Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 12 (2004) 459–467
Bioorganic & Medicinal Chemistry

Enzymatic synthesis of tea theaflavin derivatives and their anti-inflammatory and cytotoxic activities

Shengmin Sang,[a,*] Joshua D. Lambert,[a] Shiying Tian,[c] Jungil Hong,[a] Zhe Hou,[a] Jae-He Ryu,[a] Ruth E. Stark,[c] Robert T. Rosen,[b] Mou-Tuan Huang,[a] Chung S. Yang[a] and Chi-Tang Ho[b]

[a]*Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers University, 164 Frelinghuysen Road, Piscataway, NJ 08854-8020, USA*

[b]*Department of Food Science and Center for Advanced Food Technology, Rutgers University, 65 Dudley Road, New Brunswick, NJ 08901-8520, USA*

[c]*Department of Chemistry, College of Staten Island and Institute for Macromolecular Assemblies, City University of New York, 2800 Victory Boulevard, Staten Island, NY 10314-6600, USA*

Received 21 August 2003; revised 26 September 2003; accepted 14 October 2003

Abstract—Derivatives based on a benzotropolone skeleton (**9**–**26**) have been prepared by the enzymatic coupling (horseradish peroxidase/H_2O_2) of selected pairs of compounds (**1**–**8**), one with a *vic*-trihydroxyphenyl moiety, and the other with an *ortho*-dihydroxyphenyl structure. Some of these compounds have been found to inhibit TPA-induced mice ear edema, nitric oxide (NO) synthesis, and arachidonic acid release by LPS-stimulated RAW 264.7 cells. Their cytotoxic activites against KYSE 150 and 510 human esophageal squamous cell carcinoma and HT 29 human colon cancer cells were also evaluated.

1. Introduction

Tea is one of the most widely consumed beverages in the world; it has been used as a daily beverage and crude medicine in China and Japan for thousands of years. More than 300 different kinds of tea are produced from the leaves of *Camellia sinensis* by different fermentation processes and divided into three general types: green tea (non-fermented), oolong tea (semi-fermented), and black tea (fermented). Green tea and oolong tea are more popular in China, Japan, Korea and some African countries, whereas black tea is preferred in India and the Western countries. Some experimental and epidemiological studies have linked the drinking of tea to reduction in the risk of cardiovascular diseases and cancer.[1–3] These effects have been attributed to the polyphenol compounds in tea. Whereas catechins are the most abundant polyphenols in green tea, the typical pigments in black tea are theaflavins and thearubigins, which are formed by oxidation of catechins during fermentation.[4]

Theaflavins are orange or orange-red in color and possess a benzotropolone skeleton that is formed from co-oxidation of selected pairs of catechins, one with a *vic*-trihydroxyphenyl moiety, and the other with an *ortho*-dihydroxyphenyl structure.[5,6] In addition to the four major theaflavins (theaflavin, theaflavin 3-gallate, theaflavin 3′-gallate and theaflavin 3,3′-digallate), stereoisomers of theaflavins and a number of theaflavin derivatives, including theaflavic acids and theaflavates, have also been reported from black tea.[7–9] It is known that theaflavins, which account for 2–6% of the dry weight of solids in brewed black tea,[10] contribute greatly to the quality of tea in terms of color,[11] 'mouthfeel',[12] and extent of tea cream formation.[13] Recently, theaflavins have attracted considerable interest because of their potential benefits for human health, including antimutagenicity,[14] suppression of cytochrome P450 1A1 in cell culture,[15] anticlastogenic effects in bone marrow cells of mice,[16] suppression of extracellular signals and cell proliferation,[17] and anti-inflammatory and cancer chemopreventive action.[18] Theaflavins also sca-

Keywords: Black tea; Theaflavins; Benzotropolone; Enzymatic synthesis; Peroxidase; Anti-inflammatory activities; Cytotoxic activities.
* Corresponding author. Tel.: +1-732-445-3400x300; fax: +1-732-932-6776; e-mail: ssang@rci.rutgers.edu

0968-0896/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2003.10.024

venge H_2O_2[19] and have been shown to inhibit lipid oxidation,[20,21] LDL oxidation,[22] DNA oxidative damage,[19] and xanthine oxidase activity.[19]

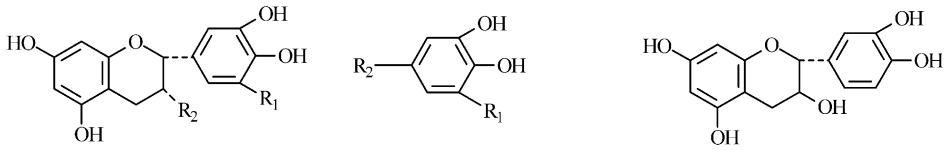
Because of their low abundance and challenging purification procedure, previous research on theaflavins has focused on using mixtures. Alternatively, chemical synthesis could allow preparation of large quantities of pure compounds for biological assays. It is well known that polyphenoloxidase (PPO) and peroxidase (POD) are key enzymes in pigment generation during the process of making black tea.[23] Many studies have been carried out on the PPO-catalyzed formation of black tea oxidation products.[24–29] Model oxidation systems have also been used to compare the oxidation products obtained with tea PPO and with horseradish POD.[30] Our recent study showed that horseradish POD can oxidize tea catechins to form theaflavin-type compounds in the presence of H_2O_2.[31] In the study presented herein, we report the synthesis of 18 theaflavin derivatives using the horseradish POD/H_2O_2 system. Their abilities to inhibit TPA-induced mouse ear edema, nitric oxide (NO) synthesis, and arachidonic acid release by LPS-stimulated RAW 264.7 cells were determined. Their cytotoxic activites against KYSE 150 and 510 human esophageal squamous cell carcinoma and HT 29 human colon cancer cells were also evaluated.

2. Results and discussion

2.1. Enzymatic synthesis of compounds 9–26

Eighteen benzotropolone derivatives (Fig. 2) have been synthesized by the reaction of selected pairs of compounds (**1**–**8**) (Fig. 1), one with a *vic*-trihydroxyphenyl moiety, and the other with an *ortho*-dihydroxyphenyl structure, using horseradish POD in the presence of H_2O_2. The reaction involves the oxidation of the B rings to quinones, followed by Michael addition of the gallocatechin quinone to the catechin quinone prior to carbonyl addition across the ring and subsequent decarboxylation (Fig. 3).[10,32] Among the products (Fig. 2), **17** (neotheaflavate B) and **22** are new compounds; **21** and **23**–**25** have been reported previously from chemical oxidation reactions but not identified in tea;[33–35] **9**–**16**, **18**–**20**, and **26** have been found in black tea. Since the major tea catechins include (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG), it is plausible that, like theaflavate A and B, neotheaflavate B may also be present as a minor compound in black tea. Work is in progress to establish the presence of neotheaflavate B in black tea using LC/MS/MS and fragment ion mass spectra of **17** and the theaflavin fraction of black tea.

