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# SYNTHESIS AND STUDIES ON ANTIOXIDANTS: ETHOXYQUIN (EQ) AND ITS DERIVATIVES

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**Abstract:** In our study ethoxyquin (EQ) and its two complexes with flavonoids were obtained from ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, EQ) and quercetin (EQ-Q, 1:1) or rutin (EQ-R, 1:1). Cytotoxicity of the tested compounds was studied using the trypan blue exclusion method and the properties of the studied compounds were also analyzed with the TUNEL method evaluating their ability to induce apoptosis. It was shown that EQ induced apoptosis in cultured human lymphocytes, especially at 0.25 and 0.5 mM concentrations. The same effects were also observed after the incubation of lymphocytes with EQ-Q and EQ-R, but the numbers of apoptotic cells observed were lower than for EQ.

**Keywords:** ethoxyquin; rutin; quercetin; TUNEL method; apoptosis; cytotoxicity

Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, EQ) is an antioxidant which is used to preserve vitamins and lipids in various food products and animal feeds. It is lipid-soluble and thus can accumulate in human and animal tissues. It is quickly absorbed from alimentary canal and its highest concentration is found in the liver, kidneys and fatty tissue (1,2). Recently, many unfavourable side-effects have been observed, mainly in dogs fed with EQ--containing feeds (3). Chromosome aberration test *in vitro* revealed that this substance induced chromosome aberrations (breaks, dicentrics, atypical translocated chromosomes, chromatid exchanges) in human lymphocytes and in Chinese hamster ovary cells (4,5). On the other hand, antimutagenic and anticarcinogenic properties of EQ were observed in different studies (6,7). However, due to the harmful effects observed in animals fed with EQ-containing feeds and to its high toxicity in many studies, analogues of EQ are searched and prepared and their properties and antioxidant efficacy are studied (8). Also, there were attempts to synthesize complexes of two biologically active molecules (9). In the present work we characterized new EQ derivatives (complexes with antioxidants: quercetin and rutin) and compared their ability to induce apoptosis.

## EXPERIMENTAL

### Chemical general methods

Preparative TLC separations were carried out on pre-coated silica gel plates (E. Merck $60F_{254}$). Flash column chromatography was performed using E. Merck Kieselgel 60 (230–400 mesh) for ethoxyquin compounds. The $^{13}C$ NMR spectra were obtained at 75 MHz with chemical shifts being referenced to TMS or the solvent peaks. Multiplicity of carbon signals was determined by DEPT experiments with a phase angle of 135°: (C) not observed; (CH) positive; ($CH_2$) negative; ($CH_3$) positive. Elementary analyses were performed in the Atlantic Microlab, Inc., Norcroos, Georgia, USA). Anhydrous solvents were freshly distilled under argon, $CH_2Cl_2$ over $CaCl_2$, and diethyl ether ($Et_2O$) over Na/benzophenone. Dry methanol was prepared by distillation from Mg $(OMe)_2$ [Mg turnings (4 g), $I_2$ (0.4 g), methanol (120 mL)]. Thin-layer chromatography was carried out on pre-coated (on plastic sheets) silica gel 60 F-254 (layer thickness 0.2 mm). The reactions were carried out in distilled water (under argon). Melting points were determined in open capillaries (1.5–1.8 mm) with a Mel-Temp (Cambridge) melting point apparatus and were uncorrected.

### Chemicals

The synthesis of ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, EQ, CAS number: 91-53-2, Figure 1) was described elsewhere (7). Crude ethoxyquin was cooled and purified twice by flash column chromatography with such solvents as $CH_2Cl_2$/methanol (50:1) and n-hexane/ethyl acetate (100:1) for elution. Most of the products were oils which were identified by NMR spectroscopy. Part of the product was a dimer – 1,8'-bis (1,2-dihydro-

