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Comparative Study of Antioxidant Activity of Stem and Leaves of *Entada Spiralis* and Their Antibacterial Properties Against *Erwinia Chrysanthemi*

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ABSTRACT

The present study was designed to compare the antibacterial effect between stem and leaves of *Entada spiralis* against soft rot bacteria *Erwinia chrysanthemi* and its antioxidant properties which could benefit to indigenous people. *E. spiralis* which locally knows as 'sintok' is a liana and it grows wildly in Malaysia. Indigenous people are utilizing this plant as natural washing agent and shampoo as well as ethnomedicine to cure diseases since they have little access to modern medicines. *E. chrysanthemi* is bacteria which commonly cause soft-rot disease of vegetables. The antibacterial activity of stem and leaves of *E. spiralis* against *E. chrysanthemi* were evaluated from disc diffusion method. The DPPH radical scavenging method and dot blot assay were utilized to see the potential of stem and leaves of *E. spiralis* as an antioxidant agent. The results indicated that the leaves part was more antioxidative than stem of *E. spiralis* with IC_{50} of 10.5 μg/mL. Ethyl acetate extract from stem part at concentration of 200 mg/mL was found to be the most active extract against *E. chrysanthemi* with the highest inhibition zone of 16.3mm. The investigation is hoped to provide basic information for the development of potent natural pesticides and natural remedy which is safer and eco-friendly.

Keywords: *Entada spiralis; Erwinia chrysanthemi; Antibacterial; Antioxidant; Soft-rot disease*

INTRODUCTION

Medicinal plants or traditional herbs contain secondary metabolites which are the assets of new medications and many of the modern medicines [1]. For many years indigenous people used traditional herbs from natural sources to treat many diseases. Nowadays, traditional herbs are still important as the

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sources of indigenous people health care even with existence of various modern medicines. In Africa, almost 80% of people used traditional herbs as their health care [2].

Antioxidants is a valued substance needs by organisms to trap the free radicals. It is a substance that overcome oxidative damage to target molecule or cellular component that arise as effect to chemical reaction that involve free radical. With that antioxidant are able to damage the development of reactive oxygen species [3] which triggers some cancer diseases. For *Entada* species, it was reported that *E. phaseoloides* has showed positive antioxidant activity through the effective concentration at which 50% of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging [4]. The radical scavenging (EC_{50}) value has been found ranging from 2.6 to 601 mg/mL for DPPH assay. The ABTS and DPPH radical assay *of E.abyssinica* had been assessed to show the antioxidant activity of the plant [4]. The IC_{50} values for the different compounds ranged from 0.48 to 2.87 µg/m L in the DPPH assay while in the ABTS assay is from 2.53 to 17.04 µg/mL.

Antimicrobial or also known as antibiotics is an agent that can reduce and destroy the activity of microorganisms such as fungi and bacteria [5]. The antimicrobial activities of medicinal plants toward bacteria were resulted from the phytochemical components such as triterpenoid and alkaloid. It was reported that free radicals, bacteria and fungi can be inhibited with the use of certain phytochemicals components from medicinal herbs through antioxidants and antimicrobial activity [6].

Erwinia chrysanthemi is one of the plant pathogen which commonly attack pineapple and cause heart rot disease where the attacking can cause death of living tissue in the leaves and at the bottom of pineapple. *E. chrysanthemi* contain pectinase, cellulose and proteases that are able to degrade the plant cell [7]. Pectate lyases (Pels), the major degradative enzymes in *E. chrysanthemi* consist of PelA, PelB, PelC, PelD and PelE which contribute to the virulence of infection. The pathogen attacks xylem and later causes the plants to wilt. *E. chrysanthemi* cause disease in many host. It can infect fleshy fruit or vegetables such as cucumber and ornamental crops such as orchid. The infection mainly happens during hot and humid conditions.

Previous studies which related to antimicrobial properties against human patogen using stem of *E. spiralis* and other plant species have been reported [8-11]. However, the investigations of antimicrobial activity against plant pathogen of soft rot disease using stem and leaves of *E. spiralis* was not reported until now as well as its antioxidant properties. Therefore, there is a need to evaluate both antimicrobial and antioxidant potency using stem and leaves of *E. spiralis* for the benefit of indigenous people.

