

Functional Properties of Oleoresin Extracted from White Pepper (*Piper nigrum* L.) Retting Waste Water

(Sifat Kefungsian Oleoresin Diekstrak daripada Air Sisa Rendaman Lada Putih (*Piper nigrum* L.))

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ABSTRACT

This study was carried out to determine the antioxidant activity, total phenolic content (TPC) and piperine in oleoresin extract of pepper retting waste water. Fresh pepper berries were soaked in distilled water (1:2 w/w) and allowed to ret at four different temperatures: 28, 35, 42 and 49°C for a total of 16 days. The retting waste water was collected on the 4th, 8th, 12th and 16th day and was then subjected to dichloromethane liquid extraction prior to analyses. The results showed that highest ($p<0.05$) oleoresin yield (271.34 ± 28.09 mg/g), antioxidant activity in DPPH ($84.58 \pm 4.93\%$), FRAP assay (49.55 ± 6.11 mM TE/g) and total phenolic content (5.64 ± 0.06 mg GAE/g) obtained at low temperature and shorter days of retting (28°C, day 4). Whilst at high temperature with longer days of retting (49°C, day 16) resulted in significantly higher ($p<0.05$) piperine content ($1.81 \pm 0.44\%$) in the waste water. Thus, the findings showed that the agro-industrial waste water produced during white pepper processing contain valuable bioactive oleoresins and other functional compounds which could be explored in details.

Keywords: Agro-industrial waste; antioxidant activity; pepper retting water; phenolic content; piperine

ABSTRAK

Kajian ini dijalankan untuk menentukan aktiviti antioksidan, jumlah kandungan fenolik (TPC) dan piperina ekstrak oleoresin daripada air sisa rendaman lada. Buah lada segar direndam dalam air suling (1:2 w/w) dan dibiarkan mereput pada empat suhu berbeza iaitu 28, 35, 42 dan 49°C selama 16 hari. Persampelan air sisa rendaman telah diambil pada hari ke 4, 8, 12 dan 16 sepanjang proses pereputan untuk analisis lanjut. Keputusan kajian menunjukkan air sisa rendaman pada suhu yang rendah dan hari rendaman yang lebih pendek (28°C, 4 hari), menunjukkan kandungan oleoresin (271.34 ± 28.09 mg/g), aktiviti antioksida tertinggi bagi ujian DPPH ($84.58 \pm 4.93\%$) dan FRAP (49.55 ± 6.11 mM TE/g) dan jumlah kandungan fenolik (5.64 ± 0.06 mg GAE/g) yang paling tinggi ($p<0.05$). Walau bagaimanapun, pada suhu tinggi dengan hari rendaman yang lebih lama (49°C, 16 hari) tetap bermanfaat kerana menunjukkan kandungan piperina yang tinggi ($1.81 \pm 0.44\%$; $p<0.05$) dalam air sisa. Oleh itu, penemuan kajian ini menunjukkan bahawa sisa agro-industri semasa penghasilan lada putih iaitu air sisa rendaman mempunyai bahan oleoresin dan sebatian aktif lain yang boleh diteroka untuk kajian lanjut.

Kata kunci: Aktiviti antioksidan; kandungan fenolik; piperina; rendaman air lada; sisa agro-industri

INTRODUCTION

The pepper (*Piper nigrum* L.) plant is where black pepper and white pepper are derived from. Compared to black pepper that only requires sun drying of the harvested pepper berries, white pepper production requires additional processes to decorticate the pepper outer pericarp (Vinod et al. 2014). The most common practice is via water retting method in which the pepper berries are immersed in water; either in river, small lakes or soaked in containers for a maximum of 8 to 15 days (Parthasarathy et al. 2007; Zachariah 2008). This is carried out in order to allow natural enzymes produced by the microflora present in the water and on the pepper berries to degrade and weaken the pepper pericarp cell wall (Akin 2010; Sivakumaar et al. 2010).

The amount of water used for the retting process is usually two to three times the weight of pepper berries and

if carried out using still water in containers, the water need to be changed every 2 to 3 days to prevent malodour due to the production of methylphenol and methylindole (Horne 2012; Steinhaus & Schieberle 2005). This large volume of water is always discarded after sufficient retting has been performed. The pepper berries obtained after the retting are rubbed or trampled to remove the soft and rotted pericarp from the pepper core which is the white pepper. Often, the retting water and pericarp waste from this process are released into natural watercourse such as the river and is considered an environmental pollution.

