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ScienceDirect
Journal of Ethnopharmacology 115 (2008) 381–386
Journal of ETHNO-PHARMACOLOGY
www.elsevier.com/locate/jethpharm

Aqueous crude extract of *Rhoeo discolor*, a Mexican medicinal plant, decreases the formation of liver preneoplastic foci in rats

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Received 29 March 2007; received in revised form 4 October 2007; accepted 9 October 2007
Available online 23 October 2007

Abstract

There are many plants in Mexico with medicinal properties, some of them used in alternative medicine to treat cancer, such is the case of *Rhoeo discolor* L. Hér Hance (*Commelinaceae* family); however, there are not scientific reports that validate their antitumoral property. The present study shows the protective effects of the *Rhoeo discolor* aqueous crude extract (ACE) against rat liver cancer using the resistant-hepatocyte model. The carcinogenesis protocol consisted on the initiation with *N*-diethylnitrosamine, followed by the promotion with 2-acetylaminofluorene and a partial hepatectomy. After 24 days, the γ-glutamyl transpeptidase positive, corresponding to altered hepatocytes foci (AHF), were quantified. Additionally to discard a possible carcinogenic effect of ACE, it was first tested as promoting agent instead 2-acetylaminofluorene, and second, ACE was administered as initiator and promoter instead of the whole carcinogenic treatment. In summary, firstly, ACE administration reduced the number and area of preneoplastic lesions with dose below 20 mg/kg body weight and secondly, ACE administration neither presented a promoting or initiator effects nor induced the development of AHF. Results of this investigation justify continuing with further studies of *Rhoeo discolor* components to develop chemoprevention strategies as an option in the treatment of cancer.

Keywords: *Rhoeo discolor*; Purple maguey; *Commelinaceae* family; Medicinal plants; Hepatocarcinogenesis; Chemopreventive effect; Hormesis

1. Introduction

In Mexico, in spite of the active practice of traditional medicine, the extraordinary floristic wealth that locates it in the fourth world place, and the knowledge of people for the curative properties of the plants, only 1–5% of plants has been validated chemically and pharmacologically (Meckes et al., 1993; Huerta, 1997). Cancer is the second cause of death in several countries, included Mexico (INEGI, 2005). Then, it is justified the search for new active agents against cancer. Since there are examples of plants as source of relevant antitumoral compounds, attention has been focused on natural products (Meckes et al., 1993; Popoca et al., 1998). Evidence obtained from experimental studies has been important to select a plant with potential antitumoral properties (Popoca et al., 1998; Seth and Sharma, 2004). To study stages of cancer development, animal models have been broadly used. That is the case of the rat resistant-hepatocyte carcinogenesis model, where in short periods and in a synchronous way, proliferation of populations of altered hepatocytes is produced (Farber and Sarma, 1987; Semple-Roberts et al., 1987). The groups of altered hepatocytes have elevated levels of detoxification enzymes such as γ-glutamyl transpeptidase; therefore, this enzyme has been employed as a tumor marker (Semple-Roberts et al., 1987; Hanigan, 1998). The antitumoral capacity of some plants described as curative has been

Abbreviations: 2-AAF, 2-acetylaminofluorene; ACE, aqueous crude extract; AHF, altered hepatocytes foci; CMC, carboxymethylcellulose; DEN, *N*-diethylnitrosamine; DMSO, dimethylsulfoxide; GGT, γ-glutamyl transpeptidase; PH, partial hepatectomy; BW, body weight.

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doi:10.1016/j.jep.2007.10.022

demonstrated experimentally, i.e., *Ardisia compressa* and *Calendula officinalis* (González de Mejía et al., 2004; Barajas-Farías et al., 2006).

