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RESEARCH ARTICLE

Brucea javanica seeds as source of potential natural antioxidants to improve biodiesel thermal and oxidative stability

Khalil Hasni [a,c], Zul Ilham [a,*], Jamaludin Mohamad [a] and Mahendra Varman [b]

[a] *Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[b] *Department of Mechanical Engineering, Faculty of Engineering, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[c] *Lasbella University of Agriculture, Water and Marine Science, 90150 Uthal, Pakistan*

* *Corresponding author: ilham@um.edu.my*

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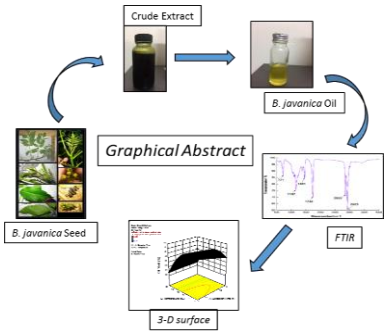
Graphical abstract



Abstract

Biodiesel is one of the most promising renewable energy derived from biological sources. Biodiesel is clean and renewable biofuel but its oxidative stability is low. To increase oxidative stability of biodiesel, natural antioxidants are considered as potential compounds to be used. The purpose of the work was to investigate and determine the antioxidation potential of *Brucea javanica* seeds by applying different antioxidant assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), metal chelating and ferric reducing antioxidant power (FRAP). Furthermore, Total phenolic content (TPC) and Total flavonoid content (TFC) tests were done. Among the three extracts of *Brucea javanica* seeds, ethyl acetate extract showed the highest total phenolic content (98.5 ±0.1mg GAE/g dry extract). The highest total flavonoid content (20 ± 0.4mg QAE/g of dry weight) and DPPH inhibition activity 90%. Ethyl acetate extract also exhibited the highest chelating activity with 59% inhibition in the metal chelating assay. However, the Rancimat showed a maximum of 69hour induction period (IP) in biodiesel when gallic acid is added.

Keywords: *Brucea javanica*, Biodiesel, Auto-oxidation, Natural Antioxidants, Total Phenolic Content, Total Flavonoid Content, Rancimat test

INTRODUCTION

Biodiesel can be produced from various sources by transesterification of oil/triglycerides with alcohols (Meher, Vidya Sagar, and Naik 2006). Biodieel is produced from plant seed oils, Jatropha curcas is most well known plant for biodiesel production (Contran et al. 2013). Some other known oil crops are palm macaw (Ciconini et al. 2013), andriba and chest-nut tree (Iha et al. 2014), cardoon [1], Ilama [2], crambe [3], parasol tree china [4] and babassu [5]. A major problem associated with biodiesel is that it is vulnerable to oxidative stress. Hence, the biodiesel oxidation is very important because when the oxidative reaction occurs, biodiesel deteriorates. The oxidation of lipids is more or less same in characteristic as biodiesel oxidation. In addition to the oxidation process, polymers may form when unsaturated fatty acids present, which in turn will lead to higher molecular weight products that will increase their viscosity. The oxidation of biodiesel could lead to many mechanical problems such as deposit formation, fuel system corrosion and filtering problems [6].

Brucea javanica belongs to Simaroubaceae family, is an evergreen plant, its seed is also known as yadanzi and was first cited in the Chinese medical monograph called Compendium of Materia Medica, published in the sixteenth century[7]. This plant is used as traditional medicine (Kim, Hitotsuyanagi, and Takeya 2004) and used for anti-diabetic treatment [8]. Several secondary metabolites have been isolated from *Brucea javanica* seed such as alkaloids, lignans, quassinoids, triterpenoids and flavonoids. The literature search suggested that *Brucea javanica* plant contains 74 quassinoid compounds, in which 33 of them are glycosides [9]. The objective of the study was to investigate antioxidants activities of *Brucea javanica* seeds and determine its potential to boost up the oxidative stability of biodiesel.

