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A Dual and Opposite Effect of *Calendula officinalis* Flower Extract: Chemoprotector and Promoter in a Rat Hepatocarcinogenesis Model

Original Paper

Abstract

Calendula officinalis extracts have protective and cytotoxic effects. We previously reported the dual activity of *C. officinalis* in primary rat hepatocyte cultures treated with *N*-nitrosodiethylamine. At nM concentrations it was anti-genotoxic while at μM concentrations it exhibited genotoxic effects. Here we tested the activity of *Calendula officinalis in vivo* in male Fischer 344 rats initiated with *N*-nitrosodiethylamine, promoted with 2-acetylaminofluorene, and 70% partially hepatectomized. Liver γ-glutamyltranspeptidase positively altered hepatocyte foci 25 days after initiation were our end point. The protective effect of *C. officinalis* started at 0.1 mg/kg concentration, increased at 0.5 mg/kg and reached its maximum at 2.5 mg/kg, when it decreased the area and number of altered foci by 55% and 49%, respectively, in comparison with rats treated only with carcinogen. At 5 mg/kg the number and area of altered hepatocyte foci were still lower, but almost reached the figures of carcinogen-treated rats. Ten and 20 mg/kg doses produced a notorious increment in the area and number of altered hepatic foci, and at 40 mg/kg of extract the increment was 40% and 53%, respectively. Additionally, when 2-acetylaminofluorene was substituted by a 40 mg/kg *C. officinalis* extract, a promoting effect was observed with increments of 175% and 266% in area and number of altered hepatocyte foci with respect to controls. When *N*-nitrosodiethylamine was substituted by 40 mg/kg of extract, the latter did not show initiator activity. In summary, we showed a protecting activity of *C. officinalis* at low doses, but doses above 10 mg/kg increased altered hepatocyte foci. This dual effect is an example of the phenomenon of hormesis. Furthermore, 40 mg/kg of dry weight extract administered instead of 2-acetylaminofluorene induced a clear promoting activity. These *in vivo* results are similar and consistent with those reported by us in primary rat liver cell cultures.

Key words
Antigenotoxicity · genotoxicity · promoter · chemopreventive effect · hepatocarcinogenesis · *Calendula officinalis* · hormesis

Abbreviations

DDT:	1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane
DEN:	N-diethylnitrosamine
AHF:	altered hepatocytes foci
RH:	resistant hepatocyte model
AEE:	Aqueous-ethanol extract
GGT:	γ-glutamyltranspeptidase
2-AAF:	2-acetylaminofluorene
PH:	partial hepatectomy

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Introduction

The effect of fruits and vegetables on reducing the risk of cancer [1] has generated great interest in the fields of food and medicine [2]. Traditional medicine is largely centered on the consumption of plants such as *Arnica montana*, *Chamomilla matricans*, *Hypericum perfoliatum*, *Calendula officinalis*, and their derivatives. Natural products containing powerful chemicals with antitumoral activity have been described, as in green or black tea [3], [4]. In spite of this information it should not be excluded that some plant extracts used as antitumorals can also present cytotoxic activity [5], [6].

C. officinalis is an annual herb from the Compositae family, 50 to 70 cm tall, with daisy-like flowers with large yellow-orange petals that close at night, and is a native of the Mediterranean region. In Europe and America it is cultivated for ornamental and medicinal purposes. The plant has been traditionally employed in folk therapy [7]. More than 35 properties have been attributed to the flower decoctions and tinctures, e.g., choleretic, anti-inflammatory, analgesic, antitumoral, antiulcer, bactericide, diuretic, tonic and useful in wound-healing and skin eruptions. Antibacterial and anti-inflammatory actions have been confirmed by experimental pharmacology [8], [9]. On the other hand, extracts of leaves, flowers and the whole plant have also been found to be cytotoxic to cell lines [5]. However, mutagenicity testing with the *Salmonella* microsome assay and the mouse marrow micronucleus test were negative [10].

