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The effect of fermentation on total phenolic, flavonoid and tannin content and its relation to antibacterial activity in jaruk tigarun (*Crataeva nurvala*, Buch HAM)

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Abstract

Jaruk tigarun is a traditional fermented food from South Borneo Indonesia, made from tigarun flower (*Crataeva nurvala*, Buch HAM) . It has been used traditionally to reduce fever and to cure disease for postpartum women. Although it has been consumed for generations and believed to have health benefit, limited scientific research have been done to evaluated it. The study aimed to determine total phenolic, tannin and flavonoid content and evaluated the antibacterial activities of fresh and fermented tigarun flower extract. The fresh and fermented flowers (jaruk tigarun) were freeze dried, powdered and extracted using methanol, ethanol and ethyl acetate solvent. The total phenolic and total tannin were determined using Folin-Ciocalteu methods while total flavonoid content by aluminium trichloride ($AlCl_3$). Antibacterial activity in each extract and MIC were evaluated using well diffusion and macrodilution methods. The result showed that the highest total phenolic content was obtained in methanolic extract of jaruk tigarun (53.24 ± 0.21 mg GAE/g). After fermentation, total phenolic and flavonoid content of tigarun flower generally increased while total tannin content was decreased. The increasing of total phenolic and flavonoid content consequently exhibited the strongest antibacterial activities. Methanolic extract of jaruk tigarun showed high inhibition compared to ethanolic and ethyl acetic extract. The highest inhibition of methanolic extract was on *Pseudomonas flourescens* (8.62 ± 0.54 mm). However the antibacterial activity is lower than that chloramphenicol. *Pseudomonas flourescens* and *Staphylococcus aureus* showed the most susceptibility to methanolic extract with MIC concentration 6,250 µg/mL. The result indicated that the fermentation increase the total phenolic and antibacterial activity of jaruk tigarun.

Introduction

Indonesia has a wide variety of unique treasures of traditional foods and the potential to be developed as a functional food product. Traditional foods are generally prepared from local raw materials with recipes that have been known to the local community (Nugroho and Susanto, 1998). It is characterized by a unique sensory and have a physiological nutritious for health. For example, tempe that has antioxidant properties and anti-diarrheal (Esaki *et al*., 1996; Hartiningrum, 2010). In South Borneo, there are also many traditional foods that have been used for generations to cope with health problems. However, limited study has been done to reveal the potential health benefits component and antibacterial activity of these food

Jaruk tigarun was made from the flower of tigarun (*Crataeva nurvala* HAM) which fermented in boiled water for a certain time without the salt addition (Nazarni, 2006). The word jaruk is derived from Banjar languange which means pickled (Hapip, 2008). It is consumed as a side dish vegetable and quite popular with locals as it is considered to increase appetite with spesific sour-bitter flavour.

In South Borneo, Jaruk tigarun is used to reduce fever and cure kalalah- the postpartum women disease, so after childbirth they are usually advised to consume tigarun. In India, the bark of *Crataeva* was used as ayurvedic to cure disorders of urinary organ, fever, vomiting and gastric irritation (Parvin *et al.*, 2012).

The use of this fermented food indicates antimicrobial or cytotoxic activity that needs to be further investigated. The components of that have been identified in the bark tigarun are flavonoids, glucosinolates, plant sterols, lupeol, saponins and tannins (Geetha and Varalaksmi, 1999) as well as

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succinic acid, lactic acid and mannitol (Paarakh *et al*., 2011). Another compound identified was flavonoid in the leaves and triterpene compounds in the fruit (Gagandep and Meera, 2006; Gagandep *et al.*, 2009). Flavonoids, tannins and lupeol known to have antioxidant and antibacterial activity (Shirwaikar *et al*., 2004; Abd- Alla *et al*., 2009; Bhaskar *et al*., 2009; Ahmed *et al.*, 2010). Another pharmacological activity of stem extracts *Crataeva nurvala* include antiinflamatory, anti arthritis (Das *et al*., 1974), anti-radical (Bhaskar *et al.*, 2009) and antibacterial activity (Parvin *et al*., 2012).

