6. E. Rubin and M. Rottenberg, *Fedn Proc.* **41**, 2465 (1982).
7. P. Seeman, *Pharmac. Rev.* **24**. 583 (1972).
8. R. A. Boigegrain, Y. Fernandez, M. Massol and S. Mitjavila, *Chem. Phys. Lip.* **35**. 321 (1984).
9. H. Rottenberg, A. J. Waring and E. Rubin, *Science* **213**. 583 (1981).
10. S. Kelly-Murphy, A. J. Waring, H. Rottenberg and E. Rubin, *Lab. Invest.* **50**, 174 (1984).
11. T. F. Taraschi and E. Rubin, *Lab. Invest.* **52**, 120 (1985).
12. F. Beaugé, C. Fleuret-Balter, F. Barin, J. Nordman and R. Nordmann, *Drug Alcohol Depend.* **10**, 143 (1982).
13. C. Fleuret-Balter, F. Beaugé, F. Barin, J. Nordmann and R. Nordmann, *Pharmac. Biochem. Behav.* **18**, suppl. 1, 25 (1983).
14. F. Beaugé, C. Fleuret, F. Barin and R. Nordmann, *Biochem. Pharmac.* **33**, 3591 (1984).
15. D. B. Goldstein, *Ann. Rev. Pharmac. Toxicol.* **24**. 43 (1984).
16. G. B. Zavoico, L. Chandler and H. Kutchaï, *Biochim. biophys. Acta* **812**, 299 (1985).
17. T. Bütcher and H. Redetzki, *Med. Klin. Wochensch.* **29**, 615 (1951).
18. C. W. Cotman and D. A. Matthews, *Biochim. biophys. Acta* **249**, 380 (1971).
19. F. Beaugé, H. Stibler and H. Kalant, *Pharmac. Biochem. Behav.* **18** Suppl. 1, 519 (1983).
20. O H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. M. Shinitzky and M. Inbar, *Biochim. biophys. Acta.* **433**, 133 (1976).
22. H. Rottenberg, *Biochim. biophys. Acta,* **855**, 211 (1986).

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Tumor promoter-induced basophil histamine release: effect of selected flavonoids

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The tumor promoter phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), has been shown to be a potent stimulus of human basophil histamine release [1, 2]. It is active in the absence of extracellular Ca^{2+}, suggesting that it mobilizes intracellular Ca^{2+} which is required for the exocytosis of basophil granules [3]. The extracellular Ca^{2+}-independence of basophil histamine release contrasts with the largely extracellular Ca^{2+}-dependent release of histamine stimulated by other agents such as antigen, anti-IgE, concanavalin A (Con A), the chemoattractant f-Met-Leu-Phe, and the Ca^{2+} ionophore A 23187 [3].

TPA is one of several recognized tumor promoters that act to promote tumor formation (papillomata) in mouse skin treated at first with an "initiator", e.g. 7,12-dimethylbenz[*a*]anthracene. In the two-stage model of carcinogenesis, both an initiator and a promoter are required for tumor development, neither agent alone being sufficient to induce tumorigenesis. Other recently recognized tumor promoters include teleocidin, an indole product of the mycelia of *Streptomyces mediocidicus,* and aplysiatoxin, a polyacetate compound derived from a blue green alga [4, 5]. Teleocidin and aplysiatoxin, like TPA, are known to stimulate rat brain protein kinase C *in vitro* [6, 7], an enzyme believed to be important in histamine release from rat basophilic leukemia cells (RBL) [8] and in the regulation of a variety of cell activation processes [9].

We decided to investigate whether teleocidin and aplysiatoxin, like TPA, were capable of stimulating human basophil histamine release. In addition, we wished to determine whether certain flavonoids known to be effective inhibitors of TPA-induced histamine release [10] would also inhibit teleocidin- and aplysiatoxin-induced histamine release. Our findings form the basis of this report.

Materials and methods

Preparation of leukocyte suspensions for histamine release and determination of histamine. Leukocyte suspensions were prepared as described previously, and histamine was determined in total leukocyte suspensions and supernatant fractions in accordance with earlier reports [10–13]. The Tris buffer contained 0.6 mM Ca^{2+}, 1.0 mM Mg^{2+} and 0.03% human serum albumin for all experiments except for studies of histamine release in the absence of buffer Ca^{2+}; in these experiments Ca^{2+} was eliminated from the Tris buffer.

