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Black garlic extract: Phytochemical characterisation and application as natural antioxidant in burgers

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Abstract

The phytochemical characteristics of black garlic extract (BGE) and its antioxidant effect on burgers were evaluated in the present work. For this, four burger formulations were produced: a negative control (without antioxidants), a positive control (with 2,6-di-tert-butyl-4-methylphenol), T3 (with the addition of 3% BGE), and T6 (with the addition of 6% BGE). BGE showed a 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity of 87% and a total phenolic content of 72.86 mg gallic acid equivalents/g extract. Twenty phytochemicals were annotated by liquid chromatography coupled to electrospray ionisation mass spectrometry in the BGE, including amino acids, peptides, and sulphur-containing derivatives. The addition of 6% BGE (T6) affected the pH and two-colour parameters (redness, *a** and yellowness, *b**) of burgers with significant differences compared with the negative and positive controls. Burgers with BGE presented similar lipid oxidation to the positive control over 12 days of storage. In conclusion, BGE could have great potential as a natural antioxidant in burgers.

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Introduction

Meat product consumers are searching for fast and pleasant products, among which, burgers stand out (Rios-Mera *et al*., 2019). However, due to their high fat, protein, and water contents, burgers are very susceptible to adverse chemical reactions such as lipid oxidation, which can negatively affect their colour and nutritional quality (Heck *et al*., 2019). To overcome these problems, synthetic antioxidants are added to most meat products. Nevertheless, due to the growing concern among consumers about the potential toxicological effects of these synthetic antioxidants, researchers and manufacturers are looking for natural extracts with similar antioxidant potential as synthetic agents (Menegali *et al*., 2019).

Many studies have shown that natural extracts, especially those obtained from plants, contain bioactive compounds that can exert an antioxidant effect on fresh meat and meat products (Reddy *et al*., 2018). From this perspective, plant extracts from different sources such as fruits, vegetables, herbs, and spices have been successfully incorporated into fresh meat and meat products to maintain their quality and oxidative stability. Some of these natural antioxidants used in fresh meat and its products have demonstrated antioxidant activity that is similar to or greater than synthetic antioxidants (Pateiro *et al*., 2021). They can also delay spoilage and extend the shelf-life of products without damaging the organoleptic properties of the foods (Manessis *et al*., 2020). In addition, these natural antioxidants can improve the functional, nutritional, and bioactive properties of meat products (Pateiro *et al*., 2021). As a functional food product that is nutritionally rich and has several bioactive properties, including antimicrobial and antioxidant activities (Kimura *et al*., 2017; Botas *et al*., 2019), black garlic could be a useful additive in burgers for both the food industry and consumers looking for more natural foods.

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Black garlic is produced by allowing natural garlic (*Allium sativum*) to age at 60 - 90°C and 70 - 90% relative humidity for 2 - 40 days, without using additional treatments or additives (Kimura *et al*., 2017). The high humidity and temperature favour a fermentation process that involves enzymatic and non-enzymatic reactions such as Maillard reaction, oxidation of phenols, and caramelisation (Afzaal *et al*., 2021). Black garlic has distinct differences from natural garlic in terms of its phytochemical composition, nutritional and organoleptic profiles, physicochemical and biological properties, and sensorial characteristics (Molina-Calle *et al*., 2017a; Afzaal *et al*., 2021). Amino acids, saccharides, lipids and their derivatives, and organosulfur compounds have been reported to be the main classes of compounds in polar extracts of black garlic (Molina-Calle *et al*., 2017a). Regarding the biological properties of black garlic, some researchers have demonstrated its hepatoprotective, anticancer, anti-obesity, anti-inflammatory, anti-allergic, antidyslipidemic, antibacterial, antidiabetic, cardioprotective, neuroprotective, and antioxidant activities (Kimura *et al*., 2017; Botas *et al*., 2019; Afzaal *et al*., 2021). Based on the literature, extracts of black garlic have higher antioxidant capacity than extracts of natural garlic, highlighting their potential use as natural antioxidant agents in foods (Jeong *et al*., 2016; Botas *et al*., 2019).

Although the health benefits of black garlic are well known (Kimura *et al*., 2017), few studies have assessed the use of black garlic in meat products, especially in burgers. The present work thus addressed this research gap, paving ways for future research to develop healthier meat products in line with the demands of consumers (Saldaña *et al*., 2021). In the first part of the present work, the antioxidant activity, phenolic content, and phytochemical compounds of a hydroethanolic black garlic extract (BGE) were determined. Then, two concentrations of BGE (3 and 6%) were used as natural antioxidants in burgers. The effect of these formulations on the physicochemical and oxidative properties was analysed during 12 days of refrigerated storage.

Materials and methods

Preparation and characterisation of black garlic

Commercial peeled and vacuum-packed black garlic bulbs were provided by Chisato Kondo Co. (Guatapará, São Paulo, Brazil). Black garlic was prepared in two stages using a forced air circulation oven, the first one corresponding to 80°C for 48 h (2 d), and the second one corresponding to 65°C for 192 h (8 d). Relative humidity was maintained at 90% during the entire processes.

After receipt, the peeled and vacuum-packed black garlic bulbs were immediately stored at -18°C for 5 d until use. Before extraction, the black garlic bulbs underwent proximate analysis (moisture: 33.9%; lipids: 2.4%; proteins: 35.1%; carbohydrates: 24.6%; and ash: 3.9%) in triplicate according to AOAC (2005). In addition, aerobic mesophilic (colony-forming units (CFU/g)) and coliforms (CFU/g) were counted at 35 and 45°C, respectively. *Salmonella* sp. and moulds and yeasts were determined following the recommendations of Downes and Ito (2001). The microbiological results showed that the black garlic bulbs were within the quality microbiological standards (aerobic mesophiles: < 10 CFU/g; coliforms at 45°C: < 10 CFU/g; moulds and yeasts: < 10 CFU/g; *Salmonella* sp.: absence in 25 g of the sample).

