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Full Length Research Paper

Antioxidant capacity of extracts from calyx fruits of roselle (*Hibiscus sabdariffa* L.)

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The antioxidant capacities of extracts of dried roselle (*Hibiscus sabdariffa* L.) calyx and fruit with distilled water ethanol (30, 60 and 95%) were determined by 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH), hydroxyl and 2-2'-azino-bis-(3-ethyl-benzthia-zoline-6-sulfonic acid) (ABTS) radical-scavenging assays. The results show that the extract in 30% ethanol always had the highest radical-scavenging activity, while the extract in 95% ethanol showed the lowest. Correlation analyses between antioxidant activities and contents of total phenols, anthocyanins and total saccharides indicated that the higher activity of 30% ethanol extract depends on its higher level of total phenol contents (20.25 mg GAE/g). The correlation coefficients R^2 were 0.9615, 0.9089 and 0.9771, respectively. Hence, the 30% ethanol extract could be used as a good natural antioxidant with potent free radical scavenging activity.

Key words: Antioxidant, roselle, total phenol, anthocyanins, total saccharides.

INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.) is a tropical/subtropical plant from West-Africa, India and Malaysia. In China, it is mainly distributed in Guangdong, Guangxi, Fujian, Taiwan and Yunnan Provinces. The biological activities of roselle, such as protection effects from atherosclerosis (Chang et al., 2005; Chen et al., 2003; Kao et al., 2009), anticarcinogenic activities (Chen et al., 2003; Chewonarin et al., 1991; Tseng, 1998; Tseng et al., 2000) especially in the field of leukemia (Chang et al., 2005; Hou et al., 2005), cyclooxygenase inhibitory activities (Christian et al., 2006), chemo-preventive properties (Liu, 2006), hepatoprotective effects and antihypertensive effects (Liu et al., 2002) have been reported.

The over production of free radicals and reactive oxygen species can induce many kinds of diseases. Nevertheless, natural antioxidants protect against these oxidative stresses and associated diseases. They have played an important role in the health care industry (López et al., 2007). Studies demonstrated that the calyx of roselle (Mourtzinos et al., 2008) was rich in phenolic compounds and anthocyanins. It was reported that those compounds could be considered as a great source of natural antioxidants. Those compounds were extracted from roselle in different studies using water (Mojiminiyi et al., 2007; Tsai et al., 2002), acidified methanol (Kao et al., 2009) and absolute ethanol (Tseng, 1998) as extract dissolvent, or sequentially with hexane, ethyl acetate and acidified methanol (Christian et al., 2006). However, to the best of our knowledge, there were no studies reported about the antioxidant activities of the aqueous extract or extracts with different concentrations of ethanol from roselle.

In the present study, the antioxidant capacities of the different extracts of roselle were studied by 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH), hydroxyl (OH) and 2-2'-azino-bis-(3-ethyl-benzthia-zoline-6-sulfonic acid) (ABTS)

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Abbreviations: DPPH, 2,2-Diphenyl-2-picrylhydrazyl hydrate; **ABTS,** 2-2'-azino-bis-(3-ethyl-benzthia-zoline-6-sulfonic acid).

assays. The contents of total phenols (TP), anthocyanins and total saccharides (TS) were also determined. Furthermore, the correlations between antioxidant capacities and contents of total phenols, anthocyanins and total saccharides were analyzed.

MATERIALS AND METHODS

Roselle (*H. sabdariffa* L.) was obtained from Danyang, Zhenjiang, Jiangsu Province in November 2008, and identified by Chen Li (Chief Pharmacist, Food and Drug Administration Bureau of Zhenjiang).

