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Safety and antiulcer efficacy studies of *Achillea millefolium* L. after chronic treatment in Wistar rats

Ana Maria Cavalcanti [a], Cristiane Hatsuko Baggio [a], Cristina Setim Freitas [a], Lia Rieck [a], Renato Silva de Sousa [b], José Eduardo Da Silva-Santos [a], Sonia Mesia-Vela [a], Maria Consuelo Andrade Marques [a,*]

[a] *Department of Pharmacology, Universidade Federal do Paraná, Caixa Postal 19031, Centro Politécnico - Jardim das Américas, Curitiba, PR 81531-990, Brazil*
[b] *Laboratory of Veterinary Pathology, Department of Veterinary Medicine, Universidade Federal do Paraná, Rua dos Funcionários 1540, Curitiba, PR 80035-050, Brazil*

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Abstract

Achillea millefolium L. (Asteraceae), popularly known as yarrow, has been used in folk medicine to treat complaints such as inflammation, pain, wounds, hemorrhages and gastrointestinal disturbances. The aim of the present study was to assess the safety and efficacy of the aqueous extract (AE) of the plant after chronic exposure. Indeed, the AE was effective in protecting the gastric mucosa against acute gastric lesions induced by ethanol and indomethacin and in healing chronic gastric lesions induced by acetic acid with (ED_{50} = 32 mg/kg, p.o.). Safety studies were performed in female and male Wistar rats treated daily with AE (0.3–1.2 g/kg, p.o./day) or vehicle (water, 10 ml/kg/day) for 28 or 90 consecutive days. Satellite groups consisted of animals sacrificed 30 days after the end of these treatments. Clinical observations, body and organ weight measurements, gross autopsy, hematology, clinical biochemical and histopathological examinations were performed. Slight changes in liver weight, cholesterol, HDL-cholesterol and glucose were observed in male and female animals. These changes were not correlated with dose or time of exposure of the animals to the AE. Overall, the results show the antiulcer potential of the aerial parts of the *Achillea millefolium* which is accompanied by no signs of relevant toxicity even at very long chronic exposure.

Keywords: *Achillea millefolium*; Yarrow; Medicinal plants; Antiulcer; Preclinical toxicity

1. Introduction

Achillea millefolium L. (Asteraceae), popularly known as "yarrow", is a widely distributed medicinal plant that has been used for over 3000 years (Mitich, 1990). Popular indications of this specie include treatment of wounds, hemorrhages, headaches, inflammation, pain, spasmodic diseases, flatulence, and dyspepsia (Correia, 1974; Chandler et al., 1982; Blumenthal et al., 2000). Some of these reputed folk effects have been determined showing the potential medicinal use of the plant. The medicinal properties of *Achillea millefolium* are worldwide recognized and the plant is included in the national *Pharmacopoeias* of countries such as Germany, Czech Republic, France and Switzerland (Mitich, 1990; Bradley, 1992; Alonso, 1998; Blumenthal et al., 2000). In Brazil, *Achillea millefolium* is included in the list of the 16 medicinal plants of the "Verde Saúde" (Green Health), a public health phytotherapy Agency supported by the Municipal Health Secretary of Curitiba (PR, Brazil) which includes the anti-inflammatory, analgesic, antispasmodic and antiseptic properties of the plant.

Preparations of *Achillea millefolium* have been shown to have anti-inflammatory (Goldberg et al., 1969; Tunon et al., 1995), antitumor (Tozyo et al., 1994), antioxidant, antimicrobial (Candan et al., 2003), and liver protective activities (Gagdoli and Mishra, 1995; Lin et al., 2002). In addition, our laboratory previously reported the potent gastric anti-secretory and gastroprotective activity of the aqueous extract of the plant in several acute models of gastric injury in rodents (Baggio et al., 2002).

* Corresponding author. Tel.: +55 41 3361 1721; fax: +55 41 3266 2042.
E-mail addresses: crisbaggio@hotmail.com (C.H. Baggio), mconsu@ufpr.br (M.C.A. Marques).

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Phytochemical studies have identified achilleine (Miller and Chow, 1954), azulene and chamazulene (Haggag et al., 1975; Kokkalou et al., 1992; Kubelka et al., 1999), achimillic acids A, B and C (Tozyo et al., 1994), 1,8-cineole (Kokkalou et al., 1992), and flavonoids such as apigenin and luteolin (Valant-Vetschera and Wollenweber, 1988; Guédon et al., 1993) in the aerial parts of *Achillea milefolium*, but only a few studies correlated these substances with pharmacological activities or toxicity. Health hazards associated with long-term exposure to *Achillea millefolium*'s extracts are not well established. Despite the Food and Drug Administration has classified the plant as non-poisonous and has approved its utilization in alcoholic drinks (Duke, 1987) some toxic effects had been reported after its use by humans and in animal experiments. Toxic effects in human include contact dermatitis (Hausen et al., 1991), headaches and dizziness (Alonso, 1998; Cáceres, 1999). In animals, *Achillea millefolium*'s preparations reduced fetal weight and increased placental weight when given to pregnant rats (Boswell-Ruys et al., 2003) and had antispermatogenic effects in mice (Montanari et al., 1998) but recent studies of our group did not identify any relevant toxicity on important reproductive biomarkers after 90-day treatments of female Wistar rats (Dalsenter et al., 2004).

Here in we investigated the efficacy of the AE of *Achillea millefolium* as a gastroprotector in a chronic model of gastric ulcer as well as the possible associated toxicity after chronic exposure of rats to the plant.

2. Methodology

2.1. Collection of plant material

Achillea millefolium L. was provided by Fazenda Solidariedade (Campo Magro, PR, Brazil) where the plant is grown under controlled conditions. There, samples of *Achillea millefolium* are selected and propagated by rhizome division and then cultivated in a specific culture area. The sample used in our experiments was collected in December 2000, the flowers were discarded and the aerial parts (leaves, stalks and stems) were allowed to dry at room temperature. The plant was identified by Msc. Olavo Guimarães, Department of Botany, Universidade Federal do Paraná (Curitiba, PR, Brazil). A specimen of the plant was deposited in the Herbarium of this institution under number 45106.

