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Inhibition of Cancer Disease in Selected Citrus Plant by *Gelonium multiflorum* Extract

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Abstract— Inhibition of *Xanthomonas campestris* pv. *Citri*: the pathogenic bacteria that caused canker disease in citrus plant were studied in lemon leaf by *Gelonium multiflora* extract. The antimicrobial activity was examined and antioxidant activities of the extracts were compared with ascorbic acid by scavenging effect with 2, 2- diphenyl-2-picrylhydrazyl radical (DPPH). Finally, the extracts were inhibited of canker disease in lemon leaves growing in the greenhouse. Dichloromethane extract of bark was purified by silica gel column with the solvent mixture of dichloromethane:methanol in polarity order from less polar to more polar. The purified fraction with 1:1 ratio at 400 – 500 mg L^{-1} followed by the ratio 1:9 (dichloromethane:methanol) purified from stem extracts at 500 mg l^{-1}provided the inhibition activities against *Xanthomonas campestris* pv. *Citri*. The diameters of clear zone were 20 and 15 mm, respectively. Purified fraction from stem eluted with 1:9 and 3:7, dichloromethane:methanol and purified fraction from bark eluted with 4:6, dichloromethane:methanol provided antioxidant activities with DPPH but lower than ascorbic acid. Purified dichloromethane: methanol; 1:9 **(**at 700 mg L^{-1} completely inhibited the growth of *Xanthomonas campestris* pv. *Citri*. Moreover, purified fraction of bark with dichloromethane:methanol; 2:3 **(**at 500 mg L^{-1} partially inhibited. All purified fractions (from column chromatography) of bark extract presented higher inhibition activities against *Xanthomonas campestris* pv. *Citri* than stem extracts of *Gelonium multiflora*.

Keywords— **Inhibition; *Xanthomonas campestris* pv. *Citri*; canker disease; *Gelonium multiflora*; antioxidant activities**

INTRODUCTION

Citrus canker was firstly found in Florida since 1912. It was widely spread throughout southeastern parts of the US. Its outbreak was originated from the imported seedlings from Japan and was eradicated from Florida and the adjacent states in 1933. Citrus canker is characterized by erumpent lesions on fruit foliage, and young stems of citrus resulting the defoliation, dieback, and fruit drop (Fig 1) after severe infection (Dospon, 1964; Civeloro, 1984; Timmer, 1984; Graham, 1984). Additionally, the citrus fruits loosed their qualities and quantities due to the infected tree debilitation. The pathogenic bacteria; *Xanthomonas axonopodis* pv. *Citri* occurred in large areas of the world's citrus growing countries including India, Pakistan; the islands of Indian Ocean, South-East Asia, China and Japan (Achor, 1996; Das, 2003).

Xanthomonas axonopodis pv. *Citri (*synonym *X. campestris* pv. *Citri*); the gram negative-straight-rod bacteria; is a polar flagellum, strictly aerobe bacteria with approximately 6x2 micron in dimension. It can hydrolyze starch and gelatin but cannot reduce nitrate to nitrite. Acid was produced when cultured in glucose, galactose, fructose, sucrose and mannose media, but not from culturing on arabinose. The different degrees of disease depend on pathogenicity and virulence of different strains. The virulent *Xanthomonas axonopodis* pv. *Citri* was isolated from citrus hystrix, tangerine and pummelo, respectively . In Thailand, citrus canker is a serious disease that affects all types of citrus and severely infects on *Citrus aurantifolia*, lime (Pinyopap, 1987; Bui, 2007).

Fig. 1 Canker disease in citrus plant. (a) Citrus canker lesions on immature fruit stems and foliage of citrus plant (b) orange tree showing fruit drop due to citrus canker infection (c) defoliation from severe canker infection (Gottwald, 2002).