Chemicals. Quercetin, fisetin and chalcone were obtained from the Aldrich Chemical Co., Milwaukee, WI; and luteolin and apigenin from Sarget Laboratoires, Merignac, France. Nobiletin and tangeretin were provided by Dr. James Tatum, Department of Citrus, Lakeland, FL; TPA, phloretin and taxifolin were from Sigma, St. Louis, MO. Teleocidin and aplysiatoxin were isolated by a procedure described previously [5].

Experimental protocol. The effects of the following flavonoids were examined: quercetin (3′,4′,3,5,7-pentahydroxyflavone), luteolin (3′,4′,5,7-tetrahydroxyflavone), taxifolin (dihydroquercetin), nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone), fisetin (3′,4′,3,7-tetrahydroxyflavone), apigenin (4′,5,7-trihydroxyflavone), tangeretin (4′,5,6,7,8-pentamethoxyflavone), phloretin (β-(*p*-hydroxyphenyl)-2,4,6-trihydroxypropiophenone) and chalcone (1,3-diphenyl-2-propene-1-one). The flavonoids were dissolved in dimethyl sulfoxide (10–20 mM stock solutions) and were diluted in buffer to provide concentrations between 5 and 50 μM in the final cell suspensions. The final concentration of DMSO did not exceed 0.5%, a concentration which did not affect the histamine release process [12]. As a matter of routine, cell suspensions were preincubated for 10 min with the flavonoids prior to addition of the tumor promoters, and the incubation was then continued for an additional 60 min. The reaction was terminated by centrifugation, and supernatant histamine was measured. The tumor promoters were dissolved in DMSO and diluted in buffer to provide final concentrations in cell suspensions of 1–200 ng/ml. All experiments were conducted at 37° in polypropylene tubes. Histamine was determined by an automated technique [13].

Results

Tumor promoter stimulation of basophil histamine release. As shown in Fig. 1, TPA, teleocidin and aplysiatoxin (1–200 ng/ml) each stimulated basophil histamine release in a concentration-dependent manner. The maximal histamine releasing effect was noted at 10–50 ng/ml. Each tumor promoter stimulated histamine release in the presence or absence of extracellular buffer Ca^{2+} although the

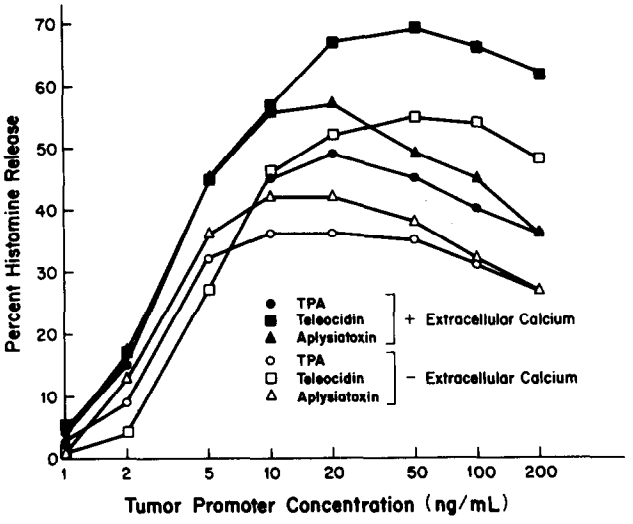


Fig. 1. Tumor promoter-induced basophil histamine release in the presence and absence of extracellular Ca^{2+} (0.6 mM). Results displayed are from one of three experiments each of which gave similar results. Total cellular histamine was 13–42 ng/ml of final cell suspension for the three experiments.

histamine releasing effect of each tumor promoter was slightly greater in the presence of extracellular Ca^{2+} than in its absence (Fig. 1), although teleocidin at higher concentrations stimulated histamine release in the absence of extracellular Ca^{2+} as well as the others in its presence.

