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Characterization and Antioxidant Activity of Portuguese Craft Beers

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Resumo

A produção e o consumo de cerveja têm aumentado, face à popularidade da cerveja artesanal. A presença de compostos fenólicos, afeta as características organoléticas e estabilidade química, sendo responsável por potenciais benefícios à saúde. O trabalho apresenta como principal objetivo a determinação de parâmetros químicos e da atividade antioxidante em cervejas artesanais e matérias-primas. Para tal, realizou-se um estudo experimental em 16 cervejas artesanais portuguesas, 10 amostras de malte e 11 amostras de lúpulo. Nas cervejas analisou-se o pH, acidez total, açucares redutores e compostos fenólicos totais (TPC). A determinação da atividade antioxidante (ensaio do radical DPPH e da ferrozina) realizou-se nas cervejas e amostras de malte e lúpulo. As cervejas artesanais apresentaram valores de pH entre 4,27 e 4,92; acidez total entre 0,10 ± 0.00% e 0,62 ± 0.01%; açucares redutores entre 2598,0 e 4446,3 mg/L. A amostra AMP-IS obteve o maior valor de TPC (2172,5 ± 170,1 mg GAE/L) e uma atividade quelante do ferro também elevada (46.2 ± 0.9%). O maior valor de inibição do radical DPPH foi de 99.4 ± 0.6% (AMP-IPA). Estes resultados realçam a possibilidade de um consumo relevante de antioxidantes, sem o consumo excessivo de álcool, através de uma seleção adequada de cervejas.

Palavras-chave: cerveja artesanal; antioxidantes; atividade biológica; compostos fenólicos; malte; lúpulo

Abstract

The production and consumption of beer has increased, stimulated by the popularity of craft beer. The presence of phenolic compounds affects organoleptic characteristics and chemical stability, being responsible for potential health benefits. This work aims to determine the chemical parameters and antioxidant activity in craft beers and raw materials. An experimental study was carried out on 16 Portuguese craft beers, 10 samples of malt and 11 samples of hops. In beers, pH, total acidity, reducing sugars and total phenolic compounds (TPC) were analyzed. The determination of antioxidant activity (DPPH and ferrozine assay) was carried out in beers and samples of malt and hops. Craft beers showed pH values ranging from 4.27 to 4.92; total acidity from 0.10 ± 0.00% to 0.62 ± 0.01%; and reducing sugars ranging from 2598.0 to 4446.3 mg/L. The AMP-IS beer sample had the highest TPC value (2172.5 ± 170.1 mg GAE/L) and an iron chelating activity also high (46.2 ± 0.9%). The highest inhibition value of the DPPH radical was 99.4 ± 0.6% (sample AMP-IPA). These results highlight the possibility of achieving a relevant antioxidants intake, without excessive alcohol consumption, through an appropriate selection of beers.

Keywords: Craft beer; antioxidants; biological activities; phenolic compounds; hops; malt

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List of abbreviations and acronyms

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ABV	Alcohol by volume
AC	Absorbance of Control
AS	Absorbance of Sample
CAM	Chorioallantoic Membrane
CAT	Catalase
CCl_4	Carbon Tetrachloride
CO_2	Dioxide carbon
COX	Cyclooxygenase
DMSO	Dimethyl sulfoxide
DNS	3,5-dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EBC	European Brewing Convention
ED_{50}	Median Effective Dose
EDTA	Ethylenediamine tetraacetic acid
EDTAE	Ethylenediamine tetraacetic acid Equivalents
$FeSO_4$	Iron (II) sulfate
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalents
GE	Glucose equivalents
GGT	Gamma-Glutamyl Transpeptidase
$GSHP_x$	Glutathione peroxidase
HDL	High-Density Lipoproteins
IBU	International Bittering Units
IC_{50}	Half maximal Inhibitory Concentration
IPA	India Pale Ale
LDL	Low-Density Lipoproteins
LS	Large Scale
MCA	Metal Chelating Activity
NaOH	Sodium Hydroxide
Na_2CO_3	Sodium Carbonate
NO	Nitric Oxide

OH·	Hydroxyl radical
ORAC	Oxygen Radical Absorbance Capacity
QE	Quercetin equivalents
PPAR α	Peroxisome proliferator–activated receptor alfa
PVP	Polyvinylpyrrolidone
RLE	Rat Lung Endothelial
ROO^{-}	Peroxyl Radical
ROS	Reactive Oxygen Species
SD	Standard Deviation
SOD	Superoxide Dismutase
SS	Small Scale
TE	Trolox Equivalents
TNF	Tumor Necrosis Factor
TPC	Total Phenolic Content
USA	United States of America
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

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1. Introduction

Beer is a very popular alcoholic beverage worldwide. Although the excessive wine and beer consumption is associated with harmful health effects, moderate one is accepted as part of a healthy diet and lifestyle. Regarding beer, its consumption is associated with informal occasions, marking the end of the workday and the beginning of a relaxation time. This beverage has a flexible consumption, since it can be associated with meals or not (Silva et al., 2017).

Although industrial beer is still the most common beer, craft beer has been gaining popularity. Craft beer is characterized by production in small and independent breweries, with an annual production of 6 million barrels or less. Also, only less of 25% of the brewery can be owned or controlled by a beverage alcohol industry member (Garavaglia & Swinnen, 2017). Another requirement for craft beers is that alcohol content must come from beer made with traditional or innovative ingredients and fermented using yeast (Rodhouse & Carbonero, 2019). In most cases, craft beers are not submitted to filtration, pasteurization or re-fermented in bottles, maintaining its original sensorial characteristics, full-bodied taste and aromas (Mastanjević et al., 2019; Mudura et al., 2016).

The popularity of craft brewers is due to its focus on organoleptic characteristics, particularly flavour. To achieve these, a careful selection of raw materials (e.g. malt, hops and yeast strains), and changes in manufacturing processes (e.g. in mashing times and temperatures, fermentation and maturation), are made to obtain a product more appealing to the consumer (Mastanjević et al., 2019; Tozetto et al., 2019). In craft breweries, the goal is not large-scale, low-cost production, but rather combining tradition with innovation, authenticity and product quality, making it more attractive to consumers (Peters et al., 2017). Although craft beers are becoming more popular, information about its potential health-positive extracts or components are still scarce, because most of the studies are still performed with industrial beers.

1.1. Craft Beer Market and Consumption

According to World Health Organisation (WHO), in 2016 beer represented 26% of alcohol consumption in Portugal, making it the second most consumed alcoholic beverage (WHO, 2016). Although industrial beer is the most common, there has been an increase in beer production and consumption, promoted mainly by the growing popularity of craft beer (Peters et al., 2017).

The number of craft breweries continues to grow each year. In the United States of America (USA), in a universe of 7450 breweries, 7346 were classified as craft breweries. In terms of sales, craft beers grew 4% compared to 2017 representing 13.2% of the total beer market in the USA (Brewers Association, 2019). In Portugal there are about 640 thousand consumers of craft beer. Portuguese craft beer market has grown five times above mean of market, between 2017 and 2019. In 2017 there were 120 breweries in

activity, 115 of which correspond to microbreweries (Marktest, 2018; Pinto, 2019). The main reasons why consumers prefer craft beer are the perception of high quality raw materials used, the image of a less industrialized product and the experience of new tastes and different types of beer (Mudura et al., 2016).

1.2. Craft Beer Production Process

Beer has four main ingredients: malted cereals, hops, water and yeast. The most used cereal is barley, however, wheat, sorghum or rye can also be used. This raw material is the primary source of protein, lipid, carbohydrate, and polyphenols present in beers. Hops (*Humulus lupulus* L.) is mainly used to provide bitterness and characteristic flavours to beer. However, this component is also important to protect beer of bacterial spoilage. Some varieties of hops are used for its ability to provide bitterness and others specifically to provide hop aromas. Water is, quantitatively, beer´s main ingredient, corresponding to more than 90% of the final product. The mineral content of the water used in brewing affects the beer properties and contributes to final product's flavour. Yeast is responsible for the fermentation process and has a fundamental impact on the beverage's quality, since the chosen strain influences the final product properties (Preedy, 2009).

Beer production process begins with malting, a stage where controlled cereal germination takes place, at low temperature. This process allows the activation of enzymes that degrade sugars, which will hydrolyze starch and convert it into fermentable sugars that will be used later by yeasts. Then, cereals are roasted in order to dry, stop germination and maintain the enzymes degradation capacity. The malted cereals are then ground and hot water is added and kept at approximately 62°C, to start the mashing process. In this phase, starch is hydrolyzed to oligosaccharides with up to four polymerization degrees as maltose, maltotriose, fructose, glucose and sucrose. At the end of the mashing step, wort (a sugar-containing liquid) is formed. It is then strained with water and brought to boiling, time when the hops are added. In the boiling phase wort is sterilized, bitter compounds from hops added early to the boil and oils and aroma compounds from late additions are extracted. In this process proteinaceous complexes precipitate and unwanted volatiles are removed. Also, wort is concentrated by water evaporation (Humia et al, 2019).

The product obtained is cooled to a temperature compatible with the growth of the selected, to be added, yeasts. During fermentation, yeasts convert sugars to alcohol and carbon dioxide (CO_2). The last step is beer maturation, which consists in storing it at low temperatures. The temperature and time of maturation depends of the beer style (Eaton, 2017; Humia et al., 2019). In industrial beers there is an extra step of filtration and pasteurization of the final product. A stabilizer, polyvinylpyrrolidone (PVP) is also added. Filtration and addition of stabilizers does not occur in most craft beers (Humia et al., 2019; Marques et al., 2017).

1.3. Beer styles

Beer classification is primarily based on fermentation process. Beer styles can be divided into two main groups: Lager beers and Ale beers. Beers of the Lager type include different styles, namely, Bock, Dortmunder, Munchener, Pilsener and Vienna (Marzen). Beers of Ale type include a variety of styles such as, Porter, Stout, Salsons, Alt, Light ale, Pale ale, Bitters and Barley wines (Caballero et al., 2003).

The main difference between these groups is in the yeast and the temperature during fermentation. Lagers, the most consumed type of beer, are produced by low fermentation, which is carried out under temperatures that range from 3.3 to 13.0°C, for 4–12 weeks. *Saccharomyces pastorianus* strains are the yeasts associated to this beer style. After fermentation, yeast cells deposit at the bottom of the fermentation tank and are usually removed (Libkind et al., 2011; Oliveira Neto et al., 2017; Wendland, 2014). Main lagers include pale lager, bock, dunkel, helles, oktoberfest, pilsner, schwarzbier, and vienna lager (Sánchez-Muniz et al., 2019). In contrast, Ale beers are produced by high fermentation, occurring between 16–24°C for shorter periods. *S. cerevisiae* is the yeast employed in this style. After fermentation yeast cells rise to the surface of fermentation tank, forming a thick film that is generally not completely removed (Libkind et al., 2011; Moura-Nunes et al., 2016).

