

## Additive Inhibitory Effect of Saffron in Combination with Sodium Selenite on Tumor Cells

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### Abstract

Both saffron and selenium have been reported to exhibit antigenotoxic and cancer chemopreventive activities. We have examined the effects of naturally occurring saffron extracts in combination with sodium selenite on colony formation and nucleic acid synthesis of different human cultured malignant cells. Results of these experiments demonstrated that saffron in combination with sodium selenite displays an additive inhibitory effect on colony formation and nucleic acid synthesis in tumor cells at low concentrations of both agents. These results suggest the potential use of saffron as an anticancer agent, alone or in combination with sodium selenite.

### INTRODUCTION

A growing body of evidence indicates that different plant extracts and synthetic compounds possess antitumor and anticancer effects and can be used as potential cancer chemopreventive agents (Abdullaev, 2001). This has strengthened the rationale for combining cancer chemopreventive agents to target multiple pathways. The process of identifying potential synergistic or additive combinations of chemopreventive agents should be based upon a systematic process of preclinical development in vitro followed by animal model carcinogenesis testing. A major problem in the use of chemopreventive drugs (especially with those of synthetic origin) is their potential toxicity to normal cells. One approach to solving this problem is to employ a combination of different natural and synthetic anticancer agents. Several reports have demonstrated that selenium in combination with different agents, such as alpha-tocopherol, ascorbic acid, interleukin-2, adriamycin, taxol, beta-carotene, vitamins E and C, significantly reduces tumor cell growth in vivo and in vitro (Dias et al., 2000; Vadgama et al., 2000; Thikkurissy et al., 2001; Whanger, 2002;). Furthermore, animal studies have shown that selenium-enriched onions, tea, broccoli, garlic, and Brazilian nuts are very effective in the inhibition of tumors. (Tang et al., 2001; Whanger, 2002; Amantana et al., 2002; Davis et al., 2002). Commercial saffron is produced from dried stigmas of *Crocus sativus* L. and used as a dye in perfume and as a spice for culinary purposes, as well as remedies against numerous health conditions (Abdullaev, 1993). Recent evidence indicates that saffron extracts and their components possess anticarcinogenic (inhibition of chemical carcinogenesis) and antitumor (inhibition of tumor growth) activities in vivo and in vitro (Abdullaev, 2002). Purified characteristic compounds of saffron: crocin, safranal, picrocrocin and  $\beta$ -carotene, inhibited different types of tumor cell growth (Tarantilis et al., 1994). Selenium compounds has been shown to possess anticancer activity in both in vivo and in vitro experimental systems (Combs and Gray, 1998). Recent data have indicated that the complementary supplementation of selenium is useful in the clinical treatment of different types of cancer (Bantzel, 1999). The dose-dependent antimutagenic effect of sodium selenite on acridine orange and 7, 12-dimethylbenzo[a] anthracene (DMBA) was demonstrated in the Ames/*Salmonella* test system (Martin and Adams, et al, 1981).

Additionally, it was shown that sodium selenate and sodium selenite had a weak mutagenic activity in Kada's rec-assay and Ames' *Salmonella* test (Noda et al., 1979). Thus, both saffron (Abdullaev and Frenkel, 1992; Abdullaev, 1993, 2002; Tarantilis et al., 1994; Nair et al., 1995; Abdullaev and González de Mejía, 1995) and selenium (Noda et al., 1979; Martin et al., 1981; Combs and Gray, 1998; Bantzel, 1999) have been reported to exhibit antigenotoxic and cancer chemopreventive activities. The aim of the present study was to examine the cytotoxic, mutagenic and antimutagenic activities of saffron *C. sativus* in combination with sodium selenite. For this purpose, we employed a number of in vitro tests in both human and bacterial cells.

## **MATERIALS AND METHODS**

### **Chemicals**

Sodium selenite was purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (D-MEM) was purchased from Gibco/BRL Products (Grand Island, NY). Fetal bovine serum (FBS) was obtained from HyClone (Road Logan, UT). Culture dishes and flasks were obtained from Costar (Corning, NY). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium selenite ( $\text{Na}_2\text{SeO}_3$ ), was dissolved in distilled water, adjusted to pH=7.4 and added directly to the culture medium. The concentration of  $\text{Na}_2\text{SeO}_3$  was modified depending on the assay as reported in the text.