The time course of tumor promoter-induced histamine release is shown in Fig. 2 in which it can be seen that each tumor promoter stimulated histamine release in a relatively gradual manner and which appeared not to be complete at 60 min. In these experiments, the ongoing histamine release reaction was stopped by the addition of luteolin (100 μM) at the different time intervals and chilling the tubes in iced water.

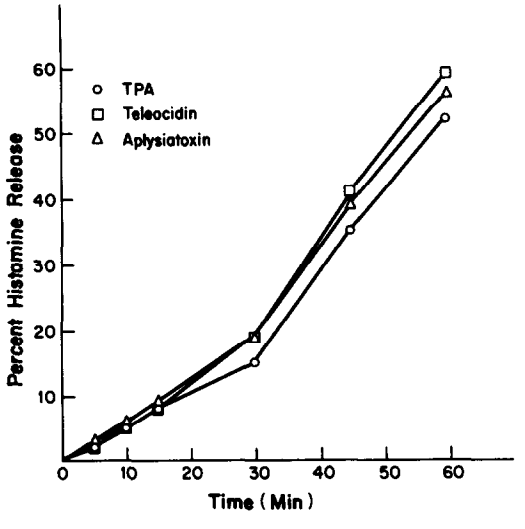


Fig. 2. Time course of tumor promoter-induced basophil histamine release. Results shown are from one of three identical experiments, each of which gave similar findings. The final concentration of each tumor promoter in the reaction mixtures was 20 ng/ml for TPA and teleocidin and 10 ng/ml for aplysiatoxin. The histamine release reaction was stopped at the designated time by addition of luteolin (final concentration, 100 μM) and rapid chilling in iced water. Total cellular histamine ranged from 34 to 37 ng/ml for the three experiments.

Effect of flavonoids on tumor promoter-induced basophil histamine release. Luteolin, nobiletin, quercetin, fisetin, apigenin and chalcone all produced concentration-dependent inhibition of tumor promoter-induced histamine release at concentrations of 5–50 μM, as shown in Fig. 3. There was a remarkable correspondence of inhibitory activity for each of these flavonoids against TPA-, teleocidin- and aplysiatoxin-induced histamine release. Taxifolin was essentially inactive against each tumor promoter, whereas tangeretin and phloretin showed moderate activity only at the higher concentrations (20 and 50 μM). Table 1 shows the IC_{50} values (μM) for the flavonoids against each tumor promoter.

Discussion

These experiments demonstrate that the more recently described tumor promoters, teleocidin and aplysiatoxin, stimulated human basophil histamine release in a manner essentially similar to the recognized histamine-releasing capability of the phorbol ester, TPA. Thus, teleocidin and aplysiatoxin stimulated basophil histamine release in the absence of extracellular buffer Ca^{2+} as previously described for TPA [1], and the time course of histamine release was similar for each tumor promoter (in the presence of extracellular Ca^{2+}) (Fig. 2). It should be noted that human basophils and rat mast cells differ in their responsiveness to these tumor promoters. Rat mast cells are unresponsive to high concentrations, but low concentrations synergistically enhance histamine release stimulated by the non-TPA-type tumor promoters, thapsigargin and palytoxin [15].

The flavonoids luteolin, nobiletin, quercetin, fisetin, apigenin, and the open chain congener, chalcone, were effective inhibitors of tumor promoter-induced histamine release. The inhibitory activities of these particular flavonoids were remarkably similar for each of the tumor promoters, suggesting that TPA, teleocidin and aplysiatoxin all act by a similar, or common, pathway of basophil activation which is sensitive to inhibition by certain flavonoids, depending on structure. Taxifolin (dihydroquercetin), on the other hand, was inactive in each case, and tangeretin and the dihydrochalcone, phloretin, were also inactive (arbitrarily defined as $IC_{50} > 50$ μM) as seen in Table 1. Luteolin, nobiletin, apigenin and tangeretin are flavones; quercetin and fisetin are flavonols; and taxifolin is a flavanonol. Chalcone and phloretin are open-chain analogs of the flavonoids. The structures and activities of

