



UNIVERSITI PUTRA MALAYSIA

***ANTIBACTERIAL AND SPORICIDAL ACTIVITY OF Syzygium grande
(Wight) Walp. AND Oenanthe javanica (Blume) DC. AGAINST
Bacillus sp.***

KHALED ABDUSALAM. B. A ATRASH

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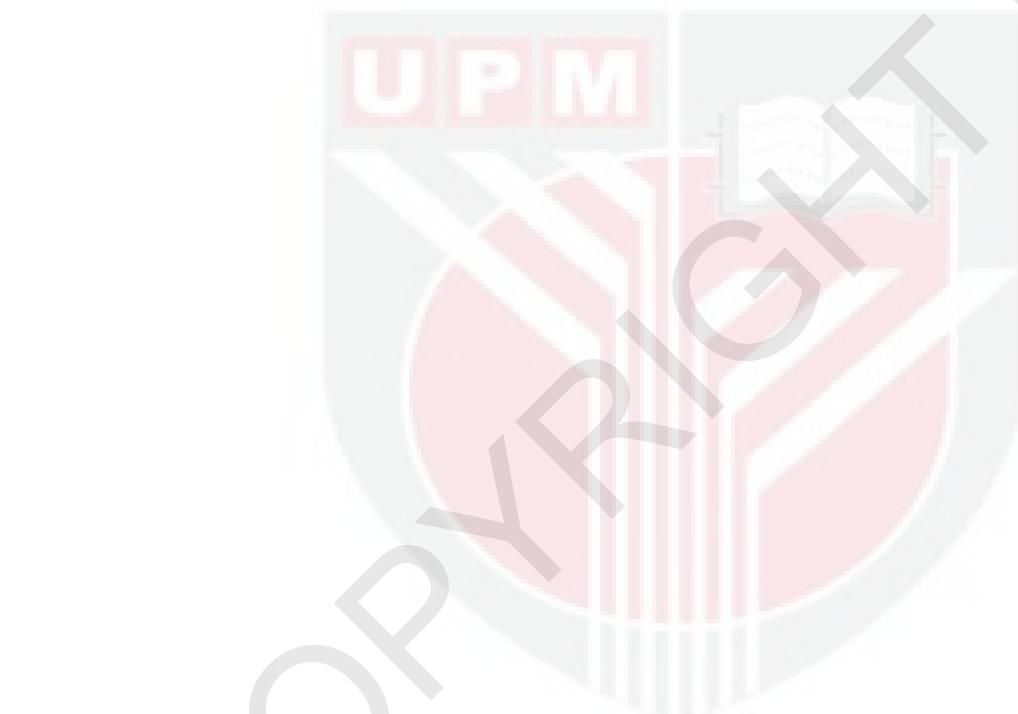
**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

June 2020

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment
of the requirement for the degree of Doctor of Philosophy

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By

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June 2020

**Chairman : Professor Khozirah binti Shaari, PhD
Institute : Bioscience**

The contamination of many products in the food industry has always been linked to spore-forming bacteria including *Bacillus* sp. which commonly found in different type of food raw materials. Although different approaches for managing bacterial endospore contamination is urgently needed in diverse industries and applications, natural products are seen as the alternative solution. Based on previous studies, both *Syzygium grande* and *Oenanthe javanica* have been reported to exhibit several biological properties including antibacterial and antifungal. Thus, the main aim of this study is evaluating antibacterial and sporicidal activities of *Syzygium grande* and *Oenanthe javanica* against vegetative cells and spores of *B. cereus* ATCC33019, *B. subtilis* ATCC6633, *B. megaterium* ATCC 14581 and *B. pumilus* ATCC 1488. A further objective is to isolate the bioactive compound(s).