### **Preparation of the Saffron Extract**

Stigmata of pure saffron (Mancha, Spain) were purchased from a local market and stored in the dark at 4°C. Herbarium vouchers are held in the "Herbario ALBA" (Index Herbariorum New York), University of Castilla-La Mancha, Spain, with number 5994. The chemical analysis of the saffron extract revealed that its characteristic compounds include three main chemical components: 1. the bright yellow coloring carotenoids (the main coloring ingredients of saffron are a water-soluble  $\alpha$ -crocin); 2. a bitter tasting picrocrocin (a glucoside of safranal); and 3. a spicy aroma, safranal (one of the main ingredients responsible for the aroma of saffron, a monoterpene aldehyde). Saffron was found to contain also sugar, alkaloids, amino acids, vitamins A, B, C,  $\text{H}_2\text{O}$ , fixed and volatile oil, wax and ash (Abdullaev, 1993). The concentrated saffron extract was prepared as previously described (Abdullaev and Frenkel, 1992). Briefly, aliquots of saffron (dried whole stigmata) were ground, and then were three times extracted with 75 % aqueous ethanol overnight with magnetic stirring while in the dark. The resulted pooled extracts were centrifuged at 30,000 g for 15 min and filtered through a Whatman filter (Gf/F, 0,7  $\mu\text{m}$ ) to separate the plant residue, which was discarded. Obtained extracts of saffron were lyophilized (24 h) in Fast Freeze and Lyth-Lock Freeze Dry Flask, LABCONCO Co., U.S.A. The saffron extract concentration was modified depending on the assay as reported in the text.

### **Colony Formation Assay**

Cells ( $2 \times 10^5$ ) were seeded in 100-mm Petri dishes with 10 ml D-MEM medium. After 2 days, different concentrations of tested agents were added and incubation continued 1 h for sodium selenite and 3 h for saffron. The cells were then trypsinized (0.05M trypsin), counted and 200 cells were seeded in 60-mm Petri dishes for colony formation determination. At least 3 replicate colony determinations were carried out for each culture. After 10 days of incubation, the resulting colonies were rinsed with 50 mM phosphate buffer, pH 7.6, containing 150 mM NaCl, fixed with methanol, stained with Giemsa (Sigma) and the number of colonies (with diameter > 0.05 mm) per dish were determined as described previously (Abdullaev and Frenkel, 1992).

### **Determination of Nucleic Acid Synthesis**

Cells were grown in monolayer cultures in 35-mm Petri dishes. Saffron extract

and selenite alone and in combination were added at concentrations as indicated in the individual experiments and incubation was continued at 37°C for sodium selenite for 1 h and for saffron, 3 h. DNA and RNA synthesis were monitored by the incorporation of their radioactive precursor (Abdullaev and Frenkel, 1992). [<sup>3</sup>H]-thymidine and [<sup>3</sup>H]-uridine (final concentration = 3μCi/ml) were added to 4 ml medium covering the cell monolayer, while incubation continued for 30 min. To stop radioactivity uptake, the medium was removed and the cells were washed twice with 50 mM phosphate buffer, pH 7.6, containing 150 mM NaCl; then 1 ml 0.1% SDS, containing 10 mM EDTA (pH 7.4) was added to each dish. After 20 min at room temperature, the lysate was poured into a tube and 1 ml of 10% cold trichloroacetic acid (TCA) was added. The resulting precipitate was collected on a Whatman GF/C filter, washed four times with 0.01 N NaCl, dried and counted in a liquid scintillation counter (Beckman LS 6500). To obtain blanks, the cultures were kept on ice for 30 min after the labeled compound was added. All determinations were done in triplicate.

### **Mutagenicity and Antimutagenicity Assays**

Dr. B.N. Ames from the University of California at Berkeley kindly provided the *Salmonella typhimurium* TA98 tester strain. The mutagenic and the antimutagenic activities of the saffron extract and sodium selenite (alone and in combination) were determined using the plate incorporation test (Maron and Ames, 1983) and indirect-acting mutagen: benzo [a] pyrene (BP). BP (10 μg/plate) was chosen as positive control for the antimutagenicity studies, since this dose is not toxic for TA98 strain. 0.5 ml of S9 mixture (10% rat liver S9 fraction, 8 mM MgCl<sub>2</sub>, 33 mM KCl, 4 mM NADP, 5 mM glucose-6-phosphate and 100 mM sodium phosphate buffer (PBS), pH 7.4) were added to the molten top agar before plating. Toxicity of the tested agents was assessed by the observation of the background bacterial growth in the minimal agar plates due to the trace of histidine in the medium. Revertant colonies were counted after a 72-h incubation period at 37°C using a MiniCount colony counter (Biotran II, New Brunswick Scientific, Edison NJ). All determinations were done in triplicate. Because the plate-incorporation reversion test used in our studies does not directly measure possible toxicity of the tested agents, a decrease in reversion could be due to a toxic effect of the agents. To examine this possibility, the background lawn growth on the plates was observed under a microscope when the revertant colonies were counted. No obvious toxic effect of saffron extract or sodium selenite at all concentrations used was noted.

