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ANTIFUNGAL ACTIVITY OF *Hevea brasiliensis* FRESH LATEX AND RUBBER PROCESSING EFFLUENT IN RELATION TO POLYPHENOL COMPOSITION AND POLYPHENOL OXIDASE ACTIVITY AS A POSSIBLE PROTECTION APPROACH AGAINST FUNGAL DISEASE

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ABSTRACT

Hevea brasiliensis, an important rubber plant in the region of South East Asia faces many pathological problems including the white root disease from *Rigidoporus micropus* that affects the latex production. In this study, C-serum from the fresh latex of *H. brasiliensis* and rubber-processing effluent from a processing plant was obtained to explore the possible antifungal activities and its relation to polyphenols. Antifungal activities of both samples were tested against the infective fungal organism of white root disease, *Rigidoporus microporus* and other soil-borne fungi including *Aspergillus niger*, *Fusarium* sp. and *Penicillium* sp. through poison plate method. Both samples were assessed for polyphenol content via total phenolic content (TPC) assay and its oxidizing enzyme, polyphenol oxidase (PPO). The C-serum showed the highest inhibition percentage on the *R. microporus* at 59% and lowest on *A. niger* at 16%, while the effluent only showed inhibition on *R. microporus* at 10%. Presence of phenolics was found higher in the C-serum (1.745 g/ml gallic acid equivalent) than the effluent (0.061 g/ml gallic acid equivalent). PPO activities were detected at 0.0145 unit/µg sample in *H. brasiliensis* and 0.0092 unit/µg sample in the effluent. This observation suggest the attribution of phenolics content towards antifungal activities in the *H. brasiliensis* which may be important in regulation of disease prevention through breeding activities. Understanding the PPO activity in *H. brasiliensis* is also important owing to the relation with protection against tapping wound in rubber plant as well as the browning of latex produced from this crop.

Key words: *Hevea brasiliensis,* antifungal activities, *Rigidoporus micropus*, polyphenol, disease resistance

INTRODUCTION

Hevea brasiliensis is the only commercial crops that produces natural rubber worldwide. Latex from the rubber plant is processed and turned into various rubber-based products that contributes to the income of several nations in the South East Asia region. However, *H. brasiliensis* that is massively planted in estates faces several threats including pathogens such as fungal infection, as well as pests infestation such as mites and bugs (Jayasinghe, 1999). The most severe problem that affect the plantation is white root disease caused by fungi *Rigidoporus micropus* (Jayasuriya & Thennakoon, 2007) and *R. lignosus* (Ogbebor *et al.*, 2015) causing a significant economic loss to the farmers in Malaysia (Sail *et al.*, 1990). The fungi is capable to decompose woody structure and spread to root of the plant by extending the hyphae to the bark surface (Farhana *et al.,* 2017). The non-rubber component of the latex is fractionated into several components including a clear serum known as C-serum that corresponds to the latex cytosol (Kongsawadworakul & Chrestin, 2003). This fraction has been reported for its antifungal effect on the *Aspergillus niger* (Daruliza *et al.*, 2011).

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Containing together in the latex from the rubber tree is polyphenol oxidase (PPO), an enzyme reported playing a major role in browning on cut or damaged and infected plant tissues due to the polymerization of PPO-generated quinones, generating phytomelamines (Araji *et al*., 2014). PPO also possessed potential roles in plant defense against pathogens and pests (Constabel & Barbehenn, 2008). Previous study have reported the role of PPO in plant defense mechanism on tapping-wound of rubber plants, specifically of *H. brasiliensis* (Wititsuwannakul *et al.*, 2002). It is the main motivation to this study for determining the PPO roles in regulating plants from pathogens.

