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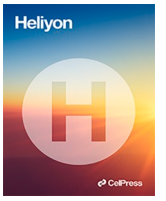

Research article

# Comparative evaluation of physicochemical and antimicrobial properties of rubber seed oil from different regions of Bangladesh

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ABSTRACT

In Bangladesh, the annual production of rubber seeds is typically left untapped although the seeds contained a high percentage of oil but underutilized without any value-added utilization. This study aims to evaluate the geographical effect on physicochemical properties, fatty acid composition and the antimicrobial activity of oil extracted from rubber seeds. Seeds were collected from three different regions of Bangladesh and the oil was extracted by the soxhlet method using *n*-hexane as a solvent. Results demonstrated that the geographical regions have some significant effect on the properties of rubber seed oil (RSO). The physicochemical properties of RSO varied from region to region. For example, the percent of yield, higher heating value, and flash point varied from 50.0 to 50.8 %, 31.8–33.3 kJ/g, and 237–245 °C, respectively. The chemical parameters, such as acid value, iodine value, and hydroxyl value varied from 13.3 to 18.2 mg KOH/g, 132–137 g $I_2$/100g, and 47.7–55.8 mg KOH/g, respectively. Chromatographic analysis showed that RSO mainly contains palmitic, linoleic, linolenic, and stearic acid. Regional variations were also seen in the composition of these fatty acids. Most notably, regardless of the rubber seeds collected from various locations, RSO exhibited inhibitory activity against only gram positive bacteria. The zone of inhibition range for different tested gram positive bacteria was 2.33–11.17 mm irrespective of different RSO samples.

## 1. Introduction

Bangladesh is a Southeast Asian country and one of the largest deltas in the world; the agricultural and industrial sectors of Bangladesh are influenced by its geographical location. Rubber tree (*Hevea brasiliensis*) belongs to the family of Euphorbiaceae had been found mostly in Asia and Africa regions, and Bangladesh is most suitable for rubber plantation. The north-eastern and south-eastern hilly regions in Bangladesh grow huge amounts of rubber trees. The land of these regions is least fertile and not appropriate for the growing of regular crops, but more suitable for rubber plantation [1–4]. Rubber plantation is more profitable than

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afforestation in terms of optimum utilization of land. In Bangladesh, around 91.8 thousand hectares of land is used for rubber plantation and these rubber gardens are mainly located at Chattagram, Cox's Bazar, Khagrachari, Bandarban, Rangamati, Sylhet, Habiganj, Moulavibazar, Mymensingh, Tangail, and Sherpur districts [5,6]. Rubber seeds are matured two times a year, the peak season for the production of seeds is from August to September and the less production season is from January to February [7]. Depending on soil property and crop density the average production of rubber seed per hectare is 150 kg/year [8]. Unfortunately, this huge amount of rubber seed does not find any value-added application and a major portion is treated as wasted or used for burning purposes. The seed contains about 35–45 % of oil which may have promising applications for industrial purposes [9].

The RSO is an abundant source of essential fatty acids and it contains more than 50 % of polyunsaturated fatty acids [10]. Up to date, it has been reported in the literature, that RSO may be utilized for the manufacture of fatty acids [11], production of biodiesel [12], soap [13] and surface coating materials, including paint, and alkyd resin [14–16]. Physicochemical characterization together with fatty acids composition [17], antifungal activity [18], antimicrobial activity [19], chemical composition [20] of different indigenous plant oils from different parts of the world were studied by various researchers. The production of alkyd resins from RSO and its suitability as binders in solvent-borne and water-reducible coatings has been also reported [21–23]. Furthermore, vegetable oils may be used as prominent raw materials for the production of edible and non-edible oils as well [24]. In addition, polymeric products obtained from vegetable oil are used for different purposes including paints, printing inks, plastic processing, fat liquors, resin, pharmaceuticals, lubricants and cosmetics [25–29]. After extraction of oil from rubber seed the remaining high protein content meal cake of these seeds could serve as feed for animals, such as poultry and cattle [30,31].

The vegetable oil showed biological activities including antimicrobial and antifungal activities. The fatty acid composition of RSO and the abundance of volatile aromatic molecules such as aldehydes, alcohols, acids, and esters are regarded to be the primary causes of its antifungal effect [18]. In the case of black seed oil, the presence of biologically active substances including α-thujene, α-pinene, limonene, thymoquinone, myristicin, thymol, etc. led to antimicrobial activities [18,32]. It was reported that thymol, eugenol, and carvacrol were responsible for the antibacterial effectiveness of oregano oil [33]. Moreover, essential oils including ginger oil, black seed oil, oregano oil and rose oil possess several mechanisms for antimicrobial activity. The primary target of the mechanism of action seems to focus mostly on disrupting the structure of cell membranes, which results in cell leakage and cell death, secondary effects could be achieved by inhibiting cell respiration and preventing the production of new membranes. The essential oils are highly volatile and lipophilic, thereby, they readily penetrate the cell membrane and exert their biological effects [34]. Besides, hydrophobicity is an important characteristic of essential oils and their components, which enables them to partition with the cell membrane's lipids of bacteria and mitochondria, rendering them more permeable by disturbing the cell structures. This results in the leakage of critical molecules and ions from the bacterial cell to a great extent eventually causing the death of bacterial cells [35].

The geographical origin of the sample is an important indicator that has a significant effect on the oil properties, percent of yield, and antimicrobial and antifungal activities [13,36]. There is a lack of sufficient information about the effect of geographical regions on RSO properties. The current study aims to investigate the physicochemical properties, fatty acid composition and antimicrobial properties of RSO. The main raw material of this study is rubber seed, which has been collected from three different regions of Bangladesh, namely, Karnajora rubber garden at Sherpur; Bhatera rubber garden, Moulavibazar and Raojan rubber garden at Chattagram. Then, oil is extracted from these three samples in parallel by the soxhlet method using n-hexane as a solvent. Afterward yield of oil percent, physicochemical properties, fatty acid composition, and antimicrobial and antifungal performances of the extracted oil are measured. This study revealed that the production of RSO from renewable sources is not only an effective utilization of waste material but also promising applications for different value-added purposes.

## 2. Materials and methods

### 2.1. Chemical and apparatus

n-hexane (purity >97 %), methanol (purity 99–100 %), ethanol (purity >99.9 %), iso-Octane, and potassium iodide (purity 99–100.5 %) were supplied from Honeywell, Germany. Acetic anhydride (purity 99.5 %), sodium thiosulfate (purity >98.0 %), sodium hydroxide pellets (purity 96 %), sodium chloride, potassium hydroxide pellets (purity >84 %), potassium dichromate (purity 99.5 %), and iodine monobromide (purity 98 %) were purchased from Sigma-Aldrich, Germany. Hydrochloric acid (purity 37 %), sulfuric acid (purity 98 %), isopropanol, glacial acetic acid, pyridine, chloroform, phenolphthalein and starch indicator were obtained from Merck, Germany. Distilled deionized water was used throughout the study. All the reagents were of analytical grade and they were used without any prior treatment.

