Stabilization of Neem Oil Biodiesel with Corn Silk Extract during Long-term Storage

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Abstract: The current study aimed to evaluate the antioxidant efficiency of different extracts of corn silk. In addition, the impact of corn silk extract on oxidative stability of neem biodiesel during storage was studied. The highest phenolics, DPPH radical scavenging and reducing power activities were recorded for methanol-water extract. The longest oxidation stability (10 h) was observed for biodiesel samples blended with 1000 ppm of corn silk extract (CSE). At the end of storage period the induction time of biodiesel samples mixed with 1000 ppm of CSE or butylated hydroxytoluene (BHT) were about 6.72 and 5.63 times as high as in biodiesel samples without antioxidants. Biodiesel samples blended with 1000 ppm of CSE had the lowest acidity at the end of storage period. Peroxide value of biodiesel samples containing 1000 ppm of CSE was about 4.28 times as low as in control sample without antioxidants.

Key words: antioxidant efficiency, corn silk extract, oxidative stability, neem oil biodiesel, long-term storage, Rancimat

1 Introduction

Biodiesel has been mainly produced from vegetable oils all over the world. More than 95% of global biodiesel production is made from vegetable oils¹. Between 2005 and 2017, biodiesel use of edible vegetable oils is expected to represent more than a third of the projected growth in edible vegetable oil use². However, there are several reasons against the use of edible vegetable oils as a feed-stock in biodiesel production.

Utilization edible oil in the production of biodiesel fuel has an impact on global imbalances and market demand and food supply by high prices, and limit the food sources and the growth of a commercial plant capability. Consequently, focus should be turned to resources of non-edible, which are not widely used in human nutrition and can grow in arid lands. Oils from these resources are, as a rule, unsuitable for human consumption due to the presence of toxic compounds. For example, the main toxic compounds in *Jatropha* plants are protein curcin and purgative agents; protein ricin in castor plant³.

Neem (*Azadirachta indica*), which belongs to the family *Meliaceae*, is an evergreen tree of potential medicinal value and typically found in most tropical countries of the world. In the recent period, it was planted nearly 4,000 neem trees in Egypt found in the Delta zone and South Sinai Governorate³. The adult of neem tree produces

about 350 kg of fruits and this amount is given about 30 kg of seeds⁴. Neem kernels contain 30 - 50% oil and many active ingredients having antifeedant, growth-inhibiting, antiviral activity and anti-ovipositional properties^{5,6}. Neem oil is widely used as insecticides, lubricant, drugs for variety of diseases such as diabetes and tuberculosis⁷⁻⁹. Average percent of fatty acid content was oleic acid 45.6%, linoleic acid 16.8%, palmitic acid 17.21%, stearic acid 15.2%, and linolenic acid 1.3%¹⁰.

Biodiesel composes of long chain fatty acid esters derived from feed stocks such as animal lipid, vegetable oils, as well as used cooking oil, etc. which contain saturated and unsaturated fatty acids which are highly susceptible to oxidation accelerated by exposure to air during storage and at high temperature can result in production of polymerized compounds. Oxidation instability may lead to produce the oxidation products such as aldehydes, shorter chain carboxylic acids, insolubles as well as sediments in the biodiesel¹¹. The oxidation products formed can cause damage to the mechanical parts of engines, such as corrosion of the metal systems, degradation of the physical characteristics of the elastomers, and blockages in the injection pumps and filters^{12,13}. Antioxidant substances scavenge free radical molecules therefore, stabilizing fatty peroxy radicals and slowing oxidation chain reactions¹⁴⁻¹⁷. Phenolic substrates are commonly found in both edible and

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non edible plants, and they have been showed to have multiple impacts, including antioxidant activity¹⁸⁾. Potential sources of antioxidant compounds have been searched in several types of plant substrates such as vegetables, fruits, leaves, oilseeds, cereal crops, barks and roots, spices and herbs, and crude plant drugs¹⁹⁾.

Corn is the world's third most important cereal crop, less than wheat and rice²⁰⁾. Corn silk, the female flower stigma of maize (*Zea mays* L.) is a yellow silky strand which is on top of corn fruit. Corn silk is a by-product obtained from maize production e.g. food, processed food and feeds. Silk of corn is discarded as waste along with the other parts of the corn like cobs as well as husk because of a lack of benefits of them. Corn silk consists of protein, carbohydrate, Ca^{2+} , K^+ , Mg^{2+} and Na^+ salts, volatile oils, steroids, alkaloids as well as some of flavonoids²¹⁻²³⁾. Several components present in corn silk have been reported to possess antioxidant activities²⁴⁾. Corn silk (*Zea mays*) is traditionally used in the treatment of cystitis, edema, gout, kidney stones, nephritis, and urological disorders²³⁾.

The primary aim of the current study is to assess the antioxidant efficiency of different extracts of corn silk (*Zea mays* hairs). Furthermore, the impact of corn silk extract on oxidative stability of neem seeds oil based biodiesel during long-term storage was assessed.

