









¹H NMR-Based Metabolomics Profiling of *Syzygium grande* and *Oenanthe javanica* and Relationship Between Their Metabolite Compositions and Antimicrobial Activity Against *Bacillus* species

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(Received January 03, 2021; Revised May 22, 2021; Accepted May 29, 2021)

Abstract: The aim of the present study was to evaluate the variations in the phytochemical compositions and the antimicrobial activities of *Syzygium grande* and *Oenanthe javanica* leaf extracts against *Bacillus* sp. The antibacterial activity of the methanol, 70% methanol, ethyl acetate, and hexane extracts of both plant species were examined *in vitro*, against various *Bacillus* species. In addition, the ¹H Nuclear Magnetic Resonance (¹H NMR) spectroscopy-based metabolomics was employed to gain insights into the correlations between the chemical constituents of the bioactive extract to the biological activity. The hexane extract of *S. grande* leaves and methanolic extract of *O. javanica* leaves showed more potent anti-*Bacillus* activity, among the test extracts. Principal component analysis (PCA) successfully differentiated the extracts on the basis of their metabolite profiles. The results of the Partial Least Square (PLS) analysis showed that the non-polar compounds of the hexane extract of *S. grande* (alanine, betulin, β -sitosterol, β -caryophyllene, acetic acid and 3-hydroxybutyric acid) were strongly correlated to its anti-*Bacillus* activity. On the other hand, choline, ellagic acid, and gallic acid, the metabolites present in the methanolic extract of *O. javanica*, were strongly correlated to its anti-*Bacillus* activity. On this basis, it was therefore concluded that these compounds could be the potential bioactive constituents, contributing to the anti-*Bacillus* activity of the individual plant species. Further in-depth investigations into the potential utilization of the two plants for useful applications in managing and control of *Bacillus* sp., will help pave the way towards their valorization.

Keywords: Anti-*Bacillus* activity; *Syzygium grande*; *Oenanthe javanica*; ¹H NMR-based metabolomics; multivariate data analysis. © 2021 ACG Publications. All rights reserved.

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1. Introduction

Bacillus species is a Gram-positive, facultative anaerobic, motile rod-shaped bacterium [1]. The bacterium is ubiquitous in nature, and occurs frequently in a wide range of food raw materials (Van Opstal *et al.*, 2004; Grande *et al.*, 2006; Rodrigo *et al.*, 2021). Thus, these bacteria are an important cause of foodborne pathogenesis, with potential to contaminate numerous food items, such as infant formulas and cereals, cooked rice, dried milk products, dehydrated potato products, meat, eggs, spices, pasta and noodles, causing food spoilage and dietary illnesses [2-5]. The heat resistant property of *Bacillus* spores and an ability to adapt to changes in the environment allow the bacterial species to survive in food materials, even for foods which have undergone moderate heat processing and normal cooking processes [5]. Meanwhile, medicinal plants are rich sources of secondary metabolites with various biological properties. They are in fact a primary source of antimicrobial agents for use against many bacterial and fungal species [6-7]. Presently, there is renewed interest in antimicrobial activities of phytochemicals due to several compounding factors such as dangerous side effects, misuse or overuse, and emergence and rapid spread of antimicrobial resistant organisms. Newer, safer and more effective antimicrobials are therefore very much needed as alternatives.

In our continued effort to find new antimicrobial agents from natural sources, preliminary antimicrobial screening of local medicinal plants were regularly conducted. Among the plant species screened, also included were several medicinal herbs which for generations have been consumed as a traditional vegetable known as 'ulam' or as an ingredient in traditional cuisines. In the screening exercise, two of the herbs, namely *Syzygium grande* (Myrtaceae) and *Oenanthe javanica* (Apiaceae) were found to have strong activity against several *Bacillus* species used in the screen. The two herbs were thus selected for further evaluation, in order to gain some insights into the unique metabolites that could be contributing to their anti-*Bacillus* activity.

In Malaysia, *S. grande* (syn. *Eugenia grandis*) is known as sea apple, jambu ayer laut, jambu laut and jamba. It is used medicinally to treat cases of coughs, piles, tooth diseases, dysentery, bronchitis, and diabetes [8]. The *Syzygium* species have been shown to have potent antibacterial, antifungal, antioxidant, anti-inflammatory, anti-nociceptive, antiviral, and anticancer activities [9-12]. On the other hand, *O. javanica* (Blume) is an aquatic perennial plant [13]. It is well known to be cultivated as traditional vegetable or "ulam" due to its strong celery-flavoured leaves. In Korean and Chinese traditional medicines, this plant is being used for treating swellings, jaundice, abscesses, hypertension, fever cold, polydipsia, and abdominal pain [14-15]. *Oenanthe javanica* has also been reported to have antibacterial and antifungal activities [16-17].

Proton nuclear magnetic resonance (^1H NMR) is a very efficient and robust technique for generating metabolic profiles. This technique is capable of identifying and discriminating a large number of differential metabolites in organisms [18]. Recently, NMR spectroscopy is widely used in plant metabolomics due to its ability for instantaneous detection of a wide variety and abundant primary and secondary metabolites. Further advantage of NMR spectroscopy over other methods is the short analysis time and simple sample preparation step [19]. Furthermore, the high reproducibility of NMR technique makes it an excellent choice of analytical platform for use in metabolomics studies. There is scarce information about the compounds extractable from *S. grande* and *O. javanica* with solvents of different polarities. Thus, the aim of this study was to examine the metabolite variation of *S. grande* and *O. javanica* extracts prepared using solvents with different polarities (hexane, ethyl acetate, methanol and 70% methanol) and to correlate these variations with their antimicrobial activities using ^1H NMR-based metabolomics approach. Principal component analysis (PCA) was used to differentiate the extracts according to the metabolite composition and to recognize the characteristic differential metabolites, while partial least square (PLS) regression analysis was applied to correlate the metabolites with the antimicrobial activities in order to predict the bioactive compounds of the plants. The results may give new biological and chemical insights for further exploration of these plants into botanical and plant-based pharmaceutical preparations.

2. Materials and Methods

2.1. Materials

Analytical grade solvents (methanol, n-hexane and ethyl acetate) were used for the extraction. Deionized water was obtained from a Milli-Q purification system (Milipore, Bedford, MA, USA). Media used for the antibacterial assays included Mueller Hinton agar (MHA; Difco, Sparks, USA) and Mueller Hinton broth (MHB; Difco, Sparks, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Leicestershire, United Kingdom).

