

# STUDY ON THE POSSIBLE PROTECTIVE EFFECT OF MELATONIN AND SILYMARIN AGAINST THE TOXIC EFFECT OF FAVISM FACTOR “DIVICINE”

MAHMOUD S. ARBID <sup>a</sup>, SAWSAN S. MAHMOUD <sup>a</sup> AND KHALED M. M. KORIEM <sup>b</sup>

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## ABSTRACT

Experiments were carried-out with Sprague Dawley rats to determine as the administration of Mt and Sy on the toxicity produced by DV, a free radical compound. IP injection of DV (250 mg/rat b.wt.) alone resulted in 100% mortality within 24 h accompanied by a rapid decrease in the concentration of GSH in RBCs. Administration of both doses of Mt (1 and 2 mg/kg) and Sy (22 mg/kg) with DV greatly reduced mortality and increased the concentration of GSH. Blood cells and hemoglobin also returned towards normal values. Liver enzymes (AST, ALT, ALP and bilirubin) and kidney function (urea and creatinine) were highly significant increased after divicine injection, while Mt and Sy in combination with DV returned these parameters to normal values.

Keywords: Divicine, GSH, melatonin, sylimarin, Favism, Rats.

## RESUMEN

El objetivo del presente estudio fue elucidar el posible efecto protector de la melatonina y la Silimarina en caso de efectos tóxicos de DV en ratas albinas. En la administración IP de DV se tuvo el 100% de mortalidad y una disminución de la concentración de GSH en RBCs. La administración de Mt y Sy con DV reducen la mortalidad y el aumento de la concentración de GSH. Asimismo se observa que contrarresta el aumento de los parámetros en el hígado y en las funciones renales después de la inyección de DV.

<sup>a</sup>Pharmacology and <sup>b</sup>Medical Physiology Departments, Medical Research Division, National Research Centre, Dokki, Giza, Egypt.

\*Corresponding Author Dr. Khaled Koriem, Medical physiology Department, Medical Research Division, National Research Centre, El-Behooth Street, Dokki, Giza, Egypt.

Mobile phone: 002 0116861722 Fax: 002 023370931E-mail: [kkoriem@yahoo.com](mailto:kkoriem@yahoo.com)  
[kkoriem@ucla.edu](mailto:kkoriem@ucla.edu)

## LIST OF ABBREVIATIONS

**Mt:** Melatonin; **Sy:** Silymarin; **DV:** Divicine; **IP:** Intraperitoneal; **ER:** Estrogen receptor **ANOVA:** Analysis of Variance; **Hb content:** Hemoglobin content; **RBCs count:** Red Blood Cells count; **WBCs count:** White Blood Cells count; **s.AST:** serum Aspartate Aminotransferase; **s.ALT:** serum Alanine Aminotransferase; **s.ALP:** serum Alkaline Phosphatase; **GSH:** Glutathione; **G6PD:** Glucose-6-Phosphate Dehydrogenase; **GSH-Px:** Glutathione Peroxidase; **B.wt.:** Body Weight; **ROS:** Reactive Oxygen Species; **IRI:** Ischemia/Reperfusion Injury.

## INTRODUCTION

DV the aglycon of vicine, which isolate from faba beans (*Vicia faba L.*) is the causative agent of favism, a hemolytic anemia in G6PD-deficient humans (Belsey, 1973 & Mager *et al.*, 1980). DV is a highly reactive compounds generating free radicals, which interact with the abundant supply of oxygen in the blood to produce super-oxide radicals (Albano *et al.*, 1984); which if not neutralized by the free radical scavenging system, cause cell damage. RBCs are irreversibly damage under the appropriate conditions and may be lead to favism like signs; where the data confirmed that DV hydroquinone agent generate ROS within erythrocytes under hemolytic conditions (McMillan *et al.*, 2005)

There is evidence which suggest that some antioxidant can ameliorate the oxidative effect of DV. Mt is considered as a good antioxidant agent. It reduces oxidative damage and increases survival of mice infected with *Schistosoma mansoni* (ElSokkary *et al.*, 2001). It has a protective effect against apoptosis induced by aflatoxin B1 in rat liver (Meki *et al.*, 2001). Mt also prevents many diabetic complications by reducing oxidative stress and protects organisms from oxidative damage (Baydas *et*

*al.*, 2002). Mt increases ER- $\alpha$  in the nuclear fraction and the binding affinity of ER- $\alpha$  for Mt was higher than ER- $\beta$  (Yoo and Jeung, 2009).

