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5

In vitro antioxidant activities of resveratrol, cinnamaldehyde and their synergistic effect against cyadox-induced cytotoxicity in rabbit erythrocytes

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Abstract

This study was conducted to explore the potential benefits of using cinnamaldehyde (CIN), resveratrol (RES) separately or in combination on cyadox (CYA)-induced alterations in isolated rabbit erythrocytes. Erythrocytes suspensions were partitioned into 7 groups (5 replicates/ group), 1st kept as control treated with phosphate buffered saline (PBS) with dimethyl sulphoxide (DMSO); 2nd group was subjected to CYA (40 mg/ml), 3rd group was incubated with CIN (40 mM), 4th group was subjected to RES (40 mM), 5th group was co-exposed to CYA (40 mg/ ml) and CIN (40 mM), 6th group was co exposed to CYA (40 mg/ml) and RES (40 mM), and 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 minutes. Our results revealed that exposure to CYA caused a significant decrease (linear and quadratic) in superoxide dismutase (SOD) and catalase (CAT) activities and the contents of reduced glutathione (GSH) and glutathione transferase (GST). Incubation of erythrocytes with CYA increased GSSG content, GSSG/GSH ratio, malonaldehyde (MDA) and protein carbonyl (PrC) concentrations while it

decreased the total protein (TP). CYA also lead to hemolysis and energy depletion of erythrocytes beside activation of caspase cascades, suggesting the pro-oxidant effect CYA that could be implicated in eryptosis. CIN and RES were able to inverse these hazardous effects of CYA. However, CIN was more effective than RES, their combination showed a positive synergistic effect in protecting the cells against oxidative injury caused by CYA.

Keywords: Cyadox, oxidative stress, cinnamaldehyde, resveratrol, apoptosis, erythrocytes

Introduction

Quinoxaline 1,4-dioxides (QdNOs) derivatives are one of the most important synthetic antimicrobial agents used world- wide as feed additives to promote growth and feeding behaviors of different animal species at subtherapeutic levels (Carta et al., 2005). Olaquindox (OLA), carbadox (CBX), mequindox (MEQ), quinocetone (QCT), and cyadox (CYA) are the known members of QdNOs class. The wide use of QdNOs derivatives in animal feed, particularly in high doses for long periods, usually causes hazardous effects for both animals and human (Markovic et al., 2000). OLA and CBX were reported to have in vitro mutagenic potential (Chen et al., 2008) in addition to carcinogenic, developmental and reproductive toxicities (Chen et al., 2009; Woodward, 2008). QCT induces DNA damage and increase the percent of micronucleated cell culture (Jin et al., 2009). MEQ has also been reported to cause adrenal toxicity in male rats due to oxidative injury (Huang et al., 2009). Compared with other QdNOs derivatives, CYA has been reported to be less toxic in mutagenicity tests (Huang et al., 2008). However, CYA showed different signs of toxicity in piglets (Nabuurs et al., 1990), displayed pro-oxidant properties in adrenocortical cells (Huang et al., 2010) and had a teratogenic potential and reproductive toxicity in rats (Wang et al., 2011). Moreover, Ihsan et al. (2013) reported a mutagenic potential of QdNOs on bacterial cells in the Ames test. 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).The involvement of ROS in the deleterious impacts caused

58 by QdNOs has been reported in some earlier studies (Azqueta et al., 2007; Chowdhury et al., 2004).
59 Due to the wide range and long-term use of CYA in food animals as a potential replacement for OLA
60 and CBX, it is necessary to perform a series of studies to understand its toxic characters, especially
61 the pro-oxidant activity as oxida- tive stress could be implicated in many toxicological, pathological
62 and physiological disturbances. Therefore, in the present study we choose the erythrocytes as a
63 biological lipid membrane model, which is very sensitive to the peroxidation process owing to the
64 high oxygen tension, high membrane concentration of polyunsaturated fatty acids and redox active
65 hemoglobin molecules as reported by Ahmad & Beg (2013). Oxidative stress resulted from exposure
66 of erythrocytes to some drugs and xenobiotics could trigger their programed death (eryptosis) and
67 disturbance of their antioxidant defense system (Lang et al., 2010). Oxidative stress could also
68 activate some cysteine proteases-caspases in erythrocytes which function either as initiators (as
69 caspase-8) in response to proapoptotic signals or as effectors (as caspase-3) present in considerable
70 amounts in erythrocytes, whereas other important mitochondrial cascades of apoptosis such as
71 caspase-9, Apaf-1 and cytochrome c are absent (Berg et al., 2001). Eryptosis can also be triggered by
72 osmotic shock and energy depletion in the cell (Lang & Qadri, 2012).
73 In an attempt to reduce the oxidative stress induced by different kinds of chemicals feed additives,
74 herbal plants and their extracts are used to provide animals with antioxidant polyphenolic
75 phytochemicals that could improve the animal health (Alagawany et al., 2015a). One of the most
76 abundant beneficial compounds is resveratrol (3,5,4'-trihydroxy- trans-stilbene; RES), a stilbenes-
77 type aromatic phytoalexin predominantly found in grapes, peanuts, berries, yucca schidigera and
78 turmeric (Alagawany et al., 2015b). The other well-known molecules, such as cinnamaldehyde (3-
79 phenyl-2-propenal; CIN) is present mostly in some herbs such as cinnamon (Faix et al., 2009).
80 CIN and RES are used particularly as feed additives in animal and poultry nutrition to reduce free
81 radicals. Numerous in vitro and in vivo studies described different biological effects of resveratrol,
82 including antioxidant, cardioprotective, anti-aging, anticancer, anti-inflammatory, imunomodulatory
83 as well as metabolic-modifying activities (Frojdo et al., 2007). The dietary supplementation of