2.2. Plant Collection and Preparation

Leaves of *S. grande* were collected from the campus ground of Universiti Putra Malaysia (UPM), while *O. javanica* was purchased from a local wet-market in Serdang, Selangor, Malaysia. Taxonomic verification of the identity of the two plant species was made *via* comparison with the plant database of the Biodiversity Unit at the Institute of Bioscience, UPM, with the help of the Institute's botanist, Dr. Mohd Firdaus Ismail. After collection, the samples were washed thoroughly with distilled water, followed by oven drying at 40°C (Memmert Universal, Schwabach, Germany). A powerful Waring blender (Model 32 BL80, New Hartford, NY, USA) was used to pulverize the dried plants into fine powder. After pulverizing, all the samples were stored in airtight polyethylene plastic bags, at 4°C.

2.3 Preparation of Extracts

For extraction, 5 g portions of the powdered leaf material were mixed with 100 mL of the respective solvents, which included 100% hexane, 100% ethyl acetate, 100% methanol, and 70% methanol. Each plant:solvent mixture was subjected to ultrasonic-assisted extraction in an ultrasonic bath for 1 h. The mixtures were subsequently filtered through Whatman No 1 filter paper, the collected filtrates concentrated using vacuum rotary evaporator (Heidolph VV2011, Schwabach, Germany), and subsequently, lyophilized in a freeze dryer to yield the crude extracts. For each solvent, the extract was prepared in six replicates (n=6), in accordance with the requirements for metabolomics analysis.

2.4 Sample Preparation for Antibacterial Assay

The stock solutions (100 mg/mL) of each solvent extracts were prepared by dissolving 10 mg of the crude extract in 100 µL DMSO. From each stock solution, 1% test solutions (10 mg/mL) were prepared by diluting 100 µL of the stock solution in 900 µL distilled water. The solutions were stored at 4°C prior to the assay.

2.5 Preparation of Bacterial Suspension

Bacillus cereus ATCC33019, *Bacillus subtilis* ATCC6633, *Bacillus megaterium* ATCC14581 and *Bacillus pumilus* ATCC14884 were obtained from American Type Culture Collection (Gaithersburg, MD, USA). The bacterial strains were cultured on fresh media (nutrient agar) and incubated for one day at 37°C. The bacterial colonies of these strains were preserved on nutrient agar (NA) for a couple of weeks prior to sub-culture [20]. Preparation of all bacterial strains was performed according to the Clinical and Laboratory Standards Institute (CLSI) standards: M02-A11 [21]. Briefly, the stock culture of bacteria was sub-cultured on Mueller Hinton agar (MHA) for 12 to 24 h at 37°C [22]. Subsequently, two to three colonies of bacteria were transferred into 1 mL MHB using a sterile cotton swab and the bacterial suspension vortex-mixed for 10 min and allowed to grow at 37°C. After 24 hours, 10 µL of the bacterial suspension was transferred into 10 mL of MHB. The turbidity of inoculum was diluted to approximately above 10⁶ CFU/mL concentrations, utilizing a standard broth microdilution and inoculum quantification methods [22]. Inoculum quantification was performed by plating 20 µL of bacterial suspension on MHA and counting the colonies formed after incubation for one day at 37°C.

2.6. Determination of Minimal Inhibitory Concentration (MIC)

The MICs were determined as outlined by CLSI [21]. The MICs of extracts of *S. grande* and *O. javanica* against *B. cereus*, *B. subtilis*, *B. megaterium*, and *B. pumilus* were performed in a 96-well microtiter plate with two-fold standard broth microdilution method with an inoculum of approximately 10^6 CFU/mL. Briefly, 100 μ L of each extract (10 mg/mL concentration) was mixed and diluted in two-folds with the test bacteria in 100 μ L of MHB. Column 12 of the microtiter plate contained the highest concentration of the extracts (500 μ g/mL) while column 3 comprises the lowest concentration (19.50 μ g/mL). Column 2, containing only MHB and inoculum, served as the positive growth control while column 1 served as negative control containing MHB media but no inoculum and antibacterial agent. The microtiter plate was incubated aerobically at 30°C for 24 h. The MIC is defined as the lowest concentration of antibacterial agent that completely inhibited visible growth [23].

2.7. Sample Preparation for NMR Measurement

Five milligrams of each of the extract was placed in Eppendorf tubes and mixed with 600 μ L of DMSO- d_6 , containing 0.1% trimethylsilyl propionic acid (TSP). The mixture was vortexed for 1 min and ultrasonicated for 15 min at room temperature. Next, the mixture was centrifuged at 13000 rpm for 10 min. A volume of 550 μ L of the supernatant was transferred to an NMR tube and submitted for NMR analysis. NMR spectra were obtained on a 500 MHz Varian INOVA NMR spectrometer (Varian Inc., Palo Alto, California, USA), running at a frequency of 499.887 MHz at room temperature (25°C), using DMSO- d_6 as an internal lock. The pre-saturation (PRESAT) pulse sequence was applied to all the samples to reduce water (H_2O) signals, and the acquisition time for every spectrum was 3.54 min with 64 scans.

2.8. Processing of 1H NMR Spectra

The 1H NMR spectra were processed, bucketed and converted to ASCII files using Chenomx software version 8.2 (Alberta, Canada). The δ 0.5–10.0 region was binned with a width of δ 0.04, generating 237 bins. The residual signal of DMSO at δ 2.43–2.63 was excluded for all spectra.

2.9. Statistical Analyses

Windows Excel 2010 and GraphPad Prism software version 5.01 (San Diego, CA, USA) were used for the analysis of antimicrobial data. The results were expressed as mean \pm SD of 6 replicates. For multivariate data analysis (MVDA), the standardized binned data was imported into SIMCA-P software version 13.0 (Umeå, Sweden). Principal component analysis (PCA) and partial least-square analysis (PLS) method were conducted with Pareto scaling technique. For PLS analysis, the NMR chemical shifts of samples were the X variables, while the antimicrobial data (1/MIC) was input as Y variables. Validation of the PLS model was performed by permutation test [24].

3. Results and Discussion

3.1. Minimal Inhibitory Concentration (MIC)

The *in vitro* minimum inhibitory concentrations (MICs) of the different extracts of both plants against *Bacillus* sp. growth were determined. The results are as shown in Figures 1 and 2.

