

*Full Length Research Paper*

# Hepatoprotective effects of antioxidants against non-target toxicity of the bio-insecticide spinosad in rats

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The bio-insecticide spinosad (SPD) is increasingly being used in pest management programmes. In order to further assess its toxic effects to non-target species, male rats were exposed sub-chronically to SPD at a dose equivalent to  $1/20$  LD<sub>50</sub> for four weeks. In order to assess the toxicity of SPD, parameters such as the activities of enzymes and concentrations of non-enzymatic antioxidant components, histopathological examination, DNA fragmentation and chromosomal aberrations in liver were analysed. Protection by an antioxidants mixture (AM), containing vitamin C, vitamin E and silymarin, against the effects of SPD was investigated. Exposure to SPD inhibited the activity of acetylcholinesterase, and depleted contents of reduced glutathione and malondialdehyde. SPD caused significant inhibition of activity of key antioxidant enzymes (GST, SOD) and induction in GPx. Treatment with AM attenuated all SPD-mediated effects. Histological examination of the liver revealed that SPD caused focal necrosis and degenerative changes in hepatocytes, along with cytoplasmic vacuolation. All of these lesions were significantly less in rats fed with AM. SPD accelerated formation of internucleosomal DNA fragmentation, which was attenuated by treatment with AM. Similarly, SPD caused significant structural chromosomal aberrations in bone marrow cells, the frequency of which was diminished by AM.

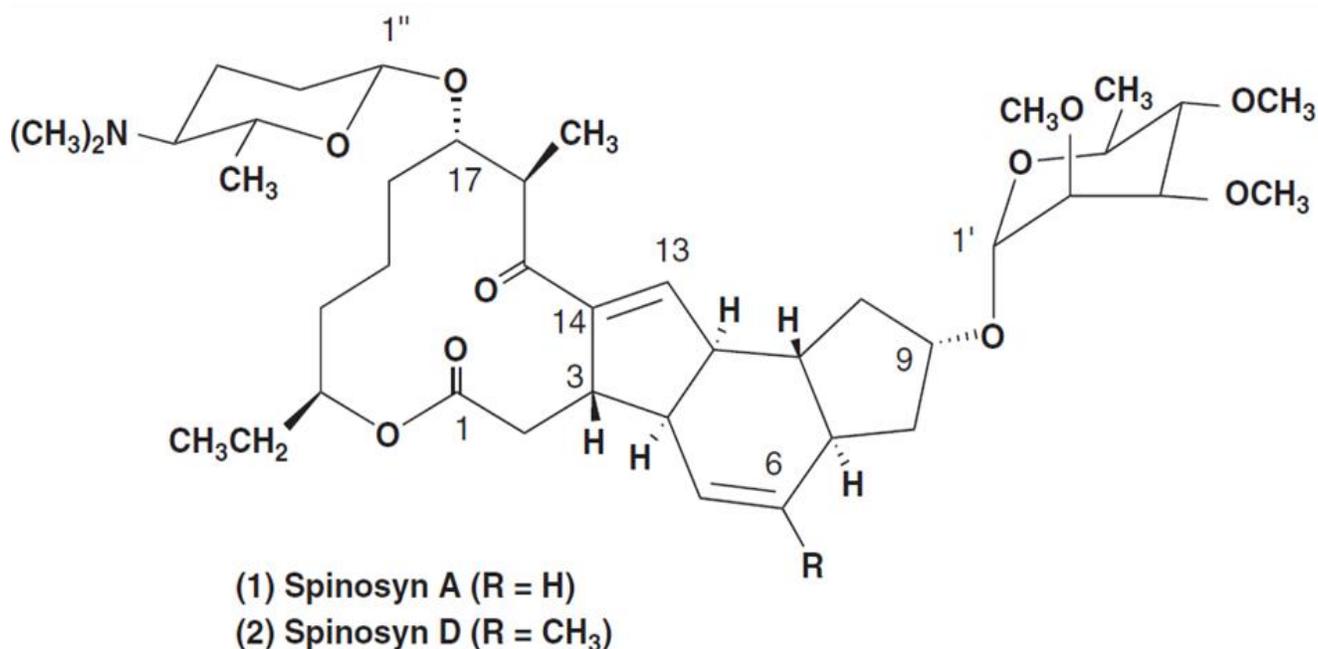
**Key words:** Antioxidants, DNA damage, silymarin, spinosad, vitamin C, vitamin E.

## INTRODUCTION

Integrated pest management (IPM) is an effective and environmentally appropriate approach to pest management that relies on a combination of biological, cultural, physical, mechanical, and chemical tools to

minimize economic, health, and environmental risks while protecting valuable crops. Thus, IPM is focused to increase the sustainability of farming systems *via* minimizing and/or preventing the use of conventional insecticides (Dent, 2000). Bio-pesticides can be particularly effective when incorporated into IPM and disease management programs. Hence, bio-pesticides are now considered as the safest insecticides and an

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**Figure 1.** Structures of spinosad. (1) Spinosyn A and (2) spinosyn D.

environmentally-friendly substitute of broad-spectrum, synthetic insecticides (Afify et al., 2009).

