT of rats exposed to DDVP for 6 weeks when compared with the control. For ALP, there was a significant decrease in the activity of ALP across the three groups and there was a significant decrease in the activity of total bilirubin of rats exposed to DDVP for 4 weeks, while there was a significant decrease in the activity of direct bilirubin in all groups compared with the control (Table 1).

There was a significant decrease in the activity of AST in the group exposed to CYP for 2 weeks, which significantly increased in week 4 and week 6. There was also a significant increase in the activity of ALT in week 2, which decreased with no significant difference in week 4 and week 6. The ALP activity increased significantly in weeks 2, 4 and 6 of exposure while the direct bilirubin activity decreased with duration of exposure of CYP (Table 2).

The AST, ALT and ALP activities significantly increased after 2-week exposure and decreased with duration of exposure of the mixture of DDVP+CYP and the bilirubin activity decreased with duration of exposure (Table 3).

Significant increase in values of K^+, Na^+, Cl^- and urea and a significant decrease in HCO_3^- were observed in the groups when compared with the control (Table 4).

The results of exposure of rats to CYP and DDVP+CYP revealed significantly increased levels of K^+, Na^+, Cl^-, HCO_3^- and urea and a significant decrease in creatinine across the groups when compared with the control (Tables 5 and 6).

Histology of the lung and liver showed moderate lymphocytic infiltration and hepatocytic steatosis thus progressing with duration of exposure to fumigants, while the kidney showed no remarkable changes.

Table 1. Mean± SD values of some liver enzymes of rats exposed to DDVP through inhalation.

Group	AST (U/L)	ALP (U/L)	ALT (U/L)	T.BILL (mg/l)	D.BILL (mg/l)
Control	80.50±7.00^{ae}	362.25±180.80^{a}	81.00±10.03^{ac}	6.05±0.42^{ac}	2.67±0.34^{a}
1B	93.75±9.28^{b}	881.25±189.21^{bf}	105.50±11.90^{ba}	6.37±0.70^{ac}	1.49±0.41^{bc}
2B	89.00±1.24^{be}	1166.00±59.93^{c}	101.25±20.17^{be}	4.62±1.80^{b}	1.55±0.12^{bc}
3B	75.75±6.65^{a}	710.50±187.96^{f}	73.75±15.94^{cf}	5.90±0.25^{ac}	1.97±0.42^{bc}

Data in the same column bearing different superscripts differ significantly. (p<0.05) DDVP: Dichlorovos. 1B = exposed to DDVP for 2 weeks, 2B = exposed to DDVP for 4 weeks and 3B = exposed to DDVP for 6 weeks.

Table 2. Mean± SD values of some liver enzymes of rats exposed to CYP DDVP through inhalation.

Group	AST (U/L)	ALP (U/L)	ALT (U/L)	T.BILL (mg/l)	D.BILL (mg/l)
Control	80.50±7.00^{ae}	362.25±180.80^{a}	81.00±10.03^{ac}	6.05±0.42^{ac}	2.67±0.34^{a}
1A	76.00±7.87^{a}	1032.25±9.53^{c}	94.25±4.03^{ab}	6.32±0.99^{ac}	2.27±0.30^{a}
2A	83.25±7.13^{ba}	902.50±47.74^{bcf}	80.00±5.29^{ac}	6.12±0.87^{a}	1.92±0.29^{bc}
3A	85.00±3.65^{ba}	1020.75±5.37^{bc}	82.75±1.50^{ac}	4.77±0.05^{b}	1.80±0.66^{bc}

Data in the same column bearing different superscripts differ significantly. (p<0.05) CYP: Cypermethrin, 1A = exposed to CYP for 2 weeks, 2A = exposed to CYP for 4 weeks and 3A = exposed to CYP for 6 weeks.

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Table 3. Mean± SD values of some liver enzymes of rats exposed to a mixture of DDVP +CYP DDVP through inhalation.

Group	AST (U/L)	ALP (U/L)	ALT (U/L)	T.BILL (mg/l)	D.BILL (mg/l)
Control	80.50±7.00[ae]	362.25±180.80[a]	81.00±10.03[ac]	6.05±0.42[ac]	2.67±0.34[a]
1C	93.00±7.25[b]	1169.50±65.22[c]	110.00±28.08[b]	5.85±1.31[ac]	1.27±0.25[bc]
2C	79.75±5.43[ae]	1009.50±37.11[bc]	82.25±5.31[ac]	4.00±0.93[b]	1.00±0.74[bd]
3C	60.25±8.13[d]	849.75±148.43[bf]	72.50±3.87[cf]	4.20±0.08[b]	1.32±0.43[bd]

Data in the same column bearing different superscripts differ significantly. ($p<0.05$) DDVP: Dichlorovos, CYP: Cypermethrin. 1C = exposed to the mixture of DDVP +CYP for 2 weeks, 2C = exposed to the mixture of DDVP +CYP for 4 weeks and 3C = exposed to the mixture of DDVP + CYP for 6 weeks.

Table 4. Mean± SD values of serum urea, creatinine and some electrolytes of rats exposed to DDVP DDVP through inhalation.

Group	K^+	Na^+	Cl^-	HCO_3^-	UREA (mg/l)	CREAT (mg/l)
Control	3.72±0.23[a]	124.50±5.19[a]	89.50±5.19[a]	25.25±3.50[a]	4.42±1.99[a]	96.25±11.44[a]
1B	6.37±0.82[bf]	136.75±4.03[bc]	89.00±4.24[a]	24.75±3.09[a]	6.45±1.42[ac]	112.00±2.94[b]
2B	5.42±0.50[c]	135.00±5.47[bc]	97.25±6.80[bc]	22.75±1.50[ab]	7.22±0.60[bd]	57.24±65.24[e]
3B	4.92±0.25[d]	135.00±3.91[bc]	96.00±5.94[bc]	19.00±5.16[bc]	6.27±0.55[ac]	6.00±2.16[g]

Data in the same column bearing different superscripts differ significantly. ($P<0.05$) DDVP: Dichlorovos, 1B = exposed to DDVP for 2 weeks, 2B = exposed to DDVP for 4 weeks and 3B = exposed to DDVP for 6 weeks.

Table 5. Mean± SD values of serum urea, creatinine and some electrolytes of rats exposed to CYP DDVP through inhalation.

Group	K^+	Na^+	Cl^-	HCO_3^-	UREA (mg/l)	CREAT (mg/l)
Control	3.72±0.23[a]	124.50±5.19[a]	89.50±5.19[a]	25.25±3.50[a]	4.42±1.99[a]	96.25±11.44[a]
1A	5.22±0.35[c]	141.50±1.29[c]	95.00±3.65[b]	26.00±3.16[a]	5.15±0.31[ad]	90.25±6.89[c]
2A	6.32±0.76[bf]	140.75±4.50[c]	105.50±7.04[c]	28.25±2.21[ac]	8.92±2.62[b]	90.00±6.83[c]
3A	4.52±0.68[d]	139.00±2.16[c]	99.25±3.36[d]	27.50±5.44[ac]	8.10±2.15[b]	5.50±3.41[g]

Data in the same column bearing different superscript differ significantly. ($p<0.05$) CYP: Cypermethrin. 1A = exposed to CYP for 2 weeks, 2A = exposed to CYP for 4 weeks and 3A = exposed to CYP for 6 weeks.

Table 6. Mean± SD values of serum urea, creatinine and some electrolytes of rats exposed to a mixture of DDVP +CYP DDVP through inhalation.

Group	K^+	Na^+	Cl^-	HCO_3^-	UREA (mg/l)	CREAT (mg/l)
Control	3.72±0.23[a]	124.50±5.19[a]	89.50±5.19[a]	25.25±3.50[a]	4.42±1.99[a]	96.25±11.44[a]
1C	6.57±0.76[bf]	134.75±6.22[bc]	89.25±4.78[a]	17.75±5.61[b]	5.17±1.73[ad]	124.00±7.16[d]
2C	5.57±0.37[c]	138.50±3.87[bd]	97.75±6.13[bc]	23.75±3.21[a]	5.60±1.00[ad]	29.00±43.42[f]
3C	4.85±0.36[d]	136.00±3.55[bc]	99.50±1.73[d]	27.00±4.69[ac]	8.77±2.60[b]	5.75±2.21[g]

Data in the same column bearing different superscripts differ significantly. ($P<0.05$) DDVP: Dichlorovos, CYP: Cypermethrin. 1C = exposed to a mixture of DDVP +CYP for 2 weeks, 2C = exposed to a mixture of DDVP +CYP for 4 weeks and 3C = exposed to a mixture of DDVP + CYP for 6 weeks

Discussion

Serum enzyme levels are considered indicators of overall health status of an individual, especially in hepatocyte injury and related stress (Khan *et al.*, 2009). Small amounts of intracellular enzymes are present in the blood as a result of normal cell turnover. When damage to cells occurs, increased amounts of enzymes will be released and their concentrations in blood will rise.

