UNIVERSITI PUTRA MALAYSIA

ANTIMICROBIAL ACTIVITY, PHYTOCHEMICAL AND TOXICITY ANALYSES OF SALAM [Syzygium polyanthum (Wight) Walp.] LEAF EXTRACT AND ITS APPLICATION IN FOOD

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By

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Chairman : Associate Professor Yaya Rukayadi, PhD
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Food products can be subjected to contaminate by bacteria and fungi. The growth of this microorganisms in food products can cause foodborne illness. To overcome this problem, the prevention should be done at the early stage of food processing such as sanitizing. Commonly, chemicals sanitizer had been apply in food industry. However, application of this chemicals for long term was affected human health. Therefore, development of natural sanitizer derived from plant sources are gaining more attention nowadays. In this study, the antimicrobial activity of salam [*Syzygium polyanthum* (Wight) Walp.] leaves extract was evaluated against 17 types of pathogenic microorganisms including *Escherichia coli* O157:H7, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus oligosporus*, Rhizopus oryzae, *Candida albicans*, *Candida krusei*, *Candida glabrata* and *Candida parapsilosis* in order to determine the ability of *S. polyanthum* as natural food sanitizer. The susceptibility test showed that all tested pathogenic bacteria were inhibited by *S. polyanthum* extract, with the range of inhibition zone between 6.67 to 9.67 mm. The extract could inhibit the growth of *L. monocytogenes* and *S. aureus* with MIC of 0.63 mg/mL, meanwhile MIC of the extract against others pathogens were 1.25 mg/mL. *L. monocytogenes* can be killed completely at MBC value of 0.63 mg/mL, *S. aureus, S.* Typhimurium, *V. cholerae* and *V. parahaemolyticus* were at 1.25 mg/mL, while the other strains were at MBC value of 2.5 mg/mL. Time-kill curve study showed that *E. coli* O157:H7, *L. monocytogenes*, *P. aeruginosa*, *S. aureus*, *V. cholerae* and *V. parahaemolyticus* can be killed by *S. polyanthum* extract at 4× MIC for 4 h, 4× MIC for 1 h, 4× MIC for 1 h, 4× MIC for 4 h, 4× MIC for 4 h, 4× MIC for 4 h, respectively. However, the population of *K. pneumoniae*, *P. mirabilis* and *S.* Typhimurium showed 3 Log reduction after treated at 4× MIC for 4 h. All filamentous fungi species showed 6.5 mm in inhibition zone, while 1.25 mg/mL and 5.0 mg/mL

for their MIC and MFC, respectively. In qualitative analysis of inhibition germination conidia, all the tested fungi species showed no growth after treated with extract started at 1× MIC for 9 days. Based on quantitative analysis, using 4× MIC for 24 h, the percentage of conidia germination were fully inhibited for *A. flavus* and *R. oryzae* (0%), meanwhile, for *R. oligosporus* and *A. niger* were reduced to 1% and 13%, respectively. Inhibition zone for *Candida* species were between 7.00-7.67 mm. For MIC and MBC, all *Candida* species can both inhibited and killed completely at range 0.63-1.25 mg/mL. *C. albicans*, *C. glabrata* and *C. parapsilosis* can be killed by *S. polyanthum* extract at 4× MIC for 4 h, 4× MIC for 2 h, 4× MIC for 4 h or 2× MIC for 4 h while the population of *C. krusei* reduced about 3 Log reduction after treated for 4× MIC for 4 h. Cell constituents release analysis and observation by using scanning electron microscope showed altering in cell wall linearity, cells ruptured and leakaged of the cytoplasm. Generally, the antimicrobial activities of *S. polyanthum* extract were not affected by different pH and temperatures. GC-MS analysis identified the presence of active compounds which responsible to contribute antimicrobial properties in *S. polyanthum* extract included pyrogallol, phytol, hexadecanoic acid, α-Tocopherol and β-Sitosterol while gallic acid, bergenin, quercetin 3-(6''-galloylgalactoside), madecassic acid, quillaic acid and asiatic acid were detected by using LC-MS. The toxicity study by using brine shrimp assay demonstrated that *S. polyanthum,* extract was not toxic to *Artemia salina* with LC_{50} was 75.85 mg/mL. Generally the significant reduction of natural microflora in tested food samples were started at 0.50% (v/v) of extract at 5 min. During storage, 5% (v/v) showed better effect in controlling the microbial survival throughout the storage time. Physical characteristics in term of colour, texture and odour, all the treated samples until the highest concentration of extract [5% (v/v)] showed not significantly different and this finding also parallel to sensory acceptability where 5% of extract was accepted by the panelists. In conclusion, *S. polyanthum* extract exhibited antimicrobial activity, thus it can be developed as natural sanitizer for washing raw food materials and prevent the food spoilage during storage.