In this context, the bio-insecticide Spinosad (SPD) is a secondary metabolite of the aerobic fermentation of the naturally-occurring soil actinomycete *Saccharopolyspora spinosa* (Bacteria: Actinobacteridae). SPD contains two insecticidal factors, spinosyns A and D, present in an approximately 85:15% ratio in the final product (Kirst, 2010). Spinosyn A, the major component in the fermentation mixture, is composed of a tetracyclic polyketide aglycone to which two different sugars are attached; a neutral saccharide substituent (2, 3, 4-tri-O-methyl-L-rhamnosyl) on the C-9 hydroxyl group and an aminosugar moiety (b-D-forosaminyl) on the C-17 hydroxyl group. The second most abundant fermentation component is Spinosyn D, which is 6-methyl-spinosyn A (Figure 1) (Kirst, 2010). SPD is a powerful neurotoxin against certain arthropods, especially lepidopterans, coleopterans, and dipterans (Bret et al., 1997). SPD has been shown to exhibit a novel mode of action through activation of nicotinic acetylcholine receptors, which provokes the over-excitation of the insect nervous system leading to involuntary muscle contractions, prostration with tremors, and finally paralysis. SPD also affects  $\gamma$ -amino butyric acid (GABA)-gated ion channels of the insect nervous system, which might further contribute to paralysis (Salgado, 1998). Although SPD has been described as an insecticide with a novel mode of activity, resistance management is acknowledged as essential to perpetuating the long-term effectiveness of this bio-insecticide (Ramasubramanian and Regupathy, 2004;

Bond et al., 2004). Moreover, SPD is considered to be a selective pesticide since it exhibits little or no effect on most beneficial insects. Also, it exhibits little toxicity to mammals and since it degrades in the environment, it has a favourable environmental profile (Schoonover and Larson, 1995; Cleveland et al., 2001). Furthermore, SPD is a natural product and currently being registered in Egypt as a safer alternative to synthetic pesticides. It has been approved for use in organic agriculture by numerous certification bodies worldwide (Racke, 2007).

The potential for effects of SPD on non-target organisms is not fully understood. In this context, an accumulating body of evidence highlighted some potential safety concerns and risks of toxicity associated with the use of SPD. The acute oral rat  $\text{LD}_{50}$  values for SPD range from 3738 to 45000 mg/kg body weight (Yano et al., 2002). Daily intakes of 2.7 to 8.6 mg/kg body weight (bw) in experimental animals has been reported to be within the established “number of observed adverse effect level” (NOAEL) safety margins (Yano et al., 2002). Alternatively, the NOAEL for topical administration to rabbits was as great as 1000 mg/kg bw. Recent reports indicated that SPD is toxic to honey bees with a  $\text{LC}_{50}$  of 7.34 mg/l (Rabea et al., 2010). Moreover, despite the fact that SPD exhibits, no acute toxicity to terrestrial birds or most aquatic invertebrates, SPD-mediated cellular vacuolation has been observed in a tissues, including the adrenal gland, liver, lymphoid cells, kidney, thyroid, stomach and lung. This is caused by cellular phospholipidosis, a condition that results from accumulation of polar lipids in lysosomes, which leads to

formation of ultra structural lysosomal lamellar inclusion bodies (Stebbins et al., 2002; Yano et al., 2002).

Furthermore, SPD decreases viability in two mammalian cell types, based on *in vitro* cytotoxicity as determined by uptake of neutral red (Pérez-Pertejo et al., 2008). In the study, the effects of sub-chronic oral exposure to SPD on albino, Sprague-Dawley rats was determined. The potential of treatment of rats with an antioxidant mixture, including vitamin E, vitamin C and silymarin to protect against effects of sub chronic exposure to SPD was assessed. The endpoints studied were: i) toxicity and oxidative stress markers (AChE, MDA, and GSH), ii) activities of antioxidant enzymes (GST, SOD, GPx and CAT), iii) DNA fragmentation profile, iv) liver histopathology and, v) Chromosomal aberration patterns in rat bone marrow cells

## MATERIALS AND METHODS

### Chemicals

All fine chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis MO, USA). All reagents were of standard laboratory grade. Spinosad, under the commercial name Tracer® (CAS No. 131929-60-7), which is a mixture of spynosin A (2-[(6-deoxy-2,3,4-tri-O-methyl- L-mannopyranosyl)oxy]-13-[[5 (dimethylamino) tetrahydro-6-methyl-2H-pyran-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a,16b-tetra-decahydro-14 methyl-1H-as-indaceno(3,2-d)oxacyclododecin-7,15-dione) and spynosin D (2-[(6-deoxy-2,3,4-tri-O-methyl- L-mannopyranosyl)oxy]-13-[(5-dimethylamino)tetrahydro-6-methyl-2H-pyran-2-yl]oxy]-9-ethyl-2, 3,3a,5a,5b,6,9,10,11,12,13,14,16a,16b-tetradecahydro-4,14-dimethyl-1H-as-indaceno(3,2-d)oxacyclododecin-7,15-dione), were purchased from Dow AgroSciences LLC (Indianapolis, IN, USA), comprising 480 g AI L<sup>-1</sup>.

