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Original Article

The component analysis of liquid smoke from rice hulls and its toxicity test on baby hamster kidney cells

[Análisis de componentes del humo líquido de las cáscaras de arroz y su prueba de toxicidad en células renales de crías de hámster]

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Abstract

Context: The pyrolysis product of rice hulls (*Oryza sativa*) has been used for the natural preservation of food and shows the potential to act as an antioxidant and anti-inflammatory product. However, the safety of this treatment has still not been studied.

Aims: To analyse liquid smoke derived from rice hulls (*Oryza sativa*) components, its physical characteristics including acidity, density, and its toxicity on baby hamster kidney (BHK) cells.

Methods: The physical characteristics of acidity and density were analysed using a digital pH meter and gravimetry. The components of liquid smoke from rice hulls were analysed using gas chromatograph mass spectrometry. Eight concentrations (1, 2.5, 5, 7.5, 10, 12.5, 15 and 17.5%) of liquid smoke from rice hulls were used and immersed in media culture for 24 hours. Then, the MTT assay method was used to investigate the cytotoxic effect on BHK cells. Optic formazan density was indicated from the number of living cells.

Results: The acidity of liquid smoke from rice hulls was 2.296 and the density is 1.0102 g/mL. The components analysed were 2-methoxyphenol (13.45%), mequinol (13.45%) and phenol (10.52%). The 10% concentration showed the highest number of living cells at 49.33%, while liquid smoke rice hulls had 12.5% of cells still alive (p=0.000), followed by the concentration of 7.5% (p=0.000), concentration of 5% (p=0.000) and liquid smoke rice hulls at 1% (p=0.000).

Conclusions: Liquid smoke from rice hulls (*Oryza sativa*) showed that it's potential of toxicity may be related its high acidity, components and the concentration.

Keywords: component analysis; liquid smoke rice hulls; *Orzya sativa*; physical characteristics; toxicity test.

Resumen

Contexto: El producto de pirólisis de la cáscara de arroz (*Oryza sativa*) se ha utilizado para la conservación natural de alimentos y muestra el potencial de actuar como un producto antioxidante y antiinflamatorio. Sin embargo, aún no se ha estudiado la seguridad de este tratamiento.

Objetivos: Analizar el humo líquido derivado de los componentes de la cáscara de arroz (*Oryza sativa*), sus características físicas, incluida la acidez, la densidad y su toxicidad en las células de riñón de cría de hámster (BHK).

Métodos: Las características físicas de acidez y densidad se analizaron mediante un pHmetro digital y gravimetría. Los componentes del humo líquido de las cáscaras de arroz se analizaron mediante espectrometría de masas con cromatógrafo de gases. Se utilizaron ocho concentraciones (1; 2,5; 5; 7,5; 10; 12,5; 15 y 17,5%) de humo líquido de cáscaras de arroz en medio de cultivo durante 24 horas. Luego, se utilizó el ensayo MTT para investigar el efecto citotóxico sobre las células BHK. La densidad óptica del formazán se indicó a partir del número de células vivas.

Resultados: La acidez del humo líquido de las cáscaras de arroz fue de 2,296 y la densidad de 1,0102 g/mL. Los componentes analizados fueron 2-metoxifenol (13,45%), mequinol (13,45%) y fenol (10,52%). La concentración del 10% mostró el mayor número de células vivas con 49,33%, seguido del humo líquido de cáscaras de arroz al 12,5% (p=0,000), 7,5% (p=0,000), 5% (p=0,000) y el humo líquido de las cáscaras de arroz 1% (p=0,000).

Conclusiones: El humo líquido de las cáscaras de arroz (*Oryza sativa*) mostró que su potencial de toxicidad puede estar relacionado con su alta acidez, componentes y concentración.

Palabras Clave: análisis de componentes; arroz cáscaras; características físicas; humo líquido; *Orzya sativa*; prueba de toxicidad.

