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Total Phenolic Content and *In vitro* Antioxidant Activity of Winged Bean (*Psophocarpus tetragonolobus*)

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Abstract: *Psophocarpus tetragonolobus* also known as the Asparagus pea or winged pea (Family: Fabaceae) most widely grown in hot, humid equatorial countries like Malaysia. It provides a source of vitamin A, vitamin C, calcium and iron. The antioxidant potential of 75% methanol extract, ethyl acetate and chloroform soluble fractions of *P. tetragonolobus* was screened for *in-vitro* antioxidant activity using total phenolic content, ferric reducing power, 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay, Ferric Thiocyanate (FTC) and Thiobarbituric Acid (TBA) tests. It was found that ethyl acetate fraction have maximum amount of polyphenolics compounds (1.69 mg/g GAE in concentration 0.5 mg/mL) higher than methanol and chloroform fractions. Antioxidant concentration was found highest in ethyl acetate extract (27.12 mM) using FRAP method, while chloroform extract has the highest antioxidant level (4.48 mM) using ABTS assay. Ethyl acetate extract of *P. tetragonolobus* was found to have highest antioxidant activity (7.27%) compared to methanol and chloroform using FTC method. While, TBA results shows no difference in antioxidant activity of all extract of *P. tetragonolobus*.

Key words: *Psophocarpus tetragonolobus*, phenolics content, antioxidant activity, different fractions, reductive potential

INTRODUCTION

Psophocarpus tetragonolobus (*Psophocarpus* is from the Greek for "noisy fruit") also known as the Asparagus pea or winged pea (Family: Fabaceae) most widely grown in hot, humid equatorial countries like Malaysia. It's a high-climbing, twining vine with trifoliate leaves. They are larger than green bean flowers, a little more than 1 in (2.5 cm) long and hang in loose clusters of 2-10 flowers. Besides Malaysia, winged bean also widely cultivated in the tropics, especially in Myanmar, India, Indonesia, Thailand, Bangladesh, West Africa, the West Indies and South Florida.

As far as our literature survey could ascertain, there are few information was available on the *in vitro* antioxidant activities of the *P. tetragonolobus* (Lee *et al.*, 2011; Amin Ismail *et al.*, 2009). Therefore, this current investigation was to evaluate the *in vitro* antioxidant capacities of the methanol, chloroform and ethyl acetate extract of *P. tetragonolobus*. The antioxidant activities of *P. tetragonolobus* were measured in a concentration of 1 mg/mL using different antioxidant assays. Furthermore, the total phenolic content, the reducing power and the antioxidant activity contents were also measured and their correlation with the antioxidant.

According to Showkat *et al.* (2011), free radicals, the partially reduced metabolites of oxygen and nitrogen, are

highly toxic and reactive. The most common reactive oxygen species are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxy radical (ROO) and highly reactive hydroxyl radical (OH). Oxidation process is one of the most vital routes for producing free radicals in food, drugs and living systems. Antioxidants are the substances that when present in low concentration significantly delay or reduce the oxidation of the substrate. Antioxidants protect the body from damaging oxidation reactions by reacting with free radicals and other reactive oxygen species within the body and hindering the process of oxidation. Hence, diseases associated with free radicals can be avoided by antioxidant therapy which gained an immense importance.

Polyphenol are the major plant compounds and are commonly found in both edible and inedible plants and they have been reported to have multiple biological effects, including antioxidant activity. Their antioxidant activity is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary

heart disease and cancer is also raising interest among scientists, food manufacturers and consumers. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g was daily ingested from a diet rich in fruits and vegetables.

Phenolic compounds are plant secondary metabolites that are derived from pentose phosphate and phenylpropanoid pathways. Epidemiological evidences have suggested that phenolic compounds possess a wide span of physiological functions including antioxidant, anti-carcinogenic, anti-inflammatory, anti-allergenic, anti-atherogenic, anti-microbial, anti-thrombotic, cardio-protective and vasodilatory effects to a greater or lesser extent (Liyana-Pathirana and Shahidi 2005; Balasundram *et al.*, 2006). Fruits, vegetables and beverages are the principal dietary sources of phenolic compounds.

