Food and Chemical Toxicology 174 (2023) 113689

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Study on the antineoplastic and toxicological effects of pomegranate (*Punica granatum* L.) leaf infusion using the K14-HPV16 transgenic mouse model

Manyou Yu [a,*], Irene Gouvinhas [a], Maria J. Pires [a,c], Maria J. Neuparth [b,c], Rui M. Gil da Costa [a,d,e,f], Rui Medeiros [e], Margarida M.S.M. Bastos [f], Helena Vala [a,h], Luis Félix [a,i], Carlos Venâncio [a,c], Ana I.R.N.A. Barros [a], Paula A. Oliveira [a,g]

[a] Centre for the Research and Technology of Agro-Environmental and Biological Sciences / Institute for Innovation, Capacity Building, and Sustainability of Agri-Food Production (CITAB / Inov4Agro), University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal
[b] Research Centre in Physical Activity, Health and Leisure (CIAFEL), Faculty of Sports of the University of Porto (FADEUP), Porto, Portugal
[c] Laboratory for Integrative and Translational Research in Population Health (ITR), TOXRUN- Toxicology Research Unit University Institute of Health Sciences, CESPU, CRL, Gandra, Portugal
[d] Postgraduate Programme in Adult Health (PPGSAD), Morphology Department and University Hospital (HUUFMA), Federal University of Maranhão (UFMA), São Luís, Brazil
[e] Molecular Oncology and Viral Pathology Group, IPO Porto Research Center (CI-IPOP)/RISEI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto)/ Porto Comprehensive Cancer Center (PortoCCC), Porto, Portugal
[f] LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Porto, Portugal
[g] Department of Veterinary Sciences, School of Agricultural and Veterinary Sciences, UTAD, Vila Real, Portugal
[h] Agrarian School of Viseu, Polytechnic Institute of Viseu, Viseu, Portugal
[i] i3S-Instituto de Investigação e Inovação em Saúde, Laboratory Animal Science, IBMC-Instituto de Biologia Molecular Celular, Universidade do Porto, 4200-135, Porto, Portugal

ARTICLE INFO

Handling Editor: Dr. Bryan Delaney

Keywords:
K14HPV16
Human papillomavirus
Neoplasia
Pomegranate leaf

ABSTRACT

Punica granatum L. (pomegranate) has been used in functional foods due to its various health benefits. However, the *in vivo* biological potential of its leaf remains little known. This study has aimed to characterize the antineoplastic and toxicological properties of using pomegranate leaf infusion (PLI) on transgenic mice carrying human papillomavirus (HPV) type 16 oncogenes. Thirty-eight mice were divided into 3 wild-type (WT) and 3 transgenic (HPV) groups, with exposure to 0.5% PLI, 1.0% PLI, and water. The animals' body weight, drink and food consumption were recorded. Internal organs, skin samples and intracardiac blood were collected to evaluate toxicological parameters, neoplastic lesions and oxidative stress. The results indicated that PLI was safe as no mortality, no behavioural disorders and no significant differences in the levels of microhematocrit, serum biochemical markers, internal organ histology, and oxidative stress was found among the WT groups. Histological analysis revealed that HPV animals that consumed PLI exhibited reduced hepatic, renal and cutaneous lesions compared with the HPV control group. Low-dose PLI consumption significantly diminished renal hydronephrosis lesions and relieved dysplasia and carcinoma lesions in the chest skin. Oxidative stress analysis showed that low-dose PLI consumption may have more benefits than high-dose PLI. These results suggest that oral administration of PLI has the potential to alleviate non-neoplastic and neoplastic lesions against HPV16-induced organ and skin injuries, though this requires further scientific research studies.

1. Introduction

Punica granatum L., commonly known as pomegranate, is a fruit-bearing deciduous shrub, belonging to the Punicaceae family. It originates from the Himalayan region and is extensively cultivated as a fruit or ornamental tree in the Mediterranean region (Puneeth and Chandra, 2020). Besides its fruit, the non-edible parts of pomegranate, such as its leaves, have been traditionally utilized for medicinal

* Corresponding author.
E-mail address: jiangymy518@163.com (M. Yu).

https://doi.org/10.1016/j.fct.2023.113689
Received 3 March 2022; Received in revised form 12 February 2023; Accepted 20 February 2023
Available online 27 February 2023

purposes. In Ethiopia, leaves crushed in water are taken orally to expel tapeworms (Ross, 2005). Hot water extract from dried pomegranate leaves is used externally for treating urinary tract infections in Belize and taken orally for irregular menses in Malaysia (Ross, 2005). Recently, pomegranate leaves (PL), which are considered to be promising in terms of developing 'functional foods' (foods which offer health benefits beyond their nutritional value), have been endowed with a variety of *in vitro* biological properties, including anti-cancer (Balamurugan et al., 2021; Deng et al., 2018; Kiraz et al., 2016; Saratale et al., 2018; Sarkar and Kotteeswaran, 2018; Toda et al., 2020), antioxidative (Yu et al., 2021b), anti-inflammatory (Toda et al., 2020), antidiabetic (Saratale et al., 2018), and antimicrobial properties (Acquadro et al., 2020; Swilam and Nematallah, 2020; Tehranifar et al., 2011).

The degree of impact of these activities depends on the structural diversity of polyphenolics, namely the number and the location of hydroxylation, galloylation, and polymerization, which has been primarily associated with their potentially chemopreventive and chemotherapeutic properties (Ismail et al., 2016; Yu et al., 2021a). Although many bioactive components still have not been recognized so far, ellagitannins (ETs) and flavonoid glycosides (FGs), ranking first and second respectively, are the most abundant polyphenols identified in pomegranate leaf infusion (PLI), contributing to its high level of free-radical scavenging capacity and ferric-reducing power (Yu et al., 2021a).

Combating cancer through a dietary intake rich in natural bioactive substances rather than synthetic drugs has been thriving. Along this line, ETs merit being part of a healthy diet as functional formulations, since they exhibit direct *in vitro* anti-proliferative impact against cancer cells, with low cell toxicity (Ismail et al., 2016; Villalba et al., 2019). PL extracts have been found to exert *in vitro* chemotherapeutic and antitumoral features against myeloma (Kiraz et al., 2016), colon (Balamurugan et al., 2021), cervical (Sarkar and Kotteeswaran, 2018), prostate (Deng et al., 2018), liver (Saratale et al., 2018), and lung (Toda et al., 2020) cancer cell lines, by inhibiting inflammation, inducing apoptosis, and stimulating cell cycle arrest.

Moreover, several assays suggest that PL extracts display hepatoprotective activity against carbon tetrachloride or high-fat diet-induced hepatotoxicity in experimental animals (Kumar et al., 2018; Rao and Krishnamurthy, 2019). Additionally, PL can also improve renal function in drug-induced nephropathy in rats (e.g. gentamicin and streptozotocin) (Ankita et al., 2015; Mestry et al., 2020). The methanolic extract of PL exhibited *in vivo* anticonvulsive properties on epilepsy in an experimental animal model (Viswanatha et al., 2016). Furthermore, clinical trials revealed that ellagic acid, as the primary hydrolysate of ETs, exhibited chemopreventive capacity in HPV-related pre-neoplastic lesions of the cervix (Morosetti et al., 2017). Given these previous findings, we performed *in vivo* research to study the therapeutic properties of PLI employing an HPV16-transgenic mouse model.

2. Materials and methods

2.1. Plant sample preparation

The plant species of *Punica granatum* L. and its leaves (PL) were authenticated and provided by a botanist from the Botanical Garden of the University of Trás-os-Montes and Alto Douro (UTAD, Vila Real, Portugal). The collected samples (harvested from the pomegranate during its blooming stage in June) were rinsed with tap water, dried until completely dehydrated, ground into a fine powder, and then subsequently stored hermetically at room temperature (RT, 20–25 °C), away from any light sources.

The PLI was prepared as previously described by Yu et al. (2021a), as follows: 5 g of PL was steeped in 1 L of boiled water (pH of around 6.0), recording the infusion at a concentration of 0.5% (w/v). The infusion was left to stand for 30 min until the temperature was reduced properly and then filtrated twice in sequence using first cotton gauze and then qualitative filter paper. Another 10 g of PL was extracted by the same procedure to obtain the 1.0% infusion. These two different concentrations of PLI were cooled down to RT before being fed to the animals for the *in vivo* studies. All drinks, including water and infusions, were freshly prepared three times (every Monday, Wednesday and Friday) per week and replenished for each cage, at the same time.

