International Food Research Journal 25(1): 321-328 (February 2018)
Journal homepage: *http://www.ifrj.upm.edu.my*

Utilization of soursop leaves as antihyperuricemic in functional beverage 'Herbal Green Tea'

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Article history

Received: 18 August 2016
Received in revised form: 25 November 2016
Accepted: 26 November 2016

Keywords

Antihyperuricemic
Herbal green tea
IC_{50}
Soursop leaves
Xanthine oxidase

Abstract

Soursop leaves contain several bioactive compounds which are important for health, including antihyperuricemic activity. Moreover, a processing into green tea could increase their active compounds activity. Therefore, a research was done to determine *in vitro* antihyperuricemic activity on soursop leaves brew which was processed into herbal green tea. Research method was done by processing soursop leaves according to green tea making procedure and brewed them at 70°C, 85°C and 100°C for 15, 30 and 45 minutes. Antihyperuricemic activity was measured in vitro using xanthine oxidase enzyme and compared to dried soursop leaves brew and fresh soursop leaves brew. Results showed that brewing of herbal green tea from soursop leaves at 100°C for 30 minutes showed the highest inhibition activity towards xanthine oxidase, with IC_{50} value of 291.11 ± 13.69 ppm. Inhibition activity of herbal green tea was higher than dried soursop leaves brew and fresh soursop leaves brew, which had IC_{50} value of 648.92±15.34 ppm and 2111.20 ± 55.50 ppm, respectively. Herbal green tea brew from soursop leaves contains total phenolic, flavonoid and condensed tannin content higher than dried soursop leaves and fresh soursop leaves. Physically, herbal green tea brew from soursop leaves has reddish yellow color, similar to original tea brew, but it was not well accepted by panelists due to specific aroma and taste from soursop leaves.

Introduction

Hyperuricemic is a condition of the increase of uric acid content in body that can cause inflammation, particularly on joints, and has a positive correlation with several degenerative diseases, such as kidney disease (Cirillo *et al.*, 2006), increase of triglycerides amount (Chen *et al*., 2007), obesity, and insulin resistance (Baldwin *et al*., 2011), heart disease (Chuang *et al*., 2012), and hypertension (Lin *et al*., 2012). Chen *et al.* (2007) reported that hyperuricemic condition could increase the risk of degenerative diseases 1.6 times higher. Uric acid problem occurs mainly because of unhealthy eating pattern, which are over-eating pattern and high consumption of foods that contain high uric acid.

Several ways can be done to reduce or prevent uric acid disease, such as by managing the eating pattern and/or consuming anti uric acid medicine. Medicine consumption often causes side effects and it is more expensive (Li *et al*., 2008). Therefore, people started using traditional treatment which uses natural ingredients, because it is considered safer and more economic. Natural ingredients that contain active compounds which give pharmacological effects are secondary metabolites from plants (Liu *et al*., 1998; Stepp and Moerman, 2001).

One of plants that contain many active compounds are soursop that flourishes in Indonesia. A part of soursop plant that has been reported to contain active compounds is its leaves. Soursop leaves have been reported to have anti uric acid activity (Wahjuni *et al*., 2012), anti inflammation (Foong and Hamid, 2012), antinociceptive and antiulcerogenic (Hamid *et al*., 2012), anticancer (Rachmani *et al*., 2012), antidiabetic (Purwatresna, 2012), anticholesterol (Uneputty *et al*., 2013), and antioxidant (Budiarti *et al*., 2014). Multifunction properties from soursop leaves extract are related to their active compounds, such as flavonoid, tannins, calcium oxalate, alkaloids, fatty acids, phytosterols and myricyl alcohols (Asprey and Thornton, 2000; Adjie, 2011).

A potency of soursop leaves as antihyperuricemic has been reported, *in vitro* using xanthine oxidase enzyme, as well as *in vivo* using rats. Xanthine oxidase enzyme catalyzes reaction of hypoxanthine into xanthine and then oxidized into uric acid. Artini *et al.* (2012) reported that butanolic extract of soursop leaves could reduce uric acid content better than allopurinol on wistar rats. A similar result was

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also reported by Wahjuni *et al.* (2012). Soursop leaves ability to lower uric acid is probably because of flavonoid content in soursop leaves. According to Sutomo (2003), flavonoid compounds could reduce uric acid content by inhibiting xanthine oxidase enzyme.

