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RESEARCH ARTICLE

Analysis of total flavonoid and antioxidant activity of coconut shell liquid smoke (*Cocos nucifera* L.) as an antibacterial

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Abstract

Background: Coconut shell is often used for cooking or treated as waste. Liquid smoke is a by-product of coconut shell processing, which results from condensation of combustion steam. It is divided into grades one, two, and three based on the characteristics and utilisation. It contains phenolic, carbonyl acids and other compounds which act as antioxidants, antibacterials, and antifungals. **Objective:** To determine the total content of flavonoids, antioxidants and antibacterial activity in liquid smoke. **Method:** Liquid smoke was produced by a pyrolysator and distilled to produce grades one, two and three. It was then tested for antibacterial activity using the minimal inhibitory concentration (MIC) method against *S. aureus* and *E. coli*. The $AlCl_3$ reagent and DPPH method were used to determine the total flavonoid and antioxidant levels, respectively. **Results:** The MIC of grade one, two, and three coconut shell liquid smoke was 10%; 10%; 5% against *S. aureus*; and 10%, 5%, and 5% against *E. coli*. The total flavonoid yields from grades one, two and three were 0.0041, 0.0174, and 0.2741 mg QE/ml, respectively. Test results for antioxidant grades with IC_{50} values were 142.82, 121.64, and 90.80 µg/mL. **Conclusion:** Coconut shell liquid smoke has antibacterial activity, contains a certain amount of flavonoids, and also possesses antioxidant activity.

Introduction

Coconut (*Cocos nucifera* L.) is one of Indonesia's more abundant natural resources. Nearly every component of the coconut fruit is reportedly useful, although some, like the shell portions, are thrown away and treated as waste (Alouw & Wulandari, 2020). Biological waste products are still considered safer for people to use when compared to chemical or synthetic materials due to their long-term adverse effects that could be highly detrimental to human health. Natural substances that can be used for both prevention and therapy are regarded as healthier and safer and have no or fewer adverse side effects (Ristiani *et al*., 2022).

Condensing smoke from burning wood creates liquid smoke, a mixture of wood smoke dispersed in water. Due to the flavouring properties of the phenolic compounds, liquid smoke is utilised commercially as a fish and meat flavouring ingredient (Muratore & Licciardello, 2005). Additionally, liquid smoke has long been used as a preservative for post-harvest agricultural, medical, and food items (Muratore and Licciardello, 2015). Liquid smoke can be produced from a variety of biomasses that contain cellulose, hemicellulose, and lignin, such as coconut shells, through pyrolysis (Mulyawanti, *et al*., 2019).

Liquid smoke contains phenolic and acidic compounds with antimicrobial and antioxidant properties. According to research by Zuraida *et al*. (2009), coconut shell liquid smoke has antibacterial activity against *Staphylococcus aureus*, which can extend the shelf life

of fish balls for 16 hours. Bacterial contamination that occurs in food can cause foodborne diseases (Zuraida, *et al*., 2009; Soares *et al*., 2016).

Escherichia coli is a gram-negative bacteria that can contaminate raw or inappropriately processed meat. *E. coli* bacteria in food and beverages that enter the human body can cause symptoms such as diarrhoea, nausea, vomiting, and diseases such as dysentery, gastroenteritis, and other digestive tract diseases. It is found in the human intestine, plays a role in removing waste products from the digestive tract, and can infect the intestines, causing diarrhoea. The growth of pathogenic bacteria that cause infection and disease needs to be inhibited with antibacterials (Bonten *et al*., 2021; Mansouri *et al*., 2021). Based on this, to increase the potential of coconut shell liquid smoke as an antimicrobial, it is necessary to research antibacterial activity tests that can inhibit the growth of *E. coli*.

Liquid smoke can be used as a food preservative due to the presence of acids, phenolic, and carbonyl compounds. It contains more than 400 components, has a function to inhibit bacterial growth, and is relatively safe to use as a natural preservative. Similarly, liquid smoke from coconut shells contains approximately the same components and functions as other types of liquid smoke. Phenolic compounds are one of the main chemical compounds with antibacterial properties (Suryanto *et al*., 2020; Wibisono, *et al*., 2022).