2.2. Polyphenolic profile and stability of PLI

The polyphenolic profile of the PLI was characterized using a the Reverse Phrase High Performance Liquid Chromatography–Diode Array Detector (RP–HPLC–DAD) system (Thermo Finnigan, San Diego, CA, USA). The methods have been reported in detail by our team as previously described (Yu et al., 2021a). The stability of the PLI was assessed by spectrophotometric analysis, also as previously described (Yu et al., 2021a). Experimental protocols included the determination of phenolic classes (total phenols, *ortho*-diphenols, and flavonoids) and the antioxidant capacities *in vitro* via 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium cation radical ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhidrazyl radical ($DPPH^{\bullet}$) scavenging assays over three consecutive days at RT.

2.3. Animals

This study was authorized by the Ethics Committee of the University of Trás-os-Montes and Alto Douro (852-e-CITAB-2020_A_1-e-CITAB-2021). The study followed domestic legislation (Decree-Law 113/2013, August 7) and European Directive 2010/63/EU on the protection of animals. Thirty-eight male mice were used in this experiment, including 20 wild-type (FVB/n) mice and 18 transgenic (hemizygotic $HPV16^{+/-}$) mice, at 20 weeks of age. These animals were generously donated by Doctors. Jeffrey Arbeit and Douglas Hanahan via the USA National Cancer Institute Mouse Repository, University of California, and maintained at our animal facilities. All animals were housed in polycarbonate cages with corncob beds (Ultragene, Santa Comba Dão, Portugal) and environmental enrichment. The cages were changed and cleaned weekly. All mice were maintained under controlled conditions, e.g., temperature (23 ± 2 °C), relative humidity (50 ± 10%), and a photoperiod cycle of 12 h light (8 a.m.–8 p.m.) and 12 h darkness. The animals were fed *ad libitum* with water and a standard diet (Mucedola S.R.L., 4RF21 GLP Certificate, Milan, Italy). The mice were genotyped as previously described (Ferreira et al., 2021) and assigned to either transgenic or wild-type groups.

2.4. Experimental design

According to their differing genotypes and treatments, the animals were divided into six groups, as shown in Fig. 1: G1 and G2 were exposed to 0.5% PLI (WT 0.5% PLI; n = 7 and HPV 0.5% PLI; n = 6); G3 and G4 were exposed to 1.0% PLI (WT 1% PLI; n = 6 and HPV 1% PLI; n = 7); and G5 and G6 were exposed to cooled boiled water (WT water; n = 7 and HPV water; n = 5). Since the ultimate aim of these infusions is for human use, PLI was prepared in natural conditions that mimic

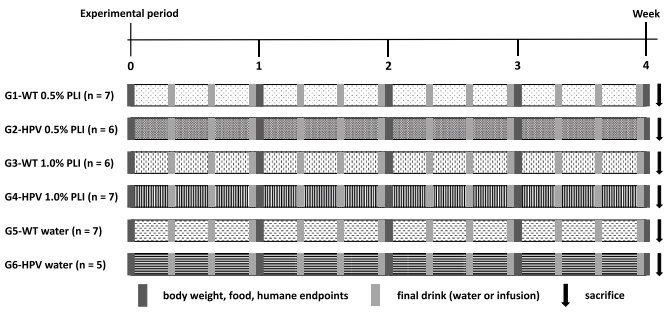

Fig. 1. Experimental protocol.

normal dietary habits for human consumption. Therefore, all drinks (including water, 0.5% and 1.0% of infusions) were orally administrated to the animals via lab-rodent water bottles attached to the breeding cages *ad libitum* that the mice were able to consume freely. Over the four consecutive weeks of the assay, all the mice had their health status checked daily. Their drink consumption (water/infusion) was registered three times (every Monday, Wednesday, and Friday) per week. The animals' body weight (BW), food consumption, body temperature and humane endpoints were recorded weekly.

At the end of the experimental protocol, all animals were sacrificed through an intraperitoneal injection of an overdose of xylazine-ketamine. Blood samples were then collected via an intracardiac puncture,following the Federation of European Laboratory Animal Science Association (FELASA) guidelines and placed in heparin tubes to block the clotting cascade. Complete necropsies were conducted. Subsequently, the heart, lungs, spleen, liver, kidneys, ear skin, chest skin, thymus, urinary bladder, testicles and adipose tissues were collected, weighed and placed in 10% neutral-buffered formalin for histological analysis. Body length (nose-to-anus) (BL), femoral length (FL), tibial length (TL) and abdominal perimeter (AP) were measured (cm) using a tapeline.

2.5. Haematology

Blood samples were placed in capillary tubes and centrifuged (12000 rpm for 5 min) for microhematocrit (Ht, %) determination. In addition, blood samples placed into heparin tubes were clotted and centrifuged at 3000 rpm for 15 min (4 °C). The serum was separated, transferred into a fresh tube and frozen at −80 °C until testing. Concentrations of creatinine and urea, both excreted by the kidneys, and two liver enzymes including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed by an automatic blood analyser (Prestige 24i, PZ Cormay S.A., Warsaw, Poland).

2.6. Histological analysis

The fixed internal organs and skin samples were transferred to histological cassettes, dehydrated in a series of graded ethanols and embedded in paraffin wax. Then embedded blocks were serially cutat a thickness of 4-μm, using a microtome and collected on glass slides. The histological sections were deparaffinized by placing the slides in xylene solution twice for 5 min, rehydrated through a sequence of graded ethanols and stained with Haematoxylin and Eosin (H&E) using automatic staining equipment. All slides were examined for routine histopathological diagnosis by light microscopy using an Axioplan 2 Microscope (Zeiss, Jena, Germany). Image processing was performed with the LAS Advanced Analysis Software Bundle (No: 12730448). The severity of non-proliferative and inflammatory lesions was scored semi-quantitively in accordance with a previous report (Mega et al., 2014).

2.7. Oxidative stress determination

Following euthanasia, the animal's liver and kidneys were harvested and rinsed with ice-cold phosphate buffer saline. A portion of each organ was homogenized in the cold HEPES buffer (ingredients: 0.32 mM of sucrose, 20 mM of HEPES, 1 mM of $MgCl_2$, and 0.5 mM of phenylmethylsulfonyl fluoride (PMSF, pH 7.4)) and separately centrifuged at 12000×*g* for 10 min at 4 °C (Deng et al., 2009). The resultant supernatants were then subjected to a biochemical investigation of the different antioxidant markers, such as superoxide dismutase (SOD) (Durak et al., 1993), catalase (CAT) (Clairborne, 1985), glutathione peroxidase (GPx) (Carlberg and Mannervik, 1975), and glutathione reductase (GR) activities (Carlberg and Mannervik, 1975), as outlined in previous studies (Félix et al., 2018).

Total glutathione (GSH and GSSG) levels (Hissin and Hilf, 1976) and glutathione *S*-transferase (GST) activity were also evaluated (Habig and Jakoby, 1981). The oxidative stress index (OSI) was indicated as the ratio of GSH/GSSG. Protein carbonyls (PC) (Mesquita et al., 2014) and lipid peroxidation (LPO) (Wallin et al., 1993) products were evaluated as oxidative biomarkers. The level of LPO in the liver and kidneys of animals was spectrophotometrically evaluated by the thiobarbituric acid reactive substances (TBARS) assay. The DCFH-DA fluorescent probe was used to estimate intracellular reactive oxygen species (ROS) levels (Deng et al., 2009). The lactate dehydrogenase (LDH) activity was also assessed (Domingues et al., 2010). Values were normalized to the levels of total protein (Bradford, 1976). For more detailed information, please refer to the Supplementary Materials.

2.8. Statistical analysis

Measurements of the PLI's polyphenolic composition and antioxidant capacity were performed in triplicate and the results were expressed as mean ± SE (standard error) (n = 3). Data related to animal tests (including BW, weight gain, relative organ weight, ratios of BW and lengths, and blood parameters) was analyzed as mean ± SE. The ponderal gain (PG) of each animal PG = (W2–W1)/W2 was calculated, with W1 being the initial BW and W2 being the final BW (FBW). Relative weight (RW) of internal organs was indicated as the ratio of the animal's organ weight by FBW. The Kolmogorov-Smirnov test was applied to verify the normal distribution of the data. These analyses were performed with IBM SPSS version 25 (Chicago, Illinois, USA), by using analysis of variance (ANOVA) followed by the Bonferroni test. The Chi-square test was conducted to analyze the histological results. The data of oxidative stress analysis was statistically carried out by using ANOVA followed by Tukey's Multiple Comparison Test (GraphPad Prism software, version 9.2.0). Data were considered statistically significant when $p < 0.05$.

