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Research Article

Study of ultrasound and enzyme assisted extraction of tannins from mangosteen peel in Vietnam

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Abstract

In Viet Nam, mangosteen is grown mainly in the Southeastern and the Mekong Delta provinces (about 4,900 ha). The peel and un-ripened fruit accounts for 68-70 %, which is now almost are discarded. Mangosteen peels content more than 20% (w/w) polyphenols (Nguyen Thi Hien et al, 2018). Many compounds of them are antioxidative, antibacterial, antifungal and protein precipitable such as xanthone, tannin, anthocyanin.

In this study, traditional extraction, enzyme assisted extraction (EAE) and ultrasonic assisted extraction (UAE) to obtain polyphenol compounds from the peels of *Garcinia mangostana* Linn collected from Can Tho, Vietnam were investigated. For traditional extraction: Ethanol (60%) acidified was used as solvent, the ratio of solvent to material was of 20 (v/w), temperature at 60°C was selected. For EAE, amylase and polygalacturonase enzymes help to increase tannins extraction efficiency in comparison to non-enzymes extraction. The condition for EAE: 0.5% concentration of enzyme (v/w) at 60°C, pH 5 for 30 minutes was selected. UAE technique has increased extraction efficiency, especially at intensity of 40KHz, at 60°C for 15 minutes and its extraction efficiency was more than 90%. Practical applications: Xanthone, anthocyanin and tannin extracted from peels of mangosteen, can be used afterward for additive in some food application as natural coloring substance and antioxidants for preservation.

Keywords: extraction, mangosteen, tannin, enzyme, Ultrasonic

Abbreviations: EAE - enzyme assisted extraction; DF - dilution factor; MSE - maceration solvent extraction; MW - molecular weight; TMA - total monomeric anthocyanins; UAE - ultrasonic assisted extraction; UEAE - ultrasonic and enzyme assisted extraction

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Introduction

Mangosteen is a tropical fruit that is grown primarily in hot, humid climates of southeast Asia such as Thailand, Malaysia, Singapore, Vietnam, and Indonesia. Mangosteen has been introduced to Vietnam since the beginning of the 19th century. It is grown mainly in the Southeastern and the Mekong Delta provinces such as Ben Tre, Soc Trang, Vinh Long, Can Tho, Dong Nai, Hau Giang and Binh Duong provinces. Currently, Southern Vietnam has about 5.500 ha of mangosteen, harvested from April to August. The weight of Viet Nam's mangosteens is around 80 grams but the fruit peels account for 68-70%, which is an inedible and hard, and almost discarded (Phuong 2016). The tropical fruit mangosteen is rich in antioxidants and micronutrients. In southeast Asia, the peel has been used for medicinal purposes for generations. According to folklore, the peel was used to make a tea for treat diarrhea, bladder infections, and gonorrhoea. An ointment made from the rind was applied to skin rashes. Today, the peel has been found to contain alpha-mangostin, beta-mangostin, garcinone B, and garcinone E which are collectively called xanthones. The xanthones are essential group having biological activity, with over 50 different xanthones (Obolskiy et al. 2009). In addition, mangosteen peels also include anthocyanin, tannin. The total anthocyanins content in the peels of the ripened mangosteen fruit was at about 4235 ± 203.5 mg.kg⁻¹, including cyanidin-sophoroside, cyanidin-glucoside, cyanidin-glucoside-pentoside, cyanidin-glucoside-X, cyanidin-X2 and cyanidin-X, (Palapol et al. 2009). The tannin group represents about 14.1% (Moosophon et al. 2010), in which tannin was predominant, such as procyanidin, propelargonidin, prodelphinidin, afzelechin/epiafzelechin, catechin/epicatechin, and gallocatechin/epigallocatechin (Fu et al. 2007). These compounds have many proven properties such as antioxidant capacity (Tjahjani et al. 2012), antibacterial, anti-fungal (Priya et al. 2010), anti-inflammatory, (Chen et al. 2008; Gutierrez-Orozco and Failla 2013; Pedraza-Chaverri et al. 2008; Fu et al. 2007) etc.