EXPERIMENTAL

Sample preparation

Entada spiralis stem and leaves were collected from Tasik Chini Pahang. The stem and leaves of *E. spiralis* were washed and chopping before drying process for about five days at room temperature (25°C). Dried leaves and stem were grounded using a grinder into a small piece. All grinded stem and leaves were subjected to consecutively soaking process using petroleum ether (PE), dichloromethane (DCM) and ethyl acetate (EA). After filtration process, the filtrates were evaporated using rotary evaporator until crude extracts of PE, DCM and EA were formed.

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FTIR analysis

The functional groups present in all types of extracts for stem and leaves parts were determined with the use of FTIR instrument. Different absorption frequency of absorption peaks from FTIR spectrum indicated the existence of different functional groups for each of extracts.

Thin Layer Chromatography (TLC) analysis

Each type of extract for each part of *E. spiralis* was subjected to thin layer chromatographic analysis using different ratio of developing solvents. The combination of petroleum ether and DCM was used as developing solvent for petroleum ether extract. Combination of DCM and methanol was used for DCM extract and combination of ethyl acetate and DCM was used as developing solvent for ethyl acetate extract.

Each extract was applied on TLC plate 1 cm x 7 cm in size and after drying, the TLC was dipped in developing solvent until it reached solvent front. The TLC plate was taking out from TLC jar and left for a while for drying process before visualized under short wave and long wave UV lamp. The process was repeated until the best separation of compounds on TLC was observed. The spot of compounds was marked and the Rf value was calculated. The best TLC separation for each extract were selected and sprayed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) to screen the presence of antioxidant. TLC plates were also sprayed with vanillin/H_2SO_4 followed by heating, with Dragendorff's reagent and Ferric chloride reagent to screen terpenoid, alkaloid and phenolic respectively.

Antioxidant assay

Dot blot assay

TLC plates were cut in a size of 10 cm x 10 cm. The crude extracts with different concentration ranging from 0.1965 mg/mL to 100 mg/mL were spotted on TLC plates. Then, the plates were sprayed with 0.05% of DPPH reagent. The formation of yellow spot against purple background on TLC plate indicated the presence of antioxidant. The process was run in triplicate.

Radical scavenging activity

1 mL of the different concentration of extracts and standard solution of ascorbic acid ranging from 3.125 until 100µg/mL was mixed with 3 mL of 0.004% of DPPH solution. Then, the mixture was shaken vigorously and placed it in the dark room for 30 minutes. The absorbance was measured at 517 nm using UV- Visible spectrophotometer. The entire assay was conducted in triplicate [13]. The Equation 1 was used to calculate the percentage of radical scavenging activities. The control was the solvent used for each extract.

Scavenging activity (%) = [(Acontrol-Asample) / Acontrol] x 100% (1)

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Antibacterial activity assay

The antibacterial activity of extracts from stem and leaves of *E.spiralis* against *E. chrysanthemii* was conducted through disc diffusion method. The effectiveness of the extracts was identified from the clear inhibition zone around the discs.

Preparation microbial inoculum

Microbial inoculum was prepared from stock cultures of *E. chrysanthemii* by streaking onto Mueller Hinton Agar (MHA). The culture was diluted using Muller Hinton Broth (MHB) and standardized to an absorbance of 0.11 to 0.12 at 600 nm by using UV spectrophotometer which corresponded to Mac Farland turbidity standard of 0.5×10^6 CFU/mL.

Disc diffusion method

Standardized inoculum (0.5×10^6 CFU/mL) was applied on solidified agar plate using sterile cotton bud and the plate was allowed to dry for 5 minutes. The impregnated discs at different concentrations (50, 100, 200 and 400 mg/mL) were place aseptically on inoculated agar plates. The clear zone of inhibition around the disc indicated the antibacterial effect of extract against bacteria. The empty disc will be used as negative control while disc of ampicillin (10µg/mL) were used as standard reference or positive control. Determinations of all assays were carried out in triplicate.

RESULTS AND DISCUSSION

FTIR analysis

The FTIR analysis was conducted for all types of extracts by means to determine the types of functional groups present according to the absorption peaks appeared at certain wavenumber. The information of functional groups was used to support antioxidant results as well as antibacterial properties of extracts. According to Table 1, common functional groups such as C-H, C=O and C-O were found in each types of extract from leaves and stem part of E. spiralis. However, the hydroxyl group OH was only appeared in leaf's extract of E. spiralis. The information about functional groups might be important to explain about antibacterial behavior of both parts of E. spiralis. This is because the presence of certain functional groups of compounds such as OH hydroxyl might interfere or may poison the bacteria's characteristic and gave different results of antibacterial activity of extracts.

