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Research paper

Antioxidant activities of oligostilbenoids from the stem bark of *Dipterocarpus verrucosus, Dipterocarpus crinitus* and *Dipterocarpus cornutus*

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Abstract

Resveratrol, a stilbene-based polyphenol, is known to be substantially beneficial for human health because of its significant roles as antioxidant agents. Its oligomers received a considerable chemical and biological interest because of their structural complexity as well as their wide array of bioactivities role and function that includes as an antioxidative agent. Based on previous report, only 17 oligomers from Dipterocapaceae have been studied for their antioxidant and other activities. In an attempt to explore new antioxidant leads, the stem bark of *Dipterocarpus* from Dipterocarpaceae family were extracted and purified to give 14 oligostilbenes. The structures of the compounds were determined by comprehensive analysis of spectroscopic data including UV, IR, MS and NMR after purification of individual compounds through a combination of chromatographic methods. The antioxidant activity was measured using four methods (Total Phenolic Content, DPPH, FTC and TBA). Fourteen compounds were screened for DPPH activity while only major compounds were screened using FTC and TBA methods in order to investigate the possible correlation between both mechanisms. The crude extracts showed high percent inhibition in TPC, FTC, TBA and DPPH methods. The TPC values for the crude extracts are within a range of 331.54 – 482.31 mg/g GAE. In DPPH radical scavenging activity, the crudes displayed lower radical scavenging activity as compared to BHT and ascorbic acid. Nevertheless, *D. verrucosus* and *D. cronutus* demonstrated a significant free radical scavenging ability with IC_{50} values of 80 and 210 µg/ml, respectively. Both species also showed low absorbance values, which indicated a high level of antioxidant activity in FTC method. *D. verrucosus*, *D. cornutus* and *D. crinitus* extracts exhibited antioxidant potential in TBA method with percent inhibition of 88.64 %, 99.13 % and 95.02 %, respectively, as compared to vitamin E which has a percent inhibition of 83.23 % at a final concentration of 0.02% w/v. Evaluation on compounds indicated that resveratrol, diptoindonesin E, 4-methoxygallocatecatechin, ampelopsin A and scopoletin possessed IC_{50} values between 75-450 µg/mL in the DPPH assay. The results suggest that oligomer resveratrol from *Dipterocarpus* are potential sources of natural antioxidants.

Keywords: *Antioxidant; Dipterocarpaceae; Dipterocarpus; Polyphenol; Resveratrol oligomers*

1. Introduction

Resveratrol are commonly found in edible food and beverages such as mulberries, peanuts and grape family [1]. However, lack of research and ongoing information about the resveratrol from Dipterocarpaceae family. Dipterocarpaceae is a large family of tropical plants. Ironically, there are only seven species in *Dipterocarpus* genus that were repeatedly isolated in terms of their resveratrol oligomer which are *Dipterocarpus hasseltii* [2], *D. retusus* [3], *D. grandiflorus* [4], *D.intricatus* [5], *D.semivestitus* [6,7], *D. alatus* [8], *D.tuberculatus* [9] and two species of *D. obtusifolius* [10] and *D.zeylanicus* [11] yielded triterpenoid. Furthermore, The Malaysian Red List of Peninsular Malaysia Dipterocarpaceae reported 35 species of Dipterocarpaceae are categorized as endangered species and 15 species are critically endangered [12]. *Dipterocarpus cornutus* is categorized as critically endangered while *Dipterocarpus crinitus* is vulnerable. The stem bark of Dipterocarpaceae plants is byproduct of timber industry and often wasted.

