Potential of Bioactive Substances of *Piper caninum* and *Piper betle* Var. Nigra Leaves as Biopesticide in Organic Bali Rice

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Abstract

Rice is consumed as a staple food by majority of the people in the world and failure in rice crop, due to any reason, poses a severe threat of starvation. Rice blast, caused by a fungus blast has been ranked among the most important plant diseases. It is by far the most threatening disease of ric crop and it is found wherever rice is grown. All of the rice blast disease management strategies that have been employed have limited success and rice blast has never been eliminated from a region in which rice is grown. Hence there is need to look for the best remedy in terms of effectiveness and organic nature of the method etc. This study was aimed to determine the plant growth promoting and biopesticidal effects of bioactive components present in a mixture of *Piper caninum* and *Piper betle* var. Nigra leaf extracts. . The extracts were applied in the field to determine their inhibition effects against blast disease, growth and yield improvement.. Extract of both the plants promoted plant growth and exhibited antifungal activity against rice blast fungus, *Pyricularia oryzae*. However the synergistic effect of the mixture of the two extracts exhibited greater effects than an effect of a single extract. . All treatments reduced the intensity of blast disease on week 15 with disease intensity by 7.90%. The extracts could increase plant height, the numbers of tillers, number of leaves, number of grains per panicle number of heads per panicle, and the full grain weight hill.. The highest potential yield (t/ha) was observed in the 2% extract treatment, and all treatment results significantly differed from that of the control. The potential grain yield was 3.23 t/ha in the control, while that in the treatment ranged from 3.81 t/ha to 5.61 t/ha. The high grain yield observed with the treatment was caused by the low intensity of blast disease.

Keywords:Bioactive substances; Biopesticide; Blast disease; Disease management; Plant growth promotion;

1. Introduction

Rice is the principle food for majority if population in the world, it is grown in every part of the world. Among the various disease, rice blast caused by *Pyricularia oryzae* is by far the most damaging disease that can reduce the crop yield by 50% and thus affects the economy and it is found wherever rice is grown. It affects rice plants with the potential to cause empty rice grains leading to reduction in crop yield [1]. Blast disease is caused by *Pyricularia oryzae* or *Magnoporthe oryzae* [2].

The challenge to produce high quality, nutritive and organic rice continues, in ever-increasing amounts at lower costs, all while in the presence of an unforgiving and unrelenting pathogen. Among the various strategies applied to manage rice blast, the use of botanical pesticides appears not only cost effective but organic too. Use of biopesticides offers numerous advantages over the chemical fungicides in terms of plant growth promotion and disease management [1,2].

Piper caninum has a bright green color, hairy, heart-shaped leaf. It thrives in bushes and is a creeping plant attached to parent plants, such as jackfruit, coconut and others. It grows in places with an altitude of 600 m above sea level [3]. *Piper betle* var. Nigra is an endemic Indonesian plant that grows in bushes; it crawls and attaches to shady plants and almost grows on all trees. Nigra has a dark green leaf color, smooth leaf surface, black stem and heart-shaped leaves and grows on places with an altitude of 500–700 m above sea level. Both these plants produce a wide range of different bioactive secondary metabolites [4]. These secondary metabolites are now gaining great significance as a in organic management of plant nutrition and disease control biopesticides due to their eco-friendly, biodegradable nature and safety to non-target organisms [5]. In addition to the *P. caninum* extract, rhiobacterial based biocontrol agents such as biopesticides *Bacillus subtilis*, *Bacillus cereus* and *Pseudomonas fluorecens* etc have been in use to suppress blast disease [6,7].

We hypothesize that the biopesticidal; preparation of *Piper caninum* and *Piper betle* var. Nigra promotes rice growth and yield parameters and possess the biocontrol potential against rice blast however, a combination of the mixture of these two extracts may act synergistically and produce more pronounce effect than the effect of a single preparation. emay have more activity than the activity of single extract [8].