3. Results

3.1. General findings

The results of chromatographic characterization and the polyphenolic profile of PLI have been reported in our previous work (Yu et al., 2021a). PLI's stability was spectrophotometrically studied considering its content of total phenols, *ortho*-diphenols and flavonoids, as well as its level of free-radical scavenging capacity, over two days (Table 1). Results indicated that these phenolic contents and the antioxidant activities of PLI maintained a high level of stability over 48 h,

Table 1
Degradation (%) of phenolic content and antioxidant capacity of pomegranate leaf infusions.

Spectrophotometric analysis	0 h	24 h	48 h	Degradation (%)
Phenolic content				
Total phenols (mg gallic acid g^{-1} DW)	156.3 ± 1.2 ^{b}	156.8 ± 0.5 ^{a}	150.5 ± 0.2 ^{c}	3.7
Total *ortho*-diphenols (mg gallic acid g^{-1} DW)	257.4 ± 2.0 ^{a}	256.0 ± 2.0 ^{a}	247.4 ± 1.9 ^{a}	3.9
Total flavonoids (mg rutin g^{-1} DW)	61.4 ± 0.3 ^{a}	58.4 ± 0.8 ^{b}	56.7 ± 0.6 ^{c}	7.7
Antioxidant capacity				
ABTS radical scavenging (mmol Trolox g^{-1} DW)	2.0 ± 0.0 ^{a}	1.9 ± 0.1 ^{b}	1.9 ± 0.1 ^{b}	6.2
DPPH radical scavenging (mmol Trolox g^{-1} DW)	1.7 ± 0.0 ^{a}	1.7 ± 0.1 ^{b}	1.6 ± 0.1 ^{b}	3.8

Values are presented as mean ± SE (n = 3) for each polyphenolic content and antioxidant activity assay. Mean values followed by different superscript lowercase letters report significant differences between different storage times, according to Tukey's multiple range test.
DW: plant dry weight; **ABTS**: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; **DPPH**: 2,2-diphenyl-1-picrylhidrazyl.

exhibiting less than 10% of degradation. But it was observed that the infusions began to degrade after 3 days' storage at RT, showing some flocculent precipitates (data not shown). Therefore, each animal's drink supply was replaced after intervals of no longer than three days, in order to reduce variations due to the compound's degradation.

During the experimental period, no mortality was recorded. Behavioural disorders were not observed, and we did not identify body temperature changes between the HPV groups (G2, G4, G6) and WT groups (G1, G3, G5) (data not shown).

The animals' BW variation and ponderal gain (PG) in all experimental groups are presented in Fig. 2. At the beginning (Week 0) of the assay (Fig. 2A), the animals' mean BW in the HPV water group (G6, 33.79 ± 1.14 g/animal) was statistically different from the WT water group (G5, 28.25 ± 0.76 g/animal) ($p = 0.001$). The same trend between these two groups was also found after the first week (Week 1) ($p = 0.002$). Moreover, the highest mean BW was observed in G6 (37.41 ± 3.73 g/animal) at the end of the study (Week 4), when compared with the HPV 0.5% PLI group (G2, 28.53 ± 0.78 g/animal) ($p = 0.003$), HPV 1.0% PLI group (G4, 30.25 ± 1.06 g/animal) ($p = 0.02$), and G5 (29.27 ± 0.77 g/animal) ($p = 0.005$). A similar tendency was indicated among these groups in Week 2 and Week 3 ($p < 0.05$), respectively. However, there was no difference in the animals' PG across the different groups (Fig. 2B).

The mean values of drink and food consumption during the observation are reported in Fig. 3. In general, the transgenic groups had higher drinking volume and food consumption than the wild-type groups. G2 exhibited both higher drink and food consumption (8.98–10.12 and 4.75–5.61 g/d/animal, respectively) than the WT 0.5% PLI group (G1, 4.02–4.44 and 1.56–3.82 g/d/animal of drink and food, respectively) and G4 (5.12–6.43 and 3.98–4.28 of drink and food, respectively) ($p < 0.01$). G4 also drank more than the WT 1.0% PLI group (G3, 4.28–4.61 g/d/animal), while less than G6 (10.84–11.22 g/d/animal) ($p < 0.05$). A significant difference ($p < 0.01$) in water intake was found between the positive (G6) and negative (G5) control groups. Furthermore, significantly ($p < 0.01$) higher food consumption was revealed in G6 (5.29–5.87 g/d/animal), in comparison to G2, G4, and G5.

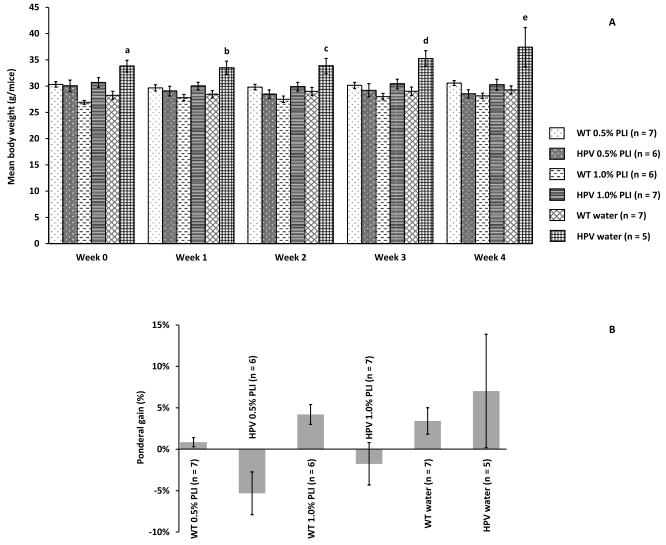

Fig. 2. Body weight variation (A) and ponderal gain (B) in all experimental groups. Results were expressed as mean ± SE.
[a] Statistically different from the WT water group ($p = 0.001$) in Week 0;
[b] Statistically different from the WT water group ($p = 0.002$) in Week 1;
[c] Statistically different from the HPV 0.5% PLI group ($p = 0.002$), HPV 1.0% PLI group ($p = 0.041$), and WT water group ($p = 0.006$) in Week 2;
[d] Statistically different from the HPV 0.5% PLI group ($p = 0.003$), HPV 1.0% PLI group ($p = 0.021$), and WT water group ($p = 0.001$) in Week 3;
[e] Statistically different from the HPV 0.5% PLI group ($p = 0.003$), HPV 1.0% PLI group ($p = 0.020$), and WT water group ($p = 0.005$) in Week 4.

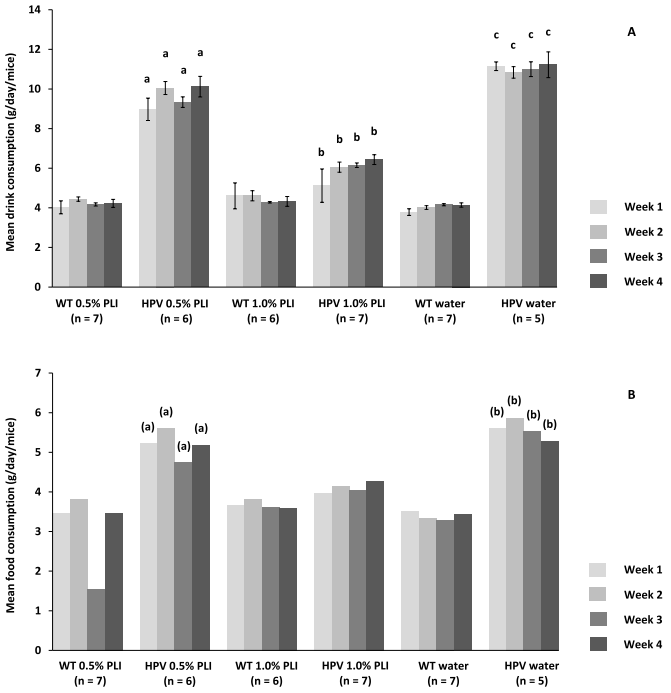

Fig. 3. Mean daily consumption of drink (water and infusion) (A) and food (B) in all experimental groups.
[a] $p < 0.01$, statistically different from the WT 0.5% PLI group and the HPV 1.0% PLI group in each week;
[b] $p < 0.05$, statistically different from the WT 1.0% PLI group and the HPV water group in each week;
[c] $p < 0.01$, statistically different from the WT water group in each week;
[(a)] $p < 0.01$, statistically different from the WT 0.5% PLI group and the HPV 1.0% PLI group in each week;
[(b)] $p < 0.01$, statistically different from the HPV 0.5% PLI group, the HPV 1.0% PLI group, and the WT water group in each week.

