

Characterization Antimicrobes of *Pliiek U*, A Traditional Spice of Aceh

NURLIANA^{1*}, LISDAR IDWAN SUDIRMAN²

¹Faculty of Veterinary Medicine, Syiah Kuala University, Darussalam, Banda Aceh 23111, Indonesia

²Departement of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia

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Pliiek u obtained by traditionally fermentation of coconut meat is a potential source of antimicrobial compounds. This research was aimed to detect of their active compounds by bioautographic method and to analyze their chemical composition by GC-MS. For this purposes, *pliek u* was extracted with ethanol 96% to get crude ethanol extract of *pliek u* (EEP) and to get ethanol extract of residual *pliek u* (EERP) which was previously extracted by hexane. Crude EEP was separated into four bioautographic spots with different R_f (0.93, 0.71, 0.19, and 0.10) which were all shown to be active against *Staphylococcus aureus*. Similar result was shown by EERP, but only resulted three bioautographic spots (R_f 0.77, 0.63, and 0.4). Crude EEP consisted of 22 components representing 99.98% with fatty acids, ester, alcohol as major constituents and aliphatic hydrocarbon. EERP consisted of 9 components representing 99.80% with alcohol as major constituents and fatty acids, ester, 4-Dibenzofuramine and amine as minor constituents. The present of many active compounds in *pliek u* supports the use of *pliek u* as spice to improve the quality of food and encourages further studies to determine those active compounds.

Key words: antimicrobe detection, chemical composition, *pliek u*

INTRODUCTION

Indonesia is one of countries rich with traditional fermented foods. They can be used as beverage, sauce, spice and proteinaceous plant food. *Pliiek u* is coconut meat fermented involuntarily during few days. It is the last product of fermentation after separation of the oil during the first, second and third steps of fermentation (Cut pipi June 2006). *Pliiek u* has extended use traditionally as spice in Nanggroe Aceh Darussalam (NAD).

Traditional fermented food are prepared from raw or heated materials and acquire their characteristic properties. They have a increasing nutritional value by reduction of toxic compounds, better digestibility and production of vitamins and antibiotics (Wolf 1997; Campbell-Platt 2000). The traditional fermented food such as tempeh reported by Wang and Heselltime (1979), Gandjar (2000) possessed antimicrobial activity.

The fermentation products were produced by biochemical process or slow decomposition process induced by microorganisms or enzymes. Fermentation process depend on the types, number and activity of microorganisms, chemical composition of the original substrates and environmental condition, therefore the raw materials will be converted to final products which are considered comparable or better than the original materials (Battcock & Azam-Ali 1998; Chisti 2000). Fermented foods containing organic acids, bacteriocins, alcohol, fatty acid, enzymes are resistant to deterioration by spoilage microorganisms. More recently, it has been established that some natural compounds produced by fermenting cultures can be extracted and purified, and they

use as preservative foods or antimicrobial agents (Hoover 2000). Recently, fermented foods are gaining popularity due to the increasing of product shelf life with a minimum preservatives.

Traditionally, coconut oil use for a number of health problems such as skin diseases, stomache, venereal diseases and influenza (Fife 2005). Based on many reports, coconut meat and its oil contain various substances with therapeutic effects. The components of lipid such as fatty acids and their derivates in coconut are very useful as functional components or antimicrobial compounds (Kabara 1993). Free fatty acids and their monoglycerides were proven as antimicrobial activity against various microorganisms such as bacteria, fungi and virus, and also did not generate resistance of microbes (Kabara 1978; Wang & Johnson 1992; Wang *et al.* 1993; Nair *et al.* 2005).

In the previous study reported by Nurliana *et al.* (2008), Nurliana *et al.* (2009), the crude ethanol extract of *pliek u* (EEP) and ethanol extract of residual *pliek u* (EERP) which was previously extracted by hexane were tested against a number of strains of microorganisms (Gram positive, Gram negative bacteria and fungi) by paper disc method. The results shows that both EEP and EERP were active against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*. However EEP was active against one species of fungi (*Candida albicans*) as well. Therefore, it was necessary to detect the active compounds of both extracts in order to understand the number and characters of components in the extract of *pliek u* by bioautographic method and GC-MS analysis. In addition this research was particularly done to support traditional food as a healthy food.