2.2. Preparation of the aqueous extract (AE)

The aqueous extract (AE) was prepared by infusion of the aerial parts of the plant (3× 30 min) in water at 70 °C (1:10, w/v). The infusion was filtered and concentrated under vacuum (at 56 °C) to 1/12 of the original volume and stored at −20 °C. The concentrated extract (yield 36%) was diluted in distilled water immediately before use.

2.3. Animals

Male and female Wistar rats (180–200 g) were supplied by Instituto de Tecnologia do Paraná (TECPAR, PR, Brazil). All animals were acclimatized to our laboratory conditions for 10 days before the beginning of the experiments. Animals were maintained under standard laboratory conditions on a constant 12 h light/dark cycle with controlled temperature (22 ± 2 °C). Standard pellet food (Nuvital®, Curitiba/PR, Brazil) and water were available ad libitum. The Institutional Ethics Committee of Universidade Federal do Paraná approved all procedures adopted in this study.

2.4. Induction of chronic gastric lesions by acetic acid

Chronic gastric ulcers were induced in female rats with acetic acid according to a modification of the method described previously (Takagi et al., 1969). A total of 30 rats were randomly distributed into 5 groups. Animals were anaesthetized, the abdomen was exposed and 0.05 ml of 2.5 and 5% acetic acid (v/v) was injected into the anterior subserose and 0.05 ml of 10% acetic acid and 0.9% saline on the posterior subserose of gastric wall. After recovery from anesthesia, animals were treated with vehicle (water, 1 ml/kg, p.o.) or AE (100 and 300 mg/kg, p.o.) twice a day for 7 days for the preventive action. To determine the healing capacity of the AE, similar doses of the extract (100 and 300 mg/kg, p.o./twice a day) were administered after the gastric lesions were installed at seventh day after injection of acetic acid. On the day following the last administration, the animals were sacrificed. The stomach was removed for analysis of gastric lesions to score the index of mucosal damage (IMD).

2.5. Acute gastric lesions

Fasted rats were treated (p.o.) with vehicle (water, 0.1 ml/100 g, $n = 6$), ranitidine (60 mg/kg, $n = 6$), AE (125–2000 mg/kg, $n = 6$). Gastric lesions were induced after 1 h by ethanol (0.5 ml/animal, 70%, p.o.), or indomethacin (20 mg/kg, s.c.) (Djahanguiri, 1969; Robert et al., 1979). Animals were sacrificed 1 and 6 h after treatments, respectively. The stomachs were removed and the mucosa washed and examined under a stereoscope to score the number of ulcers and to determine the IMD.

2.6. Hippocratic test

Non-fasted rats (both sexes) were treated with AE (3 and 10 g/kg, p.o. or 1 and 3 g/kg, i.p.). The animals were observed continuously for 1 h followed by every hour up to 6 h and by 14 days for any changes in behavior and manifestations of the toxic symptoms according to Malone (1977).

2.7. Repeated dose 28- or 90-day oral treatment

Adult male and female rats were randomly assigned to four groups ($n = 40$, 20 per sex). One group received vehicle (water, 10 ml/kg, p.o.) and the other three were treated with the AE (0.3, 0.6 and 1.2 g/kg, p.o.). The lowest dose of the extract was selected based on its protective activity against chronic gastric lesions induced in rats. The higher doses were

determined by geometrical progression according to the instructions of the Brazilian Sanitary Surveillance Agency (ANVISA, 2000). Treatments were performed daily by gastric intubations (gavage), always in the morning (WHO, 1993; OECD, 1998). At day 28, half of the animals ($n = 10$/sex/group) in the control and AE groups were killed. The remaining animals ($n = 10$/sex/group) were maintained under treatment until the 90th day, when were also killed. Two satellite groups, treated either with vehicle ($n = 5$/sex) or the highest dose of AE (1.2 g/kg; $n = 5$/sex), were maintained under standard laboratory conditions without any treatment for additional 30 days after the treatment period (28 or 90 days). Clinical and behavioral signs were observed daily throughout the study according to Guideline No. 408 of the Organization for Economic Cooperation and Development (OECD, 1998). Body weight was determined at the beginning of treatments and then weekly until the day of necropsy (day 28 or 90). Satellite groups were also observed and weighed until end of experimental period. At the end of treatment periods (28th or 90th day), animals were anesthetized with pentobarbital (60 mg/kg, i.p.), the anterior abdominal wall was surgically opened and blood samples were collected through the abdominal aorta artery. Animals were then killed by exsanguinations. All animals were subjected to a gross autopsy (WHO, 1993; OECD, 1998) and selected organs were removed and weighed. In addition, representative samples of stomach, jejunum, ileum, colon, liver, pancreas, adrenals, lung, spleen and kidneys were collected and fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5 μm, stained with hematoxylin and eosin and then examined by light microscopy.

2.8. Hematology and clinical chemistry determinations

With exception of clotting time which was determined using the first 1 ml of blood collected directly into glass tubes maintained in a water-bath at 37 °C, all other hematological and clinical chemistry analyses were carried out at the Municipal Laboratory of Curitiba (PR, Brazil). Quantitative hematological parameters determined in whole blood (2 ml) collected into tubes containing EDTA were determined using an automated cell counter (STKS Coulter, Miami, CA, USA). The parameters analyzed were: red blood cell count, hemoglobin, hematocrit, mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), total leukocyte count, and platelet count. Qualitative parameters were measured in peripheral blood films stained with an automated staining device (HEMA-TEK 2000, Bayer, Tarrytown, NY, USA). Blood cell morphology and differential leukocyte count (relative number, %) were obtained by light microscopy. Serum clinical chemistry examinations were carried out with an automated chemistry analyzer (BM/Hitachi 912, Cobas Mira, Roche, and Indianapolis, IN, USA). The parameters evaluated were: total serum protein, albumin, cholinesterase, total bilirubin, alanine aminotransferase, aspartate aminotransferase (AST), alkaline phosphatase (ALP), total cholesterol, HDL-cholesterol, triglycerides, amylase, glucose, urea nitrogen, uric acid, creatinine, sodium, and potassium.

