

Research Article

Antioxidant and antiproliferative activities of methanolic extracts of beer

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Abstract

The beneficial effects of beer on human health, such as reduced risk of heart diseases and certain types of cancer, has been associated to its polyphenolic content and resulting antioxidant activity. The aim of this study was to verify the relationship between the antioxidant and antiproliferative activity of the compounds contained in beer. In order to evaluate different antioxidant profiles, methanolic extracts of various commercial beers and of samples collected during the brewing process were analyzed. Their antioxidant power was evaluated by measuring: i) the ferric reducing activity, ii) their ability in suppressing the release of thiobarbi-

uric acid reactive substances and iii) the content of antioxidant compounds. The antiproliferative activity was detected by determining the inhibition of HeLa cancer cell growth following incubation with the extracts. The results of this study showed that most beer extracts have significant antiproliferative effects, but these effects were not due to the antioxidant power of the extracts. By contrast, they were found to be related to the extract-induced increase of the intracellular levels of reactive oxygen species. The antiproliferative action of the extracts could result from a pro-oxidant mechanism due to non-phenolic compounds.

Introduction

Beer is a natural drink containing a large number of plant derived compounds and produced by yeast during the fermentation process. Research has shown that moderate consumption of beer has beneficial effects on health due to its antioxidant characteristics (Arranz *et al.* 2012). Beer, in fact, contains compounds with high antioxidant activity such as reducing sugars, Maillard reaction products, vitamins and polyphenols. Numerous studies report that flavonoids, a class of polyphenols, exert antiproliferative effects against ovarian, leukemic, intestinal, lung, breast and bladder cancer cells (Duthie *et al.* 2000). It has also been reported that lyophilized preparations of beer with a high concentration of polyphenols and antioxidant activity exhibit cytotoxic activity on HL60 cells, in contrast to beer with low phenolic content. The presence of quercetin,

resveratrol and gallic acid is suggested to be responsible for such cytotoxicity (Tedesco *et al.* 2005). Due to its antioxidant properties, many studies are being conducted on a possible role of beer as an anticancer and chemopreventive agent. In this context we studied the antioxidant and anticancer activities of methanolic extracts of different commercial beers and of samples collected at varying steps of the brewing process. In order to evaluate the antiproliferative effects of the antioxidant compounds present in these extracts, their levels were correlated to the cell growth inhibition rate probed in cancer cell cultures incubated with the extracts. Since small antioxidant phenols, such as quercetin and gallic acid, are known to behave as pro-oxidants under certain conditions (Yordi *et al.* 2012), the alteration of the cellular redox balance in response to the extracts was also investigated to elucidate the mechanism adopted by the cell to control proliferation.

Materials and Methods

Methanolic extracts

Five commercial beers, Trappistes Rockefort (A), Budejovicky Budvar (B), Schneider Weiss (C), O'Hara's (D), Old Tom (E) and samples collected during the brewing process, mash (m), sweet wort (sw), boiled wort (bw), final wort (fw), cold wort (cw) of a Lager Cerb type beer (F) were treated with 2 volumes of cold methanol (Sigma-Aldrich) and left overnight at 4°C. The precipitate was removed by centrifugation at 8000 x g for 30 min. The supernatant was dried in vacuum at 30°C and resuspended in distilled water (same volume as sample used). The weights (gr) of dry residues, determined after drying 1 ml of each sample at 37°C overnight, were: 0.0515(A); 0.0394(B); 0.0420 (C); 0.0314(D); 0.0590(E); 0.0140(F); 0.0568(m); 0.0791(sw); 0.0457(bw); 0.0482(fw), 0.0461 (cw). Each sample was then resuspended in distilled water to obtain a final concentration of 1mg/ml.