EXPERIMENTAL WORK

Materials

Chemicals and reagents

Palm oil methyl esters (POME), butylated hydroxyanisole (BHA), ferrozine, sodium nitroferricyanide (III) dehydrate, sodium acetate trihydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid monohydrate, 2,4,6-tripyridyl-S-triazine (TPTZ), Folin Ciocalteu reagent and sodium phosphate monobasic were obtained from Sigma Chemical Company (USA). Acetic acid glacial, ascorbic acid, ferric sulfate, ferric chloride hexahydrate, sodium chloride, aluminium chloride, potassium acetate, quercetin, ethylene-diamine-tetra-acetic-acid (EDTA) and sodium bicarbonate were purchased from Merck Chemical Co. (Malaysia). Ethyl acetate, methanol and hexane (Systerm), all the grade solvent for liquid chromatography mass spectrometry (LCMS) were purchased from Fisher Scientific (Malaysia). All chemicals used are of analytical grade and were used without further purification.

Sample collection

Brucea javanica seeds were collected from areas surrounding University of Malaya's Glami Lemi Biotechnology Research Centre in Jelebu District, Negeri Sembilan, Malaysia. The seed powder was completely dried and stored at 2-8 ^{o}C in capped bottles filled with nitrogen.

Extracts preparation

Brucea javanica seeds were grounded into powder form. The extraction was done with three different solvents. A total of 1kg of powder was extracted with methanol, ethyl acetate and hexane solvent. The extraction was done by using soxhlet apparatus and was filtered with Whatman filter papers. The methanol, ethyl acetate and hexane crude extracts were later tested for their antioxidant activities.

Polyphenols determination

Total phenolic content determination

The total phenolic content was evaluated by using the Folin Ciocalteu method [10] 100µl sample extract was added to 1 mL of 0.5 M Folin–Ciocalteu reagent, and it was then stirred .1 mL of 75g/L sodium bicarbonate was added and the mixture was shaken again for 30 seconds. The incubation period was 2 hours and the sample was placed in the dark and 96 well microplate were used to measure its absorbance at 765nm. Gallic acid was used as standard. The total phenolic content was assessed utilizing the standard curve of Gallic acid and it was expressed as gallic acid equivalents (GAE) mg/g of dry extract.

Total flavonoid content determination

Total flavonoid content determination was evaluated according to a modified method. 100 mL of 10% aluminium chloride with 1M of potassium acetate was prepared as reagent. Then 3.8 mL of methanol was added at room temperature for 40 minutes. 20µl of sample extract was mixed with the reagent and analysed using 96 well microplate at 510 nm. The total flavonoid contents were determined by plotting the quercetin calibration curve with 5 different concentrations and expressed as quercetin equivalent (QAE) mg/g of dry extract.

Spectrometry Analysis

Gas chromatography mass spectrometry analysis (GCMS)

Gas chromatography mass spectrophotometry analysis was performed by using Trace GC 2000 gas chromatograph coupled to a Polaris-Q Ion trap mass spectrometer (Thermo Finnigan, Austin, TX, USA). Zebron ZB-5ms (Phenomenex, Torrance, CA, USA) fused silica capillary column (30m long x 0.25mm I.D. x 0.25 film thickness) was used as the column. The oven temperature was programmed to hold the initial temperature of 40°C. It was maintained for 5 minute before being increased gradually every 10°C for 2-10 minutes up to 280°C. Helium was used as carrier gas at 1 mL/min in constant flow mode with injection temperature of 200°C and auxiliary temperature of 250 °C.

Liquid chromatography mass spectrometry analysis (Agilent)

A broad and rapid LC/MS with MS/MS data collection based analysis is done for Bruce javanica seeds. Ionization mode was negative. Dilution of sample was done with water and nylon filter (0.2uM) was used for filtration. The column was Agilent Zorbax (C18-50mm × 5uM × 2.0mm). The buffer used were Acetonitrile (5mM ammonium format and with 0.1% formic acid) and urine. AB Sciex instrument was used for detection in LCMS. The identification of known compounds was done by crosschecked with the extension library.