84 resveratrol could decrease malonaldehyde (MDA) while increasing the levels of glutathione
85 peroxidase (GSH-Px), superoxide dismutase (SOD) (Hao et al., 2011).
86 Similarly, cinamaldehyde has been demonstrated to have an antimicrobial activity (Wang et al.,
87 2005), anti-platelet aggregating and vasodilatory action (VanderEnde & Morrow, 2001), anticancer
88 activity (Wu et al., 2005), immunomodu- latory effects (Guo et al., 2006) and could inhibit the
89 induction of nitric oxide synthase and nitric oxide in a dose-dependent manner (Lee et al, 2005).
90 To our knowledge, there is a lack of literature on the effects of RES, CIN or their combination on the
91 antioxidant defense system of the erythrocytes exposed to CYA. Thus, the present study aims at
92 exploring the potential benefits of using CIN and RES either individually or in combination on the
93 alterations that could be induced by CYA on isolated rabbit erythrocytes through measuring the
94 response of antioxidant defense system, hemoglobin and adenosine triphosphate (ATP) content,
95 adenine nucleotide pool size and adenylate energy charge in addition to the extent of lipid and protein
96 oxidation of erythrocytes, cell injury and apoptosis by measuring the LDH release and the induction
97 of caspase cascade activation. DPPH● radical has been widely used to test the radical scavenging
98 ability of various natural products (Gulcin et al., 2004). So, it is used in this study to determine the
99 free radical scavenging activity of both RES and CIN
100

101 **Materials and methods**

102 Chemicals

103 Cyadox (CYA, C12H9N5O3, molecular weight 271.23 gmol, CAS No: 65884–46-0, purity 98%)
104 was obtained from Hangzhou Uniwise International Co., Ltd. (Zhejiang, China). Resveratrol (3,5,40-
105 trihydroxy-trans-stilbene, purity of 99%) and pure cinnamaldehyde (3-phenyl-2-propenal, pur-
106 ity 98%) were purchased from Oxford Laboratory Mumbai, India. Kits of antioxidants were obtained
107 from Biodiagnostic, BD, Egypt. Caspase 3 and caspase 8 assay kits were purchased from Biovision
108 Inc. (Mountain View, CA). LDH Cytotoxicity Detection Kit (plus) (LDH) was purchased from Roche

Applied Science (Mannheim, Germany). All other chemicals were purchased from Sigma (St. Louis, MO). All other reagents used were of analytical grade.

Animals and care

Male New Zealand White rabbits (age of 3 months and initial weight of 2.00 ± 0.05 kg) were used for the experiment. They were obtained from rabbit farm of the Faculty of Agriculture of Zagazig University. Animals were individually housed in stainless steel cages at room temperature (25 ± 2 ◦C) with a relative humidity of 50–60% and on a 12 h light–darkn cycle. The animals had free access to commercial pellet diet and water ad libitum. The care and welfare of the animals conformed to the guidelines of the Animal Use Research Ethics Committee of Zagazig University, Egypt.

DPPH● free radical-scavenging activity of CIN and RES

The electron donation ability of cinnamaldehyde and resveratrol was measured by bleaching the purple-colored solution of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) according to the method of Hanato et al. (1988). For evaluation, 1 ml of cinnamaldehyde, resveratrol solution (2.5 mg dissolved in 1 ml of ethanol) was mixed with 1 mL of 0.2 mM DPPH● dissolved in ethanol. After incubation period of 30, 60 and 120 min at room temperature, the optical density of the solution was determined against a control at 517 nm using Jenway–UV–VIS Spectrophotometer. The DPPH● free radical was calculated from the absorption value by the following equation:

Antioxidant activityðInhibitionÞ%

¼ ½ðAcontrol — AsampleÞ=Acontrol] × 100

where A control is the absorbance of the control reaction and A sample is the absorbance in the presence of cinnamalde- hyde or resveratrol. Tertiary butylhydroquinone (TBHQ) was used as a positive control (40 mg/ml in absolute ethanol). Samples were analyzed in triplicate.

Preparation of erythrocytes

135 Five ml of blood was collected aseptically from the ear vein of all animals in a test-tube containing
136 heparin to avoid coagulation. Blood was centrifuged at 3000 rpm for 10 min at 4 OC, and the plasma
137 and buffy coat were carefully removed. The erythrocytes were harvested by centrifugation after
138 washing once with 0.9% NaCl solution and two times with ice-cold phosphate buffered saline (PBS:
139 145 mMNaCl, 1.9 mM NaH2PO4, 8.1 mM Na2HPO4) and finally resus- pended in PBS to obtain
140 cell suspensions at 10% hematocrit to be used for incubations. (Yang et al., 2006).
141

142 Treatment of erythrocytes
143 Cyadox (CYA), cinnamaldehyde (CIN) and resveratrol (RES) were primarily solubilized in a small
144 amount of DMSO (not exceed 0.1%) and further diluted in PBS till required concentrations (40 mg/ml
145 of CYA and 40 mM of both CIN and RES). These concentrations were selected on the basis of
146 concentration response curve (data not shown). Erythrocyte suspensions were partitioned into 7
147 groups (5 replicates/ group), 1st group was kept as control treated with PBS with DMSO; 2nd group
148 was subjected to CYA (40 mg/ml), 3rd group was incubated with CIN (40 mM), 4th group was
149 subjected to RES (40 mM), 5th group was co-exposed to CYA (40 mg/ml) and CIN (40 mM), 6th
150 group was co-exposed to CYA (40 mg/ml) and RES (40 mM), and 7th group was exposed to CYA
151 in combination with both CIN and RES at the same indicated concentrations. The reaction mixtures
152 of different groups were incubated at 37 ◦C for 3 h with gentle shaking every 15 minutes.
153

154 Assessment of antioxidant activities of erythrocytes
155 Antioxidant indices
156 After incubation, the mixtures were stored at 20 ◦C and thawed one day later for RBCs lysis by
157 osmotic pressure, then they were centrifuged and supernatants were obtained. Superoxide dismutase
158 (SOD) activity was measured accord- ing to Misra & Fridovich (1972). Catalase (CAT) activity was
159 determined according to Aebi (1984), where the decrease in hydrogen peroxide concentration was
160 measured spectrophoto- metrically at 240 nm during 1 min. Glutathione S-transferase (GST; EC

