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^{1}H-NMR Based Chemometric Approach for Phytochemicals Variation Study on *Erythrina fusca* Flower Extracts and Correlation with Their Antibacterial Activity

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Erythrina fusca Lour., (Fabaceae) is a flowering plant locally known as "Chengkering", and has been traditionally used to treat certain symptoms associated with bacterial infections. However, limited study is available to support this traditional use, particularly the activity of the flower part against bacterial strains that commonly cause infection on human skin. The present work reports on the *in vitro* antibacterial activity of *E. fusca* flower's extracts [hexane, dichloromethane (DCM), and methanol (MeOH)] against skin infectious bacterial strains namely *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Enterobacter cloacae*, and *Enterobacter aerogenes* evaluated through well diffusion method. The variation of the phytochemicals in the extracts and their correlation with the antibacterial activity were determined through proton nuclear magnetic resonance (^{1}H-NMR)-based chemometric analysis. The hexane extract was active against *S. aureus* and *S. haemolyticus* strains with 14.00 ± 0.00 and 9.67 ± 0.33 mm zone of inhibition (ZOI), respectively. MeOH extract showed activity against *E. cloacae* (8.33 ± 0.33 mm) and *E. aerogenes* (8.17 ± 0.17 mm) strains. While DCM extract was only active against *S. aureus* (9.67 ± 0.33 mm). The NMR analysis on the extracts lead to the identification of 75 phytochemicals mainly from the alkaloids, flavonoids, pterocarpans, terpenes, saponins, sterols, and phenols classes of compounds. The chemometric analysis revealed the activity against *S. haemolyticus* was correlated with the flavonoid 5,3′-dihydroxy-4′-methoxy-5′-(3-methyl-1,3-butadienyl)-2″,2″-dimethylpyrano-[5,6:6,7]-isoflavanone (**47**), and the pterocarpans sandwicensin (**58**) and neorautenol (**52**). While the activity on *E. cloacae*, and *E. aerogenes* was collectively contributed by the alkaloids erysotrine *N*-oxide (**11**), 10-hydroxy-11-oxoerysotrine (**18**), and magnoflorine (**33**). This suggests that the therapeutic value reported for the plant could be synergically contributed by the above-mentioned phytochemicals. The finding provides evidence to support the plant's traditional use and could be the source of antibacterial agents for further studies.

Keywords: Traditional use; alkaloid; flavonoid; chemometric; *Erythrina*

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The rise of antibacterial resistance in human pathogens has led to an increase in the infection's incidence of infection in hospitals and communities [1]. Though antimicrobial drugs are widely accessible, microbial infections continue to be a significant cause of illness and death globally [2]. Approximately 62 million cases of infectious diseases are reported annually, with an estimated 17 million deaths attributed to these diseases and 70% of deaths in this population are caused by pathogenic bacteria [3, 4]. Thus, there is a need to search for natural chemical agents as an alternative for developing new drugs, including those derived from plants. Plants that possess antimicrobial compounds and wound-healing properties are often used as a source of plant-based remedies for skin infections [5, 6]. Studies have shown that some plant extracts inhibit the growth of microorganisms that cause infection in the skin area [7-9].

Erythrina fusca Lour. is a flowering plant belonging to the Fabaceae family. It is locally known as "Chengkering" or "Dedap", and has been traditionally used for remedies. In Thailand, the bark and leaves of the plant are consumed orally to treat inflammation [10]. Meanwhile, the scraped inner bark was applied for dressing fresh wounds by the Indonesian people [11]. *E. fusca* was also recorded for treating certain symptoms associated with bacterial infections, such as

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itching infected wounds and fungal dermatosis [11]. Out of the seven *Erythrina* species available in Malaysia, only *E. fusca* has been recognized for its traditional use in treating wounds [12]. Most of the reported infectious-related biological activities of the plant were attributed to its erythrina alkaloids, flavonoids, pterocarpans, and terpene components [13-18]. A recent antibacterial study on the plant's aqueous leaf extract against *Porphyromonas gingivalis* gave a stronger bactericidal effect compared to the standard used, chlorhexidine [19]. However, there is no report on the antibacterial activity of the *E. fusca*'s flowers on the bacterial strains that commonly cause infections to human skin. Thus, this warrants a further antibacterial study to be conducted on the flower extracts and identification of the active phyto-chemicals through direct spectral measurement such as nuclear magnetic resonance (NMR) spectroscopy [20].

NMR is a non-destructive, sensitive, and most widely used spectroscopy technique due to its rapid and simultaneous detection of a large number of phytochemicals [21-25]. Furthermore, the correlation of the active phytochemicals in the *E. fusca* flower extracts with the antibacterial activities can be determined using a chemometric approach employing multivariate data analysis (MVDA). This paper reports on the *in vitro* antibacterial activity of the hexane, dichloromethane, and methanolic *E. fusca* flower extracts against common skin infectious bacterial strains namely *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Enterobacter cloacae*, and *Enterobacter aerogenes*. The phytochemicals variation in the extracts and the proton NMR characteristics of the different classes of identified phytochemicals in *E. fusca* are also included. The findings support the traditional use of the plant and suggest that it may serve as a potential source of antibacterial agents in future studies.

EXPERIMENTAL

Chemicals and Reagents

Organic solvents of n-hexane, dichloromethane (DCM), chloroform ($CHCl_3$), dimethylsulfoxide (DMSO), and methanol (MeOH) of analytical grade were supplied by Elite Advanced Materials Sdn Bhd (Selangor, Malaysia). Deuterated solvent DMSO-d_6 and the standard 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4) were purchased from Merck KGaA, (Darmstadt, Germany) and used for NMR analysis.

Extracts Preparation

The flowers of *E. fusca* (750 g) were collected from Hutan Simpan Bangi, Malaysia in August 2018 and botanically authenticated by a certified botanist, Mr. Ahmad Zainudin Ibrahim. The voucher specimen HTBP 5273 was deposited at the herbarium of Taman Botani Putrajaya. The sample was cleaned with laboratory wipes and shade-dried for 2-3 weeks. The dried materials were cut into small pieces and pulverized using an electric grinder (Fritsch/Pulverisette, Idar-Oberstein, Germany). The powdered sample (60.0 g) was macerated with a ratio of 1:10 (powdered sample: MeOH) for 72 hours at room temperature. After three days, the extract was filtered through Whatmann No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator (R-125 BUCHI, Flawil, Switzerland) resulted in 0.64 g dried extract.

