



## Research article

## Development of plant-based emulsion formulations to control bacterial leaf blight and sheath brown rot of rice

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## ABSTRACT

Bacterial leaf blight (BLB) and sheath brown rot (SBR), caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *Pseudomonas fuscovaginae*, respectively, are bacterial diseases that lead to substantial yield losses in rice. Natural plant-based products represent a sustainable alternative to combat bacterial diseases due to their biodegradability and overall safety. However efficient ways of delivering them are crucial to their success. In an attempt to maximize the antibacterial properties of botanical bactericides for the control of these pathogens, this study evaluated the efficiency of different emulsion formulations of *Piper sarmentosum* extracts. The emulsion formulations were demonstrated to be effective in controlling BLB and SBR of rice in *in vitro* plate assays and *in planta* under glasshouse conditions. The observed *in vitro* inhibition of the bacterial pathogens and significant disease suppression *in planta* indicate that these plant extract formulations represent promising alternatives to be adopted in management strategies for controlling rice diseases.

## 1. Introduction

Bacterial leaf blight (BLB) is a vascular disease caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), and has been reported as one of the most devastating diseases of rice growing regions in the world [1]. BLB was first observed in various parts of southern Japan in the late twentieth century, and was later reported to be of economic importance in many other countries of Asia [2] including Malaysia. Xoo causes up to 70% yield losses in irrigated and lowland rice depending on the geographic location and seasonal conditions [3]. BLB infection begins as the cells of Xoo enter the epithem, the tissue that connects the water pores (hydathodes) with the xylem [4]. The bacteria then multiply and move throughout the xylem vessels, continue to grow until these vessels are blocked with bacterial cells [5]. Infection can occur during seedling or the early tillering stage. BLB symptoms include leaf yellowing with brown stripes below the leaf tip and along the leaf margins. If infection occurs at later stages, lesions expand and leaves become greyish-brown [6].

Another rice disease of economic importance is sheath brown rot caused by *Pseudomonas fuscovaginae* and is increasingly attracting attention due to its negative impact on yield. Losses of up to 58% have been reported in Japan [7], 72.2% in Indonesia and a staggering 100% in

Madagascar [8]. It was first reported a few decades ago in Hokkaido, Japan, and at the time was ranked as the most important bacterial disease of rice in this location [9]. Although this disease has been considered relatively new in Asia, it is widespread as it has been reported in all continents of the world except Antarctica [10, 11]. Sheath brown rot occurs preferentially under conditions of high humidity, low temperature and high elevation in the tropics and subtropics. Typical symptoms include sheath lesion, grain infertility, malformation, poorly filled panicles and grain discoloration [8]. Discoloured grains may have reduced quality and nutritional value. In Italy, it is estimated that, at harvest, susceptible cultivars can have more than 30% discoloured grains and the average crop loss due to sterility was 1.1% and 0.1% in 2003 and 2004, respectively [12]. Disease symptoms can be observed as early as at the seedling stage, when the disease is often lethal. At later infection stages, the lower part of the leaf sheath turns dark brown [13], and the whole leaf sheath eventually becomes necrotic in the final stage of infection [8].

*Piper sarmentosum* is a creeping tropical herb plant affiliated to the family Piperaceae. This plant occurs in different countries of Southeast Asia including Malaysia, Thailand and Indonesia, where it is known locally as 'Pokok Kadok', 'Chaplu', and 'Sirih duduk' or 'Mengkadak', respectively [14, 15, 16]. This plant has been used traditionally in different parts of Southeast Asia for its numerous medicinal purposes [17]. Chemical analyses of

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*P. sarmentosum* have revealed bioactive compounds that are detrimental to plant pathogens, such as flavonoids, alkaloids, phenolic compounds, steroids and propenylphenols [18, 19, 20].

Despite extracts of *P. sarmentosum* have been shown to possess anti-bacterial activity against rice pathogens [18, 19, 20], the development of formulations for its use as natural plant protectants has still not been sufficiently explored. A hypothetical ternary phase diagram consisting of three components of the system can be defined as the isotropic system of a mixture of suitable surfactants, oil, and water [21, 22]. Bancroft's rule states that, for predicting micro-emulsion types, water-soluble emulsifiers tend to form oil-in-water (O/W) emulsions, and oil-soluble emulsifiers tend to form water-in-oil (W/O) emulsions [23]. Emulsions can be divided into three types: 1) W/O emulsion, where water is the dispersed phase and oil is the continuous phase; 2) O/W emulsion, in which oil is the dispersed phase and water is the continuous phase; and 3) multiphase emulsion, which consists of both W/O emulsion and O/W emulsion simultaneously [24]. Micro-emulsions are thermodynamically stable, isotopically clear dispersions of two immiscible liquids such as oil and water stabilized by the interfacial film of any surfactant and/or co-surfactant. They are known to improve the solubility, stability and efficacy of bioactive compounds [25]. Mounting evidence supports the use of formulations containing natural products as they typically have lower toxicity to non-target organisms and renewable resources, are highly biodegradable and usually more economical compared to synthetic chemical pesticides [26, 27]. Therefore, the objective of this study was to evaluate several formulated plant extracts and their effectiveness in inhibiting the growth of *P. fuscovaginae* and Xoo and disease suppression in rice plants. Effective management options for bacterial diseases in plants are scarce and often rely on chemical control of bacterial diseases with antibiotics and copper-based compounds [28]. However, development of resistant pathogens to these chemicals and effects on untargeted organisms are major environmental concerns [29]. If natural plant products are efficient in controlling disease development, formulations containing these plant extracts can potentially be environmentally safe alternatives as components in integrated pest management programs.

