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Potency of yogurt as angiotensin converting enzyme inhibitor with addition of *Ficus glomerata* Roxb fruit extract

[1,2]Wulandani, B.R.D., [2]Marsono, Y., [2]Utami, T. and [2*]Rahayu, E.S.

[1]*Faculty of Animal Science, Mataram University, Mataram- West Nusa Tenggara 83521, Indonesia*
[2]*Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*

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Abstract

Ficus glomerata Roxb was found to contain flavonoids which are known for their ability as angiotensin converting enzyme (ACE) inhibitors. The aim of this research was to determine the ability of yogurt with *F. glomerata* Roxb extract as angiotensin converting enzyme inhibitors during storage (1, 7, 14, 21 and 2 8 days). The results showed that the total amount of lactic acid bacteria, antioxidant activity, the value of o-phthalaldehyde (OPA) and angiotensin converting enzyme inhibition on yogurt with *F. glomerata* Roxb extract during storage in refrigerator (4°C) were higher and significantly different ($p<0.05$) compared to yogurt that optimally inhibit angiotensin converting enzyme inhibitors on day 7 of storage.

Introduction

Ficus glomerata Roxb. syn. *F. racemosa* L. is a plant species in the family Moraceae (Figure 1). It is originally from Australia, Malaysia, South-East Asia and Indian Subcontinent. *F. glomerata* Roxb extract has total phenolic content of 246.4 mg/g GAE (Gallic Acid Equivalent) and 65.7% antioxidant activity based on ethyl acetate fractionation. *F. glomerata* Roxb was also found to contain flavonoids (quercetin) (Verma *et al.*, 2010). Kwon *et al.* (2006) stated that the high content of flavonoid compound of fruits and leaves of *F. glomerata* Roxb could inhibit angiotensin converting enzyme (ACE) either *in vivo* or *in vitro*.

Some researches have been conducted to determine the proteolytic system of lactic acid bacteria involving extracellular proteinase and several intracellular peptides, that produces ACE inhibitor in dairy products (Yamamoto *et al.*, 1994). Fitzgerald *et al.* (2004) stated that yogurt is able to provide beneficial health effects associated with proteolytic result (mainly bioactive peptides) during fermentation and storage of yogurt as an angiotensin converting enzyme inhibitor. The extract of medicinal plant have been widely used to affect the pattern of proteolysis of lactic acid bacteria, antioxidant activity of yogurt, and enhance the ability of angiotensin converting enzyme inhibitor in yogurt (Shori and Baba 2013). The purpose of this present study was to assess the inhibitory potential of yogurt with *F. glomerata* Roxb extract as anti-hypertensive (i.e.angiotensin converting enzyme inhibitor).

Figure 1. Fruits of *Ficus glomerata* Roxb

Materials and Methods

Materials

Fruits of *F. glomerata* Roxb were obtained from Praya, Central Lombok, West Nusa Tenggara, Indonesia. Chemicals were obtained rom Sigma-Aldrich, Germany. Chemical used were Gallic acid, quercetin, rutin, flavanone, and catechin standards, 1-diphenyl-2-picrylhydrazyl (DPPH), methanol, ethanol 95%, angiotensin coverting enzyme, substrate (Hip-His-Leu), HEPES buffer, sodium chloride (NaCl), hydrogen chloride (HCl), sodium chloride (NaCl), sodium carbonate (Na_2CO_3), and distilled water (dH20). Starter culture of *Streptococcus thermophillus* FNCC 0015 and *Lactobacillus bulgaricus* FNCC 0041 obtained from Food and Nutrition Culture Collection (FNCC), Gajah Mada

*Corresponding author.
Email: *endangsrahayu@ugm.ac.id*

University, Yogyakarta, Indonesia.

Preparation of F. glomerata *Roxb fruit extraction*

Fruits of *F. glomerata* Roxb were chopped off into small size and dried at 60°C in the oven for about 12 hours. Then, the chopped fruits of *F. glomerata* were mashed using a blender, dried and powdered for extraction.

