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Antitumorigenic Activities of Chalcones. I. Inhibitory Effects of Chalcone Derivatives on ^{32}Pi-Incorporation into Phospholipids of HeLa Cells Promoted by 12-*O*-Tetradecanoyl-phorbol 13-Acetate (TPA)

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More than forty chalcone derivatives were synthesized to examine their structure–activity relationship against tumorigenesis. As a primary screening test, the inhibitory activities of the chalcones for the ^{32}Pi-incorporation into phospholipids of HeLa cells enhanced by 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) were examined. 3-Hydroxy-chalcone derivatives possessing methyl group in 3′-, 4′-, or 2′-position and isoliquiritigenin homologs showed potent inhibitory activities in the phosphorylation test, which suggests their antitumorigenic effects.

Key words 3-hydroxy-3′-methylchalcone; 3-hydroxy-4′-methylchalcone; isoliquiritigenin; antitumor promoting activity; structure–activity relationship

Many kinds of naturally occurring and synthetic chalcones showed antimicrobial,[1] antifungal[2] and anti-ulceric[3] activities as well as inhibitory effects on 5-lipoxygenase and cyclooxygenase.[4]

Licochalcone A (=3-α,α′-dimethylallyl-4,4′-dihydroxy-6-methoxychalcone), a characteristic chalcone of Xin-jiang licorice which is the root of *Glycyrrhiza inflata* BETAL, showed remarkable antiinflammatory and antitumorigenic activities. These were demonstrated on mouse ear edema induced by arachidonic acid or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and mouse skin papilloma initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoted by TPA, respectively.[5]

The *in vitro* test on phosphorylation of phospholipids promoted by TPA in HeLa cells is a screening test for the antitumorigenic activities and shows good parallelism with *in vivo* experiments.[5,6] Accordingly, a series of simple chalcone derivatives was synthesized by classical Claisen–Schmidt condensation to examine their *in vitro* antitumorigenic activities using the cellular phosphorylation test.

The only compounds showing characteristic potency in this test were examined in *in vivo* antitumorigenic experiments.

MATERIALS AND METHODS

Chemicals Chalcone (benzalacetophenone) was purchased from Nakalai Tesque Inc. (Japan). Licochalcone A and echinatin were isolated from Xin-jiang licorice, the root of *Glycyrrhiza inflata* BETAL. Other chalcone derivatives were prepared by the Claisen–Schmidt condensation[7,8] of substituted acetophenone with various benzaldehyde derivatives. General procedures for the preparation of synthetic chalcones were as follows: Substituted acetophenone (0.02 M) and substituted benzaldehyde (0.02 M) were dissolved in EtOH (7 ml) and added with aqueous 60% KOH (10 ml). The mixture was stirred for 5—24 h at room temperature and then acidified with 10% HCl. The precipitated chalcone derivatives were washed with water, chromatographed over silica gel (hexane : EtOAc = 9 : 1) and recrystallized from hexane with EtOAc or aq. MeOH.

In the case of synthesis of chalcone derivatives possessing 2′- or 3,4-dihydroxyl group, the hydroxyl group of the component was protected with methoxymethyl using chlorodimethyl ether.[9]

TPA was purchased from Sigma. Radioactive inorganic phosphate (^{32}Pi, carrier-free) was obtained from Japan Radioisotope Association.

Cell Culture HeLa cells (human cervical cancer cells) and chick embryo fibroblasts were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% calf serum.

^{32}Pi-Incorporation into Phospholipids of Culture Cells ^{32}Pi-Incorporation into phospholipids of HeLa cells was assayed by the method described previously.[6]

RESULTS AND DISCUSSION

Chemistry The chemical structures of the synthesized chalcone derivatives, the physical data and yields of the chalcones are listed in Table 1. Known chalcones are shown with supplementary reference numbers; chalcones without reference numbers are new compounds. The structures of new chalcone compounds were proved by ^{1}H- and ^{13}C-NMR spectra. Their high-resolution mass spectra (M^+) were within ±0.9 millimass unit of the calculated values (Table 2).

