Artisanal fortified beers: brewing, enrichment, HPLC-DAD analysis and preliminary screening of antioxidant and enzymatic inhibitory activities

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ABSTRACT

In this study an artisanal "Porter style" beer has been enriched with diverse natural bioactive substances, ~~low fermentation-enriched beers have been made~~ following *all-grain* brewing method. Common beer generally contains a poor ~~phenolic~~ content ~~in the class~~ of phenolic acids which confers a low antioxidant power and nutritional value, also due to the presence of ethanol. However in this work we aimed to enrich beer with different flavonoids and other food supplements like taurine, resveratrol and caffeine, thus enhancing its nutritional value and energizing properties. A series of flavonoid/phenol-enriched artisanal beers have been prepared, then sample of each has been tested *in vitro* to evaluate antioxidant activity, chelating power and enzymatic inhibition capacity. Beer samples were also analysed with HPLC-DAD system to determinate flavonoid and phenol contents. Results show increased nutritional values and significant antioxidant properties in comparison with ~~blank artisanal~~ not-fortified beer as control ~~and commercial beer, thus paving the way to the potential use of these beverages as new food supplements~~.

Keywords: beer, flavonoids, antioxidants, ~~food supplements~~, fermentation, drink

1. Introduction

Beer is one of the most popular alcoholic drinks consumed in the world and its production is known since ancient times. Beer brewing is typically realised through alcoholic fermentation of barley malt operated by different types of yeasts, with the final adjunction of hops. (Olaniran, Hiralal, Mokoena, & Pillay, 2017) The main ingredient is malt, which derives from some types of grasses, especially by barley. Malt is the result of an enzymatic digestion operated by a series of amylases produced by the cereal in the germination step, during which starch is hydrolysed into small carbohydrates such as disaccharide maltose, the trisaccharide maltotriose, monosaccharides like glucose, fructose, and sucrose. (Liu & Quek, 2016; Olaniran et al., 2017) These are fermentable sugars representing the nourishment of a great number of yeasts; *Saccharomyces cerevisiae* and *Saccharomyces carisbergensis* are the most common yeast species used to beer brewing. (Liu & Quek, 2016; Olaniran et al., 2017). Literature reports many methods for beer brewing and various type of malts, hops and yeasts that affect beer taste, aroma, colour and texture. ~~The final step before fermentation of beer production is the adjunction of hops.~~ Hopping step consists on adding dried hops to the wort during boiling step. (Olaniran et al., 2017) The addiction of hops gives the typical bitter taste which is desirable by the main consumers. (Olaniran et al., 2017) Hops contain a complex series of organic compounds *e.g.* hop acids, hop oil and various flavonoids; the hop acid class of isohumolones is particularly relevant for beer bitter taste. (Olaniran et al., 2017) Natural phenolic antioxidants ~~Flavonoids~~ like chlorogenic acid, syringic acid, resveratrol and carvacrol are also added during hopping step by fruits. (Olaniran et al., 2017) (Liu & Quek, 2016; Olaniran et al., 2017) Moreover, beer contains a series of volatile compounds responsible for taste and aroma. (Olaniran et al., 2017) In addition to ethanol other alcohols are formed during fermentation step, like propanol, hexanol, benzyl alcohol and isoamyl alcohol; different esters and carbonyl compounds like ethyl acetate, isoamyl acetate, benzyl acetate, acetaldehyde and 2,3-pentanedione derive from amino acids and fatty acid metabolism. These molecules have a fundamental role to confer different aromas and tastes, depending on their concentration. (Olaniran et al., 2017) In fact, isoamyl acetate is responsible of a banana taste, while phenyl acetate gives a honey taste; ethyl acetate confers an undesirable “solvent flavour”, high concentration of hexanol is related to a greasy aroma badly affecting beer taste. (Olaniran et al., 2017) Phenolic content is also a key factor in determining beer taste, haze and bitterness. (Piazzon, Forte, & Nardini, 2010; Shopska et al., 2021; Šibalić, Planinić, Jurić, Bucić-Kojić, & Tišma, 2021) Beer has a moderate content of phenolic compounds ~~phenols and flavonoids~~ normally present in fruits, vegetables and some beverages ~~with very high antioxidant power~~. They also exhibit a series of different biological activities like antiallergic, antiviral and anti-inflammatory activity, but the most important is the capacity to stabilize and inactivate free radical species deriving from pro-oxidative cellular stress. (Anand David, Arulmoli, & Parasuraman, 2016; Bertuzzi et al., 2020; de Gaetano et al., 2016) Flavonoids are also present in hops, but a significant fraction of them ~~flavonoids are~~ is not assimilated by human organism, in fact, intestinal villi can't adsorb flavonoid bonded with matrix such as cellulose and other insoluble fibres. ~~however acid condition associated with high temperature can hydrolyse this bond.~~ (Schulz et al., 2019) However in a recent study by Grieco and co-workers, was reported that lactic fermentation with *Lactobacillus rhamnosus* can hydrolyse this bond, thus increasing the phenolic absorbable fraction in date fruit bars. (Maisto et al., 2021) Most common flavonoids are quercetin, ~~resveratrol,~~ ~~gallic acid,~~ ~~syringic acid,~~ ~~ferulic and caffeic acid,~~ ~~but also~~ rutin, iso-quercetin, ~~chlorogenic acid~~ and catechin. Quercetin is a coumaric compound belonging to the class of tetrahydroxy-flavonols largely contained in fruit, vegetables and seeds. (Anand David et al., 2016) Different studies have highlighted numerous biological activities like anti-inflammatory activity, anti-depressive, anti-obesity, anti-allergic and anti-asthmatic activity, anti-hypercholesterolemic effects, but also anti-atherosclerotic and vasodilator activity related to this compound. (Anand David et al., 2016; Mlcek, Jurikova, Skrovankova, & Sochor, 2016) ~~Thus the scientific interest towards quercetin and his similar compounds, like rutin, iso-quercetin and catechin is growing. (Anand David et al., 2016) Numerous studies have demonstrated that quercetin can act as inhibitor of cyclooxygenase, lipoxygenase, NO synthase, and human reactive C protein leading to a reduction of inflammatory states. (Anand David et al., 2016) Some preclinical studies conducted in rats have highlighted an inhibition of both acute and chronic inflammation. (Anand David et al., 2016) Many cardiovascular positive effects are related to the consumption of quercetin: in fact, Quercetin can prevent platelet aggregation, can reduce oxidation caused by LDL and can reduce fat accumulation in human cells, also enhancing fat cell apoptosis. (Anand David et al., 2016) As a result, quercetin has the capacity to reduce atherosclerosis, hypertension and to improve the health of blood vessels. (Anand David et al., 2016) Other studies reported anticancer and antiproliferative properties, but also antibacterial and antiviral proprieties. (Anand David et al., 2016) Thanks to its antioxidant effects, quercetin consumption has been associated to minor neuroinflammation, and less reduction in cognitive performance and age-depended neurodegeneration. (Anand David et al., 2016) Many other studies report that quercetin has an anti-H1 and anti-H2 effect, so it can act as antiallergic and anti-asthmatic molecule and can inhibit gastric acid secretion with prevention of gastritis. (Anand David et al., 2016) Quercetin anti-H1, anti-H2 and antiallergic capacity is due to the inhibition of enzymes, inflammatory mediators and mast cellules activation through the block of calcium, prostaglandins, leukotrienes, and histamine releases. (Mlcek, Jurikova, Skrovankova, & Sochor, 2016)~~

