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The bioavailability report of turmeric powder and fermented turmeric powder

New Bellus Research Center and TRI NEO BIOTECHNOLOGY

The TRI NEO BIOTECHNOLOGY was commissioned to execute the assay and write the report. The TRI NEO BIOTECHNOLOGY address is 20F., No. 81, Sec. 1, Xintai 5th Rd., Xizhi Dist., New Taipei City 221, Taiwan (R.O.C.)

Abstract

The MDCK cell line is derived from canine kidney epithelial cells. It grows fast and takes only 2 to 6 days to form a complete and dense cell monolayer. In the permeability study of drug absorption, MDCK cells are seeded in an insert cell culture dish (transwell) The polyester membrane grows to form a polarized cell layer, which can be used as a model for studying two-way drug transport, rapid screening, transport mechanism and prediction of in vivo absorption.

The purpose of this study is to test the difference in permeability of test substances-turmeric powder and fermented turmeric powder in MDCK cell *in vitro* drug absorption permeability test. The test is divided into two parts. The first part is to test whether the test substance is cytotoxic to MDCK, so as to screen out the suitable test substance preparation concentration in the subsequent drug absorption and permeability test, and implant the MDCK canine kidney epithelial cell line in a 96-well plate, after the cells are attached, remove the cell culture solution and add the test substance. The cell survival rate of the test substance solution containing Fermented turmeric powder 200 μg/mL, 100 μg/mL, 50μg/mL, 25 μg/mL and 12.5 μg/mL was 105.7%, 100.2%, 99.8%, 100.3%, 102.8%, respectively. The cell survival rate of the test substance solution containing Turmeric powder 200 μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL and 12.5 μg/mL was 102.3%, 99.5%, 101.4%, 102.4%, 111.0%, respectively. The test substance solution of each concentration has no significant difference with the negative control group.

In the drug absorption and permeability test, the Papp values of solutions containing fermented turmeric powder 200 and 100 μg/mL were 0.153, 6.597, 9.053 cm/s and 0.058, 0.066, 2.730 cm/s at 4.5, 24, and 48 hours, respectively. The Papp values of the test substance solution containing turmeric powder 200 and 100 μg/mL was 0.013, 0.384, 1.445 cm/s and 0.023, 0.007, 0.004 cm/s at 4.5, 24, and 48 hours, respectively. The data showed that the fermented turmeric powder had the better drug absorption and permeability after 24 and 48 hrs.

Objectives

The objectives of this study are to test the difference in permeability of test substances-turmeric powder and fermented turmeric powder in MDCK cell *in vitro* drug absorption permeability test.

Materials and Methods

The turmeric powder and fermented turmeric powder were provided by New Bellus Enterprises Co., Ltd. The MDCK cell line was obtained from BCRC (BCRC #60004). Mycoplasma detection assay was followed by the operation standard for mycoplasma detection (SHGLP-SOP-CE-005). The cell culture incubation protocol was followed the by the cell culture operation standard book (SHGLP-SOP-CE-002).

The test substance was extracted with 95% alcohol and adjusted the concentration to 20 mg/mL. The test substance was centrifuged at ultra-high speed, and the supernatant was passed through a 0.2 μm sterile filter membrane. The extract is yellow clear liquid. Test substance group was prepared by taking the extracted stock solution and mixed with a blank cell culture solution to adjust the concentration to 200 μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL and 12.5μg/mL for cytotoxicity test. A blank cell culture solution was prepared at two concentrations of 200 μg/mL and 100 μg/mL for drug absorption and permeability tests. The negative control was 1% of 95% EtOH at cell culture liquid.

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Cell line culture

The frozen MDCK cells are thawed and cultured in DMEM medium at 37±1°C and 5±1% CO_2 in air. After the cells grow, observe the growth state with a microscope, and use the cells in the experiment after subculture for 2 to 3 generations.

