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CORRESPONDENCE TO: Ms. Joanna Molga
Polish Journal of Food and Nutrition Sciences
Institute of Animal Reproduction and Food Research
of Polish Academy of Sciences
ul. Tuwima 10, 10-747 Olsztyn, Poland
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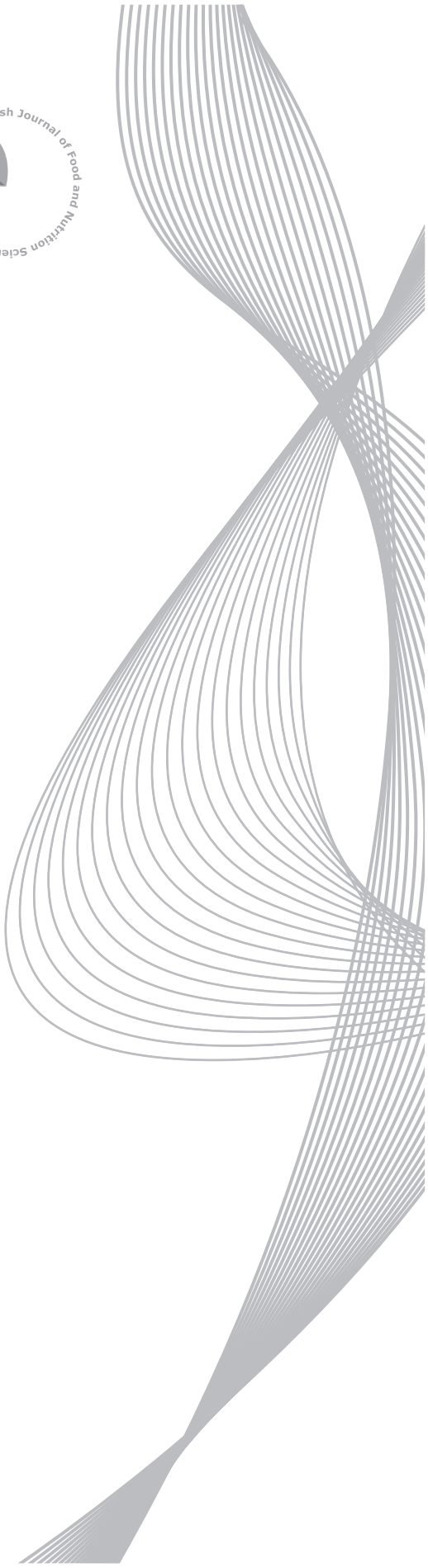

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2022, VOL. 72, NO. 2

ORIGINAL PAPERS

Control of Mould Spoilage on Apples Using Yeasts as Biological Control Agents.
Z. Gomomo, M. Fanadzo, M. Mewa-Ngongang, J.W. Hoff, M. van der Rijst, V.I. Okudoh, J. Kriel, H.W. du Plessis 119

Effect of Wheat and Barley Malt Addition on the Quality of the Baking Blend and Wheat Bread.
J. Belcar, J. Kaszuba, J. Gorzelany 129

Ultrasound-Assisted Extractions for Improving the Recovery of Phenolics and Charantin from Bitter Melon and for Increasing the Antioxidant, Antidiabetic and Anti-Obesity Activities of Its Extracts.
J.J. Lee, K.Y. Yoon 141

Soaking Soybean Seeds with *Abeliophyllum distichum* Nakai Extract Increased the Yield and Nutritional Value of Soybean Sprouts.
J.-H. Kim, Y.-H. Yoon, I.-D. Kim, S.K. Dhungana, D.-H. Shin 151

Characterization of Triterpene Saponin Composition of White, Yellow and Red Beetroot (*Beta vulgaris* L.).
A. Spórna-Kucab, A. Tekieli, K. Skalicka-Woźniak, A. Grzegorczyk, T. Świergosz, S. Wybraniec 159

Black Trumpet, *Craterellus cornucopioides* (L.) Pers.: Culinary Mushroom with Angiotensin Converting Enzyme Inhibitory and Cytotoxic Activity.
J. Radović, A. Leković, A. Tačić, M. Dodevska, T. Stanojković, T. Marinković, Č. Jelić, T. Kundaković-Vasović 171

Adsorption and Desorption Characteristics and Purification of Isoflavones from Crude Soybean Extract Using Macroporous Resins.
T.N.T. Tran, X.V. Bui, N.T.T. Loan, N.H.T. Anh, T.D. Le, T.M.H. Truong 183

Effect of Water Treatment and Immersion in Calcium Salt Solutions on the Quality of Fruits of Peumo Pink Tomato (*Solanum lycopersicum* L.) Stored under Cold Conditions.
J. Correa, A. Amorós, H. Silva, J.I. Covarrubias, V.H. Escalona 193

Instruction for Authors 203

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Original article
Section: Food Technology

Control of Mould Spoilage on Apples Using Yeasts as Biological Control Agents

*Zukisani Gomomo[1,2], Morris Fanadzo[2], Maxwell Mewa-Ngongang[1,3], JustinW. Hoff[1], Marieta van der Rijst[4], Vincent I. Okudoh[3], Johan Kriel[5], Heinrich W. du Plessis[1]**

[1]*Post-Harvest and Agro-Processing Technologies Division, ARC Infruitec-Nietvoorbij, South Africa*
[2]*Department of Agriculture, Cape Peninsula University of Technology, Wellington, South Africa*
[3]*Bioresource Engineering Research Group (BioERG), Department of Biotechnology, Cape Peninsula University of Technology, Cape Town, South Africa*
[4]*ARC Biometry, ARC Infruitec-Nietvoorbij, South Africa*
[5]*Crop Development Division, ARC Infruitec-Nietvoorbij, South Africa*

Key words: mould spoilage, fungicides, postharvest control, biocontrol agents (BCAs), apples, yeast

Considerable quantities of fruit are lost during pre- and post-harvest stages due to mould spoilage. The aim of this study was to evaluate the antagonistic effect of selected yeasts against spoilage mould *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alstroemeriae*. One hundred and four yeast isolates were screened for antagonistic activity against *B. cinerea*, *P. expansum* and *A. alstroemeriae* using radial inhibition, dual and mouth-to--mouth plate assays. Sixty-seven out of 104 yeasts showed growth inhibition activity against *P. expansum*, while 36 yeasts inhibited *B. cinerea*, 47 yeasts inhibited *A. alstroemeriae,* but only 22 yeasts displayed inhibition activity against all three moulds. *Candida pyralidae* Y63, *Meyerozyma guilliermondii* Y88 and *Zygoascus hellenicus* Y89 had the highest inhibition activity against all three moulds, when mode of inhibition was due to direct contact. Volatile organic compounds produced by *Pichia kluyveri* Y64, *C. pyralidae* Y63 and *M. guilliermondii* Y88 demonstrated the highest growth inhibition against all three moulds. These yeasts were also evaluated against all three moulds on apples. *P. kluyveri* Y64 displayed 100%, 57% and 26% growth inhibition against *A. alstroemeriae*, *B. cinerea* and *P. expansum*, respectively, on apples and performed slightly better than a commercial fungicide against *B. cinerea* and *P. expansum*. While *M. guillermondii* Y88 showed 100%, 60% and 18% inhibition on apples against *A. alstroemeriae*, *B. cinerea* and *P. expansum*, respectively. *P. kluyveri* Y64 and *M. guilliermondii* Y88 demonstrated potential as biofungicides and warrant further investigation.

INTRODUCTION

Fruits are commercially and nutritionally important commodities and play an important role in human health by supplying vitamins and minerals [Al-Hindi *et al*., 2011]. Globally, apple (*Malus domestica*) production increased from 75 to more than 85 million tonnes per annum from 2014 to 2020 [FAOstat, 2020]. South Africa is a relatively small apple grower in terms of global hectares and produces approximately 1.3 million tonnes each year, with a total value of more than 8 billion rands [Du Plessis, 2017]. Ninety-two percent of this income is generated by fresh fruit sales [Du Plessis, 2017]. Agricultural products are subject to mould spoilage before, during and after harvest, as well as during transportation and processing [Romanazzi *et al*., 2016; 2017]. Fruit losses due to spoilage mould pose several challenges to the agrifood industry [Parveen *et al*., 2016]. Each year, 25% of the total fruits produced is lost in industrialised countries and more than 50% in developing countries [Droby, 2005; Nunes, 2012]. During the pre- and post-harvest stages, considerable amounts of fruits including apples are lost due to mould diseases caused by *Botrytis cinerea* (grey mould), *Penicillium expansum* (blue mould), *Alternaria* spp. (necrotic leaf blotch), *Venturia inaequalis* (apple scab), *Cladosporium* spp. (Sooty spot) and *Colletotrichum gloeosporioides* (bitter rot) [Sharma *et al*., 2009].

Spoilage moulds need to be controlled to maintain the quality and abundance of fruit produced around the world [Mercier & Lindow, 2001]. Currently, fruit producers and exporters are using costly spraying programs incorporating synthetic chemicals, which are labour intensive and require the application of various classes of fungicides sprayed up to 20 times during the growing season. These practices, even at the lowest dose, can negatively affect the health of consumers,

* Corresponding Author:
Tel.: +27 21 809 3063;
E-mail: DPlessisHe@arc.agric.za (H.W. du Plessis)

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the environment, and the taste and aroma of the food being preserved [Benito *et al*., 2009; Contarino *et al*., 2019; Oliveira *et al*., 2014]. Some mould can become resistant to fungicides as farmers use the chemicals regularly [Fernández-Ortuño *et al*., 2008].

The desire to minimise chemical residues and to offset rising prices of new synthetic chemicals is fostering the search for alternatives to synthetic chemical fungicides [Quaglia *et al*., 2011; Robiglio *et al*., 2011]. The recent trend is shifting towards safer and environmentally friendly alternatives for the control of post-harvest decay [Sharma *et al*., 2009]. Biological control using yeasts demonstrated great potential as an alternative to chemical fungicides [Liu *et al*., 2013; Mewa-Ngongang *et al*., 2019a] and is more environmentally friendly and cost effective [Bonaterra et al., 2012]. In the last years, a considerable amount of microbial-based commercial products and patents have been developed worldwide in order to exploit the microbial strategies to counteract the growth of spoilage and/or pathogenic microorganisms in pre and post-harvest [De Simone *et al*., 2021].

Yeasts can be used as an alternative to synthetic chemicals because of their ability to compete for nutrients and space, their ability to grow faster than most fungal pathogens and the production of inhibitory growth compounds [Liu *et al*., 2013]. Yeasts, such as *Meyerozyma guilliermondii*, *Candida pyralidae* and *Hanseniaspora* species, have the ability to secrete extracellular metabolites, such as volatile organic compounds (VOCs), acetic acid, hydrogen sulphide and cell wall-degrading enzymes, which have antimicrobial properties against many fruit spoilage moulds [Al-Maawali *et al*., 2021; Cordero-Bueso *et al*., 2017; Grevesse *et al*., 2003; Han *et al*., 2021; Hua *et al*., 2014; Mewa-Ngongang *et al*., 2019b; Ruiz-Moyano *et al*., 2020; Zhou *et al*., 2018]. The aim of this study was to screen yeasts for growth inhibition activity against *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alstroemeriae* under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Culturing conditions and inoculum preparation

One hundred and four yeast isolates were obtained for evaluation from the ARC Infruitec-Nietvoorbij (the Fruit, Vine and Wine Institute of the Agricultural Research Council, Stellenbosch, South Africa), the Instituto Superior de Agronomia (Lisbon, Portugal), the Centraal Bureau voor Schimmelcultures (Utrecht, Netherlands), the Gulbenkian Institute of Science (Oeiras, Portugal) and the Council for Scientific and Industrial Research (Pretoria, South Africa) (Table S1 in the Supplementary Materials). Yeast selection was based on previous research and the fact that the yeasts were isolated from different fruits and environments. The yeasts were cultured on yeast malt agar (YMA) (1% glucose, 0.3% malt extract, 0.5% peptone, 2% bacteriological agar) for 2 days at 28°C. A wire loopful of each pure yeast colony on the plates was transferred into test tubes containing 5 mL of sterilised yeast malt broth (YMB) (Sigma-Aldrich, Saint Louis, USA) and incubated at 28°C for 2 days. Thereafter 1 mL of the culture was transferred to a sterile 2 mL microtube and centrifuged at 20,400×*g* for 5 min. The supernatant was discarded, and the pellet resuspended in 100 μL of sterile distilled water. Yeast cells were counted using a Neubauer haemocytometer (Sigma-Aldrich, Darmstadt, Germany) and a microscope (Euromex, Arnhem, Netherlands), at 400× magnification, in order to prepare the yeast inoculum (1×10^8 cells/mL).

For the apple bioassay, grape pomace extract was obtained by pressing "Chenin Blanc" grape pomace from the ARC Infruitec-Nietvoorbij research farm (Stellenbosch) at 200 kPa. The resultant grape pomace extract was frozen in a 25 L polypropylene bucket at −20°C. Prior to use, the grape pomace extract was thawed and diluted with sterile distilled water to a sugar concentration of 100 g/L. Yeast strains Y63 (*C. pyralidae*), Y88 (*M. guilliermondii*) and Y64 (*P. kluyveri*) from the ARC Infruitec-Nietvoorbij culture collection, were grown in 5 mL of YMB for 2 days at 28°C and then transferred to Erlenmeyer flasks containing 50 mL of sterile grape pomace extract broth (GPB), incubated at 28°C and agitated at 150 rpm, using a rotary shaker (LM-53OR, RKC Instrument Inc., Ohta-ku Tokyo, Japan) for 2 days. The yeast cultures were then transferred to 500 mL of GPB and grown at 28°C for 24 h under agitation. The yeast inoculum of 1×10^8 cells/mL was used, as mentioned previously.

The fruit spoilage moulds, *B. cinerea* FFD 003–15, *P. expansum* C370V59 and *A. alstroemeriae* C370V51, were obtained from the fungal genebank and Post-harvest Pathology laboratory at ARC Infruitec-Nietvoorbij and cultured for 7 to 14 days at 25°C on potato dextrose agar (PDA, Merck, Johannesburg, South Africa). Spores were harvested by gently scraping them from the surface of the agar and rinsing with sterile distilled water to attain a volume of 50 mL of the spore suspension in a sterile 250 mL Schott bottle. Prior to the dilution with sterile distilled water, a haemocytometer and a microscope (400× magnification) were used to count the spores in the initial spore solution, as previously described. The inoculum was prepared by diluting the spore suspension to 1×10^5 spores/mL.

Radial growth inhibition assay

The radial growth inhibition assay was applied as described by Núñez *et al*. [2015], with some modifications. In brief, a 5 mm mycelial disk, obtained from a 7-day old mould culture, was placed at the centre of a fresh YMA plate using a sterile cork borer. Subsequently, 15 μL of the yeast cells suspension (1×10^8 cells/mL) was spotted 25 mm away from the mycelial disk. Four different yeast isolates were spotted per plate and incubated at 25°C for 7 days. All yeast treatments had three replicates. The control plates only contained the 5 mm diameter mycelial disk of the respective mould. Results were recorded as (-) no activity, (+) mild activity, (++) medium activity, (+++) strong activity. Positive (+) growth inhibition results were observed by the presence of C-shaped growth around the yeast colonies, as shown in Figure 1. Yeasts with strong inhibition activity (+++) had the biggest C-shaped inhibtion zone and the distance between the yeast colony and the mould growth exceeded 10 mm. Yeasts with medium inhibtion activity (++) had a smaller C-shaped zone and the distance between the yeast colony and the mould growth was between 6 and 9 mm. For yeasts with mild

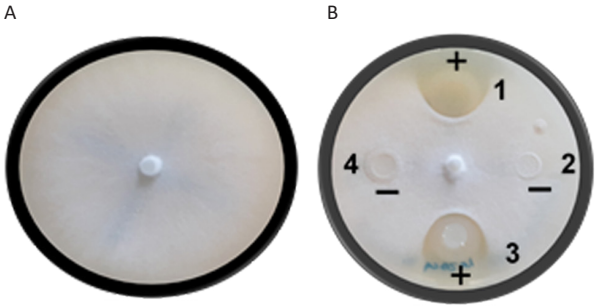

FIGURE 1. An example of the radial growth inhibition assay results. *Botrytis cinerea* growth (A) and antagonistic effect of selected yeast isolates *Candida pyralidae* Y63 (1), *Pichia kluyveri* Y64 (2), *Meyerozyma guilliermondii* Y88 (3) and *Debaryomyces hansenii* Y8 (4) against *B. cinerea* (B) on yeast malt agar. The positive sign (+) represents growth inhibition activity and the negative sign (-) represents no inhibition. This is a representative example of three replicates.

activity (+), the distance between the yeast and mould colony was less than 6 mm and no activity (-) meant that no mould growth inhibition was observed.

Diffusible metabolites assay

The dual assay described by Chen *et al*. [2018] was used to evaluate 23 yeasts (Table 1). Only those yeasts that showed growth inhibition activity against all three mould species during the radial growth inhibition assay were evaluated further. The yeast Y64 (*Pichia kluyveri*) was used in a previous study by Mewa-Ngongang *et al.* [2019a,b] and was included as the reference strain. Similar to the radial growth inhibition assay, a 5 mm mycelial disk was placed at the edge of the YMA plate. Subsequently, 20 μL of the yeast suspension (1×10^8 cells/mL) was spotted 40 mm away from the mycelial disk (Figure S1 in the Supplementary Materials) and incubated at 25°C for 9 days. The negative control (C) plates contained only the 5 mm diameter mycelial disk of the respective mould. All treatments had three replicates. The percentage inhibition was calculated as fungal radial growth inhibition (FRGI) using the following mathematical expression:

$$FRGI = (D_0 - \frac{D_t}{D_0}) \times 100 \quad (1)$$

with D_0 representing the horizontal growth average of the fungal colony on the negative control plates and D_t representing the horizontal growth average of the fungal colony on the yeast treated plates (Figure S1 in the Supplementary Materials), as described by Núñez *et al.* [2015].

Volatile organic compound (VOCs) assay

To assess the effect of VOCs produced by the 23 yeasts used in relation to their growth inhibition potential against fruit spoilage organisms, the mouth-to-mouth assay described by Medina-Córdova *et al*. [2016] was used. Two YMA plates facing each other were sealed with laboratory film, per experimental repeat. The bottom plate was spread with 100 μL of the yeast suspension (1×10^8 cell/mL), while the top plate contained a 5 mm mould mycelial disk placed at the centre. The negative control treatment (C) only contained the 5 mm diameter mycelial disk in the centre of the plate, while no yeast was spread on the second plate. The plates were incubated at 25°C for 7 days. All treatments had three replicates. The VOC inhibition activity (VOCIA) was calculated using the following mathematical expression [Núñez *et al*., 2015]:

$$VOCIA = (D_0 - \frac{D_t}{D_0}) \times 100 \quad (2)$$

with D_0 representing the average diameter of the fungal colony on the negative control plates and D_t representing the diameter of the fungal colony on the yeast treated plates, as shown in Figure S2 in the Supplementary Materials.

TABLE 1. Yeasts selected for the dual and mouth-to-mouth assays on yeast malt agar.

Yeast code	Species
Y6	*Aureobasidium melanogenum*
Y11	*Debaryomyces hansenii*
Y17	*Hanseniaspora occidentalis*
Y24	*Meyerozyma guilliermondii*
Y35	*Rhodotorula dairenensis*
Y39	*Meyerozyma guilliermondii*
Y63	*Candida pyralidae*
Y64	*Pichia kluyveri**
Y65	*Meyerozyma guilliermondii*
Y74	*Torulaspora delbrueckii*
Y75	*Saccharomyces cerevisiae*
Y83	*Brettanomyces lambicus*
Y84	*Debaryomyces hansenii*
Y88	*Meyerozyma guilliermondii*
Y89	*Zygoascus hellenicus*
Y91	*Zygosaccharomyces rouxii*
Y92	*Zygosaccharomyces rouxii*
Y93	*Zygosaccharomyces microellipsoides*
Y95	*Zygosaccharomyces florentinus*
Y96	*Zygosaccharomyces fermentati*
Y97	*Zygosaccharomyces bisporus*
Y102	*Candida magnolia*
Y103	*Saccharomyces cerevisiae*

*Used as reference yeast.

Post-harvest application using apple bioassay

The post-harvest biocontrol efficacy assay was performed on the apple cultivar "Panorama Goldens" and sixteen treatments were applied (Table 2). Each treatment had five replicates. Each replicate consisted of a rectangular fruit-packaging box containing five apples. Ethanol (70%, *v/v*) was sprayed on the apples to eradicate any microorganisms on the surface and allowed to dry completely before wound infliction.

TABLE 2. Treatments applied on apples during postharvest biocontrol trials*.

Treatment	Treatment description
Treatment 1	Sterile distilled water (Control)
Treatment 2	*Botrytis cinerea*
Treatment 3	*Penicillium expansum*
Treatment 4	*Alternaria alstroemeriae*
Treatment 5	*B. cinerea* and *Candida pyralidae* Y63
Treatment 6	*P. expansum* and *C. pyralidae* Y63
Treatment 7	*A. alstroemeriae* and *C. pyralidae* Y63
Treatment 8	*B. cinerea* and *Meyerozyma guilliermondii* Y88
Treatment 9	*P. expansum* and *M. guilliermondii* Y88
Treatment 10	*A. alstroemeriae* and *M. guilliermondii* Y88
Treatment 11	*B. cinerea* and *Pichia kluyveri* Y64
Treatment 12	*P. expansum* and *P. kluyveri* Y64
Treatment 13	*A. alstroemeriae* and *P. kluyveri* Y64
Treatment 14	*B. cinerea* and Captan
Treatment 15	*P. expansum* and Captan
Treatment 16	*A. alstroemeriae* and Captan

*Apples were incubated in rectangular fruit packaging boxes and five boxes (replicates) were used per treatment, with each box containing five apples.

Apples were uniformly wounded (approximately 5 mm diameter and 3 mm deep), with a sterile cork borer. After 15 min, 15 μL of sterile purified water was inoculated into the wound of the blank treatment, while the other treatments received 15 μL of the respective mould spore suspension (1×10^5 cells/mL) and then allowed to dry for 30 min. Subsequently, 15 μL of a yeast inoculum (1×10^8 cells/mL) or 15 μL of the commercial fungicide, *N*-trichloromethylthio-4-cyclohexene-1,2--dicarboximide, common name Captan (800 g/kg; Universal Crop Protection (Pty) Ltd, Kempton Park, South Africa) at a concentration of 0.5 g/L, was introduced into the wound. The negative control treatments were only infected with *B. cinerea*, *P. expansum* or *A. alstroemeriae* and not treated with yeast or the commercial fungicide. Treated apples were incubated at ±20°C for 7–20 days at a relative humidity of 80%. Growth inhibition results were characterised by the absence of mould development. Lesion diameters were measured, and percentage growth inhibition was calculated and analysed statistically to determine the effectiveness of the treatments.

Yeast identification

Twenty-four isolates that showed growth inhibition activity were identified during this study (Table 3). Yeast DNA was extracted using the method described by Lõoke *et al*. [2011]. Yeast identification to species level was performed by amplification of the 5.8S-internal transcriber spacer (ITS) ribosomal region, using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) [Mitchell *et al*., 1994]. PCR reaction mixture (50 μL)

TABLE 3. Species identity and growth inhibition activity* of the yeasts screened against selected mould on yeast malt agar.

Yeast codes	Species	% identity**	*Penicillium expansum*	*Botrytis cinerea*	*Alternaria alstroemeriae*
Y1	*Rhodotorula dairenensis*	***	+	–	++
Y2	*Hanseniaspora uvarum*	***	+	–	–
Y3	*Hanseniaspora uvarum*	91	–	+++	–
Y5	*Saccharomyces uvarum*	***	+	–	+
Y6	*Aureobasidium melanogenum*	99	++	+++	++
Y7	*Aureobasidium melanogenum*	***	–	++	+++
Y8	*Debaryomyces hansenii*	***	+	–	–
Y10	*Saccharomyces uvarum*	***	+	–	+
Y11	*Debaryomyces hansenii*	***	+	++	++
Y12	*Rhodotorula dairenensis*	***	++	–	++
Y13	*Hanseniaspora opuntiae*	99	–	++	–
Y14	*Saccharomyces uvarum*	97	+	–	+++
Y15	*Hanseniaspora uvarum*	***	–	–	+
Y16	*Hanseniaspora uvarum*	***	+	–	–
Y17	*Hanseniaspora occidentalis*	89	++	+++	+++
Y18	*Debaryomyces hansenii*	98	–	+	+
Y19	*Hanseniaspora uvarum*	98	+	–	+
Y20	*Hanseniaspora uvarum*	99	+++	–	–
Y21	*Debaryomyces hansenii*	***	+++	–	–
Y24	*Meyerozyma guilliermondii*	***	++	++	+++
Y25	*Hanseniaspora uvarum*	93	+++	++	–
Y26	*Hanseniaspora uvarum*	***	+	–	–
Y27	*Hanseniaspora uvarum*	***	–	–	+
Y30	*Candida oleophila*	***	+	–	+
Y31	*Candida oleophila*	92	+	+	–
Y32	*Candida oleophila*	***	+	–	–
Y34	*Candida oleophila*	99	+	–	–
Y35	*Rhodotorula dairenensis*	99	++	+++	++
Y36	*Candida oleophila*	***	++	–	–
Y37	*Candida oleophila*	99	++	–	–
Y38	*Hanseniaspora uvarum*	99	+	–	+

TABLE 3 continued.

Yeast codes	Species	% identity**	*Penicillium expansum*	*Botrytis cinerea*	*Alternaria alstroemeriae*
Y39	*Meyerozyma guilliermondii*	90	+	+++	+++
Y43	*Hanseniaspora guilliermondii*	99	+	–	–
Y45	*Zygosaccharomyces bailii*	***	+	–	–
Y47	*Hanseniaspora opuntiae*	99	++	–	–
Y50	*Candida stellimalicola*	97	+	–	+++
Y51	*Pichia kudriavzevii*	100	++	–	–
Y53	*Rhodotorula dairenensis*	***	–	–	+++
Y54	*Hanseniaspora guilliermondii*	***	+	–	–
Y55	*Pichia kudriavzerii*	94	+	–	–
Y56	*Pichia fermentans*	***	–	–	+
Y57	*Hanseniaspora valbyensis*	***	–	+	+
Y58	*Saccharomyces cariocanus*	***	+	–	–
Y61	*Dekkera anomala*	***	+	–	+
Y62	*Dekkera anomala*	***	–	+	–
Y63	*Candida pyralidae*	***	+++	+++	+++
Y65	*Meyerozyma guilliermondii*	98	+	+++	++
Y67	*Brettanomyces lambicus*	***	+	+	–
Y69	*Zygosaccharomyces bailii*	***	–	–	++
Y70	*Lanchancea thermotolerans*	***	–	–	+
Y71	*Torulaspora delbrueckii*	***	+	–	–
Y72	*Metschnikowia pulcherrima*	***	–	+	–
Y73	*Lanchancea thermotolerans*	***	+	–	–
Y74	*Torulaspora delbrueckii*	93	++	+++	++
Y75	*Saccharomyces cerevisiae*	***	+	+	++
Y76	*Zygosaccharomyces bailii*	***	+	–	–
Y78	*Meyerozyma guilliermondii*	98	+	–	–
Y79	*Pichia kluyveri*	***	+	–	–
Y80	*Zygoascus hellenicus*	***	+++	–	–
Y81	*Meyerozyma guilliermondii*	***	+	–	+
Y82	*Meyerozyma guilliermondii*	***	+	–	+
Y83	*Brettanomyces lambicus*	***	+	+++	+++

TABLE 3 continued.

Yeast codes	Species	% identity**	*Penicillium expansum*	*Botrytis cinerea*	*Alternaria alstroemeriae*
Y84	*Debaryomyces hansenii*	***	++	+++	++
Y85	*Pichia kluyveri*	98	+	–	+
Y87	*Meyerozyma guilliermondii*	***	+	–	–
Y88	*Meyerozyma guilliermondii*	***	+++	+++	+++
Y89	*Zygoascus helenicus*	***	+++	+++	+++
Y90	*Zygosaccharomyces bailii*	***	+	–	++
Y91	*Zygosaccharomyces rouxii*	***	+	+++	++
Y92	*Zygosaccharomyces rouxii*	***	++	+	++
Y93	*Zygosaccharomyces microellipsoides*	***	+	+++	++
Y94	*Zygosaccharomyces cidri*	***	+	–	–
Y95	*Zygosaccharomyces florentinus*	***	+	+++	++
Y96	*Zygosaccharomyces fermentati*	***	+	++	++
Y97	*Zygosaccharomyces bisporus*	***	+	+	++
Y98	*Zygosaccharomyces bisporus*	***	++	+	–
Y99	*Brettanomyces bruxellensis*	***	–	+	–
Y100	*Brettanomyces bruxellensis*	***	–	+	–
Y101	*Brettanomyces lambicus*	***	–	+	+
Y102	*Candida magnoliae*	***	+	+	++
Y103	*Saccharomyces cerevisiae*	95	+	++	++
Y104	*Saccharomyces cerevisiae*	***	+	–	+
Y105	*Meyerozyma guilliermondii*	***	+	–	–

*(-) no growth inhibition, (+) mild activity: inhibition zone less than 6 mm, (++) medium activity: inhibition zone 6 to 9 mm, (+++) strong activity: inhibition zone exceeded 10 mm.
**Percentage similarity compared to sequences on the NCBI database using the standard nucleotide homology search Basic Local Alignment Search Tool.
***Not identified during this study, previously identified yeasts.

contained 5 μL of SuperTherm Taq buffer, 0.2 μL of SuperTherm Taq polymerase (Separation Scientific SA (Pty) Ltd, Johannesburg, South Africa), 1.5 μL of 25 mM $MgCl_2$, 1 μL of 2.5 mM deoxynucleotide (dNTP) solution, 3 μL of each primer (2.5 mM), 0.5 μL of bovine serum albumin (BSA), 5 μL of template DNA (100 ng/μL) and 30.8 μL of sterile dH_2O. The PCR conditions used were: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were submitted to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for Sanger sequencing. The sequenced fragments were then compared to sequences on the NCBI database using the standard nucleotide homology search Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nih.gov/BLAST).

Statistical analyses

Growth inhibition data were subjected to the analysis of variance (ANOVA) using XLSTAT software (Version 18.07.39157, Addinsoft, New York, NY, USA) and the general linear model (GLM) procedure of SAS software (version 9.4, SAS Institute Inc, Cary, NC, USA). Fisher's least significant difference (LSD) values were calculated at the 5% probability level (p=0.05) to facilitate comparison between treatment means.

RESULTS AND DISCUSSION

Radial growth inhibition assay and yeast identification

Out of the 104 yeasts tested, 83 showed growth inhibition activity against the selected mould species. Sixty-seven yeasts showed growth inhibition activity against *P. expansum*, 47 against *A. alstroemeriae,* 36 against *B. cinerea*, and 22 yeasts showed inhibition activity against all three moulds (Table 3). Most of the yeasts that showed growth inhibition activity against at least one mould species belonged to the genus *Hanseniaspora*, with *Hanseniaspora uvarum* being the predominant species. However, of the 22 yeasts that showed activity against all three mould species, most of the isolates belonged to the genus *Zygosaccharomyces* (Table 1). Only 24 of the 83 isolates that showed growth inhibition activity were identified during this study (Table 3). The identities of the other 59 isolates that showed growth inhibition activity were already known.

Yeasts can inhibit the growth of mould in different ways, such as the ability to grow faster than the spoilage mould by rapidly colonising surfaces, competition for nutrients or by production of growth inhibition compounds [Banjara *et al*., 2016; Liu *et al*., 2013; Mewa-Ngongang *et al*., 2019b].

Diffusible metabolites assay

The 22 selected yeast strains and the reference strain (Y64) showed varying levels of antagonistic effects against *B. cinerea, P. expansum* and *A. alstroemeriae* (Figure 2). In general, the selected yeasts showed the highest inhibition activity against *B. cinerea* (39% mean inhibition) and lower activity against *A. alstroemeriae* (31% mean inhibition) and *P. expansum* (17% mean inhibition).

Y88 (*Meyerozyma guilliermondii*), Y63 (*Candida pyralidae*) and Y89 (*Zygoascus hellenicus*) exhibited the highest growth inhibition activity against *B. cinerea*, with 63%, 62% and 58%, respectively (Figure 2a). Y88, Y63 and Y89 showed significantly higher inhibition activity than the other 20 yeast treatments. Yeast Y64 (*Pichia kluyveri*), which was selected as the reference yeast, showed low inhibition activity (3%) against *B. cinerea*. Yeasts Y88, Y63 and Y89 also exhibited the highest growth inhibition activity against *P. expansum*, with 42%, 38% and 35%, respectively, and were significantly better than the other yeast treatments (Figure 2b). The reference yeast strain (Y64) showed 2% inhibition against *P. expansum*. The same three yeasts (Y88, Y63 and Y89) also exhibited the highest inhibition activity against *A. alstroemeriae*, with 41%, 37% and 35%, respectively (Figure 2c). The reference yeast (Y64) showed 7% inhibition activity against *A. alstroemeriae*. *Meyerozyma guilliermondii* strain Y88 had the highest inhibition activity against all three

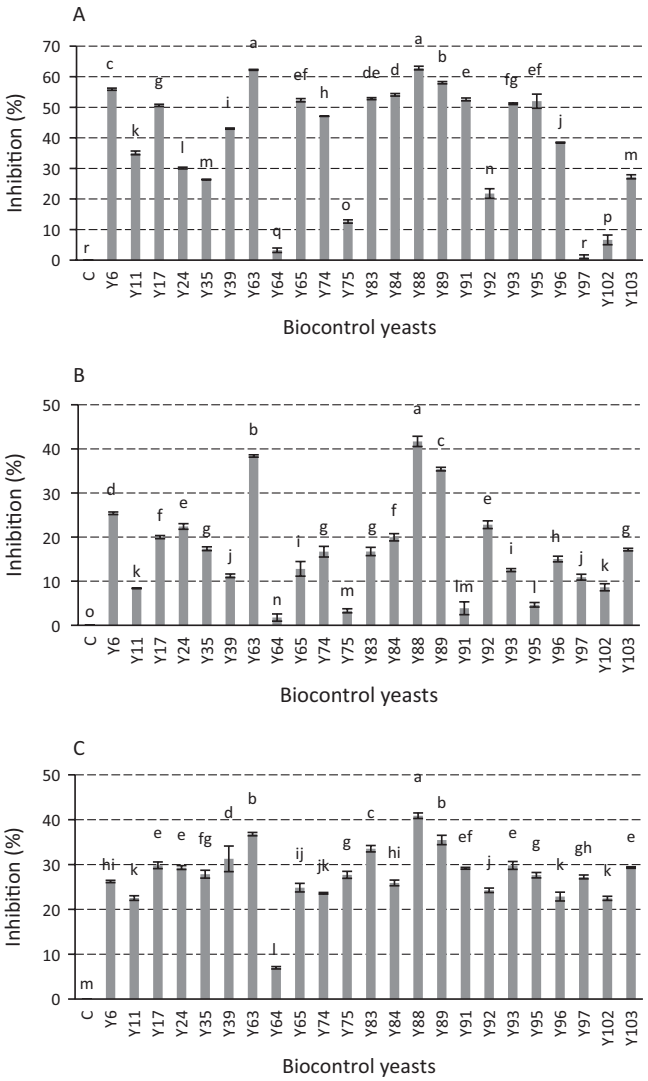

FIGURE 2. Growth inhibition activity expressed as a percentage (%) of 23 yeasts against *Botrytis cinerea* (A), *Penicillium expansum* (B) and *Alternaria alstroemeriae* (C) based on the diffusible metabolites assay results. Values are means of three replicates and the standard deviations are also shown. The different letters indicate significant differences (p<0.05) between treatments. The plates of negative control treatments (C) only contained the respective mould species and served as the reference treatment to determine growth inhibition.

mould species. This is the first report of growth inhibition activity of the *M. guilliermondii* species against *A. alstroemeriae.* Al-Rahbi *et al*. [2021] and Al-Maawali *et al*. [2021] reported that *M. guilliermondii* had an antagonistic effect against *Alternaria alternata* under *in vitro* conditions. In turn, Wang *et al*. [2018] reported that *M. guilliermondii* exerted antagonistic effects against two strains of *B. cinerea*, while inhibition of *P. expansum* growth by *M. guilliermondii* was reported by Han *et al.* [2021].

Candida pyralidae Y63 was the second-best performing yeast against all three moulds. This is in agreement with the findings of Mewa-Ngongang *et al*. [2019b], who reported the antagonistic effects of *C. pyralidae* against the germination of *B. cinerea* spores under *in vitro* conditions. This is the first report of the growth inhibition properties of *C. pyralidae* against *P. expansum* and *A. alstroemeriae,* and of the growth inhibition properties of *Z. hellenicus* against *B. cinerea, P. expansum* and *A. alstroemeriae.* Nally *et al.* [2012] and Mewa-Ngongang *et al*. [2019b] also reported that different yeast species showed growth inhibition activity at different levels against fruit spoilage mould, which is in agreement with the findings of the current study.

Volatile organic compound assay

The production of VOCs as a mode of action against mould was investigated using the mouth-to-mouth assay. Most of the 23 yeasts produced VOCs that inhibited the growth of *B. cinerea* (Figure 3a), *P. expansum* (Figure 3b) and *A. alstroemeriae* (Figure 3c), but the level of growth inhibition varied among the yeasts. Yeast isolates Y64 (*P. kluyveri*), Y63 (*C. pyralidae*), Y24 (*M. guilliermondii*) and Y92 (*Zygosaccharomyces rouxii*) showed 91%, 57%, 56% and 50% growth inhibition activity against *B. cinerea*, respectively (Figure 3a). Against *P. expansum,* the highest growth inhibition was shown by *P. kluyveri* Y64, *M. guilliermondii* Y88 and Y65, with 81%, 70% and 69%, respectively (Figure 3b). The best performing yeasts against *A. alstroemeriae* were *P. kluyveri* Y64, *C. pyralidae* Y63 and *M. guilliermondii* Y88, with 76%, 68% and 61% growth inhibition activity, respectively (Figure 3c).

Yeast isolate Y64 (*P. kluyveri*) showed the highest growth inhibition activity against all three moulds and was significantly better than the other yeast treatments during the VOC trial (Figure 3). While the opposite was observed during the diffusible metabolite assay (Figure 2). This strongly suggests that the mode of action of Y64 is linked to its ability to produce VOCs. The findings of this study are in agreement with Mewa-Ngongang *et al*. [2019b], who also reported on the ability of *P. kluyveri* and *C. pyralidae* to inhibit the growth of *B. cinerea* under *in vitro* conditions. Ruiz-Moyano *et al*. [2020] reported that *H. uvarum* also produced VOCs to control the growth of *B. cinerea* on fruits. Choińska *et al*. [2020] observed that *M. guilliermondii* produced VOCs to control the growth of *B. cinerea* and *P. expansum*, which is in agreement with the findings from this study. *Pichia kluyveri* showed the highest inhibition against *P. expansum*. Cordero-Bueso *et al*. [2017] also reported that VOCs produced by *P. kluyveri* exhibited antagonistic activity against *P. expansum.* This is the first report of VOCs from *P. kluyveri*, *C. pyralidae* and *M. guilliermondii* inhibiting the growth of *A. alstroemeriae.* However, Al-Maawali *et al*. [2021] showed that VOCs produced by *M. guilliermondii* inhibited the mycelial growth of *A. alternata.*

Post-harvest application of biocontrol yeasts on apples

The yeasts were effective in preventing mould spoilage of apples and reducing decay considerably (Figure 4). The inhibition responses were yeast and mould species-dependent. *Meyerozyma guilliermondii* Y88 and *P. kluyveri* Y64 were effective in suppressing mould growth on apples, with 100% inhibition activity against *A. alstroemeriae.* The commercial fungicide (Captan) also provided 100% inhibition. *Candida pyralidae* Y63 showed 36% inhibition against *A. alstroemeriae,* which was significantly lower compared to the other treatments. This is the first report on growth inhibition activity of *P. kluyveri*, *C. pyralidae* and *M. guillermondii* against *A. alstroemeriae* on apples. However, Al-Rahbi *et al*. [2021]

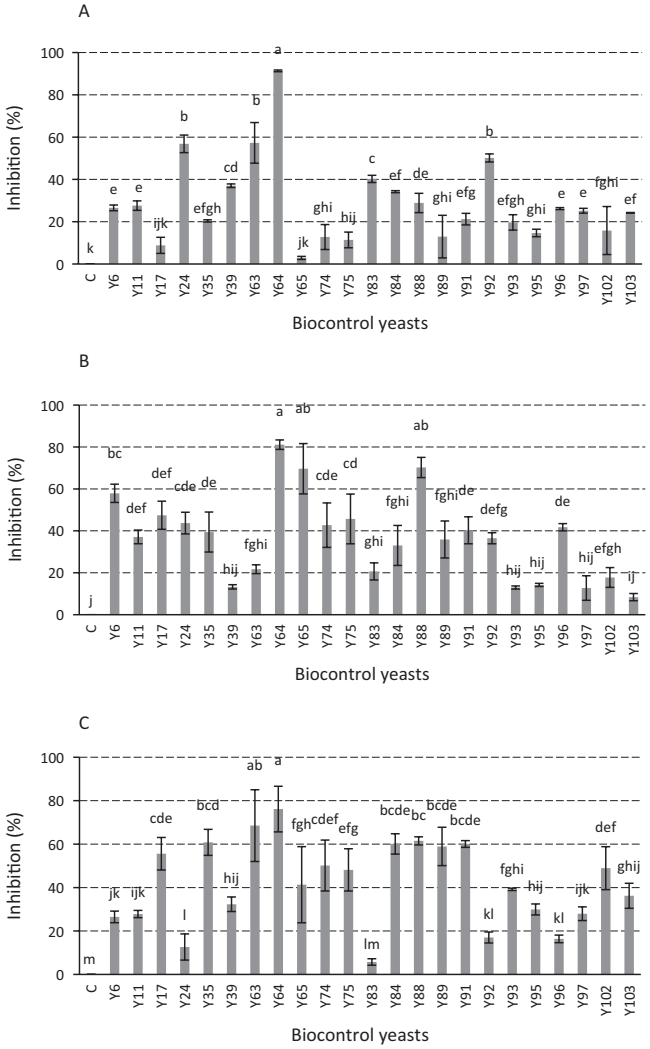

FIGURE 3. The growth inhibition activity expressed as a percentage (%) of 23 yeasts against *Botrytis cinerea* (A), *Penicillium expansum* (B) and *Alternaria alstroemeriae* (C) based on the volatile organic compound production. Values are means of three replicates and the standard deviations are also shown. The different letters indicate significant differences ($p<0.05$). The negative control treatments (C) only contained the respective mould species and served as the reference treatment to determine growth inhibition.

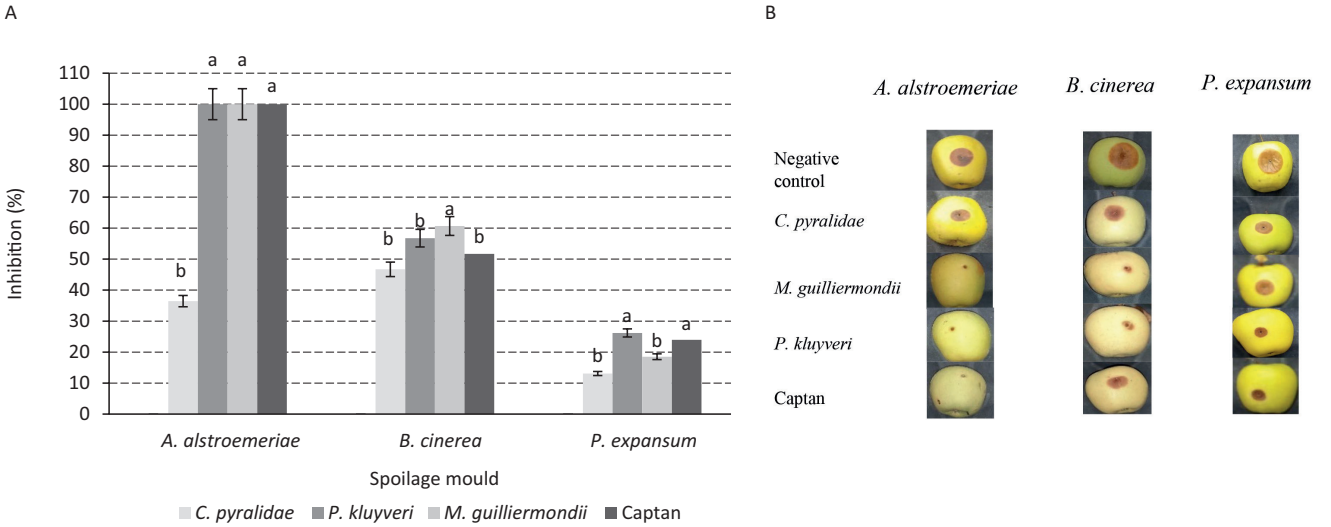

FIGURE 4. The growth inhibition activity expressed as a percentage (%) of *Candida pyralidae* Y63, *Meyerozyma guilliermondii* Y88 and *Pichia kluyveri* Y64 against *Alternaria alstroemeriae*, *Botrytis cinerea* and *Penicillium expansum* during postharvest trials on apples (A). Values are means of five replicates and the standard deviations are also shown. The different letters indicate significant differences ($p<0.05$) between treatments. (B) Photographs of apples showing lesion diameters. Each set is a representative example of 25 apples. For the negative control treatments, the apples were only infected with the respective moulds, therefore no growth inhibition.

and Al-Maawali *et al*. [2021] showed that *M. guilliermondii* reduced the fruit rot lesions of *A. alternata* on strawberries and tomatoes by 68% and 50%, respectively.

Against *B. cinerea*, *M. guilliermondii* Y88 and *P. kluyveri* Y64 showed 61% and 57% inhibition, respectively, which was higher than the 52% obtained by the commercial fungicide (Figure 4a). *Candida pyralidae* Y63 inhibited *B. cinerea* growth by 47%, which was significantly lower than the other treatments. These findings are in agreement with those of Mewa-Ngongang *et al*. [2019b] who also reported on the antagonistic effects of *C. pyralidae* against *B. cinerea* on apples. Wang *et al*. [2018] reported that *M. guilliermondii* showed an antagonistic effect against *B. cinerea* isolates on grape berries, while Mewa-Ngongang *et al*. [2021], showed that *P. kluyveri* when applied preventively, was effective in suppressing *B. cinerea* growth by 95% on apples.

All the yeasts showed the lowest growth inhibition activity against *P. expansum* (Figure 4a). *Pichia kluyveri* Y64 exhibited the highest growth inhibition activity (26%) against *P. expansum* and performed slightly better than the commercial fungicide, which ensured 24% inhibition. The commercial fungicide displayed lower activity against *B. cinerea* and *P. expansum* than expected, which could be possibly attributed to the resistance of the specific moulds. Follow up studies should include more than one fungicide. *Meyerozyma guilliermondii* Y88 and *C. pyralidae* Y63 inhibited the growth of *P. expansum* by 19% and 13%, respectively. This study confirmed the findings of Cordero-Bueso *et al*. [2017], who reported that *P. kluyveri* exhibited antagonistic activity against *P. expansum*. In turn, Han *et al*. [2021] demonstrated that *M. guilliermondii* exhibited antagonistic activity against *P. expansum* on pears. This is the first report on the growth inhibition properties of *C. pyralidae* against *P. expansum* on apples. These observations on apples could be of great importance to the agricultural industry because these biocontrol yeasts can potentially be used as alternatives to chemical fungicides.

CONCLUSIONS

The cell suspensions of yeast strains *C. pyralidae* Y63, *M. guillermondii* Y88 and *Z. hellenicus* Y89 elicited the best antagonistic effects against *B. cinerea*, *P. expansum* and *A. alstroemeriae.* The production of VOCs by *P. kluyveri* was the mechanism of inhibition against *B. cinerea*, *P. expansum* and *A. alstroemeriae. Candida pyralidae* Y63, *M. guillermondii* Y88 and *P. kluyveri* Y64 were effective inhibitors of all three mould species on apples and their efficacy was comparable to the commercial fungicide. These yeasts can potentially be considered as alternatives to chemical fungicides. However, further research is needed to determine how to apply these yeast-based biocontrol agents and to establish the most effective minimum dosage or inhibitory concentration needed. The main VOCs and other possible compounds that are responsible for inhibition should be identified and the production process needs to be optimised. Future research should also investigate other mechanisms of action and the application of yeast-based biological agents on fruit for pre-harvest control of mould.

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CONFLICTS OF INTEREST

The authors hereby declare that they have no conflict of interest.

ORCID IDs

H.W. du Plessis https://orcid.org/0000-0001-8092-6492
M. Fanadzo https://orcid.org/0000-0001-7777-7078
Z. Gomomo https://orcid.org/0000-0002-7137-323X
J.W. Hoff https://orcid.org/0000-0002-7354-6413
J. Kriel https://orcid.org/0000-0001-7744-0037
M. Mewa-Ngongang https://orcid.org/0000-0003-2588-3973
V.I. Okudoh https://orcid.org/0000-0002-8468-1338
M. van der Rijst https://orcid.org/0000-0002-4027-5194

SUPPLEMENTARY MATERIAL

The following are available online at http://journal.pan.olsztyn.pl/Control-of-Mould-Spoilage-on-Apples-Using-Yeasts-as-Biological-Control-Agents,147913,0,2.html; Yeast used in the study. Visual representation of the growth of *Botrytis cinerea* (A) and the antagonistic effect of yeast isolate *Meyerozyma guilliermondii* Y88 against *B. cinerea* (B) on yeast malt agar. Visual representation of the growth of *Botrytis cinerea* (A) and the antagonistic effect of yeast isolate *Pichia kluyveri* Y64 against *B. cinerea* (B) on yeast malt agar.

REFERENCES

1. Al-Hindi, R.R., Al-Najada, A.R., Mohamed, S.A. (2011). Isolation and identification of some fruit spoilage fungi: Screening of plant cell wall degrading enzymes. *African Journal of Microbiology Research*, *5*(4), 443–448.
2. Al-Maawali, S.S., Al-Sadi, A.M., Ali Khalifa Alsheriqi, S., Nasser Al-Sabahi, J., Velazhahan, R. (2021). The potential of antagonistic yeasts and bacteria from tomato phyllosphere and fructoplane in the control of *Alternaria* fruit rot of tomato. *All Life*, *14*(1), 34–48. https://doi.org/10.1080/26895293.2020.1858975
3. Al-Rahbi, B.A.A., Al-Sadi, A.M., Al-Mahmooli, I.H., Al-Maawali, S.S., Al-Mahruqi, N.M.T., Velazhahan, R. (2021). *Meyerozyma guilliermondii* SQUCC-33Y suppresses postharvest fruit rot of strawberry caused by *Alternaria alternata*. *Australasian Plant Pathology*, *50*(3), 349–352. https://doi.org/10.1007/s13313-021-00779-z
4. Banjara, N., Nickerson, K.W., Suhr, M.J., Hallen-Adams, H.E. (2016). Killer toxin from several food-derived *Debaryomyces hansenii* strains effective against pathogenic *Candida* yeasts. *International Journal of Food Microbiology*, *222*, 23–29. https://doi.org/10.1016/j.ijfoodmicro.2016.01.016
5. Benito, S., Palomero, F., Morata, A., Uthurry, C., Suárez-Lepe, J.A. (2009). Minimization of ethylphenol precursors in red wines *via* the formation of pyranoanthocyanins by selected yeasts. *International Journal of Food Microbiology*, *132*(2–3), 145–152. https://doi.org/10.1016/j.ijfoodmicro.2009.04.015
6. Bonaterra, A., Badosa, E., Cabrefiga, J., Francés, J., Montesinos, E. (2012). Prospects and limitations of microbial pesticides for control of bacterial and fungal pomefruit tree diseases. *Trees, 26*, 215–226. https://doi.org/10.1007/s00468-011-0626-y
7. Chen, P.H., Chen, R.Y., Chou, J.Y. (2018). Screening and evaluation of yeast antagonists for biological control of *Botrytis cinerea* on strawberry fruits. *Mycobiology*, *46*(1), 33–46. https://doi.org/10.1080/12298093.2018.1454013
8. Choińska, R., Piasecka-Jóźwiak, K., Chabłowska, B., Dumka, J., Łukaszewicz, A. (2020). Biocontrol ability and volatile organic compounds production as a putative mode of action of yeast strains isolated from organic grapes and rye grains. *Antonie van Leeuwenhoek*, *113*(8), 1135–1146. https://doi.org/10.1007/s10482-020-01420-7
9. Contarino, R., Brighina, S., Fallico, B., Cirvilleri, G., Parafati, L., Restuccia, C. (2019). Volatile organic compounds (VOCs) produced by biocontrol yeasts. *Food Microbiology*, *82*, 70–74. https://doi.org/10.1016/j.fm.2019.01.008
10. Cordero-Bueso, G., Mangieri, N., Maghradze, D., Foschino, R., Valdetara, F., Cantoral, J.M., Vigentini, I. (2017). Wild grape-associated yeasts as promising biocontrol agents against *Vitis vinifera* fungal pathogens. *Frontiers in Microbiology*, *8*, art. no. 2025. https://doi.org/10.3389/fmicb.2017.02025
11. De Simone, N., Capozzi, V., Amodio, M.L., Colelli, G., Spano, G., Russo, P. (2021). Microbial-based biocontrol solutions for fruits and vegetables: recent insight, patents, and innovative trends. *Recent Patents on Food, Nutrition & Agriculture*, *12*(1), 3–18. https://doi.org/10.2174/2212798412666210125141117
12. Du Plessis, K. (2017). Post-harvest Innovation Programme Innovate flipbook, pp. 82–83 [https://www.postharvestinnovation.org.za/PHI-2017/mobile/index.html] (Accessed: 01 March 2021).
13. Droby, S. (2005). Improving quality and safety of fresh fruits and vegetables after harvest by the use of biocontrol agents and natural materials. *I International Symposium on Natural Preservatives in Food Systems*, *ISHS Acta Horticulturae, 709,* 45–52. https://doi.org/10.17660/ActaHortic.2006.709.5
14. FAOstat. (2020). Food and Agricultural Organisation of the United Nations – Statistical Division. Production – Crops and livestock products [www.faostat.org] (Accessed: 25 March 2022).
15. Fernández-Ortuño, D., Torés, J.A., De Vicente, A., Pérez-García, A. (2008). Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. *International Microbiology*, *11*(1), 1–9.
16. Grevesse, C., Lepoivre, P., Jijakli, M.H. (2003). Characterization of the exoglucanase-encoding gene PaEXG2 and study of its role in the biocontrol activity of *Pichia anomala* strain K. *Phytopathology*, *93*(9), 1145–1152. https://doi.org/10.1094/PHYTO.2003.93.9.1145
17. Han, J., Zhao, L., Zhu, H., Dhanasekaran, S., Zhang, X., Zhang, H. (2021). Study on the effect of alginate oligosaccharide combined with *Meyerozyma guilliermondii* against *Penicillium expansum* in pears and the possible mechanisms involved. *Physiological and Molecular Plant Pathology*, *115*, art. no. 101654. https://doi.org/10.1016/j.pmpp.2021.101654

18. Hua, S.S.T., Beck, J.J., Sarreal, S.B.L., Gee, W. (2014). The major volatile compound 2-phenylethanol from the biocontrol yeast, *Pichia anomala*, inhibits growth and expression of aflatoxin biosynthetic genes of *Aspergillus flavus*. *Mycotoxin Research*, *30*(2), 71–78. https://doi.org/10.1007/s12550-014-0189-z
19. Liu, J., Sui, Y., Wisniewski, M., Droby, S., Liu, Y. (2013). Review: Utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. *International Journal of Food Microbiology*, *167*(2), 153–160. https://doi.org/10.1016/j.ijfoodmicro.2013.09.004
20. Lõoke, M., Kristjuhan, K., Kristjuhan, A. (2011). Extraction of genomic DNA from yeasts for PCR-based applications. *BioTechniques*, *50*(5), 325–328. https://doi.org/10.2144/000113672
21. Medina-Córdova, N., López-Aguilar, R., Ascencio, F., Castellanos, T., Campa-Córdova, A.I., Angulo, C. (2016). Biocontrol activity of the marine yeast *Debaryomyces hansenii* against phytopathogenic fungi and its ability to inhibit mycotoxins production in maize grain (*Zea mays* L.). *Biological Control*, *97*, 70–79. https://doi.org/10.1016/j.biocontrol.2016.03.006
22. Mercier, J., Lindow, S.E. (2001). Field performance of antagonistic bacteria identified in a novel laboratory assay for biological control of fire blight of pear. *Biological Control*, *22*(1), 66–71. https://doi.org/10.1006/bcon.2001.0957
23. Mewa-Ngongang, M., Du Plessis, H.W., Chidi, B.S., Hutchinson, U.F., Ntwampe, K.S.O., Okudoh, V.I., Jolly, N.P. (2021). Physiological and antagonistic properties of *Pichia kluyveri* for curative and preventive treatments against post-harvest fruit fungi. *Polish Journal of Food and Nutrition Sciences, 71*(3), 245–253. https://doi.org/10.31883/pjfns/13927
24. Mewa-Ngongang, M., du Plessis, H.W., Hlangwani, E., Ntwampe, S.K.O., Chidi, B.S., Hutchinson, U.F., Jolly, N.P. (2019a). Activity interactions of crude biopreservatives against spoilage yeast consortia. *Fermentation*, *5*(3), art. no. 53. https://doi.org/10.3390/fermentation5030053
25. Mewa-Ngongang, M., du Plessis, H.W., Ntwampe, S.K.O., Chidi, B.S., Hutchinson, U.F., Mekuto, L., Jolly, N.P. (2019b). The use of *Candida pyralidae* and *Pichia kluyveri* to control spoilage microorganisms of raw fruits used for beverage production. *Foods*, *8*(10), art. no. 454. https://doi.org/10.3390/foods8100454
26. Mitchell, T.G., Freedman, E.Z., White, T.J., Taylor, J.W. (1994). Unique oligonucleotide primers in PCR for identification of *Cryptococcus neoformans*. *Journal of Clinical Microbiology*, *32*(1), 253–255. https://doi.org/10.1128/jcm.32.1.253-255.1994
27. Nally, M.C., Pesce, V.M., Maturano, Y.P., Muñoz, C.J., Combina, M., Toro, M.E., De Figueroa, L.C., Vazquez, F. (2012). Biocontrol of *Botrytis cinerea* in table grapes by non-pathogenic indigenous *Saccharomyces cerevisiae* yeasts isolated from viticultural environments in Argentina. *Postharvest Biology and Technology*, *64*(1), 40–48. https://doi.org/10.1016/j.postharvbio.2011.09.009
28. Nunes, C.A. (2012). Biological control of postharvest diseases of fruit. *European Journal of Plant Pathology*, *133*(1), 181–196. https://doi.org/10.1007/s10658-011-9919-7
29. Núñez, F., Lara, M.S., Peromingo, B., Delgado, J., Sánchez-Montero, L., Andrade, M.J. (2015). Selection and evaluation of *Debaryomyces hansenii* isolates as potential bioprotective agents against toxigenic penicillia in dry-fermented sausages. *Food Microbiology, 46*, 114–120. https://doi.org/10.1016/j.fm.2014.07.019
30. Oliveira, C.M., Auad, A.M., Mendes, S.M., Frizzas, M.R. (2014). Crop losses and the economic impact of insect pests on Brazilian agriculture. *Crop Protection*, *56*, 50–54. https://doi.org/10.1016/j.cropro.2013.10.022
31. Parveen, S., Wani, A.H., Bhat, M.Y., Koka, J.A., Wani, F.A. (2016). Management of postharvest fungal rot of peach (*Prunus persica*) caused by *Rhizopus stolonifer* in Kashmir Valley, India. *Plant Pathology and Quarantine*, *6*(1), 19–29. https://doi.org/10.5943/ppq/6/1/4
32. Quaglia, M., Ederli, L., Pasqualini, S., Zazzerini, A. (2011). Biological control agents and chemical inducers of resistance for postharvest control of *Penicillium expansum* Link. on apple fruit. *Postharvest Biology and Technology*, *59*(3), 307–315. https://doi.org/10.1016/j.postharvbio.2010.09.007
33. Robiglio, A., Sosa, M.C., Lutz, M.C., Lopes, C.A., Sangorrín, M.P. (2011). Yeast biocontrol of fungal spoilage of pears stored at low temperature. *International Journal of Food Microbiology*, *147*(3), 211–216. https://doi.org/10.1016/j.ijfoodmicro.2011.04.007
34. Romanazzi, G., Feliziani, E., Baños, S.B., Sivakumar, D. (2017). Shelf life extension of fresh fruit and vegetables by chitosan treatment. *Critical Reviews in Food Science and Nutrition*, *57*(3), 579–601. https://doi.org/10.1080/10408398.2014.900474
35. Romanazzi, G., Smilanick, J.L., Feliziani, E., Droby, S. (2016). Integrated management of postharvest grey mould on fruit crops. *Postharvest Biology and Technology*, *113*, 69–76. https://doi.org/10.1016/j.postharvbio.2015.11.003
36. Ruiz-Moyano, S., Hernández, A., Galvan, A.I., Córdoba, M.G., Casquete, R., Serradilla, M.J., Martín, A. (2020). Selection and application of antifungal VOCs-producing yeasts as biocontrol agents of grey mould in fruits. *Food Microbiology*, *92*, art. no. 103556. https://doi.org/10.1016/j.fm.2020.103556
37. Sharma, R.R., Singh, D., Singh, R. (2009). Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. *Biological Control*, *50*(3), 205–221. https://doi.org/10.1016/j.biocontrol.2009.05.001
38. Wang, X., Glawe, D.A., Kramer, E., Weller, D., Okubara, P.A. (2018) Biological control of *Botrytis cinerea*: interactions with native vineyard yeasts from Washington State. *Phytopathology*, *108*(6), 691–701. https://doi.org/10.1094/PHYTO-09-17-0306-R
39. Zhou, Y., Li, W., Zeng, J., Shao, Y. (2018). Mechanisms of action of the yeast *Debaryomyces nepalensis* for control of the pathogen *Colletotrichum gloeosporioides* in mango fruit. *Biological Control*, *123*, 111–119. https://doi.org/10.1016/j.biocontrol.2018.05.014

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Original article
Section: Food Quality and Functionality

Effect of Wheat and Barley Malt Addition on the Quality of the Baking Blend and Wheat Bread

Justyna Belcar[1]*, *Joanna Kaszuba*[2], *Józef Gorzelany*[1]

[1]*Department of Food and Agriculture Production Engineering, Collegium of Natural Sciences, University of Rzeszów, Zelwerowicza St. 4, 35–601 Rzeszów, Poland*
[2]*Department of Food Technology and Human Nutrition, Collegium of Natural Sciences, University of Rzeszów, Zelwerowicza St. 4, 35–601 Rzeszów, Poland*

Key words: grain storage, wheat malt, barley malt, amylolytic activity, bread quality

Wheat grain obtained from agricultural overproduction is stored for a long time in grain warehouses, which reduces its baking value. The aim of this study was to use barley and wheat malt flours as additives to flours obtained from grain of different wheat varieties stored for 12 months under optimal conditions, to improve their baking properties. The addition of barley malt flour at a mean rate of 0.5% or wheat malt flour at a mean rate of 0.7% reduced the time needed for the wheat gel to reach its maximum viscosity by 67.2%, compared to the control sample (without malt flour added). Bread made of flour blends with malt flours added was characterised by a higher loaf volume and specific volume as well as darker crust and crumb. The addition of malt flours also led to significantly reduced hardness, gumminess, and chewiness of bread crumb. The study demonstrated that it is possible to effectively use long-stored wheat grains for bread making applying wheat malt flour or barley malt flour as enhancers.

