The effect of rooibos tea (*Aspalathus linearis*) on the survival and transformation of C3H-BT1 mouse fibroblasts

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This experiment examined the effect of rooibos tea (*Aspalathus linearis*) on the survival and transformation of C3H-BTI mouse fibroblasts which are cells that show a high propensity for expressing a transformed (cancer) phenotype. Both the fermented (red) and unfermented (green) varieties of the tea were tested. A standard solution of each tea was made and sterilized. Cells were cultured in media containing different amounts of tea ranging from 0.0 to 0.5 μL/mL for red tea and from 0.0 to 1.5 μL/mL for green tea. The experiment was duplicated using media without phenol red to rule out any effect that the pH indicator might have had. The teas did not appear to have a significant effect on transformation frequencies. The tea had a negative affect on survival. Marked aberrations in colony growth patterns were noted with skeletons that increased with dosage being the most significant abnormality.

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INTRODUCTION

Cancer is the uncontrolled proliferation of cells. Mutations or damage to a cell's DNA accumulate causing a cell to not recognize its boundaries and to make uncontrolled reproduction its primary function. In this experiment C3H-BT1 mouse fibroblasts were used to see if Rooibos tea (*Aspalathus linearis*) had an effect on their transformation into cancerous cells. This particular line of cells has a high propensity toward becoming cancerous. It also has a variable phenotype, in which some progeny from a normal colony can become cancerous and some progeny from a cancerous colony can become normal. The reason for the malleability of the phenotype is unknown, but environmental conditions do seem to play a role. The C3H-BT1 cell line was developed by Jianglan Hannah Zhang (1994) at Bemidji State University with funding by NASA. The C3H-BT1 line is a useful *in vitro* model for the study of cancer.

Cancerous transformation *in vivo* and *in vitro* exhibits a continuum of severity that is loosely placed into stages *in vivo* or types *in vitro*. *In vitro*, normal cells grow in a monolayer, showing contact inhibition. Stage I *in vivo* cancers proliferate uncontrollably but have not invaded surrounding tissues. In stage II, cancerous cells are able to break through the basement membrane and invade adjacent tissues. In cell cultures, the substrate is not a biological membrane: it is a polyethylene plate that cells cannot penetrate. A colony that no longer has a monolayer and has significant piling but has not yet progressed to the severe Type III is described as Type I or Type II based on the density of the piling. In stage III *in vivo* cancers, cells are able to metastasize to distant areas. *In vitro* Type III are defined as colonies with very dense piling extending to the periphery and greater than 50% crisscrossing of cells at the edges. These are the cells that proved to be tumorigenic *in vivo* when transplanted into immuno-compromised C3H/He and nude strains of mice (Zhang, 1994).

In this study the adhesion of fibroblasts played a key role. Adhesion of a fibroblast to the underlying

substrate occurs by means of hemidesmosomes. Attachment induces a signal in the cell that is relayed to the nucleus. Reduction in the number of these signals results in the cell undergoing apoptosis (programmed cell death).

Rooibos tea, known commonly as red tea, is a tea from South Africa that has long been considered a health aid by the people of the area. In 1996, von Gadow, Joubert, and Hansmann compared the antioxidant activity of three different preparations of rooibos tea with green, oolong, and black teas. Since that time only a few studies have been done involving red tea. Marnewick, Gelderblom, and Joubert noted unfermented rooibos tea to have a protective effect against mutagenesis derived from more than one known mutagen using the *Salmonella typhimurium* mutagenicity assay (2000). Marnewick et al (2003) studied mutagenesis in *Salmonella* grown with liver fractions from rats given a steady diet of rooibos tea. The tea protected against mutagenesis caused by some mutagens but not others. Joubert, Winterton, Britz, and Ferreira assessed the radical scavenging capacity of rooibos tea components (2003). Marnewick et al (2004) noted inhibition of tumor promotion in mouse skin by topical application of aqueous rooibos tea solutions. Although not specifically cancer research, Lamosova, Jurani, Greksak, Nakano, and Vanekova (1997) found that rooibos tea extract inhibited the growth of cultured chick embryonic skeletal muscle cultures.

Rooibos tea is available in two different preparations: fermented and unfermented. The traditional fermented preparation is called red tea, while the relatively new unfermented preparation is called green-red tea. For the purposes of this work unfermented rooibos tea will be referred to as green tea (not to be confused with *Camillia senisis*, the unfermented variety of which is commonly known as green tea). The anti-oxidant composition of rooibos tea appears to vary with preparation, with the green having more types of anti-oxidants and greater concentrations of them than the red tea.

