



Faculty of Resource Science and Technology

**Study Of Antimicrobial Activity on *Syzygium aromaticum* Extract
Against *Vibrio cholerae* Biofilm**

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(45854)

**Bachelor of Science with Honours
(Resource Biotechnology)
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**Study Of Antimicrobial Activity on *Syzygium aromaticum* Extract
Against *Vibrio cholerae* Biofilm**

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This project is submitted in partial fulfilment of requirement
For the degree of Bachelor of Science with Honours
(Resource Biotechnology)

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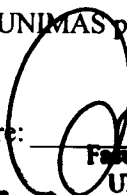
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against *Vibrio cholerae* Biofilm**

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ABSTRACT

Syzygium aromaticum (clove), able to inhibit bacterial growth through disruption of the bacterial cell membrane. *S. aromaticum* flower bud were analyzed by methanol extraction and tested against 19 samples of *V. cholerae*. The susceptibility of *V. cholerae* towards *S. aromaticum* extract evaluated using disk diffusion method showed the best zone diameter of inhibition of 12.667 mm at 100 mg/ml concentration. The MBEC₅₀ of *V. cholerae* ranged 12.5 between 25.0 mg/ml while MBEC₉₀ is 50.0 mg/ml extract concentration. Morphology change of *V. cholerae* observed through scanning electron microscope (SEM) for every treatment showed disruption of cell membrane. Thus in this study, *S. aromaticum* bud extract can be considered as potential antimicrobial agent against single cells of *V. cholerae* and able to prevent formation of *V. cholerae* in biofilm thus act as antibiofilm.

Keywords: *Syzygium aromaticum*, *Vibrio cholerae*, zone diameter inhibition, minimum biofilm eradication concentration, scanning electron microscope

ABSTRAK

Syzygium aromaticum (cengkih), dapat menghalang pertumbuhan bakteria melalui kerosakkan membran sel bakteria. Tunas bunga *S. aromaticum* dianalisis menggunakan pengekstrakan metanol dan diuji terhadap 19 sampel *V. cholerae*. Aktiviti antibakteria terhadap *V. cholerae* dengan menggunakan ekstrak *S. aromaticum* dianalisis menggunakan kaedah cakera penyebaran menunjukkan diameter zon terbaik perencatan adalah 12.667 mm pada kepekatan 100 mg / ml. MKBP₅₀ *V. cholerae* adalah antara 12.5 antara 25.0 mg / ml manakala MKBP₉₀ adalah pada kepekatan ekstrak 50.0 mg / ml. Perubahan morfologi *V. cholerae* diperhatikan melalui mikroskop imbasan elektron (SEM) untuk setiap rawatan menunjukkan kerosakkan membran sel. Oleh itu dalam kajian ini, ekstrak tunas bunga *S. aromaticum* boleh dianggap berpotensi sebagai ejen antimikrob terhadap sel-sel tunggal *V. cholerae* dan dapat mencegah pembentukan *V. cholerae* dalam bentuk biofilm dengan itu bertindak sebagai antibiofilm.

Kata kunci : *Syzygium aromaticum*, *Vibrio cholerae*, zon perencatan aktiviti antimikrob, minimum kepekatan pembasmian biofilem, mikroskop imbasan elektron

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List of Abbreviations

MBEC	Minimum Biofilm Eradication Concentration
MHA	Mueller-Hinton Agar
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
dh₂O	Distilled water
ddh₂O	Double distilled water
cfu/ml	Colony formation unit per millilitre
PBS	Phosphate buffered saline
SEM	Scanning electron microscope

1.0 INTRODUCTION

Majority of antimicrobial agents are chemically based. The main concern of using chemical antimicrobial agents is their possible side effects to the consumer. The emergence of resistant bacterial strains and consumer concerns regarding the use of chemical products, have lead to the discovery to find natural antimicrobial substitutes for synthetic agents especially in food industry. Due to certain aspect such as misused of antibiotics, increasing case of treatment failure, genetic variation of microbes and cross transfer of resistant genes among bacterial species, has lead to the emergence of drug-resistant organisms.