Soxhlet apparatus was used for the oil extraction. Fourier transform infrared (FTIR) spectrophotometer (Frontier, PerkinElmer, USA) and gas chromatography (Trace 1300, Thermo Scientific, PA, USA), equipped with flame ionization detector and a fused silica capillary column (TR-FAME, 30 m 0.25 mm 0.25 m film thickness, Thermo Scientific, PA, USA) were used for the determination of functional groups and composition of fatty acids in the oil, respectively. A heating mantle (MS-DM603, Mtops, Korea) was used to remove n-hexane from the extracted oil at around 65 °C and after cooling it was preserved at 4–6 °C in a refrigerator for further analyses. Karl-Fischer Titrator (906 Titrando, Metrohm, Switzerland) was used for the determination of water content.

### 2.2. Collection and pretreatment of raw material

Rubber seed samples were collected from three different geographical locations in Bangladesh (Karnajora rubber garden, Sherpur; Bhatera rubber garden, Moulavibazar; and Raozan rubber garden, Chattagram). The outer layers (shell) of the freshly collected seeds

were cracked manually and the kernel was collected and sliced into small pieces and then dried in an oven at 60 °C (~24 h) until constant weight. The rubber seed contains on average 54 (% w/w) of the kernel. The dried seeds kernels were ground and sieved to average particle sizes of 0.5, 1.0, 1.5, 2.0 and 2.5 mm. The ground seed kernels were stored in air-tight containers and kept in the refrigerator (4–6 °C) for further use.

### 2.3. Extraction of RSO and optimization of the process

Different batch sizes of the ground seeds kernel were loaded into a thimble and placed in the soxhlet apparatus at 65 °C and 200 mL of n-hexane was used as an extraction solvent for the optimization of maximum yield of oil extraction. In addition, the kernel of different particle sizes (0.5, 1.0, 1.5, 2.0 and 2.5 mm) was used for the optimization of the maximum yield of oil. The obtained RSO in this stage was filtered and de-solventized (to remove n-hexane) with the heating mantle attached to a condenser and collecting flask. The extracted RSO is stored in an amber glass bottle and preserved at 4–6 °C in a refrigerator for further analysis.

### 2.4. Physicochemical properties and characterization of RSO

The appearance and odor of the extracted oil were observed visually and by using sense while color value (Y+5R) was determined by instrumental method with a Lovibond colorimeter (Model F, Lovibond, Salisbury, UK) using 1-inch cell. The result was expressed as Y+5R, where Y stands for yellowness and R refers to redness [37]. The water content of RSO was determined with Karl-Fischer Titrator by taking 1.0 gm of the sample following the Ph. Eur. Method 2.5.32. Relative density was examined by the pycnometer method at 25 °C. The sulfated ash content of the oil was calculated in a muffle furnace at 650 °C. Dynamic viscosities were calculated following ASTM D 445 method by using two specific temperatures at 40 and 100 °C. A Redwood viscometer was used for the estimation of viscosity. The viscosity index was analyzed according to ASTM D 2270. The refractive index was determined by a buyer Refractometer (PR-Butyro, ATAGO, Tokyo, Japan) according to the European Pharmacopeia Method. Cloud point, pour point and flash point were analyzed by ASTM D2500, ASTM D97 and ASTM D 6450 (Close Cup) method, respectively. Higher and lower heating values of the RSO were determined by using XRY -1A Bomb Calorimeter following the ASTM D3278-21 method.

The infrared spectrums of samples were conducted using a PerkinElmer FTIR spectrometer equipped with Attenuated Total Reflection (ATR) unit. The direct measurement of samples was conducted in absorbance mode applying a spectral resolution of 4 $cm^{-1}$ and acquiring 40 scans for each spectrum in the spectral range from 4000 to 650 $cm^{-1}$.

The fatty acid composition was determined after the formation of fatty acid methyl ester (FAME) of RSO by following the published method with a minor modification [38]. In brief, an aliquot of 30 mg of oil was mixed with 2 mL of 0.5 M sodium methoxide (prepared by dissolving metallic sodium in methanol) in a screw-caped test tube and incubated for 45 min at 60 °C with occasional shaking after every 10 min. Then, 100 μL of 1 M methanolic sulfuric acid was added to it and incubated for another 30 min at the same temperature. After that 1 mL of saturated sodium chloride was added to the tube and FAME was extracted with 2 mL *iso* octane. Finally, the upper *iso* octane layer was passed through an anhydrous sodium sulfate column to remove water. The filtrate was collected in a vial and 1 μL was injected in gas chromatography in a split injection (50:1) technique. Nitrogen was used as the carrier gas at a constant flow rate of 1 mL/min. The injector temperature was 250 °C, whereas the initial oven temperature of 150 °C was kept for 5 min. After holding the temperature at 200 °C for 5 min at a rate of 5 °C/min, it was raised to 240 °C for 5 min at a rate of 10 °C/min. The results of the automated gas chromatography software (Chromeleon, version-7.00) were then displayed as relative percentages after the fatty acids were identified using the corresponding fatty acid methyl ester standards (Supelco 37 Component FAME mix, USA).

Saponification value, iodine value, peroxide value, acid value, and hydroxyl value were analyzed by following the European Pharmacopeia methods. Ester value was calculated by simply subtraction of acid value from the saponification value [39]. Free fatty acid (FFA) content was calculated by following equation (1) [40]:

$$\text{FFA} = 0.503 * \text{ Acid value} \quad (1)$$

### 2.5. Assessment of antimicrobial activities of RSO

#### 2.5.1. Test organisms and their source

The antimicrobial properties of rubber seed oil were tested against Gram-positive bacteria [*Bacillus subtilis*, *Bacillus cereus*, *Bacillus megaterium* and *Staphylococcus aureus*], Gram-negative bacteria [*Escherichia coli*, *Salmonella* sp., and *Shigella* sp.] as well as fungi [*Aspergillus niger*, *Aspergillus flavus*, *Penicellium notatum* and *Candida albicans*]. The bacteria and fungi used for this study were obtained from the microbiology laboratory of the Leather Research Institute, Bangladesh Council of Scientific and Industrial Research, Savar, Dhaka, Bangladesh. The stock cultures were sub-cultured and maintained on Nutrient agar (NA) (Himedia) and Sabouraud Dextrose agar (SDA) (Himedia) medium for bacteria and fungi, respectively.

#### 2.5.2. Preparation of inoculum

Colonies from the freshly prepared plates were suspended in Nutrient broth (NB) (Himedia) and Sabouraud Dextrose Broth (SDB) (Himedia) for bacteria and fungi and were incubated for 18–24 h at 37 °C and 30 °C respectively [18]. After the incubation period, the turbidity was adjusted using sterile NB and SDB equal to that of the standard 0.5 McFarland solution at 600 nm which is equivalent to $10^6$–$10^8$ CFU/ml with the help of a spectrophotometer [41].