INTRODUCTION

Consumers increasingly seek out baked products not only with specific taste and flavour but also containing natural ingredients used as additives. Given this, efforts are made to improve the quality of these products even at the stage of wheat grain milling, *e.g.*, by developing flour blends, and also at the later stages by modifying production technologies. The application of improvers has been shown to affect organoleptic properties (taste, aroma), crumb texture, and loaf volume of the bread [Li Vigni *et al*., 2010].

Raw materials of natural origin which may be used to improve wheat flour include malts, malt extracts, and malt flours. When added in adequate amounts, they have been shown to positively affect technological properties and sensory characteristics of bread [Honců *et al.*, 2015; Rögner *et al.,* 2021a,b]. The production of malts involves controlled germination of caryopses in specified conditions, whereby the grain is soaked which leads to enhanced enzymatic activity and partial growth of rootlets; subsequently, the material is dried and the rootlets are removed [Farzaneh *et al*., 2017; Kleinwächter *et al*., 2014; Rögner *et al.,* 2021b; Schmitt *et al*., 2013]. Wheat and barley malts are sources of α-amylase which was found responsible for increased viscosity of wheat flour dough. During the processes of dough preparation, kneading, and baking, its activity affected CO_2 production in the dough, while its excess amount led to superfluous starch dextrinization and softening of the dough; consequently causing very high viscosity of the bread crumb produced [Zarzycki *et al.,* 2012]. In bread making, malt was usually added at a rate of approximately 1% (depending on flour quality) [Honců *et al*., 2015]. In addition to increasing the rate of the fermentation process, malt contributed to enhanced rheological properties of the dough, while the increased content of reducing sugars led to greater intensity of the Maillard reaction resulting in golden-brown colour of the bread crust [Honců *et al*., 2015; Rögner *et al.,* 2021b]. The addition of malt did not modify the taste of wheat bread but contributed to greater loaf volume, better crumb texture, and extended shelf-life of the bread [Honců *et al*., 2015].

The addition of barley or wheat malt with moderate proteolytic activity (reflected by Kolbach Index) contributed to the strengthening of the gluten network formed in the dough [Honciů *et al*., 2015; Zadeike *et al*., 2018]. Notably, however, malt with high proteolytic activity expressed as the Kolbach Index >42–45% should not be used for this purpose since it may adversely affect the properties of the gluten network in the dough, and consequently the quality and stability of the finished product [Zadeike *et al*., 2018]. The addition of germinated grains was also reported to result in increased

* Corresponding Author:
E-mail: justyna.belcar@op.pl (J. Belcar)

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bioavailability of mineral substances and dietary fibre, and in reduced contents of phytic acid and tannins in the finished products [Agrahar-Murugkar *et al*., 2015].

The purpose of this study was to determine amylographic properties of the flour produced from grains of three wheat varieties stored for one year in optimum conditions, to assess the effects of wheat and barley malt flour addition on flour quality, and to examine the breads produced for their physical and textural properties.

MATERIAL AND METHODS

Material

The material selected for the study comprised grains of three winter varieties of common wheat: Reform, Findus, and Kilimanjaro, as well as flours obtained by milling these grains. The research material was provided by Farming Co-operative SAN (Łąka, Poland). The grain was produced using conventional farming methods in 2019, in the Głuchów village (50°04'54''N; 22°16'11''E) in Łańcut District, Podkarpackie Region, Poland.

The malt flours were produced from spring barley of Irina variety and winter wheat of Elixer variety provided by Farming Cooperative SAN (Poland). The grain was produced in 2019, using conventional farming methods, in the Łąka village (50°05'10''N; 22°05'48''E), Rzeszów District, Podkarpackie Region, Poland.

Experimental design

The experimental design is presented in Figure 1. The wheat grains of tree varieties (8 kg each) were cold-stored for 12 months at a temperature of 11–12°C and relative humidity of 10%. After 8 weeks and 12 months of storage, 3 representative samples of the grains (500 g each) were taken for determinations of grain quality characteristics. Moreover, 5-kg portions of 12 month-stored grain of each wheat variety were taken for milling. They were purified in an SLN3 type separator (Pfeuffer GMBH, Kitzingen, Germany), conditioned up to the moisture content of 15 g/100 g (24 h), and milled in a Quadrumat Junior mill (Brabender GmbH & Co. KG, Duisburg, Germany).

Flour yield was calculated as follows:

$$Flour\ yield = \frac{Flour\ weight\ (g)}{Grain\ subjected\ to\ milling\ (g)} \times 100\% \quad (1)$$

Three representative samples (1.5 kg each) of flour of each wheat variety were taken for determinations of the chemical composition and quality characteristics. The flours (3.5 kg of flour made from each of the wheat varieties) were also used for baking breads. The breads were prepared with the addition of wheat and barley malt flours. Flour blends and breads containing barley malt flour (B) were marked as follows: RB (Reform variety), FB (Findus variety), and KB (Kilimanjaro variety), whereas flours and breads containing wheat malt flour (W) were marked as RW (Reform variety), FW (Findus variety), and KW (Kilimanjaro variety). Controls (C) marked as RC (Reform variety), FC (Findus variety), and KC (Kilimanjaro variety) were breads without malt flour addition. Three loaves of bread of each type were baked and their physical parameters and texture prolife were analysed.

The wheat and barley malt flours were obtained at a laboratory of the Department of Agricultural and Food Engineering at the University of Rzeszów. Briefly, the grains were spread on metal germination plates covered with filter paper, and soaked to the moisture content of 45 g/100 g. Plates with the samples were placed in a climatic cabinet and kept therein at a relative air humidity of 90% and a temperature of 15°C. Following the defined germination time, the material was dried in a laboratory dryer and the rootlets were removed. Finally, the malted wheat and barley grains were milled (Cemotec mill, Foss, Hillerød, Denmark) into flour of particle size <2 mm. The commodity characteristics of grains and the assessment of the malting process and quality attributes of wheat and barley malt flours were presented in our previous publication [Belcar *et al.,* 2020].

Analysis of commodity characteristics of grains

The moisture content of the grain was determined using the Polish Committee for Standardization and International Organization for Standardization (PN-ISO) oven drying method [PN-EN ISO 712:2012, 2012]. The contents of total protein (expressed as g per 100 g of dry weight (d.w.) of grain), wet gluten (g/100 g), and starch (g/100 g d.w.) as well as Zeleny sedimentation value (mL) were determined using a near-infrared (NIR) DA 7200 spectrometer (Perten Instruments, Huddinge, Sweden) by passing the NIR light through the test grain placed in the chamber.

Determination of physical and chemical parameters as well as indirect baking quality indicators of the flours

The moisture content of the flour was assessed in accordance with the American Association of Cereal Chemists (AACC) method 44–15.02 [AACC, 2009]. The ash content of the flour was assessed by means of combustion method, in a muffle furnace (Nabetherm, Lilienthal, Germany), in accordance with AACC method 08–01.01 [AACC, 2009]. The contents of total protein and damaged starch (both expressed based on d.w. of grain) as well as Zeleny sedimentation value (mL) were determined using a NIR DA 7200 spectrometer (Perten Instruments). Measurements of wet gluten content and gluten index (GI) were performed in compliance with the PN-ISO method [PN-EN ISO 21415–2:2015–12, 2015] as well as International Association for Cereal Science and Technology (ICC) standard method no. 155 [ICC, 1994] using a Gluten Index System (Glutomatic 2200; centrifuge type

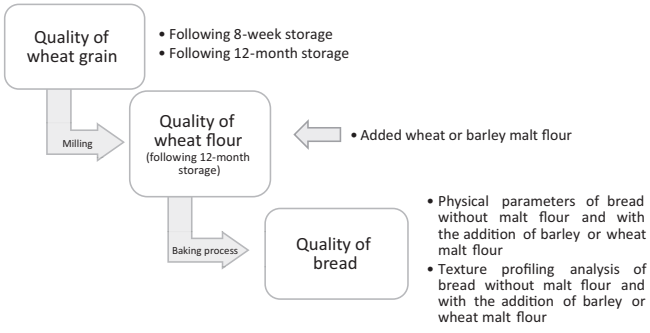

FIGURE 1. Experimental design.

2015; Perten Instruments). Flour water absorption was determined using a Farinograph-E (Brabender), in line with the ICC standard method no. 115/1 [ICC, 1992a]. The analyses were also made for dough, with a consistency of 500 farinograph units (acc. ICC [1992a]), made from flour whose weight was equivalent to the contents of dry matter in 50 g of flour with 14 g/100 g moisture content. The falling number (FN) of flour was determined based on the Hagberg-Perten ICC standard method no. 107/1 [ICC, 1995], using a Perten Falling Number 1800 apparatus (Perten Instruments). Tests were also performed to determine the proportions of malt flour and wheat flour needed to produce a baking blend with FN=250 s, which is an optimal value for wheat flour [ICC, 1995].

In order to assess starch gelatinization, amylographic analyses were performed for wheat flours and the wheat flour blends with the specified addition of wheat malt flour (RW–0.61%; FW–0.67% and KW–0.82%) or barley malt flour (RB–0.49%; FB–0.49% and KB–0.50%). The analyses were performed in accordance with the ICC standard method no. 126/1 [ICC, 1992b] using a Viscograph-E apparatus (Brabender). The flour samples with weights equivalent to the contents of dry matter in 80 g of flour with 14 g/100 g moisture content were mixed with water to achieve the final weight of 500 g. Apparent viscosity was measured at temperatures from 30 to 95°C, with intervals of 1.5°C/min. The values identified in the graph included the maximum apparent viscosity (Brabender unit, BU), gelatinization onset temperature (°C), and gelatinization peak temperature (°C).

Bread baking

Production of dough and the bread baking test were carried out in line with the direct single-phase method, in compliance with the recommendations of the Bakery Institute in Berlin [Jakubczyk & Haber, 1983]. The wheat doughs consisted of flour, yeast (at a rate of 3% relative to the weight of flour), salt (at a rate of 1% relative to the weight of flour), and water in the volume needed to achieve dough consistency of 350 FU. Corresponding samples of wheat dough were made from baking formulation consisting of flour blends with wheat malt flour or barley malt flour, mixed at specified proportions to obtain FN=250 s; the percentage of wheat malt flour was 0.61%, 0.67% and 0.82% for RW, FW, and KW, respectively, and the percentage of barley malt flour was 0.49%; 0.49%, and 0.50% for RB, FB, and KB, respectively. The ingredients were combined in a bowl of a KU2-3E multifunctional food processor (MeskoAgd, Skarżyko-Kamienna, Poland) with dough hooks attached. To achieve uniform consistency, the dough was kneaded for 2 min at 100 rpm and for 1 min at 160 rpm, and then allowed to rest for 45 s. The doughs were placed in a proofing chamber, at a temperature of 30°C and relative humidity of 80% (SvebaDahlen, Fristad, Sweden) for 60 min, with punch-down after 30 min. After the main fermentation process had been completed, the dough was weighed and divided into 250-g chunks. The chunks were manually formed into spherical shapes and inserted into greased baking pans, and placed again in the proofing chamber, to allow the dough to rise. After the proofing process had been completed, the pans with dough were placed in the baking chamber of the Classic modular electric oven (SvebaDahlen). The baking process was carried out at a temperature of 230°C for 30 min. After they had been removed from the oven, the breads were wetted with water for a shiny skin and weighed; subsequently they were left for 24 h at a room temperature.

Determination of baking quality indicators and physical parameters of bread

Dough yield (DY; %) was calculated from the following formula:

$$DY = \frac{a}{m} \times 100\% \quad (2)$$

where: a – dough weight (g) and m – flour weight (g; acc. moisture content of flour – 15 g/100 g) [Sobczyk *et al.,* 2017a].

Cold bread weight (CB; g) was measured by weighing a loaf of bread 24 h after baking (Radwag, Radom, Poland), and baking yield (BY; %) was calculated as:

$$BY = \frac{CB \times DY}{a} \quad (3)$$

The volume of bread (VB; mL) was determined with the Sa-Way apparatus (bread volumeter) (Sadkiewicz Instruments, Bydgoszcz, Poland) according to the AACC method no. 10–05.01 [AACC, 2009]. The volume of bread produced from 100 g of flour (VB100; mL) was computed using the following formula:

$$VB100 = \frac{VB - DY}{a} \quad (4)$$

Specific volume (SV, mL/g) was calculated according to the formula previously used by Krochmal-Marczak *et al.* [2020]:

$$S = \frac{VB}{CB} \quad (5)$$

Crumb porosity, reflecting the relationship between the volume of pores in bread crumb and the overall volume of bread crumb, was assessed using Jakobi's method [Jakubczyk & Haber, 1983]. The determination consisted in cutting bread crumb into cubes with an edge length of 1 cm, removing the air from the crumb by kneading, and measuring the volume of the crumb. The porosity of the crumb (P; %) was calculated using the following formula:

$$P = \frac{a - b}{a} \times 100\% \quad (6)$$

where: a – volume of the bread with the crumb intact (mL), and b – volume of the crumb after pore removal (mL) [Jakubczyk & Haber, 1983].

Loaves of bread obtained from flours of three wheat varieties, both without and with the addition of wheat malt flour or barley malt flour, were cut in half and then photos of the crumb cross-section were taken using a Sony DSC – RX100 MIII digital camera (Tokyo, Japan).

The colour of bread crust and crumb was assessed in the CIELab system (L^*, a^*, b^*) using a spectrometer (HunterLab, Reston, VA, USA) [El-Sohaimy *et al.,* 2021]. The total colour difference (ΔE) between the crumb of the control

bread and the bread with the addition of malt flour was also calculated [Martins & Silva, 2002; Patras *et al.,* 2011].

The texture profile of the breads was assessed 24 h after baking using a Brookfield CT3 texture analyzer (Brookfield Engineering Laboratories, Middleboro, MA, USA). The following parameters were measured or calculated: hardness (strength), cohesiveness, springiness, elasticity, gumminess, and chewiness [Sobczyk *et al*., 2017a]. During the test, the sample with a diameter of 10 mm was compressed twice with a 13.5 mm pressing device, with a moving speed of 2 mm/s, whereby deformation of the sample reached half of its height. Interval of 2 s was applied between the cycles.

Statistical analysis

All analyses were done in triplicate (n=3). The acquired results were subjected to statistical analyses using Statistica 13.3. (TIBCO Software Inc., Tulsa, OK, USA). A one-way analysis of variance (ANOVA) with completely randomized design was used with a significance level defined as α=0.05. Tukey HSD test was applied to compare the mean values.

RESULTS AND DISCUSSION

Quality changes of wheat grain during storage and quality characteristics of the flour produced from wheat grain following 12-month storage

Directly after it is harvested, wheat grain presents a low technological value (both for milling and baking), due to which the raw material is stored for 6–9 weeks (post-harvest dormancy) before the grain can be processed by milling, and its products can be used in the food industry. Processes taking place in the grain during this period lead to, *e.g*., improved gluten quality and suppressed amylolytic activity (including mainly α-amylase, which is the main enzyme responsible for the breakdown of starch in wheat grains and increased falling number of flour) [Baik & Donelson, 2018; Zarzycki *et al.,* 2012]. The rate of changes taking place in wheat grains during storage depends on the storage conditions, *i.e*., relative air humidity and temperature, as well as on the water content of the grain [Baik & Donelson, 2018]. Extended storage leads to grain weight loss, followed by moisture content decrease, and is linked with carbon dioxide excretion as well as suppressed activity of enzymes from the group of hydrolases (mainly amylases) and to a lesser extent with the suppressed activity of proteolytic enzymes [Baik & Donelson, 2018]. In our study, the characteristics of the grain of wheat varieties related to the quality of the protein-starch complex were determined, and the results achieved for the grain stored for 8 weeks and for 12 months since harvest are shown in Table 1.

The moisture content of the grain sampled 8 weeks after harvest varied significantly (p<0.05), ranging from 12.70 g/100 g d.w. in wheat of Reform variety to 14.90 g/100 g d.w. in wheat of Kilimanjaro variety. After one year of storage in optimum conditions, the moisture content of the grain decreased to 11.4–11.6 g/100 g d.w. (Table 1). The moisture content decrease in the stored wheat grain makes it possible to extend the storage time without compromising grain quality (under controlled storage parameters, including temperature of grain and relative air humidity in the storage facility, *e.g.*, silo). High temperature and moisture content of stored grain enhance degradation of starch contained in wheat and also increase the risk of appearance of storage pests, whose presence and activity in wheat grain significantly reduces its quality [Baik & Donelson, 2018].

The protein content of wheat grain is significantly affected by nitrogen fertilisers applied during the vegetation period, as well as by the environmental conditions and variety-related characteristics [Belcar *et al.,* 2020]. In our study, the total protein content of the Findus variety wheat grain was significantly higher (p<0.05) compared to the other two wheat varieties studied (Reform and Kilimanjaro), in which it was similar (Table 1).

Prolonged storage of wheat grain may adversely affect both the content of wet gluten (decrease in glutenin content) and Zeleny sedimentation value [Baik & Donelson, 2018]. The grain Zeleny sedimentation value is an important parameter indirectly indicating its baking quality, and more specifically the quality of gluten, which, as the main component of the flour protein complex, is responsible for bread structure formation [Sobczyk *et al.,* 2017a]. The grain of the studied wheat cultivars sampled 8 weeks after harvest was characterised by a high Zeleny sedimentation value ranging from 51.0 to even 69.0 mL, which decreased significantly (p<0.05) only in Findus grain after 12-month storage (Table 1). The content of wet gluten in wheat grain after 8 weeks of storage ranged from 27.20 to 35.70 g/100 g, while after one year of storage, it increased by 16.82% for the Kilimanjaro variety and did not change significantly (p≥0.05) for the other varieties.

TABLE 1. Quality characteristics of grain of different wheat varieties stored for 8 weeks and 12 months after harvest.

Characteristics	Reform		Findus		Kilimanjaro	
	8 weeks	12 months	8 weeks	12 months	8 weeks	12 months
Moisture content (g/100 g)	12.70±0.36^{c}	11.50±0.40^{d}	14.10±0.30^{b}	11.40±0.35^{d}	14.90±0.10^{a}	11.60±0.20^{d}
Total protein content (g/100 g d.w.)	14.00±0.17^{b}	13.60±0.36^{b}	14.80±0.20^{a}	15.00±0.10^{a}	13.60±0.36^{b}	13.90±0.26^{b}
Wet gluten content (g/100 g)	30.60±0.56^{b}	31.80±0.66^{b}	35.70±0.80^{a}	37.00±0.53^{a}	27.20±1.50^{c}	32.70±0.96^{b}
Starch content (g/100 g d.w.)	59.60±1.32^{a}	60.20±1.65^{a}	58.80±1.15^{a}	58.50±0.44^{a}	60.10±0.53^{a}	59.60±1.59^{a}
Zeleny sedimentation value (mL)	51.0±3.6^{b}	52.0±1.7^{b}	69.0±1.0^{a}	51.0±1.7^{b}	58.0±2.7^{b}	56.0±4.0^{b}

Data are expressed as mean ± standard deviation (n=3). Values within rows with different letters are significantly different (p<0.05).

TABLE 2. Quality characteristics of the flours produced from grain of different wheat varieties after 12-month storage.

Characteristics	Reform	Findus	Kilimanjaro
Flour yield (%)	80.30±0.81	79.50±0.97	80.40±0.64
Moisture content (g/100 g)	11.71±0.02^{a}	11.50±0.04^{a}	11.74±0.12^{a}
Total protein content (g/100 g d.w.)	14.60±0.35^{b}	16.70±0.42^{a}	15.30±0.32^{b}
Zeleny sedimentation value (mL)	44.0±3.5^{a}	46.0±3.0^{a}	43.0±3.0^{a}
Ash content (g/100 g d.w.)	0.64±0.04^{ab}	0.72±0.04^{a}	0.61±0.03^{b}
Damaged starch content (g/100 g d.w.)	3.77±0.05^{c}	4.58±0.05^{a}	4.12±0.05^{b}
Wet gluten content (g/100 g)	30.02±0.45^{c}	36.32±0.21^{a}	31.62±0.28^{b}
Gluten index	98.00±0.60^{a}	94.00±0.06^{c}	96.00±0.35^{b}
Water absorption (%)	56.30±0.40^{c}	63.30±0.06^{a}	57.30±0.10^{b}
Falling number (s)	571.1±2.1^{b}	533.3±1.9^{c}	596.0±1.5^{a}

Data are expressed as mean ± standard deviation (n = 3). Values within rows with different letters are significantly different (p<0.05).

The chemical and technological characteristics of the wheat flours from grain stored for 12 months were also determined and the results are shown in Table 2. The ash content of the flours obtained from wheat grains of Reform, Findus, and Kilimanjaro varieties was at a similar level (Table 2). Hrušková *et al.* [2003] analysed ash content of flours with different proportions of the outer layer of the grain (bran), and found that a high ash content was unfavourable as it slightly deteriorated rheological properties of the dough and affected bread volume.

The content of damaged starch in the flours from the investigated wheat varieties ranged from 3.77 g/100 g d.w. (Reform) to 4.58 g/100 g d.w. (Findus) (Table 2). Damage of a starch granule affects the fermentation process, bread crumb structure as well as water absorption by flour during the kneading process [Sobczyk *et al.,* 2017b]. If malt is added, highly active α-amylase contained in it attacks damaged starch and provides a substrate to baker's yeast to initiate dough fermentation (starting dose) before the hydrolytic enzymes contained in the flour provide the substrate to baker's yeast [Hrušková *et al*., 2003].

The total protein content of flour obtained from the wheat grain of the Findus variety was significantly (p<0.05) higher compared to the flours from the other wheat varieties analysed (Table 2). The quantity and quality of gluten affect the viscoelastic and rheological properties of the dough obtained. The high quality of gluten causes that a stable gluten network is formed in the dough, which retains carbon dioxide molecules and, as a result, contributes to a high bread loaf volume [Sobczyk *et al.,* 2017a]. In our study, the wet gluten content of the flours did not differ significantly (p≥0.05) between wheat varieties and ranged between 30.02 g/100 g and 36.32 g/100 g (Table 2). The flours were also not differentiated by the Zeleny sedimentation value. The gluten index is a parameter that allows for indirect determination of bread volume and elasticity [Sobczyk *et al.,* 2017a]. Flours obtained from wheat grain stored for 12 months had a gluten index above 94 (Table 2), which proved their good baking quality. Wheat flours with a gluten index above 95 are characterised by high gluten quality and are used for bread making [Sobczyk *et al.,* 2017a].

The water absorption value of flour depends mainly on the quantity and quality of gluten and the starch content and the degree of its damage [Sobczyk *et al.,* 2017a]. The water absorption of the tested flours was significantly differentiated (p<0.05), including the highest value determined for the flour obtained as a result of milling wheat grain of the Findus variety (63.3%), which is associated with the high content of gluten in this sample (Table 2).

Effect of barley malt flour or wheat malt flour addition on the falling number of the wheat flour

The falling number describes the activity of flour enzymes that affect bread crumb quality since they promote dough fermentation process. With longer storage time, the activity of α-amylase gradually decreases in wheat grains, leading to a higher falling number of the flour produced [González-Torralba *et al.*, 2013]. The rate of decrease in the activity of hydrolytic enzymes is related to the temperature at which the grain is stored. Storage of wheat grain for a certain period of time at a higher temperature (about 30°C) led to more rapid changes and decrease in the falling number compared to the grain stored for the same duration at a temperature of 15°C [González-Torralba *et al.*, 2013]. The optimum falling number of wheat flours should range from 220 to 250 s [ICC, 1995].

After 12-month storage, the flour produced from the grain had a falling number ranging from 533.3 to nearly 600 s and the value significantly (p<0.05) varied among the wheat varieties (Table 2). The addition of barley malt flour turned out to be more effective in reducing the falling number of wheat flour obtained 12 months after the grain harvest, compared to the flour blend with wheat malt flour (lower proportion of barley malt flour by 28.3% compared to wheat malt flour), which was noticed for all tested flours. If malt flour is added to wheat flour, the yeast uses the simple sugars contained in the malt flour in the initial stage of fermentation, and those contained in the wheat flour are used later; thanks to this,

the dough fermentation process is faster, and the amount of CO_2 released increases, which significantly affects the texture and moisture content of bread crumb [Hrušková *et al*., 2003]. The addition of barley malt flour or wheat malt flour is a natural method to compensate for a decreased amount of yeast used in the dough [Honciů *et al*., 2015].

Amylograph characteristics of wheat flours with malt flour added

Amylograph may be used to examine changes in the properties of a flour suspension subjected to heating. As shown by earlier research, the addition of malt flour at a rate of 0.2–0.5% led to a decrease in the temperature at which starch gelatinisation begins, and to a decrease in the maximum viscosity compared to the gels obtained from wheat flour without malt flour addition (depending on the malt diastatic power and wheat flour quality) [Hrušková *et al*., 2003]. The results of amylographic tests applied to wheat flours as well as wheat flour blends with malt flours are shown in Figure 2. The maximum viscosity of the flour suspensions varied significantly. On average, the gelatinisation of the suspensions began in the 851th s of the test, whereas the initial temperature of gelatinisation did not differ. The maximum viscosity of the gel was observed in the 1200th s of the test on average, at a temperature of 87.9°C (RC, FC) or 89.7°C (KC). The addition of barley malt flour reduced the time needed for achieving the maximum viscosity to 801 s and a mean temperature of 69.5°C, decreasing the time required to achieve the maximum viscosity by 67.2% relative to the control sample (with no malt flour added). The addition of wheat malt flour slightly increased the temperature of maximum viscosity (on average 70.0°C) compared to the gels comprising barley malt flour, with the same time needed to achieve the maximum viscosity. The maximum viscosity of the gels with wheat malt flour added was in the range from 282 BU in the case of FW to 372 BU in the case of RW sample. In a study by Hrušková *et al.* [2003], the maximum viscosity of wheat gels was found at 489 BU, and the addition of malt flour at a rate of 0.50% decreased it by 50.80% (in flour with ash content of 0.68% d.w.). These high maximum viscosity values identified in wheat gels without malt flour show that starch contained in them had good swelling power but featured suppressed activity of amylolytic enzymes, which was also confirmed by the high falling number (Table 2). The same relationship was reported by Gupta *et al.* [2009]. Amylolytic activity may be reflected by the falling number and by very high viscosity of the wheat gels produced [Zarzycki *et al.,* 2012].

Baking process of bread with malt flour added

Final proofing time of doughs with wheat malt flour added was the same ($p \geq 0.05$) as in the doughs without malt (with the exception of the dough prepared from Findus wheat), whereas the doughs enhanced with barley malt flour needed significantly ($p < 0.05$) more time for proofing compared to the control doughs (Figure 3).

Dough yields of the samples obtained from flour produced from wheat grain of the Reform variety with no malt flour added were lower than those of the samples with barley or wheat malt flour added (Table 3). The differences in dough yield in the samples with barley malt flour or wheat malt flour added were statistically insignificant ($p \geq 0.05$) for Reform and Kilimanjaro varieties, whereas the baking yield of the samples with wheat malt flour added was higher than that of the samples with barley malt flour only for Kilimanjaro variety. The addition of malt flours to the wheat flour did not significantly ($p \geq 0.05$) affect the cold bread weight (Table 3).

The volume of bread with malt flour added is affected mainly by amylase activity but also by activities of other enzymes, *i.e.*, hemicelluloses, lipases, proteases, and oxidative enzymes [Mäkinen & Arendt, 2012]. The addition of malt flour at a rate of 0.2–0.5% has been shown to increase bread volume by 4.1% [Hrušková *et al*., 2003]. Based on Gupta *et al.* [2009] research on the properties of wheat and barley starch, it can be assumed that in our study bread volume was positively affected by reduced time needed to begin the gelatinisation process of wheat flour (KC, RC or FC) by the addition of malt flour. The lowest loaf volume was characteristic for bread without malt flour addition (Table 3), while the bread enriched with malt flour had a significantly higher loaf volume, regardless of the wheat flour used (except KB). The highest loaf volume in the group of breads with wheat malt flour added was found in KW sample (613.13 mL), and in the group of breads with barley malt added in FB sample (608.33 mL). The highest volume of loaf made from 100 g of flour was identified in the case of bread produced from Findus wheat flour, both in the samples with no malt added (372.27 mL) and in those with barley malt (402.66 mL) or

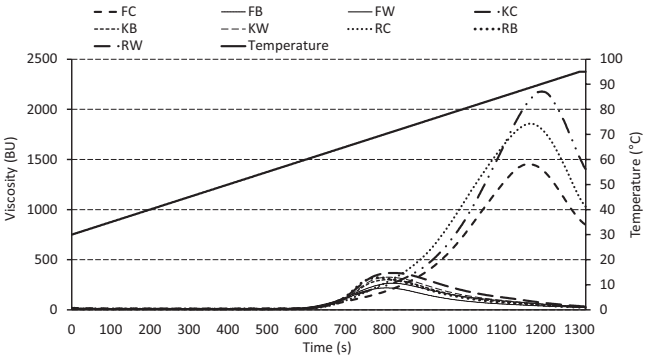

FIGURE 2. Amylogram of flours obtained from wheat of Findus variety (FC), Kilimanjaro variety (KC), Reform variety (RC), and flour blends with barley malt flour addition (B) or with wheat malt flour addition (W).

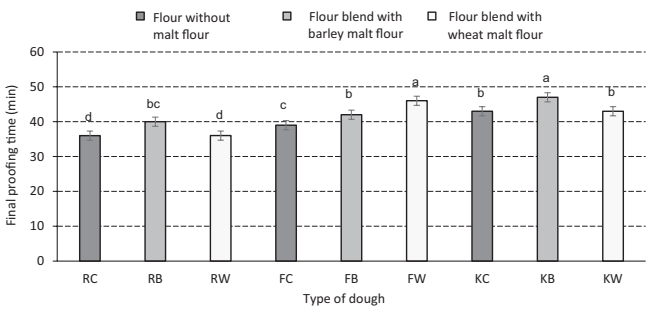

FIGURE 3. Final proofing time of dough obtained from flour of wheat of Findus variety (FC), Kilimanjaro variety (KC), Reform variety (RC), and of dough produced from flour blends with barley malt flour addition (B); or with wheat malt flour addition (W). Different letters above the bars indicate significant differences between the values ($p < 0.05$).

TABLE 3. Dough yield, baking yield, and physical parameters of bread made from flours of various wheat varieties and from flour blends with the addition of barley and wheat malt flours.

Bread	Dough yield (%)	Baking yield (%)	Cold bread weight (g)	Loaf volume (mL)	Volume of bread from 100 g flour (mL)	Specific volume (mL/g)	Porosity (%)
RC	159.2±2.8^{e}	133.8±0.2^{cd}	210.4±0.4^{a}	548.3±2.9^{c}	348.7±1.9^{c}	2.61±0.02^{c}	75.20±1.35^{ab}
RB	161.6±1.4^{e}	135.1±1.1^{bc}	209.5±1.7^{abc}	593.3±32.2^{ab}	382.7±20.3^{b}	2.83±0.16^{abc}	64.40±1.55^{f}
RW	161.5±2.1^{e}	134.8±0.1^{c}	209.1±0.2^{a-d}	570.0±13.2^{b}	367.5±8.5^{bc}	2.72±0.06^{bc}	68.70±1.48^{def}
FC	167.0±3.0^{a}	138.0±0.7^{a}	207.0±1.0^{b-e}	558.3±18.9^{b}	372.3±12.3^{bc}	2.70±0.10^{bc}	79.30±1.17^{a}
FB	165.8±2.9^{b}	136.7±0.7^{ab}	206.7±0.7^{de}	608.3±2.9^{a}	402.7±2.4^{a}	2.95±0.10^{a}	66.30±1.50^{ef}
FW	166.5±2.0^{a}	137.4±0.7^{a}	206.5±1.0^{bcd}	608.3±5.8^{a}	404.3±3.8^{a}	2.94±0.02^{ab}	69.60±1.19^{bc}
KC	161.0±2.9^{e}	134.8±0.7^{c}	209.9±1.2^{ab}	568.3±17.6^{b}	365.2±11.2^{bc}	2.71±0.08^{bc}	72.20±1.62^{bcd}
KB	160.5±1.8^{d}	132.0±0.8^{d}	206.0±1.1^{e}	580.0±26.5^{ab}	371.8±16.9^{bc}	2.81±0.12^{abc}	67.10±1.40^{ef}
KW	160.6±2.0^{d}	133.9±0.6^{c}	208.8±1.1^{a-e}	613.3±10.4^{a}	393.2±7.4^{ab}	2.94±0.06^{ab}	73.90±1.66^{bc}

Data are expressed as mean ± standard deviation (n=3). Values within columns with different letters are significantly different (p<0.05).
RC, Reform variety without malt; RB, Reform variety with barley malt; RW, Reform variety with wheat malt; FC, Findus variety without malt; FB, Findus variety with barley malt; FW, Findus variety with wheat malt; KC, Kilimanjaro variety without malt; KB, Kilimanjaro variety with barley malt; KW, Kilimanjaro variety with wheat malt.

wheat malt flour (404.28 mL). Hrušková *et al.* [2003] reported the mean volume of loaf made from 100 g of flour with 0.50% addition of malt flour at 359 mL (in the case of flour with mean ash content of 0.55%) and at 345 mL (in the case of flours with a higher mean ash content of 0.68%). On the other hand, Mäkinen & Arendt [2012] reported a slightly lower volume of loaf from 100 g of flour in the case of breads with barley (313 mL) and wheat malt flour (308 mL) added.

Bread crumb should have fine cellular structure (fine pores evenly arranged across the bread crumb), that is a group of interconnected small pores characterised by thin walls and an empty space between these pores; gluten network in the dough should have sufficient elasticity, and the rate of dough growth (release of CO_2) should be moderate so that the cell walls are not disrupted [Mäkinen & Arendt, 2012].

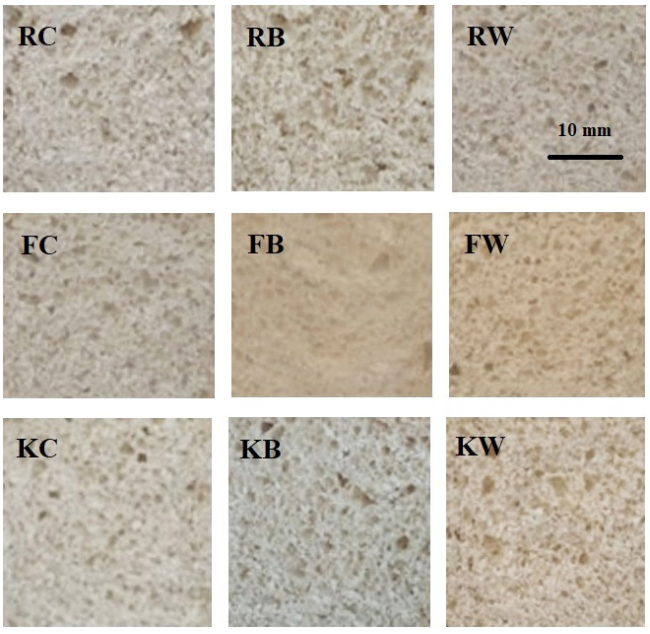

FIGURE 4. Crumb cross section of bread made from flour of wheat of Findus variety (FC), Kilimanjaro variety (KC), Reform variety (RC) and bread made from flour blends with barley malt flour (B) or with wheat malt flour (W).

Porosity of the crumb in breads without malt flour addition was better compared to the porosity of the crumb in the breads with malts added, except for KW (Table 3, Figure 4). The addition of malt leads to an increase in the number of large pores in bread crumb because of the increased activity of amylases which affect the process of fermentation and result in relatively high content of CO_2 in the dough. Amylolytic enzymes contained in malts produce such effects as gas retention in the dough, dough viscosity decrease, and product volume increase [Zadeike *et al*., 2018]. Wheat bread with the addition of barley β-glucan was characterised by a higher loaf volume, a thick-walled crumb with reduced elasticity compared to the control sample [Skendi *et al*., 2010]. Comparative assessment of breads enhanced with malt flour (Table 3, Figure 4) shows that the addition of wheat malt to a lesser degree impaired bread crumb porosity (on average decrease by 6.30% compared to the control sample), relative to barley malt (a decrease by 12.61%).

Bread colour parameters

The colour parameters of the bread crust changed under the influence of malt flour addition to the baking blend, both in the case of wheat and barley malt flours (Table 4). The addition of barley malt flour darkened the crusts of the bread obtained from the flour of each wheat variety (Findus, Kilimajaro, Reform), as did the addition of wheat malt flour, except for KW. The colour of the crust of the bread obtained from blends of flour from two wheat varieties with both malt flours (RB, RW, FB, FW) showed a significantly (p<0.05) higher value of the a^* parameter compared to the control samples. In the case of Kilimanjaro variety, the addition of malt (KB, KW) caused no significant (p≥0.05) change in a^* parameter value. On the other hand, only the b^* value of Kilimanjaro bread crust with the addition of barley malt flour (KB) was

TABLE 4. Colour parameters of crust and crumb of breads made from flours of various wheat varieties and from flour blends with the addition of barley and wheat malt flours.

Bread	Crust				Crumb			
	*L**	*a**	*b**	Δ*E*	*L**	*a**	*b**	Δ*E*
RC	60.33±2.01^{a}	12.34±0.99^{d}	32.20±0.58^{bc}	–	69.58±1.78^{ab}	2.62±0.17^{a}	20.46±0.51^{c}	–
RB	52.86±2.82^{bcd}	14.93±1.13^{bc}	30.62±0.97^{c}	8.12±1.59^{a}	67.43±1.46^{b}	2.87±0.18^{a}	20.53±0.54^{c}	2.18±0.65^{ab}
RW	52.53±1.58^{bcd}	15.37±0.50^{ab}	31.03±0.75^{c}	8.47±1.05^{a}	68.13±1.60^{b}	2.83±0.18^{a}	20.61±0.49^{bc}	1.53±0.96^{b}
KC	55.20±1.85^{bc}	16.07±0.89^{ab}	34.50±1.13^{a}	–	71.51±1.64^{a}	2.61±0.37^{a}	21.06±0.89^{a}	–
KB	51.38±1.93^{cd}	15.90±0.54^{ab}	30.96±0.83^{c}	5.41±1.30^{b}	68.78±1.23^{ab}	2.72±0.31^{a}	21.60±0.52^{ab}	2.80±2.07^{a}
KW	55.63±2.24^{b}	15.69±0.56^{ab}	33.97±1.02^{ab}	2.73±0.52^{c}	69.51±1.30^{ab}	2.65±0.19^{a}	20.82±0.53^{abc}	2.79±1.22^{a}
FC	56.52±1.62^{ab}	13.77±0.89^{cd}	31.66±0.72^{c}	–	68.57±2.16^{ab}	2.72±0.21^{a}	20.54±0.34^{c}	–
FB	51.43±2.28^{cd}	16.48±0.99^{a}	31.85±0.62^{c}	5.80±2.03^{b}	68.44±1.44^{b}	2.61±0.27^{a}	20.58±0.62^{c}	1.10±0.61^{b}
FW	49.95±2.59^{d}	16.43±0.53^{a}	30.77±1.69^{c}	7.23±3.35^{ab}	67.77±1.28^{b}	2.71±0.18^{a}	20.34±0.41^{c}	1.90±0.30^{ab}

Data are expressed as mean ± standard deviation (n=3). Values within columns with different letters are significantly different (p<0.05).
RC, Reform variety without malt; RB, Reform variety with barley malt; RW, Reform variety with wheat malt; FC, Findus variety without malt; FB, Findus variety with barley malt; FW, Findus variety with wheat malt; KC, Kilimanjaro variety without malt; KB, Kilimanjaro variety with barley malt; KW, Kilimanjaro variety with wheat malt.

significantly lower compared to the control sample. The total colour difference Δ*E* of the crust of breads with barley malt flour added ranged from 5.41 to 8.12 (Table 4). These values were high, reflecting the fact that the colour of bread crusts in this case was quite different than in the control samples. In the case of the crust of breads enhanced with wheat malt flour, values of the total colour difference were within a similar range except for the KW sample, where the Δ*E* was low (2.73) but noticeable by individuals with no related expertise. These differences in the colour parameters of bread crust could be due to the Maillard reaction between fermenting sugars derived from the malts and amino acids [Rögner *et al.,* 2021b]. In our previous study, we found that both malt flours were rich sources of reducing sugars, accounting for 1.10% in wheat malt and 1.55% in barley malt (expressed as glucose equivalents), as well as soluble proteins with contents at 3.83% d.w. in wheat malt and 3.97% d.w. in barley malt [Belcar *et al.*, 2020]. Another reason for colour differences could be the more intense caramelisation occurring at a high temperature during the baking process of breads with malts [Yang *et al*., 2020]. Moreover, the higher content of β-glucan in barley malt (456 mg/L) as compared to wheat malt (86 mg/L) [Belcar *et al.*, 2020] could result in a lighter crust and, at the same time, a slight reduction in the colour of the crumb of bread enriched with barley malt flour compared to the control product. Similar results were obtained by Hager *et al.* [2011] for wheat bread with the addition of oat β-glucan.

The values of the crumb colour parameters of the tested breads with the addition of barley and wheat malt flours and the control bread were not significantly differentiated (Table 4). Adekunte *et al.* [2010] classified differences in perceivable colour as: very distinct (Δ*E*>3), distinct (1.5<Δ*E*<3), and small difference (Δ*E*<1.5). In the case of the breads enriched with malt wheat flour, the difference in their crust color (except KW) was very distinct compared to the crust colour of the control breads (Δ*E*> 3), whereas the difference in crumb colour of the breads (except for FB) was distinct compared to the control bread crumb (1.5<Δ*E*<3). The Δ*E* value for FB was 1.10, indicating a little perceivable colour difference.

Analysis of crumb texture in the investigated breads

Results related to the texture profile of bread crumb made from flours of different wheat varieties and from baking blends with malts are presented in Table 5. Generally, the breads with both barley malt flour and wheat malt flour added presented lower values of texture parameters compared to the breads without malt flour addition. On the other hand, the value of any texture parameter of the crumb of the breads enriched with wheat malt flour did not differ significantly from that of the breads with barley malt flour. The addition of wheat malt flour and barley malt flour led a decrease in bread crumb hardness by on average 26.19% and 27.46%, respectively, compared to the control samples. In a study conducted by Zadeike *et al*. [2018], wheat malt flour was added at a rate of 2% in the process of ciabatta production, leading to 20.5% decrease in its hardness and to 12.6% decrease in its cohesiveness, compared to the control sample. Yang *et al*. [2020] reported that with an increasing amount of malt in the cookies, their hardness decreased (compared to the control sample) and they turned less brittle. The decrease in these parameters was associated with enhanced degradation of starch and protein by enzymes from the group of amylases and proteinases in the doughs with malt added. In turn, Goesaert *et al*. [2009] found that malt flour added to bread delayed the process of crumb hardening owing to both the activity of endoamylases reducing the strength of starch networks and the activity of exoamylases which cause splitting of amylopectins. In a study by Mäkinen & Arendt [2012], the hardness of breads with the addition of malt flour was found to increase with longer duration of storage (0–5 days). The increased wheat malt flour content in the baked products was

TABLE 5. Texture parameters of the crumb of breads made from flours of various wheat varieties and from flour blends with the addition of barley and wheat malt flours.

Bread	Hardness (N)	Cohesiveness	Elasticity	Springiness	Gumminess (N)	Chewiness (N)
RC	16.40±3.32^{a}	0.48±0.03^{cde}	0.25±0.02^{b-e}	11.29±0.25^{b}	7.83±1.59^{a}	6.54±1.36^{ab}
RB	11.85±2.65^{bc}	0.45±0.03^{e}	0.21±0.01^{f}	10.67±0.28^{cd}	5.25±1.04^{b}	4.15±0.84^{cd}
RW	11.57±2.37^{bc}	0.47±0.04^{de}	0.23±0.02^{ef}	10.52±0.33^{d}	5.30±1.05^{b}	4.07±0.94^{cd}
KC	14.68±3.02^{ab}	0.53±0.04^{bc}	0.28±0.02^{b}	11.47±0.34^{ab}	7.74±1.35^{a}	6.58±1.16^{a}
KB	10.33±2.26^{c}	0.51±0.06^{bcd}	0.24±0.02^{cde}	10.75±0.38^{cd}	5.26±1.09^{b}	4.20±0.94^{cd}
KW	10.89±3.72^{bc}	0.51±0.06^{bcd}	0.24±0.04^{def}	11.12±0.31^{bc}	5.60±2.52^{ab}	4.63±2.19^{bcd}
FC	10.88±2.41^{bc}	0.60±0.04^{a}	0.34±0.02^{a}	11.80±0.19^{a}	6.54±1.28^{ab}	5.71±1.12^{abc}
FB	8.16±1.78^{c}	0.55±0.05^{b}	0.27±0.03^{bcd}	11.02±0.40^{bc}	4.43±0.85^{b}	3.64±0.79^{d}
FW	8.35±1.40^{c}	0.56±0.03^{ab}	0.28±0.02^{bc}	11.29±0.27^{b}	4.68±0.81^{b}	3.92±0.71^{cd}

Data are expressed as mean ± standard deviation (n=3). Values within columns with different letters are significantly different (p<0.05)
RC, Reform variety without malt; RB, Reform variety with barley malt; RW, Reform variety with wheat malt; FC, Findus variety without malt; FB, Findus variety with barley malt; FW, Findus variety with wheat malt; KC, Kilimanjaro variety without malt; KB, Kilimanjaro variety with barley malt; KW, Kilimanjaro variety with wheat malt.

associated with a decrease in hardness, compared to the control sample and baked products enhanced with barley malt. Furthermore, these authors found that even a small addition of malts (0.5%) extended the shelf-life of the baked products even with longer storage time. In the present study, the addition of wheat malt flour led to a decrease in the gumminess and chewiness on average by 29.47% and 32.92%, respectively, and the addition of barley malt flour resulted in a mean decrease by 32.42% and 36.32%, respectively, compared to the control samples (Table 5). The values of cohesiveness, springiness, and elasticity also slightly decreased in the baked products with malt flour added, compared to the control samples, which shows that both wheat malt and barley malt flour added to the baking blend in the right proportion positively affects the texture properties of the crumb, which increases attractiveness of the finished product.

CONCLUSION

Long-term storage of surplus wheat grain (*e.g.*, resulting from abundant harvests or high wheat yield) leads to a gradual deterioration of its quality. Wheat and barley malt flours can be added as technological improvers to wheat flour made from long-stored grain. Breads with a small amount of barley malt flour (0.5% on average) or wheat malt flour (0.7% on average) featured higher values of physical parameters, such as loaf volume and specific volume compared to the control breads (without malt flour). The use of malt flour darkened the colour of bread crust and crumb and positively affected the texture profile of the bread produced. Malts are products available all year round, but are slightly more expensive than wheat grain due to the technological processes (malting and drying) used in their production. However, they are added to bread in small amounts, which should not increase the economic costs of the bread making. Wheat grain surplus stored in warehouses can be successfully used for the production of bread enriched with malts flour.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

ORCID IDs

J. Belcar https://orcid.org/0000-0002-2306-8716
J. Gorzelany https://orcid.org/0000-0002-1028-097X
J. Kaszuba https://orcid.org/0000-0002-3327-8370

REFERENCES

1. AACC (2009). AACC International Approved Methods of Analysis, 11th ed., International: St. Paul, MN, USA.
2. Adekunte, A.O., Tiwari, B.K., Cullen, P.J., Scannell, A.G.M., O'Donnell, C.P. (2010). Effect of sonication on colour, ascorbic acid and yeast inactivation in tomato juice. *Food Chemistry*, *122*(3), 500–507. https://doi.org/10.1016/j.foodchem.2010.01.026
3. Agrahar-Murugkar, D., Gulati, P., Kotwaliwale, N., Gupta, C. (2015). Evaluation of nutritional, textural and particle size characteristics of dough and biscuits made from composite flours containing sprouted and malted ingredients. *Journal of Food Science and Technology*, *52*(8), 5129–5137. https://doi.org/10.1007/s13197-014-1597-y
4. Baik, B.K., Donelson, T. (2018). Postharvest and postmilling in wheat grain and flour quality characteristics. *Cereal Chemistry*, *95*(1), 141–148. https://doi.org/10.1002/cche.10013
5. Belcar, J., Matłok, N., Gorzelany, J. (2020). Technological assessment of winter cultivar of common wheat (*Triticum aestivum* L.) and winter barley (*Hordeum vulgare* L.) for pale malt production. *Acta Universitatis Cibiniensis. Series E: Food Technology*, *24*(1), 89–98. https://doi.org/10.2478/aucft-2020-0008
6. El-Sohaimy, A., Shehata, G., Djapparovec, T.A., Mehany, T., Zeitoun, A., Zeitoun, M. (2021). Development and character-

ization of functional pan bread supplemented with quinoa flour. *Journal of Food Processing and Preservation*, *45*(2), art. no. e15180. https://doi.org/10.1111/jfpp.15180
7. Farzaneh, V., Ghodsvali, A., Bakhshabadi, H., Zare, Z., Carvalho, I.S. (2017). The impact of germination time on the some selected parameters through malting process. *International Journal of Biological Macromolecules*, *94*, Part A, 663–668. https://doi.org/10.1016/j.ijbiomac.2016.10.052
8. Goesaert, H., Slade, L., Levine, H., Delcour, J.A. (2009). Amylases and bread firming – an integrated view. *Journal of Cereal Science*, *50*(3), 345–352. https://doi.org/10.1016/j.jcs.2009.04.010
9. González-Torralba, J., Arazuri, S., Jarén, C., Arregui, L.M. (2013). Influence of temperature and r.h. during storage on wheat bread making quality. *Journal of Stored Products Research*, *55*, 134–144. https://doi.org/10.1016/j.jspr.2013.10.002
10. Gupta, M., Bawa, A.S., Semwal, A.D. (2009). Morphological, thermal, pasting and rheological properties of barley starch and their blends. *International Journal of Food Properties*, *12*(3), 587–604. https://doi.org/10.1080/10942910801947763
11. Hager, A.-S., Ryan, L.A.M., Schwab, C., Gänzle, M.G., O'Doherty, J.V., Arendt, E.K. (2011). Influence of the soluble fibres inulin and oat β-glucan on quality of dough and bread. *European Food Research and Technology*, *232*, 405–413. https://doi.org/10.1007/s00217-010-1409-1
12. Honců, I., Krejćířová, L., Příhoda, J., Sluková, M. (2015). The effect od addition of malt flour on the dough, volume and sensory properties of bread. *PARIPEX – Indian Journal of Research*, *4*(9), 152–155.
13. Hrušková, M., Švec, I., Kučerová, I. (2003). Effect of malt flour addition on the rheological properties of wheat fermented dough. *Czech Journal of Food Science*, *21*(6), 210–218. https://doi.org/10.17221/3500-CJFS
14. ICC. (1992a). International Association for Cereal Science and Technology; Standard Method No. 115/1. Method for using the Brabender Farinograph. ICC Secretariat, Vienna.
15. ICC (1992b). International Association for Cereal Science and Technology; Standard Method No. 126/1. Method for using the Brabender Amylograph. ICC Secretariat, Vienna.
16. ICC. (1994). International Association for Cereal Science and Technology; Standard Method No. 155. Determination of wet gluten quantity and quality of whole wheat meal and wheat flour. ICC Secretariat, Vienna.
17. ICC. (1995). International Association for Cereal Science and Technology; Standard Method No.107/1. Determination of the "falling number" according to Hagberg-Perten as a measure of the degree of alpha-amylase activity in grain and flour. ICC Secretariat, Vienna.
18. Jakubczyk, T., Haber, T., (Eds). (1983). Analysis of Cereals and Cereal Products, Warsaw University of Life Sciences Press, Warsaw, ISBN: 83–00–01628–7, pp. 265–270 (in Polish).
19. Kleinwächter, M., Müller, Ch., Methner, F.–J., Selmar, D. (2014). Biochemical heterogeneity of malt is caused by both biological variation and differences in processing. I. Individual grain analyses of biochemical parameters in differently steeped barley (*Hordeum vulgare* L.) malts. *Food Chemistry*, *147*, 25–33. https://doi.org/10.1016/j.foodchem.2013.09.090
20. Krochmal-Marczak, B., Tobiasz-Salach, R., Kaszuba, J. (2020). The effect of adding oat flour on the nutritional and sensory quality of wheat bread. *British Food Journal*, *122*(7), 2329–2339. https://doi.org/10.1108/BFJ-07-2019-0493
21. Li Vigni, M., Durante, C., Foca, G., Ulrici, A., Møller Jespersen, B.P., Bro, R., Cocchi, M. (2010). Wheat flour formulation by mixture design and multivariate study of its technological properties. *Journal of Chemometrics*, *24*(7–8), 523–533. https://doi.org/10.1002/cem.1286
22. Mäkinen, O.E., Arendt, E.K. (2012). Oat malt as a baking ingredient – A comparative study of the impact of oat, barley and wheat malts on bread and dough properties. *Journal of Cereal Science*, *56*(3), 747–753. https://doi.org/10.1016/j.jcs.2012.08.009
23. Martins, R.C., Silva, C.L.M. (2002). Modelling colour and chlorophyll losses of frozen green beans (*Phaseolus vulgaris* L.). *International Journal of Refrigeration*, *25*(7), 966–974. https://doi.org/10.1016/S0140-7007(01)00050-0
24. Patras, A., Brunton, N.P., Tiwari, B.K., Butler, F. (2011). Stability and degradation kinetics of bioactive compounds and colour in strawberry jam during storage. *Food and Bioprocess Technology*, *4*(7), 1245–1252. https://doi.org/10.1007/s11947-009-0226-7
25. Polish Committee for Standardization. (2015). Wheat and wheat flour – Gluten content – Part 2 Determination of wet gluten quantity and gluten index by mechanical equipment. PCS standard PN-EN ISO 21415–2:2015–12 (in Polish).
26. Polish Committee for Standardization. (2012). Cereal grains and processed cereals – Determination of moisture (Reference method). PCS standard (in Polish).
27. Rögner, N.S., Mall, V., Steinhaus, M. (2021a). Impact of malt extract addition on odorants in wheat bread crust and crumb. *Journal of Agricultural and Food Chemistry, 69*(45), 13586–13595. https://doi.org/10.1021/acs.jafc.1c05638
28. Rögner, N.S., Mall, V., Steinhaus, M. (2021b). Odour-active compounds in liquid malt extracts for the baking industry. *European Food Research and Technology*, *247*, 1263–1275. https://doi.org/10.1007/s00217-021-03707-z
29. Schmitt, M.R., Skadsen, R.W., Budde, A.D. (2013). Protein mobilization and malting–specific proteinase expression during barley germination. *Journal of Cereal Science*, *58*(2), 324–332. https://doi.org/10.1016/j.jcs.2013.05.007
30. Skendi, A., Biliaderis, C.G., Papageorgiou, M., Izydorczyk, M.S. (2010). Effect of two barley β-glucan isolates on wheat flour dough and bread properties. *Food Chemistry*, *119*(3), 1159–1167. https://doi.org/10.1016/j.foodchem.2009.08.030
31. Sobczyk, A., Pycia, K., Stankowski, S., Jaworska, G., Kuźniar, P. (2017a). Evaluation of the rheological properties of dough and quality of bread made with the flour obtained from old cultivars and modern breeding lines od spelt (*Triticum aestivum ssp. spelta*). *Journal of Cereal Science*, *77*, 35–41. https://doi.org/10.1016/j.jcs.2017.07.013
32. Sobczyk, A., Pycia, K., Jaworska, G., Kaszuba, J. (2017b). Comparison of fermentation strength of the flours obtained from the grain of old varieties and modern breeding lines of spelt (*Triticum aestivum ssp. spelta*). *Journal of Food Processing and Preservation*, *41*(6), art. no. e13293. https://doi.org/10.1111/jfpp.13293

33. Yang, B., Guo, M., Zhao, Z. (2020). Incorporation of wheat malt into a cookie recipe and its effect on the physicochemical properties of the corresponding dough and cookies. *LWT – Food Science and Technology*, *117*, art. no. 108651. https://doi.org/10.1016/j.lwt.2019.108651
34. Zadeike, D., Jukonyte, R., Juodeikiene, G., Bartkiene, E., Valatkeviciene, Z. (2018). Comparative study of ciabatta crust crispness through acoustic and mechanical methods: Effects of wheat malt and protease on dough rheology and crust crispness retention during storage. *LWT – Food Science and Technology*, *89*, 110–116. https://doi.org/10.1016/j.lwt.2017.10.034
35. Zarzycki, P., Sobota, A., Ciesielska, Ż. (2012). Effect of storage time on the falling number and apparent viscosity of wheat flour gruels. *ŻYWNOŚĆ. Nauka. Technologia. Jakość, 6*(85), 66–78 (in Polish; English abstract). https://doi.org/10.15193/zntj/2012/85/066-078

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Ultrasound-Assisted Extractions for Improving the Recovery of Phenolics and Charantin from Bitter Melon and for Increasing the Antioxidant, Antidiabetic and Anti-Obesity Activities of Its Extracts

*Jin Ju Lee, Kyung Young Yoon**

Department of Food and Nutrition, Yeungnam University, Gyeongsan, Gyeongbuk 38547, South Korea

Key words: bitter melon, ultrasound-assisted extraction, charantin, phenolic compounds, antioxidant activity, antidiabetic activity

Bitter melon is rich in bioactive compounds and has a significant potential for commercial use as a functional food material. Its bioactive compound-rich extract was prepared using probe- or bath-type ultrasound-assisted extraction (UAE) with 60% (*v/v*) ethanol or distilled water. The composition and bioactivity of the extracts prepared using UAE was compared with those obtained by conventional extraction methods, such as autoclave extraction, ethanol extraction, and hot-water extraction. Although the yield of the autoclave extraction was the highest, the extracts obtained using UAE and aqueous ethanol exhibited a higher total phenolic content, antioxidant activity, antidiabetic activity (α-amylase and α-glucosidase inhibitory activities), and pancreatic lipase inhibitory activity than the conventional extracts. In particular, UAE with probe system and aqueous ethanol (UAE-P-E) was effective for extracting bioactive compounds, such as phenolics (total phenolic content of 18.73 mg GAE/g extract) and charantin (28.56 mg/g extract). Among all extracts, this prepared by UAE-P-E showed the highest DPPH radical scavenging activity (IC_{50} of 0.55 mg/mL), ferric reducing antioxidant power (250.5 μmol TE/g extract), and pancreatic lipase inhibitory activity (76.38% at a concentration of 3.0 mg/mL). These results suggest that bioactive compound-rich extracts from bitter melon obtained using UAE, especially UAE-P-E, are expected to have high application potential as a functional food material, and are also expected to be used as natural antioxidant, antidiabetic, and anti-obesity agents.

