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RESEARCH ARTICLE

Rp- hplc facilitated quantitative analysis of tectorigenin in the different species of *iris* plant and evaluation of its invitro anticancer potential

Sajad H. Wani, Asif amin, Shahnawaz N. Sofi, Taseem A. Mokhdomi, [1]Shoiab Bukhari, Qazi Parvaiz Hassan and Raies A. Qadri

[1]Department of Biotechnology, University of Kashmir
[2]Indian Institute of Integrative Medicine (CSIR) Sanat Nagar, Srinagar

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ABSTRACT

A simple HPLC-UV-DAD method was developed for rapid identification and quantitation of tectorigenin in five species of genus *Iris* viz *Iris crocea, Iris ensata, Iris germanica, Iris kashmeriana and Iris spuria* growing wild in Kashmir valley. The analysis was performed by using Chromolith RP-18e analytical column (5 µm; 4.6 mm × 100 mm) at isocratic elution of methanol and water (30:70, v/v) with diode array detection at 265nm. The calibration curve showed good linearity ($r^2 > 0.998$) within test ranges and recoveries were 98.2 to 101.2%. The optimized method was successfully applied for the analysis of tectorigenin in 12 samples of five species of *Iris* collected from different eco-geographical zones. The tectorigenin content in investigated samples was greatly variant ranging from 1.08% to 8.84% with a maximum in *Iris germanica* collected from Gulmarg region of Kashmir valley. The method established in this paper is simple and reliable and could easily be used for the qualitative analysis of tectorigenin in *Iris* species. Furthermore, to evaluate anti-cancer properties, different concentrations of tectorigenin were tested against colon cancer cell line, Caco-2 using MTT cell proliferation assay. The results showed a prominent growth inhibition in a concentration dependent manner with maximum inhibition at a concentration of 100µM.

INTRODUCTION

Iris is the largest and most complicated genus of Iridaceae having variety of plants with showy flowers ranging in colour from gold or yellow to white, blue, lavender and purple. There are more than 60 species found in China [1], 37 in Turkey [2], 16 in Pakistan [3, 4], 12 in India [5], 3 in Egypt [6]. *Iris* species have gained a great popularity due to their sweet violet fragrance interested in the perfume and cosmetic industries [7]. Many members of the genus *Iris* have also been used as traditional folk medicine for the treatment of various diseases. Its rhizomes are commonly used in Chinese folk medicine for clearing heat and detoxifying, eliminating phlegm, swelling, and pain in the throat [8]. The peeled and dried rhizomes of *Iris* collectively known as rhizome iridis enjoyed popularity due to their emetic, cathartic, stimulant, expectorant and errhine properties (9). Rhizomes of this plant are used for fever [10] and roots are used for kidney infections [11]. *Iris* species have been used for the treatment of inflammation, bacterial infection, or cancer [12, 13]. The phytochemistry of this genus has been the subject of extensive investigation and found to be a rich source of flavones, isoflavones [14, 15] and quinones [16]. These classes of compounds have attracted considerable attention because of their antioxidant [17], cytotoxic [18], anti-microbial, anti-inflammatory and phytoesterogenic properties [19-21]. The preventive role of isoflavones in cancer, cardiovascular diseases, osteoporosis, and menopausal symptoms is well documented [22, 23, 24]. In traditional Chinese medicine, it was used as a bitter medicine to treat disorders described as *Zheng Jia Jie Ju*, which are similar to modern descriptions of tumors [25-26].

