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Efficacy of stingless bee (*Heterotrigona itama*) propolis aqueous extract in controlling anthracnose and maintaining postharvest quality of chilli (*Capsicum annuum*) during storage

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Abstract

Anthracnose, mainly caused by *Colletotrichum* spp., is one of the fungal diseases resulting in major economic losses affecting chilli production worldwide. Propolis extract from honeybee has been discovered for its antifungal properties, which can potentially reduce postharvest decay. Therefore, the present work investigated the efficiency of aqueous extract of stingless bee (*Heterotrigona itama*) propolis in controlling postharvest anthracnose and maintaining quality of chilli. *Colletotrichum capsici* was isolated and characterised from infected chilli. Stingless bee propolis extract at different concentrations of 1, 2, 5, and 10% were assessed *in vitro* to inhibit the mycelial growth of *C. capsici*. The best concentration in inhibiting *C. capsici* growth was thereafter selected for *in vivo* experiments. All tested concentrations of stingless bee propolis extract inhibited the mycelial growth of *C. capsica*, the highest being 35% inhibition from the treatment of 10% propolis extract. The extract at this concentration was therefore tested *in vivo*, and showed effectiveness in reducing the percentage of disease severity in chilli. The propolis extract was also efficient in reducing weight loss, retaining firmness, pH, total soluble solid, and colour (a* value) throughout 21-day storage at 10°C. The present work demonstrated that aqueous extract of stingless bee propolis could have the potential to control anthracnose disease and delay deterioration, thus maintaining the postharvest quality parameters of chilli. This natural product from stingless bee has the prospect to be an alternative to synthetic fungicide.

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Introduction

Anthracnose is one of the major diseases that are commonly found in tropical fruits and vegetables (Barrera *et al.*, 2015). Globally, chilli (*Capsicum annuum*) is considered one of the most prevalently infected crop commodities. Anthracnose is caused by *Colletotrichum* spp. that results in the reduction of fruit quality postharvest (Prathibha *et al.*, 2013), thus affecting many farmers due to high postharvest losses. The application of synthetic fungicides has been a common conventional method used to treat this problem. The usage of chemicals to control decay in fruits during postharvest treatment could lead to possible toxicity risk for humans and the environment if used continuously (Tripathi and Dubey, 2004). This, therefore, warrants the exploration of effective natural product to potentially substitute the synthetic fungicides.

Stingless bees are found in tropical and subtropical regions of the world such as Southeast Asia, Australia, Africa, and South America (Mohd Rafie *et al.*, 2018). *Heterotrigona itama* is one of stingless bee species in the Indo-Malayan stingless bee clade. This species is widely domesticated in Malaysia and neighbouring countries such as Indonesia and Thailand. Locals prefer to rear this species because it is widely distributed, and easy to

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manage as compared to the other stingless bee species (Abd Razak *et al.*, 2016; Omar *et al.*, 2017). Apart from honey and bee breed, this species also produces propolis that has been reported for its various bioactivities (Abdullah *et al*., 2019). Propolis is a natural substance collected by the bees (stingless bee and honeybee) originated from different parts of the plants such as tree buds, sap flows, or other sources (Pasupuleti *et al*., 2017). This natural product of bees helps in retaining optimal structure and environment of beehive (Kasote *et al.*, 2015). According to Ibrahim *et al*. (2016a), stingless bee produces higher quantity of propolis than honeybee. Hence, the extraction of propolis from stingless bee was chosen over honeybee in the present work.

Propolis has been used traditionally for medicinal purposes in humans due to its potential health benefits. Propolis provides numerous health properties including antiseptic, anti-inflammatory, antioxidant, antibacterial, antifungal, antiulcer, anticancer, and immunomodulatory properties, thus making it exceptionally applicable in the treatment of various diseases (Pasupuleti *et al.*, 2017). There are at least 300 compounds contained in propolis including flavonoids, phenolic acids, terpenoids, steroids, as well as amino acids (Bankova *et al.*, 2000).