Antioxidant activity assays

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Donating hydrogen ability was used to measure free radical assay of extracts by using DPPH radical [11] with a modification. Concisely, 40μL sample extracts with varying concentrations were blended with 50μM DPPH (200μL) solution in methanol. The mixture was shaken vigorously and incubated at room temperature for 20 minutes. Measurement of absorbance was done at 517 nm in photo-spectrometer. Ascorbic acid (5–80 μg/mL) was used as a standard and ethanol as the control. Tests were performed in triplicates (n=3) and expressed as μg/mL. Inhibition percentage was calculated as:

"Percentage Inhibition = Absorbance of Control - Absorbance of Sample / Absorbance of Control x100"

Metal chelating activity

The metal chelating activity based on ion chelating activity of ferrous was done[12] by looking at Fe+ ferrozine complex based on the method as described by Welch (1990). Crude extracts with various concentrations were mixed with dH2O (120μL) and FeCl2 (2mM) 10μL in 96-well microplate. Ferrozine (5mM, 20μL) was added to the mixture to initiate the reaction. The incubation period of reaction mixture was 20 minutes and the measurement of absorbance at 562 nm was done. EDTA Na2 (5 to 80μg/mL) was used as metal chelator with ethanol (100 μL) was used as control. The following formula was used to express the result:

"*Percentage Inhibition = Absorbance of Control - Absorbance of Sample / Absorbance of Control x100"* .

Ferric reducing antioxidant power (FRAP)

The activity of FRAP assay was determined as stated previously [13] 20μL of extracts in methanol were mixed with 200μL of FRAP assay reagent which was prepared on daily base (5mL 10mMTPTZ in 40mMHCl, 5mL of 20mM FeCl3, and 50mL of 0.3M acetate buffer (pH 4) in micro-well plate reader. A 10 minute incubation period was used. TPTZ-Fe2+ complex formation was measured at 595nm in the presence of antioxidant compounds with a 96 well microplate and methanol was used as blank. Standard curve calibration was plotted using ferrous sulphate (FeSO4) as standard. The linear regression line was used to evaluate FRAP value. The absorbance at 595 nm was measured and the results were stated as mmol Fe2+/g of dry extract from triplicated test.

Oxidative stability of biodiesel

Effect of crude extracts on biodiesel

A total of 2g of palm oil methyl esters (POME) or biodiesel were placed individually in a 10mL test tube. 200 to 600ppm of *Brucea javanica* seed crude extract were added into each tube and duration was 2h for 8h and temperature was 80oC. A DPPH is a free radical.

DPPH measurement

In DPPH method, a total 2g of oil along with a total of 2mL of DPPH (0.01mM) were mixed and kept in caped tubes along with crude extracts. DPPH was used as free radical and crude extract were used as natural antioxidant. The reduction capability was determined by examining the decrease and increase in DPPH absorbance at 517 nm induced by antioxidants.

Oxidation stability test with rancimat

Rancimat (Metrohm, Herisau, Switzerland) was used to test the oxidative stability of biodiesel in accordance with European biodiesel standard EN14112. The equipment operates under following conditions, the air flow rate was 10L/h, a total of 3g biodiesel placed in heating block with a temperature range of 100 to 120oC, the vapours discharged to a flask containing 0.06L distilled water and the conductivity change was recorded by a computer simultaneously. The induction period was determined to see time duration and antioxidants concentration was 0 to 10,000ppm.

Statistical analysis

All the data were statistically analysed using SPSS to determine whether there are significant differences between the samples that contribute to the oxidation activity. The results expressed as ± SEM (standard error mean). Statistical package for the social sciences (SPSS) software was used in statistical analysis applying ANOVA test with $P < 0.05$.