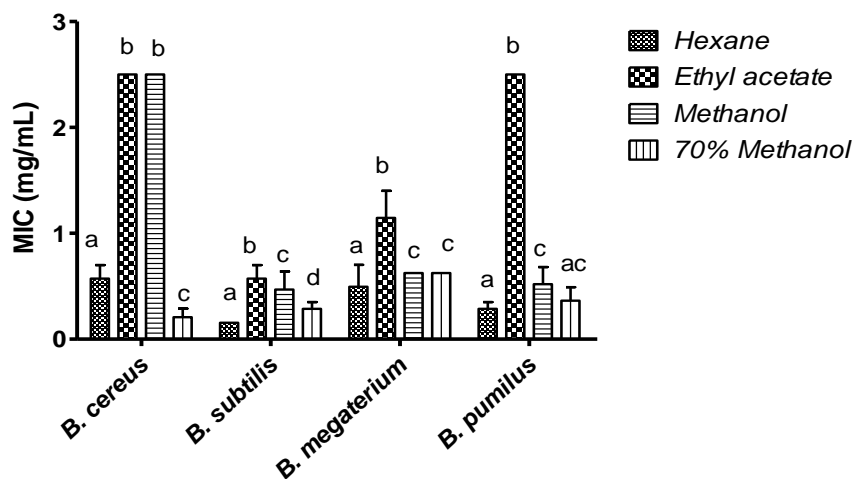


Figure 1. Minimum inhibitory concentration (MIC) of *S. grande* extracts against selected bacteria. Data shown are means of six biological replicates \pm SD. Different letters indicate significant differences at $p < 0.05$

The MIC results revealed the growth inhibition levels of the bacterial strains by the different extracts. The extracts, i.e hexane, EtOAc, MeOH and 70% MeOH, showed different growth inhibition against the *Bacillus* sp., with MIC values ranging between 0.156 to 2.5 mg/mL. The variation in antibacterial activities among the extracts is due to the different types of metabolites being extracted by the different polarity of the extraction solvent used, with the more potent metabolites extractable by hexane, which was the least polar solvent. As can be seen from Figure 1, the hexane extract of *S. grande* exhibited the strongest growth inhibitory effect than the other *S. grande* extracts since it gave the smallest MIC values against each of the tested bacteria, except for *B. cereus*. For *B. cereus*, the 70% MeOH extract exhibited the strongest inhibitory effect with MIC value of 0.156 mg/mL, while the MIC recorded by the hexane extract was 0.625 mg/mL. The results from the MIC measurements also indicated that *B. subtilis*, *B. megaterium* and *B. pumilus* were the most sensitive to the hexane extract of *S. grande*, with MIC values of 0.156, 0.312 and 0.312 mg/mL, respectively.

The literature search could ascertain there were some reports on antimicrobial activity of hexane extracts of different plants against *Bacillus* strains. A previous study on *Salvia tomentosa* Miller showed that its hexane extract significantly inhibited *B. cereus* with MIC value 14.50 mg/mL [25]. In addition, the hexane extract of *Satureja hortensis* showed significant inhibition against *B. megaterium* with MIC value 0.625 mg/mL [26]. A previous study on *Ocimum basilicum* also showed that its hexane extract has an antimicrobial effect against *B. megaterium* with MIC value of 250 μ g/mL [27]. In another study, the hexane extract of *Chlorella vulgaris* was also found to be effective against *B. subtilis* compared to other solvent extracts [28]. Thus, it can be concluded that some of the non-polar compounds present in the hexane extract of *S. grande* have substantially potent antibacterial activity, and may be contributing to a greater proportion of the overall activity of the plant, in comparison to others in the semi to highly polar extracts.

On the other hand, the extracts of *O. javanica* exhibited different antimicrobial effect against *Bacillus* sp. (Figure 2). Unlike *S. grande*, the highly polar extracts of *O. javanica* (MeOH and 70% MeOH) exhibited higher antimicrobial activity compared to the non-polar and semi-polar extracts, i.e the hexane and EtOAc extracts. The MeOH and 70% MeOH extracts exhibited the highest inhibition against *B. cereus* with a similar MIC value of 0.625 mg/mL. Meanwhile, against *B. subtilis*, *B. megaterium* and *B. pumilus*, the MeOH extract was found to be the most active extract, giving MIC values of 0.625, 0.312 and 0.625 mg/mL, respectively. A previous study has also reported the susceptibility of *Bacillus* sp to *Oenanthe javanica*, where the methanol and aqueous extracts of *O. javanica* significantly inhibited the growth of *B. cereus* [29]. Thus, it can be concluded that some of the polar compounds present in the MeOH extract of *O. javanica* have substantially potent antibacterial activity, and may be contributing to a greater proportion of the overall anti-*Bacillus* activity of the plant.

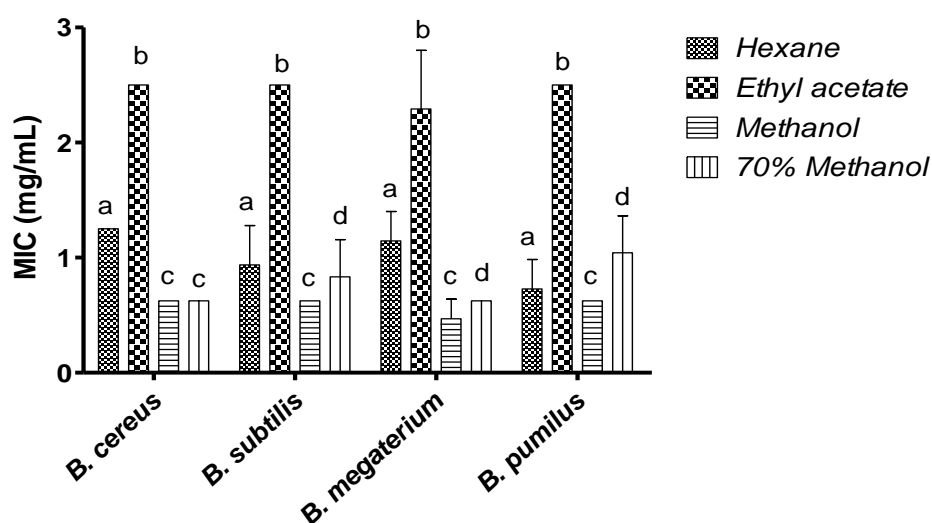


Figure 2. Minimum inhibitory concentration (MIC) of *O. javanica* extracts against selected bacteria. Data shown are means of six biological replicates \pm SD. Different letters indicate significant differences at $p < 0.05$.

3.2. ^1H NMR Spectra of *S. grande* and *O. javanica* Extracts and Metabolite Identification

An overlay of the representative ^1H NMR spectra of the different *S. grande* extracts is shown in Figure 3. Additional spectral data of the extract are provided in Supporting Information (see Figures S1 and S2). Detailed analysis of the ^1H NMR spectral data permitted the identification of compounds as listed in Table 1. The identification was carried out by comparison with Chenomx database and information obtained from previous research [30–34], further supported by *J*-resolve spectra of the extract. Meanwhile, Figure 4 presents the overlay of the ^1H NMR spectra of the different *O. javanica* extracts. Additional spectral data of the extract are also provided in Supporting Information (see Figures S3, S4 and S5). Similarly, detailed analysis of the ^1H NMR spectral data, together with comparison with Chenomx database and literature reports [35–37], as well as *J*-resolve spectra enabled the identification of compounds as listed in Table 2.

