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Hevea brasiliensis (Rubber Seed) Oil: Extraction, Characterization, and Kinetics of Thermo-oxidative Degradation Using Classical Chemical Methods. / Onoji, S. E., Iyuke, S. E., & Igbafe, A. I.

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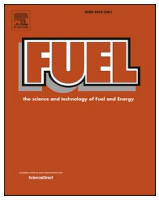

Full Length Article

Biodiesel production from *Hevea Brasiliensis* seed oil

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ABSTRACT

The *Hevea Brasiliensis* (HB) seed is a potential raw material for biodiesel production, having a high oil content. However, it is inappropriate for biodiesel production due to the free fatty acids (FFA) value, requiring an adjustment of this parameter. Thus, this work aimed to develop a treatment capable of reducing the acidity content and enabling the quality this biofuel. Firstly, the acid index was decreased by an esterification reaction using sulphuric acid and methanol. Under the best condition corresponding to 1.5% sulphuric acid and 1:2 oil: methanol (v:v), the acidity reduction achieved was a range of 99.55%, and after this, the basic transesterification method was employed to produce the *Hevea Brasiliensis* biodiesel. As a result, the condition mentioned before shows 98% of ester content and the main physicochemical characteristics required by the market as acidity index, specific gravity and corrosivity to copper.

1. Introduction

Due to constant concerns about the environment, the search for sustainable and alternatives energy sources has received great attention in the research development and commercialization field of new products. Biodiesel is a fuel derived from renewable raw materials. It has shown numerous environmental advantages over fossil fuels as a low greenhouse gas emission which are closely related to the global climate crisis [1–6]. In addition, studies show that pollutants derived from burning fossil fuels cause health problems [7,8,9].

In this way, some countries have invested in government policies that encourage biodiesel production. In 2020, the Brazilian government established that diesel fuel must contain 11% of biodiesel in its composition [10]. The vegetable lipid feedstock for biodiesel production depends on regional climate. In European countries and Canada, the principal source is rapessed oil, soybean oil in the United States, palm oil in Malaysia, and other tropical countries [11].

Although Brazil is a country with a continental dimensions the biodiesel production is concentrated in the south and centrer-west regions and has the commodities as a vegetable lipid raw material, mainly soybean [12]. Thus, one of the significant challenges of the brazilian biodiesel industry is the development of research in raw materials that do not compete with the food industry and present high profitability and an established production chain. In this context, the rubber tree seed appears as a potential raw material.

The rubber tree is originally from the Amazon region and had its cultivation expanded to all tropical climate areas due to the interest in latex production [13]. Since the 1990 s, Brazilian researchers have developed genetic selection work to obtain rubber tree clones with high production potential, resistant to diseases, with better quality products and adapted to adverse environmental conditions [14]. This genetic selection has an essential role because it's the most effective method to control the South American Leaf Blight (SALB) a Latin American endemic disease that affects plants' growth and reduces latex production a ratio between 20% and 75% [15]. Germination and viability tests of the seedlings generated from the rubber tree seed demonstrate that the seedlings have a low survival rate [16]. In 2011 the area cultivated with rubber trees was estimated in more than 9,7 million hectares worldwide, and the rubber seed yield was a ratio of 136–2000 kg/ha/year. This estimative was developed in Asiatic countries, and the production yield has a relationship with clone variety cultivated [17].

In the literature, there are few works on rubber seed biodiesel production as Zamberi, Ani, and Abdollah [18], that employed esterification followed by a transesterification process, obtaining the biodiesel ester content of 88.06%. Nevertheless, it is highlighted that still remains inadequate for marketing, because in Brazil, the National Agency for Petroleum, Natural Gas and Biofuels (ANP)[19] established the minimum acceptable ester value is 96.5%.

Silitonga et al. [20] used several steps to produce HB biodiesel with satisfactory physical–chemical characteristics, including degumming,

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esterification, neutralization, and transesterification. Nonetheless, this procedure requires considerable resources, considering the variables time and chemical reagents that increase production costs of this biofuel.

Also, different catalysts can be employed. Sebastian, Muralledharan, and Santhiagu [21] produce HB biodiesel through of transesterification by enzymatic catalysis. Ong et al. [22] performed it by heterogeneous catalysis, with CuO supported on carbon. However, both works use expensive materials and time-consuming processes.

When non-edible or residual oils are used, non-conventional methods are proposed for the production of biodiesel [23–29], such as, for example, Nadeem et al. [29] who used a heterogeneous catalyst derived from eggshells for transesterification of residual cooking, with a conversion rate of 75.2% using catalytic transesterification, while in the photocatalytic process it obtained 86.8%. Noreen et al. [28] produced biodiesel using heterogeneous base nanocatalysts with a conversion rate of 90%. These are some examples of new technologies used for the production of biodiesel derived from oils with a high acidity content, which are more expensive processes economically and in terms of reagents used, and even so, not reaching values above 96.5% of esters, such as is suggested by Brazilian quality standards.

Given the literature evidence found, this work aims at the Haveas Brasiliensis (HB) oil use to biodiesel production from an easy, effective, and economically viable process that use smaller amounts of reagents, shorter reaction times and low temperatures, in addition to being a process compatible with the usual processes in the industry. Besides to the aforementioned, the protocol described presents a high potential for scalability and commercialization.

2. Material and methods

2.1. HB oil extraction

The seeds were donated by KaiserAgro company and were washed, dried, peeled. The pult endosperm was crushed in a multiprocessor brand Philips Walita model RI7776 and then subjected to oil extraction processes, where the soxhlet equipment from Marconi brand model MA487/8/2050 was used, which performed the extraction with reflux using hexane as solvent. The solvent was refluxed for 2 h from the first cycle. Then, to perform the separation of the hexane solvent from the oil, a rotary evaporator brand IKA model RV 10D was used in vacuum, in a water bath branded IKA model HB 10D, with a temperature of 40 °C and rotation of 100 rpm, until only the oil remains. This study was registered in Sisgen (National System for the Management of Genetic Heritage), number A4C9E3E.

2.2. Physico-chemical characterization of H.B.oil

The following parameters were analyzed for the characterization of HB oil, considering acidity index, free fatty acids, and saponification index. All analyses were done following the standard established by Adolf Lutz Institute [30].

To determine moisture and seed volatiles, the method described in the Rules for Seed Analysis established by the Ministry of Agriculture, Livestock, and Supply (MAPA) was used [31]. The moisture content in vegetable oils was determined as described in IT POV 314 from MAPA [32].

Density verification was performed in a digital densimeter DMA 35 Basic, Anton Paar brand at 20 °C [33]. For the oxidation stability test, standard EN 14,112 was used, with the device Rancimat 873, Metrohn brand.

2.3. Crude HB oil pretreatment

2.3.1. Treatment of HB oil with sulphuric acid

A homogeneous treatment was carried out via an esterification process using concentrated sulphuric acid (H_2SO_4) catalyst. The acid was employed in a ratio of 0,5–1,5% in relation to the HB oil. The methanol ratio used was 1:6 (HB Oil:methanol), and the reaction mixture was kept under constant stirring maintenance at 45 °C for 45 min [34]. To determine the ideal ratio between HB oil:methanol, tests were carried out in the ratios 1:6, 1:3, 1:2 and 1:1 (v:v).

2.4. Biodiesel production

The post-treatment oil was added to methanol in molar ratio of 1:3, 1:6, and 1:9 (HB oil:methanol), using alkaline catalysis in the transesterification process, under adjustment [35].

The biodiesel samples were subjected to three washing steps: (1) with HCl or NaOH solution 0,1%; (2) saturated brine solution; (3) distilled water. Next, the samples were dried for 2 h at 100 °C in a Solab model SL-100 oven [36,37].

2.4.1. Biodiesel Physico-chemical analysis

The physicochemical analysis performed were aspect, oxidation stability at 110 °C, acidity index, specific mass at 20 °C, kinematic viscosity at 40 °C, corrosivity to copper, 3 h at 50 °C, free and total glycerol, mono, di and triglycerides according PEA 05 Rev.14, EN 14112–16, ASTM D664-18e2 - Method B, ASTM D4052-18a, ASTM D445-19, ASTM D130-19, NBR15908-15 respectively.

The Nuclear Magnetic Resonance (NMR) analyzes were performed in a Bruker model DPX 400 equipment. The rubber tree oil and biodiesel were dissolved in deuterated chloroform ($CDCl_3$), placed in a specific tube for the equipment, at 25 °C and the analysis was performed at 400 MHz. To calculate the ester content, the method was used Ruschel et al. [38].

To determine the main composition of esters present in biodiesel, Chromatography coupled to a mass detector (GCMS) was used, with a VF-5MS column measuring 30 m × 0.35 m. The biodiesel sample (9.7 mg) was diluted in ethyl acetate (5 mL). Then, the sample was added to a flask and taken to the GCMS. Inlet temperature was 280 °C, helium gas flow was 1.02 mL/min using splitless mode. For the heating ramp, an initial temperature of 80 °C for 5 min was used, followed by heating at 6 °C/min until reaching 180 °C. Then, the column was heated at a rate of 10 °C/min until reaching a temperature of 285 °C, keeping at this temperature for 5 min. The ion source was maintained at 200 °C and the interface at 280 °C. The scan mode is 35 – 300. The percentage of fatty esters was determined by the peak area on the chromatogram.

3. Results and discussions

3.1. HB oil extraction

The yield of the chemical oil extraction process was 34% (v:m). It was observed that after the extraction, the seeds presented an oily aspect, indicating that the oil content could be higher than was obtained. According to the literature, the oil concentration in H.B. seed varies in the range from 38 to 46% (v:m) [39,40,41]. In 2018, Boonnuoun [41] using optimized conditions and liquefied dimethyl ether as a solvent, obtained content of 41,20% oil in HB seeds.

It is noteworthy that the results obtained for the oil content are higher than the oilseeds most used for biodiesel production. Soybean, for example, has an oil content in the range of 18–20% [42] and cotton, 29% [43]. These results demonstrate the potential of HB seeds as an oil source for biodiesel production.

3.2. HB oil characterization

The results of the physicochemical characterization of HB oil are shown in Table 1.

In the oil acidity determination test, the value obtained was 60.19 ± 0.27 mg of KOH/g of oil and 30.27 ± 0,14% FFA (Table 1). According to

Table 1
HB oil physicochemical parameters.

Parameter	Result
Acidity index (mg KOH/g oil)	60.19 ± 0.27
Free fatty acids (FFA) express in oleic acid (%)	30.27 ± 0.14
Saponification Index (mg KOH/g oil)	177.74 ± 2.00
Density (kg/m^3)	911.00 ± 1.00
Moisture content (%)	7.46 ± 0.13
Oxidative stability (h)	1.31 ± 0.21

Da Silva and Neto [44], oils with an acid index greater than 3 mg of KOH/g of oil are not suitable for transesterification. For the FFA content, the oil must present a value lower than 0.5% to be transesterified [45]. Thus HB oil needs a treatment to minimize the acidity.

Specifically for HB oil, Silitonga et al. [20] described an acid index of 84 mg KOH/g of oil, and Ramadhas, Jayaraj, Muraleedharan [46] determined the value of 34 of acidity content for the same seed. These results suggest that environmental conditions (soil, climate, planting, and harvesting) can interfere with the HB oil acidity. However, the values presented in the surveys are higher than those recommended for biodiesel production.

The HB seed oil saponification index value was 177.74 ± 2.00 mg KOH/g of oil. This value is lower than refined oils used for biodiesel production, such as cotton, sunflower, and soybean [47]. In addition, the value is close to that found by Zamberi, Ani, and Abdollah [18], which corresponded to 180.9 mg KOH/g of HB oil.

The HB oil density is 910 kg/m^3, close to soybean oil density which can range from 914 − 922 kg/m^3 [48,49]. This value is consistent with that found by Dhawane, Kumar, Halder [50] dan Silitonga et al. [20]. The authors report that HB oil density was 920 kg/m^3 and 925.8 kg/m^3, respectively.

The crude HB oil presents oxidative stability of 1.31 ± 0.21 h. The low oxidative stability of HB oil is probably associated with its chemical composition. Onoji *et al.* [51] report that HB oil has a high content of unsaturated fatty acids such as oleic acid (12.7–42.8%), linoleic acid (39.6–52.85%), and acid alpha-linoleic (2.38–26%). According to the literature, unsaturated fatty acids are more likely to undergo oxidation [45].

3.3. Crude HB oil pretreatment

3.3.1. Treatment of HB oil with sulphuric acid

The results regarding the treatment of HB oil with sulfuric acid are listed in Table 2.

According to Table 2, all tests showed a reduction in the acidity index, initially 62.57 mg of KOH/g of oil and 31.5% to FFA. After the treatment, the met values are according to literature specification (less than 3 mg KOH/g of oil and 0.5% od FFA), indicating that it is a viable method to minimize the acidity. An esterification reaction occurs by acid catalysis, and the FAA reacts with alcohols to form esters, reducing the acidity [44,45].

Using the same process, Ahmad et al. [35], reduced the FFA content in HB oil from 42% to 0.82%. In your protocol, the authors used methanol: HB oil ratio (15:1), sulphuric acid (10%), the reaction time of 90 min, and temperature of 45 °C.

Vieira et al. [45] employed acid esterification to reduce the acidity content of several oils, including passion fruit pulp. The initial acidity index was 67.5 mg of KOH/g of oil, and after treatment, the acidity was reduced to 0,62 mg of KOH/g of oil. In this procedure, the reaction was carried out for 3 h, and the oil-methanol ratio was 1:6, 70 °C with 2% (m/m) sulphuric acid/oil.

Table 2
Results of acidity index after treatment with sulfuric acid.

Sample	Amount of sulfuric acid PA (%) (v:v)	Acidity index in mg of KOH/g of oil	Free fatty acids in % of oleic acid	Acidity reduction (%)
1	0.5	0,69 ± 0.10	0.35 ± 0.05	98.90
2	1	0,63 ± 0.08	0.32 ± 0.04	98.99
3	1.5	0.29 ± 0.08	0.14 ± 0.04	99.55

It is noteworthy that the treatment proposed in this article aims at lower amounts of reagent, shorter reaction time, and consequently less energy use, unlike the processes above, but with similar results.

Regardless of the proportions of sulfuric acid used, oils with adequate characteristics for alkaline transesterification were obtained. At 1.5%, there was the greatest reduction in absolute terms of acidity content. Thus, this percentage was used for HB oil pretreatment employed in the biodiesel synthesis described in this work. It is noteworthy that concerning the cost-benefit, the assay with 0.5% could be used.

3.3.2. Methanol influence on the HB oil pretreatment

Investigating the influence of methanol, it was observed that the ratio of 1:2 HB oil to methanol (v:v) was the most efficient (Table 3, sample 2). Under this condition, it was possible to recover the HB oil with a yield of 90.75 + 0.98% and acidity content of 0.296 + 0.04 mg KOH/g oil. In the other proportions, it was possible to recover the HB oil in lower yields. Thus, the condition of 1:2 (HB oil:methanol) was used in the other steps of this study.

3.4. Ester content o HB biodiesels

After the HB oil transesterification process, the biodiesel obtained was submitted to NMR analysis.This experiment was used to prove the conversion of triglyceride into fatty methyl esters and quantify the esters content, Fig. 1. It is observed in Fig. 1, the disappearance of the signals from glycerin moiety (4.32 ppm and 4.14 ppm) and the appearance of a singlete, in the chemical shift of 3.57 ppm and relative integral to 3 hydrogens, characteristic of the methyl ester.

From the confirmation of the HB oil transesterification, biodiesel conversion was calculated through the ^{1}H NMR spectrum, considering the hydrogens integration from methoxyl group (–OCH3), in the chemical shift (δ) of 3.57 ppm, and the methylene hydrogens alpha carbonyl, $\delta = 2.29$ ppm. The conversion to methyl esters is given by the ratio between the two integrations, corrected by the number of hydrogens in each signal, given by equation (1) [38].

$$C_{EM} = \left(\frac{\frac{Int_{OMe}}{3}}{\frac{Int_{CH2}}{2}} \right) \times 100 \quad (1)$$

C_{EM} = Conversion to methyl ester (%);
Int_{OMe} = Hydrogens from methoxy group integration ($\delta = 3.57$ ppm);
Int_{CH2} = Methylene hydrogens alpha carbonyl integration ($\delta = 2.29$ ppm).

The methanol influence promoting HB oil conversion into biodiesel was analyzed in three reaction conditions, as shown in Table 4.

Table 3
Methanol influence on acid content reduction.

Sample[a]	Methanol variation (v:v)	Acidity index (mg KOH/g oil)	Free fatty acids (FFA) in % oleic acid	Yield (%)
1	1:1	1.58 ± 0.20	0.79 ± 0.02	59.55 ± 0.85
2	1:2	0.34 ± 0.05	0.17 ± 0.03	90.75 ± 0.98
3	1:3	0.29 ± 0.03	0.14 ± 0.02	76.90 ± 0.80
4	1:6	0.29 ± 0.08	0.14 ± 0.04	44.57 ± 0.86

[a] all experiments were carried out in triplicates.

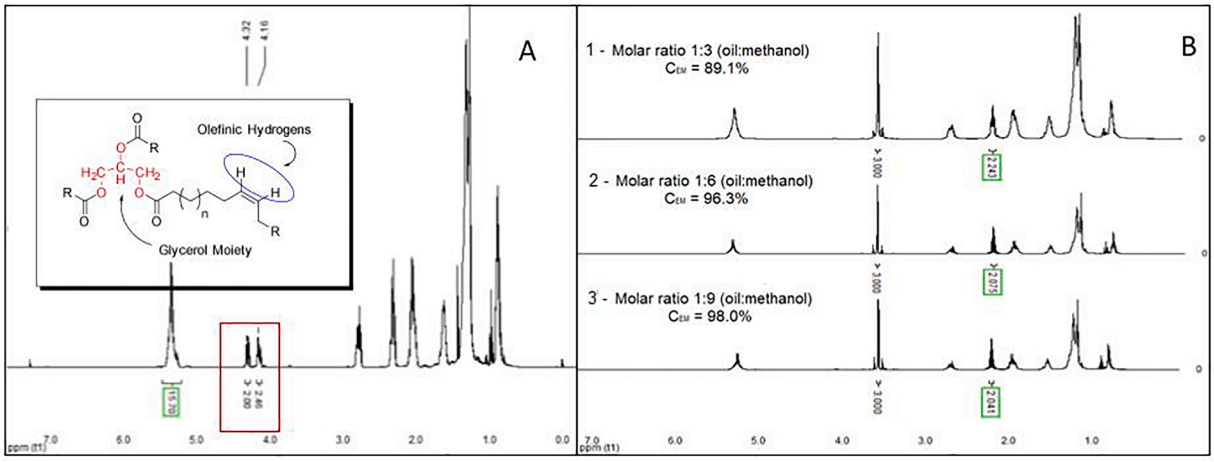

Fig. 1. ^{1}H NMR spectra of HB oil (A) and HB biodiesel (B) at 400 MHz in $CDCl_3$.

Table 4
Methanol influence study on the conversion of H.B. oil to methyl ester.

Sample	Molar ratio (H.B. oil:methanol)	C_{EM}(%)[a]	Yield(%)[b]
1	1:3	89,1	63,3
2	1:6	96,3	77,8
3	1:9	98,0	81,5

[a] Ester content was obtained from equation (1).
[b] Yield obtained after the biodiesel washing and drying step.

The resolution N° 798/2019 [19] established the minimum value for ester content is 96.5% to be considered biodiesel. Among the samples studied, the two lowest molar ratios used presented unsatisfactory values. For the highest molar ratio of methanol, 98.0% of ester content was obtained, under the provisions of the standard.

These results align with those found in the literature since the transesterification reaction can be directed towards greater biodiesel formation by adding reagents such as methanol [52].

3.5. HB biodiesel physicochemical characterization

Table 5 shows the results obtained in the physicochemical characterization for HB biodiesel produced from treated oil under 1.5% of H_2SO_4, avolumetric ratio of 1:2 (HB oil:methanol) and alkaline transesterification with molar ratio 1:9 (HB oil:methanol).

The appearance classification was clear and free of impurities following the limits established by the ANP. Also, the visual color was light yellow.

According to Table 5, HB biodiesel presents for the oxidation stability test a value of 5.72 + 0.43 h. This value is plausible due to the presence of unsaturated chains [51]. The induction time (IT) was lower than recommended by ANP, usually, biodiesel derived from different raw materials, in the absence of antioxidants, have lower values for IT, for example, Gasparin et. al. [53] presented an IT of 5.33 h. In this sense, the ANP, through resolution ANP n° 798/2019 [19], establishes the mandatory addition of antioxidants in biodiesel regardless of the raw material used to maximize stability to oxidation.

The relatively low TI can be explained by the ester composition of HB biodiesel, which according to the chromatographic tests demonstrate a high degree of unsaturation of HB seed oil biodiesel. The esters identified through GCMS assays were methyl linoleate (C18:2), methyl linolenate (C18:3), methyl palmitate (C16:0), methyl stearate (C18:0) and methyl palmitoleate (16:1). the first two in greater quantity, 35.34 and 22.46%, respectively. These results are similar to those reported by Onoji *et al.* [51].

The increase in the biofuel IT with the addition of synthetic or natural antioxidants is the target of numerous researches. Ni et al. [54] applied several synthetic antioxidants to HB seed biodiesel, which initially had a value of 0.8 h of IT and which after the addition of 2000 mg/kg of TBHQ resulted in 13.09 h of IT. The study Narayanasamy, Jeyakumar, and Manoharan [55] focused on adding natural antioxidants to HB seed oil biodiesel, thus increasing the induction time from 2.23 h to 26.5 h with the addition of 2000 ppm of ginger extracts.

According to table 3, HB biodiesel complies with the value established by the ANP for acidity index, below 0.5 mg of KOH/g of biodiesel. The value of 0.28 mg KOH/g of biodiesel converges with those obtained by De Almeida, Duarte, and Neto [56] with 0.36 mg KOH/g for soy biodiesel and Batista et al. [57] of 0.25 mg KOH/g for macaúba

Table 5
Results of HB biodiesel physicochemical characterization.

Analyses	Result	Limit[a]	Unit	Uncertainties	Methodology
Aspect	Clear and impurity-free	Clear and impurity-free	–	n/a	Visual
Oxidation stability at 110° C	5.72	12	h	0.43	EN 14112–16
Acidity index	0.28	0.50 max.	mg_{KOH}/g	0.09	ASTM D664 –18e2 –
Specific gravity at 20° C	879.9	850 a 900	kg/m^3	0.2	ASTM D4052-18a
Flash point	less than60.0	100.0 min	°C	9.3	ASTM D93 – 18 Procedure C
Kinematic viscosity at 40° C	6.7027	3.0–6.0	mm^2/s	0.021	ASTM D445 – 19
Corrosivity to copper, 3 h at 50° C	1b	1 max.	–	n/a	ASTM D130 – 19
Free glycerol	0.01	0.02 max.	% mass	0.002	NBR15908 – 15
Total glycerol	0.06	0.25 max.	% mass	0.016	NBR15908 – 15
Monoacylglycerol	0.19	0.7 max.	% mass	0.058	NBR15908 – 15
Diacylglycerol	less than0.05.	0.20 max.	% mass	0.038	NBR15908 – 15
Triacylglycerol	less than005	0.20 max.	% mass	0.016	NBR15908 – 15

[a] Reference values specified in ANP Resolution N° 798/2019.

biodiesel. Silitonga et al. [20] describe the value of 0.48 mg KOH/g of HB seed biodiesel, which is higher than observed in the present study.

The value stipulated by ANP for the specific mass of biodiesel is from 850 to 900 kg/m3, so HB biodiesel complies with the specifications, see Table 5. In addition, it was close to those found for soybean biodiesel (870 kg/m^3) [56], cotton (881.6 kg/m^3), sunflower (883.0 kg/m^3) [58] and are similar to was found by Zamberi, Ani and Abdollah [18] which obtained a value equal to 877 kg/m^3 for HB biodiesel.

The flashpoint was less than 60.0 °C, and this value is far from that predicted by the ANP standards. The values found in the literature for HB oil biodiesel were 152 °C [35] and 157 °C [43]. This discrepancy is probably associated with the non-elimination of methanol in washing processes.

After 3 h at 50 °C, the corrosivity to copper presented a value of 1b, being within the standard established by the ANP. This value is identical to that found by Silitonga et al. [20], indicating HB biodiesel is not aggressive to that metal.

The free glycerol of HB biodiesel was 0.01% and the total glycerol 0.06%, both in compliance with ANP specifications. These results confirm the effectiveness of product separation. The results are similar to other studies with the same raw material, such as Ahmad et al. [35], which obtained a free glycerol content of 0.02% and a total glycerol content of 0.35%.

The amount of monoacylglycerol corresponded to 0.19%, also the proportion of diacylglycerol and triacylglycerol was less than 0.05% for both. All those values are following the specifications of the ANP and are lower than the measured by Panichikkal et al. [59] for HB biodiesel to monoacylglycerol (0.30%) and triacylglycerol (0.18%).

The steps and methods used to produce HB biodiesel resulted in a high-quality biofuel. The methodology maximizes the incorporation of reagents into the final product, employing a low temperature, a short reaction time, promoting greater energy efficiency. Most of the physicochemical characteristics of HB biodiesel comply with the limits established by the ANP. The oxidation stability can be easily corrected adding an antioxidant, as foreseen by ANP regulations, and the flashpoint that can be raised through a more efficient washing process. In addition, given the effectiveness of this biodiesel production process, intellectual protection was requested via patent filing (BR1020200246674).

4. Conclusion

This study proposes HB seed, currently considered a forest residue, as a raw material for biodiesel production. It can contribute to the diversification of the Brazilian energy matrix and minimize the dependence of this industry on soybean oil. Although HB oil having a high acidity content, the described methodology showed a reduction of acidity by 99.55%, using the reaction conditions 1.5% sulfuric acid, 1:2 in a volumetric ratio between oil: methanol, the temperature at 45 °C and 45 min. With the treated oil, it was possible to produce biodiesel with 98% ester content showing satisfactory physical–chemical characteristics that meet other ANP specifications.

The future perspectives of this work are to test and make viable new methods to reduce the acidity of the oil, using reduced amounts of reagents and with high efficiency, aiming at a profitable production of biodiesel. Due to the national and international patent filing, the researchers intend to test the scalability of the process for later technology transfer to interested companies.

Currently, rubber tree seed is considered a forest residue and causes financial and environmental damage to the crop. The use of this seed for the production of biodiesel, a product with high added value, contributes to solving these problems in addition to diversifying the national energy matrix.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Decelerated skin aging effect of rubber (Hevea brasiliensis) seed oil in cell culture assays. / Chaikul, P., Lourith, N., & Kanlayavattanakul, M.

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Decelerated skin aging effect of rubber (*Hevea brasiliensis*) seed oil in cell culture assays

Puxvadee Chaikul[1,2]✉, Nattaya Lourith[1,2] & Mayuree Kanlayavattanakul[1,2]

Rubber seeds, the abundant by-products of rubber tree (*Hevea brasiliensis*), have been studied for sustainable utilization. Nevertheless, there is no information available regarding activity against skin aging. The study aimed to prepare rubber seed oil (RSO) and evaluate fatty acid compositions by gas chromatography - mass spectrometry (GC/MS), linamarin contamination by ultra-high performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS). Additionally, cytotoxicity assay and anti-skin aging activities, including cell proliferating stimulation, cellular antioxidant, collagen stimulation, and matrix metalloproteinase-2 (MMP-2) inhibition, were analyzed in immortalized human skin keratinocytes (HaCaT cells) and human dermal fibroblasts. RSO was pale-yellow oily liquid with an extraction yield of 35.79 ± 0.52%. Principal fatty acids were comprised of oleic (43.37 ± 0.76%), linoleic (38.49 ± 0.81%), palmitic (11.47 ± 0.12%), and stearic (6.66 ± 0.05%) acids. Linamarin contamination was not detected in 100 µg/mL RSO, demonstrating the absence of a cyanogenic glucoside. Non-cytotoxic concentrations of RSO in both cells were in the range of 0.0001–0.1 mg/mL. Activities of RSO against skin aging included the cell proliferating stimulation, the antioxidant activity, the collagen stimulation, and the MMP-2 suppression at mRNA expression level and enzymatic activity. Study results have suggested that rubber seeds can probably be employed as a promising ingredient in the preparations designed for deceleration of skin aging.

Keywords Skin aging, Antioxidant activity, Collagen stimulation, Matrix metalloproteinase-2 suppression, Rubber seeds

Abbreviations

RSO	Rubber seed oil
MMP-2	Matrix metalloproteinase-2
SRB	Sulforhodamine B
AAPH	2,2'-azobis (2-amidinopropane) dihydrochloride
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
GC/MS	Gas chromatography-mass spectrometry
UHPLC-MS/MS	Ultra-high performance liquid chromatography-tandem mass spectrometry
HaCaT cells	Immortalized human skin keratinocytes
HDF	Human dermal fibroblasts
PBS	Phosphate buffered saline
CAA	Cellular antioxidant activity

Rubber tree (*Hevea brasiliensis*), a native plant in rainforests of the Amazon, is globally cultivated more than 10 million hectares in tropical and subtropical countries, mostly in Thailand, Malaysia, Indonesia, Vietnam, China, and India. The primary purpose of tree plantation is to gather the released milky latex from mature tree cutting for commercial use[1,2]. Additionally, rubber seeds, the tree by-products, are reported to yield an approximation of 2 tons per hectare per year[3]. The abundance of seeds is conventionally used for tree propagation and is extensively studied for sustainable and effective utilization. Numerous reports of seed applications include biolubricant base stock preparation[4], environmental friendly coating[5], biodiesel production[3], animal feed[6], soap and cosmetic ingredients[7,8]. Moreover, the preparation of rubber seed extracts and the evaluation of prepared extracts, including the main components and the biological activities, have been exhibited[9–13]. Nevertheless, there is no information available regarding activity of rubber seeds against skin aging.

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Skin is the largest organ of the body that serves several functions beyond physical barrier. The vital functions of skin include the body's defense against environmental insults, temperature regulation, skin hydration, and cell renewal process[14]. Aging of skin is the progressive loss of skin regeneration capacity and functionality and is caused by a complex interplay of intrinsic and extrinsic variables. Both intrinsic factors, such as genetic factors, oxidative process, and hormones, and extrinsic factors, which are cigarette smoking, air pollution, and solar radiation, gradually affect skin changes, including skin dehydration, wrinkling, sagging, and pigmented spots[15,16]. Since the youthfulness is the desire characteristics for everyone in society, the anti-aging techniques and substances have been thoroughly studied in an effort to slow down the aging process[17]. Among these substances, natural ingredients have popularly been the most interesting issues due to the consumers' demand for safe substances as well as their multifunctional properties for application[18]. The market analysis on the global value of anti-aging is expected to surpass USD 284.8 Billion by 2028[19].

To examine the activity of rubber seeds against skin aging, this study prepared the rubber seed oil (RSO) and evaluated the fatty acid compositions by gas chromatography - mass spectrometry (GC/MS) and the contamination of linamarin, which is a reported cyanogenic glucoside in rubber seeds, by ultra-high performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS). In addition, the cytotoxicity assay and the anti-skin aging activities, including cell proliferating stimulation, cellular antioxidant, collagen stimulation, and matrix metalloproteinase-2 (MMP-2) inhibition, were tested in immortalized human skin keratinocytes (HaCaT cells) and human dermal fibroblasts (HDF) in comparison to the fatty acids found in oil and vitamin C. The study results would indicate the potential utilization of rubber seeds in the preparations designed for deceleration of skin aging.

Materials and methods

Materials

The analytical grade of dichloromethane, ethanol, hexane, hydrochloric acid, methanol, toluene, magnesium sulfate, and sodium hydroxide were purchased from RCI Labscan Limited (Bangkok, Thailand). Linamarin, sulforhodamine B (SRB), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), hydrogen peroxide, vitamin C, palmitic acid, stearic acid, oleic acid, and linoleic acid were from Sigma Aldrich Co. (Missouri, USA). Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin solution, and fetal bovine serum were obtained from Gibco (Maryland, USA). The other reagents were of analytical grade.

Preparation of RSO

Rubber seeds of trees clone RRIM 600 cultivated at Chiang Rai province, Thailand, were obtained during harvesting time in August - September 2021. The seeds were cleansed by tap water, dried, and subsequently extracted as previous study[9]. Briefly, rubber seeds were ground and extracted by a Soxhlet extractor using hexane for 6 h. The obtained seed extract was concentrated by using a rotary evaporator until the weight remained constant. Three extractions were performed and calculated the extraction yield.

Analysis of fatty acids

Fatty acid compositions in RSO was evaluated by GC/MS[9]. RSO was prepared by previous report[20]. Briefly, the mixture of RSO, toluene, methanol, and hydrochloric acid was kept at 45 °C for 24 h. The mixture was then partitioned with hexane, dried with anhydrous magnesium sulfate, and concentrated to dryness in vacuo. A sample diluted in dichloromethane was injected (220 °C) in the splitless mode into a gas chromatograph (6890 N, Agilent, USA) equipped with a HP-5MS capillary column (Agilent, 0.25 µm, 30 m 0.25 mm) and 5973 N mass spectrophotometer (Agilent). The flow rate of helium, a carrier gas, was set at a rate of 1 mL/min. The program was operated as follows: 50 °C held for 5 min, rising to 65 °C at 2 °C/min, to 200 °C at 5 °C/min held for 5 min, and then to 250 °C at 10 °C/min held for 10 min. The retention time as well as the mass spectra of oil composition were acquired by the Wiley 7n.1 database. Triplicate analyses of fatty acid compositions in RSO were performed.

Analysis of linamarin

The contamination of linamarin in RSO was investigated as previously described[21] with some modifications. Standard linamarin at concentrations of 0.01–5 µg/mL and RSO (100 µg/mL) were dissolved in methanol and filtered through 0.2 µm membrane. The assay was performed by UHPLC-MS/MS system (Nexera X2 and LCMS-8060, Shimadzu, Japan) using Shim-pack GIST C_{18} column (3 µm, 2.1 mm × 150 mm) at a rate of 0.2 mL/min. The injection volume was 10 µL. The mobile phase consisted of A, 0.1% formic acid in water, and B, acetonitrile. The linear gradient condition was operated as follows: 0–1 min isocratic of 5%B; 1–3 min linear gradient 5–20%B; 3–7 min linear gradient 20–40%B; 7–8.5 min isocratic 40%B. The column was re-equilibrated for 5 min with 5%B. Mass spectrometry, which included electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode under both positive and negative conditions, was used for detection. The standard curve of linamarin was plotted and the contamination of linamarin in RSO was evaluated.

Cytotoxicity assay

Cell culture

Immortalized human skin keratinocytes (HaCaT cells) and human dermal fibroblasts (HDF) were purchased from Elabscience (USA) and PromoCell (Germany), respectively. HDF cell line at 10th – 15th passage was used in cell culture assays. Both HaCaT cells and HDF were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% antibiotic (penicillin/streptomycin solution) and 10% fetal bovine serum in a

humidified incubator at 37 °C with 5% CO_2 (CB210, Binder, Germany). The cells were harvested, counted, and seeded in culture plates. Analysis in cultured cells was performed in triplicate.

Sample preparation
RSO was dissolved in dimethyl sulfoxide (DMSO), whereas fatty acids in RSO, including palmitic, stearic, oleic and linoleic acids, were dissolved in absolute ethanol. Vitamin C was dissolved in culture medium. All samples were sterilized by filtration.

Cytotoxicity assay
Sulforhodamine B (SRB) was performed to evaluate the cytotoxicity of samples[10]. Cells, either HDF or HaCaT cells (1×10^4 cells/well), were seeded in 96-well plates and incubated for 24 h. Several concentrations (0.0001-1 mg/mL) of samples, including RSO, fatty acids in RSO, and vitamin C, and solvent were added. After 72 h, cells were fixed by 50% trichloroacetic acid solution and washed. Thereafter, cells were stained by SRB dye and washed unbound dye away. The bound dye was dissolved in tris buffer and the absorbance was measured at 540 nm using a microplate reader (SPECTROstar Nano, BMG Labtech, Germany). The cell viability of each sample was calculated in comparison to solvent.

Analysis of anti-skin aging activities

Cell proliferating stimulation in HaCaT cells
Cell proliferating stimulation activity was analyzed in HaCaT cells as previous report[12] with some modifications. Cells (1×10^4 cells/well) were seeded in 96-well plates and incubated overnight. The non-cytotoxic concentrations of samples were added and incubated. The medium and samples were refreshed after 72 h and further incubated to 120 h. The cell fixation and staining with SRB dye were then performed. The bound dye was dissolved to measure the absorbance at 540 nm. The calculation of cell proliferating stimulation was performed in comparison to control.

Cellular antioxidant in HaCaT cells and HDF
Antioxidant activity in HaCaT cells was performed by cellular antioxidant activity (CAA) assay[22] with some modifications. HaCaT cells (2×10^4 cells/well) were seeded in black 96-well plates and incubated overnight. Cells were then rinsed with phosphate buffered saline (PBS). DCFH-DA dissolved in PBS containing glucose was added and incubated. After 1 h, the fresh medium and samples at non-cytotoxic concentrations were added to replace the fluorescent dye and further incubated for 1 h. Cells were then rinsed with PBS and AAPH was applied to the cells. The culture plate was placed in a fluorescence plate reader (FLUOstar Omega, BMG Labtech, Germany) at 37 °C. The fluorescence intensity was measured at 520 nm (emission) and 485 nm (excitation) every 5 min for 1 h. Each plate consisted of cells treated with oxidant (control), culture medium (blank), and samples at non-cytotoxic concentrations. The cellular antioxidant activity of each sample was calculated in comparison to control and blank.

Antioxidant activity in HDF was analyzed by addition of hydrogen peroxide[15]. HDF ($1x10^4$ cells/well) were seeded in 96-well plates and incubated overnight for cell adhesion. The non-cytotoxic concentrations of samples and solvent were added and incubated for 24 h. Samples were replaced with fresh medium containing 150 μM hydrogen peroxide and the mixture was incubated for an additional 4 h. The cell fixation was then performed, washed, and dyed with SRB. The dissolved bound dye was measured at 540 nm using a microplate reader. The antioxidant activity was calculated in comparison to oxidative control (hydrogen peroxide treated cells).

Collagen stimulation in HDF
Collagen stimulation analysis was performed in HDF as previous study with some modifications[23]. Cells (3×10^5 cells/well) were plated in 12-well plates and incubated overnight. Samples at the greatest non-cytotoxic concentration and solvent were added and incubated for 72 h. The mixture of RIPA lysis buffer (Thermo Fisher Scientific, USA) and protease inhibitor cocktail (Roche, Germany) was added and centrifuged to collect cell pellets. Sirius red dye was added into cell pellets and then incubated for 30 min. The obtained red precipitate was centrifuged, collected, and dissolved in sodium hydroxide. The dissolved dye was measured at 550 nm. The collagen stimulation was calculated in comparison to control.

MMP-2 inhibition in HDF
The inhibition of MMP-2 enzyme was tested at mRNA expression level by reverse transcription-polymerase chain reaction (RT-PCR) and enzymatic activity by SDS-PAGE zymography in HDF[23,24]. Cells (5×10^5 cells/well) were plated in 6-well plates and incubated overnight. The medium was replaced with fresh serum-free medium. The addition of samples at the greatest non-cytotoxic concentration and solvent were performed and further incubated for 72 h. The medium was collected to analyze MMP-2 enzymatic activity by SDS-PAGE zymography using gelatin as substrate. The zymographic bands were examined by Gel Documentation Imaging System (Bio-Rad, UK) and the inhibition of MMP-2 enzymatic activity was calculated in comparison to control. For MMP-2 mRNA expression analysis, cells were then extracted to collect RNA by PureLinkRNA mini kit (Thermo Fisher Scientific, USA) as manufacturer's instruction. Total RNA was quantified by Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies, USA). MMP-2 and β-actin genes were reverse transcribed and amplified from the extracted RNA by SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Thermo Fisher Scientific, USA). The primers for MMP-2 and β-actin, which was used as an internal control, are shown in Table 1. The RT-PCR products were separated by 1.5% agarose gel electrophoresis in the 1 × tris-acetate-EDTA buffer at 100 V for 1 h. The agarose gel was visualized by Gel Documentation Imaging System. The suppressive activity on MMP-2 mRNA expression of sample was evaluated in comparison to control.

Genes	Primers		PCR products (bp)
	Forward	Reverse	
MMP-2	5′-ATT CTA CTG ATA TCG GGG CTT TGA-3′	5′-ATG TCC TTG GGG TAT CCG TGT AG-3′	409
β-Actin	5′-TCA TGC AGT GTG ACG TTG ACA TCC GT-3′	5′-CCT AGA AGC ATT TGC GGT GCA CGA TG -3′	310

Table 1. Primers for MMP-2 and β-actin mRNA expression analysis.

Fatty acid	Content (%)
Palmitic acid (C16:0)	11.47 ± 0.12
Stearic acid (C18:0)	6.66 ± 0.05
Oleic acid (C18:1)	43.37 ± 0.76
Linoleic acid (C18:2)	38.49 ± 0.81
Unsaturated fatty acids	81.87
Saturated fatty acids	18.13
Ratio of unsaturated to saturated fatty acids (UFA: SFA ratio)	4.52

Table 2. Fatty acid compositions in RSO analyzed by GC-MS.

Statistical analysis
Data were presented as the mean ± standard error of mean of three independent experiments. The one-way analysis of variance (ANOVA) and Tukey's post hoc test were performed for data analysis at the significant level of p-value < 0.05.

Results
Preparation of RSO
Rubber tree clone RRIM 600 is the widely recommended cultivar to plant in Thailand, due to its tolerance to abiotic stress, including drought, temperature, and salinity[25]. Rubber seeds of this cultivar were extracted and exhibited the pale-yellow oily liquid. The extraction yield was 35.79 ± 0.52%.

Analysis of fatty acids
Table 2 presents the fatty acid compositions in RSO. The unsaturated fatty acids, including oleic and linoleic acids, were the major fatty acids in RSO that accounted for 81.87% of total fatty acid content. The saturated fatty acids, which were palmitic and stearic acids, were also observed. The ratio of unsaturated to saturated fatty acids was 4.52, indicating the promising benefit for application in health care products and cosmetics.

Analysis of linamarin
Figure 1 demonstrates UHPLC-MS/MS chromatogram of a standard linamarin and linamarin contamination in RSO. The retention time of linamarin was at 3.14–3.18 min with parent ion at m/z 270.00 and daughter ion at m/z 243.00. The standard curve was shown a line equation ($y = 347363x + 24870$) with R^2 value of 0.9988. Linamarin contamination in 100 µg/mL RSO was lower than the detection limit (0.01 µg/mL or 10 ppb), indicating an undetectable level.