In this study, we evaluated the effect of the extract of these plants alone and in combination on the intensity of blast disease and on the growth and yield of red rice.

2. Materials and methods

2.1. Plant source and extraction

P.caninum and *P.betle* var. Nigra leaves were collected from the village of Senganan, Tabanan Regency, Bali Indonesia (longitude 115.0; latitude -8.45 and altitude 249 m above sea level). Mature leaves (1 kg) were collected air dried for 4 days cut it into small pieces and blended until they were powdered. 300 g powder was dissolved in 3 L methanol 70% for 2 days, filtered, evaporated with a rotary evaporator, and the crude extract was obtained [5].

2.2. Source of P. oryzae

The isolate of *P. oryzae* was obtained from collection of the Biopesticide Laboratory of Udayana University, Bali, Indonesia. It was earlier identified based on homology of 18S rRNA gene sequences [5].

2.3. Gas chromatography–Mass spectrophotometry (GC–MS) analysis

GC–MS analysis was conducted to identify active compounds with antifungal activity against *Curvularia verruculosa*, which causes *Curvularia* spots disease in rice. Samples of the most active and relatively pure fractions were analyzed by GC–MS. The structure of the isolated compound was determined on the basis of their molecular weights and fragmentation patterns to the compounds in the library of the GC–MS system [9].

2.4. Research Design

This study was followed by randomised block design (RBD) with five treatments and six replicates. There were 30 experimental units, and each unit consisted of six plants. The total number of rice plants was 180. F0 (control) = without extract treatment; F1 = treatment with 0.5% extract; F2 = treatment with 1% extract; F3 = treatment with 1.5% extract; F4 = treatment with 2%. All treatments (F1-F4) were sprayed with liquid organic fertilizer 1% for 5 L/ha. This research was carried out in the rice fields of the village of Senganan, Penebel, Tabanan Bali, Indonesia [6].

2.5. Seeding

Quality seeds of Bali rice were soaked for one night, drained and germinated on a tray before seeding on soil media. The seedlings were watered every day for 15 days in the afternoon. After 15 days, the seedlings were ready for transplantation.

2.5. Transplanting

Transplanting was performed in the afternoon, and each unit was planted with one seedling. Healthy seedlings of homogeneous size were planted.

2.6. Planting Medium Preparation

The experiment was conducted in paddy fields. The rice field was mixed with compost as much as 30 kg/are. Soil was mixed with compost when ploughing, left for a week, and ploughed again and planted with red rice plants in accordance with the treatment.

2.7. Maintenance

Fertilization was conducted at 15 and 35 days after transplanting by using liquid organic fertilizer suria green with a concentration of 1% sprayed evenly on the surface of rice plants (5 liters of liquid organic fertilizer in 1 hectare. Water level was 5 cm from the soil level.

2.8. Isolate of P. oryzae

P. oryzae was inoculated to the rice plants by using a sprayer. The fungus sample with a spore density of 25×10^4 mL^{-1} was sprayed @ 20 ml for each clump.

2.9. Extract Application

The extracts were sprayed on rice plants in the afternoon a day after the pathogens were inoculated. The treatment was repeated four times with an interval of 2 weeks [6].

2.10. Harvest

Red rice were harvested at 4 months and 15 days, dried for 3 days and ground in a scrub.

2.11. Observed Parameters

The observed parameters included the intensity of blast leaf spot disease, growth parameters such as plant height, the number of leaves and the number of tillers and yield components like the number of productive tillers, the number of pithed rice per panicle, weight of pithed rice per clump, percentage of empty grain and yield achieved by weighing the rice produced, obtaining the total production and converting the values in t/ha. The leaf spot disease intensity was determined using with the following formula [6].

$$DI = \frac{\sum_{i=0}^{i}(ni.vi)}{NV} x\ 100\%$$

Where

DI = Disease intensity,

n_i = the number of leaves with a score of i,

v_i = value of each category of disease scores,

N = the number of leaves observed and

V = the highest score.