## 2. Materials and methods

### 2.1. Development of plant-based formulations

A methanol crude extract of *P. sarmentosum* was used in this study to develop the formulations. Plants extracts were prepared as previously described [18]. Concentrated liquid extracts (40% a.i) were obtained by dissolving the solid crude extracts in methanol (w/v). Termul 5030 (non-ionic blend surfactant) and Termul 1284 (ethoxylated castor oil) were selected and used as surfactants (KC Chemicals, Malaysia) and the proportion of each surfactant in the formulation is described in Table 3. Three formulations were developed, namely EF1, EF2 and EF3. The formulations were dissolved in water (1:1) before application on rice plants.

### 2.2. Chemical analysis of leaf crude extracts of *P. sarmentosum*

The crude extract was analysed using gas chromatography-mass spectrometry (GC-MS). The equipment used was a GCMS-QP2010 Ultra with a SGE-BPX5 fused silica capillary column of 30 m long with 0.25 mm inner diameter and 0.25  $\mu\text{m}$  film thickness. Injection temperature and the interface were set to 250 °C and ion source to 200 °C. The temperature was gradually increased from 50 °C for 10 min to 300 °C at a rate of 5 °C  $\text{min}^{-1}$ . The chromatograms and mass spectra were identified and interpreted using the National Institute of Standards and Technology (NIST) 09 spectral data library. Retention time and mass spectral library for automatic peak quantification of unknown compounds were applied following the NIST format. The spectra obtained from the unknown compounds were compared to the known compounds in the NIST library

and the structure, molecular weight and name of the compounds established. The percentage composition of the crude extract constituents was expressed as a relative percentage of the total peak area.

### 2.3. Construction of ternary phase diagrams

The screening of different components of the formulation (e.g. oil phase and surfactants) was performed before constructing the pseudo-ternary phase diagram. The emulsions were prepared using the aqueous titration method [30]. The three components of phase diagram systems consisted of aqueous, surfactant and oil phase [31]. To investigate the validity of Bancroft's rule, emulsions were prepared using different oil to surfactants ratios (w/w) with different solubility by centrifugation. The components consisted of plant crude extracts, Termul 1284 or Termul 5030 (surfactants) and methyl ester (oil). The ratio of the mixture included eleven combinations, 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10 (w/w). A total of 0.5 mL of oil and surfactant were weighed into 10-mL screw cap test tube. Titration of water into the mixture of oil and surfactant at which changes occurred were taken as the point to determine the phase areas. Emulsions are stabilised when a surfactant is added to the two-phase system caused by the slowdown of emulsion breaking such as coalescences [32]. The phase behaviour and formation of emulsion of the ternary mixtures prepared for each sample were evaluated visually for spontaneous emulsification based on clarity, stability and transparency of the mixtures at room temperature. The ternary phase diagram was then plotted. The points were marked on the three-component phase diagram to indicate the ratio of the surfactant, oil and water obtained. The isotropic area of emulsion in the phase diagram systems are indicated by the joining points. All the constructed phase diagram system was defined based on their surfactant phase (Table 1) with methyl ester as the oil phase and water as the aqueous phase. Samples were vortexed in test tubes and centrifuged for 15 min at 2,058 x g. The stability of the emulsion system was assessed by obtaining a transparent one-phase [33]. The conductivity for each emulsion was measured periodically with the drop test for 1 h at room temperature to ensure that there was no inversion occurred [34].

### 2.4. Selection of formulation composition from ternary phase diagram

The construction of pseudo-ternary phase diagrams was conducted using Chemix software, version 3.6 phase diagram plotter (UK). Based on the isotropic part in the phase diagram, several points of different oil/surfactant ratios within isotropic area in the phase diagram were chosen so that the increasing size of proportions in the system is dynamically stable. Different formulations were selected from each of the constructed phase diagrams to incorporate plant crude extracts into the solution. Formulations that were miscible with the crude extract and remained homogeneous were selected. Selected formulations were subjected to particle size determination, stability and thermal stability tests, polydispersity index test and zeta potential as described below.

