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Antibacterial activity of *Nicolaia speciosa* fruit extract

Naufalin, R. and Herastuti, S.R.

Faculty of Agriculture, Departement of Agricultural Technology, Jenderal Soedirman University, Purwokerto Central Java - Indonesia 53123

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Abstract

Nicolaia speciosa fruit contains bioactive compounds which have potentials as natural antibacterial agents. Two extraction methods of bioactive compounds of *N. speciosa* fruit, i.e. single and multistage extractions, were evaluated. The multistage extraction utilized series of solvents in sequence involving hexane (nonpolar solvent), ethyl acetate (semipolar) and ethanol (polar). The antibacterial activities of the extracts were evaluated against *Escherichia coli*, *Bacillus cereus* and *Pseudomonas aeruginosa*, using agar Well diffusion method in combination with Thin Layer Chromatography (TLC) method and by observing the cell membrane leakage. The multistage extraction provided higher yields and reduced extraction time compared to the single step method. Ethanolic extract of *N. speciosa* showed the strongest inhibitory effect against bacteria. The minimum inhibitory concentration (MIC) of the ethanolic extract ranged from 20 mg/mL to 32 mg/mL. The TLC method showed that the fractions of ethanolic extract of *N. speciosa* with Rf 4= 0.33; Rf 8 = 0.82 and Rf 9 = 0.83 showed better antibacterial activity. The release of cells materials, measured at 260 and 280 nm using UV - Vis spectrophotometer was observed, indicative of leakage of the cytoplasmic membrane.

Introduction

Nicolaia speciosa, known as kecombrang Indonesia and torch ginger in Malaysia, is one of tropical spice plants that has a unique taste and flavour. Its flowers are commonly used as a flavouring ingredient for local culinary in Indonesia, but the fruit is not optimally utilized. *N. speciosa* contains a considerable amount of alkaloids, flavonoids, polyphenols, steroids, saponins and essential oils (Naufalin *et al*., 2005; Naufalin and Herastuti, 2012). These compounds can be extracted by various solvents and are potential to be used as antimicrobial agents (Naufalin and Herastuti, 2013). The study of *N. speciosa* antimicrobial activity was initiated by Naufalin *et al.* (2005) employing multistage extraction method using series of solvents including hexane (nonpolar solvent), ethyl acetate (semipolar) and ethanol (polar). The multistage extraction method using a nonpolar solvent and the residue was extracted back with semipolar, and polar solvents. The results showed that the ethanol extracts of *N. speciosa* flowers had an antibacterial activity against *Escherichia coli* and *Bacillus subtilis* that represent Gram-positive and Gram-negative bacteria, respectively (Naufalin *et al.*, 2006). Numerous studies have shown that torch ginger flower *(N. speciosa)* exhibited rich antioxidants, anticancer and antimicrobial activities (Naufalin and Herastuti, 2016; Habsah *et al*., 2005; Chan *et al*., 2007, 2008; Lachumy *et al*., 2010; Wijekoon *et al.*, 2011a, 2011b).

N. speciosa flower powder has a crude lipid content of 10.81% and its fruit powder has a lipid content of 13.8% (Naufalin and Herastuti, 2012). According to Kanazawa *et al.* (1995) fats and other oils having large molecules can not enter the cell wall. Fats and oils can also interact with essential oils or phenolic compounds resulting in a lowered antibacterial activity. The mutistage extraction method can reduce fats and oils contained in the plant powder. As indicated by Houghton and Roman (1998), extractions using petroleum ether or hexane could be used to obtain plant waxes, fats and vegetable oils (Houghton and Raman, 1998).