Corresponding author: raies@kashmiruniversity.ac.in

Phytochemistry of *Iris* species is dominated by the presence of polyphenols especially isoflavones [27]. Tectorigenin, one of the most important isoflavone isolated from different *Iris* species has got diverse pharmacological properties [28,29]. The present work describes the qualitative, quantitative as well as Bioevaluation analysis of this magic molecule in some selected species of genus *Iris* growing wild in Kashmir valley. The analysis was performed using HPLC coupled with diode array detection. The present work is the first report of its kind in which tectorigenin was quantified in five species of *Iris* growing in Kashmir. Further the effects of tectorigenin were tested against colon cancer cell line, Caco-2 using MTT cell proliferation assay.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The optimization of HPLC conditions was performed using the standard solution of tectorigenin first and then solution of IC-3 sample. Peak resolutions were tested and compared using different solvent systems (acetonitrile-methanol, acetonitrile-water, and methanol-water) in varying proportions. The separation of tectorigenin was achieved on a RP-18e column using methanol-water (30:70; v/v) at a flow rate of 0.6 ml/min with a runtime of 55 minutes. Photodiode array detector (DAD) was used in HPLC analysis and the optimum monitor wavelength at 265nm was selected from the full range spectra.

Calibration curves and recovery

Methanol stock solution of the standard reference compound tectorigenin was prepared and diluted to appropriate concentrations for the construction of calibration curve. At least four concentrations

of tectorigenin were injected in the concentration range of 0.08µg/ml to 0.96µg/µl. The calibration curve was constructed by plotting the peak area versus the concentration of each analyte with detector wavelength set at 265 nm. Excellent calibration curve was obtained for the standard within the concentration range of 0.08 µg to 0.96 µg/µl (r^2 = curve coefficients > 0.998). The recovery study was within the concentration range of the calibration curve. The recovery of the method was estimated by spiking IK-1 sample with 2.0 µg/3.8 mg of the standard tectorigenin. The data from the recovery studies revealed that the recovery of the method was in the range of 98.2 to 101.2% evaluating the accuracy of the method.

Quantification of tectorigenin in five different species of *Iris*

Tectorigenin was separated in all the samples using the developed HPLC method. Typical chromatograms of the different extracts and the mixed standard are shown in Fig. 2. The identification of the investigated compound was carried out by comparison of retention times and UV spectra with those obtained by injecting standards in the same conditions.

The developed HPLC method was applied to analyse tectorigenin in twelve samples of five species of *Iris*. The data are summarized in Table 2. The results showed that the content of tectorigenin was greatly variant in the rhizomes of five different *Iris* species (Fig. 2) and the content also varied in samples from different eco-geographical zones. The concentration of tectorigenin ranged from 1.08 mg/g in IC-2 sample (collected from IIIM field in June) to 8.84 mg/g in IG-2 sample (collected from Gulmarg in June 2010).

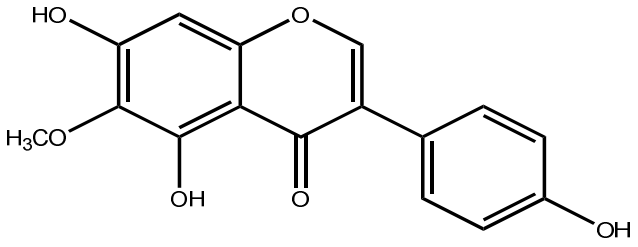


Fig. 1. Tectorigenin (5,7,4'-trihydroxy-6-methoxyisoflavone)

Antiproliferative Effects

The effect of different concentrations of tectoregenin on the proliferation of Caco-2 cells was seen to be dose dependent. The results demonstrate that percentage of growth inhibition increased with increasing concentration of tectoregenin from 15 µM to 100 µM. Tectorigenin exhibited maximum antiproliferative potential at a concentration of 100 µM and lowest at 15 µM.

Experimental

Chemicals and reagents

Methanol and water were of HPLC grade and were purchased from Merck (Mumbai, India). Tectorigenin purchased from Sigma Aldrich, India was used as an external standard for HPLC in the current study.

Plant materials

The raw plant materials of five *Iris* species were collected from different eco-geographical zones of Kashmir valley (Table 1) and after proper identification the specimens were deposited in the Herbarium of the institute under voucher specimens (No. I001/2010-I005/2010).

