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# Mutagenic and antimutagenic effects of *Heterotheca inuloides*

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**The antioxidant and hepatoprotective effects of *Heterotheca inuloides* have been reported before, nevertheless its use as a possible chemopreventive agent has not been documented. The aim of this study was to evaluate the mutagenic and antimutagenic activities of *H. inuloides* extracts using the Ames test. Both, the methanolic and acetonic extracts, were mutagenic in the TA98 but not in TA100 or TA102 strains. On the other hand, the methanolic extract reduced the mutagenicity of norfloxacin, benzo[a]pyrene and 2-aminoanthracene. Quercetin, one of the main components in the methanolic extract, also presented a mutagenic/antimutagenic dual effect and is an inhibitor of Cytochrome P450 (CYP) 1A. The antigenotoxic properties of *H. inuloides* could be due to the antioxidant properties previously reported and to its CYP inhibitory effect mediated by quercetin. Further studies with *in vivo* systems will afford information about *H. inuloides* beneficial and detrimental properties.**

It is recognized that a side effect of industrial activity has been the production of genotoxic chemicals with the potential of inducing cancer and other chronic diseases in exposed individuals[1]. Polycyclic aromatic hydrocarbons (PAHs) generated by fossil fuel combustion engines and tobacco consumption are a good example of such unintended side effects[2]. Short term assays were developed to identify hazardous chemicals with mutagenic/carcinogenic activity in order to regulate their use[3]. At the same time, the same methodologies have been proposed to identify natural and synthetic molecules that interfere with the mutagenic processes thus creating the concept of chemoprevention[4] meaning that substances of natural or synthetic origin are used to lower the risk of cancer development. The use of medicinal plants is common in Latin-American countries. It has been reported that plant material contain complex mixtures of natural components like polyphenols, polyamines, chlorophylls and constitutes natural sources of active ingredients that can be used for therapeutic treatment[5]. Several molecules of plant origin have been reported to possess antimutagenic activity[6–8] or hepatoprotective and antioxidant potential[9,10]. Nevertheless, other plants commonly used in the mexican diet like *Gnaphalium* spp., *Poliomintha longiflora and Piper sanctum* are capable to produce frameshifs mutations in bacteria or a high mortality rate in the lethal toxicity test using *Artemia salina*[11]. Due to the fact that the use of plants for medical reasons is not regulated in most of the Latin-American countries, it is of vital importance to evaluate the safety of traditional medicine products as well as their beneficial properties. The Ames test is a well known mutagenicity assay able to detect base pair or frameshift point mutations and its use has been recommended for the assessment of the antimutagenic effect of molecules from natural origin including plants[4]. *Heterotheca inuloides* (Asteraceae) is a plant native to México that grows in hot and temperate regions of the central area of the country[12]. The beneficial properties of this plant are well known; the aerial parts including leaves and flowers are used in folk medicine for the topical treatment of inflammation and contusions, skin wound healing and bruises[13,14]. Several metabolites from *H. inuloides* have been isolated and its antioxidant[9,15–17], cytotoxic, analgesic, and antimicrobial activities were extensively reported[15,18–21]. We have previously reported the hepatoprotective effect of the acetonic and methanolic extracts of *H. inuloides* as well as quercetin using a $CCl_4$ toxicity model in rat[10]. The objective of the present work was to evaluate the mutagenic/antimutagenic effects of the ethanolic and acetonic extracts from *H. inuloides* in the Ames test, using mutagens that follow different DNA damaging pathways including: alkylation, oxidation and formation of bulky adducts. The mutagenic/antimutagenic activity of the extracts and one of its main components, quercetin (Figure 1), were evaluated.

