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# Assessment of the Potential Impact of Aqueous and Ethanolic Malt Extracts on HepG2 Liver Cells

Running title: Craft Beer and Malt Liver Impact

Diana Santos[1], Maria João Pereira[1], Ângelo Jesus[2], Rita Ferraz de Oliveira[2,3], Ana Isabel Oliveira[2] and Cláudia Pinho[2*]

[1]ESS, Polytechnic of Porto, Rua Dr. António Bernardino de Almeida 400, 4200-072 Porto, Portugal
[2]REQUIMTE/LAQV, ESS, Polytechnic of Porto, Rua Dr. António Bernardino de Almeida 400, 4200-072 Porto, Portugal
[3]Center for Health Technology and Services Research at the Associate Laboratory RISE-Health Research Network (CINTESIS@RISE), Department of Education and Psychology, University of Aveiro (UA), Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

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## SUMMARY

*Research background.* Malt is the second most abundant raw material in beer and the main source of phenolic compounds. However, information on the specific contribution of different types of malt to the biological activity of beer and its effects on liver function remains limited. Therefore, this study aims to evaluate the antioxidant potential and effects on liver function of a Portuguese craft beer (Imperial Stout - IS-N) and the aqueous and ethanolic extracts of malts present in IS-N beer (Carafa III, Caramunich III, Carapils and Pilsner).

*Experimental approach.* The aqueous and ethanolic extracts of malt were obtained by solid-liquid extraction at a ratio of 1 g of powder to 9 mL of distilled water and 1 g of powder to 10 mL of 95

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[*]Corresponding author:
Phone: +351 222061000
Fax: +351 222061001
E-mail: clp@ess.ipp.pt

%(*V*/*V*) ethanol solution, respectively. The total phenolic content (TPC) was determined by the Folin–Ciocalteu method, and antioxidant activity was evaluated by ABTS, DPPH, and metal chelating capacity (ferrozine) assays. Cytotoxicity was evaluated in HepG2 cells using the MTT assay after exposing the cells to different concentrations of the samples (1–500 µg/mL) for 24 and 48 hours. Liver function was evaluated by determining alanine aminotransferase (ALT) activity in the cell supernatant.

*Results and conclusions.* TPC values ranged from (7.12±0.54) mg GAE/g to (28.19±0.53) mg GAE/g, with the aqueous extract of Caramunich III ((23.61±0.61) mg GAE/g) standing out for its high TPC and greater antioxidant capacity ($IC_{50}$ ABTS=(17.32±0.77) µg/mL; $IC_{50}$ DPPH=(152.64±9.34) µg/mL). Malt extracts showed no cytotoxicity up to 250 µg/mL, while IS-N beer showed cytotoxicity in ethanolic solvent at 500 µg/mL after 24 hours. IS-N beer induced lower ALT levels compared to single malts, suggesting a possible synergistic effect between its bioactive compounds. Thus, evaluating the biological activity of malts could contribute to the production of beers with a better functional profile and less hepatic impact.

*Novelty and scientific contribution.* This study breaks new ground by exploring the role of malts in the antioxidant activity and hepatic impact of craft beer, offering new data that could guide the development of beverages with a functional profile that is more beneficial for health.

**Keywords**: craft beer; malt; antioxidant; metabolic activity; alanine aminotransferase (ALT)

## INTRODUCTION

Beer is the most popular alcoholic beverage in the world, widely consumed and with an estimated global production, in 2023, of around 1.88 billion hectolitres per year [*1*]. Historically, beer has been valued not only for its sensory characteristics, but also for its cultural and social role in different societies.

In recent decades, the craft beer sector has experienced remarkable growth [*1*], driven by consumers who value products with unique sensory profiles, differentiated composition, sustainable appeal and potential health benefits [*1*,*2*]. The basic composition of beer includes four main ingredients: water, malt, hops (*Humulus lupulus* L.) and yeast [*3*]. However, the final product can be enriched with other ingredients that confer specific sensory and functional properties [*4*].

Among the essential components of the traditional formulation, malt and hops play central roles in both the sensory quality and bioactive profile of beer. Hops impart characteristic bitterness and aroma, contribute to foam stability, and have antimicrobial action that aids in the preservation of the beverage [*5*]. In addition to these technological functions, hops contain resins, essential oils, and a variety of phenolic compounds with recognised antioxidant activity, representing approximately one-third of the phenolic compounds present in beer [*5*,*6*].

Malt, usually obtained from barley (*Hordeum vulgare* L.), also contributes decisively to the flavour, aroma, colour and body of the beverage [*7*]. In addition, malt is an important source of phenolic compounds with recognised antioxidant activity, contributing about two-thirds of the phenolic content of beer. The composition of these compounds varies according to the type of cereal used, the degree of roasting and the malting process [*8*,*9*]. Thus, the chemical diversity between different types of malt can have an impact not only on the sensory properties of beer, but also on its physiological effects, including its impact on liver function [*10*].

The liver is a critical organ responsible for multiple functions, including detoxification, and metabolism of exogenous substances, including ethanol and bioactive compounds present in beer. [*10*]. As the main site of alcohol metabolism, this organ is particularly exposed to the action of toxic byproducts generated during this process [*11*]. Among these substances are acetaldehyde and reactive oxygen species (ROS), which can cause cellular damage and compromise liver function when produced in excess. Although moderate beer consumption has been associated with beneficial effects such as antioxidant capacity, neuroprotective properties, improved lipid profile, reduced risk of atherosclerosis and decreased inflammatory markers [*12*,*13*], excessive beer consumption is associated with hepatotoxicity, which can lead to hepatic steatosis, alcoholic hepatitis, fibrosis and cirrhosis [*10*]. Hepatotoxicity is often assessed through the analysis of serum biomarkers, especially the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), whose elevation indicates damage to the integrity of liver cells [*14*].

Thus, this work aims to characterize the extract of a Portuguese craft beer and its malts (aqueous and alcoholic extracts) in terms of their antioxidant potential and impact on liver function, by analysing cell toxicity and the activity of the ALT enzyme in human hepatocarcinoma cells (HepG2 cells).

## MATERIALS AND METHODS

### *Chemicals*

Gallic acid (GA) and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). Absolute ethanol, disodium phosphate, and ethylenediaminetetraacetic acid (EDTA) were supplied by VWR Chemicals (Ohio, USA). Quercetin was obtained from Sigma-Aldrich (St. Louis, USA), while potassium persulphate came from Biochem (Cosne-Cours-sur-Loire, France). Ferrozine, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1 % antibiotic/antifungal solution were purchased from Thermo Scientific (Kandel, Germany). Monosodium phosphate was supplied by J. T. Baker (Deventer, Netherlands), and dimethyl sulfoxide (DMSO) and sodium chloride were obtained from Fisher Scientific (Loughborough, United Kingdom). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) came from Acros Organics (Geel, Belgium), while sodium carbonate was purchased from Atom Scientific (Manchester, UK). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from TCI (Zwijndrecht, Belgium). Trypsin, phosphate-buffered saline (PBS) and minimum essential medium (MEM) were supplied by Corning (Manassas, USA), and foetal bovine serum (FBS) was obtained from Biochrom KG (Berlin, Germany). Finally, the ALT enzyme assay kit was purchased from Randox Laboratories (UK).

### *Beer and malt samples*

Portuguese craft beer Imperial Stout (IS-N) (Porto, Portugal) was chosen because of its potential antioxidant and hepatoprotective activity demonstrated in our previous study [*15*], and also because of the availability of its malts. IS-N beer is made with Carafa Special III, Caramunich III, Carapils and Pilsner malts (selected malts), provided by the brewery that produces the craft beer (IS-N).

