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Preliminary Observation on the Cytotoxic Activity of New Chlorophyllin Derivative RCD on Human Tumour Cell Lines *In Vitro*

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Abstract. *Background: Chlorophyllin is used in traditional Chinese medicine because of its anticancer properties. This article describes the preparation and cytotoxic activity of a reduced chlorophyllin derivative (RCD) on tumour cell lines. Materials and Methods: RCD was prepared by reducing chlorophyllin with lithium aluminium hydride, and its solubility in the alkaline and organic phases are different from that of the parent compound chlorophyllin. Results: RCD also has different chromatographic behaviour from chlorophyllin on thin-layer chromatography and high-pressure liquid chromatography, and excitation and emission spectra. RCD has cytotoxic activity against ZR-75, MCF-7 and HT-29, human tumour cell lines. A clonogenic assay showed that the growth of tumour colonies on soft agar was reduced by RCD in a dose-dependent manner. Conclusion: RCD is a novel compound exhibiting anticancer activity.*

Chlorophyllin, is a water soluble semi-synthetic derivative of chlorophyll whose anticancer effects have been extensively reviewed Nagini *et al*. (1). It was first extracted from *Bombyx mori* excreta, which were used against cancer in traditional Chinese medicine, and it has been successfully tested on HL60 human promyelocyte leukaemia, K-562 myelogenous leukaemia and MCF-7 breast carcinoma cell lines (2). Nano-capsulated chlorophyllin was found to significantly delay the progression of lung cancer *in vitro* and *in vivo* (3).

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Chlorophyllin is poorly absorbed by the gut (4), but Sato *et al*. demonstrated that it is well distributed in rats when administered intraperitoneally (5). Furthermore, when administered orally, the lipophilic chlorophyllin ester is well absorbed, passes into total body water and gives serum a green colour (4).

It appears from that free carboxyl groups appear to hinder the absorption of chlorophyllin (4, 5), therefore, these carboxyl groups need to be modified. In particular it is important to modify the chlorophyllin structure (Figure 1) into a lipophilic compound by reducing the carboxylic groups to alcoholic groups (6) and to test whether the reduced chlorophyllin derivative (RCD) exerts cytotoxic activity on human tumour cell lines *in vitro*.

Materials and Methods

Chlorophyllin sodium copper salt (Figure 1) was obtained from Sigma (Milan, Italy) and chromatography solvents were purchased from E. Merck (Darmstadt, Germany).

Preparation of RCD. Chlorophyllin sodium copper salt (1.5 g) was dissolved in water (50 ml), washed with chloroform, brought to pH 3 with 0.1 N HCl and extracted with chloroform (50 ml) twice. The organic phase was dried with anhydrous Na_2SO_4 and evaporated to dryness under vacuum. The yield was 80%. The product (1 g) was suspended in tetrahydrofuran (100 ml) and treated with an excess (1 g) of lithium aluminium hydride ($LialH_4$) (6). After 12 hours, excess $LiAlH_4$ was quenched by adding ethyl acetate (50 ml) at 0˚C and then 6 N HCl (50 ml) and water (50 ml) were added; the supernatant was removed and the precipitate was washed with ethyl acetate. The organic phases were combined, washed with 1 N NaOH, water and dried with anhydrous Na_2SO_4 and evaporated to dryness under vacuum to give 760 mg of crude RCD. The RCD produced was analysed by means of thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and spectrofluorimetry.

