The role of natural compounds in rat mammary cancer: the beneficial effects of *Santolina chamaecyparissus* L. aqueous extract

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Abstract

Breast cancer is the most diagnosed cancer among women, and a leading cause of death worldwide. *Santolina chamaecyparissus* L. is a plant with multiple health benefits, including anticancer and anti-diabetic properties. This study aimed to assess the chemopreventive effects of *S. chamaecyparissus* aqueous extract (SCE) in an animal model of mammary cancer. A total of 28 four-week-old female Wistar rats were divided into four groups: control, MNU-induced (IND), SCE-supplemented (SCE), and SCE+IND. SCE was added to drinking water (12.72 mg/kg body weight) *ad libitum*. MNU was administered via the intraperitoneal route at 50 days of age. Weekly monitoring of body weight, food/drink intake, humane endpoints, and number of mammary tumours were recorded. Twenty weeks after MNU administration, animals were sacrificed by anaesthetic overdose and a necropsy was performed. Blood samples were used to determine blood count and serum biochemistry analysis, while kidney and liver samples were analysed for oxidative stress. Tumour samples were collected for gene expression and histology studies. SCE chemical composition was analysed by LC-MS and contained 19 phenolic compounds, with the most abundant being myricetin-*O*-glucuronide and 1,3-*O*-dicaffeoylquinic acid. Two animals in the IND group were sacrificed due to exceeding the humane endpoint limits. SCE supplementation delayed mammary tumour development, reducing its volume and weight. SCE had a positive impact on haematological parameters, particularly the neutrophil-lymphocyte ratio (P=0.026). No significant differences were observed in serum biochemistry, except for creatinine kinase MB, or in oxidative stress markers. Gene expression analysis showed significantly reduced *VEGF* expression levels (P=0.0158) in tumours from SCE+IND. These findings suggest that SCE is deserving of further study to identify the individual compounds and to understand its influence on animal models during cancer development.

Key words: *Breast cancer; Wistar rats; Santolina chamaecyparissus; Chemoprevention; Animal model*

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Introduction

In vitro and *in vivo* studies with specific dietary extracts obtained from natural sources have been shown to interfere with molecular mechanisms and signalling pathways linked to the different stages of breast cancer development (Mokbel and Mokbel, 2019). Most of these extracts demonstrate inhibitory effects on tumour growth, angiogenesis, proliferation, invasion and metastasis (Ko and Moon, 2015). Natural compounds are considered advantageous for cancer chemoprevention because they have fewer side effects, lower toxicity and provide a sustainable and cost-effective alternative to synthetic drugs (Ko and Moon, 2015).

Santolina chamaecyparissus L., commonly known as cotton-lavender, is a small, evergreen plant native to the Mediterranean region, Europe, and America (Tundis and Loizzo, 2018). It belongs to the Asteraceae family and has greyish leaves and yellow inflorescences that bloom in the summer (Saygideger et al., 2021a). Despite limited studies, researchers have reported its analgesic (Giner et al., 1988), anti-cancer (Saygideger et al., 2021b), anti-inflammatory (Djarmouni et al., 2018), antidiabetic (Ali et al., 2021), antimicrobial (AlMotwaa and Al-Otaibi, 2022), antioxidant and hepatoprotective (Messaoudi et al., 2018) properties.

Evaluating new therapies against breast cancer often involves the use of animal models (Faustino-Rocha et al., 2017), namely by administering *N*-methyl-*N*-nitrosourea (MNU) to female rats. The induced mammary tumours resemble their human counterparts in morphology, histopathology, and molecular signatures (Boix-Montesinos et al., 2021), making them useful for evaluating chemopreventive compounds (Mollard et al., 2011).

The main aim of this study was to assess the chemopreventive effects of a *Santolina chamaecyparissus* L. aqueous extract (SCE) in the development of mammary cancer induced by *N*-methyl-*N*-nitrosourea in female Wistar rats.

Material and methods

Preparation of Santolina chamaecyparissus aqueous extract

Extraction

S. chamaecyparissus aqueous extract preparation was performed with the macer-

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ation of plant material (1 g) with 30 mL water at 25ºC and 150 rpm, during 1 h. It was then filtered through Whatman No. 4 paper and the residue was once again extracted with an additional portion (30 mL) of water. The extract was concentrated at 35ºC under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then lyophilised (FreeZone 4.5, Labconco, Kansas City, MO, USA).

Chemical characterisation

Chemical characterisation was performed in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) as previously described by Barros et al. (2013). Compound detection was performed using a Diode Array Detector (DAD, at 280, 330 and 370 nm) and an Ion Trap Linear LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) with an electrospray ionisation (ESI) source. Data acquisition, processing and interpretation were performed with Xcalibur software version 2.2 (ThermoFinnigan, San Jose, CA, USA). For the identification of compounds, retention time (Rt), wavelength of maximum absorption (λmax), pseudomolecular ion ($[M-H]^-$), UV-Vis spectra, mass spectra and patterns of the ion breakdown (MS^2) were compared with the literature and commercially available standards. For component quantification (expressed in µg/mL of extract), calibration curves ($R^2 \geq 0.999$) obtained from available standards were used. The stability of drinking water was evaluated during five consecutive days, at room temperature in the light. In this study, the aqueous extract was prepared at the feeding concentration and analysed daily through an LC-DAD-ESI/MS system, to detect the degradation of any compounds.