2.9. Statistical analysis

Data were tested for normal distribution and then subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test or the Kruskal–Wallis' test followed by Dunns' multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of AE on acetic acid-induced chronic gastric lesions

The oral administration of AE (100 and 300 mg/kg/7 days), 7 days after the ulcer induction, healed the gastric ulcer induced by acetic acid in 75 and 90%, respectively compared to control group (lesion index value = 132 ± 16) (Fig. 1A). The ED_{50} for the healing effect was 32.4 mg/kg. However, the oral administration of AE (100 and 300 mg/kg/7 days), 1 day after the acetic acid injection, did not prevent the formation of gastric ulcer when compared to control group (lesion index value = 129 ± 16) (Fig. 1B).

3.2. Effects of AE on acute gastric lesions induced by ethanol and indomethacin

The pre-treatment of the animals with AE decreased gastric lesions induced by ethanol with ED_{50} 936 mg/kg (Table 1). Oral

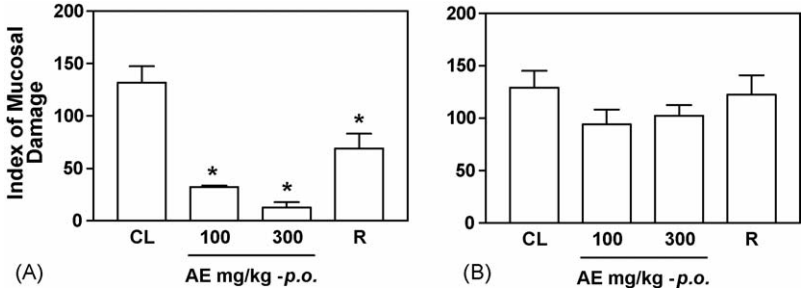

Fig. 1. Protective effect of aqueous extract of *Achillea millefolium* against chronic gastric lesions induced by acetic acid. The animals were orally treated with vehicle (C: water, 0.1 ml/100 g), ranitidine (R: 60 mg/kg) and AE (100 and 300 mg/kg) during 7 days and 1 day after the gastric lesions induction (Panels A and B). The results are expressed as mean ± S.E.M. ($n = 6$). Statistical comparison was performed using analysis of variance (ANOVA) followed by Tukey's test. $^{*}p < 0.05$ when compared to damaged control group.

Table 1
Effect of aqueous extract of *Achillea millefolium* against acute gastric lesions induced by ethanol and indomethacin

Treatment	Index of mucosal damage (IMD)	
	70% Ethanol (0.5 ml/animal, p.o.)	Indomethacin (20 mg/kg, s.c.)
Control (water, 0.1 ml/100 g)	112.6 ± 7.0	113.6 ± 9.3
AE (125 mg/kg)	78.0 ± 16.0*	91.3 ± 8.0
AE (1500 mg/kg)	23.8 ± 5.6*	91.6 ± 6.9
AE (2000 mg/kg)	15.8 ± 4.3*	57.5 ± 9.0*
Ranitidine (60 mg/kg)	47.9 ± 4.7*	9.3 ± 2.2*

The results are expressed as mean ± S.E.M. ($n = 6$). Statistical comparison was performed using analysis of variance (ANOVA) followed by Tukey's test.
* $p < 0.05$ when compared to control group.

administration of AE 1 h before the induction of gastric lesions by indomethacin reduced (by 49%) the IMD only with the highest dose tested (2000 mg/kg) (Table 1).

3.3. Hippocratic test

Acute administration of AE in doses up to 10 g/kg (p.o.) or 3 g/kg (i.p.) did not cause death of animals.

3.4. Clinical observations and body and organ weights

All rats survived until the end of the 90-day period of treatment with the AE. Animals under treatment with AE either for 28 or 90 days had similar mobility, reflexes, muscular tonus and breathing patterns than animals in control group treated with vehicle. Also, gain of weight was similar in all groups tested. No important changes in organ weight were observed with exception of a decrease of about 9% in liver weight in female animals treated with 0.3 g/kg for 90 days and in male animals treated with 0.6 g/kg for 90 days and with 1.2 g/kg for both 28 and 90 days (Table 2).

3.5. Gross autopsy and histopathology

Histopathological evaluation did not reveal changes in the stomach, jejunum, ileum, colon, liver, pancreas, adrenals, lung, spleen or kidneys of AE-treated animals when compared to control animals.

3.6. Hematology parameters

Tables 3 and 4 show hematology parameters measured in male and female rats treated with AE. In males it was observed

Table 2
Organ weights (%)

Weight (%)	AE (g/kg)							
	0		0.3		0.6		1.2	
	28 days	90 days	28 days	90 days	28 days	90 days	28 days	90 days
Male								
Liver	3.9 ± 0.1	3.3 ± 0.1	3.7 ± 0.1	3.4 ± 0.1	3.7 ± 0.1	3.5 ± 0.1*	3.5 ± 0.1*	3.5 ± 0.1*
Kidneys	0.36 ± 0.01	0.30 ± 0.01	0.36 ± 0.01	0.30 ± 0.01	0.35 ± 0.01	0.30 ± 0.01	0.34 ± 0.01	0.30 ± 0.01
Female								
Liver	3.8 ± 0.1	3.3 ± 0.1	3.8 ± 0.1	3.0 ± 0.1*	3.6 ± 0.1	3.2 ± 0.1	3.7 ± 0.1	3.2 ± 0.1
Kidneys	0.33 ± 0.01	0.28 ± 0.01	0.32 ± 0.01	0.27 ± 0.01	0.33 ± 0.01	0.27 ± 0.01	0.33 ± 0.01	0.28 ± 0.01

The results are expressed as mean ± S.E.M. ($n = 10$). Statistical comparison was performed using analysis of variance (ANOVA) followed by Tukey's test.
* $p < 0.05$ when compared to control group.