In this study, the metabolomics analysis of the 70% methanol, methanol, ethyl acetate and hexane extracts from the both plant was performed and showed clear discrimination between the different solvent extracts due to presence of different classes of compounds. Twenty compounds of *S. grande* have been assigned, including amino acids, carbohydrate, triterpene, terpenoids, and organic compounds. Compounds responsible for the differentiation were identified by comparison of their ¹H-NMR chemical shifts. The PLS results indicated that many non-polar compounds from *S. grande* hexane extract included betulin, β -sitosterol, oleanolic acid, β -caryophyllene, acetic acid and 3-hydroxybutyric acid were strongly correlated to anti-Bacillus. While, eighteen compounds of *O. javanica* have been assigned, including amino acids, carbohydrate, an organic acid terpenoids, and organic compounds. The PLS results showed the different polar compounds included sugars, glycine, choline, proline, ellagic acid, and gallic acid from *O. javanica* methanol extract are the potential contributors for the antimicrobial activities. Various solvent fractions obtained from solvent partitioning of the methanolic extracts of *S. grande* and *O.*

javanica were screened for antimicrobial and sporicidal activity against of target *Bacillus* species, respectively. The hexane fraction of both plant showed the highest antimicrobial and sporicidal activities against all *Bacillus* sp. The results showed that the growth of vegetative cells of all tested *Bacillus* species were inhibited by *S. grande* hexane fraction at 1.0% with MIC values ranged between 0.039 to 0.625 mg/mL. While, MBC values ranging between 0.312 to 1.25 mg/mL. *Bacillus* species was also inhibited by *O. javanica* hexane fraction with MIC between 0.078 to 0.625 mg/mL. While, MBC values between 0.625 to 2.5 mg/mL. From time-kill assays, 4×MIC and 8×MIC of the hexane fractions of *S. grande* and *O. javanica*, respectively, were found to kill 99.90% of *Bacillus* sp. after 2 to 4 h incubation. Hexane fraction of *S. grande* was found to completely eradicate all *Bacillus* spores at 1%, after 2 to 3h of incubation. Similarly, *O. javanica* hexane fraction completely eradicate of all *Bacillus* spores at 1% after 3 to 4h of incubation. From the GCMS analysis, a total of 33 and 29 compounds could be putatively identified in the *S. grande* and *O. javanica* hexane fractions, respectively, which included hydrocarbons, fatty acid, diterpenoids and triterpenoids. Meanwhile, 18 compounds from *S. grande* and 21 compounds from *O. javanica* ethyl acetate fractions were putatively identified using LC-MS/MS analysis. Most of the compounds had antibacterial activities based on previously reported.

Isolation of bioactive constituents was then attempted on the active fractions using bioautographic assay and column chromatography. The hexane fraction of *S. grande* yielded β -sitosterol and ursolic acid, while lupeol was isolated from the hexane fraction of *O. javanica*. All compounds were found able to inhibit the growth of all the *Bacillus* species at 100 μ g/mL. Both β -sitosterol and lupeol gave lowest MIC value (31.25 μ g/mL) against *B. pumilus*, and gave equally low MIC values (31.25 to 62.5 μ g/mL) against the other *Bacillus* species. Overall the MBC values ranged from 125 to 250 μ g/mL. While, ursolic acid was slightly less bioactive, with MIC values of 62.5 to 250 μ g/mL against the test organisms, while the MBC values ranged from 125 to 500 μ g/mL. Finally, at concentrations 100 to 1000 μ g/mL, the three compounds were investigated for their abilities to reduce the viability of *Bacillus* sp. spores. β -sitosterol was found to kill all *Bacillus* spores at 1000 μ g/mL, after 2 to 3 h of incubation while, ursolic acid at same concentration was killed all *Bacillus* spores after 2 h of incubation. Similarly, lupeol at 1000 μ g/mL, gave complete elimination of *Bacillus* spores after 1 or 2 h of incubation. In conclusion, *S. grande* and *O. javanica* hexane fractions and its compounds which isolated from these fractions exhibited antimicrobial and sporicidal activity against *Bacillus* species, thus it can be developed as anti-*Bacillus* agent.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**AKTIVITI ANTIBAKTERIA DAN SPORISID *Syzygium grande* (Wight) Walp.
DAN *Oenanthe javanica* (Blume) DC. TERHADAP *Bacillus* sp.**

Oleh

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Pencemaran pelbagai produk dalam industri makanan sering dikaitkan dengan bakteria pembentuk spora seperti *Bacillus* sp. yang kebiasaananya dijumpai dalam pelbagai jenis bahan mentah makanan. Walaupun pendekatan yang berbeza amat diperlukan dalam pelbagai industri dan aplikasi bagi menguruskan pencemaran endospora bakteria, produk semulajadi dilihat sebagai penyelesaian alternatif. Berdasarkan kajian-kajian sebelum ini, kedua-dua *Syzygium grande* dan *Oenanthe javanica* telah dilaporkan menunjukkan beberapa ciri biologi termasuk antibakteria dan antikulat. Oleh itu, matlamat utama kajian ini adalah untuk menilai aktiviti antibakteria dan aktiviti sporisidal *Syzygium grande* dan *Oenanthe javanica* terhadap sel-sel vegetatif dan spora *B. cereus* ATCC33019, *B. subtilis* ATCC6633, *B. megaterium* ATCC 14581 dan *B. pumilus* ATCC 1488. Selain itu, kajian ini juga bertujuan untuk mengasingkan sebatian-sebatian bioaktifnya.

