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Evaluation of *In-Vitro* Free Radicals Scavenging Activity of Edible Insects

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ABSTRACT

This research evaluates the *in vitro* free radicals scavenging activities of edible insects. The insects used in this research were Silkworm *(Bombyx mori)* and Crickets *(Gryllidae)*. The insects ento-chemicals compounds were extracted by using water, ethanol, and hexane. The extraction yields of the two insects studied range from 3.32%-18.57%. The antioxidant value of the insects was also studied. The DPPH assay was utilized to study the antioxidant value and scavenging activities of the insects. According to the findings, Crickets and Silkworm show a significant amount of antioxidant values, with Crickets have generally higher free radical scavenging activity compound ranging from 67-82%, compared to the Silkworm which ranges from 61-81%. In this work, the antioxidant activity of edible insects was examined in vitro, with a particular emphasis placed on the insects' capacity to quench the activity of free radicals. This study highlights the potential of edible insects as a source of natural antioxidants and suggests that they may be a valuable addition to the diet as a means of reducing the risk of chronic diseases associated with oxidative stress.

1. Introduction

Insects are being sought not just for traditional consumption and high nutritional value, but also as possible sources of bioactive components such as peptides, polysaccharides, fatty acids, and polyphenols [1]. Insects are also highly desired for their medicinal properties. According to the history of research studies, insects have been researched and utilized as a source of medicinal products since ancient times. Insects and insect-derived products have been used as medicinal products in various regions of the world since ancient times. Honey, for example, is used to heal burns. Honey and beeswax were used together to treat various dermatological conditions, including tinea, pityriasis

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versicolor, atopic dermatitis, and diaper dermatitis [2]. In ancient cultures, insects were found and used as medicine, and scientists are currently researching and aiming to find various natural products derived from insects [2].

Entomophagy, or the eating of insects and invertebrates, has been part of human history for generations, playing an important role in cultural and religious practices. According to the Food and Agriculture Organization [3], the insect is consumed by at least two billion people worldwide. Beetles (Coleoptera), African caterpillars (Lepidoptera), and bees, wasps, and ants (Hymenoptera) account for 31.8%, 18.4%, and 14% of global insect consumption, respectively. However, in most Western countries, eating insects is often regarded as a primitive practice that causes disgust due to insect morphologies. A renewed global interest in entomophagy and invertebrates emerges from a dire need to preserve agricultural resources and drastically reduce the ecological impact of the consumption of animals on the planet. The composite nutritional content, which is a direct result of plant-based feeding, along with the undeniably ecological attributes, suggests that insects could play a role as sustainable and functional foods [4].

Free radicals are constantly produced by the body during its regular metabolic activities, but oxidative stress develops when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of cells to neutralize them. The harmful effect of ROS/RNS that causes biological damage is known as oxidative stress and nitrosative stress [5]. Excess ROS can damage cellular lipids, proteins, or DNA, interfering with their normal function [6]. Oxidative stress can occur when cells are unable to eliminate an overabundance of free radicals. To put it another way, oxidative stress is caused by an imbalance in the generation and neutralization of ROS/RNS [7]. Excess hydroxyl radicals and peroxynitrite, for example, can damage cell membranes and lipoproteins through a process known as lipid peroxidation. This reaction produces malondialdehyde (MDA) and conjugated diene molecules, both of which are cytotoxic and mutagenic. Lipid peroxidation is caused by a radical chain reaction, which implies that once it begins, it spreads quickly and impacts a large number of lipid molecules [8]. Proteins can also be harmed by ROS/RNS, resulting in structural alterations and decreased enzyme function [9]. When DNA is oxidatively damaged, distinct oxidative DNA lesions occur, which can cause mutations. The body has many mechanisms in place to defend itself against these attacks, including DNA repair enzymes and/or antioxidants [10]. If not effectively controlled, oxidative stress can cause an array of chronic and degenerative diseases, as well as the aging process and some acute pathologies such as trauma and stroke [11].

Oxidative stress has been correlated to the development of "civilization diseases" such as cancer, stroke, myocardial infarction, or inflammation, as well as the degenerative process associated with ageing, such as Parkinson's and Alzheimer's disease. The consumption of antioxidant-rich foods plays a vital role in the prevention of multiple illnesses. Numerous studies have shown that antioxidant and anti-inflammatory peptides contain anti-ROS properties and may lead to a major decrease in oxidative stress [12].

