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Genotoxic and anti-genotoxic properties of *Calendula officinalis* extracts in rat liver cell cultures treated with diethylnitrosamine

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Abstract

Calendula officinalis flower extracts are used to cure inflammatory and infectious diseases, for wound healing and even cancer with partial objective evidence of its therapeutic properties or toxic effects, many of which can be attributed to the presence of flavonols. We studied whether *C. officinalis* extracts induce unscheduled DNA synthesis (UDS) in rat liver cell cultures, and if these extracts can reverse diethylnitrosamine (DEN)-induced UDS. Four different flower extracts were prepared: aqueous (AE), aqueous–ethanol (AEE), ethanol (EE) and chloroform (CE). AE and AEE were evaporated to 6.72 and 4.54 mg of solid material per ml, respectively and final ethanol concentration in AEE was 0.8%. EE and CE were dried and resuspended in dimethyl sulfoxide (DMSO) to 19.2 and 10 mg of solid material per ml. Ethanol residue of EE was 0.34%. In the UDS assay in liver cell cultures, DEN at 1.25 μM produced a maximal increase of 40% ^{3}H-thymidine (^{3}HdTT) incorporation, and both, AE and AEE showed complete reversion of the DEN effect at around 50 ng/ml and between 0.4 to 16 ng/ml, respectively. In the absence of DEN, these two polar extracts induced UDS at concentrations of 25 μg for AE and 3.7 μg/ml for AEE to 100 μg/ml in rat liver cell cultures. Concentrations producing genotoxic damage were three orders of magnitude above concentrations that conferred total protection against the DEN effect. Thus, at the lower end, ng/ml concentrations of the two polar extracts AE and AEE conferred total protection against the DEN effect and at the higher end, g/ml concentrations produced genotoxic effects. These results justify the study of *C. officinalis* flower extracts to obtain products with biological activity and to define their genotoxic or chemopreventive properties.

Keywords: Chemoprotection; Unscheduled DNA synthesis; Natural products; Primary liver cell cultures; Polyphenolic plants

1. Introduction

The study of compounds of plant origin has generated great interest in the fields of food and medicine (Barcelo et al., 1996; García-Gasca et al., 1998; Kim et al., 1998). Many of them have chemopreventive properties and in principle they could exert an immediate impact in high-risk cancer populations, since these compounds are proposed in protocols as preventive and even as therapeutic tools (Yuspa, 2000).

Crude extracts have been used in medicinal research of natural products, and they have been the source of many human chemopreventive agents (Hyun et al., 1987). Many of these compounds are present in the diet, and a variety of mechanisms have been described for their chemoprotective properties (Wattenberg, 1992; Rogers et al., 1993; Guengerich, 1995; Barcelo et al., 1996). Further description of new chemical substances with protective or preventive properties against cancer is a goal emerged from studies of the biological effects of natural products.

Abbreviations: AE, aqueous extract; AEE, aqueous–ethanolic extract; CE, chloroformic extract; DEN, diethylnitrosamine; DMSO, dimethyl sulfoxide; EE, ethanolic extract; EGTA, ethylene glycol-bis (β-amino ethyl ether) *N,N,N′,N′*-tetraacetic acid; 3HdTT, 3H-deoxythymidine; HU, hydroxyurea; PCA, perchloric acid; UDS, unscheduled DNA synthesis.

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The antiviral, antitumoral and antimutagenic properties of *Calendula officinalis* have been described (Elias et al., 1990; Pietta et al., 1992; Kalvatchev et al., 1997; Boucard-Maitre et al., 1988). Nevertheless, a genotoxic effect has been reported of a 60% hydroalcoholic flower extract at concentrations from 100 to 1000 g of solids/ml in the mitotic segregation assay of the heterozygous diploid D-30 of *Aspergillus nidulans* (Ramos et al., 1998), although the same extracts were not mutagenic in the Ames assay, at concentrations of 50–5000 μg/plate with *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, either with or without the S9 fraction, and in the mouse bone marrow micronucleus test, doses up to 1 g/kg for 2 days were not genotoxic either.