Sixteen compounds of *S. grande* extracts could be assigned, comprising 9 primary and 7 secondary metabolites. These compounds included amino acids, carbohydrates, triterpene, triterpenoids, and aromatic acids (Figure 3). In the aliphatic region (δ 0.50–3.00), compounds including alanine, glutamine, acetic acid, pyruvic acid, 3-hydroxybutyric acid, 2,3-butanediol, and β -sitosterol were identified with the signals at δ 1.48 (d, $J = 6.5$ Hz), δ 2.20 (m), δ 2.01 (s), δ 2.39 (s), δ 1.18 (d, $J = 6.5$ Hz), δ 1.12 (d, $J = 4.5$ Hz) and δ 0.88 (d, $J = 8.0$ Hz) and δ 1.05 (s), respectively. In the sugar region (δ 3.0–5.5), fructose and α -glucose were detected at δ 4.29 (d, $J = 8.0$ Hz) and δ 5.20 (d, $J = 3.5$ Hz), respectively. In addition, the presence of choline was detected with the singlet at δ 3.27 (s), whilst the signal at δ 4.70 (s) and δ 4.58 (s) were ascribed to betulin. Meanwhile, the signals at δ 3.21 (dd, $J = 4.0, 3.5$ Hz) and δ 5.37 (m) were ascribed to oleanolic acid and β -caryophyllene, respectively. In the aromatic region (δ 5.5–8.5), the presence of fumaric acid, cinnamic acid and gallic acid were indicated at δ 6.46 (s), δ 7.40–7.44 (m) and δ 6.94 (s), respectively.

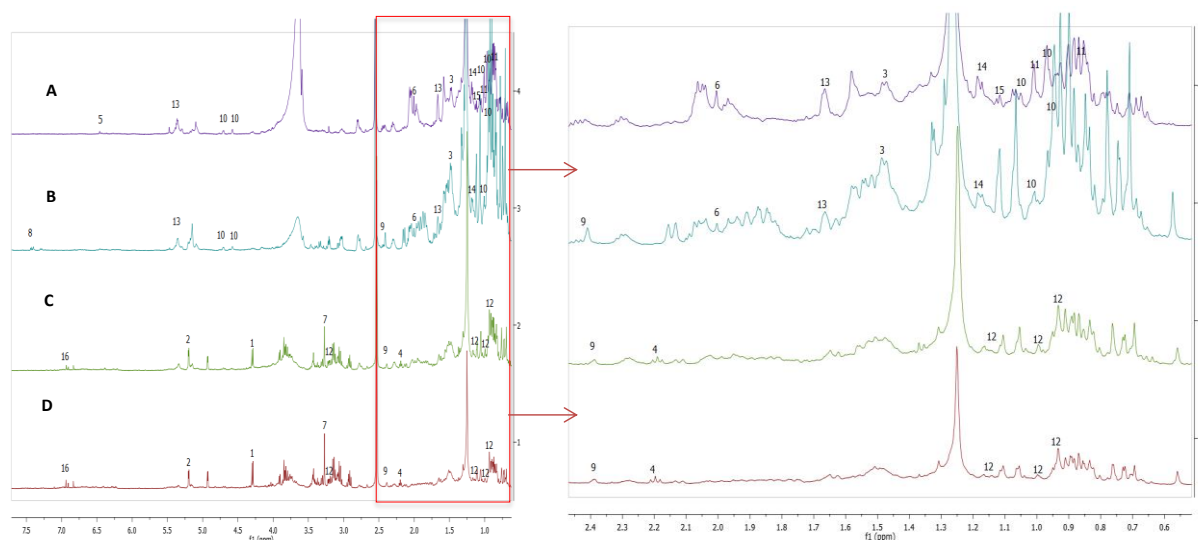


Figure 3. ^1H NMR spectra (δ 0.5 to 7.5 ppm) of hexane (A), EtOAc (B), MeOH (C) and 70% MeOH (D) extracts of *S. grande* leaves

Fructose (1), α - glucose (2), alanine (3), glutamine (4), fumaric acid (5), acetic acid (6), choline (7), cinnamic acid (8), pyruvic acid (9), betulin (10), β -sitosterol (11), oleanolic acid (12), β -caryophyllene (13), 3-hydroxybutyric acid (14), 2,3-butanediol (15) and gallic acid (16)

Table 1. ^1H NMR signals of metabolites identified in *S. grande* extracts

No	Metabolites	^1H NMR signals	Hex.	EtOAc	MeOH	70%MeOH
Primary metabolites						
1	Fructose	δ 4.29 (d, J = 8.0 Hz)	-	-	+	+
2	α - Glucose	δ 5.20 (d, J = 3.5 Hz)	-	-	+	+
3	Alanine	δ 1.48 (d, J = 6.5 Hz)	+	+	-	-
4	Glutamine	δ 2.20 (m)	-	-	+	+
5	Fumaric acid	δ 6.46 (s)	+	-	-	-
6	Acetic acid	δ 2.01 (s)	+	+	-	-
7	Choline	δ 3.27 (s)	-	-	+	+
8	Cinnamic acid	δ 7.40-7.44 (m)	-	+	-	-
9	Pyruvic acid	δ 2.39 (s)	-	+	+	+
Secondary metabolites						
10	Betulin	δ 4.70 (s), δ 4.58 (s) δ 1.01 (s), δ 0.97 (s), δ 0.93 (s)	+	+	-	-
11	β -sitosterol	δ 0.88 (d, J = 8.0 Hz), δ 1.05 (s)	+	-	-	-
12	Oleanolic acid	δ 0.89 (s), 0.91(s), 0.93 (s), 1.00 (s), δ 1.17 (s), δ 3.21 (dd, J = 4.0, 3.5 Hz)	-	-	+	+
13	β -caryophyllene	δ 5.37 (m), δ 1.67 (s)	+	+	-	-
14	3-Hydroxybutyric acid	δ 1.18 (d, J = 6.5 Hz)	+	+	-	-
15	2,3-Butanediol	δ 1.12 (d, J = 4.5 Hz)	+	-	-	-
16	Gallic acid	δ 6.94 (s)	-	-	+	+

Hex.: hexane; EtOAc: Ethyl acetate; MeOH: methanol; 70% MeOH: 70% methanol-30 % Su.

The + and - signs indicate the presence and absence of a particular metabolite in the extract, respectively.