The structures of the prepared compounds have been confirmed by MS and NMR spectroscopic analyses; in particular, the structure of the new compound, neotheaflavate B (**17**), which was the products of the reaction between (+)-catechin (C) (**5**) and (−)-epicatechin gallate (ECG) (**3**).[36,37] The molecular formula of **17** was determined to be $C_{36}H_{28}O_{15}$ by positive-ion APCI-MS ($[M+H]^+$ at m/z 701) as well as from its ^{13}C NMR data, which was the same as that of theaflavate B (**16**). Its ^1H and ^{13}C NMR data are very similar to those of theaflavate B. The ^{13}C NMR spectrum of **17** also displayed 36 carbon signals, 18 of which were assigned to the A and C rings of flavan-3-ols. In addition, the ^1H NMR spectrum exhibited two sets of signals, due to protons, at the 2-, 3-, 4-, 6-, and 8-positions of the flavan-3-ol nucleus. These observations also indicate that the A and C rings of **17** did not undergo any change during oxidation. In comparison with the ^1H NMR spectra of compounds **3** and **5**, compound **17** is distinguished by the absence of galloyl ester signals of **3** and the B-ring signals of **5**, a large downfield shift of H-3, and three more olefinic proton signals (δ 7.61 brs H-c; 7.64 s H-g; 8.77 brs H-e). In the ^{13}C NMR spectrum of **17**, besides the A and C ring signals, there were observed 18 carbon signals including one carbonyl (δ 186.6 s C-a), one ester carbonyl (δ 167.7 s C-l) and 16 olefinic carbons (Table 1). All of these spectral features support the presence of benzotropolone groups in **17**. Thus, the galloyl ester group on **3** can react with the B-ring of **5** to form the benzotropolone. This assertion is supported by the 2D HMBC NMR spectrum. The HMBC spectral analysis yielded correlation peaks between H-c (δ 7.61) and C-a (δ 186.6), C-b (δ 154.7), C-d (δ 124.0), C-e (δ 134.3), C-l (δ 167.7); H-e (δ 8.77) and C-c (δ 115.8), C-d (δ 124.0), C-f (δ 135.0), C-l (δ 167.7), C-j (δ 121.8); H-g (δ 7.64) and C-2 (δ 79.7), C-h (δ 149.8), C-i (δ 152.3), C-k (δ 128.8). Thus, the correlation peaks of H-c and H-e with the ester carbonyl carbon C-l, and H-g with C-2 of the flavan-3-ol unit all indicate the presence of a benzotropolone group formed by the galloyl ester group on **3** with the B-ring of **5**. Thus, the structure of **17** was deduced as shown (Fig. 2) and named neotheaflavate **B**. The complete interpretation of the NMR data was based on the results of HMQC and HMBC experiments (Table 1).



(**1**) EC : R_1=R_2=H
(**2**) EGC : R_1=OH, R_2=H
(**3**) ECG : R_1=H, R_2=Gallate
(**4**) EGCG : R_1=OH, R_2=Gallate
(**6**) Catechol : R_1=R_2=H
(**7**) Pyrogallol : R_1=OH, R_2=H
(**8**) Gallic acid: R_1=OH, R_2=COOH
(**5**) C

Figure 1. Structures of compounds **1**–**8**.

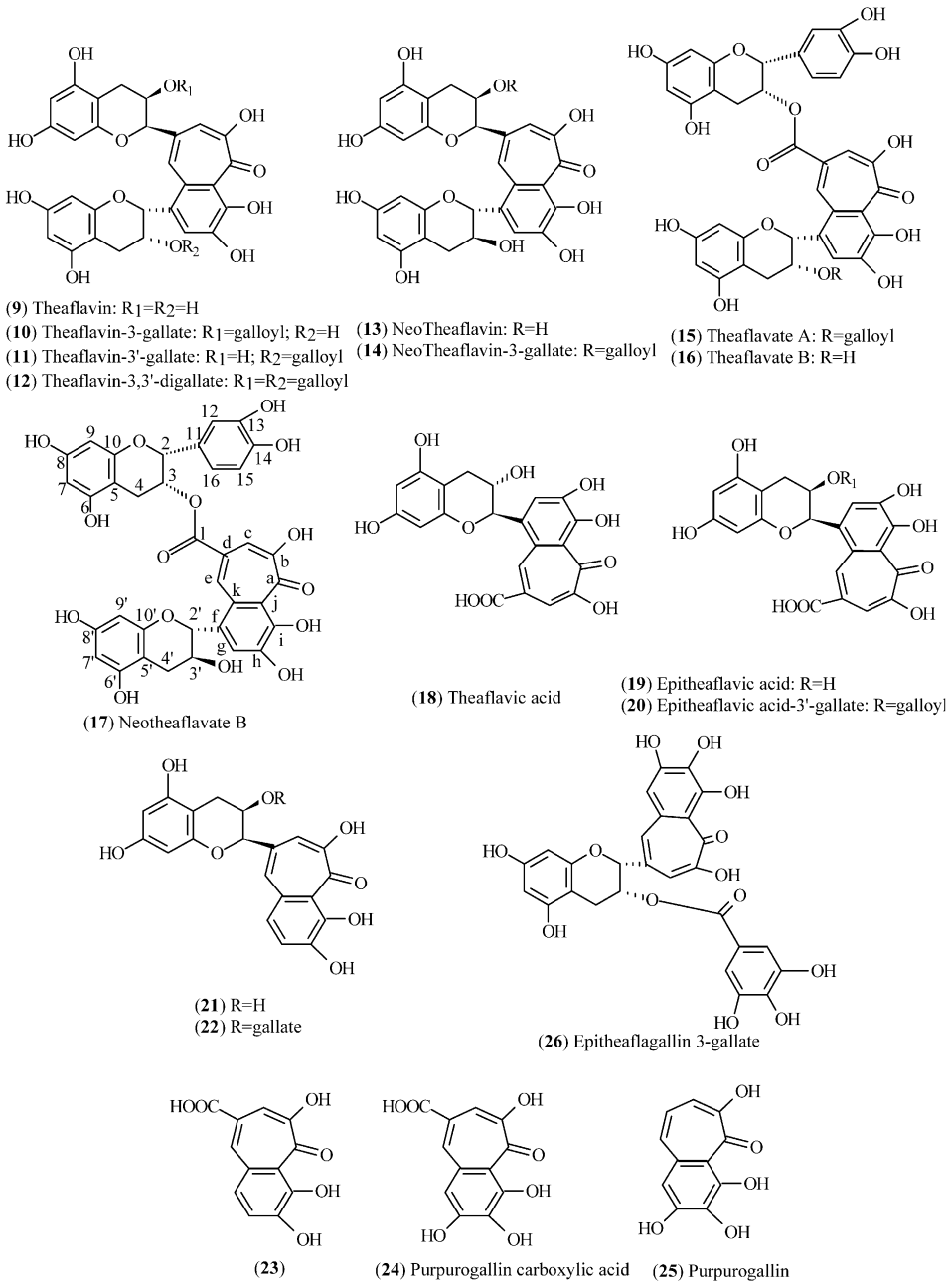


Figure 2. Structures of compounds **9**–**26**.