Each category was scored as follows:

0 = no attack at all,

1 = very mild attack (0%–10% broken leaf surface),

2 = mild attack (10%–30% broken leaf surface),

3 = moderate attack (30%–50% of the leaf surface is damaged),

4 = severe attack (50%–75% of the leaf surface is damaged) and

5 = very heavy attack (75%–100% of the leaf surface is damaged).

2.12. Data Analysis

Data were analysed with analysis of variance, and different values were analysed by Duncan's multiple-range test at 5% level [9].

3. Results

3.2. Gas Chromatography–Mass Spectrometry (GC–MS) of extract

GC-MS analysis of the mixture of leaf extract of *P. caninum* and *P. betle* var. Nigra (Figure 1) revealed the presence of nine peaks for bioactive compounds, which differed from those found in the extract of single plant alone (Table 1). New compounds that caused different reactions were detected in the extract mixture (Table 1).

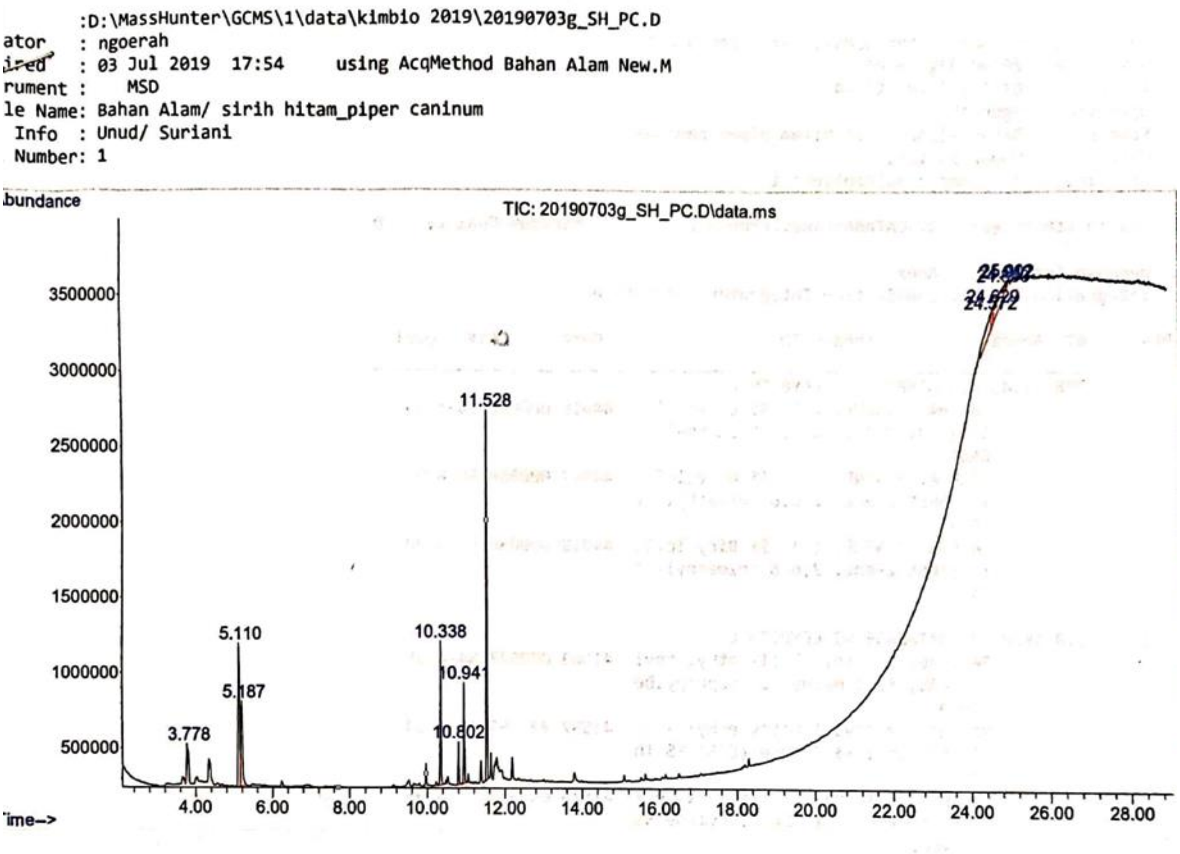

Figure 1. Chromatograms *Piper caninum* and *Piper betle var nigra* mixture leaf extract. Nine peaks of active compounds were detected in the mixture of *P.caninum* and *P. betle* var. Nigra leaf extracts: peak 1: α-pinene, peak 2:benzene, peak 3:γ-terpinene, peak 4: copaene, peak 5: benzenemethanol, peak 6: acetamide, peak 7: α-gurjunene, peak 8:5,8-epoxy-15-nor-labdane and peak 9: ethyl 5-formyl-3-(2-ethoxycarbonyl).

Table 1. Bioactive compounds of *P. Caninum, P. betle*. Var. Nigra and mixture of leaf extracts

Peak	Retention time (min)			Area (%)			Bioactive compound		
	PC	Pb	Mix.	PC	Pb	Mix.	PC	Pb	Mix.
1	3,300	3.791	3.778	47688	5.67	1.45	Benzene	Alpha.-pinene	Alpha.-pinene
2	3,457	5.115	5.110	40974	16.21	10.07	Xylene	Benzene	Benzene
3	11,32	5.185	5.187	111881	10.55	12.04	Tetradecane	dl-Limonene	Gamma.-terpinene