Dalam kajian ini, analisis metabolomik 70% metanol, metanol, etil asetat dan ekstrak heksana dari kedua-dua tumbuhan tersebut telah dilakukan dan telah menunjukkan diskriminasi yang jelas antara ekstrak pelarut yang berbeza kerana terdapat kelas-kelas sebatian yang berlainan. Dua puluh sebatian dari *S. grande* telah dikenalpasti, termasuk asid amino, karbohidrat, triterpena, terpenoid, dan sebatian organik. Sebatian yang mempengaruhi pembezaan tersebut telah dikenalpasti berdasarkan perbandingan nilai anjakan kimia $^1\text{H-NMR}$ mereka. Hasil keputusan PLS menunjukkan sebatian tidak polar dari ekstrak heksana *S. grande* termasuk betulin, β -sitosterol, asid oleanolik, β -kariofailina, asid asetik dan asid 3-hidroksibutirik sangat berkait dengan anti-*Bacillus*. Manakala, lapan belas sebatian dari *O. javanica* telah dikenalpasti termasuk asid amino, karbohidrat, asid organik, dan sebatian organik. Hasil keputusan PLS menunjukkan sebatian polar yang berlainan termasuk gula, glisin, kolin, prolin, asid ellagik, dan asid gallik dari ekstrak metanol *O. javanica* adalah penyumbang yang berpotensi untuk aktiviti antimikrob. Pelbagai pecahan

pelarut yang diperolehi daripada pembahagian pelarut ekstrak metanol *S. grande* dan *O. javanica* telah diuji untuk aktiviti antimikrob dan sporisid terhadap spesies *Bacillus* yang disasarkan. Pecahan heksana dari kedua-dua tumbuhan tersebut menunjukkan aktiviti antimikrob dan sporisidal yang tertinggi terhadap semua *Bacillus sp.* Hasil keputusan menunjukkan bahawa pertumbuhan sel-sel vegetatif dari semua spesis *Bacillus* yang diuji telah dihalang oleh pecahan heksana *S. grande* pada 1.0% dengan nilai MIC dalam lingkungan antara 0.039 hingga 0.625 mg / mL. Manakala, nilai MBC adalah dalam lingkungan antara 0.625 hingga 2.5 mg / mL. Dari ujian masa-pembunuhan, $4 \times$ MIC dan $8 \times$ MIC pecahan heksana *S. grande* dan *O. javanica*, masing-masing didapati membunuh 99.90% *Bacillus sp.* selepas inkubasi selama 2 hingga 4 jam. Pecahan heksana dari *S. grande* didapati benar-benar membasmikan semua spora *Bacillus* pada 1%, selepas 2 hingga 3 jam inkubasi. Begitu juga, pecahan heksana *O. javanica* telah membasmikan semua spora *Bacillus* pada 1% selepas 3 hingga 4 jam inkubasi. Daripada analisis GCMS, sejumlah 33 dan 29 sebatian telah dikenalpasti di dalam pecahan heksana *S. grande* dan *O. javanica*, masing-masing, termasuk hidrokarbon, asid lemak, diterpenoid dan triterpenoid. Sementara itu, 18 sebatian dari *S. grande* dan 21 sebatian dari pecahan *O. javanica* etil asetat telah dikenalpasti dengan menggunakan analisis LC-MS / MS. Majoriti sebatian-sebatian tersebut mempunyai aktiviti antibakteria berdasarkan laporan yang dilaporkan sebelum ini.

