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Original Research Article

Phenolic Compounds from Cricket Powder and Its Protein Extract after Passing through the Simulated *In vitro* Digestion

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ABSTRACT

Phenolic compounds found in edible insects is due to plant-derived phenolics present in their diet and the ability of insects to synthesize through the sclerotization process. This study aimed to examine the loss of phenolic compounds from cricket powder (CP) and cricket crude protein extract (CCP) after passing through the simulated *in vitro* digestion. CCP was prepared by alkali extraction of CP, followed by isoelectric point precipitation at pH 4.0. The protein content of CP and CCP was determined as 54.95 and 61.12% dry basis (d.b.), respectively, with moisture content approx. 3-5%. The total phenolic content (TPC) via Folin-Ciocalteu's reagent of both samples were measured. CP contained less TPC than CCP at the respective 5.87 and 30.64 mg gallic acid equivalent (GAE)/g. Gallic acid, isoquercetin, quercetin and tannic acid were phenolic compounds found in both CP and CCP. During the simulated *in vitro* digestion, TPC in both samples dramatically reduced after passing the gastric condition at pH 3. The TPC at small intestinal condition at pH 7 of CCP reduced whereas that of CP slightly increased. As phenolic compounds have been widely known for their antioxidant activity, thus DPPH and FRAP antioxidant assays were used for the assessment in this study. The results showed that the reduction of antioxidant activity was in proportion to TPC. Suggesting that the pH and digestive enzymes could affect the loss of phenolic compounds in CP and CCP.

INTRODUCTION

Phenolic compounds are phytochemicals which are mostly found in plants. Their biological functions in plant tissues have been attributed to the resistance against several external harms, for instance, microbial pathogens, virus, solar radiation, including playing a role in pollination processes (Cheynier, 2012; Quideau et al., 2011). As plants are food for edible insects, thus certain phenolic compounds have been reported to be found in them (Nino et al., 2021). Phytochemicals are known as health beneficial

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compounds towards humans and animals. Their bioactivities are, such as antioxidative, anti-inflammatory and antimicrobial activities (Carocho and CFR Ferreira, 2013; Di Carlo et al., 1999; Farhadi et al., 2019; Gomes et al., 2008). During the mid-20th century, scientists discovered the presence of phenolic compounds in the insect cuticle, wings, and intestinal tract (Simmonds, 2003). Groups of phenolic compounds found in edible insects are varied among insect species because of different types of feed they consume and the differed biosynthesis per specie (Nino et al., 2021). Sclerotization is the process by which the insect cuticle is hardened. During this process, the incorporation of phenolic compounds in the cuticular matrix occurred with the involvement of structural proteins and chitin (Andersen, 2010). These non-dietary phenolic compounds are synthesized through a series of enzymes taking place in cuticle, however, phenoloxidase enzymes are key players in this phenolic synthesize process (Sugumaran, 2010).

The novelty of this work is the knowledge on total phenolic content (TPC) present in cricket powder and its protein crude extract. The bioavailability of TPC of edible insect products in the form of cricket powder (CP) and cricket crude protein extract (CCP) at each gastrointestinal digestive phase have not been yet reported elsewhere. The high TPC content of CP and CCP could be a source of natural antioxidant, therefore understanding the change of polyphenolic compounds and antioxidant activity through gastrointestinal tract could be valuable for further study.

MATERIALS AND METHODS

Materials

Cricket powder (CP) was purchased from Global Bugs Asia Co., Ltd. (Thailand). Folin-Ciocalteu's reagent was purchased from BDH (Paris, France). Sodium carbonate and acetic acid were purchased from Merck (Darmstadt, Germany). All enzymes for *in vitro* digestion and other chemical reagents including: gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox and 2,3,5-Triphenyltetrazolium chloride (TPTZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol was purchased from QRec (Auckland, New Zealand).

Proximate analysis

Moisture content was measured in CP and CCP according to the Association of Official Analytical Chemists (AOAC, 2000) and (AOAC, 2019). As the moisture content was determined using the oven drying method, the loss weight obtained after drying was used to calculate the moisture content (AOAC, 2000). Crude fat (AOAC, 2019), crude fiber (AOAC, 2019) and crude ash content (AOAC, 2019) were measured in CP. Crude protein content (AOAC, 2019) was determined in both CP and CCP. Carbohydrate content was calculated using the method of difference. Polyphenolic compounds profile was analyzed by Liquid chromatography-mass spectrometry (LC/MS) (in-house method based on Bolivian Journal of Chemistry) (Peñarrieta et al., 2007).