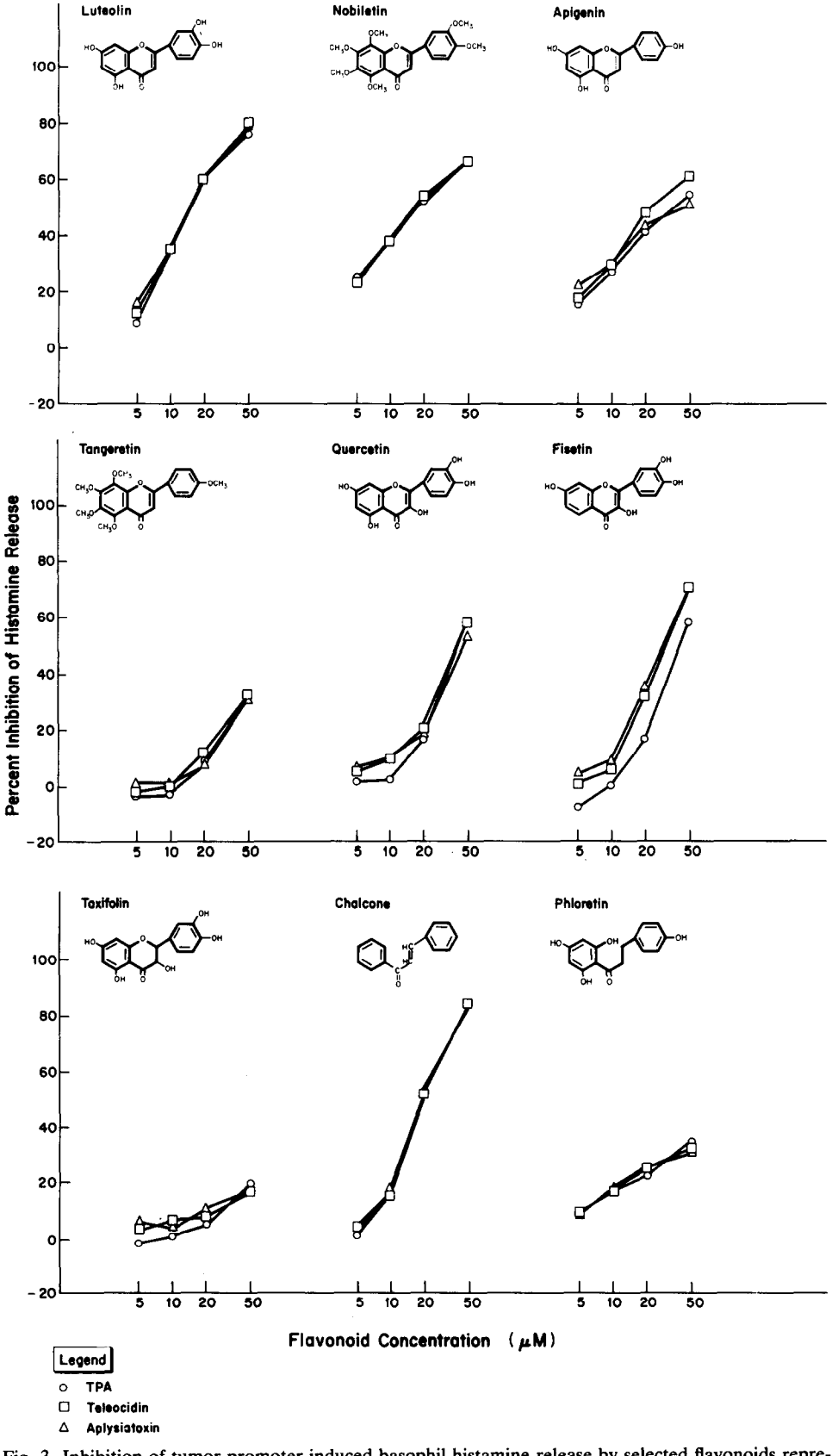
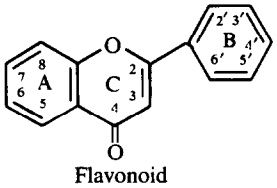


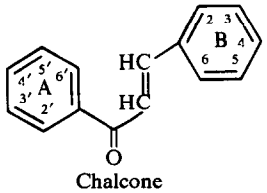
Fig. 3. Inhibition of tumor promoter-induced basophil histamine release by selected flavonoids representing several different structural classes (see Table 1). The data presented are based on at least three experiments for each point. Total cellular histamine for each experiment ranged from 14 to 67 ng/ml, and the histamine release stimulated by the tumor promoters ranged from 19 to 71%.