Propolis from honeybee is reported to be able to retard the growth of phytopathogenic fungi *in vitro* such as *Botrytis cinerea*, *Penicillium expansum*, *Penicillium digitatum*, and *Colletotrichum* spp. (Tripathi and Dubey, 2004; Meneses *et al.*, 2009; Abo-Elyousr *et al*., 2021). However, the possible antifungal activity of stingless bee propolis has not been largely explored. Investigation on the potential of this natural product as an alternative strategy towards controlling postharvest losses, particularly in reducing anthracnose, is therefore crucial. The present work thus aimed at assessing the potential of stingless bee propolis in reducing anthracnose and retaining postharvest quality in chilli.

Materials and methods

Stingless bee propolis and chilli collection

The propolis sample from the *H. itama* species was collected from Sekayu, Hulu Terengganu District in Malaysia, and immediately transported to the laboratory. Healthy, mature (maturity index of 2), and uniform sized chillies free from any detectable signs of infection of infection were harvested from a commercial orchard located in Kuala Terengganu, Malaysia.

Preparation of aqueous extract of stingless bee propolis

Stingless bee propolis was frozen and grinded in a chilled blender to prepare a powdered propolis (Soylu *et al.*, 2008). Propolis powder (25 g) was mixed with 150 mL of distilled water, and shaken continuously by orbital shaker (LM-570D, Taiwan) for 5 d at 27°C. The mixture was then centrifuged at 5,000 rpm for 15 min, and filtered with Whatman No. 1 filter paper to remove any insoluble wax. The filtrate was then evaporated (Büchi Rotavapor R-215, Germany) to obtain the crude extract prior to the preparation of four different concentrations of propolis extract (1, 2, 5, and 10%).

Isolation and characterisation of Colletotrichum capsici

The isolation of *C. capsici* was carried out from infected chilli samples showing anthracnose symptoms. Small slices of the chilli skin measuring 1 × 1 cm were cut using disinfected blade. Then, the surface was soaked with 1% sodium hypochlorite (v/v) for 2 min to disinfect. The surface was then rinsed with sterile distilled water, and dried on sterile filter paper. The sterile diseased tissue was inoculated by using scalpel to the centre of Petri dishes containing potato dextrose agar (PDA), and incubated at room temperature (28 ± 2°C) for 1 w. Single spore isolate was prepared according to Kimaru *et al.* (2018).

In vitro antifungal effect of stingless bee propolis aqueous extract

The assessment of *in vitro* antifungal activity of stingless bee activity of stingless bee propolis was prepared by using cork borer to transfer 5-mm diameter disc of *C. capsici* pure culture in the centre of Petri dishes containing PDA supplemented with propolis aqueous extract at concentrations of 1, 2, 5, and 10%. For control experiment, Petri dishes containing original PDA were used. PDA with and without propolis extracts were incubated for 6 d at 28°C (Long *et al.*, 2018). The percentage of mycelium inhibition was then taken by measuring the diameter of mycelium growth in control agar and agar plate supplemented with propolis extracts. The percentage of mycelium inhibition was calculated

using Eq. 1:

$$\%\ \text{Mycelium inhibition} = \frac{Diameter\ of\ mycelium\ growth\ (Control - Treatment)}{\text{Control}} \times 100 \quad \text{(Eq. 1)}$$

Assessment on the effect of stingless bee propolis aqueous extract on chilli

Ninety chillies were soaked in 1% sodium hypochlorite for 2 min, and rinsed with sterilised distilled water. Then, they were air-dried at room temperature. The selection of propolis concentration used for this assessment was based on the highest inhibition obtained through *in vitro* antifungal assessment. Half the number of chillies was immersed in 10% aqueous extract of propolis for 3 min, and air-dried (15 chillies per each replicate). The other half served as the control group, and was immersed in distilled water for a similar duration (15 chillies per each replicate). Chillies were then wounded using sterile needle at 2-mm depth, and inoculated with mycelial plug of *C. capsici*. Control chilli was inoculated with clean PDA only. Inoculated chillies were stored at 10°C for 21 d, and measured for postharvest quality parameter at 3-d intervals. The assessment included physical, chemical, and disease severity evaluations.