### *Preparation of beer and malt extracts for analysis*

The preparation of malt samples was performed as described by Mareček *et al.* [*16*] and Wu *et al.* [*17*], with slight modifications. The malt cereals were ground in an electric mill (Taurus®; Aromatic, Oliana, Spain) for 40 seconds, subsequently proceeding to its granulation with a 500 µm mesh sieve. To obtain the aqueous malt extract, 25 g of powder was added to 225 mL of distilled water, placing in a water bath at 45 °C for 15 min. After cooling, a gravity filtration was performed (Whatman filter paper, Nº. 1) and the filtrates obtained were stored at −80 °C until completely frozen. The extracts were then freeze-dried (LABCONCO®; FreeZone®, Kansas, USA) under freeze-drying conditions of 0.007 kPa, with a condenser surface temperature of −72 °C for 3 days, and storage at −80 °C.

To obtain the ethanolic malt extract, 1 g of the powder was added to 100 mL of 95 % (*V/V*) ethanol solution. The extraction was carried out at room temperature by magnetic stirring (VWR®, 985VW0CHSEUA, Leuven, Belgium) at 400 rpm for 30 min. Then, it was filtered by gravity (Whatman filter paper, Nº. 1) and the filtrates obtained were placed in a rotary evaporator (VWR®; Ika® RV8, Staufen, Germany), with reduced pressure (90 kPa), at 60 rpm and controlled temperature (40 °C) (VWR®; Ika® HB10, Staufen, Germany), until complete ethanol evaporation. The extracts were freeze-dried under the same conditions described above.

The IS-N craft beer (ABV 8.50 % (*V/V*)) was provided by the brewery responsible for its production. Upon receipt, the sample underwent the necessary treatments prior to experimental testing, following the procedures described in our previous study [*15*]. The contents of the bottle were initially mixed uniformly for 10 s and then subjected to gas removal by sonication (Bandelin Sonorex®, Bandelin, Berlin, Germany) for 40 min at a frequency of 35 kHz at room temperature. After that, the alcohol present was removed using a rotary evaporator (VWR®; Ika® RV8, Staufen, Germany) operating at 40 °C, 60 rpm and a pressure of 90 kPa for 1 hour. The sample was then freeze-dried (LABCONCO®; FreeZone®, Kansas, USA) under a pressure of 0.007 kPa, with the condenser surface cooled to −72 °C, for a period of three days.

### *Determination of total phenolic content and antioxidant capacity*

The determination of total phenolic content (TPC) (Follin-Ciocalteau method) and antioxidant activity using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferrozine (metal chelating activity) assays in aqueous and ethanolic extracts of malts under study was carried out as described in our previous study [*15*].

For TPC quantification, 250 µL of the sample (1 mg/mL), distilled water (blank) or standard gallic acid solutions (GA) (5–100 µg/mL) were mixed with 2.5 mL of Folin-Ciocalteu reagent (0.2 M) and incubated for 5 min at room temperature in the dark. Subsequently, 2 mL of sodium carbonate solution (75 g/L) was added, adjusting the final volume to 5 mL with distilled water. After 1 hour of incubation under the same conditions, the absorbance was measured at 760 nm in a UV-Vis spectrophotometer (Model VWR UV-1600PC, Leuven, Belgium). The total concentration of phenolic compounds was determined by comparison with the standard curve of gallic acid, expressing the results in milligrams of gallic acid equivalents (GAE) per gram of sample.

The $ABTS^{+}\cdot$ assay was conducted from the preparation of the radical by the reaction between ABTS (7.0 mM) and potassium persulphate (2.45 mM) solutions kept in the dark for 16 h at room temperature. The obtained solution was diluted in PBS until it reached an absorbance of 0.7±0.05 at 734 nm. Next, 0.3 mL of the sample (malt extracts, IS-N craft beer), Trolox standard (1–1000 µg/mL) or distilled water (blank) were added to 2.7 mL of the $ABTS^{+}\cdot$ solution. After 30 min of incubation at room temperature in the dark, the absorbance was measured at 734 nm in a UV-Vis spectrophotometer (model VWR UV-1600PC, Leuven, Belgium), and the antioxidant activity was expressed as inhibitory average concentration $IC_{50}$ (µg/mL) and the percentage of inhibition was calculated according to the established formula:

$$\mathrm{ABTS^{+}\cdot\ inhibition}=(A_{\mathrm{blank}}-A_{\mathrm{sample}})/(A_{\mathrm{blank}})\cdot 100 \qquad /1/$$

In the ferrozine assay, 50 µL of the sample (beer or positive control, EDTA) was added to a 0.15 mM ferrous sulphate solution and left to stand for 5 min, protected from light. Next, 50 µL of 0.5 mM ferrocyanide was added, and the mixture was shaken vigorously and left for 10 min at room temperature and protected from light. The absorbance was measured at 562 nm in a microplate reader (Thermo Scientific; Multiskan FC, Woodlands, Singapore). Metal chelating activity was expressed as $IC_{50}$ (µg/mL), and the percentage inhibition was calculated according to the following formula:

$$\mathrm{Chelating\ activity}=(A_{\mathrm{control}}-A_{\mathrm{sample}})/(A_{\mathrm{control}})\cdot 100 \qquad /2/$$

Determination of the antioxidant potential using the 2,2-diphenyl-1-picrylhydrazyl assay (DPPH) was carried out as described by Silva *et al.* [*18*], with slight modifications. Briefly, 19.4 µL of sample (1, 5, 10, 25, 50, 100, 250 and 500 µg/mL of malt extracts, craft beer (IS-N) or quercetin - positive control) was added to 175 µL of light-protected DPPH radical (100 µM). The absorbance was measured in a microplate reader (Thermo Scientific; Multiskan FC, Woodlands, Singapore) at a wavelength of 520 nm. The readings were repeated every minute for 1 hour. The ability to neutralize the DPPH radical was expressed as $IC_{50}$ (µg/mL) and the percentage of inhibition was calculated using the following formula:

$$\mathrm{Chelating\ activity}=(A_{\mathrm{blank}}-A_{\mathrm{sample}})/(A_{\mathrm{blank}})\cdot 100 \qquad /3/$$

*Cell line maintenance*

HepG2 cells were maintained as described by Oliveira *et al.* [*19*]. The cells were cultured in 25 $cm^3$ flasks in MEM medium supplemented with 10 % (*V/V*) FBS and 1 % (*V/V*) antibiotic (ampicillin and streptomycin) and incubated at 37 °C with 5 % $CO_2$ (Advantage-Lab® AL01-01-100, Schilde, Belgium). The culture medium was changed every 2 days, and sub-culture took place when 60/80 % confluence was reached, with the addition of 0.25 % trypsin-EDTA.

### *Evaluation of liver toxicity*

To study the cytotoxicity of craft beer and 95 % (*V/V*) aqueous and ethanolic malt extracts, the methods described by Carvalho *et al.* [*20*] and Viegas *et al.* [*21*] were used, with slight modifications. Briefly, HepG2 cells were incubated in 96-well plates (VWR®, Radnor, USA) at a density of $2.0 \cdot 10^5$ cells/well, 48 h before incubation with the samples. The cells were then treated with different concentrations of the samples (1−500 μg/mL) for 24 and 48 h (100 μL final volume/well), and cytotoxicity was estimated using the MTT assay. Briefly, after the incubation period, 10 μL of the MTT solution (5 g/mL) was added to the wells and left for 1 h in an atmosphere of 5 % $CO_2$ at 37 °C (Advantage-Lab® AL01-01-100, Schilde, Belgium). Afterwards, the medium was removed and the formed formazan crystals were dissolved in a 50:50 (*V/V*) DMSO:ethanol absolute solution. The absorbance was measured at a wavelength of 570 nm using a microplate reader (Thermo Scientific; Multiskan FC, Woodlands, Singapore). The results were expressed as a percentage of cell viability in relation to the control (cells without extract) using the equation:

$$\text{Cell viability}=(A_{\text{sample}}/A_{\text{blank}})\cdot 100 \qquad /4/$$

### *Alanine aminotransferase activity measurement*

Measurement of alanine aminotransferase (ALT) enzyme activity was performed based on the method described by González *et al.* [*22*], using assay kits obtained from Randox Laboratories. HepG2 cells were treated with aqueous and ethanolic extracts of the malts, and with craft beer (IS-N) in aqueous and ethanolic solvents (corresponding to the original percentage of alcohol). The concentrations were selected based on cell viability results, choosing, for each sample, the concentration that induced the highest and lowest cell viability. After the incubation period of 24 and 48 h, the supernatant was removed from the wells, and the enzyme activities were determined immediately. The results were expressed in units per litre (IU/L).