Rhoeo discolor L. Hér Hance [syn. *Tradescantia spathacea* Swartz, *Rhoeo spathacea* (Swartz) Stearn] is a plant of Mexico that is in use in traditional medicine. This plant belongs to the *Commelinaceae* family and localizes in Caribbean and Central America. In the Southeastern of Mexico, it is known as "Maguey Morado" (Purple Maguey) and the decoction of the leaves is daily free-consumed as curative of cancer, without existing scientific evidence of such property (Del Amo, 1979; Argueta and Cano, 1994). It is known that the aqueous extract of *Rhoeo spathacea* blocks the antiadrenergic action of bretylium (García et al., 1971) and is contraceptive in rats (Weniger et al., 1982). The extracts of *Rhoeo discolor* have been incorporated in cosmetics to improve the appearance of skin (Meybeck et al., 1999). Some chemicals detected in *Rhoeo discolor* are flavonoids, anthocyanins, saponins, carotenoids, waxes, terpenoids, and coumarinic and steroidal compounds (Idaka et al., 1987; Domínguez-Ortiz, 2002). On the other hand, *Rhoeo discolor* ethanolic crude extract evaluated in an *in vitro* system, showed antimutagenic, antigenotoxic and antioxidative activities (González-Ávila et al., 2003).

Due to the absence of scientific reports *in vivo* that corroborate the antitumoral property of *Rhoeo discolor*, it is evident the importance of the exploration of this plant. It is also important as part of a permanent screening program to search new agents to treat cancer. Therefore, the present investigation was focused to study the antitumoral effect of the *Rhoeo discolor* ACE through the resistant-hepatocyte model, using Fischer 344 rats and the γ-glutamyl transpeptidase preneoplastic enzymatic marker.

2. Materials and methods

2.1. Plant material

The ethnomedical bibliography and the popular use regarding traditional practices by herbalists were the base for selecting the plant studied in this investigation. Blooming plants of *Rhoeo discolor* were obtained from Dr. Miguel Ángel Domínguez-Ortiz, of the Universidad Veracruzana, Ver., México. The collection was carried out in the months of September and October in the State of Veracruz, México. Botanical identification of the plant was obtained, and classified, reference voucher No. CIB 5811, were deposited at the Herbarium of the Instituto de Investigaciones Biológicas of Universidad Veracruzana (Veracruz, México).

2.2. Preparation of aqueous crude extract

The extract was elaborated in the Laboratory of Vegetal Biotechnology of ITTJ, Jal., Mexico. Clean fresh leaves (100 g) were cut and placed in 100 ml of boiling distilled water for 30 min. The liquid was filtered through Whatman paper No. 4, and dried by aspersion in a Mini Spray Dryer Büchi model B-191 (inlet temperature 180 °C, outlet temperature 96 °C, feed flow of 330 ml/h), yielding 8.37% extract.

2.3. Animals

Male Fischer 344 rats weighing 180–200 g (14 weeks old) were obtained from the Production Unit of Experimental Laboratory Animals (UPEAL-CINVESTAV, México D.F., Mexico). Rats were housed in a room with controlled temperature (23 ± 2 °C), humidity (55 ± 5%), under a 12 h light–dark cycle and fed with a standard laboratory diet (Purina 5053) and water ad libitum. All animals received human care and the study protocols were in compliance with the institutional guidelines for the use of laboratory animals.

2.4. Experimental protocol

Chemical reagents were purchased to Sigma Chemical, Co. (St. Louis, MO). The antitumoral effect of *Rhoeo discolor* was determined following the modified rat resistant-hepatocyte model protocol (Carrasco-Legleu et al., 2004). Rats were randomly distributed into 14 groups: with complete carcinogenic treatment (groups I–IX), only with DEN (groups XIII and XIV) with 10 rats each, and without carcinogens (groups X–XII) with 5 rats each. Experimental groups are shown in Fig. 1. As it has been described by Farber and Sarma (1987), in order to generate liver preneoplastic lesions, it is obligatory to perform the three interventions: administration of an *initiator* (DEN) on day 1, a *promoter* in three successive doses (2-AAF), and at day 10 a partial hepatectomy as a stimulus of cellular proliferation. Thus, on day 1, animals of the positive control (group I), as well as groups II to IX, XIII and XIV, were injected intraperitoneally with 200 mg/kg BW of DEN. On days 7–9 post-initiation, groups from I to IX also received via gavage 20 mg/kg BW per day of