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INTRODUCTION

Rice (*Oryza sativa*) is a staple food in Indonesia (Panuju et al., 2013). Rice husk is a waste product that is created from rice processing, and it is estimated to produce 12.76 × 10^6 tons of waste each year from 22 provinces in Indonesia (Anshar et al., 2014). The main composition of rice hulls consists of cellulose, hemicellulose and lignin. These ingredients will produce liquid smoke in the pyrolysis process (Risfaheri et al., 2018). The chemical components of liquid smoke consist of phenol, guaiacol and 2-methoxy-5-methylphenol (2-EMP) (Surboyo et al., 2019a). Because of these components, liquid smoke from rice hulls has been shown to have antioxidant properties due to its ability to bind to reactive oxygen species (ROS) (Kim et al., 2011). It is also an anti-inflammatory agent because of its ability to inhibit pro-inflammatory cytokines (Yang et al., 2012a) and an antidiabetic agent due to its ability to reduce blood glucose levels (Yang et al., 2012b). These properties are necessary for diabetic patients because patients with diabetes are more susceptible to developing oral mucosa ulcerations, such as traumatic ulcers and recurrent aphthous stomatitis (Rohani, 2019). The oral ulcerations of diabetic patients have difficulty healing because the hyperglycaemia causes changes in the immune responsiveness (Tripathi and Tripathi, 2015). Other types of liquid smoke produced from coconut shells showed the potential to stimulate the healing of oral ulcerations in diabetics by increasing ulcer healing (Surboyo et al., 2019b), increasing the number of collagens (Surboyo et al., 2017) and decreasing pro-inflammatory cytokines (Surboyo, et al., 2019a). Based these effects, liquid smoke from rice hulls has the potential to be developed as a medicine because it has anti-inflammatory and antioxidant properties.

The possible development of liquid smoke from rice hulls into medicine is very promising. The production levels of rice plants in the world are very high, and raw materials are easily obtained in order to process them into medicine to improve health status.

One of the requirements for a natural product to become a drug is that must be non-toxic, which can be proven through toxicity testing. Toxicity tests occur on an in *vitro* and *in vivo* level. Toxicity tests need to be carried out for any drug product that will be marketed. This initial test is very important pharmacologically and toxicologically because it will be used to determine the dose, the time span of administration and its application (Parasuraman, 2011). On the *in vitro* level, the effects are observed in the cell culture, and on the *in vivo* level, the acute toxicity test needs to be performed. The acute toxicity test is part of the preclinical tests designed to measure the toxic effect of a compound on a natural product (Valle, 2018). An acute toxicity test must be performed after the *in vitro* cytotoxicity test (Erhirhie et al., 2018).

The paradigm that develops in society today is that medicines derived from natural ingredients are harmless and have no side effects. This assumption is not true. Each ingredient has the potential to be toxic, depending on how high of a dose makes its way into the body (Zhou et al., 2019). Therefore, traditional medicine needs to be ensured of its safety and an acute toxicity test is the main test that must be conducted (Ekor, 2014). Although liquid smoke derived from rice hulls has been proven to have antioxidant, anti-inflammatory and antidiabetic properties, there has been no research on the toxicity of liquid smoke from rice hulls. The toxicity test is fundamental for the development of new drugs (Dorato and Buckley, 2007). Therefore, this study aims to analyse the components, physical characteristics including acidity, density, and toxicity of liquid smoke from rice hulls (*Oryza sativa*).

One of the goals of this research is to provide information about other potential uses of liquid smoke from rice hulls. Besides functioning as a natural food preservative, it can also be used as a new drug candidate for treating of oral ulcerations. As a result, the processing of rice waste in the form of rice husks can be utilised as much as possible to produce products that have a therapeutic effect.

MATERIAL AND METHODS

This research was conducted with a post-test only control group designed in Pusat Veterinaria Farma (PUSVETMA), Surabaya (ISO 45001: 2018). Ethical approval was obtained from the Ethical Clearance Section of the Health Experiment Committee, Faculty of Dental Medicine, Universitas Airlangga with registration no. 285/HRECC. FODM/X/2018. The liquid smoke from rice hulls used in this study was made by Forest Products Research and Development Center Laboratory, Bogor.

