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Elemental Analysis, Extraction Conditions, and Brine Shrimp Toxicity of Defatted *Hylocereus polyrhizus* Seed

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Extracts prepared from *Hylocereus polyrhizus* have received attention in pharmacological research due to their potent anti-inflammatory, antioxidant, anti-cancer, anti-diabetic, and anti-microbial effects. This current study aims to determine the level of multi-element contents, the effect of different extraction conditions on the total amount of polyphenolic content, the antioxidant potential, and the toxicity of the Defatted *H. polyrhizus* Seed Extract (DHPSE). The phenolic content was evaluated through Total Phenolic Content (TPC) and Total Flavonoid Content (TFC), while the antioxidant levels were measured through 2,2-diphenyl -1- picrylhydrazyl (DPPH) radical scavenging activity. The toxicity of the DHPSE extract was assessed through a brine shrimp lethality assay. The DHPSE exhibited the presence of potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), aluminum (Al), chromium (Cr), nickel (Ni), lead (Pb), cobalt (Co), and selenium (Se). The current study also revealed that all the various extracting conditions of DHPSE possessed a significant influence ($p < 0.05$) on the antioxidant capacity and polyphenolic content and presented no toxicity effect. The optimized conditions determined for ethanol concentration, extraction time, and temperature were 80%, 60 minutes, and 80°C, respectively. These ideal parameters gave 120.33 mg GAE/g sample and 10.76 mg QE/g sample, respectively, with 92% inhibition of DPPH scavenging. Therefore, these findings provide valuable insight for the future utilization of defatted *H. polyrhizus* seed.

Keywords: *Hylocereus polyrhizus*; mineral; antioxidant; phenolic content; solvent; time; temperature; defatted seed extract

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Pitaya, sometimes referred to as dragon fruit, is a cactus species native to Central and Northern South America that has emerged as a new commercial crop in Malaysia. Two varieties of pitaya are extensively cultivated: *Hylocereus polyrhizus* (red flesh pitaya) and *Hylocereus undatus* (white flesh pitaya). It has recently become the preferred fruit in the market due to its sugary taste, attractive texture, and numerous health benefits [1]. The outer skin (exocarp) of the pitaya has a scaly structure, and the flesh of the fruit contains plentiful black seeds [2]. In the past few years, researchers primarily aimed at pitaya seed oil extraction, which was reported to be rich in linoleic acid. It is an essential fatty acid demanded by food and cosmeceutical industries since it is reported to help alleviate cracked skin and keep it moist.

In contrast, studies regarding the antioxidant compounds contained in pitaya defatted seeds, a by-product of oil extraction, have drawn less attraction. Antioxidant components are naturally formed in plants, such as phenolics, flavonoids, lignans, and tannins [3]. Notably, phenolic compounds are known for their health-benefiting properties. They demonstrate a wide range of pharmacological properties, including anti-microbial, anti-inflammatory, antioxidant, antimutagenic, and antiallergenic effects [4]. The extraction of phytochemicals from the plant matrix is considered crucial, as it is regarded as the first step in separating

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desired natural products from raw materials. Various factors, including type of extraction, extraction time, type of solvent, pH, and temperature, impact the extraction process [5]. Thus, optimizing the extraction process to better isolate phenolic compounds from various food matrices is crucial.

To the best of our knowledge, no studies have been reported regarding the factors that influenced the extraction condition of antioxidant compounds from the Defatted *H. polyrhizus* Seed Extract (DHPSE). Consequently, the optimal state for extracting natural antioxidants was developed using a single-factor experiment. The current research aims to investigate the impacts of ethanol concentration, extraction time, and temperature on Total Phenolics Compound (TPC), Total Flavonoid Compounds (TFC), and antioxidant potential based on 2,2-diphenyl -1- picrylhydrazyl (DPPH) radical scavenging activity. In addition, the multi-element analysis and toxicity of DHPSE are also explored throughout this study.