MATERIALS AND METHODS

Extraction of crude extract: Fifty grams of winged beans, vegetable is washed under running tap water and dried with a soft cloth. Fresh flesh was then cut into small pieces, approximately 1.5 cm x 1.5 cm x 1.5 cm wide in size. Sample was then macerated in hexane, DMS and methanol for one hour with occasional shaking. All the procedures are carried out in a dark environment and the samples were left in dark conditions for 4 days. Universal bottles are weighed and the weights are recorded. Universal bottles are put into oven at 45°C for 30 mins. The extraction solvents are filtered with Whatman No. 41 filter paper (pore size 20-25 µm). The filtered solvent is put into the rotavapor flask and concentrated under reduced pressure at 40°C using rotary vacuum evaporator (Buchi Rotary Evaporator R-200, Switzerland) and store at -20°C until were used for the analysis.

Total phenolics content: The amount of total phenolics in extract was determined with Folin-Ciocalteu reagent. Standard for TPC test were using gallic acid and alpha-tocopherol. 4 mg of samples were weighed and dissolved in 4 ml of 70% ethanol. 0.75 mL of Folin-Ciocalteu's reagent (previously diluted 10 fold with distilled water) was added into test tube containing samples and stand at room temperature for 5 mins. 0.75 mL of sodium carbonate (60g/L) were added and

left at room temperature around 90 minutes. The mixture was read at 725 nm using UV-Vis spectrophotometer.

Total antioxidant status (FRAP): The FRAP Substrate Working Solution was then prepared by adding 25 µL of 3% Hydrogen Peroxide Solution to 10 mL of FRAP substrate solution. It was used within 20-30 mins. Then, assays were prepared in the 96 well plates. In wells for the Trolox standard curve, 10 µL of a Trolox Standard (from tube 1-6) and 20 µL of Myoglobin Working Solution are added. Meanwhile, in wells for the Test samples, 10 µL of a Test sample and 20 µL of Myoglobin Working Solution are added. After that, 150 µL of FRAP Substrate Working Solution was added to each well. Then, they were incubated for 5 mins at room temperature (27°C). The 70 µL of stop solution was added to each well. The Stop Solution was warmed to room temperature (27°C) and mixed until homogenous prior used. The endpoint absorbance was read at 405 nm using a plate reader.

ABTS assay

Trolox standard well: Trolox standard well is prepared by adding 10 µL of Trolox standard, 10 µL of Metmyoglobin and 150 µL of Chromogen per well in the designated wells on the plate.

Sample well: Sample well is prepared by adding 10 µL of Trolox standard, 10 µL of Metmyoglobin and 150 µL of Chromogen to wells. The reactions are initiated by adding 40 µL of hydrogen peroxide working solution to all the wells being used, as quickly as possible. Then, the plate is covered with plate cover, followed by incubate on a shaker for 3-5 min at room temperature. The cover is removed and the absorbance is read at 750 nm or 405 nm using plate reader. The antioxidant concentration was then calculated.

Reductive potential: The standards used in this assay were gallic acid, alpha-tocopherol and L-ascorbic acid. 6 mg of samples extract (ethyl acetate, chloroform and methanol of long beans extraction) were weighed and dissolved in 6 mL of distilled water. Then, the sample solutions were added in each test tube/facon tube followed the concentration as in Table 1. 2.5 mL, 0.2 M, pH 6.6 of phosphate buffer and 2.5 mL of 1% potassium ferricyanide solution were added. The mixture was incubated 50°C for 20 mins. 2.5 mL of 10%

Table 1: Total phenolic content in ethyl acetate, chloroform, methanol and alpha-tocopherol extracts of *Psophocarpus tetragonolobus*

Concentration (mg/mL)	Total phenolic content (mg/g) GAE			
	alpha-tocopherol	Methanol	Ethyl acetate	Chloroform
0.1	1.659	0.000	0.864	0.215
0.2	1.745	0.031	0.907	0.567
0.3	1.748	0.137	1.444	1.211
0.4	1.796	1.127	1.621	1.223
0.5	1.920	1.089	1.687	1.651

trichloroacetic acid were added. Then, centrifugation was carried out for 10 minutes at 1000 g. 2.5 mL supernatant was taken and mixed with 2 mL dH₂O and 0.5 mL of 0.1% FeCl₃. The absorbance was measured at 700 nm using a UV-Vis spectrophotometer.