The identified metabolites in *O. javanica* extracts were similarly diverse, comprising 11 primary and 6 secondary metabolites. The compounds identified range from amino acids, fatty acids, carbohydrates, terpenoids, and aromatic acids (Figure 4). Visual inspection of the aliphatic region (δ 0.50–3.00) revealed the presence of glutamine, valine, acetic acid, isoleucine, leucine, and fatty acid with the signals at δ 2.10–2.20 (m), δ 1.03 (d, $J = 6.0$ Hz) and δ 0.95 (d, $J = 6.0$ Hz), δ 1.96 (s), δ 0.81 (t, $J = 6.0$ Hz), δ 0.88 (m), δ 1.29 (m), respectively. Palmitic acid was also detected at δ 2.29 (m) and 1.52 (m). In the sugar region (δ 3.0–5.5), fructose and α -glucose were detected at δ 4.29 (d, $J = 8.0$ Hz) and δ 5.20 (d, $J = 3.5$ Hz), respectively. Choline was detected based on the singlet at δ 3.27, whilst the signal at δ 3.58 (s), δ 3.55 (s) were ascribed to glycine, while betaine was detected by the signals at δ 3.29 (s) and δ 3.85 (s). The signals at δ 4.73 (s) and δ 4.67 (s) were ascribed to lupeol. Moreover, the δ 3.21 (dd, $J = 3.5, 3.5$ Hz) and δ 5.36 (m) were assigned to oleanolic acid. In the aromatic region (δ 5.5–8.0), the presence of maleic acid was detected at δ 6.39 (s), and ellagic acid was detected at δ 7.91 (s), δ 7.55 (s), δ 7.45 (s). Gallic acid was also detected based on the signal at δ 7.08 (s).

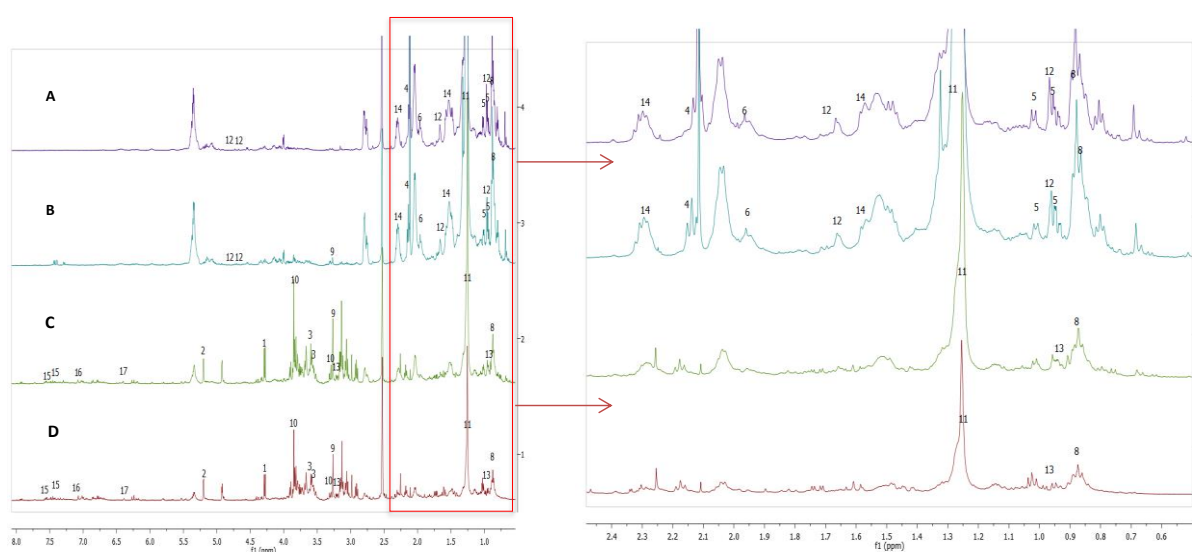


Figure 4. ^1H NMR spectra of hexane (A), EtOAc (B), MeOH (C) and 70% MeOH (D) extracts of *O. javanica* from δ 0.5 to 8.0 ppm

Fructose (1), α - glucose (2), glycine (3), glutamine (4), valine (5), acetic acid (6), isoleucine (7), leucine (8), choline (9), betaine (10), fatty acid (11), lupeol (12), oleanolic acid (13), palmitic acid (14), ellagic acid (15), gallic acid (16) and maleic acid (17)

Table 2. ^1H NMR signals of metabolites identified in *O. javanica* extracts

No	Metabolites	^1H NMR signals	Hex.	EtOAc	MeOH	70% MeOH
Primary metabolites						
1	Fructose	δ 4.29 (d, $J = 8.0$ Hz)	-	-	+	+
2	α - Glucose	δ 5.20 (d, $J = 3.5$ Hz)	-	-	+	+
3	Glycine	δ 3.58 (s), δ 3.55 (s)	-	-	+	+
4	Glutamine	δ 2.10–2.20 (m)	+	+	-	-
5	Valine	δ 1.03 (d, $J = 6.0$ Hz), δ 0.95 (d, $J = 6.0$ Hz)	+	+	-	-
6	Acetic acid	δ 1.96 (s)	+	+	-	-
7	Isoleucine	δ 0.81 (t, $J = 6.0$ Hz)	+	+	-	-
8	Leucine	δ 0.88 (m)	+	+	+	+
9	Choline	δ 3.27 (s)	-	+	+	+
10	Betaine	δ 3.29 (s), δ 3.85 (s)	-	-	+	+
11	Fatty acid	δ 1.29 (m)	+	+	+	+

Table 2 continued..

Secondary metabolites						
12	Lupeol	δ 4.73 (s), δ 4.67 (s), δ 1.67 (s), δ 1.03 (s), δ 0.90 (s)	+	+	+	+
13	Oleanolic acid	δ 0.89 (s), 0.91 (s), δ 1.12 (s), δ 3.21 (dd, J = 3.5, 3.5 Hz)	-	-	+	+
14	Palmitic acid	2.29 (m), 1.52 (m)	+	+	-	-
15	Ellagic acid	δ 7.91 (s), δ 7.55 (s), δ 7.45 (s)	-	-	+	+
16	Gallic acid	δ 7.08 (s)	-	-	+	+
17	Maleic acid	δ 6.39 (s)	-	-	+	+

3.3. Differentiation of *S. grande* and *O. javanica* Extracts

The variation in metabolite contents of *S. grande* and *O. javanica* samples extracted with different solvents were evaluated using multivariate data analysis (MvDA). The principal component analysis (PCA) was applied to understand the clustering features of the four different solvents samples and the metabolites that contributed to the variability. The PCA score plot showed the clustering of the samples, and loading plot gave the variable contributions to the sample differences.

The processed ^1H NMR data was subjected to PCA and the results are shown in Figure 5. As shown in the PCA score plot, the first principal component (PC1) accounted for 42.4% of the variation in the data, whereas PC2 was able to explain 35.4% of the variation (Figure 5A). The score plot (Figure 5A) revealed that the different *S. grande* extracts were separated into three clusters. The MeOH and 70% MeOH extracts were clustered together, having negative PC1 and positive PC2 scores respectively; while the hexane and ethyl acetate extracts were well separated, having positive and negative PC1 scores, respectively. These results indicated that the MeOH and 70% MeOH extracts have similar metabolite composition but may differ slightly in their concentrations. The loading plot (Figure 5B) showed that the metabolites contributing to the separation of MeOH and 70% MeOH extracts from the other extracts were fructose, glucose, glutamine, choline, pyruvic acid, and gallic acid. Meanwhile, the hexane extract was separated by having higher contents of alanine, betulin, β -sitosterol, acetic acid, 3-hydroxybutyric acid and β -caryophyllene. Cinnamic acid, fumaric acid and 2,3-butanediol were found to be responsible for the separation of the EtOAc extract from the rest of the groups. The distribution of these compounds could possibly be due to the polarity of these compounds, i.e their hydrophobic and hydrophilic characteristics.