EXPERIMENTAL

Chemicals

Sodium carbonate 99% (Na_2CO_3: CAS 497-19-8), Folin –Ciocaltue's reagent, gallic acid 97% (CAS 149-91-7), quercetin 95% (CAS 117-39-5), aluminum chloride 98% ($AlCl_3$: CAS 7446-70-0), 2,2-Diphenyl -1-picrylhydrazyl (DPPH: CAS 1898-66-4), ascorbic acid (CAS 50-81-7) and ethanol 95% (CAS 64-17-5) were purchased from Sigma-Aldrich. All chemicals used throughout the experiment were of analytical grade.

Plant Material

The red pitaya fruit was purchased from a local farm in Sepang, Malaysia. The seed was removed from the flesh and rinsed under running water. The dried seeds were crushed into tiny pieces and sealed in a bottle. The seeds were then extracted using n-hexane via a maceration process for defatting purposes. Then, the derived samples were placed in a fume hood overnight to ensure the remaining n-hexane was fully evaporated prior to the extraction process.

Multielement Analysis

The 500 g mass of dried seed was weighed and transferred into the digestion vessel. The mixture of 8 mL of 65% nitric acid (HNO_3) and 2 mL volumes of 30% hydrogen peroxide (H_2O_2) was added. The vessel was closed and taken to the microwave oven model ETHOS UP (Milestone MLS, Sorisole, BG, Italy) for sample digestion according to the following heating program: 10 minutes to reach 180°C and 15 minutes to hold at 180°C. The digested samples were filtered using filter paper (0.45 µm, Avantec MFS Inc., Pleasanton, CA, USA). Subsequently, the samples were made up to a final volume of 50 mL with distilled water. Finally, the prepared samples proceeded to analyze multi-element composition using Perkin-Elmer Sciex Elan 900 quadrupole-based ICP-MS [6].

Extraction of *H. polyrhizus* Seed

To obtain DHPSE, the defatted samples were extracted using ethanol at a 1:20 solid-to-liquid ratio. Shaking water bath extraction was performed at constant shaking of 90 rpm at designated temperatures and times. Then, the homogenized mixture was centrifuged at 25°C for 5 minutes. Consequently, the supernatant was filtered through a Whatman No. 1 filter paper and subjected to vacuum drying using a rotary evaporator to obtain the crude extract. The obtained sample was kept at 4°C until it was utilized.

Experimental Design

An experiment with a one-factor-at-a-time method was used in this study to identify the ideal conditions for extracting antioxidative polyphenols from DHPSE. The study investigated three parameters: ethanol proportion (ranging from 20% to 95%), time of extraction (ranging from 30 to 240 minutes), and extraction temperature (ranging from 20 to 80°C). Each parameter was examined by varying only one variable at a time while keeping the other variables constant. The optimum extraction conditions were decided based on the response of the experiment, which comprised TPC, TFC, and DPPH radical scavenging activity.

Ethanol Concentration

The effect of ethanol concentration ranging from 20% to 95% (20%, 40%, 60%, 80%, and 95%) was studied by maintaining a constant extraction duration of 90 minutes and a stable temperature of 40°C.

Extraction Time

By applying the best ethanol concentration determined before, the extraction was performed by setting the extraction temperature at 40°C and different extraction times (30, 60, 120, 180, and 240 minutes).

Extraction Temperature

The extraction of defatted seed samples was performed using the optimal concentration of solvent and extraction time, which had been previously identified. The extraction temperature varied from 20 to 80°C (20, 40, 60, 80°C).

Total Phenolic Content (TPC)

The concentration of TPC in DHPSE was determined using the Follin-Ciocalteu reagent, following the method [7] with minor modifications. Note that 100 µL of the diluted DHPSE was added to the vial containing 50 µL of Folin. Then, it is followed by the addition of sodium carbonate. The absorbance was measured at 765 nm after incubating for 2 hours by a microplate spectrophotometer. TPC was determined by utilizing the gallic acid calibrated curve and reported as Gallic Acid Equivalent (GAE) in milligrams per gram of dry weight.

Total Flavonoid Content (TFC)

The amount of flavonoid was measured according to the aluminum chloride colorimetric method, as explained by [8], with several modifications. Consequently, 100 µL (1 mg/mL) of the sample was mixed with 100 µL of (2%, w/v) aluminum chloride. The reaction mixture was allowed to stand for 15 minutes at room temperature. The absorption at 415 nm was then measured with a microplate reader. TFC was determined using the standard calibration curve and expressed as mg quercetin equivalent (mg QE/g) of the extracted sample.