The reportedly high antioxidant content of this tea sparked this investigation. Antioxidants have long been under investigation as possible anti-cancer agents. Oxidative damage to DNA is one major source of cancer-causing mutations. Normal respiratory processes in living cells produce reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide (H_2O_2). Oxidative damage occurs when ROS initiate a chain of reactions in which a single electron is stripped from a random molecule. This creates a new radical which in turn reacts indiscriminately with any other surrounding molecule, which creates yet another radical. The nitrogenous bases of DNA are highly susceptible to damage from free radicals, and damage to certain areas of DNA turn off the cell's natural checkpoints that prevent cancer-causing growth. Antioxidants prevent damage by neutralizing the highly-reactive oxygen radicals without becoming radicals themselves, thus breaking the chain of propagation. This indicates that regular anti-oxidant consumption could be a preventative therapy for cancers that are caused by oxidative damage. Not all anti-oxidants that have been investigated have been shown to have anti-cancer properties, and there are probably many anti-oxidants that remain to be investigated.

Preventative therapies are undertaken by those who have a high risk for a disease but have not yet been diagnosed with it. Quite often these therapies must be continued for the rest of the individual's life in order to maintain their effectiveness. Generally, therapies that are unpleasant, time-consuming, or inconvenient are not complied with in the long term; whereas therapies that are benign and easily integrated into the daily routine are more likely to be followed. A possible therapy for individuals with a family history of certain types of cancer would be the regular consumption of an anti-oxidant known to prevent that type of cancer. Teas of various kinds have been indicated as good sources of several different anti-oxidants. A convenient, pleasant, and uninvolved therapy could be as simple as having a cup of tea each day.

Cell cultures are normally grown in media containing phenol red to indicate the pH of the media since cells have a narrow range of pH tolerance. In a previous experiment by Kjerstin Johnson (2002), phenol red was found to react with green tea (*Camilla senisis*) to form H_2O_2. As H_2O_2 is damaging to cells, it was postulated that the decrease in cell survival that the experimenter noted may have been due to the presence of phenol red in the media. Phenol red is not present in vivo, thus the results were not an accurate model of what occurs in a living body. Both media with phenol red and without phenol red were used in this experiment to eliminate this error.

This experiment sought to determine if either the fermented (red) or unfermented (green) preparations of rooibos tea could affect the survival and transformation rates of C3H-BT1 cells.

MATERIALS AND METHODS

Reagents and Equipment

The following reagents were used and were obtained from Sigma Chemical Company: Phosphate buffered saline (PBS) without Mg^{++} or Ca^{++}, Giemsa stain, trypsin, Fetal Bovine Serum (FBS), 2mM glutamine, gentamicin, and Minimal Essential

Medium (MEM) with and without phenol red. Methanol was obtained from VWR.

Equipment used include the following: NUAIRE sterility hood (NU-420-400); Olympus CK 2 inverted phase-contrast micropscope; Leitz Fluovert inverted phase-contrast microscope; Olympus ST-SZ 4060 dissecting stereoscopes; Olympus LMS-225R dissecting microscope; Diagnostics Instruments 11.5 Color Mosaic microscope camera; Pall VacuCap 60 Filter Units with 0.2 μm Supor Membrane; Corning cell culture dishes (100 mm x 20 mm, treated non-pyrogenic, polystyrene); Drumond and Eppendorf pipettors; Forma Scientific CO_2 water-jacketed incubator (3110); Microsoft Word; Microsoft Excel.

Red Rooibos and Green Rooibos teas were obtained through the Strand Tea Company. A standard solution for each tea was made by boiling 260 mL distilled water on a hot plate. The water was removed from the hot plate and 6 tea bags were added. The tea was allowed to steep for ten minutes. At the end of this time, the tea bags were removed and the bags were pressed with a spoon to recover excess water. The tea solution was measured in graduated cylinders to determine the amount of water lost through evaporation. Distilled water was added to bring the volume back to 260 mL. The tea solutions were centrifuged at 4,000 rpm for 10 minutes. The supernatants were pipetted off into clean bottles, and the solutions were frozen until the next lab period. Centrifugation had to be repeated after the first freezing due to the formation of precipitates. A 0.2 μm filter was utilized to sterilize the solutions. The filtered tea solutions were separated into aliquots and frozen. Freezing sometimes resulted in precipitation of a brown substance resembling tea grounds. These were eliminated by re-filtration.