Table 1. IC_{50} Values (μM) of flavonoids for inhibition of tumor promoter-induced basophil histamine release*



Flavonoid

Compound	Substituent positions: Ring A 5	6	7	Ring B 8	2′	3′	4′	5′	6′	Ring C 3	C2–C3	IC_{50}: TPA	Teleocidin	Aplysiatoxin
Flavones														
Luteolin	OH	—	OH	—	—	OH	OH	—	—	—	u†	18	17	16
Nobiletin	OCH_3	OCH_3	OCH_3	OCH_3	—	OCH_3	OCH_3	—	—	—	u	20	19	19
Apigenin	OH	—	OH	—	—	—	OH	—	—	—	u	37	26	41
Tangeretin	OCH_3	OCH_3	OCH_3	OCH_3	—	—	OCH_3	—	—	—	u	>200	>100	>300
Flavonols														
Quercetin	OH	—	OH	—	—	OH	OH	—	—	OH	u	51	49	63
Fisetin	—	—	OH	—	—	OH	OH	—	—	OH	u	49	32	31
Flavanonol														
Taxifolin	OH	—	OH	—	—	OH	OH	—	—	OH	s	>1000	>1000	>1000



Chalcone

Compound	Substituent positions: Ring A 2′	3′	4′	5′	6′	Ring B 2	3	4	5	6	IC_{50}: TPA	Teleocidin	Aplysiatoxin
Chalcone													
Chalcone	—	—	—	—	—	—	—	—	—	—	20	20	20
Dihydrochalcone													
Phloretin	OH	—	OH	—	OH	—	—	OH	—	—	>200	>200	>200

* The term "flavonoids" as used here includes both the 2-phenyl-γ-benzopyrones used in these experiments as well as the open-chain chalcone congeners [14].

† u stands for an unsaturated bond at C2–C3, and s stands for the saturated C2–C3 bond.

these compounds are displayed in Fig. 3 and Table 1. It is evident that small differences in molecular structure have striking effects on inhibitory capacity, e.g. (1) nobiletin vs tangeretin, (2) quercetin vs taxifolin (dihydroquercetin), and (3) chalcone vs the dihydrochalcone, phloretin. The significance of these structural determinants with respect to inhibitory activity is the subject of a future publication.

When luteolin (100 μM) was added to tumor promoter-stimulated cell suspensions at 5, 10, 15, 30, 45 and 60 min after addition of promoters (20 ng/ml), the ongoing release of histamine was totally abrogated. This finding is in keeping with the effects of quercetin on antigen-induced histamine release [12] and indicates that basophil activation by either the tumor promoters or antigen (and possibly other activators) is accompanied by the generation of a flavonoid-sensitive product or products. Interaction of this putative product with the flavonoid totally inhibits the further outcome of the activation process. The time course of TPA-induced basophil histamine release is slow as compared to that stimulated by anti-IgE as described by Schleimer *et al.* [1]. The time course of histamine release elicited by teleocidin and aplysiatoxin was essentially identical to that of TPA, indicating that all three tumor promoters probably act by a common mechanism. The findings strongly suggest that the biochemical pathways utilized in tumor promoter- and anti-IgE-induced histamine release differ significantly.

Precisely how the flavonoids affect tumor promoter-induced histamine release is unknown. However, each of the tumor promoters in the present study is known to

stimulate protein kinase C [6, 7] and, therefore, it is tempting to speculate that human basophil histamine release stimulated by the tumor promoters is a protein kinase C-dependent process. The validity of this possibility is suggested by the experiments of Sagi-Eisenberg *et al.* [8] with RBL cells. Also, since quercetin has been shown to inhibit protein kinase C [16, 17], it is possible that this flavonoid and the other active flavonoids in this study cause inhibition of tumor promoter-induced histamine release by an effect on protein kinase C activation.