DPPH-Radical Scavenging Activity (DPPH-RSA)

The test was evaluated using a method adapted from [9]. Briefly, 50 µL of sample and 150 µL of ethanolic DPPH solution were mixed in a 96-well microtiter plate and kept in the dark for 30 minutes at room temperature. The decrease in absorbance reading was measured at wavelength 515 nm using a microplate reader. For this test, ethanol and DPPH solutions without sample extract were used as blanks and controls, respectively. The capacity of the extract to scavenge the DPPH radical was represented by a percentage of inhibition, calculated using the subsequent equation:

$$Percentage\ of\ Inhibition = \frac{A_{control} - A_{sample}}{A_{control}}\ x\ 100$$

Cytotoxicity Bioassay

The brine shrimp lethality assay was conducted according to the protocols described by [10] with slight modifications. The brine shrimp (*Artemia salina*) eggs used in this method were in the form of a sea salt premix (Ocean Nutrition Europe) containing 28 g of sea salt and 2 g of Artemia cysts. The decapsulation of brine shrimp cysts started with the hydration of artemia cysts. Subsequently, 200 mg of Artemia cysts were added into 18 mL of tap water in a 50 mL falcon tube and left for about an hour. Then, a mixture of 660 µL of sodium hydroxide (32%) and 10 mL of 5% sodium hypochlorite (Clorox) was added to the hydrated egg suspension. The mixture was lightly shaken and followed by adding 14 mL of sodium thiosulphate (10 g/L) after 2 minutes of mixing. Next, the mixture was filtered using a sieve cloth after 2 minutes and washed with 20 mL filtered and autoclaved natural seawater collected from Port Dickson, Negeri Sembilan, Malaysia. Subsequently, the egg seawater was resuspended into 30 mL of filtered and autoclaved natural seawater and left for 1 day in the incubator shaker at room temperature to allow the shrimp to be hatched and mature as nauplii. In brief, various concentrations of DHPSE varied from 62.5, 125, 250, 500, and 1,000 µg/mL were tested in vials containing 10 nauplii and incubated for 24 hours with the addition of 2 µL of 1% of autoclaved yeast suspension as a live feed for nauplii. The same concentrations of sodium dodecyl sulfate were used as a positive control. The experiments were run in triplicate for each concentration. The total viable count of nauplii in each vial was enumerated and documented at 6, 12, 18, and 24 hours. The concentrations that killed 50% of nauplii (LD_{50}) were calculated from the regression equation of graph percentage of mortality vs logarithm of concentration.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism software (Version 8.0; San Diego, CA, USA). The values were expressed as the mean ± Standard Deviation (SD). The groups were compared using the analysis of variance (ANOVA) test followed by Tukey's post hoc. A probability level of $P < 0.05$ was used to determine statistical significance.

RESULTS AND DISCUSSION

Multielemental Analysis

The elements potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), aluminum (Al), chromium (Cr), nickel (Ni), lead (Pb), cobalt (Co), and selenium (Se) were detected in DHPSE. The concentration of these elements was then quantitatively measured, as presented in Table 1.

Ethanol Concentration

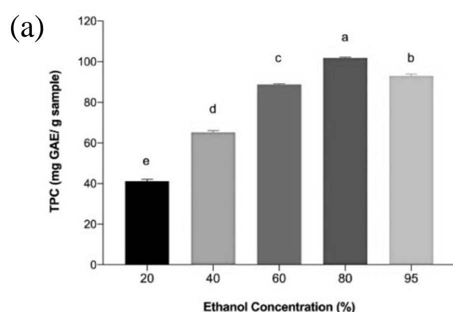
The experiment results demonstrated that all ethanol concentrations ranging from 20% to 95% were able to extract antioxidative polyphenols with significant differences ($p < 0.05$). Ethanol was selected due to its safety and low toxicity compared to other solvents used in the extraction processes, such as methanol, acetone, and others. Based on the fundamental principle of similarity and impermissibility ("like dissolve like"), the phytochemicals in plant matrices (solute) will be extracted better with the solvents

