ORIGINAL ARTICLE

Effects of different heat treatments on the antioxidant activity and ascorbic acid content of bitter melon, *Momordica charantia*

Efeitos de diferentes tratamentos térmicos na atividade antioxidante e no conteúdo de ácido ascórbico de melão amargo (Momordica charantia)

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Abstract

This study investigated the effects of different heat treatments, namely boiling, microwaving and pressure cooking, on the reduced ascorbic acid content and antioxidant activity in *Momordica charantia* extracts. The total antioxidant activity of *M. charantia* extracts was determined by ferric reducing-antioxidant power and 2,2-diphenyl-1-picrylhydrazyl radical scavenging assays. The reduced ascorbic acid content was estimated by ferric reducing-antioxidant power-ascorbic acid assay and confirmed by high performance liquid chromatography. Regardless of the cooking method, all cooked *M. charantia* extracts had significantly ($p < 0.05$) higher total antioxidant activity, radical scavenging potency and reduced ascorbic acid content than the uncooked sample. The decreasing order of reduced ascorbic acid in *M. charantia* was as follows: microwaved > boiled > pressure-cooked > uncooked. The reduced ascorbic acid in *M. charantia* strongly correlated with the total antioxidant activity ($r = 0.958$, $p < 0.05$) and radical scavenging potency ($r = 0.951$, $p < 0.05$). The principle component analysis showed that 87% of the variation in the antioxidant values of *M. charantia* extracts was attributed to the different cooking methods. Heat treatment either by boiling or microwaving for a short period of time (10 minutes) improved the reduced ascorbic acid content in the *M. charantia* extract. This study showed that the reduced ascorbic acid could be retained under high temperature and pressure in *M. charantia* extracts.

Keywords: Ascorbic acid; Antioxidant activity; Thermal processing; High performance liquid chromatography; *Momordica charantia*.

Resumo

Este estudo investiga o efeito de diferentes tratamentos térmicos, a saber, fervura, cozimento em micro-ondas e cozimento sob pressão sobre o ácido ascórbico e a atividade antioxidante no extrato de *Momordica charantia*. A atividade antioxidante total dos extratos de *M. charantia* foi determinada pelo poder redutor-antioxidante férrico e pelos ensaios de eliminação de radicais 2,2-difenil-1-picrifirazol. O teor de ácido ascórbico foi estimado pelo ensaio de redução do poder férrico-antioxidante-ácido ascórbico e confirmado por cromatografia líquida de alta eficiência. Independentemente do método de cocção, todos os extratos de *M. charantia* cozidos apresentaram ($p < 0,05$) maior atividade antioxidante total, potência de eliminação de radicais livres e teor de ácido ascórbico, quando comparados com a amostra não tratada termicamente. A ordem decrescente de ácido ascórbico reduzido em *M. charantia* foi a seguinte: micro-ondas > fervura > cozimento sob pressão > não tratado termicamente. O ácido ascórbico reduzido em *M. charantia* correlacionou-se fortemente com a atividade antioxidante total ($r = 0,958$, $p < 0,05$) e potência de eliminação de radicais livres ($r = 0,951$, $p < 0,05$). A análise dos componentes principais mostrou que 87% de variação nos valores antioxidantes do extrato de *M. charantia* foi atribuída aos diferentes métodos de cozimento. O tratamento térmico por ebulição ou micro-ondas por um curto período de tempo (10 minutos) melhorou o teor de ácido ascórbico reduzido no extrato de *M. charantia*. Este estudo mostra que o ácido ascórbico pode ser retido sob alta temperatura e pressão no extrato de *M. charantia*.

Palavras-chave: Ácido ascórbico; Atividade antioxidante; Processamento térmico; Cromatografia líquida de alta performance; *Momordica charantia*.

1 Introduction

Antioxidants are chemical compounds that prevent or delay the oxidation of other molecules either by scavenging or preventing free radical formation (Apak et al., 2007). The natural antioxidants found in fruits and vegetables, such as polyphenols, are reported to correlate with the reduced risk of chronic human diseases such as cancer, cardiovascular diseases, hypertension and diabetes mellitus (Zhang & Hamauzu, 2004). Ascorbic acid is commonly known as vitamin C. This water-soluble antioxidant protects the cell membrane against lipid oxidation and helps to regenerate other antioxidants (Valko et al., 2006). In fresh fruits and vegetables, the most common form of ascorbic acid is L-ascorbic acid (L-AA) (Munyaka et al., 2010).

