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RESEARCH

Untargeted ^{1}H NMR Metabolomics of *Elephantopus Scaber* Reveals Correlation with Anti-*Vibrio* Activity

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Abstract

Background *Elephantopus scaber* (*E. scaber*) is a medicinal plant frequently used in traditional medicine for its health-promoting properties, as it contains numerous physiologically active compounds. However, scientific research on the metabolite profile of this plant remains limited.

Purpose This study aims to establish chemical profiles of *E. scaber* leaf extracted by different polarity solvents (hexane, chloroform, acetone and 70% (v/v) ethanol) and to correlate the chemical markers to anti-*Vibrio* activities using the ^{1}H NMR-metabolomics approach.

Methods A portion of the powdered *E. scaber* leaf sample was extracted using different solvent systems, namely hexane, chloroform, ethyl acetate, and 70% (v/v) ethanol. Each extract was evaporated to dryness and subsequently lyophilized before being subjected to NMR analysis. For multivariate data analysis, all samples were prepared in six replicates.

Results The application of principal component analysis effectively distinguished the extracts based on their metabolite profiles. Acetone and 70% (v/v) ethanol were determined to have good extraction efficiency. The findings from the partial least squares analysis revealed a significant correlation between the polar chemicals present in the acetone extract of *E. scaber* leaf and its anti-*Vibrio* activity. According to the loading plot, acetone extracts contain a more diversified spectrum as compared to the other extracts, with a total of 42 metabolites comprising carbohydrates, amino acids, flavonoids, terpenoids, organic and fatty acids.

Conclusion The identification of these metabolites in *E. scaber* leaf extracts indicate that they may play a significant role in the anti-*Vibrio* action.

Keywords Elephantopus scaber · Chemical profile · Anti-*Vibrio* · Principal component analysis · Partial least squares

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Introduction

Vibrio species are Gram-negative, curved rod-shaped, motile bacteria that have a strong preference for environments with varying levels of salinity and temperatures ranging from 10 °C to 30 °C [1]. A number of species within this genus have been linked to various infections, including septicemia, gastroenteritis and wound infections [2]. *Vibrio parahaemolyticus* (*V. parahaemolyticus)* and *Vibrio vulnificus* (*V. vulnificus)*, which are bacteria found in seawater, can cause inflammation of the small intestine, diarrhoea, cramps and septicemia when they enter the human body through wounds or when undercooked seafood is consumed [3–6]. *Vibrio campbellii* (*V. campbellii)* is also seawater bacteria that is responsible for inducing luminous illness in farmed shrimp, leading to their death [7]. It also causes mortality in cultured fish and shellfish and may have a role in the occurrence of coral bleaching [8, 9]. Despite the fact that infections induced by *Vibrio* species are treatable with a variety of antibiotics, the pursuit of novel broad-spectrum antibiotics is imperative due to the multidrug resistance exhibited by these pathogens. Alternative approaches could be developed by focusing on natural anti-*Vibrio* agents derived from natural resources.

In recent years, various research has reported that the *Elephantopous scaber* (*E. scaber*) Lam plant, which belongs to the Asteraceae family, contains an extensive range of phytoconstituents and exhibits numerous promising medicinal properties. In Malaysia, this plant is generally referred to as Tutup Bumi, whereas in other countries it is known as Elephant's Food or Didancao [10]. Additionally, it is frequently applied as a therapeutic intervention for the management of gastropathy, hepatitis, nephritis, scabies, arthralgia, chest pain, fever and cough associated with pneumonia, bronchitis, arthritis and carbuncle. Moreover, it effectively eliminates bladder stones [11]. The plant's extracts or components have been shown to possess antibiosis, antiviral and cytotoxicity actions [12].

By combining NMR with multivariate data analysis, the metabolomic profiles of numerous plant species and traditional phytomedicines can be determined [13]. Principal component analysis (PCA), orthogonal partial least squares-discriminant analysis (OPLS-DA) and partial least squares-discriminant analysis (PLS-DA) are multivariate methods that were created specifically for NMR data analysis because of the highly complex sets of NMR data. While 1D and 2D NMR metabolomics provide high reproducibility, minimal sample preparation and quantitative capability, they are generally less sensitive than mass spectrometry–based platforms and may exhibit overlapping signals that limit detection of very low-abundance metabolites [14]. Through a combination of anti-*Vibrio* activity, ^{1}H NMR data and metabolomics approach, the biomarkers and mechanism of action exerted by *E. scaber* leaf extracted should be better understood. Hence, the focus of this work is to differentiate the metabolite profiles of *E. scaber* leaf extracted by varying polarity solvents and to correlate the chemical markers to anti-*Vibrio* activities using ^{1}H NMR-metabolomics approach. Potentially useful information for developing effective treatments for *Vibrio* infections may emerge from this study.

Experimental

Materials

The extraction process utilized analytical grade solvents, including ethanol, n-hexane, acetone and chloroform. Methanol, deuterated methanol-d_4 (CD_3OD, 99.8%), non-deuterated potassium dihydrogen phosphate (KH_2PO_4, pH 6.0), deuterium oxide (D_2O, 99.9%) and trimethylsilylpropionic acid-d_4 sodium salt (TSP) were acquired from Merck (Darmstadt, Germany). The deionized water used was from a PURELAB Chorus 2 system (ELGA Lab Water, USA). *V. vulnificus* (ATC.33147), *V. parahaemolyticus* (ATC.17802) and *V. campbellii* (ATC.BAA-1116) were obtained from American Type Culture Collection (Manassas, VA, USA). Mueller Hinton agar (MHA; Difco, Sparks, USA) and Mueller Hinton broth (MHB; Difco, Sparks, USA) were used as media for the antibacterial assays. Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich.

Plant Collection and Preparation

E. scaber leaf were harvested from the Universiti Putra Malaysia (UPM) campus. The harvested leaf samples were rinsed with flowing distilled water and delicately dried using paper towels. Then the samples were kept in sealed containers and stored overnight in ultra-low temperature freezer. The frozen samples were dried in a freeze-dryer (Scanvac Labogene) for 72 h and pulverized into fine powder using a grinder (Waring, 32 BL80, New Hartford, NY, USA). The powder samples were kept in a −80 °C freezer until the further analysis.