Animals

This study was conducted in accordance with the Portuguese (Decree-Law No. 113/2013) and European (Directive 2010/63/EU) legislation on the protection of animals used for experimental purposes. Ethical issues were reviewed by an Ethics Review Body ("*Órgão Responsável pela Bem-Estar e Ética Animal*"), under reference 834-e-CITAB-2020, and by *Direção Geral de Alimentação e Veterinária* (DGAV), under reference 04583.

A total of 28 four-week-old Wistar female rats (*Rattus norvegicus*) were obtained from Envigo RMS Spain S.L. (Spain). The exact date of birth for the IND and SCE+IND group animals was known, since carcinogen administration must be performed at 50 days of age (Russo and Russo, 1998). Animals were kept at the animal facilities of the University of Trás-os-Montes and Alto Douro, under controlled conditions of temperature (23±2°C), humidity (50±10%), air system filtration (10-20 ventilations/hour) and on a 12h:12h light:dark cycle. A standard diet (4RF21, Mucedola, Italy) and water were provided *ad libitum* and animals were weighed weekly to estimate food and water consumption.

Experimental procedures

After one week of acclimation, during which the animals were handled daily, animals were randomly assigned into four groups (n=7/group): Control (CTRL), MNU-induced (IND), SCE-supplemented (SCE) and SCE-supplemented and MNU-induced (SCE+IND). SCE efficacy was previously evaluated using the MCF-7 cell line (breast cancer cell line) and the GI_{50} was 60 µg/mL. Animals in the SCE groups received SCE at a dose of 12.72 mg/kg body weight (b.w.) dissolved in drinking water for 140 days.

N-methyl-N-nitrosourea induction protocol

At seven weeks of age (50 days old), animals from the IND and SCE+IND groups

were administered the carcinogen *N*-methyl-*N*-nitrosourea (MNU) by the intraperitoneal route (50 mg/kg). MNU (Fluorochem, UK) was dissolved in saline solution (NaCl 0.9%, B. Braun, Germany) and administered within one hour of preparation. Non-induced groups were injected intraperitoneally with 0.5 mL saline solution (NaCl 0.9%).

Humane endpoints

A previously established table of humane endpoints was used weekly to assess animal well-being (Faustino-Rocha et al., 2019), using evaluation parameters such as body condition, body weight, body posture, hair appearance, grooming, colour of the mucous membranes, eyes, ears, mental status, responsiveness to external stimuli, and hydration status were registered. Each parameter was assigned a score ranging from zero to three and animals were sacrificed if they reached a total score of four (Silva-Reis et al., 2021).

Necropsy

After being fasted for twelve hours, animals were sacrificed through an administration of ketamine (75 mg/kg, Imalgene 1000, Merial SAS, Lyon, France) and xylazine (10 mg/kg, Rompun 2%, Bayer Healthcare S.A., Kiel, Germany) by the intraperitoneal route, followed by exsanguination via cardiac puncture, as indicated by the Federation for Laboratory Animal Science Associations (2007). Blood samples were collected into lithium-heparin and EDTA tubes. Lithium-heparin tubes were centrifuged (Heraeus Labofuge 400R, USA) for 15 min at 1500 x g, plasma was obtained and stored at -80°C for biochemical analysis. EDTA tubes were used for haemogram analysis. All animals were scalped to collect the following information: number, size, localisation, and weight of mammary tumours. Some tumours were sampled for genetic analysis and quickly frozen at -80°C. A systematic necropsy was performed and organs (heart, thymus, spleen, liver, lungs, kidneys), and visceral adipose tissue (VAT) were collected and weighed. Mammary tumours, organs and scalped skin were immersed in phosphate-buffered formaldehyde for histological analysis.

Haematological analysis

Hemogram

A complete blood count (CBC) was performed (IDEXX ProCyte Dx Hematology system) and included: erythrocytes, haematocrit, haemoglobin, mean cell volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), reticulocyte, leucocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelets, mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT) counts.

Serum marker analysis

Serum concentrations of albumin, cholesterol, urea, glucose, triglycerides, alanine aminotransferase (ALAT) and creatinine kinase MB (CK-MB) were determined in an autoanalyser (Prestige 24i, Cormay PZ).

Oxidative stress parameters

Sample preparation

A portion of each liver lobe and half of each kidney were removed to evaluate oxidative stress markers, *i.e.*, the activity of superoxide dismutase (SOD) and catalase (CAT). After thawing each sample, the material was homogenised in cold phosphate (100 mM)-EDTA (1 mM) Buffer at pH 7.4 with a Potter homogeniser. Subsequently, the solution was homogenised in an ice bath with an ultrasonic processor (4 x 20 sec, intermittent 20 sec). The solution was then centrifuged at 2000 x g for

10 min. Following that, the supernatant was transferred to another microtube. Another centrifugation was performed, at 12000 x g for 10 min to remove any cellular waste still in the solution. The supernatant was then transferred to another tube and reserved for further analysis. The results of the enzymatic activities were expressed as a function of the protein content of the samples, after their quantification on the BioTek Gen5™ (Powerwave XS2, BioTek Instruments, Inc. USA) based on the absorbance measurement at 280 nm.

