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Research Article

ANTIBACTERIAL, *IN VITRO* CYTOTOXIC, AND ANTIOXIDANT ACTIVITIES OF ELECTROLYZED OXIDIZING/REDUCING WATER

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ABSTRACT

Objective: Electrolyzed oxidizing/reducing water is popular as health beneficial water in Indonesia. In this study, we examined the level of antibacterial, anticancer, and antioxidant activity of the electrolyzed water.

Methods: The efficacy of electrolyzed water produced by Enagic® at six level pH (2.5, 6.0, 7.0, 8.5, 9.0, and 9.5) was investigated. Antibacterial activity was evaluated using a macrodilution method. The anticancer activity was performed against human breast cancer (T47D) cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Moreover, the antioxidant activity was determined using antioxidant model, 2,2-diphenyl-1picrylhydrazyl radical scavenging activity.

Results: The results show that electrolyzed water exhibited antibacterial activity against *Propionibacterium acnes* and *Staphylococcus epidermidis*. Among six level pH, electrolyzed water at pH 2.5 showed the highest antibacterial activity. The *in vitro* cytotoxic activity of electrolyzed water showed potential moderate cytotoxicity. The activity tends to be higher in alkaline electrolyzed water. However, the electrolyzed water showed free radical scavenging activity.

Conclusion: Electrolyzed water that marked in Indonesia has some potential health benefits. The activity is dependent on pH.

Keywords: Antibacterial activity, Antioxidant, Anticancer, Electrolyzed water, A healthy water.

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INTRODUCTION

Today, people are very concerned about consuming drinks that have some health beneficial effects, including the Indonesian community. The functional water can be obtained by irradiation of light, ultrasonication, treatment with a magnetic field, and electrolysis. Among these methods, electrolyzed water has been widely used for functional water.

Electrolyzed oxidizing/reducing water that has been developed in Japan is one option that has some health beneficial effects. This water is made through an ionization process by flowing electric current, which ultimately produces alkaline and acidic water. The alkaline water or electrolyzed reduced water (ERW) is rich in hydroxyl ions (OH^-). On the other hand, the acidic water or electrolyzed oxidized water (EOW) is rich in hydrogen ions (H^+). Its water has advantages because it has smaller molecular cluster compared to its raw material, making it more simply absorbed by the body. Electrolyzed water is made by passing the aqueous solution of NaCl or KCl solution on an electrolyzing machine. This machine consists of anodes and cathodes separated by a special diaphragm which separates the alkaline fraction at the cathode and the acid fraction at the anode [1]. The negatively charged particles and ions (Cl^- and OH^-) move toward the anode where electrons are released, and hydrochloric acid (HCl), hypochlorous acid (HOCl), hypochlorite ion (^-OCl), oxygen gas (O_2), and chlorine gas (Cl_2) are produced. However, positively charged ions and particles (H^+ and Na^+) move toward the cathode, where they obtain electrons, resulting in the generation of sodium hydroxide (NaOH) and hydrogen gas [2].

Previous studies have shown that EOW effectively kills *Escherichia coli, Salmonella enteritidis*, and *Listeria monocytogenes* [3,4]. The electrolyzed water shows bactericidal, virucidal, and fungicidal effects in numerous areas such as medicine and dentistry as well as on fruits, vegetables, and agriculture [2,5]. ERW is known to suppress oxidative stress associated with the disease, such as diabetes [6], cancer [7-10], arteriosclerosis, and neurodegenerative diseases [11]. On the other hand, neutral electrolyzed water has been shown to be effective for the inactivation of human norovirus [12].

One of the most popular electrolyzed water in Indonesia is produced by Enagic. This water is available on the market at various pH values. The pH is 2.5, 6.0, 7.0, 8.5, 9.0, and 9.5 with different uses. This research was conducted to determine the electrolyzed water potential for health marked in Indonesia by conducting antibacterial, anticancer, and antioxidant activity tests.

MATERIALS AND METHODS

Materials

Water electrolyzed used for this study was purchased from local producers in Indonesia. The water was generated using a water ionization machine made by the Japanese company Enagic. Six different pH of electrolyzed waters were used in the present research. Human breast cancer T47D cells lines were provided from cancer and stem cells research center, Universitas Muhammadiyah Purwokerto. *Propionibacterium acnes* and *Staphylococcus epidermidis* were obtained from the American type culture collection (ATCC; Rockville, MD, USA) as well as the culture collection of the Laboratory Microbiology, Universitas Muhammadiyah Purwokerto. Fetal bovine serum (FBS), Dulbecco's modified eagles medium (DMEM), penicillin-streptomycin, trypsin-EDTA, and fungizone were procured from Gibco (Invitrogen, USA). Phosphate-buffered saline (PBS) 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl

(DPPH), and nutrient agar (NA) were purchased from Sigma-Aldrich. Furthermore, sodium dodecyl sulfate (SDS) and HCl were obtained from Merck.