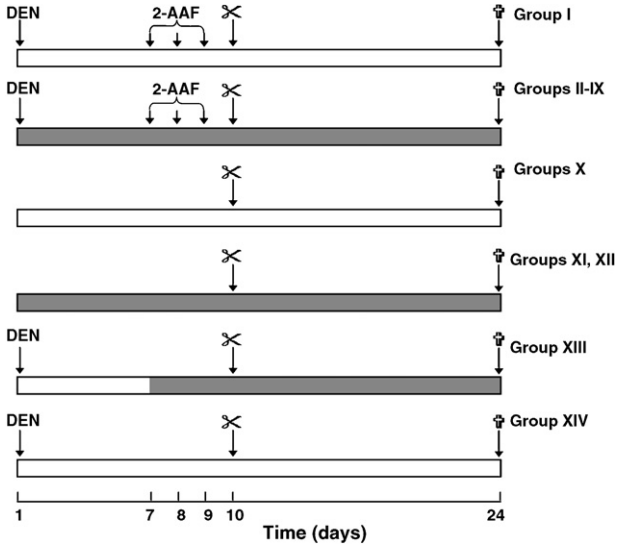

Fig. 1. Experimental protocol. Positive control (group I); groups treated with *Rhoeo discolor* at doses of 0.0025 (II), 0.025 (III), 0.125 (IV), 0.25 (V), 2.5 (VI), 5.0 (VII), 7.5 (VIII) and 20.0 mg/kg BW (IX); negative control (group X); groups without carcinogens: with ACE at doses of 2.5 (XI) and 20 mg/kg BW (XII); *Rhoeo discolor* at dose of 2.5 mg/kg BW in substitution of 2-AAF (XIII); without 2-AAF (XIV). □ = distilled water to drink; ■ = ACE dissolved in distilled water to drink; ✂ = partial hepatectomy; ✞ = sacrifice; DEN = *N*-diethylnitrosamine; 2-AAF = 2-acetylaminofluorene.

2-AAF dissolved in DMSO, in 1% aqueous solution of CMC to a final concentration of 10 mg/ml. At day 10, animals of all groups were subjected to 2/3 partial hepatectomy.

In respect to the protecting effect of *Rhoeo discolor,* the groups treated with the carcinogenic protocol were subjected to a wide range of ACE concentrations in drinking water. Group II received 0.0025, group III: 0.025, group IV: 0.125, group V: 0.25, group VI: 2.5, group VII: 5.0, group VIII: 7.5 and group IX: 20.0 mg/kg BW. As it is unknown whether ACE prevents carcinogenesis, or it has carcinogenic capability, in respect to the latter, the potential of ACE as carcinogen was tested at two doses: group XI: 2.5 and group XII: 20.0 mg/kg BW. In group XIII ACE was tested only as a promoter, substituting 2-AAF for the extract at dose of 2.5 mg/kg BW, starting at day 7 until sacrifice day.

Rats in the group X (negative control without carcinogens or ACE); they were administered distilled water instead DEN, and DMSO instead 2-AAF.

All animals were subjected to a partial hepatectomy in day 10 and sacrificed on day 24 post-initiation. Livers were excised, sliced, immersed in methyl-isobutane, quickly frozen in liquid nitrogen and stored at −80 °C until analysis.