## Methods

**Chemicals and bacterial strains.** D-Glucose-6-phosphate, β- nicotinamide adenine dinucleotide phosphate (NADP), N-Methyl-N'-nitro-N-nitrosoguanidine, 2-Aminoanthracene, 2- Aminofluorene, Benzo[a]pyrene, L-histidine, D-biotin, D-glucose, potassium phosphate,

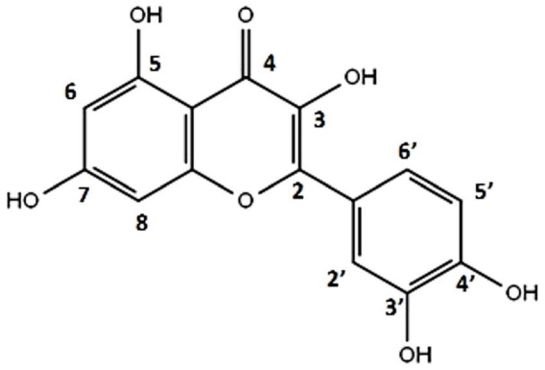


**Figure 1 | Chemical structure of quercetin.**

magnesium sulphate and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA), Arochlor 1254 was obtained from Supelco (Bellefonte, PA, USA). We also used nutrient broth No. 2 (Oxoid Inc., Ogdensburg, NY, USA). *Salmonella typhimurium* strains TA100, *(hisG46, pKm101, rfa, uvvB);* TA98 *hisD3052, pKm101, rfa, uvrB)* and TA102 *(hisG48, Pq1, pKm101, rfa)* were donated by Dr. Takehiko Nohmi National Institute of Health Sciences, Tokyo, Japan.

**Preparation of S9 mix.** Arochlor 1254 in corn oil (200 mg/ml) was administered intraperitoneally (500 mg/Kg body weight) to four male Sprague Dawley rats acording to Maron and Ames[22]. The rats were euthanized on day five after inoculation, and their livers were obtained aseptically. The S9 fraction was obtained according to the procedure used by Ames[23]. The liver S9 mix was prepared according to the procedure used by Ames[23].

**Plant material and extraction.** *H. inuloides* flowers were collected in Puebla, México and authenticated by personnel at the Department of Medicinal Plants of the Botanical Garden (Institute of Biology, Universidad Nacional Autónoma de México). A voucher sample (Myrna Mendoza C 15,375) was deposited in the National Herbarium at Instituto Mexicano del Seguro Social, México City. 2.0 kg of ground and dried plant material was extracted three times for 24 h each with acetone at room temperature. Plant residue was then extracted in the same way with methanol (3 times/24 h). After solvent evaporation, 12 and 15 g of acetonic and methanolic residues were obtained, respectively. Phosphate buffer pH 7.4 was used as the solvent for quercetin and the methanolic residue and olive oil for the acetonic residue.

**Total polyphenols determination.** The Folin-Ciocalteu method was used to determine the polyphenol content in both extracts[24]. The equivalent to 100 mg of each plant extract was mixed with100 μL of Folin-Ciolcateu reagent and incubated at room temperature for 1 min. After that, 300 μL of a 200 g/L sodium carbonate solution was added and incubated for 15 min at 50°C. The mixture was ice cooled and the absorbance was scored in a spectrophotometer at λ=765 nm. Catechin was used as standard and the polyphenol content of each extract was expressed as mg of catechin equivalents/g of extract.

**Mutagenicity and antimutagenicity tests.** We used the standard plate-incorporation method of the Ames test[23], with or without the liver S9 mix. 2 mL of molten top agar, which contained 0.6% of agar, 0.5% of NaCl, 0.5 mM of biotin, and 0.05 mM of l-histidine, were successively mixed with 0.1 mL of overnight culture (approximately 1 $\times 10^8$ cells), methanolic or acetonic extract (125–1000 μg/plate) or quercetin (2–10 μg/plate), and 0.5 mL of S9 mix or 0.5 mL of phosphate buffer. In the antimutagenicity experiments, methanolic extract at three different concentrations (500, 750, 1000 μg/plate) were added to 2 ml of molten top agar along with either 2AA (1.5 μg/plate), BP (5 μg), MNNG (5 μg) or norfloxacine (70 ng) and 0.1 mL of the overnight culture. When 2AA or BP was used, 0.5 ml of the S9 mix or 0.5 ml of phosphate buffer was added. The test components were mixed in a vortex for three seconds at low speed, and the mixture was distributed over the surface of the minimal agar plates. The plates were incubated at 37°C for 48 h, and the number of revertant colonies was counted. The extracts were considered as mutagenic when the number of induced revertant colonies doubled that found in control plates and a dose-dependent mutagenic effect was found[25].