2 Materials and methods

2.1 Materials

Neem seeds used in the current investigation were harvested in July 2014 from a 15 year-old trees located at Giza, Egypt. The seeds were dried under shade. The dried seeds were ground in an electric grinder (Braun Model 1021), passed through a 150- μm mesh sieve, and stored in plastic bags and stored in a freezer (-25°C) until further use.

Corn silk was collected from Agricultural Research Center, Giza, Egypt. Corn silk was detached, cleaned and washed with distilled water. The collected corn silk was dried in an electric air draught oven (Isotemp Oven, Fisher Scientific) at 50°C for 24 hours. The dried corn silk was ground in an electric grinder (Braun Model 1021) and stored in glass containers at 4°C for further analysis.

Folin-Ciocalteu reagent, sodium carbonate, citric acid, aluminum chloride, ferric chloride, copper sulfate, tween 80 and Butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co., Ltd (St. Louis, MO, USA).

2.2 Extraction of Antioxidant Material

The ground corn silk materials (100 g) were separately mixed with 1000 mL of each of the extraction solvents, 100% methanol, 80% methanol (methanol: water, 80:20 v/v), 100% acetone, 80% acetone (acetone : water, 80:20 v/v) and distilled water in conical flasks. Extraction was exe-

cuted on a shaker for 48 h. Each extract was filtered through Whatman No 1 paper. The collected filtrates were individually dried at 45°C by using a rotary evaporator. The concentrated extracts were stored in kept at -18°C until further use.

2.3 Neem oil extraction

The extraction of oil of neem seeds was carried out by using chloroform: methanol (2:1, v: v) according to the procedures proposed by Bligh and Dyer²⁵⁾.

2.4 Purification of neem oil

Purification process was performed to improve the quality attributes (i.e., free fatty acid content, peroxide value) of crude oil. Magnesol XL was added to crude neem oil at level of 2% (w/v) then mechanically stirred for 1 h at 105°C . The slurry was filtered through a Whatman No. 1 filter paper using vacuum filtration. The vacuum filtration facilitates the oil flow through the filter paper²⁶⁾. Filtrated neem oil was heated at 70°C and hot water $80-85^\circ\text{C}$ was added (1:1 v/v). The mixture has been mixed using rod of glass for 15 minutes, and left for cooling at room temperature. The upper oil phase was centrifuged at 10,000 rpm for 20 min. The centrifuged oil was stirred with the anhydrous sodium sulfate for ca. 5 min, and then filtered (vacuum filtration) through a Whatman No 1 paper²⁷⁾.

2.5 Biodiesel production

One thousand grams of purified neem oil were placed into a 2000 mL round-bottom flask equipped with a condenser. After the oil was heated to 65°C , the solution of sodium hydroxide (5.0 g) in methanol (144.82 mL, 6:1 molar ratio of methanol to oil) was slowly added into the reaction. The reaction temperature of the transesterification treatment was appointed at 60°C . The reaction continued for 110 min. By the end of the incubation, the mixture was transferred into a reparatory funnel, left for 24 h for separation the glycerin and impurities from the biodiesel. The produced biodiesel was washed with deionized hot water (50°C) several times for removing the catalyst, glycerol, and impurities²⁸⁾.

2.6 Storage experiment

The obtained biodiesel sample has been divided into 5 groups each one 200 mL, the first one represents the control, without additives. The second and third portions were blended with 500 and 1000 ppm of BHT. The fourth and the last portion were blended with corn silk extracts at levels of 500 and 1000 ppm, respectively. Biodiesel samples have been stored at room temperature of approximately 25°C and relative humidity 65% in a 100 mL glass bottle and were tightened with a cap to ensure that the biodiesel samples were free from air contact²⁹⁾. Biodiesel samples were evaluated and analyzed by using various procedures

at the start of the experiment and at 1, 2, 3, 4, 5 and 6 months from the start of the experiment.

2.7 Analytical methods

2.7.1 Determination of total phenolic content

The total phenolic content was assayed by using Folin–Ciocalteu reagents with analytical grade gallic acid as the standard. 1 mL of extract or standard solution (0–500 mg/L) was added to deionized water (10 mL) and Folin–Ciocalteu phenol reagents (1.0 mL). After 5 minutes, 20% sodium carbonate (2.0 mL) has been added to the mixture. After being kept in darkness for 1 h, the absorbance was measured at 750 nm. Amounts of total phenolics were calculated using gallic acid calibration curve. The results were expressed as mg of gallic acid equivalent per 100 g dry weight (DW)³⁰⁾.

2.7.2 Determination of Individual Phenolic Compounds by HPLC

Phenolic compounds of methanolic extract of corn silk were quantified using the previously reported procedures by Ahmed and Ali³¹⁾.