According to the research by Xin *et al*. (2022), the test to assess the total flavonoids in liquid smoke is rarely reported. Nevertheless, coconut shell liquid smoke has potent antioxidant activity with an IC_{50} value of 91.27 µg/ml. Flavonoids are the most diverse group of phenolic compounds and can be found in almost all plants, generally in the epidermal tissue of leaves and fruit peels. As polyphenols, many studies have proven the benefits of flavonoids for human health, including anti-cancer, anti-inflammatory, antioxidant, antiallergic, antiviral, and anti-melanogenesis. Consumption of foods, especially vegetables and fruits rich in flavonoids, can prevent the risk of cardiovascular disease (Suryanto *et al*., 2020; Xin, *et al*., 2021).

From these references, it can be seen that there is a good correlation between the high levels of phenolic compounds and acids contained in coconut shell liquid smoke and the total flavonoid content, as well as the antibacterial and antioxidant activity of the liquid smoke. This study aims to analyse the total flavonoids and antioxidant activity of coconut shell liquid smoke as an antibacterial.

Methods

The sample used in this study was coconut shell waste (*Cocos nucifera* L.) taken from a traditional market located on Jl. Halat, Medan, North Sumatra. The bacteria used in this study were *S. aureus* and *E. coli*.

Liquid smoke process

The coconut shell was cleaned and then crushed using a hammer into small pieces so that the sample could enter the pyrolysis reactor. It was put into the burner until solid and then closed tightly. It was then put into the furnace, which has a smoke funnel connected to the condenser, and baked for four hours and 15 minutes at a temperature of 400^{o}C.

Liquid smoke purification

The lowest grade three liquid smoke was obtained from pyrolysis. Purification to obtain grade two liquid smoke, which is better than grade three, was carried out by distilling the grade three liquid smoke. Grade one liquid smoke was obtained by the redistillation of grade two liquid smoke so that the chemical constituents, such as benzopyrene, could be separated. Liquid smoke is distilled by putting it into a distillation flask that has been assembled with a Liebig condenser, then heated using a hot plate until it reaches a maximum temperature of 150^{o}C for grade three to grade two and a temperature of 125^{o}C for grade two to grade one. These are the ideal temperatures to obtain suitable distillates from liquid smoke (Suryanto *et al*., 2020).

Phytochemical screening and antibacterial activity

Phytochemical screening of coconut shell liquid smoke included examination of alkaloids, phenols, flavonoids, glycosides, saponins, steroids/triterpenoids, and tannins. Antibacterial activity was tested using the paper disc diffusion method by dripping the paper with 100 µL of different test solution concentrations (from a concentration of 5, 10, 15, 20, 40, 60, 80, and 100%). The positive control was 50 µL of 200 µg tetracycline, and the negative control was 50 µL of distilled water on solid media inoculated with bacteria. The media is incubated at 37^{o}C for 18-24 hours. The diameter of the inhibition area (clear zone) around the paper tray was measured using a vernier calliper. These procedures were done for the liquid smoke from grades one to three.

Determination of total flavonoids

Total flavonoids were determined by colourimetric method using UV-Vis spectrophotometry. The samples were prepared using a 0.5 ml solution with a concentration of 100% v/v (grade one and two) and

25% v/v (grade three), added with 1.5 ml methanol pro analysis, 0.1 ml of aluminium chloride solution ($AlCl_3$), 0.1 ml of 1M sodium acetate, and 2.8 ml of double distilled water. It was then incubated for 30 minutes at room temperature. Absorbance was measured at a maximum wavelength of 429 nm. A similar procedure was done across the grade of the liquid smoke. Quercetin with various concentrations ranging from 35-85 µg/mL was prepared as a calibration curve. The total flavonoid content obtained was expressed as mg quercetin equivalent (QE) /ml of the sample.