The RW of animals' internal organs in the different groups tested is analyzed in Table 2. G2 showed the highest mean RW of all organs studied, which was statistically different from G1 ($p = 0.004$) and G4 ($p = 0.032$) for the heart, different from G1 and G6 for the lung and right kidney ($p < 0.05$), and different from G1, G4, and G6 for the spleen, liver, and left kidney ($p < 0.05$).

The ratios of different body-size parameters are indicated in Table 3. G6 had a significantly higher ratio of FBW/BL than G5 ($p = 0.004$), and a higher ratio of FBW/TL than G2 ($p = 0.038$). Concerning the FL to abdominal perimeter (AP) ratio, there was a significant difference between G2 and G4 ($p = 0.012$). Additionally, the lowest AP/FBW ratio was found in G6 and was significantly different from G2 and G4 ($p < 0.05$). When comparing groups that consumed the same drink volumes, HPV groups displayed higher ratios of FBW/BL and FBW/TL versus the WT groups, although some ratios did not achieve statistical significance across groups.

3.2. Haematology

The microhematocrit (Ht) and serum biochemical results are registered in Table 4. There were no significant differences in the Ht values between groups. However, all groups exposed to PLI (G1, G2, G3, G4) displayed higher Ht than groups exposed to water alone (G5, G6) ($p > 0.05$). Moreover, no significant differences were detected in the serum levels of creatinine, urea, and AST among the groups. That said, the concentrations of creatinine, urea, and ALT in the HPV groups that

Table 2
Absolute weight (AW, g) and relative weight (RW, %) of internal organs, and animal's final body weight (FBW, g) in the experimental groups (mean ± SE).

	G1 WT 0.5% PLI (n = 7)	G2 HPV 0.5% PLI (n = 6)	G3 WT 1.0% PLI (n = 6)	G4 HPV 1.0% PLI (n = 7)	G5 WT water (n = 7)	G6 HPV water (n = 5)
Heart						
AW	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
RW	0.4 ± 0.0	0.6 ± 0.0 [a]	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Lung						
AW	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
RW	0.6 ± 0.0	0.8 ± 0.0 [b]	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
Spleen						
AW	0.1 ± 0.0	0.6 ± 0.2	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0
RW	0.4 ± 0.0	2.1 ± 0.6 [c]	0.4 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.8 ± 0.1
Liver						
AW	1.6 ± 0.0	2.1 ± 0.3	1.4 ± 0.0	1.5 ± 0.0	1.5 ± 0.1	2.1 ± 0.2
RW	5.2 ± 0.1	7.4 ± 0.7 [c]	5.1 ± 0.2	5.1 ± 0.2	5.2 ± 0.2	5.8 ± 0.2
Left kidney						
AW	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
RW	0.7 ± 0.0	0.9 ± 0.0 [c]	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0
Right kidney						
AW	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
RW	0.7 ± 0.0	0.9 ± 0.0 [b]	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0
FBW	30.6 ± 0.5	28.5 ± 0.8	28.1 ± 0.5	30.3 ± 1.1	29.3 ± 0.8	37.4 ± 3.7 [d]

[a] Statistically different from the WT 0.5% PLI group and the HPV 1.0% PLI group ($p < 0.05$).
[b] Statistically different from the WT 0.5% PLI group and the HPV water group ($p < 0.05$).
[c] Statistically different from the WT 0.5% PLI group, the HPV 1.0% PLI group, and the HPV water group ($p < 0.05$).
[d] Statistically different from the HPV 0.5% PLI group, HPV 1.0% PLI group, and WT water group ($p < 0.005$).

Table 3
Relationship among final body weight and different length parameters (mean ± SE).

Group	FBW/BL (g/cm)	FBW/TL (g/cm)	FL/AP (cm/cm)	AP/FBW (cm/g)
G1-WT 0.5% PLI (n = 7)	3.0 ± 0.0	18.2 ± 1.2	0.2 ± 0.0	0.3 ± 0.0
G2-HPV 0.5% PLI (n = 6)	3.3 ± 0.1	19.1 ± 1.0 [b]	0.2 ± 0.0 [c]	0.3 ± 0.0
G3-WT 1.0% PLI (n = 6)	2.9 ± 0.1	20.4 ± 0.5	0.2 ± 0.0	0.3 ± 0.0
G4-HPV 1.0% PLI (n = 7)	3.2 ± 0.1	24.1 ± 0.9	0.2 ± 0.0	0.3 ± 0.0
G5-WT water (n = 7)	3.0 ± 0.1 [a]	20.6 ± 0.8	0.2 ± 0.0	0.2 ± 0.0
G6-HPV water (n = 5)	3.7 ± 0.3	24.9 ± 2.4	0.2 ± 0.0	0.2 ± 0.0 [d]

FBW: final body weight; **BL**: body length; **TL**: tibial length; **FL**: femoral length; **AP**: abdominal perimeter.
[a] Statistically different from the HPV water group ($p = 0.004$).
[b] Statistically different from the HPV water group ($p = 0.038$).
[c] Statistically different from the HPV 1.0% PLI group ($p = 0.012$).
[d] Statistically different from the HPV 0.5% PLI group and HPV 1.0% PLI group ($p < 0.05$).

Table 4
Microhematocrit (Ht, %) and serum parameters (mean ± SE).

Group	Ht (%)	Creatinine (mg/dL)	Urea (mg/dL)	ALT (U/L)	AST (U/L)
G1-WT 0.5% PLI (n = 7)	39.9 ± 1.1	0.5 ± 0.2	46.8 ± 2.7	27.8 ± 2.4 [a]	85.8 ± 10.1
G2-HPV 0.5% PLI (n = 6)	38.7 ± 1.0	0.3 ± 0.1	41.4 ± 1.4	36.9 ± 3.0	52.4 ± 6.9
G3-WT 1.0% PLI (n = 6)	39.4 ± 0.6	0.7 ± 0.3	44.3 ± 2.4	49.0 ± 1.5	88.8 ± 5.1
G4-HPV 1.0% PLI (n = 7)	40.6 ± 0.7	0.4 ± 0.2	45.5 ± 2.2	40.7 ± 2.3	81.6 ± 7.3
G5-WT water (n = 7)	38.2 ± 0.6	0.4 ± 0.2	40.8 ± 2.4	42.5 ± 2.8	94.8 ± 10.4
G6-HPV water (n = 5)	36.4 ± 3.8	0.6 ± 0.2	48.1 ± 2.3	45.6 ± 6.2	78.7 ± 11.5

ALT: alanine aminotransferase; **AST**: aspartate aminotransferase.
[a] Statistically different from the WT 1.0% PLI group and the WT water group ($p < 0.01$).

consumed PLI (G2, G4) were lower than the positive control group (G6) ($p > 0.05$). Although the differences were not statistically significant, AST concentrations were lower in HPV groups than in WT groups, when the compared groups took the same drink volumes ($p > 0.05$). It may be observed that G1 had a significantly lower ALT level than G3 and G5 ($p < 0.05$).

3.3. Histological analysis

The hepatic and renal lesions identified in each experimental group are listed in Table 5 and Fig. 4. Regarding the liver samples (Fig. 4A), although all groups developed hydropic degeneration, G4 showed the lowest level at 42.9% (3/7) ($p > 0.05$). No (0%) WT animals developed congestion, and very few (33.3%, 2/6) WT animals (G3) showed inflammatory infiltrate, while these lesions were found in all HPV groups. Among all HPV groups, G4 displayed the lowest value of congestion (14.3%, 1/7) ($p > 0.05$).

Concerning the kidneys (Table 5 and Fig. 4B), G6 showed a significantly higher value of hydronephrosis (100%, 5/5) ($p < 0.05$) and exhibited a visually higher value of cylinder (40%, 2/5), whereas the other groups were characterized by no (0%) or rare (28.6%, 2/7) cylinder and hydronephrosis lesions. Moreover, all groups developed a low grade of inflammatory infiltrate; G2 had a lower value than G4 ($p < 0.05$). In this regard, the three WT groups presented only rare and small foci of inflammatory cells, whereas almost all animals from G6, as well as three animals from G4, revealed mild damage with few and small inflammatory cell infiltrate.

Histological results for the ear pavilion skin samples can be consulted in Table 6 and Fig. 4C. Overall, skin lesions varied from notorious epidermal hyperplasia, some with papillomatosis and dysplasia, accompanied by sebaceous hyperplasia. As expected, the ear pavilion lesions were only registered in HPV groups, while all WT groups exhibited normal ear skin (0%) in this study. No significant differences in hyperplasia and dysplasia were found among any of the HPV groups. None of the studied ear pavilion skin lesions was observed in the WT groups. Neoplastic lesions were only registered in G4, including two papillomas (28.6%) and a small invasive carcinoma (14.3%), characterized by cytologic atypia with loss of cell polarity, single-cell dyskeratosis, mitotic figures, and papillary sprouting basal-growth pattern.

