ELSEVIER

International Journal of Pharmaceutics 131 (1996) 1–7

international journal of pharmaceutics

Research papers

Anti-tumour and free radical scavenging activity of synthetic curcuminoids

Ruby John Anto[a], Girija Kuttan[a], K.V. Dinesh Babu[b], K.N. Rajasekharan[b], Ramadasan Kuttan[a,*]

[a] *Amala Cancer Research Centre, Amala Nagar, Thrissur, 680 553 Kerala, India*
[b] *Department of Chemistry, University of Kerala, Thiruvananthapuram, Kerala, India*

Received 17 May 1995; accepted 5 July 1995

Abstract

Eight synthetic curcuminoids were investigated for their cytotoxic and tumoricidal activities as well as for their free radical scavenging activity. All the curcuminoids were found to be cytotoxic to cultured L929 cells; concentration needed for 50% inhibition being around 1 μg/ml (3.9–2.5 μM). As antitumour agents, veratryl curcuminoid and salicyl curcuminoid increased the life span of animals by 100.6 and 86.9%, respectively. All the curcuminoids inhibited in vitro lipid peroxidation and scavenged superoxides and hydroxyl radicals. Curcuminoids with a free hydroxyl group on the phenyl ring, such as salicyl curcuminoid, were found to be most active. Compounds which did not have free hydroxyl group such as veratryl curcuminoid, had lower activity in vitro but showed comparable activity in vivo. The results indicated that synthetic curcuminoids, like natural curcumin are potent antioxidants.

Keywords: Curcumin; Synthetic curcuminoid; Antitumour agent; Antioxidant

1. Introduction

Curcumin, an active ingredient from *Curcuma longa* is reported to have cytocidal activity towards tumour cells (Kuttan et al., 1985) and is reported to have antitumour activity in animals (Soudamini and Kuttan, 1988). It inhibited production of oxygen free radicals (Elizabeth and Rao, 1990) and is a potent inhibitor of lipid peroxidation (Nishigaki et al., 1992; Soudamini et al., 1992). It has been reported to be anticarcinogenic (Huang et al., 1988; Soudamini and Kuttan, 1989) and antimutagenic (Nagabushan et al., 1987). It is being tried as a chemopreventive drug by National Cancer Institute, USA (Kelloff et al., 1994). Its activity against HIV virus also has been reported recently (Li et al., 1993). Other natural curcuminoids obtained from *Curcuma longa* were also found to have similar or even higher activity as compared to curcumin (Ruby et al., 1995). These findings indicated that the general structure of curcumin i.e., the conjugated diene with an adjacent phenolic group has the general property to act as a potent antioxidant compound. In the

* Corresponding author.

SSDI 0378-5173(95)04254-8

and antitumour activity of several synthetic curcuminoids.

2. Materials and methods

Eagle's minimum essential medium and trypsin were obtained from Hi-media Laboratories Private Ltd., Bombay. L-Phosphatidyl choline was purchased from Sigma Chemical Company, St. Louis, MO. Phorbol-12-myristate-13-acetate was a gift from Dr. Allan Conney, USA, and nitroblue tetrazolium (NBT) from Sisco Research Laboratories Pvt. Ltd., Bombay. *p*-Nitrosodimethyl aniline (PNDA) and thiobarbituric acid was obtained from BDH Chemicals, Poole, England. All other chemicals and reagents used were of analytical reagent quality. The curcuminoids were synthesised by the method reported previously (Dinesh Babu and Rajasekharan, 1994). Structure of curcuminoids used in the present study is shown in Fig. 1. As the molecular weights of the curcuminoids did not vary very much (Mol. wt. 256–396) all the concentrations were expressed as μg/ml.

2.1. Determination of in vitro cytotoxicity of curcuminoids

Ehrlich ascites carcinoma cells and Dalton's lymphoma ascites cells which were originally procured from the Cancer Research Institute, Bombay and the Cancer Institute, Madras, respectively, were maintained as ascites tumours in Swiss albino mice. L929 Cells obtained from the National Facility for Animal Tissue and Cell Culture, Pune, were maintained in culture using minimum essential medium containing 10% goat serum.