Many different methods have been studied to exploit bioactive compounds from mangosteen peels. The traditional method is the maceration solvent extraction (MSE). In recent years, ultrasonic assisted extraction (UAE), enzyme assisted extraction (EAE), have been used to extract bioactive compounds from plant materials has shown tremendous research interest and potential. The ultrasound has formed the cavitation bubbles and the collapse of this bubbles produces forces that disrupt plant cell membrane and subsequently provoke the release of bioactive compounds without at the same time destroying them (Sanderson 2004). There are some published studies on the extraction of bioactive compounds from mangosteen using ultrasound-assisted methods, including quantitative and qualitative determination of xanthones (Ji et al. 2007), fast screening and fractionation of major xanthones (Destandau et al. 2009), and the effects of drying methods on assay and antioxidant activity of xanthones (Suvarnakuta et al. 2011), the effect of ultrasound sonication time and amplitude on total phenolic and anthocyanin yield (Cheok et al. 2013). Compared with the traditional methods, UAE has many advantages, such as shorter time extraction, less solvent used, higher extraction yield and better products (Cheok et al. 2013; Zhang et al. 2015). Bioactive compounds in plants exist in complex forms, which bind to cellulose, pectin, starch and protein molecules in the cell wall. Enzymes have been used particularly for the treatment of plant material prior to conventional methods for extraction. Various enzymes such as cellulases, pectinases, hemicellulase, protease and amylase has been reported on the possibility to disrupt the structural integrity of the plant cell wall, thereby enhancing the extraction of bioactive from plants. These enzymes hydrolyze cell wall components thereby increasing cell wall permeability and release bioactive compounds from plant cells while being extracted and the yield released is higher respectively (Puri et al. 2012; Wang et al. 2010). Our recent publication showed that the mangosteens contain a significant amount of cellulose (41.56 ± 0.41 %), starch (8.86 ± 0.14 %) and pectin ($2.79 \pm$

0.75%) (Hien et al. 2018). They have prevented the release of active substances out of plant cells and the material treated with amylase or polygalactonase enzymes gave the extraction efficiency of tannins from the mangosteen peels higher than without enzyme pre-treatment. In this study, traditional extraction, enzyme assisted extraction (EAE) and ultrasonic assisted extraction (UAE) to obtain tannins from the peels of *Gacinia mangostana* peels collected from Can Tho, Vietnam were investigated. The objective of this study was to select the EAE condition and the UAE condition for the highest extraction efficiency of polyphenols from mangosteen peels. Various factors affecting the processing of enzymatic materials such as enzyme concentration, time treatment, temperature treatment and pH were investigated (Pinelo et al. 2006). Factors affecting the ultrasound process include ultrasonic amplitude, ultrasonic duration and temperature ultrasound were investigated, too. The best conditions of UAE and EAE were selected based on the comparison of extraction performance with traditional extraction conditions.

Materials and Methods

Materials.

Fifty kg mangosteen fruits were collected from Can Tho province of Viet Nam in June 2017. The mangosteen peel was separated from the fresh ripe mangosteen fruit. The peels were sliced in small pieces, then dried by fan in dark, at room temperature in two days. Then it was blended into small size of 20 mesh and packed in a plastic bag and preserved at -20°C before using.

Termamyl SC and Pectinex Ultra SP-L enzymes of Novozymes's have been used to treat of material prior to conventional methods for extraction.

Termamyl SC: In this product the key enzyme activity is provided by endo-amylase that hydrolyzes (1.4)-alpha-D-glucosidic linkages in starch polysaccharides. Declared activity is 120 KNU-S.g⁻¹.

Pectinex Ultra SP-L: In this product the key enzyme activity is provided by polygalactonase that

hydrolyzes (1.4)-alpha-D-galactosiduronic linkages in pectate and other galacturonans. Declared activity is 3800 PGNU.ml⁻¹

Chemicals

Ethanol 99%, hydrochloric acid 35%-37%, methanol 99%, potassium chloride, sodium acetate, trifluoroacetic acid, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Wako Pure Chemical Industries, Ltd., Osaka, Japan); DPPH (1,1-diphenyl-2-picrylhydrazyl) (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan).