161 2.5.1.18) activity was determined spectrophoto- metrically according to Habig et al. (1974) using S-
162 2,4- dinitrophenyl glutathione (CDNB) as a substrate. Total reduced glutathione (GSH) contents were
163 measured by Ellman's reaction using 5,5-dithiobis 2-nitrobenzoic acid (Ellman, 1959), while
164 oxidized glutathione (GSSG) was measured by Hissin & Hilf's (1976) method, the results expressed
165 as mmol/g Hb and the ratio of GSSG and GSH were calculated by specific values in the samples.
166 Lipid peroxidation
167 Lipid peroxidation was assessed by determining erythrocyte MDA levels using the thiobarbituric acid
168 method (Bartosz, 2004) and the reaction product was measured spectrophoto- metrically at 535 mm.
169
170 Protein oxidation
171 Protein carbonyl content was measured as an index of protein oxidation, as described by Uchida &
172 Stadtman (1993). In brief, the experimental tube included 0.8 ml of the lysate/membrane sample in
173 isotonic buffer with an equal volume of 0.1% (w/v) 2,4-DNPH in 2N HCl, and the control tube
174 contained equal volume of the sample and 2N HCl. Both the sets were incubated at room temperature
175 for 60 min. After the incubation, 20% trichloroacetic acid was added and the contents centrifuged at
176 1900g. After washing with etha- nol:ethylacetate mixture (50:50), the residue was dissolved in 8 M
177 guanidine hydrochloride in 133 mMTris solution (pH 7.2) containing 13 mM EDTA and centrifuged
178 at 1900g. The absorbency of each sample was read at 365 nm in an UV/VIS spectrophotometer
179 (ELICO, Hyderabad, India, Model SL 159) against the control. The results were expressed as mmoles
180 of 2,4-DNPH-incorporated/mg protein based on a molar extinction coefficient of 2.1 104 M-1 cm-1
181 for aliphatic hydrazones.
182
183 Hemoglobin (Hb) and protein determination
184 Hemoglobin (Hb) and protein concentrations in the hemoly- sates were determined usingy standard
185 kits spectrophotomet- rically. The Hb assay was based on the colorimetric cyanomethemoglobin
186 method according to Drabkin (1946). Erythrocytes pellets were diluted in an alkaline medium

187 containing potassium cyanide and potassium ferricyanide (Drabkin's reagent). Hb oxidized to
188 methemoglobin combines with cyanide to form cyanomethemoglobin, which was measured at 540
189 nm using the Varian Cary 50 UV–Vis spectrophotometer. The protein assay was based on the
190 colorimetric biuret method, according to Bradford (1976).
191
192 Measurement of erythrocyte ATP content, adenylate pool size and adenylate energy charge
193 The procedure for measurement of the ATP content was based on the reactions described by Adams
194 (1963). 1 ml of the reaction mixture and 1 ml of TCA (12%) were placed into an Eppendorf tube,
195 mixed well and then cooled for 5 min (ice bath), before centrifugation at about 800g for 10 min to
196 obtain a clear supernatant. The supernatant (0.5 ml), 0.3 mg NADH (reduced form of a-nicotinamide
197 adenine dinucleotide), and 1.0 ml H2O were added into 1.0 ml of phosphoglyceric acid (PGA)
198 buffer.
199 Glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglyce- ric phosphokinase (GAPD/PGK)
200 enzyme mixture (0.04 ml) was combined with the above solution. After 10 min, absorbance was
201 measured against the blank at 340 nm. By ascertaining the absorbance decrease at 340 nm caused by
202 oxidation of the NADH to NAD, the original quantity of ATP could be gauged. These calculations
203 were then used to determine ATP concentration, with ATP expressed as mmol/ g Hb. To determine
204 the adenylates contents, erythrocytes were sedimented (3000 rpm at 4 ◦C for 3 min) and lysed in ice-
205 cold perchloric acid. The lysates were centrifuged at 13 000 g for 3 min and the supernatants were
206 neutralized with cold KOH. Intracellular ATP concentrations were quantified using luciferin–
207 luciferase assay kit, and ADP and AMP were measured as the difference after enzymatic conversion
208 to ATP (Yoshino et al., 1992). The adenylate energy charge (EC) was calculated by the equation
209 defined by Atkinson & Walton (1967) as follows: EC ([ATP] + 1/ 2[ADP])/([ATP] + [ADP] +
210 [AMP)].
211
212 Membrane toxicity assay

213 The membrane toxicity can be rated by quantifying the liberation of the intracellular enzyme lactate
214 dehydrogenase (LDH) into the supernatant. Briefly, after incubation Packed erythrocytes were
215 collected by centrifugation and 100 ml of supernatant from each treatment was transferred into a 96-
216 well flat bottom microtiter plate, which was then warmed to 37 ◦C. To assess the LDH liberation, the
217 Cytotoxicity Detection Kit (plus) (LDH) (Roche Applied Science, Mannheim, Germany) was used
218 according to the manufacture's instruction.
219
220 Determination of caspase activities
221 Erythrocytes were suspended in 100 ml of lysis buffer (50 mMTris–Hcl containing protease inhibitor
222 cocktail) and centrifuged at 2000 g for 10 min at 4 ◦C. The proteolytic activity of caspase 3 and
223 caspase 8 were evaluated in erythrocyte lysates using the fluorometric substrates Ac (N-acetyle)-
224 DEVD (Asp-Glu-Val-Asp) -AFC (7-amino-4- trifluoromethylcoumarin; Ac-DEVD-AFC) as
225 (caspase-3 substrate) and N-acetyl-L-isoleucyl-L-alpha-glutamyl-L- threonyl-N-[2-oxo-4-
226 (trifluoromethyl)-2H-1-Penzopyran7-yl]- L-alpha-asparagine; Ac-IETD-AFC) as (caspase-8
227 substrate), following the protocols of the Caspase Activity Assay kits. The activity was monitored as
228 the linear cleavage followed by release of the AFC side chain and was compared with a linear standard
229 curve generated on the same microplate.
230
231 Statistical analysis
232 The experiment was carried out as a completely randomized design. Data were statistically analyzed
233 using GLM procedure SAS (SAS Institute Inc., 2001). Orthogonal polynomial contrasts were used to
234 test the linear and quadratic effects of the increasing levels of CYA supplementation to isolated rabbit
235 erythrocytes.
236
237 **Results**
238 DPPH radical-scavenging activity of CIN and RES