Solid-Liquid Extraction (SLE): Trituration Technique

The flower crude extract (0.64 g) was then triturated with three different solvents in increasing polarity manner: n-hexane, DCM, and MeOH, and each extract was concentrated using vacuum evaporator yielded three triturated extracts: flower-hexane (FH, 65 mg), flower-DCM (FD, 110 mg), and flower-MeOH (FM, 664 mg). The extracts were then stored in 4°C prior to analyses.

In Vitro Antibacterial Activity

Bacterial Strains

The evaluation of the antibacterial activity of the extracts followed a standard protocol [26] where four bacterial strains were employed: *Staphylococcus aureus* (ATCC25923), *Staphylococcus haemolyticus* (ATCC 29970), *Enterobacter aerogenes* (ATCC13048) and *Enterobacter cloacae* (ATCC35030). They were all acquired from Dr. Normala from Bioassay Laboratory, AuRIns in 2020. The test bacteria inocula were prepared using nutrient broth (NB) and stored at 4°C prior to subsequent experiments.

Samples Preparation

Fifty mg of each extract (FH, FD, and FM) was weighed into a sterile Eppendorf tube and was fully dissolved in 1 mL of DMSO (100%). Then, 30 µL of each solution was used in the antibacterial assay.

Antibacterial Assay

The evaluation of the antibacterial activity was conducted through the agar-well diffusion method [27]. The experiment was conducted in triplicate (n=3) for each bacterial strain. Bacterial culture media Mueller-Hinton agar was used as a culture medium in the preparation of agar plates while nutrient broth (NB) was used in the preparation of bacterial inoculum and adjusted to 0.5 McFarland standard. Bacteria obtained from CDDR and AuRIns' laboratories underwent sub-culturing in sterilized NB overnight, serving as the source for the inoculum. The McFarland standardized inoculum (1 x 10^8 CFU/mL) was prepared using a spectrophotometric method where the optical density (OD) of 0.08 – 0.1 at 600 nm wavelength was adjusted for the bacterial suspension

in sterilized NB. A volume of 100 µL of the standardized inoculum was applied to the agar plate surface using a sterile cotton swab. Then, 30 µL of the sample was pipetted into each well of 6 mm diameter punched in the agar plates using a sterile cork borer. Ciprofloxacin (CIP30) 30 µg/well was used as a positive control while DMSO 30 µL/well was used as the negative control. The agar plates were incubated for 24 hours at 37 °C. The diameter, zone of inhibition (ZOI) was measured in mm after 24-hour period had elapsed and recorded for the samples and ciprofloxacin.

Determination of Minimal Inhibitory Concentration (MIC)

The determination of minimum inhibitory concentration (MIC) for the samples against *S. aureus* and *S. haemolyticus* followed precisely the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) protocol [28]. The samples and bacteria were examined according to their positive results in the antibacterial assay. Ciprofloxacin was as used a positive control and DMSO as a negative control. Microdilution was performed in triplicates (n=3) for each bacterial species. In a 96-well round-bottom microtiter plate, each bacterial culture was arranged to include a vertical row for broth sterility control, three vertical rows for 50 mg/mL extract sterility control, one vertical row for growth control, one vertical row for antibiotic control, and finally, three vertical rows for 50 mg/mL of each test sample. All eight wells in a vertical row were filled with 100 µl Mueller Hinton broth. Subsequently, the mixture in the first well of each vertical row was mixed thoroughly. Then, a separate and sterile pipette was used to transfer 100 µL of the mixture in the first well into the second well (2 – 2) and mixed thoroughly. Again, 100 µL of the mixture was transferred from the second well into the third well (2 – 3) and mixed thoroughly. The serial dilution was continued to the eighth well (2 – 8). Subsequently, 100 µL was removed from the eighth well. The final concentration of antibiotics and the sample in each well was one-half of the original concentration. Following this, 5 µL of diluted bacterial suspension (1.5×10^6 cell/ mL) was added to all wells (excluding the broth sterility and sample sterility control column) and mixed thoroughly with a micropipette. After overnight incubation at 37 °C, the colour change was observed. The lowest concentration prior to colour change was considered the MIC.

Statistical Analysis of Variance (ANOVA)

Data were analysed using One-way analysis in variance (ANOVA) using Microsoft Excel and presented as "mean ± standard deviation'. Groups were compared against each other. F values with $P < 0.05$ were considered significant.

Proton Nuclear Magnetic Resonance (NMR) Analysis

Sample Preparation

The ^{1}H-NMR analysis was conducted with slight modifications from a previous report [29]. Each extract (20.0 mg) was accurately weighed in 2.0 mL Eppendorf tubes and dissolved in 600 µL of DMSO-d_6 containing 2.90 mM of TSP-d_4 as a standard. This was replicated five times (n=5) for each extract. The mixture was vortexed for 10 min, followed by ultrasonication for an additional 10 min at room temperature. Next, the mixture was centrifuged at 13000 rpm for 10 min to obtain a clear supernatant and 0.6 mL of the supernatant was transferred to a 5 mm NMR tube and labeled accordingly to perform NMR analysis immediately using a predetermined setting for all the measurements.

Sample Measurement

A Fourier Transform Nuclear Magnetic Resonance (FT-NMR) 600 MHz with Cryoprobe (Bruker, USA), runs at a frequency of 600 MHz at room temperature was used in this analysis. A pulse width and deuterated solvent DMSO-d_6 were selected as the internal lock for each sample. The 1D-NMR Nuclear Over Hauser Effect with GPPR pulse sequence (NOESYGPPR) experiment was set to 64 scans and 2 prior dummy scans of 4 k points with a spectral width of 20.5488 ppm, a receiver gain of 181, and an acquisition time of 4.9480 s. For all samples, the FIDs were Fourier transformed with line broadening (LB) of 0.4 Hz. The data were acquired automatically under the control of ICON-NMR (Bruker BioSpin, Rheinstetten, Germany), requiring about 10.11 min acquisition time. The resulting spectra were manually phased, baseline corrected, and calibrated to TSP at 0.0 ppm.

For two-dimensional *J*-resolved ^{1}H-NMR analysis, the spectra were obtained from spectral widths of 5.208 kHz in F2 (chemical shift axis) and 50 Hz in F1 (spin-spin coupling constant axis) of 16 k data points using 8 scans for each increment. The total acquisition time was 14.52 min, including a 1.0 s relaxation delay. In F1, the datasets were zero-filled to 512 points and a sine bell-shaped function to double complex Fourier transformation was applied. The results of spectra titled along the rows by 45°, symmetrized about the central line along F2. The baseline was manually corrected and calibrated to the internal standard (TSP = 0.0 ppm). Data were exported as the 1D projection (F2 axis) of the 2D *J*-resolved spectra.