Cytotoxicity MTT assay

The cells were seeded in a 96-well plate with a density of 1×10^5/well for 22 to 26 hrs at 37±1°C and 5±1% CO_2 in air. Remove the cell culture medium, added the prepared culture medium of different concentrations of test substance (200 μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL and 12.5 μg/mL), and incubate in a CO_2 incubator for 24±2 hours(5±1% CO_2 , 37±1°C). After reaction, 10 μL of 5 mg/mL MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] solution was added (final concentration 0.5 mg/mL and stock solution 5 mg/mL MTT in PBS), for 3-4 hrs. The MTT solution and medium were removed and 100 μL DMSO was added to each well. Absorbance was measured at 570 nm using the ELISA microplate reader.

$$\text{Survival rate (\%)} = \frac{OD_{570\,e}}{OD_{570\,b}} \times 100\%$$

$OD_{570\,e}$ is the average absorbance of the test substance.
$OD_{570\,b}$ the average absorbance of the negative control.

The curcumin mass spectrometry detection assay

Use LC/MS/MS (Thermo TSQ Quantum Access MAX), carried out negative ion electrospray ionization (ESI-) scanning mode, liquid chromatography system is Accela 1250 pump and Accela Open Autosampler. The chromatography column used is XSelect™ CSH™ Phenyl Hexyl, 2.5 μm, with an inner diameter of 2.1 mm × 10 cm, the temperature of the chromatography column is set to 40°C, and the injection volume of each needle is 10 μL. Mobile phase solution: A solution (0.2% FA in H_2O) and B solution (ACN) are analyzed at a mixing ratio of 45:55. The ion pair parameters of curcumin compounds are set as the following table :

Compoun	Parent ion	Molecular ion	Collision energy
Curcumin	366.87	134.00	35
	366.87	149.00	20
	366.87	217.00	14

Permeability models for drug transport

Take fast-growth cells, adjust the cell concentration to $5x10^4$/mL, added the suspended test cells into the upper layer of polyester membrane of a 24-well culture dish, added 400 μL per well, and added the 800 μL blank culture medium in lower layer, incubate in an environment containing 5±1% carbon dioxide and a temperature of 37±1°C for 4 days, and wait until the cells grow full. Observe with a microscope before the start of the experiment to confirm that the cells are in normal condition then start the experiment. Remove the cell culture medium, add the prepared culture medium of different concentrations of test substance (200 μg/mL, 100 μg/mL), and culture in a CO_2 incubator (5±1% CO_2, 37±1 °C). At different time points (4.5, 24, and 48 hours), take 50 μL from the lower cell fluid, and added 450 μL of MeOH to precipitate the protein and collect the supernatant by centrifugation and analyze the concentration of curcumin by LC-MS/MS.

$$\text{Papp (cm/s)} = \left(\frac{dQ}{dt}\right) \times \left(\frac{1}{A}\right) \times \left(\frac{1}{C0}\right)$$

dQ/dt: the volume of curcumin transported per unit time point

dQ: the total amount of the substance to be tested; the sample to be tested collected per unit time, multiply the dilution factor and the volume of the culture medium in the well (mL) to calculate the total amount of the sample (ng)

dt: time; the total number of seconds per unit time to collect samples

A: transport membrane area (fixed value; 0.33 cm^2)

C0: initial drug concentration; the peak area of curcumin is measured by LC-MS/MS and then brought back to the calibration line to calculate the initial sample concentration.

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Results

Cytotoxicity assay

The cell survival rate data was showed as in Table 2. The cell survival rates of the test substance solutions containing fermented turmeric powder 200 μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL and 12.5 μg/mL were 105.7%, 100.2%, 99.8%, 100.3%, 102.8%, respectively. The cell survival rate of the test substance solution containing turmeric powder 200 μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL and 12.5 μg/mL was 102.3%, 99.5%, 101.4%, 102.4%, 111.0%, respectively. There was no significant difference between the test substance solution of each concentration and the negative control group, and the result showed that the test substance had no toxic reaction. Based on the results of this test, the concentration of the test substance which is suitable for the subsequent drug absorption permeability test was screened out.