The introduction of the heat treatment of fruits and vegetables has been reported to affect the nutritional content and antioxidant activity of the fruits and vegetables (Zhang & Hamauzu, 2004). Previously, the present authors showed that the appropriate heat treatment may improve or preserve the nutritional values of selected vegetables and mushrooms (Ng et al., 2011; Ng & Rosman, 2019; Ng & Tan, 2017). Interestingly, boiling significantly increased the phenolic content (> 30%) and total antioxidant activity (> 200%) of bitter melon (*Momordica charantia*). Although the phenolic content was shown to be a significant contributor to the antioxidant activity of the cooked *M. charantia* extracts, the total antioxidant activity determined was higher than could be explained on the basis of the phenolic content, and the authors did not exclude the likelihood that the high antioxidant activity detected in the cooked *M. charantia* extracts could also be attributed to L-AA, since *M. charantia* is rich in L-AA (Grover & Yadav, 2004).

Since L-AA is more labile than other antioxidants, its concentration in fruits and vegetables could be altered by different heat treatments such as cooking (Munyaka et al., 2010). In the Asian cuisine, *M. charantia* is usually cooked by different methods prior to consumption, such as steaming, microwaving and boiling. The aim of this study was to investigate the effects of different types of wet heat treatments, namely boiling, microwaving and pressure cooking on the L-AA content of *M. charantia* extracts, and to determine the contribution of L-AA to the antioxidant activity of the cooked *M. charantia* extracts. The findings of this study could provide an insight to the heat processing techniques that could preserve the L-AA and antioxidant activity in the fruits and vegetables.

2 Material and methods

2.1 Chemicals

All the chemicals used in this study were of analytical grade except for those used in the HPLC analysis. Absolute methanol (CH_4O), ethanol (C_2H_6O), fuming hydrochloric acid (HCl), glacial acetic acid ($C_2H_4O_2$) and ferric (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), were purchased from Merck (Darmstadt, Germany). Ferrous (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$), L-ascorbic acid, L-ascorbic oxidase (EC 1.10.3.3), sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Sample preparation and processing

Bitter melons (*Momordica charantia*) (approximately 2000 g) were purchased from TESCO™ Extra hypermarket in Seremban, Malaysia and processed as previously described (Ng et al., 2011, 2014). Briefly, the seeds were removed, and the edible parts of the fruits washed, dab dried and sliced into small pieces prior to weighing. 150 g of sample in 200 mL of distilled water were used for each cooking method. A cooking time of 10 minutes was applied to simulate the usual domestic cooking processes (Suresh et al., 2007). For microwave cooking, the vegetable sample was microwaved in the centre position of a commercial 1000W-domestic microwave oven (model: NN-CT579S, Panasonic Co., Osaka, Japan) at a high power setting operating of 2450 MHz for 10 minutes. For boiling, a hot plate was used to boil the vegetable at 100 °C for 10 minutes and the water temperature was monitored using a mercury thermometer. For pressure cooking, the vegetable was pressure cooked in a commercial pressure cooker (model: Buffalo QWP403, Ni Hsin Co. Sdn Bhd, Selangor, Malaysia) at a high pressure setting (15 pounds per square inch or 103.4 kPa) at 121 °C for 20 minutes (including 5 minutes for the water to boil, 10 minutes of cooking and 5 minutes for the release of the steam pressure). The glass beakers were loosely covered to prevent water evaporation during cooking. The internal temperatures of the boiled, microwaved and pressure-cooked vegetable samples (triplicates), measured using a digital food thermometer, were 79.3 ± 1.1 °C, 84.3 ± 1.6 °C and 72.4 ± 0.9 °C respectively. All the cooked vegetable samples were cooled rapidly on ice to prevent further cooking by the residual heat. The cooked vegetable samples were homogenised with the cooking water in an electric blender. For the raw (uncooked) control samples, 150 g of the vegetable were homogenized in 200 mL of distilled water. The homogenates were filtered through cheese cloth and centrifuged at 2400x*g* for 15 minutes. The clear vegetable extracts were freeze dried and maintained at -20 °C until analysed. The freeze dried extracts were reconstituted with distilled water and analysed in triplicate in a single run.