### Animals and experimental design

Sixty male albinos Sprague-Dawley rats, weighing 150 to 200 g, were obtained from the animal house of the National Organization of Drug Control and Research (NODCAR), Egypt. Animals were housed under standard lighting and relative humidity (50 ± 15%) conditions in a temperature-controlled room (25 ± 2°C). Animals were allowed to acclimatize to the laboratory environment for 10 days, and were allowed free access to standard dry pellet diet and water *ad libitum*. All animal experiments reported upon here complied with the ethics and regulations of the Animal Care and Use Committee of Cairo University, Egypt. Subsequently, rats were randomly separated into three polypropylene cages of twenty individuals each and were administered solutions orally *via gavage*, daily for a four-week period. In this study, the median lethal dose (LD<sub>50</sub>) of spinosad (SPD) in rats was determined to be 6949.8 mg/kg (~7 g/kg). Experimental design was as follows: the first group served as a control in which rats were fed on adequate standard laboratory diet, the second was the experimental group in which rats received a sub-chronic dose of SPD at the level of 1/20 LD<sub>50</sub> (347.49 mg kg<sup>-1</sup> bw), for 4 weeks, and the third was the experimental group in which rats were administered a mixture of three antioxidants (vit C, 50 mg kg<sup>-1</sup> bw; vit E, 50 mg kg<sup>-1</sup> bw and silymarin, 50 mg kg<sup>-1</sup> bw) daily; at the beginning of the day concomitant with SPD at the end of the day. Antioxidant mixtures

were dissolved in 0.5% gum-acacia solution to give aqueous suspensions (Sabina et al., 2009).

### Histology of liver

Tissue preparation for the biochemical determination of enzymatic activities and histological examination of liver samples was conducted as previously described (Aboul-Soud et al., 2011).

### Protein estimation

The protein contents of various samples were determined according to the method of Bradford (1976) by using bovine serum albumin as a standard.

### Toxicity and oxidative stress parameters

Acetylcholinesterase (AChE, EC 3.1.1.7) activity was assayed as previously described (Ellman et al., 1961). The extent of lipid peroxidation (LPO) in liver homogenate was estimated as the concentration of malondialdehyde (MDA), which is the end product of LPO, and reacts with thiobarbituric acid (TBA) as a TBA reactive substance (TBARS) according to Ohkawa et al. (1979).

### Antioxidant enzymes

Estimation of glutathione-S-transferase (GST) activity was undertaken spectrophotometrically by following the increase of yellow colour developed as a result of the conjugation of 1-chloro 2, 4-dinitrobenzene (CDNB) with GSH by GST, using the method of Habig et al., (1974). Measurement of the enzymatic activities for catalase (CAT, EC 1.11.1.6), superoxide dismutase (CuZnSOD, EC 1.15.1.1) and glutathione peroxidase (GPx, EC 1.11.1.9) were conducted according to previously described methods (Aboul-Soud et al., 2011; Al-Othman et al., 2011), by use of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), nitroblue tetrazolium (NBT) and GSH as substrates, respectively.

### Non-enzymatic antioxidants

Concentrations of reduced glutathione (GSH) in liver was estimated by use of a colorimetric technique, based on the development of a yellow colour when DTNB [(5, 5 dithiobis-(2-nitrobenzoic acid))] was added to compounds containing sulfhydryl groups, according the method described by Sedláč and Lindsay (1968). Ascorbic acid (ASC) concentration was determined according to the method of Jagota and Dani (1982) that uses Folin phenol reagent and measuring the resulting colour at 670 nm.

### Chromosomal aberrations in bone marrow cells

Rat bone marrow cells and chromosomal preparations were conducted according to the method of Yosida and Amano (1965). After 24 h from the last treatment, rats were injected intraperitoneally with colchicine at a final concentration of 3.0 mg kg<sup>-1</sup> bw, 2 h prior to being euthanized by decapitation. The scanning of slides for mitotic spread metaphases was conveniently accomplished with a 25 × magnification objective, and analyzed with 100 × oil objective. Five rats were exposed to each dose, while structural alterations of chromosomes were evaluated in 100 metaphases per animal. Metaphases with gaps, chromosome or

**Table 1.** Effect of antioxidant mixture (vit E, vit C and silymarin) treatment on poisoning and oxidative stress parameters in liver from negative control and spinosad-treated experimental groups.