Antioxidant enzymes

The levels of SOD were determined according to Durak et al. (1993), based on its ability to reduce and slow the photochemical reduction of a chromophore, nitroblue tetrazolium chloride (NBT). NBT allows for the detection of superoxide ion production through the oxidation of hypoxanthine to xanthine and then to uric acid by xanthine oxidase (Rodriguez et al., 2020). The reaction mixture (170 µL per well) consisted of potassium phosphate buffer (50 mM KH_2PO_4, 1 mM EDTA pH 7.4), hypoxanthine (0.6 mM) and NBT (0.2 mM). The well plates were monitored spectrophotometrically (560 nm for 2 min) to obtain the blank value. The reaction was then initiated by adding xanthine oxidase (23 mU/moL) to the enzymatic extract at 25–30°C and the well plates were read for 3 min. Results were expressed in U activity/min/mg protein, where one unit of SOD activity (1U) is defined as the amount of SOD that inhibits 50% of the reduction of NBT to formazan. The levels of CAT were determined as described by Claiborne (1985). The reaction mixture consisted of 190 µL sodium buffer (100 mM, pH 7.4) with H_2O_2 (20 mM). The well plates were read (240 nm for 2 min) to obtain the blank value. Following that, a 10 µL sample was added to each well and read for 3 min. Results were expressed in µmol H_2O_2 consumed/min/mg protein.

Histopathological analysis

Organs and tumours collected during necropsy were sectioned and processed for light microscopy. Paraffin sections (thickness 2 µm) were stained with haematoxylin and eosin; the histopathological analysis was done by two experienced pathologists (FS and AG).

Gene expression analysis

RNA isolation, quantification and cDNA synthesis

Total RNA was extracted from mammary tumours using the GF-1 Total RNA Extraction Kit (Vivantis Technologies, Selangor Darul Ehsan, Malaysia) according to the manufacturer's instructions. RNA quantification was performed on BioTek Gen5™ (Powerwave XS2, BioTek Instruments, Inc. USA) using the Well-to-Well blanking method. RNA integrity was analysed in agarose gel electrophoresis at 0.8% (80 V for 30 min), using Green Safe staining followed by detection under ultraviolet light. Samples were diluted to 1000 ng/µL before reverse transcribing the total RNA using the NZY M-MuLV First-Strand cDNA Synthesis Kit™ (Nzytech, Lisbon, Portugal). Obtained samples were then diluted in a 1:10 ratio and kept at -20°C.

Quantitative single step real-time polymerase chain reaction

Quantitative real-time PCR (qRT-PCR) was used to determine the gene expression levels of *VEGF* (vascular endothelial growth factor), *PCNA* (proliferating cell nuclear antigen), *ER-α* (oestrogen receptor alpha) and *ER-β* (oestrogen receptor beta). Table 1 shows the displays primer sequences, annealing temperatures (Ta), and amplicon size for each gene.

Table 1. List of primers used for qRT-PCR (adapted from Kumar et al. (2020)

Gene	Primer sequence (5'-3')	Ta	Amplicon size	Reference
GAPDH	F: GTTACCAGGGCTGCCTTCTC R: GGGTTTCCCGTTGATGACC	56°C	168 bp	Shibata et al. (1999)
PCNA	F: GAGTGGGGAGCTTGGCAAT R: ACAACAAGGGGTACATCTGC	56°C	198 bp	Kumar et al. (2020)
VEGF	F: CAGCTATTGCCGTCCAATTGA R: CCAGGGCTTCATCATTGCA	56°C	131 bp	Gómez et al. (2002)
ER-α	F: TAAGAACCGGAGGAAGAGTTG R: TCATGCGGAATCGACTTG	56°C	623 bp	Al-Bader (2006)
ER-β	F: AAGTAGCCGGAAGCTGACAC R: CCGGGACCACATTTTTGCAC	56°C	197 bp	Kumar et al. (2020)

F: Primer Forward; **R**: Primer Reverse.

qRT-PCR was conducted in a StepOnePlus Real Time PCR system (Applied Biosystems, Foster City, USA). The final volume of each PCR reaction was 10 µL, consisting of 5 µL Sybr® Select Master Mix (Applied Biosystems), 4.2 µL cDNA and 0.4 µL of each primer. The programme used for qRT-PCR was initial denaturation for 3 min at 95°C, followed by 40 cycles of 10 sec at 95°C, 20 sec at 56°C and 15 sec at 72°C. To rule out interference from primer dimers, DNA impurities, and other non-specific products, a melting curve analysis was conducted. Amplifications were carried out in duplicate, and analysis was performed using StepOnePlus Real Time PCR software (Applied Biosystems). On each plate, a negative control was added to rule out any contamination or the presence of primer-dimers. *GAPDH* gene expression was used as an internal control to normalise the data. Relative expression levels were calculated using the ΔCT method.

Statistical analysis

The formulas used in this study are presented in Table 2. All data were analysed using Statistical Package for Social Sciences (SPSS), version 26.0 (SPSS Inc, Chicago, IL, USA). The Shapiro-Wilk test was used to evaluate the normal distribution and the Levene test was used to assess the homogeneity of the variances. To assess statistical differences, an analysis of variance (ANOVA) was used, followed by Bonferroni multiple comparison test. Values with $P<0.05$ were considered statistically significant. Graphs were obtained using GraphPad Prism®, version 9.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Santolina chamaecyparissus aqueous extract

The phenolic profile of the *S. chamaecyparissus* aqueous extract is presented in Table 3. Nineteen compounds were detected, from which 12 were phenolic acids and 7 were flavone and flavonols. These compounds were previously described by Aourach et al. (2021) in methanol extracts (microwave and ultrasound), where 1,3-*O*-dicaffeoylquinic acid, followed by 1,5-*O*-dicaffeoylquinic acid, were the most

Table 2. Formulas for each parameter evaluated in the experimental work.