2.7.3 Antioxidant activity

a. Scavenging DPPH radicals

The free radical scavenging capacity of different extracts of corn silk was determined by using 1, 1-diphenyl-picrylhydrazil (DPPH) assay³²⁾. Butylated hydroxytoluene (BHT) was used as positive control. The radical-scavenging activity was expressed as inhibition percentage and calculated using the following equation:

$$\% \text{ Radical scavenging activity} = [(\text{Abs control} - \text{Abs test}) / (\text{Abs control})] \times 100.$$

Where, Abs control is the absorbance of DPPH radical + methanol.

Abs sample is the absorbance of DPPH radical + sample extract or standard.

b. Reducing power assay

The reducing power of the extracts was conducted according to previously reported procedures by Chou *et al.*³³⁾. 1 mL of extract was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] (2.5 mL, 1%). After incubation at 50°C for 25 min., 2.5 mL of trichloroacetic acid (10 g/100 mL) were added and the mixture was centrifuged at 650 rpm for 10 min. Finally, 2.5 mL of the upper layer was mixed with 2.5 mL of deionized water and 0.5 mL of aqueous FeCl_3 (0.1 g/100 mL). The absorbance was measured at 700 nm. Higher absorbance indicates higher reducing power. Butylated hydroxytoluene (BHT) was used as positive control.

2.7.4 Moisture content

The amount of moisture was determined according to the AOAC official method³⁴⁾.

2.7.5 Density

The density of the samples was determined by a mass over volume measurement at 25°C.

2.7.6 Acid value

Acid value was determined according the A.O.A.C. method (969.17)³⁵⁾. Acid value is expressed as mg KOH required to neutralize one gram of sample.

2.7.7 Peroxide value

Determination of peroxide value was performed according to the procedures of A.O.A.C method (965.33)³⁵⁾. Peroxide value is expressed as milliequivalent peroxides kg^{-1} oil sample.

2.7.8 Oxidative stability of biodiesel samples

The oxidative stability of the biodiesel samples was evaluated using Rancimat 679 instrument (Metrohm, Herisau, Switzerland). Essentially, the oxidation process happens inside a reaction vessel heated at 110°C (with 3 g of biodiesel sample) under air flow of 10 L h^{-1} . The volatiles oxidation products formed during the oxidation experiment are collected in a vessel containing 60 mL of distilled water, and the water conductivity is monitored continuously. A sudden change in electric conductivity of this water is noticed in the induction period point, indicating the propagation launch of an oxidation process.

2.8 Statistical analysis

Results are expressed as mean $\pm$ SD. Data were statistically analyzed according to the procedures described by Gomez and Gomez³⁶⁾. SPSS Version 18.0 (SPSS Inc., Chicago, IL, USA) was used to analyze data.

3 Results and discussion

3.1 Total phenolic compounds (TPCs) of different solvent extracts of corn silk

Phenolic compounds are a class of antioxidant agents acting as free radical terminators³⁷⁾. Phenolic substances have been shown to be responsible for the antioxidant activity of plant materials. TPC may contribute directly to antioxidative activity³⁸⁾ of the plant extracts. **Figure 1** shows the total phenolic compounds of different solvent extracts of corn silk. TPCs of corn silk extracts ranged from 274.81 to 510.60 mg / 100 g dry material (DW). Similar results were reported by Tian *et al.*³⁹⁾, who studied the contents of total phenolic of six corn silk varieties (Denghai6702, Delinong988, Tunyu808, Zhongdan909, Liangyu208, and Jingke968). They showed that the highest content of total phenolics was detected in Zhongdan909 (4.87 mg g^{-1}), whereas the lowest content was found in Liangyu208 (1.99 mg g^{-1}). Solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations were used for the extraction of phenolics from plant substrates, often with various levels of water. Selecting the right solvent affects

Fig. 1 Total phenolic content of different solvent extracts of corn silk (LSD at 0.05 = 0.47). Data are expressed as mean $\pm$ SD. Values given represent means of three determinations. Values followed by different letters are significantly different ($p < 0.05$).

the amount and rate of polyphenols extracted⁴⁰. There are significant effects of various solvents on the TPC ($p < 0.05$). The highest ($p \leq 0.05$) levels of TPC (510.60 mg / 100 g DW) were recorded for methanol-water (80:20 v/v) extract followed by pure methanol extract (420.10 mg / 100 g DW) and distilled water extract (360.15 mg / 100 g DW). The variability in the TPC caused by different solvents may be attributed to the varying solubility of the phenolic compounds, which is directly related to the compatibility of the compounds with the solvent system⁴¹. Mixtures of alcohols and water at different levels have shown to be more effective in extracting phenolic compounds compared to mono-component solvent system⁴². Mixing a small quantity of water with organic solvent usually leads to a more polar medium, which facilitates the polyphenols extraction⁴³. In addition, the mixture viscosity decreased because of the presence of water, which might have improved the mass transfer⁴⁴. Similar, observations were noted by Agbangnan *et al.*⁴⁵, who found that the high yield of phenolics can be extracted from sorghum leaf using water, while extraction of phenolics from wheat bran requires 80% aqueous ethanol⁴⁶. However, moderate amounts (317.16 and 274.81) of TPCs were detected for 80% acetone (acetone: water, 80:20 v/v) and 100% acetone, respectively. The type of phenolics soluble in each of the extracting solutions tested is mainly different, because the phenolics include one or more of hydroxyl groups (polar side) attached directly to an aromatic ring (non polar side). This stereochemistry differentiates phenolics according to their polarity variance, which affects the recovery of phenol from natural sources, when accomplished with solvent extraction, being the yield of the process depends strongly on the nature of the solvent⁴⁷. In particular, methanol alcohol has been demonstrated to be more efficient in extraction of polyphenols with lower molecular weight while flavones