Antibacterial activity of electrolyzed water

Agar macrodilution was adopted for measuring the antibacterial activity of the electrolyzed water. Clindamycin (20 µg/mL) was used as the positive control; however, sterile distilled water was used as a negative control. Electrolyzed waters with different pH (2.5, 6.0, 7.0, 8.5, 9.0, and 9.5) were collected from Purwokerto, Indonesia. A 1 mL bacterial inoculum (1×10^8 colony-forming unit [CFU/mL]) was added to 9 mL aliquot of each electrolyzed water sample in 15 mL sterile conical tubes (5 tubes per samples). The tubes were incubated at 35°C for 6 min. The treatment electrolyzed waters were diluted serially and plated (100 mL per plate) onto NA. Then, agar plates were incubated in an anaerobic environment at 37°C for 24 h. After incubation, the colony was counted by colony counter.

***In vitro* cytotoxic activity of electrolyzed water**

Three different pH of electrolyzed waters (2.5, 6, and 9) were used in the experiment. T47D cells are regenerated in the medium when used *in vitro* tests. T47D cells were grown in DMEM growing media containing FBS 10% (v/v) and penicillin-streptomycin 1% (v/v). The cells (10^4 cells/well) were planted into the 96 plates and incubated at 37°C for 24 h, with 5% CO_2. After incubation, the culture medium was removed and cells were washed using PBS. The cells were treated by electrolyzed water and then incubated again for 24 h. The concentration of each electrolyzed water was 100.0, 75.0, 50.0, 25.0, and 12.5% (v/v) in DMEM. Cell viability was determined by MTT assay. The cells were added with MTT-containing medium (0.5 mg/mL) for 4 h at 37°C, with 5% CO_2. Viable cells will react with MTT to generate purple colored formazan crystals. After 4 h of incubation, the reaction was stopped with 10% SDS in 0.1 M HCl solution. Then, the plate was incubated overnight at room temperature and protected from light. At the end of incubation, the plates were shaken, and cells absorbance was measured by ELISA reader (Bio-Rad) at λ 595 nm.

Antioxidant activity of electrolyzed water

Antioxidant activity of electrolyzed water was determined using the DPPH radical scavenging assay adopted by Brand-Williams *et al.* [13] with a minor modification. Briefly, the DPPH solution 0.004% (w/v) 2 mL was added on to electrolyzed water in concentration from 6.25 to 100% (v/v). The mixture was allowed to stand at room temperature in the dark for 30 min. Then, the absorbance is read at the maximum wavelength. After incubation, the absorbance was measured at 516 nm against a blank by spectrophotometer Ultraviolet (UV) Vis (Shimadzu UV-1201). The ability to scavenge the DPPH• was determined using the formula given by Brand-Williams *et al.* [13]. The activities were compared to L-ascorbic acid [14].

RESULTS AND DISCUSSION

In this research, six different commercially available pH of electrolyzed water were evaluated by pH meter (metrohm). These pH values are shown in Table 1. It can be observed that the pH values found for samples 2-5 are in close agreement with the labeled pH. Sample 1 presented pH value above the labeled pH, whereas sample 6 presented pH value under the labeled pH.

The highest difference of pH value is in the sample with a pH label 2.5. Based on data from chemical analysis and spectroscopic data, the main components of electrolyzed acidic water are Cl_2/HClO, which is in equilibrium according to the pH of the solution [4,15]. The Cl_2 gas is susceptible to decrease due to the storage; this will cause the pH of the water to increase.

Antibacterial activity of electrolyzed water

Antibacterial activity of electrolyzed water was investigated against skin poisoning bacteria, including *P. acnes* and *S. epidermidis*. The bacterial cells have been recognized as bacteria initiating inflammation in acne. It has been claimed that electrolyzed water, especially marked in the strong acid water, can be used to treat infections caused by bacteria on the skin. Clindamycin antibiotics are used as a positive control. The results of electrolyzed water in killing *P. acnes* and *S. epidermidis* are reported in Table 2.