PARAMETER	FORMULA	REFERENCE
Mean food/drink consumption (g)	$\frac{Initial\ food/drink\ weight - Final\ food/drink\ weight}{Number\ of\ animals\ in\ the\ cage\ \times number\ of\ days}$	Oliveira et al. (2017)
Ponderal Gain (%)	$\frac{Final\ body\ weight - Initial\ body\ weight}{Final\ body\ weight \times 100}$	Arantes-Rodrigues et al. (2011)
Relative organ/VAT weight (mg/g BW)	$\frac{\text{Organ/muscle/VAT}\ mass\ (mg)}{Final\ animal\ BW\ (g)}$	Santos et al. (2019)
Tumour volume (cm^3)	$\frac{Width\ (cm)^{\,2} * Length\ (cm)}{2}$	Faustino-Rocha et al. (2013a)

VAT: visceral adipose tissue; **BW**: Body weight.

Table 3. Identification and quantification (µg/mL) of phenolic compounds present in the *S. chamaecyparissus* aqueous extract via LC-DAD-ESI/MS (mean ± standard deviation).

Peak	Rt (min)	Λmax (nm)	[M-H] m/z	MS^2 (m/z)	Tentative identification	µg/mL of extract
1	4.35	325	353	191(100),179(28),173(2),135(9)	3-*O*-Caffeoylquinic acid	7.7±0.1
2	4.90	325	515	353(11), 341(6), 323(100), 191(61), 179(6), 161(18)	5-*O*-Caffeoylquinic acid hexoside	6.8±0.1
3	5.99	323	353	191(22),179(52)173(100), 135(13)	5-*O*-Caffeoylquinic acid	6.81±0.02
4	6.41	326	707	353(100)	dimer of 3-*O*-Caffeoylquinic acid	45.1±0.5
5	6.68	327	707	353(100)	dimer of 5-*O*-Caffeoylquinic acid	2.85±0.03
6	8.85	335	593	503(33),473(100),383(26),353(53),341(6)	Apigenin-C-hexoside-C-hexoside	8.6±0.1
7	14.02	352	479	317(100)	Myricetin-3-*O*-hexoside	4.1±0.04
8	15.08	339	463	301(100)	Quercetin-3-*O*-galactoside	12.3±0.2
9	15.65	338	677	515(100), 353(21)	1,3,5-*O*-tricaffeoylquinic acid	4.1±0.1
10	16.71	342	463	301(100)	Quercetin-3-*O*-glucoside	2.17±0.02
11	17.16	341	565	521(100),479(23),317(18)	Myricetin-*O*-malonylhexoside	3.8±0.04
12	17.68	350	493	331(100),317(10)	Myricetin-*O*-glucuronide	38.5±0.5
13	19.14	321	515	353(100),335(4),253(5),191(12),179(2)	1,3-*O*-Dicaffeoylquinic acid	165±4
14	20.26	327	515	353(100),317(5),335(3),299(8),255(5), 191(2),179(5)	1,4-*O*-Dicaffeoylquinic acid	13±1
15	21.58	328	515	353(100),335(2),191(15),179(2),173(2)	1,5-*O*-Dicaffeoylquinic acid	27±1
16	22.54	328	549	387(100)	Medioresinol-*O*-hexoside	4.6±0.2
17	23.17	329	515	353(100),335(3),299(2),191(12),179(5), 173(2)	4,5-*O*-Dicaffeoylquinic acid	8.79±0.5
18	26.26	335	725	563(100),389(26),341(21),193(13),173(8)	Apigenin-6-C-Pentoside-8-C-Hexoside-7-*O*-Hexoside	2.3±0.1
19	29.64	325	457	295(100)	Coutaric acid hexoside	9.4±0.5
					Total Phenolic Acids	301±7
					Total Flavanoids	71.7±1
					Total Phenolic Compounds	373±8

Standard curves: 1- chlorogenic acid (y = 168823x – 161172; LOD = 0.20 µg/mL; LOQ = 0.68 µg/mL); 2- apigenin-6-C-glucoside (y = 107025x + 61531; LOD = 0.10 µg/mL; LOQ = 0.53 µg/mL); 3- quercetin-3-*O*-glucoside (y = 34843x – 160173; LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL); 4- naringenin (y = 18433x + 78903; LOD = 0.17 µg/mL; LOQ = 0.81 µg/mL).

abundant compounds. Moreover, myricetin-*O*-glucuronide was the most abundant flavonoid. The stability of these extracts was studied during five consecutive days and the concentration of phenolic compounds did not decrease on day five (*data not shown*). Therefore, the drinking water was maintained up to a maximum of three days, to avoid compound degradation.

Mortality rate

Two animals from the IND group were sacrificed in the 13th and 18th weeks as they reached the critical limit determined by the humane endpoint evaluation, and therefore these animals were excluded from all analyses.

General findings

The initial and final mean body weights and ponderal weight gain were not significantly different between the groups (Table 4).

The mean values for food and drink consumption showed higher final values in the SCE groups, and lowest values in the IND group (P>0.05) (Table 5).

Relative organ and visceral adipose tissue weight

No significant differences were found regarding relative organ weight (Table 6).

Tumour development

The percentage of mammary tumours per group is summarised in Table 7. Tumour frequency in the IND group was 57% (n=4) and in the SCE+IND group was 29% (n=2). The first tumour appeared in the IND group 10 weeks after MNU administration, in an animal that achieved the humane endpoints score. The first tumour in the SCE+IND group appeared 16 weeks after MNU administration.

The number of palpable mammary tumours and the percentage of tumour-bearing rats during the weeks of the experimental protocol are shown in Figure 1.

Haematology

Haemogram

Haemoglobin, mean platelet volume (MPV), platelet distribution width (PDW),

Table 4. Anthropometric parameters in all experimental groups (mean±SE).

