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Inducing the cell cycle arrest and apoptosis of oral KB carcinoma cells by hydroxychavicol: roles of glutathione and reactive oxygen species

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1 Hydroxychavicol (HC; 10–50 μM), a betel leaf component, was found to suppress the 2% H_2O_2-induced lucigenin chemiluminescence for 53–75%. HC (0.02–2 μM) was also able to trap superoxide radicals generated by a xanthine/xanthine oxidase system with 38–94% of inhibition. Hydroxyl radicals-induced PUC18 plasmid DNA breaks was prevented by HC (1.6–16 μM).

2 A 24-h exposure of KB cells to HC (0.5, 1 mM) resulted in 54–74% cell death as analysed by a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. HC (10, 50 μM) further suppressed the growth of KB cells (15 and 76%, respectively). Long-term colony formation of KB cells was inhibited by 51% with 10 μM HC.

3 Pretreatment of KB cells with 100 μM HC inhibited the attachment of KB cells to type I collagen and fibronectin by 59 and 29%, respectively.

4 Exposure of KB cells to 0.1 mM HC for 24 h resulted in cell cycle arrest at late S and G2/M phase. Increasing the HC concentration to 0.25 and 0.5 mM led to apoptosis as revealed by detection of sub-G_0/G_1 peaks with a concomitant decrease in the number of cells residing in late S and G_2/M phase.

5 Inducing the apoptosis of KB cells by HC was accompanied by marked depletion in reduced form of GSH (>0.2 mM) and the increasing of reactive oxygen species production (>0.1 mM) as analysed by CMF- and DCF-single cell fluorescence flow cytometry.

6 These results indicate that HC exerts antioxidant property at low concentration. HC also inhibits the growth, adhesion and cell cycle progression of KB cells, whereas its induction of KB cell apoptosis (HC>0.1 mM) was accompanied by cellular redox changes.
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Keywords: Betel quid; piper betle; hydroxychavicol; reactive oxygen species; apoptosis; cell cycle; redox status.

Abbreviations: AN, areca nut; BQ, betel quid; CMF-DA, 5-chloromethylfluorescein diacetate, DCFH-DA, dichlorofluorescein-diacetate, DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl-sulphoxide; ECM, extracellular matrix, FN, fibronectin; GSH, glutathione; GSSG, oxidized glutathione; HC, hydroxychavicol; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PBL, *piper betle* leaf; PBS, phosphate buffered saline; ROS, reactive oxygen species

Introduction

Epidemiological studies have reported a close association between a betel quid (BQ)-chewing habit and oral cancer (IARC, 1985; Thomas & Wilson, 1993; Ko *et al*., 1995). Since there are about 600 million BQ-chewers in the world, it seems that an oral health hazard associated with this habit is a major concern. A chewing mixture of BQ comprised of the ingredients such as areca nut, lime and betel leaf (PBL, *piper betle* leaf). Tobacco or *piper betle* influorescence is also part of different BQ preparations around the world (IARC, 1985; Ko *et al*., 1995; Thomas & Maclennan, 1992). Epidemiological study suggests that chewing BQ containing PBL and excluding *piper betle* influorescence is less risky for oral cancer than mixtures containing *piper betle* influorescence (Ko *et al*., 1995); however how PBL exerts its anticarcinogenic potential is not well understood.

Most of the previous animal-model experiments dealing with the effects of BQ-chewing have revealed the potential anticarcinogenic effects of PBL (Bhide *et al*., 1979; Ranadive *et al*., 1979; Shirname *et al*., 1983; Rao *et al*., 1985; Padma *et al*., 1989a, b; Azuine & Bhide, 1992). The intragastric intubation of Swiss mice with a PBL-extract infusion has failed to induce any tumour amongst experimental animals (Bhide *et al*., 1979). The administration of PBL extract also decreases the incidence of acetoxymethyl nitrosamine-induced hamster oral tumour and tumour burden (Azuine & Bhide, 1992). Moreover, PBL extract inhibits the areca nut (AN) extract-induced mutation in *Salmonella typhimurium* TA100 and AN extract-induced tumors amongst Swiss mice

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(Shirname *et al*., 1983), and the presence of PBL reduces the carcinogenicity of BQ on the hamster cheek pouch (Ranadive *et al*., 1979). Further, PBL also inhibits the carcinogen-induced tumors of the oral cavity (Rao, 1984), mammary tissue (Rao *et al*., 1985) and skin (Azuine & Bhide, 1992). In addition, PBL exerted chemopreventive effects against NNN and NNK-induced lung and forestomach tumours (Padma *et al*., 1989b). Used in *in vitro* models, PBL extract demonstrates to be non-mutagenic to four strains of *Salmonella typhimurium* (TA100, TA1535, TA98, TA1538), and even in the presence of S9 liver homogenate (Shirname *et al*., 1983). Furthermore, PBL extract inhibits the mutagenicity of a large array of environmental mutagens and carcinogens (Nagabhushan *et al*., 1987), such as two tobacco-specific nitrosamines (Padma *et al*., 1989a). On the contrary, a 24-h exposure of human white blood cells to PBL extract leads to pronounced chromatid aberrations (Sadasivan *et al*., 1978). In addition, an organic PBL extract exerts cytotoxicity in human buccal fibroblasts when given in concentrations ranging from 3 μg ml^{-1} to 1 mg ml^{-1}. Concomitantly, PBL extract also noticeably suppresses O^6-methyl-guanine-DNA methyltransferase (MGMT) activity by 44 and 48% at concentrations of 130 and 320 μg ml^{-1}, respectively (Liu *et al*., 1997). Thus, PBL ingredients can reduce the functional MGMT activity and hence increase the risk of oral cancer. Therefore, the roles of PBL in BQ-elicited carcinogenesis warrants further evaluation.

Hydroxychavicol (HC), a major phenolic compound in PBL and inflorescence *piper betle* (IPB), has been hypothesized to be responsible for the anticarcinogenic effects of PBL. At a concentration of 25 μM, HC has been observed to inhibit the 3(H)benzopyrene-DNA interactions in the presence of S9 mouse- and rat-liver homogenate (Lahiri & Bhide, 1993). Furthermore, HC also inhibits the nitrosation reaction (Nagabhushan *et al*., 1989), DMBA and tobacco-specific nitrosamine-induced mutations in *Salmonella typhimurium* TA100 and bone-marrow micronucleated cells for Swiss male mice (Padma *et al*., 1989a; Amonkar *et al*., 1986; 1989). In addition, HC has been shown to enhance mouse-liver glutathione S-transferase activity *in vivo* (Lahiri & Bhide, 1993). The chemical species 1′-Hydroxychavicol (1′-HC, 810 nM), structurally similar to HC, inhibits the TPA-induced H_2O_2 production and inflammatory response in mouse skin (Nakamura *et al*., 1998). However, recent studies have found that HC induces oxidative stress and leads to DNA strand breaks and 8-OH-dG formation for cultured Chinese hamster ovary (CHO) cells (Lee-Chen *et al*., 1996; Chen *et al*., 2000). Further tests focusing on the roles of HC in the pathogenesis of oral mucosal diseases in BQ-chewers, the antioxidant, prooxidant and anticarcinogenic effects of HC were evaluated using cultured oral KB carcinoma cells.