*Corresponding author. Phone/Fax: +62-651-7551536,
E-mail: nunayafiq@yahoo.com

MATERIALS AND METHODS

Pliek u. *Pliek u* was obtained from home industry, at Redeup village in Aceh Besar, Nanggroe Aceh Darussalam.

Preparation of Ethanol Extract from *Pliek u.* *Pliek u* of 20 g was macerated in 200 ml of ethanol 96% and stirred in incubator shaker (Innova 4230, New Brunswick scientific, Edison, USA) at 28 °C and 130 rpm for 24 hours. This extraction procedures were repeated three times. Extracts were filtered using fritted glass filter which was connected with vacuum pump. The extracts were pooled and concentrated using rotary evaporator (Büchi, Switzerland) with waterbath temperatures of 40-50 °C and under pressure of 175 mBAR. The extracts were concentrated with air from air compressor. The crude ethanol extract of *pliek u* was named as EEP.

Preparation of Ethanol Extract from Residual *Pliek u.* *Pliek u* of 20 g was macerated in 200 ml of hexane and stirred in incubator shaker (Innova 4230, New Brunswick scientific, Edison, USA) at 28 °C and 130 rpm for 24 hours. This extraction procedures were repeated three times. Extracts were filtered using fritted glass filter which is connected with vacuum pump. The extracts were pooled and concentrated using rotary evaporator (Büchi, Switzerland) with waterbath temperatures of 40-50 °C and under pressure of 335 mBAR. The hexane extract was kept for further study. Furthermore, its residue (residual *pliek u*) was macerated in 200 ml of ethanol 96% and stirred in incubator shaker (Innova 4230, New Brunswick scientific, Edison, USA) at 28 °C and 130 rpm for 24 hours. This extraction procedures were also repeated three times. The extracts were pooled and concentrated using rotary evaporator (Büchi, Switzerland) with waterbath temperatures of 40-50 °C and under pressure of 175 mBAR. The extracts were concentrated with air from air compressor. The crude ethanol extract of residual *pliek u* was named as EERP.

Detection of Antimicrobial Compounds by Bioautographic Method. The bioautographic method was carried out according to Sudirman (2005). Thin-Layer Chromatography (TLC) was carried out for bioautographic purpose with silica gel plates (Merck 60 F 254, 0.1 mm thick, 20 x 5 cm). The target microorganism *Staphylococcus aureus* (Nakamura *et al.* 1999) was isolated from clinical cases of bovine mastitis at the Bacteriology Laboratory, Faculty of Veterinary Medicine, Bogor Agricultural University. The inoculums of *S. aureus* were obtained by inoculating a *ose* of a black colony grown on Vogel Johnson Agar (VJA) into 10 ml of Mueller Hinton broth and incubated at 37 °C for 24 hours.

Ten microliters of each EEP or EERP was deposited as spots on TLC plates. The plates were developed in a butanol-acetic acid-water mixture (3:1:1). The solvent was removed from plates by drying in laminar flow cabinet with exhausted fan for 24 hours. The spots were detected by being viewed under UV light at 366 nm. The chromatograms were then sterilized with UV light at 254 nm for one hour. The plates were placed on a support in sterile polyethylene box in which a moist filter paper was put at the bottom. About 12 ml of molten Vogel Johnson Agar media (VJA added with tellurite of 0.1%) containing *S. aureus* (10^6 - 10^7 cfu/ml) were uniformly spread using sterile Pasteur pipette on the plates. The

chromatograms were incubated at 10 °C for three hours in order to diffuse of the compounds into agar media, and then incubated at 37 °C for 24 hours. Plates with inhibition spots were documented and active spots R_f values were measured.