Resveratrol, largely present in grapes and red wine (Mollica et al., 2021) has been also recently identified in beer in lower concentration; it is a stilbene derivate with high antioxidant power and potential preventing activity in several human diseases. (Chiva-Blanch et al., 2011) Resveratrol glycoside piceid, was found in some hop varieties and pellets used to produce beer in a concentration between 0.5 to 11.7 mg/Kg; it was also detected in a concentration between 0.3 to 1.2 mg/kg, as free form of *cis/trans*-resveratrol. (Chiva-Blanch et al., 2011) ~~However,~~ Hops are added in small amount during beer production thus a low quantity of resveratrol is transferred in beer, that's why ~~content of *cis/trans*-resveratrol in beer is very low; thus,~~ normal beers can't be considered a dietary source of resveratrol. (Chiva-Blanch et al., 2011) The polyphenol content in beer confers an interesting nutritional value, ~~because it contains a significant polyphenol content,~~ depending on the quality and quantity of malt and hops, and to the production method used. (Piazzon et al., 2010; Šibalić et al., 2021) ~~Besides the aforementioned compounds beer contains many different phenolic substances, principally resveratrol, quercetin, rutin and other phenolic acids, but also tannins, proanthocyanins and amino phenolic acids. (Deng et al., 2019)~~ Phenols malt wort content depends to the temperature applied during the mashing step, in fact a too long boiling step (> 60 min) decreases the total phenol content and antioxidant activity of beer, because phenols polymerise during this passage. (Piazzon et al., 2010; Šibalić et al., 2021) On the other hand, total phenolic content depends also to the type of beer considered; in fact, there was found significant differences in phenol content of lager beer, ale beer and other commercial beers. (Piazzon et al., 2010; Šibalić et al., 2021) Dealcoholized beers have a lower antioxidant power and phenolic content than strong and dark beer according to the following order: dealcoholized beer < lager < pilsner < wheat < ale < abbey < bock; probably because dealcoholized beer is usually brewed with a minor quantity of original wort extract. (Piazzon et al., 2010; Šibalić et al., 2021) Malt has a fundamental role in beer's production as antioxidant molecules, phenols and starch source. (Piazzon et al., 2010; Šibalić et al., 2021) Malt provides between 86% and 95% of total antioxidant activity and about 80% of total phenolic content, instead hops provide less amount of phenolic content without significantly affecting antioxidant capacity of beer. (Shopska et al., 2021) Germination, kilning and roasting are the main processes that influence the phenolic profile and antioxidant power of malts, thus determining beer properties. (Shopska et al., 2021) It's possible to significantly increase beer phenolic content using different raw materials or by adding one or more of them for example, diverse cereals instead of barley. Many studies described the production of beer by buckwheat. (Deng et al., 2019) The best benefit obtained with buckwheat beer brewing is to increase its quercetin content of about 60 times. (Deng et al., 2019) On the other hand, buckwheat is gluten-free food and it is also a source of proteins, amino acids, lipids and dietary fibres. (Deng et al., 2019) However, phenolic content and nutritional value of buckwheat strongly depend on different factors, like buckwheat species, cultivars and thermal processes. (Bai et al., 2015; Ge & Wang, 2020;

