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Antioxidant activity and genotoxic effect on HeLa cells of phenolic compounds from infusions of *Quercus resinosa* leaves

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ABSTRACT

Water infusions of mature and fresh *Quercus resinosa* leaves were evaluated for antioxidant activity and genotoxic effects on HeLa cells. Native Mexicans used to drink *Q. resinosa* leaves tea as a refreshing beverage. The air dried leaves were pulverised and boiled in water, then their phenolic content and condensed tannins were determined. The chromatographic profile of 15 phenolic components in *Quercus* leaves infusions was also determined by HPLC. In vitro analysis of antioxidant capacity of leaves infusion extracts were performed by the DPPH method and the deoxyribose assay. The genotoxicity of *Q. resinosa* leaves extracts was evaluated on HeLa cells as well as its underlying mechanism by the single-cell electrophoresis assay (comet assay). Results show that fresh leaves infusions increase the oxidative process and other damage to DNA in transformed human cells. Fresh leaves from *Q. resinosa* may serve as a potential source of phenolics with anticancer activity.

1. Introduction

The association between consumption of tea and the risk of cancer has been studied in different epidemiological studies (Su & Arab, 2002; Sun, Yuan, Koh, & Yu, 2006). The effects of dietary polyphenols are currently investigated due to their antioxidative and anticarcinogenic activities (Fresco, Borges, Diniz, & Marques, 2006; Scalbert, Johnson, & Saltmarsh, 2005). Proanthocyanidin content is a reasonable and important parameter for evaluating quality of green tea and related brand products. Proanthocyanidins is a family of complex polyphenol polymers widespread in nature, which occur in plant leaves. They are composed of monomeric flavan-3-ol units as catechins; in green tea the common compounds are epicatechin, epicatechi-3-gallate, epigallocatechin and epigallocatechi-3-gallate (Adhami & Mukhtar, 2006). Several studies have identified positive physiological effects and health promotion characteristics of tea polyphenols (Freese et al., 1999; Yang & Landau, 2000).

Plant polyphenols are well recognised for their antioxidant activities. These compounds scavenge free radicals and disrupt the free-radical chain reaction of lipid peroxidation. Oxygen free radicals are continuously formed as intermediates of enzymatic reactions during normal cellular functions and some may be involved in growth regulation and intercellular signalling (Rahman, Biswas, & Kirkham, 2006). As antioxidants, polyphenols may protect cell constituents against oxidative damage and therefore, limit the risk of various degenerative diseases associated with oxidative stress.

Polyphenols may act as suppressing agents, and inhibit the formation and growth of tumours from initiated cells because they inhibit cell proliferation *in vitro*. Aberrations in the regulation of a number of key pathways controlling cell proliferation are necessary for establishment of all tumours. Several antioxidants in plants have been suggested to contribute to the anticarcinogenic effect, and others such as flavanols, have also been able to inhibit cancer cell proliferation *in vitro* (Scalbert et al., 2005).

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Quercus resinosa leaves are used in the north of Mexico as a refreshing beverage. This tree plant is commonly known in Mexico as *Encino* (oak). Luna-José, Montalvo-Espinosa, and Rendón-Aguilar (2003) reported the use of oak leaves as food by several native Mexicans; *Tarahumaras* mix oak leaves with maize to make tortillas; *Otomies* use to cook scrambled eggs with oak leaves; *Tepehuanos* flavour *tesguino*, an ancient fermented corn drink, with oak leaves.

The presence of flavanols was studied recently as non-timber products from several species of *Quercus* (Almeida, Fernandes, Lima, Costa, & Bahia, 2008). However, the antioxidant and anticancer activities of *Q. resinosa* leaves are still unexplored. The therapeutic actions of herbal drugs, such as antimutagenicity, anticarcinogenicity, and antiaging, may be related to the antioxidant activity of their constituents. Therefore, it is important to evaluate the antioxidant effect of *Q. resinosa* leaves infusions. This paper reports the radical scavenging activity of lyophilised infusions of *Q. resinosa* leaves and their effect on proliferation and survival of HeLa cells. A preliminary evaluation of the phenolic composition of infusions was also made.