The increase in activity of serum AST observed in DDVP exposure for 2 and 4weeks and the decrease observed in week 6 of exposure (Table 1) suggest toxic and harmful effects of DDVP on the liver. The decreased activity of serum AST observed after 2-week exposure of CYP and then rapid increase in 4 and 6weeks of exposure (Table 2) showed toxicity of CYP on the liver. Similar

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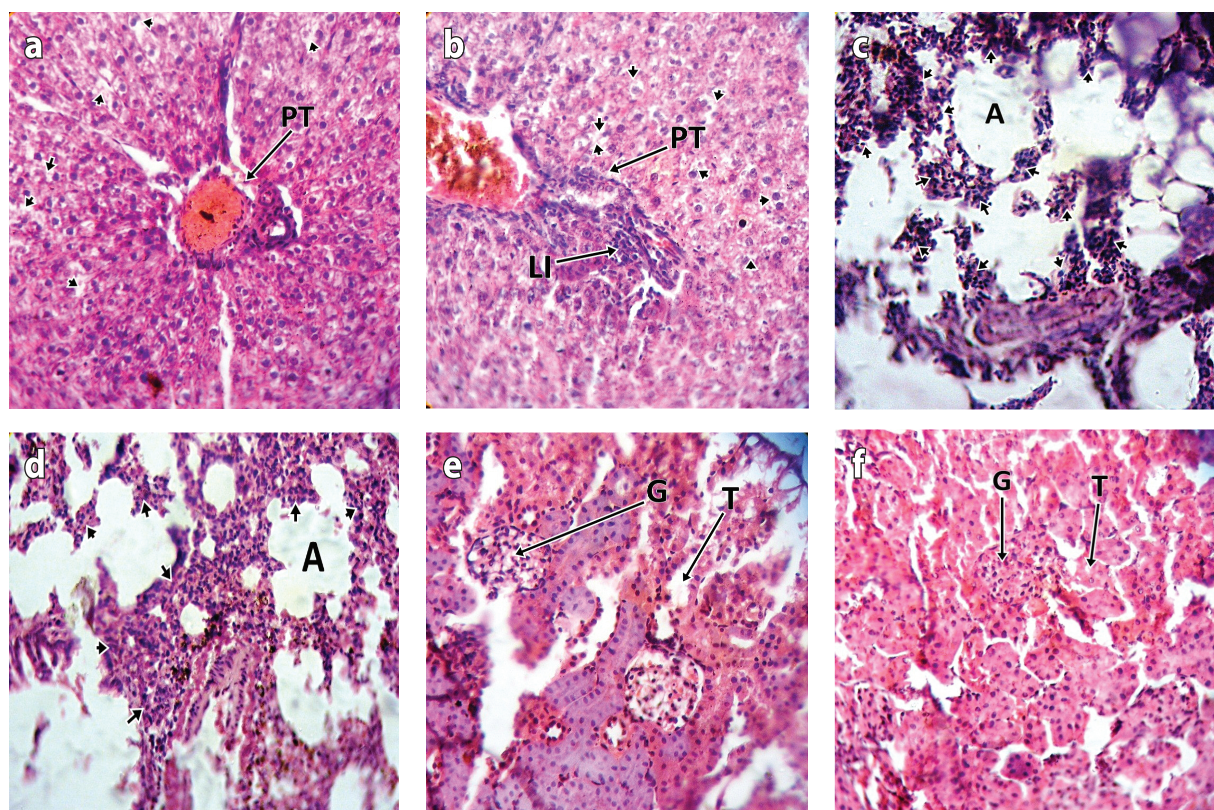

Figure 1. a: Photomicrograph of the liver of test animal treated with cypermethrin for 2 weeks showing mild steatosis of hepatocytes (short arrows) around portal tract (PT) (H & E stain ×400). **b:** Photomicrograph of the liver of test animal treated with dichlorvos for 2 weeks showing moderate steatosis of hepatocytes (short arrows) and mild lymphocytic infiltration (LI) of portal tract (PT) (H & E stain ×400). **c:** Photomicrograph of the lung of test animal treated with cypermethrin for 2 weeks showing moderate lymphocytic infiltration (short arrows) of the interstitium (H & E stain ×400). **d:** Photomicrograph of the lung of test animal treated with dichlorvos for 2 weeks showing moderate lymphocytic infiltration (short arrows) of the interstitium (H & E stain ×400). **e:** Photomicrograph of the kidney of test animal treated with cypermethrin for 2 weeks showing unremarkable glomeruli (G) and renal tubules (T) (H & E stain ×400). **f:** Photomicrograph of the kidney of test animal treated with dichlorvos for 2 weeks showing unremarkable glomeruli (G) and renal tubules (T) (H & E stain ×400).

effect was also observed in the exposure of DDVP+CYP. The increase in AST activity of the groups exposed to DDVP observed in this study agrees with earlier investigations by Atef (2010) and Ajiboso (2012) on effects of a class of organophosphate insecticides in rats.

High levels of ALT usually indicate a damaged liver while most low ALT levels indicate malnutrition as a major cause of low blood ALT levels (Gimson, 1996). The mild decrease in activity of liver ALT in groups exposed to DDVP and DDVP+CYP for 6weeks may be due to malnutrition. According to Lum (1995), malnutrition is one of the conditions that leads to low serum ALT. The decreased serum activity of ALT in this study may be attributed to malnutrition that resulted from withdrawal from food by animals due to continuous and prolonged exposure to DDVP and DDVP+CYP. ALT activity was not altered in the groups exposed to CYP for 4 and 6weeks (Table 2). Impairment in alkaline phosphatase of rats exposed to other classes of insecticides such as DDT, malathion, phosalone and elsan, as reported by Saigal *et al.* (1982), was also observed in this study with altered activity of serum ALP of groups exposed to DDVP, CYP and DDVP+CYP for 2, 4, 6 weeks.

The increased bilirubin levels observed in rats exposed to DDVP and CYP for 2 weeks may be attributed to the breakdown of hemoglobin and the subsequent mild decrease may be due to the withdrawal from food in the exposed groups. A similar finding was reported by Ajiboso *et al.* (2012).

Increased creatinine levels of rats exposed for 2 weeks showed the idiosyncratic hepatotoxic properties of DDVP and DDVP+CYP. The increase in creatinine levels of groups exposed to DDVP in this study agrees with the result obtained by Atef (2010). However the decrease in creatinine levels for groups exposed to CYP for 2, 4, and 6weeks may be attributed to malnutrition. It was the same for the rats exposed to DDVP+CYP and DDVP for 4 and 6 weeks, which agrees with the report of Ajiboso *et al.* (2012). The kidney maintains blood creatinine within a normal range. Creatinine has been found to be a fairly reliable indicator of kidney function and an elevated creatinine levels suggests impaired kidney function or kidney disease (Al-Jassabi *et al.*, 2011). In this study, there was an increase in the levels of urea for all groups exposed to DDVP, CYP and DDVP+CYP for 2 weeks, 4 weeks and 6 weeks, that may be attributed to high body muscle

mass breakdown generating more waste nitrogen due to prolonged inhalation of toxic compounds. The balance of electrolytes in the body is essential for normal body function of cells and organs. The observed increase in values of sodium, potassium, chloride and bicarbonate ions was as a result of impaired kidney function due to prolonged exposure of toxic compounds (DDVP and CYP).

The histology report of the lungs and liver showed moderate lymphocytic infiltration and hepatocytic steatosis which progressed with duration of exposure to DDVP and CYP that could be attributed to the toxic effect of the fumigants on the organs. Notably, lungs are the first site of metabolism of gaseous poison, followed by the liver. However the kidneys showed no remarkable changes which did not correspond with the result of biochemical analysis of this study, and the reports by Owoeye *et al.* (2012) and Blair *et al.* (1976). The changes observed in the biochemical analysis may be attributed to malnutrition because these fumigants could cause loss of appetite in the experimental animals.

In conclusion, exposure to fumigants (DDVP and CPY) caused no mortality but induced biochemical alterations in Wistar rats and these disturbances in biochemical parameters and histology of vital organs, such as the liver and kidneys, could be attributed to toxic effects of DDVP and CPY.

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ORIGINAL ARTICLE

The hepatoprotective and antioxidative effect of saffron stigma alcoholic extract against vincristine sulfate induced toxicity in rats

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ABSTRACT

Vincristine (VCR) is an important anti-cancer drug, which is highly toxic for the liver. This study aimed at evaluating the protective effect of alcoholic extract of saffron stigma against vincristine hepatotoxicity in the rat. A total number of 50 rats were randomly divided into 10 groups, including controls, rats receiving 0.25 mg/kg (A group), 0.5 mg/kg (B group), 0.75 mg/kg (C group) VCR, 0.25 mg/kg VCR + 0.5 mg/kg saffron (D group), 0.5 mg/kg VCR + 0.5 mg/kg saffron (E group), 0.75 mg/kg VCR + 0.5 mg/kg saffron (F group), 0.25 mg/kg VCR + 1mg/kg saffron (G group), 0.5 mg/kg VCR + 1 mg/kg saffron (H group), and 0.75 mg/kg VCR + 1 mg/kg saffron (I group) groups. Serum level of liver enzymes, including aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and bilirubin were measured using specific kits at the end of the experimental period. Serum total antioxidant capacity (TAC) and malondialdehyde (MDA) values were measured using ferric reducing antioxidant of power (FRAP) and thiobarbituric acid reaction (TBAR) methods, respectively. Administration of VCR, especially at the concentration of 0.75mg/kg, caused severe hepatic injury with significant increase in the levels of AST (582.0±39.45 UI), ALT (124.0±5.92 UI), ALP (939.8±89.8 UI) enzymes and bilirubin (0.17±0.008). VCR administration also significantly increased the serum MDA level (0.49±0.021 nmol/ml), while TAC value was declined significantly (241.27±18.27 µmol/l). These effects were dose-dependent. Treatment with saffron extract decreased the activity of liver enzymes and MDA values in hepatotoxic rats with a significant enhancement in serum TAC content. These effects were notable for rats that received 1mg/kg plant extract. Administration of saffron, especially at higher concentration, can reduce VCR-induced hepatotoxicity, antioxidant depletion and lipid peroxidation, presumably due to its antioxidative properties.