Methods

Synthesis of hydroxychavicol

Hydroxychavicol was synthesized from eugenol by modifying the method described by Grieco *et al*. (1976; 1977). Briefly, BBr_3 (40 ml, 1.0 M in CH_2Cl_2) was slowly added to a solution containing eugenol (5 ml, 32.5 mmol) and dichloromethane (100 ml) while keeping the mixture uniform by constant stirring at -78°C. The mixture was stirred for an additional 30 min then warmed to -10°C and maintained at this temperature for 3 h. Thereafter the disappearance of the initial substance was observed by using thin layer chromatography (TLC) trace. The reaction was quenched by the addition of saturated aqueous sodium bicarbonate solution and neutralized to a final pH of around 4–5 following which, the aqueous layer was extracted with dichloromethane (3×20 ml). The combined organic layers were washed with brine, dried over anhydrous sodium sulphate, and concentrated *in vacuo*. The residue was purified by silica-gel column chromatography eluted with ethyl acetate-hexanes (1 : 4) to give HC (3.21 g, 94%). The purity of HC was confirmed by infra-red spectroscopy and NMR.

Scavenging of H_2O_2 by HC

This reaction was carried out in a volume of 250 μl, consisting of a mixture of 133 μl of 50 mM Tris buffer (pH = 7.4), 100 μl of 2 mM lucigenin, and varying concentrations of vitamin C or HC (0.01–1 mM). A similar volume of DMSO diluent (solvent) was used for the control. Subsequently, the reaction was commenced by the auto-injection of 17 μl of 30% H_2O_2 (final H_2O_2 concentration was 2%). The H_2O_2-induced lucigenin chemiluminescence was measured using a Microplate Luminometer (Orion Microplate Luminometer, Berthold DS, Tforzheim, Germany).

Scavenging of superoxide radicals

This reaction was carried out in a mixture of 250 μl containing 150 μl of 50 mM Tris (pH = 7.4), 60 μl of 2 mM lucigenin and varying concentrations of vitamin C or HC (5 μl, final concentration = 0.02–20 μM). Subsequently, 10 μl of xanthine oxidase (0.02 u ml^{-1}) was added. The reaction was commenced by the injection of 30 μl of xanthine (0.33 M). The superoxide-induced lucigenin chemiluminescence was measured over a period of 10 s by a Microplate Luminometer. Thereafter, the chemiluminescence level was recorded and averaged.

Effects of HC on hydroxyl radical-induced DNA breaks

The reaction was conducted in a total volume of 30 μl containing 5 μl of 50 mM Tris buffer (pH 7.4), 5 μl of PUC18 plasmid DNA (5 μg), 5 μl of 0.1–100 μM HC or DMSO diluent (as control). Then 10 μl of 30% H_2O_2 and 5 μl of 500 μM $FeCl_2$ was added at 37°C for 30 min. After 30 min, the reaction mixture was placed in 0.8% agarose gel electrophoresis and run at 100 V for 30 min, using a Mupid-2 electrophoretic equipment. The DNA was visualized and photographed by a UV view-box.

Cytotoxicity assay

Oral KB carcinoma cells were inoculated into 24-well culture plates at a density of 1×10^5 cells well^{-1} in DMEM supplemented with 10% FCS. Following incubation of the KB cells for a period of 24 h, the medium was changed whereas containing additions such as DMSO (as a control, below 0.5%, v v^{-1}) and varying concentrations of HC (0.1–2 mM). Then the cells were further incubated for another

24 h. The relative cytotoxicity of the medium was enumerated by the use of a modified MTT assay (Chang *et al*., 1998, Jeng *et al*., 1999a, b; Jeng *et al*., 2000). In addition, colony formation assay was used for further evaluation the effect of HC on the long-term survival of KB cells. Briefly, 1000 KB cells were inoculated into 100 mm culture dishes and exposed to various concentrations of HC and DMSO (control) for 24 h. Thereafter, medium was changed with fresh DMEM containing 10% FCS for 10–15 days, with medium changes each 3 days. The number of colony was counted following methanol fixation and methylene blue staining.

Effect of HC on the growth of KB cells

Briefly, 5×10^3 KB cells were seeded into each well of a 24-well culture plate in DMEM containing 10% FCS. The cells were then incubated for a period of 24 h. Cells were exposed to fresh medium containing DMSO or various concentrations of HC and incubated under the same conditions as mentioned previously for 5 days. The viable cell number was enumerated using the MTT assay as described above (Chang *et al*., 1998, Jeng *et al*., 1999a, b; Jeng *et al*., 2000).

Adhesion of KB cells to collagen and fibronectin

The assay for the cell attachment to matrix proteins was conducted (Chang *et al*., 1998, Jeng *et al*., 1999a). KB carcinoma cells were detached from culture plates by the application of trypsin/EDTA. These cells were then washed with PBS and resuspended in DMEM without serum, and pre-incubated for 1 h at a concentration of 1×10^5 cells ml^{-1} with varying concentrations of HC (50–500 μM) or DMSO. The cells were then added into 24-well culture plates, the wells were precoated with collagen and fibronectin, and given 1 h for attachment. Floating cells were then aspirated and the remaining cells washed with PBS. Attached cells were cultured in DMEM with 10% FCS and 0.5 mg ml^{-1} MTT for 2 h under standard incubating conditions. The formazan so-elicited was eluted with DMSO and the level read against a blank at OD_{540} using a Dynatech microplate reader.

Effect of HC upon the cell cycle control for KB cells

In order to determine whether HC can modulate the cell-cycle progression of incubated KB cells, 5×10^5 KB cells were plated into 100-mm cell-culture dishes containing DMEM with 10% FCS, then incubate under standard conditions for 24 h. Fresh medium containing different concentrations of HC (final concentration of 0.05 to 0.75 mM) was added and then incubated for another 24 h. Any morphological changes can be observed and photographed using a phase contrast microscope.

Flow cytometry was used for analysing cellular DNA content (Jeng *et al*., 1999a; Zamai *et al*., 1993). Chemical-induced cell death can be mediated by either a necrosis or an apoptosis pathway (Wyllie, 1997; Renvoize *et al*., 1998); thus both floating and attached KB cells were collected and mixed together in a centrifuge tube. KB cells of two separate culture dishes with similar exposure conditions were collected together, re-suspended and fixed over a period of 30 min in 70% ice-cold ethanol containing RNase at a concentration of 2 mg ml^{-1}. The cells were then washed twice with PBS and finally stained with propidium iodide (PI) (40 μg ml^{-1}) for 10 min at room temperature. The PI-elicited fluorescence of individual KB cells was measured by a FACSCalibur Flow Cytometer (Becton Dickinson, Worldwide Inc., San-Jose, CA, U.S.A.) supplemented with an Argon ion laser. The wavelength of laser excitation was set at 488 nm and the emission collected was set at greater than 590 nm. The FL2 fluorescence was collected in a linear/log scale fashion. A total of 20,000 cells were analysed for the control sample and for each HC-treated sample. The percentage of cells in G_0/G_1 phase, S phase, G_2/M and sub-G_0/G_1 phase were determined using standard ModiFit software programs.

Growth of KB cells were also evaluated by directly measuring the viable cell number. Briefly, 5×10^5 cells were inoculated into 100 mm culture dishes. After 24 h, cells were exposed to HC (10, 50 and 100 μM) or DMSO (control) for 1 and 3 days. Cells were therefore trypsinized and viable cells that excluded trypan blue dye were counted as described previously (Jeng *et al*., 1994).

Analysis of cellular reduced form of GSH and the generation of reactive oxygen species

Since HC has both catechol and allyl moieties, it is crucial to evaluate whether HC exerts its cytotoxic effect via metabolic activation and induction of oxidative stress. For elucidation of this question, 5×10^5 KB cells in DMEM containing 10% FCS were exposed to HC or DMSO (as control) for 24 h. To evaluate whether HC may generate reactive oxygen species (ROS) intracellularly, cells treating with HC or DMSO diluents (control) were stained with 10 μM of 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37°C, detached with trypsin/EDTA, washed with phosphate buffered saline (PBS), resuspended in PBS and subjected immediately for flow cytometry (Wang *et al*., 1999; Chang *et al*., 2001). For determining the intracellular level of reduced GSH, KB cells were treated with HC and DMSO, stained with 25 μM of 5-chloromethylfluorescein diacetate (CMF-DA) for 30 min at 37°C, trypsinized, resuspended in PBS, and immediately used for flow cytometric analysis.