*2.5.3. Antimicrobial activity of rubber seed oil (agar well diffusion assay)*

The antibacterial and antifungal activity of extracted RSO was assessed by agar well diffusion method on NA and SDA medium for bacteria and fungi, respectively [35,42]. All culture media was sterilized at 121 °C for 15 min in an autoclave. The homogenized fluid agar media was poured into sterile petri dishes and allowed to solidify the medium within 30 min. After solidification, the inoculum suspension of examined organisms was swabbed uniformly and the inoculum was left to dry for 5 min according to a previous study [41]. Five wells per plate were developed by a standard cork borer of 5 mm in diameter was used to cut the surface of agar media of multiple plates. 100 μL of each extract was poured into separate wells. Four different concentrations of RSO in n-hexane (500 mg/mL, 250 mg/mL, 125 mg/mL, and 62.5 mg/mL) were used [34]. Negative control was prepared using respective solvents such as n-hexane.


**Fig. 1.** Schematic representation of the extraction of RSO.

The plates were kept at room temperature for 30 min to allow proper diffusion of the oil into the agar and then incubated at 37 °C for 24 h and 30 °C for 3–5 days for bacteria and fungi, respectively. Triplicates were prepared for each sample. The oils having antimicrobial action inhibit the microbial growth and the clear zones were formed. After incubation the diameter of circular inhibitory zones formed around each well were evaluated in millimeter (mm) and recorded [35].

### 2.6. Statistical analysis

Triple measurements were carried on for all the characterization techniques, and a mean value was reported. For the antimicrobial

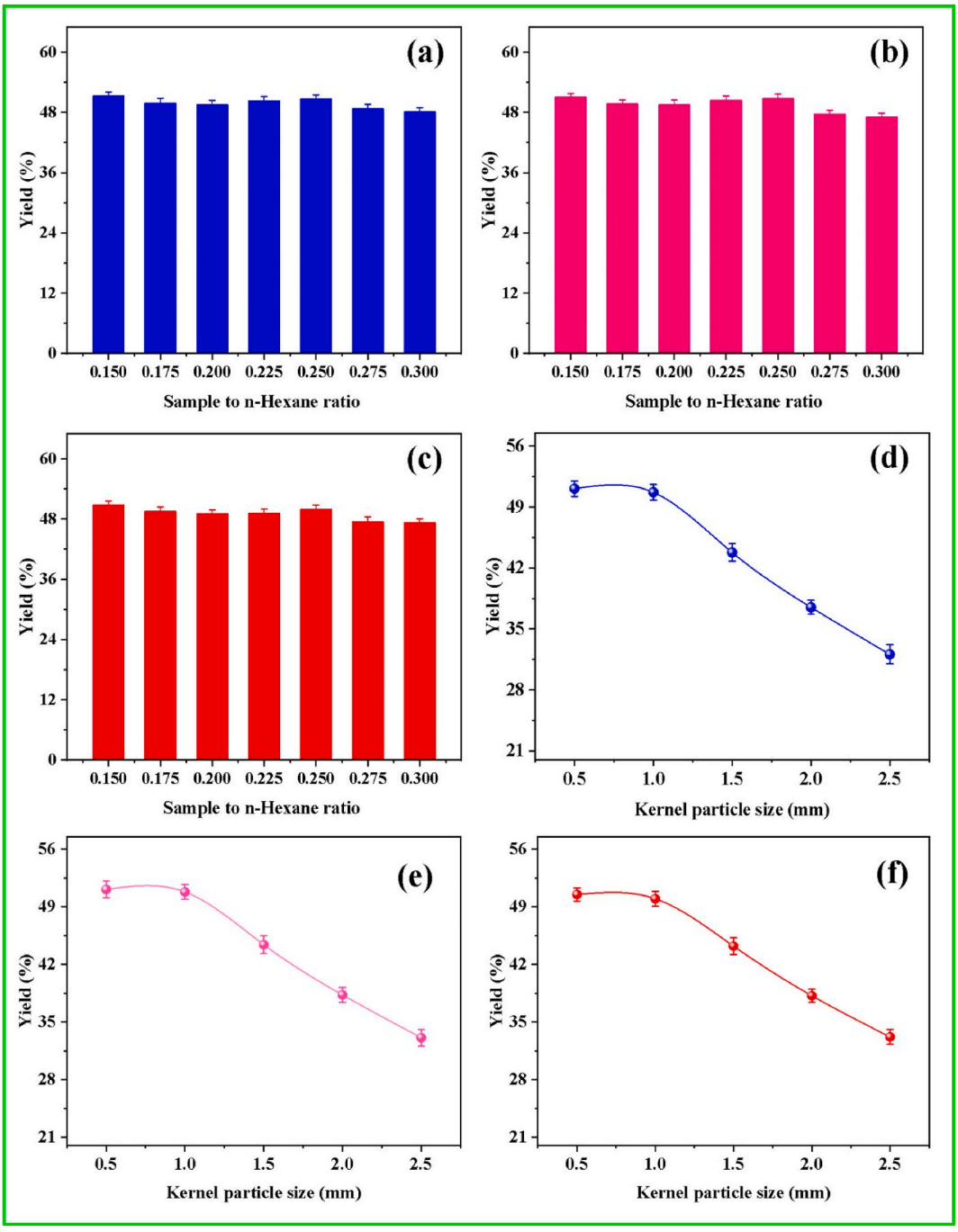


**Fig. 2.** Optimization of the oil extraction process. Optimize solvent to solute ratio of (a) Karnajora, Sherpur (b) Bhatera, Moulavibazar (c) Raojan, Chattagram; Optimize of particle size (d) Karnajora, Sherpur (e) Bhatera, Moulavibazar (f) Raojan, Chattagram.

assay, the data were analyzed by using MS Excel 2016 and presented as mean ± standard error (SE) of three replicates.

## 3. Results and discussion

### 3.1. The design concept of RSO

Rubber seeds are an ample by-product in rubber plantations, with annual production between 136 to 2000 kg per hectare of land. It is estimated that every year approximately $7.7 \times 10^9$ kg of rubber seeds are produced globally [19]. But unfortunately, these huge amounts of rubber seeds are being underutilized without any valorization. However, proper utilization of rubber seed to produce oil can become a clean energy resource and can be exploited to its true potential. Moreover, the physicochemical properties of RSO are an important indicator for the addition of its potential values. It is essential to study the physicochemical properties of RSO for the production of bio-diesel, surface coating and other preserving materials. Rubber seeds contain a high percentage (35–45 %) of oil and the oil is richest in aromatic molecules such as aldehydes, alcohols, acids, esters and among other [9].

These aromatic molecules or phenolic structures in RSO can destroy the cell membrane of microorganisms [17]. In addition, some biologically active substances including α-thujene, α-pinene, limonene, thymoquinone, myristicin, thymol, etc. in RSO contribute to the antimicrobial activities [18]. The extraction process of oil from rubber seeds is presented in Fig. 1. This facile method to produce RSO from rubber seeds is not only a green approach for the potential utilization of waste biomass but also an effective way to synthesize oil from renewable sources.