Unlike electrolyzed water at pH 6.0, 7.0, 8.5, 9.0, and 9.5, the results of the antibacterial activity test against *P. acnes* and *S. epidermidis* at pH 2.5 showed that there were no bacterial colonies growing (Fig. 1). This result is in line with previous studies which indicated that electrolyzed oxidizing water might be potential as a disinfectant [16] This is possible because electrolyzed water at low pH contains a high concentration of HOCl. The concentration of HOCl is known to correlate with the ability of electrolyzed water to kill bacterial cells [15]. In water with a low pH can damage the outer membrane of bacterial cells so that HOCl can enter bacterial cells more efficiently [17]. HOCl is the most active chlorine compound, apparently killing microbial cells through inhibition of glucose oxidation by chlorine sulfide-oxidizing groups of certain enzymes that are important in carbohydrate metabolism [18].

Table 1: pH measurements of electrolyzed water

Sample	Labelled pH	Found pH[a]	pH differences
1	2.5	2.76±0.04	0.36
2	6.0	6.03±0.05	0.03
3	7.0	7.01±0.02	0.01
4	8.5	8.47±0.04	0.03
5	9.0	8.99±0.02	0.01
6	9.5	9.38±0.21	0.12

[a]Mean±SD, n=3

Table 2: Viability of *P. acnes* and *S. epidermidis* after exposure to electrolyzed water measurements of electrolyzed water

Treatment	CFU/mL	
	P. acnes	*S. epidermidis*
Positive control	0±0	0±0
pH 2.5	0±0	0±0
pH 6.0	1420±148	398±69
pH 7.0	557±103	1927±661
pH 8.5	403±67	1116±117
pH 9.0	1168±175	228±21
pH 9.5	1503±122	24±3

[a]Mean±SD, n=5, *P. acnes*: *Propionibacterium acnes*, *S. epidermidis*: *Staphylococcus epidermidis*, CFU: Colony-forming unit

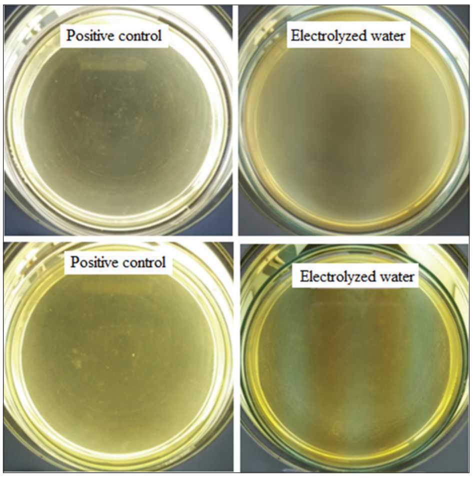

Fig. 1: Effect of electrolyzed water at pH 2.5 on the growth of *Propionibacterium acnes* (top) and *Staphylococcus epidermidis* (bottom) by agar macrodilution

Table 3: Antioxidant activity of electrolyzed water, using DPPH free radical scavenging method

Treatment	EC_{50}±SD (%)	EC_{20}±SD (%)
pH 2.5	227.4±9.7	102.5±8.0
pH 6.0	293.0±34.4	130.3±16.5
pH 7.0	607.4±28.7	219.6±13.1
pH 8.5	195.8±4.5	63.3±2.3
pH 9.0	177.4±1.6	63.2±4.0
pH 9.5	167.6±2.8	56.0±3.2
Ascorbic acid	9.3±0.4 (µg/mL)	3.4±0.3 (µg/mL)

DPPH: 2,2-diphenyl-1picrylhydrazyl, EC_{50}: 50% efficient concentration

P. acnes grew well in neutral or slightly alkaline. This result is in line with previous studies [19]. We observed that *P. acnes* was still survived even at pH 9.5 of electrolyzed water. Furthermore, *S. epidermidis* grew much better in the pH of 7.0–8.5. However, minor changes in the pH in the pH 9.0–9.5 range can decrease the number of *S. epidermidis*. *S. epidermidis* did not survive at pH 9.5 of electrolyzed water. A substantial population reduction of *S. epidermidis* at pH 9.5 (24 CFU/mL) was observed after 24 h incubation. The reduction population of *S. epidermidis* is known to correlate with the high concentration of OCl^- in electrolyzed water at pH 9.0–9.5 [20].