Equipment

UV-Visible spectrophotometer UV-1800, Shimadzu, Japan; Rotavapor R-300, B'U'CHI, Switzerland; ultrasonic tank

Methods

Total monomeric anthocyanins (TMA) determination. The total monomeric anthocyanin (TMA) content of Mangosteen pericarp was determined using pH differential method which required two types of buffer solution, potassium chloride and sodium acetate buffers. The preparation of potassium chloride buffer of 0,025M, pH 1,0 and sodium acetate buffer of 0,4M, pH4,5 followed the method of Giusti and Wrolstad (2001) (Giusti and Jing 2008). The total monometric anthocyanin were calculated as cyanidin-3-glucoside using the following equation:

$$TMA = \frac{A \times MW \times DF \times 1000}{(\epsilon \times l)}, \left(\frac{mg}{L} \right)$$

where:

A (absorbance) = (A₅₁₀ - A₇₀₀)_{pH1} - (A₅₁₀ - A₇₀₀)_{pH 4.5}; MW (molecular weight) = 449.2 g.mol⁻¹ for cyanidin-3-glucoside; DF (dilution factor); 1000 = conversion from g to mg; ε (molar absorptivity coefficient in L.mol⁻¹.cm⁻¹ for cyanidin-3-glucoside (C-3-G)) = 26900; l = pathlength in cm.

Determination of total polyphenols content. Total polyphenol content in mangosteen pulp was determined by Colorimetric method using Folin-Ciocalteu reagent. 100 µl of samples at different

concentrations were added to the plastic plate (96 wells), then 10 µl of Folin-Ciocalteu reagent and after 5 minutes 100 µl 7% Na₂CO₃, they all reacted in the shade dark within 90 minutes. Samples were measured optical density meter on the Biotek, at a wavelength of 750 nm. Gallic acid was used as standard and the calibration curve was built on the concentration range of 0, 20, 40, 60, 80, µg.mL⁻¹ (Singleton 1965).

Determination of tannin content by Lowenthal method. In H₂SO₄, the polyphenol (tannin) compound is easily oxidized by KMnO₄ with an indigocarmin indicator. The titration ends when the indicator color changes from green to gold. Tannin content was determined by the amount of KMnO₄ used for titration. Get 5 mL extract, 5 mL sufoindigocamin reagents, 150 ml distilled water into 250 ml flasks. Stir well and titrate with 0.1 N KMnO₄ until golden color appears. Tannins content is calculated by the formula:

$$X = \frac{(a - b) \times V2 \times 0,004157 \times 100}{V1 \times G} \quad (1)$$

where:

X: tannin content (% dry weight); 100: Conversion factor %; V1: volume of sample solution (5 mL); V2: volume of extract (ml); a: amount of KMnO₄ used to titrate the sample (ml); b: amount of KMnO₄ used to titrate control samples (mL); 0.004157: tannin calculation factor corresponding to 1mL KMnO₄ 0.1N (g); G: mass of dry material (g).

The extraction efficiency (y %) was calculated by the following formula:

$$y = \frac{a}{b} \times 100, \quad (\%) \quad (2)$$

where:

a is the content of tannins in extract which have been determined by the Lowenthal method (%);

b is the total content of tannins in mangosteen peel powder which have been determined by the Lowenthal method (%)

Antioxidant property of the extract. 5 mM ascorbic acid from which diluted concentrations: 0; 0.05; 0.1; 0.25; 0.5; 0.8; 1 mM. DPPH 0.1 mM solution in ethanol. Free radical scans of samples are determined based on the DPPH free radical scans (SC%).

$$SC\% = 100 \times (1 - (OD_{Sp} - ODB_{Isp}) / (OD_{Ct} - ODB_{Ict})) \quad (3)$$

where:

OD_{sp}, ODB_{Isp}, ODC_t, ODB_{Ict} are the optical absorbance value of the sample, blank sample, control sample and blank of control, respectively.

Ascorbic acid is used as a positive control. Samples are kept in the dark, incubated at room temperature for 30 minutes. Then measured optical absorption on the Biotek, US visible UV spectrophotometer at 517 nm. Antioxidant activity of the sample was evaluated based on the IC50 value, which express the amount of antioxidant to reduce 50% of free radical. The IC50 is determined through the correlation function between the concentration of solute and the percentage of DPPH free radical scans (Kim et al. 2003).

