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Cytinus hypocistis (L.) L. extract effects in an animal model of papillomavirus-induced neoplasia

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Abstract

Infections with certain types of papillomavirus, such as the human papillomavirus 16 (HPV16), are associated with the development of preneoplastic lesions and cancers of the anogenital, and head and neck regions. *Cytinus hypocistis* (L.) L. extracts are composed of substances presenting antiproliferative, antioxidant, anti-inflammatory and antibacterial properties, which might be promising as new therapeutic compounds. This study analysed the influence of topical application of an extract obtained from *C. hypocistis* (CH) on K14-HPV16 and FVB/n mice to evaluate its therapeutic and toxicological properties. To achieve the study goals, 30 female mice, 33–37 weeks old, were divided into six groups (n=5/group): I (HPV+CH3.1); II (HPV+CH6.2); III (HPV+CH12.4); IV (FVB/n+CH12.4); V (HPV+control) and VI (FVB/n+-control). CH was applied topically to both ears for 28 days. After this period, all animals were sacrificed for samples collection. Skin lesions were classified histologically. Toxicological parameters included haematological and biochemical blood markers, and hepatic oxidative stress analysis. Transgenic animals showed a decrease in mean body weight regardless of the concentration of extract applied. The extract had no influence on physiological parameters, organ weight, or biochemical and oxidative stress parameters. Histology demonstrated the presence of proliferative epithelial lesions in the skin and oral mucosa of K14-HPV16 mice, with no association with the application of this extract. Overall, the application of CH extract had no influence on the skin lesions and was well tolerated by the animals in these concentrations.

Key words: *animal model; mouse; natural compound; toxicology*

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Introduction

Medicinal plants have contributed greatly in the development of formulations/products with therapeutic potential, as many of their extracts exhibit antitumor, anti-inflammatory and antimicrobial properties (Medeiros et al., 2018). Several studies have demonstrated that many of the substances isolated from plants, or their crude extracts, show promising pharmacological properties (Medeiros et al., 2018; Santos et al., 2019). The genus *Cytinus* is composed of rootless, stemless, and leafless parasites. Their vegetative body is reduced to an endophytic system that lives within the roots or stems of the host, from which nutrients and water are absorbed (De Vega et al., 2007, 2009). Four subspecies of *C. hypocistis* (CH) have been recognized, each with a distinct host range (De Vega et al., 2008). This genus occurs in the Mediterranean regions, South Africa and Madagascar (Roquet et al., 2016).

During times of famine, the nectar of CH was sucked and spread over bread (Melcek and Rop, 2011). From a nutritional point of view, pollen, nectar, and petals are the three main components of CH flowers (Silva et al., 2019). The second most important component is the nectar, which contains a balanced mixture of sugars, amino acids, proteins, inorganic ions, lipids, organic acids, phenolic substances, alkaloids and terpenoids. Galoyl-bis-hexahydroxydiphenol (HHDP)-glucose, digaloyl-bis-HHDP-glucopyranose and trigaloyl-bis-HHDP-glucose were the most abundant of the 17 phenolic compounds identified in CH (Silva et al., 2020). CH extracts have antioxidant properties

(Zucca et al., 2015), broad-spectrum microbial inhibition against Gram-positive (Maisetta et al., 2019) and Gram-negative bacterial species, antiproliferative activity in four tumour cell lines (NCI-H460, HeLa, HepG2 and MCF-7) and no toxicity in a non-tumour-cell line (PLP2) (Silva et al., 2020). The reported bioactive properties of CH extracts, especially the antiproliferative activity against the HeLa cell line (cervical carcinoma induced by human papillomavirus) (HPV) highlighted the potential of this plant as a source of natural bioactive compounds for new drug development. Therefore, it is important to conduct studies on its bioactive properties and investigate the therapeutic properties in an animal model that reflects the pathogenesis of cervical carcinoma caused by HPV.

In this context, this study was designed to evaluate the influence of the topical application of a CH extract on K14-HPV16 mice to understand its therapeutic effects and toxicological properties. In this mouse model, the genes of the HPV early region are expressed under the promoter of cytokeratin 14 specifically targeting epithelial basal cells, promoting the development of typical lesions of the various stages of carcinogenesis (Medler et al., 2018), thus providing a suitable experimental model for studying HPV-induced carcinogenesis.

Materials and methods

Sample preparation

Cytinus hypocistis (L.) L. subspecies *macranthus* Wettst plants were collected in July 2018 from the host species *Halimium lasianthum* subspecies *alyssoides* (Lam.) Greuter at three different locations in Castro Daire, Portugal. Plant identification and extract preparation were conducted as described previously (Silva et al., 2021). Briefly, after lyophilisation, plant specimens were milled to a fine powder (~40 mesh) and extracted using Ultrasound-Assisted Extraction (UAE) at the optimum global condition. Three different concentrations of the final extract were then incorporated in a base cream (Versatile™, Base cream evanescent O/W-500 g, Fagron Iberica): CH3.1: 3.1 mg extract (E)/g cream; CH6.2: 6.2 mg E/g cream, and CH12.4: 12.4 mg E/g cream (Silva et al., 2020). These concentrations were selected based on the IC_{50} concentration of the anti-inflammatory activity of *CH* extracts tested in a murine macrophage cell line (RAW 264.7) (Silva et al., 2020). The phenolic compounds' stability in the formulation was monitored by HPLC-DAD-ESI/MS^n (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) throughout the experiment (Taofiq et al., 2018).