Antioxidant activity by DPPH method

Determination of antioxidant activity was done using the DPPH method. UV-VIS spectrophotometry was used to measure the maximum absorption of DPPH solution with a concentration of 35-85 µg/mL in methanol. These measurements showed that DPPH in methanol produces maximum absorption at a wavelength of 516 nm. Then, a series of samples with a concentration of 20-70 µg/ml was made. Into each flask, 0.5 ml of DPPH 200 µg/ml was added and diluted with methanol up to the mark line. The absorption of the solution was measured at a wavelength of 516 nm for each solution concentration using a UV-VIS spectrophotometer. To determine the activity of quercetin as a comparison, a quercetin concentration of 500 µg/ml was made, and then a series of concentrations of two, four, six and eight micrograms per millilitre was also made. Into each flask, 1 ml of DPPH 200 µg/ml was added and diluted with methanol up to the mark line. Similarly, the absorption was measured at 516 nm for each concentration using a UV-Vis spectrophotometer (Khodijah *et al*., 2022).

Statistic test

The research findings are displayed using the mean ± standard deviation format. Statistical analysis was conducted using version 22.0 of SPSS software and one-way analysis of variance (ANOVA) and Kruskall Wallis. If a significant difference exists, the analysis is continued with the Tukey test. Statistical analysis was performed at a 95% level of confidence.

Results

Grade three liquid smoke was obtained from the pyrolysis of coconut shell waste (*Cocos nucifera* L.) by a pyrolysator. The grade three liquid smoke had a dark colour and a powerful smoky smell and still contained tar and Polycyclic Aromatic Hydrocarbon (PAH) compounds, which are carcinogenic. Grade two liquid smoke was obtained by purification of grade three liquid smoke using the distillation method, while grade one liquid smoke resulted from the purification of grade two liquid smoke through redistillation (Figure 1).

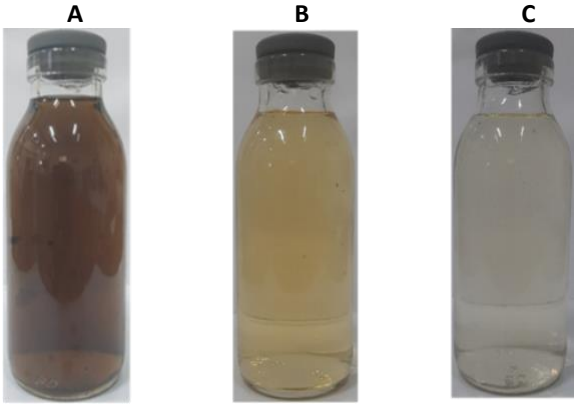

Figure 1: Liquid smoke. A=Grade Three; B=Grade Two; C= Grade One

Phytochemical screening on the liquid smoke grade one to three was carried out to identify the presence of alkaloids, phenols, flavonoids, saponins, and steroids/triterpenoids. The coconut shell liquid smoke positively contained phenolic compounds, flavonoids, aglycone glycosides, steroids/triterpenoids, and tannins. This agreed with the study of Mulyawanti *et al*. (2019), which stated similar findings.

The results of the antibacterial activity test showed that coconut shell liquid smoke could inhibit the growth of *S. aureus and E. coli.* The statistical test findings for the antibacterial activity of Grades one, two, and three coconut shell liquid smoke against *Staphylococcus aureus* and *Escherichia coli* showed a significance value of $p > 0.05$ in the normality test. Hence, it can be inferred that the data is normally distributed. The data was then analysed using a one-way ANOVA test, which revealed significance levels of 0.519 and 0.155 for the two bacteria ($p > 0.05$). Therefore, it is determined that grades one, two, and three of coconut shell liquid smoke with varying concentrations, as well as the positive controls and negative controls, did not significantly differ regarding the diameter of the inhibitory zones.

The MIC for gram-positive *S. aureus* was 10% in grades one and two, with an inhibition zone diameter of 7.65 ± 1.27 mm and 7.87 ± 1.44 mm, respectively, and 5% in grade three, a diameter of 6.42 ± 0.10 mm. Also, the MIC for the gram-negative *E. coli* was 10% in grade one with an inhibition zone diameter of 8.88 ± 0.50 mm, 5% in grades two and three, with an inhibition zone diameter of 6.11 ± 0.02 mm and 8.30 ± 2.82 mm, respectively. These showed that the increase in the concentration of liquid smoke is directly proportional

to the diameter of the inhibition zone. Distilled water as the negative solvent control did not provide an inhibition zone, while the positive control Tetracycline with a concentration of 200 µg gave an inhibition zone diameter of 14.38 ± 0.64 mm and 15.71 ± 1.32 mm for the gram-positive *S. aureus* and gram-negative *E. coli*, respectively. The optimum concentration of liquid smoke that effectively inhibited the growth of *S. aureus* and *E. coli,* respectively, was at 40% and 40% with inhibition zone diameter of 17.93 ± 1.41 mm and 14.45 ± 1.21 mm in grade one, 40% and 40% with diameter of 19.98 ± 3.11 mm and 14.21 ± 1.03 mm in grade two, and 20% and 20% with diameter of 14.00 ± 2.25 mm and 14.08 ± 3.08 mm in grade three (Table I).