KEY WORDS: vincristine; saffron; hepatotoxicity; liver enzymes; oxidative stress

Introduction

Vincristine (VCR) is a natural alkaloid compound that can be extracted from the leaves of field grown *Catharanthus roseus* plant (Kumar *et al.*, 2013). Evidence revealed that it is a highly active cell cycle-dependent compound that targets tubulin, causing depolymerization of microtubule, M-phase arrest, and apoptosis in mitotic cells (van Tellingen *et al.*, 1992; Gidding *et al.*, 1999; Silverman & Deitcher, 2013). The interaction between vincristine-spindle microtubules changes spindle structure and function in a dose-dependent manner (Silverman & Deitcher, 2013). At low concentrations and short-term exposure, it stimulates reversible mitotic arrest, prevents chromosome segregation and causes of some abnormalities in morphology or polymerization of spindle microtubules (Blajeski *et al.*, 2002). Higher VCR concentration and long-term exposure can be associated with disruption and total depolymerization of microtubule and subsequently lethal cytotoxicity (Jordan *et al.*, 1992; Takano *et al.*, 1993; Geyp *et al.*, 1996). For this reason, VCR is now considered a potential anticancer compound that has been widely used for therapeutic goals, particularly for childhood and

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adult hematologic malignancies. However, the antitumor activity of VCR is dependent on the concentration and duration of exposure and the number of cells transiting through mitosis during the period of drug exposure (Silverman & Deitcher, 2013).

Vincristine sulfate is a novel formulation of VCR used extensively in the chemotherapeutic management of a variety of pediatric malignancies (Thakur *et al.*, 2016). Despite its potent anti-tumor activity, it has cytotoxicity effects on normal cells. Many studies have reported the cytotoxicity effect of VCR on different cells such as hepatic, pancreatic, and lymphocyte cells (Nevalainen, 1975; Schrek & Stefani, 1976; el Saghir & Hawkins, 1984; Ogunc *et al.*, 2017). It is thus necessary to enhance the therapeutic activity of VCR to increase the VCR dose while limiting free-drug-associated toxicity.

To diminish the cytotoxic effect of VCR, this study aimed at considering the protective effects of saffron extract against vincristine sulfate-induced hepatotoxicity in the rat. Saffron, the dried stigma of the flowers of the saffron crocus (*Crocus sativus*), is now classified as a potent plant antioxidant (Mashmoul *et al.*, 2013). Many studies demonstrated the antioxidative and positive effects of saffron on human health (Nair *et al.*, 1995; Verma & Bordia, 1998; Samarghandian *et al.*, 2017). A great number of studies have also considered saffron as a potential therapeutic drug in clinical trials (Assimopoulou *et al.*, 2005; Kamalipour & Akhondzadeh, 2011). Thus the application of saffron extract in different types of diseases such as neuronal and cardiovascular disorders as well as cancer has been studied (He *et al.*, 2005). The health promoting properties of saffron are primarily due to the existence of a bioactive compound known as "crocin" (Mashmoul *et al.*, 2013). This is a unique carotenoid compound with a potential antioxidant capacity that makes the distinctive bright yellow color of the stigma (Mashmoul *et al.*, 2013).

Although several studies have considered positive effects of saffron on human health, less information is available about its hepatoprotective effect after VCR treatment. We hypothesize that saffron administration may help maintain liver health by decreasing oxidative stress status and antioxidant depletion in rats exposed to VCR sulfate. Therefore, the present study was designed to investigate for the first time the effects of saffron on oxidative stress status and liver injuries in rats that received VCR-sulfate.

Materials and methods

Plant material and extraction preparation

Saffron stigma was purchased from the Novin Saffron Company, Mashhad, Iran. Saffron extract was provided using maceration method, in which 50 g of stigmas were ground to powder and macerated in 1000 ml distilled water for 48 h. The mixture was then filtered within 72 h and subsequently concentrated under vacuum at room temperature. The extract yield was 50% w/w.

Study design

In this experimental study, 50 male Wistar rats (30–35 weeks of age) with a body weight of 200–250 g were provided from the laboratory animal research center of Tehran University of Medical Sciences. After a period of one week adaptation with lab environment, the rats were randomly allocated into 10 groups (n=5 for each group) including control. The rats received 0.25 mg/kg (A group), 0.5 mg/kg (B group), 0.75 mg/kg (C group) VCR, 0.25 mg/kg VCR + 0.5 mg/kg saffron (D group), 0.5 mg/kg VCR + 0.5 mg/kg saffron (E group), 0.75 mg/kg VCR + 0.5 mg/kg saffron (F group), 0.25 mg/kg VCR + 1 mg/kg saffron (G group), 0.5 mg/kg VCR + 1 mg/kg saffron (H group), and 0.75 mg/kg VCR + 1 mg/kg saffron (I group). In each group the rats were housed 3 per cage (30×15×15 cm) in a climate controlled room (ambient temperature of 22±2 °C, humidity 50±5, and a 12:12 light/dark cycle) and had free access to food (10g/kg/day) and tab water. The study was approved by the Animal Care and Use Committee at the Islamic Azad University of Damghan.

Vincristine sulfate was injected intraperitoneally for a period of 8 weeks. All injections were carried out at 10 a.m. After one week from the last injection, the rats were anesthetized with diethyl ether and blood samples were provided from the aorta. Rats in D, E, F, G, H and I groups were subsequently treated orally with different concentrations of saffron for 8 weeks. Blood samples were collected one week after the last administration of saffron for measurement of liver enzymes. The normal control group was injected with sterile saline via the tail vein and with intragastrically administered distilled water.

Biochemical analysis

Serum samples were separated by centrifugation at 3000 rpm for 10 min for the assessment of aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) enzymes and bilirubin. The activity of liver specific enzymes including AST (Cod: 11531), ALT (Cod: 11533) and ALP (Cod: 11593) was evaluated with the commercial ELISA kits provided from BioSystems Company. An Auto Analyzer apparatus (Roche Hitachi 911 and 912 models) was applied for the assessment of these enzymes. Total bilirubin was measured using the Diazotized Sulfanilic method (Fossati *et al.*, 1989). Bilirubin reacts with diazotized sulfanilic acid and to form a blue azopigment that can be measured at 540 nm.

Malondialdehyde (MDA) measurement

Malondialdehyde (MDA) level was measured in order to estimate the lipid peroxidation in serum of rats. MDA content was assayed using thiobarbituric acid reactive substance (TBARS) method as described previously by Tavilani and colleagues (Tavilani *et al.*, 2005). Briefly, 100 µl of the serum samples were mixed with 500 µl of trichloroacetic acid (TCA) and then 10 µl of hydroxytoluene was added to the prepared solution and centrifuged at 3 000×*g* for 10 min. 500 µl of the supernatant was removed from the solution, 400 ml of TBARS (144.14 g/mol;

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Merck, Germany) was added to it and the mixture was preserved at 95 °C for one hour. Samples were stored and cooled down at room temperature for 15 min. Then they were re-centrifuged at 4000×*g* for 10 min and the light absorption of the supernatant was determined by a spectrophotometer at the wavelength of 532 nm. Finally, MDA concentration was calculated, using the standard curve.

Total antioxidant capacity (TAC) measurement

Serum TAC was measured according to Benzie method (Benzie & Strain, 1996). Briefly, 100 µl of serum samples were diluted 10-fold with distilled water and then immediately used for TAC assay. 1.5 ml of FRAP reagent (including acetate buffer 300 mM, pH 3.6, TPTZ 10 mM and ferric chloride 20mM) was added to each tube and kept in water bath at 37 °C for 5 min. Then, 50 µl of diluted serum sample was added to each tube, and again kept in water bath at 37 °C for 10 min. After 10 min, the absorbance of blank, standards (125, 250, 500 and 1000 µM/l $FeSO_4$) and samples was assayed by spectrophotometer at 593 nm.

Statistical analysis

All data are reported as means ±SD. The mean of all parameters between different groups was compared using the one-way ANOVA: *post-hoc* Tukey test. Data were analyzed using SPSS, version 19. The value of $p<0.05$ was considered significant.

Results

The mean ± SD activity of AST, ALT, ALP enzymes and bilirubin contents in serum of all groups is shown in Figure 1. A significant difference was found in the mean values of all factors between groups ($p<0.0001$).

VCR administration to the experimental rats caused severe hepatic injury with considerable increase in the levels of AST, ALT and ALP enzymes. The mean of these enzymes in serum of rats received VCR alone, especially at higher concentration (0.5 and 0.75 mg/kg), was significantly higher than that in the other groups ($p<0.0001$). This effect was dose-dependent, and rats treated with the dose of 0.75 mg/kg VCR had the highest mean value of AST, ALT and ALP enzymes compared to the other groups (p=0.00000, Figures 1A–C).

Pre-treatment of rats with saffron extract reduced the level of these enzymes to normal. Mixed treatment with saffron reduced the mean of AST levels compared with the non-saffron groups (Figure 1A). The saffron treated group of F (V=0.75+S=0.5) showed the lowest mean value of AST (145.60±33.17 U/I). Although mixed treatment with saffron reduced the mean activity of ALT in C and I groups, we did not find a significant alteration in mean activity of this enzyme between A, B, D, E, G and H groups. Similarly, there were no significant difference in mean activity of ALP in A, B, D, E, F, G and I groups. However, combinational treatment with vincristine 0.5 mg/kg + saffron 1 mg/kg (group H) declined the activity of ALP compared to the other groups.

The mean value of bilirubin in different groups can be seen in Figure 1D. Rats that received 0.75 mg/kg VCR and those in control groups showed the significantly highest mean concentration of bilirubin (0.17±0.008 and 0.19±0.02 U/I, respectively) compared to the other groups. Vincristine treatment increased the mean level of bilirubin in a dose-dependent manner. Saffron treatment, especially at higher concentration (1 mg/kg), declined significantly the mean of bilirubin in G, H and I groups.

VCR treatment decreased the TAC content (Figure 1E) and declined the MDA value (Figure 1F) in a dose-dependent manner. Rats that received 0.75 mg/kg of VCR showed significantly ($p<0.001$) the lowest mean concentration of TAC (241.27±18.27 µmol/l) compared to the other groups. In contrast, rats treated with 0.75 mg/kg of VCR demonstrated the highest mean level of MDA (0.49±0.021 nmol/ml) compared to the other groups (Figure 1F). A trend was observed toward increased value of TAC and decreased level of MDA after treatments with saffron extract, especially at the concentration of 1 mg/kg.