The short term in vitro cytotoxicity studies were done using Ehrlich ascites cells (Kuttan et al., 1985). Different concentrations of the curcuminoids were incubated with tumour cells (1 million) suspended in PBS and cytotoxicity was determined after 3 h by the trypan blue exclusion method.

2.2. Determination of cytotoxicity of curcuminoids in tissue culture

The effect of curcuminoids on the growth inhibition of tissue cultured cells were studied using L929 cells (Kuttan et al., 1988). Different concentrations of the curcuminoids (2–20 μg/ml) were incubated with 5×10^4 cells in minimum essential medium containing 10% goat serum. After incubation, the cells were detached by trypsinization (0.2%) and the percentage of live cells were calculated and compared with that of the control.

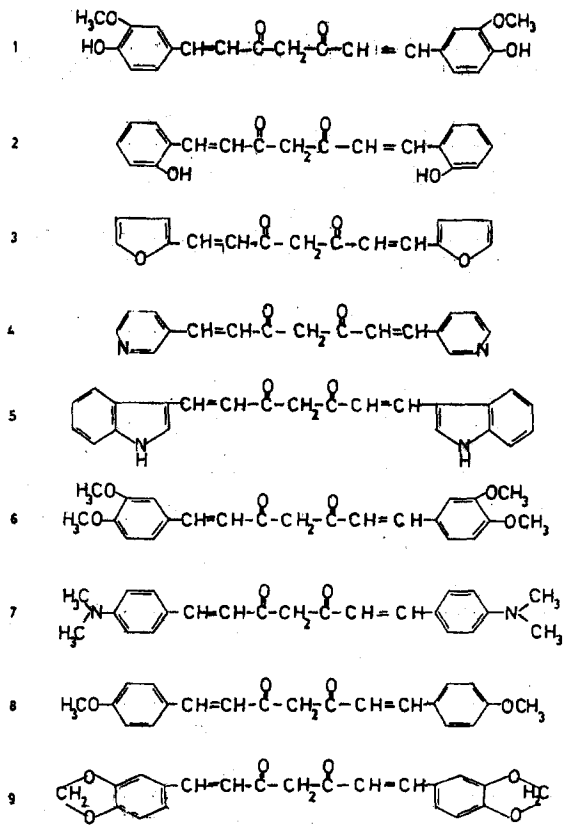

Fig. 1. Structure of curcuminoids. (1) Curcumin. (2) Salicyl curcuminoid. (3) Furfural curcuminoid. (4) Pyridine-3. (5) Indole-3. (6) Veratryl. (7) Dimethylamino. (8) *p*-Anisyl. (9) Piperonal.

Table 1
Cytotoxicity of curcuminoids towards tissue cultured L929 cells

Name of the compound	Percentage control ($T/C \times 100$)			
	10 (μg/ml)	5 (μg/ml)	2 (μg/ml)	1 (μg/ml)
Furfural curcuminoid	3.8	9.6	17.2	26.1
Salicyl	1.27	10.5	12.03	26.09
Pyridine-3	27.4	36.8	45.9	51.5
Indole-3	15.2	37	47.8	63.04
Veratryl	0.725	6.7	34.8	48.6
Dimethylamino	5.8	21.01	43.48	53.62
p-Anisyl	15.04	25.19	32.5	44.4
Piperonal	15.7	27.4	32.9	36

2.3. Liposome encapsulation of curcuminoids

Neutral unilamellar liposomes of the curcuminoids were prepared by the method of Bangham et al. (1974).