Materials

Propidium iodide, calf-skin type I collagen, bovine plasma fibronectin (FN), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl-sulphoxide (DMSO), vitamin C, dichlorofluorescein-diacetate (DCFH-DA) and eugenol were purchased from Sigma (Sigma Chemical Company, St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS) and trypsin/EDTA were from Gibco (Life Technologies, Grand Island, NY, U.S.A.). Reagents for flow cytometry were obtained from Becton Dickinson (U.S.A.). Ethidium bromide and agarose were acquired from HT Inc., (U.K.). 5-chloromethylfluorescein diacetate (CMF-DA) was obtained from Molecular Probes (Eugene, Oregon, U.S.A.).

Statistical analysis

Two or more separate experiments were performed for each case. Statistical analysis was conducted using a paired

Student's *t*-test. A P value <0.05 was considered to constitute differences between experimental and control groups.

Results

HC as a scavenger of H_2O_2

Reactive oxygen species (ROS) such as H_2O_2, superoxide and hydroxyl radicals have been shown to play a central role in the pathogenesis of BQ-chewing-related oral mucosal diseases (Thomas & MacLennan, 1992; Nair *et al*., 1987; 1990; Stich & Anders, 1989). Since 1′-HC inhibits the TPA-induced H_2O_2 formation in the skin of ICR mice (Nakamura *et al*., 1998), we first checked to determine whether HC could act as an H_2O_2 scavenger. HC proved to be effective in entrapping the H_2O_2. As shown in Figure 1a, HC at a concentration of 10 μM and 50 μM decreased the H_2O_2-induced chemiluminescence by 53 and 75%, respectively. The value of relative light unit (RLU) decreased from 1177 (control) down to 563 and 296, due to the presence of HC at concentrations of 10 μM and 50 μM. In this reaction, DMSO diluent control revealed little effect upon H_2O_2-elicited chemiluminescence (data not shown). Vitamin C also inhibited the H_2O_2-elicited chemiluminescence by about 20% at a concentration of 570 μM (Figure 1b).

HC as a superoxide radical scavenger

Hydroxychavicol demonstrated to be a potent superoxide radical scavenger. As illustrated in Figure 1c, HC effectively scavenges xanthine/xanthine oxidase-induced superoxide radicals. At a concentration of 0.02 μM, HC reduced the xanthine/xanthine oxidase-induced chemiluminescence by 38% (Figure 1c). The maximal scavenging effect of HC was obtained at a concentration range of between 0.2 and 2 μM with an associated inhibition of 96 and 94% respectively.

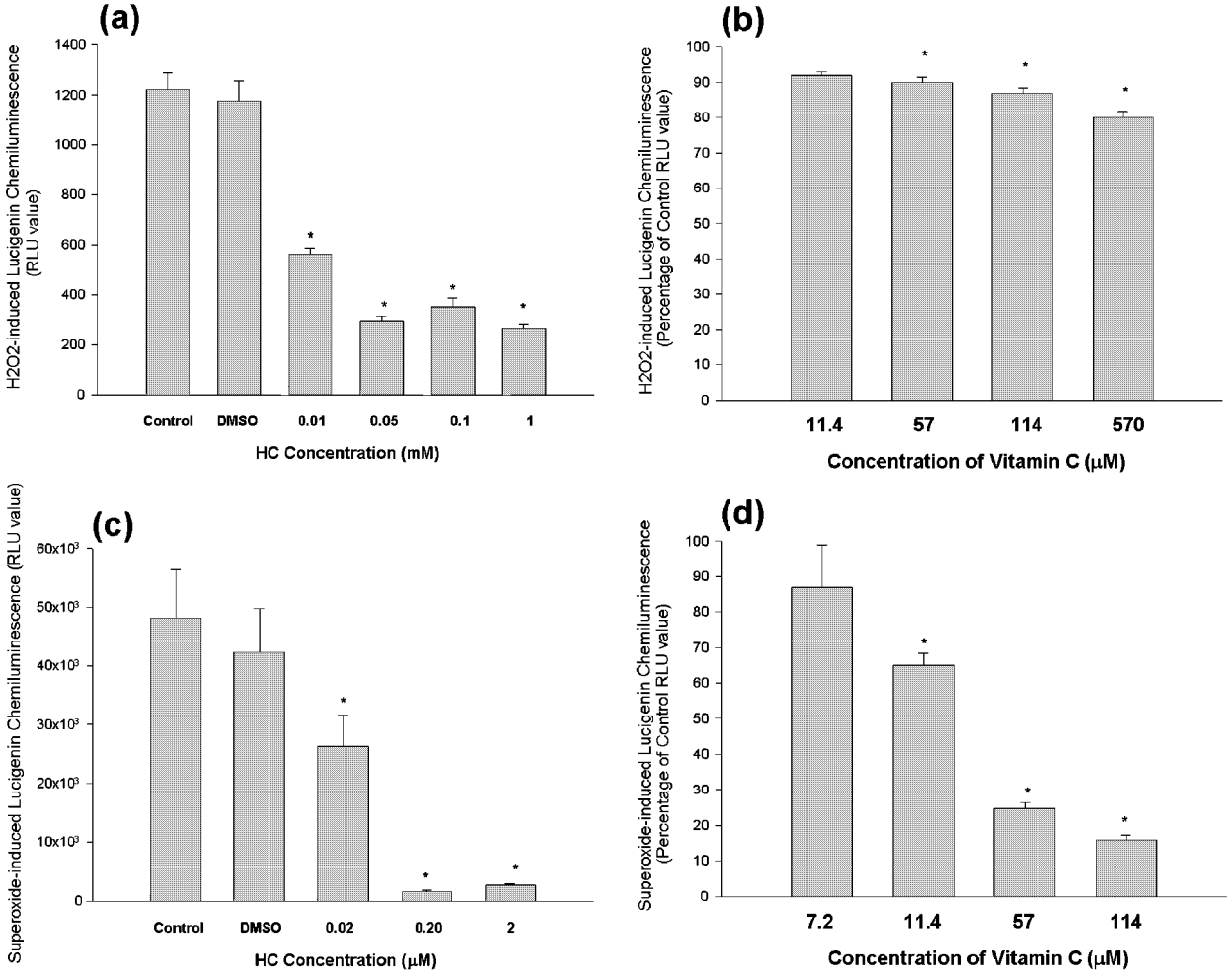

Figure 1 Hydroxychavicol (HC) as a H_2O_2 and superoxide radical scavenger. (**a**) Externally-added H_2O_2 was used to generate lucigenin-enhanced chemiluminescence and its inhibition by HC. The peak of chemiluminescence (RLU) in each test was recorded. Results were expressed as RLU (mean$\pm$s.e.m.), ($n=4$). (**b**) effect of vitamin C on H_2O_2-induced chemiluminescence. (**c**) Xanthine and xanthine oxidase were used to generate superoxide radicals. The emitted chemiluminescence during the first 10-s period was averaged for data-presentation purposes. The relative light units (RLU) were measured and expressed as percentage of control (mean$\pm$s.e.m.) ($n=11$). (**d**) effect of vitamin C on xanthine-xanthine oxidase-induced superoxide chemiluminescence. *Denotes significant difference when compared with DMSO group.