Discussion

In this study, we considered the effect of 8-week treatment with saffron extract on VCR sulfate-induced hepatotoxicity in male rats. Our data revealed that administration of VCR to the experimental rats led to severe hepatic injury with significant enhancement in the levels of AST, ALT and ALP enzymes as well as bilirubin. It not only decreased the mean value of TAC, it also increased the mean of MDA levels in serum of VCR exposed rats. This agent induced hepatotoxicity in a dose-dependent manner. Interestingly, treatment with saffron extracts, especially at higher concentration (1 mg/kg), decreased hepatic injury with considerable increase in TAC value and significant reduction in the mean level of MDA. These data suggest that saffron extract can prevent VCR-induced hepatotoxicity through inhibition of oxidative stress and antioxidant depletion.

Our findings are in agreement with other research results. Several lines of studies indicated hepatoprotective effects of saffron. For example, Shati *et al.* considered the effect of saffron on aluminum ($AlCl_3$)-induced hepatotoxicity (Shati & Alamri, 2010). Their results showed that saffron treatment minimized the toxic effect of $AlCl_3$ with significant improvement in liver biochemical markers (cholesterol levels, triglycerides, GGT, ALT, AST and ALP) and lipid peroxidation. Another study investigated the protective effect of saffron extract (40 and 80 mg/kg for 8 weeks) on fatty liver tissue of high-fat diet induced obese rats (Mashmoul *et al.*, 2016). The results demonstrated that saffron extract dose-dependently alleviated the levels of liver enzymes and histopathological changes in these rats. The authors concluded that saffron extract has hepatoprotective effect against non-alcoholic fatty liver disease and high-fat diet-induced liver damage

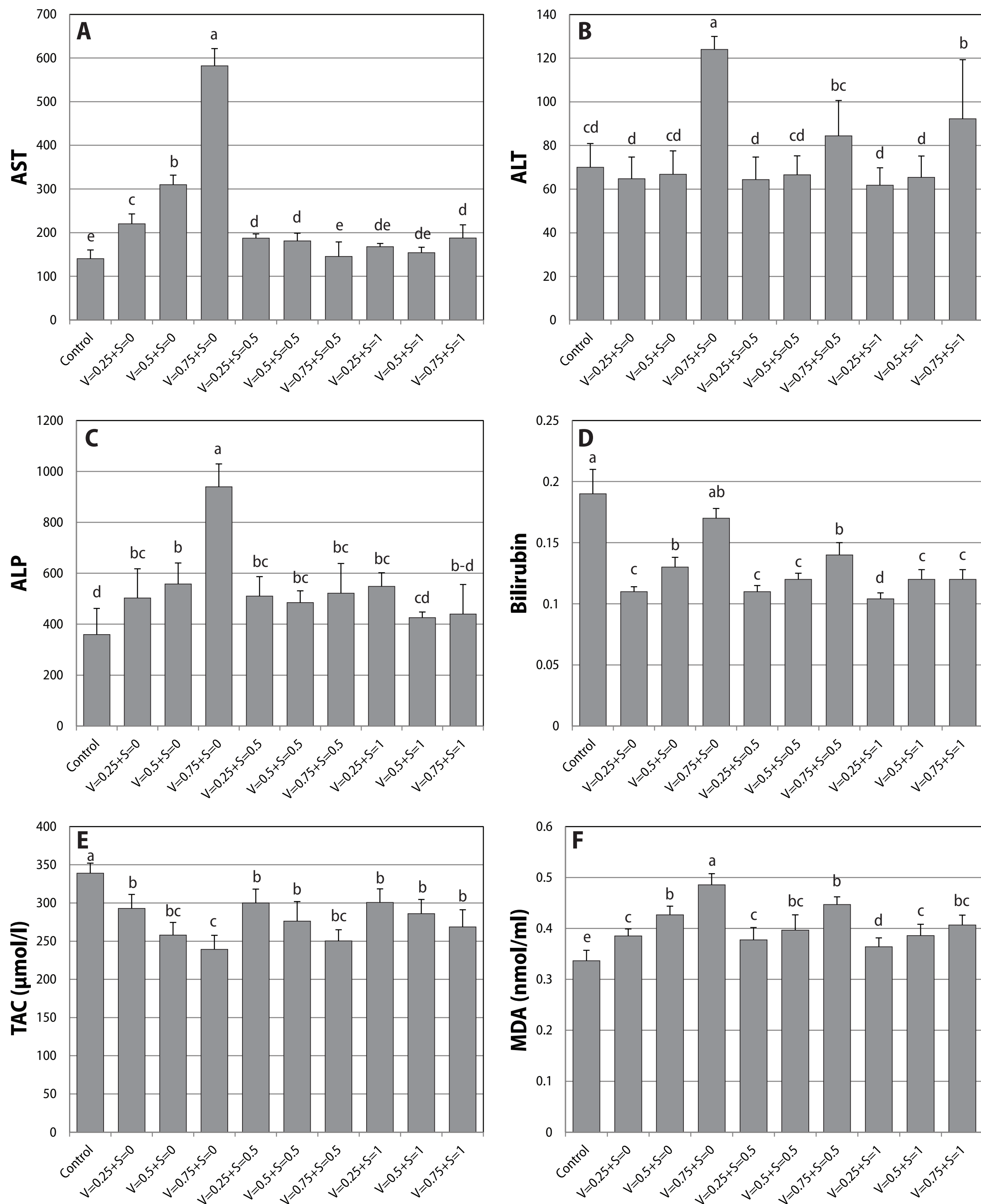

Figure 1. A: Comparison of AST activity between all groups (mean and SEM). There is a significant difference in mean AST activity between all groups (p<0.001). Saffron treatment decreased the vincristine-induced AST activity especially at higher concentration. V: vincristine; S: saffron. **B:** Comparison of the ALT activity between all groups (mean and SEM). There is a significant difference in mean of ALT activity between all groups (p<0.001). Saffron treatment decreased the vincristine-induced ALT activity. V: vincristine; S: saffron. **C:** Comparison of ALP activity between all groups (mean and SEM). There is a significant difference in mean ALP activity between all groups (p<0.001). Saffron treatment decreased the vincristine-induced ALP activity especially at higher concentrations. V: vincristine; S: saffron. **D:** Comparison of the bilirubin levels between all groups (mean and SEM). There is a significant difference in mean bilirubin concentration between all groups (p<0.001). Saffron treatment decreased the vincristine-induced bilirubin concentration. V: vincristine; S: saffron. **E:** Comparison of TAC mean levels between all groups (mean and SEM). There is a significant difference in mean TAC concentration between all groups (p<0.001). Saffron treatment increased the TAC values in VCR treated rats. V: vincristine; S: saffron; TAC: total antioxidant capacity. **F:** Comparison of MDA mean levels between all groups (mean and SEM). There is a significant difference in mean MDA concentration between all groups (p<0.001). Saffron treatment reduced the MDA values in VCR treated rats. V: vincristine; S: saffron; MDA: malondialdehyde.

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(Mashmoul *et al.*, 2016). The protective effect of saffron on liver cancer was also considered in previous studies (Harrington, 2011; Amin *et al.*, 2016). In a clinical trial study, Hosseini *et al.* evaluated the effects of saffron capsules (50 mg, twice daily) on the response to treatment in patients suffering from liver metastases (Hosseini *et al.*, 2015). They suggested that saffron might be useful in these patients. The potential protective effect of saffron ethanol extract on hepatic ischemia-reperfusion injury was also reported in a previous study (Pan *et al.*, 2013). In another research, the hepatoprotective effect of saffron extract was evaluated against acetaminophen toxicity in male Wistar rats (Omidi *et al.*, 2014). The administration of saffron with a dose of 20 mg/kg was found to be associated with lower levels of AST, ALT and bilirubin, with a significantly higher concentration of total protein and albumin (Omidi *et al.*, 2014). These data are consistent with our findings, as we showed that saffron extract can reduce the mean value of ALT, AST, ALP and bilirubin. In addition to the hepatoprotective effects of saffron, numerous studies have reported its protective effect on different tissues, such as kidney (Hosseinzadeh *et al.*, 2005), brain (Berger *et al.*, 2011), skin (Das *et al.*, 2004), against a wide range of chemicals. Thus the protective effect of saffron extract against doxorubicin-induced acute cardiotoxicity in rabbit was also reported (Chahine *et al.*, 2014).

Although saffron treatments can protect the liver against vincristine sulfate, the mechanism in which saffron extracts improve these abnormalities is not well clear. Recent evidence has indicated that overproduction of free radicals and oxidative stress is one of the significant mechanisms in which vincristine sulfate causes tissue injuries (Martins *et al.*, 2011). Inhibition of oxidative stress (OS) induced by vincristine sulfate and reactive oxygen species (ROS) seem to be one of the mechanisms by which saffron extract improves liver injury. Interestingly, Pan *et al.* proposed that saffron can reduce hepatic injury through regulating protein oxidation (Pan *et al.*, 2013). Many studies have also revealed that saffron extract has an antioxidative property and prohibits OS with considerable increase of different antioxidants (Das *et al.*, 2010; El-Beshbishy *et al.*, 2012; Samarghandian *et al.*, 2014; Ghaffari *et al.*, 2015). For example, Koul and Abraham demonstrated that saffron pretreatment reduced the level of lipid peroxidation with concomitant increase in antioxidants such as glutathione content (GSH) and the activity of glutathione S-transferase (GST), glutathione peroxidase (GPX) and catalase (CAT) (Koul & Abraham, 2017). In an *in vitro* study, saffron treatment significantly increased the survival of HCT116 cells, inhibited the ROS generation, regulated the activity of CAT and superoxide dismutase (SOD) and reduced the lipid peroxidation biomarkers. A reduction in mitochondrial membrane potential, DNA fragmentation and caspases activation were also observed (Ben Salem *et al.*, 2016). In another research, the protective effect of saffron was considered on genotoxins-induced oxidative stress in Swiss albino mice (Premkumar *et al.*, 2003). The findings revealed a significant reduction in the content of lipid peroxidation with a concomitant enhancement in the liver enzymatic (SOD, CAT, GST, GPx) and non-enzymatic antioxidants (GSH) in saffron pretreated animals (Premkumar *et al.*, 2003). Recent evidence has also shown that administration of saffron extracts significantly reduced oxidative myocardial damage through antioxidant and antiapoptotic mechanisms (Chahine *et al.*, 2014). Mahmoudzadeh *et al.*, evaluated the anti-inflammatory and protective effects of saffron extract (5, 10 , and 20 mg/kg) against ischemia/reperfusion-induced renal disturbances (Mahmoudzadeh *et al.*, 2017). They demonstrated that saffron extract can decrease the plasma creatinine concentration as well as lipid peroxidation biomarker level, TNF-α and intercellular adhesion molecule-1 expression and leukocyte infiltration in a dose-dependent manner (Mahmoudzadeh *et al.*, 2017). Therefore, these findings support the idea that saffron may protect liver against vincristine sulfate toxicity through inhibition of oxidative stress.