Preparation of *E. scaber* leaf extracts

The *E. scaber* freeze-dried leaf powder (10 g) was extracted using sonication in 200 mL of the respective solvents i.e., 70% (v/v) ethanol, acetone, chloroform and hexane (20 min, 25 °C). Crude extracts were obtained by repeating the extraction process twice and then filtering the combined supernatant through 125 mm Whatman filter paper. The

remaining supernatant was evaporated using a rotary evaporator. A total of six replicates for each solvent was prepared.

Preparation of samples for anti-*Vibrio* testing

To prepare a stock solution of each plant extract at a concentration of 100 mg/mL, 10 mg of extract was dissolved in 100 µL of DMSO. In order to produce a 1% test solution (10 mg/mL), 100 mg/mL of each stock solution was diluted in 900 µL of distilled water.

Disc Diffusion Assay

The disc diffusion assay was applied to ascertain the antibacterial efficacy [15]. The *Vibrio* strains were cultivated on Mueller-Hinton agar medium added with 1% NaCl from the culture stock. Then, 0.5 McFarland turbidity was made from pure colonies. To inoculate fresh Petri dishes, a cotton swab was used. The *E. scaber* leaf extracts were dissolved with DMSO to make a 10 mg/mL concentration. Prior to use, Whatman grade 6 mm filter paper discs were impregnated with 10 µL of this solution. After incubating the inoculated plates for 24 h at 37 °C, inhibition zones were examined. Six replicates were utilised in each experiment and the inhibition zone diameter was determined in millimetres (mm) for each experiment. Tetracycline (5 µg) was used as standard drugs against the tested *Vibrio* strains. Standard drugs, i.e., tetracycline (5 µg), were employed against the investigated strains of *Vibrio*.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Values

The minimum inhibitory concentrations (MICs) for *Vibrio* bacteria that are susceptible to the *E. scaber* leaf extracts were determined using the broth microdilution method. Filtered sterile distilled water was used to generate a series of two-fold serial dilutions. These dilutions were made from a 10 mg/mL stock of plant extracts, resulting in test concentrations ranging from 10 to 0.0195 mg/mL for each solvent extract. A 100 µL aliquot of double strength Mueller-Hinton broth containing 1% NaCl was placed into each well of the 96-well plates. Subsequently, 50 µL of the extract at different concentrations was added in a decreasing order, along with 50 µL of the test bacteria suspension. The positive control well contains of 100 µL Mueller-Hinton broth with 1% NaCl, 50 µL of tetracycline and 50 µL of the test bacteria. The negative control well contains of 100 µL Mueller-Hinton broth with 1% NaCl, 50 µL of filtered sterile distilled water and 50 µL of the test bacteria. After incubating the inoculated plates aerobically for 24 h at 37 °C, the MIC of the test extract was evaluated. The MIC value represents the minimum concentration of the test sample at which complete inhibition of bacterial growth occurred.

To determine the MBC, a 10 µL sample of the suspension from each of the 12 wells used in the MIC test was transferred to an MHA plate for sub-culturing. After incubating the inoculated plates for 24 h at 37 °C, the MBC value of the test extract was evaluated. The MBC is the concentration of the test material at which the bacterial strain was eliminated. The MIC and MBC values were each calculated in triplicate.

NMR Spectroscopy and Multivariate Data Analysis

The ^{1}H NMR spectra were obtained at a temperature of 25˚C using a 500 MHz Varian INOVA NMR spectrometer, which operates at a proton NMR frequency of 499.887 MHz. Preparation of samples followed the previously described method [16]. In a 2.0 mL Eppendorf tube containing 50 mg of each extract, a volume of 0.75 mL was added to a 1: 1 mixture of methanol-d_4 and potassium dihydrogen phosphate buffer (pH 6.0) in deuterium oxide, which also included 0.1% trimethylsilylpropionic acid-d_4 sodium salt. The mixture was vortexed for 1 minute, ultrasonication for 20 min and centrifugation at 10,000 rpm for 10 min. The supernatant (0.6 mL) was immediately placed to 5 mm NMR tube to perform ^{1}H-NMR measurement employing pre-saturation sequence. The obtained spectra were processed using the Chenomx software (v.5.1, Alberta, Canada) with a uniform setting applied to all spectra. The spectral intensities were grouped into bins of similar width (0.04) within the range of 0.50–10.00. The areas between 4.70 and 4.90, which correspond to water at 3.23 and 3.36, which represent residual methanol, were omitted. SIMCA-P software (v. 13.0, Umetrics, Umeå, Sweden) was utilized to analyse multivariate data using the PCA and PLS analysis methods. Pareto scaling method was applied in this analysis. The *X*-variables used in the in PLS analysis were NMR chemical shifts, while the *Y*-variable represented the inhibition zone value as determined by the disc diffusion method. Model validation technique such as permutation test was used to verify that the PLS model obtained was accurate and predictive. The identification of metabolites was conducted by comparing the distinctive signals detected in the ^{1}H-NMR spectra of the extract produced from *E. scaber* leaf with those documented in the literature, the Chenomx database (Chenomx NMR Suite 7.7 library) and the human metabolome database (www.hmdb.ca). Additionally, the 2D *J*-resolved was utilized to aid in the identification of metabolites.