The hydroalcoholic *C. officinalis* extracts contain saponins, glycosides of sesquiterpene and flavonol glycosides (Yoshikawa et al., 2001). The main flavonols are iorhamnetin, quercetin and kaempferol (Pietta et al., 1992), and they have several biological activities, namely bactericidal, anti-inflammatory, antiviral, antitumoral and antimutagenic (Asad et al., 1998). These effects suggest that flavonols can act as chemopreventive agents in cancer through a variety of mechanisms; it has been reported that they can inhibit mitochondrial succinoxidase (Hodnick et al., 1986), act as biotrasformation enzyme modulators (Eaton, 1996; Zhai et al., 1998), and that they have potent antioxidant properties (Laughton et al., 1989; Asad et al., 1998). Despite these benefits, some reports indicate that flavonoids such as quercetin have pro-oxidant actions and bind to DNA and degrade it, like the known anticancer drugs bleomycin and duanomycin (Laughton et al., 1989). Furthermore, the same flavonols are genotoxic to prokaryotic and eukaryotic cells in circumstances modulated by chemical features, such as pH, high concentration, appearance of antioxidants, presence of transition metals such as Cu^{2+} and metabolism (Duarte-Silva et al., 2000).

These controversial results of the genotoxic or anticarcinogenic action of flavonoids and of the plant extracts that contain them, justify the study to clarify the mechanisms and conditions that mediate their biological effects before considering them therapeutical agents. In the UDS assay, a measurement of inducible DNA repair in rat hepatocyte cultures (Mitchell et al., 1983; Swierenga et al., 1991), the genotoxic and antigenotoxic activity can be studied in parallel, the first, expressed as an increment of ^{3}HdTT incorporation in DNA, and the second expressed as a reversion of the increment of ^{3}HdTT in DNA induced by the known genotoxic carcinogen, diethylnitrosamine (DEN) (Verna et al., 1996).

These precedents prompted us to assay *C. officinalis* extracts for possible genotoxic or anti-genotoxic properties in the UDS assay in primary rat liver cell cultures.

2. Material and methods

2.1. Flower gathering

In February 1998, *C. officinalis* was cultivated in a patch of 100 m^2 at the experimental fields of the Universidad Nacional Autónoma de México (UNAM). During the months of May through August, well-developed flowers were collected, washed with water and dehydrated in an air forced oven at 60 °C in a dark room.

2.2. Flower extracts

2.2.1. Aqueous flower extract (AE)

One liter of deionized water was boiled (at Mexico City altitude), and 15 g of dried flowers were added and left to stand for 15 min. The extract was sterilized by filtration through a membrane with pore size 0.22 μ, and at the same time insoluble materials were removed. One dried aliquot of the filtered extract contained 6.72 mg/ml of solid material.

2.2.2. Aqueous–ethanol flower extract (AEE)

Five g of dried flowers were stirred in 500 ml of 96% ethanol for 24 h in the dark at room temperature, then 500 ml of deionized water were added and agitated again for 72 h. Insoluble material was removed by filtration and extract was evaporated to a final volume of 400 ml. This final extract was sterilized by filtration through a membrane with pore size 0.22 μ. Solid concentration was 4.54 mg/ml and ethanol concentration was 0.8%. Alcohol concentration was determined by gas chromatography HP4890D adapted with a Headspace Sampler HP7694E.

2.2.3. Ethanol flower extract (EE)

To a liter of absolute ethanol, 15 g of dried flowers were added and, avoiding light, it was agitated for 72 h. Insoluble material was removed by filtration and the extract solvent was distilled under reduced pressure to a maximal temperature of 60 °C, until almost dry. The solid material was weighed and dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 19.2 mg/ml, with an ethanol residue of 0.34% that was determined as in AEE.

2.2.4. Chloroform flower extract (CE)

This extract was prepared in the same way as EE, but with chloroform. Concentration of solids was 10 mg/ml and in this case chloroform residue was not determined.