The fractionation of *N. speciosa* flower powder extract was performed using thin layer chromatography. Thin Layer Chromatography (TLC) is one method of quickly separation with simple equipment and a lot of trial parameters which can be varied to obtain good separation. TLC is an adsorption separation system and partitions. Separating layer consists of a stationary phase placed in a holder in the form of plate glass, metal or a suitable layer and the mobile phase in the form of developer solution. The mixture to be separated in the form of a solution focused on the plate, to produce spots or early band,

*Corresponding author.
Email: *rnaufalin@yahoo.co.id*
Tel: +62 281 638791; Fax : +62 281 638791

then the plates were developed with a significant solids. Separation occurs during impregnation in the capillaries, then formed separate spots. If the spot is not visible, can be seen with the aid of ultraviolet light or iodine vapor was used to detect the compounds (Naufalin and Herastuti, 2012). Selection of hexane and ethyl acetate as the mobile phase based on research conducted by Naufalin *et al.* (2005) that perform phytochemical screening rhizomes *N. speciosa* chemically, by fractionation of *N. speciosa* rhizome using preparative TLC with a stationary phase of silica gel GF254 and as a developer solution is n-hexane and ethyl acetate (96: 4 v / v). The aims of this research is to determine the part of N. speciosa fruit that used as antibacterial through diffusion test and to determine MIC, fractionation using TLC and observe the leakages of microbial membrane cell.

Materials and Methods

N. speciosa *fruit powder processing*

The *N. speciosa* fruit was cut and spread on trays and dried to a water content of 8% (w/w) with a blower dryer at a temperature of 50ºC. Prior to the extraction, dried *N. Speciosa* fruit was crushed in a blender until homogeneous powder was obtained (Naufalin, 2008; Naufalin and Herastuti, 2016).

Extraction process of N. speciosa *fruit powder*

The extraction was carried out by using hexane, ethyl acetate and ethanol as solvents. In addition, a multistep sequential extractions using hexane, followed by ethyl acetate and the final step using ethanol as solvents was carried out as follows. The powdered fruit of *N. speciosa* was dissolved in hexane (1:4 w/v), then shaken at a rotation speed of 150 rpm for 2 hours, before being filtered with filter paper (Whatman No. 42) to obtain extract 1 and pulp 1. The remaining extract 1 solvent was removed with a rotary evaporator to obtain hexane fraction. Air-dried pulp 1 was extracted again using ethyl acetate to obtain extract 2 and pulp 2. Air-dried pulp 2 was extracted again using ethanol to obtain extract 3 and pulp 3. *N. speciosa* fruit extracts were then flowed with N_2 gases (Naufalin *et al.*, 2013).

Phytochemical analysis

The qualitative tests for the identification of phytochemical compounds such as phenols, steroids, triterpenoids, tannins and flavonoids were carried out according to the procedures as described by Harborne (2006).

Microorganism preparation

The tested bacteria were *Bacillus cereus* (FNCC 057), *Pseudomonas aeruginosa* (FNCC 063) obtained from The Center for Food and Nutrients, Gadjah Mada University, and *E. coli* (ATCC 25922). The bacterial stock cultures were maintained on nutrient agar slants and stored at 4°C. The bacterial strains were grown in the Nutrient Broth at 37°C for 24 hr before being used for the antimicrobial activity tests.

Antibacterial activities analysis

The *N. speciosa* fruit extract antibacterial activity against *B. cereus, P. aeruginosa* and *E. coli* were carried out using the agar well-diffusion method. The antibacterial activity was expressed as the diameter of the zone of inhibition (mm) formed by the bacteria (Carson and Riley, 1995) and streptomycin used as positive control.

Determination of minimum inhibitory concentration (MIC)

MIC determination was done with a 10-35 mg/mL concentrate according to the method used by Kubo *et al*. (1992; 1993) with some modifications. The extracts were mixed with 10 uL of the bacteria test culture in a shaker incubator at a speed of 150 rpm for 24 hours. MIC value is the minimum extract concentration that inhibits 90% of the growth of bacteria during a 24-hour incubation.

Thin layer chromatography

The ethanol extracts of *N. speciosa* showing significant antimicrobial activity were analysed using thin layer chromatography (TLC). About 10 µl of each extract was applied on precoated aluminium silica gel G 25 plates. The developing solvents used were hexane and ethyl acetate (7:3 v/v). The TLC plates were run in triplicates. The reference chromatogram was used to determine the spots and visualised by UV light to see if the separate spots were UV active prior to spraying with vanillin sulphuric acid (2%) spray reagent (Dahiya and Purkayastha, 2012).