In vitro cytotoxic activity of electrolyzed water

The *in vitro* cytotoxic activity of electrolyzed waters with different pH was evaluated against T47D breast cancer cell. T47D cell is a continuous cell line isolated from ductal breast tumor tissue. This cell expresses the p53 protein, which has a mutation. This causes p53 cannot bind to the response of elements to DNA. Thus, the ability of p53 to regulate cell cycle is reduced and can even remove [21]. The treatment of electrolysis water samples for 24 h resulted in a decrease in percentage of T47D cell viability compared to cell control (Fig. 2). Under acidic, slightly acidic, and slightly alkaline conditions, electrolyzed waters had *in vitro* cytotoxic activity. The electrolyzed water at pH 2.5 had the lowest cytotoxicity on T47D cell lines, with an the half maximal inhibitory concentration (IC_{50}) of 59.4%, followed by electrolyzed water at pH 6 with an IC_{50} value of 47.0%, and electrolyzed water at pH 9 with an IC_{50} value of 46.6%.

Morphological alteration of T47D cell lines on exposure using electrolyzed water was demonstrated under an inverted microscope (Carl Zeiss) with a magnification of ×20 (Fig. 3). The cells showed the most noticeable effect after incubation to the electrolyzed water. At high electrolyzed water concentration (100%), a shrunken and rounder of the cells were conspicuously observed. Most of the cells showed signs of detachment from the surface of the well plates indicating cell death.

Antioxidant activity of electrolyzed water

The previous study has found that ERW exhibits reactive oxygen species (ROS)-scavenging activity. It can scavenge not only H_2O_2 and superoxide radical but also singlet oxygen and hydroxyl radical. ERW has an alkaline pH. In the current study, we actually observed that electrolyzed water can scavenge free radical species not only at alkaline but also at pH 2.5 and 6.0. The DPPH radical scavenging activity results are presented in Table 3 as compared with known antioxidant ascorbic acid.

According to observed experimental data in term of 50% efficient concentration (EC_{50}), unfortunately, we cannot categorize an antioxidant capacity of electrolyzed water regarding Blois's category [22]. A DPPH method could not detect radical-scavenging activity in electrolyzed water sensitively [23]. The intracellular ROS scavenging activity method (fluorescent stain method and immuno-spin trapping assay) and electrochemical techniques seem to be able to observe the activity specifically [23,24].

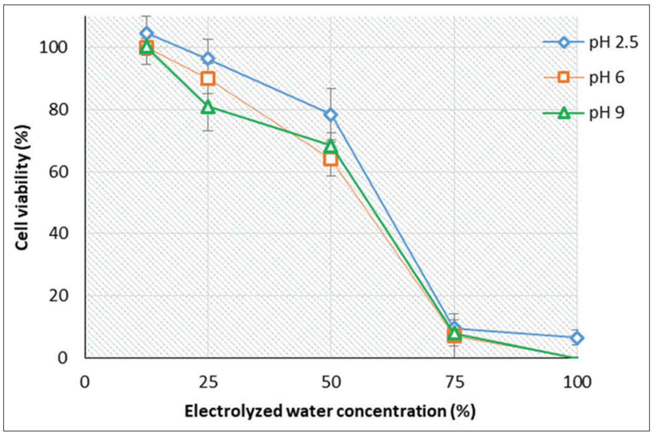

Fig. 2: *In vitro* cytotoxic activity of electrolyzed water against T47D cell lines

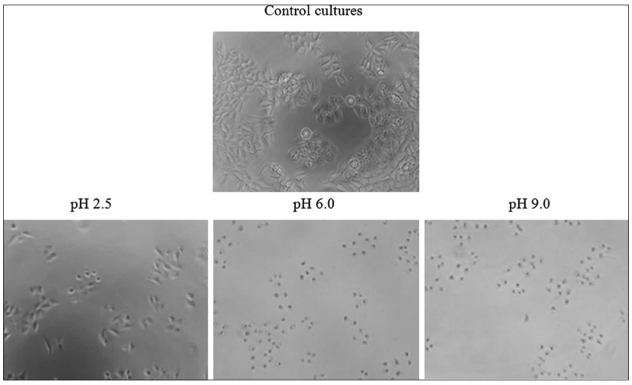

Fig. 3: Phase contrast images of T47D cells after incubation with 100% of electrolyzed waters at pH 2.5, 6.0, and 9 for 24 h (×20)

CONCLUSION

Some Indonesian people have a positive opinion about electrolyzed water marketed in Indonesia. The water has various pH with different uses. The results provide information that electrolyzed water has antibacterial, cytotoxic, and antioxidant activities. The activity is dependent on pH. The electrolyzed water at pH 2.5 showed the highest antibacterial activity. The alkaline electrolyzed water showed the highest cytotoxicity on T47D cell lines, with an IC_{50} of 46.6%. Furthermore, the electrolyzed water showed free radical scavenging activity with an EC_{50} of 167.6% at pH 9.5.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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