Lactic acid bacteria (LAB) have been traditionally used for vegetables fermentation since they are naturally present in it. Lactic acid bacteria present a small part (2.0 - 4.0 log cfu g^{-1}) of the autochthonous microbiota of raw vegetables and fruits (Di Cagno *et al*., 2013). The main species isolated from raw or spontaneously fermented vegetables were *L. plantarum, L. paraplantarum, L. fermentum* and *L. pentosus* which have β-glucosidase, tannase and decarboxilase activity (Osawa *et al.*, 2000; Sánchez *et al.*, 2000; Di Cagno *et al.*, 2008). Natural and induced fermentation by *L. plantarum* and other LAB are sufficient to improve the concentration of phenolic compounds in fermented plant product by release these enzyme (Ciafardini *et al.*, 1994; Duenas *et al.*, 2005). Complex polyphenols are hydrolysed to simpler and more biologically active compounds during fermentation, while β-glucosidase might cleavage of inter-sugar linkages releasing the corresponding glycosides that were hydrolysed liberating the phenolic aglycon moieties (Martins e*t al.*, 2011). Phenolic compounds are known to have membrane-active properties against microorganisms causing leakage of cell constituents (Johnston *et al*., 2003).

The fermentation process can increase the biological activity of the active compound and nutritional component in the food ingredients (Parvez *et al*., 2006; Zhu *et al*., 2007; Chelule *et al*., 2010), so study on the potential and health benefit of jaruk tigarun as a traditional food product is important. This study aims to determine the total phenolic, flavonoid and tannin content of fresh and fermented tigarun flower as well as evaluate the antibacterial activity of jaruk tigarun extracted with methanol, ethanol and ethyl acetate.

Materials and Methods

Materials

Tigarun fresh flower obtained from five district in South borneo and harvested when the flowers bloom season from November 2012 – March 2013. Flower was collected and identified in Plant Systematic Laboratory, Faculty of Biology, Gadjah Mada University, Yogyakarta. Methanol, ethanol and ethyl acetate (analytical grade, Merck). Quercetin, gallic acid and tannic acid were purchase from Sigma chemical Co. Folin-Ciocalteu Reagent, sodium bicarbonate, aluminium chloride were purchased from Merck (Darmstadt, Germany). Nutrient broth and nutrient agar were purchased from Oxoid. Pathogenic bacteria from FNCC (Food and Nutrition of Culture Collection): *Escherichia coli* FNCC 0091, *Salmonella* sp. FNCC 0050, *Pseudomonas flourescens* FNCC 0070, *Staphylococcus aureus* FNCC 0047, and *Bacillus subtilis* FNCC 0059 obtained from the Center for the Study of Food and Nutrition, Gadjah Mada University, Yogyakarta.

Preparation of fresh and jaruk tigarun extract

Fresh flowers were freeze dried and crushed to a powder. To produce jaruk tigarun, the fresh flower fermented for 7 days in warm water until it became red-brown in colour, wilt but crunchy and had a specific sour-bitter flavour (Nazarni *et al*., 2013). Jaruk was drained, freeze dried and crushed to a powder. The powders were sieved to obtain the size of 30 mesh and stored at -20ºC for further extraction. Jaruk and fresh flower powders each macerated in methanol, ethanol and ethyl acetate (1:20) at a temperature of 60ºC, shaken at 100 rpm, for 96 hours. The mixture was filtered by whatman no.1 and evaporated to dryness using vacuum rotary evaporator at 60ºC.

Analysis of extract

Total phenolic content (TPC)

Total phenols of the extract of fresh flowers and jaruk tigarun performed according to the protocol of Hung and Yen. (2000). Extract (0.1 mg) were dissolved in 0.1 mL distilled water. One mL solution was added to 2 mL of 2% Na_2CO_3. After 3 min, 50% Folin-Ciocalteau reagent (0.1 mL) was added to the mixture which was then left for 30 min. Absorbance was measured at 750 nm using Shimadzu 1650 UV vis spectrophotometer. TPC was expressed as gallic acid equivalents (GAE) in mg per g of extract.

Total tannin content

Total tannin determined according to Rangana. (1977). One mL extract dissolved in 7.5 mL distilled water was added to 0.5 mL Folin-Ciocalteu's reagent (1:1) and 1.0 mL saturated Na_2CO_3 (35%). The mixtures were vortex and kept in dark for 30 min. The absorbance was measured at 760 nm. Standard curve

prepared with tannic acid and total tannin expressed as tannic acid equivalent in mg/g of extract.