Chemicals and reagents

DPPH, ABTS and Folin-Ciocalteu reagent were obtained from Sigma-Aldrich (Milwaukee, WI, USA). All solvents and chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Preparation of roselle extracts

The calyx and fruit were dried in a hot-air dryer (Yi-heng Science and Technique Co. Ltd., China) at 70°C and triturated before solvent extraction. Fifteen grams of roselle calyx and fruit powders were extracted with 300 ml of water or 30% ethanol, 60% ethanol, 95% ethanol at 75°C for 1 h, followed by filtration with filter paper (30 to 50 μM, Hangzhou Xinhua Paper Industry Co., Ltd., Hangzhou, Zhejiang, China), respectively, and then the residues were re-extracted with 300 ml of the same solvent as described above. The combined supernatants were concentrated in a rotary evaporator at 55°C, and lyophilized to afford the various extracts. The extraction yield was calculated as:

$$\text{Extraction yield (\%)} = \frac{\text{Sample extract weight}}{\text{Total sample weight}} \times 100$$

DPPH assay

The DPPH free radical-scavenging activity of each sample was determined according to the method described by Scherer and Godoy. (2009). Briefly, 2.0 ml of roselle extract samples or ascorbic acid standards at different concentrations were added to 2.0 ml DPPH dehydrated ethanol solution (0.2 mM) separately. After a 30-min incubation at room temperature in the dark, the absorbance was measured at 517 nm. The radical scavenging activity was calculated as follows:

$$\text{Inhibition (\%)} = [1- (A_i - A_j) / A_0] \times 100$$

Where, A_0 is the absorbance of the blank sample, A_i is the absorbance in the presence of the test compound at different concentrations and A_j is the absorbance of the blank reagent. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration vs. the corresponding scavenging effect, and ascorbic acid was used for comparison.

Hydroxyl radical-scavenging assay

According to the method described by Su et al. (2009), 2.0 ml $FeSO_4$ (6 mM) aqueous solution was added to 2.0 ml sample extracts, and then 2.0 ml H_2O_2 (6 mM) was added. After a 10-min incubation period at room temperature, 2.0 ml salicylic acid (6 mM) were added, and incubated for 30 min. The absorbance was measured at 510 nm. The radical scavenging activity was calculated as above. All tests were carried out in triplicate.

ABTS assay

The ABTS assay was performed according to the method described by Li et al. (2009) with slight modification. ABTS radical cation ($ABTS^+$) was produced by mixing the 7 mM ABTS solution with 2.45 mM potassium persulfate aqueous solution leaving them in the dark at room temperature for 12 to 16 h. The $ABTS^+$ solution was diluted with phosphate buffer to the absorbance of 0.70 ± 0.02 at 734 nm. Then 6 min after adding 0.2 ml of sample aqueous solution to 3.8 ml of diluted $ABTS^+$ solution, the A_{734} absorbance was measured. For the blank, 0.2 ml water was used instead of the sample. The radical scavenging activity was calculated as above. All tests were carried out in triplicate.

Determination of total phenols

Total phenols were determined by the Folin-Ciocalteu method of Chew et al. (2009). Briefly, 1.5 ml Folin-Ciocalteu reagent (10%, v/v) was mixed with 1.2 ml 7.5% (w/v) Na_2CO_3 solution, then 0.3 ml sample solution was added. After a 30-min incubation at room temperature in dark, the absorbance was measured at 765 nm. Gallic acid was used as a standard compound for the standard curves. The results were presented in mg gallic acid equivalent (GAE)/g extract. All the experiments were carried out in triplicate.

Determination of anthocyanins

Anthocyanins were determined by the pH differential method of Chew et al. (2009) with slight modifications. Briefly, 1.0 ml 0.2 mol/L potassium chloride solution (pre-adjusted to pH 1.0 with 1.0 mol/L HCl) or 1.0 ml 1 mol/L sodium acetate buffer (pre-adjusted to pH 4.5 with 1.0 mol/L HCl) was added into 2.0 ml of extracts respectively. The absorbances were then measured at 517 and 700 nm. The sample absorbance equals A_{517} value minus A_{700} value. The concentration of anthocyanins was calculated with the following formula:

$$\text{Concentration of anthocyanins (mg/L)} = A / \varepsilon L \times MW \times 10^3 \times \text{Dilution factor}$$

Where, A is the difference of sample absorbance between pH 1.0 and 4.5, ε is molar extinction coefficient for cyanidin-3-glucoside (26,900); L is the path length of the spectrophotometer cell (1.0 cm), and MW is molecular weight of cyanidin-3- glucoside (449.2 g/mol). The result was expressed as mg cyanidin-3-glucoside (cy-3-glu) equivalent/g extract. All the experiments were carried out in triplicate

Determination of total saccharides

Total saccharides were determined by the phenol-sulfuric method of Chinese Pharmacopoeia (2005). Sixty milligrams of anhydrous dextrose was dissolved in 100 ml water (0.6 mg anhydrous dextrose /ml) and it was then used as standard solution. Next, 1.0, 1.5, 2.0, 2.5 and 3.0 ml of standard solution were diluted to 50 ml, respectively, and then 2.0 ml aliquot of each solution was immediately mixed with 1.0 ml 4% phenol and 7.0 ml sulfuric acid.