Group	Treatment period/parameter							
	2 weeks		4 weeks		2 weeks		4 weeks	
	AChE (U/g protein)		GSH ( $\mu\text{g}/\text{mg}$ protein)		MDA (nmol/mg protein)		ASA (ng/mg protein)	
1 <sup>st</sup> (Control)	4.41 $\pm$ 0.31		1.20 0.06		15.6 $\pm$ 0.36		641.6 $\pm$ 49.5	
2 <sup>nd</sup> (SPD)	3.08 <sup>**</sup> $\pm$ 0.10	2.98 <sup>**</sup> $\pm$ 0.25	0.84 <sup>*</sup> 0.04	0.55 <sup>**</sup> $\pm$ 0.05	19.3 <sup>**</sup> $\pm$ 0.67	18.8 <sup>*</sup> $\pm$ 1.35	553.9 <sup>ns</sup> $\pm$ 6.70	642.6 ns $\pm$ 19.2
3 <sup>rd</sup> (SPD+AM)	3.61 <sup>**</sup> $\pm$ 0.24	3.82 <sup>**</sup> $\pm$ 0.03	1.34 <sup>ns</sup> 0.09	1.67 <sup>*</sup> 0.14	15.0 <sup>ns</sup> $\pm$ 1.32	14.6 <sup>ns</sup> $\pm$ 0.85	567.9 ns $\pm$ 4.40	647.6 ns $\pm$ 16.9

Each value represents the mean of 8 rats  $\pm$  SE. ns: value is not significant, that is  $P > 0.10$ . For significance, asterisks were used according to the significance level: \* $< 0.05$ ; \*\* $< 0.01$ ; as compared with the negative untreated control. Spinosad (SPD), antioxidants mixture (AM), acetylcholinesterase activity (AChE), malondialdehyde (MDA), reduced glutathione (GSH) and ascorbic acid (ASA).

chromatid breakage, fragments, deletions, centric fusions, centromeric attenuations as well as numerical aberrations (hyperdiploidy) were recorded.

#### DNA fragmentation

DNA fragmentation in hepatocytes was assayed as previously described (El-Shemy et al., 2010) with some modifications. Briefly, approx. 10 mg of liver tissues were transferred to an Eppendorf tube, containing 600  $\mu\text{l}$  lysing buffer (50 mM NaCl, 1 mM Na<sub>2</sub> EDTA, 0.5% SDS), squeezed with disposable plastic pestle and incubated overnight at 37°C. Next, 200  $\mu\text{l}$  of NaCl solution were added, shaken gently and centrifuged at 12,000 rpm for 10 min. Then, the supernatant containing DNA was treated with RNase (100  $\mu\text{g}$  ml<sup>-1</sup>), which was then incubated at 50°C for 30 min, followed by the addition of proteinase K (200  $\mu\text{g}$  ml<sup>-1</sup>) and further incubated at 37°C for 1 h. Finally, DNA was purified with phenol, chloroform and isoamyl alcohol (25 : 24 : 1, v/v/v), and precipitated at -20°C with ice-cold 100% ethanol, sodium acetate (0.3 M) (2v : 1v). The DNA fragments were electrophoresed on a 2.0% agarose gel containing 0.1 mg ml<sup>-1</sup> ethidium bromide.

#### Statistical analyses

The calculations and statistical analysis were conducted by use of the Statistical Package for Social Sciences (SPSS) for Windows version 17.0. All data were represented as

mean  $\pm$  standard deviation (SD). Data were subjected Student's *t*-test for comparison of the means between treatments and the negative control. Statistical probabilities of Type I error ( $P < 0.05 = *$  and  $P < 0.01 = **$ ) were considered to be significant.

## RESULTS

### Effects of spinosad on AChE activity and oxidative stress markers in liver

The effect of sub-chronic exposure of rats to SPD, at a dose equivalent to  $1/20$  LD<sub>50</sub>, on toxicity and oxidative stress markers was investigated in the liver after two durations of exposure: 2- and 4-week-post-treatment (*wpt*) (Table 1). SPD resulted in significantly ( $P < 0.01$ ) less acetylcholinesterase (AChE) activity, compared to the untreated negative control, both at 2 and 4 *wpt* (Table 1). Treating the SPD-exposed rats with an antioxidant mixture AM, comprised of vitamin C, vitamin E and silymarin (SPD + AM), resulted in a significant amelioration of effects of SPD on AChE activity in liver at both 2 and 4 *wpt*, relative to that in livers of rats treated with SPD alone. As a marker of lipid peroxidation of biological membranes, the concentration malondialdehyde

(MDA) in liver was evaluated. Exposure to SPD resulted in significantly greater concentrations of MDA at both 2 and 4 *wpt*, relative to the negative control. Treatment with AM resulted in significant reductions in concentrations relative to the positive control (SPD alone). The concentration of MDA in livers of rats treated with both SPD and MA were not significantly different from that of the controls or initial values (Table 1). Exposure to SPD resulted in significantly less reduced glutathione (GSH), relative to the negative control at both time points examined. Again, the AM treatment was capable of recovering the concentration of GSH to normal, concentrations that were comparable those of the negative controls (Table 1). However, rats exposed to SPD exhibited no significant changes in the levels of ascorbic acid (ASA) as compared to the negative control. Similarly, AM treatment of SPD-exposed rats had no effect on concentrations of ASA at either time point (Table 1).

### Effect of spinosad on activities of antioxidant enzymes in liver

SPD resulted in a significant ( $P < 0.05$ ) inhibition

**Table 2.** Effect of antioxidant mixture (vit E, vit C and silymarin) treatment on the activity of antioxidant enzymes in liver from negative control and spinosad-treated experimental groups.

