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Antiviral activity of *Eucheuma cottonii* to control *Pepper yellow leaf curl virus*

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Abstract. *Temaja IGRM, Selangga DGW, Sudiarta IP, Listhiani L. 2024. Antiviral activity of* Eucheuma cottonii *to control* Pepper yellow leaf curl virus. *Biodiversitas 25: 1615-1622.* The chili pepper plant in Bali is often infected with yellow curl symptoms caused by *Pepper yellow leaf curl Indonesia virus* (PYLCIV). The virus is always present in the field and no control measure has been found effective to suppress the PYLCIV infection. Control efforts using macroalgae extract have been frequently used and are able to suppress plant virus infection, but no report on its effectiveness in suppressing PYLCIV infection. This research aimed to analyze the effect of *Eucheuma cottonii* Weber Bosse macroalgae extract in suppressing PYLCIV infection on chili pepper plants in Bali. The observed parameters were symptom changes, disease incidence and severity, virus confirmation by PCR, and phytochemical analysis. Chili pepper plants infected by PYLCIV before *E. cottonii* application showed moderate to severe symptoms, but after *E. cottonii* application plants turned symptomless or showed light symptoms. The PYLCIV disease incidence in chili pepper Seret and Pelita8 cultivars before application was 100%, and after application ranged from 45-55%. The PYLCIV disease severity in chili pepper Seret and Pelita 8 cultivars before application ranged between 68-72%, and after application ranged between 25-34%. PYLCV on chili pepper Seret and Pelita 8 cultivar that were treated with *E. cottonii* produced lighter DNA band compared to positive control (C+). Major compounds found in *E. cottonii* extract were propanoic acid, ethyl ester; n-propyl acetate; sec-Butyl acetate; Toluene; Bicyclo [4.2.0] octa-1, 3, 5-triene; and styrene. The *E. cottonii* extract was able to suppress the PYLCIV infection on chili pepper plants.

Keywords: Antiviral activity, chili pepper, disease incidence, disease severity, *Eucheuma cottonii*, PYLCIV

INTRODUCTION

Chili pepper (*Capsicum frutescens* L.) is one of the important horticulture commodities in Indonesia. In 2020, through President of Indonesia decree number 59, chili pepper has been considered as a staple agricultural product (Presidential of Indonesia Regulation 2020). Several compounds within chili pepper are essential oil, capsaicin, vitamins A, C, and E, which exist in abundance for health purposes. According to Wahyuni et al. (2013), metabolite compounds in chili pepper plants, such as flavonoid compound groups (quercetin, apigenin and luteolin) and a large group from capsaicinoids compounds (acyclic diterpenoid glycosides) can represent chili pepper quality, such as the taste and the plant's resistance against disease. Moreover, it also contains several compounds, such as ascorbic acid, carotenoid, and a high content of β-carotene and antioxidants (Wahyuni et al. 2013) which are beneficial for human health. *Capsicum annuum* var. *Llanerón* known as red sweet pepper, contains antioxidants and cytotoxic compounds which act as anticancer for humans (Yazdizadeh et al. 2013).

The main problems faced by chili pepper producers in tropical regions are pest infestation and plant disease which caused loss in chili production. One of the plant diseases that often cause losses is yellow disease, bulai disease, or stunt disease caused by a Geminivirus (Geminiviridae), According to Yang et al. (2017), Geminivirus is the main group of viruses with single-strand DNA and has the ability to multiply genetic material without directly integrating with plant genome. This virus is spread by a vector, *Bemisia tabaci* Gennadius 1889 (Gennadius) (Hemiptera: Aleyrodidae) or whitefly. Whitefly gets the virus when feeding from infected plants (acquisition). The virus spread by whitefly replicated in the nucleus and moved between cells by using plasmodesmata (Krapp et al. 2017). Whitefly infestation in plants can also reduce the plant metabolism process due to leaf damage suffered by infested plants (Li et al. 2022; Temaja et al. 2022).

Geminivirus infection can drastically reduce photosynthesis, growth, fruit growth, and fruit quality of the host plant, although the effect is influenced by the number of plants infected and the age of the plants when infected (Listihani et al. 2019; Selangga and Listihani 2021; Listihani et al. 2022c; Selangga and Listihani 2022; Selangga et al. 2022a, b; Selangga et al. 2023). Chili pepper plants with a disease caused by Geminivirus, *Begomovirus* genus, or often called *Pepper yellow leaf curl virus* may suffer up to 100% of production loss (Cania et al. 2021). Production loss due to Geminivirus in tomato plants reached up to 100% with a high whitefly infestation (Inoue-Nagata et al. 2016). Plants infected by yellow curl disease are also known to produce

disease-carrying seedlings (Fadhila et al. 2020), which may cause prolonged production loss due to disease.