2.5. Histochemical γ-glutamyl transpeptidase staining and analysis

Liver sections of 20 μm thickness were obtained in a cryostat (Slee Cryostat MTC), fixed in absolute ethanol during 5 min at −20 °C and stained according to Rutenburg et al. (1969). In order to detect the preneoplastic cells induced by the carcinogenic treatment, the marker γ-glutamyl transpeptidase (GGT) was determined. γ-glutamyl-4-methoxy-2-naphtylamine at a ratio of 1:20 with 25 mM Tris pH 7.4 containing 0.5 mg/ml glycyl-glycine and 0.5 mg/ml 4-benzoylamino-2,5-diethoxybenzene-diazonium chloride hemi[zinc chloride] salt (fast blue bb salt) were added and the reaction kept 20 min at

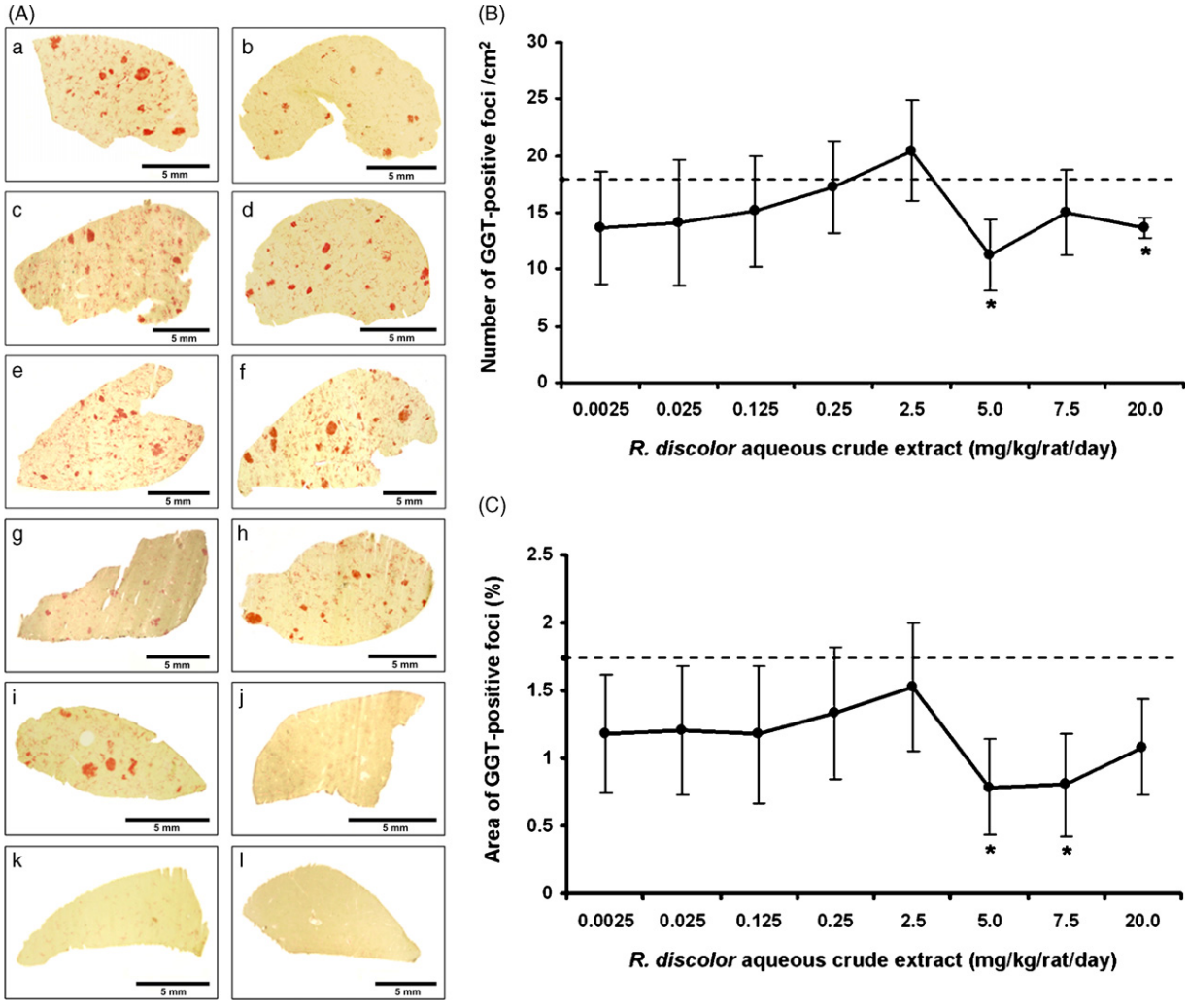

Fig. 2. Effect of *Rhoeo discolor* aqueous crude extract on the induction of GGT-positive altered hepatocytes foci. (A) Liver sections representative of each treatment. (a) Group I, (b) II, (c) III, (d) IV, (e) V, (f) VI, (g) VII, (h) VIII, (i) IX, (j) X, (k) XI, (l) XII. (B) Effect of ACE on the number of GGT-positive foci/cm^2. (C) Effect of ACE on area of GGT-positive foci. Each value represents the mean ± S.D. from at least three animals (except group IX, $n = 2$). *Denotes significance at $p \leq 0.05$ vs. positive control.

room temperature. Next, tissue sections were washed with distilled water and the precipitate was fixed with 100 mM cupric sulfate for 2 min. The tissue was stained of a dark-red color in areas where the enzyme showed activity. Finally, images from liver sections were captured with a digital camera (Color View 12, Soft Imaging System GmbH) coupled to a stereoscopic microscope (Olympus model SZ40). The clonal proliferation of initiated cells corresponds to GGT-positive hepatocyte foci and nodules that were quantified with image analysis software (AnalySIS Soft Imaging System GmbH, ver. 3.00). Three histological liver sections were randomly selected for each rat.