### **Statistical Analysis**

Data were analyzed using Statistical Analysis System software (SAS Institute Inc., Cary, NC 27511, U.S.A. Release 6.02). Values were considered significant when  $p < 0.05$ .

## **RESULTS**

### **Cytotoxic Effect of Saffron Extract and Sodium Selenite Alone and in Combination on Tested Cells**

We have examined the effect of two concentrations (50 and 100 μg/ml) of the saffron extract on colony formation of normal and malignant human cells (CCD-18Lu, HeLa, A-204 and HepG2 cells) in vitro. Results of these experiments (Figure 1) demonstrated that the saffron extract had no effect on normal cells, but inhibited colony formation of all tested malignant cell lines in a dose-dependent manner. A-204 cell line showed the higher sensitivity to the inhibitory effect of saffron extract than the other malignant cell lines tested. The effect of low concentration (10 μM) of sodium selenite on colony formation of normal human cells (CCD-18Lu) and human malignant (HeLa, A-204 and HepG2) cells in vitro was tested. Results (Figure 1) indicated that sodium selenite had no effect on normal cells but inhibited colony formation of malignant cells. HeLa cells were more sensitive to sodium selenite than other tested malignant cells. Figure 1 shows that the treatment of tested tumor cells with saffron extract in combination

with sodium selenite caused an additive inhibitory effect on colony formation, in comparison to the effect of these agents alone at the same concentrations. A more effective inhibition (54%, 80% and 52% for HeLa, A-204 and HepG2 cells, respectively) was found when these tumor cells were treated with the combination of saffron (100 µg/ml) and sodium selenite (10 µM). The effect of the saffron extract and sodium selenite alone and in combination on DNA and RNA synthesis was also examined (Figure 3). Similar to the effects of these agents on colony formation, the combination of saffron with sodium selenite inhibited more effectively nucleic acid (both DNA and RNA) synthesis in all tumor cells tested.

#### **Mutagenic and Antimutagenic Activities of Saffron Extract and Sodium Selenite, Alone and In Combination**

No marked effect of the saffron extract on BP-induced mutagenic activity up to the highest concentration used (1500 µg/plate) was seen (Table 1) in the Ames/*Salmonella* test system. Although all tested concentrations of saffron extract slightly reduced BP mutagenicity, no clear dose-response effect was obtained. Data reveal that increasing the amount of sodium selenite per plate up to 672 µg was dose-dependently effective in reducing the mutagenicity of BP (Table 1). In these experiments 10 µg BP per plate gave the greatest mutagenicity, resulting in 405 revertants per plate. Higher concentration of sodium selenite (672 µg/plate, approx. 44 ppm) induced approximately a 37 % reduction in BP mutagenicity. Previously, it was shown that sodium selenite had no mutagenic effect in the Ames/*Salmonella* test system (16). We also examined the effect of the saffron extract (1500 µg/plate) in combination with different concentrations of sodium selenite on the mutagenicity induced by BP. Results presented in Table 1 indicated that the saffron extract did not have a marked influence on the antimutagenic activity of sodium selenite. A higher concentration of sodium selenite in combination with a higher concentration (1500 µg/plate) of saffron induced approximately a 41 % reduction in the number of revertants, which was almost similar (37.1 %) to effect by sodium selenite (672 µg/plate) alone.

#### **DISCUSSION**

The prospect that the combination of different chemopreventive agents may confer protection against cancer has drawn substantial attention. It was recently reported that the combination of different micronutrients, such as alpha-tocopherol, selenium and ascorbic acid, reduced the incidence of DMBA-induced mammary tumors in rats (Abdullaev and González de Mejía, 1995); interleukin-2 and selenium inhibited the growth of squamous carcinoma cell (Abdullaev, 2001); adriamycin or taxol and selenium caused synergistic inhibition of different lung, prostate, colon small intestine and liver cancer cells in vitro (Abdullaev, 2002). It was also demonstrated that selenium-enriched green tea enhances selenium's antimutagenic effect (Amantana et al., 2002); selenium-enriched broccoli inhibits chemically induced colon tumor development in rats and spontaneous tumorigenesis in Min mice (Bantzel, 1999); selenium-enriched garlic reduces the growth of human gastric carcinoma cells (Combs and Gray, 1998).