***In Vitro* Screening Test for Antitumorigenic Activity** A structure–activity relationship among the chalcones was shown in their inhibitory effects on ^{32}Pi-incorporation to the phospholipids of HeLa cells promoted by TPA (Table 3), which were obviously parallel with the antitumorigenic activities *in vitro* and *in vivo*.[5,6] The inhibitory potency was evaluated as +(26—50%), ++(51—75%), +++ (76—100%). 3-Hydroxy-3′-methylchalcone (3′Me-3-C), 3-hydroxy-4′-methylchalcone (4′Me-3-C), 3-

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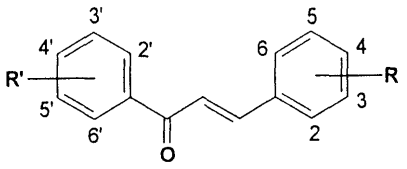
Table 1. Structure and Physical Data of Chalcone Derivatives



Compound	Abbr.	Substitution R′	Substitution R	Formula	Crystal	mp	Yield (%)	Reference
Chalcone	C			$C_{15}H_{12}O$	Pale yellow needle	57—58	com.[b]	
2′-Hydroxychalcone	2′-C	2′-OH		$C_{15}H_{12}O_2$	Yellow plate	82—84	58	1, 2, 4, 10*a*
4′-Hydroxychalcone	4′-C	4′-OH		$C_{15}H_{12}O_2$	White powder	181—182	42	1, 2, 4, 10*b*
3-Hydroxychalcone	3-C		3-OH	$C_{15}H_{12}O_2$	Pale yellow powder	160—161	47	1, 2
4-Hydroxychalcone	4-C		4-OH	$C_{15}H_{12}O_2$	Yellow powder	184	38	1, 2, 10*b*
2′,4′-Dihydroxychalcone	2′,4′-C	2′,4′-OH		$C_{15}H_{12}O_3$	Yellow needle	145—146	52	4, 10*c*
2′,3-Dihydroxychalcone	2′,3-C	2′-OH	3-OH	$C_{15}H_{12}O_3$	Yellow needle	159—160	25	2
2′,4-Dihydroxychalcone	2′,4-C	2′-OH	4-OH	$C_{15}H_{12}O_3$	Orange yellow needle	155—158	22	2, 4, 10*d*, *e*
2,4′-Dihydroxychalcone	2,4′-C	4′-OH	2-OH	$C_{15}H_{12}O_3$	Yellow needle	227—229	31	1
3′,3-Dihydroxychalcone	3′,3-C	3′-OH	3-OH	$C_{15}H_{12}O_3$	Brownish yellow powder	168—169	38	1, 10*f*
3,4′-Dihydroxychalcone	3,4′-C	4′-OH	3-OH	$C_{15}H_{12}O_3$	Brownish yellow needle	237	33	1, 2
4′,4-Dihydroxychalcone	4′,4-C	4′-OH	4-OH	$C_{15}H_{12}O_3$	Pale yellow prism	202	27	1, 10*b*
2′,4′,4-Trihydroxychalcone (isoliquiritigenin)	2′,4′,4-C	2′,4′-OH	4-OH	$C_{15}H_{12}O_4$	Yellow needle	209—212	40	4, 10*d*, *g*
3,4′,4-Trihydroxychalcone	3,4′,4-C	4′-OH	3,4-OH	$C_{15}H_{12}O_4$	Yellow needle	208—212	18	4, 10*b*
4′-Hydroxy-2-methoxychalcone	2MeO-4′-C	4′-OH	2-MeO	$C_{16}H_{14}O_3$	White yellow needle	194—198	51	10*h*
4′-Hydroxy-3-methoxychalcone	3MeO-4′-C	4′-OH	3-MeO	$C_{16}H_{14}O_3$	White yellow needle	161—163	47	
4′-Hydroxy-4-methoxychalcone	4MeO-4′-C	4′-OH	4-MeO	$C_{16}H_{14}O_3$	Yellow needle	188—190	35	2
4′-Hydroxy-2-methylchalcone	2Me-4′-C	4′-OH	2-Me	$C_{16}H_{14}O_2$	Pale yellow needle	105—187	13	
4′-Hydroxy-3-methylchalcone	3Me-4′-C	4′-OH	3-Me	$C_{16}H_{14}O_2$	Brownish yellow needle	161—162	35	