### *Statistical analysis*

Statistical analysis was done using GraphPad Prism® v. 8.0 software [*23*]. The results of the assays were analysed in triplicate and expressed as mean±standard deviation. The comparison of the samples in terms of TPC and antioxidant potential was determined using a simple statistical analysis of variance (one-way ANOVA) with Tukey's multiple comparison test for comparisons between multiple pairs. The correlation between the mean TPC and the antioxidant assays was determined by the correlation coefficient (r), calculated by Pearson's product-moment correlation.

Regarding the cell viability assay, the one-way ANOVA with Dunnett's multiple comparison test was used to compare each concentration with the control (cells without extract). The comparison of the metabolic activity of the extracts under study (aqueous and ethanolic) of each malt and beer sample for the same concentration was made using Sidak's multiple comparison test (one-way ANOVA). The same test was used to compare the levels of liver enzymes produced by incubating the cells with two concentrations of the same extract under study. For all assays, statistical differences were considered significant when $p<0.05$.

## RESULTS AND DISCUSSION

### *Total phenolic content*

Malt is the main source of phenolic compounds in beer (75–80 %) [*18*]. Phenolic compounds are secondary metabolites containing at least one aromatic ring linked to hydroxyl groups or other structural elements. These compounds represent a group of substances with different chemical structures, differing in terms of their resistance to free radicals and metal chelation, as well as other reactions that occur in beer or living cells [*5*]. Recognized as potent antioxidants, phenolic compounds play critical roles in the sensory properties, colour and colloidal stability of beer, as well as contributing to antioxidant activity [*24*,*25*]. Table 1 shows the results of the TPC determined in the four malts. Regarding TPC of craft beer (IS-N), those values were already determined in our previous study (TPC=(8.27±0.16) mg GAE/g of extract) [*15*].

In this study, the TPC of malts varied considerably between the extracts analysed ((7.12±0.54)–(28.19±0.53) mg GAE/g). The ethanolic extract of Caramunich III had the highest value ((28.19±0.53) mg GAE/g) and was statistically superior to the others ($p<0.05$), followed by the aqueous extract of the same malt. On the other hand, the ethanolic extract of Carapils had the lowest TPC ((7.12±0.54) mg GAE/g), significantly lower than the others ($p<0.05$). These results indicate a tendency for TPC to increase with the intensity of the malt colour, showing that darker malts such as Caramunich III and Carafa III have a higher concentration of phenolic compounds compared to lighter malts such as Pilsner and Carapils.

At a European level, the colour of malts and beer is expressed in units of the European Brewery Convention (EBC). Pilsner malt (3 and 4 EBC) is the lightest of the malts analysed and is classified as a base malt, used mainly to provide fermentable sugars, given its high enzymatic activity [*18*,*26*–*28*]. Meanwhile, Carapils (10−20 EBC), Caramunich III (130−160 EBC) and Carafa III (1250−1400 EBC) are considered specialty malts [*26*,*28*]. Due to the heat treatment they undergo, a loss in enzymatic activity is verified and for this reason these malts are traditionally used in smaller quantities (usually around 5 %) compared to base malts [*27*]. Its main function is to contribute to specific sensory characteristics of the beer, such as colour, aroma and flavour [*18*,*27*].

The variation in TPC observed between different malts and types of extract (aqueous *vs* ethanolic) corroborates the literature, which highlights the influence of the solvent on the extraction of phenolic compounds [*9*,*29*]. Other factors, such as the barley variety, the growing region, the use of fertilizers, as well as the germination and drying stages, also have an impact on the phenolic content and antioxidant activity of malts [*27*,*30*,*31*]. The main phenolic compounds present in malt are (+)-catechin, protocatechuic acid, quercetin, ferulic acid and gallic acid [*31*].

According to the literature, the amount of phenolic compounds in malt tends to increase with increasing colour intensity, especially up to around 450-500 EBC [*32*]. This is partly due to the polymerization and preservation of phenolic compounds during moderate drying regimes. In addition, products of the Maillard reaction, such as melanoidins, also contribute to the antioxidant potential and are formed in greater quantities during drying and roasting. However, in malts with a colour above 500 EBC, such as the Carafa III analysed in this study, there is a decrease in the content of phenolic compounds, which can be explained by the ability of melanoidins to retain simple phenolic compounds in their structure, as well as the possible inactivation of enzymes (such as ferulic acid esterase) responsible for their release from barley cell walls [*32*].

The literature also shows variations in TPC between different types of malt and barley. Zhao *et al.* [*9*] reported TPC values ranging from 2.17 to 2.56 mg GAE/g dry barley for 14 varieties of Chinese malted barley (Gan4, Gan3 e Wupi1, Ken2 e Ken3, Humai8, Humai16, Gangpi1, Suyin1, Huaimai19, Linnong, Nongmai, KA4B e Gang2), extraction with acetone 80 % (*V/V*). In the study by Gąsior *et al.* [*27*], the wort obtained from Pilsner malt had a TPC of (192.58±8.66) mg GAE/L. In contrast, in the study by Oliveira Neto *et al.* [*33*], the aqueous extract of Pilsner malt revealed a considerably lower TPC of only (0.91±0.00) µg/mL.

In the study performed by Šimić *et al.* [*34*], which evaluated nine varieties of barley and their respective malts (Barun, Bravo, Bingo, Premium, Vanessa, Tiffany, Maxim, Gazda, Rex e Average), extraction with acidified methanol (HCl/methanol, 1:100, *V/V*), demonstrated a higher content of total phenolics and antioxidant activity in malts compared to their corresponding barleys. The TPC values for the malts ranged from (1.53±0.09) to (1.82±0.00) mg GAE/g dry mass (DM), while for the barleys, they ranged from (1.27±0.03) to (1.67±0.09) mg GAE/g DM. These results are in accordance with the study performed by Dvořáková *et al.* [*30*], which found that most of the ten varieties of aqueous extract from the malts studied (Prestige, Jersey, KM1910, Malz, Merlim, Sebastian, Tolar, Bojos, Amulet) had higher antioxidant activity compared to their respective barleys, ranging from 1.1 to 2.9 mg GAE/g DM and from 0.6 to 1.5 mg GAE/g DM, respectively.

In our previous study [*15*] IS-N beer had a TPC of (8.27±0.16) mg GAE/g of extract, lower than that observed in the malt extracts analysed, with the exception of the ethanolic extract from Carapils. This difference may be related to the technological brewing process, such as malting, the milling method, as well as the method and mode of mashing, which influence the final phenolic content of the wort and beer [*27*,*32*]. Furthermore, the difference may also be related to the fact that, in beer formulation, dark (or special) malts are traditionally used in smaller quantities compared to the base malt (in this case, Pilsner), which has lower TPC values [*27*]. This may explain the lower TPC values observed in beer compared to the malts analysed individually.

The study performed by Censi *et al.* [*29*] corroborates the differences in TPC between the different extracts used and the final beer. In the aqueous starter malts, TPC ranged from (11.672±1.814) to (16.568±2.412) mg GAE/g, and the ethanolic extracts ranged from (28.101±1.052) to (72.143±1.866) mg GAE/g. The beers analysed ranged from (18.961±1.082) to (35.822±0.147) mg GAE/g.