Fruit of F. glomerata *Roxb extraction*

F. glomerata Roxb fruit powder was weighed (50 g), then diluted with 500 ml of distilled water in a 1-liter Erlenmeyer and stored in water bath at a temperature of 50^0C for ±5 hours. The supernatant was then stored in the refrigerator (temperature 4^0C) until next use.

Preparation of yogurt

Each ampoule of starter bacteria Lactobacillus bulgaricus FNCC 0041 and Streptococcus thermophillus FNCC 0015 was opened aseptically by cutting the ampoule, then five (5) drops of sterile physiological sodium chloride solution/broth was added. The suspension formed was then inoculated into each of MRS broth (50 mL) and incubated at 37°C for 24 hours. Then, 12.5 mL of each starter cultures of *L. bulgaricus* FNCC 0041 and *S. thermophillus* FNCC 0015 from the MRS broth were added into the UHT milk (250 ml) and incubated at 37°C for 24 hours.

Plain yogurt and yogurt with *F. glomerata* Roxb extract were prepared on the same day. *F. glomerata* Roxb fruit extract (100 mL) was added to the full cream milk that has been pasteurized before (820 mL) and skim milk (30 mL), then followed by addition of mix starter culture of *S. thermophillus* FNCC 0015 and *Lactobacillus bulgaricus* FNCC 0041 (50 mL). Distilled water (100 mL) was used as replacement of *F. glomerata* Roxb fruit extract for plain yogurt. Yogurt was then fermented in a water bath (43°C) until pH 4.5 was reached. The prepared yogurt samples were stored in the refrigerator for 1; 7; 14; 21 and 28 days prior to analyses.

Preparation of yogurt water extract

Plain- and herbal-yogurts (10 grams) were homogenized with 2.5 mL of sterile distilled water. The pH of the yogurts was determined and the yogurts subsequently acidified to pH 4.0 with Hydrochloric acid solution (0.1 M). The acidified yogurts were then heated in water bath (45°C) for 10 min followed by centrifugation (5000 x g, 10 min 4°C). Sodium hydroxide (0.1 M) was added to adjust the pH of supernatant to 7.0. The neutralized supernatants were re-centrifuged (5000 x g, 10 min 4°C) and the supernatant was harvested and stored in a -20°C freezer forfurther analysis.

pH and total titratable acid (TTA) determination

Yogurt was initially homogenized in water (1:9 ratio) prior to pH determination. The pH of the homogenized yogurt was read using a digital pH meter. TTA was determined by titration using 0.1 N Sodium hydroxide. Yogurt sample (1 mL) was transferred into an Erlenmeyer flask containing 9 ml dH_2O. A few drops (3 - 5) of 0.1% phenolphthaleinas pH indicator were added. The yogurt mixture was then titrated with 0.1 N Sodium hydroxide with continuous stirring until theconsistent pink color appeared. The amount of acid produced during fermentation was calculated as follows:

Percentage of Lactic Acid = Dilution factor (10) x V NaOH x 0:1 N x 0:009 x100%

WhereV is volume of Sodium hydroxide required to neutralize the acid.

Total phenolic assay

Total phenolic compounds were determined by Shetty *et al.* (2005). Briefly, water extract of herbs (diluted to similar extent as for herbal-yogurt) or yogurts (1.0 mL) were mixed with 1.0 mL of 95% ethanol and 5 mL of distilled water. Folin ciocalteu reagent (diluted 1:1 with distilled water) was added to each sample followed by thorough mixing using a vortex mixer. Sodium carbonate (1.0 mL, 5% or 5g/100 mL) was added to the reaction mixtures and these were left to stand for 60 min incubation at room temperature. The absorbance of 725 nm (using UV/Vis Spectrophotometer, Shimadzu model 1601) values were converted to total phenolics expressed in microgram equivalents of gallic acid (µg gallic acid equivalent, GAE) per mL sample. Standard curves were simultaneously established for each assay using various concentrations of gallic acid (5–60 µg/mL) in methanol.

Antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical inhibition assay

Yogurt extracts (250 µL) were added into 3.8 mL of 60 mmol/L DPPH (Sigma-Aldrich,Germany)/L in ethanol. The decrease in absorbance was monitored at 517 nm until a constant reading was obtained (using UV/Vis Spectrophotometer, Shimadzu model 1601). The constant reading for the yogurt extracts and control (consisting of 250 µL of water as a replacement of extract) was used in calculating the

% inhibition of DPPH oxidation (Apostolidis *et al*., 2007) as follows:

$$280\% \text{ inhibition} = \frac{[A_{517}^{control} - A_{517}^{extract}]}{[A_{517}^{control}]} \times 100$$

O-Phthalaldehyde (OPA) assay

This assay was used to evaluate proteolysis of milk proteins. The OPA reagent was done as described by Church *et al*. (1983). A small aliquot of yogurt extract (10–50 µl containing 5–100 µg protein) was added directly into 1.0 ml of OPA reagent. The solution was mixed briefly by inversion and incubated for 2 min. at room temperature. The absorbance readings were taken at 340 nm (using UV/Vis Spectrophotometer, Shimadzu model 1601). The peptide concentration was estimated against the tryptone standard curve.

Angiotensin-I Converting Enzyme (ACE) inhibitory activity

The ACE reagent prepared as described by Chusman and Cheung, (1971) with slight modification by Byun and Kim (2002). A yogurt extract (50 µl) with 50 µl of ACE solution (25 munits/mL) was pre-incubated at 32°C for 10 min, and the mixture was subsequently incubated with 50 µl substrate (8 mM Hip His Leu in 50 mM HEPES) for 30 min at the similar temperature. The reaction was terminated with addition of 250 µl 1 M hydrogen chloride. The solution was extracted with 1.5 mL ethyl acetate and then centrifuged (4000 x g) for 15 min. The supernatant was transferred into a room temperature in a vacuum dryer for 2 hours and then dissolved in 3 mL distilled water and absorbance was measured at 228 nm using (using UV/Vis Spectrophotometer, Shimadzu model 1601).

Results and Discussion

Effect of fruit extract of F. glomerata *Roxb on pH and TTA during fermentation and storage of yogurt*

The result of the pH observation during the fermentation process shows that both plain yogurt and yogurt with *F. glomerata* Roxb extract has similar initial pH, that was 5.85±0.03 (plain yogurt), 5.83±0.04 (yogurt with fruit extracts of *F. glomerata* Roxb 5 %) and 5.80±0.02 (yogurt with fruit extracts of *F. glomerata* Roxb 10%) and showed significant difference ($p<0.05$) on the seventh hours of fermentation, with the pH values of each treatment were 4:58±0.01 (in plain yogurt), 4:57±0.00 (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 4:55±0.01 (yogurt with fruit extracts of *F. glomerata* Roxb 10%). Furthermore, the values of TTA (total titratable acid) on the seventh hours of fermentation were 0.70 ± 0.01 (plain yogurt), 0.74 ± 0.03 (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 0.76 ± 0.04 (yogurt with extracts fruit *F. glomerata* Roxb 10%) and did not show significant differences ($p>0.05$). Storage in the refrigerator for 28 days lead to further reduction of the pH that were as follows: 4.28±0.01 (plain yogurt), 4.20±0.01 (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 4.02±0.02 (yogurt with fruit extracts of *F. glomerata* Roxb 10%) with corresponding TTA values as follows: 1.06±0.01 (plain yogurt), 1.22±0.01 (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 1.31±0.01 (in yogurt with fruit extracts of *F. glomerata* Roxb10%) at day 28 stored in a refrigerator.