Leakage of membrane cell analysis

Cultures were transferred to 100 mL of fresh sterile TSB and incubated at 37°C for 18-24 h. After incubation, cultures were centrifuged at 10.000 rpm for 10 min, resuspend in sterile sodium chloride solution (0.85 g/100 mL). Suspensions were then adjusted to achieve a concentration of approximately 10^{10} CFU/mL. The N. speciosa fruit extract at concentration of 1x MIC was put into each test tube containing 4 mL of the above bacterial suspensions.

These suspensions were incubated at 37°C for 0, 20, 40, 60, 100, and 120 min. Bacterial suspension of pure culture that had been grown for 24 hour. After incubation, cultures were centrifuged at a speed of 3,500 rpm for 20 minutes. The filtrate was removed and phosphate buffer is added to the cell deposition in a test tube (On metta-aree *et al.*, 2006 with some modifications; Imelda *et al*., 2013).

Statistical analysis

Quantitative data was analyzed used variance test (F test) in 5%, continued in Duncan's Multiple Range Test (DMRT) if it finds the significant difference. The results were expressed as mean ± SD.

Results and Discussion

Yields of extraction

The multistage extractions was performed in this research since it has been known as an efficient procedure for extraction of limited number of samples and effective to separate the active components of extract based on its polarity (Houghton and Raman, 1998). The extraction process was carried out starting with a non polar condition using hexane, followed by a semi-polar condition using ethyl acetate and finally using a polar condition using ethanol as solvent. The yield of multistep extractions was significantly higher than single step extraction and ethanol extract give higher yields of kecombrang fruit extract (Table 1). This indicated that kecombrang fruit contains polar compound, i.e., in order to obtain polar component, it is necessary for the preparation material with a non-polar solvent extraction followed by polar extraction. This observation is supported with the previous research on bioactive compound of kecombrang fruit, whereby polar compound were more abundant (Naufalin *et al.*, 2003, Naufalin dan Herastuti, 2012)

Results of the analysis of the phytochemical components of *N. speciosa* fruit (Table 2) extracted with ethyl acetate yields steroids, terpenoids, alkaloids, flavonoids and glycosides, whereas extraction with ethanol yields phenolics, steroids, terpenoids, alkaloids, and glycosides. Phenolic components are generally soluble in organic solvents that are polar such as ethanol. Phenolic compounds are substances which have aromatic rings with one or more hydroxyl groups so it's easily soluble in polar solvents (Houghton and Raman, 1998).

Phenolic compounds extracted from kecombrang flower has been reported to exhibit anti-bacterial activity against selected Gram-negative bacteria, such as *E. coli* and *B. subtilis* (Naufalin *et al*., 2003). In general, plant phenolic compounds have been shown to have antibacterial activity. For example, the antimicrobial activity in germinated fenugreek seeds which may be due partly to the presence of flavonoids and polyphenols has been reported (Norziah *et al*., 2015).

Steroids are extracted in hexane, ethyl acetate and ethanol solvent, as steroid is partially non polar to semipolar. Steroids of Alstonia macrophylla plants have been reported to possessed antibacterial activity against *E. coli, Salmonella* Typhi, *Staphylococcus aureus* and *B. subtilis* (Chattopadhyay *et al.* 2001). In addition, Quinlan *et al*. (2000) have reported on the antibacterial activities of steroidal extracts obtained from various medicinal.

Alkaloids are known to have antimicrobial compounds, which are beneficial in inhibiting infection caused by microorganisms. The alkaloids from Sida acuta had a good antimicrobial activity against the test microorganisms. The broth microdilution assay gave minimal inhibitory concentration values ranging from 16 to 400 µg/ml and minimal bactericidal concentration values ranging from 80 to up to 400 µg/ml (Karou *et al.*, 2006). This is in agreement with the study of Scazzocchio *et al.* (2001), who reported on the antibacterial activity of alkaloids (berberine, beta-hydrastine, canadine and canadaline) of *Hydrastis canadensis* L. (Ranunculaceae) against 6 strains of microorganism: *Staphylococcus aureus, Streptococcus sanguis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Scazzocchio *et al.*, 2001). Alkaloids which are one of the largest groups of phytochemicals in plants have amazing effects on humans and this has led to the development of powerful pain killer medications (Kam and Liew, 2002).