Linear relationship of Curcumin calibration curve

Take the curcumin standard stock solution, dilute it with MeOH and prepare the sample solution with the concentration of 1, 5, 10, 50, 100 and 200 ng/mL, and analyze it by LCMS/MS. Using curcumin concentration as the abscissa and the ratio of curcumin to internal standard peak area as the ordinate, the calibration curve colud be drawn. The R2 value obtained from the linear regression of the calibration curve was 0.9955, which was indicated that the concentration of curcumin has a good linear relationship between 1-200 ng/mL (Figure 1 and Table 1).

Drug absorption and permeability assay

The lower curcumin concentration data was showed as in Table 3 and Figure 2. The curcumin concentration of the test substance solution containing fermented turmeric powder 200 and 100 μg/mL were 0.21, 48.15, 132.15 ng/mL and 0.04, 0.24, 19.93 ng/mL at 4.5, 24, and 48 hours respectively. The curcumin concentration of the test substance solution containing turmeric powder 200 and 100 μg/mL were 0.02, 3.15, 23.73 ng/mL and 0.02, 0.03, 0.03 ng/mL at 4.5, 24, and 48 hours respectively.

In addition, Apparent Penetration Coefficient (Papp) is an internationally accepted indicator of drug permeability. In order to calculate the Papp, this test also detected that the initial concentrations of 200 and 100 μg/mL curcumin of fermented turmeric

powder were 204.80 and 102.41 ng/mL, respectively; and the initial concentration values of 200 and 100 μg/mL curcumin for turmeric powder were 230.41 and 97.65 ng/mL, respectively.

The Papp values calculated from the lower curcumin concentration and the initial curcumin concentration are showed as in Table 4 and Figure 3. The Papp values of test substance solutions containing fermented turmeric powder 200 and 100 μg/mL were 0.153, 6.597, 9.053 cm/s and 0.058, 0.066, and 2.730 cm/s at 4.5, 24, and 48 hours, respectively. The Papp values of test substance containing turmeric powder 200 and 100 μg/mL were 0.013, 0.384, 1.445 cm/s and 0.023, 0.007, 0.004 cm/s at 4.5, 24, and 48 hours, respectively. According to the above data, it was indicated that the fermented turmeric powder has better drug absorption and permeability after 24 and 48 hours.

References

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5. *In vitro* metabolism- and transporter- mediated drug-drug interaction studies guidance for industry (2017). Clinical Pharmacology.