Therefore, a continuous research to seek for new, effective and affordable antimicrobial agent is crucial to prevent increasing cases caused by those organisms. Alternatives antimicrobial agent must be safe for consumers and harmless to the environment. Such antibacterial agents are often to be found within the plant kingdom (Burt, 2004; Bakkali *et al.*, 2008).

Numerous studies have been conducted to evaluate the potential of plant extracts and essential oils to become one of the antimicrobial agent (Smith-Palmeret *et al.*, 1998). According to Zakaria (1991), even in developing countries nowadays, plant materials play a major role in primary health care as therapeutic remedies. Hence, to seek for a solution to find a substitute for chemically based antimicrobial agents, research has rapidly conducted to develop and employ natural antimicrobial agents derived from plant extracts or essential oils (Appendini and Hotchkiss, 2002; Guillard *et al.*, 2008). Herbal plant species and spices are known to have a great potential to become an antimicrobial agent since they have wide range of organic compounds which capable to enhance antimicrobial activity to exhibit the presence of microorganisms

especially food borne microorganism (Deans and Ritchie, 1987; Deans *et al.*, 1993; Appendini and Hotchkiss, 2002; Stahl-Biskup, 2004; Guillard *et al.*, 2008).

Syzygium aromaticum, commonly known as cloves, have a great potential to be one of an antimicrobial agent to control the emergence of microorganisms related to food borne pathogens. According to previous studies on cloves extract, clove have been identified to have several biological properties such as antibacterial, anti-fungal, insecticidal and antioxidant properties, and have been traditionally used as flavoring agent and antimicrobial material in food (Lee and Shibamoto, 2001; Huang *et al.*, 2002; Velluti *et al.*, 2003). Since clove extract has been used widely as pharmaceuticals, flavoring and antimicrobial agents in food industry, it is necessary to asses their specific reaction towards food borne pathogenic microorganisms such as *Vibrio cholerae*.

One of the global health problem is the increasing cases due to multi-drug resistance bacteria which could result in mortality of millions of people annually due to infections. From all the infectious diseases occur, more than half of the percentages are related with bacterial communities which capable to proliferate by forming biofilms (Lewis, 2007). Therefore, further understanding in the process of biofilms formation is required starting from the early stages to the maturation and then their relationship in biotic and abiotic environment.

Majority of studies related to herbs and spices as antimicrobial agents conducted were tested toward single cells of bacteria. The research of its activity in the biofilms is still need to be evaluates. Thus in this study, *S. aromaticum* bud extract have be screened to become potential antimicrobial agent against single cells of *V. cholerae* then the evaluation of antibiofilm were tested against of *V. cholerae* biofilm. .

The objectives of this study are as follows :

1. To evaluate the susceptibility of *Syzygium aromaticum* bud extract towards *Vibrio cholerae*
2. To determine the minimum biofilm eradication concentration (MBEC) of *Syzygium aromaticum* extract towards *Vibrio cholerae* biofilm.
3. To study on the morphologies of *Vibrio cholerae* after treated with several concentrations of *Syzygium aromaticum*

2.0 LITERATURE REVIEW

2.1 Background of clove (*S. aromaticum*)

The scientific classification of *S. aromaticum* (clove) is shown in Table 1.

Table 1: Scientific classification of *S. aromaticum*

Kingdom	Plantae
Phylum	Angiosperms
unranked	Eudicots
unranked	Rosids
Order	Myrtales
Family	Myrtaceae
Genus	<i>Syzygium</i>
Species	<i>aromaticum</i>

Syzygium aromaticum, or commonly known as clove is one of the most valuable spices that have been used for a very long time ago especially in India and China. 'Cloves' are given to that species based on their morphological characteristic of the flower bud that shape like a broad-headed nail and it originates from the French and English word for 'nail' 'le clou' and 'clout'.

The dried bud of *S. aromaticum* (clove) purchased from local market in Kuching is shown in Figure 1.