Characterization of Antimicrobial Compounds. The work was done according to Simonsen *et al.* (2006) by Laboratory of Doping Investigation and Public Health, Jakarta. The chemical composition of ethanol extracts (EEP and EERP) were determined by gas chromatography-mass spectrometry (GC-MS) analysis (FAMES1 M). Samples were prepared according to AOAC (1995). They were dissolved in appropriate solvent to get 150 μ l/ml of concentration. The GC (Agilent Technologies 6890N) inlet temperature was 250 °C. The oven temperature was held at 130 °C for 2 minutes, then with a rate of 6 °C per minute increased to 170 °C which was held for 2 minutes, furthermore 3 °C per minute to 215 °C held for 1 minute, 40 °C per minute increased to 250 °C for 10 minutes. The injection volume was 5 μ l with Helium as carrier. Total run time was 20 minutes with a constant flow at 1.5 ml/minute. The column was a capillary column (Innowax), length 30 m x diameter 0.25 mm, film thickness 0.25 μ m. The flow was split by 1:100 before introduction into Mass Selective detector (Agilent 5973, electron impact (EI)) and Chemstation data system apparatus. The ion source temperature was 230 °C, interface temperature was 280 °C and quadrupole temperature was 140 °C. Components were recognized by retention times of the chromatogram peaks and by their mass spectra. Data were descriptively analysed according to Fessenden and Fessenden (1986).

RESULTS

Detection of the Antimicrobes on Thin-Layer Chromatography. *Pliek us* were extracted with ethanol giving 72.0% of crude EEP, but only 33.24% of EERP. Then both extracts of *pliek u* were subjected to bioautographed.

On silica gel thin-layer chromatograms, crude EEP and EERP were separated into four and three bioautographic spots or pink growth inhibition zones respectively. These inhibition zones were surrounded by black colony of *S. aureus*. The R_f values of the inhibition zones of crude EEP were 0.93, 0.71, 0.19, and 0.10 and those of EERP of 0.77, 0.63, and 0.4 (Figure 1 b,d). Based on these results, the biggest second inhibition zone (R_f 0.71) of crude EEP and the small first one of EERP (R_f 0.77) were fluorescens under UV light at 366 nm (Figure 1 a,c).

Components Identification of Ethanol Extracts of *Pliek u.* A GC-MS analysis of ethanol extracts of *pliek u* enabled the identification of 22 components which were representing 99.89% of crude EEP and 9 components which were representing 99.80% of EERP (Table 1). The principal components of crude EEP was a large amount of fatty acids (caprylic acid; capric acid; lauric acid; miristic acid; palmitic acid; palmitoleic acid; stearic acid; oleic acid; linoleic acid; 7,10,13-hexadecatrienoic acid; 9,12,15-octadecatrienoic acid; and tetradecanedioic acid) representing 43.64% of crude EEP and their derivates, ester and alcohol, representing 30.99,

2.81% of crude EEP respectively, and other components in small constituents, aliphatic hydrocarbon representing 22.45% of crude EEP. Based on Fessenden and Fessenden (1986) fatty acids were grouped in carboxylic acid. The constituents of EERP were small amount of fatty acids (lauric acid, miristic acid, and oleic acid) representing 4.25% and their derivatives, ester and alcohol, representing 14.89, 45.13% of EERP respectively, and other components 4-Dibenzofuramine (22.82%) and Etyl-2,2-difluoro-2-(4-propen-3' (piperadine) (12.71%).