Typical symptom of the infected plant is the occurrence of a necrotic lesion surrounding with yellow halo on leaves (about 2 – 10 mm circular spot) but halo seldom occurs on fruit and

stem. The pathogens can induce a blister-like (spongy postulate) lesion in both epidermal surfaces from tissue hyperplasia. These white or pale yellow postulates were then darken and thicken from light tan to brown corky canker with roughly touch (Leksonboon, 2003) .

Since *Xanthomanas campestris* pv. *Citri* can frequently be detected in soil, citrus roots, and 17 various weed species collected in citrus orchards. The bacteria can survive for 2 to 3 months before declining to nondetectable levels after burying lesion infected citrus. To control the disease requires integrated cultural practices and chemical sprays. The trees must be planted using disease-free stocks, pruning, burning all infected, and applying copper formulations sprays (Goto, 2003; Lee, 1920; Agrios, 1997). The limitation of applying controlled chemicals is to develop a resistance by phytopathogenic bacteria to these chemical control agents (Sigee, 1993). The mixed antibiotics such as Kanker-X (20 % streptomycin-sulfate + 2.5 % oxytetracycline + 2.5 % procain penicilin G, 200 ppm) is the best antibiotic that can control citrus canker at the different degrees followed agrimycin (18.8 % streptomycin-sulfate + 1.5 % oxytetracycline, 400 ppm) and tetracycline (800 ppm), respectively. Recently, the herbal extract becomes an alternative natural pesticide for controlling of plant disease including canker instead of chemical pesticides or synthetic antibiotic. The herbal extracts are freed from chemical residue in both plant and soil and safe fruits for human health. The ethanolic extracts from guava leaf, beleric myrobalan fruit, pomegranate fruit peel, nut gall fruit and myrobalan wood fruit have potentially inhibited the growth of *Xanthomanas campestris* pv. *Citri* (Vudhivanich, 2003). Canker disease on *Citrus aurantifolia* (lime) caused by *Xanthomonas axonopodis* pv. *Citri* (Xci12) could be controlled by five aqueous extracts of *Hibiscus subdariffa* Linn., *Psidium guajava* Linn., *Punica granatum* Linn., *Spondias pinnata* (Linn.f.) Kurz, and *Tamarindus indica* Linn. from 18% to 52%.under greenhouse condition.

Gelonium multiflorum (Fig.2) is a plant belonging to Euphorbiaceae family; it is found in the tropical rain forests around central, eastern and southern Thailand (Daubenfeld, 2005). The beautiful-straight-stem with white to pale yellow bark and dark green leaves, the flowers bloom around March to May, come to the fruits on April to June. Isolation of chemical constituents from *Gelonium multiflorum* consisted of various compounds of alkaloids, cardiac glycocides, flavanoid, saponin, terpinenoids, lactone, gelonin and so on. The gelonin located in the vacuole of *Gelonium multiflorum* seeds composed of amino acid sequence and the glycosylation motif of the ribosome inactivation proteins. The homologue of terpenoids and related compounds-III (bauerenol and multiflorenol) were isolated from its bark (Sengupta, 1963). Two flavone diglycosides along with 7, 4′-O-dimethylscuttelarein and 6-O-beta-D-glucopyranocides were extracted from seeds of *Gelonium multiflorum* and the chemical structures were also provided [18]. The minor constituents, i.e., diterpine lactones and gelomulides G-J, were isolated from leaves. In medical purposes, the MAP30 (Momordica anti-HIV protein of 30 kDa) and GAP31 (Celonium anti-HIV protein of 31 kDa) from *Gelonium multiflorum* potentially inhibited the infection of human immuno-deficiency virus type I (HIV-1) in T lymphocytes and monocytes. *Gelonium multiflorum* extracts could also be applied for phytopathogenic bacteria. The polar compositions extracted from various parts of *Gelonium multiflorum* inhibited canker bacterium; *Xanthomonas campestris* pv. *Citri* as well as *Staphylococcus aureus, Bacillus subtilis Escherichia coli, Pseudomonas aeruginosa* and *Mycobacterium lacticola* (Khuntong, 2008)

Fig. 2 *Gelonium multiflorum* A Juss

The research aims were to separate the crude extract by silica gel column chromatography. The separated fractions were examined for antioxidant activity with DPPH assay and *in vitro* antimicrobial activities. The inhibition of canker disease in lime causing from infection of *Xanthomonas campestris* pv. *Citri* was studied in greenhouse.