Data on the histological analysis of chest skin is summarized in Table 6 and Fig. 4D. Like the result regarding ear skin, WT groups showed normal chest skin (0% lesions in G1 and G3; 16.7% congestion in G5). Epidermal lesions of hyperplasia were registered (100%) in all HPV animals. G2 (33.3%) showed significantly lower dysplasia than G4 (86%) and G6 (100%) ($p < 0.05$). Moreover, all animals in G6 had congestion while the other HPV groups showed 0% (G4) or 16.7% (G2) ($p < 0.01$). Neoplastic lesions were registered in all HPV groups. A small

Table 5
Number of animals (%) with histological liver and kidney lesions in all experimental groups.

	G1 WT 0.5% PLI (n = 7)	G2 HPV 0.5% PLI (n = 6)	G3 WT 1.0% PLI (n = 6)	G4 HPV 1.0% PLI (n = 7)	G5 WT water (n = 7)	G6 HPV water (n = 5)
Liver						
Hydropic degeneration	6 (100%)	4 (66.7%)	5 (83.3%)	3 (42.9%)	7 (100%)	4 (80%)
Inflammatory infiltrate	0 (0%) [a]	4 (66.7%)	2 (33.3%)	4 (57.1%)	0 (0%)	1 (20%)
Congestion	0 (0%) [b]	3 (50%)	0 (0%)	1 (14.3%)	0 (0%)	2 (40%)
Kidney						
Inflammatory infiltrate	5 (71.43%)	3 (50%) [c]	6 (100%)	7 (100%)	7 (100%)	5 (100%)
Hydronephrosis	0 (0%)	0 (0%)	0 (0%)	2 (28.6%)	0 (0%)	5 (100%) [d]
Cylinder	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (40%)

[a] Significantly different from the HPV 0.5% PLI group ($p = 0.009$).
[b] Significantly different from the HPV 0.5% PLI group ($p = 0.033$).
[c] Significantly different from the HPV 1.0% PLI group ($p = 0.033$).
[d] Significantly different from the HPV 0.5% PLI group, HPV 1.0% PLI group, and WT water group ($p < 0.05$).

papilloma was observed in one animal in G4 (14.3%). In G4 there was also one malignant tumour (14,3%), a carcinoma *in situ*, characterized by severe dysplasia, with pale atypical cells, enlarged nuclei, marked loss of cellular polarity, forming nests inside the epidermis without invading the basement membrane. G6 revealed two malignant neoplastic lesions (40%), one carcinoma *in situ* and one invasive squamous cell carcinoma, poorly differentiated, characterized by ulceration, necrosis, marked cellular atypia, enlarged nuclei, pleomorphism, anisokaryosis, mitotic figures, abundant and strongly eosinophilic cytoplasm, revealing individual keratinization. These proliferating cells were organized into islands, extending onto the dermal layer and forming acantholytic cysts.

3.4. Oxidative stress evaluation

The results of the hepatic stress analysis are presented in Fig. 5A. G2 showed a decrease (35%) in ROS level when compared to G1 ($p = 0.021$). For the determination of antioxidant enzymes, significant differences were not found in the levels of SOD, CAT and GPx among the groups studied ($p > 0.05$). Moreover, in G4 consumption of high-dose PLI led to a decline (58%) in GR level compared to G2 ($p = 0.037$) and G3 ($p = 0.018$). Whereas in G4 the GST concentration raised 54% and 39% when compared to G2 ($p < 0.001$) and G5 ($p = 0.009$), respectively.

On the other hand, the consumption of infusions by the WT (G1, G3) and HPV groups (G2, G4) did not promote any significant changes in GSH, GSSG and OSI levels ($p > 0.05$), when compared to the negative (G5) and positive (G6) control group, respectively. No significant alterations of PC and LPO levels were detected between groups ($p > 0.05$), while G3 showed higher LDH concentration than G1 ($p = 0.004$).

Results concerning renal stress analysis are shown in Fig. 5B. Significant changes were observed in the GR level, with an increase of 57% and 62% in G4 compared to G2 ($p = 0.038$) and G6 ($p = 0.033$), respectively. In WT animals, G3 showed an increased of 43% in PC concentration, compared with G1 ($p = 0.005$). In general, there were no significant variations reported in the remaining parameters of oxidative stress analysis among groups.

4. Discussion

Human papillomavirus (HPV) is the most common viral infection of the reproductive tract and oncological diseases associated with HPV responsible for 5% of all human cancers (Estêvão et al., 2019). The non-oncogenic HPV types (low-risk HPVs) can lead to warts. Oncogenic HPV types (high-risk HPVs, e.g. type 16 and 18) can trigger pre-malignant and malignant lesions, which evolve into cervical, vaginal and vulval cancers in women, penile cancer in men, as well as anal and oropharyngeal cancers in both sexes, if the infection persists (Estêvão et al., 2019). The genome of HPV16 can be segmented into three sections: the early gene-coding region (E), the late gene-coding region (L), and the long control region (LCR) (Pal and Kundu, 2020). The E6 and E7 oncoproteins take charge of several cellular checkpoints to establish cancer hallmarks (Pal and Kundu, 2020).

In this study, a K14-HPV16 transgenic mouse model was employed, by which the human *cytokeratin 14* (K14) gene promoter targets the expression of all early genes, including E6 and E7, to the basal cells in the keratinized squamous epithelia, driving multi-step carcinogenesis (Ferreira et al., 2021). These multi-step lesions developing in the animal model are histologically and molecularly similar to the lesions observed in HPV-related human diseases (Ferreira et al., 2021; Smith-McCune et al., 1997). So this model can hopefully be used to study the pathophysiological development of HPV16-induced carcinogenesis and to evaluate the effects of different natural compounds and drugs on the disease's development.

Strategies involving effective diagnosis, intervention and prevention can markedly mitigate the mortality associated with cancers (Usha et al., 2020). In reality, an increasing numbers of researchers have suggested that the intake of natural products can reasonably reduce the risk of cancer incidence (Usha et al., 2020). Moreover, the growing world population and market demands entail a necessary increase in agricultural by-products, and numerous studies have proved that organic agricultural wastes can potentially be a profitable source of natural antioxidants, particularly phenolic compounds (Jimenez-Lopez et al., 2020). In this regard, pomegranate leaves, containing high amounts of ellagitannins and flavonoids, have been shown to exhibit promising anticancer activities over the last decade (Balamurugan et al., 2021; Deng et al., 2018; Kiraz et al., 2016; Saratale et al., 2018; Sarkar and Kotteeswaran, 2018; Toda et al., 2020). However, studies on the anti-tumoral capacity of pomegranate leaves (PL) against HPV-induced cancer in animal models remain scarce.

In this work, we evaluated the chemo-preventive effects of pomegranate leaf infusion (PLI) for the first time in a K14-HPV16 transgenic mouse model. According to our results, no mortality and no significant behavioural (alertness, restlessness, irritability, and fearfulness), neurological (bleeding, convulsions, gait, and pain), breathing, and gastrointestinal (stomach ache, diarrhoea) changes were observed, showing that the PLI was non-toxic under the present experimental conditions.