with the higher molecular weight are better extracted with aqueous acetone^{48–50}. Acetone has also been found to be more efficient than other organic solvents for the extraction of phenolics from different raw materials such as brewer's spent grains⁴⁷, *Syzygium cumini*⁵¹. Acetone: water mixtures (50–95% v/v) have been reported to be one of the most effective solvents for extraction of phenolics from different natural sources, especially from protein matrices, since this mixture is able to degrade polyphenol–protein complexes⁵². These differences could be attributed to the different methods of extraction and standards used, but also to different varieties, genomics and harvest season of the samples (all these parameter affect the synthesis and accumulation of phenolics in some parts of the plant)^{41, 53}. In the present investigation, the higher phenolics content of aqueous methanol (80% methanol) extract might be responsible for the enhanced antioxidative activities compared to the other extracts. Similar studies reported that the enrichment of phenolic compounds within plant extracts is correlated with their enhanced antioxidant activity^{54–56}.

3.2 Antioxidant activity

An antioxidant may be defined as a substance which, in relatively low concentration, markedly inhibits the rate of the reaction with oxygen^{57, 58}. The stable free radical α , α -diphenyl- β -picrylhydrazyl (DPPH) assay is rapid and sensitive way to survey the antioxidant activity of plant extracts²³. The radical scavenging activity of different extracts of corn silk was compared with that of BHT at the same concentration and expressed as % of inhibition against DPPH (Fig. 2). All extracts of corn silk exhibited appreciable scavenging activity ranging from 53.24% to 83.7% (Fig. 2). Free radical scavenging activities of extracts depends mainly on the structural configuration of these compounds and the capacity of these components to lose hydrogen^{59, 60}. Aqueous methanol (80%) extract of corn silk has significantly higher DPPH radical scavenging activities than those from other extracts and greater than BHT. The highest DPPH radical scavenging activities (83.7%) was observed for aqueous methanol (80%) extract of corn silk followed by BHT (78.1%), pure methanol extract (74.9%) and water extract (71.02%). Aqueous acetone (acetone: water, 80:20 v/v) and 100% acetone showed weaker DPPH radical scavenging activities (59.3 and 53.24%, respectively) than methanolic extract. The different antioxidant activities of phenolic extracts could be attributed to different extracting solvents, as the antioxidant activity depended mainly on polarity and the type of the solvent, the procedures of isolation, the purity of active components and the method of determination⁶¹. Interestingly, Sultana *et al.*⁶² showed that among the four solvent extracts [ethanol, methanol, aqueous ethanol (ethanol: water, 80:20 v/v) and aqueous methanol (metha-

Fig. 2 DPPH free radicals scavenging activity of different extracts of corn silk compared to BHT (LSD at 0.05 = 0.49).

Data are expressed as mean $\pm$ SD.

Values given represent means of three determinations.

Values followed by different letters are significantly different ($p < 0.05$).

nol: water, 80:20 v/v) used, the aqueous methanol and aqueous ethanol extracts had the greatest phenolic content. This might be attributed to the fact that phenolics have been extracted in larger quantities with more polar solvents such as aqueous ethanol / methanol as compared to absolute ethanol / methanol^{63, 64}. The results of Tomsone *et al.*⁶⁵, on horseradish roots showed that that acetone comparing to other solvents is an acceptable solvent, however it is not the best. The reducing power assay is usually used to evaluate the ability of an antioxidant to donate an electron⁶⁶. The presence of reductants in samples leads to reduce the ferric ion/ferricyanide complex to its ferrous form. The amount of ferrous ion complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm⁴¹. Results of reducing power activity of different solvent extracts of corn silk are presented in Fig 3. The reducing power of corn silk extracts ranged from 0.46 to 0.840 (absorbance value). The order of reducing power of the extracts is aqueous methanol(80%) > pure methanol extract > water extract > aqueous acetone extract > 100% acetone extract. In the same time the reducing power activity of aqueous methanol(0.840, absorbance value) was higher than that of BHT(0.763, absorbance value) at the same concentration. The reducing power of certain compounds is associated with the presence of reductones^{67, 68}. The antioxidant action of reductones is based on the breaking of the free-radical chain by donating a hydrogen atom⁶⁹.