**Survival assay.** *S. typhimurium* cell treated with Arnica extracts along with the different mutagens as described above, were diluted in isotonic saline solution and seeded in nutrient agar plates. After incubation for 24 hr. at 37°C the colony forming units (cfu) were scored and the percent of surviving cells were calculated taking as 100% survival the number of cfu in control plates. A result was considered a positive toxic effect when up to 20% of bacterial death occurred.

**Inhibition studies in rat liver microsomes.** CYP1A1 ethoxyresorfin *O*-deethylase (EROD) associated activity was measured as described[26]. Different concentrations of quercetin dissolved in dimethylsulfoxide (DMSO) were added to a mixture containing rat liver microsomes (0.1 mg), substrate (1 μM ethoxyresorufin), and buffer pH 7.6 (50 mM Tris-HCl and 25 mM MgCl2) and incubated for 3 min at 37°C. NADPH (0.5 mM) was added and the fluorescence signal was recorded every 15 s for 3 min. Activities were calculated from a standard curve of resorufin (5–50 pmol/mL).

**Statistics.** Antimutagenesis assays were analyzed by a two tail ANOVA test with the Graph-pad Sigma 2.01 sofware.

## Results

**Standardization of the extracts.** Acetonic and methanolic extracts contained 19.35 and 50.03 mg/mL of catechin equivalents respectively. The total polyphenol content of the extracts provide a reference of its antioxidant capacity.

**Mutagenesis.** None of the two extracts were mutagenic to TA100 or TA102 *S. typhimurium* strains (data not shown). Nevertheless, the two extracts were mutagenic to the frameshift TA98 strain in the absence or presence of S9 mix (Figure 2). The highest mutagenic effect was obtained with the acetonic extract in the presence of metabolic activation. Nevertheless, the acetonic extract showed toxic effects resulting in a bacterial dead above 20% (data not shown) especially in TA98 and TA100 strains bearing the uvr$^-$ genotype. On the other hand, the methanolic extract was not toxic to any of the tester strains. Quercetin was also mutagenic to the TA98 strain in the presence and absence of metabolic activation (Figure 3). The presence of S9 mix potentiated the mutagenic activity of quercetin observed without metabolic activation.

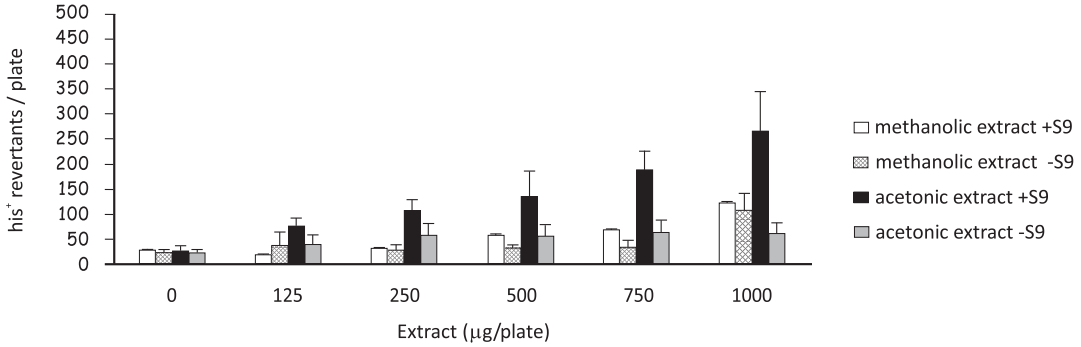


**Figure 2 | Mutagenicity of *H. inuloides* acetonic and methanolic extracts in *S. typhimurium* TA98 strain in the presence and absence of S9 mix.** *H. inuloides* methanolic and acetonic extracts was added at different concentrations to petri dishes as described in material and methods. After incubation at 37°C for 48 h, the number of revertant colonies was scored. Each data represent the mean number of revertant colonies found in two independent experiments (3 plates/experiment).The mean number of spontaneous TA98 revertants and standard deviation were 23.5 ± 5 and 27.7 ± 9 in the absence or presence of S9 mix respectively.