### 3.2. Optimization of the extraction process of RSO

To obtain maximum yield, the extraction process of RSO was optimized based on the solute to solvent ratio and particle size of the kernel. At first, the effect of the solute (g) to solvent (mL) ratio on oil yield was studied by several experiments using different amounts of the kernel (1 mm particle size) with a fixed amount (200 mL) of n-hexane. The result shows that the maximum oil yield was found at low solute to solvent ratios compared to high ratios (Fig. 2(a-c)) for all the samples. When the solute to solvent ratio is 0.15 the yield is highest and then decreased gradually up to 0.225. Interestingly, the yield is again increased when the solute to solvent ratio is 0.25 then decreased. Generally, the extraction yield should be higher at a lower solute to solvent ratio. This trend of oil yield concerning solute to solvent ratio may be due to the higher agglomeration of the kernel occurring at a low ratio [34]. In addition, the solvent could be reused in the next cycle, for this reason, the solute to solvent ratio was chosen at 0.25 in the whole study.

The effect of particle size of the kernel on oil yield was investigated using the solute to solvent ratio of 0.25 followed by 6 h refluxing time. The obtained results are summarized in Fig. 2(d–f). From the result, it was observed that the yield percentage decreased with increasing the particle size. When the average particle size of the kernel was increased up to 2.5 mm the resultant yield of oil was lower (~32 %). The particle size of kernel 0.5 mm provided a slightly higher yield (~51 %) compared to the 1.0 mm particle size (~50) [34]. The smaller particle size generates a larger surface area that increases oil yield by facilitating solvent diffusivity in the seed powder. For this reason, a 1.0 mm particle size kernel was used for all the samples to extract oil from rubber seed. In the whole study, an average particle size of 1.0 mm, extraction time of 6 h and 0.25 solute to solvent ratios was chosen to obtain the maximum oil yield.

### 3.3. Physicochemical properties and spectrochemical analyses of RSO

#### 3.3.1. Appearance, odor and color

The extracted RSO from three regions were liquid at room temperature and unpleasant to odor. The physical appearances of RSO


**Fig. 3.** Physical appearance of extracted RSO from different regions: (1) Karnajora, Sherpur, (2) Bhatera, Moulavibazar, (3) Raojan, Chattagram.

are different for different regions, such as brownish yellow, reddish brown, and yellowish (Fig. 3). Different colors of RSO may be due to the presence of different plant pigments like tocopherols, carotenoids and derivatives [43]. The color values of RSO are also determined with a Lovibond colorimeter using 25.4 mm cell (path length) and results found that the values are different from region to region (Table 1). For example, the Lovibond color values of most edible vegetable oils are less than 10 for yellow (Y), 2.5 for red (R) and 20 for (Y+5R) score [37]. Although, color and appearance are important quality parameters for edible oil. It is found that, the color values of extracted RSO: Y is less than 10 for all samples but the R values are greater than 2.5, and (Y+5R) values are greater than 20, indicating the RSO are not useable as edible oil.

### 3.3.2. Water content, heating value and sulfated ash content

The water content, heating value and sulfated ash content of RSO were presented in Table 2. According to the National Soybean Processors Association, the water content of crude oil is less than 0.30 % [44], where the extracted RSO fulfill the requirement. Furthermore, the higher and lower heating values of RSO from different regions varied insignificantly. The higher heating value of RSO from Bhatera, Moulavibazar region shows higher than the other two regions. The water content of RSO from different regions is varied slightly due to geographical locations and consistent with the heating values. The sulfated ash content of RSO is very much lower, less than 0.10 % which implies that the RSO does not contain heavy elements.

### 3.3.3. Relative density, viscosity, viscosity index and refractive index

The relative densities of RSO from different regions at 25 °C were found at the ranges of 0.885–0.901 (Table 2). These values were similar in the reported literature [45]. It is assumed that RSO is lighter than water and absence of heavy elements [46]. Although, comparatively higher values of relative densities have been reported by the authors [34]. The viscosities of the bio-lubricant at 40 °C and 100 °C are important lubricity properties useful in determining the applicability of the lubricant at low and high temperatures. They can also be used to evaluate the thermal stability of the bio-lubricant. Table 2 shows that the viscosities of RSO and the viscosity are significantly lower than those of the lubricant oil. Furthermore, the viscosities of RSO have a significant effect on geographical location. A similar observation was reported in the published literature [9]. The viscosity of RSO may improve by adding some derivatives those can be improved the molecular weight of the oil [35]. The refractive indexes of RSO were 1.4574–1.4665, which is similar to those reported by previous research and near to refined soya oil, castor Oil [47,48].

### 3.3.4. Flash point, pour point and cloud point

The flash point, pour point and cloud point of the RSO are presented in Table 2. Results show that the flash point varied from 237 to 245 °C for three different sources of rubber seeds. Similar results were reported by Onoji et al. [49]. Interestingly, the oil from Bhatera, Moulavibazar region shows the highest value of flash point among the other sources, which is consistent with heating value and water content as well. Higher flash points for any type of vegetable oil may be further used for the production of biodiesel or lubricants. The pour point and cloud point of RSO varied insignificantly from different regions of sources (Table 2).

The pour point is the temperature at which the oil ceases to flow. This is important as it gives an indication of the temperature over which the oil will perform best particularly in mechanical applications. A low pour point is indicative of the use of the lubricant in cold temperatures. The pour point of RSO is comparatively lower (−4 °C to −3 °C) [34] suggesting that it can be employed even under cold conditions (Table 2).

The cloud point of a bio-oil is the temperature below which the oil forms a cloudy appearance. The values cloud point of RSO from different regions was consistent with earlier reported literature [50]. Although, according to ASTM standard D 6751, no limit is specified for cloud point. The reason is that the climate conditions in the world vary considerably, thus affecting the bio-oil properties in a specific region [51].

### 3.3.5. Acid value, iodine value, peroxide value, saponification value, hydroxyl value and ester value

Different chemical parameters of RSO, such as acid value, iodine value, peroxide value, saponification value, and hydroxyl value are determined by following the standard procedure and results are presented in Table 2. Acid values of RSO are found from 13.3 to 18.2 mg KOH/g for three different sources. Similarly, iodine value, peroxide value, saponification value, and hydroxyl value for different regions show insignificant changes for three different sources. Ester value and FFA content of RSO are calculated from saponification and acid value parameters.