4′-Hydroxy-4-methylchalcone	4Me-4′-C	4′-OH	4-Me	$C_{16}H_{14}O_2$	Pale yellow needle	193—195	10	10*i*
2-Hydroxy-4′-methylchalcone	4′Me-2-C	4′-Me	2-OH	$C_{16}H_{14}O_2$	Yellow green powder	152—153	41	2
3-Hydroxy-4′-methylchalcone	4′Me-3-C	4′-Me	3-OH	$C_{16}H_{14}O_2$	Brownish yellow needle	142—143	36	2
4-Hydroxy-4′-methylchalcone	4′Me-4-C	4′-Me	4-OH	$C_{16}H_{14}O_2$	Pale yellow powder	155—156	36	2
3-Hydroxy-2′-methylchalcone	2′Me-3-C	2′-Me	3-OH	$C_{16}H_{14}O_2$	Pale yellow needle	93—95	32	
3-Hydroxy-3′-methylchalcone	3′Me-3-C	3′-Me	3-OH	$C_{16}H_{14}O_2$	Pale yellow needle	106—107	23	
3-Hydroxy-3′-methoxychalcone	3′MeO-3-C	3′-MeO	3-OH	$C_{16}H_{14}O_3$	Yellow prism	99—101	47	
3-Hydroxy-4′-methoxychalcone	4′MeO-3-C	4′-MeO	3-OH	$C_{16}H_{14}O_3$	Pale yellow needle	163—165	40	2
3′-Hydroxy-3-methylchalcone	3Me-3′-C	3′-OH	3-Me	$C_{16}H_{14}O_2$	Pale yellow powder	93—94	17	
4′,4-Dihydroxy-2-methoxychalcone (echinatin)	2MeO-4′,4-C	4′-OH	2-MeO,4-OH	$C_{16}H_{14}O_4$	Yellow prism	209—211	n.p.[c]	10*j*
2′,3-Dihydroxy-4′-methylchalcone	4′Me-2′,3-C	4′-Me,2′-OH	3-OH	$C_{16}H_{14}O_3$	Yellow needle	168—170	10	
2′,3-Dihydroxy-5′-methylchalcone	5′Me-2′,3-C	2′-OH,5′-Me	3-OH	$C_{16}H_{14}O_3$	Yellow needle	157—158	34	
3,4-Dihydroxy-4′-methoxychalcone	4′MeO-3,4-C	4′-MeO	3,4-OH	$C_{16}H_{14}O_4$	Yellow powder	170—172	13	4
3,4-Dihydroxy-4′-methylchalcone	4′Me-3,4-C	4′-Me	3,4-OH	$C_{16}H_{14}O_3$	Yellow green powder	198—199	11	4
4-Isopropyl-4′-hydroxychalcone	4isoPr-4′-C	4′-OH	4-isoPr	$C_{18}H_{18}O_2$	White plate	149	33	
4′-Chloro-4-hydroxychalcone	4′Cl-4-C	4′-Cl	4-OH	$C_{15}H_{11}ClO_2$	Yellow needle	168	31	2
3-Hydroxy-4′-*tert*-butylchalcone	4′*tert*-Bu-3-C	4′-*tert*-Bu	3-OH	$C_{19}H_{20}O_2$	White yellow needle	128	50	
4-Hydroxy-4′-*tert*-butylchalcone	4′*tert*-Bu-4-C	4′-*tert*-Bu	4-OH	$C_{19}H_{20}O_2$	Yellow needle	171—172	27	
2′,3,4′-Trihydroxy-3′-methylchalcone	3′Me-2′,3′,4′-C	3′-Me,2′,4′-OH	3-OH	$C_{16}H_{14}O_4$	Yellow powder	228—230	26	
2′,3,4-Trihydroxy-5′-methylchalcone	5′Me-2′3,4-C	5′-Me,2′-OH	3,4-OH	$C_{16}H_{14}O_4$	Orange needle	177—180	18	4
3-Methoxy-3′-methylchalcone	3MeO-3′Me-C	3′-Me	3-MeO	$C_{17}H_{16}O_2$	Oil		21	
3-Methoxy-4′-methylchalcone	3MeO-4′Me-C	4′-Me	3-MeO	$C_{17}H_{16}O_2$	Pale yellow prism	65	32	
4-Methoxy-3′-methylchalcone	4MeO-3′Me-C	3′-Me	4-MeO	$C_{17}H_{16}O_2$	Oil		16	
4-Methoxy-4′-methylchalcone	4MeO-4′Me-C	4′-Me	4-MeO	$C_{17}H_{16}O_2$	Pale yellow prism	89—91	42	2
3-α,α′-Dimethylallyl-4,4′-dihydroxy-6-methoxychalcone (licochalcone A)	Lico A	4′-OH	6-MeO,4-OH, 3-R[a]	$C_{21}H_{22}O_4$	Yellow needle	99—100	n.p.[c]	10*k*