Triterpenoids was extracted from the *N. speciosa* fruit. Pentacyclic triterpenoids have shown anti-staphylococcal activities and although individually weaker than common antibiotics produced from bacteria and fungi, synergistically these compounds may use different mechanism of action or pathways to exert their antimicrobial effects, as implicated in the lowered MICs (Chung *et al.,* 2011). Triterpenoids compounds that have antimicrobial activity include merediol, linalool, indole and kadinen that effective to inhibit the growth of *B. subtilis, S. aureus* and *E. coli* (Kubo *et al*., 1993)

Antibacterial activity of N. speciosa *extracts*

Hexane extract did not exhibit inhibitory effects against all bacteria tested. Table 2 shows that the phytochemical components of hexane extract contains steroids, triterpenoids, alkaloids and glycosides.

Table1. The yield of *N. speciosa* fruit extract

Extraction technique	Single extraction			Multistage extraction		
	Hexane extract (%)	Ethyl acetate extract (%)	Ethanol extract (%)	Hexane extract (%)	Ethyl acetate extract (%)	Ethanol extract (%)
Yield	9.2±0.01	3.1±0.01	13.5±0.02	9.1±0.03	3.4±0.02	15.1±0.02

Table 2. Compounds of *N. speciosa* fruit by phytochemical analysis

Extraction technique	Single extraction			Multistage extraction		
	Hexane extract (%)	Ethyl acetate extract (%)	Ethanol extract (%)	Hexane extract (%)	Ethyl acetate extract (%)	Ethanol extract (%)
Phenolic	-	-	+	-	-	+
Steroid	+	+	+	+	+	+
Triterphenoid	+	+	+	+	+	+
Alkaloid	+	+	+	+	+	+
Tanin	-	-	-	-	-	-
Flavonoid	-	+	-	-	+	-
Glycosida	+	+	+	+	+	+

Note : + = positive - = negative

Hexane extracts also contain essential oils, which hindered the contact between the antimicrobial compound and essential oils with bacterial cells. Oils and other fats interfere with the process of diffusion and protects the bacteria from antibacterial compounds, rendering the hexane extract to be uneffective in inhibiting the growth of bacteria. Non polar extract extracted from the roots of *Terminalia sericea* plants also showed no inhibition against *S. aureus*, *E. coli, B. anthracis, Salmonella* Typhi and *P. aeruginosa* (Moshi and Mbwambo, 2005).

Ethyl and ethanol extracts of *N. speciosa* were found to show antibacterial activity against *E. coli, B. cereus, P. aeruginosa,* with zone of inhibition ranging between 8-15.8 mm for ethyl acetate extract and 8-24 mm for ethanol extract (Table 3). Ethanol extract gives a higher antibacterial activity than the extract of ethyl acetate. Phytochemical component analysis of *N. speciosa* fruit extracted with ethyl acetate contains steroids, terpenoids, alkaloids, flavonoids and glycosides, while extraction with ethanol contains phenolic, steroids, terpenoids, alkaloids, and glycosides. Flavanoid have semipolar to polar properties (Harborne, 2006), whereas in the ethanol extract, phenolic is more polar than flavonoid. Phenolic has higher antimicrobial activities than flavonoid (Naufalin dan Herastuti, 2012), synergism of the phytochemical components (phenolic, steroids, terpenoids, alkaloids, and glycosides) in the ethanol extract, which has optimum polarity to enable it to diffuse more easily and is able to inhibit the growth of bacteria.

Further testing of the extract of ethanol were conducted to determine the value of MIC against *E. coli, B. cereus* and *P. aeruginosa* with the direct method on nutrient broth media. MIC values of extracts of *N. speciosa* fruit ranged from 20-32 mg/ml, with *E. coli* showing MIC of 32 mg/ml and *P. aeruginosa* showing MIC of 20 mg/ml. The differences observed in the susceptibilities of the bacteria tested against the ethanol extracts could due to their cell wall component, such as the porin protein. For example, the porin protein in *P. aeruginosa* PAO1 has a diameter of 2 nm, larger than Porin Omp F and OMPC protein with a diameter of 1.2 nm in *E. coli* K-12 (Nikaido, 2003)