Cytotoxicity assay
Figure 2 presents the cytotoxic effect of RSO, fatty acids in RSO, and vitamin C in HaCaT cells and HDF. The changes in the viability of HaCaT cells agreed with those in HDF and exhibited in the concentration-dependent manner. The viability of HaCaT cells and HDF treated with RSO at 0.0001–0.1 mg/mL was greater than 80%, while the decrease in viability was shown when addition of 1 mg/mL RSO. When fatty acids in RSO, including palmitic, stearic, oleic, and linoleic acids, were treated at concentrations of 0.0001–0.01 mg/mL, both HaCaT cells and HDF demonstrated more than 80% vitality. However, the significantly reduced viability of cells was observed when concentration of all fatty acids was 0.1 mg/mL. The viability of both HaCaT cells and HDF treated with vitamin C at 0.0001–0.1 mg/mL was shown to be greater than 80%, whereas 1 mg/mL vitamin C treatment significantly decreased cell viability.

Analysis of anti-skin aging activities
The non-cytotoxic concentrations of RSO, fatty acids in RSO, including palmitic, stearic, oleic, and linoleic acids, and vitamin C were evaluated the activities against aging in HaCaT cells and HDF.

Cell proliferating stimulation in HaCaT cells
Figure 3 shows the cell proliferating stimulation of RSO, fatty acids in RSO, and vitamin C in HaCaT cells. The concentration-dependent response demonstrated the stimulation of cell proliferation when treated with RSO, fatty acids in RSO, and vitamin C. At 0.01 mg/mL, the proliferating stimulation of cells treated with RSO was 23.23 ± 4.36%, that exhibited the comparable results with palmitic acid (24.05 ± 3.06%), stearic

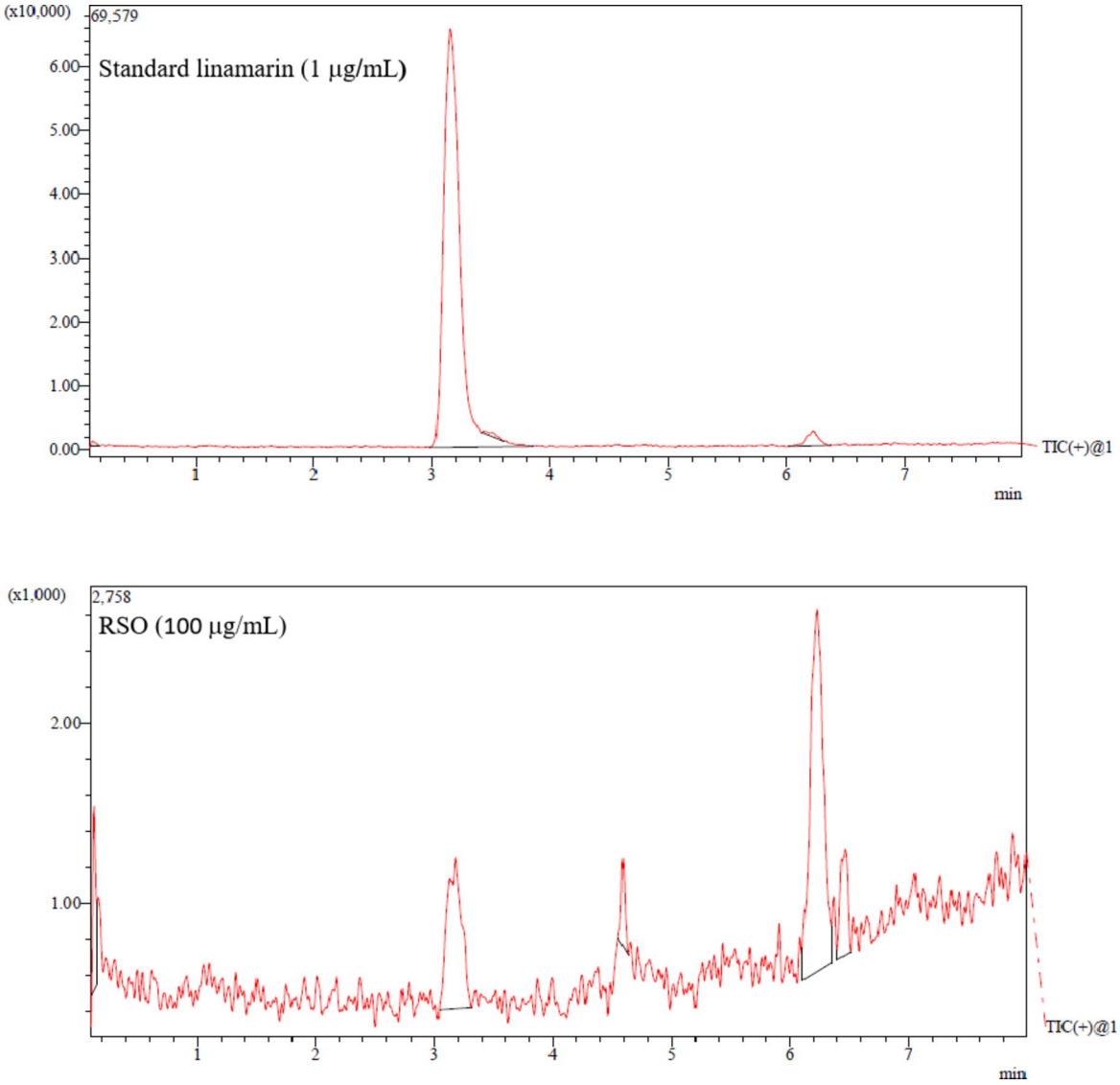

Fig. 1. UHPLC-MS/MS chromatogram of a standard linamarin (1 µg/mL) and linamarin contamination in RSO (100 µg/mL).

acid (20.25 ± 1.40%), oleic acid (23.16 ± 0.74%), and linoleic acid (33.62 ± 1.94%) treatment. An increase in proliferating stimulation effect (33.81 ± 1.76%) was observed when treated with 0.1 mg/mL RSO. The cell proliferating stimulation of 0.1 mg/mL vitamin C was 49.84 ± 0.46%.

Antioxidant activity in HaCaT cells and HDF

Figure 4 presents the antioxidant activity of RSO, fatty acids in RSO, and vitamin C in HaCaT cells and HDF. Although the different test methods were employed, the finding results of antioxidant activity in both cells exhibited the free radical scavenging ability. In HaCaT cells, the content of cellular free radicals was monitored following sample treatment and addition of AAPH, an oxidant. Treatment with RSO, oleic acid, linoleic acid, and vitamin C resulted in a decrease in cellular free radicals; however, treatment with palmitic acid and stearic acid did not significantly affect the formation of free radicals. In HDF, the cellular oxidative damage was formed after addition of hydrogen peroxide, which led to a significantly decrease in cell viability (76.08 ± 1.34%). Treatment with substances possessing antioxidant activity exhibited greater cell viability than hydrogen peroxide treatment. RSO, oleic acid, linoleic acid, and vitamin C demonstrated the free radical scavenging effect that subsequently protected oxidative damage-induced cell death and led to the greater cell viability than hydrogen peroxide treatment. Palmitic acid and stearic acid did not hold antioxidant activity; therefore, viability of cells was comparable to the hydrogen peroxide treatment.

Collagen stimulation in HDF

Figure 5 demonstrates the collagen stimulation of RSO, fatty acids in RSO, and vitamin C. A significant increase in collagen was observed in cells treated with RSO, fatty acids in RSO, and vitamin C. At 0.01 mg/

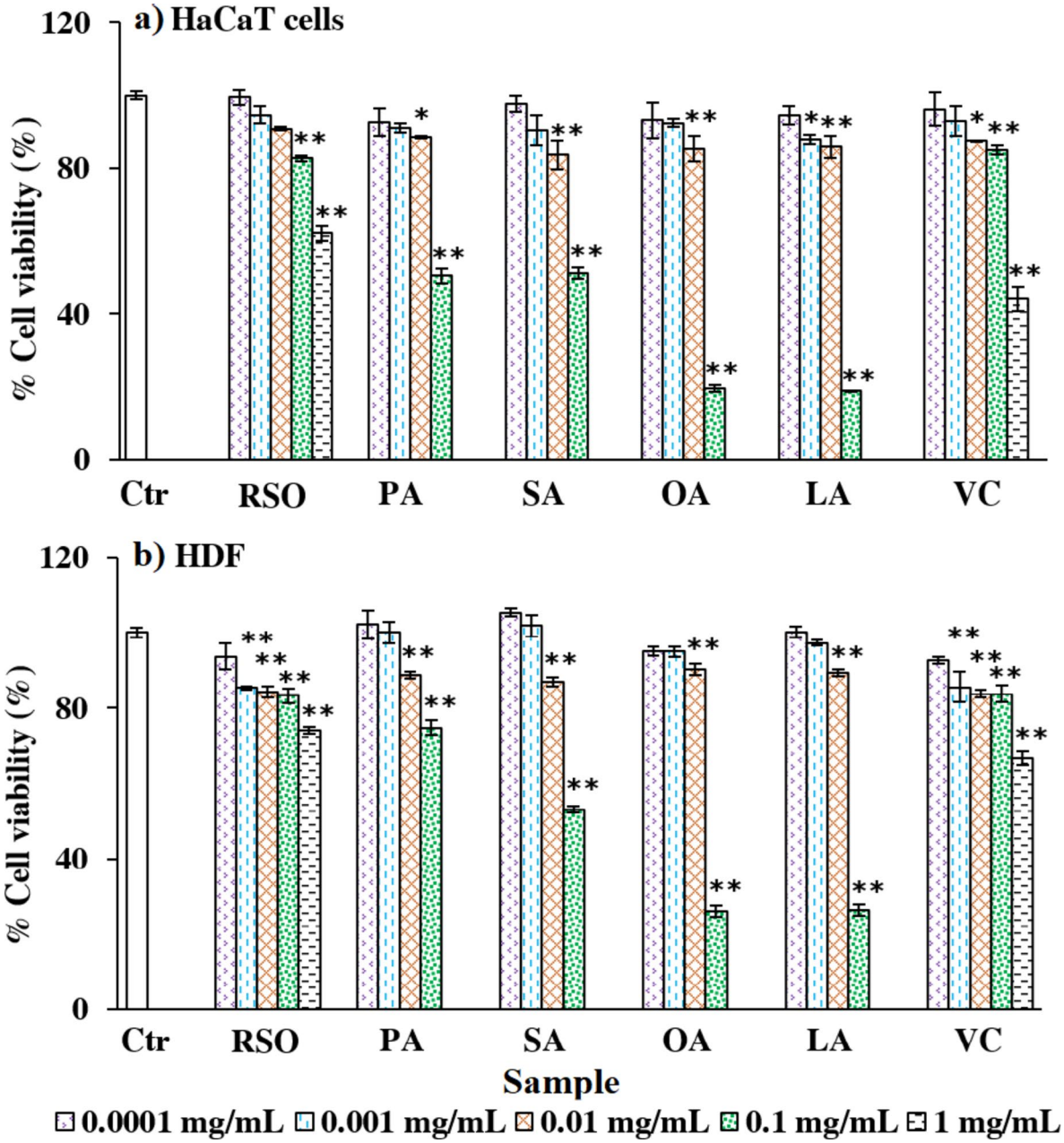

Fig. 2. Cytotoxicity assay of RSO, fatty acids in RSO, including palmitic (PA), stearic (SA), oleic (OA), and linoleic (LA) acids, and vitamin C (VC) at concentrations of 0.0001–1 mg/mL in HaCaT cells and HDF. * and ** indicate the significant difference compared to control (Ctr) at $p < 0.05$ and 0.01, respectively.

mL, the collagen stimulation of RSO (12.92 ± 1.09%) was comparable to that of palmitic acid (13.47 ± 2.27%), stearic acid (12.70 ± 0.78%), and oleic acid (17.45 ± 2.25%), but was significantly less than that of linoleic acid (26.21 ± 2.16%, $p = 0.002$). Cells treated with 0.1 mg/mL RSO exhibited collagen stimulation of 22.72 ± 3.67%, that was comparable to 0.1 mg/mL vitamin C (36.07 ± 3.89%, $p = 0.067$).

MMP-2 inhibition in HDF

The inhibition of MMP-2 mRNA expression analyzed by RT-PCR and MMP-2 enzymatic activity performed by SDS-PAGE zymography are presented in Figs. 6 and 7, respectively. The full length of agarose gels (Fig. S1 and S2) and zymogram (Fig. S3) are available in Supplementary information. The results of MMP-2 mRNA expression were corresponded to those of enzymatic activity. The significant MMP-2 suppression on mRNA expression level and enzymatic activity was shown in RSO, oleic acid, linoleic acid, and vitamin C treatment, whereas that of palmitic acid and stearic acid treatment was not observed. Among the effect on MMP-2 mRNA expression and enzymatic activity, linoleic acid demonstrated the highest suppression of MMP-2 mRNA expression (47.97 ± 1.95%) with the undetectable band of enzymatic activity. RSO at 0.1 mg/mL exhibited the suppressive

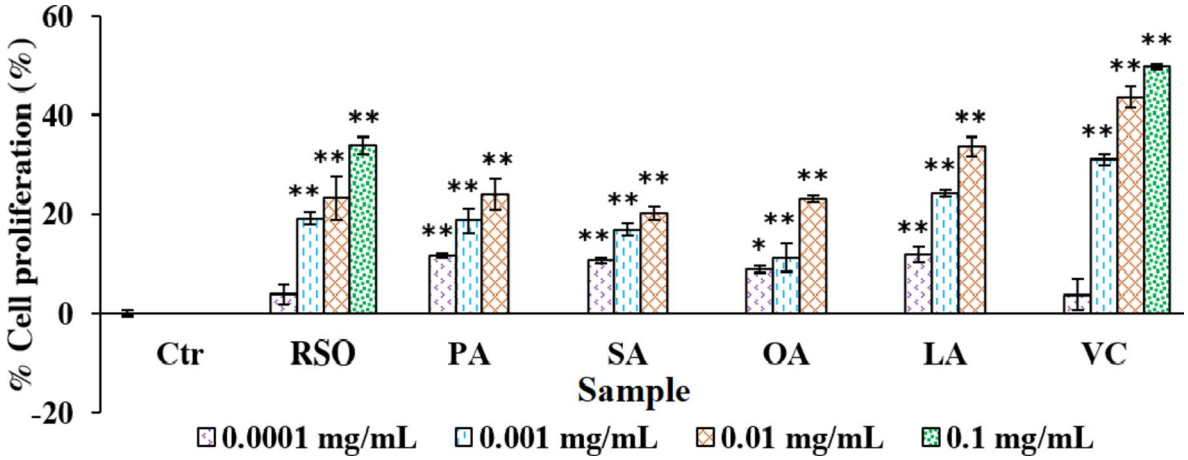

Fig. 3. Cell proliferating stimulation of RSO, fatty acids in RSO, including palmitic (PA), stearic (SA), oleic (OA), and linoleic (LA) acids, and vitamin C (VC) at non-cytotoxic concentrations of 0.0001–0.1 mg/mL in HaCaT cells. * and ** indicate the significant difference compared to control (Ctr) at $p < 0.05$ and 0.01, respectively.

effect on mRNA expression (20.07 ± 0.76%) and enzymatic activity (15.44 ± 0.44%), which were comparable to that of 0.1 mg/mL vitamin C (22.60 ± 2.82% for mRNA expression and 17.85 ± 1.76% for enzymatic activity).

Discussion

Rubber seeds were extracted following Lourith et al. (2014). However, the extraction yield in this study was higher than that in previous report. The different yield may be due to the difference in stages of growth rubber trees and agronomic conditions as well as the harvesting time[26].

The fatty acid compositions in RSO were in agreement with previous study[9]. But the difference in unsaturated fatty acid content was noted. This may be owing to plant growing stages and environmental factors of plantation, including soil composition, light intensity, and the planting area[26,27]. Among the oils used as cosmetics or cosmetic ingredients, the fatty acid components of those oils comprise of high content of unsaturated fatty acids which have been increasingly interested as the quality fats and are associated with the medicinal and cosmetic potential[28].

Linamarin is one of the general cyanogenic glucosides in agricultural products, including lima bean (*Phaseolus lunatus*) and cassava (*Manihot esculenta*). The hydrolysis of linamarin by β-glycosidase leads to the production of hydrogen cyanide, a highly poisonous substance[21]. Previous research has demonstrated the contamination of linamarin in fresh rubber seeds[29], which influences the safety consideration for seed application as a part of animal feeding compositions. In this study, linamarin contamination in RSO was analyzed and indicated an undetectable level. The lack of linamarin in RSO corresponded with previous report that has indicated the lack of cyanide presence in rubber seed oil extracts analyzed by Fourier transforms infrared spectroscopy (FTIR) and colorimetric methods[30].

Since skin aging is manifested in several components of skin, so two distinct cell models were used in the present study. HaCaT cell line represents the main cells responsible for skin epidermis, while HDF cell line refers to the cells found in skin dermis[15,31]. Cytotoxicity assay is performed to determine the non-cytotoxic concentrations of RSO, fatty acids in RSO, and vitamin C in order to analyze the anti-skin aging activities of these substances. The non-cytotoxic treatment exhibits cell viability of more than 80%, while cytotoxicity is evident when cell viability is less than 80%[32]. The cytotoxic results indicated that the non-cytotoxic concentrations of RSO, all fatty acids in RSO, and vitamin C were in the range of 0.0001–0.1, 0.0001–0.01, and 0.0001–0.1 mg/mL, respectively. The safe concentrations of RSO and fatty acids in HaCaT cells and HDF were in good agreement with those in B16F10 melanoma cells and 3T3-L1 cells[10]. The cytotoxicity of RSO at high concentration may be attributed to the fatty acid components. Previous study has shown that the acidity of fatty acids at high concentration can diffuse across cell membrane, interfere intracellular pH, and lead to the decreased cell viability[10]. Additionally, unsaturated fatty acids are associated with the inhibition of cancer cell growth by inducing cell apoptosis[33]. The high concentration of vitamin C has been shown to cause prooxidant effects, which in turn induce cell death[34].

The loss of skin thickness in aged skin is associated with several factors, including the decrease in cell numbers, cell proliferation, and contact area between dermal and epidermal cells (dermal-epidermal junction), and the alteration of degradation enzymes and tissue inhibitors of metalloproteinases (TIMPs)[16]. Cell proliferation is vital for tissue growth, repair, and regeneration. Analysis of cell proliferation refers to total cells involved in cell growth and cell division, whereas cytotoxicity assay indicates the healthy and viable cells after exposure to tested samples. Previous reports have shown that the aged cells lose their proliferative capacity and contribute to age-related skin changes[16,35]. The prolonged contact time of cells to the sample at non-cytotoxic concentrations has been applied to study the proliferative effect[36,37]. The substance with an ability for skin cell proliferating stimulation may be employed as a promising agent to slow down skin aging. The findings showed that fatty acids included in RSO may be responsible for cell proliferative activation. Previous studies have shown that

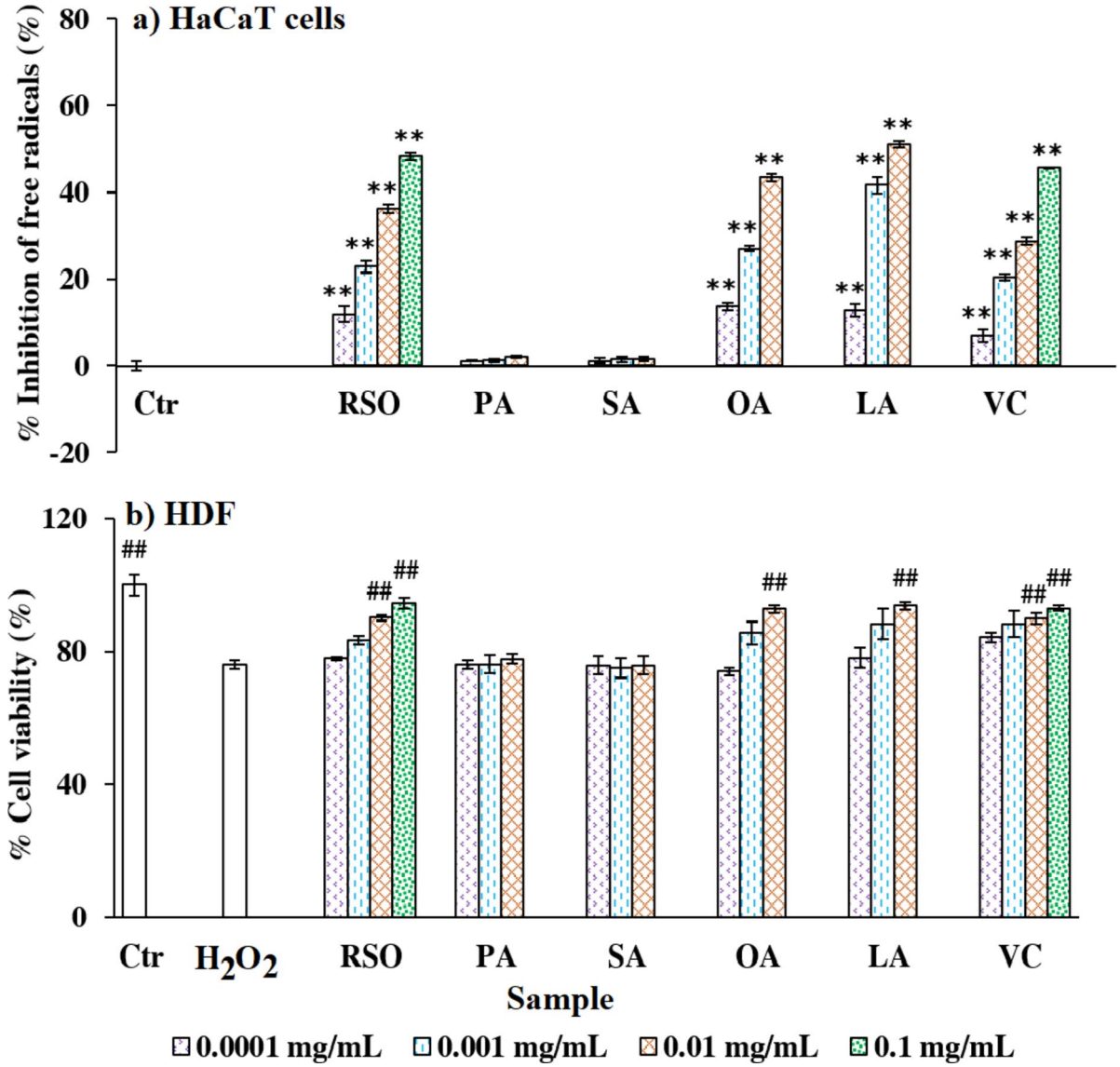

Fig. 4. Antioxidant activity of RSO, fatty acids in RSO, including palmitic (PA), stearic (SA), oleic (OA), and linoleic (LA) acids, and vitamin C (VC) at non-cytotoxic concentrations of 0.0001–0.1 mg/mL in HaCaT cells by cellular antioxidant activity and HDF by addition of hydrogen peroxide (H_2O_2). * and ** indicate the significant difference compared to control (Ctr) at $p < 0.05$ and 0.01, whereas # and ## indicate the significant difference compared to oxidative control (H_2O_2) at $p < 0.05$ and 0.01, respectively.

fatty acids, including palmitic, stearic, oleic, and linoleic acids, are crucial for cell growth and play several roles in cellular functions, that comprise cell growth and proliferation, cell membrane constituents, cell signaling, and cell energy[38,39]. The cell proliferation promoting activity of vitamin C is reported to mediate via activating extracellular signal-regulated-kinase (ERK) pathway[40].

Free radicals are normally produced via endogenous cellular functions, such as inflammation, infection, and aging, and exogenous sources, including pollution, tobacco smoking, and radiation. Currently, free radicals are associated with the cause of health problems and aging process[41]. Several studies have reported strategies to balance and/or reduce free radicals via increasing antioxidant system, including antioxidant enzymes and small-molecule exogenous antioxidants[16,23,41]. Among exogenous antioxidants, nature-derived substances are widely adopted as safe to use[42]. Antioxidant activity of an exogenous compound can be assessed in several approaches, including measurement of intracellular free radicals by CAA assay, measurement of cell viability after induction of oxidative damage by hydrogen peroxide, and measurement of cellular antioxidant enzymes[15,18,43]. Different analyses are combined to explain the antioxidant pathway of a potential compound. The measurement of intracellular free radicals by CAA assay was performed in HaCaT cells, due to these cells are representative for cells at skin epidermis. In general, the skin epidermis serves as physical barrier that is vulnerable to environmental insults and causes the free radicals to develop[16]. The addition of peroxyl radicals, AAPH, mimics the oxidative damage occurred at lipid bilayer membrane and leads to the increased cell permeability and cell

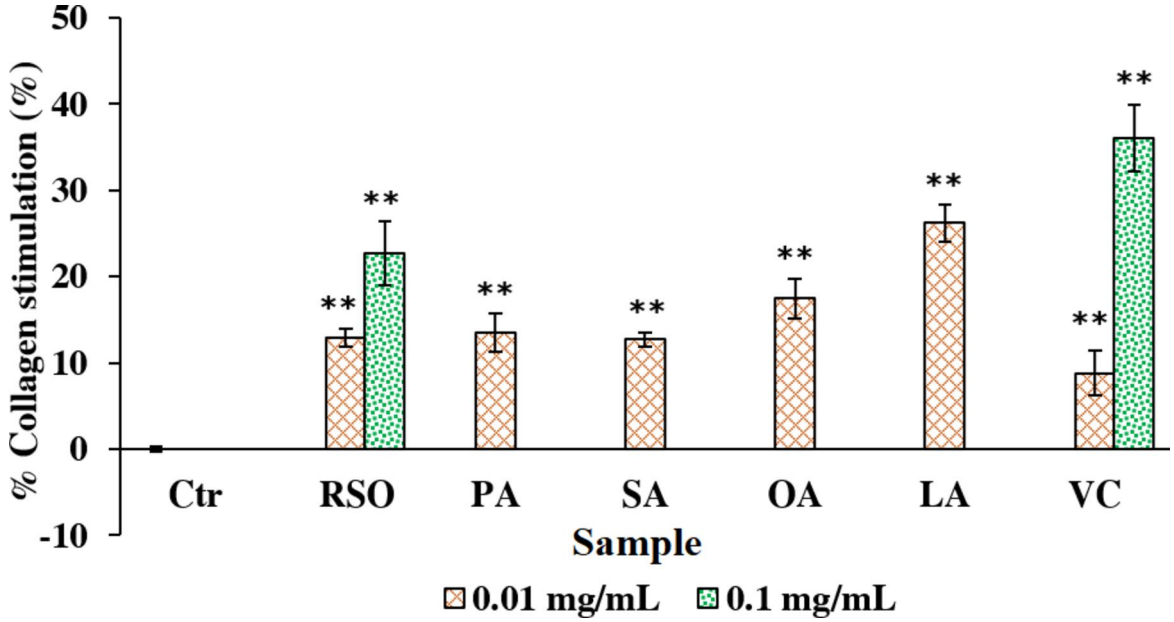

Fig. 5. Collagen stimulation activity of RSO, fatty acids in RSO, including palmitic (PA), stearic (SA), oleic (OA), and linoleic (LA) acids, and vitamin C (VC) at non-cytotoxic concentrations of 0.01–0.1 mg/mL in HDF. * and ** indicate the significant difference compared to control (Ctr) at $p < 0.05$ and 0.01, respectively.

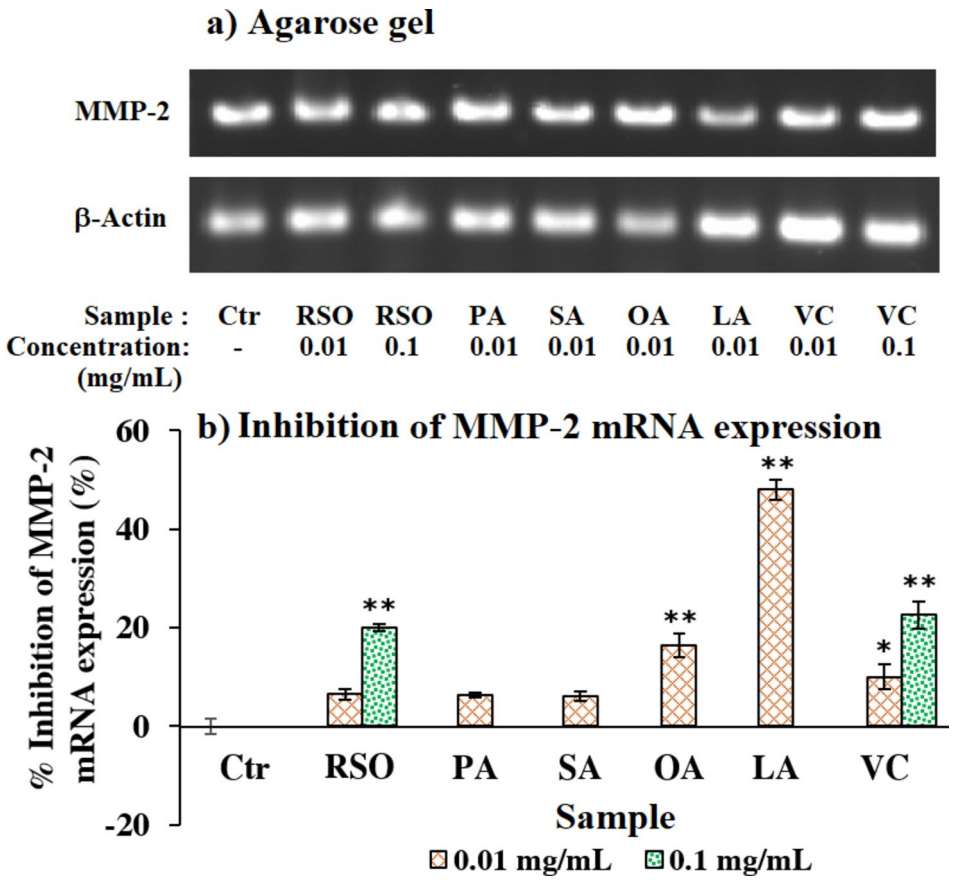

Fig. 6. Suppressive activity on MMP-2 mRNA expression level of RSO, fatty acids in RSO, including palmitic (PA), stearic (SA), oleic (OA), and linoleic (LA) acids, and vitamin C (VC) at non-cytotoxic concentrations of 0.01–0.1 mg/mL in HDF. * and ** indicate the significant difference compared to control (Ctr) at $p < 0.05$ and 0.01, respectively.

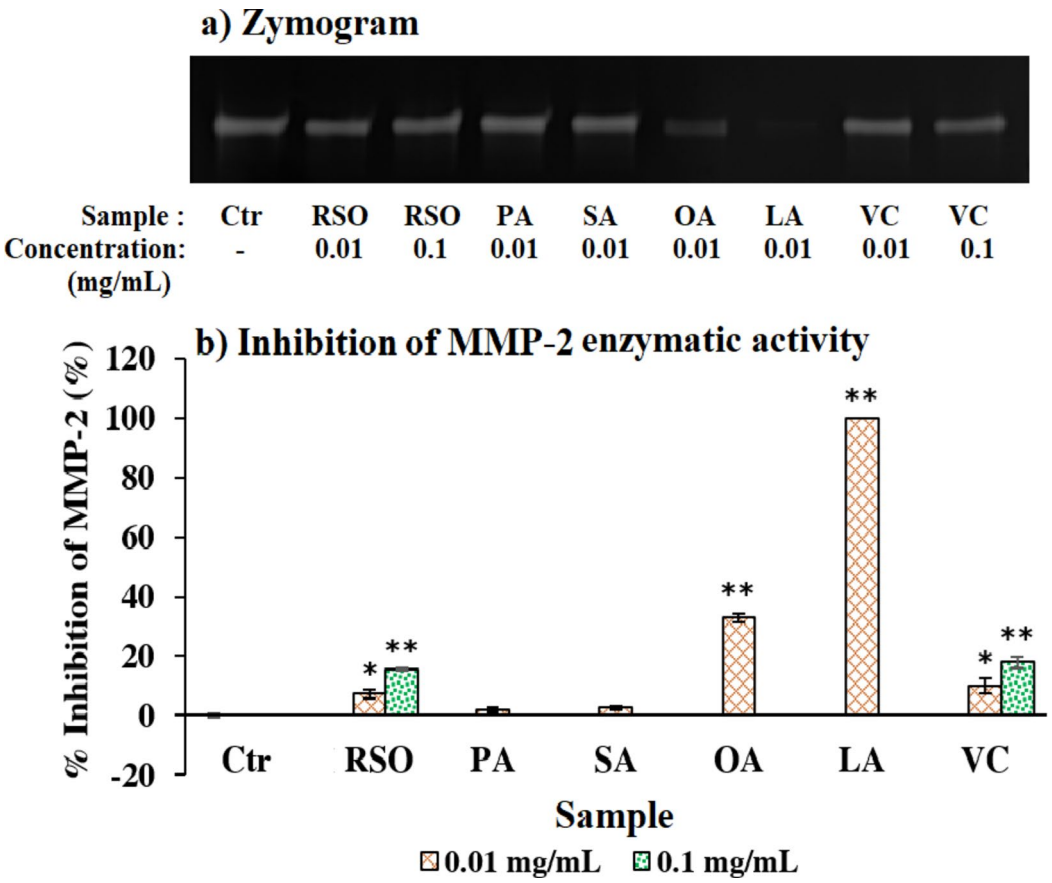

Fig. 7. Inhibitory activity on MMP-2 enzymatic activity of RSO, fatty acids in RSO, including palmitic (PA), stearic (SA), oleic (OA), and linoleic (LA) acids, and vitamin C (VC) at non-cytotoxic concentrations of 0.01–0.1 mg/mL in HDF. * and ** indicate the significant difference compared to control (Ctr) at $p < 0.05$ and 0.01, respectively.

death[22]. Antioxidant compounds could scavenge and reduce the formation of free radicals. The measurement of cell viability after induction of oxidative damage by hydrogen peroxide was analyzed in HDF. After addition of hydrogen peroxide, the free radicals are generated and subsequently induce cell death via apoptosis pathway[44]. Antioxidant substances could exhibit the free radical scavenging and protect the cellular damage, resulting in the increased cell viability. In this study, the non-enzymatic antioxidant activity of RSO was analyzed and revealed that RSO displayed the free radical scavenging via reducing intracellular free radicals as well as cellular protecting against oxidative damage. Antioxidant activity of RSO agreed with the previous studies performed the *in vitro* antioxidant assays and in 3T3-L1 culture cells[9,10]. Oleic and linoleic acids, two major unsaturated fatty acids found in RSO, may be in charge of the antioxidant activity. The activity of vitamin C is reported to involve in the suppression of free radical formation[45].

The decreased skin integrity in aged skin is associated with the markedly reduced collagen content. Collagen is an abundant structural protein that supports human skin. The colorimetric collagen analysis using Sirius red is specific staining technique, which binds the anionic dye to the triple helix structure [Gly-x-y] of collagen type I and III and is concentration-dependent assay[23,46]. The increased collagen content of RSO may be attributed to a variety of fatty acids, particularly unsaturated fatty acids. Previous study has shown that fatty acids enhance collagen content through increasing transforming growth factor as well as being components of collagen production[47,48]. The involvement of vitamin C in collagen stimulation is mediated via acting as cofactor of enzymes used for collagen synthesis as well as increasing mRNA expression of procollagen[23,49].

The increased activity of MMPs in aged skin is strongly correlated with the signs of aging appearance, including skin wrinkling and sagging[15,16]. MMPs play a crucial role in regulation of cell-cell and cell-extracellular matrix interaction, modification of matrix structure, and function of cell surface signaling system. MMP-2 (gelatinase A) is responsible to cleave gelatins, collagen type IV, and other extracellular matrices, including elastin and laminin. The SDS-PAGE zymography has been shown as a sensitive approach to quantify gelatinase enzyme activity. The secreted gelatinase in serum-free medium is separated in the denaturing condition by gel electrophoresis, and then analyzes enzyme activity via degradation of gelatin, an enzyme substrate mixed in polyacrylamide gel, in the renaturing and developing condition[50]. The upregulation of MMP-2 enzyme in aged skin is associated with skin wrinkles and impairment of tissue homeostasis[51]. Thus, substances possessing MMP-2 inhibitory activity can possibly be indicated to decelerate the aging signs. The MMP-2 inhibitory activity of

RSO at mRNA expression level and enzymatic activity may result from the unsaturated fatty acids, both oleic acid and linoleic acid. Previous study has shown that antioxidant activity of unsaturated fatty acids can counteract the generated free radicals, prevent the lipid peroxidation, and increase activity of antioxidant enzymes, resulting in the inhibitory activity of MMPs[15,49,52].

In conclusion, rubber seeds of trees clone RRIM 600 were extracted by Soxhlet apparatus and given the extraction yield of 35.79 ± 0.52%. The principal fatty acids in RSO comprised of saturated fatty acids, which were palmitic and stearic acids, and unsaturated fatty acids, including oleic and linoleic acids. Cytotoxicity assay and the anti-skin aging activities of RSO were performed in HDF and HaCaT cells. The non-cytotoxic concentrations of RSO, which were in the range of 0.0001–0.1 mg/mL, exhibited the cell proliferating stimulation, the antioxidant activity, the collagen stimulation, and the MMP-2 inhibition at mRNA expression level and enzymatic activity. The study results indicate that RSO can potentially be employed as a functional ingredient to decelerate the aging process. The further study of RSO is recommended to study the molecular mechanisms of antioxidant activity, including nuclear factor erythropoietin-2-related factor 2/heme oxygenase 1 (Nrf2/HO-1) and antioxidant enzymes, such as glutathione peroxidase, superoxide dismutase, and catalase. Currently, Nrf2/HO-1 signaling pathway plays an essential role in cellular response against oxidative damage. Nrf2, a transcriptional factor, translocates into the nucleus to activate function by binding to antioxidant response element (ARE) pathway and consequently induces the downstream of gene expression, such as HO-1, glutathione peroxidase, and other antioxidant enzyme production[53,54]. Additionally, the clinical efficacy of RSO against skin aging is suggested to perform for claim substantiation.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Conceptualization, P.C; methodology, P.C., N.L. and M.K; investigation, P.C., N.L. and M.K; data analyses, P.C., N.L. and M.K; writing-original draft, P.C., N.L. and M.K; writing-revise and editing, P.C., All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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ARTICLES FOR FACULTY MEMBERS

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Hevea brasiliensis (Rubber Seed) Oil: Extraction, Characterization, and Kinetics of Thermo-oxidative Degradation Using Classical Chemical Methods

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Supporting Information

ABSTRACT: In the present study, nonedible seed oils from underutilized Nigerian NIG800 clonal rubber seeds were extracted using a solvent method to obtain a yield of 43 wt % after extraction for 1 h using a 0.5 mm kernel particle size. The oil was characterized by GC-MS, FT-IR, and NMR analyses, and found to possess several potential industrial applications. The physicochemical properties determined agreed with reported values in the literature. The low ash content (0.001 wt %) indicates the absence of trace metals that catalyze oxidation reactions. The low moisture (1.73 wt %) and carbon residue (0.4 wt %) contents, high volatile matter (97.869 wt %), and low freezing point (−18 °C) properties of the oil indicate a better source material for biodiesel synthesis for use in cold regions compared to other vegetable oils. The higher heating value of 39.37 kJ/kg for the oil is within the range of values reported by researchers for other nonedible vegetable oils. The high content of saturated fatty acids (30.67 wt %) and moderately low monounsaturated fatty acids (69.33 wt %) confer a good shelf life compared to other oils. A closer examination of results of the NMR and GC-MS show a satisfactory agreement that these genetically modified rubber seeds have an insignificant proportion of polyunsaturated fatty acids (linoleic, linolenic, etc.). This insignificant presence of polyunsaturated fatty acids supports higher thermal stability, and slower rate of oxidation of the oil compared to other vegetable oils. The kinetics of thermal oxidative degradation follows a first-order reaction. The activation energy of 13.07 kJ/mol was obtained within the temperature range 100−250 °C.

1. INTRODUCTION

The depletion of fossil materials and the concern for the degraded environment motivated researchers in the global community to search for alternative source routes for energy. Vegetable oils are seen as plausible source materials for renewable energy production.[1] To avert a food-fuel crisis, nonedible vegetable oils, considered as low-grade oils that are not suitable for food uses, could be an attractive preference to edible oils for industrial applications.[2] These nonedible oil plants can be grown in rural, unproductive, and degraded lands, as they are well adapted to a semiarid and low moisture/fertility environment.[3]

Vegetable seed plants are abundant in every region of the world, and over 350 of them have been identified with domestic and industrial uses. One of the few versatile bioenergy crops with nonedible oil that has attracted the attention of researchers in recent times is the rubber tree (*Hevea brasiliensis*). The tree belongs to the plant family *Euphorbiaceae* and the subfamily genus *Hevea*, and is native to the Amazon region of Brazil in South America, but is mainly grown in Southeast Asia countries (90.5%), sub-Saharan Africa (7%), South America (1.5%), and others (1%).[1]

Hevea brasiliensis is economically cultivated for the production of latex as a source of natural rubber for the production of various rubber products in use globally, while the seeds are underutilized.[1,4,5] However, the oil from the underutilized seeds is the second most valuable product after the latex.[6] The combined production capacity of rubber seed oil in the sub-Saharan Africa countries is projected at about 18 kiloton annually.[1] This value is expected to increase substantially with the expansion of rubber plantations to generate nonedible oils that will make up the greatest proportion of renewable raw materials for the chemical industries in the future. Rubber seed oil has several industrial applications, such as biodiesel synthesis,[1,7,8] biolubricant and hydraulics oil formulations,[9,10] linseed oil substitute,[10] oleochemicals production,[11] and cosmetic and pharmaceutical products.[5] Other uses include paint and coating formulations, as semidrying oil,[3] alkyd resin, and polyurethane biocomposites production.[12,13] The reported rubber seed oil (RSO) yield of 35−50 wt % in the literature is high to encourage its extraction and commercialization for industrial uses.[1]

Industrially, several methods are employed for oil extraction, such as mechanical pressing, solvent extraction, enzymatic, aqueous, and their combinations.[1,14,15] Mechanical pressing (batch or continuous) is widely used and well adapted to rural areas with moderate initial and operating costs, but oil yield is low.[16−18] Recent studies revealed solvent extraction using *n*-

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hexane, as commonly practiced industrially, because of its inherent advantages, such as higher oil yield, low operating cost, shorter time, reduced volume usage, and lower turbidity.[1,7] Some of the recent work on oil seed extraction employing *n*-hexane as solvent included oils from seeds of rubber,[1,7] yellow horn,[19] *terminalia catappa L*,[20] and hempseed.[14]

Generally, seed oils deteriorate when handled defectively, with the major decomposition reaction being oxidation in the presence of atmospheric air (oxygen). The oxidative stability of oils is an important indicator of performance and shelf life, and it depends on the fatty acid compositions and conditions to which it is subjected.[21] Poor oxidative stability, unpleasant odor, filter clogging tendency, low temperature fluidity, and other conditions that make them unsuitable for long-term storage before use are some of the characteristics of vegetable oils.[22] Thermal oxidation of seed oil on prolonged heating occurs through a free radical mechanism. This is characterized by the initial emergence of a sweetish and unpleasant odor that becomes progressively worse until it attains a characteristic smell of rancid fat, yielding peroxides and unstable hydroperoxides as primary products.[21,23] The primary products degrade easily to produce secondary products such as aldehydes, ketones, acids, and alcohols.[21,23,24] Oxidative stability of vegetable oils can be improved by use of various antioxidant additives available in markets or more importantly by increasing the saturated fatty acids and oleic acid contents in oil by genetic modification of oil bearing plants.[25] Such thermal oxidative reactions can be investigated using thermoanalytical methods such as differential scanning calorimetry (DSC), thermogravimetry analysis (TGA), derivative thermogravimetry (DTG), and Fourier transform-infrared (FT-IR) spectroscopy.[21,23−25] However, simple and low-cost classic chemical analysis, such as determination of the concentration of the peroxide value, iodine value, acid value, and refractive index, can be effectively employed to evaluate the oxidation reaction state of oils under thermal conditions that can influence the mechanism of the degradation process.[21,22]

Aravind et al.[3] in their study carried out TGA on RSO under an oxygen environment in the temperature range 0−500 °C, and reported RSO to be thermally stable up to 250 °C, and gradual degradation occurs after 300 °C. The findings agreed with the reports of Reshad et al.[7] on the TGA of RSO and observed negligible weight loss (<0.2%) at 90 °C due to the presence of moisture (free water), but they were thermally stable up to 250 °C.