The effluent from the rubber processing can affect the water surface quality by increasing biochemical oxygen demand (BOD) index when it is released untreated (Kumlanghan *et al.*, 2008). Presence of polyphenols in the latex C-serum, as well as in the effluent from rubber processing industry which was measured via spectroscopy and spectrophotometry method has been previously reported (Ismun *et al*., 2018). The presence of this valuable compound not only provides the potential to be isolated, but also offer the possibility of utilizing the effluent for applications such as in crop irrigation. This finding has therefore created an interest to investigate the antifungal activities and the PPO levels in both effluent and latex samples.

In this study, four strains of fungi including the white root disease fungal *R. micropus*, environment presence fungi *Aspergillus niger* and *Penicillium* sp. and postharvest product threat fungal *Fusarium* sp. were tested against C-serum latex and effluent from rubber processing via poisoned plate technique to determine the antifungal activities in the samples, and its relation to the phenolic composition.

MATERIALS AND METHODS

Sample collection and preparation

Latex from *H. brasiliensis* was obtained freshly from Bukit Putera rubber plantation in Setiu, Terengganu and rubber-processing effluent sample from Pond 1, MARDEC factory in Kuala Berang, Terengganu, Malaysia. Both samples were placed on ice to preserve the polyphenols content. The latex was then centrifuged at 19,000 rpm for 60 minutes at 4°C to separate into three-layer form containing latex at the top, followed by C-serum and B-serum at the bottom layer, according to Wang *et al.* (2010). The effluent was then filtered to discard the suspended debris and centrifuged at 9,000 rpm at 4°C. Then C-serum and supernatant from effluent were obtained and freeze-dried for further analysis.

Determination of polyphenol oxidase activity

Polyphenol oxidase (PPO) activity of both samples were measured spectrophotometrically via the oxidation of 5 mM catechol as a substrate at UV 410 nm, according to Wititsuwannakul *et al*. (2002). Two ml of 50 mM phosphate buffer pH7 and 1 ml of 5 mM catechol were added in test tube at 25°C. Enzyme-containing sample (0.1 ml) were added and placed in spectrophotometer. The absorbance readings were recorded every 10 seconds for 1 min. The absorbance then were plotted against time. Bradford assay for protein content determination were done using bovine serum albumin as standard. The enzyme were expressed as unit/mg sample.

Quantification of total phenolic content

Total phenolic concentration (TPC) was measured in C-serum of latex and the effluent from the processing industry via UV-Vis spectrophotometer according to the Folin and Ciocalteu (1927). Gallic acid was used as standard in this assay with concentrations between 0 to 100 mg/L. One mL of 70% ethanol was added to 20 mg of the dried C-serum and effluent sample and heated at 70°C. Then the mixture was centrifuged at 200 × g for 10 min. The supernatant was collected and diluted up to 20 mL of volume with distilled water. One mL of standards, blank and samples extracts were loaded in cuvette and 5 mL of diluted Folin Ciocalteu reagent (1:10 with ultrapurified water) were added and mixed. The mixtures were incubated at room temperature for 5 min. Then, 80 µl of 7.5% sodium carbonate were added to the mixtures in purpose to stop the reaction and incubated at 45°C for 30 min in dark. The mixtures were then read at 765 nm using the UV-Vis spectrophotometer. The area under the curve was calculated against the gallic acid standard curve, and results were expressed as gallic acid equivalent (GAE).

Assessment of antifungal activities

The antifungal activity was assessed according to the procedure reported in Ahanjan and Raveesha (2009). Potato dextrose agar (PDA) medium was prepared by adding 39 g of the powder into 1 L of distilled water then stirred and boiled prior to sterilization at 121°C for 15 min. Latex serum of 500 ppm and effluent was prepared by dissolving 50 mg dried serum or effluent respectively into 100 ml methanol. Then 15 ml of PDA was poured into the petri dish and 5 ml of dissolved latex serum or effluent was added as treatments. Petri dish representing controls were also prepared which includes 20 ml PDA as the positive control, and 15 ml PDA added with 5 ml methanol as the negative control. A 5 mm disc of 7 days-old culture of the test fungi was placed at the center of the each petri