The phenolic profile of beer is diverse, encompassing catechins and proanthocyanidins, prenylchalcones and their flavanone derivatives, as well as flavonols, hydroxybenzoic acids, hydroxycinnamic acids and stilbenes [*35*].

It should be noted that the Folin-Ciocalteu method, despite being widely used for drinks and plant extracts, is not specific for phenolic compounds and can be affected by other compounds with reducing activity [*9*,*25*]. In this way, the possibility of overestimating the TPC results cannot be ruled out [*25*].

### *Antioxidant assays*

Beer and malts represent a heterogeneous matrix that has antioxidants with different mechanisms of action, which translates into different contributions to their antioxidant capacity [*36*,*37*]. The antioxidant activity of the Imperial Stout craft beer (IS-N) under study was determined previously by the research group, using different colorimetric antioxidant assays (ABTS and ferrozine assays) [*15*], as there is no standard method capable of objectively characterizing the total antioxidant capacity of the samples [*38*].

ABTS and DPPH assays allow the antioxidant potential of an extract to be measured by its ability to donate hydrogen atoms and electrons, neutralizing the respective radicals with a consequent decrease in absorbance [*39*]. Ferrozine assay, based on metal chelating activity, estimates the antioxidant potential of the extract by its ability to chelate ferrous ions ($Fe^{2+}$), making it impossible to oxidize them to ferric ions ($Fe^{3+}$), preventing the formation of the hydroxyl radical ($OH^{\cdot}$), the initiator of the oxidative chain [*40*]. Some of the assays, such as ABTS and DPPH, are common in beers and cereals due to their sensitivity, convenience, and ease [*34*]. The results of the assays in malts are shown in Table 2. Regarding the antioxidant activity of the craft beer (IS-N) determined by the DPPH assay, it was not possible to determine $IC_{50}$ values for the range of concentrations analysed.

The antioxidant potential of the malt extracts according to the ABTS assay ranged from (17.32±0.77) µg/mL (Caramunich III aqueous extract) to (133.59±2.01) µg/mL (Caramunich III ethanolic extract). It should be noted that the aqueous extract of Caramunich III had the lowest $IC_{50}$ value, showing high antioxidant capacity ($IC_{50}$<50 µg/mL), with significant differences compared to other samples ($p<0.05$).

Analyzing the results, it can be verified that the aqueous malt extracts generally showed greater antioxidant potential (from (17.32±0.77) µg/mL for Caramunich III aqueous extract to (57.11±1.21) µg/mL for Pilsner aqueous extract) compared to the ethanolic extract (from (38.15±0.78) µg/mL for Pilsner ethanolic extract to (546.94±9.38) µg/mL for Carapils ethanolic extract). This suggests that the antioxidant compounds with the greatest potential to neutralize 50 % of the initial amount of $ABTS^{+}\cdot$ free radicals are more soluble in water than in 95 % (*V/V*) ethanol, except for the ethanolic extract of Pilsner malt (activity of the ethanolic extract higher than the aqueous extract,

$p<0.05$). The study performed by Censi *et al.* [*29*] corroborates the obtained results, as the aqueous extracts of the initial malt of the analyzed beers presented a higher antioxidant potential (ranging from (21.389±2.694) µmol TE/g to (46.823±0.031) µmol TE/g), determined by the ABTS assay, than the ethanolic extracts with 70 % ethanol (*V/V*), with an antioxidant potential ranging from (41.305±2.778) µmol TE/g to (97.207±19.118) µmol TE/g.

In our previous study [*15*], IS-N craft beer showed an IC$_{50}$ of (80.09±1.11) µg/mL determined by the ABTS assay, showing moderate antioxidant activity (50<IC$_{50}$<100 µg/mL) [*41*], similar to what was found in the present work for the ethanolic extract of Carafa III, the ethanolic extract of Caramunich III, and the aqueous extract of Pilsner.

In the study by Gąsior *et al.* [*27*], the wort obtained from Pilsner malt was able to inhibit (22.17±0.87) % of ABTS$^{+\cdot}$ radicals. Also, in the study performed by Oliveira Neto *et al.* [*33*], (42.82±13.32) µL of aqueous extract from Pilsner malt was able to neutralize 50 % of ABTS$^{\cdot+}$ radicals. Zhao *et al.* [*9*] found that the antioxidant potential of acetone extracts (80 %(*V/V*)) from 14 varieties of Chinese malt, determined by to the ABTS assay, ranged from 11.39 to 13.58 µmol TE/g of dry barley. Finally, Dvořáková *et al.* [*30*] demonstrated that the antioxidant capacity of aqueous malt extracts of 10 varieties determined using the ABTS assay ranged from 0.20 to 0.45 mg GAE/g dry extract.

Regarding the DPPH assay, the antioxidant potential of the malt extracts ranged from (152.64±9.34) µg/mL in the aqueous extract of Caramunich III to (441.23±9.38) in the ethanolic extract of Carapils. The aqueous extract of Caramunich III was the only aqueous extract in which it was possible to determine the IC$_{50}$ value in the range of concentrations tested (1–500 µg/mL). However, its antioxidant capacity is considered low, given that its IC$_{50}$>100 µg/mL [*41*].

On the other hand, as previously mentioned, it was not possible to determine IC$_{50}$ in the IS-N craft beer, as well as in the aqueous extract of Carafa III and both Pilsner malt extracts, at the concentrations tested (1–500 µg/mL), indicating low antioxidant efficacy determined by the DPPH method.

In the study by Özcan *et al.* [*42*], it was observed that methanolic extracts from malt showed greater antioxidant activity than the same solvent extracts from barley, with inhibition percentages of (67.31±0.00) % for malt and (66.48±0.00) % for barley. These results were corroborated by Šimić *et al.* [*34*], where, using acidified methanol extraction, DPPH inhibition ranged from (63.06±1.88) % to (68.37±2.01) % for malts, and from (58.19±2.15) % to (65.19±1.49) % for barleys.

The data obtained in this study do not corroborate the results observed by Silva *et al.* [*18*], in which the beers showed better antioxidant activity than the raw materials. In the present study, aqueous extracts of Carafa III, Caramunich III, Carapils and Pilsner had greater antioxidant activity compared to the beer (IS-N), in ABTS assay. Using the DPPH assay, ethanolic extracts of Carafa III and Carapils, as well as the extracts of Caramunich III (ethanolic and aqueous), demonstrated greater antioxidant potential than beer.

These differences in the results can be attributed to various biotic and abiotic factors that influence plant physiology and the production of secondary metabolites with antioxidant activity, as well as the type of solvent used in the extraction, which plays a crucial role in the efficiency of phenolic compound extraction [*9*].

In addition, the selection of barley varieties with greater metal chelating activity is essential for beer quality, as it contributes to the stability of its flavour. This is because transition metal ions can activate the oxygen present in the drink, promoting oxidation reactions that result in the formation of compounds responsible for undesirable flavours [*9*].

In the present study, using the ferrozine assay, it was not possible to determine the $IC_{50}$ values in the range of concentrations tested (1–500 µg/mL) for the craft beer or the malt extracts. These results indicate that the samples studied had low antioxidant capacity ($IC_{50}$>100 µg/mL) [*41*].

In the study performed by Zhao *et al.* [*9*], values of metal chelating activity in Chinese malts extracted with 80 % (*V/V*) acetone varied between 1.15 and 2.06 µmol EDTAE/g dry barley, with a weak correlation between TPC and metal chelating capacity. In the study by Silva *et al.* [*18*], the chelating activity of metals in the aqueous extracts of the evaluated malts varied between (12.0 ± 0.5) and (24.8 ± 0.6) %, with the aqueous extract of Carapils malt showing a chelating percentage of (20.5±0.7) %.