2.6. Statistical analysis

The results were expressed as mean ± standard deviation (S.D.). Number and area of GGT-positive AHF of the groups treated with *Rhoeo discolor* extract were compared to the positive control using one way ANOVA, in a SigmaStat (ver. 2.03) program with Dunnett test. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

The effect of the aqueous crude *Rhoeo discolor* extract (ACE) on the prevention of GGT-positive Altered Hepatocyte Foci (AHF) is shown in comparison with the carcinogenesis and non-treated groups in Fig. 2A. Administration of ACE alone did not induce AHF, which indicates that the extract "per se" is not able to generate genotoxic lesions, even at 20 mg/kg BW (Fig. 2A, panel l). Moreover, when ACE was administered instead of the promoting carcinogen 2-AAF, it did not present promoting activity (Fig. 3A, panel a). AHF of the carcinogenic control (group I) was 1.7% of the examined area (Fig. 2C); the number of AHF was 17.5/cm^2 (Fig. 2B). As shown in Fig. 2, all groups that received a complete carcinogenic treatment and different ACE concentration showed a decrease in GGT+ area with respect to the carcinogenic control [Fig. 2A, panels b–i; Fig. 2B and C (11.6–54.6% reduction)]; most of them (6/8) showed a lower number of AHF than positive control level (Fig. 2B and C, 1.5–35.6% reduction). The minimum value for area and number of AHF was obtained at 5.0 mg ACE/kg BW (35.6% of AHF/cm^2 and 54.6% of area); the maximum value was obtained at 2.5 mg ACE/kg BW (17% above the control in number, however, 11.6%