Study of anti uric acid activity from soursop leaves is mostly in form of extract form certain solvents. This is considered unpractical to be applied on food products. Therefore, study is done in form of functional beverage which is considered to be more practical and natural. One of food products that need to be improved is herbal tea. The considerations are 1) tea is a product that is widely consumed in the world as well as in Indonesia; it can be seen from consumption rate of tea is about 527 gram per capita per year and consumption rate of tea in Indonesia is about 457 gram per capita per year (Euromonitor, 2014); 2) tea making process is relatively simple; 3) tea making process can improve its bioactive activity for example: a) black tea making could increase total polyphenol and total catechin content, and antioxidant activity (Karori *et al*., 2007); b) black tea making form soursop leaves could improve anti uric acid activity; c) green tea making from soursop leaves could improve antioxidant activity (Adri and Hersoelistyorini, 2013) and antidiabetic activity (Hardoko *et al*., 2015).

Compared to black tea, green tea has 1.5 times higher antioxidant activity (Langley-Evan, 2000), and 2 times higher antidiabetic activity compared to black tea from soursop leaves (Hardoko *et al*., 2015). Based on this comparison, there is an assumption that anti uric acid activity of herbal green tea from soursop leaves could be higher as well. Besides, high consumption of green tea in Asian countries, such as China, Taiwan, Japan and Korea (Cabrera *et al.*, 2006), as well as high prevalence of uric acid disease in Indonesia on 2013 (Kemenkes, 2014), support the development of herbal green tea from soursop leaves. Thus, it is required to study the anti uric acid activity from herbal green tea brew of soursop leaves.

Materials and Methods

Materials used in herbal green tea from soursop leaves making were fresh soursop leaves (*Annona muricata* L.) which had green color with 10-11 cm in length and 4-5 cm in width, which were obtained from Indramayu, Karangmalang Village, Indramayu District, West Java, Indonesia. Major ingredients used for *in vitro* antihyperuricemic analysis were xanthine oxidase enzyme (Sigma Aldrich Chemical. Co.), xanthine substrate (Sigma Aldrich Chemical. Co), uric acid medicine "allopurinol" (PT. Kalbe Farma Indonesia), and phosphate buffer (Merck).

Herbal green tea from soursop leaves making

The making of herbal green tea from soursop leaves was based on research by Mulyawan (2007) and Preedy (2013). The process started by sorting or choosing fresh soursop leaves which had no defects and had relatively similar size. The chosen soursop leaves were cleaned from physical contamination and dirt by using running water, and then drained. Then, soursop leaves were fixated by steaming at 100^{o}C for 6 minutes, cooled and hand-rolled for 20 minutes. Before dried, leaves were size-reduced using a scissor until they reached 0.5 cm in width. The last step was drying at 70^{o}C for approximately 5 hours or until the moisture content reached 5 percents. In the end, herbal green tea from soursop leaves were ready to be brewed.

Dried soursop leaves making

The making of dried soursop leaves was also based on research by Mulyawan (2007). Fresh soursop leaves were sorted to obtain leaves with no defects and had relatively similar size, separated from physical contamination and washed with running water, drained, and dried in cabinet dryer at 70^{o}C for approximately 5 hours. Then, they were size-reduced until they reached 0.5 centimeters in width and dried soursop leaves were ready to be brewed.

Brewing of soursop leaves products

Brewing of soursop leaves was conducted according to Coe *et al.* (2013). One point five (1.5) gram of herbal green tea from soursop leaves was brewed in 200 mL demineralized water at temperature combination of 70°C, 85°C and 100°C for 15, 30, and 45 minutes. The brew was stirred 6 times, screened and cooled for approximately 17 minutes.

One point five (1.5) gram of dried soursop leaves, and/or 6 gram of fresh soursop leaves, each was brewed in 200 mL of demineralized water at the best combination of time and temperature based on the lowest IC_{50} value of xanthine oxidase from herbal green tea from soursop leaves brew. The brew was analyzed for its inhibition activity towards xanthine oxidase, total phenolic content, total flavonoid content, total condensed tannin, color using chromameter, and hedonic organoleptic test.