MATERIAL AND METHODS

Plant collection and preparation.

G*elonium multiflorum* was planted in the botanical garden, Kasetsart University, Si Racha Campus and identified by comparison with Vecsommai and Kavduengtain (Vecsommai, 2004). The branches (about 10 centimeters in diameter) were collected and removed all the leaves. After cutting into about 30-cm-long, the stem was cleaned and the bark was peeled off. The stem and bark were air-dried separately at room temperature and ground.

Stem and bark extraction of Gelonium multiflorum.

Dried bark powder was initially soaked in hexane for 7 days and filtered. The filtrates were dried under vacuum by using rotary evaporator. The bark residues were continuously extracted twice with hexane using the same procedures as the first extraction. The dried crude of two extracts was combined and weighted. The bark residue from hexane extract was further extracted with dichloromethane followed by methanol using the same procedures as hexane extraction. The crude extracts were stored at 4 ^{0}C until further use.

Dried stem powder of 2 x 1000 g was initially soaked with hexane (2 x 4 l) and followed the same procedures as bark extraction. Stepwise of extraction procedures was shown in Fig. 3. Discard the nonpolar components from hexane extraction. The moderately polar to polar extracts from dichloromethane and methanol were then separated through silica gel column chromatography.

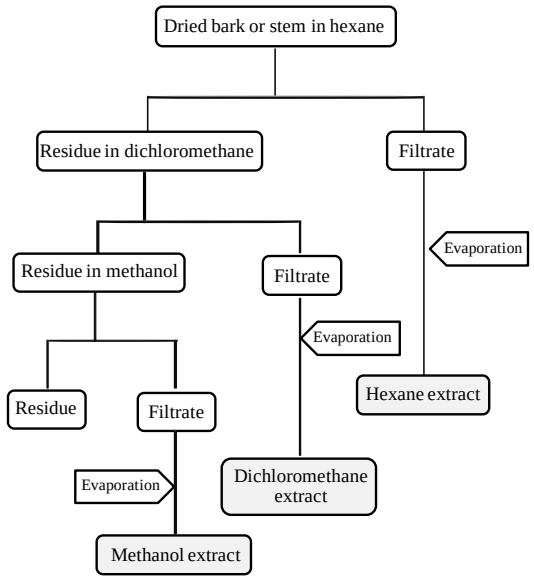

Fig. 3 *Gelonium multiflorum* extraction procedures.

Preliminary separation of crude extracts by thin layer chromatography

The chemical compositions in crude extracted from dichloromethane and methanol were roughly separated by thin layer chromatography (TLC aluminum sheets silica gel $60F_{254}$, 20 x 20 cm^2). The mixed solvent (hexane, dichloromethane and methanol) in various ratios from less polar to more polar was used as mobile phase. The mobile phase was initiated from the mixture of hexane and dichloromethane, the ratio was varied from 100:0, 90:10, 80:10,…, 100 v/v, respectively. The mobile phase was further changed to dichloromethane and methanol with the same manner. The separation was held in chromatographic tank saturated with gaseous mobile phase. Each composition was detected under ultraviolet light at the fixed wavelength of 254 nm. The separated compositions were identified by fluorescent spots of different retention factor; R_f.

Separation of crude extracts by using column chromatography.

The nonpolar fractions may not be quantitatively purified due to contain very small amount of active ingredients to microorganisms [19]. The dichloromethane extracts of bark and stem were chromatographed over silica gel column (5 x 60 centimeter) using solvent mixtures of increasing polarity starting from the solvent mixture of hexane:dichloromethane to dichloromethane:methanol. as eluant. Every 50-ml of eluant was collected and dried under vacuum with rotary evaporator. The dried fractions of same compositions (characterized on TLC aluminum sheets silica gel $60F_{254}$ and eluted with a mixture of chloroform and petroleum ether; 30:70; v/v) were combined in addition to obtain the yield of separated fractions. The separated fractions were used to study antioxidant activity, *in vitro* evaluation of bacterial inhibition. The inhibition of canker disease in lime was also examined in greenhouse.