Qin, Wang, Shan, Hou, & Ren, 2010) Another strategy to improve the phenolic content and antioxidant power of beer is the adjunction of some fruits like cherry, peach, orange, and many others during fermentation step. (Deng et al., 2020) In a recent study conducted by Deng and co-workers, ale beers have been enriched with omija fruit (*Schisandra chinensis*) at diverse boiling times. (Deng et al., 2020). Adding omija fruit at initial boiling step produces a typical red colour beer due to some phenolic compounds formed during *Maillard reactions*, among them high quantity of lignans; for this reason, omija fruit can be considerate as a valid solution to improve nutritional value of beer. (Deng et al., 2020) Similar results have been obtained by Adamenko and co-workers with Cornelian cherry (*Cornus mas* L.) (Adamenko, Kawa-Rygielska, & Kucharska, 2020) Authors have developed a non-alcoholic beer brewed with special yeast *saccharomyces ludwigii* added to cornelian cherry juice, characterised by natural aroma and sour taste. (Adamenko et al., 2020) The final beer presents a significant content of phenolic compounds, anthocyanins and iridoids, lower energy value, major antioxidant capacity and new sensory attributes compared to standard beer, thanks to the presence of cornelian cherry juice. (Adamenko et al., 2020)

Beer brewing methods can be promptly selected to prepare gluten-free beers for coeliac. Common beer contains gluten, which derives from the cereal used to prepare malts, (Cela et al., 2020) however beer can be brewed with a gluten-free malt obtained by natural gluten-free cereals, to afford a gluten-free beer. (Cela et al., 2020) An example of gluten-free cereal is teff. (Gebremariam, Zarnkow, & Becker, 2013) Teff has a good malting characteristic and brewing potential. (Gebremariam et al., 2013) Its attitude for malting was investigated by Grebremarian and co-workers, which have tested five different teff varieties for malting procedure revealing that *Kuncho* teff variety has the best enzyme activity and fermentable sugar content. (Gebremariam et al., 2013) Malting conditions, like temperature and germination time are also important to obtain a high quality of teff malt. (Di Ghionno et al., 2017) It was found that optimal malting condition for teff is 4 days of germination at 48% of steeping degrees under 24° C. (Di Ghionno et al., 2017) Furthermore, the total di-methyl-sulphur teff content can be below 7 mg/Kg to ensure a good quality malt for brewing. (Gebremariam et al., 2013) Other examples of natural gluten-free cereals that can be malted are rice, maize, oat, sorghum, millet, buckwheat, quinoa, and amaranth. (Cela et al., 2020).

Beer's nutritional value is due to the content of micro and macro-nutrients, vitamins, phenolic substances, minerals, and fibres, ~~Phenolic compounds confer to beer numerous beneficial proprieties like anti-inflammatory and antioxidant activity but also a protective activity on cardiovascular and neuro-protective systems. These properties~~ that make beer an excellent base for nutritional enriched drinks ~~beverages brewing~~. The main intent of this research is to develop a new beer-based ~~beverage~~ drink enriched with flavonoids (*e.g.* quercetin and rutin), resveratrol, caffeine and taurine according to the dosages contained in food supplements in order to ~~preserve the reintegrative propriety and to~~ enhance its nutritional value (Figure 1).

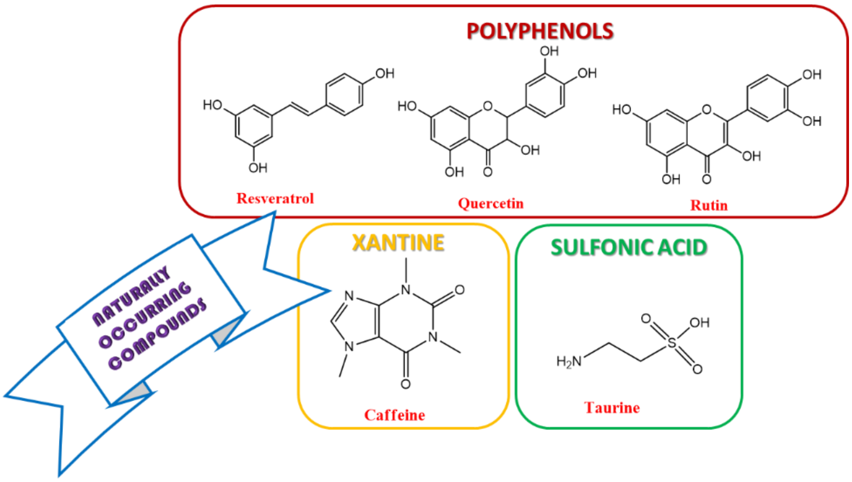

Figure 1. Naturally occurring compounds selected for artisanal beer's fortification.