Table 1 Inhibition zones of *E. scaber* leaf extracted with different solvents against *Vibrio* strains

Diameter of inhibition zones in mm (including disc). Values are expressed as means ± standard deviation (SD)

Vibrio Strain	Inhibition Zone Diameter (mm)				
	Tetracycline (5 µg)	70% (v/v) Ethanol	Acetone	Chloroform	Hexane
V. vulnificus (ATC.33147)	22.00 ± 0.00	18.87 ± 0.30	18.90 ± 0.07	15.27 ± 0.44	9.23 ± 0.36
V. parahaemolyticus (ATC.17802)	17.00 ± 0.00	8.50 ± 0.32	8.55 ± 0.43	8.43 ± 0.31	7.64 ± 0.21
V. campbellii (ATC.BAA-1116)	24.33 ± 0.52	18.61 ± 0.60	18.69 ± 0.19	17.82 ± 0.33	13.69 ± 0.69

Table 2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values (mg/mL) of different solvent extracts of *E. scaber* leaf against *Vibrio* strains

Vibrio spp.		70% (v/v) Ethanol	Acetone	Chloroform	Hexane
V. vulnificus (ATC.33147)	MIC (mg/mL)	0.625 ± 0.000	0.313 ± 0.000	1.250 ± 0.000	2.500 ± 0.000
	MBC (mg/mL)	0.625 ± 0.000	0.313 ± 0.000	1.250 ± 0.000	5.000 ± 0.000
V. parahaemolyticus (ATC.17802)	MIC (mg/mL)	1.250 ± 0.000	1.250 ± 0.000	1.250 ± 0.000	2.500 ± 0.000
	MBC (mg/mL)	1.250 ± 0.722	1.250 ± 0.000	5.000 ± 1.443	5.000 ± 0.000
V. campbellii (ATC.BAA-1116)	MIC (mg/mL)	0.313 ± 0.000	0.156 ± 0.000	0.313 ± 0.000	1.250 ± 0.000
	MBC (mg/mL)	0.313 ± 0.180	0.156 ± 0.000	0.625 ± 0.000	1.250 ± 0.000

Values are expressed as means ± standard deviation (SD)

Results and Discussion

Antibacterial activity of *E. scaber* leaf extract

The disk diffusion susceptibility test was used to screen the *E. scaber* leaf extracted with different solvents for measurement of antimicrobial activity against *Vibrio* strain. The extract with the highest potency was evaluated by measuring the size of the inhibition zone resulting from its anti-*Vibrio* activity. A larger zone of inhibition, the more potent of the extract with higher anti-*Vibrio* activity. Inhibition zones of *E. scaber* leaf extracted with different solvents i.e., 70% (v/v) ethanol, acetone, chloroform and hexane against *Vibrio* strains (*V. vulnificus*, *V. parahaemolyticus* and *V. campbellii*) is shown in Table 1. (Extended data are shown in Table S1 and S2 of the supplementary material. Among the different solvent of the extracts, acetone exhibited the highest activity against all the *Vibrio* strains, producing inhibition zones of 18.90, 18.69 and 8.55 mm at 10 mg/mL against *V. vulnificus*, *V. campbellii* and *V. parahaemolyticus*, respectively. It can be observed that solvents with different polarities have a significant influence on the inhibition zone. Acetone and 70% (v/v) ethanol extracts exhibited greater antibacterial activities against *Vibrio* strain. It is probably due to the capability of high-polarity solvents dissolving more secondary metabolites [17]. The potential antibacterial activity and extraction efficiency of active metabolites are both affected by the solvent's polarity.

MIC and MBC values of *E. scaber* leaf extract

The anti-*Vibrio* activity of the *E. scaber* leaf that were extracted using different solvents was further investigated by determining the MIC and MBC values, in addition to the total activity (Table 2 and Table S3 of the supplementary material). The results show that the level of growth inhibition exhibited by the various extracts towards the *Vibrio* strains.

The MIC and MBC values of the acetone extract ranged between 0.313 and 0.156 mg/mL, respectively. The acetone extract was more potent against *V. campbellii* (MIC 0.156 mg/mL; MBC 0.156 mg/mL) and *V. vulnificus* (MIC 0.313 mg/mL; MBC 0.313 mg/mL) in comparison to the *V. parahaemolyticus* strain, which showed similar value for MIC and MBC, i.e., 1.250 mg/mL, respectively. The MIC and MBC for the 70% (v/v) ethanol extract were 0.625–1.250 mg/mL and 0.313–1.250 mg/mL, respectively, while the MIC and MBC recorded by chloroform extracts were 0.313–1.250 mg/mL and 0.625–5.000 mg/mL, respectively. Meanwhile, hexane extracts showed the MIC and MBC values in the range of 1.250–2.500 mg/mL and 1.250–5.00 mg/mL, respectively. Therefore, the current findings indicate that the *E. scaber* leaf acetone and 70% (v/v) ethanol extracts exhibit moderate to significant activity against the evaluated *Vibrio* strains.

The MIC and MBC values of the acetone extract varied from 0.313 to 0.156 mg/mL, respectively. The potency of the acetone extract was higher against *V. campbellii* (MIC 0.156 mg/mL; MBC 0.156 mg/mL) and *V. vulnificus* (MIC

0.313 mg/mL; MBC 0.313 mg/mL) compared to the *V. parahaemolyticus* strain, which exhibited the same values for MIC and MBC, notably 1.250 mg/mL, respectively. The MIC and MBC of the 70% (v/v) ethanol extract were found to be between 0.625 and 1.250 mg/mL and between 0.313 and 1.250 mg/mL, respectively. On the other hand, the MIC and MBC values obtained from the chloroform extracts ranged from 0.313 to 1.250 mg/mL and from 0.625 to 5.000 mg/mL, respectively. Hexane extracts exhibited MIC values ranging from 1.250 to 2.500 mg/mL and MBC values ranging from 1.250 to 5.00 mg/mL. Hence, the present results suggest that the acetone and 70% (v/v) ethanol extracts of *E. scaber* leaf have moderate to substantial efficacy against the tested *Vibrio* strains.

Tamokou et al. reported that, plant extracts or their components are considered highly active if they exhibit MIC values below 100 µg/ml [18]. They are considered considerably active if the MIC falls between 100 and 512 µg/ml, moderately active if the MIC is between 512 and 2048 µg/ml, and not active if the MIC exceeds 2048 µg/ml. Hence, the current findings demonstrate that the extracts from *E. scaber* leaf exhibit a moderate to significant level of activity against the *Vibrio* strains that were determined. Furthermore, some polar metabolites found in the leaf' extracts have significant anti-*Vibrio* properties and could contribute strongly to the overall plant's anti-*Vibrio* activity.

