Investigation of antioxidant activity of active compounds in ethyl acetate crude extract from stem of *Paederia foetida* Linn.

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ABSTRACT

This research presents the antioxidant activity of active compounds in ethyl acetate crude extract from the stem of *Paederia foetida* Linn.by using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. Antioxidant activity of ethyl acetate crude was investigated and the percentage of inhibition was 74.72%, indicating a good antioxidant activity. The ethyl acetate crude was then isolated by a silica gel column chromatography method. It was found that one separated fraction showed high percentage (59.15%) in the inhibition. The functional group and structure of the compound in this fraction was then analyzed *via* Fourier Transform Infrared Spectroscopy (FT-IR), Nuclear Magnetic Resonance (NMR) spectroscopy and Thin Layer Chromatography (TLC). These evidences implied that the fraction containing active compounds possibly composed of the flavonoid derivatives having hydroxyl and methyl groups as the substituent groups.

Keywords: 2,2-diphenyl-1-picrylhydrazyl free scavenging, antioxidant, *Paederia foetida* Linn.

INTRODUCTION

Nowadays, people largely put emphasis on the importance of the study about free radicals (Halliwell, 2009; Jirum and Srihanam, 2011), since free radicals are the main cause of diseases. Free radicals can be formed in human bodies; for example, from respiration, and they are caused by the metabolism process or even stress, which can also stimulate the formation of free radicals. If the number of free radicals is high, various diseases like heart disease, brain disease or cancer may follow. Additionally, free radicals can result from factors outside the body such as irregular eating patterns, alcohol consumption, air pollution, certain types of medicine, etc. Generally, human bodies have their own substances that can help to get rid of free radicals. These substances can be divided into 2 main groups; i.e., 1) substances which control or prevent the formation of free radicals, including enzymes like superoxide dismutase, glutathione peroxidase, catalase and peroxidase and, 2) antioxidants (Velioglu *et al.*, 1998; Chanwitheesuk *et al.*, 2005) which help to destroy the chain reaction of the formation of reactive oxygen species (ROS), a major substance which can produce free radicals. Antioxidants are, for example,

phenolic compounds, vitamin C, vitamin E and beta-carotene, which can be found in different kinds of vegetables, fruit or herbs. At present, researchers have conducted many studies to find antioxidants in many types of herbs due to the fact that herbs are easy to find and inexpensive compared to modern medicines that have similar properties in the treatment of disease. It has been discovered that different types of herbs contain different types of antioxidants. Therefore, this research is interested in finding antioxidants in *Paederia foetida* Linn. , since this plant is a herb that can be used to treat a variety of basic illnesses like herpes, diabetes, bloating, diseases in the digestive tract, tooth cavity problems, alcohol and tobacco intoxication, upset stomach, and food poisoning, etc. It can also be used as diuretic, an appetite stimulant, an elixir and as treatment for dyspepsia (Kumar, *et al*.,2009).

According to previous research, there have already been some studies regarding biological effects of the extract from the stalk of *Paederia foetida* Linn. In 2006, Afroz and Alamgir reported research about the efficiency of *Paederia foetida* Linn. extract in ethanol as a treatment for diarrhea and found out that it could help to stop the symptom. Then, in 2007, Nayapan studied the efficiency of *Paederia foetida* Linn. crude extract in methanol in resisting *Streptococcus mutans*, which is the cause of a tooth cavity occurring. It was discovered that the extract could resist *Streptococcus mutans* quite efficiently.

Besides, there has also been some research concerning the efficiency of *Paederia foetida* Linn. extract as an antioxidant. For example, in 2009, Osman and co-workers studied the antioxidant property of the crude extract of *Paederia foetida* Linn.'s fresh and dry leaves in methanol. It was discovered that the extract's efficiency as an antioxidant was quite satisfactory.

As can be seen from the previous research as mentioned above, it is obvious that the studies in the antioxidant properties of the chemical compositions of different parts of *Paederia foetida* Linn. are still limited. Thus, this research has an objective to study the antioxidant properties of the chemical *composition* of crude extract from stem of *Paederia foetida* Linn. in ethyl acetate. It is expected that there should be quite a lot of antioxidant substances in the ethyl acetate crude extract. This research also aims to study the basic structure of the antioxidant chemical composition of the extract from step of *Paederia foetida* Linn. Using a 2,2–diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging method. Also, this method is facile, convenient, rapid and cost effective (Mathew and Hbraham, 2006).

