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CYTOTOXICITY OF PLANT FLAVONOIDS AGAINST HeLa CELLS

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Abstract—(−)-Epigallocatechin and 28 other plant flavonoids were tested for cytotoxic activity against HeLa cells Flavones and flavanones were active and several compounds with planar and non-planar ring systems showed high cytotoxic activities Although no clear structure–activity relationship was deduced, hydroxyl groups on the A- and B-ring affected the cytotoxic potency positively or negatively, depending on the position of substitution The uptake of thymidine was predominantly inhibited by myricetin, whereas the uptake of uridine was inhibited by (−)-epigallocatechin, the uptake of both thymidine and uridine were inhibited by 7,8-dihydroxyflavone

INTRODUCTION

Since (−)-epigallocatechin, an antibiotic of the Okinawan medicinal plant, *Elaeagnus glabra* Thunb (Elaeagnaceae) [1, 2], showed cytotoxicity against HeLa cells (uterine carcinoma cell) [1], 28 related flavonoids were tested for the cytotoxic activity against the cells in this study

Edwards *et al.* [3] have tested a large number of flavonoids for cytotoxicity against KB cells (cells from human carcinoma of the nasopharynx), and have also shown *in vivo* activity of the flavonoids with other tumour systems other than HeLa clls Several workers [4–7] have isolated cytotoxic flavonoids from plants by biological assay with KB cells However, cytotoxicity against HeLa cells has not been tested systematically with plant flavonoids Furthermore, no work has been reported for effect of flavonoids on uptake of macromolecule precursors in the uterine carcinoma cell Therefore, this paper reports the cytotoxic activity of the flavonoids, their structure–activity relationships and effect of several cytotoxic flavonoids on the uptake of the precursors in HeLa cells.

RESULTS AND DISCUSSION

The structures and cytotoxic activity (IC_{50}) against HeLa cells for 29 flavonoids are shown in Table 1. The conformations of selected (**1**, **2**, **7**, **16**, **17**, **22** and **27**) were analysed by computer graphics. The results indicated that the A/C ring system of the flavones and flavonols is planar, while that of the other flavonoids is non-planar (Fig. 1 shows the computer graphics of **16** and **27**) Among the flavonoids tested for the cytotoxicity, nine showed significant activities at less 10 μg/ml, and nine others medium activities at 10–20 μg/ml dosages

Structure–activity relationships in flavones and flavonols

On the A-ring of the flavone skeleton which, itself, had the cytotoxic activity (see **1**), 2 vicinal hydroxyl (OH) much enhanced activity (**2** and **3**). In the case of the 5,7-dihydroxy flavones, catechol substitution in the B-ring was required for high activity (compare **4** with **5**). However, in the flavonol series 3′,4′-dihydroxylation was not needed for activity (**7**). 2′-OH and 6-OH substitution in the flavonol series reduced actvity (**8**, **13** and **14**). The 4′-OH, 3′,4′-OHs and 3′,4′,5′-OHs decreased, in this order, the activity in the range 6–20 μg/ml dosages (**7** > **9** > **11** > **16** in the activity).

Structure–activity relationships in flavanones, flavanonols and catechins

The 2,3-double bond was favorable for activity (compare the following pairs: **1** > **17**, **5** ≫ **19**; **10** ≫ **22**, **11** ≫ **23**; and **15** > **24**) However, when a B-ring hydroxyl was methylated (**20** and **21**), the 2,3-double bond did not increase activity (compare **5** with **20** and **21**). In non-planar flavonoids, the activity was diminished by 3-hydroxylation (flavanones > flavanonols and catechins). For greater activity, a 3′- or 4′-methoxyl was advantageous (**20** and **21**)

Comparison of the activities of the catechins, **26** and **27**, suggests that a 5′-hydroxyl group enhances activity. In the planar flavonoids, 5′-hydroxylation had no effect on activity (see **11** and **16**). 3′,4′,5′-Trihydroxylation is only effective for cytotoxic activity in the catechin series.

Inhibition of uptake of macromolecule precursors

For the incorporation experiments, **2**, **16** and **27** were employed as test flavonoids. **2** was selected because of its high activity. Myricetin (**16**) with a planar structure was selected for comparison with **27**.