a) R = α,α′-dimethylallyl; *b*) com.: commercial substance; *c*) n.p.: natural product.

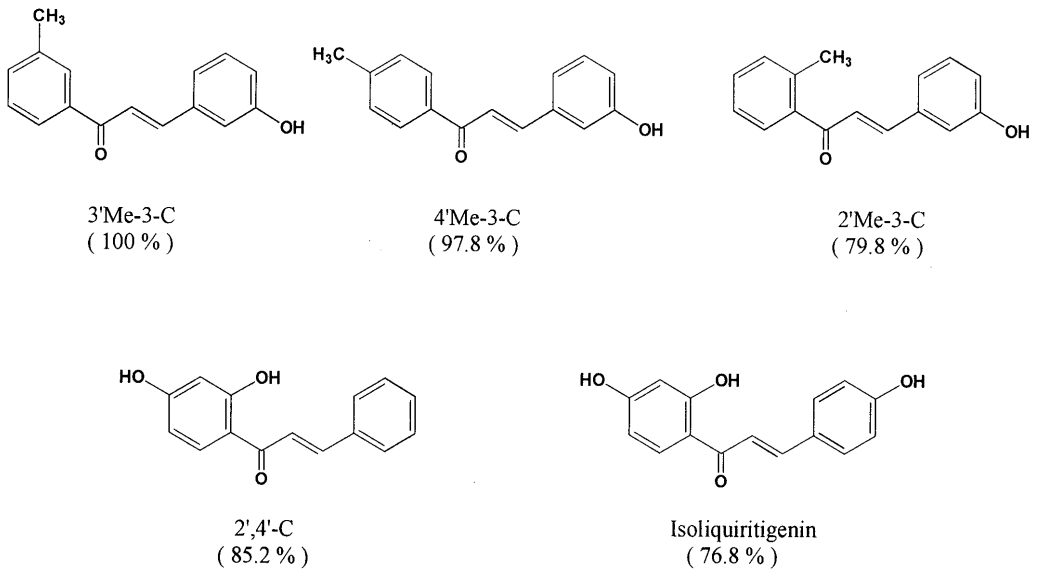
Table 2. High Resolution Molecular Mass Numbers $[M]^+$ of the Chalcones Newly Synthesized

Compound	$[M]^+$ Calcd	$[M]^+$ Found	Compound	$[M]^+$ Calcd	$[M]^+$ Found
4′-Hydroxy-3-methoxychalcone	254.0943	254.0952	2′,3-Dihydroxy-5′-methylchalcone	254.0943	254.0944
4′-Hydroxy-2-methylchalcone	238.0994	238.0995	4-Isopropyl-4′-hydroxychalcone	266.1306	266.1310
4′-Hydroxy-3-methylchalcone	238.0993	238.0990	3-Hydroxy-4′-*tert*-butylchalcone	280.1464	280.1470
3-Hydroxy-2′-methylchalcone	238.0994	238.0990	4-Hydroxy-4′-*tert*-butylchalcone	280.1463	280.1460
3-Hydroxy-3′-methylchalcone	238.0994	238.0992	2′,3,4′-Trihydroxy-3′-methylchalcone	270.0892	270.0898
3-Hydroxy-3′-methoxychalcone	254.0943	254.0940	3-Methoxy-3′-methylchalcone	252.1151	252.1157
3′-Hydroxy-3-methylchalcone	238.0994	238.0994	3-Methoxy-4′-methylchalcone	252.1151	252.1150
2′,3-Dihydroxy-4′-methylchalcone	254.0943	254.0947	4-Methoxy-3′-methylchalcone	252.1151	252.1151