-6-ethoxy-2,2,4-trimethylquinoline), and some part of it was a polymeric solid. The blue fluorescent band from TLC identified a colourless solid as 2,4-dimethyl-6-ethoxyquinoline (m.p. 85–87°C). The initial impurities were eluted by using flash chromatography (a gradient elution with n-hexane/diethyl ether (1 to 5%)). TLC (in n-hexane/ethyl acetate, 50: 1) of distilled EQ sample (Kugelrohr Büchi apparatus distillation) gave a yellow-coloured band at the top of the plate. EQ was a yellow oil; $n_D^{20}$ 1.570; IR (film, $cm^{-1}$) 3362s, 3023v, 2973s, 2870s, 1652v, 1579m, 1500s, 1477s, 1446s, 1389m, 1381m, 1261s, 1200s, 1155s, 1116m, 1051s, 956v, 930v, 869v, 804m; $^1$H NMR in $CDCl_3$ EQ (2 % solution) $\delta_H$ in ppm: 6.69 (dd, EQ-C<u>H</u>-5; J= 2.7 Hz; J= 0.7 Hz, 1H); 6.59 (dd, EQ-C<u>H</u>-7; J= 8.2 Hz; J= 2.7 Hz, 1H); 6.39 (dd, EQ-C<u>H</u>-8; J= 8.2 Hz; J= 0.7 Hz, 1H); 5.35 (m, EQ-C<u>H</u>-3; 1H); 3.95 (dd, EQ-C<u>H$_2$</u>-O-, 2H); 3.43 (bs, EQ-N<u>H</u>-, 1H); 1.96 (m, EQ-C<u>H$_3$</u>-4; 3H); 1.36 (t, EQ-C<u>H$_3$</u>-CH$_2$-O-; J= 7.0 Hz, 3H); 1.24 (s, EQ-C<u>H$_3$</u>--2, 6H); $^{13}$C NMR in $CDCl_3$ EQ (8% solution) $\delta_C$ in ppm: 151.2 (EQ-C-6); 137.6 (EQ-C-4); 129.5 (EQ--C-3); 128.5 (EQ-C-9); 122.8 (EQ-C-10); 114.6 (EQ-C-8); 113.6 (EQ-C-7); 111.1 (EQ-C-5); 64.2 (EQ-<u>C</u>H$_2$-O); 51.6 (EQ-C-2); 30.4 (EQ-<u>C</u>H$_3$-4); 18.5 (EQ-<u>C</u>H$_3$-4); 15.0 (EQ-<u>C</u>H$_3$-CH$_2$-O-); $^{13}$C NMR in $CF_3$-COOD EQ (10% solution) $\delta_C$ in ppm: 131.1 (EQ-C-6); 130.0 (EQ-C-9); 123.6 (EQ-C-3); 118.8 (EQ-C-4); 117.3 (EQ-C-10); 113.5 (EQ-C-8); 112.7 (EQ-C-7); 112.4 (EQ-C-5); 64.2 (EQ-<u>C</u>H$_2$-O); 57.8 (EQ-C-2); 24.0 (EQ-<u>C</u>H$_3$-4); 17.8 (EQ-<u>C</u>H$_3$-2); 14.4 (EQ-<u>C</u>H$_3$-CH$_2$-O-).

The following two compounds were synthesized: EQ with quercetin dihydrate (Q, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one dihydrate, Fluka, >98%, CAS number: 117–39–5; m.p. 314–315.5°C dec.) complex (1:1, EQ-Q) and EQ with rutin trihydrate (R, Fluka, ~90%, CAS number: 153–18–4, crystallized twice from dry methanol and once from dry ethanol, m.p. 188–189.5°C dec.) complex (1:1, EQ-R) (Figure 1). These compounds were obtained in the reaction of EQ (0.01 M; 2.17 g) in water-methanol solution (1:4, 250 mL) with quercetin (0.01 M, 3.38 g) or rutin (0.01 M, 6.01 g), respectively. The obtained solutions were heated to 55°C and mixed with activated carbon (3 g/100 mL). Then the hot solutions were filtered off and concentrated to about 20% initial volumes. After cooling (5–10°C), the compounds slowly crystallized. The crystalline products were filtered off and washed with cold dry methanol and again with diethyl ether and a dry methanol mixture (1:1) and then were nitrogen-dried (yield: EQ-Q 67%; EQ-R 71%). EQ-Q and EQ-R analytical samples (1 g) were obtained by lyophilisation from water-ethanol solutions (2:1; 20 mL) with the use of high vacuum system. The obtained complexes: EQ-Q (grey-yellow product, m.p. >165°C dec., 210°C strong dec.) and EQ-R (yellow-brown product, m.p. >180°C dec., > 200°C red-brown oil) slowly changed to brown (7–10 days) at room temperature. The compounds had broad melting points probably because of rearrangements during melting operation and loss of some water (first at over 80°C, and second time at over 140°C).