having a polarity near them [11]. The highest TPC and TFC results were obtained when using 80% ethanol, and the value reported was (101.81 mg GAE/g sample) and (9.4 mg QE/g sample), respectively. Meanwhile, the lowest values were recorded at 20% ethanol with 41.14 mg GAE/g sample and 1.28 mg GAE/g sample for TPC and TFC, respectively (Figure 1). Different solvent concentrations also resulted in a significant effect ($p < 0.05$) on the DPPH-radical scavenging activity of DHPSE (Figure 1). The maximum percentage of inhibition of DPPH radical acquired was recorded at 80% and 95% ethanol concentration. This occurrence can be related to the fact that the presence of water in the solvent can help improve extraction efficiency [12]. Furthermore, ethanol may enhance TPC yield by breaking the interaction between solutes and the plant matrix, whereas water will facilitate cell swelling [13]. Our present data align with previous studies [14] and [15] that demonstrated that the binary solvent system could assist in extracting more phenolic compounds from flaxseed and Japanese edible herbal *Angelica keiskei*. Therefore, by considering the highest amount of TPC and TFC in the extract, 80% ethanol concentration was selected as the best extraction solvent for the next experiment.

Table 1. Mineral contents in the DHPSE.

Mineral	Concentration value (mg/L)
Potassium (K)	8.65 ± 0.26
Calcium (Ca)	1.77 ± 0.04
Magnesium (Mg)	4.38 ± 0.29
Sodium (Na)	0.43 ± 0.29
	Concentration value (μg/L)
Iron (Fe)	6042.22 ± 52.73
Zinc (Zn)	1793.78 ± 376.70
Copper (Cu),	770.83 ± 15. 88
Manganese (Mn)	3464.48 ± 38.61
Aluminium (Al)	3775.86 ± 9.02
chromium (Cr)	133.87 ± 0.41
Nickel (Ni)	128.92 ± 0.57
Lead (Pb)	12.95 ± 2.55
Cobalt (Co)	3.23 ± 0.48
Selenium (Se)	1.11 ± 0.01

(a)



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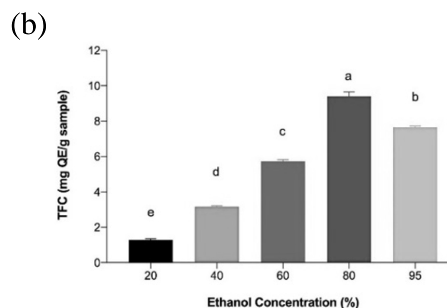

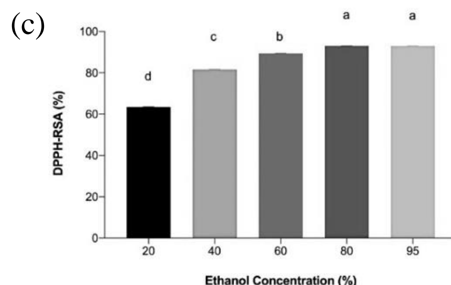

Figure 1. TPC (a), TFC (b), and DPPH-radical scavenging activity (c) of DHPSE at different concentrations of ethanol. Means with the same letter are not significantly different at $p < 0.05$.