Despite the fact that some people still see eating as an unappealing habit, insects and invertebrates are commonly fed whole, entomophagy is becoming an increasing trend in human nutrition in European countries. Meanwhile, entomophagy is not a foreign dietary practice in Malaysia, but acceptance of insects as food among Malaysians is still unclear [13]. Although it is widely acknowledged that insects offer a rich source of valuable proteins, minerals, vitamins, and fatty acids while having little environmental impact, little is known about their therapeutic value as a source of natural antioxidant. This research shows that commercially available edible insects can be beneficial sources of antioxidants, with efficiency based on taxonomy and feeding behaviors. Consumption of antioxidant-rich foods such as fruits and vegetables help in the prevention of oxidative stress-related diseases such as cardiovascular disease, diabetes, and cancer [14].

The nutritional value of edible insects varies greatly due to a broad range of edible insect species. Insects, on the other hand, have a high protein content that ranges from 20% to 76% of dry matter depending on the species and stage of development [15]. As a result, eating insects may be an excellent source of bioactive peptides. However, there is insufficient proof in the literature of a screening of the antioxidant properties of insects and invertebrates that is appropriate for human consumption. The purpose of this research is to investigate the oxidative stress generated by commercially available edible insects from various species and eating habits in order to determine whether they have an antioxidant impact in vitro.

2. Methodology

2.1 Sample Preparation

The sample preparation process included two primary steps: drying and grinding. The edible bug samples were dried for 48 hours in a convection oven dryer set to 40°C. Then, it was grinded with the D3V-10 Wonder Crush/Mill Tilt. The powdered insects then were stored in the 50ml centrifuge tube to use.

2.2 Extraction of Edible Insects

The extraction of insects involves numerous processes and techniques. The Buamard and Benjakul (2015) [16] method was used in this study. The insects were extracted using the Rapid Solvent Extraction Method using 80% ethanol. In amber-colored extraction vials, 34g of powdered insects were soaked in a 100ml solution of 80% ethanol (v/v) in a 70:30 ratio at room temperature (26°C to 30°C). The conical flask will be sealed with aluminum foil sheets and labeled. The Silkworm and Cricket extract solutions were then incubated for 72 hours with sporadic stirring and shaking at 5°C. Following that, the insect extracts will be separated and filtered using Whatman No.1 filter sheets. To get 30g extracts of samples, the filtrates will be concentrated under a reduced pressure at 50 °C using a rotary evaporator (Bibby Sterlin Ltd, UK). The previously described processes will next be performed with ethanol and water as solvents.

$$Y\ extract\ (\%) = \frac{M\ extract}{m\ feed} \times 100 \quad (1)$$

where, Y extract is the extraction yield, M extract is mass of crude extract (g) and M feed is the mass of the feed (g).

2.3 Total Phenolic Content of Edible Insects

The total phenolic content was determined using the calorimetric method as described by Annissworth *et al.,* [17]. The reaction mixture was prepared by mixing 0.25 mL sample/standard, 0.25 mL of 10% Folin-Ciocalteu's reagent dissolved in 2.25 mL of deionized water and let it stand for 5 mins. Followed by the addition of 2.5 mL of 7% Sodium Carbonate (Na_2CO_3) solution. The mixture was mixed thoroughly and kept in the dark at room temperature for 1.5 h. The blank solution was also prepared by using gallic acid at a concentration range of 50-500mg/L. The absorbance was recorded using a plate reader at 760 nm. All the analysis was repeated three times and the mean value of absorbance was obtained. Total phenolic content was determined by extrapolating the

calibration line which was construed by gallic acid solution. The TPC was expressed as gallic acid equivalent (mg GAE) per gram of the dried sample.

2.4 Total Flavonoid Content of Edible Insects

The total flavonoid content of Bombyx mori and *Gryllus bimaculatus* was determined by using aluminum chloride calorimetric method based on the methodology reported by Afify *et al.,* [18] with some modifications. 0.5 mL of sample (1mg/mL) was mixed with 1mL of 10% aluminum chloride, 1mL of potassium acetate (1M) and 2.5 mL of distilled water. Rutin was used to make the calibration curve. The absorbance of the mixtures was measured at 405nm. The total flavonoid content was expressed in terms of rutin equivalent (mg QE/g of sample). All the analyses were repeated three times and the mean value of absorbance was obtained.