Within these two large groups, there are several styles originated from variations in process, formulations and ingredients. Beer styles can be differentiated by their own characteristics such as color, aroma, bitterness and alcohol content (Humia et al., 2019; Moura-Nunes et al., 2016). An additional difference between lager and ale strains is related to the capacity to produce different types and levels of flavoring compounds. Lager yeasts are acknowledged by the lack of an ester-derived fruity or floral aroma, while ale yeasts produce higher concentrations of esters and higher alcohols (Krogerus et al., 2015). The following figure (Figure 1) shows the main existing beer styles.

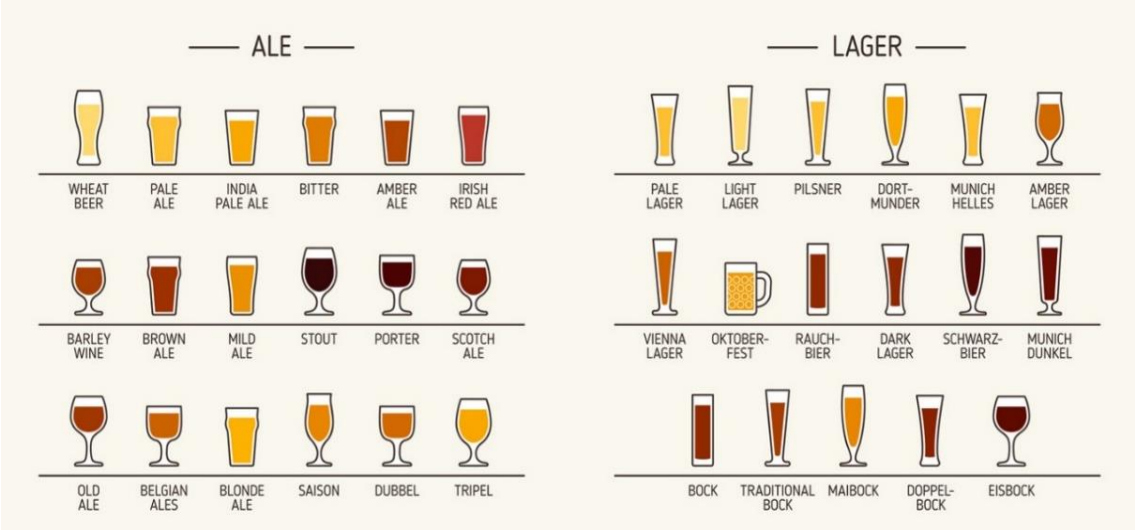

Figure 1 – Main beer styles (Image retrieved from "Estilos de Cerveja Variedades de Cerveja Tipos de Cerveja Estilos de Cerveja," n.d.).

1.4. Craft Beer Composition

Beer has a higher nutritional value than other alcoholic beverages, because of the numerous compounds (like vitamins and essential nutrients), originated, mainly, from brewing materials and the processes of malting and fermentation (Tafulo, Queirós, Delerue-Matos, & Sales, 2010).

The main component of beer is water (approximately 90%), followed by alcohols resulting from the fermentation process (on average 3.5–10%), carbohydrates (1-6% w/V), and minerals. Beer is also composed by CO_2, inorganic salts, nitrogen, organic acids, higher alcohols, aldehydes, esters, sulphur compounds, hop derivates and B complex vitamins. The main inorganic salts present in beer are calcium, magnesium, sodium, potassium, sulphate, chloride, phosphate, carbonate, and nitrate. Other elements are present but only in small amounts and are known as trace elements (Cortese et al., 2020). In general, organic acids belong to yeast and bacterial fermentation, while the inorganic compounds found in beer are metal cations, trace metals, and anions, which influence the drink's clarity and salty taste (Quesada-Molina et al., 2019).

Malt is the primary source of proteins, lipids, carbohydrates, and polyphenols present in beer. As previous described, in mashing step, proteins and peptides are broken down to aminoacids thereby continuing the enzymatic degradation started during malting operations. Most of these aminoacids will be used by the yeast in fermentation step. Some wort aminoacids are metabolized by yeast to form higher alcohols, which are important flavour compounds in beer. Lipids present in beer have a very low concentration (less than 0.1%) and can be considered a fat-free drink. Although yeast ferment most of the sugar, converting it to alcohol, it is still possible to find some carbohydrates in the final product. The main carbohydrates present in beer are fructose, glucose, sucrose, maltose and maltotriose. Malt is rich in several vitamins, that are solubilized into wort. Complex B vitamins (specially biotin, inositol and panthotenic acid) showed to be a key as growth factor for yeast. For this reason, lower concentration of vitamins in the final product are found, but beer is still considered a valuable source of many water-soluble vitamins (particularly folate, riboflavin, panthotenic acid, pyridoxine, and niacin) (Buiatti, 2008).

Yeast plays an important role in the formation of components found in beer. Besides alcohol and CO_2, fermentation also produces higher alcohols, organic acids, esters, aldehydes, ketones and sulphur compounds, which are very important in the definition of the sensorial profile of beer (Buiatti, 2008).

Hops, another important ingredient of craft beer, are used to provide bitterness and characteristic hoppy flavours. Hops contain a range of components, but the most important in brewing are resins and essential oils. Soft resins can be divided in two groups of compounds known as humulones (α-acids) and lupulones (β-acids). During the wort boiling, α-acids are converted to iso-α-acids (iso-humulones), which are the most important bitter compounds in beer. The amount of iso-α-acids is measured by the

International Bitterness Units (IBU). Hops essential oils are responsible for beer's aroma and flavour (Buiatti, 2008).

Beer also contains several phenolic compounds which are derived from malt (two-thirds) and hops (one-third). These compounds contribute to beer's flavour, aroma, and chemical stability. In general, craft breweries use high quality raw materials, during beer production, which may be related to the presence of different and more abundant phenolic compounds in craft beers. Phenolic compounds can act as antioxidants and prevent the oxidative degradation of beers. The mostly reported phenolic compounds in beer include flavonoids (e.g. flavanols, flavones), hydroxycoumarins, phenolic acids (e.g. 4-hydroxyphenylacetic, vanillic, caffeic, syringic, p-coumaric, ferulic, and synaptic acids), tannins, proanthocyanidins, and amino phenolic compounds, all of which have been related to antioxidant activity, as well as other biological effects (Humia et al., 2019; Jardim et al., 2018). The European Prospective Investigation into Cancer and Nutrition cohort study reported that beer is the main contributor to hydroxybenzoic acid intake, being a good source of phenolic compounds (Zamora-Ros et al., 2013). Hops also contain many polyphenolic compounds, like xanthohumol and related prenylflavonoids as isoxanthohumol, desmethylxanthohumol, 6-prenylnaringenin, 8-prenylnaringenin and 6-geranylnaringenin (Česlová et al., 2009).

1.5. Biological Activities of Craft Beer and its Bioactive Compounds

It has been demonstrated that beer is rich in bioactive compounds, with a protective role in human health. Beer also has a lower alcohol content compared to other popular alcoholic drinks. Diverse physiological effects and health benefits in cardiovascular disease, diabetes, specific cancer types, inflammation, neurodegenerative diseases and osteoporosis have been associated with beer consumption (Ristivojević & Morlock, 2018; Sanna & Pretti, 2015).

1.5.1. Cardiovascular diseases

Moderate and regular consumption of alcoholic beverages is associated to cardioprotective effects (Jastrzebski et al., 2007; Piazzon, Forte, & Nardini, 2010). This effect can be noted in adults including higher risk populations (individuals with diabetes, hypertension, hypercholesterolemia, heart disease, or who are overweight and smokers) (Krenz & Korthuis, 2012). This inverse correlation between cardiovascular mortality and alcoholic beverages consumption is known as the 'French Paradox'. Alcohol is the main responsible for this protective role, for its effect on blood lipid profile and coagulation. At low doses, alcohol consumption leads to an increase in High-Density Lipoproteins (HDL) cholesterol and appears to decrease

Low-Density Lipoproteins (LDL) cholesterol. Also, decreases platelet aggregation, fibrinogen and some procoagulant factors other than fibrinogen. There is a positive relation between alcohol consumption and plasmatic concentration of tissue plasminogen activator (Schlienger, 2001). Brenner et al., (2001) concluded that the inverse relation between alcohol consumption and cardiovascular disease risk was particularly strong among subjects who consumed exclusively or predominantly beer. However, additional mechanisms may account for the strong protective effect of moderate beer consumption (Brenner et al., 2001). Increased levels of HDL-cholesterol and positive effect on blood lipid profile were confirmed among moderate beer consumers (Romeo, González-Gross, Wärnberg, Díaz, & Marcos, 2008).

Beyond alcohol, other possible protective mechanisms should be considered such as specific estrogenic and antioxidant activity of beer and prevention of an alcohol-induced rise in serum homocysteine due to pyridoxin present in beer (Brenner et al., 2001). The presence of antioxidants in beer protects the vascular endothelium and prevents LDL oxidation, which is an important step in atherogenesis (Schlienger, 2001). Also, the hop-derived bittering agents are valuable components due to its sedative and hypnotic effects (Sohrabvandi, Mortazavian, & Rezaei, 2012).

Beer compounds showed a protective effect on cardiovascular system, including ischemic stroke, congestive heart failure, peripheral arteriopathy, and coronary heart disease. Beer phenolic content reduced leukocyte adhesion molecules and inflammatory biomarkers, whereas alcohol mainly improves the lipid profile and reduced some plasma inflammatory biomarkers related to atherosclerosis (De Gaetano et al., 2016).

Finally, beer is a source of folate, vitamin B6 and B12, molecules involved in the pathways of homocysteine, a risk factor for cardiovascular disease. Rossi et al., (2020) evaluated if a consumption of craft or industrial beer could reduce serum homocysteine. Contrary to industrial beers, the consumption of craft beer did not modify homocysteine levels but increased the level of gamma-glutamyl transpeptidase (GGT) (16.6 vs. 18.6 U/L) and reduced the concentration of vitamin B6 (20.9 vs. 16.9 ng/ml). The two types of beer used in the study had different amounts of alcohol (23.8 g in craft beers vs. 11.9 g in industrial beers) which could explain the absence or effect on homocysteine by craft beers. However, it is known that other parameters can affect homocysteine blood levels like physical activity or diet (Rossi et al., 2020).

1.5.2. Diabetes

Moderate alcohol consumption is associated with a significantly reduced risk of type 2 diabetes. The mechanisms are not entirely clear, but it seems to be related with modulation of changes in the endocrine functioning of fat tissue, modulation of the inflammatory status of several organs, or modulation of metabolism, leading to an increase of insulin sensitivity (Hendriks, 2007; Joosten et al., 2012).

Protein glycation, a normal part of the aging process, has been implicated in various complications of diabetes mellitus and many phenolic compounds are important inhibitors of this process. Since beer is rich in phenolic compounds, Elrod et al., (2017) studied the hypothesis of this beverage inhibit the protein glycation. The common craft beer styles studied were American pale ale, porter, stout, India pale ale (IPA), and Imperial IPA. Also, a major, mainstream commercial American beer (American lager style) was purchased for comparison purposes. Most beers decreased glycation by approximately 30–40%, whereas the industrial beer produced an increase in glycation by approximately 12% over controls. All styles inhibited protein glycation on a volumetric (4 μL/mL) basis, and all but one sample of Imperial IPA inhibited glycation based on phenolics (4 μg of phenols/mL). Different styles of beer have different antioxidant and protein glycation effects, which opens the possibility for craft beer to be designed as a functional beverage, inhibiting protein glycation and potentially preventing the development of complications in type 2 diabetes (Elrod et al., 2017).