Various control efforts have been attempted, from physical control methods, such as insecticide spraying to other methods like designing resistant varieties by crossbreeding and biotechnology. Various research was performed to overcome the problem, such as inducing resistance with antivirus contained in macroalgae extract (Santosa 2017). Macroalgae with high virus suppression ability are *Dictyota cervicornis* Kütz., *Sargassum* sp., *Padina australis* Hauck, and *Laurencia majuscule* (Harv.) A.H.S.Lucas (Santosa 2017). Macroalgae contains flavonoids, saponins, and steroids which are able to suppress virus infection. *Eucheuma spinosum* J.Agardh and *Eucheuma cottonii* Weber Bosse are able to suppress disease incidence by 80% and 40% by lowering disease severity by 71% and 48% in diseases caused by Begomovirus infection (Listihani et al. 2023c). According to Listihani et al. (2023c), the alternative solution in controlling viruses is by using extracts containing compounds that improve plant resistance against viral infections or their carrying vector. Microalgae usage has a lot of advantages and has economic value to support local community's life (Setiawati et al. 2017), such as reducing the cost of plant pest organism control, no dangerous chemical residue, and can be received, and used by the farmers for a long period of time.

Echeuma cottonii has the ability to suppress SPLCV infection in sweet potatoes (Listihani et al. 2023). Thus, this research aimed to analyze the effect of *E. cottonii* extract in suppressing PYLCV in chili pepper plant.

MATERIALS AND METHODS

Virus inoculum propagation

The virus source plant (isolate) for PYLCV was obtained from previous research collection originating from Sekaan, Bangli, Bali, Indonesia (Temaja et al. 2022), which was then nurtured in a greenhouse in Agriculture Faculty, Universitas Udayana. PYLCV inoculum propagation was performed via *B. tabaci* whitefly infection on chili pepper plants of the Seret cultivar. *B. tabaci* was given an acquisition feeding period for 24 hours on the chili pepper plant as the initial inoculum source (from the previous research) and then moved to healthy chili pepper plants aged 6 weeks after sowing (10 whiteflies/plant) for 24 hours of the inoculation feeding period. Afterwards, *B. tabaci* was taken away from the chili plants, and the plants were nurtured until symptoms appeared. Symptomatic plants were then used as virus inoculum.

Research plant preparation

The two chili pepper genotypes used were cv Pelita 8 and cv Seret. The chili pepper seeds were sown on commercial media (a mixture of manure, compost, and rice husk). After 3-5 weeks since sowing or after the seedlings had 3-4 leaves, were moved into plastic bags (30×35 cm) filled with a mixture of soil and manure (2:1 w/w) weighing 5 kg. PYLCV inoculation by *B. tabaci* was performed with steps as previously explained which consist of 24 hours acquisition feeding period and 24 hours inoculation feeding period with 10 whiteflies/plant.

Sampling and macroalgae extract-making

Macroalgae samples were collected from uprooting whole thallus in Serangan Island. Thallus were washed with water and stored in a strapped plastic container containing sterile seawater. Macroalgae rough extract was made for 2 g wet weight of each sample. The samples were ground in liquid nitrogen by mortar and pestle and then diluted three times with 30 mL methanol before being filtered by Whatman No. 41 filter paper. The rough extract was then evaporated by a rotary evaporator to separate methanol and extract, obtaining the rough extract of the macroalgae.

Macroalgae extract application to test plants

Macroalgae active compound extract was diluted in 5% 2-methoxyethanol to obtain 10 µg/µL liquid extract. The macroalgae extract was then sprayed on the leaf of the test plant in three sprays by using a mini sprayer. Treated plants were nurtured and observed until mild symptoms or symptomless appeared from severe symptoms.

The research was conduted in one factor and 20 repetitions with a uniform testing environment. The factor consists of two treatments (with *E. cottonii* extract and without *E. cottonii* extract) applied on the test plants. Positive control (test plants confirmed positive for PYLCV without treatment) and negative control (test plants confirmed negative for PYLCV without treatment) were also used in the research. The observed variables were symptom variations, disease incidence, and disease severity obtained by using disease scoring, and viral load in the plants was detected by PCR.

