Prophylactic Resveratrol Ameliorates Thioacetamide Hepatotoxicity in A Dose-Dependent Fashion Through the Regulation of Gene Expression Levels of MiR-155 and MiR-21

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ABSTRACT

Objectives: Evaluation of the prophylactic effect of resveratrol (RSV) on thioacetamide (TAA)-induced hepatotoxicity. **Experimental Protocol:** 50 albino rats have divided into a control group that received no medications, TAA group that received TAA intraperitoneal injection (200 mg/kg trice weekly for 4 weeks) and three groups that received 10, 20, and 40 mg/kg RSV for 6-wk and on the 3rd week TAA was injected as for TAA animals. Blood samples were collected for spectrophotometric estimation of serum activity levels of aspartate (AST) and alanine transaminase (ALT), total bilirubin (TB) and albumin, ELISA estimation of serum levels of tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6), IL-10, Malondialdehyde (MDA) and activity level of superoxide dismutase (SOD), and for estimation of gene expression levels of miR-21 and miR-155 using the quantitative reverse transcriptase polymerase chain (qRT-PCR). **Results:** Exposure to TAA disturbed liver functions, increased levels of inflammatory cytokines, initiates tissue lipid peroxidation and induced upregulation of expression levels of miR-21 and miR-155. RSV prophylaxis improved the TAA-induced effects and prevented the overwhelming TAA-upregulation of microRNAs in a dose-dependent manner, but this effect is more manifest in miR-155. Regression analysis suggested that an RSV dose of 28.2 mg/kg (95% CI: 23.72-32.68) as a prophylactic dose can reduce the hazard of TAA hepatotoxicity to 30%. **Conclusion:** TAA-hepatotoxicity might be mediated through the upregulation of gene expression levels of miR-155 and miR- 21 initiating a cascade that ended in liver fibrosis. Resveratrol prophylaxis may protect or at least ameliorate the TAA-hepatotoxicity mostly through improving the deregulated expression levels of microRNAs.

INTRODUCTION

Liver fibrosis (LF) is the abnormal replacement of hepatic parenchymal cells by fibrous tissue and can progress to cirrhosis and cause liver failure or even cancer (Zhu *et al.,*2020). Hepatic stellate cells activation (HSCs) plays an important role in the pathogenesis of LF (Zaafan and Abdelhamid.,2022) and activation of communication between HSCs and dendritic cells (DCs), the regulators of the pathologic inflammatory milieu in LF results in the expression or secretion of pro-fibrotic proteins (Xiang *et al.,*2022).

MicroRNAs (MiR-) are small, non-coding RNAs, which regulate the expression of the target mRNA, and alteration of MiR expressions is associated with pathological conditions of the affected organs (Ariyachet *et al.,*2022). Specific microRNAs was suggested to affect the HSCs functions; MiR-378 and MiR-17 play a role in the pathogenesis of LF through activation of HSCs via altering TGF-β/smads and Wnt/β-catenin pathways (Zaafan and Abdelhamid.,2022). Also, neutrophil/myeloid-specific miR-223 has a key regulator role in the development of LF through the activation of HSCs (Ariyachet *et al.,*2022). However, miR-122 suppresses liver cirrhosis through its inhibitory effect on HSCs by targeting the EphB2 expression (Ma *et al.,*2022).

Resveratrol (RSV) is a polyphenol natural plant extract and can be found in trans- or cis-configurations (Zheng *et al.,*2022). RSV; the trans-3, 4, 5-trihydroxystilebene form can be isolated and purified from multiple natural sources especially grapes (Shaito *et al.,*2020). RSV has strong antioxidant and anti-inflammatory properties (Xu et al.,2022) and is an activator of AMP-activated protein kinase with many reported health benefits (Zhao *et al.,*2022).

Currently, treatment of LF depends on the management of the primary disease or trying to lower inflammation, prevent oxidative stress, and increase collagen breakdown (Damiris *et al.,*2020). Unfortunately, no effective antifibrotic therapy is devoid of adverse effects, thus prophylaxis, if possible, maybe the possible route, especially with chemical hepatic toxicity, and thus the present study has been developed to evaluate the prophylactic effect of RES on thioacetamide-induced hepatic toxicity as a model of occupational hepatotoxicity.

MATERIALS AND METHODS

Design:

Prospective comparative animal-model study.

Setting:

Departments of Forensic & Clinical Toxicology and Medical Biochemistry, Faculty of Medicine in conjunction with Zoology Department, Faculty of Science, Benha University.

