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Alleviative effect of some phytochemicals on cyadox-induced oxidative damage in rabbit erythrocytes

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Abstract

This study was carried out to evaluate the effects of different concentrations of cyadox (CYA), cinnamaldehyde (CIN) and resveratrol (RES) on rabbit isolated erythrocytes in two experiments. Experiment 1 evaluated the effects of different concentrations (2.5, 5, 10, 20, 40, 60, 80 and 100) μg/ml of CYA and μM of both CIN and RES separately on isolated erythrocytes in order to make the concentration response curve. Results indicated that CYA caused a significant depletion of reduced glutathione (GSH) content compared to control at 40 μg/ml and the depletion increased by increasing the concentration. On the other hand, both RES and CIN showed a highly significant elevation of GSH content at 40 μM being more effective by increasing concentrations. Experiment 2 investigated the potential benefits of using CIN and RES separately or in combination on CYA induced alterations in isolated rabbit erythrocytes (the used concentrations were based on the results of experiment 1). Results revealed that exposure to CYA caused a significant decrease in superoxide dismutase (SOD) and catalase (CAT) activities and reduced glutathione (GSH) and total protein (TP) contents, CYA also elevated extracellular hemoglobin (Hb) and adenosine triphosphate (ATP), increased the malonaldehyde (MDA) and protein carbonyl (PrC) contents with increasing caspase3 and caspase8 activities suggesting CYA pro-oxidant effect. Both CIN and RES were able to inverse these hazardous effects of CYA. However, CIN was more effective than RES, and their combination showed a positive synergistic effect in protecting cells against oxidative injury caused by CYA.

Key Words: Cyadox, phytochemicals, oxidative stress, response curve, erythrocytes.

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Introduction

Quinoxaline 1,4-dioxides (QdNOs) derivatives are one of the most important synthetic antimicrobial agents used worldwide as feed additives to promote growth and feeding behaviors of different animal species at sub therapeutic levels since the use of high doses for long periods usually causes hazardous effects for both animals and human (Carta *et al.*, 2005). Olaquindox (OLA), carbadox (CBX), mequindox (MEQ), quinocetone (QCT), and cyadox (CYA) are the known members of QdNOs class. QdNOs derivatives were reported to have in vitro mutagenic potential, induce DNA damage and increase the percent of micro-nucleated cell culture (Jin *et al.*, 2009), carcinogenic, developmental and reproductive toxicities and adrenal toxicity in male rats due to oxidative injury (Huang *et al.*, 2009). Compared with other QdNOs derivatives, CYA has been found to be less toxic in mutagenicity tests (Huang *et al.*, 2008). However, CYA showed different signs of toxicity in piglets (Nabuurs *et al.*, 1990) and had a teratogenic potential and reproductive toxicity in rats (Wang *et al.*, 2011). CYA has caused sub-chronic toxic effects in dogs (Wang *et al.*, 2015). Some primary metabolites of CYA showed significant decrease in body weight and change in clinical serum biochemistry in addition to pathological changes in liver of rats (Huang *et al.*, 2016). These undesirable effects could be attributed to the generation of reactive oxygen species (ROS) as a result of QdNOs reduction during their metabolism (Liu *et al*, 2009).

In an attempt to reduce the oxidative stress induced by different types of chemical feed additives, herbal plants and their extracts are used to provide animals with antioxidant polyphenols to improve their health. Among the most abundant beneficial compounds that are used as feed additives for animal and poultry to reduce free radicals are resveratrol (3, 5, 4'-trihydroxy-trans-stilbene; RES), a stilbenes type aromatic phytoalexin which was predominantly found in grapes, peanuts, berries, *Yucca schidigera* and turmeric (Alagawany *et al.*, 2015) and cinnamaldehyde (3-phenyl-2-propenal; CIN) which presents in cinnamon (Faix *et al.*, 2009). Numerous in vitro and in vivo studies described different biological effects of resveratrol, including antioxidant, cardio-protective, anti-aging, anticancer, anti-inflammatory, immunomodulatory, antioxidants and metabolic modifying activities (Hao *et al.*, 2011).