^{1}H NMR Spectra Processing

Chenomx software (v.8.2, Alberta, Canada) was implemented to perform the processing of the ^{1}H-

NMR spectra. All the spectra were automatically binned to ASCII files with the same parameters: width (δ 0.04) between of 0.5- 10.0 ppm. The chemical shift ranges of δ 2.40–2.60 and δ 3.25–3.55, which corresponded to DMSO signals were excluded and the total variables of the 243 bins were generated for each ^{1}H-NMR spectrum.

Multivariate Data Analysis (MVDA)

The Soft Independent Modelling by Class Analogy (SIMCA)-P program (v.14.0, Umetrics, Umeå, Sweden) was used to perform the MVDA on the normalized NMR dataset that had been binned. The principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) method were conducted with the Pareto scaling technique to visualize and discriminate the samples according to their phyto-chemicals, thus providing a general idea of the data-set structure. Both were also used to observe possible outliers within the model. The partial least square (PLS) analysis was performed with the Pareto scaling technique to identify the phytochemicals that significantly contribute to the intergroup differentiation. The NMR chemical shifts of samples were set as the X variables, while the antibacterial data (zone inhibition diameter) was input as the Y variables. Validation of the PLS model was performed by regression test, as considered the goodness of fit and predictability values more than 0.5 to be a valid model.

RESULTS AND DISCUSSION

Antibacterial Activity

Based on the results shown in Table 1, it can be seen that each extract has selectivity against different tested bacterial strains with inhibition zones ranging from 8 to 14 mm. The extracts, nevertheless, mostly showed activity against the *S. aureus* strain, where the flower-hexane (FH) extract demonstrated the highest inhibitory effect (14.00 ± 0.00 mm). This extract is also active against *S. haemolyticus* resulting inhibition diameter of 9.67 ± 0.33 mm. However, only the flower-methanol (FM) extract displayed significant antibacterial activity against both Gram-positive strains (*E. aerogenes* and *E. cloacae*), with a close ZOI of 8.33 ± 0.33 and 8.17 ± 0.17, respectively. The minimum inhibitory concentration (MIC) of the active extracts was 50 mg/mL which was less potent than the standard antibiotic (CIP30) used, in which the bacteria were highly susceptible at 0.23 µg/mL. The variability of the antibacterial activity of the extracts can be attributed to their different types of phytochemicals extracted by different polarities of solvents [6]. This obeys the principle of "like dissolves like" which implies that polar solvents dissolve polar molecules effectively, while non-polar solvents dissolve non-polar molecules [30, 31].

Phytochemicals Profiling and Identification in *E. fusca* Flower Extracts Using Proton NMR-based Analysis

Previous studies have reported a number of phyto-chemicals from *E. fusca* including 12 alkaloids, 21 flavonoids, and 10 pterocarpans [32-34]. However, there are still limited data available on the phyto-chemical profile of the flower part extracted using different solvents. In this study, ^{1}H-NMR-based analysis was conducted to profile and identify the phytochemicals in the FH, FD, and FM extracts of *E. fusca*'s leaves. Assignment of peak signals in the ^{1}H-NMR spectra was based on previously reported literature [35-75] and by comparison with standard online databases, such as the Reaxys web at https:// www.reaxys.com/, Dictionary of Natural Products (DNP) at https://dnp.chemnetbase.com/, PubChem at https://pubchem.ncbi.nlm.nih.gov/, Simulate and Predict NMR Spectra database at https://www. nmrdb. org/, and ChemDraw.

Table 1. Inhibition zone of *E. fusca* flower extracts against common bacterial skin infection strains.

	FH	FD	FM	CIP30
[a]***S. aureus* ATCC 25923**	14.00 ± 0.00	9.67 ± 0.33	n.z.	40.00 ± 0.00
[a]***S. haemolyticus* ATCC 29970**	9.67 ± 0.33	n.z.	n.z.	45.00 ± 0.00
[b]***E. aerogenes* ATCC 13048**	n.z.	n.z.	8.33 ± 0.33	32.00 ± 0.00
[b]***E. cloacae* ATCC 35030**	n.z.	n.z.	8.17 ± 0.17	30.00 ± 0.00

[a]Gram-positive strain; [b]Gram-negative strain; FH, flower-hexane; FD, flower-DCM; FM, flower-MeOH; ±, Std. variation value; n.z., no inhibition zone.