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Table 1: Analysis of functional groups of stem and leaves of *E. spiralis*

Extract	Leaves		Stem	
	WN (cm^{-1})	FG	WN(cm^{-1})	FG
PE	2923	C-H	2924.14	C-H stretch
	1738	C=O	1709.42	C=O stretch
	1228	C-O	1251.82	C-O stretch
	3457	O-H		
DCM	2925	C-H	2917.11	C-H stretch
	1738	C=O	1708.76	C=O stretch
	1365	C-O	1264.98	C-O stretch
	2854	O-H		
EA	2927	C-H	2925.67	C-H stretch
	1737	C=O	1738.11	C=O stretch
	1365	C-O	1228.87	C-O stretch
	3357	O-H		

WN: wavenumber; FG: functional group; PE: petroleum ether; DCM: dichloromethane; EA: ethyl acetate

Qualitative Phytochemical and Antioxidant Screening using Thin layer chromatography (TLC) analysis

In this analysis, the developed TLC was sprayed with DPPH reagent to screen the presence of antioxidant in each extracts of stem and leaves of *E. spiralis*. The emerging of yellow color after spraying with DPPH demonstrated the antioxidant existence. The developed TLC of each extract of both parts was also sprayed with certain reagents to detect the presence of terpenoid, alkaloid or phenolic and the results are illustrated in Table 2.

Table 2 reveals both parts of *E. spiralis* contained terpenoid, alkaloid and phenolic but not all are antioxidatives. In stem part, all types of extract contained terpenoid which antioxidative while alkaloid and phenolic were only found in EA extract which also antioxidative. For leaves part, PE extract was found to have antioxidative terpenoid and phenolic whereas DCM extract just contained antioxidative phenolic. EA extract were found to contain alkaloid and phenolic but unfortunately they were not antioxidative. As a conclusion, the stem and leaves part of *E. spiralis* were qualitatively antioxidative which mostly came from the contribution of terpenoid and phenolic compounds.

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Table 2: TLC Screening of selected phytochemicals and antioxidant of stem and leaves of *E. spiralis*

TLC plate of extract	Stem			Leaves		
	Terpenoid	alkaloid	phenolic	Terpenoid	Alkaloid	phenolic
PE	√*	x	x	√*	x	√*
DCM	√*	x	x	x	x	√*
EA	√*	√*	√*	x	√	√

*antioxidative; √ = present; x = absent

Antioxidant Activity

DPPH Staining Dot Blot Assay

This method explained how different concentration preparation corresponded to the intensity of yellow spot capacity on the TLC plate. It was an eye-detected semi-quantitatively rapid screening method and only based on the inhibition of the accumulation of oxidized products and the yellow color represented the nature of radical scavenger presented in selected concentration of extract. This semiquantitative dot blot assay was conducted using three different extract of PE, DCM and EA. Extracts with serially diluted ranging from 100 mg/mL to 0.195 mg/mL were singly spotted on TLC plate as illustrated in Figure 3.