Chromatography. Chlorophyllin and RCD were determined by means of HPLC using a Spectra System P2000 liquid chromatograph (Thermo Scientific, Waltham, Massachusetts, MA, USA) equipped

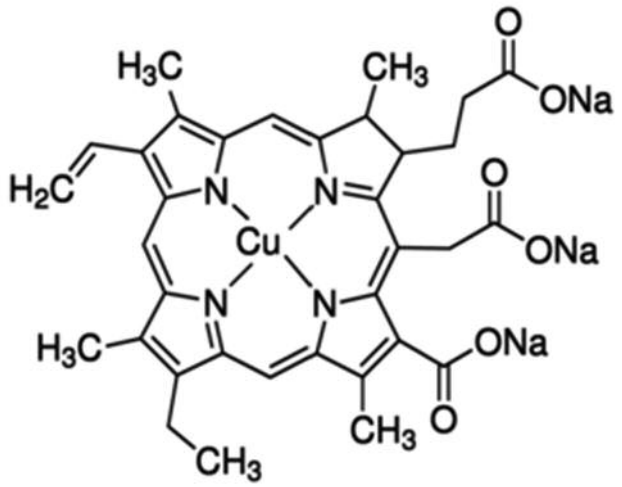

Figure 1. *Chemical structure of the chlorophyllin sodium copper salt.*

Table I. *Partitioning of chlorophyllin and reduced chlorophyllin derivative (RCD) (%) between organic phase (ethyl acetate) and aqueous phase (0.1 N sodium hydroxide). The samples were analysed by high-performance liquid chromatography. Data are the mean±S.E. for three individual determinations. While chlorophyllin was found to be concentrated in the aqueous phase, RCD was largely partitioned into the organic lipophilic layer.*

Compound	Organic phase	Aqueous alkaline phase
Chlorophyllin	2.1±0.4	94.3±5.2***
RCD	87.8±6.2***	6.4±1.9

***Significantly different at $p<0.0001$.

Table II. *Relative mobilities (Rf) of reduced chlorophyllin derivative (RCD) and chlorophyllin on thin-layer chromatography plates.*

Mobile phase	Chlorophyllin (R_f)	RCD (R_f)
Chloroform/methanol/NH_4OH (80/20/1)	0.12±0.02	0.20±0.03
Ethyl acetate/methanol/NH_4OH (50/50/1)	0.16±0.01	0.37±0.03

with an injection valve (model 7125 Rheodyne, Supelco-Merck KGaA, Darmstadt, Germany) and a Waters 474 Scanning Fluorescence Detector (Waters, Milford, Massachusetts, MA, USA). The system was connected to a Hitachi-Merck D-2000 chromato-integrator (Hitachi-Merck, Darmstadt, Germany). A Waters Symmetry C18 3.5 μm (150×4.6 mm i.d.) column coupled to a Waters Sentry Symmetry C18 guard column was used at room temperature (Waters S.p.a., Sesto San Giovanni, Milan, Italy). The mobile phase was 50/50 (v/v) acetonitrile: 50 mM potassium phosphate buffer (K_2HPO_4 pH 3.50). The flow