Also, xanthohumol and isohumulones, extracted from hops, have demonstrated positive effects against obesity and diabetes by regulation of glucose and cholesterol metabolism. Yajima et al. (2004) demonstrated a significant reduction in fasting blood glucose and hemoglobin A1c after 8 weeks of intervention in the isohumulone treated group (by 10.1 and 6.4%, respectively, vs. week 0), in diabetic humans (Yajima et al., 2004). Other studies indicated that the isohumulones from hops reduced elevated triglycerides associated with insulin resistance and raised HDL cholesterol levels while reducing liver fat stores in diabetic animal models. The modulatory effects of isohumulones on lipid metabolism seems to be mediated by peroxisome proliferator-activated receptor α (PPARα), which is a nuclear receptor of clinical interest as a drug target in various metabolic disorders and also exhibits marked anti-inflammatory capacities(Miura et al., 2005; Shimura et al., 2005).

Miranda et al., (2016) found that dietary xanthohumol reduced body weight gain and ameliorated hyperglycemia, dyslipidemia, insulin resistance and leptin resistance in diet-induced obesity in mice. Furthermore, dietary xanthohumol decreased the plasmatic levels of inflammatory cytokines which may contribute to the mitigation of obesity and insulin resistance in these mice (Bland et al., 2015; Miranda et al., 2016).

1.5.3. Cancer and inflammation

Although heavy drinking is related with carcinogenesis (especially to cancers of the mouth, pharynx, larynx, esophagus, and liver), moderate beer drinking has potential cancer preventive effects. Epidemiological and *in vivo* studies, in animal models, showed significantly decrease in the risk of prostate cancer, renal cell cancer and colorectal tumorigenesis. Also, beer possesses antimutagenic effects, protecting against various carcinogens (Gerhäuser, 2005). Hop compounds appear to be the main

responsible for cancer preventive potential of beer by its apoptosis-inducing, anti-estrogenic, anti-angiogenic, anti-inflammatory, antiproliferative, and antioxidant activities as well as modulation of carcinogens metabolism (Machado, Faria, Melo, & Ferreira, 2017).

Humulones were described as potent angiogenesis inhibitor, presumably through the regulation of cyclooxygenases (COX's). Humulone demonstrated to suppress angiogenesis *in vivo* in Chick embryo chorioallantoic membrane (CAM) in a dose-dependent manner with an ED_{50} of 1.5 mg/CAM. The antiangiogenic activity of humulone was 40 times more potent when compared to a specific COX-2 inhibitor (NS-398) (ED_{50} of 65 mg/CAM). This compound also inhibited vascular endothelial cell tube formation in rat lung endothelial (RLE) cells, endothelial cell proliferation in mouse endothelial cell line (KOP2.16), and vascular endothelial growth factor (VEGF) production in KOP2.16 endothelial cells and Co26 colon cancer cells. Since angiogenesis plays a key role in the development of malignant tumours, its inhibition prevents tumour growth and metastasis (Shimamura et al., 2001).

Xanthohumol demonstrated anti-inflammatory potential by inhibition of Cox-1 (IC_{50} = 16.6 and μM), Cox-2 (IC_{50} = 41.5 μM) activity and prevented nitric oxide (NO) release stimulated by lipopolysaccharides. NO generation mediated by iNOS has a relevant role in epithelial carcinogenesis, since it is involved in the production of vascular epidermal growth factor, accelerating tumour development. Xanthohumol was reported as a potential inhibitor of phase 1 Cyp1A activity, which is indicative of a possible important chemopreventive behaviour in the initial phase of carcinogenesis. Xanthohumol was identified as a monofunctional inducer of NAD(P)H:quinone reductase activity (Gerhäuser, 2005). Xanthohumol (10 μM) was capable to significantly decrease prostaglandin E2 production in cholangiocarcinoma cells, M139 and M214 (0.43 ± 0.13 nM and 0.39 ± 0.17 nM, respectively), but the underlying mechanism is still under investigation. These findings present xanthohumol as a potential anti-cancer or chemopreventive agent (Jongthawin, Techasen, Loilome, & Yongvanit, 2012).

Humulone demonstrated glucocorticoid-like suppression activity in Tumor Necrosis Factor α (TNFα)-induced COX-2 transcription and its signal transduction may be independent from glucocorticoid receptor (Yamamoto, Wang, Yamamoto, & Tobe, 2000). However, alcohol consumption, regardless of drink, is associated with an increased risk of breast cancer (Li et al., 2009).

1.5.4. Neurological disorders

Beer is one of the main dietary sources of silicon, in the bioavailable form (silicic acid or orthosilicic acid) (Sripanyakorn et al., 2004). Silicon seems to decrease aluminium bioavailability by blocking its uptake through the gastrointestinal tract and by preventing reabsorption. González-Muñoz et al., (2008) showed that the inclusion of silicon in the diet in the form of silicic acid or beer lowered aluminium levels in mice brains in about 40% when compared to the group that received only aluminium nitrate ($p < 0.01$), leading

to reduction of harmful effects of increased cerebral peroxidation. Cerebral peroxidation is linked with neurodegenerative diseases development. Also, aluminium appears to play an active role in the pathogenesis of critical neuropathologic lesions in Alzheimer's disease and other related disorders, through cross-linking hyperphosphorylated proteins (Gonzalez-Muñoz et al., 2008; González-Muñoz, Peña, & Meseguer, 2008; Perl & Moalem, 2006)

Hops iso-α-acids, have a role in prevention of Alzheimer's disease-like symptoms by enhancing microglial phagocytosis, suppressing inflammation, and improving cognitive function. At 0.3 and 1 μM iso-α-acids, trans-isohumulone and cis-isohumulone significantly enhanced microglial phagocytosis ($p < 0.01$ in all concentrations and compounds studied but 0.3 μM iso-α-acids ($p < 0.05$)), in dose-dependent manner. Trans-isohumulone and cis-isohumulone, both with high concentrations in beer, had potent activity when compared with the other stereoisomers (Ano et al., 2017). Xanthohumol, another compound from hops, demonstrated neuroprotective effect on cerebral ischemic damage in rats. Treatment with xanthohumol (0.4 mg/kg) markedly reduced the infarct area to about 20%. This protection is probably mediated by inhibition of inflammatory responses, apoptosis and platelet activation (Yen et al., 2012).

Moderate alcohol consumption is associated with a reduced risk of dementia and Alzheimer's disease. However, no clear association between specific type of alcohol beverage and dementia has been established. The existing literature on beer consumption is limited and reports are not clear on associating beer consumption and dementia (De Gaetano et al., 2016; Mukamal et al., 2003; Ruitenberg et al., 2002). Also, regular beer, due to its alcohol content, might not be adequate for consumption in all human beings (e.g. pregnancy, children, people affected by liver diseases) (Sánchez-Muniz et al., 2019).

1.5.5. Menopause and Osteoporosis

Hot flashes, anxiety, insomnia, and osteoporosis are the major complications often associated with menopause. A prenylated flavanone from hops, 8-prenylnaringenin, is one of the most potent phytoestrogens and can be used to improve symptoms associated with menopause. It seems therefore safe to assume that human exposure to phytoestrogens through beer consumption causes no detrimental health effects (Stevens & Page, 2004).

Elderly postmenopausal women experience high rates of osteoporotic fractures, caused by an imbalance between bone formation by osteoblasts and bone resorption by osteoclasts, mediated by hormonal changes. Calcitonin secretion decreases in postmenopausal women, which has been linked to osteoporosis, since calcitonin both inhibits the resorption and stimulates the formation of bone. Moderate alcohol consumption is a powerful stimulant of calcitonin secretion, being positively correlated with bone mass and bone mineral density in women (Pedrera-Zamorano et al., 2009).

Beer contains high levels of silicon, in the form of biologically active orthosilicic acid [$Si(OH)_4$]. Malted barley provides the major source of silicon content in beer (Bertuzzi et al., 2020). Silicon supplementation of postmenopausal women with osteoporosis inhibits bone resorption and increases trabecular bone volume and bone mineral density. Beer could be useful in stimulating bone formation and/or reducing the postmenopausal loss of bone mass (Pedrera-Zamorano et al., 2009). Pedrera-Zamorano et al. (2009) found that women who drink beer have greater bone density, maybe due to synergic effect of a combination of silicon and isoflavonoids (daidzein, genistein, and others) present in beer (Pedrera-Zamorano et al., 2009).

Despite xanthohumol and humulone don't show appreciable estrogenic activity, they were identified as strong inhibitors of bone resorption (Stevens & Page, 2004). Li et al., (2015) demonstrated that xanthohumol inhibited osteoclast differentiation and bone resorption in a dose-dependent manner, *in vitro*, using mouse and human models. The half maximal inhibitory concentration (IC_{50}) value of mouse osteoclast is about 1 μM, while the IC_{50} value of human is between 0.25 and 0.5 μM. It was also tested the *in vivo* effect of xanthohumol in ovariectomy-induced bone loss, using mices. Treatment with xanthohumol significantly inhibited the ovariectomy-induced bone loss ($p < 0.001$). Inhibition of osteoclasts formation is related with suppression of RANK/TRAF6 interaction, and by blocking NF-κB and Ca^{2+}/NFATc1 signalling pathway. The results suggest that xanthohumol is a potential therapeutic agent in the treatment of osteoclast-related diseases such as osteoporosis (Li et al., 2015). Xanthohumol appears to induce osteoblast differentiation by activating RUNX2, the main osteogenic master gene for bone formation (Jeong et al., 2011).

1.5.6. Hepatoprotection

Despite lack of studies, beer may be associated with hepatoprotective properties. Zhou et al., (2018) demonstrated a direct hepatoprotective effect of the total flavonoids from hop against carbon tetrachloride (CCl_4)-induced acute liver injury, in mices liver tissue. The potential mechanism might be through antioxidant and anti-inflammatory effects on reducing liver toxicity because the activities of the antioxidant enzymes catalase (CAT), glutathione peroxidase ($GSHP_x$) and superoxide dismutase (SOD) in liver tissues were significantly restored by hop flavonoids treatment. Also, hop flavonoids decreased TNF-α levels in serum, compared to normal control group. Results also revealed higher amounts of total flavonoids including chalcones and dihydroflavones in hop, which may explain its hepatoprotective and antioxidant activity against CCl_4-induced liver damage (Zhou et al., 2018).

The activation of hepatic stellate cells plays a critical pathophysiological role in the progression of chronic liver disease. Xanthohumol has been shown to inhibit the activation of primary human hepatic stellate cells *in vitro* even in low concentrations (5 μM). Furthermore, xanthohumol induced apoptosis in

activated hepatic stellate cells *in vitro* in a dose-dependent manner (0–20 μM). Moreover, xanthohumol reduced expression of the pro-inflammatory factors and pro-fibrogenic genes, improving several mechanisms which play a critical role in the pathogenesis of acute and chronic liver injury, and its therapeutic application appears as a promising strategy. However, pharmacologically relevant xanthohumol concentrations cannot be reached by beer consumption (Weiskirchen, Mahli, Weiskirchen, & Hellerbrand, 2015).