Enzyme assisted extraction (EAE). 10 grams of mangosteen peel powder was added into 250 ml Erlenmeyer flask, mixed with 45ml purified water (with various pH: 4; 5; 6; 7) and enzyme (with various concentration enzymes: 0.25; 0.5; 0.75 and 1.0 % (v/w dry material)). The Erlenmeyer flask was incubated in a water bath at the selected temperature (40°C; 50°C; 60°C and 70°C), for the selected time (15; 30; 60; 90 minutes). When the enzyme pre-treatment was completed, the ethanol (96%) and purified water were added to the extracted concentration (ethanol 45%) with the ratio of solvent to material of 20 (v/w), temperature at 60°C for the duration of 120 minutes. The extracts then were obtained after vacuum filtration. The tannin content has been determined by the

Lowenthal method. The mode selected is which has the highest tannin extraction efficiency. Repeat the experiment using the selected mode with ethanol 60% solvents to compare the tannin extraction efficiency.

Ultrasonic assisted extraction (UAE). 10 grams of mangosteen peels powder was added into 250 ml Erlenmeyer flask and mixed with 200ml ethanol 45% (v/v). The Erlenmeyer flask was incubated in the ultrasonic tank at the selected ultrasonic amplitude (Max ultrasonic amplitude = 40kHz, 2/3 max ultrasonic amplitude and 1/3 max ultrasonic amplitude) and the selected temperature ultrasound (30°C, 40°C, 50°C and 60°C) for the ultrasonic time (10; 15; 20 and 30 minutes). The extracts then were obtained after vacuum filtration. The extract has been determined of tannin content by the Lowenthal method. The mode has been selected which has the highest tannin extraction efficiency. Repeat the experiment using the selected ultrasound mode with 60% ethanol solvents to compare the tannin extraction efficiency and antioxidant activity of the extract.

Ultrasonic and enzyme assisted extraction (UEAE). 10g of mangosteen peels powder was added into 250 ml Erlenmeyer flask and mixed with 45ml water which was pH=5 and 0.5% enzyme. The Erlenmeyer flask was incubated in the ultrasonic tank at 40KHz, 60°C for 15 minutes. At the end of the ultrasound time, the sample was maintained at 60°C for 15 minutes. When the treatment of material was completed, the extraction process has been done according to the mode we have chosen (Hien et al. 2018). The ethanol (96%) and purified water were added to the extracted concentration (ethanol 60%) with the ratio of solvent to material of 20 (v/w), temperature at 60°C for the duration of 120 minutes. The extracts then were obtained after vacuum filtration. The extract has been determined of tannin content by the Lowenthal method and test antioxidant activity.

Results and Discussion

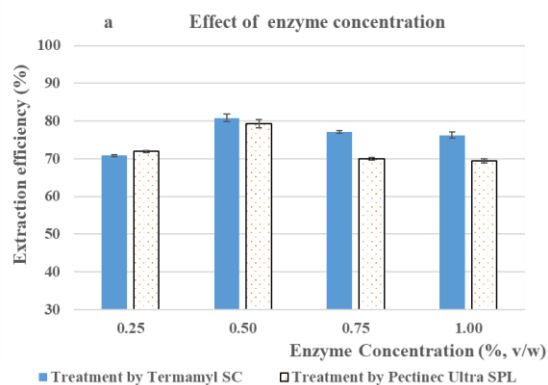
Mangosteen peels composition. 50 kg of mangosteen fruit has been collected in Can Tho city of Vietnam in 8/2017. The average weight of the fruit was 87.22g. The intestinal fruit (white, edible) was 26.76% (w/w) and no anthocyanin. The stem was 3.11% (w/w) and anthocyanins content 0.02% (dry weight)). The fruit peel (dark purple, not edible) was 70.13% (w/w), including 81.94 % (w/w) outer pericarp and 18.06% (w/w) inner pericarp. The results of the study (Table 1) showed that polyphenols, tannins and anthocyanins were concentrated mainly in the fruit peel (27.73%, 14.27% and 0.66%, respectively). The anthocyanins content in mangosteen peels is high compared to some plants that have been used in anthocyanin extraction such as *Mirabilis jalapa* flower (338.61 mg/kg) (Vankar and Srivastava 2010), eggplant (450,1mg.g⁻¹) (Sadilova et al. 2006), Purple Sweet Potato (687.58 ppm) (Wicaksono et al. 2016) and fresh blueberries (7.2 ± 0.5 mg.g⁻¹ dry matter) (Lohachoompol et al. 2004). The content of tannin (14.27% dry weigh) is relatively higher than that of some traditional sources for tannin extraction as buds and young leaves of tea (10 %) and black tea 13.6 % (Khasnabis et al. 2015).