INTRODUCTION

Bitter melon *(Momordica charantia* L.) is an annual vine plant belonging to the Cucurbitaceae family and is widely grown in tropical and subtropical regions, such as Asia, Africa, and central Europe [Jia *et al*., 2017]. Bitter melon is also called bitter gourd because of its characteristic bitter taste, as well as has various other names such as wild cucumber in Africa, karela in India, goya in Japan, and yoeju or bitter cucumber in South Korea [Grover & Yadav, 2004]. The large quantities of bioactive compounds, such as charantin, alkaloids, triterpenoids, phenolic acids, flavonoids, saponins, and carotenoids have been determined in this fruit [Day *et al*., 1990; Pugazhenthi & Murthy, 1995]. Among them, phenolic compounds were recognized as important. The type and content of phenolic compounds in bitter melon differs depending on the fruit part and its degree of ripening. The major phenolic compounds of green and ripe fruits are gallic acid, (+)-catechin, tannic acid, and caffeic acid [Kubola & Siriamornpun, 2008]. Horax *et al.* [2010] found that gallic acid, *p*-coumaric acid, quinic acid, 4-hydroxybenzyoic acid, caffeic acid, and gentisic acid were the main phenolic acids of pulp, peel, and seeds. The charantin is another main bioactive component of bitter melon. It exists as a mixture of two steroidal saponins, β-sitosteryl glucoside and stigmasteryl glucoside and is known for its hypoglycemic activity by acting on β-cells of the pancreas and promoting insulin secretion [Grover & Yadav, 2004]. The charantin content of bitter melon varies depending on the plant variety, genetic resource, fruit maturity, growing region, and cultivated environment [Goo *et al*., 2016; Lee *et al*., 2016; Mahwish *et al*., 2021].

The extraction method influences the content of bioactive compounds and the biological activity of the extract; thus, it is an important step in the process of recovering bioactive constituents from plant materials. Conventional extraction techniques, such as Soxhlet extraction, maceration, and steam distillation, have been used to extract bioactive compounds from plants [Ajila *et al*., 2011]. However, they have certain drawbacks, such as low yield, degradation of the target compounds, large solvent requirements, and long extraction times; therefore, elicit adverse impact on the environment and human health [Chemat *et al*., 2012]. Recently, green extraction methods, such as pressurized liquid extraction, pressurized hot-water extraction, supercritical extraction, microwave-assisted extraction, and ultrasound-assisted extraction (UAE), have been used as alternatives to the conventional methods [Ajila *et al.*, 2011; Lončarić *et al*., 2020].

* Corresponding Author:
Tel.: +82-53-810-2878; Fax: +82-53-810-4768;
E-mail: yoonky2441@ynu.ac.kr (K.Y. Yoon)

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UAE is used for the efficient recovery of bioactive compounds, including phenolics, from various plant materials [Horzic *et al*., 2012]. There are two types of ultrasonic systems: bath and probe types, depending on the location of the ultrasound. Ultrasound generates cavitation bubbles in the extraction solvent, and the collapse of these bubbles leads to the extreme conditions of high temperature and high pressure [Rostagno *et al*., 2003]. During ultrasound treatment, the plant cell wall located near the bubble collapses, facilitating the penetration of the solvent into the plant tissue, and thereby increasing the release of organic compounds inside the plant cell [Sharmila *et al*., 2016]. The UAE methods provide a simple alternative to the conventional extraction methods, increase the extraction speed, and minimize the volume of solvent used, thereby enabling the efficient extraction of useful components [Vilkhu *et al.*, 2008]. The proposed study aimed to produce an extract rich in bioactive compounds from bitter melon for further application in the food industry as a functional ingredient with potent biological activities. For this purpose, bioactive compound-rich extracts were prepared using UAE (in bath and probe systems), a green extraction technique, and the bioactive compound contents and health-related properties, including antioxidant, antidiabetic, and pancreatic lipase inhibitory activities of the extract, were measured. In addition, the studied bioactive compound compositions and activities were compared with those of the extracts obtained by conventional extraction methods, such as autoclave extraction (AE-W), aqueous ethanol extraction (EE), and hot-water extraction (HWE).

MATERIALS AND METHODS

Materials and chemicals

Bitter melon (15–20 cm), cultivated in Gyeungsan (South Korea), was purchased, washed, and had its seed removed. The pericarp was lyophilized and ground into a fine powder (approximately 0.2 mm). Ground samples were stored at –40°C in a deep freezer (MDF, Sanyo, Tokyo, Japan).

HPLC standards of phenolic compounds (gallic acid, chlorogenic acid, (–)-epicatechin, (+)-catechin, caffeic acid), naringin, Folin–Ciocalteu phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, porcine pancreatic α-amylase (≥5 units/mg solid), *p*-nitrophenyl-α-D-glucopyranoside (*p*-PNG), α-glucosidase (from *Saccharomyces cerevisiae,* ≥5 units/mg protein using *p*-PNG), porcine pancreatic lipase (100–650 units/mg protein using olive oil), Trolox, ascorbic acid, and acarbose were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Charantin (mixture of stigmasta-5,25-dien-3β-yl β-D-glucopyranoside and β-sitosteryl glucoside; $C_{35}H_{60}O_6$ and $C_{35}H_{58}O_6$; molecular weight 576.85 and 574.83; purity: 91.2% *w/w*) used as an HPLC standard was purchased from ChromaDex (Santa Ana, CA, USA). Orlistat was purchased from Tokyo Chemical Co. (Tokyo, Japan). Ethanol and other chemicals were of analytical grade.

Extract preparation

The extracts of freeze-dried and ground bitter melon were prepared using ultrasound-assisted extraction with a probe system and 60% (*v*/v) ethanol (UAE-P-E) or water (UAE-P-W), ultrasound-assisted extraction with an ultrasonic bath and 60% (v/v) ethanol (UAE-B-E), autoclave extraction with water (AE-W), 60% (*v*/v) ethanol extraction (EE), and hot-water extraction (HWE). The extraction conditions were selected based on previously reported optimized methods for the extraction of bioactive ingredients from various natural materials [Lee & Yoon, 2021; Suh *et al.*, 2017]. For UAE with the probe system, 2 g of ground sample was added to a certain amount of solvent (60% (*v/v*) ethanol or distilled water) corresponding to 1:20 (*w/v*), and extraction was performed using an ultrasonic probe device (KFS-600N, Korprotech, Seoul, South Korea) for 15 min at a constant frequency of 20 kHz and an ultrasonic intensity of 270 W. For UAE-B-E, a ground sample of bitter melon (2 g) was mixed with 40 mL of 60% (*v*/v) ethanol (1:20, *w*/v) in an Erlenmeyer flask (100 mL). The flask was placed in an ultrasonic bath (5510-DTH, Brason, Danbury, CT, USA), with circulating water, set at 40°C and 20 kHz, with an ultrasound intensity of 270 W for 15 min. To prepare the extract using AE-W, the sample and distilled water were mixed in a ratio of 1:20 (*w/v*), and the mixture was autoclaved at 121°C at 0.13 MPa for 15 min. For EE and HWE, a ground sample (2 g) was mixed with 60% (*v/v*) ethanol or distilled water in an Erlenmeyer flask at a 1:20 (*w/v*), and the mixture was then placed in a shaking water bath (BS-11, JeioTech, Seoul, South Korea) for 3 h at 60°C and 90°C, respectively. The mixture obtained using each extraction method was centrifuged at 12,000×*g* and 4°C for 20 min, and the supernatant was condensed using an evaporator (N-1000, EYELA, Tokyo, Japan) at 35°C and freeze-dried. All the above extraction procedures were performed in triplicate. The extract powders were stored in a deep freezer until further use, and then dissolved in distilled water to prepare a constant concentration and used as an analytical sample.

Determination of total phenolic content and total flavonoid content

The total phenolic content (TPC) of the extracts was measured using the Folin–Ciocalteu method [Folin & Ciocalteu, 1927] with gallic acid as the standard. Briefly, 0.1 mL of the extract was mixed with the Folin–Ciocalteu's phenol reagent (0.1 mL) and allowed to react for 3 min, followed by the addition of 2 mL of distilled water and 0.2 mL of 10% sodium bicarbonate. The absorbance of the mixture was measured at 725 nm (U-2000 spectrophotometer, Hitachi, Tokyo, Japan) after incubation at room temperature for 1 h. The results were expressed as mg gallic acid equivalents (GAE)/g extract.

The total flavonoid content (TFC) was determined using the method reported by Sembiring *et al.* [2018]. Extract (0.5 mL, 10 mg/mL) was added to 0.1 mL of 10% aluminum nitrate and 0.1 mL of 1 M potassium acetate. Then, 4.3 mL of 80% (*v/v*) ethanol was added, the sample was then mixed and left in the dark for 40 min, and its absorbance was measured at 415 nm (U-2000 spectrophotometer, Hitachi). Naringin was used as the standard, and the results were expressed as mg naringin equivalents (NE)/g extract.

HPLC ANALYSIS OF CHARANTIN

The charantin content of the extracts was determined by the HPLC method described previously by Goo *et al*. [2016] with some modifications. The Waters 2695 HPLC device (Waters Co., Milford, MA, USA) equipped with a Waters 2489 UV detector and an Atlantis dC18 column (4.6×150 mm, 5 μm; Waters Co.) was used. The extract dissolved in distilled water was passed through membrane filters (0.45 μm pore size, Millipore, Billerica, MA, USA) and injected into the HPLC column. The column temperature was maintained at 30°C, and the injection volume was 10 μL. The mobile phase consisted of water (solvent A) and 100% acetonitrile (solvent B). The following gradient program was used for the separation of charantin: 85–95% B (0–15 min), 95% B (15–20 min), 95–85% B (20–35 min), 85% B (35–45 min). The analysis was performed at a flow rate of 1 mL/min, with the detection wavelength set at 197 nm. Peak identification was done by comparing the retention time with that of a standard solution, and the charantin content was expressed as mg/g extract.

HPLC ANALYSIS OF PHENOLIC COMPOUNDS

The content of phenolic compounds in the extract was measured according to the method described in the study of Nour *et al*. [2013]. The phenolic compounds were analyzed using HPLC under the same conditions as those used for the charantin analysis described above, except for the mobile phase, which consisted of 1% (*v/v*) phosphoric acid (solvent A) and 100% acetonitrile (solvent B), and the detection wavelength which was 280 nm. The following gradient program was used for the separation of phenolic compounds: 10–30% B (0–27 min), 30–44% B (27–55 min), 44–10% B (55–60 min). Peak identification was performed using phenolic standards, and the content of phenolic compounds was expressed as mg/g extract.

Determination of antioxidant activity

The DPPH radical scavenging activity of extracts was determined as described by Brand-Williams *et al.* [1995]. The bitter melon extracts were dissolved in distilled water with different concentrations ranging from 250 to 2,500 μg/mL. Sample solution (100 μL) and 0.2 mM DPPH radical solution (200 μL) were added to each well of a 96-well-plate. The plate was incubated at 37°C for 30 min, and the absorbance was measured at 517 nm using a microplate reader (Epoch, BioTek Instrument Inc., Winooski, VT, USA). Distilled water was used in the control sample instead of the sample solution, and ascorbic acid was used as a positive control. The following formula was used to calculate the DPPH radical scavenging activity:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100 \quad (1)$$

where: A_{sample} is the absorbance with the test sample, and $A_{control}$ is the absorbance with distilled water instead of test sample. DPPH radical scavenging activity was expressed as half-maximal inhibitory concentration (IC_{50}) defined as the extract concentration, that is required to inhibit 50% of the DPPH radicals.

Ferric reducing antioxidant power (FRAP) was measured using the method described by Benzie & Strain [1996] with some modifications. Sodium acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ, 10 mM), and ferric chloride (20 mM) were prepared and mixed in a ratio of 10:1:1 (*v/v/v*) and used as the FRAP reagent. Next, 175 μL of FRAP reagent was added to 25 μL of extract solution (concentration of 250–2,000 μg/mL), and after reacting at 37°C for 30 min, the absorbance was measured at 590 nm using a microplate reader (Epoch, BioTek Instrument Inc.). Standard curve was prepared using different concentrations (0.63, 0.125, 0.250, 0.5, 1.0. μM) of Trolox. The results were expressed as μmol Trolox equivalents (TE)/g extract.

Determination of antidiabetic activity

α-Amylase inhibitory activity

The α-amylase inhibitory activity of the extract was evaluated according to the method described by Kazeem *et al*. [2013]. Sample solution (20 μL; 250–2,000 μg/mL), phosphate buffer (50 μL, 100 mM, pH 6.8), and 0.1 mL of α-amylase (from porcine pancreas, 1 U/mL) were placed in a tube. This mixture was pre-incubated at 37°C for 5 min, after which 0.1 mL of 1% (*w/v*) soluble starch was added, and the mixture was incubated at 37°C for 5 min. The reaction was terminated by adding 0.1 mL of aliquot of 3,5-dinitrosalicylic acid color reagent solution and then boiled at 95°C for 15 min. Finally, 0.9 mL of distilled water was added to the reaction mixture and vortexed, and the absorbance was measured at 540 nm. Acarbose (concentration of 100––1,000 μg/mL) was used as a positive control. Inhibitory activity was calculated using equation (2) and curves of inhibitory activity *vs* concentration were plotted. α-Amylase inhibitory activity was expressed as IC_{50} defined as the extract concentration, which is required to inhibit 50% of the enzyme activity.

$$\text{Inhibitory activity (\%)} = \left(1 - \frac{A_{sample} - A_{blank}}{A_{control}}\right) \times 100 \quad (2)$$

where: A_{sample} is the absorbance with the test sample, A_{blank} is the absorbance with distilled water instead of substrate, and $A_{control}$ is the absorbance with distilled water instead of test sample.

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was determined using the method reported by Kim *et al.* [2004]. The sample solution (50 μL; 250–2,000 μg/mL) was blended with 50 μL of α-glucosidase (0.2 U/mL) dissolved in 0.2 M potassium phosphate buffer (pH 6.8), followed by pre-incubation at 37°C for 15 min. Then, 0.1 mL of 3 mM *p*-NPG was added to initiate the enzymatic reaction, and the mixture was incubated at 37°C for 10 min. NaOH (50 μL, 0.1 M) was added to stop the reaction, and the absorbance was measured at 405 nm. Acarbose (concentration of 100–1,000 μg/mL) was used as a positive control. The inhibitory activity was calculated using equation (2), as described above. α-Glucosidase inhibitory activity was expressed as IC_{50} – the extract concentration that required to inhibit 50% of the enzyme activity.

Determination of pancreatic lipase inhibitory activity

The pancreatic lipase inhibitory activity was determined to investigate the anti-obesity effect of the extract. Porcine pancreatic lipase (6 μL, 10 mg/mL) dissolved in enzyme buffer (10 mM 3-(*N*-morpholino)propanesulfonic acid, 1 mM ethylene-diamine-tetraacetic acid, pH 6.8), 170 μL of Tris buffer (100 mM Tris, 5 mM $CaCl_2$, pH 7.0), and 20 μL of each concentrated sample (0.5, 1.0, 2.0, and 3.0 mg/mL) was added to a 96 well-plate, the mixture was mixed well and incubated at 37°C for 15 min. Next, 4 μL of 10 mM *p*-nitrophenyl butyrate was added to the reaction solution, which was incubated at 37°C for 60 min. The absorbance of the reaction solution was measured at 400 nm using a microplate reader (Epoch, BioTek, Instrument Inc.). Orlistat (0.5 mg/mL) was used as a positive control and the lipase inhibitory activity was calculated using equation (2) as described above.

Statistical analysis

All experiments were performed in triplicate, and the experimental results were expressed as the mean± and standard deviation. One-way analysis of variance (ANOVA) was performed using the SPSS version 23.0 (SPSS Inc., Chicago, IL, USA) and Duncan's multiple range test comparisons were performed at $p<0.05$ to determine the statistically significant differences.

RESULTS AND DISCUSSION

Extraction yield

The yields of extraction of bitter melon by various methods are shown in Figure 1. The yield of AE-W was the highest (50.22%), followed by HWE (42.60%), UAE-P-W (37.72%), UAE-P-E (34.84%), UAE-B-E (34.38%), and EE (33.69%). There was no significant ($p\geq0.05$) difference between the yields of UAE-P-E and UAE-B-E. The yields of extraction with water were higher than those with aqueous ethanol. The extraction efficiency of functional ingredients from plants is affected by various factors, such as the solvent type, material to solvent ratio, temperature, and pH [Vilas-Boas *et al*., 2020]. In particular, the type and concentration of solvent are important factors that have the most significant influence on the extraction efficiency [Gunathilake *et al*., 2019]. Water is the most polar solvent and it can easily extract many polar plant components that dissolve in it. There are bioactive compounds among them. However, undesirable compounds, such as carbohydrates and proteins, can also be eluted into the water together with bioactive compounds [Do *et al.*, 2014]. The combination of water and organic solvents can facilitate the extraction of bioactive compounds that are soluble in water and/or organic solvents. Therefore, aqueous ethanol is most often used to extract bioactive components, including phenolic compounds, from various plants [Sun *et al*., 2020]. Temperature also significantly affects the extraction efficiency, as higher temperatures increase solubility of the solute in the solvent and increase the rate at which the solute diffuses into the solvent bulk, resulting in a higher mass transfer rate [Cacace & Mazza, 2003]. In our study, the highest extraction yield of AE-W could be due to the high temperature and high dissolving ability of water.

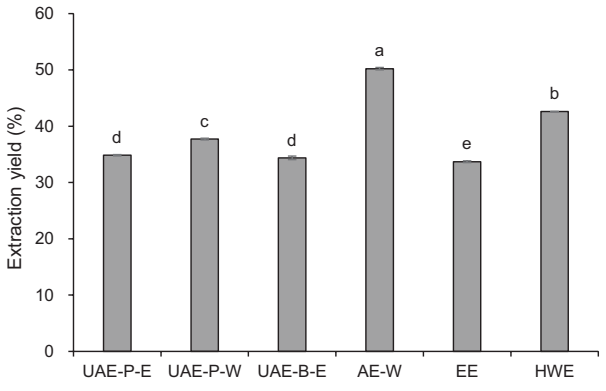

FIGURE 1. Yield of extraction of bitter melon by various extraction methods. UAE-P-E, ultrasound-assisted extraction with a probe system and 60% (*v*/v) ethanol; UAE-P-W, ultrasound-assisted extraction with a probe system and water; UAE-B-E, ultrasound-assisted extraction with an ultrasonic bath and 60% (*v*/v) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v*/*v*) ethanol extraction; HWE, hot-water extraction. Bar represents the mean and standard deviation ($n=3$). Values with different letters are significantly different at $p<0.05$.

Total phenolic, total flavonoid, and charantin contents

The contents of total phenolics, total flavonoids, and charantin in the extracts obtained from bitter melon by various methods were determined, and the results are shown in Table 1. Phenolics are a diverse group of compounds with various structures and molecular weights that exist in abundance as secondary metabolites in plants. Phenolic compounds are found in various plants and exhibit various biological activities such as anticancer, anti-allergic, antibacterial, anti-inflammatory, and antithrombotic, as well as acting as antioxidants [Sen *et al*., 2013]. The TPC of the bitter melon extract was in the range of 11.08–18.73 mg GAE/g extract, and the highest TPC was found in the extract obtained using UAE-P-E (Table 1). For the remaining extracts, TPC decreased in the following order of extraction methods: UAE-B-E > UAE-P-W > EE > AE-W > HWE. These results are consistent with findings published by Dobrincic *et al.* [2020], who reported that the TPC of extracts obtained from olive leaves using UAE was higher than those of extracts obtained by high pressure-assisted extraction and conventional heat-reflux extraction. The TFC of extracts obtained using ethanol was higher than that of the extract obtained using water as a solvent (Table 1). The highest TFC was found in the extract obtained using UAE-P-E (8.29 NE/g extract). In other extracts, TFC decreased in the following order of extraction methods: UAE-B-E > EE > UAE-P-W = AE-W = HWE.

Generally, UAE produced the extract with higher TPC and TFC than the other extraction methods. This phenomenon can be explained by the destruction of the cell wall by the bubbles generated by cavitation caused by ultrasonic waves during the UAE, which increased the elution and solubility of substances inside the cell [Horzic *et al.*, 2012]. In addition, UAE-P-E produced the extract with higher TPC and TFC than UAE-B-E. This could be because the ultrasonic generator was located at the bottom of the ultrasonic bath and indirectly transmitted energy to the sample through

TABLE 1. Total phenolic, total flavonoid, and charantin contents of bitter melon extracts obtained by various extraction methods.

Extraction method	Total phenolics (mg GAE/g extract)	Total flavonoids (mg NE/g extract)	Charantin (mg/g extract)
UAE-P-E	18.73±0.18[a]	8.29±0.12[a]	28.56±0.36[a]
UAE-P-W	14.45±0.26[c]	3.58±0.03[d]	Nd
UAE-B-E	16.34±0.24[b]	7.39±0.13[b]	17.79±0.45[b]
AE-W	12.66±0.30[e]	3.99±0.12[d]	Nd
EE	13.53±0.22[d]	5.37±0.29[c]	14.60±0.24[c]
HWE	11.08±0.06[f]	3.62±0.11[d]	Nd

Results are shown as mean ± standard deviation (n=3).
UAE-P-E, ultrasound-assisted extraction with a probe system and 60% (*v/v*) ethanol; UAE-P-W, ultrasound-assisted extraction with a probe system and water; UAE-B-E, ultrasound-assisted extraction with an ultrasonic bath and 60% (*v/v*) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v/v*) ethanol extraction; HWE, hot-water extraction; GAE, gallic acid equivalent; NE, naringin equivalent; Nd, not detected.
Values with different letters in the same column are significantly different at $p<0.05$.

the medium during UAE-B, whereas in UAE-P, energy was directly transferred to the sample by ultrasonic waves generated from the probe, which increased the ultrasonic efficiency [Capelo *et al*., 2005].

The content of charantin was the highest in the bitter melon extract obtained using UAE-P-E (28.56 mg/g extract), followed by UAE-B-E and EE (Table 1). Charantin was detected only in the aqueous ethanolic extract, presumably because charantin, a saponin component, has a higher affinity for ethanol than water. Kole *et al*. [2013] reported a difference in charantin content of 5.01–8.29 mg/g of lyophilized and powdered bitter melon depending on the extraction conditions, and Lee *et al.* [2016] reported charantin content in the range of 13.3–24.7 mg/g dry weight (DW), depending on the harvest period. Mahwish *et al*. [2021] also reported that the charantin content of bitter melon differed depending on the part of the fruit, and was higher in the flesh part (0.16 mg/g DW) than in the whole fruit (0.11 mg/g DW) and skin (0.08 mg/g DW). As mentioned earlier, charantin demonstrates insulin-like activity by increasing insulin release from the pancreas and decreasing gluconeogenesis [Wang *et al.*, 2014]. Therefore, the high charantin extract obtained using UAE-P-E is expected to exhibit a significant antidiabetic activity.

Content of individual phenolics

The composition of phenolic compounds of bitter melon extracts was shown in Table 2. Five phenolic compounds (gallic acid, chlorogenic acid, (-)-epicatechin, (+)-catechin, and caffeic acid) were identified. Gallic acid was present in the highest content in all extracts, and chlorogenic acid had the second highest content. All extracts showed the highest gallic acid content, followed by chlorogenic acid content. The gallic acid content in the extracts obtained using UAE-P-E, UAE-B-E, UAE-P-W, EE, AE-W, and HWE was 4.55, 3.68, 3.35, 3.05, 2.12, and 2.11 mg/g extract, respectively, and the chlorogenic acid content was 1.08, 0.86, 0.76, 0.69, 0.69, and 0.52 mg/g extract, respectively. The content of total phenolic compounds detected by HPLC was 7.05 mg/g extract (UAE-P-E), 5.73 mg/g extract (UAE-B-E), 5.19 mg/g extract (UAE-P-W), 4.59 mg/g extract (EE), 3.30 mg/g extract (AE-W), and 3.28 mg/g extract (HWE). Thus, the total phenolic content in the extract obtained using UAE-P-E was 2.2 times higher than that in the extract obtained using HWE. Horax *et al.* [2010] reported that the main phenolics of the 80% ethanolic extract of bitter melon were catechin (1.54 mg/g extract), gentisic acid (0.72 mg/g extract), gallic acid (0.49 mg/g extract), chlorogenic acid (0.66 mg/g extract), epicatechin (0.29 mg/g extract), *o*-coumaric acid (0.27 mg/g extract), and procatechuic acid (0.12 mg/g extract). Thus, most of the identified phenolic compounds were similar to those found in this study, but their content was lower. Lopes *et al.* [2020] reported that a wider variety of phenolic compounds were extracted from bitter melon by UAE than by conventional extraction, and the content was also found to be higher than that in conventional extracts. Gallic acid is a representative phenolic that is widely distributed in plants and exhibits antioxidant, anti-inflammatory, and antibacterial activities [Bai *et al.*, 2020]. Chlorogenic acid has various bioactivities such as antibacterial, anti-inflammatory, antipyretic,

TABLE 2. Content of phenolic compounds of bitter melon extracts (mg/g extract) obtained using various extraction methods.

Extraction method	Gallic acid	Chlorogenic acid	(–)-Epicatechin	(+)-Catechin	Caffeic acid
UAE-P-E	4.55±0.28[a]	1.08±0.08[a]	0.61±0.06[a]	0.65±0.02[a]	0.16±0.01[a]
UAE-P-W	3.35±0.15[bc]	0.76±0.08[b]	0.51±0.03[a]	0.45±0.01[c]	0.12±0.01[c]
UAE-B-E	3.68±0.02[b]	0.86±0.04[ab]	0.54±0.04[a]	0.51±0.01[b]	0.14±0.01[b]
AE-W	2.12±0.09[d]	0.69±0.07[bc]	0.22±0.02[b]	0.19±0.02[f]	0.08±0.02[de]
EE	3.05±0.14[c]	0.69±0.05[bc]	0.43±0.02[b]	0.32±0.01[d]	0.10±0.01[d]
HWE	2.11±0.14[d]	0.52±0.02[c]	0.28±0.02[b]	0.26±0.01[e]	0.06±0.01[e]

Results are shown as mean ± standard deviation (n=3).
UAE-P-E, ultrasound-assisted extraction with a probe system and 60% (*v/v*) ethanol; UAE-P-W, ultrasound-assisted extraction with an probe system and water; UAE-B-E, ultrasound-assisted extraction with an ultrasonic bath and 60% (*v/v*) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v/v*) ethanol extraction; HWE, hot-water extraction.
Values with different letters in the same column are significantly different at $p<0.05$.

TABLE 3. Antioxidant activity of bitter melon extracts obtained using various extraction methods.

Extraction method	IC_{50} of DPPH radical scavenging activity (mg/mL)	Ferric reducing antioxidant power (μmol TE/g extract)
UAE-P-E	0.55±0.03^{e}	250.5±2.7^{a}
UAE-P-W	1.17±0.01^{bc}	170.0±2.9^{c}
UAE-B-E	1.01±0.03^{d}	214.3±6.2^{b}
AE-W	1.20±0.10^{b}	168.8±7.4^{c}
EE	1.07±0.04^{cd}	176.7±3.8^{c}
HWE	2.19±0.11^{a}	130.1±2.5^{d}
Ascorbic acid	0.12±0.12^{f}	–

Results are shown as mean ± standard deviation (n=3).
UAE-P-E, ultrasound-assisted extraction with a probe system and 60% (*v/v*) ethanol; UAE-P-W, ultrasound-assisted extraction with a probe system and water; UAE-B-E, ultrasound-assisted extraction with a ultrasonic bath and 60% (*v/v*) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v/v*) ethanol extraction; HWE, hot-water extraction; TE, Trolox equivalent.
Values with different letters in the same column are significantly different at $p<0.05$.

neuroprotective, anti-obesity, antiviral, antibacterial, and antihypertensive as well as antioxidant activity [Naveed *et al*., 2018]. In turn, (+)-catechin is a strong antioxidant cholesterol esterase inhibitor, arteriosclerosis preventing agent, as well as antibacterial and antiulcer agent [Pedro *et al.*, 2020]. Therefore, the phenolic-rich extracts of bitter melon, specially these obtained using UAE, are expected to exhibit high biological activity, including antioxidant activity.

Antioxidant activity

To evaluate the antioxidant activity of extracts of bitter melon, the DPPH radical scavenging activity and FRAP were determined and results are shown in Table 3. The IC_{50} values of the extract for DPPH radical scavenging activity ranged from 0.55 to 2.19 mg/mL, with the lowest value determined for the extract obtained using UAE-P-E followed by UAE-B-E, EE, UAE-P-W, AE-W, and HWE. IC_{50} of ascorbic acid was 0.12 mg/mL. These results of antioxidant activity was very high compared to that reported by Aljohi *et al.* [2016], who found that the DPPH radical scavenging activity of bitter melon extract was 50% at a concentration of 15 mg/mL. Nam & Kim [2015] reported that the ethanolic extract of dried unripe bitter melon demonstrated higher DPPH radical scavenging ability than the hot water extract at all tested concentrations, which was consistent with the results of this study.

The effect of the extraction method on FRAP of the extracts was similar as on DPPH radical scavenging activity; the highest FRAP was found for the extract obtained by UAE-P-E (250.5 μmol TE/g extract), followed by UAE-B-E (214.3 μmol TE/g extract), and the lowest by HWE (130.1 μmol TE/g extract). The FRAP values for EE, UAE-P-W, and AE-W samples were in the range from 168.8 to 176.7 μmol TE/g extract and did not differ significantly ($p\geq0.05$). A study by Alothman *et al*. [2009] found that ethanolic extracts of pineapples, banana, and guava had higher FRAP than those prepared using water. Ahmad-Qasem *et al.* [2013] reported that the extract obtained from olive leaves by UAE with ethanol had significantly higher FRAP than that obtained by EE, which is consistent with the results of this study.

Among the UAE samples, aqueous ethanolic extracts demonstrated higher antioxidant activity than that obtained using water. It is assumed that the phenolic compounds have a high affinity for ethanol compared to water, and the high antioxidant activity of the ethanolic extracts were due to the high phenolic content. In addition, the highest radical scavenging activity and reducing power of the extract produced with UAE-P-E were likely due to the high gallic acid and caffeic acid contents, which, according to literature reports, provide excellent radical scavenging activity [Chalas *et al.*, 2011].

Anti-diabetic activity

α-Amylase is an enzyme that breaks down α-D-(1,4)-glucoside bonds of polysaccharides composed of α-linked glucose, such as starch or glycogen, and is an essential enzyme for carbohydrate metabolism. Intestinal α-glucosidase is a membrane-bound enzyme located in the epithelium of the small intestine and breaks down starch and disaccharides into absorbable monosaccharides [Patel & Ghane, 2021]. For the treatment of diseases, such as non-insulin-dependent diabetes mellitus and hyperglycemia, in which carbohydrate metabolism does not proceed normally and is manifested by high blood sugar, due to insulin resistance, it is necessary to control the digestion and absorption of sugar [Nam & Kim, 2015]. Blood glucose is regulated when the activity of enzymes, which play an important role in carbohydrate digestion, is inhibited, and the breakdown and absorption of starch entering the body through meals decreases, causing the amount of glucose released into the blood to decrease [Kajaria *et al.*, 2013]. Therefore, in patients with non-insulin-dependent diabetes mellitus, obesity, and hyperglycemia, the activity levels of α-amylase and α-glucosidase serve as indicators of the suppression of blood glucose levels.

The antidiabetic activity of bitter melon extract was determined by α-amylase and α-glucosidase inhibitory activity assays, and results are shown in Table 4. The IC_{50} values for the α-amylase inhibitory activity of the bitter melon extract were 0.81 and 0.83 mg/mL for UAE-P-E and UAE-B-E samples, respectively. In increasing order, the IC_{50} values for other extracts were 0.92 mg/mL (UAE-P-W), 1.02 mg/mL (EE), 1.49 mg/mL (AE-W), and 1.62 mg/mL (HWE). The IC_{50} value of acarbose, a positive control, was 0.25 mg/mL, and the extract obtained using UAE-P-E with the highest α-amylase inhibitory activity, had approximately 31% of the activity of acarbose. α-Glucosidase inhibitory activity of extracts exhibited the same trend as the α-amylase inhibitory activity, and the UAE-P-E extract demonstrated the lowest value at 0.96 mg/mL, which corresponds to 74% of the activity of acarbose (0.71 mg/mL). These results were higher than those reported by Kang *et al.* [2018], who found that α-glucosidase inhibitory activity of hot water and ethanolic extracts from bitter melon were 29.65% and 66.88%, respectively, at a concentration of 2.5 mg/mL. In turn, Nam & Kim [2015] reported that the ethanolic extract obtained from

TABLE 4. Antidiabetic activity of bitter melon extracts obtained using various extraction methods.

Extraction method	α-Amylase inhibitory activity	α-Glucosidase inhibitory activity
	IC_{50} (mg/mL)	
UAE-P-E	0.81 ± 0.02^{e}	0.96 ± 0.02^{b}
UAE-P-W	0.92 ± 0.05^{cd}	1.26 ± 0.02^{d}
UAE-B-E	0.83 ± 0.01^{e}	1.02 ± 0.00^{bc}
AE-W	1.49 ± 0.04^{b}	1.66 ± 0.05^{e}
EE	1.02 ± 0.04^{c}	1.06 ± 0.03^{cd}
HWE	1.62 ± 0.03^{a}	1.88 ± 0.04^{f}
Acarbose	0.23 ± 0.01^{f}	0.71 ± 0.02^{a}

Results are shown as mean ± standard deviation (n=3).
UAE-P-E, ultrasound-assisted extraction with an probe system and 60% (*v/v*) ethanol; UAE-P-W, ultrasound-assisted extraction with an probe system and water; UAE-B-E, ultrasound-assisted extraction with an ultrasonic bath and 60% (*v/v*) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v/v*) ethanol extraction; HWE, hot-water extraction.
Values with different letters in the same column are significantly different at $p<0.05$.

immature dried bitter melon demonstrated significantly higher α-glucosidase inhibitory activity than the hot water extract, which was consistent with the results of the present study.

These results demonstrate that the antidiabetic activity of bitter melon extracts was correlated with TPC, suggesting that the TPC may have a significant effect on the antidiabetic activity. Polyphenols can be used to prevent and manage diabetes mellitus *via* insulin-dependent and independent approaches: the former protects pancreatic islet β-cells, reduces β-cell apoptosis, promotes β-cell proliferation, activates insulin signaling, and stimulates pancreas to secrete insulin, the latter inhibits glucose absorption, inhibits digestive enzymes, regulates intestinal microflora, and inhibits the formation of advanced glycation end products [Sun *et al.*, 2020]. The mechanism of the high inhibitory activity of the ultrasonicated extracts involves modifying the α-amylase molecular structure by the hydroxyl radical generated by the ultrasound action [Kadkhodaee & Povey, 2008]. The extract obtained using aqueous ethanol as a solvent exhibited a higher antidiabetic activity than the water extract, presumably due to the effect of charantin, an antidiabetic component of bitter melon.

Pancreatic lipase inhibitory activity

Pancreatic lipase, secreted from the pancreas and stomach, plays an important role in fat digestion by decomposing triglycerides into 2-monoacylglycerol and two fatty acids. Therefore, by inactivating pancreatic lipase activity, triglycerides and cholesterol are excreted outside the body without being digested and absorbed in the body, thereby preventing the accumulation of fat [Bitou *et al.*, 1999]. Orlistat, mainly used as a treatment for obesity, irreversibly binds to pancreatic lipase and inactivates the enzyme. Due to the enzyme inhibitory action of Orlistat, it reduces intestinal triglyceride and cholesterol absorption and increases excretion, thereby acting as an anti-obesity agent, but causing side effects such as abdominal pain, diarrhea, and headache [El-Korany *et al.*, 2020]. Therefore, pancreatic lipase inhibitors as dietary components are still being searched for.

The pancreatic lipase inhibitory activity of bitter melon extracts obtained using various extraction methods and Orlistat as a positive control, is shown in Figure 2. The pancreatic lipase inhibitory activity of Orilstat was 87.38% at a concentration of 0.5 mg/mL. For extracts, the inhibitory activity increased as the sample concentration increased. The extract obtained using UAE-B-E showed the highest pancreatic lipase inhibitory activity ($p<0.05$) between samples at a concentration of 0.5 mg/mL. In turn, values for UAE-P-E, UAE-B-E, and EE (40.43%-41.47%) were significantly ($p<0.05$) higher than those for other extracts at 1.0 mg/mL. In addition, at concentrations of 2.0 and 3.0 mg/mL, the highest pancreatic lipase inhibitory activity ($p<0.05$) was demonstrated by the extract obtained using UAE-P-E (62.93 and 76.38%,

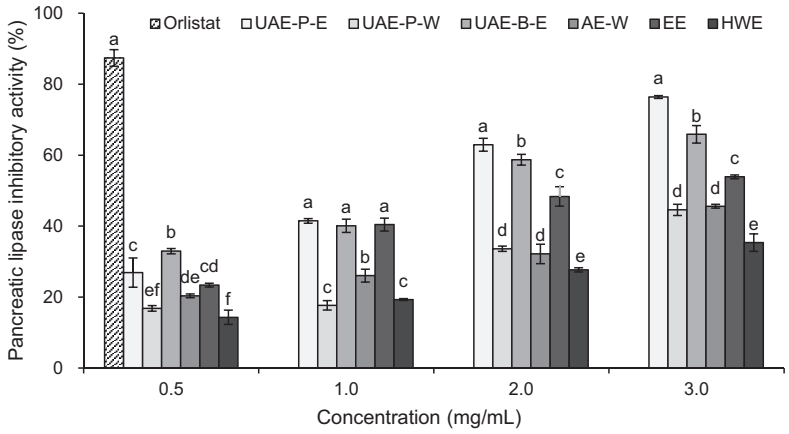

FIGURE 2. Pancreatic lipase inhibitory activity of bitter melon extracts obtained by various extraction methods. UAE-P-E, ultrasound-assisted extraction with a probe system and 60% (*v/v*) ethanol; UAE-P-W, ultrasound-assisted extraction with a probe system and water; UAE-B-E, ultrasound-assisted extraction with an ultrasonic bath and 60% (*v/v*) ethanol; AE-W, autoclave extraction with water; EE, 60% (*v/v*) ethanol extraction; HWE, hot-water extraction. Pancreatic lipase inhibitory activity of Orlistat was tested at a concentration of 0.5 mg/mL. Bar represents the mean and standard deviation (n=3). Values with different letters separately for each concentration are significantly different at $p<0.05$.

respectively). However, all water extracts exhibited low pancreatic inhibitory activity at all sample concentrations. Fan *et al*. [2019] reported that the major bioactive compounds of bitter melon showing the anti-obesity activity were triterpenoids, saponins, and phenolics, which inhibit fat synthesis, promote glucose utilization, and stimulate auxiliary lipid-lowering activity. In turn, McDougall *et al*. [2009] found that polyphenol fractions of strawberries and raspberries exhibited lipase inhibitory activity and reported that this was due to the polyphenol compounds, such as tannin, contained in berries. Reports also state that the hydroxyl groups present in polyphenol compounds enter into hydrophobic interactions with pancreatic lipase and inhibit enzyme activity [Deavile *et al.*, 2007].

CONCLUSIONS

In the present study, we compared the TPC, TFC, phenolic composition, charantin content, antioxidant, antidiabetic, and pancreatic lipase inhibitory activities of extracts of bitter melon obtained using five extraction methods. The results demonstrate the feasibility of producing extracts with high bioactivity. In particular, the extract obtained using an ultrasonic probe with 60% ethanol (UAE-P-E) showed not only high TPC and TFC, but also excellent DPPH radical scavenging activity, FRAP and pancreatic lipase inhibitory activity. In addition, it had a high charantin content and showed α-glucosidase inhibitory activity equivalent to 74% of acarbose used in diabetes treatment. Therefore, the extract obtained from bitter melon using UAE-P-E is a practical new material with antioxidant, antidiabetic and anti-obesity activities, and has the potential to be applied to manufacture functional foods in the food industry. Furthermore, the UAE-P-E extract is expected to be a useful agent in diabetes treatment; however, extensive research is needed for effective drug development in the future.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests

ORCID ID

K.Y. Yoon https://orcid.org/0000-0003-0626-5563

REFERENCES

1. Ahmad-Qasem, M.H., Canovas, J., Barrajon-Catalan, E., Micol, V., Carcel, J.A., Garcia-Perez, J.V. (2013). Kinetic and compositional study of phenolic extraction from olive leaves (var. Serrana) by using power ultrasound. *Innovative Food Science & Emerging Technologies, 17*, 120–129. https://doi.org/10.1016/j.ifset.2012.11.008
2. Ajila, C.M., Brar, K., Verma, M., Tyagi, R.D., Godbout, S., Valero, J.R. (2011). Extraction and analysis of polyphenols: Recent trends. *Critical Reviews in Biotechnology, 31*(3), 227–249. https://doi.org/10.3109/07388551.2010.513677
3. Aljohi, A., Matou-Nasri, S., Ahmed, N. (2016). Antiglycation and antioxidant properties of *Momordica charantia*. *PLOS ONE, 11*(8), art. no. e0159985. https://doi.org/10.1371/journal.pone.0159985
4. Alothman, M., Bhat, R., Karim, A.A. (2009). Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents. *Food Chemistry, 115*(3), 785–788. https://doi.org/10.1016/j.foodchem.2008.12.005
5. Bai, J., Zhang, Y., Tang, C., Hou, Y., Ai, X., Chen, X., Zhang, Y., Wang, X., Meng, X. (2020). Gallic acid: Pharmacological activities and molecular mechanisms involved in inflammation-related diseases. *Biomedicine & Pharmacotherapy, 133*, art. no. 110985. https://doi.org/10.1016/j.biopha.2020.110985
6. Benzie, I.F.F., Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry, 239*(1), 70–76. https://doi.org/10.1006/abio.1996.0292
7. Bitou, N., Nimomiya, M., Tsjita, T., Hsjita, H. (1999). Screening of lipase inhibitors from marine algae. *Journal of Lipid Research, 34*(5), 441–445. https://doi.org/10.1007/s11745-999-0383-7
8. Brand-Williams, W., Cavalier, M.E., Berset, C. (1995). Use of free radical method to evaluate antioxidant activity. *LWT – Food Science and Technology, 28*(1), 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5
9. Cacace, J., Mazza, G. (2003). Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering, 59*(4), 379–389. https://doi.org/10.1016/S0260-8774(02)00497-1
10. Capelo, J.L., Maduro, C., Vilhena, C. (2005). Discussion of parameters associated with the ultrasonic solid-liquid extraction for elemental analysis (total content) by electrothermal atomic absorption spectrometry. *Ultrasonics Sonochemistry, 12*(3), 225–232. https://doi.org/10.1016/j.ultsonch.2003.10.010
11. Chalas, J., Claise, C., Edeas, M., Messaoudi, C., Vergnes, L., Abella, A., Lindenbaum, A. (2011). Effect of ethyl esterification of phenolic acid on low-density lipoprotein oxidation. *Biomedicine & Pharmacotherapy, 55*(1), 54–60. https://doi.org/10.1016/S0753-3322(00)00011-1
12. Chemat, F., Vian, M.A., Cravotto, G. (2012). Green extraction of natural products: Concept and principles. *International Journal of Molecular Sciences, 13*(7), 8615–8627. https://doi.org/10.3390/ijms13078615
13. Day, C., Cartwright, T., Provost, J., Bailey, C.J. (1990). Hypoglycemic effect of *Momordica charantia* extracts. *Planta Medica, 56*(5), 426–429. https://doi.org/10.1055/s-2006-961003
14. Deavile, E.R., Green, R.J., Muller-Harvey, I., Willoughby, I., Frazier, R.A. (2007). Hydrolyzable tannin structures influence relative globular and random coil protein binding strengths. *Journal of Agricultural and Food Chemistry, 5*(11), 4554–4561. https://doi.org/10.1021/jf063770o
15. Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S., Ju, Y.H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatic. Journal of Food and Drug Analysis, 22*(3), 296–302. https://doi.org/10.1016/j.jfda.2013.11.001

16. Dobrincic, A., Repajic, M., Garofulic, I.E., Tuden, L., Dragovic-Uzelac, V., Levaj, B. (2020). Comparison of different extraction methods for the recovery of olive leaves polyphenols. *Processes, 8*(9), art. no. 1008. https://doi.org/10.3390/pr8091008
17. El-Korany, S.M., Helmy, O.M., El-Halawany, A.M., Ragab, Y.E., Zedan, H.H. (2020). Kojic acid repurposing as a pancreatic lipase inhibitor and the optimization of its production from a local *Aspergillus oryzae* soil isolate. *BMC Biotechnology, 20*(1), art. no. 52. https://doi.org/10.1186/s12896-020-00644-9
18. Fan, M., Kim, E.K., Choi, Y.J., Tang, Y., Moon, S.H. (2019). The role of *Momordica charantia* in resisting obesity. *International Journal of Environmental Research and Public Health, 16*(18), art. no. 3251. https://doi.org/10.3390/ijerph16183251
19. Folin, O., Ciocalteu, V. (1927). On tyrosine and tryptophane determinations in proteins. *Journal of Biological Chemistry, 73*(2), 627–650. https://doi.org/10.1016/S0021-9258(18)84277-6
20. Goo, K.S., Ashari, S., Basuki, N., Sugiharto, A.N. (2016). The bitter gourd *Momordica charantia* L.: Morphological aspects, charantin and vitamin C contents. *IOSR Journal of Agricultural and Veterinary Science, 9*(10), 76–81. https://doi.org/10.9790/2380-0910017681
21. Grover, J.K., Yadav, S.P. (2004). Pharmacological actions and potential uses of *Momordica charantia*: a review. *Journal of Ethnopharmacology, 93*(1), 123–132. https://doi.org/10.1016/j.jep.2004.03.035
22. Gunathilake, K.D.P.P., Ranaweera, K.K.D.S., Rupasinghe, H.P.V. (2019). Response surface optimization for recovery of polyphenols and carotenoids from leaves of *Centella asiatica* using an ethanol-based solvent system. *Food Science & Nutrition, 7*(2), 528–536. https://doi.org/10.1002/fsn3.832
23. Horax, R., Hettiarachchy, N., Chen, P. (2010). Extraction, quantification, and antioxidant activities of phenolics from pericarp and seeds of bitter melons (*Momordica charantia*) harvested at three maturity stages (immature, mature, and ripe). *Journal of Agricultural and Food Chemistry, 58*(7), 4428–4433. https://doi.org/10.1021/jf9029578
24. Horzic, D., Jambrak, A.R., Belscak-Cvitanovic, A., Komes, D., Lelas, V. (2012). Comparison of conventional and ultrasound assisted extraction techniques of yellow tea and bioactive composition of obtained extracts. *Food and Bioprocess Technology, 5*(7), 2858–2870. https://doi.org/10.1007/s11947-012-0791-z
25. Jia, S., Shen, M., Zhang, F., Xie, J. (2017). Recent advances in *Momordica charantia*: Functional components and biological activity. *International Journal of Molecular Sciences, 18*(12), art. no 2555. https://doi.org/10.3390/ijms18122555
26. Kadkhodaee, R., Povey, M.J.W. (2008). Ultrasonic inactivation of *Bacillus* alpha-amylase. I. effect of gas content and emitting face of probe. *Ultrasoics Sonochemistry, 15*(2), 133–142. https://doi.org/10.1016/j.ultsonch.2007.02.005
27. Kajaria, D., Ranjana, Tripathi, J., Tripathi, Y.B., Tiwari, S. (2013). *In-vitro* α amylase and glycosidase inhibitory effect of ethanolic extract of antiasthmatic drug – Shirishadi. *Journal of Advanced Pharmaceutical Technology & Research, 4*(4), 206–209. https://doi.org/10.4103/2231-4040.121415
28. Kang, J.R., Kang, M.J., Kim, G.W., Shin, J.H. (2018). Physicochemical characteristics and physiological activity of bitter melon (*Momordica charantia* L.) from different regions. *Journal of the Korean Society of Food Science and Nutrition, 47*(3), 253–262 (in Korean). https://doi.org/10.3746/jkfn.2018.47.3.253
29. Kazeem, M.T., Adamson, J.O., Oqunwande, I.A. (2013). Modes of inhibition of α-amylase and α-glucosidase by aqueous extract of *Morinda lucida* Benth leaf. *BioMed Research International, 2013*, art. no. 527570. https://doi.org/10.1155/2013/527570
30. Kim, Y.M., Wang, M.H., Rhee, H.I. (2004). A novel alpha glucosidase inhibitor from pine bark. *Carbohydrate Research, 339*(3), 715–717. https://doi.org/10.1016/j.carres.2003.11.005
31. Kole, C., Kole, P., Randunu, K.M., Choudhary, P., Podila, R., Ke, P.C., Rao, A.M., Marcus, R.K. (2013). Nanobiotechnology can boost crop production and quality: First evidence from increased plant biomass, fruit yield and phytomedicine content in bitter melon (*Momordica charantia*). *BMC Biotechnology, 13*(1), art. no. 37. https://doi.org/10.1186/1472-6750-13-37
32. Kubola, J., Siriamornpun, S. (2008). Phenolic contents and antioxidant activities of bitter gourd (*Momordica charantia* L.) leaf, stem and fruit fraction extracts *in v*itro. *Food Chemistry 110*(4), 881–890. https://doi.org/10.1016/j.foodchem.2008.02.076
33. Lee, H.J., Lee, S.G., Kim, S.K., Choi, C.S. (2016). Variation on charantin contents of various organs and harvest seasons in bitter gourd. *Horticultural Science and Technology, 34*(5), 701–707 (in Korean). https://doi.org/10.12972/kjhst.20160073
34. Lee, J.J., Yoon, K.Y. (2021). Optimization of ultrasound-assisted extraction of phenolic compounds from bitter melon (*Momordica charantia*) using response surface methodology. *CYTA – Journal of Food, 19*(1), 721–728. https://doi.org/10.1080/19476337.2021.1973110
35. Lončarić, A., Jozinović, A., Kovač, T., Kojić, N., Babić, J., Šubarić, D. (2020). High voltage electrical discharges and ultrasound-assisted extraction of phenolics from indigenous fungus-resistant grape by-product. *Polish Journal of Food and Nutrition Sciences, 70*(2), 101–111. https://doi.org/10.31883/pjfns/117716
36. Lopes, A.P., Galuch, M.B., Petenuci, M.E., Oliveira, J.H., Canesin, E.A., Schneider, V.V.A., Visentainer, J.V. (2020). Quantification of phenolic compounds in ripe and unripe bitter melons (*Momordica charantia*) and evaluation of the distribution of phenolic compounds in different parts of the fruit by UPLC–MS/MS. *Chemical Papers, 74*, 2613–2625. https://doi.org/10.1007/s11696-020-01094-5
37. Mahwish, Saeed, F., Sultan, M.T., Riaz, A., Ahmed, S., Bigiu, N., Amarowicz, R., Manea, R. (2021). Bitter melon (*Momordica charantia* L.) fruit bioactives charantin and vicine potential for diabetes prophylaxis and treatment. *Plants, 10*(4), art. no. 730. https://doi.org/10.3390/plants10040730
38. McDougall, G.J., Kulkarni, N.N., Stewart, D. (2009). Berry polyphenols inhibit pancreatic lipase activity *in vitro*. *Food Chemistry, 115*(1), 193–199. https://doi.org/10.1016/j.foodchem.2008.11.093

39. Nam, S.W., Kim, M.R. (2015). A study on inhibitory activities on carbohydrate and anti-inflammatory activities of hot-water and ethanol extracts from immature dried bitter melon (*Momordica charantia* L.). *Journal of the East Asian Society of Dietary Life, 25*(6), 999–1006 (in Korean). https://doi.org/10.17495/easdl.2015.12.25.6.999
40. Naveed, M., Hejazi, V., Abbas, M., Kamboh, A.A., Khan, G.J., Shumzaid, M., Ahmad, F., Babazadeh, D., FangFang, X., Modarresi-Ghazani, F., WenHua, L., XiaoHui, Z. (2018). Chlorogenic acid (CGA): A pharmacological review and call for further research. *Biomedicine & Pharmacotherapy*, *97*, 67–74. https://doi.org/10.1016/j.biopha.2017.10.064
41. Nour, V., Trandfir, I., Cosmulescu, S. (2013). HPLC determination of phenolic acids, flavonoids and Juglone in walnut leaves. *Journal of Chromatographic Science, 51*(9), 883–890. https://doi.org/10.1093/chromsci/bms180
42. Patel, S.B., Ghane, S.G. (2021). Phyto-constituents profiling of *Luffa echinata* and *in vitro* assessment of antioxidant, anti-diabetic, anticancer and anti-acetylcholine esterase activities. *Saudi Journal of Biological Sciences, 28*(7), 3835–3846. https://doi.org/10.1016/j.sjbs.2021.03.050
43. Pedro, A.C., Maciel, G.M. Ribeiro, V.R., Haminiuk, C.W.I. (2020). Fundamental and applied aspects of catechins from different sources: a review. *International Journal of Food Science and Techn*ology, *55*(2), 429–442. https://doi.org/10.1111/ijfs.14371
44. Pugazhenthi, S., Murthy, S.P. (1995). Partial purification of a hypoglycemic fraction from the unripe fruits of *Momordica charantia* Linn (bitter group). *Indian Journal of Clinical Biochemistry, 10*(1), art. no. 19. https://doi.org/10.1007/BF02873663
45. Rostagno, A., Palma, M., Barroso, C.G. (2003). Ultrasound-assisted extraction of soy isoflavones. *Journal of Chromatography. A*, *1012*(2), 119–128. https://doi.org/10.1016/S0021-9673(03)01184-1
46. Sembiring, E.N., Elya, B., Sauriasary, R. (2018). Phytochemical screening, total flavonoid and total phenolic content and antioxidant activity of different parts of *Caesalpinia bonduc* (L.) Roxb. *Pharmacognosy Journal, 10*(1), 123–127. https://doi.org/10.5530/pj.2018.1.22
47. Sen, S., De, B., Devanna, N., Chakraborty, R. (2013). Total phenolic, total flavonoid content, and antioxidant capacity of the leaves of *Meyna spinosa* Roxb., an Indian medicinal plant. *Chinese Journal of Natural Medicines, 11*(2), 149−157. https://doi.org/10.1016/S1875-5364(13)60042-4
48. Sharmila, G., Nikitha, V.S., Ilaiyarasi, S., Dhivya, K., Rajasekar, V., Kumar, N.M., Muthukumaran, K., Muthukumaran, C. (2016). Ultrasound assisted extraction of total phenolics from *Cassia auriculata* leaves and evaluation of its antioxidant activities. *Industrial Crops and Products, 84*, 13–21. https://doi.org/10.1016/j.indcrop.2016.01.010
49. Suh, S., Kim, Y.E., Yang, H.J., Ko, S., Hong, G.-P. (2017). Influence of autoclave treatment and enzymatic hydrolysis on the antioxidant activity of *Opuntia ficus-indica* fruit extract. *Food Science and Biotechnology, 26*(3), 581–590. https://doi.org/10.1007/s10068-017-0085-3
50. Sun, C., Zhao, C., Guven, E.C., Paoli, P., Simal-Gandara, J., Ramkumar, K.M., Wang, S., Buleu, F., Pah, A., Turi, V., Damian, G., Dragan, S., Tomas, M., Khan, W., Wang, M., Delmas, D., Portillo, M.P., Dar, P., Chen, L., Xiao, J. (2020). Dietary polyphenols as antidiabetic agents: Advances and opportunities. *Food Frontiers, 1*(1), 18–44. https://doi.org/10.1002/fft2.15
51. Vilas-Boas, A.A., Campos, D.A., Nunes, C., Ribeiro, S., Nunes, J., Oliveira, A., Pintado, M. (2020). Polyphenol extraction by different techniques for valorisation of non-compliant Portuguese sweet cherries towards a novel antioxidant extract. *Sustainability, 12*(14), art. no. 5556. https://doi.org/10.3390/su12145556
52. Vilkhu, K., Mawson, R., Simons, L., Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry – A review. *Innovative Food Science & Emerging Technologies, 9*(2), 161–169. https://doi.org/10.1016/j.ifset.2007.04.014
53. Wang, H.Y., Kan, W.C., Cheng, T.J., Yu, S.H., Chang, L.H., Chuu, J.J. (2014). Differential anti-diabetic effects and mechanism of action of charantin-rich extract of Taiwanese *Momordica charantia* between type 1 and type 2 diabetic mice. *Food and Chemical Toxicology, 69*, 347–356. https://doi.org/10.1016/j.fct.2014.04.008

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Original article
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Soaking Soybean Seeds with *Abeliophyllum distichum* Nakai Extract Increased the Yield and Nutritional Value of Soybean Sprouts

*Jeong-Ho Kim[1], Yong-Han Yoon[1], Il-Doo Kim[2], Sanjeev Kumar Dhungana[3], Dong-Hyun Shin[4]**

[1]*Department of Green Technology Convergence, Konkuk University, Chungju 27478, Korea*
[2]*International Institute of Research & Development, Kyungpook National University, Daegu 41566, Korea*
[3]*Department of Southern Area Crop Science, National Institute of Crop Science, Rural Development Administration, Miryang 50424, Korea*
[4]*School of Applied Biosciences, Kyungpook National University, Daegu 41566, Korea*

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Abeliophyllum distichum Nakai, an ornamental plant, contains a wide range of phytochemicals having pharmaceutical properties. The use of different plant-based extracts for the enhancement of yield and/or quality of soybean sprouts, one of the most inexpensive but nutritious food products, is common. The objective of this study was to examine the effect of *A. distichum* flower extract (ADE) on the yield and nutritional value of soybean sprouts. Soybean seeds were soaked in ADE solutions with concentrations (*w/v*) of 0.5% (ADE-0.5), 1% (ADE-1), 3% (ADE-3), and 5% (ADE-5). The effect of ADE concentration on the yield and different nutrient components varied. The highest soybean sprout yield and vitamin C content were found with ADE-3. The most abundant essential amino acid content was detected in ADE-1, whereas the greatest amounts of total isoflavones and total minerals were determined in ADE-5 and the ADE-untreated control, respectively. Overall results of yield, color, and contents of vitamin C, amino acids, and isoflavones suggest that 1% or 3% of *A. distichum* extract could be an optimum concentration to soak the soybean seeds for higher sprout yield and nutrient content.