Preparation of black garlic extract (BGE)

The black garlic cloves were dried in an oven (TE-395, Tecnal, São Paulo, Brazil) with air circulation at 100°C for 5 h, and then ground (TE-648, Tecnal). Then, 56 g of ground dried black garlic was subjected to extraction with 672 mL of a mixture containing ethanol and water (12:1, v/v). The mixture was homogenised (1 h at 25°C) and vacuum filtered. The hydroethanolic extract was concentrated in a rotary evaporator at 45°C under reduced pressure until it reached 7% of the initial volume (Campagnol *et al*., 2011). The concentrated extract was frozen and lyophilised, yielding 14.5 g of dry extract (25.8%). Subsequently, the powder was diluted to 50 mL with distilled water (0.29 g/mL), and stored at -18°C in amber glass. The total soluble solid content was determined in triplicate using a refractometer (Reichert, AR200, Buffalo, NY, USA), with an average result of 29° Brix.

Antioxidant activity and total phenolic content of BGE

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of BGE was measured according to Gao and Wang (2012) at a final concentration of 48.3 mg/mL. The total phenolic content was determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965), using gallic acid

as the standard; the results are expressed as milligrams of gallic acid equivalents (GAE) per gram of the sample (mg GAE/g). These experiments were performed in triplicate.

Determination of phytochemicals present in BGE by high-performance liquid chromatography coupled with electrospray ionisation mass spectrometry (HPLC-ESI-MS)

The phytochemical constituents of BGE were determined with an HPLC instrument (Infinity 1260, Agilent®) coupled with a high-resolution quadruple time of flight mass spectrometer (6520B - Agilent®) with an ESI source following the method described by Silva *et al*. (2019). The chromatographic conditions were as follows: 1.0 µL of the sample was injected into the column (Agilent Zorbax C18; 2.1 mm inner diameter, 50 mm length, 1.8 µm particles). The mobile phase was ultrapure water acidified with formic acid (0.1%, v/v) (A) and methanol (B). The solvent gradient system started with 2% B (0 min), followed by 98% B (0 - 15 min), and 98% B (15 - 17 min), at a flow rate of 0.4 mL/min. The sample was analysed at a concentration of 2.0 mg/mL. The mass spectrometer operated with a nebuliser pressure of 58 PSI, a drying gas flow rate of 8.0 L/min, and at 220°C. The energy applied to the capillary was 4.5 kV. The high-resolution mass for each ion (MS) was obtained in the positive $[M+H]^+$ and negative $[M-H]^-$ modes. Sequential mass spectrometry (MS^2) analyses were performed at different collision energies (5 - 30 eV).

The proposed molecular formula for each compound was selected based on the smallest difference between the experimental and theoretical mass, the error in parts per million (ppm), double bond equivalence, and the nitrogen rule. Suggestions for possible structures and classes of metabolites were determined based on comparison to the METLIN and PubChem databases, and by analysing the solvent system, retention times, and sequential mass spectra from other published studies.

Production of burgers

Four burger formulations were prepared: a negative control (C-, without antioxidants), a positive control (C+, with 2,6-di-tert-butyl-4-methylphenol [BHT]), and two formulations with 3% (T3) and 6% (T6) BGE (Table 1). The BGE concentrations were determined based on preliminary tests.

Table 1. Burger formulations.

(%)	C-	C+	T3	T6
Raw material				
Beef	90	90	90	90
Pork back fat	10	10	10	10
Ingredient/additive*				
Salt	2	2	2	2
Water	6	6	3	-
Black garlic extract	-	-	3	6
2,6-Di-tert-butyl-4-methylphenol	-	0.01	-	-

C- = negative control; C+ = positive control; T3 = burger made with the addition of 3% black garlic extract; T6 = burger made with the addition of 6% black garlic extract; and (*) added relative to the meat mixture.

Beef and pork back fat was ground in a meat grinder (PJ22, Jamar Ltda., São Paulo, Brazil) using an 8 mm plate. First, beef was mixed with salt to extract the myofibrillar proteins, and then pork back fat, water, and antioxidants (BGE or BHT) were added. Following complete manual homogenisation of the ingredients, the burgers (100 g, 2.5 cm thick, and 11 cm in diameter) were formatted with a burger-maker (HP 112, Picelli, São Paulo, Brazil), packed individually in polyethylene plastic bags, sealed in a sealer (Selovac, São Paulo, Brazil), and immediately stored at 2 ± 1°C for 12 d. Three independent replicates were performed for each treatment, and 100 samples (10 kg) were produced per treatment.

Physicochemical and oxidative characteristics of raw burgers

The pH, water activity (a_w), colour parameters, and thiobarbituric acid reactive substances (TBARS) were determined in triplicate in the raw burgers after 0, 3, 6, 9, and 12 d of storage. A pH meter (HI 221, Hanna Instruments, Barueri, Brazil) was used to determine the pH of the samples. The pH meter was calibrated with pH 4.00 and 7.00 buffers before use,

and temperature compensation was performed. The reading was carried out in a homogenate of sample and water (1:10). The a_w was determined at 25°C using a digital thermohygrometer (Aqua Lab 4TE, Decagon, Pullman, WA, USA) with approximately 1 g of sample. Three colour parameters—lightness (*L**), redness (*a**), and yellowness (*b**)—were measured in three pieces per treatment using a colorimeter (model CR-400, Konica Minolta, Ramsey, NJ, USA), with a standard D65 illuminant, a standard observer of 2°, and an aperture size of 8 mm. The TBARS assay was performed with malonaldehyde (MDA) as the main lipid peroxidation product following the method described by Sinnhuber and Yu (1958) and Buege and Aust (1978).

Statistical analysis

The experiment was carried out using a randomised block design (considering as blocks of three independent processing) arranged in a factorial scheme with four treatments and five storage times, with different batches each day. After confirming the adherence of the assumptions of analysis of variance (ANOVA)—homoscedasticity based on the Breusch-Pagan test and normality of the residuals based on the Shapiro-Wilk test at 5% significance—the model shown in Eq. 1 was used to study the two effects (executed at 5% significance).

$$y_{ijk} = \mu + b_k + \beta_i + \gamma_j + \beta\gamma_{ij} + \varepsilon_{ijk} \quad \text{(Eq. 1)}$$

where, μ = constant inherent in all observations, y_{ijk} = response variable measured along time, b_k = associated with the k-th block (k = 2) arranged to the β_i treatment (I = 4), γ_j = storage time point (j = 5), $\beta\gamma_{ij}$ = interaction between treatments and storage times, while ε_{ijk} = experimental error associated with the ANOVA model distributed with a mean of zero and constant variance. Linear regression was performed to compare quantitative factors, while Tukey's test was applied for the pairwise comparison of qualitative factors. The data analysis was conducted using the R software.