### 2.5. Phase behaviour analysis of the emulsion formulation

**Particle size determination:** the particle size of the formulation system was measured by Nanophox Sympatec (Germany) particle size analyser equipped with Photon Cross Correlation Spectrophotometer (PCCS) using Windox 5 Software via 3D cross correlation technique. Each sample was diluted with distilled water. Samples concentration from 0.0001

**Table 1.** Amount of emulsion components in the formulations.

Emulsion components	Function	Amount (in %)
Crude extracts and water	Aqueous phase	90
Methyl ester	Oily phase	0–5
Termul 1284/5030	Surfactants	5–10

Vol.-% to approx. 20 Vol.-%. The samples were transferred into 1-cm<sup>s</sup> cuvettes and placed in a thermostat chamber, illuminated by vertical polarized helium neon laser (632.8 nm) at 25 °C.

**Stability test:** the selected formulations were centrifuged at 3,500 rpm for 30 min. They were then maintained at room temperature for four weeks and evaluated for their ability to keep a transparent single-phase form after four weeks of storage to confirm the presence of an emulsion [35, 36].

**Thermal stability study:** aliquots of the selected pre-formulation concentrates were monitored at a constant temperature of 25 °C for 2 months and at 54 °C for 2 weeks, by monitoring an aliquot of the emulsion. This test estimates the emulsion quality.

**Polydispersity index (PDI):** this index was calculated to estimate the particle size distribution of nanoparticles obtained from the photo-correlation spectroscopic analysis. It is a dimensionless number inferred from the autocorrelation function. It ranges from a value of 0.01 for mono dispersed particles and up to values of 0.5–0.7. Samples of very wide size distribution usually have polydispersity index values of >0.7 [37].

**Zeta potential analysis:** this represents the surface charge that was determined by measuring the electrophoretic mobility of the nanoparticles using a Malvern zetasizer (Malvern Instruments, UK). For this particular analysis, formulation samples were prepared by dilution in distilled water [38].

## 2.6. *In vitro* assessments of antimicrobial activity of emulsion formulations against rice pathogenic bacteria

Bacterial cultures of *P. fuscovaginae* (JX915743.1) and *X. oryzae* pv. *oryzae* (CP000967.1) were obtained from the bacterial collection from the Microbiology Laboratory, Department of Plant Protection, UPM, Selangor, Malaysia. The bacterial cultures were prepared as previously described [18].

The antibacterial activities of methanol leaf extracts were evaluated by agar well diffusion [39] and sterile disc diffusion assay [40]. An aliquot of a bacterial culture in Mueller Hinton broth (MHB) (Merck) was inoculated onto the surface of Mueller Hinton agar (MHA) (Merck) using a sterile inoculation loop. Four wells with the diameter of 8 mm were punched into the agar using a sterile cork borer. A volume of 50 µL of formulation emulsion was added to one well, and, to the other three wells, the same volume of crude extract, methanol (as negative control) and streptomycin sulfate (100 µM) (as positive control) were added, respectively. Plates with *P. fuscovaginae* and Xoo, were incubated at 30 °C and 28 °C, respectively for 24 h. The antibacterial activity of the formulation emulsions was evaluated and compared to the crude extract by measuring the zone of inhibition. The experiment was conducted in triplicate and repeated twice.

In the sterile disc diffusion assay, sterile paper discs (10 mm) were saturated with each crude extract, emulsion formulation, methanol and streptomycin. The paper discs were then dried at room temperature under the laminar flow to avoid contamination. Streptomycin sulphate was used as positive control, and methanol was used as negative control. The inoculum (50 µL) was pipetted onto the media and spread using an L-shaped glass rod. All the sterile disc was carefully placed on the surface of MHA containing the bacterial inoculum. Four discs were placed at a uniform distance and firmly pressed down using sterile forceps. The plates of *P. fuscovaginae* and Xoo, were incubated at 30 °C and 28 °C, respectively for 24 h. The experiment was conducted in triplicates and repeated twice.

## 2.7. Effect of plant extract formulations on the incidence of BLB and SBR under glasshouse conditions

Our previous studies demonstrated that crude extracts of *P. sarmentosum* and their emulsion formulation were highly effective in suppressing *in vitro* growth of *P. fuscovaginae* and Xoo (Syed Ab Rahman et al. 2014a, b) (Table 3). Therefore, we tested the suppressive potential