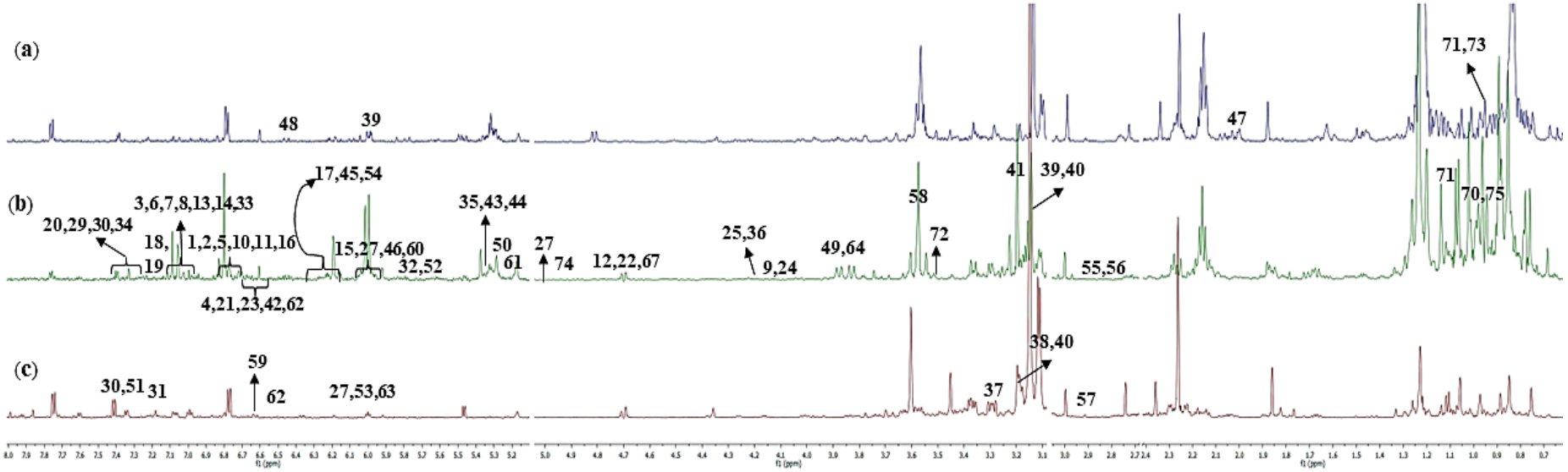

Figure 1. a) Hexane extract (FH); **b**) DCM extract (FD); **c**) MeOH extract (FM). Identified phytochemicals: **1**, Erysodine; **2**, Erysovine; **3**, Erysopine; **4**, Erysoline; **5**, Erysotrine; **6**, 11*α*-Hydroxyerythravine; **7**, 11*β*-Hydroxyerysotrine; **8**, 11*α*-Hydroxyerysotrine; **9**, Erythristemine; **10**, Erysovine *N*-oxide; **11**, Erysotrine *N*-oxide; **12**, 11*β*-Hydroxyerysotramidine; **13**, 11*β*-Methoxyerythraline; **14**, Erythrinine; **15**, 8-Oxoerythraline; **16**, 11-Methoxyerythratine; **17**, Erytharbine; **18**, 10-Hydroxy-11-oxoerysotrine; **19**, Erytharborine; **20**, Sodium Erysovine 15-*O*-sulfate; **21**, Erysosalvine; **22**, 11-Hydroxyerysosalvine; **23**, Erysotine; **24**, Erythratidine; **25**, Epierythratidine; **26**, 11*β*-Hydroxyerythratidine *N*-oxide; **27**, Erysotinone; **28**, 11-Hydroxyerysotinone; **29**, 10,11-Dioxoerythratine; **30**, 10,11-Dioxoepierythratidine; **31**, Hypaphorine; **32**, Erythbidin; **33**, Magnoflorine; **34**, 5,4′-Dihydroxy-8-(3,3-dimethylally)-2″-methoxyisopropylfurano- [4,5:6,7]-isoflavone; **35**, 5,4′-Dihydroxy-8-(3,3-dimethylally)-2″-hydroxymethyl-2″-methylpyrano-[5,6:6,7]-isoflavone; **36**, Indicanine; **37**, Warangalone; **38**, Lupinifolin; **39**, Citflavanone; **40**, Erythrisenegalone; **41**, Lonchocarpol A; **42**, Liquiritigenin; **43**, Fuscaflavanone B; **44**, Sigmoidin H; **45**, Eryzerin D; **46**, Atalantoflavone; **47**, 5,3′-dihydroxy-4′-methoxy-5′-(3-methyl-1,3-butadienyl)-2″,2″-dimethylpyrano-[5,6:6,7]-isoflavanone; **48**, 5,3′-dihydroxy-5′-(3-hydroxy-3-methyl-1-butenyl)-4′-methoxy-2″,2″-dimethylpyrano-[5,6:6,7]-isoflavanone; **49**, Catechin; **50**, Calopocarpin; **51**, Demethylmedicarpin; **52**, Neorautenol; **53**, Cristacarpin; **54**, Phaseollidin; **55**, Dolichin A; **56**, Dolichin B; **57**, Orientanol A; **58**, Sandwicensin; **59**, Erythbidin D; **60**, Hydroxycristacarpone; **61**, Sandwicarpin; **62**, Erycristanol B; **63**, Erythbidin C; **64**, Methyl gallate; **65**, Loliolide; **66**, Erythrodiol; **67**, Lupeol; **68**, Ursolic acid; **69**, Stigmasterol; **70**, Stigmast-3-en-4-one; **71**, Stigmasta-4,22-dien-3-one; **72**, β-sitosterol; **73**, Sigmoiside A; **74**, Sigmoiside B; **75**, Sigmoiside F.

