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ANTIOXIDANT ACTIVITY AND TOTAL POLYPHENOL CONTENT OF INSECTS USED AS FEED AND FOOD

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ABSTRACT

This study aimed to evaluate the antioxidant activity, total polyphenol content (TPC), and correlation between TPC and antioxidant activity of eight insect species allowed as feed and three of them as food in the European Union. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was used to measure antioxidant activity, and the Folin-Ciocalteu method was used to measure TPC. The results showed significant variability in both antioxidant activity and TPC among the tested insect species. *Bombyx mori* (1.64±0.01 mg TEAC/g DM; TEAC- Trolox equivalent antioxidant capacity, DM-dry matter) among all tested insects and *Acheta domesticus* (1.30±0.14 mg TEAC/g DM) among the insects allowed as food exhibited the highest ($p<0.05$) antioxidant potential, which may be attributed to their polyphenol content. On the other hand, the *Hermetia illucens* sample had the lowest ($p<0.05$) antioxidant activity (0.20±0.01 mg TEAC/g DM). The highest ($p<0.05$) TPC was observed in the sample of *Gryllus assimilis* (14.51±0.94 mg GAE/g DM; GAE- gallic acid equivalent) among the tested samples, except for the *B. mori* sample (13.39±0.35 mg GAE/g). Significantly ($p<0.05$), the lowest TPC was in the *H. illucens* sample (5.18±0.06 mg GAE/g DM). Mean antioxidant activity and TPC were determined to be 0.87 mg TEAC/g DM and 10.63 mg GAE/g DM, respectively. Evaluating insects grouped by their development stages (adults, larvae, pupae), pupae had the highest ($p<0.05$) antioxidant activity (1.64 mg TEAC/g DM), and the lowest ($p<0.05$) TPC was in the larvae group (8.23 mg GAE/g DM). A moderate positive correlation ($r=0.625$, $p<0.01$) was observed between TPC and antioxidant activity. The results confirmed that insects may serve as significant sources of natural antioxidants. Also, the total polyphenol content and antioxidant activity were influenced by the developmental stage.

Keywords: alternative sources, DPPH, sustainability, bioactive compounds

INTRODUCTION

Antioxidants are substances that can prevent or slow down cell damage by free radicals **(Sevindik *et al.*, 2020).** Oxidative stress has been associated in humans with many civilizational diseases, such as inflammation, cardiovascular disease, stroke, and cancer **(Ali *et al.*, 2008; Stadtman, 2006)**. In animals, oxidative stress has a negative impact on reductions in animal performance and product quality. For example, it has been shown to negatively affect the number of somatic cells in milk, as well as the sensory quality of meat **(Ponnampalam *et al.*, 2017)**. The results of **Mihok *et al.* (2024)** suggest the significant role of oxidative stress in the pathogenesis of canine babesiosis and propose that antioxidant therapy could be useful in its treatment. Numerous studies have demonstrated that antioxidants and anti-inflammatory peptides can protect against reactive oxygen species (ROS) and may also lower levels of oxidative stress **(Torres-Fuentes *et al.*, 2011; Carrasco-Castilla *et al.*, 2012; Karaś *et al.*, 2015; Zielińska *et al.*, 2017)**. The UN predicts that the world's population will reach 9.7 billion by 2050. This means that food production must increase to satisfy rising food demand, while arable land availability will be limited **(Imathiu, 2020)**. One option to meet the increasing need for food and feed is insects. Entomophagy, the consumption of insects and invertebrates, has been integral to human culture and religion for centuries **(Costa-Neto, 2005; Rzymski *et al.*, 2021)**. Insects are the natural food of many animals, including fish, wild boar, and free-ranging poultry, so it can be assumed that these animals are evolutionarily adapted to eating them. Despite being part of the natural diet of livestock, it has hardly been used as feed in the past **(Sealey *et al.*, 2011; van Huis *et al.*, 2013)**. Insects provide a significant source of high-quality protein, fats, amino acids, and fatty acids **(Benzertiha *et al.*, 2020; da Silva Lucas *et al.*, 2020; Šťastník *et al.*, 2021; Hawkey *et al.*, 2021)**, as well as essential nutrients, including minerals and vitamins **(Pal and Roy, 2014; Ojha *et al.*, 2021; Weru *et al.*, 2021)**. Moreover, various beneficial compounds found in edible insects can significantly enhance health. These compounds can be steroids, interferons, cordycepin, polysaccharides, microelements, chitin/chitosan, sex-attractive hormones, antimicrobial peptides, lecithin, and various other nutrients **(Ivanišová *et al.*, 2023)**. Insects are also considered promising due to their rapid reproductive cycles and high conversion efficiency of feed. Insects can also grow and thrive on poor-quality food (substrate), converting it into high-quality, concentrated protein and other nutrients. The bioconversion of waste materials can leverage these advantages to establish a sustainable circular economy. Besides being a quality source of nutrients, its mass production is also promising from an environmental perspective **(Poma *et al.*, 2017; Ruskova et al., 2023; Sogari *et al.*, 2023)**. Several recent studies have investigated the polyphenol content and antioxidant activity of insects or foods enriched with insect-derived ingredients. Alagappan et al. (2021) describe that whole *Oecophylla smaragdina* ants exhibit the highest antioxidant capacity in methanol extracts and the greatest total phenolic content in water extracts, compared to the ant nest, anterior part of the body, and gaster, indicating their potential as a rich source of natural antioxidants. Hong et al. (2024) highlight the higher DPPH antioxidant and antimicrobial activity of hydrolyzed *Tenebrio molitor* protein compared to its non-hydrolyzed form. Also, Keil et al. (2022) present a comparison of various drying methods of *T. molitor* larvae and their impact on antioxidant capacity and volatile compounds. Kowalski et al. (2023) evaluated the incorporation of *Tenebrio molitor*, *Alphitobius diaperinus*, and *Acheta domesticus* at two inclusion levels in biscuits and reported an increase in total phenolic content and DPPH antioxidant activity with higher insect addition. They also observed a strong positive correlation between phenolic content and antioxidant capacity in sponge cake samples enriched with insect ingredients.