Materials and Apparatus

Plant Materials : The stem of *Paederia foetida* Linn. used in this study was collected from Amphoe Maung, Tak province, Thailand, in August 2011. The plant was deposited in Department of Chemistry, Naresuan University and was kept at -4 °C until used.

Materials : Silica gel (Kiesel gel 60, Merck), TLC plate (silica gel 60F254, Merck), Methanol (CH_3OH, Lab scan), Hexane (C_6H_{14}, Lab scan,) Ethyl acetate ($C_4H_8O_2$, Lab scan) Acetone ($C_3H_2O_6$, Lab scan), 2,2-Diphenyl-1-picrylhydrazyl (DPPH, Fluka) Butylated hydroxytoluene (BHT, Fluka).

Apparatus: Fourier transform infrared spectrophotometer (FT-IR , Perkin-Elmer). UV-VIS Spectrophotometer (FT-IR Perkin-Elmer), Nuclear Magnetic Resonance spectroscopy (NMR, Brucker, 400 MHz), Rotary evaporator (Buchi).

Methods

1.The extraction of stem of Paederia foetida Linn. *using various solvents*

Firstly, the sun-dried stem of *Paederia foetida* Linn. (600 g.) was mashed up and put into three different solvents with different polarities: hexane (3 x 800 mL.), ethyl acetate (3 x 800 mL.), and methanol (3 x 800 mL.). Then, these extracts had their solvents evaporated by means of a rotary evaporator, yielding hexane crude extracts as yellow green oil (16.53 g.) ethyl acetate crude extracts as brown oil (17.83 g.) and methanol crude extracts as dark-red gummy residue (12.97 g.).

2. The study in the antioxidant activity using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method

1.5 mL of sample solutions in ethanol (2mg/mL) was pipetted into a test tube. Then, 1.5 mL of DPPH solution in ethanol at the concentration of 5×10^{-4} M was added. The solution was shaken and placed in dark at room temperature for 30 min. Afterwards, the solution's absorbance of substance at 517 nm wavelength was measured. The result was used in the calculation to find out the percentage of inhibition in comparison to the absorbance of DPPH solution fromthe formula below:

$$\% \text{ Inhibition} = 100 - \%\text{Activity}$$

$$\% \text{ Activity} = (\text{Abs of the solution}) / (\text{Abs of DPPH}) \times 100$$

3. Separation of the crude extract by Column Chromatography.

The crude extract was separated by column chromatography technique. Silica gel was used as the stationary phase, while hexane-ethyl acetate and ethyl acetate-methanol solvent at different proportions were used as the mobile phase. When the separation of chemical composition at different proportions of the mobile phase was complete, each separated fraction had its elementary identity tested by Thin Layer Chromatography (TLC) technique in order to combine the fractions that had the same elementary chemical composition. Then, the antioxidant activity of

each combined fraction was studied in order to find the fraction with the best antioxidant activity

4. Structural elucidation of the separated chemical composition.

The separated chemical constitution with the best antioxidant activity had its elementary chemical structure was studied by Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared Spectroscopy (FT-IR) technique, and Thin Layer Chromatography (TLC) technique. The TLC technique was tested by various reagents to detect functional groups and elementary structure of the separated chemical composition.

5. Determination of functional groups and structure of the separated chemical composition

The functional groups and structure of the composition having the best antioxidant activity were investigated by TLC technique with the following reagents:

A. Potassium permanganate ($KMnO_4$): to test alkene, alkyne and aromatic compounds.

B. Iodine (I_2): to test aromatic or double covalent bond substances and aldehydes or ketones.

C. Ferric chloride ($FeCl_3$): to test phenolic, ester and amide substances.

D. Aluminium chloride ($AlCl_3$): to test flavonoid substances.

Results and Discussion

Extraction of stem of Paederia foetida Linn. *and the study in the antioxidant activity of the crude extracts by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method*

After the dried stem of *Paederia foetida* Linn. was ground up, it was found that the amount of mashed-up sample was 600 g. The sample powder was mixed with hexane, ethyl acetate and methanol in separate aliquot. This gave rise to three types of crude extracts from the stem of *Paederia foetida* Linn.; hexane crude extract, ethyl acetate crude extract and methanol crude extract.

Three types of crude extracts were then tested to study their antioxidant activity by a DPPH free radical scavenging method. In this method, butylated hydroxytoluene (BHT) and ethanol were used as the positive control and blank solution, respectively. The percentage of free radical inhibition of each type of crude extracts was demonstrated in Figure 1.