^{1}H NMR spectra of *E. scaber* leaf extract and metabolites identification

The metabolites of *E. scaber* leaf extracted with various solvents (70% (v/v) ethanol, acetone, chloroform and hexane) have been identified using ^{1}H NMR analysis. Figure 1 shows an overlay image of the ^{1}H NMR spectra of different leaf extracts obtained from *E. scaber*. Forty-two metabolites were identified in *E. scaber*, including carbohydrates, amino acids, flavonoids, terpenoids, organic and fatty acids. Table 3 (Fig. S1 of the supplementary material) present the identified metabolites and their related unique signals derived from several *E. scaber* leaf extracts. The compounds were ascribed by comparing their NMR spectra with those of the reference metabolites i.e., Chenomx, and the human metabolome database which were measured under identical conditions as the extracts. The 2D *J*-resolved experiment was used to further confirm the identification of the metabolites (Fig. S2 of the supplementary material).

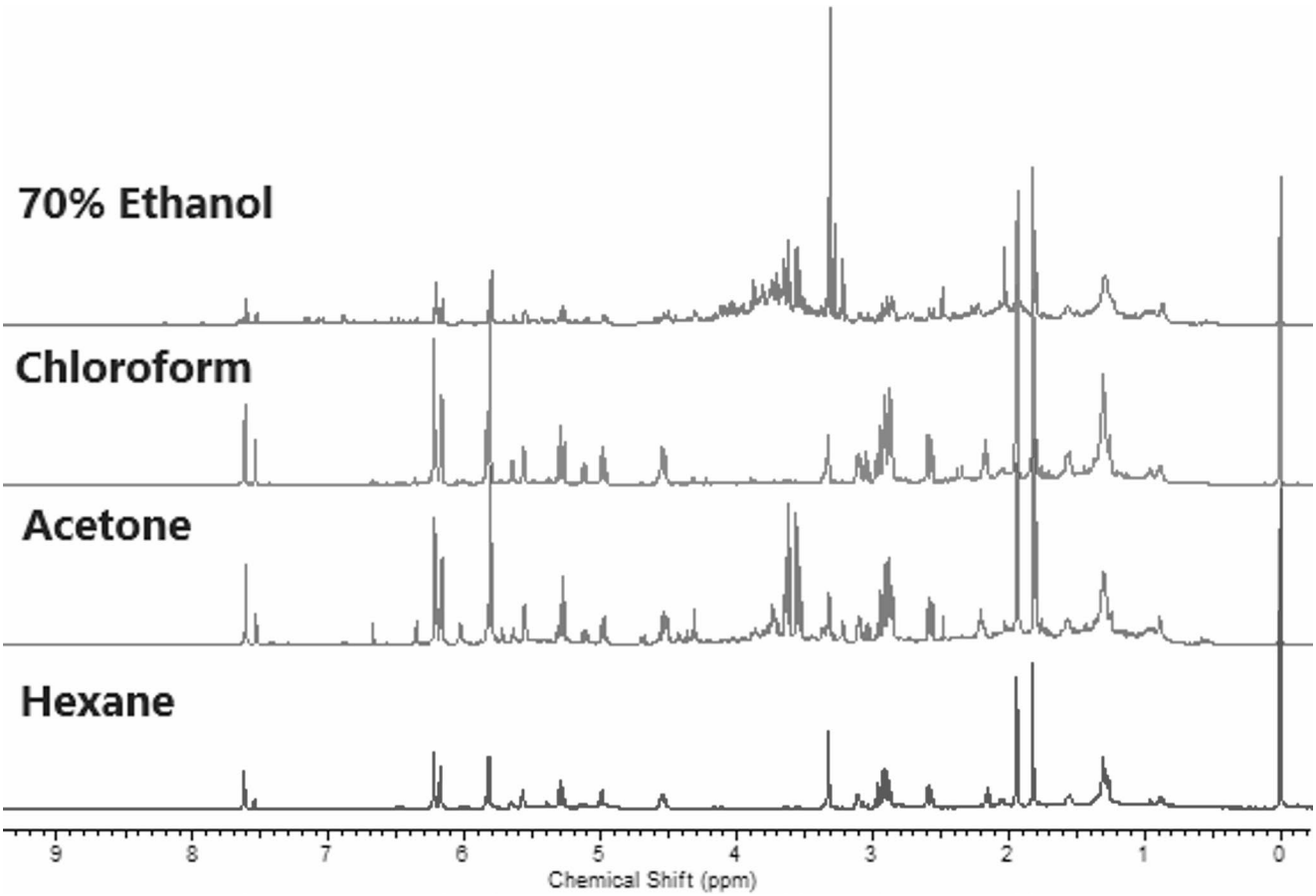

Fig. 1 Representative 500 MHz ^{1}H NMR spectra of different solvent extracts of *E. scaber* leaf

Table 3 Assignments of NMR signals for metabolites identified in ^{1}H and 2*D* NMR spectra of *E. scaber* leaf extract with corresponding multiplicity (s: singlet; d: doublet; dd: doublet of doublet; t: triplet; m: multiplet) and scalar coupling constant (*J* (Hz)) values