Table 3
Hematology parameters of male rats

Hematology parameters	AE (g/kg)							
	0		0.3		0.6		1.2	
	28 days	90 days	28 days	90 days	28 days	90 days	28 days	90 days
Clotting time (s)	110.2 ± 5.7	143.1 ± 7.8	112.5 ± 7.5	138.4 ± 5.0	99.7 ± 4.4	118.1 ± 2.0*	121.1 ± 4.2	137.4 ± 8.1
RBC count ($\times 10^6$ mm^{-3})	8.1 ± 0.1	8.4 ± 0.1	8.3 ± 0.1	8.6 ± 0.1	8.3 ± 0.1	8.6 ± 0.1	8.0 ± 0.1	8.5 ± 0.1
Hemoglobin (g/dl)	15.9 ± 0.1	16.2 ± 0.1	16.3 ± 0.1	16.1 ± 0.2	16.2 ± 0.1	16.3 ± 0.2	16.0 ± 0.1	16.1 ± 0.2
Hematocrit (%)	43.8 ± 0.2	44.7 ± 0.3	45.3 ± 0.5	43.9 ± 0.4	44.1 ± 0.3	44.7 ± 0.4	43.5 ± 0.6	44.5 ± 0.3
MCV (fl)	54.0 ± 0.2	53.3 ± 0.3	54.3 ± 0.2	51.0 ± 0.3*	53.3 ± 0.4	52.1 ± 0.4	54.0 ± 0.3	51.9 ± 0.5
MCH (pg)	19.5 ± 0.1	19.1 ± 0.1	19.9 ± 0.2	18.7 ± 0.2	19.6 ± 0.2	19.0 ± 0.1	19.9 ± 0.2	18.9 ± 0.1
MCHC (%)	36.1 ± 0.2	36.2 ± 0.4	36.6 ± 0.2	36.6 ± 0.4	36.8 ± 0.2	36.4 ± 0.3	36.8 ± 0.3	36.4 ± 0.4
WBC count ($\times 10^3$ mm^{-3})	10.0 ± 0.3	8.9 ± 0.5	10.7 ± 0.7	9.5 ± 0.5	11.3 ± 0.7	8.1 ± 0.5	10.5 ± 0.6	8.5 ± 0.4
Lymphocytes (%)	67.0 ± 1.5	64.4 ± 4.2	64.2 ± 2.7	68.3 ± 1.8	63.9 ± 4.5	60.9 ± 1.4	65.5 ± 2.1	66.6 ± 2.6
Neutrophils (%)	20.5 ± 1.2	26.1 ± 3.1	24.9 ± 2.9	23.2 ± 0.9	21.3 ± 4.0	27.3 ± 1.5	19.7 ± 1.7	24.8 ± 2.3
Monocytes (%)	10.0 ± 1.1	6.3 ± 1.2	8.3 ± 0.8	5.3 ± 0.3	8.0 ± 0.7	5.6 ± 0.5	11.1 ± 0.9	5.8 ± 0.8
Eosinophils (%)	1.0 ± 0.3	1.4 ± 0.4	1.3 ± 0.3	1.4 ± 0.5	0.6 ± 0.3	1.3 ± 0.2	1.2 ± 0.2	1.7 ± 0.3
Platelets count ($\times 10^3$ mm^{-3})	830.1 ± 25.3	949.2 ± 30.9	877.6 ± 16.4	955.8 ± 19.7	888.8 ± 23.1	886.6 ± 12.1	852.3 ± 21.1	923.0 ± 13.5

The results are expressed as mean ± S.E.M. ($n = 10$). Statistical comparison was performed using Kruskal–Wallis' test followed by Dunns' test.
* $p < 0.05$ when compared to control group.

Table 4
Hematology parameters of female rats

Hematology parameters	AE (g/kg)							
	0		0.3		0.6		1.2	
	28 days	90 days	28 days	90 days	28 days	90 days	28 days	90 days
Clotting time (s)	106.5 ± 5.6	130.0 ± 4.5	107.6 ± 5.8	114.2 ± 4.0	103.8 ± 5.8	113.2 ± 4.6	100.8 ± 6.6	135.8 ± 7.1
RBC count ($\times 106\,mm^{-3}$)	7.7 ± 0.1	7.9 ± 0.1	7.6 ± 0.1	7.7 ± 0.1	7.6 ± 0.1	7.7 ± 0.1	7.4 ± 0.1*	7.8 ± 0.1
Hemoglobin (g/dl)	14.7 ± 0.1	15.3 ± 0.2	15.0 ± 0.1	15.3 ± 0.1	14.7 ± 0.1	14.9 ± 0.2	14.9 ± 0.1	15.1 ± 0.1
Hematocrit (%)	42.3 ± 0.3	42.3 ± 0.4	41.7 ± 0.4	42.9 ± 0.5	41.0 ± 0.6	41.9 ± 0.4	40.6 ± 0.4*	41.9 ± 0.4
MCV (fl)	55.3 ± 0.2	54.4 ± 0.4	55.1 ± 0.2	54.9 ± 0.4	54.6 ± 0.2	54.1 ± 0.6	54.9 ± 0.3	53.7 ± 0.5
MCH (pg)	19.1 ± 0.2	19.7 ± 0.1	19.8 ± 0.1	19.8 ± 0.2	19.7 ± 0.2	19.6 ± 0.1	20.0 ± 0.2*	19.4 ± 0.1
MCHC (%)	34.7 ± 0.3	36.0 ± 0.4	35.8 ± 0.1*	36.1 ± 0.4	36.4 ± 0.2*	35.9 ± 0.4	36.8 ± 0.1*	36.1 ± 0.4
WBC count ($\times 103\,mm^{-3}$)	7.4 ± 0.4	6.4 ± 0.4	7.7 ± 0.4	6.9 ± 0.4	8.4 ± 0.5	5.6 ± 0.5	7.8 ± 0.4	6.9 ± 0.7
Lymphocytes (%)	64.5 ± 2.1	68.2 ± 1.2	66.0 ± 2.3	67.5 ± 3.4	66.7 ± 1.8	56.2 ± 3.5*	70.8 ± 2.0	63.4 ± 2.6
Neutrophils (%)	23.2 ± 1.9	19.9 ± 2.0	23.6 ± 1.9	22.2 ± 2.8	23.2 ± 1.7	34.5 ± 3.5*	19.3 ± 1.6	25.7 ± 2.8
Monocytes (%)	9.6 ± 1.5	5.3 ± 0.8	7.7 ± 1.3	5.4 ± 0.9	9.1 ± 1.1	4.2 ± 0.8	7.8 ± 0.9	6.0 ± 0.8
Eosinophils (%)	1.4 ± 0.3	1.5 ± 0.5	1.6 ± 0.2	1.6 ± 0.4	1.0 ± 0.2	1.6 ± 0.5	0.6 ± 0.3	1.6 ± 0.3
Platelets count ($\times 103\,mm^{-3}$)	878.3 ± 25.1	913.8 ± 29.4	875.9 ± 30.4	920.2 ± 16.4	860.6 ± 24.6	927.8 ± 38.9	844.4 ± 27.2	987.4 ± 33.2