Barley, the main cereal used in beer, blocked increased levels of the liver injury markers such as hepatic lipid accumulation, in alcohol-fed mice, showing hepaprotective activity. This protection appears to be related to the phenolic compounds present in barley composition (Lee et al., 2016; Shah, Parmar, Thakkar, & Gandhi, 2010).

1.5.7. Oxidative Stress

The high concentration of free radicals in the body origins tissue damage and pathologies caused by oxidative stress. Free radicals are produced as a result of biological processes, but they can also be induced by external factors, such as pollution, solar radiation, stress and tobacco (Jemia et al., 2013; Neagu, Păun, Moroeanu, & Radu, 2010). Oxidative stress is involved in the pathology of many diseases, such as atherosclerosis, diabetes, neurodegenerative diseases, aging and cancer (Liguori et al., 2018). Studies shown that beer contributes to inhibiting or delaying oxidative stress by increasing the total antioxidant capacity of plasma (Ghiselli et al., 2000; Maldonado, Moreno, & Calvo, 2009; Rodrigues et al., 2016). The high content of bioactive compounds, especially phenolic compounds, in beer is the most possible explanation of its high antioxidant activity (Gorinstein et al., 2007). For example, xanthohumol (1 μM) was 8.9 fold more powerful in scavenging hydroxyl radical ($OH^{\cdot}$), and 2.9 fold more powerful in scavenging peroxyl ($ROO^{\cdot}$) radicals, than the reference compound (Trolox) and also showed an inhibition property on superoxide anion radical production (Stevens & Page, 2004).

In a study conducted by Ruiz-Ruiz et al., (2020), it was evaluated the antioxidant activity of polyphenols extracted from hop provided by a craft brewery. Hop methanolic extract exhibited adequate amounts of Total Phenolic Content (TPC), expressed in g Gallic Acid Equivalents (GAE)/Kg (1.54 g GAE/kg of discarded hop) and flavonoids (0.61 g CE/kg of discarded hop). Also, the methanolic extract from hop discarded inhibited 4.24% of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical (Ruiz-Ruiz et al., 2019).

Koren et al., (2017) evaluated 40 Hungarian retail beers, including some craft beers, for folic acid content, antioxidant profile and physicochemical parameters. Results showed that higher antioxidant activity observed in dark and craft beers may be due to special malts, like crystal or caramel malts, and other coloring malts used in its production (Koren et al., 2017).

Bertuzzi et al., (2020) evaluated parameters like TPC and antioxidant capacity in small (SS) and large-scale (LS) brewed beer samples. Comparing SS and LS beers only considering regular beers (the most representative style) significantly higher values for both TPC and antioxidant capacity were found for SS beer (506.6 ± 162.3 mg_{GAE}/L for TPC ($p < 0.01$); 1.6 ± 0.5 mM Trolox ($p < 0.001$) and 0.54 ± 0.08 mol_{Trolox}/mol_{GAE} ($p < 0.01$), for the ABTS assay; 5.4 ± 1.7 mM Fe^{2+} ($p < 0.001$) and 1.8 ± 0.2 $mol_{Fe2+/}mol_{GAE}$ ($p < 0.01$) for the Ferric Reducing Antioxidant Power (FRAP) assay. These values can be explained by the fact that, in Italy, legislation bans pasteurisation and microfiltration processes for SS beer (Bertuzzi et al., 2020).

1.6. Beer as functional beverage

Microbiota plays a well-established role in infectious gastrointestinal diseases. Recent research has linked intestinal microbiota imbalance to gastrointestinal disorders such as antibiotic-associated diarrhoea, ulcers, inflammatory bowel disease, irritable bowel syndrome and colon cancer. Furthermore, the microbiota has been proposed as a major regulator of the immune system outside the gut. Probiotics are microorganisms used as dietary supplements that in a correct dosage are potentially beneficial for human health, especially for the intestinal microbial balance (Czerucka et al., 2007; Mulero-Cerezo et al., 2019).

While health benefits of lactic acid bacteria as probiotics are well known, few data are available on probiotic yeasts in fermented food (Capece et al., 2018). Some studies approached the use of yeasts of probiotic species, like *S. cerevisiae var.* boulardii, *Lactobacillus paracasei* and Kefir. In these studies, it was found that beer could be a vehicle for probiotic delivery, once they find an adequate number of viable cells in the final product. Because viability is crucial for the efficacy of probiotics, a craft beer could be more suitable to be produced as probiotic beer than an industrial beer since craft beer is unpasteurized and unfiltered (Alcine Chan, Chua, Toh, & Liu, 2019; Capece et al., 2018; Mulero-Cerezo et al., 2019; Poveda, Ruiz, Seseña, & Palop, 2017; Rodrigues et al., 2016).

Craft breweries do not only produce classic beer styles, but also innovative beers brewed with unusual ingredients like fruits, vegetables, and spices, with a particular focus on environmental-friendly ingredients (Graefe, Mowen, & Graefe, 2017). Also, the use of by-products from the food industry can be an interesting alternative in beer brewing, adding economic value to wasted raw materials, reducing costs and environmental problems (Bharat Helkar, Sahoo, & Patil, 2016).

For example, the use of goji berries, Umbrian legumes, *Parastrephia lucida*, *sapa*, propolis and olive (*Olea europaea* L.) leaves as beer ingredients has been studied. The incorporation of these ingredients has shown to increase the concentration of bioactive compounds in beer. Ducruet et al., (2017) added whole and ground goji berries (50 g/L) along the brewing process. The addition of goji berries at the beginning of

wort boiling allowed the extraction of more phenolic compounds (335 mg GAE/L for the standard amber ale beer vs. 623 mg GAE/L for the beer with goji berries) and the production of a beer with 60% to 80% more antioxidant activity and the best sensorial characteristics. No significant differences were found between beers with ground or whole goji berries (Ducruet et al., 2017).

Luneia et al., (2018) produced and analysed beers with local spelt and barley malt for which lentils and chickling were added. It was found that beers had a high content of total phenolic compounds (358 mg/L to 636 mg/L), 1.01 and 1.85 mg/L of isoflavones and more than 15% of recommended dietary allowance of magnesium and potassium. However, authors did not compare their beers with standard or commercial ones (Luneia, Zannoli, Farchioni, Sensidoni, & Luneia, 2018).

Bustos et al., (2019) produced porter beer enriched with *Parastrephia lucida* with four different concentrations (5, 1, 0.5, and 0.1% w/V). It was observed that TPC values of the four enriched beers (480.16 to 800.64 mg GAE/L) was significantly higher ($p < 0.05$) than the control beer (413.21 mg GAE/L). The increase in bioactive compounds was linear with the increase in plant concentration. Also, the results of antioxidant activity for enriched beers showed increased values (2.17 ± 0.08 to 5.46 ± 0.04 mmol TE/L for FRAP; 1.38 ± 0.03 to 3.34 ± 0.11 mmol/ TE/L for ABTS; 10.14 ± 0.76 to 30.58 ± 1.20 mmol TE/L for Oxygen Radical Absorbance Capacity (ORAC)), when compared to control (1.88 ± 0.05 mmol TE/L for FRAP; 1.15 ± 0.10 mmol/ TE/L for ABTS, 7.86 ± 0.14 mmol TE/L for ORAC) (Bustos et al., 2019).

Also, Sanna & Pretti, (2015) studied the effect of addition of *sapa*, a cooked must from wine grapes, and wine barrel ageing in polyphenol content and antioxidant activities of craft beers. The obtained results suggested that storage of beer in wood barrels that contained red wines or the addition of *sapa* from red wine grapes contributed to enhance total phenolic content in beers and improved free radical scavenging ability and ferric reducing activity. For control beers TPC ranged from 331.9 to 496.3 mg GAE/L, for beer enriched with *sapa* the observed values were between 362.8 and 974.9 mg GAE/L and for wine barrel ageing beer 536.0 to 1035.3 mg GAE/L (Sanna & Pretti, 2015).

Ulloa et al., (2017) studied the influence of the addition of propolis ethanolic extract to beer at different concentrations (0.05, 0.15, and 0.25 g/L). Results showed that bioactive compounds, expressed as TPC and total flavonoids compounds, were higher than control. The concentrations of phenolic compounds in enriched beers were 253.0 to 306.5 mg GAE/L, whereas the total flavonoid content (expressed as milligrams of quercetin equivalents (QE) per liter of beer) ranged from 19.6 to 26.9 mg QE/L. For control beer phenolic content was 242 mg GAE/L and flavonoid content 16.9 mg QE/L. The concentration of bioactive compounds increased with the concentration of propolis in the ethanolic extract (Ulloa et al., 2017).

Guglielmotti et al., (2020) studied the contribution of olive leaves as beer ingredient to bitterness and antioxidant activity of this beverage. Thirteen beer samples were produced, adding olive leaves during boiling at different boiling times (60 and 5 minutes before the end of boiling), in different forms (dry

crumbled, infusion, and powder) and concentrations (low and high olive leaves-containing beer) sample. The addition of olive leaves highly increased polyphenol content of beers but antioxidant activity was not influenced. High olive leaves-containing beer samples showed significantly higher values ($p \leq 0.05$), between 525.8 and 795.5 mg/L, when compared to 228.1 mg/L of reference beer. Polyphenols extraction from leaves was favoured by heat and boiling time (Guglielmotti, Passaghe, & Buiatti, 2020).

Pereira et al., (2020) studied wheat craft beers brewed with cashew peduncle (*Anacardium occidentale*) and orange peel (*Citrus sinensis*) to evaluate its physicochemical characterization, antioxidant activity, and sensory analysis. The results showed that formulations containing 10% (m/m) of cashew peduncle possessed a higher increase in polyphenol content (722.3 ± 13.8 and 726.6 ± 2.6 GAE mg/L), in comparison with formulations which contained 5% (m/m) (640.9 ± 58.2 and 652.2 ± 28.7 GAE mg/L) and significantly ($p < 0.05$) higher antioxidant activity (1725.1 ± 24.7 and 1736.9 ± 58.8 μM/L, respectively) (Pereira et al., 2020). The use of new plant materials and production techniques can increase the concentration of antioxidant compounds in craft beer, obtaining a beverage with potential healthy value added and improved stability.

Although craft beers are becoming more popular, information about its potential health-positive extracts or components are still scarce, because most of the studies are still performed with industrial beers. Also, as far as we know, no researches have been conducted with Portuguese craft beers related to the characterization and antioxidant activity, evidencing the importance of this work.

Therefore, the study aims to:

1. Describe the craft beer market, consumption, production process, and styles, with a special focus on articles that discuss the biological activities of craft beer and its bioactive compounds.
2. Determine the chemical characterization such as pH, total acidity, and reducing sugars, and also evaluate the total phenolic content and antioxidant activity of Portuguese craft beers and raw materials used in its production, mainly hops and malt.