In this study, regarding malt extracts, there was a significant correlation only between the ABTS and DPPH antioxidant assays ($p<0.05$) when analysing the ethanolic extracts (Table 3), as would be expected given that these assays are based on the transfer of electrons or hydrogen atoms. Furthermore, there was a negative correlation between TPC and the ABTS test in the aqueous extracts, as well as between TPC and the ABTS and DPPH assays in the ethanolic extracts, which attests to the fact that phenolic compounds contribute to the elimination of ABTS and DPPH radicals, *i.e.* antioxidant capacity.

In the study performed by Zhao *et al.* [*9*], a weak correlation was observed between TPC of malt extracts and metal chelating capacity, but a significant correlation of TPC with the ABTS and DPPH assays ($p<0.01$), as well as a significant correlation ($p<0.01$) between the ABTS and DPPH assays themselves, as observed in the present study. In turn, the study by Gąsior *et al.* [*27*] reported a strong correlation between TPC of malt extracts and the ABTS assay ($r=0.75$).

### *Toxicity of craft beer and malt extracts*

#### Metabolic activity

In the present study, HepG2 cells were incubated with IS-N beer in aqueous and ethanolic solvents corresponding to the original alcohol percentage (8.5 %(*V*/*V*)), as well as with the aqueous and ethanolic malt extracts, for 24 and 48 h, in order to ascertain their cytotoxicity (cell viability of less than 80 %) (Fig. 1 and Fig. 2) and the concentration of ALT enzyme (Fig. 3) [*43*,*44*].

The HepG2 cell line, derived from human hepatocarcinoma, is widely used as an *in vitro* alternative to primary human hepatocytes. HepG2 cells maintain several specialized functions of normal human hepatocytes, including the ability to express liver enzymes, and are considered a suitable model for *in vitro* studies of xenobiotic metabolism and liver toxicity [*45*,*46*].

The aqueous and ethanolic extracts of Caramunich III, Carafa III and Carapils showed cytotoxic effects only at a concentration of 500 µg/mL, with the exception of the aqueous extract of Carafa III after 24 h of incubation, which showed cytotoxicity from 250 µg/mL (Fig. 1) [*43*,*44*].

On the other hand, the aqueous and ethanolic extracts of Pilsner showed no significant cytotoxicity at any of the concentrations tested, regardless of the incubation time (Fig. 2a and Fig. 2b).

When analysing the effect of malt extracts on cell viability, it was found that the aqueous and ethanolic extracts of Carafa III (24 and 48 h) and Caramunich III (48 h) did not promote any significant increase in cell viability compared to the control at any of the concentrations tested. In contrast, in the remaining samples, it was possible to observe at least a significant increase ($p<0.05$) in cell viability compared to the control in one of the concentrations evaluated, which suggests a possible protective or stimulating action on cell metabolism on the part of certain compounds present.

In general, the ethanolic malt extracts showed greater cell viability compared to the corresponding aqueous extracts. However, an exception was observed in the aqueous extract of

Pilsner after 48 h of incubation, where there was a clear superiority to the ethanolic extract. In this case, cell viability with the aqueous extract ranged from 96.3 to 312.8 %, while with the ethanolic extract the values ranged from 86.2 to 206.1 %. These results suggest that, specifically for Pilsner malt, the aqueous solvent may have favored the extraction of compounds with greater protective potential, thus promoting a more beneficial cellular response.

These results are in accordance with the work of Yao *et al.* [*47*], which showed that highland barley extracts exert lipid-lowering effects on HepG2 cells, but concentrations above 1000 µg/mL significantly compromise cell viability, suggesting that there is a maximum concentration limit to maintain the integrity and survival of liver cells *in vitro*.

The cytotoxicity assessment of IS-N craft beer revealed that cell viability in aqueous solvent ranged from 98.3 to 114.7 % after 24 h of incubation. After 48 h of incubation, viability varied between 86.5 and 113.4 % in aqueous solvent and between 94.0 and 121.0 % in ethanolic solvent. In other words, always above 80 % under the conditions reported (Fig. 2c and Fig. 2d) [*43*,*44*].

IS-N beer in both solvents was shown to have the ability to increase cell viability compared to the control up to a concentration of 25 mg/mL, when incubated for 24 h ($p<0.01$) and 48 h ($p<0.001$).

However, in the 24 h incubation, IS-N beer in ethanolic solvent, at a concentration of 250 µg/mL, significantly reduced cell viability compared to the control ($p<0.01$), also showing a significant decrease compared to the same concentration in aqueous solvent ($p<0.001$). At 500 µg/mL, there was an even more marked reduction in cell viability ($p<0.0001$), which means that cytotoxicity is present, given that cell viability was less than 80 % [*43*,*44*].

After 48 h of incubation, it was observed that IS-N beer in aqueous solvent, at a concentration of 500 µg/mL, also significantly reduced cell viability compared to the control ($p<0.0001$), showing no cytotoxicity (Fig. 2d) [*43*,*44*]. This reduction in cell viability is less pronounced when compared to the same concentration of IS-N in ethanolic solvent ($p<0.05$).

The increase in cell viability observed in the presence of ethanol, in some concentrations, can be explained by the hormesis phenomenon associated with moderate beer consumption. This effect translates into the ability of low concentrations of ethanol to exert beneficial actions without inducing oxidative stress, unlike what occurs with excessive exposure [*48*,*49*]. Furthermore, the phenolic compounds present in beer, recognized for their antioxidant and bioactive properties, may play an additional protective role on liver cells, especially in situations of limited exposure to ethanol [*48*,*49*].

### Enzyme alanine aminotransferase

The enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are widely used as biochemical markers of liver damage, given their high concentration in the liver [*14*,*50*]. ALT is found predominantly in the cytosol of hepatocytes, while AST is mainly a microsomal enzyme. Increased levels of these aminotransferases in the extracellular environment characterize a hepatocellular pattern of damage, generally associated with damage to the plasma membrane or hepatocyte death. However, AST is less specific to the liver, as it is also present in extra-hepatic tissues such as skeletal muscle, myocardium and kidneys. For this reason, although both are useful in assessing liver cytotoxicity, ALT is considered a more sensitive and specific marker for identifying direct liver damage [*14*,*50*]. Fig. 3 shows the results relating to ALT levels after incubation, 24 and 48 h, of HepG2 cells with the samples under study.

The study of the concentrations where the highest and lowest cell viability of HepG2 cells was found for each sample revealed a clear dose-dependent relationship between ALT levels and the concentrations of the samples tested (Table S1). The lower concentrations induced significantly reduced ALT levels when compared to the same sample at a concentration of 500 µg/mL ($p<0.0001$). The significant increase in ALT activity at 500 µg/mL suggests a more pronounced cytotoxic effect associated with the higher concentrations.

After 48 h of incubation, a further increase in ALT levels was observed in most samples, with the exception of a few situations, namely the aqueous extract of Carafa III (25 µg/mL), the ethanolic extract of Carafa III (100 µg/mL) and the aqueous extract of Pilsner (500 µg/mL). These results may indicate a possible protective effect or slower toxicity kinetics associated with certain compounds present in these formulations.

When comparing samples tested at 500 µg/mL, the ethanolic extract of Carafa III stood out, inducing the smallest increase in ALT, both after 24 and 48 h of incubation. In contrast, the aqueous extract of Carapils caused the greatest increase in enzymatic activity at the same concentration.

In general, the ethanolic extracts induced lower ALT concentrations than the corresponding aqueous extracts at a concentration of 500 µg/mL, except for IS-N beer and Pilsner malt after 48 hours, in which the ethanolic extracts induced higher levels. These results suggest that the solvent can influence the extraction of compounds with different toxicity potentials.

It should be noted that although the aqueous and ethanolic extracts of Pilsner at 500 µg/mL promoted greater cell viability, this result did not translate into lower ALT activity. On the contrary, there was an increase in ALT levels compared to the lower concentrations, where, paradoxically, cell viability was lower.