PYLCV confirmation by PCR and sequencing analysis

The virus detection method by PCR consists of several phases, like total DNA extraction, DNA amplification, and amplification product visualization. Total DNA extract was manually performed by following the CTAB method. Virus DNA amplification was performed by using universal primer pairs for *Begomovirus* (SPG2-_5'-ATCCVAAYWT YCAGGGAGCTAA-3'/ SPG1_5'-CCCKGTGCGWRAA TCCAT-3'). The nucleotide sequence similarity analysis to determine the virus species was performed by Blast software on the NCBI site (www.ncbi.nlm.nih.gov).

Identification of bioactive compounds using gas chromatography-mass spectrometry (GC-MS)

The present investigation was carried out to characterize the bioactive compounds present in extract of *E. cottonii* using Gas Chromatography-Mass Spectrum (GC-MS). The GC-MS procedure of Denpasar Police Forensic Laboratory was adopted for the identification of bioactive compounds. For this, 1 mg of thick extract dissolved in 900 µL of ethyl acetate. Analysis of *Eucheuma cottonii* extract samples using N_2 gas which was used as a carrier in constant flow with a flow rate of 1 mL/minute. Next, placed it in a centrifuge for 3 minutes with 10 times dilution so that the sample was homogeneous, then 1 µL was taken and injected into the GC column. The injection temperature was set at

290°C and maintained for 27 minutes. The resulting graph peaks at retention times were matched to a reference (PubChem Database).

RESULTS AND DISCUSSION

Disease symptoms appeared on chili pepper plants (cv Pelita 8 and cv Seret) after PYLCIV inoculation with whitefly in the greenhouses were yellow mosaic, green mosaic, curling, stunting, and both upward leaf curl or downward leaf curl. The treated plants showed different symptom variations. Plant response can be grouped into two types, those that showed no symptoms and those with mild symptoms (Figures 1 and 2). Symptomless plants had the same leaf shape, color, and size as healthy plants. Chili pepper plants with mild symptoms had normal-sized leaf, but uneven green color area on leaves, or slightly darker color on the leaf surface. The symptoms observed in the present research are the same as reported by Selangga et al. (2022b), which are yellow curled leaves, mosaic and stunting. The spread of the virus is not caused by wind or direct interaction between infected plants and healthy plants without a vector. The vector of this virus, especially the yellow leaf curl virus in chili peppers, is the insect *B. tabaci* also known as the whitefly. Whitefly is a plant pest with a wide range of hosts, a life cycle that depends on the temperature and its host, and is virulent since its nymph stage (instar) (Subagyo and Hidayat 2014; Chintkuntlawar et al. 2016).

The PYLCIV infection on cv Pelita 8 tends to be more severe compared to cv Seret. The PYLCIV infection on chili pepper plants (cv Pelita 8 and cv Seret) in Bali has been reported by Selangga et al. (2019), Selangga and Listihani (2021), Selangga et al. (2022b). The initial disease symptoms appeared between 7 to 28 days after inoculation (dai). The longest incubation period, was between 14 to 28 dai occurred in cv Pelita 8 (Ayu et al. 2021). The short incubation period may be caused by a high virulence factor (Wu et al. 2022). Selangga et al. (2021) reported that PYLCIV Bali isolate can be considered a virulent isolate. Several chili pepper varieties had been checked for resistance against PYLCIV in Bali. Seret and Pelita 8 can be considered as a vulnerable variety because the disease incidence reached 100% in all locations in Bali.

The efforts to improve chili pepper commodity agricultural products are still being fought for to fulfill the consumption demand in Indonesia as well as to make it an export commodity. The main hindrance in the production improvement effort is the yellow curl disease problem in chili peppers (pepper yellow leaf curl disease) which is caused by *Pepper yellow leaf curl virus*. This yellow curl disease in chili pepper can cause a drop in chili pepper production and even can cause up to 100% production loss due to harvest failure (Fadhila et al. 2020).

The chili pepper plants inoculated by PYLCIV and was treated with *E. cottonii* extract showed different symptom variations. Plant response can be grouped into two types, those that showed no symptoms and those with mild symptoms (Figures 1 and 2). Symptomless plants have the same leaf shape, color, and size as healthy plants. Chili pepper plants with mild symptoms have a normal-sized leaf, and on the surface of the leaf an uneven green coloring can be found, or a slightly darker color that only appears slightly on the leaf surface. The application of *E. cottonii* extract influenced the yellow leaf curl disease incidence and severity in cv Pelita 8 (Table 1).