Assessment of physical quality of chillies

Physical quality was measured through percentage weight loss, change in colour (a* value; redness), and firmness of chillies. Weight loss of chillies from each treatment was measured throughout 21-d storage at every 3-d intervals (Barzegar *et al*., 2018). The difference between initial and final weight was considered as the total weight loss during the storage interval, and expressed as percentage weight loss using Eq. 2:

$$\text{Percentage weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \quad \text{(Eq. 2)}$$

The colour changes (redness) of chilli skin were analysed non-destructively using Konica Minolta Chroma meter Model CR400X (Chahbani *et al*., 2018). The a* values were taken from three different spots around the top, middle, and bottom of chillies.

Chilli firmness was measured at 3-d storage intervals using TA-XT Plus Texture Analyser (Stable Micro Systems, United Kingdom) (Chaple *et al*., 2016). The data were obtained using a 2-mm diameter stainless needle probe P/2N, a penetration speed of 5 mm/s, and a penetration depth of 30 mm. The firmness readings were taken on the upper, middle, and bottom part of chillies, and the readings were expressed in Newton (N).

Assessment of chemical quality of chillies

Chemical quality parameters measured included total soluble solid (TSS) and lycopene content in chillies. Total soluble solid of chilli pulp was determined using a hand-held refractometer. The chillies were crushed by mortar and pestle to obtain the juice. Then, the juice was filtered through a muslin cloth. A drop of the filtrate was placed on the prism glass of the refractometer. The refractometer was calibrated with distilled water before analysis. The TSS was recorded and expressed as °Brix.

Lycopene was measured according to Anthon and Barrette (2007) with slight modifications. Chilli (1 g) was added with 100 mL of hexane:ethanol:acetone (2:1:1), and incubated for 10 min. Thereafter, 15 mL of water was added to reach phase separation. The upper phase was collected and measured for absorbance on UV-Vis spectrophotometer UV-1800 (Shimadzu Corporation, Japan) at 503 nm wavelength. The lycopene concentration was calculated using Eq. 3:

$$\text{Lycopene (mg/g)} = \frac{\text{Abs} \times 537 \times 100 \times 0.55}{172 \times \text{W}} \quad \text{(Eq. 3)}$$

where, Abs = absorbance at 503 nm; 537 g/mol = the molecular weight of lycopene; 100 mL = volume of mixed solvent; 0.55 = the volume ratio of the upper layer to the mixed solvent; 172 mM^{-1} = the extinction coefficient for lycopene in hexane; and W = weight of sample (g).

Assessment of disease severity index of chillies

The disease severity was assessed at the end of the storage period (day 21, based on the following scale (from 0 to 5) according to Yadav *et al.* (2017)), and was categorised as follows:

0 = healthy, no disease;
1 = 1 - 5% of fruit area were affected;
2 = 5.1 -10% of fruit area were affected;
3 = 10.1 - 25% of fruit area were affected;
4 = 25.1 - 50% of fruit area were affected; and
5 = > 50.1% of fruit area were affected.

Then, the percentage of disease severity (DSI) was calculated using Eq. 4:

$$\text{DSI (\%)} = \frac{\text{Total number of symptomatic fruits} \times \text{Severity index}}{\text{Total number of fruit examined} \times \text{maximum rating scale number}} \times 100 \quad \text{(Eq. 4)}$$

Statistical analysis

A complete randomised experimental design was performed with three replicates per treatment. Data were subjected to analysis of variance, *t*-test, and Wilcoxon signed-rank test using IBM SPSS Statistics 23. Statistical significance was considered at $p < 0.05$.