The PCA results for *O. javanica* extracts are presented in Figure 6. Three clearly separated clusters can be seen in the PCA score plot of the *O. javanica* extracts (Figure 6A). An eigenvalue of about 92.7% was described by the two PCs, accounting for a variance of 81.6 % by PC1 and 11.1 % by PC2 (Figure 6A). The polar (MeOH, and 70% MeOH) and non-polar (hexane) extract samples were clearly separated by PC1. The MeOH, and 70% MeOH clusters were projected to the negative side of PC1, whereas the non-polar and semi-polar extracts (hexane and ethyl acetate) were clustered together on the positive PC1 side. Meanwhile, the MeOH and 70% MeOH extracts were separated by PC2, having positive and negative PC2 score, respectively.

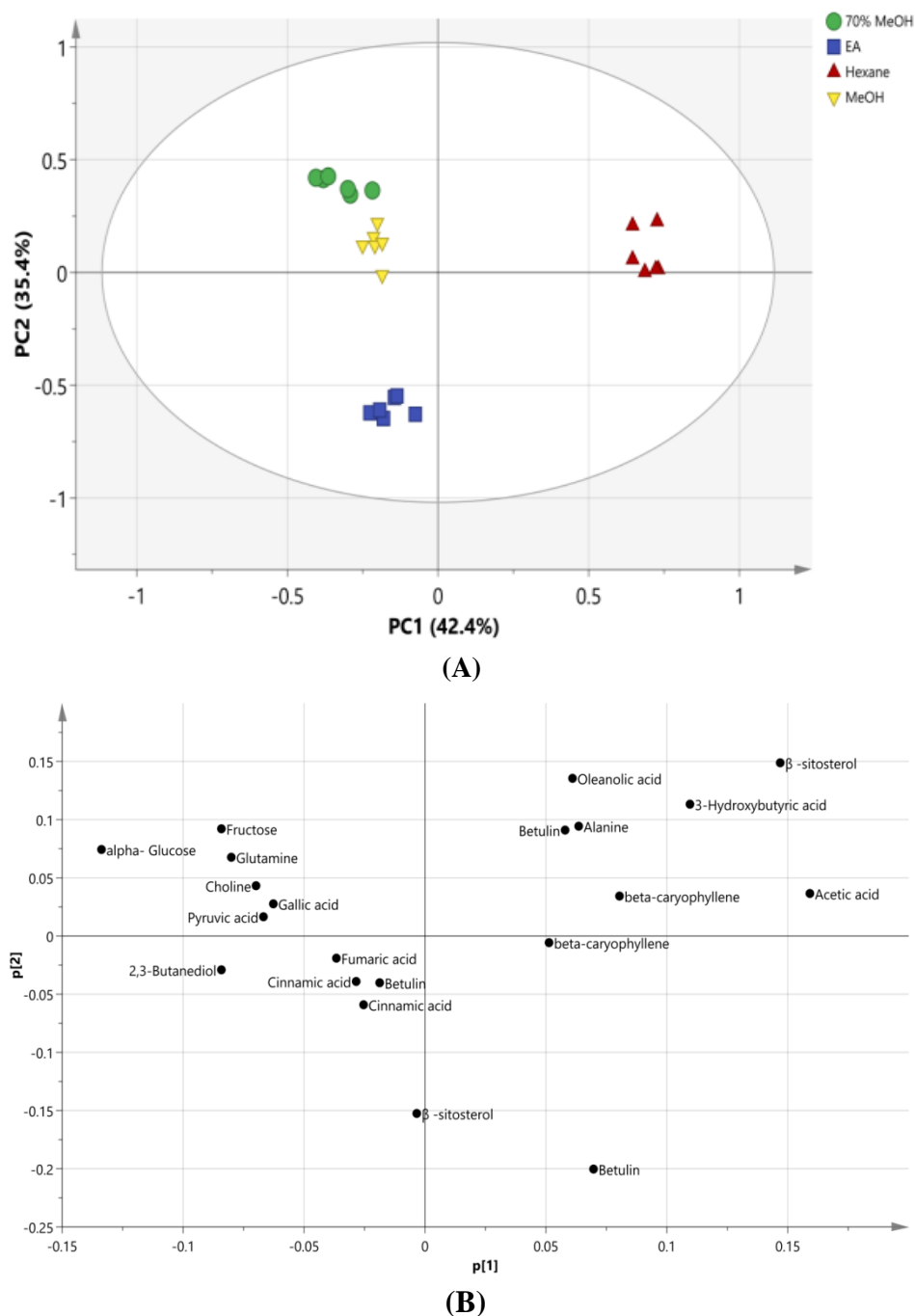
Anti-Bacillus activity of *S. grande* and *O. javanica*

Figure 5. The PCA score (A) and loading (B) plots of the different solvent extracts of *S. grande*

The loading plot (Figure 6B) showed that the hexane and EtOAc extracted mostly similar metabolites. The EtOAc and hexane extracts were richer in the contents of alanine, isoleucine, leucine, valine, glutamine, acetic acid, fatty acid, palmitic acid and oleanolic acid, while the MeOH and 70% MeOH extracts contained higher levels of choline, ellagic acid, gallic acid and maleic acid. In addition, fructose, glucose, glycine, and betaine also contributed to the separation of the two polar extracts from the two less polar extracts.