Table 3. Effect of Chalcone Derivatives on TPA-Enhanced ^{32}Pi-Incorporation into Phospholipids of Cultured Cells

Compound	Abbr.	Inhibition	Compound	Abbr.	Inhibition
Chalcone	C	+	4-Hydroxy-4′-methylchalcone	4′Me-4-C	
2′-Hydroxychalcone	2′-C	+	3-Hydroxy-2′-methylchalcone	2′Me-3-C	+ + +
4′-Hydroxychalcone	4′-C	+	3-Hydroxy-3′-methylchalcone	3′Me-3-C	+ + +
3-Hydroxychalcone	3-C	+	3-Hydroxy-3′-methoxychalcone	3′MeO-3-C	+
4-Hydroxychalcone	4-C	+	3-Hydroxy-4′-methoxychalcone	4′MeO-3-C	
2′,4′-Dihydroxychalcone	2′,4′-C	+ + +	3′-Hydroxy-3-methylchalcone	3Me-3′-C	+
2′,3-Dihydroxychalcone	2′,3-C	+	Echinatin		+ +
2′,4-Dihydroxychalcone	2′,4-C	+ +	2′,3-Dihydroxy-4′-methylchalcone	4′Me-2′,3-C	+
2,4′-Dihydroxychalcone	2,4′-C	+	2′,3-Dihydroxy-5′-methylchalcone	5′Me-2′,3-C	
3′,3-Dihydroxychalcone	3′,3-C	+	3,4-Dihydroxy-4′-methoxychalcone	4′MeO-3,4-C	+
3,4′-Dihydroxychalcone	3,4′-C	+	3,4-Dihydroxy-4′-methylchalcone	4′Me-3,4-C	+
4′,4-Dihydroxychalcone	4′,4-C		4-Isopropyl-4′-hydroxychalcone	4isoPr-4′-C	+
Isoliquiritigenin		+ + +	4′-Chloro-4-hydroxychalcone	4′Cl-4-C	+ +
3,4′,4-Trihydroxychalcone	3,4′,4-C		3-Hydroxy-4′-*tert*-butylchalcone	4′*tert*-Bu-3-C	+
4′-Hydroxy-2-methoxychalcone	2MeO-4′-C		4-Hydroxy-4′-*tert*-butylchalcone	4′*tert*-Bu-4-C	+ +
4′-Hydroxy-3-methoxychalcone	3MeO-4′-C		2′,3,4′-Trihydroxy-3′-methylchalcone	3′Me-2′,3,4′-C	+
4′-Hydroxy-4-methoxychalcone	4MeO-4′-C	+	2′,3,4-Trihydroxy-5′-methylchalcone	5′Me-2′,3,4-C	+
4′-Hydroxy-2-methylchalcone	2Me-4′-C	+	3-Methoxy-3′-methylchalcone	3MeO-3′Me-C	+
4′-Hydroxy-3-methylchalcone	3Me-4′-C		3-Methoxy-4′-methylchalcone	3MeO-4′Me-C	
4′-Hydroxy-4-methylchalcone	4Me-4′-C	+ +	4-Methoxy-4′-methylchalcone	4MeO-4′Me-C	+ +
2-Hydroxy-4′-methylchalcone	4′Me-2-C	+	4-Methoxy-3′-methylchalcone	4MeO-3′Me-C	+
3-Hydroxy-4′-methylchalcone	4′Me-3-C	+ + +	Licochalcone A	Lico A	+ +

HeLa cells were incubated with or without test compound (5 μg/ml), and after 1 h ^{32}Pi (370 kBq per culture) was added with or without TPA (50 nM). Incubation was continued for 4 h, and then the radioactivity incorporated into the phospholipid fraction was measured. Inhibition rates were calculated as percentages with respect to the control value: Less than 25% inhibition = no mark, 26—50% inhibition = +, 51—75% inhibition = + +, 76—100% inhibition = + + +.