2.3. Reagents

Collagenase IV, ethylene glycol-bis (β-amino ethyl ether) *N,N,N′N′*-tetraacetic acid (EGTA), phenol red,

hydroxyurea (HU), diethylnitrosamine (DEN) and Tris–HCl were obtained from Sigma Chemical Co. DMSO, perchloric acid (PCA) and most of the salts used were from Merck Co. The media and culture reagents were obtained from Gibco BRL. Calf serum was from HyClone Laboratories. Tritiated deoxythymidine ^{3}HdTTP was obtained from Amersham Life Sciences. KCl, NaCl, Na_2HPO_4, Trizma base and $CaCl_2$ were purchased from J. T. Baker. Insulin (U-100) was from Lilly.

2.4. UDS assay in primary hepatocyte culture

Hepatocytes were isolated from male Wistar rats (180–200 g) by the collagenase perfusion method as previously described (Mendoza-Figueroa et al., 1979) and three replicates of 8×10^5 cells were seeded onto 35-mm culture dishes. Cells were cultured in Eagle's medium modified by Dulbecco-Vogt and supplemented with 7% fetal bovine serum and 5 g insulin/ml and placed in a 37 °C humidified incubator in atmosphere of 90% air/10% CO_2.

After 2 h, detached cells were washed out; the dishes were refilled with 1 ml of fresh medium containing 10 μM of a DNA replication inhibitor, HU and incubated for 1 h. The medium was removed and 1 ml of serum-free medium containing 10 μM HU, 5 Ci of ^{3}HdTTP and 10 μl of DMSO were added. DEN was dispensed to final concentrations of 0.625–10 μM per dish. Finally, different concentrations of the four *C. officinalis* extracts were dispensed from 0.2 ng up to 100 μg per dish. Control dishes received the vehicle of the corresponding extract. Hepatocytes were cultured for 4 h.

Cells were processed according to Mendoza-Figueroa et al. (1979) and after 4 h of incubation hepatocytes were harvested. To account for variation in the number of cells between dishes, results were normalized as a function of DNA concentration. For this purpose DNA hydrolysate was obtained as in Leyva and Kelly (1974). Dishes were washed three times with saline solution, cells detached with a rubber policeman, collected and centrifuged in a total volume of 0.8 ml of saline during 5 min at 10,000 rpm. Supernatants were discarded and pellets resuspended in ice-cold, 0.35 ml of 0.01 M Tris–HCl, pH 7.4, and frozen and thawed twice. The lysate was centrifuged at 10,000 rpm for 10 min. The supernatant was removed and the pellet resuspended in 0.35 ml of 0.01 M Tris–HCl, with 0.05 ml of albumin solution, 1 mg/ml. After 30 min in ice, an equal volume of 0.4 M PCA was added and left in the cold for 30 min. Tubes were centrifuged 5 min at 10,000 rpm. The supernatant was discarded and the pellet resuspended in 0.33 ml of 1 M PCA. The suspension was kept at 70 °C for 30 min and centrifuged for 5 min at 10,000 rpm. The supernatant was divided in two: one 0.06-ml aliquot for DNA determination by the diphenylamine method described by Burton (1956) and another 0.25-ml aliquot which was placed in a plastic vial containing 3 ml of Insta-gel (Packard Instrument Co., Inc. Downers Grove, IL, USA). Incorporation of tritiated thymidine in dpm per mg DNA was determined and results were expressed as percent of control incorporation. Differences in the mean of ^{3}HdTT incorporation for each treatment were analyzed by Student's *t*-test and statistical significance defined as $P\leqslant0.05$.