Table 1. Disease score based on visual symptoms in plant

Score	Symptoms
0	Symptomless
1	Yellow leaves
2	Yellow and curly leaves
3	Yellow and cupping with leaf edges curl upward or downward
4	Yellow, curly and cupping of the leaves
5	Plant dwarfing with leaf yellowing and malformation

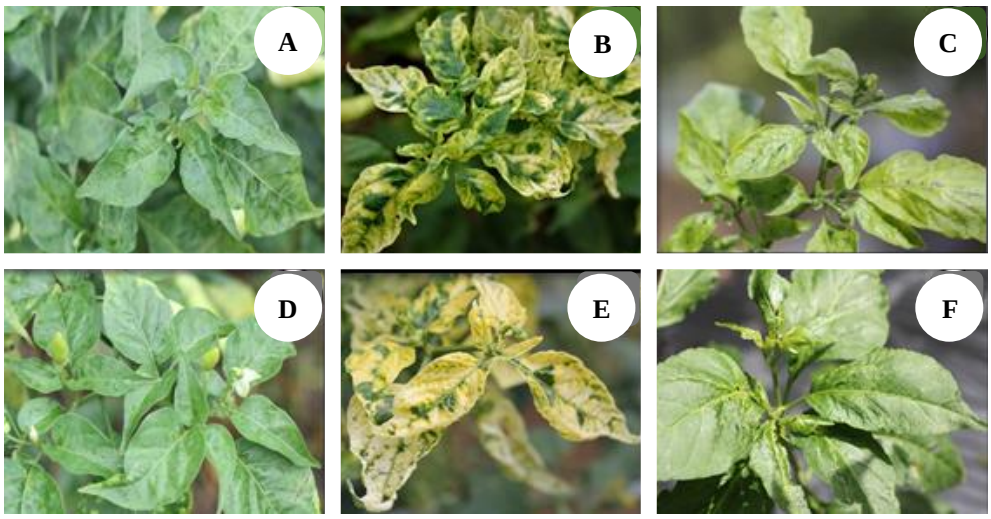

Figure 1. Symptoms of PYLCV infection in chili var. Seret before and after macroalgae treatment: A and D. Negative control; B and E. Positive control; C. PYLCV symptoms before *E. cottonii* treatment; F. PYLCV symptoms after *E. cottonii* treatment

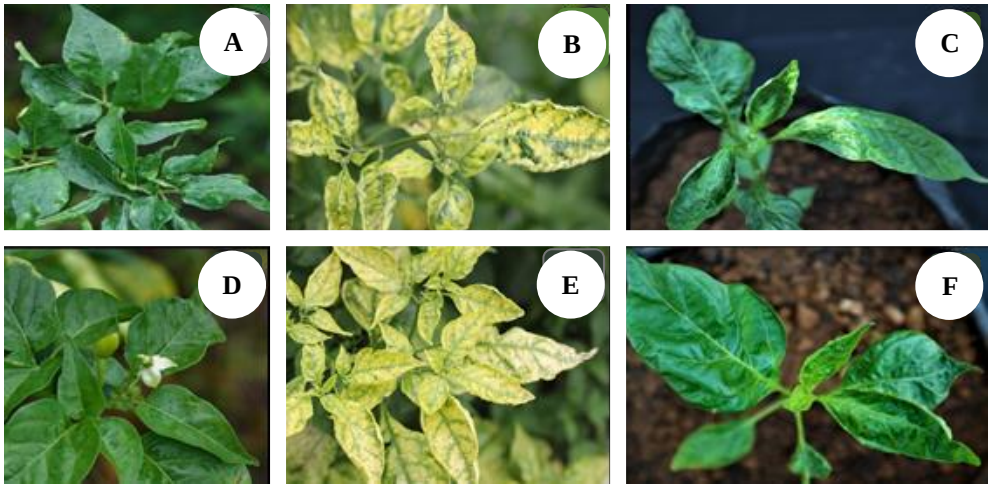

Figure 2. Symptoms of PYLCV infection in chili var. Pelita 8 before and after macroalgae treatment: A and D. Negative control; B and E. Positive control; C. PYLCV symptoms before *E. cottonii* treatment; F. PYLCV symptoms after *E. cottonii* treatment