3'Me-3-C (100 %) 4'Me-3-C (97.8 %) 2'Me-3-C (79.8 %)

2',4'-C (85.2 %) Isoliquiritigenin (76.8 %)

Chart 1. Chalcone Derivatives Showing High Inhibitory Effect on ^{32}Pi-Incorporation to the Phospholipids Promoted by TPA in HeLa Cells

hydroxy-2′-methylchalcone (2′Me-3-C), 2′,4′-dihydroxychalcone (2′,4′-C) and isoliquiritigenin gave the potency in grade + + + (Chart 1). Especially, 3′Me-3-C and 4′Me-3-C showed the strongest inhibitory effect, 100% and 97.8% (5 μg/ml), respectively, among the chalcones so far tested.

Three chalcones, which give the + + + potency, possess a hydroxyl at 3-position in B-ring and methyl at 2′-, 3′- or 4′-position, respectively, in A-ring (2′Me-3-C, 3′Me-3-C and 4′Me-3-C). The presence of free hydroxyl at 3-position seems to be essential, since *O*-methylation of these compounds decreased the inhibitory potency (3MeO-3′Me-C, 3MeO-4′Me-C). The presence of hydroxyl or methoxyl on the A-ring of chalcone possessing 3-hydroxyl on the B-ring also decreases the inhibitory activity (2′,3-C, 3′,3-C, 3,4′-C(+), 3′MeO-3-C(+), 4′MeO-3-C).

2′,4′-Dihydroxychalcone (2′,4′-C) and isoliquiritigenin (2′,4′,4-C), which possess neither 3-hydroxy nor methyl on the A-ring, gave fairly high potency (+ + +85.2% and + + +76.8% (5 μg/ml), respectively). Yamamoto *et al.*[11] reported that 2′,4′-C prevented gastric ulcer formation in rats induced by water-immersion stress and by acetic acid. Isoliquiritigenin (2′,4′,4-C) has been noted to have other biological effects such as the inhibition of monoamine oxidase,[12] aldose reductase,[13] c-AMP-phosphodiesterase,[14] and allergic reaction in animals.[15] Recently, Kato and his coworkers[16] reported the anti-tumorigenic activity of isoliquiritigenin on the DMBA-

Table 4. Effect of Isoliquiritigenin and Related Compounds on TPA-Enhanced ^{32}Pi-Incorporation into Phospholipids of Cultured Cells

Compound	Inhibition (%)
Isoliquiritigenin	76.8
2′,4-C	73.2
2′,4′-C	85.2
2,4′-C	27.0
4′,4-C	18.3
2′-C	49.3
4′-C	26.7
4-C	36.2

initiated and TPA-promoted skin papilloma of CD-1 mice. The presence of a chelated hydroxyl at 2′-position on the A-ring seems to enhance the antitumorigenic activity of isoliquiritigenin related chalcones. The decrease of hydroxyl from isoliquiritigenin resulted in suppression of inhibitory potency (2′,4-C(++), 2,4′-C(+), 2′-C(+), 4′-C(+), 4-C(+)) (Table 4).

In the *in vitro* cell culture experiments, some synthetic chalcones showed remarkable inhibition of proliferation of the human malignant tumor cells such as HGC-27 (gastric cancer), HeLa (cervical carcinoma), PANC-1 (pancreatic cancer) and GOTO (neuroblastoma). 3′Me-3-C showed the highest potency of antiproliferation of tumor cells, and the inhibitory activity was dose dependent.

By the analysis of cell cycle, 3′Me-3-C was proved to cause an arrest in the G_0/G_1 phase, delaying passage through the *S* phase. 3′Me-3-C also modulated protein synthesis and reduced phosphorylation of proteins in tumor cells HGC-27.

The *in vivo* antitumorigenic activity of 3′Me-3-C was demonstrated in mice skin papilloma by the two-stage DMBA/TPA model. Tumor bearing mice (%) and the average number of tumors per mouse at week 18 were 20 and 0.5, respectively, while those of the control were 90 and 5.1.[17]

In connection with the findings of Markaverich *et al.*[18] that some antitumorigenic active compounds including quercetin, luteolin and 4,4′-dihydroxy chalcone occupy nuclear type-II estrogen binding sites (EBS) competitively with estradiol and diethylstilbestrol, 3′Me-3-C was tested for its binding affinity to type-II EBS of HGC-27 cells and showed a stronger affinity than quercetin.[17] This result must be studied further as one factor in the antitumorigenic mechanism of this type of compound.

Part of this study was reported earlier.[19,20]

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