Finally, IS-N craft beer induced lower ALT levels than those observed in single malt extracts such as Carapils and Pilsner. This effect may be related to the presence of other typical beer components, such as hops and fermentation products, which together can modulate and attenuate the toxic effect of individual ingredients.

The literature has demonstrated the hepatoprotective potential of extracts derived from barley, although largely through experimental models other than the one used in this study. Hosseini *et al.* [*50*], evaluated the effect of aqueous barley extract on rats fed a high-fat diet. The results revealed a significant reduction in the concentration of the AST and ALT enzymes ($p<0.05$) in the experimental group, indicating a beneficial effect of barley extracts in reducing the risk of fatty liver disease.

Quan *et al.* [*31*] demonstrated that pre-treatment with free phenolic extract of barley (FPEB) exerted a significant protective effect in *in vivo* and *in vitro* models. In rats exposed to carbon tetrachloride ($CCl_4$), FPEB significantly reduced serum levels of the enzymes ALT and AST ($p<0.05$), while promoting an increase in hepatic antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. In addition, in BRL cells (rat hepatocytes) treated with $CCl_4$, FPEB significantly attenuated ALT and AST levels ($p<0.001$), as well as reducing apoptosis and induced cell damage. These results reinforce the hepatoprotective potential of barley phenolic compounds.

Park *et al.* [*51*] evaluated the effects of barley sprout extract supplementation in individuals with fatty liver induced by habitual alcohol consumption. After 12 weeks of supplementation, a significant reduction was observed in liver fat content ($p<0.001$) and in levels of the liver enzyme GGT (γ-glutamyl transpeptidase) ($p<0.05$), indicators of improved liver function. Although the reductions in ALT and AST levels did not reach statistical significance, there was a downward trend (ALT: (37.8±2.2) IU/L to (35.9±2.3) IU/L), suggesting a possible beneficial effect of barley sprout extract in modulating the liver's response to alcohol-induced chronic oxidative stress.

Therefore, the results obtained corroborate the relevance of barley-derived compounds in modulating the hepatocellular response. The lower release of ALT observed at the lowest concentrations tested, and in IS-N craft beer compared to single malts, suggests that phenolic compounds and other bioactive compounds present in malt may confer properties with potential benefits for liver health, or at least be less toxic on human liver cells [*31*,*35*].

## CONCLUSIONS

Malt is the second most abundant raw material in the composition of beer and is the main source of fermentable sugars, phenolic compounds and others with antioxidant potential. This study found that TPC and antioxidant activity were influenced by both the variety of malt and the type of solvent used in the extraction process. IS-N craft beer showed lower TPC and antioxidant activity values than most of the malt extracts analysed. However, it was observed that most of the dark malts presented a higher TPC and greater antioxidant potential, especially in the aqueous extracts.

Regarding metabolic activity, the malt extracts showed no cytotoxicity up to a concentration of 250 µg/mL, while IS-N beer presented cytotoxicity when in ethanolic solvent at a concentration of 500 µg/mL, in the 24 h period incubation.

In addition, incubation of HepG2 cells with IS-N beer resulted in lower levels of the ALT enzyme compared to single malts, despite its lower phenolic and antioxidant content. These results suggest a possible synergistic effect between the various bioactive compounds in beer, which could mitigate the toxic effects of the individual ingredients.

In summary, prior assessment of the biological activity of malts can enable careful selection of varieties for beer production, thereby optimising not only the antioxidant and functional profile of the beverage, but also mitigating potential adverse effects on liver function. This optimisation can be achieved by using malts with a higher content of phenolic compounds and high antioxidant capacity, especially dark malts, which have shown better performance in *in vitro* assays, as well as by the appropriate choice of solvents and extraction conditions, favouring the incorporation of more stable and biologically relevant bioactive compounds in the final product. However, to confirm and further these observations, it will be important to expand the analysis to different beer styles and apply complementary extraction and antioxidant methods, in addition to performing phytochemical characterisation of the samples and developing *in vivo* studies to assess the bioavailability, efficacy, and safety of the identified compounds. These advances will allow for a more comprehensive understanding of the functional potential of craft beer and validate the real impact of its antioxidant and cytoprotective properties on liver health.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## SUPPLEMENTARY MATERIALS

Supplementary materials are available at: www.ftb.com.hr.

## AUTHORS’ CONTRIBUTION

D. Santos, M. J. Pereira, A. I. Oliveira and C. Pinho developed the conceptualization and methodology of the study. D. Santos and M. J. Pereira wrote and prepared the original draft. A. I. Oliveira, R. Oliveira, A. Jesus, and C. Pinho validated the data and results, as well as conducted the revision and editing of the manuscript. All authors read and approved the final version of the manuscript.

## ORCID ID

D. Santos https://orcid.org/0009-0002-9855-0686
M.J. Pereira https://orcid.org/0009-0009-5161-8488
A. Jesus https://orcid.org/0000-0003-3972-8253
R.F. Oliveira https://orcid.org/0000-0003-3933-5740
A.I. Oliveira https://orcid.org/0000-0003-4420-6476
C. Pinho https://orcid.org/0000-0002-3157-3712

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Table 1. Results of total phenolic content (TPC) in malt extracts

| Malt extract | TPC (mg de GAE/g) |
|---|---|
| Carafa III aqueous | (22.19±1.10)$^{a,b,c}$ |
| Carafa III ethanolic | (20.69±0.44)$^{a,b}$ |
| Caramunich III aqueous | (23.61±0.61)$^{a,c}$ |
| Caramunich III ethanolic | (28.19±0.53)$^{d}$ |
| Carapils aqueous | (14.04±0.86)$^{e}$ |
| Carapils ethanolic | (7.12±0.54)$^{f}$ |
| Pilsner aqueous | (10.89±0.27)$^{g}$ |
| Pilsner ethanolic | (8.83±0.20)$^{h}$ |

The different letters indicate statistically significant differences ($p<0.05$)

Table 2. Antioxidant activity of malt extracts with ABTS, DPPH and ferrozine assays

| Malt extract | ABTS ($IC_{50}$ µg/mL) | DPPH ($IC_{50}$ µg/mL) | Ferrozine ($IC_{50}$ µg/mL) |
|---|---|---|---|
| Carafa III aqueous | (40.34±0.94)$^{a,e,h}$ | ND | ND |
| Carafa III ethanolic | (118.25±4.93)$^{b}$ | (229.26±9.73)$^{a,c}$ | ND |
| Caramunich III aqueous | (17.32±0.77)$^{c}$ | (152.64±9.34)$^{b}$ | ND |
| Caramunich III ethanolic | (133.59±2.01)$^{d}$ | (227.01±7.02)$^{a,c}$ | ND |
| Carapils aqueous | (41.69±0.13)$^{a,e,h}$ | ND | ND |
| Carapils ethanolic | (546.94±5.29)$^{f}$ | (441.23±9.38)$^{d}$ | ND |
| Pilsner aqueous | (57.11±1.21)$^{g}$ | ND | ND |
| Pilsner ethanolic | (38.15±0.78)$^{a,e,h}$ | ND | ND |

ABTS=2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay, DPPH=2,2-diphenyl-1-picrylhydrazyl assay, $IC_{50}$=concentration able to inhibit by 50 %. The different letters indicate statistically significant differences ($p<0.05$). ND=not detected $IC_{50}$ at the tested concentrations