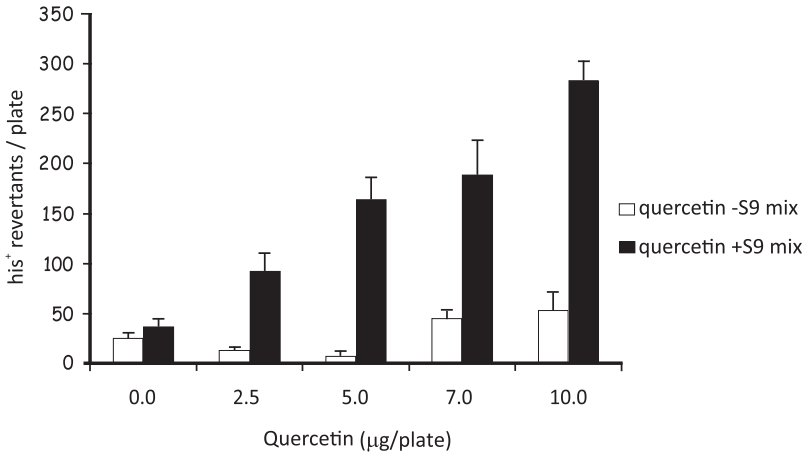


**Figure 3 | Mutagenicity of quercetin in *S. typhimurium* TA98 strain in the presence and absence of S9 mix.** Quercetin was added at different concentrations to petri dishes as described in material and methods. After incubation at 37°C for 48 h, the number of revertant colonies was scored. Each data represent the mean number of revertant colonies found in two independent experiments (3 plates/experiment).The mean number of spontaneous TA98 revertants and standard deviation were 25.7 ± 5 and 36.7 ± 9 in the absence or presence of S9 mix respectively.

**Antimutagenesis.** Due to the toxicity observed with the acetonic extract, antimutagenesis assays were carried out only with the methanolic one. This extract showed an antimutagenic effect against the genotoxic damage exerted by 2AA in the presence of S9 mix (Figure 4) to the TA98 strain. On the other hand, the extract was also able to interfere with the mutagenicity of norfloxacin to the TA102 strain (Figure 5) and MNNG, to the TA100 (figure 6) strain in the absence of metabolic activation. Quercetin showed an antimutagenic effect below 2 μg/plate dose against the mutagenicity of benzo[a]pyrene in the presence of S9 mix to the TA98 strain (figure 7).

**Inhibition of CYP1A activity.** Quercetin inhibited the ethoxyresorufin-*O*-deethylase activity associated to CYP1A1 in doses ranging from 0.05 to 1.6 μg/mL (Figure 8).

## Discussion

The characterization of the acetonic and methanolic extracts of *H. inuloides* were previously reported to contain a mixture of molecules such as catenanes, sterols, polyacetylenes, triterpenes, sesquiterpenes, flavonoids and flavonoid glycosides[20,27]. The two extracts gave positive result for mutagenesis as detected in the TA98 strain of *S. typhimurium* in the presence and absence of metabolic activation (Figure 2). These results indicate the presence of frameshift direct mutagens and those mediated by metabolic activation. Quercetin is one of the main constituents in the methanolic extract accounting for up to 0.19% although it is also present in the acetonic extract[10], therefore we proceeded to test the mutagenicity of this flavonoid obtaining a pattern of mutagenicity similar to that found in the extracts (Figure 3). Rietjens et al.[28], has reported that a flavonoid structure with a free hydroxyl group at position 3, a double bond linking positions 2 and 3, and a keto-group at position 4, allowing the proton of the hydroxyl group at 3 to tautomerise to a 3-keto moiety, favors mutagenic activity. Among the compounds that are present in *H. inuloides* extracts, quercetin (Figure 1) and kaempferol, display these structural features. Furthermore, the mutagenic activity of these flavonoids was previously reported by Aparecida et al.[29], supporting our results for quercetin.