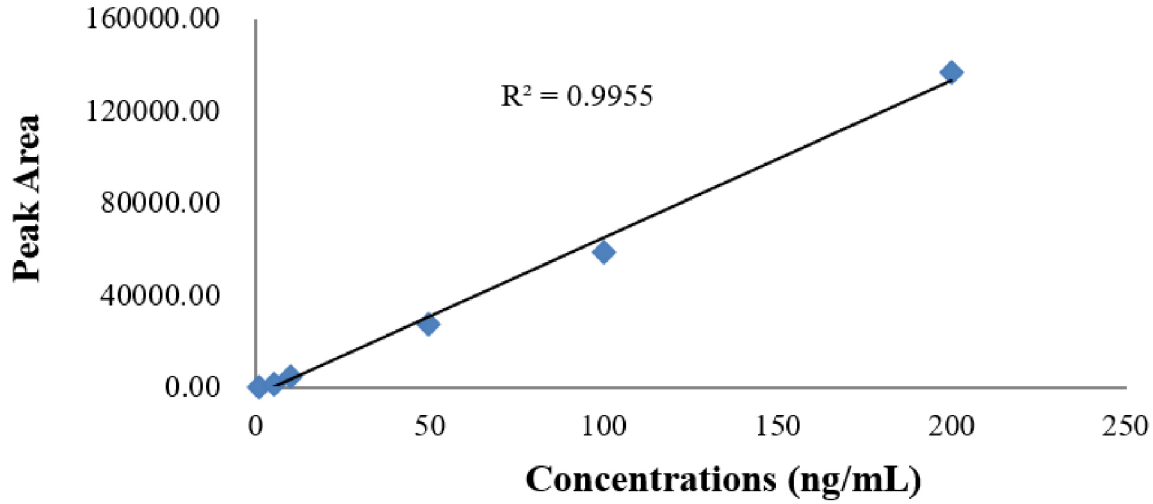

Figure 1. The calibration line of curcumin.

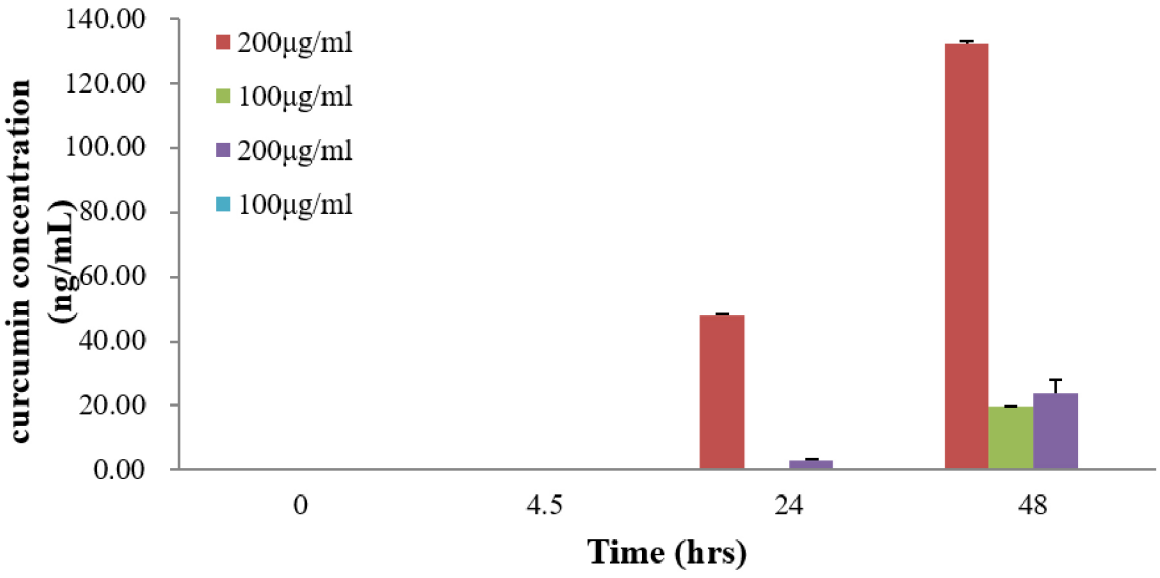

Figure 2. The curcumin concentration in lower layer fluid in MDCK cell absorption and permeability test.