INTRODUCTION

Abeliophyllum distichum Nakai is the single species in the *Oleaceae* family [Oh *et al.*, 2003]. *A. distichum*, commonly known as white forsythia, is mainly used as an ornamental deciduous plant. It has recently drawn attention of many researchers due to a wide range of bioactive phytochemicals, such as acteoside, eutigoside B, dendromoniliside D, forsythoside B, isoacteoside, isomucronulatol 7-*O*-glucoside, plantamajoside, wighteone, rutin, cornoside, hirsutrin, chlorogenic acid, caffeic acid, gentisic acid, ferulic acid, and quercetin [Ahn & Park, 2013; Choi *et al.*, 2017; Ju *et al.*, 2021; Lee *et al.*, 2021; Li *et al.*, 2013; Oh *et al.*, 2003; Yoo *et al.*, 2021]. So far, these phytochemicals have been reported to exhibit antioxidant activity [Choi *et al.*, 2017], anti-proliferative activity against human colorectal cancer cells [Park *et al.*, 2014], antidiabetic effect [Li *et al.*, 2013], and antihypertensive activity [Oh *et al.*, 2003]. Kwon *et al*. [2014] have determined the nutrient components and evaluated the safety of *A. distichum* leaves and stems to be used as a natural constituent in the preparation of nutritional and functional foods. They found that *A. distichum* leaves and stems, which contained proteins, lipids, sugars, vitamins, minerals, organic acids, and phenolic compounds, were safe to be used as a food material.

Soybeans are a rich source of proteins, lipids, and several phytochemicals, including phenolics (isoflavones and anthocyanins) and saponins [Brummer *et al.*, 1997; Hubert *et al.*, 2008; Lee *et al.*, 2009; Lee & Cho, 2012]. Germination may further enhance the nutritional value of soybean seeds [Paucar-Menacho *et al.*, 2010] because it not only modifies the existing nutrients but also produces new compounds [Spanier *et al.*, 2001]. Generally, a week is sufficient to prepare soybean sprouts, which can be grown year-round using simple and inexpensive technologies.

Various studies have shown that the seed soaking and/or treatment with irrigating solutions could enhance the quality and nutritional values of soybean sprouts. The soaking and spraying of seeds with a zinc sulfate solution has been reported to enhance the zinc content [Zou *et al.*, 2014], while in tap water with persimmon fruit powder to increase the yield and the contents of vitamin C, isoflavones, and total phenolics

* Corresponding Author:
E-mail: dhshin@knu.ac.kr (Dong-Hyun Shin)

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of soybean sprouts [Kim *et al.*, 2017]. The use of Pu-erh tea extracts for seed soaking also augmented the yield and nutritional values of soybean sprouts [Kim *et al.*, 2020].

Considering the pharmaceutical properties of *A. distichum* and the use of different plant-based extracts, including persimmon fruit powder [Kim *et al.*, 2017], lacquer stem [Kwak *et al.*, 2017], and Pu-erh tea [Kim *et al.*, 2020] for soybean sprout cultivation, this study aimed to investigate the effect of seed soaking in *A. distichum* extracts on the quality characteristics and yields of soybean sprouts.

MATERIALS AND METHODS

Chemicals and experimental materials

The following chemicals: metaphosphoric acid, 2,6-dichloroindophenol, indophenol dye, and isoflavone standards were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) and amino acid standards were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All the chemicals used in this study were of analytical grade.

Sowonkong, one of the common sprout cultivars of soybean (*Glycine max* L.) in Korea, was used to produce sprouts. The seeds were obtained from the Agricultural Research and Extension Services, Gyeongsangbuk-do, Korea. The mean 1000-seed weight of the cultivar was 120 g. Flowers from the 5-year-old *Abeliophyllum distichum* Nakai plants, grown in Youngdong-gun, Chungcheongbuk-do, Korea, were collected for this study.

Preparation of *Abeliophyllum distichum* extracts

The *A. distichum* flowers were oven-dried (50°C until constant weight). The oven-dried flowers were ground into powder using a commercial grinder (HIL-G-501, Hanil Co., Seoul, Korea). Initially, 10% (*w/v*) extract of *A. distichum* (ADE) was prepared with 70% (*v/v*) ethanol by extracting at room temperature using a shaking incubator (250 rpm) for 24 h. The ethanol was removed at 60°C using a rotary evaporator (RV 10D, IKA, China). The concentrated extracts were freeze-dried. Later, four different concentrations (0.5, 1, 3, and 5%; *w/v*) of *A. distichum* extracts (ADEs) were prepared using tap water.

Cultivation of soybean sprouts

One kilogram of seeds, in three replicates, were washed with tap water, and then the excess water was drained. The seeds were soaked in tap water or four different concentrations (0.5, 1, 3, and 5%; *w/v*) of ADE for 6 h [Kim *et al.*, 2020]. The sprout samples were named with the concentration of the seed soaking extract used *i.e.*, control, ADE-0.5, ADE-1, ADE-3, and ADE-5 for that soaked in tap water alone, 0.5, 1, 3, and 5% of ADE, respectively. After 6 h of soaking, the seeds were thoroughly rinsed with tap water and put into the bottom-perforated 15-L plastic buckets, and covered with double-layered black landscape fabric to minimize light exposure during sprout growing [Kim *et al.*, 2017]. The germinating seeds and sprouts of all five treatments were periodically sprinkled with tap water for 2 min every 3 h using two hoses of 1-cm diameter. Soybean sprouts were cultivated at 20±1°C for 6 days.

Measurement of sprout yield and preparation of sprout powders

Sprout yield, assessed as the fresh weight of soybean sprouts, was measured on day 6 by subtracting the weight of the empty bucket from the gross weight of each bucket containing sprouts. The freshly harvested sprouts with cotyledons, hypocotyl, and roots were kept at –70°C for 24 h, followed by freeze-drying. The freeze-dried sprouts were ground into powder using a commercial grinder (HIL-G-501, Hanil Co., Seoul, Korea) and passed through a 100-mesh sieve [Kim *et al.*, 2017].

Determination of vitamin C content

The vitamin C content in the sprouts was measured following the method of AOAC [1990]. One gram of sprout powder was mixed with 7.5 mL of 3% (*w/v*) metaphosphoric acid and homogenized (AM-8, Nihonseike Kaisha, Tokyo, Japan). The mixture was filtered through 0.45 μm membrane filter (Millipore, Bedford, MA, USA) and made to the final volume of 12 mL. A half volume (6 mL) of the mixture was titrated with 0.025% (*w/v*) 2,6-dichloroindophenol. The vitamin C present in the mixture is oxidized, and the indophenol dye was reduced to a colorless compound. The ascorbic acid (vitamin C) content of sprout powders was determined by an external standard of ascorbic acid with an aqueous solution of 0.025% (*w/v*) 2,6-dichloroindophenol. The absorption was read at 250 nm using a spectrophotometer (OPTIZEN POP-V, LAB Keen Innovative Solutions, Daejeon, Korea). A calibration standard curve of ascorbic acid was plotted and used to calculate the vitamin C content as milligrams of ascorbic acid per 100 g fresh weight (FW).

Color parameters measurement

Hunter's color of soybean sprout powder was measured following the procedures described earlier [Kim *et al.*, 2014]. The *L* (lightness), *a* (redness), and *b* (yellowness) values were determined using a Chroma Meter (CR-300, Minolta Corp., Tokyo, Japan). The instrument was calibrated using a calibration plate (Minolta Corp.; YCIE=94.5, XCIE=0.3160, YCIE=0.330) and a standard plate (Hunter Associates Laboratory Inc., Reston, VA, USA; $L=97.51$, $a=-0.18$, $b=1.67$).

Determination of free amino acid content

The free amino acid content was assayed following the method described by Je *et al*. [2005]. Sprout powder (1.5 g) was homogenized (12,000 rpm, 2 min) with 10 mL of ice-cold 6% (*v/v*) perchloric acid in an ice bath using an ACE homogenizer (Nissei AM-7, Nihonseikei Kaisha Ltd., Tokyo, Japan), followed by incubation in ice for 30 min and centrifugation (3913×*g*, 15 min). The supernatant was filtered through a filter paper (Whatman No. 41) and adjusted to pH 7.0 using a KOH solution (33%, *w/v*). The precipitate of potassium perchlorate was removed by centrifugation (3913×*g*, 10 min). Then, the pH of the supernatant was adjusted to 2.2 with hydrochloric acid (10 M), and distilled water was added to make the final volume of 50 mL. Two milliliters of sample aliquot were mixed with 1 mL of a lithium citrate buffer (pH 2.2) to prepare the reaction mixture for the free amino acid determination. The content of free amino acids was analyzed using

an automatic amino acid analyzer (Biochrom-20, Pharmacia Biotech Co., Uppsala, Sweden) and expressed as mg per g sprout dry weight (DW).

Determination of mineral content

The mineral content of soybean sprouts was analyzed according to the procedures described in a previous report [Skujins, 1998] with some modifications. Sprout powder (0.5 g) was digested in a mixture of 65% HNO_3 (15.0 mL) and 35% H_2O_2 (2 mL). An equal volume of distilled water was added to dilute the mixture. The mineral content of the samples was estimated using an inductively coupled plasma atomic emission spectrometer (ICP AES, Varian Vista, Victoria, Australia) after calibrating the instrument with a working standard prepared from a commercially available multielement standard solution (Merck, Darmstadt, Germany). The results were expressed as mg per kg sprout DW.

Determination of isoflavone content

Two hundred micrograms of sprout powder were extracted with 6 mL of methanol (80%, *v/v*) using an ultrasonic-assisted method at 40°C for 30 min, followed by centrifugation (3913×*g*, 15 min) and filtration of the supernatant through a membrane filter (0.45 μm, Millipore, Bedford, MA, USA). The filtrate was used for the isoflavone analysis using a high-performance liquid chromatography (HPLC) system with a UV detector (Prostar 230, Varian Co., Palo Alto, CA, USA) by following a previously described method [Jiao *et al.*, 2016]. A Nova-Pak C18 reversed-phased column (150×3.9 mm, 4 μm particle size) and Adsorbosphere C18 direct-connect guard column (Waters Co., Milford, MA, USA) were used. The flow rate of the mobile phase was 1 mL/min and the gradient elution of solvents A and B (solvent A – aqueous acetic acid (0.1%, *v/v*) and solvent B – acetic acid in acetonitrile (0.1%, *v/v*)) was used as follows: 13–35% of B for 52 min. The oven temperature was set to 35°C. The injection volume was 20 μL. The eluted isoflavones were detected at 260 nm. Each peak was identified by the retention time and the characteristic UV spectrum in comparison with the corresponding standards. The isoflavone content was calculated using the calibration curve of an internal standard 2,4,4'-trihydroxydeoxybenzoin (THB) and expressed as mg of isoflavone per kg of freeze-dried soybean sprouts (mg/kg DW).

Statistical analysis

Analysis of variance was carried out using SAS 9.4 software (SAS Institute, Cary, NC, USA) to compare the treatments. Three batches of soybean sprouts were produced for each treatment. Two replicates for free amino acid and three replicates for the other analyses were carried out. The significant differences among the treatment means were identified at 5% probability using Tukey's test.

RESULTS AND DISCUSSION

Yield of sprouts and their moisture and vitamin C contents

Vitamin C is an important nutrient with a strong antioxidative capacity. The yield and vitamin C content of soybean sprouts were significantly affected by ADE treatment; however, the moisture content remained unaffected (Table 1). A significantly ($p<0.05$) high yield increment was obtained in ADE-3 (11.2%), followed by ADE-1 (9.3%) compared to that of the control. ADE-0.5 (5.4%) and ADE-5 (6.3%) had the least yield increment and these values did not differ significantly ($p\geq0.05$). ADE-3, which showed the utmost yield, had the greatest vitamin C content of 18.20 mg/100 g FW. In turn, the contents of vitamin C of ADE-0.5 (16.59 mg/100 g FW) and the control (16.07 mg/100 g FW) were not significantly ($p\geq0.05$) different.

TABLE 1. Yield of soybean sprouts grown after seed soaking in different concentrations (0.5%–5%, *w/v*) of *Abeliophyllum distichum* extracts (ADE) and their moisture and vitamin C contents.

Sample*	Total fresh weight (g)	Moisture (g/100 g)	Vitamin C (mg/100 g fresh weight)
Control	5425±32^{d} (100.0%)	87.31±0.03^{a}	16.07±0.31^{c}
ADE-0.5	5718±21^{c} (105.4%)	87.21±0.21^{a}	16.59±0.52^{bc}
ADE-1	5930±30^{b} (109.3%)	88.00±1.12^{a}	16.99±0.32^{b}
ADE-3	6031±40^{a} (111.2%)	87.42±0.08^{a}	18.20±0.20^{a}
ADE-5	5768±45^{c} (106.3%)	88.02±1.01^{a}	16.81±0.21^{b}

* The values at ADE correspond to its concentrations in the seed soaking solution. Values are expressed as mean ± standard deviation of three replicates. Values followed by different letters in the same column are significantly different ($p<0.05$). Values, after total fresh weight, in the brackets indicate the sprout yield difference in comparison to the control (100%).

Although the availability of plant growth regulators in ADE was not measured, it can be stated from the previous reports on the plant-based extracts, including persimmon fruit powder [Kim *et al.*, 2017], lacquer stem [Kwak *et al.*, 2017], and Pu-erh tea [Kim *et al.*, 2020] that some growth-promoting substances could be present in ADE and that might have played a role in changes observed in the yield and nutritional value of the soybean sprouts. Wang *et al*. [2016] reported that the supplementation of soybean sprout growth with calcium increased both the sprout yield and vitamin C content, and claimed these effects could be caused by the Ca-induced increased content of plant hormones, like indoleacetic acid and gibberellin. A high calcium content was found in *A. distichum* [Kwon *et al*., 2014], which seems to be important for increasing the yield and vitamin C content of the ADE-treated soybean sprouts. We could not exactly explain the reasons for the higher vitamin C content in ADE-3 than in ADE-5. There might be some kinds of stress in soybean sprouts due to a higher concentration of ADE. The elevated yield and nutrient content might be due to the absorption of *A. distichum* phytochemicals [Ahn & Park, 2013; Choi *et al.*, 2017; Ju *et al.*, 2021; Lee *et al.*, 2021; Li *et al.*, 2013; Kwon *et al.*, 2014; Oh *et al.*, 2003; Yoo *et al.*, 2021] during seed-soaking [Lintschinger *et al.*, 2000]. Wang *et al*. [2016] hypothesized that the regulation of enzyme activity promoted growth and increased the nutritional value of soybean by calcium would involve hormones.

TABLE 2. Hunter's color parameters of 6-day-old soybean sprouts grown after seed soaking in different concentrations (0.5%–5%, *w/v*) of *Abeliophyllum distichum* extracts (ADE).

Sample*	Lightness (*L*)	Redness (*a*)	Yellowness (*b*)
Control	77.56±0.86[a]	1.51±0.06[a]	21.35±0.33[b]
ADE-0.5	77.50±0.66[a]	0.25±0.03[d]	22.00±0.29[a]
ADE-1	77.75±0.92[a]	0.74±0.16[c]	22.15±0.28[a]
ADE-3	77.49±0.61[a]	0.81±0.04[b]	21.33±0.09[b]
ADE-5	77.68±0.42[a]	0.94±0.11[b]	21.46±0.05[b]

* The values at ADE correspond to its concentrations in the seed soaking solution. Lightness (100, white; 0, black); redness (−, green; +, red); yellowness (−, blue; +, yellow). Values are expressed as mean ± standard deviation of three replicates. Values followed by different letters in the same column are significantly different ($p<0.05$).

Color parameters of soybean sprouts

Redness and yellowness values of soybean sprout color were significantly ($p<0.05$) influenced by seed-soaking in ADE during sprout growth; however, lightness values remained unaffected ($p\geq0.05$) (Table 2). The redness value of all the ADE-treated samples was significantly ($p<0.05$) lower compared to the control. The yellowness values of ADE-0.5 and ADE-1 were increased by ADE treatment. The lowest redness value was obtained in ADE-0.5 (0.25), which was one of the samples with the greatest yellowness value.

Although the reason behind the color variations in soybean sprouts was not well known, ADE treatment affected the color appearance of soybean sprouts. Treatment of soybean seeds with different plant extracts could alter the color parameters of soybean sprouts [Kim *et al.*, 2017, 2020; Kwak *et al.*, 2017]. The color of a food product is an influencing factor to determine the alacrity of consumers to pay for the product [Udomkun *et al.*, 2018]. The soaking of soybean seeds with ADE enhanced the yellowness of the sprouts, which is one of their looked-for characteristics [Park *et al.*, 1995].

Free amino acid content

The free amino acid compositions of soybean sprouts grown after seed-soaking in tap water and in different concentrations of ADE are shown in Table 3. The essential amino acid content of ADE-0.5 (14.47 mg/g DW) and ADE-1 (14.92 mg/g DW) was higher than that of the control (13.99 mg/g DW). However, the soaking of soybean seeds with higher concentrations of *A. distichum* extracts *i.e.*, ADE-3 (11.08 mg/g DW) and ADE-5 (8.41 mg/g DW), reduced the content of essential amino acids compared to the control. On the other hand, although contents of some of the individual amino acids, such as proline, were increased in the soybean sprouts, the contents of non-essential and total amino acids decreased upon the seed treatment with *A. distichum* extracts. The total content of other free amino acids also decreased upon the ADE treatment; however, the contents of L-α-aminoadipic acid and L-α-amino-*n*-butyric acids were found to increase.

Similar results of a higher content of essential amino acids, like leucine, isoleucine, lysine, methionine, and valine, were found in the soybean sprouts treated with Pu-erh tea extracts [Kim *et al.*, 2020]. Seed soaking in ADE reduced the non-essential and other amino acids of soybean sprouts compared to the control except for the content of a few amino acids. It can be speculated that calcium, which is present in *A. distichum* [Kwon *et al*., 2014], might have induced conversion among some amino acids [Wang *et al*., 2016]. Additionally, there might be some kinds of stress at higher ADE concentrations resulting in reduced contents of some of the amino acids. ADE treatment has increased the content of a few amino acids in the soybean sprouts, making them more nutritive. Fortification of food products with certain nutrients to make them more nutritious is not uncommon. Soybean sprouts were fortified with zinc sulfate to increase zinc availability [Zou *et al.*, 2014]. Fortification and consumption of wheat flour with lysine significantly improved the sensitive indicators of nutritional status of a studied population [Hussain *et al.*, 2004]. Functional non-essential amino acids, like proline, the content of which was significantly high in ADE-1 (Table 3), play a key role in the metabolic pathways associated with maintenance, growth, reproduction, and immunity [Wu, 2009].

Mineral content

Like the total amino acid content, the total mineral content of soybean sprouts decreased as a result of seed-soaking in ADE solutions; however, contents of some of the individual mineral elements increased upon the ADE treatment (Table 4). Among the eight minerals measured, K (11322––14913 mg/kg DW) was the most abundant, while Cu (16.3––36.3 mg/kg DW), followed by Mn (38.2–38.8 mg/kg DW) was the least abundant mineral in the sprout samples. Content of four minerals (Ca, Fe, Na, and Zn) were higher in at least one of the ADE-treated soybean sprouts, whereas contents of two minerals (Cu and K) were higher in the control. The Mg content of the control, ADE-0.5, and ADE-1 did not differ significantly ($p\geq0.05$). The Mn content of the control did not differ significantly ($p\geq0.05$) from those of the ADE--treated soybean sprouts.

The higher mineral content in the ADE-treated soybean sprouts compared to the control might be due to the mineral-rich ADE [Kwon *et al.*, 2014]. Similar results of higher mineral content were found in previous studies with zinc sulfate-treated soybean sprouts [Xu *et al.*, 2012; Zou *et al.*, 2014], zinc sulfate-fortified germinated brown rice [Wei *et al.*, 2012], selenium-applied cereal sprouts [Lintschinger *et al.*, 2000], iron-fortified soybean sprouts [Kujawska *et al.*, 2016], and Pu-erh tea-treated soybean sprouts [Kim *et al.*, 2020]. Fe, Zn, and Ca, which increased in soybean sprouts upon the ADE treatment, are some of the most commonly lacking minerals in human diets [White & Broadley, 2009].

Isoflavone content

Isoflavones are considered a type of bioactive dietary supplement that elicits a number of health benefits. The contents of total isoflavones and individual isoflavones, except genistein and glycitein, increased significantly ($p<0.05$) in soybean sprouts with ADE treatments (Table 5). Daidzin (332––362 mg/kg DW), followed by genistin (292–335 mg/kg DW),

TABLE 3. Free amino acid composition (mg/g dry weight) of 6-day-old soybean sprouts grown after seed soaking in different concentrations (0.5%–5%, *w/v*) of *Abeliophyllum distichum* extracts (ADE).

Amino acid	Control	ADE-0.5	ADE-1	ADE-3	ADE-5
Essential amino acids					
L-Histidine	3.34±0.1[a]	3.30±0.4[a]	3.26±0.2[b]	2.21±0.1[c]	1.66±0.1[d]
L-Isoleucine	1.25±0.2[c]	1.30±0.1[b]	1.39±0.1[a]	1.11±0.1[d]	0.87±0.1[e]
L-Leucine	0.95±0.1[b]	0.98±0.2[b]	1.06±0.2[a]	0.77±0.2[c]	0.57±0.1[d]
L-Lysine	2.44±0.3[c]	2.55±0.2[b]	2.66±0.1[a]	2.01±0.1[d]	1.25±0.1[e]
L-Methionine	0.33±0.1[a]	0.35±0.2[a]	0.34±0.2[a]	0.25±0.1[b]	0.16±0.2[c]
L-Phenylalanine	1.75±0.1[b]	1.87±0.1[a]	1.86±0.1[a]	1.11±0.2[c]	1.08±0.1[c]
L-Threonine	1.66±0.1[b]	1.82±0.2[a]	1.79±0.1[a]	1.51±0.2[c]	1.09±0.1[d]
L-Valine	2.27±0.1[c]	2.30±0.1[b]	2.56±0.2[a]	2.11±0.1[d]	1.73±0.2[e]
Sub-Total	13.99	14.47	14.92	11.08	8.41
Non-essential amino acids					
Glycine	0.32±0.2[a]	0.31±0.1[a]	0.32±0.1[a]	0.21±0.2[b]	0.19±0.1[b]
L-Alanine	3.07±0.1[c]	3.51±0.3[b]	3.78±0.2[a]	3.01±0.1[d]	2.01±0.2[e]
L-Arginine	17.4±1.2[a]	15.21±0.9[b]	12.78±0.8[c]	10.12±1.2[d]	6.28±0.5[e]
L-Aspartic acid	2.54±0.2[a]	2.50±0.1[b]	2.54±0.1[a]	2.01±0.1[c]	1.82±0.1[d]
L-Serine	3.49±0.1[b]	0.35±0.2[b]	3.58±0.2[a]	2.01±0.1[c]	1.98±0.1[d]
L-Tyrosine	0.35±0.2[a]	0.34±0.2[a]	0.35±0.1[a]	0.22±0.1[b]	0.16±0.3[c]
Proline	1.69±0.1[b]	1.72±0.2[b]	1.83±0.2[a]	1.12±0.2[c]	0.82±0.1[d]
Sub-total	28.86	23.94	25.18	18.70	13.26
Other free amino acids					
Aminoisobutyric acid	0.26±0.1[a]	0.27±0.1[a]	0.26±0.1[a]	0.20±0.1[b]	0.10±0.1[c]
Ethanolamine	0.57±0.2[a]	0.50±0.2[b]	0.45±0.3[c]	0.21±0.3[d]	0.19±0.1[d]
L-α-Aminoadipic acid	0.47±0.2[b]	0.50±0.2[b]	0.56±0.3[a]	0.38±0.3[c]	0.28±0.2[d]
L-α-Amino-*n*-butyric acid	0.14±0.1[b]	0.15±0.1[a]	0.15±0.1[a]	0.11±0.1[c]	0.09±0.2[c]
β-Alanine	0.52±0.2[a]	0.51±0.1[a]	0.53±0.2[a]	0.42±0.1[b]	0.39±0.1[b]
γ-Amino-*n*-butyric acid	1.96±0.2[a]	1.16±0.2[b]	0.93±0.2[c]	0.66±0.2[d]	0.45±0.1[e]
Sub-total	3.92	3.09	2.88	1.98	1.50
Total	46.77	41.50	42.98	31.76	23.17

The values at ADE correspond to its concentrations in the seed soaking solution. Values are expressed as mean ± standard deviation of two replicates. Values followed by different letters in the same row are significantly different ($p<0.05$).

was the most abundant isoflavone in the sprout samples. Interestingly, the amount of these two isoflavones increased with the concentration of ADE used to soak the seeds. Glycitein, which was the least abundant isoflavone in the sprout samples, was not affected by the ADE treatment.

Although the mechanism behind the isoflavone variation due to the ADE treatment was not clear, presumably, the enhanced phenyl-alanine and isoflavone synthetase activities due to the effect of minerals [Jung *et al.*, 2000; Wang *et al.*, 2016] might have increased the isoflavone content in sprouts grown after seed-soaking in ADE solutions. During seed germination, carbohydrates are consumed rapidly, and water-soluble metabolites are removed, which increases the isoflavone content [Kim *et al.*, 2013]. Variation in isoflavone content of germinated soybean seeds could be due to the conversion of other flavonoids to isoflavones and isoflavones to other flavonoids [Zhu *et al.*, 2005]. The flavonoid content of ADE [Kwon *et al.*, 2014; Lee *et al.*, 2020] might have played a role in increasing the isoflavone content in the ADE-treated soybean sprouts. Moreover, a high calcium content of ADE might also have contributed to an increase in the isoflavone content in the ADE-treated soybean sprouts as in calcium-treated soybean sprouts [Wang *et al.*, 2016]. Similar results of a high isoflavone content were also found in soybean sprouts grown

TABLE 4. Mineral content (mg/kg dry weight) of 6-day-old soybean sprouts grown after seed soaking in different concentrations (0.5%–5%, *w/v*) of *Abeliophyllum distichum* extracts (ADE).

Element	Control	ADE-0.5	ADE-1	ADE-3	ADE-5
Ca	2897±11^{b}	2782±10^{c}	2522±12^{d}	2533±8^{d}	3210±7^{a}
Cu	36.3±1.2^{a}	29.6±1.6^{b}	25.3±1.4^{c}	20.2±1.6^{d}	16.3±1.2^{e}
Fe	60.3±0.9^{e}	72.1±0.8^{d}	89.3±1.2^{c}	112.0±1.6^{b}	131.4±1.5^{a}
K	14,913±21^{a}	13,222±1^{b}	12,213±30^{c}	12,002±40^{c}	11,322±35^{d}
Mg	1611±9^{a}	1601±8^{a}	1599±12^{ab}	1580±10^{b}	1548±1^{c}
Mn	38.2±0.7^{a}	38.7±0.8^{a}	38.2±2.1^{a}	38.4±2.9^{a}	38.8±0.9^{a}
Na	477±4^{e}	531±4^{d}	582±5^{c}	612±9^{b}	633±9^{a}
Zn	46.6±1.2^{c}	47.2±1.0^{c}	49.3±1.1^{b}	53.3±1.7^{a}	55.2±0.8^{a}
Total	20,079.4	18,323.6	17,118.1	16,950.9	16,954.7

The values at ADE correspond to its concentrations in the seed soaking solution. Values are expressed as mean ± standard deviation of three replicates. Values followed by different letters in the same row are significantly different ($p<0.05$).

TABLE 5. Isoflavone content (mg/kg dry weight) of 6-day-old soybean sprouts grown after seed soaking in different concentrations (0.5%–5%, *w/v*) of *Abeliophyllum distichum* extracts (ADE).

Sample*	Daidzin	Daidzein	Genistin	Genistein	Glycitin	Glycitein	Total
Control	332±10^{d}	16±3^{c}	292±4^{d}	35±3^{a}	84±4^{c}	10±1^{a}	769
ADE-0.5	342±11^{c}	20±2^{b}	300±3^{c}	23±1^{b}	90±3^{b}	11±2^{a}	786
ADE-1	351±10^{b}	22±2^{b}	303±8^{c}	21±2^{bc}	90±3^{b}	11±2^{a}	798
ADE-3	356±6^{b}	20±3^{b}	325±3^{b}	20±2^{c}	95±2^{a}	10±1^{a}	826
ADE-5	362±1^{a}	25±1^{a}	335±4^{a}	18±1^{d}	99±2^{a}	10±2^{a}	849

* The values at ADE correspond to its concentrations in the seed soaking solution. Values are expressed as mean ± standard deviation of three replicates. Values followed by different letters in the same column are significantly different ($p<0.05$).

after the seed soaking with persimmon fruit powder and Puerh tea extracts [Kim *et al.*, 2017, 2020]. Soy isoflavones are found to be beneficial against a number of health disorders. Their coupled intake with vitamin D has been reported to mitigate the irritable bowel disease in female patients [Jalili *et al.*, 2016]. In addition, they have been shown to provide protection against breast and prostate cancers, osteoporosis, cardiovascular diseases, and diabetic conditions, and also to assuage menopause-related symptoms [Abdelrazek *et al.*, 2019; Sathyapalan *et al.*, 2018].

CONCLUSIONS

The effects of seed soaking with different concentrations of the *A. distichum* extract on the growth and nutritional value of soybean sprouts were examined. The yield of soybean sprouts increased by up to 11.2% compared to the control. The vitamin C content also significantly improved with 1 to 5% of ADE treatment. Although the total free amino acid content was lower in the ADE-treated sprouts compared to the control, the amount of essential amino acid increased upon the 0.5 and 1% ADE treatments. The contents of minerals: Ca, Fe, Na, and Zn, were higher in at least one of the ADE-treated soybean sprouts. The isoflavone content in all the ADE-treated soybean sprouts was higher than that of the control. Overall results suggest that soybean seed soaking with 1 or 3% of *A. distichum* extract could be a good option to improve the sprout yield and quality despite a reduction in some nutrient components.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ORCID IDs

S.K. Dhungana https://orcid.org/0000-0002-2495-9078

REFERENCES

1. Abdelrazek, H.M.A., Mahmoud, M.M.A., Tag, H.M., Greish, S.M., Eltamany, D.A., Soliman, M.T.A. (2019). Soy isoflavones ameliorate metabolic and immunological alterations of ovari-

ectomy in female wistar rats: antioxidant and estrogen sparing potential. *Oxidative Medicine and Cellular Longevity*, *2019*, SI, art. no. 5713606.
https://doi.org/10.1155/2019/5713606

2. Ahn, J., Park, J.H. (2013). Effects of *Abeliophyllum distichum* Nakai flower extracts on antioxidative activities and inhibition of DNA damage. *Korean Journal of Plant Resources, 26*(3), 355–361.
https://doi.org/10.7732/kjpr.2013.26.3.355
3. AOAC Association of Official Analytical Chemists. (1990). *Official Methods of Analysis of the Association of Official Analytical Chemists*; *Volume 15*; AOAC International: Arlington, VA, USA.
4. Brummer, E.C., Graef, G.L., Orf, J., Wilcox, J.R., Shoemaker, R.C. (1997). Mapping QTL for seed protein and oil content in eight soybean populations. *Crop Science*, *37*(2), 370–378.
https://doi.org/10.2135/cropsci1997.0011183X003700020011x
5. Choi, J.H., Seo, E.-J., Sung, J., Choi, K.M., Kim, H., Kim, J.-S., Lee, J., Efferth, T., Hyun, T.K. (2017). Polyphenolic compounds, antioxidant and anti-inflammatory effects of *Abeliophyllum distichum* Nakai extract. *Journal of Applied Botany and Food Quality*, *90*, 266–273.
https://doi.org/10.5073/JABFQ.2017.090.033
6. Hubert, J., Berger, M., Nepveu, F., Paul, F., Daydé, J. (2008). Effects of fermentation on the phytochemical composition and antioxidant properties of soy germ. *Food Chemistry*, *109*(4), 709–721.
https://doi.org/10.1016/j.foodchem.2007.12.081
7. Hussain, T., Abbas, S., Khan, M.A., Scrimshaw, N.S. (2004). Lysine fortification of wheat flour improves selected indices of the nutritional status of predominantly cereal-eating families in Pakistan. *Food and Nutrition Bulletin*, *25*(2), 114–122.
https://doi.org/10.1177/156482650402500202
8. Jalili, M., Hekmatdoost, A., Vahedi, H., Poustchi, H., Khademi, B., Saadi, M., Zemestani, M., Janani, L. (2016). Co-administration of soy isoflavones and vitamin D in management of irritable bowel disease. *PLOS One*, *11*, art. no. e0158545.
https://doi.org/10.1371/journal.pone.0158545
9. Je, J.-Y., Park, P.-J., Jung, W.-K., Kim, S.-K. (2005). Amino acid changes in fermented oyster (*Crassostrea gigas*) sauce with different fermentation periods. *Food Chemistry*, *91*(1), 15–18.
https://doi.org/10.1016/j.foodchem.2004.05.061
10. Jiao, C., Yang, R., Zhou, Y., Gu, Z. (2016). Nitric oxide mediates isoflavone accumulation and the antioxidant system enhancement in soybean sprouts. *Food Chemistry*, *204*, 373–380.
https://doi.org/10.1016/j.foodchem.2016.02.147
11. Ju, H.J., Lim, H.B., Hyun, T.K. (2021). The chemical composition and anti-inflammatory effect of the essential oil obtained from *Abeliophyllum distichum* flowers. *Botanica Serbica, 45*(1), 137–142.
https://doi.org/10.2298/BOTSERB2101137J
12. Jung, W., Yu, O., Lau, S.-M.C., O'Keefe, D.P., Odell, J., Fader, G., McGonigle, B. (2000). Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. *Nature Biotechnology, 18*, 208–212.
https://doi.org/10.1038/72671
13. Kim, I.-D., Dhungana, S.K., Park, Y.-S., Kim, D., Shin, D.-H. (2017). Persimmon fruit powder may substitute Indolbi, a synthetic growth regulator, in soybean sprout cultivation. *Molecules*, *22*(9), art. no. 1462.
https://doi.org/10.3390/molecules22091462
14. Kim, I.-D., Lee, J.-W., Kim, S.-J., Cho, J.-W., Dhungana, S.K., Lim, Y.-S., Shin, D.-H. (2014). Exogenous application of natural extracts of persimmon (*Diospyros kaki* Thunb.) can help in maintaining nutritional and mineral composition of dried persimmon. *African Journal of Biotechnology*, *13*, 2231–2239.
https://doi.org/10.5897/AJB2013.13503
15. Kim, J.-H., Yoon, Y.-H., Kim, I.-D., Dhungana, S.K., Shin, D.-H. (2020). Pu-erh tea extract treatment could be an efficient way to enhance the yield and nutritional value of soybean sprout. *Molecules*, *25*(17), art. no. 3869.
https://doi.org/10.3390/molecules25173869
16. Kim, S.-L., Lee, J.-E., Kwon, Y.-U., Kim, W.-H., Jung, G.-H., Kim, D.-W., Lee, C.-K., Lee, Y.-Y., Kim, M.-J., Kim, Y.-H., Hwang, T.-Y., Chung, I.-M. (2013). Introduction and nutritional evaluation of germinated soy germ. *Food Chemistry*, *136*(2), 491–500.
https://doi.org/10.1016/j.foodchem.2012.08.022
17. Kujawska, M., Ewertowska, M., Ignatowicz, E., Adamska, T., Szaefer, H., Zielińska-Dawidziak, M., Piasecka-Kwiatkowska, D., Jodynis-Liebert, J. (2016). Evaluation of safety of iron-fortified soybean sprouts, a potential component of functional food, in rat. *Plant Foods for Human Nutrition*, *71*, 13–18.
https://doi.org/10.1007/s11130-016-0535-8
18. Kwak, H.-S., Dhungana, S.K., Kim, I.-D., Shin, D.-H. (2017). Effect of lacquer (*Toxicodendron vernicifluum*) extract on yield and nutritional value of soybean sprouts. *Korean Journal of Food Science and Technology*, *49*(4), 415–420.
https://doi.org/10.9721/KJFST.2017.49.4.415
19. Kwon, S., Kang, H., Kim, M., Kim, J., Shin, H., Kim, K. (2014). Analysis on the components and safety evaluation of *Abeliophyllum distichum* Nakai leaves and stems. *Journal of Environmental Health Sciences*, *40*(3), 234–244 (in Korean; English abstract).
https://doi.org/10.5668/JEHS.2014.40.3.234
20. Lee, H.-D., Kim, J.H., Pang, Q.Q., Jung, P.-M., Cho, E.J., Lee, S. (2020). Antioxidant activity and acteoside analysis of *Abeliophyllum distichum*. *Antioxidants*, *9*(11), art. no. 1148.
https://doi.org/10.3390/antiox9111148
21. Lee, J.H., Cho, K.M. (2012). Changes occurring in compositional components of black soybeans maintained at room temperature for different storage periods. *Food Chemistry*, *131*, 161–169.
https://doi.org/10.1016/j.foodchem.2011.08.052
22. Lee, J.H., Kang, N.S., Shin, S.-O., Shin, S.-H., Lim, S.-G., Suh, D.-Y., Baek, I.-Y., Park, K.-Y., Ha T.J. (2009). Characterisation of anthocyanins in the black soybean (*Glycine max* L.) by HPLC--DAD-ESI/MS analysis. *Food Chemistry*, *112*, 226–231.
https://doi.org/10.1016/j.foodchem.2008.05.056
23. Lee, Y.G., Choi, W.S., Yang, S.O., Bo, J.H., Kim, H.G., Fang, M., Yi, T.Y., Kang, S.C., Lee, Y.H., Baek, N.I. (2021). Volatile profiles of five variants of *Abeliophyllum distichum* flowers using headspace solid-phase microextraction gas chromatography--mass spectrometry (HS-SPME-GC-MS) analysis. *Plants, 10*, art. no. 224.
https://doi.org/10.3390/plants10020224
24. Li, H.M., Kim, J.K., Jang, J.M., Cui, C.B., Lim, S.S. (2013). Analysis of the inhibitory activity of *Abeliophyllum distichum* leaf constituents against aldose reductase by using high-speed counter current chromatography. *Archives of Pharmacal Research*, *36*, 1104–1112.
https://doi.org/10.1007/s12272-013-0127-1

25. Lintschinger, J., Fuchs, N., Moser, J., Kuehnelt, D., Goessler, W. (2000). Selenium-enriched sprouts. A raw material for fortified cereal-based diets. *Journal of Agricultural and Food Chemistry*, *48*, 5362–5368. https://doi.org/10.1021/jf000509d
26. Oh, H., Kang, D.-G., Kwon, T.-O., Jang, K.K., Chai, K.-Y., Yun, Y.-G., Chung, H.-T., Lee, H.-S. (2003). Four glycosides from the leaves of *Abeliophyllum distichum* with inhibitory effects on angiotensin converting enzyme. *Phytotherapy Research*, *17*, 811–813. https://doi.org/10.1002/ptr.1199
27. Park, G.H., Park, J.H., Eo, H.J., Song, H.M., Woo, S.H., Kim, M.K., Lee, J.W., Lee, M.H., Lee, J.R., Koo, J.S., Jeong, J.B. (2014). The induction of activating transcription factor 3 (ATF3) contributes to anti-cancer activity of *Abeliophyllum distichum* Nakai in human colorectal cancer cells. *BMC Complementary and Alternative Medicine*, *14*, art. no 487. https://doi.org/10.1186/1472-6882-14-487
28. Park, M., Kim, D., Kim, B., Nahmgoong, B. (1995). Studies on pollution-free soybean sprout production and circulation market improvement. *Korea Soybean Digest*, *12*, 51–67.
29. Paucar-Menacho, L.M., Berhow, M.A., Mandarino, J.M.G., Chang, Y.K., Mejia, E.G. de. (2010). Effect of time and temperature on bioactive compounds in germinated Brazilian soybean cultivar BRS 258. *Food Research International*, *43*, 1856–1865. https://doi.org/10.1016/j.foodres.2009.09.016
30. Sathyapalan, T., Aye, M., Rigby, A.S., Thatcher, N.J., Dargham, S.R., Kilpatrick, E.S., Atkin, S.L. (2018). Soy isoflavones improve cardiovascular disease risk markers in women during the early menopause. *Nutrition, Metabolism and Cardiovascular Diseases*, *28*, 691–697. https://doi.org/10.1016/j.numecd.2018.03.007
31. Skujins, S. (1998). *Handbook for ICP-AES (Varian-Vista). A Short Guide to Vista Series ICP-AES Operation*. Varian Int. AG, Zug, Switzerland.
32. Spanier, A.M., Shahidi, F., Parliment, T.H., Mussinan, C., Ho, C.-T., Tratras Contis, E., Kayahara, H., Tsukahara, K., Tatai, T. (2001). Flavor, health and nutritional quality of pre-germinated brown rice. In A.M. Spanier, F. Shahidi, T.H. Parliment, C. Mussinan, C.-H. Ho., C.T. Contis (Eds.), *Food Flavors and Chemistry: Advances of the New Millennium*, Royal Society of Chemistry, Cambridge, UK, pp. 546–551. https://doi.org/10.1039/9781847550859
33. Udomkun, P., Ilukor, J., Mockshell, J., Mujawamariya, G., Okafor, C., Bullock, R., Nabahungu, N.L., Vanlauwe, B. (2018). What are the key factors influencing consumers' preference and willingness to pay for meat products in Eastern DRC? *Food Science & Nutrition*, *6*, 2321–2336. https://doi.org/10.1002/fsn3.813
34. Wang, X., Yang, R., Jin, X., Shen, C., Zhou, Y., Chen, Z., Gu, Z. (2016). Effect of supplemental Ca_{2+} on yield and quality characteristics of soybean sprouts. *Scientia Horticulturae*, *198*, 352–362. https://doi.org/10.1016/j.scienta.2015.11.022
35. Wei, Y., Shohag, M.J.I., Wang, Y., Lu, L., Wu, C., Yang, X. (2012). Effect of zinc sulfate fortification in germinated brown rice on seed zinc concentration, bioavailability, and seed germination. *Journal of Agricultural and Food Chemistry*, *60*, 1871–1879. https://doi.org/10.1021/jf205025b
36. White, P.J., Broadley M.R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytologist*, *182*, 49–84. https://doi.org/10.1111/j.1469-8137.2008.02738.x
37. Wu, G. (2009). Amino acids: metabolism, functions, and nutrition. *Amino Acids*, *37*, 1–17. https://doi.org/10.1007/s00726-009-0269-0
38. Xu, N., Zou, T., Pang, J.W., Hu, G.L. (2012). Effects of exogenous zinc soaking on the seed germination, sprout growth of soybean and zinc accumulation in the sprouts. *Soybean Science*, *31*, 932–936.
39. Yoo, T.-K., Jeong, W.T., Kim, J.G., Ji, H.S., Ahn, M.-A., Chung, J.-W., Lim, H.B., Hyun, T.K. (2021). UPLC-ESI-Q-TOF-MS-based metabolite profiling, antioxidant and anti-inflammatory properties of different organ extracts of *Abeliophyllum distichum*. *Antioxidants*, *10*, art. no. 70. https://doi.org/10.3390/antiox10010070
40. Zhu, D., Hettiarachchy, N.S., Horax, R., Chen, P. (2005). Isoflavone contents in germinated soybean seeds. *Plant Foods for Human Nutrition*, *60*, 147–151. https://doi.org/10.1007/s11130-005-6931-0
41. Zou, T., Xu, N., Hu, G., Pang, J., Xu, H. (2014). Biofortification of soybean sprouts with zinc and bioaccessibility of zinc in the sprouts. *Journal of the Science of Food and Agriculture*, *94*, 3053–3060. https://doi.org/10.1002/jsfa.6658

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Original article
Section: Food Quality and Functionality

Characterization of Triterpene Saponin Composition of White, Yellow and Red Beetroot (*Beta vulgaris* L.)

Aneta Spórna-Kucab[1], Anna Tekieli[1], Krystyna Skalicka-Woźniak[2], Agnieszka Grzegorczyk[3], Tomasz Świergosz[1], Sławomir Wybraniec[1]*

[1]Department of Chemical Technology and Environmental Analysis, Faculty of Chemical Engineering and Technology, Cracow University of Technology, Warszawska Str. 24, 31–155 Kraków, Poland
[2]Department of Natural Products Chemistry, Medical University of Lublin, Chodźki Str. 1, 20–093 Lublin, Poland
[3]Department of Pharmaceutical Microbiology with Laboratory for Microbiological Diagnostics, Medical University of Lublin, Chodźki Str. 1, 20–093 Lublin, Poland

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Beta vulgaris L. is an important source of bioactive saponins – a group of secondary metabolites – that have spurred a growing interest due to their health-promoting properties. This study aimed to gain information on triterpene saponin profile of the peel and flesh of white, yellow and red beet of six cultivars – Snow Ball, Boldor, Ceryl, Chrobry, Forono and Tytus – harvested in Poland, in the same region. Twenty four saponins with oleanolic acid, hederagenin, akebonoic acid and gypsogenin as aglycons were identified and quantified by liquid chromatography/tandem mass spectrometry (LC--ESI-MS/MS). Among them, betavulgaroside I, II, III and IV were the major compounds, but the quantitative profile of saponins was found to be dependent on beet cultivar and root part, respectively. The highest content of saponins was found in the peel of yellow *B. vulgaris* Boldor (20812 mg/kg fresh weight, fw), while the lowest saponin content was determined in the flesh of white *B. vulgaris* Snow Ball (497 mg/kg fw). In addition, the total saponin content in peel and flesh in yellow beet (26054 mg/kg fw) was much higher than the total content in peel and flesh in red beet Tytus (8364 mg/kg fw) and white beet Snow Ball (1204 mg/kg fw). This is the first report on the profile of saponins in white and yellow beets.

INTRODUCTION

Beetroot (*Beta vulgaris* L.) belongs to Chenopodiaceae family and has several varieties ranging from the white to yellow and red [Bárta *et al*., 2020]. *B. vulgaris* is a vegetable consumed worldwide due to its health benefits. It can be eaten as raw, boiled, steamed and roasted [Kavalcová *et al*., 2015]. Many studies confirm that the consumption of this vegetable helps protect against several diseases due to the presence of compounds implicated in numerous health benefits [Chhikara *et al*., 2019; Clifford *et al*., 2015]. These bioactive compounds include betalains [Pietrzkowski *et al*., 2010; Wybraniec *et al*., 2011], carotenoids, polyphenols (including flavonoids) [Chhikara *et al*., 2019], inorganic nitrate [dos Santos Baião *et al*., 2021; Lidder & Webb, 2013] and saponins [Mikołajczyk-Bator *et al*., 2016; Sparg *et al*., 2004].

Saponins are natural glycosides that are known for their physicochemical (biosurfactant) properties and biological activities; therefore, they are commercially significant compounds with applications in the food, cosmetic and pharmaceutical industries [Güçlü-Üstündağ & Mazza, 2007; Rai *et al*., 2021]. A recent study has indicated multiple and complex activities of triterpene saponins, including anti-inflammatory, anti-bacterial, anti-allergic, hepatoprotective and anti-tumor ones; therefore, foods rich in saponins may reduce the risk of development of selected diseases [Fang *et al*., 2020].

Due to the considerable application potential of saponins, in recent years there has been a significant increase in research on these compounds in terms of their uses as eco-friendly, biodegradable biosurfactants [Muhammad & Khan, 2018; Schmitt *et al*., 2014] and pharmaceuticals [Mbaveng *et al*., 2018; Xu *et al*., 2018]. Beetroot, as a readily available and inexpensive vegetable, appears to be a good source of saponins [Mroczek *et al*., 2012; Spórna-Kucab & Wybraniec, 2020]. Triterpene saponins present in *Beta vulgaris* L. are complex molecules consisting of the aglycone – hederagenin, akebonoic acid, oleanolic acid or gypsogenin coupled to sugar chain units. The number/type of sugars and different possibilities of sugar chain composition cause great natural diversity of saponin structures in *Beta vulgaris* L. [Mikołajczyk-Bator *et al*., 2016]. The glycosidic bond is present between the aglycone and one (monodesmoside) or two (didesmoside) sugar

* Corresponding Author:
E-mail: aneta.sporna-kucab@pk.edu.pl (A. Spórna-Kucab)

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chains at C-28 (*via* an ester) or C-3 (*via* an ether). The glycone can consist of hexoses, pentoses, 6-deoxyhexoses and uronic acids [Mikołajczyk-Bator *et al*., 2016].

Saponins are present in the leaves and roots of *Beta vulgaris* L. [Mroczek *et al*., 2019] and were found in the roots of red beet cultivars [Mikołajczyk-Bator *et al*., 2016; Mroczek *et al*., 2019] as well as in the sugar beets. Beetroot varieties differ in terms of their profile and content. Moreover, the differences in the content of saponins are also noticed in roots and leaves of the same variety [Edelmann *et al*., 2020].

White and yellow beet saponins have never been studied so far, which might be due to the low availability of these varieties compared to red beet. Thus, for a comprehensive characterization of the composition of triterpene saponins, their accurate profiles were studied in selected red (Ceryl, Chrobry, Forono, Tytus) as well as yellow (Boldor) and white (Snow Ball) varieties harvested in Poland.

MATERIALS AND METHODS

Sample materials

Red, yellow and white *B. vulgaris* cultivars – Ceryl, Chrobry, Forono, Tytus, Boldor and Snow Ball, were collected in Poland. The red cultivars (Ceryl, Chrobry, Forono, Tytus) were harvested from Spójnia company (Nochowo, Poland) and the yellow cultivar from Bejo company (Ożarów Mazowiecki, Poland) in September 2019. The seeds of white cultivar were harvested from Torseed company (Toruń, Polska) and grown in September 2021. The fresh roots were washed under running water and peeled out to obtain the peel and flesh for further quantitative and qualitative analyses of saponins. Individual plant parts, the peel and flesh of studied *B. vulgaris* cultivars, were weighed before extraction and stored in a freezer.

Solvents and reference compounds

Respective standards from a previous study on *B. vulgaris* (Red Sphere cultivar) were used for the saponin identification [Spórna-Kucab & Wybraniec, 2020]. Ethanol was purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland). LC-MS-grade acetonitrile and formic acid (purity ≥98%) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Oleanolic acid standard was purchased from Sigma-Aldrich. All chemicals and solvents were of analytical grade and used as received. Water utilized throughout the experiments with a resistivity of 18.0 mΩ/cm at 21°C was deionized in a Milli-Q purification system (Merck, Darmstadt, Germany).

Sample extraction

A 100-g portion of each peel or flesh was extracted with 300 mL of 80% (*v/v*) ethanol by ultrasound-assisted maceration for 30 min. The procedure was repeated three times, and each time extracts were combined, partially concentrated at 25°C under reduced pressure and freeze-dried. Finally, the freeze-dried extracts were weighed and used for further studies on the quantitative and qualitative profile of saponins by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) system.

LC-MS – instrumentation and conditions

Qualitative and quantitative analyses were performed by high-performance liquid chromatography and electro-spray ionization mass spectrometry (LC-ESI-MS/MS) using an LCMS-8030 system (Shimadzu, Kyoto, Japan). The LC system consisted of a SIL-20ACXR autosampler, a degasser, and a Nexera LC-20ADXR binary pump equipped with a gradient controller. LC separation was performed using a 100×4.6 mm, 5.0 μm Kinetex C_{18} chromatography column (Phenomenex, Torrance, CA, USA) protected by a 4×2 mm guard column of the same material (Phenomenex) and thermostated at 40°C. LabSolutions software version 5.91 SP1 was used for data acquisition in LC -ESI-MS/MS.

In the LC system, the mobile phase was composed of acetonitrile (A) and 2% (*v/v*) aqueous formic acid (B). The flow rate was kept constant at 0.5 mL/min. Oleanolic acid and extracts were analyzed in different gradient elutions. For extracts, the gradient elution was programmed as follows: from 5% to 60% A, 0–62 min; from 60% to 80% A, 62–65 min, 80% to 5% A, 65–66 min. For oleanolic acid, the gradient elution was as follows: from 20% to 99% A, 0–7 min; 99% A, 7–15 min.

Triple quadrupole mass spectrometer with an electrospray ion source coupled to the LC system described above was used for MS/MS experiments. The following instrumental parameters were applied in ESI-MS/MS analysis of saponins: curved desolvation line (CDL) and heat block temperature of 230°C, nebulizing gas flow rate of 1.5 L/min, electrospray voltage of 4.5 kV and the capillary temperature at 250°C. The relative collision energies for MS/MS analyses were set at -35 V, and argon was used as the collision gas. Data were recorded in a negative ion mode using a scan mode with *m/z* ranging from 100 to 2000 Da and the selected ion monitoring (SIM).

Quantitative and qualitative analysis of saponins

Oleanolic acid is an aglycone of the most saponins identified in the *B. vulgaris* roots, thus, it was utilized to plot the calibration curve. A stock solution of the external standard – oleanolic acid (20.0 μg/mL), was prepared in water and stored at 8°C until further use. Calibration solutions were prepared by 1:1 (*v/v*) stepwise dilution of the stock solution in order to get five calibration points over a concentration range from 1.25 to 20.0 μg/mL. Then, 10 μL of the standard was injected three times to the LC-ESI-MS/MS system. The calibration curve was prepared by plotting the peak area ratios of the standard from MS chromatograms against their concentrations showing a linear response with the coefficient of determination (R^2) of 0.9996.

In the analysis of saponins, freeze-dried extracts (100 mg) were diluted in demineralized water (1 mL) and centrifuged at 2504×*g* for 5 min, then 20 μL of each sample was injected three times to the LC-ESI-MS/MS system. The content of individual saponins as well as their total content in the studied extract was estimated from the external standard calibration curve plotted for oleanolic acid. The peak areas from LC-ESI-MS chromatograms were used for quantification. The use of aglycone in the analysis of glycosides bears the risk of receiving a less accurate result than in the case of using single, separated standards of saponins. However, at present, such standards are not easily available and obtaining them

from the plant is time-consuming and challenging. Therefore, using the calibration curve based on oleanolic acid seems to be a good solution in saponin analysis.

Three independent LC-ESI-MS/MS analyses were run for each sample to evaluate instrumental precision. The MS chromatograms allowed identifying compounds below the LOQ. Compounds with signal-to-noise ratio lower than 10:1 were below LOQ.

Content of saponins was expressed in mg/kg fresh weight (fw) of peel or flesh.

Statistical analysis

Data were reported as the mean ± standard deviation (SD) of three measurements. One-way analysis of variance (ANOVA) was used for the statistical analysis of the data with the help of Statistica, version 7.1 (StatSoft, TIBCO Software Inc. Palo Alto, CA, USA). Differences between means were determined using Fisher's test and were found significant at $p<0.05$.

RESULTS AND DISCUSSION

Identification of triterpene saponins in *B. vulgaris*

The complete structural characterization of saponins in plant materials is complicated and time-consuming. The LC-ESI-MS/MS is a viable method used to characterize natural compounds based on their fragmentation [Spórna-Kucab *et al*., 2019, 2020; Wybraniec *et al*., 2010].