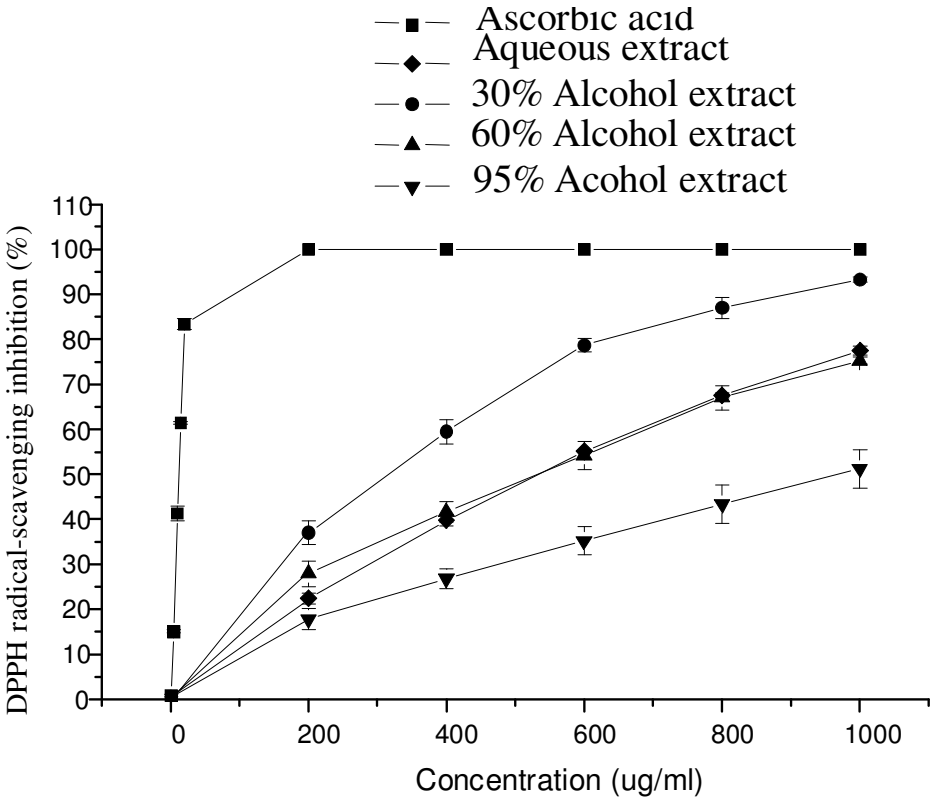

Figure 1. The antioxidant activities of the four extracts of roselle in DPPH free radical-scavenging assays. Ascorbic acid was used as a positive control. Data were presented as mean ± SD (n = 3). DPPH, 2,2-Diphenyl-2-picrylhydrazyl hydrate.

After a 30-min-incubation at 40°C, the mixture was left in an ice-bath for 5 min and then the absorbance was measured at 490 nm. The tests were carried out in triplicate. The total saccharides of sample were quantified with the standard curve mentioned above. The total saccharide values were presented in milligram (mg) anhydrous dextrose equivalent / g extract.

Statistical analysis

Data were reported as mean ± standard deviation (SD) of triplicate determinations. Statistical calculations by SPSS version 16.0 software (SPSS Inc, Chicago, USA) were carried out. One way analysis of variance was applied for determining differences between results of samples. Values of $p < 0.05$ were considered as significantly different.

RESULTS AND DISSCUSSION

Antioxidant capacity

In this study, the antioxidant properties of the aqueous and different ethanol extracts of calyx and fruit of roselle were tested, and then compared with the activity of the well known antioxidants ascorbic acid. Moreover, the IC_{50} values, which refer to the smallest concentration of the antioxidants necessary for 50% of inhibition, were calculated.