Our results indicate that the saffron extract in combination with sodium selenite caused an additive inhibitory effect on tumor cells growth (Figure 1) and nucleic acid synthesis (Figure 2) only in tested malignant cells in vitro, and might be an effective inhibitor of human tumor cells growth in vivo. It is evident from our results that the saffron extract exhibited non-mutagenic and non-antimutagenic (Table 2) effects on BP mutagenicity in the *Salmonella* tester strain TA98. We also observed that sodium selenite itself reduces mutagenicity (Table 1) of BP and this antimutagenic effect is highly dependent on the concentration of selenium. Finally, we demonstrated that the combination of saffron extract with sodium selenite had no marked influence on the antimutagenic effect of selenium against BP. The lack of synergistic effect of saffron in combination with sodium selenite on sodium selenite-induced antimutagenicity may be due to the fact that the saffron itself has no mutagenic or antimutagenic activities. In

conclusion, the present study supports the increasing evidence that naturally occurring saffron extracts in combination with synthetic sodium selenite play an important role in cancer chemoprevention. Additional studies are needed to determine the biological activity of ingredients of saffron, as well as the most effective approach of treatment and concentrations of these natural and synthetic agents and the molecular mechanism(s) of their anticancer activity.

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## Literature Cited

- Abdullaev, F.I. 1993. Biological effects of saffron. *BioFactors* 4: 83-86.
- Abdullaev, F.I. 2001. Plant-derived agents against cancer. In: Gupta, S.K. (ed.). *Pharmacology and Therapeutics in the New Millennium*. Narosa Pub. House, New Delhi, India. pp. 345-354.
- Abdullaev, F.I. 2002. Cancer chemopreventive and tumoricidal properties of saffron (*Crocus sativus* L.). *Exp. Biol. Med.* 247: 20-25.
- Abdullaev, F.I. and Frenkel, G.D. 1992. Effect of saffron on cell colony formation and cellular nucleic acid and protein synthesis. *BioFactors* 3:201-205.
- Abdullaev, F.I. and González de Mejía, E. 1995. Inhibition of colony formation of HeLa cells by naturally occurring and synthetic agents. *BioFactors* 5: 133-138.
- Amantana, A., Santana-Rios, G., Butler, J.A., Xu, M., Whanger, P.D, and Dashwood, H. 2002. Antimutagenic activity of selenium-enriched green tea toward the heterocyclic amine 2-amino-methylimidazo[4,5-f]quinoline. *Biol. Trace Elem. Res.* 86: 177-191.
- Bantzel, J. 1999. Experiences with sodium selenite in treatment of acute and late adverse effects of radio chemotherapy of head-neck carcinomas. *Cytoprotection Working Group in AK Supportive Measures in Oncology within the scope of MASCC and DKG. Med Klin.* 94: 49-53.
- Combs, G.F. and Gray, W.P. 1998. Chemopreventive agents: Selenium. *Pharm. Therapy* 79: 179-192.
- Davis, C.D., Zeng, H. and Finley, J.W. 2002 Selenium-enriched broccoli decreases intestinal tumorigenesis in multiple intestinal neoplasia mice. *J. Nutr.* 132: 307-309.
- Dias, M.F., Sousa, E., Cabrita S., Patricio, J. and Oliveria, C.F. 2000. Chemoprevention of DMBA-induced mammary tumors in rats by a combined regimen of alpha-tocopherol, selenium and ascorbic acid. *Breast J.* 6: 14-19.
- Maron, D.M. and Ames, B.N. 1983. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* 113: 173-215.
- Martin, S.E., Adams, G.H., Schillaci, M. and Milner, J.A. 1981. Antimutagenic effect of selenium on acridine orange and 7,12-dimethylbenz(a)anthracene in the Ames *Salmonella*/microsomal system. *Mutat. Res.* 82: 41-46.
- Nair, S.C., Kurumboor, S.K. and Hasegawa, J.H. 1995. Saffron chemoprevention in biology and medicine: a review. *Cancer Biother.* 10: 257-264.
- Noda, M., Takano, T. and Sakurai, H. 1979. Mutagenic activity of selenium compounds. *Mutat. Res.* 66: 175-179.
- Rockwell, P. and Raw, J. 1979. A mutagenic screening of various herbs, spices and food additives. *Nutr. Cancer* 1: 10-15.
- Tang, F., Zhou, J. and Gu, L. 2001. In vivo and in vitro effects of selenium-enriched garlic on growth of human carcinoma cells. *Zhonghua Zhong Liu Za Zhi* 23: 461-464.
- Tarantilis, P.A., Morjani, H., Polissiou, M. and Manfait, M. 1994. Inhibition of growth and induction of differentiation promyelocytic leukemia (HL-60) by carotenoids from *Crocus sativus* L. *Anticancer Res.* 14: 1913-1918.
- Thikkurissy, S., Pavone, A., Rega, A., Bae, R., Roy, M., Wishe, H.I. and Kiremidjian-Shimacher, L. 2000. Effect of interleukin-2 and selenium on the growth of squamous cell carcinoma cells. *Otolaryngol. Head Neck Surg.* 124: 142-149.
- Vadgama, J.V., Wu, Y., Shen, D., Hsia, S. and Block, J. 2000. Effect of selenium in