Results and discussion

Annotation of phytochemicals present in BGE

The chemical composition of commercial black garlic produced by Chisato Kondo Co. is being reported herein for the first time. The hydroethanolic BGE was analysed with LC-ESI-MS. The annotated chemical constituents are shown in Table 2.

Twenty compounds were annotated, including amino acids, di- and tripeptides, a monoglucoside, organic acids, phenolics, ester, sulphur-containing compounds, and sphingolipids. These classes have previously been reported in fresh garlic (Farag *et al*., 2017) and black garlic (Choi *et al*., 2014; Kimura *et al*., 2017; Molina-Calle *et al*., 2017a; 2017b).

The amino acids such as arginine, phenylalanine, valine, *S*-allyl-L-cysteine sulfoxide (alliin), and *S*-allyl-cysteine; myo-inositol saccharide; malic and citric acids; and the dipeptides *N*-glutamyl-*S*-allylcysteine, *N*-glutamyl-phenylalanine, and *N*-glutamyl-*S*-allylthiocysteine annotated in BGE have been reported in polar extracts of fresh garlic and/or black garlic (Farag *et al*., 2017; Molina-Calle *et al*., 2017a; 2017b).

Regarding the phenolic compounds, only 2,7-dihydroxy-2H-1,4-benzoxazin-3-one or 2,4-dihydroxy-2H-1,4-benzoxazin-3-one was annotated in BGE, and there were no flavonoids. In a methanol and water (50:50, v/v) extract of black garlic analysed by Molina-Calle *et al*. (2017a), there were no phenolic compounds, and only the flavonoid styraxin or uralenol was present. Phenolic compounds such as caffeic and ferulic acids were found in a methanolic extract of fresh garlic (Farag *et al*., 2017), but not in the present BGE or in the study by Molina-Calle *et al*. (2017a).

Different extraction and analytical methods can promote qualitative and quantitative variations in the chemical composition of a natural product. Factors such as the storage conditions (including time), seasonality, growth stage, climate, and soil type also influence the chemical composition of metabolites (Bagudo and Acheme, 2014; Farag *et al*., 2017; Najman *et al*., 2021). In addition to these variables, the different processes involved in producing black garlic must also be considered (Botas *et al*., 2019). These factors make it difficult to compare and define the chemical composition of a food product.

Choi *et al*. (2014) analysed the content of some amino acids, total polyphenols, and total flavonoids in the aqueous extract of black garlic produced in Korea. They found that the content of these compounds may increase or decrease during the black garlic production stages. These variations are related to several reactions that can occur during the aging of

Table 2. Annotation of compounds in hydroethanolic black garlic extract by high-performance liquid chromatography coupled with electrospray ionisation mass spectrometry.

N.	Rt (min)	$[M - H]^-$ MS	$[M + H]^+$ MS	Error (ppm) - / +	Fragment ions (*m/z*) MS^2 $[M-H]^-$ / $[M+H]^+$	Molecular formula	Annotated compound	Reference
1	0.57	173.1050	175.1190	3.4/ 0.0	20 eV: 131, 114, 112 / 10 eV: 175, 158, 130	$C_6H_{14}N_4O_2$	Arginine	Molina-Calle *et al*. (2017a); Farag *et al*. (2017)
2	0.57	--	337.1718	0.0	5 eV: 337, 320, 319, 301, 291, 283, 273, 187, 175	$C_{12}H_{25}N_7O_4$	Arginine hexoside	Wang *et al*. (2008)
3	0.60	--	118.0863	0.0	--	$C_5H_{11}NO_2$	Valine	Molina-Calle *et al*. (2017a); Choi *et al*. (2014)
4	0.61	179.0574	--	0.0	10 eV: 179, 161, 149, 143, 141, 131, 129, 125	$C_6H_{12}O_6$	Myo inositol	METLIN; Molina-Calle *et al*. (2017a)
5	0.66	--	178.0530	-1.1	--	$C_6H_{11}NO_3S$	Allyl-L-cysteine sulfoxide (Alliin)	Molina-Calle *et al*. (2017a)
6	0.71	--	292.1503	-2.0	20 eV: 262, 232, 150, 127, 117, 112	$C_{11}H_{21}N_3O_6$	Threonine-threonine-alanine	METLIN
7	0.73	133.0145	--	2.2	10 eV: 133, 115	$C_4H_6O_5$	Malic acid	Molina-Calle *et al*. (2017a)
8	0.81	191.0198	--	0.5	10 eV: 173, 155, 147, 129, 117, 111	$C_6H_8O_7$	Isocitric or citric acid	Farag *et al*. (2017); Molina-Calle *et al*. (2017a)
9	0.81	128.0356	130.0498	2.3/ -0.7	10 eV: 130, 117, 115, 106, 102, 100	$C_5H_7NO_3$	Pyroglutamic acid	PubChem
10	1.04	--	182.0447	-0.5	20 eV: 182, 173, 164, 154, 140, 136	$C_8H_7NO_4$	2,7-Dihydroxy-2H-1,4-benzoxazin-3(4H)-one or 2,4-Dihydroxy-2H-1,4-benzoxazin-3(4H)-one	PubChem
11	1.16	--	162.0583	0.0	10 eV: 154, 145, 136, 120, 116	$C_6H_{11}NO_2S$	*S*-allyl-cysteine	Molina-Calle *et al*. (2017a)
12	1.31	205.0354	--	0.0	10 eV: 189, 173, 143, 111	$C_7H_{10}O_7$	Methylisocitric acid or methylcitric acid	Tienaho *et al*. 2019)