Despite its mutagenicity, the methanolic extract displayed an antimutagenic action against a pro-mutagen (2AA), a free radical inducer (norfloxacin) and an alkylating agent (MNNG) (Figures 4,5,6). Regarding the mechanism behind 2-AA reduced mutagenicity, the methanolic extract capacity to inhibit the metabolism of this promutagen by CYP1A was tested, but the extract interfered with the chemical determination of the CYP1A associated alkoxyresorufin-dealkylase activities. Nevertheless, we were able to identify the capacity of quercetin to inhibit the CYP1A1 activity as shown in figure 8. Based on these results we propose that the presence of this flavonoid in the extract accounts for the antimutagenic activity through a mechanism involving CYP inhibition. It is important to note that quercetin inhibits the mutagenicity of BaP, a promutagen that also needs to be activated by CYP1A (Figure 7). However, although quercetin was able to inhibit CYP1A1 activity almost completely at the highest dose tested, it was unable to inhibit 100% of the BaP muta-

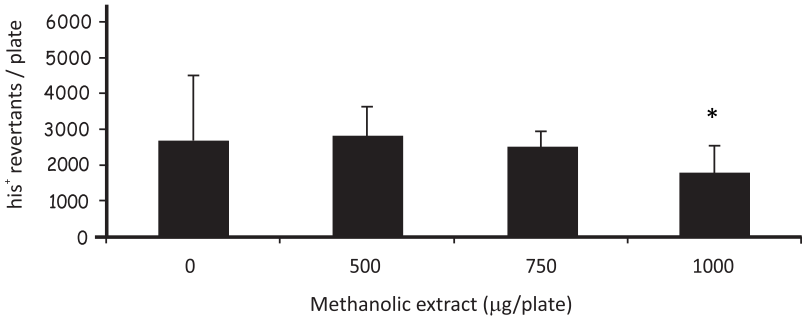


**Figure 4 | Antimutagenic effect of *H. inuloides* methanolic extract in the presence of 2AA.** *H. inuloides* methanolic extract was added at different concentrations to petri dishes containing 2AA (10 μg/plate) plus liver S9 mix as described in Material and methods. After incubation at 37°C for 48 h, the number of revertant colonies was scored. Each data represent the mean number of revertant colonies found in two independent experiments (3 plates/experiment).The mean number of spontaneous TA98 revertants and standard deviation were 13.2 ± 4. (*significant, $P < 0.001$).

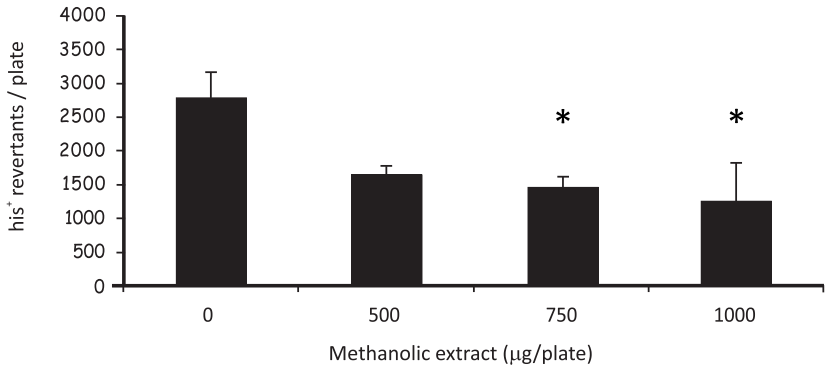


**Figure 5** | **Antimutagenesis of *H. inuloides* methanolic extract on the mutagenicity of norfloxacine.** Different concentrations of *H. inuloides* methanolic extract were added to petri dishes containing norfoxacin (70 ng/plate) as described in material and methods. Each data represent the mean number of revertant colonies found in two independent experiments (3 plates/experiment).The mean number of spontaneous TA102 revertants and standard deviation were 271.5 ± 74. *P<0.05.