Peak No.	Metabolites	^{1}H Chemical Shifts (multiplicity, J)
1	Valine	1.07 (d, J = 5.0 Hz), 1.02 (d, J = 5.0 Hz)
2	Alanine	1.50 (d, J = 10.0 Hz)
3	Aspartic acid	2.86 (dd, J = 10.0 Hz, 5.0 Hz), 2.67 (dd, J = 10.0 Hz, 10.0 Hz)
4	*N*, *N*-Dimethylglycine	2.94 s
5	Isodeoxyelephantopin	1.93 s, 2.74 (d, J = 15.0 Hz)
6	Glutaric acid	2.33 (t, J = 10.0 Hz)
7	Succinic acid	2.49 s
8	Citric acid	2.59 (dd, J = 5.0 Hz, 5.0 Hz)
9	Kaempferol	6.17 (d, J = 5.0 Hz), 6.36 (d, J = 5.0 Hz)
10	Chlorogenic acid derivatives	3.57 (dd, J = 10.0 Hz, 5.0 Hz), 3.87 (dd, J = 3.0 Hz, 2.0 Hz), 7.66 (d, J = 10.0 Hz), 6.21 (d, J = 5.0 Hz), 3.75 (dd, J = 2.0 Hz, 5.0 Hz), 5.29 (dd, J = 12.0 Hz, 10.0 Hz), 5.65 s
11	Choline	3.22 s, 3.50 (dd, J = 5.0 Hz, 5.0 Hz)
12	Catechin	6.48 (d, J = 15.0 Hz), 4.60 (d, J = 10.0 Hz), 2.91 (dd, J = 5.0 Hz, 5.0 Hz)
13	Luteolin	6.55 s
14	Ascorbic acid	4.54 (d, J = 5.0 Hz)
15	Lactic acid	1.35 (d, J = 5.0 Hz), 4.09 (dd, J = 2.5 Hz, 5.0 Hz)
16	*p*-Hydroxybenzoic acid	7.94 (d, J = 10.0 Hz), 6.90 (d, J = 10.0 Hz)
17	γ-Aminobutyric acid	3.03 (t, J = 10.0 Hz)
18	Fructose	4.19 (d, J = 10.0 Hz), 4.04 (dd, J = 0.5 Hz, 1.0 Hz)
19	Ferulic acid	7.18 (d, J = 15.0 Hz)
20	Myricetin	7.30 s, 7.04 s
21	Trigonelline	4.45 s, 9.15 s
22	Quercetin derivatives	6.32 (d, J = 20.0 Hz), 6.39 (d, J = 15.0 Hz)
23	Asparagine	2.98 (dd, J = 5.0 Hz, 2.5 Hz)
24	Lupeol	4.71 s, 4.65 s, 2.03 s, 0.93 s, 0.87 s
25	*α*-Glucose	5.18 (d, J = 5.0 Hz), 5.24 (d, J = 5.0 Hz)
26	Taxifolin	6.82 (d, J = 10.0 Hz), 5.91 s, 5.85 s, 4.99 (d, J = 10.0 Hz)
27	Azelaic acid	1.26 (t, J = 7.0 Hz)
28	2-Octenoic acid	5.82 (d, J = 5.0 Hz)
29	Sucrose	5.42 (d, J = 5.0 Hz)
30	Betaine	3.28 s
31	Malic acid	6.02 s
32	Stigmasterol	5.13 (dd, J = 10.0 Hz, 10.0 Hz)
33	Deoxyelephantopin	5.15 (dd, J = 0.5 Hz, 0.5 Hz), 1.86 s
34	Scabertopin	1.79 (d, J = 1.5 Hz)
35	Betulinic acid	1.68 s, 0.96 s, 0.95 s
36	Threonine	1.30 (d, J = 5.0 Hz)
37	Isoorientin	6.67 (s)
38	*β*-Glucose	4.32 (d, J = 1.5 Hz), 3.10 (m)
39	Glycerol	3.64 (dd, J = 5.0 Hz, 5.0 Hz)
40	5-*O*-Caffeoylquinic acid	7.61 (d, J = 3.0 Hz)
41	Acetic acid	1.76 s
42	Vanillic acid	7.39 (dd, J = 3.5, 3.0 Hz)

Valine, alanine, aspartic acid, *N*, *N*-dimethylglycine, isodeoxyelephantopin, glutaric acid, succinic acid, citric acid, asparagine, azelaic acid, scabertopin, betulinic acid, threonine and acetic acid were recognized based on the signals observed in the aliphatic region of δ 0.50–3.00. Sucrose, *α*-glucose, *β*-glucose and fructose were observed in the sugar region (δ 3.00–5.50.00.50). Other metabolites present in the sugar area were chlorogenic acid derivatives, choline, ascorbic acid, γ-aminobutyric acid, betaine, stigmasterol and glycerol. Trigonelline was detected with the singlet at δ 4.45 and δ 9.15, whilst the signal at δ 1.35 (d) and δ 4.09 (dd) were ascribed to lactic acid. Moreover, the singlet at δ 4.71, δ 4.65, δ 2.03, δ 0.93 and δ 0.87 were assigned to lupeol whereas the signal at δ 5.15 (dd) and δ 1.86 (s) were detected as deoxyelephantopin. In the aromatic region (δ 5.50–8.50), luteolin, ferulic acid, 2-octenoic acid, malic acid, isoorientin, 5-*O*-caffeoylquinic acid and vanillic acid were identified with the signals at δ 6.55 (s), δ 7.18 (d), δ

5.82 (d), δ 6.02 (s), δ 6.67 (s), δ 7.61 (d) and δ 7.39 (dd), respectively. *p*-Hydroxybenzoic acid was observed with the signals of doublet at δ 6.90 and δ 7.94, whereas myricetin was detected as singlet at δ 7.04 and δ 7.30 s. The identified peaks of catechin were detectable at δ 6.48 (d), δ 4.60 (d) and δ 2.91 (dd). Meanwhile, the signal of doublet at δ 6.17 and δ 6.36 were ascribed to kaempferol, whilst the signals at δ 6.32 (d) and δ 6.39 (d) were recognized as quercetin derivatives. Taxifolin was ascribed based on the signals at δ 6.82 (d), δ 5.91 (s), δ 5.85 (s) and δ 4.99 (d).

Discrimination of *E. scaber* leaf extracts by PCA model from NMR analysis

The variation in metabolite contents of *E. scaber* samples extracted with different solvents (70% (v/v) ethanol, acetone, chloroform and hexane) were analyzed using multivariate data analysis. To identify the grouping characteristics of the four distinct solvent extracts and the compounds that influenced the variability, PCA was implemented. The score plot of PCA displayed the grouping of the *E. scaber* extracts according to their similarity, while the loading plot illustrated the contributions of the variables to the differences observed among the *E. scaber* extracts.