The aim of this study was to evaluate the antioxidant activity using the DPPH method and the determination of total polyphenol content in the samples of 8 insect species, namely *Hermetia illucens, Tenebrio molitor, Alphitobius diaperinus, Musca domestica, Acheta domesticus, Gryllodes sigillatus, Gryllus assimilis*, and *Bombyx mori*. The EU **(Regulation EU 2017/893; Regulation EU 2021/1925)** permits the use of all eight tested species for animal nutrition. **Regulation 2015/2283 and Regulation (EU) 2017/2470** allow the use of *T. molitor, A. domesticus*, and *A. diaperinus* as novel food. Another objective was to determine the antioxidant activity and total polyphenol content (TPC) of the three insect groups based on their developmental stage. Also, the correlation between TPC and antioxidant activity was evaluated.

MATERIAL AND METHODS

Samples

In our experiment, we tested 8 species of insects. For each insect species, we tested three samples (n=3). Specifically, these were the larvae of the black soldier fly (*H. illucens*), mealworm (*T. molitor*), lesser mealworm (*A. diaperinus*), and housefly larvae (*M. domestica*). Next, there were 3 species of adult crickets: house cricket (*A. domesticus*), tropical house cricket (*G. sigillatus*), and Jamaican field cricket (*G. assimilis*). The last species tested was silkworm pupae (*B. mori*). All samples were purchased commercially. The purchased samples were whole full fat insects, and all samples were purchased dried. The manufacturers do not specify the drying method on the product packaging. Before analysis, the samples were mechanically processed in a laboratory mill (FRITSCH, Idar-Oberstein, Germany) with 1 mm openings. Except for grinding, the samples were not processed in any way prior to extraction. All tested insect species are allowed as feed in the EU (**Regulation EU 2017/893; Regulation EU 2021/1925**), and three of the tested insects (*T. molitor, A. domesticus*, and *A. diaperinus*) are allowed as novel food in the EU (**Regulation EU 2015/2283; Regulation EU 2017/2470**).

Antioxidant characteristics

Extract preparation

20 mL of 80% ethanol was used to extract one gram of homogenized samples (3 samples from each species; n=3) for two hours on a pulse shaker (LT 3, NEDFORM ltd., Benešov, Czech Republic) at laboratory temperature. Then the solution was filtered through filter paper (Whatman n. 4). The resulting liquid was then used to measure the concentration of TPC and DPPH antioxidant activity.

DPPH antioxidant activity

The samples' ability to remove free radicals was determined by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test, described by **Sánchez-Moreno *et al.* (1998)**. After dissolving 0.012 g of DPPH in 100 mL of ethanol, 4 mL of the DPPH solution was added to 1 mL of extracts. The BioTek Microplate Reader (ELx800, Agilent Technologies, Santa Clara, CA, USA) was used to measure the extracts' absorbance at 510 nm. Trolox equivalent antioxidant capacity (TEAC), measured in milligrams per gram of dry matter, was used to express the antioxidant activity of the samples.