N.	Rt (min)	$[M - H]^-$ MS	$[M + H]^+$ MS	Error (ppm) - / +	Fragment ions (*m/z*) MS^2 $[M-H]^-$ / $[M+H]^+$	Molecular formula	Annotated compound	Reference
13	1.31	--	144.0656	0.7	10 eV: 144, 129, 116, 102	$C_6H_9NO_3$	Methylpyroglutamate	METLIN
14	1.49	--	166.0863	0.0	10 eV: 152, 141, 134, 125, 120, 103	$C_9H_{11}NO_2$	Phenylalanine	Molina-Calle *et al*. (2017a)
15	2.34	289.0867	291.1010	1.0/ 0.3	10 eV: 271, 215, 171, 128	$C_{11}H_{18}N_2O_5S$	N-Glutamyl-S-allylcysteine	Farag *et al*. (2017); Molina-Calle *et al*. (2017a)
16	3.49	293.1151	295.1288	0.3/ 0.0	10 eV: 275, 257, 165, 164, 128	$C_{14}H_{18}N_2O_5$	N-Glutamyl-phenylalanine	Farag *et al*. (2017); Molina-Calle *et al*. (2017a)
17	4.20	321.0579	323.0730	-1.5/ 0.0	5 eV: 249, 215, 171, 128	$C_{11}H_{18}N_2O_5S_2$	N-Glutamyl-S-allylthiocysteine	Farag *et al*. (2017); Molina-Calle *et al*. (2017a)
18	8.19	--	274.2743	0.7	20 eV: 274, 256, 230, 212, 106	$C_{16}H_{35}NO_2$	Hexadecaesfinganine	Huang *et al*. (2016); Qu *et al*. (2018)
19	8.30	--	230.2482	1.7	20 eV: 230, 212, 175, 129	$C_{14}H_{31}NO$	Xestoaminol C	Huang *et al*. (2016)
20	8.40	--	290.2691	0.3	20 eV: 272, 242, 158, 122	$C_{16}H_{35}NO_3$	Hexadecafitoesfinganine	Qu *et al*. (2018)

MS = mass spectrum; MS^2 = sequential mass spectrum; Rt = retention time; and -- = Not obtained. METLIN: online library available at: https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage. PubChem: online database on chemical compounds available at: https://pubchem.ncbi.nlm.nih.gov/.

garlic promoted by a temperature increase, mainly the Maillard reaction (Kimura *et al*., 2017). During aging, the Maillard reaction decreases some amino acids and peptides, and reduces sugars *via* the reactions between their amino groups and carbonyl groups, respectively (Choi *et al*., 2014). On the other hand, the high relative humidity and temperature favour hydrolytic decomposition of polysaccharides, justifying the considerable increase in the sugar content in processed black garlic (Najman *et al*., 2021). Most studies have reported higher phenolic and flavonoid contents in black garlic compared with fresh garlic. However, other work has shown that processing fresh garlic at high temperatures for an extended period of time could decrease the phenolic content (Afzaal *et al*., 2021).

Fresh garlic has a volatile fraction consisting of lipophilic (non-polar) sulphur-containing metabolites with a low molecular weight. Generally, diallyl disulphide, allyl methyl trisulphide, 3-vinyl-1,2-dithiacyclohex-5-ene, and diallyl trisulphide are the major components of this fraction (Satyal *et al*., 2017). These compounds can be extracted by steam dragging techniques or with low-polarity solvents. The BGE evaluated in the present work was not expected to contain these compounds, given that the extraction was performed with a hydroalcoholic solvent with high polarity. Another point to be considered regarding this volatile fraction is that some organosulphur compounds characteristic of fresh garlic are highly sensitive to heating, and can quickly degrade or volatilise during stages involving heating (Molina-Calle *et al*., 2017b).

The concentration of some metabolites that are characteristic of fresh garlic, such as alliin, allicin, and methiin, decrease considerably or disappear after 30 - 36 days of fermentation (Molina-Calle *et al*., 2017b). The organosulphur compounds annotated in BGE were the amino acids *S*-allyl-cysteine and allyl-L-cysteine sulfoxide (alliin) correlated to the presence of $[M+H]^+$ ions at *m/z* 162.0583 and 178.0530, respectively, and the dipeptides *N*-glutamyl-*S*-allylcysteine and *N*-glutamyl-*S*-allylthiocysteine correlated to the $[M+H]^+$ ions at *m/z* 291.1009 and 321.0579, respectively. Based on comparison of the MS^2 fragmentation pattern with data from Huang *et al*. (2016) and Qu *et al*. (2018), the $[M+H]^+$ ions at *m/z* 274.2743, 230.2482, and 290.2691 in BGE were annotated as the sphingolipids hexadecaesphinganine, xestoaminol C, and hexadecaphytosphinganine, respectively.

The findings from the present work were consistent with the tentative identification made by Molina-Calle *et al*. (2017a) who also proposed the presence of these classes of compounds in black garlic. In addition, the amino acid arginine hexoside, the tripeptide threonine-threonine-alanine, pyroglutamic acid, methylcitric or methylisocitric acid, and methylpyroglutamate were annotated in BGE. In summary, the chemical composition of BGE mainly comprises amino acids and their derivatives, as well as organosulphur compounds, which was consistent other studies (Choi *et al*., 2014; Kimura *et al*., 2017; Molina-Calle *et al*., 2017a).

Antioxidant activity and total phenolic content of BGE

The antioxidant activity of BGE in terms of the percentage of DPPH free radical scavenging and the total phenolic content are shown in Table 3.

Table 3. Total phenolic content and antioxidant activity of black garlic extract.

Extract	Total phenol content (mg GAE/g extract)	Antioxidant activity (% DPPH free radical scavenging)
Black garlic extract Ethanol:water (12:1, v/v)	72.86 ± 1.75	87.04 ± 1.00

DPPH = 2,2-diphenyl-1-picrylhydrazyl; and GAE = gallic acid equivalents.

The DPPH free radical scavenging activity (87.04%) and the total phenolic content (72.86 mg GAE/g extract) for BGE were consistent with what has been reported in other studies with black garlic. Lishianawati *et al*. (2022) reported that an acetone and water (70:30, v/v) extract of black garlic showed DPPH radical scavenging activity of 80.91%, and a total phenolic content of 62.5 mg GAE/g. Jang *et al*. (2018) investigated the ethanolic and aqueous extracts of black garlic, obtaining an antioxidant activity of 51.16 and 79.21%, and a phenolic content of 43.01 and 147.58 mg GAE/g, respectively. Choi *et al*. (2014) evaluated aqueous extracts of black garlic produced under different heat treatment times. The DPPH free radical scavenging activity ranged from 37.32% (25.8 mg GAE/g) to 74.48% (35.28 mg

GAE/g), and was significantly higher than that of fresh garlic (4.65%), with a phenolic content of 13.91 mg GAE/g. Setiyoningrum *et al*. (2021) produced black garlic under different treatment times, and found variations for the hydroethanolic extracts (ethanol:water 70:30, v/v) in the antioxidant activity (from 26.02 to 90.54%) and the total phenolic content (from 101.3 to 157.3 mg GAE/g). Kim *et al*. (2012) reported that a hydroethanolic extract of black garlic (ethanol:water 70:30, v/v) had an antioxidant activity of 82.53%, and a total phenolic content of 0.22 mg GAE/g. Najman *et al*. (2021) studied the total phenolic content in hydromethanolic extracts (methanol:water 80:20, v/v) of conventional fresh garlic (6.06 mg GAE/g), organic fresh garlic (8.08 mg GAE/g), conventional black garlic (13.64 mg GAE/g), and organic black garlic (17.24 mg GAE/g). The extracts of black garlic showed higher phenolic content compared with extracts of fresh garlic, with substantially higher content in the extract of organic black garlic.