dish and incubated at 25°C for 3 days for *A. niger*, *Fusarium* sp. and *Penicillium* sp. and 7 days for *R. micropus*. *R. micropus* which is a phytopathogenic fungus requires a longer incubation period to reach a maximum growth. Established fungi cultures of *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. were obtained from the Laboratory of Agri-Food pest and Disease Management (LAPDiM), Universiti Malaysia Terengganu, while *R. microporus* was previously isolated from infected rubber tree clones. The colony diameter was measured in mm after the incubation process to determine the inhibition effectiveness. All the treatment and controls were done in five replicates. The percentage of mycelial growth was determined by using the following formula:

$$Percentage\ inhibition = \frac{DC - DT}{DC} \times 100$$

Where DC = average mycelial growth in control and DT = average mycelial growth in treatment.

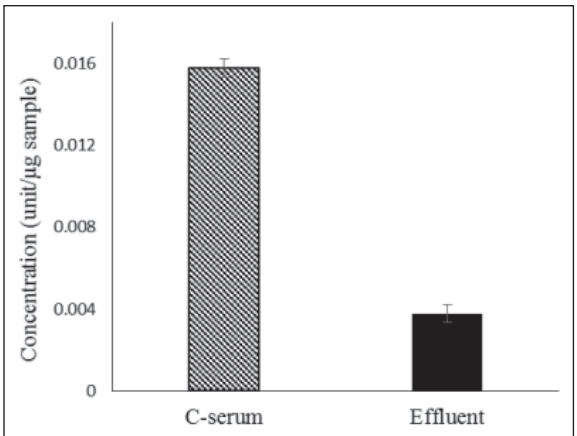
RESULTS AND DISCUSSION

Polyphenol oxidase activity

Figure 1 shows the PPO activities contained in the C-serum latex from *H. brasiliensis* and rubber processing effluent. PPO activities in latex C-serum was found to be 0.015779 unit/mg sample which was 76% higher than PPO activity in the effluent (0.003779 unit/mg sample). The enzyme activities were determined by measuring catechol concentration reacted with the enzyme. Polyphenol oxidase (PPO) is the enzyme that is responsible in the browning effect on damaged and exposed tissues on plants (Vaughn & Duke, 1984; Waleed *et al*., 2009). A study done by Coseteng and Lee (1987) proved that the degree of browning changed on apples parallel with PPO activities increment. PPO was also reported to be negatively correlated with extension of rotting on wound surface of potato (Wegener, 2002). Wititsuwannakul *et al*. (2002) reported that PPO plays a role in increasing polyphenols on the tapping wound of rubber plant as a defense mechanism to protect the tapping wound from pathogens. PPO is reported to induce oxidation of phenolics to free radicals which creates a hostile environment that retards the development of pathogens (Ngadze *et al*., 2012).

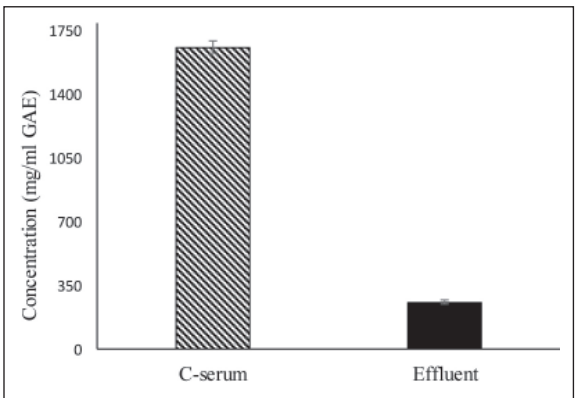
Total phenolic content

The total phenolic content in the C-serum fraction of latex and the effluent from the processing industry has been previously reported (Ismun *et al*., 2018) (Figure 2). The total polyphenol content of C-serum and effluent were expressed as mg/ml gallic acid equivalent (GAE) (standard equation of the curve: y = 0.002x; R^2 = 0.9967). There was a 6-fold higher of total polyphenol content in the C-serum (1665.1 mg/ml GAE) compared to the effluent (261.9 mg/ml GAE). Total phenols together with PPO has been reported to be important in disease resistance (Ngadze *et al*., 2012). Assessing these two components in *H. brasiliensis* could help provide a picture of the defense capability of this crop. Comparison with other resistant or disease-susceptible varieties of *Hevea* is therefore becoming our interest in order to expand the knowledge on protecting this crop against related diseases.