of crude extracts and emulsion formulations against SBR and BLB under glasshouse conditions. Temperature was set to 30 ± 2 °C with 80% humidity. Trials were conducted at the experimental farm of the Faculty of Agriculture, University Putra Malaysia. Rice seeds were surface-sterilised by a soaking treatment for 1 min in absolute ethanol and followed by 0.1% sodium hypochlorite solution for 20 min. Seeds were then thoroughly rinsed with sterilised distilled water, and subsequently imbibed in sterile distilled water for 24 h. Sterilised seeds were sown in plastic pots (W: 500 H: 380 L: 500) containing clayey loam soil (10 seeds/pot). Cultures of *P. fuscovaginae* and Xoo (grown at 30 °C and 28 °C) were centrifuged and bacterial pellets were resuspended in water at a concentration of 1 × 10<sup>8</sup> CFU mL<sup>-1</sup>. A volume of 500 mL of the emulsion formulations, streptomycin sulphate (100µM) and sterile distilled water (negative control) was applied on the stem and leaves of rice plants by spraying, two days prior the inoculation of pathogens. Inoculation of the Xoo and *P. fuscovaginae* was conducted on leaves of seven day-old plants by two methods: clip and pin-prick inoculations. By the clip-inoculation method, sterile scissors were dipped in the inoculum solution and used to cut three cm at the leaf tips [41]. The pin-prick inoculation consists of dipping a sterile pin in the inoculum solution and subsequently to prick the leaves with this pin on the sides and in the centre [10]. Three biological replicates consisting of five pots each were included per treatment in a completely randomised design under glasshouse conditions. Each pot contained ten plants. Observation of disease symptoms were recorded seven days after the pathogen inoculation. Disease symptoms and infection were assessed on the infected leaf sheath and the length of the lesion measured. The severity of disease was evaluated in each treatment based on the score chart scale of 0–9, (0 = healthy; 1 = 1–5%; 2 = 6–10%; 3 = 11–25%; 5 = 6–50%, 7 = 51–75%, and 9 = > 76% of the leaf area infected) with some modifications [8]. Disease severity for each treatment was calculated using the formula = (Sum of all disease rating/Total no. of rating x maximum disease score) x 100.

## 2.8. Statistical analysis

Glasshouse trials were repeated twice. Hence, results from duplicate tests were collected for the final data analysis. Analyses of variance were performed using JMP12 software. Data obtained from this experiment were subjected to one-way analysis of variance (ANOVA) using Tukey's honest significant difference test (HSD). Differences were assigned significant at  $P < 0.05$ .

## 3. Results

### 3.1. Chemical compounds detected in leaf crude extracts of *P. sarmentosum*

GC-MS analyses of crude leaf extracts of *P. sarmentosum* revealed 50 different organic compounds (Table 2). Chromatograms obtained from the GC-MS analysis of crude extracts are shown in Figure 1. The main identified compounds were Asarone (15.93 %), beta-asarone (16.70 %) and 1, 3-Benzodioxole, 4-methoxy-6-(2-propenyl) (7.43%). Eleven unknown compounds were detected. Another compound identified in the crude extract was 1, 3-Benzodioxole, 4-methoxy-6-(2-propenyl) (Myristicin).

### 3.2. Pseudo-ternary phase diagram of emulsion formulation systems

Oil in water (O/W) emulsion was formed using surfactant with hydrophilic and lipophilic balance (HLB) value greater than 10, namely Termul 1284 (HLB = 12.6) and Termul 5030 (HLB = 13.6). Two ternary phase diagrams were constructed (Figure 2 and Figure 3). Two phase systems were observed in the diagrams. Both diagrams showed one out of two phase regions in the emulsion formulation systems. One phase of isotropic area was observed towards the aqueous rich apex as shown in Figure 2 and Figure 3. Three points were selected from their isotropic