Animals

A total of 30 female *Mus musculus* were used: 20 K14-HPV16 transgenic (hemizygotic HPV^+) and ten FVB/n (homozygous HPV^-) mice aged 33-37 weeks old. The K14-HPV16 strain was generously donated by Dr. Jeffrey Arbeit and Dr. Douglas Hanahan, from the University of California, through the USA National Cancer Institute Mouse Repository. Genotyping of animals was performed at the Portuguese Oncology Institute of Porto using previously described methods (Paiva et al., 2015) to confirm that these animals were hemizygotic for HPV16. This experimental study was approved by the University of Trás-os-Montes and Alto Douro (UTAD) Animal Welfare Committee (approval number 852-e-CITAB-2020) and by the Portuguese Veterinary Directorate (approval number 014139-2022). Animals were kept in accordance with Portuguese (Decree-Law 113, August 7) and European (Directive 2010/63/EU) legislation.

Experimental conditions

Animals were kept under controlled experimental conditions of temperature (23±3°C), relative humidity (50±10%) and light-dark cycle (12/12 h light/dark). Throughout the experiment, mice were fed with a standard diet (Diet Standard 4RF21®, Mucedola, Italy) and drank tap water *ad libitum*. The animals were identified individually and housed in hard polycarbonate cages using corncob for bedding (Ultragene, Santa Comba Dão, Portugal) and environmental enrichment was provided with paper rolls. Cages were cleaned and bedding replaced weekly.

Experimental design

Animals were divided into six groups (*n*=5 per group) according to their phenotype. Groups with transgenic animals were exposed to CH in three different concentrations 3.1, 6.2 and 12.4 mg - I (HPV+CH3.1); II (HPV+CH6.2); III (HPV+CH12.4), respectively. Another group with FVB/n animals was exposed to the maximum concentration (IV - FVB/n+CH12.4) and two control (CR) groups of both phenotypes: V (HPV+CR) and VI (FVB/n+CR), in which only the base cream was applied.

CH extract was applied topically to both ears with a spatula to form a thin and homogeneous layer, five days a week over 28 days, at approximately the same time of day and by the same researcher. Animals in the CR groups received application of only the base cream under the same conditions. Humane endpoints were assessed weekly by the same researcher, using a previously published scoring sheet (Oliveira et al., 2017). Animals that reached a total score equal to or greater than four at any time point were designated for euthanasia. Animal weight and food and water consumption were recorded weekly at the same time using a precision scale (KERN® PLT 6200-2A, Dias de Sousa S.A., Alcochete, Portugal).

At the end of the 28-day period, all animals were sacrificed by intraperitoneal administration of an overdose of xylazine and ketamine, followed by cardiac puncture exsanguination, according to the FELASA guidelines (Forbes et al., 2007). Skin samples (ear and chest skin), tongue, and internal organs (spleen, heart, liver, lungs, kidneys, and thymus) were collected. The internal organs were immediately weighed. Liver and ear skin were used for histological analysis and oxidative stress determination. The left kidney was used for histological analysis and the right kidney was used for oxidative stress determination.

Microhematocrit and serum biochemistry

Microhematocrit tubes were centrifuged in a PrO-Vet centrifuge (Centurion, Scientific Limited) at 1200 rpm for 5 min. The ratio (%) of the volume of packed red blood cells to the volume of whole blood was calculated using a ruler.

Blood for biochemical analysis was stored in heparinized tubes, centrifuged at 3000 rpm for 10 min (at 4°C), and the serum was separated and frozen at -80°C until further analysis. The determination of serum concentrations of creatinine, urea, albumin (Alb), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) was performed using spectrophotometric methods using an Autoanalyzer (Prestige 24i, Cornay PZ).

Histopathological analysis

Samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and then 2 μm histological sections were obtained. The slides were stained with haematoxylin and eosin (H&E) and examined by optical microscopy. The chest skin,

ear and tongue samples were histologically classified as normal skin, moderate to marked hyperplasia (increased number of cells and layers of the epidermis/mucosa), dysplasia (morphological change of cells, with moderate cytonuclear atypia), carcinoma *in situ* (CIS), or invasive carcinoma (IC) (Araújo et al., 2018). The liver samples were evaluated for: presence of necrosis, inflammation (assessed as absent, presence of less than five multifocal aggregates of inflammatory cells, or presence of five or more multifocal aggregates of inflammatory cells) and cytomegalocytosis. The kidneys were evaluated for inflammation (assessed as absent, presence of less than five multifocal aggregates of inflammatory cells, or presence of five or more multifocal aggregates of inflammatory cells).