Anal. Calcd for EQ-Q $C_{29}H_{29}NO_8$ plus 1/4$H_2O$ ($C_{29}H_{29.5}NO_{8.25}$): C, 66.46; H, 5.67; N, 2.67, found: C, 66.36; H, 5.30; N, 2.62. Anal. Calcd for EQ-R $C_{41}H_{49}NO_{17}$ plus 1/2$H_2O$ ($C_{41}H_{50}NO_{17.5}$): C, 58.84; H, 6.02; N, 1.67, found: C, 58.64, H, 5.93, N, 1.55.

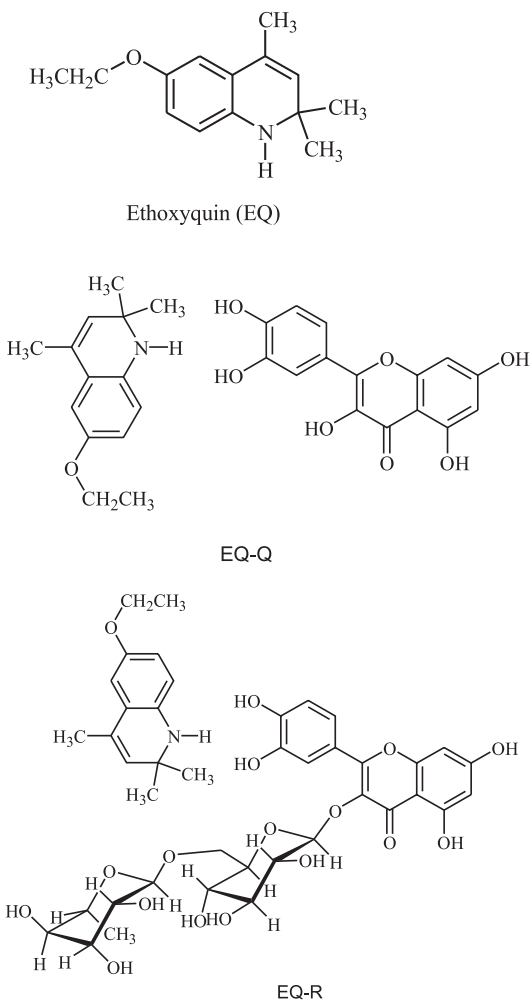


Figure 1. Chemical structures of the synthesized compounds: EQ, EQ-Q and EQ-R.

IR (KBr, $cm^{-1}$) EQ-R: 3362 (strong, 3600–3400 broad absorption; $NH_2^+$ stretching vibration), 2974 bs, 2929s, 2220–2550 bm ($NH_2^+$ combination band, broad weak absorption), 1653s, 1606 ($NH_2^+$ deformation vibration, sharp), 1580s, 1498s, 1477s, 1447s, 1357s, 1301s, 1261s, 1201s, 1156s, 1116s, 1054s, 957m, 870m, 807s.

$^{13}C$ NMR DMSO-$d_6$ (10% solution) EQ-R $\delta_C$ in ppm:

Aromatic rings – rutin: 177.6 (R-C-4); 164.3 (R-C-7); 161.4 (R-C-9); 156.9; 156.3; 148.6; 144.9; 141.8; 133.5 (R-C-3); 115.5 (R-C-2'); 115.3; 113.2; 104.1 (R-C-10); 98.9 (R-C-6); 93.8 (R-C-8).

Sugar carbons (glucose G and rhamnose Rh) – rutin: 101.8 (G-C-1); 100.9 (Rh-C-1); 76.6 (G-C-3); 76.0 (G-C-5); 74.3 (G-C-2); 72.1 (Rh-C-4); 70.8 (Rh-C-3); 70.5 (Rh-C-2); 70.0 (G-C-4); 68.3 (Rh-C--5); 66.9 (G-$\underline{C}H_2$-6); 18.2 (Rh-$\underline{C}H_3$).

Ethoxyquin carbons: 149.9 (EQ-C-6); 138.4 (EQ-C-9); 129.6 (EQ-C-3); 127.6 (EQ-C-4); 121.6 (EQ-C-10); 115.2 (EQ-C-8); 114.6 (EQ-C-7); 110.2 (EQ-C-5); 63.5 (EQ-$\underline{C}H_2$-O); 51.1 (EQ-C-2); 30.2 (EQ-$\underline{C}H_3$-4); 18.3 (EQ-$\underline{C}H_3$-2); 14.9 (EQ-$\underline{C}H_3$-$CH_2$--O-).