These findings indicate that the aqueous methanol (80%) extract of corn silk possessed greater phenolic content, DPPH radical scavenging activity and reducing power activity than those from other extracts and BHT. Thus, the stabilization effects of aqueous methanol(80%)

Fig. 3 Reducing power activity of different solvent extracts of corn silk compared to BHT (LSD at 0.05 = 0.01).

Data are expressed as mean $\pm$ SD.

Values given represent means of three determinations.

Values followed by different letters are significantly different ($p < 0.05$).

extract were evaluated by mixing different levels of this extract into neem biodiesel during long storage process.

3.3 Qualitative analysis of Phenolic compounds in methanolic extract of corn silk

Based on the abovementioned results, HPLC analysis was employed to define qualitative and quantitative content of phenolic acids in methanolic extract of corn silk. HPLC is the preferred technique for quantification of phenolic compounds⁷⁰. Several polyphenolic compounds were identified in methanolic extract of corn silk. These included gallic, pyrogallol, catechin, protocatechuic acid, catechol, chlorogenic acid, rutin, caffeic acid, vanillic acid, quercetin, ferulic acid, coumaric acid, cinnamic, and kaempferol (Table 1). The quantities of the identified compounds are expressed in milligrams per 100 g of dry sample. The predominant phenolics of aqueous-methanolic extract of corn silk are protocatechuic acid (178.5), chlorogenic acid (26.71), gallic acid (12.5) pyrogallol (11.7), vanillic acid (10.52), coumaric acid (7.92), kaempferol (15.32) and ferulic acid (5.90) mg/100 g DW, respectively (Table 1). These findings in agreement with previous studies showed that protocatechuic, gallic, cinnamic, *p*-coumaric acids are the most representative phenolic acids in the phenolic composition of corn silk extracts⁷¹⁻⁷³.

3.4 Physicochemical properties of neem biodiesel

The physicochemical properties of the biodiesel obtained from neem seeds oil were shown in Table 2. The moisture content of fresh neem oil biodiesel was 0.05%, this low level of moisture content reflects the quality of the obtained biodiesel. The greatest level of water content allowed in biodiesel as defined in the ASTM standard

Table.1 HPLC analysis of phenolic compounds (mg / 100 g DW) of methanolic extract of corn silk.

Phenolic compounds (mg/100 g)	Corn silk extract
Gallic	12.5
Pyrogallol	11.7
Catechin	2.01
Protocatechuic	178.5
Catechol	2.85
Chlorogenic acid	26.71
Rosmarinic acid	ND
Rutin	1.37
Caffeic acid	0.87
Vanillic acid	10.52
ferulic acid	5.90
Coumaric acid	7.92
Cinnamic	0.78
Kaempferol	15.32

Values are presented as mean value (n = 2) and expressed as mg/100 g of dry weight.

ND means not detected.

D6751 is 0.050% vol⁷⁴). High moisture content in biodiesel may cause problems in handling, transportation, and storage⁷⁵). Density is an important characteristic of fuel, because the injection systems, pumps, as well as injectors must provide the amount of fuel specifically adjusted to provide proper combustion⁷⁶). Density of the obtained biodiesel was 0.868 g cm⁻³. This value satisfies the value of the density identified by the American Society for Testing and Materials (ASTM D6751) and European standard (EN 14214). The induction period of fresh neem oil biodiesel

without adding any antioxidants was 5.2 h. ASTM D6751-07 includes an oxidation stability standard of a three-hour minimum induction period (IP) as measured using the Rancimat test (EN14112)⁷⁷). The European Committee for standardization adopted a six-hour minimum IP as the specification⁷⁸). The acid value is a facile method for monitoring fuel quality. Acid value of neem oil biodiesel was 0.17 mg KOH/g. The greatest value of AV for pure biodiesel, as identified in ASTM standard D6751 is 0.8 mgKOH/g⁷⁴). However EN 14112⁷⁸) sets a limit of acid value 0.58 mg KOH/g for biodiesel. The peroxide value measures the presence and amount of unstable hydroperoxide which is a portion of deteriorated biodiesel formed when oxygen of the air react with fatty esters (oxygenated fatty esters), in other word it measures how much oxygen is incorporated into biodiesels chemical make up as oxygenated fatty compounds. These oxygenated fatty compounds are inherently unstable, thus it is usually the first step in the oxidative degradation pathway of biodiesel fuel. Peroxide value of fresh neem oil biodiesel was 2.4 milliequivalent peroxides/kg (Table 2). The iodine number is an index of the number of double bonds in biodiesel, and therefore is a parameter that quantifies the degree of unsaturation of biodiesel²⁶). Iodine value of fresh neem oil biodiesel is 71.9 g I₂/100 g. The European EN 14214 biodiesel standard sets a limit for iodine value of <120. These findings indicate that the density, moisture content, acid value, iodine number, and oxidation stability at 110°C of the biodiesel obtained from neem seeds oil agree with the approved international standards.