Parameter	CTRL	IND	SCE	SCE+IND
Initial BW (g)	137.47 ± 4.54	122.69 ± 3.77	132.9 ± 5.48	127.08 ± 7.63
Final BW (g)	278.72 ± 3.96	276.17 ± 13.82	280.29 ± 12.4	254.72 ± 2.66
Δ Weight (g)	141.25 ± 6.64	151.86 ± 11.29	147.40 ± 10.79	127.64 ± 7.83
PWG (%)	50.59 ± 1.90	54.77 ± 1.34	52.29 ± 1.98	50.10 ± 2.99

BW: Body weight; **PWG**: Ponder weight gain.

Table 5. Food and drink consumption per day and per animal for all experimental group

Parameter	FOOD (g)		DRINK (mL)	
	Initial	Final	Initial	Final
CTRL	14.95	15.56	22.32	28.06
IND	14.06	14.00	21.55	23.17
SCE	15.20	16.71	25.44	30.08
SCE+IND	14.86	18.21	26.08	37.44

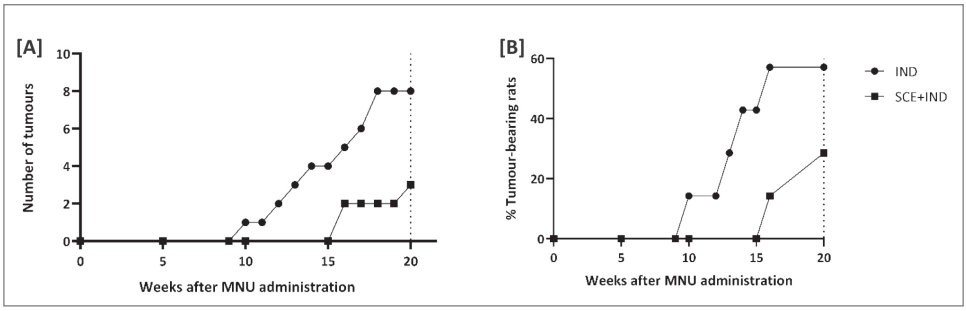

Figure 1. Number of palpable mammary tumours **[A]** and percentage of tumour-bearing rats along the weeks after MNU administration **[B]**.

Table 6. Relative organ and visceral adipose tissue (VAT) weight (mg weight/g body weight) for each experimental group (mean ± SE).

PARAMETER	CTRL	IND	SCE	SCE+IND
Thymus	1.400 ± 0.205	1.325 ± 0.176	1.390 ± 0.185	1.536 ± 0.132
Spleen	2.471 ± 0.138	2.493 ± 0.366	2.465 ± 0.161	2.674 ± 0.174
Heart	2.873 ± 0.061	2.819 ± 0.070	2.809 ± 0.094	2.817 ± 0.099
Liver	25.671 ± 1.237	27.368 ± 3.001	24.420 ± 0.930	25.837 ± 0.906
Lungs	5.122 ± 0.184	5.010 ± 0.312	4.947 ± 0.275	5.491 ± 0.240
Right Kidney	3.591 ± 0.22	3.945 ± 0.354	4.017 ± 0.196	3.826 ± 0.148
Left Kidney	3.506 ± 0.215	3.567 ± 0.302	3.744 ± 0.172	3.650 ± 0.174
VAT	40.70 ± 2.91	59.59 ± 5.03	30.18 ± 5.42	25.41 ± 4.51 a

[a] significantly different from the IND group ($P<0.001$).

Table 7. Effect of SCE supplementation on the development of MNU-induced mammary tumours (Data regarding tumour weight and volume are presented as mean ± SE).

Group	Tumour incidence (%)	Latency period	Tumour weight (g)	Tumour volume (cm^3)
IND	57.14	10^{th} week	2.31 ± 1.13	2.02 ± 1.23
SCE+IND	28.57	16^{th} week	0.39 ± 0.02	0.57 ± 0.15

and neutrophil-lymphocyte ratio (NLR) parameters showed statistically significant differences between groups (Table 8).

Serum biochemistry

The only biochemistry marker that showed statistical differences between groups was creatine kinase-myocardial band (CK-MB) (Table 9).

Antioxidant enzymes

There were no statistically significant differences between the experimental groups in the analysis of oxidative stress markers (Table 10).

Histopathology

The histological classification of mammary tumours is summarised in Table 11.

Table 8. Haemogram parameters for each experimental group (mean±SE).