Catalase enzymatic activity

For determining catalase activity, samples (liver, kidney, and ear skin) were homogenized in cold Tris-HCl buffer (10 mM, pH 7.4) using a Potter-Elvehjem homogenizer. The mixture was placed in an ice bath in an ultrasonic processor (2x10s, 10s intermittent) to ensure a higher yield in enzymatic extraction. Post-mitochondrial supernatant was obtained after two cycles of centrifugation (Sigma Laborzentrifugen™ 2-16K centrifuge, Osterode am Harz, Germany) at 4°C: 4000 rpm for 10 min and 16000 rpm for 10 min. The supernatant obtained in the last centrifugation was used to evaluate the catalase enzymatic activity (CAT), which was determined by measuring the decrease in hydrogen peroxide concentration at 240 nm[1]. The reaction mixture (2 mL) consisted of phosphate buffer (50 mM Na_2HPO_4, pH 7.4), 50 µL H_2O_2, (3% *w/v*) and 50 µL sample. The reaction was initiated by adding 50 µL H_2O_2 to the phosphate buffer mixture with 50 µL sample at 30ºC. Enzyme kinetics were monitored spectrophotometrically (Varian Cary® 100 UV-Vis Spectrophotometer) at 240 nm for three minutes. The results of the enzymatic activities were expressed in H_2O_2 µmol/min/mg. Protein was measured at 260 nm and 280 nm using a Take3 Multi-Volume plate (Take3 plate, BioTek Instruments, USA); the final protein amount was determined by the ratio between 260 nm and 280 nm, according to the manufacturer's instructions. All samples were analysed in duplicate using 20 µL tissue at 30°C on a PowerWave XS2 microplate UV/VIS reader (Bio-Tek Instruments, USA).

Statistical analysis

Body weight gain (%) was calculated according to the following formula:

$$\frac{\text{Final body weight-Initial body weight}}{\text{Final body weight}} \text{x } 100$$

Statistical analysis of the scores of the evaluated humane endpoints was performed according to the following equation (Oliveira et al., 2017):

$$\text{Humane endpoints} = \frac{\text{Sum of the total scores for each animal}}{\text{Number of animals in the group}}$$

Statistical analysis was performed using the SPSS program (Statistical Package for Social Sciences, Chicago, IL, USA) version 20. A Chi-squared test was performed to study the distribution of histological lesions among groups. For other variables, a one-way Analysis of Variance (ANOVA) followed by the Bonferroni test was performed. The results were expressed as mean ± standard error (S.E.), and *p* values lower than 0.05 were considered statistically significant.

Table 1. Humane endpoints mean scores *per* group (mean ± S.E.).

Group (*n*=5)	Group I (HPV+CH3.1)	Group II (HPV+CH6.2)	Group III (HPV+CH12.4)	Group IV (FVB/n+CH12.4)	Group V (HPV+CR)	Group VI (FVB/n+CR)
Humane endpoints' scores	2.160±0.407	2.680±0.120	1.880±0.287 [a]	0.000±0.000	1.880±0.393 [b]	0.000±0.000

[a] Statistically different from group IV ($p<0.005$); [b] Statistically different from group VI ($p<0.05$); CR - Control; CH3.1 - 3.1 mg E/g base cream; CH6.2 - 6.2 mg E/g base cream; CH12.4 - 12.4 mg E/g base cream; HPV - human papillomavirus.

Results

General results

The stability of the phenolic compounds incorporated in the base cream was preserved during the *in vivo* experiments.

A total score of humane endpoints equal to or greater than four was not reached during the study (Supplementary Table S1). The animals showed no signs of behavioural changes and no mortality was observed. The mean scores and the respective statistical analysis of the humane endpoints *per* group can be seen in Table 1. The transgenic animals showed higher mean scores when compared with FVB/n animals.

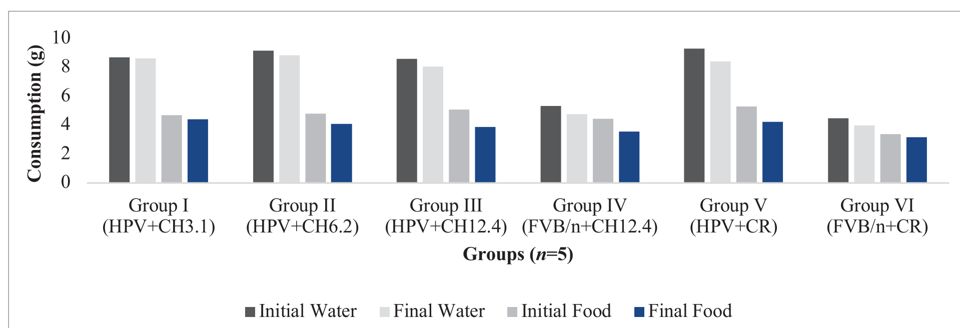

Figure 1. Means of food and water consumption (g) at the beginning and at the end of the experiment. Statistically significant differences were not found ($p>0.05$). CR - Control; CH3.1 - 3.1 mg E/g base cream; CH6.2 - 6.2 mg E/g base cream; CH12.4 - 12.4 mg E/g base cream; HPV - human papillomavirus.

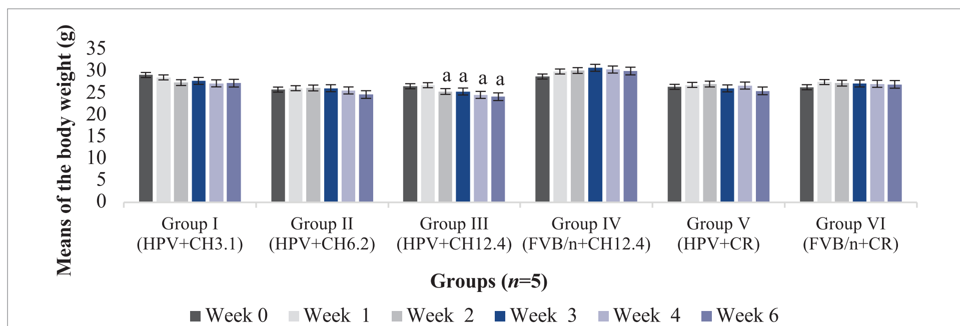

Figure 2. Mean ±S.E. (g) of the body weight of the animals during the trial. (a) Statistically different from group IV ($p<0.005$). CR - Control; CH3.1 - 3.1 mg E/g base cream; CH6.2 - 6.2 mg E/g base cream; CH12.4 - 12.4 mg E/g base cream; HPV - human papillomavirus.