Cricket crude protein extraction

Briefly, 500 g of cricket powder was mixed with 5L of deionized distill (DI) water and adjusted pH to 13 by 2 M NaOH while stirring. The mixture was stirred at 85 ºC for 30 min and then cooled down in an ice bath for 10 min. After that, the mixture was centrifuged at 6,000 rpm, 4 ºC for 30 min. The supernatant was collected, adjusted to pH 4 using 2 M HCl and centrifuged again at the same condition. The precipitate was collected and freeze dried to obtain cricket crude protein extract (CCP) and was kept at 4 ºC until use.

The simulated *in vitro* digestion

The simulated *in vitro* digestion protocol was adopted from the work of Minekus et al (2014). At oral phase, 5 g of either a mixture of CP or CCP was mixed with simulated salivary fluid (SSF) and 0.5 mL salivary α-amylase solution (1,500 U/mL), then incubated in a shaking water bath at 37 ºC for 2 min. At gastric phase, 10 mL of oral bolus from the oral phase were added with simulated gastric fluid (SGF) and 1.6 mL pepsin solution (25,000 U/mL), then the mixture was adjusted to pH 3 and incubated at 37 ºC for 2 h. At small intestinal phase, 20 mL of gastric chyme from the gastric phase were mixed with simulated intestinal fluid (SIF) and 2.5 mL fresh bile (160 mM). The mixture was adjusted to pH 7 and then 5 mL pancreatin (800 U/mL) was added, thereafter incubated in a shaking water bath at 37 ºC for 2 h. The liquid digesta was immediately snap frozen in liquid nitrogen and stored at – 20 ºC for further analysis. The thaw digesta were centrifuged and collected the supernatant for the analysis of TPC, DPPH and FRAP.

Determination of total phenolic content (TPC)

Briefly, 1 mL of samples or blank or gallic acid standard was mixed with 50 mL of DI water and 5 mL of 2 N Folin-Ciocalteu's reagent. After incubation for 5 min in dark at room temperature, 20 mL of 20% sodium carbonate solution and 24 mL of DI water were added. The reaction was mixed and continued to incubate for another 30 min in dark at room temperature. The absorbance was measured at 750 nm (Bobo-Garcia et al., 2015).

Determination of antioxidant activities

- **DPPH radical scavenging activity (DPPH assay)**

DPPH radical scavenging assay was determined according to Herald et al (2012); Huang et al (2014). Two hundred µL of DPPH solution (150 mM) was mixed with 25 µL of samples or blank or trolox standard (6.25-100 ppm). The reaction was incubated in dark

at room temperature for 30 min. The absorbance was read at 517 nm.

- **Ferric reducing antioxidant power activity (FRAP assay)**

FRAP was determined according to Sharopov et al (2015); Huang et al (2014). One hundred eighty µL of FRAP solution (300 mM acetate buffer pH 3.6, 10 mM TPTZ, 20 mM $FeCl_3$; at volume ratio 10:1:1, v/v/v) was added into 20 µL of samples or blank or trolox standard (10-100 ppm). The reaction was incubated in dark at 37 °C for 30 min. The measured absorbance was accessed at 595 nm.

Statistical analysis

All statistical tests were performed using SPSS software version 25 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to analyze statistical differences among treatment. Differences between before and after *in vitro* digestion of treatments was tested by Paired-samplet- test. A statistically significant difference was set at 95% confidence interval ($p \leq 0.05$).

RESULTS AND DISCUSSION

Chemical composition of CP and CCP

The chemical compositions of CP are shown in Table 1. The moisture and protein content of CCP were 5.46 ± 0.08% and 61.12 ± 0.37% dry basis (d.b.), respectively. The defatting step using hexane for 1h at room temperature was applied to CP prior to alkali protein extraction as it might help to increase protein content in CCP. The result showed no significant difference of protein content in CCP with and without the defatting step (data not shown). Therefore, non-defatted CP powder was used to extract CCP in this study.