Determination of Folin Ciocalteu reactive compounds

Total phenolic content was determined in the methanolic extracts following a procedure described by Singleton and Rossi (Singleton & Rossi 1965) with a few modifications. 20 µl of sample was reacted with 50µl of 0.2 mol/l Folin-Ciocalteu reagent and 100µl of 20% Na₂CO₃ (Sigma-Aldrich) and 830 µl H₂O. The samples were incubated for 30 minutes at room temperature and the absorbance readings were taken at 700 nm. The measurement was carried out in triplicate and the results were expressed as mean O.D./mg dry weight.

Cell Culture

HeLa cells (from ATCC cell lines, catalogue number CCL-2), derived from human cervical carcinoma, were grown in 25 cm² flasks (12000 cells/cm²) with 5 ml of Dulbecco's modified Eagle Medium with 10% fetal calf serum, penicillin at 100 units/ml, streptomycin at 100 µg/ml and 2mM glutamine (Invitrogen Paisley UK) at 37°C humidified atmosphere of 5% CO₂/ 95% air.

MTT test

Cell growth was evaluated by MTT test (Sylvester 2011). Cells were seeded in 96 well plates (2000 cells/well). 24 hours after seeding, 4µl of each extract were added per well, for a final concentration of 40µg/ml. Following 24 hours of treatment, the culture medium was removed and cells were incubated for 3 hours with MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich) at 0.5 mg/ final concentration. The culture medium was then removed and the formazan salt, pro-

duced by mitochondrial enzyme succinate dehydrogenase, was solubilized with 200µl Dimethyl Sulfoxide (Sigma-Aldrich) per well. The absorbance, measured at 550 nm by using a Titertek Multiskan MC plate reader (ICN/Flow Biochemicals, Huntsville, AL), was directly related to the number of viable cells. Cell growth inhibition was calculated as 100 – (mean O.D. of treated cells/ mean O.D. of control cells). Each point was the mean of five repetitions.

Determination of O₂⁻ release

Intracellular super oxide anion (O₂⁻) production in HeLa cells was assayed using a colorimetric method (Rauen et al 2000) based on the reaction between Nitro Blue Tetrazolium (NBT Sigma-Aldrich) chloride and O₂⁻, with the consequent formation of Formazan salt. Cells were seeded in 96 well plates (10000 cell/well) and 4µg of each extract were added to the culture medium (40µ/ml) 24 hours after seeding. Following 24 hours of treatment, the culture medium was removed and cells were incubated with 1 mg/ml medium culture NBT at 37°C for 3 h. The medium was then removed, the cells were dissolved in dimethyl sulphoxide and the absorbance of the produced Formazan salt was measured at 550 nm. The (O₂⁻) production was determined as mean O.D./10000 cells. Each point was the mean of five repetitions. The percentage values vs control are calculated as 100- (O₂⁻ production of treated cells/O₂⁻ production of control cells).

Determination of hydrogen peroxide production

Hydrogen peroxide (H₂O₂) generation in HeLa cells was assayed using a colorimetric method involving the oxidation of iodide in the presence of ammonium molybdate and photometric analysis of the resulting blue starch-iodine complex (Graf & Penniston 1980). Cells were seeded in 96 well plates (10000 cells/well) and 4µg of each extract were added to the culture medium 24 hours after seeding (40µg/ml final concentration). Following 24 hours of treatment, the culture medium was removed and the cells were treated with 38.5 mM HCl, 80 mM potassium iodide, 80 mM ammonium molybdate in H₂SO₄ and 0.38% starch (Sigma-Aldrich). Absorbance was then measured, after 20 min, at 570 nm using a Titertek Multiskan MC plate reader (ICN/Flow Biochemicals, Huntsville, AL). The H₂O₂ production was determined as mean O.D./10000 cells. Each point was the mean of five repetitions. The percentage values vs control are calculated as 100- (H₂O₂ production of treated cells/ H₂O₂ production of control cells).