The disease incidence in cv Seret before *E. cottonii* treatment was 100%, while the disease incidence after treatment was 45% (Table 2). The disease severity before *E. cottonii* treatment was 68%, whereas after the treatment it was 25%. The lowest disease incidence and severity were shown by the group with *E. cottonii* extract treatment. The disease incidence in cv Pelita 8 chili pepper plants before *E. cottonii* treatment was 100%, while after treatment it was 55%. The disease severity before the *E. cottonii* extract treatment was 72%, whereas after treatment it was 34%. These observations showed that *E. cottonii* extract treatment can suppress disease incidence and severity in both tested variants. The result showed that the application of *E. cottonii* extract was found effective in suppressing disease incidence and severity. Several factors that influence the effectiveness of *E. cottonii* treatment are environmental factors, the plant resistance, and nature of virus.

The disease severity shows how severe the yellow disease phenotypic effect is on the plant caused by the virus infection. The more severe the symptoms, the higher the virus activity in the plant cells and the more detrimental it is to the plant. High virus activity inside the plant can disrupt the plant's physiological processes, which affects the plant's metabolism. The symptoms that occur, such as yellow mosaic, is a clearing of leaf vein tissue due to a certain protein induction from virus DNA which causes damage to the plant's chloroplast and disrupt the photosynthesis process (Bhattacharyya et al. 2015). The disruption of plant's physiological mechanism caused the plant's growth and development to be hampered, which impacted production. Research has been done to obtain a control strategy for yellow leaf curl disease in chili pepper due to PYLCV infection. *Serangium japonicum* Chapin 1940 beetle with its high preference is an effective predator of *B. tabaci* (Tian et al. 2017). Intercropping patterns can lower the reproduction and spread of *B. tabaci* compared to monoculture planting patterns. Intercropping planting between chili pepper and cabbage can suppress the *B. tabaci* population by up to 60.72% (Gadzekpo et al. 2020).

The newest research stated that virus DNA was found in plant seedlings which have been previously infected (Fadhila et al. 2020), which makes virus spread prevention in plants extremely crucial to prevent the production of infected seedlings. The confirmation of Begomovirus in the plant was performed 30 days after *E. cottonii* extract application was implemented. DNA fragments of 912 bp in size were amplified from the cv Seret and Pelita 8 after *E. cottonii* was applied by using the Begomovirus universal primers SPG1/SPG2 (Figure 3). The PYLCV on chili var. Seret and Pelita 8 treated with *E. cottonii* showed thinner DNA bands compared to the positive control (C+). This showed that PYLCIV DNA concentration was very low in plants treated with *E. cottoni* extract compared to the positive control. *E. cottonii* may have the ability to suppress PYLCIV replication. The amplification DNA product was then used for sequencing to determine the species of the virus. Sequencing analysis confirmed that the virus infecting the test plants in a greenhouse was PYLCIV with 97% and 99% homology against Blanga, Bangli isolates from Bali.

The chemical compound analysis of the seaweed produced 27 chromatogram peaks which can be seen in Figure 4. In the seaweed extract test, there were six compounds with quality >70 and high AUC values which were propanoic acid, ethyl ester; n-Propyl acetate; sec-Butyl acetate; Toluene; Bicyclo [4.2.0] octa-1, 3, 5- triene; and Styrene (Table 3). The GC-MS analysis of seaweed extract showed that compounds with the highest AUC (Area Under the Curve) value (major compound) were propanoic acid, and ethyl ester which were found in a retention period of 2.476 minutes (Table 3). The compounds reported in Table 3 are dominated by the ester functional group and hydrocarbon aromatic compound. The ester functional group is known to be an antimicrobial agent (Nowak et al. 2021). The propanoic acid, ethyl ester compound was the highest AUC value of 37.71% which included as an ester compound group with antimicrobial activity, used as food seasoning, and often used in industry.