2.3 Ferric-reducing Antioxidant Power (FRAP) assay

The total antioxidant activities of the *M. charantia* extracts were determined by the FRAP assay according to Benzie & Strain (1996). A standard curve was prepared using a ferrous sulphate solution (0-1000 µM). The FRAP values were expressed as mmol ferrous sulphate equivalent per 100 g freeze dried extract (mmol FE/100 g).

2.4 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay

The radical scavenging activities of the *M. charantia* extracts were determined using DPPH radicals as outlined by Gerhäuser et al. (2003). A standard curve was prepared using a L-AA solution (0-1000 µM). The half maximal inhibitory concentration (IC_{50}) for the L-AA standard was 23.45 mmol/l. The DPPH radical scavenging activity was expressed as the percentage inhibition of the DPPH radicals (%).

2.5 Ferric-reducing antioxidant power-ascorbic acid (FRASC) assay

The L-AA contents of the *M. charantia* extracts were determined according to Szeto et al. (2002) with some modifications. In a paired treatment, the *M. charantia* extracts (100 μl of 5 mg/mL) were incubated with and without L-ascorbic acid oxidase (L-AAO) (EC 1.10.3.3) (5 IU/mL) at 37 °C for 5 minutes, and then chilled on ice to terminate the L-AAO reaction. The total antioxidant activities of the samples were then determined using the FRAP assay. The difference in the absorbance values between the L-AAO treated and untreated *M. charantia* extracts corresponded to the L-AA content in the sample extracts. A standard curve was prepared using a L-AA solution (0-150 μg/mL) in order to calculate the L-AA content of the *M. charantia* extracts. The L-AA content was expressed as mg per 100 g of freeze dried extract (mg/100 g).

2.6 HPLC analysis of the L-AA

The L-AA content was confirmed using high performance liquid chromatography (HPLC) according to De Quirós et al. (2001), with some modifications. The *M. charantia* extracts (5 mg/mL) were reconstituted using distilled water and filtered before the HPLC analysis. The filtered extracts (20 μL) were injected into an HPLC system (Waters Delta Prep, Waters Co., Milford, Massachusetts, United States) equipped with a dual λ absorbance detector (ultraviolet detector), and a Purospher STAR column (100 mm x 4.6 mm I.D. Purospher, 5 μm, LiChrocart RP-18) (Merck® KGaA, Darmstadt, Germany), and using HPLC-grade methanol/water (15/85; v/v) as the mobile phase at a flow rate of 0.90 mL/min. The chromatograms were recorded at 254 nm and an external linear L-AA standard curve ($R^2 = 0.9367$) was used to quantify the L-AA in the raw (uncooked) and cooked *M. charantia* extracts (Figure 1). The L-AA standards used for quantification ranged from 0.50 to 20.0 μg/mL (Figure 1a), based on the area under the peak. The L-AA content was expressed as mg per 100 g of freeze dried extract (mg/100 g).
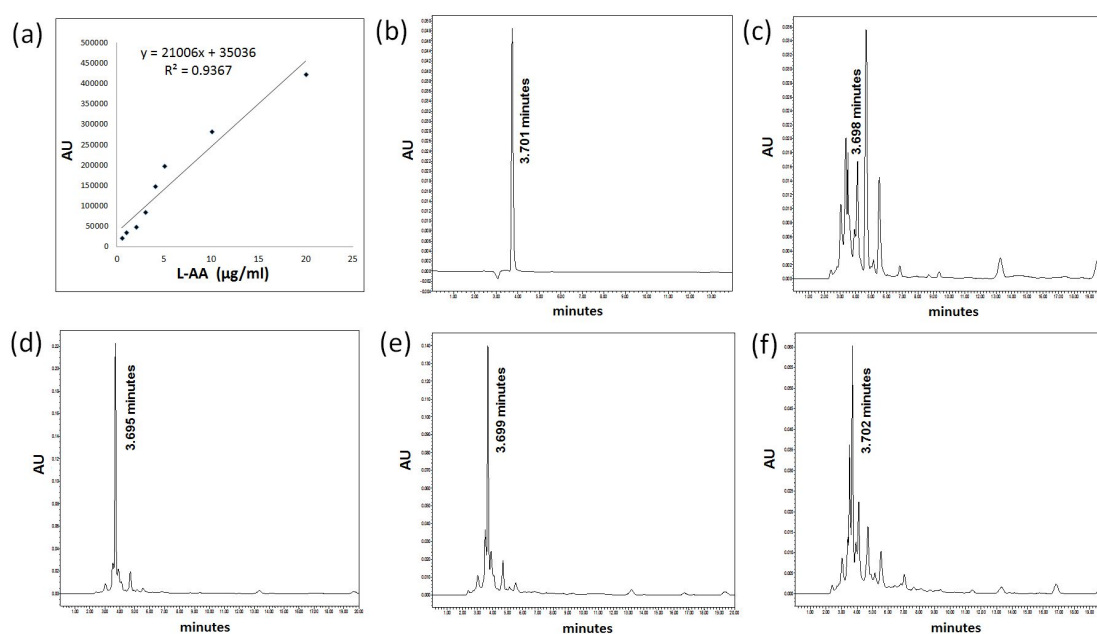