Ethical considerations:

The study protocol was approved by the Local Ethical Committee, Faculty of Medicine, Benha University by RC: 25-11-2022. The study was conducted according to the Guidelines for the Care and Use of Laboratory Animals (The National Academy of Sciences: Guide for the Care and Use of Laboratory Animals, 1996).

Experimental Protocol:

Fifty normal healthy growing adult male albino rats, weighing 200-250 gm and aged 8-10 weeks, were purchased from The Animal Farm, Faculty of Veterinary Medicine, Zagazig University and were grown in the animal house, Faculty of Veterinary Medicine, Benha University. Animals were kept under standard living conditions at a temperature of 20ºC, humidity rate of 60%, and 12-hs day/night cycle. Animals were maintained on a

standard diet and free water supply till the start of the study regimens. Animals were divided into five separate cages (n=10):

1. Control group (Group C) received no medications.
2. TAA group had intraperitoneal injection of TAA (Sigma–Aldrich Co., St. Louis, MO, USA; 25 g vial) in a dose of 200 mg/kg three times weekly for 4- wks (Algandaby *et al.,*2017).
3. Study groups: 30 rats were divided equally into three separate cages and were given RSV solution; 10, 20 and 40 mg/kg, prepared in a mixture with sodium carboxymethyl cellulose and ionized water (1:200 by vol.) (Huang *et al.,*2013; Zhang *et al.,*2019). RSV therapy was started and on the 3rd week of RSV administration, TAA (200 mg/kg) was injected 3 times weekly for 4-wks.

Blood Sampling:

The rat was restrained for a very short time, the shaved hind limb was immobilized in the extended position, a puncture of the saphenous vein was made by a fine needle to collect the blood sample and gentle finger pressure above the puncture site was applied to control blood flow and after complete stoppage of blood, the animal was returned to the cage (Kumar *et al.,*2017). Blood was collected into two tubes:

1. The 1st part of the blood sample was allowed to clot and after centrifugation at 3000 rpm, the supernatant was collected in a dry clean Eppendorf tube and kept frozen at -20°C till being assayed.
2. The 2nd part was put in a dry clean tube with ethylene diamine tetra-acetic acid, and kept at -20°C till being assayed using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for estimation the gene expression levels of miR-21 and miR-155.

Investigations:

1. Serum levels of activity of aspartate transaminase (AST) and alanine transaminase (ALT) (Huang *et al.,*2006), total bilirubin (TB) (Watson.,1961), and albumin (Rees *et al.,*2012) levels using a spectrophotometric automated chemical analyzer (The Biotechnica BT3000 Plus, Italy).
2. Serum levels of studied biomarkers were measured using quantitative sandwich enzyme immunoassay technique by ELISA kits (Abcam Inc., San Francisco, USA) as described in the enclosed pamphlet and were read using Dynatech MR 7000 reader). The studied biomarkers included human tumor necrosis factor-α (TNF-α; Cat. No. ab181421) (Coughlan *et al.,* 2001), Interleukin-6 (IL-6, catalog no. ab46042) (Gaines-Das and Poole., 1993), IL-10 (Cat. No. ab46034) (Fei *et al.,*2016), Malondialdehyde (MDA, Cat. No. ab233471) (Liu *et al.,* 2014) and activity level of superoxide dismutase (SOD, Cat. No. ab65354), (Mu *et al.,* 2015).
3. qRT-PCR estimation of gene expression levels of miR-21 and miR-155 was performed as described previously (Yang *et al.,* 2021) as follows: Total RNA including microRNA was extracted from samples using the miRNeasy Mini Kit (QIAGEN, Germany), and the relative quantitation of miR-21 and miR-155 by two-step Real-time PCR Using Maxima SYBR Green (QuantiTect SYBR Green PCR Kit: QIAGEN, Catalog no. 218073, Str. 1 - 40724). The cDNA was synthesized using miScript II RT Kit (QIAGEN, Germany). The mixture of RNA starting amounts, buffers for reverse transcription reactions for quantization of miRNAs, and the recommended RNA input were incubated for 60 min at 37°C, for 5 min at 95°C to inactivate the miScript Reverse Transcriptase Mix then placed on ice and later diluted by 40 µl RNase-free water to the 10 µl reverse transcription reaction and mixed gently then briefly centrifuged and continued with real-time PCR using QuantiTect SYBR Green PCR Kit3 according to manufacturer's instructions (SYBR). The total volume of PCR reaction mix was 25µl/tube. The real-time cycler was programmed using ABI 7900HT Fast Real-Time PCR System, (Applied

Biosystem, Singapore). The amplification level miR-155 was programmed with a denaturation was performed at 95°C for 30 sec and then 40 cycles were conducted at 95°C for 10 sec and 60°C for 30 sec. For miR-21, the reaction was activated initially at 95ºC for 15-s, denaturation at 94ºC for 15-sec, annealing at 55ºC, for 30-sec and extension at 72ºC for 30-sec, and the process is repeated for 40 cycles. The expression levels of microRNAs were determined after correction with the GADPH expression level. Controls were chosen as the reference samples, and fold changes in the levels of microRNAs were determined by the $2^{-\Delta\Delta CT}$ (cycle threshold) method and expressed as fold change using the Step One software (Applied Biosystems, USA).