Pengasingan sebatian bioaktif kemudiannya dilakukan pada pecahan yang aktif dengan menggunakan kromatografi assay dan kromatografi kolumn. Pecahan heksana *S. grande* menghasilkan β -sitosterol dan asid ursolik, sementara lupeol diasingkan dari pecahan heksana *O. javanica*. Ketiga-tiga sebatian tersebut didapati mampu menghalang pertumbuhan semua spesies *Bacillus* pada 100 μg / mL. Kedua-dua β -sitosterol dan lupeol memberikan nilai MIC terendah (31.25 μg / mL) terhadap *B. pumilus*, dan memberikan nilai MIC sama rendah (31.25 hingga 62.5 μg / mL) berbanding spesies *Bacillus* yang lain. Secara keseluruhan, nilai MBC adalah dalam lingkungan antara 125 hingga 250 μg / mL. Sementara itu, asid ursolik sedikit kurang bioaktif, dengan nilai MIC dalam lingkungan antara 62.5 hingga 250 μg / mL terhadap semua organisme yang diuji, manakala nilai MBC adalah dalam lingkungan antara 125 hingga 500 μg / mL. Akhir sekali, pada kepekatan antara 100 hingga 1000 μg / mL, ketiga-tiga sebatian telah diselidik untuk kebolehan mereka mengurangkan daya maju spora *Bacillus*. β -sitosterol didapati membunuh semua spora *Bacillus* pada 1000 μg / mL, selepas 2 hingga 3 jam inkubasi, manakala, asid ursolic pada kepekatan yang sama telah membunuh semua spora *Bacillus* selepas 2 jam inkubasi. Begitu juga, lupeol pada 1000 μg / mL memberikan penghapusan spora *Bacillus* yang lengkap selepas 1 atau 2 jam inkubasi. Kesimpulannya, pecahan heksana *S. grande* dan *O. javanica* dan sebatian-sebatian yang diasingkan daripada pecahan ini menunjukkan aktiviti antimikrob dan sporisidal terhadap species *Bacillus*, oleh itu ia boleh dibangunkan sebagai agen anti-*Bacillus*.

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This thesis was submitted to the Senate of the 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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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
Ca ²⁺	Calcium cation
Ca ²⁺ -DPA	Calcium cation and dipicolinic acid chelate
CC	Column Chromatography
CD3OD	Methanol-d4
CDCl ₃	Deuterated Chloroform
CFU	Colony forming unit
CH ₃ OH- <i>d</i> 4	Deuterated Methanol-d4
CHCl ₃	Chloroform
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
COSY	Correlations Spectroscopy
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance Spectroscopy
DMSO	Dimethyl sulfoxide
DMSO- <i>d</i> 6	Deuterated Dimethyl sulfoxide
EtOAc	Ethyl acetate
G	Gram
GC-MS	Gas Chromatography – Mass Spectrometry
¹ H-NMR	Proton Nuclear Magnetic Resonance Spectroscopy
H	Hour
Hex	Hexane
HMBC	Heteronuclear Multiple-bond Correlation
HSQC	Heteronuclear Single-quantum Correlation

Hz	Hertz
IR	Infrared Spectroscopy
J	Coupling constant in Hz
LC-MS	Liquid Chromatography–Mass Spectrometry
m	Multiple
m.p.	Melting point
m/z	Mass per Charge
MBC	Minimum Bactericidal Concentration
MeOH	Methanol
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
mHz	MegaHertz
MIC	Minimum Inhibitory Concentration
Min	Minute
mL	Milliliter
mm	Millimeter
MS	Mass Spectrometry
MS/MS	Mass / Mass Spectrometry
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
n- BuOH	n-Butanol
NA	Nutrient agar
NaCl	Sodium chloride
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PLS	Partial Least Squares

Ppm	Part Per Million
ppm	Part per million
Rpm	Revolutions per minute
Rt	Retention Time
s	Singlet
Sp	Species
t	Triplet
TLC	Thin layer chromatography
WHO	world health organization
α	Alpha
β	Beta
δ	Chemical Shift in ppm
μg	Microgram
μL	Microliter

CHAPTER 1

INTRODUCTION

1.1 Background

In the late 1700s, Nicolas Appert invented the appertization process, currently known as canning, to prolong food quality, shelf-life, in addition to prevent food spoilage over an extended period of time. Accordance with his conviction, the eradication of air was responsible for canned food stability. Subsequently, Cohn & Koch (1875) discovered spores for the first time, while Pasteur discovered spores-producing bacteria, causing food spoilage. Both these discoveries connected microbial activities with food spoilage and safety. Globally, these food spoilage microorganisms extensively affected a wide range of food, causing substantial food wastage and losses, even in the developed countries. According to Gustavsson *et al.* (2011), the annual losses of world food caused by different factors including spoilage microorganisms, can reach up to 40%.