Keyword: Antimicrobial activity, *S. polyanthum* leaves, pathogenic microorganisms, toxicity, natural sanitizer.

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AKTIVITI ANTIMIKROBIAL, FITOKIMIA DAN TOKSISITI ANALISIS EKTRAK DAUN SALAM [*Syzygium polyanthum* (Wight) Walp.] DAN PENGGUNAANNYA DI DALAM MAKANAN

Oleh

SUZITA BINTI RAMLI

Mac 2018

Pengerusi : Profesor Madya Yaya Rukayadi, PhD
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Produk makanan mempunyai risiko untuk terdedah dengan pencemaran bakteria dan kulat. Pertumbuhan mikroorganisma di dalam produk makanan boleh menyebabkan penyakit bawaan makanan. Untuk mengatasi masalah ini, langkah pencegahan harus dilakukan pada peringkat permulaan semasa pemprosesan makanan seperti mencuci dengan ejen pembasuh. Kebiasaannya, ejen pembasuh kimia digunakan di dalam industri makanan. Walaubagaimanapun, penggunaan bahan kimia ni untuk jangka masa yang panjang akan menjejaskan kesihatan manusia. Oleh itu, penghasilan ejen pembasuh semulajadi daripada sumber tumbuh-tumbuhan semakin mendapat perhatian masa kini. Dalam kajian ini, aktiviti antimikrobial ekstrak daun salam [*Syzygium polyanthum* (Wight) Walp.] dinilai terhadap 17 jenis mikroorganisma patogenik termasuk *Escherichia coli* O157:H1, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus oligosporus*, *Rhizopus oryzae*, *Candida albicans*, *Candida krusei*, *Candida glabrata* dan *Candida parapsilosis* untuk menentukan keupayaan ekstrak *S. polaynthum* sebagai ejen pembasuh makanan semulajadi. Hasil kajian menunjukkan bahawa semua patogen bawaan makanan yang diuji, terencat apabila didedahkan kepada ekstrak *S. polyanthum*, dengan lingkungan zon perencatan antara 6.67 - 9.76 mm. Ekstrak *S. polyanthum* boleh menghalang pertumbuhan *L. monocytogenes* dan *S. aureus* dengan MIC, 0.63 mg/mL, sementara itu MIC ekstrak terhadap pathogen yang lain adalah 1.25 mg/mL. *L. monocytogenes* boleh dibunuh sepenuhnya pada nilai MBC 0.63 mg/mL, *S. aureus*, *S.* Typhimurium, *V. cholerae* dan *V. parahaemolyticus* adalah 1.25 mg/mL, manakala patogen jenis lain adalah pada nilai 2.5 mg/mL. Analisis keluk-masa pembunuhan menunjukkan bahawa, *E. coli* O157: H7, *L. monocytogenes*, *P. aeruginosa*, *S. aureus*, *V. cholerae* dan *V. parahaemolyticus* boleh dibunuh oleh ektrak *S. polyanthum* pada 4× MIC untuk 4 jam, 4× MIC untuk selama 1 jam, 4× MIC selama 1 jam, 4× MIC untuk 4 jam,