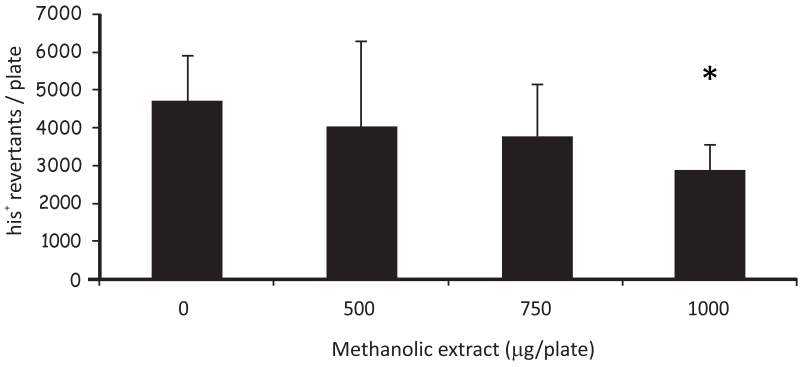


**Figure 6** | **Antimutagenesis of *H. inuloides* methanolic extract on the mutagenicity of MNNG.** Different concentrations of *H. inuloides* methanolic extract were added to petri dishes containing MNNG (5 μg/plate) as described in material and methods. Each data represent the mean number of revertant colonies found in two independent experiments (3 plates/experiment). The mean of spontaneous TA100 revertants and standard deviation were 144.3 ± 70. *P<0.05.

genic activity. At least two mechanisms could be involved in the generation of these results: i) other CYP subfamilies besides 1A could be responsible for BaP activation. It has been reported that subfamilies 2C8, 2C9 and 3A4 are able to oxidize BaP to BaP 7,8-oxide which is the first step in BaP activation[30]; and ii) the mutagenic/antimutagenic dual effect of quercetin leads to the induction of a similar number of revertant colonies in the presence of different doses of the flavonoid and BaP.

The methanolic extract showed a clear dose –response antimutagenesis effect against the alkylating compound MNNG, although only the highest extract concentration showed a statistically significant difference compared to the negative control (Figure 6). Additional mechanisms of action could be involved including the modulation of the expression of DNA repair genes and the capacity of some plant molecules to trap mutagenic compounds. Arriaga-Alba et al.[31] demonstrated that the presence of the DNA-repair *ogt*

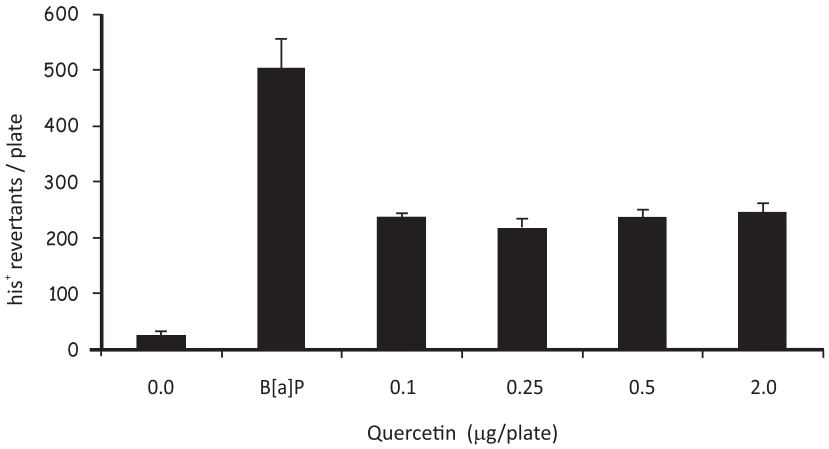


**Figure 7** | **Antimutagenesis of quercetin on the mutagenicity of benzo[a]pyrene.** Different concentrations of quercetin were added to petri dishes containing benzo[a]pyrene (5 μg/plate) plus S9 mix as described in material and methods. Each data represent the mean number of revertant colonies found in two independent experiments (3 plates/experiment).The mean of spontaneous TA98 revertants and standard deviation were 25.3 ± 6.4.