Ferric thiocyanate (FTC) method: The sample extract was weighed 4.0 mg and mixed with 4.0 mL absolute ethanol, 4.1 mL of 2.52% linolenic in absolute ethanol, 4.0 mL of 0.05 M phosphate buffer (pH 7) and 3.9 mL distilled water. The mixture solution is mixed in the tube with a screw cap and then placed in the dark oven at 40°C for about 10 minutes. 0.1 mL of the solution is taken and is added with 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate. 0.1 mL of 0.02 M ferrous chloride is added in 3.5% HCl to the reaction mixture and the absorbance is read at 532 nm precisely 3 minutes after the addition. Gallic acid, L-ascorbic acid and alpha-tocopherol were used as standards. The percentage of inhibition is calculated using the following formula:

$$\text{Inhibition (\%)} = 100 - [(A_1/A_0) \times 100]$$

Whereby, A₀ is the absorbance of the control reaction and A₁ is the absorbance in the presence of the sample extract. (Santanu *et al.*, 2010).

Thiobarbituric Acid (TBA) method: One mL sample solution from FTC method was added with 2.0 mL of 20% trichloroacetic acid (TCA) +2.0 mL 0.67% thiobarbituric acid (TBA) in the test tube. The mixture was placed in water bath (95°C) for 10 minutes. After cooling, it was then centrifuged at 3000 rpm for 20 minutes. The absorbance of the supernatant was read at 532 nm using UV-Vis spectrophotometer.

Statistical analysis: All data was reported as mean ± standard deviation (SD) of triplicate determination and analyzed using one-way analysis of variance (ANOVA) with significant differences between means determined at p<0.05, measured with Duncan's multiple range tests using Statistical Package for Social Science Research Version 17.0 (SPSS).

RESULTS AND DISCUSSION

Determination of total phenolic compounds: Phenolic compounds are known as powerful chain breaking antioxidants. These compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. Therefore, it is worthwhile to determine the total amount of phenolic content in the plant chosen for the study. Based on the Fig. 1, the phenolic content can be determined from the regression equation of calibration curve ($y = 0.3949x + 1.1659$, $R^2 = 0.9636$) and

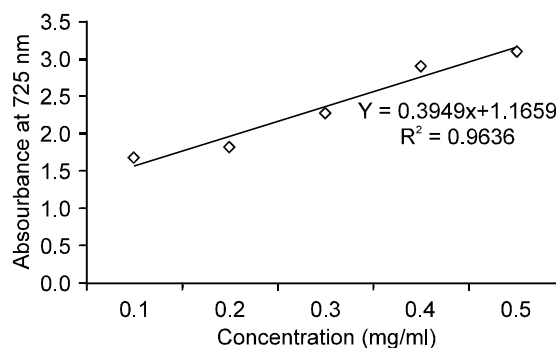


Fig. 1: A graph of standard gallic acid for total phenolic contents. By using equation of gallic acid standard, antioxidant concentration (mM) = $[(y - 1.1659)/0.3949] \times 1 \text{ mg/mL}$

expressed as Gallic Acid Equivalence (GAE). Alpha-tocopherol is used as positive control which believed to have the highest amount of phenolic compound and is commercially used in pharmaceutical production.

The total phenolic content of extract dissolved in ethyl acetate shows higher value compared to chloroform and methanol. Methanol shows the least total phenolic content when using this Folin-Ciocalteu method. The total phenolic content increases with the concentration. In the simplest term, if the concentration of the extract is high, the phenolic content also will be higher. The highest phenolic content analyzed is 1.687 mg/g GAE in concentration 0.5 mg/mL ethyl acetate solvent. Alpha-tocopherol which act as positive control show the highest phenolic content than *Psophocarpus tetragonolobus* extract.

Ethyl acetate extraction was found as the highest phenolics content fraction. The high concentration of phenolics in the ethyl acetate fraction may be due to purification and concentration of phenolics throughout the fractionation procedure and it is probably responsible for its high free radical scavenging activity. The FCR reducing capacity of different fractions is due to presence of hydroxyl groups present in the polyphenolics and flavonoids. It was reported that, the presence of hydroxyl groups is contribute directly to antioxidant effect of the system and it also has an important role in preventing lipid oxidation.

The usage of Folin-Ciocalteu reagent also was measured based on the colour measurement which was non-specific on phenol. In fact, Folin-Ciocalteu reagent measures the ability of any mixture to reduce phosphomolybdic and phosphotungstic acids in the compound into a blue complex. The presence of ascorbic acid or other very easily oxidized substances which not considered as phenolic compounds, may also result in the formation of blue color with Folin-Ciocalteu reagent, causing an overestimation of total phenolic content (Shahidi and Nacz, 2004). So, it can be said