Due to the prolong soaking of pepper berries during the water retting process, there are possibilities that the bioactive compounds and organic matter naturally present in pepper such as oleoresins (containing piperine and phenolic compounds) may have leached out into the soaking water. Oleoresin is a lipophilic mixture containing

volatile and non-volatile component that deliver the aroma and pungency characteristics of pepper (Premi 2000). The quality of pepper oleoresin depends on the volatile oil, piperine and other non-volatile constituents. Piperine is a bioactive compound due to its effects on cells and tissues of living organism. This naturally occurring alkaloid has demonstrated plenty of beneficial health and therapeutic effects (Gorgani et al. 2017). Piperine found in pepper *Piper nigrum* has major pharmacological impacts on neuromuscular systems, exercises sedative effect and helps in digestion (Andrade & Ferreira 2013). It also reported to have immunomodulatory, anti-carcinogenic, anti-asthmatic, stimulatory, hepatoprotective, anti-inflammatory antimicrobial and anti-ulcer activities (Bai & Xu 2000; Darshan & Doreswamy 2004; Yang et al. 2002). Pepper has been widely reported to possess radical scavenging activities and phenolic compounds (Lu et al. 2011). The presence of plant polyphenols as functional compounds supplies dietary antioxidants and helps in human health by reducing disease risks (Arcaro et al. 2014; Meghwal & Goswami 2013). Currently there is no study done to investigate the active compound in retting waste water. The extraction of bioactive and functional components present in agro-industrial waste supports greener and sustainable process and the recovered compounds can be utilized as drugs, functional food ingredients or nutraceuticals (Joana Gil-Chávez et al. 2013; Kumar et al. 2017). Hence, this study aimed to evaluate the presence of the bioactive and functional compounds in the pepper retting waste water at different temperatures and days.

MATERIALS AND METHODS

SAMPLE PREPARATION

Retting of pepper berries in water (1:2 w/w) was carried out in conical flasks placed in a temperature controlled shaking water bath at 28, 35, 42 and 49°C. Retting waste water was changed daily to avoid microbial contamination and spoilage. The retting waste water were collected at 4, 8, 12 and 16 days and kept in seal bag at -4°C before subjected to dichloromethane (DCM) liquid extraction.

EXTRACTION OF OLEORESIN

Water sample (80 mL) was filtered by using filter paper (Whatman No. 1, 11 µm) before being poured into separatory funnel and added with 5 mL of saturated sodium chloride (NaCl). Fifteen mL of dichloromethane (DCM) was gradually added and mixture was gently shaken for about 3 min before resting. The step was repeated two times with addition of 15 mL DCM. DCM layer with small amount of emulsion was then removed into clean 125 mL conical flask and anhydrous sodium sulfate was added to dry the solution. Extractant was then evaporated using rotatory evaporator at 50°C for 25 min and the weight recorded as oleoresin yield and kept at -18°C until further analyses.

FREE RADICAL SCAVENGING ACTIVITY (DPPH) ASSAY

DPPH was prepared in methanol to give a 0.15 mM solution and 2.9 mL DPPH was allowed to mix with 100 µL sample. Solution was kept in dark for 30 min. Amount of 300 µL solution was pipetted into 96-well plat and was read at 517 using spectrophotometer (BioTek Epoch). The result was expressed as percent of radical scavenging activity; where A1 is the absorbance of the DPPH blank and A2 is the absorbance of the sample with DPPH. Reading was blanked with methanolic solution of DPPH.

$$\text{% Free radical scavenging activity} = \frac{(A_1 - A_2)}{A_1} \times 100$$

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

FRAP assay was conducted according to Benzie and Strain (1996) with modification. The FRAP reagent consists of 10 mM TPTZ solution in 40 mM HCl, 20 mM FeCl₃ solution and 0.3M acetate buffer (pH3.6) in proportions of 1:1:10 (v/v/v). One mL of sample was mixed with 2 mL of freshly prepared FRAP reagent and the reactive mixture was incubated in the dark for 30 min. The absorbance was measured at 593 nm using a UV spectrophotometer. Standard curve of 5, 10, 15, 20 and 25 ppm were made with Trolox and the results were expressed as mM TE/g in Trolox equivalents.