	PARAMETER	CTRL	IND	SCE	SCE+IND
Red blood cell parameters	Erythrocytes (M/μL)	8.28 ± 0.24	8.39 ± 0.27	8.94 ± 0.28	8.41 ± 0.11
	Haematocrit (%)	44.60 ± 0.99	47.05 ± 0.93	47.68 ± 1.20	46.00 ± 0.62
	Haemoglobin (g/dL)	15.18 ± 0.29	15.73 ± 0.31	16.38 ± 0.41 [b]	15.53 ± 0.19
	MCV (fL)	53.99 ± 0.55	56.23 ± 1.09	53.48 ± 1.29	54.75 ± 0.73
	MCH (pg)	18.35 ± 0.23	18.78 ± 0.34	18.36 ± 0.45	18.50 ± 0.19
	MCHC (g/dL)	34.05 ± 0.22	33.43 ± 0.14	34.36 ± 0.14	33.78 ± 0.13
	RDW (%)	20.52 ± 0.57	19.68 ± 1.29	20.50 ± 0.76	20.06 ± 0.16
	Reticulocytes (K/μL)	243.53 ± 18.96	281.75 ± 28.96	173.18 ± 14.17	193.43 ± 20.05
White blood cell parameters	Leucocytes (K/μL)	1.87 ± 0.24	2.55 ± 0.33	2.35 ± 0.36	2.86 ± 0.41
	Neutrophils (K/μL)	0.42 ± 0.06	0.59 ± 0.11	0.26 ± 0.04	0.35 ± 0.08
	Lymphocytes (K/μL)	1.34 ± 0.20	1.81 ± 0.27	2.01 ± 0.32	2.38 ± 0.34
	Monocytes (K/μL)	0.10 ± 0.02	0.14 ± 0.03	0.08 ± 0.01	0.12 ± 0.03
	Eosinophils (K/μL)	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
	Basophils (K/μL)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
Platelet parameters	Platelets (K/μL)	613.72 ± 31.71	578.25 ± 59.07	626.80 ± 36.71	518.15 ± 89.89
	MPV (fL)	8.72 ± 0.08	9.03 ± 0.26	8.34 ± 0.10	8.29 ± 0.15 [a]
	PDW (fL)	9.86 ± 0.17	10.58 ± 0.42	8.78 ± 0.16 [b]	9.06 ± 0.14 [a, b]
	PCT (%)	0.54 ± 0.03	0.53 ± 0.06	0.53 ± 0.04	0.56 ± 0.07
	NLR	0.34 ± 0.06	0.35 ± 0.08	0.13 ± 0.01 [b]	0.16 ± 0.02 [b]

[a] statistically significant compared to IND; [b] statistically significant compared to CTRL.

Table 9. Serum biochemical parameters for each experimental group (mean ± SE).

Parameter	CTRL	IND	SCE	SCE+IND
Albumin (g/L)	4.77 ± 0.26	4.52 ± 0.01	4.52 ± 0.38	4.25 ± 0.14
Cholesterol (mg/dL)	104.78 ± 11.15	90.2 ± 3.32	78.72 ± 7.09	69.56 ± 10.25
Urea (mg/dL)	43.73 ± 3.84	34.13 ± 4.31	39.12 ± 1.92	46.89 ± 4.10
Glucose (mg/dL)	263.63 ± 49.88	359.70 ± 58.09	211.32 ± 29.28	203.80 ± 14.96
Triglycerides (mg/dL)	77.83 ± 13.76	122.45 ± 30.98	90.9 ± 16.32	91.03 ± 17.15
ALAT (U/L)	62.69 ± 22.04	53.23 ± 12.82	31.40 ± 6.14	34.85 ± 10.14
CK-MB (U/L)	471.16 ± 60.48	558.94 ± 214.81	119.36 ± 17.03 [a]	155.48 ± 19.04 [a, b]

[a] statistically significant compared to CTRL; [b] statistically significant compared to IND.

The total number of mammary lesions was lower in SCE+IND compared to the IND group. There were no preneoplastic lesions identified in animals from the IND group. All malignant lesions developed in both groups were non-invasive.

Molecular tumour markers

The qRT-PCR analysis on the tumours from the groups IND (n=4) and SCE+IND (n=2) are presented in Figure 2. All tumours used in this analysis from the

Table 10. Oxidative stress parameters for each experimental group (mean±SE).

Group	SOD (U activity/min/mg protein)		CAT (μmol H_2O_2 consumed/min/mg protein)	
	Liver	Kidney	Liver	Kidney
CTRL	2.60 ± 0.27	2.82 ± 0.03	111.60 ± 16.26	91.72 ± 2.13
IND	1.82 ± 0.59	3.23 ± 0.57	92.86 ± 11.81	125.90 ± 32.91
SCE	3.02 ± 0.22	2.98 ± 0.39	119.60 ± 19.28	114.00 ± 5.808
SCE+IND	2.50 ± 0.58	3.11 ± 0.81	106.50 ± 10.60	103.80 ± 6.25

Table 11. Histological classification of mammary tumours developed by animals in the IND and SCE+IND groups.

Lesions	Number of lesions	
	IND	SCE+IND
Preneoplastic lesions		
Intraductal proliferation	0	3
Benign lesions		
Fibroadenoma	0	1
Malignant lesions		
Papillary non-invasive carcinoma	2	0
Solid and cribriform non-invasive carcinoma	6	2
Total	8	2
Total number of lesions	**8**	**6**

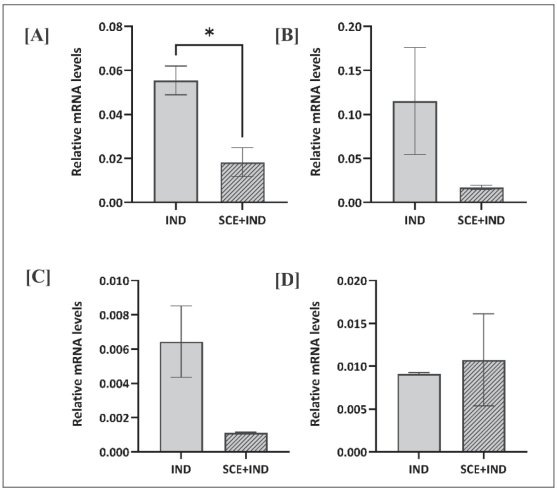

Figure 2. Effect of SCE on the relative mRNA levels of four tumour markers in MNU-induced tumours from female Wistar rats (mean±SE): **[A]** *VEGF*; **[B]** *PCNA*; **[C]** *ER-α*; **[D]** *ER-β*. * $P<0.05$.

IND group were cribriform non-invasive carcinoma, while the tumours from the SCE+IND group were intraductal proliferation and cribriform non-invasive carcinomas.