Aslam [13] have documented all ethnomedicinal use of *Dipterocarpus* all over the world. Most of the isolation compounds process obtained from the bark of the plant. *Dipterocarpus gracillis* or locally known as 'keruing kesat' is used as an antiseptic for gonorrhoea; urinary disease. *D. tuberculatus* have been used in treating leishmanicidal, or as antiseptic and anti-inflammatory purposes [14] and also traditionally prescribed as a cure to skin inflammation, bronchial infection, colitis and anxiety. The leave and stem of *D. turbinatus* which is classified as toxic are traditionally used to cure gonorrhoea, gleets, ulcer and rheumatism. The bark of *D. alatus* which is commonly distributed in Thailand, Cambodia, Laos, Vietnam and Philippines contains medicinal values to treat rheumatism, diseases of liver besides enhancing appetite stimulation of cattle in animal husbandry. *D. costatus* or vernacularly known as Keruing bukit is used to treat ulcer. The

bark of *D. indicus* can also cure rheumatism. Interestingly, another report on *Dipterocarpus* extract; *D. obtusifolius* Teijsm ex Miq have reported may cured against AIDS. The formulation was created by combination of *D. obtusifolius* Teijsm ex Miq with *Melastoma villosum Lodd, Lyophyllum aggregatum, Dictyophora indusiata,* pu-erh tea, mentha and stevia [13]. Despite of the wide spectrum in plant's active properties, it is suggested that collective and synergistic participation facilitate such chemical compounds resulting in effective healing procedure.

Dipterocarpaceae is an important source of resins such as oleoresin and dammars. Interestingly, this stem is proven to produce a variety of chemical constituents such as volatile oil, triterpenoid, flavonoid, arylpropanoid and oligomeric resveratrol. The ongoing research on the chemical constituents of resinous part are focusing on the harvesting and utilization of terpenoid and sesquiterpenes. Since hopeaphenol and polyphenol compound from oligomer resveratrol has been isolated from two species of *Hopea odorata* and *Balanocarpus heimee* in early 1950, the research on resveratrol has been carried out profusely. Since its discovery, scientists have found the chemical structures of the derivatives of resveratrol and its effect on biological activities such as its cytotoxicity [14,15,16], antiviral [17], antibacterial [18,19,20,21], antioxidant [15,22] and anti-inflammatory [23]. For that reason, there is a growing demand, interest and opportunity in exploring resveratrol produced by plants. This compound is patented and marketed globally to supply a growing demand. This study was conducted to evaluate the antioxidant activity of the crudes and the compounds itself. Antioxidants can be defined as bioactive compounds that inhibit or delay the oxidation of molecules [24]. Antioxidants are categorized as natural or synthetic antioxidants. Some synthetic antioxidants commonly used are: BHT, BHA, propyl gallate, and tertbutylhydroquinine. Many scientists have concerns about safety because synthetic antioxidants have recently been shown to cause health problems such as liver damage, due to their toxicity and carcinogenicity. Therefore, the development of safer antioxidants from natural sources has increased, and plants have been used as a good source of traditional medicines to treat different diseases [25,26]

2. Materials and Methodology

2.1. Plant extract preparation

Stem bark of dried *Dipterocarpus verrucous* (DV), *D.cornutus* (DC) and *D.crinitus* (DCJ) plant (5kg) were ground and extracted with methanol. Further fractionation using various chromatography techniques were carried out consecutively. The compounds were identified and confirmed by ^{1}H NMR, ^{13}C NMR, LCMS, UV and IR and were compared with previous data in literature.

2.2. Total Phenolic Content (TPC):

Gallic acid was used as a standard with varied concentration from 100 ppm to 500 ppm. Both samples and standards were mixed with Folin-Ciocalteu reagent (1:1), 7.5% (w/v) sodium carbonate and diluted with water before observed under UV-Vis spectrophotometer at 760 nm absorbance. Each measurement was repeated five times and TPC was expressed as mg Gallic acid equivalent per gram extract weight (mg GAE/g extract weight). This assay was conducted as described by Velioglu [27].

2.3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Method

The antioxidant activity of the plant extracts and pure compounds were evaluated with radical scavenging activity of the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radicals according to the previous procedure described by Zain [22]. Stock solutions of extracts and pure compounds were prepared at 1000 ppm in methanol. The stock solutions were diluted to different concentrations (500 ppm to 7.8125 ppm in methanol) in a 96-well microtiter plate. Then, 5 µl DPPH solutions (prepared as 5 mg DPPH in 2 ml methanol) were added to each well. The plate was shaken gently and placed in the dark for 30 minutes at room temperature. The absorbance was then measured at 517 nm using Elisa Reader. Percentage inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{(\text{Abs control} - \text{Abs sample} / \text{Abs control})}{} \times 100$$

where Abs control is the absorbance of DPPH solution without samples.