Table 1. Chemical compositions of cricket powder

Composition	(% d.b.)
Protein	54.95 ±0.30
Fiber	3.33 ± 0.02
Ash	0.81 ± 0.01
Fat	13.34 ± 0.04
Carbohydrate	27.57 ± 0.13**
Total	100
Moisture	3.39 ± 0.37

*Values (means ± SD) are from two replications (n=2)

**Calculated by difference.

Total phenolic content (TPC)

The presence of TPC in CP and CCP was found at 5.87±0.14 and 30.64±4.12 mg GAE/g, respectively. The 5 folds higher in phenolic content of CCP than CP can be explained by the fact that alkali condition can liberate polyphenols from their embedded structure through the destruction of covalent bonds (Acosta-Estrada et al., 2014), therefore protein-phenolic complexes were exposed to the water phase (Hall et al., 2017). These complexes were then precipitated at isoelectric point of cricket protein at pH 4. Our finding is in accordance with the work of Kim et al (2020) who reported that TPC in crude protein extract from *Gryllus bimaculatus* was higher than its powder. Pérez-Jiménez et al (2010) showed a long list of TPC from different raw materials. Apparently, CCP contains higher TPC than any sources in that report. It is worth noted that this is the first finding to indicate a very high TPC in cricket crude protein extract.

As shown in Figure 1, TPC in CP and CCP is different at the beginning of *in vitro* digestion as of the original content. It seems that the matrix of CCP is sensitive towards gastrointestinal digestion due to the more loosely structure of CCP as discussed above, particularly at the gastric condition pH 3. It can be speculated that the alkali extraction at pH 13 can rupture or weaken some chemical bonds inside CCP, thus it becomes sensible for enzyme accessibility and for gastric condition (Qin et al., 2022 and Ma et al., 2020). Alminger et al (2014) mentioned that acidic condition causes breaking of bonds between the bioactive compounds and nutrients by the acid medium, which helps to the release polyphenolic compounds from the food matrix. With the low stability polyphenols, the pH change during digestion can have a remarkable impact (Ortega et al., 2011).

TPC of CP after gastric and intestinal conditions was slightly decreased ($p \geq 0.05$), which can be implied that the complex structure of CP involving non-covalent and covalent bonding between polyphenol-protein, protein-carbohydrate, or carbohydrate-lipid is able to protect phenolic compounds embedded inside (Debelo et al., 2020). Domínguez-Avila et al (2017) reported that lipid and carbohydrate may protect phenolic compounds at small intestinal phase better than gastric phase. At small intestinal phase, total phenolic content of CCP significantly decreased from the gastric phase ($p \leq 0.05$), while CP did not, probably due to the structural differences of the two samples. The antioxidant activity of the digesta should be derived from TPC but not the protein hydrolysate, otherwise the activity should have been increased due to the protein digestion.

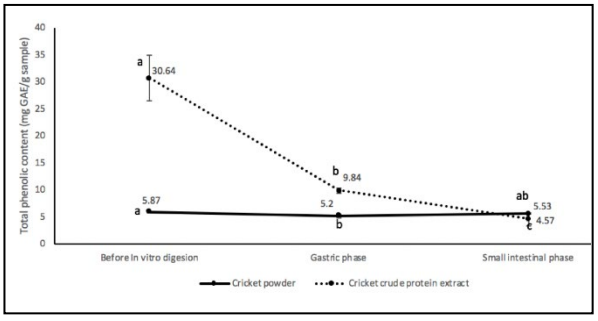

Figure 1. Total phenolic content of cricket protein (CP) and cricket crude protein extract (CCP) before digestion, after gastric and small intestinal phase. *Values (means ± SD) are from three independent x 3 replications (n=9). Different superscript letters in the same line are significantly different ($p \leq 0.05$).

Polyphenolic profile

Polyphenolic profile was analyzed by liquid chromatography-mass spectrometry (LC/MS). The values are from one replication due to cost of analysis. Gallic acid, isoquercetin, quercetin and tannic acid were found in both CP and CCP, while apigenin could only be found in CP (Table 2). There are many phenolic compounds which have been reported in edible insect species, for instance, tricin, luteolin, orientin, iso-orientin, vitexin, isovitexin, isorhamnetin, myricetin, ferulic acid etc. (Nino et al., 2021). In our study, although TPC was found to be higher in CCP over CP, but the sum of individual analyzed phenolic compounds in Table 2 is lower in CCP. This might be due to the limited knowledge available regarding phenolic compounds in an individual insect species, therefore it was very challenging to include all phenolic compounds in edible insects reported in literature in this measurement. The digesta of CP and CCP could not be measured the polyphenolic profile because of too low TPC concentration in the digesta.