Discussion

Several studies have evaluated the effects of *S. chamaecyparissus* extracts on the MCF-7 breast cancer cell line (Ali et al., 2021; Saygideger et al., 2021b). This plant's extracts have the capacity to reduce cell proliferation and have potential therapeutic properties. Our study aimed to evaluate the chemopreventive properties of a *S. chamaecyparissus* aqueous extract in MNU-induced mammary tumours in female rats, contributing to the field of natural chemopreventive agents in breast cancer (BC) research.

In a previous study lasting 18 weeks and using Sprague-Dawley female rats, the induced group had a 60% frequency of mammary tumours (Faustino-Rocha et al., 2019), which is similar to the frequency observed in our study. However, even though the concentration of MNU was the same, tumour number and size were smaller in our study. These differences could be explained by the rat strain (Wistar), as it is known to develop fewer spontaneous neoplasms than other strains (Taylor and Mowat, 2020).

When analysing a chemopreventive agent, the time of tumour appearance, its size, number, and histological grade have been highlighted as important factors to consider (Shankar et al., 2022). In this research, the first tumour appeared six weeks later in the SCE+IND group compared to the IND group, and fewer animals developed tumours in SCE+IND. Natural compounds have shown the potential for cancer chemoprevention, with fewer side effects, low toxicity, and efficacy against cancer cells (Khazaei Koohpar et al., 2015). The LC-MS analysis of SCE revealed the presence of 19 polyphenolic compounds, mostly known for their anticancer activity, namely caffeoylquinic acid (Jafari et al., 2018), quercetin (Rauf et al., 2018), and myricetin (Devi et al., 2015).

Humane endpoints are used to minimise animal pain during experimental protocols (Tannenbaum and Bennett, 2015). In this study, two animals in the IND group were sacrificed as they reached the critical limit of four. Both animals, belonging to the IND group, developed mammary tumours with adverse effects on their well-being. These animals presented self-induced trauma in the mammary region and a tumour burden that exceeded 10% of their body weight. The rats in the SCE+IND group did not show similar severity according to humane endpoints analysis, possibly due to the modulation of the neoplastic process. The assessment of food and drink intake values can also help estimate animal wellbeing (Seyidoglu et al., 2021). Our study found that food and drink intakes were lower in IND, which is consistent with other studies (Mansingh et al., 2020).

Several organs were collected during the necropsy, but no statistically significant differences were found, which is in accordance with the literature (Faustino-Rocha et al., 2013). In our study, differences were observed regarding the relative weight of VAT between the induced groups. Accumulation of VAT has been linked to BC progression, which can lead to insulin resistance, inflammation, and growth factor production that support tumour development (van Kruijsdijk et al., 2009). A higher accumulation of VAT in animals from the IND group could be attributed to discomfort, which appears to be addressed by SCE, improving animal overall wellbeing or directly impacting

VAT accumulation. Some major phenolic compounds, such as anthocyanins, resveratrol, and curcumin, have shown anti-obesity effects (Meydani and Hasan, 2010).

Regarding the haemogram analysis, there were some differences between the groups, but all parameters were within the normal range (de Kort et al., 2020). Haemoglobin levels in the SCE group were higher than in the control group, but were not deemed clinically significant. The decrease in the neutrophil-lymphocyte ratio (NLR), an indicator of systemic inflammation (Hosseini et al., 2018), in the supplemented groups was interesting, seeing as previous studies have shown that SCE extracts have anti-inflammatory activity (Djarmouni et al., 2018). Inflammation can impact cancer progression (Zhang et al., 2020) and anti-inflammatory agents, reduce tumour growth, angiogenesis, metastasis, and increased survival rate in a murine BC model (Hosseini et al., 2018). Another parameter that was significantly altered was mean platelet volume (MPV), which is linked to larger tumours, higher stage, distant metastases, and poor prognosis in BC patients (Gu et al., 2016), and was increased in IND compared to SCE+IND. Similarly, platelet distribution width (PDW), whose increase is linked with a poor prognosis in BC (Huang et al., 2018), showed the same trend with IND having the highest values in comparison with SCE+IND.

In this experimental work, several serum biochemistry parameters were also determined, with no significant differences except for creatine kinase MB (CK-MB). Cholesterol and alanine aminotransferase (ALAT) levels were not statistically significant, but were lower in SCE groups. Higher cholesterol levels have been linked to the development of hormone receptor-positive BC, cardiovascular and metabolic disorders (Lee et al., 2016) and this decrease may be important for the prevention of these diseases. Higher levels of ALAT suggest hepatocellular injury (Contreras-Zentella and Hernández-Muñoz, 2016); the decrease observed for SCE groups grants it special interest. In a study by Messaoudi et al. (2018), following liver damage due to CCl_4 intoxication in male Wistar rats, ALAT levels were restored to normality with the supplementation of *S. chamaecyparissus* extracts. Creatine kinase-MB (CK-MB), a form of CK found in heart muscle cells, is a marker of myocardial injury (Zaki et al., 2019). CK-MB was significantly decreased for the SCE and SCE+IND groups, but no injury was induced in the non-supplemented groups. Seeing as treatment with doxorubicin (DOX) leads to increased CK-MB levels (Zaki et al., 2019), co-administration of SCE and DOX could reduce the induced cardiotoxicity.