The IC50 values of DPPH mean how much of a particular substance or what concentrations is needed to scavenge 50% DPPH free radicals. Butylated hydroxyanisole (BHA) serves as positive control, while methanol was the negative control.

2.4. Ferric Thiocyanate Method (FTC):

The antioxidant activity analysis using ferric thiocyanate method was performed based on Osawa and Namiki [28]. 10 mg/mL of the crude extracts and pure compounds (final concentration 0.02% w/v) with 1 mg/mL were dissolved in 4.0 ml of ethanol absolute, followed by the addition of 4.1 ml of a 2.51% linoleic acid solution in EtOH and 8 ml of a 0.05M phosphate buffer (pH 7.0). The mixture was incubated at 40ºC in a screw-cap vial. During the incubation, a 1.0 ml aliquot was taken from the mixture, and diluted with 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloric acid, the absorbance for the red color was measured at 500 nm in a spectrophotometer.

2.5. Thiobarbituric Acid Method (TBA):

This assay was performed based on the method by Kikuzaki and Nakatani [29]. The same samples prepared for FTC method were used. 2.0 ml of the mixture solution from the FTC method was added with 2.0 ml of 0.67% thiobarbituric acid and 1.0 ml of 20% trichloroacetic. The mixture was then heated in a boiling water bath for 10 min. After the mixture was cooled, the mixture was centrifuged at 3000 rpm for 20 min, and the absorbance was measured at 532 nm.

2.6. Statistical analysis

All data were expressed as mean ± standard deviation with three replicates and averaged. A significant difference was considered at the level of $p < 0.05$.

3. Result and Discussion

Antioxidants activities (AOA) were determined with two indirect methods; which are ferric thiocyanate (FTC) and thiobarbituric acid (TBA) and one direct method; 1,1-diphenyl-2- picrylhydrazyl (DPPH). Besides the direct and indirect methods, total phenolic content (TPC) which is not designed specifically for AOA also has been used to estimate antioxidant potential on crude extracts. 14 compounds; resveratrol (1), ε-viniferin (2), laevifonol (3), ampelopsin A (4), α-viniferin (5), davidiol A (6), isohopeafenol (7), vaticanol B (8), diptoindonesin E (9), bergenin (10), scopoletin (11), 4'-*O*-methylgallocatechin (12), β-sitosterol (13), β-sitosterolglucoside (14). Fig. 1 showed the structures of the compounds isolated. All the compounds were tested on DDPH methods, while TPC, FTC and TBA were evaluated on three major compounds; α-viniferin (5), laevifonol (3), vaticanol B (8).

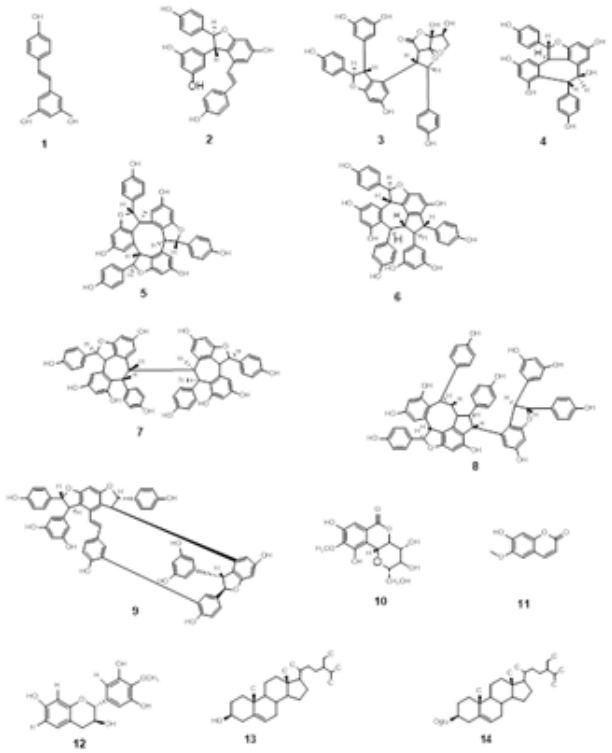

Fig. 1: Compounds isolated (1-14) from *D..verrucosus*, *D.crinitus*, *D.cournutus*