The sequences of the used primers for the detection of the expression levels of the studied microRNAs

Items	Primers Sequences
MiR-155-F	GGGGGTTAATGCTAATTGTGAT
MiR-155-R	AGTGCGTGTCGTGG
MiR-21-F	CGCTAGCTTATCAGACTGATG
MiR-21-R	GAGGTATTCGCACCAGAGGA
GAPDH-F	CCACCCATGGCAAATTCCATGGCA
GAPDH-R	TCTAGACGGCAGGTCAGGTCCAC

Statistical Analysis:

Statistical analyses by IBM® SPSS® Statistics (Version 22, 2015; Armonk, USA) were performed using by application of the One-way ANOVA and Chi-square test (X^2 test). Pearson's correlation analysis was applied to evaluate correlations between the used RSV dose and the plasma levels of gene expression of the studied microRNAs. The receiver characteristic curve was used to determine the best dose of RSV to provide the maximal downregulation of plasma levels of gene expression of the studied microRNAs as judged by the significance of the difference between the area under the curve (AUC) for the studied variable and that under the reference line (AUC=0.5). Kaplan-Meier regression analysis was applied to suggest the dose of RSV that probably induces the maximum reduction of the hazard of TAA hepatotoxicity. The significance of the results was evaluated at the cutoff point for *P* value of <0.05.

RESULTS

Estimated serum activity levels of AST and ALT, and serum levels of TB were significantly higher, while serum albumin levels were significantly lower in TAA samples in comparison to samples of animals of other groups. The estimated liver function tests showed non-significant differences between samples of R40 and control animals, while the differences were significant between samples of R10 and R20 animals compared to control samples with non-significant differences between samples of R10 and R20 animals. Serum activity levels of AST and ALT in the R40 samples were significantly lower than in the R10 and R20 samples. Only serum activity levels of AST were significantly higher in R20 samples than in R40 samples, while other estimated variables showed non-significant differences (Table 1).

Table 1: Liver function tests estimated in samples of the study animals.

Parameter		Control	TAA	R10	R20	R40
AST (IU/L)		25±6.4	69.5±10.9	49.5±9.2	40.5±10.8	27.5±5.3
	Vs. control		<0.001	<0.001	<0.001	0.956
	Vs. TAA			<0.001	<0.001	<0.001
	Vs. R10				0.105	<0.001
	Vs. R20					0.0057
ALT (IU/L)		32.5±7.4	63±8.3	45±9.6	38±7.4	33±4.3
	Vs. control		<0.001	0.0017	0.404	0.908
	Vs. TAA			<0.001	<0.001	<0.001
	Vs. R10				0.178	0.0027
	Vs. R20					0.502
Albumin (g/ml)		4.6±0.3	3.23±0.7	4.01±0.4	4.19±0.4	4.45±0.4
	Vs. control		<0.001	0.014	0.164	0.917
	Vs. TAA			0.0005	0.00001	<0.001
	Vs. R10				0.852	0.115
	Vs. R20					0.598
Total bilirubin (mg/dl)		0.91±0.02	2.26±0.7	1.55±0.3	1.18±0.2	0.98±0.08
	Vs. control		<0.001	0.0002	0.309	0.987
	Vs. TAA			0.00004	<0.001	<0.001
	Vs. R10				0.074	0.0013
	Vs. R20					0.607

Estimated levels of TNF-α and IL-6 were increased significantly, while IL-10 levels were decreased significantly in TAA than control samples. In comparison to control samples, serum cytokines' levels showed non-significant differences in samples of R40 group. Further, serum TNF-α and IL-6 levels estimated in R20 and R40 samples showed significant decrease than in R10 samples, with a non-significant difference between R20 and R40 samples. On contrary, estimated levels of IL-10 showed insignificant differences between R groups despite being in favor of the higher RSV doses (Table 2).