2.5 Determination of Antioxidant Capacity of Edible Insects

A single antioxidant test model should not be used to determine antioxidant activity. In practice, numerous in vitro test techniques are used to assess antioxidant activity in the samples of interest. Another consideration is that antioxidant test models differ in various ways. As a result, it is difficult to thoroughly compare one procedure to another. There are several in-vitro ways of assessing free radical scavenging activities. In this research, the Silkworm and Crickets will be extracted by using different solvent extraction. The crude extracted sample will also be analysed for the percentage of antioxidant activities using DPPH assay.

2.5.1 Determination of Antioxidant Capacity by DPPH Assay

The inhibitory activity of the samples extracted will be compared to that of well-characterized antioxidants. The molecule 2,2-Diphenyl-1-picrylhydrazyl (,-diphenyl—picrylhydrazyl; DPPH) is characterized as a persistent free radical due to the delocalization of the spare electron over the molecule as a whole, preventing dimerization, as most other free radicals do. Without direct light exposure, the assay produces a concentrated violet solution that is constant at room temperature. The deep violet colour is also caused by electron delocalization, which is characterized by an absorption band in ethanol solution centred at about 517 nm. When a DPPH solution is combined with a substrate (AH) that may donate a hydrogen atom, the reduced form is formed with the loss of the violet colour. Figure 1 show the scavenging activity as below.

Fig 1. DPPH scavenging activity

The DPPH solution is prepared by dissolving 4mg of DPPH in 100 mL of methanol. The change in optical density of DPPH radicals is measured to evaluate the antioxidant capability of the test samples through free radical scavenging. The sample extract (1 mL) is diluted with methanol before being

mixed with 2 mL DPPH solution (0.5 mM). The absorbance is measured at 517 nm after 30 minutes [19]. The following equation is used to compute the percentage of DPPH radical scavenging:

$$\%\ inhibition\ of\ DPPH\ radical\ = \frac{(Abr - Aar)}{Abr} \times 100 \quad (2)$$

where *Abr* is the absorbance with standard and *Aar* is the absorbance with insect sample extract.

Antioxidants engage in a reaction with DPPH, a stable free radical. As a result of this reaction, DPPH is converted to DPPH-H, and the absorbance of the DPPH radical is lowered in comparison to the DPPH-H form. The degree of discoloration indicates the antioxidant chemicals' or extracts' radical-scavenging capacity, as evaluated by their ability to donate hydrogen. Because of its capacity to absorb light, the stable 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical has a brilliant violet hue. This is due to its (separated) conjugated structure, which consists of phenyl groups joined by unpaired electrons from the radical group. When anti-oxidants consume unpaired electrons, the conjugated system appears to lose its ability to absorb (at least in the optical range) and turns colourless [20].

3. Results

3.1 Extraction of Edible Insects

Three solvents such as 80% ethanol, water, and methanol were used during the extraction procedure to successfully extract from two different types of edible insects. Table 1 shows the amount of edible insect extract collected from each of the two different types of insects.

Table 1
Extraction Yield of Edible Insects Extract

Variety of Insect	Solvent Used (100 ml)	Weight of Extracts (g)	Extraction Yield (%)
Silkworm	Ethanol 80%	34	7.78
	Distilled Water	34	3.32
	Hexane	34	7.82
Crickets	Ethanol 80%	34	18.01
	Distilled Water	34	18.57
	Hexane	34	18.09

3.2 Total Phenolic and Flavonoid Contents of the Edible Insects

Both crickets and silkworm moth show a noticeable amount of total phenolic and flavonoid content. Both insects that were extracted using hexane showed lower amounts of TPC and TFC compared to the samples that were extracted using other solvents, proving the theory that TFC and TPC in edible insects are solvent-dependent, as well as the correlation between the polarity of the solvent and amount of TPC and TFC, contained in the extract (Figure 2). Since hexane is a non-polar solvent, thus the total phenolic and flavonoid content was lower than the insects that were extracted using higher polarity solvents such as distilled water and ethanol 80%.