4	12,909	10.339	10.338	454456	8.65	10.19	Dodecanoic acid	Copaene	Copaene
5	13,851	10.803	10.802	24038	2.63	2.99	Heptadecane	Benzaldehyde	Benzenemethanol
6	17,420	10.942	10.941	36460	5.78	7.18	Hexadecanoic acid	Acetamide	Acetamide
7	17,963	11.485	11.528	12973	14.41	25.59	Octadecamethylcyclononasiloxane	Benzoic acid	Alpha.-Gurjunene
8	18,426	11.529	24.572	34013	28.91	9.27	Phthalic acid	Alpha-gurjunene	5,8-Epoxy-15nor-labdane
9	19,136	24.975	24.629	8641	4.48	2.38	8,11, 14-Docosatrienoic acid	Tetrasiloxane	Ethyl 5-formyl-3-(2-ethoxycarbonyl
10	22,935	---	---	82,531	---	---	1,2-Benzenedicarboxy1lic acid	---	---

GC–MS analysis of individual extract and mixture of both the extracts was carried out by using GC-MS.

3.3. Intensity of Blast Disease

Significantly different intensities of blast disease between the control and treatment groups were observed on week 8. The lowest intensity was observed at 2% extract concentration with 86.87% inhibitory activity (Table 2).

Table 2. Intensity of blast disease and inhibiting activity after 8, 12, and 15 weeks.

Treatment (Extract)	Intensity of blast disease (%)			Inhibitory activity (%)		
	8 weeks	12 weeks	15 weeks	8 weeks	12 weeks	15 weeks
F0 (Control)	69.98a*	76.43a*	80.18a*	-	-	-
F1 (0.5%)	53.88b	40.12b	37.29b	23.01	47.51	53.49
F2 (1%)	34.29c	23.14c	21.20c	51.00	69.72	73.60
F3 (1.5.%)	19.37d	15.01d	13.09d	72.32	80.36	83.67
F4 (2.0%)	9.19e	8.98e	7.90e	86.87	88.25	90.15

*Values followed by the same letters at the same column show no significant different based on Duncan's Multiple Range Test at the P = 5%.

On week 12, the intensity of blast disease in the control increased from 69.98 (week 8) to 76.43% and reached to 80.18% on week 15. Minimum disease intensity (8.98%) was observed in the treatment with 2% which further declined to 7.90% on week 15 (Table 2).