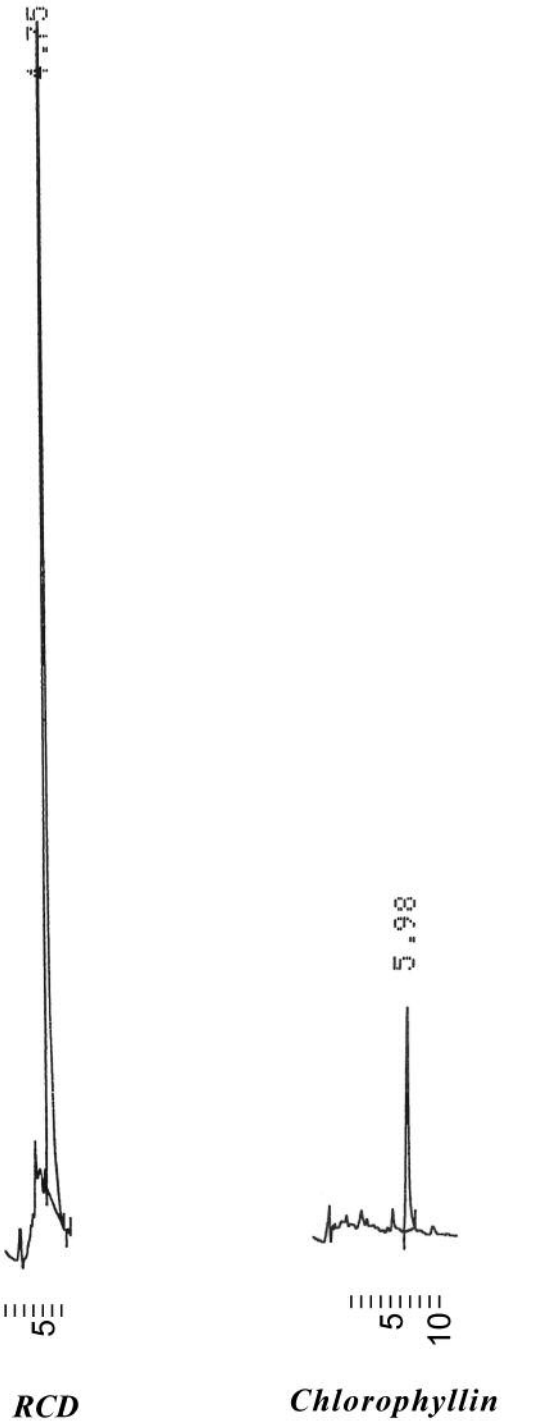

Figure 2. *Chromatogram of reduced chlorophyllin derivative (RCD) and chlorophyllin. The separation was carried out on an RP18 (3.5 μm, 150 mm ×4.6 mm I.D.) column with 50:50 acetonitrile–0.05 M phosphate buffer, pH 3.5, as mobile phase at a flow rate of 1 ml/min. The eluate was monitored at λ_{ec}=388 nm of excitation and λ_{em}=585 nm of emission for RCD and λ_{ec}=405 nm of excitation and λ_{em}=675 nm of emission for chlorophyllin. RCD was found to have a retention time of 4.75 min and appeared to be homogeneous and pure (95%). Retention time of chlorophyllin was 5.98 min.*