Sample preparation for HPLC

Stock solutions of extracts and the standard were obtained by dissolving 5 mg of each of the extract in 1.0 ml MeOH and 1.0 mg of the standard in 1.0 ml MeOH respectively. The resulting solutions were filtered through 0.45µm filter membrane. Working solutions of appropriate concentrations were prepared by diluting stock solutions. The stability of stock as well as working solutions was monitored and no change in concentration was observed.

HPLC conditions for quantitative analysis

Quantitative HPLC analysis was performed on a Schimadzu Class VP HPLC system equipped with a binary pump (LC-10AT), an autosampler (SIL-10AD), a column oven (CTO-10AS), a Diode array detector (SPD-M10A), vacuum membrane degasser (DGU-14A) and a system integrator (SCL-10A) controlled by a Class VP software which was used for data analysis and processing. Separation was carried out on a Chromolith RP-18e column (5 µm; 4.6 mm × 100 mm) with column oven temperature of 30 ^{o}C using an isocratic solvent system consisting of methanol and water (30:70; v/v). Elution was performed at a flow rate of 0.6 ml/min and the injection volume was 5.0 µl. The analytes were monitored at 265 nm.

Cell lines and cell culture conditions

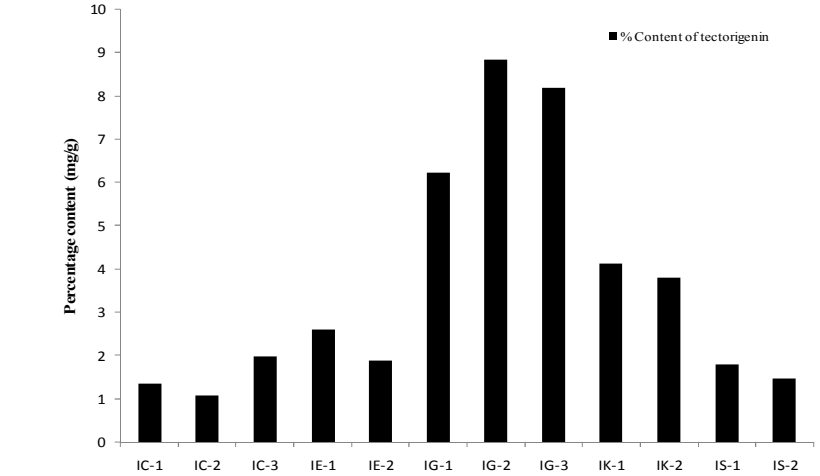
Caco-2 (Colon adenocarcinoma) cell line was kindly provided by Hybridoma Laboratory, National Institute of Immunology, India. Cells were grown in DMEM (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) under standard culture conditions at 37% in 5% $Co_{2\ in}$ a humidified incubator. 2×10^4 cells were plated per well in a 96 well plate. The media was also supplemented with 250 IU/ml penicillin (invitrogen, USA)

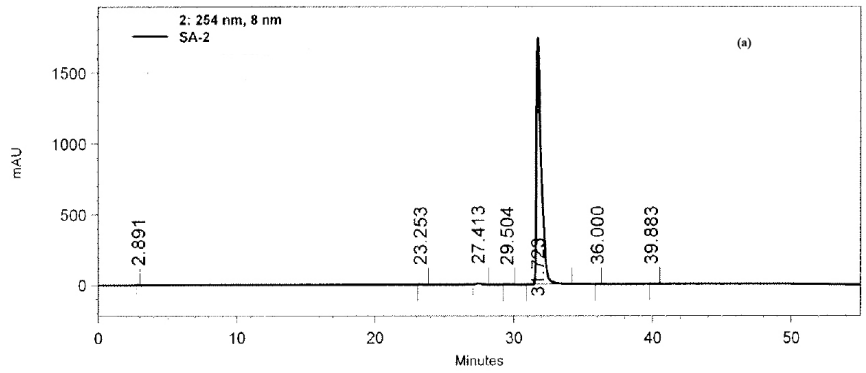
Table 1. Summary for the tested samples of *Iris*