In the present study, RSO was extracted using an *n*-hexane/Soxhlet extractor. The oil was characterized to determine the physicochemical properties, and gas chromatography−mass spectrometry (GC-MS) was performed to reveal the fatty acid composition profile. The functional groups present in the oil and the modes of vibration at room temperature were revealed using an FTIR technique, and they were subsequently confirmed using nuclear magnetic resonance (NMR) spectroscopy. Vegetable oils degrade through three main chemical reactions: thermolytic (heating in the absence of oxygen), oxidative (in the presence of oxygen), and hydrolytic (in the presence of water). The kinetics of thermal oxidation of the oil under an oxygen environment at isothermal conditions was determined; employing less cost-effective simple chemical analysis to evaluate the kinetic parameters of the reaction.

2. EXPERIMENTAL SECTION

2.1. Materials and seed preparation. The rubber seeds used in this study were handpicked from the NIG800 clones at the plantations of the Rubber Research Institute of Nigeria (RRIN), Iyanomo, Benin City (Figure S1). The seeds were examined for freshness, washed, cleaned, and dried at room temperature for 48 h. Approximately 3 kg of seeds were deshelled manually using a laboratory mortar and pestle to free the kernels from the shells. The seed shells and kernels were weighed separately, and their weight percentage recorded. A portion of the seed kernels was milled and sieved into five-particle sizes (0.5, 1, 1.5, 2, and 2.5 mm) to determine the size that will give the best oil yield. All chemical reagents used in this study were of analytical grades manufactured by BDH Chemicals Ltd., Poole England, and GFS Chemicals, Inc., 867 McKinley Ave., Columbus, OH 43223.

2.2. Methods. *2.2.1. Particle size determination for best rubber seed oil yield.* Several process parameters, such as type of solvent, kernel particle size, seed/solvent ratio, extraction time, and temperature, affect the yield of seed oil extraction.[20] The choice of solvent is based on cost, behavior toward the matrix, and toxicity. In this study, five-particle sizes of rubber seed kernel (0.5, 1, 1.5, 2, and 2.5 mm) were considered to determine the particle size that will give the maximum oil yield. For each particle size considered, the extractor was charged with 50 g of milled rubber seed kernel packed in a muslin cloth, and placed in a thimble of the extractor. A 500-mL round-bottom glass flask was filled with 225 mL of *n*-hexane and tightly fixed to the end of the extractor (Figure S2).

Heating was provided for the setup by a heating mantle regulated at 60 °C for 45 min. Extraction time was counted when the first drop of the extracting solvent recycled back into the thimble. Each experiment was conducted in three replicates, and the mixture was concentrated at 65 °C (*BUCHI Rotavapor R-200*) under vacuum to remove solvent and recover the extracted oil. The oil yield was gravimetrically determined using eq 1, and the average values are displayed (Figure 1). The particle size that gave the maximum oil yield was employed for further oil extraction.

$$RSO\ yield\ (wt\ \%) = \frac{mass\ of\ extracted\ oil\ (g)}{mass\ of\ rubber\ seed\ kernel\ used\ (g)} \times 100 \quad (1)$$

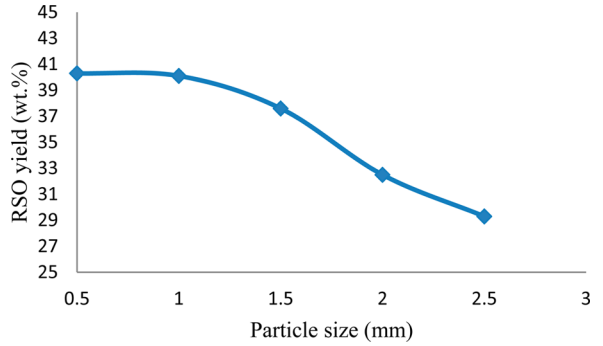

Figure 1. Effects of particle sizes on rubber seed oil yield.

2.2.2. Conradson carbon residue of rubber seed oil. The conradson carbon residue was determined following the procedures described in ASTM D189-IP 13. The determination of the carbon residue left after evaporation and pyrolysis of oil is intended to provide some indication of its relative coke-forming propensity when used as source material for fuel. In this study, 3 ± 0.1 g of oil was weighed and placed in a porcelain crucible containing 0.3149 g of glass bead to maintain uniform heating. The crucible was placed in the center of the skidmore iron crucible (Stanhope-Seta apparatus) with a lid. Heat was applied, and the preignition period observed was 30 ± 5 min when smoke appeared above the chimney. The experiment lasted for 60 ± 5 min in an air-free environment. At the end of the specified heating period, the test crucible containing the carbonaceous residue was

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cooled in a desiccator and weighed again. The residue remaining was calculated as a percentage of the original sample, and reported as noncombustible carbon residue. The experiment was carried out in duplicate, and the average value was recorded (Table 1).

Table 1. Compositional Analysis of Rubber Seed and Fresh Seed Oil[a]

Parameter	Composition (wt %)
Rubber seed:	
Kernel	40.47 ± 0.07
Kernel moisture	9.7 ± 0.07
Shell	49.83 ± 0.14
	100
Rubber seed oil:	
Conradson carbon residue	0.4 ± 0.028
Moisture content	1.73 ± 0.028
Ash content	0.001 ± 0.0001
Volatile matter	97.869 ± 0.056
	100

[a]Values are mean ± standard deviation of duplicate data.

2.2.3. Moisture content analysis of rubber seed oil. The moisture content was determined using a standard laboratory U CLEAR oven (*Model: DHG-9053A*) operated at 105 °C. About 20 g of the sample was placed in a clean porcelain crucible and weighed. The process of heating and cooling was repeated every 10 min after weighing until constant weight was obtained. All analyses were done in duplicate, and moisture content was determined by calculating the weight difference of the sample before and after oven drying (Table 1).

2.2.4. Ash content analysis of rubber seed oil. The ash content was determined by burning 20 g of oven-dried sample in a porcelain crucible placed in an electric muffle furnace (Carbolite, Parson Lane, Hope Valley S33 6RB, England, *Model: RWF 12/5*) maintained at 550 ± 25 °C for an initial 20 min, cooled in a desiccator, and weighed again. The heating and cooling process was repeated every 10 min interval until the carbonaceous residue was reduced to an ash. All analyses were done in duplicate, and the results were expressed on a dry weight basis according to procedures prescribed in ASTM D 482-IP 4.

2.2.5. Physico-chemical characterization of rubber seed oil. The extracted rubber seed oils were analyzed for density, specific gravity, saponification value, acid value, iodine value, peroxide value, kinematic viscosity, refractive index, and pH (Table 2) using standard procedures as described by ASTM and AOAC (1990).[26]

The acid value was determined by a titration method, while the iodine value was obtained by Wijs' method. The specific gravity measurement was carried out at room temperature using standard specific gravity bottles. The state and color of the oil were noted using visual inspection at room temperature. Other parameters evaluated are cetane number,[27] higher heating value (HHV),[28] mean molecular mass of fatty acids,[29] and average molecular mass of oil.[30] ASTM standard procedures were followed to determine cloud, flash, freezing, boiling, pour, and aniline points, respectively. The cold filter plugging point (CFPP) was estimated using the correlation cited by Verma et al.[31]

2.2.6. GC-MS, FT-IR, and NMR characterization of rubber seed oil. The extracted oil was characterized by gas chromatography−mass spectroscopy (GC-MS), Fourier transform infrared spectroscopy (FT-IR), and nuclear magnetic resonance (NMR) techniques.

2.2.6.1. Fatty acid profile of rubber seed oil. The fatty acid profile was determined qualitatively using a GC-MS (*QP2010 Plus Shimadzu, Japan*) system equipped with a flame ionization detector (FID) with a capillary column DB-1 (length 30 m × diameter 0.25 mm × film thickness 0.25 μm). Helium was used as carrier gas with a linear velocity of 49.2 cm/s and a purge flow of 3 mL/min. The column oven temperature was programmed at 70 °C and was quickly ramped at 10 °C/min until it reached 280 °C and was held for 5 min. A sample of the oil was mixed with 1 mL of HPLC grade *n*-hexane. A portion of

Table 2. Physicochemical Properties and Other Characteristics of Rubber Seed Oil[a]

Parameters	refs 1, 3, 7	This study
Color	Golden yellow, light/dark brown	Dark brown
Density, g/cm^3 @ 25 °C	0.857−0.943	0.886 ± 0.002
Specific gravity @ 15 °C	0.91	0.909 ± 0.002
°API gravity @ 15 °C	NA	24.1 ± 0.282
pH	6	6 ± 0.141
Oil content (wt %)	40−50	43 ± 0.141
Iodine value, g I_2/100 g oil	113−146	137.02 ± 0.028
Peroxide value, mequiv O_2/kg oil	1.6−16	10.46 ± 0.098
Saponification value, mg KOH/g oil	183.91−235.28	195.30 ± 0.282
Acid value, mg KOH/g oil	1.68−42.41	18.20 ± 0.141
Free fatty acid (%FFA as oleic acid)	0.84−42.412	9.10 ± 0.07
Kinematic viscosity, mm^2/s @ 40 °C	6−66	40.18 ± 0.028
Refractive index @ 20 °C	1.46−1.47	1.4707 ± 0.00028
Pour point, °C	−9 to −1.5	−6
Cloud point, °C	3−4	5.5
Cold filter plugging point, °C	NA	−0.025
Flash point, °C	72−295	240.3
Fire point, °C	298	256
Aniline point, °C (°F)	NA	21 (69.8)
Boiling point, °C	NA	119
Freezing point, °C	NA	−18
Cetane number	45−49.73	43.42
Higher heating value (HHV), MJ/kg	36.1−44	39.37
Mean mol. weight of fatty acids (g/mol)	NA	286.74
Average molecular weight of RSO (g/mol)	NA	898
Diesel index	NA	15.71

[a]NA means not available; values are mean ± standard deviation of duplicate data.

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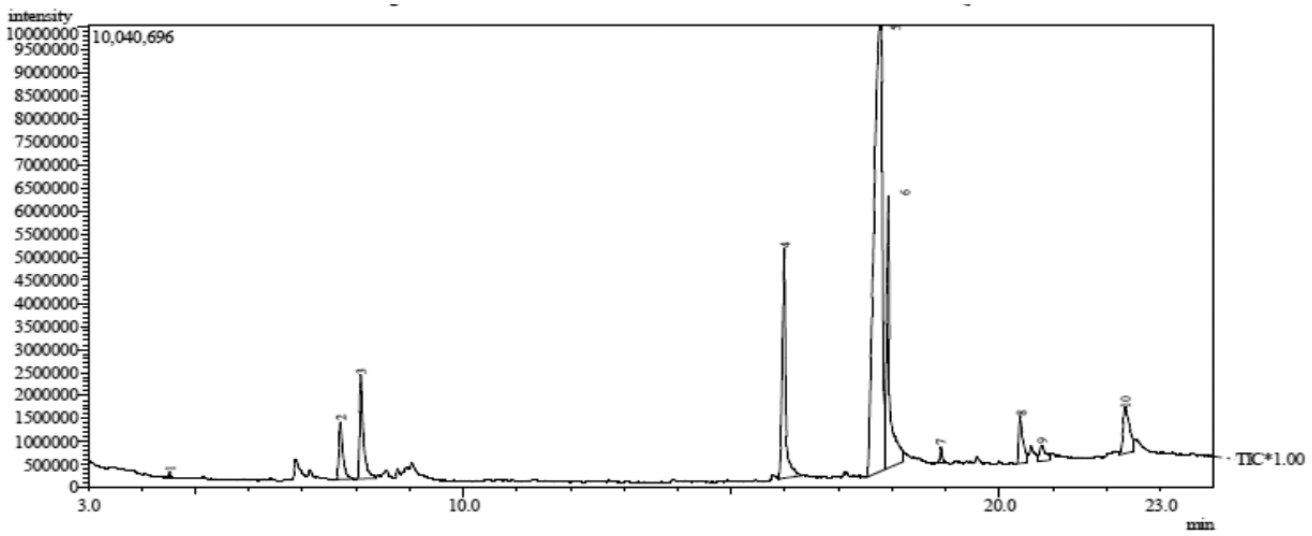

Figure 2. Gas chromatogram of rubber seed oil with identified peaks: peak 4 (palmitic acid), peak 5 (oleic acid), peak 6 (stearic acid), and peak 10 (*cis*-erucic acid).

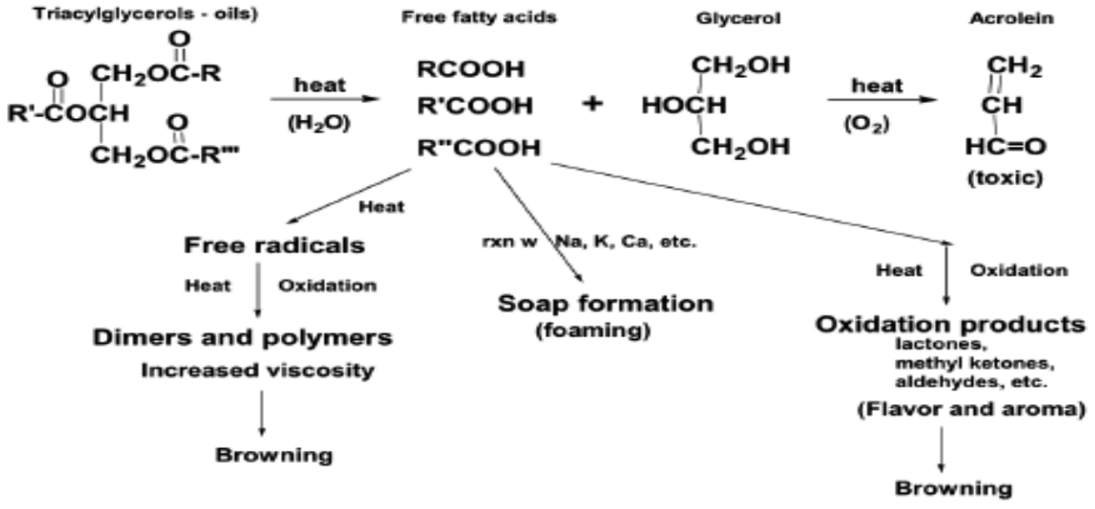

Figure 3. Overall process of oil degradation.[34]

this mixture (0.5 μL) was injected into the GC at 250 °C at a split ratio of 20:1. A column pressure of 116.9 kPa and column flow rate of 1.80 mL/min were maintained. The mass spectrometer (MS) was operated in electron ionization mode with the following parameters: ion source temperature 200 °C, interface temperature 250 °C, and solvent cut time 2.5 min. It was scanned from m/z 30 to 350, and identification of the peaks was performed by comparing retention times with those of the National Institute of Standards and Technology (NIST) library analyzed under the same conditions. The area percentage method was used to estimate the fatty acid compositions of the oils.[32] The results of fatty acid profile and gas chromatogram analysis are presented in Table 3 and displayed in Figure 2, respectively.

2.2.6.2. Fourier transform infrared (FT-IR) spectroscopy. FT-IR spectroscopy can be employed to identify the various functional groups present in oils and gives an insight into the formation of primary and secondary oxidation products when the oil is under thermal stress.[33] In this study, FT-IR was used to characterize the oil at room temperature (28 °C) in order to assess the oxidation state of the oil prior to thermal treatment using an FTIR spectrometer (Model: TENSOR 27, Bruker Optics Inc., USA) equipped with a detector having the spectral range 4000−500 cm^{-1}. The FTIR was analyzed using OPUS spectroscopy software supplied with the instrument.

2.2.6.3. Nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy is a useful nondestructive tool that complements GC-MS in the characterization of lipids and identification of compounds present. In this study, the ^{1}H NMR spectrum was recorded on a *Bruker* ultrashield TM 500-MHz NMR spectrophotometer using deuterated chloroform ($CDCl_3$) as solvent (δ = 7.26 ppm) containing a small amount of tetramethylsilane (TMS) as an internal standard (δ = 0 ppm) and put in a 5 mm diameter tube. The ^{13}C NMR experiments were performed using the same equipment, and the carbon atom in $CDCl_3$ was taken as 77.42 ppm. All other peaks were assigned with respect to it. About 25−30 mg of RSO was dissolved in 1 mL of $CDCl_3$, and the experiments were performed at 25 °C.

2.2.7. Mechanisms of thermal oxidation and hydrolysis of oil. Nonedible RSO is a plausible source material for biodiesel production and other industrial uses. Hydrolysis of the ester linkage of triacylglycerols due to the presence of moisture releases long-chain free fatty acids as shown in Figure 3.[34] Thermal hydrolysis takes place mainly within the oil phase rather than at the water−oil interface.

Other factors that influence the oxidation process include heating duration, fatty acids compositions, and presence of a catalyst/antioxidant. The mechanisms of thermal oxidation depicted in Figure 4[34] involve the following chemical steps: initiation, propagation, and termination reactions.

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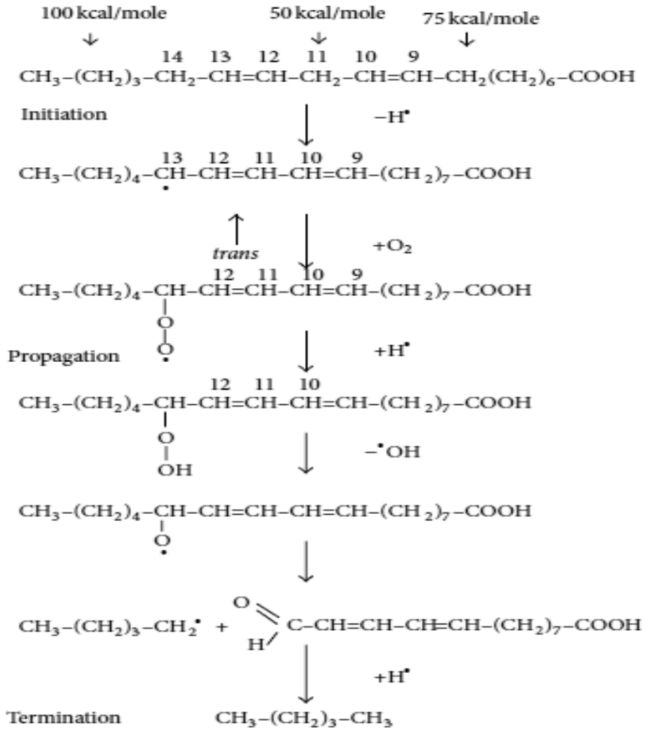

Figure 4. Mechanisms of initiation, propagation, and termination of thermal oxidation of oils.[34]

However, the strengths of the C−H bonds of saturated and unsaturated fatty acids identified in the oil determine its oxidation rates. During the initiation stage, free radicals are generated from the weakest C−H bond in the oil (Carbon 11−linoleic acid) through energy addition (50 kcal/mol).[34] In the propagation stage, the free radical at carbon 11 will be rearranged to form a conjugated pentadienyl radical at carbon 9 or carbon 13 with a trans double bond. The double bonds at carbon 9 and carbon 12 decrease the C−H bond at carbon 11 by electron withdrawal. The C−H bond on carbon 8 or carbon 11, which is α to the double bond of oleic acid, is about 75 kcal/mol. The C−H bond on the saturated carbon without any double bond next to it is approximately 100 kcal/mol.[34] Termination of the reaction occurs when radicals react with each other to produce nonradical compounds (volatile or nonvolatile).

2.2.8. Kinetics of thermal oxidative degradation of rubber seed oil. Vegetable oils (mainly triglycerides) undergo chemical reactions such as oxidation, isomerization, polymerization, and hydrolysis when subjected to a prolonged heating process. These reactions affect the physicochemical properties of these oils and their quality. The thermal study of oils before their industrial applications permits data to be obtained on the influence of time and temperature on their thermal oxidation.[23]

In this study, classical chemical analysis methods were used to determine the change in the peroxide value, iodine value, and refractive index of RSO when subjected to heat treatment.[22] RSO has been reported to be thermally stable up to 250 °C under heat treatment.[3] For this reason, the peroxide value, iodine value, and refractive index were determined at 100, 150, 200, and 250 °C at various time intervals using methods prescribed by ASTM and AOAC (1990).[26] The iodine value is a measure of the degree of unsaturation to quantify the amount of double bonds present in the oil that reflects its susceptibility to oxidation[35] and was used in the present study to generate data for kinetic plots.

The broad reaction rate expression proposed by several researchers for the kinetics of thermo-oxidative degradation of RSO is expressed in eq 2.[22,24,36,37]

$$-dC/dt = kC^n \quad (2)$$

where C is the iodine value at any time, n is the order of the reaction, and k is the reaction rate constant, whose temperature dependence is commonly described by Arrhenius eq 3.

$$k = A_o \exp(-E_a/RT) \quad (3)$$

T is the absolute temperature, R is the universal gas constant, E_a is the activation energy, and A_o is the pre-exponential factor. The thermo-oxidative degradation of vegetable oils generally follows a first-order reaction as given in eq 4.[36]

$$\ln C_t/C_o = kt \quad (4)$$

where C_t is the iodine value at time t and C_o is the initial iodine value.

3. RESULTS AND DISCUSSION

3.1. Effects of particle sizes on rubber seed oil yield.

The results depicted in Figure 1 showed that the particle size

Table 3. Fatty Acids Profile of Rubber Seed Oil

Fatty acid/Systemic name	Chemical formulas	Composition (wt %) ref 1	Composition (wt %) This study
Myristic ($C_{14:0}$)/Tetradecanoic	$C_{14}H_{28}O_2$	2.2	
Palmitic ($C_{16:0}$)/ Hexadecanoic	$C_{16}H_{32}O_2$	0.23−10.6	13.85
Palmitoleic ($C_{16:1}$)/ Hexadec-9-enoic	$C_{16}H_{30}O_2$	0.23−0.25	
Stearic ($C_{18:0}$)/ Octadecanoic	$C_{18}H_{36}O_2$	5.69−12	16.82
Oleic ($C_{18:1}$)/*cis*-9-Octadecenoic	$C_{18}H_{34}O_2$	12.7−42.08	64.11
Linoleic ($C_{18:2}$)/*cis*-9-*cis*-12-Octadecadienoic	$C_{18}H_{32}O_2$	39.6−52.84	
α-Linolenic ($C_{18:3}$)/*cis*-9-*cis*-12-cis-15-Octadecatrienoic	$C_{18}H_{30}O_2$	2.38−26	
Arachidonic ($C_{20:0}$)/Eicosanoic	$C_{20}H_{40}O_2$	0.66−0.97	
Erucic acid ($C_{22:1}$)/*cis*-13-Docosenoic	$C_{22}H_{42}O_2$		5.22
Total saturated		8.78−23.57	30.67
Total monounsaturated		12.93−42.33	69.33
Total polyunsaturated (linoleic, linolenic, etc.)		41.98−78.84	trace

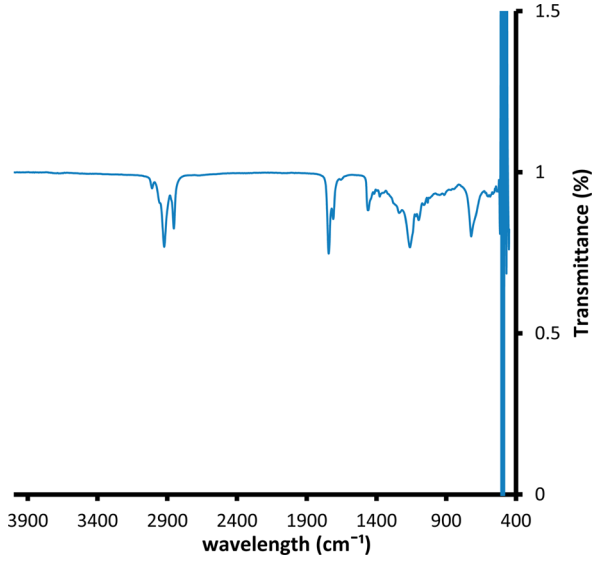

Figure 5. FTIR spectrum of rubber seed oil.

0.5 mm yielded a maximum oil yield of 40.3 wt % among the available sizes. The smaller particle size creates a larger surface area that enhances the oil yield by facilitating the solvent diffusivity in the seed powder. This agreed with the findings of

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Table 4. Wavenumbers and Their Corresponding Functional Groups for FTIR Analysis of Rubber Seed Oil

Wavenumber (cm^{-1})	Functional group	Description of vibration
3009	Alkenes	This is due to =C−H stretching of nonconjugated unsaturation (methylene group)
2921−2853	Alkanes	These strong peaks are attributed to the stretching of C−H (methyl group)
1742	−Carbonyl	This strong and sharp peak is attributed to −C=O stretching of esters
1710	Carboxylic acids	This assigned peak is due to stretching vibration of C=O
Fingerprint region (1461−585 cm^{-1})		
1461	Alkenes	The medium signal can be attributed to bending frequency of C−H
1377	Alkenes	The assigned peak is due to C−H bending vibrations of alkenes (CH_2 group)
1238	Carboxylic acids, Esters	The assigned peak is attributed to stretching vibration of C−O
1160	Esters	C−O stretching vibration attributed to ester groups
1118−1033	Esters	=C−O−C stretching vibration of ester groups
721	Aromatics	Assigned to out-of-plane bending vibration of saturated carbon atom (C−H)
585	Alkanes	The weak signal is due to C−H vibration

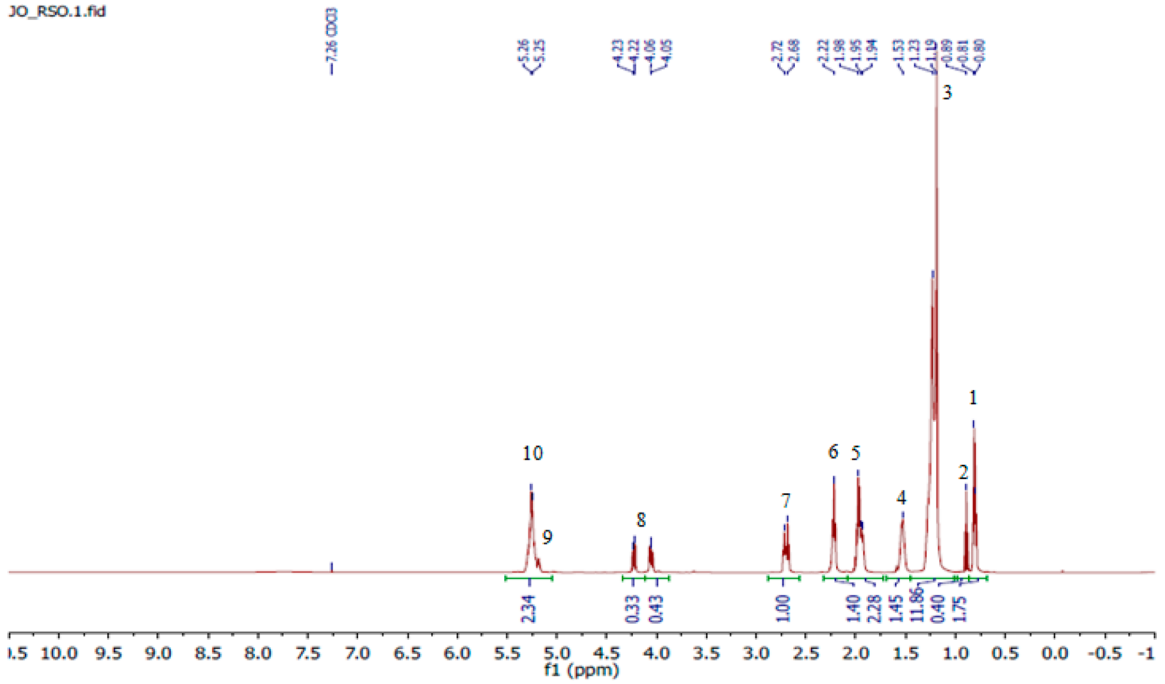

Figure 6. ^{1}H NMR spectrum of rubber seed oil.

Reshad et al.,[7] where the maximum RSO yield of 49.36 wt % was obtained from the smallest particle size (1 mm) that was considered after an 8 h extraction time. The findings of this study concur with those of Menkiti et al.,[20] who extracted *Terminalia catappa L* seed oil using *n*-hexane solvent, and obtained a yield of 60.45 wt % from the smallest kernel particle size 0.5 mm compared to the other sizes (1, 1.5, 2, and 2.5 mm) considered. In this study, the particle size 0.5 mm was used for further oil extraction for chemical analyses.

3.2. Analysis of fresh rubber seed and seed oil. The compositions of fresh rubber seed and seed oil analysis are presented in Table 1. The kernels moisture content (9.7 wt %) of the rubber seed evaluated was higher than the reported value of 6.5 wt % for yellow horn seed kernels.[19] The determined rubber seed oil moisture content is low (1.73 wt %) compared to that of castor oil (8 wt %) and shea nut oil (10 wt %),[38] *Afzelia africana* oil (2.10 wt %), and *Hura crepitans* oil (1.90 wt %)[33] High moisture content initiates the oxidation process that reduces the oil's shelf life. The low value for this oil is an indication that it could be stored for a long period without appreciable deterioration in value. The ash content of the rubber seed oil (0.001 wt %) is lower than the prescribed ASTM ash limit (0.01 wt % max) and those of *Afzelia africana* oil (0.006 wt %) and *Hura crepitans* oil (0.004 wt %).[33] The low ash value is an indication that the oil lacks trace metals that catalyze oxidation reactions that cause rancidity, high acidity, and other unpleasant characteristics during storage. The carbon residue (0.4 wt %) is close to the ASTM standard limit of 0.35 wt % max for diesel fuels,[28] but higher than those of other vegetable oils such as sunflower (0.03 wt %), rapeseed (0.05 wt %), and olive (0.09 wt %).[39] The high volatile content of the oil suggests that it could be a plausible source material for biodiesel synthesis for use in internal compression ignition engines.

3.3. Physico-chemical analysis of rubber seed oil. The extracted rubber seed oil was subjected to physicochemical analysis following the procedures prescribed in the AOAC (1990)[26] and ASTM methods. The results of the analyses presented in Table 2 indicate RSO as an alternative material to edible oils for commercial production of biodiesel. Such transesterification processes on esterified oil are often carried out below 65 °C and 1 atm using methanol as reactant.

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Table 5. ^{1}H NMR Spectrum Analysis of Rubber Seed Oil[a]

Signal	Chemical shift (ppm)	Functional group	Assignments
1	0.80 and 0.81	$-CH_3$ (terminal methyl protons) (saturated and oleic acids)	all acyl chains except linolenyl
2	0.89	$-CH_3$ (terminal methyl protons) (*cis*-erucic acid)	acyl chains
3	1.19 and 1.23	$-(CH_2)_n-$ (saturated methylene proton)	all acyl chains
4	1.53	$-OCO-CH_2-CH_2-$ (β-methylene protons) (carbonyl)	acyl chains
5	1.94−1.98	$-CH_2-CH=CH$ (allylic methylene protons)	all acyl chains
6	2.22	$-OCO-CH_2-$ (α-methylene protons)	acyl chains
7	2.68 and 2.72	$=HC-CH_2-CH=$ (bis-allylic methylene protons)	acyl chains
8	4.05−4.23	$-CH_2OCOR$ (methylene protons on carbons 1, 3)	glyceryl group
9	5.25	>CHOCOR (proton on carbon atom 2). It overlaps with signal 10	glyceryl group
10	5.26	$-CH=CH-$ (olefinic protons)	acyl chains

[a]The ^{1}H NMR chemical shift (2.8 ppm),[51] usually attributed to the presence of linolenic and linoleic chains ($CH=CHCH_2CH=CH$), is not present in rubber seed oil used in this study (Figure 6). This is in satisfactory agreement with the GC-MS and ^{13}C NMR analyses of the oil.

3.3.1. Color, pH, density, and specific gravity. The color of the RSO was dark brown after clarification, and it remained liquid at room temperature. The pH value of 6.0 obtained for RSO compares favorably with those reported for castor oil (6.8) and luffa cylindrica seed oil (3.93).[38] This value is an indication of the presence of a reasonable amount of free fatty acid and the advantageous utilization of the oil in soap making.[11]

The density at 25 °C was determined using a pycnometer and was found to be 0.886 g/cm^3, which implies that RSO is less than water with the absence of heavy elements. The specific gravity at 15 °C was determined as 0.909 using standard specific gravity bottles, and the °API gravity of 24.1 was calculated for the oil. Specific gravity is an indication of the

Table 6. ^{13}C NMR Analysis of Carbon Type Present in Rubber Seed Oil

Group	Carbon environment	Chemical shift, δ (ppm)	Functional group
A	aliphatic	13.91−33.97	CH_3
B	glyceryl	61.93−77.16	CH_2-OCOR
C	unsaturated alkenes	126.99−131.99	$CH=CH$
D	carbonyl	172.41−172.82	$C=O$
	carboxylic acid	178.64	COOH

Table 7. Effect of Heating on Peroxide Value, Iodine Value, and Refractive Index of Rubber Seed Oil[a]

Temp (°C)	Time (min)	Peroxide value (mequiv O_2/kg oil)	Iodine value (g I_2/100 g oil)	Refractive index @ 20 °C
100	30	4.02 ± 0.25	117.72 ± 1.10	1.4651 ± 0.013
	60	4.50 ± 0.12	117.43 ± 0.16	1.4322 ± 0.048
	120	4.81 ± 0.01	117.24 ± 0.05	1.4147 ± 0.018
	180	5.00 ± 0.22	117.02 ± 0.02	1.3514 ± 0.043
	240	5.40 ± 0.28	116.98 ± 0.66	1.3011 ± 0.001
	300	5.61 ± 0.15	116.84 ± 0.05	1.2430 ± 0.011
150	30	4.38 ± 0.04	117.56 ± 0.36	1.4630 ± 0.004
	60	4.66 ± 0.08	117.32 ± 0.16	1.4290 ± 0.001
	120	4.98 ± 0.12	117.12 ± 0.24	1.4130 ± 0.019
	180	5.24 ± 0.05	116.84 ± 0.32	1.3850 ± 0.005
	240	5.56 ± 0.35	116.60 ± 0.87	1.3651 ± 0.006
	300	5.82 ± 0.16	115.40 ± 0.84	1.3436 ± 0.002
200	30	4.62 ± 0,12	117.38 ± 0.09	1.3647 ± 0.002
	60	4.88 ± 0.08	117.02 ± 0.02	1.3230 ± 0.001
	120	5.00 ± o.12	116.61 ± 0.38	1.2991 ± 0.002
	180	5.34 ± 0.05	116.32 ± 0.31	1.2911 ± 0.001
	240	5.76 ± 0.08	115.68 ± 0.98	1.2834 ± 0.002
	300	5.94 ± 0.04	115.24 ± 0.05	1.2433 ± 0.001
250	30	4.74 ± 0.02	117.21 ± 0.50	1.3020 ± 0.002
	60	4.90 ± 0.07	116.60 ± 0.28	1.2750 ± 0.001
	120	4.98 ± 0.11	116.20 ± 0.28	1.2331 ± 0.001
	180	5.20 ± 0.07	115.40 ± 0.28	1.2311 ± 0.001
	240	5.50 ± 0.07	114.82 ± 1.32	1.2212 ± 0.005
	300	5.70 ± 0.14	114.48 ± 0.22	1.2011 ± 0.010

[a]Values are mean ± standard deviation of duplicate data.

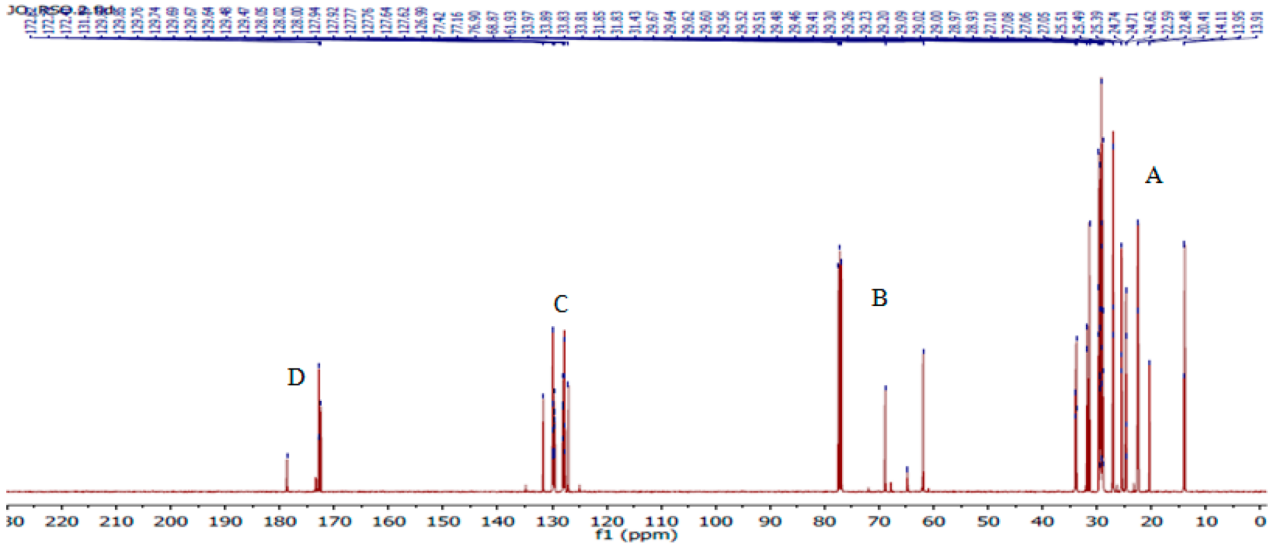

Figure 7. ^{13}C NMR spectrum of rubber seed oil.

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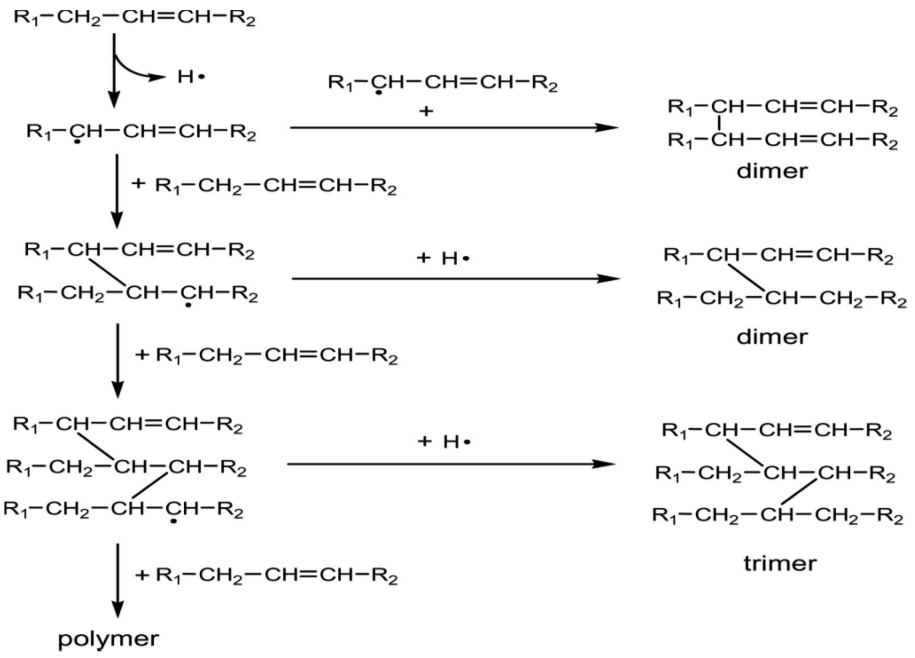

Figure 8. Acyclic polymer formation from oleic acid during thermal oxidation of oils.[34]

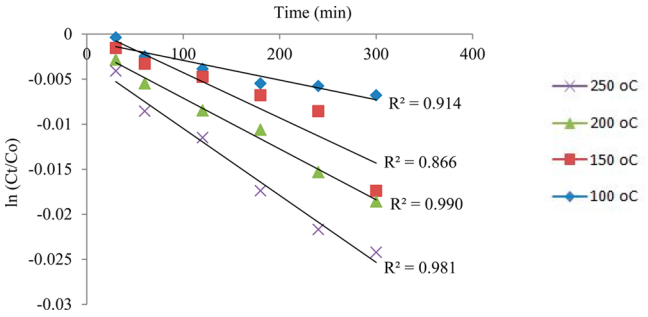

Figure 9. $\ln(C_t/C_o)$ vs time (min) for iodine values at 100, 150, 200, and 250 °C.

Table 8. Kinetic Parameters for Degradation of Rubber Seed Oil[a]

Parameter/ Temp	373 K	423 K	473 K	523 K
k (s^{-1})	2.0×10^{-3}	5.0×10^{-3}	6.0×10^{-3}	7.0×10^{-3}

[a] E_a = 13.07 kJ/mol. A_0 = 161×10^{-3} s^{-1}

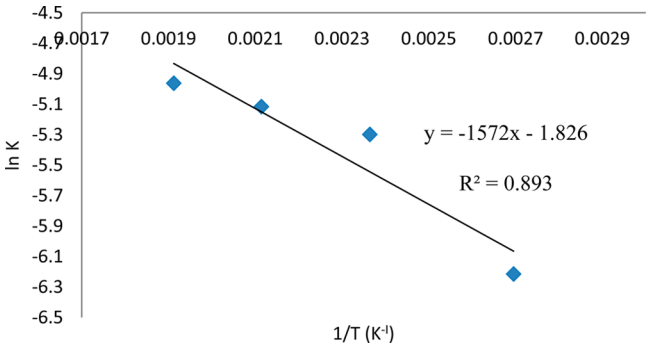

Figure 10. Arrhenius plot for kinetics of thermo-oxidative degradation of rubber seed oil.

energy content of a fuel. A fuel with a high specific gravity (low °API) has a higher heating value than a fuel with a low specific gravity (high °API). The physical characteristics seem to be similar to those of other common vegetable oils, and the oil was validated as environmentally friendly.