239 The results of DPPH● radical-scavenging activities of CIN and RES are represented in Figure 1. The
240 results clearly indicated that CIN and RES exhibited antioxidant activity. CIN showed relatively high
241 antioxidant activity (93.00%) than RES (90.12%) compared to the radical scavenging activity of
242 TBHQ (98.65%) after 120 min.
243
244 The effect of CYA on antioxidant indices
245 The influences of CYA, RES, CIN and their combinations on antioxidant indices of the normal rabbit
246 erythrocytes are shown in Table 1. CYA at a dose of 40 mg/ml inhibited the activities of SOD and
247 CAT and significantly decreased the GSH and GST concentrations linearly and quadratically
248 (p50.001) compared to control and other treated groups, while increasing the GSSG and GSSG/GSH
249 ratio. Incubation of erythrocytes with CIN or RES (40 mmol/L) separately resulted in a linear and
250 quadratic (p50.001) increase in the activities of SOD and CAT and GSH and GST concentrations to
251 levels better than control itself, where CIN showed the heights values followed by RES then control.
252 In addition, incubation of erythrocytes with CIN in combination with CYA resulted in a linear and
253 quadratic (p50.001) improve- ment in the antioxidant capacity of erythrocytes than CYA + RES
254 where GSH concentration in this group did not statistically differ than the CYA group; however, both
255 the treatments did not reach the control values. Moreover, treatment of erythrocytes with CYA in the
256 presence of both CIN and RES showed results comparable with that of control. Incubation of
257 erythrocytes with RES, CIA or their combin- ations with CYA also significantly decreased the GSSG
258 and GSSG/GSH ratio than CYA alone in a considerable level, however did not reach the control value
259 (Table 1).
260
261 The effect of CYA on lipid and protein oxidation
262 Lipid peroxidation measured by the MDA concentration and protein oxidation represented by PrC
263 content were both linearly and quadratically (p50.001) increased in erythro- cytes incubated with
264 CYA (40 mg/ml) compared with control and all other treated groups (Table 1). Incubation of

265 erythrocytes with CIN or RES (40 mmol/L) individually resulted in a linear and quadratic (p50.001)
266 decrease in the MDA and PrC content to levels lower than control values, where CIN resulted in a
267 much reduction in their concentra- tions than RES. Incubation of erythrocytes with CIN + CYA
268 resulted in a linear and quadratic (p50.001) decrease in the MDA and PrC content than CYA + RES,
269 however both the treatments resulted more than the control levels. Meanwhile, treatment of
270 erythrocytes with CYA + CIN + RES succeeded to minimize the MDA and PrC content to the control
271 values.
272
273 The effect of CYA on hemoglobin content
274 The effect of CYA RES, CIN and their combinations on Hb content in the hemolysate of isolated
275 rabbit erythrocytes are summarized in Table 2. Incubation of erythrocytes with CYA (40 mg/ml) for
276 3 h linearly and quadratically (p50.001) increased the Hb content in the hemolysate compared to the
277 control. On the other hand, Hb content was both linearly and quadratically (p50.001) decreased by
278 incubation with CIN or RES, where the lowest Hb content (11.29 g Hb/dl hemolysat) was obtained
279 after incubation with CIN compared to control and all other treated groups. Treatment of erythrocytes
280 with CIN + CYA linearly and quadratically (p50.001) decreased the Hb content than CYA + RES;
281 however, both the treat- ments are still higher than the control levels. CIN and RES showed a
282 synergistic effect against CYA, where treatment of erythrocytes with CYA + CIN + RES reduced the
283 Hb content to the control level (Table 2).
284
285 The effect of CYA on protein content
286 Incubation of erythrocytes with CYA (40 mg/ml) alters the protein content of isolated erythrocytes
287 linearly and quad- ratically (p50.001). On the other hand, the protein content was both linearly and
288 quadratically ($p<0.001$) increased by incubation with CIN or RES, where the CIN achieved the
289 highest TP content compared to the control and all other treated groups (8.25 g/dL hemolysate).
290 Treatment of erythro- cytes with CIN + CYA linearly and quadratically ($p<0.001$) increases the TP

291 content than CYA + RES, however both the treatments resulted in levels lower than the control. CIN
292 and RES exhibited a good synergistic effect by elevating the reduced TP content induced by CYA,
293 where treatment of erythrocytes with CYA + CIN + RES increased the TP con- tent to be similar to
294 that of control (Table 2).
295

296 The effect of CYA on ATP content, adenylate pool size and adenylate energy charge

297 Incubation of erythrocytes with CYA (40 mg/ml) for 3 h produced 7.60 mmol/gHb linear ($p< 0.001$)
298 and quadratic ($p<0.001$) increase in the ATP release compared to the control which increased by 4.62
299 mmol/gHb. (Table 2). Incubation of erythrocytes with CIN or RES (40 mmol/L) in the absence of
300 CYA resulted in a linear ($p< 0.001$) and quadratic ($p<0.001$) decrease in the amount of released ATP
301 to levels lower than control values; however, RES showed lower inhibition than CIN. Incubation of
302 erythrocytes with CIN + CYA resulted in a linear and quadratic ($p<0.001$) decrease in the ATP
303 content than CYA + RES; however, both the treatments did not reach the control values. Treatment
304 of erythrocytes with CYA in combination with both CIN and RES showed a linear ($p < 0.001$) and
305 quadratic ($p<0.001$) decrease in the ATP content released, which was better than CYA + CIN or CYA
306 + RES.