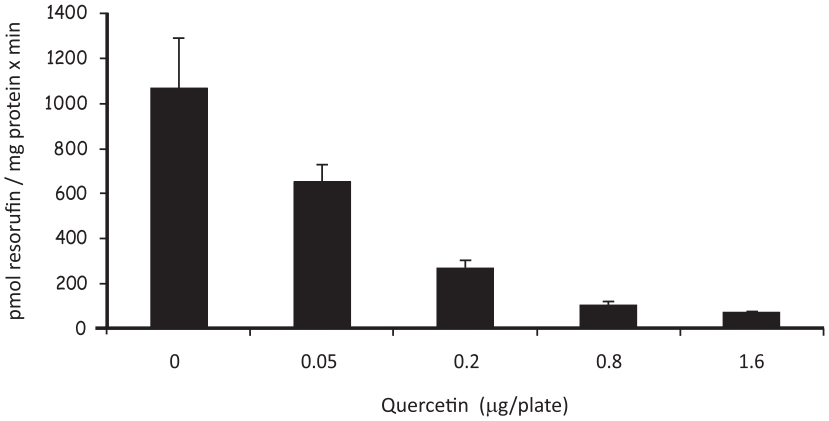


**Figure 8 | Concentration-dependent inhibitory effect of quercetin and on the rat microsomal CYP1A1 associated ethoxyresorufin-*O*-dealquilase activity.** Quercetin was added at different concentrations to the incubation mixture containing rat liver microsomes and ethoxyresorufin (1 μM) as described in material and methods.

gene is necessary for the antimutagenic action of *Rhoeo dischlor* extracts suggesting that these extracts may improve the $O^6$ alkylguanine DNA alkyltransferase DNA repair enzyme. On the other hand, Mezougg et al.[32], reported that the extracts of *Laurus nobilis, Rosmarinus officinalis, Trigonella foenum-graecum, and Myristica fragrens* could be acting as desmutagens by binding the direct acting alkyl radical and preventing DNA damage.

Norfloxacin is a mutagenic fluoroquinolone detectable with the *Salmonella* strain TA102 that detects DNA oxidative damage[33]. Additionally, norfloxacin modifies the activity of some markers of oxidative damage such as GPx, GST and CAT in zebrafish[34]. These results evidenced the ability of norfloxacin to generate oxygen free radicals able to interact with DNA and proteins. Results in figure 5 demonstrate that *H. inuloides* methanolic extract reduced the number of revertant colonies induced by norfloxacin and we propose that the mechanism behind this effect involves free radical scavenging. *In vivo* and *in vitro* antioxidant properties of *H. inuloides* were previously reported, the methanolic extract showing the greatest antioxidant capacity[10,11]. Althoug *H. inuloides* contains mutagenic molecules able to induce base pair and frameshift mutations; it is a complex mixture that also holds methanol extractable polar compounds with antioxidant and CYP inhibitory properties. These compounds are able to cope with the presence of free radicals as well as with polycyclic aromatic hydrocarbons that need CYP activation in order to produce nucleophilic entities that react with DNA or proteins leading to premutagenic lesions. An evaluation of the protective and deleterious effects of *H. inuloides* in *in vivo* animal models is needed in order to gain insight of its potential use as a chemopreventive mixture.


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## Author contributions

J.E., R.C. and M.A. wrote the main manuscript text. N.R., J.S. and S.H. participated in the experimental design and preparation of figures 1–7. All authors reviewed the manuscript.

## Additional information

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