From these results, HC is a better potent superoxide scavenger than a H_2O_2 scavenger. However, elevating the concentration of HC to 20 μM resulted in an increase in the level of the emitted chemiluminescence, the reasons are not yet clear. Vitamin C a well known antioxidant, also scavenged the superoxide radicals with 35–75% of inhibition at concentrations of 11.4 to 57 μM (Figure 1d).

Effects of HC on the hydroxyl radical-induced DNA breaks

HC by itself induced little DNA breaks even in the presence of 100 μM of $FeCl_2$ and $CuCl_2$ (data not shown). In addition, HC was also effective in scavenging the hydroxyl radicals. As shown in Figure 2, most of the purchased PUC18 plasmid DNA was in form I (supercoil form). Exposure of PUC18 plasmid DNA to H_2O_2 and $FeCl_2$ led to the increasing of form II (open circular), complimenting the decreasing of form I. Presence of HC (1.6 and 16 μM) showed a more protective effect than DMSO diluent (control) in preventing the hydroxyl radical-induced DNA breaks.

HC-elicited growth inhibition of and cytotoxicity to KB cells

Since the work of many researchers has demonstrated that HC exerts anticarcinogenic potential (Padma *et al*., 1989b; Lahiri & Bhide, 1993; Amonkar *et al*., 1986; 1989; Bhide *et al*., 1991; 1994), we proposed that this reported effect of HC was probably due to its potential antioxidant properties and/or its direct suppressive effects upon cancer cells. In order to address this question, we first attempted to determine whether HC could be toxic to oral KB carcinoma cells using both short-term and long-term cell survival assay. At concentrations greater than 0.5 mM, HC exerted a marked growth inhibition and cytotoxicity effect upon KB cells. As shown in Figure 3a, HC reduced the viable KB cell number by 54 and 74%, respectively, at a concentration of 0.5 and 1 mM. Hydroxychavicol may also effectively suppress the growth of KB cells, plated at a low cell density. As depicted in Figure 3b, exposure of KB cells to HC at a concentration of 10 and 50 μM for 5 days markedly inhibited the growth of KB cells by a figure of 15 and 76%, respectively. At concentrations greater than 100 μM, HC led to virtually complete cell death. Eugenol, the other PBL component that exhibited structural similarity with HC, also inhibited the growth of KB cells, although its potency was less than that of HC. As shown in Figure 3c, eugenol inhibited the growth of KB cells by 25 and 71%, at concentrations of 0.25 and 0.5 mM, respectively. Nevertheless, we used the colony formation capacity to determine the long-term survival of KB cells. As shown in Figure 3d, at a concentration of 10 μM, HC decreased the colony forming capacity of KB by 51%. At concentrations higher than 50 μM, no viable colony can be detected (data not shown).

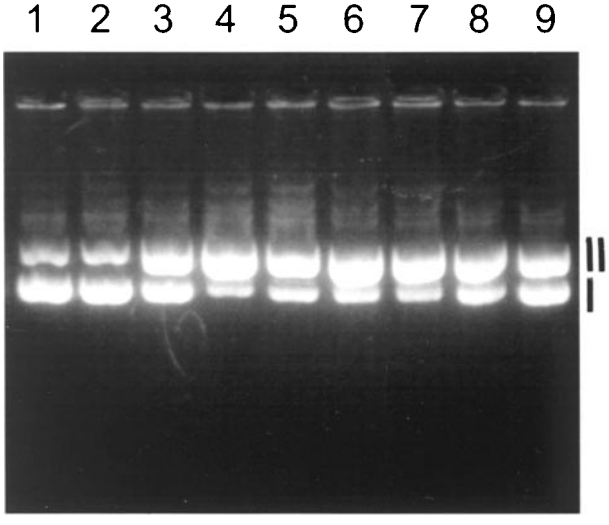

Figure 2 Effect of HC on the hydroxyl radical-induced DNA breaks in PUC18 plasmid DNA. H_2O_2 and $FeCl_2$ (Fenton reaction) was used to generate hydroxyl radicals, leading to DNA breaks on PUC18 plasmid DNA. Various concentrations of HC or DMSO diluent (control) were tested for its prevention of DNA breaks. Lane 1, control PUC18 DNA; Lane 2, DNA + 5% H_2O_2; Lane 3, DNA + 100 μM $FeCl_2$; Lane 4 DNA breaks induced by H_2O_2 with $FeCl_2$; Lane 5–9, DNA breaks induced by Fenton reaction in the presence of sequentially DMSO diluent control and varying concentrations (0.016, 0.16, 1.6 and 16 μM) of HC. Both form I (supercoil) and form II (open circular) DNA bands can be detected from gels.

Morphological changes to KB cells following exposure to HC

The toxic effects of HC upon KB cells was associated with morphological changes. As observed under phase-contrast microscopy, untreated KB cells were cuboid or polygonal in appearance, and some clustering of cells into a 'nest' could be observed (data not shown). However, following exposure to HC at a concentration of 0.1 mM, some KB cells retracted and became much more rounded in appearance, and, an overall decrease in cell number and a loss of cell nests (data not shown). Further elevating the exposure concentration of HC to 0.25 mM led to further cell retraction, rounding and a trend towards cell suspension (rather than adhesion) for most of the cultured KB cells.

HC-elicited inhibition of the adhesion of KB cells to collagen and fibronectin

The adherence of cancer cells to an extracellular matrix is associated with enhanced invasion and metastasis (Bernstein & Liotta, 1994; Lozano *et al*., 1996). From our study, the pretreatment of KB cells with HC pronouncedly inhibited the cells' adhesion to matrix proteins. As shown in Figure 4a, HC at a concentration of 100 μM and 250 μM suppressed the attachment of KB cells to type I collagen by 59 and 78%, respectively. A similar inhibitory effect of HC upon the adhesion of KB cells to fibronectin (FN) was also noted (Figure 4b). Moreover, HC at a concentration of 100 and 250 μM, inhibited the adhesion of KB cells to FN by 29 and 73% respectively.

Effects of HC on the cell cycle control

The growth of cancer cells is strictly regulated by the cell-cycle progression (Eastman & Rigas, 1999; Shackelford *et al*., 1999). Since we have demonstrated that HC inhibits the