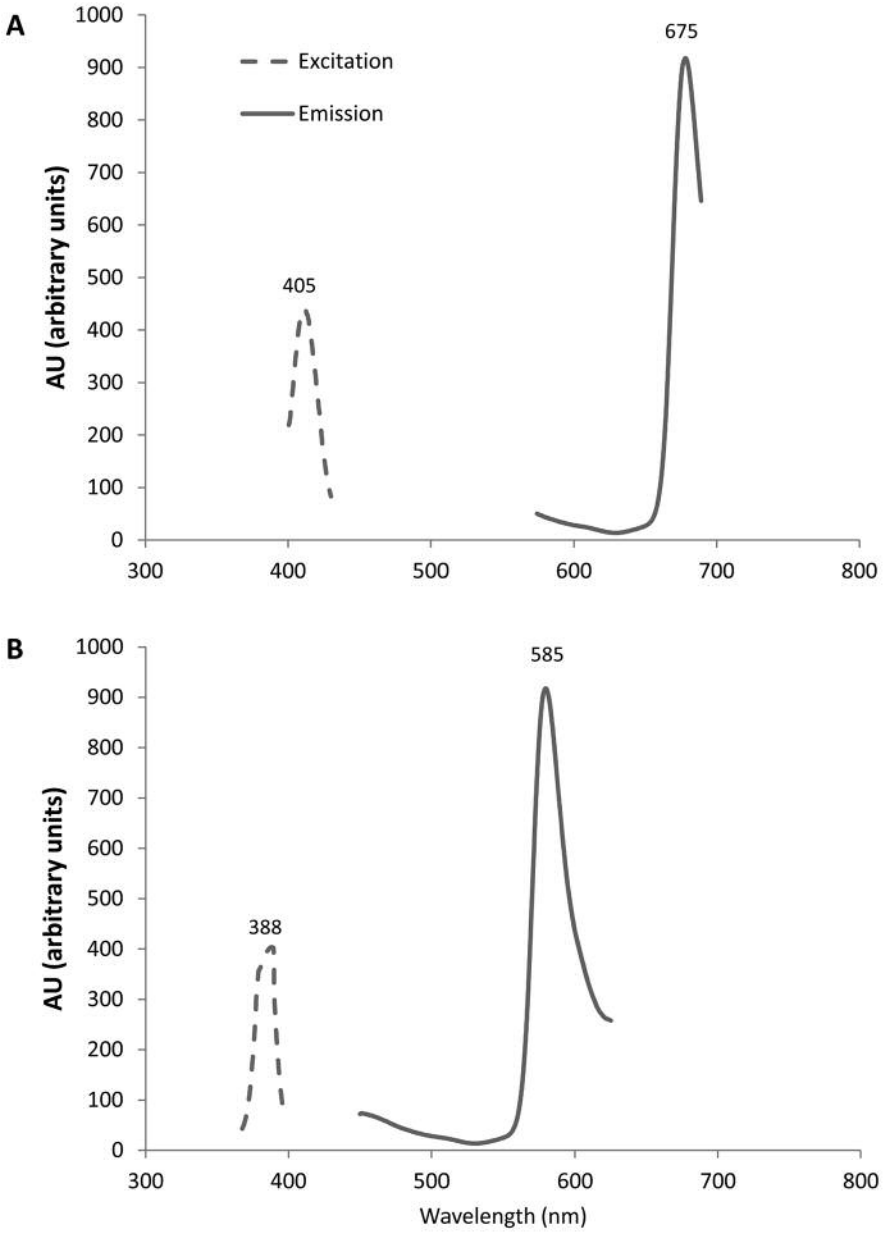

Figure 3. *Excitation and emission spectra of chlorophyllin (A) and reduced chlorophyllin derivative (RCD) (B) dissolved in methanol.*

rate was 1.0 ml/min and the column effluent was monitored at excitation and emission wavelengths of 388 and 585 nm for RCD or 405 nm excitation and 675 nm for chlorophyllin. The major peak present was analysed.

Thin layer chromatography. TLC absorption plates pre-coated with 0.25 mm silica gel 60 were obtained from Sigma-Aldrich (Milan, Italy). The samples were spotted on the plates which were then developed in Camag tanks using mobile phases of chloroform/ methanol/NH_4OH (80/20/1) and ethyl acetate/ methanol/NH_4OH (50/50/1). R_f values were then calculated.

Spectroscopy. Chlorophyllin and RCD were dissolved in methanol. The absorption and emission spectra were recorded on a Perkin-Elmer spectrometer (Milan, Italy).

Partitioning into aqueous and organic phases. Chlorophyllin and RCD (0.1 mg/ml) were partitioned into aqueous and organic phases. They were dissolved in ethyl acetate and shaken with equal volumes of 0.1 N NaOH. After centrifugation, aliquots of the organic and aqueous phases were submitted to spectrophotofluorimetric and HPLC analysis and the results were compared using non-extracted samples as reference standards.