Exposure to TAA deleteriously affected the oxidative status as manifested by significantly higher serum MDA levels with the significantly suppressed activity of serum SOD in TAA samples compared to control and RSV samples. RSV prophylaxis significantly ameliorated the effects of TAA on the redox milieu as shown by the comparable levels of estimated parameters in samples of control and RSV groups and in samples of RSV groups, despite of the marvelous effects of higher RSV doses (Table 3).

Plasma microRNAs expression levels were significantly increased in TAA, R10, and R20 samples than in control samples, while in R40 samples there was significant increase of miR-21 levels, but non-significant increase of miR-155 levels than in control samples. Moreover, plasma levels of both microRNAs were significantly lower in R40 samples than in R10 and R20 samples with an insignificant difference in favor of R20 to R10 samples (Table 4).

Table 2: Estimated cytokines' levels in the sera of animals of different groups

Parameter		Control	TAA	R10	R20	R40
TNF-α (ng/ml)		1.06±0.33	6.43±1.3	3.14±1.1	1.7±0.6	1.15±0.5
	Vs. control		<0.001	<0.001	0.349	**0.937**
	Vs. TAA			<0.001	<0.001	**<0.001**
	Vs. R10				0.0009	**<0.001**
	Vs. R20					**0.503**
IL-6 (ng/ml)		13.8±2.7	77.7±24.3	41.5±12.5	26.2±6.2	17±3.1
	Vs. control		<0.001	<0.001	0.131	**0.971**
	Vs. TAA			<0.001	<0.001	**<0.001**
	Vs. R10				0.035	**0.0002**
	Vs. R20					**0.396**
IL-10 (ng/ml)		18±3.1	13.1±2.9	13.8±2.2	14.5±2.3	16.2±2.8
	Vs. control		0.0004	0.003	0.019	**0.479**
	Vs. TAA			0.968	0.707	**0.049**
	Vs. R10				0.968	**0.200**
	Vs. R20					**0.536**

Table 3: Serum levels of MDA and SOD activity in samples of studied

Parameter		Control	TAA	R10	R20	R40
MDA (µmol/L)		7.08±0.84	28±13	14.2±4.2	11.95±1.7	9.55±1.3
	Vs. control		<0.001	0.049	0.317	**0.865**
	Vs. TAA			<0.001	<0.001	**<0.001**
	Vs. R10				0.889	**0.363**
	Vs. R20					**0.877**
SOD (U/ml)		6.15±0.5	3.15±0.6	4.1±0.4	4.8±0.5	5.8±0.8
	Vs. control		<0.001	<0.001	<0.001	**0.591**
	Vs. TAA			0.0019	<0.001	**<0.001**
	Vs. R10				0.036	**<0.001**
	Vs. R20					**0.001**

Table 4: The expression levels of miR-21 and miR-155 in samples of studied animals

Parameter		Control	TAA	R10	R20	R40
miR-21		0.86±0.17	3.41±0.56	3.04±0.66	2.65±0.44	1.82±0.64
	Vs. control		<0.001	<0.001	<0.001	0.0004
	Vs. TAA			0.427	0.007	<0.001
	Vs. R10				0.373	<0.001
	Vs. R20					0.0026
miR-155		1.03±0.2	4.155±0.8	2.48±0.7	2.26±0.65	1.365±0.3
	Vs. control		<0.001	<0.001	<0.001	<0.635
	Vs. TAA			0.0019	<0.001	<0.001
	Vs. R10				0.890	0.0002
	Vs. R20					0.004

Correlation analysis showed a negative relation between RSV dose and the plasma levels of gene expression of miR-21 and miR-155 (r=-0.727 & -0.784, p<0.001, respectively) as shown in Figures 1&2. ROC curve analysis to define the dose of RSV as a prophylactic dose against the overregulating effect of TAA on gene expression of microRNAs defined 40 mg/kg as a prophylactic dose with a significant area under the curve (AUC) in comparison

to the area under the reference line, while AUCs for RSV 10 and 20 mg/kg for both microRNAs showed a non-significant difference in relation the area under the reference line as shown in Table 5 and Figures 3-5. The downregulating effect of RSV in a dose of 40 mg/kg was more manifested on the expression of miR-155 than on miR-21 with a significant (p=0.042) difference between AUCs for both microRNAs according to the paired-sample analysis of the difference between AUCs.