3.5 Effect of antioxidants on the oxidative stability of neem oil biodiesel

Oxidative stability is one of the main issues that affect the use of biodiesel because of its content of esters of linoleic and linolenic acids, which methylene bis allylic positions are particularly susceptible to oxidation. Rancimat

Table 2 Physicochemical properties of the obtained biodiesel compared to ASTM and EN 14214 standards.

Properties	Values	ASTM specification	EN 14214
moisture content %	0.05	0.05 maximum	—
Density (g cm ⁻³)	0.868	0.87 to 0.90	0.86 to 0.90
Acid value mg KOH/g	0.17	maximum 0.8	maximum 0.50
Iodine value (g I ₂ /100 g)	71.9	—	120 maximum
Oxidation Stability 110°C (hours)	5.2	minimum 3	minimum 6
peroxide value (milliequivalent peroxides/kg)	2.4	—	—
Physical state at 25°C	Liquid		

Data are expressed as mean ± SD.

Values given represent means of three determinations.

method, described in EN 14112, has been included in the European biodiesel standards. It is nearly identical to the AOCS Oil Stability Index (OSI) method Cd12b-92³⁴). As shown in Table 2, the oxidation stability of fresh biodiesel was 5.2 h, this finding indicates that biodiesel sample met the standard ASTM D6751 (3 h) specification, however do not meet the EN 14214 (6 h) specification., which making it necessary to add antioxidants to the biodiesel samples to satisfy the quality specification for oxidation stability. Figure 4 shows the effect of two different antioxidants (BHT and corn silk extract) with concentrations of 500 and 1000 ppm, on the oxidation stability of neem biodiesel. In general, all of the tested additives had positive influence on the oxidation stability of biodiesel samples produced from neem oil and increased the induction time of samples. The increase of oxidation stability was gradually increased with increasing the concentration of antioxidant. The extension of IP by adding an antioxidant has been associated with the efficiency of antioxidant, which is sometimes called antioxidant index or protection factor. The oxidation stability of biodiesel samples blended with tested antioxidants varied from 7.6 to 10 h. This finding indicates that the six-hour limit was reached when 500 ppm of corn silk extract or BHT were separately added at the same concentration. The longest oxidation stability (10h.) was observed for biodiesel samples blended with 1000 ppm of corn silk extract (CSE) followed by those samples treated with 1000 ppm of BHT (9.3 h), biodiesel sample blended with 500 ppm of CSE (7.8 h) and those samples mixed with BHT at concentration 500 ppm (7.6 h), respectively. These findings indicate that the extract of corn silk and BHT at level 1000 ppm improved the oxidation stability of the tested biodiesel by 92.30 and 78.84%, respectively. Since the speed of oxidation of biodiesel depends upon factors i.e., temperature, light and intensity of radiation, studies should be conduct-

ed to determin an appropriate concentrations of antioxidants that can effectively provide long term storage stability of biodiesel and blends under various conditions⁷⁹). Phenol itself is chemically inert as an antioxidant. Hydrogen substitution by alkyl groups in ortho- and para-position increases the density of electron of the OH moiety by inductive effect. This reinforces its reactivity toward fat radicals. Furthermore, the basic structural orientation of the compounds identifies the antioxidant activity of phenolics, such as how easily a hydrogen atom from a hydroxyl group can be donated to a free radical, and the ability of the compounds to support an unpaired electron⁸⁰). The position of hydroxyl groups appears more important than their number for the antioxidant capacity of phenolics; for example, hydroxyl groups in the ortho position of the B ring can greatly enhance the antioxidant capacity, such as in catechins⁸¹). Corn silk extract contains a large variety of phenolic derivatives, including protocatechuic, gallic, cinnamic, *p*-coumaric acids flavonoids, and tannins (Table 1). Protocatechuic acid and other structurally analogous dihydroxy and trihydroxyphenolic acids, 3,4-dihydroxyphenylacetic acid, hydrocaffeic acid, caffeic acid, gallic acid, 3,4,5-trihydroxyphenylacetic acid, and 3-(3,4,5-trihydroxyphenyl)propanoic acid show antioxidant activity by both chelating metal transition ions by scavenging free radicals through the donation of a hydrogen atom (H·) or electron (e⁻)⁸²). Phenolic acids also have a high antioxidant capacity, which decreases in the order: protocatechuic acid > caffeic acid > *p*-hydroxybenzoic acid > ferulic acid > vanillic acid > *p*-coumaric acid^{81, 83}). Corn silk is rich in phenolic compounds, particularly flavonoids and tannins^{84, 85}). Flavanols and tannins have a variety of phenolic hydroxyl groups and exhibit the greatest antioxidant ability and free radical-scavenging activity among about one hundred of phenolics^{81, 86, 87}).

Fig. 4 Effect of two different antioxidants (BHT and corn silk extract) with concentration of 500 and 1000 ppm, on the oxidation stability of neem biodiesel. Data are expressed as mean $\pm$ SD. Values given represent means of three determinations. Values followed by different letters are significantly different ($p < 0.05$).