The sequence for DPPH radical-scavenging abilities were shown in Figure 1: 30% ethanol extract > 60% ethanol extract > aqueous extract > 95% ethanol extract. The IC_{50} values of DPPH radicals scavenging for the aqueous and different ethanol extracts from calyx and fruit of roselle were shown in Table 1. The IC_{50} values of these four extracts were 486.52 ± 10.56, 289.01 ± 16.68, 465.88 ± 36.94 and 1051.72 ± 184.20 µg/ml, respectively. In the hydroxyl radical-scavenging assay, the antioxidant activities were in the similar order: 30% ethanol extract > aqueous extract > 95% ethanol extract > 60% ethanol extract (Figure 2), and IC_{50} values were 1097.76 ± 237.63, 1238.50 ± 108.40, 1268.66 ± 71.24 and 1540.83 ± 140.97 µg/ml, respectively (Table 1). The scavenging effects on the ABTS radical also showed that the 30% ethanol extract had the lowest IC_{50} value (423.25 ± 31.38 µg/ml) and highest antioxidant activity (Figure 3 and Table 1). Meanwhile the 95% ethanol extract has the highest IC_{50} value (2087.08 ± 201.55 µg/ml) in ABTS assay (Table 1).

Extract yields and content determination

Distilled water, 30% ethanol, 60% ethanol and 95%

Table 1. The yields of four extracts from calyx and fruit of roselle and antioxidant activity of four extracts of calyx and fruit of roselle.

Sample	Yield (%)	IC_{50} (μg / ml)		
		DPPH	OH	ABTS
Aqueous extract	36.75 ± 0.92[a]	486.52 ± 10.56[b]	1238.50 ± 108.40[a]	564.24 ± 25.59[b]
30% Ethanol extract	37.35 ± 1.06[a]	289.01 ± 16.68[c]	1097.76 ± 237.63[b]	423.25 ± 31.38[c]
60% Ethanol extract	35.11 ± 0.40[a]	465.88 ± 36.94[d]	1540.83 ± 140.97[a]	916.70 ± 71.97[d]
95% Ethanol extract	20.88 ± 0.69[b]	1051.72 ± 184.20[a]	1268.66 ± 71.24[a]	2087.08 ± 201.55[a]
Ascorbic acid	-	10.91 ± 0.15[e]	30.70 ± 2.22[c]	20.87 ± 0.38[e]

Data were presented as mean ± SD (n=3).[a, b, c, d, e] Values in the same column followed by a different letter are significantly different ($p<0.05$, Student's *t*- test). DPPH, 2,2-Diphenyl-2-picrylhydrazyl hydrate; OH, hydroxyl; ABTS, 2-2'-azino-bis-(3-ethyl-benzthia-zoline-6-sulfonic acid).