2. Methods

2.1. Chemicals

Sodium hydroxide (NaOH), phenolphthalein and Folin–Ciocalteu reagent, dimethyl sulfoxide (DMSO), and iron (II) sulphate heptahydrate, were purchased from VWR (Portugal). Potassium sodium tartrate tetrahydrate, 3,5-dinitrosalicylic acid (DNS), glucose, sodium carbonate, gallic acid, absolute ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrozine and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma-Aldrich (USA).

2.2. Beer Samples and Raw Materials

A total of 16 craft beer samples were selected. Sample collection was mainly random and depending on access and availability. The main goal was to collect as much craft beer samples as possible and different beer styles. All craft beers were produced in Portugal and were obtained from the donation of craft breweries. Three industrial beers, used for comparison purposes, were collected in supermarkets. Table 1 shows the characteristics and packaging specifications of all the different beers used in the study.

During the study, together with craft beers, the starting malts (10 samples) and hops (11 samples), from different varieties, offered from Portuguese craft breweries, were also analysed.

2.3. Samples Preparation

For craft beers, contents of each bottle was homogenized (stirring with a glass rod for 10 s), in order to incorporate the foam in beer samples, and avoid loss of bitter substances. Then, samples were degassed by sonication (Sonorex Super RK 100/H, Bandelin) for 40 minutes at 35 kHz, and room temperature to avoid contributions from dissolved CO_2 to the signals. The disappearance of the bubbles were indicative of the absence of CO_2 (Granato, Branco, Faria, & Cruz, 2011; Popescu et al., 2013). Beer bottles were stored in the dark and analysed immediately after opening. Aliquots were frozen at -80 ˚C until analysis.

Malt samples were prepared according to Mareček et al., (2017). Briefly, the malt was grounded in an electric mill and 25 g of the sample was weighed. Then, 225 mL of distilled water was added to the malt and placed in a 45 ˚C water bath for 15 minutes. After cooling, it was filtered and kept at -20 ˚C until use.

Hop samples were prepared according to Krofta et al., (2008), with minor modifications. The hop pellets were grounded with an electric mill and 5 g of dry hop material were weighed. The ground hops were transferred to an Erlenmayer with boiling water and allowed to boil for 30 minutes. After cooling, the

flask content was transferred to a 1000 mL volumetric flask and completed the volume with distilled water. The extract was filtered and kept at -20 ˚C until use.

Table 1. Characteristics of each beer samples regarding its production, packaging type and source.

Beer samples	Craft vs. Industrial	Beer Style	Beer Color	Packing	Packing Volume (mL)	Source
BG-SB	Craft Beer	Strong Bitter	Pale Amber	Bottle	330	Brewery
BG-OS	Craft Beer	Oatmeal Stout	Black	Bottle	330	Brewery
PS-P	Craft Beer	Pilsner	Deep Gold	Bottle	330	Brewery
PS-IS	Craft Beer	Imperial Stout	Black	Bottle	330	Brewery
PS-HN	Craft Beer	Special (Honey Beer)	Amber Brown	Bottle	330	Brewery
N-LAG	Craft Beer	Lager	Deep Gold	Bottle	330	Brewery
N-IPA	Craft Beer	India Pale Ale	Pale Amber	Bottle	330	Brewery
B-MD	Craft Beer	Munich Dunkel	Brown	Bottle	330	Brewery
B-BA	Craft Beer	Blond Ale	Pale Gold	Bottle	330	Brewery
B-IPA	Craft Beer	Indian Pale Ale	Pale Amber	Bottle	330	Brewery
AL-W	Craft Beer	Witbier*	Pale Gold	Bottle	330	Brewery
AL-OS	Craft Beer	Oatmeal Stout**	Black	Bottle	330	Brewery
AL-IPA	Craft Beer	India Pale Ale	Medium Amber	Bottle	330	Brewery
AMP-IS	Craft Beer	Imperial Stout	Black	Bottle	330	Brewery
AMP-VL	Craft Beer	Vienna Lager	Pale Amber	Bottle	330	Brewery
AMP-IPA	Craft Beer	India Pale Ale	Pale Amber	Bottle	330	Brewery
SB-P	Industrial Beer	Pilsner	Pale Gold	Bottle	250	Supermarket
SB-S	Industrial Beer	Pilsner	Straw	Bottle	250	Supermarket
SG-P	Industrial Beer	Munich Dunkel	Brown	Bottle	250	Supermarket

* With honey, coriander, orange peel and pennyroyal; ** With carob and fig

2.4. Chemical analysis of craft beer samples

The pH determination, total acidity, reducing sugar content, and TPC were performed in all beer samples. Data regarding beer color, expressed in European Brewing Convention (EBC); bitterness, expressed as International Bittering Units (IBU); and alcohol content listed by volume (ABV) were provided by craft breweries (Table 2).

2.4.1. pH determination

The pH was measured in 100 mL of the degassed beer and using a calibrated pH meter (Symphony, VWR, Portugal).

2.4.2. Total Acidity (TA)

The acidity was measured by titration (50 mL of degassed beer) with a 0.1 M NaOH solution in the presence of phenolphthalein as the indicator until the appearance of pale pink color that persisted for 1 min (Popescu et al., 2013). In darkest samples, titration was performed with a calibrated pH meter, measuring the amount of sodium hydroxide required to raise the pH to 8.2. Total acidity of beer was calculated using the formula: $TA\ (as\ Lactic\ Acid) = \frac{V \times 0.9}{50}$, where V = volume in mL of 0.1 M NaOH used (Chemists, n.d.; Howat, Carter, Pixley, & Castagno, 2018).

2.4.3. Reducing sugar content

The analysis of reducing sugar content was carried out using the Dinitrosalicylic acid (DNS) method, proposed by Başkan et al., (2016), with minor modifications. Briefly, a solution of DNS (1%) was prepared by dissolving 1 g of DNS in 20 mL of NaOH (2 M). After that, 30 g of sodium and potassium tartrate were added, and the mixture diluted with distilled water (1 L). After that, 500 µL of beer sample was mixed with 500 µL of the DNS solution and vortexed vigorously. Then, it was incubated in a water bath at 100 °C, for 5 minutes. As a standard solution, different concentrations of glucose solution were used (50, 100, 200, 400, 600, 800 and 1000 mg / L). The absorbance was read at 540 nm in a UV-Vis spectrophotometer (Model VWR UV-1600PC). With the data obtained, a linear regression line was calculated using the standard glucose solutions. Results were expressed in glucose equivalents (GE)/100 mL of beer sample.

2.5. Parameters related to antioxidant capacity

2.5.1. Total Phenolic Content (TPC)

TPC were determined according to the spectrophotometric method Folin-Ciocalteu, described by Marques et al., (2017). Briefly, 1.25 mL of Folin-Ciocalteu reagent (0.2 M) was added to 250 µL of degassed beer sample or GA (standard solution) and allowed to stand for 5 minutes. Then, 2 mL of sodium carbonate (Na_2CO_3) solution (75 g/L) was added. Distilled water was added until 5mL. The solution was incubated for 1 h at room temperature in the dark and then the absorbance was read at 760 nm using a UV-Vis spectrophotometer (Model VWR UV-1600PC). Absorbance values were converted to GAE mg/L craft beer through a calibration curve obtained with standard GA in water.

2.5.2. DPPH scavenging activity

For DPPH scavenging activity (Lima et al., 2007), in a 96-well plate, a 19.4 µL aliquot of sample was added to 175 µl of DPPH (100 µM) radical, and the absorbance was measured at 517 nm, using a microplate reader (Model MR133T, DYnex). Reading was repeated every 5 minutes, until the end of the reaction. DPPH scavenging activity was calculated using the following formula: $Inhibition(\%) = \frac{(AC-AS)}{AC} \times 100$, where AC is the absorbance of the control and AS represents the absorbance of the sample.

2.5.3. Metal Chelating Activity (MCA)

Metal Chelating Activity or Ferrozine assay was carried out according to the methodology described by Russo et al., (2005). Briefly, a 96-well plate was prepared by adding 50 µl of sample or EDTA (positive control) and 50 µl of 0.15 mM ferrous sulfate solution ($FeSO_4$) to each well. The plate was left to stand for 5 minutes and then added 50 µl of 0.5 mM ferrozine to each well. The mixture was vigorously stirred and left for 10 minutes at room temperature and protected from light. Absorbance was measured at 562 nm using a microplate reader (Model MR133T, DYnex). With the data obtained, the chelating capacity of the samples was calculated using the following formula: $Chelating\ Activity(\%) = \frac{(AC-AS)}{AC} \times 100$, where AC is the absorbance of the control and AS represents the absorbance of the sample.

2.6. Statistical Analysis

Data are presented as the mean ± standard deviation (SD) for triplicate determinations, and samples were collected from the same production lot. The results were assessed through statistical analysis of simple variance (one-way ANOVA), to detect statistically significant differences, using SPSS® software (version 26.0). The Tukey test was also applied to identify samples with significant differences between them (95% significance). Correlation coefficient (r) was calculated using Pearson Product Moment Correlation, to determine the correlations among means. Differences with a $p < 0.05$ were considered significant.

3. Results and Discussion

3.1. Chemical analysis of craft beer samples

The classification of beer in different styles is based on properties like alcohol content, color, bitterness, clarity, flavor, and ingredients. There are several parameters related to the quality of craft beer like pH, total acidity, reducing sugars, total phenolic compounds, among others.

Table 2 shows the parameters analysed in sample beers related to alcohol content, color, bitterness, pH, acidity and reducing sugar content. Beer is an alcohol source, although its content is variable depending on the type, ingredients, and fermentation. Beer ABV typically ranges from 3 to 14% when normal fermentation is used, but the most commonly consumed styles, do not exceed 6% (Rodhouse & Carbonero, 2019). According to Bamforth & Charles (2002), many beers have alcohol content ranging from 3% to 6% (v/v), which represents 56.2% of all the craft beers analysed in our study (Table 2).

Table 2. Principal chemical parameters of each analysed beer.