3.1. Total phenolic content (TPC)

DV (*D.verrucosus*), DCJ (*D.cornutus*) and DC (*D.crinitus*) crude extracts that were tested displayed a TPC with a range of 331.54 – 482.31 mg/g GAE. Among all the samples, DV extracts showed the highest significant ($p < 0.05$) TPC 482.31 mg/g followed by DC extracts 405.38 mg/g and DCJ extract with 331.54 mg/g of GAEs. According to Duh [30], TPC that exceeded 16.71 mg GAE/100g indicates high antioxidant activity. Therefore, from the results above, it can be suggested that all *Dipterocarpus* species possessed high antioxidant activity. According to Xu and Chang [31] the exact chemical nature of the reductions in TPCs are still not fully understood. However, this could be attributed to the chemical transformation, decomposition of phenolics and formation of phenolic-protein complex under thermal and pressure conditions that have been known to clarify. It has been known that phenolic compounds possess diverse biological activities such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities and these activities might be related to their antioxidant activity. Thus, from the results obtained, the value of TPC can be correlated with compounds isolated that have been studied in this research. In our phytochemical study, compounds in DV crude extract possess more stilbene compounds which is polyphenol compounds as compared to DC and DCJ which contain less polyphenols and more triterpenoid. As a result, extracts that contain more stilbene compounds resulted in high TPC. The Folin-Ciocalteu procedure also has the reputation of being less prone to interference by non-phenolics [32]. Table 1 indicated total phenolic content and radical scavenging activity of the crude and compounds.

3.2. Radical scavenging activity of free radical (DPPH)

The DPPH method is commonly used due to their relatively stable radicals, good reproducibility, easy measurement, economic efficiency and its simple reaction system; direct reaction between the radical and an antioxidant. The assay is based on visually noticeable decolourisation from purple to yellow as the radical loses this absorption feature when accepting an electron or a free radical species [32]. This can be monitored spectrophotometrically since it provides a strong absorption at 517 nm. The mechanism on the reduction of DDPH radical has been extensively investigated and reported. The IC_{50} value is defined as the concentration that causes a decrease in the initial amount of DPPH radicals by 50% [23]. It is the concentration where the active crude extract will exhibit 50% of antioxidant activity [33]. Crude extracts exhibit 50% of inhibition at concentration less than 20 µg/ml and these concentrations are considered positive for antioxidant activity [34]. Percent scavenging activity of DPPH and IC_{50} are the most widely used parameters. Table 1 indicates scavenging of DPPH radicals by BHT and ascorbic acid standards as compared to *Dipterocarpus* extracts. All of the samples displayed lower scavenging activity as compared to BHT and ascorbic acid. However, as compared to the samples, sample DV and DC demonstrated significant free radical scavenging with IC50 values of 180 and 210 µg/ml, respectively. While the extract of DCJ does not gave any value of IC_{50}. The lower value of IC_{50} indicates higher antioxidant power. According to Atun [35], the percentage of scavenging activity for the extract of DV and DC are moderate as compared to standard BHT and ascorbic acid and can be classified as active. The extract possessed high activity as free radical scavenger could be due to the presence of polyhydroxyl compounds in the respective extract.