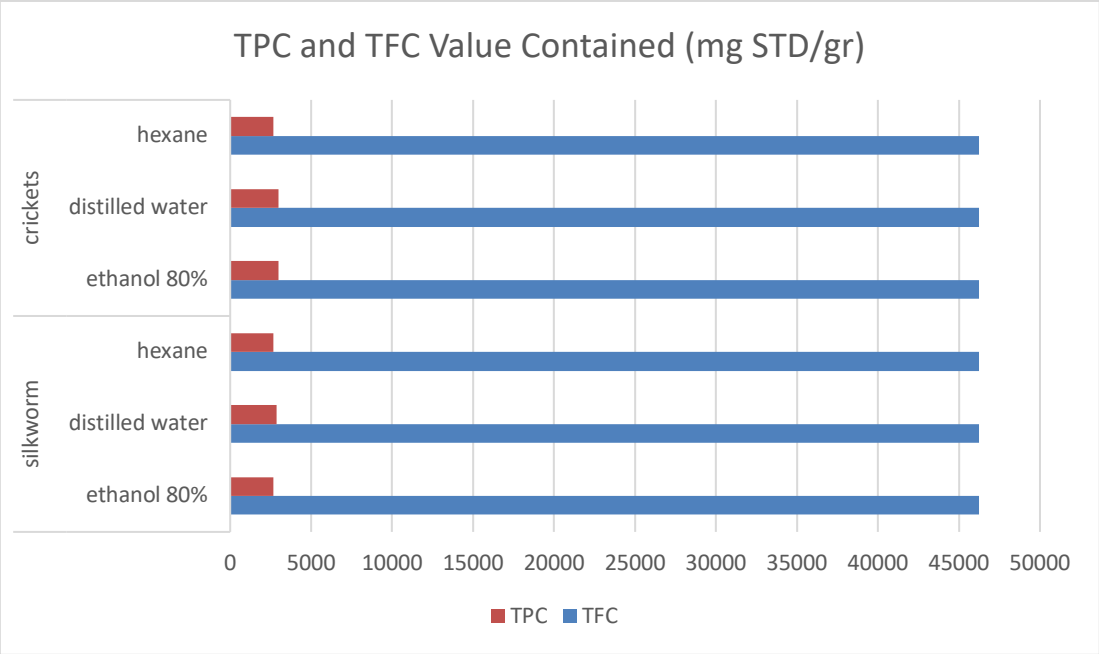

Fig. 2. Total phenolic and flavonoid compound contained in silkworm and crickets

To fully comprehend the possible health advantages of the insects, it is important to understand the correlation between total phenolic and flavonoid levels and antioxidant activity. Increased antioxidant activity is often indicated by increased total phenolic and flavonoid levels. As a result, the results imply that extracts from silkworms and crickets that were prepared with ethanol at 80% and distilled water ought to have greater antioxidant capacity than extracts that were prepared using hexane.

The number of flavonoids and phenolic chemicals in the extracts can vary greatly depending on the extraction solvent used. When it relates to extracting such compounds, polar solvents like ethanol 80 percent and distilled water perform more effectively than non-polar solvents like hexane. This is due to the fact that polar solvents are easier to dissolve and extract flavonoids and other polar, hydrophilic phenolic chemicals from insect tissues [21].

It has been established that phenolic chemicals contribute significantly to the antioxidant capacity of a wide range of foods and biological systems. These substances can serve as a buffer against oxidative stress and damage from free radicals, which are unstable chemicals that can injure tissues and cells. Since phenolic compounds have the ability to effectively neutralize free radicals and prevent oxidative damage, bigger amounts of these compounds are often associated with higher levels of antioxidant activity [21].

A specific type of phenolic compound that is well-known for its strong antioxidant capabilities is flavonoids. They are present in a wide range of plant-based diets and have been linked to several health advantages, such as lowering inflammation and enhancing cardiovascular function. Since flavonoids may effectively neutralize free radicals and prevent oxidative damage, larger amounts of these chemicals are often associated with higher levels of antioxidant activity [21].

The value of TPC and TFC were calculated by using the calibration curve that was obtained from the Figure 3 and 4 below by plotting the data by using the Eq. (3) below;

$$y = mx + c \tag{3}$$

In which that x was 25, y-axis was the absorbance value at respective values, and x-axis were the concentration range in μg/l.