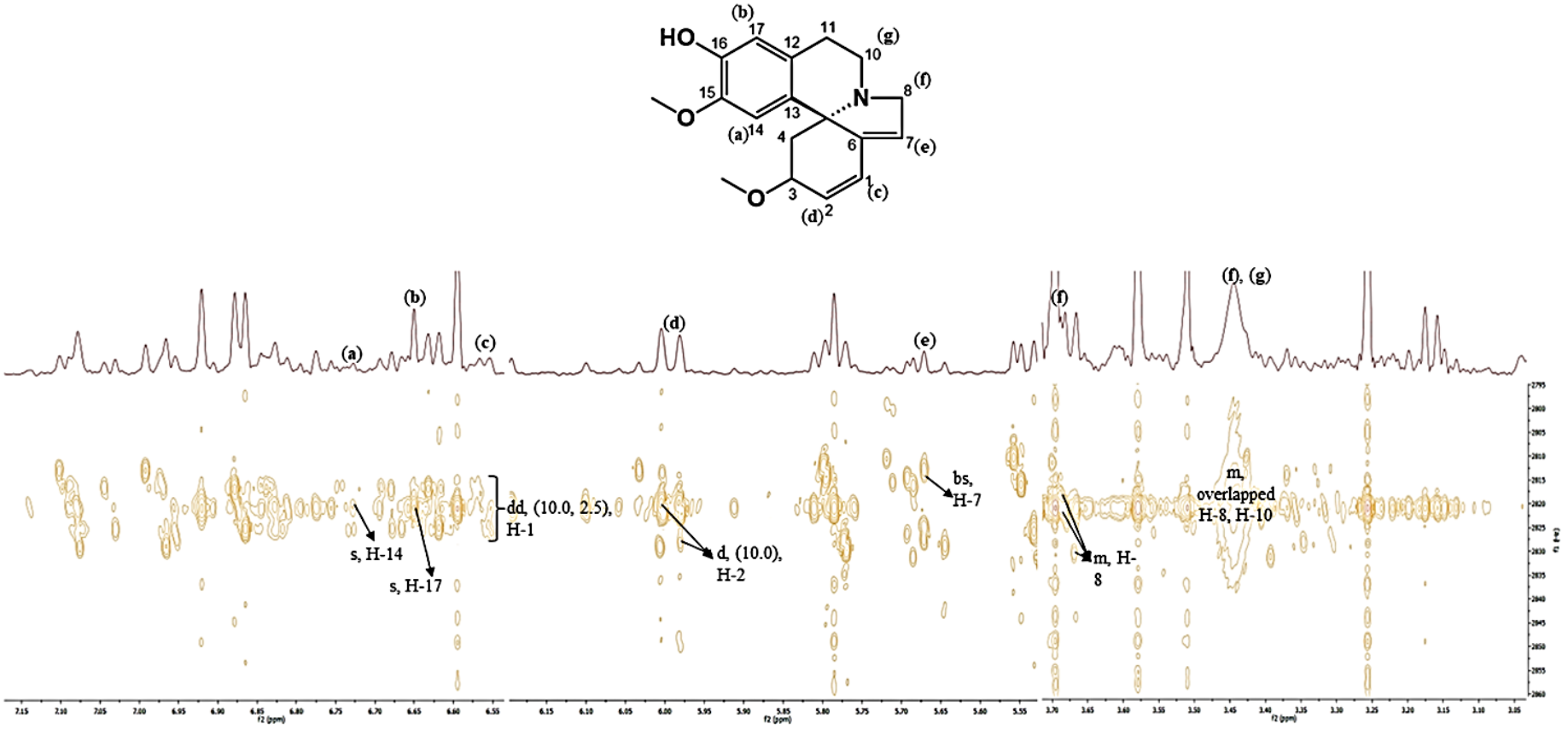

Figure 2. *J*-Resolved nuclear magnetic resonance (NMR) spectrum of the diene system in the region of δ 5.60 – 6.60 ppm and the aromatic moiety in the region δ 6.60-6.75 ppm of erysodine (**1**). (**a**) – (**g**) were the assigned peaks to represent the significant protons of erysodine

Table 2. Classified phytochemicals with their proton NMR characteristics in different extracts of *E. fusca* flowers.

.	Phytochemicals (Unique chemical shift, multiplicity)	Type of extract			Reference
		FH	FD	FM	
	Alkaloids				
1	Erysodine (6.73, *s*)		✓		[44]
2	Erysovine (6.71, *s*)		✓		[45]
3	Erysopine (6.94, *s*)		✓		[46]
4	Erysoline (6.46, *s*)		✓		[47]
5	Erysotrine (6.76, *s*)		✓		[44]
6	11*α*-Hydroxyerythravine (6.91, *s*)		✓		[48]
7	11*β*-Hydroxyerysotrine (6.89, *s*)		✓		[48]
8	11*α*-Hydroxyerysotrine (7.01, *s*)		✓		[48]
9	Erythristemine (4.14, *dd*, *J* = 3.2, 3.6 Hz)		✓		[49]
10	Erysovine *N*-oxide (6.62, *s*)		✓		[50]
11	Erysotrine *N*-oxide (6.83, *s*)		✓		[51]
12	11*β*-Hydroxyerysotramidine (4.77, br *s*)		✓		[51]
13	11*β*-Methoxyerythraline (6.99, *s*)		✓		[52]
14	Erythrinine (7.10, *s*)		✓		[52]
15	8-Oxoerythraline (6.02, *s*)		✓		[53]
16	11-Methoxyerythratine (6.64, *s*)		✓		[53]
17	Erytharbine (6.22, *s*)		✓		[54]
18	10-Hydroxy-11-oxoerysotrine (7.26, *s*)		✓		[54]
19	Erytharborine H (7.23 *s*)		✓		[55]
20	Sodium Erysovine 15-*O*-sulfate (7.40, *s*)		✓		[45]
21	Erysosalvine (6.55, *s*)		✓		[47]
22	11-Hydroxyerysosalvine (4.74, *m*)		✓		[56]
23	Erysotine (6.48, *s*)		✓		[47]
24	Erythratidine (4.10, *m*)		✓		[47]
25	Epierythratidine (4.21, *m*)		✓		[43]
26	11*β*-Hydroxyerythratidine *N*-oxide (5.15, *d*, *J* = 3.3 Hz)		✓		[43]
27	Erysotinone (6.18, *m*)		✓	✓	[47]
28	11-Hydroxyerysotinone (6.26, s)			✓	[47]
29	10,11-Dioxoerythratine (7.38, *s*)		✓		[57]
30	10,11-Dioxoepierythratidine (7.45, *s*)		✓	✓	[57]
31	Hypaphorine (7.15, *s*)			✓	[58]
32	Erythbidin B (5.77, *s*)		✓		[51]
33	Magnoflorine (7.02, *s*)		✓		[59]
	Flavonoids				
34	5,4′-Dihydroxy-8-(3,3-dimethylally)-2″-methoxyisopropylfurano-[4,5:6,7]-isoflavone (7.47, *d*, *J* = 8.8 Hz)		✓		[60]
35	5,4′-Dihydroxy-8-(3,3-dimethylally)-2″-hydroxymethyl-2″-methylpyrano-[5,6:6,7]-isoflavone (5.49, *d*, *J* = 10.0 Hz)		✓		[60]
36	Indicanine D (4.20, *m*)		✓		[38]
37	Warangalone (3.35, *d*, *J* = 7 Hz)				[61]
38	Lupinifolin (3.20, *d*, *J* = 7.2 Hz)		✓	✓	[62]
39	Citflavanone (5.95, s)	✓			[62]
40	Erythrisenegalone (3.25, *d*, *J* = 7.2 Hz)		✓	✓	[62]
41	Lonchocarpol A (3.34, *d*, *J* = 6.4 Hz)		✓		[62]
42	Liquiritigenin (6.58, *dd*, *J* = 8.6, 2.4 Hz)		✓		[62]
43	Fuscaflavanone B (5.45, *t*, 5.3)		✓		[62]
44	Sigmoidin H (5.44, *d*, *J* = 10 Hz)		✓		[63]
45	Eryzerin D (6.30, *d*, *J* = 2.2 Hz)		✓		[64]
46	Atalantoflavone (6.19, *s*)		✓		[64]