Total phenolic and flavonoid content

The extractable phenolic compound content in *Brucea javanica* seed extract was determined by using the linear Gallic acid standard curve (R^2=0.966). Total flavonoid and phenolic contents amount in *Brucea javanica* seeds extract is listed in Table 1. Among the three extracts of *Brucea javanica* seed, ethyl acetate extract exhibits the highest capacity of total phenolic at 98.5±0.1mg GAE/g of dry extract. The data implies that ethyl acetate extract is the more extractable solvent to extract phenolic content.

Table 1. Total flavonoid and phenolic contents in *B. javanica* seed.

Fractions	Ethyl Acetate	Methanol	Hexane
TFC (QAE/g dry extract)	20 ± 0.4[a]	12.9 ± 0.3[b]	10 ± 0.4[c]
TPC (GAE/g dry extract)	98.5 ±0.1[a]	85.3 ±0.6[b]	36.9 ±0.8[c]

Data are mean ± SD (n=3). Mean with different lower cases (a, b, c) in the same column are significantly different at P < 0.05 using ANOVA

The total phenolic content extracted from methanol extract was 85.3±0.6mg GAE/g of dry weight. Among the three extracts, hexane has the smallest total of phenolic content at 36.9±0.8mg GAE/g of dry weight. The data indicate that the quantity of total phenolic content varies among the three selections. The deviation may be expected due to the different phenols present in the extracts. This can be supported up by the previous finding which stated that different solvents extract different phenolic compounds and presents a different reaction in the Folin-Ciocalteu method [14].

The total flavonoids content suggests that ethyl acetate extract exhibits the highest total flavonoid content at 20.0 ±0.4 mg QAE/g of dry weight and methanol extract exhibits at 12.9 ± 0.3mg QAE/g of dry weight. Hexane extract presented the lowest amount of flavonoid content at 10.0 ±0.4 mg QAE/g of dry weight. Ethyl acetate extracts showed highest TPC and TPC because of its polarity to extract more compounds as compared to methanol and hexane.

The most commonly distributed antioxidant groups found in plants are flavonoids which are characterized by a ring structure known as benzo-y-pyrone, which is distributed in most fruits and vegetables [15]. Flavonoids contain an aromatic ring which lets them to take over and donate electrons from free radicals [16]. Flavonoids have been proven to protect lipids against oxidation. In the proposed work, the total flavonoids content in *Brucea javanica* seed extracts was assessed using linear quercetin standard curve (R2=0.996). The flavonoids content found were little in total compared to the total phenolic content.

Mass spectrometry analysis

GCMS identification and analysis was carried out in order to identify compounds that are possibly present inside *Brucea javanica* seed extracts. Hence, information was provided on key compounds that may be responsible for the antioxidant activity such as phenols and flavonoids. Phytochemical profile was determined using mass analysis through direct infusion technique in n-hexane extract of *Brucea javanica* seed. Chemical compounds suggested and detected in n-hexane extract along with the corresponding values for comparison crosschecked with the reference library. The compounds detected in GCMS with their retention time are listed in Table 2.

To identify the chemical compounds LCMS was conducted and hence confirmation of antioxidant compounds present in *Brucea javanica* seed was done.

The analysis was done using LCMS technique for methanol and ethyl acetate extracts of *Brucea javanica* seed and chromatogram is shown in Figure 1. The compound with their retention time and molecular formula are listed in Table 3. The compounds detected via LCMS was gallic acid, ellagic acid, quinic acid, brevifolin and strictinin.

Antioxidant activity

2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

The summary of DPPH assay is shown in Figure 2. The results indicate that ethyl acetate extract of *Brucea javanica* seed shows the highest DPPH inhibition (90% with IC_{50}= 31.2 μg/mL) using different concentrations of seeds extract followed by methanol extract with 82 % inhibition (IC_{50}= 184 μg/mL) using different concentrations of seeds extract (62.5, 125, 250, 500 and 1000μg/mL) and the lowest inhibition is shown by n-hexane extract, up to 78 % DPPH inhibition with (IC_{50}= 319μg/mL) using different concentrations of seeds extract. Ascorbic acid was used as control.