2. Materials and methods

2.1. Chemical reagents

Catechin, gallic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), DMEM (Dulbeccós Modified Eaglés Medium), Folin-Ciocalteu reagent, deoxy-D-ribose, were purchased from Sigma–Aldrich (Toluca, México).

2.2. Plant material

All *Q. resinosa* leaves were obtained from 10 trees located at Km 9.2–9.4 of Mezquital-Charcos Road in southern Durango, Mexico. Voucher Specimen No. 7144 was deposited at CIIDIR-IPN Durango Herbarium. Fresh (Qr1) and mature (Qr2) leaves were collected.

2.3. Preparation of herb infusions

Herb sample (10 g) was added to 250 mL boiling water and kept stirring for 10 min. Crude herb infusions were obtained by subsequent filtration and employed in the ensuing experiments. Two infusion extracts were obtained, EQr1 from fresh leaves, and EQr2 from mature leaves.

2.4. Polyphenol content

Total phenolic content in herb infusions was determined by the Folin-Ciocalteu method modified by Heimler, Vignolini, Dini, Vincieri, and Romani (2006) using catechin and gallic acid as the most representative phenolic standards, and expressing the results as catechin or gallic acid equivalents (mg/g of sample).

2.5. Proanthocyanidin content

It was determined as described by Brand-Williams, Cuvelier, and Berset (1995) using catechin as standard and expressing the results as catechin equivalents (mg catechin/g of sample).

2.6. High performance liquid chromatographic (HPLC) analysis

Individual phenolic acids and other simple phenolic compounds were separated and quantified using a high-performance liquid chromatography (HPLC) system with automatic injection (Agilent HP 1100 Systems, Agilent Technologies Inc., Santa Clara, CA, USA). HPLC chromatograph was equipped with an 1100 quaternary gradient pump, in-line degasser autosample, dual wavelength UV/VIS detector and acquisition system (Agilent Sofware 1100, Agilent Technologies Inc., Santa Clara, CA, USA). A reversed-phase Zorbax octadecylsilane column (150 × 4.6 mm, ODS-C18) from Agilent (Agilent Technologies Inc., Santa Clara, CA, USA) was used and operated at room temperature. The phenolic acids were eluted at 1 mL/min using a gradient systems consisting of two solvents: (A) acetic acid–water (2:98, v/v) and (B) acetic acid–acetonitrile–water (2:30:68, v/v/v). The starting mobile phases ratio was 90% A and 10% B; and 100% B at time 30 min. The separated compounds were identified at 280 nm, and quantified by diode array detection on the basis of chromatographic retention times and co-elution with pure standards. Twelve pure phenolic acids (*p*-coumaric, ferulic, benzoic, salicylic, syringic, gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, chlorogenic, 4-hydroxy-3-metoxybenzoic, caffeic) as well as catechin, epicatechin and vanillin were used for calibration and quantification.

2.7. Antioxidant capacity assays

2,2-Diphenyl-1-picrylhydrazyl radical cation assay. The antioxidant capacity was measured applying the DPPH scavenging method (Brand-Williams et al., 1995) with some modifications (Manzocco, Anese, & Nicoli, 1998). A calibration curve at 515 nm (UV/Vis Spectrophotometer Varian, Cary 50, Varian, Palo Alto, CA, USA) was made to calculate the remaining DPPH concentration in the reaction medium at several concentrations (0–1000 μg/mL). The EC_{50} value was obtained by plotting the percentage of residual DPPH at steady state as a function of the antioxidant concentration. A volume of 1.9 mL of DPPH in methanol was used. The reaction was started by the addition of 30 μL of experimental samples at EC_{50} for 60 min.