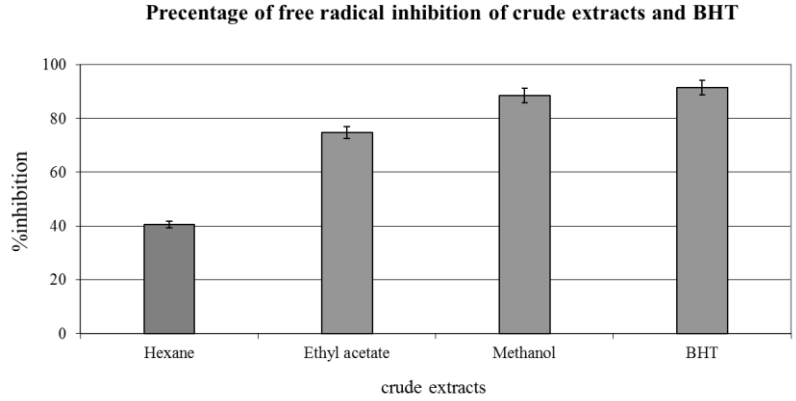

Figure 1. Percentage of free radical inhibition of crude extracts and BHT (the positive control)

From Figure 1, the result showed that methanol crude extract had the greatest antioxidant activity (88.54 % of inhibition), followed by ethyl acetate crude extract (74.72% of inhibition) and hexane crude extract (40.52% of inhibition), respectively. According to the study of the elementary chemical constitution by TLC technique, it was found out that the chemical composition of the ethyl acetate crude extract was not complicated compared to that of the methanol crude extract. Also, the ethyl acetate crude extract had quite a satisfactory percentage inhibition, and it was a crude extract in a solvent of which polarity was not too high. It was expected that the separation of the chemical composition of this crude extract should be easily accomplished. Therefore, the ethyl acetate crude extract would be focused for separation of its chemical composition by column chromatography in the next step.

The separation of the ethyl acetate crude extract by Column Chromatography

The separation of the ethyl acetate crude extract (17.83 g.) was fractionated using silica gel column chromatography. The column was initially eluted with 100% hexane and the polarity of eluent was gradually increased from 100% hexane to ethyl acetate in hexane and then 5% methanol in ethyl acetate. The volume of each fraction was approximately 300 mL and each was evaporated to about 30 mL. The similar fractions were combined together according to the TLC profile. The results showed that the extract could be separated into 6 fractions (1A-6A) according to their structural polarity and TLC profile. Then, the antioxidant activity of every fraction (1A-6A) was tested and the result was demonstrated in Figure 2.

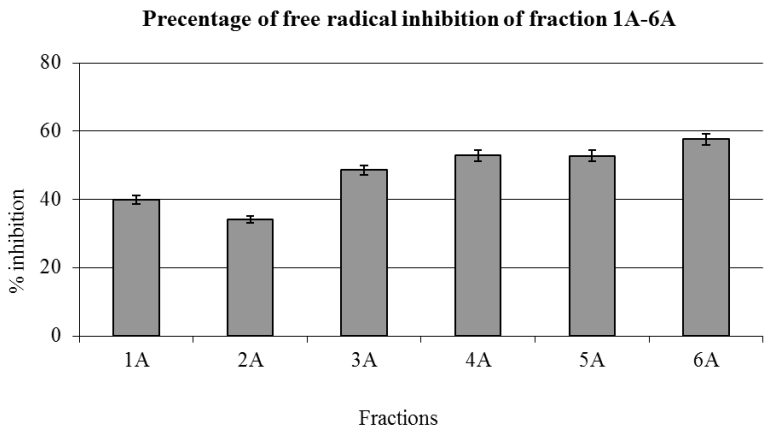

Figure 2. Percentage of free radical inhibition of fraction 1A-6A

According to Figure 2, fraction 6A was isolated as yellow oil (5.34 g.). It was obtained after eluting it with 70% ethyl acetate in hexane and showed the highest percentage of antioxidation (57.52%). Thus, fraction 6A would be focused and used for chemical composition separation by the column chromatography and initially eluted with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 100% ethyl acetate and then 5% methanol in ethyl acetate to provided 4 fractions (1B-4B) according to the TLC profile. Again, the antioxidant activity of all 4 fractions was studied and the result was shown in Figure 3.