3.6 Impact of storage time on oxidation stability of neem oil biodiesel

Oxidation stability is an important biodiesel fuel quality parameter. It is expressed by the induction period (IP). IP can be defined as the length of time before the rapid acceleration of oxidation. It is the measure of resistance to oxidation. The induction period is also known as the oxidative stability index. Generally, every fuel has its own induction period. Based on several studies using the Rancimat test at various temperatures, the induction period is a linear function of the test temperature^{88, 89}). In order to avoid degradation, the biodiesel must be stored at a low temperature for a long storage time²⁹). Figure 5 shows the changes in oxidation stability of neem oil biodiesel with and without antioxidants during storage at 25°C for 6 months. Generally, oxidation stability values of all biodiesel samples were gradually and significantly ($p \leq 0.05$) decreased with increasing storage period. This means that the relationship

Fig. 5 Changes in the oxidation stability of biodiesel samples treated with different concentration (ppm) of BHT and CSE during storage at room temperature. LSD at 0.05 = 0.15.

Data are expressed as mean $\pm$ SD.

Values given represent means of three determinations.

between oxidation stability and storage time is inversely proportional, and the slope of the graph dropped. The highest decreases were observed for control samples (without additives). However, the rates of decrease in oxidation stability are slower for those biodiesel samples blended with different concentrations of antioxidants. At the end of storage time the induction period of treated samples ranged from 3.6 to 7.4 h. These findings indicate that biodiesel samples blended with antioxidants at the end of storage time met the standard ASTM D6751 (3 h) specification, but only biodiesel samples mixed with 1000 ppm of BHT or CSE met the EN 14214 (6 h) specification. Here again, at the end of storage period the induction time of biodiesel samples mixed with 1000 ppm of corm silk extract or BHT were about 6.72 and 5.63 times as high as in biodiesel samples without antioxidants. A similar trend has been reported in the oxidation stability of biodiesel⁷⁹ over an 8-week storage period. The impact of antioxidant concentration on autooxidation rates depends upon several factors, e.g. antioxidant structure, storage conditions, as well as the composition of the sample being oxidized³⁷. Treatment with oxidation inhibitors is a promising way because it facilitates the use of existing storage tanks and fuel handling systems without requiring upgrades or redesign. Antioxidants such as butylated hydroxytoluene (BHT) or tert-butylhydroquinone (TBHQ) are known to retard effects of oxidation on AV and PV of biodiesel⁹⁰. Other antioxidants known to enhance resistance to oxidation of vegetable oils include , tocopherols, ascorbyl palmitate, butylated hydroxyanisole (BHA) as well as propyl gallate (PrG)⁹¹. Oxidation may be interrupted by several types of antioxidants which can react with either chain-carrying

peroxyl radicals or the alkyl radical intermediates⁹². The first class of antioxidants (AH), called primary antioxidants, includes hindered phenols such as butylated hydroxyanisole, butylated hydroxytoluene as well as α -tocopherol. To be effective, these compounds must compete with the unsaturated lipid substrate which is normally present in the highest concentration. The second class of antioxidants (Q^*), called secondary antioxidants, includes quinones such as α -tocopherolquinone and ubiquinone which must compete with O_2 in a fast reaction. These compounds may therefore only be active in biological systems in which the oxygen content is relatively low⁹³.

3.7 Impact of storage time on acid value of neem oil biodiesel

The oxidation process produces hydroperoxides, aldehydes, ketones, and acids that change the fuel's properties. Therefore, the acid value should be monitored, and fuel-enhancing additives may need to be added. Acid value is defined as the mass of potassium hydroxide (KOH) (milligram) that is required to neutralize one gram of a chemical substance. It is also called the acidity number. Generally, the acid value describes the amount of acid in the oil. There are many factors that influence the value of acidity, such as the temperature, storage time, and so forth. Figure 6 shows the changes in acid value of neem oil biodiesel with and without antioxidants during storage at 25°C for 6 months. The acid value of neem biodiesel initially was 0.17 mg KOH/g. The results presented in Fig. 6 showed that the acid values of all biodiesel samples under investigation were significantly and gradually increased ($p \leq 0.05$) with increasing the storage time. Acid can be formed through the oxidation reaction and hydrolysis reaction when exposed to oxygen and moisture in the air, respectively.

Fig. 6 Changes in acid value of biodiesel samples treated with different concentration (ppm) of BHT and CSE during storage at room temperature. LSD at 0.05 = 0.013

Data are expressed as mean $\pm$ SD.

Values given represent means of three determinations.