**Table 2.** Composition of chemical constituents in the methanol extract of fresh leaves of *P. sarmentosum*.

ID#	Name of compounds	Ret. time	Ret. index	Conc. unit	S/N
1	Butanal, 2-methyl-	2.693	696	0.359 %	104.72
2	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	17.883	1158	1.880 %	181.37
3	4-vinylphenol	21.543	1234	2.221 %	196.99
4	Hydrocinnamate < methyl->	24.087	1288	0.497 %	132.22
5	Naphthalene <1,2,3,4,4a,7-hexahydro-, 1,6-dimethyl-, 4-(1-methylethyl)-, (1alpha,4beta,4beta)-(±)->	28.373	1380	0.695 %	23.35
6	Cubebene < alpha->	28.92	1392	0.750 %	8.87
7	Methyl eugenol	29.925	1415	1.810 %	1.01
8	Caryophyllene	30.44	1427	4.037 %	3.26
9	alpha.-Humulene	32.063	1464	0.450 %	147.47
10	Myristicin	32.345	1470	0.474 %	7.31
11	Copaene < alpha->	32.87	1482	0.439 %	2.01
12	Cubebene < alpha->	33.187	1489	2.136 %	141.49
13	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4a.alpha. 7. alpha., 8a.beta.)]-	33.582	1498	0.294 %	29.01
14	bicyclogermacrene	33.817	1504	1.664 %	5.47
15	<b>1,3-Benzodioxole, 4-methoxy-6-(2-propenyl)</b>	34.232	1514	<b>7.426 %</b>	23.22
16	Unknown	34.57	1522	0.529 %	58.73
17	Cadinene < delta->	34.73	1526	1.229 %	0.34
18	Unknown	35.185	1537	0.449 %	9.49
19	Benzenepropanoic acid, 4-methoxy-, methyl ester	35.588	1547	1.227 %	5.25
20	Cyclohexanemethanol, 4-ethenyl-.alpha., .alpha, 4-trimethyl-3-(1-methylethenyl)-, [1R-(1.alpha. 3. alpha., 4.beta.)]-	36.132	1560	1.539 %	4.88
21	Benzene, 1,2,3-trimethoxy-5-(2-propenyl)-	36.205	1561	0.563 %	60.92
22	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, (E)-	36.455	1567	0.702 %	0.37
23	<b>Asarone</b>	37.073	1582	<b>15.926 %</b>	855.98
24	Caryophyllene oxide	37.642	1596	0.356 %	11.26
25	4-(4-Methoxyphenyl)-1-butanol	37.94	1603	1.150 %	17.27
26	Trans (.Alpha.)-asarone	38.942	1629	1.958 %	48.85
27	Apiol	40.422	1666	2.970 %	74.04
28	<b>beta-asarone</b>	41.585	1696	<b>16.703 %</b>	606.46
29	Unknown	43.077	1735	8.339 %	134.44
30	Unknown	45.928	1812	1.051 %	605.26
31	Unknown	46.225	1820	0.358 %	20.70
32	Neophytadiene	46.782	1836	0.462 %	10.16
33	Unknown	48.335	1879	0.329 %	55.09
34	Hexadecanoic acid, methyl ester	50.143	1931	0.387 %	36.70
35	n-Hexadecanoic acid	51.538	1972	0.829 %	2.52
36	Unknown	52.432	1998	2.631 %	25.36
37	Linolenate < methyl->	55.955	2107	0.338 %	61.77
38	Phytol	56.212	2115	8.227 %	422.07
39	9,12-Octadecadienoic acid (Z,Z)-	57.117	2144	0.324 %	19.53
40	7-Tetradecenal, (Z)-	57.33	2151	0.851 %	9.45
41	Unknown	58.06	2174	0.216 %	2.74
42	Octanoic acid, 2-dimethylaminoethyl ester	61.525	2289	0.493 %	48.57
43	Fumaric acid, 2-dimethylaminoethyl heptyl ester	66.64	2470	0.323 %	28.37
44	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	68.265	2530	0.990 %	34.89
45	Unknown	73.028	2714	0.282 %	0.53
46	Benzene, 1-methoxy-4-(phenylethynyl)	73.518	2734	0.806 %	13.70
47	Vitamin E	83.075	3147	0.519 %	30.26
48	Unknown	85.693	3267	0.337 %	3.58
49	Unknown	86.3	3295	0.462 %	3.50
50	Stigmast-5-en-3-ol, (3.beta.)	88.002	3373	1.017 %	8.31

S/N: signal-to-noise ratio.

regions as emulsion formulations and coded as shown in Table 3 (EF1, EF2, and EF3). All formulations comprise of 5–10% surfactant, 0–5% oil, and 90% crude extracts after addition to the *P. sarmentosum* extract.

Termul 1284 and Termul 5030 showed an adequate miscibility when mixed with water where only 2-phase regions were observed in the system.



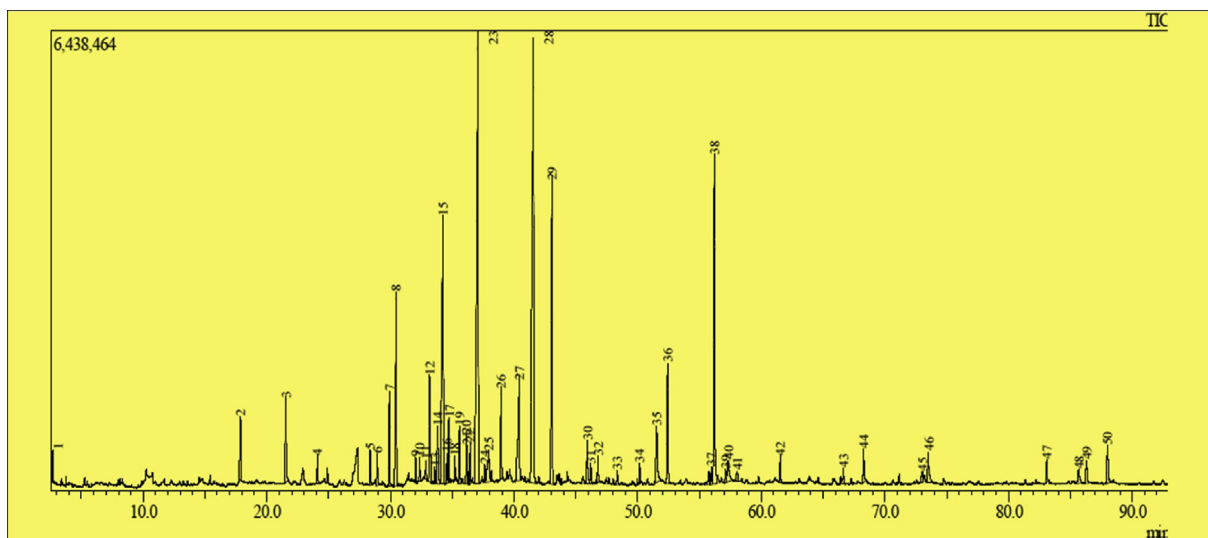


Figure 1. Total-ion current (TIC) of GC-MS chromatogram of 50 major compounds in a *P. sarmentosum* leaf extracts.