Beer Sample	Beer Style	Alcohol (ABV%)	Color (EBC)	Bitterness (IBU)	pH	Total Acidity (%)	Sugar (mg/L)
BG-SB	Strong Bitter	5.2	17	40	4.43±0.02	0.18±0,00	2598.0±0.0
BG-OS	Oatmeal Stout	6.5	123	42	4.55±0.01	0.51±0.02	2598.0±0.0
PS-P	Pilsner	4.5	7	36	4.81±0.01	0.15±0.00	2598.0±0.0
PS-IS	Imperial Stout	11.6	130	60	4.37±0.03	0.62±0.01	3844.2±438.8
PS-HN	Special (Honey Beer)	7.1	37	26	4.28±0.04	0.32±0.01	2598.0±0.0
N-LAG	Lager	5.0	12	18	4.57±0.00	0.18±0.01	2598.0±0.0
N-IPA	India Pale Ale	6.0	15	50	4.61±0.01	0.23±0.00	4070.5±23.8
B-MD	Munich Dunkel	5.2	40	20	4.78±0.05	0.37±0.00	3077.2±94.1
B-BA	Blond Ale	4.8	10	13	4.43±0.03	0.17±0.00	2239.7±62.6
B-IPA	India Pale Ale	6.5	17	40	4.92±0.02	0.28±0.01	3798.0±39.3
AL-W	Witbier	5.0	8	17	4.46±0.01	0.17±0.00	2598.0±0.0
AL-OS	Oatmeal Stout	5.5	88	39	4.49±0.01	0.44±0.01	4406.3±25.2
AL-IPA	India Pale Ale	6.5	23	55	4.69±0.03	0.33±0.00	4294.2±28.8
AMP-IS	Imperial Stout	10.0	122	67	4.77±0.02	0.48±0.02	4446.3±31.1
AMP-VL	Vienna Lager	5.4	21	25	4.27±0.03	0.27±0.00	2598.0±0.0
AMP-IPA	India Pale Ale	7.0	21	80	4.84±0.04	0.44±0.02	4049.7±18.8
SB-P	Pilsner	5.2	8	16	4.50±0.07	0.15±0.00	2598.0±0.0
SB-S	Pilsner	5.0	6	30	4.45±0.06	0.11±0.00	2598.0±0.0
SG-P	Munich Dunkel	4.1	39	13	4.56±0.05	0.10±0.00	2598.0±0.0

In this study, the alcohol content varied between 4.5 (Pilsner) and 11.6 (Imperial Stout). It is a fact that there is considerable variability in alcohol content within and across beverage type (e.g., beer, wine, and

distilled spirits). For example, some light beers contain half as much alcohol as a regular beer, while some craft and specialty beers contain twice as much (Institute on Alcohol Abuse, 2018). The higher values were seen in a specialty beer (Honey Beer – 7.1) and in both Imperial Stout beers (10.0 and 11.6). The alcohol present in beers, apparently, can exert a neuroprotective effect, which can be linked to signal transduction activation processes potentially involving Reactive Oxygen Species (ROS), several key protein kinases, and increased heat shock proteins (Collins et al., 2009). Also, the presence of alcohol provides protection against several heart diseases and along with polyphenols can reduce oxidative stress (Arranz et al., 2012).

Two Imperial Stout and one Oatmeal Stout beer showed the higher values of color, expressed in EBC (122, 130 and 123, respectively). These samples represent dark colored beers.

Bitterness is measured in IBUs which gives an approximate value of iso-α-acids present in milligram of iso-α-acid per liter of beer (Oladokun et al, 2017). Beer IBUs typically range between 5 and 120, and the popular use of higher quantities of more bitter hops in craft beers leads to higher IBU levels (Rodhouse & Carbonero, 2019). In this study, bitterness values range from 13 (Munich Dunkel) to 80 (one India Pale Ale), expressed as IBU. It has been shown that for beers, darker brown colors are associated with stronger, or more bitter, tastes/flavors (Guinard et al., 1998). This fact is, in part, in accordance with our results which demonstrated that the two Imperial Stout beers showed high values for color and bitterness. Also, Imperial Stout beers are known for its medium to aggressively high bitterness (Burnham et al., 2018). Finally, it is important to noticed that the time of hop addition and hop variety used for beer production have been suggested as factors that may impact on bitterness quality, explaining the variety of values observed in this study (Oladokun et al, 2017).

In general, craft beers presented similar values of pH, ranging from 4.27±0.03 to 4.92±0.02. B-IPA (India Pale Ale beer) was the least acidic of all samples analysed with a pH of 4.92, while AMP-VL (Vienna Lager beer) was the most acidic with a pH of 4.27. These results are in accordance with Granato et al., (2011) which pH values of the samples ranged from 4.13 to 4.97. The total acidity of beer samples ranges from 0.10 ± 0.00% to 0.62 ± 0.01% lactic acid equivalent (Table 2). The pH and total acidity are important criteria for brewers due to its influence on the sensory attributes, biological and chemical stability. In the case of light lager beers, brewing industry usually prefer pH in a range of 3.90 – 4.20 (Pai et al., 2015).

Reducing sugar content in analysed beers ranged from 2598.0±0.0 to 4446.3±31.1 mg/L glucose equivalents. Considering that beers are usually presented in 0.33 L bottles, these results translate into a sugar content of 0.857 to 1.467 g per serving. As expected, these beers had a low sugar content., which are in accordance with other studies. For example, Pai et al., (2015) presented a reducing sugar content in the beer samples studied ranging from 0.469 ± 0.021 mg/mL to 2.682 ± 0.008 mg/mL. Reducing sugar concentrations are an important parameter in fermentation of beer because it provides information on optimization and regulation of the fermentation process to increase the yield and quality of the product

(Zhang et al., 2019). Different contents of total and fermentable sugars are reported according to beer type. Yeast can only use selected lower molecular weight sugars, such as fructose, glucose, maltose, sucrose and maltotriose (Pai et al., 2015).

3.2. Total phenolic content and antioxidant activity of beer samples

In this study 16 samples of craft beer and 3 samples of the most consumed industrial beers in the Portuguese market were analysed in terms of parameters related to antioxidant capacity. So far more than 50 polyphenolic compounds have been identified in beer, of which 75% to 80% are derived from malt and 15% to 25% from hops (Gerloff, Singer, & Feick, 2010). These compounds improve the quality and acceptance of craft beers, influencing flavor and product stability, and contribute to the overall antioxidant activity of the beverage (Granato et al., 2011).

The main method to determine TPC is Folin–Ciocalteu assay. This is a colorimetric method based on electron transfer reactions between phenolic compounds and Folin–Ciocalteu reagent, resulting in blue color formation, proportional to the concentration of phenolic compounds (Sánchez-Rangel et al., 2013).

Table 3. Phenolic content, DPPH radical scavenging activity and metal chelating activity in analysed beers.

Beer Sample	Beer Style	TPC (mg GAE/L)	DPPH (% inhibition)	Metal Chelating Activity (%)
BG-SB	Strong Bitter	555.1 ± 49.4^{b}	58.3 ± 1.1^{a}	14.1 ± 1.1
BG-OS	Oatmeal Stout	$1416.0 \pm 80.4^{a,b,c}$	ND	30.6 ± 0.7^{a}
PS-P	Pilsner	343.8 ± 22.2	87.8 ± 5.0^{b}	22.2 ± 1.9^{a}
PS-IS	Imperial Stout	$1749.3 \pm 227.1^{a,b,c}$	82.2 ± 4.4	$3.4 \pm 0.5^{b,c}$
PS-HN	Special (Honey Beer)	$950.4 \pm 59.7^{a,b,c}$	96.8 ± 8.2^{b}	$1.6 \pm 0.4^{b,c}$
N-LAG	Lager	484.5 ± 40.1	73.4 ± 4.6^{a}	21.7 ± 0.2^{a}
N-IPA	India Pale Ale	513.8 ± 26.5^{b}	76.7 ± 2.8	27.6 ± 0.3^{a}
B-MD	Munich Dunkel	$752.2 \pm 83.3^{a,b,c}$	ND	$67.0 \pm 1.2^{a,b,c}$
B-BA	Blond Ale	449.9 ± 18.0	80.1 ± 6.7	22.9 ± 1.4^{a}
B-IPA	Indian Pale Ale	$588.9 \pm 70.0^{a,b}$	95.8 ± 3.4^{b}	17.0 ± 0.7
AL-W	Witbier	410.7 ± 37.4	95.4 ± 3.0^{b}	22.8 ± 1.3^{a}
AL-OS	Oatmeal Stout	$757.8 \pm 30.1^{a,b,c}$	97.9 ± 1.3^{b}	23.5 ± 2.4^{a}
AL-IPA	India Pale Ale	$758.0 \pm 89.1^{a,b,c}$	59.5 ± 3.8^{a}	$8.6 \pm 0.6^{b,c}$
AMP-IS	Imperial Stout	$2172.5 \pm 170.1^{a,b,c}$	ND	$46.2 \pm 0.9^{a,b,c}$
AMP-VL	Vienna Lager	$658.4 \pm 23.4^{a,b,c}$	88.8 ± 7.0^{b}	12.5 ± 0.7
AMP-IPA	India Pale Ale	$936.4 \pm 53.1^{a,b,c}$	$99.4 \pm 0.6^{a,b}$	$113.4 \pm 15.8^{a,b,c}$
SB-P	Pilsner	312.6 ± 28.7	85.7 ± 2.1^{b}	$7.6 \pm 0.3^{b,c}$
SG-S	Pilsner	255.3 ± 69.6	70.4 ± 0.7	23.1 ± 1.3^{a}
SG-P	Munich Dunkel	394.0 ± 48.7	ND	21.3 ± 0.5^{a}

ND – Not determined

Values are means ± SD (n = 3). [a] Significantly different compared with SB-P ($p < 0.05$); [b] Significantly different compared with SG-S ($p < 0.05$); [c] Significantly different compared with SG-P ($p < 0.05$) (ANOVA followed by post-tests).

In craft beers TPC varied between 343.8± 22.2 mg GAE/L and 2172.5± 170.1 mg GAE/L. In industrial beers TPC varied between 255.3± 69.6 mg GAE/L and 394.0± 48.7 mg GAE/L. In craft beers, both Imperial Stout (AMP-IS, PS-IS) and one Oatmeal Stout (BG-OS) showed significantly higher values for TPC (2172.5 ± 170.1 mg GAE/L, 1749.3 ± 227.1 mg GAE/L and 1416.0 ± 80.4 mg GAE/L, respectively). The lowest values were observed in two industrial beers, both Pilsner (SB-S, 255.3 ± 69.6 mg GAE/L and SB-P, 312.6 ± 28.7 mg GAE/L). In their study, García-Guzmán et al., (2018) also observed that the highest polyphenol indices were obtained in stout beers.

Comparing our results with other studies, TPC values are higher in Portuguese craft beers. For example, Piazzon et al., 2010 analysed five different brands for each of the seven beer types and found different values depending on the beer type, ranging from 366 GAE mg/L for dealcoholized beers and 875 GAE mg/L for bock beers. Marques et al., (2017) examined the TPC of four craft beers and values ranged from 448.57 to 531.30 mg GAE/L. Granato et al., (2011) studied 29 beers (11 brown ale and 18 lager) and TPC ranged from 119.96 to 525.93 mg GAE/L, for laboratory produced beers. Finally, Zhao et al., (2010) analysed 34 commercial beer samples and found values varying from 152.01 mg GAE/L to 339.12 mg GAE/L. These differences can be explained by beers with high original mash and with more dark/brown color, which tends to increase the value of phenolic compounds (Piazzon et al., 2010) like craft beer samples BG-OS, AL-OS (Oatmeal stout), PS-IS, AMP-IS (Imperial stout), B-MD (Munich dunkel).