Figure 1. HPLC analysis of L-AA in the *M. charantia* extracts (a) 8-point standard calibration curve (b) L-AA standard 20 μg/mL (c) raw sample (d) boiled sample (e) microwaved sample (f) pressure cooked sample. AU denotes arbitrary units.

2.7 Statistical analysis

All the data were expressed as the mean ± standard deviation (SD) (triplicates). The differences between the means for the antioxidant activities and L-AA contents of the *M. charantia* subjected to the different heat treatments, were evaluated by a ONE-WAY ANOVA with Tukey's post hoc test, and the association of L-AA with the antioxidant activity was analysed by Pearson's correlation test. The original variables in the samples cooked by the different methods and their antioxidant activities were subjected to the Principal Component Analysis (PCA) by transforming them into a new set of principal components which determine their contribution towards the variation in the whole data set. The statistical significance was set at a p-value of less than 0.05. All analyses were carried out using the GraphPad Prism® for Windows® version 6.0 (GraphPad® Software Inc., San Diego, CA, U.S.A) and XLSTAT 2014 software for Microsoft Excel program (Addinsoft Inc., New York, NY, USA).

3 Results and discussion

Amongst the plant organs, the fruit is the main source of ascorbic acid followed by the leaf (Asensi-Fabado & Munné-Bosch, 2010). *M. charantia*, a fruit commonly found in tropical areas of Asia, Africa and Amazon, has been regarded as a food and medicinal plant (Grover & Yadav, 2004). L-AA is commonly found in the cytosols, cell walls, vacuoles, chloroplasts, apoplasts and mitochondria (Munyaka et al., 2010). As a potent cellular antioxidant, L-AA has excellent reactive oxygen species scavenging properties (Asensi-Fabado & Munné-Bosch, 2010) but is vulnerable to non-enzymatic and enzymatic degradation. It can be oxidized to dehydroascorbic acid by ascorbic acid peroxidase (EC 1.11.1.11) and L-ascorbic acid oxidase (L-AAO) (EC 1.10.3.3), commonly found in the cell wall, extracellular matrix and vacuoles (Munyaka et al., 2010). Table 1 shows the extraction efficiencies, antioxidant activities and L-AA contents of the raw (uncooked) and cooked *M. charantia* samples. Boiling and microwaving significantly increased the total antioxidant activities (FRAP values) of the *M. charantia* samples. In addition, the radical scavenging potency of the microwave-cooked *M. charantia* extract also showed a similar increasing trend. In the present study, the L-AA content determined by HPLC (Figure 1) in the raw (uncooked) *M. charantia* (82.0 ± 2.9 mg/100 g) was similar to the levels reported in other studies, which were 79.8 ± 9.3 mg/100 g (Malik et al., 2011) and 72.5 ± 22.3 mg/100 g (Somsub et al., 2008). Although higher levels of L-AA were detected in all the samples using the HPLC method when compared to the FRASC assay, the order of the L-AA contents for the cooked *M. charantia* extracts remained the same: microwaved > boiled > pressure-cooked > uncooked (Table 1). HPLC is a better analytical tool to quantify the L-AA when compared to the FRASC assay. However, the FRASC assay provides an alternative tool for the simple and fast screening of the L-AA content in biological fluids.