3.3.2. Iodine, peroxide, saponification, and acid values. The iodine value (IV) measures the degree of unsaturation in a fat or vegetable oil and determines its oxidative stability. The iodine value of RSO (137.02 g I_2/100 g oil) was estimated by Wijs' method and falls within the range specified for semidrying oils (100−150 g I_2/100 g).[38] The higher iodine value may be due to high-unsaturated fatty acid content, as observed in GC-MS analysis of oil (Table 3). The peroxide value (PV) determines the extent to which the oil has undergone rancidity when stored, heated, or in contact with air and could be used to assess the quality and stability of oils. Oils become rancid when the PV ranges from 20 to 40 mequiv O_2/kg oil.[40] The Standard Organization of Nigeria (SON, 2000)[41] and the Nigerian Industrial Standards (NIS, 1992)[42] specify a peroxide value of 10 mequiv O_2/kg oil for edible oils. The PV obtained for RSO in this study was 10.46 mequiv O_2/kg oil, and it is within the range reported in the literature for RSO (1.6−16 mequiv O_2/kg oil),[1] and close to Nigerian standards for edible oils. This indicates that RSO can be stored for a long period without deterioration. The saponification value (SV) indicates the amount of alkali required to convert the oil into soap and is an index of the average molecular mass of fatty acid in the oil sample.[39,43] As oil is mainly triglycerides, it allows for comparison of the average fatty acid chain length. The SV of 195.3 mg KOH/g RSO obtained in this study lies between those of sunflower (186) and coconut oils (265),[3] and within the range of 195−205 mg KOH/g oil for edible palm oils as specified by SON (2000)[41] and NIS (1992).[42] This shows that RSO has a lesser tendency to saponify at elevated temperatures than coconut oil. The acid value (AV) of RSO was 18.20 mg KOH/g oil, which indicates high levels of free fatty acids (9.10 wt %), a limiting factor in its use as a source of food. The acceptable limit for edible oils is ≤10 mg KOH/g oil.

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Therefore, chemical re-esterification or physical refining would be required to deacidify the oil.[44] However, this value is low enough and is within the limits for industrially useful oils.[45]

3.3.3. Kinematic viscosity. Kinematic viscosity (KV) is a measure of the resistance of oil to shear. The dynamic (absolute) viscosity for the RSO was digitally determined at 40 °C using a Brookfield DV - I + Viscometer (*Model LVDV −|+*) manufactured by Brookfield Engineering Laboratories Inc., Middleboro, MA 02346 USA. It was operated at 100 rpm with spindle No. 61 and a Torque of 59.2%. The average KV of the RSO was calculated as 35.6 cP and converted to 40.18 mm^2/s based on eq 5. This is low compared to jatropha curcas L. oil (77.4 mm^2/s) but higher than that of conventional diesel (<5 mm^2/s).[7]

$$\text{Kinematic viscosity } (mm^2/s) = \frac{\text{Absolute viscosity } (cP)}{\text{Density } (g/cm^3)} \quad (5)$$

3.3.4. Cold flow properties. The flow properties of RSO were observed under low temperatures, and the parameters considered are pour point (PP), cloud point (CP), and cold filter plugging point (CFPP), respectively.

3.3.4.1. Pour point (PP). The PP is the lowest temperature at which the oil suffers from gel formation and attains semisolid state and becomes deprived of its flow ability.[31] The value of −6 °C observed for this RSO is lower than that of Jatropha oil (4 °C)[7,46] and castor oil (−2 °C),[7] comparable to waste cooking oil (−7 °C), but higher compared to canola oil (−18 °C).[7] The value obtained is close to the reported value of −9 °C for RSO.[3] This suggests that it can be employed under cold climatic conditions as a source material for a lubricating oil formulation.

3.3.4.2. Cloud point (CP). CP is the temperature at which a cloud or haze first appears in a liquid when cooled under carefully controlled conditions. The observed CP for RSO was 5.5 °C. This value is lower than 10−11 °C reported for Jatropha oil.[46] This suggests that RSO is mainly unsaturated and a good source material for biodiesel synthesis for use in cold regions compared to Jatropha oil.

3.3.4.3. Cold filter plugging point (CFPP). The CFPP measures the lowest temperature at which a fuel gives trouble-free flow in a fuel system. The CFPP value of −0.025 was estimated from eq 6 cited by Verma et al.[31]

$$CFPP = 0.8537 \times CP - 4.72 \quad (6)$$

The low CFPP confirms that the RSO is mainly unsaturated fatty acids and its use is favorable in cold climatic regions.

3.3.5. Aniline point. The aniline point (AP) of oil is defined as the minimum temperature at which equal volumes of aniline and oil are miscible. It is a measure of the aromatic content of oil and an evaluating factor for diesel index determination and cetane number. At room temperature (25 °C), complete miscibility of RSO with an equal volume of aniline was observed. The oil was cooled to 10 °C in an ice-bath and mixed with an equal volume of aniline to form a 2-phase mixture. The mixture was heated gradually until complete miscibility was observed at 21 °C (69.8 °F) as the aniline point.

3.3.6. Boiling, freezing, flash and fire points. The boiling point was determined as 119 °C using a Stanhope−Setastill apparatus (*Model: 11860-3U*). This suggests that moisture present could be removed at 105 °C without affecting its properties prior to industrial applications such as biodiesel and lubricant synthesis. The freezing point was observed at −18 °C using Stanhope-Seta Cloud and Pour Point Refrigerator Surrey, England (Test Bath No. 4) calibrated to −51 °C. The fire and flash points were determined by using Stanhope-Seta Pensky-Martens (A and B closed cup-Model 34100-2/34000-0U) multiflash equipment. The flash and fire points were observed as 240.3 and 256 °C, respectively. This shows that the oil can be stored safely at room temperature.

3.3.7. Refractive index (RI). The refractive index (RI) is the ratio of the velocity of light in vacuum to the velocity of light in a medium (in this case, RSO) and an indication of the level of saturation of the oil. RI was determined at 20 °C using an Abbe Refractometer (*Model: 60/ED*) and a calibrated chart. The value of 1.4707 calculated for RSO falls within the range reported in the literature for vegetable oils.[7]

3.3.8. Other parameters. Other parameters determined for RSO are cetane number, diesel index, higher heating value, and average molecular weight.

3.3.8.1. Cetane number (CN). Cetane number is a measure of the tendency of a fuel to knock in a diesel engine. It is an indication of the ignition quality of a fuel. CN for RSO was calculated as 43.42 using the correlation developed by Mofijur et al.[27] (eq 7).

$$CN = 46.3 + (5458/SV) - (0.225 \times IV) \quad (7)$$

This value compares favorably with those reported in the literature (45−49.73) for RSO.[1] However, with the recent price instability of crude oil in the world markets, there is renewed interest in the use of vegetable oils such as RSO to produce biodiesel for use in diesel engines.

3.3.8.2. Diesel index (DI). The diesel index of RSO is computed using eq 8 as follows:

$$\text{Diesel index } (DI) = \frac{\text{Aniline point } (°F) \times \text{API gravity } (60°F)}{100} \quad (8)$$

The value 15.71 obtained is below the minimum standard of 50 specified for diesel fuel. Therefore, this oil cannot be used directly on diesel engines without chemical conversion.

3.3.8.3. Higher heating value (HHV). The HHV of vegetable oils can be calculated by using SV and IV obtained from simple chemical analyses using common laboratory equipment. In this study, a simple correlation (R^2 = 0.9999) developed by Demirbas[28] (eq 9) was used to estimate the HHV for RSO.

$$HHV\ (MJ/kg) = 49.43 - [0.041(SV) + 0.015(IV)] \quad (9)$$

The value obtained (39.37 MJ/kg) is within the range reported in the literature for RSO (36.1−44 MJ/kg)[1,7] and comparable to those reported for jatropha curcas L. oil (38.66).[47]

3.3.8.4. Average molecular weight of rubber seed oil. Vegetable oils are mainly triglycerides of three fatty acid chains with a glycerol backbone. The average molecular weight of vegetable oils can be estimated using simple analytical processes in laboratories. Higher molecular mass indicates higher heating values for the oil. The determination of the molecular weight of vegetable oil is important for biodiesel production reactions because the quantity of reagents used is based on the molecular weight of the vegetable oil. The average molecular weight of the RSO was calculated using eq 10 reported by Fillières et al.[30]

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$$\text{Avg molecular weight of oil } (g/mol) = 3M_{w_{FA}} + M_{w_{gly}} - 3M_{w_{water}} \quad (10)$$

where M_{WFA} is the mean molecular weight of fatty acids present, M_{Wgly} is the molecular weight of glycerol (92.09), and M_{Wwater} is the molecular weight of water (18.01).

The mean molecular weight of fatty acids of RSO was estimated from eq 11 reported by Ajiwe et al.[29] and calculated as 286.74.

$$\text{Mean molecular weight of fatty acids} = (56/SV) \times 1000 \quad (11)$$

The average molecular weight of the RSO estimated from eq 10 is 898 g/mol. The value obtained compares favorably with those reported in the literature for sunflower oil (876), rapeseed oil (992), olive oil (857), and used frying oil (882).[39]

3.4. Fatty acid profile of rubber seed oil. Gas chromatography−mass spectrometry (GC-MS) analysis was employed to characterize the oil to determine its fatty acid profile. The identification of compounds and their structures was based on data from the NIST library. The results presented in Table 3 and Figure 2 indicate that the oil is mainly unsaturated. The fatty acids present are palmitic (13.85%), stearic (16.82%), oleic (64.11%), and *cis*-erucic (5.22%). The total unsaturated fatty acid composition of the RSO was 69.33% with a saturated content of 30.67%. Although the composition of this oil did not follow the trend of some of the reported fatty acid profile for RSO in the literature,[1] genetic variations could be responsible for the disparity observed in the fatty acid profile of seed oil from the NIG 800 clonal series of Nigeria rubber seeds. The high content of oleic acid and the presence of *cis*-erucic acid indicate the potential use of seed oils from clone NIG800 series as a source of oleochemicals. This could replace the oleochemicals from petroleum currently used for the oleochemical and steel industries. The mean molecular weight of fatty acids present in the RSO used in this study was calculated as 280.81 using eq 12. This value compares favorably with 286.74 obtained using the chemical analysis method earlier shown in eq 11 with 2.06% deviation.

$$\text{Mean molecular weight of fatty acids} = \frac{\sum f_i}{\sum \frac{f_i}{M_{wi}}} \quad (12)$$

where f_i = % composition of fatty acid from GC-MS analysis and M_{wi} = molecular weight of a fatty acid.

3.5. FT-IR and NMR spectroscopy of rubber seed oil. The structural characterizations of RSO were further performed by FT-IR, ^{1}H NMR, and ^{13}C NMR analyses to confirm results from GC-MS analysis. These analyses are essential for industrial applications of the oil.

3.5.1. FT-IR analysis of rubber seed oil. Further insight into the chemical composition of RSO was provided by the FTIR spectrum at room temperature (28 °C) based on Table S1. It has been reported that very low proportions of compounds present in a mixture exhibit very weak bands which are not detectable in the IR spectrum.[21] The absorption bands of vegetable oils at intervals of 3200 and 3600 cm^{-1} are usually due to the O−H stretching vibrations of alcohols, carboxylic acids, and hydroperoxides.[33] These bands range is conspicuously absent at room temperature for the oil under study. This indicates the absence of a free hydroxyl functional group (O−H) for alcohols, phenols, and primary and secondary oxidation products for the extracted oil, as shown in the FTIR spectrum (Figure 5). Data showed that triglyceride (TG) was the main component in this RSO. The strong absorption band of the ester carbonyl functional group of TG C═O was observed around 1742 cm^{-1}. The stretching vibration of C−O at 1160 cm^{-1} is attributed to the presence of ester groups. The FTIR spectrum presents a fingerprint region (1461−585 cm^{-1}) that can be used as an analytical tool to detect RSO adulteration. Table 4 shows the functional groups and modes of vibration present in this RSO at room temperature.

3.5.2. ^{1}H NMR analysis of rubber seed oil. The ^{1}H NMR spectrum of RSO shown in Figure 6, with ten different signals of variably significant intensity depending on the proportions of the different acyl groups, is comparable with those of most vegetable oils reported in the literature[48] and with that of RSO reported by other researchers.[7,49,50] The signals are due to the protons of the triacylglycerols and are proportional to the number of hydrogen atoms of each kind that are present in the oil sample. The assignments of the signals of the RSO spectrum to the different kinds of hydrogen atoms of the acyl chains based on the literature provided by Table S2[48] are presented in Table 5.

Signal 1 (Figure 6, Table 5) is produced by overlapping of the doublet signals of the methyl group protons of the saturated and omega-9 (i.e., oleic acid) acyl groups, and it appears at 0.80 and 0.81 ppm, respectively. Signal 2 is a singlet due to methyl the protons of the erucyl group (i.e., *cis*-erucic acid) and appears at 0.89 ppm. Signal 3 is due to the protons of the saturated methylene group of all acyl chains and appears at 1.19 and 1.23 ppm. Signals 4 and 6 are due to methylene protons in the β and α positions and appear at 1.53 and 2.22 ppm, respectively. Signal 5, between 1.94 and 1.98, is due to α-methylene protons in relation to a single double bond (allylic protons). Signal 7, an overlapping signal between 2.68 and 2.72 ppm, is due to signals from α-methylene protons in relation to double bonds (biallylic protons). Signal 8, which appears between 4.05 and 4.23 ppm, is due to the protons on carbon atoms 1 and 3 of the glyceryl group. Signal 9, which appears at 5.25, is due to the proton on carbon atom 2 of the glyceryl group that overlaps with signal 10 (olefinic protons of different acyl groups) at 5.26 ppm.

3.5.3. ^{13}C NMR analysis of rubber seed oil. The ^{13}C NMR spectrum for the oil under study is depicted in Figure 7 and Figure S3. The spectrum could be grouped into four distinct spectral regions (A, B, C, and D) as presented in Table 6. The results are similar to previous studies by other researchers on RSO.[49] When the ^{13}C NMR is compared with spectra in Figure S4,[51] it shows the possible absence of linolenic and linoleic acids as confirmed by the data from GC-MS analysis (Table 3).

3.6. Kinetics, hydrolysis, and byproducts of thermo-oxidative degradation of rubber seed oil. When vegetable oils are heated under extreme conditions experienced in frying or in industrial applications, they undergo thermal oxidative reactions that are reflected in remarkable changes in peroxide, iodine, and refractive index values, respectively.[22] The rate of production of peroxide in *Hevea brasiliensis* seed oil increases with temperature and time (Table 7). This shows that rubber seed oil undergoes thermal degradation that results in oxidative rancidity and formation of unstable hydroperoxides. Moisture present in RSO hastens its oxidation process (hydrolysis) that generates free fatty acids (FFAs) and their oxidized compounds, as depicted in Figure 3. During thermal hydrolysis, the oil decomposed to produce di- and monoacylglycerol,

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glycerol, and FFAs that accelerate further the hydrolysis of the oil. The major thermal degraded byproducts of RSO are low molecular volatile compounds, such as aldehydes, ketones, carboxylic acids, and short chain alkanes and alkenes (Tables 4, 5, and 6) and nonvolatile dimers and polymers. The RSO used in this study is rich in monounsaturated oleic acids (64.11 wt %) and could form dehydroxydimer, ketohydrodimer, monohydrodimer, and dehydrodimer of oleic acid. Dimers and polymers are large molecules with the molecular weight range 692−1600 Da. These compounds are formed during thermal oxidation by a combination of −C−C−, −C−O−C−, and −C−O−O−C− bonds and have hydroperoxy, epoxy, hydroxy, and carbonyl groups, and −C−O−CC and −C−O−O−C− linkages.[34] The aforementioned groups and linkages were present in the oil used for this study as deduced from FTIR and NMR results (Tables 4, 5, and 6). This establishes the possibility of generating dimers and polymers from RSO at the elevated temperatures considered in the kinetic study. The mechanism for the formation of acyclic polymers generated from the oleic acid component of the RSO during thermal degradation is shown in Figure 8. The degradation process also results in loss of unsaturation (iodine value) in the fatty acids as shown in Table 7. The reaction follows first order as depicted in Figure 9. The double bonds in vegetable oils increase the refractive index, and the progressive decrease in refractive index on heating as seen in Table 7 confirms loss of unsaturation. The kinetic parameters for degradation of RSO at 100, 150, 200, and 250 °C are given in Table 8. The Arrhenius plot ($\ln k$ vs $1/T$) for the degradation kinetics in the temperature range 100−250 °C is shown in Figure 10 with coefficient of determination R^2 equals 0.893. The activation energy (E_a) and the pre-exponential factor determined are 13.07 kJ/mol and 161 × 10^{-3} s^{-1}, respectively. The high activation energy suggests that the oil is less susceptible to thermal deterioration than other vegetable oils, such as *Hura crepitans* seed oil (E_a = 1.989 kJ/mol).[22] The proposed Arrhenius equation for the kinetics of degradation of rubber seed oil in the temperature range 100−250 °C is given in eq 13.

$$k\ (s^{-1}) = 161 \times 10^{-3} \exp(-13.07/RT) \qquad (13)$$

4. CONCLUSIONS

With the current instability in petroleum prices and uncertainties concerning petroleum availability in the future, there is renewed interest in industrial use of vegetable oils. Nonedible rubber seed oil that does not compete with food uses was chemically extracted from underutilized seeds of Nigerian NIG800 rubber plantation clones. The oil was characterized and found to possess several potential industrial applications. The physicochemical properties determined were in the range previously reported by researchers for rubber seed oil. The IR and NMR spectra of rubber seed oil confirm the presence of both saturated and unsaturated long chain fatty acid groups in the oil, as seen in other vegetable oils. The high content of oleic acid (64.11%) and the presence of *cis*-erucic acid (5.22%) indicate their potential use as source materials for oleochemical and steel industries in the future. The kinetics of thermal oxidative degradation of the oil using a chemical analysis method follows a first-order reaction with coefficients of determination greater than 80%. A closer examination of the results of the NMR (Figures S3 and S4, and Tables S2 and 5) and GC-MS (Figure 2 and Table 3) shows a satisfactory agreement that these genetically modified high oleic rubber seeds have and insignificant proportion of polyunsaturated fatty acids (linoleic, linolenic, etc.). This insignificant presence of polyunsaturated fatty acids, and high oleic acid content support higher thermal stability and slow rate of oxidation of the oil compared to other vegetable oils. The high activation energy (13.07 kJ/mol) suggests that the oil is less susceptible to thermal deterioration than other vegetable oils, such as *Hura crepitans* seed oil (E_a = 1.989 kJ/mol). The results show that the oil is thermally stable and its properties are retained up to 250 °C.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.energyfuels.6b02267.

Infrared group absorption frequencies (Table S1). Assignment of signals of ^{1}H NMR spectra for vegetable oils—hazenut and walnut (Table S2). Rubber plantation and seeds in various processing stages (Figure S1). *n*-Hexane-Soxhlet extraction processes (Figure S2). ^{13}C NMR spectra of rubber seed oil in the study (Figure S3). Double bond peaks in the ^{13}C NMR spectrum for pure oleic acid, linoleic acid, and linolenic acid. Peaks for each characteristic double bond carbon are assigned the letters **A** for the peak found in all acids, **B** for the peak found in both linoleic and linolenic acid, and **C** and **C′** for the peaks found only in linolenic acid (Figure S4).[51] (PDF)

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Notes

The authors declare no competing financial interest.

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SUSTAINABLE PROCESSING OF RUBBER (HEVEA BRASILIENSIS) SEED OIL: PHYSICOCHEMICAL INSIGHTS INTO EXTRACTION AND ANTIOXIDANT PRESERVATION

In vitro antioxidant extracts evaluation from the residue of the Hevea brasiliensis seed. / Oleinik, G., Dario, P. P., de Morais Gasperin, K., Benvegnú, D. M., Lima, F. O., Soares, L. C., & Gallina, A. L.

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In vitro antioxidant extracts evaluation from the residue of the *Hevea brasiliensis* seed

Giovanna Oleinik[1], Priscila Paola Dario[2], Katiane de Morais Gasperin[3], Dalila Moter Benvegnú[1], Fernanda Oliveira Lima[1], Letiére Cabreira Soares[1] & André Lazarin Gallina[4]✉

The antioxidants used in the food industry are essential to inhibit the formation of free radicals, preserving the existing properties in the different matrices. However, the insecurity of the synthetic antioxidants regarding human health propels search for natural substrates with potential antioxidant activity as an alternative to synthetic compounds. In this way, the work had as objective obtaining extracts from the seed pomace of the *Hevea brasiliensis* (rubber tree), relating the contents of flavonoids and total phenols in the application as an antioxidant. The methodology consisted of the extraction using four solvents, varying extractive methods, time, and seed concentrations. The antioxidant activity in vitro was evaluated by capturing the DPPH (2,2-diphenyl-1-picryl-hydrazil) radical. The optimized results demonstrate that the aqueous extracts produced in the Soxhlet in the concentrations of 85 g L^{-1} and retention time of 4 h reached 37.73 ± 1.69% in the antioxidant tests of the free radical DPPH capture, 1405.15 mg EAC 100 g^{-1} in the quantification of phenolic compounds and 223.34 mg 100 g^{-1} of total flavonoids. Thus, this work may contribute to the realization of studies and future research for characterization and identification concerning which phenolic compounds and flavonoids attribute the antioxidant characteristic to the extracts produced, enabling the discovery of products with high added value in the production chain. In addition, because the water used as a solvent showed greater antioxidant potential between the extracts, the non-toxic and environmentally friendly character is highlighted, allowing a wide variety of applications in the food industry.

Antioxidants accompany the history of life's emergence on earth. In this way, due to the inappropriate environment with extreme conditions responsible for the free radical formation, the antioxidants were fundamental for the complex molecules constitution[1]. It could capture and stabilize free radicals in the oxidation process, and according to their source, they can be classified as synthetic, petroleum-derived, and natural from biomass[2].

Synthetic antioxidants are used as preservatives to prolong the products shelf life, highlighting the butylated hydroxyanisole (BHA), the butylated hydroxytoluene (BHT), the tertiary butylhydroquinone (TBHQ), and the propyl gallate (PG), which can exhibit, besides the antioxidant potential by capturing free radicals via hydrogen transfer, the chelating effect of metals. Although widely used by the food industry in Brazil, the application of synthetic antioxidants is argued due to the evidence that these substances' continued consumption can pose health risks[3].

Currently, in the food sector, the oxidation process of oils and fats poses economic challenges since oxidation leads to the appearance of unpleasant tastes and odors, the reduction of the nutritional properties, and the formation of toxic compounds for humans, which can cause cerebrovascular accidents, and cancer[4]. Despite its use, animal toxicological studies show synthetic antioxidants related to carcinogenic effects, increasingly putting natural antioxidants as an alternative[5].

Regarding antioxidants from renewable raw materials, industrial by-products and food waste show potential for conversion into bioproducts with antioxidant properties, with the challenges caused by the availability of the material, the degree of toxicity of the extract obtained, and the search for methodologies that allow the greater extraction efficiency[6,7].

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Variables|levels	− 1	0	+ 1
Extraction time (h)	2	4	6
Extract concentration (g L^{-1})	20	40	60

Table 1. Levels and variables used in the experimental design.

In the Brazilian context, the use of rubber seed bagasse *Hevea brasiliensis* to obtain antioxidant extracts is a promising alternative. The extensive cultivation of the species in Brazil, include the states of Amazonas, São Paulo, Espírito Santo, Mato Grosso, Mato Grosso do Sul, Goiás, Bahia and Paraná[8]. The biorefinery concept could be applied to better use the substrate, which integrates processes for converting the raw material into compounds with added value[9]. With waste generated by the food, beverage, feed, and agriculture industries, the raw material is the most suitable for the biorefinery approach due to the constancy of supply, size, and nutritional content[10].

From the above, to increase the applications of the same matrix for different purposes, the use of tree seed coproduct from *Hevea brasiliensis* becomes to besides latex exploitation also, an additional value for farmers who cultivate this species. Moreover, aspects such as the circular economy and the maximum use of the matrix during the production chain stages could reduce dependence on fossil sources[6]. Therefore, the concepts of circular economy and bioeconomy come together with common goals of adding value to waste[11].

The current use of rubber trees consists mainly of extracting the latex used for the production of natural rubber, obtaining the oil from the seeds destined for the paint and varnish industry sector, and, after the latex production cycle, the exploration of the wood[8]. Additionally, there are studies on *Hevea Brasiliensis* latex as a bioactive material in tissue repair in cattle. For this purpose, Nellore cattle were submitted to experimental subcutaneous implants of fragments of natural latex membranes[12]. Seeking to obtain products with greater added value from *Hevea brasiliensis*, Raknam, Pinsuwan, and Amnuaikit[13] investigated different methods of extracting rubber seed oil as an unconventional oil source for cosmetic products. Fawole et al.[14] produced a protein isolate from a defatted rubber seed meal. Hassan et al.[15] performed the characterization of rubber seed bark and rubber seed to make biofuel production. Widyarani et al.[16] investigated protein and oil production methods from rubber tree seed kernels, focusing on protein recovery. Despite the numerous applications, the literature lacks research exploring the proprieties antioxidant from coproduct of this tree seed.

However, the importance of using plant extracts is a long-standing one, being the first reports of this use as antioxidant related in Ancient Egypt, where one of the factors contributing to the mummification process was this activity attributed to secondary plant metabolites[1], including vitamins C and E, carotenoids, and phenolic compounds[1,17]. The literature presents numerous studies using different extracts and matrices that correlate the antioxidant activity with the concentration of phenols and flavonoids[18] and the composition of polyphenols with in vitro antioxidant capacity[19]. In addition, many studies quantify the content of total phenolics[20–24] and flavonoids in plant matrices[22,25,26].

From the above, considering the importance of the Hevea brasiliensis species in the Brazilian context, no studies refer to the antioxidant potential of this seed bagasse. Therefore, the challenges concerned synthetic antioxidants and the need to dispose of waste from the production chain with potential for application in rubber tree bioproducts, the present work carried out the study of the optimal extraction conditions for obtaining extracts with in vitro antioxidant potential, considering the presence of total phenols and flavonoids. The varied parameters were different concentrations of rubber seed bagasse, solvents, methods, and extraction times, suggesting apply these antioxidant extracts in the food, pharmaceutical, and bioenergy fields. This study may open paths for research as characterization of extracts with greater potential and identified which phenolic and flavonoid compounds provide this antioxidant characteristic for each extract, valorizing the native species.

Materials and methods

Plant materials. The use of plant material in the study complies with relevant institutional, national, and international guidelines and legislation. The samples of rubber seed *Hevea brasiliensis* were collected from the city of Paranaíba, Mato Grosso do Sul, Brazil, by the Kaiser Agropecuária Company, which plants rubber trees. The seeds that fell to the ground were later collected by the company and sent to the Federal University of Fronteira Sul in Realeza. The seeds of *Hevea brasiliensis* would be discarded. However, they were used for research. This research using *Hevea brasiliensis* seed was registered in SisGen (National System for the Management of Genetic Heritage and Associated Traditional Knowledge) under registration number A4C9E3E. The raw material was crushed in a Britannia brand BPM900P multiprocessor to standardize the size of the particles and facilitate oil extraction. Then, the substrate of this extraction called bagasse was used in the experiment.

Preparation of antioxidant extracts. The extracts were obtained using two extraction methods (infusion and Soxhlet) with different solvents (water, methanol 99.5%, absolute ethanol, and hexane). The solvent was heated to boiling temperature in the first method and then added to the raw material. This mixture was stored in a closed environment, a period described in the experimental planning. For the second method, the sample was inserted into the Soxhlet apparatus[17].

Experimental design. The experimental design for the statistical study considered the two extraction methods and four different solvents as descriptive variables. Regarding the quantitative variables, the extraction time and the concentrations of the rubber seed residue were selected, as described in Table 1. The tests were

performed in duplicate, and the antioxidant capacity was obtained by the percentage of the capture of the DPPH radical (2,2-diphenyl-1-picryl-hydrazil), determination of flavonoids, and total phenols.

Determination of the extracts antioxidant activity. The method for determining the total antioxidant activity consists of the reduction reaction of the DPPH organic free radical, which presents a violet color, forming the yellow-colored compound 2,2-diphenyl-1-picrylhydrazine (DPPH-H). The elimination of DPPH results in a decrease in absorbance (A) at a wavelength of 515 nm[27].

To carry out this method of determining antioxidant activity, first, the 0.1 mmol L^{-1} DPPH ethanolic solution was prepared and subsequently diluted in ethanol by a factor of 10. The determinations were carried out with the addition of 2.7 mL of DPPH in 0.3 mL of the solvent used in the extract for the control ($A_{control}$).

Regarding the samples, the procedure was repeated. However, instead of the solvent, 0.3 mL of the obtained extract (A_{sample}) was added, and, as a blank for the samples, extract, and ethanol was added. The sample blank was used to obtain xr7the final absorbance of the DPPH reduction only. Afterward, the absorbance was analyzed using a Thermo Scientific brand UV/VIS spectrophotometer, model Evolution 201, at a wavelength of 515 nm after 30 min of reaction. All assays were performed in duplicate. The percentage of DPPH free radical capture corresponding to antioxidant activity (AA_{DPPH}) was performed based on Eq. (1)[28].

$$\%AA = \frac{A_{control} - A_{sample}}{A_{control}} 100 \quad (1)$$

where $A_{control}$ is the absorbance of the DPPH control solution and A_{sample} the absorbance of DPPH with the respective sample.

Determination of the total flavonoids. For total flavonoids quantification, a solution was prepared by adding 0.32 mL of the extract of interest, 0.32 mL of aluminum chloride solution ($AlCl_3$), 2% (m/v), and 3.36 mL of ethanol P.A. After 25 min, absorbance was measured in triplicate, through a spectrophotometer at a wavelength of 413 and 427 nm, using 0.32 mL of just sample solvent, 0.32 mL of $AlCl_3$ solution and 3.36 mL of ethanol as a blank.

In the analytical curve, the procedure described above was repeated using standard rutin solutions in 60% ethanol, at concentrations from 0.05 to 0.5 mg L^{-1}, with a variation of 0.05 mg L^{-1} at each point, to analyze the extracts[29]. The equation of the line was $y = 1.32860606x - 0.00746667$ with $R^2 = 0.98185$.

Determination of the total phenolics. Phenolic compounds can capture free radicals and metal chelators, working at the beginning of oxidation and the propagation process. According to the methodology described by Sousa et al.[30] for determining total phenolics in a solution containing 0.1 mL of the extract, 2.5 mL of 0.1 mol L^{-1} Folin Ciocalteau solution and 2.0 mL of saturated sodium carbonate (Na_2CO_3) solution were added. To analyze the absorbance of the samples, the tests were stored on a bench for a period of 1 h, and then measurements were performed in triplicate at a wavelength of 720 nm, using water as a blank.

In the construction of the analytical curve, gallic acid was used as a standard at concentrations of 0.1; up to 4.0 mg L^{-1}, with variations of 0.5 mg L^{-1}, and then, determining the total phenolic content in mg of gallic acid per 100 g^{-1} of the sample[31], obtaining the equation of the line equal to $y = 0.449428x + 0.020706$ with $R^2 = 0.99973$.

Physicochemical characterization of extracts: density and pH. According to the values obtained in the statistical analysis of the experimental design, the result with the highest antioxidant capacity was submitted to physical–chemical characterizations. A graduated stem densimeter from Anton Paar, model DMA 35, was used to determine the density of the extracts. The pH of the extracts was measured using a bench-top pH meter brand MS Tecnopon and model mPA 210, previously calibrated.

Statistical analysis. The t test with a confidence level of 95% performed in the Microsoft Excel® package was used as the first selection criterion for the results related to DPPH radical capture through different extraction methods and extracts. For the initial experimental design analysis, the qualitative variables (solvent and extraction method) and the quantitative variables mentioned in the experimental design were considered.

After verifying the solvent and the method with the greatest antioxidant potential, the response surface methodology was used for the condition that presented the best results, considering a 2^3 planning using Design Expert® software with the levels and quantitative variables previously described.

In the validation process of the equation represented by the model, the residual dispersion graphs and the analysis of variance table (ANOVA) were considered. The percentage of variation was calculated from the latter, and maximum deviation explained R^2 the F value of the regression F_R and the lack of adjustment F_{la}, using Eqs. (2), (3), (4) and (5)[32], respectively. Also, Pearson's correlation test was performed in the Statistica 13 software®, where the data were correlated with different methods, time, substrate concentration, % DPPH, flavonoids, and phenols results.

$$R = \frac{SQ_R}{SQ_T} 100 \quad (2)$$

where SQ_R is the ratio between the quadratic sum of the regression and SQ_T the total quadratic sum.

Time (h)	Concentration (g L^{-1})	Water[a]	Methanol[b]	Ethanol[a]	Hexane[c]
Infusion[A]					
2	20	4.16 ± 0.59	8.03 ± 3.39	8.49 ± 0.26	3.51 ± 1.17
2	40	21.68 ± 0.69	–	15.12 ± 0.09	5.55 ± 0.27
2	60	20.78 ± 0.20	9.91 ± 2.07	16.87 ± 0.17	–
4	20	12.54 ± 0.29	–	13.19 ± 0.09	–
4	40	15.24 ± 0.20	1.67 ± 0.74	8.98 ± 0.26	–
4	60	20.85 ± 0.29	2.40 ± 0.44	10.18 ± 0.09	–
6	20	3.05 ± 0.08	2.71 ± 0.15	7.41 ± 0.09	–
6	40	1.45 ± 0.49	6.10 ± 0.22	7.05 ± 0.26	–
6	60	12.67 ± 0.49	8.08 ± 0.66	10.36 ± 0.00	–
Soxhlet[B]					
2	20	9.35 ± 0.69	8.71 ± 0.81	10.54 ± 0.09	–
2	40	22.65 ± 0.10	19.76 ± 1.40	8.80 ± 0.17	–
2	60	34.00 ± 0.69	13.61 ± 1.25	12.11 ± 0.09	–
4	20	19.18 ± 0.49	12.72 ± 1.92	1.27 ± 1.96	–
4	40	36.36 ± 0.88	13.56 ± 0.74	14.76 ± 1.11	–
4	60	49.45 ± 0.39	10.79 ± 1.11	–	–
6	20	6.58 ± 0.29	10.85 ± 1.03	5.06 ± 2.90	–
6	40	30.13 ± 0.49	12.20 ± 0.15	–	–
6	60	36.43 ± 1.18	19.08 ± 0.30	10.78 ± 0,26	–

Table 2. Results of the t-test, for the percentage of DPPH radical capture, for the different extracting solvents and extraction methods. – Did not show antioxidant activity. *Equal letters represent that there was no significant difference between the means, with 95% confidence.

$$R^2 = \frac{SQ_T - SQ_{ep}}{SQ_T} 100 \tag{3}$$

where SQ_{ep} corresponds to the quadratic sum of the pure error.

$$F_R = \frac{MQ_R}{MQ_r} \tag{4}$$

where MQ_R is the root mean square of the regression and MQ_r the root mean square of the residuals.

$$F_{la} = \frac{MQ_{la}}{MQ_{pe}} \tag{5}$$

where MQ_{la} is the square mean of the regression and the square mean of the residuals MQ_{pe}.

Results and discussions

Antioxidant activity of extracts using different extraction methods. Table 2 shows the data referring to the mean antioxidant activity of the tests at times of 2, 4, and 6 h, for all extracting solvents and the extraction methods with the t-test application, for comparison of means.

The t test was used to compare the test means for different solvents. Thus, all tests performed with the aqueous solvent and the other solvents were compared, as the aqueous extract was the one that presented the highest average of antioxidant activity. Thus, the t test shows no statistical difference between the solvent water and ethanol for the infusion method. Concerning water and hexane and water and methanol, there was a significant difference.

In the soxhlet method, the water solvent presents a significant difference when compared to all other solvents. Thus, water can be suggested as the best solvent, regardless of the extraction system used, since the average results were higher for this solvent, which is relevant for the study. It is justifiable that aqueous extracts have more significant evolutions since most antioxidant compounds, such as phenolics and flavonoids, are polar, having greater solubility in water. Hence, the extraction with this solvent is more effective. Also, according to Mokrani and Madani[33], the solubility of phenolic compounds is established according to the polarity of the solvent. That is, each solvent extracts a mixture of phenolic compounds. Flavonoids, for example, are extracted mainly by water, ethanol, or ethyl acetate.

Vizzotto and Pereira[34] tested solvents with little (hexane) and higher polarity (acetone, methanol, and ethanol). The results obtained by the authors corroborate that less polar solvents presented the lowest effects for the extraction of phenolic compounds, in this case, blackberry. On the contrary, polar solvents were more effective in extracting polyphenols.

Polyphenols and flavonoids can interact whit the DPPH radical by electron ou hydrogen transfer mechanism. The radical specie promotes the homolytic breaking of the H–O bond and generating radicals from polyphenols

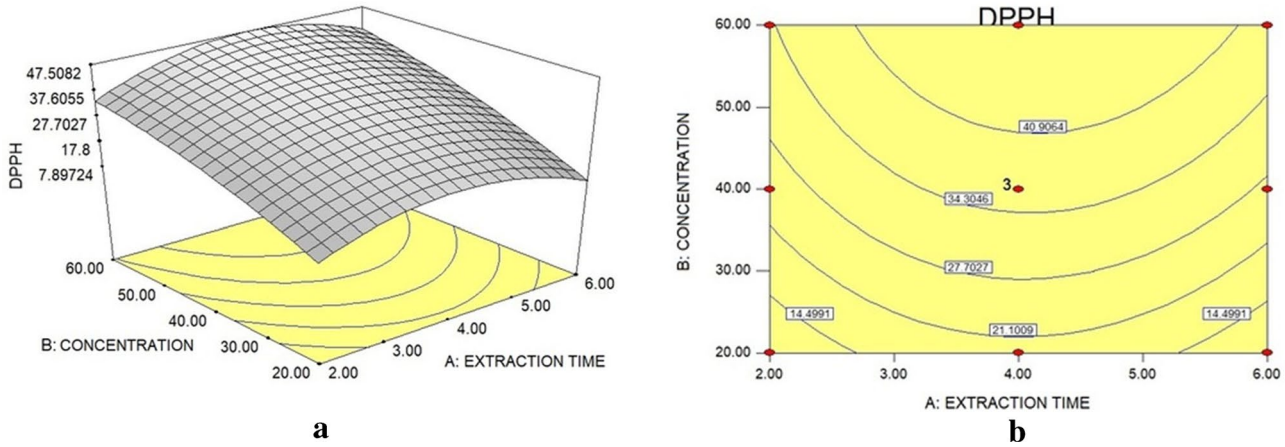

Figure 1. Response surface (**a**) and contour lines (**b**) of the DPPH statistical analysis of the aqueous extract obtained via Soxhlet.

Factor	Quadratic sum (SQ)	Degrees of freedom (DF)	Square mean (MQ)	F	*P*
Regression (R)	1628.02	5	325.60	48.52	0.0003
Time (linear)	8.50	1	8.50	1.27	0.3116
Time (quadratic)	312.46	1	312.46	46.56	0.0010
Concentration (linear)	1197.94	1	1197.94	178.53	< 0.0001
Concentration (quadratic)	25.39	1	25.39	3.78	0.1093
Interaction time × concentration	6.73	1	6.73	1	0.3624
Residue (r)	33.55	5	6.71		
Lack of adjustment (la)	30.06	3	10.02	5.75	0.1518
Pure error (ep)	3.49	2	1.74		
Total quadratic sum (SQ_T)	1661.57	10			

Table 3. ANOVA for the results statistical treatment related to the planning for obtaining extracts by Soxhlet in aqueous solvent.

and flavonoids. The new radicals are stabilized by electronic delocalization along with the aromatic units, interrupting the radical reaction propagation step[27].

Another observed relationship was temperature since the solvent that came closest to water in terms of antioxidant activity was ethanol (Table 2), which also has a specific polarity and a relatively high boiling point compared to other solvents. It can be suggested high temperatures contribute to the extraction. And several studies have shown results in which elevated temperatures favor the extraction of antioxidant compounds[35–39].

Regarding the extraction method, according to the t test results, there was a significant difference between Soxhlet and infusion, and the first one presented extracts with a greater antioxidant capacity compared to the infusion results. From the data described in Table 2, it is possible to observe for the two extraction methods, considering the same time, the highest percentage of DPPH capture occurred in the high concentrations of rubber tree seed bagasse, except hexane, which for both methods only showed efficiency for the infusion method at the extraction time of 2 h, with concentrations of 20 and 40 g L^{-1}.

The most notable advantages of the soxhlet method are the non-permanent contact of the sample with the solvent, constant renewal, avoiding solvent saturation, and the system temperature remains relatively high, as the heat applied to the evaporation process is continuous. These advantages contribute to higher antioxidant rates extracted in the soxhlet method than the infusion method, which is static and not isothermal[35–39].

Optimization of the experimental condition with the highest percentage of DPPH capture. From the conditions established in the first experimental design and the application of the t test to verify the significant difference of samples from solvents and extraction methods, it was found that the best antioxidant activity occurred using water extraction in Soxhlet. Therefore, from the extracts obtained in this condition, the response surface and the contour line graphs presented in Fig. 1a,b were calculated, seeking the pre-optimization of the performed points.

From Fig. 1b, which presents the contour lines, it is possible to observe a tendency to the optimum is using the extraction time from 4 h and extract concentration greater than 40 g L^{-1}. In this perspective, according to the response surface to describe the model, Eq. (6) was considered of the quadratic type suggested in the analysis. The ANOVA values are shown in Table 3.

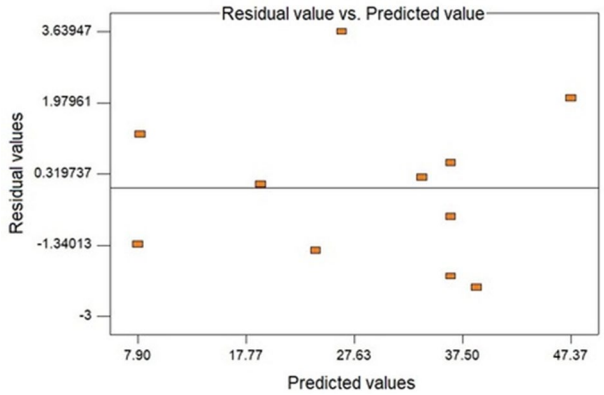

Figure 2. Plot of residuals versus predicted for the regression of the DPPH statistical analysis of the aqueous extract obtained via Soxhlet.