Sy is a well known plant product; it extracted from *Silybum Marianum* (Milk thistle); it chiefly consisting of silibinin, silydamin and silychristine (Wagner, 1986). *Silybum Marianum* extract (usually standardized to contain 70% silymarin) have been shown to protect the liver from wide range of toxins (Vogel *et al.*, 1975). Sy mostly explain as an excellent antioxidant agent, it inhibits phosphatidyl choline synthesis or stimulation of hepatic RNA and protein synthesis (Li *et al.*, 2003 and Schumann, 2003). Dietary supplementation of Sy protects against chemically induced nephrotoxicity, inflammation and renal tumor promotion response (Kaur *et al.*, 2009).

The objective of the present work was to determine if certain free radical scavenging compounds such as Mt and Sy could protect against the toxic effect of DV in rats.

## MATERIALS AND METHODS

### Animals

Male Sprague Dawley albino rats weighing 100 $\pm$ 10g were used throughout the experiment. All animals were obtained from the animal house colony of the National Research Centre, Dokki, Giza, Egypt. The animals were allowed free access to water and were fed uniform stander diet formula (Rogers, 1979). The experiment was carried-out in accordance with the national regulations on animal welfare and Institutional Animal Ethical Committee (IAEC), "National Research Centre (NRC), Cairo - Egypt".

### Drugs

Mt was purchased from Sigma Chemical Co., USA. It was injected IP at a dose of 1mg/kg b.wt dissolved in 0.5ml distilled

water, which equivalent to maximum therapeutic human dose once daily. On the other hand, Sy was obtained from SEDICO-Pharmaceutical Co., Egypt. It was injected IP at a dose of 22mg/kg b.wt dissolved in 1ml distilled water, which equivalent to medium therapeutic human dose. On the other side, Pure DV was prepared as described by Marquardt *et al.* (1983) and DV by acid hydrolysis of Vicine as described by Arbid and Marquardt (1986).

#### Preparation of DV for injection into rats

DV was prepared as suspension on degassed PBS (0.05 Phosphate buffer, pH 7.4, 0.075 M with respect to NaCl) at 26°C just prior injection.

#### Experimental Design

A total number of 160 male albino rats were divided into two major groups as follows:

**Experiment I.** 80 rats were divided to eight groups each of 10 rats. The first group acts as control and injected IP with PBS. Group 2 injected IP with DV (250 mg/kg body weight). Groups 3 and 4 injected IP with 1 and 2mg Mt, respectively. Group 5 injected IP with 22mg/kg b.wt. Sy. Group 6 and 7 injected IP with 1 and 2mg Mt in combination with 250mg/kg DV. Group 8 injected with 22mg Sy in conjugation with 250mg/kg DV. Blood samples were collected at the end of 30 days of experimental study from retro-orbital plexus (Helperin *et al.*, 1951) in heparinized tubes mixed gently and kept on ice. GSH was immediately determined. Rats were checked for mortality after 24 and 72 hours after injection.

**Experiment II.** 80 rats were divided into eight groups similar to that in experiment I. The dose of DV injected in the rats was 50mg/kg b.wt (Equivalent to 1/5 LD<sub>50</sub> of DV). The rats injected with combinations of DV and either Sy or Mt for 30 days. The blood samples were collected at the end of 30 days in sterile tubes and immediately WBCs count, RBCs count and Hb content were determined. AST, ALT, serum biliru-

bin and ALP were estimated as markers of the status of liver. Blood urea and serum creatinine were determined as markers for the status of the kidney.