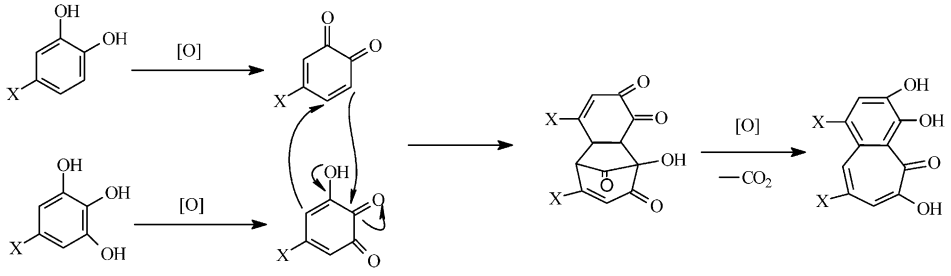


Figure 3. Possible mechanism for the formation of benzotropolone structures.

Table 1. δ_H (600 MHz) and δ_C (150 MHz) NMR spectral data for compound **17** (CD_3OD) (δ in ppm, J in Hz)

	δ_H	δ_C		δ_H	δ_C
2	5.38 m	79.7 d		2.91 dd 15.6, 4.8	
3	5.59 brs	72.0 d	5′		101.2 s
4	2.95 d 17.4	26.6 t	6′		157.3 s
	3.03 dd 17.4, 4.8		7′	6.03 d 1.8	95.9 d
5		99.2 s	8′		157.9 s
6		157.7 s	9′	5.98 d 1.8	97.0 d
7	5.96 s	96.1 d	10′		157.0 s
8		157.7 s	a		186.6 s
9	5.96 s	96.8 d	b		154.7 s
10		156.8 s	c	7.61 brs	115.8 d
11		131.2 s	d		124.0 s
12	6.88 d 1.8	114.4 d	e	8.77 brs	134.3 d
13		145.8 s[a]	f		135.0 s
14		146.0 s[a]	g	7.64 s	122.5 d
15	6.64 d 8.4	116.2 d	h		149.8 s
16	6.82 dd 1.8, 8.4	119.0 d	i		152.3 s
2′	5.04 brs	78.0 d	j		121.8 s
3′	4.06 m	69.6 d	k		128.8 s
4′	2.65 dd 15.6 9.0	29.6 t	l		167.7 s

[a] Assignments in these rows may be interchanged.

Another new compound (**22**), the product of the reaction between EGCG (**4**) and catechol (**6**), was assigned a molecular formula of $C_{27}H_{20}O_{12}$ determined by positive-ion APCI-MS ($[M+H]^+$ at m/z 537) as well as from its ^{13}C NMR data. The only difference between the NMR spectra of compounds **22** and **21**, the product of the reaction between EGC (**2**) and catechol (**6**), was the presence of the gallate group signals in **22**, just like the difference between EGCG and EGC. Thus, the structure of **22** was determined as the gallate ester of **21** (Fig. 2).

Taken together, our findings support the hypothesis that peroxidase plays an important role in the formation of theaflavin-related compounds during black tea fermentation. Moreover, our synthetic method should be useful for the preparation of theaflavin derivatives for laboratory study or industrial development of high-content theaflavin products.

3. Anti-inflammatory activities of compounds 9–26

Three different anti-inflammatory models were used to evaluate the activities of these theaflavin derivatives. In the TPA-induced mice ear edema assay, the individual compounds and TPA were applied topically to mouse ears. Application of TPA on skin induces epidermal hyperplasia and inflammation. The induction of inflammation in skin is thought to involve cyclooxygenase (COX).[38] Muller-Decker and coworkers[39] reported that topical treatment of mouse skin with TPA increased COX-2 expression and led to increased production of prostaglandin E_2 at the site. The weight of ear punctures increased with TPA treatment: 0.5 μM theaflavins significantly reduced the punctured ear weight (Table 2). Most of the synthesized theaflavin-related compounds (**9**–**26**) displayed greater inhibition of inflammation than EGCG, the major catechin in green tea. This result suggests a rationale for the finding that black tea polyphenols have similar biological activities to green tea polyphenols, even though green tea has higher catechin content than black tea.[40] It is quite reasonable that some of these compounds that have higher molecular weight and have more phenol groups than EGCG, such as compounds **9**–**12**, **14**–**17**, showed higher anti-inflammation activity. However, some these compounds that have similar or lower molecular weight and have similar or less phenol group than EGCG, such as compounds **18**–**19**, **21**–**23**, showed even higher activity. Since the common figure of these compounds is the presence of their benzotropolone moiety, so these may be explained that the benzotropolone moiety contributes significantly to their anti-inflammatory activities. Further structure and activity relationship studies are required. Secondly, theaflavin 3,3′-digallate (**12**), theaflavate A (**15**), **22**, and purpurogallin (**25**), at 50 μM, were found to inhibit nitric oxide (NO) synthesis by 82.4, 86.3, 49.3, and 65.3%, respectively (Table 3). Finally, theaflavin 3-gallate (**10**), theaflavic acid 3′-gallate (**20**), and purpurogallin (**25**), at 50 μM, showed the largest inhibition (62.7, 72.7 and 69.5%, respectively) of arachidonic acid release by LPS-stimulated RAW 264.7 cells.

Both arachidonic acid metabolites and NO are important mediators of oxidative stress and inflammation in vivo. The present results demonstrate that the newly-synthesized theaflavin-like compounds are capable of inhibiting both of these processes in LPS-induced murine macrophages. Given the potential role of COX-2 in TPA-medicated ear edema in mice, the presently observed inhibition of arachidonic acid release after

Table 2. Anti-inflammatory activity in TPA-induced mice ear edema assay of compounds **9**–**19** and **21**–**23**[a,b]

Treatment	Average weight of ear punctures (mg) (mean ± SE)	Inhibition (%)
Acetone	7.44 ± 0.08*	—
Acetone + TPA	11.7 ± 1.38	—
9 + TPA	7.90 ± 0.42*	89.2
10 + TPA	7.86 ± 0.24*	91.5
11 + TPA	7.44 ± 0.18*	100.0
12 + TPA	7.03 ± 0.18*	100.0
4 + TPA	8.79 ± 0.54*	68.3
Acetone	7.44 ± 0.0.7*	—
Acetone + TPA	15.91 ± ± 0.51	—
13 + TPA	10.58 ± 0.73*	62.9
14 + TPA	8.42 ± 0.42*	88.4
15 + TPA	8.52 ± 0.26*	87.2
16 + TPA	8.35 ± 0.32*	89.2
17 + TPA	8.65 ± 0.64	85.7
18 + TPA	9.28 ± 0.37*	78.3
Acetone	7.17 ± 0.18*	—
Acetone + TPA	15.85 ± 0.86	—
18 + TPA	8.95 ± 0.43*	79.5
19 + TPA	9.39 ± 0.43*	74.4
21 + TPA	7.86 ± 0.18*	92.1
22 + TPA	7.49 ± 0.12*	96.3
23 + TPA	8.61 ± 0.54*	83.3
9 + TPA	8.23 ± 0.40	87.8

*$p < 0.05$, significantly different from TPA-treated group by the Student's t-test.
[a] Data are expressed as the mean ± SE, $n = 6$.
[b] $n = 5$, TPA (1 nmol).

treatment of macrophages with theaflavin derivatives suggests that inhibition of this pathway are involved in the observed anti-inflammatory effects in vivo. The inhibition of arachidonic acid release assay may be caused by a blockade of phospholipase A_2 activation or activity and further mechanistic studies are required. Similarly, the mechanism for the inhibition of NO formation by LPS-activated macrophages is also not clear. The compounds may inhibit either iNOS activity or LPS induction of the enzyme. Further studies are needed to determine the mechanism(s) of action of these compounds.