Table 1. Extraction efficiencies, antioxidant activities and reduced ascorbic acid (L-AA) contents of the *M. charantia* extracts submitted to different wet heat treatments.

M. charantia samples	Extraction efficiency		Antioxidant activity		L-AA	
	Freeze dried extract (g)	Percentage [#]	FRAP (mmol FE/100 g)	DPPH [##] (% scavenging)	FRASC (mg/100 g)	HPLC (mg/100 g)
Raw (uncooked)	2.5	1.6	2.7 ± 0.1 [a]	5.1 ± 0.3 [a]	20.0 ± 6.9 [a]	82.0 ± 2.9 [a]
Boiled	3.7	2.5	8.7 ± 0.5 [b]	7.0 ± 2.4 [a]	496.0 ± 10.6 [b]	550.6 ± 25.9 [b]
Microwaved	2.0	1.3	7.8 ± 0.3 [b]	9.1 ± 1.7 [b]	514.7 ± 68.4 [b]	671.3 ± 21.1 [c]
Pressure cooked	2.5	1.7	3.3 ± 0.1 [a]	5.4 ± 1.9 [a]	54.7 ± 4.6 [a]	159.3 ± 27.3 [d]

Values presented as the mean value ± SD (n=3) unless otherwise indicated. [#]The extraction efficiency percentage of the freeze dried extract was calculated based on the amount of fresh sample used (150 g). [##]The half maximal inhibitory concentration (IC_{50}) for the ascorbic acid standard was 23.45 mmol/l. Values in the same column followed by different superscript letters are significantly different ($p < 0.05$).

Interestingly, a significant increase in L-AA content was detected in the *M. charantia* samples after pressure cooking, microwaving and boiling, when compared to the raw (uncooked) sample (Table 1). This

was in agreement with another recent finding (Subramaniam et al., 2017). A previous study showed that heat attenuated the antioxidant capacity and caused a loss of ascorbic acid in several cooked vegetables (Zhang & Hamauzu, 2004; Somsub et al., 2008). In the present study, the increased L-AA contents in the pressure-cooked, microwaved and boiled *M. charantia* samples, as compared to the raw (uncooked) sample, could be attributed to matrix softening by the heat, resulting in greater extractability of the L-AA. Munyaka et al. (2010) showed that L-AAO was completely inactivated in vegetables cooked for 10 minutes above 70 °C, and this was further supported by a recent similar finding (Wawire et al., 2011). Therefore, the inactivation of L-AAO and other L-AA oxidizing enzymes in the cooked vegetable could contribute to the stability of the L-AA in the cooked *M. charantia*. The low L-AA content in the raw (uncooked) *M. charantia* extract could be due to the enzymatic degradation of L-AA to dehydroascorbic acid during the processing of the vegetable at a temperature below 70 °C. However, the L-AA content was significantly lower in the pressure-cooked sample when compared to the boiled and microwaved samples, due to the different heating mechanisms involved in pressure cooking and the degradative effect of prolonged heat treatment (20-minute pressure cooking) on the L-AA. Nevertheless, the L-AA of the pressure-cooked sample was higher than that of the raw (uncooked) sample (Table 1).

In the correlation analysis, the L-AA was strongly correlated with the total antioxidant activity ($r = 0.958$, $R^2 = 0.917$, $p < 0.05$) and the DPPH radical scavenging potency ($r = 0.951$, $R^2 = 0.905$, $p < 0.05$). This finding supports the contribution of the L-AA to the total antioxidant activity and free radical scavenging potency observed in the raw and cooked *M. charantia* extracts. The above strong correlation also suggests the good reducing power and radical scavenging potency of the L-AA. However, there is also the possibility that the improvement in the total antioxidant activity in the cooked *M. charantia* extracts could be attributed to the interaction of other antioxidant compounds not investigated in this study, such as the Maillard products, polyphenols and antioxidant enzymes. A previous study showed that customised cooking could induce the extraction of water-soluble phenolic compounds from the tissues of *M. charantia* (Ng et al., 2011). The upregulation of several antioxidant enzymes such as superoxide dismutase and quinone oxidoreductase during heat stress could also contribute to the increased antioxidant activity observed in the cooked *M. charantia* samples (Ng et al., 2014).