In summary, lipid peroxidation and total antioxidant depletion is one of the major mechanism by which VCR causes severe hepatic injury. Administration of saffron, especially at higher concentration, can reduce VCR-induced hepatotoxicity, possibly due to its antioxidative properties.

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ORIGINAL ARTICLE

The effect of venlafaxine on blood pressure and ECG in rats fed with high-fat-fructose diet

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ABSTRACT

Metabolic syndrome represents one of the major health, social and economic issues nowadays, and affects more than 25% people worldwide. Being a multifactorial health problem, metabolic syndrome clusters various features, such as obesity, dyslipidemia, hyperglycemia and hypertension. Each of these disturbances represents a risk factor for developing cardiovascular disease. Moreover, patients with metabolic syndrome are more likely to suffer from depression, thus treatment with antidepressants (*e.g.* venlafaxine) is often neccessary. However, many of the antidepressants themselves may contribute to worsening or even development of the metabolic syndrome, thus creating a "vicious circle". The aim of this work was to investigate on the animal model of metabolic syndrome, i.e. on hypertriacylglycerolemic rats fed high-fat-fructose diet (HFFD): 1) the effect of a change in diet from HFFD to a standard diet (SD) and the effect of venlafaxine treatment, 2) during HFFD, 3) as well as during a changed diet to SD. We focused on biometric parameters, blood pressure and selected ECG parameters. We observed the reversibility of the present metabolic and cardiovascular changes by switching the HFFD to SD in the last 3 weeks of the experiment. Switch to the standard diet led to decrease of body weight, even in the presence of venlafaxine. Administration of venlafaxine caused the decrease of heart weight/body weight index in rats fed with HFFD compared to the untreated group fed with HFFD for 8 weeks. Blood pressure, which was increased in the HFFD group showed a tendency to decrease to control values after switching to the standard diet . Administration of venlafaxine led to significant increase in all parameters of blood pressure when rats were fed with HFFD throughout the whole experiment. In untreated rats fed with HFFD for 8 weeks, we observed a shorter PQ interval and prolonged QRS complex as well as QTc interval compared to untreated rats with diet switched to SD. This effect was potentiated by venlafaxine administered not only during HFFD but even after switch to SD. Our results point to the fact that metabolic syndrome is clearly affecting the function of the cardiovascular system by modifying blood pressure and electrical activity of the heart. Moreover, administration of venlafaxine may lead to worsening of the observed changes, especially in the presence of high-fat-fructose diet.

KEY WORDS: metabolic syndrome; high-fat-fructose diet; antidepressants; venlafaxine; blood pressure; ECG

Introduction

Metabolic syndrome (MetS) belongs to the most widespread disorders around the world. Its prevalence is rising from year to year, currently affecting more than 25% of the human population and reaching the dimension of an epidemy. MetS is comprised of several independent features – central obesity, hypertension, dyslipidemia, and hyperglycemia, as the result of insulin resistance and impaired glucose tolerance (Gancheva *et al.*, 2017). The pathophysiology of MetS is most likely an interplay between various factors – genetic, metabolic, and environmental (Watanabe *et al.*, 2008; Knezl *et al.*, 2017). Patients suffering from MetS are at higher risk of developing cardiovascular diseases (CVD), such as coronary heart disease, left ventricular dysfunction, myocardial electrical disturbances or sudden cardiac death, as each individual sign and symptom of MetS represents a risk factor for CVD development (Yilmaz *et al.*, 2015; Kurl *et al.*, 2016; Knezl *et al.*, 2017).

A further global health and socio-economic problem with rising prevalence are psychiatric disorders, especially

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depression. Patients with serious mental illnesses have 2–3 times higher mortality rates and an approximately 30 years shorter life expectancy than the general population. More than 60% of psychiatric patients die mostly due to a non-psychiatric disorder, such as CVD (Alosaimi *et al.,* 2017), which could be, in turn, caused by MetS.

Epidemiological studies have shown an association between MetS and depression (Simon *et al.,* 2006), the prevalence of depression among patients with MetS being as high as 31% (Vancampfort *et al.,* 2014). Obesity is considered to increase the risk of depression, and depression is believed to be a predictive factor for the development of obesity (Gariepy *et al.,* 2010). Various mechanisms have been proposed to mediate the association between depression and MetS, including inflammation, leptin resistance, hypothalamic-pituitary-adrenal (HPA) axis dysregulation, sympatho-adrenal activation, sleep disturbances and poor diet that can result in a decrease in physical activity (Pan *et al.,* 2012). Treatment with antidepressants is therefore often necessary in these patients. However, there is evidence that antidepressants, including venlafaxine, are independently associated with the worsening or even development of MetS, by an increase in systolic and diastolic blood pressure, as well as in LDL cholesterol levels and body weight (McIntyre *et al.,* 2010). Moreover, venlafaxine itself may cause cardiac electrical disturbances, such as QT interval and QRS complex prolongation or lethal forms of dysrhythmias (Khalifa *et al.,* 1999; Bavle, 2015; Vicen *et al.,* 2016; Sasváriová *et al.,* 2018).

Since MetS is a disorder characterized by the dysbalance in energy storage and utilization (Gancheva *et al.,* 2017), one of the most important factors playing a role in the development of MetS is diet. The rising prevalence of MetS may be caused by the general change of lifestyle and eating habits in the modern society, with the trend of high caloric, carbohydrate and fat intake, together with avoidance of physical activity (Finucane *et al.,* 2011), often connected with increased risk of CVD and mortality (Flegal *et al.,* 2007). For experimental observations of the impact of MetS on the cardiovascular system, it is necessary to develop a suitable animal model that would be reliable and would combine as much risk factors of MetS as possible. In the present work, we used as an animal model of MetS hereditary hypertriacylglycerolemic (hHTG) rats fed high-fat-fructose diet (HFFD). Our aim was to investigate 1) the effect of a change in the diet from HFFD to a healthy/standard diet (SD), 2) further the effect of venlafaxine treatment during HFFD, or during achanged diet to SD. We focused on biometric parameters, blood pressure and selected ECG parameters. According to available scientific literature, we expect that we are the first who investigated the effect of venlafaxine treatment in relation to the intake of different dietary composition. We focused on the possibility to reverse the changes by switching HFFD to the standard diet, thus simulating the change of regimen in patients diagnosed with MetS.

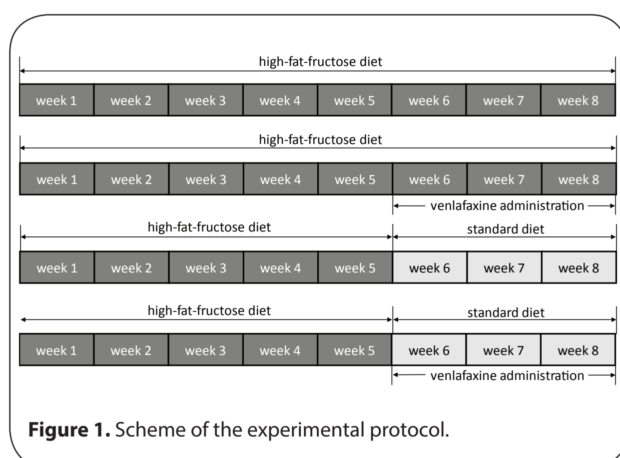

Figure 1. Scheme of the experimental protocol.

Materials and methods

In our 8-week lasting experiment male hereditary hypertriacylglycerolemic rats (hHTG; age: 12 weeks, n=38) were divided into: group HFFD 8 – rats fed 8 weeks with high-fat-fructose diet (HFFD); group HFFD 5+3 – rats fed 5 weeks with HFFD followed by 3 weeks of standard diet (SD); group HFFD 8+VE – rats fed 8 weeks with HFFD and within the last 3 weeks of experiment under the HFFD treated with venlafaxine; group HFFD 5+3+VE – rats fed 5 weeks HFFD, then 3 weeks SD and treated with venlafaxine during the switched diet (Figure 1). HFFD was enriched with 10% of fructose, 7.5% of lard and 1% of cholesterol. The experimental model of metabolic syndrome was designed and prepared at the Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine of the Slovak Academy of Sciences (Kaprinay *et al.,* 2016; 2017).

All of the procedures with animals were performed according to the Principles of Laboratory Animal Care executed by the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, CEM SAS and by the State Veterinary and Food Administration of Slovak Republic (No. 3635/14-221).