Chemicals

MTT reagent (thiazole blue tetrazolium, M2128, Sigma Aldrich, USA); phosphate buffer saline (PBS, Bioenno Tech, California, USA); dimethyl sulfoxide (DMSO) (AnalaR, BDH limited, Poole, England) and sterile water (API IPHA, IPHA laboratory, Bandung, Indonesia).

Production of liquid smoke from rice hulls

The rice hulls that produced the liquid smoke used in this study were obtained from a local farmer in Malang, Indonesia (7°58'46.92"S, 112° 37'49.44"E).

A quantity of 1860 g of liquid smoke from rice hulls (*Oryza sativa*) was acquired from the pyrolysis process. Pyrolysis is a condensation process that occurs when steam from rice hulls combusts at temperatures around 400°C without oxygen (Surboyo et al., 2019b).

Characteristics of liquid smoke from rice hulls

Acidity was analysed using a digital pH meter (Mettler Toledo S220, Malaysia) and a density test was conducted using a pycnometer (Brand, Germany).

Component analysis of liquid smoke from rice hulls

The components were determined by using a GC-MS model 6890N (Agilent Technologies, Inc., Santa Clara, CA), then by using a mass spectrometer detector 5975B and DB-5MS UI column (Agilent Technologies, stationary phase; polyethylene glycol, 30 m × 0.25 mm; i.d. 0.25 µm). The injector's temperature was 250°C, as previously described (Surboyo et al., 2019a). Helium was used as a carrier gas at a constant flow rate of 1 mL/min. The initial oven temperature of the column was raised from 50° to 300°C at a rate of 20°C/min then maintained for 4 minutes at 300°C. The mass spectrometer detector used a temperature of 270°C with conditions of a capillary direct interface. The MS Source temperature was 230°C and MS Quad temperature was 150°C. Column flow was 1 mL/min, the linear velocity was 36.445 cm/sec, and the total flow was 104 mL/min. The comparison between mass spectra and retention time index of authentic reference compounds was stored in the NIST14 mass spectral data library and was used for the identification of the individual constituents.

Cytotoxicity test

The baby hamster kidney (BHK-21) cells were cultivated in a roux bottle and harvested with trypsin versene solution. The BHK cells were each put into 96 microplate wells filled with D-MEM media, which contained 10% fetal bovine serum albumin and was incubated at 37°C for 24 hours (Meilena et al., 2018).

The liquid smoke from rice hulls was prepared in eight different concentrations that were diluted by sterile water. Liquid smoke from rice hulls was added into 96 microplate wells with concentrations of 1, 2.5, 5, 7.5, 10, 12.5, 15 and 17.5%. The concentration microplate wells were incubated for 24 hours and after that time period, a solution containing MTT reagent in PBS was added to each well. The wells were then re-incubated for 6 hours. Furthermore, DMSO was added to each well and shaken with a plate shaker. The wells were read using an Elisa Reader with a 620 wavelength. The calculation results were said to be non-toxic if ≥60% of the BHK cells lived (Paramitha and Tukiran, 2018). The percentage of the live cells was calculated by using the following formula [1] (Paramitha and Tukiran, 2018):

$$\%\ Live\ cell = \frac{(\text{test group} + \text{test media}) \times 100\%}{\text{cell} + \text{media}} \quad [9]$$

Where:

% live cells: value of Optical Density (OD) for each sample; test group: value of Optical Density (OD) after each test; media: value of Optical Density (OD) on the average of each media control; cell: value of Optical Density (OD) on average of cell control.

Statistical analysis

The toxicity data of liquid smoke from rice hulls was presented as the mean standard values deviation for each concentration. The differences of live cells in each concentration of liquid smoke from rice hulls were then analyzed using analysis of variance (one-way analysis of variance). This was followed by a post-hoc analysis in which $p<0.05$ was considered to be a significant difference. The SPSS version 24 (IBM SPSS Statistic 24 for Mac, USA) was used for the analysis.