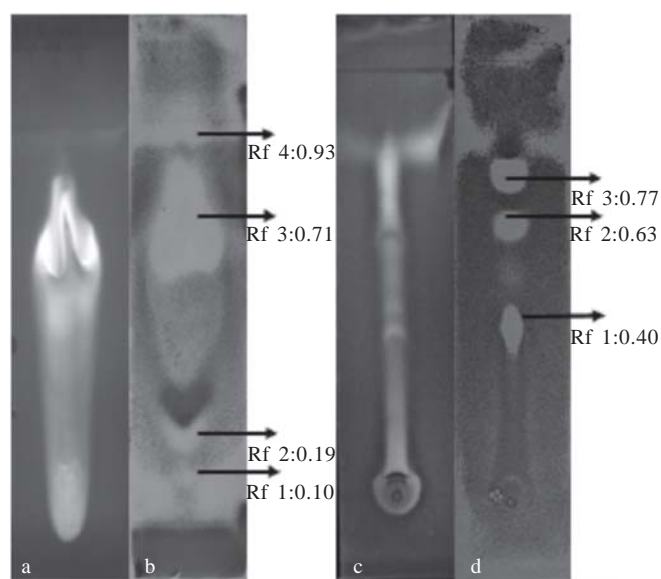


Figure 1. Chromatograms in UV light at 366 nm of crude EEP (a), bioautograms of crude EEP (b), EERP (c), and EERP (d).

DISCUSSION

Bioautographic method is considered the most efficient method for detection of antimicrobial compounds. It uses very little amount of sample compared to disc diffusion method. Bioautographic method combine two methods, there are chemical (chromatogram) and microbiological method. Thus the extract is separated into its difference active components, therefore the process of identification and isolation of the active compounds might be in accurate direction (Rosner & Aviv 1980; Rahalison *et al.* 1991; Sudirman 2005). The active compounds diffuse from the stationary phase into the agar layer giving the inhibition zones as shown in Figure 1.

The bioautographic detection on silica gel thin-layer chromatograms with *S. aureus* exhibited that crude EEP and EERP contained at least four and three active compounds respectively. This different results were due to the separation of components previously isolated by hexane from EERP. Based on previously study that crude EEP was more active than EERP tested by paper disc method. It was due to crude EEP active against bacteria and fungi. EERP and hexane extract only active against bacteria and fungi respectively (Nurliana *et al.* 2009).

The active compounds of crude EEP were different with those of EERP, only the compound or spot of R_f value of about 0.71 obtained from crude EEP might be similar to the compound of R_f value of about 0.77 of EERP. This result was supported by detection of both spots with UV light of 366 nm giving fluorescens spots. However both spots should be revealed with chemical revelators to confirm this results as done by Sudirman (1994).

Table 1. Chemical composition of crude ethanol extract of *pleik u* (EEP) and ethanol extract of residual of *pleik u* (EERP)

Compound	Grouping*	Peak area (%)	
		EEP	EERP
Octanoic acid (caprylic acid) (C8:0)	Carboxylic acid	0.32	
Decanoic acid (capric acid) (C10:0)	Carboxylic acid	0.91	
Decanoic acid methyl ester	Ester	0.49	
Dodecanoic acid (lauric acid) (C12:0)	Carboxylic acid	10.76	0.85
Dodecanoic acid, methyl ester	Ester	8.05	
Dodecanoic acid, 2 hydroxy-1	Alcohol		31.47
Tetradecanoic acid (miristic acid) (C14:0)	Carboxylic acid	5.24	0.55
Tetradecanoic acid methyl ester	Ester	7.34	
Hexadecanoic acid (palmitic acid) (C16:0)	Carboxylic acid	10.24	0.01
Hexadecanoic acid methyl ester	Ester	6.15	
Hexadecanoic acid 2,3-dihydroxy	Alcohol		13.66
Hexadecanoic acid, 2 hydroxy-1	Alcohol	2.81	
9-Hexadecenoic acid (palmitoleic acid) (C16:1)	Carboxylic acid	2.39	
Octadecanoic acid (stearic acid) (C18:0)	Carboxylic acid	1.55	
Octadecanoic acid methyl ester	Ester	3.70	14.89
9-Octadecenoic acid (oleic acid) (C18:1)	Carboxylic acid	9.69	2.84
9-Octadecenoic acid methyl ester	Ester	4.57	
9,12-Octadecadienoic acid (linoleic acid) (C18:2)	Carboxylic acid	0.74	
9,12-Octadecadienoic acid methyl ester	Ester	0.69	
7,10,13-hexadecatienoic acid	Carboxylic acid	1.06	
9,12,15-octadecatienoic acid	Carboxylic acid	0.23	
3-Dodecendiene	Aliphatic hydrocarbon	19.36	
1,4-cyclononadiene	Aliphatic hydrocarbon	3.09	
Tetradecanedioic acid	Carboxylic acid	0.51	
4-Dibenzofuramine			22.82
Etyl-2,2-difluoro-2-(4-propen-3' (piperadine)	Amine		12.71
Totally components identified		99.89	99.80

*Grouping of components according to Fessenden and Fessenden (1986).