Antihyperuricemic activity test using xanthine oxidase

The procedure for antihyperuricemic test was modified from a method used by Muthiah (2012)

and Sivashanmugam and Chatterjee (2012). Zerro point five (0.5) mL sample in a reaction tube was added with 1.45 mL of phosphate buffer and xanthine substrate, homogenized using vortex, and then incubated at 25°C for 15 minutes. Incubation result was added with 0.05 mL of xanthine oxidase enzyme, homogenized using vortex and incubated again at 25°C for 60 minutes. Moreover, 0.5 mL of 1N HCl was added to stop enzymatic reaction and the absorbance was measured at wavelength of 290 nm to obtain sample absorbance (S). The same procedure was used to prepare control, i.e. without addition of xanthine oxidase enzyme, to obtain absorbance of sample control (KS). Blank (without sample) and blank control (without sample and enzyme) were also prepared to obtain blank absorbance (B) and blank control absorbance (KB). Inhibition (%) was calculated based on formula: [(B - KB) – (S - KS) / (B-KB)] x 100%.

Total phenolic content determination

Total phenolic content determination was conducted according to Anesini *et al.* (2008). Zero point three (0.3) mL of herbal green tea from soursop leaves brew (diluted 10 times) was added with 1.5 mL of Folin-Ciocalteu and 1.2 mL of 7.5% Na_2CO_3 solution. The solution was then incubated at room temperature for 60 minutes. The absorbance was measured at wavelength of 765 nm. A standard curve was prepared using gallic acid solution with concentration of 10 –100 ppm. Phenolic content was expressed in mg GAE/L sample, based on standard curve equation. Total phenolic was calculated based on formula :

Total Phenolic = x / dilution factor.

Total flavonoid determination

Total flavonoid content determination was conducted according to Choudhary *et al.* (2013). One (1.0) mL brew of herbal green tea from soursop leaves was mixed with 1.0 mL of methanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1.0 M CH_3COOK, and 2.8 mL of distilled water. The mixtured was homogenized using vortex and incubated at room temperature for 30 minutes. The absorbance was measured at wavelength of 415 nm. Standard solution of quercetin was solubilized in methanol and diluted to concentrate of 20, 40, 60 and 80 ppm. Flavonoid content was expressed in mg QE/L sample, based on standard curve equation. Total Flavonoid = x / dilution factor.

Total condensed tannins determination

Total condensed tannins determination was conducted according to Rabeta and Lai (2013). Zero point five (0.5) mL of sample or standard solution was mixed with 3 mL of 4% vanillin in methanol (w/v) and 1.5 mL of concentrated HCl. The mixture was incubated at dark room with room temperature for 15 minutes. The absorbance was measured at wavelength of 500 nm. Standard solution was made from catechin at concentration of 40, 60, 80, 100 and 120 ppm. Condensed tannin content was expressed in mg CE/L sample, based on standard curve equation. Total tannin was calculated based on formula: Total Tannin = x / dilution factor

Color assessment using chroma meter

Color assessment determination was conducted according to Vondoni and Rossi (2009). Color assessment was done using Chroma meter Minolta CR-400. Color parameter was expressed in L^*, a^*, and b^*. L^* shows brightness intensity, a^* shows green-red color intensity, b^* shows blue-yellow color intensity. a^* dan b^* value was then calculated to obtain °Hue value, using formula: °Hue = arctan (b^*/a^* x [360°/ (2 x 3.14)]

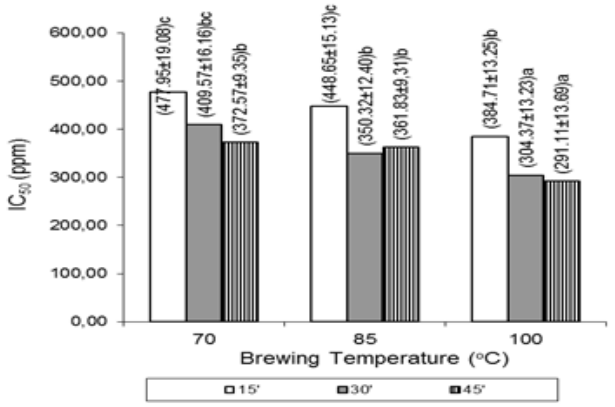
Organoleptic analysis

Organoleptic test performed on herbal green tea from soursop leaves was hedonic test, according to Meilgaard *et al*. (1999). The parameters assessed were color, aroma, taste and overall acceptance, using 70 semi-trained panelists and hedonic score of 1 to 7, where 1 = dislike extremely, 2 = dislike moderately, 3 = dislike slightly, 4 = neither like nor dislike, 5 = like slightly, 6 = like moderately, 7 = like very much.