Group	Treatment period/parameter							
	2 weeks		4 weeks		2 weeks		4 weeks	
	SOD (U/mg protein)		GST (U/mg protein)		GPx (U/mg protein)		CAT (U/mg protein)	
1 <sup>st</sup> (Control)	2.960 ± 0.169		19.4 ± 0.78		9.28 ± 0.140		0.212 ± 0.002	
2 <sup>nd</sup> (SPD)	2.359 ± 0.135*	2.231 ± 0.122*	12.0 ± 1.07***	13.6 ± 0.45***	10.9 ± 0.16	10.7 ± 0.29	0.208 ± 0.008 <sup>ns</sup>	0.219 ± 0.004 <sup>ns</sup>
3 <sup>rd</sup> (SPD+AM)	3.188 ± 0.149 <sup>ns</sup>	3.271 ± 0.071 <sup>ns</sup>	16.5 ± 1.05*	17.7 ± 0.64*	9.71 <sup>ns</sup> ± 0.07	9.15 <sup>ns</sup> ± 0.52	0.219 ± 0.013 <sup>ns</sup>	0.209 ± 0.013 <sup>ns</sup>

Each value represents the mean of 8 rats' ±SE. ns: value is not significant, that is, P > 0.10. For significance, asterisks were used according to the significance level: \* < 0.05; \*\* < 0.01; as compared with the negative untreated control. Spinosad (SPD), antioxidants mixture (AM), acetylcholinesterase activity (AChE), malondialdehyde (MDA), reduced glutathione (GSH) and ascorbic acid (ASA).

of superoxide dismutase (SOD) activity at the two time points observed, 2 and 4 *wpt*, compared to the untreated control group. Treatment with AM resulted in a significant recovery of activity of SOD activity to approximately normal values (Table 2). Similarly, the activity of glutathione S-transferase (GST) was significantly ( $P < 0.01$ ) less in livers of rats fed with SPD. Normal GST activities were recovered to an appreciable extent upon treatment of SPD-exposed rats with AM (Table 2). Significantly ( $P < 0.05$ ) greater activity of glutathione peroxidase (GPx), relative to controls, was observed in response to SPD exposure. The greater activity of SOD was not significantly different from that of the negative controls upon treatment with AM (Table 2). Neither the SPD exposure alone or in combination with AM caused any changes in the activity of catalase (CAT), when compared to the negative control (Table 2).

### Histological findings

Observations with light microscopy revealed regular morphology of the liver with well-designed hepatic cells, the central vein, portal area and no

pathological alterations in surrounding hepatocytes in the first, control group (Figure 2 A). In the second group, which had received SPD at a dose of  $1/20$  of LD<sub>50</sub>, severe focal necrosis and degenerative changes in the hepatocytes, along with cytoplasmic vacuolation, were observed (Figure 2B and C). Notably, these histological changes in the liver were significantly less in the third group, which had been co-administered with the same SPD dose concomitant to AM. Supplementation with AM provided protection with better arrangement of well-formed polygonal hepatocytes and relatively reduced cytoplasmic vacuolation, in comparison to SPD-treated specimens (Figure 2D).

### Effect of SPD on DNA fragmentation

Exposure of hepatocytes to an SPD dose equivalent to  $1/20$  of LD<sub>50</sub> resulted in significant incidences of internucleosomal DNA fragmentation, as evidenced by the formation of a DNA ladder on agarose gel, which is indicative of cells undergoing apoptosis (Lane 2) (Figure 3). No laddering of DNA was detected in the sample from untreated control (Lane 1). Treatment with AM significantly reduced the extent of the observed

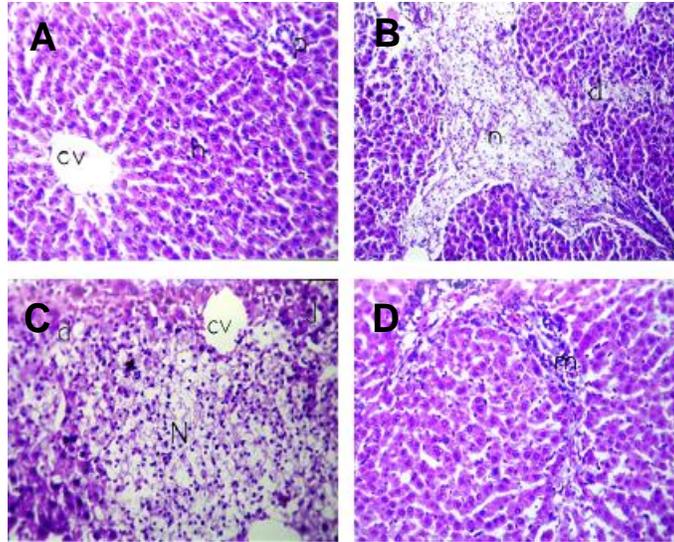
DNA laddering in hepatocytes co-exposed with SPD (Lane 3).