3. Results

Analysis of the spectra of four *C. officinalis* extracts in the UV range showed that the aqueous (AE) and aqueous–ethanol (AEE) extract gave typical spectra of flavonols, with two absorbing bands at 255 nm and at 335 nm (Markham, 1982) (Fig. 1). Genotoxicity was evaluated in primary rat liver cultures treated with extracts. Only the polar extracts AE and AEE, at high concentration, from 50 and 3.7 μg/ml, respectively, to 100 μg/ml, induced dose–response DNA repair as an incorporation of ^{3}HdTT to DNA, approximately 40% above the control value (Fig. 2); in contrast, at low concentrations of approximately 0.8 μg/ml, AE showed incorporation of ^{3}HdTT below control values. To determine the anti-genotoxic properties of the extracts, a DEN dose–response curve in rat liver cell cultures was assayed. As shown in Fig. 3, the response of the liver cell cultures to different concentrations of DEN

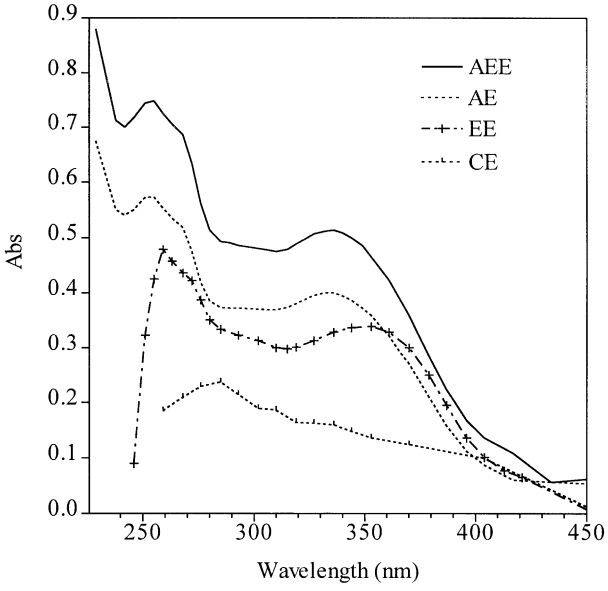

Fig. 1. UV spectra of *C. officinalis* flower extracts. Concentration of four extracts, AE = aqueous, AEE = aqueous–ethanol, EE = ethanol and CE = chloroform was adjusted to 225 μg of solid material per ml of solvent. AE and AEE were dissolved in water and EE and CE in DMSO. Extract solutions were adjusted with their respective solvents. These solutions did not absorb at wavelengths between 400 and 800 nm.

increased proportionally until the dose was 1.25 μM, when maximal UDS effects occurred and produced a ^{3}H dTT incorporation 40% above control values. Taking into account the results of UDS, 1.25 μM DEN was used to test extract anti-genotoxicity. Fig. 4 indicates the anti-genotoxic effect of the *C. officinalis* flower extracts; here again the extracts obtained with polar solvents AE and AEE are the active extracts. The AEE protects 70% of the DEN genotoxic damage even at 0.2 ng/ml, and this protection is maintained up to 0.2 μg/ml, and at approximately 0.4 ng/ml the reversion of DEN-induced UDS is complete. A similar effect is observed with the aqueous extract; it is anti-genotoxic at low concentration and approximately at 0.05 μg/ml, DEN-induced genotoxicity is abolished. Likewise, ethanol extracts showed a similar effect approximately at the same concentration; however, differences with respect to DEN-treated values are not statistically significant. The chloroform extract had slight anti-genotoxic activity at 6.25 and 50 μg/ml. The striking features shown in Figs. 2 and 4 are: (a) ethanol or chloroform extracts are not genotoxic even at concentrations of 50 μg of solid material per ml and they partially protect liver cells of DEN-induced UDS; (b) aqueous or AEE extracts, at ng of solid material per ml concentrations, protect completely against DEN-induced UDS; however,