IR (KBr, $cm^{-1}$) EQ-Q: 3289 ($NH_2^+$ stretching vibration, broad strong absorption), 2972m, 2822–2520 ($NH_2^+$ combination band, broad weak absorption), 1660m, 1616s ($NH_2^+$ deformation vibration, strong), 1558m, 1513s, 1501s, 1445m, 1357s, 1319s, 1248s, 1201m, 1158s, 1092m, 1051vs, 1008v, 999m, 932m, 798m, 789m;

$^{13}C$ NMR DMSO-$d_6$ (8% solution) EQ-Q $\delta_C$ in ppm:

175.7 (Q-C-4); 163.7 (Q-C-7); 160.6 (Q-C-9); 156.0 (Q-C-2); 147.5; 146.7 (Q-C-3'); 144.9; 135.5 (Q-C-3); 121.9 (Q-C-6'); 119.8 (Q-C-1'); 115.5; 115.0; 105.2 (Q-C-10); 98.0 (Q-C-6); 93.2 (Q-C-8).

Ethoxyquin carbons: 151.9 (EQ-C-6); 138.1 (EQ-C-9); 130.3 (EQ-C-3); 128.9 (EQ-C-4); 123.2 (EQ-C-10); 114.8 (EQ-C-8); 114.7 (EQ-C-7); 110.8 (EQ-C-5); 64.5 (EQ-$\underline{C}H_2$-O); 51.5 (EQ-C-2); 28.8 (EQ-$\underline{C}H_3$-4); 17.8 (EQ-$\underline{C}H_3$-2); 14.5 (EQ-$\underline{C}H_3$-$CH_2$--O-).

**Lymphocyte isolation and culture**

Lymphocytes were isolated from peripheral blood obtained from two healthy non-smoking donors by the density gradient centrifugation with Histopaque-1077 (15 min., 280 g). The separated lymphocytes were washed in the culture medium RPMI 1640 and added to the medium ($0.8 \times 10^6$ cells per mL) containing 85% RPMI 1640, 15% fetal bovine serum, 100 IU/mL penicillin (each of Sigma) and 0.1 mg/mL PHA (Gibco).

**Cytotoxicity assay**

The viability of the cells after their 24-h treatment with the tested compounds was determined by the trypan blue exclusion assay. The compounds were added to lymphocyte cultures after 24 h from their beginning.

**The TUNEL assay**

The DeadEnd™ Fluorometric TUNEL System (Promega) was used to study the ability of the tested compounds to induce apoptosis. This system detects the fragmented DNA of apoptotic cells labelled at 3'-OH ends with fluorescein. The lymphocytes after 24 h in culture were treated with the tested compounds used at four concentrations. Lymphocytes were fixed on the slides with 4% paraformaldehyde (Polysciences) and then incubated (1 h, 37°C) with a mixture of terminal deoxynucleotidyl transferase (TdT) and fluorescein-labelled nucleotides. The analysis was performed using a fluorescence microscope (OLYMPUS, 520 nm filter). The nuclei of apoptotic cells were dyed green. The treatment of the slides with propidium iodide was used as a counterstaining for all cells. The percentage of apoptotic cells was calculated for 1000 cells in two independent experiments. Statistical analysis was performed using the exact Fisher test. Differences were considered as significant when $p < 0.05$.

## RESULTS

**Cytotoxicity assay**

To compare cytotoxicity of all tested compounds, the trypan blue exclusion assay was perfor-

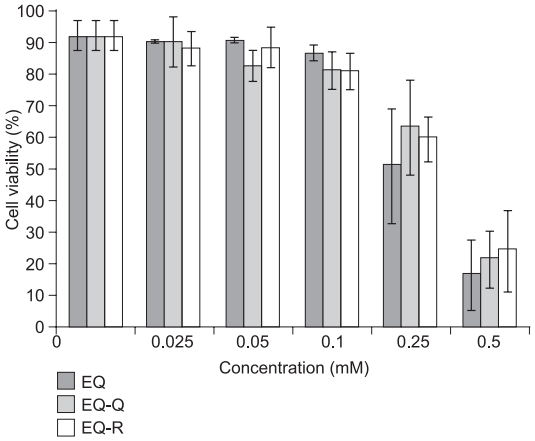


Figure 2. Lymphocyte viability (%) after 24 h treatment with EQ and its complexes with flavonoids determined by trypan blue exclusion assay.

med. The 24-h incubation of the lymphocytes with the agents showed that the cell viability was significantly decreased after their treatment with EQ, EQ--R and EQ-Q (Figure 2) used at the two highest concentrations (0.25 and 0.5 mM); the differences in comparison with the negative control were statistically significant ($p < 0.05$). The most cytotoxic compound was EQ, but the differences between cytotoxicity of the tested compounds were not very significant.