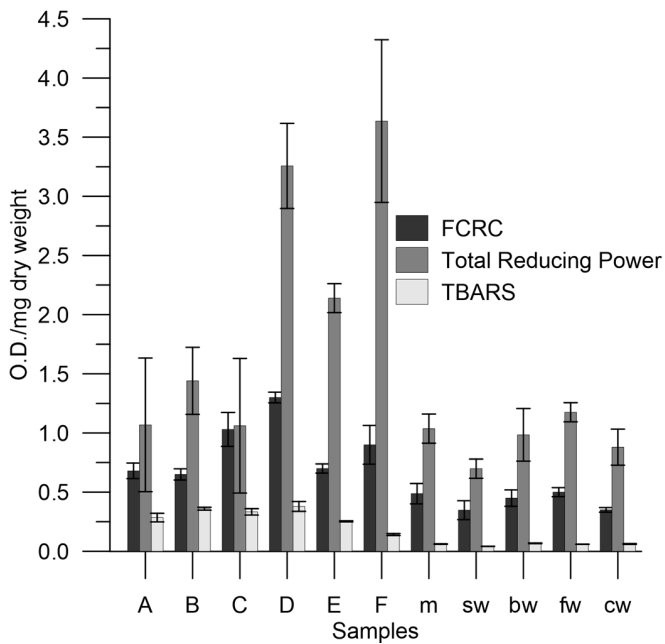


Figure 1. Content of Folin Ciocalteu reactive compound (FCRC) and antioxidant activity level (total reducing power and inhibition of TBARS production) determined in methanol extracts of commercial beers (A, B, C, D, E) and of samples drawn during the brewing process: mash (m), sweet wort (sw), boiled wort (bw), final wort (fw), cold wort (cw) and final beer (F).

Determination of thiobarbituric acid reactive substances (TBARS)

The measure of the antioxidative activity (AOA) of the extracts was carried out by determining their ability to inhibit the TBARS production following a procedure developed by Koracevic *et al.* (2001). A solution of Fe-EDTA complex reacts with hydrogen peroxide to form hydroxyl radicals which, in turn, degrades benzoate leading to TBARS release. Antioxidants present in the methanolic extracts suppress the production of TBARS. This reaction can be determined spectrophotometrically and results in an inhibition of colour development which is related to the AOA.

Determination of total reducing power

The determination of the total reducing power was carried out according to Zhao *et al.* (2006). Briefly, 2.5 ml of 0.2 mol/l of phosphate buffer pH 6.6 and 2.5 ml of 1% $K_3Fe(CN)_6$ (Sigma-Aldrich) were added to 1 ml of sample. The mixture was incubated at 50°C for 20 minutes. 2.5 ml of 10% trichloroacetic acid (Sigma-Aldrich) was then added and the mixture was centrifuged at 10,000 x g for 10 minutes. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ (Sigma-Aldrich). The absorbance was taken at 700 nm and the results were expressed as O.D./mg of dry weight.