Supplementary Table S1. Total score of humane endpoints for each experimental group.

Group (*n*=5)		Body Condition							Coat and grooming							Eyes, Ears, and Whiskers					
		S0	S1	S2	S3	S4	S5		S0	S1	S2	S3	S4	S5		S0	S1	S2	S3	S4	S5
I (HPV+CH3.1)	0	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	0	5(100%)	5(100%)	1(20%)				0	3(60%)	3(60%)	2(40%)	2(40%)	1(20%)	1(20%)
	1							1			4(80%)	5(100%)	5(100%)	5(100%)	1	2(40%)	2(40%)	3(60%)	3(60%)	4(80%)	4(80%)
	2							2							2						
	3							3							3						
II (HPV+CH6.2)	0	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	0	5(100%)	5(100%)					0	1(20%)	1(20%)	1(20%)	1(20%)		
	1							1			5(100%)	5(100%)	5(100%)	5(100%)	1	4(80%)	4(80%)	4(80%)	4(80%)	5(100%)	5(100%)
	2							2							2						
	3							3							3						
III (HPV+CH12.4)	0	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	0	5(100%)	5(100%)	1(20%)		1(20%)		0	2(40%)	2(40%)	2(40%)	2(40%)	1(20%)	1(20%)
	1							1			4(80%)	5(100%)	4(80%)	5(100%)	1	3(60%)	3(60%)	3(60%)	3(60%)	4(80%)	4(80%)
	2							2							2						
	3							3							3						
IV (FVB/n+CH12.4)	0	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	0	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	0	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)
	1							1							1						
	2							2							2						
	3							3							3						
V (HPV+CR)	0	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	0	5(100%)	5(100%)	1(20%)				0	2(40%)	2(40%)	1(20%)	1(20%)	1(20%)	1(20%)
	1							1			4(80%)	5(100%)	5(100%)	5(100%)	1	3(60%)	3(60%)	4(80%)	4(80%)	4(80%)	4(80%)
	2							2							2						
	3							3							3						
VI (FVB/n+CR)	0	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	0	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	0	2(40%)	2(40%)	1(20%)	1(20%)	1(20%)	1(20%)
	1							1							1	3(60%)	3(60%)	4(80%)	4(80%)	4(80%)	4(80%)
	2							2							2						
	3							3							3						

Transgenic animals consumed more water than FVB/n mice, but no differences were observed for food consumption ($P>0.05$) (Figure 1).

Figure 2 shows the variation of the mean body weight for each group during the protocol. From the second week until the end of the experiment, animals from group III (HPV+CH12.4) had a significantly lower body weight when compared with animals from group IV (FVB/nCH12.4) ($P<0.05$).

The body weight gain for each experimental group is displayed in Figure 3. Regardless of the extract concentration, all animals from the transgenic groups (HPV animals) treated with CH extract (groups I, II and III) cream or CR base cream (group V) showed a negative mean of body weight gain.

Table 2 shows the relative mean weight of internal organs for the different groups in the study. There was a statistically significant increase of kidney weight between group III (HPV+CH12.4) and group IV (FVB/n+CH12.4) ($P<0.05$). Statistically significant differences were not found in the weight of the remaining organs ($P>0.05$).

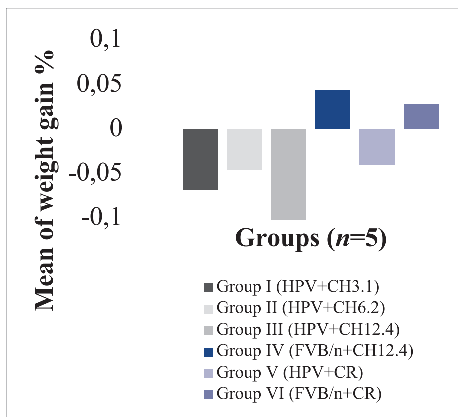

Figure 3. Mean body weight gain (%) in all experimental groups. CR - Control; CH3.1 - 3.1 mg E/g base cream; CH6.2 - 6.2 mg E/g base cream; CH12.4 - 12.4 mg E/g base cream; HPV - human papillomavirus.

Microhematocrit and serum biochemical parameters

The microhematocrit (%) and serum biochemical parameters are shown in Table 3. No significant differences were found between groups treated with CH extract and the control groups ($P>0.05$).

Histopathological analysis

The results of the histological analysis of chest and ear skin, and tongue samples

Table 2. Relative mean weight (g) of the internal organs (mean ± S.E.).