Table 3. Correlation between total phenolic content (TPC) and antioxidant assays in malt extracts

| | TPC aqueous extract | TPC ethanolic extract | ABTS aqueous extract | ABTS ethanolic extract | DPPH aqueous extract | DPPH ethanolic extract |
|---|---|---|---|---|---|---|
| TPC aqueous extract | 1.000 | - | -0.841 | - | - | - |
| TPC ethanolic extract | - | 1.000 | - | -0.451 | - | -0.940 |
| ABTS aqueous extract | - | - | 1.000 | - | - | - |
| ABTS ethanolic extract | - | - | - | 1.000 | - | 0.999* |
| DPPH aqueous extract | - | - | - | - | - | - |
| DPPH ethanolic extract | - | - | - | - | - | 1.000 |

TPC=total phenolic content, ABTS=ABTS$^{+\cdot}$ neutralization assay, DPPH=2,2-diphenyl-1-picrylhydrazyl assay. *Correlation is significant at the 0.05 level (two-tailed)

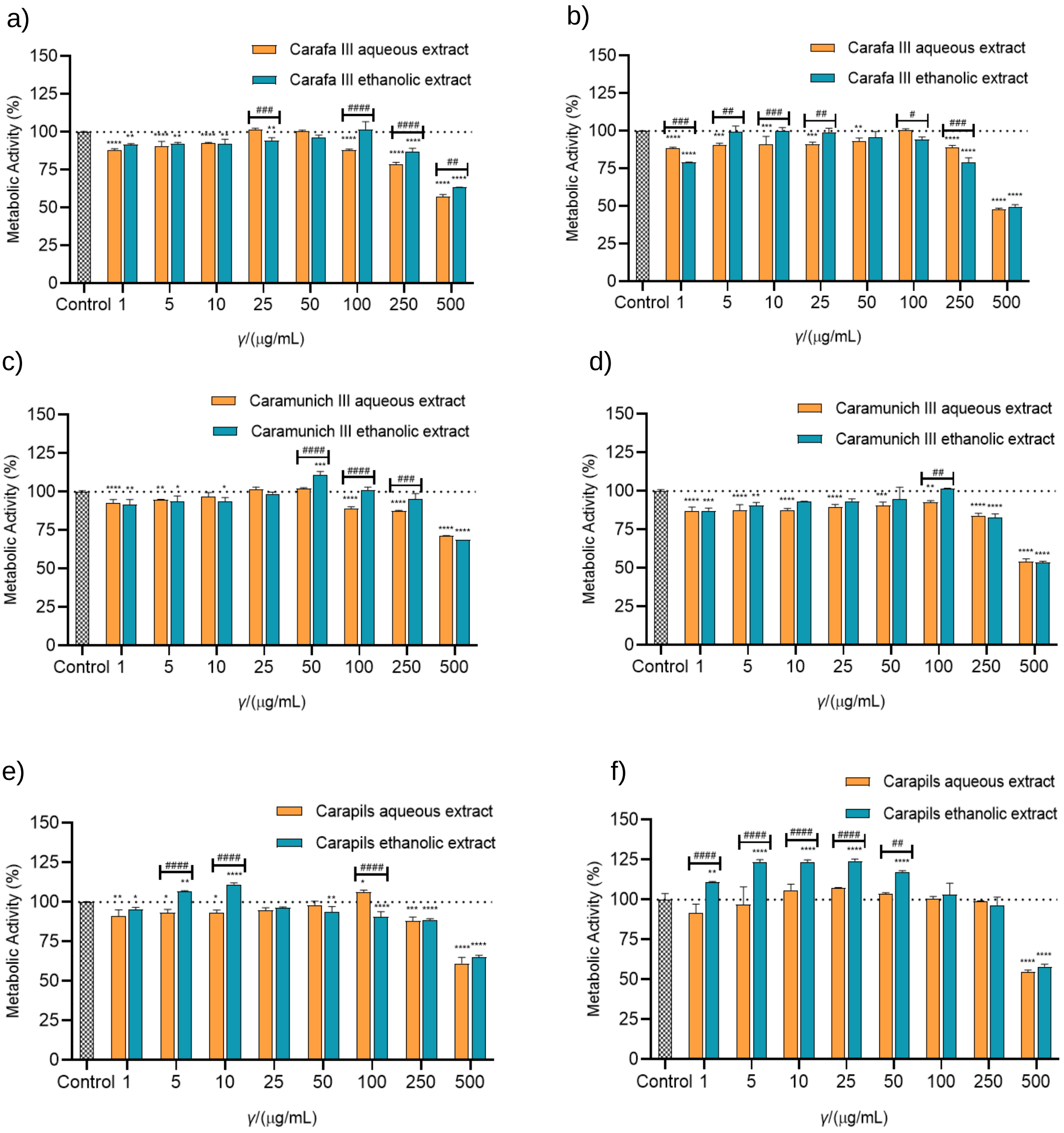

Fig. 1. Cytotoxicity, determined by the MTT assay, of different concentrations of Carafa III, Caramunich III and Carapils malt, in HepG2 cells. Aqueous and ethanolic extract of Carafa III malt after: a) 24 h and b) 48 h. Aqueous and ethanolic extract of Caramunich III malt after: c) 24 and d) 48 h. Aqueous and ethanolic extract of Carapils malt after: e) 24 h and f) 48 h. Data are presented as mean±standard deviation of three independent samples (*N*=3), in triplicate. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$ when compared to the control (cells without extract). #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001 when comparing two similar concentrations