Acid value is an important parameter for any type of oil. Although, it is reported that the acid value of RSO is varied in a significant

**Table 1**
Physical parameter of RSO from different regions.

| Physical Parameter | | Regions of collecting rubber seed | | |
|---|---|---|---|---|
| | | Karnajora, Sherpur | Bhatera, Moulavibazar | Raojan, Chattagram |
| Physical state | | Liquid | Liquid | Liquid |
| Physical appearance | | Brownish yellow | Reddish brown | Yellowish |
| Odor | | Unpleasant | Unpleasant | Unpleasant |
| Color | Yellow (Y) | 9.5 | 9.9 | 6.1 |
| | Red (R) | 6.9 | 10.9 | 2.9 |
| | Score (Y+5R) | 44 | 64.4 | 20.6 |

**Table 2**
Physicochemical properties of RSO from different regions.

| Parameter | Method | Regions of collecting rubber seed | | |
|---|---|---|---|---|
| | | Karnajora, Sherpur | Bhatera, Moulavibazar | Raojan, Chattagram |
| Percent of oil yield, % (w/w) | – | 50.7 | 50.8 | 50.0 |
| Water content, % (w/w) | Ph. Eur. Method 2.5.32 | 0.201 | 0.178 | 0.221 |
| Relative Density at 25 °C | Ph. Eur. Method 2.2.5 | 0.889 | 0.885 | 0.901 |
| Viscosity, cSt @ 40 °C | ASTM D 445 | 18.9 | 17.0 | 34.4 |
| @ 100 °C | ASTM D 445 | 6.11 | 6.01 | 8.79 |
| Viscosity index | ASTM D 2270 | 314 | 356 | 251 |
| Refractive index | Ph. Eur. Method 2.2.6 | 1.46 | 1.46 | 1.47 |
| Iodine value, (g $I_2$/100g) | Ph. Eur. Method 2.5.4 | 134 | 132 | 137 |
| Peroxide Value, (meq $O_2$/1000g) | Ph. Eur. Method 2.5.5 | 3.85 | 3.86 | 3.78 |
| Saponification Value, (mg KOH/g) | Ph. Eur. Method 2.5.6 | 180 | 183 | 178 |
| Acid value, (mg KOH/g) | Ph. Eur. Method 2.5.1 | 15.5 | 18.2 | 13.3 |
| Ester Value, (mg KOH/g) | – | 164 | 165 | 164 |
| Hydroxyl Value, (mg KOH/g) | Ph. Eur. Method 2.5.3 | 47.7 | 55.8 | 48.4 |
| Higher heating value, (kJ/g) | ASTM D3278-21 | 32.3 | 33.3 | 31.8 |
| Lower heating value, (kJ/g) | ASTM D3278-21 | 30.7 | 31.7 | 30.2 |
| Cloud Point, (°C) | ASTM D2500 | 8.00 | 10.0 | 9.00 |
| Pour Point, (°C) | ASTM D97 | −3.00 | −4.00 | −3.00 |
| Flash Point, (°C) | ASTM D 6450 (Close Cup) | 237 | 245 | 241 |
| FFA, %(w/w) | – | 7.80 | 9.16 | 6.69 |
| Sulfated ash content, % (w/w) | Ph. Eur. Method 2.4.14 | 0.0690 | 0.0861 | 0.0780 |

range [18,40]. In our study, it shows that the oil obtained from Raojan, Chattagram region is slightly lower compared to the other two regions. In addition, oil obtained from Bhatera, Moulavibazar region is slightly higher than other regions. However, the acid values for the RSO obtained from three different regions are in the reported results [47].

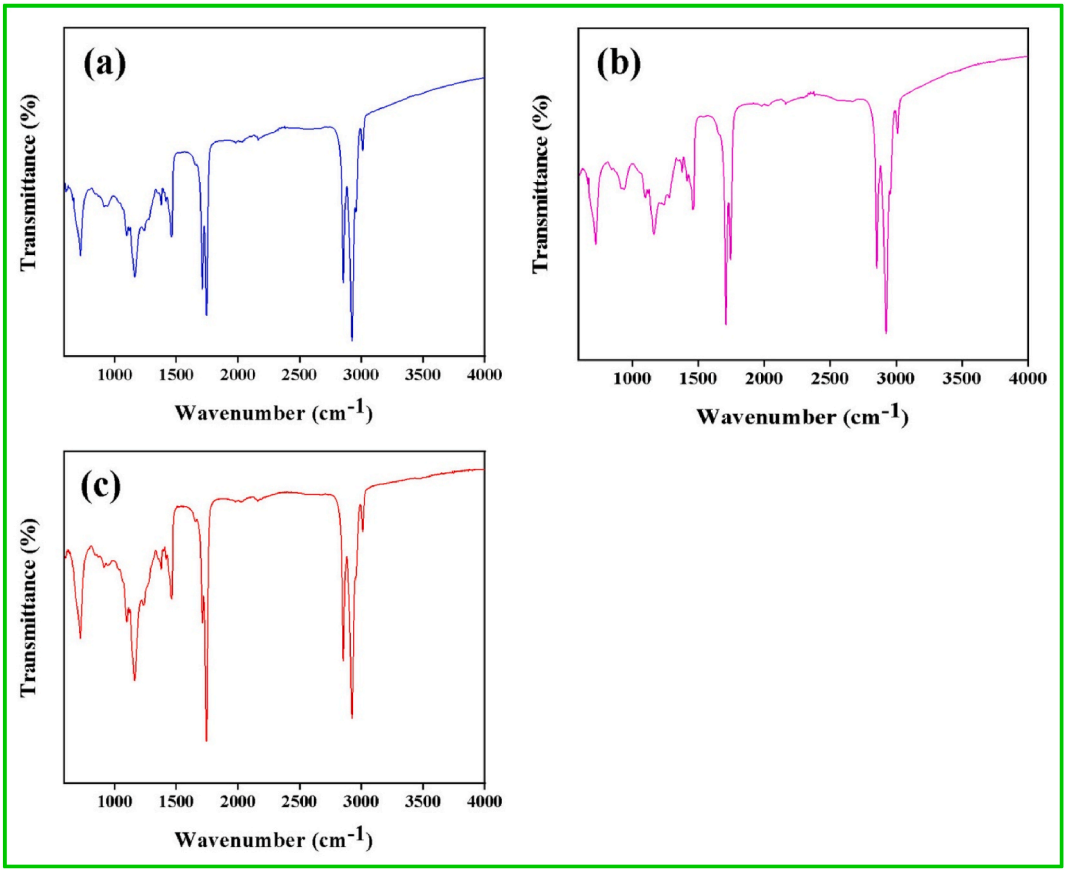


**Fig. 4.** FTIR spectra of RSO extracted from (a) Karnajora, Sherpur (b) Bhatera, Moulavibazar (c) Raojan, Chattagram.

The iodine value of RSO from different regions shows between 132 $gI_2$/100 g to 137 $gI_2$/100 g indicating that RSO is a semi-drying oil and gives an assumption about the degree of unsaturation [18]. The saponification value obtained for RSO was 178–183 mg KOH/g (Table 2), which is similar to those reported by previous research [52]. The peroxide value of oils gives an idea about the quality, stability and extent to which rancidity reactions have occurred. The peroxide value of this study was found less than 4 meq $O_2$/1000g and this value is in the range for a series of vegetable oils [47,53]. The hydroxyl value corresponds to the number of milligrams of potassium hydroxide (KOH) required to neutralize the amount of acetic acid combined with hydroxyl groups in 1 g of a sample on acetylation. The result found in this study are about 47.7–55.8 mg KOH/g this high value suggests that the total amount of free polyols-derived cyclic ethers as well as the acid values in the samples will also be comparably high [54]. The calculated ester value and FFA content were found from 164 to 165 mg KOH/g and 6.69–9.16 %, respectively (Table 2). These results are also similar to previously reported studies [18,40].