Antioxidant activities by scavenging with 1,1-dipheny picrylhydrazyl (DPPH) assay.

DPPH• is a stable free radical because its spare electrons delocalized over the whole molecule. A deep violet color of DPPH• is detected at the maximum absorption, λ_{max} at 520 nm. A non-radical form of DPPH is obtained from the reaction of the radical scavenger and DPPH radical resulting (Szabo, 2007) the appearance of the pale yellow. The experimental procedures were modified from Brand-Williams et al. by mixing the solution of each fraction from column chromatography (25 mg) in methanol (10 ml) with DPPH solution (6×10^{-5} M). The ratios of separated fraction and DPPH were varied from 3:1, 1.5:1 and 0.75:1, the mixed solutions were kept in darkness for 15 min. The appearance of violet (DPPH) turned to pale yellow (DPPH). The absorbance of pale yellow solution was measured in a well-calibrated Biochrome S112 double beam spectrophotometer at λ_{max} 520 nm over blank (3 replicates). The antioxidant activity of herbal extract was compared with ascorbic acid. Radical scavenging activity was calculated by equation:

$$\% \text{ DPPH Scavenging} = 100 \times \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \quad (1)$$

Where absorbance of control was the absorbance in absence of extracts, absorbance of sample is the absorbance in presence of standards or extracts [24].

In vitro evaluation of bacterial inhibition

1. Test microorganisms. The bacterium *Xanthomonas campestris* pv. *Citri* in SX agar (special solid medium for *X. Campestris*) was obtained from Department of Plant Pathology, Faculty of Agriculture Kamphaeng Saen, Kasetsart University. The stock culture was maintained in nutrient glucose agar (NGA) slant at 4°C and sub-cultured monthly.

2. Evaluation of antibacterial activity. Antibacterial activity of the fractions from bark and stem were evaluated by paper disc diffusion method [25]. *Xanthomonas campestris* pv. *Citri* was cultured by inoculating a loopful of test organism in 50 ml of nutrient glucose broth (NGB) from NGA slant. Broth was incubated on a shaker at 200 rpm, 30°C for 24 hours. The optical density (OD) was adjusted to 0.1 at wavelength 590 nm . A suspension of the tested microorganism 100 µl of 10^5 cells per ml is spread on the NGA agar plate. Filter paper discs (6 mm in diameter) were impregnated with 10 µl of each herbal fraction at the range between 20 – 300 ppm and placed on the NGA agar. Discs impregnated with methanol were used as negative controls. These plates were incubated at 30 ^oC for 24 h. The diameters of the inhibition zone are measured in millimeter (mm). All tests were performed in triplicate.

3. Inhibition of Xanthomonas campestris pv. Citri in lime: greenhouse experiment. Lime trees about 2-feet high (free from infection) were planted to a pot of 8 inched diameter in greenhouse. The leaves of six-month-ages lime were inoculated by leaf puncture method. After cleaning, the lime leaves were prickled with a sterile sharp needle and then immediately inoculated with a drop of *Xanthomonas campestris* pv. *Citri* with concentration of 10^5 CFU/ml (at least five leaves/plant) were infected, totally ten plants. The tested plants were grown in a greenhouse with the average temperature around 30 ^oC, the leaves were infected after approximately 2 weeks. The various fractions of herbal extract with concentration range from 100, 300, 500, 700 mg L^{-1} were sprayed to the infected leaves, the bacterial inhibition was observed from the microscopic images, the size of the incident lesions and the reduction of infection.

RESULT AND DISCUSSION

Percentage yields of Gelonium multiflorum extracts.