Table 5: Receiver operating characteristic curve analysis of varied doses of RSV concerning their effect on plasma gene expression levels of studied microRNAs

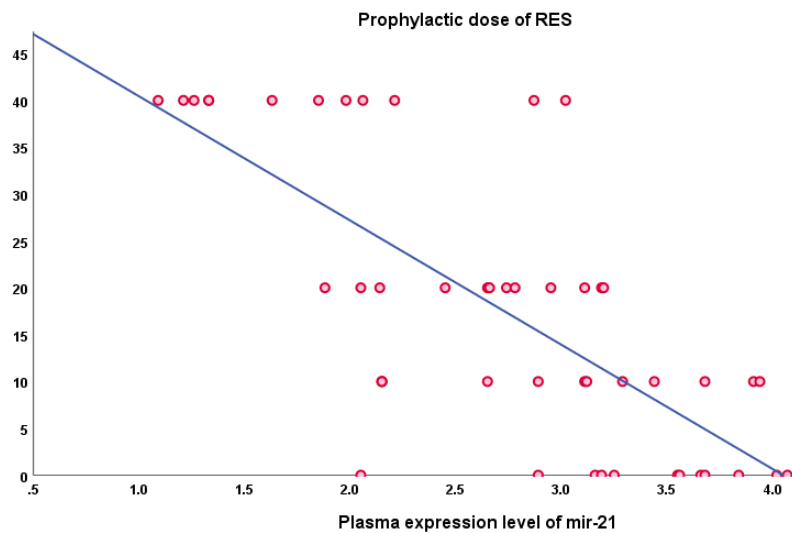| Dose	Variate	Area under curve	Standard error	P value	95% Confidence interval
R10	**miR-21**	0.350	0..084	0.122	0.184-0.515
	miR-155	0.486	0.087	0.886	0.316-0.656
R20	**miR-21**	0.579	0.080	0.418	0.423-0.735
	miR-155	0.545	0.082	0.634	0.386-0.706
R40	**miR-21**	0.907	0.049	<0.001	0.812-1.000
	miR-155	0.923	0.036	<0.001	0.863-1.000

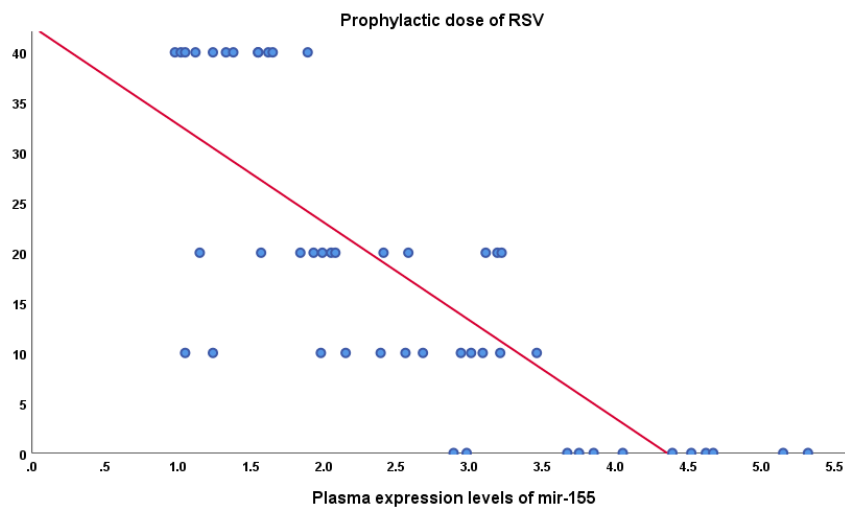**Fig. 1:** The relation between plasma levels of miR-21 and the used prophylactic RSV dose.

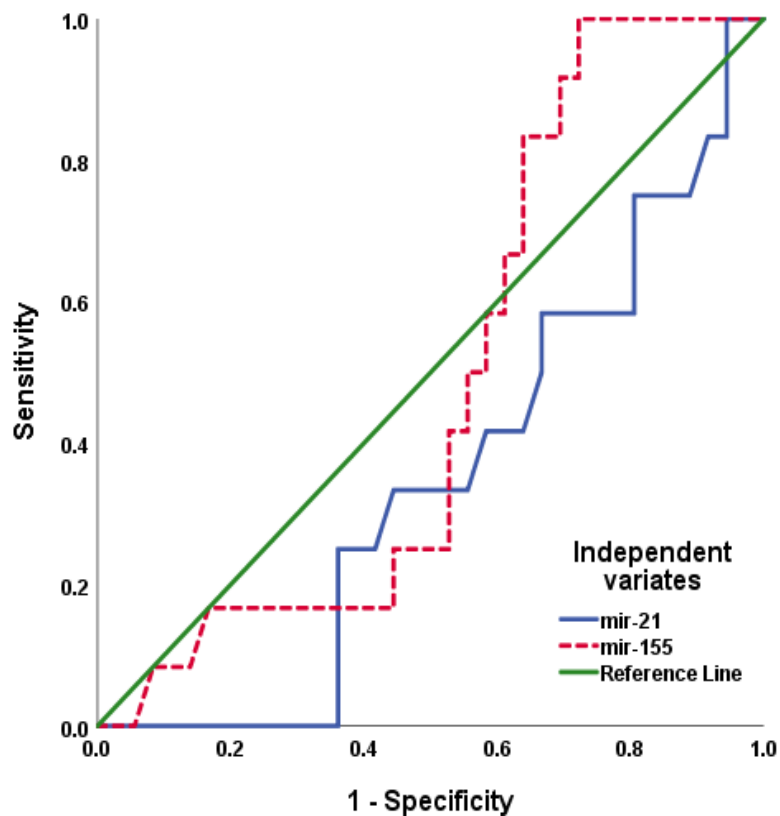**Fig. 2:** The relation between estimated levels of miR-155 and the used prophylactic RSV dose.