Similarly, CIN has been demonstrated to have an antimicrobial, anti-platelet aggregating, vasodilatory, anticancer and immunomodulatory effects and it could inhibit the induction of nitric oxide synthase and nitric oxide in a dose-dependent manner (Vangalapati *et al.*, 2012).

The present study aims at exploring the benefits of using CIN, RES and their combination on CYA induced alterations in isolated rabbit erythrocytes where rabbits are one of the most important animals exposed to treatment by CYA as antimicrobial agent and the oxidative damage of could result in some pathological conditions like anaemia and cardiovascular diseases especially with using CYA for long periods. Erythrocytes were used as it is very sensitive to the peroxidation process owing to its high oxygen tension, membrane concentration of polyunsaturated fatty acids and redox active Hb molecules as recently reported by Ahmed and Beg (2013). Moreover, GSH was used for studying the concentration response curve as it is known to provide the primary antioxidant defense for the stored erythrocytes, protect membrane lipids and proteins and keep the stability of the membrane skeleton and survival of erythrocytes against oxidative damage.

Materials and methods

Chemicals: Cyadox (CYA, $C_{12}H_9N_5O_3$, molecular weight 271.23 gmol, CAS No: 65884-46-0, purity 98%) was obtained from Hangzhou Uniwise International Co., Ltd. (Zhejiang, China (Mainland). Resveratrol (3, 5, 4'-trihydroxy-trans-stilbene,

purity of 99%) and pure cinnamaldehyde (3-phenyl-2-propenal, purity ≥ 98%) were purchased from Oxford Laboratory Mumbai, India. All other chemicals were purchased from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade. Caspase3 and caspase8 assay kits were purchased from Biovision Inc. (MountainView, CA, USA). LDH Cytotoxicity Detection Kit (plus) (LDH) (Roche Applied Science, Mannheim, Germany).

Animals and care: Male New Zealand White rabbits obtained from rabbit farm of the faculty of agriculture of Zagazig University (aged 3 months with initial body weight of 2.00 ± 0.05 kg) were used.

DPPH• free Radical-Scavenging Activity of CIN and RES: The electron donation ability of cinnamaldehyde and resveratrol was measured by bleaching of the purple colored solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH˙) according to the method of Hanato *et al.* (1988).

Preparation of erythrocytes: Five ml of blood was collected aseptically from the ear vein of all animals in a graduated test-tube containing heparin to avoid coagulation. Blood was centrifuged at 3000 rpm for 10 min at 4°C in graduated tubes and the plasma and buffy coat were carefully removed. The erythrocytes were harvested by centrifugation after washing once with 0.9% NaCl solution and two times with ice-cold phosphate buffered saline (PBS: 145 mMNaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4) and finally suspended in PBS to obtain cell suspensions at 10 % hematocrit to be used for incubations (Yang *et al.*, 2006).

Treatment of erythrocytes:
Experiment 1: Concentration response curve: CYA, CIN and RES were primarily solubilized in a small amount of DMSO (not exceed 0.1%) and further diluted in PBS to get different concentrations (2.5, 5, 10, 20, 40, 60, 80 and 100) μg /ml of CYA and μM of both CIN and RES, where each concentration was incubated with erythrocyte suspension individually in groups of 5 samples at 37°C for 3 h and compared with control group that was treated with PBS with DMSO (0.1% v/v) in order to make the concentration response curve.

Experiment 2: This experiment was carried out based on the data obtained from Experiment 1. Seven groups (n = 5) of erythrocytes suspension were used. In particular, the 1st group kept as control treated with PBS with DMSO, 2nd group was subjected to CYA (40 μg /ml), 3rd group was incubated with CIN (40 μM), 4th group were subjected to RES (40 μM) while the 5th group was co exposed to CYA (40 μg /ml) and CIN (40 μM). The 6th group was co exposed to CYA (40 μg /ml) and RES (40 μM). The 7th group was exposed to CYA in combination with both CIN and RES at the same indicated concentrations. The reaction mixtures of different groups were incubated at 37°C for 3 h with gentle shaking every 15 min.

Assessment of antioxidant activities of erythrocytes:
Antioxidant indices: For measuring the antioxidant parameters, after incubation the mixtures were stored at −20°C and thawed one day later for RBCs lysis by osmotic pressure, then they were centrifuged and supernatants obtained and used for measuring the activities of superoxide dismutase (SOD) according to Misra and Fridovich, (1972), catalase (CAT) according to Aebi (1984), total reduced glutathione (GSH) according to Ellman, (1959).