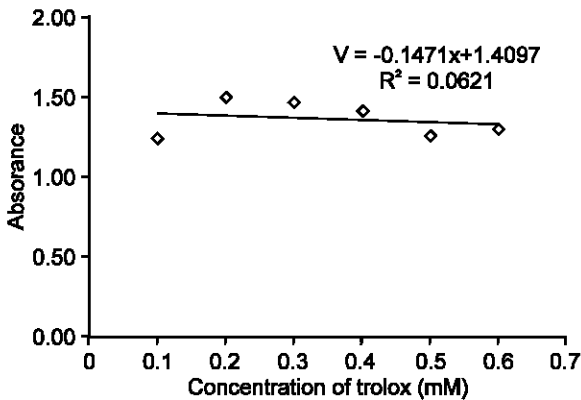


Fig. 2: Antioxidant analysis by FRAP method shows the absorbance reading for different concentrations. By using equation of trolox standard, antioxidant concentration (mM) = $[(y-1.4097)/(-0.1471)] \times 1$ mg/mL

that Folin reagents may be inappropriate for plant extracts with high levels of other easily oxidisable substances.

Antioxidant assays (FRAP): The Ferric Reducing Ability of Plasma (FRAP) method measures the antioxidant capacity of a given substance, as compared to the standard. The principle FRAP method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants. Reducing power is to measure the reductive ability of antioxidant and it is evaluated by the transformation of Fe (III) to Fe (II) in the presence of the sample extracts (Gülçin *et al.*, 2007).

Figure 2 shows that absorbance changes are linear over a wide concentration range with antioxidant mixtures. Measured of trolox standard by using line graph is resulted with sloped down. The antioxidant concentration decreased with an increased in extracts' absorbance. The data was shown that all the samples decreased their antioxidant concentration when the absorbance of extracts was increased. Meanwhile, reducing power decreased with a slightly decreased in extracts concentration. Therefore, the result shows that all the samples decreased their reducing ability when the concentration of extracts was decreased. The greatest power of reducing ability of the extracts is on trolox concentration 0.2, whereby the antioxidant concentration is highest at this point.

Table 2 shows the FRAP values of sample winged beans. The antioxidant activity of *P. tetragonolobus* was highest in ethyl acetate extract as compared to methanol and chloroform. The concentration value for ethyl acetate is 27.12 ± 0.116 mM; methanol is 16.51 ± 0.178 mM and chloroform with 6.44 ± 0.591 mM.

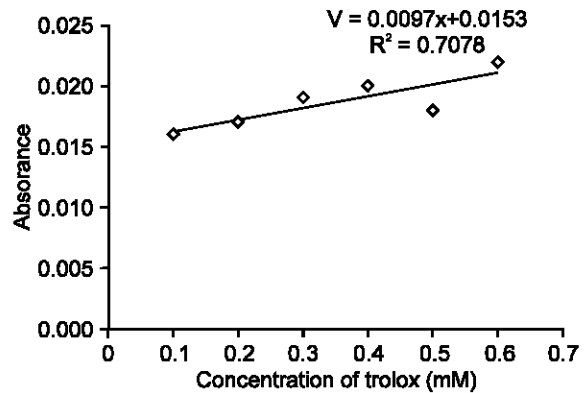


Fig. 3: Trolox standard graph

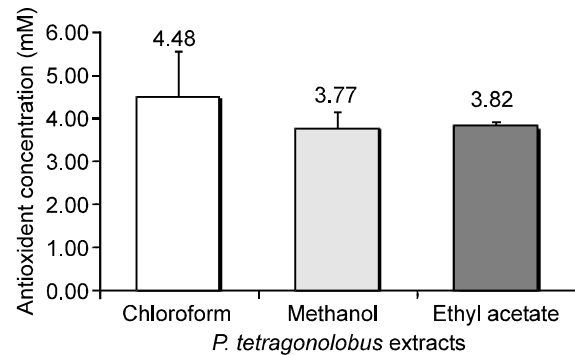


Fig. 4: Antioxidant concentration of *P. Tetragonolobus* in chloroform, methanol and ethyl acetate extracts. The values are presented as mean \pm SD

Table 2: Antioxidant concentration of extract

Extract	Antioxidant concentration (mM)
Chloroform	6.44 ± 0.591
Methanol	16.51 ± 0.178
Ethyl acetate	27.12 ± 0.116

Result presented in mean \pm SD

Antioxidant assay (ABTS): ABTS assay is designed to measure the overall antioxidant capacity within a given sample. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS in comparison to trolox, a water-soluble tocopherol analogue. The concentration of the antioxidant in the sample is inversely proportional to the absorbance of the radical caption produced by 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sul-fonate) (ABTS). This assay is often referred to as the Trolox Equivalent Antioxidant Capacity (TEAC) assay (Fig. 3). The reactivity of the various antioxidants tested is compared to that of Trolox which is a water-soluble analog of vitamin E. It is an antioxidant, like vitamin E and is used in biological or biochemical applications to reduce oxidative stress or damage.