The results are expressed as mean ± S.E.M. ($n = 10$). Statistical comparison was performed using Kruskal–Wallis' test followed by Dunns' test.
* $p < 0.05$ when compared to control group.

a slight decrease (30%) in clotting time and in MCV (4%) in animals treated with 0.6 g/kg and 0.3 g/kg of AE for 90 days (Table 3). In female animals, many other changes were observed such as an increase in MCHC in animals treated with the three doses of AE for 28 days. Lymphocytes were decreased while neutrophils were increased in animals treated with 0.6 g/kg for 90 days. In female animals receiving 1.2 g/kg it was observed a decrease in RBC count and hematocrit and an increase in MCH after 28 days of exposure only (Table 4).

3.7. Serum biochemistry

Tables 5 and 6 show biochemistry parameters determined in animals treated with AE for 28 and 90 days. In males, it was observed an increase in HDL-cholesterol in animals treated with 0.3 and 0.6 g/kg for 28 days (18 and 32%, respectively). Cholesterol was also increased (19%) only with 0.6 g/kg of the AE for 28 days. Triglycerides were reduced (35%) after 28 days treatment with 1.2 g/kg (Table 5). These changes were not observed in male animals treated for 90 days (Table 5). Alkaline phosphatase was also increased in male rats after treatment with 0.3 g/kg for 90 days while it was decreased after treatment with 1.2 g/kg for 28 days. Amylase was decreased with 0.6 g/kg treatment for 90 days while blood glucose was decreased with 0.3 and 1.2 g/kg for 90 days (Table 5). In females, cholesterol was increased after 0.3 g/kg treatment for 28 days as well as HDL-cholesterol with treatments with AE 0.3 and 1.2 g/kg (Table 6). In addition, 0.6 g/kg treatment of female animals increased blood glucose after 28 days while it was decreased after 90 days of treatment. Urea nitrogen was decreased by treatment of animals with 0.3 and 1.2 g/kg after 28 days while it increased after 90 days treatment with 1.2 g/kg. Uric acid was also decreased in animals receiving 0.3 g/kg for 90 days. Sodium was also increased by 0.6 and 1.2 g/kg for 28 days (Table 6).

4. Discussion

Achillea millefolium L is widespread species used in Brazilian folk medicine to treat diverse diseases including inflammation, pain and gastrointestinal disturbances. Screening of gastroprotective potential against acute and chronic ulcers showed positive correlation with the folk medicinal use. Since the aqueous extract of the plant was more potent in the necrotizant model of gastric lesion induced by ethanol when compared to lesion induced the inhibition of prostaglandins through indomethacin indicates that other cytoprotective mechanism may be involved in the effect. A "topical" effect of the AE can be discarded since the intraperitoneal administration of 300 mg/kg of AE also protected the animals from gastric lesions induced by ethanol by 54% (control values = 99.9 ± 23).

Comparatively to the acute models (ED_{50} of 936 mg/kg, p.o.), the potency of the AE in healing chronic gastric ulcers (ED_{50} of 32 mg/kg, p.o.) showed to be about 28-fold more potent. Increase potential of the extract on healing chronic gastric lesions induced by acetic acid was not correlated to a prevention of the installation of gastric ulcers in the same model which indicates that cytoprotective mechanisms independent of acid may be activated by the AE of *Achillea millefolium*. Gastric lesions by this acid are induced by alteration of various factors including prostaglandins, growth factors, adherent mucus, microcirculation submucosal, nitric oxide and cytokines (Kobayashi et al., 2001; Shahin et al., 2001).

The gastroprotective activity of the AE was accompanied by no signs of toxicity in any relevant parameters linked to liver, kidney or hematological systems for periods up to 90 days in either female or male rats. Overall, the results show that high doses of the AE prepared from *Achillea millefolium* given daily for 28 or 90 days does not alter the nutritional status either induce systemic toxicity in rats. The absence of changes in body weight gain, clinical and behavioral patterns of AE-treated animals,