Lipid peroxidation: Lipid peroxidation was assessed by determining erythrocyte MDA levels using the thiobarbituric acid method (Bartosz, 2004) and the reaction product was measured spectrophotometrically at 535 mm.

Protein oxidation: Protein carbonyl content was measured as an index of protein oxidation as described by Uchida and Stadtman, (1993).

Hemoglobin (Hb) and protein determination: The hemolysis process of erythrocytes was monitored by hemoglobin (Hb) release. After incubation time periods, 50 ml of cell suspension was added to 1 ml of PBS and centrifuged (1000 g, 7 min). The Hb content of supernatants was measured by absorbance at 540 nm using the Varian Cary 50 UV–vis spectrophotometer. While total protein (TP) concentrations in the hemolysates was determined spectrophotometrically by standard kits based on the colorimetric biuret method.

Measurement of erythrocyte release of ATP: The procedure for measurement of the ATP content in the reaction mixture was based on the reactions described by Adams, (1963).

Membrane toxicity assay: The membrane toxicity can be rated by quantifying the liberation of the intracellular enzyme lactate dehydrogenase (LDH) into the supernatant using cytotoxicity detection Kit (plus) (LDH) according to the manufacturer's instruction.

Determination of caspase activities: The proteolytic activities of caspase3 and caspase8 were evaluated in erythrocyte lysates using the fluoro-metric substrates DEVD-AFC (caspase-3 substrate) and IETD-AFC (caspase-8 substrate), following the protocols of the caspase activity assay kits.

Statistical analysis: The experiment was carried out as a completely randomized design. Data were statistically analyzed using GLM procedure SAS (SAS Institute Inc., 2001). Orthogonal polynomial contrasts were used to test the linear and quadratic effects of the increasing levels of supplements to isolated rabbit erythrocytes. The differences among means were determined using the post-hoc Tukey's test. Statements of statistical significance are based on $P < 0.05$, unless otherwise stated.

Results

DPPH· Radical-Scavenging Activity of CIN and RES

The results of DPPH· radical-scavenging activities of CIN and RES are represented in (Fig. 1). The results indicated that CIN and RES exhibited antioxidant activity. The CIN showed relatively high antioxidant activity (93.00%) than RES (90.12%) compared to the radical scavenging activity of TBHQ (98.65%) after 120 min.

Results of experiment 1: Concentration response curve

The results indicated that the first concentration of CYA caused a significant depletion of GSH content compared to control (40 μg/ml) and the degree of depletion increased by increasing concentration. Thus, from the toxicological point of view 40 μg/ml was used in the second experiment to study the toxic effect of CYA. On the other hand, both RES and CIN began to show a highly significant elevation of GSH level at 40 μM being more effective by increasing the concentrations, but from the economical point of view we choose this concentration to study their modulatory effects on CYA exposed erythrocytes (Fig. 2).

Results of experiment 2

The effect of CYA on antioxidant indices

Results in Table 1 showed that CYA at a dose of (40 μg/ ml) inhibited the activities of SOD and CAT and decreased GSH concentration linearly and quadratically ($P < 0.001$) compared to control and other experimental groups. Incubation of erythrocytes with CIN or RES (40 μM) separately significantly ($P < 0.001$) increased the activities of SOD and CAT as well as the concentration of GSH to levels better than control, where CIN showed the height values followed by RES then control. In addition, CIN+CYA resulted in a linear and quadratic ($P < 0.001$) improvement in the antioxidant capacity of erythrocytes than CYA+RES in which GSH concentration did not