Hydroalcoholic *C. officinalis* extracts contain sugars, carotenoides, phenolic acids, sterols, saponins, flavonoids, resins, sterins, quinines, mucilages, vitamins, polyprenylquinones and essential oils. The main flavonols in *C. officinalis* are isorhamnetin, quercetin and kaempferol [11] and these active principles are known to have different biological activities such as bactericidal, anti-inflammatory, antiviral, antitumoral and antimutagenic [12]. There are also reports that some flavonols like quercetin can present both antioxidant and pro-oxidant activities [13].

However, an opposite effect can be produced with *C. officinalis* extracts depending on the administered dose. This phenomenon, called hormesis, has been typified for several drugs and seems to be of great pharmacological importance. It occurs when the same drug produces stimulation at low and inhibition at high doses. Graphics of this phenomenon adopt an inverted "U" or "J" shape depending on the endpoint [14]. Hormetic phenomena have been the object of systematic research; e.g., 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT) [15] and phenobarbital at low doses decrease and at high doses increase preneoplastic lesions in liver [16]; acetylsalicylic acid at low doses increases and at high doses decreases blood clotting [17]; some antibiotics increase bacterial proliferation at low doses and decrease it at high doses [18].

In previous work we reported genotoxic and anti-genotoxic properties of *C. officinalis* extracts in unscheduled DNA synthesis in rat liver cell cultures induced with *N*-diethylnitrosamine (DEN). At the lower end, ng/mL concentrations of extracts conferred total protection against the effect of DEN and, at the higher end, μg/mL doses produced genotoxic effects [19]. These controversial effects of *C. officinalis* extract justified the study *in vivo,* and for this purpose we chose to investigate its effects on the induction of altered hepatocyte foci (AHF) in the resistant hepatocyte (RH) model in rat.

Materials and Methods

Animals

Male Fischer 344 rats, weighing between 180 – 200 g, were obtained from the Production and Experimentation Unit of Laboratory Animals (UPEAL-CINVESTAV, México D.F, Mexico) and had access to food and water ad libitum. During treatment rats were transferred to the holding room, under controlled conditions of temperature and light. All animals received humane care and the study protocols were in compliance with the institutional guidelines for use of laboratory animals.

Experimental protocol

We followed the modified RH model protocol with the variant proposed by Carrasco et al. [20]. This model presents the following features: a) AHF are generated rapidly and initiator dose-dependently, b) our laboratory records show that all animals sacrificed 40 weeks or more after initiation presented liver tumors; both AHF and tumors expressed the positive marker γ-glutamyltranspeptidase (GGT). Rats were initiated with 200 mg/kg of (Sigma-Aldrich) DEN in a single *i.p.* dose. Three doses of 20 mg/kg of the promoter (Sigma-Aldrich) 2-acetylaminofluorene (2-AAF) dissolved in dimethyl sulfoxide and mixed in a 1% aqueous solution of carboxymethylcellulose to a final concentration of 10 mg/mL were subsequently administered. 2-AAF was administered at days 7, 8 and 9. At day 10 the proliferative stimulus of a partial hepatectomy was performed. Animals were killed 25 days after DEN administration, livers excised, sliced, immersed in methyl-isobutane, quickly frozen in liquid nitrogen and stored at –80 °C until analysis.

Aqueous-ethanol extract (AEE) of flowers

An AEE of flowers was prepared as previously described. Briefly, *C. officinalis* was cultivated in a patch of 100 m^2 at the experimental fields of the National Autonomous University of Mexico (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. Five g of dried flowers were stirred for 24 h in 500 mL of 96% ethanol, in the dark at room temperature. Then, 500 mL of deionized water were added and the mixture was stirred again for 72 h. Insoluble material was removed by filtration and the extract was evaporated to a final volume of 400 mL. This final extract was sterilized by filtration through a membrane of 0.22 μ pore size. The solid concentration was 4.54 mg/mL and the ethanol concentration was 0.8%. The alcohol concentration was determined by gas chromatography using an HP4890D adapted with a Headspace Sampler HP7694E [19].