Although the mean BW of G6 was significantly higher than the other groups and the PG of G2 and G4 declined, no statistical changes in PG were found among different groups, suggesting the growth of animals may not be affected by the consumption of PLI. The trend in PG between the transgenic group exposed to plant extracts (G2 or G4 decreased) and the positive control group (G6 increased) was similar to the previous

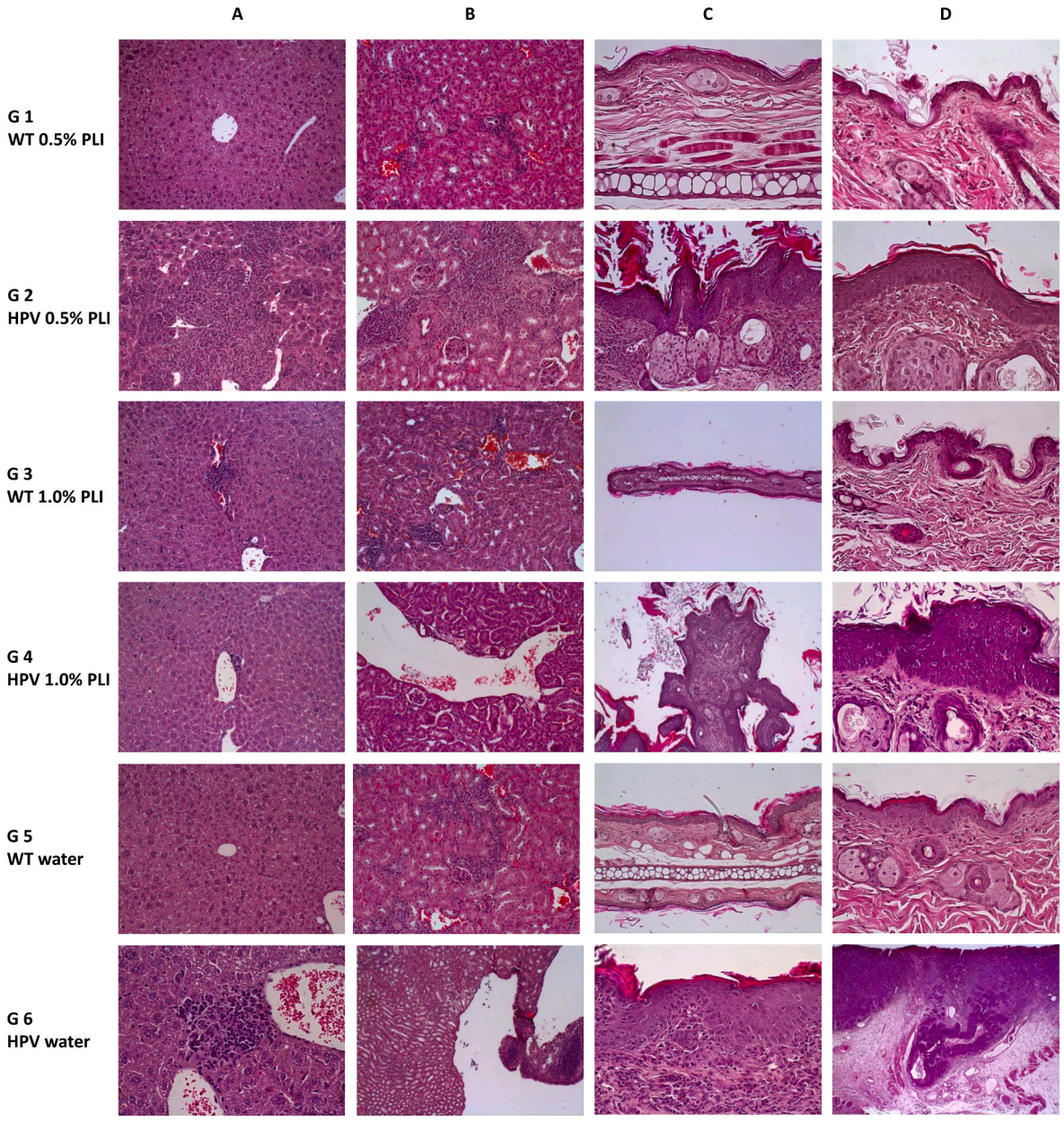

Fig. 4. Histological analysis of (A) liver, (B) kidney, (C) ear pavilion skin, and (D) chest skin in each experimental group. (A) Hydropic degeneration in all groups (A-G1 to G3; A-G5 to G6), being less evident in G4 (A-G4). WT groups showing minimal inflammatory infiltrate, only in two animals (A-G3), HPV groups showing inflammatory infiltrate (A-G2, A-G4) with infiltrate aggravation and congestion (A-G6); (B) all groups showing inflammatory infiltrate, two HPV groups showing hydronephrosis (B-G4, B-G6); (C) normal ear pavilion skin (C-G1, C-G3, C-G5), HPV groups showing papillary epidermal hyperplasia (C-G2), papilloma (C-G4), and invasive carcinoma (C-G6); (D) WT groups showing normal skin (D-G1, D-G3, D-G5), HPV groups showing regular epidermal hyperplasia with sebaceous hyperplasia (D-G2), severe dysplasia (D G-4), invasive squamous cell carcinoma (D-G6) characterized by ulceration, necrosis, extending onto dermal layer. H&E.

studies performed by our team, in which different natural substances were studied with the same animal model (Ferreira et al., 2021; Medeiros-Fonseca et al., 2018). In addition, animals in G2 exhibited a slimmer figure, based on the significantly lower FBW/TL ratio and higher FL/AP ratio found. However, the association of PLI with the growth of mice was still not clear, and therefore requires further verification programs.

Higher water consumption was observed in the transgenic mice, corresponding to findings previously described (Gil da Costa et al., 2017), but G4 animals consumed nearly half the volume of the other HPV groups. This observation regarding the drink consumption of voluntary oral administration of PL extracts was not discussed in previous reports (Ankita et al., 2015; Mestry et al., 2020; Patel et al., 2014). Accordingly, it was speculated that the unfavourable flavour of high-dose PLI could be one of the main factors which led to lower drink consumption.

Rao and Krishnamurthy (2019) indicated that the ethanolic extract of PL combined with gallic acid restored liver function more effectively than the ethanolic extract of PL alone or orlistat (control). Thus, the addition of some non-toxic and/or healthy ingredients to PLI could be beneficial for promoting the acceptance of high-dose PLI, that is, eliminating the influence of taste favouring and better supporting a dose-response-study. However, these authors (Rao and Krishnamurthy, 2019) did not report the specific setup of the oral experiments. Another path to reduce the variations in drink volume was to adopt the oral gavage (Ankita et al., 2015; Mestry et al., 2020; Patel et al., 2014) which will be considered in our next validation assay.

As we mentioned previously, in G2 the animals' bodies were thinner.

Table 6
Number of animals (%) with histological cutaneous lesions in all experimental groups.

	G1 WT 0.5% PLI (n = 7)	G2 HPV 0.5% PLI (n = 6)	G3 WT 1.0% PLI (n = 6)	G4 HPV 1.0% PLI (n = 7)	G5 WT water (n = 7)	G6 HPV water (n = 5)
Ear pavilion lesions						
Hyperplasia	0 (0%) [a]	5 (83.3%)	0 (0%) [b]	5 (71.43%)	0 (0%) [c]	4 (80%)
Dysplasia	0 (0%) [a]	6 (100%)	0 (0%) [b]	7 (100%)	0 (0%) [c]	4 (80%)
Papilloma	0 (0%)	0 (0%)	0 (0%)	2 (28.6%)	0 (0%)	0 (0%)
Carcinoma	0 (0%)	0 (0%)	0 (0%)	1 (14.3%)	0 (0%) [c]	0 (0%)
Sebaceous hyperplasia	0 (0%) [a]	6 (100%)	0 (0%) [b]	5 (71.43%)	0 (0%) [c]	5 (100%)
Congestion	0 (0%) [a]	6 (100%)	0 (0%) [b]	4 (57.1%)	0 (0%) [c]	5 (100%)
Inflammatory infiltrate	0 (0%) [a]	6 (100%)	0 (0%) [b]	6 (86%)	0 (0%) [c]	5 (100%)
Chest skin lesions						
Hyperplasia	0 (0%) [a]	6 (100%)	0 (0%) [b]	7 (100%)	0 (0%) [c]	5 (100%)
Dysplasia	0 (0%)	2 (33.3%) [d]	0 (0%) [b]	6 (86%)	0 (0%) [c]	5 (100%)
Papilloma	0 (0%)	0 (0%)	0 (0%)	1 (14.3%)	0 (0%)	0 (0%)
Carcinoma	0 (0%)	0 (0%)	0 (0%)	1 (14.3%)	0 (0%) [c]	2 (40%) [e]
Sebaceous Hyperplasia	0 (0%) [a]	5 (83.3%)	0 (0%) [b]	7 (100%)	0 (0%) [c]	4 (80%)
Congestion	0 (0%)	1 (16.7%) [c]	0 (0%)	0 (0%) [c]	1 (16.7%) [c]	5 (100%)
Inflammatory infiltrate	1 (16.7%) [a]	6 (100%)	0 (0%) [b]	6 (86%)	0 (0%) [c]	5 (100%)

[a] Significantly different from the HPV 0.5% PLI group ($p < 0.05$).
[b] Significantly different from the HPV 1.0% PLI group ($p < 0.05$).
[c] Significantly different from the HPV water group ($p < 0.01$).
[d] Significantly different from the HPV 1.0% PLI group and the HPV water group ($p < 0.05$).
[e] Significantly different from the HPV 0.5% PLI group and the HPV 1.0% PLI group ($p < 0.05$).