3.4. Plant Growth

3.4.1. Number of Tiller

Plant growth is considerably influenced by various genetic and environmental factors. The results showed that the extract significantly affected the growth and the number of red Balinese tillers. Table 3 shows that the number of tillers in control 4 on week 4 increased by 8 and 12 tillers on weeks 8 and 1, respectively. The largest number of tillers (13.72) was observed in the 2% extract treatment group on week 4. The mixture of extracts of *P.caninum* and *P.betle* var. Nigra leaves significantly increase the number of tillers.

Table 3. The effect of extract on the average number of tillers

Fertilizer	Number of Tillers	Number of Leaf	Height (cm)

extract mixture formulation	4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
F0 (Control)	8.56a*	9.09a*	8.99a*	29.09a*	44.90a*	41.39a*	28.35a*	118.11a*	178.97a*
F1 (0.5%)	10.12b	12.15b	12.09b	29.50a	46.77b	39.45a	38.92b	121.29b	175.98b
F2 (1%)	10.23b	12.59b	12.49bc	33.23b	49.86c	43.22b	39.02b	122.50b	175.67b
F3 (1.5.%)	10.51b	13.01b	13.00c	38.33c	69.80e	62.76c	40.59b	122.45b	174.87b
F4 (2.0%)	13.72c	13.15b	13.11c	42.98d	71.51e	65.91d	40.61b	121.98b	172.99c

*Values followed by the same letters at the same column show no significant different based on Duncan's Multiple Range Test at the P = 5%.
*Values followed by the same letters at the same column show no significant different based on Duncan's Multiple Range Test at the P = 5%.

3.4.2. Number of Leaves

Plant leaves greatly determine plant productivity given that their main function is to carry out photosynthesis. If leaf growth is affected, then plant productivity is also reduced. Table 3 shows that the highest number of leaves was observed in the 2% extract treatment on week 8. The average from week 4 to week 8 increased and decreased after week 12 because most leaves were old, and growth had decreased. The numbers of leaves between the control and treatment groups differed, indicating that the extract can increase the leaf number.

3.4.3. Plant Height

Red Balinese rice plants have a higher average height compared with hybrid rice. Thus, red Bali rice is vulnerable and may droop once the grain becomes heavy. If this plant is extremely fertile, then it will droop before the yellowing of rice. Thus, the effect on quality of rice will reduce rice production. The treatment of 2% mixture of extracts of *P.caninum* and *P. betle* var Nigra for 12 weeks incubation shortened the stem (172.99cm) over the long stem obtained with control (178.97 cm), 0.5% (175.98 cm), 1.0% (175.67 cm) and 1.5% (174.87 cm) concentration of extracts resulted (Table 3).

3.5. Yield

One of the problems in rice agriculture is fungal diseases, such as the blast disease caused by *P. oryzae*, which result in up to 90% loss in rice yield [10]. The rice plants in the control group (Figure. 2a), while those in the treatment group did not exhibit any change (Figure. 2b) due to the action of the extract, which enhanced the stem strength. The highest number of productive tillers (13) was observed in F3 (1.5% extract) and F4 treatments (2.0% extract), with respectively, whereas the number of productive tillers in the control (Table 4). The F3 (1.5% extract) and F4 treatments (2.0% extract) produced more numbers of grains per panicle (219.09) as compared to the control other treatments. The highest full grain weight/clump was observed in in the treatment with 2% extract treatment (81.20 g) as compared the lowest full grain weight obtained in control treatment (41.56 g). Application of 1.5% and 2.0% of extract helped in reducing the percentage of empty grain/clump from 12.03% obtained in control to 2.45 and 2.41% respectively (Table 4). The leaf extracts of *Piper caninum* and *Piper betle* var. Nigra were combined. The treatment effectively decreased the percentage of empty grains and increased yield components.