Group (*n*=5)	Thymus	Heart	Lungs	Spleen	Liver	Right kidney	Left kidney
Group I (HPV+CH3.1)	0.0005 ± 0.0003	0.005 ± 0.0003	0.006 ± 0.0003	0.006 ± 0.0007	0.054 ± 0.0018	0.0065 ± 0.0004	0.006 ± 0.0003
Group II (HPV+CH6.2)	0.0014 ± 0.0004	0.004 ± 0.0003	0.007 ± 0.0005	0.008 ± 0.0009	0.062 ± 0.0025	0.0066 ± 0.0003	0.006 ± 0.0004
Group III (HPV+CH12.4)	0.0018 ± 0.0005	0.005 ± 0.0009	0.007 ± 0.0004	0.007 ± 0.0017	0.058 ± 0.0024	0.0077± 0.0003 [a]	0.006 ± 0.0002 [a]
Group IV (FVB/n+CH12.4)	0.0020 ± 0.0003	0.003 ± 0.0003	0.006 ± 0.0003	0.003 ± 0.0002	0.051 ± 0.0020	0.0056± 0.0002	0.004 ± 0.0003
Group V (HPV+CR)	0.0014 ± 0.0006	0.005 ± 0.0003	0.006 ± 0.0003	0.006 ± 0.0007	0.054 ± 0.0029	0.0065± 0.0005	0.006 ± 0.0002
Group VI (FVB/n+CR)	0.0023 ± 0.0003	0.004 ± 0.0001	0.006 ± 0.0004	0.003 ± 0.0002	0.045 ± 0.0018	0.0061± 0.0003	0.005 ± 0.0002

[a] Statistically different from group IV ($p<0.005$). CR - Control; CH3.1 - 3.1 mg E/g base cream; CH6.2 - 6.2 mg E/g base cream; CH12.4 - 12.4 mg E/g base cream; HPV - human papillomavirus.

Table 3. Microhematocrit (%) and serum biochemical parameters (mean ± S.E.).

Group (*n*=5)	Group I (HPV+CH3.1)	Group II (HPV+CH6.2)	Group III (HPV+CH12.4)	Group IV (FVB/n+CH12.4)	Group V (HPV+CR)	Group VI (FVB/n+CR)
Ht (%)	43.62 ± 0.85	40.93 ± 1.44	41.48 ± 1.61	43.30 ± 1.30	42.90 ± 0.53	45.33 ± 0.25
Albumin (g/L)	27.30 ± 1.04	22.94 ± 3.00	23.70 ± 4.02	27.66 ± 3.31	30.12 ± 0.48	32.04 ± 0.85
Creatinine (mg/dl)	0.54 ± 0.83	0.27 ± 0.16	0.52 ± 0.31	0.27 ± 0.07	0.36 ± 0.15	0.72 ± 0.31
Urea (mg/dl)	46.42 ± 2.52	47.96 ± 2.63	60.20 ± 6.14	56.44 ± 4.88	70.12 ± 7.12	51.84 ± 6.13
ALT (U/L)	33.76 ± 5.14	36.60 ± 4.83	35.90 ± 5.93	36.60 ± 5.59	33.74 ± 3.52	27.88 ± 3.15
AST (U/L)	46.76 ± 6.55	47.42 ± 2.71	37.64 ± 4.67	39.54 ± 2.65	32.50 ± 3.54	29.80 ± 4.03

ALT - alanine aminotrsnfersase; AST - aspartate aminotransferase; CR - Control; CH3.1 - 3.1 mg E/g base cream; CH6.2 - 6.2 mg E/g base cream; CH12.4 - 12.4 mg E/g base cream; HPV - human papillomavirus; Ht - hematocrit. Statistically significant differences were not found ($p>0.05$).

Table 4. Incidence of skin lesions in each experimental group, number (%).

Group (*n*=5)		Group I (HPV+CH3.1)	Group II (HPV+CH6.2)	Group III (HPV+CH12.4)	Group IV (FVB/n+CH12.4)	Group V (HPV+CR)	Group VI (FVB/n+CR)
Ear skin	Normal	0 (0%)	0 (0%)	0 (0%)	5 (100%)	0 (0%)	5 (100%)
	Hyperplasia	5 (100%)	5 (100%)	5 (100%) [a]	0 (0%)	4 (80%) [b]	0 (0%)
	Dysplasia	5 (100%)	5 (100%)	5 (100%) [a]	0 (0%)	4 (80%) [b]	0 (0%)
	CIS	5 (100%)	5 (100%)	4 (80%) [a]	0 (0%)	2 (40%)	0 (0%)
	IC	0 (0%)	0 (0%)	1 (20%)	0 (0%)	1 (20%)	0 (0%)
Chest skin	Normal	0 (0%)	0 (0%)	0 (0%)	4 (80%)	0 (0%)	5 (100%)
	Hyperplasia	5 (100%)	5 (100%)	5 (100%) [a]	1 (20%)	5 (100%) [b]	0 (0%)
	Dysplasia	4 (80%)	5 (100%)	5 (100%) [a]	0 (0%)	4 (80%) [b]	0 (0%)
	CIS	4 (80%)	5 (100%)	4 (80%) [a]	0 (0%)	2 (40%)	0 (0%)
	IC	1 (20%)	1 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Tongue	Normal	0 (0%)	0 (0%)	0 (0%)	5 (100%)	0 (0%)	5 (100%)
	Hyperplasia	4 (80%)	5 (100%) [b]	5 (100%) [a]	0 (0%)	2 (40%)	0 (0%)
	Dysplasia	1 (20%)	2 (40%)	1 (20%)	0 (0%)	0 (0%)	0 (0%)
	CIS	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
		1 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

[a] Statistically different from group IV ($p<0.005$); [b] Statistically different from group VI ($p<0.005$). CR - Control; CH3.1 - 3.1 mg E/g base cream; CH6.2 - 6.2 mg E/g base cream; CH12.4 - 12.4 mg E/g base cream; HPV - human papillomavirus; CIS - carcinoma *in situ*; IC - invasive carcinoma.

are summarised in Table 4. Macroscopically, these lesions were observed as crusting and thickened multifocal skin areas associated with erythema, predominantly in the auricular pavilions and cephalic region. Epidermal hyperplasia and high-grade dysplasia were observed in the transgenic groups (groups I, II, III and V), but not in control animals (groups IV and VI).