### 3.3.6. Spectroscopic properties of RSO

The FTIR spectra of RSO from different regions are presented in Fig. 4(a–c). From the FTIR spectrum of RSO (Fig. 4 & Table 3), the characteristic peak at 3010 $cm^{-1}$ was due to the stretching vibration band of C–H [55]. The peaks obtained at 2924 $cm^{-1}$ and 2854 $cm^{-1}$, which derived from the C–H asymmetric stretch vibrations in methylene and methyl groups on the chain. The characteristic strong peak at around 1745 $cm^{-1}$ was assigned to the ester carbonyl (C═O) groups [56]. In addition, the absorption band at 1710 $cm^{-1}$ and 1459 $cm^{-1}$ for the carboxylic acid (C═O) and C═C bending vibration of the aliphatic double bond, respectively. The stretching vibration (ester) peak of the C–O–C and C–H groups show at 1163 $cm^{-1}$ and 722, respectively [57].

The fatty acid composition is a main indicator of the purity and quality of the vegetable oils because the type and quantity of each fatty acid vary from one vegetable oil to another. The main fatty acid composition and content of RSO are shown in Fig. 5 and Table 4. Fig. 5(a–c) represents that the fatty acid compositions of RSO obtained from different regions are almost similar.

The main fatty acid composition of RSO was palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid for all the samples but their content is varied. The RSO contained about 17.5 ± 0.5 % saturated fatty acid and about 82 ± 0.5 % unsaturated fatty acid. The unsaturated fatty acids are two types, thus, 25.5 ± 1 % correspond to the monounsaturated and 56 ± 2 % for polyunsaturated. Linoleic and alpha-linolenic fatty acids were the main components of polyunsaturated fatty acids. In this study, their content in different regions of oil was varied from 31.2 to 44.8 % and 12.1–26.2 %, respectively. Several factors such as geographical location, weather, and soil property may be responsible for this variation. Similar factors were reported to influence the content of fatty acid in a previous study [34]. The unsaturated fatty acid content of RSO was near to that of flaxseed oil (85.29 % w/w). Furthermore, the linolenic acid of RSO has highest compared to soybean oil and olive oil [58].

## 3.4. Antimicrobial activity of RSO

Results of the antimicrobial activity of rubber seed oils against bacteria and fungi were assessed both quantitatively and qualitatively through the presence or absence of inhibition zones and zone diameters including the well diameter are shown in Table 5.

Antimicrobial activity was found against Gram-positive bacteria, in particular, *Bacillus cereus*, *Bacillus megaterium* and *Bacillus subtilis* as shown in Fig. 6(a–i) and Table 5. No activity was found against *Staphylococcus aureus,* tested Gram-negative bacteria and fungi. Moderate to lower concentrations of oil samples were effective against *Bacillus* species. The highest zone of inhibition of 11.0 ± 0.5 mm, 9.83 ± 0.60 mm and 10.3 ± 0.92 mm was determined at the concentration of 125 mg/ml of Karnajora, Sherpur; Bhatera, Moulavibazar and Raojan, Chattagram; respectively against *B. cereus* (Table 5). The same concentration performed the best against *B. megaterium* with inhibition zone of 9.83 ± 0.88 mm, 7.0 ± 3.51 mm and 3.17 ± 3.17 mm for Karnajora, Sherpur; Bhatera, Moulavibazar and Raojan, Chattagram; respectively (Table 5). In the case of *B. subtilis*, Karnajora, Sherpur exhibited the highest inhibition zone of 11.17 ± 1.01 mm at the concentration of 250 mg/mL. While, Bhatera, Moulavibazar and Raojan, Chattagram produced the maximum inhibition zone of 9.5 ± 0.28 mm and 10.67 ± 0.66 mm of diameter respectively at 62.5 mg/ml against *B. subtilis* (Table 5). Negative control i.e., n-hexane did not show any inhibitory effect on the growth of all tested microorganisms (Table 5).

In this study, the antimicrobial activity of rubber seed oils against Gram (+) bacteria, Gram (−) bacteria and fungi was investigated using four different concentrations of each oil sample by calculating the growth inhibition zone around the agent's well in millimeters shown in Table 5. The results found from the study demonstrated that the rubber seed oil possesses antibacterial activity against Gram (+) bacteria. When the extracted rubber seed oil was assayed against the tested microorganisms, the mean zone of inhibition found was between 2.33 ± 2.33 mm and 11.17 ± 1.01 mm (Table 5). On the whole, in this study rubber seed oil showed higher activity against

**Table 3**
The main peaks in the FTIR spectrum of RSO and their assignments.

| Regions of collecting rubber seed | Assignment of peaks ($cm^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | C–H stretching vibration (aliphatic) | C═O stretching vibration (ester) | C═O stretching vibration (carboxylic acid) | C═C bending vibration (aliphatic) | C–*O*–C stretching vibration (ester) | C–H group vibration (aliphatic) |
| Karnajora, Sherpur | 3010, 2923, 2854 | 1745 | 1710 | 1459 | 1163, 1099 | 722 |
| Bhatera, Moulavibazar | 3010, 2924, 2854 | 1745 | 1710 | 1459 | 1163, 1099 | 722 |
| Raojan, Chattagram | 3010, 2923, 2854 | 1744 | 1712 | 1463 | 1161, 1098 | 722 |