Incorporated radioactivity was counted depending on flavonoid concentrations (12 5, 25 and 50 μg/ml) and

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Table 1 Cytotoxicity of plant flavonoids against HeLa cells

Flavonoid	Position of OH group	Other substituent	Cytotoxicity (IC_{50}, μg/ml)
Flavone			
Flavone (**1**)			10 7
7,8-Dihydroxyflavone (**2**)	7,8		2 9
6,7-Dihydroxyflavone (**3**)	6,7		2 6
Chrysin (**4**)	5,7		56 3
Luteolin (**5**)	5,7,3',4'		4 0
Apigenin-7,4'-dimethyl ether (**6**)	5	7,4'-diOMe	54 4
Flavonol			
Galangin (**7**)	5,7		6 3
Datiscetin (**8**)	5,7,2'		30 4
Kaempferol (**9**)	5,7,4'		8 6
Fisetin (**10**)	7,3',4'		9 9
Quercetin (**11**)	5,7,3',4'		16 9
Rhamnetin (**12**)	5,3',4'	7-OMe	7 0
Morin (**13**)	5,7,2',4'		60 5
Quercetagetin (**14**)	5,6,7,3',4'		53 3
Robinetin (**15**)	7,3',4',5'		20 0
Myricetin (**16**)	5 7,3',4',5'		18 9
Flavanone*			
(±)-Flavanone (**17**)			15 9
(±)-5,7,4'-Trihydroxy-flavanone (**18**)	5,7,4'		10 9
(±)-Eriodictyol (**19**)	5,7,3',4'		15 3
(±)-Homoeriodictyol (**20**)	5,7,4'	3'-OMe	4 0
(±)-Hespertin (**21**)	5,7,3'	4'-OMe	7 9
Flavanonol†			
(−)-Fustin(2β,3α) (**22**)	7,3',4'		25 3
(+)-Taxifolin(2α,3β) (**23**)	5,7,3',4'		49 1
(+)-Dihydrorobinetin(2α,3β) (**24**)	7,3',4',5'		23 3
Catechin			
(+)-Catechin(2α,3β) (**25**)‡	5,7,3',4'		>100
(−)-Epicatechin(2α,3α) (**26**)‡	5,7,3',4'		57 4
(−)-Epigallocatechin(2α,3α) (**27**)‡	5,7 3',4',5'		18 4
Acetate of **27**(2α,3α) (**28**)§		3,5,7,3',4',5'-hexaOAc	18 0
Methyl ether of **27**(2α,3α) (**29**)§		5,7,3',4',5'-pentaOMe	32 4

*Racemates

†$[\alpha]_D$ values (*c* 1 0, 50% Me_2CO–H_2O, 25°) are **22**, −14°, **23**, +49°, and **24**, +17°

‡$[\alpha]_D$ values (*c* 1 0, EtOH, 25°) are **25**, +59°, **26**, −64°, and **27**, −64°

§$[\alpha]_D$ values (*c* 1 0, Me_2CO, 25°) are **28**, −20°, and **29** −14°

culture periods (24 and 48 hr) (Table 2) Since reliable data for the inhibitory action of the flavonoids on the incorporation of the precursors (labelled thymidine, uridine and leucine) was observed at 25 μg/ml concentration, degree of inhibition (%) of the flavonoids for incorporation was calculated on the basis of concentration, and is shown in Fig 2

2 inhibited uptake of thymidine and uridine, and additionally inhibited that of leucine at 48 hr (Fig 2a) The high cytotoxicity of **2** may be due to this non-specific inhibitory action on the uptake of these precursors In **16**, the uptake of thymidine was inhibited predominantly (Fig 2b), while that of uridine was in **27** despite weak inhibitory action (Fig 2c)

From the data in Table 2, unexpected high radioactivities were counted in the experiment with 50 μg/ml of **27** on leucine incorporation (data with † in the table), and were reproducible in a second experiment (the data in Table 2 were from the first experiment) These high incorporation values into HeLa cells are, therefore, assumed to be due to an increase in the cells beyond the cytotoxic effect of **27**

Recently, Mookerjee *et al* [8] reported effects of some flavonoids on lymphocyte proliferative responses, and have concluded that the flavonoids inhibited the uptake of thymidine into phytomytogen-stimulated lymphocytes but did not affect incorporation of the transported thymidine into newly synthesized DNA It is, therefore, impossible