combination with adriamycin or taxol on several different cancer cells. *Anticancer Res.* 20(3A): 1391-1414.  
 Whanger, P.D. 2002. Selenocompounds in plants and animals and their biological significance. *J. Am. Coll. Nutr.* 21: 223-232.

## **Tables**

Table 1. Antimutagenicity of saffron extract and sodium selenite, alone and in combination, assayed in the *Salmonella typhimurium* plate incorporation test, with metabolic activation (S<sub>9</sub>mix) and 10 µg per plate of BP.

His <sup>+</sup> REVERTANTS PER PLATE (mean ± SD) <sup>a</sup>	
TA98	
Test agents (µg/plate)	His <sup>+</sup> Revertants/plate (mean ± SD) <sup>a</sup>
None	36±2.3
Saffron extract	
500	347±30
1000	344±30.4
1500	343±32.7
BP (10 µg per plate)	391±19.5
None	45±5.6
Sodium selenite	
173	350±8.3
346	289±22.4
519	269±11.7
672	255±9.7
BP (10 µg per plate)	405±16.2
None	39±8.7
Saffron extract + sodium selenite	
1500+173	310±13.2
1500+346	242±20.1
1500+519	190±11.7
1500+672	186±10.8
BP (10 µg per plate)	315±12.9

<sup>a</sup> Each value represents the mean ± Standard Deviation (SD) of triplicate plates