47	5,3′-dihydroxy-4′-methoxy-5′-(3-methyl-1,3-butadienyl)-2″,2″-dimethylpyrano-[5,6:6,7]-isoflavanone (1.98, *br s)*	✓			[54]
48	5,3′-dihydroxy-5′-(3-hydroxy-3-methyl-1-butenyl)-4′-methoxy-2″,2″-dimethylpyrano-[5,6:6,7]-isoflavanone (6.45, *d*, *J* = 16.1 Hz)	✓			[54]
49	Catechin (3.76, *m*)		✓		[61]
	Pterocarpans				
50	Calopocarpin (5.36, *br t*)		✓		[65]
51	Demethylmedicarpin (7.32, *d*)			✓	[65]
52	Neorautenol (5.66, *d*)		✓		[65]
53	Cristacarpin (6.31, *d*, *J* = 2.3 Hz)			✓	[67]
54	Phaseollidin (6.38, *d*, *J* = 8.0 Hz)		✓		[65]
55	Dolichin A (2.87, 3.01, *q*)		✓		[66]
56	Dolichin B (2.81, 3.02, *q)*		✓		[66]
57	Orientanol A (3.15, *d*, *J* = 5.0 Hz)			✓	[67]
58	Sandwicensin (3.63, *d*, *J* = 11 Hz)		✓		[68]
59	Erythbidin D (6.07, *d*, *J* = 2.2 Hz)			✓	[70]
60	Hydroxycristacarpone (6.05, *dd*, *J* = 10.1, 1.7 Hz, 5.27 *d*, *J* = 1.7 Hz)		✓		[69]
61	Sandwicarpin (5.26, *s*)		✓		[69]
	Phenols				
62	Erycristanol B (6.43, *m*, 2.72, *dd*, *J*= 15, 4 Hz)	✓	✓	✓	[66]
63	Erythbidin C (6.36, *dt*, *J* = 15.9, 6.2 Hz, 2.63, *t*, *J* = 7.4 Hz)			✓	[67]
64	Methyl gallate (3.79, *s*)		✓		[69]
	Terpenes				
65	Loliolide (5.74, *s*)	✓			[73]
66	Erythrodiol (3.23, 3.57, *d*, *J* = 11.0 Hz)		✓		[69]
67	Lupeol (4.70, 4.55, *s*)		✓		[71]
68	Ursolic acid (0.84, *d*, *J* = 10 Hz)				[71]
	Sterols				
69	Stigmasterol (1.07, *s*)		✓		[72]
70	Stigmast-3-en-4-one (0.94, *d*, *J* = 6.4 Hz)		✓		[72]
71	Stigmasta-4,22-dien-3-one (1.04, *d*, *J* = 6.8 Hz)	✓			[72]
72	β-sitosterol (3.52, *s*)		✓		[73]
	Saponins				
73	Sigmoiside A (0.90, s), (4.89, *d*, *J* = 7.1 Hz, H-l' of galactose)	✓			[74]
74	Sigmoiside B (0.98, *s*), (4.85 d, J = 6.8 Hz, H-l' of glucose)		✓		[74]
75	Sigmoiside F (0.78, *s*), (4.62, d, J = 7.8 Hz, 1-H of glucose)		✓		[75]

Note: *s*, singlet; *d*, doublet; *dd*, doublet of doublet; *q*, quartet; *m*, multiplet and t, triplet; FH, flower hexane; FD, flower DCM; and FM, flower MeOH.

As shown in Figure 1, the FH (**a**), FD (**b**), and FM (**c**) extracts of *E. fusca* flower showed a diversity of resonances in all of its spectra. Resonances are intense in the low-, mid- and high-field regions of both the FH and FD extracts. Of the extracts, the most phytochemicals were managed to be identified in the FD extract (60). The phytochemicals identified in the extracts (Table 2) belong to a diverse class of compounds including alkaloids (32), flavonoids (16), pterocarpans (12), terpenes (4), saponins (3), phenols (3), and sterols (4), where most of them have been reported as the constituent of *Erythrina* species [32-34]. Notably, the phytochemicals citaflavanone (**39**), neorautenol (**52**), and hydroxycristacarpone (**60**) were first time identified in the flower part. Magnoflorine (**33**) and loliolide (**65**) have not been previously reported in *E. fusca* or other *Erythrina* species. Consequently, this highlights the effectiveness of using [1]H-NMR in the identification of unreported compounds from samples.

Regardless of the identification of specific chemical shift characteristics for a particular phytochemical, its assignment was still difficult due to overlapping peaks, especially the leftover water signals which overlap with the anomeric protons of sugars or glycosides at δ 4.8–5.2 ppm [76]. An important factor to take into consideration when using NMR spectroscopy in complex samples is resonance overlap. Thus, 2D experiments (*J*-resolved) were performed to provide additional information on signal splitting [77]. In Figure 2, the *J*-resolved NMR spectrum of the diene system in the region δ 5.60-6.60 ppm and the aromatic moiety in the region δ 6.60-6.75 ppm are the

characteristics of the alkaloid, erysodine. It can be observed that the signals are resolved, particularly those where the coupling constant can be easily calculated, i.e., doublet of doublets (*dd*) and doublet (*d*) signals at *ca*. 6.55 and 6.98 ppm, respectively. A similar concept has been employed to solve and confirm the identification of the other compounds in the extracts.

Multivariate Data Analyses (MVDA)

MVDA technique was applied to assess the variation in phytochemicals content in the extracts. The method of partial least squares (PLS) projection to latent structures was employed to identify the phytochemicals that are correlated to the antibacterial activity. PLS facilitates the linking of data from independent factors (X-matrix and ^{1}H NMR spectral data) to dependent variables (antibacterial activity), which enables the prediction of potential biomarkers. The score plot was constructed to illustrate the differences in the exacts while the PCA loading plot showed the phytochemicals that contributed to the cluster's separation. The models were validated through internal cross-validation using cumulative values either R^2, R^2Y or R^2X, and Q^2 for score plot and permutation tests (n=100). The R^2 values indicate how well the model fits the data, with a value closer to one indicating a better fit. The Q^2 value, on the other hand, is a measure of the model's predictive ability where values exceeding 0.5 and 0.9 are considered to have good and excellent predictability, respectively [77, 78].

Phytochemicals Variation in E. fusca Flower Extracts PCA

Based on Figure 3**a** PCA score plot, the flower extracts were clustered into three groups based on 15 samples with clear separation between FD and FM extracts by PC1 46.7% of the variance, and FM and FH by PC2 35.3% of variance, cumulatively describing a total variance of 82.0% with excellence value of fit goodness (R^2X = 0.999) and predictability (Q^2 = 0.983). However, the plots for FH and FD extracts overlapped and did not show a clear separation between the two principals. This situation might be due to the extracted phytochemicals having preferability towards both solvents used (Hx, and DCM) as they are close polarity-wise.