307 Concerning the effects of the different treatments on the intracellular ATP contents, the only change
308 observed was in the CYA group where incubation of erythrocytes with CYA (40 mg/ml) for 3 h
309 produced 1.7 mmol/gHb linear (p 0.001) and quadratic (p50.001) decrease in the ATP content
310 intracellular compared to control which decreased by
311 2.4 mmol/gHb, while the ADP and AMP contents and adenylate energy charge (Figure 2) did not
312 significantly change among all the treated groups. Incubation of erythro- cytes with CYA
313 significantly decreased the adenylate pool size; however, it did not change in the other groups. RES,
314 CIA or their combination with CYA succeeded in restoring the total adenylate to the normal values,
315 suggesting their antioxidant capacity (Table 2).
316

317 The effect of CYA on LDH release

318 The effect of CYA, CIN and RES on the integrity of cell membrane was evaluated by measuring the
319 LDH release, as shown in Figure 3. Erythrocytes incubated with CYA for 3 h exhibited marked
320 significant elevation of LDH release (560.66; p50.005) compared to all the experimental groups. On
321 contrary, CIN and RES significantly decrease LDH release from erythrocytes, where the lowest
322 measured LDH (120 and 130 IU) was obtained after incubation with CIN and RES, respectively,
323 compared to control and all other treated groups. Treatment of erythrocytes with CIN + CYA
324 significantly reduced the release of LDH than CYA + RES and both are higher than the control levels.
325 The synergistic effect of CIN and RES against CYA was also observed, where the treatment of
326 erythrocytes with CYA + CIN + RES reduced the LDH release to the level control.

327

328 The effect of CYA on caspase activity

329 The effects of CYA on erythrocyte caspase activity are showed in Figures 4 and 5. Exposure of
330 erythrocytes to CYA leads to the activation of caspases (caspase 3 and caspase 8). Incubation of
331 erythrocytes with CIN or RES individually or in combination of CYA significantly decreased caspase
332 activities (caspase 3 and caspase 8); however, CIN showed advantage over RES in both the
333 treatments. The synergistic effect of CIN + RES on erythrocytes treated with CYA was clear on
334 decreasing the caspase activities; however, their activities did not return to normal values.

335

336 **Discussion**

337 Oxidative stress has been reported to be implicated in many alterations in the structure and function
338 of different body cells. It causes loss of membrane integrity and glycoproteins, alterations in signal
339 transduction pathways and triggers eryptosis caspase cascades, energy depletion, leading to cellular
340 abnormalities. Therefore, oxidative stress is very important in studies aimed at evaluating the drug
341 toxicity as well investigating the antioxidant capacity of natural com- pounds used as feed additives.

342 Under normal physiological conditions, internal antioxi- dant enzymes, such as superoxide dismutase
343 (SOD), catalase (CAT), act as defense mechanisms against intracellular oxidative stress. SOD and
344 CAT could protect hemoglobin from peroxidation by their free radical scavenging activities and
345 elimination of ROS via decreasing the potentially dangerous formation of H2O2 (Aebi, 1984; Evans
346 & Halliwell, 2001). Our results revealed that exposure of erythrocytes to CYA caused a significant
347 (linear and quad- ratic) decrease in both SOD and CAT activities, suggesting the ability of CYA to
348 produce a short and unstable release of ROS, which was reported to be the key mediator of QdNOs-
349 induced cell death (Huang et al., 2010; Liu et al., 2008, 2009). CYA also induced a significant
350 decrease of antioxidant potential as indicated by increasing progressive loss of GST concentration
351 associated with the depletion of GSH level and increased GSSG content and GSSG/GSH ratio in the
352 exposed erythrocytes, where GSH is known to protect membrane lipids and proteins against oxidation
353 and maintain the stability of the membrane skeleton and survival of erythrocytes against oxidative
354 damage by binding with malonldehyde (MDA) and other deleterious endogenous substances
355 preventing their harmful effects (Bukowska, 2003; Cimen, 2008; Yamamoto et al., 1985). The
356 increased GSSG and GSSG/GSH ratio could be returned to the increased oxidation of GSH by the
357 attack of free radicals that could be produced by CYA or by consump- tion of GSH during repair
358 processes such as the reduction of oxidized membrane-protein thiol groups, as described by Ko et al.
359 (1997).

360 Depletion of GSH was accompanied with increased MDA content-exposed group (a marker of lipid
361 peroxidation; LPO) in CYA (40 mg/ml), indicating the capability of CYA in producing a state of
362 considerable erythrocytic oxidative injury as MDA is a highly reactive bifunctional molecule that
363 cross- links erythrocyte phospholipids and proteins to alter the functions of cell membrane leading to
364 decreased erythrocytic survival, and has been proposed as a general mechanism for cell injury and
365 eryptosis (i.e. induce hemolysis) (Banerjee et al., 2008; Sugihara et al., 1991). This comes on line
366 with the results obtained in the present study concerning the effects of CYA on increasing the
367 extracellular Hb and ATP content and confirmed by the increased release of LDH, which is

368 considered as indicator of membrane toxicity. These results suggest the role of CYA in the induction
369 of ROS in erythrocytes membrane which is often the initial site of damage, and peroxidation of
370 membrane lipids causes hemolysis and alters the protein and lipid content to diGerent extents, in
371 agreement with May (1998). Additionally, the interaction between MDA and CuZn–SOD of
372 erythrocytes leads to the modification of histidine amino acid residues and the production of protein–
373 protein cross-linked derivatives, where each type of ROS gives a different protein oxidation pattern
374 (Kwon et al., 2000). This could explain the generation of protein carbonyl derivatives, which could
375 also be accounted for the decreased protein content of the rabbit erythrocytes after incubation with
376 CYA.
377 Oxidative stress and impairment of antioxidant defense system observed in CYA-exposed
378 erythrocytes could be the main cause of activating the caspase 8, which is a membrane- bound
379 mediator initiating the cellular cascade for apoptosis and caspase 3, that is, the effector mediator
380 leading to proteolysis of cellular proteins as reported (Mandal et al., 2012). The significant increase
381 in caspase 3 and 8 could be considered as a good indicator for the eryptotic effect of CYA. ATP is
382 used by erythrocytes to control deformation, maintain membrane shape, osmotic stability, asymmetry
383 of the membrane phospholipids, synthesis of glutathione and other metabolites, and to protect
384 hemoglobin, enzymes and membrane proteins against oxidative impairment (Van Wijk & Van
385 Solinge, 2005). This explains the decreased intracel- lular ATP and consequently the adenylate pool
386 size in erythrocytes exposed to CYA, where cells consumed ATP to compensate the reduced GSH
387 level. On the other hand, adenylate energy charges did not significantly differ among the different
388 groups.
389 The ATP depletion indicates the ability of CYA to induce changes in erythrocyte shape and alterations
390 in the submembrane skeletal-network proteins, these alterations have been reported to cause decrease
391 in filterability, deformations and increase in viscosity of blood, and consequently damage the tissue
392 by microvascular occlusion and local tissue ischemia (Rendell et al., 1992; Somer & Meiselman,
393 1993).