Antibacterial compounds of fractions

N. speciosa fruit extract was fractionated by thin layer chromatography silica gel using hexane and ethyl acetate solvents, with fractions 4, 8 and 9 showing inhibition activities against all tested bacteria, while the 9th fraction is the fraction that is most active (Figure 1). The diameter of bacterial inhibition test of all the ethanol extract fraction was lower than the ethanol extract, which means that the mechanism of action of the main antibacterial compounds in the extract are synergistic. Panizzi *et al.* (2002) reported that the methanol extract of *Rubus ulmifolius* have greater antibacterial activity against

Table 3. Antibacterial activity (Inhibition zone in mm) from *N. speciosa* fruit extract

Inhibition zone (mm) of *Nicolaia speciosa* fruit extract									
Test Bacteria	Concentration of *Nicolaia speciosa* fruit extract (mg/ml)								
	Kontrol	5	10	15	20	25	30	40	50
	Solvent : Hexane								
E. coli	30.28±0.01	0	0	0	0	0	0	0	0
B. cereus	25.13±0.01	0	0	0	0	0	0	0	0
P. aeruginosa	25.58±0.02	0	0	0	0	0	0	0	0
	Solvent : ethyl acetate								
E. coli	30.28±0.01	0	9±0.01	10±0.01	11.1±0.01	11.7±0.01	12.5±0.02	10.8±0.01	10.9±0.01
B. cereus	25.13±0.01	8±0.01	9.9±0.03	9.9±0.01	9.9±0.01	10.6±0.02	11.1±0.01	13.7±0.01	15.8±0.03
P aeruginosa	25.58±0.02	10.5±0.01	12±0.01	12.8±0.01	13.3±0.01	13.1±0.01	13±0.01	14±0.01	14.1±0.01
	Solvent : Ethanol								
E. coli	30.28±0.01	12±0.01	12±0.01	14.8±0.01	15.3±0.01	18.5±0.01	19.4±0.01	24±0.01	24±0.01
B. cereus	25.13±0.01	8.6±0.01	16±0.02	16±0.01	18±0.01	18±0.01	22.9±0.03	22.2±0.01	22.6±0.01
P. aeruginosa	25.58±0.02	8±0.02	8±0.01	14±0.01	15.9±0.01	16.4±0.01	16.7±0.01	16±0.01	16.1±0.02

Control : Streptomycin

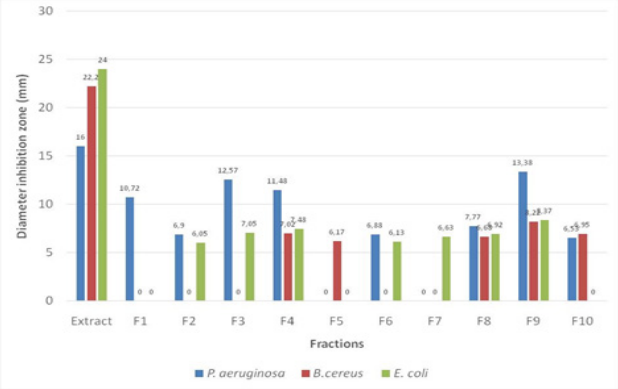

Figure 1. Inhibition zone (mm) of control (*N. speciosa* extract (30 mg/ml)) and *N. speciosa* fractions agains test bacteria (A : *E. coli,* B : *B. cereus*, C : *P. aeruginosa*)

P. aeruginosa, E. coli and *Staphylococcus aureus* than its fractions.

Leakage of membrane cell

The antibacterial mechanism of ethanolic extract of *N. speciosa* fruit at a concentration of 1 MIC causes leakage of the cell components. Figure 2 A shows the increase in absorbance of the cell supernatant, which indicates an increase in compound that can be absorbed at a wavelength of 260 nm and 280 released by the bacterial cell. The compounds that can be absorbed at a wavelength of 260 nm is RNA and RNA derivatives, namely nucleotides, while those detected at a wavelength of 280 nm are proteins (Gilbert, 1984). Park *et al.* (2003) showed that the spectrophotometer at 260 nm detect purine, pyrimidine and ribonucleotide, while at 280 nm detect tyrosine and tryptophan. Nucleic acid secretion and cell signaling proteins are leaking due to damage to the cell membrane or a change in permeability of the cell membrane. The increase in absorbance at a wavelength of 280 nm is greater than at 260 nm, it means indicating bacterial cells leak intracellular protein compound (in the cytoplasm or periplasm). These results are consistent with research by Davidson *et al.* (2005), that leakage of bacterial cells are treated with butylated hydroxyanisole (BHA) was detected more at a wavelength of 280 nm than at 260 nm.