RESULTS

Production of liquid smoke from rice hulls

The pyrolysis process of 860 g of rice hulls was carried out at a final temperature of 400°C (heating rate 3.33°C/min) for 4 hours and 20 minutes. The water content of the rice hulls was about 7.14%. The product of the pyrolysis process was 38.49% liquid smoke, 47.34% charcoal and 5.10% heavy tar (Table 1). Then, the liquid smoke from the pyrolysis process was distilled at 120°C and the final yield of liquid smoke from the pyrolysis process was 87%.

Characteristics of liquid smoke from rice hulls

The liquid smoke that was obtained from rice hulls through the pyrolysis process has a yellow color. The acidity of the liquid smoke from rice hulls was 2.296 and the density was 1.0102 g/mL.

Component analysis of liquid smoke from rice hulls

The GC-MS analysis showed that 28 components were identified in the liquid smoke derived from rice hulls (Fig. 1). The major components analysed were 2-methoxyphenol (guaiacol) (13.45%), mequinol (13.45%) and phenol (10.52%). Other components included 6-octadecenoic acid, oleic acid, 9-octadecenoic acid (7.81%) and 2-methoxy-5-methylphenol (2-EMP) (4.88%) (Table 2).

Cytotoxicity test of liquid smoke from rice hulls

The liquid smoke from rice hulls concentration of 10% (49.33 ± 2.15) has the highest amount of living cells compared to the 12.5% (43.59 ± 1.14), 7.5% (37.71 ± 3.15), 5% and 1% concentrations (29.85 ± 0.72) ($p=0.000$). The living cell count was not different in the concentration of 10% (49.33 ± 2.15) compared to the 2.5% (29.66 ± 0.58) ($p=0.854$), 15% (39.48 ± 1.38) ($p=0.086$) and 17.5% concentrations (43.56 ± 1.05) ($p=0.967$) (Fig. 2).

Table 1. Product of pyrolysis process of rice hull.

No	Product	Weight (g)	(%)
1	Liquid smoke	309	38.49
2	Heavy tar	41	5.10
3	Charcoal	380	47.34