Moderate to marked hyperplasia of the epidermis, in most cases associated with foci of dysplasia and CIS, was observed in the animals of the treated and untreated transgenic groups (groups I, II, III and V).

Table 5. Histological classification of liver and kidney samples.

		Group (*n*=5)	Group I (HPV+CH3.1)	Group II (HPV+CH6.2)	Group III (HPV+CH12.4)	Group IV (FVB/n+CH12.4)	Group V (HPV+CR)	Group VI (FVB/n+CR)
Liver	Inflammatory Infiltrate	Absent	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)	1 (20%)
		<5 AL	0 (0%)	2 (40%)	3 (60%)	5 (100%)	3 (60%)	4 (80%)
		≥5 AL	5 (100%) [a]	3 (60%)	2 (40%)	0 (0%)	1 (20%)	0 (0%)
	Necrosis		4 (80 %) [a]	3 (60%)	3 (60%)	1 (20%)	0 (0%)	0 (0%)
	Cytomegalocytosis		0 (0%) [b]	3 (60%)	4 (80 %) [c]	0 (0%)	1 (20%)	3 (60%)
Left kidney	Inflmmatory Infiltrate	Absent	1 (20%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)	0 (0%)
		<5 AL	1 (20%)	2 (40%)	3 (60%)	5 (100%)	2 (40%)	4 (80%)
		≥5 AL	3 (60%)	2 (40%)	2 (40%)	0 (0%)	2 (40%)	1 (20%)

[a] Statistically different from group V ($p<0.05$); [b] Statistically different from group III ($p<0.05$); [dc] Statistically different from group IV ($p<0.05$). CR - Control; CH3.1 - 3.1 mg E/g base cream; CH6.2 - 6.2 mg E/g base cream; CH12.4 - 12.4 mg E/g base cream; <5 AL - less than five lymphoid aggregates; ≥5 AL - five or more lymphoid aggregates; HPV - human papillomavirus.

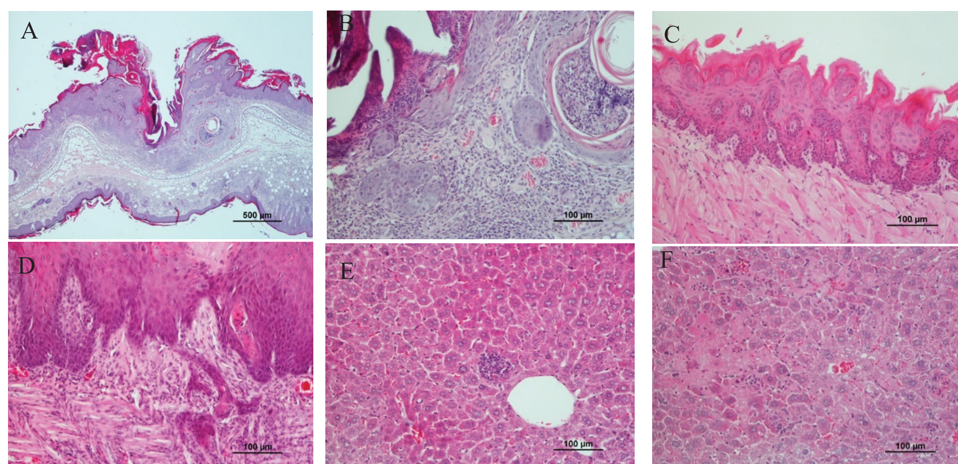

Figure 4. Lesions observed in K14-HPV16 mice: ear skin characterized by the presence of invasive carcinoma, characterized by malignant proliferation of epithelial cells of the epidermis, with the invasion of the adjacent dermis at depth. The adjacent epidermis showed marked hyperplasia of the epidermis, and the dermis showed marked inflammation (HPV+CR) (A and B); Hyperplasia of the tongue mucosa (HPV+CH3.1) (C); Tongue characterized by the presence of invasive carcinoma (HPV+CH3.1) (D); Liver characterized by the presence of an inflammatory infiltrate focus (HPV+CH6.2) (E); Liver characterized by focal necrosis (HPV+CH6.2) (F). Hematoxylin and eosin (H&E) staining.

One animal from group IV (HPV+CH12.4) and one animal from group V (HPV+CR) had an IC in the ear skin (Figures 4A and B). Mixed inflammatory infiltrates in the adjacent dermis and sebaceous gland hyperplasia were also frequently observed in these animals. Regarding the tongue, the treated transgenic animals (groups I, II and III) often showed lesions of moderate hyperplasia. One animal from group I (HPV+CH3.1) showed an IC (Figures 4C and D). Control animals treated and untreated (groups IV and VI) did not exhibit changes in the ear skin or the tongue,

Table 6. Oxidative stress parameters (mean ± S.E.).