Our results also showed a value of TPC, for sample PS-HN (beer with honey), significantly higher than in industrial beers (950.4 ± 59.7 mg GAE/L). Recent *in vitro* and *in vivo* studies have confirmed that honey possesses a range of antioxidant, antimicrobial, antiviral, anticancer, and antidiabetic properties (Cianciosi et al., 2018; Samarghandian et al., 2017). Most of the biological activities of honey are attributed to its constituent phenolic and flavonoid compounds (Olas, 2020). Nardini & Foddai (2020) referred that most special beers (six out of seven), including a beer with the addition of honey, showed total polyphenols content considerably and significantly ($p < 0.05$) higher (range 464–1026 mg/L of beer) as compared with that of the conventional beers (range 274–446 mg/L of beer).

The best TPC results observed in dark craft beers may be due to the special malts, like crystal or caramel malts, and other colouring malts used in its production (Koren et al., 2017). TPC of craft beers is generally higher comparing to the values observed for industrial beers. This can be explained by large breweries that often use less expensive raw materials and/or different techniques in brewing process to produce a more cost-effective product. Craft breweries use raw materials, such as barley and hops, during beer production, which may be related (in part) to the presence of different and more abundant phenolic compounds in these beverages (Humia et al., 2019). Also, craft beers are not submitted to filtration or pasteurization, processes that affect TPC (Humia et al., 2019; Mastanjević et al., 2019).

The DPPH method is widely used to assess antioxidant capacity. This method is based on the elimination of the stable free radical DPPH. The use of this radical has advantages: good stability in the absence of light, applicability, simplicity, viability and possibility to use in studies of antioxidant evaluation of pure substances, mixtures or complex matrices (Oliveira, 2015).

Antioxidants with DPPH radical scavenging activity can donate hydrogen to free radicals, particularly to the lipid peroxides or hydroperoxide radicals that are the major propagators of the chain autoxidation of lipids, and to form non-radical species, resulting in the inhibition of propagating phase of lipid peroxidation.

Beer with higher DPPH radical scavenging activity are important to flavor stability, which is the major determinant of the shelf life of this beverage, because beer staling is generally considered as the formation of saturated and unsaturated aldehydes, due to lipid oxidation (Zhao et al., 2010).

Antioxidant capacity of craft beers was evaluated by measuring DPPH radical scavenging activity and metal chelating activity. For DPPH assay, the inhibition percentages varied between 58.3 ± 1.1 % and 99.4 ± 0.6%. These values are in accordance to those reported by Pai et al., (2015) who found values ranging from 68.34 ± 0.85 % to 89.90 ± 0.71 %. On the other hand, the values obtained are higher than those reported by Granato et al., (2011) who found values between 4.75 and 59.98 % for Brazilian commercial beers, and Marques et al., (2017) who had values ranging from 29.4% to 48.5% in craft beers (produced in laboratory).

Regarding craft beers, the sample AMP-IPA (India Pale Ale) showed the best value of DPPH radical inhibition (99.4 ± 0.6%) and significantly higher comparing to the industrial beers analysed, $p < 0.05$. However, values for DPPH radical inhibition in craft and industrial beers were similar. In industrial beers, the higher value was 85.7± 2.1% (SB-P) and the lowest was 70.4± 0.7% (SB-S). Overall, the results indicate that samples have a high DPPH radical scavenging activity, which indicates a good beer stability and high antioxidant capacity. Samples with more DPPH radical scavenging do not necessarily present more TPC. Therefore, the amount of certain phenolic compounds rather than the quantity of TPC seems to determine the biological activity of beers with respect to antioxidant activity(Gorinstein et al., 2001). For example, phenolic acids strongly contribute to the antioxidant activity of beer (Piazzon et al., 2010), and flavonoids have been reported to be free radical scavengers, metal chelators, and strong antioxidants (Kumar & Pandey, 2013).

The metal chelating activity allows to evaluate the inhibition of the ferrozine-Fe^{2+} complex. Through Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$) the Fe^{2+} cation originates a hydroxyl radical. If the sample is able to chelate iron, it will prevent this reaction from happening, decreasing the production of free radicals (Berker et al., 2010). Phenolic compounds in beer can act as chelating agents of metallic catalysts (Zhao, 2014). In this study, the metal chelating activity of samples varied from 1.6± 0.4% to 113.4± 15.8%. The highest chelating activity was seen in sample AMP-IPA, an India Pale Ale (113.4 ± 15.8%), which also presented the highest DPPH scavenging activity (99.4 ± 0.6%) and a good TPC value (936.4 ± 53.1%).

Samples AMP-IS (Imperial Stout) and B-MD (Munich Dunkel) also presented values of metal chelating activity (46.2 ± 0.9% and 67.0 ± 1.2%, respectively) significantly higher than industrial beers.

Other authors also evaluated metal chelating activity in beer samples. Zhao et al., (2010) presented values ranging from 0.12 μmol EDTAE/L to 54.57 μmol EDTAE/L and referred that the raw materials and the brewing process can have a major impact on the metal chelating activity.

In this study, beer color has a positive, statistically significant correlation with TPC (Table 4). This result was expected, since it is known that darker beers have an higher content of polyphenols (Saura-Calixto et al., 2008). Also, alcohol content and TPC have a positive, statistically significant correlation. The effect of ethanol content might be explained by the higher solubility of phenolic compounds in this solvent in comparison to water, increasing their extraction from raw materials during brewing (Moura-Nunes et al., 2016). Higher phenolic compounds in dark beers along with higher alcohol content is due to the fact that these beers are brewed from wort with higher extract content. Also, beers with higher original gravity generally showed higher TPC (>180mg GAE/L), indicating that phenolic compounds in beer mainly originated from barley malt and hops, although brewing process itself may influence the final polyphenols content and antioxidant activity of beers (Zhao, 2014). Gorjanović et al., (2010) also reported that polyphenols content is lower in alcohol-free beers because they are usually brewed with lower original wort extract and inhibition of alcohol formation.

Table 4. Correlations among beer parameters, antioxidant activity evaluation indices and total phenolic contents.

	TPC	ABV	EBC	IBU	PH	RS	TA	DPPH	MCA
TPC	1	0,880**	0,868**	0,628**	0,069	0,407**	0,858**	0,154	0,168
ABV	-	1	0,719**	0,691**	0,023	0,457**	0,801**	0,107	0,036
EBC	-	-	1	0,446**	-0,026	0,326*	0,847**	0,144	0,034
IBU	-	-	-	1	0,406**	0,734**	0,659**	0,013	0,440**
PH	-	-	-	-	1	0,501**	0,110	0,120	0,552**
RS	-	-	-	-	-	1	0,518**	0,127	0,356**
TA	-	-	-	-	-	-	1	0,289	0,262
DPPH	-	-	-	-	-	-	-	1	0,302*
MCA	-	-	-	-	-	-	-	-	1

ABV, alcohol by volume; IBU, bitterness; EBC, colour; RS, Reducing Sugars Content; TA, Total Acidity; TPC, Total Phenolic Content; DPPH, DPPH radical scavenging activity; MCA, Metal Chelating Activity. * Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed)

The correlation between parameters related to antioxidant activity (DPPH scavenging activity and metal chelating activity) stands out, suggesting that overall antioxidant activity evaluation results were consistent although these assays involved different reaction mechanisms. Therefore, compounds that

can inhibit DPPH radicals are capable of chelating ferrous ions. The lack of correlation between TPC and the antioxidant activity methods, was also reported by other authors (Oliveira Neto et al., 2017; Zhao et al., 2010).

Significant positive correlations between alcohol content, color, or bitterness and TPC were observed. Beer labels contain information of its alcohol content, and in some samples, they also included colour indication (EBC) and/or bitterness (IBU). The presence of this information on the label can be important for the consumer, helping to choose a product with a greater presence of phenolic compounds and a beverage with increased health value. A significant positive correlation between bitterness and metal chelating activity was also found, reinforcing the importance of indicating bitterness on labels.

3.3. Antioxidant activity of raw materials

The antioxidant activity of raw materials, namely malt and hop, used in the production of craft beers was also analysed. The malt and hop samples used in this study were analysed as pure ingredients and not as the specific mixtures actually used to brew specific beers, and this is because the receipt was confidential and thus it was unable to reproduce the mixture.

Malted barley is the second highest ingredient, in proportion, after water in brewing. Barley contains high levels of β-glucans and phenolic compounds with antioxidant properties. During malting, the extractability of phenolic compounds increases mainly due to enzymatic processes and better friability (Koren et al., 2019). Malts are not all equal and its chemical composition largely depends on the time and temperatures of the process (Briggs et al., 2004). Specialty malts are produced not for its enzyme content but to provide extra color and flavor to beer (Carvalho et al., 2016).

For malt, DPPH scavenging activity varied between 36.5± 2.8% and 96.0± 2.1% (Table 5). These results are similar to that observed by Coghe et al., (2005). In their study, the authors reported 16% to 89% of radical scavenging activity for wort samples. On the contrary, Koren et al. (2019)found values above 80% for all their malt samples, however, malt extraction was performed with a solution of 80% acetone and 20% water. Antioxidants from malt are able to scavenge oxygen-free radicals and prevent oxidative reactions, avoiding the addition of exogenous antioxidant (Vanderhaegen et al., 2006). Different phenolic compounds have been identified in barley and malt, including flavan-3-ols, proanthocyanidin oligomers, hydroxycinnamic acid derivatives, and flavonols (Carvalho et al., 2016), which may be related to the antioxidant activity observed in our samples, regarding DPPH assay.

Barley and wheat are the most common grains used in brewing. During the process, the grain is malted, milled, and mashed to convert starch to sugar, to be used for fermentation (Rodhouse & Carbonero, 2019). In this study, 9 of the 10 malt were barley malts and only one was wheat malt. The highest value was seen in Pale Ale malt (96.0%), which corresponds to a barley malt, and the lowest value was observed for Wheat

malt (36.5%). Also, in their study, Fogarasi et al., (2015) tested different cereals, namely, one organic einkorn wheat (*Triticum monococcum* L.), one organic barley (*Hordeum vulgare* L.), and eight bread wheat (*T. aestivum*) varieties. In all cases the barley sample had the highest antioxidant potential and polyphenol content. Values for DPPH scavenging activity observed in two base malts (Wheat malt, 36.5 ± 2.8% and Pils malt, 47.5 ± 5.8%) were significantly lower than those observed in all the other malts. Base malts are used mainly to add fermentable sugars to the beers instead of adding aroma, flavour, and colour.

Metal chelating activity in malt samples varied between 12.0± 0.5% and 24.8± 0.6%, with significantly higher value corresponding to Munich malt (24.8 ± 0.6%). Munich-style barley malt is known to exhibit antioxidant properties that are beneficial in stabilizing beer flavor (Briggs, 1998). The existence of metal chelating activity can be explained by the presence of possible chelating agents, like some phenolic compounds, that can inhibit radical generation by stabilizing transition metals, and consequently reducing free radical damage (Lu et al., 2007).

Table 5. Antioxidant Activity of Malt Samples.