TOTAL PHENOLIC COUNT (TPC)

Phenolic content was measured using Folin-Ciocalteu method. One mL of sample extract was added to 5 mL of Folin-Ciocalteu reagent and 4 mL solution of 7.5% Na₂CO₃. After 90 min of incubation 200 µL was pipetted into 96 well plate and measured at 760 nm absorbance by BioTek Epoch microplate spectrophotometer. Standard curve of Gallic acid was done at concentration of 1 - 50 ppm and phenolic activity was determined and expressed as mg Gallic acid equivalent (GAE)/g.

PIPERINE DETERMINATION

Two mL of sample extract was filtered using disposable microfilter (0.22 µm) into HPLC amber vial and 20 µL of the diluent was injected into HPLC equipped with a Photodiode Array Detector (Waters, 2998). Column used was Water XBridge (C18 4.6 × 150 mm, 4.5 µm) with flow rate of 1 mL min⁻¹ and mobile phase of methanol: deionized water (2.3:1). Piperine analytical standard (Sigma Aldrich) was prepared at 1, 2, 4, 6, 8 and 10 ppm in 95% methanol to establish the standard curve.

STATISTICAL ANALYSIS

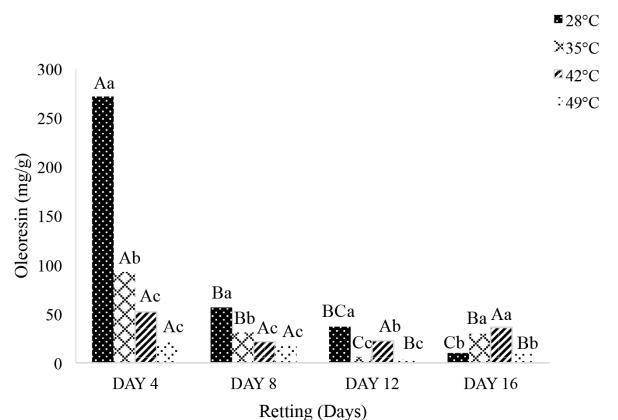
All analyses were performed in triplicate (*n*=3) and data obtained were reported as mean ± standard deviation. One way ANOVA was carried out to compare the difference in the

mean values, whereby means were considered significant if $p<0.05$. Pearson's correlation was carried out to investigate the correlation between oleoresin yield, DPPH, FRAP, TPC and piperine content. All statistical analyses were carried out using SPSS statistical package version 22.

RESULTS AND DISCUSSION

YIELD OF OLEORESIN

Pepper oleoresins extracted by using solvent possess flavour profile similar to freshly ground black pepper. Due to the ease of handling as well as free from microbial contamination and deterioration the oleoresins have the potential application as natural flavouring substances in food (Srinivasan 2007). Figure 1 shows the oleoresin extracted from retting waste water accumulated at different days and temperature. Oleoresin from day 4 (28°C) gives highest yield ($p<0.05$) compared to other days and temperature with $271.34 \pm 28.09 \text{ mg/g}$. Most of the leaching out happened during the earlier days and lower temperature retting as the amount of oleoresin is significantly higher ($p<0.05$) on day 4 of temperature 28 and 35°C compared to other days. It is also postulated that the oleoresin yield may be higher if the waste water was collected in beginning of retting day of 1, 2 and 3. From the previous study, oleoresin extracted from black pepper has been reported to be in the range 5-15% (Shaikh et al. 2006). Retting at higher temperature may affect the yield of oleoresin obtained since the oleoresin compound is sensitive to heat, light and oxygen (Shaikh et al. 2006). Pearson correlation in Table 1 shows that oleoresin content in waste water is positively correlated with antioxidant activity DPPH ($r=0.877, p<0.01$), FRAP ($r=0.819, p<0.01$) and total phenolic content ($r=0.902, p<0.01$). This shows that extracted oleoresin in the waste water still contain important antioxidant and phenolic compounds as it leach out from the pepper pericarp.



^{a-b} Means \pm standard error with different uppercase letters are significantly different ($p<0.05$) at the same temperature of different retting days

^{a,b} Means \pm standard error with different lowercase letters are significantly different ($p<0.05$) on the same day at different retting temperature

FIGURE 1. Oleoresin yield extracted from pepper retting waste water at different retting temperature and days