394 From all the above mentioned observations, we can conclude that CYA has the ability to trigger the
395 eryptosis of erythrocytes, and this effect is mainly related to oxidative stress exerted upon cells and
396 activation of caspases and energy depletion.
397 Interestingly, incubation of erythrocytes with CIN or RES either separately or in combination in the
398 presence of CYA resulted in increased concentration of GSH and GST and the activities of SOD and
399 CAT and protein content in the rabbit erythrocytes, while decreased the levels of MDA and PrC and
400 the markers of hemolysis (Hb and ATP) and reduced the LDH release and decreased GSSG/GSH
401 ratio and the caspase cascade activities while preserving the energy of the cells, suggesting the CIA
402 and RES antioxidant and modulatory properties which come in line with the obtained DPPH● radical
403 scavenging activities of both the polyphenolic compounds.
404 Resveratrol could provide cell protection against oxidative- stress-induced injury by increasing the
405 activities of antioxi- dant enzymes, including SOD and CAT, glutathione S-transferase and NADPH
406 quinine oxido reductase, as described by Young et al. (2000). Effective elimination of superoxide,
407 hydroxyl and metal-induced free radicals bal- ances the hydroxyl phenolic groups (Lopez-Velez et
408 al., 2003), decreasing ROS generation (Das, 2011).
409 RES has also been suggested to exert its antioxidant protection effects on erythrocytes by improving
410 the GSH content and reducing lipid peroxidation (Mikstacka et al., 2010). RES can activate redox
411 system of erythrocytes plasma membrane (Rizvi & Pandey, 2010), and act as a potent modulator of
412 erythrocytes membrane transporters (Pandey & Rizvi, 2014) and preserve the normal functioning of
413 erythro- cytes membrane ATPase (Wang et al., 2016).
414 Antioxidant protection effects of RES by decreasing the generation of ROS and hydrogen-peroxide-
415 induced cell death has also been reported in some in vitro studies on different types of cells (Brito et
416 al., 2006; Kode et al., 2008; Li et al., 2006; Sayin et al., 2011; Vieira de Almeida et al., 2008).
417 Similarly, RES could increase the oxidative enzyme activities, enhance antioxidant status, reduce the
418 lipid peroxidation level as well as improve the total antioxidant capacity in vivo (Liu et al., 2014;
419 Sahin et al., 2010; Sridhar et al., 2015). RES antioxidant activity is parallel with its DPPH● radical

420 scavenging activity observed in the present work, which agrees with the results of Lopez-Velez et al.
421 (2003) and Ioanna et al. (2015).
422 Similarly, CIN could reverse the undesirable effects of CYA upon erythrocytes by enhancing the
423 activities of SOD and CAT as well as increasing the GSH content with a significant reduction in lipid
424 and protein oxidation. These results are in agreement with Subash-Babu et al. (2014), who
425 demonstrated that cinnamaldehyde enhances the activity of antioxidant defense system against ROS
426 produced under hyperglycemic conditions in animal providing protection to pancreatic b-cells. These
427 effects may help cinnamaldehyde to act as a potential antioxidant, as it exhibited radical scavenging
428 activities in different in vitro models like DPPH, superoxide, nitric oxide, H2O2 scavenging activity
429 and reducing power (Haripriya et al., 2013). Cinnamaldehyde also exhibits strong antioxidant
430 capacity to scavenge free radicals of oxygen and lipids, as reported by Mathew & Abraham (2006).
431 Additionally, ROS release from lipopoly- saccharide (LPS)-stimulated J774A(0).1 macrophages was
432 reduced by cinnamaldehyde (Chao et al., 2008).
433 The obtained improvement in the antioxidant capacity of erythrocytes observed in this study after the
434 addition of RES and CIN separately or in combination in the presence of CYA suggests the
435 antioxidant effect of these phytochemical addi- tives and their important role in maintaining the
436 normal physiological conditions of the erythrocytes that are required for antioxidant defense systems
437 in eliminating ROS. The oxidative damage exerted by CYA upon erythrocytes in the form of
438 hemolysis and eryptosis could be due to some pathological conditions like anemia and cardiovascular
439 dis- eases, especially CYA could be used for long periods in animal feed.
440

441 **Conclusion**

442 The impacts noted in the present study indicated that CYA have hazardous pro-oxidant effects on
443 body cells. These results may be attributed to the possibility of this type of feed additive to generate
444 ROS and a state of oxidative injury and increasing the cell damage. CIN and RES as natural
445 phytogenic additives could be helpful in reducing the hazardous effects of CYA, keeping the normal

446 function of the body cells and efficiently protecting the cells against oxidative injury, suggesting their
447 role in eliminating ROS which are responsible for lipid peroxidation, peroxidative hemolysis and
448 aging of cells. CIN and RES also showed valuable synergistic effects against the pro-oxidant activity
449 of CYA.