The main sources of variability in the *M. charantia* extracts were evaluated using PCA by focusing on the relationship between the antioxidant activity variables (FRAP, DPPH and L-AA) and sample treatment (different heat treatments) (Figure 2). The results, shown as a biplot (score plot and loading plot), depicted the distribution and correlation of the samples with the four original variables in two principle components. The first principle component (F1, x-axis) and second principle component (F2-y-axis) corresponded to the largest cumulative variance of 98% in the original variables. The first principal component separated the raw (uncooked) and pressure cooked bitter melon samples (negative area) from the boiled and microwaved counterparts (positive area). This finding shows that in this study, 87% of the variation corresponding to the antioxidant values of the sample, was attributed to the cooking effect. This was in agreement with Domínguez et al. (2014), who reported that the changes in nutrient composition in cooked foods were mainly dependent on the heat and mass transfer in the different cooking methodologies.
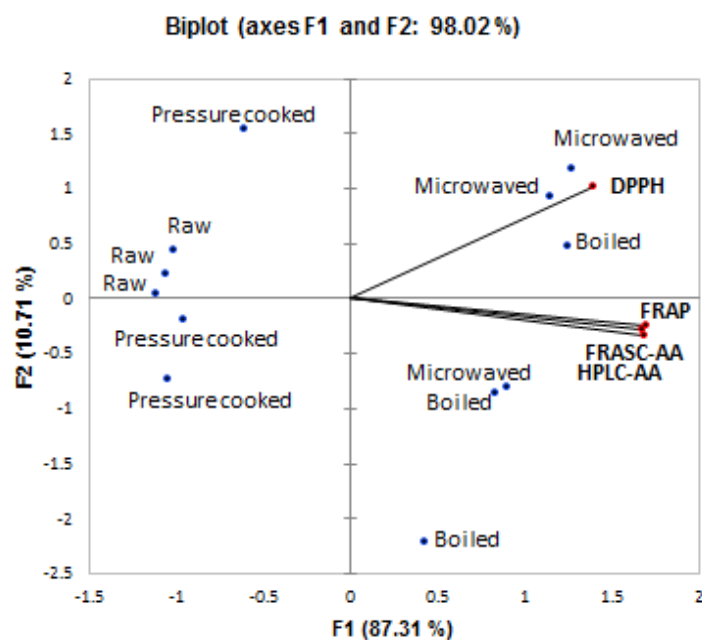
Figure 2. Principal component analysis map showing the relationships between the different types of *M. charantia* sample (raw, boiled, microwaved and pressure-cooked) and the antioxidant activities. DPPH = 2,2-Diphenyl-1-picryl-hydrazyl radical scavenging activity; FRAP = Ferric reducing antioxidant power; FRASC-AA = ferric reducing antioxidant power-ascorbic acid; HPLC-AA = High performance liquid chromatography-ascorbic acid.

On the contrary, a clear separation of DPPH radical scavenging value from the other antioxidant values (FRAP and L-AA) in the second principle component made up 11% of the observed variation. This can be explained by the different mechanisms (DPPH versus FRAP) used to measure *in vitro* sample antioxidant activity in this study (Ng & See, 2019). Nevertheless, the FRAP and L-AA variables (FRASC-AA and HPLC-AA) showed similar vector directions, indicating a strong relationship between these values. Amongst the different cooked samples, the boiled and microwaved samples showed stronger positive correlations with the antioxidant values (FRAP, DPPH and L-AA) when compared to the raw and pressure cooked samples. The PCA data in this study confirmed that the main variation in the antioxidant activities observed in the *M. charantia* extracts was dependent on the different heat treatment applied.

4 Conclusion

This study provided evidence that heat treatment either by boiling or microwaving for a short period of time (10 minutes) improved the L-AA content of the *M. charantia* extracts. It also showed that the L-AA content of *M. charantia* could be retained under high temperature and pressure. The effects of different temperatures and pressures on the stability of both L-AA and other L-AA oxidizing enzymes in other vegetables deserve further investigation. The findings of this study could serve as a reference for both the domestic and industrial heat processing of fruits and vegetables, particularly *M. charantia*, in preserving their nutritional value.

Acknowledgement

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