3.1. Growth inhibitory activity of compounds 9–26 on HT29 human colon cancer cells, and KYSE 150 and 510 human esophageal squamous cells

The growth inhibitory activity of compounds **9–26** (50 μM) was determined after treatment for 24 h in KYSE 150 and 510 human esophageal squamous cell carcinoma cells and in HT29 human colon cancer cells. All of the synthetic compounds showed weak inhibition (10% or less) of the HT29 cell growth (Table 4). However, KSYE 150 and KYSE 510 cells showed varying degrees of sensitivity to the compounds. Theaflavin 3,3′-digallate (**12**), theaflavate A (**15**), purpurogallin (**25**), and epitheaflagallin 3-gallate (**26**) showed the highest activity toward both cell lines, with IC_{50} values of 18, 18, 7 and 17 μM, respectively, in KYSE 510 cells (Table 4).

4. Experimental

4.1. General procedures

^1H (600 MHz), ^{13}C (150 MHz) and all 2D NMR spectra were acquired on a Varian ^{Unity}INOVA 600 NMR spectrometer (Palo Alto, CA, USA) equipped with a z-gradient inverse-detection triple resonance probe. ^1H–^{13}C HMQC and HMBC experiments were performed as described previously.[41] Mass spectrometry was performed on a VG Platform II single quadrupole mass spectrometer (Micromass Co., MA,USA) equipped with an atmospheric pressure ion source and atmospheric pressure chemical ionization (ApCI) interface. The effluent from the LC column was delivered to the ion source (150 °C) through a heated nebulizer probe (450 °C) using nitrogen as drying gas (300 L/h) and sheath gas (150 L/h). Positive ions were acquired in full scan (m/z 450–1300 for quantification and m/z 200–1000 for identification, 1s scan time). RP C-18 silica gel and Sephadex LH-20 gel were purchased from Sigma Chemical Co. (St. Louis, MO,USA). Thin-layer chromatography was performed on Sigma-Aldrich TLC plates (250 μm thickness, 2–25 μm particle size), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in ethanol solution. Catechol, (+)-catechin, (−)-epicatechin (EC), gallic acid, pyrogallol, horseradish peroxidase, H_2O_2 and CD_3OD were purchased from Aldrich Chemical Co. (Milwaukee, WI,USA). (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG) were isolated from green tea polyphenol extract, which was a gift from A. M Todd Company (Montgomeryville, PA,USA).

HT-29 human colon adenocarcinoma cells and RAW 264.7 murine macrophages were obtained from American Type Tissue Culture (Manassas, VA, USA). KYSE 150 and KYSE 510 human esophageal squamous cell carcinoma cells were a gift from Dr. Y. Shimada (Kyoto University, Kyoto, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO,USA).

4.2. Preparation of tea catechins

After filtration, the crude green tea polyphenol was loaded directly onto a Sephadex LH-20 column and

Table 3. Inhibition (%) of NO synthesis and arachidonic acid release by LPS-stimulated RAW 264.7 cells as a bioassay marker of compounds **9–26**

Compd	NO synthesis	Arachidonic acid release
9	7.4	29.4
10	17.3	62.7
11	26.3	30.7
12	82.4	0.0
13	0.0	5.9
14	20.3	47.0
15	86.3	0.0
16	14.0	26.1
17	19.0	26.6
18	27.0	0.0
19	15.3	16.4
20	35.0	72.7
21	15.7	43.9
22	49.3	50.5
23	4.7	27.8
24	4.7	13.7
25	65.3	69.5
26	1.0	41.6

Table 4. Growth inhibitory activity of compounds **9–26** on several types of cancer cells

Compd	HT-29 (24 h)	KYSE510 (24 h)	KYSE150 (24 h)	KYSE510 (24 h, IC_{50}) (μM)
9	112.2	71.9	41.9	
10	109.2	30.8	17.9	
11	107.0	18.5	9.7	
12	107.6	2.2	6.5	18
13	103.4	78.1	37.7	
14	104.3	27.1	23.3	
15	103.6	8.8	10	18
16	105.7	22.8	14.6	
17	108.5	28.7	20.9	
18	91.3	47.1	49.9	
19	105.1	107.8	56.6	
20	112.1	38.2	32.5	
21	106.4	77.8	48	
22	112.3	28.2	22.9	
23	96.8	99.6	98.1	
24	97.5	88.9	85.1	
25	98.4	13.8	N/D	7
26	113.1	9.3	13.9	17

Results are expressed as % of control growth.

eluted with 95% ethanol to give three catechins: epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatecin gallate (EGCG). Each catechin was further purified by HPLC using an RP C-18 column eluted with a methanol–water solvent system.

4.3. Synthesis of theaflavin-type compounds

4.3.1. Theaflavin 9. EC (1.0 g) and EGC (1.0 g) were dissolved in a mixture of acetone-pH 5.0 phosphate–citrate buffer (1:10 v/v, 50 mL), which contained 4 mg horseradish peroxidase. Four 2.0-mL aliquots of 3.13% H_2O_2 were added during a period of 45 min while stirring. The resulting reaction mixture was extracted with ethyl acetate (3×50 mL). After concentration, the residue was applied to a Sephadex LH 20 column and eluted with acetone-water (2:3 v/v) to obtain 250 mg of theaflavin. ^{1}H NMR (600 MHz, CD_3OD, TMS internal reference): δ_H 7.97 1H s, 7.85 1H s, 7.34 1H s, 6.02 1H d, J=2.4 Hz, 5.99 1H d, J=2.4 Hz, 5.97 1H d, J=2.4 Hz, 5.96 1H d, J=2.4 Hz, 5.64 1H brs, 4.91 1H brs, 4.45 1H m, 4.32 1H m, 2.98 1H dd, J=4.8, 16.8 Hz, 2.94 1H dd, J=4.8, 16.8, 2.84 1H brd, J=16.8 Hz, 2.82 1H brd, J=16.8 Hz; ^{13}C NMR (150 MHz, CD_3OD): δ_C 185.1, 158.1, 158.0, 157.6, 157.5, 157.3, 156.6, 155.1, 150.9, 146.1, 134.4, 131.2, 129.0, 126.6, 123.7, 121.9, 118.3, 100.3, 99.8, 96.8, 96.7, 96.1, 96.0, 81.2, 77.1, 66.7, 65.6, 30.0, 29.4 ppm; positive APCI-MS m/z 565 $[M+H]^+$.