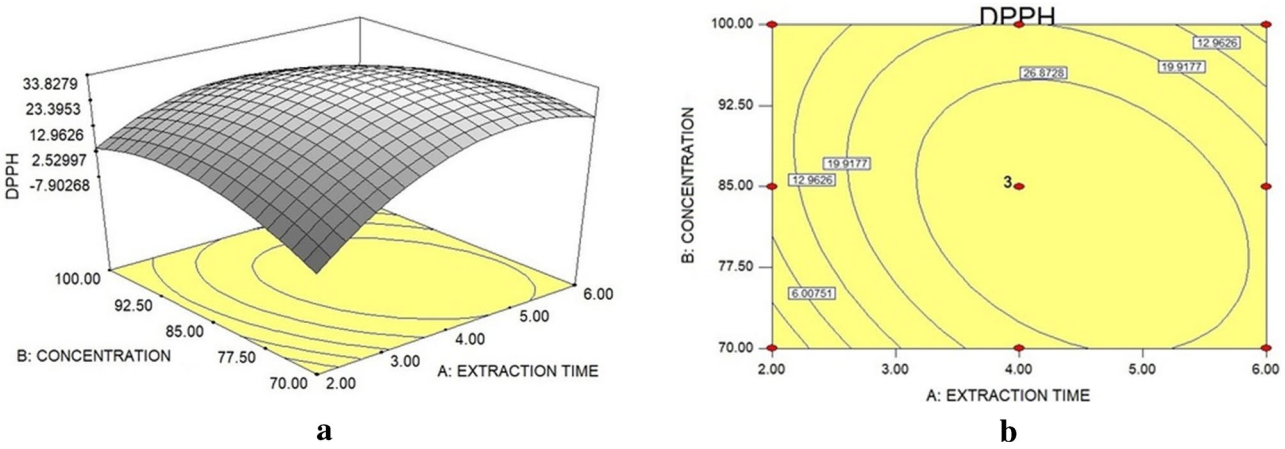

Figure 3. Response surface (**a**) and contour lines (**b**) of the DPPH statistical analysis of the aqueous extract obtained via Soxhlet for extraction optimization.

$$\begin{aligned} \text{DPPH} = &-46.13 + 21.51 \times \text{Time} + 1.21 \times \text{Concentration} - 2.78 \times \text{Time}^2 \\ &- 7.91.10^{-3} \times \text{Concentration}^2 + 0.032 \times \text{Time} \times \text{Concentration} \end{aligned} \quad (6)$$

In the validation process of the model equation for the response surface, the percentage of maximum variation explained by the quadratic equation corresponded to R = 99.79%, while the obtained variance presented $R^2 = 97.98\%$, allowing a predictive capacity with relation to the trend of the experimental points for the set of variables considered in the experiment.

Another aspect considered is the statistical significance presented by the values $F_R = 48.52$ and $F_{la} = 5.76$ for the reference for the percentage of the F 5% distribution, corresponding to 5.05 and 19.16 respectively, the first being much higher and the second smallest about the value of F_R and F_{la} obtained in the model[32].

The graph presented in Fig. 2 describes the random dispersion of the residuals, concluding that the quadratic equation used to represent the response surface according to the conditions used does not follow a trend. Therefore, a second analysis was performed looking for the path of the improved condition to obtain a greater antioxidant capacity of the extract from the aqueous solution using the Soxhlet system[24,40–42].

From the results obtained in the first response surface, the path to the optimum happened through the second experimental design with the same time values but increased concentrations to 70, 85, and 100 g L^{-1}. According to the methodology described for the generated response surface, data analysis is presented in Fig. 3a and the contour lines graph in Fig. 3b.

The result for the best antioxidant activity of the rubber tree seed bagasse present in the extract corresponded to 85 g L^{-1}, with an extraction time of 4 h. Under these conditions, the percentage of antioxidant activity via DPPH capture was 37.73 ± 1 0.69%. The fact that the highest antioxidant capacity value is obtained for an intermediate concentration can be explained by the extraction method, which consists of a purely physical process, with no chemical reaction in getting the extract, that is, the extractive release process by the penetration of the solvent into the plant matrix is one of the determining factors in the extraction[43]. Therefore, one of the hypotheses is that the increase of the vegetal matrix above the concentration of 85 g L^{-1} may have affected the penetration of the solvent and, consequently, decreasing the concentration of antioxidant compounds.

Factor	Quadratic sum (SQ)	Degrees of freedom (DF)	Square Mean (MQ)	F	*P*
Regression (R)	1923.01	5	284.60	5.10	0.0491
Time (linear)	305.31	1	305.31	4.05	0.1005
Time (quadratic)	716.76	1	716.76	9.50	0.0274
Concentration (linear)	21.55	1	21.55	0.29	0.6160
Concentration (quadratic)	289.26	1	289.26	3.83	0.1076
Interaction time × concentration	251.70	1	251.70	3.33	0.1274
Residue (r)	377.37	5	75.47		
Lack of adjustment (la)	362.78	3	120.93	16.58	0.0574
Pure error (ep)	14.59	2	7.29		
Total quadratic sum (SQ_T)	2300.38	10			

Table 4. ANOVA for the statistical treatment of the results related to the planning to obtain the optimization of the extraction.

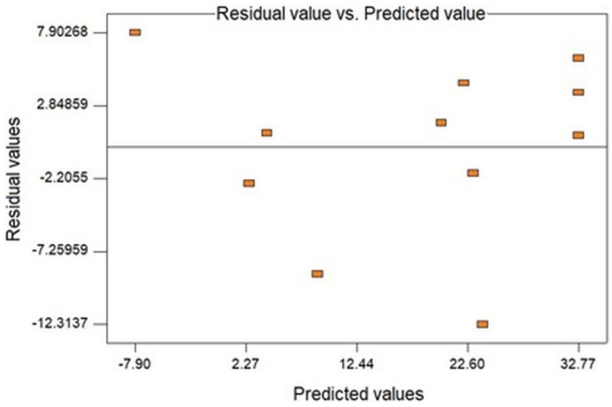

Figure 4. Graph of residues versus predicted for the regression of the DPPH statistical analysis, of the aqueous extract obtained via Soxhlet for the second planning developed.

Also, regarding the second planning, the ANOVA results are shown in Table 4, and Eq. (7) describes the quadratic model for the behavior of data according to the variables in the analysis.

$$\begin{aligned} \text{DPPH} = &-471.06 + 59.68 \times \text{Time} + 9.00 \times \text{Concentration} - 4.21 \times \text{Time}^2 \\ &- 0.05 \times \text{Concentration}^2 - 0.27 \times \text{Time} \times \text{Concentration} \end{aligned} \quad (7)$$

In the model validation for the response surface, R^2 showed 83.60% confidence, while the maximum variation reached 99.37%. In this context, the other parameters were evaluated, seeking greater reliability in predicting the results. Regarding the F_R and F_{la} values, the values found corresponded to 5.10 and 16.59. Thus, considering the percentage of the F distribution equal to 5%, these values show statistical significance.

The last parameter considered the residue scatter plot (Fig. 4), where random behavior was observed. Therefore, from the evaluation carried out, it is possible to conclude that the second model presents an adjustment to the variables selected for the analysis but with lower reliability when compared to the first planning.

According to Sousa[31], percentages lower than 50% of DPPH free radical capture are classified as weak antioxidant activity. Therefore, considering that the highest percentage of DPPH capture achieved in the second planning was 37.73%, the extracts produced with the seed of *Hevea brasiliensis* have weak antioxidant activity. However, it is essential to highlight that the substrate used to carry out the extracts is the coproduct of this tree seed.

About the literature, the study by Zain et al.[44] found a value of 34.84% of DPPH free radical capture for methanol extract of seeds of the RRIM 2025 rubber tree clone *Hevea brasiliensis*. Machado et al.[22] found for *Garcinia cochinchinensis Choisy* fruit extracts 90.6 ± 2.52% of DPPH capture. Bryan-Thomas[45] found 46.24% of antioxidant activity for *M. zapota* extract and 9.39% for *A. muricata* extract. Bispo et al.[46] evaluated the antioxidant capacity of coffee wood extracts, and the Catuaí variety showed the best results, with 34.5% of DPPH sequestration. Pereira et al.[47] evaluated the antioxidant profile of a mixed juice containing kale (*Brassica oleracea* L.), yam (*Dioscorea* spp.), and orange (*Citrus sinensis*) and presented a sequestration percentage of 94.81%. Da Silva Acácio et al.[48] found a percentage of 84.89% of DPPH free radical capture for the extract of *Melochia tomentosa L.* at a concentration of 75 μg mL^{-1}.

The antioxidant activity of rubber tree seed bagasse is weak but has higher or similar values than some other matrices, being those seeds and fruits. Considering there are no reports in the literature of using this material as

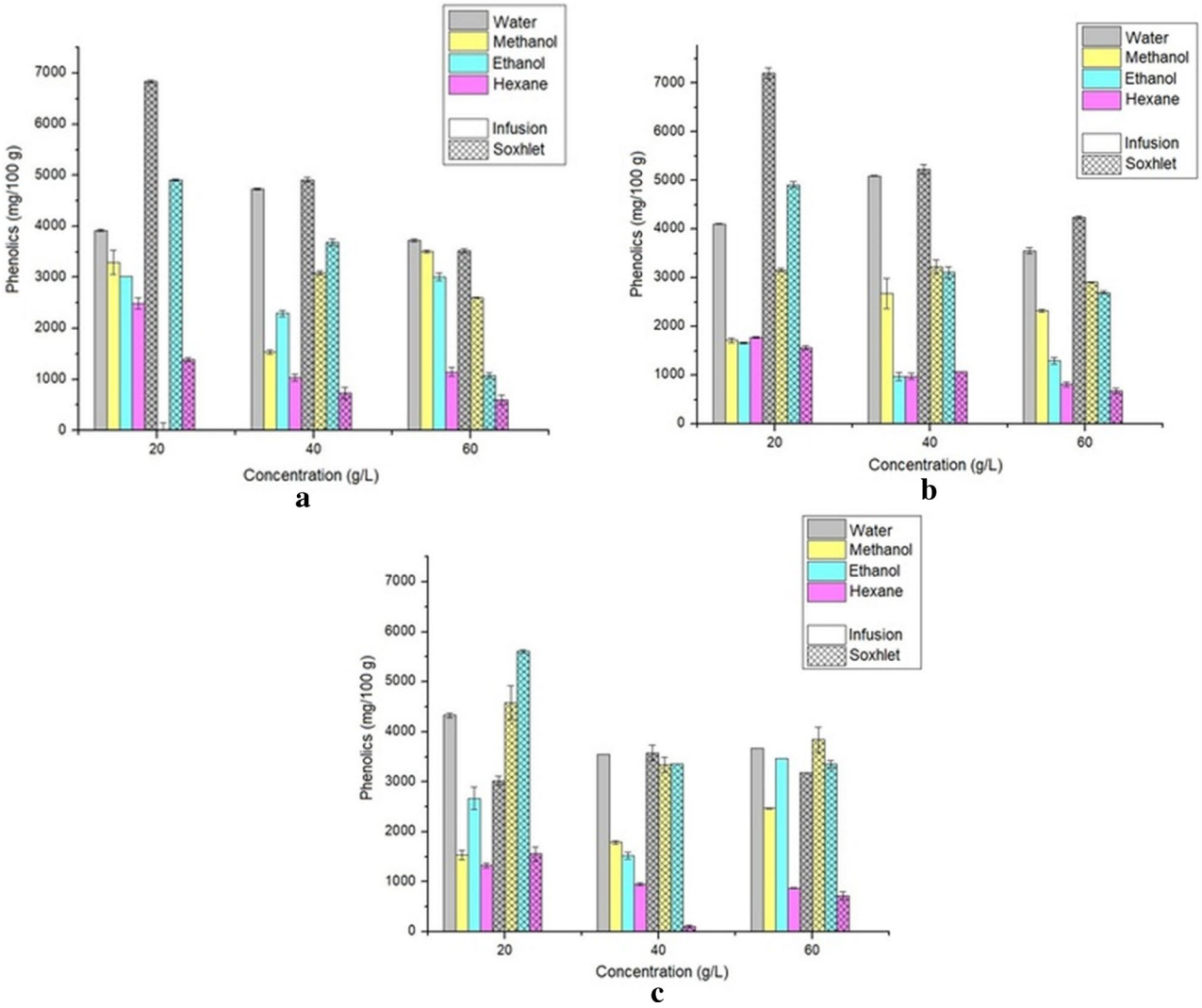

Figure 5. Concentration of total phenols using 2 h (**a**), 4 h (**b**), and 6 h (**c**) as extraction time.

an antioxidant and the low cost of acquiring the raw material because it is derived from an oil extraction process, the indices found are satisfactory and promising, adding value in the production chain.

Determination of the total phenolics and flavonoids concentration. *Total phenolics and flavonoids from the first planning.* The evaluation of the total phenolic concentration is shown in Fig. 5, where the different methods, solvents, and concentrations were analyzed according to each extraction time.

Compared with other works performed in the literature on the species, the content of total phenolic compounds is satisfactory, although there are no studies that analyze the antioxidant potential of *Hevea brasiliensis* seed bagasse. The extracts in this study reached 7201.32 mg of gallic acid equivalents 100 g^{-1} (mg GAE 100 g^{-1}) in the aqueous extract in the Soxhlet, in the concentration of 20 g L^{-1} and 4 h of extraction, while Agbai et al.[49] found for the sample of raw rubber seed meal *Hevea brasiliensis* (RRSM) a content of phenolic compounds of 2.77 ± 0.06 mg GAE g^{-1}. Ismun et al.[50] determined the polyphenol content in *Hevea brasiliensis* latex serum C and in the effluent for rubber processing, finding a 0.0393 g GAE mL^{-1} in latex serum C and a content of 0.0099 g GAE mL^{-1} in the effluent. Zain et al.[44] found content of 0.010 mg GAE mL^{-1} of phenolic compounds for the methanol extract of seeds of the rubber tree clone RRIM 2025 *Hevea brasiliensis*.

Concerning other studies with different matrices, Sarkis[23] obtained 690 mg GAE 100 g^{-1} of total phenolics for pecan extract, using a water/ethanol solution as solvent (20:80, v/v). Wang et al.[24] found 92.96 ± 1.47 mg of GAE g^{-1} of phenolic compounds for nutshell ethanol extracts. Da Silva et al.[21] evaluated the total phenols for jilo extracted with different solvents, and the optimized result was 830.6 ± 16.2 mg GAE 100 g^{-1} with extraction with 20% methanol/water (v/v). Alasalvar and Bolling[20] obtained a total of 112–310 mg 100 g^{-1} for Brazil nuts, 137–274 mg 100 g^{-1} for cashew, 47–418 mg 100 g^{-1} for almonds, 46–156 mg 100 g^{-1} for Macadamia and 1285–2016 mg 100 g^{-1} for Pecan nut. Machado et al.[22] found extracts (with 80% acetone) of the pulp of *Garcinia cochinchinensis Choisy* a total phenolic content of 469.6 ± 114.9 mg gallic acid 100 g^{-1}, and in leaves 3739.7 ± 310.5 mg gallic acid 100 g^{-1}.

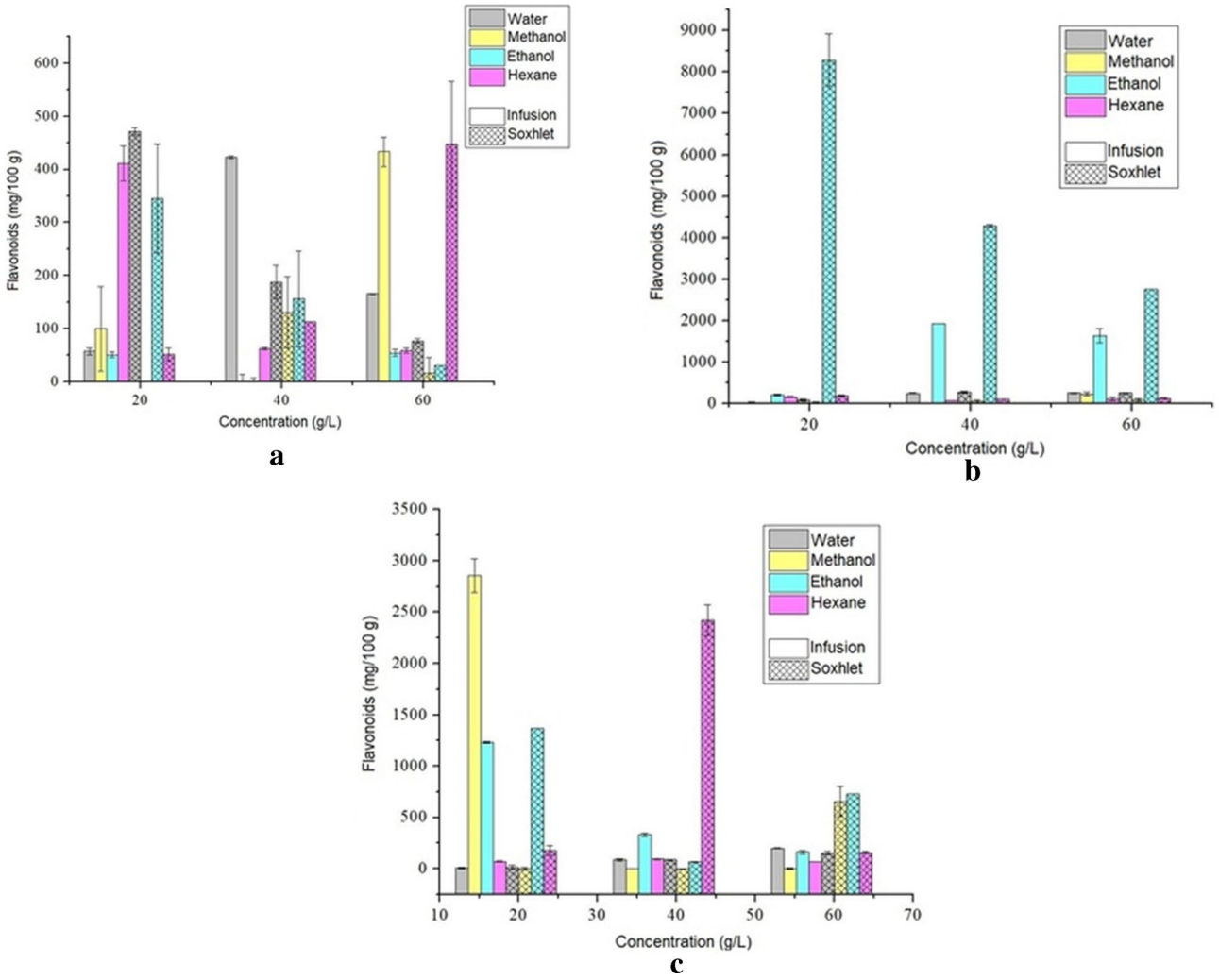

Figure 6. Total flavonoids concentration using 2 h (**a**), 4 h (**b**), and 6 h (**c**) as extraction time.

According to Assis et al.[51], phenolic compounds, particularly phenolic acids, are found mainly in higher polarity extracts. They investigated the extraction of phenolic compounds from the microalgae *Spirulina platensis* and *Chlorella pyrenoidosa* with methanol and ethanol solvents, and the methanol extracts from the two microalgae showed higher content of phenolic compounds (MES (methanolic extracts of Spirulina) = 2.62 mg GAE g^{-1}; MEC (methanolic extracts of Chlorella) = 0.69 mg GAE g^{-1}) compared to ethanol extracts (EES (ethanolic extracts of Spirulina) = 1.37 mg GAE g^{-1}; EEC (ethanolic extracts of Chlorella.) = 0.41 mg GAE g^{-1}). Manivannan et al.[52] studied methanol, diethyl ether, and hexane solvents to extract phenolic compounds from the microalgae *Chlorella marina* and obtained the best results for methanol extracts (0.64 mg GAE g^{-1}). Hajimahmoodi et al.[53] found for *Chlorella vulgaris*, 3.69–19.14 (mg GAE g^{-1}) for the aqueous fraction, 0.02–3.59 (mg GAE g^{-1}) for the aceto-ethyl fraction and 0.02–0.49 (mg GAE g^{-1}) for the hexane fraction. These results corroborate the results presented, which indicated the polar solvents extracted more phenolic compounds.

Figure 6 shows the different methods, solvents, and concentrations analyzed according to each extraction time to flavonoids.

According to the results obtained, the maximum concentration of total flavonoids was extracted using the soxhlet method in 4 h and with the ethanol solvent, reaching 8290.52 mg of rutin 100 g^{-1}. For the aqueous extract, the best extraction method was Soxhlet at a concentration of 20 g L^{-1} and 2 h, with a value of 471.23 mg of rutin 100 g^{-1}. The extract from the first planning, which showed more significant activity in DPPH capture (4 h of extraction, Soxhlet, 60 g L^{-1}, water as solvent), has a significantly higher concentration of phenolic compounds (4241.67 mg GAE 100 g^{-1}) in the composition of the extract compared to the concentration of flavonoids (254.92 mg of rutin 100 g^{-1}). Thus, the capture of DPPH was almost exclusively due to the activity of phenolic compounds. The phenolic compounds obtained during plant extracts have different structures, such as phenolic acids, coumarins derivatives, tannins, and flavonoids[54].

It is possible to verify, in the present work, flavonoid quantification results superior to those of Agbai et al.[49], which were obtained for the sample of raw rubber seed meal (RRSM) *Hevea brasiliensis* a flavonoid content of 60.00 ± 3.53 mg quercertin 100 g^{-1}. Zain et al.[44] found 0.200 mg of catechin mL^{-1} for methanol extract with leaves of the rubber tree clone RRIM 3001 *Hevea brasiliensis*. Nevertheless, it did not find flavonoids for methanol

	Method	Concentration	Time	% DPPH capture	Flavonoids	Phenolics
Method		0.00	− 0.00	0.5647*	0.0727	0.2489
		p = 1.00	p = 1.00	p = 0.015*	p = 0.774	p = 0.319
Concentration	0.00		0.00	0.6265*	0.2410	− 0.4536
	p = 1.00		p = 1.00	p = 0.005*	p = 0.335	p = 0.059
Time	− 0.00	0.00		− 0.1172	− 0.4310	− 0.3821
	p = 0.00	p = 1.00		p = 0.643	p = 0.074	p = 0.118
% DPPH capture	0.5647*	0.6265*	− 0.1172		0.2626	− 0.0458
	p = 0.015*	p = 0.005*	p = 0.643		p = 0.293	p = 0.857
Flavonoids	0.0727	0.2410	− 0.4310	0.2626		0.4482
	p = 0.774	p = 0.335	p = 0.074	p = 0.293		p = 0.062
Phenolics	0.2489	− 0.4536	− 0.3821	− 0.0458	0.4482	
	p = 0.319	p = 0.059	p = 0.118	p = 0.857	p = 0.062	

Table 5. Pearson correlation for aqueous solvent. *Results statistically significant.

	Method	Concentration	Time	% DPPH capture	Flavonoids	Phenolics
Method		0.00	− 0.00	0.7927*	− 0.2274	0.3204
		p = 1.00	p = 1.00	p = 0.000*	p = 0.364	p = 0.195
Concentration	0.00		0.00	0.2459	− 0.1597	0.2239
	p = 1.00		p = 1.00	p = 0.325	p = 0.527	p = 0.372
Time	− 0.00	0.00		− 0.0119	0.2944	0.2341
	p = 0.00	p = 1.00		p = 0.963	p = 0.236	p = 0.350
% DPPH capture	0.7927*	0.2459	− 0.0119		− 0.1505	0.5475*
	p = 0.000*	p = 0.325	p = 0.963		p = 0.551	p = 0.019*
Flavonoids	− 0.2274	− 0.1597	0.2944	− 0.1505		− 0.1575
	p = 0.364	p = 0.527	p = 0.236	p = 0.551		p = 0.532
Phenolics	0.3204	0.2239	0.2341	0.5475	− 0.1575	
	p = 0.195	p = 0.372	p = 0.350	p = 0.019	p = 0.532	

Table 6. Pearson correlation for methanol extract. *Results statistically significant.

extracts with rubber tree clone seeds. Gullón et al.[25] presented for purple corn *Zea mays L.* 18.6 mg of rutin g^{-1} of flavonoids. Rico et al.[26] found a flavonoid content of peanut shell self-hydrolysis liqueurs of 10.30 mg of rutin.g^{-1} of raw material. Machado et al.[22] presented for *Garcinia cochinchinensis Choisy* leaf extract a flavonoid content of 665.1 ± 122.9 mg of rutin 100 g^{-1}. The presence of flavonoids is an indication of the improvement in antioxidant activity[55]. However, as the rubber tree seed pomace is a complex matrix, synergistic activity could happen between the antioxidant compounds.

Using the t test to verify the statistical difference in the concentration of phenols and flavonoids, the extraction methods using infusion and Soxhlet with aqueous solvent were considered, at concentrations 40 g L^{-1} and 60 g L^{-1}, these tests being related to the higher DPPH values found in Table 2. The results showed that the extraction methods do not differ statistically for the contents of phenols and flavonoids for these conditions. However, for the latter, in the soxhlet system, the difference was significant between the concentrations ($p = 0.0169$). For the concentration of 60 g L^{-1}, the average was higher, corroborating the choice for the development of the second planning, the increase in concentrations.

Total phenolics and flavonoids from second planning. Afterward, a second analysis was performed from the condition that showed the best antioxidant activity, corresponding to 85 g L^{-1} and the concentration of 100 g L^{-1}. At this concentration, the phenolic compounds content was 1405.17 mg GAE 100 g^{-1}, and the flavonoid content 223.34 mg 100 g^{-1}. Statistically, differences were found between the content of phenols ($p = 0.024$) and flavonoids ($p = 0.0013$). For both compounds, the content was higher in the experiment containing 85 g L^{-1} and 4 h of extraction, which is correlated with the antioxidant capacity data using the DPPH. As in the initial planning, the phenols content was higher than flavonoids, following the same behavior already demonstrated, suggesting that the antioxidant capacity is linked to the phenols content or synergism of the compounds in the sample.

Pearson correlation. Person correlation tests were performed to verify the existence of a linear correlation between the variables and results obtained for each solvent. In this way, the Pearson correlation for aqueous solvent is shown in Table 5.

As shown in Table 5, most variables do not demonstrate a linear correlation with the results of flavonoids and phenolics for the aqueous extract, except for the % DPPH capture, which showed a median and positive

	Method	Concentration	Time	% DPPH capture	Flavonoids	Phenolics
Method		0.00	− 0.00	− 0.3984	0.3398	0.5478*
		p = 1.00	p = 1.00	p = 0.101	p = 0.168	p = 0.019*
Concentration	0.00		0.00	0.2038	− 0.2046	− 0.4127
	p = 1.00		p = 1.00	p = 0.417	p = 0.415	p = 0.089
Time	− 0.00	0.00		− 0.4445	0.1088	0.1036
	p = 0.00	p = 1.00		p = 0.065	p = 0.667	p = 0.683
% DPPH capture	− 0.3984	0.2038	− 0.4445		− 0.3778	− 0.3157
	p = 0.101	p = 0.417	p = 0.065		p = 0.122	p = 0.202
Flavonoids	0.3398	− 0.2046	0.1088	− 0.3778		0.2954
	p = 0.168	p = 0.415	p = 0.667	p = 0.122		p = 0.234
Phenolics	0.5478*	− 0.4127	0.1036	− 0.3157	0.2954	
	p = 0.019*	p = 0.089	p = 0.683	p = 0.202	p = 0.234	

Table 7. Pearson correlation for the ethanol extract. *Results statistically significant.

	Method	Concentration	Time	% DPPH capture	Flavonoids	Phenolics
Method		0.00	− 0.00	− 0.3439	0.2805	− 0.3191
		p = 1.00	p = 1.00	p = 0.162	p = 0.168	p = 0.197
Concentration	0.00		0.00	− 0.1632	− 0.0107	− 0.6927*
	p = 1.00		p = 1.00	p = 0.518	p = 0.967	p = 0.001*
Time	− 0.00	0.00		− 0.4212	0.2360	− 0.2440
	p = 0.00	p = 1.00		p = 0.082	p = 0.346	p = 0.329
% DPPH capture	− 0.3439	− 0.1632	− 0.4212		− 0.0479	0.3321
	p = 0.162	p = 0.518	p = 0.082		p = 0.850	p = 0.178
Flavonoids	0.2805	− 0.0107	0.2360	− 0.0479		− 0.4004
	p = 0.168	p = 0.967	p = 0.346	p = 0.850		p = 0.100
Phenolics	− 0.3191	− 0.6927*	− 0.2440	0.3321	− 0.4004	
	p = 0.197	p = 0.001*	p = 0.329	p = 0.178	p = 0.100	

Table 8. Pearson correlation for the hexane extract. *Results statistically significant.

correlation statistically significant with the variables method and concentration. These results indicate that the Soxhlet method contributes to the greater extraction of antioxidant compounds responsible for capturing free radicals or their non-degradation. By this method, the solvent does not come into contact with the sample at the boiling temperature. This ability to neutralize free radicals cannot be related to phenolic compounds or phenols by this analysis. Pearson's correlation analysis was also performed for the methanol extract, and the results are shown in Table 6.

In Table 6, as well as in the aqueous extract, the methanol solvent extract presented a linear correlation with the result of % DPPH capture, which is a statistically significant, high, and positive correlation with the employed method. For this extract, it was also observed that the Soxhlet method contributes to the increase in DPPH capture. This ability to neutralize free radicals can be related to phenolic compounds by this analysis, as there was a statistically significant and positive correlation between % DPPH capture and the amount of phenols. Table 7 shows the results of the Pearson correlation for the extract obtained in the ethanol solvent.

There is a linear correlation between the extraction method and the amount of phenols for the ethanol solvent, and this correlation is statistically significant, positive, and intermediate level. This result indicates that the Soxhlet method amplifies the amount of phenols extracted by this solvent, enhancing the antioxidant capacity. Also, Table 8 shows the Pearson correlation for the hexane extract.

According to the Pearson correlation statistical test for the hexane solvent, the only statistically significant linear correlation was phenols with concentration. There is a minimization of the amount of phenols when increasing the study concentration, indicating that hexane is not the ideal solvent for extracting phenolic compounds for this biomass.

Physicochemical characterization of the extract: density and hydrogenic potential. The physicochemical analyzes were carried out considering the extract with the most significant antioxidant potential was the aqueous extract at the concentration of 85 g L^{-1}, in a time of 4 h. For the sample, the pH and density (ρ) values reached 6.21 and 0.999 g mL^{-1}, respectively, at a temperature of 25 °C, values close to those found in the literature for similar plant species. For example, Cardoso et al.[56] found pH indices for aqueous extracts of Brazil nuts of 6.34 without preservatives and 5.87 with added preservatives.

Conclusions

Brazil's rubber tree has been explored for latex production, but few studies about other commercial applications are described in the literature, mainly for its residues. This work showed that the aqueous extracts, made from rubber tree seed bagasse residue, are rich in phenolic compounds and flavonoids, superior to several antioxidant extracts produced from other seeds showed in other works during the paper. The results of statistical correlation showed that DPPH radical capture, reaching 37.73%, is closely linked to the presence of phenols, attributed to ease of extraction by aqueous solvent. It was evidenced that the method Soxhlet is one of the variables that most correlates with the antioxidant activity, mainly to DPPH, thus suggesting that it is suitable for extracts that intend to be used as antioxidants. The antioxidant activity allows the application of the extract in different industry sectors, contributing to future research on the characterization and identification of phenolic compounds and flavonoids responsible for the activity of the antioxidant extract. Besides that, the valorization of this industrial coproduct makes the productive chain economically and environmentally attractive, favoring the development of integrated processes at the laboratory, pilot, and industrial scale.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Competing interests

The present work will be sent to the patent deposit in Inpi-Brazil.

Additional information

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ARTICLES FOR FACULTY MEMBERS

SUSTAINABLE PROCESSING OF RUBBER (HEVEA BRASILIENSIS) SEED OIL: PHYSICOCHEMICAL INSIGHTS INTO EXTRACTION AND ANTIOXIDANT PRESERVATION

Microwave-Assisted Extraction and Physicochemical Evaluation of Oil from Hevea brasiliensis Seeds./ Creencia, E. C., Nillama, J. A. P., & Librando, I. L.

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Article

Microwave-Assisted Extraction and Physicochemical Evaluation of Oil from *Hevea brasiliensis* Seeds

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Abstract: The rubber tree (*Hevea brasiliensis*) is exploited mainly for latex in view of its economic importance. However, one of its auxiliary products, the rubber seed, does not find any major applications, and hence, even the natural production of seeds itself remains underutilized. In this study, microwave-assisted Soxhlet extraction is used as a green alternative to extract the oil from seeds at a reaction time of 90 min and microwave power of 300 W. The objective of the study is to evaluate the effects of the processing conditions, including drying time, temperature, solid–solvent ratio, and extraction solvent, on the yield of rubber seed oil. Moreover, the microwave-assisted aqueous extraction (MAAE) under acidic conditions is also investigated. Based on the results, n-hexane gave the best yield at an optimized 1:20 seed–hexane ratio at 72 °C compared with the conventional Soxhlet method and the acidic MAAE. Furthermore, the chemical characteristics of the oil showed a high value of free fatty acids (% FFA) (1.15–7.61%) and an iodine value (IV) that ranges from 100–150. As a semi-drying oil, rubber seed oil (RSO) can be used as an ingredient for surface coating and in the formulation of products where the presence of unsaturation is important.

Keywords: rubber seed oil; microwave-assisted extraction; Soxhlet extraction; microwave-assisted aqueous extraction

1. Introduction

The Philippine government enacted a law in 2010, Republic Act 10089, creating the Philippine Rubber Research Institute (PRRI), which is tasked with developing the rubber industry in the country. The law mandates the PRRI to "monitor and evaluate the rubber research programs and identify the immediate needs and essential concerns in the rubber industry in consonance with the local and national economic development." The PRRI is also tasked with "coordinating with other government agencies in order to formulate strategies that would jump-start the growth of the rubber industry". With the PRRI in place, the country will see more research that will investigate the development of other applications for the rubber tree.

Rubber tree (*Hevea brasiliensis*) is one of the leading commercial agricultural trees in the world, and one of the most important revenue-generating trees in the Philippines. Apart from its use in latex production for foreign exchange, the tree produces oil-bearing seeds whose oil content in dried kernel form varies from 35% to 45% [1]. Rubber seed oil (RSO) is currently being studied as a candidate component in the manufacture of various products, such as soap, paints, and alkyd resin due to its semi-dried nature. Moreover, RSO has been investigated as an effective and "green" ingredient in production of biodiesel [2]. Although the rubber seed has only been traditionally used as a planting material and is generally neglected, along with the wood from the rubber tree, studies for the utilization of the seed oil have generally increased [3]. Consequently, it is necessary to find an efficient method of extracting the oil from its natural source.

Throughout the years, numerous methods, such as expeller pressing and organic solvent extraction, have been developed in order to extract oil from seed materials [4]. However, regulatory problems associated with the use and disposal of organic solvents have undesirable effects on oil and costs [5]. Driven by these purposes, advances in sample preparation have resulted in a number of techniques that both improve the extraction yield, as well as minimize or even eradicate the drawbacks posed by conventional techniques.

Microwave-assisted extraction (MAE) is a novel technology, which has been studied extensively in recent years, mainly to reduce extraction time and solvent consumption [6,7]. This new technology involves lower energy consumption, higher extraction yields, and less production of waste, which results in a more economically favorable extraction procedure [8]. It has been well-documented that microwave treatment of oilseeds during solvent extraction leads to the protein denaturation of cells, resulting in an improved extraction [9]. Furthermore, microwave-assisted extraction can also reduce the use of organic solvents, because it can generally make use of aqueous solvents as the extraction media. This technique is specifically called microwave-assisted aqueous extraction (MAAE).

Thus, this study will put forward the use of microwave-assisted extraction as a green alternative method for the extraction of rubber seed oil, as well as the evaluation of its physicochemical properties in terms of appearance, free fatty acid content, iodine value, and peroxide value. In addition, this study will also be able to promote the valorization of rubber seeds, as well as its utilization as a renewable source to produce seed oil, which can be used for a variety of applications, particularly to produce biofuel.

2. Materials and Methods

2.1. Materials and Reagents

Fresh rubber (*Hevea brasiliensis*) seeds were collected from rubber plantations in Zamboanga Sibugay, Philippines (7°47′24.19″ N, 122°35′37.78″ E). The seeds were subjected to shell/husk removal in order to separate the kernel. The seeds were cut into smaller pieces and oven-dried at 105 °C for 2, 3, 4, and 18 h, respectively. Then, the dried kernels were ground to fine powder (40-mesh) for oil extraction. All chemicals and reagents were purchased commercially, of analytical grade, and were used as received.

2.2. Microwave-Assisted Soxhlet Extraction

Five g of ground rubber seeds were placed into the extractor through a thimble, and the oil was extracted with 100 mL of four different solvents (n-hexane, n-hexane–acetone (3:1 v/v), n-hexane–ethanol (3:1 v/v), and absolute ethanol) for 90 min at a power of 300 W using the Milestone RotoSYNTH microwave reactor (Milestone Srl, Bergamo, Italy). Low-polar and nonpolar extractants were heated to their boiling points while stirring with a Weflon magnetic bar (Milestone Srl, Bergamo, Italy) to aid absorption of microwave radiation. After extraction, the extract was filtered to remove particles that were entrained during the process. The solvent was separated from the crude rubber seed oil using a rotary evaporator.

The oil yield obtained was expressed in terms of mass percentage of the samples and calculated as follows:

$$\text{Oil yield (wt \%)} = \frac{\text{mass of oil extract (g)}}{\text{mass of ground rubber seed (g)}} \times 100 \quad (1)$$

2.3. Acidic pH-Based Microwave-Assisted Aqueous Extraction

About five g of ground rubber seeds were placed into the extracting flask through a thimble and added with a specified volume of the extracting solvent (1×10^{-3} M HCl, 1×10^{-4} M HCl, 1×10^{-6} M HCl, and distilled water) to produce a solid–solvent ratio of 1:12 m/v. The initial temperature and time were then set based on the results from the performed optimization.

The microwave power was also set to 500 W, and the stirring was kept at 70%. After the extraction, the sample was removed from the flask and heated in a boiling water bath for 15 min for demulsification. After cooling to room temperature, the mixture was then washed twice with 15-mL portions of n-hexane, producing an aqueous layer and a foam layer. The obtained foam layer was afterwards subjected to the rotary evaporator and centrifuged at 3000 rpm for 30 min to completely break the layer, producing an aqueous phase and an upper organic phase. The organic phase was then collected and subjected to the rotary evaporator to recover the extracted seed oil, which was then dried to constant weight. The oil yield was then calculated using Equation (1).

2.4. Conventional Soxhlet Extraction

The conventional Soxhlet extraction was performed using the official method for oil extraction from the Association of Official Analytical Chemists (AOAC, 1997) [10] with few modifications. About 10 g of ground rubber seeds were placed into the extractor through a thimble, and the oil was extracted with approximately 120 mL of n-hexane. The extraction process was allowed to progress for 6 h, and the solvent–oil mixture was filtered and subjected to solvent removal by rotary evaporator to recover the crude oil extract.

2.5. Evaluation of Physicochemical Properties

The characterization of the obtained seed oils was performed using the American Oil Chemists' Society (AOCS) standard methods.

- Free fatty acid (FFA)—The FFA content was evaluated using AOCS Official Method Ca 5a-40.
- Iodine Value (IV)—The IV was carried out following the AOCS Official Method Cd 1d-92.
- Peroxide Value (PV)—The PV was measured following the AOCS Official Method Cd 8b-90.

3. Results and Discussion

3.1. Microwave-Assisted Soxhlet Extraction

Observations have been made regarding the physical characteristics of rubber (*Hevea brasiliensis*) seed oil extracted from the four different solvents. The oil from n-hexane was clear and golden yellow in color, with the original odor of the rubber seed. This is because n-hexane has low polarity, which is miscible in oil and extracts only the glycerides in the seeds. On the other hand, white particles were observed to be extracted together with the oil upon the addition of a polar solvent to n-hexane. Hron et al. [11] and Ferriera-Dias et al. [12] reported that polar solvents, such as ethanol, have the capability to co-extract other polar compounds in oilseeds, which are insoluble in non-polar solvents. The oil extracted using absolute ethanol was viscous, yellowish to brownish in color with an emulsion-like appearance.

3.1.1. Effect of Solid–Solvent Ratio on Oil Yield

In this study, three mass–volume ratios of seeds to solvent (n-hexane) were tested using the 18-h oven-dried, ground rubber seeds. As shown in Figure 1, the highest oil yield was obtained at the 1:20 solid–solvent ratio. Therefore, it can be said that increasing the ratio also increases the yield. This is because the concentration gradient between the solid and the liquid phase becomes greater, which favors good mass transfer and increased extraction efficiency [13]. Subsequent experiments on microwave-assisted Soxhlet extraction were carried out using the 1:20 ratio.

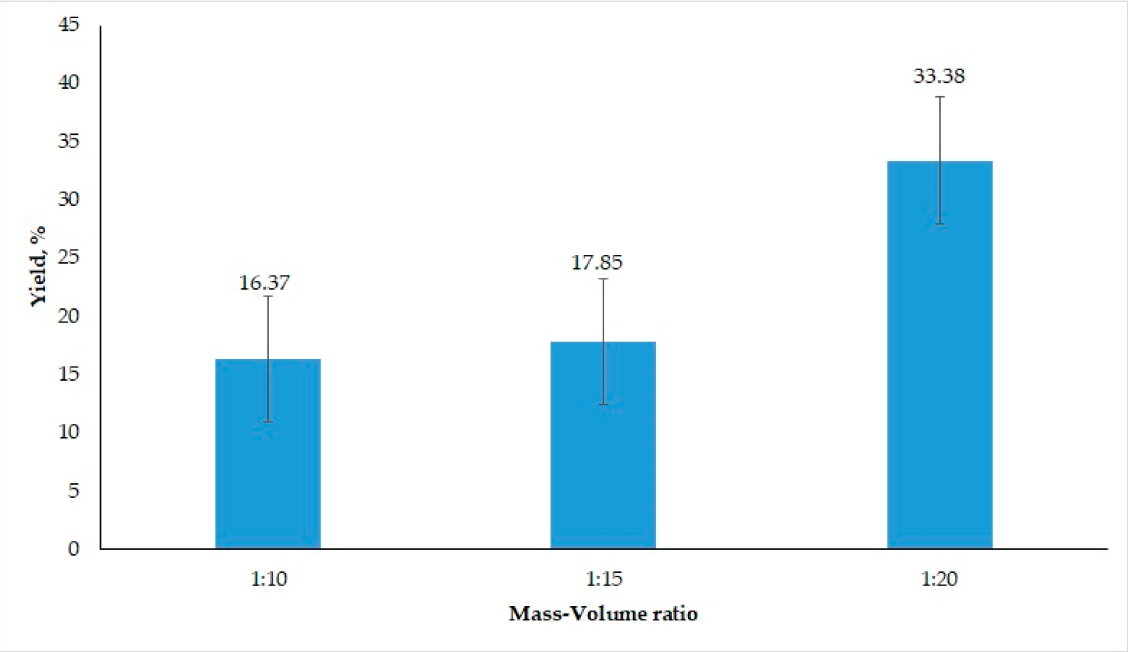

Figure 1. Effect of mass–volume (n-hexane) ratio on the extraction efficiency with a reaction time of 90 min at 72 °C and microwave power of 300 W.

3.1.2. Influence of Oven-Drying Time

The effect of oven-drying the sample at 2, 4, and 18 h on the oil yield is graphically illustrated in Figure 2. It is observed that, irrespective of the solvent used, high oil yields were obtained at the shortest oven-drying time, which was the 2-h period. This result can be attributed to the moisture content of the ground rubber seeds prior to extraction. Previous studies have shown that moisture in seeds plays a significant role during oil extraction. Accordingly, protein coagulation is promoted as heat is transferred through the moisture in the oilseeds, and this has been shown to facilitate the release of oil. Moreover, as the drying time of the samples is increased, so is moisture loss. Relatively long drying times are therefore associated with harder seed particles as a result of the very low moisture content after heating. This consequently causes the oil to be more attached to the seeds and hinders its release, thereby lowering the oil extraction yield [14]. This may account for the reduction in oil yield for the 18-h, oven-drying period.