In summary, the tumor promoters, teleocidin and aplysiatoxin, like TPA, stimulated human basophil histamine release both in the presence or absence of extracellular Ca^{2+} and in a kinetically similar fashion. The flavonoids luteolin, nobiletin, quercetin, fisetin, apigenin and chalcone inhibited histamine release by each promoter in a concentration-dependent manner, but taxifolin and the dihydrochalcone derivative, phloretin, lacked activity, indicating that the flavonoid effects were structure dependent and stereoselective. The mechanism of flavonoid action is still unknown, but an effect on protein kinase C is a distinct possibility.

Allergy Division ELLIOTT MIDDLETON, JR.*
Departments of Medicine and Pediatrics
State University of New York at Buffalo
Buffalo, NY 14214, U.S.A.

National Cancer Center Research Institute HIROTA FUJIKI
Tsukiji 5-chome, Chuoku
Tokyo 104, Japan

Allergy Division MOHAMMEDI SAVLIWALA
Departments of Medicine and Pediatrics GARY DRZEWIECKI
State University of New York at Buffalo
Buffalo, NY 14214, U.S.A.

* Correspondence to: Elliott Middleton, Jr., M.D., Buffalo General Hospital, 100 High St., Buffalo, NY 14203, U.S.A.

REFERENCES

1. R. P. Schleimer, E. Gillespie and L. M. Lichtenstein, *J. Immun.* **126**, 570 (1981).
2. R. P. Schleimer, E. Gillespie, R. Daiuta and L. M. Lichtenstein, *J. Immun.* **128**, 136 (1982).
3. J. C. Foreman, in *Biochemistry of the Acute Allergic Reactions* (Eds. E. L. Becker, A. S. Simon and K. F. Austen), p. 315. Alan R. Liss, New York (1981).
4. H. Fujiki, T. Sugimura and R. E. Moore, *Environ. Hlth Perspect.* **50**, 85 (1983).
5. H. Fujiki and T. Sugimura, *Cancer Surv.* **2**, 539 (1983).
6. H. Fujiki, Y. Tanaka, R. Miyake, U. Kikkawa, Y. Nishizuka and T. Sugimura, *Biochem. biophys. Res. Commun.* **120**, 339 (1984).
7. I. B. Weinstein, J. Argoleo, J. Backer, A. Jeffrey, W-L. W. Hsiao, S. Gattoni-Celli, P. Kirschmeier and E. Okin, in *Cellular Interactions by Environmental Tumor Promoters* (Eds. H. Fujiki, E. Hecker, R. E. Moore, T. Sugimura and I. B. Weinstein), p. 59. Japan Scientific Societies Press, Tokyo (1984).
8. R. Sagi-Eisenberg, H. Lieman and I. Pecht, *Nature, Lond.* **313**, 59 (1985).
9. Y. Nishizuka, *Science* **233**, 305 (1986).
10. E. Middleton, Jr. and G. Drzewiecki, *Biochem. Pharmac.* **33**, 3333 (1984).
11. E. Middleton, Jr. and D. Drzewiecki, *Biochem. Pharmac.* **31**, 1449 (1982).
12. E. Middleton, Jr., G. Drzewiecki and D. Krishnarao, *J. Immun.* **127**, 546 (1981).
13. R. P. Siraganian, *J. Immun.* **119**, 2078 (1977).
14. J. B. Harborne, T. J. Mabry and H. Mabry, *The Flavonoids.* Academic Press, New York (1975).
15. K. Ohuchi, N. Hirasawa, C. Takahashi, M. Watanabe, S. Tsurufuji, H. Fujiki, M. Suganuma, H. Hakii, T. Sugimura and S. Brogger Christensen, *Biochim. biophys. Acta* **887**, 94 (1986).
16. M. Gschwendt, F. Horn, W.Kittstein and F. Marks, *Biochem. biophys. Res. Commun.* **117**, 444 (1983).
17. H. Fujiki, T. Horiuchi, K. Yamashita, H. Hakii, M. Suganuma, H. Nishino, A. Iwashim, Y. Hirata and T. Sugimura, in *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure–Activity Relationships* (Eds. V. Dody, E. Middleton, Jr. and J. B. Harborne), p. 429. Alan R. Liss, New York (1986).