### Cytogenetic effects of spinosad on rat bone marrow cells

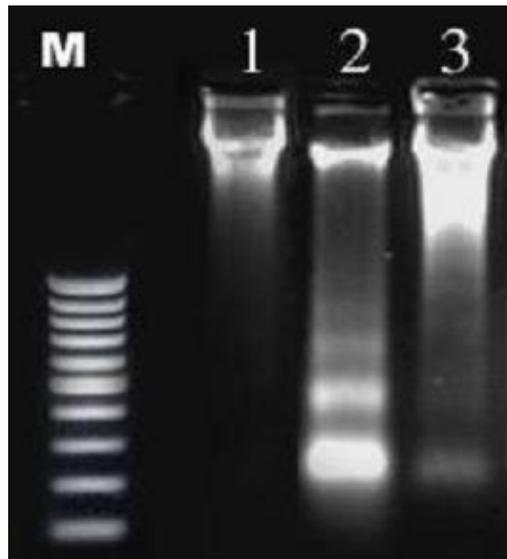
The number and percentage of the structural and numerical aberrations induced in rats exposed to SPD, compared to untreated control rats and the effect of the AM treatment is given (Table 3). Some examples of the chromosomal aberrations observed are shown (Figure 4). Notably, bone marrow cells of rats which had been exposed to SPD for four weeks exhibited 133% and 270% increase in the chromosomal aberration with and without chromatid gaps respectively, as compared to untreated control. The treatment of SPD-exposed rats with AM resulted in fewer chromosomal aberrations by 26.5% and 32.4% with and without chromatid gaps respectively, as compared to the positive control group which has received SPD alone (second group).

### DISCUSSION

Some pesticides, such as organophosphorous



**Figure 2.** Paraffin sections stained by haematoxylin and eosin (H&E) for histological examination of hepatocytes. A, image of liver tissue in untreated control showing normal structure, central vein (cv), portal area (p) and surrounding hepatocytes (h) (x64); B&C, liver sections of rat treated with SPD daily for 4 weeks, A, liver section showing sever focal necrosis (n) and degenerative changes (d) in the hepatic parenchyma in (x40). C, magnification of the SPD-mediated necrosis (N) in hepatocytes (x64); D, liver section of rats treated with antioxidants mixture (vitamin C, vitamin E and silymarin) after having been sub-chronically exposed to SPD for 4 wk showing significantly reduced degenerative changes in hepatocytes and few inflammatory cells infiltration (x64).