The loading plot in Figure 3**b** revealed that the flavonoids 5,3′-dihydroxy-4′-methoxy-5′-(3-methyl-1,3-butadienyl)-2″,2″-dimethylpyrano-[5,6:6,7]-iso flavanone (**47**) and 5,3′-dihydroxy-5′-(3-hydroxy-3-methyl-1-butenyl)-4′-methoxy-2″,2″-dimethylpyrano-[5,6:6,7]-isoflavanone (**48**) were the significant phytochemicals contributing to the separation of the FH extract from the other extracts. Conversely, the FD extract showed separation based on the most significant phytochemicals sterols stigmasterol (**69**), and the saponins sigmoiside A (**73**) and sigmoiside B (**74**). The discrimination of FM extract from the others was based on the phytochemical's flavonoids lupinifolin (**38**) and erythrisenegalone (**40**) and pterocarpan orientanol A (**57**). Interestingly, the only compound 8-oxoerythraline (**15**) in the centre of the plot indicates the marker for the extracts' similarities. The flavonoids **38** and **40** were previously isolated from the same methanol extract of *E. fusca* and *E. crista* [62]. While the flavonoids **47** and **48** were isolated from the less polar DCM soluble portion of an acetone extract of *E. herbacea* [54]. Some of the other phytochemicals including **57**, **68**, **73**, and **74** were reported from a close polarity of solvents to the present study [67, 71, 74]. This is not surprising as the solubility of compounds could be vary depending on the complexity of their matrix [30].

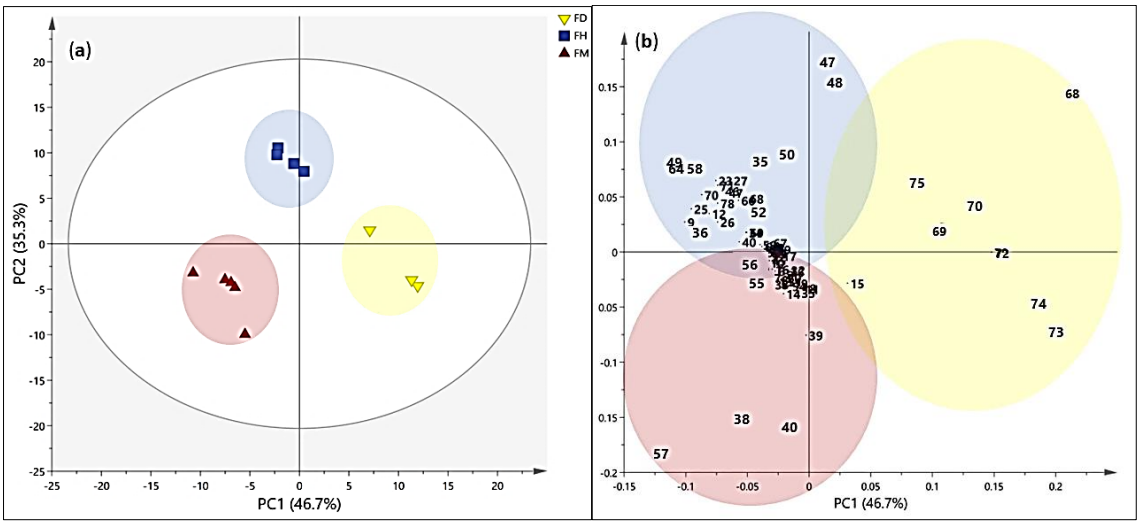

Figure 3. (a) PCA and (b) Loading scatter plot of coloured by the most important factors: blue, flower-triturated hexane extract; yellow, flower-triturated DCM extract; and red, flower-triturated MeOH extract. The assigned numbers for phytochemicals are based on Table 2.

Correlation Between Phytochemicals and Antibacterial Activity Through Partial Least Square (PLS) Projection

The excellent fitness (R^2Y = 0.995) and predictive ability (Q^2 = 0.931) PLS biplot shown in Figure 4 represents the correlation between antibacterial activity and phytochemicals in the extracts. The FH extract demonstrated the strongest correlation with the antibacterial activity against both Gram-positive strains of *S. aureus* and *S. haemolyticus* at the positive sides of PLS C1 and C2 justifying its inhibition zone of 14.00 ± 0.00 and 9.67 ± 0.33 mm, respectively, observed in the antibacterial activity discussed earlier in Table 2. However, the model only depicts the activity against *S. haemolyticus* and the phytochemicals flavonoid 5, 3′-dihydroxy-4′-methoxy-5′-(3-methyl-1,3-butadienyl) -2″,2″-dimethylpyrano-[5,6:6,7]-isoflavanone (**47**), and the pterocarpans sandwicensin (**58**) and neorautenol (**52**) were collectively contributed to the activity. On the other hand, the FM extract (located at the negative side of PLS C1) showed the strongest correlation on both the Gram-negative strains of *E. aerogenes* and *E. cloacae* supporting their earlier ZOI of 8.33 ± 0.33 and 8.17 ± 0.17 mm, respectively. The activity of FM extract also contributed a collective phytochemical from the class of alkaloids, namely erysotrine *N*-oxide (**11**), 10-hydroxy-11-oxoerysotrine (**18**), and magnoflorine (**33**). The structures of these active phytochemicals are shown in Figure 5. A thorough literature research revealed that the active phytochemicals have never been reported for any antibacterial activity before. However, a study by Chitopoa et al. has found that the hexane extract of *E. abyssinica* exhibited the highest inhibition zone (23 mm) against *S. aureus* compared to its DCM extract [79]. Although they did not identify the compounds responsible for the activity, *E. fusca* and *E. abyssinica* are chemotaxonomically related. Thus, their antibacterial activity on *S. aureus* could be attributed to the same class of compounds.

CONCLUSION

The present study found different extraction solvents used for *E. fusca*'s flower resulted in different antibacterial activity on the tested strains. The hexane extract demonstrated notable activity against *S. aureus* and *S. haemolyticus* strains, the MeOH extract displayed efficacy against *E. cloacae* and *E. aerogenes* strains, while the DCM extract specifically targeted *S. aureus*. Through NMR analysis, 75 phytochemicals were identified, mainly from the classes' alkaloids, flavonoids, pterocarpans, terpenes, saponins, sterols, and phenols. MVDA analysis revealed only the correlation between the activity against *S. haemolyticus* with the flavonoid 5,3′-dihydroxy-4′-methoxy-5′-(3-methyl-1,3-butadienyl)-2″,2″-dimethylpyrano-[5,6:6,7]-isoflavanone (**47**), and the pterocarpans sandwicensin (**58**) and neorautenol (**52**). Meanwhile, the activity against *E. cloacae* and *E. aerogenes* was attributed to the alkaloids erysotrine N-oxide (**11**), 10-hydroxy-11-oxoerysotrine (**18**), and magnoflorine (**33**). This collective evidence highlights the potential therapeutic value of the plant, suggesting a synergistic contribution from the identified phytochemicals. These findings not only support the plant's traditional use but also propose it as a promising source for further antibacterial studies.