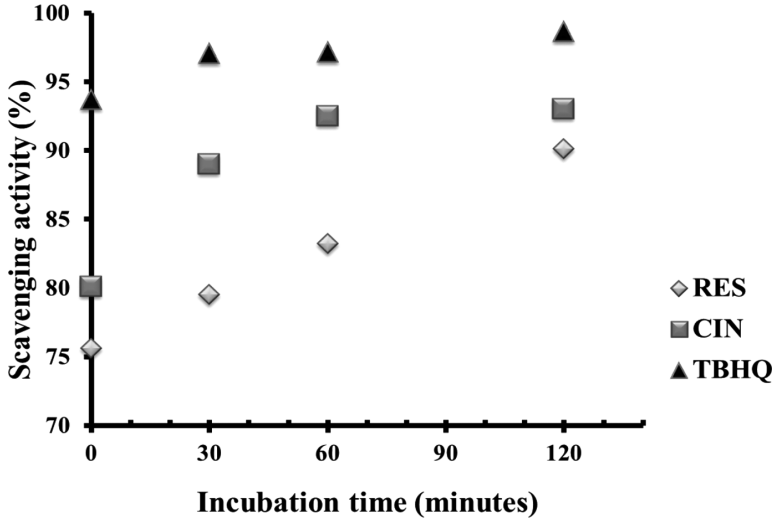

Fig. 1. Scavenging activity of CIN and RES against DPPH radical compared with TBHQ. (n = 5/group).

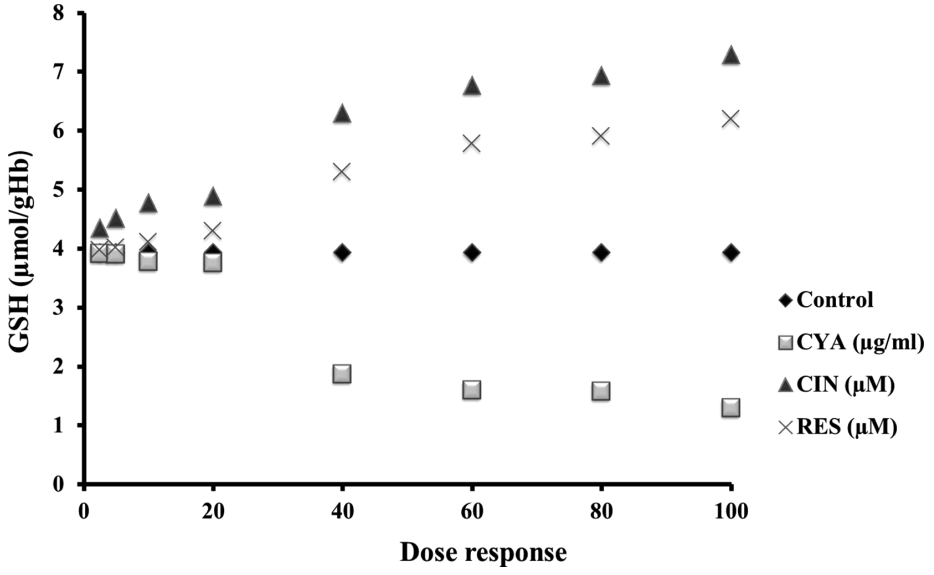

Fig. 2. The concentration response curve of erythrocytes exposed to cyadox (CYA), cinnamaldehyde (CIN), resveratrol (RES) in comparison with control. (n = 5/treatment).

statistically differ than CYA group. Meanwhile, CYA+CIN+RES showed results comparable to control.

The effect of CYA on lipid and protein oxidation

Lipid peroxidation measured by the MDA concentration and protein oxidation represented by PrC content were significantly ($P < 0.001$) increased in erythrocytes incubated with CYA compared with control and the other groups (Table 1). Incubation of erythrocytes with CIN or RES (40 µM) individually resulted in a linear and quadratic ($P < 0.001$) decrease in the MDA and PrC contents to being lower than control values, where CIN resulted in a much reduction in concentration than RES. CIN+CYA resulted in a linear and quadratic ($P < 0.001$) decrease in the MDA and PrC contents than CYA+RES. Meanwhile, CYA+CIN+RES succeeded to minimize the MDA and PrC contents to control values.