Results and discussion

Morphological characteristics of Colletotrichum capsici isolates

The morphological characteristics of isolated fungi showing colony characteristics, type of conidia, and setae were identified, and *C. capsici* isolates was recognised (Figure 1). Colonies of *C. capsici* showed grey to white colour with thin white cottony mycelia. Reverse colony shows concentric ring with black averculi orange conidia. The shape of conidia of the isolate agreed with previous description by Sharma *et al.* (2005).

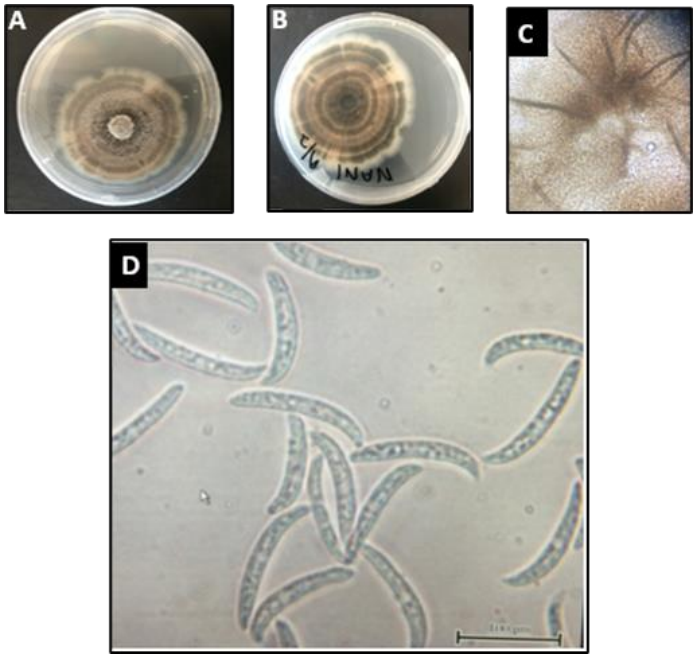


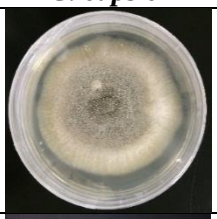
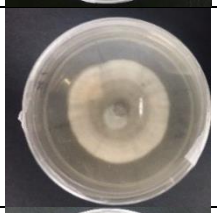
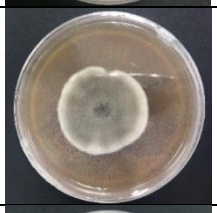
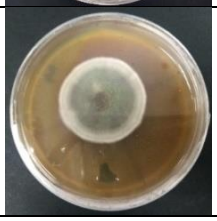
Figure 1. *Colletotrichum capsici*, **(A)** obverse view showing grey to white colony with thin cottony mycelium, **(B)** reverse view showing concentric ring with black averculi orange conidial masses, **(C)** setae, **(D)** falcate conidia.

The effect of different concentrations of stingless bee propolis aqueous extract on percentage of in vitro mycelial inhibition of C. capsici

Table 1 illustrates the inhibition of *C. capsici* mycelial growth on PDA supplemented with different concentrations of aqueous extract of propolis after 6-d incubations. Mycelial growth inhibition was positively related to the tested concentration of propolis where inhibition values for concentrations of 1, 2, 5, and 10% were 5.18, 13.08, 24.14, and 34.63%, respectively. Stingless bee propolis showed valuable antifungal activity against *C. capsici* alongside those reported from the honeybee propolis (Maysoun *et al*., 2014; Ali *et al*., 2014; 2015). Honeybee propolis have been reported for its effectiveness in inhibiting the growth *C. capsici* (Ali *et al*., 2014) and other important agricultural fungi species such as *Botrys cinerea*, *Fusarium* sp., *Alternaria alternata* (Curifuta *et al*., 2012), *Penicillium expansum* (Matny *et al*. 2015), and *Clostridium musae* (Dudoit *et al.*, 2020). These studies utilised ethanolic extract of honeybee propolis, showing a greater mycelial inhibition of the tested fungi, for instance 40% mycelial inhibition of *Fusarium* sp. and 89% inhibition of *C. capsici* by 1 and 0.5% honeybee propolis, respectively (Curifuta *et al*., 2012; Ali *et al.*, 2014). Therefore, the difference of capacity in inhibiting the mycelial growth found in the present work could be attributed to the more efficient extraction of bioactive substances from the propolis using ethanol (Pobiega *et al*., 2019). However, aqueous extraction of propolis has the advantage of lower production cost, and the absence of ethanol in the solution may be a preferred choice for food applications (Pobiega *et al*., 2019). The present work thus added to the scarce body of literature on the potential of aqueous propolis extract as an agricultural antifungal agent.