HPLC-DAD analysis was performed on each sample using not-fortified ~~blank~~ beer as control ~~reference~~. Their antioxidant and enzyme inhibitory activities were also investigated and compared to the control ~~standard and commercial Porter style~~.

2. Materials and methods

2.1 Reagents, solvents and standard phenolic and flavonoid compound

Gallic acid, catechin, chlorogenic acid, *p*-OH benzoic acid, vanillic acid, epicatechin, syringic acid, 3-OH benzoic acid, 3-OH-4-MeO benzaldehyde, *p*-coumaric acid, rutin, sinapinic acid, *t*-ferulic acid, naringin, 2.3-diMeO benzoic acid, benzoic acid, *o*-coumaric acid, quercetin, harpagoside, *t*-cinnamic acid, naringenin, resveratrol, and carvacrol were purchased from Sigma Aldrich (Milan, Italy). Methanol, acetonitrile (both HPLC-grade) and formic acid (pure 99%) were obtained from Carlo Erba Reagenti (Milan, Italy). Double-distilled water was obtained using a Millipore Milli-Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA, USA).

2.2 Beer brewing

Pale Ale malts, Crystal 100 and 300 malts, East Kent Golding hop and Safale-4 yeast were bought to MrMalt (Prato, IT). Quercetin, rutin, taurine and resveratrol were purchased ~~by~~ from Sigma (Milano, IT). Coffee been extract was prepared starting from 5 g of commercial coffee been (Castroni, Rome, IT), which was added in boiling water at 100 °C and left to stand for 5 minutes. The suspension was filtered and the solution was lyophilized, the so obtained brown powder was used as such for beer's fortification. Beer brewing (*all-grain*) was developed using KLARSTEIN (di Chal-Tec GmbH

Wallstr.16, 10179 Berlin-Deutschland) masher according to the following procedure: Malts (4.5 Kg) were ground and put into KLARSTEIN masher with mineral water (25 L); temperature was brought to 68 °C for 60 minutes during *meshing* step. Then temperature was raised to 72 °C for 10 minutes, and to 78 °C for 5 minutes during the *mesh out* step. Then *sparging* step was raised at 78 °C for 15 minutes, the grain waste was removed and the first portion of hop was added (30 g). Temperature was raised to 100 °C for 50 minutes (*hopping* step), then a second portion of hop was added ~~and left to boil in must~~ boiling for 10 minutes. Finally, the must was cooled ~~at~~ to 24-25 °C. Hydrated yeast (7.5 g) was added. Must was then left to ferment for 7 days at 20 °C or until completeness; after fermentation step, sugar (10 g/L), resveratrol (250 mg/ 330 mL), quercetin dihydrate (200 mg/ 330 mL), rutin (300 mg/ 330 mL), coffee been extract (150 mg/ 330 mL), coffee been extract *plus* taurine (150 mg + 105 mg/ 330 mL) were added to crude beer (20 L) during bottling and refermented for 30 days. After refermentation, 100 mL of each ~~mixture beer type~~ sample was lyophilized and analysed by HPLC-DAD to evaluate their total phenol and flavonoid contents, antioxidant activity and enzyme inhibitory activity.

2.3 HPLC-DAD analysis

HPLC analyses were performed on a Waters liquid chromatograph equipped with a model 600 solvent pump and a 2996 photodiode array detector (PDA). *Empower v.2* Software (Waters Spa, Milford, MA, USA) was used for data acquisition. A C18 reversed-phase packing column (Prodigy ODS(3), 4.6 × 150 mm, 5 µm; Phenomenex, Torrance, CA, USA) was used for the separation. The column oven (Jetstream2 Plus) was set at 30 ± 1°C. The UV/Vis acquisition wavelength was set in the range of 200 - 500 nm. The quantitative analyses were achieved at maximum wavelength for each compound. The injection volume was 20 µL. The mobile phase was directly *on-line* degassed by using Biotech DEGASi, mod. Compact (LabService, Anzola dell'Emilia, Italy). Gradient elution was performed using the mobile phase water-acetonitrile (93:7, *v:v*, 3% acetic acid) as reported in Table 1. Samples for HPLC-PDA analysis were prepared as follows: the lyophilized sample was weighted and solubilize in mobile phase A (milliQ water + 3% acetic acid): B (acetonitrile +3% acetic acid) (93:7, *v: v*). The samples were prepared at concentration of 1 mg/250 µL. All samples were vortexed for 1/2 min, sonicated for 15 min and then an aliquot of 20 µL was injected in the chromatographic system for the analysis. Table 2 reports ~~while in Table 2 were reported~~ the retention times and the maximum wavelengths used for the quantitative analyses.