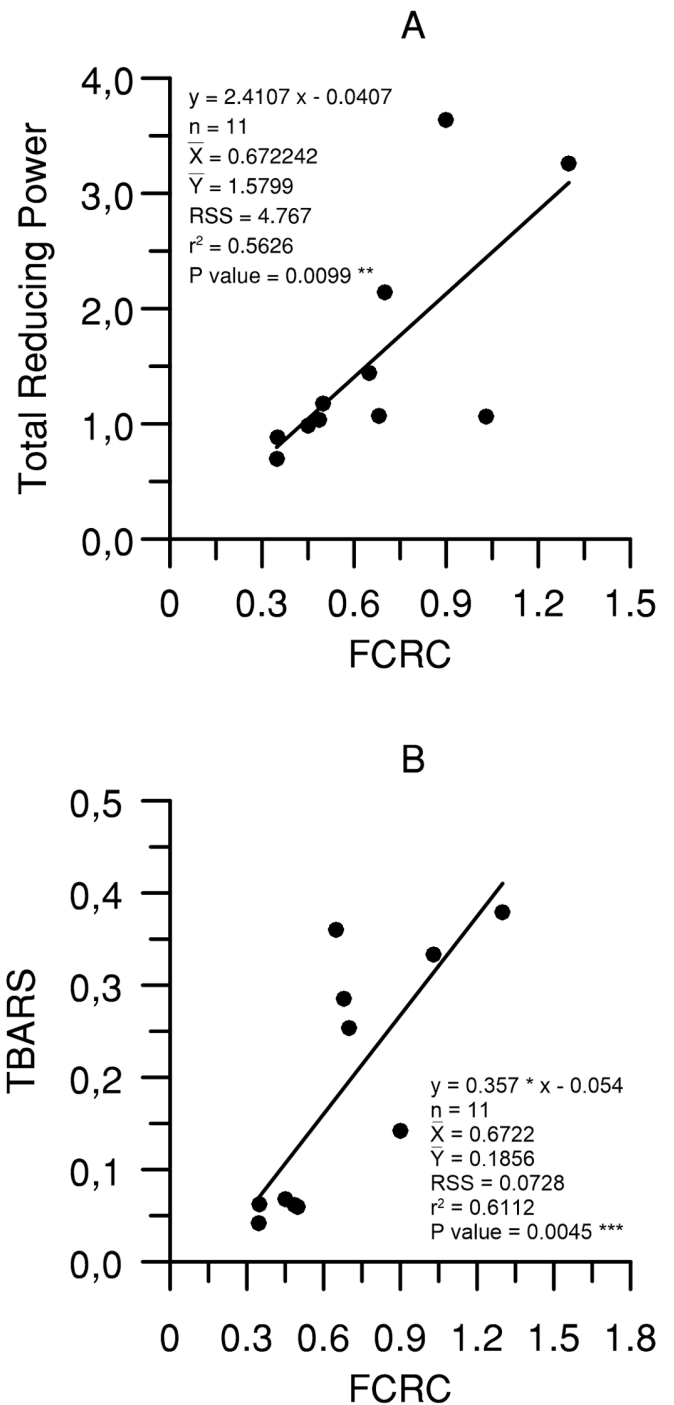


Figure 2. Correlation between antioxidant properties and the content of Folin Ciocalteu reactive compounds (FCRC) in the methanol extracts of commercial beers and of samples of different phases of the brewing process. A: FCRC content vs total reducing power. B: FCRC content vs inhibition of TBARS production.

Statistical analysis

Statistical analysis of the level of Folin-Ciocalteu reactive compounds, antioxidant properties (total antioxidant power and thiobarbituric acid reactive substances (TBARS)) and percentage of O_2^- and H_2O_2 production were analyzed by the one Way ANOVA with Newman

-Keuls test for Multiple Comparison Test (Statistica 8.0 Statsoft, Tulsa, USA).

The correlation between antioxidant properties, antiproliferative activity, percentage of O_2^- and H_2O_2 production and the content of Folin Ciocalteu reactive compounds (FCRC) has been investigated through the analysis of the coefficient of determination (r^2) and the Pearson index (r Pearson) (Statistica 8.0 Statsoft, Tulsa, USA). Where appropriate, data are presented as mean \pm S.D. A $p < 0.05$ was considered to be significant.