Furthermore, water in oil itself can cause hydrolysis of the esters and turn it into alcohol and acids^(26, 94). The highest level of acidity at the end of storage period (0.49 mg KOH / g) was recorded for biodiesel samples which were stored without antioxidants. Biodiesel samples blended with various levels antioxidants had lower levels of acidity at the end of storage time, ranging from 0.25 to 0.31 mg KOH/g oil. In the same time, these values were significantly ($p \leq 0.05$) less than the maximum allowed value of EN 14214 and ASTM D6751 specifications. Mixing biodiesel samples with different concentrations of CSE or BHT led to a significant ($p < 0.05$) decrease in acid values during the storage process. Acid value of biodiesel sample without antioxidants was about 1.68, 1.58, 1.88 and 1.96 times as high as in biodiesel samples mixed with 500ppm BHT, 500 ppm CSE, 1000 ppm BHT and 1000 ppm of CSE, respectively. This finding indicates that the highest reductions of FFA content were observed for biodiesel samples treated with 1000 ppm of BHT or CSE. Corn silk extract has high levels of phenolic compounds and flavonoids (Table 1). Flavonoids are the most common and widely distributed group of phenolic compounds, occurring in corn silk parts. The mechanisms of the actions of flavonoids are through scavenging or chelating processes^(95, 96). Polyphenols include many classes of compounds (phenolic acids, flavanols, xanthones, kaempferol etc.) and act as free radical scavengers. Flavonoid which is the major class of polyphenols exhibits strong antioxidant capacities⁽⁹⁷⁾. In this respect, Five flavones glycosides (i.e., 2"-O- α -l-rhamnosyl-6-C-3" deoxyglucosyl-3'-methoxyluteolin, ax-5'-methane-3'-methoxymaysin, ax-4"-OH-3'-methoxymaysin, 6,4'-dihydroxy-3'-methoxyflavone-7-O-glucoside, and 7,4'-dihydroxy-3'-methoxyflavone-2"-O- α -l-rhamnosyl-6-C-fucoside) have been successfully isolated from corn silk and identified by Ren *et al.*⁽⁹⁸⁾. They noted that most of the flavones glycosides showed a high antioxidant activity in a lecithin liposome system, as well as a strong scavenging activity against radicals such as 1, 1-diphenyl-2-picrylhydrazyl (DPPH), superoxide, and hydroxyl radicals.

3.8 Impact of storage time on peroxide value of neem oil biodiesel

The peroxide value (PV) is an indicator of the state of primary oxidation⁽⁹⁹⁾. The PV determination measures the presence of oxidative moieties in a sample. The oxidative moieties typically found in biodiesel are hydroperoxides formed when oxygen of air reacts with fatty esters. This usually is the first step in the oxidative degradation process of biodiesel⁽¹⁰⁰⁾. Peroxide values indicate the extent of intermediate hydroperoxide formation and are expressed as milliequivalents of active oxygen per kilogram of biodiesel sample. Figure 7 shows the changes of peroxide values of neem oil biodiesel with and without antioxidants during storage at 25°C for 6 months. The peroxide value of fresh

biodiesel without antioxidant (control) was 2.4 meq/kg oil. A gradual increase in peroxide values as a function of storage time was observed for all biodiesel samples and at all intervals (Fig. 7). This is attributed to the fact that the double bonds in the fatty acid esters are present in methylene-interrupted configuration rather than conjugated structures. The bis-allylic hydrogen in these structures is susceptible to abstraction by free radicals, which renders the molecule reactive with elemental oxygen. Peroxyl radicals formed from reaction with oxygen abstract hydrogen from the bis-allylic site of other polyunsaturated esters, forming hydroperoxides and additional radical esters that propagate a chain reaction, or react with an ester radical to form peroxylinked dimers. The peroxides formed are relatively unstable and react intramolecularly, cleaving the compound adjacent to the double bond into lower molecular weight radicals that then form acids, aldehydes, ketones, alcohols, olefins, and alkanes⁽¹⁰¹⁾. The highest value of peroxide at the end of storage period was observed for antioxidant-free biodiesel. It was observed that the increase in PV of control sample without antioxidants was from 2.4 meq/kg to 38.45 meq/kg during the 6 months of storage. The peroxide values at the end of storage time were significantly ($p < 0.05$) lower in biodiesel samples containing antioxidants than in control sample without antioxidants. Blending biodiesel samples with different concentrations of CSE or BHT resulted in a marked decline of their PV values during storage process, thus showing enhancement of the oxidative stability of neem biodiesel. Peroxide value of biodiesel samples containing 500ppm BHT, 500 ppm CSE, 1000 ppm BHT and 1000 ppm of CSE were about 2.59, 2.51, 4.10 and 4.28 times as low as in control

Fig. 7 Changes in peroxide value of biodiesel samples treated with different concentration (ppm) of BHT and CSE during storage at room temperature.

LSD at 0.05 = 0.13

Data are expressed as mean $\pm$ SD.

Values given represent means of three determinations.

sample without antioxidants. The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities²³⁾. The storage stability of biodiesel was first studied by DuPlessis *et al.*¹⁰²⁾, who monitored the production of acids, peroxides, and aldehydes, as well as increase in viscosity and decline in Rancimat induction time over a period of 90 days. They found that exposure to heat and air greatly accelerated degradation of biodiesel, but when stored at 20°C in closed containers or stored after the addition of an antioxidant, the biodiesel remained stable.

Conflict of Interests

None of the authors have any competing interests.

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