Total polyphenol content

The Folin–Ciocalteu method, which was modified by **Singleton *et al.* (1965)**, was used to measure the samples' TPC using spectrophotometry. The reaction mixture was prepared by adding 0.1 mL of the Folin–Ciocalteu reagent, 0.1 mL of the sample extract, and 1 mL of a 20% sodium carbonate solution. A BioTek Microplate Reader (ELx800) was used to detect absorbance at a wavelength of 700 nm. The gallic acid equivalent (GAE) milligrams per dry matter was used to express the overall polyphenol content.

Statistical analysis

Results were statistically analyzed using the statistical program SPSS 26.0 (IBM) and one-way ANOVA. The Tukey test was used for evaluating statistical significance between variables at a 0.05 level. The Pearson correlation coefficient was used to evaluate the correlation between DPPH antioxidant activity and total polyphenol content, with significance at the 0.01 level. All sample analyses were performed in duplicate.

RESULTS AND DISCUSSION

DPPH antioxidant activity

The DPPH antioxidant activity assay is a widely utilized method for assessing antioxidant activity (**Li *et al.*, 2017**). This characterization is accomplished, for instance, by evaluating hydrolysates produced through several enzymatic treatments, as well as by examining peptides sourced from insects or aqueous or organic solvent extracts of insects (**Lee *et al.*, 2021**). Due to the various ways of action of antioxidants, various test methodologies must be applied to assess antioxidant and antiradical activity (**Gumul *et al.*, 2023**). Historically, the antioxidant action of insect extracts has been ascribed only to their phenolic constituents, predominantly flavonoids, which serve as antioxidants. It is acknowledged that bioactive peptides, proteins, vitamins, and free amino acids may also demonstrate antioxidant activities (**Nino *et al.*, 2021a**). In the eight examined insect samples, DPPH antioxidant activity was evaluated. The highest ($p<0.05$) antioxidant activity was found in the *B. mori* sample (1.64 mg TEAC/g DM; DM-dry matter; Table 1) compared to the rest of the samples. The difference ($p<0.05$) between the *B. mori* and *H. illucens* samples with the lowest antioxidant activity was 1.44 mg TEAC/g DM. The *A. diaperinus* and *T. molitor* samples had the strongest antioxidant activity among the larvae, whereas *A. domesticus* exhibited the highest activity among the adult crickets. Also, **Gumul *et al.* (2023)** reported the DPPH antioxidant activity in insect meal samples of *A. diaperinus* (15.67 mg Tx/g; Tx-Trolox equivalents antioxidant capacity), *A. domesticus* (16.27 mg Tx/g), and *T. molitor* (15.85 mg Tx/g). **Nino *et al.* (2021b)** evaluated the DPPH antioxidant activity of *A. domesticus* extracts, showing IC_{50} (half-maximal inhibitory concentration) values of 0.346 mg/mL for those fed on a commercial diet and 0.275 mg/mL for those fed on organic feed. **Del Hierro *et al.* (2020)** evaluated the antioxidant potential of *T. molitor* and *A. domesticus* extracts to inhibit DPPH using various extraction methods and solvents. The *in vitro* antioxidant activity of most tested extracts, evaluated by the DPPH assay, demonstrated 80% inhibitory activity. Although there were no substantial differences (mean values of 72% and 57%, respectively), it was shown that extracts from crickets have greater antioxidant activity than those from mealworms, independent of the extract type. Among the three tested insects allowed as food in the EU, the highest ($p<0.05$) antioxidant activity was in the *A. domesticus* sample compared to the *T. molitor* and *A. diaperinus* samples. In addition to the three tested species that are allowed as novel food in the EU, *Locusta migratoria* is also allowed as novel food in the European Union (**Regulation EU 2021/1975**). The mean antioxidant activity of the analyzed samples was 0.87 mg TEAC/g DM. In the evaluation of antioxidant activity between developmental stages, the pupal extracts (1.64 mg TEAC/g DM) showed the highest ($p<0.05$) antioxidant activity in comparison with adults and larvae (Table 2). These findings suggest that the developmental stage has a significant effect on the antioxidant activity of insects. **Sadat *et al.* (2022)** present a comprehensive overview of the bioactive compounds identified in methanolic extracts of silkworm (*B. mori*) pupae. Several polyphenols were identified, including quercetin, resveratrol, kaempferol, myricetin, and naringenin, recognized for their significant antioxidant properties.