Cell Cultures

Four sets of cell cultures were prepared using BT1 medium consisting of 10% FBS, 1% 2mM glutamine, and 0.25% gentamicin in MEM. Two sets used MEM without phenol red while 2 used MEM with phenol red. Each set consisted of 6 control plates with no tea and 5 sets of 4 plates with differing amounts of tea. For the 2 sets using red tea (one with phenol red and one without phenol red), the following amounts of tea were added to 10mL of media: 0.50 μL, 1.63 μL, 2.75 μL, 3.88 μL, and 5.00 μL. For the 2 sets using green tea (one with phenol red and one without phenol red), the following amounts were used: 1.00 μL, 4.50 μL, 8.00 μL, 11.50 μL, and 15.00 μL.

The cultures were prepared as follows: Culture dishes (plates) were labeled in a sterile environment. To each was added 10 mL BT1 culture medium and the appropriate amount of tea. A volume of C3H-BT1 cell suspension was added to each plate such that 100 colonies would result, assuming a plating efficiency of 70%.

The cultures were incubated at 37° C and 5% CO_2 for two weeks. In pilot experiments the colonies washed off the plates during the fixation process. At the end of the incubation period sample plates were examined under an inverted phase-contrast microscope, and the colonies were determined to be only delicately adhered to the plates. As rinsing the plates with PBS would cause the colonies to disengage from the base, this step was eliminated. The medium was pipetted off the colonies, and just enough methanol to cover the plate was pipetted on and left for 10 minutes. The methanol was pipetted off and the plates were allowed to dry. A second treatment of methanol was pipetted on and after 10 minutes pipetted off, and this too was allowed to dry. The plates were rinsed with distilled water 3X to remove salts from the media that had precipitated when the methanol was added. The plates were stained with Giemsa stain for 15 minutes. This was poured off and each plate was rinsed 3X with distilled water. The colonies were examined under a dissecting microscope and staged as Normal, Type I/II (moderately transformed), or Type III (highly transformed).

RESULTS

Survival

Comparative survival of all four culture sets is shown in Fig. 1. Survival (% control) for each plate was calculated by dividing the total number of colonies by the mean of the total number of colonies on the control plates. Skeletons (discussed later) were included in these counts.

Cultures grown in red tea without phenol red consistently showed greater survival than cultures in red tea with phenol red. For cultures grown in green tea without phenol red survival was initially greater than that with phenol red, however at concentrations greater than 0.8 μL/mL survival was similar.

For cultures grown in red tea with phenol red survival decreased with concentration to 40% at the highest dose.

For cultures grown in green tea survival decreased as a function of concentration, reaching a low of 50-60% at concentrations $\geq$ 0.8 μL/mL.

Transformation

In all four sets, normal colonies ranged from 11-33%, type I/II colonies ranged from 52-77%, type III colonies ranged from 3-22%.