Table 1. Chemical composition of fresh mangosteen in Can tho province of Vietnam

Content	Fresh mangosteen peel	Fan to dry, avoid sunlight (DS)	Fruit stalk	Flesh fruit
Moisture (%)	63.33 ± 0.17	11.74± 0.47	65.69 ± 0.16	81.26 ± 0.04
Polyphens (g AGE/100g dry matter)	27.73 ± 0.09	21.91 ± 0.04	9.88 ± 0.05	11.40 ± 0.04
Tannins (% dry weight)	14.27 ± 0.07	13.91 ± 0.44	3.22 ± 0.02	0.76 ± 0.05
Anthocyanins (g C-3-G/100g dry matter)	0.66 ± 0.02	0.11±0.00	0.02±0.00	0±0.00

The results of the study (Table 1) showed that the total content of polyphenols and tannins in the dry material samples (DS) is not much reduced compared to fresh samples (FS) (the reduction level is 20.99% and 2.52% respectively). But the treatment regime has a great influence on the anthocyanin content. The mono-anthocyanin content decreased further after the material was dried (from 0.66% to 0.11%). Although the mono-anthocyanin content in the dry material is low, the red purple color of the extract obtained from FS and DS has not been significantly different. Thus, the pigment that makes up the red purple color of the extract has been preserved in the material when dried. This indicates that all of the fresh and dry mangosteen peel are a potential source for extract compounds of polyphenols such as tannins, anthocyanins

Enzyme assisted extraction (EAE). The effect of the factors on the extraction with the amylase enzyme (Termamyl SC) and with the polygalactonase enzymes (Pectinex Ultra SPL) was shown in Figure 1.



The results showed that the changes in enzyme concentration, pH, treatment temperature and time affected the efficiency of tannin extraction. The result (Figure.1a) has shown that when the concentration of enzyme increases from 0.25% to 0.5%, the extraction efficiency has increased markedly. Amylase enzyme hydrolyzed the (1.4) -

alpha-D-glucosidic linkages in starch polysaccharides. Meanwhile, polygalactonase

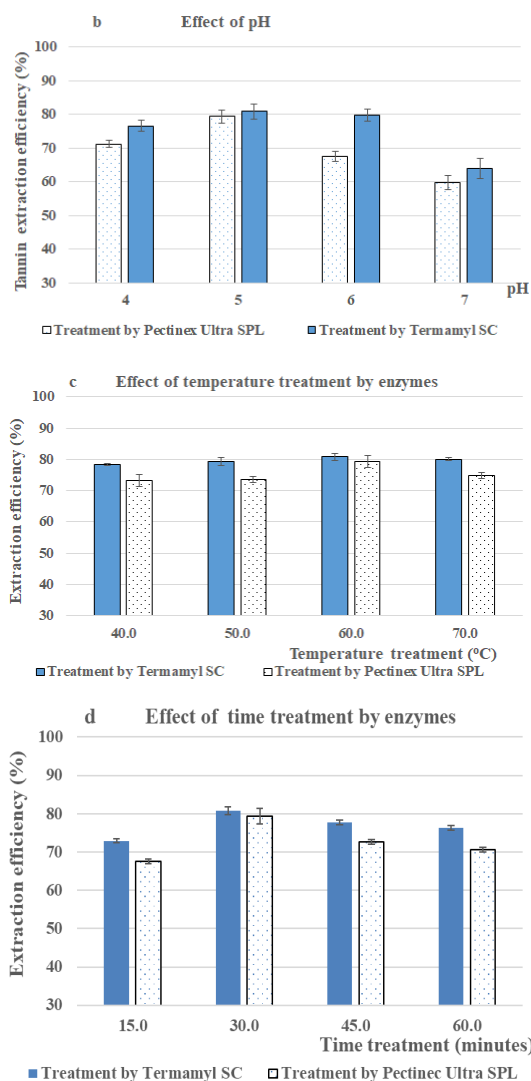


Figure 1. Enzyme assisted extraction (EAE)