#### Diagnostic kits

All reagent kits were used for rat serum. All chemicals were purchased from Bio-diagnostic Co., Egypt. White and red blood cells were determined by trunk's fluid according to Mitruka *et al.* (1977). Hb content was determined by the method described by Van Kampen and Zijlstra (1961). Serum AST and ALT were determined according to the method of Reitman and Frankel (1957). Serum total bilirubin determination was performed with Walter and Gerard method (1970). Serum ALP was determined by the colorimetric method of *Kind and King* (1954). Blood urea was determined by the enzymatic method according to the method of Patton and Crouch (1977) and serum creatinine was performed by kinetic method (Houot 1985). GSH concentration was determined according to Beutler *et al.* (1963)

#### Statistical analysis

Data were evaluated with Statistical Package for Social Science (SPSS/10) software for Windows. These hypothesis include one way ANOVA followed by Least significant difference (LSD) at P<0.0001. Tukey's test was carried-out to determine differences between groups. Data were tabulated in tables and figures; where significant difference according to control groups at P<0.05.

## RESULTS

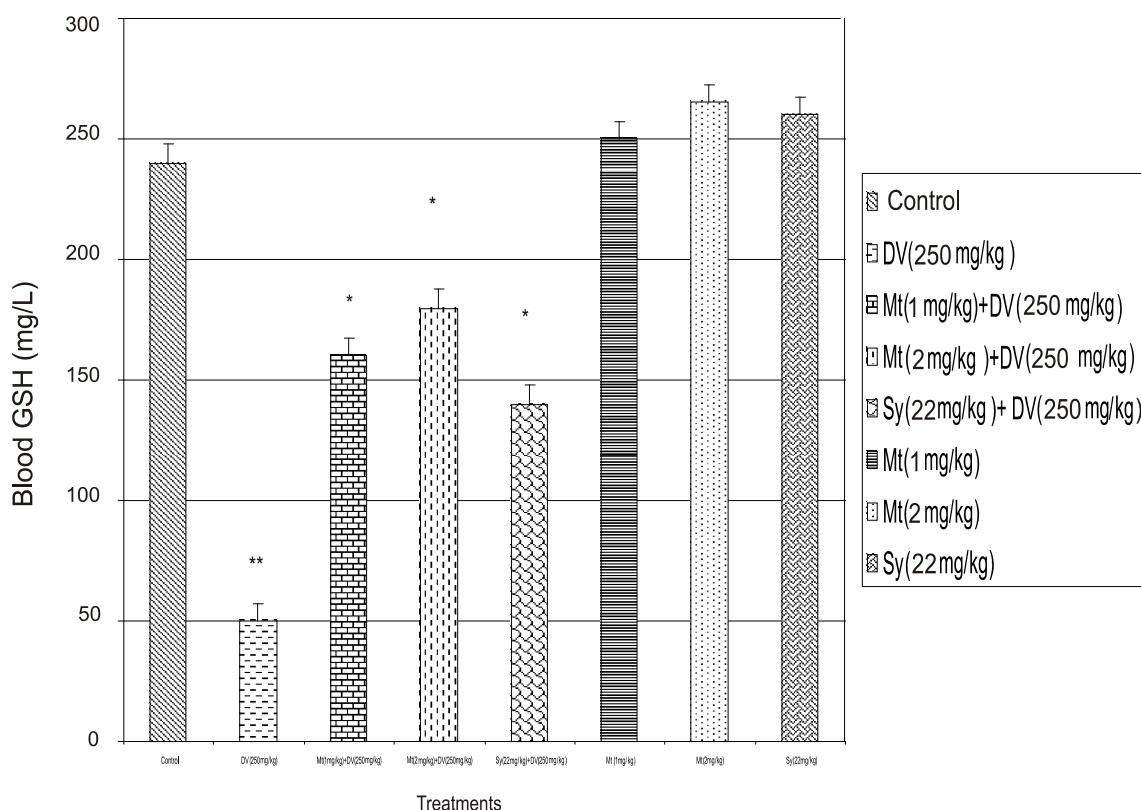
Table (1) demonstrated that DV (250mg/kg b.wt.) caused toxic effect and 100% mortality after 24 hours of the injection. No mortality was observed after IP injection of both Mt (1 and 2mg/kg b.wt.) and Sy (22mg/kg b.wt.). Mortality was decreased