Table 1. Gradient elution program used for HPLC analyses

<table>
<tr><th>TIME (min)</th><th>FLOW (mL min^{-1})</th><th>%A</th><th>%B</th></tr>
<tr><td>0</td><td rowspan="8">1</td><td>93</td><td>7</td></tr>
<tr><td>0.1</td><td>93</td><td>7</td></tr>
<tr><td>30</td><td>72</td><td>28</td></tr>
<tr><td>38</td><td>75</td><td>25</td></tr>
<tr><td>45</td><td>2</td><td>98</td></tr>
<tr><td>47</td><td>2</td><td>98</td></tr>
<tr><td>48</td><td>93</td><td>7</td></tr>
<tr><td>58</td><td>93</td><td>7</td></tr>
</table>

Table 2. Analytes, retention times, and maximum wavelengths used for quantitative analyses

Analytes	Retention Times (min)	λ max
Gallic acid	4.99	271 nm
Catechin	13.36	278 nm
Chlorogenic acid	14.29	324 nm
4-hydroxybenzoic acid	14.71	256 nm
Vanillic acid	17.31	260 nm
Epicatechin	18.30	278 nm
Syringic acid	18.50	274 nm
3-hydroxybenzoic acid	19.41	275 nm
3-hydroxy-4-methoxybenzaldehyde	22.08	278 nm
p-coumaric acid	22.65	310 nm
Rutin	25.38	256 nm
Sinapinic acid	26.18	324 nm
t-ferulic acid	27.75	315 nm
Naringin	29.78	285 nm
2,3-dimethoxybenzoic acid	30.36	299 nm
Benzoic acid	31.20	275 nm
o-coumaric acid	34.81	276 nm
Quercetin	40.57	367 nm
Harpagoside	45.49	280 nm
t-cinnamic acid	45.87	276 nm
Naringenin	46.74	290 nm
Carvacrol	49.95	275 nm

2.4 Biological activity

2.4.1 *In vitro* antioxidant assays

The antioxidant assays [1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline) 6-sulfonic acid (ABTS) radical scavenging, cupric ion reducing antioxidant capacity (CUPRAC), ferric ion reducing antioxidant power (FRAP), metal chelating ability (MCA) and phosphomolybdenum assay (PBD)] were previously described (Zengin and Aktumsek, 2014; Mollica et al. 2021). For DPPH, ABTS, CUPRAC and FRAP assays data were expressed as mg Trolox equivalents (TE)/g extract. ~~whereas in~~ MCA and PBD were expressed as mg EDTA equivalents (EDTAE)/g extract and mmol TE/g extract respectively ~~were used~~. Total phenolic content (TPC) and total flavonoid content (TFC) were determined as previously described (Zengin and Aktumsek, 2014), and expressed as mg gallic acid equivalents (GAE)/g extract (TPC) and mg rutin equivalents (RE)/g extract (TFC), respectively.

2.4.2 Enzyme inhibitory activity

The protocols used for acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase, amylase and glucosidase assays were previously provided (Zengin, 2016; Sinan, 2021). In cholinesterase assays, data were expressed as mg galanthamine equivalents (GALAE)/g extract, whereas mg kojic acid equivalents (KAE)/g extract were used in tyrosinase assay. For amylase and glucosidase assays, the results were reported as mmol acarbose equivalents (ACAE)/g extract.

2.5. Statistical analysis

All the experiments were performed in three replicates, with the results presented as mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) with Turkey's post-hoc test was conducted; $p < 0.05$ was considered statistically significant. The correlation analysis between TPC, TFC and biological activities was reported as Pearson's coefficients, calculated using Graph software (9.0).

3. Results and discussion

3.1 Determination of the total content of flavonoid and phenol compounds

The total content of phenols and flavonoids in beer samples was examined using spectrophotometric methods. The results are shown in the Table 3.

Table 3. Beers antioxidant activity and total content of flavonoid and phenol compounds

Samples	T~~C~~PC (mg GAE/g)	TFC (mg RE/g)	DPPH (mg TE/g)	ABTS (mg TE/g)	FRAP (mg TE/g)	CUPRAC (mg TE/g	PHD (mmol TE/g)	MCA (mg EDTAE/g)
~~Blank Beer~~ control	3.21±0.07^{f}	0.03±0.01^{e}	1.09±0.25^{d}	2.63±0.25^{d}	7.94±0.25^{c}	15.37±0.39^{c}	0.27±0.03^{ab}	na
Rutin beer	3.43±0.10^{e}	0.11±0.01^{d}	2.13±0.24^{bc}	2.77±0.19^{d}	6.89±0.06^{e}	14.04±0.24^{d}	0.27±0.02^{ab}	na
Coffee beer	3.37±0.03^{ef}	0.01±0.01^{e}	0.39±0.02^{e}	0.14±0.02^{f}	7.39±0.05^{d}	14.05±0.23^{d}	0.22±0.01^{b}	na
Quercetin beer	5.21±0.04^{c}	0.64±0.03^{a}	2.65±0.29^{b}	4.65±0.25^{c}	8.70±0.17^{b}	16.91±0.47^{b}	0.25±0.01^{b}	2.28±0.29^{c}
Resveratrol beer	5.77±0.04^{b}	0.25±0.04^{c}	2.01±0.23^{c}	7.19±0.41^{b}	8.85±0.14^{b}	17.26±0.20^{b}	0.27±0.04^{ab}	3.60±0.53^{b}
Coffee and taurine beer	7.18±0.04^{a}	0.40±0.04^{b}	5.62±0.30^{a}	8.59±0.39^{a}	13.14±0.09^{a}	26.18±0.45^{a}	0.32±0.02^{a}	5.17±0.63^{a}
~~Commercial beer~~	~~3.89±0.03^{d}~~	~~0.13±0.03^{d}~~	~~0.68±0.08^{de}~~	~~1.03±0.08^{e}~~	~~6.54±0.07^{e}~~	~~13.33±0.20^{d}~~	~~0.21±0.02^{b}~~	~~0.34±0.05^{d}~~