Blood pressure was measured by non-invasive tail-cuff method on the dorsolateral tail artery of a rat, with the MLT125/R cuff (Pulse transducer pressure cuff for rats), using NIBP Controller and Powerlab 8/30 (equipments used were from ADInstruments, Spechbach, Germany) using a rat-friendly procedure (Lipták *et al.* 2017a). Blood pressure, as well as standard ECG were monitored in conscious rats, using Seiva EKG Praktik Veterinary (Seiva s.r.o., Prague, Czech Republic) (Kraľova *et al.,* 2008). ECG recordings were analyzed offline (Seiva Database Veterinary, Prague, Czech Republic) and we focused on the duration of PQ interval, QTc interval and of the QRS complex. Corrected QT (QTc) interval was used for elimination of the variability in heart rate and calculated according to the following formula:

$$QTc=(QT)/(\sqrt{(RR/200)})$$

where QT – duration of QT interval, RR – the time (ms) between two consecutive R peaks, 200 – physiological

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heart rate in isolated spontaneously beating rat heart. The susceptibility of the heart to the development of sustained ventricular tachycardia and ventricular fibrillation was evaluated under conditions of isolated spontaneously beating heart perfused according to Langendorff.

After the isolation, hearts were perfused retrogradely through the aorta at constant pressure (80 mmHg). For the assessment of basal diastolic pressure, water-filled latex balloon (size 5; 0.1 ml volume) was inserted into the left ventricle and adjusted to diastolic pressure 8–10 mmHg. The threshold for the development of sustained fibrillation or tachycardia was detected by stimulation (Electrostimulator ST-3, Medicor, Budapest, Hungary) with the pair of electrodes attached to the right ventricle and ECG-like recording was monitored, by pair of eletrodes positioned on the wall of left ventricle. The current intensity was increasing by 5 mA/30 s from 10 mA to 50 mA until the induction of 2 minutes lasting ventricular tachycardia (VT) or fibrillation (VF). Consequently, the flow of the perfusion medium was stopped and time needed for the restoration of the sinus rhythm was recorded (Tribulova *et al.*, 2002; Knezl *et al.*, 2017; Liptak *et al.*, 2017b). The basic parameters of stimulation used were: 10 mA, train duration: 2 s, stimulation rate: 100 pps, delay: 0.1 ms, duration: 0.2 ms. The system BioLab F ver.1 (Institute of Measurement Science, Slovak Academy of Sciences, Bratislava, Slovakia) was used for data collection and offline analysis.

Solutions and chemicals

Composition of the Krebs-Henseleit solution used for isolated heart perfusion was (in mmol/1): NaCl, 118; KCl, 4.75; $CaCl_2 \times 2H_2O$, 2.5; $MgSO_4 \times 7H_2O$, 1.2; KH_2PO_4, 1.18; $NaHCO_3$, 20.0; glucose, 11.1; saturated by the mixture of 95% O_2 + 5% CO_2, pH=7.4, temperature 37 °C. The chemicals used were from Centralchem (Bratislava, Slovakia) and mikroCHEM (Pezinok, Slovakia). Venlafaxine administered p.o. in dose 10mg/kg/day was from Chemos (Regenstauf, Germany).

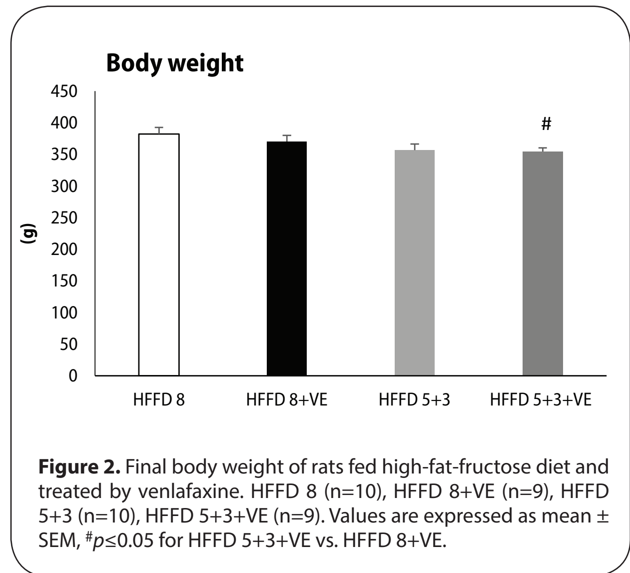

Figure 2. Final body weight of rats fed high-fat-fructose diet and treated by venlafaxine. HFFD 8 (n=10), HFFD 8+VE (n=9), HFFD 5+3 (n=10), HFFD 5+3+VE (n=9). Values are expressed as mean ± SEM, $^{\#}p \leq 0.05$ for HFFD 5+3+VE vs. HFFD 8+VE.

Data analysis

All of the obtained data were statistically analyzed in Excel (Microsoft Excel 2016) with standard statistical functions (mean ± SEM) and one-way analysis of variance (ANOVA) was used to evaluate the difference among all experimental groups (SPSS 16.0), using the LSD, Bonferroni and Tukey *post-hoc* tests. The level of $p \leq 0.05$ was considered as a statistically significant difference.

Results

hHTG rats fed with HFFD for 8 weeks had the highest body weight among all of the tested groups. Switch of the HFFD to standard diet caused a decrease in body weight, and moreover, administration of venlafaxine together with standard diet caused a significant lowering of body weight compared to HFFD 8 group (Figure 2). We observed the same trend also in heart weight, when switch to standard

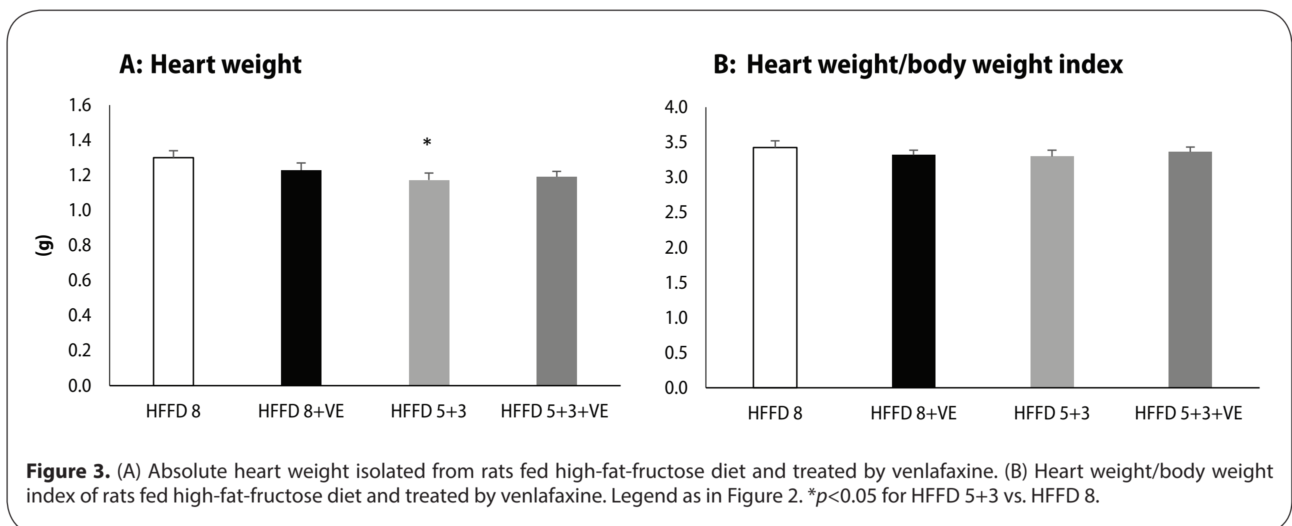

Figure 3. (A) Absolute heart weight isolated from rats fed high-fat-fructose diet and treated by venlafaxine. (B) Heart weight/body weight index of rats fed high-fat-fructose diet and treated by venlafaxine. Legend as in Figure 2. $^{*}p<0.05$ for HFFD 5+3 vs. HFFD 8.

Table 1. Effect of venlafaxine administered during HFFD or standard diet in tested rats on blood pressure parameters.

Group	sBP			dBP		
	baseline	**5th week**	**8th week**	**baseline**	**5th week**	**8th week**
HFFD 8	119.66±6.24	133.17±3.16 †	125.88±3.52	75.14±2.06	81.98±4.22	77.54±4.18
HFFD 8+VE	116.27±4.29	125.27±4.3 £	132.34±3.32 *	77.36±2.2	82.38±2.03 £	84.46±1.35 *
HFFD 5+3	124.3±10.36	143.6±3.29 ¤	129.46±4.19 €	73.18±6.09	86.24±1.91	78.48±8.05
HFFD 5+3+VE	122.22±5.96	139.73±6.44	126.18±5.47 #	79.94±2.79	88.7±3.02 §	80.92±2.72 #
	Pulse pressure			**Mean arterial pressure**		
HFFD 8	44.52±4.56	51.19±2.86	48.32±3	89.98±3.34	99.04±3.65 †	93.67±3.71
HFFD 8+VE	39.52±2.36	42.88±2.62	47.88±2.1	90.53±2.85	96.67±2.72 £	100.42±1.98 *
HFFD 5+3	51.12±4.73	57.36±2.95	50.98±3.17	90.22±7.45	105.36±2.03 ¤	95.47±2.94 €
HFFD 5+3+VE	42.28±3.26	51.04±3.57 §	45.26±2.98	94.04±3.83	105.71±4.13 §	96±3.59 #

Values of systolic (sBP), diastolic (dBP) blood pressure, pulse pressure and mean arterial pressure at the beginning of the experiment ("baseline"), after 5 weeks of HFFD (5th week) and after 3 weeks of venlafaxine administration (8th week). HFFD 8 (n=10), HFFD 8+VE (n=9), HFFD 5+3 (n=10), HFFD 5+3+VE (n=9). Values are expressed as mean ± SEM, †*p*≤0.05 for HFFD 8; 5th week vs. 8th week; £*p*≤0.05 for HFFD 8+VE, 5th week vs. 8th week; **p*≤0.05 for HFFD 8+VE, 8th week vs. baseline; €*p*≤0.05 for HFFD 5+3, 8th week vs. 5th week; ¤*p*≤0.05 for HFFD 5+3, 5th week vs. baseline; §*p*≤0.05 for HFFD 5+3+VE, 5th week vs. baseline; #*p*≤0.05 for HFFD 5+3+VE, 5th week vs. 8th week.