Table 1. Effects of CIN, RES and their combinations on antioxidant enzymes and lipid and protein peroxidation of rabbit erythrocyte exposed to CYA, (n = 5)

Treatment[1]	Parameters[2] (Mean ± SE)				
	CAT (IU/g Hb)	SOD (IU/g Hb)	GSH (μmol/g Hb)	MDA (μmol/g Hb)	PrC (μmol/g Hb)
Control	1465 ± 12.37[c]	879 ± 8.76[d]	3.94 ± 0.03[c]	2.10 ± 0.03[b]	5.70 ± 0.03[c]
CYA	1372 ± 10.06[d]	729 ± 9.35[e]	1.87 ± 0.02[f]	3.74 ± 0.05[a]	7.72 ± 0.03[a]
CIN	2069 ± 17.32[a]	1262 ± 13.25[a]	6.31 ± 0.05[a]	1.40 ± 0.03[d]	3.80 ± 0.01[e]
RES	1742 ± 15.63[b]	1113 ± 10.59[b]	5.30 ± 0.05[b]	1.70 ± 0.01[c]	4.35 ± 0.05[d]
CYA with CIN	1462 ± 13.96[c]	1052 ± 10.79[c]	2.18 ± 0.03[e]	2.78 ± 0.01[b]	6.90 ± 0.01[b]
CYA with RES	1436 ± 15.37[c]	847 ± 11.41[d]	1.87 ± 0.01[f]	3.69 ± 0.06[a]	7.61 ± 0.06[a]
CYA with CIN+RES	1462 ± 11.66[c]	890 ± 12.94[d]	2.39 ± 0.02[d]	2.05 ± 0.01[b]	5.72 ± 0.01[c]
P-value[3]					
Linear	<0.001	<0.001	<0.001	<0.001	<0.001
Quadratic	<0.001	<0.001	<0.001	<0.001	<0.001

[1]CYA: Cyadox; RES: Resveratrol; CIN: Cinnamaldehyde
[2]CAT: catalase; SOD: superoxide dismutase; GSH: reduced glutathione; MDA: malonaldehyde; PrC: protein carbonyl
[3]Linear and quadratic effects of treatments.

Table 2. Effects of CIN, RES and their combinations on total protein (TP), hemoglobin (Hb) and adenosine triphosphate (ATP) of rabbit erythrocyte exposed to CYA, (n = 5)

Treatment[1]	Parameters[2] (Mean ± SE)		
	TP g/dl hemolysate	Hb g/dl hemolysate	ATP release (μmol/g Hb)
Control	7.24 ± 0.06[c]	12.75 ± 0.23[d]	4.62 ± 0.01[e]
CYA	3.59 ± 0.16[f]	16.27 ± 0.13[a]	7.60 ± 0.02[a]
CIN	8.25 ± 0.01[a]	11.92 ± 0.02[f]	3.50 ± 0.07[f]
RES	7.72 ± 0.01[b]	12.37 ± 0.05[e]	4.30 ± 0.07[e]
CYA with CIN	6.73 ± 0.01[d]	13.93 ± 0.01[c]	6.31 ± 0.06[c]
CYA with RES	5.83 ± 0.10[e]	14.39 ± 0.03[b]	7.17 ± 0.01[b]
CYA with CIN+RES	7.36 ± 0.15[c]	12.76 ± 0.01[d]	5.10 ± 0.27[d]
P-value[3]			
Linear	<0.001	<0.001	0.001
Quadratic	<0.001	<0.001	<0.001

[1]CYA: Cyadox; RES: Resveratrol; CIN: Cinnamaldehyde.
[2]TP: total protein; Hb: hemoglobin, ATP: adenosine triphosphate.
[3]Linear and quadratic effects of treatments.

The effect of CYA on hemoglobin content

The effect of CYA, RES, CIN and their combinations on Hb release are summarized in Table 2. Incubation of erythrocytes with CYA (40 μg/ml) showed linear and quadratic significant ($P < 0.001$) increase in Hb release in the reaction mixture compared to control. On the other hand, Hb was linearly and quadratically ($P < 0.001$) decreased by incubation with CIN or RES. CIN+CYA linearly and quadratically ($P < 0.001$) decreased the Hb content than CYA+RES. Both CIN and RES showed a synergistic effect against CYA, where treatment of erythrocytes with CYA +CIN+RES reduced the Hb content compared to control.