Group (*n*=5)		Group I (HPV+CH3.1)	Group II (HPV+CH6.2)	Group III (HPV+CH12.4)	Group IV (FVB/n+CH12.4)	Group V (HPV+CR)	Group VI (FVB/n+CR)
CAT (H202 µmol /min/mg)	Liver	479.16 ± 20.05	484.56 ± 50.27	439.81 ± 56.74	367.46 ± 21.86	465.24 ± 47.31	353.45 ± 47.65
	Right kidney	69.44 ± 2.86	69.18 ± 3.98	70.62 ± 10.80	67.23 ± 3.81	70.46 ± 6.60	68.20 ± 16. 84
	Ear	17.77 ± 6.76	17.27 ± 1.00	17.66 ± 2.44	16.59 ± 2.63	17.30 ± 3.57	17.08 ± 3.71

CAT - catalase; CR - Control; CH3.1 - 3.1 mg E/g base cream; CH6.2 - 6.2 mg E/g base cream; CH12.4 - 12.4 mg E/g base cream; HPV - human papillomavirus. Statistically significant differences were not found ($p>0.05$).

though one control animal (FVB/n+CH 12.4) showed moderate epidermal hyperplasia on the chest skin.

The histological lesions identified in the left kidney and liver are shown in Table 5. It was possible to observe that the application of CH extract did not influence the percentage of lesions identified in the kidneys and liver, because the transgenic animals (groups I, II, III and V) developed spontaneous lesions regardless of the concentration of the extract applied.

The K14-HPV16 mice showed multifocal inflammatory infiltrate (Figure 4E) and areas of necrosis in the liver (Figure 4F), while the FVB/n animals did not show changes (except for one treated animal from group IV that showed areas of necrosis in the liver). The hepatocyte size increase was frequently observed in both transgenic and FVB/n groups, regardless of treatment. Foci of mononuclear inflammatory infiltrate were also commonly observed in the kidneys. Furthermore, no differences were observed in the kidney or the liver between FVB/n and control groups that could be associated with the application of the extract.

Catalase enzymatic activity

The CAT in the liver, kidney and ear did not differ significantly among the groups ($P>0.05$). Despite this, CAT activity was slightly higher in FVB/n mice when compared with K14-HPV16 mice ($P>0.05$) (Table 6).

Discussion

Medicinal plants have contributed to the development of new therapeutic strategies through the use of their compounds and secondary metabolites. The antioxidant, antibacterial and antiproliferative properties of CH extract *in vitro* have been previously reported (Magiatis et al., 2001; Zucca et al., 2015), but have not previously been evaluated in an animal model. This extract has previously been shown to contain a total of 17 phenolic compounds, with galloyl-bis-HHDP-glucose as most abundant (Silva et al., 2020). In the K14-HPV16 transgenic mice model, the expression of HPV16 early region genes is driven by the cytokeratin 14 promoter/enhancer, specifically targeting epithelial basal cells, including the key drivers of malignant transformation E6 and E7, to basal epithelial cells in keratinized squamous epithelia (Arbeit et al., 1996; Paiva et al., 2015). The HPV16 E6 and E7 oncoproteins induce the degradation of the cellular p53 and retinoblastoma proteins, allowing unchecked proliferation, survival, and accumulation of genetic mutations and driving carcinogenesis (Hoppe-Seyler et al., 2018). Consequently, these animals develop multi-step lesions associated with

progressive inflammation, such as hyperplasia, dysplasia, CIS and IC in the skin of the chest and ears (Santos et al., 2017), and have also been used to study cervical (Arbeit et al., 1996), oropharyngeal (Mestre et al., 2020), penile (Medeiros-Fonseca et al., 2020) and anal (Stelzer et al., 2010) carcinogenesis. This model is well characterised, facilitating the study of genetic and epigenetic factors that coordinate malignant conversion, regulate neoplastic progression, and evaluating different therapeutic approaches. To the extent of the authors' knowledge, this is the first experimental study to evaluate the effects of CH extract in a murine model. Therefore, the main goal of this study was to assess the efficacy of topical application of the CH extract against cutaneous lesions of K14-HPV16 transgenic mice and to evaluate its toxicity *in vivo*.

During the experimental, we evaluated several physiological and behaviour parameters. No mortality was recorded, and there were no changes in mouse behaviour or other clinical symptoms associated with toxicity. These results are interesting, since signs of toxicity have been commonly described with the consumption or administration of natural compounds, particularly in terms of mortality and behavioural changes (Oliveira et al., 2017; Dennis, 2000). Thus, the CH extract application at these concentrations did not cause noticeable toxicity. No animals reached the critical limit of four and no changes in animal physiology were observed to justify their euthanisation prior to the scheduled end date. The parameter that showed the most change in this study was coat appearance and grooming. Self-cleaning in animals is an innate behaviour involved in hygiene maintenance and other physiologically essential processes, such as thermoregulation, and social and stimulus communication (Kaleuff et al., 2016; Islam et al., 2022). All transgenic animals showed a decrease in self-cleaning after the first application of the cream, and HPV1+CH6.2 animals showed a greater lack of grooming than the other groups, which contributed to a higher mean endpoint score. Food consumption of the animals remained constant regardless of the group and the extract concentration used. However, as expected, the transgenic animals showed a higher water consumption compared to the FVB/n, because K14-HPV16 mice dehydrate due to the HPV-developed epithelial lesions, which compromise the barrier functions of the skin (Gil da Costa et al., 2017); therefore, this higher water consumption is related to the phenotype of these animals and not to the concentration of extract applied.