4.3.2. Theaflavin 3-gallate 10. Following the procedure for the synthesis of **9**, EC (1.0 g) and EGCG (1.0 g) were used to synthesize 220 mg of **10**. ^{1}H NMR (600 MHz, CD_3OD): δ_H 7.91 1H s, 7.80 1H s, 7.38 1H s, 6.80 2H s, 6.02 1H d, J=1.8 Hz, 6.00 1H d, J=2.4 Hz, 5.99 2H s, 5.78 1H m, 5.55 1H brs, 5.11 1H s, 4.16 m, 3.07 dd, J=4.8, 16.8 Hz, 2.99 dd, J=4.2, 16.8, 2.91 brd, J=16.8 Hz, 2.83 brd, J=16.8 Hz; ^{13}C NMR (150 MHz, CD_3OD): δ_C 185.6, 167.4, 158.0, 157.9, 157.8, 157.3, 156.4, 156.3, 155.4, 151.2, 146.4, 146.3×2, 139.9, 133.5, 131.3, 128.7, 125.7, 123.8, 121.9, 121.0, 117.5, 110.1×2, 100.2, 99.3, 96.9, 96.8, 96.2, 95.8, 79.8, 77.0, 69.0, 65.7, 30.1, 27.1 ppm; positive APCI-MS m/z 717 $[M+H]^+$.

4.3.3. Theaflavin 3′-gallate 11. Following the procedure for the synthesis of **9**, ECG (1.0 g) and EGC (1.0 g) were used to synthesize 110 mg of **11**. ^{1}H NMR (600 MHz, CD_3OD): δ_H 7.88 1H s, 7.87 1H s, 7.37 1H s, 6.84 2H s, 6.06 d, J=2.4 Hz, 6.00 1H d, J=2.4 Hz, 5.98 1H d, J=2.4 Hz, 5.97 1H d, J=2.4 Hz, 5.87 1H brs, 5.81 1H m, 4.94 1H brs, 4.33 1H brs, 3.09 1H dd, J=4.8, 17.4 Hz, 2.96 1H dd, J=4.8, 16.8, 2.88 1H brd, J=17.4 Hz, 2.861H dd, J=2.4, 16.8 Hz; ^{13}C NMR (150 MHz, CD_3OD): δ_C 185.6, 167.2, 158.0, 157.9, 157.8, 157.7, 157.0, 156.6, 156.0, 155.5, 151.1, 146.2×2, 139.7, 134.8, 130.3, 128.8, 125.9, 123.0, 121.9, 120.9, 118.3, 110.1×2, 99.8, 99.6, 96.8, 96.7, 95.8, 95.7, 81.2, 75.8, 68.3, 66.5, 29.3, 27.2 ppm; positive APCI-MS m/z 717 $[M+H]^+$.

4.3.4. Theaflavin 3,3′-digallate 12. Following the procedure for the synthesis of **9**, ECG (1.0 g) and EGCG (1.0 g) afforded 100 mg of **12**. ^{1}H NMR (600 MHz, CD_3OD): δ_H 7.79 1H s, 7.76 1H s, 7.47 1H s, 6.88 2H s, 6.80 2H s, 6.07 1H d, J=2.4 Hz, 6.03 2H d, J=2.4 Hz, 6.00 1H d, J=2.4 Hz, 5.86 1H brs, 5.76 1H m, 5.67 1H m, 5.21 1H s, 3.17 1H dd, J=4.8, 16.8 Hz, 3.09 1H dd, J=4.8, 17.4, 2.91 2H m; ^{13}C NMR (150 MHz, CD_3OD): δ_C 185.7, 167.6, 167.1, 158.0, 157.9×3, 157.2, 156.5, 155.5, 151.3, 146.5, 146.22×2, 146.21×2, 139.8, 139.7, 134.2, 130.4, 128.9, 125.8, 122.8, 122.1, 121.1, 121.0, 117.5, 110.2×2, 110.1×2, 99.7, 99.3, 97.0×2, 96.1, 95.9, 80.1, 75.7, 69.4, 68.2, 27.4, 27.0 ppm; positive APCI-MS m/z 869 $[M+H]^+$.

4.3.5. Neotheaflavin 13. Following the procedure for the synthesis of **9**, Catechin (1.0 g) and EGC (1.0 g) afforded 120 mg of **13**. ^{1}H NMR (600 MHz, $(CD_3)_2CO$): δ_H 8.26 1H s, 7.46 1H s, 7.63 1H s, 6.06 1H d, J=2.4 Hz, 6.03 1H d, J=2.4 Hz, 5.96 1H d, J=2.4 Hz, 5.95 1H d, J=2.4 Hz, 5.62 1H d, J=7.8 Hz, 5.01 1H s, 4.39 1H m, 4.15 1H m, 2.97 1H dd, J=5.4, 15.6 Hz, 2.91 1H dd, J=4.2, 16.8, 2.84 1H dd, J=1.2, 16.8 Hz, 2.66 1H dd, J=9.6, 15.6 Hz; ^{13}C NMR ($(CD_3)_2CO$, 150 MHz): δ_C 184.8, 157.6, 157.5, 157.4, 157.0, 156.7, 156.6, 154.4, 150.5, 146.2, 134.8, 132.2, 130.8, 128.6, 122.3, 121.6, 119.2, 100.7, 99.2, 96.4, 96.3, 95.6, 95.4, 81.5, 79.1, 69.5, 66.6, 30.0, 29.3 ppm; positive APCI-MS m/z 565 $[M+H]^+$.

4.3.6. Neotheaflavin 3-gallate 14. Following the procedure for the synthesis of **9**, Catechin (1.0 g) and EGCG (1.0 g) were used to synthesize 170 mg of **14**. ^{1}H NMR (600 MHz, CD_3OD): δ_H 8.04 1H s, 7.59 1H s, 7.49 1H s, 6.92 2H s, 6.01 2H d, J=2.4 Hz, 5.98 1H d, J=2.4 Hz, 5.97 1H d, J=2.4 Hz, 5.67 1H brs, 5.56 1H m, 5.11 1H s, 4.22 1H m, 3.03 1H dd, J=4.8, 17.4 Hz, 2.92 1H brd, J=16.8 Hz, 2.83 1H dd, J=4.8, 16.8, 2.66 dd, J=8.4, 16.8; ^{13}C NMR (150 MHz, CD_3OD): δ_C 185.8, 167.4, 158.0, 157.9, 157.8, 157.7, 156.7, 156.5, 155.3, 151.6, 146.9, 146.2×2, 139.9, 134.0, 132.0, 130.4, 127.7, 122.3, 121.3, 121.0, 117.6, 110.2×2, 100.6, 99.2, 96.9, 96.7, 95.9, 95.6, 80.5, 77.1, 69.9, 68.8, 28.5, 27.0 ppm; positive APCI-MS m/z 717 $[M+H]^+$.