The active ingredients for bacterial inhibition including *Xanthomonas campestris* pv. *Citri* were found in moderately polar to polar components and the bacterial inhibition from column separated fractions was better than crude extracts [19]. Column chromatographic separation of crude extracts from bark and stem was focused on the moderately polar (dichloromethane extracts) to polar components (methanol extracts), Percentage yields from bark and stem of *Gelonium multiflorum* extracts.

The values in the blanket stand for solvent ratio.

The moderately polar components from dichloromethane extracts of stem and bark consisted of variety compounds (Fig. 4 and 5) due to their different R_f values (data not shown). Conversely, the polar compositions from methanol extracts consisted of three groups causing from the same R_f values:

Group I (R_f = 1.10): SDG 7, SDG 8, SDG 9, SMG 4 (stem)
BDG 6, BDG 7, BMG 5, BMG 6 (bark)
Group II (R_f = 1.20); SMG 5 (stem)
BMG 4, BMG 7 (bark);
Group III (R_f = 1.50) SDG 10 (stem);

The percentage yields of each fraction were also provided in Fig 4 and 5. The yields by crude were almost 10%, it could be possibly extracted by agriculturist but the extracts were less active by the purified fraction. However, the extracts could be routinely used with conventional extraction because of low effective dose.

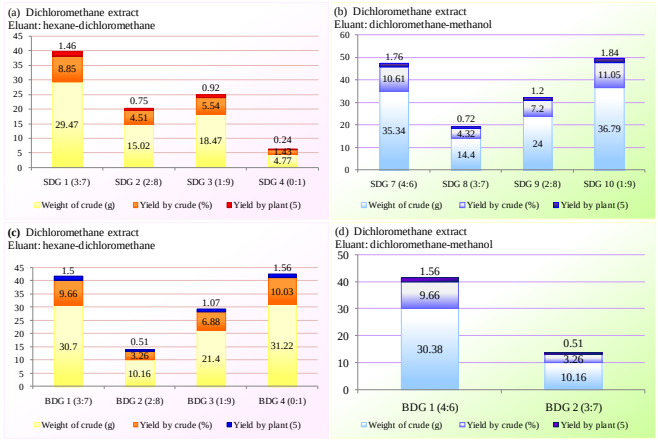

Fig. 4 Yield of dichloromethane extract of stem and bark of *Gelonium multiflorum*.

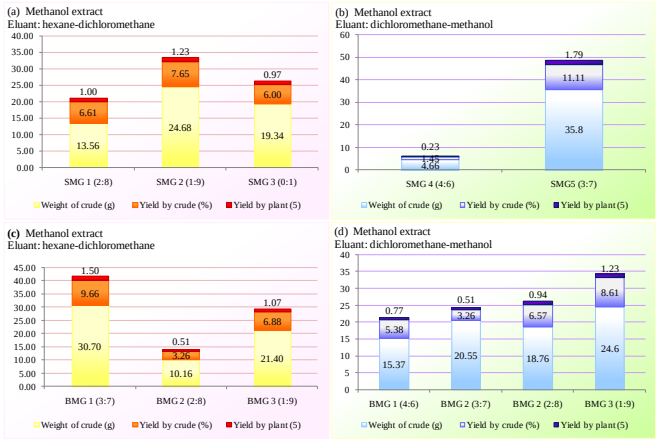

Fig. 5 Yield of methanol extract of stem and bark of *Gelonium multiflorum*.

Note: S (stem); B (bark); D (dichloromethane); M (methanol); G (*Gelonium multiflorum*)

Antioxidant activity of Gelonium multiflorum extracts to Xanthomonas campestris pv. Citri.