The absorbance at 260 nm of supernatant of *E. coli, B. cereus* and *P. aeruginosa* suspensions increased obviously after 20 min exposure to the ethanolic extract of the *N. speciosa* fruit. These results indicated that cell materials were released outside the cells. Similarly, the release of cell materials into the supernatant, indicated by the increasing absorbance at 280 nm, from *E. coli, B. cereus* and *P. aeruginosa* suspensions was also observed after 20 min exposure (Figure 2B). The absorbance values of *P. aeruginosa* suspensions were higher than that of *E. coli* and *B. cereus* suspensions at the same exposure time, for all treatments. These results indicated that treatment with ethanolic extract of *N. speciosa* resulted in disruption of membrane permeability which induced leakage of the cytoplasmic membrane. It occurred due to severe and irreversible damage of cytoplasmic membrane (Carson *et al.*, 2002). The cytoplasmic membrane may become damaged and functionally disabled when bacterial suspensions are exposed to antibacterial agents. Various vital intracellular materials including small ions such as K^+ and $(PO_4)^{3-}$ tend to leach out, followed by leaching of large molecules such as DNA, RNA and other materials (Shan *et al.*, 2008; Xing *et al.,* 2009). Phenolic compounds of *N. speciosa* fruit extract as the major compounds of the extract have been reported can disturb the cytoplasmic membrane, disrupt the proton motive force, electron flow and active transport (Naufalin *et al.*, 2005).

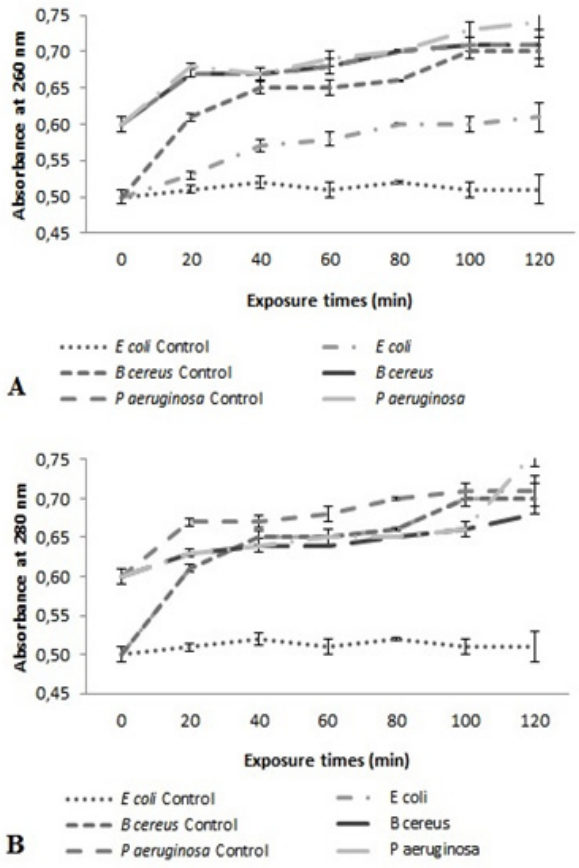

Figure 2. Absorbance of supernatant at (A) 260 nm and (B) 280 nm from *E. coli, B. cereus* and *P. aeruginosa* suspensions without (control) and after exposure to the ethanolic extract of *N. speciosaat* concentration of 1x MIC, measured at different exposure times

Conclusion

The multistage extractions produce the higher yield than single extraction. Ethanol extract of kecombrang fruits have bioactive compound as antimicrobial than ethyl acetate extract and the damage of microbial cell from ethanol extract kecombrang fruit is shown on leakage of cell bacteria test.

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References

Carson, C. F. and Riley, T. V. 1995. Antimicrobial Activity of the Major Components of the Essential Oil of Melaleuca alternifolia. Journal of Applied Bacteriology 78: 264-269.

Carson, C. F., Mee, B. J. and Riley, T. V. 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. Antimicrobial Agents and Chemotherapy 46(6): 1914–1920.