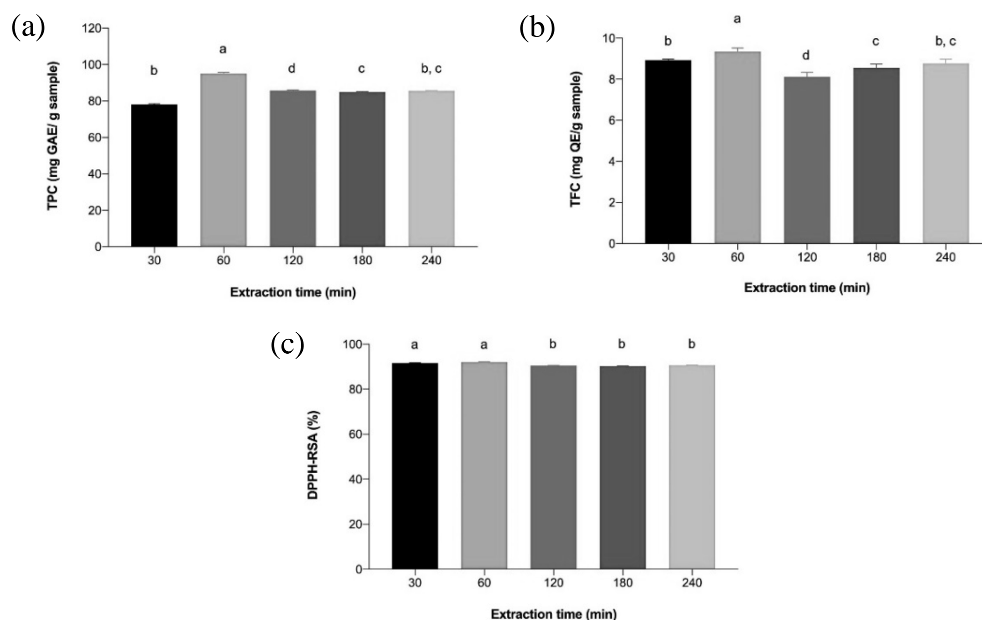

Figure 2. TPC (a), TFC (b), and DPPH-radical scavenging activity (c) of DHPSE at various extraction time. Means with the same letter are not significantly different at $p < 0.05$.

Extraction Time

The duration of the extraction process is a crucial element to be considered in order to minimize energy usage and extraction costs. The extraction duration can range from a few minutes to a maximum of 24 hours [16]. In this study, the range between 30 to 240 minutes revealed an increment in TPC, TFC, and DPPH-radical scavenging activity as extraction time increases from 30 to 60 minutes (Figure 2). The amount recorded for this period time is 95.10 mg GAE/g sample of TPC and 9.34 mg QE/g sample of TFC, which allowed 92% of scavenging activity on DPPH radical. After 60 minutes, further prolonged process duration did not affect the phenolic yields. This situation may be attributed to Fick's second law of diffusion, which states that a final equilibrium between solute concentrations in the plant matrix and the bulk solution would be accomplished after a specific time, as previously reported by [17]. Thus, extending the extraction period is unlikely to enhance the retrieval of phenolic content.

Moreover, the reduction in the percentage of inhibition of DPPH radicals was noticed after reaching 180 minutes, which reduced from 92.05% to 90.24%. This occurrence is possibly due to the oxidation of phenolic compounds due to exposure to light and oxygen [18]. Similar trends have been reported by [19] and [20] in which prolonged extraction time beyond 60 minutes led to a decrease in bioactive components of mangosteen (*Garcinia mangostana*) and mulberry leaf, respectively. Therefore, 60 minutes was selected as the extraction time for the following experiment.

Extraction Temperature

The effect of temperature on the TPC, TFC, and antioxidant activity of DHPSE is presented in Figure 3. It can be observed that temperature significantly impacts the recovery of phenolic content but not DPPH scavenging activity. Theoretically, high temperatures will soften the

plant tissue and interrupt the cell membrane integrity due to the weak interactions between phenol-protein and phenol-polysaccharide molecules. Thus, phenolic compounds can be easily migrated into the solvent [21]. This correlates with the current result obtained that revealed TPC and TFC were sharply increased from 20°C to 80°C, which corresponded to the value of TPC (58.67 mg GAE/g sample to 120.33 mg GAE/g sample) and TFC (6.37 mg QE/g sample to 10.76 mg QE/g sample), respectively. Several studies reported that temperature could enhance the phenolic recovery from Taiwan short-leaf pine [22] and annatto seeds [23]. This outcome, perhaps due to the rise in the heat, had improved the solubility of phenolic compounds and reduced solvent viscosity [24].

However, an increase in extraction temperature from 40°C to 80°C resulted in a slight reduction in antioxidant activity of DHPSE from 92.76% to 92.22%, respectively. The increase of phenolic content observed in the extracts with the increasing temperature was not accompanied by a corresponding increase in antioxidant activities. Our results align with the findings of [25], who discovered that the antioxidant properties of *Clinacanthus nutans* extract were reduced at 80°C and 120 extraction time. According to [26], the decrease in the percentage of inhibition of DPPH radicals may be attributed to the degradation of the antioxidant compound linked to the phenolic compound, which cannot tolerate high temperatures. The extraction temperature of 80°C was selected as the optimal extraction temperature to extract polyphenol due to the highest phenolic content yield reported.