Data are mean of five-replicate extractions of a pool of latex from five trees; and five-replicate extractions of the effluent. Error bars represent standard deviation of the replicates.

Fig. 1. Polyphenol oxidase (PPO) activity in C-serum of *H. brasiliensis* latex and rubber processing effluent expressed as unit/µg sample.



Data are mean of five-replicate extractions of a pool of latex from five trees; and five-replicate extractions of the effluent. Error bars represent standard deviation of the replicates.

Fig. 2. Total phenolic content in C-serum of *H. brasiliensis* latex and rubber processing effluent expressed as mg/ml gallic acid equivalents.

The phenolic content in the effluent was only 15% than the value quantified in the C-serum, but as a byproduct or waste, the phenolic quantified is considered to be valuable. Many polyphenol compounds have been identified from industrial effluent including catechins, proanthocyanidins, glycosylated flavonols, rutin, luteolin and tyrosol found in wine and olive oil production wastewater (Torres *et al*., 2002; Trifunschi *et al.*, 2015). The phenolics found in the rubber processing effluent therefore reflect the possibility of isolation and minimizing loss through waste reprocessing. The findings also open up a possibility for utilizing the wastewater from rubber processing in applications that can be benefited from the various bioactivities of phenolics such as agricultural irrigation.

Antifungal activities

Antifungal activities was assessed by testing four strains of fungi including *R. micropus*, *A. niger*, *Fusarium* sp. and *Penicillium* sp. via poisoned plate technique. The growth of mycelia of *R. micropus* after 7 days, and *A. niger, Fusarium* sp. and *Penicillium* sp. after 3 days in PDA or PDA incorporated with 5 ppm of latex serum or effluent are shown in Figure 3. The percentage of inhibition in mycelia growth on the four tested strains of fungi cultures by the samples of latex serum and effluent were quantified by comparison to the controls (Figure 4). This study observed an antifungal activity in latex C-serum on all four strains cultures, with the highest inhibition on *R. micropus* (59%) and followed by *Penicillium* sp. (53%), *Fusarium* sp. (36%) and *A. niger* (16%). On the other hand, the effluent only inhibited the mycelia growth of *R. micropus* at a rate of 10%.

Rigidoporus is a common phytofungal that is capable to cause white root disease started by decomposing woody bark and secreting a wide range of hydrolytic and oxidative enzymes involved in plant polymer alteration before colonizing the root system (Nandris *et al.*, 1987). The rhizomorphs are also capable to spread up to several long without woody substrate and infected other plant