2.4. Determination of the activity of curcuminoids in reducing ascites tumour

Swiss albino mice (six per group) were injected intraperitoneally with Ehrlich ascites tumour (10^6 cells). Liposomally encapsulated curcuminoids (12.5 mg/kg) were injected intraperitoneally 24 h after tumour implantation and continued on alternate days for 10 days. The death pattern of animals due to tumour burden was noted and the percentage increase in life span was calculated (Kuttan et al., 1988).

2.5. Determination of superoxide scavenging activity

Superoxide scavenging activity of the curcuminoids were determined by the method of McCord and Fridovich (1969) which depends on the light induced superoxide generation by riboflavin and the corresponding reduction of NBT. The assay mixture contained different concentrations of the curcuminoids and EDTA (6 mM containing 3 μg NaCN), NBT (50 μM) riboflavin (2 μM) and phosphate buffer (58 mM. pH 7.8) in a total volume of 3 ml. The tubes received uniform illumination for 15 min and, thereafter, optical density was measured at 560 nm. The percentage inhibition of superoxide production by the curcuminoids was evaluated by comparing the absorbance of the control and experimental tubes.

2.6. Determination of lipid peroxidation inhibiting activity

Different concentrations of the curcuminoids were incubated at 37°C with a 25% mice liver homogenate (0.1 ml) containing 30 mM KCl, Tris–HCl buffer (0. 04 M, pH 7.0), ascorbic acid (0.06 mM) and ferrous iron (0.16 mM) in a total volume 0.5 ml for 1 h (Bishayee and Balasubramaniam, 1971). Inhibition of lipid peroxidation was determined by the thiobarbituric acid method (Ohkawa et al., 1979).

2.7. Determination of hydroxyl radical scavenging activity

Hydroxyl radicals generated through Fenton reaction bleach *p*-nitrosodimethyl aniline (PNDA) specifically. The activity of the curcuminoids in scavenging these free radicals was measured by the extent of inhibition of bleaching of PNDA in presence of the curcuminoids (Unnikrishnan and Rao, 1990).

2.8. Inhibition of superoxide generation by macrophages activated with PMA

Peritoneal macrophages elicited by sodium ca-

seinate were activated on the fifth day by injection of PMA (100 ng/animal/kg, i.p.) in the presence and absence of the curcuminoids (50 mg/kg, i.p). After 3 h the peritoneal macrophages were harvested. The inhibition produced by the curcuminoids on the superoxide generation by the macrophages was measured by the nitroblue tetrazolium reduction (Dwivedi et al., 1992). In short, 0.5 ml of the mixture (6:2:4) of NBT (0.2% in PBS pH 7.4), dextrose (5%) and Hank's balanced salt solution was mixed with 0.5 ml of peritoneal macrophages (1×10^6 cells/ml) and incubated for 45 min at room temperature. The mixture was centrifuged and the cell pellet was boiled with 2 ml pyridine for 10 min. The optical density of the supernatant was measured at 515 nm which is a measure of the amount of formazan produced by the peritoneal macrophages.

3. Results

Salicyl curcuminoid produced 50% cell death at a concentration of 8.5 μg/ml and indole-3-curcuminoid produced the same effect at a concentration of 18.6 μg/ml. All the other curcuminoids required 50 μg/ml or more to produce 50% cell death (data not shown). The insolubility of the curcuminoids in PBS may be the reason for the low activity. However, all of them were found to be cytotoxic to tissue-cultured L929 cells at concentrations around 1 μg/ml (Table 1).

Most of the curcuminoids when administered as liposomes intraperitoneally could increase the life span of mice bearing ascites tumour. The increase in life span produced by veratryl, salicyl, piperonal, *p*-anisyl and dimethyl amino curcuminoids was 100.6, 86.9, 69.7, 61.5 and 55.2% respectively (Table 2).

The compounds were investigated for their ability to inhibit the ferrous ion-induced peroxidation of rat liver homogenate. Salicyl and piperonal curcuminoids produced 50% inhibition of lipid peroxidation at concentrations of 19.5 and 20 μg/ml, while *p*-anisyl and indole-3- curcuminoids produced the same inhibition at concentrations of 31 and 37 μg/ml, respectively. The concentration required by all other curcuminoids was around 50 μg/ml (Fig. 2).