To establish the extract dose per rat we assumed that one person of 70 kg takes 1 L of *C. officinalis* per day. This infusion contains a total of 1.8 g of solid material. From these data we extrapolated the dose to a 200 g rat. Initially we investigated the effect of a 20 mg/kg/day dose of AEE on the induction of AHF. Results ob-

served with this dose were the starting point to define further doses. To investigate the effect of AEE taking into account the above considerations, it was orally administered 7 days before initiation, at doses of, 0.1, 0.5, 2.5, 5, 10, 20 or 40 mg/kg. To test the effect of the extract as initiator it was administered in substitution of DEN for the first 7 days. To test its promoting activity in substitution of 2-AAF, the extract was administered during 18 days starting at day 7; in both cases the dose was 40 mg/kg. Enough AEE was dissolved in water depending on the dose needed, knowing that *ad libitum* administration will allow each rat to consume 25 mL of water per day.

Histochemical GGT staining analysis

Histological liver sections of 15 μm thickness were obtained with a cryostat (Slee cryostat MTC, Germany) fixed in absolute ethanol during 5 min at 4 °C and stained according to Rutenburg [21]. γ-Glutamyl-4-methoxy-2-naphthylamine (GMNA) at a ratio of 1 : 20 with 25 mM Tris, pH 7.4, 0.5 mg/mL glycyl-glycine and 4-benzoylamino-2,5-diethoxybenzene-diazonium chloride hemi (zinc chloride) salt (fast blue bb salt) were added to sections and the reaction maintained during 30 min. Next, the slides were washed with phosphate buffer solution and added with 0.1 M cupric sulfate solution for 2 min. Finally, images of the GGT-positive foci were captured with a digital camera (Color View 12, Soft imaging System GmbH) and preneoplastic lesions were quantified with image analysis software (Analysis Soft Imaging System GmbH). Three slices were randomly selected from each rat liver and analyzed.

Statistical analysis

Area and number of GGT positive foci or AHF of the groups treated with *C. officinalis* AEE were compared with the complete carcinogenic treatment group. Results were analyzed by Student's test. Differences were considered statistically significant at $p < 0.05$.

Results

The first negative control group of rats did not receive treatment, the second negative control group only underwent partial hepatectomy (PH) and the third group only received AEE extract at 0.1, 0.5, 2.5, 5, 10, 20 and 40 mg/kg during 32 days and later underwent PH. The liver sections of these three control groups did not present AHF (Figs. **1a** – **c**). In a positive group for reference, rats received the complete treatment, DEN, 2-AAF and HP and a parallel group received the carcinogenic treatment plus the vehicle. The area and number of the AHF in the positive group for reference were 5.5% and 17.5 AHF/cm^2, respectively, in the RH model (Fig. **1d**). The complete carcinogenic treatment with the AEE vehicle did not change the results (Fig. **1e**).

When rats treated with the carcinogens received an additional 0.1 mg/kg/rat/day of AEE, the area and number of AHF decreased from 5.5% of the liver area to 3.3% and from 17.5 to 12.5 AHF/cm^2, respectively. When the extract was administered at 0.5 mg/kg/rat/day of AEE, the area and number of AHF further decreased to 2.9% and to 16.8 AHF/cm^2, respectively. This represented a reduction of 47% of the area with respect to the RH model. When the extract was administered at 2.5 mg/kg/rat/day, the area and number of AHF decreased even more, to 2.5% and 8.9 AHF/cm^2, respectively. This amounted to the greatest reduction: 55% of the area and 49% of the number of AHF with respect to the RH model. When the extract was administered at 5 and 10 mg/kg/rat/day, no significant changes occurred compared to the reference group. Livers of rats administered 20 mg/kg/rat/day showed an important increment in the area and number of AHF; the area increased from 5.5% to 8.03% and the number from 17.5 to 24.8 AHF/cm^2, this was an increment of 46% and 42%, respectively; at 40 mg/kg/rat/day the area was 7.7% with 26.7 AHF/cm^2, which represent a 40% and 53% increment with respect to the reference group (Fig. **2**).