450

451 **Declaration of interest**

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

454

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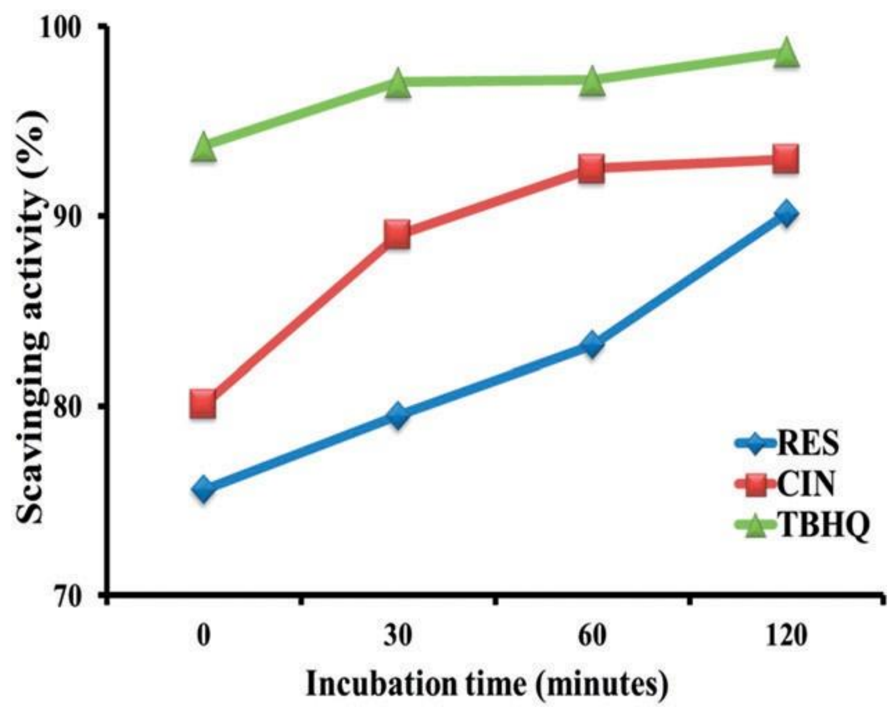

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640 **Figure 1.** Scavenging activity of cinnamaldehyde (CIN) and resveratrol (RES) (%) against DPPH radical compared with TBHQ.

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643 T

644 able 1. Effects of CIN, RES and their combinations on antioxidant enzymes and lipid and protein peroxidation of rabbit erythrocyte exposed to
645 CYA.

646

647

	Parameters (Mean ± SE)							
Treatment	CAT (IU/g Hb)	SO D (IU/g Hb)	GST (mmol/g Hb)	GSH (mmol/g Hb)	GSSG (mmol/g Hb)	GSSG/GS H ratio	MDA (mmol/g Hb)	PrC (mmol/g Hb)
Control	1465 ± 12.37^{c}	879 ± 8.76^{e}	2.10 ± 0.01^{b}	3.94 ± 0.03^{c}	0.55 ± 0.02^{c}	0.13 ± 0.01^{d}	2.10 ± 0.03^{b}	5.70 ± 0.03^{c}
CYA	1372 ± 10.06^{d}	729 ± 9.35^{f}	0.95 ± 0.02^{e}	1.87 ± 0.02^{f}	1.98 ± 0.01^{a}	1.05 ± 0.03^{a}	3.74 ± 0.05^{a}	7.72 ± 0.03^{a}
CIN	2069 ± 17.32^{a}	1262 ± 13.25^{a}	4.95 ± 0.03^{a}	6.31 ± 0.05^{a}	0.46 ± 0.03^{d}	0.07 ± 0.03^{e}	1.40 ± 0.03^{d}	3.80 ± 0.01^{e}
RES	1742 ± 15.63^{b}	1113 ± 10.59^{b}	4.73 ± 0.03^{a}	5.30 ± 0.05^{b}	0.43 ± 0.05^{d}	0.08 ± 0.01^{e}	1.70 ± 0.01^{c}	4.35 ± 0.05^{d}
CYA with CIN	1462 ± 13.96^{c}	1052 ± 10.79^{c}	1.89 ± 0.06^{c}	2.18 ± 0.03^{e}	0.68 ± 0.04^{b}	0.31 ± 0.05^{b}	2.78 ± 0.01^{b}	6.90 ± 0.01^{b}
CYA with RES	1436 ± 15.37^{c}	847 ± 11.41^{e}	1.76 ± 0.01^{d}	1.87 ± 0.01^{f}	0.71 ± 0.03^{b}	0.37 ± 0.04^{b}	3.69 ± 0.06^{a}	7.61 ± 0.06^{a}
CYA with CIN + RES	1462 ± 11.66^{c}	890 ± 12.94^{e}	2.00 ± 0.03^{b}	2.39 ± 0.02^{d}	0.62 ± 0.01^{b}	0.25 ± 0.01^{c}	2.05 ± 0.01^{b}	5.72 ± 0.01^{c}
P-value*								
Linear	50.001	50.001	50.001	50.001	50.001	50.001	50.001	50.001
Quadratic	50.001	50.001	50.001	50.001	50.001	50.001	50.001	50.001

648 CYA: Cyadox; RES: Resveratrol; CIN: Cinnamaldehyde; CAT: catalase; SOD: superoxide dismutase; GST: glutathione transferase; GSH: reduced glutathione; GSSG: oxidized glutathione; MDA:
649 malonaldehyde; PrC: protein carbonyl. *Linear and quadratic effects of treatments. Means in the same column within each classification bearing different letters are significantly ($P \leq 0.05$) different.

650

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

	Parameters (Mean ± SE)						
	Intracellular				Extracellular		
Treatment	ATP (mmol/ml RBCs)	ADP (mmol/ml RBCs)	AMP (mmol/ml RBCs)	Adenylate pool size	ATP release (mmol/g Hb)	TP g/dl hemolysate	Hb g/dl hemolysate
Control	2.4 ± 0.01[a]	0.25 ± 0.01	0. 028 ± 0.00	2.55 ± 0.02[a]	4.62 ± 0.01[e]	7.24 ± 0.06[c]	12.75 ± 0.23[d]
CYA	1.7 ± 0.02[b]	0.21 ± 0.01	0. 023 ± 0.00	1.9 ± 0.03[b]	7.60 ± 0.02[a]	3.59 ± 0.16[f]	16.27 ± 0.13[a]
CIN	2. 22 ± 0.01[a]	0.23 ± 0.03	0.027 ± 0.01	2.41 ± 0.07[a]	3.50 ± 0.07[f]	8.25 ± 0.01[a]	11.92 ± 0.02[f]
RES	2. 31 ± 0.03[a]	0.22 ± 0.01	0.022 ± 0.00	2.4 ± 0.06[a]	4.30 ± 0.07[e]	7.72 ± 0.01[b]	12.37 ± 0.05[e]
CYA with CIN	2. 29 ± 0.02[a]	0.23 ± 0.02	0.025 ± 0.00	2.5 ± 0.05[a]	6.31 ± 0.06[c]	6.73 ± 0.01[d]	13.93 ± 0.01[c]
CYA with RES	2. 16 ± 0.08[a]	0.20 ± 0.01	0.023 ± 0.00	2.49 ± 0.12[a]	7.17 ± 0.01[b]	5.83 ± 0.10[e]	14.39 ± 0.03[b]
CYA with CIN + RES	2. 30 ± 0.13[a]	0.24 ± 0.04	0. 026 ± 0.01	2.53 ± 0.09[a]	5.10 ± 0.27[d]	7.36 ± 0.15[c]	12.76 ± 0.01[d]
P-value*							
Linear	0.001	0.065	0.064	0.041	0.001	50.001	50.001
Quadratic	50.001	0.054	0.057	0.034	50.001	50.001	50.001

CYA: Cyadox; RES: Resveratrol; CIN: Cinnamaldehyde; ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphateTP: total protein; Hb: hemoglobin. *Linear and quadratic effects of treatments. Means in the same column within each classification bearing different letters are significantly ($P \leq 0.05$) different.