Table 2
Effect of curcuminoids on reducing ascites tumour

Name of the sample	Average lifespan	Increase in lifespan (%)
Control	17.2 ± 1.2	
Furfural curcuminoid	22.83 ± 4.3	30.5
Salicyl	32.7 ± 13.7	86.9
Pyridine-3	25.3 ± 6.4	44.6
Indole-3	20.7 ± 3	18.3
Veratryl	34.5 ± 8	100.6
Dimethylamino	26.7 ± 1.2	55.2
p-Anisyl	27.8 ± 2.1	61.5
Piperonal	29.2 ± 4.6	69.7

Values are means ± S.D. of six determinations. Dose of the sample given: 12.5 mg/kg body weight/day for 5 alternate days.

Superoxides produced by the photosensitization of riboflavin was inhibited by salicyl, *p*-anisyl and piperonal curcuminoids. Salicyl curcuminoid produced 50% inhibition of superoxide production at a concentration of 2.5 μg/ml while *p*-anisyl and piperonal curcuminoids produced the same effect at a concentrations of 3.3 and 5.2 μg/ml, respectively. All the others required more than 24 μg/ml to produce 50% inhibition (Fig. 3).

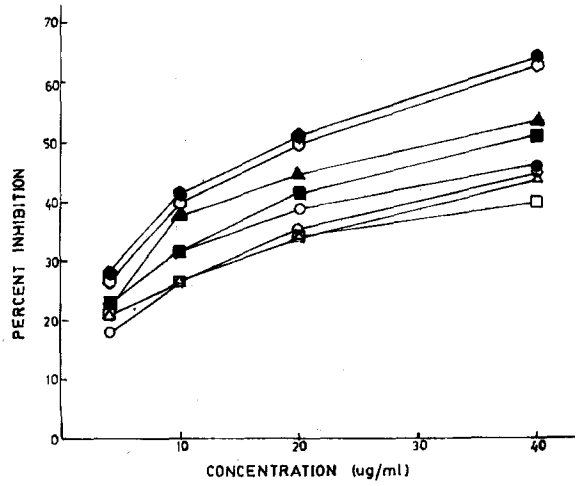

Fig. 2. Effect of curcuminoids on inhibition of lipid peroxidation. Salicyl curcuminoid (◆). Piperonal (◇). Pyridine-3 (●). Furfural (○). *p*-Anisyl (▲). Veratryl (△). Indole-3 (■). Dimethylamino (□).

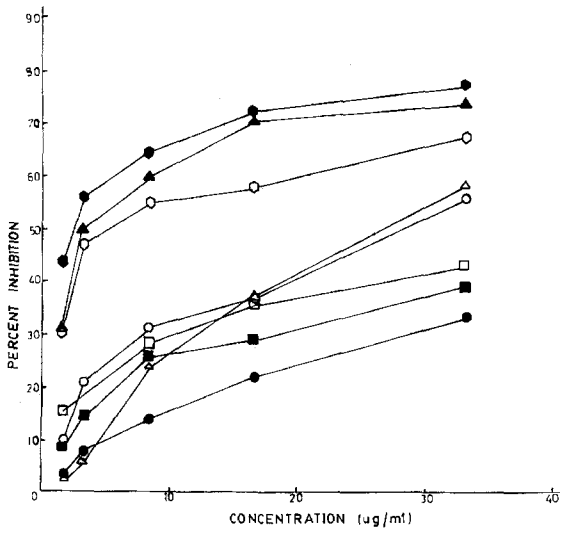

Fig. 3. Effect of curcuminoids on inhibition of superoxide production (refer to Fig. 2 for the symbols).