PCA score plot (Fig. 2A) obtained from ^{1}H-NMR data spectra showed that the four solvent extracts show different metabolite profiles. The PCA score plot demonstrates that PC1 accounted for 56.5% of the data's variation, while PC2 explained 32.5% of the variation. The different extracts of *E. scaber* were divided into four main clusters. Principal Component 1 (PC1) accounted for 56.5% of the total variance, and PC2 contributed an additional 32.5%, totaling 89.0% of explained variance—highlighting the robustness of the model in capturing the major differences among

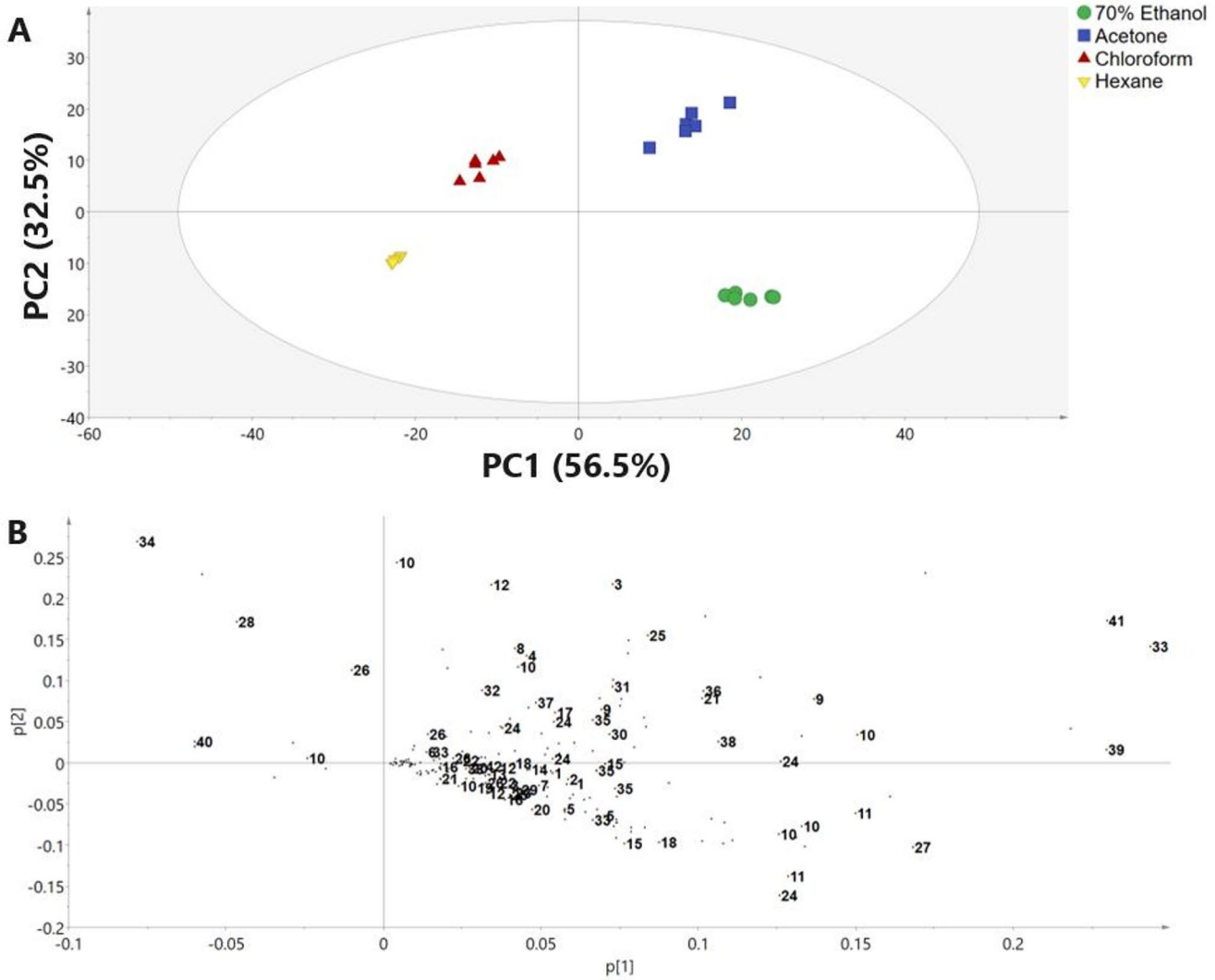

Fig. 2 The PCA score (**A**) and loading (**B**) plots of different extracts of *E. scaber*. For the interpretation of the numbers assigned to the metabolites, refer to Table 3

extracts. The clear separation into four clusters reflects the distinct metabolite signatures of each extraction solvent.

Specifically, the chloroform extract was isolated in the upper-left quadrant, the hexane extract in the lower-left, the acetone extract in the upper-right, and the 70% ethanol extract in the lower-right quadrant. This spatial arrangement indicates that chloroform and hexane extracts share some similarities (both non-polar), while acetone and ethanol extracts (more polar) group separately, consistent with solvent polarity effects on metabolite solubility.

The PC1 loading plot (Fig. 2B) showed that taxifolin, 2-octenoic acid, and 5-*O*-caffeoylquinic acid had the highest positive loadings, driving the clear separation of the chloroform extract from the others. In contrast, numerous metabolites displayed strong negative loadings, reflecting their greater abundance in the more polar extracts and highlighting the influence of solvent polarity on metabolite distribution. Specifically, the acetone extract was enriched in aspartic acid, *N*,*N*-dimethylglycine, glutaric acid, citric acid, kaempferol, chlorogenic acid, catechin, *γ*-aminobutyric acid, trigonelline, lupeol, *α*- and *β*-glucose, betaine, malic acid, stigmasterol, deoxyelephantopin, threonine, isoorientin, acetic acid, and ascorbic acid, whereas the 70% (v/v) ethanol extract contained higher levels of fructose, sucrose, alanine, asparagine, succinic acid, luteolin, lactic acid, *p*-hydroxybenzoic acid, ferulic acid, myricetin, quercetin, betulinic acid, vanillic acid, and isodeoxyelephantopin.

This distribution aligns with the expected polarity-driven extraction behavior: polar solvents (ethanol and acetone) enrich sugars, amino acids, and organic acids, whereas less polar phenolics and terpenoids accumulate in the chloroform extract. Further insights from Partial Least Squares Discriminant Analysis (PLS-DA) Variable Importance in Projection (VIP) plots (Fig. S3 of the supplementary material) confirmed these findings. Metabolites abundant in acetone and ethanol extracts showed significant VIP contributions, underscoring their importance in differentiating these extracts.