According to Vedamuthu (1982), the extension of yogurt storage time to 28 days led to decreased of pH values (4.2 – 4.4) and was likely to occur due to an accumulation of acetic acid, acetaldehyde, formic acid and lactic acid. Organic acids during fermentation and storage times which are produced in yogurt (lactic acid, citric acid, formic acid, acetic acid and butyric acid) have a linear relationship with the accumulation of TTA (Ostlie *et al.*, 2005; Billard *et al*., 2007). The values of TTA which is higher at the end of fermentation and storage times on *F. glomerata* Roxb yogurt compared to plain yogurt may indicate the different microbial populations during the fermentation and storage time of yogurt.

Total phenolic content

Total phenolic content (TPC) on plain yogurt and yogurt with *F. glomerata* Roxb during fermentation were 6.72±0.21 µgGAE/mL (plain yogurt), 19.06±0.36 µgGAE/mL (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 22.05±0.50 01 µgGAE/ml (yogurt with fruit extracts of *F. glomerata* Roxb 10%) and showed significant differences ($p<0.05$) on the initial time of fermentation. As for TPC value at the end of fermentation, the values were 11.72±0.52 µgGAE/mL (plain yogurt), 23.77±0.60 µgGAE/ml (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 26.73±1.05 µgGAE/mL (yogurt with fruit extracts of *F. glomerata* Roxb 10%) which showed significant differences ($p<0.05$).

The result showed a gradual increase of total phenolic content during storage time on refrigerator. The highest value of total phenolic content were 3 3.52±1.86 µgGAE/mL (in yogurt with fruit extracts of *F. glomerata* Roxb10%) on 28 days of storage in 4°C. TPC values in plain yogurt reflected the phenolic compounds that were associated with milk protein breakdown (Damin *et al.*, 2009). The amino acid tyrosine for example, has a phenolic side chain that detects the presence of phenolic compounds during

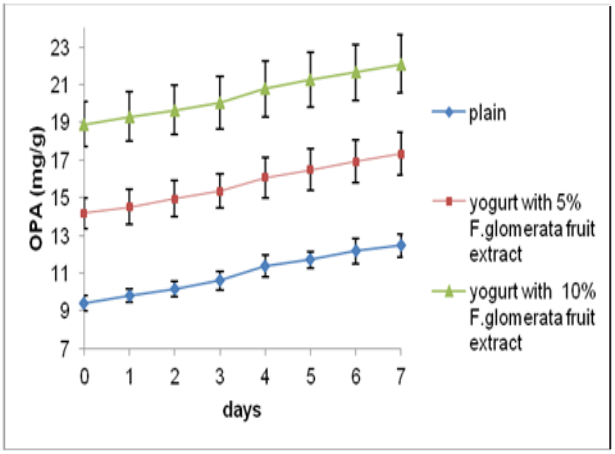

Figure 2. Evaluation of proteolysis by o-phthalaldhyde (OPA) assay of yogurt during fermentation.

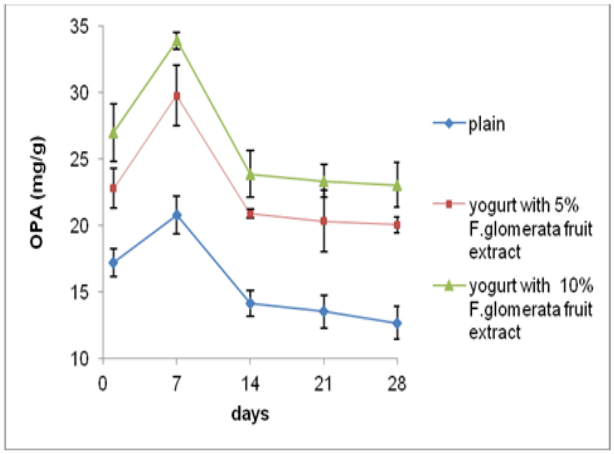

Figure 3. Evaluation of proteolysis by o-phthalaldhyde (OPA) assay of yogurt during storage times.