The analysis of extracts of peel and flesh of four red (Ceryl, Chrobry, Forono, Tytus), one yellow (Boldor) and one white (Snow Ball) *B. vulgaris* cultivars by LC-ESI-MS/MS revealed the presence of 24 triterpene saponins consisting of oleanolic acid, hederagenin, akebonoic acid and gypsogenin as the aglycones (Figure 1). The compounds identified as well as their retention times, $[M–H]^-$ and MS/MS data are summarized in Table 1.

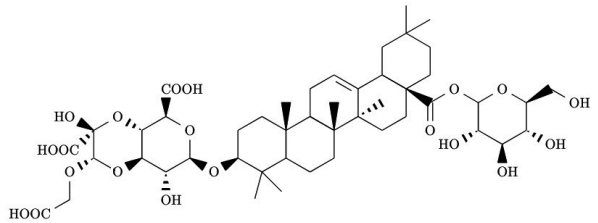
Ten compounds (saponins **3**, **5**, **7–9**, **17**, **18**, **21**, **22**, **24**) were identified by comparison with the reference compounds isolated in a previous research [Spórna-Kucab & Wybraniec, 2020]. In contrast, the remaining 14 compounds were tentatively identified by the interpretation of their fragmentation patterns obtained from mass spectra (MS/MS experiments) and by comparison with the previous experiments reported in the literature [Mikołajczyk-Bator *et al*., 2016; Mroczek *et al*., 2012, 2019].

Saponins with oleanolic acid as the aglycone

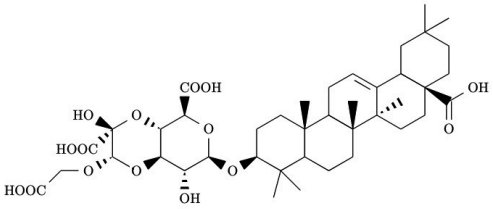
Previous research on saponins show that *B. vulgaris* contains significant amounts of oleanolic acid derivatives, which we also confirmed in our research. Here, seventeen saponins as oleanolic acid derivatives were identified in the peel and flesh samples of all analyzed beetroot cultivars (Table 1, Figure 2, Figure 3, Figure 4, Figure 5).

Oleanolic acid as well as saponins containing oleanolic acid are of great interest to the food, cosmetic and pharmaceutical industries because they exhibit various biological properties [Dubois *et al*., 1990; Hikino *et al*., 1985; Lemmich *et al*., 1995; Parus, 2013; Yoshikawa *et al*., 1994]. Oleanolic acid itself exhibits anti-inflammatory, antibacterial and antiseptic [Ismaili *et al*., 2001] as well as hepatoprotective [Hikino *et al*., 1985] and hypoglycemic effects [Yoshikawa *et al*., 1994].

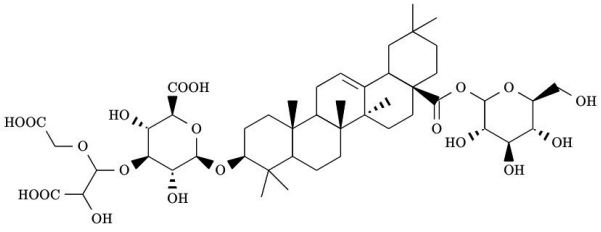
The following sugar moieties were found in the identified *B. vulgaris* saponins: uronic acid (UrA), deoxyhexose (dHex), pentose (Pen), hexose (Hex), substituted sugar residues of the acetal (Act) and dioxolane (Diox) type (Table 1). Different sugar moieties as well as a different number of sugar units were noticed. The previous research results show that the activity of saponins containing oleanolic acid depends on the structure of the sugar chain [De Tommasi *et al*., 1991; Yoshikawa *et al*., 1994] and can exhibit wider spectrum of activities then oleanolic acid, inlcuding *e.g.* immunostimulating [Dubois *et al.,* 1990], cytotoxic, anti-carcinogenic, anti-mutagenic, antiviral and protozoicidal. Moreover, saponins are well known for their hemolytic properties, which are not always desired; however, this strongly depends on the type of sugar, the number of sugar groups and the spatial arrangement of sugar chains [Lemmich *et al*., 1995]. Moreover, the tumor-specificity of the cytotoxic action seems to be influenced by the structure of the sugar portion of the saponins [Kuroda *et al*., 2001].



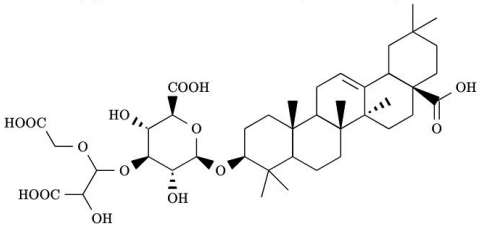
(a) betavulgaroside I (saponin **8**)



(b) betavulgaroside II (saponin **23**)



(c) betavulgaroside III (saponin **9**)



(d) betavulgaroside IV (saponin **22**)

FIGURE 1. Chemical structures of the main saponins detected in *Beta vulgaris* L.: (a) betavulgaroside I, (b) betavulgaroside II, (c) betavulgaroside III, and (d) betavulgaroside IV.

TABLE 1. Chromatographic and mass-spectrometric data of saponins identified in *Beta vulgaris* L. extracts.

No.	Saponin	Trivial name	t_R (min)	*m/z* $[M–H]^-$	*m/z* from MS^2 of $[M–H]^-$
1	Act-Hex-Hex-UrA-oleanolic acid	betavulgaroside V	36.3	1117	997; 955; 793; 631; 455
2	Act-Hex-UrA-akebonoic acid	betavulgaroside VIII	38.2	939	777; 615; 439
3	Act-Hex-Hex-UrA-oleanolic acid	betavulgaroside V	38.4	1117	997; 955; 793; 631; 455
4	Hex-Pen-Hex-UrA-oleanolic acid		38.6	1087	967; 925; 763; 631; 455
5	Act-Hex-Pen-UrA-oleanolic acid	betavulgaroside IX	39.4	1087	925; 763; 631; 455
6	Act-Hex-Hex-UrA-oleanolic acid	betavulgaroside V	40.1	1117	997; 955; 793; 631; 455
7	Hex-Pen-UrA-oleanolic acid		41.0	925	763; 631; 455
8	Diox-Hex-UrA-oleanolic acid	betavulgaroside I	41.5	953	909; 793; 631; 455
9	Act-HexUrA-oleanolic acid	betavulgaroside III	41.8	955	835; 793; 673; 631; 455
10	Act-dHex-UrA-oleanolic acid		45.7	939	777; 631; 455
11	Hex-Pen-UrA-akebonoic acid	betavulgaroside X	46.3	909	747; 615; 439
12	Act-dHex-UrA-oleanolic acid		47.0	939	777; 631; 455
13	Pen-UrA-hederagenin		47.2	779	647; 471
14	$C_5H_4O_5$-Hex-UrA-akebonoic acid		47.8	921	439
15	Act-UrA-hederagenin	betavulgaroside VII	48.0	809	647; 471
16	Hex-UrA-gypsogenin		48.4	807	645; 469
17	Hex-Pen-UrA-oleanolic acid		49.7	925	763; 631; 455
18	Act-Hex-UrA-oleanolic acid	betavulgaroside III	49.8	955	835; 793; 673; 631; 455
19	Act-UrA-akebonoic acid		51.0	777	615; 439
20	$C_4H_4O_5$-Hex-UrA-oleanolic acid		51.4	925	763; 631; 455
21	Pen-UrA-oleanolic acid		54.3	763	631; 455
22	Act-UrA-oleanolic acid	betavulgaroside IV	55.6	793	673; 631; 455
23	Diox-UrA-oleanolic acid	betavulgaroside II	56.0	791	631; 455
24	UrA-oleanolic acid		56.4	631	455

Act – acetal substituent, Diox – dioxolane substituent, Hex – hexose, dHex – deoxyhexose, Pen – pentose, UrA – uronic acid, t_R – retention time.

Oleanolic acid in the unbound form has not been identified, but its derivative with uronic acid was detected. The main and simplest saponin with the pseudomolecular ion $[M–H]^-$ at *m/z* 631 was tentatively identified as saponin **24** based on its fragmentation pattern and by comparison to the reference compound previously isolated from *B. vulgaris* cultivar Red Sphere [Spórna-Kucab & Wybraniec, 2020]. Compound **24** fragmented to *m/z* 455, which corresponded to oleanolic acid. The presence of a daughter ion for the studied compounds at *m/z* 455 indicated the loss of the uronic acid (631–455=176) for saponin **24** [Mroczek *et al*., 2012, 2019; Spórna-Kucab & Wybraniec, 2020]. Previous research indicates that uronic acid attached to the oleanolic acid may significantly enhance its activity against retinovirus, which is responsible for most colds [De Tommasi *et al*., 1991]. This saponin was also identified previously in the *B. vulgaris* cultivar Red Sphere [Mroczek *et al*., 2012, 2019; Spórna-Kucab & Wybraniec, 2020].

Saponin profiles in *B. vulgaris* revealed the presence of three monosubstituted with pentose (saponin **21**), acetal (saponin **22**) or dioxolane (saponin **23**) derivatives of saponin **24**. The presence of daughter ions for compounds at *m/z* 631 indicated the loss of pentose (763–631=132) for saponin **21**, acetal-type substituent (793–631=162) for saponin **22** and dioxolane-type substituent (791–631=160) for saponin **23**, respectively. The structures of saponin **21** and **22** were confirmed with the reference compound from *B. vulgaris* cultivar Red Sphere [Spórna-Kucab & Wybraniec, 2020]. Compound **21** has been previously identified in leaves of *B. vulgaris* cultivars Red Sphere, Forono, Egyptian and Round Dark [Mroczek *et al*., 2019]. The structure and fragmentation pattern of saponin **22**, commonly known as betavulgaroside IV, was previously described [Mikołajczyk-Bator *et al*., 2016; Yoshikawa *et al*., 1996]. Saponin **23** was detected with $[M–H]^-$ at *m/z* 791 and the fragmentation daughter ions at *m/z* 631 and 455. Previous research, revealed two saponins with *m/z* 791 [Mikołajczyk-Bator *et al*., 2016]. These

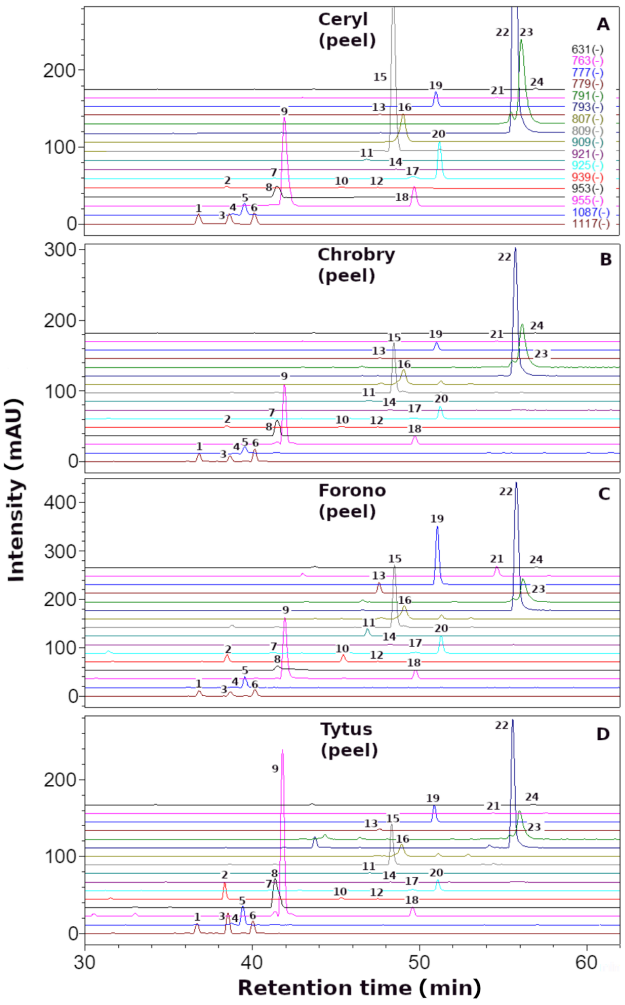


FIGURE 2. Single-ion monitoring chromatograms obtained by LC-ESI--MS in a negative ion mode for saponins of red *Beta vulgaris* L. peels: (a) Ceryl, (b) Chrobry, (c) Forono, and (d) Tytus.

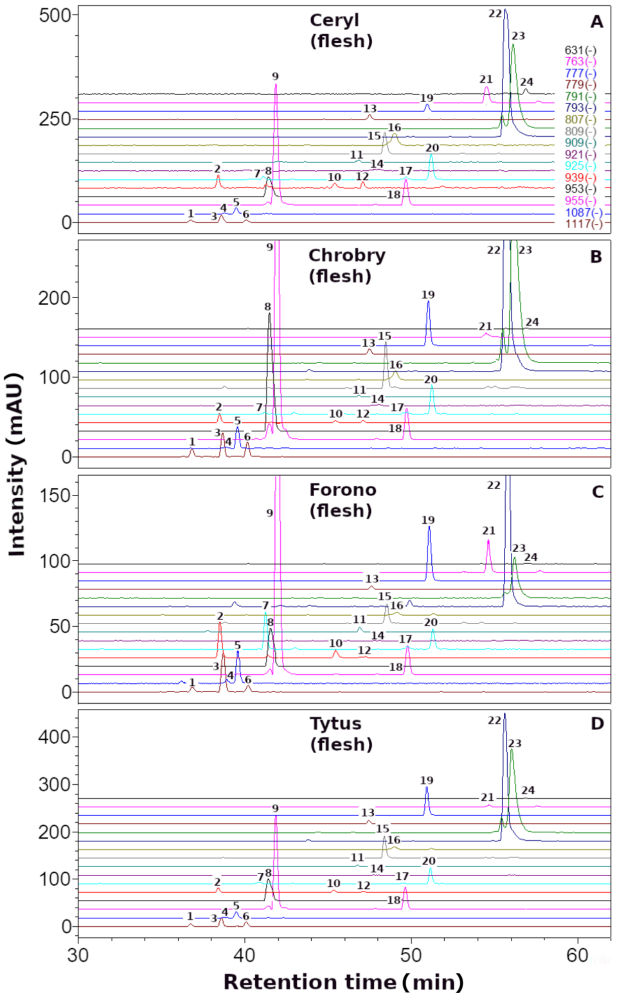


FIGURE 3. Single-ion monitoring chromatograms obtained by LC-ESI--MS in a negative ion mode for saponins of red *Beta vulgaris* L. flesh: (a) Ceryl, (b) Chrobry, (c) Forono, and (d) Tytus.

saponins differ in the presence of the aglycones: oleanolic acid (*m/z* 455) or akebonoic acid (*m/z* 439). Herein, the MS/MS spectrum of **23** exhibited an aglycone at *m/z* 455; therefore, this saponin has been identified as betavulgaroside II. Previous research showed that hexose attachment to oleanolic acid and glucuronic acid enhanced the antiviral activity of saponins [De Tommasi *et al*., 1991]; however, to the best of our knowledge, there is no research on the effect of a single substituent of another sugar on the properties of saponins.

The monosubstituted with hexose saponin **24** was not detected in any peel and flesh sample but simultaneous presence of hexose and dioxalane (saponin **8**), hexose and acetal-type (saponin **9**, **18**) or hexose and $C_4H_4O_5$ (saponin **20**) substituents was noticed.

Saponins **8** ([M–H]$^-$ at *m/z* 953), **9** and **18** ([M–H]$^-$ at *m/z* 955) were confirmed with the authentic standards isolated and described in previous experiments [Spórna-Kucab & Wybraniec, 2020]. These saponins were thoroughly described using NMR by Yoshikawa *et al*. [1996], which named them as betavulgaroside I (saponin **8**) and III (saponin **9**, **18**) (Table 1). Betavulgaroside I and III were also isolated from *Achyranthes fauriei* and named achyranthoside B and C, respectively [Ida *et al*., 1994]. Achyranthoside B may find application in the treatment of cancer, as research shows that it is toxic to human colorectal cancer cells and murine melanoma cells [Ida *et al*., 1994].

The fragmentation pattern of saponin **20** ([M–H]$^-$ at *m/z* 925) was already described in the studied *B. vulgaris* cultivar Nochowski [Mikołajczyk-Bator *et al*., 2016]; therefore, its tentative identification was feasible.

Hexose-hexose and hexose-pentose substituents in saponin **24** ([M–H]$^-$ at *m/z* 631) were not identified. But hexose--hexose-acetal and hexose-pentose-hexose substituents were detected in saponins **1**, **3** and **6** ([M–H]$^-$ at *m/z* 1117) as well as saponin **4** ([M–H]$^-$ at *m/z* 1087). It is worth noting that tetraglycoside saponins have stronger bactericidal properties than triglycoside saponins [Francis *et al*., 2002; Konishi *et al*., 1998]. Moreover, antibacterial properties generally depend on the attached sugar; therefore, these compounds may be very interesting for further studies of their antimicrobial activities. Saponin **6** was named betavulgaroside V and was also isolated from *Achyranthes fauriei* and named achyranthoside D

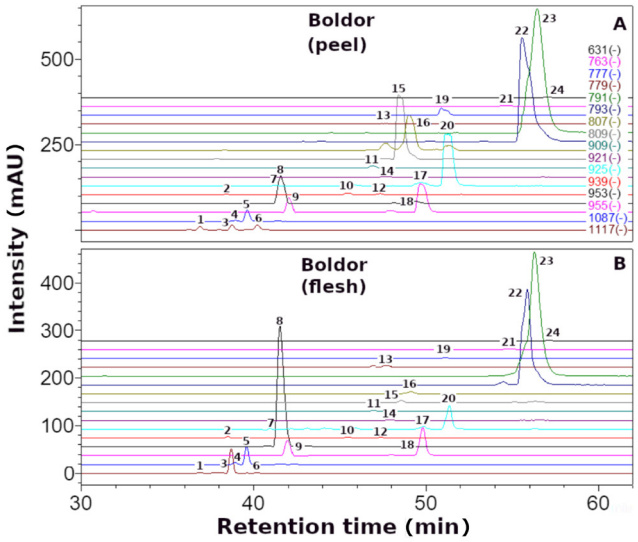


FIGURE 4. Single-ion monitoring chromatograms obtained by LC-ESI--MS in a negative ion mode for saponins of yellow *Beta vulgaris* L.: (a) peel, and (b) flesh.

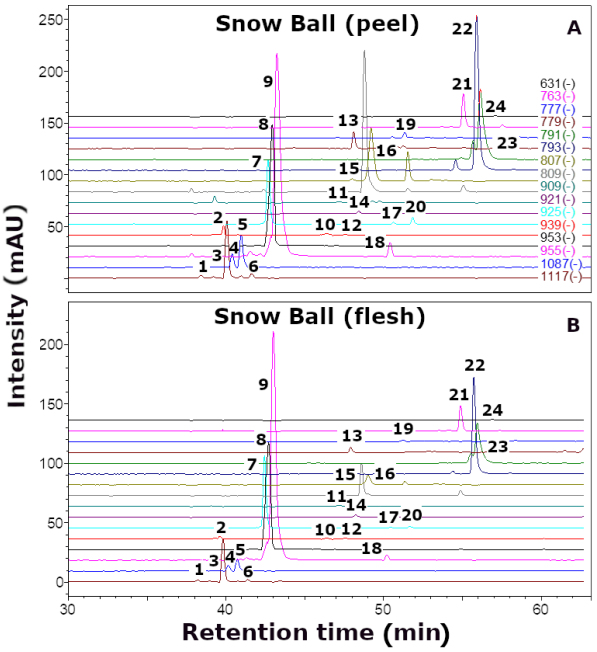


FIGURE 5. Single-ion monitoring chromatograms obtained by LC-ESI--MS in a negative ion mode for saponins of white *Beta vulgaris* L.: (a) peel, and (b) flesh.

[Kuwada *et al*., 2020]. Saponin **3** was isolated in previous research and used here as a reference compound [Spórna--Kucab & Wybraniec, 2020]. For saponins detected with $[M–H]^-$ at *m/z* 1117, the previous study [Mikołajczyk-Bator *et al*., 2016] of *B. vulgaris* cultivars indicated the presence of four positional isomers of two hexose, one uronic acid moiety, and an acetal-type substituent attached to the oleanolic acid by an ester bond at the C-28 position or another bond at the C-3 position. Saponin **4** was tentatively identified by comparison with previously reported data [Mikołajczyk--Bator *et al*., 2016]. Previous study found that saponin **4** had a positional isomer with identical aglycone ions detected at *m/z* 455 and identical moieties (*i.e*., uronic acid, two hexoses and pentose) connected at different positions. Hence, the more polar isomer of **4** was not identified, presumably because of its low content.

The saponin **24** might also be bisubstituted with pentose-hexose (saponin **7**, **17**) and trisubstituted with pentose-hexose-acetal (saponin **5**). The signal detected with $[M–H]^-$ at *m/z* 1087 (saponin **5**) gave a base peak in the MS chromatogram. Saponin **5** showed molecular negative ions at *m/z* 925, 763, 631, and 455, suggesting the presence of substituted sugar residues of acetal (1087–925=162 Da), hexose (925–763=162 Da), pentose (763–631=132) and uronic acid (631–455=176), respectively. The two saponins detected with $[M–H]^-$ at *m/z* 925 (saponin **7**, **17**), which were already described in *B. vulgaris* cultivar Nochowski [Mikołajczyk--Bator *et al*., 2016], were found in this analysis. Saponins **5**, **7**, **17** were identified by comparison with the reference compounds isolated previously from *B. vulgaris* cultivar Red Sphere [Spórna-Kucab & Wybraniec, 2020]. The chemical structure of compound **5**, named betavulgaroside IX, was thoroughly described by H-NMR and C-NMR [Yoshikawa *et al*., 1998].

The saponin **24** bisubstituted with deoxyhexose and acetal substituent was detected (saponins **10**, **12**) and these compounds showed a pseudomolecular $[M–H]^-$ ion at *m/z* 939. Similar retention times of saponins **10** and **12** may indicate that these compounds might be isomers. Previous research [Mikołajczyk-Bator *et al*., 2016] indicates three saponins with oleanolic acid in *B. vulgaris*, with two of them having a similar structure and polarity. Therefore, these compounds were tentatively identified based on the interpretation of their fragmentation patterns.

Saponins with akebonoic acid as the aglycone

Four saponins, which comprised akebonoic acid as an aglycone, were identified and profiled using LC-ESI-MS/MS in the peel and flesh samples of all analyzed beetroot cultivars (Table 1, Figure 2, Figure 3, Figure 4, Figure 5).

The simplest saponin with a pseudomolecular ion $[M–H]^-$ detected at *m/z* 615 corresponded to akebonoic acid (*m/z* 439), whereas uronic acid (*m/z* 176) was not detected. Herein, the MS/MS spectrum revealed akebonoic acid as the aglycone for saponins with $[M–H]^-$ at *m/z* 777 (saponin **19**), 909 (saponin **11**), 921 (saponin **14**) and 939 (saponin **2**). These saponins were previously identified in *B. vulgaris* cultivar Nochowski [Mikołajczyk-Bator *et al*., 2016]. Additionally, saponin with a pseudomolecular ion detected at *m/z* 777 was identified in Swiss chard (*B. vulgaris*) [Mroczek *et al*., 2021].

Saponin **19** had a negative pseudomolecular ion at *m/z* 777 and daughter ions at *m/z* 615 and 439. The MS spectrum of compound **19** revealed an aglycone ion at *m/z* 439, which corresponded to akebonoic acid. Saponins with akebonoic acid were previously identified and profiled by LC-ESI-MS/MS in *B. vulgaris* cultivar Nochowski [Mikołajczyk-Bator *et al*., 2016]. Findings of research suggest two positional isomers of saponins with $[M–H]^-$ at *m/z* 777 differing in polarity. The fragmentation pattern as well as the polarity of saponin **19** indicate the presence

of an acetal-type substituent attached to the akebonoic acid (777–615=162) and uronic acid moiety (615–439=176).

Saponin **11** had a negative pseudomolecular ion detected at *m/z* 909, yielding fragmentation ions at *m/z* 747, 615 and 439. The *m/z* fragments corresponded to the loss of hexose (–162 Da) and pentose moieties (–132 Da) and further uronic acid (–176 Da), respectively. The ion detected at *m/z* 439 suggested akebonoic acid to be the aglycone, which allowed identifying compound **11** as betavulgaroside X. This compound was previously detected in the *B. vulgaris* cultivar Nochowski [Mikołajczyk-Bator *et al*., 2016]. Moreover, the structure of compound **11** was determined by Yoshikawa *et al*. [1996].

Saponin **14** $[M–H]^-$ ion was detected at *m/z* 921, which provided the product ion in the MS/MS spectra at *m/z* 439 characteristic for akebonoic acid. The review of previous findings [Mikołajczyk-Bator *et al*., 2016] indicates only one saponin in the *B. vulgaris* cultivars with $[M–H]^-$ at *m/z* 921; therefore, saponin **14** was tentatively identified by comparison with previously reported data.

Saponin **2** yielded a product ion in the MS/MS spectra at *m/z* 439 characteristic for akebonoic acid. Analysis of *B. vulgaris* cultivar Nochowski indicates the presence of three akebonoic acids as the aglycones [Mikołajczyk-Bator *et al*., 2016]. The dominant saponin in *B. vulgaris* including akebonoic acid glycosides is a well-known betavulgaroside VIII [Yoshikawa *et al*., 1998]. The remaining saponins with akebonoic acid were identified in trace amounts in the previous research [Mikołajczyk-Bator *et al*., 2016]; therefore, we did not detect these compounds in the studied material.

To the best of our knowledge, there are no studies on the bioactivity of purified saponins with akebonoic acid as the aglycone. The saponins might be very useful for the pharmaceutical industry because akebonoic acid is considered a potential candidate for the treatment of type-2 diabetes due to its antidiabetic activity [Dirir *et al*., 2021]. The previous study showed that fractions containing saponins with akebonoic acid and gypsogenin obtained from *Chenopodium bonus-henricus* L. exhibited hepatoprotective activity [Kokanova-Nedialkova *et al*., 2020].

Saponins with hederagenin and gypsogenin as the aglycones

In the present study, hederagenin and gypsogenin as the aglycones were detected in three saponins identified with $[M–H]^-$ at *m/z* 779 (saponin **13**), 807 (saponin **16**) and 809 (saponin **15**) (Table 1, Figure 2, Figure 3, Figure 4, Figure 5). Compound **13** detected with $[M–H]^-$ at *m/z* 779 was identified based on its fragmentation pattern (MS/MS experiments) and by comparison with the literature data [Mikołajczyk-Bator *et al*., 2016]. Literature findings indicate only one saponin detected with $[M–H]^-$ at *m/z* 779. Moreover, the fragmentation of saponin **13** to ions at *m/z* 647 and hederagenin as an aglycone at *m/z* 471 confirms the presence of pentose (779–647=132) including uronic acid (647–471=176) in the structure.

Saponin **15** presented a pseudomolecular ion $[M–H]^-$ at *m/z* 809 releasing fragment ions at *m/z* 647 (loss of acetal-type substituent) as well as *m/z* 471 (loss of uronic acid) and was tentatively identified as betavulgaroside VII. Saponin **15** was earlier identified in *B. vulgaris* [Mikołajczyk-Bator *et al*., 2016; Mroczek *et al*., 2012]. Previous findings [Mikołajczyk-Bator *et al*., 2016; Mroczek *et al*., 2012] indicate two saponin isomers detected with $[M–H]^-$ at *m/z* 809, significantly differing in their polarity, consequently, the confirmation of saponin **15** was more convenient.

Correspondingly, previous research [Mikołajczyk-Bator *et al*., 2016] suggested only one saponin with a molecular ion $[M–H]^-$ detected at *m/z* 807. A high content of saponin **16** in yellow *B. vulgaris* cultivar enabled its fragmentation, which indicated the loss of hexose (162 Da) and uronic acid (176 Da) from gypsogenin as an aglycone ($[M–H]^-$ at *m/z* 469), respectively.

The beneficial effect of hederagenin derivatives has been repeatedly confirmed [Fang *et al*., 2020; Kuljanabhagavad & Wink, 2009]. They have been proven to exhibit the following activities: antimicrobial, antioxidant, molluscidal, fungicidal [Kuljanabhagavad & Wink, 2009], anti-inflammatory, anti-arthritic, anticomplementary and cytotoxic [Fang *et al*., 2020]. Cytotoxic activity was also confirmed for gypsogenin derivatives [El Hazzam *et al*., 2020]; however, they have not been thoroughly tested for their effects. Therefore, their activity can be very interesting; hence it is important to look for their new sources. Hederagenian glycosides were detected in a previous study of saponins from the *B. vulgaris* cultivars Red Sphere, Rocket, Wodan and Nochowski [Mikołajczyk-Bator *et al*., 2016; Mroczek *et al*., 2012].

Saponin quantitative analysis

The quantification of the individual saponins of beetroot peel and flesh was carried out using oleanolic acid as a standard. The oleanolic acid was the aglycone of the most identified saponins. The qualitative profiles of the peel and flesh saponins of all analyzed beetroot cultivars were identical, but the content of individual compounds was highly varied across the samples (Table 2, Table 3). The total saponin content ranged from 707 mg/kg fw (Snow Ball) to 6834 mg/kg fw (Forono) for peel samples and from 497 mg/kg fw (Snow Ball) to 6864 mg/kg fw (Boldor) for flesh samples. The quantitative profiles of saponins divided into four groups according to their aglycone – oleanolic acids, akebonoic acid, gypsogenin or hederagenin – are discussed below.

Saponins with oleanolic acid as the aglycone

Among 17 derivatives of oleanolic acid, compounds **8**, **9**, **22** and **23** (betavulgaroside I–IV) were the main saponins determined in the peel and flesh of *B. vulgaris* cultivars (Table 2, Table 3). The content of saponin **22** was the highest in most of beetroot peel (452–7361 mg/kg fw) and flesh (423–2090 mg/kg fw) samples with the exception of the peel of Tytus cultivar, flesh of Boldor cultivar and flesh and peel of Snow Ball cultivar wherein saponins **9** (675 mg/kg fw), **8** (2603 mg/kg fw) and **9** (164–173 mg/kg fw), respectively, were dominant. In a previous study, a high content of compound **9** (9543 mg/kg dw) was found in beetroot flesh of red Red Sphere cultivar [Mroczek *et al*., 2019]. The content of saponin **23** was high in most of the analyzed cultivars (36.1–5516 mg/kg fw), except for cultivars Chrobry (peel) (6.7 mg/kg fw) and Forono (4.5–5.2 mg/kg fw).

TABLE 2. Content of individual saponins and total saponins in fresh peel of *Beta vulgaris* L. cultivars (mg/kg fresh weight) analyzed by LC-DAD-ESI-MS/MS.

No.	Saponin	Red cultivars				Yellow cultivar	White cultivar
		Ceryl	Chrobry	Forono	Tytus	Boldor	Snow Ball
1	Act-Hex-Hex-UrA-oleanolic acid	0.021±0.003^{f}	19.4±0.60^{d}	82.7±1.2^{b}	37.9±1.1^{c}	97.3±2.0^{a}	1.2±0.05^{e}
2	Act-Hex-UrA-akebonoic acid	2.9±0.15^{e}	3.4±0.14^{c}	101±2.5^{a}	53.2±1.8^{b}	1.6±0.09^{f}	3.2±0.16^{d}
3	Act-Hex-Hex-UrA-oleanolic acid	32.5±0.78^{d}	15.3±0.30^{f}	65.8±2.1^{c}	77.8±2.1^{a}	67.8±1.2^{b}	29.3±0.73^{e}
4	Hex-Pen-Hex-UrA-oleanolic acid	0.61±0.030^{e}	0.023±0.003^{f}	1.3±0.08^{d}	2.7±0.12^{c}	16.7±0.6^{a}	6.8±0.26^{b}
5	Act-Hex-Pen-UrA-oleanolic acid	41.4±0.65^{d}	17.8±0.63^{e}	140±3.8^{b}	74.3±1.4^{c}	369±5.1^{a}	17.4±0.35^{e}
6	Act-Hex-Hex-UrA-oleanolic acid	38.2±0.69^{c}	33.6±0.70^{d}	1.2±0.06^{f}	48.5±1.7^{b}	96.1±2.4^{a}	1.9±0.09^{e}
7	Hex-Pen-UrA-oleanolic acid	0.25±0.012^{d}	0.012±0.002^{e}	0.37±0.02^{c}	0.33±0.02^{c}	1.2±0.06^{b}	30.5±0.79^{a}
8	Diox-Hex-UrA-oleanolic acid	11.8±0.15^{f}	54.8±0.80^{e}	55.2±1.0^{d}	154±3.8^{b}	1200±16^{a}	152±3.0^{c}
9	Act-HexUrA-oleanolic acid	2.2±0.11^{f}	174±2.3^{c}	1069±22^{a}	675±14^{b}	118±2.2^{e}	173±3.5^{d}
10	Act-dHex-UrA-oleanolic acid	0.51±0.022^{f}	0.85±0.04^{e}	98.1±2.0^{a}	3.3±0.14^{d}	30.4±0.6^{b}	22.6±0.67^{c}
11	Hex-Pen-UrA-akebonoic acid	0.072±0.009^{e}	0.050±0.007^{c}	0.20±0.01^{d}	2.8±0.16^{b}	105±2.4^{a}	<LOQ
12	Act-dHex-UrA-oleanolic acid	0.81±0.040^{c}	0.35±0.022^{d}	1.40±0.06^{b}	0.33±0.02^{e}	15.3±0.4^{a}	0.023±0.004^{f}
13	Pen-UrA-hederagenin	42.0±0.90^{b}	0.14±0.008^{e}	150±4.1^{a}	1.8±0.10^{d}	32.6±0.60^{c}	0.032±0.004^{f}
14	$C_5H_4O_5$-Hex-UrA-akebonoic acid	0.032±0.004^{c}	0.032±0.004^{c}	0.053±0.006^{a}	0.044±0.006^{b}	0.12±0.008^{d}	<LOQ
15	Act-UrA-hederagenin	639±8.5^{c}	133±4.2^{d}	989±20^{b}	152±1.8$^{e, a}$	2283±44^{a}	71.0±1.4^{f}
16	Hex-UrA-gypsogenin	152±5.5^{b}	35.7±1.3^{e}	16.2±0.21^{f}	65.2±1.5^{c}	856±14^{fa}	37.4±0.74^{d}
17	Hex-Pen-UrA-oleanolic acid	6.7±0.21^{d}	0.86±0.04^{e}	21.1±0.90^{b}	8.3±0.20^{c}	137±2.0^{a}	0.023±0.004^{f}
18	Act-Hex-UrA-oleanolic acid	73.5±1.2^{c}	25.6±0.50^{e}	150±2.2^{b}	35.6±0.90^{d}	415±9.1^{a}	6.2±0.31^{f}
19	Act-UrA-akebonoic acid	46.8±1.1^{d}	20.4±0.38^{e}	881±11^{a}	63.6±0.90^{c}	274±6.1^{b}	2.1±0.11^{f}
20	$C_4H_4O_5$-Hex-UrA-oleanolic acid	255±5.4^{c}	70.6±1.3^{e}	528±12^{b}	82.3±1.6^{d}	1735±20^{a}	2.4±0.12^{f}
21	Pen-UrA-oleanolic acid	45.5±0.48^{c}	0.071±0.008^{f}	62.3±1.4^{b}	2.3±0.10^{e}	68.0±1.9^{a}	15.3±0.46^{d}
22	Act-UrA-oleanolic acid	1194±15^{c}	452±5.0^{e}	2410±51^{b}	547±34^{d}	7361±117^{a}	88.2±1.7^{f}
23	Diox-UrA-oleanolic acid	497±13^{b}	6.7±0.30^{e}	5.2±0.16^{f}	173±4.0^{c}	5516±160^{a}	44.9±0.90^{d}
24	UrA-oleanolic acid	6.1±0.30^{b}	0.073±0.009^{e}	5.2±0.22^{c}	1.9±0.10^{d}	16.3±0.44^{a}	<LOQ
	Total	3089±72^{c}	1065±12^{e}	6834±98^{b}	2263±29^{d}	20812±228^{a}	707±18.4^{f}

Data are expressed as mean ± standard deviation (n=3). Values in each row having the same letter are not significantly different (p>0.05). Results are expressed as oleanolic acid equivalents. LOQ – limit of quantification. The assigned saponin numbers correspond to those listed in Table 1.

Among the remaining compounds, a higher content of saponin **20** was found in selected *B. vulgaris* samples. The cultivar with the highest content of this saponin was Boldor (peel) (1735 mg/kg fw). Saponin **20** was previously determined in a high content in beetroot of an Egyptian cultivar (1052 mg/kg dw) [Mroczek *et al*., 2019].

Saponins **4**, **12**, **17**, **24** were found in *B. vulgaris* cultivars in low amounts (0.02–137.56 mg/kg fw). Moreover, the content of saponins **12**, **17**, **24** in Snow Ball cultivar (flesh) was below the limit of quantification (LOQ). Interestingly, a previous report [Mroczek *et al*., 2019] indicates that compounds **4** (200–3933mg/kg dw) and 17 (170–1123 mg/kg dw) were present mainly in leaves of *B. vulgaris* cultivars [Mroczek *et al*., 2019], whereas compound **24** was detected in a low content (46.9–482 mg/kg dw) in leaves and roots.

According to Mroczek *et al*. [2019], saponin **1** mainly occurred in leaves of *B. vulgaris* cultivars (37.6–780 mg/kg dw). Here, a low content of saponin **1** (0.021–97.3 mg/kg fw) was found in the peel and flesh of *B. vulgaris* cultivars, which may confirm a high content of this compound in the leaves.

The content of saponins **5**, **6** and **18** in the peel (96.1––415 mg/kg fw) and saponin **3** in the flesh (318 mg/kg) of yellow *B. vulgaris* was higher than in white (0.83–29.3 mg/kg fw) and red (0.21–150 mg/kg fw) cultivars. Therefore, yellow *B. vulgaris* seems to be a rich source of these compounds.

The content of saponins **7**, **10** and **21** in *B. vulgaris* cultivars was low. The highest content of saponin **7**, **10** and **21** was determined in red cultivars: Forono (saponin **7** and **10**) and Ceryl (saponin **21**). Saponins **7** (563–2954 mg/kg dw) and **21** (172–623 mg/kg dw) seem

TABLE 3. Content of individual saponins and total saponins in fresh flesh of *Beta vulgaris* L. cultivars (mg/kg fresh weight) analyzed by LC-DAD-ESI-MS/MS.

No.	Saponin	Red cultivars				Yellow cultivar	White cultivar
		Ceryl	Chrobry	Forono	Tytus	Boldor	Snow Ball
1	Act-Hex-Hex-UrA-oleanolic acid	6.8±0.28^{c}	33.8±0.50^{a}	4.9±0.16^{d}	24.4±0.50^{b}	2.3±0.09^{e}	0.52±0.02^{f}
2	Act-Hex-UrA-akebonoic acid	5.9±0.24^{c}	31.4±0.90^{b}	0.23±0.011^{f}	42.8±0.83^{a}	5.7±0.13^{d}	0.92±0.04^{e}
3	Act-Hex-Hex-UrA-oleanolic acid	35.6±0.55^{e}	99.2±2.2^{b}	58.8±1.4^{d}	93.1±1.0^{c}	318±9.0^{a}	19.5±0.39^{f}
4	Hex-Pen-Hex-UrA-oleanolic acid	3.6±0.16^{c}	4.5±0.22^{b}	3.6±0.20^{c}	1.5±0.06^{e}	21.3±0.44^{a}	2.9±0.14^{d}
5	Act-Hex-Pen-UrA-oleanolic acid	28.8±0.53^{e}	91.0±4.4^{b}	45.3±1.4^{d}	67.7±1.5^{c}	234±3.1^{a}	6.1±0.27^{f}
6	Act-Hex-Hex-UrA-oleanolic acid	9.8±0.16^{c}	63.1±2.7^{a}	0.29±0.016^{f}	50.2±1.4^{b}	2.2±0.08^{d}	0.83±0.04^{e}
7	Hex-Pen-UrA-oleanolic acid	15.4±0.28^{d}	0.14±0.008^{e}	50.4±2.4^{a}	0.041±0.005^{f}	0.21±0.01^{c}	32.2±0.64^{b}
8	Diox-Hex-UrA-oleanolic acid	166±5.1^{d}	784±22^{b}	52.8±2.6^{f}	279±4.0^{c}	2603±43^{a}	133±2.6^{e}
9	Act-HexUrA-oleanolic acid	799±10^{c}	1905±35^{a}	2.7±0.14^{f}	1120±22^{b}	36.3±0.42^{e}	164±2.9^{d}
10	Act-dHex-UrA-oleanolic acid	0.18±0.009^{f}	6.2±0.20^{d}	11.6±0.20^{c}	23.0±0.30^{a}	0.55±0.03^{e}	18.1±0.58^{b}
11	Hex-Pen-UrA-akebonoic acid	1.2±0.070^{d}	5.4±0.20^{c}	6.0±0.16^{b}	14.5±0.40^{a}	0.61±0.03^{e}	0.031±0.005^{f}
12	Act-dHex-UrA-oleanolic acid	2.9±0.14^{c}	7.5±0.30^{a}	0.47±0.03^{e}	3.0±0.16^{b}	1.9±0.08^{d}	<LOQ
13	Pen-UrA-hederagenin	2.7±0.12^{c}	0.24±0.014^{e}	4.1±0.12^{b}	4.2±0.14^{a}	0.43±0.02^{d}	<LOQ
14	$C_5H_4O_5$-Hex-UrA-akebonoic acid	0.032±0.004^{d}	0.053±0.007^{c}	0.035±0.004^{d}	0.071±0.009^{b}	0.083±0.009^{a}	<LOQ
15	Act-UrA-hederagenin	120±1.4^{c}	202±5.6^{b}	28.6±0.60^{d}	234±4.2^{a}	2.8±0.09^{f}	12.5±0.26^{e}
16	Hex-UrA-gypsogenin	0.36± 0.020^{f}	5.9±0.20^{c}	0.37±0.02^{f}	10.3±0.20^{b}	1.8±0.06^{e}	3.1±0.10^{d}
17	Hex-Pen-UrA-oleanolic acid	4.1±0.20^{d}	15.4±0.34^{b}	2.5±0.12^{e}	8.3±0.20^{c}	19.1±0.32^{a}	<LOQ
18	Act-Hex-UrA-oleanolic acid	0.21±0.011^{f}	141±5.6^{a}	45.7±1.3^{b}	0.42±0.02^{e}	2.9±0.08^{c}	2.4±0.12^{d}
19	Act-UrA-akebonoic acid	38.0±1.4^{d}	193±4.5^{c}	80.3±1.7^{d}	309±4.2^{a}	1.1±0.06^{e}	0.11±0.007^{f}
20	$C_4H_4O_5$-Hex-UrA-oleanolic acid	0.52±0.030^{e}	245±5.0^{c}	54.1±2.0^{b}	322±4.2^{b}	873±16^{a}	0.59±0.04^{e}
21	Pen-UrA-oleanolic acid	105±2.8^{a}	0.17±0.009^{f}	41.7±1.0^{b}	0.49±0.03^{e}	24.4±0.34^{c}	16.4±0.98^{d}
22	Act-UrA-oleanolic acid	1260±12^{c}	1998±55^{b}	423±4.5^{e}	2090±22^{a}	532 ±9.1^{d}	47.7±1.4^{f}
23	Diox-UrA-oleanolic acid	889±22^{c}	1032±18^{b}	4.5±0.15^{f}	1403±24^{a}	558±8.1^{d}	36.1±0.72^{e}
24	UrA-oleanolic acid	2.3±0.12^{a}	0.12±0.008^{e}	0.032±0.005^{d}	0.39±0.02^{c}	0.45±0.03^{b}	<LOQ
	Total	3497±52^{d}	6864±82^{a}	922±14^{e}	6101±85^{b}	5242±63^{c}	497±11^{f}

Data are expressed as mean ± standard deviation (n=3). Values in each row having the same letter are not significantly different (p>0.05). Results are expressed as oleanolic acid equivalents. LOQ – limit of quantification. The assigned saponin numbers correspond to those listed in Table 1.

to be present mainly in the leaves of *B. vulgaris* cultivars; therefore their content in beet roots may be at a quite low level [Mroczek *et al*., 2019].

Saponins with akebonoic acid as the aglycone

Saponin **19** was the main akebonoic acid derivative determined in the peel and flesh of *B. vulgaris* cultivars (0.11––881 mg/kg fw) (Table 2, Table 3). Its content was the highest in the peel of Forono cultivar, whereas the lowest in the flesh of Snow Ball cultivar. The contents of the remaining saponins **2**, **11** and **14** with akebonoic acid were much lower. The content of saponin **14** was very low in all studied samples (0.032–0.12 mg/kg fw), especially in the peel and flesh of Snow Ball cultivar, where the determined value was below the limit of quantification. In turn, higher contents of saponins **2** and **11** were determined in the peel of Forono (101 mg/kg fw) and Boldor (105 mg/kg fw) cultivars.

Saponins with hederagenin and gypsogenin as the aglycones

Three saponins, which consisted of hederagenin (saponins **13** and **15**) or gypsogenin (saponin **16**) as an aglycone, were identified in the studied cultivars. Among these compounds, saponin **15** was dominant (Table 2, Table 3) and its highest content was determined in the peel of the yellow cultivar Boldor (2283 mg/kg fw). Interestingly, its content in flesh of yellow cultivar was merely 2.8 mg/kg fw. The peels of the studied cultivars turned out to be the richest sources of saponin **16**, the highest content of which was detected in the peels of yellow cultivar Boldor (peel) (856 mg/kg fw). The highest content of saponin **13** was determined in Forono

(peel) (150 mg/kg fw) but still it was lower than contents of saponins **15** and **16**.

CONCLUSIONS

In summary, a simple procedure for saponin quantification with oleanolic acid, hederagenin, akebonoic acid and gypsogenin glycosides was developed. Herein, 24 saponins were detected in six *B. vulgaris* cultivars, of which saponins of the white and yellow cultivars were identified for the first time.

Our results report that the qualitative profile of saponins was identical for the peel and flesh of red, white and yellow cultivars, while the quantitative profile depended on the cultivar and part of the root. Among the identified compounds, betavulgaroside I, II, III and IV were dominant in the studied cultivars.

B. vulgaris seems to be a good source of these compounds for future research because their contents in peels and flesh were high, which is uncommon. The highest content of saponins was found in the peels of the yellow *B. vulgaris* cultivar (Boldor). In contrast, the lowest content was determined in the flesh of the white cultivar (Snow Ball).

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

ORCID IDs

A. Grzegorczyk https://orcid.org/0000-0002-2812-5046
K. Skalicka-Woźniak https://orcid.org/0000-0002-9313-5929
A. Spórna-Kucab https://orcid.org/0000-0002-6653-3702
T. Świergosz https://orcid.org/0000-0002-5317-1597
A. Tekieli https://orcid.org/0000-0003-1147-9136
S. Wybraniec https://orcid.org/0000-0002-1263-4188

REFERENCES

1. Bárta, J., Bártová, V., Šindelková, T., Jarošová, M., Linhartová, Z., Mráz, J., Bedrníček, J., Smetana, P., Samková, E., Laknerová, I. (2020). Effect of boiling on colour, contents of betalains and total phenolics and on antioxidant activity of colourful powder derived from six different beetroot (*Beta vulgaris* L.). *Polish Journal of Food and Nutrition Sciences*, *70*(4), 377–385. https://doi.org/10.31883/pjfns/128613
2. Chhikara, N., Kushwaha, K., Sharma, P., Gat, Y., Panghal, A. (2019). Bioactive compounds of beetroot and utilization in food processing industry: a critical review. *Food Chemistry*, *272*, 192–200. https://doi.org/10.1016/j.foodchem.2018.08.022
3. Clifford, T., Howatson, G., West, D.J., Stevenson, E.J. (2015). The potential benefits of red beetroot supplementation in health and disease. *Nutrients*, *7*(4), 2801–2822. https://doi.org/10.3390/nu7042801
4. De Tommasi, N., Conti, C., Stein, M.L., Pizza, C. (1991). Structure and *in vitro* antiviral activity of triterpenoid saponins from *Calendula arvensis*. *Planta Medica*, *57*(3), 250–253. https://doi.org/10.1055/s-2006-960084
5. Dirir, A.M., Daou, M., Yousef, A.F., Yousef, L.F. (2021). A review of alpha-glucosidase inhibitors from plants as potential candidates for the treatment of type-2 diabetes. *Phytochemistry Reviews, 2021*, 1–31. https://doi.org/10.1007/s11101-021-09773-1
6. dos Santos Baião, D., da Silva, D.V.T., Paschoalin, V.M.F. (2021). A narrative review on dietary strategies to provide nitric oxide as a non-drug cardiovascular disease therapy: beetroot formulations – A smart nutritional interaction. *Foods*, *10*(4), art. no. 859. https://doi.org/10.3390/foods10040859
7. Dubois, M.-A., Benze, S., Wagner, H. (1990). New biologically active triterpene-saponins from *Randia dumetorum*. *Planta Medica*, *56*(5), 451–455. https://doi.org/10.1055/s-2006-961009
8. Edelmann, M., Dawid, C., Ralla, T., Stark, T. D., Salminen, H., Weiss, J., Hofmann, T. (2020). Fast and sensitive LC-MS/MS method for the quantitation of saponins in various sugar beet materials. *Journal of Agricultural and Food Chemistry*, *68*(50), 15027–15035. https://doi.org/10.1021/acs.jafc.0c05836
9. El Hazzam, K., Hafsa, J., Sobeh, M., Mhada, M., Taourirte, M., El Kacimi, K., Yasri, A. (2020). An insight into saponins from *Quinoa* (*Chenopodium quinoa* Willd): a review. *Molecules*, *25*(5), art. no. 1059. https://doi.org/10.3390/molecules25051059
10. Fang, Z., Li, J., Yang, R., Fang, L., Zhang, Y. (2020). A review: the triterpenoid saponins and biological activities of *Lonicera Linn*. *Molecules*, *25*(17), art. no. 3773. https://doi.org/10.3390/molecules25173773
11. Francis, G., Kerem, Z., Makkar, H.P.S., Becker, K. (2002). The biological action of saponins in animal systems: a review. *British Journal of Nutrition*, *88*(6), 587–605. https://doi.org/10.1079/BJN2002725
12. Güçlü-Üstündağ, Ö., Mazza, G. (2007). Saponins: properties, applications and processing. *Critical Reviews in Food Science and Nutrition*, *47*(3), 231–258. https://doi.org/10.1080/10408390600698197
13. Hikino, H., Kiso, Y., Kinouchi, J., Sanada, S., Shoji, J. (1985). Antihepatotoxic actions of ginsenosides from *Panax ginseng* roots. *Planta Medica*, *51*(1), 62–64. https://doi.org/10.1055/s-2007-969395
14. Ida, Y., Satoh, Y., Katoh, M., Katsumata (nee Ohtsuka), M., Nagasao, M., Yamaguchi, K., Kamei, H., Shoji, J. (1994). Achyranthosides A and B, novel cytotoxic saponins from *Achyranthes fauriei* root. *Tetrahedron Letters*, *35*(37), 6887–6890. https://doi.org/10.1016/0040-4039(94)85032-1
15. Ismaili, H., Tortora, S., Sosa, S., Fkih-Tetouani, S., Ilidrissi, A., Della Loggia, R., Tubaro, A., Aquino, R. (2001). Topical anti-inflammatory activity of *Thymus willdenowii*. *Journal of Pharmacy and Pharmacology*, *53*(12), 1645–1652. https://doi.org/10.1211/0022357011778250
16. Kavalcová, P., Bystrická, J., Tomáš, J., Karovičová, J., Kovarovič, J., Lenková, M. (2015). The content of total polyphenols and antioxidant activity in red beetroot. *Potravinárstvo – Slovak Journal of Food Sciences*, *9*(1), 77–83. https://doi.org/10.5219/441

17. Kokanova-Nedialkova, Z., Nedialkov, P., Kondeva-Burdina, M. (2020). Ultra-high-performance liquid chromatography – high-resolution mass spectrometry profiling and hepatoprotective activity of purified saponin and flavonoid fractions from the aerial parts of wild spinach (*Chenopodium bonus-henricus* L.). *Zeitschrift Für Naturforschung C*, *76*(7–8), 261–271. https://doi.org/10.1515/znc-2020-0144
18. Konishi, M., Hano, Y., Takayama, M., Nomura, T., Hamzah, A.S., Ahmad, R.B., Jasmani, H. (1998). Triterpenoid saponins from *Hedyotis nudicaulis*. *Phytochemistry*, *48*(3), 525–528. https://doi.org/10.1016/S0031-9422(98)00038-7
19. Kuljanabhagavad, T., Wink, M. (2009). Biological activities and chemistry of saponins from *Chenopodium quinoa* Willd. *Phytochemistry Reviews*, *8*(2), 473–490. https://doi.org/10.1007/s11101-009-9121-0
20. Kuroda, M., Mimaki, Y., Sashida, Y., Kitahara, M., Yamazaki, M., Yui, S. (2001). Securiosides A and B, novel acylated triterpene bisdesmosides with selective cytotoxic activity against M-CSF-stimulated macrophages. *Bioorganic & Medicinal Chemistry Letters*, *11*(3), 371–374. https://doi.org/10.1016/S0960-894X(00)00674-0
21. Kuwada, K., Kawase, S., Nakata, K., Shinya, N., Narukawa, Y., Fuchino, H., Kawahara, N., Kiuchi, F. (2020). LC–MS analysis of saponins of *Achyranthes* root in the Japanese market. *Journal of Natural Medicines*, *74*(1), 135–141. https://doi.org/10.1007/s11418-019-01355-y
22. Lemmich, E., Cornett, C., Furu, P., Jørstian, C.L., Knudsen, A.D., Olsen, C.E., Salih, A., Thiilborg, S.T. (1995). Molluscicidal saponins from *Catunaregam nilotica*. *Phytochemistry*, *39*(1), 63–68. https://doi.org/10.1016/0031-9422(94)00866-R
23. Lidder, S., Webb, A. (2013). Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) *via* the nitrate--nitrite-nitric oxide pathway. *British Journal of Clinical Pharmacology*, *75*(3SI), 677–696. https://doi.org/10.1111/j.1365-2125.2012.04420.x
24. Mbaveng, A.T., Ndontsa, B.L., Kuete, V., Nguekeu, Y.M.M., Çelik, İ., Mbouangouere, R., Tane, P., Efferth, T. (2018). A naturally occuring triterpene saponin ardisiacrispin B displayed cytotoxic effects in multi-factorial drug resistant cancer cells *via* ferroptotic and apoptotic cell. *Phytomedicine*, *43*, 78–85. https://doi.org/10.1016/j.phymed.2018.03.035
25. Mikołajczyk-Bator, K., Błaszczyk, A., Czyżniejewski, M., Kachlicki, P. (2016). Characterisation and identification of triterpene saponins in the roots of red beets (*Beta vulgaris* L.) using two HPLC–MS systems. *Food Chemistry*, *192*, 979–990. https://doi.org/10.1016/j.foodchem.2015.07.111
26. Mroczek, A., Kapusta, I., Janda, B., Janiszowska, W. (2012). Triterpene saponin content in the roots of red beet (*Beta vulgaris* L.) cultivars. *Journal of Agricultural and Food Chemistry*, *60*(50), 12397–12402. https://doi.org/10.1021/jf303952x
27. Mroczek, A., Kapusta, I., Stochmal, A., Janiszowska, W. (2019). MS/MS and UPLC-MS profiling of triterpenoid saponins from leaves and roots of four red beet (*Beta vulgaris* L.) cultivars. *Phytochemistry Letters*, *30*, 333–337. https://doi.org/10.1016/j.phytol.2019.02.015
28. Mroczek, A., Klimczak, U., Kowalczyk, M. (2021). Determination of saponins in leaves of four swiss chard (*Beta vulgaris* L.) cultivars by UHPLC-CAD/QTOF-MS/MS. *Polish Journal of Food and Nutrition Sciences*, *71*(2), 147–159. https://doi.org/10.31883/pjfns/134623
29. Muhammad, M.T., Khan, M.N. (2018). Eco-friendly, biodegradable natural surfactant (*Acacia Concinna*): an alternative to the synthetic surfactants. *Journal of Cleaner Production*, *188*, 678–685. https://doi.org/10.1016/j.jclepro.2018.04.016
30. Parus, A. (2013). Właściwości farmakologiczne saponin. *Postępy Fitoterapii*, *3*, 200–20204 (in Polish).
31. Pietrzkowski, Z., Nemzer, B., Spórna, A., Stalica, P., Tresher, W., Keller, R., Jimenez, R., Michałowski, T., Wybraniec, S. (2010). Influence of betalain-rich extract on reduction of discomfort associated with osteoarthritis. *New Medicine*, *1*, 12–17.
32. Rai, S., Acharya-Siwakoti, E., Kafle, A., Devkota, H. P., Bhattarai, A. (2021). Plant-derived saponins: a review of their surfactant properties and applications. *Sci*, *3*(4), art. no. 44. https://doi.org/10.3390/sci3040044
33. Schmitt, C., Grassl, B., Lespes, G., Desbrières, J., Pellerin, V., Reynaud, S., Gigault, J., Hackley, V.A. (2014). Saponins: A renewable and biodegradable surfactant from its microwave-assisted extraction to the synthesis of monodisperse lattices. *Biomacromolecules*, *15*(3), 856–862. https://doi.org/10.1021/bm401708m
34. Sparg, S.G., Light, M.E., Van Staden, J. (2004). Biological activities and distribution of plant saponins. *Journal of Ethnopharmacology*, *94*(2–3), 219–243. https://doi.org/10.1016/j.jep.2004.05.016
35. Spórna-Kucab, A., Kumorkiewicz, A., Szmyr, N., Szneler, E., Wybraniec, S. (2019). Separation of betacyanins from flowers of *Amaranthus cruentus* L. in a polar solvent system by high--speed counter-current chromatography. *Journal of Separation Science*, *42*(9), 1676–1685. https://doi.org/10.1002/jssc.201801172
36. Spórna-Kucab, A., Wróbel, N., Kumorkiewicz-Jamro, A., Wybraniec, S. (2020). Separation of betacyanins from *Iresine herbstii* Hook. ex Lindl. leaves by high-speed countercurrent chromatography in a polar solvent system. *Journal of Chromatography A*, *1626*, art. no. 461370. https://doi.org/10.1016/j.chroma.2020.461370
37. Spórna-Kucab, A., Wybraniec, S. (2020). High-speed countercurrent chromatography in separation and identification of saponins from *Beta vulgaris* L. cultivar Red Sphere. *Polish Journal of Food and Nutrition Sciences*, *70*(1), 67–74. https://doi.org/10.31883/pjfns/116416
38. Wybraniec, S., Stalica, P., Spórna, A., Mizrahi, Y. (2010). Profiles of betacyanins in epidermal layers of grafted and light-stressed cacti studied by LC-DAD-ESI-MS/MS. *Journal of Agricultural and Food Chemistry*, *58*(9), 5347–5354. https://doi.org/10.1021/jf100065w
39. Wybraniec, S., Stalica, P., Spórna, A., Nemzer, B., Pietrzkowski, Z., Michałowski, T. (2011). Antioxidant activity of betanidin: electrochemical study in aqueous media. *Journal of Agricultural and Food Chemistry*, *59*(22), 12163–12170. https://doi.org/10.1021/jf2024769
40. Xu, J., Wang, S., Feng, T., Chen, Y., Yang, G. (2018). Hypoglycemic and hypolipidemic effects of total saponins from *Stauntonia chinensis* in diabetic db/db mice. *Journal of Cellular and Molecular Medicine*, *22*(12), 6026–6038. https://doi.org/10.1111/jcmm.13876

41. Yoshikawa, M., Matsuda, H., Harada, E., Murakami, T., Wariishi, N., Yamahara, J., Murakami, N. (1994). Elatoside E, a new hypoglycemic principle from the root cortex of *Aralia elata* Seem.: Structure-related hypoglycemic activity of oleanolic acid glycosides. *Chemical and Pharmaceutical Bulletin*, *42*(6), 1354–1356. https://doi.org/10.1248/cpb.42.1354
42. Yoshikawa, M., Murakami, T., Kadoya, M., Matsuda, H., Muraoka, O., Yamahara, J., Murakami, N. (1996). Medicinal Foodstuffs. III. Sugar Beet. (1) : Hypoglycemic oleanolic acid oligoglycosides, betavulgarosides, I, II, III, and IV, from the root of *Beta vulgaris* L. (*Chenopodiaceae*). *Chemical and Pharmaceutical Bulletin*, *44*(6), 1212–1217. https://doi.org/10.1248/cpb.44.1212
43. Yoshikawa, M., Murakami, T., Kadoya, M., Yamahara, J., Matsuda, H. (1998). Medicinal foodstuffs. XV. Sugar beet. (2): Structures of betavulgarosides V, VI, VII, VIII, IX, and X from the roots and leaves of sugar beet (*Beta vulgaris* L., *Chenopodiaceae*). *Chemical and Pharmaceutical Bulletin*, *46*(11), 1758–1763. https://doi.org/10.1248/cpb.46.1758

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Original article
Section: Food Quality and Functionality

Black Trumpet, *Craterellus cornucopioides* (L.) Pers.: Culinary Mushroom with Angiotensin Converting Enzyme Inhibitory and Cytotoxic Activity

*Jelena Radović[1], Aleksandra Leković[1], Ana Tačić[2], Margarita Dodevska[3], Tatjana Stanojković[4], Tijana Marinković[5], Čedomir Jelić[5], Tatjana Kundaković-Vasović[1]**

[1]*Department of Pharmacognosy, University of Belgrade, Faculty of Pharmacy, Vojvode Stepe 450, 11322 Belgrade, Serbia*
[2]*Department of Organic Chemistry, Faculty of Technology, University of Niš, Bulevar Oslobođenja 124, 16000 Leskovac, Serbia*
[3]*Institute of Public Health of Serbia "Dr Milan Jovanovic Batut", dr Subotića 5, 11000 Belgrade, Serbia*
[4]*Institute for Oncology and Radiology of Serbia, Pasterova 11, 11000 Belgrade, Serbia*
[5]*Institute of Public Health Kragujevac, Nikole Pašića 1, 34000 Kragujevac, Serbia*

Key words: *Craterellus cornucopioides*, β-glucan, nutrients, angiotensin converting enzyme inhibition, cytotoxicity

Nutritional value and chemical composition, including the content of vitamins, fatty acids, 5'-nucleotides and nucleosides and amino acids, as well as biological activities, including antioxidant, angiotensin converting enzyme (ACE) inhibitory and cytotoxic activity of black trumpet (*Craterellus cornucopioides* (L.) Pers.) were tested *in vitro*. *C. cornucopioides* was low in energy, fat and carbohydrate contents, but rich in dietary fibre, especially β-glucan as well as niacin and α-tocopherol. The content of essential and non-essential free amino acids was 1.49 and 5.48 mg/g dry weight (dw). The nucleosides and 5'-nucleotides were determined at 1.84 and 3.99 mg/g dw, respectively. The share of unsaturated fatty acids (UFAs) was 75.92% with oleic acid as the major UFA. Cyclohexane and dichloromethane extracts expressed significant cytotoxic activity against selected cell lines, human epithelial cervical cancer cells (HeLa), adenocarcinomic human alveolar basal epithelial cells (A549), colorectal cancer cells (LS174) and normal MRC-5 human embryonic lung fibroblast cells (IC_{50} of 78.3–155.6 μg/mL). ACE inhibitory activity of the aqueous extract was strong with an IC_{50} of 0.74 μg/mL. It can be concluded that black trumpet is a good source of nutrients, such as vitamins, dietary fibres, amino acids, nucleotides and fatty acids, which contribute to the overall nutritional value of this fungus with potential for ACE inhibitory activity and use in anti-hypertensive diet.