Sample	Type of Malt	DPPH (%inhibition)	Metal Chelating Activity (%)
Munich	Base	92.5 ± 7.2^{b}	24.8 ± 0.6^{d}
Pils	Base	47.5 ± 5.8^{a}	19.8 ± 1.7^{c}
Wheat	Base	36.5 ± 2.8^{a}	13.5 ± 1.9^{a}
Pale Ale	Base	96.0 ± 2.1^{b}	20.3 ± 0.1^{c}
Biscuit	Specialty	90.7 ± 2.2^{b}	$18.8 \pm 2.8^{b,c}$
Carapils	Specialty	85.1 ± 3.5^{b}	20.5 ± 0.7^{c}
Crystal Light	Specialty	90.5 ± 5.4^{b}	$14.9 \pm 1.0^{a,b}$
Chocolate	Specialty	ND	14.2 ± 0.6^{a}
Chateau Special	Specialty	ND	19.0 ± 1.5^{c}
Cara Ruby	Specialty	ND	12.0 ± 0.5^{a}

ND – Not determined; Values are means ± SD (n = 3). Means with different superscript letters in the same column are significant differences ($p < 0.05$, ANOVA followed by post-tests).

The amount of hops (*Humulus lupulus* L.) required in beer production is significantly smaller. However, it has a crucial impact on beer quality. The brewing industry uses various hop varieties differing in content and composition of bioactive compounds, which can be associated to differences in its antioxidant properties (Krofta et al., 2008). The importance of hop polyphenols in the brewing process is due to protein–polyphenol interaction of nonbiological haze, which limits the shelf life of bottled beers (Almaguer et al., 2014). Some polyphenols are unique in hops like multifidol glucosides and prenylflavonoids, such as xanthohumol, desmethylxanthohumol, 6-prenylnaringenin, and 8-prenylnaringenin (Biendl, 2009).

In this study, hop samples were prepared to simulate the brewing process. In DPPH scavenging activity assay, results range from 64.7% (Magnum variety) to 79.6% (Organic sample), all values above 50% of

inhibition (Table 6). Mudura et al., (2010), reported values between 3.54% and 13.45% for DPPH scavenging activity for hops cultivated in Romania. The differences observed in both studies may be explained by the fact that antioxidant content of hops can be influenced, for example, by soil and weather conditions during vegetation and ripening, hop plant age and harvest time, geographic and/or cultivar differences (Mikyška & Jurková, 2019).

Table 6. Antioxidant Activity of Hops Samples.

Hop varieties	Brewing Use	DPPH (% inhibition)	Metal Chelating Activity (%)
Organic	-	79.6 ± 2.6^{b}	$16.2 \pm 3.9^{b,c}$
Magnum	Bittering	64.7 ± 6.5^{a}	$14.2 \pm 1.7^{b,c}$
Mittelfruh	Aromatic	76.3 ± 3.3^{b}	3.8 ± 0.5^{a}
Hersbrucker	Aromatic	78.2 ± 1.0^{b}	23.5 ± 2.4^{d}
Celeia	Aromatic	77.9 ± 2.6^{b}	11.9 ± 4.0^{b}
East Kent	Aromatic	$73.8 \pm 1.8^{a,b}$	12.1 ± 0.3^{b}
Citra	Dual Purpose	76.4 ± 2.4^{b}	$18.3 \pm 1.6^{c,d}$
Mosaic	Dual Purpose	78.3 ± 0.5^{b}	$12.3 \pm 1.1^{b,c}$
Simcoe	Dual Purpose	78.0 ± 5.4^{b}	$16.2 \pm 0.7^{b,c}$
Centennial	Dual Purpose	76.8 ± 1.7^{b}	$15.4 \pm 1.9^{b,c}$
Perle	Dual Purpose	76.5 ± 2.6^{b}	$14.7 \pm 0.2^{b,c}$

Values are means ± SD (n = 3). Means with different superscript letters in the same column are significant differences ($p < 0.05$, ANOVA followed by post-tests).

The results showed that bittering hops had the lowest scavenging activity (significantly lower inhibition percentage - 64.7 ± 6.5%, $p < 0.05$), when compared with the majority of aromatic and dual-purpose hops. The results are consistent with findings published by Mudura et al., (2010), where aromatic varieties showed higher content of polyphenolic compounds and higher anti-radical activity (Huller Bitterer, 13.45%), when compared with bittering hops (e.g. Magnum, 3.54%).

For metal chelating activity, and using water as solvent, the results varied between 3.8± 0.5 % (Mittelfruh hop) to 23.5± 2.4% (Hersbrucker hop). Hersbrucker, an aromatic hop, was the sample with the highest value of metal chelating activity (significantly higher inhibition percentage - 23.5 ± 2.4%). In their study, Kobus-Cisowska et al., (2019) reported higher values for metal chelating ability in samples, above 20% in water extracts. However, a different methodology in sample preparation was employed, since it was used a three-step extraction method and the extracts were centrifuged. Other possible explanation is the possibility that the hops used in both studies have different origins and cultivation conditions. Also, in their study, Kobus-Cisowska et al., (2019) showed that among the analysed hop cone extracts, the highest amount of iron ions (55.43–88.76%) was chelated by the ethanol extracts of the Magnum cultivar, and the lower metal chelating ability was demonstrated for water extracts.

Comparing the results from DPPH assay in malt and hop samples, values were higher in malt samples (excluding two base malts). It is known that around 80% of the phenolic compounds identified in beer is derived from malt, while the remaining 20% comes from hop (De Keukeleirc, 2000; Quifer-Rada et al., 2015). Moreover, malt can contribute to about 95% and 86% of the antioxidant capacity of dark and pale beers, respectively (Čechovská et al., 2012).

Finally, comparing the results obtained for raw materials (malts and hops) with craft beers, beers have a significant higher DPPH scavenging activity and metal chelating activity ($p = 0.049$ and $p = 0.002$). Two possible explanations are a synergistic effect of phenolic compounds present in raw materials and the increased solubility of these compounds in hydroalcoholic solutions (Saura-Calixto et al., 2008).

4. Conclusion

Craft beer consumption is increasing, mainly due to distinct characteristics comparing to industrial beers. Many effects of beer compounds on biological systems have received special attention from the scientific community. It has been demonstrated that bioactive compounds present in beer, such as phenolic compounds, have antioxidant activity and an important role in human health. It was found that craft beer and its components can act in cardiovascular disease, diabetes, cancer, inflammation, neurological disorders, menopause, osteoporosis, hepatoprotection and oxidative stress. However, despite the several interesting bioactive compounds present in craft beer, there are still few studies addressing the benefits of a final product for human health. Most of literature focus on the raw materials, industrial beers, or on the brewing process, with few studies evaluating the biological activities of the craft beers available on the market. The addition of innovative raw materials and the improvement of production techniques give uniqueness and additional value to beer. Therefore, further investigation on the health potential of craft beer is needed. More *in vitro* studies regarding its potential mechanisms of action, future clinical and *in vivo* studies concerning the bioavailability, distribution, efficacy, and safe are needed, since all compounds present in craft beer might act in synergy.

The present study describes the variations in chemical parameters, phenolic content and antioxidant activities of Portuguese craft beers and raw materials used in its production.

Regarding the chemical parameters, in this study, craft beers presented similar values of pH, ranging from 4.27±0.03 (Vienna Lager) to 4.92±0.02 (India Pale Ale); and the total acidity of beer samples ranges from 0.10 ± 0.00% (industrial beer – Munich Dunkel) to 0.62 ± 0.01% lactic acid equivalent (Imperial Stout). As already mentioned, pH and total acidity are important criteria for brewers due to its influence on the sensory attributes, biological and chemical stability. Also, reducing sugar content ranged from 2598.0 to 4446.3 mg/L glucose equivalents.

Beer contains several healthy compounds and, in this study, the content of total phenolics was determined in several craft beer samples. These compounds improve the quality and acceptance of craft beers (for example, by adding a bitter and astringent taste to the drink) and contribute to its overall antioxidant activity. In this study, there were considerable variations in phenolic profiles and antioxidant activities of commercial craft beers across different brands and styles. However, several beers showed a high content of phenolic compounds and good antioxidant activity.

In craft beers the TPC varied between 343.8 ± 22.2 mg GAE/L and 2172.5 ± 170.1 mg GAE/L. In industrial beers TPC, values were lower, and varied between 255.3 ± 69.6 mg GAE/L and 394.0 ± 48.7 mg GAE/L. It is known that TPC values vary depending on beer color, style, and composition of the raw materials. Craft breweries use materials, such as barley and hops, during beer production, which may influence the presence of different and more abundant phenolic compounds in these beverages. Also, the

best TPC results observed in dark craft beers may be related to the special malts. In general, industrial beers exhibited a lower phenolic content than craft beers.

Regarding antioxidant activity, for DPPH assay, the inhibition percentages varied between 58.3 ± 1.1 % (Strong Bitter) and 99.4 ± 0.6 % (India Pale Ale). The best value of DPPH radical inhibition was seen in a craft beer and it was significantly higher comparing to the industrial beers analysed. Overall, the results indicate that samples have a high DPPH radical scavenging activity, which indicates a good beer stability and high antioxidant capacity. However, there was no correlation between DPPH radical scavenging and TPC, which indicate that the amount of certain phenolic compounds rather than the quantity of TPC may influence the antioxidant activity. The highest chelating activity was seen in sample AMP-IPA, an India Pale Ale (113.4 ± 15.8 %), which also presented the highest DPPH scavenging activity (99.4 ± 0.6 %) and a good TPC value (936.4 ± 53.1 GAE/L).

It was observed that many beer characterization parameters (like alcohol content, colour, bitterness, total acidity or reducing sugars content) were positively correlated with TPC, which can be used as an indicator in consumer's choice.

The contribution of beer to antioxidants or phenolic compounds intake may vary significantly on the basis of the different beer types. It is also known that the antioxidant potential of the final product depends on the raw materials used. For that reason, in this study different malts and hops used in the production of the Portuguese craft beers were analysed. In general, beer samples revealed higher antioxidant capacity than the raw materials. However, raw materials showed different antioxidant activities, reinforcing that a careful selection is needed to obtain a final product with higher antioxidant capacity.

Future Perspectives

In the light of the complexity of beer compounds and wide choice of raw materials, further research is needed to understand the effect of these compounds in biological activities and chemical characteristics of craft beers.

- The types and concentration of phenolic compounds present in craft beer can vary according to beer style. Therefore, in future research, it might be interesting to identify the phenolic compounds present in craft beers by, for example, high-performance liquid chromatography (HPLC).
- It would be also important to characterize the specific phenolic compounds mainly responsible for beer antioxidant activity, providing a good way for brewers to increase selectively certain phenolic content during brewing, for improvement on flavour stability and biological activities of the final product.
- Some compounds present in craft beer may be related to liver protection. Therefore, in future research, it might be interesting to *in vitro* evaluate the hepatoprotection of different Portuguese craft beers and raw materials, using, for example, an HepG2 cell line.

- Phenolic compounds present in beers are originated mostly from barley malt and to a minor extent from hop. Also, in craft beers, producers commonly use mixtures of raw materials. Therefore, it might be also interesting in the future to study the TPC and antioxidant activity of the mixtures of hop and malts used in each beer analyzed.

Limitations

There were, however, some limitations in this study mostly related with the sample size. Therefore, additional work with a larger craft beer sample that is representative of Portuguese craft beers is needed to strengthen this work.

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