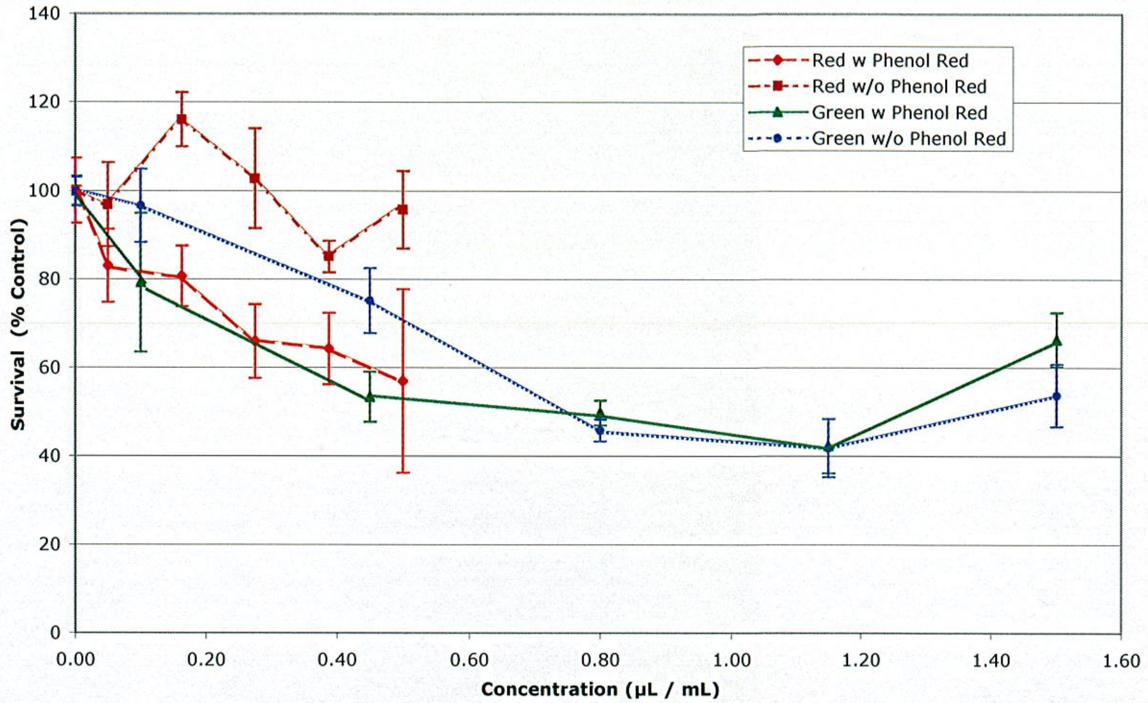


Figure 1: Comparative survival of C3H/BT1 cells as a function of concentration in media with red and green rooibos teas with or without phenol red. Each data point represents the mean value ± 1 SEM for 4-6 cultures.

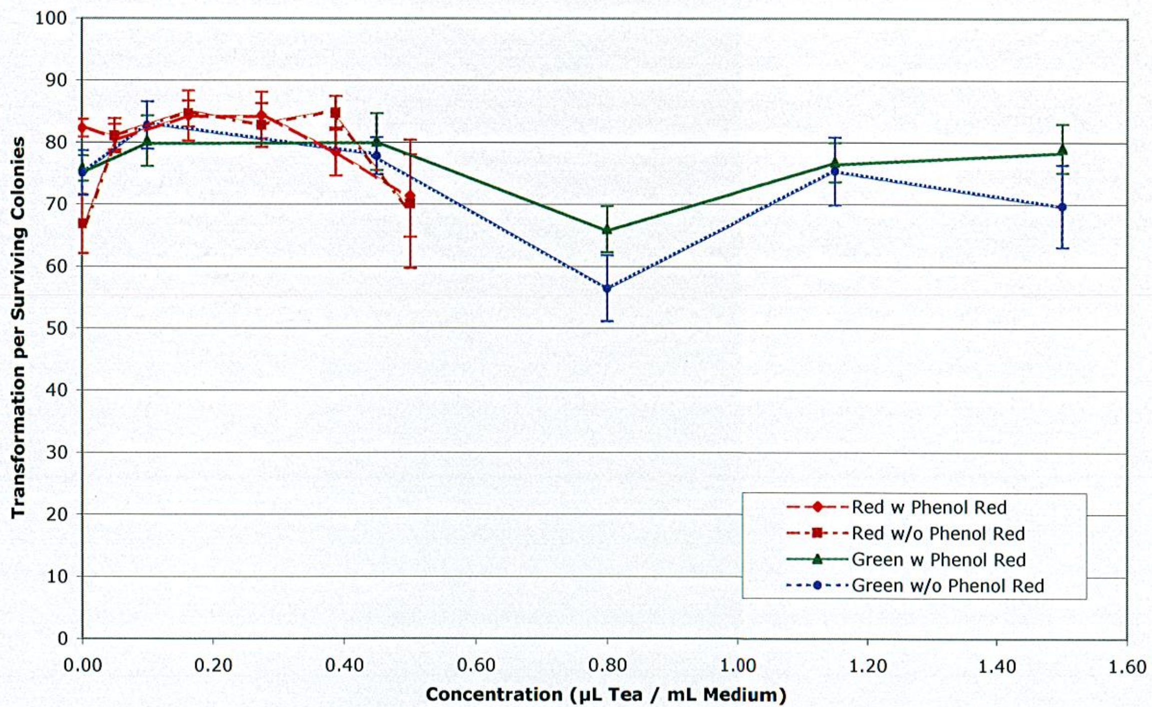


Figure 2: Comparative transformation of C3H/BT1 cells as a function of concentration in media with red and green rooibos teas with or without phenol red. Each data point represents the mean value ± 1 SEM for 4-6 cultures.

Comparative transformation of the four culture sets is shown in Fig. 2. Transformation was calculated by dividing the number of transformed colonies on a plate by the total number of colonies evaluated on the plate. Skeletons were not included in either number.