### 3.3. Phase behaviour analysis of the emulsion formulations

Oil in water (O/W) and water in oil (W/O) microemulsions usually have a size range of 0.1–5 μm with an average of 1–2 μm [42]. The particle size of the emulsion formulations ranged from 0.0783 to 0.198 μm (Table 4). All the formulations remained stable after the exposure at 25 °C and 54 °C. The polydispersive index (PDI) varied between 0.0 and 1.0. PDIs closer to 0.0 indicate narrow size distribution of the formulation. The PDIs of the developed formulation were 0.59, 1.00 and 0.29, which means that the prepared microemulsion is monodispersive in nature, stable and the particles will not coalesce to form bigger droplet size.

The storage stability of colloidal dispersions can be assessed by measuring the zeta potential or particle size distribution of the formulation system. In general, due to electric repulsion, particle aggregation will probably not occur for charged particles. The value of zeta potential obtained for all the emulsion formulations were above 30 mV (Table 4). Zeta potential values over 30 mV (positive or negative values) create

### Termul 5030-Oil-Water

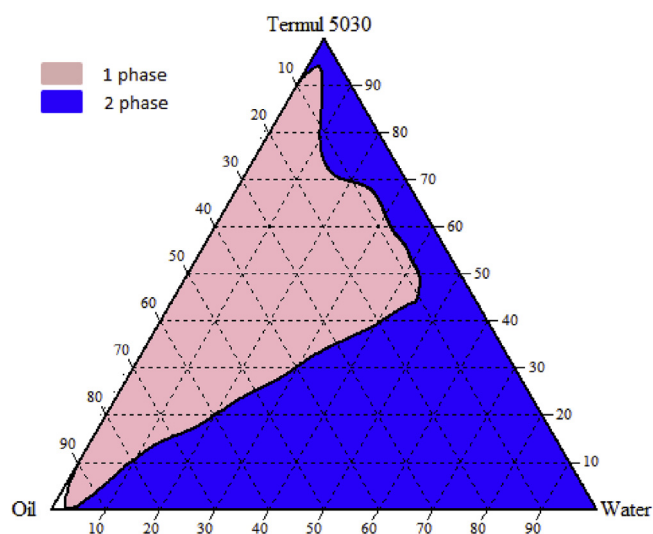


Figure 3. Phase diagram system of Termul 5030/methyl ester/water with 20% (w/w) active ingredient.

### Termul 1284-Oil-Water

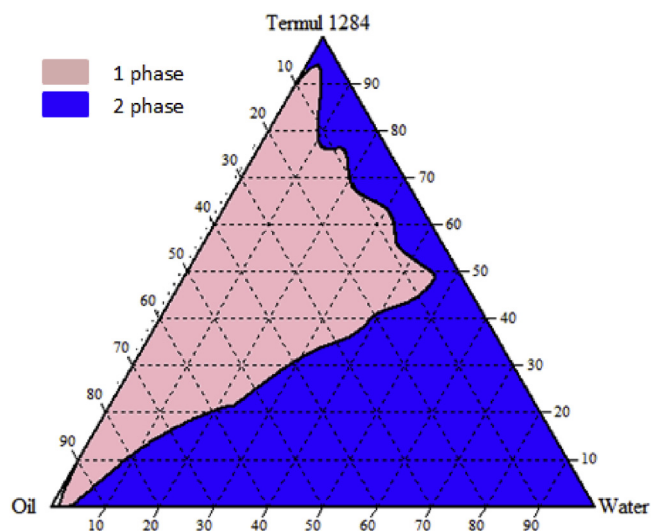


Figure 2. Phase diagram system of Termul 1284/methyl ester/water with 20% (w/w) active ingredients.

Table 3. Ratios of the mixture for each formulation.

No.	Compounds	Proportion	Code
1	Termul 5030 (1)/Methyl ester/Crude extract	(4.9:5.1:90)	EF1
2	Termul 5030 (2)/Methyl ester/Crude extract	(5.9:4.1:90)	EF2
3	Termul 1284/Crude extract	(10:90)	EF3

Table 4. Characterization of the emulsion formulations of *P. sarmentosum* crude extract.

Formulations	Particle size (d.nm)	Polydispersive index (PDI)	Zeta potential (mV)	Cond. (mS/cm)
EF1	78.3	0.294	-30.9	0.95
EF2	112	1.0	-32.5	1.9
EF3	198	0.595	-31.2	0.83

more stable suspensions due to repulsion between the particles that prevents their aggregation [43].