Collectively, these results highlight the significant influence of solvent polarity on metabolite partitioning and demonstrate that comprehensive metabolomic profiling of *E. scaber* requires a combination of solvents to capture its full range of bioactive compounds.

A PLS analysis was performed to establish the relationship between the identified metabolites in the extracts and their respective biological activity against the selected *Vibrio* strains. The NMR signals correspond to the *X* variables and the value of inhibition zone by disc diffusion method of the anti-*Vibrio* activities against the *Vibrio* species were represent as the *Y* variables. *R2* and *Q2* cumulative internal cross validation were performed to validate the PLS model. The *R2* metric quantifies the level of fitness of a model, while *Q2* measures the prediction accuracy of the model.

In general, an indication of better predictive quality and fairness of fit is implied when the *R2* and *Q2* values approach 1 [19]. The autofit analysis of the PLS model for *E. scaber* extracts indicated that the model can be well described by four principal components (PCs), as evidenced by the cumulative goodness of fit (*R2Y*) value of 0.965. Additionally, the model demonstrates strong predictive ability, as indicated by the cumulative *Q2* value of 0.936. Permutation tests were utilised to validate the PLS model. The cross validation and permutation tests showed no PLS model overfitting (Fig. S4 of the Supplementary material). Given that the *Y*-intercepts of *Q2* and *R2* were both lower than 0.5 and 0.05, respectively, it can be concluded that the PLS model was valid and did not exhibit any signs of over-fitting with the data [20]. Based on these results, the PLS model show an adequate fit of the model. The PLS biplot analysis (Fig. 3) revealed a significant correlation between the acetone extract of *E. scaber* and its anti-*Vibrio* activity against the *V. parahaemolyticus*, *V. vulnificus* and *V. campbelli*.

The compounds found in the acetone extract that contribute to the anti-*Vibrio* activity against *Vibrio* strains include alpha-glucose, aspartic acid, *N*, *N*-dimethylglycine, γ-aminobutyric acid, betaine, threonine, glutaric acid, citric acid, acetic acid, chlorogenic acid, malic acid, ascorbic acid, stigmasterol, kaempferol, catechin, taxifolin, isoorientin, lupeol, betullinic acid, scabertopin, deoxyelephantopin, isodeoxyelephantopin and trigonelline. These metabolites have been shown to be antibacterial against numerous bacteria, including *Vibrio* species.

Flavonoid such as catechin, kaempferol, taxifolin and isoorientin were proven to be effective against some bacteria. It has been reported that catechin was responsible for inhibiting the growth of many microorganisms, including *V. parahaemolyticusn*, *V. vulnificus* and *Aeromonas* hydrophila [21]. Kaempferol and some of its derivatives were found to be effective against *Vibrio* species, showing potent antibacterial properties [22–26]. Taxifolin has demonstrated antibacterial activity, exhibiting efficacy against an extensive range of bacteria, including *Streptococcus sobrinus* and *V. parahaemolyticus* [27, 28]. Furthermore, it is employed to improve the effectiveness of specific antibiotics, including ceftazidime and levofloxacin, which are recognised for their potential as combined therapies for patients harbouring a strain of *S. aureus* that is resistant to methicillin [29].

Isoorientin is a natural flavonoid of luteolin glycosides, found in variety of plants. This metabolite generating a lot of interest due to its multiple pharmacological activities. It has been reported that isoorientin inhibits the growth of *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas*