Total flavonoid content

Total flavonoid content was determined using aluminium chloride ($AlCl_3$) according to known method (Ordonez *et al*., 2006), using quercetin as a standard. The extract (0.1 mL) was added to 0.3 mL distilled water followed by 5% $NaNO_3$(0.03 mL). After 5 min at 25ºC, $AlCl_3$ (0.03 mL, 10%) was added. After further 5 min, the reaction mixture was treated with 0.2 mL of NaOH 10%. Finally, the reaction mixture was diluted to 1 mL with water and the absorbance was measure at 510 nm. The result were expressed as mg quercetin (QE)/ g of extract.

Antibacterial assay

Antibacterial activity of the extracts was measured by well difusion (Perez *et al.*, 1990). Pathogenic bacteria refreshed and grown in nutrient broth for 20 -24 hours. One mL culture pathogens (10^6 -10^7 CFU / mL) were taken and poured in petri contains 6-10 mL nutrient agar temperature of 45ºC and cooled. Wells (8 mm diameter) were cut out from agar plates using a sterilized stainless steel borer and filled with 50 µL of the extract. The plates inoculated with different bacteria were incubated at 37ºC for 24-48 hours. Clear zone wells indicate the presence of antibacterial activity. Cloramphenicol was used as a positive control.

Determination of minimum inhibitory concentration

The selected extract were screen to determine minimum inhibitory concentrations (MIC's) by modified standard two-fold broth in macrodilution methodology given by CLSI (CLSI, 2012). A stock solution of methanolic extract was serially diluted in steril test tube with nutrient broth to obtain a concentration ranging from 195 µL/mL to 50.000 µL/mL. A minimum final volume of 1 mL of each dilution is needed for the test. A standardized inoculum for each bacterial strain was prepared so as to give inoculum size approximately 5 x 10^6 CFU/ mL in each tube. Add 1 mL of the inoculum to each tube containing 1 mL of antimicrobial agent in the dilution series (and a positive control tube containing only broth), and mix. Tubes were kept at 37ºC for an overnignt incubation. The MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacterial strain.

Statistical analysis

All the analysis was perform in triplicate, result were expressed as means ± standard deviation. The differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) with tukey methods range tests using SPSS statistics 22 software. ANOVA data with a $P<0.05$ was considered statistically significant.

Results and Discussion

Total phenolic, tannin and flavonoid content of fresh and fermented tigarun flower

Significant differences were found in total phenolic content (TPC) among fresh and fermented flower (jaruk tigarun), ranging from 12.58±0.09 to 53.24±0.73 mg GAE/g extract (Table 1). The highest result was 53.24±0.73 mg GAE/g obtained from fermented flower in methanolic extract. However, TPC in flower was lower than that of the other part of tigarun. Kumari and Kakkar. (2008) reported that bark of *C. nurvala* has a high total phenolic content about 195.2±0.30 mg GAE/g. So far our finding is the first time on the TPC reported of *Crataeva* flower.

The phenolic content of fermented flower were higher than that of the fresh form in each extract. This shows an increase in phenolic content during the fermentation. Fermentation process causes release of microbial enzyme which in turn produce more freely available form of plant chemicals like flavonoid, tannin, alkaloid and phenylpropanoids (Messen and Vuyst, 2002). The presence of lactic acid bacteria in fermentation contributes to the simple phenolic conversion and the depolymerization of high molecular weight phenolic compounds (Othman *et al.*, 2009). Ng *et al.* (2011) also reported that plant parts have an increase in total phenols after fermentation.

The highest TPC was observed in methanolic extract of fermented flower followed by ethanolic and ethyl acetic extract. Methanol was more efficient to extract polyphenolic compounds compared to ethanolic and ethyl acetic solvent. Phenolic compounds are generally more soluble in polar organic solvents. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction (Zhou and Yu, 2004; Mohsen and Ammar, 2008).