These findings are similar in some ways with previous findings which suggested that variations in extract polarity alter extract ability to extract specific phenolic and flavonoid compounds [17].

Metal chelating activity assay

The procedure used to determine metal chelating activity was the ion chelating activity of ferrous. The summary of metal chelating antioxidant activity is given in Figure 3.

The resolution suggests that the ethyl acetate extract of seed shows the highest inhibition up to 59% with (IC_{50}=299 μg/mL) followed by methanol extract with 37% inhibition with (IC_{50}=656 μg/mL) and the lowest activity is shown by hexane extract with 27% inhibition (IC_{50}= 1100 μg/mL) at different concentrations of seeds extract.

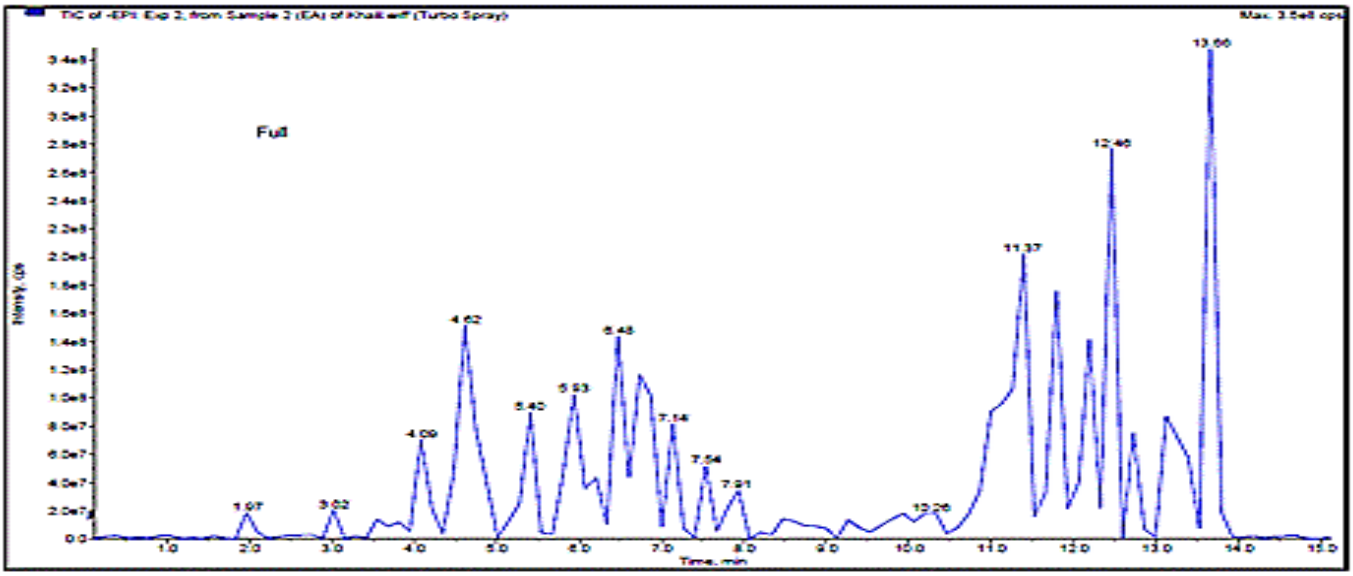


Figure 1. LCMS analysis of Ethyl acetate extract of *B. javanica* seeds.