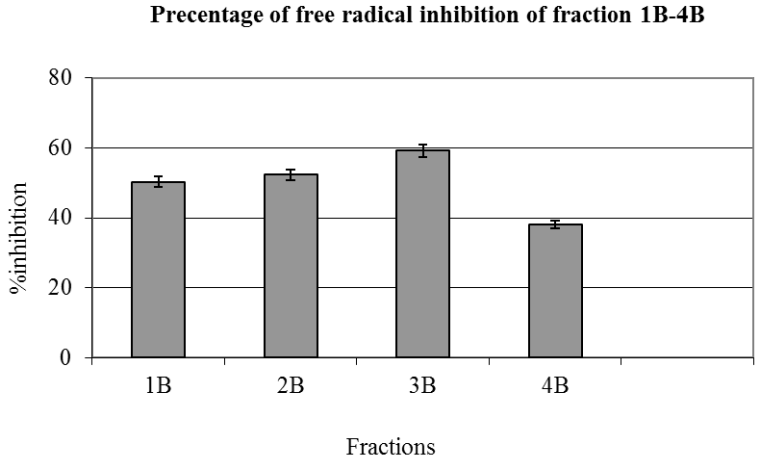

Figure 3. Percentage of free radical inhibition of fraction 1B-4B

According to the study in anti-oxidation of fractions 1B-4B, the fraction 3B was obtained as yellow oil (2.15 g.), which was eluted with 80% ethyl acetate in hexane and showed the highest antioxidation percentage at 59.15%. Consequently, this fraction was further studied to find its elementary chemical constitution by FTIR and NMR spectroscopies as well as TLC in order to further explore the chemical structure of the active substances.

Structural elucidation of active compounds in fraction 3B by NMR, FT-IR and TLC techniques

According to FT-IR spectrum, fraction 3B revealed the presence of outstanding absorption bands at 3393 cm^{-1}, suggesting the presence of hydroxyl groups and showed the strong absorption band at 2958 cm^{-1}, 2914 cm^{-1} and 2848 cm^{-1} due to methyl and methelene group stretching vibration. In addition, a strong absorption band at 1711 $cm^{-1}$was observed and this corresponds to carbonyl groups. Also, strong absorption bands appear at 1255 cm^{-1} and 1095 cm^{-1} corresponding to C-O stretching vibration. The spectrum was showed in Figure 4.

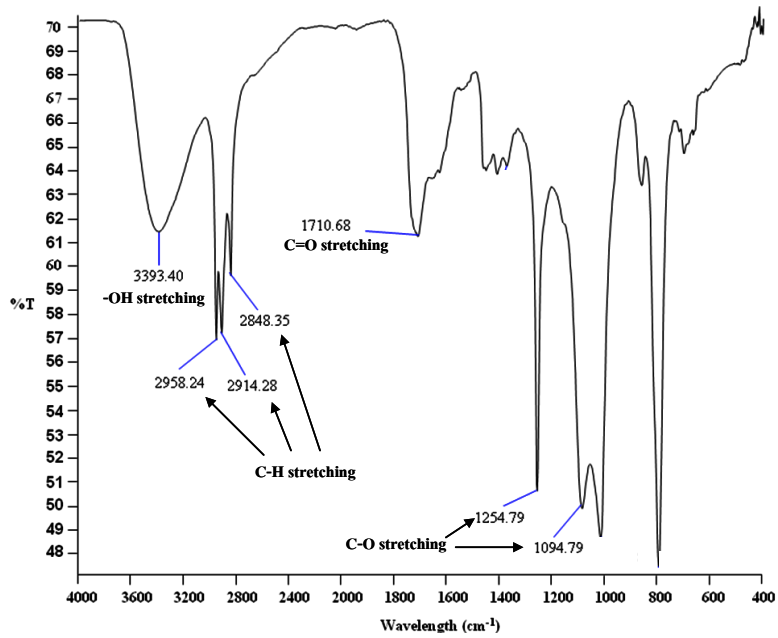

Figure 4. FT-IR spectrum of fraction 3B

The ^{1}H-NMR spectrum showed the signal of methyl protons attached to aromatic groups (Ar-C<u>H</u>$_3$, δH 2.50 ppm). The signal of methyl protons attached to aliphatic groups (R–C<u>H</u>$_3$) was also found at δ_H 1.30 ppm. In addition, the aromatic proton showed the signal at δ_H 6.8 ppm and those of the hydroxyl group (-O<u>H</u>) appeared a singlet signal at δ_H 7.8 ppm (Figure 5).