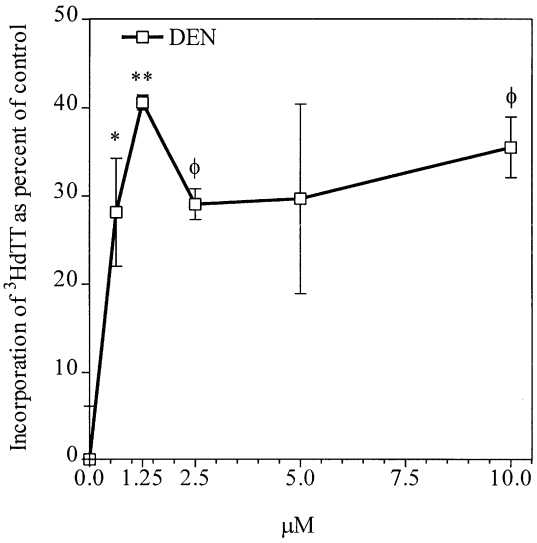

Fig. 3. Evaluation of DEN genotoxic effect on primary rat liver cell cultures. 35 mm-culture dishes were added with 8×10^5 hepatocytes. They were incubated for 1 h with 10 μM HU. The medium was removed and 1 ml of a serum-free medium containing 10 μM HU, 5 μCi of ^{3}HdTT and 10 μl of DMSO and different concentrations of DEN were added and incubated for 4 h. Incorporation of ^{3}HdTT was estimated as dpm/μg of DNA and results are expressed as percent of control samples. Each concentration was assayed by triplicate, mean±S.D. significantly different from control sample values are shown. $P\leqslant0.05$ *, 0.01$^\Phi$ and 0.005**.

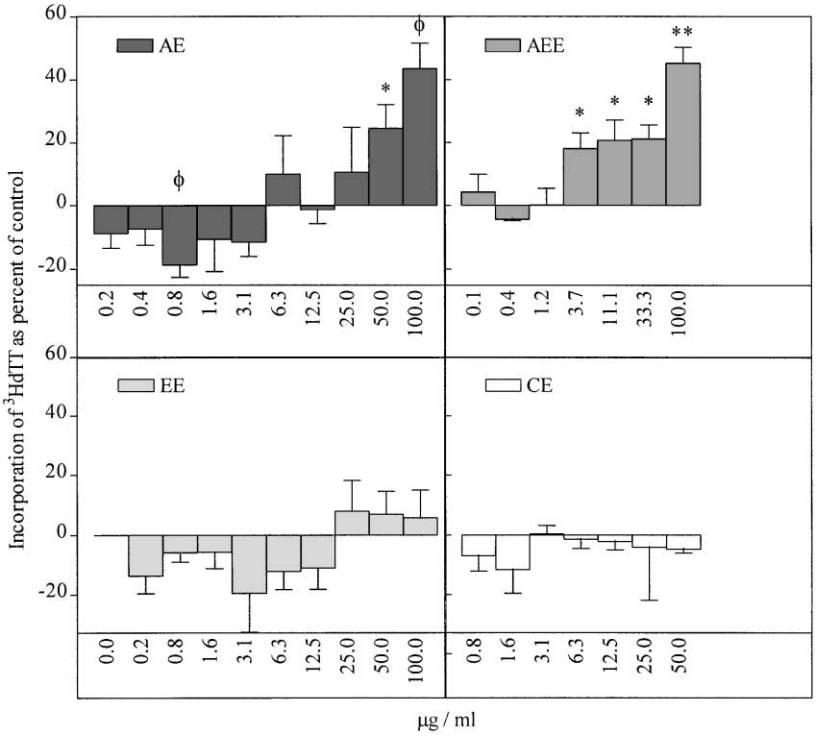

Fig. 2. Evaluation of the genotoxic effects of *C. officinalis* flower extracts on primary rat liver cell cultures. 35 mm-culture dishes were added with 8×10^5 hepatocytes. They were incubated for 1 h with 10 μM hydroxyurea (HU). The medium was removed and 1 ml of a serum free-medium containing 10 μM HU, 5 Ci of ^{3}HdTT and different concentrations of a given flower extract in 10 μl of DMSO were added and incubated for 4 h. Incorporation of ^{3}HdTT was estimated as dpm/g of DNA and results are expressed as percent of control samples. Each concentration was assayed by triplicate, mean±S.D. significantly different from control sample values are shown. $P\leqslant0.05$*, 0.01$^\Phi$ and 0.005**.