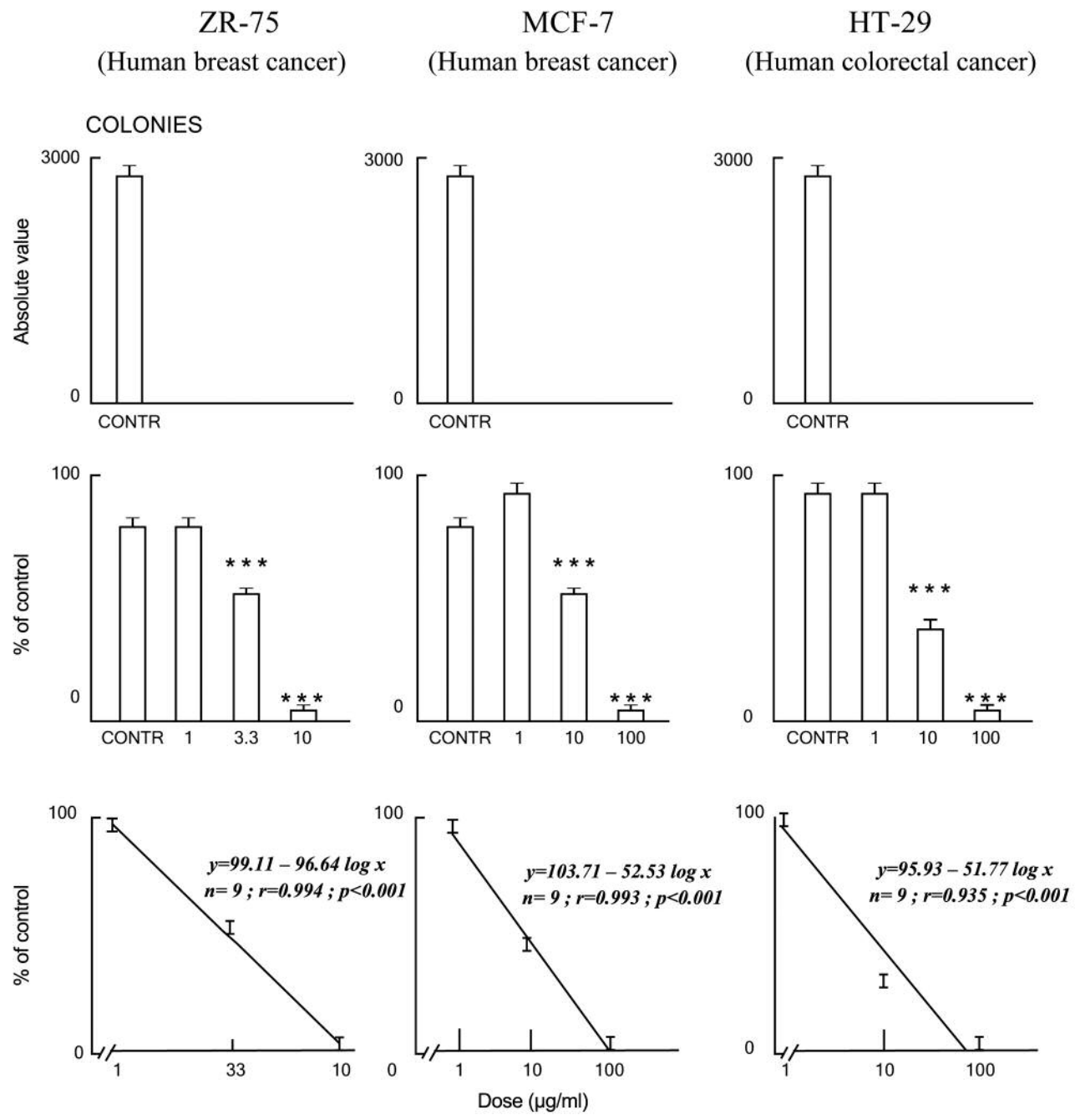

Figure 4. *Effects of reduced chlorophyllin derivative (RCD) on tumour cell lines: ZR-75 (human breast cancer), MCF-7 (human breast cancer) and HT-29 (human colorectal cancer) as evaluated by clonogenic assay. The values are the mean of three individual determinations±S.E., represent the survivors and are expressed as a percentage that of controls. The absolute values at day 14 are expressed as the number of colonies.*

Cell lines. RCD was screened for cytotoxic activity against ZR-75, MCF-7 (human breast cancer) and HT-29 (human colorectal cancer) cell lines cultured *in vitro*. These cell lines were gifts from Dr. C. Kent Osborne (University of Texas, Health Science Centre at San Antonio, TX, USA) and were maintained in minimal essential medium plus 10% foetal calf serum.