2.7.1. Deoxyribose assay

The hydroxyl radical scavenging was evaluated using the deoxyribose degradation assay (Re et al., 1999). The assay mixtures, containing the sample at several concentrations (1, 10, 100 and 1000 μg/mL) was used at a final volume of 1 mL, that is, 1 mM in deoxyribose, 24 mM in sodium phosphate (containing 15 mM NaCl, pH 7.4), 0.1 mM in $FeCl_3$, 0.1 mM in ethylendiaminetetraacetic acid (EDTA), 1 mM in H_2O_2 and 0.1 mM in ascorbic acid. After incubation at 37 °C for 1 h, colour development was promoted via addition of 1.5 mL of 28% (w/v) trichloroacetic acid (TCA) and 1.0 mL of 1% (w/v) thiobarbituric acid (TBA) in 0.05 M NaOH, followed by heating at 100 °C for 15 min. Inhibition of deoxyribose degradation was expressed as a percent of the decrease in absorbance, when compared to the control (assay without sample).

2.8. Cancer cells proliferation and survival studies

HeLa cells were grown in Dulbeccós Modified Eaglés Medium (DMEM, Hyclone) with 5% calf serum (Hyclone) and antibiotics. Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For cell proliferation assays, HeLa cells were transferred into 24 well plates at a density of 5×10^3 per well in 0.5 mL of DMEM with serum and antibiotics. After 24 h, the medium was changed by serum-free DMEM supplemented with 0.5% of bovine serum albumin (BSA) and antibiotics (proliferation control cells) and 5, 20, 40, 60, 80, 100, 120, 140, 160 and 200 μg/mL of extract. At this time, cells of two wells were counted in order to calculate the survival rate (survival control cells) (Garcia-Gasca, Paz-Gonzalez, Moncada-Alvarez, Blanco-Labra, & Salazar-Olivo, 2002). Incubation was continued for 24 h more and cells were

trypsinised and the cell suspension was counted with a Coulter Counter.

2.9. Comet assay

Two controls were used: dimethyl sulphoxide (1.5%) as negative control, and H_2O_2 (1000 μM) as positive control, both in PBS at pH 7. Each sample was added with 70 μL of ethidium bromure (2 μg/mL) and observed under a fluorescent microscope (Carl Zeiss, AxiosKop 40). The tail moment was reported according to Singh, McCoy, Tice, and Schneider (1988). Apoptosis results were evaluated by the comet assay method (Singh, 2000).

3. Results and discussion

Q. resinosa fresh leaves showed a distinctive reddish-deep green upper face and light green-reddish fuzzy lower face, while mature leaves showed an uniform light brown colour with minute fuzz at the lower face. Extract yields are reported in Table 1, indicating that infusions from EQr1 (45.32 ± 2.08%) produced 2.8 times more extractables than EQr2 (15.98 ± 0.63%). Recent experimental studies have recognised that infusions from tea (*Camellia sinensis*) exhibit significant health protecting activity due to its high flavonoid content (Stangl, Lorenz, & Stangl, 2006). Results from our experiments show a significant higher total phenolic and proanthocyanidin contents in EQr1 than in EQr2. Similar results for extracts from young and mature leaves of *Aegiceras corniculatum* were also reported (Lin, Liu, & Mau, 2008). The loss of phenolics in mature leaves extracts was attributed to degradation of phenols.

The phenolic composition of infusions from *Quercus* leaves is presented in Table 2 according to the HPLC profile with selected standards. When comparing the chemical composition of infusions from fresh and mature leaves, we can observe an increase of epicatechin concentration in the infusions from mature leaves (EQr2) and the appearance of catechin as a representative flavanol derived from hydrolysis reactions of glycosides originally present in the green material. Catechin (0–9.7 mg/g) and epicatechin (5.6–9.1 mg/g) were found in *Quercus* leaves at similar concentration range as in commercial green (catechin 0–5.8 mg/g; epicatechin 6.5–15.4 mg/g) and black teas (catechin 2.7–15.4 mg/g; epicatechin 1.1–9.0 mg/g) as reported by Henning et al. (2003). However, this flavanol contribution seems to be in balance with the total phenolic content (Table 1) because the ratio of catechin to gallic acid equivalents remains in the same order of magnitude (about two), signifying a balance between condensed and hydrolysable tannins, as the relatively constant concentration of gallic acid also suggests (Makkar & Becker, 1994). In the case of vanillin its depletion can be explained through oxidative demethoxylation reactions that give place first to vanillic acids and later to hydrobenzoic acids. Cinnamic acids did not change significantly but some caffeic acid may proceed esterification reactions with quinic acids (not evaluated) to produce the chlorogenic acid detected in mature leaves (Clifford, 2003).