INTRODUCTION

Black trumpet, *Craterellus cornucopioides* (L.) Pers. is a delicious edible mushroom from the family Cantharellaceae. It is black-brown or almost black mushroom without determined separation of fruiting body between stalk and cap. It looks like a trumpet at the bottom of the tank and slowly extends to the edges. This mushroom grows in groups, in deciduous forests especially on oak and beech [Wheeler & Jordan, 2007]. Primary metabolites (fatty acids, lipids, carbohydrates, amino acids, 5'-nucleotides) and secondary metabolites (phenolic compounds and sterols) were previously identified in *C. cornucopioides* [Beluhan & Ranogajec, 2011; Liu *et al*., 2012; Palacios *et al*., 2011]. Considerable amounts of vitamin C, lycopene, β-carotene, α- and γ-tocopherols, and β-glucan were determined as well [Gil-Ramirez *et al*., 2011; Liu *et al*., 2012; Vamanu & Nita, 2014; Watanabe *et al*., 2012]. It was found that the bioactive compounds of *C. cornucopioides* were responsible for several of its biological activities, inlcuding: anti-inflammatory, antimutagenic, cytotoxic, hypoglycemic and antioxidant ones [Kosanić *et al*., 2019; Liu *et al*., 2012; O'Callaghan *et al*., 2015; Palacios *et al*., 2011; Vamanu & Nita, 2014].

High blood pressure and cardiovascular disease are one of the risk factors for morbidity in both developed and underdeveloped countries. The main risk factors for hypertension that can be influenced are unhealthy diet (excessive salt intake, diet rich in fats and trans fats, low intake of fruits and vegetables), insufficient physical activity and sedentary lifestyle, smoking and alcohol abuse, overweight or obesity [World Health Organization Cardiovascular diseases factsheet, 2021, https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)]. According to the recommendations of the European Society of Cardiology and the British Society of Hypertension, therapy of hypertension begins with either angiotensin converting enzyme (ACE)

* Corresponding Author:
Tel.: +381 11 3951351; Fax: +381 11 3972840;
E-mail: tatjana.kundakovic@pharmacy.bg.ac.rs (Tatjana Kundaković-Vasović)

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inhibitors or angiotensin II receptor (AT_1) antagonists in patients who have normal or increased plasma renin, thiazide diuretics or calcium antagonists [Williams *et al*., 2018]. If an adequate antihypertensive effect is not achieved and the drug is well tolerated, an antihypertensive agent from another group is added (thiazide diuretics, Ca^{2+} antagonists, β-adrenoceptor antagonists, α_1-adrenoceptor antagonists) [Williams *et al*., 2018]. Previous studies have shown significant ACE inhibitory activity of different mushrooms, their extracts and different isolates *in vitro* and *in vivo* [Kundaković & Kolundžić, 2013], whereas clinical trials have confirmed the effects of individual mushrooms on lowering blood pressure [Khatun *et al*., 2007; Lo *et al*., 2012]. Mushrooms represent a significant source of bioactive peptides and other compounds that show ACE inhibitory activity. A large number of ACE inhibitory peptides have been isolated from edible mushrooms in the last twenty years, *e.g. Grifola frondosa* [Choi *et al*., 2001], *Mycoleptodonoides aitchisonii* [Sakamoto *et al*., 2001], *Tricholoma giganteum* [Lee *et al*., 2004], and *Ganoderma lucidum* [Wu *et al*., 2019]. In addition to peptides and oligopeptides, triterpenoids (ganoderic acid F) and D-mannitol of mushrooms have also been shown to elicit an inhibitory effect on ACE activity [Kundaković & Kolundžić, 2013].

Regarding antitumor activity, the effects have been shown for various mushrooms and their isolated compounds on different cell lines. For example, 3β,5α,9α-trihydroxy-ergosta-7,22-dien-6-one isolated from *Valvariella volvacea* showed significant antitumor activity against human liver cancer cell line (HepG-2) and human gastric cancer cell line (SGC-7901), while ergosterol and ergosterol peroxide expressed significant activity against human prostatic carcinoma cell line (PC-3M) [Chen *et al*., 2020]. Also, mushroom polysaccharides (*e.g.*, lentinan isolated from *Lentinula edodes*) have been reported to elicit the antitumor effect *via* different mechanisams including cancer-preventing activity, immuno-enhancing activity and direct tumor inhibition activity [Pandya *et al*., 2019]. Therefore, guided by the promising findings of previous research, we aimed to evaluate the ACE inhibitory activity of aqueous extracts of wild growing *C. cornucopioides* collected in Bosnia and Hercegovina and to test antitumor activity of its different extracts on several cell lines, in addition to the evaluation of nutritional value, chemical composition and radical scavenging activity.

MATERIAL AND METHODS

Materials and reagents

The following reagents were used: mushroom and yeast β-glucan assay kit K-YBGL and total dietary fibre assay kit K-TDFR obtained from Megazyme Int. (Wicklow, Ireland); ACE Kit-WST obtained from Dojindo Laboratories (Kumamoto, Japan). Analytical grade methanol was obtained from Macron Fine Chemicals (Avantor, Radnor, PA, USA); analytical grade cyclohexane, HPLC grade acetonitrile, methanol and ethanol, as well as sulfuric acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Analytical grade dichloromethane, hexane and petroleum ether, tetrabuthylammonium bromide (TBAB), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), Folin-Ciocalteu reagent, as well as analytical standards of vitamins (thiamine hydrochloride (B_1), riboflavin (B_2), nicotinamide (B_3) and pyridoxine hydrochloride (B_6)), amino acids and 5'-nucleotides and nucleoside analytical standards, RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, streptomycin, penicillin, HEPES buffer solution and *cis*-diaminedichloroplatinum (*cis*-DDP) were purchased form Sigma-Aldrich (St. Louis, MO, USA). Cholecalciferol (vitamin D_3), vitamin A palmitate and DL-α-tocopherol (vitamin E) analytical standards were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany); dimethylformamide and triethylamide were obtained from Acros Organics (Geel, Belgium); gallic acid and dimethyl sulfoxide from Carl Roth (Karlsruhe, Germany). Potassium dihydrogen phosphate, sodium bicarbonate, sodium carbonate and sodium phosphate were purchased form Centrohem (Stara Pazova, Serbia); and hydrochloric acid from Zorka Pharma (Šabac, Serbia). Fatty acid methyl esters (Supelco 37 Component FAME Mix) and dabsyl chloride solution were obtained from Supelco (Bellefonte, PA, USA). Formic acid was provided by Honeywell (Honeywell International, Inc., Morristown, NJ, USA) and ammonium formate by Agilent Technologies, Inc. (Santa Clara, CA, USA).

Sample collection

Fruiting bodies of the mushroom *Craterellus cornucopioides* (L.) Pers. (Cantharellaceae family) were collected in Bosnia and Herzegovina, on the northern slopes of mountain Kozara (Kozarska Dubica) in July 2014 (GPS coordinates were: 45°05'25.7"N; 16°51'33.0"E), near the Monastery Moštanica.

Before extraction, the mushroom was dried at room temperature, pulverized in a sample laboratory mill (MRC SM-450, MRC-Laboratory Equipment, Essex, United Kingdom) and stored at 4°C. The voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Belgrade, Republic of Serbia (No 29).

Extract preparation

Extraction was carried out as described previously by Kolundžić *et al*. [2017] with solvents of different polarity including methanol, cyclohexane and dichloromethane. The dry powdered mushroom (20 g) was weighed and extracted once for two days with each mentioned solvent, at room temperature with a powder to solvent ratio of 1:10 (*w/v*). The organic solvents were evaporated under low pressure and dried to obtain the black-colored extracts: 0.34 g of cyclohexane extract (CCC), 0.11 g of dichloromethane extract (CCD) and 0.14 g of methanol extract (CCM). Aqueous extract was prepared with 1 g of dried, powdered mushroom mixed with 120 mL of distilled water, heated in a water bath for 30 min at 100°C with occasional shaking. After extraction, water was removed using an Alpha 1–4 LD plus freeze dryer (Martin Christ, Osterode am Harz, Germany) to obtain 0.45 g of dry extract (CCA).

Determination of nutritional value

The dried mushroom was analyzed for the contents of moisture, crude protein, crude fat, total dietary fibre and crude ash. Its nutritional value was determined by using the procedures described by the Association of Official

Analytical Chemists [AOAC, 1995]. Gravimetric method was deployed to determine the moisture and crude ash contents after drying at 105°C and incineration at 550°C, respectively. The crude protein content was determined by the Kjeldahl method using a nitrogen conversion factor of 4.38 for protein calculation. The crude fat content was determined using the Soxhlet extraction with petroleum ether as a solvent, after the treatment with HCl. Total carbohydrate content was calculated as the residual difference after subtracting crude protein, crude ash, moisture, total dietary fibre and crude fat content from 100. The content of total dietary fibre was determined according to the instruction manual of the assay kit (K-TDFR, Megazyme Int.). All results were expressed in g/100 g of dry weight (dw) of mushroom.

Determination of the content of glucans

The content of glucans (including α-glucan, β-glucan and total glucan) was determined according to the instruction manual of the mushroom and yeast β-glucan assay kit (K-YBGL) (Megazyme Int.), in dried, powdered mushroom (DM), in mushrooms after thermal treatment (cooking at 100°C for 30 min) (DMAC) and in CCA, CCM, CCC and CCD. Contents of total glucans and α-glucan were determined measuring absorbance at 510 nm on an Evolution 300 UV-Vis spectrophotometer (Thermo Scientific, Madison, WI, USA). The content of β-glucans was calculated indirectly from the difference between the content of total glucans and α-glucan. Results were expressed in g/100 g dw of mushroom or extract.

Determination of vitamin contents

The content of thiamine, riboflavin, niacin, pyridoxine, as water-soluble vitamins B_1, B_2, B_3 and B_6, respectively, was determined in CCA and CCM, whereas the content of retinol, cholecalciferol and α-tocopherol as fat-soluble vitamins A, D_3 and E, respectively, was determined in CCC. Before the analysis, methanol and aqueous extracts were diluted with water, whereas cyclohexane extract was diluted with hexane, followed by the filtration of solutions through a 0.45 μm filter. An Agilent 1200 series HPLC with diode aray detector (DAD) and a Zorbax Eclipse XDB-C18 (4.6×250 mm, 5 μm) reverse phase column (Agilent), thermostated at 25°C, was used for determinations.

Five microliters of polar extracts, used for the analysis of water-soluble vitamins, were injected into the column. The solvent A consisting of 0.1 M phosphate buffer, pH 2.8 and acetonitrile (90:10, *v/v*) was used as an eluent for 10 min at the flow rate of 1.0 mL/min. The absorbance for thiamine and niacin was measured at 254 nm, for riboflavin at 268 nm and for pyridoxine at 291 nm. For the analysis of fat-soluble vitamins, the non-polar extract was injected at the volume of 10 μL into the same column. The gradient solvent system, which consisted of mobile phase A and acetonitrile (B), was applied (1% of B for 4.5 min, and then 90% of B until 6 min) with the flow rate of 0.5 mL/min. The absorbances for retinol, cholecalciferol and α-tocopherol determination were measured at 326, 266 and 210 nm, respectively. Identification of vitamins in the samples was performed based on retention times (t_R) and UV spectra relative to t_R and UV spectra of the standards. Quantification was performed based on peak areas using baseline construction method. All standard solutions were prepared with a range from 1 to 100 mg/L, except for α-tocopherol with a range of 5–200 mg/L. The content of vitamins was expressed in g/100 g of dry extract.

Determination of the contents of 5'-nucleotides and nucleosides

For analysing the content of 5'-nucleotides and nucleosides, the samples were prepared according to Beluhan & Ranogajec [2011], with slight modifications. Dried homogenized mushroom weighing 500 mg was mixed with 10 mL of deionized water in a round bottom flask. The suspension was heated on a water bath for 5 min and then stirred with a vortex stirrer for 30 s and cooled to room temperature. The samples were filtered through a 0.45 μm diameter filter before analysis. Stock standard solutions of single 5'-nucleotides (5'-adenosine monophosphate, 5'-AMP; 5'-citidine monophosphate, 5'-CMP; 5'-uridine monophosphate, 5'-UMP; and 5'-guanosine monophosphate, 5'-GMP) and nucleosides (adenosine, cytidine, uridine, guanosine) were prepared at a concentration of 0.5 mg/mL. A series of dilutions (0.25, 0.125, 0.0625, 0.0312 and 0.0156 mg/mL) were prepared from the stock standard solution for the construction of the calibration curves of each of the eight listed substances. All standard solutions were filtered through a 0.45 μm diameter filter. The stock standard solutions were stored at 4°C protected from light until the beginning of the analysis.

Identification and content determination of 5'-nucleotides and nucleosides was performed according to a modified method of Ranogajec *et al*. [2009] on an Agilent 1100 HPLC system with a binary pump, a G1315B DAD and a Zorbax Eclipse XDB-C18 column (4.6×250 mm, 5 μm). Twenty microliters of the test sample and standard solutions were injected. The solvent system was composed of two mobile phases: 0.1 M KH_2PO_4 and 4 mM tetrabuthylammonium bromide, pH 6 (A) and a mixture of mobile phase A with methanol (70:30, *v/v*), pH 7.2 (B). Elution started at the flow rate of 1.2 mL/min with 5% B until 9 min, 25% B at 15 min, 90% B at 17.5 min, 100% B at 19 min and 5% B at 24 min. The absorbance was recorded at 254 nm. Identification of 5'-nucleotides and nucleosides in the sample was performed based on t_R and UV spectra relative to t_R and UV spectra of the standards. Quantification was performed based on peak areas using baseline construction method. The content of 5'-nucleotides and nucleosides was expressed in mg/g dw of mushroom.

Analysis of free amino acid composition and umami potential

Samples of dried mushroom were prepared as described above for the analysis of the contents of 5'-nucleotides and nucleosides. An amino acid standard solution containing L-alanine, L-arginine, L-glutamic acid, L-cystine, L-aspartic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine was used to prepare stock standard solutions and their dilutions (250, 125, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.0678 μM).

Before the analysis, derivatization of the standards and the amino acids of the test sample was performed

according to Ribeiro *et al*. [2008]. Briefly, 20 μL of both the test solution and standard solutions was mixed with 180 μL of a reaction buffer (0.15 M $NaHCO_3$, pH 8.6), stirred, and 200 μL of dabsyl chloride solution (12.4 mM) was added. The vials were stirred again and heated to 70°C for 15 min. The reaction was ended by cooling on ice for 5 min. Then, 400 μL of a dilution buffer was added, containing 50 mL of acetonitrile, 25 mL of ethanol and 25 mL of an elution buffer (9 mM sodium phosphate, 4% dimethylformamide, 0.15% triethylamine, pH 6.5). The mixture was centrifuged for 5 min at 1,700×*g*. The supernatant was filtered and stored at –20°C until the analysis.

The analysis of amino acids was performed using the LC--MS-ESI system (Agilent 1260/6130) equipped in an Agilent Zorbax SB-Aq column (3.0×150 mm, 3.5 μm) thermostated at 25°C. The volume of injection was 3 μL. The mobile phase system was composed of two solvents: 0.1% (*v/v*) ammonium formate in water (A) and 0.1% (*v/v*) formic acid in acetonitrile (B). Elution started at the flow rate of 0.3 mL/min with 15% B until 3 min, 85% B at 63 min and 15% B at 74 min. Detection wavelengths were at 436 nm. Electrospray ionization (ESI) mass spectra were recorded in the positive ion mode in a range of 50–600 *m/z*; fragmentator voltage of 250, nitrogen flow of 10 mL/min at 350°C, under pressure of 40 psi and capillary voltage of 3500 V. Individual amino acids were identified in the mixture based on their t_R and mass spectra in relation to the t_R and mass spectra of the corresponding standards, analysed under the same chromatographic conditions. The amino acid contents were calculated based on the peak area using baseline construction method. The content of individual amino acids was expressed in mg/g dw of mushroom. Also, to evaluate the umami potential of our tested mushroom sample, the equivalent umami concentration (EUC) value was calculated according to the formula given by Yamaguchi *et al*. [1971]. The EUC value of 100% indicates that the umami intensity of fruit bodies or mycelia per 1 g dry weight is equivalent to the umami intensity given by 1 g of monosodium glutamate (MSG).

Determination of fatty acid composition

Fatty acid composition of CCC was analyzed according to the method described by Ušjak *et al*. [2019] using an Agilent 6890N gas chromatography (GC) system equipped with a split/splitless injector (260°C), a flame ionization detector (FID) and a capillary column (Agilent J&W HP-88, 100 m × 0.25 mm, 0.20 μm film thickness), coupled with an Agilent 5975C mass spectrometry (MS) detector operating in the electron ionization mode at 70 eV. Saponification was achieved with 50% KOH at 90°C for 1 h. Petroleum ether was used to separate unsaponifiable fractions. Fatty acid methyl esters (FAME) were obtained by esterification of fatty acids with 98% H_2SO_4/anhydrous MeOH. The carrier gas was He, used at the flow rate of 1.2 mL/min. The oven temperature was initially held at 140°C for 5 min, then increased linearly from 140°C to 240°C at 4°C/min, and finally held at 240°C for 10 min. The FID and MS transfer line temperatures were 260°C and 250°C, respectively. Split ratio was 1:25 and the injected volume was 1 μL of 1% solution of the FAME in dichloromethane.

The identification of the FAME was based on the comparison of their t_R and mass spectra to those of the representative standards ran under the same chromatographic conditions and to those from the NIST/NBS 05 and Wiley (8th edition) libraries, and the literature. Relative percentages of the compounds were calculated based on the peak areas.

Determination of total phenolic content

The content of total phenolics in CCM and CCA was determined spectrophotometrically, as described previously by Kolundžić *et al*. [2017]. To this end, 100 μL of methanol solution of CCM (11 mg/mL) and CCA (12 mg/mL) was mixed with 750 μL of the diluted Folin-Ciocalteu reagent (1:1, *v/v*, in distilled water). After 5 min, 750 μL of 60 g/L Na_2CO_3 solution was added to each sample. Firstly, the mixtures were shaken and then left for 90 min in the dark at room temperature. Absorbance was measured at 725 nm using UV--Vis spectrophotometer Evolution 300 (Thermo Scientific). As a blank, 100 μL of the solvent was used. Series of gallic acid dilutions (1–10 mg/mL) were reacted in parallel, the absorbance was measured and the calibration curve was constructed. The content of total phenolics was expressed as mg of the gallic acid equivalent (GA)/g of dry extract.

Determination of DPPH radical scavenging activity

The ability of CCM and CCA to scavenge free radicals was measured in a DPPH test [Kolundžić *et al*., 2017]. Briefly, six volumes (10, 30, 50, 70, 90, 100 μL) of stock methanol solutions of extracts (CCM 60 mg/mL; CCA 16 mg/mL) were mixed with methanol, so that the total volumen became 2 mL, and 0.5 mL of 0.5 mM DPPH radical solution in methanol was added. The concentration range was 240, 720, 1200, 1680, 2160 and 2400 μg/mL for CCM and 64, 192, 320, 448, 576 and 640 μg/mL for CCA. The mixtures were left in the dark at room temperature for 30 min. The absorbance of the solution was measured at 517 nm using an Evolution 300 UV-Vis spectrophotometer (Thermo Scientific) and the percent of inhibition of DPPH radical was calculated. The results were expressed as a IC_{50} value, *i.e.* concentration of the extract that reduced 50% of the DPPH radicals.

Determination of ACE inhibitory activity

The ACE Kit-WST (Dojindo Laboratories) was used to determine ACE inhibiting activity of CCA. In the assay, 3-hydroxybutyric acid (3HB), which is released from 3-hydryoxybutyryl-Gly-Gly-Gly (3HB-GGG), was enzymatically detected. The concentrations ranging between 0.1 and 20 mg/mL of the aqueous extract were used and the procedure of the manufacturer was completely followed. The experiment was done in the 96-well microtiter plate. The absorbance of the solutions was measured on an EL×800 absorbance microplate reader (BioTek Instruments, Winooski, VT, USA) at 450 nm and the extract concentration needed to inhibit 50% of ACE activity (IC_{50}) was calculated.

Cytotoxicity assay

Treatment of cell lines and determination of cell survival rate were done according to the procedure described by Kolundžić *et al*. [2017]. Human epithelial cervical cancer

cells HeLa, adenocarcinomic human alveolar basal epithelial cells A549, colorectal cancer cells LS174 and normal MRC-5 human embryonic lung fibroblast cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). All cancer cell lines were maintained in the recommended RPMI-1640 medium supplemented with 10% heat-inactivated (56°C) FBS, L-glutamine (3 mM), streptomycin (100 mg/mL), penicillin (100 IU/mL), and 25 mM HEPES buffer and adjusted to pH 7.2 by bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Stock solutions (100 mg/mL) of four extracts were made in dimethyl sulfoxide and dissolved in corresponding medium to the required working concentrations. Final concentrations achieved in the treated wells were 12.5, 25, 50, 100 and 200 μg/mL. The effects on cancer cell survival were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) test, 72 h upon extract addition, as described previously [Kolundžić *et al*., 2017]. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was then read in an ELISA plate reader (Thermo Labsystem Multiskan EX, Thermo Fisher scientific, Waltham, MA, USA) at 570 nm. IC_{50} was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. Cisplatin (*cis*-DDP) was used as a positive control.

Statistical analysis

All measurements were done in three analytical repetitions. Data are presented as mean ± standard deviation (SD). Statistical analyses were conducted using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Data were statistically analysed using Student's *t*-test (for two groups) or one-way analysis of variance (ANOVA) with post-hoc Tukey's test (for three or more groups), where appropriate. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Nutritional value, contents of total dietary fibre and glucans

Fruiting bodies of *C. cornucopioides* were low in crude fat (5.02±0.16 g/100 g dw), total carbohydrates (11.97±0.87 g/100 g dw) and crude proteins (11.51±0.16 g/100 g dw), which resulted in a low energy value (248.03±9.84 kcal/100 g dw; 1037.63±31.00 kJ/100 g dw). The moisture content was 6.84±0.10 g/100 g dw, and crude ash was 10.20±0.12 g/100 g dw. Results obtained in this study are in correlation with previous results in terms of the contents of crude ash and crude fat, but the protein content was far lower than 47.21%, 69.45% and 50.10% reported by Beluhan & Ranogajec [2011], Barros *et al*. [2008] and Colak *et al*. [2009], respectively. *C. cornucopioides* was rich in total dietary fibre (54.46±0.90 g/100 g dw). β-Glucan content in our sample (dried mushroom) was 15.65 g/100 g dw, which accounted for approximately 98% of total glucans (Table 1) and one third of the total dietary fibre. A similar result (16.20 g/100 g dw) has been reported for black trumpet growing in Poland of [Mirończuk-Chodakowska *et al*., 2017]. Cooking has been reported to influence the proximate glucan content in mushrooms [Kolundžić *et al*., 2017].

TABLE 1. The content of total, α- and β-glucan (g/100 g dw) in dried *Craterellus cornucopioides* before and after cooking and in extracts of mushrooms.

	Total glucan	α-Glucan	β-Glucan
Dried mushroom	15.96±0.07^a	0.31±0.01^a	15.65±0.06^a
Dried mushroom after cooking	1.53±0.02^d	0.08±0.01^c	1.44±0.03^c
CCA	2.54±0.10^b	0.15±0.01^b	2.39±0.10^b
CCM	1.61±0.04^c	0.13±0.01^b	1.47±0.04^c

Results are expressed as mean ± standard deviation ($n=3$). Values with different superscript letters in column differ significantly at $p<0.05$. CC, *C. cornucopioides* extracts (A, aqueous; M, methanol); dw, dry weight.

In the present study, the content of total glucans decreased significantly ($p<0.05$) after cooking to 1.53 g/100 g dw, indicating approximately 90% reduction (Table 1). The content of glucans was also determined in extracts. The presence of glucans was detected in polar (aqueous and methanol) but not in non-polar extracts (cyclohexane and dichloromethane). The significantly ($p<0.05$) highest content of total glucans, α- and β-glucan was detected in dried mushrooms compared to extracts. In turn, CCA had a significantly ($p<0.05$) higher content of all three glucan types (total glucans, α- and β-glucan) compared to DMCA and CCM, except for α-glucan in CCM. Therefore, it can be concluded that glucan contents in mushrooms depend not only on the cooking process, but also on the extraction method as well as the choice of solvent used for extraction.

Vitamins

The contents of water-soluble vitamins B_1, B_2, B_3 and B_6 were determined in polar extracts (CCA and CCM), while those of fat-soluble vitamins A, D_3 and E were determined in the non-polar extract (CCC), (Table 2). The most abundant water-soluble vitamin was B_3 – niacin with the content of 249.0 mg/100 g dry CCA and 367.0 mg/100 g dry CCM, followed by vitamin B_2 – riboflavin, vitamin B_1 – thiamine and vitamin B_6 – pyridoxine. ANOVA showed a significantly higher vitamin B_1 content in CCA compared to CCM ($p<0.05$), and significantly higher contents of vitamin B_3 and B_6 in CCM compared to CCA ($p<0.05$). So far, the content of B-group vitamins in *C. cornucopioides* has not been determined extensively. Çağlarırmak [2011] reported thiamine, riboflavin, pyridoxine and niacin contents of 0.17–0.63, 0.26–0.90, 0.14–0.56 and 6.38–8.37 mg/100 g dw, respectively, in dried cultivated shiitake, white and brown button mushrooms and oyster mushroom.

α-Tocopherol is the most active form of vitamin E in humans and as a free radical scavenger it protects our bodies against degenerative diseases [Tucker *et al.,* 2005]. α-Tocopherol was found as the most abundant fat-soluble vitamin in the cyclohexane extract of *C. cornucopioides*, with the content of 2816 mg/100 g dry extract corresponding to 47.90 mg/100 g dw of mushroom. The content of α-tocopherol previously reproted in *C. cornucopioides* was 0.115 mg/100 g dw [Liu *et al*., 2012], which is much lower than the value reported in our study. In turn, contents of total tocopherols have

TABLE 2. The content of vitamins in extracts of *Craterellus cornucopioides* (mg/100 g dry extract).

Vitamin	CCC	CCM	CCA
B_1	–	19.4±1.4[b]	54.7±2.9[a]
B_2	–	81.2±8.2[a]	76.3±6.2[a]
B_3	–	367.0±4.5[a]	249.0±5.0[b]
B_6	–	17.0±1.7[a]	8.3±1.0[b]
E	2816±36	–	–
D_3	89.3±8.9	–	–
A	16.1±1.8	–	–

Results are expressed as mean ± standard deviation (n=3). Values with different superscript letters in row differ significantly at p<0.05. CC, *C. cornucopioides* extracts (A, aqueous; M, methanol; C, cyclohexane).

been determined in mushrooms of *Boletus* species, revealing 2533 μg/100 g dw in *B. reticulatus* [Heleno *et al*., 2011].

Group-D vitamins occurred in two forms: ergocalciferol – vitamin D_2 being the major form of vitamin D found in edible mushrooms, and cholecalciferol – vitamin D_3 found in lesser amounts [Cardwell *et al*., 2018]. Also, ergosterol (one vitamin D precursors) is commonly present in fungi [Venditti *et al.*, 2017]. Considerable amount of vitamin D_3 has been found in the cyclohexane extract of *C. cornucopioides*, *i.e*. 89.3 mg/100 g dry extract which corresponded to 1.52 mg/100 g dw of mushroom. There is no literature data that support this founding. So far, only vitamin D_2 has been detected in the wild growing species of the genus Cantharellaceae, *i.e.* at 10.70 mg/100 g dw in *Cantharellus cibarius* and 21.10 mg/100 g dw in *C. tubaeformis* [Teichmann *et al*., 2007]. In the case of vitamin A, it was detected in the CCC at 16.1 mg/100 g dw (0.27 mg/100 g dw of mushroom), and here likewise, there is no literature data to compare this value.

5'-Mononucleotides and nucleosides

Nucleosides, being precursor molecules for DNA and RNA synthesis, are vital for cellular function. Also, both 5'-nucleotides and nucleosides were reported to exert various positive impacts on brain function, immune system, fatty acid metabolism, and gastrointestinal tract [Phan *et al*., 2018]. The most common nucleotides found in mushrooms are 5'-adenosine monophosphate (5'-AMP), 5'-cytidine monophosphate (5'-CMP), 5'-guanosine monophosphate (5'-GMP), 5'-inosine monophosphate (5'-IMP), 5'-uridine monophosphate (5'-UMP) and 5'-xantosine monophosphate (5'-XMP) [Phan *et al*., 2018].

The total content of 5'-nucleotides in dried body of *C. cornucopioides* was 3.99 mg/g dw, while the content of nucleosides was 1.84 mg/g dw. The 5'-nucleotide content could be ordered as follows: 5'-UMP > 5'-AMP > 5'-GMP > 5'-CMP (1.55±0.03, 0.87±0.47, 0.83±0.15 and 0.74±0.13 mg/g dw, respectively). In turn, the content of nucleosides decreased in the following order: uridine > adenosine = guanosine > cytidine (0.50±0.01, 0.47±0.07, 0.47±0.03 and 0.40±0.04 mg/g dw, respectively). The findings obtained from this research are different than the results published before. Ranogajec *et al*. [2009] reported lower contents of the same 5'-nucleosides and nucleotides in *C. cornucopioides* sample, *i.e.* 0.30 and 0.91 mg/g dw, respectively, while the content of 5'-nucleosides in *C. cornucopioides*, reported later by the same research group, was much higher, reaching 10.48 mg/g dw [Beluhan & Ranogajec, 2011]. These differences in the results are due to the influence of different factors on 5'-nucleotide content, such as the use of different parts of the fungus, the stage of fungal development, and collection time, as shown in the example of *Tricholoma matsutake* where the contents of 5'-AMP and 5'-GMP were higher in its pileus than stalk [Cho *et al*., 2010].

5'-Nucleotides, such as 5'-GMP, 5'-IMP,5'-XMP and 5'-AMP, in synergy with glutamic and aspartic acid contribute to the specific umami taste of mushrooms, which is the taste induced by monosodium glutamate (MSG) [Phat *et al*., 2016]. Among these compounds, 5'-GMP is the one responsible for the meaty taste of mushrooms, and it is a stronger flavor enhancer than MSG [Beluhan & Ranogajec, 2011]. The umami intensity of the mentioned compounds is discussed below in the Free amino acids section.

Free amino acids

The contents of individual free amino acids in dried *C. cornucopioides* are shown in Table 3. All amino acids, except histidine and cystine, were identified in the sample. The total amino acid content was 6.97 mg/g dw. According to the literature, the content of free amino acids in mushrooms is low and varies from 7.14 mg/g to 12.3 mg/g in dried edible mushrooms, with glutamic acid (21.7–23.7%) and alanine (17.7–17.9%) found as major ones [Cheung, 2010]. The content of amino acids in *C. cornucopioides* has been previously examined, where higher values were obtained for amino acids (67.48 mg/g dw), with glutamic acid as the most major amino acid [Beluhan & Ranogajec, 2011].

The content of essential amino acids in *C. cornucopioides* was 1.49 mg/g dw, while non-essential amino acids were present in a higher content of 5.48 mg/g dw (Table 3). The calculated ratio between essential and non-essential amino acids was 0.27, which was below the value of 0.6 recommended by FAO/WHO [Wang *et al*., 2014].

Another classification of amino acids can be made according to their characteristic taste [Beluhan & Ranogajec, 2011]. Among the *C. cornucopioides* amino acids were those with sweet taste, including alanine, glycine, serine and threonine; bitter taste, including arginine, isoleucine, leucine, methionine, phenylalanine and valine; tasteless, including lysine and tyrosine; and monosodium glutamate-like (MSG-like) taste, including glutamic and aspartic acids (Table 3). The predominant amino acids were those with bitter taste (3.14 mg/g dw) followed by MSG-like tasting (1.83 mg/g dw) and tasteless (1.00 mg/g dw). The low content of sweet-tasting amino acids (0.68 mg/g dw) was not surprising and it was in accordance with the low content of carbohydrates. Nevertheless, it has been found that sweet and MSG-like amino acids are so-called taste-active amino acids in mushrooms, unlike the bitter-testing ones, and so bitter taste might be suppressed by the sweet components [Mau, 2005].

TABLE 3. Free amino acid content in dried *Craterellus cornucopioides* and taste of amino acids.

Type of amino acid	Amino acid	Taste*	Content (mg/g dw)
Essential amino acid	Histidine	Bitter	nd
	Lysine	Tasteless	0.38±0.02
	Threonine	Sweet	0.21±0.01
	Methionine	Bitter	0.01±0.00
	Valine	Bitter	0.04±0.01
	Leucine/ isoleucine	Bitter	0.68±0.04
	Phenylalanine	Bitter	0.17±0.05
Non-essential amino acid	Cystine	/	nd
	Arginine	Bitter	2.24±0.01
	Serine	Sweet	0.15±0.06
	Aspartic acid	MSG-like	0.43±0.11
	Glutamic acid	MSG-like	1.40±0.10
	Glycine	Sweet	0.22±0.03
	Alanine	Sweet	0.10±0.03
	Tyrosine	Tasteless	0.62±0.01
	Proline	/	0.32±0.01

*according Beluhan & Ranogajec [2011].
Results are expressed as mean ± standard deviation (n=3). MSG-like, monosodium glutamate-like; nd, not detected; dw, dry weight.

TABLE 4. Fatty acid composition (% total fatty acids) of *Craterellus cornucopioides* cyclohexane extract.

Fatty acid	Content
C 15:0	0.33±0.05
C 16:0	9.96±0.25
C 16:1	0.34±0.20
C 17:0	0.19±0.01
C 18:0	12.43±0.37
C 18:1*n*9c	60.83±1.46
C 18:2*n*6c	10.85±0.10
C 20:0	0.41±0.03
C 20:5*n*3	0.70±0.03
C 22:0	0.36±0.01
C 22:6*n*3	2.93±0.21
C 24:0	0.41±0.08
C 24:1*n*15c	0.26±0.06
SFAs	24.08±0.79
MUFAs	61.44±1.73
PUFAs	14.48±0.34

Results are expressed as mean ± standard deviation (n=3). SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

As mentioned before, the synergistic effect of umami 5'-nucleotides (5'-AMP, 5'-IMP, 5'-GMP, and 5'-XMP) and umami amino acids (glutamic and aspartic acid) may enhance the umami taste of mushrooms. Hence, the EUC value was calculated to evaluate the umami potential of the mushroom sample. The EUC values are grouped into four levels: >1000% (>10 g MSG/g dw); 100–1000% (1–10 g MSG/g dw); 10–100% (0.1–1 g MSG/g dw) and 10% (<0.1 g MSG/g dw) [Mau, 2005]. The EUC value for the tested *C. cornucopioides* was 36.20%, which classifies it as the mushroom with EUC values at the third level. According to Mau [2005], mushrooms with EUC values at the second and third levels are those that do not contribute to umami taste but are still used for culinary purposes for their flavor, consistency and texture.

Fatty acids

The fatty acid composition of the cyclohexane extract of *C. cornucopioides* is shown in Table 4. Saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) have been identified and quantified. The most abundant group of fatty acids were MUFAs (61.44%), followed by SFAs (24.08%) and PUFAs (14.48%). This is considered to be a positive ratio since the SFAs are recognized to be associated with cardiovascular disorders and atherosclerosis, while the high intake of PUFAs and MUFAs is considered to prevent these disorders [Silva Figueiredo *et al*., 2017]. The obtained data are in accordance with findings from previously conducted studies where MUFAs were also detected as the most represented class of fatty acids in *C. cornucopioides* (59.85%), followed by PUFAs (23.79%) and SFAs (16.36%) [Barros *et al*., 2008].

The main fatty acid in the tested mushroom was oleic acid (Table 4). This monounsaturated but not an essential fatty acid is effective in lowering cholesterol level, which affects high blood pressure [Abugri *et al*., 2016]. Essential fatty acids include linoleic acid (C18:2n6c) and α-linolenic acid (C18:3n3c). Since the human organism is not able to synthesize these fatty acids, they must be provided with diet. In our study, only linoleic acid was found in *C. cornucopioides* with a relative content of 10.85% (Table 4). In turn, stearic acid was the most abundant (12.43%) among the identified saturated fatty acids. However, it has no impact on increasing serum low density lipoprotein (LDL) cholesterol level, due to its rapid conversion into oleic acid in the organism [Grundy, 2013]. Nevertheless, there was a higher content of unsaturated than saturated fatty acids in the tested *C. cornucopioides*, which can be classified as a healthy food.

Total phenolic content and DPPH radical scavenging activity

Phenolic compounds are very good antioxidants and possess very good radical scavenging ability [Zhao *et al.,* 2014]. The content of total phenolics in the methanol and aqueous extracts of *C. cornucopioides* is shown in Table 5. The methanol extract contained almost twice less total phenolics than the

TABLE 5. The total phenolic content, DPPH radical scavenging activity and angiotensin converting enzyme (ACE) inhibitory activity of *Craterellus cornucopioides* extracts.

	Total phenolic content (mg GA/g extract)	DPPH scavenging activity (IC_{50}; μg/mL)	ACE inhibitory activity (IC_{50}; mg/mL)
CCA	16.96±0.22[a]	473±5[b]	0.74±0.07
CCM	8.95±0.06[b]	887±15[a]	–

Results are expressed as mean ± standard deviation (n=3). Values with different superscript letters in column differ significantly at p<0.05. CC, *C. cornucopioides* extracts (A, aqueous; M, methanol); GA, gallic acid equivalent.

aqueous extract and thus exhibited lower DPPH radical scavenging activity (IC_{50} of 887 μg/mL for CCM and 473 μg/mL for CCA). Previous results of Liu *et al*. [2012] have shown significant, but lower than the results in this study, DPPH radical scavenging activity of *C. cornucopioides* extracts with the IC_{50} value of 40.30 mg/mL for ethanol extract and 26.37 mg/mL for aqueous extract and with quercetin as a major compound in the ethanol extract. Vamanu & Nita [2014] have used a different extraction method with a fluidized bed extractor, but the extract produced also had a lower total phenolic content (88.4 mg GA/100 g of extract) than in this study, whereas DPPH radical scavenging activity was about 60% for the extract concentration of 1 mg/mL. Different extraction procedure leads to the extraction of other compounds with antioxidative power as ascorbic acid, lycopene, β-carotene and tocopherols [Vamanu & Nita, 2014], but in our study, lipophilic compounds could accumulate in non-polar, cyclohexane extract which was not suitable for antioxidant activity estimation using the DPPH method.

ACE inhibitory activity

The results indicated dose-dependent ACE inhibitory activity of the *C. cornucopioides* aqueous extract with an IC_{50} of 0.74 mg/mL (Table 5). According to the manufacturer of the ACE kit (Dojindo Laboratories), IC_{50} of captopril – well-known ACE inhibitor – was 2.14 nM (0.465 ng/mL), in the same test [https://dojindo.com/product/ace-kit-wst-a502/]. The results of Kolundžić *et al*. [2017] study with *Cantharellus cibarius* from the same family Cantharellaceae showed 68% ACE inhibitory activity at a dose of 1.25 mg/mL. In turn, Jang *et al*. [2011] isolated and characterized two ACE inhibitors with IC_{50} values of 0.46 and 1.14 mg/mL from fruiting bodies of *Pleurotus cornucopiae*, and demonstrated an antihypertensive effect *in vivo* of an aqueous extract of the same fungus. Aqueous and ethanolic extracts of haesongi mushroom (*Hypsizigus marmoreus*) showed dose-dependent ACE inhibitory activity *in vitro* (aqueous extract had strong activity: 95.34% at a concentration of 40 mg/mL) [Jung *et al*., 2009]. Lee *et al*. [2004] showed ACE inhibitory activity of an aqueous extract of *T. giganteum* with an IC_{50} of 0.31 mg/mL and of an isolated peptide consisting of proline, glutamic acid and glycine (IC_{50} of 0.04 mg/mL), as well as different ACE inhibitory activities for aqueous, ethanolic and methanolic extracts of several mushrooms: *Pleurotus sajor-caju*, *P. ostreatus*, *Flammulina velutipes*, *T. giganteum*, *Agaricus bisporus*, *Poria cocos*, *Grifolia umbellata*, and *Lentinus edodes*, but the best activity was shown for the aqueous extract of *T. giganteum*.

Cytotoxic activity

The cytotoxic effect of four *C. cornucopioides* extracts was tested against human epithelial carcinoma cells, human lung carcinoma cells, human colon carcinoma cells, as well as on normal cell line. The respective results are presented in Table 6. From the obtained data, it can be concluded that all four extracts of *C. cornucopioides* expressed moderate cytotoxic activity against the tested cell lines. Non-polar extracts (cyclohexane and dichloromethane) were significantly (p<0.05) more active against the tested cell lines, including normal ones. The highest activity was observed in the cyclohexane extract against human epithelial carcinoma cells (IC_{50} of 78.3 μg/mL). Polar extracts (aqueous and methanol) showed almost no activity against the tested cell lines.

The anti-tumor effect of some non-polar compounds has been confirmed before, including fatty acids [Sagar *et al*., 1992]. Linoleic acid exhibited antimicrobial activity and cytotoxic activity against the HeLa tumor cell line. This fatty acid found as abundant fatty acid in the cyclohexane extract (Table 4) could be responsible for its high cytotoxic activity. Seven illudin sesquiterpenoids, *i.e.* craterellin A, B and C, illudin F, M, T and illudalenol, and one gymnomitrane sesquiterpenoid, gymnomitr-3-en-10β,15-diol, have been isolated by Guo *et al*. [2017a] from an ethyl acetate extract of *C. cornucopioides* as colorless oils and tested against several cancer cell lines (breast cancer SK-BR-3, hepatocellular carcinoma SMMC-7721, human myeloid leukemia HL-60, pancreatic cancer PANC-1 and lung cancer A-549). Only craterellin C (sesquiterpenoid) isolated from

TABLE 6. Cytotoxic activity (IC_{50}, μg/mL) of *Craterellus cornucopioides* extracts.

	CCA	CCM	CCD	CCC	*cis*-DDP*
HeLa	>200	>200	135.6±9.7[a]	78.3±0.3[b]	2.37±0.28
LS174	191.5±7.2[a]	186.6±19.0[a]	135.7±9.9[b]	139.1±9.9[b]	4.83±0.35
A549	>200	>200	153.2±7.0[a]	141.9±8.0[a]	11.6±1.6
MRC5	189.2±9.4[a]	>200	148.5±3.6[b]	155.6±4.9[b]	14.3±1.3

Results are expressed as mean ± standard deviation (n=3). Values for extracts with different superscript letters in row differ significantly at p<0.05. CC, *C. cornucopioides* extracts (A, aqueous; M, methanol; C, cyclohexane; D, dichloromethane); HeLa, human epithelial cervical cancer cells; LS174, colorectal cancer cells; A549, adenocarcinomic human alveolar basal epithelial cells; MRC5, human embryonic lung fibroblast cells; *cis*-DDP, *cis*-diaminedichloroplatinum. **cis*-DDP was used as positive control.

the ethyl acetate extract of black trumpet, expressed moderate cytotoxity against A-549 (21.0 μM). Craterellin D and E, as well as menthane monoterpene, 4-hydroxy-4-isopropenyl--cyclohexanemethanol acetate, have also been isolated from the ethyl acetate extract of *C. cornucopioides*, and tested against the same cell cultures, but none of the compounds exhibited cytotoxic activity [Guo *et al*., 2017b]. In turn, methanolic extracts of twenty nine mushroom species, including *C. cornucopioides*, were tested by MTT assay against human lung adenocarcinoma A549 [Vasdekis *et al*., 2018]. Black trumpet extract was among the extracts with the most prominent activity with IC_{50} <1 mg/mL. Piceatannol, the natural stilbene with antioxidant, anticancer and anti-inflammatory activities, was identified in all tested extracts with promising cytotoxic activity. Similar research has recently been conducted by Kosanić *et al*. [2019]. This research group tested the cytotoxic potential of an acetone extract of *C. cornucopioides* against the same cell lines as in our research. The tested extract showed a moderate effect, and HeLa were the most sensitive. These authors concluded that this effect might have been due to the presence of polyphenols. The non-polar extract analyzed in our study (CCC) was also the most active against HeLa cell line.

CONCLUSION

Based on the results of this study, it can be concluded that black trumpet was a good source of nutrients, suitable for daily diet, primarily due to its low fat content and high content of dietary fibre, especially β-glucan. The nutritional potential of this fungus was also ascribed to the presence fatty acids, especially oleic acid and linoleic acid, which would be very important for people with hypertension or metabolic syndrome. The most promising result was the inhibitory effect of the aqueous extract on ACE. This was the first time that ACE inhibitory activity of black trumpet extract was tested. Concerning cytotoxicity, the cyclohexane extract, which was rich in fatty acids and vitamin E, exhibited the greatest potential.

In terms of the content of other nutrients and bioactive compounds, this mushroom was rich in vitamins and, to the best of our knowledge, this is the first time that their content was determined in extracts of different polarities, according to the solubility of the vitamins themselves, and not in fresh mushrooms. The content of amino acids, nucleotides and fatty acids, was obviously lower than in previous studies, but it was still correlated with the overall nutritional potential of this fungus. As expected, the content of total phenolics was also low, which resulted in poor antioxidant activity. Black trumpet should be analyzed in more detail for identification and isolation of specific compounds that contribute to the ACE inhibitory and cytotoxic activity.

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CONFLICT OF INTERESTS

The authors confirm that this article content has no conflict of interest.

ORCID IDs

M. Dodevska https://orcid.org/0000-0001-6030-459X
T. Kundaković-Vasović https://orcid.org/0000-0001-5777-8612
J. Radović https://orcid.org/0000-0001-7324-1647
T. Stanojković https://orcid.org/0000-0001-9178-9200
A. Tačić https://orcid.org/0000-0003-3119-033X

REFERENCES

1. Abugri, D., McElhenney, W., William, K. (2016). Fatty acid profiling in selected cultivated edible and wild medicinal mushrooms in Southern United States. *Journal of Experimental Food Chemistry*, *2*(1), art. no. 100108. https://doi.org/10.4172/2472-0542.1000108
2. AOAC. (1995). Official Methods of Analysis (16th ed.). Arlighton, VA: Association of Official Analytical Chemists.
3. Barros, L., Cruz, T., Baptista, P., Estevinho, L., Ferreira, I. (2008). Wild and commercial mushrooms as source of nutrients and nutraceuticals. *Food and Chemical Toxicology*, *46*(8), 2742–2747. https://doi.org/10.1016/j.fct.2008.04.030
4. Beluhan, S., Ranogajec, A. (2011). Chemical composition and non-volatile components of Croatian wild edible mushrooms. *Food Chemistry*, *124*(3), 1076–1082. https://doi.org/10.1016/j.foodchem.2010.07.081
5. Cağlarirmak, N. (2011). Chemical composition and nutrition value of dried cultivated culinary–medicinal mushrooms from Turkey. *International Journal of Medicinal Mushrooms*, *13*, 351–356. https://doi.org/10.1615/IntJMedMushr.v13.i4.50
6. Cardwell, G., Bornman, J.F., James, A.P., Black, L.J. (2018). A review of mushrooms as a potential source of dietary vitamin D. *Nutrients*, *10*(10), art. no. 1498. https://doi.org/10.3390/nu10101498
7. Chen, P., Qin, H.J., Li, Y.W., Ma, G.X., Yang, J.S., Wang, Q. (2020). Study on chemical constituents of an edible mushroom *Volvariella volvacea* and their antitumor activity *in vitro*. *Natural Product Research, 34*(10), 1417–1422. https://doi.org/10.1080/14786419.2018.1509324
8. Cheung, P.C.K. (2010). The nutritional and health benefits of mushrooms. *Nutrition Bulletin*, *35*(4), 292–299. https://doi.org/10.1111/j.1467-3010.2010.01859.x
9. Cho, I.H., Choi, H.K., Kim, Y. (2010). Comparison of umami-taste active components in the pileus and stipe of pine-mushrooms (*Tricholoma matsutake* Sing.) of different grades. *Food Chemistry*, *118*(3), 804–807. https://doi.org/10.1016/j.foodchem.2009.05.084
10. Choi, H.S., Cho, H.Y., Yang, H.C., Ra, K.S., Suh, H.J. (2001). Angiotensin I-converting enzyme inhibitor from *Grifola frondosa*. *Food Research International*, *34*(2–3), 177–182. https://doi.org/10.1016/S0963-9969(00)00149-6
11. Colak, A., Faiz, Ö., Sesli, E. (2009). Nutritional composition of some wild edible mushrooms. *Turkish Journal of Biochemistry*, *34*(1), 25–31.