The effect of CYA on protein content:

Incubation of erythrocytes with CYA alters

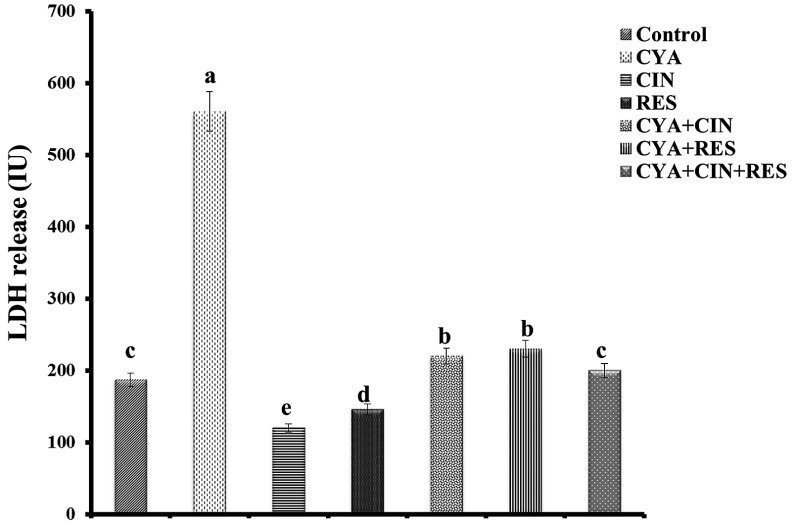

Fig. 3. Effects of CIN, RES and their combinations on LDH release of rabbit erythrocyte exposed to CYA. (n = 5/treatment).

the TP content of isolated erythrocytes ($P < 0.001$). On the other hand, TP content was both linearly and quadratically ($P < 0.001$) increased by incubation with CIN or RES, where the CIN achieved the highest TP content compared to the other groups. CIN+CYA linearly and quadratically ($P < 0.001$) increased the TP content than CYA+RES. CIN and RES exhibited a good synergistic effect by elevating the reduced TP content induced by CYA by increasing its level to be similar to that of control (Table 2).

The effect of CYA on ATP release

Incubation of erythrocytes with CYA (40 μg/ml) for 3 h produced linear ($P = 0.001$) and quadratic ($P < 0.001$) increase in the ATP content to be compared to control (Table 2). Incubation of erythrocytes with CIN or RES (40 μM) in absence of CYA resulted in a linear ($P = 0.001$) and quadratic ($P < 0.001$) decrease in the amount of released ATP to levels lower than control values however RES showed lower inhibition than CIN. CIN+CYA resulted in a linear and quadratic ($P < 0.001$) decrease in the ATP content than CYA+RES. Treatment of erythrocytes with CYA in combination with both CIN and RES showed a linear ($P = 0.001$) and quadratic ($P < 0.001$) decrease in ATP content better than CYA+CIN or CYA+RES.

The effect of CYA on LDH release

Erythrocytes incubated with CYA for 3 h exhibited marked significant elevation of LDH release ($P < 0.005$) compared to the experimental groups. Contrarily, CIN and RES significantly decreased LDH release from erythrocytes, where the lowest measured LDH (120 and 130 IU) was obtained after incubation with CIN and RES respectively compared to control and the other groups. CIN+CYA significantly reduced the release of LDH than CYA+RES and both were higher than control. The synergistic effect of CIN and RES against CYA was also observed where treatment of erythrocytes with CYA+CIN+RES reduced the LDH release to the level control (Fig. 3).

The effect of CYA on caspase activity

Exposure of erythrocytes to CYA led to the activation of caspase3 and caspase8. Incubation of erythrocytes with CIN or RES individually or in combination with CYA significantly decreased caspases activities. However, CIN showed advantage over RES in both treatments. The synergistic effect of CIN+RES on erythrocytes treated with CYA was clear on decreasing of both