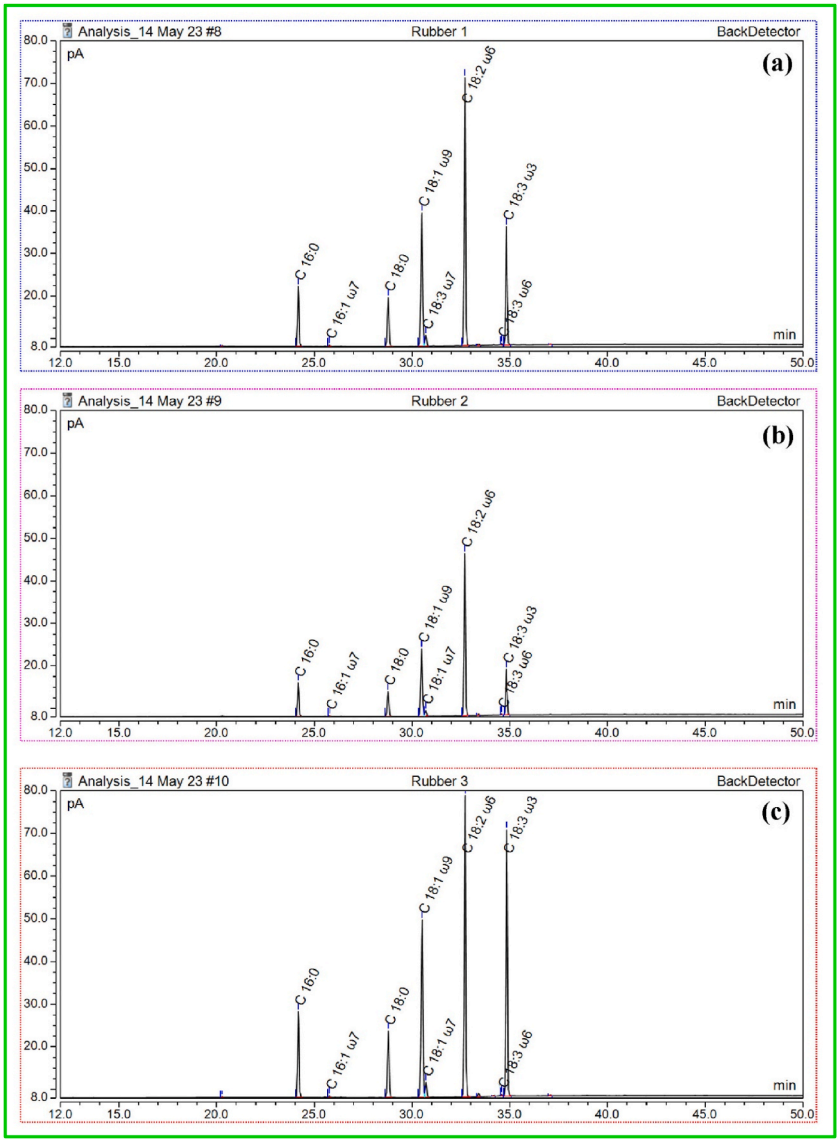


**Fig. 5.** Gas chromatogram of extracted RSO from different regions (a) Karnajora, Sherpur (b) Bhatera, Moulavibazar (c) Raojan, Chattagram of Bangladesh.

**Table 4**
Fatty acid composition of RSO.

| Name of fatty acid | Content of fatty acid (%) | | |
|---|---|---|---|
| | Karnajora, Sherpur | Bhatera, Moulavibazar | Raojan, Chattagram |
| Palmitic acid (C 16:0) | 8.67 | 9.19 | 8.62 |
| Palmitoleic acid (C 16:1 ω7) | 0.190 | 0.180 | 0.170 |
| Stearic acid (C 18:0) | 8.72 | 8.42 | 8.32 |
| Oleic acid (C 18:1 ω9) | 24.1 | 23.0 | 22.9 |
| cis Vaccenic acid (C 18:3 ω7) | 1.85 | 1.80 | 1.73 |
| Linoleic acid (C 18:2 ω6) | 39.2 | 44.8 | 31.2 |
| Gamma Linolenic acid (C 18:3 ω6) | 0.160 | 0.180 | 0.140 |
| Alpha Linolenic acid (C 18:3 ω3) | 16.7 | 12.1 | 26.2 |
| Unknown | 0.410 | 0.330 | 0.720 |
| Total | 100 | 100 | 100 |

**Table 5**
Antimicrobial assessment of RSO.

| Microorganisms | Diameter of zone of inhibition (mm) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n-hexane | Karnajora Rubber Garden, Sherpur | | | | Bhatera Rubber Garden, Moulavibazar | | | | Raojan Rubber Garden, Chattogram | | | |
| | | Concentration (mg/mL) | | | | Concentration (mg/mL) | | | | Concentration (mg/mL) | | | |
| | | 500 | 250 | 125 | 62.5 | 500 | 250 | 125 | 62.5 | 500 | 250 | 125 | 62.5 |
| *B. cereus* | N | 5.5 ±2.75 | 9.67 ±0.92 | 11.0 ±0.5 | 9.83 ±0.16 | 8.17 ±0.16 | 8.5 ±0.28 | 9.83 ±0.60 | 9.16 ±0.16 | N | 3.0 ±3 | 10.3 ±0.92 | 3.3 ±3.3 |
| *B. megaterium* | N | 5.17 ±2.58 | 9.17 ±0.16 | 9.83 ±0.88 | 9.67 ±0.66 | 5.0 ±2.5 | 6.67 ±3.38 | 7.0 ±3.51 | 6.0 ±3.0 | N | 2.33 ±2.33 | 3.17 ±3.17 | 3.0 ±3.0 |
| *B. subtilis* | N | 4.83 ±2.42 | 11.17 ± 1.01 | 11.0 ±0.0 | 10.5 ±0.76 | 2.33 ±2.33 | 9.5 ±0.5 | 9.17 ±0.60 | 9.5 ±0.28 | 2.5 ±2.5 | 9.83 ±2.58 | 10.33 ± 0.33 | 10.67 ±0.66 |
| *S. aureus* | N | N | N | N | N | N | N | N | N | N | N | N | N |
| *E. coli* | N | N | N | N | N | N | N | N | N | N | N | N | N |
| *Salmonella* sp. | N | N | N | N | N | N | N | N | N | N | N | N | N |
| *Shigella* sp. | N | N | N | N | N | N | N | N | N | N | N | N | N |
| *Aspergillus flavus* | N | N | N | N | N | N | N | N | N | N | N | N | N |
| *Aspergillus niger* | N | N | N | N | N | N | N | N | N | N | N | N | N |
| *Penicillium notatum* | N | N | N | N | N | N | N | N | N | N | N | N | N |
| *Candida albicans* | N | N | N | N | N | N | N | N | N | N | N | N | N |

Values are means of triplicate determination (n = 3) ± standard error. N, no zone of inhibition was found.