Clonogenic assay for cytotoxic activity. A soft-agar clonogenic system was used (7). The cells were suspended in 0.3% agar in tissue culture medium and 1 ml of the resulting mixture was pipetted into 1 ml McCoy's medium plus 15% heat-inactivated foetal calf serum and a variety of nutrients in 35 mm plastic Petri dishes. The density of cells was 3,000 cells/plate. The plates were

incubated at 37˚C in a humidified atmosphere with 7% CO_2 in the dark. Experiments on drug treated and untreated cells were run in triplicate. Colonies with a diameter >100 μm, which usually appeared by day 14, were counted under an inverted stage microscope at ×30. The survival of tumour colony-forming units was expressed as a percentage of the ratio between drug-treated plates and untreated control plates.

Statistical analysis. The mean values and standard errors of triplicate determinations of controls and treated cells (as a percentage of controls) were calculated and Student's *t*-test was used to establish the significance of the difference (8). Linear regression of the data (as a percentage of controls) *vs*. log dose was calculated using the least-squares method (8).

Results

Partitioning into aqueous and organic phases. Spectrophotofluorimetric analysis showed that chlorophyllin accumulated in the alkaline phase (94.3%), whereas RCD was extracted into the organic phase (87.8%). RCD was found to be a lipophilic compound, and chlorophyllin a hydrophilic one (Table I).

Chromatography. Table II and Figure 2 show the chromatographic behaviour of the compounds. TLC showed that chlorophyllin and RCD migrated differently (Table II), and HPLC showed that they had different retention times (RCD 4.75 min *vs*. chlorophyllin 5.98 min) (Figure 2), thus indicating they were different compounds.

Excitation and emission spectra. Figure 3 shows that the excitation and emission spectra of chlorophyllin and RCD were different.

Cytotoxic activity. Figure 4 shows the effects of RCD on ZR-75, MCF-7 and HT-29 tumour cell lines. It reduced the growth of tumour colonies on soft agar and the regression of effect (percentage of controls) *vs*. log dose showed that the dose–response curve was linear (Figure 4). The ED_{50} values (μg/ml), which was calculated from Figures 4 and 5, were in the low μg/ml range and varied among the different cell lines. The cytotoxic effect of RCD against the normal MRC-9 human embryonic lung fibroblast cell line was relatively small about 30 μg/ml (Figure 5). Chlorophyllin did not have any cytotoxic activity on the tested cell lines at doses of <100 μg/ml.

Discussion

The structure of chlorophyllin was modified by the reduction of carboxyl functions to obtain RCD, a highly lipophilic compound that, unlike chlorophyllin, does not dissolve in the alkaline phase.

The different partitioning of chlorophyllin and RCD into alkaline and organic phases respectively demonstrated that RCD is different from its parent compound chlorophyllin, and