Antioxidant activity

Antioxidant activity was expressed as DPPH and FRAP values (TEAC mg Trolox/mL) of CP and CCP. They were determined before and after passing through the *in vitro* digestion. Different weight of CP and CCP (0.25, 0.5 and 1 g) were tested to assure the capability of the assays to deal with *in vitro* digested cricket protein. As shown in Table 3 and 4, DPPH and FRAP antioxidant activity increased when higher concentration of samples was used at the start of *in vitro* digestion. Antioxidant activity assessment from both assays of CCP at 0.25, 0.5 and 1 g is higher than those of CP due to the higher presence of phenolic compounds. Although higher TPC in CCP, but after digestion, the activity is relatively similar to CP. This trend is in accordance with the decrease of TPC which have previously been discussed. Our finding is in accordance with Zhou et al (2009) who reported the direct relationship between DPPH and TPC.

Table 2. Polyphenolic compounds found in cricket powder and cricket crude protein extract

Phenolic compounds	Cricket powder (mg/kg)	Cricket crude protein extract (mg/kg)
Gallic acid	1.68	0.74
Eriodictyol	nd	nd
Apigenin	0.51	nd
Isoquercetin	0.62	0.53
Kaempferol	nd	nd
Quercetin	1.07	0.67
Hydroquinin	nd	nd
Rutin	nd	nd
Catechin	nd	nd
Tannic acid	0.86	1.02
Total	4.74	2.96

*nd: not detected

Table 3. Antioxidant activity assessment via DPPH assay before-after passing through the simulated *in vitro* digestion

Weight of samples	DPPH TEAC (mg Trolox/ mL sample)			
	CP (Before *in vitro* digestion)	CP (After *in vitro* digestion)	CCP (Before *in vitro* digestion)	CCP (After *in vitro* digestion)
0.25 g	11.81 ± 0.55^{Aa}	0.32 ± 0.13^{Ba}	16.57 ± 0.73^{Aa}	0.77 ± 0.28^{Ba}
0.5 g	23.62 ± 1.10^{Ab}	0.66 ± 0.06^{Bab}	33.14 ± 1.46^{Ab}	0.96 ± 0.06^{Ba}
1 g	47.24 ± 2.20^{Ac}	0.95 ± 0.37^{Bb}	66.29 ± 2.93^{Ac}	1.94 ± 0.64^{Bb}

*Values (means ± SD) are from three independent x 3 replications (n=9). Values in the same row before-after in vitro digestion superscripted with different capital letters are significantly different ($p \leq 0.05$). Values in the same column superscripted with different lowercase letters are significantly different ($p \leq 0.05$).

Table 4. Antioxidant activity assessment via FRAP assay before-after passing through the simulated *in vitro* digestion

Weight of samples	FRAP TEAC (mg Trolox/ mL sample)			
	CP (Before *in vitro* digestion)	CP (After *in vitro* digestion)	CCP (Before *in vitro* digestion)	CCP (After *in vitro* digestion)
0.25 g	3.76 ± 0.03^{Aa}	0.82 ± 0.14^{Ba}	9.64 ± 0.24^{Aa}	1.06 ± 0.04^{Ba}
0.5 g	7.53 ± 0.05^{Ab}	1.8 ± 0.70^{Ba}	19.27 ± 0.47^{Ab}	2.32 ± 0.18^{Bb}
1 g	15.07 ± 0.09^{Ac}	2.83 ± 0.49^{Bb}	38.55 ± 0.94^{Ac}	4.05 ± 0.47^{Bc}

*Values (means ± SD) are from three independent x 3 replications (n=9). Values in the same row before-after in vitro digestion superscripted with different capital letters are significantly different ($p \leq 0.05$). Values in the same column superscripted with different lowercase letters are significantly different ($p \leq 0.05$)

CONCLUSIONS

In summary, there is a considerable amount of phenolic compounds presence in cricket powder (CP). Protein extraction using alkali condition followed by isoelectric point precipitation could enrich protein yield approximately 5% and phenolic content by up to 5 times. However, the loss of phenolic compounds in cricket crude protein extract (CCP) was dramatically decreased at the gastric phase, was remarkable. It seems that gastric condition and digestive enzymes play a critical impact on the loss of phenolic compounds in CCP but not CP. As high concentration of polyphenolic compounds was found in CCP, techniques to help protecting those compounds from food processing condition or from gastro-intestinal digestion could be done in the future.

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