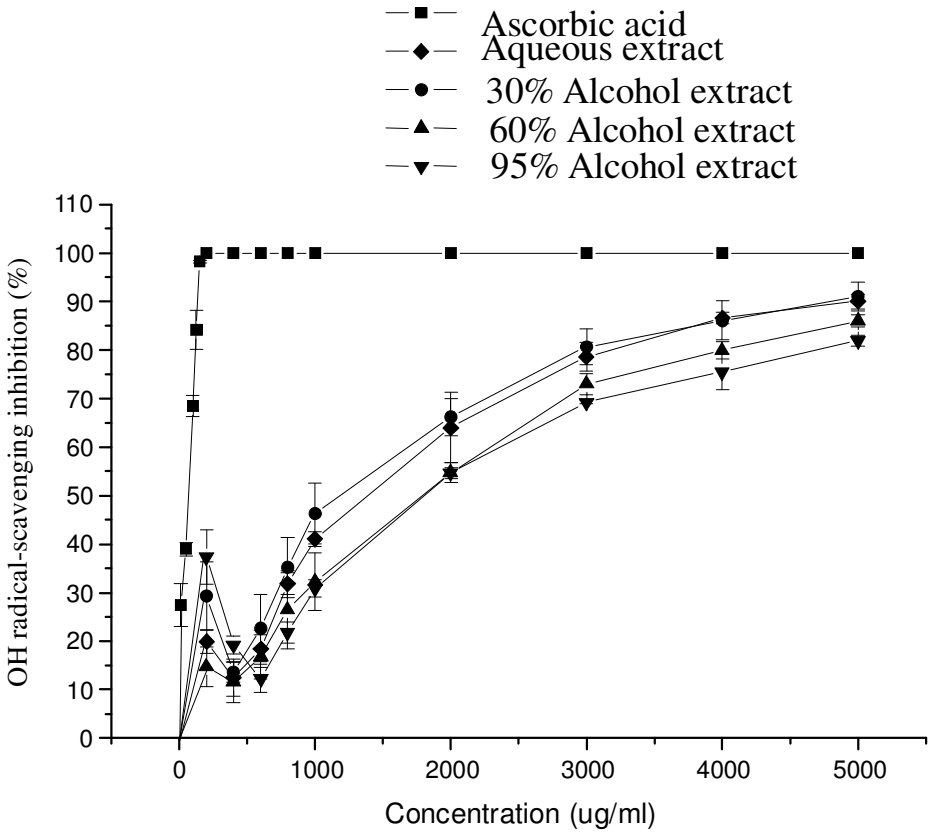

Figure 2. Antioxidant activities of the four extracts of roselle in OH free radical-scavenging assays. Ascorbic acid was used as a positive control. Data were presented as mean ± SD (n = 3). OH, Hydroxyl.

ethanol were used as the extraction solvents, and the yields were 36.75 ± 0.92, 37.35 ± 1.06, 35.11 ± 0.40 and 20.88 ± 0.69%, respectively. The contents of total phenols, anthocyanins and total saccharides of different extracts of calyx and fruit of roselle are presented in Table 2. Results indicate that the 30% ethanol extraction exhibited the highest total phenols content (20.25 mg GAE/ g). The content of anthocyanins decreased in the following order: 60% ethanol > 30% ethanol > aqueous > 95% ethanol. Furthermore, the aqueous extract showed the highest content of total saccharides (261.00 ± 16.51 mg anhydrous dextrose equivalent/100 g), followed by 30% ethanol extract and 60% ethanol extract. Our results reveal that 30% ethanol extract contained remarkable levels of total phenols, anthocyanins and total saccharides. Various solvents would affect extraction