The antifungal activity against *C. capsici* reported in the present work could be attributed to the bioactive compounds present in the stingless bee propolis. Ibrahim *et al.* (2016b) have previously reported the presence of various bioactive compounds in *H. itama* propolis such as terpenoids, flavonoids, phenols, essential oils, steroids, saponin, and coumarin. These bioactive compounds might have synergistically hindered the access of *C. capsici* to obtain nutrients from the PDA, thus restricting the mycelial growth (Ali *et al.*, 2014).

Table 1. Percentage (%) of mycelium inhibition of *C. capsici* with different concentrations of propolis aqueous extract.

Treatment	Percentage of mycelium inhibition (%)	Inhibition zone of *C. capsici*
Control (0% propolis extract)	0	
1% propolis extract	5.18	
2% propolis extract	13.08	
5% propolis extract	24.14	
10% propolis extract	34.63	

The effect of 10% propolis aqueous extract on postharvest quality assessment of chillies

Aqueous extract of stingless bee propolis at a concentration of 10% was selected for the *in vivo* analysis following the observation of the highest antifungal activity against *C. capsici in vitro*.

The effect of 10% propolis aqueous extract on physical quality of chillies

The weight loss of chillies increased steadily with storage time (Figure 2A). Weight loss is associated with water loss of most fruits by transpiration and respiration (Abbasi *et al.*, 2011). The higher rate of weight loss in control chillies could have been a result of membrane damage and skin surface cracking (Lara *et al.*, 2014) caused by the disease development which was detected on day 6 of storage. The propolis-treated chillies, on the other hand, had a significantly reduced rate of weight loss ($p < 0.05$) over 21-d storage, thus suggesting that propolis could create an efficient moisture barrier that helps to protect epidermal tissue of chillies (Ali *et al*., 2015). A previous study investigating the addition of propolis into an edible film reported the effectiveness of the propolis in enhancing the water vapour barrier properties of the film (Pastor *et al*., 2010). This suggested that the stingless bee propolis could create a barrier on the skin of chillies that helps prevent the loss of moisture, which therefore aids in preserving them. Previous studies have reported similar observations on the effectiveness of honeybee propolis to delay weight loss of dragon fruits and cherry tomatoes (Zahid *et al.*, 2013; Pobiega *et al.* 2020). Propolis was also found to delay the reduction