enzyme hydrolyzed the (1.4)-alpha-D-galactosiduronic linkages in pectate and other galacturonans. Hydrolysis which broke the plant cell structure of the material, has facilitated the erosion of solvents into the plant cells and dissolved active substances (Puri et al. 2012). The enzyme's activity depends on the pH of the reaction medium. The experimental results (Figure.1b) showed that between 4 and 6 pH, both amylase and polygalactonase enzymes have strong activity. The

extraction efficiency increased and reached the highest value at pH = 5. However, pH more than 6 has shown disadvantage for these two enzymes, the extraction efficiency has been reduced. The results of Figure 1c showed that the temperature increased gradually from 40 to 60°C gave gradual extraction efficiency. High temperatures have increased the rate of hydrolysis reaction and it has increased the ability of solvent to penetrate which has increased the solubility of active substances. At a temperature of 60°C for the highest extraction efficiency. However, temperatures higher than 60°C have been detrimental to the activity of enzymes amylase and polygalactunase, thus the effective support for extraction has decreased. The process of contact between enzyme and substrate or hydrolysis reaction under enzymatic catalysis requires time. The results of the study (Figure. 1d) have shown that 30 minutes of enzymatic treatment was the time required for the highest extraction efficiency. 0.5% enzyme amylase or polygalactunase enzyme, pH 5, at 60°C for 30 minutes was selected to treatment raw materials in tannins extraction from dried mangosteen peels. This condition makes the tannins extraction efficiency increases 16.03% with Pectinex Ultra SPL and 17.5% with Termamyl SC (The tannin extraction efficiency was 79.33% và 80.80%, respectively). Experiments applied this enzyme treatment conditions and then extracted with 60% ethanol (which we published in 2018) had the same results (Hien et al. 2018). The tannin extraction efficiency was 94.83% which was more than 7.48% (with Termamyl SC) and 93.11% which was more than 5.76% (with Pectinex Ultra SPL) This condition is similar to some published research works such as (Heo et al. 2005) or (Wang et al. 2014).

Ultrasonic assisted extraction (UAE). The results shown in Figure 2 show that the ultrasonic intensity decreases from value max = 40kHz to 1/3max for gradual extraction efficiency. The greater the intensity of ultrasound, which has created greater force on the plant cell wall. High intensity focused ultrasound has broken the cell wall structure easier. As a result, the solvent's cavitation into the cell is easier, the extraction yield is higher. The increase in temperature and ultrasound time also increased the

ability of solvent to penetrate and the process of dissolving the active substance into the solvent was faster (Cheok et al. 2013).

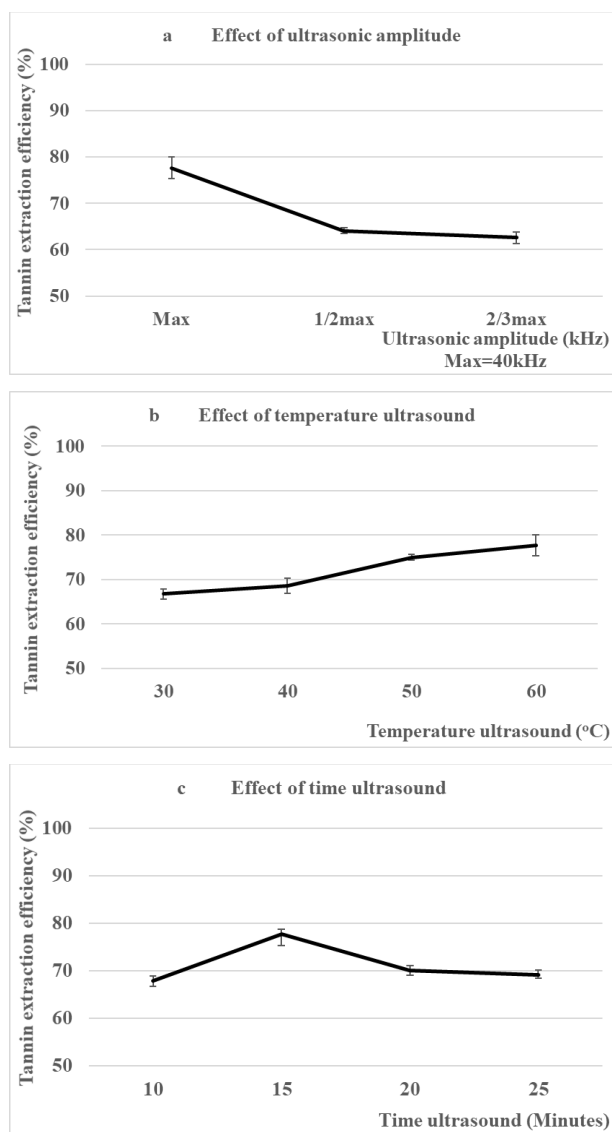


Figure 2. Ultrasonic assisted extraction (UAE)