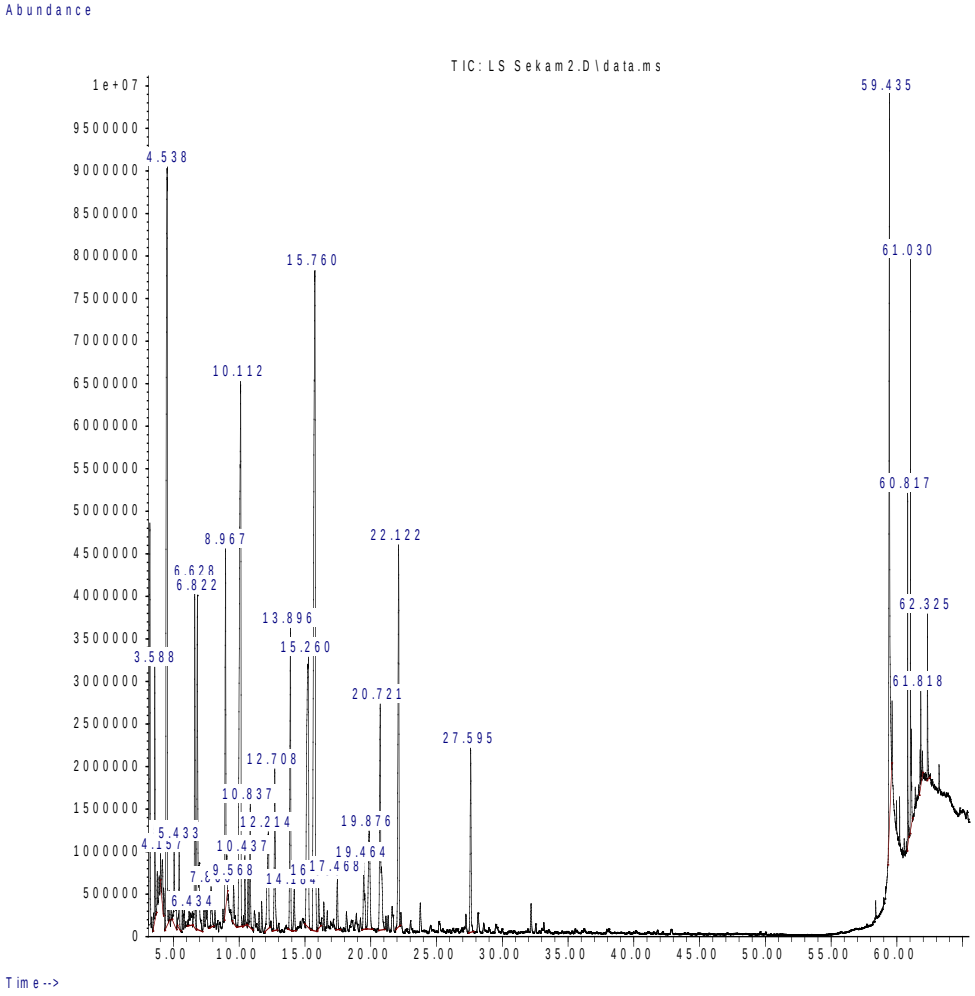

Figure 1. GC-MS component spectra of liquid smoke from rice hulls.

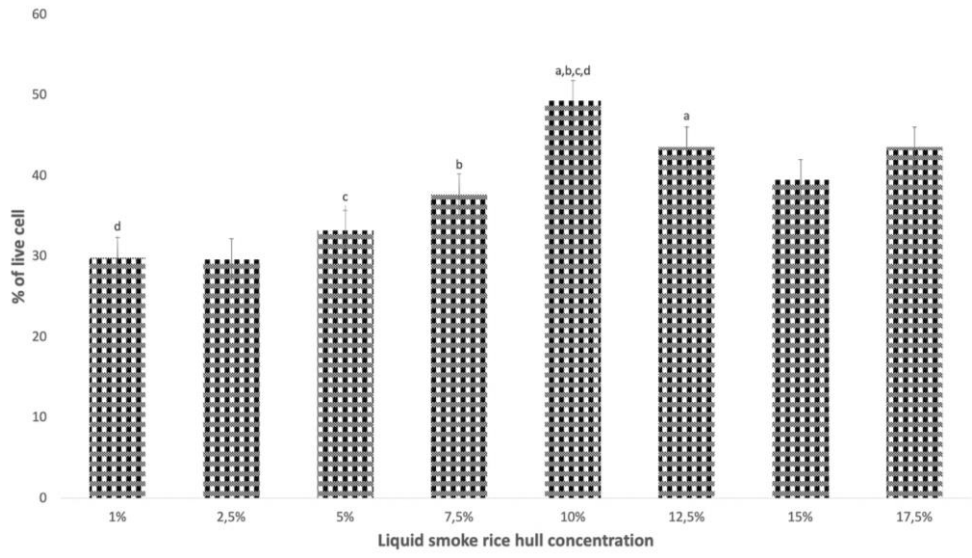

Figure 2. Percentage of BHK-21 in MTT assay.

The same character on the top of the bar is indicated differently for each group with one-way ANOVA and LSD tests ($p<0.05$).

Table 2. Component analysis of liquid smoke rice hull by GC-MS.

No	Component	RT	Peak area (%)
1	Cyclopentanone	3.588	1.76
2	1-Methylcycloheptene	7.860	0.54
3	5-Methyl-2-furancarboxaldehyde	8.967	3.91
4	Phenol	10.112	10.52
5	2,5-Dihydro-3,5-dimethyl-2-furanone	10.837	1.56
6	2,3-Dimethyl-2-cyclopenten-1-one	12.708	2.09
7	3-Methyl-1,2-cyclopentanedione	12.214	2.14
8	3-Methyl-1,2-cyclopentanedione	12.214	2.14
9	2-Hydroxy-3-methyl-2-cyclopenten-1-one	12.214	2.14
10	2-Methyl-phenol	13.896	3.55
11	Creosol	15.260	6.72
12	2-Methoxy-phenol	15.760	13.46
13	Mequinol	15.760	13.46
14	2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-	17.468	0.63
15	2,4-Dimethyl-phenol	19.464	1.10
16	3,5-Dimethyl-phenol	19.464	1.10
17	4-Ethyl-2-methoxy-phenol	19.876	2.18
18	4-Ethyl-phenol	20.721	3.90
19	2-Methoxy-5-methylphenol	22.122	4.88
20	6-Octadecenoic acid	59.435	7.81
21	Oleic acid	59.435	7.81
22	9-Octadecenoic acid	59.435	7.81
23	Glycidyl oleate	61.030	2.63
24	(Z)- 9-Octadecenal	61.030	2.63
25	2-Octyl-cyclopropaneoctanal	61.030	2.63
26	(Z)-9-Octadecenal	60.817	1.33
27	7-Pentadecyne	61.818	0.66
28	trans-13-Docosenamide	62.325	0.89