Figure 2a Control. Rice stalks are not strong and fall at the age of 4 months

Figure 2b. Treatment F4. Rice stalks are strong and the rice grains are bigger

Table 4. Effect of leaf extract treatment on yield components and variety of rice plants

Treatment	No. of productive tillers	No .of grain / panicle	Full grain weight /clump (g)	Empty grain / clump (%)	Potential yield (ton/ha)	Yield increase vis-a-vis control (%)	Rice quality (mm)		
							Long	Wide	Diameter
Fo	8.7a*	219.09a*	41.56a*	12.03a*	3.23 a*	-	0.51a*	0.20a*	0.30a*
F1	11.04b	235.10b	53.98b	9.01b	3.81a	17.97	0.59b	0.23b	0.35b
F2	12.87c	238.43c	61.76c	6.91c	4.24b	31.27	0.61b	0.25b	0.35b
F3	13.00c	242.19d	75.98d	2.45d	5.49c	69.97	0.68c	0.29c	0.38c
F4	13.00c	242.22d	81.20e	2.41d	5.61c	70	0.79d	0.31c	0.43d

*Values followed by the same letters at the same column show no significant different based on Duncan's Multiple Range Test at the P = 5%.

3.5.1. Potential yield

The highest potential yield (5.61t/ha) was observed in the 2% extract treatment vis-a-vis 3.23 t/ha obtained in control treatment, and all treatments resulted in less yield (Table 4). The F3 (1.5% extract) and F4 treatments (2.0% extract) improved the rice yield by 69.97% and 70% respectively.

3.6. Rice quality

The sizes of rice grains between the control and treatment groups significantly differed. Application of 2.0% extract resulted in significant improvement in rice quality it yielded rice grain size with maximum length (0.79 mm), maximum width (0.31 mm) and a larger diameter (0.43 mm) (Table 4).

4. Discussion

GC_MS analysis of mixture of *P.caninum* and *P. betle* var Nigra revealed the presence of various bioactive compounds such as an cyclooxygenase-2 by methylated flavones9,10, α-pinene ($C_{10}H_{16}$), 1,2-dimethylbenzene (C_8H_{10}), [11,12]. γ-Terpinene ($C_{10}H_{16}$), copaene ($C_{15}H_{24}$) [13], benzene methanol ($C_7H_{10}O$), ccetamide (CH_3CONH_2), [14] α-Gurjunene, [15]. 5,8-Epoxy-15-nor-labdane ($C_{20}H_{38}$) [16,17]. The presence of characteristic functional groups, retention time and the properties of these bioactive compounds resembled with those of standard compounds as per WILEY7 library LIB [18].

Inhibition of rice blast pathogen *P.oryzae* is due to its presence of antifungal components found in the mixture of *P.caninum* and *P.betle* var. Nigra leaf extracts. Potent antifungal activity of mixture of extracts is due to the synergistic action of the preparation that resulted in a greater inhibition compared with a single extract Terpinene has been reported to inhibit the growth of *Enterococcus faecalis* (1.3 cm), *Lactococcus plantarum* (0.9 cm), *Proteus vulgaris* (0.6 cm), *Candida tropicalis* (1.3 cm), *C. albicans* (0.8 cm), *Rhizomucor miehei* (0.6 cm) and *C. glabrata* (0.6 cm) [17].

The chemical content of *P. caninum* extract can act as an antibacterial [19]; essential oils from *P. caninum* contain antioxidants and antimicrobials and anticancer compounds [20].