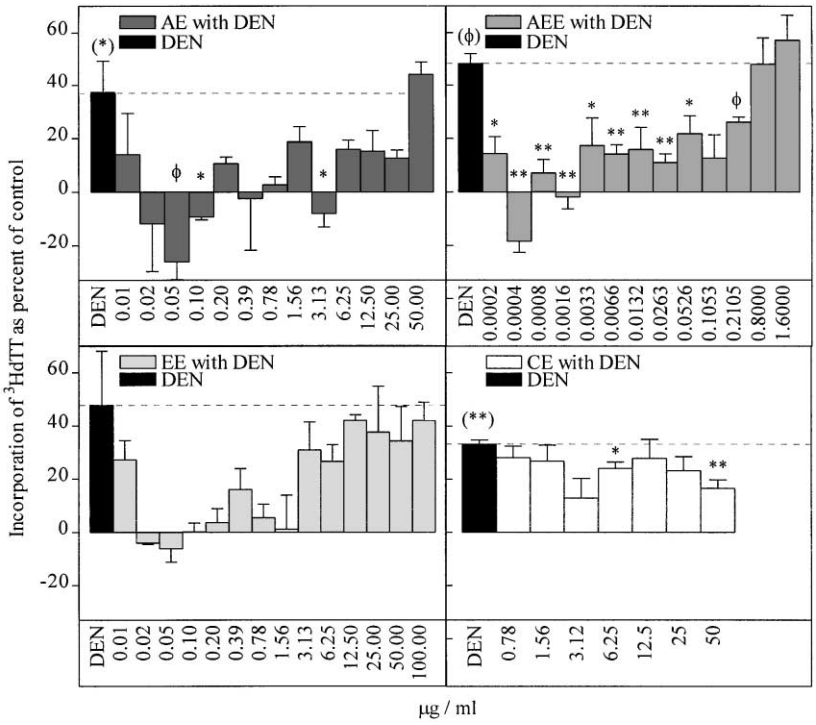

Fig. 4. Evaluation of anti-genotoxic effects of *C. officinalis* flower extracts on primary rat liver cell cultures treated with DEN. 35 mm-culture dishes were added with 8×10^5 hepatocytes. They were incubated for 1 h with 10 μM HU. The medium was removed and 1 ml of a serum-free medium containing 10 μM HU, 5 Ci of ^{3}HdTT, DEN to a final concentration of 1.25 μM and different concentrations of a given flower extract in 10 μl of DMSO were added and incubated for 4 h. Incorporation of ^{3}HdTT was estimated as dpm/μg of DNA and results are expressed as percent of control. The dotted line represents thymidine incorporation induced by 1.25 μM DEN. Each concentration was assayed by triplicate, mean±S.D. significantly different from DEN sample value are shown. $P\leqslant0.05$*, 0.01^{Φ} and 0.005**. As indicated, DEN values are significantly different from control values as shown. $P\leqslant0.05$ (*), $0.01^{(\phi)}$ and 0.005(**).

concentrations three orders of magnitude higher are genotoxic.