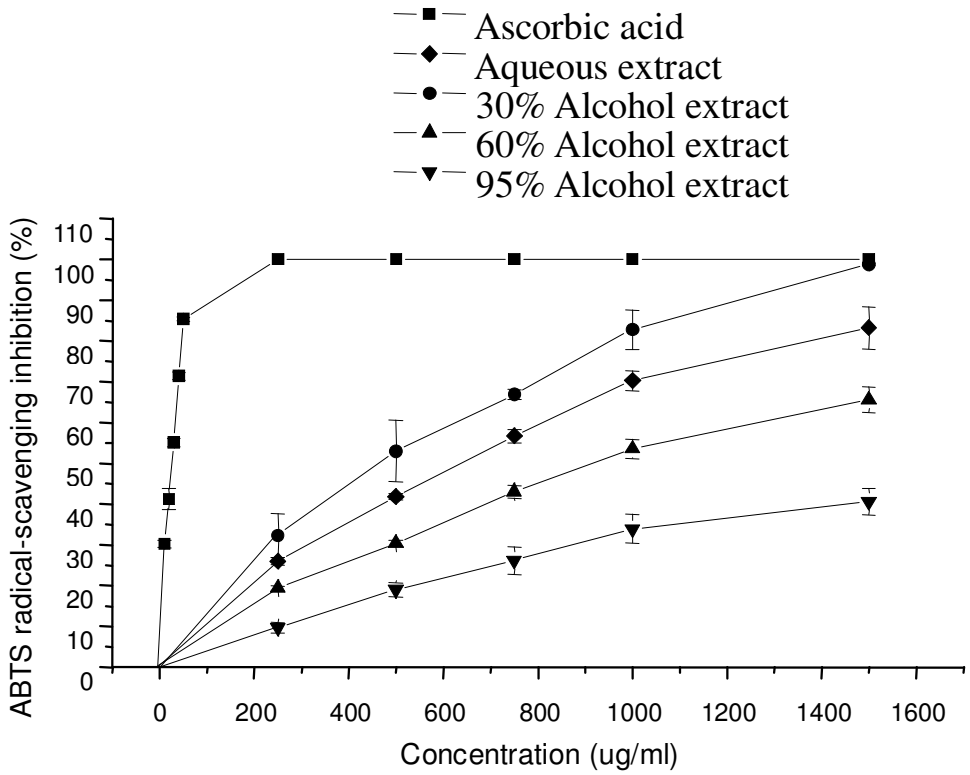

Figure 3. Antioxidant activities of the four extracts of roselle in ABTS free radical-scavenging assays. Ascorbic acid was used as a positive control. Data were presented as mean ± SD (n = 3). ABTS, 2-2'-Azino-bis-(3-ethyl-benzthia-zoline-6-sulfonic acid).

Table 2. The contents of total phenols, anthocyanins and total saccharides of four extracts of calyx and fruit of roselle.

Sample	Total phenol[d] (mg GAE /g)	Anthocyanin (mg cy-3-glu equivalent /g)	Total saccharides[e] (mg anhydrous dextrose equivalent/g)
Aqueous extract	19.51 ± 0.48[a]	0.18 ± 0.012[a]	261.00 ± 16.51[a]
30% Ethanol extract	20.25 ± 0.74[b]	0.20 ± 0.017[a]	211.71 ± 37.89[a]
60% Ethanol extract	18.33 ± 0.44[a]	0.24 ± 0.032[b]	188.22 ± 29.60[a]
95% Ethanol extract	7.05 ± 0.38[c]	0.12 ± 0.012[c]	138.61 ± 18.56[b]

Data were presented as mean ± SD (n=3).[a, b, c]Values in the same column followed by a different letter are significantly different ($p<0.05$, Student's *t*- test). [d]Standard curve for total phenols: $y = 8.3820 x + 0.1153$, $r^2 = 0.9992$; [e]standard curve for total saccharides: $y = 0.0095 x + 0.0053$, $r^2 = 0.9999$.

efficiencies of these components.

Correlations between antioxidant activity and contents of total phenols, anthocyanins and total saccharides

Correlations were studied between the antioxidant activities and contents of total phenols, anthocyanins and total saccharides. It was observed that 30% ethanol extract had the highest radical-scavenging activities among the four extracts, while the 95% ethanol extract had much lower results. Correlation analysis (Table 3) showed that the higher activities of 30% ethanol extract were probably based on its higher contents of total phenols ($R^2 = 0.9615$, 0.9089 and 0.9771, respectively).

Conclusion

Our results clearly show that 30% ethanol extract of calyx and fruit of roselle had strong antioxidant activities. Our study suggests that this extract could be used as an effective and safe antioxidant source. Notably, our correlation study indicates that the total phenol contents of this extract played major roles in the antioxidant

Table 3. Correlations established between the antioxidant activity (IC_{50} values) and the contents of total phenols, anthocyanins and total saccharides.

Value	Equation[a]		
	Total phenols	Anthocyanins	Total saccharides
IC_{50} of DPPH assay	Y= -0.0523x + 1.4249, R^2 = 0.9615, p < 0.05	Y = -5.4448x + 1.5806, R^2 = 0.6763, p > 0.05	Y = -0.0047x + 1.5186, R^2 = 0.5283, p > 0.05
IC_{50} of Hydroxyl assay	Y= -0.0284x + 1.7497, R^2 = 0.9089, p < 0.05	Y = -2.6805x + 1.7823, R^2 = 0.5235, p > 0.05	Y = -0.0026x + 1.8152, R^2 = 0.5279, p > 0.05
IC_{50} of ABTS assay	Y = -0.1203x + 2.9564, R^2 = 0.9771, p < 0.05	Y = -10.895x + 3.0134, R^2 = 0.5203, p > 0.05	Y = -0.0128x + 3.5484, R^2 = 0.7389, p > 0.05

[a]x represents the content of total phenols, anthocyanins or total saccharides. Y represents IC_{50} (mg / ml).

activities.

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