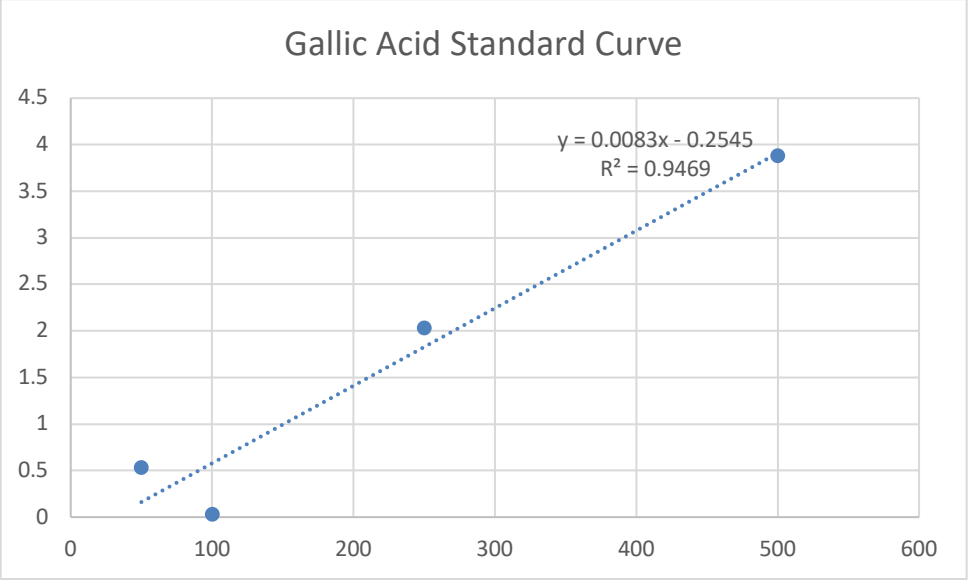

Fig 3. Total phenolic content calibration curve at 760nm absorbance

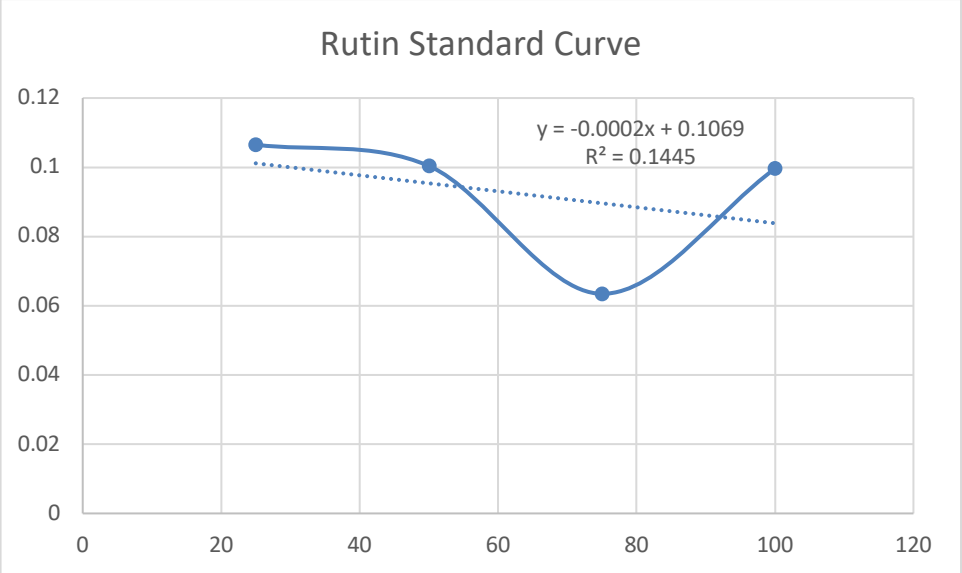

Fig 4. Total flavonoid content calibration curve at 405nm absorbance

3.3 Antioxidant of Edible Insects (DPPH Assay)

The medicinal use of insects is linked to their ability to scavenge free radicals and act as an antioxidant. Insects have been found to be beneficial to human health. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) technique was used in this work to assess the antioxidant activity of edible insect extracts. The DPPH radical is a stable organic nitrogen radical, and the method is relatively straightforward to perform; all of these features may contribute to the test's prominence in antioxidant screening. Figure 5 shows the DPPH radial scavenging activity obtained from two different types of edible insect extracts.