RT: retention time

DISCUSSION

Pyrolysis products mostly contain phenolic compounds such as phenol, guaiacol and 2-methoxy-5-methylphenol (EMP) (Isahak et al., 2012). In this study, the liquid smoke from rice hulls contains 2-methoxyphenol (guaiacol) (13.45%), mequinol (13.45%), phenol (10.52%) and 2-methoxy-5-methylphenol (2-EMP) (4.88%). The acidity was 2.296 and the density was 1.0102 g/mL. Other research found that liquid smoke from rice hulls produced 2-methoxyphenol (guaiacol) (3.75%) and phenol (10.99%) (Kim et al., 2011). The different components are determined by raw materials including water content (Stefanidis et al., 2014), final temperature during pyrolysis (Budara-

ga et al., 2016), oxygen admission into reactor (Peterson et al., 2020) and pyrolysis reactor design (Kan et al., 2016).

One of the pyrolysis products that is widely used to preserve food is liquid smoke (Rozum, 2014). Many studies have reported that liquid smoke has better value as a food preservative compared to synthetic preservatives (Achmadi et al., 2013). Liquid smoke is usually used as a preservative for poultry, fish, meat and processed meats. Liquid smoke is able to maintain the meat's protein (Yusnaini et al., 2012) and fat content (Swastawati et al., 2012). Liquid smoke can not only be applied by itself, but it can also be developed with other ingredients such as chitosan. Application of a liquid smoke concentration of 1.5% and chitosan 2.5% can make tofu and meatballs last for three days and provides better colour, aroma and texture (Purba et al., 2014).

Research on the effects of liquid smoke toxicity has not been widely reported. Research on the toxicity of liquid smoke derived from coconut shells was conducted by Budijanto et al. (2008), who reported that liquid smoke did not show toxic effects on experimental animals (Budijanto et al., 2008). Other studies have shown that liquid smoke from coconut shells has an analgesic effect (Surboyo et al., 2012). Surboyo et al. (2019a) also proved that liquid smoke can be used as an alternative medicine for oral ulcerations because it can reduce the inflammation caused by inhibiting macrophages (Ernawati et al., 2020; Surboyo et al., 2020), NF-κB activation, reduce TNF-α production (Surboyo et al., 2019a) and increase the amount of fibroblasts (Ayuningtyas et al., 2020) and collagen in a dose of 1 µL (Surboyo et al., 2017). Tarawan et al. (2017) also confirmed that liquid smoke from coconut shells has the benefit of accelerating the healing of skin burns by increasing the fibroblast formations.