Antioxidant activity of flavonoids and their metabolites *in vitro* depends upon the arrangement of functional groups about the nuclear structure (Khlebnikov, Schepetkin, Domina, Kirpotina, & Quinn, 2007). The scavenging activity of freeze-dried leaves infusions was determined by the DPPH assay and the median effective concentrations (EC_{50}) shown in Table 3. DPPH is not a naturally occurring radical, and is relatively stable compared to the highly reactive superoxide and hydroxyl species primarily responsible for oxidative damage in biological systems. Therefore, the free-radical scavenging capacity shown by *Quercus* samples may be primarily attributed to the high reactivity of their phenolic hydroxyl substituents. EQr1 presented a strong antioxidant capacity due to the scavenging activity towards free radicals. The antioxidant capacities of extractables were statistically different from each other ($p < 0.05$); EQr1 exhibited the highest chain-breaking activity ($k = 5.0577$) when EC_{50} was used, followed by catechin ($k = 4.1406$), gallic acid ($k = 3.010$) and finally EQr2 ($k = 0.0356$), which showed the lowest reaction rate.

Moreover, antioxidant capacity was assessed via inhibition of deoxyribose degradation (expressed as percent). Once hydroxyl radicals are formed, they can degrade deoxyribose into fragments that on heating with thiobarbituric acid generate a pink chromogen. When a scavenger molecule is added to the reaction mixture, it competes with the deoxyribose for hydroxyl radicals and can inhibit deoxyribose degradation depending on its concentration and rate constant for reaction with **.**OH. Fig. 1 shows the effect of EQr1 and EQr2 upon the degradation of deoxyribose in the presence of ethylenediaminetetraacetic acid (EDTA). EQr1 at the concentrations tested, appeared better than catechin in protecting deoxyribose damage due to **.**OH.

When the antioxidant concentration was increased to 1000 μg/mL, the inhibition effect of EQr1 was 100%, similar to gallic acid (99.16%), while EQr2 was 92.05%. These results are similar to those reported for alcoholic extracts of *Quercus infectoria* galls that have

Table 2
Phenolic compounds profile (±S.D.) in infusions of *Quercus resinosa* fresh (EQr1) and mature (EQr2) leaves ($n = 2$) by HPLC analysis.

Compound (MW)	EQr1 (mg/g sample)	EQr2 (mg/g sample)
Epicatechin (290.28)	5.64 ± 0.39	9.10 ± 0.03
Vanillin (152.14)	9.15 ± 0.53^{b}	–
p-Coumaric acid (164.15)	3.25 ± 0.21^{c}	3.38 ± 0.20^{c}
Ferulic acid (194.18)	–	–
Benzoic acid (122.12)	6.45 ± 0.40	10.35 ± 0.60
Salicylic acid (138.12)	9.21 ± 0.43^{b}	22.92 ± 1.24
Syringic acid (198.17)	–	2.98 ± 0.23^{cd}
Gallic acid (170.12)	13.83 ± 0.54^{a}	13.42 ± 1.05
Protocatechuic acid (154.12)	–	–
p-Hydroxybenzoic acid (138.13)	–	8.56 ± 0.19
Catechin (290.28)	–	9.69 ± 0.33^{ab}
Vanillic acid (168.14)	41.46 ± 3.20	10.37 ± 0.07^{a}
Chlorogenic acid (354.30)	–	2.08 ± 0.07^{d}
4-Hydroxy-3-methoxybenzoic acid (168.2)	2.78 ± 0.07^{c}	–
Caffeic acid (180.15)	12.20 ± 0.65^{a}	8.78 ± 0.10^{b}

Means in the same column with a common letter are not significantly different ($p < 0.05$).

Table 1
Yield, total phenolic and proanthocyanidin contents (mean ± S.D.) in infusions of *Quercus resinosa* fresh (EQr1) and mature (EQr2) leaves ($n = 4$).