Table I: Antibacterial activity

Sample	Concentration (%)	Diameter zone inhibition (mm)	
		Staphylococcus aureus	*Escherichia coli*
Grade one	100	23.46 ± 1.05	20.48 ± 2.08
	80	21.65 ± 0.32	19.31 ± 1.84
	60	20.83 ± 0.63	17.40 ± 0.67
	40	17.93 ± 1.41	14.45 ± 1.21
	20	12.96 ± 2.57	12.68 ± 1.17
	15	9.20 ± 1.57	10.81 ± 1.37
	10	7.65 ± 1.27	8.88 ± 0.50
	5	-	-
Grade two	100	24.00 ± 0.98	16.25 ± 1.38
	80	22.90 ± 1.43	15.28 ± 1.41
	60	21.33 ± 0.68	14.78 ± 1.12
	40	19.98 ± 3.11	14.21 ± 1.03
	20	10.88 ± 1.31	11.55 ± 0.47
	15	9.61 ± 0.68	9.9 ± 0.55
	10	7.87 ± 1.44	8.01 ± 1.02
	5	-	6.11 ± 0.02
Grade three	100	23.83 ± 0.85	24.35 ± 0.54
	80	22.61 ± 2.03	22.73 ± 1.07
	60	21.66 ± 2.06	21.93 ± 1.58
	40	18.53 ± 1.94	18.56 ± 0.79
	20	14.00 ± 2.25	14.08 ± 3.08
	15	9.18 ± 1.20	9.36 ± 2.62
	10	7.40 ± 0.65	8.80 ± 1.94
	5	6.42 ± 0.10	8.30 ± 2.82
Positive control (Tetracycline)	200 µg	14.38 ± 0.64	15.71 ± 1.32
Negative control (Distilled water)	-	-	-

The total levels of flavonoids obtained in grades three, two, and one were 0.2741, 0.0174, and 0.0041 mg QE/mL samples, respectively. The sequence of total flavonoid content is grade three > grade two > grade one, with the lowest content in grade one (Table II).

Table II: Total flavonoid content

Sample	Concentration (% v/v)	Total flavonoids content (mg QE/ mL sample)	Mean total flavonoids content (mg QE/ mL sample)
Grade one	100	0.0041 0.0041 0.0041	0.0041
Grade two	100	0.0174 0.0173 0.0174	0.0174
Grade three	25	0.2741 0.2740 0.2740	0.2741

The total flavonoid content data was put through normality, and the findings showed a significance level of $p < 0.05$, indicating that the data is not normally distributed. The Kruskall-Wallis test was used to continue analysing the data, and a significance value of 0.023 was found for the total flavonoid content at the 0.05 level of significance. Therefore, it is clear that the total flavonoid content in grades one and two (both of which have a concentration of 100%) is vastly different from that in grade three (25%).

Based on Table III, the analysis of the inhibitor of free radicals in various grades of liquid smoke and quercetin as a comparison showed that increasing concentration will increase DPPH scavenging activity and more hydrogen atoms are paired with electrons in DPPH free radicals, which decreases the absorption (Mirzadeh, Arianejad & Khedmat, 2020).

Table III: Percent inhibitor

Sample	Concentration (µg/ mL)	Inhibitor (%)
Grade one	0	0
	40	12.24
	50	17.35
	60	21.69
	70	24.20
Grade two	0	0
	40	15.23
	50	19.59
	60	24.68
	70	29.27
Grade three	0	0
	40	10.90
	50	16.13
	60	22.66
	70	27.22
Positive control (Quercetin)	0	0
	2	9.57
	4	17.76
	6	27.18
	8	35.21

The smaller the IC_{50} value, the stronger the antioxidant activity of the compound. A compound is considered a very strong antioxidant when the IC_{50} value is less than 50 µg/ml, strong at 50-100 µg/ml, moderate at 101-150 µg/ml, and weak at 151-200 µg/ml. The IC_{50} values obtained for grade one and grade two liquid showed moderate antioxidant activity, while grade three had a strong antioxidant activity (Table IV).