Concerning mean body weight, we observed that animals from the HPV+CH12.4 group showed a significant decrease in mean body weight when compared to animals from FVB/n+CH12.4 after the second week of CH application. This decrease in body weight is due to phenotype, since the same extract concentration was applied to both groups. This means that it was not the CH extract that interfered with body weight variation, but the fact that these animals were transgenic and showed clinical signs of HPV16 oncogenes. Significant differences were observed in the mean weight of internal organs. The HPV+CH12.4 animals (group III) showed a significant increase in the mean relative weight of the kidneys compared to FVB/n+CH12.4 animals (group IV), suggesting that this difference is phenotype-based and not due to CH. No statistically significant differences were observed in the mean weight of the liver and other organs. A comparison of the mean organ weight between treated and untreated animals is used to predict the compound's toxic effect and identify the

organs most severely affected by exposure (Wolfsegger et al., 2009).

The CH extract did not induce detectable hepatotoxicity at the biochemical or histological levels. The liver was selected for histological observation because this organ has been previously described to suffer severe inflammation in this animal model (Roquet et al., 2016; Medeiros-Fonseca et al., 2018) and it is thus particularly susceptible to additional toxic damage. Hepatomegaly is a typical finding for this strain (Gil da Costa et al., 2017) reflecting chronic hepatic inflammation. The analysis of the histological results of the liver and kidney allowed for identification of the presence of inflammatory infiltrate in both organs of animals of different groups exposed to different concentrations of the tested cream. This mouse strain shows particular fragilities, such as chronic hepatic and liver inflammation, which were not aggravated by CH. Determining liver and kidney function is critical to understanding the impact of laboratory animal exposure to different compounds and determining whether or not they have associated toxicity (Pizzo et al., 2015). In this study, the liver (ALT, AST, and Alb) and the kidney (creatinine and urea) showed no differences among the groups. The microhematocrit values were similar among groups. This means that this extract did not influence the total number of red blood cells. Similar results have been reported in other studies evaluating natural compounds in this animal model (Almeida et al., 2021; Santos et al., 2019). The evolution of skin lesions in this animal model is progressive. Progression from epithelial hyperplasia to dysplasia typically occurs between 20 and 30 weeks of age (Medeiros-Fonseca et al., 2018). In this context, due to the advanced age of our K14-HPV16 animals (33-37 weeks), CIS and IC were also observed in the ear and chest skin, and in the tongue, regardless of whether the extract was applied or not. The tongue was collected to assess the effects of CH extract due to self-cleaning by animals. A similar percentage of skin lesions among groups revealed that the application of this extract did not stop the evolution of these lesions, nor did it have a promoting effect. When applied to the skin of healthy animals, the extract did not induce the development of skin lesions, further supporting the hypothesis that it was safe in these experimental conditions.

The topical application of this extract did not influence the evaluated oxidative stress parameters. However, transgenic animals showed higher values of CAT enzyme activity when compared to FVB/n animals. This was likely because papillomavirus increases the activity of protective mechanisms, such as CAT (Camini et al., 2017; Georgescu et al., 2018). Also, chronic inflammation associated with papillomavirus leads to the production and release of reactive oxygen species (ROS), and the expression of viral oncoproteins induces oxidative stress (Calaf et al., 2018; Erturan et al., 2019). Accordingly, the differences, although not significant between the transgenic and FVB/n groups, can be explained by the fact that cancer cells exhibit higher ROS levels than normal cells. Again, the application of this extract was observed to be safe.

Conclusion

The application of CH extract under the described conditions was safe, and no signs of toxicity were observed. The percentage of CIS lesions was lower in animals treated with CH. Different concentrations of CH and during a longer period of exposure should be evaluated in future research.

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Učinci ekstrakta *Cytinus hypocistis* (L.) L. u životinjskom modelu neoplazije inducirane papiloma virusom

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Infekcije određenim vrstama papiloma virusa, poput humanog papiloma virusa 16 (HPV16), povezane su s razvojem preneoplastičnih lezija i karcinoma anogenitalnog područja i područja glave i vrata. Ekstrakti *Cytinus hypocistis* (CH) sadrže tvari koje pokazuju antiproliferativna, antioksidativna, protuupalna i antibakterijska svojstva te bi stoga mogle predstavljati nove, obećavajuće terapijske spojeve. Cilj je ovog rada bio analizirati utjecaj topikalne primjene ekstrakta dobivenog iz *C. hypocistis* (L.) L. na K14-HPV16 i FVB/n miševima za procjenu njegovih terapijskih i toksikoloških svojstava. Za postizanje ciljeva istraživanja, trideset ženki miševa starosti 33-37 tjedana podijeljeno je u šest skupina (*n*=5/skupini): I (HPV+CH3,1); II (HPV+CH6,2); III (HPV+CH12,4); IV (FVB/n+CH12,4); V (HPV+kontrola) i VI (FVB/n+kontrola). CH je tijekom 28 dana topikalno primijenjen na oba uha. Nakon tog razdoblja sve životinje su žrtvovane u svrhu prikupljanja rezultata. Lezije kože su histološki klasificirane. Toksikološki parametri uključivali su hematološke i biokemijske markere krvi te analizu oksidativnog stresa jetre. Transgenične životinje pokazale su smanjenje srednje tjelesne mase, bez obzira na primijenjenu koncentraciju ekstrakta. Ekstrakt nije utjecao na fiziološke parametre, masu organa ili parametre biokemijskog i oksidativnog stresa. Histološki je dokazana prisutnost proliferativnih epitelnih lezija na koži i oralnoj sluznici K14-HPV16 miševa, bez povezanosti s primjenom ovog ekstrakta. Općenito, primjena CH ekstrakta nije utjecala na lezije kože te su ga životinje dobro podnosile u primijenjenim koncentracijama.

Ključne riječi: životinjski *model, miš, prirodni spoj, toksikologija*