DPPH is used as an indicator of the free radical that is available to evaluate the free radical scavenging activity of various compounds including phenolic, flavanoid and terpines. The odd electron was paired to bind with hydrogen donating antioxidant (AH) in equation 1 or with R-radical in Fig 6. The violet color with absorbance at 520 nm was rapidly declined following by slow depreciation in absorbance. The rapid reaction caused from transferring of the most labile H atoms to the radicals, while the simultaneous slow step depended on the residual H-donating capacity of antioxidant degradation products. For any antioxidants, the reaction mechanisms between the antioxidant and DPPH• depended on the structural configuration of the antioxidant (Bondet, 1997)

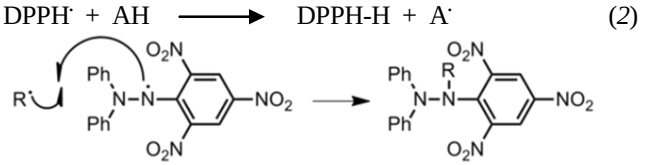

Fig. 6 Reaction of DPPH radical with antioxidant (Prakash).

Gelonium multiflorum extracts presented antioxidant activities since its compositions were mostly consisted of flavanone, glucosides and terpenes (Das, 1993; Parveen, 1987; Das, 1994). The compounds in SDG 1 and SDG 10 at the minimum concentration of 20 mg/l were the highest antioxidant activities against DPPH radical from within 3 second. The compounds in Group II provided the antioxidant activity within 60 min at the minimum concentration of 60 mg/l. The compounds in Group I provided antioxidant activities at minimum concentration 40 mg/l of within 60 min. Since the reaction time was long comparing with the others two (G I and G II), they may proposed their steric hindrance to DPPH radicals. Comparing with ascorbic acid, the herbal extracts had lower antioxidant activities, the concentration of ascorbic acid was less than 3.5 mg L^{-1} but the reaction mechanisms were the same: rapid at the beginning and delayed at the end.

In vitro evaluation of antibacterial activity of Gelonium multiflorum extract.

The fractions BMG 4 and BMG 6 from methanol extracts of bark were able to inhibit the growth of *Xanthomonas compestris* pv *Citri* for *in vitro* test. Minimum antibacterial activities of these fractions was 500 mg/l, the diameter of clear zone was 50 mm. At the concentration of 700 mg/l, the growth of *Xanthomonas compestris* pv *Citri* was completely inhibited. No clear zone appeared with any fractions of *Gelonium multiflorum* extracts. The bacterial inhibition from *Gelonium multiflorum* extract was greater than the extract from nut gall fruit, pomegranate fruit peel, beleric myrobalan fruit myrobalan (1,000 mg/l) wood fruit (10,000 mg/l) and guava leaf (50,000 mg/l). The diameters of clear zone were 16.8, 16.6, 14.2, 12.1 and 10.2 mm for nut gall fruit, pomegranate fruit peel, beleric myrobalan fruit myrobalan, wood fruit and guava leaf, respectively.

Inhibition of Xanthomonas campestris pv. Citri in lime: greenhouse experiment.

In greenhouse experiment, leaves of lime were infected with canker disease caused by *Xanthomonas compestris* pv *Citri* after the lesion appeared for 7 days. The mean diameter of lesion was approximately 3 mm. The infected leaf turned to pale yellow V-shaped chlorotic to necrotic foliar lesions, vascular blackening, wilting and finally dieback. The pathogen was inoculated from the leaf margins towards the veins followed water stress and developing chlorotic symptoms. The bacteria caused the occlusion of water-conducting vessels and producing exopolysaccharides and components that degraded plant cell wall. The vascular tissues was darken following by bacterial invasion, the black rot disease was seen in Fig.7 (Seebold, 2008).

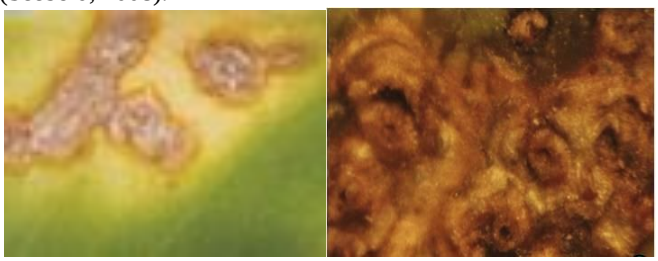

Fig. 7 Infected lime leaf from *Xanthomonas campestris* pv. *Citri* (a) and microscopic image (45X)