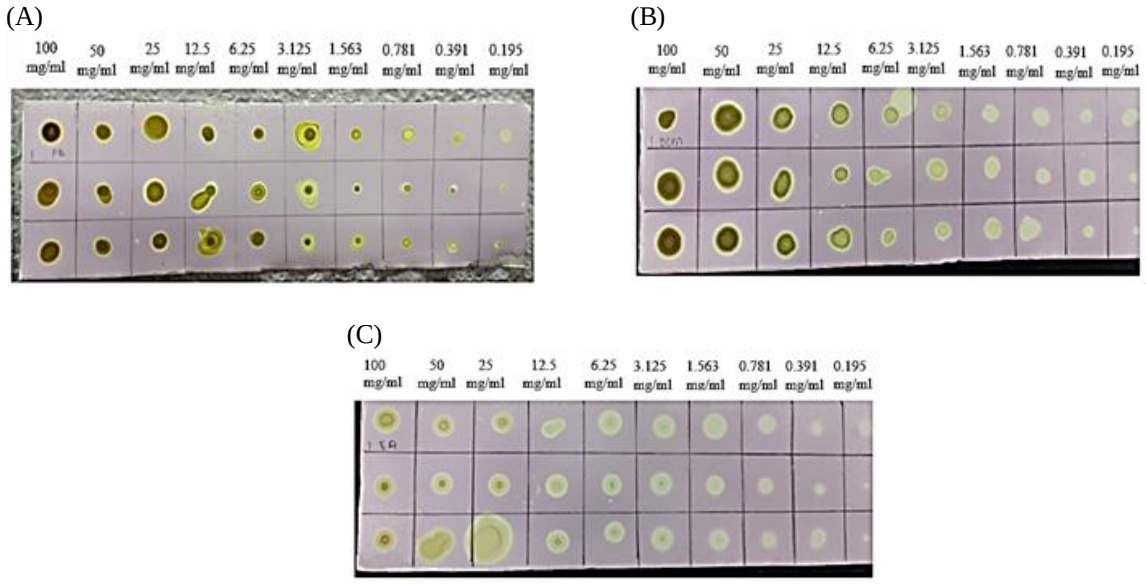

Figure 1: Dot blot assay of *E. spiralis* leaves. Chromatogram A: PE extract; Chromatogram B: DCM extract; Chromatogram C: EA extract

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Figure 1 demonstrated the results of semiquantitative dot blot assay for each extract of *E. spiralis* leaves. As illustrated, each chromatogram exhibited the yellow color spot up until 0.195 mg/mL in which chromatogram A of PE extract showed the most intense yellow color compared to chromatogram B and C. This could be due to the presence of two major antioxidative components of terpenoid and phenolic in PE extract as furnished in Table 2. The intensity of yellow color seemed to slightly reduce in chromatogram B of DCM extract and it might be due to lower concentration of antioxidants as concentration of extract decreased. Thus it was said to behave in concentration dependent manner. On the other hand, the intensity of yellow color looks like the same in chromatogram C of EA as concentration decreased.

However, the dot blot assay result of EA extract was not consistent with the TLC screening result of EA from Table 3, whereby EA extract was antioxidative according to dot blot assay but it was not showing any antioxidant behavior from TLC antioxidant screening result. One of the reasons was the function of synergistic effect of phytochemicals in EA extract makes it possible to reveal antioxidant characteristic. However, when EA extract was individually separated to a single phytochemical, their antioxidant diminished and this phenomenon might be due to the synergistic effect did not work well enough in solitary condition.