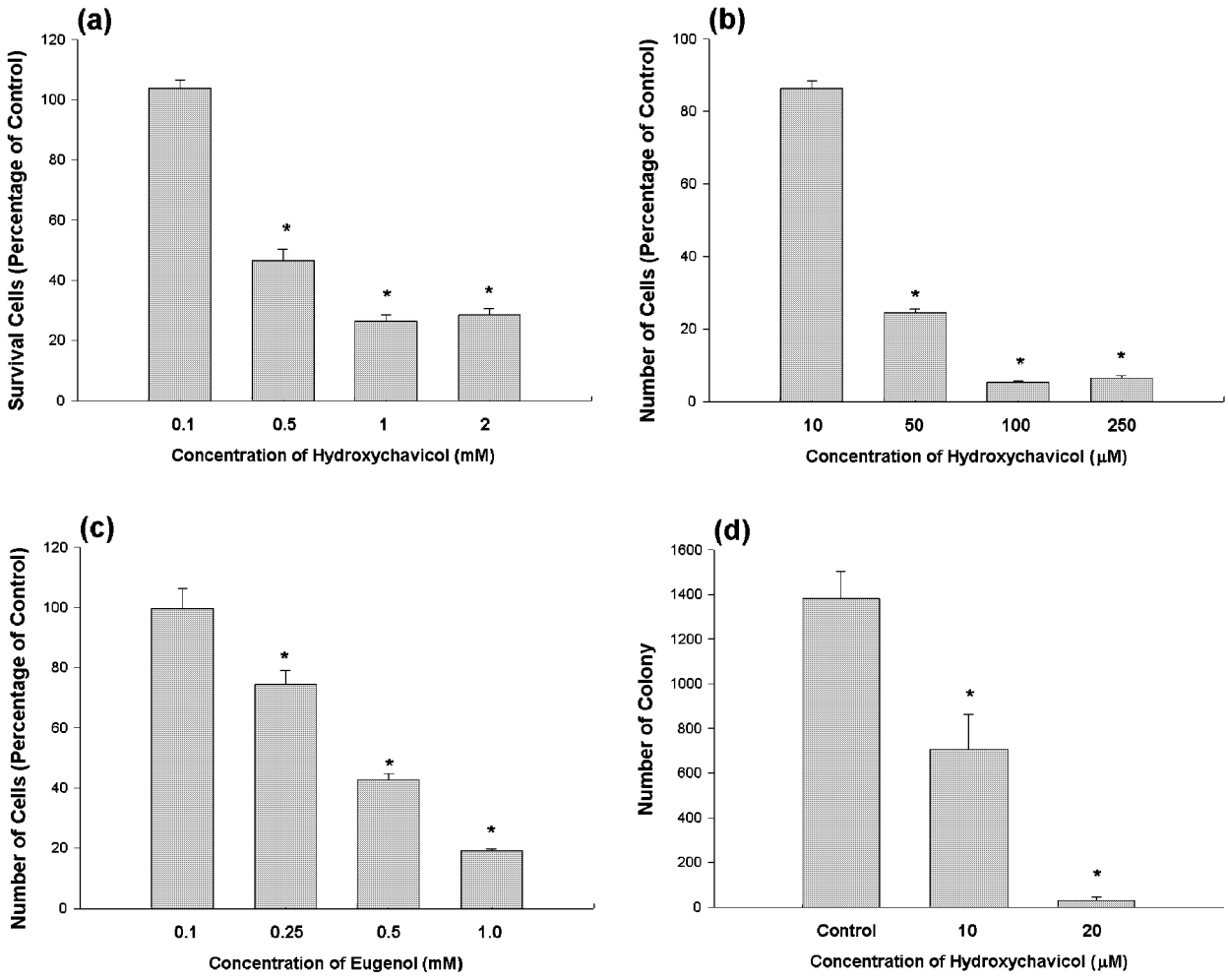

Figure 3 Cytotoxicity and the cytostatic effects of HC upon cultured KB cells. (**a**) confluent KB cells (1×10^5 cells) in 24-well culture plates were exposed to HC (0.1 to 1 mM) for a period of 24 h. The degree of cytotoxicity was evaluated with an MTT assay. (**b**) Proliferating KB cells (5×10^3 cells well^{-1}) were exposed to HC (10–500 μM) or (**c**) eugenol (0.1–1 mM) for a period of five days. Cell numbers were measured with an MTT assay. Results were expressed as percentage of control (as 100%) (Mean ± s.e.m.). (**d**) effect of HC on colony forming capacity of KB cells. *Denotes marked differences when compared with control.

growth of KB cells, an intriguing question arises, as to whether the effects of HC are due to its dysregulation of KB cell-cycle control. Using a flow-cytometry technique, we found that the exposure of KB cells to HC at a concentration of 0.05 mM and 0.1 mM for a period of 24 h led to cell cycle arrest at late S and G_2/M phases (Figure 5b). The proportion of cells in late S and G_2/M phases rised from 21% for control cells (Figure 5a) to 34 and 40.7% for KB cells treated with HC at a concentration of 0.05 and 0.1 mM respectively (Table 1). Further increasing the concentration of HC to 0.25–0.5 mM led to marked apoptosis, as revealed by the presence of a sub-G_0/G_1 peak (Figure 5c). Concomitantly, HC (0.1–0.75 mM) also induced S-phase cycle arrest for KB cells (Table 1). For further evaluation whether induction of cell cycle arrest and apoptosis may lead to changes in viable cell number, we directly measured the number of survival KB cells following exposure to HC (10–100 μM). A 3-day exposure of KB cells to HC 50 and 100 μM of HC markedly decreased the viable cell number. After 3 days of incubation, KB cells may proliferate from 1×10^5 cells to 4.92×10^5 cells. Exposure to 50 and 100 μM of HC decreased the cell number to 3.15 and 1.38×10^5 cells, respectively (Figure 5d).

Effect of HC on the reduced GSH levels of oral KB cells

Cellular GSH level is crucial for the growth, cell cycle progression and apoptosis in a number of cells (Poot *et al*., 1995; Benard *et al*., 1999; Vahrmeijer *et al*., 1999; Schnelldorfer *et al*., 2000). We therefore measured whether depletion of reduced GSH was associated with HC toxicity on KB cells by single cell flow cytometric analysis of CMF fluorescence. This method has been used to estimate the cellular level of residual GSH in cultured thymocytes and granulosa cells (Chikahisa *et al*., 1996; Burghardt *et al*., 1992). In this study, most of the control KB cells showed high reduced GSH content as demonstrated in flow cytometric histogram (Figure 6a). The M2 population of KB cells have higher reduced GSH content, as revealed by high CMF fluorescence, whereas M1 population exert lower reduced form of GSH. Exposure of KB cells to HC for 24 h

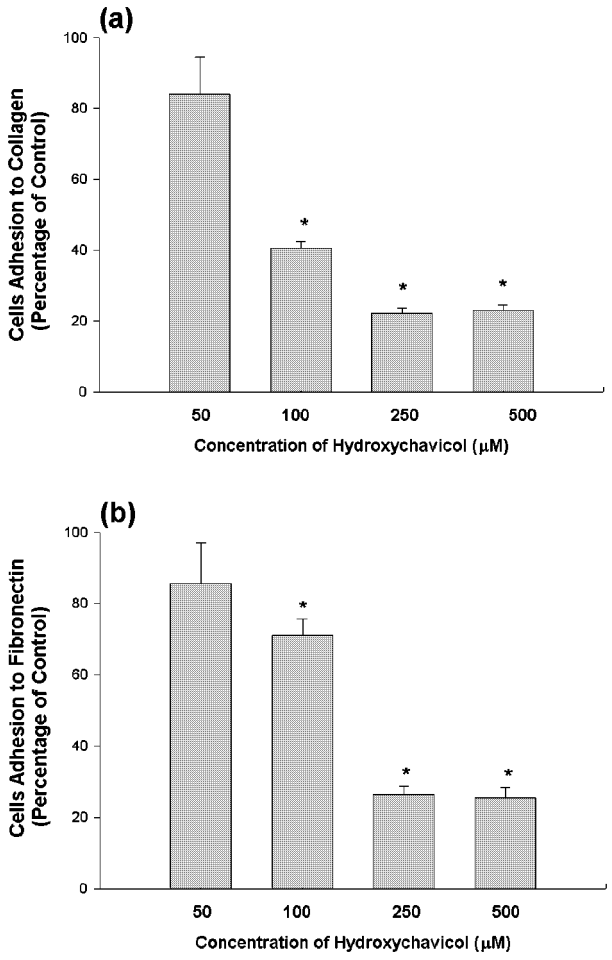

Figure 4 Effects of HC upon the attachment of KB cells to collagen and fibronectin. (**a**) inhibition of the adhesion of KB cells to collagen-coated wells by HC (50–500 μM), (**b**) inhibition of the adhesion of KB cells to fibronectin-coated wells by HC (50–500 μM). Results were expressed as a percentage of the viability of test cells to control cells (Mean$\pm$s.e.m.). ($n=4$) *Denotes marked difference when compared with control.

markedly increased the percentage of cells residing in the M1 population from 2% (control) to 19.3% (0.2 mM HC) and 53.2% (0.3 mM HC), respectively (Figure 6b, Table 2).