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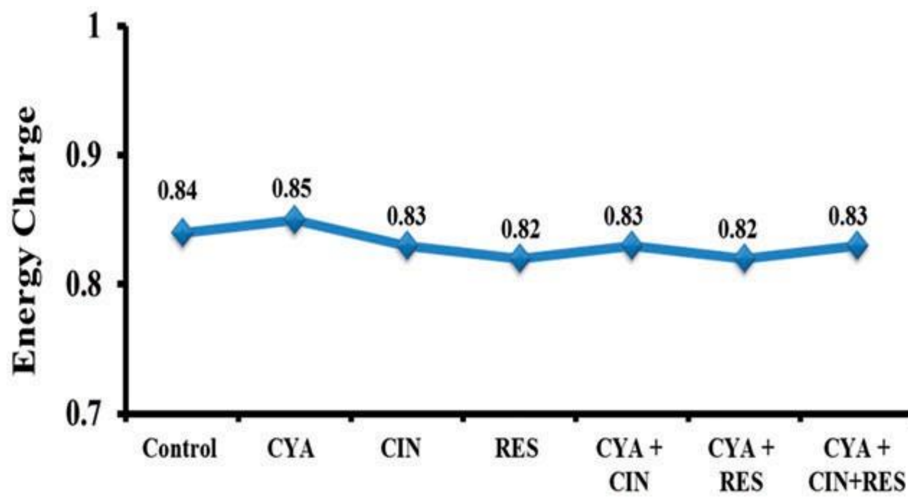

660 Figure 2. Effects of cinnamaldehyde (CIN), resveratrol (RES) and their combinations on adenylate

661 energy charge of rabbit erythrocyte exposed to CYA.

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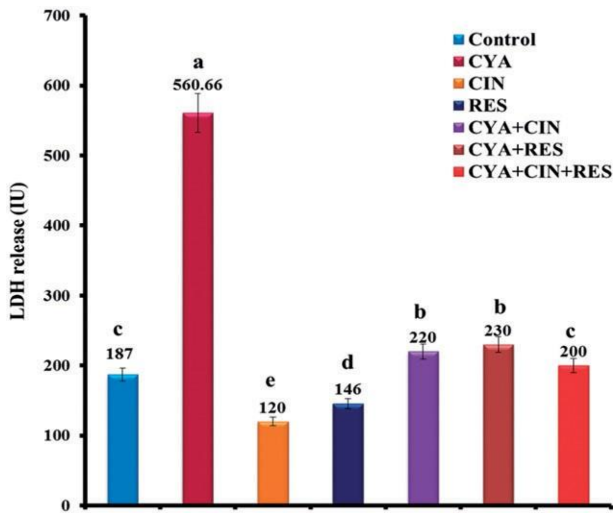

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664 Figure 3. Effects of cinnamaldehyde (CIN), resveratrol (RES) and their combinations on LDH (IU)

665 release of rabbit erythrocyte exposed to CYA.

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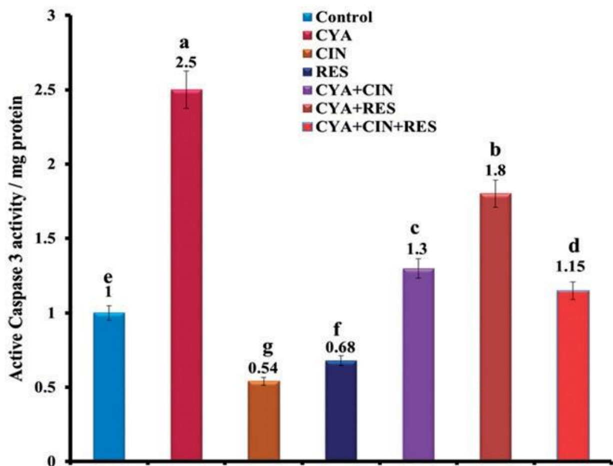


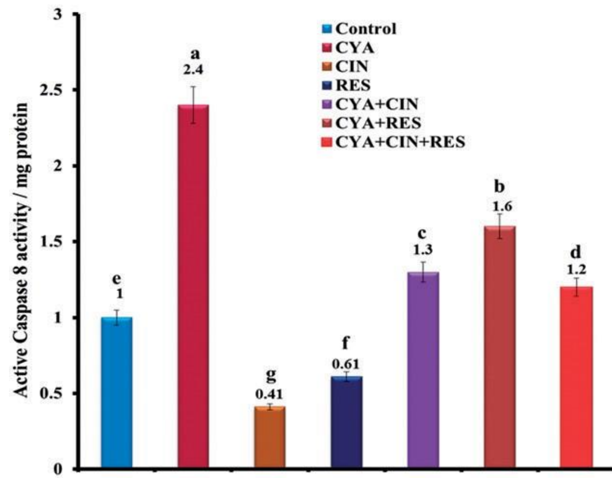
668 Figure 4. Effects of cinnamaldehyde (CIN), resveratrol (RES) and their combinations on active

669 caspase3 activity/mg protein of rabbit erythro- cyte exposed to CYA.

670

671 Figure 5. Effects of cinnamaldehyde (CIN), resveratrol (RES) and their combinations on active
672 caspase8 activity/mg protein of rabbit erythro- cyte exposed to CYA.

673



674