Results and Discussion

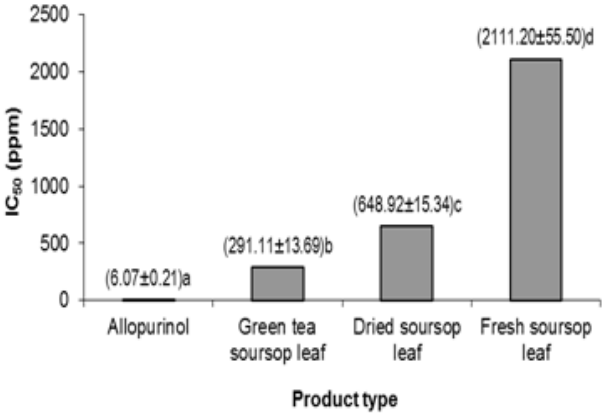
Moisture content and phytochemical of soursop leaves and their product

Fresh soursop leaves used had moisture content of 71.89 ± 1.22%, whereas dried soursop leaves and herbal green tea from soursop leaves had moisture content of 5.46±0.48% and 3.18 ± 0.80%, respectively. Based on Indonesian National Standard (SNI) SNI No. 01-3945-1995 (BSN, 1995) about green tea, the maximum moisture content of green tea is 12%. Thus, the moisture content of dried soursop leaves and herbal green tea from dried soursop leaves are in accordance with SNI of green tea criteria. The lower moisture content on herbal green tea from soursop leaves compared to dried soursop leaves could be due to rolling step in herbal green tea making step that causes disruption in cell membrane. As a result,



Notes: different superscript notation on each factor shows significant difference ($p<0.05$).

Figure 1. IC_{50} value towards xanthine oxidase based on different brewing time and temperature of herbal green tea from soursop leaves



Notes: different superscript notation shows significant difference ($p<0.05$).

Figure 2. IC_{50} value of Allopurinol and soursop leaves brew that was brewed at 100°C for 30 minutes

with the same drying time and temperature, water evaporation process will be faster compared to dried soursop leaves that do not undergo rolling process (Sato *et al*., 2007). Other than that, size reducing prior to drying process increases the surface area and water evaporation rate from the leaves (Hernani and Nurdjanah, 2009).

Qualitatively, phytochemical compounds in fresh soursop leaves, dried soursop leaves, and herbal green tea from soursop leaves are tannins, flavonoids and steroids, but they do not contain alkaloids and terpenoids. These compounds were presumed to play role in inhibition towards xanthine oxidase enzyme. This is related to a statement from Owen and Johns (1999) that tannins were potential in inhibiting xanthine oxidase with mechanism of non-selective binding. Flavonoids have also inhibition activity towards xanthine oxidase using competitive mechanism (Lin *et al*., 2002). The presence of steroid in soursop leaves was also reported by Florence *et al.* (2014).

The influence of brewing time and temperature of soursop leaves towards xanthine oxidase inhibition

Inhibition level of xanthine oxidase is expressed as IC_{50}. In this case, IC_{50} is defined as 50% inhibition activity towards xanthine oxidase (enzyme that forms uric acid) by herbal green tea from soursop leaves brew. The lower the IC_{50} value, the higher the inhibition towards enzyme activity. IC_{50} value of herbal green tea from soursop leaves based on different brewing time and temperature can be observed on Figure 1.

Figure 1 shows that the brewing time of 30 and 45 minutes at 70°C, 85°C and 100°C had no significant effect on IC_{50} value (did not decrease IC_{50} value significantly) ($p<0.05$). However, brewing temperature at 100°C for 30 and 45 minutes had lower IC_{50} value compared to other treatments. It means inhibition activity of soursop leaves brew at 100°C for 30 and 45 minutes is higher compared to other brews. For efficiency consideration, brewing at 100°C for 30 minutes was selected to obtain the best inhibition activity towards xanthine oxidase enzyme.