ANTIOXIDATIVE ACTIVITY

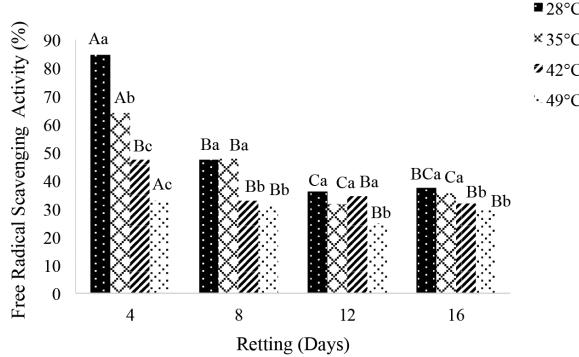
FREE RADICAL SCAVENGING ACTIVITY ASSAY

DPPH or 1, 1 Diphenyl 2- Picryl Hydrazyl assay is widely used to test the free radical scavenging activity of plant extract. The antioxidant activity of retting waste water was tested by the ability of the extract to inhibit stable free radical DPPH. Figure 2 shows the DPPH scavenging activity of retting waste water on day 4 at 28°C was significantly highest ($p<0.05$) at $84.58 \pm 4.93\%$. High value of DPPH in retting waste water suggested that the functional components from the pepper berries skin or pericarp might have leached out into the water due to the degradation of the pericarp cell wall during earlier days of retting process. Gülcin (2005) reported DPPH radical scavenging activity of 55 and 48% in water and ethanol extracts of black pepper respectively compared to their BHT

TABLE 1. Pearson correlation between antioxidant activity, total phenolic content, piperine content and oleoresin yield obtained from pepper retting waste water

		Oleoresin	DPPH	FRAP	TPC	Piperine
Oleoresin	Pearson Correlation, r Sig. (2-tailed), p		0.877* 0.000	0.819* 0.000	0.902* 0.000	-0.363 0.160
DPPH	Pearson Correlation, r Sig. (2-tailed), p	0.877* 0.000		0.625* 0.002	0.875* 0.000	-0.407 0.067
FRAP	Pearson Correlation, r Sig. (2-tailed), p	0.819* 0.000	0.625* 0.002		0.820* 0.000	-0.094 0.687
TPC	Pearson Correlation, r Sig. (2-tailed), p	0.902* 0.000	0.875* 0.000	0.820* 0.000		-0.191 0.394
Piperine	Pearson Correlation, r Sig. (2-tailed), p	-0.363 0.160	-0.407 0.067	-0.094 0.687	-0.191 0.394	

*Correlation is significant at the 0.01 level (2-tailed)



^{a-b} Means \pm standard error with different uppercase letters are significantly different ($p<0.05$) at the same temperature of different retting days

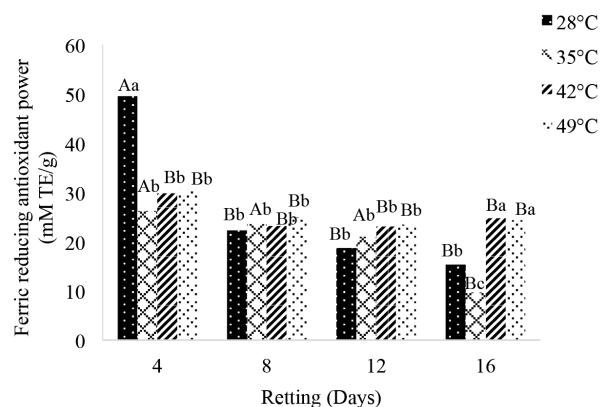
^{a-b} Means \pm standard error with different lowercase letters are significantly different ($p<0.05$) on the same day at different retting temperature

FIGURE 2. Free radical scavenging activity (%) of retting waste water at different retting temperature and days

control with 76% activity. The DPPH scavenging activity started to decrease as the retting days and temperature increase; showing lower ability of antioxidant compound to scavenge the radical compound at higher temperature and longer retting day. Lowest DPPH scavenging activity was shown at temperature 49°C; day 12 which has no significant different ($p>0.05$) with 49°C; day 16 and 49°C; day 8. This is also probably due to the significant physical changes observed on pepper berries. At high temperature retting of 49°C the skin of pepper berries become dark, tough and stiff. Strengthen pericarp skin of pepper berries eventually cease to release active compound into the water hence reduce the scavenging activity.