The result of total flavonoid content from extracts of tigarun flower is given in Table 1. The total flavonoid content varied from 1.96±0.88 to 6.53±0.00 mg QE/g. Methanolic extract from fresh flower had the highest flavonoid content (6.53±0.00 mg QE/g). There are no significant differences ($P>0.05$) in TFC among fresh and fermented methanolic extract, but on the contrary for ethanolic and ethyl acetic

Tabel 1. Total phenolics, flavonoid and tannin content of extract of tigarun

sample	Extract	Phenolic content (mg GAE/g)	Total flavonoid (mg QE/g)	Total tannin (mg TAE/g)
Fresh flower	Methanolic	50.37±0.22^{e}	6.53±0.00^{c}	6.56±0.22^{d}
	Ethanolic	35.49±0.12^{c}	3.61±0.07^{b}	0.54±0.05^{a}
	Ethyl acetic	12.58±0.09^{a}	1.96±0.88^{a}	0.99±0.03^{b}
Jaruk	Methanolic	53.24±0.73^{f}	6.28±0.36^{c}	0.55±0.92^{a}
	Ethanolic	44.86±0.90^{d}	5.83±1.00^{c}	1.97±0.10^{c}
	Ethyl acetic	23.95±0.13^{b}	3.41±0.57^{b}	1.17±0.03^{b}

*Different letter in the same coloum indicates significant difference ($P < 0.05$)

extract ($P<0.05$). The TFC from ethanolic extract increased from 3.61±0.07 to 5.83±1.00 mg QE/g after fermentation, similar result were found for ethyl acetic extract which increased from 1.96±0.88 to 3.41±0.57 mg QE/g. Microbial enzymes, such as glucosidase, amylase, cellulase, tannase, esterase, invertase or lipase produced during fermentation can hydrolyse glucosides, and break down plant cell walls or starch. These enzymes play a role in disintegrating the plant cell wall matrix and consequently facilitating the flavonoids extraction (Hur *et al*., 2014). Another mechanism is along fermentation the β-glucosidases of microbial origin could also be used to hydrolyze the phenolics and flavonoids. *L. plantarum* were documented as having strong glucosidase activity (Duenas *et al*., 2005) therefore, increased active compounds might be converted from the enzymatic cleavage of corresponding glucosides.

Total tannin content varied from 0.54±0.05 to 6.56±0.22 mg TAE/g (Table 1). The highest total tannin content was observed in methanolic extract of fresh flower (6.56±0.22 mg TAE/g) which have decreased after fermentation (0.55±0.92 mg TAE/g). These result suggested that along fermentation, tannic acid degrades into smaller component. Tannic acid is one of the most abundant reserve materials of plants. Tannase (tannin acyl hydrolase, EC. 3.1.1.20) catalyzes the hydrolysis of ester and depside linkages in hydrolyzable tannins like tannic acid. The products of tannic acid hydrolysis are glucose and gallic acid (Rodríguez *et al*., 2008). Osawa *et al.* (2000) reporting the occurrence of *lactobacilli* capable of degrading hydrolyzable tannin in foodstuffs such as pickled vegetables and cheese. Kachouri and Hamdi. (2004), also reported tannic acid degradation into monomeric products by *L. plantarum* is in accordance to the depolymerisation of high molecular weight of phenolic compounds and a reduction of low molecular weight phenolics compounds in olive mill wastewater.

In contrast, total tannin content in ethanolic and ethyl acetic extract were increase after fermentation. It is more caused by extraction ability from solvents and solubility of tannic acid. Fresh biological material are mostly stored in protected state such as binding to membranes, compartmentalization, protection by lipophilic material and so on (Shimizu, 1998). Polar organic solvent such as methanol are often used in extraction even if they may not be the best solvent to dissolve the target molecules. It is thought that alcoholic solvents can break up the compartmental structures and efficiently penetrate cell membran, permitting the extraction of high amount of endocellular component (Gloria *et al*., 1998). Different from another solvent which more difficult to break up the compartmental structures. However, adapt with the principle, "like dissolves like" (Shimizu, 1998), tannic acid is lower solubility in non polar organic solvent, with that result low concentration obtained in extract. Along fermentation in a line with the cell wall degradation, tannic acid released and carried over by non polar organic solvent.