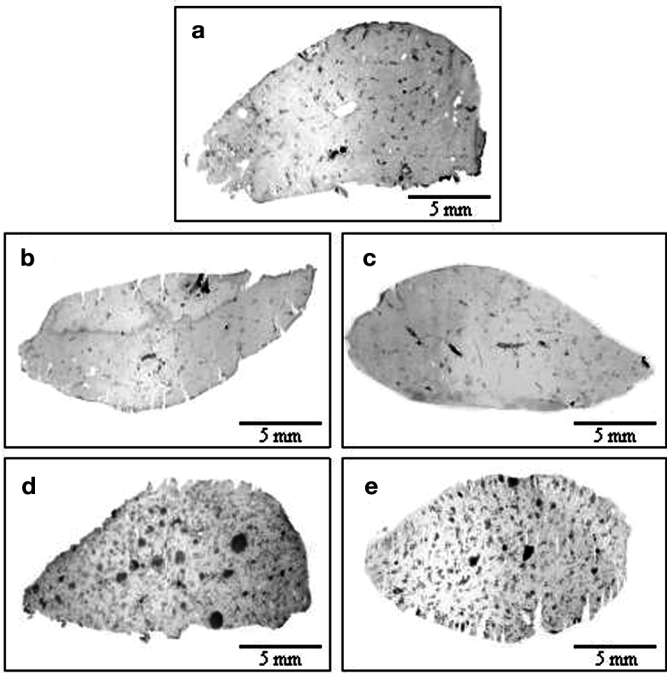
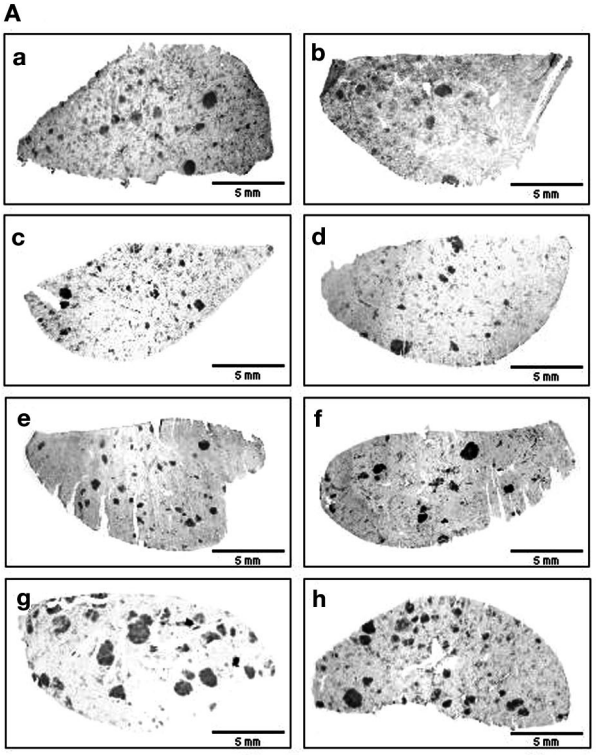


Fig. **1** Positive and negative controls. **a**, **b** and **c** Histochemical rat liver sections from negative controls: **a** rats without treatment (n = 4), **b** rats with only PH (n = 4) and **c**) rats with PH and *Calendula officinalis* extract 40 mg/kg during 32 days (n = 4). **d** and **e** Histochemical rat liver sections from positive controls: **d** rats received DEN, 2 AAF and PH (n = 9) and **e** rats as in **d** plus vehicle (n = 9). In **a**, **b** and **c**, there were no preneoplastic lesions. **d** Preneoplastic lesions, 5.5% of area and 17.5 of AHF/cm^2. Results in **e** are similar to **d**.