Results

Five commercial beers and samples from various steps of the brewing process were treated with methanol for

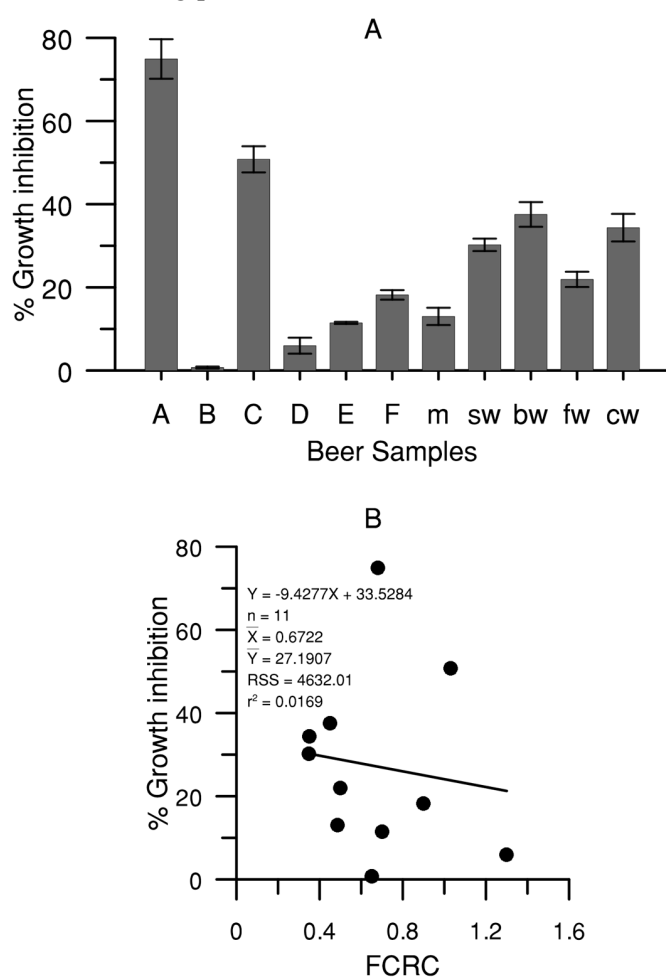


Figure 3. Antiproliferative activity of various methanol extracts and its relationship with the content of Folin Ciocalteu reactive compounds (FCRC). A: percentage of cell growth inhibition with respect to the controls after treatment with methanol extracts of commercial beers (A,B,C,D,E) and with samples drawn during the production process of lager type beer: mash (m), sweet wort (sw), boiled wort (bw), final wort (fw), cold wort (cw) and final beer (F). B: correlation between the percentage of cell growth inhibition and the polyphenol contents in the various methanol extracts.

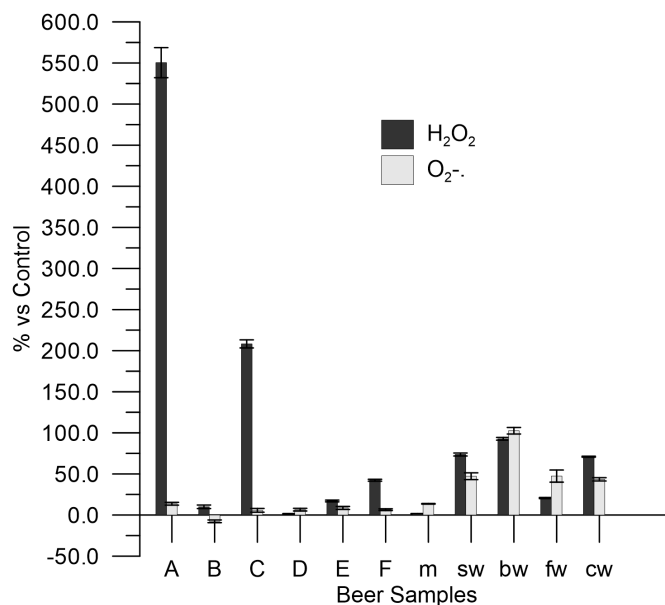


Figure 4. Percentage of O_2^- and H_2O_2 production with respect to the controls, in cells treated with methanol extracts obtained from commercial beers (A,B,C,D,E) and from mash (m), sweet wort (sw), boiled wort (bw), final wort (fw), cold wort (cw) and final beer (F).