Before applying with *Gelonium multiflorum* extracts, the microscopic images were brown, waxy and roughly moist lesion. After applying of the extracts, it was found that the lesion became dry and the diameter of the lesion did not increase. The extract from *Gelonium multiflorum* was the alternative method to control canker disease. Since the active ingredient was a polar compound, the convenient extraction procedure was to soak the ground, dried bark of *Gelonium multiflorum* in commercial ethanol. The fractions BMG 4 and BMG 6 were effective for bacterial inhibition nearly 100% at the lowest concentration of 100 mg/l in greenhouse conditions. The inhibition of *Xanthomona campestris* pv. *Citri* reached completely at the concentration of herbal extract 700 mg/l. Same as aqueous extract from five plant; *Hibiscus subdariffa* Linn., *Psidium guajava* Linn., *Punica granatum* Linn., *Spondias pinnata* (Linn.f.) Kurz, and *Tamarindus indica* Linn. The polar fractions of these plant extracts were evaluated for control of canker disease on *Citrus aurantifolia* (lime) caused by *Xanthomonas campestris* pv. *Citri.* from 18% to 52% (Leksomboon, 2001). However, the canker lesion was still remain after the bacteria *Xanthomonas campestris* pv. *Citri* was completely inhibited (Fig. 7). To spray the herbal extract before infection with canker disease may increase productivity of citrus fruit. The percentage of extraction yield was high enough to use this extracted product instead of chemical pesticide or antibiotic medicines.

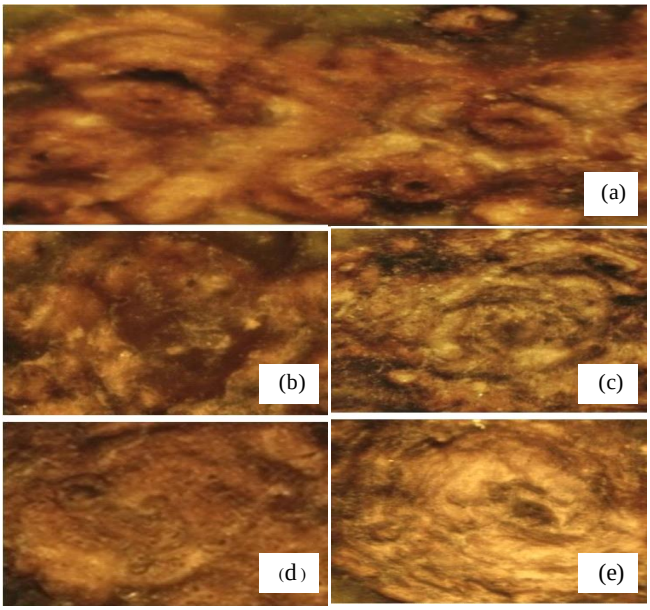

Fig. 7 (a) canker lesion infected from *Xanthomonas campestris* pv. *Citri* on lime leaf. (b), (c), (d) and (e) canker lesions after applying *Gelonium multiflorum* extracts at the concentrations varying from 100, 300, 500 and 700 mg/l, respectively.

Gelonium multiflorum was extracted with dichloromethane followed by methanol with discard the nonpolar compositions. The active ingredients consisted about 2% yield of dried bark with low applied concentration (about 10 to 500 times lower than other herbal plants. Antioxidant activities against DPPH radical were taken place with higher concentration than ascorbic acid. The polar compounds extracted from bark of *Gelonium multiflorum* were able to apply as natural pesticides instead of chemicals. The extraction process did not complicate, commercial ethanol could be used as extracted solvent with only single step. The separation and purification may be unnecessary since the bacterial inhibition was applied for plant pathogen. However, *Gelonium multiflorum* extracts were suitable for prevention infection. Since the lesion from canker disease cannot be removed after infection with disease bacteria and the bacteria were already destroyed.

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