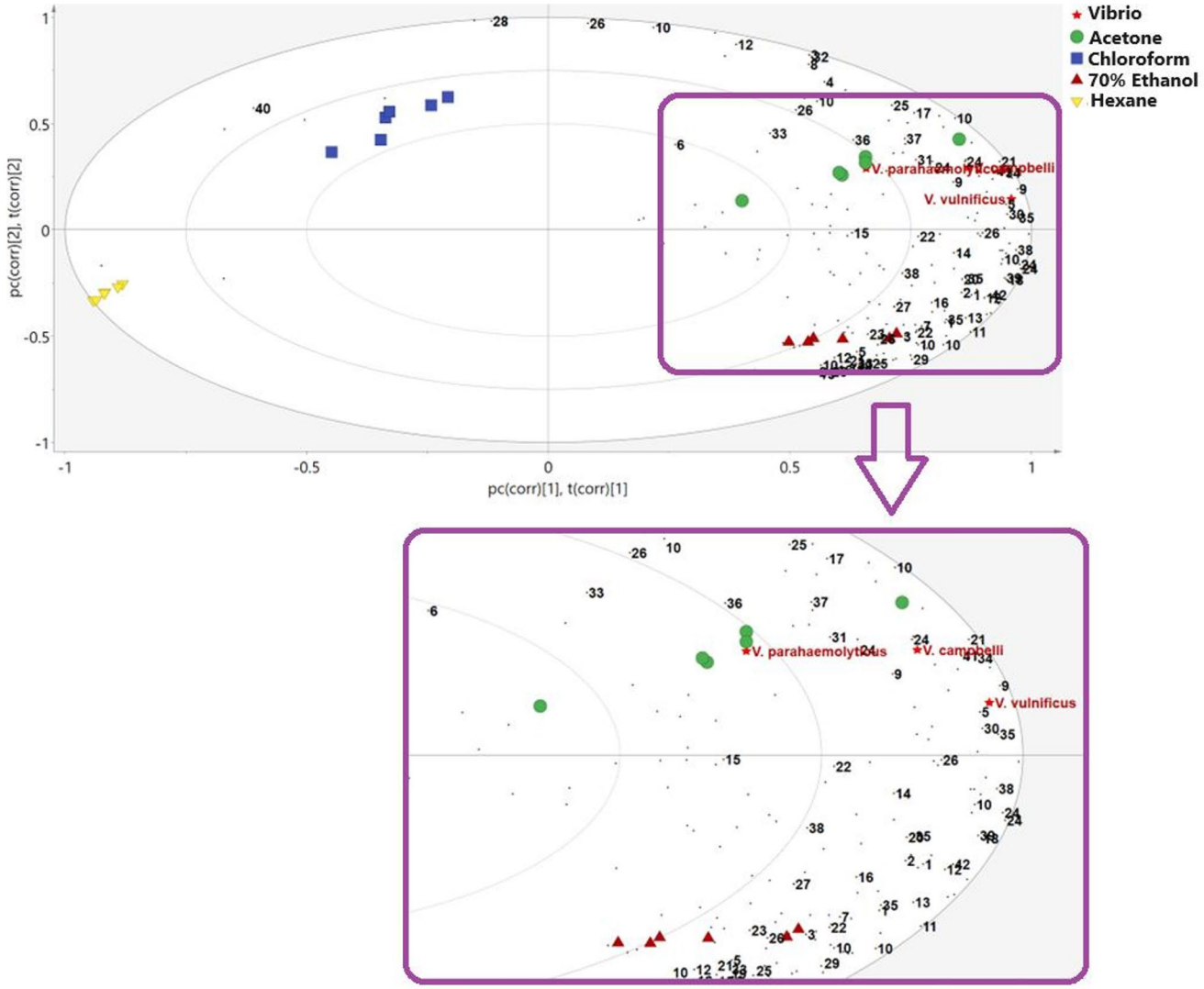

Fig. 3 The PLS biplot describing the correlation among the phytochemical constituents of different *E. scaber* extracts and their antimicrobial activities against selected *Vibrio* species. For the interpretation of the numbers assigned to the metabolites, refer to Table 3

aeruginosa and *Escherichia coli* [30]. Meanwhile, lupeol and stigmasterol isolated from the stem bark of *Delonix regia* displays potent antibacterial properties towards *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Shigella boydii*, *Shigella dysenteriae*, *Vibrio mimicus* and *V. parahemolyticus* [31].

Sesquiterpene lactones are a group of plant terpenoids that exist naturally and are known for their diverse and distinctive characteristics as natural products. They have gained significant attention for their potent antibacterial properties [32]. Scabertopin, deoxyelephantopin, isodeoxyelephantopin are sesquiterpene lactones which has been proven to be effective against some bacteria i.e., *Staphylococcus aureus*, *Staphylococcus epidermidi*, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum canis* [33]– [34].

Trigonelline is a naturally occurring quaternary alkaloid and plant hormone found in a wide variety of plants [35]. Its potent antibacterial efficacy against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Bacillus subtilis*, *Candida albicans* and *Candida parapsilosis* has garnered significant interest [36].

Chlorogenic acid is a natural phenolic chemical that is present in numerous plant species. Due to its low toxicity level, it is often used in various fields of food and conventional medicine [37]. A study done by Acosta-Smith revealed that chlorogenic acid shows antimicrobial action against *Vibrio cholerae*, *Vibrio alginolyticus*, *Vibrio furnissii*, *Vibrio fluvialis* and *V. parahaemolyticus* [38].

Antibacterial activity of betullinic acid has been reported against *Vibrio cholerea*. The compound possesses significant antibacterial against the strain [39]. A study on screening of antibacterial activity of betulin and its derivatives including betulinic acid was conducted towards *Enterobacter aerogenes*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albican.* The results revealed that betulinic acid shown antibacterial properties against the tested strains [40].

Malic acid is a metabolite that exists in several plants and used as a flavouring agent in beverages and food. It has exhibited antimicrobial efficacy against a diverse array of bacterial species, such as *Listeria monocytogenes*, *Salmonella enteritidis* and *Escherichia coli* [41]. Meanwhile, some studies revealed that malic acid is effective in preventing infection with *Vibrio* species such as *V. harveyi* and *V. parahaemolyticus* [42, 43].

Ascorbic acid has been employed for the treatment of bacterial infections, specifically *Helicobacter pylori*-associated gastritis and peptic ulcers [44]. It also could inhibit the quorum sensing mechanism in *Pseudomonas aeruginosa* and *V. campbellii*, hence decreasing the generation of virulence factors [45, 46].

Inhibitory effect of citric acid from lemon, lime and sudachi juices on the growth of *Vibrio cholerae*, *Vibrio alginolyticus*, *Vibrio mimicus*, *Vibrio anguillalum* and *V. parahaemolyticus* were investigated by Tomotake et al. [43]. The citric acid from the juices shows potent antibacterial properties against the tested *Vibrio* strains. It has been reported that *Vibrio* strains were more sensitive to acids compared to other enteric bacteria and the acid sensitivity of *Vibrio* varied from species to species [47].

Gamma-aminobutyric acid (GABA) is a crucial metabolite that plays a significant role in plant growth, development and the response to stress [48]. Phong et al. determined the GABA content in lactic acid bacteria strains from seven types of Nem Chua products and tested their antibacterial efficacy against *Bacillus subtilis* [49]. They found that the GABA level in the lactic acid bacteria strains increased and inhibited antibacterial activity against *Bacillus subtilis*.

Conclusion

The utilisation of the NMR-metabolomics approach has facilitated an effective way to investigate the variation in metabolites of *E. scaber* in relation to its diverse solvent extractions (ranging from polar to non-polar) with a biological activity i.e., anti-*Vibrio*. The use of PLS facilitated the differentiation and association between the anti-*Vibrio* activity and the specific phytochemicals present, which was attributed to the various solvents used for extraction. The acetone extract of *E. scaber* leaf shows the good extraction efficiency possessing the highest anti-*Vibrio* activity. Additionally, higher concentrations of valuable metabolites were found in the acetone extracts, which may account for their anti-*Vibrio* activity.

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Author Contributions A.I., N.S. and M.A.Z. collected samples, conducted data analysis and interpretation; A.I. prepared and finalized the manuscript; Y.R. and A.F.A.R. supervision. All authors approved the final version of the manuscript.

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Data Availability All data generated or analysed during this study are included in the Supplementary file.

Declarations

Compliance with Ethics Requirements This article contains no studies with human or animal subjects.

Consent To Participate Not applicable.

Consent To Publish Not applicable.

Competing interests The authors declare no competing interests.

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