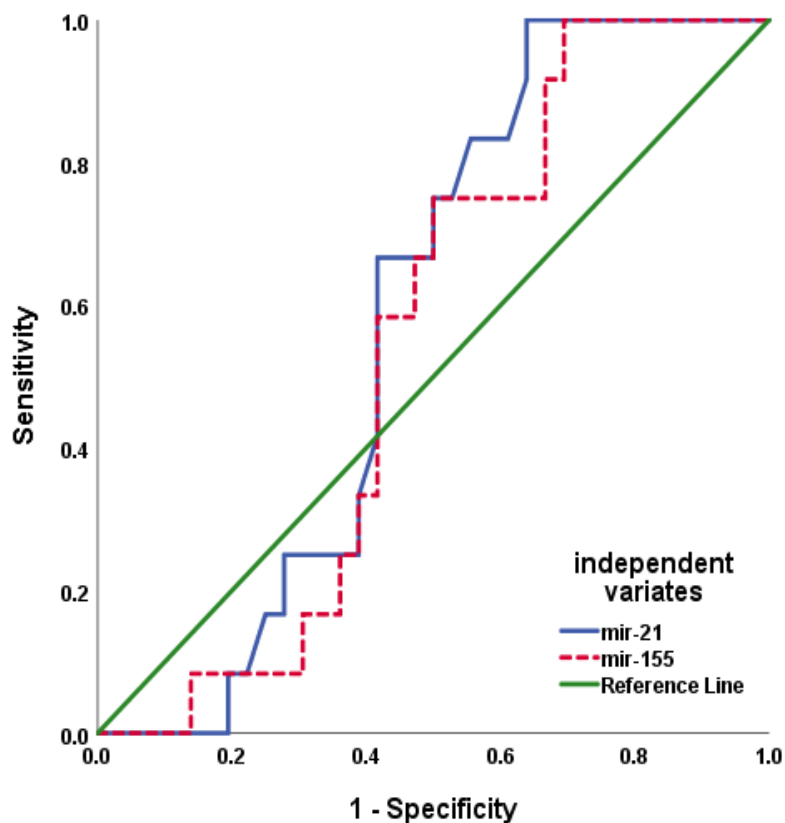**Fig. 3:** The ROC curve analysis of the effect of RSV at dose of 10 mg/kg on the plasma levels of studied microRNAs

Fig. 4: The ROC curve analysis of the effect of RSV at dose of 20 mg/kg on the plasma microRNAs
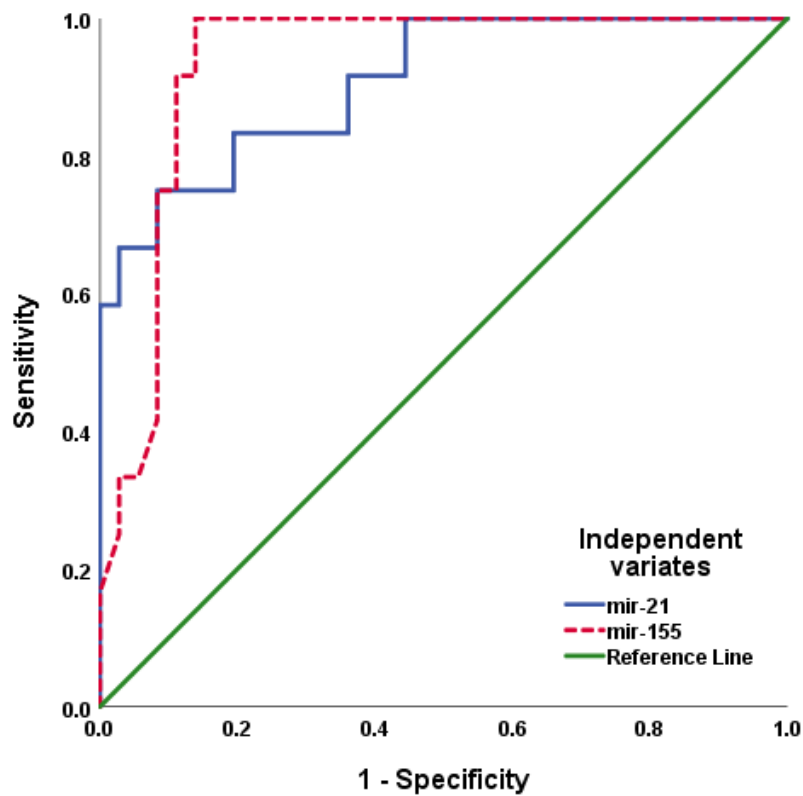
Fig. 5: The ROC curve analysis of the effect of RSV at dose of 40 mg/kg on the plasma levels of the studied microRNAs