4.3.7. Theaflavate A 15. Following the procedure for the synthesis of **9**, ECG (0.85 g) itself was used to synthesize 60 mg of **15,** and 600 mg ECG was recovered. ^{1}H NMR (600 MHz, CD_3OD): δ_H 8.33 1H s, 7.81 1H s, 7.65 1H s, 6.87 1H dd, J=1.8, 7.8 Hz, 6.85 1H d, J=1.8 Hz, 6.80 2H, s, 6.53 1H d, J=7.8 Hz, 6.15 1H d, J=2.4 Hz, 6.11 1H d, J=2.4, 6.09 1H d, J=2.4 Hz, 5.98 1H, d, J=2.4 Hz, 5.69 1H brs, 5.64 1H brs, 5.52 1H, m, 5.11 1H s, 3.32 1H dd, J=4.8, 18.0 Hz, 3.10 1H dd, J=4.8, 18.0 Hz, 3.05 dd, J=1.8, 16.8 Hz, 2.91 d, J=16.8 Hz; ^{13}C NMR (150 MHz, CD_3OD): δ_C 186.8, 167.8, 167.3, 158.2, 158.1, 158.0, 157.9, 157.2, 157.1, 155.3, 149.5, 146.4×2, 146.3, 146.0, 140.0, 133.5, 131.6, 131.2, 126.6, 124.8, 122.9, 122.6, 121.0, 119.0, 116.4, 115.8, 114.2, 110.2×2, 100.0, 99.4, 97.3, 97.2, 96.5, 96.4, 78.0, 75.6, 72.1, 68.9, 27.3, 26.7 ppm; positive APCI-MS m/z 853 $[M+H]^+$.

4.3.8. Theaflavate B 16. Following the procedure for the synthesis of **9**, Catechin (1.0 g) and EGCG (1.0 g) were used to synthesize 200 mg of **16**. ^{1}H NMR (600 MHz, CD_3OD): δ_H 8.26 1H s, 7.88 1H s, 7.59 1H s, 6.87 1H dd, J=1.8, 7.8 Hz, 6.86 1H d, J=1.8 Hz, 6.55 1H d,

J = 7.8 Hz, 6.16 1H d, J = 2.4 Hz, 6.08 1H d, J = 2.4 Hz, 6.05 1H d, J = 2.4 Hz, 5.98 1H, d, J = 2.4 Hz, 5.66 1H brs, 5.46 1H brs, 5.08 1H s, 4.14 1H brs, 3.34 1H dd, J = 4.8, 16.8 Hz, 3.21 1H dd, J = 4.8, 16.8 Hz, 3.17 1H dd, J = 3.6, 16.2 Hz, 2.88 1H d, J = 16.8; ^{13}C NMR (150 MHz, CD_3OD): δ_C 186.4, 167.8, 158.3, 158.0, 157.9, 157.7, 157.4, 157.1, 154.9, 151.8, 149.5, 146.3, 146.0, 134.8, 132.3, 131.3, 126.5, 124.4, 123.7, 122.4, 119.2, 116.3, 115.8, 114.4, 100.7, 99.5, 97.2, 97.1, 96.6, 96.5, 78.1, 77.0, 72.1, 66.7, 30.0, 26.7 ppm; positive APCI-MS m/z 701 $[M+H]^+$.

4.3.9. Neotheaflavate B 17. Following the procedure for the synthesis of **9**, Catechin (1.0 g) and EGCG (1.0 g) were used to synthesize 90 mg of **17**. ^{1}H NMR (600 MHz, CD_3OD): Table 1; ^{13}C NMR (150 MHz, CD_3OD): Table 1; positive APCI-MS m/z 701 $[M+H]^+$.

4.3.10. Theaflavic acid 18 and purpurogallin carbolic acid 24. Following the procedure for the synthesis of **9**, Catechin (1.0 g) and gallic acid (0.5 g) were used to synthesize 60 mg of **18** and 10 mg of **24**. ^{1}H NMR (600 MHz, CD_3OD): δ_H 9.00 1H s, 7.82 1H s, 7.66 1H s, 5.98 1H d, J = 2.4 Hz, 5.91 1H d, J = 2.4 Hz, 5.43 1H brd, J = 7.2 Hz, 4.21 1H, m, 2.94 1H dd, J = 4.8, 16.2 Hz, 2.64 1H dd, J = 4.8, 16.2 Hz; ^{13}C NMR (150 MHz, CD_3OD): δ_C 186.6, 170.3, 157.9, 157.6, 156.6, 154.7, 152.2, 149.3, 139.5, 134.4, 132.2, 128.9, 125.0, 122.7, 116.5, 100.7, 96.8, 96.1, 80.0, 69.1, 29.3 ppm; positive APCI-MS m/z 429 $[M+H]^+$. **24:** ^{1}H NMR (CD_3OD, 600 MHz): δ_H 8.17 1H s, 7.66 1H s, 6.94 1H s; ^{13}C NMR (150 MHz, CD_3OD): δ_C 184.0, 170.0, 156.6, 154.4, 153.2, 152.2, 137.8, 134.2, 125.9, 123.0, 116.3, 114.5 ppm; positive APCI-MS m/z 265 $[M+H]^+$.

4.3.11. Epitheaflavic acid 19 and purpurogallin carbolic acid 24. Following the procedure for the synthesis of **9**, Epicatechin (EC) (0.5 g) and gallic acid (1.0 g) were used to synthesize 70 mg of **19** and 25 mg of **24**. **19:** ^{1}H NMR (600 MHz, CD_3OD): δ_H 8.60 1H s, 7.95 1H s, 7.80 1H s, 6.09 1H s, 6.00 1H s, 5.88 1H s, 5.77 1H m, 3.17 1H dd, J = 4.8, 17.4 Hz, 2.94 1H d, J = 17.4 Hz; ^{13}C NMR (150 MHz, CD_3OD): δ_C 186.5, 170.1, 158.2, 157.7, 157.2, 155.0, 151.6, 149.2, 134.2, 132.2, 126.7, 125.2, 123.4, 122.6, 116.2, 99.2, 96.8, 96.1, 77.0, 66.4, 30.0 ppm; positive APCI-MS m/z 429 $[M+H]^+$.

4.3.12. Epitheaflavic acid 3-gallate 20, theaflavate A 15 and purpurogallin carbolic acid 24. Following the procedure for the synthesis of **9**, ECG (0.5 g) and gallic acid (1.0 g) were used to synthesize 20 mg of **20**, 40 mg of **15** and 10 mg of **24**. **20:** ^{1}H NMR (600 MHz, CD_3OD): δ_H 8.64 1H s, 7.84 1H s, 7.83 1H s, 6.83 2H s, 6.02 1H d, J = 2.4 Hz, 5.98 1H d, J = 2.4, 5.61 1H s, 4.37 1H, m, 3.03 1H dd, J = 4.8, 16.8 Hz, 2.87 d, J = 16.8 Hz; ^{13}C NMR (150 MHz, CD_3OD): δ_C 186.5, 170.1, 167.1, 158.0, 156.9, 155.1, 151.7, 148.9, 146.2×2, 139.8, 132.8, 131.6, 126.8, 122.6, 122.5, 120.8, 116.3, 110.0×2, 99.3, 96.9, 96.0, 75.7, 68.6, 27.2 ppm; positive APCI-MS m/z 581 $[M+H]^+$.