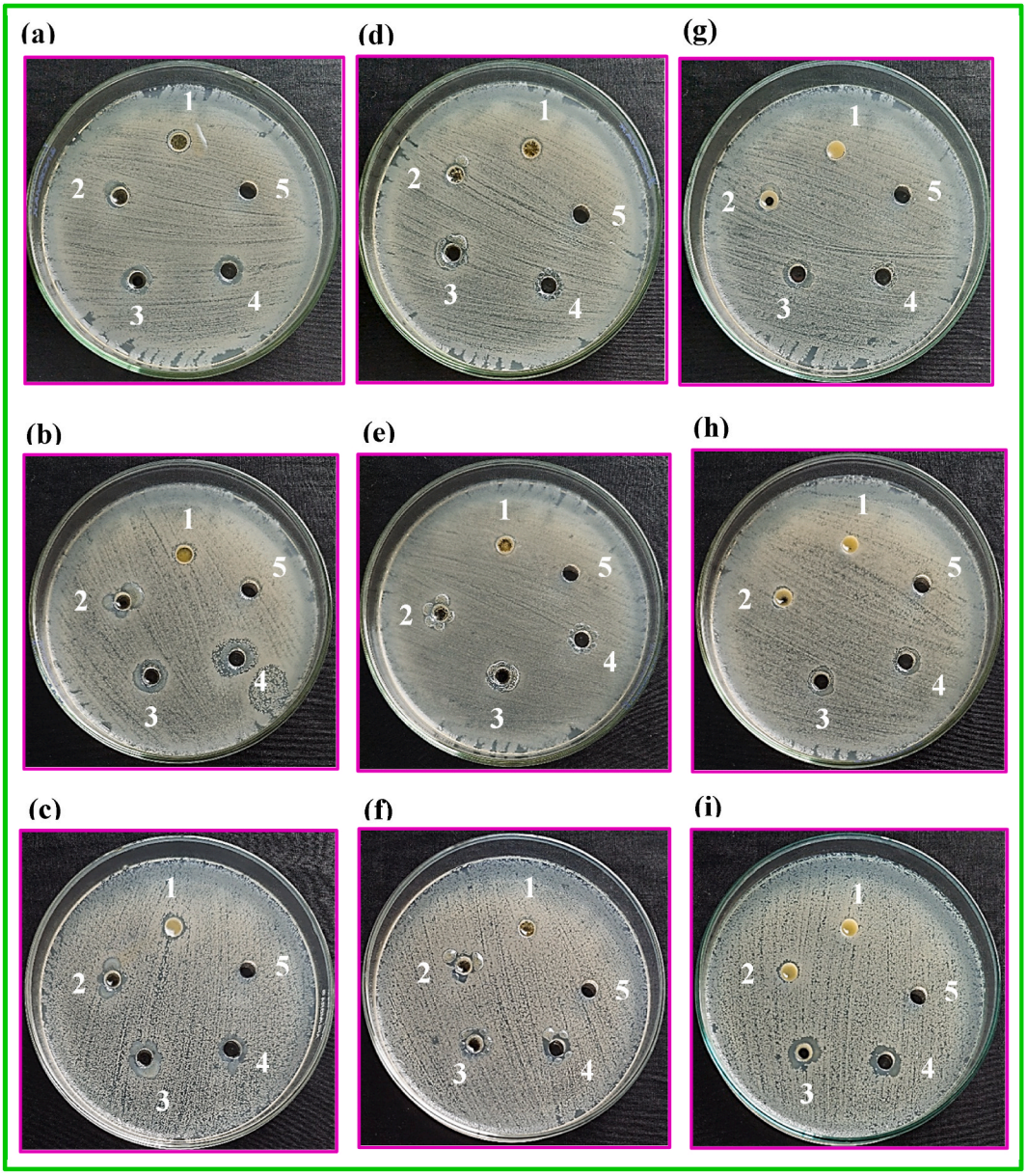


**Fig. 6.** The inhibition zone (mm) of Karnajora, Sherpur (a, b, c) against *B. cereus* (a), *B. megaterium* (b), *B. subtilis* (c), Bhatera, Moulavibazar (d, e, f) against *B. cereus* (d), *B. megaterium* (e), *B. subtilis* (f) and Raojan, Chattagram (g, h, i) against *B. cereus* (g), *B. megaterium* (h), *B. subtilis* (i). Here, 1,2,3,4 and 5 denote oil concentrations of 500 mg/mL, 250 mg/mL, 125 mg/mL, 62.5 mg/mL and n-hexane respectively.

*B. subtilis,* moderate activity against *B. cereus,* and lower activity against *B. megaterium.* Oil extracted from rubber seeds collected from the Sherpur region showed higher activities regardless of tested microorganisms compared to that of the other two regions (Table 5). The highest activity was recorded as $11.17 \pm 1.01$ mm for oil extracted from seeds collected from the Sherpur region against *B. subtilis*. *B. subtilis* was more susceptible to oil extracted from seeds collected from the Chattagram region irrespective of tested concentrations compared to two other tested *Bacillus* spp. (Table 5). None of the tested seed oils showed activities against tested Gram (−) bacteria and fungi.

However, the oil extracted from the rubber seeds showed antifungal activity on the fungal strain of *Fusarium* sp [18]. Besides, *Croton macrostachyus* seed oil and neem seed oil also showed activities against the *Aspergillus Niger*, *Candida albicans* and *Penicillium verrucosum*. *Penicillium brevicompactum* respectively [59,60]. In a previous study, it was found that *Nigella sativa* Linn. seed oil was more effective on Gram (+) bacteria than Gram (−) bacteria [18], which is in line with the findings of the present study. Non-edible *Ricinus communis* L. seed oil exhibited antimicrobial activity against *Bacillus subtilis, Aspergillus fumigatus* and *Aspergillus flavus* [61]. In this study, moderate to lower concentrations of rubber seed oil showed higher activities against tested microorganisms. Nevertheless, the

antibacterial and antifungal action of *Croton macrostachyus* seed oil increased with increasing its concentration [59].

## 4. Conclusions

In this study, RSO is extracted from rubber seeds using n-hexane as a solvent after collecting the seeds from three different regions in Bangladesh. Our choice is three rubber gardens that are situated in different parts of Bangladesh, thus, Karnajora rubber garden, Bhatera rubber garden, and Raojan rubber garden are situated at Sherpur, Maulavibazar and Chattagram districts, respectively. The percentage of yield and flash point for three different samples varied from 50.0 to 50.8 %, and 237–245 °C, respectively. The higher heating value of RSO obtained from Sherpur, Maulavibazar and Chattagram are found 32.3; 33.3 and 31.8 kJ/kg, indicating that the RSO may be utilized for the production of biodiesel. The chemical parameters, such as acid value, iodine value and hydroxyl value varied from 13.3 to 18.2 mg KOH/g, 132–137 g $I_2$/100g and 47.7–55.8 mg KOH/g, respectively. Gas chromatographic results indicated that RSO contains different types of fatty acids and they are mainly palmitic, linoleic, linolenic and stearic acid. Most importantly, RSO contains many biologically active substances and aromatic molecules such as aldehydes, alcohols, acids, esters, etc. Due to the presence of these functional groups in RSO, it shows an inhibitory action against fungi and bacteria, respectively. These functions of RSO might play a new role in the preservation of many raw materials and the biomedical sector as well. This study revealed that the production of RSO from renewable sources is not only an effective utilization of waste biomass but also promising applications of RSO for different value-added purposes.

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## Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable requests.

## CRediT authorship contribution statement

**Md. Ashraful Alam:** Writing – original draft, Methodology, Formal analysis, Conceptualization. **Md. Tushar Uddin:** Writing – review & editing, Validation, Supervision. **Khandokar Tahmina Tasnim:** Data curation. **Shashanka Shekhar Sarker:** Formal analysis, Data curation. **Md. Abdur Razzaq:** Formal analysis, Data curation. **Md. Alamgir Kabir:** Formal analysis, Data curation. **SM Asaduzzaman Sujan:** Formal analysis, Data curation. **Ajoy Kanti Mondal:** Writing – review & editing, Validation, Supervision.

## 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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