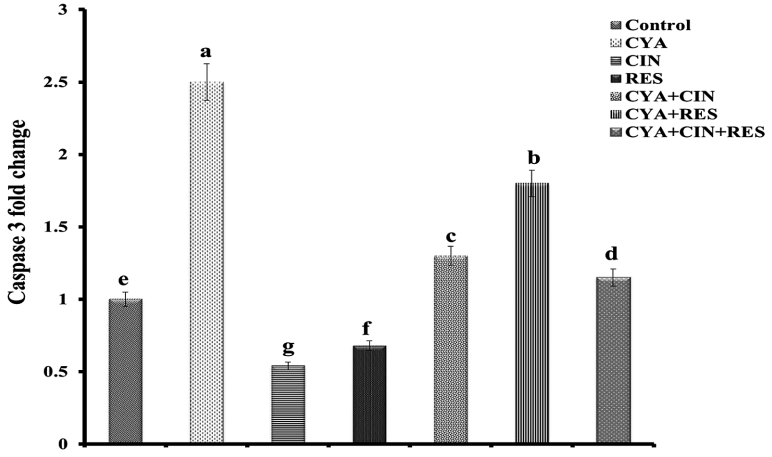

Fig. 4. Effects of CIN, RES and their combinations on caspase3 activity of rabbit erythrocyte exposed to CYA. (n = 5/treatment).

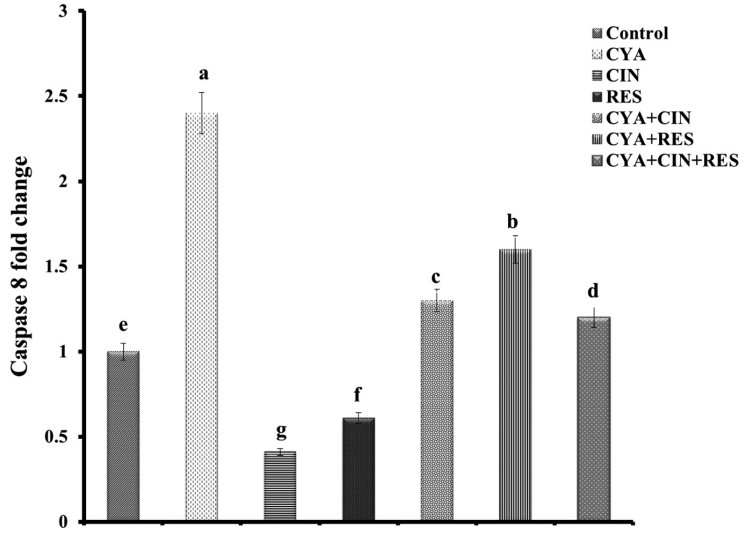

Fig. 5. Effects of CIN, RES and their combinations on caspase8 activity of rabbit erythrocyte exposed to CYA. (n = 5/treatment).

caspases (Fig. 4 and 5).

Discussion

This study was conducted to investigate the possible modulatory effects of CIN and RES as natural phytogenic additives against the hazardous impacts of CYA in isolated rabbit erythrocytes. Under normal physiological conditions, internal antioxidant enzymes such as SOD and CAT act as defense mechanisms against intracellular oxidative stress and provide cell protection by their free radical scavenging activities and elimination of ROS. In the present work, CYA caused a significant decrease in SOD and CAT activities in rabbit erythrocytes. This decrease may be returned to the ability of CYA to produce a short and unstable release of ROS, which was the key mediator of QdNOs-induced cell death and this agrees with the study of Liu *et al.*, (2009) who suggested that QdNOs displayed prooxidant activities on adrenocortical cells.

Furthermore, CYA induced depletion of the GSH level which is required to protect important proteins of RBCs against oxidation by maintaining

SH groups in Hb and enzymes in the reduced state, and binding with MDA and other deleterious endogenous substances (Hao *et al.*, 2011).

Depletion of GSH is accompanied with increased MDA content in CYA exposed group indicating the capability of CYA in producing a state of considerable erythrocytic oxidative injury as MDA is a highly reactive bifunctional molecule, that cross-link erythrocyte phospholipids and proteins to alter the functions of cell membrane leading to decreased erythrocytic survival and has been proposed as a general mechanism for cell injury (i.e induce hemolysis) (Banerjee *et al.*, 2008). This comes on line with the results obtained in the present study concerning the effects of CYA on increasing the released Hb and ATP and confirmed by the increased release of LDH which is considered as indicator of membrane toxicity. Moreover, these results suggest the role of CYA in induction of ROS in erythrocytes membrane and peroxidation of membrane lipids leading to hemolysis and altering the protein and lipid contents to different extents. Additionally, the interaction between MDA and CuZn-SOD of erythrocytes leads to the modification of histidine amino acid residues and the production of protein–protein cross-linked derivatives as a result each type of ROS gives a different protein oxidation pattern (Kwon *et al.*, 2000). This could explain the generation of protein carbonyl derivatives which could be also account for the decreased protein content of the rabbit erythrocytes after incubation with CYA.