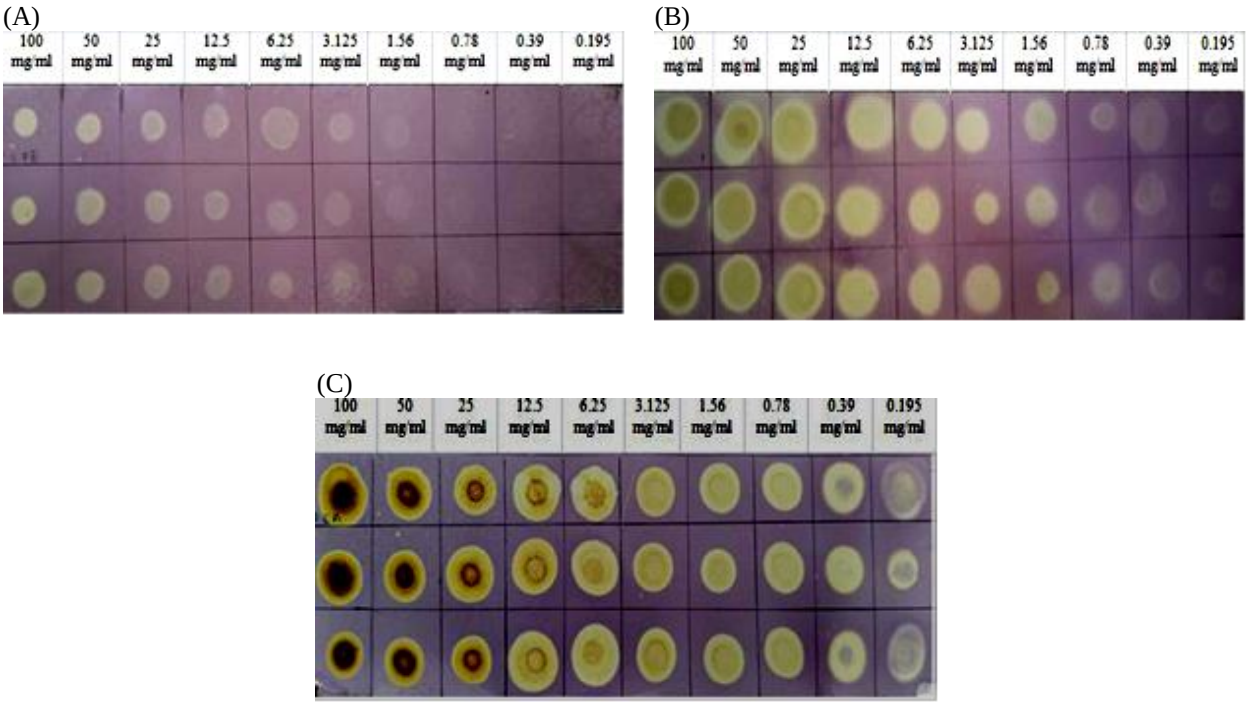

Figure 2: Dot blot assay of *E. spiralis* stem. Chromatogram A: PE extract; Chromatogram B: DCM extract; Chromatogram C: EA extract

Figure 2 illustrates the results of dot blot assay *of E. spiralis's* stem extracts. According to figure 2, the EA extracts was found to be the most antioxidative than extracts of DCM and PE since the intensity of yellow spots were still can be seen at low concentration of 0.195 mg/mL. For DCM and PE extract, the intensity of yellow spots decreased as concentration decreased.

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In PE extract, the yellow spots started to disappear at concentration 1.56 g/mL until 0.195 mg/mL. This observation could be due to the concentration of antioxidant started to reduce as concentration of extract was getting lower and resulted less power to scavenge DPPH radicals. It was concluded that the EA extract was the most powerful antioxidant source and the least powerful was PE extract. As furnish in Table 2, the EA extract of *E. spiralis* stem contained three major antioxidative phytochemicals namely as terpenoid, alkaloid and phenolic. The contribution of synergistically action of antioxidative constituents in the extract might be the reason why EA extract acted as powerful scavenger.

Quantitative DPPH Radical Scavenging Activity

The antioxidant properties of stem and leaves of *E. spiralis* also can be quantified by evaluating inhibition concentration, IC_{50}. Generally, IC_{50} value denotes the concentration of sample required to scavenge 50% of DPPH radicals. Experimentally, the purple color of DPPH solution decolorized into yellow color as hydrogen from the antioxidant source was accepted [12]. The lower the IC_{50} value, the higher the antioxidant properties of the extracts sample and vice versa.

Table 3: IC_{50} values for stem and leaves of *E. spiralis*

Extract sample	Leaves	Stem
	IC_{50}	**IC_{50}**
PE	10.5 µg/mL	42 µg/mL
DCM	65.3 µg/mL	>100 µg/mL
EA	12 µg/mL	13 µg/mL
Ascorbic acid(standard)	10 µg/mL	10 µg/mL

As illustrated in Table 3, the polarity of extract increases from PE to EA but the trend of IC_{50} value is different between leaves and stem part. In leaves part, the PE extract which is non polar extract exhibited lower IC_{50} value of 10.5 µg/mL which considered as good antioxidant because it scavenged 50% DPPH radicals at lower concentration than DCM and EA extract although DCM and EA were more polar than PE. According to Table 4, the presence of phenolic and terpenoid in PE extract might be the reason of its best performance. The result was also in agreement with previous report [12] who reported that the presence of phenolic compound in extract contributed to high antioxidant activity.

However, in stem part, the polar extract of EA had become the most antioxidative than PE and DCM since its IC_{50} value was 13 µg/mL. The higher antioxidant activity of EA extract was probably due to the presence of terpenoid, alkaloid and phenolic (Table 4) whereby all of them were effectively scavenged the DPPH radicals. According to previous study [13], higher DPPH reductions indicated higher antioxidant occurred in the extract whereby polar extract strongly bleach the DPPH color thus, giving the lowest IC_{50} reading compared to the other extract at the same concentration. The finding was quite consistent with previous research since EA extract is medium polar extract [12]. Other researchers also reported that stronger antioxidants properties of EA of *E. spiralis*'s stem extract than DCM and PE extract was probably due to high activity of its phenolic content. Phenolic activity of MeOH extract relatively due to its redox ability that enable them to work as hydrogen donating agents, reducing agents and also singlet oxygen quenching agents [13].