Figure 1 : Dried *S. aromaticum* bud purchase from local market in Kuching

As describe by S. Nehra (2007), clove have a cylindrical base shape of its hypanthium, ball-like, unopened corolla, which are surrounded by the four-toothed calyx. The corolla consists of unopened membranous petals with several stamens and single stiff prominent style averagely 10.0 to 17.5mm length, 4.0mm width, 2.0mm thickness. The hypanthium is a small angular peduncle, flattened at the base and well supplied with oil glands that impart a characteristic aromatic odour. Hypanthium is surmounted with 4 thick acute divergent surrounded by ball-like corolla. Good quality cloves are brownish black in colour, with full and plump crown, should not contain more than 12 per cent moisture, and 2-3 per cent foreign matter. Those foreign matter include khoker cloves, mother cloves, headless cloves and other extraneous matter. Khoker cloves are those cloves which undergone fermentation due to improper drying and recognized by

pale brown colour and whitish mealy appearance. Mother cloves are clove fruits produced when the fertilization occurred. Headless cloves are cloves without the ball-shaped unopened flower bud at the top .

Clove tree is believed to be indigenous to the Moluccas or Spice Islands, a group of volcanic islands in eastern Indonesia. Clove also produced in Malaysia, Sri Langka and Haiti, but not in commercially significant qualities. Clove tree are suitable to be grown in deep sandy, red acid loams.

Cloves tree have a straight trunk and grows up to a height of 10 to 12m. The buds are ready to harvest after six month of the bud appear. Flower buds are greenish or reddish in colour when fresh and it will turns brown and brittle on drying. For cloves storage, both whole and ground cloves should be stored in an airtight container in a cool, dark, and dry place. According to D. Grotto (2007), whole clove can be stored up to one year, while ground cloves can only be stored for six months.

2.1.1 Active constituent of clove

As reported by H.K. Bakhru (1992), the clove bud extract contains 3 major components which are eugenol, eugenol acetate and caryophyllene. Majority of the clove extract constituent is Eugenol. Approximately 72-90% of their active constituent is Eugenol. Other components of clove extract are Acetyl eugenol, Beta-caryophyllene, Vanillin, Crategolic acid, Tannins, Gallotannic acid, Methyl salicylate, Flavonoids eugenin, Kaempferol, Rhamnetin, Eugenitin, Triterpenoids. (D. Bhowmik *et al.*, 2002). Specifically, Free eugenol, eugenol acetate, caryophyllene, sesquetrepane ester, phenyl propanoid, and β -caryophyllene are the

pharmacologically active compounds can be extracted from clove bud. (Kurkawa M. *et al.*, 1998, Miyazawa M. and Hisama M., 2003).

2.1.2 Nutritional and Medicinal value of cloves

Cloves have many uses especially in medicinal uses and as a flavourant. Cloves can also be used as a culinary spice, for flavouring pickles, curries, ketchups and sauces, as an ingredient of many toothpastes and mouthwashes. Cloves has many industrial applications and is extensively employed in perfumes and in scenting soaps (S. Nehra, 2007). Cloves has been used in India and China, for a very long time ago as a spice to check both tooth decay and counter halitosis which is bad breath (H.K. Bakhru, 1990). Cloves has high source of manganese which vital for metabolism, enhance enzymes and helps to strengthen bone. Cloves also high in fiber. Omega-3 and other phytonutrients for enhance the immune system was reported abundance in clove. Therefore, clove helps to boost immunity in human body. Besides, it has been discovered to help prevent adult onset diabetes by tripling insulin levels (D. Bhowmik *et al.*, 2012).