Inhibition phenomenon by herbal green tea from soursop leaves might be related to a statement from Harbourne *et al.* (2009) that different inhibition capacity towards xanthine oxidase is related to extracted bioactive compounds. Heat at high temperature causes damage on leaves cell membrane; therefore it increases cell membrane permeability and facilitates water penetration into the leaves to extract intracellular bioactive components. The higher the extraction temperature, the higher the damage and cell membrane permeability. This makes extraction process becomes faster and increases extraction yield (He *et al*., 2012; Settharaksa *et al*., 2012). The more the extracted bioactive compounds, the better the inhibition activity towards xanthine oxidase.

Comparison of inhibition activity towards xanthine oxidase between allopurinol and soursop leaves brew

Allopurinol is a generic medicine that is widely used to cure uric acid disease or lower the hyperuricemic condition. The comparison of inhibition activity towards xanthine oxidase between allopurinol and soursop leaves brew can be observed on Figure 2. Based on Figure 2, herbal green tea brew from soursop leaves gives the lowest IC_{50} value compared to other products, although it is still higher than allopurinol. This indicates that herbal green tea brew from soursop leaves has higher inhibition activity towards xanthine oxidase (antihyperuricemic) compared to dried soursop leaves or fresh soursop

leaves brew. Compared to 'black tea' soursop leaves that has IC_{50} value of 382.74 ± 55.50 ppm (Hardoko *et al.*, 2015), IC_{50} value of green tea soursop leaves is still lower.

Thus, soursop leaves processing into herbal green tea is proven to be able to increase inhibition activity towards xanthine oxidase or antihyperuricemic activity. Increase of antihyperuricemic activity or inhibition towards xanthine oxidase from herbal green tea from soursop leaves brew is 2.2 times higher than dried soursop leaves brew and even 7.3 times higher than fresh soursop leaves brew. These phenomena are assumed to be related to enzyme inactivation at fixing process that can prevent oxidation of bioactive compounds (Green, 2008; Preedy, 2013) and rolling step that can cause disruption of cell, therefore it increases extraction rate of bioactive compounds during brewing (Kumara and Amarakoon, 2006). Besides, drying process can also increase solubility of bioactive compounds during brewing because there is a cell disruption and porous structure formation on leaves that can increase cell permeability (Fazaeli *et al*., 2012).

The increase of inhibition activity towards xanthine oxidase enzyme by herbal green tea from soursop leaves compared to dried soursop leaves brew and fresh soursop leaves brew is supported by total phenolic, total flavonoid and total condensed tannin content that are higher on herbal green tea from soursop leaves. This is supported by Spanou *et al*. (2012) who stated that flavonoid is also potential in inhibiting xanthine oxidase. Polyphenolic compounds, such as geraniin, corilagin and gallic acid, have also inhibition activity towards xanthine oxidase (Wu *et al.*, 2010).

The high inhibition activity towards xanthine oxidase by allopurinol is related to higher amount or dosage of bioactive compounds, i.e. 700000 ppm, whereas bioactive compounds of herbal green tea from soursop leaves that consist of phenolic, flavonoid and condensed tannin are about 572.52 ± 9.74 mg GAE/L sample, 41.37 ± 8.84 mg QE/L sample, and 519.10 ± 45.12 mg CE/L sample, respectively (Table 1).

Based on Figure 2, it can also be observed that IC_{50} value of allopurinol is 49 times lower than herbal green tea from soursop leaves. For medicinal purpose, the average dosage of allopurinol that is usually consumed by a gout patient is 300 mg/tablet. Based on this dosage, the amount of herbal green tea from soursop leaves that has to be consumed to obtain the similar inhibition activity to 300 mg allopurinol is around 10 portion with serving size of 1.5 gram per 200 mL of water.

High phenolic and flavonoid content of herbal green tea from soursop leaves is related to enzyme inactivation process (fixing step), rolling and drying that can increase water permeability into leaves cell, therefore the amount of extract obtained is optimized. Besides, fixing step can also prevent oxidation on condensed tannin to increase the amount of condensed tannin on herbal green tea from soursop leaves (Green, 2008; Preedy, 2013).