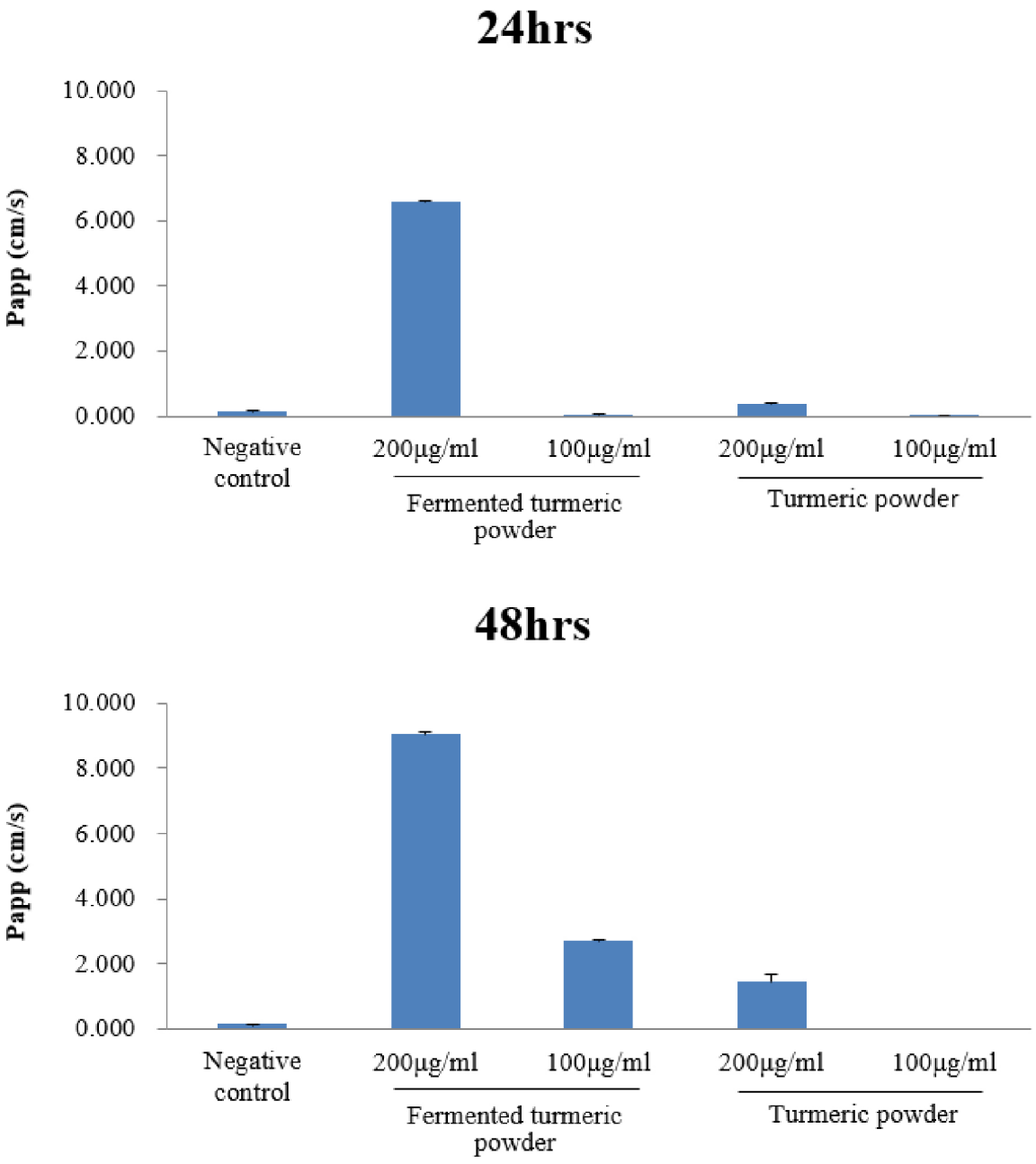

Figure 3. The Papp of curcumin at 24 hours and 48 hours.

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Table 1. The calibration curve value of curcumin.

	Standard concentrations (ng/mL)	Peak area
Standard 1	1	154.00
Standard 2	5	1406.97
Standard 3	10	4181.74
Standard 4	50	27682.76
Standard 5	100	59075.26
Standard 6	200	136349.26

Table 2. The survival rate in cytotoxicity MTT assay.