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

In the ferric reducing antioxidant power (FRAP) assay conducted, $\text{Fe}^{3+}(2,4,6\text{-tripyridyl-s-triazine})_2\text{Cl}_3$ was used as oxidant probe to measure the reducing power of antioxidant compounds. Reduced oxidant probe in the reaction will change colour and stop changing upon completion (Huang et al. 2005). Referring to Figure 3, the waste water collected exhibit highest ferric reducing capacity (49.55 ± 6.11 mM TE/g, $p<0.05$) at lower temperature (day 4; 28°C) of retting equate to the result of DPPH assay. Water uptake by osmosis during the soaking of pepper berries weaken the matrix cells and cause the pericarp cells to stretched and swelled (Yadav & Tyagi 2008). Prolong immersion in water causes the cell wall structure to be damaged and collapsed releasing various active compound into the water. Pearson correlation showed that antioxidant activity of waste water by DPPH ($r=0.877$, $p<0.01$) and FRAP ($r=0.819$, $p<0.01$) was strongly correlated with the oleoresin yield ($p<0.01$). A significant correlation ($p<0.01$) was observed for FRAP ($r=0.820$) and DPPH ($r=0.875$) assay with TPC. These results indicated the extracts from waste water exhibited a noticeable effect on scavenging free radical and was mainly contributed by the phenolic compounds (Mahdavi et al. 2017).



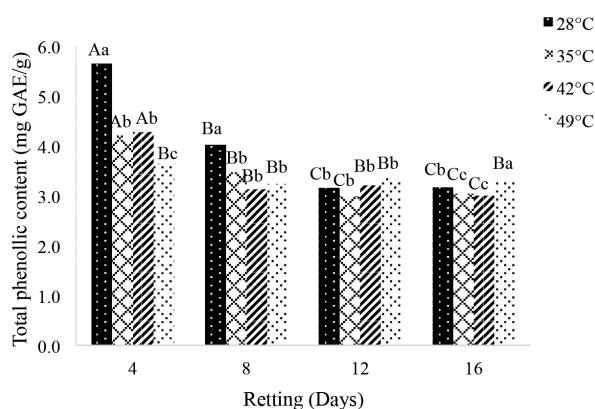
^{a-b} Means \pm standard error with different uppercase letters are significantly different ($p<0.05$) at the same temperature of different retting days

^{a-b} Means \pm standard error with different lowercase letters are significantly different ($p<0.05$) on the same day at different retting temperature

FIGURE 3. Ferric reducing antioxidant power in Trolox equivalent (mM TE/g) of waste water after retting process at different temperature and days

TOTAL PHENOLIC CONTENT (TPC)

Phenolic compounds are naturally found in fruits, vegetables, cereals, legumes and beverages in abundant amount (Kochhar 2016; Prakash & Sharma 2014). Besides providing beneficial effects on the plant host, these compounds can function as free radical scavengers thus gives important biological properties in promoting human health such as anti-aging, antioxidant activity and anti-carcinogenic effects (Dai & Mumper 2010; Działo et al. 2016; Pandey & Rizvi 2009). Figure 4 shows the TPC in the oleoresin extracted from retting waste water (expressed as mg gallic acid per g of sample). Retting waste water on day 4 ranging from 5.64 ± 0.06 to 3.63 ± 0.11 mg GAE/g show a highest value ($p<0.05$) at 28°C compared to other temperature tested in the same day. Significantly higher ($p<0.05$) TPCs were obtained on day 4 compared to other days on the respective temperature. The phenolic content in retting waste water reduced as the day of retting increase for each temperature. The phenolic content in retting waste water was found to be positively correlated ($p<0.01$) with oleoresin and DPPH activity as discussed previously. A highly positive correlation between TPC and antioxidant activity has been reported in various plant species and plant-based food products (Esmaeili et al. 2016; Wern et al. 2016). Gülçin (2005) reported 0.054 and 0.043 mg/g TPC in water extracts and ethanol extracts of black pepper, respectively. In Agbor et al. (2006) study, polyphenolic concentration of hydrolyzed and non-hydrolyzed white pepper was about 7.0 - 8.0 and 5.0 - 6.0 mg/g catechin equivalent. Andrade and Ferreira (2013) found much higher yield of phenolics on black pepper with 27 ± 3 mg GAE/g by Soxhlet method and 26 ± 3 mg GAE/g by ultrasound method. Variyar and Bandyopadhyay (1994) earlier work has identified syringic, vanillic and ferulic acid in the



^{a-b} Means ± standard error with different uppercase letters are significantly different ($p<0.05$) at the same temperature of different retting days

^{a-b} Means ± standard error with different lowercase letters are significantly different ($p<0.05$) on the same day at different retting temperature