Although several factors make it difficult to compare these works—geographic location; climatic conditions; seasonality; and differences in obtaining black garlic, extraction techniques, and analytical methodologies—the results showed that BGE had higher phenolic content and antioxidant potential (based on DPPH free radical scavenging) than other previously reported extracts of black garlic, confirming the suitability of BGE as a good source of natural antioxidants. The phenolic content of BGE can be attributed to the high temperature used during the production of black garlic, which damages cell walls and vacuoles, facilitating the release of these compounds from the tissue matrix (Najman *et al*., 2021).

The antioxidant activity found in BGE in the present work may be related to the presence of phenolic compounds and the contribution of constituents from other classes of metabolites that were annotated by LC-ESI-MS. The amino acids annotated in BGE are well known for their antioxidant capacity, especially arginine and phenylalanine (Masek *et al*., 2014; Xu *et al*., 2017). Sulphur-containing amino acids (cysteine and methionine) and their derivatives have also shown relevant antioxidant effects in different assays, including DPPH free radical scavenging (Kim *et al*., 2018). Between the two sulphur-containing amino acids, cysteine is a more potent antioxidant agent (Masek *et al*., 2014; Xu *et al*., 2017; Kim *et al*., 2018). In the present work, some cysteine-derived amino acids and peptides were annotated in BGE.

Free radicals can be stabilised by two main mechanisms: electron transfer and hydrogen atom transfer. In addition to well-known groups for antioxidant activity such as carboxyl and phenols, the sulfhydryl group (-SH) present in sulphur-containing amino acids can also be highlighted. The -SH group can contribute to the antioxidant activity by donating a hydrogen atom or by losing an electron from the sulphur atom (Zou *et al*., 2016). In the present work, some peptides were also annotated in BGE, and they have been shown to scavenge free radicals (Zou *et al*., 2016). So, in addition to amino acids and cysteine derivatives (allyl-L-cysteine sulfoxide and *S*-allyl-cysteine), the dipeptides (*N*-glutamyl-*S*-allylcysteine, *N*-glutamyl-phenylalanine, and *N*-glutamyl-*S*-allylthiocysteine) and the tripeptide (threonine-threonine-alanine) annotated may contribute to the antioxidant effect of BGE.

Organic acids (malic, citric or isocitric, and pyroglutamic acids) were also found in BGE. Organic acids are considered to be weaker antioxidant agents than phenolic compounds and amino acids (Zhang *et al*., 2019), but they do exert antioxidant actions (Liu *et al*., 2019). Hence, together with phenolic compounds, amino acids, and peptides, they may also be responsible for the observed antioxidant activity.

Overall, BGE is a rich source of compounds with recognised antioxidant potential, particularly those containing a cysteine unit. Sulphur-containing amino acids and peptides with good antioxidant effects can be considered extremely useful to the food industry as additives in other foods to increase shelf-life and, at the same time, offer benefits against cell damage caused by oxidative stress (Kim *et al*., 2018).

Water activity, pH, and colour parameters of burger formulations throughout storage period

Table 4 shows the a_w, pH, and colour parameter results of the burger formulations throughout the storage period.

There were no significant interactions for these measures, with no relationship between the treatments and storage period. The addition of BGE did not significantly affect a_w of the burgers. The addition of BGE affected pH; it was significantly different for T6 burgers compared with C- burgers. However, the pH of T3 burgers did not differ from the pH of C- and C+ burgers ($p > 0.05$). The lower pHs of the samples containing BGE might have been due

Table 4. Mean colour parameters (*L**, *a**, and *b**), water activity (a_w), and pH of burgers with different concentrations of black garlic extract throughout 12 days of refrigerated storage.

Treatment	Time (day)					Mean
	0	3	6	9	12	
			pH			
C-	5.81 ± 0.03	5.72 ± 0.12	5.88 ± 0.08	5.80 ± 0.00	5.72 ± 0.12	5.79 ± 0,07^{a}
C+	5.83 ± 0.02	5.83 ± 0.01	5.88 ± 0.11	5.81 ± 0.02	5.75 ± 0.29	5.82 ± 0,05^{a}
T3	5.69 ± 0.03	5.52 ± 0.28	5.70 ± 0.01	5.57 ± 0.18	5.71 ± 0.34	5.64 ± 0,09^{ab}
T6	5.60 ± 0.03	5.45 ± 0.23	5.65 ± 0.07	5.67 ± 0.15	5.54 ± 0.05	5.58 ± 0,09^{b}
			L*			
C-	51.51 ± 1.69	53.73 ± 1.65	49.66 ± 1.41	50.70 ± 1.54	49.61 ± 4.27	51.04 ± 1.69^{a}
C+	50.36 ± 1.18	52.34 ± 3.97	50.06 ± 0.69	49.51 ± 3.38	49.98 ± 5.64	50.45 ± 1.09^{a}
T3	46.15 ± 1.34	46.51 ± 0.10	49.32 ± 0.06	46.30 ± 6.68	45.88 ± 6.96	46.83 ± 1.40^{a}
T6	47.71 ± 3.75	47.04 ± 2.12	48.65 ± 0.99	48.58 ± 4.60	49.66 ± 10.41	48.33 ± 0.99^{a}
			a*			
C-	14.76 ± 0.35	11.07 ± 3.61	8.00 ± 0.13	7.40 ± 0.15	7.00 ± 0.75	9.64 ± 3.27^{a}
C+	14.15 ± 0.51	12.09 ± 0.19	10.71 ± 2.42	7.44 ± 1.38	6.92 ± 0.17	10.26 ± 3.07^{a}
T3	13.63 ± 1.23	8.88 ± 2.63	7.16 ± 0.66	6.74 ± 0.99	8.11 ± 1.99	8.9 ± 2.76^{ab}
T6	9.86 ± 1.44	8.19 ± 1.59	7.17 ± 0.65	6.03 ± 0.15	5.92 ± 0.22	7.44 ± 1.64^{b}
			b*			
C-	8.82 ± 0.96	8.40 ± 0.12	8.09 ± 0.41	6.98 ± 1.66	6.77 ± 0.24	7.81 ± 0.90^{bc}
C+	7.48 ± 0.37	7.95 ± 0.11	7.39 ± 0.03	7.33 ± 2.10	7.83 ± 1.16	7.59 ± 0.27^{c}
T3	8.91 ± 0.23	8.14 ± 0.16	9.88 ± 1.09	9.16 ± 0.45	8.52 ± 0.88	8.92 ± 0.66^{b}
T6	10.23 ± 1.55	11.10 ± 0.49	11.30 ± 0.73	11.09 ± 1.03	11.72 ± 2.19	11.09 ± 0.54^{a}
			a_w			
C-	0.983 ± 0.005	0.981 ± 0.004	0.979 ± 0.006	0.977 ± 0.004	0.975 ± 0.003	0.979 ± 0.003^{a}
C+	0.984 ± 0.002	0.981 ± 0.003	0.980 ± 0.005	0.979 ± 0.007	0.974 ± 0.003	0.980 ± 0.003^{a}
T3	0.980 ± 0.005	0.981 ± 0.002	0.980 ± 0.002	0.976 ± 0.008	0.976 ± 0.003	0.978 ± 0.002^{a}
T6	0.985 ± 0.001	0.980 ± 0.003	0.978 ± 0.003	0.975 ± 0.009	0.974 ± 0.005	0.978 ± 0.004^{a}