Using ethanol and methanol as solvent for extraction, many polar components were dissolved and showed activity with broad spectrum as done by Okeke *et al.* (2001), Barbour *et al.* (2004), Voravuthikunchai *et al.* (2004), Shah *et al.* (2004), Nkere and Iroegbu (2005), Runyoro *et al.* (2006), Duraipandiyani *et al.* (2006), Gupta *et al.* (2006), Rojas *et al.* (2006), Nurliana *et al.* (2008, in publishing process).

Based on GC-MS analysis, saturated fatty acids and their derivatives (grouping in carboxylic acid and ester) with carbon chain lengths of C8, C10, C12, C14, C16, and C18 had the greatest peak area in crude EEP (33.11%), but less in EERP (1.44%) which were only of C12, C14 and without ester group. Monoglycerides of medium-chain saturated fatty acids particularly the monoglyceride of lauric acid are more active as antibacteria compared with free fatty acids, but various free fatty acids, especially their derivatives have been reported having broad spectrum antimicrobial activity against various microbes (Kabara 1978; Kabara 1993; Nair *et al.* 2005). Monolauric (MC12, monoglyceride) obtained from coconut oil had stronger antimicrobial activity than other monoglycerides such as monocaprilic (MC8), monocapric (MC10), monomiristic (MC14), meanwhile monopalmitic (MC16), monostearic (MC18), monooleic (MC18:1), and monolinoleic (MC18:2) did not have antimicrobial activity (Wang & Johnson 1992; Wang *et al.* 1993).

In addition, fatty acid with carbon chain lengths of C12, C14, C16, C16:1, C18, C18:1, C18:2, and C18:3 were inactive against negative Gram bacteria and only fatty acids with carbon C12 and C16:1 were active against positive Gram bacteria (Quattara *et al.* 1997). Only the lauric acid derivatives were active against bacteria, fungi and virus. Antimicrobes could be more active in combination of several antimicrobial compounds (Wang & Johnson 1992; Kabara 2000; Øihåková *et al.* 2001; Nair *et al.* 2005).

Both crude EEP and EERP contain of carboxylic acids and hydroxylated components. The site and number of hydroxyl groups of both extracts might be related to their activity against microorganism. The evidence was reported by Cowan (1999) that the increased hydroxylation results in increasing of antimicrobial activity, similar to extracts of herb, spice and plants medicine which contain carboxylic acids (Chen Xie *et al.* 2004; Paraschos *et al.* 2007).

In general, the solubility of fatty acid was more higher in non polar solvents than their glyceride components. The longer the carbon chain more difficult to dissolve. The unsaturated fatty acids dissolve easier than saturated fatty acids in non polar solvents. Based on fatty acid characters, it is suggested the hexane extract (non polar extract) separated from *pliek u* is further investigated.

Many compounds found in *pliek u* supported its use in food. Many spices are synergists and when combined exhibit antibacterial action much stronger than they do alone. Thus, herbs used in our foods might protect us from infectious and pathogenic disease due to a much more complete chemistry than antibiotics. Garlic for instance, has been found to contain at least 33 sulfur compounds, 17 amino acids, and a dozen other compounds. Extracts of garlic have shown a broad-

spectrum antibiotic including against *Staphylococcus aureus* and *Candida albicans* (Elnima *et al.* 1983; Block 1985).

Traditional fermented food is a hygienic safety food, but it should be taken into consideration to problem of mycotoxin produced by fungi during fermentation. It is necessary for future studies should be undertaken to determine microorganisms involved in the first, second and third steps of *pliek u* fermentation under natural condition. On the basis of experimental evidence in this investigation it appears that *pliek u* is a useful source for exploiting the pharmacologically interesting components and it suggested to complete chemical characterization of the active substances.

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