4. Discussion

Epidemiological studies have shown that frequent consumption of fruits and vegetables is associated with low risks of various cancers (Wattenberg, 1992). This protective effect has been attributed in part to flavonoids, through modulation of several enzymes of the P450 family involved in precarcinogen metabolism (Zhai et al., 1998). The hydroalcoholic extracts of *C. officinalis* contain saponins, triterpenes and flavonols; for this reason, it is not surprising that extracts of this polyphenolic plant have been used for multiple medicinal purposes in folk therapy (Ramos et al., 1998), included as antitumorals (Boucaud-Maitre et al., 1988). We found that polar aqueous and aqueous-ethanol *C. officinalis* flower extracts at nanogram (ng/ml) concentrations are capable of protecting against the genotoxic effects of the known carcinogen DEN. We propose that this effect is caused by flavonols. Congruent with this assumption is that our two polar extracts showed the typical spectra of flavonols and these compounds have been isolated from hydroalcoholic extracts (Pietta et al., 1992). Evaluation of the putative genotoxicity of extracts without DEN showed that the same extracts at microgram (μg/ml) concentrations; three orders of magnitude above the protective concentration were, in fact, genotoxic. Similar controversial effects have been shown in flavonols such as quercetin, which acts as an antioxidant and at higher concentrations can act as prooxidant (Laughton et al., 1989). Duarte-Silva et al. (2000), found that flavonols with a structure similar to quercetin are genotoxic to prokaryotic and eukaryotic cells and this depends on their auto-oxidative ability that is promoted by alkaline pH and by the metabolic system. Altogether, these results indicate that the active compounds of the *C. officinalis* polar extracts are flavonols; however, we cannot exclude a possible synergistic or antagonistic effect of flavonols with other compounds in the extracts. It would be interesting to verify the protective effects of extracts in a more complex assay such as the inhibition of preneoplastic nodule induction in some model of hepatocarcinogenesis, as has been done with other plant extracts (Ramírez-Mares et al., 1999). In short, our results show a clear anti-genotoxic effect at low concentrations of two *C. officinalis* extracts that contain flavonols, and genotoxic effects of

the same extracts at high concentrations. These opposite effects at different extract concentrations is a feature that should be defined to consider plant polyphenols as therapeutic agents.

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References

Asad, D.F., Singh, S., Ahmad, A., Hadi, S.M., 1998. Flavonoids: antioxidant in diet and potential anticancer agent. Medical Science Research 26, 723–728.

Barcelo, S., Gardiner, J.M., Gesher, A., Chipman, J.K., 1996. CYP2E1-mediated mechanism of antigenotoxity of the broccoli constituent sulforaphane. Carcinogenesis 17, 277–282.

Boucaud-Maitre, Y., Algernon, O., Raynaud, J., 1988. Cytotoxic and antitumoral activity of *Calendula officinalis* extracts. Pharmazie 43, 220–221.

Burton, K., 1956. A study of the conditions and mechanism of the diphenylamine reaction for colorimetric estimation of deoxyribonucleic acid. Journal of Biochemistry 62, 315–319.

Duarte-Silva, I., Gaspar, J., Gomes-da-Costa, G., Rodríguez, A.S., Laires, A., Rueff, J., 2000. Chemical features of flavonols affecting their genotoxicity. Potential implications in their use as therapeutical agents. Chemico–Biological Interactions 124, 29–51.

Eaton, E.A., 1996. Flavonoids, potent inhibitors of the human P-form phenolsulfotransferase: Potential role in drug metabolism and chemoprevention. Drug Metabolism and Disposition 24, 232–237.

Elias, R., De Méo, M., Vidal-Ollivier, E., Laget, M., Balansard, G., Dumenil, G., 1990. Antimutagenic activity of some saponins isolated from *Calendula officinalis* L., *C. arvensis* L. and *Hedera helix* L. Mutagenesis 5, 327–331.

García-Gasca, T., Fattel, S., Villa-Treviño, S., González de Mejía, F., 1998. Effect of carotenoids against genotoxicity of diethylnitrosamine on rat hepatocytes. Toxicology in Vitro 12, 691–698.

Guengerich, F.P., 1995. Influence of nutrients and other dietary materials on cytochrome P-450 enzymes. American Journal Clinical Nutrition 61, 651s–658s.

Hodnick, W.F., Kung, F.S., Roettger, W.J., Bohmont, C.W., Pardini, R.S., 1986. Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. Biochemical Pharmacology 35, 2345–2357.

Hyun, K.L., Young, K.K., Young-Hwa, K., Jung, K.R., 1987. Effect of bacterial growth-inhibiting ingredients on the Ames mutagenicity of medicinal herbs. Mutation Research 192, 99–104.

Kalvatchev, Z., Walder, R., Garzaro, D., 1997. Anti-HIV activity of extracts from *Calendula officinalis* flowers. Biomedical Pharmacotherapy 51, 176–180.