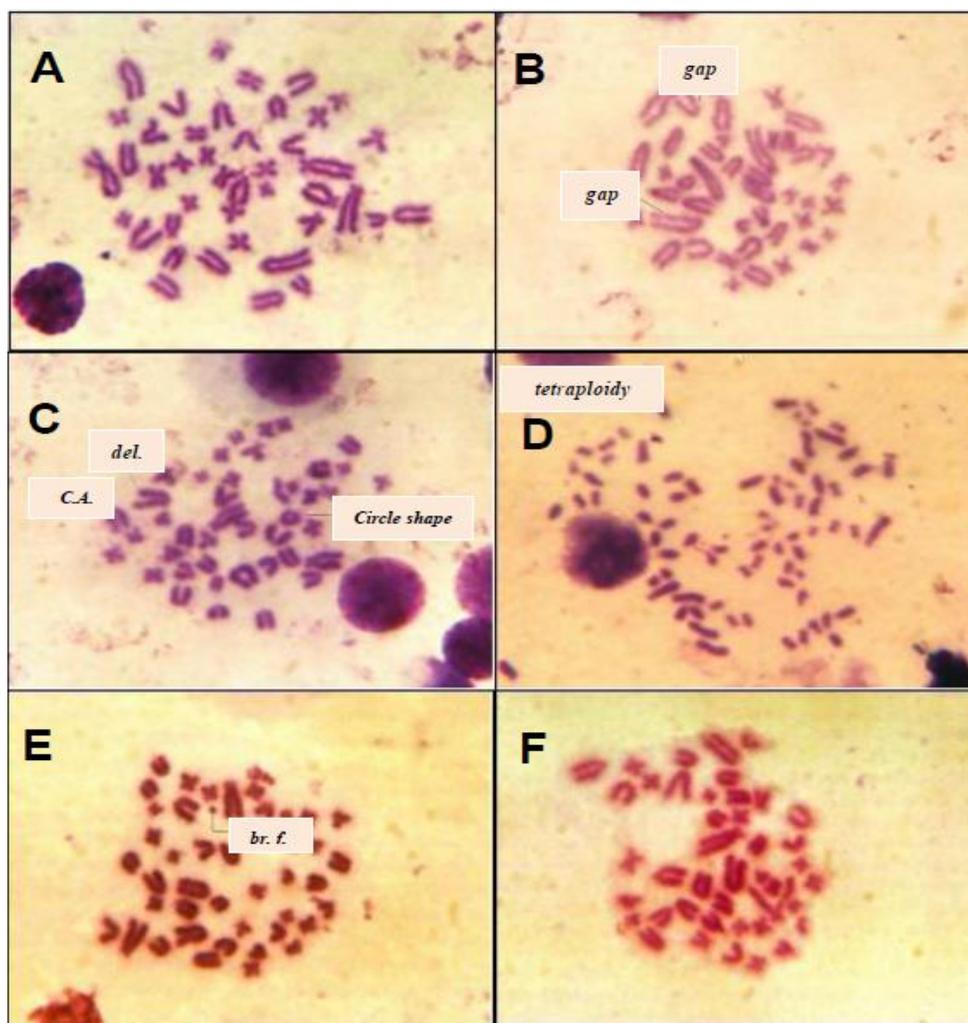


**Figure 3.** Internucleosomal DNA fragmentation in spinosad-exposed rats and the protective effect of the treatment with antioxidant mixture (AM). Lane 1, untreated control; Lane 2, DNA from rat hepatocytes exposed to SPD for 4 weeks; Lane 3, DNA from rat hepatocytes treated with antioxidant mixture (AM) after having been exposed to SPD for 4 weeks; M: represents molecular DNA maker expressed in base pairs.

**Table 3.** The number and percentage of metaphase with chromosomal aberrations in bone marrow cells of rats treated with spinosad alone or with antioxidants mixture (vit C, vit E and silymarin) after 4 weeks of treatment.

Group	Mean $\pm$ S.E		No. and (%) of metaphase				
	Including gaps	Excluding gaps	Chromatid gap	Break or fragmentation	Deletion	Centromeric attenuation	Tetraploidy
1 <sup>st</sup> = Control	4.2 $\pm$ 0.60	2.0 $\pm$ 0.45	11 (2.4)	3 (0.6)	2 (0.4)		5 (1.0)
2 <sup>nd</sup> = SPD	9.8 $\pm$ 0.80**	7.4 $\pm$ 1.08*	12 (2.4)	20 (4.0)	6 (1.2)	3 (0.6)	8 (1.6)
3 <sup>rd</sup> = SPD+AM	7.2 $\pm$ 0.37*	5 $\pm$ 0.31*	11 (2.2)	9 (1.8)	3 (0.6)	3 (0.6)	10 (2.0)

P values were: \* $P < 0.05$ ; \*\* $P < 0.01$  student's *t*-test. Each value represents the mean of 8 rats  $\pm$ SE. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; as compared with control. SPD= spinosad, AM= antioxidants mixture.



**Figure 4.** Effects of spinosad (SPD) on metaphase chromosomal aberrations in bone marrow cells of rat. A) metaphase image of showing normal chromosomes, B) image showing chromatid gaps caused by subchronic exposure to SPD for 4-week post-treatment, C) image showing deletions (del.), centromeric attenuation (C.A.) and circle shaped-chromosomes caused by subchronic exposure to SPD for 4-week post-treatment, D) image showing tetraploidy caused by subchronic exposure to SPD for 4-week post-treatment, E) image showing chromosomal break fragments (br. fr.) gaps caused by subchronic exposure to SPD for 4-week post-treatment, and F) image showing normal chromosome shapes in bone marrow cells treated with antioxidant mixture (AM) after having been exposed to SPD.

insecticides (OPIs), exert significant adverse toxicity in non-target species through the inhibition of AChE (Aboul-Soud et al., 2011; Al-Othman et al., 2011). The mode of action of SPD is characterized by an excitation of the nervous system with activation of nicotinic acetylcholine receptors (nAChRs), accompanied by effects on  $\gamma$ -amino butyric acid (GABA) receptor function and GABA-gated chloride channels (Salgado, 1998). It was observed in this study that, AChE enzyme activity was inhibited, as a result of the sub-chronic exposure of rats to SPD at  $1/20$  of  $LD_{50}$  for four weeks (Table 1), is consistent with the mode of action of chemicals that inhibit AChE (O'Brien et al., 1974). Inhibition of the AChE results in accumulation of acetylcholine (ACh) at cholinergic synapses to toxic levels leading to over-stimulation muscarinic and nicotinic receptors. This "cholinergic syndrome" includes signs and symptoms such as greater sweating and salivation, bronchoconstriction, and bronchial secretion, miosis, bradycardia, loss of reflexes, generalized convulsions, coma and central respiratory paralysis (Lassiter et al., 2003). The results of the study reported here suggest that supplementation with an antioxidants mixture (AM; vit C, vit E and sylimarin) exerted significant reversions in AChE inhibition (Table 1), particularly four *wpt*, which is indicative of the potential protective effect of the AM. The toxicity of OPIs might be due, at least in part, to formation of reactive oxygen species (ROS), which can result in lipid peroxidation (LPO). LPO is estimated by greater concentrations of thiobarbituric acid reactive substance (TBARS), which is assessed by its end product malondialdehyde (MDA) (Aboul-Soud et al., 2011). The observation of oxidative damage to the liver in rats exposed to a subchronic dose of SPD, as reflected by the significant increase in MDA production and the substantial inhibition in reduced glutathione (GSH) content (Table 1), is in agreement with the results of previous studies, which have also demonstrated a decrease in GSH content after exposure to OPIs and a significant correlation between AChE and GSH (Aboul-Soud et al., 2011). The results of the present study indicated that the 4 week SPD exposure induced oxidative stress, as demonstrated by compromised enzymatic antioxidant defences (that is Glutathione S-transferase, GST; superoxide dismutase, SOD) (Table 2). While activity of catalase (CAT) remained unchanged, significant induction in glutathione peroxidase (GPx) activity was observed (Table 2). Similar to the results observed in this study, SPD caused significant alterations in the glutathione-redox cycle in the form of significant decrease in total and reduced glutathione, large increase in GPx activity, little induction of glutathione reductase, and significant decline of GST activities, in two *in vitro* mammalian cellular models (Pérez-Peretejo et al., 2008). The presence of antioxidant agents reversed the toxic effect of SPD on enzymatic and non-enzymatic antioxidant component in the liver of rats (Table 1 and 2), which suggests a protective effect of AM against the