Total polyphenol content

In the tested samples, the TPC was highest ($p<0.05$) in the *G. assimilis* sample at 14.50 mg GAE/g DM, compared to the other samples, except for the *B. mori* sample (Table 1). The difference ($p<0.05$) between the *G. assimilis* and *H. illucens* samples with the lowest TPC was 9.33 mg GAE/g DM. **Nino *et al.* (2021b)** found that *A. domesticus* fed organic feed had a TPC of 2.1 g GAE/100g, while those fed commercial feed had a content of 1.9 g GAE/100g, which was lower compared to the tested *A. domesticus*. Compared to the examined species, the results published by **Gumul *et al.* (2023)** had lower values of the TPC in meals from *A. diaperinus* (283.26 mg GAE/100g), *A. domesticus* (292.37 mg GAE/100g), and *T. molitor* (273.15 mg GAE/100g). In the group of insects allowed as food in the EU, the significantly ($p<0.05$) highest TPC has been confirmed in the *A. domesticus* sample in comparison with samples of *T. molitor* and *A. diaperinus*. The mean polyphenol concentration of the analyzed samples was 10.63 mg GAE/g DM. When the TPC was evaluated by developmental stage (Table 2), the lowest ($p<0.05$) polyphenol content was found in the larvae (8.23 mg GAE/g DM) in comparison with pupae and adults. These results suggest that the developmental stage of the insect influences the polyphenol content. **Raza *et al.* (2022)** reported the TPC for the weaver ant *Oecophylla smaragdina*, with values of 369.69 mg GAE/100g for adults and 486.04 mg GAE/100g for brood. Additionally, the termite *Odontotermes sp.* had a TPC of 626.92 mg GAE/100g. Various studies have also been published that have looked at the TPC and antioxidant activity following the addition of insects to various foods (**Ivanišová *et al.*, 2023; Gumul *et al.*, 2023; Kowalski *et al.*, 2023**). **Ivanišová *et al.* (2023)** tested the antioxidant activity and TPC of crackers with 5% insect addition. Crackers with mealworm, grasshopper, and cricket powder had DPPH antioxidant activity of 2.51, 2.64, and 2.56 mg TEAC/g versus 1.93 mg TEAC/g in the control. Furthermore, the TPC was higher in crackers with 5% insect addition (0.78, 0.61, and 0.71 mg GAE/g, respectively) compared to 0.49 mg GAE/g in control. Furthermore, **Kowalski *et al.* (2023)** evaluated the 15% and 30% addition of *T. molitor, A. diaperinus*, and *A. domesticus* to biscuits and tested the TPC and antioxidant activity of DPPH. TPC ranged from 82.42 to 155.73 mg catechin/100 g DM. DPPH ranged from 6.63 to 13.2 mg Tx/100g DM. These results indicate that adding insects to foods enhances their polyphenolic content and antioxidant activity.

Table 1 The antioxidant activity and total polyphenol content of the analyzed individual insect samples (n=3)

Insect species	Food/Feed	Developmental Stage	DPPH [mg TEAC/g DM]	Min.	Max.	TPC [mg GAE/g DM]	Min.	Max.
Alphitobius diaperinus	Food Feed	Larvae	0.85±0.03 [a]	0.82	0.87	8.49±0.23 [a]	8.33	8.65
Hermetia illucens	Feed	Larvae	0.20±0.01 [b]	0.20	0.20	5.17±0.07 [b]	5.13	5.22
Musca domestica	Feed	Larvae	0.65±0.03 [a]	0.63	0.68	9.56±0.13 [a]	9.47	9.66
Tenebrio molitor	Food Feed	Larvae	0.85±0.03 [a]	0.83	0.87	9.70±0.13 [a]	9.60	9.79
Acheta domesticus	Food Feed	Adult	1.30±0.15 [c]	1.19	1.40	12.00±0.13 [c]	11.91	12.10
Gryllodes sigillatus	Feed	Adult	0.74±0.02 [a]	0.73	0.75	12.20±0.39 [c]	11.93	12.47
Gryllus assimilis	Feed	Adult	0.72±0.02 [a]	0.70	0.73	14.50±1.01 [d]	13.79	15.22
Bombyx mori	Feed	Pupae	1.64±0.01 [d]	1.64	1.64	13.38±0.37 [cd]	13.12	13.65
Mean (n=24)	-	-	0.87±0.42	0.20	1.64	10.63±2.92	5.13	15.22

DPPH-2,2-diphenyl-1-picrylhydrazyl, DM-dry matter, TEAC-Trolox equivalent antioxidant capacity, TPC-total phenolic content, GAE-gallic acid equivalent.
[abcd] Statistically significant differences were observed with different indexes in columns ($p<0.05$).