2.1.3 Research related to cloves

Lee and Shibamoto (2001), claimed that cloves extract have a great potential to be used as an anticarcinogenic agent since cloves extract contain small percentage of antioxidant properties. They also reported that cloves extract can be used as a potential chemopreventative agent. Cloves have been used as a carminative, which helps to increase hydrochloric acid in the stomach and to improve peristalsis (Phyllis and James, 2000). In addition, cloves also have potential as anti-mutagenic (Miyazawa and Hisama, 2003), antioxidant (Chaieb *et al.*, 2007), anti-ulcerogenic (Li *et al.*, 2005) and anti-parasitic (Yang *et al.*, 2003). Studies have been conducted and reported clove extract have high anti-microbial properties against important

human pathogenic microorganisms and microorganisms which able to cause food spoilage, due to a high concentration of eugenol in cloves extract (Wenqiang *et al.*, 2007; Bhuiyan *et al.*, 2010; Ultee *et al.*, 2002; Chaieb *et al.*, 2007; Lee and Shibamoto, 2001). Previous research have found several benefits of clove. List of several benefits of *S. aromaticum* (clove) is summaries in Table 2.

Table 2 : Benefits of clove (Debjit Bhowmik *et al.*, 2012)

Description	
Antiseptic Benefits	Remedy for common problem such as cuts, fungal infections, burns, wounds, athlete's foot and bruises. It helps to boost the immune system by purifying the blood and help to fight against various diseases.
Digestive Health Benefits	Helps to cure flatulence, loose stools, indigestion and nausea. Also useful in relieving the symptoms of diarrhea, gastric irritability and vomiting.
Anti-Inflammatory Benefits	Aromatic smell of clove helps to relieve certain respiratory conditions like coughs, colds, asthma, bronchitis and sinusitis.
Analgesic Benefits	Used for treatment of various dental problem like tooth aches. Also used to relieve pain from sore gums and improves overall dental health.
Natural Herbal Remedies	Helps to relieve toothaches, earaches, nausea, hypertension and pain from burns and wounds. Also helps in respiratory problems, great air freshener, mosquito repellent, fly deterrent, and ant killer.

2.2 Background of *Vibrio cholerae*

Vibrio cholerae, a gram negative bacteria, can be classified as toxigenic or nontoxigenic based on the presence or absence of the operon which encode for cholerae toxin, ctxAB operon. With an estimated infectious dose of ~ 1 000 000 cells toxigenic strains of *V. cholerae*, the person can be suspected to have cholera disease. Cholerae is a rapid, ranging from mild to severe gastroenteritis characterized by “rice water” stools that may be accompanied by vomiting. According to Centers for Disease Control and Prevention (2016), disease cause from toxigenic *V. cholerae* rarely result in death in developed countries (<1% of cases), but cholerae generally has a global fatality rate approximately 1-5%.

2.2.1 Sources of *V. cholerae*

According to V. Bergen (1996), the outbreak cases of cholera usually occur when people use poor hygiene and poor sewage treatment that contaminate the river water supply. According to World Health Organization (WHO), this case commonly occurred particularly in the squatter areas of towns and the poorer rural areas. Cholera transmission can occur due to inadequate access to clean water and sanitation facilities. Typical at-risk areas include certain rural area where basic infrastructure is not available, as well as camps for internally displaced persons or refugees, where minimum requirements of clean water and sanitation have not been met. Environment can be the most common route of transmission for cholera, whether from its natural occurrence or contamination of waters in human feces. Shellfish may also serve as an important vehicle in the transmission of cholera.

2.2.2 Diseases cause by *V. cholerae*

According to World Health Organization (WHO), cholera can be either endemic or epidemic. A cholera-endemic area is an area where confirmed cholera cases were detected during 3 out of the last 5 years with evidence of local transmission (originally from that particular area). A cholera epidemic is defined by the occurrence of at least 1 confirmed case of cholera with evidence of local transmission in an area where there is not usually cholera. The use of water contaminated with *V. cholerae* in daily routine significantly contribute to increase the risk of infection. According to Centre for Disease control and Prevention (2016), severity of cholera has increased dramatically in recent years. There is no particular or specific antimicrobial agent that can cure diarrheal disease until now. The treatment is depends on simple rehydration therapy, sometimes in combination with chemical based antimicrobial agents (Sack *et al.*, 2004).