Table 5
Biochemical parameters of male rats

Biochemical parameters	AE (g/kg)							
	0		0.3		0.6		1.2	
	28 days	90 days	28 days	90 days	28 days	90 days	28 days	90 days
Serum protein (g/dl)	6.0 ± 0.1	6.2 ± 0.1	6.1 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	6.3 ± 0.1	5.6 ± 0.1	6.2 ± 0.1
Albumin (g/dl)	3.4 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.8 ± 0.1	3.5 ± 0.1	4.4 ± 0.3*
Cholinesterase (U/l)	207.7 ± 26.2	305.6 ± 21.1	212.9 ± 17.4	303.8 ± 32.6	226.6 ± 12.4	250.3 ± 23.2	226.5 ± 25.7	239.9 ± 21.3
TGL (mg/dl)	74.8 ± 8.5	94.3 ± 6.3	50.8 ± 5.9	82.9 ± 4.9	59.0 ± 5.9	112.5 ± 9.7	48.6 ± 4.5*	107.1 ± 9.4
Cholesterol (mg/dl)	65.8 ± 3.3	79.6 ± 4.6	77.7 ± 3.4	77.6 ± 2.7	78.5 ± 2.7*	76.5 ± 2.5	68.1 ± 2.9	71.6 ± 1.9
HDL-cholesterol (mg/dl)	50.7 ± 2.8	62.8 ± 3.3	59.9 ± 2.0*	62.1 ± 1.8	67.1 ± 2.5*	62.1 ± 1.6	58.2 ± 1.8	58.1 ± 1.8
Total bilirrubin (mg/dl)	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
ALP (U/L)	307.4 ± 6.9	271.3 ± 9.8	278.6 ± 24.2	358.2 ± 19.1*	247.8 ± 11.7	291.4 ± 19.0	231.7 ± 11.4*	298.7 ± 20.6
AST (U/L)	107.7 ± 7.3	115.6 ± 10.7	92.6 ± 4.9	126.3 ± 10.0	111.9 ± 6.6	138.7 ± 6.5	101.2 ± 5.0	128.6 ± 6.4
ALT (U/L)	67.8 ± 2.3	66.9 ± 3.7	57.4 ± 3.2	75.2 ± 2.3	65.7 ± 3.3	69.4 ± 2.5	55.0 ± 1.4*	71.5 ± 2.6
Amylase (U/L)	2994.0 ± 115.0	2591.0 ± 46.0	2947.0 ± 157.0	2839.0 ± 50.0	3066.0 ± 56.0	2898.0 ± 71.0*	2610.0 ± 103.0	2749.0 ± 130.0
Blood glucose (mg/dl)	175.6 ± 6.3	162.5 ± 5.1	187.5 ± 4.6	142.2 ± 5.6*	174.1 ± 6.6	146.9 ± 5.3	175.5 ± 4.2	143.3 ± 3.1*
Uric acid (mg/dl)	1.3 ± 0.1	2.7 ± 0.6	1.0 ± 0.1	1.7 ± 0.2	1.5 ± 0.1	2.3 ± 0.3	1.2 ± 0.1	2.6 ± 0.5
Urea nitrogen (mg/dl)	46.1 ± 1.5	43.6 ± 2.7	51.0 ± 3.1	47.4 ± 1.7	42.1 ± 0.6	46.6 ± 1.7	41.9 ± 1.8	47.4 ± 1.6
Creatinine (mg/dl)	0.50 ± 0.01	0.54 ± 0.02	0.49 ± 0.01	0.52 ± 0.01	0.53 ± 0.02	0.52 ± 0.01	0.51 ± 0.01	0.53 ± 0.02
Sodium (mequiv./l)	140.3 ± 0.3	140.9 ± 0.2	141.0 ± 0.3	141.1 ± 0.3	141.6 ± 0.1*	140.8 ± 0.6	141.5 ± 0.2*	142.3 ± 0.5
Potassium (mequiv./L)	4.6 ± 0.1	4.8 ± 0.2	4.4 ± 0.1	5.0 ± 0.3	4.7 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	4.7 ± 0.2

The results are expressed as mean ± S.E.M. ($n = 10$). Statistical comparison was performed using Kruskal–Wallis' test followed by Dunns' test.
* $p < 0.05$ when compared to control group.

Table 6
Biochemical parameters of female rats

Biochemical parameters	AE (g/kg)							
	0		0.3		0.6		1.2	
	28 days	90 days	28 days	90 days	28 days	90 days	28 days	90 days
Serum protein (g/dl)	6.2 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.0 ± 0.1	6.5 ± 0.1	6.1 ± 0.1	6.4 ± 0.1
Albumin (g/dl)	3.6 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.9 ± 0.1	3.7 ± 0.1	3.8 ± 0.1
Cholinesterase (U/l)	1060.0 ± 75.0	1645.0 ± 131.0	1082.0 ± 65.0	1678.0 ± 113.0	973.0 ± 48.0	1535.0 ± 141.0	1195.0 ± 48.0	1393.0 ± 67.0
TGL (mg/dl)	45.6 ± 3.0	62.8 ± 4.9	47.7 ± 2.2	62.6 ± 7.3	42.6 ± 3.9	67.3 ± 6.7	45.6 ± 4.5	61.5 ± 4.6
Cholesterol (mg/dl)	69.5 ± 2.8	74.4 ± 4.0	82.5 ± 3.9*	70.5 ± 4.2	73.2 ± 2.3	74.4 ± 3.6	79.0 ± 1.7	75.6 ± 2.9
HDL-cholesterol (mg/dl)	61.2 ± 1.8	62.6 ± 3.2	71.7 ± 2.7*	61.5 ± 3.1	67.7 ± 1.7	64.1 ± 3.1	70.8 ± 1.6*	63.9 ± 2.2
Total bilirrubin (mg/dl)	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
ALP (U/L)	219.5 ± 11.6	184.8 ± 17.9	246.1 ± 20.0	207.6 ± 15.4	244.3 ± 17.5	226.5 ± 11.7	199.5 ± 13.8	236.7 ± 20.1
AST (U/L)	96.5 ± 5.0	137.8 ± 6.7	91.4 ± 6.7	108.8 ± 12.6	91.3 ± 4.6	122.7 ± 8.9	90.8 ± 5.4	127.5 ± 6.2
ALT (U/L)	60.4 ± 2.1	62.9 ± 4.8	58.1 ± 1.6	66.2 ± 6.5	60.2 ± 3.1	74.3 ± 7.9	58.4 ± 2.6	80.0 ± 7.9
Amylase (U/L)	2288.0 ± 99.0	1922.0 ± 97.0	2348.0 ± 77.0	2004.0 ± 83.0	2269.0 ± 65.0	2140.0 ± 114.0	2172.0 ± 58.0	2005.0 ± 87.0
Blood glucose (mg/dl)	169.5 ± 4.7	154.9 ± 3.3	184.8 ± 4.4	153.9 ± 4.2	189.4 ± 5.3*	138.9 ± 4.5*	174.3 ± 4.9	154.4 ± 3.2
Uric acid (mg/dl)	1.3 ± 0.1	2.0 ± 0.2	1.2 ± 0.1	1.2 ± 0.1*	1.1 ± 0.1	2.0 ± 0.2	1.1 ± 0.1	1.9 ± 0.4
Urea nitrogen (mg/dl)	47.4 ± 1.3	44.1 ± 1.9	40.8 ± 1.4*	43.2 ± 2.0	42.6 ± 1.6	49.4 ± 2.9	39.0 ± 1.4*	50.7 ± 1.8*
Creatinine (mg/dl)	0.54 ± 0.01	0.54 ± 0.01	0.55 ± 0.01	0.56 ± 0.01	0.54 ± 0.01	0.57 ± 0.02	0.55 ± 0.01	0.59 ± 0.02
Sodium (mequiv./L)	139.9 ± 0.2	140.9 ± 0.3	140.1 ± 0.3	140.9 ± 0.3	141.0 ± 0.1*	140.2 ± 0.4	141.2 ± 0.1*	141.1 ± 0.4
Potassium (mequiv./L)	4.2 ± 0.1	4.9 ± 0.2	3.8 ± 0.2	4.2 ± 0.2	4.2 ± 0.1	4.4 ± 0.2	4.0 ± 0.1	4.7 ± 0.2