Kaplan-Meier regression analysis showed that animals that were devoid of prophylaxis had a 100% hazard risk of TAA hepatotoxicity, while administration of 10 mg/kg RSV reduced this risk to 18%, administration of 20 mg/kg reduced the risk by 48% and the dose of 40 mg/kg reduced the risk down to 5%. Further, the analysis suggested that the appropriate prophylactic dose of RSV against TAA hepatotoxicity is 28.2($\pm$2.26; 95% CI: 23.72-32.68), which can reduce the hazard of TAA hepatotoxicity to 30% (Fig. 6).
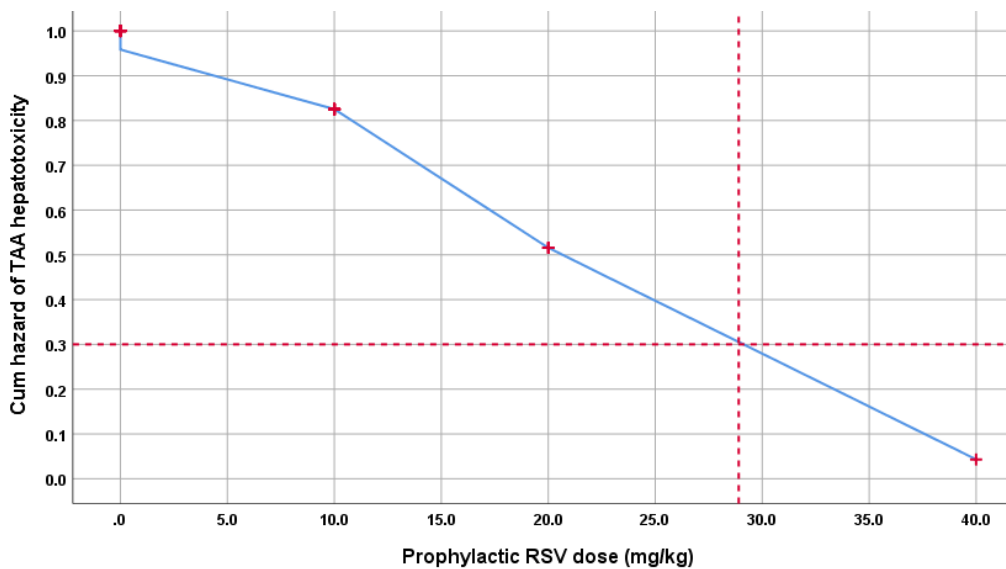

Fig. 6: The Kaplan-Meier cumulative hazard curve for the optimal dose of RSV that minimize the TAA hepatotoxic effect of

DISCUSSION

Chronic exposure to thioacetamide (TAA) induced intense inflammatory and oxidative stress as manifested by significantly higher serum levels of inflammatory cytokines and serum MDA levels with a concomitant decrease of serum anti-inflammatory cytokines and antioxidants in samples of all animals even those received prophylaxis. In line these results, multiple animal models documented the deleterious effects of TAA on the immune and redox milieus (Bashandy *et al.,*2018; Yang *et al.,*2019; Ahmed *et al.,*2022; Ayoub *et al.,*2022). Moreover, TAA-hepatotoxicity was associated with increased serum levels of AST, ALT, bilirubin, and decreased serum albumin levels, and these changes paralleled the disturbed inflammatory and redox milieus for TAA-induced hepatotoxicity. Similarly, Hussein *et al., (*2020) reported that TAA induces significant increases of activities of hepatic enzymes with decreased activities of the hepatic antioxidant enzymes and increased MDA content compared to normal levels.