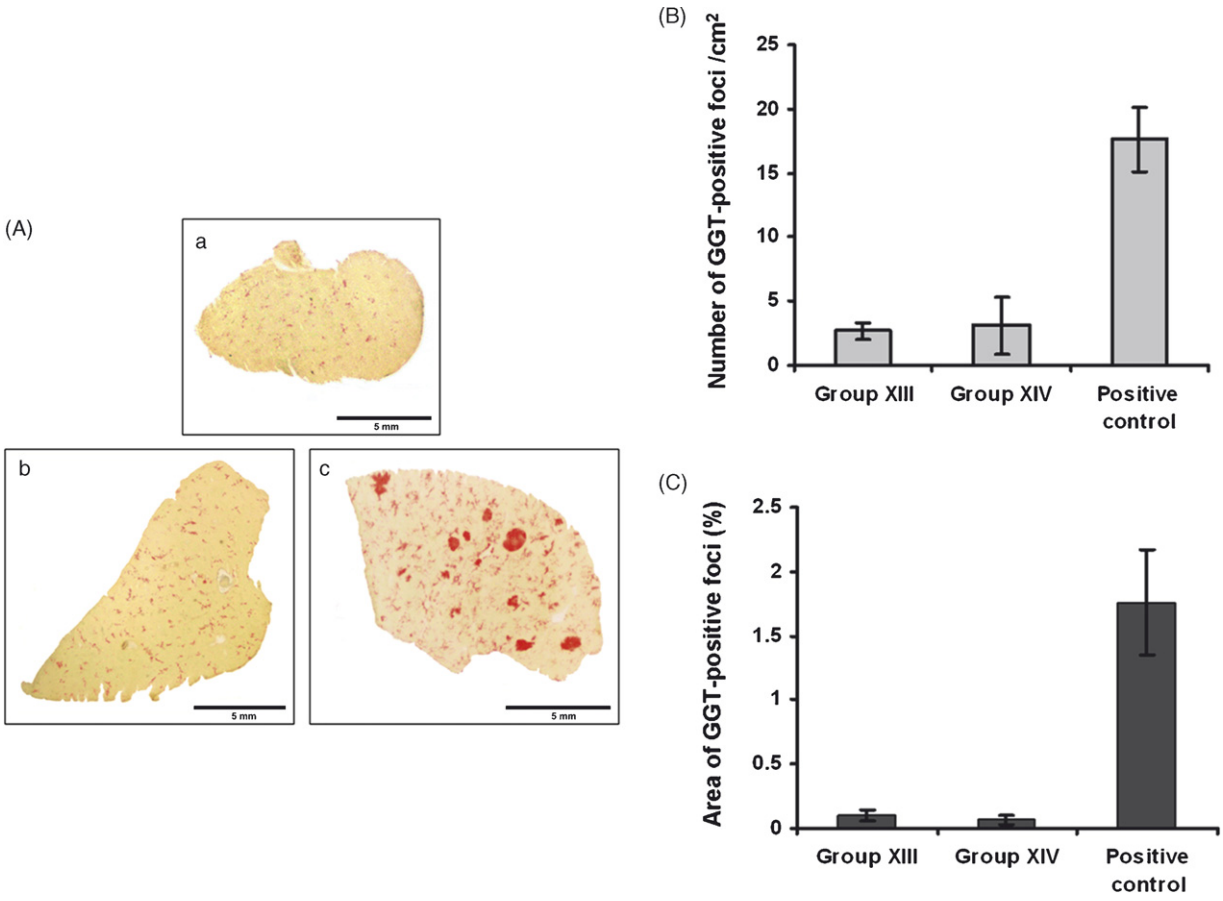

Fig. 3. Effect of *Rhoeo discolor* aqueous crude extract as possible promoter in the carcinogenesis protocol. (A) Representative GGT stained rat liver sections: (a) rats received ACE at 2.5 mg/kg BW, starting at day 7, in place of 2-AAF (group XIII); (b) treatment with DEN (XIV); (c) carcinogenic positive control (I). (B) Effect of ACE on the number of GGT-positive foci/cm^2. (C) Effect of ACE on area of GGT-positive foci. Each value represents the mean ± S.D. from at least three rats. There are not significant differences between group XIII and group XIV (Dunnett test, $p \leq 0.05$).

below the control level in area). In general, it was observed a tendency to increase and then to decrease in number and area of GGT-positive foci when ACE dose was increased. The differences were statistically significant in groups VII and IX on number of AHF/cm^2 and in groups VII and VIII on area, both versus positive control. The liver sections of the groups X–XII did not present foci (Fig. 2A, panel j, k and l). When ACE was tested as promoter, rat livers did not show AHF (Fig. 3). It is important to mention that at dose of 20.0 mg/kg BW (group XIII) the animal survival was 100%; nevertheless, this ACE concentration, in combination with the complete carcinogenic treatment (group IX) had a survival of only 20% of the animals, in comparison with 60% registered for the complete carcinogenic treatment (group I). Moreover, for the groups II–VIII a survival of the animals was higher (70%) mainly in the treatments with low concentrations of *Rhoeo discolor* (data not shown).