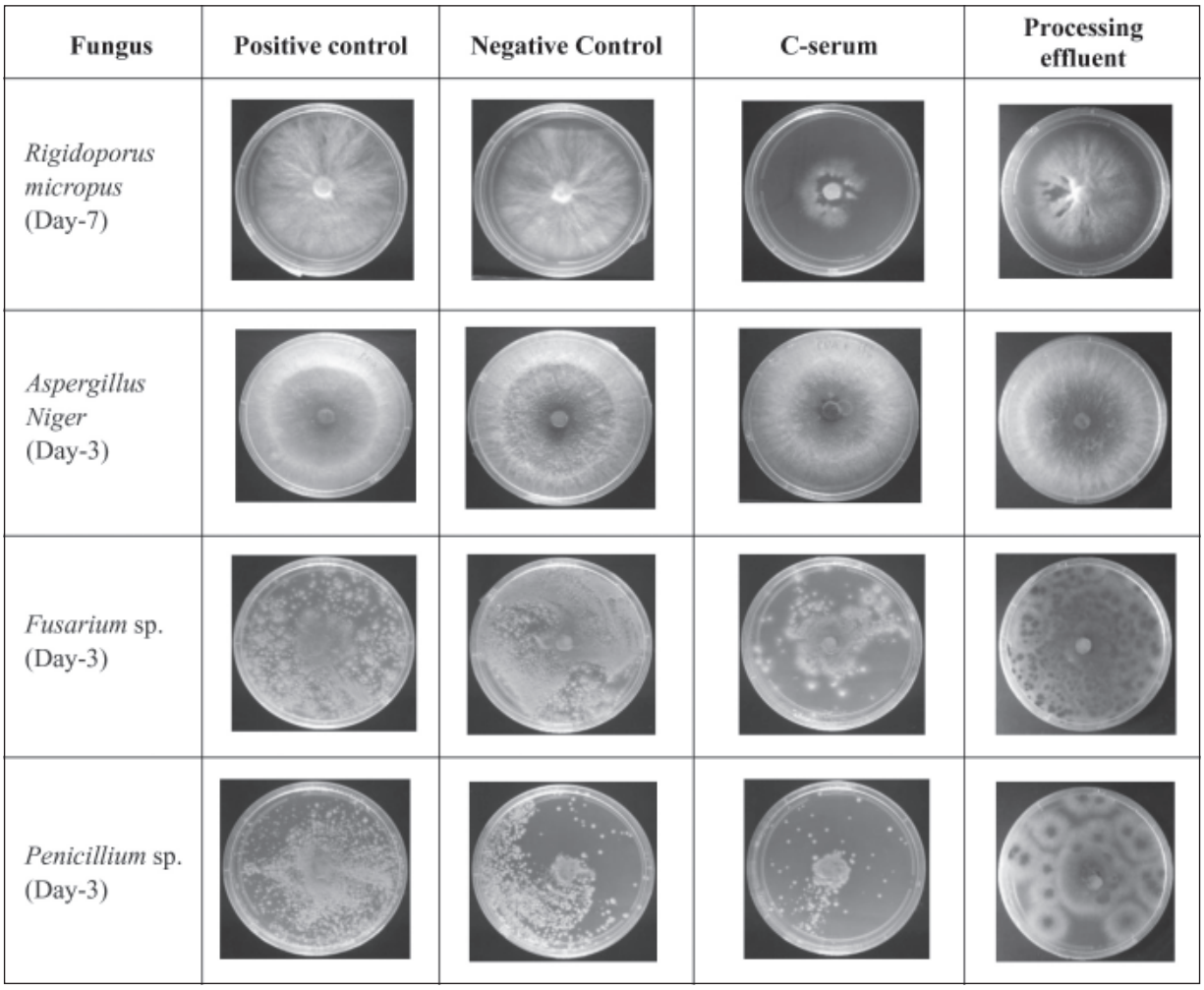


Fig. 3. Fungi cultures of *R. micropus*, *A. niger*, *Fusarium* sp. and *Penicillium* sp. in positive control, negative control, C-serum of *H. brasiliensis* latex and rubber processing effluent incorporated into the PDA.