toxicity of SPD, including oxidative damage. It has been previously reported that the presence of antioxidant agents, such as GSH, vitamin C and E, were correlated with a significant reduction of the cytotoxic effect of SPD, in two *in vitro* mammalian cellular models (Pérez-Peretejo et al. 2008). This result is consistent with the histological findings in the present study, indicating that SPD-mediated liver injuries could be prevented by the treatment with the AM (Figure 2A to D).

In the present study it was demonstrated that concurrent treatment with AM prevents damage, caused by SPD, which suggests that the toxic effect of SPD on hepatocytes is mediated primarily through the formation of ROS. ROS are thought to be neutralized with AM, since damage was not observed in SPD-treated individuals that previously received AM. ROS can be detoxified by a battery of enzymatic defences, including SOD, CAT, and selenium-dependent GPx, or non-enzymatic systems by the scavenging action of GSH, while organic peroxides can be detoxified by the activity of GST (Halliwell and Gutteridge, 1999). Modulation of these enzymes and concentrations of GSH is important in the balance of the redox status through the reduction of ROS and peroxides produced in the organism, as well as in the detoxification of xenobiotics (Ramiro-Puig et al., 2007; Ramos, 2008).

ROS generation can, affect cell activity and function leading to apoptosis or necrosis inducing DNA damage or fragmentation. Our experimental findings indicate that SPD induced DNA damage in liver, as manifested by severe internucleosomal DNA fragmentation and the formation of a DNA ladder on agarose gel, hallmark of cells undergoing apoptosis (Figure 3). The mixture of antioxidants attenuated fragmentation of DNA, thus indicating a protective role of antioxidants in protecting genetic material. ROS can have diverse effects on mammalian cell growth and, even small quantities, are capable of directing cells to undergo apoptosis or programmed cell death (Ray et al., 1999; Finkel and Holbrook, 2000). Oxidative stress might be a common mediator of apoptosis (Bagchi et al., 1999) and might be promoting apoptotic-type DNA fragmentation (Ray et al., 1991; Sandstrom et al., 1994). Thus, the observed protective effect of AM might be mediated through ROS neutralizing capacity, whereby preventing damage to DNA and cellular components leading to the attenuation of apoptosis-related pathway (Sies and Murphy, 1991; Packer, 1991).

The observation of cytogenotoxic effects of SPD (Figure 4A to F) such as the 133% and 270% increase in the chromosomal aberration (CA) with and without chromatid gaps, in bone marrow cells, respectively, as compared to untreated control (Table 3) is consistent with the results of other studies (Hagmar et al., 1994). Those authors found that, SPD is mutagenic to male albino rats, including chromosomal aberrations, damage which has been associated with increased cancer risk. Genotoxic

risks of human exposure to pesticides remain a worldwide concern, because some pesticides are mutagenic and linked to the development of cancers (Leiss and Savitz, 1995). CA can be used as an early warning signal for cancer development. Greater incidences of CA have been associated with increased cancer risk and it has been suggested that the detection of an increase in CA related to an exposure to genotoxic agents, may be used to estimate cancer risk (Hagmar et al., 1994). Treatment of SPD-exposed rats with AM resulted in reduction in the frequency of CA by 26.5% and 32.4% with and without chromatid gaps respectively, as compared to the positive control group which has received SPD alone (second group) (Table 3). It has been reported that ascorbate and alpha-tocopherol both inhibited the chromosomal aberrations induced by both particulate and soluble chromate compounds (Blankenship et al., 1997), thus demonstrating the role of antioxidants in modulating the genotoxicity of xenobiotics. In summary, exposure of rat to SPD inhibited the activity of acetylcholinesterase, and depleted contents of reduced glutathione and malondialdehyde. Moreover, SPD caused significant inhibition of activity of key antioxidant enzymes (GST, SOD) and induction in GPx. Treatment with antioxidant mixture (AM, containing vitamin C, vitamin E and silymarin) attenuated all SPD-mediated effects. Histological examination of the liver revealed that SPD caused focal necrosis and degenerative changes in hepatocytes, along with cytoplasmic vacuolation. All of these lesions were significantly less in the rats fed AM. SPD accelerated formation of internucleosomal DNA fragmentation, which was attenuated by treatment with AM. Similarly, SPD caused significant structural chromosomal aberrations in bone marrow cells, the frequency of which was diminished by AM.

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