absorbance spectrophotometer reading (Shah, 2000). Another possibility was that the microorganism could have utilized phenolic acids such as ferulic acid and p-caumaric acid during fermentation and post acidification that produces phenolic acids such as vanilic acid and p-hydroxybenzoic before the aromatic ring structure was broken down (Blum, 1998). Ishikawa *et al.* (2002) stated that improved TPC values at *F. glomerata* Roxb yogurt can be explained by the presence of phytochemical indigenous compounds in *F. glomerata* Roxb fermentation and storage at 4°C (e.g. flavonoid and phenolic compounds)

Antioxidant activity of yogurt

The results showed that at the end of fermentation and during the storage period in the refrigerator (4°C). *F. glomerata* yogurt has higher antioxidant activity which was significantly different ($p<0.05$) compared with plain yogurt. Antioxidant activity of yogurt at the end of fermentation were 26.01±0.49% (plain yogurt), 31.29±0.70% (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 34.22±0.71% (yogurt with fruit extracts of *F. glomerata* Roxb 10%). Optimal inhibitor was achieved during the seventh day of storage which was 32.93±2.48% (plain yogurt) and showed significant differences ($P<0.05$) with *F. glomerata* Roxb yogurt, that were4 5.54% (yogurt with fruit extracts of *F. glomerata* Roxb 5%), and 50.70±0.54% (yogurt with fruit extracts of *F. glomerata* Roxb 10%). The higher antioxidant activity in *F. glomerata* Roxb yogurt was most likely because of contribution of each phytochemical compounds and as a result of microbial metabolic activity (Thompson *et al.*, 2007).

The high inhibition of DPPH on the seventh day of storage in the refrigerator (4°C) associated with the metabolic activity of yogurt bacteria even at low temperatures. The growth of bacteria that constantly occur during storage of yogurt in refrigerator (4°C) possibility of changed the antioxidant activity (Blum, 1998). Yildiz and Eyduran (2009) stated that reduction of antioxidant activity during storage in the refrigerator was associated with increased of phenolic compounds degradation.

Evaluation of proteolysis by o-phthalaldhyde (OPA) assay

Yogurt with fruit extracts 10% had higher OPA values (18.89±1.20 mg/mL) than yogurt with fruit extracts 5% (14.17±0.82 mg/mL) and plain yogurt (9.35±0.41 mg/mL) on initial time of fermentation and increased to 17.17±1.04 (plain yogurt), 22.79±1.51 (yogurt with fruit extracts of *F. glomerata* Roxb 5%), and 27.00±2.18 (yogurt with fruit extracts of *F. glomerata* Roxb10%). Proteolysis in plain yogurt, yogurt with fruit extracts of *F. glomerata* Roxb 5% and yogurt with fruit extracts of *F. glomerata* Roxb 10% increased 20.98%, 36.54% and 25.60%, respectively, on day 7 of storage with peptide concentration recorded as 20.77 mg/mL, 29.80±2.32 mg/mL and 33.91±2.64 mg/mL respectively ($P<0.05$) (Figure 2). *L. bulgaricus* and *S. thermophillus* are metabolically active even at 4°C.

According to Amirdivani and Baba (2011), the level of proteolysis by lactic acid bacteria is potentially enhanced by the presence of phenolic compounds lead to increased production of bioactive peptide that occur in the first weeks of storage in the refrigerator. It decreased during the second week until the fourth week of storage due to the decline in the ability of phenolic compounds to breakdown of proteins (Figure 3).

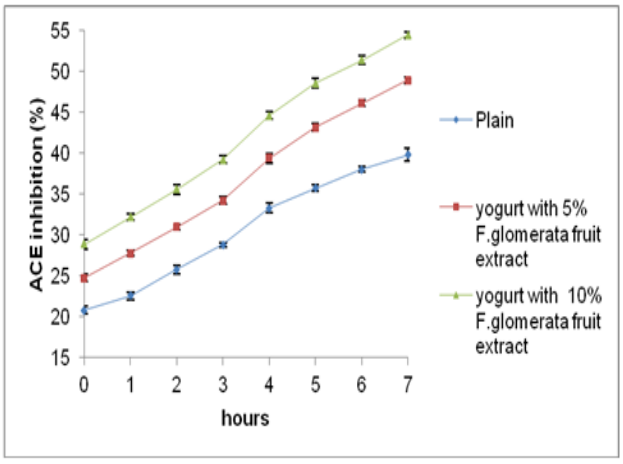

Figure 4. Angiotensin converting enzyme (ACE assay of yogurt during fermentation.