12. Gil-Ramirez, A., Clavijo, C., Palanisamy, M., Soler-Rivas, C., Ruiz-Rodrigez, A., Martin, F., Reglero, G., Perez, M. (2011). Edible mushrooms as potential sources of new hypocholesterolemic compounds. (2011). *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products* (ICMBMP7), 4–7 October 2011, Arcachon, France, 110–119.
13. Grundy, S.M. (2013). Cholesterol: Factors determining blood levels. In B. Caballero (ed.), *Encyclopedia of Human Nutrition* (3rd edition). London, United Kingdom: Academic Press, pp. 335–340. https://doi.org/10.1016/B978-0-12-375083-9.00051-9
14. Guo, H., Diao, Q., Hou, D., Li, Z., Zhou, Z., Feng, T., Liu, J. (2017a). Sesquiterpenoids from cultures of the edible mushroom *Craterellus cornucopioides*. *Phytochemistry Letters*, *21*, 114–117. https://doi.org/10.1016/j.phytol.2017.06.007
15. Guo, H., Diao, Q.P., Zhang, B., Feng, T. (2017b). Two new illudane sesquiterpenoids and one new menthane monoterpene from cultures of *Craterellus cornucopioides*. *Journal of Asian Natural Products Research*, *21*(2), 123–128. https://doi.org/10.1080/10286020.2017.1394293
16. Heleno, S.A., Barros, L., Sousa, M.J., Martins, A., Santos-Buelga, C., Ferrerira, I.C.F.R. (2011). Targeted metabolites analysis in wild *Boletus* species. *LWT – Food Science and Technology*, *44*(6), 1343–1348. https://doi.org/10.1016/j.lwt.2011.01.017
17. https://dojindo.com/product/ace-kit-wst-a502/ [last visited 15.4. 2022]
18. Jang, J.H., Jeong, S.C., Kim, J.H., Lee, Y.H., Ju, Y.C., Lee, J.S. (2011). Characterisation of a new antihypertensive angiotensin I-converting enzyme inhibitory peptide from *Pleurotus cornucopiae*. *Food Chemistry*, *127*(2), 412–418. https://doi.org/10.1016/j.foodchem.2011.01.010
19. Jung, E.B., Jo, J.H., Cho, S.M. (2009). Hypoglycemic and angiotension converting enzyme inhibitory effect of water and ethanol extracts from Haesongi mushroom (*Hypsizigus marmoreus*). *Food Science and Biotechology*, *18*(2), 541–545. https://www.earticle.net/Article/A105510
20. Khatun, K., Mahtab, H., Khanam, P.A., Sayeed, M.A., Khan, K.A. (2007). Oyster mushroom reduced blood glucose and cholesterol in diabetic subjects. *Mymensingh Medical Journal*, *16*(1), 94–99. https://doi.org/10.3329/mmj.v16i1.261
21. Kolundžić, M., Stanojković, T., Radović, J., Tačić, A., Dodevska, M., Milenković, M., Sisto, F., Masia, C., Farronato, G., Nikolić, V., Kundaković, T. (2017). Cytotoxic and antimicrobial activities of *Cantharellus cibarius* Fr. (Cantarellaceae). *Journal of Medicinal Food*, *20*(8), 790–796. https://doi.org/10.1089/jmf.2016.0176
22. Kosanić, M., Ranković, B., Stanojković, T., Radović-Jakovljević, M., Ćirić, A., Grujičić, D., Milošević-Djordjević, O. (2019). *Craterellus cornucopioides* edible mushroom as source of biologically active compounds. *Natural Product Communications*, *14*(5), 1–6. https://doi.org/10.1177/1934578X19843610
23. Kundaković, T., Kolundžić, M. (2013). Therapeutic properties of mushrooms in managing adverse effects in the metabolic syndrome. *Current Topics in Medicinal Chemistry*, *13*(21), 2734–2744. https://doi.org/10.2174/15680266113136660196
24. Lee, D.H., Kim, J.H., Park, J.S., Choi, Y.J., Lee, J.S. (2004). Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom *Tricholoma giganteum*. *Peptides*, *25*(4), 621–627. https://doi.org/10.1016/j.peptides.2004.01.015
25. Liu, Y.T., Sun, J., Luo, Z.Y., Rao, S.Q., Su, Y.J., Xu, R.R., Yang, Y.J. (2012). Chemical composition of five wild edible mushrooms collected from Southwest China and their antihyperglycemic and antioxidant activity. *Food and Chemical Toxicology*, *50*(5), 1238–1244. https://doi.org/10.1016/j.fct.2012.01.023
26. Lo, H.C., Hsu, T.H., Lin, F.Y., Wasser, S.P., Chen, Y.H., Lee, C.H. (2012). Effects of yellow brain culinary-medicinal mushroom, *Tremella mesenterica* Ritz.:Fr. (Higher basidiomycetes), on immune function in normal and type 1 diabetic rats. *International Journal of Medicinal Mushrooms*, *14*(5), 447–457. https://doi.org/10.1615/IntJMedMushr.v14.i5.20
27. Mau, J.L. (2005). The umami taste of edible and medicinal mushrooms. *International Journal of Medicinal Mushrooms*, *7*(1–2), 119–126. https://doi.org/10.1615/IntJMedMushr.v7.i12.120
28. Mirończuk-Chodakowska, I., Witkowska, A.M., Zujko, M.E., Terlikowska, K.M. (2017). Quantitative evaluation of 1,3,1,6 β-D--glucan contents in wild-growing species of edible Polish mushrooms. *Roczniki Państwowego Zakładu Higieny*, *68*(3), 281–290.
29. O'Callaghan, Y.C., O'Brien, N.M., Kenny, O., Harrington, T., Brunton, N., Smyth, T.J. (2015). Anti-inflammatory effects of wild Irish mushroom extracts in RAW264.7 mouse macrophage cells. *Journal of Medicinal Food*, *18*(2), 202–207. https://doi.org/10.1089/jmf.2014.0012
30. Palacios, I., Lozano, M., Moro, C., D'Arrigo, M., Rostagno, M.A., Martínez, J.A., García-Lafuente, A., Guillamón, E., Villares, A. (2011). Antioxidant properties of phenolic compounds occurring in edible mushrooms. *Food Chemistry*, *128*(3), 674–678. https://doi.org/10.1016/j.foodchem.2011.03.085
31. Pandya, U., Dhuldhaj, U., Sahay, N.S. (2019). Bioactive mushroom polysaccharides as antitumor: an overview. *Natural Product Research, 33*(18), 2668–2680. https://doi.org/10.1080/14786419.2018.1466129
32. Phan, C., Wang, J., Cheah, S., Naidu, M., David, P., Sabarantam, V. (2018). A review on the nucleic acid constituents in mushrooms: nucleobases, nucleosides and nucleotide. *Critical Reviews in Biotechnology*, *38*(5), 762–777. https://doi.org/10.1080/07388551.2017.1399102
33. Phat, C., Moon, B., Lee, C. (2016). Evaluation of umami taste in mushroom extracts by chemical analysis, sensory evaluation, and an electronictongue system. *Food Chemisty*, *192*, 1068–1077. https://doi.org/10.1016/j.foodchem.2015.07.113
34. Ranogajec, A., Beluhan, S., Šmit, Z. (2009). Analysis of nucleosides and monophosphate nucleotides from mushrooms with reversed-phase HPLC. *Journal of Separation Science*, *33*(8), 1024–1033. https://doi.org/10.1002/jssc.200900516
35. Ribeiro, B., Andrade, P., Silva, B., Baptista, P., Seabra, R., Valentão, P. (2008). Comparative study on free amino acid composition of wild edible mushroom species. *Journal of Agricultural and Food Chemistry*, *56*(22), 10973–10979. https://doi.org/10.1021/jf802076p

36. Sagar, P.S., Das, U.N., Koratkar, R., Ramesh, G., Padma, M., Kumar, G.S. (1992). Cytotoxic action of *cis*-unsaturated fatty acids on human cervical carcinoma (HeLa) cells: relationship to free radicals and lipid peroxidation and its modulation by calmodulin antagonists. *Cancer Letters*, *63*(3), 189–198. https://doi.org/10.1016/0304-3835(92)90260-3
37. Sakamoto, Y., Takeuchi, A., Sato, T., Obara, K., Takai, K., Fujino, K., Hirose, T., Inagaki, Y. (2001). Identification of antihypertensive substance in an aqueous extract from fruit body of *Mycoleptodonoides aitchisonii*. Oyo Yakuri/*Pharmacometrics*, *61*, 221–229.
38. Silva Figueiredo, P., Carla Inada, A., Marcelino, G., Maiara Lopes Cardozo, C., de Cássia Freitas, K., de Cássia Avellaneda Guimarães, R., Pereira de Castro, A., Aragão do Nascimento, V., Aiko Hiane, P. (2017). Fatty acids consumption: the role metabolic aspects involved in obesity and its associated disorders. *Nutrients*, *9*(10), art. no. 1158. https://doi.org/10.3390/nu9101158
39. Teichmann, A., Dutta, P.C., Staffas, A., Jägerstad, M. (2007). Sterol and vitamin D_2 concentrations in cultivated and wild grown mushrooms: Effects of UV irradiation. *LWT – Food Science and Technology*, *40*(5), 815–822. https://doi.org/10.1016/j.lwt.2006.04.003
40. Tucker, J.M., Townsend, D.M. (2005). Alpha-tocopherol: roles in prevention and therapy of human disease. *Biomedicine & Pharmacotherapy,* 59(7), 380–387. https://doi.org/10.1016/j.biopha.2005.06.005
41. Ušjak, L., Sofrenić, I., Tešević, V., Drobac, M., Niketić, M., Petrović, S. (2019). Fatty acids, sterols, and triterpenes of the fruits of 8 *Heracleum* taxa. *Natural Product Communications, 14*(6). https://doi.org/10.1177/1934578X19856788
42. Vamanu, E., Nita, S. (2014). Biological activity of fluidized bed ethanol extracts from several edible mushrooms. *Food Science and Biotechnology*, *23*(5) 1483–1490. https://doi.org/10.1007/s10068-014-0203-4
43. Vasdekis, E.P., Karkabounas, A., Giannakopoulos, I., Savvas, D., Lekka, M. (2018). Screening of mushrooms bioactivity: piceatannol was identified as a bioactive ingredient in the order *Cantharellales*. *European Food Research and Technology*, *244*, 861–871. https://doi.org/10.1007/s00217-017-3007-y
44. Venditti, A., Frezza, C., Sciubba, F., Serafini, M., Bianco, A. (2017). Primary and secondary metabolites of an European edible mushroom and its nutraceutical value: *Suillusbellinii* (Inzenga) Kuntze. *Natural Product Research, 31*(16), 1910–1919. https://doi.org/10.1080/14786419.2016.1267731
45. Wang, X.M., Zhang, J., Wu, L.H., Zhao, Y.L., Li, T., Li, J.Q., Wang, Y.Z., Liu, H.G. (2014). A mini-review of chemical composition and nutritional value of edible wild-grown mushroom from China. *Food Chemistry*, *151*, 279–285. https://doi.org/10.1016/j.foodchem.2013.11.062
46. Watanabe, F., Schwarz, J., Takenaka, S., Miyamoto, E., Ohishi, N., Nelle, E., Hochstrasser, R., Yabuta, Y. (2012). Characterization of vitamin B12 compounds in the wild edible mushrooms black trumpet (*Craterellus cornucopioides*) and golden chanterelle (*Cantharellus cibarius*). *Journal of Nutritional Science and Vitaminology*, *58*(6), 438–441. https://doi.org/10.3177/jnsv.58.438
47. Wheeler, S., Jordan, P. (2007). A Practical Encyclopedia of Mushrooms (Praktična enciklopedija pečuraka). JRJ: Zemun-Belgrade, Serbia, p. 54.
48. Williams, B., Mancia, G., Spiering, W., Rosei, E.A., Azizi, M., Burnier, M., Clement, D.L., Coca, A., De Simone, G., Dominiczak, A., Kahan, T., Mahfoud, F., Redon, J., Ruilope, L., Zanchetti, A., Kerins, M., Kjeldsen, S.E., Kreutz, R., Laurent, S., Lip, G.Y.H., McManus, R., Narkiewicz, K., Ruschitzka, F., Schmieder, R.E., Shlyakhto, E., Tsioufis, C., Aboyans, V., Desormais, I. (2018). ESC Scientific Document Group. 2018 ESC/ESH Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH). *European Heart Journal*, *39*(33), 3021–3104. https://doi.org/10.1093/eurheartj/ehy339
49. World Health Organization Cardiovascular diseases factsheet, 11 June 2021. https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) [last visited 20.5.2022].
50. Wu, Q., Li, Y., Peng, K., Wang, X. L., Ding, Z., Liu, L., Xu, P., Liu, G.Q. (2019). Isolation and characterization of three antihypertension peptides from the mycelia of *Ganoderma lucidum* (Agaricomycetes). *Journal of Agricultural and Food Chemistry*, *67*(29), 8149–8159. https://doi.org/10.1021/acs.jafc.9b02276
51. Yamaguchi, S., Yoshikawa, T., Iikeda, S., Ninomiya, T. (1971). Measurement of the relative taste intensity of some L-amino acids and 5'-nucleotides. *Journal of Food Science*, *36*(6) 846–849. https://doi.org/10.1111/j.1365-2621.1971.tb15541.x
52. Zhao, H., Zhang, H., Yang, S. (2014). Phenolic compounds and its antioxidant activities in ethanolic extracts from seven cultivars of Chinese jujube. *Food Science and Human Wellness,* 3(3–4), 183–190. https://doi.org/10.1016/j.fshw.2014.12.005

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Section: Food Technology

Adsorption and Desorption Characteristics and Purification of Isoflavones from Crude Soybean Extract Using Macroporous Resins

Thi Ngoc Thu Tran[1,a], Xuan Vung Bui[2], Nguyen Thi Truc Loan[3], Nguyen Huu Thuan Anh[4,5], Truong Dang Le[4,5,a], Thi Minh Hanh Truong[3,]*

[1]*University of Technology and Education, The University of Danang, 48 Cao Thang St., 550000 Danang, Vietnam*
[2]*University of Education, The University of Danang, 459 Ton Duc Thang St., 550000 Danang, Vietnam*
[3]*University of Science and Technology, The University of Danang, 54 Nguyen Luong Bang St., 550000 Danang, Vietnam*
[4]*Institute of Environmental Technology and Sustainable Development, Nguyen Tat Thanh University, Ho Chi Minh City 700000, Vietnam*
[5]*Faculty of Food and Environmental Engineering, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam*

Key words: soybean isoflavones, macroporous resin, purification, adsorption and desorption characteristics, daidzin, genistin

Isoflavones in soybean have been well-known with many health-promoting effects on humans. This study aimed to purify isoflavones from the crude soybean extract by the static adsorption/desorption process on macroporous resins. A screening test of four commercial resins: D101, AB-8, Amberlite® XAD4, and Diaion HP20 according to their adsorption/desorption characteristic for isoflavones was investigated. All four resins showed high adsorption and desorption characteristics in which D101 resin was chosen as the most suitable resin for purifying isoflavones. Compositional analysis showed that daidzin and genistin were the main isoflavones in the crude soybean extract. The adsorption isotherms data of total isoflavones, daidzin, and genistin fitted well with the Langmuir model with $R^2>0.98$. The dynamic adsorption conditions for the purification process of isoflavones on the D101 resin-packed column were selected at the bed volume (BV) of 200 mL, feed volume of 3.75 BV, and flow rate of 1.5 BV/h. The dynamic desorption was carried out with the elution solution of 70% (*v/v*) ethanol, elution volume of 2.5 BV, and flow rate of 1 BV/h. The total isoflavone content in the purified extract was 8.70-fold higher than its initial content in the crude soybean extract with a recovery yield of nearly 80%. The study results reveal a strong possibility for large-scale production of isoflavones for further application in functional food products or pharmaceutical products.

INTRODUCTION

Isoflavones, well-known as phytoestrogens, have been closely related to the structure of estrogen, a hormone released in a woman's body. Isoflavone compounds are naturally found in the members of the bean family (Fabaceae (Leguminosae)) [Bennetau & Pelissero, 2013; Bustamante-Rangel *et al.*, 2018; Rostagno *et al.*, 2010]. Isoflavones are a subclass of flavonoids exhibiting high antioxidant, anticancer, or anti-inflammatory activities [Lee *et al.*, 2005; Shim *et al.*, 2008]. Soybean isoflavones have been reported to induce a strong antioxidant activity both in the *in vitro* and *in vivo* studies [Li *et al.*, 2018]. Therefore, the use of these naturally-derived isoflavones as functional food supplements has been continuously gaining attraction due to the high demand of health-conscious customers [Almeida *et al.*, 2015; Uifălean *et al.*, 2015].

However, a very small amount of isoflavones in the crude soybean extract might limit its applicability or feasibility in functional food products [Li *et al.*, 2018]. Meanwhile, crude soybean extract mostly comprises polysaccharides and other impurity parts [Liu *et al.*, 2005]. Therefore, it should be subjected to the purification process to concentrate the isoflavones to be further applied on an industrial scale. Various fractionation methods have been applied to purify these bioactive compounds, such as fractional distillation, fractional crystallization, or preparative HPLC, which are not suitable on the industrial scale as they are cost-ineffective, time-consuming and applicable only in the a small-scale production [Yang *et al.*, 2016]. Meanwhile, using column chromatography with the stationary phase of macroporous resin beads to purify isoflavones has been found to offer many advantages, such as high selectivity in adsorption, cost-effectiveness, or less consumption of solvents [Kammerer *et al.*, 2019; Li & Chase, 2009; Soto *et al.*, 2011].

Many macroporous resins, such as D101, AB-8, Amberlite® XAD4, Diaion HP20, and H103, which are safe for food

* Corresponding Author:
E-mail: ttmhanh@dut.udn.vn
[a] These authors equally contributed to the work

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TABLE 1. Physical and chemical properties of the macroporous resins.

Trade name	Polarity	Matrix	Surface area (m^2/g)	Mean pore size (Å)	Particle size (mm)
AB-8	Weak polar	Styrene-Divinylbenzene	480–520	130–140	0.3–1.25
D101	Non-polar	Styrene-Divinylbenzene	500–550	90–100	0.3–1.25
Diaion HP-20	Weak polar	Styrene-Divinylbenzene	500	260	0.25–0.85
Amberlite® XAD4	Non-polar	Styrene-Divinylbenzene	750	100	0.25–0.85

application [Gao *et al.*, 2018], have been studied to purify isoflavone compounds from crude extracts of soybean hypocotyls [Choi & Kim, 2007], defatted soy flakes [Wu & Lai, 2007], kudzu root (*Puerariae lobatae* Radix) [Guo *et al.*, 2015], or okara (soy pulp) [Li *et al.*, 2012; Sevillano *et al.*, 2014]. AB-8 resin, which exhibited high adsorption and desorption abilities, was reported to be a proper resin for the purification process of genistein, an isoflavone in soybean [Li *et al.*, 2012]. SP-825 and SP-207 resins were successfully used to purify catechol, 4-ethylguaiacol, 4-ethylphenol, and daidzein of soy sauce with high adsorption and desorption potentials [Kim *et al.*, 2014]. In turn, D101 resin showed high adsorption/desorption capability for purifying steroidal saponins from the *Rhizoma paridis* [Wu & Lai, 2007].

In Vietnam, soybean is a commonly used food material due to its health benefits. Therefore, functional products enriched with soybean isoflavones can gain the attention of health-conscious consumers. In this study, four macroporous resins (D101, AB-8, Amberlite® XAD4, and Diaion HP20) were screened for the purification process of isoflavones from the soybean aqueous ethanol crude extract. Besides, static adsorption and desorption characteristics of not only total isoflavones but also the predominant isoflavones from the extract were investigated. The purification of isoflavones from soybean crude extracts by an appropriate resin column was carried out and key isoflavone constituents were also identified. The result is expected to show the high feasibility of using macroporous resins in the purification process of isoflavones on a large scale so that it facilitates the development of new functional food products with the purified extract.

MATERIALS AND METHODS

Materials

Soy (*Glycine max* L. Merr) beans were collected at local farms in Dai Loc district – Quang Nam province, Vietnam. Damaged beans were removed to avoid affecting the quality of the resulting soybean flour. Soybeans were dried at 60°C until reaching the moisture content of 9.04±0.04 g/100 g. The dried beans were homogeneously ground into a powder form and stored in closed boxes at the temperature of 20±2°C to be prepared for subsequent experiments.

Six isoflavone standards (genistin, glycitin, daidzin, genistein, glycitein, daidzein) with the purity of 99% were supplied from the United States Pharmacopeia (North Bethesda, MD, USA). Ethanol and acetonitrile (HPLC grade) were supplied from Merck KGaA (Darmstadt, Germany). D101 and Amberlite® XAD4, two non-polar resins, were from Anhui Sanxing Resin Technology Co., Ltd (Anhui, China), and Thermo Fisher Scientific Inc. (Waltham, MA, USA), respectively. Meanwhile, the two weak polar resins of AB-8 and Diaion HP20 were supplied from Anhui Sanxing Resin Technology Co., Ltd, and Mitsubishi Chemical Corporation (Tokyo, Japan), respectively. The characteristics of the studied macroporous resins are shown in Table 1.

Macroporous resins pretreatment

The resins were pretreated to remove impurity particles trapped inside the pores. First, they were soaked and allowed to swell for 24 h in 96% (*v/v*) ethanol solution followed by a washing step with distilled water to completely remove ethanol. The resins were then treated with HCl solution (5%, *v/v*) and NaOH solution (5%, *w/v*). Finally, distilled water was used again to wash these resins until neutralization of washing water [Guo *et al.*, 2015].

Crude soybean extract preparation

The crude soybean extract was obtained following our previous study [Tran *et al.*, 2019]. Briefly, soybean flour with an accurate weight was added to an 80% (*v/v*) ethanol solution. The sample was incubated in a water bath (HH-2, Wincom Company Ltd., Shanghai, China) at the temperature of 50°C under stirring (JJ-1, Hinotek Group, Ningbo, China). The mixture was then transferred to a rotary evaporator (model DHWEV01001V, Daihan Scientific Co., Ltd, Kangwon-do, South Korea) at 48–52°C to completely remove ethanol. The obtained residue was freeze-dried using an Alpha 1–2 Ldplus freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at -40°C for 16 h [Guo *et al*., 2015]. A stock solution of the extract was prepared by diluting the freeze-dried crude soybean extract in distilled water. The initial total isoflavone content of the stock solution was tentatively determined as 432.34 μg/mL. The term "total isoflavones" is defined as the mixture of six isoflavones including the glycoside isoflavones (genistin, glycitin, daidzin) and the corresponding aglycone forms (genistein, glycitein, and daidzein).

HPLC analysis of isoflavones

Reversed phase HPLC was used to quantify the content of daidzin, genistin, and total isoflavones. Briefly, an HPLC system (1200 Agilent, Agilent Technologies, Inc., Santa Clara, CA, USA) was connected with a quaternary gradient pump (including degasser), an autosampler, a diode-array detector, and ChemStation software for controlling chromatographic

parameters and quantitative analysis. A reversed phase column (Lichrospher® 100 RP-18, 4.6×250 mm, 5 μm, Merck) was placed in the column compartment with temperature maintained at 40°C to separate the isoflavones. The mobile phase at a flow rate of 1.5 mL/min comprised eluent A (0.05% (*v/v*) phosphoric acid) and eluent B (acetonitrile) which were filtered through a 0.45 μm membrane. The gradient elution used for the mobile phase was in the following order: starting with 100% of A for 5 min, changing from 10% to 30% of B (linear) for over 60 min, washing with 90% of B for 5 min, equilibrating with 100% of A for 10 min. All the compounds were detected at 260 nm. Quantification of isoflavones was conducted by constructing an external calibration curve. Each isoflavone peak was compared with isoflavone standards according to its spectrum and retention time [Tran *et al.,* 2019].

Static adsorption and desorption characteristics of isoflavones on four macroporous resins

Static adsorption and desorption tests

Four adsorption resins, including Diaion HP20, Amberlite® XAD4, D101, and AB-8, were selected to evaluate the purification capacity of isoflavones from the crude ethanolic soybean extract. The static adsorption was conducted in 250 mL conical flasks with stoppers which contained 10 g of resins and an exact volume of crude extract solutions. The flasks were shaken for 1 h at 25°C with the speed of 120 rpm, then allowed to stand for 1 h. This step was repeated in 3 cycles to attain the adsorption equilibrium. The resins were washed with distilled water followed by the addition of 50 mL of a 70% (*v/v*) ethanol solution to desorb isoflavones [Tang *et al.*, 2018; Yang *et al.*, 2016]. The extracts from the desorption process were concentrated under a vacuum rotary evaporator then diluted to 50 mL by acetonitrile and double distilled water mixture at a 1:1 ratio (*v/v*). The equilibrium concentrations of daidzin, genistin, and total isoflavones in adsorption solutions (C_e, mg/mL) and desorption solution (C_d, mg/mL) were determined by HPLC. The adsorption capacity (q_e, mg/g dry resin) adsorption ratio (A, %), desorption capacity (q_d, mg/g dry resin), desorption ratio (D, %) and recovery ratio (H, %) of the resins were calculated as follows:

$$q_e = \frac{(C_0 - C_e) \times V_0}{m} \quad (1)$$

$$A = \frac{(C_o - C_e)}{C_o} \times 100 \quad (2)$$

$$q_d = \frac{C_d \times V_d}{m} \quad (3)$$

$$D = \frac{C_d V_d}{C_o \times V_o - C_e \times V_e} \times 100 \quad (4)$$

$$H = \frac{C_d \times V_d}{C_0 \times V_0} \times 100 \quad (5)$$

where: C_0 is the initial concentration of daidzin, genistin and total isoflavone in sample solutions (mg/mL); V_0 is the volume of initial sample solution (mL); m is the weight of dry resin used (g); V_d is the volume of desorption solution (mL); and V_e is the volume of elution solution (mL).

Equilibrium adsorption isotherms

To plot the adsorption isotherms of daidzin, genistin, and total isoflavone on the D101 resin, 50 mL of crude extract solution were added at varying concentrations to 2.5 g of the resin at 25°C. The content of daidzin, genistin, and total isoflavones in the sample was measured after reaching their equilibrium concentration. Langmuir and Freundlich models were used to describe the isoflavones adsorption behaviors on D101 resin [Duran *et al.*, 2011; Guo *et al.,* 2015; Liu *et al*., 2010; Shazeli *et al.*, 2020].

The Langmuir adsorption equation is as follows:

$$\frac{q_e}{q_{max}} = \frac{K_L \times Ce}{1 + K_L \times Ce} \quad (6)$$

where: C_e (mg/mL) and q_e (mg/g dry resin) are equilibrium concentrations of analytes in the adsorption solutions and adsorption capacity, respectively; K_L (L/mg) is the Langmuir constant; q_{max} (mg/g dry resin) is the theoretical maximum adsorption capacity. Another important characteristic of Langmuir isotherms is a dimensionless constant (separation factor, R_L). It was calculated as follows:

$$R_L = \frac{1}{1 + K_L C_0} \quad (7)$$

where: C_0 (mg/mL) is the initial concentration of sorbate.

The Freundlich equation can be expressed as:

$$q_e = K_F \times C_e^{\frac{1}{n}} \quad (8)$$

where: K_F (mg/g) is the Freundlich constant, and 1/n is an empirical constant having the value of $0.1 < 1/n < 1$ or $1 < n < 10$.

Static desorption ratio at different ethanol concentrations

After the adsoprtion process, the D101 resin was washed by distilled water, then soaked in 50 mL of an ethanol solution at diferent concentrations, 30, 40, 50, 60, 70, 80, and 90% (*v/v*), for 2 h to reach the equilibrium desorption. The mixtures were then filtered and subjected to the composition analysis using the HPLC. The desorption ratio (D, %) was calculated according to the previous papers [Ma *et al.*, 2015; Tungmunnithum *et al.*, 2020] from the content of eluted daidzin, genistin, and total isoflavones.

Dynamic adsorption and desorption of isoflavones on the D101 resin-packed column

Dynamic adsorption test

Dynamic adsorption tests were evaluated by using a glass column (inner diameter of 3 cm, length of 50 cm) wet-packed with 100 g of D101 resins and with the bed volume (BV) of 200 mL. The stock extract solution (C_0=432.34 mg/mL) was gradually loaded on the resin column at a flow rate

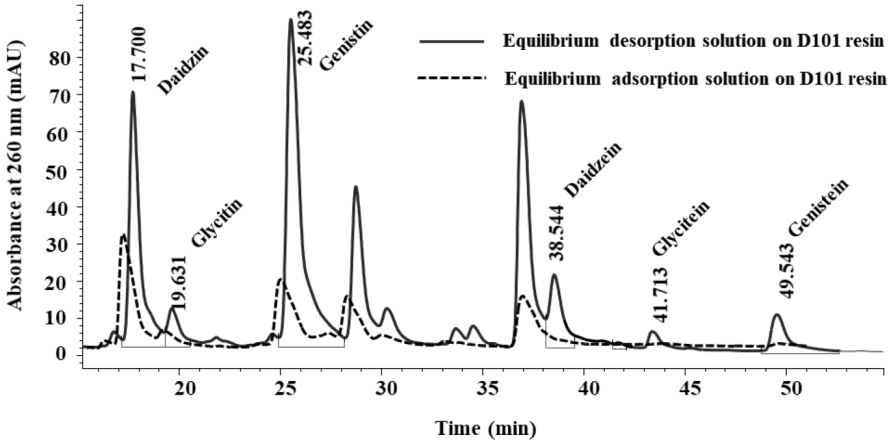

FIGURE 1. HPLC chromatograms of both equilibrium adsorption and equilibrium desorption solutions on the D101 macroporous resin.

of 1.5 BV/h. The effluents were taken at intervals of 50 mL to determine the concentrations of daidzin, genistin, and total isoflavones (C_e). These results were used to plot the adsorption breakthrough curves of daidzin, genistin, and total isoflavones. The adsorption capacity (q_e, mg/g) and the adsorption ratio (A, %) on the D101 resin-packed column were also calculated according to the previous papers [Li *et al.*, 2012; Liu *et al.*, 2010].

Dynamic desorption test

After attaining the adsorption equilibrium, the impurities in the resin column were removed by washing with double distilled water at a flow rate of 3 BV/h. The isoflavones were desorbed by 70% (*v/v*) ethanol solution at a flow rate of 1 BV/h. Eluted solution was collected at intervals of 50 mL until no isoflavones were left in the sample. The eluted sample was subjected to composition analysis to determine the concentrations of daidzin, genistin, and total isoflavones (C_d). These results were used to build up the desorption curves of isoflavones on the D101 resin packed column followed by the determination of desorption capacity (q_d, mg/g), desorption ratio (D, %), and recovery ratio (H, %) using the procedure described by Li *et al.* [2012] and Liu *et al.* [2010].

Statistical analysis

The results were analyzed by using a Minitab 18 software (Minitab, LLC, State College, PA, USA). Experimental values were expressed as mean ± standard deviation of three replicates. Analysis of variance (ANOVA) and Fishers least significant difference (LSD) test were utilized to compare the mean values with the significance level at $p<0.05$.

RESULTS AND DISCUSSION

Static adsorption and desorption characteristics of four macroporous resins

A suitable macroporous resin for the purification process of isoflavones was evaluated based on the adsorption and desorption performance. Adsorption capacity depicts the amount of isoflavones absorbed on resins, whereas the desorption capacity describes the amount of isoflavones desorbed from resins in the desorption solution, respectively [Gao *et al.*, 2018]. In this study, the HPLC results showed that all six isoflavones, including glycitin, daidzin, genistin, daidzein, glycitein, and genistein, were present in both equilibrium adsorption and desorption solutions on the four evaluated resins. That was, all macroporous resins exhibited the capacity in the adsorption and desorption of six isoflavones, indicating their applicability in the purification of isoflavones from the crude soybean extract. A typical chromatogram for the recognition of these isoflavones on the D101 resin is depicted in Figure 1. The peaks of isoflavones in the chromatography were consistent with a previous study of Tran *et al.* [2019]. As shown in Figure 1, the predominant compounds in the adsorption/desorption solution were daidzin and genistin, thus the total isoflavones and two of these dominant compounds were selected as the responses for the adsorption/desorption performances on the four resins.

The adsorption and desorption characteristics of total isoflavones, daidzin, and genistin on the four resins were distinct, as presented in Figure 2. The adsorption capacities of the total isoflavones, daidzin, and genistin were 4.89–5.32 mg/g, 1.31––1.45 mg/g, and 2.19–2.35 mg/g, respectively. The adsorption ratios of the resins were 76–82% for total isoflavones, 64–71% for daidzin, and 79–85% for genistin. The adsorption of phenolics on macroporous resins is a result of physical interaction via van der Waals forces, hydrogen bonds, or the π-π conjugation between phenolic compounds and benzene rings of macroporous resins [Gao *et al.*, 2018; Yang *et al.*, 2016]. It was found that the adsorption and desorption capacities were dependent on many noticeable variables such as surface area, pore diameters, and polarity of resins [Gao *et al.*, 2018; Sun *et al*., 2015; Tang *et al.*, 2018], or molecular weight of absorbed substances [Dong *et al.*, 2015]. The chemical constituents of macroporous resins were also considered a key factor influencing the adsorption and desorption characteristics [Fu *et al*., 2006]. In our study, the adsorption performances of D101 and Amberlite® XAD4 resins were considerably better than the others, and there was no significant difference in adsorption capacity between these two resins ($p\geq0.05$) (Figure 2a). This phenomenon could be due to the different

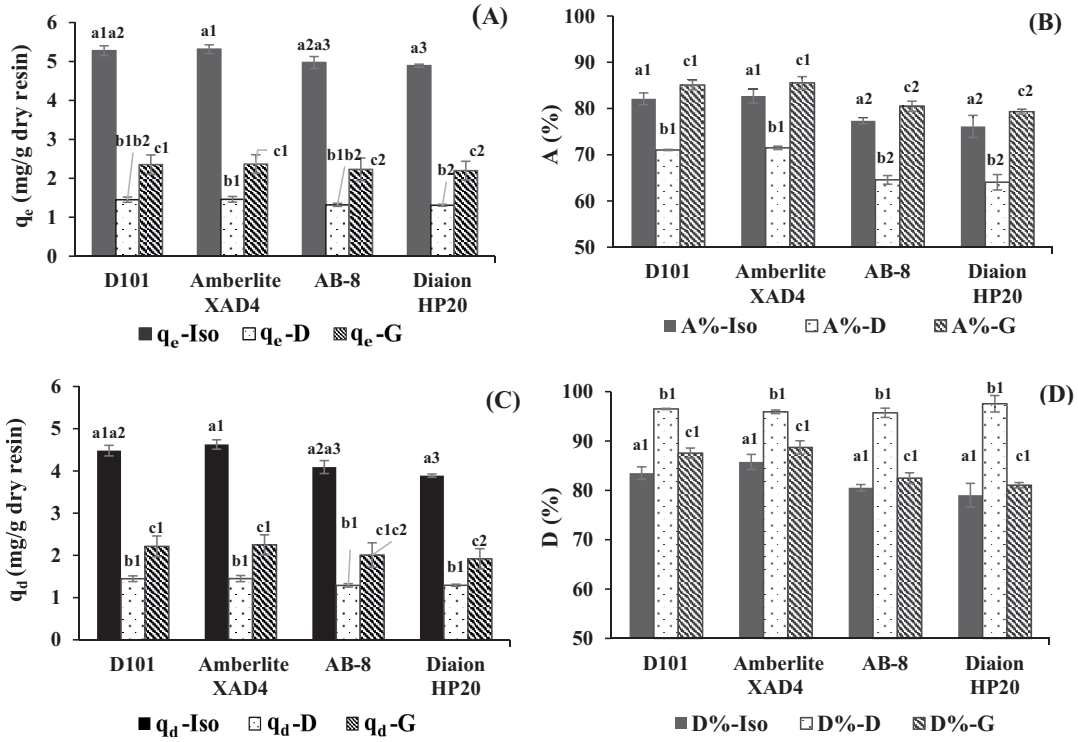

FIGURE 2. Adsorption capacity, q_e (A), adsorption ratio, A (B), desorption capacity, q_d (C), and desorption ratio D (D) of total isoflavones (-Iso), daidzin (-D), genistin (-G) on the four resins.

Error bars show standard deviations. Different numbers for the same letter above bars indicate significant differences between resins ($p<0.05$).

polarity of these resins. The D101 and Amberlite® XAD4 resins with non-polar property could better absorb non-polar isoflavones compared to AB-8 and HP-20, which are weak polar resins. This complied with the principle of "like dissolve like" indicating which non-polar compounds are easier absorbed by non-polar resins [Yan & Tang, 2003]. The desorption capacities of daidzin, genistin, and total isoflavones on D101 resin were 1.45 mg/g, 2.21 mg/g, and 4.48 mg/g, respectively, and there were insignifican differecest ($p\geq0.05$) between D101 and Amberlite® XAD4 resins (Figure 2c). The significantly ($p<0.05$) lower desorption capacity was determined on AB-8 and Diaion HP20 resins for total isoflavones. The desorption ratios of total isoflavone for four resins were from 79 to 86%. Interestingly, inversely to the adsorption ratios, the desorption ratios of daidzin (95–98%) were higher than those of genistin (81–88%).

Generally, both two non-polar resins (Amberlite® XAD4, D101) and two weak polar resins (Diaion HP20, AB-8) showed good adsorption and desorption characteristics of isoflavones from crude soybean extract and only slightly better parameters were found for the non-polar ones. Our results support previous report showing that Amberlite® XAD4 resin was efficient in purifying the major isoflavones from the defatted soy flakes [Wu & Lai, 2007]. However, compared to high-priced Amberlite® XAD4 resin, the D101 resin used in this study, which is cheaper, fairly possessed the same adsorption and desorption properties. Therefore, D101 resin could be a proper material for the purification process of isoflavones from crude soybean extract; therefore, subsequent experiments were conducted on the D101 resin.

Adsorption isotherms of isoflavones on the D101 resin

Adsorption isotherms are usually described by the Langmuir and Freundlich equations due to their simplicity and high accuracy [Jia & Lu, 2008; Yin *et al.*, 2010]. The adsorption isotherms of daidzin, genistin, and total isoflavones on the D101 resin at 25°C, described by the Langmuir and Freundlich models, are presented in Figure 3. The Langmuir model depicts the adsorption behavior of the mono-molecular layer, whereas the Freundlich model is applied to describe that of both mono-molecular layer and multi-molecular layer [Sun *et al.*, 2015]. The adsorption capacity of total isoflavones, daidzin, and genistin increased with increasing equilibrium concentration (C_e) (Figure 3). This was possible because the initial concentration served as a driving force to attenuate the resistance of mass transfer for the isoflavones movement from the crude extract to the D101 resin surface [Gao *et al.,* 2018]. The parameters of Langmuir and Freundlich isotherms at the temperature of 25°C are listed in Table 2. Both models showed a very good fit to the experimental data with the high coefficient of determinations (R^2). However, this coefficient for the Langmuir model was higher for daidzin, genistin, and total isoflavones ($R^2>0.98$) compared to the Freundlich model (0.862–0.951). Hence, the Langmuir model was found to better describe the adsorption capacity of isoflavones on the resin and this finding was in agreement with a study of Tang *et al.* [2018]. In other words, the adsorption behavior of soybean isoflavones on the D101 resin was based on the formation of a mono-molecular layer. The R_L values of Langmuir model, in this study, ranged from 0.019 to 0.155, indicating the favorable isotherms of isoflavones

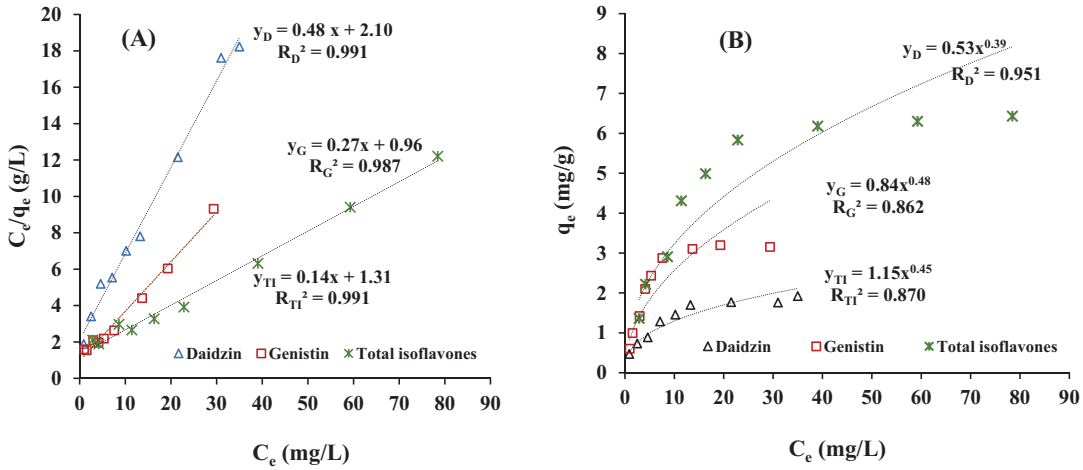

FIGURE 3. Langmuir (A) and Freundlich (B) adsorption isotherms of daidzin, genistin, and total isoflavones on the D101 resin at 25°C. C_e: equilibrium concentration of analytes in the adsorption solutions, q_e: adsorption capacity.

on the D101 resin. Previous studies also highlighted that R_L values in the range of 0–1 showed the favorable isotherms of analytes adsorbed on the macroporous resins [Duran *et al.*, 2011; Tang *et al.*, 2018]. In addition, the low values of R_L indicated the strong interaction between bioactive compounds and macroporous resins [Duran *et al.*, 2011]. Besides, the 1/n value in the range from 0.220 to 0.483 in the Freundlich model (Table 2) was adequate. The 1/n value from 0.1 to 0.5 indicates that it is easy to carry out the adsorption process but it is impossible when this value exceeds 1.0 [Jia & Lu, 2008; Liu *et al.*, 2010].

Static desorption of isoflavones on the D101 resin by different ethanol solution

The desorption process is the second step to purify the isoflavones. The proper ethanol concentration is vital to obtain better recovery of isoflavones. The effects of ethanol concentrations on the desorption ratio of total isoflavones, daidzin, and genistin on the D101 resin are presented in Figure 4. The effect of ethanol concentration on the desorption ratio was significant ($p<0.05$). The increment in ethanol concentration was found to increase the desorption ratio, which reached the maximal value at the ethanol concentration of 70% (*v/v*). Further increase in the ethanol concentration did not promote better desorption ratio. This finding was in agreement with previous studies [Guo *et al.*, 2015; Jia & Lu, 2008; Shazeli *et al.*, 2020; Tungmunnithum *et al.*, 2020] that most flavonoids were desorbed by aqueous-ethanol solution at the ethanol concentration range from 50% to 90% (*v/v*). In this context, the weak polarity of isoflavones facilitates their dissolution in ethanol [Tran *et al.*, 2019]. However, the increment in the ethanol concentration led to a reduction in the polarity of the desorption solution, and the polarity of ethanol at the concentration above 70% (*v/v*) was too low to effectively extract isoflavones from the resin. A similar problem was previously discussed when desorbing dioscin from *Dioscorea nipponica* on the D101 resin [Yin *et al.*, 2010]. Finally, the ethanol concentration of 70% (*v/v*) was considered a proper concentration to desorb isoflavones on the D101 resin in the studied conditions.

Purification of isoflavones on the D101 resin-packed column

Dynamic adsorption breakthrough curves for total isoflavones, daidzin, and genistin on the D101 resin are the function of the effluent volume and the concentration ratio (C_e/C_o) of isoflavones herein, as presented in Figure 5. Normally, the 10% ratio of solutes to the initial concentration of the feed in the effluent is defined as the breakthrough point or leak point. After reaching the leak point, the adsorption affinity reduces significantly, leading to solute leakage from the resins [Liu *et al.*, 2013; Tang *et al.*, 2018]. The breakthrough volume is defined as the volume of feed extract when

TABLE 2. The parameters of Langmuir and Freundlich adsorption isotherms of isoflavones on the D101 resin.

Compound	Langmuir model				Freundlich model		
	q_{max} (mg/g dry resin)	K_L L/mg)	R_L	R^2	1/n	K_F	R^2
Daidzin	2.121	0.224	0.032–0.154	0.991	0.390	0.523	0.951
Genistin	3.672	0.283	0.019–0.102	0.987	0.483	0.843	0.862
Total isoflavones	7.369	0.104	0.023–0.119	0.991	2.220	0.450	0.870

q_{max}, theoretical maximum adsorption capacity; K_L, Langmuir constant; R_L, separation factor; 1/n, empirical constant; K_F, Freundlich constant.

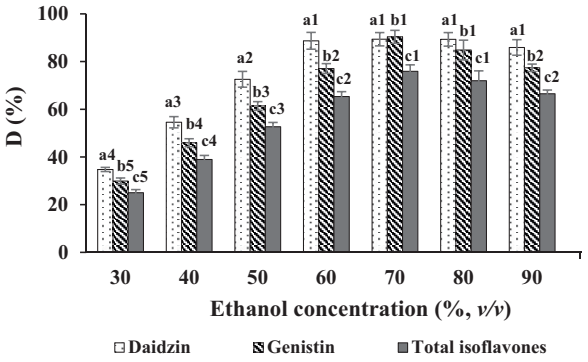

FIGURE 4. Effect of ethanol concentrations on the static desorption ratio (D) of total isoflavones, daidzin, and genistin on the D101 resin. Error bars show standard deviations. Different numbers for the same letter above bars indicate significant differences between resins ($p<0.05$).

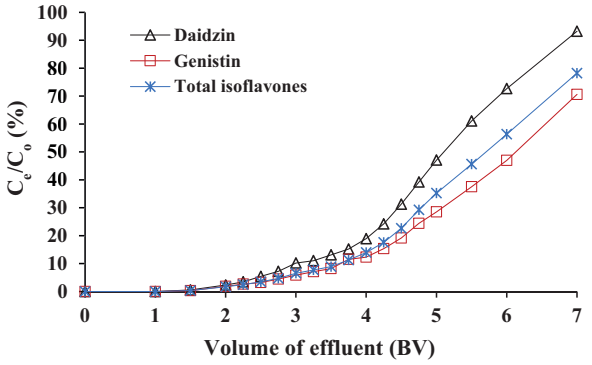

FIGURE 5. Dynamic adsorption breakthrough curves for total isoflavones, daidzin, and isoflavones on the D101 resin packed column.

C_0: concentration of isoflavones in stock extract solution, C_e: concentration of isoflavones in effluent.

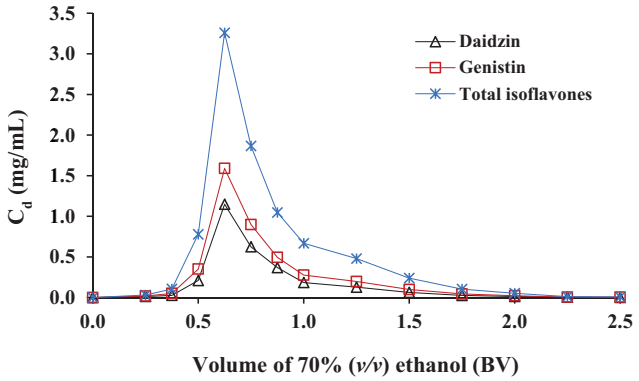

FIGURE 6. Dynamic desorption curves for total isoflavones, daidzin, and genistin on the D101 resin packed column.

C_d: concentration of isoflavones in the eluted solution.

the leak point is reached [Gao *et al.*, 2018]. Breakthrough volume helps determine when the sample volume can be preconcentrated to limit the loss of analytes [Liu *et al.*, 2013]. As shown in Figure 5, the leak points of daidzin and genistin were approximately 3.0 BV and 3.75 BV, respectively. The breakthrough point of total isoflavones was nearly the same as that

TABLE 3. Isoflavone contents in the crude soybean solid extract and purified isoflavone solid extract.

Compound	Crude soybean solid extract (mg/g)	Purified isoflavone solid extract (mg/g)	Purified/crude ratio of isoflavones
Daidzin	5.40±0.14^{b}	49.69±0.24^{b}	9.20±0.19
Glycitin	0.89±0.02^{e}	7.35±0.13^{d}	8.21±0.12
Genistin	8.01±0.02^{a}	68.51±0.36^{a}	8.56±0.24
Daidzein	1.18±0.10^{c}	11.16±0.43^{c}	9.47±0.37
Glycitein	0.10±0.01^{d}	0.47±0.01^{e}	7.94±0.12
Genistein	1.05±0.03^{c}	7.49±0.14^{d}	7.12±0.01
Total isoflavones	16.63±0.12	144.67±1.21	8.70±0.01

Different superscripts (a-e) indicate the significant differences between the analyzed compounds within the same column ($p<0.05$).

TABLE 4. Dynamic adsorption and desorption characteristics of isoflavones on the D101 resin packed column.

Compound	Dynamic adsorption		Dynamic desorption		
	q_e (mg/g)	A (%)	q_d (mg/g)	D (%)	H (%)
Daidzin	0.91±0.01	94±2	0.762±0.01	84±2	79±1
Genistin	1.40±0.02	98±1	1.11±0.01	79±1	77±1
Total isoflavones	2.95±0.02	97±2	2.39±0.02	81±1	78±2

q_e, adsorption capacity; A, adsorption ratio; q_d, desorption capacity; D, desorption ratio; H, recovery ratio.

of genistin due to the fact that the genistin content in the crude soybean extract was the highest, as shown in Table 3. Daidzin leaked with a lower volume compared to the others. This was possibly attributed to the discrepancies in polarity, initial concentrations, and adsorption rates [Tang *et al.*, 2018]. In summary, all isoflavones in the feed extract were mostly absorbed by the resin before 3.75 BV, thus it was selected as the feed volume at the fixed flow rate of 1.5 BV/h as it reached the maximal treating capacity of the resin. Parameters of dynamic adsorption of isoflavones on the D101 resin-packed column are presented in Table 4. The adsorption ratios for total isoflavones, daidzin, and genistin were high (>94%), confirming the suitability of D101 resin in the purification process of isoflavones.

The dynamic desorption curves of total isoflavones, daidzin, and genistin are illustrated in Figure 6. The concentration of isoflavones increased with the elution volume, reaching a maximum at the elution volume of 0.68 BV. Then, it decreased gradually. Similar behaviors were also discussed in many previous reports [Gao *et al.*, 2018; Li *et al.*, 2012; Wu & Lai, 2007]. The desorption process was completed when reaching the elution volume of 2.5 BV (Figure 6). The hydrogen bonds and hydrophobic interactions between the functional groups of phenolic compounds and the resin

are broken during their elution with ethanol [Gao *et al.*, 2018]. The dynamic desorption parameters are presented in Table 4. As a result, the desorption ratios (ranging from 79 to 84%) were fairly lower than the adsorption ratios. The recovery ratio of the adsorption/desorption process achieved a relatively high percentage, *i.e.* around 79%. Therefore, the desorption volume of 70% (*v/v*) ethanol as eluate was selected at 2.5 BV at the fixed flow rate of 1 BV/h.

The composition analysis results of the purified isoflavones solid extract compared to the crude soybean solid extract are shown in Table 3. The total isoflavone content in the purified isoflavones solid extract was 144.67 mg/g, being more than 8.70-fold higher than that in the crude soybean solid extract. The contents of daidzin and genistin increased by 9.20 and 8.56 times, respectively. The obtained results were possibly comparable with prior reports that the phenolic compound content was 3.5–11-fold higher than their initial extracts with the recovery yield of 70–95% [Gao *et al.*, 2018; Liu *et al.*, 2010; Liu *et al.*, 2013; Shazeli *et al.*, 2020; Sun *et al.*, 2015; Tang *et al.*, 2018]. For example, the flavonoids from the extract of Chinese wolfberry were concentrated from 0.58% to 10.77% when using D101 resin [Wu *et al.*, 2015]. The podophyllotoxin content in the extract from *Sinopodophyllum hexandrum* purified using D101 resin was 7.41-fold higher than that of the crude extract with a recovery ratio of 74.6% [Wang *et al.*, 2018].

CONCLUSION

Static adsorption and desorption characteristics of isoflavones from crude soybean extract on four resins were successfully evaluated. The D101 resin was the best resin to purify the isoflavones due to its high adsorption and desorption characteristics. Daidzin and genistin were the main isoflavones in the crude soybean extract. The experimental data of adsorption isotherms of total isoflavones, daidzin, and genistin showed better fit with the Langmuir model compared to the Freundlich model. The use of the D101 resin-packed column allowed an 8.70-fold increase in the content of total isoflavones in the extract with the recovery yield of nearly 80%. The result suggested the high feasibility of D101 resin in the purification process of isoflavones from the crude soybean extract in large-scale production. This result highlighted the practical aspect of using macroporous resins in the purification process so that the purified isoflavones could be easily applied to develop functional food products instead of using crude extract.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

ORCID IDs

Nguyen Thi Truc Loan https://orcid.org/0000-0002-0152-2981
Truong Dang Le https://orcid.org/0000-0003-0335-1140

REFERENCES

1. Almeida, I.M.C., Rodrigues, F., Sarmento, B., Alves, R.C., Oliveira, M.B.P.P. (2015). Isoflavones in food supplements: chemical profile, label accordance and permeability study in Caco-2 cells. *Food & Function, 6*(3), 938–946. https://doi.org/10.1039/C4FO01144A
2. Bennetau-Pelissero, C. (2013). *Isoflavonoids and Phytoestrogenic Activity*. *Natural Products*. Springer Berlin Heidelberg, Germany. pp. 2381–2416. https://doi.org/10.1007/978-3-642-22144-6_80
3. Bustamante-Rangel, M., Delgado-Zamarreño, M.M., Pérez-Martín, L., Rodríguez-Gonzalo, E., Domínguez-Álvarez, J. (2018). Analysis of isoflavones in foods. *Comprehensive Reviews in Food Science and Food Safety*, *17*(2), 391–411. https://doi.org/10.1111/1541-4337.12325
4. Choi, Y.B., Kim, K.S. (2007). Purification of isoflavone from soybean hypocotyls using various resins. *Korean Journal of Environmental Health*, *31*(3), 221–226.
5. Dong, Y., Zhao, M., Sun-Waterhouse, D., Zhuang, M., Chen, H., Feng, M., Lin, L. (2015). Absorption and desorption behaviour of the flavonoids from *Glycyrrhiza glabra* L. leaf on macroporous adsorption resins. *Food Chemistry*, *168*, 538–545. https://doi.org/10.1016/j.foodchem.2014.07.109
6. Duran, C., Ozdes, D., Gundogdu, A., Senturk, H.B. (2011). Kinetics and isotherm analysis of basic dyes adsorption onto almond shell (*Prunus dulcis*) as a low cost adsorbent. *Journal of Chemical and Engineering Data*, *56*(5), 2136–2147. https://doi.org/10.1021/je101204j
7. Fu, Y., Zu, Y., Liu, W., Efferth, T., Zhang, N., Liu, X., Kong, Y. (2006). Optimization of luteolin separation from pigeonpea [*Cajanus cajan* (L.) Millsp.] leaves by macroporous resins. *Journal of Chromatography A*, *1137*(2), 145–152. https://doi.org/10.1016/j.chroma.2006.08.067
8. Gao, C., Zhao, S., Yagiz, Y., Gu, L. (2018). Static, kinetic, and isotherm adsorption performances of macroporous adsorbent resins for recovery and enrichment of bioactive procyanidins from cranberry pomace. *Journal of Food Science*, *83*(5), 1249–1257. https://doi.org/10.1111/1750-3841.14142
9. Guo, H.D., Zhang, Q.F., Chen, J.G., Shangguang, X.C., Guo, Y.X. (2015). Large scale purification of puerarin from Puerariae Lobatae Radix through resins adsorption and acid hydrolysis. *Journal of Chromatography B, 980*, 8–15. https://doi.org/10.1016/j.jchromb.2014.12.014
10. Jia, G., Lu, X. (2008). Enrichment and purification of madecassoside and asiaticoside from *Centella asiatica* extracts with macroporous resins. *Journal of Chromatography A, 1193*(1–2), 136–141. https://doi.org/10.1016/j.chroma.2008.04.024

11. Kammerer, D.R., Kammerer, J., Carle, R. (2019). Adsorption and Ion Exchange for the Recovery and Fractionation of Polyphenols: Principles and Applications. Chapter 19, In *Polyphenols in Plants* (2nd ed.), Elsevier Inc., The Netherlands, pp. 327–339. https://doi.org/10.1016/B978-0-12-813768-0.00018-9
12. Kim, J., Yoon, M., Yang, H., Jo, J., Han, D., Jeon, Y.J., Cho, S. (2014). Enrichment and purification of marine polyphenol phlorotannins using macroporous adsorption resins. *Food Chemistry*, *162*, 135–142. https://doi.org/10.1016/j.foodchem.2014.04.035
13. Lee, C.H., Yang, L., Xu, J.Z., Yeung, S.Y.V., Huang, Y., Chen, Z.Y. (2005). Relative antioxidant activity of soybean isoflavones and their glycosides. *Food Chemistry*, *90*(4), 735–741. https://doi.org/10.1016/j.foodchem.2004.04.034
14. Li, H., Liu, J., Li, D., Wang, H. (2012). Study on separation and purification of genistein in the soybean residue using macroporous resin adsorption. *Industrial & Engineering Chemistry Research, 51*(1), 44–49. https://doi.org/10.1021/ie200057e
15. Li, H., Lin, L., Feng, Y., Zhao, M., Li, X., Zhu, Q., Xiao, Z. (2018). Enrichment of antioxidants from soy sauce using macroporous resin and identification of 4-ethylguaiacol, catechol, daidzein, and 4-ethylphenol as key small molecule antioxidants in soy sauce. *Food Chemistry, 240*, 885–892. https://doi.org/10.1016/j.foodchem.2017.08.001
16. Li, J., Chase, H.A. (2009). Use of expanded bed adsorption to purify flavonoids from *Ginkgo biloba* L. *Journal of Chromatography A, 1216*, 8759–8770. https://doi.org/10.1016/j.chroma.2009.03.002
17. Liu, J., Chang, S.K.C., Wiesenborn, D. (2005). Antioxidant properties of soybean isoflavone extract and tofu *in vitro* and *in vivo*. *Journal of Agricultural and Food Chemistry, 53*(6), 2333–2340. https://doi.org/10.1021/jf048552e
18. Liu, W., Zhang, S., Zu, Y.G., Fu, Y.J., Ma, W., Zhang, D.Y., Kong, Y., Li, X.J. (2010). Preliminary enrichment and separation of genistein and apigenin from extracts of pigeon pea roots by macroporous resins. *Bioresource Technology, 101*(12), 4667–4675. https://doi.org/10.1016/j.biortech.2010.01.058
19. Liu, Z., Wang, J., Gao, W., Man, S., Wang, Y., Liu, C. (2013). Preparative separation and purification of steroidal saponins in *Paris polyphylla* var. *yunnanensis* by macroporous adsorption resins. *Pharmaceutical Biology, 51*(7), 899–905. https://doi.org/10.3109/13880209.2013.770537
20. Ma, T., Sun, X., Tian, C., Luo, J., Zheng, C., Zhan, J., Ferreira, I.C.F.R., Turner, N.D. (2015). Enrichment and purification of polyphenol extract from *Sphallerocarpus gracilis* stems and leaves and *in vitro* evaluation of DNA damage-protective activity and inhibitory effects of α-amylase and α-glucosidase. *Molecules, 20*(12), 21442–21457. https://doi.org/10.3390/molecules201219780
21. Rostagno, M.A., Manchón, N., Guillamón, E., García-Lafuente, A., Villares, A., Martínez, J.A. (2010). Methods and techniques for the analysis of isoflavones in foods. In T.J. Quintin (Ed.), *Chromatography: Types, Techniques and Methods* (1st ed.), Nova Science Publishers, Inc., New York, USA, pp. 157–198.
22. Sevillano, D.M., Jankowiak, L., van Gaalen, T.L.T., van Wielen, L.A.M., Hooshyar, N., van der Goot, A.J., Ottens, M. (2014). Mechanism of isoflavone adsorption from okara extracts onto food-grade resins. *Industrial & Engineering Chemistry Research, 53*(39), 15245–15252. https://doi.org/10.1021/ie5026419
23. Shazeli, M., Zain, C., Lee, S.Y., Teo, C.Y., Shaari, K. (2020). Adsorption and desorption properties of total flavonoids from oil palm (*Elaeis guineensis jacq.)* mature leaf on macroporous adsorption resins. *Molecules, 25*(4), art. no. 778. https://doi.org/10.3390/molecules25040778
24. Shim, J., Kim, Y.J., Lee, H.S. (2008). Effects of soybean isoflavone extract on the plasma lipid profiles and antioxidant enzyme activity in streptozotocin-induced diabetic rats. *Nutrition Research and Practice, 2*(4), 218–226. https://doi.org/10.4162/nrp.2008.2.4.218
25. Soto, M.L., Moure, A., Domínguez, H., Parajó, J.C. (2011). Recovery, concentration and purification of phenolic compounds by adsorption: A review. *Journal of Food Engineering, 105*(1), 1–27. https://doi.org/10.1016/j.jfoodeng.2011.02.010
26. Sun, P.C., Liu, Y., Yi, Y.T., Li, H.J., Fan, P., Xia, C.H. (2015). Preliminary enrichment and separation of chlorogenic acid from *Helianthus tuberosus* L. leaves extract by macroporous resins. *Food Chemistry, 168*, 55–62. https://doi.org/10.1016/j.foodchem.2014.07.038
27. Tang, D., Zhu, J.X., Nie, H., He, B., Xu, Y.H., Zhu, Q. (2018). Simple and efficient approach for enrichment of major isoflavonoids from *Astragalus membranaceus* with macroporous resins and their nephroprotective activities. *Industrial Crops and Products, 125*, 276–283. https://doi.org/10.1016/j.indcrop.2018.08.023
28. Tran, T.N.T., Chew, K.W., Bui, X.V., Nguyen, T.D.P., Le, T.T.A., Truong, T.M.H., Show, P.L. (2019). Optimization of isoflavones extraction from soybeans using full factorial design. *Journal of Food Processing and Preservation, 43*(9), art. no. e14078. https://doi.org/10.1111/jfpp.14078
29. Tungmunnithum, D., Drouet, S., Kabra, A., Hano, C. (2020). Enrichment in antioxidant flavonoids of stamen extracts from *Nymphaea lotus* L. using ultrasonic-assisted extraction and macroporous resin adsorption. *Antioxidants, 9*(7), art. no. 576. https://doi.org/10.3390/antiox9070576
30. Uifălean, A., Farcaş, A., Ilieş, M., Hegheş, S.C., Ionescu, C., Iuga, C.A. (2015). Assessment of isoflavone aglycones variability in soy food supplements using a validated HPLC-UV method. *Clujul Medica, 88*(3), 373–380. https://doi.org/10.15386/cjmed-468
31. Wang, Y., Zhang, G., Chi, X., Chen, S. (2018). Green and efficient extraction of podophyllotoxin from *Sinopodophyllum hexandrum* by optimized subcritical water extraction combined with macroporous resin enrichment. *Industrial Crops and Products*, *121*, 267–276. https://doi.org/10.1016/j.indcrop.2018.05.024
32. Wu, C., Lai, S. (2007). Preparative isolation of isoflavones from defatted soy flakes. *Journal of Liquid Chromatography & Related Technologies, 30*(11), 1617–1640. https://doi.org/10.1080/10826070701224796
33. Wu, S., Wang, Y., Gong, G., Li, F., Ren, H., Liu, Y. (2015). Adsorption and desorption properties of macroporous resins for flavonoids from the extract of Chinese wolfberry (*Lycium barbarum* L.). *Food and Bioproducts Processing, 93*, 148–155. https://doi.org/10.1016/j.fbp.2013.12.006

34. Yan, B., Tang, Q. (2003). Comparison of kinetics of organic reactions carried out on resin beads of different diameters. *Industrial & Engineering Chemistry Research*, *42*(24), 5964–5967. https://doi.org/10.1021/ie030149u
35. Yang, Q., Zhao, M., Lin, L. (2016). Adsorption and desorption characteristics of adlay bran free phenolics on macroporous resins. *Food Chemistry, 194*, 900–907. https://doi.org/10.1016/j.foodchem.2015.08.070
36. Yin, L., Xu, Y., Qi, Y., Han, X., Xu, L., Peng, J., Sun, C.K. (2010). A green and efficient protocol for industrial-scale preparation of dioscin from *Dioscorea nipponica* Makino by two-step macroporous resin column chromatography. *Chemical Engineering Journal, 165(*1), 281–289. https://doi.org/10.1016/j.cej.2010.09.045

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Original article
Section: Food Quality and Functionality

Effect of Water Treatment and Immersion in Calcium Salt Solutions on the Quality of Fruits of Peumo Pink Tomato (*Solanum lycopersicum* L.) Stored under Cold Conditions

*Julio Correa[1], Asunción Amorós[2], Herman Silva[3,4], Jose I. Covarrubias[4], Víctor H. Escalona[1,4]**

[1]Centro de Estudios Postcosecha (CEPOC), Facultad de Ciencias Agronómicas, Universidad de Chile, 8820808, Av. Santa Rosa 11315, La Pintana, Santiago, Chile
[2]Centro de Investigación e Innovación Agroalimentario y Agroambiental (CIAGRO-UMH), Universidad Miguel Hernández. Ctra. Beniel, Km 3.2, 03312, Orihuela, Alicante, Spain
[3]Laboratorio de Genómica, Funcional & Bioinformática, Facultad de Ciencias Agronómicas, Universidad de Chile, 8820808, Av. Santa Rosa 11315, La Pintana, Santiago, Chile
[4]Departamento de Producción Agrícola, Facultad de Ciencias Agronómicas, Universidad de Chile, 8820808, Av. Santa Rosa 11315, La Pintana, Santiago, Chile

Key words: calcium chloride, calcium lactate, calcium propionate, postharvest, heat treatment, heat shock protein

Tomato is one of the most consumed vegetable crops worldwide. In the break stage, it is perishable, and it has a postharvest life that does not exceed three weeks at 12 to 15°C. The aim of this study was to evaluate the effect of immersion in water and in calcium salt solutions on the metabolism and quality of tomato of traditional Peumo Pink cultivar stored for 28 days at 10°C plus a simulated trading period of 2 days at 20°C. Fruits were treated in water at 5, 45 and 60°C at two immersion times of 1 and 4 min or in 2% solutions of calcium chloride, lactate and propionate at 10 and 45°C for 4 min. The respiration rate, ethylene production and heat shock protein gene expression as well as firmness, total phenolic content and antioxidant capacity of tomato were determined. Dipping in water at 60°C reduced the loss of firmness and respiratory rate of tomatoes up to 21 days at 10°C + 2 days at 20°C. Treatment in water at 45°C for 4 min and at 60°C for 1 or 4 min stimulated heat shock protein gene expression. However, fruit treated at 60°C for 1 or 4 min showed uneven ripening; hence, the immersion in water at 45°C for 4 min was the most recommended to extend the postharvest life of tomatoes. In turn, the immersion in calcium lactate and propionate solutions at 45°C increased total calcium content and maintained firmness after 28 days at 10°C + 2 days at 20°C. The use of high temperature and calcium salts for dipping would be beneficial to provide the vegetal tissues with calcium and reduce the softening of the tomato after prolonged cold storage.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a vegetable of Western South and Central America origin. In 2018, its global production was 182 million tons, and it is grown annually on 4.76 million hectares. The main world producers are: China, the European Union, India, the United States, and Turkey [FAOSTAT, 2020]. One of the ancestral tomato cultivars still grown by small farmers in the central area of Chile is the Peumo Pink tomato (Spanish: Rosado de Peumo). It is a non-genetically modified cultivar, characterized by fruits with a bright pink color, juicy flesh and large size (500 to 600 g per fruit).