Chan, E. W. C., Lim, Y. Y. and Omar, M. 2007. Antioxidant and antibacterial activity of leaves of *Etlingera* species (Zingiberaceae) in Peninsular Malaysia. Food Chemistry 104: 1586-1593.

Chan, E. W. C., Lim, Y. Y., Wong, L. F., Lianto, F. S.,Wong, S. K., Lim, K. K., Joe, C. E. and Lim, T.Y. 2008. Antioxidant and tyrosinase inhibition properties of leaves and rhizomes of ginger species. Food Chemistry 109: 477-483.

Chattopadhyay, D., Maiti, K., Kundu, A. P., Chakraborty, M. S., Bhadra, R., Mandal, S. C. and Mandal, A. B. 2001. Antimicrobial activity of *Alstonia macrophylla*: a folklore of bay islands. Journal of Ethnopharmacology 77(1): 49-55.

Chung, P. Y., Navaratnam, P. and Chung, L. Y. 2011. Synergistic antimicrobial activity between pentacyclic triterpenoids and antibiotics against *Staphylococcus aureus* strains. Annals of Clinical Microbiology and Antimicrobial 10(25): 1-6.

Church, G. M. and Gilbert, W. 1984. Genomic sequencing. Proceedings of the National Academy of Sciences 81(7): 1991-1995.

Dahiya, P. and Purkayastha, S. 2012. Phytochemical screening and antimicrobial activity of some medicinal plants against multi-drug resistant bacteria from clinical isolates. Indian Journal of Pharmaceutical Sciences 74(5): 443-450.

Davidson, P. M., Sofos, J. N. and Branen, A. L. 2005. Antimicrobials in food. CRC press.

Gilbert P. 1984. The revival of microorganisms sublethally injured by chemical inhibitors. Andrew M. H. E., and Russell, A. D. (Eds). The revival of injured microbes. Academic Press, London.

Habsah, M., Ali, A. M., Lajis, N. H., Sukari, M. A., Yap, Y.H., Kikuzaki, H. and Nakatani, N. 2005. Antitumour promoting and cytotoxic constituents of Etlingera elatior. Malaysian Journal of Medical Science 12: 6-12.

Harborne, J. B. 2006. Phytochemicals technique. Bandung (ID): ITB Press.

Houghton, P. J. and Raman. 1998. Laboratory Handbook for The Fractonation of Natural Extract. Chapman and Hall. London.

Imelda, F., Faridah, D. N. and Kusumaningrum, H. D. 2014. Bacterial inhibition and cell leakage by extract of *Polygonum minus* Huds. leaves. International Food Research Journal 21(2): 553-560.

Kam, P. C. A. and Liew. 2002. Traditional Chinese herbal medicine and anaesthesia. Anaesthesia 57(11): 1083-1089. (give name of second author)

Kanazawa, A., Ikada, T. and Endo, T. 1995. A Novel Approach to Made of Action of Cationic Biocides Morphological Effect on Antibacterial Activity. Journal of Applied Bacteriology 78: 55-60.

Karou, D., Savadogo, A., Canini, A., Yameogo, S., Montesano, C., Simpore, J. and Traore, A. S. 2006. Antibacterial activity of alkaloids from *Sida acuta*. African Journal of Biotechnology 5(2): 195-200.

Kubo, I., Muroi, H. and Himejima, M. 1992. Antimicrobial activity of green tea flavor components and their combination effects. Journal of Agricultural and Food Chemistry 40(2): 245-248.

Kubo, A,, Lunde, C.S. and Kubo, I. 1993. Antimicrobial activity of olive oil flavour compounds. Journal of Agricultural and Food Chemical 40(6): 999-1003.

Lachumy, S. J. T., Sasidharan, S., Sumathy, V. and Zuraini, Z. 2010. Pharmacological activity, phytochemical analysis and toxicity of methanol extract of *Etlingera elatior* (torch ginger) flowers. Asian Pacific Journal of Tropical Medicine 3: 769-774.

Moshi, M. J. and Mbwambo, Z. H. 2005. Some pharmacological properties of extracts of *Terminalia sericea* roots. Journal of Ethnopharmacology 97(1): 43-47.