The liver and kidneys are crucial for drug metabolism, and enzymes such as superoxide dismutase (SOD) and catalase (CAT) help eliminate reactive oxygen metabolites (Kurutas, 2016). MNU administration reduces the levels of antioxidant enzymes in rat liver through the inactivation and exhaustion caused by oxidative stress (Kakkar et al., 1997). Our findings, although not statistically significant, showed a trend consistent with the literature, with the IND group having the lowest levels of hepatic SOD and CAT (Gal et al., 2020). SCE appeared to normalise the affected antioxidant defence system, as evidenced by a slight increase in hepatic SOD and CAT activities.

Histopathological analysis revealed that tumours from SCE+IND were mostly preneoplastic or benign lesions, while all tumours from IND were malignant. *S. chamaecyparissus* extracts have been reported to have anticancer properties *in vitro* (Saygideger et al., 2021b), and it appears to

also interfere or delay the progression of tumours from benign to malignant. Compared to IND, SCE+IND presented a lower frequency of malignant lesions, and a higher latency period, as well as increased overall wellbeing and survival, suggesting that SCE may be a promising therapeutical option, especially for chemoprevention.

Based on the literature and their importance to cancer treatment and prevention (Kumar et al., 2020), we evaluated the gene expression of *VEGF*, *PCNA*, *ER-α* and *ER-β*. The best prognosis for BC occurs when *PCNA*, *VEGF*, and *ER-α* expression levels are lower (Kumar et al., 2020) and *ER-β* expression is higher (Treeck et al., 2008). *GAPDH* was used as a housekeeping gene because it has been shown to be effective (Vermani et al., 2020). SCE's effect on gene expression may explain its ability to prevent BC, due to its rich chemical composition. Our study showed a significant reduction of *VEGF* expression in SCE+IND, which resulted in smaller and fewer tumours, due to inadequate blood supply (Kumar et al., 2020). Angiogenesis is a driver of BC aggressiveness (Heer et al., 2020), which may explain why tumours from the IND were classified as malignant lesions and most tumours from SCE+IND group were classified as preneoplastic. *PCNA*, *ER-α* and *ER-β* expression levels, although not statistically different, presented trends consistent with the literature regarding a better prognosis in BC.

Conclusion

This study suggests that the aqueous flower extract of *Santolina chamaecyparissus* L. has a significant effect on mammary cancer in female Wistar rats. Supplementation with SCE delayed the onset of tumours and reduced tumour frequency, size and number. The animals showed a notable improvement in overall health. Furthermore, the study observed an absence of adverse side effects in liver or renal functions. Our study also observed a reduction of *VEGF* expression levels in tumours from SCE+IND, which is a key factor in cancer progression. A reduction of VAT, PDW and MPV in SCE+IND could also explain the better prognosis observed. The results regarding *ER-α* expression levels and CK-MB serum levels could prove valuable in future studies, particularly using tamoxifen and doxorubicin, revealing the potential for decreased dosages and side effects.

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Uloga prirodnih spojeva kod raka mliječnih žlijezda u štakora; blagotvorni učinci vodenog ekstrakta *Santolina chamaecyparissus* L.

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Rak dojke najčešće je dijagnosticiran rak u žena i vodeći uzrok smrti na svijetu. *Santolina chamaecyparissus* L. je biljka s višestrukim blagotvornim učincima za zdravlje, uključujući antitumorska i antidijabetička svojstva. Cilj je ove studije bio procijeniti kemopreventivne učinke vodenog ekstrakta *S. chamaecyparissus* (SCE) na životinjama oboljelim od raka mliječnih žlijezda. Dvadeset i osam četiri ženki starih tjedna wistar štakora podijeljeno je u četiri skupine: kontrolnu, MNU-inducirano (IND), s dodatkom SCE (SCE) i SCE+IND. Skupini SCE je dodan vodi za piće (12,72 mg/kg tjelesne mase) *ad libitum*; MNU je primijenjen intraperitonealnim putem u 50. danu života. Tjedno je bilježeno praćenje tjelesne mase, unosa hrane/tekućine, humano usmrćivanje i broj tumora mliječnih žlijezda. Dvadeset tjedana nakon primjene MNU, životinje su žrtvovane predoziranjem anestetikom i obavljena je razudba. Uzorci krvi su rabljeni za određivanje krvne slike i analizu biokemije seruma, dok su uzeti uzorci bubrega i pluća rabljeni za analize oksidativnog stresa. Uzorci tumora su prikupljeni za studije ekspresije gena i histološke studije. Analiziran je kemijski sastav skupine SCE pomoću LC-MS i otkriveno je da sadrži 19 fenolnih spojeva od kojih su najobilniji bili miricetin-*O*-glukuronid i 1,3-*O*-dikafeoilkina kiselina. Dvije životinje iz IND skupini žrtvovane su zbog prekoračenja ograničenja za humano usmrćivanje. Skupini SCE dodatak je odgodio razvoj tumora mliječnih žlijezda, smanjujući njegov volumen i masu. Skupina SCE je imala pozitivni učinak na hematološke parametre, posebice na omjer neutrofila i limfocita (P=0,026). Nikakve značajne razlike nisu otkrivene u biokemiji seruma, osim kreatinin kinaze MB, niti u markerima oksidativnog stresa. Analiza ekspresije gena pokazala je značajno smanjene razina ekspresije *VEGF* (P=0,0158) u tumora iz skupine SCE+IND. Ovi nalazi ukazuju da bi skupinu SCE trebalo dodatno ispitati da bi se identificirali pojedinačni spojevi i razumio njegov utjecaj na životinjama oboljelih od raka mliječnih žlijezda.

Ključne riječi: *rak dojke, wistar štakori, Santolina chamaecyparissus, kemoprevencija, životinjski model*