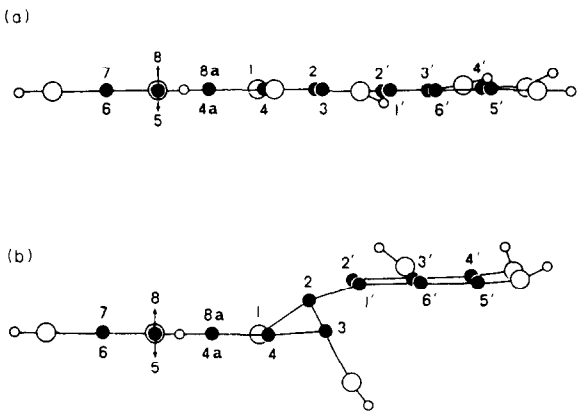

Fig 1 Computer graphics of myricetin (**16**) (a) and (—)-epigallocatechin (**27**) (b), in which large open circle (○), small open circle (o) and closed circle (●) indicate oxygen, hydrogen (of OH) and carbon atoms, respectively The viewpoint is adjusted to view from C-5 to C-8 of the A-ring

ible to state whether or not the effects of the flavonoids studied here to decrease the incorporation of the precursors is due to inhibition of uptake of the precursors or to inhibition of uptake into HeLa cells The difference in activity between **16** (planar) and **27** (non-planar) is interesting, but it is not clear whether this is related to conformational differences Other workers [e g 9] have suggested the participation of planar flavonoids with nucleic acid bases by intercalating interaction into the stacking of the bases.

Edwards *et al* [3] have tested the cytotoxicity of **4**, **5**, **7**, **9–16**, **18** and **21–24** with KB cells, and recognized cytotoxicities [<30 μg/ml (ED_{50})] in **4**, **9** and **14**, **15**. These activities are different from the cytotoxicities against HeLa cells Several flavonoids (mainly **11**) have been reported to inhibit DNA and RNA synthesis in Ehrlich ascite cells by increasing cyclic AMP level [10] or by inhibiting RNA polymerase activity [11]

EXPERIMENTAL

Chemicals Flavonoids **1–25** were obtained commercially From optical rotations (Table 1) and Cotton effect in the CD curves of **17–25** [12], it was estimated that the flavanones (**17–21**) were racemates, and the others (**22–25**) were optically active with the configurations at the 2 and 3 positions as shown in Table 1 Catechins, **26** and **27** were isolated from *E glabra* [1], and, **28** and **29** were prepared from **27**

Acetate of **27** (**28**) Treatment of **27** with Ac_2O and pyridine (room temp, overnight) gave **28**, mp 189–190°, $[\alpha]_D^{25}$ −20° (*c* 1 0, Me_2CO), EIMS *m/z* 558 $[M]^+$ (2), 516 $[M-H_2CO]^+$ (7), 498 $[M-AcOH]^+$ (15), 139 (100) Found C, 58 33, H, 4 77 $C_{27}H_{26}O_{13}$ requires C, 58 07, H, 4 69, IR, ^1H and ^{13}C NMR data were consistent with the structure

Methylether of **27** (**29**) MeOH soln of **27** was methylated with diazomethane (room temp, overnight) to afford **29**, mp 182°; $[\alpha]_D^{25}$ −14° (*c* 1.0, Me_2CO), EIMS *m/z* 376 $[M]^+$ (37), 358 $[M-H_2O]^+$ (6), 167 (100) Found· C, 64 10, H, 6 21. $C_{20}H_{24}O_7$ requires C, 63 82; H, 6 34 IR, ^1H and ^{13}C NMR data were consistent with thus structure

Radioactive precursors [2-^{14}C]Thymidine (51 mCi/mM), [2-^{14}C]uridine (51 mCi/mM) and L-[U-^{14}C]leucine (348 mCi/mM) were purchased from Radiochemical Centre, Amersham, Buckinghamshire

Cytotoxicity test The method essentially that of Kurobane *et al* [13] HeLa-S_3 cells were maintained in a suspension culture of RPMI 1640 [Nissui Pharm Co (Tokyo)] supplemented with 10% calf serum [Gibco 200–6170 (U S.A)] containing L-glutamine and a penicillin-streptomycin mixture (Gibco 600–5070, 5000 U/ml) A 100 μl aliquot of 1×10^5 cells/ml was mixed with a 100 μl aliquot of two-fold serial dilution of a flavonoid, and the mixture was incubated on a well of a microtiter plate [96-well, Costar (U S A)] (37°, 96 hr) The cells stained with gentian violet was solubilized with EtOH Absorbance of the coloured EtOH soln was measured at 620 nm to determine cytotoxicity (IC_{50}) of the flavonoid on the calibration curve