Table (1): Protective effect of Melatonin and Silymarin against the toxicity of Divicine

Treatments	Mortality Rate (%)	
	Mortality (24 h)	Mortality (72 h)
Control	0	0
DV(250mg/kg)	100	100
Mt (1mg/kg)+DV(250mg/kg)	40	60
Mt (2mg/kg)+DV(250mg/kg)	60	65
Sy (22mg/kg)+DV(250mg/kg)	35	70
Mt (1mg/kg)	0	0
Mt (2mg/kg)	0	0
Sy (22mg/kg)	0	0

10 animals occurred per each group. Number of replicates (n) was 3.

Fig. 1 Protective effect of Melatonin and Silymarin of the decreasing Blood GSH induced by Divicine



10 animals occurred per each group. Number of replicates (n) was 3. \*P<0.05 significant difference compared to control group, \*\*P<0.01 highly significant difference compared to control.

in groups injected with Mt (1mg/kg b.wt.) combined with 250mg/kg b.wt. DV after 24 hours mortality was found to be 40%; while 60% mortality appeared after 72 hours of Mt (1mg/kg b.wt.) injection. Treatment with Mt (2mg/kg) with DV revealed 60% mortality after 24 hours of injection, while 65% mortality found after 72 hours of Mt (2mg/kg b.wt.) injection. But in the case of Sy (22mg/kg b.wt.) with DV mortality was found to be 35% and 70% after 24 and 72 hours, respectively of Sy injection.

Fig (1) showed that the value of GSH in control group was 240mg/litre. IP injection with DV (250 mg/kg b.wt.) induced highly significant decrease ( $P<0.01$ ), but Mt(1 and 2 mg/kg b.wt.)+DV(250mg/kg b.wt.) and Sy(22mg/Kg b.wt.)+DV(250mg/Kg b.wt.) re-

vealed significant decrease ( $P<0.05$ ); where glutathione values increased from 50mg/litre (DV) to be 160 mg/litre (1mg Mt/kg b.wt.), 180 mg/litre (2mg Mt/kg b.wt.) and 140 mg/litre (22mg Sy/Kg b.wt.). On the other side, IP injection of Mt (1 and 2mg/kg b.wt) and Sy (22mg/kg b.wt) showed insignificant increase ( $P>0.05$ ) and GSH values between 250 and 265mg/litre.

Table (2) revealed Tukey's test analysis; where DV (50mg/kg b.wt.) significantly decreased ( $P<0.05$ ) WBCs count, RBCs count and Hb content, respectively. But groups injected with Mt (1 and 2mg/kg b.wt.)+DV(50mg/kg b.wt.) and Sy(22mg/kg b.wt.) + DV(50mg/kg b.wt.); these parameters almost returned close to normal values ( $P>0.05$ ) were occurred. On the other hand,

Table (2): Protective effect of Melatonin and Silymarin against blood toxicity induced by Divicine

Treatments	Parameters		
	WBCs ( $\times 10^3/\text{mm}^3$ )	RBCs ( $\times 10^6/\text{mm}^3$ )	Hb content (mg/dl)
Control	7.7 $\pm$ 0.41	8.2 $\pm$ 0.43	14.8 $\pm$ 0.81
DV (50mg/kg)	5.2 $\pm$ 0.30*	5.2 $\pm$ 0.61*	12.2 $\pm$ 0.66*
Mt (1mg/kg)+DV(50mg/kg)	7.0 $\pm$ 0.56	8.6 $\pm$ 0.81	13.9 $\pm$ 0.71
Mt (2mg/kg)+DV(50mg/kg)	7.3 $\pm$ 0.60	8.3 $\pm$ 0.75	14.1 $\pm$ 0.63
Sy (22mg/kg)+DV(50mg/kg)	7.2 $\pm$ 0.66	8.8 $\pm$ 0.71	14.5 $\pm$ 0.81
Mt (1mg/kg)	8.8 $\pm$ 0.39*	7.9 $\pm$ 0.85	14.1 $\pm$ 0.82
Mt (2mg/kg)	12.2 $\pm$ 0.61*	8.2 $\pm$ 0.69	14.6 $\pm$ 0.85
Sy (22mg/kg)	8.9 $\pm$ 0.55	8.3 $\pm$ 0.66	13.8 $\pm$ 0.75

Results were expressed as Mean  $\pm$  SE and \* $P<0.05$  significant difference compared to control. 10 animals occurred per each group. Number of replicates (n) was 3.