* Values expressed are means ± S.D. of three parallel measurements. TPC: Total phenolic content; TFC: Total flavonoid content; PHD: Phosphomolybdenum; MCA: Metal chelating; FRAP: Ferric reducing antioxidant power; CUPRAC: Cupric ion-reducing activity; TE: Trolox equivalent; EDTAE: EDTA equivalent. GAE: Gallic acid equivalent; RE: Rutin equivalent; nt: not tested; na: no active. Different letters in the same column indicate significant differences in the tested samples ($p<0.05$)

The content of ~~best result in terms of~~ total phenols follows this order of magnitude: ~~content is in~~ coffee / taurine-enriched beer ~~was determined as~~ (7.18 mg GAE/g) > ~~followed by~~ resveratrol-enriched beer (5.77 mg GAE/g) > quercetin-enriched beer (5.21 mg GAE/g). In terms of total flavonoid content, the richest sample was quercetin fortified beer (0.64 mg RE / g), followed by coffee / taurine (0.40 mg RE/g) and resveratrol (0.25 mg RE/g). The lowest values for total flavonoid were found in control beer (0.03 mg RE/g) and coffee beer (0.01 mg RE/g). The enrichment with quercetin, resveratrol or coffee / taurine clearly increased the total phenol content compared to the control beer. Diverse antioxidant activities could be explained with total content of phenolic compounds, different preparation techniques and geographical locations of malt and hops (Vidyalakshmi et al., 2022; Rahman et al., 2020; Zhao et al., 2010; Nardini and Garaguso, 2020; Nescitelli et al. 2016).

3.2 Antioxidant activity

The antioxidant properties of the tested beer samples were evaluated by different chemical assays. The results are depicted in Table 3. DPPH and ABTS radicals are widely used to assess the radical scavenging abilities of plant extracts. In both assays, the best scavenging ability was determined in coffee extract/taurine enriched beer (DPPH: 5.62 mg TE/g and ABTS: 8.59 mg TE/g), followed by quercetin enriched beer (2.65 mg TE/g) in the DPPH assay and resveratrol enriched beer (7.19 mg TE/g) in the ABTS assay. The electron-donating abilities of the tested beer samples were examined by reducing power assays, namely CUPRAC and FRAP. The assays are based on the reduction of cupric/ferric to copper/ferrous form by antioxidant compounds. In both of them, the best reduction ability was observed for coffee extract/taurine enriched beer (CUPRAC: 26.18 mg TE/g and FRAP: 13.14 mg TE/g). The phosphomolybdenum assay consists in the conversion of Mo (VI) to Mo (V) by antioxidant compounds, thus forming a blue Mo (V) / phosphate complex which has a maximum of absorbance at 695 nm. In this sense, the assays could be considered as a reducing power assay, as well CUPRAC and FRAP but all antioxidant components are susceptible to this reaction, thus the assay is considered as an antioxidant assay. The best ability was observed for coffee extract/taurine enriched beer in phosphomolybdenum assay (0.32 mmol TE/g), while other beer samples showed very similar activities (0.21-0.27 mmol TE/g). These results are consistent with the total phenolic content in the extracts, further confirming that they actively contribute to the free radical and reducing abilities of the tested beer samples. The correlation analysis was performed between the test systems and the obtained results (Figure 2).