No.	Code	Samples	Sources	Collection date
1	IC-1	*Iris* croceae	Sonamarg, Kashmir, India	May, 2010
2	IC-2	*Iris* croceae	IIIM field, Srinagar, Kashmir, India	April, 2010
3	IC-3	*Iris* croceae	Gulmarg, Kashmir, India	June, 2010
4	IE-1	*Iris* ensata	Naranag, Kashmir, India	May, 2010
5	IE-2	*Iris* ensata	IIIM field, Srinagar, Kashmir, India	April, 2010
6	IG-1	*Iris* germanica	IIIM field, Srinagar, Kashmir, India	April, 2010
7	IG-2	*Iris* germanica	Gulmarg, Kashmir, India	June, 2010
8	IG-3	*Iris* germanica	Sonamarg, Kashmir, India	May, 2010
9	IK-1	*Iris* kashmiriana	Local graveyard, Srinagar, Kashmir India	April, 2010
10	IK-2	*Iris* kashmiriana	Naranag, Kashmir, India	May, 2010
11	IS-1	*Iris* spuria	Gulmarg, Kashmir, India	June, 2010
12	IS-2	*Iris* spuria	Sonamarg, Kashmir, India	May, 2010

Table 2. Content of tectorigenin in different tested samples of *Iris* on plant dry weight basis

No.	Code	% Content of tectorigenin
1	IC-1	1.36
2	IC-2	1.08
3	IC-3	1.99
4	IE-1	2.62
5	IE-2	1.89
6	IG-1	6.24
7	IG-2	8.84
8	IG-3	8.19
9	IK-1	4.13
10	IK-2	3.80
11	IS-1	1.81
12	IS-2	1.47


% Content of tectorigenin
Percentage content (mg/g)
IC-1
IC-2
IC-3
IE-1
IE-2
IG-1
IG-2
IG-3
IK-1
IK-2
IS-1
IS-2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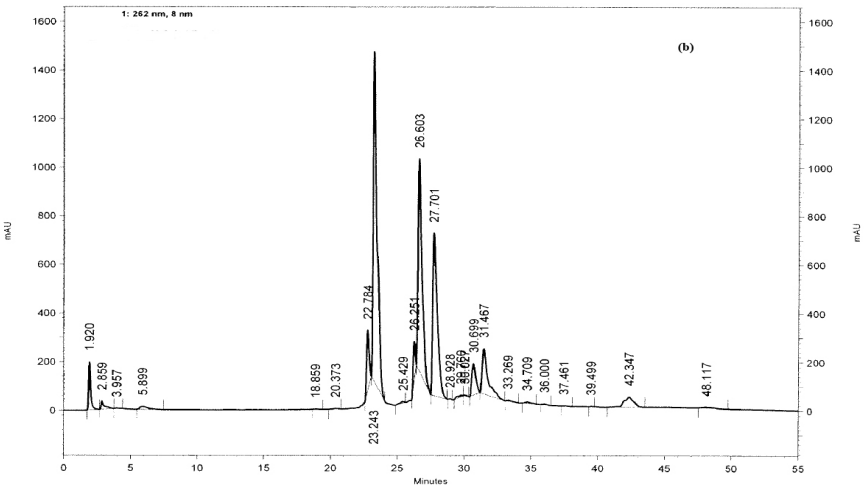
SA-2
(a)
mAU
Minutes
2.891
23.253
27.413
29.504
31.723
36.000
39.883