When the AEE extract was tested as initiator, at 40 mg/kg/rat/day instead of DEN, rat livers did not show AHF. The livers of rats, which only received DEN, PH and vehicle during 18 days starting from seven day, showed 0.8% of affected area and 6.8 AHF/cm^2. Nevertheless, when rats received 40 mg/kg/rat/day of extract during 18 days instead of 2 AAF, the livers showed 2.2% of affected area and 24.9 AHF/cm^2. These results represent an increment of 175% and 266%, respectively. The p values were 0.01 and 0.006, respectively (Fig. **3**).

Discussion

Calendula officinalis is a plant used in traditional medicine and it has been shown to have antitumoral, anti-inflammatory, healing and antiseptic properties. Of these properties: the antiviral, anti-



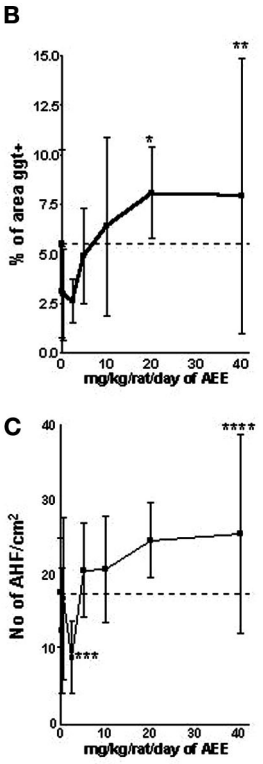


Fig. **2** Effects of *Calendula officinalis* extract on the induction of liver gamma glutamyl transpeptidase+ altered hepatocytes foci of rats treated with carcinogens. **A** Representative GGT stained rat liver sections. All rats received DEN, 2 AAF and PH. Additionally *Calendula officinalis* extract was administered at different doses during 32 days. **a** without *C. officinalis*, **b** 0.1 mg/kg, **c** 0.5 mg/kg, **d** 2.5 mg/kg, **e** 5 mg/kg and **f** 10 mg/kg, **g** 20 mg/kg and **h** 40 mg/kg. **B** Effect of *Calendula officinalis* extract on area ggt+ at different doses: without *C. officinalis*, 5.51%; with 0.1, 3.3%; with 0.5, 2.9%; with 2.5, 2.5%; with 5, 4.8%; with 10, 6.3%; with 20, 8.0%; and with 40, 7.8%. **C** Effect of *Calendula officinalis* extract on the number of altered hepatocyte foci/cm^2 at different doses: without *C. officinalis* 17.5; with 0.1, 14.2; with 0.5, 16.8; with 2.5, 8.9; with 5, 20.6; with 10, 20.7; with 20, 24.6 and with 40, 26.9. The dotted line shows the control group, those rats didn't receive AEE. P value < 0.05: * 20 versus 0.1, 0.5 and 2.5; ** 40 versus 2.5; *** 2.5 versus without *C. officinalis*, 5, 10,40; **** 40 versus 0.1.