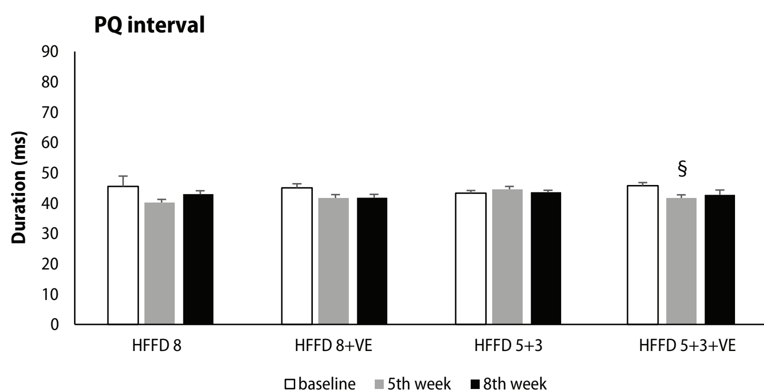

Figure 4. Changes in PQ interval duration, measured at the beginning of the experiment, after 5 weeks of HFFD and after venlafaxine administration during the next 3 weeks in rats fed HFFD or SD. Legend as in Figure 2. §p<0.05 for HFFD 5+3+VE, 5th week vs. baseline. Legend as in Figure 2.

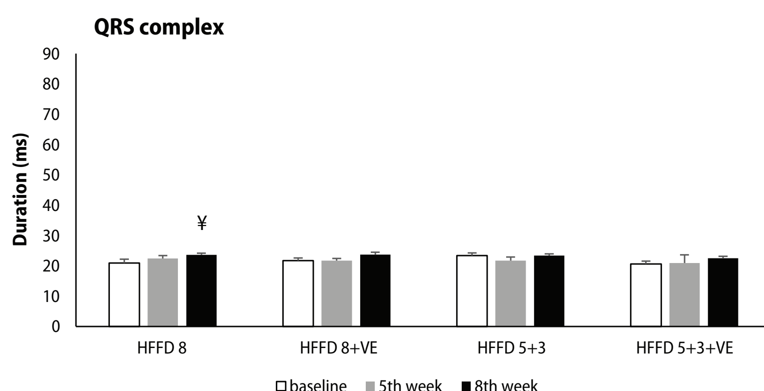

Figure 5. Changes in QRS complex duration, measured at the beginning of the experiment, after 5 weeks of HFFD and after venlafaxine administration during the next 3 weeks in rats fed with HFFD or SD. Legend as in Figure 2. ¥*p*<0.05 for HFFD 8 8th week vs. baseline.

diet led to a significant decrease in absolute heart weight (Figure 3A) and heart weight/body weight index (Figure 3B). Venlafaxine showed a tendency to lower heart weight and heart weight/body weight index in the presence of both HFFD and standard diet, however these changes were not statistically significant.

Analysis of blood pressure (Table 1) showed a significant increase in systolic, diastolic and mean arterial pressure values after 5 weeks of HFFD. Interestingly, values of blood pressure decreased after additional 3 weeks of HFFD close to the control values and also after the switch to standard diet. Administration of venlafaxine potentiated the effect of HFFD on systolic, diastolic, mean arterial and pulse pressure values and further increased these values significantly, yet only in the presence of HFFD.

ECG analysis was focused on selected parameters of cardiac electrical activity, including PQ interval, QRS complex and QTc interval duration. High-fat-fructose diet without treatment showed a tendency to shorten PQ interval during 8 weeks, while this change was significant in groups HFFD 8+VE and HFFD 5+3+VE after 5 weeks of HFFD. Administration of venlafaxine displayed only a minor effect on PQ interval duration (Figure 4). Significant prolongation of QRS complex was seen after 8 weeks of HFFD compared to baseline values.

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Venlafaxine non-significantly potentiated the effect of 5-week lasting HFFD on QRS complex duration, independently from the diet applied during its administration in the following 3 weeks (Figure 5). Significant prolongation of QTc interval was induced by five weeks lasting HFFD in almost all of the groups tested (except HFFD 5+3+VE group). This change was not modified by the switch to standard diet. Moreover, longer duration of HFFD either without or with venlafaxine administration led to further QTc interval prolongation (Figure 6).

Detection of the threshold for the development of sustained ventricular tachycardia or fibrillation showed that administration of venlafaxine to rats fed HFFD for 8 weeks led to higher susceptibility to life-threatening dysrhythmias (Figure 7A). However, the proarrhythmogenic potential of venlafaxine was not observed in the group where HFFD was changed for the last 3 weeks to standard diet. On the other hand, administration of venlafaxine shortened the time needed for the recovery of the sinus rhythm after VT/VF and consequent „stop-flow“ (discontinuation of the flow of perfusion medium), compared to HFFD 8 group (Figure 7B). Return to the standard diet led to shortening of the „stop-flow“ time, even in the presence of venlafaxine, in comparison with rats fed with the high-fat-fructose diet for 8 weeks.

Discussion

Hereditary hypertriacylglycerolemic rats fed with a high-fat diet were previously defined by our colleagues as a non-obese animal model of metabolic syndrome (MetS). The model was characterized by disturbance in glucose and lipid metabolism, hepatic steatosis, oxidative stress and endothelial damage (Kaprinay *et al.,* 2016). In this study, it has been proposed that addition of fructose to the diet could better simulate the western-type diet and lead to major changes not only in biochemical parameters but also in biometric parameters of the heart, blood pressure, and cardiac electrical activity. Moreover, forasmuch as patients with MetS are more likely to suffer from depression and take antidepressants, it is necessary to know if the antidepressants (e.g. venlafaxine) could work as protection or potentiate the changes caused by MetS, or if the changes could be attenuated by turning back to the standard diet.

Obesity is one of the key symptoms of MetS. Even though the experimental model used by us is considered as a non-obese, animals fed with HFFD for 8 weeks had the highest body weight and by switching to the standard

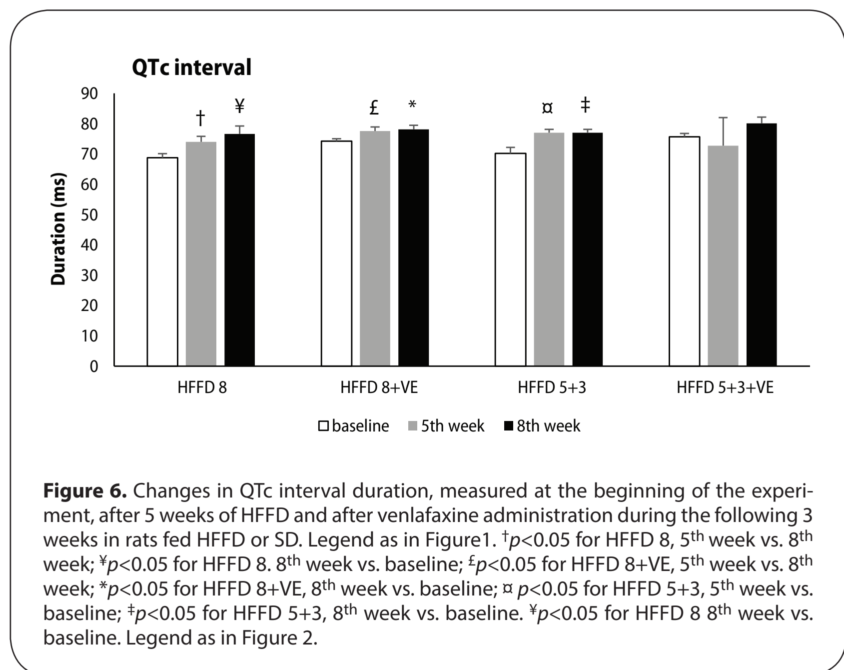

Figure 6. Changes in QTc interval duration, measured at the beginning of the experiment, after 5 weeks of HFFD and after venlafaxine administration during the following 3 weeks in rats fed HFFD or SD. Legend as in Figure1. †$p<0.05$ for HFFD 8, 5th week vs. 8th week; ¥$p<0.05$ for HFFD 8. 8th week vs. baseline; £$p<0.05$ for HFFD 8+VE, 5th week vs. 8th week; *$p<0.05$ for HFFD 8+VE, 8th week vs. baseline; ¤ $p<0.05$ for HFFD 5+3, 5th week vs. baseline; ‡$p<0.05$ for HFFD 5+3, 8th week vs. baseline. ¥$p<0.05$ for HFFD 8 8th week vs. baseline. Legend as in Figure 2.

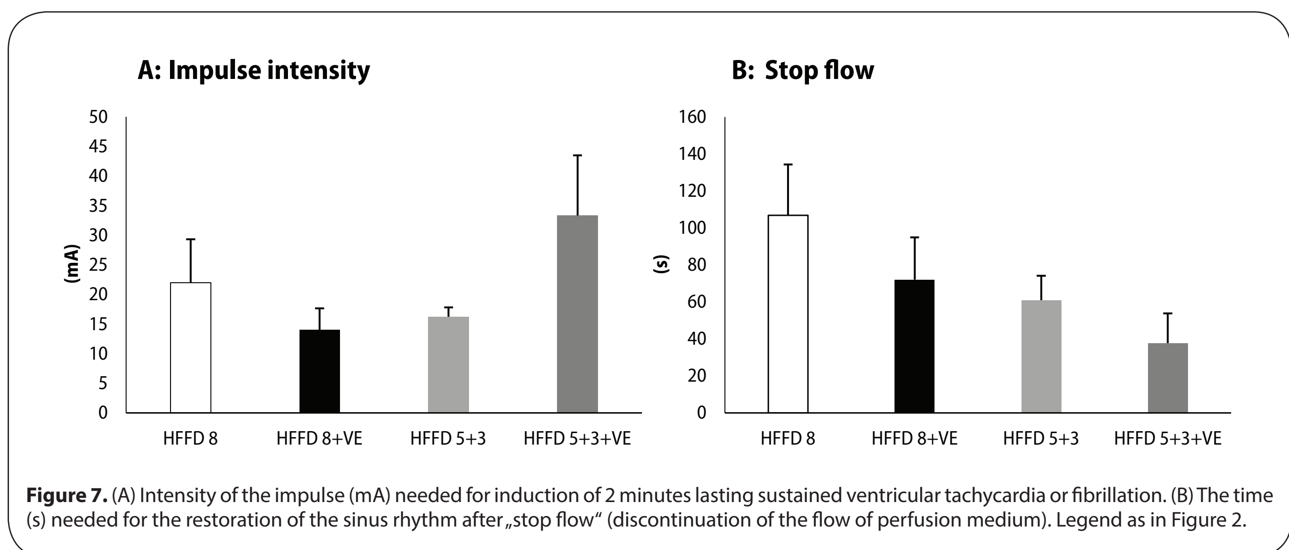

Figure 7. (A) Intensity of the impulse (mA) needed for induction of 2 minutes lasting sustained ventricular tachycardia or fibrillation. (B) The time (s) needed for the restoration of the sinus rhythm after „stop flow“ (discontinuation of the flow of perfusion medium). Legend as in Figure 2.