1: 262 nm, 8 nm
(b)
mAU
Minutes
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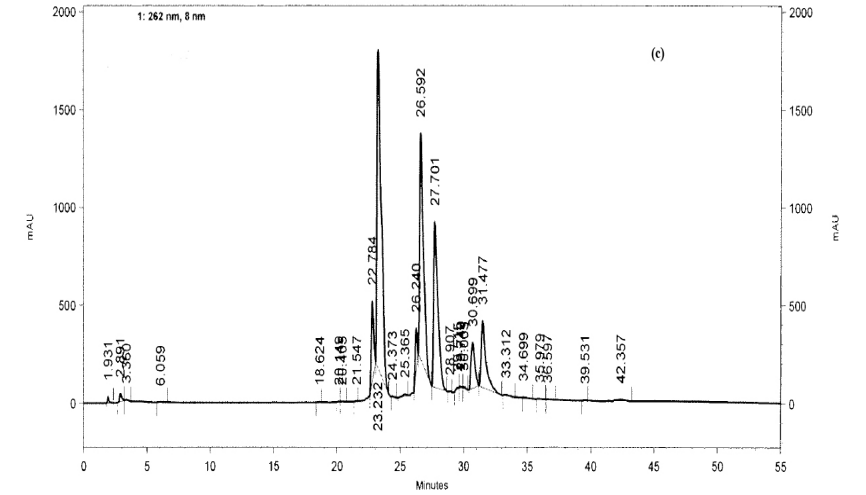
2.859
3.957
5.899
18.859
20.373
22.784
23.243
25.429
26.251
26.603
27.701
28.928
29.769
30.699
31.467
33.269
34.709
36.000
37.461
39.499
42.347
48.117


1: 262 nm, 8 nm
(c)
mAU
Minutes
1.931
6.059
18.624
21.547
22.784
23.232
24.373
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34.699
39.531
42.357