C- = negative control; C+ = positive control; T3 = burger made with the addition of 3% black garlic extract; T6 = burger made with the addition of 6% black garlic extract. Means followed by different lowercase superscripts in the same column differ significantly ($p < 0.05$) by Tukey's test.

to the pH of black garlic bulbs (4.19), which was lower compared the other ingredients used in the burger formulations. This effect was more evident in the treatment containing higher BGE concentration (T6; Table 4). However, the pHs found in all treatments in the present work were within the range considered normal for burgers (Silva *et al*., 2015; Heck *et al*., 2019).

The addition of BGE did not significantly affect the colour parameter *L** of the burgers. In addition, T3 burgers showed *a** and *b** values similar to C- burgers. However, T6 burgers showed significantly decreased ($p < 0.05$) *a** values, and increased *b** values compared with C- and C+ burgers. Kim *et al*. (2019) reported similar results: The addition of black garlic powder to raw pork patties decreased redness (*a**), and increased yellowness (*b**). These changes are probably due to the natural colouring pigments of black garlic (*L** = 19.266; *a** = 0.072; *b** = 1.768). Only the redness value was significantly lower, and other indexes had no significant differences. As expected, there was a significant decrease in *a** values during the storage period ($p < 0.05$). This change could have been due to the oxidation of the myoglobin during refrigerated storage, as observed by Fernandes *et al*. (2016). Those authors reported that the addition of a natural antioxidant as well as BHT helped to preserve the reddish colour of the burgers, although there was a slight decrease in *a** values during refrigerated storage.

TBARS of burger formulations throughout refrigerated storage

Figure 1 shows the results of lipid oxidation determined with the TBARS assay for each burger formulation.

There was a significant interaction between the treatment and storage time ($p < 0.05$). T3 burgers presented lower TBARS values compared with C- burgers throughout the storage period. Although T3 burgers showed similar TBARS values to C+ burgers on days 0 and 3, they had lower values on day 6, indicating lower lipid oxidation. Surprisingly, T6 burgers presented similar TBARS values as C- burgers at the beginning of storage (days 0 and 3), with lower values compared with C- burgers on days 6, 9, and 12, thus evidencing the antioxidant effect of BGE during storage. Some treatments, especially C+, showed a decrease in TBARS values during refrigerated storage.

Considering the storage period (Figure 1), there was no difference in TBARS values for T3 burgers, while C+ burgers reached the maximum TBARS value on day 6, and C- burgers reached the maximum TBARS value on day 9. T6 burgers presented higher TBARS values during the first days of storage, followed by a decrease from day 6. T3 burgers showed the best results, with lower TBARS values at all evaluated time points, except day 0. In general, the TBARS assay demonstrated that 3 and 6% BGE might have the potential to replace synthetic antioxidants in burgers. These results were closely related to the phenolic and phytochemical compounds annotated in BGE (Table 2), which can neutralise free radicals due to hydroxyl groups attached to the aromatic rings (Krishnan *et al.*, 2014). Kim *et al.* (2019) observed a similar trend, and reported that adding aged garlic powder decreased the TBARS values of pork patties during refrigerated storage.