24 hours at -20°C to remove high molecular weight compounds. The commercial beers used differed in the raw materials and the fermentation process, while the different phases (wort, boiled wort, final wort, cold wort and beer) we analyzed were collected during the production process of lager type beer.

In order to characterize the biological activity of these extracts, we evaluated their antioxidant properties by measuring their ferric reducing activity (total antioxidant power), and their ability in inhibiting, by the antioxidant action, the release of thiobarbituric acid reactive substances (TBARS) from benzoate. The content of antioxidants in the extracts was determined by assessing the level of Folin-Ciocalteu reactive compounds (FCRC), mostly represented by polyphenols that constitute the main class of antioxidant compounds present in beer (Figure 1). Notably their level correlated with both the total antioxidant power (Figure 2A) and the capacity to prevent TBARS formation (Figure 2B), thus indicating that the antioxidant activity detected in the methanolic extracts was mostly due to these components.

In order to evaluate the relationship between antioxidant activity and control of cell proliferation, HeLa cell cultures were incubated for 24 hours with the extracts and a possible correlation between cell growth and the level of antioxidant compounds was checked. The MTT test showed (Figure 3A) that all the methanol extracts exhibited antiproliferative action, but such effect was not correlated with the level of Folin-Ciocalteu reactive antioxidant compounds