Sample	Yield (%)	Total phenolic content		Proanthocyanidin content (mg catechin equivalents per g of sample)
		mg catechin equivalents per g of sample	mg gallic acid equivalents per g of sample	
EQr1	45.32 ± 2.08*	0.44 ± 0.04*	0.25 ± 0.03*	0.16 ± 0.05*
EQr2	15.98 ± 0.63**	0.08 ± 0.00**	0.04 ± 0.06**	0.02 ± 0.01**

*,**Means values in the same column are significantly different ($p < 0.05$).

Table 3
Chain-breaking activity and half maximal concentrations for DPPH scavenging (EC_{50}), inhibition of deoxyribose degradation (IC_{50}), inhibition of cell proliferation (ICP_{50}) and cell survival (LC_{50}) of infusions from *Quercus resinosa* fresh (EQr1) and mature (EQr2) leaves.

Sample	Chain-breaking activity ($-O.D.^{-3}/min/mg_{dm}$)	EC_{50} (μg/mL)	IC_{50} (μg/mL)	ICP_{50} (μg/mL)	LC_{50} (μg/mL)
EQr1	5.0577	507	1.69 ± 0.41[a]	67.65 ± 9.7[a]	115.23 ± 10.0[c]
EQr2	0.0356	548	36.29 ± 4.52[b]	78.90 ± 5.1[b]	127.54 ± 4.9[c]
Catechin	4.1406	140	48.08 ± 12.77[b]	75.91 ± 6.3[b]	121.95 ± 5.6[c]
Gallic acid	3.0100	33	6.42 ± 0.96[a]	69.34 ± 8.1[a]	110.80 ± 10.0[c]

Means followed by a common letter are not significantly different ($p < 0.05$).

potently scavenged free hydroxyl radicals (Kaur, Athar, & Alam, 2008).

Phenolic compounds detected in infusions have been reported with antioxidant activity and antiproliferative effects on human cancer cells via apoptosis (Mohan, Devaraj, Prathiba, Hara, & Nagini, 2006). Many studies have investigated the antioxidant effects of flavonoids and phenolic compounds in the context of a variety of cell functions. In particular, epicatechin that was found in the infusions, has been studied for its ability to attenuate oxidative stress-induced cell damage and to understand the mechanism of polyphenols protective action. This flavanol is involved in the mechanism of suppression of caspase 3 activity as a marker for apoptosis (Spencer et al., 2001). Furthermore gallic acid, also found in the infusions, is an endogenous product in plants that possesses anticarcinogenic activity (Shahrzard, Aoyagi, Winter, Koyama, & Bitsch, 2001), and in free or bound forms, it is present in large amounts in tea leaves, from which it is easily extracted by hot water infusions.

Different tests were used to assess cell viability and genotoxic effects of infusions. The potential cytotoxic effect of infusions from fresh leaves of *Q. resinosa* (EQr1), mature leaves (EQr2), gallic acid and catechin was evaluated on HeLa cells. The cell proliferation inhibition and cell survival after 24 h of treatment was dose-dependent with all assayed compounds and extracts of *Quercus* leaves (Fig. 2). Gallic acid treatment showed a dramatic reduction in the cell viability with an estimated ICP_{50} (50% of inhibition of proliferation) value of 69.34 ± 8.1 μg/mL (Table 3). Similarly, González-Laredo et al. (2007) have reported that catechin showed cytotoxicity against HeLa cells with 6 h treatment. In this case, treatment with catechin at 24 h displayed an ICP_{50} value of 75.91 ± 6.3 μg/mL at the same times assayed in this study. As to the effect of infusions, ICP_{50} values were of 67.65 ± 9.7 and 78.90 ± 5.1 μg/mL for EQr1 and EQr2, respectively. EQr2 presented an ICP_{50} value similar to catechin while EQr1 effect was alike the gallic acid treatment.

In the same way, when LC_{50} data (50% of cell death) were calculated (Table 3), it was observed that similar values were obtained for EQr1, gallic acid, EQr2 and catechin. In all cases, LC_{50} were about 1.6 times higher than ICP_{50} values, showing that it is almost necessary to double the extract concentration to provoke cell death rather than cell proliferation inhibition.