In general, tomato is classified as a climacteric fruit and shows a rise in the respiratory rate (40 mg CO_2/kg×h at 25°C) and ethylene production (10 μL C_2H_4/kg×h at 25°C) during ripening [Li *et al.,* 2020]. These metabolic processes cause physicochemical effects after harvesting and affect color, texture and taste, generating significant losses associated mainly with deteriorated sensory and microbiological quality [Sun *et al.,* 2015]. The recommendation for a tomato is to store it for two to four weeks at temperatures between 12 and 15°C and 90% relative humidity (RH) to avoid chilling injury symptoms, particularly for immature fruits [Polenta *et al.,* 2015]. In the case of Peumo Pink tomatoes, no formal postharvest studies have been reported to date. However, preliminary experiments carried out at our laboratory demonstrated a very short shelf life of 5 to 7 days at 18°C and sensitivity to chilling injury at temperatures below 10°C [Escalona, data unpublished].

There are many reports pointing to heat treatment as an effective way to reduce decay and chilling injury of sensitive fruits like tomatoes [Aguayo *et al.*, 2008; 2015]. However, the optimal combination of temperature and exposure time to heating for each species must be defined in order to ensure

* Corresponding Author:
E-mail: vescalona@uchile.cl (V.H. Escalona)

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the benefits of the method [Wang, 1998]. Hot water treatment is cheap and easily applied on a small commercial scale, and is a good solution for small growers lacking access to advanced technology. Postharvest calcium salt solution dips are another way to extend the shelf-life of the sensitive fruits. Immersion in different calcium salts, such as chloride, lactate, citrate, ascorbate, propionate and silicate, has been used before fruit storage at low temperatures [Aguayo *et al.,* 2008]. The effects of such treatments included a reduction of chilling injury or mechanical damage during cold storage [Serrano *et al*., 2004], a reduction of firmness loss and resistance to attack by rotting [Lara *et al.,* 2004; Silveira *et al.,* 2011b]. Short-time calcium dips (1–3 min) were recommended for application in combination with high temperature (40–60°C) treatments [Valero & Serrano, 2010]. On the other hand, it is not clear whether thermal shock and calcium salt treatments affect the total phenolic content and the antioxidant capacity of tomatoes, although they seem to decrease with storage time [Aguayo *et al.*, 2015].

Heat causes changes in gene expression and protein synthesis during fruit ripening. These changes cause the inhibition of ethylene synthesis and the action of the cell wall-degrading enzymes [Paull & Chen, 2000]. Heat shock proteins (HSPs) are a group of conserved proteins that function as molecular chaperones and accumulate under heat shock. Previous studies have suggested that HSPs play a pivotal role in heat shock response [Xu *et al*., 2020]. Specifically, accumulation of heat shock proteins has been found in chilling-injured pepper, cucumber, orange, lime and lemon [Valero & Serrano, 2000].

Therefore, the objective of this study was to apply different calcium salts at different temperatures for the treatment of Peumo Pink tomatoes in order to extend their storability at chilling temperature by reducing the metabolic activity, softening and decay of the fruit for more than three weeks. This study was divided into two experiments: in the first, water treatments at different temperatures were carried out on the tomatoes to verify which temperature and exposure time ensured the best fruit quality, which were then applied in calcium salt treatments in the second experiment. The novelty of this study was the combination of heat treatment with calcium salts being able to extend the shelf life of traditional and perishable Chilean 'Peumo Pink' tomatoes.

MATERIALS AND METHODS

Plant material, treatments and experimental design

The Peumo Pink tomato plants were grown in an open field in Pichidegua at 34°S 37' and 71°W 34' in the Libertador Bernardo O'Higgins Region, Chile. In February, 6 to 8 kg of fruit were harvested per plant and placed in clean plastic boxes. Those 11–14 cm in size and whose skin was green breaking to pink were selected (30% in color pink). The fruits were transported immediately to the laboratory (within 2 h) in the Centro de Estudios Postcosecha (CEPOC), Facultad de Ciencias Agronómicas, Universidad de Chile in Santiago de Chile, Chile. Each fruit was cleaned with a damp cloth to remove field dust and dirt. Damaged tomatoes were disposed of and those with a green-pink external color (lightness: 51.6±5.1; chroma: 31.0±3.7; hue angle: 93.9±6.0) and with firmness of 16.9±1.1 N were selected and stored in a cold room at 15°C until the application of treatments the next day.

The fruits were sanitized by immersing them for 2 min in a solution of 150 mg/L of sodium hypochlorite at 10°C and pH 6.5 to eliminate dirt and possible pathogenic microorganisms. About 10 L of sanitized solution was used for every 3 or 4 fruits. The fruits were placed on stainless steel meshes to remove excess water. For water treatments (Experiment 1), fruits were immersed in water at 5, 45 and 60°C for 1 and 4 min. To this end, 60 L of water at the different temperatures were used for every 5 fruits. The water was placed in an 80 L stainless container and heated on an industrial stove. The water temperatures were monitored permanently at different levels by digital thermometers and only a few fruits were treated at the same time to avoid changes in this parameter during the treatments. After the immersion, fruits were placed on stainless steel meshes and each fruit was dried individually with blotting paper. Then, the fruits were weighed on a precision electronic balance (EMB3000–1, Kern, Balingen, Germany) and individually packaged in 20×25 cm polyethylene (PE) bags with a thickness of 0.04 mm and 10 perforations of 1 mm^2 to get a high humidity and air atmosphere. The bags were heat sealed with a manual sealer (FR400, Plastic Film Sealer, Zhejiang, China) and placed on trays in a cold room at 10°C and 85 to 90% RH.

In Experiment 2, the fruits were immersed in calcium salt solutions at 10 and 45°C for 4 min. Calcium chloride, calcium lactate and calcium propionate were dissolved in tap water in concertation of 2% (*w/v*). Tomatoes immersed in tap water at 10°C for 4 min (without calcium salt) were used as a control. The fruits were placed on stainless steel meshes as described for Experiment 1 and packed in the same perforated bags.

The storage time for both experiments was 28 days at 10°C and 85–90% RH plus 2 days at 20°C and 70% RH (simulated commercial conditions). Fruits were taken for analyses after each period of 7, 14, 21 and 28 days (Experiment 1) or 14, 21 and 28 days (Experiment 2) at 10°C followed by 2 days at 20°C (storage period codes: 7+2; 14+2; 21+2; 28+2, respectively). Fifteen bagged tomatoes for both experiments were stored for each period and treatment.

Determinations made in Experiment 1

Respiratory rate and ethylene production

Determinations of respiratory rate and ethylene production were carried out for the treated tomatoes stored at 10°C and for the treated and non-stored fruits. Each fruit was placed in a 500 mL hermetically-sealed plastic jar for 1 h at 10°C. Gas samples were taken from the headspace by a portable gas analyzer (PBI Dansensor, Check Point, Ringsted, Denmark) to determine the concentration of CO_2 and O_2. The values were expressed in mg of CO_2/kg×h [Silveira *et al*., 2017]. An aliquot of 1 mL of a gas sample was also injected into a gas chromatograph (6890 N GC System, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector with a Porapak QN 80/100 column (1.2 m × 3.18 mm) (Supelco, Norwalk, CT, USA). The injector, oven and detector temperatures were 200, 50 and 200°C,

respectively. Helium gas was used as the carrier gas with a flow rate of 55 mL/min. The GC was calibrated daily with a standard gas of 0.5 μL/L (Indura, Santiago, Chile). The ethylene production was expressed in μL C_2H_4/kg×h. Five fruits were used as repetitions for each treatment.

Firmness measurement

A texture analyzer was used to measure fruit firmness (TA. XT express, Stable Micro Systems, Godalming, UK) by a 50 N load cell and a 1.27 cm diameter probe. The penetration test was performed at 3 mm/s speed and 15 mm penetration on three equidistant points of the equatorial zone (after the peel was removed). The results were expressed in Newtons (N). At the beginning of the storage, five individual untreated fruits were used to obtain an initial value. After the storage, five fruits after each treatment were analyzed and three analytical repetitions were performed for each sample (n=15).

Heat shock protein gene expression

Five whole tomatoes after treatments at 5, 45 and 60°C for 1 and 4 min and another five tomatoes not immersed in water as a control were taken for analysis. The fruits were cut into wedges, then frozen in liquid nitrogen and stored in an ultra--low temperature freezer (MDF-U33V, Sanyo, Osaka, Japan) at -80°C until analysis.

The method proposed by Polenta *et al*. [2015] was used with slight modifications for RNA extraction. A piece of frozen tomato with peel was ground in a mortar. Next, 0.1 g of ground sample was weighed and placed in a 1.5 mL Eppendorf tube, then 1 mL of a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added. The solution was left for 10 min at room temperature, then 200 μL of chloroform was added, and the mixture was stirred manually for 20 s and left for 15 min at room temperature. Subsequently, the sample was centrifuged at 12,000×g for 15 min at 4°C (Heraeus Megafuge 16R, Thermo Fisher Scientific, Waltham, MA, USA). The sample was separated into a red lower phase with phenol-chloroform, an interface and an aqueous upper phase containing RNA. The aqueous phase was transferred to a new 1.5 mL Eppendorf tube, to which 500 μL of 100% isopropanol was added, and the sample was left for 15 min at room temperature. Subsequently, it was centrifuged at 12,000×g for 10 min at 4°C and then the supernatant was removed. The RNA pellet was washed in 1 mL of 75% (v/v) ethanol and stirred for 15 s. Then, it was centrifuged at 12,000×g for 5 min at 4°C, the supernatant was discarded, and the RNA pellet was dried for 5 min. Afterward, 30 μL of RNase-free water (DEPC water) was added and finally incubated at 55°C for 10 min in a thermal water bath. The quality of the extracted RNA was determined according to the method proposed by Meisel *et al.* [2005]. Two μg were first loaded on an agarose gel in an electrophoresis chamber and the presence of the band was verified, and then 1 μg was loaded and the A_{260}/A_{280} ratio was calculated using a microplate spectrophotometer (Epoch, BioTek Instruments Inc., Winooski, VT, USA).

After RNA extraction, genomic DNA was removed from the samples to perform reverse transcription. To this end, 1 μg of the sample was taken and mixed with 1 μL of 10× reaction buffer $MgCl_2$, 1 μL of DNase I free of RNase and 7 μL of DEPC water. Samples were incubated for 30 min at 37°C in a thermal cycler (Multigene Gradient Labnet, Edison, NY, USA). After a completed cycle, 1 μL of 50 mM ethylenediaminetetraacetic acid (EDTA) was added, and the sample was incubated for 10 min at 65°C. Subsequently, 1 μL of first oligo(dT), 1 μL of random first, 4 μL of 5× reaction buffer, 1 μL of RiboLock RNase inhibitor (20 U/μL), 2 μL of 10 mM dNTP Mix and 1 μL of RevertAid M-MuLVRT (200 U/μL) were added. The sample was immediately incubated for 5 min at 25°C, followed by a cycle of 60 min at 42°C and ended at 70°C for 5 min cycle.

After reverse transcription, the gene expression was assayed for different heat stress proteins. Primers that amplify the actin gene were used to check the effectiveness of reverse transcription and the presence of cDNA. For the polymerase chain reaction (PCR) amplification, 1 μg of cDNA was mixed with 1.5 μL of 50 mM $MgCl_2$; 1 μL of 10 mM dNTP Mix; 5 μL of 10X PCR buffer; 1.5 μL of first forward; 1.5 μL of first reverse; 14 μL of DEPC water and 0.5 μL of Platinum Taq DNA Polymerase (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). The PCR was used to amplify the gene encoding for HSP21 and HSP70 proteins (Table 1). Finally, 6 μL of the PCR reaction product were loaded on 1% agarose gel to verify the presence or absence of the expressed gene.

Determinations made in Experiment 2

Each analysis was performed for the treated and stored tomatoes and, for five individual untreated fruits (at the beginning of the storage) to obtain an initial value.

Firmness measurement

The firmness of fruits was measured using a texture analyzer as described before. The results were expressed in N. Five fruits for each treatment were analyzed in triplicate (n=15).

Total calcium content

The method described by Carvajal *et al.* [1999] was used to determine the total calcium content with slight modifications. The fruits were lyophilized in a freeze-dryer (FD5508, IlShin BioBase, South Korea). A portion of 1 g of lyophilized tomato from three fruits was placed in a 200 mL glass flask, then acid digestion was performed by adding 6 mL of nitric acid (65%) and 4 mL of hydrogen peroxide (33%). Samples were placed in an orbital shaker (KS Model 125, IKA, Staufen im Breisgau, Germany) for 15 h. Then, they were autoclaved at 125°C and 147.1 kPa for 70 min, cooled and filtered

TABLE 1. PCR steps used to amplify gene expression for heat shock HSP21 and HSP70 proteins.

Steps	Temperature (°C)	Time (s)	Number of cycles
Initial denaturation	94	120	1
Denaturation	94	30	
Hybridization	54	30	40
Elongation	72	45	

through a 0.22 μm nylon syringe. The calcium content was determined by using microwave plasma atomic emission spectroscopy (MP-AES 4200, Agilent Technologies, USA). Calcium calibration standards were prepared in concentrations of 5, 10, 25, 50 and 75 mg/L in 1% HNO_3 (*v/v*) medium. Calcium content was quantified by measuring peak area at 396.847 mm emission wavelength. The total calcium content was expressed in mg Ca/100 g fresh weight (FW) of fruits.

Extract preparation for determination of total phenolic content and antioxidant capacity

The extracts were obtained from lyophilized (as mentioned above) tomatoes. An aliquot of 10 mL of 70% (*v/v*) methanol was added to 2 g of the lyophilizate following the adapted method of Swain & Hills [1959], and the resulting suspension was homogenized in an Ultra-Turrax disperser (T18 basic, IKA, USA) for approximately 30 s until a uniform consistency was obtained. Then, the sample was centrifuged (Z326K centrifuge, Hermle Labortechnik, Wehingen, Germany) for 15 min at 6,037×*g*. The supernatant was filtered through a 0.45 μm polyvinylidene fluoride (PVDF) filter and the extract was stored for one week at –20°C until the analysis.

Determination of total phenolic content

Total phenolic content (TPC) was measured using the method proposed by Singleton & Rossi [1965]. In a 2 mL Eppendorf tube, 200 μL of the Folin-Ciocalteu reagent were added to 100 μL of the extract, which was left for 5 min. Then, 800 μL of Na_2CO_3 solution were added and after 1 h of reaction time, the tube was centrifuged (Z326K centrifuge, Hermle Labortechnik, Germany) for 2 min at 6,037×*g*. The absorbance of supernatant was read at 765 nm using a UVM340 micro-plate reader (Biochrom Asys, Cambridge, United Kingdom). The total phenolic content was calculated through a calibration curve with gallic acid. The results were expressed as μg of gallic acid equivalents (GAE) per g FW of tomatoes.

Determination of antioxidant capacity

DPPH radical scavenging activity was determined using the method proposed by Brand–Williams *et al*. [1995]. In an Eppendorf tube, 250 μL of the extract and 1 mL of the DPPH radical solution (0.2·mM) were mixed. After 30 min, 200 μL of the sample was transferred to a 96-well plate to measure the absorbance at 517 nm in a spectrophotometer micro-plate reader (UVM340, Biochrom Asys). The results were calculated based on a Trolox calibration curve [Silveira *et al*., 2017] and expressed as μg Trolox equivalents (TE) per g FW of tomatoes. Ferric reducing antioxidant power (FRAP) was determined following the method proposed by Benzie & Strain [1996]. The FRAP reagent was prepared by adding 300 mM acetate buffer (pH 3.5), 200 mM ferric chloride hexahydrate solution to 10 mM 2,4,6-tripyridyl-*s*-triazine in 40 mM HCl. Subsequently, 20 μL of the extract and 600 μL of the FRAP reagent were added in a 96-well plate and measured every 30 min for 120 min at 593 nm in a microplate reader (UVM340, Biochrom Asys). The FRAP was calculated by a Trolox calibration curve. The results were expressed as μg Trolox equivalents (TE) per g FW of fruits.

Statistical analysis

The results were expressed as mean ± standard error. After each storage evaluation period, one-way analysis of variance (one-way ANOVA) was performed for data of both experiments with InfoStat software [Di Rienzo *et al*., 2013]. In the case of finding statistically significant differences between treatments, Tukey's multiple comparisons test was used. All analyses were performed at 5% significance.

RESULTS AND DISCUSSION

Experiment 1

Respiratory rate and ethylene production

Water temperature (5, 45 and 60°C) and immersion time (1 and 4 min) were observed to elicit significant effects on the respiratory rate of tomatoes before storage (Figure 1). The highest values were reached for the treatment at 60°C for 4 min (31.6 mg CO_2/kg×h). At the same time, fruits treated at 5°C for 1 or 4 min had the lowest values of 18.9 to 19.1 mg CO_2/kg×h, respectively. Compared to the immersion at 5°C, at temperatures of 45 and 60°C the respiratory rates

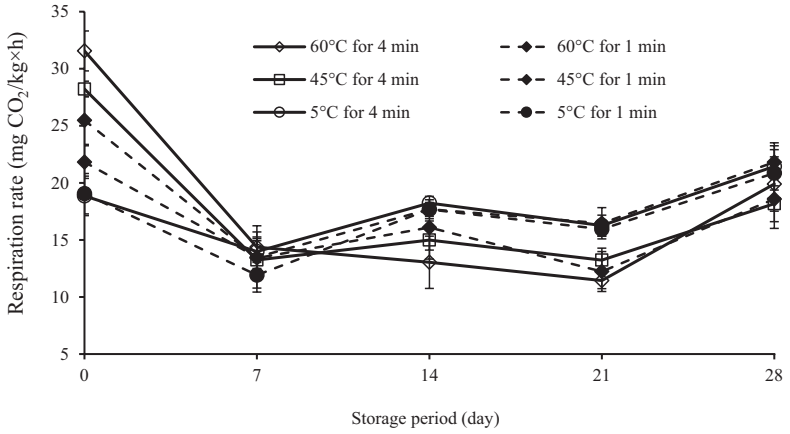

FIGURE 1. Respiratory rate of Peumo Pink tomato fruits treated in water at different temperatures (5, 45 and 60°C) and immersion times (1 and 4 min), and stored for 28 days at 10°C. The values are the mean ± standard error (n=5).

were higher, particularly when exposure time increased from 1 to 4 min. These increased values for the treatments at high temperatures and longer exposure times could be due to heat stress affecting the fruit. This behavior has also been observed in other heat-treated fruits such as mango, melon and pears immersed at 46°C for 75 min, 60°C for 1 min and 45°C for 6 min, respectively [Dea *et al.,* 2010; Silveira *et al.,* 2011a].

After 7 days of cold storage at 10°C, respiratory rates of tomatoes decreased for all treatments (Figure 1) because low temperatures retarded metabolism, as evidenced by reduced respiratory rate and ethylene production values [Oms-Oliu *et al.,* 2008; Silveira *et al.,* 2011a]. After 14 and 21 days of storage at 10°C, a lower respiration rate was found for tomatoes treated in water at 45°C and 60°C than those treated at 5°C (Figure 1); however, no clear differences were found between 1 and 4 min of exposure time. These results are consistent with those obtained by Silveira *et al*. [2011b], who stored cut melon at 5°C for 10 days after dipping in 2% peracetic acid solutions for 1.0, 1.5 and 2.0 min at 5 and 60°C, and found that the respiration rates of all samples treated at 60°C and stored for 3 or more days were lower than those of the samples dipped at 5°C.

In the same storage conditions, ethylene production showed no significant differences among treatments on days 0 and 7 with values between 1.0 to 1.5 μL C_2H_4/kg×h (Figure 2). On day 14, the ethylene production by tomatoes treated at 5°C for 1 min was higher (2.0 μL C_2H_4/kg×h) compared to those immersed at 45°C for 4 min (1.2 μL C_2H_4/kg×h). On day 21, the values for all treatments increased, and ethylene production by fruits after heat treatments, particularly at 60°C for 4 min, was lower compared to the tomatoes treated at 5°C for 1 min. After 28 days no differences were found among treatments (3.4 to 4.5 μL C_2H_4/kg×h).

Firmness

Firmness of tomatoes after immersion in water at different temperatures (5, 45 and 60°C) and times (1 and 4 min) and cold-storage are shown in Figure 3. At the beginning of the storage, the firmness of untreated tomatoes was 16.9±1.09 N. Fruits treated at 45 and 60°C for 4 min maintained the highest firmness on days 14+2 and 21+2, which could be a direct effect of heat stress from hot water immersion. In general, firmness of fruits treated at 5°C for 1 or 4 min was similar to those immersed at 45 and 60°C for 1 min.

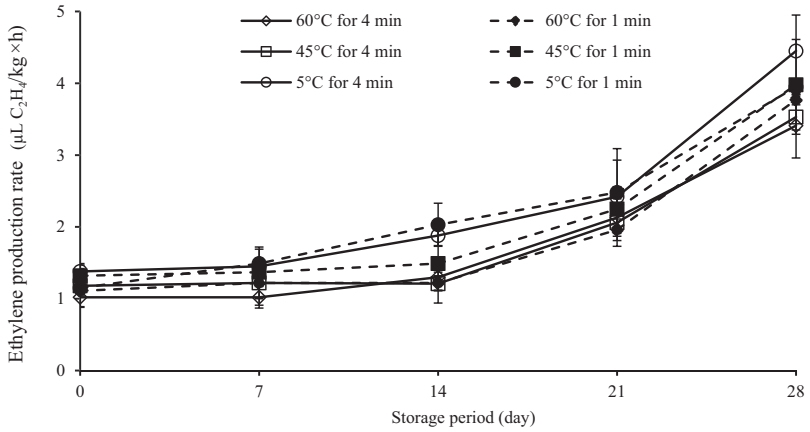

FIGURE 2. Ethylene production rate of Peumo Pink tomato fruits treated in water at different temperatures (5, 45 and 60°C) and immersion times (1 and 4 min), and stored for 28 days at 10°C. The values are the mean ± standard error (n=5).

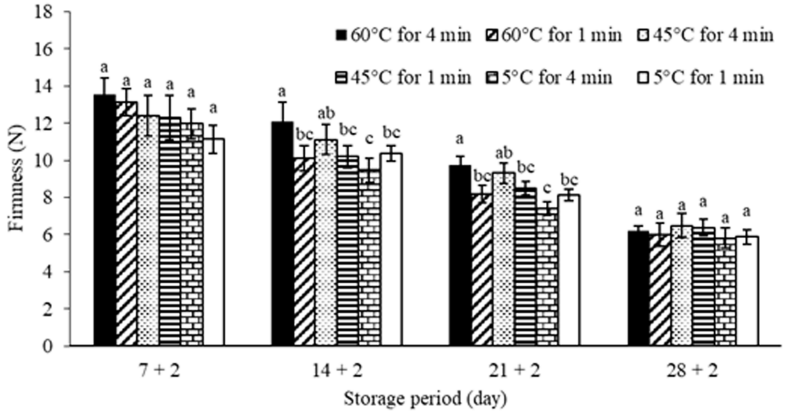

FIGURE 3. Firmness of Peumo Pink tomato fruits treated in water at different temperatures (5, 45 and 60°C) and immersion times (1 and 4 min), and stored for 28 days at 10°C + 2 days at 20°C. The values are the mean ± standard error (n=15). Different letters above bars separately for each storage period indicate significant differences (p<0.05).

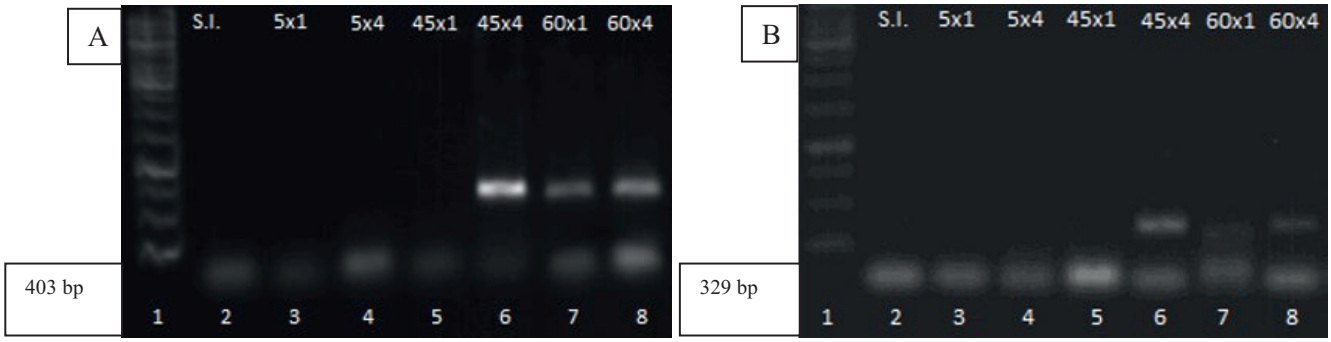

FIGURE 4. PCR for gene expression of heat shock proteins HSP21 (A) and HSP70 (B) in Peumo Pink tomato fruits treated in water at different temperatures (5, 45 and 60°C) and immersion times (1 and 4 min). Lane 1 corresponds to a molecular weight standard GeneRuler 1kb DNA Ladder; lane 2: no immersion (SI); lane 3: 5°C for 1 min (5×1); lane 4: 5°C for 4 min (5×4); lane 5: 45°C for 1 min (45×1); lane 6: 45°C for 4 min (45×4); lane 7: 60°C for 1 min (60×1) and lane 8: 60°C for 4 min (60×4). Samples were run in 1% agarose gel. bp: base pairs.

These responses could indicate that more than 1 min of exposure time is required at temperatures between 45 to 60°C to delay firmness losses. The decrease in fruit firmness as a result of heat treatment could be due to the fact that the high temperature caused a direct inhibition of the activity of pectin methylesterase and polygalacturonase [Akbudak *et al*., 2007; Pinheiro *et al.*, 2015].

Other authors have also demonstrated the effect of applying hot water immersion to firmness retention. Pinheiro *et al.* [2015] treated whole tomatoes in water at 40°C for 30 min and during 14 days of storage at 10°C the firmness of treated and untreated fruits decreased by 2.1 N and 4.3 N, respectively. Meanwhile, Lurie *et al*. [1997] used hot air at 48°C for 2 min on whole tomato and after 1 day of storage at 5°C, firmness of heat-treated fruits decreased by 2 N. On the other hand, Akbudak *et al.* [2007] found that whole tomatoes immersed in water at 54°C for 5 min had firmness of 6.87 N compared to the control with 1.67 N after 28 days of storage.

Gene expression for heat shock proteins

In Figure 4, the presence of bands shows the gene expression for the HSP21 protein in fruits treated at 45°C for 4 min and 60°C for 1 or 4 min. However, in the case of the fruit treated at 5°C for 1 or 4 min, 45°C for 1 min and untreated fruit (no immersion, SI), there was no expression, likely due to the exposure time or the temperature not being sufficient to provoke a gene response. A similar trend was found for HSP70, where the gene was expressed in fruits treated at 45 or 60°C for 4 min, while the treatment at 60°C for 1 min contributed to a very low expression compared to the other heat treatments. Finally, no expression was observed after tomato treatments at 5°C for 1 or 4 min and 45°C for 1 min.

According to Lurie *et al.* [1997] and Zhang *et al*. [2005], temperatures above 30°C produce a rapid induction and increase of the gene expression that encodes heat shock proteins (HSPs), which exert a protective role against heat stress in the tissues.

Cruz-Mendívil *et al*. [2015] found that the most important heat shock proteins of tomato fruits are HSP21 and HSP70, which have an effect of thermal tolerance to chilling injury. This effect was linked to the chaperone action of these proteins in which it aided the correct folding of other partially denatured proteins, preventing them from irreversibly aggregating with other cell proteins. Other authors, such as Zou *et al*. [2012], mentioned the important role of these proteins as cell membrane stabilizers, since they help maintain their fluidity and integrity, as well as reduce the leakage of electrolytes in the membrane at the time of stress. The same authors observed a significant decrease in cell membrane electrolyte loss in transgenic rice plants that overexpress genes encoding for HSP23 and HSP17. In addition, HSP70 induced the activity of antioxidant enzymes, which would protect against oxidative stress. According to Zhang *et al.* [2011], there is a synergistic relationship between HSP70 and the antioxidant capacity in the plant cell that could mitigate the effect of chilling injury on grape berries by applying forced air heat stress at 39°C for 10 h. This response was achieved due to the decrease in electrolyte leakage in the cell membrane and increased activity of catalase (CAT) and superoxide dismutase (SOD). The same authors suggested that exposure to forced air induced an expression of HSP70 in these berries, which enhanced the activity of antioxidant enzymes, maintained greater membrane integrity and, subsequently, caused resistance to chilling injury.

After high-temperature treatment, an increase in gene expression for HSP was reported by Polenta *et al.* [2015], who, by applying forced air at 39°C for 24 h to tomato fruit, observed a higher expression of HSP21 and HSP70. By contrast, Guidi *et al.* [2008] obtained a higher expression of HSP21, HSP23 and HSP70 in tomatoes treated in water at 42°C for 60 min or forced air at 39°C for 24 h compared to the untreated fruits.

Summarizing, the tomatoes treated at 45°C showed less respiration, the same firmness and a slightly higher expression of the HSP21 and HSP70 proteins than the tomatoes treated at 60°C. In addition, tomatoes immersed in water at 60°C presented uneven ripening that did not occur in tomatoes treated at 45°C (data not shown). Therefore, 45°C was chosen as the treatment temperature in the second experiment.

Experiment 2

Firmness

The initial firmness of untreated tomatoes before calcium application was 18.2±2.1 N. However, the firmness of Peumo Pink tomatoes treated in solutions of calcium salts (calcium

TABLE 2. Firmness (N) and total calcium content (mg Ca/100 g fresh weight) of Peumo Pink tomato fruits treated in solutions of calcium salts at two immersion temperatures (10 and 45°C), and stored for 28 days at 10°C + 2 days at 20°C.

Treatment	Firmness	Ca content	Firmness	Ca content	Firmness	Ca content
	14+2 days		21+2 days		28+2 days	
10°C + NSA	13.6±0.9^{c}	6.2±0.4^{b}	7.4±0.5^{de}	6.4±0.4^{NS}	5.9±0.5^{b}	6.4±0.4^{cd}
10°C + CaCh	8.0±0.8^{e}	5.5±0.7^{b}	9.4±0.3^{cd}	6.6±0.2	5.7±0.3^{b}	7.1±0.5^{b}
10°C + CaLa	11.5±0.7^{d}	5.3±0.7^{b}	8.2±0.4^{de}	6.0±0.2	7.0±0.3^{b}	7.5±0.2^{ab}
10°C + CaPr	17.5±0.5^{a}	6.2±0.5^{b}	7.5±0.8^{de}	7.7±0.3	6.2±0.4^{b}	6.3±0.2^{d}
45°C + NSA	16.0±0.7^{ab}	6.0±0.2^{b}	6.5±0.8^{e}	7.1±0.2	6.3±0.3^{b}	5.5±0.4^{e}
45°C + CaCh	17.5±0.7^{a}	8.9±0.5^{a}	14.7±0.8^{a}	8.4±0.3	6.8±0.3^{b}	7.6±0.4^{ab}
45°C + CaLa	15.1±1.0^{bc}	8.4±0.6^{a}	12.6±0.8^{ab}	7.7±0.3	9.4±0.4^{a}	7.5±0.1^{ab}
45°C + CaPr	13.8±0.8^{c}	7.5±0.5^{a}	10.4±0.8^{bc}	8.1±0.6	9.4±0.4^{a}	8.3±0.3^{a}

The values are the mean ± standard error (n=15 for firmness and n=5 for total calcium content). Different letters vertically indicate significant differences (p<0.05). NS, not significant. CaCh, calcium chloride; CaLa, calcium lactate; CaPr, calcium propionate; NSA, no salt application.

chloride, propionate and lactate) and two immersion temperatures (10 and 45°C) changed significantly during storage for 28 days at 10°C + 2 days at 20°C (Table 2). In general, after day 14+2, the heat-treated fruit showed higher firmness than those dipped in corresponding cold calcium solution or water, except the treatment with calcium propionate. On day 21+2, firmness of tomatoes treated in all heat calcium solutions was higher and the highest values were reached for calcium chloride (14.7 N) and calcium lactate (12.6 N). At the end of storage, after day 28+2, the fruits treated in propionate and lactate calcium at 45°C had the lowest firmness losses. According to Magee *et al.* [2003], the treatment of tomatoes with a 2% calcium chloride solution at 50°C maintained their firmness for 20 days as opposite to dipping in water at 10°C. On the other hand, Mahmud *et al.* [2008] reported that firmness of papaya dipped in 2% and 2.5% calcium chloride was maintained after storage for 24 days at 12°C. A previous report by Luna-Guzman & Barrett [2000] showed a higher firmness in fresh-cut melon immersed in a 2.5% calcium lactate solution at 60°C after 12 days at 5°C compared to controls without salt.

An increase in temperatures caused the activation of the pectin methylesterase. This enzyme catalyzes the demethoxylation of pectin of the cell wall favoring adhesion of free carboxyl groups by calcium ions known as calcium pectates. The calcium pectates provide firmness to the tissue, reducing softening and water loss [Aguayo *et al*., 2008; Luna-Guzman & Barrett, 2000; Ni *et al*., 2005].

Total calcium content

The total calcium content of untreated Peumo Pink tomatoes at the beginning of storage was 6.6±0.4 mg Ca/100 g FW. In tomatoes treated in solutions of calcium salts (calcium chloride, propionate and lactate) and two immersion temperatures (10 and 45°C), it changed significantly during storage for 28 days at 10°C + 2 days at 20°C. The total calcium content was similar for fruits treated in water and calcium salt solutions at 10°C. However, the fruit treated with calcium chloride, lactate and propionate at 45°C had higher total calcium contents, although significant differences were noted only at 14+2 days (Table 2). According to Silveira *et al.* [2011a], the application of calcium salts at high temperatures increased the diffusion of calcium in porous apoplasts and increased calcium retention in the cell wall. The increased fruit calcium contents upon the treatment with heat and calcium salts were also reported by Aguayo *et al.* [2015] in fresh-cut apples. Naser *et al*. [2018] increased total calcium in persimmon by applying 2% and 4% calcium lactate at 45°C. According to Manganaris *et al.* [2005], applications of Ca in the form of calcium chloride, calcium propionate and calcium lactate to canned peaches caused a 2.5-fold increase in Ca content in the treated fruit, saturating the Ca content of the cell wall, leading to increased firmness and crispiness of canned peaches.

Total phenolic content

Before calcium treatments, TPC of tomatoes was 214±58 μg GAE/g FW. The TPC of tomatoes after immersion in calcium solutions and in water (control) at two immersion temperatures and during cold-storage is shown in Table 3. Peumo Pink tomatoes immersed in 2% calcium chloride at 45°C and control at 10°C had the highest total phenolic content on days 21+2; however, on days 28+2, the highest TPC was found in tomatoes treated in a calcium lactate solution at 45°C and calcium propionate at 10°C followed by calcium chloride at 45°C. However, no clear trend was observed in each evaluated storage period. Nevertheless, higher total phenolic contents along with cold storage time (Table 3). The increasing TPC during storage may be due to the activity of the phenylalanine ammonium lyase (PAL), a key enzyme in the synthesis of phenylpropanoid, and some secondary metabolites [Son *et al.,* 2012], including phenolic compounds. According to Aghdam *et al.* [2013] and Nisar *et al*. [2015], calcium salts can increase PAL activity, and Nasef [2018] found that immersion in water at 45°C raised TPC in pumpkin samples compared to those treated at 10°C. At the same time, the increased TPC in our study found in Peumo Pink tomatoes during storage at 10°C can be caused by a stress

TABLE 3. Total phenolic content (TPC, μg GAE/g fresh weight, FW) and antioxidant capacity determined as ferric-reducing antioxidant power (FRAP) and DPPH radical scavenging activity (both as μg TE/g FW) of Peumo Pink tomato fruits treated in solutions of calcium salts at two immersion temperatures (10 and 45°C), and stored for 28 days at 10°C + 2 days at 20°C

Treatments	TPC	FRAP	DPPH assay	TPC	FRAP	DPPH assay	TPC	FRAP	DPPH assay
	14+2 days			21+2 days			28+2 days		
10°C + NSA	288±10^{NS}	248±22^{ab}	286±10^{NS}	680±26^{a}	448±30^{ab}	456±10^{a}	650±15^{b}	520±1de	563±20^{cd}
10°C + CaCh	277±23	151±23^{c}	278±10	493±26^{b}	247±41^{cd}	417±15^{b}	668±30^{b}	505±30^{e}	547±19^{d}
10°C + CaLa	226±8	196±28^{bc}	282±9	588±18^{ab}	347±33^{bc}	414±18^{b}	577±11^{c}	363±26^{f}	521±29^{e}
10°C + CaPr	222±16	207±16^{ab}	278±12	427±30^{c}	217±39^{d}	401±6^{c}	747±20^{a}	631±31^{b}	593±24^{b}
45°C + NSA	228±9	148±26^{c}	277±12	474±15^{bc}	331±35^{c}	411±12^{bc}	679±41^{b}	569±20^{cd}	580±13^{bc}
45°C + CaCh	217±12	148±30^{c}	276±11	705±22^{a}	509±36^{a}	455±18^{a}	708±23^{ab}	600±46^{bc}	582±20^{bc}
45°C + CaLa	266±21	268±11^{a}	286±9	552±25^{b}	347±20^{bc}	416±15^{bc}	746±33^{a}	696±26^{a}	615±13^{a}
45°C + CaPr	218±26	156±21^{c}	279±6	551±30^{b}	342±15^{bc}	429±13^{ab}	680±35^{b}	592±16^{bc}	574±9^{bc}

The values are the mean ± standard error (n=5). Different letters vertically indicate significant differences (p<0.05). NS, not significant. CaCh, calcium chloride; CaLa, calcium lactate; CaPr, calcium propionate; NSA, no salt application; GAE, gallic acid equivalent; TE, Trolox equivalent.

affecting the fruit at this low temperature. Many authors recommended a minimum temperature of 12 to 15°C to avoid chilling injury of the tomatoes [Polenta *et al.,* 2015; Zhang *et al.,* 2016]; thus, after a prolonged storage, increased activity of the PAL enzyme could occur as a response to chilling stress [Silveira & Escalona, 2014].

Antioxidant capacity

The antioxidant capacity of Peumo Pink tomatoes before treatments with calcium salt solutions were 71.8±7.7 and 247±15 μg TE/g FW determined as FRAP and DPPH radical scavenging activity, respectively. After treatments with calcium salt solutions. the FRAP of tomatoes at both immersion temperatures and cold-stored for 28+2 days ranged from 363 to 696 μg TE/g FW (Table 3). No clear trend was found between treatments for each storage period; however, like TPC, the FRAP of tomatoes after individual treatments increased as their storage time increased. A similar trend was found for DPPH radical scavenging activity (Table 3). The differences between the treatments, as for the FRAP, did not show clear trends. On days 14+2, 21+2 and 28+2, the values were between 276 to 286, 401 to 456 and 521 to 615 μg TE/g FW, respectively.

As previously mentioned, a clear trend was observed for all treatments during cold storage at 10°C where higher values were found at a longer storage period. This increased antioxidant capacity, which as related to total phenolic content, may be as a response to chilling injury at temperatures below 10°C. Several studies have shown that fruit treated in calcium salts, such as peach [Zhi *et al.,* 2017] or cherry [Aghdam *et al.,* 2013], reached a higher antioxidant capacity. According to Naser *et al*. [2018], persimmon fruits dipped in 2% and 4% calcium lactate at 45°C and stored for 40 days had a higher antioxidant capacity compared to untreated fruit stored at 10°C. Use of a calcium salt solution at elevated temperatures activated the ionic calcium channels in the plasma membrane allowing calcium to enter the cytosol. Calcium could bind to the calmodulin protein, causing intracellular signaling and enhancing PAL enzyme activity, which would increase the antioxidant capacity [De Freitas & Mitcham, 2012; Wang *et al*., 2014]. Additionally, the increase in antioxidant capacity in all treatments throughout cold storage could also be due to a natural ripening evolution of the tomato fruit, where antioxidant compounds, such as lycopene, β-carotene and flavonoids, are synthesized [Dorais *et al*., 2008].

CONCLUSIONS

The immersion in water at 45 and 60°C for 4 min caused a delay in the ripening of the Peumo Pink tomato, keeping firmness for 21 days at 10°C plus 2 days at 20°C without symptoms of rotting. Additionally, the gene expression for the heat shock proteins found for these combinations of temperatures and exposure times could be an interesting alternative to predict the effectiveness of heat treatment in such fruit as Peumo Pink tomatoes.

Applications of calcium salts at 45°C for 4 min caused changes in the physicochemical properties of Peumo Pink tomatoes. Immersion in calcium lactate and propionate at 45°C for 4 min proved best to maintain the firmness of the fruit for 28 days at 10°C plus 2 days at 20°C. The TPC and antioxidant capacity increased with the storage time of tomatoes. Although calcium applications showed an increase in TPC, no clear trend was observed on each evaluation day. The use of high temperatures with calcium salts for dipping would be beneficial to provide the vegetal tissues with calcium and reduce the softening of the tomato after prolonged cold storage.

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CONFLICT OF INTEREST

The authors declare that the study was conducted in absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ORCID IDs

A. Amorós https://orcid.org/0000-0002-5817-2898
J.I. Covarrubias https://orcid.org/0000-0002-8414-8963
V.H. Escalona https://orcid.org/0000-0001-8651-801
H. Silva https://orcid.org/0000-0003-1007-74429

REFERENCES

1. Aghdam, S.M., Dokhanieh, A.Y., Hassanpour, H., Fard, J.R. (2013). Enhancement of antioxidant capacity of cornelian cherry (*Cornus mas*) fruit by postharvest calcium treatment. *Scientia Horticulturae, 161*, 160–164. https://doi.org/10.1016/j.scienta.2013.07.006
2. Aguayo, E., Escalona, V.H., Artés, F. (2008). Effect of hot water treatment and various calcium salts on quality of fresh-cut 'Amarillo' melon. *Postharvest Biology and Technology, 47*(3), 397–406. https://doi.org/10.1016/j.postharvbio.2007.08.001
3. Aguayo, E., Requejo-Jackman, C., Stanley, R., Woolf, A. (2015). Hot water treatment in combination with calcium ascorbate dips increases bioactive compounds and helps to maintain fresh-cut apple quality. *Postharvest Biology and Technology, 110*, 158–165. https://doi.org/10.1016/j.postharvbio.2015.07.001
4. Akbudak, B., Akbudak, N., Seniz, V., Eris, A. (2007). Sequential treatments of hot water and modified atmosphere packaging in cherry tomatoes. *Journal of Food Quality, 30*(6), 896–910. https://doi.org/10.1111/j.1745-4557.2007.00168.x
5. Benzie, I.F., Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. *Analytical Biochemistry, 239*(1), 70–76. https://doi.org/10.1006/abio.1996.0292
6. Brand–Williams, W., Cuvelier, M.E., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT – – Food Science and Technology, 28*(1), 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5
7. Carvajal, M., Martínez, V., Cerda, A. (1999). Influence of magnesium and salinity on tomato plants grown in hydroponic culture. *Journal Plant Nutrition, 22*(1), 177–190. https://doi.org/10.1080/01904169909365616
8. Cruz-Mendívil, A., López-Valenzuela, J., Calderón-Vázquez, C., Vega-García, M., Reyes-Moreno, C., Valdez-Ortiz, A. (2015). Early transcriptional responses to chilling stress in tomato fruit with hot water pre-treatment. *Postharvest Biology and Technology, 109*, 137–144. https://doi.org/10.1016/j.postharvbio.2015.06.015
9. De Freitas, S.T., Mitcham, E.J. (2012). Factors involved in fruit calcium deficiency disorders. *Horticultural Revision, 40*, 107–146.
10. Dea, S., Bretch, J.K., Nunes, M.C.N., Baldwin, E.A. (2010). Quality of fresh-cut "Kent" mango slices prepared from hot water or non-hot water-treated fruit. *Postharvest Biology and Technology, 56*(2), 171–180. https://doi.org/10.1016/j.postharvbio.2010.01.007
11. Di Rienzo J.A., Casanoves, F., Balzarini, M.G., González, L., Tablada, M., Robledo, W. (2013). InfoStat versión 2013. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.
12. Dorais, M., Ehret, D.L., Papadopoulos, A.P. (2008). Tomato (*Solanum lycopersicum* L.) health components: from the seed to the consumer. *Phytochemistry Reviews, 7*, 23–50. https://doi.org/10.1007/s11101-007-9085-x
13. FAOSTAT. 2020. Available at: [http://www.fao.org/faostat/en/#home].
14. Guidi, S., Nanni, M., Polenta, G. (2008). Evaluation of heat stress proteins in fruits subjected to postharvest heat treatments (English). Evaluación de proteínas de estrés térmico en frutos sometidos a tratamientos térmicos en postcosecha (Spanish original title). Instituto Nacional de Tecnología Agropecuaria, p. 5. [https://inta.gob.ar/sites/default/files/script-tmp-inta__evaluacin_de_protenas_de_estrs_trmico_en_frutos.pdf].
15. Lara, I., García, P., Vendrell, M. (2004). Modifications in cell wall composition after cold storage of calcium-treated strawberry (*Fragaria* × *ananassa* Duch.) fruit. *Postharvest Biology and Technology, 34*(3), 331–339. https://doi.org/10.1016/j.postharvbio.2004.05.018
16. Li, S., Zhu, B., Pirrello, J., Xu, C., Zhang, B., Bouzayen, M., Chen, K., Grierson, D. (2020). Roles of RiN and ethylene in tomato fruit ripening and ripening-associated traits. *New Phytologist, 226*(2), 460–475. https://doi.org/10.1111/nph.16362
17. Luna-Guzmán, I., Barrett, D.M. (2000). Comparison of calcium chloride and calcium lactate effectiveness in maintaining shelf stability and quality of fresh-cut cantaloupes. *Postharvest Biology and Technology, 19*(1), 61–72. https://doi.org/10.1016/S0925-5214(00)00079-X
18. Lurie, S., Laamin, M., Lapsker, Z., Fallik, E. (1997). Heat treatments to decrease chilling injury in tomato fruit. Effects in lipids, pericarp lesions and fungal growth. *Physiology Plantarum, 100*(2), 297–302. https://doi.org/10.1111/j.1399-3054.1997.tb04786.x
19. Magee, R.L., Caporaso, F., Prakash, A. (2003). Effects of exogenous calcium salt treatments on inhibiting irradiation–induced softening in diced Roma tomatoes. *Journal Food Science, 68*(8), 2430–2435. https://doi.org/10.1111/j.1365-2621.2003.tb07041.x
20. Mahmud, T.M.M., Al Eryani-Raqeeb, A., Syed Omar, S.R., Mohamed, Z., Abdul-Rahman, A.E. (2008). Effects of different concentrations and applications of calcium on storage life and physicochemical characteristics of papaya (*Carica papaya* L.). *American Journal of Agricultural and Biological Sciences, 3*(3), 526–533. https://doi.org/10.3844/ajabssp.2008.526.533
21. Manganaris, G.A., Vasilakakis, M., Diamantidis, G., Mignani, I. (2005). Effect of calcium additives on physicochemical aspects of cell wall pectin and sensory attributes of canned peach (*Prunus persica* L. Batsch cv. Andross). *Journal of the Science of Food and Agriculture, 85*(10), 1773–1778. https://doi.org/10.1002/jsfa.2182
22. Meisel, L., Fonseca, B., González, S., Baeza-Yates, R., Cambiazo, V., Campos, R., Gonzalez, M., Orellana, A., Retamales, J., Silva, H. (2005). A rapid and efficient method for purifying high quality total RNA from peaches (*Prunus persica*) for functional genomics analyses. *Biological Research, 38*(1), 83–88. https://doi.org/10.4067/s0716-97602005000100010.

23. Nasef, I. (2018). Short hot water as safe treatment induces chilling tolerance and antioxidant enzymes, prevents decay and maintains quality of cold-stored cucumbers. *Postharvest Biology and Technology, 138*, 1–10. https://doi.org/10.1016/j.postharvbio.2017.12.005
24. Naser, F., Rabiei, V., Razavi, F., Khademi, O. (2018). Effect of calcium lactate in combination with hot water treatment on the nutritional quality of persimmon fruit during cold storage. *Scientia Horticulturae, 233,* 114–123. https://doi.org/10.1016/j.scienta.2018.01.036
25. Ni, L., Lin, D., Barret, M.D. (2005). Pectin methylesterase catalyzed firming effects of low temperature blanched vegetables. *Journal of Food Engineering, 70*(4), 546–556. https://doi.org/10.1016/j.jfoodeng.2004.10.009
26. Nisar, R., Baba, W.N., Masoodi, F.A. (2015). Effect of chemical and thermal treatments on quality parameters and antioxidant activity of apple (pulp) grown in high Himalayan regions. *Cogent Food & Agriculture, 1*(1), 10–16. https://doi.org/10.1080/23311932.2015.1063797
27. Oms-Oliu, G., Aguiló-Aguayo, I., Soliva-Fortuny, R., Martin-Belloso, O. (2008). Effect of ripeness at processing on fresh-cut "Flor de invierno" pears packaged under modified atmospheres conditions. International *Journal of Food Science and Technology, 44*(5), 900–909. https://doi.org/10.1111/j.1365-2621.2007.01635.x
28. Paull, R.E., Chen, N.J. (2000). Heat treatment and fruit ripening. *Postharvest Biology and Technology, 21*(1), 21–37. https://doi.org/10.1016/S0925-5214(00)00162-9
29. Pinheiro, J., Alegria, C., Abreu, M., Sol, M., Goncalves, E.M., Silva, C.L.M. (2015). Postharvest quality of refrigerated tomato fruit (*Solanum Lycopersicum*, cv. Zinac) at two maturity stages following heat treatment. *Journal of Food Processing and Preservation, 39*(6), 697–709. https://doi.org/10.1111/jfpp.12279
30. Polenta, G., Budde, C., Sivakumar, D., Nanni, M., Guidi, S. (2015). Evaluation of biochemical and quality attributes to monitor the application of heat and cold treatments in tomato fruit (*Licopersicum esculentum* Mill.). *Journal of Food Quality, 38*(3), 153–163. https://doi.org/10.1111/jfq.12139
31. Serrano, M., Martínez-Romero, D., Castillo, S., Guillén, F., Valero, D. (2004). Role of calcium and heat treatments in alleviating physiological changes induced by mechanical damage in plum. *Postharvest Biology and Technology, 34*(2), 155–167. https://doi.org/10.1016/j.postharvbio.2004.05.004
32. Silveira, A.C., Aguayo, E., Chisari, M., Artés, F. (2011a). Calcium salts and heat treatment for quality retention of fresh-cut 'Galia' melón. *Postharvest Biology and Technology, 62*(1), 77–84. https://doi.org/10.1016/j.postharvbio.2011.04.009
33. Silveira, A.C., Aguayo, E., Escalona, V.H., Artés, F. (2011b). Hot water treatment and peracetic acid to maintain fresh-cut Galia melon quality. *Innovative Food Science and Emerging Technologies, 12*(4), 569–576. https://doi.org/10.1016/j.ifset.2011.02.010
34. Silveira, A., Escalona, V.H. (2014). The use of the physical treatment on fresh-cut produce. *Stewart Postharvest Review, 10*(3), 1–5.
35. Silveira, A.C., Oyarzún, D., Sepúlveda, A., Escalona, V. (2017). Effect of genotype, raw-material storage time and cut type on native potato suitability for fresh-cut elaboration. *Postharvest Biology and Technology, 128*, 1–10. https://dx.doi.org/10.1016/j.postharvbio.2017.01.011
36. Singleton, V.L., Rossi, A.J. (1965). Colorimetry of total phenolics with phosphomolybdic – phosphotungstic and reagents. *American Journal of Enology and Viticulture, 16*, 144–157.
37. Son, K-H., Park, J-H., Kim, D., Oh, M.-M. (2012). Leaf shape index, growth, and phytochemicals in two leaf lettuce cultivars grown under monochromatic light-emitting diodes. *Korean Journal Horticultural Science & Technology, 30*(6), 664–672. https://doi.org/10.7235/hort.2012.12063
38. Sun, Q., Zhang, N., Wang, J., Zhang, H., Li, D., Shi, J., Li, R., Weeda, S., Zhao, B., Ren, S., Guo, Y.-D. (2015). Melatonin promotes ripening and improves quality of tomato fruit during postharvest life. *Journal of Experimental Botany, 66*(3), 657–668. https://doi.org/10.1093/jxb/eru332
39. Swain, T., Hills, W.E. (1959). The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *Journal of the Science of Food and Agriculture, 10*(1), 63–68. https://doi.org/10.1002/jsfa.2740100110
40. Valero, D., Serrano, M. (2010). *Postharvest Biology and Technology for Preserving Fruit Quality*. 1st edition, CRC Press. Taylor & Francis Group, pp. 109–124.
41. Wang, C. (1998). Heat treatment affects postharvest quality of kale and collard, but not of brussels sprouts. *HortScience, 33*, 881–883. https://doi.org/10.21273/HORTSCI.33.5.881
42. Wang, Y., Xie, X., Long, L. (2014). The effect of postharvest calcium application in hydro cooling water on tissue calcium content, biochemical changes, and quality attributes of sweet cherry fruit. *Food Chemistry, 160*, 22–30. https://doi.org/10.1016/j.foodchem.2014.03.073
43. Xu, L., Gao, J., Guo, L., Yu, H. (2020). Heat shock protein 70 (HmHsp70) from *Hypsizygus marmoreus* confers thermotolerance to tobacco. *AMB Express, 10*, art. no. 12. https://dx.doi.org/10.1186%2Fs13568-020-0947-6
44. Zhang, J., Huang, W., Pan, Q., Liu, Y. (2005). Improvement of chilling tolerance and accumulation of heat shock proteins in grape berries (*Vitis vinifera* cv. Jingxiu) by heat pretreatment. *Postharvest Biology and Technology, 38*(1), 80–90. https://doi.org/10.1016/j.postharvbio.2005.05.008
45. Zhang, B., Tieman, D.M., Jiao, C., Xu, Y.M., Chan, K.S., Fe, Z.J., Giovannoni, J.J., Klee, H.J. (2016). Chilling-induced tomato flavour is associated with altered volatile synthesis and transient changes in DNA methylation. *Proceedings of the National Academy of Sciences of the United States of America, 113*(44), 12580–12585. https://doi.org/10.1073/pnas.1613910113
46. Zhang, L., Yu, Z., Jiang, L., Jiang, J., Luo, H., Fu, L. (2011). Effect of post-harvest heat treatment on proteome change of peach fruit during ripening. *Journal Proteomics, 74*(7), 1135–1149. https://doi.org/10.1016/j.jprot.2011.04.012
47. Zhi, H.H., Liu, Q., Dong, Y., Liu, M., Zong, W. (2017). Effect of calcium dissolved in slightly acidic electrolyzed water on antioxidant system, calcium distribution, and cell wall metabolism of peach in relation to fruit browning. *Journal Horticultural Science and Biotechnology, 92*(6), 621–629. https://doi.org/10.1080/14620316.2017.1309994
48. Zou, J., Liu, C., Liu, A., Zou, D., Chen, X. (2012). Overexpression of OsHsp17.0 and OsHsp23.7 enhances drought and salt tolerance in rice. *Journal Plant Physiology, 169*(6), 628–635. https://doi.org/10.1016/j.jplph.2011.12.014

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Ms. Joanna Molga
Editorial Office of Pol. J. Food Nutr. Sci.
Institute of Animal Reproduction and Food Research
Tuwima 10 Str., 10–748 Olsztyn 5, Poland
phone (48 89) 523–46–70, fax (48 89) 523–46–70;
e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

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