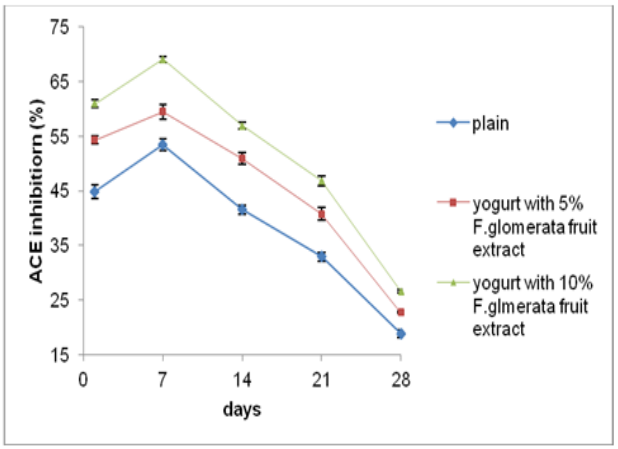

Figure 5. Angiotensin converting enzyme (ACE) assay of yogurt during storage times

Angiotensin converting enzyme (ACE) inhibition activity

The angiotensin converting enzyme (ACE) assay was carried out to evaluate the ACE inhibitory activity of bioactive peptides produced during yogurt formation and refrigerated storage (Gobbetti *et al*., 2007). At the beginning of fermentation, ACE inhibition activity were 20.82±0.45% (plain yogurt), 24.75±0.48% (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 28.87± 0.56% (yogurt with fruit extracts of *F. glomerata* Roxb 10%) and increased at the end of fermentation were 9.83±0.78% (plain yogurt), 48.94±0.42% (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 54.48±0.44% (yogurt with fruit extracts of *F. glomerata* Roxb 5%), respectively (P<0.05). These results indicate that during the fermentation process, flavonoid compounds contained in extracts of *F. glomerata* Roxb fruit has been able to improve the ability of lactic acid bacteria as an ACE inhibitor. The highest ACE inhibitory activity was 69.11±0.50% (yogurt with fruit extracts of *F. glomerata* Roxb 10%), then 59.49±1:35% (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 53.47±1:07% (plain yogurt) (P<0.05) (Figure 4).

ACE inhibitory activity had decreased in accordance with the storage time (Figure 5). The changes in ACE inhibitor activity in relation to OPA values with fermentation and storage time for both plain and yogurt with fruit extracts of *F. glomerata* Roxb (Figures 2 and 3). The respective values of ACE inhibitory activity on the 28th day of storage were 18.82±0.69% (in plain yogurt), 22.71±0.69% (yogurt with fruit extracts of *F. glomerata* Roxb 5%) and 26.61±0:32% (yogurt with fruit extracts of *F. glomerata* Roxb 10%). This suggest that the relatively less specific peptides produced during fermentation were further cut into smaller peptides and much more bioactive peptides were present during the first 7 days of storage in the refrigerator (Figure 3). Further proteolysis of the protein during longer storage time (28 days) produced peptides that are smaller may have less ACE bioactivity. The addition of *F. glomerata* Roxb extract may thus change the manner in which the microbial enzymes affected proteolysis and subsequently the formation and deactivation of proteins with anti- ACE activities.

Conclusion

F. glomerata Roxb fruit extract in yogurt fermentation process was able to increase the formation of peptides with ACE inhibitory activity. Proteolytic activity of yogurt bacteria during fermentation and storage (4^{o}C) was the highest in the presence of *F. glomerata* Roxb fruit extract along with the increase in peptide formation during fermentation and achieve its maximum extent on the seventh day of storage in the refrigerator.

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