3.1.3. Influence of Different Extraction Solvents

In the method used, four different extraction solvents of varying polarities were used. The low-polar solvents were n-hexane, n-hexane–acetone (3:1 v/v), and n-hexane–ethanol (3:1 v/v), while the polar solvent used was absolute ethanol. Because the extracted RSO is not intended for human consumption, but rather for other industrial applications, particularly biofuel production, the use of the aforementioned solvents was considered safe for this utilization. Furthermore, the purpose of using solvents with different polarities was to investigate the influence of solvent polarity on the yield and the physicochemical characteristics of the oil. As reported in Figure 2, the yield of oil extracted by n-hexane was the highest despite being dependent only on the Weflon magnetic bar to absorb microwave radiation due to its nonpolar nature. This suggests that nonpolar rubber seed oil is easier to be extracted by a nonpolar solvent such as n-hexane. Even though absolute EtOH absorbs microwave radiation efficiently, the polar nature of this solvent conceivably destroys intracellular compartmentalization in the seeds, allowing solubilization of more unsaponifiable matter and leading to lower oil yields [15].

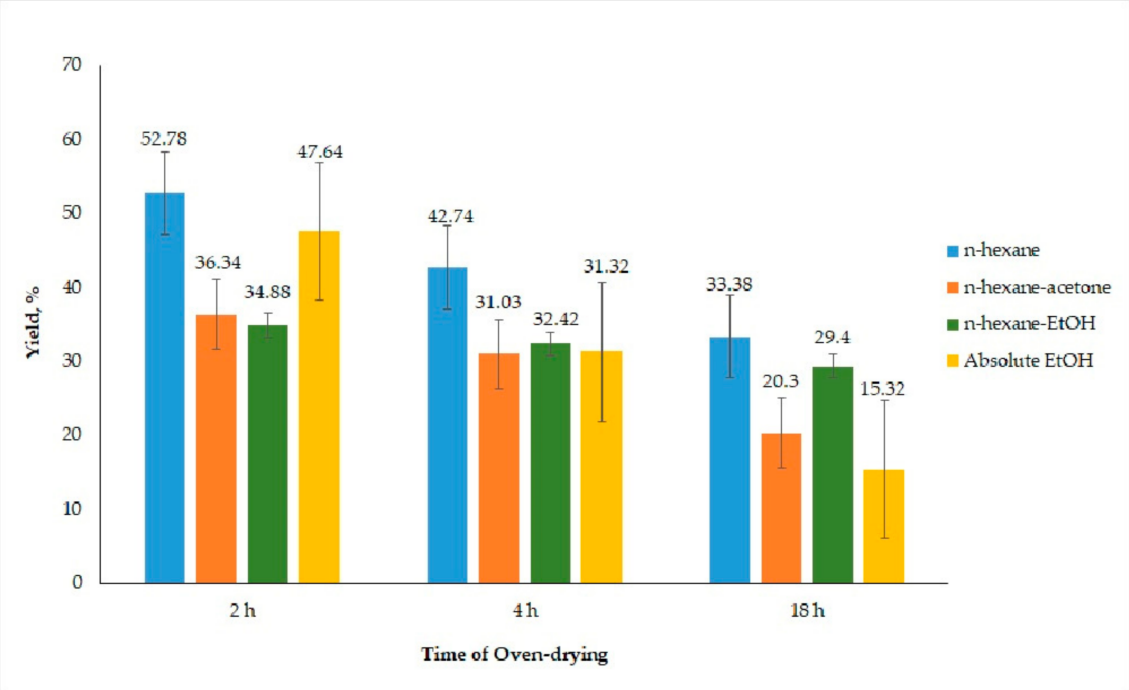

Figure 2. Variation of oil yields at 2, 4, and 18 h of oven-drying time with a reaction time of 90 min and microwave power of 300 W.

3.1.4. Influence of Temperature

The effect of the boiling temperature of solvents on the yield is presented in Figure 3. The optimum oil yield was obtained at 72 °C, which corresponds to the boiling temperature of n-hexane. It is observed that oil yield increased initially with increasing temperature. However, the continuous increase in temperature resulted in the decrease in attained oil yields. These results can be accounted for by the idea that at elevated temperatures, the disruption of the cell walls in oil-bearing cells can be promoted. This allows the oil content in the cells to easily escape from the cells into the extracting solvent, which increases the extraction yield. However, upon doing the extraction at even more elevated temperatures, the degradation of the oil is very much likely to occur. Moreover, the samples can lose a very significant amount of their moisture content. This, as mentioned previously, leads to seed hardening and subsequently, to lower extraction yields [16–18]. This may account for the decrease in oil yield from 70 to 80 °C.

Figure 4 shows the oil yields of the MW-assisted extraction method using the 2 h oven-dried samples with four different extraction solvents and irradiated at 300 W for 90 min, while the oil yield for the conventional Soxhlet extraction was obtained from a 2 h oven-dried sample with n-hexane, extracted for 6 h. The data presented shows that the yield obtained from the MW-assisted extractions using n-hexane and absolute EtOH as solvents gave higher yields than the conventional Soxhlet extraction. This signifies that the MW-assisted extraction is more efficient in terms of oil yield and time consumption as it can achieve a maximum extraction within 90 min, which cannot be achieved through the conventional Soxhlet method even after 6 h of extraction.

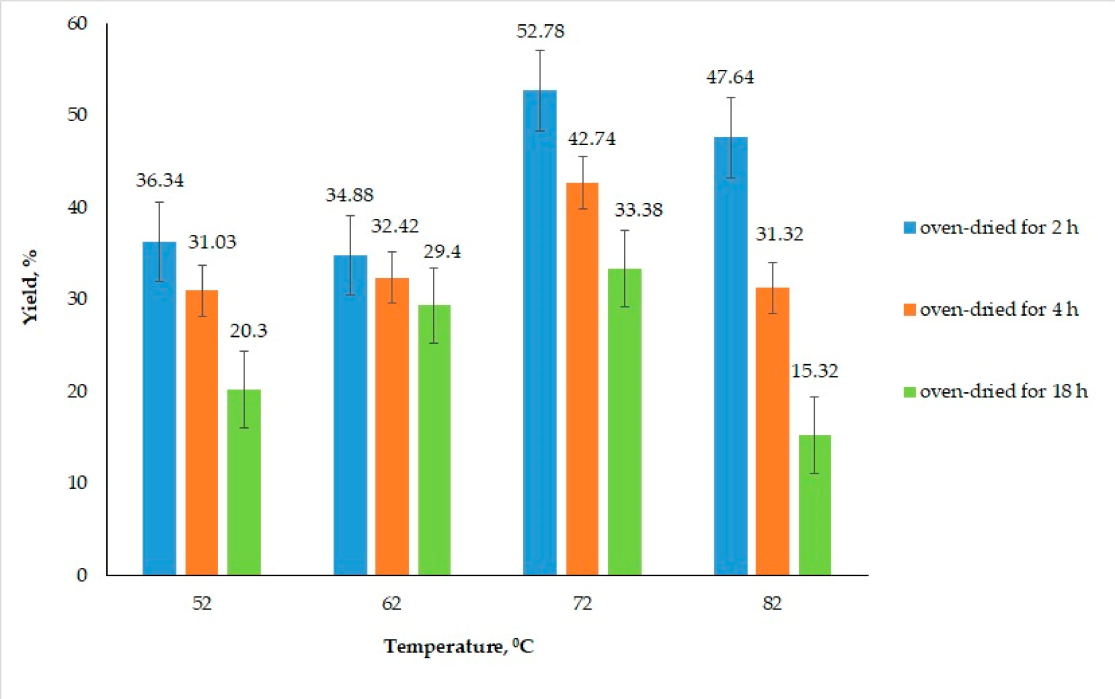

Figure 3. Effect of temperature on the extraction efficiency with reaction time of 90 min and microwave power of 300 W.

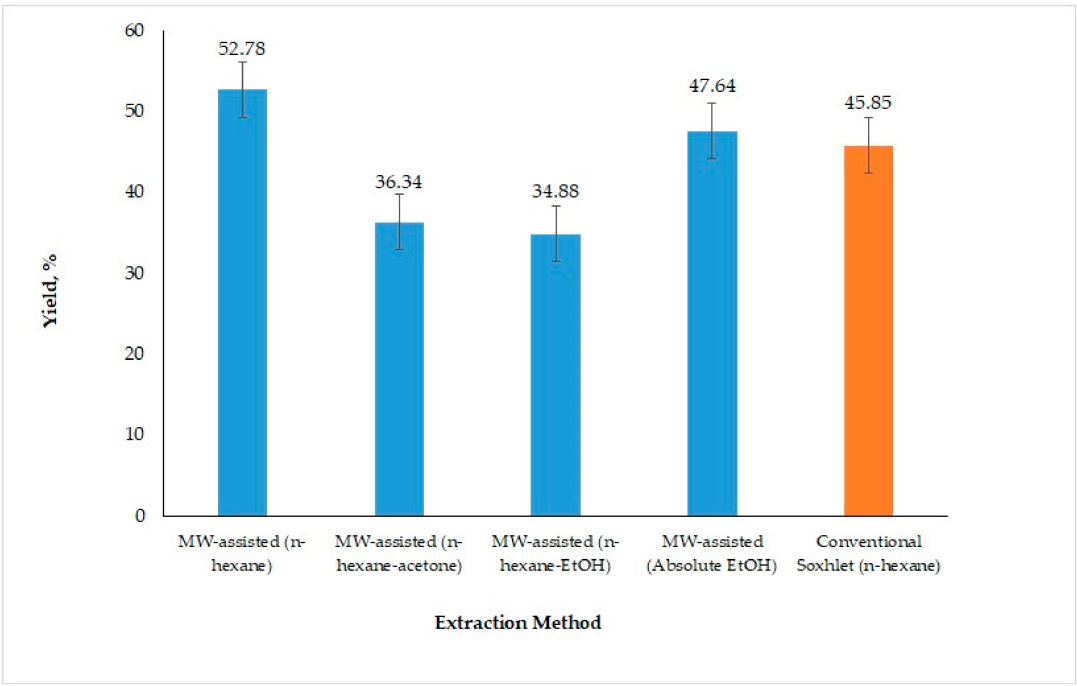

Figure 4. Comparison of the oil yields for the MW-assisted (oven-dried for 2 h) and the conventional Soxhlet extraction methods.

3.2. Acidic pH-Based Microwave-Assisted Aqueous Extraction

Aqueous extraction of rubber seed oil under the influence of microwave radiation was carried out after performing the necessary optimization procedures for the selected extraction parameters, namely extraction temperature and time. The best conditions were then identified from these optimizations

and used for the extraction procedure. Moreover, due to the aqueous nature of the extracting solvent used for the acidic MAAE, the solid–solvent ratio was reduced to 1:12 (g/mL).

3.2.1. Determination of Best Conditions for Oil Extraction

From the results presented in Figure 5, it can be seen that the highest extraction yield, with a value of 10.09%, was attained using the temperature setting of 80 °C. A general trend of increasing oil extractability with increasing temperature can be observed as the temperature is elevated from 60 °C to 80 °C. However, it can also be inferred from the data that only a very small increase in extraction yield can be observed as the temperature increases within the stated range. This is very evident in the 60 °C and 70 °C settings where the extraction yield remained at approximately 8%. This is an indication that within this range, temperature changes do not have any significant effect on the oil yield. In addition, since 90 °C is substantially close to the boiling point of water, which is the main component of the extracting solvent, gradual evaporation of the solvent may have taken place to a considerable extent as the extraction progressed. This can also be considered as a contributing factor to the decrease in the extractability of the seed oil, because this alters the initial solid–liquid ratio of the system.

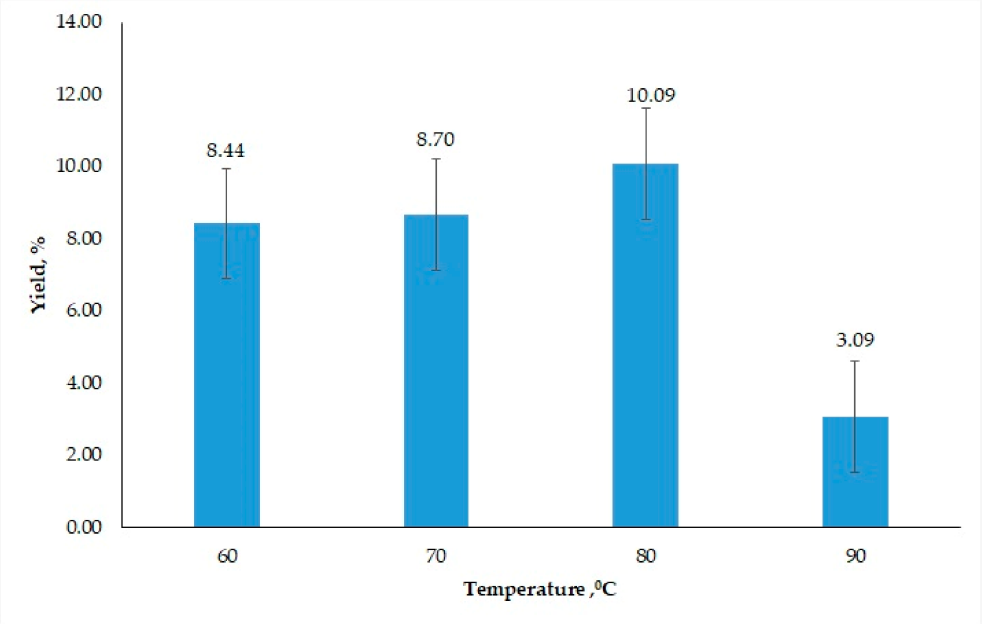

Figure 5. Variation of extraction yield with temperature.

Nonetheless, because the temperature setting of 80 °C gave the best results, this setting was used in the succeeding optimization for extraction time. As shown in Figure 6, it is very evident that carrying out the extraction for 90 min gave the highest oil yield, with a value of 10.09%. This suggests that exposure of the sample to the microwave irradiation at this time interval caused a relatively sufficient disruption of the cell walls, thereby allowing better release of the oil from the sample cells. At a shorter extraction time (60 min), the length of the exposure of the sample to the microwave radiation was not enough to cause a complete disruption of the cell walls and hence, the ineffective release of oil. Furthermore, lowest extraction yield was obtained at the 150-min extraction time. This may be due to emulsification promoted by the longer contact of the released oil with water-soluble proteins in the seed, which exhibit surfactant activity upon denaturation.

The extraction temperature and time were set at 80 °C and 90 min, respectively, since these settings gave the highest yield in the preceding optimization procedures. The acidity of the extracting solvent was regulated from pH 3 to pH 7 using diluted hydrochloric acid for solutions of pH 3–6 and commercial distilled water for pH 7. The effect of pH on oil extractability is shown in Figure 7.

The general trend that can be observed from the data is that the % yield increases with the increasing pH of the extracting solvent until pH 5. At a lower pH, lower extraction yields were observed, which may be due to proteins occurring at the isoelectric point, which aggregate with oil bodies, hindering the release of oil [4]. Beyond pH 5, a decrease in oil extractability was also observed. This is primarily due to the lesser amount of acidic species in the solvent for these pH, which means that only few acidic species can participate in the disruption of the oleosins covering the oil bodies in the seeds, and therefore, ineffective release of oil occurs.

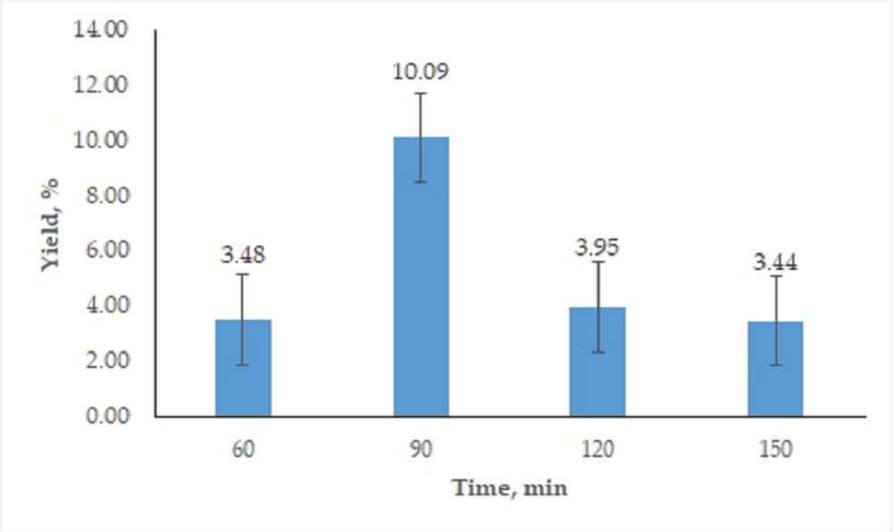

Figure 6. Variation of extraction yield with time.

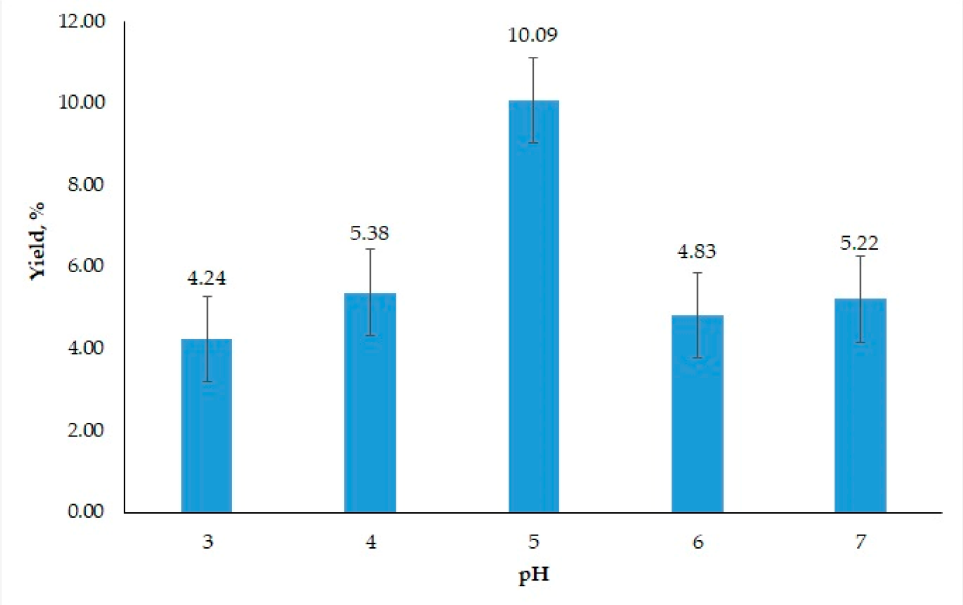

Figure 7. Variation of the extraction yield with pH of the extracting solvent.

3.2.2. Comparison of Acidic pH-Based MAAE and Conventional Soxhlet Method

It is evident from the data presented in Figure 8 that the extraction yield for the acidic pH-based MAAE is only about 25% of the extraction yield for the conventional method. This signifies that the

effect of an acidic environment was not enough in preventing the aggregation of proteins with the oil during extraction.

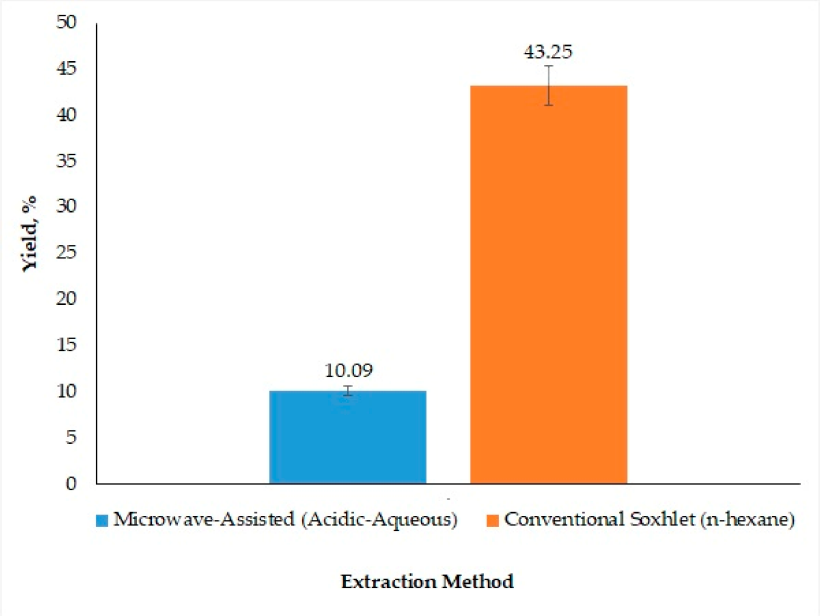

Figure 8. Comparison of extraction yields for the acidic pH-based microwave-assisted aqueous extraction (MAAE) and conventional Soxhlet method.

Moreover, this significantly large difference in extraction yields can be attributed to the fact that the determination of the best extraction conditions was only limited to the optimization of two extraction parameters. In the method used, only the effect of temperature and time were examined. As a result, the possible contributions of the other parameters in the extractability of the sample were no longer accommodated, which may have served as a contributing factor to the observed smaller yield for the MAAE. Furthermore, from these results, it can be inferred that in terms of extraction yield, the conventional method was a more effective technique.

3.3. Evaluation of Physicochemical Properties

The physicochemical properties of seed oils obtained from the MW-assisted Soxhlet extraction, the acidic pH-based MAAE, and the conventional Soxhlet methods were evaluated in order to compare their quality. The results for these determinations are summarized in Tables 1 and 2.

Table 1. Physicochemical properties of the extracted *Hevea brasiliensis* seed oil.

Parameter	Conventional Soxhlet Method [b]	MW-Assisted Soxhlet Extraction					
		n-Hexane			n-Hexane–Acetone (3:1 *v*/*v*) [a]	n-Hexane–Ethanol (3:1 *v*/*v*) [a]	Absolute Ethanol [a]
		2 h	4 h	18 h			
FFA (% Oleic)	7.61	5.16	3.91	1.19	5.43	5.20	3.67
Iodine Value	132.8	107.7	119.9	121.9	150.2	148.3	147.7
Peroxide Value (mEq/kg)	15.13	b	b	b	b	b	b

[a] Oven-dried for 2 h; [b] Cannot be determined.

Table 2. Physicochemical properties of the extracted *Hevea brasiliensis* seed oil (oven-dried for 3 h).

Parameter	Conventional Soxhlet Method	Acidic pH-Based MAAE		
		pH 3	pH 5	pH 7
FFA (% Oleic)	2.54	1.66	1.15	1.64
Iodine Value	131.9	131.2	126.4	129.7
Peroxide Value (mEq/kg)	[b]	19.50	5.18	5.62

[b] Cannot be determined.

From the results, it is observed that the seed oil extracted using the conventional Soxhlet method exhibited a higher FFA value compared with the oil extracted using the MW-assisted methods. The higher FFA content obtained from the conventional Soxhlet method implies that this oil has a higher tendency of becoming rancid than the oil extracted from the MAE. Factors, such as longer time of extraction (6 h) and no control of temperature in a conventional extraction, may have reinforced each other in promoting the hydrolysis of the triacylglycerols in the oil to occur, thereby causing greater FFA results. On the other hand, as shown in Table 1, the values of free fatty acid decreased with an increase in the oven-drying time of the seed from 2 h to 18 h. This means that the longer the oven-drying time of the seeds, agents of rancidity, such as moisture, are much reduced than at a shorter oven-drying time.

The iodine value is often used to assess the drying property of oils. The IV of the samples ranges from 100–150 as shown in Tables 1 and 2, indicating that the oil samples contain high amounts of unsaturated fatty acids. Moreover, these results verify the semi-drying property of RSO, which signifies that it may be a potential ingredient in the formulation of surface coatings. According to Warra et al. [19], oils with IVs that fall within the 100–150 range display the ability to absorb oxygen when exposed to air, which thickens them and allows them to remain sticky without forming a hard, dry film. In addition, unsaturation in vegetable oil is a desirable property in vulcanized oil synthesis. Thus, the high IV recorded for RSO is a strong indication that it would be suitable for vulcanized oil synthesis.

The observed high PV for RSO (19.50 and 15.13 mEq/kg) may be attributed to the heating during extraction, as heat favors the oxidation of fatty acids and most especially polyunsaturated fatty acids. The lower values of PV (5.18 and 5.62 mEq/kg) imply that these oils have lower degree of rancidity. From the results given, the PV of some of the samples could not be determined because they were unable to produce a deep blue color upon the addition of a starch indicator; thus, they could not be titrated to their endpoints. This suggests that only small amounts of peroxides were present in these samples.

4. Conclusions

The extraction of oil from *Hevea brasiliensis* seeds was investigated using microwave-assisted extraction methods. In general, n-hexane gave the best yield at an optimized 1:20 seed–hexane ratio at 72 °C. This method is more efficient in terms of yield and time consumption as it can achieve a maximum extraction within 90 min, which is much faster than the conventional Soxhlet method that requires 6 h of extraction. The results also showed that extraction using MAAE under acidic conditions is best carried out at 80 °C for 90 min at pH 5. However, the results revealed that the extraction yield for this method was only 25% of the conventional Soxhlet method. This signifies that the conditions used for this method are not sufficient to prevent the aggregation of the oil with seed proteins. From the obtained results for chemical analysis, the high iodine and FFA values for rubber seed oil serve as an indication of its potential as an ingredient in soap and surface coatings, as well as in biodiesel and vulcanized vegetable oil (VVO).

Overall, the method presented in this study was found to be more efficient due to the higher oil extraction yield of 52%, which only required 90 min, compared with other techniques for RSO extraction, which required longer extraction time but produced lower yields [3]. Aside from

demonstrating the opportunity to use MAE to replace the time-consuming conventional extraction process, the research findings also generated new and vital information for the exploitation of the capability of the rubber seed as a promising raw material for oleochemical processes. This, in turn, can be a helpful reference for the rubber seed agriculture industry in expanding profit. Moreover, the method used in this study may be used as a reference for future research studies that focus on RSO extraction particularly for biodiesel applications. Other possible future developments for the study could also include the refinement of the extracted RSO using methods such as esterification, as well as the assessment of the chemical properties of the biodiesel produced from the refined RSO.

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ARTICLES FOR FACULTY MEMBERS

SUSTAINABLE PROCESSING OF RUBBER (HEVEA BRASILIENSIS) SEED OIL: PHYSICOCHEMICAL INSIGHTS INTO EXTRACTION AND ANTIOXIDANT PRESERVATION

Optimization of oil yield from Hevea brasiliensis seeds through ultrasonic-assisted solvent extraction via response surface methodology./Mabayo, V. I. F., Aranas, J. R. C., Cagas, V. J. B., Cagas, D. P. A., Ido, A. L., & Arazo, R. O.

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Original Research Article

Optimization of oil yield from *Hevea brasiliensis* seeds through ultrasonic-assisted solvent extraction via response surface methodology

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ABSTRACT

The demand for oil has been increasing vastly over time, and the source of this has slowly been diminishing. The use of non-food feedstock is seen as a promising alternative source for the production of bio-based fuel. In this study, rubber (*Hevea brasiliensis*) seeds were utilized as biomass in bio-oil production considering that these are non-edible and considered wastes in rubber tree plantations. In the oil extraction process, the rubber seed kernels were oven dried at 100 °C for 24 h, powdered and then dried further at 105 °C for 4 h. After characterization, optimization study was done using Design Expert 7.0 software through central composite design of the response surface methodology. Ultrasonication technology was employed in the oil extraction process which significantly reduced the reaction time needed for extraction to 15 min compared the conventional extraction method of at least 8 h. An optimum rubber seed oil (RSO) yield of 30.3 ± 0.3% was obtained using 15 g biomass, 5:1 n-hexane to biomass (mL g^{-1}) ratio, 50 μm resonance amplitude and 60 ± 5 °C temperature at 15 min reaction time. The oil yield at optimum condition was found to have 0.89 g mL^{-1} density at room temperature, 26.7 cSt kinematic viscosity at 40 °C and high heating value of 39.2 MJ kg^{-1}. The Fourier Transform Infrared Radiation spectroscopy analysis of the RSO, at optimum condition, showed the presence of carboxylic acid and ester carbonyl functional groups which are good indicators as a potential source of biodiesel.
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1. Introduction

Fossil fuels are considered to be the principal source of energy supplying the largest percentage of global demands in more than a century. However, because of the growing demand for energy brought by industrialization and rapid population growth, some conventional energy sources including the fossil fuels have slowly been diminishing [1,2]. By 2030, the energy demand is estimated to rise to 60% compared today. This condition will eventually bring global energy crisis if we continually depend on the diminishing fossil fuel reserve which is forecasted to last only in the next 45 years [3]. Besides, the world's dependence on fossil-based fuels has been challenged in the current generation because of environmental and health threats [4] brought by the emission of toxic pollutants such as carbon monoxide, carbon dioxide (CO_2), sulfur oxides (SO_x) and hydrocarbons (HC). By these reasons, the search for cleaner and renewable energy sources that could replace fossil fuels and other non-renewable energy sources has been initiated [5].

As one of the immediate solutions, the extraction of oil from biomass has been on track. It is because oils from biomass are convertible into biodiesel, an environmentally helpful answer when used as alternative engine fuels due to less CO_2, SO_x and HC emissions [6]. Currently, biodiesel is blended with the conventional diesel fuels. In the United States, B20 (20% biodiesel blend) is applied [7], while Indonesia mandates a maximum of 10% biodiesel blend and 5% in Malaysia, Singapore, Thailand and Vietnam [8]. The

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Philippines with coconut as the primary raw material of biodiesel [9], mandates a minimum of 2% blend to all diesel engine fuels since 2009 and up to 20% in the year 2030.

However, there has been an emerging issue on the utilization of food-based biomass like coconut (first-generation feedstock) because of the "food versus fuel" crisis. That is why many researchers at present undertake further studies on the use of the second-generation feedstocks like jatropha [8] and mahogany [9]. Seeds of trees like that of rubber are categorized as a second-generation biomass. Related literature shows that rubber seed oil (RSO) contains 10.2% palmitic acid, 8.7% stearic acid, 24.6% oleic acid, 39.6% linoleic acid and 16.3% linolenic acid [10], which are suitable raw materials for biodiesel production.

There are already published studies on biodiesel production using the said biomass such as the study of Ramadhas et al. [10], Bokhari et al. [11], and Onoji et al. [12]. However, it was found that there has been no established study yet on the optimization of oil yield from rubber seed which is indeed necessary to maximize the potential of the said biomass in the production of biodiesel. The primary objective of this study, therefore, is to study on the optimization of oil extraction from rubber seeds using ultrasonic-assisted solvent extraction (UASE) and central composite design (CCD) of the response surface methodology (RSM). The ultrasonic technology was applied since it was found to be an efficient process while significantly reducing the reaction time needed compared to the conventional extraction process like Soxhlet extraction and stirring [13].

2. Materials and methods

2.1. Chemicals

The n-hexane (95%, GR grade, Duksan Pure Chemicals) used in this study was purchased from Harnwell Chemical Corporation, Cagayan de Oro City, Philippines.

2.2. Preparation and characterization of rubber seeds

The rubber seeds were collected from Poblacion, Claveria, Misamis Oriental, Philippines for uniformity of fruits variety. The kernels derived from cracking the fruit capsule shells were then oven dried at 100 °C for 24 h. The dried biomass was powdered using mortar and pestle. The powdered biomass was sieved, and the particles which passed through 20-μm mesh were oven dried further at 105 °C for 4 h and utilized in the experiment. The moisture content analysis was performed using drying oven DY610C (Yamato Scientific Chongqing Co.) and the ash analysis was done using LabTech LEF-304P-2 muffle furnace. The functional groups were determined through Fourier Transform Infrared Radiation (FTIR) analysis using Shimadzu FTIR 8400S.

2.3. Experimental design for optimization

The CCD of the RSM was employed using the Design Expert 7.0 software. Two variables were optimized such as n-hexane to biomass (HB) ratio and reaction time. The range and level employed in the experiment are shown in Table 1. The levels or range of values of the variables followed here were based on the results of the parametric study previously conducted.

Table 1
Experimental range and levels of independent variables in UASE optimization study.

Variable	Coded level				
	−2	−1	0	1	2
n-Hexane to biomass ratio (mL g^{-1})	2	3	4	5	6
Reaction time (min)	10	15	20	25	30

2.4. UASE setup and experimental procedure

The experiment was based on the suggested values designed by CCD. The recommended combinations of the independent variables were carried out to extract the oil from the rubber seeds. All runs were done inside the fume hood at ambient conditions (25 ± 1 °C) using an ice-cold water bath.

The UASE was done in a flat-bottom cylindrical jar (~10.2 cm high, 6.4 cm diameter) using ultrasonic processor UP400S (400 W, 24 kHz). This equipment has a 45 mm long and 22 mm diameter tip sonotrode with 85 W cm^{-2} acoustic power density, and a maximum amplitude of 100 μm. The ultrasonication process was conducted by inserting the probe tip approximately 5 cm from the top of the liquid for experimental runs with a larger amount of sample. For those runs with a minimal amount of sample, the probe tip was inserted in such a way that the ultrasonic irradiation will be evenly distributed throughout the sample.

The combinations of all the runs generated by CCD were followed maintaining 50 μm resonance amplitude and 60 ± 5 °C temperature. After reactor cooling of each run, 60 mL of distilled water was added and diluted the mixture while slowly mixing it using a stirring rod to facilitate the recovery of the n-hexane-oil phase. The mixture was set aside for 3 h to allow biphasic layer separation. The upper phase composing n-hexane and RSO was carefully aspirated and transferred to a flask for distillation. A 20 mL n-hexane was then added to the cell residue and stirred to extract recoverable oil. The mixture was set aside for 10 min, and the upper phase was aspirated and added to the collected n-hexane-RSO suspension. It was then heated at 70 ± 5 °C to make sure that n-hexane would evaporate and the RSO would remain. The experimental setup is presented in Fig. 1.

2.5. Analytical methods

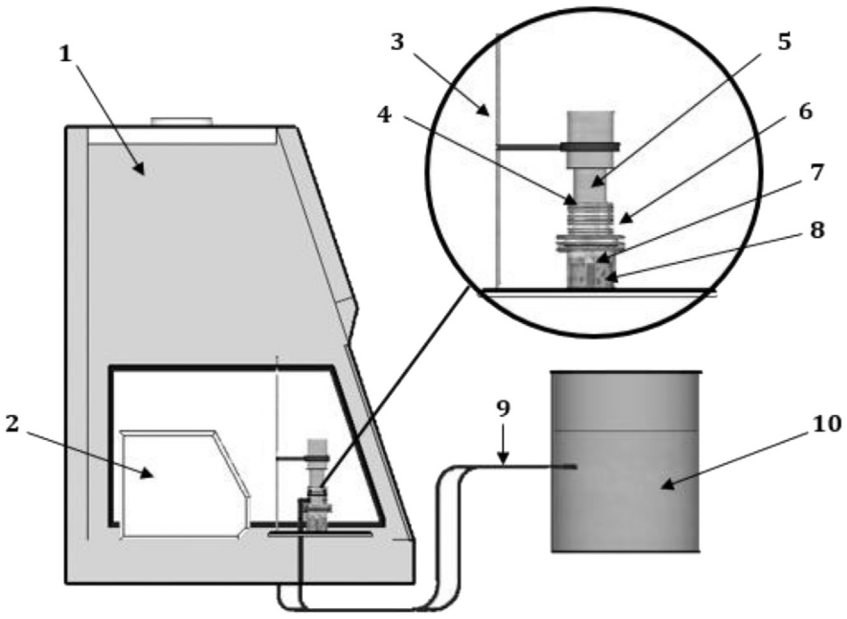
The oil extracted from the rubber seeds underwent analysis to make sure that it meets the required specifications. The density of the RSO was analyzed following the standard method for density determination. Kinematic viscosity was analyzed by ASTM D-445-71 (Kinematic Viscosity of Transparent and Opaque Liquids) using Glass Capillary Viscometer. The high heating value (HHV) of the RSO was determined using ASTM D4809 (Standard Test Method for Heat of Combustion of Liquid Hydrocarbon Fuels by Bomb Calorimeter Precision Method). The functional groups of rubber seed powder and RSO were analyzed using the FTIR spectroscopy (Shimadzu FTIR 8400S).

3. Results and discussion

3.1. Characteristics of rubber seed

The moisture content of the fresh samples and the dried samples were obtained as well as its ash content on a dry basis. Three experimental runs were done for each analysis to derive a reasonable standard deviation. Table 2 shows the results of the moisture and ash content analysis of the biomass.

The recorded rubber seed moisture content of 2.6 ± 0.3% is comparable and even slightly lower than the moisture content reported in the study of Eka et al. [14] which is 4.0%. This moisture content result is also lower than 5% moisture content, with no impact on the oil extraction efficiency [15]. The ash content of 9.8 ± 0.5% falls within the accepted range of 5–10% for diverse agricultural crop materials [16]. Based on the work of Hassan et al.



Legend

1 - Fumehood	6 - Copper tube
2 - Ultrasonic processor	7 - Sonotrode
3 - Ultrasonicator stand	8 - Rubber seed-solvent suspension
4 - Thermometer inlet	9 - Water pipe
5 - Reactor	10 - Chiller/Cold water source

Fig. 1. Experimental setup for the ultrasonication process.

Table 2
Characterization of rubber seeds.

Characteristics	Value
Moisture content (fresh)[a]	32.4 ± 0.9
Moisture content (dry)[b]	2.6 ± 0.3
Ash content[b]	9.8 ± 0.5

Mean ± standard deviation (n = 3).
[a] As received.
[b] Dried biomass.

[17], the rubber seed kernel (biomass) has a high heating value of 27.5 MJ kg^{-1}, volatile combustible matter of 89.4% and fixed carbon of 6.1%.

The functional groups found in the biomass (rubber seed powder) were also analyzed using FTIR spectrometry. Fig. 2 shows the FTIR spectra of the biomass. The rubber seed powder sample was scanned 45 times within the range of 400–4000 cm^{-1} wavelengths. The result of the FTIR analysis showed the presence of carbonyl (C═O) functional groups represented by the peak found between 1690–1850 cm^{-1} wavelength with actual peak at 1743 cm^{-1}. This also showed the presence of ketones, aldehydes and esters caused by the C═O stretching vibrations observed at peaks between 1650–1750 cm^{-1} which signifies the possible presence of these necessary compounds which are essential components of a good quality RSO.

3.2. Optimization studies and modeling

Experimental runs generated by the CCD were followed, and RSO yields were recorded. As shown in Table 3, the RSO yield varies from 21.3 wt% (run 8) to 32.0 wt% (runs 5 and 11). The optimum yield of 32 wt% was way higher than the reported yield of 24 wt% in the study of Roschat et al. [18]. This result means that oil yield from rubber seeds could be maximized using UASE via response surface modeling. Besides, the reaction time is significantly reduced which means saving of energy requirement.

The analysis of variance (ANOVA) of the response surface quadratic model for the percentage oil yield of rubber seeds is presented in Table 4. The result shows a model p-value of < 0.0001 implying that the model generated could significantly predict the RSO yield given the values of the chosen parameters. It means that there is only < 0.01% chance that error could occur due to the unpredictable data swings and variation from one value to another. The lack of fit p-value of 0.5009 implies that the lack of fit is not significant relative to pure error.

The analysis of fit summary suggested that response surface quadratic model is well suited in predicting the percentage RSO yield. The coefficient of determination (R^2) value of 0.9618 is high which denotes that the model in predicting the RSO yield is accurate and strong. This result also means that there is 96.18% certainty that the generated model can explain the variability of the data. Eq. (1) shows the reduced quadratic model equation in terms of actual factors where A represents the HB ratio (v/w) and B accounts for the reaction time (min).

$$RSO\ Yield = 15.772 + 6.709A - 0.436B + 0.133AB - 0.865A^2 - 0.001B^2 \quad (1)$$

The regression analysis results showed that A is a significant term ($p < 0.05$). This result was justified in the established equation

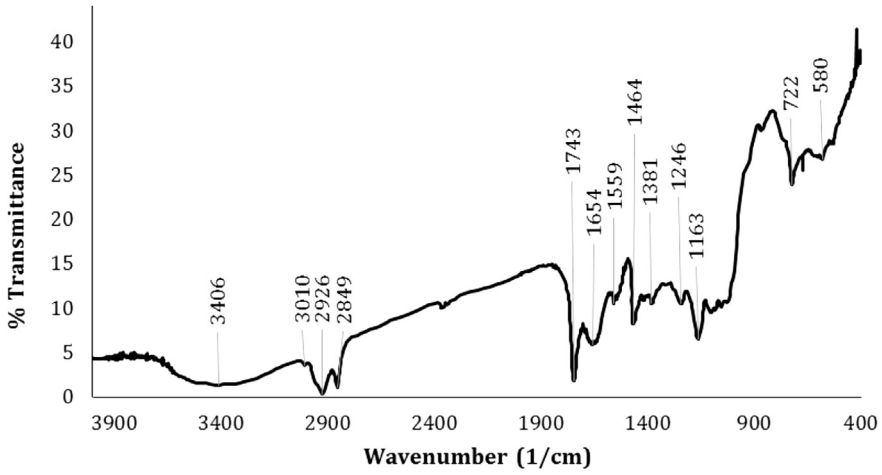


Fig. 2. FTIR spectra of rubber seed powder.

Table 3
Oil yield extracted from rubber seeds.

Experiment[a]	Operating variables		Oil yield (wt%)	
	n-Hexane (mL)	Reaction time (min)	Actual	Predicted
1	60	20	30.7	30.2
2	60	20	30.7	30.2
3	45	15	28.0	27.3
4	75	15	30.7	30.8
5	90	20	32.0	31.6
6	45	25	26.7	26.4
7	60	10	29.3	29.6
8	30	20	21.3	21.8
9	60	30	30.7	30.5
10	60	20	29.3	30.2
11	75	25	32.0	32.6
12	60	20	30.7	30.2
13	60	20	29.3	30.2

[a] Biomass = 15 g.

Table 4
ANOVA of response surface quadratic model for percentage oil yield from rubber seeds.

Source	Sum of squares	df	Mean square	F value	p-value Prob > F
Model	92.41	5	18.48	35.24	< 0.0001[a]
A-HB ratio	71.74	1	71.74	136.80	< 0.0001[a]
B-Reaction time	0.60	1	0.60	1.14	0.3208[b]
AB	1.77	1	1.77	3.37	0.1089[b]
A^2	17.16	1	17.16	32.73	0.0007[a]
B^2	0.02	1	0.02	0.04	0.8398[b]
Residual	3.67	7	0.52		
Lack of fit	1.52	3	0.51	0.94	0.5009[b]
Pure error	2.15	4	0.54		
Cor total	96.08	12			
$R^2 = 0.9618$					

[a] Significant.
[b] Not significant.

where variable A has the highest coefficient value. This result means that the increase in HB ratio could lead to significant increase in the RSO yield. On the other hand, term B is not important having a negative numerical coefficient which implies that the increase in the reaction time does not give any significant increase in the oil yield and might even reduce if time is increased longer. This result could be attributed to the fact that, at longer reaction time, the temperature in the UASE setup increases which eventually leads to the evaporation of solvent used and the deduction of the solvent which is needed in the extraction process [19]. Negative coefficients determine the terms which could lead to the decrease in the RSO yield during production.