4.3.13. Compound 21. Following the procedure for the synthesis of **9**, EGC (1.0 g) and catechol (1.5 g) were used to synthesize 226 mg of **21**. ^{1}H NMR (600 MHz, C_5D_5N): δ_H 8.15 1H s, 7.99 1H s, 7.66 1H d, J = 8.4 Hz, 7.41 1H d, J = 8.4 Hz, 6.75 1H brs, 6.74 1H brs, 5.23 1H s, 4.76 1H, s, 3.67 1H d, J = 16.2 Hz, 3.44 1H dd, J = 3.6, 16.2 Hz; ^{13}C NMR (C_5D_5N, 150 MHz): δ_C 184.9, 158.9, 158.8, 157.2, 151.8, 151.4, 147.8, 135.0, 134.5, 132.0, 126.4, 123.3, 121.2, 119.9, 99.9, 97.1, 96.0, 81.8, 66.6, 30.3 ppm; positive APCI-MS m/z 385 $[M+H]^+$.

4.3.14. Compound 22. Following the procedure for the synthesis of **9**, EGCG (1.0 g) and catechol (1.5 g) were used to synthesize 230 mg of **22**. ^{1}H NMR (600 MHz, C_5D_5N): δ_H 8.01 1H s, 7.97 1H s, 7.54 1H d, J = 8.4 Hz, 7.29 1H d, J = 8.4 Hz, 6.74 1H d, J = 2.4 Hz, 6.70 1H d, J = 2.4, 6.18 1H s, 5.38 1H, s, 3.71 1H d, J = 17.4 Hz, 3.51 1H dd, J = 3.0, 17.4 Hz; ^{13}C NMR (C_5D_5N, 150 MHz): δ_C 185.0, 166.8, 159.0, 158.8, 156.9, 155.4, 151.9, 148.1, 147.7×2, 141.4, 134.5, 133.0, 131.6, 126.4, 123.2, 121.2, 120.8, 118.8, 110.4×2, 98.9, 97.5, 96.0, 80.1, 69.1, 27.5 ppm; positive APCI-MS m/z 537 $[M+H]^+$.

4.3.15. Compound 23. Following the procedure for the synthesis of **9**, Gallic acid (2.0 g) and catechol (2.0 g) were used to synthesize 400 mg of **23**. ^{1}H NMR (600 MHz, C_5D_5N): δ_H 8.08 1H brs, 7.68 1H dd, J = 1.2, 8.4 Hz, 7.56 1H d, J = 8.4 Hz, 7.19 1H s; ^{13}C NMR (C_5D_5N, 150 MHz): δ_C 186.0, 169.6, 154.8, 152.6, 150.5, 140.0, 130.2, 128.8, 125.6, 123.0, 121.6, 117.9 ppm; positive APCI-MS m/z 249 $[M+H]^+$.

4.3.16. Purpurogallin 25. Following the procedure for the synthesis of **9**, Pyrogallol (1.0 g) and catechol (1.5 g) were used to synthesize 300 mg of **25.** ^{1}H NMR (600 MHz, C_5D_5N): δ_H 7.35 1H d, J = 11.4 Hz, 7.29 1H s, 7.24 1H d, J = 9.6 Hz, 6.66 1H dd, J = 9.6, 11.4 Hz; ^{13}C NMR (C_5D_5N, 150 MHz): δ_C 183.4, 156.3, 154.0, 153.3, 137.1, 135.1, 134.4, 123.8, 116.8, 116.2, 112.0 ppm; positive APCI-MS m/z 221 $[M+H]^+$.

4.3.17. Epitheaflagallin 3-gallate 26. Following the procedure for the synthesis of **9**, EGCG (1.0 g) and garlic acid (1.5 g) were used to synthesize 300 mg of **26.** ^{1}H NMR (600 MHz, CD_3OD): δ_H 7.44 1H s, 7.32 1H s, 6.90 2H s, 6.76 1H s, 6.03 1H d, J = 1.8 Hz, 6.00 1H d, J = 1.8, 5.66 1H, m, 5.03 1H s, 3.06 1H dd, J = 4.8, 18.0 Hz, 2.91 1H d, J = 18.0 Hz; ^{13}C NMR (150 MHz, CD_3OD): δ_C 183.3, 167.3, 157.9, 156.7, 155.0, 152.6, 152.5, 152.4, 146.3×2, 139.9, 135.7, 134.4, 134.2, 133.5, 121.0, 116.4, 116.0, 112.0, 110.2×2, 99.2, 96.9, 95.9, 80.3, 69.3, 27.1 ppm; positive APCI-MS m/z 553 $[M+H]^+$.

4.4. TPA-induced mice ear edema assay of compounds 9–19 and 21–23

The anti-inflammatory activity of compounds **9**–**19** and **21**–**23** was examined using the TPA-induced mice ear edema assay. Both ears of female CD-1 mice (five mice per group; 35 days old) were treated topically with 20 μL acetone or a test compound (0.5 μM) in 20 μL acetone, 20 min prior to topical treatment of

20 μL acetone or TPA (1.0 nM) in 20 μL acetone. Five hours later, the mice were killed by cervical dislocation, and ear punches (6 mm in diameter) were taken and weighed.

4.5. Maintenance and subculture of cell lines

HT-29 cells, RAW 264.7 cells, and KYSE cells were maintained in log phase growth in McCoy's 5A medium, Dulbecco's modified Eagles Medium, or RPMI 1640:Ham's F12 (1:1), respectively. All media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, except the RPMI 1640/Ham's F12 which was supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin.

4.6. Inhibition of nitric oxide (NO) synthesis

RAW 264.7 cells were plated in 24-well plates (3.0×10^5 cells per well) and stimulated for 1 h with 2 μg/mL LPS. The medium was then replaced with serum-free medium containing 50 μM compound and cells were cultured for 18 h. NO production was determined spectrophotometrically using previously reported methods.[42]

4.7. Inhibition of arachidonic acid release from LPS-stimulated RAW 264.7 cells

To determine inhibition of arachidonic acid release, RAW 264.7 cells were plated into a 24-well plate (3×10^5 cells per well). After 24 h, the media were removed and replaced with 1 mL of serum-free DMEM media containing 0.1 μCi/mL [5,6,8,9,11,12,14,15-^{3}H](N) arachidonic acid (NEN Life Science, Boston, MA, USA). The cells were incubated overnight, resulting in over 90% arachidonic acid absorption, and washed two times with PBS containing 0.1% BSA. The cells were stimulated with 2 μg/mL LPS for 1 h and the media was replaced with serum-free medium containing the test compounds (50 μM). After incubation of 18 h, the media was collected and centrifuged for 10 min at 12000 rpm. Radioactivity in the extracellular fluid was measured with a scintillation counter.

4.8. Growth inhibitory against human colon cancer cells and human esophageal squamous cell carcinoma cells

The growth inhibitory activity of the compounds was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which correlates cell number with mitochondrial reduction of MTT to a blue formazan precipitate.[43] In brief, cells were plated in 96-well plates ($2-10\times10^3$ per well) and allowed to attach overnight. The medium was then replaced with serum-free medium containing the test compound (0–50 μM) and the cells were incubated at 37 °C for 24 h. The medium was then replaced with fresh medium containing 1 mg/mL MTT. Following incubation at 37 °C for 2–4 h, the wells were aspirated, the dye was solubilized in DMSO, and the absorbance was measured at 595 nm. The viability of cells was compared with that of medium-control treated cells.