Naufalin, R., Jenie, B.S.L. and Herastuti. S.R. 2005. Antibacterial activity of kecombrang *(Nicolaia speciosa)* flower extract toward pathogenic and spoilage bacteria. Jurnal Teknologi dan Industri Pangan 16(2): 119-125.

Naufalin, R., Jenie, B.S.L., Kusnandar, F., Mirnawati, S. and Herastuti, S.R. 2006. Effect of pH, NaCl and heating on the antibacterial stability of kecombrang (*Nicolaia speciosa* Horan) flower extract and its application in mincet meet. Jurnal Teknologi dan Industri Pangan 17(3): 197-203.

Naufalin, R. 2008. Aktivitas dan mekanisme kerja antibakteri ekstrak bunga kecombrang (*Nicolaia speciosa* Horan). Makalah Seminar Nasional Perhimpunan Mikrobiologi Indonesia, Purwokerto 22-23 Agustus 2008.

Naufalin, R. dan Herastuti, S.R. 2012. Pengawet Alami Pada Produk Pangan. UPT. Percetakan dan Penerbitan Universitas Jenderal Soedirman, Purwokerto.

Naufalin, R. dan Herastuti, S.R. 2013. Microcapsule application of kecombrang *(Nicolaia speciosa)* flower extract: effect of concentration, type of fraction, pH of medium and NaCl on microbiological properties of minced beef. Journal Animal Production 15(1): 8-14.

Naufalin, R. dan Herastuti, S.R. 2016. Antioxidant activity and physicochemical properties of *Nicolaia speciosa* flower extract. Agriculture and Agricultural Science Procedia 09: 297-303

Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiology and Molecular Biology Reviews 67(4): 593-656.

Oonmetta-aree, J., Suzuki, T., Gasaluck, P. and Eumkeb, G. 2006. Antimicrobial properties and action of galangal (*Alpinia galanga* Linn.) on Staphylococcus aureus. Food Science and Technology 39: 1214–1220.

Panizzi, L., Caponi, C., Catalano, S., Cioni, P. L. and Morelli, I. 2002. In vitro antimicrobial activity of extracts and isolated constituents of Rubus ulmifolius. Journal of Ethnopharmacology 79(2): 165-168.

Park, S. J., Park, H. W. and Park, J. 2003. Inactivation kinetics of food poisoning microorganisms by carbon dioxide and high hydrostatic pressure. Journal of Food Science 68(3): 976-981.

Puupponen-Pimiä, R., Nohynek, L., Meier, C., Kähkönen, M., Heinonen, M., Hopia, A., and Oksman-Caldentey, K. M. 2001. Antimicrobial properties of phenolic compounds from berries. Journal of Applied Microbiology 90(4): 494-507.

Quinlan, M. B., Quinlan, R. J. and Nolan, J. M. 2000. Ethnophysiology and herbal treatments of intestinal worms in Dominica, West Indies. Journal of Ethanopharmacology 80: 75-83.

Scazzocchio, F., Cometa, M. F., Tomassini, L. and Palmery, M. 2001. Antibacterial activity of Hydrastis canadensis extract and its major isolated alkaloids. Planta Medica 67(6): 561-564.

Shan, B., Cai, Y., Brooks, J. D. and Corke, H. 2008. Antibacterial properties of *Polygonum cuspidatum* roots and their major bioactive constituents. Food Chemistry 109: 530-537.

Wijekoon, M. J. O., Bhat, R. and Karim, A. A. 2011. Effect of extraction solvents on the phenolic compounds and antioxidant activities of bunga kantan (*Etlingera elatior* Jack.) inflorescence. Journal of Food Composition and Analysis 24(4): 615-619.

Wijekoon, M. M. J. O., Karim, A. A. and Bhat, R. 2011. Evaluation of nutritional quality of torch ginger (*Etlingera elatior* Jack.) inflorescence. International Food Research Journal 18(4): 1415-1420.

Xing, K., Chen, X. G., Kong, M., Liu, C. S., Cha, D. S. and Park, H. J. 2009. Effect of oleoyl-chitosan nanoparticles as a novel antibacterial dispersion system on viability, membrane permeability and cell morphology of *Escherichia coli* and *Staphylococcus aureus*. Carbohydrate Polymers 79: 17-22.