4× MIC untuk 4 jam, 4× MIC untuk 4 jam, masing-masing. Walau bagaimanapun, populasi *K. pneumoniae*, *P. mirabilis* dan *S.* Typhimurium hanya menurun sebanyak 3 Log selepas dirawat di 4× MIC untuk 4 jam. Semua spesies kulat menunjukkan 6.5 mm untuk zon perencatan, manakala 1.25 mg/mL dan 5.0 mg/mL untuk MIC dan MFC, masing-masing. Analisis kualitatif melalui pemerhatian visual untuk perencatan percambahan konidia, secara amnya, semua spesis kulat yang diuji menunjukkan tiada pertumbuhan selepas dirawat dengan ekstrak bermula pada 1× MIC selama 9 hari. Berdasarkan analisis kuantitatif, menggunakan 4× MIC selama 72 jam, peratusan percambahan konidia telah menurun kepada 0% untuk *A. flavus* dan *R. oryzae*, sedangkan percambahan *R. oligosporus* dan *A. niger* adalah 1% dan 13% masing-masing. Zon perencatan untuk *Candida* spp. adalah di antara 7.00-7.67 mm. Untuk MIC dan MBC, *Candida* spp. boleh direncat dan dibunuh sepenuhnya pada 0.63-1.25 mg/mL. *C. albicans*, *C. glabrata* dan *C. parapsilosis* boleh dibunuh oleh ekstrak *S. polyanthum* pada 4× MIC untuk 4 jam, 4× MIC untuk 2 jam, 4× MIC untuk 4 jam atau 2× MIC untuk 4 jam manakala populasi *C. krusei* hanya berkurang sebanyak 3 Log selepas dirawat selama 4× MIC untuk 4 jam. Analisis pelepasan sel dan pemerhatian menggunakan SEM pada wakil patogen yang dirawat dengan ekstrak *S. polyanthum* pada nilai MIC menunjukkan perubahan dalam kelicinan dinding sel, sel pecah dan kebocoran sitoplasma. Secara umumnya, aktiviti antimikrobial ekstrak *S. polyanthum* tidak terjejas dengan ketara oleh pelbagai nilai pH dan suhu. Analisis GC-MS mengenal pasti kehadiran sebatian aktif yang menjadikan *S. polyanthum* bersifat antimikrobial termasuk pyrogallol, phytol, asid hexadecanoic, α-Tocopherol dan β-Sitosterol sementara asid gallic, bergenin, quercetin 3-(6"-galloylgalactoside), asid madecassic, asid quillaic and asid asiatic telah dikenal pasti menggunakan LC-MS. Kajian menunjukkan bahawa ektrak *S. polyanthum* tidak toksik kepada *Artemia salina* dengan nilai LC_{50} ialah 75.85 mg/mL. Umumnya, penurunan populasi mikroflora semulajadi bermula pada ekstrak kepekatan 0.50% (v/v) dalam tempoh 5 min. Untuk menyimpan makanan, 5% (v/v) ekstrak telah menunjukkan kesan terhadap perencatan pertumbuhan mikroorganisma. Ciri-ciri fizikal dari segi warna, tekstur dan bau, sampel makanan yang dirawat dengan ekstrak yang berkepekatan paling tinggi [5% (v/v)] menunjukan tidak signifikan dan ini selari dengan penerimaan ahli panel semasa saringan deria. Kesimpulannya, ekstrak *S. polyanthum* mempunyai aktiviti antimikrobial, oleh itu ia boleh dibangunkan sebagai ejen pembasuh semula jadi untuk membasuh bahan makanan mentah dan untuk mencegah kerosakan semasa penyimpanan makanan.

Kata kunci: Aktiviti antimikrobial, *S. polyanthum*, mikroorganisma patogenik, toksisiti, ejen pembasuh semulajadi.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ANOVA	Analysis of variance
BP	Baird-Parker
CFU	Colony forming unit
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethylsulfoxide
GC-MS	Gas Chromatography – Mass Spectrometry
GRAS	Generally Regarded as Safe
IBS	Institute of Bioscience
LC	Lethality concentration
LC-MS	Liquid Chromatography – Mass Spectrometry
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MHB	Mueller Hinton broth
MIC	Minimum Inhibitory Concentration
MOH	Ministry of Health
m/z	Mass/charge ratio
NIST	National Institute of Standards and Technology
OD	Optical density
PBS	Phosphate buffered saline
PCA	Plate Count Agar
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
Ppm	parts per million
Rpm	Revolutions per minute
SDA	Sabouraud Agar
SDB	Sabouraud Broth
SEM	Scanning Electron Microscopy
spp.	Species
TCBS	Thiosulfate citrate bile salts sucrose

UPM	Universiti Putra Malaysia
WHO	World Health organization
XLD	Xylose lysine deoxylate

CHAPTER 1

INTRODUCTION

1.1 Background

The problem of foodborne pathogens is one of major public concerns in both developed and developing countries worldwide. They become a threat to food safety especially in developing countries with poor hygiene and sanitation facilities. The rise of foodborne pathogens are responsible for millions of infectious gastrointestinal disease cases each year which increase the cost of medical care and causing loss of productivity (Bloomfield et al., 2007). Symptoms of foodborne diseases were include nausea, vomit, diarrhea and fever, whereas severe foodborne diseases may lead to death (Gandhi and Chikindas, 2007).