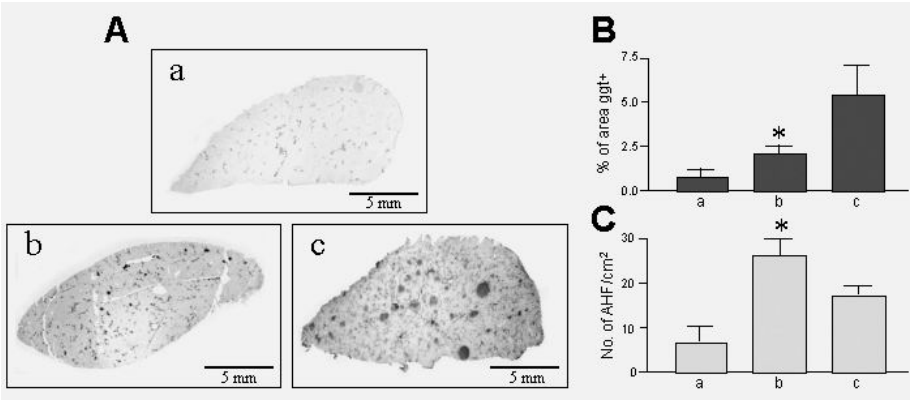


Fig. **3** Effects of *Calendula officinalis* extract as promoter on the induction of liver gamma glutamyl transpeptidase+ altered hepatocytes foci. **A** Histochemical slides of rat liver ggt+ **a** Rats received DEN, vehicle and HP (n = 5). **b** The rats received DEN, *Calendula officinalis* extract 40 mg/kg during 18 days in place of 2 AAF and HP (n = 10). **c** The rats received DEN, 2 AAF and HP (n = 9). **B** Effect of *Calendula officinalis* extract on area ggt+: **a** 0.86%, **b** 2.22% and **c** 5.51%. **C** Effect of *Calendula officinalis* extract on the number of altered hepatocyte foci (AHF)/cm^2: **a** 6.8, **b** 24.9 and **c** 17.5. * P value < 0.05, **b** versus **a**.

bacterial, fungicide [22], antitumoral [5], [23], antiedematous [9], and anti-inflammatory [8] effects have been experimentally confirmed. No reports inform of carcinogenic properties *of Calendula officinalis.* The only study, that we know of, that reports genotoxic activity at high concentration is our previous work [19], which contrasts with published results in which the Ames test was found negative [10]. These conflicting results can be conciliated by our findings of the dual opposite activity of *C. officinalis* extracts. A high dose of *C. officinalis* extracts administered to the RH model showed a clear dose-dependent tendency to increase the area and number of AHF. At low concentrations, these clearly tended to decrease. These profile changes are characteristic of a phenomenon called hormesis, described as an effect of some substances which show biphasic dose responses, displaying either an inverted "U" or "J" shaped response depending on the endpoint measured [14]. The *C. officinalis* extract effect clearly shows a hormetic response. This dual effect, the hormetic dose-response, has also been reported for arsenic, ethanol, acetylsalicylic acid, cadmium, DDT and phenobarbital, among others [24]. In studies similar to ours with the rat model used by the US National Program, DDT has been demonstrated to reduce the tumor incidence significantly below that of the control group at low doses, while being a carcinogen at higher doses [15]. One of the most striking observations related to this phenomenon is that phenobarbital, a very well known and effective cancer promoter, at low concentrations inhibits the formation of placental glutathione *S*-transferase positive foci and liver tumors, suppresses MAP kinase gene expression, and prevents generation of oxidative DNA damage as shown by 8-hydroxy-2′-deoxyguanosine levels. On the contrary, with phenobarbital at high doses, placental glutathione *S*-transferase positive foci, tumor multiplicity, hydroxyl radicals and 8-hydroxy-2′-deoxyguanosine levels were greatly elevated [16]. This is a clear description of the hormetic effect.

In the same context are the experimental model reports of the enhancement of cellular adaptive processes with low and ultra-

low doses of metal toxins [25]. Renal immature T-cells pre-exposed to ultralow levels of cadmium produce higher levels of metallothionein protein, the primary protective protein in heavy metal toxicity [26]. Of the active principles described in *C. officinalis,* very likely present in our extract, the candidates that can be pointed out as responsible for this effect, are the flavonoids, since some of them, like quercetin, has been described as anti-oxidant, pro-oxidant [13] and free radical scavenger [27].

In this study, the chemopreventive or genotoxic activities of *C. officinalis* were found to be related to the concentration. However, most striking was the promoter activity of *C. officinalis* when the extract was administered instead of the 2AAF. This dual effect of *C. officinalis* is very important for clinical practice. Many people use plants indiscriminately and drink *C. officinalis* infusion as a daily drink. According to our results, depending on the dose, *C. officinalis* can promote or decrease precancerous lesions. Thus, its use can be either harmful or beneficial.

In summary, this is the first *in vivo* study that manifests a dual dose-dependent effect of the *Calendula officinalis* extract, which shows two very striking activities, prevention at low doses, and promoting activities at high doses of AHF in the RH model.

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