**TUNEL method**

Figure 3 shows a comparison of the ability of the tested compounds to induce apoptosis. The number of apoptotic cells observed was the highest after EQ application; the statistically significant increase in the number of apoptotic cells in comparison with the negative control was observed after using EQ at 0.1–0.5 mM concentrations. The dose-effect relationship was observed. Also, EQ-Q and EQ-R very effectively induced apoptotic cells (especially at 0.25 and 0.5 mM concentrations; statistically significant differences as compared with the control), but the numbers of apoptotic cells observed were lower than those after EQ application. The statistically significant differences between EQ and EQ-Q action were observed after two highest concentrations used (0.25 and 0.5 mM). Comparison of EQ and EQ-R effects revealed significant differences at all concentrations tested.

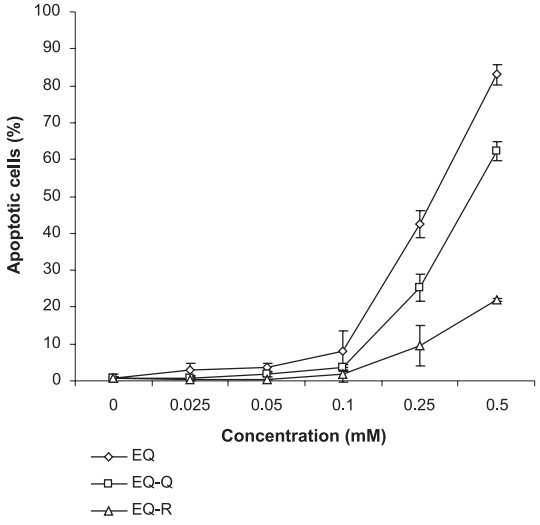


Figure 3. Comparison of the ability of the compounds tested (EQ, EQ-Q and EQ-R) to induce apoptosis in cultured human lymphocytes (the exposure time – 24 h).

**DISCUSSION**

EQ is the third class toxicity (moderate toxicity) compound according to Chemical Toxicology of Commercial Products list. The adverse health effects were observed in animals fed with EQ-containing feeds and in people working with it (10, 11). However, due to its high antioxidant properties and very low costs of production it is still used at reduced doses. A lot of research aims at finding new compounds, EQ analogues included, with similar antioxidant properties to replace EQ (8, 12). In the present paper we presented the results of the studies on cytotoxicity and the ability of EQ and two complexes of EQ with flavonoids to apoptosis induction in lymphocyte cultures. Apoptosis can be induced with many anticancer drugs (13) and this may be helpful in tumor treatment. In numerous researches, EQ inhibited anticarcinogenic properties e.g., preventing aflatoxin $B_1$-induced tumor in liver (15).

In our experiments, EQ was the most effective in induction of apoptosis of the compounds tested. Apoptosis was induced especially by high EQ concentrations used (0.1 – 0.5 mM). These EQ concentrations used caused also a significant decrease in viability of lymphocytes which was observed after their staining with trypan blue; thus decreased lymphocyte viability probably resulted from apoptosis process. Our present results obtained for EQ are in accordance with those observed in chromosome aberration test performed in our laboratory earlier (5). Statistically significant decrease (>50%) in mitotic index was observed in lymphocyte cultures following 24-h treatment with 0.25 mM EQ as compared to negative control. Decrease in nuclear division index caused by EQ was also observed in micronuclei test (data unpublished).

Synthesis of EQ complexes with flavonoids (rutin and quercetin) which are also antioxidants was prompted by the hope that combinations of the two compounds could increase antioxidative effect. This effect is examined in our other studies (data unpublished). In the present ones, it was observed that these compounds similarly as EQ alone induced apoptosis (mainly at 0.25 and 0.5 mM, 24-h exposure) but to a lesser degree than EQ. The lymphocyte viability observed after staining of cells with trypan blue was similar as for EQ, so some discrepancy is noticeable concerning the results of the two methods used. It should be noted that the TUNEL assay allows detection of early stages of apoptosis by labelling 3'OH ends of fragmented DNA. The late stages of apoptosis are not detected with this method; we also cannot observe necrotic cells. Thus further rese-

arch is necessary to elucidate cytotoxic effects of EQ and complexes of EQ with flavonoids.

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