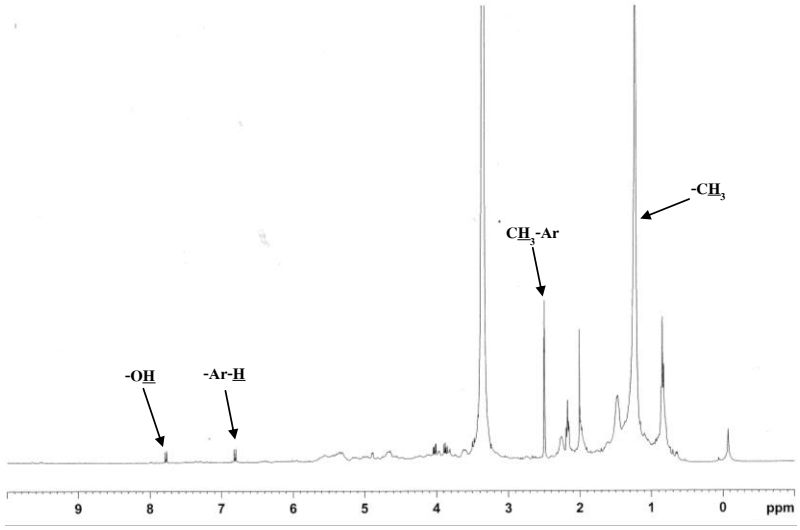

Figure 5. ^{1}H NMR spectrum of fraction 3B (solvent: DMSO-*d6*),

TLC technique of fraction 3B was then performed by detecting the active compounds with different types of reagents. Four types of reagents were used: iodine (I_2), ferric chloride ($FeCl_3$), potassium permanganate ($KMnO_4$) and aluminium chloride ($AlCl_3$). The results were displayed in Figure 6.

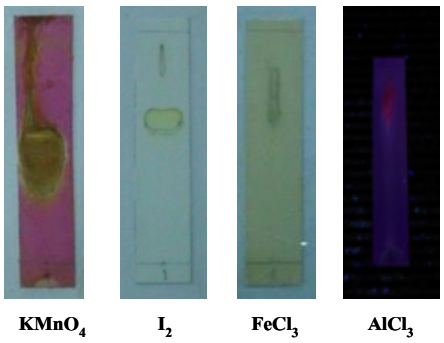

Figure 6. TLC plate testing of fraction 3B with different types of testing reagents

From Figure 6, when fraction 3B was tested with the $KMnO_4$ solution, a brownish-yellow spot appeared, indicating that the chemical composition in fraction 3B possibly composed of unsaturated bonds. When it was tested with iodine, a yellow spot appeared, meaning that fraction 3B consisted of aromatic compound. Besides, when it was tested with $FeCl_3$ and $AlCl_3$ solutions, a light brown spot and a pink spot appeared, respectively, implying that the chemical composition of fraction 3B probably contained phenolic compound and flavonoid.

For chemical test for flavonoid compound, fraction 3B was also test with sodium hydroxide following Bello's method (Bello, *et al*., 2011). The result indicated that the color was changed from yellow to colorless on addition of dilute hydrochloric acid. It was indicated the presence of flavonoids.

From the study of the basic structure of chemical composition of fraction 3B by the FT-IR, NMR TLC techniques and chemical test, it can be concluded that the chemical composition of fraction 3B might consist of flavonoid derivatives (Fig.7), which have hydroxyl groups and methyl groups in the composition.

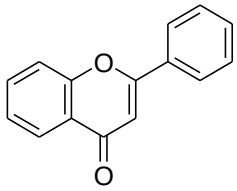

Figure 7. Core structure of flavonoid compound

CONCLUSIONS

According to the study of the antioxidant activity of the ethyl acetate crude extract from stem of *Paederia foetida* Linn. by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free scavenging method, the result showed that the antioxidant activity of the ethyl acetate crude extract was 74.72%. The ethyl acetate crude extract was further separated by the column chromatography technique. Its chemical composition which had the best antioxidant activity was fraction 6A, which had the percentage of free-radical inhibition about 58.62. After the separation of fraction 6A was performed by column chromatography in the next step, the result exhibited that fraction 3B showed the best antioxidant activity with 59.15% in free-radical inhibition. From the analysis by FT-IR, NMR and TLC techniques, fraction 3B probably contained flavonoid derivatives with hydroxyl and methyl groups in the composition.

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