Effects of HC on ROS production of KB cells

Cellular GSH is the principal detoxifying system, capable of scavenging ROS and maintaining the cellular redox status (Meister & Anderson, 1983). Depletion of cellular thiol may potentially lead to cellular oxidative stress (Ratan *et al*., 1994). Moreover, metabolic activation of plant phenolics such as quercetin and HC are shown to produce toxic prooxidants (Metodiewa *et al*., 1999; Lee-Chen *et al*., 1996). It is thus interesting to know whether HC may induce oxidative stress on oral KB cells. DCF fluorescence has been successfully used to monitor the total oxidative stress in cells (Wang & Joseph, 1999). By using this method, intracellular ROS production of KB cells was found to be slightly suppressed by 0.01 mM of HC ($P<0.05$). The mean DCF fluorescence decreased from 93.5 (control) to 78 (by 0.01 mM HC). However, exposure to HC (>0.1 mM) for 24 h led to intracellular accumulation of ROS. Mean DCF fluorescence of KB cells increased from 93.5 (control) to 144.5, 247.8 and 343.5, respectively, by 0.1, 0.2 and 0.3 mM of HC (Figure 7).

Discussion

In the experiment, HC is found to be an effective H_2O_2, superoxide radical and hydroxyl radical scavenger at concentrations ranging from 0.2 to 50 μM. HC was also found to inhibit the growth and attachment of KB cells concomitant with the induction of cell-cycle arrest and apoptosis (>100 μM). The presence of a sub-G_0/G_1 peak in flow cytometric histogram is concomitant with a reduction in the proportion of cells in G_2/M phase, this observation reveals that apoptosis is present in HC-treated KB cells residing in G_2/M phase. Since higher concentrations of HC (>0.1 mM) may induce redox changes of KB cell (GSH depletion and oxidative stress), HC may have potential antioxidative, anticarcinogenic properties and even carcinogenic effects that depend on the exposed concentrations.

Hydroxychavicol has no marked mutagenicity as assayed by the Ame's test (using strains TA98, TA100, TA1535 and TA1538), even in the presence of metabolic activation (Amonkar *et al*., 1986). Hydroxychavicol is able to enhance mouse-liver glutathione S-transferase activity *in vivo* (Lahiri & Bhide, 1993). At a concentration of 25 μM, HC is more effective than eugenol, catechin and curcumin in the suppression of rat-liver mitochondria-activated benzo(a)pyrene-DNA adduct(s) formation (Lahiri & Bhide, 1993). Moreover, HC inhibits the mutagenicity of DMBA as revealed in the Ames test (Amonkar *et al*., 1986). These chemopreventive effects have been partially ascribed to the antioxidant potential of HC. However, the antioxidant effects of HC have not been directly confirmed. It has been previously reported that HC (0.37–6 mM) blocks the nitrosation of methylurea *via* its scavenging of available nitrite ions (Nagabhushan *et al*., 1989). In the present study, HC was found to be a H_2O_2 scavenger, at concentrations ranging from 10–50 μM. Further, 1′-HC, a chemical with structural similarity to HC, has been found to be negative as regards the TPA-induced activation of EBV (Nakamura *et al*., 1998). Pretreatment of ICR mice skin with 1′-HC (810 nmol) inhibits the TPA-induced H_2O_2 formation by 49%, but 1′-HC (<100 μM) exhibits no marked effect upon the level of externally-added H_2O_2 (Nakamura *et al*., 1998). 1′-HC is also inactive in scavenging O_2^- radicals generated by HL-60 leukemia cells (<80 μM) and those generated by a xanthine/xanthine oxidase system (Nakamura *et al*., 1998). Although 1′-HC does exert strong antioxidative effects upon the propagation of lipid peroxidation, 1′-HC (<100 μM) is ineffective in the inhibition of TPA-induced H_2O_2 production by differentiated HL-60 cells (Nakamura *et al*., 1998). Contrasting this, we have found that HC (0.02–20 μM) is effective in scavenging the superoxide radicals generated by xanthine/xanthine oxidase and hydroxyl radical-induced DNA breaks. These apparently differential chemopreventive effects of HC and 1′-HC can be partially explained by a difference in the position of functional hydroxyl group in the molecular structure of these two molecules. Thus, HC is a

potential antioxidative ingredient in the PBL and IPB species. The production of H_2O_2, superoxide and hydroxyl radicals in the oral cavity has previously been noted during the chewing of BQ (Nair *et al*., 1987; 1990; 1992; 1995; Stich & Anders, 1989). Thus, HC is a potential antioxidant in the BQ mix, and it may be capable of preventing the attack of various ROS produced during BQ-chewing. Whether in an alkaline condition, or in the presence of transition metals, the potential for HC to exert some degree of genotoxicity is an issue which should be addressed further. However, HC has recently been shown to induce DNA breaks, oxidative stress and DNA damage to cultured HepG2 cells and Chinese hamster ovary cells (Lee-Chen *et al*., 1996; Chen *et al*., 2000). The reasons for such differential effects of HC are not yet clear. Since HC is not auto-oxidized in the absence of metabolic enzymes, such as monoxygenase, peroxidase and tyrosinase, (Bolton *et al*., 1994; Krol & Bolton, 1997), it seems reasonable to propose that the metabolic activation of HC in cultured CHO cells is perhaps necessary for the generation of ROS.

By adding PBL into drinking water, PBL has been shown to decrease the DMBA-induced tumour incidence and tumour burden for Wistar rats (Bhide *et al*., 1994), benzo(a)pyrene-induced forestomach tumors in mice (Bhide *et al*., 1991), and methyl(acetoxymethyl)nitrosamine-induced hamster oral carcinogenesis (Azuine & Bhide, 1992). In