The value of R^2 is 0.7078. The value of R^2 is to determine how closely the data conform to a linear relationship. The equation got from the graph used to calculate the

value of antioxidant crude extract. Hence, antioxidant concentration (mM) = $[(y-0.0153)/0.0097] \times 1 \text{ mg/mL}$. ABTS values of sample winged beans; result showed that antioxidant activity for chloroform ($4.48 \pm 1.06 \text{ mM}$) is the highest compared to methanol ($3.77 \pm 0.36 \text{ mM}$) and ethyl acetate ($3.82 \pm 0.08 \text{ mM}$). The mechanism of antioxidant action in extract was as a hydrogen donor that could terminate the oxidation process by converting the free radicals to the more stable form. Thus, the higher absorbance value of chloroform extract indicates the high content of antioxidant. Some other studies shows that

Determination of reductive potential: Reducing antioxidant power is to measure the reductive potential of antioxidant in the solution of sample extract and it is determined by the conversion of Fe^{3+} to Fe^{2+} (Gülçin *et al.*, 2003). The test solution turns from yellow turns to green and blue depending on the reductive potential of antioxidant content in the samples (Gülçin *et al.*, 2003). The reducing antioxidant of *Psophocarpus tetragonolobus* is summarized in Fig. 5. Increased absorbance reading of the reaction mixture indicates with increase in reducing power or reductive potential. From Fig. 5, methanol and chloroform showed higher than both standard of Gallic acid and L-ascorbic acid. The extract of *P. tetragonolobus* has reported significantly higher reducing potential than the Gallic acid standard. The reducing power of the sample may indicate as a significant indicator of its potential antioxidant activity. The antioxidant action of reductants is based on the breaking of the free radical chain by donating a hydrogen atom. Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. The reductive power of different fractions of long bean extract and gallic acid may be the reason for their antioxidant activity.

Ferric thiocyanate (FTC) method: The FTC method was used to measure the amount of peroxide produced at the beginning of the lipid peroxidation while TBA test is used to measure the second product of peroxide oxidation such as aldehyde and ketone (Frag *et al.*, 1989). During the linoleic acid oxidation, peroxides are formed and react with ferrous chloride to form ferric ion. The ferric ion then combines with ammonium thiocyanate and produce ferric thiocyanate which result red in colour. The higher the colour intensity indicated the higher linoleic acid emulsion oxidation as well as the absorbance. The darker the colour, the higher will be the absorbance (Huda-Faujan *et al.*, 2009). *P. tetragonolobus* crude extract showed the lowest absorbance at the first day. Ethyl acetate showed the lowest absorbance values at 0.051, followed by methanol and ascorbic acid with absorbance at 0.053, gallic acid and alpha-tocopherol (0.055) and chloroform (0.057). Absorbance of each samples increase

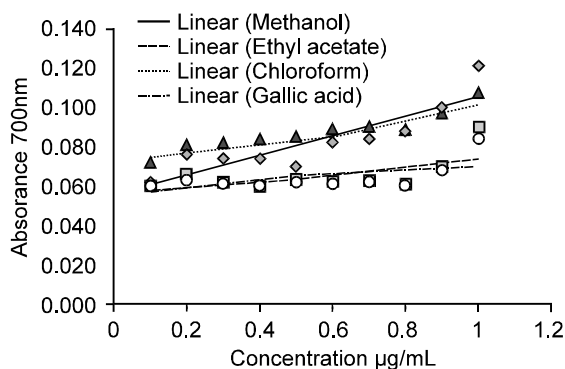


Fig. 5: Reducing power of the samples extract/fractions by Fe^{3+} - Fe^{2+} transformation