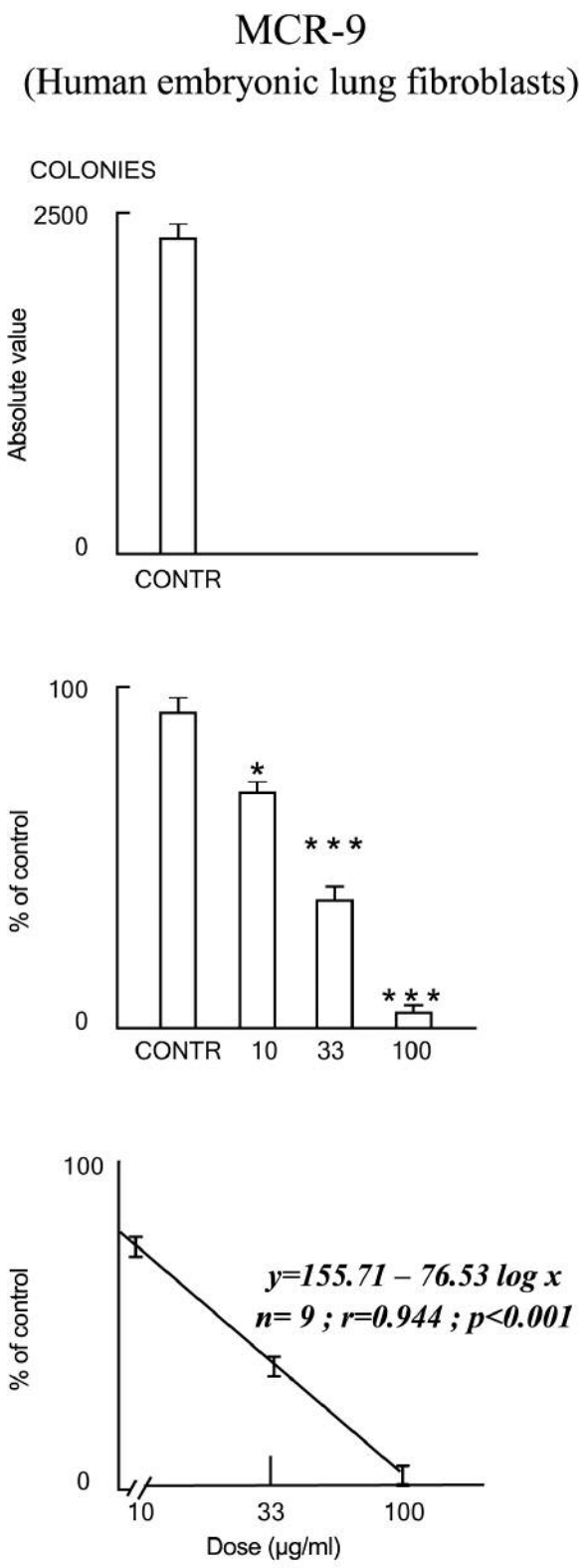

Figure 5. *Effects on human embryonic lung fibroblast (MCR-9) as evaluated by a clonogenic assay. The values are the mean of three individual determinations±S.E. The absolute values at day 14 are expressed as the number of colonies.*

their solubilities are consistent with the preparation method, which involves an exhaustive lithium aluminium hydride reduction that converts the carboxyls into hydroxyl groups.

The chromatographic behaviour of the two compounds on TLC (Table II) and HPLC (Figure 1), and their different excitation and emission spectra (Figure 2) also indicate that RCD is a novel compound and a single compound chromatographically. The structure of this molecule is currently under further investigation by mass spectrometry and nuclear magnetic resonance.

RCD had cytotoxic activity against ZR-75 and MCF-7 human breast cancer and HT-29 human colon cancer cell lines cultured *in vitro* and reduced the growth of cell colonies on soft agar in a dose-dependent manner (Figure 3).

The cytotoxic effects of RCD are in the low concentration range (μg/ml), thus making RCD more active than other chlorophyll derivatives, which are active at doses of between 25-400 μg/ml (2).

From the analysis of the ED_{50}, it appears that RCD is more active on tumour cell lines (Figure 4) then on the normal fibroblast cell line (Figure 5).

It is important to remember that chlorophyllin is reported to affect tumorigenesis and tumour progression (9); it may mediate S-phase arrest in colon cancer cells (10); it may control cell proliferation and expression of apoptosis in breast cell (11) and to prevent genotoxicity of mycotoxins (12).

It is quite important to observe that the reduction of the carboxyl groups to produce RCD, as reported in this paper, did not appear to affect the anticancer properties of chlorophyllin but exhibits a new way to prepare a class of new anticancer agents.

We are currently developing a project to elucidate the anticancer mechanism associated with reduced chlorophyllin structure.

Conflicts of Interest

The Authors declare that there is no conflict of interests regarding the publication of this article.

Authors' Contributions

AP wrote the manuscript and is the guarantor for the accuracy of the data and contributed to literature screening, ST provided data for TLC, Tables I and II, spectrophotofluorimetric analysis, all statistical analyses and tests on cellular trials. PF provided data for HPLC analysis. All Authors have agreed to the final version of the article.

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