Hydroxyl radicals produced by Fenton's reaction were effectively scavenged by Salicyl, piperonal and *p*-anisyl curcuminoids which produced 50% inhibition at 2.8, 3.4 and 5.2 μg/ml, respectively. Fig. 4 shows the inhibition produced by various curcuminoids to the hydroxyl radical production.

The antioxidant activity was further evaluated in vivo by the inhibition of the production of superoxides by macrophages activated with phorbol-12-myristate-13-acetate (PMA). Administration of 50 mg/kg body weight of salicyl curcuminoid into the peritoneal cavity of mice, pre-treated with 0.2% casein (i.p.) and PMA (100 ng; i.p.) to elicit and activate macrophages, produced 70.5% inhibition on the production of superoxides while veratryl curcuminoid produced 62.7% inhibition at this dose. With the exception of dimethyl amino curcuminoid (11.9%) all other curcuminoids produced an inhibition of 40–50% (Table 3).

4. Discussion

Earlier studies from our laboratory have shown that the natural curcuminoids isolated from *Curcuma longa* reduced animal tumours and are good antioxidants (Ruby et al., 1995). Because of this we conducted further studies on synthetic curcuminoids having similar structure, but different substituents. Salicyl curcuminoid, which is the ortho isomer of curcumin III was found to be the most active compound in this group. The latter was the most active of the curcuminoids isolated from *Curcuma longa* (Ruby et al., 1995). Some compounds such as veratryl curcuminoid which was found to reduce ascites tumour and scavenge the superoxides produced by activated macrophages in vivo, did not show significant activity in the in vitro experiments. This suggests the metabolism of this compound to an active intermediate in the body.

p-Anisyl curcuminoid, having only one methoxy group was found to be active, both in vitro and in vivo. Removal of a methyl group from the methoxy radical can change the com-

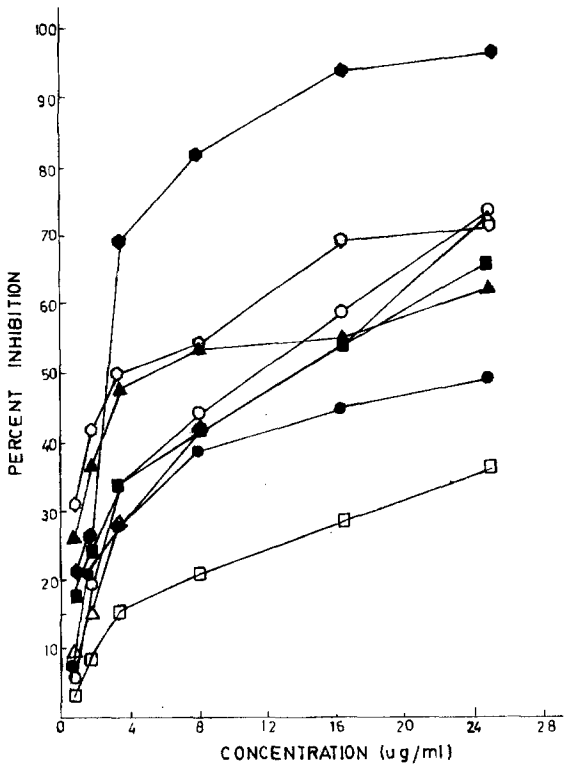

Fig. 4. Effect of curcuminoids on inhibition of hydroxyl radical production (refer to Fig. 2 for the symbols).

Table 3
Effect of curcuminoids on inhibiting superoxides produced by activated macrophages

Name of the curcumnioid	Percentage inhibition
Furfural curcuminoid	47.5 ± 6.6
Salicyl	70.5 ± 4.4
Pyridine-3	41.3 ± 3.8
Indole-3	43.3 ± 7.6
Veratryl	62.7 ± 2.8
Dimethylamino	11.9 ± 4.2
p-Anisyl	48.4 ± 2.1
Piperonal	46.3 ± 3.6

Values are means ± S.D. of three determinations. Curcuminoids (50 mg/kg body weight) were given intraperitoneally in a liposome-encapsulated form to each experimental mouse just before administration of 100 ng of PMA (i.p). Peritoneal macrophages were collected 3 h later.

pound to curcumin III. Piperonal curcuminoid, whose –OH groups are bridged by a $-CH_2$ group was also found to be a potent anticancer agent and antioxidant. The breakage of the methylene bridge can yield two free hydroxy groups on each benzene ring. In general, compounds which are shown to be active may yield phenolic derivatives upon metabolism and compounds which cannot produce phenols have lower activity.