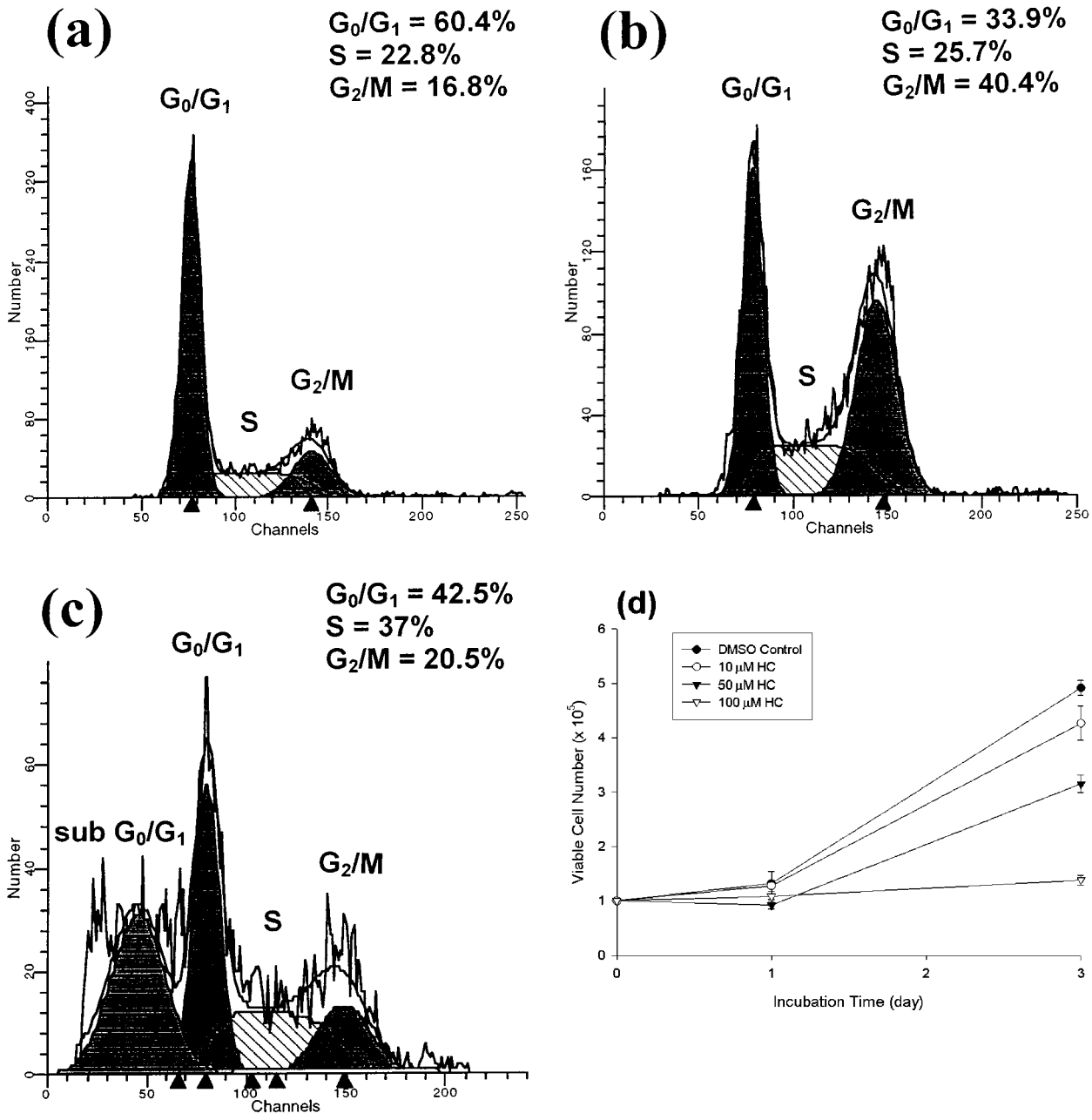

Figure 5 Effects of HC upon the cell-cycle progression of KB cells. (**a**) Untreated KB cells, (**b**) KB cells exposed to 0.1 mM HC for a period of 24 h, (**c**) KB cells exposed to 0.25 mM HC for a period of 24 h, (**d**) number of viable KB cells following exposure to HC for 1 and 3 days ($n=3$).

Table 1 Effects of HC on the cell cycle kinetics of cultured oral KB cells

Chemicals	*G_0/G_1 (%)*	*S (%)*	*Late $S+G_2/M$ (%)*
DMSO Control	55.9±1.7	23.0±0.6	21.1±1.8
0.05 mM HC	36.7±4.7*	29.5±3.8	33.8±1.1*
0.1 mM HC	26.6±2.6*	32.4±3.3*	40.7±2.8*
0.25 mM HC	45.3±4*	34.0±2.5*	24.8±2.6
0.5 mM HC	39.1±1.9*	33.4±2.1*	27.6±2.0
0.75 mM HC	38.0±0.6*	33.1±0.9*	29.0±1.2

Results are expressed as mean percentage of cells in G_0/G_1, $S+G_2/M$ phase (Mean±s.e.m.). * Denotes marked difference when compared with control group ($P<0.05$) ($n=7$).

Table 2 Effects of HC on the reduced form of GSH content in cultured KB cells

Chemicals	*% of cells in M2 region*	*% of cells in M1 region*
DMSO control	98.0±0.3	2.0±0.3
0.01 mM HC	98.0±0.2	1.7±0.2
0.05 mM HC	97.8±0.3	2.2±0.3
0.1 mM HC	95.0±1.7	5.0±1.7
0.2 mM HC	80.7±5.5*	19.3±5.5*
0.3 mMHC	46.8±9.9*	53.2±9.9*

Results are expressed as mean percentage of cells in M1 (low CMF fluorescence) and M2 region (high CMF fluorescence) (Mean±s.e.m.). *Denotes marked difference when compared with control group ($P<0.05$) ($n=6$).

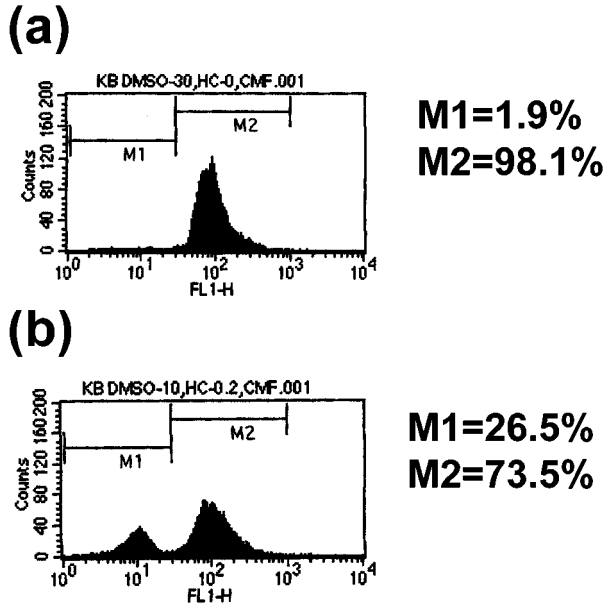

Figure 6 Effects of HC on the single cell fluorescence detection of cellular reduced GSH. KB cells (5×10^5 cells) in 100 mm culture dishes (10 ml, DMEM with 10% FCS) were exposed to HC or DMSO diluent for 24 h. Cells were stained with CMF for 30 min, trypsinized, resuspended in PBS and immediately subjected to flow cytometry immediately. Histogram of CMF fluorescence of (**a**) untreated KB cells and (**b**) KB cells exposure to 0.2 mM HC. Two population of KB cells (M1 and M2) with differential intracellular GSH content were noted. Result of one representative experiment was shown as histogram.

addition to the chemopreventive effects of PBL, the anticarcinogenic potential of various PBL ingredients can partly explain the results of a decreased tumor induction by PBL amongst experimental animals. Supporting this concept, in this study, HC was observed to be cytotoxic to KB carcinoma cells, and it inhibited their proliferation in short-term MTT assay. This is likely due to the formation of more electrophilic cytotoxin, quinone methide, via the oxidative metabolism of HC (Bolton *et al*., 1994; Krol & Bolton, 1997). The cytotoxicity of HC to KB cells was more potent than that of eugenol. This greater cytotoxicity of HC as compared to eugenol can be attributed to an additional hydroxyl group present in the aromatic ring of HC, increasing its anticarcinogenic properties. Similarly, the additional hydroxyl group is crucial for the nitrosation inhibition and antimutagenicity demonstrated by HC (Nagabhushan *et al*., 1989; Amonkar *et al*., 1986). Long-term survival of KB carcinoma cells was further suppressed by HC, as indicated by decreasing of viable colonies after 15-days recovery from a 24-h exposure to HC. Inhibiting clonal growth of cancer cells by HC may partly explain the anticarcinogenic effects of HC to experimental animals.

To proceed through invasion and metastasis, cancer cells need to adhere to an extracellular matrix (ECM), produce degrading proteolytic enzymes for matrix dissolution, remain motile and proceed through/undergo angiogenesis (Bernstein & Liotta, 1994). Interactions between cancer cells and matrix proteins such as collagen and fibronectin have been shown to be critical for the invasion and metastasis of head-and-neck squamous-cell carcinomas (Lozano *et al*., 1996; Okumura *et al*., 1996). Growth factors (hepatocyte growth factor and autocrine motility factor etc.) and inflammatory mediators such as PGE_2 have been shown to promote such cancer-cell attachment to ECM and eventually enhance tumour invasion (Lozano *et al*., 1996; Selletti *et al*., 1998; Trusolino *et al*., 1998). In contrast, the anti-carcinogenicity *via* the inhibition of tumor cell attachment by epidermal growth factor and conophylline has also been reported (Irie *et al*., 1999; Cao *et al*., 2000). In our study, HC effectively suppressed the adhesion of KB carcinoma cells to FN and collagen, suggesting that the anti-carcinogenic effects of HC may involve the differential stages of tumor invasion and metastasis.