Cytotoxicity Study

The death rate of the brine shrimp nauplii in the current study exhibits a positive correlation with increasing concentrations of the ethanolic extract of DHPSE (Table 2). The LC_{50} value was 4.57×10^3 µg/mL. Extracts are classified as toxic if LC_{50} value < 1,000 (1×10^3) µg/ mL and non-toxic if it exceeds that threshold [27]. These findings indicate that the ethanolic extract of DHPSE did not exhibit any harmful effects on the brine shrimp, confirming that the DHPSE is physiologically safe and non-toxic to humans.

CONCLUSION

The result revealed that ethanol concentration, extraction time, and extraction temperature significantly influence the extraction of phenolics compound and antioxidant capacity of defatted *H. polyrhizus* seed. The optimal condition for better recovery of phenolics compound and antioxidant capacity was 80% ethanol, 60 minutes at 80°C. In general, temperature plays a vital role in the better recovery of phenolic compounds. This is most likely attributed to heat, which can weaken the binding that phenolic chemicals have with macromolecules (such as proteins and polysaccharides), increasing the extraction yield. The cytotoxicity study suggested that the defatted *H. polyrhizus* seed had no toxicity effects. The findings of the current work are beneficial for future research on improving the polyphenol extraction from defatted *H. polyrhizus* seed. This can be achieved by exploring alternative methods, such as response surface methodology.

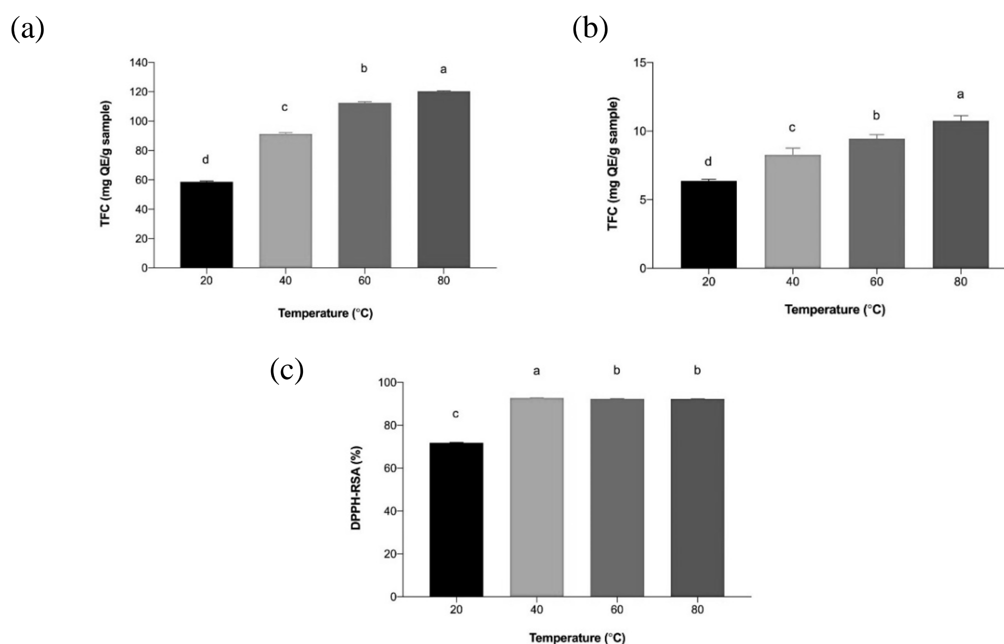

Figure 3. TPC (a), TFC (b), and DPPH-radical scavenging activity (c) of DHPSE at different extraction temperature. Means with the same letter are not significantly different at $p < 0.05$.

Table 2. Brine shrimp toxicity of DHPSE.

Concentrations (μg/mL)	Percentage mortality (%)	Regression line equation	LC_{50} (μg/mL)
62.5	0.00		
125.0	0.00		
250.0	3.30	$y = 0.011x – 0.2871$	4.57×10^3
500.0	6.67		
1000.0	10.00		

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