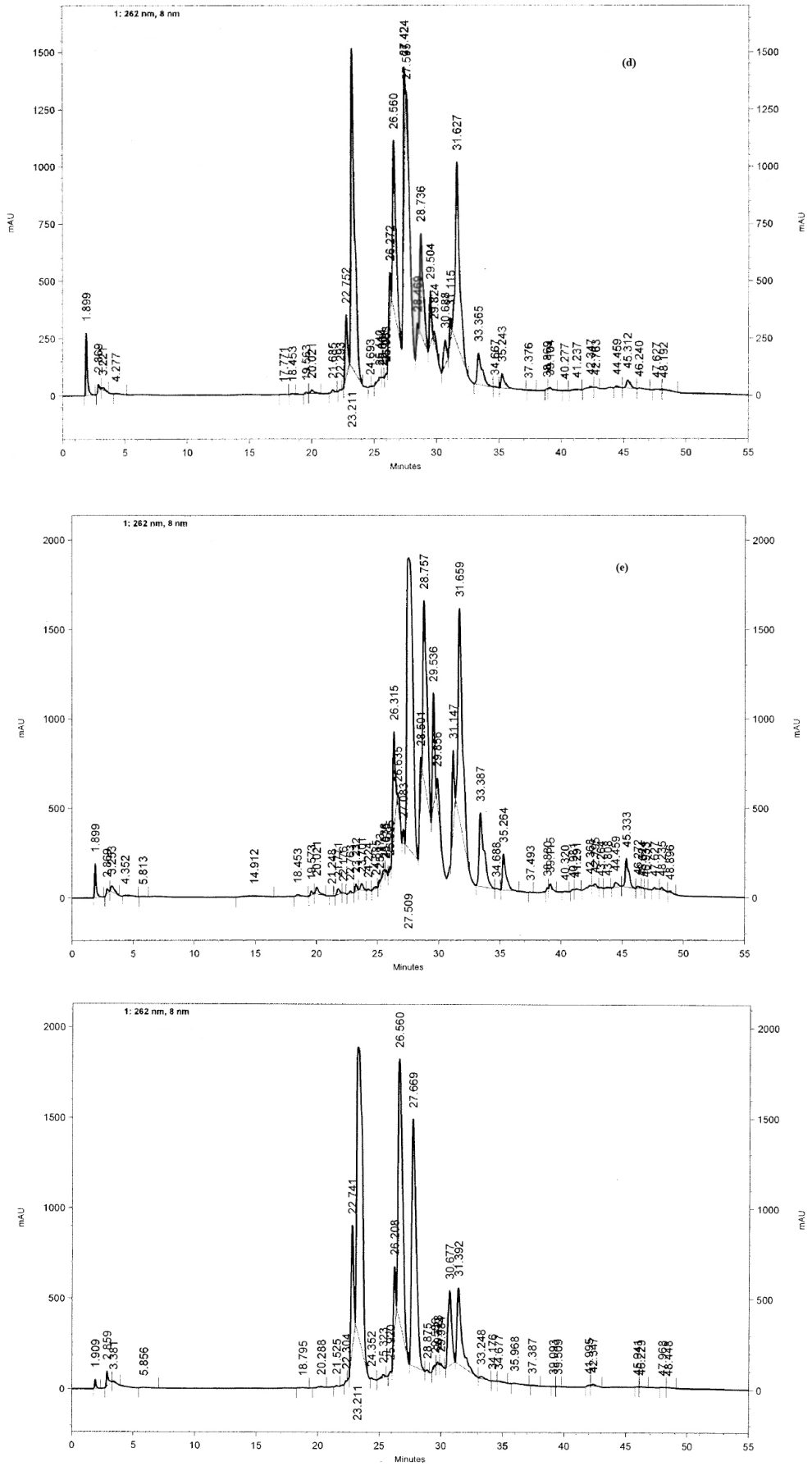

Fig. 2. Typical hplc chromatograms of (a) tectorigenin (b) IC-3 sample (c) IE-1 sample (d) IK-1 sample (e) IG-2 sample (f) IS-1 sample

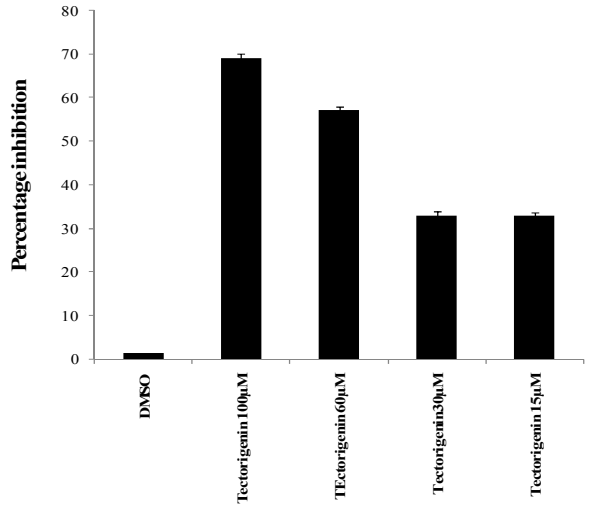

Fig.3. Effect of Tectorigenin on Caco2 cell line. The Values represent percentage inhibition of Caco2 cell lines compared to control ± SD (N=3)

Proliferation assay

The effect of tectorigenin on Caco2 cells was evaluated using MTT cell proliferation assay. The assay is based on the ability of mitochondrial succinate-tetrazolium reductase system to convert yellow tetrazolium salt MTT (sigma Aldrich, USA) to purple formazan dye that reflects the cell viability. Cell suspension (200ul) containing 2×10^4 cells per well was seeded into a 96 well microtiter plate. After 24 hours of seeding, different concentration of compound dissolved in DMSO (100µM, 60µM, 30µM, 15µM) were added. Controls consisted of either cells alone or cells treated with DMSO (solvent control). MTT solution was added to the cells at 0.1mg/ml concentration followed by incubation for 4h in 37^{o}C in dark. The supernatant was removed and an equal volume of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 565 nm (EPOCH Microplate Reader, Bio-Tek Instruments, USA).

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