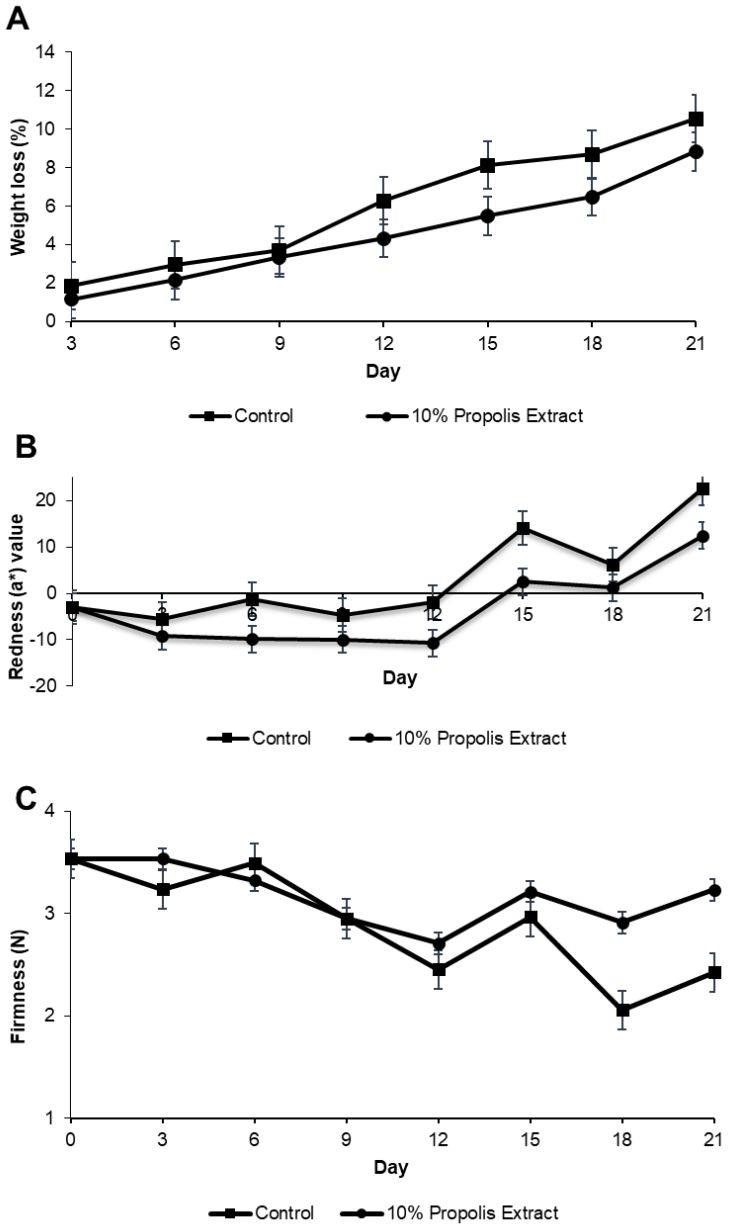


Figure 2. Effect of 10% stingless bee propolis aqueous extract on physical quality, **(A)** percentage weight loss, **(B)** colour (redness, a*), and **(C)** firmness of chilli stored at 10°C for 21 days. Data are mean ± standard error.

of weight in many fruits including bananas, grapefruits, grapes, mangoes, oranges, papayas, pomegranates, bell peppers, and tomatoes (Passos *et al.*, 2016). This, therefore, suggested the effectiveness of propolis extract in creating a protective layer on the chillies which reduced the loss of moisture, thus lowering the rate of weight loss.

Colour is an important attribute in determining the quality of fruits, especially in determining the ripeness and consumers' acceptability. Figure 2B illustrates the effect of propolis extract on the redness (a*) intensity of chillies. Redness (a* values) increased gradually throughout the storage period in both control and propolis-treated chilli from negative to positive values, thus demonstrating the colour changes from green to red throughout the storage period. The colour changes were associated with the increase in carotenoid due to the conversion of chloroplast to chromoplast during ripening stages (Pola *et al*., 2019). The present work demonstrated that propolis-treated chillies had a significantly lower redness value as compared to control ($p < 0.05$). The lower increase in a* value with application of propolis extract indicated the potential of propolis treatment in delaying the ripening process of chillies. The delayed change in colour of chillies was also in

accordance with a previous report by Ali *et al*. (2014) which used honeybee propolis in an edible coating.

Firmness, which evaluates the hardness or crispness of fruits, is one of the important parameters in determining the fruit quality during storage. Figure 2C shows the effect of stingless bee propolis extract on maintaining the firmness of chillies. Control group of chillies had a gradual decrease in firmness from day 6 to 12 of storage due to the observed disease development on them. Tissue softening occurs when fruits reach ripening-cell wall degradation and starch hydrolysis (Khaliq *et al.*, 2015). In the present work, it was observed that propolis aqueous extract significantly delayed reduction in firmness over 21-d storage period ($p < 0.05$). Propolis may have affected the insolubility of pectic material which inhibited the degradation of pectin by the action of cell-wall hydrolytic enzymes such as polygalacturonase (da Cunha *et al.*, 2018). Data obtained in the present work suggested the effectiveness of propolis in retaining structural quality and delaying deterioration of chillies. The possible protection of chilli epidermal tissue by the propolis may have been able to reduce their moisture loss, thus supporting the observation of reduced weight loss observed in the present work.