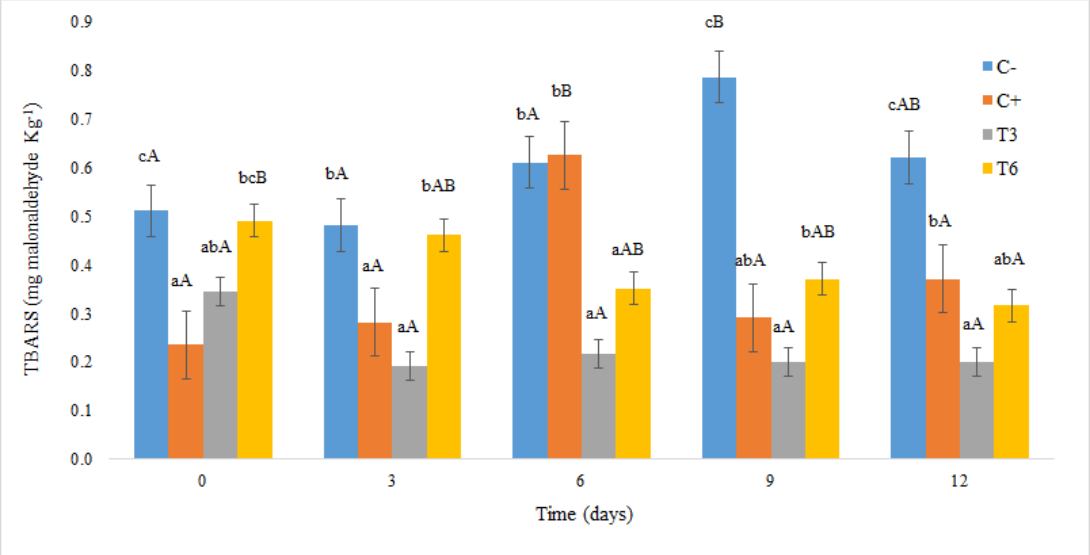

Figure 1. Mean ± standard error of thiobarbituric acid reactive substances (TBARS) of burgers with different black garlic extract concentrations during 12 days of refrigerated storage. C- = negative control; C+ = positive control; T3 = burger made with the addition of 3% black garlic extract; and T6 = burger made with the addition of 6% black garlic extract. Different lowercase letters among treatments, in similar storage period, differ significantly ($p < 0.05$) by Tukey's test. Different uppercase letters in similar treatment during storage differ significantly ($p < 0.05$) by Tukey's test.

Conclusion

In the present work, 20 phytochemical compounds were annotated in BGE, including amino acids, di- and tripeptides, a monoglucoside, organic acids, phenolics, acid esters, sulphur-containing compounds, and sphingolipids. Many of these compounds have recognised antioxidant activity. The addition of BGE to the burger formulations impacted the pH and colour parameters (a^* and b^*) of the products. The addition of 3 and 6% BGE markedly inhibited lipid oxidation. Therefore, BGE could be a promising ingredient with a considerable antioxidant potential to be used in meat products; it can meet the consumers' demand for natural products free of synthetic additives. However, additional studies are necessary to evaluate the antimicrobial and sensory effects of BGE in burgers.

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References

Afzaal, M., Saeed, F., Rasheed, R., Hussain, M., Aamir, M., Hussain, S., … and Anjum, F. M. 2021. Nutritional, biological, and therapeutic properties of black garlic: A critical review. International Journal of Food Properties 24(1): 1387-1402.

Association of Official Analytical Chemists (AOAC). 2005. Official methods of analysis of AOAC International. United States: AOAC.

Bagudo, B. U. and Acheme, O. D. 2014. Chemical analysis of locally cultivated garlic and its oil. Der Chemica Sinica 5(1): 128-134.

Botas, J., Fernandes, Â., Barros, L., Alves, M. J., Carvalho, A. M. and Ferreira, I. C. F. R. 2019. A comparative study of black and white *Allium sativum* L.: Nutritional composition and bioactive properties. Molecules 24(11): 2194-2205.

Buege, J. A. and Aust, S. D. 1978. Microsomal lipid peroxidation. Methods in Enzymology 52: 302-304.

Campagnol, P. C. B., Fries, L. L. M., Terra, N. N., Santos, B. A., Furtado, A. S., Toneto, E. R. L. and Campos, R. M. L. 2011. The influence of *achyrocline satureioides* ("Marcela") extract on the lipid oxidation of salami. Ciência e Tecnologia de Alimentos 31(1): 101-105.

Choi, I. S., Cha, H. S. and Lee, Y. S. 2014. Physicochemical and antioxidant properties of black garlic. Molecules 19(10): 16811-16823.

Downes, F. P. and Ito, K. 2001. Compendium of methods for the microbiological examination of foods. 4th ed. Washington: American Public Health Association.

Farag, M. A., Ali, S. E., Rodaya, R. H., El-Seedi, H. R., Sultani, H. N., Laub, A., … and Wessjohann, L. A. 2017. Phytochemical profiles and antimicrobial activities of *Allium cepa* Red cv. and *A. sativum* subjected to different drying methods: Comparative MS-based metabolomics. Molecules 22(5): 1-18.

Fernandes, R. P. P., Trindade, M. A., Lorenzo, P. E. S. and Melo, M. P. 2016. Effects of oregano extract on oxidative, microbiological and sensory stability of sheep burgers packed in a modified atmosphere. Food Control 63: 65-75.

Gao, L. W. and Wang, J. W. 2012. Antioxidant potential and DNA damage protecting activity of aqueous extract from *Armillaria mellea*. Journal of Food Biochemistry 36: 139-148.

Heck, R. T., Saldaña, E., Lorenzo, J. M., Correa, L. P., Fagundes, M. B., Cichoski, A. J., … and Campagnol, P. C. B. 2019. Hydrogelled emulsion from chia and linseed oils: A promising strategy to produce low-fat burgers with a healthier lipid profile. Meat Science 156: 174-182.

Huang, H., Tong, T. T., Yau, L. F., Chen, C. Y., Mi, J. N., Wang, J. R. and Jiang, Z. H. 2016. LC-MS based sphingolipidomic study on A2780 human ovarian cancer cell line and its taxol-resistant strain. Scientific Reports 6: 34684.

Jang, H. J., Lee, H. J., Yoon, D. K., Ji, D. S., Kim, J. H. and Lee, C. H. 2018. Antioxidant and antimicrobial activities of fresh garlic and aged garlic by-products extracted with different solvents. Food Science and Biotechnology 27(1): 219-225.

Jeong, Y. Y., Ryu, J. H., Shin, J. H., Kang, M. J., Kang, J. R., Han, J. and Kang, D. 2016. Comparison of antioxidant and anti-inflammatory effects between fresh and aged black garlic extracts. Molecules 21(4): 430.

Kim, J. H, Jang, H. J., Cho, W. Y., Yeon, S. J. and Lee, C. H. 2018. *In vitro* antioxidant actions of sulfur-containing amino acids. Arabian Journal of Chemistry 13(1): 1678-1684.

Kim, J. H., Nam, S. H., Rico, C. W. and Kang, M. Y. 2012. A comparative study on the antioxidative and anti-allergic activities of fresh and aged black garlic extracts. International Journal of Food Science and Technology 47: 1176-1182.

Kim, J., Jang, H. and Lee, C. 2019. Effect of aged garlic powder on physicochemical characteristics, texture profiles, and oxidative stability of ready-to-eat pork patties. Animal Bioscience 32(7): 1027-1035.

Kimura, S., Tung, Y. C., Pan, M. H., Su, N. W., Lai, Y. J. and Cheng, K. C. 2017. Black garlic: A critical review of its production, bioactivity, and application. Journal of Food and Drug Analysis 25(1): 62-70.