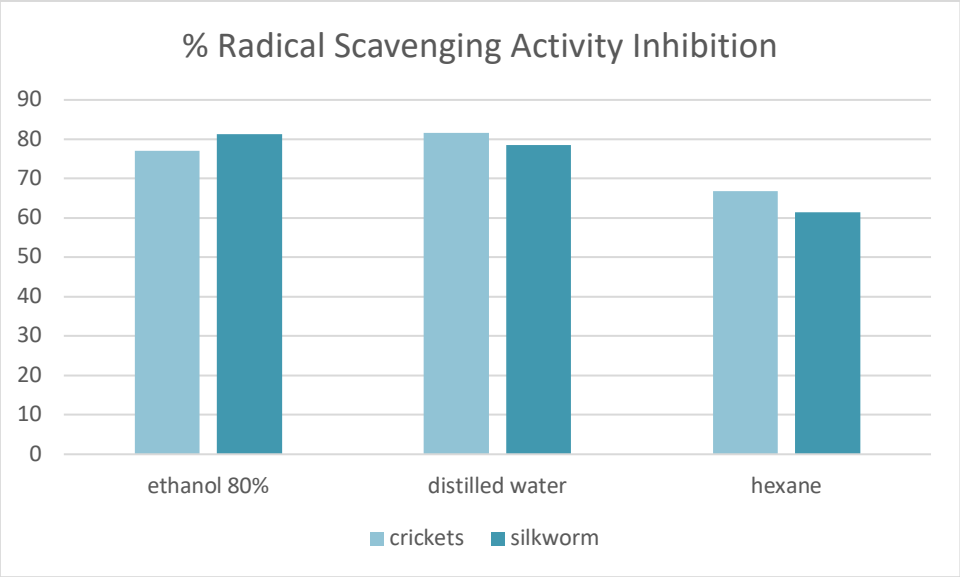

Fig. 5 DPPH free radical scavenging capacities of insects

The data indicates that both crickets and silkworm moth DPPH radical scavenging activity varies depending on the solvent used. Both insects that were extracted using ethanol 80% and distilled water showed higher DPPH radical scavenging activity compared to the samples that were extracted using hexane, proving that polar solvents and water are more effective in extracting antioxidant compounds from the insects compared to non-polar solvents such as hexane.

The difference of DPPH scavenging activity value between crickets and silkworm may be due to various reasons such as protein content, hydrolysis of proteins, solubility of antioxidants, and different antioxidant compounds. According to Kurdi *et al.,* [22], higher protein content can contribute to higher antioxidant activity, since crickets have a higher protein content compared to silkworms.

Hydrolysis of proteins in crickets can release free oligopeptides and amino acids which are known to release free radical scavenging activity. The process might be more effective in crickets due to their high protein content compared to silkworms.

The difference in antioxidant compounds that are present in the extract may be one of the reasons that cause cricket to be more effective in scavenging DPPH radicals. For example, silkworms have been found to contain melanin, which contributes to their antioxidant value, whereas crickets have a different set of antioxidant compounds that might be more effective in scavenging DPPH radicals.

4. Conclusions

It can be concluded that Crickets and Silkworm Moth are a rich source of bioactive compounds with antioxidant and anti-inflammatory activities. Higher total phenolic and flavonoid compound generally indicates higher antioxidant activity. The choice of solvent impacts the total phenolic compounds, total flavonoid compounds, DPPH radical scavenging activity, and also the extraction of the insects significantly. Polar solvents like ethanol and water are more effective in extracting these antioxidant compounds, which may lead to higher antioxidant activity. The TPC ranges from 2656-2989 mg GAE/g. Whereas the TFC ranges from 46242-46248 mg RT/g. The DPPH radical scavenging activity inhibition ranges from 61%-82%, with crickets extracted by water being the highest inhibitory of radical scavenging activity compound. From the data discussed in the previous section, it is found

that edible insects have antioxidant impacts *in-vitro*. In conclusion, the data obtained from this research shows promising results that commercially available edible insects can be beneficial sources of antioxidants, with efficiency based on taxonomy and feeding behaviours, which have the possibility to become one of the replacement sources of antioxidants besides fruits.

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