### 3.4. *In vitro* assay of the antimicrobial activity of emulsion formulations against rice pathogenic bacteria

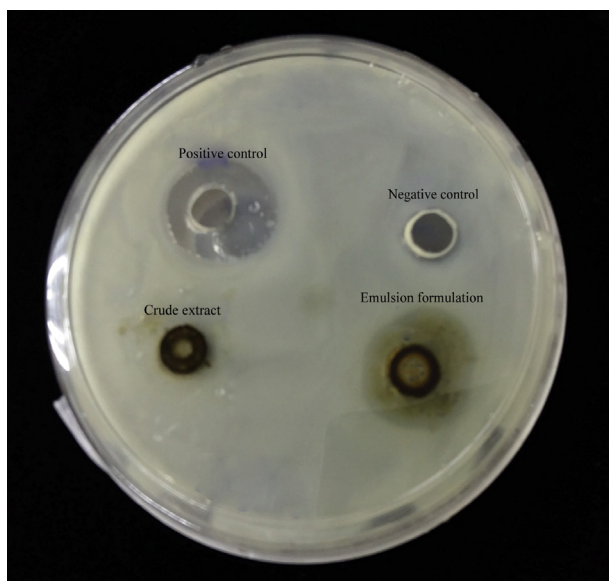
The emulsion formulation of *P. sarmentosum* significantly inhibited the growth of *P. fuscovaginae* and Xoo in plate assays. In agar well diffusion assay, the emulsion formulations have larger inhibition zones compared to crude extracts alone for both bacteria (Figure 4; Table 5). Similar results were observed in the disc diffusion assay (Table 5).

### 3.5. Effect of plant extracts formulation on the incidence of BLB and SBR in rice under glasshouse conditions

The glasshouse experiments indicated that spraying rice plants with the emulsion formulations resulted in a significant reduction in sheath brown rot and bacterial leaf blight symptoms in rice (Table 6). Disease severity of SBR (between 25 to 30%) had a reduction of 55–61% after the treatment with emulsion formulations. For BLB, the disease severity was 14.8% for all the emulsion formulations, and the disease was reduced by 80.89% with the application of emulsion formulations.

## 4. Discussion

Rice diseases have a significant effect on rice production. Considering the large rice production area in the world, even a conservative estimate of 1–5% annual yield loss from a disease would translate into millions of tons of rice and billions of dollars lost in growers' incomes [44]. Rice diseases cause a significant impact on rice supply [45]. The use of plant products and biocontrol agents for the control of plant diseases is a pressing need for agriculture due to its readily available nature, antimicrobial activity, biodegradability, non-phytotoxicity, systemicity after application, besides minimised risks and harmful effects when compared to toxic synthetic pesticides [46, 47, 48]. This study describes the formulation of plant extracts of *P. sarmentosum* oil in water emulsion with natural surfactants for the biocontrol of BLB and SBR. The observed reduction in plant symptoms under glasshouse conditions indicates that these formulations of plant extracts and oils represent potential resources for alternative management strategies for control of *P. fuscovaginae* and Xoo.



**Figure 4.** Inhibition zones on Mueller Hinton agar plates by agar well diffusion assay of EF1 emulsion formulation of *P. sarmentosum* crude extract against Xoo.

**Table 5.** Antibacterial activity of emulsion formulations of *P. sarmentosum* crude extract against rice pathogenic bacteria, *Pseudomonas fuscovaginae* and *Xanthomonas oryzae* pv. *oryzae*.

Bacteria Treatments	<i>P. fuscovaginae</i>		Xoo	
	Agar well diffusion (cm)	Disc diffusion (cm)	Agar well diffusion (cm)	Disc diffusion (cm)
EF1	19.6 ± 0.86 b	15.42 ± 0.97 bc	20.8 ± 0.52 a	15.78 ± 0.86 b
EF2	19.73 ± 0.77 b	15.7 ± 0.81 b	19.48 ± 0.5 b	15.55 ± 0.83 b
EF3	19.67 ± 0.58 b	15.88 ± 0.9 b	20.25 ± 0.51 ab	15.62 ± 0.94 b
CE	17.52 ± 0.85 c	14.3 ± 0.44 c	17.43 ± 1.05 c	14.57 ± 0.69 b
PC	21.12 ± 0.44 a	20.97 ± 0.27 a	21.18 ± 0.4 a	20.08 ± 0.61 a
NC	0 d	0 d	0 d	0 c

Within columns, values with different letters indicate significantly significant differences (Tukey Kramer HSD,  $P < 0.05$ ). All the tests were performed in triplicate. The experiment was repeated twice. EF: Emulsion formulation CE: Crude extract PC: Positive control (Streptomycin sulphate, 100 ppm) NC: Negative control (Methanol (v/v) IC: Infected control.

**Table 6.** Effect of *P. sarmentosum* emulsion formulation on sheath brown rot (SBR) and bacterial leaf blight (BLB) disease severity and reduction of rice plants under glasshouse conditions.