Moreover, bacteria, yeast, as well as moulds are considered to be the common microorganisms causing substantial amount of food spoilage along with food products (Lianou *et al.*, 2016). As soon as these microorganisms get into food items, they will grow by consuming produce metabolites and nutrients, causing food spoilage accordingly (Parlapani *et al.*, 2017). The contaminated food products consumption causes foodborne illnesses, which is an important safety matter in public health (Azziz *et al.*, 2005; Kirk *et al.*, 2017). Moreover, microorganisms predominantly exist among natural surroundings, and hence, they could easily contaminate food through processing, slaughtering harvesting along with packaging (Hatab *et al.*, 2016). Thus,

microorganisms could persist in unfavourable circumstances including pasteurization, packaging, modified atmosphere packaging, vacuum, and low temperature (Dimitrijević *et al.*, 2007; Provincial *et al.*, 2013; Saraiva *et al.*, 2016; Säde *et al.*, 2017). Apart from mold fungi, spoilage bacteria (*Bacillus* sp.) are also closely related to food spoilage. Once bacteria break down food, acids and other waste products are produced in the process. Moreover, the genus *Bacillus* could be defined as a group of rod-shaped, gram-positive, aerobic or anaerobic bacteria (Kim *et al.*, 2014), which are predominantly found in a variety of raw materials food (Opstal *et al.*, 2004). Therefore, these bacteria are potential causal agents for foodborne pathogenesis.

Bacillus sp. contaminate a variety of food and food products such as infant formula, new-born child cereal, cooked rice, dried milk, eggs, meat, flavours, pasta and noodles (Altayar & Sutherland, 2006; Kim *et al.*, 2013). Heat resistivity of *Bacillus* spores and its adaptability to different environments enable them to survive in foods that have gone through moderate cooking processes. It was reported that gaseous swelling and unpleasant odours in canned peas were mainly caused by the heat resistant spores'

growth of the bacteria (Russell, 1990). It has been found that disease and spoilage are associated with thermally processed foods, as heat causes on killing the vegetative cells but permits the growth of spore-producing bacteria. Comparing to other food-borne pathogenic bacteria, spores have better endurance under usual food processing conditions, therefore, causing an immense problem for food industries. Thus, sterilizing food procedures, pharmaceutical, medical, and other products need to consider this significant level of resistance by bacterial spores. Several chemical compounds, such as glutaraldehyde, formaldehyde, chlorine-releasing agents, peroxygens, ethylene oxide, and ozone have been applied as sporicidal agents

(Russell, 1990). According to Lawrence & Palombo, (2009), these chemical compounds kills the spores through the inactivation of different phases of the germination process including the damage it causes to the bacterial DNA, and the irreversible disorder of the spore reliability. Although, glutaraldehyde shows great sporicidal activity, the pungent, rotten-like the chemical smell which makes it unacceptable to use in food products. Additionally, high concentrations and expansive contact time are considered necessary to observe significant log reductions in spore numbers (Lawrence and Palombo, 2009). Furthermore, the exposure to glutaraldehyde has also been associated with different health impacts such as skin, eyes and respiratory irritations (Ballantyne and Jordan, 2001). Similarly, formaldehyde can be utilized in both liquid and gaseous stages of the chemical. However, the rate of sporicidal effect of formaldehyde is significantly slower than glutaraldehyde (Lawrence & Palombo, 2009). Moreover, its utilization in food products has been banned by the United States Occupational Safety and Health Act because it was potentially found to be naturally carcinogenic (Lawrence & Palombo, 2009).)

In the same way, chlorine gas can be efficiently used as sporicide. However, its application could be harmful to human health (Lawrence & Palombo, 2009). Meanwhile, hydrogen peroxide is also beneficial in this context, however, chemicals are generally unstable, and its application requires high concentrations and extended contact time. Generally, spores are resistant to hydrogen peroxide, producing protective clumps and catalase to destroy them. On the other hand, ultraviolet (UV) light and microwave radiation are found to be effective in killing *Bacillus* spores. However, they are extremely risky to use within food industry (Lawrence & Palombo, 2009). Therefore, natural products are remarkably beneficial to help during the revelation and development of new sporicides. Although, the antimicrobial properties of various chemical compounds and essential oils derived of plants against vegetative cells have been largely reported and its utility for preservation purposes is also acknowledged, their further action against endospores have not been fully explored. Thus, there is a great potential to discover new sporicides from natural products.