4. Discussion

It is important to evaluate the activity of plant crude extracts, since they are the nearest form to preparation used in the traditional medicine, and also because the pure compounds not always behave the same way as in the natural products (Meckes et al., 1993; Liu, 2004). In this study, it is clearly shown the beneficial effect of the *Rhoeo discolor* aqueous extract (ACE) to prevent a full induction of liver preneoplastic lesions induced by a carcinogenic treatment. Also, it is shown a clear tendency to increase and then decrease the number and area of GGT-positive foci when ACE dose was increased, generating a pattern that assumes a "J" shape and falls under the general category of hormesis. This is a biological phenomenon characterized by dose–response relationships displaying low-dose stimulation and high-dose inhibition (Calabrese and Baldwin, 2003; Hunt and Bowman, 2004). The hormetic responses have been reported for a wide range of inorganic agents and in many biological models, i.e., plants, invertebrate and vertebrate animals (Calabrese and Baldwin, 2003).

Carcinogenic protocol used is characterized by 60% survival. Interestingly, a dose of 5 mg/kg BW of ACE had the highest protecting effect against the carcinogenic treatment (70% survival). At 20 mg ACE/kg BW it was non-toxic, strikingly, that dose but in combination with carcinogens DEN and 2-AAF, registered 20% animal survival, suggesting that this combination is toxic. A close relationship between survival and toxicity has been also observed with other compounds by Padma et al. (1989).

Many phytochemicals classified as phenolics and carotenoids present in *Rhoeo discolor*, have been associated with the antitumoral and/or antioxidant activity present in some plants (Ho et al., 2002; Liu, 2004). Due to the presence of flavonoids in *Rhoeo discolor* and the demonstration of its antioxidant capacity (Domínguez-Ortíz, 2002; González-Ávila et al., 2003), we cannot discard the possibility that those compounds are responsible for the ACE protecting effect. A similar protective effect on GGT-positive AHF has been reported in *Calendula officinalis*, and also an association with flavonoids was suggested as responsible for this effect (Barajas-Farías et al., 2006).

The mechanisms of the protective action of ACE are unknown. According to its chemical composition and biological properties, it could be: (a) a blockade of initial DNA damage, related with the presence of phenols, coumarins and flavones (Bailey and Williams, 1993; Liu, 2004); (b) an inactivation/detoxication of carcinogens, which is related to its antimutagenic activity (Bailey and Williams, 1993); (c) the compounds of ACE elevate intracellular glutathione levels to induce the antioxidant system, as observed for quercetin and kaempferol (Martínez-Flores et al., 2002). Further studies are required to identify the components responsible of its antitumoral activity.

The results obtained in this work are the first scientific *in vivo* evidence referred to antitumoral activity of aqueous crude extract of *Rhoeo discolor*. Lastly, it is convenient to stand out the importance of continuing with the search and development of new strategies for treatment and prevention of cancer, since the incidence and mortality of this illness continue increasing annually in spite of the advances that science has had in this respect. For this purpose, the countries that have preserved a traditional medical culture, can contribute with their knowledge to provide therapeutic proposals for the resolution of health problems and also for the search of new drugs.

Acknowledgements

We are very grateful to Dr. Miguel Ángel Domínguez-Ortiz for providing *Rhoeo discolor* plants; Dr. Jorge Fernández (head of Animal House) and the M.V.Z. Rafael Leyva, Ricardo Gaxiola and Manuel Flores for the excellent technical support; Q.F.B. Leticia Alemán-Lazarini for her invaluable technical assistance; M.C. Jaime Arellanes-Robledo for excellent assistance in handling of AnalySIS program; M.C. Salvador González-Palomares for his kind support throughout the experiment. First author (TRR) is a recipient of a fellowship from CoSNET (402003075 MP), México.

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