Table (3): Protective effect of Melatonin and Silymarin against liver toxicity induced by Divicine

Treatments	Parameters			
	s.AST (u/ml)	s.ALT (u/ml)	s.Bilirubin (mg/dl)	s.AL.P (u/l)
Control	92.1 $\pm$ 3.34	68.3 $\pm$ 2.15	0.54 $\pm$ 0.08	76.4 $\pm$ 2.48
DV (50mg/kg)	130.4 $\pm$ 3.25**	80.1 $\pm$ 2.39**	0.90 $\pm$ 0.05**	104.3 $\pm$ 2.62**
Mt (1mg/kg)+DV(50mg/kg)	102.4 $\pm$ 3.33	70.1 $\pm$ 3.51	0.71 $\pm$ 0.06	82.8 $\pm$ 3.41
Mt (2mg/kg)+DV(50mg/kg)	93.6 $\pm$ 3.82	57.4 $\pm$ 3.1	0.52 $\pm$ 0.08	79.3 $\pm$ 2.44
Sy (22mg/kg)+DV(50mg/kg)	107.7 $\pm$ 4.10	66.2 $\pm$ 3.3	0.70 $\pm$ 0.05	83.1 $\pm$ 3.2
Mt (1mg/kg)	92.2 $\pm$ 3.81	66.8 $\pm$ 3.31	0.61 $\pm$ 0.07	75.8 $\pm$ 3.31
Mt (2mg/kg)	95.3 $\pm$ 3.42	58.8 $\pm$ 2.24	0.56 $\pm$ 0.04	78.8 $\pm$ 2.57
Sy (22mg/kg)	94.5 $\pm$ 4.05	55.8 $\pm$ 3.04	0.50 $\pm$ 0.08	77.3 $\pm$ 3.17

Results were expressed as Mean  $\pm$  SE and \*\* $P<0.01$  highly significant difference compared to control. 10 animals occurred per each group. Number of replicates (n) was 3.

Table (4): Protective effect of Melatonin and Silymarin against kidney toxicity induced by Divicine

Treatments	Parameters	
	Blood Urea (mg/dl)	Serum Creatinine (mg/dl)
Control	24.5±1.53	0.68±0.06
DV (50mg/kg)	35.4±1.42**	1.30±0.08**
Mt (1mg/kg)+DV(50mg/kg)	28.8±2.21	0.80±0.05
Mt (2mg/kg)+DV(50mg/kg)	25.2±1.75	0.82±0.08
Sy (22mg/kg)+DV(50mg/kg)	27.6±1.93	0.75±0.06
Mt (1mg/kg)	26.6±1.88	0.78±0.07
Mt (2mg/kg)	23.8±1.79	0.72±0.06
Sy (22mg/kg)	22.3±1.67	0.67±0.08

Results were expressed as Mean ± SE and \*\*P<0.01 highly significant difference compared to control. 10 animals occurred per each group. Number of replicates (n) was 3.

Mt (1 and 2mg/kg b.wt.) induced significant increase (P<0.05) in WBCs count without any change in both RBCs count and Hb content.

Table (3) exhibited tukey's test analysis; where serum AST, ALT, bilirubin and ALP were highly significant elevated (P<0.01) in groups injected with DV (50mg/kg b.wt.) for 30 days, but groups injected with Mt (1 and 2mg/kg b.wt.)+ DV(50mg/kg b.wt.) and Sy(22mg/kg b.wt.) + DV(50mg/kg b.wt.); the values of AST, ALT, bilirubin and ALP decreased towards control group and insignificant increase (P>0.05) in AST, ALT, bilirubin and ALP were found. On the other hand, Mt in both doses (1 and 2mg/kg b.wt.) and Sy (22mg/kg b.wt.) showed values approximately similar to that of control.