Relationship between total polyphenol content and DPPH antioxidant activity

Del Hierro *et al.* (2020) found that the relationship between TPC and DPPH antioxidant activity in extracts of *T. molitor* and *A. domesticus* highlights the dependence of antioxidant activity on polyphenol levels. Increased TPC in the samples correlates with enhanced antioxidant activity. In the experiment conducted, a moderate positive correlation of 0.625 (significant at the 0.01 level) was observed between TPC and DPPH antioxidant activity. **Kowalski *et al.* (2023)** found a significant positive correlation (r=0.939) between TPC and DPPH levels in sponge cake samples with added insects. Insects can be considered a potential source of polyphenols in animal and human nutrition with a low environmental impact. However, it should be said that the polyphenol content in insects is dependent on the food intake and taxonomy. The main source of polyphenols for insects is plant food, which may cause differences in the polyphenol content of insect samples **(Di Mattia *et al.*, 2019; Nino *et al.*, 2021a)**. The method used to determine polyphenols in the insect samples tested may influence the differences in polyphenol content, as well as the method of extraction **(Mokrani and Nadani, 2016; Gumul *et al.*, 2023)**. The Folin-Ciocalteu method is commonly used for the determination of TPC in different samples. However, it is important to say that the reaction in this method is not specific for phenolic substances, but it can react with other reducing components such as ascorbic acid, some free amino acids, alkaloids, proteins, polysaccharides, as well as organic acids or fatty acids, which can lead to overestimation of the total polyphenol content in the samples (**Huang *et al.*, 2005; Magalhães *et al.*, 2010; Everette *et al.*, 2010**).

Table 2 The antioxidant activity and total polyphenol content of the three insect groups based on developmental stage

Insect group	DPPH [mg TEAC/g DM]	Min.	Max.	TPC [mg GAE/g DM]	Min.	Max.
Adults (n=9)	0.92±0.30 [a]	0.70	1.40	12.90±1.33 [a]	11.91	15.22
Larvae (n=12)	0.64±0.28 [a]	0.20	0.87	8.23±1.96 [b]	5.13	9.79
Pupae (n=3)	1.64±0.01 [b]	1.64	1.65	13.38±0.37 [a]	13.12	13.65

DPPH-2,2-diphenyl-1-picrylhydrazyl, DM-dry matter, TEAC-Trolox equivalent antioxidant capacity, TPC-total phenolic content, GAE-gallic acid equivalent.
[ab] Statistically significant differences were observed with different indexes in columns ($p<0.05$).

CONCLUSION

The aim of this study was to evaluate the antioxidant activity, TPC, and the relationship between TPC and DPPH antioxidant activity of eight insect species allowed to be used as feed and three of them as food in the European Union. Significant differences in antioxidant activity were observed among the tested insect species. *B. mori* had significantly the highest DPPH antioxidant activity, followed by *A. domesticus* and *T. molitor,* from all tested samples. In contrast, *H. illucens* showed significantly the lowest antioxidant activity of all species. Similarly, the TPC varied, with *G. assimilis* displaying the highest TPC, whereas *H. illucens* had significantly the lowest TPC of all tested samples. *A. diaperinus* had significantly the highest antioxidant activity and TPC among the novel foods (*A. diaperinus*, *A. domesticus*, and *T. molitor*). The mean antioxidant activity and TPC of the analyzed samples were 0.87 mg TEAC/g DM and 10.63 mg GAE/g DM, respectively. When evaluating the different groups of insects based on their growth stage, the pupae showed significantly the highest antioxidant activity and polyphenol content. The lowest antioxidant activity and TPC ($p < 0.05$) were observed in the larvae group. These findings suggest that the developmental stage of the insect influences the polyphenol content and antioxidant activity. A moderate positive correlation (r=0.625, $p<0.01$) was observed between TPC and antioxidant activity, confirming the role of polyphenols as key contributors to the antioxidant potential of these insects. These findings emphasize the potential of insects as natural sources of antioxidants, which could enhance animal and human health. Given their bioactive properties, insects may serve as functional ingredients in feed and novel food formulations in order to enhance antioxidant activity. This knowledge can guide the selection of specific insect species for developing functional foods and feeds. Further research is needed to explore processing methods that could optimize their antioxidant capacity and to evaluate their long-term effects on animal health and human nutrition.

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