Table 1: Total Phenolic content, radical scavenging and FTC and TBA activity of crude and compounds

Crudes/Compounds	TPC (mg/g GAE)	DPPH (IC_{50} µg/mL)	FTC %	TBA %
D.verrucosus (DV)	482.31	80	92.97	99.13
D. crinitus (DCJ)	331.54	-	76.62	88.64
D.cornutus (DC)	405.38	210	88.36	95.02
a-viniferin		-	77.77	86.47
Vaticanol B		-	65.00	33.87
Laevifonol		-	53.35	21.32
Vitamin E			95.63	83.23
*Resveratrol		175		
*Diptoindonesin E		180		
*4-methoxygallocathecin		200		
*Ampelopsin A		210		
*Scopoletin		325		
*Davidiol A		450		
*BHT (standard)				
*Ascorbic acid		30		
		70		

*Test was done on DPPH only due to limited amount of compound

Out of 14 compounds, six compounds were classified as having active antioxidant activity with IC_{50} values between 175-450 µg/ml [35]. Resveratrol has the most radical scavenging activity comparable to BHT standard with IC_{50} value of 175 µg/ml and 173 µg/ml, respectively followed with diptoindonesin E; 180 µg/ml, catechin; 200 µg/ml ampelopsin A; 210 µg/ml, scopoletin ; 325 µg/ml and davidiol A; 450 µg/ml while the other compounds possessed IC_{50} below than 50%. Fig. 2 illustrated scavenging effect on compounds towards DPPH radicals.

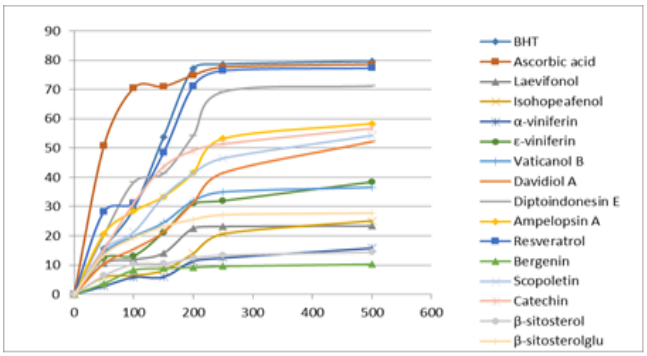

Fig. 2: Scavenging effect on compounds towards DPPH radicals. Value are expressed as mean ± standard deviation (n=3) with p< 0.05,

From the comparison with previous reports, resveratrol, diptoindonesin E, 4-methoxygallocatechin and ampelopsin A has never been evaluated by any researcher while scopoletin and davidiol A has better result as compared with previous studies. Both compounds AOA indicated 325 /413 µg/mL in *Melicope glabra* (Rubiaceae) [36] and 450/nd µg/mL in *Shorea macroptera* respectively [37]. In addition, scopoletin used in this study is less concentration with 5 µl while [36] used 30 µl. Meanwhile for davidiol, the concentration used in same concentration with 30 µl. The oxidative effect could be exerted by different mechanisms of the different phenolic compounds and/ or due to the synergistic mechanisms of the different compound [36]. Furthermore, the bioactivity of the phenolics have been reported to be associated with the ability to chelate metals, inhibit lipoxygenase, scavenge free radicals and contribute directly to oxidative action. The antioxidant activity of naturally occurring phenolic acids and flavanoids were suggested due to the presence of free hydroxyl group [38].

From this finding, it depicts that molecular structure was not the main factor that influences the AOA. This is supported from data provided because resveratrol, a monomer structure shown to be more active as a free radical scavenger with an IC_{50} value of 175 µg/ml as compared to diptoindonesin E, tetramer structure with an IC_{50} value of 180 µg/ml. In contrast to this, diptoindonesin E, tetramer has shown to be more active as compared to ampelopsin A, a dimer stucture with an IC_{50} value of 180 µg/ml and 210 µg/ml, respectively. Moreover, from Fig. 2, it showed that vaticanol B, a tetramer compound did not give any IC_{50} value. Our finding also correlates with the study conducted [39,40,35,37, 41] which revealed that oligomer molecular structure does have antioxidant activities.