In red tea, transformation per evaluated cell (%) was approximately 80% throughout with an insignificant decrease to about 70% at the highest concentration. No significant difference was noted between cultures with phenol red and those without phenol red.

In green tea, transformation per evaluated cell stayed between 75% and 85% in the first three concentrations. It then decreased to about 55% for cultures without phenol red and 65% for cultures with phenol red. The remaining two concentrations yielded between 70% and 80% transformed per evaluated cell. A slight difference was noted between the plates with phenol red and those without, but the difference was insignificant.

Colony Morphologies

Fig. 3 shows examples of typical colonies observed on control plates. The normal colony (A) exhibits a monolayer of cells that were sensitive to contact with neighboring cells. Resulting cell signaling had caused the cells to stop dividing. A Type I transformed colony (B) exhibits the beginnings of piling that occurs when cells are not sensitive to contact. The Type II transformed colony (C) shows a much denser degree of piling over most of the surface but shows some control at the edges. The Type III colony (D) shows the most severely transformed phenotype characterized by extremely dense piling even to the edges and crisscrossing at greater than 50% of the periphery.

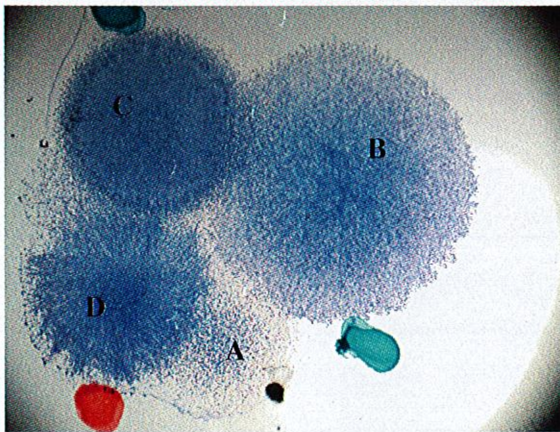


Figure 3: Examples of typical colony phenotypes observed on control plates: Normal (A), Type I (B), Type II (C), Type III (D). (picture courtesy of Dr. Alice Lindgren)

Furrows

Figure 4 shows furrows. Several plates had colonies showing streaks of no to light growth. These were very straight and thin and often veered off at sharp angles. Each furrow was lined with a wall of densely packed cells. Furrows were only noted after fixation, however, microscopic examination of the furrows showed that the cells were undisturbed. This suggests that furrows occurred while the cells were still living. Furrows occurred in all concentrations of tea including the controls.

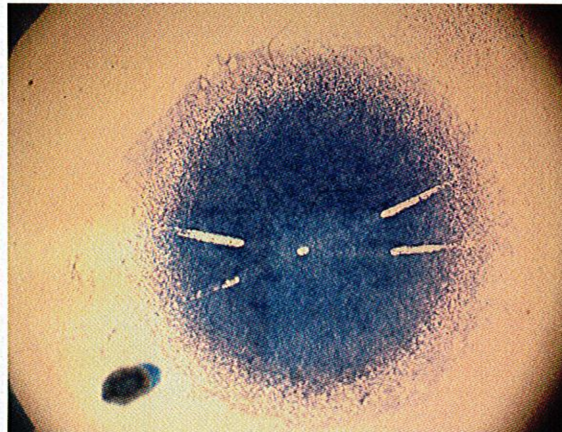


Figure 4: An example of Furrows (Red 0.275 µL/mL w/o Phenol Red). This is a typical Type II colony with dense growth in the center but controlled growth on the periphery.