2.2.3 Biofilm of *V. cholerae*

Biofilm is the community of micro-organisms living together in amorphous extracellular matrix composed of polysaccharides, extracellular DNA, and proteins. In the nature, biofilm are found to be able to develop both on abiotic and biotic surfaces. Communities of *Vibrios* have the ability to attach on abiotic surfaces or to biotic substrate such as the human intestinal mucosa or the chitinous exoskeleton of crustaceans, *Vibrio* aggregates in suspension, floccules, and pellicles formed at the liquid–air interface of static cultures (Anisia J. Silva and Jorge A. Benitez, 2016). Due to their complexity, biofilm makes microbial cells inside the matrix confer high level of antibiotic resistance. The bacteria in biofilms are slow-growing and were found to be one of the bacteria that resistant to antibiotic treatment

(D. Davies, 2003). This lead to the ineffectiveness in the inhibition and eradication of biofilm infections. Evidence has grown suggesting that *V. cholerae* can form biofilm-like aggregates during infection that could play a critical role in pathogenesis and disease transmission.

3.0 MATERIALS AND METHODS

3.1 Materials

Dried *Syzygium aromaticum* bud, absolute methanol, *Vibrio cholerae*, Mueller-Hinton Agar (MHA), Tryptic soy agar (TSA), Tryptic soy broth (TSB), distilled water (dH₂O), double distilled water (ddH₂O), PBS, gluteraldehyde, absolute ethanol

3.2 Methods

3.2.1 Collection and identification of plant materials

The cloves was purchased from market in Kuching on september 2016. The cloves was ground into fine powder form by using a blender (MODEL: Snova™ Heavy Duty Commercial Blender). The powder form of the clove was stored in an air tight bottle and stored in a dark place.

3.2.2 Extraction of clove extract by Soxhlet apparatus

The powder formed of clove bud samples was weighed for 100g and soaked with 300ml of absolute methanol. The mixture was incubated in a dark place at ambient temperature for a week. The mixture must be stirred everyday to ensure the solvent completely dissolved into the clove powder. After a week, the mixture was transferred into a filter paper extraction thimble and inserted into a 500ml reflux flask, then extracted with 250ml methanol for 6 to 8 hours in Soxhlet apparatus (Quan *et al.*, 2004), with slightly some modification. After Soxhlet extraction, extracts were concentrated using rotary vacuum evaporator at 50° C.

3.2.3 Preparation of inoculum

A total of 19 sets of *V. cholerae* from the glycerol stock was subcultured onto slant agar and was incubated for 24h. List of 19 sets of *V. cholerae* were attached on Appendix 1 and 2. After 24h, colonies from these slant agar was suspended in Tryptic Soy Broth (TSB) and was incubated for 24h. After incubation, the inoculum was diluted 5x by using Tryptic Soy Broth (TSB) to a turbidity matching 0.5McFarland standard (10^8 colony forming units (cfu/ml)). McFarland Standards can be prepared by mixing barium chloride with sulfuric acid (McFarland Standards 0.5: 0.05ml 1% barium chloride with 9.95ml 1% sulfuric acid).

3.2.4 Disc diffusion test

The media that was used for antimicrobial susceptibility test is Mueller - Hinton agar (MHA). The extract was tested to determine their antibacterial activity using the standard paper blank disc diffusion assay described by Bopp *et al.* (1999). Then, clove extract was diluted to concentration 1 mg/ml, 10 mg/ml and 100 mg/ml with 10% DMSO solution. Each of the *V. cholerae* culture was swabbed onto agar medium using sterile cotton swab. Sterile filter paper discs that were 6.0 mm in diameter was loaded with 20 μ l of extract then allowed to dry and placed on the agar plates which inoculated testing bacteria. The plates were incubated at 37°C for 24 hours and observed for zone of inhibition in millimeter (Seeley *et al.*, 2001). Result obtained for concentration 10mg/ml and 100mg/ml of clove extract were attached on Appendix 5 and 6.