Antioxidant activity of oligomer resveratrol rely on three main factors; existence of phenol ring, stability of molecular structure, and existence of double bonds of olefinic unit. From the results, resveratrol and diptoindonesin E both structures still maintaining their double of olefinic unit. Thus, both compounds have greater antioxidant value than other compounds. It is well accepted that the antioxidant ability of polyphenols is related to their hydroxyl group. The presence of a second hydroxyl group in the *ortho* or *para* position is known to increase the antioxidative ability due to additional resonance stability and *o*-quinone or *p*-quinoneformation [39,40,35]. Phenol ring can trap hydroxyl radical by releasing hydrogen radicals, that, when undergo condensation with hydroxyl radical form water molecules while radical phenol are stabilized by resonance. This is one of the reasons why resveratrol compound is referred for development as antioxidant. An antioxidant is a substance that can prevent or slowdown the reactions of radical oxidation. Further study should be continued as it indicated that plants that belonging to Diptercarpaceae family are very rich in polyphenols compounds especially resveratrol oligomers. In the perspective of flavonoid, Das and Pereira [42] have revealed that polyhydroxylated substitutions on ring A and B, a double bond between C-2 and C-3, a free 3-hydroxy substitution and a carbonyl group at C-4 moiety are significant characteristics contributes for high antioxidant activity in flavonoids.

3.3. Inhibition of lipid peroxidation (FTC and TBA)

The antioxidant activity was measured by two methods; ferric thiocyanate (FTC) and thiobarbituric acid (TBA). FTC method was used to measure and determine the peroxide level during the initial stage of lipid oxidation which indicates higher antioxidant potential if it has lower absorbance while the TBA method was used to measure and determine the amount of malondialdehyde (MDA) produced after the decomposition of the lipid peroxide during the oxidation process. MDA is a very unstable compound causing mutagenic and cytotoxic events [43]. At a low pH and high temperature (100 ◦C), MDA binds TBA to form a red complex that can be measured at 523 nm. Basically, this method was accomplished within seven days because the data of absorbance for the control will gently drop after the sixth day. In FTC method, vitamin E was used as a standard to compare with samples tested. The results revealed that DV and DC extract gave low absorbance values, which indicated a high level of antioxidant activity. However, it contradicts with DCJ where the absorbance was quite high and indicated that the antioxidant was moderate. Meanwhile as can be seen, the absorbance value of the control was the highest since it did not contain antioxidants. In parallel to this, the concentration of the peroxide decreased as the antioxidant activity increased, thus the intensity of reddish pigment will reduce. This will lead to a lower absorbance value which indicated a high level of AOA. Fig. 3 illustrated the FTC absorbance of *Dipterocarpus* extract during 6 days of incubation at 40ºC, in comparison with vitamin E

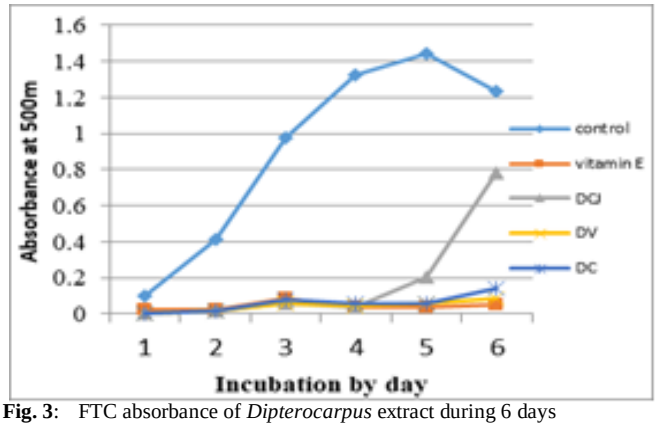

Fig. 3: FTC absorbance of *Dipterocarpus* extract during 6 days of incubation at 40ºC,in comparison with vitamin E

The TBA method was extended on the day that the absorbance value for control dropped which is normally dropped on day seven. Theoretically, the TBA method is used to measure the secondary product of oxidation such as aldehyde and ketone [44]. When compared to the percent inhibition between FTC and TBA method, (Table 1) percent inhibition for TBA method is higher than FTC method. This result does not support the theory which indicated that with FTC, the amount of peroxide in the initial stage of lipid peroxidation is much greater than the amount of peroxide in the secondary stage. Secondary products such as malonaldehyde are not stable for a long period of time. It will eventually turn into alcohol and acid which cannot be detected by a spectrophotometer. However, the TBA result showed that all the *Dipterocarpus* extracts exhibited antioxidant potential with percent inhibition 88.64 %, 99.13 % and 95.02 % each as compared to the standard; Vitamin E with 83.23 % (Table 1). The total antioxidant activity values of *Dipterocarpus* extracts in descending order are as follows: DV>DC>DCJ.