The results are expressed as mean ± S.E.M. ($n = 10$). Statistical comparison was performed using Kruskal–Wallis' test followed by Dunns' test.
* $p < 0.05$ when compared to control group.

even 30 days after withdrawal (satellite groups) of treatment indicated no signs of relevant toxicity. Liver function was determined in order to detect possible hepatic dysfunction, tissue damage or changes in biliary excretion evoked by prolonged exposure to AE. Well known markers of liver function such as levels of serum albumin, cholinesterase, cholesterol and triglycerides were unchanged in animals chronically exposed to AE, clearly demonstrating that liver function was preserved in these animals. Frequently, when hepatic injury occurs, serum aminotransferase (AST and ALT) activity increases. Chronic treatment of female and male animals did not change these parameters indicating that the AE treatment did not induce liver tissue damage. Similarly, no changes in total bilirubin after treatments with the AE indicate that the extract does not alter hepatic metabolism or biliary excretion. Alterations observed in relative liver weight were not correlated with clinical biochemical changes and could not be characterized as a hepatotoxic effect of the AE. Indeed, a previous report showed liver protection against carbon tetrachloride- and acetaminophen-induced liver injury by *Achillea millefolium* (Gagdoli and Mishra, 1995). In the same way, renal function tests were performed to assess the possible nephrotoxicity of chronic treatment with *Achillea millefolium* extract. Besides a slight change in uric acid levels in blood samples from animals exposed to AE for 90 days, none of the other parameters evaluated (urea nitrogen, sodium, potassium and creatinine) were changed in these animals, indicating that renal function was unaffected by AE treatment. Other alterations such as in hematological parameters were found just in female rats subjected to 90-day treatment with AE and were restrict to lymphocyte and neutrophil number. Chronic administration of AE reduced the glycemia rate in both male and female groups (Tables 5 and 6) which was previously observed and correlated with the presence of sesquiterpene lactones in *Achillea millefolium* (Peris et al., 1995). A single intravenous dose of achilleine, an alkaloid isolated from *Achillea millefolium*, reduced the clotting time of rabbits with similar potency than the observed with the AE in our studies (Miller and Chow, 1954).

Any of the changes observed after treatments with AE were not correlated with dose or time of exposure in neither female nor male animals and did not exceed the reference range of variation observed in the course of several experiments performed with control groups with similar number of animals (data not shown) indicating that long-term exposure to AE does not generate relevant abnormalities in rats, at least at doses and periods evaluated.

5. Conclusions

On the basis of the present findings, we conclude that after repeated dose 90-day oral exposure to AE, rats exhibited no treatment-related toxicological or histopathological abnormalities. The doses of AE tested in rats were higher than that anticipated for human consumption. Thus, it is likely that no long-term toxicological risk would occur with the doses of AE commonly consumed by humans. However, this extrapolation should be made with caution, since the real human risk cannot be assessed on the basis of the present study.

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References

Alonso, J.R., 1998. Milenrama. In: Tratado de Fitomedicina: bases clínicas y farmacológicas. Isis, Buenos Aires, pp. 725–729.

ANVISA, Agência Nacional de Vigilância Sanitária, 2000. Resolução, RCD 17. Dispõe sobre o registro de medicamentos fitoterápicos. Diário Oficial da União da República Federativa do Brasil, Brasília, pp. 25–27.

Baggio, C.H., Freitas, C.S., Nhaducue, P.F., Rieck, L., Marques, M.C.A., 2002. Action of crude aqueous extract of leaves of *Achillea millefolium* L. (Compositae) on gastrointestinal tract. Revista Brasileira de Farmacognosia 12, 31–33.

Blumenthal, M., Busse, W.R., Goldberg, A., Gruenwald, J., Hall, T., Riggins, C.W., Rister, R.S. (Eds.), 2000. Yarrow. In: Herbal Medicine Expanded Commission E Monographs. Integrative Medicine Communications, Boston, pp. 419–423.

Boswell-Ruys, C.L., Ritchie, H.E., Brown-Woodman, P.D., 2003. Preliminary screening study of reproductive outcomes after exposure to yarrow in the pregnant rat. Birth Defects Research. Part B. Developmental and Reproductive Toxicology 68, 416–420.

Bradley, P.R. (Ed.), 1992. British Herbal Compendium, vol. 1. British Herbal Medicine Association, Bournemouth, pp. 190–191.

Cáceres, A., 1999. Plantas de Uso Medicinal en Guatemala. Ed. Universitária, Guatemala, pp. 268–270.

Candan, F., Unlu, M., Tepe, B., Daferera, D., Polissiou, M., Sokmen, A., Akpulat, H.A., 2003. Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae). Journal of Ethnopharmacology 87, 215–220.