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Conceptually, the DPPH free radical scavenging activity is basically referring to the ability of 2,2-diphenyl-1-picryl-hydrazyl(DPPH), a stable free radical to be decolorized in the presence of antioxidants sources. The DPPH is decolorized when it accepts an electron donated by an antioxidant compound. Thus, the higher the antioxidant compound, the more the deep purple color decolorized, the faster the changing of purple color to yellow. With that, terpenoid and phenolic from the findings scavenged the radicals and decolorized the DPPH by donating electrons to DPPH. The rate of the formation of yellow color of each extract might be different since the quantity of antioxidants appeared on TLC was not measured, but it was suggested that the less the quantity of antioxidant, the longer the time for the color of DPPH decolorized.

Antibacterial Activity

This assay was conducted to see the effectiveness of different types of extract from leaves and stem of *E. spiralis.* The effectiveness of extracts was determined by means of the size of inhibition zone measured from the experiment. Table 4 depicts the size of inhibition zone for each extract of stem and leaves at different concentration.

Table 4: Antibacterial effect between stem and leaves of *Entada spiralis* against soft rot bacteria *Erwinia chrysanthemi*

Extract	Concentration (mg/mL)	Leaves	Stem
		Inhibition zone (mm)	Inhibition zone (mm)
PE	400	6	6
	200	6	6
	100	6	6
	50	6	6
DCM	400	12	6
	200	6	6
	100	6	8
	50	6	13
EA	400	11	6
	200	6	9.3
	100	6	16.3
	50	6	19
Ampicillin (µg/mL)	-	10	10

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Result revealed that all *E. spiralis* leaves and stem extracts show significant antibacterial activity against *E. chrysanthemi*. *E. spiralis* ethyl acetate leaves (400mg/mL) and stem (50mg/mL, 100mg/mL and 50mg/mL) extracts shows greater inhibition zone as compared to positive control. *Entada spiralis* dichloromethane leaves extracts (400 mg/mL) also can inhibit more *E. chrysanthemi* compared to positive control, ampicillin. It shows that these extracts have potential to be commercialized as biopesticide to treat *E. chrysanthemi* infection.

The positive result of antibacterial activity of *E. spiralis* leaves and stems extract against *E. chrysanthemi* may due to the strongly present of some bioactive compounds. TLC phytochemical screening in Table 2 revealed the existence of terpenoid saponin, phenolic and alkaloid in both parts. These compounds can serve as protection agent against microorganism. Saponins can penetrate the microorganisms' cell membranes which led to the high effects of antibacterial and antifungal [14, 15] where the main group in saponin which responsible for bacterial inhibition is hydroxyl OH group. Thus our FTIR result (Table 1) was quite consistent with previous study since the OH group was also found in the extracts. While terpenoid participate in the plant defense which important in controlling pest, pathogen and weed [16].

E. spiralis of petroleum ether leaves and stem extracts shows the same inhibition zone (6mm) for all concentration. Meanwhile, *E. spiralis* of dichloromethane and ethyl acetate leaves and stem extracts showed variety of inhibition zone and it may attribute to the different phytochemicals contribution in the extract. It was reported that the difference in polarity responsible for the difference in solubility of the plants bioactive compounds and attributed to the variation of inhibition zone [17].

CONCLUSIONS

All extracts from *E. spiralis* leaves and stem have shown its potent antioxidative behavior. EA extract of *E. spiralis's* stem exhibited the highest antioxidant activity with IC_{50} value of 13 ug/mL whereas PE extract of *E. spiralis's* leaves demonstrated highest antioxidant activity with IC_{50} value of 10.5 ug/mL. Regarding antibacterial activity against *E. crysanthemi*, the stem of *E. spiralis* revealed the highest antibacterial activity with inhibition zone of 16.3 mm. This finding is hope to provide a basic information to assist indigenous people in using natural sources as natural remedies and natural pesticides which is much saver, cheaper and environmental friendly.

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