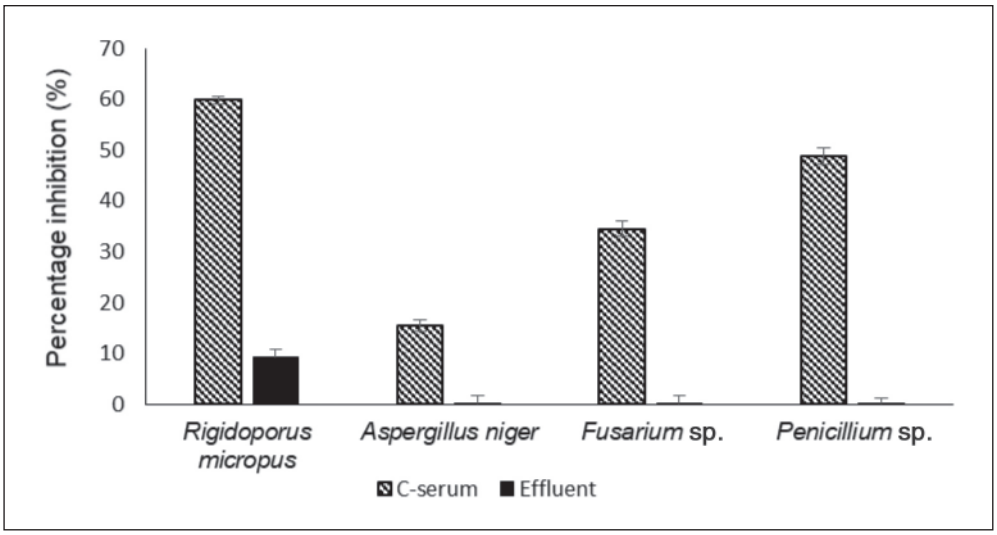


Fig. 4. Percentage inhibition of mycelia growth on the four selected strains of fungi by the C-serum of *H. brasiliensis* latex and rubber processing effluent.

(Wattanasilakorn *et al.*, 2012). It can cause death to rubber tree and huge loss to rubber plantations especially in Malaysia. To date, there are still very few studies reporting on antifungal activities in *Hevea* plants. Daruliza *et al*. (2011) outlined the antifungal activities in *H. brasiliensis* that affected the growth of *A. niger* and *Candida albicans* but they did not report involvement of polyphenol in the study.

However, previous studies have reported the association between phenolics with antifungal activity in other plants (Yang & Jiang, 2007; Morais-Braga *et al.,* 2017). Polyphenols has been successfully extracted and profiled in the latex C-serum and effluent from rubber processing using an optimized solid phase extraction and chromatographic method (Ismun *et al.,* 2018). Several polyphenols presented in both samples were identified including gallic acid, quercetin and naphtoic acid.

The capability of latex C-serum and the effluent to inhibit the mycelia growth of *R. micropus* may become an important factor in this crop that is crucial for the defense against the white root disease. Currently the white root disease is controlled by encouraging the growth of *Trichoderma*, which act as antagonist organism to *R. micropus* (Mohammed *et al*., 2014). Additionally, chemical control has also become the common approach towards protection against the disease (Gohet *et al.*, 1991; Nam *et al*., 2017).

The phenolics identified especially in the C-serum could be a marker for alteration in breeding programs for enhanced defense mechanism in this crop and other varieties. This could be an alternative approach to the chemical control for prevention of diseases in this crop. The level of PPO activity presented in the samples also might influence the strength of antifungal activities to fight against *R. micropus* and other microorganisms, as well as resistance from pests affecting this crop (Daruliza *et al*., 2011; Wititsuwannakul *et al.*, 2002). It will be useful to observe the PPO and phenolics substrate in other resistant or susceptible varieties of *Hevea* to further understand the relation between PPO and phenols to the antifungal activities in crops. Assessment of other enzymes related to plant resistance such as peroxidase and phenylalanine ammonia lyase is also important to be measured.

In addition to the antifungal activity observed from the C-serum, the smaller antifungal activity shown in the effluent was deliberated as valuable which has a prospect to be utilized prior disposal as waste to the environment. The findings from this study can provide aid to solve problems faced by the rubber industry.

CONCLUSION

In conclusion, the study successfully determined the antifungal activities against growth of four strains of fungi in the C-serum of latex from *H. brasiliensis* and the effluent from rubber processing industry as well as the presence of polyphenol oxidase activity. These data are supported with the previous analysis on total phenolic contents in both samples. The findings show promising observation for further investigation of the relation between phenolics and the plant defense activities in *H. brasiliensis.*

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