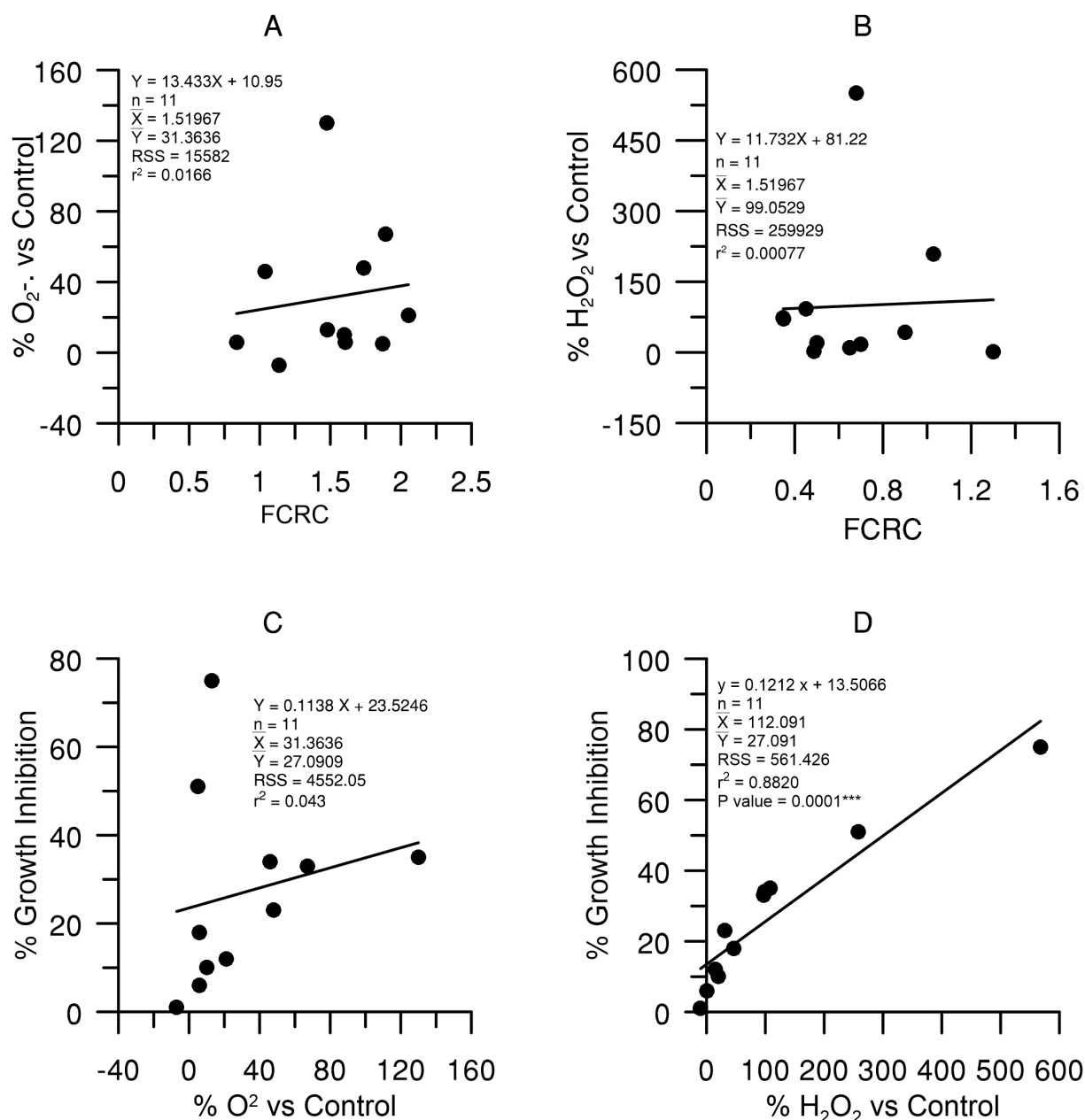


Figure 5. Correlation between the level of Folin Ciocalteu reactive compounds and the O₂⁻ (A) and H₂O₂ (B) intracellular production in the treated cells. O₂⁻ and H₂O₂ levels were calculated per 10⁴ cells, as percentages with respect to the controls. (C) and (D): correlation between the percentage of cell growth inhibition and O₂⁻ (C) and H₂O₂ (D) production in HeLa cells treated with the various methanol extracts.

(Figure 3B) which suggested that the effect of beer extracts on cell proliferation was not due to an antioxidant action. We then tested the ability of the extracts to affect the redox balance of the cells by evaluating the intracellular production of Reactive Oxygen Species (ROS) after 24 hours of incubation with the extracts. The cellular production of O₂⁻ and H₂O₂ were affected by the treatment (Figure 4), but their levels did not correlate with the content of Folin-Ciocalteu reactive compounds (FCRC) in the extracts, i.e., with their antioxidant activity (Figure 5A, B).

Notably the extracts obtained from the samples

collected during the brewing process induced cellular O₂⁻ levels markedly higher than the extracts from commercial beers. However no correlation was found when the whole set of O₂⁻ levels was matched with cell growth inhibition (Figure 5C). In contrast, a strong correlation was found between cellular growth inhibition and the intracellular H₂O₂ level induced by the various extracts (Figure 5D).

Discussion

Antioxidant compounds are thought to offer, by vari-

ous mechanisms, protection against chronic diseases and some types of cancer that have been associated with cellular damage induced by oxidative stress (Russo *et al.* 2010). Plant-derived food and beverages contain a large variety of these antioxidant molecules. Among these polyphenols, largely present in fruits, vegetables and cereal crops, have been well investigated for their antioxidant and antitumor properties (Godbole *et al.* 2013, Link *et al.* 2010, Moore *et al.* 2015). High amounts of polyphenols are found in malted grains, a key element in the process of beer production. These compounds with high antioxidant activity are retained even after malting, and end up in the beer which therefore can be considered a good source of anticancer and chemopreventive agents (Zhao *et al.* 2010).

Besides polyphenols, beer contains a large variety of compounds such as reducing sugars, Maillard reaction products and proteinaceous material, that exhibit antitumor activity and could account for the potential anticancer properties of its components. In this context we wanted to verify the relationship between antioxidant and antiproliferative activity of the compounds present in the beer. In order to obtain different antioxidant profiles, we performed methanol extracts from various commercial beers and samples collected during the brewing process.