In France, foodborne incidence was 1210 cases per 100,000 inhabitants, meanwhile in United Kingdom and United State were 2600 cases and more than 25,000 cases per 100,000 inhabitants, respectively (Teisl and Roe, 2010).Besides that, in Australia, about 5.4 million cases were reported with 15,000 people got hospitalized and 120 deaths annually (Soon et al., 2011). In the United States, the reported foodborne cases have shown an increase of approximately 48 million illnesses with 128,000 hospitalizations while death cases was about 3,000 each year (Scallan et al., 2011). In Malaysia, the incidence rate of reported food poisoning in 2013 is 47.79 per 100,000 populations, with mortality rate of 0.04% (MOH, 2014). There also food poisoning cases among pupils in school has increased by 57% in four months in 2016 compared to 2015 (MOH, 2016).

Generally, foodborne illnesses occur are due to improper food handling and poor hygienic practices, especially during food preparation and storage period (Scott, 2003). Common examples of widely reported foodborne pathogens include *Bacillus cereus*, *Campylobacter*, *Clostridium*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* and *Staphylococcus aureus* (Singh et al., 2003). In addition, deterioration of food produce by spoilage fungi at various levels of raw food processing and production affect the supply chains of agri-food supply to the customers. Its has been estimated 5-10% of food lossess were caused by fungal food spoilage (Pitt and Hocking, 2009). Various reports claimed *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp., and *Rhizopus* spp. related to the spoilage of perishable products (Tournas, 2005; Pitt and Hocking, 2009; Birhanu et al., 2014; Khokhar and Bajwa, 2014).

Food contamination can occur during post-harvest processing or even earlier which is during pre-harvesting period due to the contamination from a farm environment, water irrigation systems, manure and so on (Doyle and Erickson, 2006). Without proper

decontamination techniques, pathogens will keep on growing especially during food storage period and during food preparation caused by cross contamination (Scott, 2003).

1.2 Problem statements

Eventhough, there are many food decontamination treatments and also food preservatives that have been applied to eliminate or substantially decrease bacterial populations both in fresh produce and raw poultries including salt, sugar, chlorine, organic acids, hydrogen peroxide, ozonation and irradiation (Leistner, 2002), however, there was important to eliminate or reduce the microbial population at the early stage of food processing. Therefore, washing the food materials with any food sanitizer was crucial especially for ready to eat food such as fruits and vegetables. There was several chemical sanitizer that had been applied in food industry including chlorine, hypochlorite, iodine, quaternary ammonium, hydrogen proxide and fatty acid sanitizer. However, this chemical can be harmful to health and also to the environment (Neo et al., 2013). In addition, washing with tap or chlorinated water is the common decontamination practice used at home. However, this practice is being questioned due to the formation of carcinogenic by-products such as trihalomethanes when chlorine reacts with organic matter such as decayed leaves (Chang and Fang, 2007). Besides that, organic substance in foods can be oxidized by chlorine whereas in water carcinogenic and mutagenic by product such as haloforms and haloacetic acids can be formed (Perez-Gregorio et al., 2011).

Due to disadvantage of chemical food sanitizer, study on natural antimicrobial agents from plants is the alternative way. Furthermore, commercial natural food sanitizer is still scarce. Plant antimicrobials are gaining wide interest because most of them are classified as Generally Recognized as Safe (GRAS) status which has been discovered to have higher levels of food safety (Alzarokey and Nakahara, 2003). Many studies have reported that most of the medicinal plants, including spices and herbs have strong antimicrobial compounds (Limsuwan et al., 2009).

1.3 Objectives

A study was undertaken to discover the antimicrobial activity of *Syzygium polyanthum* extract against several types of pathogenic microorganisms and also to measure the ability of this plant extract as natural food sanitizer. The specific objectives were:

1. To determine antimicrobial activity of *S. polyanthum* leaves extract against pathogenic microorganisms.
2. To analyse modes of action of *S. polyanthum* leaves extract by cell constituents release analysis and observation using scanning electron microscope (SEM).

3. To determine the stability of *S. polyanthum* extract at different pHs and temperatures.
4. To identify the present of bioactive compounds in *S. polyanthum* leaves extract by Gas Chromatography Mass - Spectrometry (GC-MS) Liquid Chromatography Mass - Spectrometry (LC-MS).
5. To determine toxicity of *S. polyanthum* leaves extract by using brine shrimp (*Artemia salina*) lethality assay.
6. To examine the effect of *S. polyanthum* leaves extract on natural microflora in food samples at different concentrations of sanitizer solution and different storage times and also its sensory attributes acceptability.

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