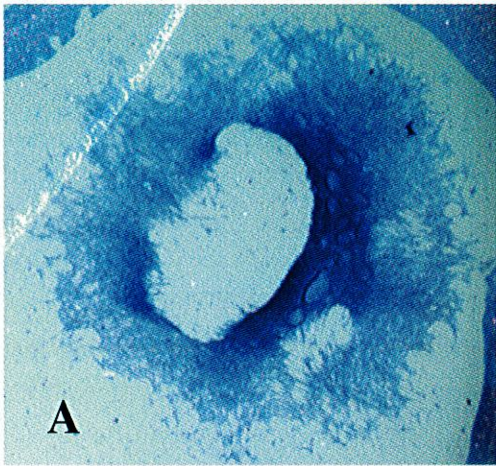
Abnormal Colony Morphologies

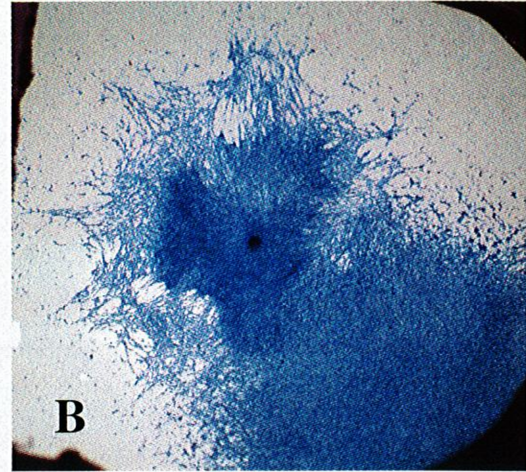
The most striking phenotypic effect caused by both teas was abnormal colony morphologies. At higher concentrations of tea marked aberrations of colony shape and growth patterns were noted. Transformed colonies were more likely than normal colonies to have a strange shape. As the concentration of tea increased regular round colonies typical of those in control plates became rare. Examples of abnormal colonies typical to our experiment are shown in Fig. 5 *A* and *B*.

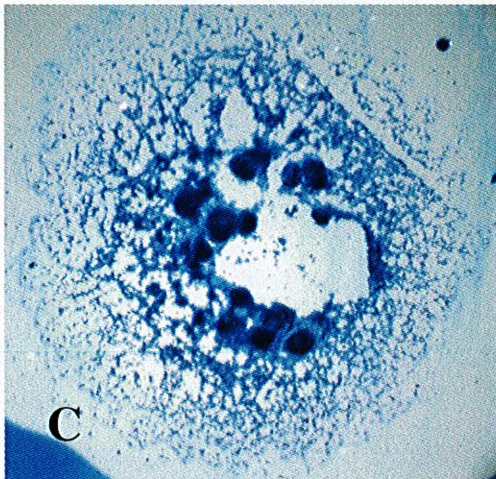
Piling

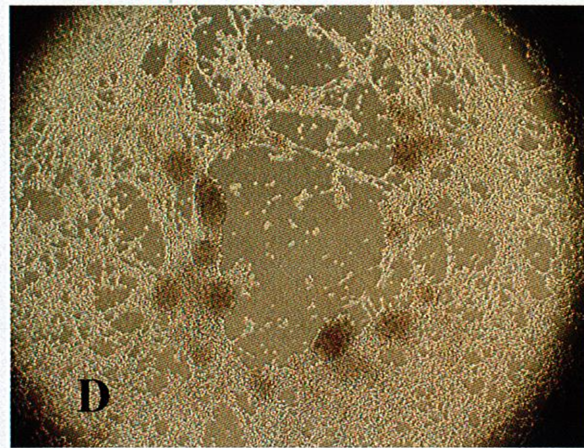
Fig. 5 *C* and *D* show piling in fixed and living cultures respectively. Piling appears as discrete nodules of densely piled cells. These occurred in both red and green tea sets. Generally the piled nodules are small as compared to the colony, chimney-like, and arranged in concentric circles about the focus of each colony.

Piling occurred in all concentrations of tea including the controls, but were significantly more prominent in experimentals than in controls. Often the piling on the controls adopted a more sheet-like appearance.









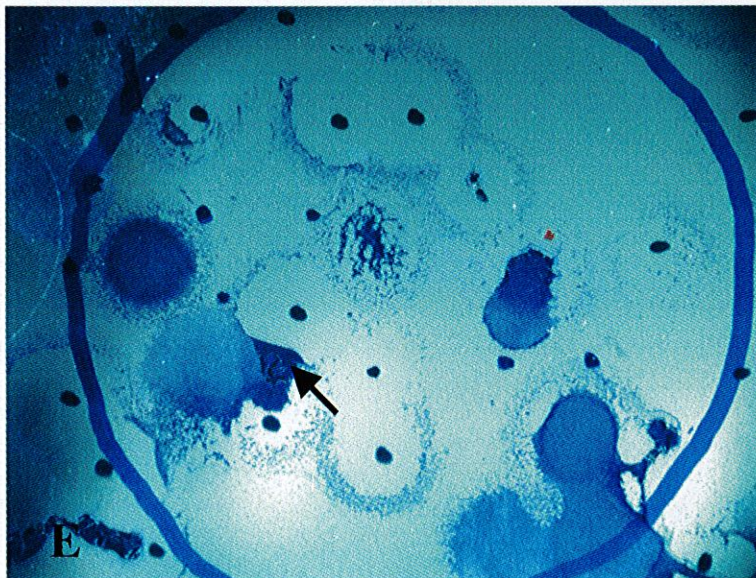


Figure 5: Examples of abnormal colony morphologies found on the following experimental plates:

A. **Green 0.45 μL/mL w/o phenol red**
Note: abnormal growth pattern

B. **Red 0.275 μL/mL w/o phenol red**
Note: abnormal growth pattern

C. **Green 0.8 μL/mL w/ phenol red**
Note: Piling (chimneys)

D. **Red 0.5 μL/mL**
Note: Piling in a living culture

E. **Green 1.15 μL/mL w/ phenol red**
Note: Skeletons indicated by dots in centers and a flap indicated by arrow

At the higher concentrations the area around each nodule was sparsely populated, whereas on control plates the area around each nodule was evenly populated. Nodules at higher dosages often occurred around an open center.

Skeletons and Flaps

Fig. 5 *E* shows examples of skeletons. Skeletons are remains of colonies in which the center has washed away, leaving a ring of attached cells. These were noted after the cultures were fixed and stained. In the process of transferring culture dishes from the incubator, colonies in experimental plates were noted to easily detach from the plate with very little agitation.

The number of skeletons on each plate was divided by the total number of colonies on the plate to determine the skeleton percentage. Fig. 6 compares the skeletons of the four culture sets.

No skeletons were noted in any of the controls. The number of skeletons initially increased almost linearly with the concentration of tea. In green tea they reached a maximum at 1.15 μL/mL and then decreased at the final concentration.

Fig. 5 *E* shows an example of a flap. Flaps were noted in both living and fixed cultures. These were colonies that had been washed up from the plate, but still adhered to each other and to part of the previously described ring. No flaps were observed in any controls.

ANALYSIS AND DISCUSSION

Furrows are believed to be resultant from a defect in the manufacturing of the plates. Straight lines rarely occur in biology but are not unusual at all in industrial processes. Culture plates need to be etched to give the fibroblasts an attachment surface. During or after the etching process a machine may have scored or abraded the surface of the plates. The polystyrene that is scratched away would form a wall on either side of the abrasion. When colonies grow on plates with these abrasions, they seem to grow up to the furrow, grow along the side of the wall, and then build a bridge of less densely-packed cells.

Phenol red appeared to have had an effect on survival. In green tea it seemed to have had a negative effect up until 1.15 μL/mL. At the concentration of red tea used in this experiment phenol red always had a negative affect on survival; however, if higher concentrations had been examined it seems probable that phenol red in red tea would have also ceased to have an effect. It is possible that phenol red always had an effect, but at higher concentrations of tea the effect was masked due to the tea's toxicity. Survival of colonies in red tea without phenol red seemed to be erratic. The cause for this is unknown. In the future it might be