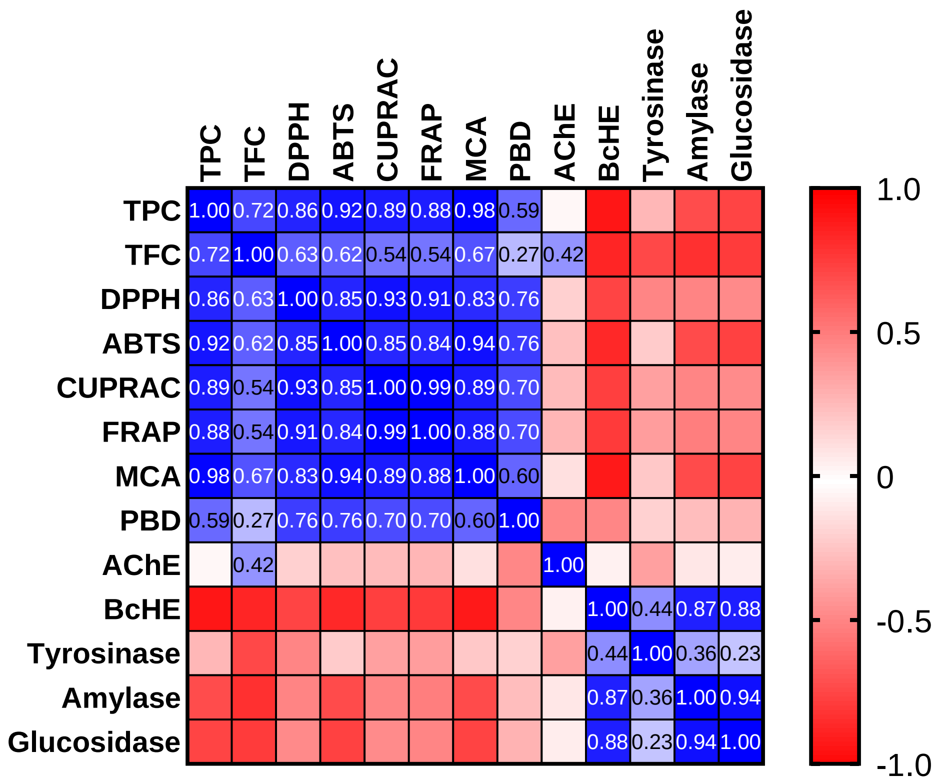

Figure 2. Pearson's correlation values (R) in the performed biological activity assays; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CUPRAC, cupric ion reducing antioxidant capacity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric ion reducing antioxidant power; MCA, metal chelating activity; PBD, phosphomolybdenum assay; TFC, total flavonoid content; TPC, total phenolic content.

A strong correlation between total phenolic and radical scavenging/reducing power assays (R>0.7) was found, in line with several literature reports (Nardini and Garaguso, 2020; Moura-Nunes et al., 2016; Baigts-Allende et al., 2021). Chelation of transition metals is closely linked to manage the production of hydroxyl radicals in Fenton reaction. In the current study, the best chelating ability was detected by coffee/taurine enriched beer (5.17 mg EDTAE/g), followed by resveratrol (3.60 mg EDTAE/g)~~,~~ and quercetin-enriched beer~~s~~ ~~and commercial beer (0.34 mg EDTAE/g)~~ (2.28 mg EDTAE/g). Overall our data demonstrate that the enriched-beer samples exhibit stronger antioxidant abilities than the ~~blank~~ control ~~and commercial beers~~. The use of quercetin and coffee extract/taurine in the artisanal beer's fortification increases its antioxidant properties, representing an extremely appealing starting point for the development of low-fermentation beer based-functional drinks.

3.3 Identification and quantification of flavonoid and phenol compounds by HPLC-DAD

~~Samples for HPLC-PDA analysis were prepared as follows: the lyophilized sample was weighted and solubilize in mobile phase A (milliQ water + 3% acetic acid): B (acetonitrile +3% acetic acid) (93:7, *v: v*). The samples were prepared at concentration of 1mg/250 µL. All samples were vortexed for 1/2 min, sonicated for 15 min and then an aliquot of 20 µL was injected in the chromatographic system for the analysis.~~ In Table 4 are reported the phenolic quantitative data (in µg/mg dry extract) observed for the different beer samples: beer control, rutin beer, coffee beer, quercetin beer, resveratrol beer, and coffee-taurin beer, respectively. The reported values are mean ± standard deviation of three independent measures. The value below Limit of Quantification (0.02 µg/mL) have been reported as BLQ (Below Limit of Quantification). Detectable quantity of rutin (0.83 ± 0.7 µg/mg) is in rutin beer sample, *p*-coumaric acid (0.05 ± 0.01µg/mg) and sinapinic acid (0.19 ± 0.01µg/mg) are in coffee beer sample, quercetin (0.19 ± 0.01µg/mg) in quercetin enriched-beer and chlorogenic acid (0.24 ± 0.01µg/mg) in coffee extract/taurine beer sample.

Table 4. Flavonoids and phenols quantification by HPLC-DAD analysis

Concentration (µg/mg)	Gallic acid	Catechin	Chlorogenic acid	*p*-OH benzoic acid	Vanillic acid	Epicatechin	Syringic acid	3-OH benzoic acid	3-OH-4-MeO benzaldehide	*p*-coumaric acid	Rutin	Sinapinic acid	*t*-ferulic acid	Naringin	2,3-diMeO benzoic acid	Benzoic acid	*o*-coumaric acid	Quercetin	Harpagoside	*Trans*-cinnamic acid	Carvacrol
BEER SAMPLES																					
Beer control																					
Rutin beer											0.83 (±0.07)										
Coffee beer										0.05 (±0.01)		0.19(±0.01)									
Quercetin beer										BLQ								0.27(±0.02)			
Resveratrol beer																					
Coffee-taurin beer			0.24(±0.01)							BLQ											

BLQ: below Limit of Quantification.