The effect of 10% propolis aqueous extract on chemical quality of chillies

Figure 3A shows the effect of propolis extract on TSS of chillies. TSS in both control and treated chillies showed a gradual increase during storage, which was similarly reported by Putra *et al.* (2017) on the effect of propolis treatment on tomatoes. The increase in TSS value at the end of storage could be associated with the conversion of carbohydrates into sugars (Tsegay *et al.*, 2013) during ripening. The present work showed that the increase in TSS in chillies treated with propolis was significantly delayed as compared to control ($p < 0.05$), which suggested a slower conversion of starch into water-soluble sugars (Samira *et al*., 2013). This observation was in conformity to previous studies reporting on a lower TSS values of other fruits treated with propolis, such as bananas (Passos *et al*., 2016) and peppers (Ekhuemelo *et al*., 2018). A slower increase in TSS in propolis-treated chillies in the present work thus indicated the potential of stingless bee propolis in delaying their ripening.

Lycopene is a bright red pigment highly found in most fruits and vegetables, especially in ripe chillies. During ripening, chlorophyll content

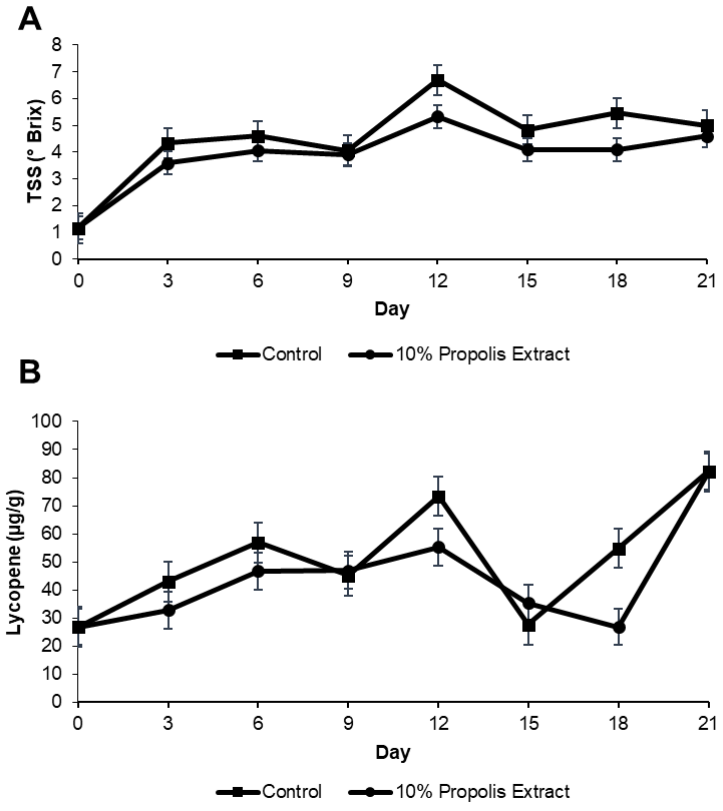


Figure 3. Effect of 10% stingless bee propolis aqueous extract on chemical quality, **(A)** total soluble solid, and **(B)** lycopene content of chilli stored at 10°C for 21 days. Data are mean ± standard error.

reduces, and the synthesis of the red pigments lycopene begins (Pola *et al.*, 2019). Figure 3B shows the lycopene content of chillies, with and without the treatment of propolis extract, throughout the storage period. Chillies treated with propolis extract showed a tendency for a lower increase in lycopene level as compared to control, but no significant difference was observed. This observation was in accordance with a report by Putra *et al.* (2017) who observed no significant effect on lycopene level of tomatoes following treatment with propolis. In relation to the significantly lower redness intensity (a* values) of propolis-treated chillies discussed earlier, a more predominant carotenoid in chillies, which is capsanthin (Pola *et al.*, 2020), could be more affected by the propolis treatment. Therefore, it is suggested that the value of capsanthin should be measured to assess the effectiveness of any potential postharvest treatments in the future.