DNA strand break assay has been used to test the genotoxicity of various chemical and physical agents in different cell types. When DNA single strand breaks the largest numbers of lesions are by far the exposed alkali labile sites (Singh, 2000). In order to elucidate the underlying mechanism on the cytotoxicity effects

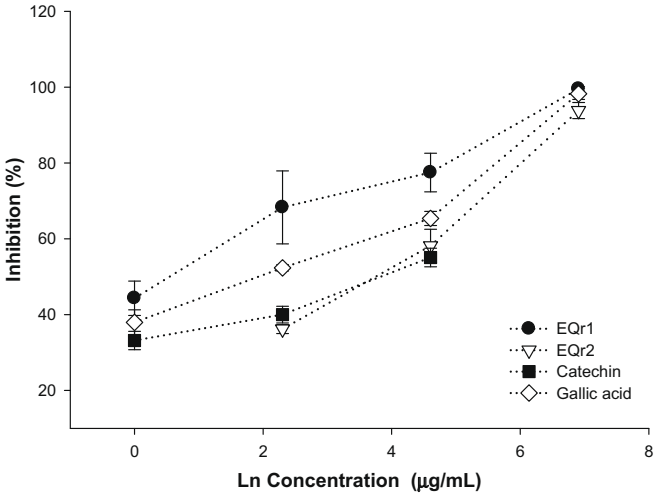

Fig. 1. Antioxidant activity (mean ± S.D.) assessed via inhibition of deoxyribose degradation (%) at different assay concentrations. EQr1 means extractables from *Quercus resinosa* fresh dry leaves; EQr2 means extractables from *Q. resinosa* mature dry leaves.

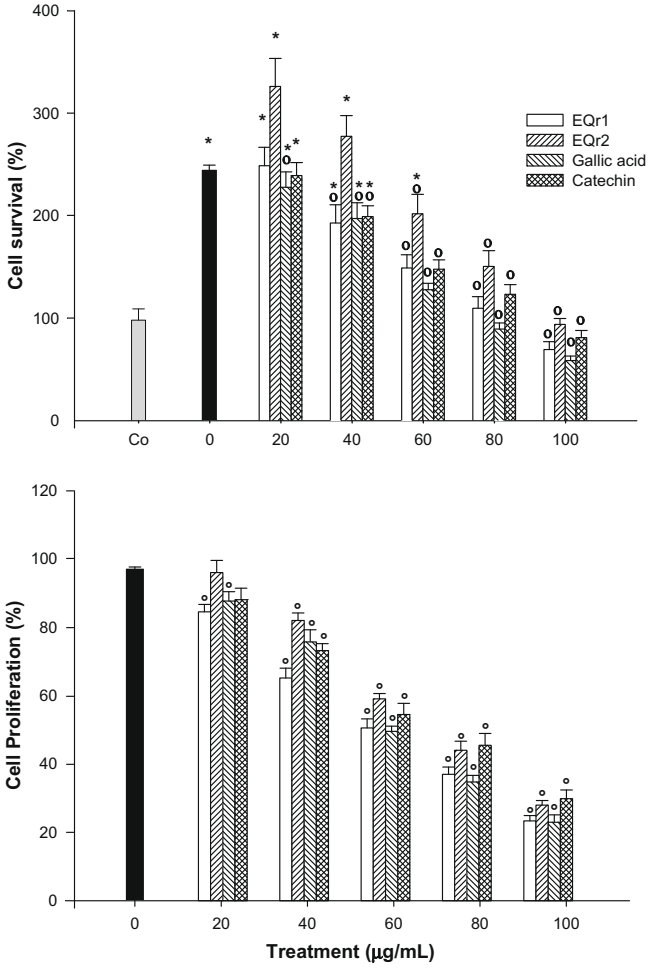

Fig. 2. Antiproliferative activity of infusions from *Quercus resinosa* fresh (EQr1) and mature leaves (EQr2), and the standards gallic acid and catechin. Data are expressed as percentage of untreated cells, mean ± S.D. ($n = 3$). Co is the control at time zero.