Table (4) showed tukey's test analysis; however a highly significant increase (P<0.01) in the levels of blood urea and serum creatinine in group injected with DV(50mg/kg b.wt.). But groups injected with Mt (1 and 2mg/kg b.wt.)+ DV(50mg/kg b.wt.) and Sy(22mg/kg b.wt.) + DV(50mg/kg b.wt.); the values of these parameters decreased towards control values and insignificant increase (P>0.05) in blood urea and serum creatinine were observed. On the other side, both doses of Mt and Sy revealed values nearly similar to that of control.

## DISCUSSION

Neither control rats nor those injected with two doses of Mt or one dose of Sy were died, on the contrary, 100% of mortality was found in those injected with DV (250mg/kg b.wt.). Most of death following DV injection occurred between 1-4 hours and all the death completed after 24 hours. A similar result occurred in a previous study in which only vicine (Arbid and Marquardt., 1986) was administrated. GSH was greatly depressed by DV treatment alone which was similar to results obtained previously by Yannai y Marquardt (1985) and Arbid y Marquardt (1986), it has been well documented that DV decrease GSH concentrations as reported in vitro by Mager *et al.* (1980) and Arese *et al.* (1981).

On the basis of the previous studies with rats, it appears that DV first caused reduction of GSH concentration in the RBCs. If the dose is high; complete depletion of GSH occurred, which then followed by peroxidative changes in hemoglobin and other components of RBCs. However, administration of both doses of Mt and a dose of Sy with DV not only reduced mortality, but also prevented the associated decrease in GSH concentration in the blood. Presumably Mt and Sy treatment interferes with DV by acting as a free radical scavenger (Bayda *et al.*, 2001), thereby preventing GSH deple-

tion and the subsequent per-oxidative effect. The decrease in the WBCs count, RBCs count and Hb content may be attributed to the depleted GSH concentration followed by per-oxidative changes in the components of hemoglobin and other blood cells (Arbid and Marquardt., 1986). Presumably effect of Mt and Sy are considered to be a free radical scavenger (Sies, 1985), and it would be expected to scavenge the super-oxide radicals or other free radicals that are formed when oxygen reacted with DV (Chevion., 1982 and Albano., 1984).

In the present study, injection of rats with Mt in both doses and a dose of Sy decrease DV induced elevation in AST, ALT, bilirubin and ALP, urea and creatinine. The protection provided as compared to enzymes levels in DV treated rats (Bayda *et al.*, 2001) reported that Mt was not only a direct scavenge of toxic radicals but also stimulates and increased the anti-oxidative enzyme GSH-Px activity in the brain and kidney, where activity of GSH-Px, was significantly increased by Mt (Pan *et al.*, 2009). On the other hand, Martin *et al.* (2002) described that Mt increased the activity of the oxidative phosphorylation enzymes and the production of ATP in rat brain and liver mitochondria. Also, the present study showed a significant therapeutic effect of

Mt and Sy on liver and kidney tissue of rats affected by DV. The mechanism of action of Sy involved different biochemical events, such as the stimulation of synthetic rate of ribosomal RNA transcription, protecting cell membrane from radical induced damage and blocking the uptake of toxins; so the administration of Sy significantly reduced the liver toxicity (Wellington and Jarvis., 2001 and Shaarawy *et al.*, 2009). Also, Mt protected kidney grafts from IRI-induced renal dysfunction and tubular injury through its anti-oxidative and anti-apoptotic capacity (Li *et al.*, 2009).

This paper provides direct evidence *in vivo* that free radical scavenging compounds specifically Mt and Sy protect against DV induced death and associated biochemical changes. The effect of DV in rats is analogous in many respects to those of favism in humans (Arbid and Marquardt., 1986). It may nevertheless, be concluded that certain compounds may be capable of greatly modifying individual susceptibility to DV and presumably to favism. These may be one of the important additional factors proposed by Mager *et al.* (1980) along with G6PD-deficiency and exposure to faba beans, which influence the degree and severity of the hemolytic attack.

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