Resveratrol (RSV) therapy before and during the exposure to TAA significantly improved the estimated levels of liver function parameters and controlled the altered levels of cytokines and MDA and SOD. In line with these data, a previous study reported that RSV administration protected against TAA toxicity through the reduction of levels of inflammatory cytokines and oxidative stress parameters and minimized DNA damage down to no significant difference in comparison to control animals (Zargar *et al.,*2019). Another study found RSV significantly decreased the bile duct ligation-induced hepatic increased levels of MDA, IL-17a, and blood liver markers (ShamsEldeen *et al.,*2021). In a similar recent study, RSV was found to inhibit TAA-induced hepatotoxicity and liver fibrosis through inhibition of TAA-induced upregulation of TNF-α, nuclear factor-kB, and the profibrotic biomarkers (Ebrahim *et al.,*2022). Further, in-vivo experiments on the TAA model assured that RSV administration decreased serum levels of transaminases and raised serum albumin levels (Liang *et al.,*2022).

In a trial to explain the pathogenesis of TAA-induced hepatotoxicity, the current study detected upregulation of miR-155 and miR-21 in blood samples of TAA-exposed animals and detected a significant correlation between these expression levels and estimated cytokines, enzyme activities, and serum MDA levels. These data allowed the assumption of a possible cascade for TAA-induced hepatotoxicity starting with exposure to TAA that induced overexpression of microRNAs which in turn induced overexpression of inflammatory mediators and initiated an oxidative stress state and the resultant disturbed inflammatory and redox milieus leading to the affection of function of hepatocyte and/or destruction of hepatocyte and initiation of fibrosis.

The detected increased plasma levels of miR-155 and miR-21 and their relation to TAA-toxicity are coincident with the results evaluated the role of another microRNA in TAA toxicity; where one study reported the presence of higher levels of microRNAs; 122, 192, and 194 in the experimental group has given TAA (Teksoy *et al.,*2020). Further, other studies detected deregulated expression levels of MiR-17 and 378 due to TAA toxicity, and this induced hepatic fibrosis through activation of hepatic stellate cells via altering Wnt/β-catenin and TGF-β/smads pathways (Zaafan and Abdelhamid.,2022; Abdelhamid *et al.,*2021).

Concerning the studied microRNAs, the obtained results and the suggested assumptions go in hand with a study that detected upregulation of miR-21, while the expression of other microRNAs was downregulated in TAA-induced liver fibrosis in rats, and attributed this to the activation of tissue growth factor-β1 (Hussein *et al.,*2020). Another study attributed TAA-induced liver fibrosis to activation of Toll-like receptor-4/miR-155/NFκB p65 pathway with increased production of inflammatory cytokines, TGF-β1, α-

SMA and downregulation of E-Cadherin (Ali et al., 2021). More recently, a study found TAA toxicity was associated with increased tissue levels of miR-155, p53, and reactive oxygen species (Dawood *et al.,*2022).

In support of these suggestions, the reported decreases of the toxic effects of TAA on liver functions, inflammatory status, and oxidative stress in samples of animals that received RSV were mostly through the control of the expression of microRNAs expressions as evidenced by the lower expression levels of both microRNAs in plasma of animals that received RSV compared to samples of animals exposed to TAA without prophylaxis and by the detected inverse relation between the received dose of RSV and expression levels of microRNAs on one side and inflammatory markers and MDA on the other side with a positive relation to the serum levels of anti-inflammatory cytokine and activity level antioxidant enzymes. In line with the assumed role of RSV prophylaxis through manipulation with expression levels of microRNAs, an animal model of RSV prophylaxis in acute liver injury reported that RSV protection was associated with inhibition of TGFβ1-Smad3-miR21 axis, and inflammatory and profibrotic cytokines (ShamsEldeen *et al.,*2021). Thereafter, another study detected a reduction of hepatocyte apoptosis with RSV administration and attributed this effect to decreasing the expression levels of miR-190a-5p and increasing the in-vitro expression of hepatocyte growth factor (Liang *et al.,*2022).

Conclusion:

Chronic exposure to TAA induced hepatotoxicity that was associated with disturbed immune and redox milieus. TAA-hepatotoxicity might be mediated through the upregulation of gene expression levels of miR-155 and miR-21 initiating a cascade that ended in liver fibrosis. Resveratrol prophylaxis may protect or at least ameliorate the TAA-hepatotoxicity mostly through improving the deregulated expression levels of microRNAs.

Recommendations:

Estimation of the levels of the studied parameters in blood samples of workers exposed to TAA was required to confirm these results for humans and trials of RSV therapy may be provided to control these changes if it was confirmed.

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