Treatments	Disease severity (%)		Reduction (%)	
	SBR	BLB	SBR	BLB
EF1	29.63 ± 1.03 ab	14.81 ± 0.82 a	55.56 ± 0.12 a	80.96 ± 0.54 a
EF2	25.9 ± 0.82 b	14.81 ± 0.82 a	61.15 ± 0.18 a	80.96 ± 0.54 a
EF3	29.63 ± 1.03 ab	14.81 ± 0.82 a	55.56 ± 0.09 a	80.96 ± 0.54 a
PC	11.11 ± 1.1 a	9.26 ± 0.82 a	83.34 ± 1.3 b	88.09 ± 0.9 b
IC	66.67 ± 0.21 c	77.78 ± 0.41 b	0.0	0.0

Values in the column represent the mean ± SE. Different letters within columns indicate statistically significant differences between treatments according to the least significant difference test ( $P < 0.05$ ). PC: positive control, IC: infected control, SBR: sheath brown rot, BLB: bacterial leaf blight.

Surfactants are important in stabilizing different inclinations of homogeneous regions in phase diagram [49]. Termul 1284 is an ethylated castor oil, a non-ionic plant-derived surfactant that functions as emulsifiers and solubilizers [50]. Termul 5030 is a blend surfactant made of a mixture of non-ionic emulsifiers, alky aryl sulfonates and iso-butanol. The best and most efficient emulsifiers are non-ionic surfactants that can be used to emulsify O/W or W/O. Furthermore, they are able to stabilize the emulsion against flocculation and coalescence [42]. In this study, non-ionic surfactants were used because of their compatibility, relatively low toxicity compared to ionic ones and negligible environmental impact [51].

The emulsion formulation was more effective in suppressing bacterial growth in comparison to the crude extract in both assays. Biological membranes are comprised of lipids, proteins, and lipoproteins. Several antimicrobial agents can disrupt bacterial membranes [52]. The emulsion formulation of *P. sarmentosum* extracts may have facilitated this process by the presence of surfactants and increased the susceptibility of the bacterial cells to antimicrobial compounds present in the extract. Radulovic et al. (2013) described that antibacterial mode of action usually includes the interaction of plant secondary metabolites with the cell membrane, diffusion through the membrane and interaction with intracellular constituents/processes.

The most predominant volatile compounds identified in this study are chemically and biologically important. Beta-asarone from *Acorus calamus* exhibits very strong antimicrobial activities against fungi [53]. Beta-asarone obtained from the extract of *Acorus gramineus* exhibited antifungal activity *in vitro* and *in vivo* against the plant fungal pathogens *Magnaporthe grisea* and *Colletotrichum orbiculare*. This extract also inhibited the growth of *Bacillus subtilis*, *Erwinia carotovora* subsp.

*carotovora*, *Ralstonia solanacearum*, and *Xanthomonas campestris* pv. *vesicatoria* [54]. Myristicin isolated from *Myristica fragrans* was demonstrated to have antibacterial activity against Gram-positive and Gram-negative bacteria [55].

Our previous study showed that *in vitro* antibacterial activity of *P. sarmentosum* leaf and fruit extracts were highly inhibitory to the growth of *P. fuscovaginae* and Xoo [19]. Later, another study demonstrated that *P. sarmentosum* plant extracts and essential oils exhibited the antibacterial activity against Xoo and other rice pathogens [56]. Leaf extracts of *Allium sativum*, *Azadirachta indica* and *Zingiber officinale* were also reported to be antagonistic towards Xoo [57]. Our recent study has also shown that preliminary laboratory experimental of the bio-formulations improved seed germination and seedling vigour. It also enhanced seedling height, root length, seedling and root dry weight compared to the control and a significantly lower disease incidence and severity were observed in rice treated with the bio-formulations under glasshouse condition [58]. The inhibition of *P. fuscovaginae* and Xoo by *P. sarmentosum* extracts may be attributed to the bacteritoxic principles: (i) bacteriostatic action which implies a continuous interference as long as the organism is in the contact of toxicant or (ii) bactericidal action which denotes a persistent action even after the withdrawal of toxicant and toxic substances present in the plant extracts. The antibacterial effect of the tested emulsion formulations could also be related to their direct toxic effect on the pathogens [59]. Mechanisms of disease suppression of plant products have been associated with chemically active compounds that may either affect the pathogen directly [60] or elicit systemic resistance in host plants which results in the interruption of disease development [61].

## 5. Conclusion

Further research is necessary to explore the efficacy of natural plant products against plant diseases in crop management systems. It has been increasingly important that factors such as sustainability, production efficiency and environmental protection are taken into account. Increasing crop yields and preventing plant diseases and pests through sustainable approaches such as the application of formulations with plant-based compounds are paramount to meet future food demands without further damage to the environment.

## Declarations

### Author contribution statement

S. F. Syed-Ab-Rahman: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

L. Costa Carvalhais: Analyzed and interpreted the data; Wrote the paper.

D. Omar: Contributed reagents, materials analysis tools or data.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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