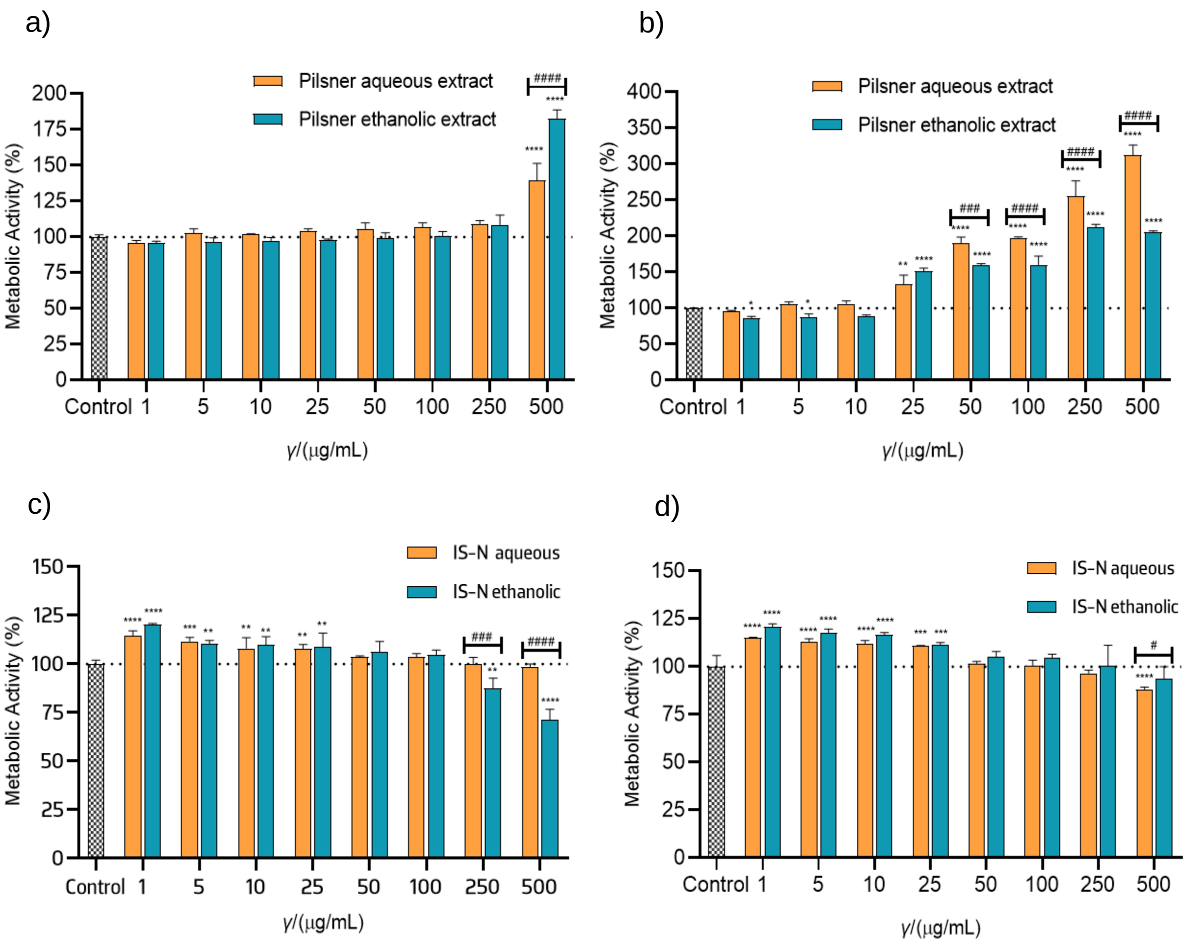

Fig. 2. Cytotoxicity, determined by the MTT assay, of different concentrations of Pilsner malt and IS-N craft beer, in HepG2 cells. Aqueous and ethanolic extract of Pilsner malt after: a) 24 h and b) 48 h. IS-N craft beer in aqueous solvent and in ethanolic solvent (ABV 8.5 % (V/V)) after: c) 24 h and d) 48 h. Data are presented as mean±standard deviation of three independent samples (*N*=3), in triplicate. $*p<0.05$, $**p<0.01$, $***p<0.001$ and $****p<0.0001$ when compared to the control (cells without extract). #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001 when comparing two similar concentrations

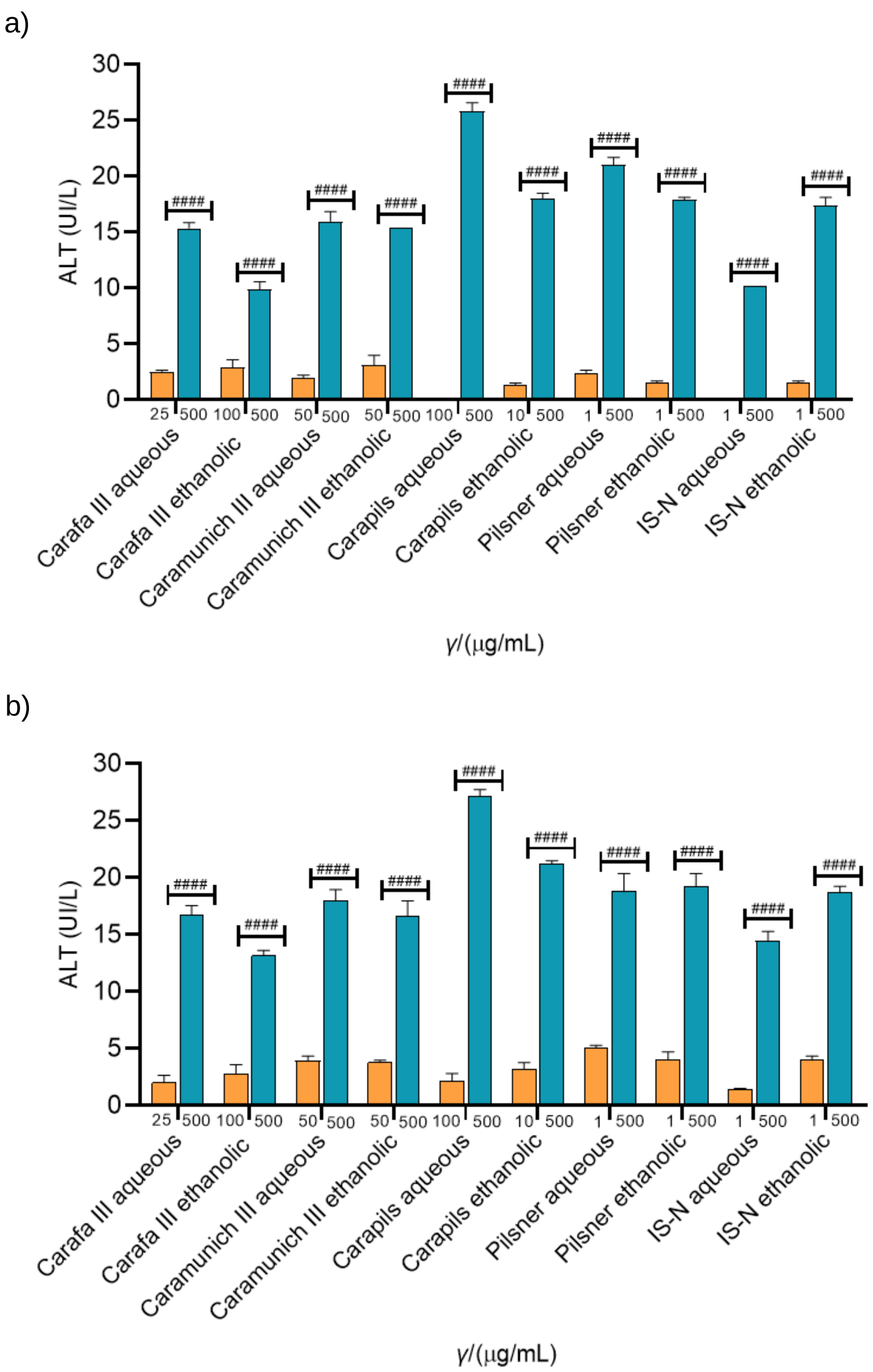

Fig. 3. In vitro determination of ALT of different concentrations of IS-N beer and aqueous and ethanolic extracts of malts, in HepG2 cells. Aqueous and ethanolic extract of malts and IS-N craft beer in aqueous solvent and in ethanolic solvent (ABV 8.5 % (V/V)) after: a) 24 h and b) 48 h. Data are presented as mean±standard deviation of three independent samples (*N*=3), in triplicate. #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001 when comparing different concentrations of the same sample

## SUPPLEMENTARY MATERIAL

Table S1. Results of determination of ALT of different concentrations of IS-N beer and aqueous and ethanolic extracts of malts, in HepG2 cells

| Malt extract | $\gamma$/(μg/mL) | *t*(incubation)/h | |
|---|---|---|---|
| | | 24 | 48 |
| | | ALT/(UI/L) | |
| Carafa III aqueous | 25 | (2.57±0.09) | (2.00±0.66) |
| | 500 | (15.40±0.42) | (16.72±0.85) |
| Carafa III ethanolic | 100 | (2.94±0.66) | (2.85±0.75) |
| | 500 | (9.93±0.66) | (13.16±0.47) |
| Caramunich III aqueous | 50 | (1.98±0.26) | (3.93±0.42) |
| | 500 | (15.99±0.87) | (18.01±0.95) |
| Caramunich III ethanolic | 50 | (3.13±0.85) | (3.79±0.19) |
| | 500 | (15.49±0.00) | (16.62±1.36) |
| Carapils aqueous | 100 | (0.00±0.00) | (2.19±0.66) |
| | 500 | (25.87±0.75) | (27.19±0.57) |
| Carapils ethanolic | 10 | (1.34±0.19) | (3.23±0.57) |
| | 500 | (18.04±0.47) | (21.21±0.29) |
| Pilsner aqueous | 1 | (0.00±0.00) | (5.11±0.19) |
| | 500 | (10.21±0.00) | (18.79±1.60) |
| Pilsner ethanolic | 1 | (1.53±0.19) | (4.08±0.66) |
| | 500 | (17.94± 0.19) | (19.26±1.13) |
| IS-N aqueous | 1 | (0.68±0.09) | (1.43±0.09) |
| | 500 | (12.66±1.13) | (14.45±0.85) |
| IS-N ethanolic | 1 | (1.53±0.19) | (3.98±0.38) |
| | 500 | (17.38±0.75) | (18.70±0.57) |