## Figures

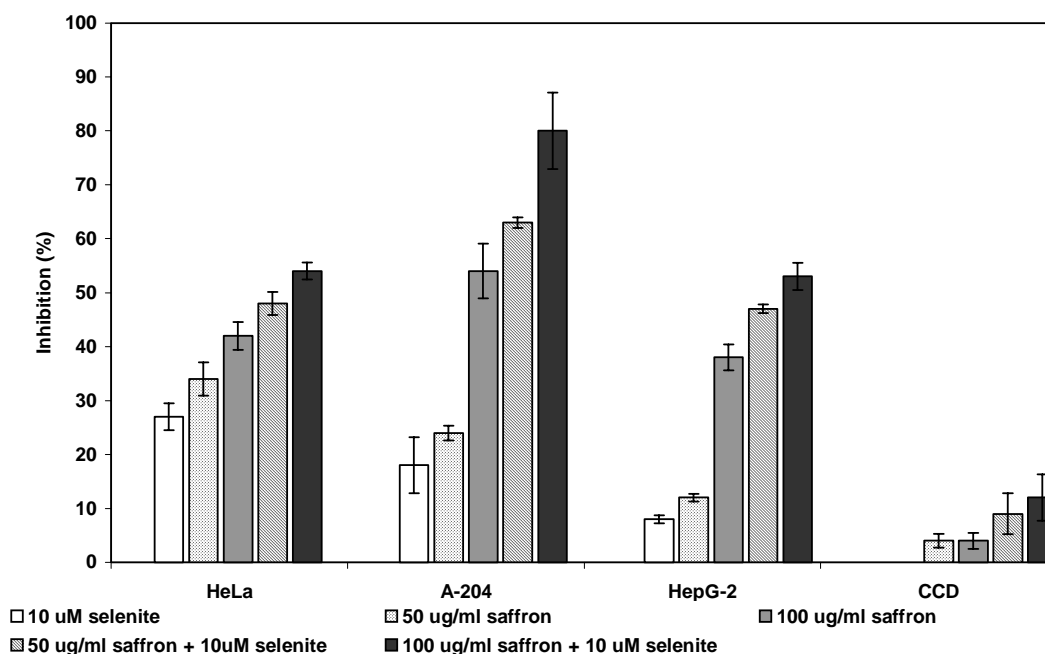


Fig. 1. Inhibitory effect of saffron extract in combination with selenite on colony formation of different malignant cells (%).

Cells [HeLa (A); A-204 (B) and HepG2 (C)] were exposed to the indicated concentrations (alone or in combination) of sodium selenite, for 1 h and of saffron extract, for 3h. The number of cells that were able to form colonies was determined as described in Materials and Methods. Results are presented as the percentage of inhibition of colony formation (colonies/dish formed by untreated cells accepted as 100%; for CCD-18Lu = 27; HeLa cells = 70; for A-204 cells = 65; for HepG2 cells = 58). Data were obtained from the number of colonies in four culture dishes per treatment.  $p < 0.05$

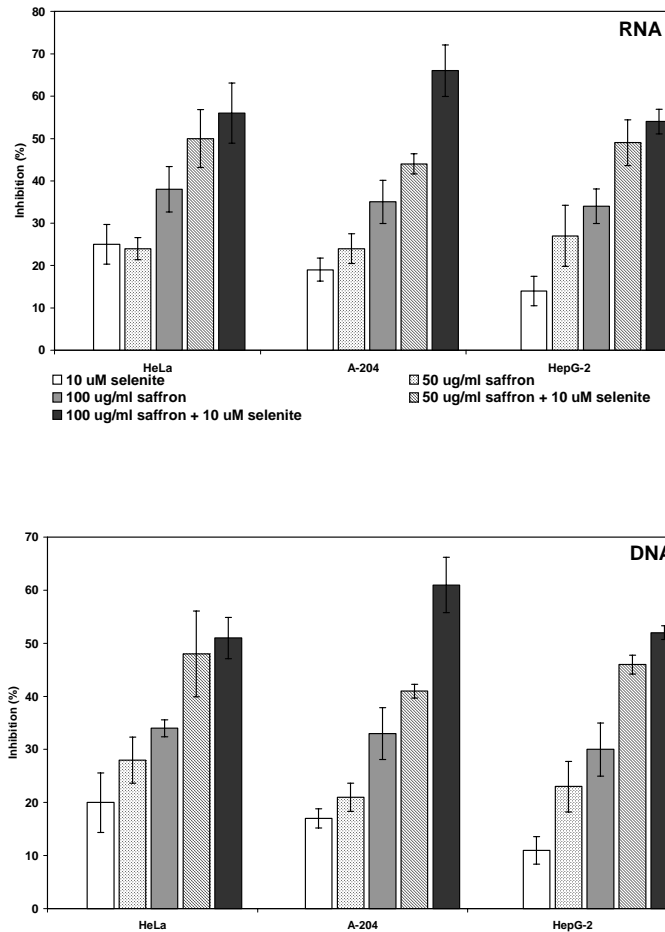


Fig. 2. Inhibitory effect of saffron extract in combination with selenite on nucleic acid synthesis in different malignant cells (%).

Cells [HeLa ; A-204 and HepG2 ] were exposed to the indicated concentrations (alone or in combination) of selenite for 1 h and of saffron extract for 3 h, after which [<sup>3</sup>H]-thymidine or [<sup>3</sup>H]- uridine was added and incubation was continued for 30 min. The radioactivity incorporated into RNA (A) or DNA (B) was then determined as described in Materials and Methods. The results are presented as a percentage of incorporation inhibition related to untreated controls (incorporation in untreated cells accepted as 100%. HeLa cells: for RNA= 42,000 c.p.m., and for DNA = 74,000 c.p.m.; A-204 cells: for RNA = 51,000 c.p.m., and for DNA = 67,000 c.p.m.; HepG2 cells: for RNA = 38,000c.p.m., and for DNA = 54,000 χ.π.μ.)

Δατα ωρε οβταινεδ φρομ τη νυμβερ οφ χολονιεσ ιν φουρ χυλτυρε δισηεσ περ τρεατμεντ. π<0.05