Incorporation of radioactive precursors into HeLa macromolecules HeLa cells (1.5×10^4 cells/400 μl) were distributed in each well of a tissue culture plate (48-well, Costar), and incubated in the same culture medium as used in the cytotoxicity test (37°, 24 hr) The cells in the exponential growth phase were used for the incorporation experiment After addition of a 100 μl aliquot of different concns [0 (control), 12 5, 25 and 50 μg/ml] of **2**, **16** or

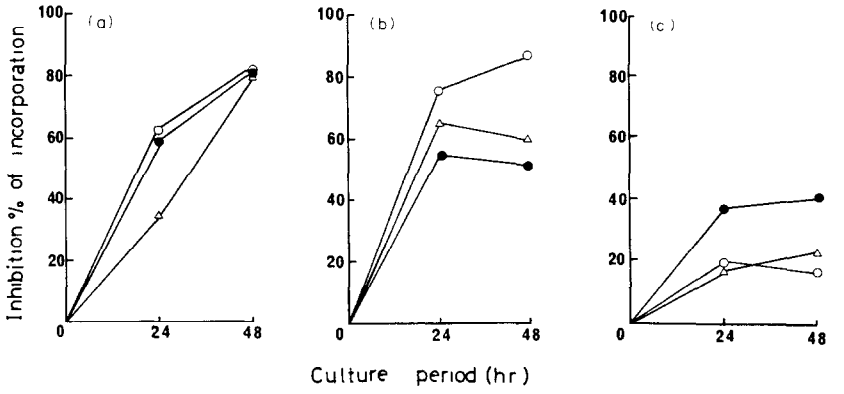

Fig. 2 Inhibitory effect of **2** (a), **16** (b) and **27** (c) on the uptake of thymidine (○–○), uridine (●–●) and leucine(△–△) in HeLa cells The cells were treated with 25 μg/ml of the flavonoids together with 5 μCi/0 1 ml of [2-^{14}C]thymidine, [2-^{14}C]uridine and L-[U-^{14}C]leucine Inhibition per cent was calculated on the basis of the incorporation data at 24 and 48 hr (Table 2)

Table 2 Radioactivity of precursors incorporated into HeLa cell macromolecule at 24 and 48 hr culture periods

Flavonoid	Concentration (μg/ml)	Radioactivity (cpm) [2-^{14}C]Thymidine 24 hr	[2-^{14}C]Thymidine 48 hr	[2-^{14}C]Uridine 24 hr	[2-^{14}C]Uridine 48 hr	L-[2-^{14}C]Leucine 24 hr	L-[2-^{14}C]Leucine 48 hr
7,8-Dihydroxyflavone (**2**)	0	12024*	35632*	80147*	177041*	4800*	16530*
	12.5	6508	10494	46856	53317	4359	6284
	25	4619*	6883*	33905*	34614*	3131*	3473*
	50	1047	1706	8644	10589	1123	1563
Myricetine (**16**)	0	10430*	34480*	73748*	157585*	6698*	15194*
	12.5	8857	30219	61313	154460	6009	14168
	25	2639*	4960*	33429*	75735*	2225*	6274*
	50	117	138	108	201	117	136
(−)-Epigallocatechin (**27**)	0	6146*	20480*	30881*	115507*	2507*	11621*
	12.5	6105	20184	29547	97461	2408	10140
	25	4944*	17073*	19246*	70254*	2049*	8796*
	50	4086	15158	16141	35903	3261†	10451†

*Based on these data, inhibition % of incorporation is calculated and shown in Fig 2
†See text

27 and a 100 μl aliquot (5 μCi) of a precursor to each of the culture wells, they were incubated for 24 or 48 hr at 37°

At the end of the incubation period (24 or 48 hr), the wells were washed with a PI buffer (Gibco 310-4190), trypsinized, and rinsed with ice-cold 5% TCA Radioactivity of the acid-insoluble was counted in a toluene based scintillation liquid

Computer graphics The program 'GONCHAN' [14] made by Dr S Ando, Tokyo Institute of Technology, was used for illustrating computer graphics (Fig 1) of **1**, **2**, **7**, **16**, **17**, **22** and **27**

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