FIGURE 4. Total phenolic content (mg GAE/g) of waste water after retting process at different temperature and days

phenolic acid fraction. Lower temperature immersion seems to leach some of these phenolic compounds from pepper berries into the water. According to Prabhakaran Nair (2011), source of phenolic compound is from the skin of pepper berries. Pepper berries have been reported to contain 3,4-dihydroxyphenyl ethanol glucoside, 3,4-dihydroxy-6-(N-ethylamino) benzamide and phenolic acid glycosides as the main phenolic compounds (Chatterjee et al 2007; Pradhan et al. 1999). According to Chatterjee et al. (2007), among the pepper phenolics fractions 3,4-dihydroxyphenyl ethanol (hydroxytyrosol) glucoside had the highest DPPH radical scavenging activity approximately 60% higher than standard Trolox. However, these compounds are lacking in black pepper due to oxidation during drying (Variyar & Bandyopadhyay 1994). The phenolic compounds in pepper have been reported to prevent coronary diseases and cancer, as well as age-related degenerative brain disorders, inhibit lipid peroxidation and reducing plasmid DNA damage induced by UV and gamma radiation. These effects were mainly as the consequences of the phenolic compounds ability to scavenge (Chatterjee et al 2007).

PIPERINE CONTENT IN WASTE WATER

Pepper piperine has been reported to exhibit numerous health benefits including stimulating digestive enzymes of pancreas (Tiwari & Singh 2008), as antidiabetic (Rauscher et al. 2000), antidepressant (Li et al. 2007), antidiarrhoeal activity (Reshma et al. 2010) and anticancer (Yaffe et al. 2015). The amount of piperine in white pepper from Piperaceae family (*Piper nigrum* L.) varies from 2% to 7.4% (Peter 2006; Ravindran 2003). In another report, white pepper piperine constitutes of 2.70 to 5.85% for 15 selected cultivars (Parthasarathy et al. 2007). However, there is no report on piperine percent of waste water from the retting of pepper berries. Table 2 shows days of retting had no significant ($p>0.05$) effect whereas temperature exhibited significant ($p<0.05$) effect on the piperine content although no apparent trend was observed. This indicates that piperine in the waste water is more temperature dependent compared to time. The highest piperine content obtained in waste water was $1.81 \pm 0.44\%$ at 49°C on day 16 and was significantly different ($p<0.05$) compared with other temperature of 28°C , 35°C and 42°C on the same day. The value of piperine found in waste water ($1.81 \pm 0.44\%$) was lower compared to the piperine found in white pepper seed (2.70 to 5.85%). However, this indicated that the piperine did leach out from the pepper seed during water retting. Low piperine content in water at lower temperature probably due to its low solubility in water (Chavarria et al. 2016; Gorgani et al. 2017). At 49°C retting temperature on each 4, 8 and 16 days show higher piperine content ($p<0.05$) compared to lower temperature retting. Meanwhile the lowest piperine content was $0.36 \pm 0.11\%$ at 35°C on day 4. Piperine was found to have no significant ($p>0.05$) correlation with TPC, antioxidant activity and oleoresin yield of waste water.

CONCLUSION

Waste water from the water retting process of pepper berries was found to contain amount of extractable oleoresin, exhibit antioxidant activity and phenolic compounds. Piperine was also detected in the waste water. The agro-industrial waste water from pepper industry can be potentially utilized for its bioactive components as well as serving greener and sustainable process for the environment.

TABLE 2. Piperine content (%) in waste water after water retting process at different temperature and days

	28°C	35°C	42°C	49°C
Day 4	0.60 ± 0.10 ^{bB}	0.36 ± 0.11 ^{bB}	1.00 ± 0.21 ^{bA}	1.41 ± 0.24 ^{bA}
Day 8	0.99 ± 0.13 ^{bA}	0.45 ± 0.14 ^{bB}	0.37 ± 0.23 ^{bB}	1.39 ± 0.23 ^{bA}
Day 12	0.91 ± 0.16 ^{bB}	0.85 ± 0.32 ^{bB}	0.78 ± 0.29 ^{bB}	1.39 ± 0.29 ^{bB}
Day 16	0.59 ± 0.13 ^{bB}	0.75 ± 0.13 ^{bB}	0.68 ± 0.44 ^{bB}	1.81 ± 0.44 ^{bA}

^{a-b} Means ± standard error with different lowercase letters in the same column are significantly different ($p<0.05$)

^{A-B} Means ± standard error with different uppercase letters in the same row are significantly different ($p<0.05$)

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