Using the CCD established equation (Eq. (1)), the results of the actual runs were validated with predicted values as shown in Table 3. As seen in the table, the actual and predicted responses were almost the same which supported the claim that the model fitted the data with high certainty of correctness and reliability. Fig. 3 shows the diagnostic graph of the actual versus predicted responses of the experimental runs. It can be observed that the actual responses plotted were near the trend line representing the predicted responses. This result means that the actual runs were carefully conducted. This result also supports the claim that response surface quadratic model best fitted the data of this study.

3.3. Effects of operating variables

According to Eq. (1), HB ratio affects the RSO yield significantly during the UASE. Fig. 4 shows the contour and 3D plots supporting that HB ratio is influential on the RSO yield. As illustrated in the figure, high HB ratio resulted in a high yield of RSO, and it was even increasing at rising reaction time. This trend is due to enough solvent amount even if vaporization might happen at longer reaction

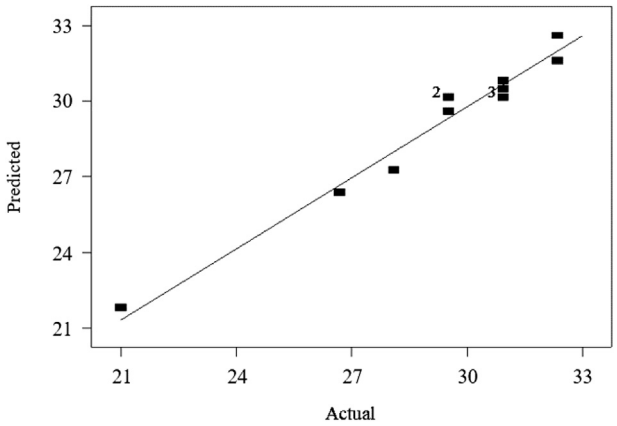


Fig. 3. Diagnostic graph on the actual versus predicted RSO yield (%) of the experimental runs.

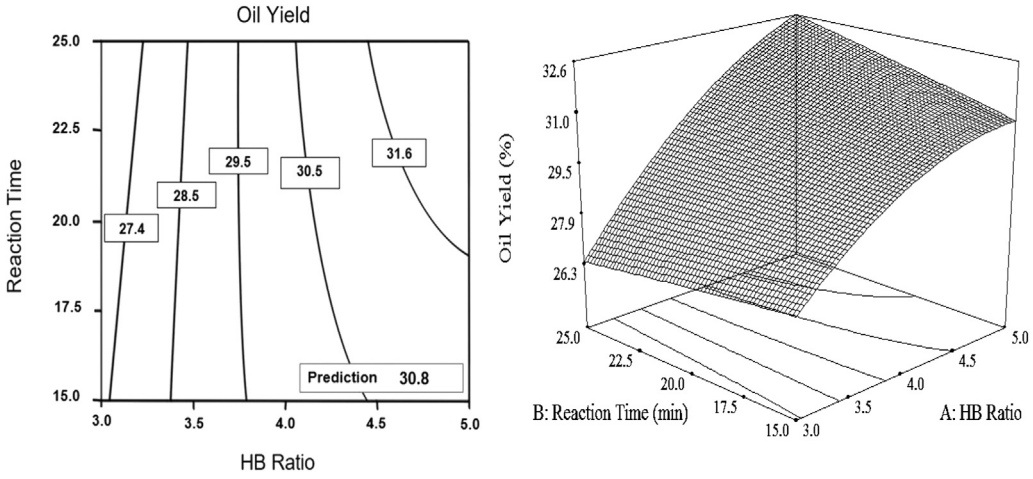


Fig. 4. Contour and 3D plots showing the effects of HB ratio and reaction time to RSO yield.

period. On the other hand, little amount of RSO yield was observed at low HB ratio, and the trend was even decreasing as the reaction time was increased. This result could be attributed to the inevitable rise in temperature caused by the ultrasonication process which caused the gradual evaporation of the solvent. The same observation was recorded in the study of Latheef [19] where it was found out that, at higher treatment time, a significant amount of solvent is lost due to evaporation and the amount of solvent left is not enough for efficient extraction.

3.4. Numerical optimization and validation

Four solutions of the RSO yield optimization were suggested by the RSM with the following criteria:

1. HB ratio was held in the acceptable range since it was found to be a significant term and that minimizing it would surely decrease the RSO yield. Maximizing it, on the other hand, means an increase in the cost of the extraction process.
2. Reaction time was minimized since the primary goal of this study is to reduce the time of oil extraction through the use of ultrasonication.
3. The response, RSO yield, was maximized since another goal of this study is to extract a high amount of oil at a short time.

The solution with the highest desirability (0.94) was selected and was verified in the actual runs (Fig. 5). The results of the actual runs were compared to the CCD prediction as summarized in Table 5.

Three verification runs were conducted, and the mean plus the standard deviation is recorded in Table 5. The error of 1.8 ± 1.0% is below the 5% acceptable error. This result supported the claim that response surface quadratic model used is valid.

3.5. Product analysis and characterization

The extracted RSO was analyzed and characterized. Its color, density (g mL^{-1}), kinematic viscosity (cSt) and HHV (MJ kg^{-1}) were determined and compared to related RSO studies as shown in Table 6.

Table 5
Optimization and validation of the oil yield from rubber seeds.

Experiment	Operating variables			Response	Error
	Biomass (g)	Hexane (mL)	Reaction time (min)	Bio-oil yield (%)	%
$CCD_{(theoretical)}$	15	75	15	30.8	
$Validation_{(actual)}$	15	75	15	30.3 ± 0.3	1.8 ± 1.0

Optimization criteria: minimize – reaction time; maximize – yield; in range – HB ratio, Mean ± standard deviation (n = 3).

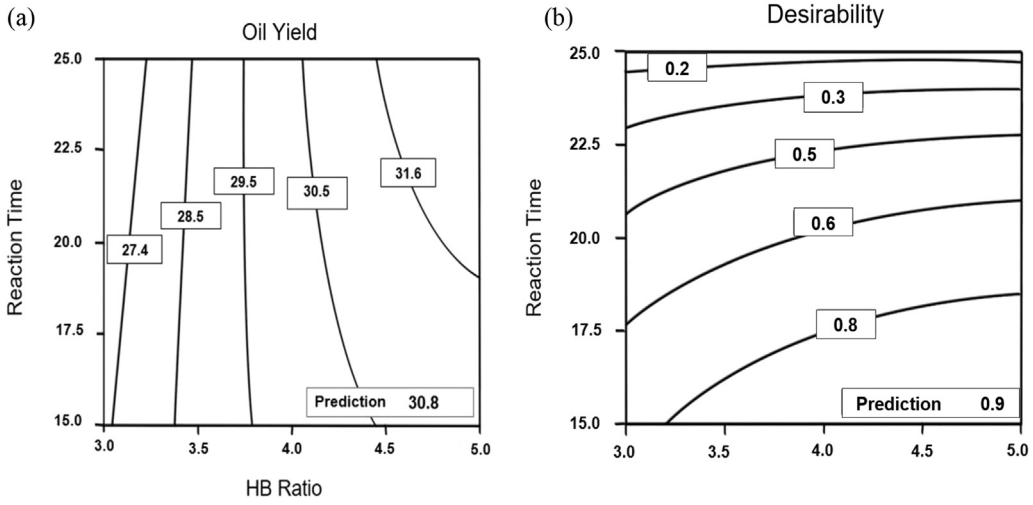


Fig. 5. Graph of profiles for predicted values of (a) extraction yield, and (b) desirability level for different variables for optimum yield.

Table 6
Characterization of produced oil in comparison to related RSO studies.

Characteristics				Reference
Color	Density (g mL^{-1})	Kinematic viscosity @ 40 °C (cSt)	High heating value (MJ kg^{-1})	
Golden brown	0.89	26.7	39.2	This study
_	–	66.2	37.5	[10]
_	0.87	36.1	37.2	[20]
Dark brown	–	40.9	39.7	[24]
Golden yellow	0.91	30[a]	39.3	[21]
_	–	41.1	39.5	[25]

[a] Tested at 24 °C.

The density of 0.89 g mL^{-1} is just comparable and slightly higher than the reported RSO density in the study of Bokhari et al. [20], and lower than in the study of Reshad et al. [21]. Its kinematic viscosity of 26.7 cSt is way lower than the reported viscosity in the related RSO studies presented in Table 6. This low viscosity record could be attributed to the ultrasonic waves which generated heat and cavitation on the rubber seed during the extraction process [22]. High viscosity is not desirable because it could cause poor fuel atomization, incomplete combustion and carbon deposition on the injector and valve seats resulting in severe engine fouling [23].

The HHV of 39.2 MJ kg^{-1} is comparable to the study of Singh et al. [24], Meena Devi et al. [25], and Reshad et al. [21] and higher compared to recorded value in the study of Bokhari et al. [20] and Ramadhas et al. [10]. This result means that the RSO extracted in this study has a high-energy content which is significant in biodiesel production. This result is supported by the moisture and ash content results which are low considering that these mentioned parameters affect the HHV of the oil. Less ash and moisture content means high HHV [26]. Furthermore, the ultimate analysis of rubber seed kernel in the study of Hassan et al. [17] showed 64.5% carbon, 8.2% hydrogen, 3.6% nitrogen and 0.3% sulfur content. The high value of carbon could be attributed as one of the reasons of the high HHV of the RSO.

The functional groups found in the RSO were also analyzed via FTIR spectrometry. Fig. 6 shows the FTIR spectra of the crude RSO extracted via UASE. The actual peaks of the RSO FTIR spectra were also identified, and the assigned functional groups were determined. The FTIR spectra indicated the presence of the carboxylic acid carbonyl functional group at wavelength peak 1720 cm^{-1} with C═O stretching vibration [27]. Wavelength peak 1749 cm^{-1} of C═O stretching vibration was also observed which is attributed to an ester carbonyl functional group. Moreover, C–O–C stretching vibrations attributed to ester were also observed at actual wavelength peaks 1162 and 1239 cm^{-1} [28]. The peaks in wavelength 3016, 2925 and 2857 cm^{-1} could be attributed to C–H stretching vibration indicating the presence of alkene and alkyl compounds. The C–H bending vibrations observed in peaks 1458 and 1374 cm^{-1} indicate the presence of CH_2 and CH_3 compounds [27]. In general, the functional groups found in the FTIR spectra of RSO showed similar functional groups present in other vegetable oils [29].

3.6. Comparison of UASE, Soxhlet extraction, and extraction via magnetic stirring

The conventional oil extraction method, Soxhlet extraction, and oil extraction via magnetic stirring were conducted to compare the efficiency of the UASE to the aforementioned ones. Fig. 7 shows the comparison of the results of the oil yield of the three methods.

At optimum condition, the UASE using n-hexane solvent had 30.3% RSO yield which is higher than the yield via magnetic stirring extraction with only 24.2%. Soxhlet extraction, meanwhile, recorded 34.4% RSO yield which is greater than the UASE method. The use of centrifugation could help in maximizing the yield in UASE method because it could improve oil retrieval along the process. The oil extraction efficiency can be arranged as follows: Soxhlet extraction > UASE > magnetic stirring.

Although Soxhlet extraction recorded 4% higher RSO yield than the UASE, extraction time and solvent volume used in UASE were far way less compared to Soxhlet extraction. Only 75 mL of n-hexane and 15 min extraction time were utilized through UASE, while 300 mL of n-hexane and 8 h extraction time were used in the Soxhlet extraction – both were employed in the same amount of biomass (15 g).

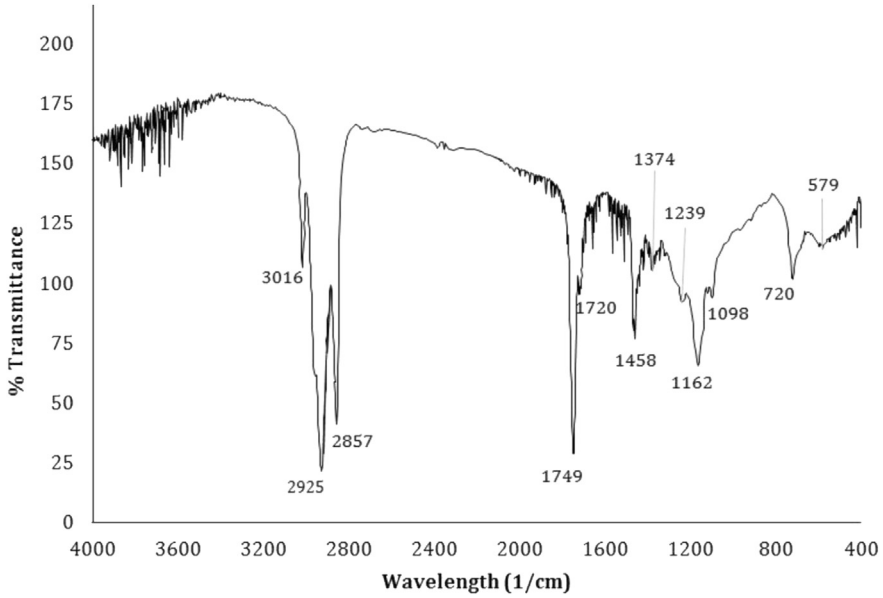
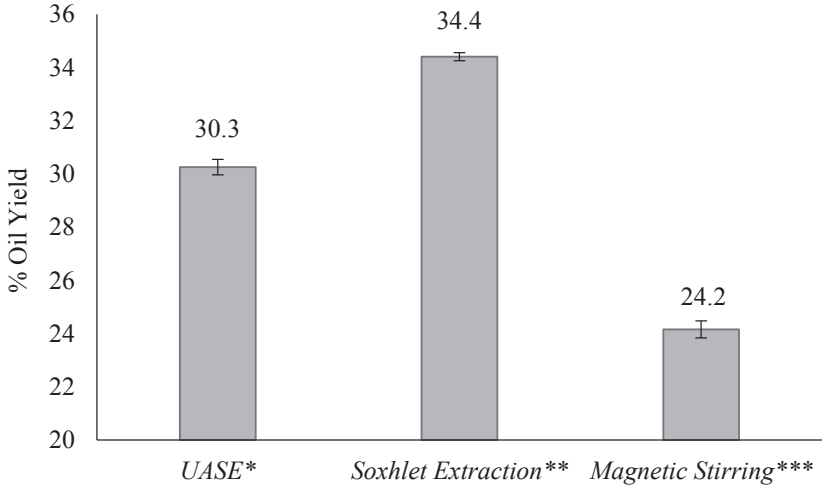


Fig. 6. FTIR spectra of RSO produced via UASE.



*optimum condition: 5:1 n-hexane to biomass ratio (75 mL), 15 g biomass, 15 min, 50 μm amplitude
**15 g biomass, 300 mL n-hexane, 8 h, 80 °C
***5:1 n-hexane to biomass ratio (20 mL), 4 g biomass, 37 min, room temperature

Fig. 7. Comparison of oil yield of UASE, Soxhlet extraction and extraction via magnetic stirring.

4. Conclusions

The results of this study show that, at optimum condition, the oil yield from rubber seed was recorded at 30.3 ± 0.3 wt% using 15 g biomass, 5:1 HB ratio (mL g^{-1}), 50 μm resonance amplitude and 60 ± 5 °C temperature at 15 min reaction time. A significant amount of extraction time was reduced in UASE compared to the conventional Soxhlet and stirring extraction methods with comparable efficiency. Furthermore, the characteristics of the RSO derived from the optimum condition are of noteworthy values. Response surface quadratic equation modeled the predicted oil yield of rubber seeds through CCD of the RSM with a p-value of < 0.0001 implying 99.99% accuracy. The R^2 value of 0.9618 indicates that there is 96.18% certainty that the generated model can explain the variability of the data.

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ARTICLES FOR FACULTY MEMBERS

SUSTAINABLE PROCESSING OF RUBBER (HEVEA BRASILIENSIS) SEED OIL: PHYSICOCHEMICAL INSIGHTS INTO EXTRACTION AND ANTIOXIDANT PRESERVATION

Rubber tree (Hevea brasiliensis) seed shell extracts as a promising green antioxidant alternative to increase biodiesel oxidation stability./ Oleinik, G., Soares, L. C., Benvegnú, D. M., Lima, F. O., Rodrigues, P. R. P., & Gallina, A. L.

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Rubber tree (*Hevea brasiliensis*) seed shell extracts as a promising green antioxidant alternative to increase biodiesel oxidation stability

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ABSTRACT

Biodiesel is a viable and advantageous alternative to fossil fuels, as its use contributes to reducing the emission of polluting greenhouse gases, in addition to being a biodegradable fuel and presenting economic advantages. However, it has low oxidative stability, quickly degrading when in contact with oxygen being important to use antioxidant additives to keep this biofuel stable. Thus, this work aimed to produce a natural antioxidant for soybean oil biodiesel from rubber tree seed shell extracts. First, was made the production of the extracts and the comparison of their antioxidant activity. The extracts were produced via Soxhlet, using two solvents (water and hydroalcoholic containing water and methanol 50 % v/v). The DPPH (2,2-diphenyl-1-picryl-hydrazyl) free radical capture test was performed to compare the antioxidant activity and the determination of total flavonoids and phenols. After, soybean biodiesel was produced, and the extracts were added to the biofuel to increase its oxidative stability. Antioxidants were added to biodiesel during the washing process. To evaluate biodiesel's oxidative stability with antioxidants, the induction time (IT) was verified with the Rancimat equipment. Biodiesel treated with hydroalcoholic extract increased the induction time of control biodiesel from 3.43 h to 6.14 h, and biodiesel treated with aqueous extract increased the induction time to 6.12 h. The extracts produced reached 92.79 % of DPPH free radical capture for the hydroalcoholic extract, and 78.97 % for the aqueous extract. Regarding the determination of total flavonoids and phenols, extracts reached up to 191.07 $\pm$ 2.90 mg.100 g^{-1} of total flavonoids, and 3278.05 $\pm$ 152.91 mg GAE.100 g^{-1} of phenolic compound. Thus, the antioxidant extracts produced under sustainable conditions from the rubber tree seed husk, which is a by-product, are a promising green alternative, with potential for application as additives in the biodiesel industry.

1. Introduction

Considering the current global energy matrix, fossil fuels constitute the vast majority of primary energy consumed worldwide. Besides that, fossil fuels will still be the most used energy source for at least one generation. In this way, research involving renewable energy sources and biomass, such as biofuels, has been taking space in the energy demand, due to the economical and cleaner aspect (Borges et al., 2017). The energy transition allowing to produce biofuels from the biomass transformation with a cust decrease and environmental sustainable way, which has the potential to reduce CO_2 emissions in the atmosphere (Grassi and Pereira, 2019).

Biodiesel has been expanding in the field of research as an alternative to fossil fuels. This long-chain fatty acid ester is derived from biomass and produced from the triglycerides transesterification or free fatty acids esterification (Arumugam and Ponnusami, 2019). This biodiesel has advantages, such a fuel made from renewable raw materials, contributing to reducing polluting gas emissions. In addition, biodiesel is biodegradable, has low or no sulfur and aromatic content, a high flash point, and increases engine life, considering it is an excellent lubricant (Knothe and Razon, 2017).

However, biodiesel has a low oxidative stability, due to the composition of the oils and fats used as raw material, which have unsaturated fatty acids susceptible to oxidation, especially when exposed to light and oxygen (Meher et al., 2006). To increase the oxidative stability of biodiesel, antioxidant additives are used. The process involves substrates

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that do not react with the oxygen molecule's free radicals, thereby preventing the formation of oxidation products. To this, the antioxidant donates an electron to the radical to neutralize its harmful activity, protecting the target material from oxidizing (Kumar and Singh, 2018).

Depending on their origin, these antioxidant substances can be classified into synthetic and natural. Synthetic antioxidants are widely used at low dosages as an antioxidant additive for biodiesel, to eliminate oxidative species. The main synthetic antioxidants are BHA (butylhydroxyanisole), BHT (2,6-di-tertbutylhydroxytoluene), PG (propylgallate), TBHQ (tert-butylhydroquine), PY (pyrogallol), and OG (octyl gallate). However, these substances are expensive and, according to studies, have carcinogenic and toxic properties, which encourages the development of cheap alternatives, less harmful to the environment and non-toxic, as natural antioxidants, which come from plant material, rich in compounds with antioxidant properties, as phenolic compounds (Kumar and Singh, 2018).

In the study by (De Sousa and Leanne Silva, 2014) the antioxidant activity of curcumin and b-carotene was evaluated as a method to control the oxidative process of soy methyl biodiesel during a storage period of 180 days, and curcumin increased the biodiesel induction period by 83 %. Schaumlöffel et al. (2021) used tannin extracts from acacia (*Acacia mearnsii*), chestnut (*Castanea sativa*) and uvacho (*Schinopsis lorentzii*) in mixture with triethanolamine as antioxidants for soy biodiesel, and as a result they obtained that, when applied to biodiesel at a concentration of 350 mg.L^{-1} in a mixture with 1.59 % by weight of triethanolamine, tannins were highly effective in increasing oxidative stability, including superior performance of the synthetic antioxidant TBHQ. Freitas et al. (2019) evaluated the antioxidant power of the natural extracts catechin, curcumin and quercetin on the oxidation stability of cotton oil methyl biodiesel, and found that all additives had a positive effect on the oxidative stability of biodiesel, in addition, catechin and quercetin were more efficient than synthetic antioxidants, while curcumin showed similar results.

The rubber tree (*Hevea brasiliensis*) is native from South America, and cultivated worldwide, with a total area of about 10 million hectares destined for harvesting and latex production (Chaikul, Lourith and Kanlayavattanakul, 2017). Latex is currently the main use of the rubber tree and it is destined for various branches of industries, for example, tires, rubber gloves and medical devices (Chaikul, Lourith and Kanlayavattanakul, 2017).

New applications of materials from rubber trees have been published in recent years. Recently, studies on the biomedical applications of latex have shown that it is capable of stimulating tissue repair (Guerra et al., 2021). Chaikul, Lourith and Kanlayavattanakul (2017) extracted rubber tree seed oil to analyze the fatty acid components and evaluate the biological activity and cytotoxicity by the sulforhodamine B assay, in addition to the melanogenesis assay and antioxidant activity, in cell culture. Aravind, Joy and Nair (2015) studied the physical-chemical, thermal and tribological properties of rubber seed oil as a possible potential for use as a biolubricant. Dhawane, Kumar and Halder (2016) in their study, presented the parametric effects in the production of biodiesel from *Hevea brasiliensis* oil using carbonaceous heterogeneous catalyst derived from flamboyant pods. (Lüneburger, 2022) enabled the production of biodiesel from rubber tree seed oil.

Despite the numerous applications of the species, so far the literature lacks research that explores the antioxidant properties of the rubber tree seed shell as an antioxidant additive to biodiesel. Oleinik, et al. (2022) studied the antioxidant activity of extracts only from the kernel of the respective seed. Thus, the aim of this study was to evaluate *Hevea brasiliensis* seed shell extracts as antioxidant additives for biodiesel. The antioxidant employed constitutes a promising green alternative, as in addition to being derived from biomass, valuing a by-product. The antioxidant tests performed on the extracts were the determination of total phenolic compounds and flavonoids and the DPPH free radical capture test. To evaluate the oxidative stability of biodiesel with antioxidants, the induction time (IT) was verified with the Rancimat device, model 873 from Metrohm.

2. Material and methods

2.1. Materials

Methanol (Synth – Brazil), Ethanol (Synth – Brazil), DPPH (Sigma-Aldrich – Brazil), $AlCl_3$ (Dinâmica – Brazil), Rutin (Êxodo Científica – Brazil), Folin Ciocalteau (Dinâmica – Brazil), Na_2CO_3 (Dinâmica – Brazil), Gallic acid (Êxodo Científica – Brazil), KOH (Dinâmica – Brazil), HCl (Êxodo científica – Brazil) and NaCl (Êxodo Científica – Brazil).

2.2. Obtaining and manipulating seed shells of Hevea brasiliensis

Hevea brasiliensis rubber tree seeds that fell to the ground and would be discarded were used in this research. Those were collected in Paranaíba, Mato Grosso do Sul, Brazil, by the company Kaiser Agro Florest according institutional norms, guidelines and national legislation (Law No. 13,123, of May 20, 2015). Subsequently, they sent them to the Federal University of Fronteira Sul in Realeza. This research with *Hevea brasiliensis* seeds was registered in SisGen (National System for the Management of Genetic Heritage and Associated Traditional Knowledge) under registration number A4C9E3E. The seed shells were crushed in a knife mill in 20 mesh to standardize the particle size and facilitate the extracts preparation.

2.3. Preparation of extracts

From the ground and dried rubber tree seed shell, extractions were performed with Soxhlet equipment at concentrations of 40, 60, and 80 g.L^{-1}, at 2, 4 and 6 hours of extraction. The solvents used for extraction were an aqueous solvent, and another hydroalcoholic solvent consisting of 50 % water and 50 % methanol (Silva et al., 2010).

Considering the different concentration conditions and extraction time mentioned, and the two solvents used, a total of 18 extracts were produced.

2.4. Experimental design

The experimental design for the statistical study considered the two different solvents as descriptive variables. Regarding the quantitative variables, extraction times of 2, 4, and 6 hours and concentrations of rubber tree seed shells of 40, 60, and 80 g.L^{-1} were selected. The tests were performed in triplicate, and the antioxidant capacity was obtained by the percentage of free radical capture DPPH (2,2-diphenyl-1-picrylhydrazyl), determination of flavonoids, and total phenols.

2.5. Determination of antioxidant activity through DPPH free radical capture

Determination of antioxidant activity by DPPH free radical capture was developed according to the methodology from Oleinik et al., (2022). An ethanolic solution of 0.1 mmol.L^{-1} of DPPH was prepared, diluted in ethanol by a factor of 10. For the determinations, 2.7 mL of the DPPH solution were added to 0.3 mL of the obtained extract (A_{sample}). In addition, the same procedure was performed by adding 2.7 mL of DPPH in 0.3 mL of the solvent used in the extract for control ($A_{control}$). The blank of the samples was obtained from the combination of 0.3 mL of the extract and 2.7 mL of ethanol. Comparative tests with TBHQ and ascorbic acid were carried out at a concentration of 1000 ppm.

After 30 minutes of reaction, the absorbance was analyzed in a UV/Vis spectrophotometer by Thermo Scientific, model Evolution 201, at a wavelength of $\lambda = 515$ nm. Assays were performed in triplicate.

Scavenging activity was verified by calculating the DPPH free radical capture percentage based in the Eq. 1 (Oleinik et al., 2022).

$$\%AA = \frac{Acontrol - (Asample - Ablank)}{Acontrol} \times 100\% \quad (1)$$

Where $A_{control}$ corresponds to the absorbance of the DPPH control solution and A_{sample} the absorbance of DPPH with the respective sample.

2.6. Total flavonoids determination

To determine total flavonoids, was prepared a solution content an aliquot of 0.32 mL of extract, 0.32 mL of aluminum chloride solution ($AlCl_3$) 2 % (m/v) and 3.36 mL P.A. ethanol that transferred to an amber flask.

After 25 minutes, the absorbance was measured in a UV/Vis spectrophotometer by Thermo Scientifc, model Evolution 201, at λ = 413 and 427 nm. As blank, was utilized a solution containing 0.32 mL of sample solvent, 0.32 mL of solution of $AlCl_3$ and 3.36 mL of P.A ethanol.

The analytical curve was carried out using standard rutin solution in P.A. ethanol for analysis of the extracts (Chabariberi et al., 2009). The equation of the line was y=1.32860606x − 0.00746667 with R^2=0.98185.

2.7. Total phenolics determination

For the total phenolics determination, first 0.1 mL of each extract was taken, added 2.5 mL of Folin Ciocalteau solution, and 2.0 mL of saturated Na_2CO_3 solution. After those steps, the mixture was kept to stand for 1 hour to react. Then, the absorbance measurements at λ=720 nm were performed in triplicate, using the UV-Visible by Thermo Scientifc, model Evolution 201, where water was applied as a blank (Sousa, 2007). Finally, gallic acid was used to build the standard curve. The total phenolic content was calculated, expressed in mg of gallic acid (GAE) per 100 g^{-1} of the sample (Sousa, Vieira, and Lima, 2011). The equation of the line obtained was y=0.449428x+0.020706 with R^2=0.99973.

2.8. Biodiesel production

Soybean oil transesterification was carried out with methanol and KOH (potassium hydroxide) as catalysts in the proportion of (1:0.3:1 %) v/v/m. Soybean oil was heated to 80°C, and the methanol and KOH mixture was heated to 40°C. After, the methanol and potassium hydroxide solution was added to the oil vessel and kept the temperature at 60 °C under stirring for 1 hour. Then, the solution was transferred to a decanting funnel for 24 hours to separate the glycerin (Gallina et al., 2010).

2.9. Biodiesel washing and drying

The biodiesel washing took place in three stages. The first step was washing with a solution of HCl (0.1 %) to neutralize the catalyst, the second was washing with a NaCl solution (which, being polar, also helps in removing the catalyst), and the third was washing with water for removing impurities (Schlindwein, 2017; Atadashi et al., 2011). Following, 30 % (v/v) of washing solutions were used about the total biodiesel volume. The washings occurred in a decantation funnel, where they remained for 24 hours. The addition of antioxidants happened during the third stage of biodiesel washing (washing with water), employing either mechanical agitation or ultrasound.

Biodiesel drying took place in an oven at 110°C for 3 hours (Lima et al., 2007).

2.10. Antioxidant evaluation effect in biodiesel

The evaluation of the antioxidant effect of the extracts on the biodiesel was carried out in a Rancimat 873 apparatus, from Metrohm, according to the European Norm EN 14112, at 110°C and an air insufflation rate of 10 L/h to obtain the induction time (TI) (Ferrari and Souza, 2009), which should be 12 hours according to the rules pre-established by the ANP in 2019 (National Agency of Petroleum, Natural Gas and Biofuels)(Anp, 2019).

2.11. Statistical analyzes

As the first selection criterion for the results related to the antioxidant activity of the extracts, the t-test was used with a confidence level of 95 %, carried out in the Microsoft Excel® package.

After verifying the extraction conditions that obtained the highest antioxidant activity, the response surface methodology was used in the second experimental design, considering a 3^2 design in the Statistica version 8 software with the levels and quantitative variables (concentration and time) described in Tables 1 and 2. In the validation process of the equation represented by the model, the graphs of the dispersion of the residues and the ANOVA table were considered, calculated from the latter the percentage of the variation (R) and the maximum variation explained (R^2), the F value of the F_R regression and the lack of adjust F_{la} (Neto et al., 2003).

3. Results and discussion

3.1. Antioxidant activity of extracts

3.1.1. Antioxidant activity of aqueous extracts

Table 3 presents the data regarding the average antioxidant activity of the tests at times of 2, 4, and 6 hours, and concentrations of 40, 60, and 80 $g.L^{-1}$, for the aqueous solvent with the application of the t-test, with a reliability of 95 %, for comparison of means.

Based on the t-test, used to compare the averages of the tests for the different extraction times and the different concentrations of the aqueous extracts, the results were statistically equal when comparing the other extraction times. However, when analyzing the average value for the times of 2 hours (82.99 %), 4 hours (79.60 %), and 6 hours (83.72 %), was observed the time of 6 hours with a higher average, which justifies carrying out a new study with longer extraction times. Considering the difference between the times 2, 4, and 6 hours was not significant, for an optimization of the experiment, the study of 2 and 6 hours was kept, and the time of 10 hours was added to analyze whether there will be an increasing trend.

Regarding the analysis of the concentrations of the shell of the rubber tree seed (*Hevea brasiliensis*), the result obtained in the t-test, demonstrated the results for the concentrations were not statistically different from each other. However, analyzing the means for the concentrations of 40 $g.L^{-1}$ (79.86 %), 60 $g.L^{-1}$ (83.20 %), and 80 $g.L^{-1}$ (85.25 %), it can be seen that for concentrations of 60 and 80 $g.L^{-1}$ the average results were higher than for 40 $g.L^{-1}$, the which justifies an optimization of the study with concentrations greater than 80 $g.L^{-1}$, but including the range already studied for possible differences.

Therefore, for an optimization of the experimental conditions with higher averages of antioxidant activity for the aqueous solvent, the extraction times analyzed were 2, 6 and 10 hours, and the concentrations will vary between 40, 80 and 120 $g.L^{-1}$.

3.1.2. Antioxidant activity of hydroalcoholic extracts

Table 4 shows the respective data for the average antioxidant activity of the tests at times of 2, 4 and 6 hours, and concentrations of 40, 60, and 80 $g.L^{-1}$ for the hydroalcoholic solvent, also with the *t*-test application.

Table 1
Levels and variables used in the experimental design of Fig. 1.

Variables	Level −1	Level 0	Level 1
Time (h)	2	6	10
Concentration ($g.L^{-1}$)	40	80	120

Table 2
Levels and variables used in the experimental design of Fig. 3.

Variables	Level −1	Level 0	Level 1
Time (h)	3	5	7
Concentration (g.L^{-1})	45	65	85

Table 3
Results of the t-test, for the percentage of DPPH radical capture, at different concentrations and extraction times for the aqueous extract.

Time (h)	Concentration (g.L^{-1})	% DPPH capture
2^{a}	40 ^{A}	83.41±1.65
2^{a}	60 ^{A}	77.15±0.27
2^{a}	80 ^{A}	79.03±0.54
4^{a}	40 ^{A}	79.65±0.72
4^{a}	60 ^{A}	85.45±0.94
4^{a}	80 ^{A}	84.50±4.63
6^{a}	40 ^{A}	85.91±2.04
6^{a}	60 ^{A}	76.21±4.21
6^{a}	80 ^{A}	87.64±1.95

* The letters a, A are used to represent that there were no statistically significant differences in the *t*-test between different times, or between different concentrations.
* Equal letters was no significant difference between means.

Table 4
T-test results for the DPPH radical capture percentage, at different concentrations and extraction times for the hydroalcoholic extract.

Time (h)	Concentration (g/L)	% DPPH capture
2^{a}	40 ^{A}	21.65±3.70
2^{a}	60 ^{A,B}	41.52±3.34
2^{a}	80^{B}	63.17±20.77
4^{b}	40 ^{A}	69.22±7.17
4^{b}	60 ^{A,B}	77.24±1.56
4^{b}	80^{B}	52.51±25.23
6^{a,b}	40 ^{A}	68.46±19.23
6^{a,b}	60 ^{A,B}	75.19±6.44
6^{a,b}	80^{B}	74.08±2.52

* The letters a, b, A, B are used to represent statistically significant differences in the *t*-test between different times, or between different concentrations.
* Equal letters was no significant difference between means.

When comparing the extraction times for hydroalcoholic solvent using t-test, was observed the times of 2 h and 4 h are statistically different. However, the times of 2 h and 6 h and, the times of 4 h and 6 h are statistically equal. Those results show the trend towards the optimum for this study is reached between 4 h and times longer than 6 h. Even when analyzing the result of the averages for the times 2 h (53.11 %), 4 h (64.65 %), and 6 h (63.26 %), was verified close values of averages to 4 and 6 hours, could justify performing an optimization including those times.For the concentration of the shell of the rubber tree seed (*Hevea brasiliensis*) average, the value of 80 g.L^{-1} (72.58 %) was the best, followed by 60 g.L^{-1} (66.32 %), and, 40 g.L^{-1} (42.12 %). The concentrations of 60 and 80 g.L^{-1} were equal statistically, while the concentration of 40 g.L^{-1} was different, which justifies the exclusion of this concentration from the next study, but maintaining the concentrations of 60 and 80 g.L^{-1} inserted in the experimental design, as these had a higher average result among the samples analysed. Therefore, in optimizing the experimental conditions of the extracts produced with the hydroalcoholic solvent, 45, 65, and 85 g.L^{-1} concentrations will be verified, varying the extraction time between 3, 5, and 7 hours.

3.2. Optimization of the experimental condition with the highest percentage of DPPH capture

Based on the results of the conditions established in the first experimental planning and the performance of the t-test to verify the significant difference of the samples from different concentrations and extraction times, experimental extraction conditions were defined to repeat the antioxidant tests. From the extracts obtained under these conditions, the response surfaces shown in Figs. 1 and 3 were calculated, with Fig. 1 referring to extracts with an aqueous solvent and Fig. 3 referring to extracts with a hydroalcoholic solvent.

Based on the statistical treatment represented in Fig. 1, it is noted that as the concentration and extraction time varies, at one point it is possible to reach an optimal point with highest antioxidant activity. The contour lines shown in Fig. 1B demonstrated the tendency towards the optimum consist of an extraction time close to 6 h and an extract concentration near to 120 g.L^{-1}. According to the response surface for the model description, Equation 2 of the quadratic type suggested in the analysis was considered, with the ANOVA values presented in Table 5.

$$\textbf{DPPH} = 77{,}37 + 5{,}34 * \text{Time} + 3{,}04 * \text{Concentration} - 12{,}66 * \text{Time}^2 - 2{,}61 * \text{Concentration}^2 - 0{,}07 * \text{Time} * \text{Concentration} \quad (2)$$

According to Table 5, it can be seen that the variables that were significant ($p<0.05$) were linear and quadratic time, and linear concentration. Based on the data in Table 5, and to validate the model equation for the response surface, the percentage of maximum variation explained by the quadratic equation calculated was R^2= 90.73 %, and the maximum explainable variation found was R= 99.98 %, which indicates the predictive capacity regarding the trend of the experimental points for the variables considered in the study.

Regarding statistical significance, the values of F_R= 7.82 and F_{la}= 138.75 were determined, with the reference for a percentage of the F 5 % distribution corresponding to 6.26 for F_R and 215.77 for F_{la}. The first calculated value (F_R) was greater than F 5 % value, indicating that the mathematical model is suitable for representing the data. The second calculated value (F_{la}) smaller than the model references value, indicating that there was no lack of fit in the model (Neto et al., 2003).

The graphs shown in Fig. 2 describes the random dispersion of the residuals and data normality, indicating that the quadratic equation, represents the response surface according to the conditions employed does not follow a trend.

Fig. 3 shows the response surface and the contour line plot for the DPPH free radical capture results under optimized conditions for the hydroalcoholic solvent.

According to Fig. 3, it can be seen that there came a time when the relationship between concentration and extraction time reached the greatest efficiency in antioxidant activity. This Figure showed the trend towards the optimum of better antioxidant activity for the hydroalcoholic extract corresponded to the concentration of 85 g.L^{-1}, with an extraction time of 5 h, under these conditions, the antioxidant activity percentage, via capture of the DPPH, was 92.79 ± 0.70 %. Eq. 3, of the quadratic type, describes the model for the response surface, and the ANOVA values are shown in Table 6.

$$\textbf{DPPH} = 90{,}05 - 5{,}85 * \text{Time} - 0{,}54 * \text{Concentration} - 11{,}07 * \text{Time}^2 - 3{,}61 * \text{Concentration}^2 - 4{,}91 * \text{Time} * \text{Concentration} \quad (3)$$

In the model validation process for the response surface, the R^2 was 70.29 %, and the R was also the same value. In addition, the F_R parameter was evaluated seeking greater reliability in predicting the results, corresponding to F_R= 5.68, to the value of the 5 % F distribution for F_R= 3.11. This value demonstrates statistical significance since it was higher than the reference (Neto et al., 2003).

Fig. 4 shows the graph with the random dispersion of the residues and data normality, indicating that the chosen model is sufficient to analyze the data.

According to the response surface with the antioxidant activity results via a DPPH free radical capture percentage, in the second planning for the aqueous extract (Fig. 1), the result for the best antioxidant activity of the rubber tree shell corresponded to 120 g.L^{-1} with an

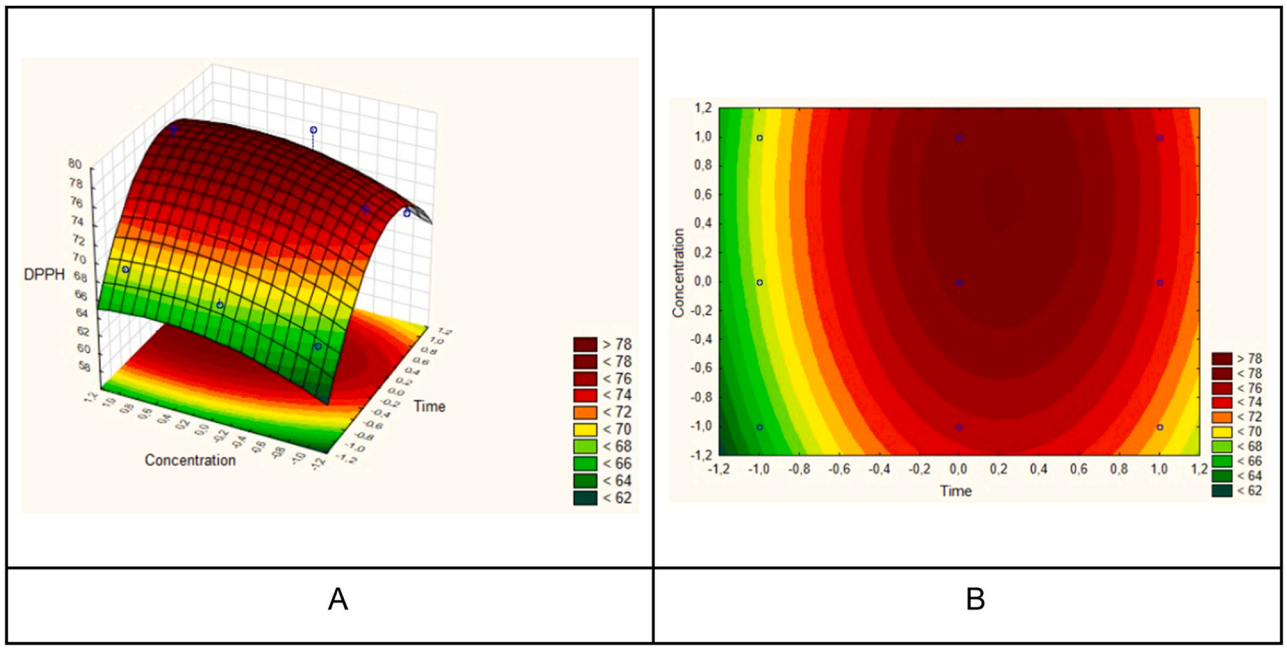

Fig. 1. Response surface (1 A) and contour lines (1B) of the statistical analysis for DPPH radical capture using the aqueous extract.

Table 5
ANOVA for the statistical treatment of the results referring to the planning for extracts in an aqueous solvent.

Factor	Quadratic sum (SQ)	Degrees of freedom (DF)	Square mean (MQ)	F	*P*
Regression (R)	163.49	5	32.70		
Time (linear)	42.698	1	42.69	1171.29	0.0186
Time (quadratic)	93.54	1	93.54	2566.15	0.0126
Concentration (linear)	13.89	1	13.89	381.15	0.0326
Concentration (quadratic)	3.97	1	3.97	108.84	0.0608
Interaction time × concentration	0.0046	1	0.00456	0.13	0.7837
Residue (r)	16.7	4	4.18		
Lack of adjustment (la)	16.66	3	5.55	152.39	0.0595
Pure error (ep)	0.04	1	0.04		
Total quadratic sum (SQ_T)	180.19	9			

extraction time of 6 h. Under these conditions, the antioxidant activity percentage was 78.97 $\pm$ 0.56 %. Also, for the hydroalcoholic extract (Fig. 3), in the second planning was 85 $g.L^{-1}$ with an extraction time of 5 h, resulting in an antioxidant activity percentage of 92.79 $\pm$ 0.70 %.