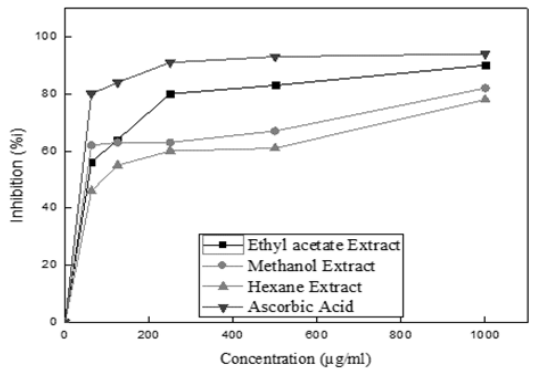


Figure 2. DPPH test of ethyl acetate, methanol and hexane extract of *Brucea javanica* seed.

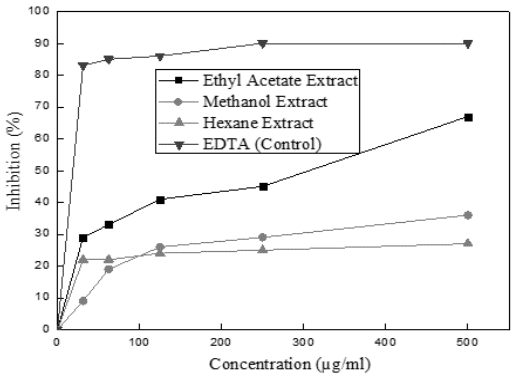
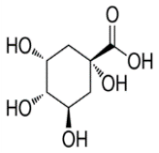
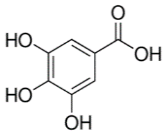
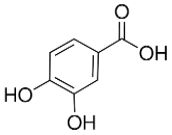
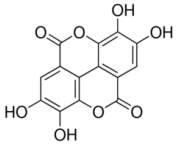
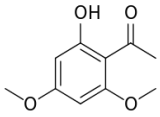
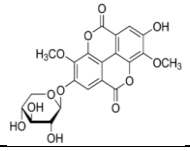


Figure 3. Metal Chelating activity assay of ethyl acetate, methanol and n-hexane extracts of *Brucea javanica* seed

Table 2. GCMS Analysis of *B. javanica* seed extract.

Peak No	Retention Time	%Area	Chemical Compound	Reference
1	5.1239	0.0521	2-Decene,6-methyl	27063
2	7.3211	0.1454	2-Decenal	26669
3	7.7503	0.0588	2,4-Decadienal	25129
4	8.0707	0.0775	2,4-Decadienal, (E,E)	25126
5	8.8775	0.7042	n-Decanoic acid	39473
6	10.102	0.3125	Cyclopentane, decyl	69587
7	10.6113	0.0564	Dodecanoic acid, methyl ester	72688
8	10.9889	0.039	Cyclohexene, 1-nonyl	67950
9	11.2693	0.1815	Cyclohexadecane	81247
10	11.8243	0.0204	11-Hexadecyn-1-ol	92516
11	12.4022	0.5184	8-Heptadecene	92565
12	12.9401	0.2045	Tridecanoicacid,12-methyl ester	95899
13	13.4837	0.7112	3-Octadecene	104188
14	13.9815	0.0318	Naphthalene	16914
15	14.5651	0.1061	1-Nonadecene	115904
16	14.7769	0.1189	cis-9-Hexadecenal	92517
17	15.0858	6.0428	Hexadecanoic acid, methyl ester	119407
18	15.8068	5.2165	n-Hexadecanoic acid	107549
19	16.8082	7.6304	9-Octadecenoic acid, methyl ester, (E)	141310
20	17.5234	22.5493	Oleic Acid	129337
21	17.9697	3.5004	Octadecanoic acid	131262
22	18.2386	8.5806	9-Eicosyne	126189
23	19.2171	4.9914	10-Heneicosene (c,t)	139793
24	19.755	4.6028	9,17-Octadecadienal, (Z)	114272
25	20.6075	8.2593	1,13-Tetradecadiene	56485
26	20.7792	5.6151	(Z)-14-Tricosenyl formate	194460
27	21.317	1.179	9,12-Octadecadienoic acid (Z,Z)	127648
28	21.689	0.456	cis-Vaccenic acid	129339
29	22.1525	0.6666	Z,E-2,13-Octadecadien-1-ol	115874
30	22.244	0.7581	Triacontyl acetate	232691
31	23.1195	1.6968	Docosane	153223
32	24.7388	9.6426	Heneicosane	141424
33	25.1107	4.1019	Stigmastan-3,5-diene	210541
34	26.8044	0.7648	Erucic acid	175491
35	27.2336	0.4068	9,12-Octadecadienoic acid	127647