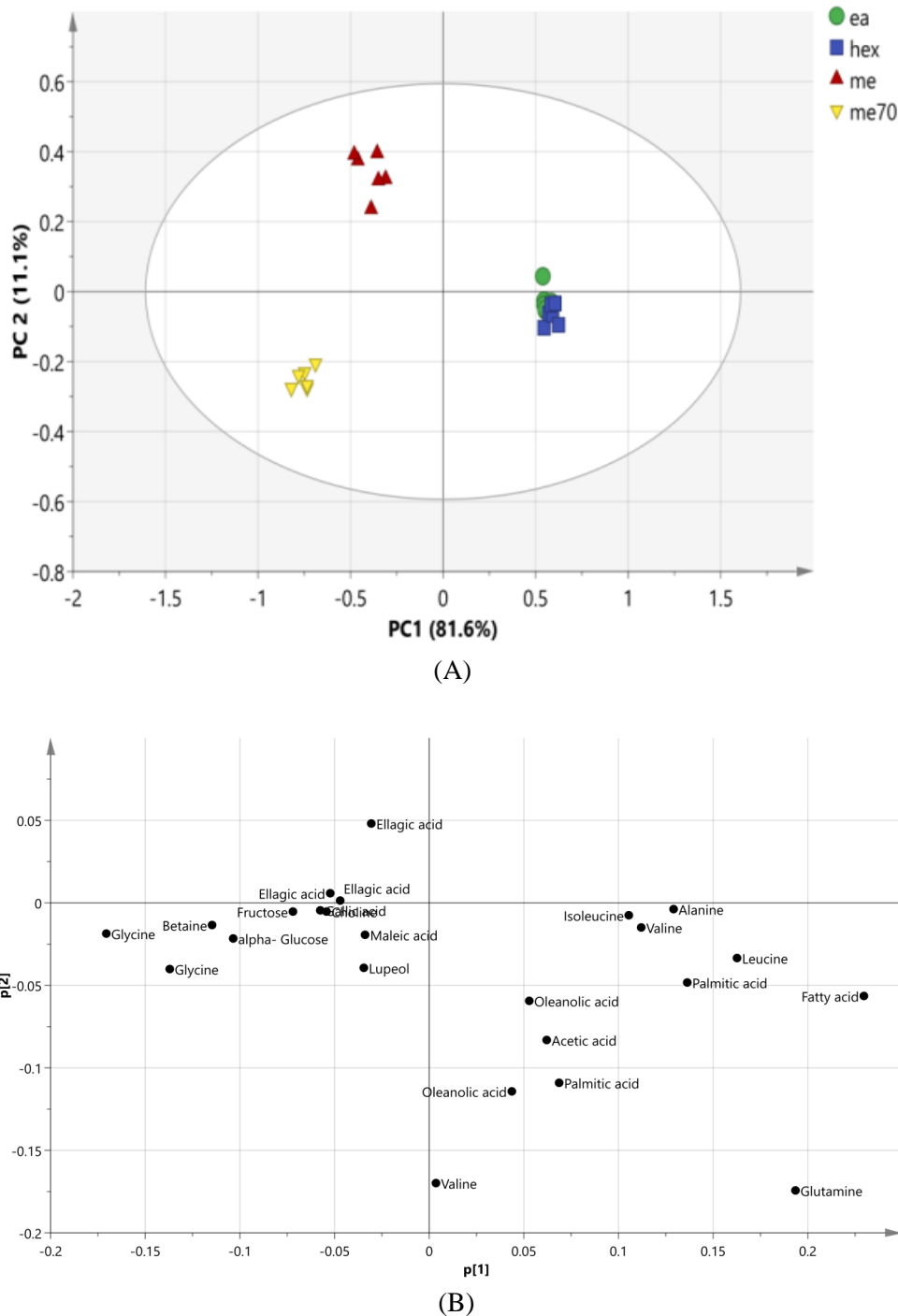


Figure 6. The PCA score (A) and loading (B) plots of different extracts of *O. javanica*

The Partial Least Square (PLS) model was built to observe the relationship between the identified metabolites in extracts with their respective bioactivities against the selected *Bacillus* strains. The NMR signals represent the X variables and the 1/MIC values of the antimicrobial activities against the different bacteria were added as the Y variables. The PLS model was validated by internal cross validation by means of R^2 and Q^2 cumulative. The R^2 shows the fitness of model, whereas Q^2 provides the predictive quality of the model. In general, the closer the R^2 and Q^2 values are to 1, the better is the performance of the model in relation to goodness of fit along with the predictive quality [38]. Autofit of the PLS model of *S. grande* extracts revealed that the model can be explained by 4 PCs with goodness of fit R^2Y cumulative value of 0.904, and good predictive ability with Q^2 cumulative value of 0.564. The validation of the PLS model was further confirmed by

permutation tests. The cross validation and permutation tests confirmed that there were no over-fitting of the PLS model (Supporting Information, Figures S6 to S9). Autofit of the PLS model for *O. javanica* extracts revealed that the model can be explained by 3 PCs with goodness of fit R^2Y cumulative value of 0.967, and good predictive ability with Q^2 cumulative value of 0.708. The cross validation and permutation tests confirmed that there were no over-fitting of the PLS model (Supporting Information, Figures S10 to S13). The Y-intercepts of Q^2 and R^2 were less than 0.5 and 0.05, respectively, which also proved that PLS model was acceptable and did not reveal over-fitting [39]. Consequently, the PLS model satisfied the criteria of a good performing model and the PLS biplot (Figure 8) was also in good agreement with the results of PCA.

From the PLS biplot (Figure 7), the 70% MeOH extract of *S. grande* was closer to the antimicrobial activity against *B. cereus*, while the hexane extract was correlated more towards the antimicrobial activity against the other *Bacillus* strains, as previously shown by the bioactivity results. It can be observed that among the compounds of the hexane extract contributing to the antimicrobial activity to the *Bacillus* strains were betulin, β -sitosterol, 3-hydroxybutyric acid, β -caryophyllene, acetic acid, and alanine. Some of these compounds have been previously reported to be antimicrobial against many type of bacteria including *Bacillus* species. Betulin have been reported to show strong antimicrobial activity against *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* [40]. Likewise, β -sitosterol was also reported to show antibacterial activity against many microorganisms, including *B. subtilis*, *S. aureus*, *Streptococci pyrogene*, *Klebsiella pneumonia* and *Shigella dysenteriae* [41]. Additionally, 3-hydroxybutyric acid was also reported to exhibit strong antimicrobial activity against *S. aureus*, *K. pneumoniae* and *C. albicans* [42], while β -caryophyllene was shown to be active against *B. cereus*, *B. subtilis*, *K. pneumonia*, *Pseudomonas aeruginosa* and *E. coli* [43].