According to Sousa et al., (2011), degradation above 70 % of DPPH radicals is classified as strong antioxidant power. Oleinik et al., (2022) report the antioxidant activity of *Hevea brasiliensis* seed bagasse and found a percentage of 37.73±1.69 % DPPH free radical capture for aqueous extracts at a concentration of 85 $g.L^{-1}$ of the respective plant material. Wan Mohd Zain (2021) obtained the seeds methanol extract of the rubber tree *Hevea brasiliensis* clone RRIM 2025 a value of 34.84 % of antioxidant activity. Siriwong et al., (2015) studied the antioxidant activity via the DPPH method of natural rubber leaf extracts *Hevea brasiliensis* and presented results with a percentage from 57.10±0.073 % of antioxidant activity for the air-dried leaf extract (ADS) and 81.90 ±0.001 % for the smoked leaf extract with ribs (RSS3), both at a concentration of 37.5 µg/µl and with cyclohexane and methanol as solvent. Lourith et al, (2014) obtained 8.90±1.15 % DPPH free radical capture for rubber tree seed oil *Hevea brasiliensis* extracted in Soxhlet. Such variations in antioxidant activity in different studies are due to the different compositions of each raw material. Each plant source has a different composition and quantity of compounds with antioxidant activity. Furthermore, different factors can influence the phytochemical profile and biological activity of an extract derived from a plant source, such as genetic and environmental factors (Tungmunnithum et al., 2018; Koczka et al., 2018). Thus, it appears that the results of antioxidant activity obtained in the present study were superior to those it found in the literature, indicating the viability of using the extracts produced with the bark of rubber tree seeds.

Furthermore, the antioxidant activity of the extracts was experimentally compared with the natural antioxidant ascorbic acid and the synthetic antioxidant TBHQ, at a concentration of 1000 ppm. For ascorbic acid, the percentage of DPPH free radical capture obtained was 92.20±0.13 %, and for TBHQ it was 91.75±0.11 %. Based on the results, it can be seen that the results obtained in this study for *Hevea brasiliensis* extracts were similar to those of the other two antioxidants, which means that the use of the extracts is viable, since both TBHQ and ascorbic acid It already has proven antioxidant activity and are widely used in industry.

3.3. Total phenolics and flavonoids concentration for the optimized experimental conditions

The total phenols and flavonoids concentrations of extracts produced with *Hevea brasiliensis* seed shell under optimized experimental conditions are presented in Tables 7 and 8, where the different solvents and concentrations were analyzed according to each extraction time.

From the above, the phenolic compounds in the composition of the extracts were significantly higher when compared to the flavonoids concentration alone. Flavonoids are a type of phenolic compound, but as the results of flavonoid quantification were low, it can be suggested that the capture of DPPH was mainly due to the activity of other types of phenolic compounds. This corroborates the results of Wan Mohd Zain, et al. (2021), when analyzing the total content of phenolic compounds and flavonoids in leaves and seeds of rubber tree clones, found

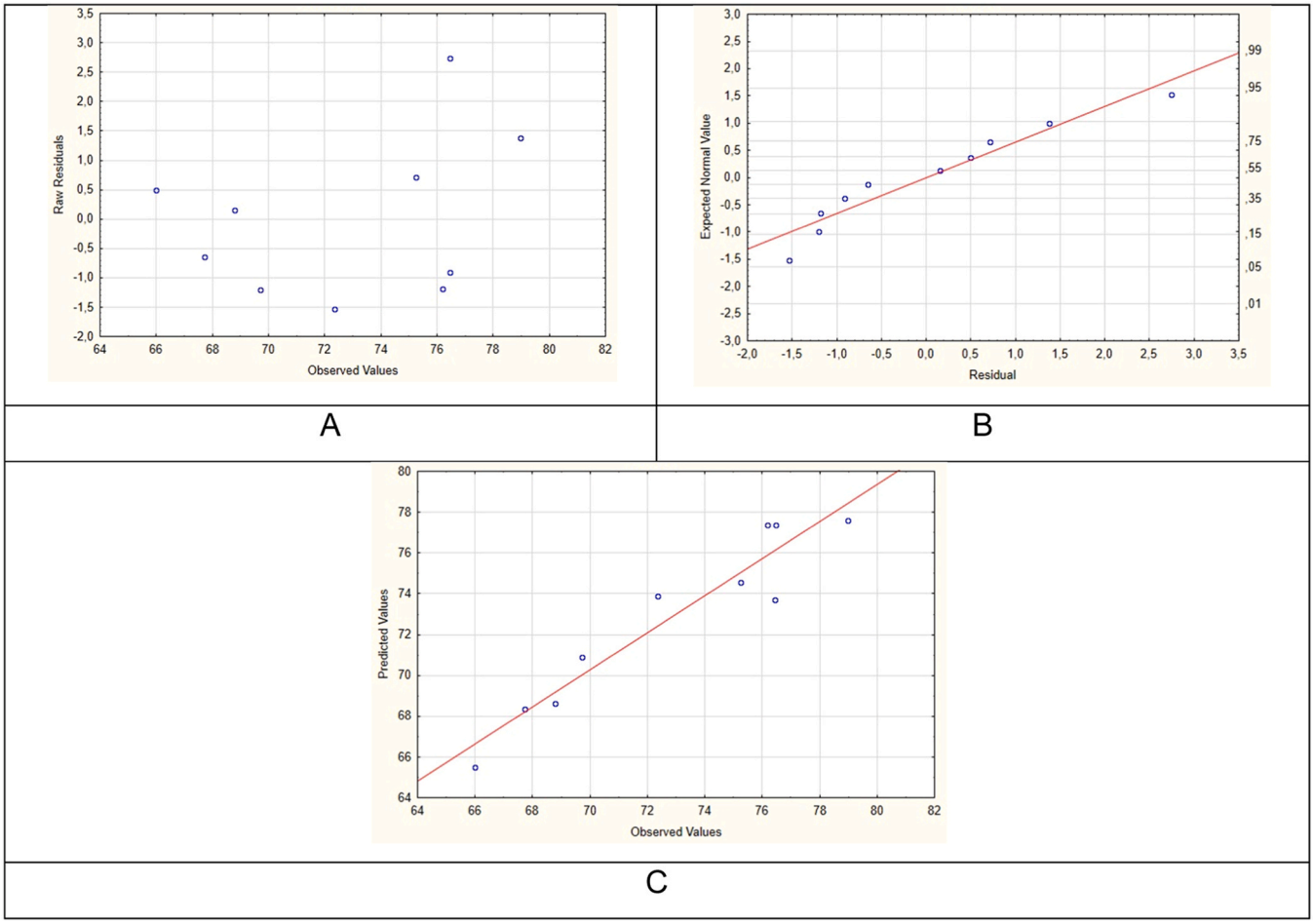

Fig. 2. (A) Graph of residuals versus predicted, (B) graph of normality test and (C) graph predicted vs. observed values, for the regression of the DPPH radical capture statistical analysis using the aqueous extract.

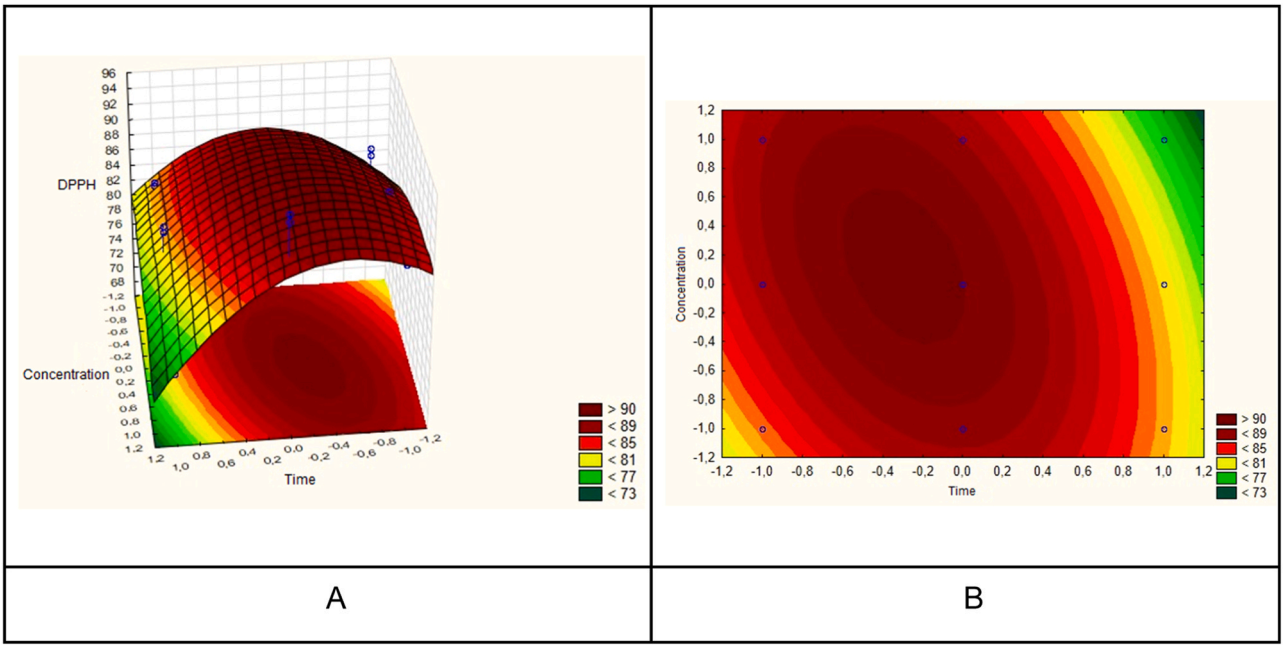

Fig. 3. Response surface (3 A) and contour lines (3B) of the DPPH statistical analysis of the hydroalcoholic extract.

Table 6
ANOVA for the statistical treatment of the results referring to the planning to obtain extracts in hydroalcoholic solvent.

Factor	Quadratic sum (SQ)	Degrees of freedom (DF)	Square mean (MQ)	F	*p*
Regression (R)	287.27	5	57.45		
Time (linear)	102.55	1	102.55	10.14	0.0079
Time (quadratic)	122.54	1	122.54	12.11	0.0045
Concentration (linear)	0.87	1	0.87	0.09	0.7737
Concentration (quadratic)	13.03	1	13.03	1.29	0.2786
Interaction time × concentration	48.27	1	48.27	4.77	0.0495
Error	121.40	12	10.12		
Total quadratic sum (SQT)	408.67	17			

flavonoids only in the leaves of the species, while the presence of these compounds was not detected in the seeds. According to the literature, the phenolic compounds obtained during the plant extract preparation have varied structures, such as phenolic acids, coumarin derivatives, tannins, and flavonoids (Ferreira et al., 2016).

Regarding total phenolics and flavonoids quantification, the results obtained were similar or superior to those found in the literature. Oleinik et al., (2022) verified for aqueous extracts at a concentration of 85 g.L^{-1} produced with *Hevea brasiliensis* seed bagasse, a value of 1405.15 mg EAC.100 g^{-1} in the quantification of phenolic compounds and 223.34 mg.100 g $^{-1}$ of total flavonoids. Ismail and Ali (2016) showed results of total phenolics and flavonoids quantification for hydroalcoholic extracts (ethanol:water, 80:20 v/v) from leaves of *Lantana camara L* (Verbenaceae). Total phenolic contents of 46.50 mg GAE.g^{-1} dry weight and total flavonoids of 16.06 mg catechin.g^{-1} were later added to the. Chaalal et al., (2019) evalueted total phenolic contents varying between 0.30 g GAE.100 g^{-1} for peanuts and 1.65 g GAE.100 g^{-1} for nuts, and flavonoid contents with values between 0, 17 g of quecertin.100 g^{-1} for peanut and 0.41 g of quecertin.100 g^{-1} for hazelnut. Ozer (2017) found a total phenolic content of 2467 ± 85 mg GAE/100 g for *Brosimum alicastrum* (Maya nut).

3.4. Evaluation of the antioxidant effect in biodiesel

The Rancimat method based on the European standard EN14112 is a way of evaluating the oxidation stability of biodiesel, and is currently considered a reference method. This is an accelerated oxidation method, in which a continuous air flow of 10 L/h passes through a compartment with a biodiesel sample, keeping the temperature constant at 110 °C. As the biofuel oxidation process takes place, volatile acids are formed that pass to another compartment containing distilled water and an electrode for measuring conductivity. Therefore, as biodiesel oxidizes, conductivity increases. The Rancimat equipment generates a graph of conductivity versus time, and, at the moment when the graph curve shows a rapid and sharp increase in conductivity, the inflection point occurs. The time it takes to reach the inflection point is called the induction period (IP), and this is a measure of oxidative stability (Focke et al., 2016; Jain and Sharma, 2010; Viomar, 2013).

Table 9 shows the induction times obtained in the Rancimat

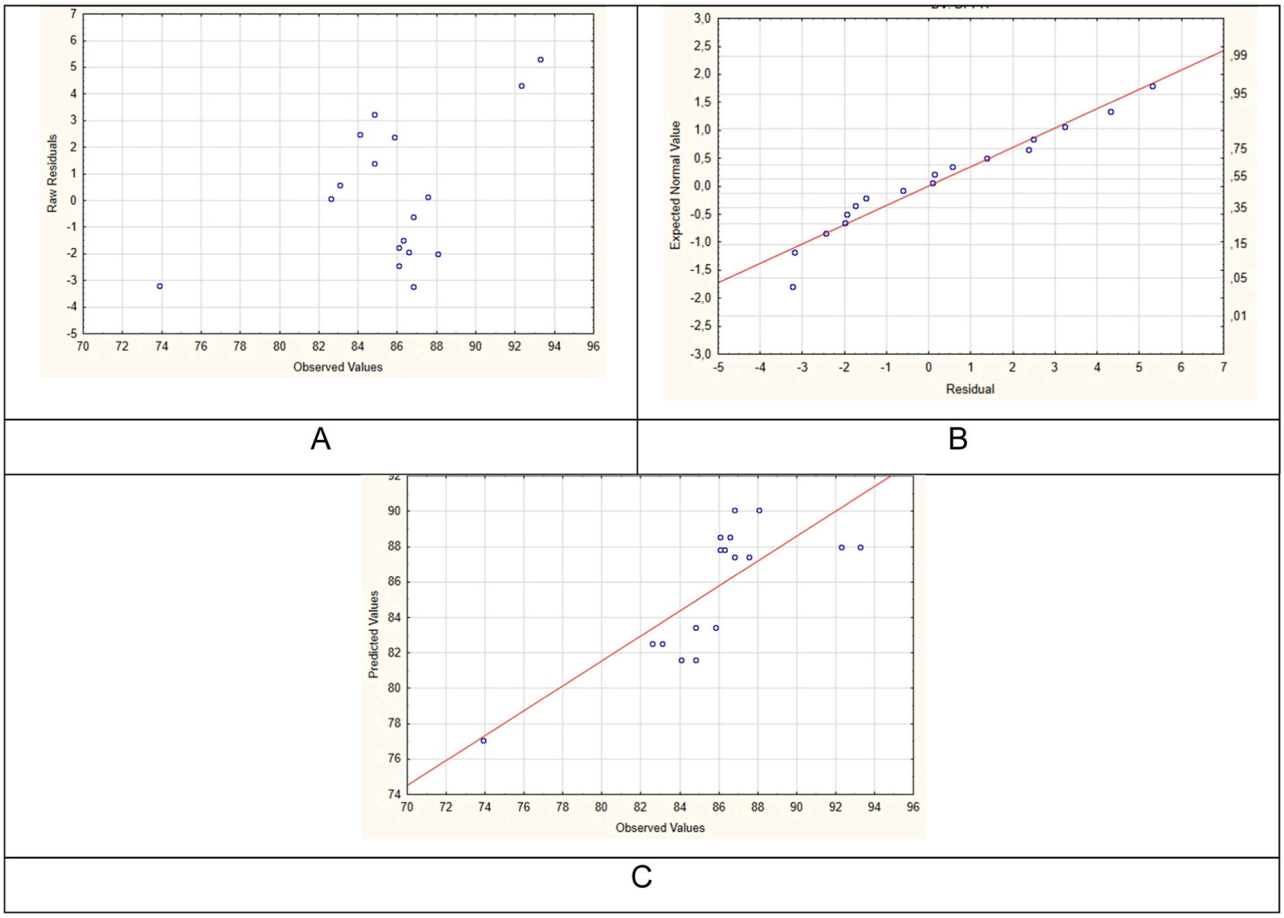

Fig. 4. – (A) Graph of residuals versus predicted, (B) graph of normality test and (C) graph predicted vs. observed values for the regression of the DPPH radical capture statistical analysis using the hydroalcoholic extract.

Table 7
Total phenolics concentration (mg GAE.100 g^{-1}) for extracts produced under optimized experimental conditions.

Time (h)	Concentration ($g.L^{-1}$)	Aqueous (mg GAE.100 g^{-1})	Time (h)	Concentration ($g.L^{-1}$)	Hydroalcoholic (mg GAE.100 g^{-1})
2	40	871.26 ± 101.41	3	45	1404.06 ± 86.40
2	80	966.86 ± 14.27	3	65	1730.84 ± 37.55
2	120	900.45 ± 48.26	3	85	1744.16 ± 70.44
6	40	1924.45 ± 37.86	5	45	1557.34 ± 59.40
6	80	1702.98 ± 22.08	5	65	2412.05 ± 74.40
6	120	1509.87 ± 42.77	5	85	1221.50 ± 30.68
10	40	1559.17 ± 43.45	7	45	3278.05 ± 152.91
10	80	2033.96 ± 62.99	7	65	2750.94 ± 103.68
10	120	2037.09 ± 47.72	7	85	2193.54 ± 47.14

Table 8
Total flavonoids concentration (mg.100 g^{-1}) for extracts produced under optimized experimental conditions.

Time (h)	Concentration ($g.L^{-1}$)	Aqueous (mg.100 g^{-1})	Time (h)	Concentration ($g.L^{-1}$)	Hydroalcoholic (mg.100 g^{-1})
2	40	94.33 ± 0.54	3	45	13.05 ± 9.91
2	80	123.22 ± 0.47	3	65	36.82 ± 8.99
2	120	66.15 ± 6.81	3	85	43.51 ± 14.42
6	40	137.61 ± 0.54	5	45	1.28 ± 3.86
6	80	100.79 ± 2.99	5	65	66.35 ± 20.73
6	120	93.85 ± 2.63	5	85	17.09 ± 2.67
10	40	117.23 ± 0.54	7	45	108.66 ± 17.40
10	80	129.65 ± 3.53	7	65	78.32 ± 9.76
10	120	191.07 ± 2.90	7	85	68.89 ± 23.99

Table 9
Induction time via Rancimat equipment.

Sample	Induction time (h)	Temperature (°C)
Biodiesel control	3.43	110
Biodiesel + aqueous extract (addition by mechanical agitation)	5.56	110
Biodiesel + aqueous extract (addition by ultrasound)	6.12	110
Biodiesel + hydroalcoholic extract (addition by mechanical agitation)	6.14	110
Biodiesel + hydroalcoholic extract (addition by ultrasound)	5.96	110

equipment for the control biodiesel samples and the biodiesel samples containing the aqueous and hydroalcoholic antioxidant extracts added via mechanical agitation or by ultrasound.

Based on the tests for the oxidative stability of the control biodiesel and the biodiesel added with the antioxidant extracts carried out in the Rancimat equipment, there was a difference in the induction time between the samples, considering that the biodiesel added with antioxidant extracts obtained induction times higher than control biodiesel, signaling an increase in oxidation stability. In this way, the efficiency of the rubber tree seed shell extract as an antioxidant is demonstrated.

However, none of the samples reached the induction period predicted by the ANP, of 12 hours. One way to get around this result is to mix the extract with other natural or synthetic antioxidants (but in smaller quantities than usual), obtaining a synergistic effect (which occurs when substances interact in order to increase or magnify one or more of their effects) as already described in the literature, for example Boschen et al. (2019) found a mixture of barley residue extract 11 $g.L^{-1}$ and citric acid 0.1 % (w/v) added to biodiesel resulted in an induction time of 14.67 ± 2.10 hours. When adding only citric acid 0.1 % (w/v) or barley residue extract 11 $g.L^{-1}$, the induction times obtained were 5.46 ± 0.07 hours and 5.76 ± 0.11 hours, respectively.

In terms of synergism, there are potentiation and addition synergism, which are mainly described in pharmacology. Potentiation synergism is when the combined effect of two drugs is greater than the sum of the isolated effects. Addition synergism is when the combined effect of two drugs is equal to the sum of the isolated effects of each of them (Do Vale, 2020). In the case of a mixture of antioxidants, the synergistic potential in most cases is not an additive value of the antioxidant properties of the individual substances. Regarding the synergism that occurred in these cases, it is difficult to elucidate the mechanism, according to the authors Krupelnytskyi (2023), Uduwana, Abeynayake and Wickramasinghe (2023) e Olszowy-Tomczyk (2020), due to the complex nature of the plant extracts. Therefore, it is suggested for future research to investigate how the mechanism of the synergistic effect of these antioxidant compounds derived from plant sources occurs.

4. Conclusion

Although numerous applications reported *Hevea brasiliensis* species in the literature, research on the use of its seed is still limited. Therefore, this study evaluated the antioxidant activity of extracts produced from the shell of the rubber tree seed as an antioxidant additive to soybean biodiesel. The rubber tree seed shell extraction in aqueous and hydroalcoholic solvent optimized of the variables concentration and extraction time.The extracts characterized radical scavenging activity and the total phenolics and flavonoids. For the aqueous extract, the optimized conditions were 120 $g.L^{-1}$ and 6 hours of extraction (78.97 % DPPH radical capture) and for the hydroalcoholic extract were 85 $g.L^{-1}$ and 5 hours (92.79 % inhibition of DPPH activity). The extracts produced do not showed a significant amount concerning total flavonoids, being rich in phenolic compounds and reaching up to 3278.05 ± 152.91 mg GAE.100 g^{-1}. Those values that are higher than several antioxidant extracts described in the literature. In this way, the extracts produced could delay the onset of biodiesel oxidation during the accelerated Rancimat analysis. Biodiesel treated with hydroalcoholic extract increased the induction time of control biodiesel from 3.43 h to 6.14 h, and biodiesel treated with aqueous extract increased the induction time to 6.12 h. The results obtained are highly promising and it have potential for application in the biodiesel production industry, in addition to being a green alternative capable of adding value to the *Hevea brasiliensis* co-product.

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CRediT authorship contribution statement

André Lazarin Gallina: Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Conceptualization. **Dalila Benvegnú:** Conceptualization. **Letiére Soares:** Writing – original draft, Supervision. **Paulo Pinto Rodrigues:** Resources. **Fernanda Lima:** Conceptualization. **Giovanna Oleinik:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Giovanna Oleinik has patent PROCESSO DE PRODUÇÃO DE BIODIESEL EMPREGANDO EXTRATO A POLPA E/OU DA CASCA DE SEMENTE DE SERINGUEIRA (Hevea brasiliensis) COM EFEITO ANTIOXIDANTE pending to INPI. Andre Lazarin Gallina has patent PROCESSO DE PRODUÇÃO DE BIODIESEL EMPREGANDO EXTRATO A POLPA E/OU DA CASCA DE SEMENTE DE SERINGUEIRA (Hevea brasiliensis) COM EFEITO ANTIOXIDANTE pending to INPI. LETIERE CABREIRA SOARES has patent PROCESSO DE PRODUÇÃO DE BIODIESEL EMPREGANDO EXTRATO A POLPA E/OU DA CASCA DE SEMENTE DE SERINGUEIRA (Hevea brasiliensis) COM EFEITO ANTIOXIDANTE pending to INPI. Dalila Moter Benvegnu has patent PROCESSO DE PRODUÇÃO DE BIODIESEL EMPREGANDO EXTRATO A POLPA E/OU DA CASCA DE SEMENTE DE SERINGUEIRA (Hevea brasiliensis) COM EFEITO ANTIOXIDANTE pending to INPI. Fernanda Oliveira Lima has patent PROCESSO DE PRODUÇÃO DE BIODIESEL EMPREGANDO EXTRATO A POLPA E/OU DA CASCA DE SEMENTE DE SERINGUEIRA (Hevea brasiliensis) COM EFEITO ANTIOXIDANTE pending to INPI. Paulo Rogerio Pinto Rodrigues has patent PROCESSO DE PRODUÇÃO DE BIODIESEL EMPREGANDO EXTRATO A POLPA E/OU DA CASCA DE SEMENTE DE SERINGUEIRA (Hevea brasiliensis) COM EFEITO ANTIOXIDANTE pending to INPI.

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ARTICLES FOR FACULTY MEMBERS

SUSTAINABLE PROCESSING OF RUBBER (HEVEA BRASILIENSIS) SEED OIL: PHYSICOCHEMICAL INSIGHTS INTO EXTRACTION AND ANTIOXIDANT PRESERVATION

The In-Situ Epoxidation of Rubber Seed Oil (Hevea brasiliensis) by Peroxyacids./ Majid, A., Anggono, A. D., Siswanto, W. A., Widodo, T., Riyadi, B., Mustapa, M. S., Syamsiyah, N. R., Faishal, A., Budiyati, E., Rahmah, A., & Fauzi, N. A.

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The In-Situ Epoxidation of Rubber Seed Oil (*Hevea brasiliensis*) by Peroxyacids †

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Abstract: This paper adds to the sustainable materials field by in-situ epoxidation of rubber seed oil (RSO), a highly underutilized resource that has been sporadically used, using an optimized combination of 30% hydrogen peroxide and acetic/formic acid sulfuric acid. Most of the previous studies deal with more common vegetable oils, where the main focus in most is given to the epoxidation of these oils and their derivatives. The RSO contained a high iodine value around 135.36 g–I2/100 g. The central to this work is the systematic study of the oxirane number as a function of reaction temperature and the double bond:RCOOH:H_2O_2 molar ratios. By testing the temperatures of 40, 50, 60, and 70 °C and three specific molar ratios (1:0.6:1.4, 1:1:2, and 1:1.5:3), this research not only found the optimal conditions for epoxidation but also gave valuable information on the reaction kinetics of rubber seed oil. The results showed that a temperature of 60 °C with a 1:1:2 molar ratio gave the highest oxirane number, especially with performic acid, which was 3.200 mmol/g. Then, overall, formic acid consistently outperforms acetic acid in terms of product yields, facilitating a more effective epoxidation process.

Keywords: in-situ epoxidation; rubber seed oil; *Hevea brasiliensis*; peroxyacids

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1. Introduction

The growing interest in sustainable and green-based materials has encouraged the potential of using renewable resources, particularly plant oils, as substitutes for petroleum materials [1,2]. Among numerous oils, the rubber tree's rubber seed oil, *Hevea brasiliensis*, is uniquely and necessarily advantageous depending on natural availability and differing molecular structure. Furthermore, the properties of the oils can be significantly improved by performing reactions based on epoxidation, which will significantly broaden the class of applications (bioplastics to coatings) for which processing is feasible. The use of epoxidation under conditions that have been measured in time and safety simply becomes a bonus of using peroxyacids as an in-situ reaction for renewable resources [3].

The in-situ epoxidation of vegetable oils has emerged as a primary area of research for renewable resources based on oils, specifically including rubber seed oil. These oils are chemical feedstock for the unsaturated fatty acids contained in can be effectively epoxidized that ultimately improve the oils' chemical character and facilitate radically novel applications. Generally, peroxyacids will be the reactant for the addition process and established that efficacy will apply mild conditions [4–6]. Further studies are attached to optimizing epoxidation processes toward an efficient, safer, and sustainable methodology of future sustainability through decreasing risk and enhancing the controllability for safety

issues. In particular, there are studies on conducting temperature and molar ratios of the reactants, i.e., the feedstock double bonds, carboxylic acids (RCOOH), and hydrogen peroxide (H_2O_2). Previous studies have demonstrated that increasing cooking temperature variables increases the rates of conversion by increasing the rate of reaction variables, but taking the temperature too high could also increase undesired side reactions and thus decrease the chemical selectivity and overall yield [7–10]. Peroxyacids are preferential for selective epoxidation by the introduction of oxirane groups in unsaturated fatty acids. A recent study also used different carboxylic acids: acetic, formic, and other fatty acids in the optimization of epoxidation. The carboxylic acids were found to have influenced the kinetics of the epoxidation reaction [8,11].

This paper is a significant contribution to the field of green materials, with a focus on the in-situ epoxidation of rubber seed oil with peroxyacids with an investigation and optimization on reaction parameters. Unlike recent works by other researches, which focused more on the epoxidation of vegetable oils extracted predominantly from plant sources that are used in synthetic rubber production, our study casts a different perspective, considering the properties of rubber seed oil that were less understood, despite the enrichment of unsaturated fatty acid in this material. In this study, we first investigate the effect of temperature, the type of carboxylic acids, the molar ratio of double bonds, carboxylic acids (RCOOH), and hydrogen peroxide (H_2O_2) related to conversion. We then optimize these three reaction parameters to maximize the conversion and, in a more delicate way, address the problem of selectivity and minimize the by-products formed as discussed in the previous epoxidation procedures. The molar ratio of the reactants, crucial to our investigation, contributes much to the conversion. For example, compared to those in the previous study working under similar experimental conditions, we explore styles to increase the amount of RCOOH, which stabilizes the reaction media and increases both production of epoxy group and reduction in the other two by-products obtained from the rubber seed oil.

2. Materials and Methods

2.1. Materials

Rubber seed oil (RSO) was purchased directly from local farmers. RSO was degummed and purified using phosphoric acid. The researchers used a variety of chemicals, including analytical grade glacial acetic acid, potassium hydrogen phthalate, Wijs solution, 98% sulfuric acid, 30% aqueous hydrogen peroxide, potassium iodide, 47% hydrobromic acid in acetic acid, and crystal violet indicator.

2.2. Epoxidation Process

In a three-neck flask, a predetermined amount of rubber seed oil (70 g) was mixed with varying amounts of carboxylic acids (such as acetic or formic acid) and hydrogen peroxide, following specific molar ratios of double bond:RCOOH: H_2O_2 (e.g., 1:0.6:1.4, 1:1:2, and 1:1.5:3). The mixture was constantly mixed and heated to various temperatures (40 °C, 50 °C, 60 °C, and 70 °C) while the reaction was allowed to continue for specified time intervals (0, 30, 60, 120, and 240 min). Samples were taken at each time point for spectroscopic analysis, typically using FTIR, to assess the conversion rates of double bonds and the formation of epoxide groups, providing insights into the optimization of the epoxidation process.

3. Results and Discussion

3.1. Temperature Influence

Figure 1 describes the oxirane number of epoxidized rubber seed oil at various temperatures for a molar ratio of double bond:CH_3COOH: H_2O_2 of 1:1:2. The findings showed that the oxirane number was clearly affected by the changes in temperature. For all temperatures, at a certain reaction time (up to 150 min), an increase in temperature will increase the oxirane number. At temperatures of 40 °C and 50 °C, the oxirane number increases continuously from the beginning of the reaction to the end of the reaction, with the optimum oxirane number being 1.333 mmol/g and 1.507 mmol/g, respectively. This implies that the increased temperatures support the reaction rate for epoxidation processes and facilitate a quicker generation of oxirane units. When the temperature was set at 60 °C, the optimum oxirane value was obtained at a reaction time of 210 min, namely 3200 mmol/g. After this time, the oxirane number decreased. This tendency also occurs at a temperature of 70 °C, the oxirane number increases with increasing reaction time (up to 150 min) until the highest point is 3.093 mmol/g, after which the oxirane number decreases drastically. A trend consistent with observations from previous studies showed that higher temperatures (above 60 °C) can have an adverse effect on oxirane number. This decrease in oxirane number could be due to the fact that oxirane is very reactive, so it easily undergoes further reactions with the remaining reactants. This statement is in line with previous research findings [7,10,12]. Overall, the highest oxirane number was obtained at a temperature of 60 °C, namely 3200 mmol/g.

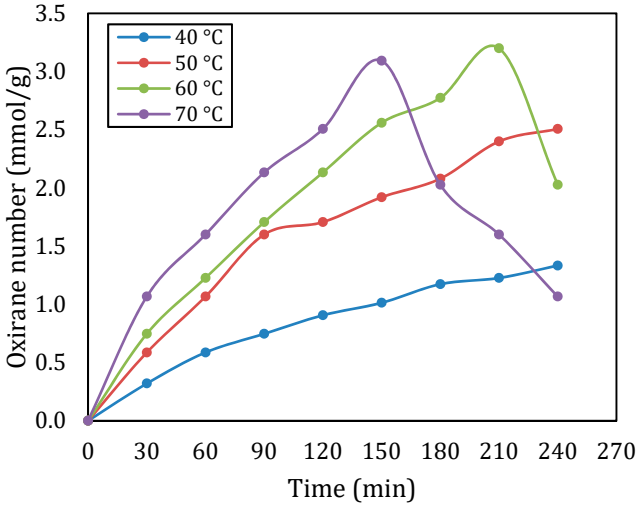

Figure 1. Oxirane number at various temperatures for a molar ratio of double bond:CH_3COOH:H_2O_2 of 1:1:2.

The data also show that the oxirane number increases over time in a time manner. There was a significant rise in conversion particularly within the 30 min, at temperatures of 50 °C and above. However, the oxirane number tended to level off after 150 min at 70 °C with values declining, suggesting a point of saturation where extended reaction times result in diminishing returns. The plateau aligns with the research by Budiyati et al. (2024), indicating that extended response times may decrease effectiveness by causing the creation of byproducts or the deterioration of the epoxide structure [13].

When comparing these findings to existing literature, several trends become evident. Many previous studies have documented optimal temperature ranges for epoxidation processes, typically around 50–60 °C. The drastic decline in oxirane number observed at 70 °C in this study inlines with some reports where adverse effects were noted at similar temperatures [7,13,14].

3.2. Molar Ratio Influence

Figure 2 illustrates the oxirane number at various molar ratios for a temperature of 60 °C. The type of carboxylic acid is acetic acid (CH_3COOH). The molar ratios of reactants had an impact on how the reactions turned out for this experiment we did with epoxidation processes at different temperatures and time intervals. We found that using the ratio of 1:1:2 consistently gave us results compared to other ratios we tested. The oxirane number for the 1:1:2 ratio came out to be around 3.200 mmol/g, while the 1:0.6:1.4 ratio only reached 2.880 mmol/g. This indicates that having a high mole of CH_3COOH and H_2O_2 in a ratio of 1:1:2 improves the reaction conditions for epoxidization and underscores the significance of maintaining stoichiometric balance in this process. Past research has consistently demonstrated that increasing the proportions of RCOOH and H_2O_2 leads to conversion rates, a finding that is well supported by the results of this study [8,15]. On the other hand, overall, the oxirane number produced at a molar ratio of 1:1:2 was slightly higher than at a molar ratio of 1:1.5:3. These small differences are telling the possibility of (1) the oxirane number approaching the maximum value, and (2) an increase in molar ratios from 1:1:2 to 1:1.5:3 did not have a significant effect. Therefore, it can be concluded that 1:1:2 was the optimum molar ratio.

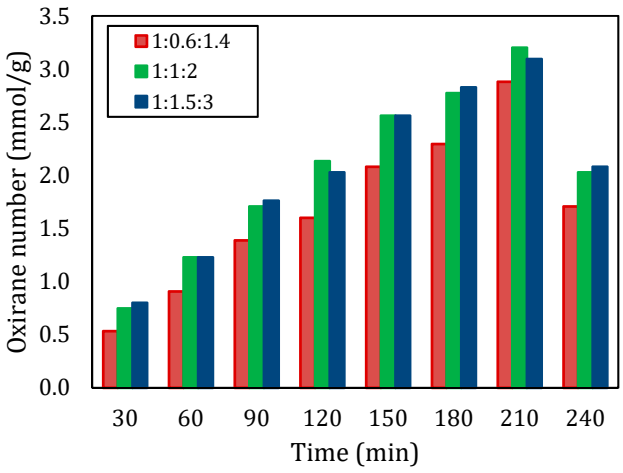

Figure 2. Oxirane number with different molar ratios of double bond:CH_3COOH:H_2O_2 at a temperature of 60 °C.

3.3. Influence of the Type of Carboxylic Acid

Figure 3 shows the effect of the type of carboxylic acid on the oxirane number in the epoxidation of rubber seed oil. From Figure 3, it can be seen that at a temperature of 50 °C, the oxidation number of rubber seed oil based epoxy using acetic acid is consistently lower than that using formic acid. Meanwhile, at a temperature of 60 °C, formic acid provides a higher oxirane number up to a reaction time of 180 min. After this time, the oxirane value with formic acid decreased significantly, the value was even lower than the oxirane value of epoxy produced from epoxidation of rubber seed oil with acetic acid. The highest oxirane number obtained in epoxidation with formic acid and acetic acid were 3.093 mmol/g and 3.200 mmol/g, respectively.

The type of carboxylic acid used in the epoxidation of rubber seed oil significantly impacts the conversion rates and the overall efficiency of the reaction. In this context, formic acid and acetic acid were examined to understand their respective influences on the epoxidation process. When formic acid is employed, it exhibits high reactivity due to its strong oxidizing characteristics. The introduction of peroxyformic acid leads to a rapid conversion of double bonds in rubber seed oil, resulting in higher epoxide yields. Studies have shown that reactions utilizing formic acid can achieve higher conversion within

shorter reaction times, particularly at optimized temperatures. The effective formation of reactive intermediates allows for a swift introduction of oxirane groups, making formic acid a superior choice for enhancing the efficiency of the epoxidation process [8,14].

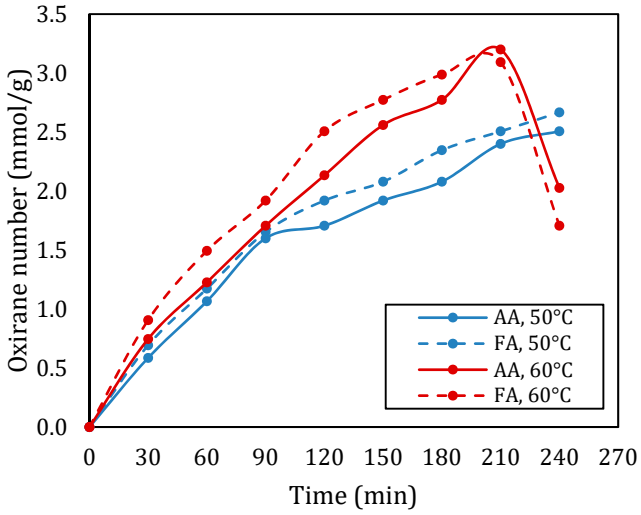

Figure 3. Oxirane number with different types of acid for the molar ratio of double bond:RCOOH:H_2O_2 of 1:1:2 at temperatures of 50 °C and 60 °C.

3.4. The FTIR Spectroscopy Data

Figure 4 presents the result of FTIR analysis of epoxidized rubber seed oil. The examination of FTIR data for the epoxidation process of rubber seed oil offers insights into the molecular transformations that take place during the reaction process. The presence of absorption bands in the spectrum is linked to functional groups identified in the oil structure and aids in a thorough comprehension of its structural alterations. The FTIR analysis highlights absorption bands that signify the existence of functional groups, within the epoxidized rubber seed oil. The detection of O–H stretching vibrations, between 3474.91–3571.35 cm^{-1}, signals the presence of hydroxyl groups formed during the epoxidation process—an observation, with the research conducted by Budiyati et al. focusing on tung seed oil epoxidation [9], and by Nwosu-Obieogu et al. studying melon seed oil properties [16].

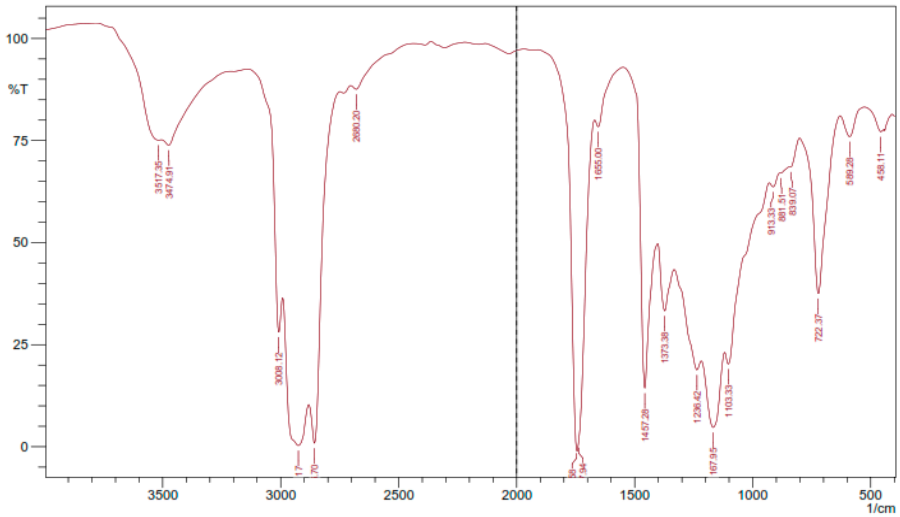

Figure 4. FTIR analysis results.

The absorption bands linked to C–H stretching at 2925.17 cm^{-1} and 2856. 7 cm^{-1} indicate the existence of hydrocarbons. These bands are also supported by the peak at

1457.28 cm^{-1}, which strengthens the idea that aliphatic structures are common in the product. The C = O stretching identified between 1737.94 cm^{-1} and 1747.58 cm^{-1} hints, at the presence of carboxylate groups originating from the fatty acids found in rubber seed oil, a discovery that suggests partial oxidation of these functional groups during epoxidation. The absorption peak, at 1167.95 cm^{-1} corresponding to C–O bonds, provides evidence for the production of alcohols commonly found in epoxidized oils. The detection of C–O–C bonds in the range of 839.07 to 881.51 cm^{-1} affirms the presence of epoxy groups in the structure, indicating the successful incorporation of oxirane rings into the fatty acid chains. The formation of hydroxyl and epoxy groups in the FTIR spectra of epoxidized vegetable oils reinforces the idea that such modifications are common across different feedstocks. This study's specific absorption values fall within the ranges reported in the literature, further validating the consistency of FTIR as an analytical tool for monitoring chemical modifications in oils [17].

4. Conclusions

The in situ epoxidation process on rubber seed oil can be carried out well. This study discusses the influence of several important parameters in epoxidation, namely: temperature, time, and molar ratio of double bond:RCOOH: H_2O_2. The research results showed that epoxidation with performic acid at a reaction temperature of 60 °C and a molar ratio of 1:1:2 produced optimum conversion. The choice of the type of carboxylic acid (formic acid or acetic acid) for epoxidation of rubber seed oil has significant implications for the resulting conversion. Formic acid consistently outperforms acetic acid in terms of reaction kinetics and product yield, thereby facilitating a more effective epoxidation process.

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