Table 1. Total phenolic, flavonoid and condensed tannin content of soursop leaves brew

Soursop leaves brew	Total Phenolic (mg GAE/L sample)	Total Flavonoid (mg CAE/L sample)	Total Condensed Tannin (mg CE/L sample)
Fresh	(194.33±27.99)^{a}	(14.65±1.48)^{a}	(41.46±3.39)^{a}
Dried	(253.25±12.74)^{b}	(37.71±5.60)^{b}	(181.32±31.62)^{b}
Herbal green tea	(572.52±9.74)^{c}	(41.34±6.84)^{b}	(519.10±45.12)^{c}

Notes: Superscript notation on each column shows significant difference ($p<0.05$).

Table 2. Color characteristic of soursop leaves brew based on °Hue and L*

Brew	Color (°Hue)	Brightness (L* value)
Fresh soursop leaves	90.00±0.05	54.09±0.40^{c}
Dried soursop leaves	89.66±0.11	49.19±1.33^{a}
Herbal green tea from soursop leaves	90.01±0.01	52.34±0.39^{b}

Notes: Different superscript notation on L* value shows significant difference ($p<0.05$).

Table 3. Organoleptic characteristics based on hedonic test (color, aroma, taste and overall acceptance) on soursop leaves brew

Soursop leaves brew	Hedonic			
	Color	Aroma	Taste	Overall acceptance
Fresh	(2.97±0.56)^{a}	(3.40±0.45)^{a}	(2.69±0.45)^{a}	(2.77±0.32)^{a}
Dried	(4.96±0.37)^{b}	(3.96±0.60)^{b}	(3.20±0.63)^{a}	(3.46±0.38)^{b}
Herbal green tea	(4.97±0.42)^{b}	(4.61±0.44)^{c}	(3.86±0.55)^{b}	(4.04±0.45)^{c}

Notes: Different superscript notation on each column shows significant difference ($p<0.05$)
1 = dislike extremely; 7 = like extremely

Color and organoleptic characteristics of soursop leaves brew

Color characteristic of soursop leaves brew which is based on oHue value and brightness (L* value) can be observed on Table 2, while organoleptic characteristics based on hedonic test can be observed on Table 3. Based on °Hue color scale, i.e. 54° – 90° is yellow-red color and 900 – 1260 is yellow color (Hutchings, 1999), then color of soursop leaves brew

is reddish yellow (Table 2), the only difference is on its brightness (L^*). The brightness of fresh soursop leaves brew is highest, followed by herbal green tea from soursop leaves and then dried soursop leaves brew. The difference in brightness might be related to enzymatic browning reaction by polyphenol oxidase and non-enzymatic browning on dried soursop leaves, in which Maillard reaction and ascorbic acid oxidation occur (Lin *et al*., 2010).

Based on hedonic test in general (Table 3), it can be seen that soursop leaves processing based on green tea processing could increase its preference on color, aroma, taste and overall acceptance, but the overall acceptance has not reached 'slightly like' score yet (score 5). Preference towards herbal green tea from soursop leaves is better than fresh soursop leaves brew and dried soursop leaves brew. This might be because there are rolling and drying steps during the making of herbal green tea from soursop leaves brew that could increase evaporation of volatile compounds that contribute to aroma. As a result, intensity of unwanted aroma and taste would decrease (Teshome *et al*., 2013). The slightly unpreferred soursop leaves brew is caused by distinctive aroma and taste from soursop leaves that are perceived as foreign aroma and taste. Therefore, it requires further product formulation to mask unwanted organoleptic attributes of soursop leaves brew.

Conclusion

Herbal green tea brew from soursop leaves has a better in vitro potency as antihyperuricemic agent compared to dried soursop leaves brew and fresh soursop leaves brew. Brewing process which resulted in the best inhibition activity towards xanthine oxidase enzyme was brewing at 100°C for 30 minutes. This process resulted in IC_{50} value of 297.70±39.73 ppm. Soursop leaves processing into herbal green tea could increase its antihyperuricemic activity for about 2.2 times higher than dried soursop leaves and 7.3 times higher than fresh soursop leaves. Herbal green tea brew from soursop leaves had reddish yellow color, similar to original tea brew, but based on organoleptic test, it was not well accepted by the panelists.

Acknowledgement

We would like to say thank you to Pelita Harapan Foundation for funding this research based on research scheme of Faculty of Science and Technology No. P-23-FAST/X/2014

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