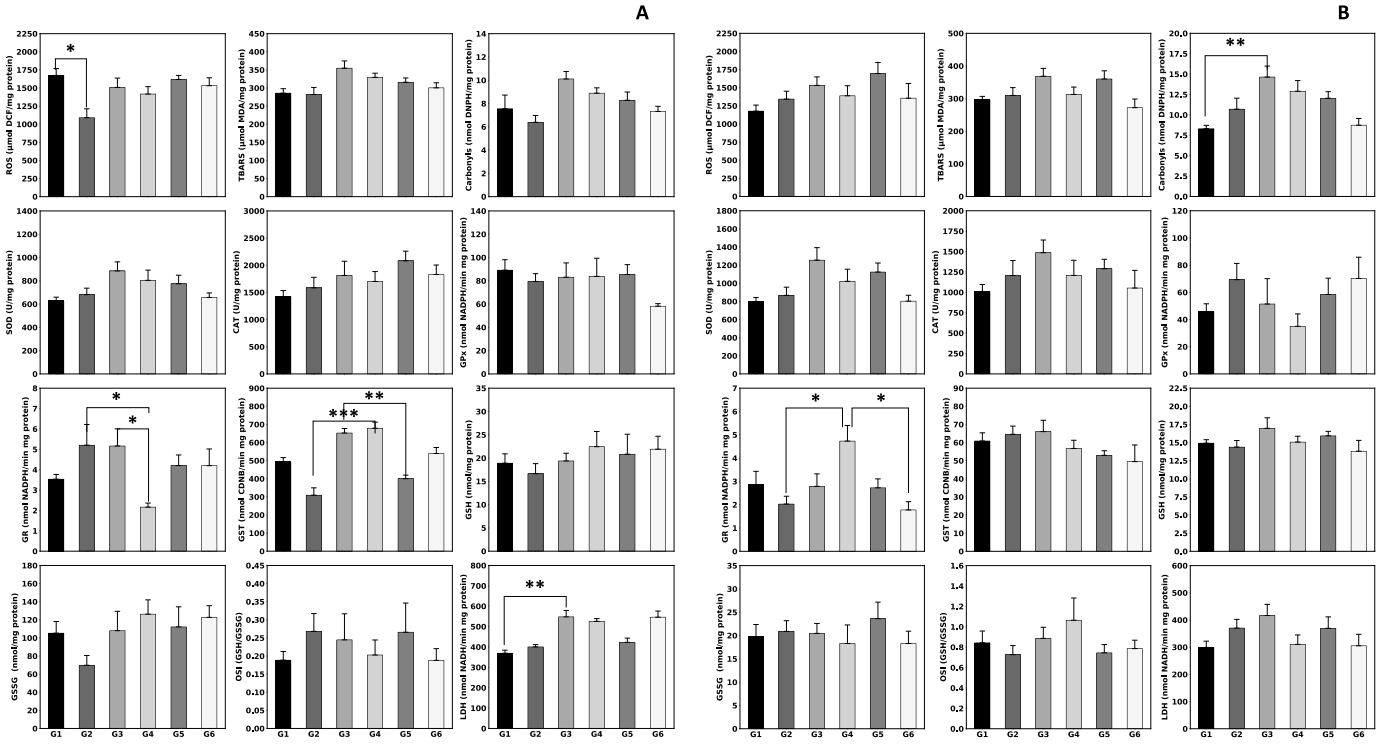

Fig. 5. Evaluation of oxidative stress in the liver (A) and kidney (B) in each experimental group. G1-WT 0.5% PLI; G2-HPV 0.5% PLI; G3-WT 1.0% PLI; G4-HPV 1.0% PLI; G5-WT water; G6-HPV water. Columns with different asterisks presented the statistical differences between groups at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Results were expressed as mean ± SE.

However, G2 showed the highest RW of the internal organs tested, possibly related to the hydropic degeneration microscopically observed in this group. Generally, hydropic changes are linked to Na^+/K^+ pump dysfunction (Nascimento-Gonçalves et al., 2021). Nevertheless, since this situation occurred in the transgenic group, it was not possible to explain with certainty the precise physical mechanism involved. So, the reasons for the increasing RW of internal organs in G2 still require further clarification. In addition, it was difficult to deduce the protective effect of PLI on the internal organs, because there were no significant changes in RW between G4 and G6. Neither dosage exhibited better efficacy. But it may be suggested that PLI caused no toxic impact on the internal organs of normal FVB/n mice in this study, because no

differences in RW were observed among all WT groups.

Apart from the PG, differences in the Ht along with the renal (creatinine, urea) and hepatic (AST) markers were also not detected between the groups, indicating that PLI did not cause toxicity under these experimental conditions. Several other studies also demonstrated the safety of PL extracts using acute toxicity tests using diverse animal models, such as female mice, and male or female rats (Ankita et al., 2015; Pottathil et al., 2020). Moreover, higher concentrations of creatinine, urea and ALT in G6 suggest harm caused by the HPV16 virus, whereas the administration of PLI seemed to promote a reduction in these biomarkers in G2 and G4, though data was not statistically significant. This can provide a clue to the hepatic and renal protective effects of PLI, and is in agreement with other reports (Kumar et al., 2018; Mestry et al., 2020).

The K14-HPV16 transgenic mouse model was developed from FVB/n mice to study the pathophysiology of HPV-induced lesions. These animals developed cutaneous lesions that started as hyperplasia, progressed to dysplasia and evolved to carcinoma *in situ* (Coussens et al., 1996; Santos et al., 2019). Concerning their hepatic histology, differences between three WT groups or between three HPV groups did not reach statistical significance, thus eliminating the possibility of toxicological effects on the liver associated with the infusion and its dosages.

Other authors (Rao and Krishnamurthy, 2019) showed that the addition of gallic acid to the ethanolic extract of PL could enhance its effectiveness in relieving liver dysfunction, thus gaining better hepatoprotective activity. Accordingly, further analyses can be designed to confirm PLI's hepatic toxicology and therapeutic capacity, using high-dose or additional substrate approaches. Preventing the development of nephropathy or blocking the progression of glomerulosclerosis plays a pivotal role in the exploitation of therapeutic agents against renal dysfunction (Ankita et al., 2015). In this study, treatment with low-dose PLI significantly diminished renal hydronephrosis lesions in HPV animals, indicating a nephroprotective effect against HPV16-induced tissue damage.

Lesions in the K14-HPV16 transgenic mouse model show characteristical neoplastic progression in the skin with advancing age (Arbeit et al., 1994; Coussens et al., 1996; Sethi and Palefsky, 2004). As previously said, ear pavilion skin and chest skin samples were examined, since these skin sites developed progressively more severe lesions (Ferreira et al., 2021; Medeiros-Fonseca et al., 2018; Santos et al., 2019) and squamous cell carcinomas (SCC) were typically observed on the epidermis of this animal strain (Arbeit et al., 1994; Coussens et al., 1996; Sethi and Palefsky, 2004). Concerning all cutaneous lesions analyzed, three WT groups exhibited normal ear skin or chest skin (0%) in this study, indicating that PLI caused no harm to the development of ear pavilion skin or chest skin in wild-type animals. The G6 (positive control group) developed hyperplastic and dysplastic lesions, in line with previous reports (Coussens et al., 1996; Santos et al., 2019). Globally, G6 showed a higher percentage of tumours and the most aggressive ones, followed by G4. This trend can mean that low-dose PLI had a better preventive effect for ear pavilion and chest skin lesions, namely in preventing neoplastic progression.

Reactive oxygen species (ROS) as the overall indicator of oxidative stress (OS) is a term that includes reactive oxygen-containing molecules, such as free radicals. The generation of ROS depends on the consumption and utilization of oxygen in diverse physiological processes, such as vigorous exercise, chronic inflammation, exposure to allergens, drugs, toxins, infections, etc. (Krishnamurthy and Wadhwani, 2012). Low levels of ROS can act as second messengers for modulating gene expression, whereas high levels, if not neutralized or eliminated, may induce OS, causing cellular damage in membrane lipids, nucleic acids, proteins and enzymes (Sáez and Están-Capell, 2017).

Malondialdehyde (MDA) serves as the end product of LPO (Nascimento-Gonçalves et al., 2021). Oxidative damage to proteins is usually accompanied by progress of carbonyl residues (Dalle-Donne et al., 2003). Antioxidant enzymes, like endogenous antioxidants, represent the most important defence mechanism against OS-induced cell damage, as they transform ROS into stable non-toxic molecules (Krishnamurthy and Wadhwani, 2012). SOD, CAT, GPx, and GR, in particular, form the first line of defence. SOD and CAT are responsible for catalysing the reduction of superoxide and hydrogen peroxide, respectively (Krishnamurthy and Wadhwani, 2012). GPx catalyses the removal of hydroperoxides using GSH as an antioxidant co-substrate, in which GSH is oxidized to GSSG (oxidized glutathione) while GR can reduce GSSG back to GSH (Sáez and Están-Capell, 2017).