Based on the statistical analysis of the antioxidant activity of coconut shell liquid smoke of grades one, two, and three, the normality test yielded a significance value of $p < 0.05$, indicating that the data do not follow a normal distribution. Data testing proceeded with the Kruskall-Wallis test, and a significance value for antioxidant activity was obtained of 0.000 when $p < 0.05$. Therefore, it is safe to say that the antioxidant activity of Grade one, Grade two, and Grade three liquid coconut shell smoke differs significantly across concentrations and when compared to quercetin.

Table IV: IC_{50} value

Sample	IC_{50} value (µg/mL)
Grade 1	142.82
Grade 2	121.64
Grade 3	90.80
Quercetin	11.28

Discussion

The total flavonoid levels decreased in grade two and one liquid smoke that has undergone distillation and redistillation processes. The boiling points of flavonoid compounds range from 134°C - 178°C, and during the distillation and redistillation processes, some of the flavonoid compounds with higher boiling points above the distillation and redistillation temperatures did not evaporate, hence retained (Wijayanti *et al*., 2020). In this study, the temperature used during the distillation process was 150°C, which is the optimum temperature to obtain grade two liquid smoke, while the redistillation process was done at 125°C to meet the optimum temperature to obtain grade one liquid smoke (Maulina & Sinaga, 2020). This is different from the results of the research conducted by Adinda *et al*. (2023), where a temperature of 190°C was reached to eliminate tar in liquid smoke.

In addition, heating at 75°C could have damaged the flavonoid compounds, thus allowing a decrease in total flavonoid levels in grade one and two liquid smoke. The highest total flavonoid content was found in grade three. This is consistent with the results of the total phenolic content in grade three, as flavonoids are part of the phenolic compounds due to the presence of -OH groups. Greater levels of flavonoid compounds in the sample give higher phenolic levels (Suryanto *et al*., 2020; Suryani *et al*., 2022).

The antioxidant activity in the test sample was lower when compared to quercetin - the positive control (11.28 µg/ml). This might be due to the pure quercetin used despite the presence of several secondary metabolites, such as phenolic compounds, flavonoids, and tannins, which have antioxidant properties, and there might also be some other compounds that could

interfere with the antioxidant activity in the test samples. The antioxidant activity of flavonoids, phenolics, and tannins is due to the presence of –OH groups attached to the aromatic carbons, which are excellent at capturing free radicals (Budaraga & Putra, 2021). The more the number of hydroxyl groups in the phenolic and flavonoid compounds, the greater the antioxidant activity.

When assessing the structure and the number of hydroxyl groups, the order of antioxidant strength from the highest to the lowest is tannins > flavonoids> phenolics. Phenolic compounds can donate hydrogen atoms; therefore, the DPPH radicals can be reduced to a more stable form (Budaraga & Putra, 2021; Suryani *et al*., 2023). Further research is recommended to carry out toxicity tests to determine the safety of using coconut shell liquid smoke.

Antibacterial activity testing is when the concentration can provide a barrier between 14 mm and 16 mm. Grade one and grade two liquid smoke, which is effective in inhibiting the growth of S. *aureus* and E. *coli* bacteria, is at a concentration of 40-100%. while in grade three, the concentration is 20-100%. This is because liquid smoke contains phenol and acid compounds with strong antimicrobial properties (Zuraida *et al*., 2009; Soares *et al*., 2016). According to Zuraida *et al*. (2009), coconut shell liquid smoke has antimicrobial activity against *Pseudomonas aeruginosa* which can extend the shelf life of fish balls for 16 hours.

Conclusion

Coconut shell liquid smoke has antibacterial and antioxidant activity and contains several flavonoids. The flavonoid content of grade one, two, and three liquid smoke increased, and the strongest IC_{50} value in grade three was 90.80 µg/ml. While there was no significant difference in the antibacterial activity of various grades, they had strong antibacterial activity.

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