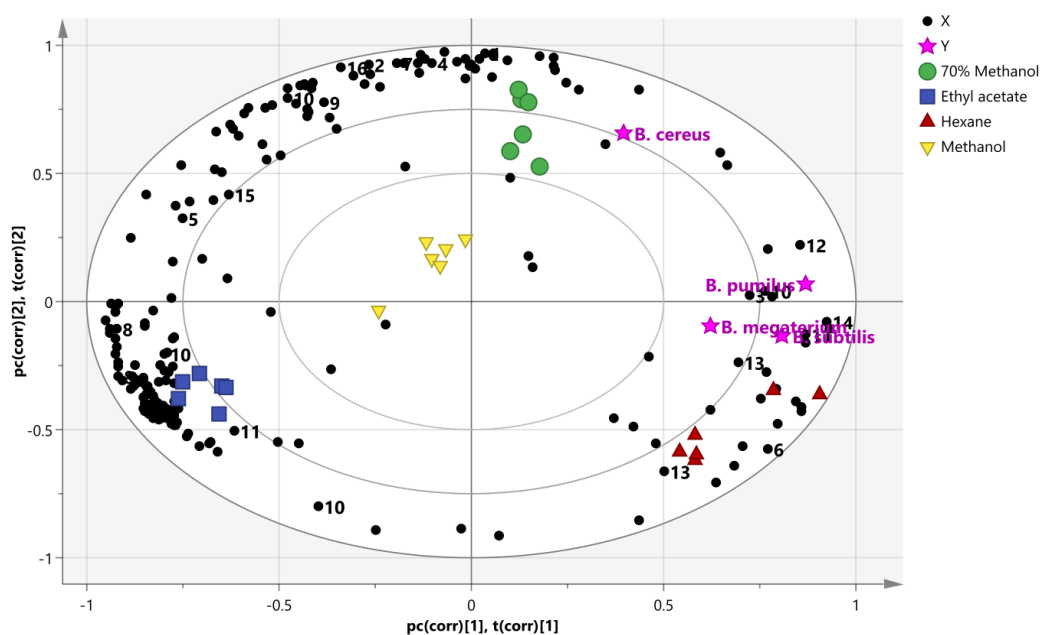


Figure 7. The PLS biplot describing the correlation among the phytochemical constituents of different *S. grande* extracts and their antimicrobial activities against selected *Bacillus* species fructose (1), α - glucose (2), alanine (3), glutamine (4), fumaric acid (5), acetic acid (6), choline (7), cinnamic acid (8), pyruvic acid (9), betulin (10), β -sitosterol (11), oleanolic acid (12), β -caryophyllene (13), 3-hydroxybutyric acid (14), 2,3-butanediol (15) and gallic acid (16).

From the PLS biplot (Figure 8), the methanol extract of *O. javanica* was strongly correlated to the antimicrobial activity against all the *Bacillus* strains, as previously shown by the bioactivity results. Thus, the polar metabolites in this extract are therefore concluded to be contributing to the potent anti-*Bacillus* activity of *O. javanica*. These metabolites included ellagic acid, maleic acid, choline, and gallic acid. The positive relationship between antibacterial activity and these compounds have also been reported previously. Ellagic acid has been reported to show antibacterial activity

towards *S. epidermidis*, *B. cereus*, *K. pneumonia* and *Salmonella typhi* [44]. Maleic acid demonstrated activity against *B. subtilis*, *E. coli*, and *Streptococcus suis* [45]. Similarly, gallic acid exhibited strong effect towards *E. coli*, *P. aeruginosa*, *S. aureus*, and *Listeria monocytogenes* [46].

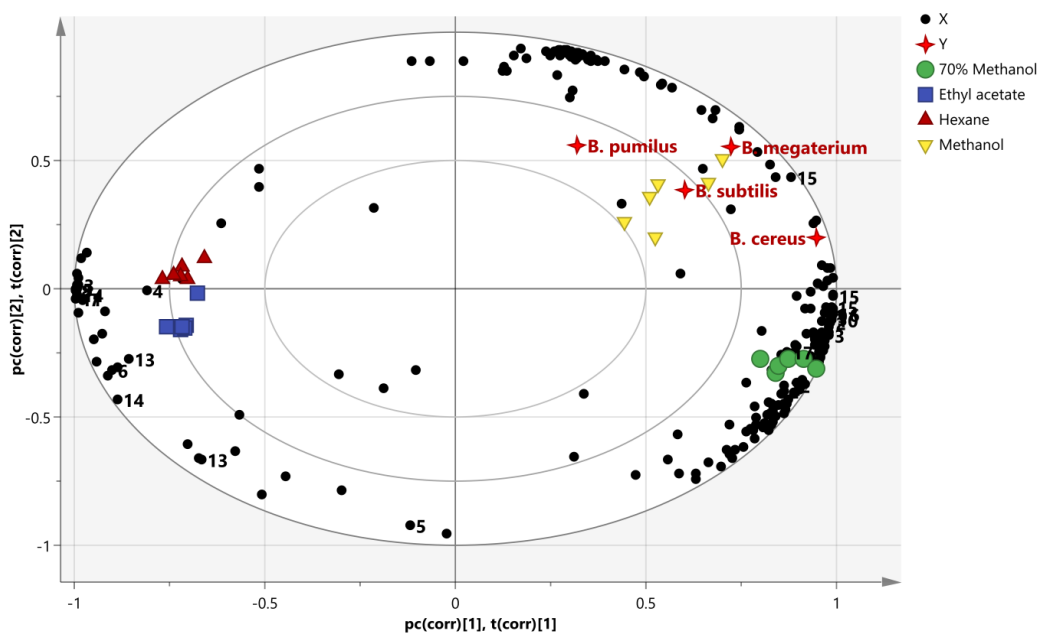


Figure 8. The PLS biplot describing the correlation among the phytochemical constituents of different *O. javanica* extracts and their antimicrobial activities against selected *Bacillus* species fructose (1), α -glucose (2), glycine (3), glutamine (4), valine (5), acetic acid (6), isoleucine (7), leucine (8), choline (9), betaine (10), fatty acid (11), lupeol (12), oleanolic acid (13), palmitic acid (14), ellagic acid (15), gallic acid (16) and maleic acid (17).

4. Conclusion

In the present study, the antimicrobial activity and metabolite variation were evaluated for different solvent extracts of *S. grande* and *O. javanica*. Hexane extract of *S. grande* and methanol extract of *O. javanica* were found to be very effective against anti-*Bacillus* compared to the other solvent extracts. In total, 16 compounds from *S. grande* and 17 compounds were identified in *O. javanica* leaf extracts. Betulin, β -sitosterol, β -caryophyllene, acetic acid, alanine and 3-hydroxybutyric acid were strongly correlated to the antimicrobial activity of *S. grande* hexane extract against *B. cereus*, *B. subtilis*, *B. megaterium* and *B. pumilus*. Whereas, choline, ellagic acid, gallic acid and maleic acid were identified as the potential contributors to the antimicrobial activity of *O. javanica* methanolic extract against the four *Bacillus sp.* NMR-based metabolomics proved to be a useful tool to differentiate between metabolite profiles of extracts obtained using solvents of varying polarity. Further, correlations between metabolites and bioactivity, visualized using PLS models of the extracts, were useful in predicting the candidate bioactive constituents of the respective plants. In conclusion, results from this metabolomics study have revealed that *S. grande* and *O. javanica* have useful antibacterial properties, and merit further in depth chemical and pharmacological investigations into their potential development into useful applications in managing and control of *Bacillus* species.

Acknowledgments

Khaled Bashir Abdusalam acknowledges University of Gharyan, Libya, for the award of PhD scholarship.

Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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