Oxidative stress and impairment of antioxidant defense system observed in CYA exposed erythrocytes could be the main cause of activating the caspase8 which is a membrane-bound mediator initiating the cellular cascade for apoptosis and caspase3 that is the effector mediator leading to proteolysis of cellular proteins as reported (Mandal *et al.*, 2012).

Interestingly, incubating erythrocytes with CIN or RES with CYA either separately or in combination resulted in increased concentration of GSH and the activities of SOD and CAT and protein content in the rabbit erythrocytes while decreased the levels of MDA and PrC and the markers of hemolysis (Hb and ATP) and reduced the LDH release and decreased the caspase activities.

The results concerning the effects of RES on erythrocyte antioxidant defense system could be attributed to its $DPPH^{\cdot}$ radical scavenging activity observed in the present work and also by Lopez-Velez *et al.* (2003) who stated that resveratrol could provide cell protection due to antioxidant capacity. RES could act directly as an effective eliminator of free radicals and through balancing of hydroxyl phenolic groups, increasing the activities of antioxidant enzymes including SOD and CAT, glutathione S-transferase and NADPH quinine oxido-reductase, reducing lipid peroxidation or scavenging the generated ROS (Das, 2011). Our results are also in agreement with Mikstacka *et al.* (2010) where resveratrol prevented the reduction in GSH content and reduced lipid peroxidation in human erythrocytes.

RES has been also suggested to exert its antioxidant protection effects through decreasing the generation of ROS in some in vitro studies like (Vieira de Almeida *et al.*, 2008; Sayin *et al.* 2011) and in vivo (Liu *et al.*, 2014; Sridhar *et al.*, 2015). RES antioxidant activity is parallel with its $DPPH^{\cdot}$ radical scavenging activity observed in the present work which agrees with Ioanna *et al.* (2015).

In a like manner, CIN could reverse the undesirable effects of CYA by enhancing the activities of SOD and CAT, increasing GSH content and reducing lipid and protein oxidation. These results are in agreement with Subash-Babu *et al.* (2014) where CIN enhances the activity of antioxidant defense system against ROS produced under hyperglycemic conditions in animal providing protection to pancreatic β-cells. These effects may be returned to the ability of CIN to act as a potential source of antioxidant scavenging free radicals as reported in different in vitro models like $DPPH^{\cdot}$, superoxide, nitric oxide, H_2O_2 scavenging activity and reducing

power (Haripriya *et al.*, 2013). CIN also exhibit strong antioxidant capacity to scavenge free radicals of oxygen and lipids. Additionally, ROS release from lipopolysaccharide (LPS) stimulated J774A.1 macrophages was reduced by CIN (Chao *et al.*, 2008).

Our findings also showed that CIN was more effective than RES as antioxidant. Agents inducing lipid peroxidation cause the release of iron from the biological complexes that enhance peroxidative damage to lipids and consequent hemolysis. It is suggested that the protection against oxidative damage to erythrocyte membrane by CIN may be due to its antioxidant activity, and independently, to intracellular chelation of iron. Intra cellular chelation of iron could be excluded as a mechanism of antioxidant activity of RES where it inhibited Fe (II)-catalyzed lipid peroxidation mainly by scavenging lipid peroxyl radicals within the membrane, and that it cannot change the oxidation state of the metal in a way that would alter the Fe[II]/Fe[III] ratio (Tadolini *et al.*, 2000). This could explain the differences in response to the two phytogenic additives.

In conclusion, the impacts noted in the present study indicated that CYA have hazardous prooxidant effects on body cells. These results may be attributed to the possibility of this type of phytochemicals to generate ROS and a state of oxidative injury. Both, CIN and RES as phytochemical additives could be helpful in reducing the hazardous effects of CYA, keeping the normal function of the body cells and efficiently protecting the cells against oxidative injury suggesting their role in eliminating ROS which are responsible for lipid peroxidation, peroxidative hemolysis and aging of cells. Moreover, it was found that CIN and RES have valuable synergistic effects against the prooxidant activity of CYA.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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