95 % EtOH extraction of fermented turmeric powder	Absorbance (OD_{570} nm)	Survival rate (%)
Negative control (NC)	0.445±0.039	100.0
Test sample extract (12.5 μg/mL)	0.457±0.075	102.8
Test sample extract (25 μg/mL)	0.446±0.025	100.3
Test sample extract (50 μg/mL)	0.444±0.016	99.8
Test sample extract (100 μg/mL)	0.446±0.028	100.2
Test sample extract (200 μg/mL)	0.470±0.024	105.7
95% EtOH extraction of turmeric powder	**Absorbance (OD_{570} nm)**	**Survival rate (%)**
Negative control (NC)	0.395±0.009	100.0
Test sample extract (12.5 μg/mL)	0.438 ±0.055	111.0
Test sample extract (25 μg/mL)	0.404±0.014	102.4
Test sample extract (50 μg/mL)	0.400±0.014	101.4
Test sample extract (100 μg/mL)	0.393±0.025	99.5
Test sample extract (200 μg/mL)	0.404±0.037	102.3

$$\text{Survival rate (\%)} = \frac{OD_{570\,e}}{OD_{570\,b}} \times 100\%$$

$OD_{570\,e}$ is the average absorbance of the test substance.
$OD_{570\,b}$ the average absorbance of the negative control.

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Table 3. The curcumin concentration value in lower layer fluid in MDCK cell absorption and permeability test.

Group	Time (hrs)	LC-MS/MS (ppb)			
		0	4.5	24	48
Negative control		0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00
	Mean±SD	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Fermented turmeric powder	200 μg/mL	0.00	0.22	48.30	132.80
		0.00	0.20	48.00	131.50
	Mean±SD	0.00 ± 0.00	0.21 ± 0.01	48.15 ± 0.21	132.15 ± 0.92
	100 μg/mL	0.00	0.05	0.25	19.90
		0.00	0.03	0.23	19.95
	Mean±SD	0.00 ± 0.00	0.04 ± 0.01	0.24 ± 0.01	19.93 ± 0.04
Turmeric powder	200 μg/mL	0.00	0.02	2.90	20.85
		0.00	0.02	3.40	26.60
	Mean±SD	0.00 ± 0.00	0.02 ± 0.00	3.15 ± 0.35	23.73 ± 4.07
	100 μg/mL	0.00	0.01	0.02	0.02
		0.00	0.02	0.03	0.03
	Mean±SD	0.00 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01

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Table 4. The Papp calculated value in MDCK cell absorption permeability test.

Group	Time (hrs)	LC-MS/MS (ppb)		
		4.5	24	48
Negative control		0.000	0.000	0.000
		0.000	0.000	0.000
	Mean±SD	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Fermented turmeric powder	200 μg/mL	0.161	6.617	9.097
		0.146	6.576	9.008
	Mean±SD	0.153±0.010	6.597±0.029	9.053±0.063
	100 μg/mL	0.073	0.068	2.726
		0.044	0.063	2.734
	Mean±SD	0.058 ± 0.021	0.066± 0.004	2.730±0.005
Turmeric powder	200 μg/mL	0.013	0.353	1.270
		0.013	0.414	1.620
	Mean±SD	0.013 ± 0.000	0.384±0.043	1.445±0.248
	100 μg/mL	0.015	0.006	0.003
		0.031	0.009	0.004
	Mean±SD	0.023 ± 0.011	0.007 ± 0.002	0.004 ± 0.001

$$\text{Papp (cm/s)} = \left(\frac{dQ}{dt}\right) \times \left(\frac{1}{A}\right) \times \left(\frac{1}{C0}\right)$$

dQ/dt: the volume of curcumin transported per unit time point

dQ: the total amount of the substance to be tested; the sample to be tested collected per unit time, multiply the dilution factor and the volume of the culture medium in the well (mL) to calculate the total amount of the sample (ng)

dt: time; the total number of seconds per unit time to collect samples

A: transport membrane area (fixed value; 0.33 cm^2)

C0: initial drug concentration; the peak area of curcumin is measured by LC-MS/MS and then brought back to the calibration line to calculate the initial sample concentration.

The initial concentration of curcumin:
Fermented turmeric powder (200 μg/mL): 204.80 ng/mL;
Fermented turmeric powder (100 μg/mL): 102.41 ng/mL;
Turmeric powder (200 μg/mL): 230.41 ng/mL;
Turmeric Powder (100 μg/mL): 97.65 ng/mL.