Table 2. The effect of *Eucheuma cottonii* application on the symptoms and severity of vein clearing disease in chili plants

Treat-ments	Before treatment			Four weeks after treatment			Begomovirus detection by PCR after treatment
	Disease symptom	Disease incidence (%)	Disease severity (%)	Disease symptom	Disease incidence (%)	Disease severity (%)	
Chili var. Seret							
C-	Symptomless	0 (0/20)	0	Symptomless	0 (0/20)	0	-
C+	Yellow leaves, Yellow and curly leaves, Yellow and cupping with leaf edges curl Upward or downward, Curly and cupping of the leaves, Plant dwarfing with leaf yellowing and Malformation	100 (20/20)	77	Yellow and curly leaves, Yellow and cupping with leaf edges curl Upward or downward, Curly and cupping of the leaves, Plant dwarfing with leaf yellowing and Malformation	100 (20/20)	86	+
E.cottonii	Yellow leaves, Yellow and curly leaves, Yellow and cupping with leaf edges curl Upward or downward, Curly and cupping of the leaves, Plant dwarfing with leaf yellowing and Malformation	100 (20/20)	68	Symptomless, Yellow leaves, Yellow and curly leaves	45 (9/20)	25	+
Chili var. Pelita 8							
C-	Symptomless	0 (0/20)	0	Symptomless	0 (0/20)	0	-
C+	Yellow leaves, Yellow and curly leaves, Yellow and cupping with leaf edges curl Upward or downward, Curly and cupping of the leaves, Plant dwarfing with leaf yellowing and Malformation	100 (20/20)	81	Yellow and curly leaves, Yellow and cupping with leaf edges curl Upward or downward, Curly and cupping of the leaves, Plant dwarfing with leaf yellowing and Malformation	100 (20/20)	87	+
E.cottonii	Yellow leaves, Yellow and curly leaves, Yellow and cupping with leaf edges curl Upward or downward, Curly and cupping of the leaves, Plant dwarfing with leaf yellowing and Malformation	100 (20/20)	72	Yellow leaves, Yellow and curly leaves	55 (11/20)	34	+

Note: Negative control, C- (PYLCVV negative test plants without treatment); positive control, C+ (PYLCV positive test plants without treatment)

Table 3. Phytochemical content in *Eucheuma cottonii* extract

Compounds	Molecular formula	Rf (minute)	AUC (%)	Uses of compounds
Propanoic acid, ethyl ester	$C_5H_{10}O_2$	2.476	37.71	Antimicrobial
n-Propyl acetate	$C_5H_{10}O_2$	2.500	10.00	As flavoring food, as a solvent for nitrocellulose and other cellulose derivatives and as a laboratory reagent
sec-Butyl acetate	$C_6H_{12}O_2$	2.961	8.63	As an extraction solvent in the process of various oils and medicines
Toluene	C_7H_8	3.070	6.41	As an industrial solvent, as a medicine, and as an antibacterial
Bicyclo [4.2.0] octa-1, 3, 5- triene	$C_{19}H_{18}O_6$	4.868	0.22	Ingredients for making medicines
Styrene	C_8H_8	4.868	0.22	Ingredients for making medicines

Note: Rf (retention time): The time required for a compound from being injected until it is detected by the detector; AUC (*area under curve*): abundance of compounds in a sample

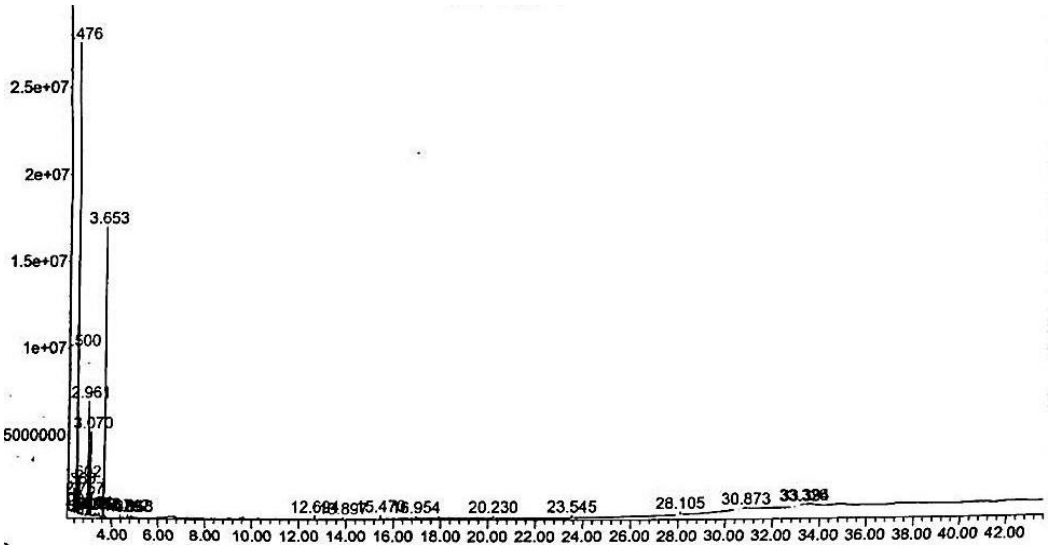

Figure 4. Chromatogram of *Eucheuma cottonii* seaweed extract from Serangan Island, Bali, Indonesia