The growth of cancer cells is tightly regulated by the cell-cycle progression (Eastman & Rigas, 1999; Shackelford *et al*., 1999). The inhibition of KB cell growth by HC may be explained by its induction of cell-cycle arrest at late S and G2/M phase. This delay of cell cycle by HC was further substantiated by the decreasing in viable cell number (Figure 5d). Untreated KB cell continue to proliferate during 3 days of culture, whereas no marked increasing of cell number was noted following exposure to 0.1 mM of HC for 3 days. HC has been shown to produce oxidative stress, leading to DNA breaks and 8-OH dG formation in cultured CHO cells (Lee-Chen *et al*., 1996). These oxidative stress and DNA-damaging effects may be one possible explanation for the induction of cell-cycle arrest, thus offering greater opportunity for the repair of DNA damage (Eastman & Rigas, 1999; Shackelford *et al*., 1999). Hydroxychavicol is also able to form conjugate with glutathione (GSH) (Bolton *et al*., 1994; Krol & Bolton, 1997) that is crucial for the regulation of cell-cycle progression and apoptosis (Poot *et al*., 1995; Vahrmeijer *et al*., 1999;

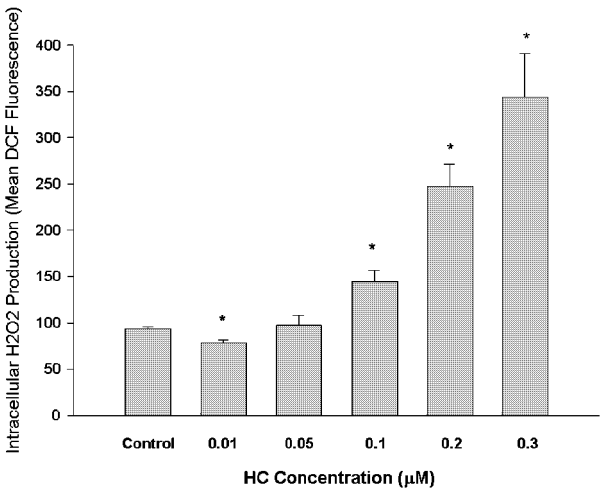

Figure 7 Effects of HC on the cellular production of ROS. KB cells (5×10^5 cells) in 100 mm culture dishes (10 ml, DMEM with 10% FCS) were exposed to HC. Cells were stained with DCFH-DA for 30 min, collected in PBS and subjected to flow cytometry immediately. Results are expressed as mean of DCF fluorescence (mean ± s.e.m.). *Denote marked difference when compared with control ($P<0.05$).

Schnelldorfer *et al*., 2000). Similarly, HC also induced evident decreasing of reduced GSH and stimulated intracellular ROS production in KB cells at concentrations comparable to its induction of cell cycle arrest and apoptosis. The induction of cellular GSH depletion and oxidative stress by HC may be a possible explanation for HC's ability to elicit cell-cycle arrest and apoptosis of KB cells. This may be attributed to the production of o-quinone during HC metabolism that subsequently induces the ROS production *via* redox cycling (Iverson *et al*., 1995; Krol & Bolton, 1997). Measurement of DCF fluorescence is very useful in quantifying overall production of ROS. However, DCFH can be oxidized by H_2O_2, lipid hydroperoxide, peroxynitrite and superoxide radicals (Banan *et al*., 2000; Catchart *et al*., 1983; Taguchi *et al*., 1996; Crow, 1997; Wang & Joseph, 1999). Further studies are needed to clarify the nature of the toxic species using specific scavengers. On the other hand, CMF fluorescence has been widely used to measure the intracellular levels of reduced GSH, but not GSH conjugate and oxidized glutathione (GSSG) (Chikahisa *et al*., 1996; Burghardt *et al*., 1992). Decreasing the cellular content in reduced form of GSH by HC can be due to conjugation between reduced form of GSH with reactive HC metabolites such as quinones, quinone methide and imine methide (Nikolic *et al*., 1999; Iverson *et al*., 1995; Krol & Bolton, 1997; Bolton *et al*., 1994) or the conversion of reduced GSH to the oxidized GSSG by the GSH peroxidase (Li *et al*., 2000). However, the concentrations of HC that affect cellular GSH content are at least one log higher than those required to affect H_2O_2 and cytotoxicity in the present study. Thus the biologic effects of HC cannot be fully explained by its depletion of cellular reduced GSH. The presence of additional toxic mechanism should be considered.

Hydroxychavicol has consistently been observed to induce late S and G_2/M cell-cycle arrest, indicating HC's potential effects upon specific cell-cycle regulatory genes. Chemical-induced cell death can be mediated by necrosis and/or apoptosis (Wyllie, 1997; Renvoize *et al*., 1998; Eastman & Rigas, 1999), the extent of which varies with the test chemicals and cell types used. Apoptosis is a critical component of cellular responses to injuries to cell membranes, mitochondria and DNA, or to a dysregulation in the cell cycle (Wyllie, 1997; Renvoize *et al*., 1998). Many chemotherapeutic and chemopreventive agents have been shown to induce apoptosis in target tumor cells (Lyons & Clarke, 1997). In the present study, the exposure of KB cells to HC at a concentration of 0.1–0.25 mM led to cell retraction and cell rounding with a loss of contact with adjacent cells. These morphological changes parallel early events of cell apoptosis (Renvoize *et al*., 1998). In addition, the induction of apoptosis of HepG2 hepatoma cells by HC having been recently reported (Chen *et al*., 2000). The induction of apoptosis of KB cells by HC has consistently been demonstrated. Interestingly the process of apoptosis was accompanied by a visible decrease in the number of cells in G_2/M phase as compared to controls, indicating that apoptotic cells are derived mainly from cells residing in G_2/M phase. The induction of KB-cell apoptosis by HC may be due to the arrest of the normal cell-cycle process, given that a similar correlation between staurosporin-induced apoptosis and cell-cycle arrest for oral KB carcinoma cells has been reported previously (Swe *et al*., 1996).

Taken all together, many phenolic antioxidants can also display pro-oxidant properties and cause cell injury under different circumstances (Stader *et al*., 1995; Cao *et al*., 1997). Frequently, at low concentrations these drugs have an antioxidant effect, but at higher doses they also exert pro-oxidant properties. Accordingly, HC may induce the intracellular production of ROS at concentrations higher than 0.1 mM. This indicates that HC is an antioxidant at low concentrations, whereas at high concentrations (>0.1 mM), HC may elicit redox status changes (decreasing in reduced form of GSH and induce oxidative cell damage). These experiments highlight that HC possesses antioxidative properties and chemopreventive potential at concentrations below 0.1 mM. Hydroxychavicol also inhibits the growth, attachment, and cell-cycle progression of KB carcinoma cells, indicating that the presence of HC in the BQ may provide some protective effects. Since betel leaf and inflorescence piper betle are two major components of BQ, BQ chewers may be continuously exposed to HC during their lives. Additional studies are required to elucidate whether enzymatic activation, the presence of transition metals in alkaline condition or changes in cell types may modulate the metabolism of HC. These investigations will facilitate our understanding of whether HC is beneficial or harmful to humans.

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