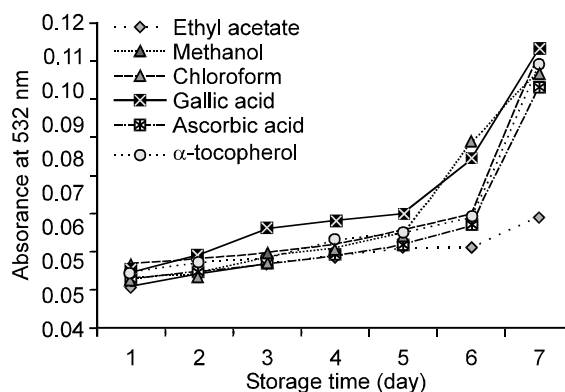


Fig. 6: Antioxidant properties of crude extract determined with the FTC method

progressively by time of incubation. At the end of the experiment, all samples showed higher absorbance values than the day 1 (Fig. 6). Ethyl acetate extract showed the lowest absorbance value with 0.069, followed by ascorbic acid (0.103), methanol (0.107) and alpha-tocopherol (0.109). Compared to standards, gallic acid displays the highest absorbance (0.113), followed by chloroform (0.112).

Higher absorbance values showed lower antioxidant activities. FTC method was used to measure the amount of peroxide formed at the primary stage of linoleic acid peroxidation. The peroxide reacts with ferrous chloride to form a reddish ferric chloride pigment. In this method the concentration of peroxide decreases as the antioxidant activity increases (Mohd Adzim Khalili *et al.*, 2012; Emynur *et al.*, 2012). The control showed increase in absorbance values from day 1 and reached on day 7. Antioxidant activities are higher during the initial of the experiment than the end of the experiment.

Thiobarbituric acid (TBA) method: Thiobarbituric acid (TBA) test is used to measure the second product of peroxide oxidation such as aldehyde and ketone (Frag

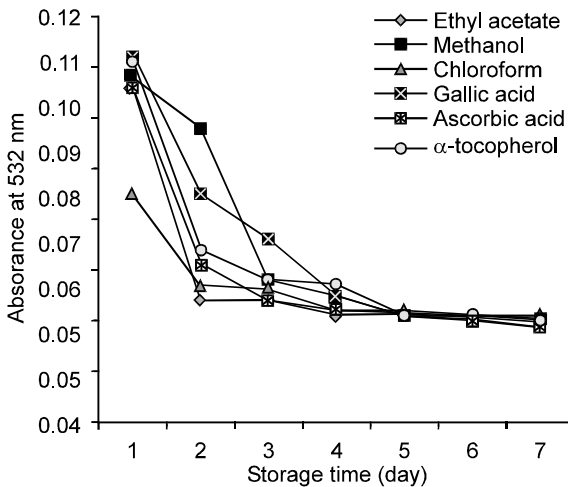


Fig. 7: Antioxidant properties of crude extract determined with the TBA method

et al., 1989). During the linoleic acid oxidation, peroxides are formed and react with ferrous chloride to form ferric ion. The ferric ion then combines with ammonium thiocyanate and produce ferric Thiocyanate which result red in colour. The higher the colour intensity indicated the higher linoleic acid emulsion oxidation as well as the absorbance. One such compound is malonaldehyde which is measured by the TBA method (Mohd Zin *et al.*, 2002). Figure 7 shows the TBA analyses of *P. tetragonolobus* extract in seven days.

Generally, all extracts showed decreasing trend from day 1 to day 7 of the TBA analysis. On day 1, chloroform recorded the lowest level (0.085) as compared to the other extracts which showed higher level ranged from 0.106-1.112. Methanol and chloroform extracts showed slow decreasing rate on day 2 but declined dramatically on the next day, while ethyl acetate, gallic acid, ascorbic acid and alpha-tocopherol extracts decreased at approximately similar rate. All extracts stop decreasing on day 5, in which they achieved equal level ranged 0.061-0.062 and the levels were not changed until day 7. Low absorbance correlate to high antioxidant activity (Sharipah Ruzaina *et al.*, 2009).

Conclusion: The results expressed in this study are the first information on the antioxidant activities of *P. tetragonolobus*. Total phenolic contents are influenced by the concentration of extract. Among all the fractions, the ethyl acetate fractions exhibited the highest total phenolic content comparing to methanol and chloroform fraction. The antioxidant capacity using FRAP method shows ethyl acetate has the highest antioxidant activities and followed by methanol and chloroform. In contrast, ABTS method shows chloroform extract to be the highest antioxidant activities compared to methanol and ethyl acetate. Ethyl acetate extract of *P. tetragonolobus* was

found to have highest antioxidant activity compared to methanol and chloroform using FTC method. While, TBA results shows no difference in antioxidant activity of all extract of *P. tetragonolobus*. From this study, it is suggested that *P. tetragonolobus* content high in phenolic compound and antioxidant capacity in ethyl acetate extracts as compared to methanol and chloroform.

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