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diet, we observed a decrease in body weight. According to Alosaimi *et al.* (2017), administration of venlafaxine and mirtazapine lead to the development of central obesity, and together with high correlation with other independent symptoms of MetS, represents a potential cardiometabolic risk factor for depressive patients. In our study, administration of venlafaxine led to a decrease in body weight, slightly in rats fed with HFFD for 8 weeks (HFFD 8+VE group) and significantly in the group fed for the last 3 weeks with the standard diet (HFFD 5+3+VE).

Lower heart weight/body weight index was observed in group HFFD 5+3, and the administration of venlafaxine caused a decrease in heart weight independently from the length of HFFD. Other studies showed that in animal models of metabolic syndrome, with a high fructose diet, abnormalities in myocardial structure (Cheng *et al.*, 2014) and cardiac hypertrophy were present (Delbosc *et al.*, 2005; Jover *et al.*, 2017). Due to a high intake of fat and fructose, hepatic and intracardial lipid accumulation was previously observed, which could affect not only morphology but also the function of the organs. On the other hand, antidepressant paroxetine was shown to decrease the heart weight by reduction of myocyte size (Toscano *et al.*, 2008).

In the present work, analysis of blood pressure showed a significant increase of blood pressure already after 5 weeks of HFFD compared to baseline values, however, further 3 weeks of modified diet resulted in a decrease in blood pressure in the HFFD 8 group, what may be a sign of some kind of adaptation. The development of prehypertension or even hypertension is consistent with other studies using similar models. Wong *et al.* (2018) used a special diet consisting of fructose, sweetened condensed milk, Hubble Mendel and Wakeman salt mixture, ghee and powdered rat chow. After 8 weeks, they observed a significant increase in blood pressure and the development of MetS. Combination of high fat and high fructose diet caused an increase in systolic blood pressure after 28 weeks in male Sprague-Dawley rats (Gradel *et al.*, 2018). On the other hand, change of a lifestyle could reverse individual components of MetS, as well as hypertension. Hinderliter *et al.* (2014) examined overweight hypertensive or prehypertensive patients who underwent intervention on their lifestyle. Only the change of the diet caused a decrease in blood pressure by 11.2 mmHg after 16 weeks, and together with weight management intervention led to weight loss about 8.7 kg. This is in agreement with our results when blood pressure, which was increased in HFFD group, showed a tendency to decrease after switching to the standard diet. We observed a decrease in systolic, diastolic, mean arterial pressure and pulse pressure after the switch to the standard diet.

Venlafaxine has been reported to accelerate hypertension (Kivrak *et al.*, 2014) or even lead to the development of hypertension in previously healthy patients (Mbaya *et al.*, 2007), and in the worst cases it resulted in a hypertensive crisis (Khurana *et al.*, 2003). Hypertensive effect of venlafaxine is connected mainly with higher doses or overdose, hypertensive effect in lower doses is rare. This could be explained by the character of venlafaxine, which is an antidepressant with a dual mechanism of action – it inhibits the reuptake of both serotonin and noradrenaline. However, the affinity of venlafaxine to serotonin receptors is 30-fold higher than to noradrenaline receptors in therapeutic doses, and its adrenergic effect could be observed mainly in higher doses (Harvey *et al.*, 2000). Some studies evaluated the association between various antidepressants and MetS in depressive patients, but the majority of them are focused on selective serotonin reuptake inhibitors (citalopram, paroxetine) or tricyclic antidepressants (imipramine, clomipramine). This study showed that even though venlafaxine did not cause weight gain, it aggravated the effect of the modified diet and had a hypertensive effect only in the presence of HFFD (HFFD 8+VE group).

HFFD caused also changes in cardiac electrical activity. We observed PQ interval shortening after 5 weeks of HFFD in all groups (except HFFD 5+3 group), but there were no further changes either after another 3 weeks of the diet or after venlafaxine administration. Even though PQ interval prolongation is considered a risk factor for the development of atrial fibrillation (Schumacher *et al.*, 2018), short PQ interval may be observed due to conduction abnormalities in the atrioventricular node and/or His-Purkyne network and may predispose the individuals to dysrhythmias (Moro & Cosio, 1980). PQ interval was found to be shortened in obese patients with no cardiovascular disease (Bilora *et al.*, 1999).

Significant prolongation of the QRS complex was present only in the group exposed to HFFD during whole experiment (HFFD 8 rats). This is consistent with the study by Axelsen *et al.* (2015), who observed prolongation of the QRS complex in rats fed with high fructose diet (FFFR), used as a model of diet-induced pre-diabetes. QRS complex widening has been previously seen also in other studies focused on experimental diabetes, such as ZDF rats (Howarth *et al.*, 2008) or type 2 diabetic Goto-Kazaki rats (Yang *et al.*, 1990). Cardiac electrophysiological abnormalities found in FFFR and ZDF rats may be caused by intramyocardial lipid accumulation found in these two models. Ectopic lipid accumulation could lead to lipotoxicity and cardiac energy alterations together with morphological and functional mitochondrial disturbances, as well as arrhythmogenesis due to abnormal cardiac conduction (van de Weijer *et al.*, 2011; Elezaby *et al.*, 2015). Khalifa *et al.* (1999) have shown, that venlafaxine blocks the fast inward sodium current (I_{Na}) in guinea pig ventricular myocytes, which would explain the prolongation in QRS complex, however, in our experiment the duration of QRS complex was not further significantly increased in other experimental groups.

ECG analysis showed significant prolongation of QTc interval as soon as after 5 weeks of HFFD and the values were even higher after further 3 weeks in the group fed HFFD during the whole experiment (HFFD 8). Moreover, switch to the standard diet and also venlafaxine administration did not reverse the effect of HFFD and the QTc interval duration was still increased. This points to the

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fact, that administration of HFFD to hHTG rats leads to irreversible prolongation of ventricular repolarization, representing a risk factor for the development of ventricular dysrhythmias and sudden cardiac death. Patients with MetS are more likely to have a prolonged QTc interval, which increases the risk of cardiovascular mortality (Li *et al.*, 2009). Additionally, venlafaxine may increase the duration of QTc interval in therapeutic doses, which could occur due to inhibition of the outward delayed rectifier potassium current (I_{Kr}) in ventricular myocytes (Lestas *et al.*, 2005; Bavle, 2015).

A long QTc interval represents a risk factor for the development of dysrhythmias, especially life-threatening ventricular tachycardia (VT) or fibrillation (VF). By electrical stimulation, we evaluated the threshold for the development of sustained VT/VF (i.e. lasting more than 2 minutes). The lower the intensity of the impulse needed, the more susceptible is the heart for VT/VF occurrence. Our results showed that venlafaxine lowered the fibrillation threshold, but also shortened the time needed for the restoration of the sinus rhythm in the presence of high-fat fructose diet, in comparison to the HFFD 8 group. On the other hand, after the switch to the standard diet, the threshold for sustained VT/VF was markedly higher and the restoration of the sinus rhythm was more rapid. Vicen *et al.* (2016) showed that venlafaxine administered in a single dose to Wistar rats caused a significantly higher incidence of life-threatening dysrhythmias, compared to the control group. In clinical practice, venlafaxine showed a potential to induce tachycardia in a dose-dependent manner (Abozguia *et al.*, 2006). Moreover, administration of high-fat diet even without fructose significantly decreased the fibrillation threshold in hHTG rats (Knezl *et al.*, 2017).

Conclusions

From our present results, we could conclude that the administration of high-fat-fructose diet to hHTG rats led to the development of MetS symptoms and altered the cardiac electrical function. Switch to the standard diet, as a non-pharmacological way of alleviating the manifestations of metabolic syndrome, was partly able to reverse the cardiovascular alterations of animal MetS, however, some of them, such as QTc interval prolongation, persisted. Venlafaxine mostly potentiated changes caused by the modified diet, especially in the presence of 8-week HFFD and seems to be potent in the progression of MetS. Improvement of a life-style, at least by modification of diet, may decrease the cardiometabolic risk of venlafaxine, nevertheless clinicians should be aware of the potential danger.

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Sasváriova M. contributed by measuring of ECG and blood pressure, administration of venlafaxine and by obtaining biometric data of rats. She is corresponding author and the main author of the paper. Micháliková D. contributed by measuring blood pressure, administration of venlafaxine. Tyukos Kaprinay B. contributed by measuring blood pressure and administration of venlafaxine. Salvaras L. contributed by obtaining the biometric data. Knezl V. supervised the isolated and perfused heart experiments by Langendorff. Gáspárová Z. contributed as Head of the project related with metabolic syndrome. She revised the manuscript. Stankovičová T. contributed by the idea to test venlafaxine effect in different diet composition. She supervised the blood pressure and ECG analysis (MS) and biometric data collection (LS). She revised the manuscript.

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