Kim, J.M., Araki, S., Kim, D.J., Park, C.B., Takasuka, N., Baba-Toriyama, H., Ota, T., Nir, Z., Khachik, F., Shimidzu, N., Tanaka, Y., Osawa, T., Uraji, T., Murakoshi, M., Nishino, H., Hiroyuki, T., 1998. Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation. Carcinogenesis 19, 81–85.

Laughton, M.J., Halliwell, B., Evans, P.J., Hoult, J.R., 1989. Antioxidant and pro-oxidant actions of plant phenolics quercetin, gossypol and myricetin. Effects on lipid peroxidation, hidroxil radical generation and bleomycin dependant DNA damage. Biochemical Pharmacology 38, 2859–2865

Leyva, A., Kelly, W.N., 1974. Measurement of DNA in cultured human cells. Analytical Biochemistry 62, 173–179.

Markham, K.R., 1982. Techniques of Flavonoid Identification. Academic Press, London. (Chapter 3).

Mendoza-Figueroa, T., López-Revilla, R., Villa-Treviño, S., 1979. Dose-dependent DNA ruptures induced by the procarcinogen dimethylnitrosamine on primary rat liver cultures. Cancer Research 39, 3254–3257.

Mitchell, A.D., Casciano, M.L., Meltz, M.L., Robinson, D.E., San, R.H.C., Williams, G.M., 1983. Von Halle, E.S., Unscheduled DNA synthesis test a report of the US. Environmental Protection Agency Gene-Tox Program Mutation Research. 123, 363–410.

Pietta, P., Bruno, A., Mauri, P., Rava, A., 1992. Separation of flavonol-2-O-glycosides from *Calendula officinalis* and *Sambucus nigra* by high-performance liquid and micellar electrokinetic capillary chromatography. Journal of Chromatography 593, 165–170.

Ramírez-Mares, M.V., Fattel, S., Villa-Treviño, S., González de Mejía, E., 1999. Protective properties of extract from leaves of *Ardisia compressa* against benomyl-induced cytotoxicity and genotoxicity in cultured rat hepatocytes. Toxicology in Vitro 13, 889–896.

Ramos, A., Edreira, A., Vizoso, A., Betancourt, J., López, M., Decalo, M., 1998. Genotoxicity of an extract of *Calendula officinalis* L. Journal of Ethnopharmacology 61, 49–55.

Rogers, A.E., Zeisel, S.H., Groopman, J., 1993. Diet and carcinogenesis. Carcinogenesis 14, 2205–2221.

Swierenga, S., Bradlaw, J.A., Brillinger, R.L., Gilman, J.P., Nestmann, E.R., San, R.C., 1991. Recommended protocols based on a survey of current practice in genotoxicity testing laboratories: unscheduled DNA synthesis assay in rat hepatocyte cultures. Mutation Research 246, 235–253.

Verna, L., Whysner, J., Williams, G.M., 1996. N-Nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacology Therapy 71, 57–81.

Wattenberg, L., 1992. Inhibition of carcinogenesis by minor dietary constituents. Cancer Research 52, 2085s–2091s.

Yuspa, S.H., 2000. Overview of carcinogenesis: past present and future. Carcinogenesis 21, 341–344.

Yoshikawa, M., Murakami, T., Kishi, A., Kageura, T., Matsuda, H., 2001. Medicinal flowers III. Marigold. (1): hypoglycemic, gastric emptying inhibitory, and gastroprotective principles and new oleanane-type triterpene oligoglycosides, calendasaponins A, B, C, and D, from Egyptian *Calendula officinalis*. Chemical and Pharmaceutical Bulletin, (Tokyo) 49, 863–870.

Zhai, S., Dai, R., Friedman, F.K., Vestal, R.E., 1998. Comparative inhibition of human cytochromes P450 1A1 and 1A2 by flavonoids. Drug Metabolism and Disposition 26, 989–992.