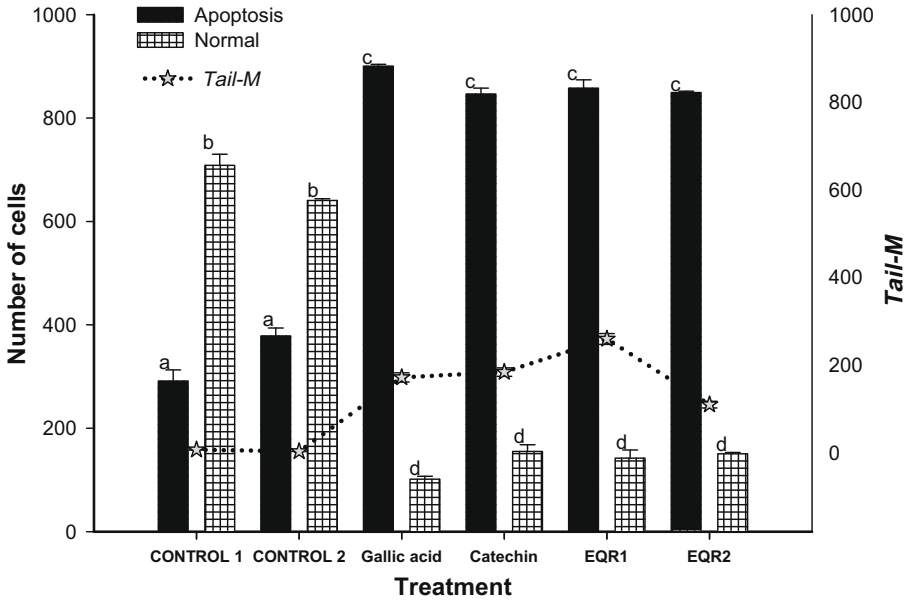

Fig. 3. Apoptotic cells results and tail moment average of HeLa cells at LC_{50}. Control 1 is DMEM supplemented with 5% of calf serum; Control 2 is DMEM supplemented with 0.5% of albumin. Standards gallic acid and catechin, and infusions of *Quercus resinosa* fresh (EQr1) and mature (EQr2) leaves, were assayed at LC_{50} in DMEM supplemented with 0.5% of albumin.

shown by extractables of *Quercus* leaves, the alkaline single-cell gel electrophoresis (comet) assay was performed. The assays were realised at LC_{50} for 24 h and tail moment average (TMA) was measured (Fig. 3). When checking comet assay results, it is possible to observe the effect of *Q. resinosa* infusions in DNA breakage of HeLa cells forming the characteristic comet tail. It was observed that EQr1 produced the highest TMA, suggesting higher genetic damage from the fresh *Quercus* leaves infusion as promotor of DNA breakage in transformed human cells. Furthermore, it shows that other polyphenols such as catechins and gallic acid are also able to catalyse DNA breakage in HeLa cells. This result agrees with other authors for gallic acid (Hadi et al., 2007) and is a significant cause of the consequent degradation of cellular DNA as a general mechanism for anticancer properties of plant phenolics.

Further, it has been proposed that most clinically used anticancer drugs can activate late events of apoptosis for which many assays are available. The DNA diffusion assay is a simple one that measures accurately necrotic and apoptotic cells enhancement due to higher sensitivity from alkaline treatment and from the numerous alkali labile sites present in the DNA of apoptotic cells. In comparing the treatment utilised and the effect elicited in the comet assay, it can be noted, with exception of the negative control, that the LC_{50} produced high genotoxicity, regard a higher number of damaged cells (Fig. 3). Cells with apoptotic vesicles were counted from 1000 cells per sample (Fig. 3) showing that all treatments with phenolics induced apoptosis in HeLa cells. EQr1 and EQr2 did not show statistical differences with gallic acid and catechin, but showed statistical differences to the controls.

In summary, our *in vitro* data provide important evidence that polyphenols from *Quercus* leaves infusion are linked to a reduced risk of cancer, via a decrease in the oxidative process and other damage to DNA in transformed human cells. It is possible that fresh leaves infusions with their higher concentration of phytochemicals may be a potent source of protective chemicals against oxidative process.

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