These observations suggest that the activity of these curcuminoids is essentially due to the phenolic group, which can react with a free radical to form the phenoxyl radical. The high activity of these compounds may be also due to the presence of a double bond in conjugation with the phenyl ring, through which the stability of the phenoxyl radical is increased by electron delocalization.

Free radical intermediates such as superoxide anion and hydroxyl radical are produced in living system by various sources such as ionization of water, by X-rays and by inflammatory phagocytes. These activated oxygen species induce DNA strand breaks and chromosomal aberrations (Cerutti, 1991) in mammalian cells.

Curcumin has been reported to be a potent inhibitor of arachidonic acid metabolism (Conney et al., 1991) and ornithine decarboxylase activity (Rao et al., 1993). It also inhibits phorbol-12-myristate-13-acetate-induced DNA synthesis (Huang et al., 1988) and BP-DNA adduct formation in vitro (Mukundan et al., 1993). It is also suggested that curcumin, as an antioxidant, facilitates or enhances the activity of the natural antioxidant system protecting the cells against the oxidative challenge involved in carcinogenesis (Perchellet and Perchellet, 1989). The well known anti-inflammatory activity (Satoskar et al., 1986) of curcumin may be attributed to its antioxidant activity.

As the synthetic curcuminoids, especially those having the phenolic structure, possess the anticancer and antioxidant activities evinced by curcumin, they may also act as potent antipromoters. In fact, preliminary work done in our laboratory indicated that salicyl and piperonal curcuminoids inhibit carcinogenesis (data not published) in mice.

Acknowledgements

The authors thank the Indian Council of Medical Research, New Delhi, for funding this project.

References

Bangham, A.D., Hill, M.W. and Miller, N.G.A., Preparation and use of liposomes as models of biological membranes. In: Korn, E.D. (Ed.), *Methods in Membrane Biology*. Vol.1, Chapter 1, Plenum Press, New York, 1974, pp. 1–64.

Bishayee, S. and Balasubramaniam, A.S., Assay of lipid peroxide formation. *J. Neurochem.*, 18 (1971) 909–920.

Cerutti, P.A., Oxidative stress and carcinogenesis. *Eur. J. Clin. Invest.*, 21 (1991) 1–5.

Conney, A.H., Lysz, T. and Abidi, T.F., Inhibitory effect of curcumin and some related dietary compounds on tumour promotion and arachidonic acid metabolism in mouse skin. *Adv. Enzyme Regul.*, 31 (1991) 385–396.

Dinesh Babu, K.V. and Rajasekharan, K.N., Simplified conditions for synthesis of curcumin I and other curcuminoids. *Org. Prep. Procedure Int.*, 26 (1994) 674–677.

Dwivedi, P.D., Verma, A.S. and Ray, P.K., Induction of immune rejection, of tumours by Protein A in mice bearing transplantable solid tissue Dalton's lymphoma tumours. *Immunopharmacol. Immunotoxicol.*, 14 (1992) 105–128.

Elizabeth, K. and Rao, M.N.A., Oxygen radical scavenging activity of curcumin. *Int. J. Pharm.*, 58 (1990) 237–240.

Huang, M.T., Smart, R.C., Wong, C.Q. and Conney, A.H., Inhibitory effect of curcumin, chlorogenic acid, caffeic acid and ferulic acid on tumour promotion in mouse skin by

12-0-tetradecanoyl phorbol-13-acetate. *Cancer Res.*, 48 (1988) 5941–5946.