Effect of 10% propolis aqueous extract on disease severity index (%) in chillies

Figure 4 illustrates that the percentage of disease severity in chillies treated with propolis extract was significantly lower as compared to control ($p < 0.05$). Lower disease severity (35%) was recorded in chillies treated with propolis extract as compared to control (45%) at the end of storage period. The *in vitro* antifungal activity observed from 10% stingless bee propolis extract as shown earlier in Table 1 supports this *in vivo* assessment. Ali *et al.* (2015) also reported the efficiency of honeybee propolis in reducing disease caused by *C. capsici* on bell peppers. Inhibition of disease severity in raspberries was also observed from the application of honeybee propolis extract as reported by Moreno *et al.* (2020). Phenols and flavonoids present in stingless bee propolis (Ibrahim *et al.*, 2016a; Abdullah *et al.*, 2020) could be attributed for the inhibition of disease development due to their active antifungal capacity (Kanwal *et al.*, 2010; Matei *et al.*, 2018). Zulhendri *et al.* (2021) reviewed several published articles on the attribution of phenolics and flavonoids towards antifungal activity against many fungal species. These compounds could also be responsible against plant pathogenic fungi such as *C. capsici* which causes deterioration of chillies.

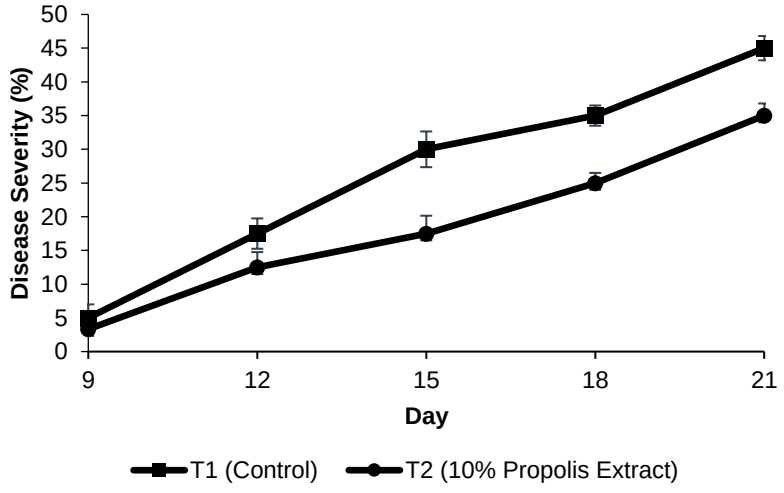


Figure 4. Effect of 10% stingless bee propolis aqueous extract on percentage of disease severity of chilli stored at 10°C for 21 days. Data are mean ± standard error.

Conclusion

The present work revealed that aqueous extract of stingless bee propolis could have the potential to be an antifungal agent that could be used against anthracnose disease in chilli. *In vitro* assessment showed that the aqueous propolis extract inhibited mycelial growth of *C. capsici*. Additionally, *in vivo* assessment exhibited a significant impact of the extract on improving select measures of postharvest quality in chilli which included decreased weight loss and disease severity, and retained firmness, a* value, and total soluble solid. The application of stingless bee propolis can potentially substitute the use of synthetic fungicide in agriculture in ensuring safety of the harvested produce. Moreover, the application

of aqueous propolis extract could be combined with other treatments for potential synergistic effect in controlling anthracnose. The prospect of utilising this natural product from stingless bee could also increase its commercial value, and economically benefit stingless beekeepers.

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