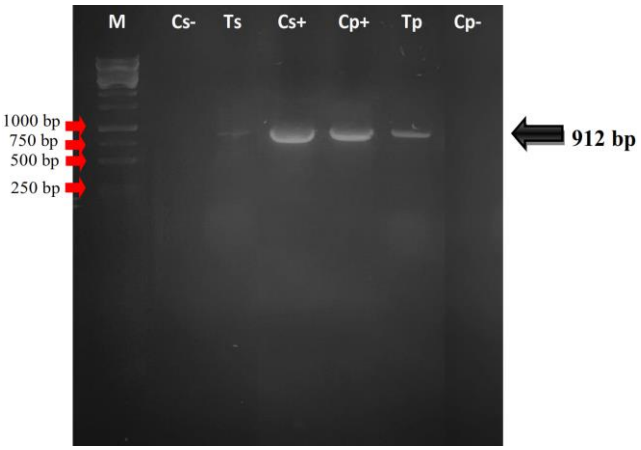

Figure 3. Visualization of Begomovirus specific DNA fragments amplified from leaf samples using universal primers SPG1/SPG2 on 1% agarose gel. (Cs-)- negative control on chili var. Seret, (Ts)- PYLCV on chili var. Seret treated with *Eucheuma cottonii*, (Cs+)- positive control on chili var. Seter, (Cp+)- positive control on chili var. Pelita 8, (Tp)- PYLCV on chili var. Pelita 8 treated with *E. cottonii*, negative control on chili var. Pelita 8 (Cp-)

The GC-MS analysis showed that other compounds in seaweed extract consisted of toluene and styrene, each with an AUC value of 6.41% and 0.22%, respectively. The toluene compound was used as an industrial diluter, drug component, and antibacterial agent (Cruz et al. 2014). Styrene compound on the other hand is a drug mixture component (Wu et al. 2019). The aromatic hydrocarbon compound in low amounts can lower the growth and development of microbes. *E. cottoni* also contains flavonoids, saponins, steroids, phenol hydroquinone, and triterpenoids (Listihani et al. 2023c). The active compounds in *E. cottonii* extracts can suppress the Begomovirus disease incidence up to 40% and suppress the disease severity to 60.76% (Listihani et al. 2023c). The bioactive compounds contained in *E. cottonii* have been proven to function as an antivirus. The macroalgae contained polyphenols, alkaloids, terpene, pigments, sterols, fatty acid, carrageenan, fucan, and several other compounds useful as an antivirus, antibacterial, and anticancer (Kalitnik et al. 2013; Barbosa et al. 2014; Prajapati et al. 2014; Hentati et al. 2020).

The disrupting mechanism of polyphenols depends on the type and structure of the virus. Polyphenol influences the virus replication cycle by interacting with the caspase protein synthesis active sites (Besednova et al. 2021). The mechanism behind the disruption by polyphenol extract depends on the type of virus and the origin of certain compounds. Most research showed that antivirus activity occurs through disrupting virus replication or limiting virus replication during the early stage of infection (Chojnacka et al. 2021; Listihani et al. 2022a, b; Selangga et al. 2022a, b; Hutasoit et al. 2023; Listihani et al. 2023a, b, c). Several research suggest the use of polyphenols as a form of prevention, allowing the blocking of virus entry and release of progeny virion (Denaro et al. 2020).

In conclusion the application of *E. cottonii* can reduce the PYLCIV infection symptoms from mild to severe symptoms into symptomless to mild symptoms. *E. cottonii* was also able to suppress the disease incidence and severity up to 55% and 43%, respectively. It caused lower virus concentration compared to positive control based on the PCR result. The major compound found in *E. cottonii* extract were propanoic acid, ethyl ester; n-Propyl acetate; sec-Butyl acetate; Toluene; Bicyclo [4.2.0] octa-1, 3, 5-triene; and styrene.

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