Synergistic compounds will increase the inhibitory effect against pathogens. *Mansoa alliacea* L. and *Allamanda cathartica* L. leaf extracts have greater inhibitory effects than a single extract against the fungus *Athelia rolfsii*, which causes peanut sprouts [5]. New compounds formed after mixing extracts; for instance, *P. caninum* contains hexadecanoic acid which is an antifungal [21], whereas *P.betle* var Nigra contains pyridine [22]; after *P.caninum* and *P. betle* var Nigra were extracted, these compounds were not detected, but new ones with more severe inhibitory effects were found. Formulation of mixture of biopesticide preparation of *Beauveria bassiana* and *Azadirachta indica* has been found as a more potent biocontrol agent than a single preparation [23]. Decrease in the attack of blast disease result in the healthy growth of rice plants. *P. caninum* leaf extract suppress blast disease and increase the number of tillers of certain rice hybrids [24]. The number of tillers is very important to increase rice production but if the tillers are too much it is also not good because there is competition in getting nutrition, so the grain quality is not good because the nutrients obtained by each tillers are not enough. Inhibition of growth of *P.oryzae* as well as growth promotion in rice is due to antifungal phytochemicals that can suppress blast disease and presence of hormones that stimulate plant growth [25]. The mixture of *P. caninum* and *P. betle* extracts contains phytochemicals that can inhibit *Pyricularia oryzae* by damaging its cell wall, causing the cell fluid to leak out and resulting in lysis and damage. Significant increase in the number of tillers following the application of the mixture of extracts of *P.caninum* and *P.betle* var. Nigra leaves is because this mixture can eliminate *P. oryzae* that causes rice blast disease.

Pyricularia oryzae causes blast disease in rice and results in empty grains. The higher the intensity of blast disease, the higher will be the percentage of empty grains. The high grain yield obtained with the treatments was caused by the low intensity of blast disease, which causes empty grains.

Decrease in the number of leaves at higher concentration (2.0%) of extract may be due to toxic effect of high concentration of the preparation that caused endurance of plant and increased the intensity of blast disease [1].

Rice blast caused by *P. oryzae* results in empty grains and the severity of disease leads to higher percentage of empty grains. Decrease in the number of empty grains following the application of mixture of the two extracts is due to the inhibition of growth of pathogen that otherwise produce more empty grains [1-3].

Various factors, such as genetics, influence plant growth; three varieties of red rice are found in Sumatra, and each variety showed a different growth [26]. This phenomenon is attributed to the toxicity that decreased plant resistance. *Piper caninum* extract can result in the short height of Bali rice plants; for instance, the stem height of the control (187.77) was shorter than that of the group treated with 2% extract (157.88); this finding proves that the rice plants did not droop [24].

The grain yield (tha^{-1}) is mostly determined by the intensity of blast disease. The higher the intensity of the disease, the more the yield loss because blast disease causes empty rice grains that result in failure of harvest. Rice quality is largely determined by genetic and environmental factors. The extract used influences the quality of rice in terms of taste. Since rice quality depends considerably on grain size [27], the application of mixture of *P. caninum* and *P. betle* can be effective method to improve the quality of Bali rice.

Increased yield on treatment (F1-F4) in addition to control, is also likely due to the addition of liquid organic fertilizer with the same dose. Liquid organic fertilizer added contains microbes such as *Enterobacter cloaceae*, *Bacillus subtilis*, and *Stenothropomonas maltopilia*. This microbial consortium stimulates plant growth as well as exhibit biocontrol activity, protecting plants from disease, thus the disease free plant can give more yield [28]. *B. subtilis* is known to promote plant growth and inhibit blast disease outbreaks in rice plants [29, 30]. *E. cloaceae* also promote plant growth and inhibit plant pathogens [28].

5. Conclusion

Nine phytochemical compounds, including α-pinene, benzene, γ-terpinene, copaene, benzenemethanol, acetamide, α-gurjunene, 5,8-epoxy-15-nor-labdane and ethyl 5-formyl-3-(2-ethoxycarbonyl), were obtained from the mixture of *Piper caninum* and *Piper betle* var. Nigra leaf extracts. These phytochemicals can reduce the intensity of blast disease by 90.15% extract concentration of 2%, promoted the growth, increased the yields by 70% and improve the quality of Bali red rice.

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