Fig. 4 illustrated FTC absorbance of major compounds isolated in this work, FTC showed all the major compound tested exhibited

moderate antioxidant potential compared to vitamin E as standards. From Fig. 4 and Table 1 it is revealed that α-viniferin possessed higher antioxidant activity (77%) as compare to vaticanol B (65%) and laevifonol (53%) which have lower absorbance. Lower absorbance values indicated a high level of AOA.

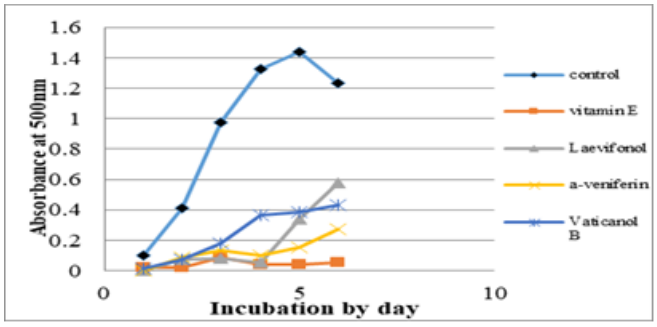

Fig. 4: FTC absorbance of major compounds vaticanol B, a-viniferin and laevifonol during 6 days of incubation at 40ºC, in comparison with vitamin E

From Table 1 also summarized that vaticanol B has lower percent inhibitions of both tests (FTC and TBA) as compared to α-viniferin even though the pattern of antioxidant process of vaticanol B supports the theory that revealed the percent inhibition for FTC method is higher than the TBA method [45]. This may indicate that the amount of peroxide in the initial stage of lipid peroxidation is much greater than the amount of peroxide in the secondary stage. This is due to malondialdehyde (MDA) which is produced on the final day of the incubation period (1 day after the control reached maximum). By determining the amount of MDA, the correlation between both FTC and TBA methods can be verified. MDA a secondary product of lipid peroxidation changes into alcohol and acid. The alcohol and acid cannot be detected with a spectrophotometer. The FTC method indicates the amount of peroxide in the initial stages of lipid peroxidation, whereas, the TBA method shows the amount of peroxide in the secondary stage of lipid peroxidation. Therefore, the higher antioxidant activity found from the ferric thiocyanate method indicated that, the amount of peroxide in the initial stage of lipid peroxidation was greater than the amount of peroxide in the secondary stage [46]. In contrast to vaticanol B, α-viniferin gave the higher percent inhibiton in TBA method as compared to FTC method. Measurement of secondary oxidation products as an index of a lipid oxidation is sometimes more appropriate because secondary products of oxidation are generally flavoure-active, whereas primary oxidation products are colourless and flavourless, thus the flavour-active will lead to the formation of MDA as a breakdown product. The intensity of pink coloured complex is directly related to the concentration of TBA reagent with reactive substances in the original sample [32]. Furthermore, the secondary product is much more stable for a period of time [47]. Secondary oxidation products include aldehydes, ketones, hydrocarbons and alcohols but the assay measures the concentration of relatively polar. Zin [43] studied on *Vigna sinensis* (Fabaceeae) revealed that several compounds of different polarity may contribute to the antioxidative activity. In addition, antioxidative activities observed in these plants could be the synergistic effect of more than two compounds that may present in the plant. It has been reported that most natural antioxidative compounds often work synergistically with each other to produce a broad spectrum of antioxidative activities that creates an effective defence system against free radical attack.

4. Conclusion

In conclusion, it was observed that all the crude extracts possessed good antioxidant activities. *Dipterocarpus verrucosus* showed the strongest antioxidant potential since it posseses more stilbene compounds. In future, waste product of timber that contain valuable resveratrol derivatives as obtained in this study could be used in pharmacological industries.

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