A few studies have demonstrated that natural product compounds, for instance, oleuropein from olives suppresses the germination and succeeding growth of *B. cereus* spores (Tassou, 1993; Tassou *et al.*, 1991). Similarly, catechins from green tea (*Camellia sinensis*) displayed inhibition activity against both *Clostridium botulinum*

and *C. butyricum* spores (Hara-Kudo *et al.*, 2005). Moreover, some cardamom essential oils, the tea plant, along with juniper leaves showed antibacterial activities against *B. subtilis* spores (Lawrence & Palombo, 2009). Extract of *Torilis japonica* can effectively suppress the germination of *B. subtilis* spores (Cho *et al.*, 2008) whilst fruit and leaf extracts of *Myristica fragrance* (nutmeg) plant has inhibited germination and succeeding growth of *B. cereus* spores (Rukayadi *et al.*, 2009).

Syzygium grande (Wight) which is known as “Jambu Laut or Sea apple” in Malaysia. It belongs to the Myrtaceae family, reportedly know to be rich in volatile oils and found to have both culinary and medicinal uses (Mohanam *et al.*, 2002). Meanwhile, *Oenanthe javanica DC*, vernacularly which is recognized as water dropwort or “selom”, is a perennial aromatic herb of the Apiaceae family. The consumption of *O. javanica* as a traditional vegetable (*ulam*) and its use as food seasoning are widely practiced in Asian countries. For example, in Korea, a herb soup is consumed as a treatment for alcohol intoxication hangovers (Kim *et al.*, 2009). Both *S. grande* and *O. javanica* were reported to demonstrate numerous biological properties which includes the antioxidant, antibacterial and antifungal (Raja *et al.*, 2015; Ajam, 2014; Kwon *et al.* 2006; Shin, 2004; Narzary *et al.*, 2018).

The latter property indicated that both plants and their chemical constituents could be useful as antibacterial and/or antisporule agents and it would be more interested to know more details about the antibacterial and sporicidal properties of these plants.

1.2 Problem Statements

Nowadays, the development of natural, plant-derived food sanitizers and food preservatives has been gaining more attention. This is due to the fact that synthetic chemicals could cause more harm than good when applied for human use, especially for food products. Certain plants contain phytochemical compounds which exhibit the antimicrobial activity that may be antibacterial, antifungal and/or antisporule. Therefore, these plants can be used in food industry as a natural preservers or food sanitizers, providing potential alternatives to synthetic chemicals (Cho *et al.*, 2008). It is equally important to develop natural preservatives that are effective and efficient, not only to eradicate the microorganism, but also to reduce the regrowth possibility of the microorganism. Thus, identifying natural sporicidal agents that can minimize or totally eradicate the *Bacillus* sp. population, this is equally important and much needed by the food industry. Furthermore, realizing the great potential of natural products in yielding useful chemicals as possible alternatives to synthetic chemicals, the antibacterial and antisporule properties of the two-essential oil-bearing plants have thus been investigated. Specifically, the plants antibacterial and antisporule activities were investigated compared to vegetative cells along with spores of *B. cereus* ATCC33019, *B. subtilis* ATCC6633, *B. megaterium* ATCC 14581 in addition to *B. pumilus* ATCC 14884.

1.3 Objectives of the Study

The specific objectives of the present study are:

1. To analyse metabolite variation of *S. grande* and *O. javanica* extracts prepared using different solvents and correlation with antimicrobial activities using ^1H NMR based metabolomics approach
2. To determine the effect of the methanolic extract and various solvent fractions of *S. grande* and *O. javanica* towards vegetative cells of *Bacillus* species.
3. To determine the effect of methanolic extract and various solvent fractions of *S. grande* and *O. javanica* towards spores of *Bacillus* species.
4. Identification the phytochemicals compounds in *S. grande* and *O. javanica* hexane and ethyl acetate fractions which had the highest antibacterial and sporicidal activity
5. To isolate and characterize the bioactive (antibacterial and antispore) chemical constituents of hexane fraction of *S. grande* and *O. javanica*.

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