Krishnan, K. R., Babuskin, S., Babu, P. A. S., Fayidh, M. A., Sabina, K., Archana, G., … and Sukumar, M. 2014. Bio protection and preservation of raw beef meat using pungent aromatic plant substances. Journal of the Science Food and Agriculture 94(12): 2456.

Lishianawati, T. U., Yusiati, L. M. and Jamhari. 2022. Antioxidant effects of black garlic powder on spent duck meat nugget quality during storage. Food Science and Technology 42: 62220.

Liu, Q., Tang, G. Y., Zhao, C. N., Gan, R. Y. and Li, H. B. 2019. Antioxidant activities, phenolic profiles, and organic acid contents of fruit vinegars. Antioxidants 8(4): 1-12.

Manessis, G., Kalogianni, A. I., Lazou, T., Moschovas, M., Bossis, I. and Gelasakis, A. I. 2020. Plant-derived natural antioxidants in meat and meat products. Antioxidants 9(12): 1215.

Masek, A., Chrzescijanska, E. and Zaborski, M. 2014. Estimation of the antioxidative properties of amino acids - An electrochemical approach. International Journal of Electrochemical Science 9: 7904-7915.

Menegali, B. S., Selani, M. M., Saldaña, E., Patinho, I, Diniz, J. P., Melo, P. S., ... and Contreras-Castillo, C. J. 2019. Pink pepper extract as a natural antioxidant in chicken burger: Effects on oxidative stability and dynamic sensory profile using Temporal Dominance of Sensations. LWT - Food Science and Technology 121: 108986.

Molina-Calle, M., Medina, V. S., Calderón-Santiago, M., Priego-Capote, F. and Castro, M. D. L. 2017b. Untargeted analysis to monitor metabolic changes of garlic along heat treatment by LC–QTOF MS/MS. Electrophoresis 38(18): 2349-2360.

Molina-Calle, M., Medina, V. S., Priego-Capote, F. and Castro, M. D. L. 2017a. Establishing compositional differences between fresh and black garlic by a metabolomics approach based on LC-QTOF MS/MS analysis. Journal of Food Composition and Analysis 62: 155-163.

Najman, K., Sadowska, A. and Hallmann, E. 2021. Evaluation of bioactive and physicochemical properties of white and black garlic (*Allium sativum* L.) from conventional and organic cultivation. Applied Sciences 11(2): 874.

Pateiro, M., Gómez-Salazar, J. A., Jaime-Patlán, M., Sosa-Morales, M. E. and Lorenzo, J. M. 2021. Plant extracts obtained with green solvents as natural antioxidants in fresh meat products. Antioxidants 10(2): 181.

Qu, F., Zhang, H., Zhang, M. and Hu, P. 2018. Sphingolipidomic profiling of rat serum by UPLC-Q-TOF-MS: application to rheumatoid arthritis study. Molecules 23(6): 1-14.

Reddy, D. M., Reddy, G. V. B. and Mandal, P. K. 2018. Application of natural antioxidants in meat and meat products - A review. Food and Nutrition Journal 3: 173.

Rios-Mera, J. D., Villa, E. S., Bravo, M. L., Patinho, I., Selani, M. M., Valentin, D. and Contreras-Castillo, C. J. 2019. Reducing the sodium content without modifying the quality of beef burgers by adding micronized salt. Food Research International 121: 288-295.

Saldaña, E., Merlo, T, C., Patinho, I., Rios-Mera, J. D., Contreras-Castillo, C. J. and Selani, M. M. 2021. Use of sensory science for the development of healthier processed meat products: a critical opinion. Current Opinion in Food Science 40: 13-19.

Satyal, P., Craft, J. D., Dosoky, N. S. and Setzer, W. N. 2017. The chemical compositions of the volatile oils of garlic (*Allium sativum*) and wild garlic (*Allium vineale*). Foods 6(8): 63.

Setiyoningrum, F., Priadi, G., Afiati, F., Herlina, N. and Solikhin, A. 2021. Composition of spontaneous black garlic fermentation in a water bath. Food Science and Technology 41: 557-562.

Silva, F. L., Silva, T. D. S., Vargas, F. C., Franzolin, R. and Trindade, M. A. 2015. Scientific note: Physicochemical parameters and sensory acceptance of buffalo burgers as compared to beef burgers. Brazilian Journal of Food Technology 17(4): 340-344.

Silva, T. C., Justino, A. B., Prado, D. G., Koch, G. A., Martins, M. M., Santos, P. S., ... and Oliveira, A. 2019. Chemical composition, antioxidant activity and inhibitory capacity of α-amylase, α-glucosidase, lipase and non-enzymatic glycation, *in vitro*, of the leaves of *Cassia bakeriana* Craib. Industrial Crops and Products 140: 111641.

Singleton, V. L. and Rossi, J. A. 1965. Colorimetry of total phenolics with phosphomolybidic-phosphotungstic acid reagent. American Journal of Enology and Viticulture 16: 144-158.

Sinnhuber, R. O. and Yu, T. C. 1958. Thiobarbituric acid method for the measurement of rancidity in fishery products. II - The quantitative determination of malonaldehyde. Food Technology 12: 9-12.

Tienaho, J., Karonen, M., Muilu-Mäkelä, R., Wähälä, K., Denegri, L. E., Franzén, R., … and Sarjala, T. 2019. Metabolic profiling of water-soluble compounds from the extracts of dark septate endophytic fungi (DSE) isolated from scots pine (*Pinus sylvestris* L.) seedlings using UPLC-Orbitrap-MS. Molecules 24(12): 2330.

Wang, J., Lu, Y. M., Liu, B. Z. and He, H. Y. 2008. Electrospray positive ionization tandem mass spectrometry of Amadori compounds. Journal of Mass Spectrometry 43(2): 262-264.

Xu, N., Chen, G. and Liu, H. 2017. Antioxidative categorization of twenty amino acids based on experimental evaluation. Molecules 22(12): 2066.

Zhang, B., Xia, T., Duan, W., Zhang, Z., Li, Y., Fang, B., … and Wang, M. 2019. Effects of organic acids, amino acids and phenolic compounds on antioxidant characteristic of Zhenjiang aromatic Vinegar. Molecules 24(20): 3799.

Zou, T. B., He, T. P., Li, H. B., Tang, H. W. and Xia, E. Q. 2016. The structure-activity relationship of the antioxidant peptides from natural proteins. Molecules 21(1): 72.