Chandler, R.F., Hooper, S.N., Hooper, D.L., Jamieson, W.D., Flinn, C.G., Safe, L.M., 1982. Herbal remedies of the maritime Indians: sterols and triterpenes of *Achillea millefolium* L. (yarrow). Journal of Pharmaceutical Sciences 71 (6), 690–693.

Correia, P.M., 1974. Dicionário de plantas úteis do Brasil e das exóticas cultivadas. vol. 5. Rio de Janeiro: Ministério da Agricultura e Instituto Brasileiro de Desenvolvimento Florestal, 687 p.

Dalsenter, P.R., Cavalcanti, A.M., Andrade, A.J., Araujo, S.L., Marques, M.C.A., 2004. Reproductive evaluation of aqueous crude extract of *Achillea millefolium* L. (Asteraceae) in Wistar rats. Reproductive Toxicology 18, 819–823.

Djahanguiri, B., 1969. The production of acute gastric ulceration by indomethacin in the rat. Scandinavian Journal of Gastroenterology 4, 265.

Duke, J.A., 1987. *Achillea millefolium* L. (Asteraceae): yarrow. In: Handbook of Medicinal Herbs. CRC Press, Florida, pp. 9–10.

Gagdoli, C., Mishra, S.H., 1995. Preliminary screening of *Achillea millefolium, Cichorium intybus* and *Capparis spinosa* for antihepatotoxic activity. Fitoterapia 66, 319–323.

Goldberg, A.S., Mueller, E.C., Eigen, E., Desalva, S.J., 1969. Isolation of the anti-inflammatory principles of *Achillea millefolium* (Compositae). Journal of Pharmaceutical Sciences 58, 938–941.

Guédon, B., Abbe, P., Lamaison, J.L., 1993. Leaf and flower head flavonoids of *Achillea millefolium* L. subspecies. Biochemical Systematics and Ecology 21, 607–611.

Haggag, M.Y., Shalaby, A.S., Verzar-Petri, G., 1975. Thin layer and gas-chromatographic studies on the essential oil from *Achillea millefolium*. Planta Medica 27, 361–366.

Hausen, B.M., Breuer, J., Weglewski, J., Rucker, G., 1991. Alpha-peroxyachifolid and other new sensitizing sesquiterpene lactones from

yarrow (*Achillea millefolium* L. Compositae). Contact Dermatitis 24, 274–280.

Kobayashi, T., Ohta, Y., Yoshino, J., Nakazawa, S., 2001. Teprenone promotes the healing of acetic acid-induced chronic gastric ulcers in rats by inhibiting neutrophil infiltration and lipid peroxidation in ulcerated gastric tissues. Pharmacology Research 43, 23–30.

Kokkalou, E., Kokkini, S., Handilou, E., 1992. Volatile constituents of *Achillea millefolium* in relation to their intraspecific variation. Biochemical Systematics and Ecology 20, 665–670.

Kubelka, W., Kastner, U., Glasl, S., Saukel, J., Jurenitsch, J., 1999. Chemotaxonomic relevance of sesquiterpenes within the *Achillea millefolium* group. Biochemical Systematics and Ecology 27, 437–444.

Lin, L.T., Liu, L.T., Chiang, L.C., Lin, C.C., 2002. In vitro anti-hepatoma activity of 15 natural medicines from Canada. Phytotherapy Research 16, 440–444.

Malone, M.H., 1977. Pharmacological approaches to natural products screening and evaluation. In: Wagner, H., Wolf, P. (Eds.), Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity. Springer-Verlag, Berlin, pp. 23–53.

Miller, F.M., Chow, L.M., 1954. Alkaloids of *Achillea millefolium* L.: isolation and characterization of achilleine. Journal of the American Chemical Society 76, 1353–1354.

Mitich, L.W., 1990. Intriguing World of Weeds: Yarrow – the herb of Achilles. Weed Technology 4, 451–453.

Montanari, T., de Carvalho, J.E., Dolder, H., 1998. Antispermatogenic effect of *Achillea millefolium* L. in mice. Contraception 58 (5), 309–313.

OECD, Organization for Economic Co-Operation and Development, 1998. Guideline 408: subchronic oral toxicity – Rodent: 90-days. Paris.

Peris, J.B., Stübing, G., Vanaclocha, B., 1995. Fitoterapia aplicada. Colegio Oficial de Farmacéuticos, Valencia, pp. 374–375.

Robert, A., Nezamis, J.E., Lancaster, C., Hauchar, A.J., 1979. Cytoprotection by prostaglandins in rats. Prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl and thermal injury. Gastroenterology 77, 433–443.

Shahin, M., Konturek, P.W., Pohle, T., Schuppan, D., Herbst, H., Domschke, W., 2001. Remodeling of extracellular matrix in gastric ulceration. Microscopy Research and Techique 15, 53, 396–408.

Takagi, E., Okabe, S., Saziki, R., 1969. A new method for the production of chronic gastric ulcer in rats and the effect of several drugs on healing. Japanese Journal of Pharmacology 19, 416–426.

Tozyo, T., Yoshimura, Y., Sakurai, K., Uchida, N., Takeda, Y., Nakai, H., Ishii, H., 1994. Novel antitumor sesquiterpenoids in *Achillea millefolium*. Chemical & Pharmaceutical Bulletin 42, 1096–1100.

Tunon, H., Olavsdotter, C., Bohlin, L., 1995. Evaluation of anti-inflammatory activity of some Swedish medicinal plants. Inhibition of prostaglandin biosynthesis and PAF-induced exocytosis. Journal of Ethnopharmacology 48, 61–76.

Valant-Vetschera, K.M., Wollenweber, E., 1988. Leaf flavonoids of the *Achillea millefolium* group. Part II. Distribution patterns of free aglycones in leaf exudates. Biochemical Systematics and Ecology 16, 605–614.

WHO, World Health Organization, 1993. Council for International Organizations of Medical Sciences (CIOMS). International Ethical Guidelines for Biomedical Research Involving Human Subjects. Geneva.