Table 3. LCMS analysis of *B.javanica* seed extracts

Peak	R.Time	Compound	Molar Mass g/mol	Structure
1	1.97	Quinic acid	192.1	
2	3.02	Gallic acid	170.1	
3	3.82	Protocatechuic acid	154.1	
4	4.62	Ellagic acid	302.1	
5	4.63	Brevifolin	196.2	
6	5.40	Ellagic Acid Isomers	--------	------
7	7.54	Di-O-methyl Ellagic acid	462.3	

Ferric reducing antioxidant power (FRAP) assay

Frap assay react with ferric tripyridyltriazine [Fe3+-TPTZ] complex and measure the reducing potential of antioxidant by producing [Fe2+-TPTZ]. Frap assay treats the antioxidants in the sample as a reductant. In the proposed study, the trend for ferric ion reducing activities of *Brucea javanica* is shown in Table 4.

Table 4. FRAP assay of *Brucea javanica* seed extract.

Seed Extracts	FRAP (mmol Fe2+/g extract)
Methanol Seed Extract	0.180 ±0.003
Ethyl acetate Seed Extract	0.163 ±0.002
Hexane Seed Extract	0.15 ±0.004

The increase in the absorbance of *Brucea javanica* is due to the formation of the Fe2+-TPTZ complex with increasing concentration of seeds extracts. The methanol extracts of *Brucea javanica* seeds showed increased ferric reducing power with the increased concentration as standard antioxidants.

Hence, The outcome indicates that the methanol extract of *Brucea javanica* seed showed the highest FRAP activity up to 71% inhibition followed by ethyl acetate and n-hexane. The result is expressed as mill mole of iron per gram of extract. Therefore, they should be able to donate electrons to free radicals stable.

Addition of *Brucea javanica* seed crude extract to biodiesel

The DPPH assay has been shown to be a good predictor of the oxidative stability of oils as determined using thermal oxidations of oil. Palm oil methyl ester (POME) was tested for the antioxidant activity at 80 °C. Absorbance of DPPH in methanol with adding of *Brucea javanica* seed extract residue in different concentrations (200, 400 and 600mg/L) to Palm oil biodiesel is shown in Figure 4.

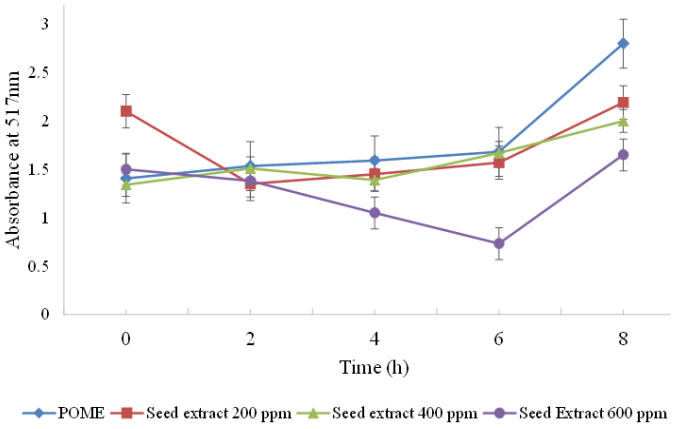


Figure 4. Effect of *Brucea javanica* seed crude extracts on Biodiesel.