The efficiency of extracting tannins was highest at 40kHz, at 60°C for 15 minutes. The tannins extraction efficiency was $77.62 \pm 2.35\%$ that was higher 14.29% than MSE method with solvent ethanol 45%. The efficiency of extracting tannins was 91.87% which higher 4.52% than MSE method

with solvent ethanol 60%. Experimental results (Figure.3) showed that the support of ultrasound and enzymatic techniques to treatment raw materials has excellent tannins efficiency extraction from dry mangosteen.

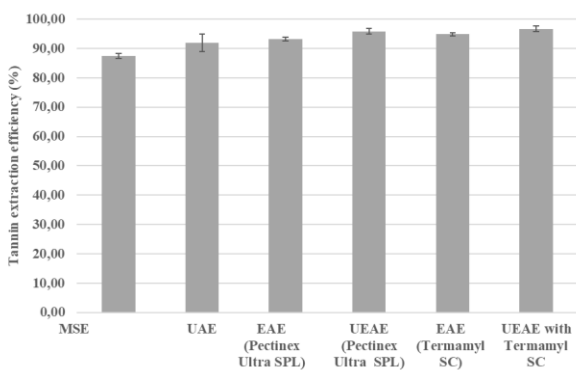


Figure 3. MSE, UAE, EAE and U-EAE of mangosteen peels

Tannins extraction efficiency was higher than MSE method (13.96% with Termamyl SC; 14.11% with Pectinex Ultra SPL). This result also has similarities with the results of Wu's research (Wu et al. 2015) who have demonstrated the combination of ultrasound and enzymes have increased the extraction yield of phenolics. The IC50 value of MSE, UAE, UEAE with Pectinex Ultra SPL, UEAE with Termamyl SC extract and vitamin C were 78.37; 59.84; 97.50; 92.22 and 50.15 ($\mu\text{g}\cdot\text{ml}^{-1}$), respectively (Table 2).

Table 2. Evaluate the antioxidant activity of the extracts on the IC50 value

Sample s	MSE	UAE	UEAE(Pectine x Ultra SPL)	UEAE (Termamy 1 SC)	VT M C
IC50 ($\mu\text{g}/\text{ml}$)	78.37	59.84	97.50	92.22	50.15

These results have shown that ultrasound and enzyme catalysts have increased the extraction efficiency of tannins and the extracts have marked

antioxidant activity. This suggests a good and safe technology for tannin extraction from mangosteen peels for application in food technology.

Conclusions

The tannins was concentrated mainly in the fruit peel. The fresh and dry mangosteen peel are a potential source for extract compounds of tannins. We suggest the treatment condition by enzymes Termamyl SC or Pectinex Ultra SPL with 0.5% enzymes, pH 5, at 60 ° C for 30 minutes and then extract by solvent ethanol 60%, at a temperature of 60° C for 120 minutes to extract tannins from dried mangosteen peels. Amylase enzym increases tannins extraction efficiency 7.48% (The tannins extraction efficiency was 94.83%). Polygalactonase enzymes increases tannin extraction efficiency 5.76% (The tannins extraction efficiency was 93.11%). Ultrasonic intensity of 40kHz, at 60°C for 15 minutes was selected for material treatment. This condition has increased the efficiency of tannin extraction 4.52% (The tannins extraction efficiency was 91.87%). The combination of enzymatic and ultrasonic material treatment was a perfect suggestion in extracting tannins from dried mangosteen peels. Tannin extraction efficiency was higher than MSE method (9.23% with Termamyl SC; 8.45% with Pectinex Ultra SPL). The extracts have marked antioxidant activity: IC50 value of U-EAE extract with Termamyl SC was 92.22 ($\mu\text{g}/\text{ml}$). IC50 value of U-EAE extract with Pectinex Ultra SPL was 97.50 ($\mu\text{g}\cdot\text{ml}^{-1}$).

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