3.4 Enzyme inhibitory activity

The enzyme inhibition theory plays an important role in treating global health problems such as diabetes mellitus, Alzheimer's disease and obesity. In particular, the inhibition of some key enzymes (*e.g.* cholinesterase, amylase, and lipase) can reduce the pathological observations in these diseases (Rauf and Jehan, 2017). Several drugs have been manufactured as enzyme inhibitors in the pharmaceutical industry, but most of them show side effects such as toxicity and gastrointestinal diseases (Jagadeesan et al., 2022; Meziant et al., 2021), thus the discovery of new and natural enzyme inhibitors has become one of the most attractive topic in the scientific platform (Mollica et al. 2018). According to these, we investigated the enzyme inhibitory properties of the tested beer samples using cholinesterases, tyrosinase, amylase and glucosidase as target enzymes. The results are shown in Table 5. In AChE inhibition assay, two beer samples (quercetin enriched ~~and commercial~~ beer) are able to inhibit the enzyme at 2.66 and 3.07 mg GALAE/g, respectively. All beer samples are active against BChE, the best result was obtained by rutin-enriched beer (7.95 mg RE/g) while the lowest BChE inhibitory effect was observed for quercetin-enriched beer (5.29 mg GALAE/g). Tyrosinase is a main enzyme involved in the synthesis of melanin; its inhibition represents a key strategy in the treatment of hyperpigmentation (Della Valle et al. 2020). The best tyrosinase inhibitory effect was detected in resveratrol-enriched beer (59.19 mg KAE/g), while the weakest ability was provided by quercetin-enriched beer (49.90 mg KAE/g). Other beer samples exhibited similar tyrosinase inhibitory effects (53.00-56.74 mg KAE/g). All samples showed very similar abilities against amylase and glucosidase enzymes. To the best of our knowledge, the information regarding the enzyme inhibitory effect of beer samples are very scarce in literature (Merino et al., 2018; Szwajgier, 2013), therefore the present study provides insightful data in this field, thus paving the way to the development of low-fermentation functional beers ~~samples~~ in the food and drink industries.

Table 5. Beer's enzymatic inhibition activity.

Samples	AChE (mg GALAE/g)	BChE (mg GALAE/g)	Tyrosinase (mg KAE/g)	Amylase (mmol ACAE/g)	Glucosidase (mmol ACAE/g)
~~Beer~~ Control	na	7.87±0.16[ab]	55.16±0.52[b]	0.17±0.01[ab]	1.20±0.01[a]
Rutin Beer	na	7.95±0.22[a]	55.86±0.60[b]	0.17±0.01[a]	1.20±0.01[a]
Coffee Beer	na	7.52±0.10[b]	56.74±0.19[b]	0.17±0.01[ab]	1.20±0.01[a]
Quercetin Beer	2.66±0.03[b]	5.29±0.24[cd]	49.90±0.85[d]	0.11±0.01[c]	1.14±0.01[c]
Resveratrol Beer	na	5.69±0.03[c]	59.19±0.44[a]	0.12±0.02[c]	1.14±0.01[c]
Coffee and taurine beer	na	5.14±0.07[d]	53.00±0.45[c]	0.14±0.01[bc]	1.17±0.01[b]
~~Commercial beer~~	~~3.07±0.01[a]~~	~~7.83±0.06[ab]~~	~~56.72±1.30[b]~~	~~0.18±0.01[a]~~	~~1.20±0.01[a]~~

* Values expressed are means ± S.D. of three parallel measurements. AChE: Acetylcholinesterase; BChE: Butirylcholinesterase; GALAE: Galatamine equivalent; KAE: Kojic acid equivalent; ACAE: Acarbose equivalent; na: not active. Different letters in the same column indicate significant differences in the tested samples ($p<0.05$)

4. Conclusions

In this study artisanal beers brewed by *all grain* method and low fermentation ~~with fresh and pleasant flavour~~ have been ~~prepared. Samples were~~ analysed by HPLC-DAD system in order to determine the polyphenolic profile. The highest total polyphenol content was found in resveratrol, quercetin and in taurine /plus coffee extract enriched beers. HPLC-DAD analysis reveals that flavonoid enriched beer has a content of bioactive substances like common food supplement and this result highlights the possibility to use enriched beer as vehicle of bioactive compounds. ~~Antioxidant activity and chelation capacity was evaluated *in vitro* trough DPPH scavenging and FRAP test;~~ The major antioxidant activity was found in resveratrol beer and coffee-taurine beer. ~~Enzymatic inhibition activity was detected *in vitro* to estimate the inhibition capacity against some enzymes involved in glucose metabolism and neurodegeneration.~~ According to our study flavonoids-enriched beer has a good inhibitory activity on enzyme tyrosinase, moderate activity against BChE and very low activity on other enzymes. These data encourage further studies on the development of enriched beers and in the optimization of ~~food supplements~~; the procedure applied in this work ~~can be extended to~~ for the production of invigorating or energizing drinks ~~beverages, as well as to prepare a lyophilized matrix for cosmetics and anti-aging products~~.

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