Research on the medicinal potential of liquid smoke derived from rice husks has not been widely reported. The therapeutic effects of liquid smoke that have been observed are anti-inflammatory (Kim et al., 2011) and antidiabetic effects (Yang et al., 2012a). Liquid smoke derived from rice hulls is able to inhibit the release of tumour necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) in the inflammatory model induced with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Yang et al., 2012a). Another asset of liquid smoke from rice hulls is its ability to reduce blood glucose and increase insulin levels in serum and liver glycogen levels (Yang et al., 2012b). The results of this research are contrary to the research explained above. Toxicity tests on the in-vitro level shows that liquid smoke from rice hulls has toxic properties, meaning the BHK cells that were used as test material have a viability of less than 60%. The highest living cell count was observed in liquid smoke from rice hulls at 10% (49.23%). The major components in liquid smoke from rice hulls are 2-methoxyphenol (guaiacol) (13.45%), mequinol (13.45%) and phenol (10.52%). Most of the pyrolysis products are dominated by the phenolic compound (Fagbemi et al., 2001). Because of its applicability to human health, the phenols toxicity to biological systems, especially cell cultures, has been the subject of current investigation (Wright and Shadnia, 2008). The toxicity of phenolic compounds is measured by qualitative structure-activity relationship (QSAR) descriptors, namely log *P*, p*K*a and OH bond dissociation enthalpy (BDE) (Reis et al., 2007). Another factor that plays a role in the toxicity of liquid smoke from rice hulls is its high acidity (Yang et al., 2012a). This approach explains that the phenoxy radical is disruptive to the cell and factors increasing its production rate that embellish the toxicity (Campos et al., 2009). The phenol mechanism at the cellular level has the highest logged *P* values, which may accumulate in the cellular membrane bilayer and not be transported into the cell. At some point, this could lead to inhibition of cellular transport and toxicity will be induced (Wright and Shadnia 2008). A wide range of literature shows that the toxicity of phenolic compounds is related to polyphenol-mediated cell death and this is attributed to the reactive oxygens species (ROS) production induced by polyphenols (Coccia et al., 2016).

Another component in liquid smoke from rice hulls is guaiacol. The guaiacol proved to be toxic

in doses of 12.5 – 200 µL. Based on Martinez Enriquez et al. (2009), this concentration will manifest as haematuria, gastric distention, and hepatic necrosis. These toxicity manifestations were distinct in 100% of animals; pulmonary oedema appeared in 87.5% and unilateral or bilateral blindness appeared in 62.5% of mice (Martinez Enriquez et al., 2009). On the cellular level, guaiacol induced sister chromatid exchange (SCE) and morphological transformation. Guaiacol is also very toxic and mutagenic to humans, but it is non-carcinogenic (Jansson et al., 1988).

Mequinol is the other dominant component in liquid smoke from rice hulls. Mequinol is a powerful melano-cytotoxic agent, which is metabolised intracellularly by tyrosinase to form catechol and then o-quinone, which is very cytotoxic. Mequinol 2% is often used to treat hyperpigmentation because it is able to inhibit melanogenesis by binding to the tyrosinase enzyme, or by oxidizing radicals that damage the lipoprotein membrane of melanocytes (Couteau and Coiffard, 2016). Liquid smoke derived from rice hulls was proven to have lower toxicity in the acute toxicity test and no hepatotoxic (Arundina et al., 2020). The other forms of liquid smoke, which have similar components to liquid smoke derived from rice hulls, have proven their ability to stimulate fibroblasts by increasing the response of macrophages (Ernawati et al., 2020). The underlying reason for this is that liquid smoke has been tested on fibroblast cells such as BHK cells rather than skin cells such as keratinocytes.

This research concludes that the liquid smoke from rice hulls showed toxicity even in the lowest concentration. The result of this research contributes to the development of processed waste products such as rice hulls to produce liquid smoke that can be used as an alternative medicine. The limitation of this study is that it only focused on the *in vitro* test of toxicity. Further observation is necessary to confirm the toxicity of liquid smoke derived from rice hulls through experiments with animals using models of acute and chronic exposure.

CONCLUSIONS

The liquid smoke from rice hulls *(Oryza sativa)* showed potential for toxicity. The 10% concentration of liquid smoke from rice hulls showed that only 49.23% BHK cells were living. The toxicity may be caused by its high acidity, component and the concentration.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Arundina I	Diyatri I	Surboyo MDC
Concepts or ideas		x	x
Design	x		x
Definition of intellectual content	x	x	
Literature search		x	x
Experimental studies	x	x	
Data acquisition	x	x	
Data analysis			x
Statistical analysis		x	
Manuscript preparation		x	x
Manuscript editing	x		x
Manuscript review	x	x	x

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