For thermally oxidized Palm oil biodiesel with adding of 200mg/L residue (*Brucea javanica* seed extract) at 0h to 2h a decrease in DPPH absorbance indicating biodiesel with little radical scavenging compounds. As the time of oxidation increased from 2h to 8h, a gradual increase in DPPH absorbance is shown indicating lack of oxidative stability. Changes of DPPH absorbance due to thermally oxidized Palm biodiesel with addition of 400mg/L residue (*Brucea javanica* seed extract) at 0h to 6h were insignificant showing a steady state pattern

indicating stability of Palm oil biodiesel. As the time of oxidation increased at 6 h to 8 h an increase in DPPH absorbance is shown indicating a reverse pattern. A steady state pattern is shown at 0h to 8h thermally oxidized Palm biodiesel with 600mg/L addition of residue (*Brucea javanica* seed extract). The pattern indicated the equivalent antioxidant dose to be used in Palm biodiesel for oxidative stability needed. Adding 200mg/L, 400mg/L and 600mg/L residue (*Brucea javanica* seed extract) with Palm biodiesel showed the optimum oxidation stability needed from natural extract to be 600mg/L. Results indicated that residue adding of (*Brucea javanica* seed extract) can be used as antioxidant to improve the oxidation stability of biodiesel. The results agreed with previous stated finding that Jatropha plant methanol crude extract shows stability up to 6hours when added to biodiesel in crude form [18].

Rancimat test

The oxidation stability of biodiesel can be extended by adding antioxidants [19]. Plants and plant derived product are rich sources of natural antioxidants Plant extracts are considered as a potential natural antioxidants because of the presence of phenolic, flavonoids and anthocyanin compounds [20]. In order to confirm autoxidation effect of phenolic compounds of *Brucea javanica* seed, gallic acid was identified and detected in both extracts of *Brucea javanica* seed confirmed by LCMS profiling. After confirming the presence of gallic acid as clear peak in LCMS. The gallic acid was applied as a natural antioxidant for biodiesel to test its oxidative stability in the Rancimat machine with different concentrations from 2000 to 10,000ppm is shown in Figure 5.

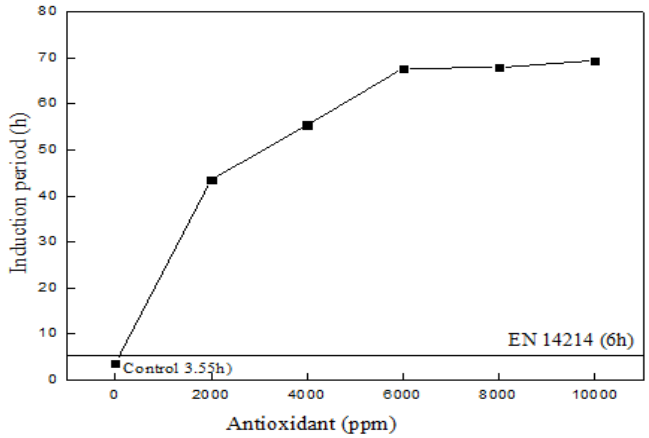


Figure 5. Influence of natural antioxidant on oxidative stability of biodiesel at 110^{0}C with concentration (2000-10,000 ppm) in Rancimat.

The effect was substantial and positive. It was noted that a substantial increment in the initiation period with gallic acid as antioxidant and which in turn prolong the oxidative stability of biodiesel. The induction period (IP) of Sample I was 43hours with 2000ppm gallic acid as antioxidants followed by Sample II (IP: 55hour, 4000ppm) followed by Sample III (IP: 67hour, 6000ppm) which is followed by Sample IV (IP: 67hour, 8000ppm) and maximum peak was obtained with Sample V (IP: 69hour, 10,000ppm.).

CONCLUSION

The information obtained from these results indicated that *Brucea javanica* seed contains many interesting antioxidant compounds such as gallic acid, ellagic acid, quinic acid, brevifolin and strictinin and showed strong antioxidant activities, which can be isolated and used as natural antioxidants to improve the oxidative stability of biodiesel.

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