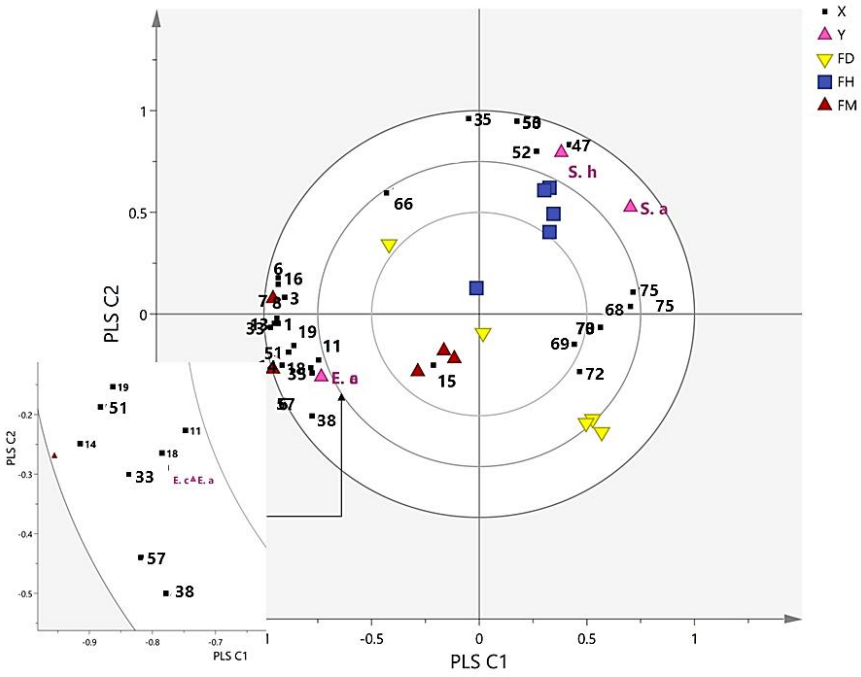

Figure 4. The PLS biplot describing the variation between *E. fusca* flower extracts with an excellent fitness (R^2Y = 0.995) and predictive ability (Q^2 = 0.931). S.a, *S. aureus*; E. a, *E. aerogenes* and E. c, *E. cloacae*. The assigned numbers of phytochemicals are based on Table 2.

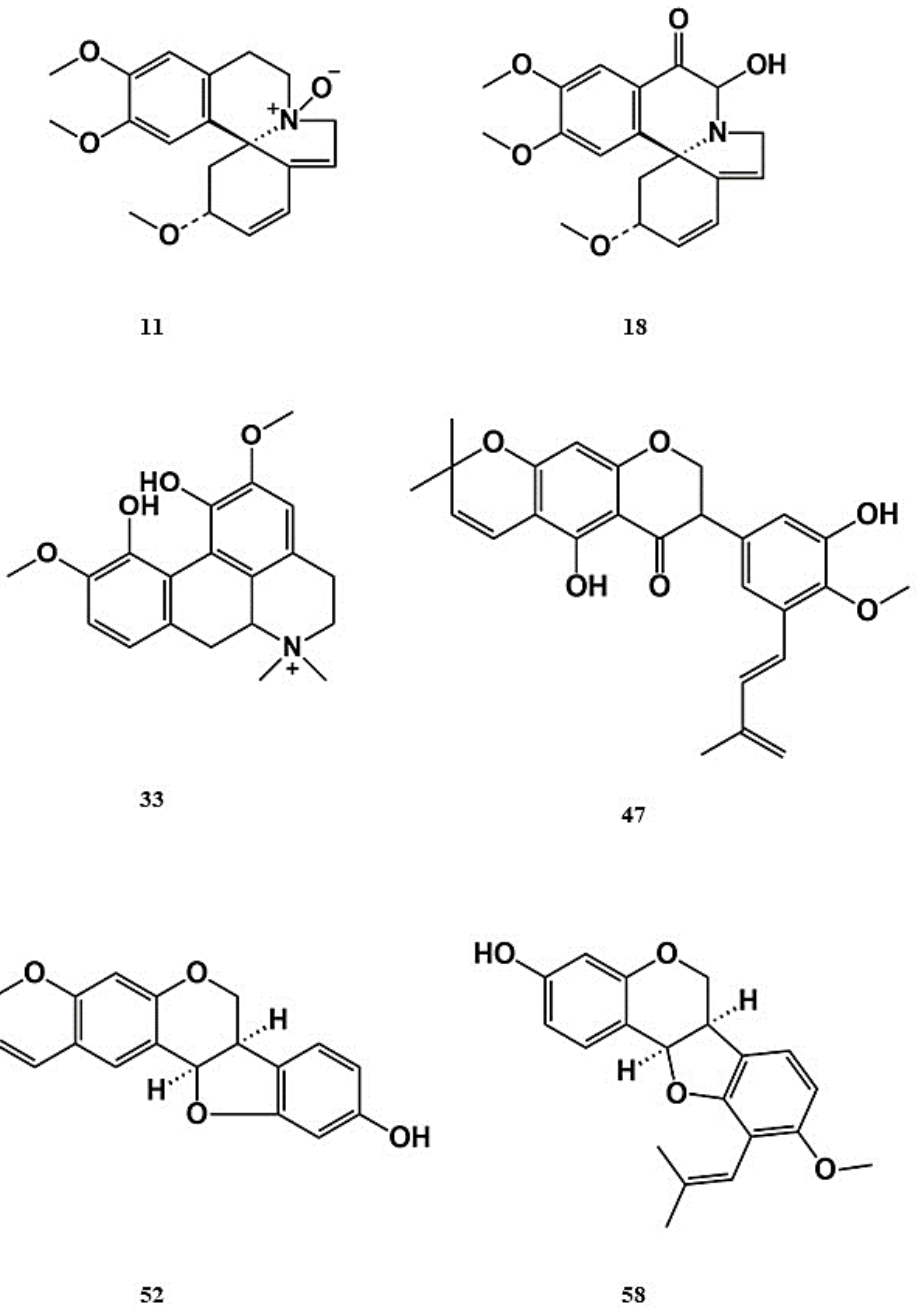

Figure 5. Structures of antibacterial active compounds: **11** erysotrine N-oxide, **18** 10-hydroxy-11-oxoerysotrine, **33** magnoflorine, **47** 5,3′-dihydroxy-4′-methoxy-5′-(3-methyl-1,3-butadienyl)-2″,2″-dimethylpyrano-[5,6:6,7]-isoflavanone, **58** sandwicensin, and **52** neorautenol.

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