The antioxidant properties of the extracts were characterized by valuating: 1) the total reducing power which is generally linked to the antioxidant activity, 2) the inhibiting action by antioxidant agents against the TBARS release and 3) the content of antioxidant compounds by Folin Ciocalteu method.

We found a significant positive correlation between the level of Folin-Ciocalteu reactive compounds and the measured antioxidant activities, which indicated that the antioxidant power of our extracts was mainly due to these components. We then tested whether their content was related to the ability of the extracts to inhibit cancer cell proliferation. The data obtained showed that most extracts significantly inhibited cell growth of the HeLa cancer cell line, but the degree of inhibition activity was not related to the antioxidant power of the extracts. We also found that the treatment with the methanolic extracts induced a marked increase of cellular production of H₂O₂. Such increase was not related to the antioxidant content of the extracts, but it was significantly correlated to the cell growth inhibition rate. Higher O₂⁻ levels were also detected in the treated cells respect to the controls, thus supporting an impairment of the cellular redox balance, but they were not correlated with cell growth arrest and antioxidant compound content. These data demonstrated that the ability of our methanolic extracts

to inhibit the proliferation of a cancer cell line was not dependent on the antioxidant activities we measured. Treatment of the cells, rather induced a more oxidative status inside cells following ROS production with a positive correlation between H₂O₂ production and cell growth inhibition. O₂⁻ production was mostly detected following incubation of cells with the extracts from samples collected during beer production, thus suggesting that it was due to the action of components present in the raw material and lost in the final beer product. By contrast H₂O₂ increase induced by most of the extracts resulted from other compounds derived from the starting material, but which are maintained during the process of beer production. Such H₂O₂ inducing factors could be responsible of the potential anticancer activity of the beer extracts. H₂O₂ accumulation has been reported to be a crucial step leading to cell death following treatment with the anticancer agent paclitaxel (Alexandre *et al.* 2006) and to trigger apoptosis in HeLa cells via the mitochondrial pathway (Sing *et al.* 2007).

It is now accepted that free radicals play a critical role, not only as carcinogens, but also as signaling molecules involved in the control of cell proliferation (Sainz *et al.* 2012). Several studies have showed an increased production of ROS in cancer cells as compared to normal cells, presumably associated with uncontrolled cell proliferation, defective metabolic regulation and oncogenic signals. A further ROS production, following exposure of cancer cells to oxidative stress generating agents, impairs the cancer cell antioxidant system and cause apoptosis induction (Watson 2013). Since cancer cells with an endogenous ROS stress should be more sensitive to such anticancer agents as compared to normal cells, this biochemical difference provides therapeutic implications. Many anticancer agents, such as arsenic trioxide, anthracyclines, cisplatin, bleomycin and irradiation, currently used in cancer therapy, act by such a mechanism that involves ROS accumulation and apoptosis (Pelicano *et al.* 2004). It is likely that the HeLa growth arrest, obtained following incubation with methanolic extracts in this study, involves a pro-oxidant mechanism that does not seem to be related to the action of phenols and other Folin-Ciocalteu reactive compounds. Beer contains certain peptidic materials mostly originating from the hydrolysis of the cereal storage proteins which occurs during the malt production process. These proteins contain sequences possessing many potential biological properties including anticancer activities (Cavazos & De Mejia 2013). Since many peptides originating from food sources are known to exhibit antitumor properties, it is possible that the peptidic fraction could contribute to the anticancer and

chemopreventive activity of beer.

In conclusion we demonstrated that beer contains, besides phenols, potential anticancer agents that inhibit HeLa cancer cell growth presumably by causing cellular ROS accumulation. The peptidic fraction present in the beer could possess this anticancer property. Peptides from natural sources have attracted much interest as drug candidates for cancer treatment owing to their fewer side effects and good effectiveness respect to the current chemotherapy molecules (Buthia & Maiti 2008). Beer could represent a good source of such bioactive peptides, and studies aimed at their identification might lead to the discovery of new tumor-targeting agents.

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