Kelloff, G.J., Boone, C.W., Crowell, J.A., Steele, V.E., Lubet, R. and Sigman, C.C., Chemopreventive drug development: perspective and progress: *Cancer Epidemiol. Biomarkers. Prev.*, 3 (1994) 85–98.

Kuttan, R., Bhanumathy, P., Nirmala, K. and George, M.C., Possible anticancer activity of turmeric. *Cancer Lett.*, 29 (1985) 197–202.

Kuttan, G., Vasudevan, D.M. and Kuttan, R., Isolation and identification of a tumour reducing component from mistletoe extract (Iscador). *Cancer Lett.*, 41 (1988) 307–315.

Li, C.J., Zhang, L.J., Dezube, B.J., Crumpacker, C.S. and Pardee, A.B., Three inhibitors of type 1 human immunodeficiency virus long terminal repeat-directed gene expression and virus replication. *Proc. Natl. Acad. Sci. USA*, 90 (1993) 1839–1842.

McCord, J.M. and Fridovich, I., Superoxide dismutase: an enzymatic function for erythrocuprein. *J. Biol. Chem.*, 244 (1969) 6049–6055.

Mukundan, M.A., Chacko, M.C., Annapurna, V.V. and Krishnaswami, K., Effect of turmeric and curcumin on BP-DNA adducts. *Carcinogenesis.*, 14 (1993) 493–496.

Nagabushan, M., Amonkar, A.J. and Bhide, S.V., In vitro antimutagenicity of curcumin against environmental mutagens. *Food Chem. Toxicol.*, 25 (1987) 545–547.

Nishigaki, I., Kuttan, R., Oku, H., Ashoori, F., Abe, H. and Yagi, K., Suppressive effect of curcumin on lipid peroxidation induced in rats by carbon tetracloride or ^{60}Co-irradiation. *J. Clin. Biochem. Nutr.*, 13 (1992) 23–29.

Ohkawa, H., Ohishi, N. and Yagi, K., Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, 95 (1979) 351–358.

Perchellet, J.P. and Perchellet, E.M., Antioxidants and multistep carcinogenesis in mouse skin. *Free Rad. Biol. Med.*, 7(1989) 377–408.

Rao, C.V., Simi, B. and Reddy, B.S., Inhibition by dietary curcumin of azoxymethane-induced ornithine decarboxylase, tyrosine protein kinase, arachidonic acid metabolism and aberrant crypt foci formation in the rat colon. *Carcinogenesis*, 14 (1993) 109–112.

Ruby, A.J., Kuttan, G., Dinesh Babu, K.V., Rajasekharan, K.N. and Kuttan, R., Antitumour and antioxidant activity of natural curcuminoids. *Cancer Lett.*, 94 (1995) 79–83.

Satoskar, R.R., Shah, S.J. and Shenoy, S.G., Evaluation of antiinflammatory property of curcumin (diferuloyl methane) in patients with postoperative inflammation. *Int. J. Clin. Pharmacol. Ther. Toxicol.*, 24 (1986) 651–654.

Soudamini, K.K. and Kuttan, R., Cytotoxic and tumour reducing properties of curcumin. *Indian J. Pharmacol.*, 20 (1988) 95–101.

Soudamini, K.K. and Kuttan, R., Inhibition of chemical carcinogenesis by curcumin. *J. Ethnopharmacol.*, 27 (1989) 227–233.

Soudamini, K.K., Unnikrishnan, M.C., Soni, K.B. and Kuttan, R., Inhibition of lipid peroxidation and cholesterol levels in mice by curcumin. *Indian. J. Physiol. Pharmacol.*, 36 (1992) 239–243.

Unnikrishnan, M.K. and Rao, M.N.A., Anti-inflammatory activity of methionine, methionine sulfoxide and methionine sulfone. *Agents Actions*, 32 (1990) 110–112.