Antibacterial activity

Five species of microorganism were used for antibacterial activity test, namely *E.coli, Salmonella, Pseudomonas flourescens* (representing gram negative) and *Staphylococcus aureus* and *Bacillus subtilis* (representing gram positive). Table 2 showed the antimicrobial test result against these bacteria by well diffusion methods. There were no inhibition against patogen in fresh flower extract except for *S. aureus* in methanolic extract (2.33±0.32 mm). In contrast, all of the solvent from jaruk extract showed the existence of inhibiton against all of bacteria tested. Methanolic and ethanolic extract of jaruk tigarun had a similar inhibition, where as the ethyl acetic extract showed a weak inhibition. The methanolic extract showed highest activity against *P.flourescens* (8.62±0.54 mm) and ethyl acetic extract showed lowest activity against *Salmonella* (3.50±0.71 mm). This result showed that fermentation brought several advantages since it improved nutrient digestability and the biological activity.

Fermentation is an ancient technology used to enhance the shelf-life and nutritional and organoleptic qualities of food (Frias *et al.*, 2005). Many biochemical changes occur during fermentation, leading to an altered ratio of nutritive and anti-nutritive components and, consequently, affects the products properties, such as bioactivity and digestibility (Zhang *et al*., 2012). Degradation of chemical components during the fermentation process thought to be related to

Tabel 2. Inhibition zone of tigarun extract that have antibacterial activity

Material	Ekstract	Inhibition zone (mm)				
		E.coli	*Salmonella*	*S. aureus*	*P.flourescens*	*B.subtilis*
Fresh flower	Methanolic	-	-	2.33±0.32	-	-
	Ethanolic	-	-	-	-	-
	Ethyl acetic	-	-	-	-	-
Jaruk	Methanolic	7.18±0.53	4.00±0.35	6.90±0.14	8.62±0.54	8.43±0.11
	Ethanolic	6.75±0.35	4.25±0.35	6.75±0.99	7.25±0.35	8.50±0.71
	Ethyl acetic	5.00±0.00	3.50±0.71	5.00±0.00	3.85±0.49	6.25±0.35
Chloramphenicol (250µg/mL)		18.50±0.71	12.0±0.0	14.5±0.71	21.0±1.41	12.50±0.71

Table 3. Minimun Inhibition Concentration in the macrodilution assay using the methanolic jaruk extract.

Microorganism	Gram	MIC (µg/mL)
E.coli FNCC 0091	-	25,000
Salmonella FNCC 0050	-	25,000
P.Flourescens FNCC 0070	-	6,250
Staphylococcus aureus FNCC 0047	+	6,250
Bacillus subtilis FNCC 0059	+	50,000

* (-) represents 'No Growth Observed'; (+) represents 'Growth Observed

the bioactivity of these components. Bioactivity of compounds such as condensed tannins, hydrolysable tannins and flavonoids are generally increased in line with the degradation of these compounds into smaller components and release its aglicon (Ming *et al.*, 2006; Kim *et al*., 2010). Degradation of complex into simple components will improve the biological and pharmacological activity of these compounds. So, the fermentation process in tigarun flower increases its antibacterial activity.

The methanolic extract of jaruk which showed the highest inhibition, were subjected to determine minimum inhibitory concentration (MIC) by two fold macrobroth dilution method. Table 3 showed that all antimicrobial activities accured in a concentration-dependent manner which range 6,250 – 50,000 µg/ml. *P.flourescens* and *S. aureus* were most susceptible and inhibited by methanolic extract at 6,250 (µg/ml). This data showed that extract of jaruk tigarun had a broad spectrum for pathogen inhibiton, either Gram positive and Gram negative. Control experiments using standard solvents showed no inhibition of any bacteria, indicating that tigarun extract itself and not solvent inhibited the growth of the Gram positives and Gram-negatives. However, the antibacterial activity of tigarun extract was lower than standard chloramphenicol. The inhibitory activities exhibited by jaruk tigarun extract agree with the previous report that showed antibacterial properties of plants due to the presence of tannins, alkaloids, flavonoids, terpenoids or essential oils (Bassole *et al*., 2003; Viljoen *et al*., 2003; Erasto *et al*., 2004). The present study suggests that the extract from jaruk tigarun possess remarkable antibacterial activity.

Conclusion

Total phenolics and antibacterial activitiy of the extract of jaruk were higher that of fresh form. Moderate antibacterial activity exhibited by the methanolic extract of jaruk tigarun indicated that jaruk tigarun had a more biological activities compared it fresh from. The results of the present study clearly indicated the antibacterial potential in the fermented flower of *Crataeva nurvala* HAM (tigarun). Furthermore, active plant extracts may be subjected to isolation of the therapeutic antimicrobials and the development of functional food by several methods.

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