Acknowledgements

This work was supported by NIH Grant PO1 CA88961 and New Jersey Commission on Science and Technology. Funds to purchase the 600 MHz NMR spectrometer at CUNY College of Staten Island were provided by the New York State Dormitory Authority and the New York State Higher Education Applied Technology Program.

References and notes

1. Yang, C. S.; Chung, J. Y.; Yang, G.; Chhabra, S. K.; Lee, M. J. *J. Nutr.* **2000**, *130*, 472 S.
2. Yang, C. S.; Maliakal, P.; Meng, X. F. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 25.
3. Mckay, D. L.; Blumberg, J. B. *J. Am. Coll. Nutr.* **2002**, *21*, 1.
4. Gross, G. G.; Hemingway, R. W.; Yoshida, T. *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*; Kluwer Academic/Plenum: New York, 1999; p 697.
5. Geissman, T. A. *Chemistry of Flavonoid compounds*; Pergamon: Oxford, UK, 1962; p 468.
6. Runeckles, V. C.; Tso, T. C. *Recent Advances in Phytochemistry*; Academic: New York, 1972; Vol. 5, p. 247.
7. Coller, P. D.; Bryce, T.; Mallows, R.; Thomas, P. E. *Tetrohedron* **1973**, *29*, 125.
8. Wan, X. C.; Nursten, H. E.; Cai, Y.; Davis, A. L.; Wilkins, J. P. G.; Davies, A. P. *J. Sci. Food Agric.* **1997**, *74*, 401.
9. Lewis, J. R.; Davis, A. L.; Cai, Y.; Davies, A. P.; Wilkins, J. P. G.; Pennington, M. *Phytochemistry* **1998**, *49*, 2511.
10. Takino, Y.; Imagawa, H.; Horikawa, H.; Tanaka, A. *Agric. Biol. Chem.* **1964**, *28*, 64.
11. Roberts, E. A. H. *Two and A Bud* **1962**, *9*, 3.
12. Millin, D. J.; Crispin, D. J.; Swaine, D. *J. Agric. Food Chem.* **1969**, *17*, 717.
13. Powell, C.; Clifford, M. N.; Opie, S.; Robertson, A.; Gibson, C. *J. Sci. Food Agric.* **1992**, *63*, 77.
14. Apostolides, Z.; Balentine, D. A.; Harbowy, M. E.; Hara, Y.; Weisurger, J. H. *Mutat. Res.* **1997**, *389*, 167.
15. Feng, Q.; Torh, Y.; Uchida, K.; Nakamura, Y.; Hara, Y.; Osawa, T. *J. Agric. Food Chem.* **2002**, *50*, 213.
16. Gupta, S.; Chaudhuri, T.; Ganguly, D. K.; Giri, A. K. *Life Sci.* **2001**, *69*, 2735.
17. Liang, Y. C.; Chen, Y. C.; Lin, Y. L.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. *Carcinogenesis* **1999**, *20*, 733.
18. Pan, M. H.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. H.; Lin, J. K. *Biochem. Pharm* **2000**, *59*, 357.
19. Lin, J. K.; Chen, P. C.; Ho, C. T.; Lin-Shiau, S. Y. *J. Agric. Food Chem.* **2000**, *48*, 2736.
20. Shiraki, M.; Hara, Y.; Osawa, T.; Kumon, H.; Nakayama, T.; Kawakishi, S. *Mutat. Res.* **1994**, *323*, 29.
21. Yoshino, K.; Hara, Y.; Sano, M.; Tomita, I. *Biol. Pharm. Bull.* **1994**, *17*, 146.
22. Yoshida, H.; Ishikawa, T.; Hosoai, H.; Suzukawa, M.; Ayaori, M.; Hisada, T.; Sawada, S.; Yonemura, A.; Higashi, K.; Ito, T.; Nakajima, K.; Yamashita, T.; Tomiyasu, K.; Nishiwaki, M.; Ohsuzu, F.; Nakamura, H. *Biochem. Pharmacol.* **1999**, *58*, 1695.
23. Dix, M. A.; Fairley, C. J.; Millin, D. J.; Swaine, D. *J. Sci. Food Agric.* **1981**, *32*, 920.
24. Robertson, A. *Phytochemistry* **1983**, *22*, 883.
25. Robertson, A. *Phytochemistry* **1983**, *22*, 889.
26. Robertson, A. *Phytochemistry* **1983**, *22*, 897.
27. Opie, S. C.; Robertson, A.; Clifford, M. N. *J. Sci. Food Agric.* **1990**, *50*, 547.
28. Opie, S. C.; Clifford, M. N.; Robertson, A. *J. Sci. Food Agric.* **1993**, *63*, 435.

29. Opie, S. C.; Clifford, M. N.; Robertson, A. *J. Sci. Food Agric*. **1995**, *67*, 501.
30. Finger, A. *J. Sci. Food Agric*. **1994**, *66*, 293.
31. Sang, S. M.; Tian, S. Y.; Meng, X. F.; Stark, R. E.; Rosen, R. T.; Yang, C. S.; Ho, C. T. *Tetrahedron Lett*. **2002**, *43*, 7129.
32. Tanaka, T.; Mine, C.; Inoue, K.; Matsuda, M.; Kouno, I. *J. Agric. Food Chem*. **2002**, *50*, 2142.
33. Collier, P. D.; Bryce, T.; Mallows, R.; Thomas, P. E. *Tetrahedron* **1973**, *29*, 125.
34. Bailey, R. G.; Nursten, H. E. *J. Sci. Food Agric*. **1993**, *63*, 455.
35. Nonaka, G. I.; Hashimoto, F.; Nishioka, I. *Chem. Pharm. Bull*. **1986**, *34*, 61.
36. Hurd, R. E.; John, B. K. *J. Magn. Reson*. **1991**, *91*, 648.
37. Rinaldi, P. L.; Keifer, P. A. *J. Magn. Reson*. **1994**, *108A*, 259.
38. Katiyar, S. K.; Mukhtar, H. *J. Cell. Biochem. Suppl*. **1997**, *27*, 59.
39. Muller-Decker, K.; Scholz, K.; Marks, F.; Furstenberg, G. *Mol. Carcinog*. **1995**, *12*, 31.
40. Leung, L. K.; Su, Y.; Chen, R.; Zhang, Z.; Huang, Y.; Chen, Z. Y. *J. Nutr*. **2001**, *131*, 2248.
41. Fang, X.; Qiu, F.; Yan, B.; Wang, H.; Mort, A. J.; Stark, R. E. *Phytochemistry* **2001**, *57*, 1035.
42. Ryu, J. H.; Ahn, H.; Lee, H. *Fitoterapia* **2003**, *74*, 350.
43. Mosmann, T. *J. Immun. Methods* **1983**, *65*, 55.