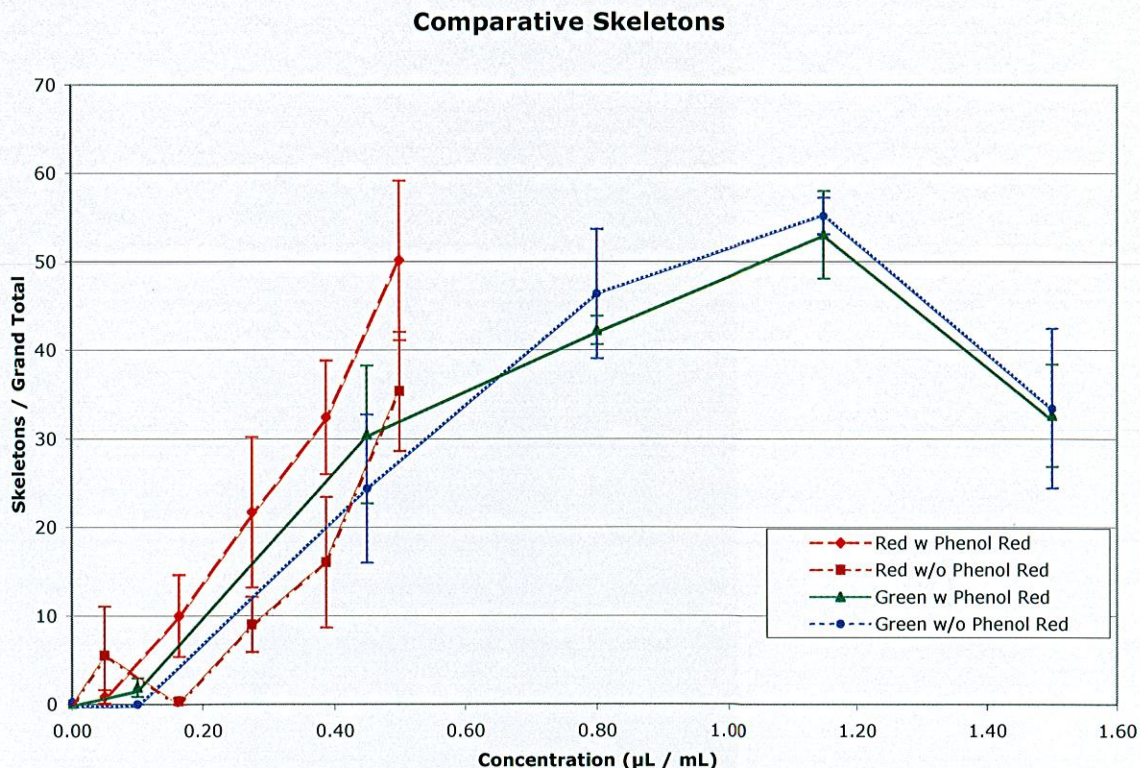


Figure 6: Comparative skeletons of C3H-BT1 cells as a function of concentration of red and green rooibos teas in media with or without phenol red. Each data point represents the mean value ± 1 SEM for 4-6 cultures.

beneficial to look at survival using smaller increments of concentrations.

In the majority of antioxidant experiments, survival increases to a point and then decreases dramatically. In this experiment, survival decreased nearly linearly with concentration in three out of the four sets, with the green tea sets reaching a low of 50-60% at concentrations ≥ 0.8 μL/mL. These results do not resemble other antioxidant experiments, thus a protective antioxidant effect was not seen here.

Phenol red appeared to have had no effect on transformation percentage. This indicates that the effects phenol red had on survival do not discriminate according to transformation stage.

The tea does not seem to have had a positive or negative effect on the progression of transformation from type I/II to type III. Type III colonies were always less numerous than normal colonies at each concentration. From the percentages stated in the results section, it can be seen that type I/II colonies were always the most numerous. The tea does not seem to have had a positive or negative effect on total number of transformed colonies except at 0.8 μL/mL of green tea, at which a decrease is shown both with and without phenol red.

The most significant finding of this experiment was the occurrence of skeletons and piling. It is possible that skeletons and piling are related. Most piling was observed in colonies staged as type I/II. One of the features of cancer cells is the ability to divide and live without adhesion. Piling on a colony could have created an unstable base that caused the colony center to pull up away from its substrate. Since cellular material was not noted in the media at any time, it is believed that the dissociated colonies could settle down again on the plate and form new attachments.

It was noted that colonies that came up during fixation and staining did not break up. Furthermore, flaps often had a stretched appearance. These observations suggest that only cell-substrate adhesion was affected and not cell-cell adhesion. Flaps seemed to be the centers of skeletons that had not completely dissociated from the bottom of the dish. Direct observation of flaps in living cultures using an inverted phase-contrast microscope revealed the cells of the flap to be still living. Under normal conditions, fibroblasts require adhesion to the substrate. Lack of the signals induced by adhesion brings about apoptosis. That these do not undergo apoptosis suggests that they are still receiving a signal to survive. Two possible mechanisms for the survival of cells in such flaps are proposed as follows:

1. The tea affects transcription or translation of any of the following proteins:
   a. adhesion proteins, producing proteins that are always in the on-switch mode;
   b. apoptosis-inducing proteins, stopping production or producing degenerate proteins that do not work;
   c. survival proteins, producing products that are more profuse or always active.
2. The tea acts as either a competitive inhibitor or an allosteric inhibitor of any of the above proteins.

When cells enter mitosis they lose their adhesions and round up. After division is complete they regain their adhesions. If very rapid cell division was initiated, there may have been a piling of cells without the development of adequate adhesion in between division times. Another possible explanation for the decreased adhesion seen in this experiment is that the tea may have stimulated rapid cell division and adequate cell adhesion never formed. In this case, adhesion proteins were not affected by the tea, but elements involved in division timing were affected. Such loss of adhesion and rounding up is also characteristic of apoptosis. Apoptotic effects have been reported in green tea (*Camilla senisis*) (Paschka et al, 1998; Li et al, 2000). Undoubtedly, questions arising from the results of this experiment warrant further research.

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