Besides this, GST is involved in the second phase of biotransformation reactions, and is capable of catalysing GSH conjugation, thus binding glutathione to endo- or exogenous toxins, detoxifying them and enabling their excretion from the organism. In addition, the advance of LDH activity would be more associated with the acceleration of anaerobic glycolysis, like in cancer cells, where the overproduction of lactate leads to the acidification of the environment (Ganapathy-Kanniappan and Geschwind, 2013). In the present study, no significant differences in the levels of ROS, LPO, PC, and LDH were found between the negative (G5) and positive (G6) control groups. Similarly, previous studies by our team also reported no changes in ROS and LPO when comparing control and induced groups featuring either male or female animals using the same transgenic mouse model (Ferreira et al., 2021; Medeiros-Fonseca et al., 2018). This may suggest that the current protocol needs to be improved if the aim is to study OS in HPV16 transgenic animals.

Moreover, we did not find significant changes in the aforementioned parameters across any of the HPV groups, which does not support the presence of protective effects of PLI against HPV16-related hepatic or renal oxidative damage. This is inconsistent with other reports that used different models (Ankita et al., 2015; Dassprakash et al., 2012; Mestry et al., 2020; Patel et al., 2014; Pottathil et al., 2020). On the other hand, results also showed that PLI had no signs of toxicity, since there were no differences between WT groups that consumed PLI (G1, G3) and the negative control group (G5). The decreased renal PC and hepatic LDH levels in G1 implied that low-dose PLI may have more benefits than a high dosage.

Recently, several studies have identified ethanolic or methanolic extracts of PL as promising protective agents against drug-induced (gentamicin or streptozotocin) renal or hepatic injuries in rats (Ankita et al., 2015; Mestry et al., 2020; Patel et al., 2014; Pottathil et al., 2020). These benefits were associated with the antioxidant potential of PL extracts, especially with high dosages (400–600 mg/kg), by improving levels of SOD, CAT, GSH or GPx, while alleviating LPO. Moreover, extract of PL in water, up to 800 mg/kg, presented no signs of toxicity and higher levels of hepatic SOD, CAT, GSH and GST, when used on cyclophosphamide-induced OS in mice (Dassprakash et al., 2012).

Nevertheless, results from our investigation did not adequately prove that drinking PLI could restore levels of SOD, CAT, GPx, GSH or GST in the HPV16-induced groups. Consequently, from the OS point of view, it cannot prove that PLI had nephro- and hepato-therapeutic potential under these experimental conditions. The discrepancy between our results and previous descriptions could possibly be put down to the samples being from diverse regions or cultivars, different animal models applied (e.g. rats), methodology of plant extraction, the concentration of plant extracts, administration routes (e.g. oral gavage), or the prolonged experimental period (e.g. 45 d) (Mestry et al., 2020; Patel et al., 2014; Pottathil et al., 2020). Additional studies are recommended to confirm the real antioxidant efficacy of PLI.

5. Conclusion

This study reported the potentially antineoplastic and chemotherapeutic properties of pomegranate leaf infusion (PLI) in a K14-HPV16 transgenic mouse model. The consumption of PLI is suggested to have no associations with the growth of animals under the experimental protocol. Moreover, PLI is proposed as being non-toxic for the studied animals, since no mortality, no behaviour disorders and no significant

differences in the levels of microhematocrit, serum biochemistry, histopathology and oxidative stress were found between wild-type groups provided with PLI and the wild-type group that drank only water.

Furthermore, oral administration of PLI as a dietary supplement can have anti-tumoral and chemopreventive potential against HPV16-induced non-neoplastic (dysplasia) and neoplastic (carcinoma) lesions. In particular, low-dose PLI exhibits more beneficial effects than high-dose on renal and chest skin lesions. However, the capacity of PLI to enhance antioxidant defence when acting against K14HPV16-related hepatic and renal oxidative damage remains unclear and requires additional investigations. Further studies could consider modifying the animal model, clarifying the efficacy of high dosages by ameliorating palatability, assessing the differences by comparison via the parenteral administration route, or testing PLI effectiveness by prolonging experimental periods. More analytical techniques, particularly those from immunohistochemistry, should be incorporated to obtain more accurate descriptions of lesions and oxidative stress effects, thus achieving more detailed experimental results.

CRediT authorship contribution statement

Manyou Yu: prepared and characterized the pomegranate leaf infusions, conducted the experiments with live animals, their sacrifice and sample processing, carried out all data analysis, and wrote the manuscript. **Irene Gouvinhas:** participated in the experimental process and the sacrifice of animals. **Maria J. Pires:** participated in the sacrifice of animals and performed blood sample tests. **Maria J. Neuparth:** participated in the sacrifice of animals and performed blood sample tests. **Rui M. Gil da Costa:** performed the histological analysis. **Rui Medeiros:** performed the histological analysis. **Margarida M.S.M. Bastos:** performed the histological analysis. **Helena Vala:** performed the histological analysis. **Luis Félix**: performed the oxidative stress analysis. **Carlos Venâncio:** performed the oxidative stress analysis. **Ana I.R.N.A. Barros:** supervised the study's objectives. **Paula A. Oliveira:** designed the experiments, supervised data interpretation, and revised the manuscript. All authors reviewed the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by National Funds from FCT – Portuguese Foundation for Science and Technology, under the projects UIDB/04033/2020 (CITAB), NORTE-01-0145-FEDER-000017 (Interact R&D), UIDB/00511/2020 (LEPABE), NORTE-01-0145-FEDER-000054 (NORTE 2020) and PI127-CI-IPOP-118-2019 (ERDF). Manyou Yu received a PhD studentship from FCT (PD/BD/135333/2017) under the Doctoral Programme "Agricultural Production Chains – from fork to farm" (PD/00122/2012).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2023.113689.

References

Acquadro, S., Civra, A., Cagliero, C., Marengo, A., Rittà, M., Francese, R., Sanna, C., Bertea, C., Sgorbini, B., Lembo, D., Donalisio, M., Rubiolo, P., 2020. Punica granatum leaf ethanolic extract and ellagic acid as inhibitors of Zika virus infection. Planta Med. 86, 1363–1374.

Ankita, P., Deepti, B., Nilam, M., 2015. Flavonoid rich fraction of Punica granatum improves early diabetic nephropathy by ameliorating proteinuria and disturbed glucose homeostasis in experimental animals. Pharm. Biol. 53, 61–71.

Arbeit, J.M., Münger, K., Howley, P.M., Hanahan, D., 1994. Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. J. Virol. 68, 4358–4368.

Balamurugan, C., Karuppasamy, R., Sivaraj, C., Saraswathi, K., Arumugam, P., 2021. Cytotoxic activity of leaves extract of pomegranate (*Punica granatum* L.). J. Pharmacogn. Phytochem. 10, 1982–1985.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480.

Clairborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), Handbook of Methods for Oxygen Radical Research, first ed. CRS press, Boca Raton, pp. 283–284.

Coussens, L.M., Hanahan, D., Arbeit, J.M., 1996. Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. Am. J. Pathol. 149, 1899–1917.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 329, 23–38.

Dassprakash, M.V., Arun, R., Abraham, S.K., Premkumar, K., 2012. In vitro and in vivo evaluation of antioxidant and antigenotoxic potential of Punica granatum leaf extract. Pharm. Biol. 50, 1523–1530.

Deng, J., Yu, L., Liu, C., Yu, K., Shi, X., Yeung, L.W.Y., Lam, P.K.S., Wu, R.S.S., Zhou, B., 2009. Hexabromocyclododecane-induced developmental toxicity and apoptosis in zebrafish embryos. Aquat. Toxicol. 93, 29–36.

Deng, Y.-l., Li, Y.-L., Zheng, T.-T., Hu, M.-X., Ye, T.-H., Xie, Y.-M., Yin, W.-Y., 2018. The extract from *Punica granatum* (pomegranate) leaves promotes apoptosis and impairs metastasis in prostate cancer cells. Sichuan Da Xue Xue Bao Yi Xue Ban 49, 8–12.

Domingues, I., Oliveira, R., Lourenço, J., Grisolia, C.K., Mendo, S., Soares, A.M.V.M., 2010. Biomarkers as a tool to assess effects of chromium (VI): comparison of responses in zebrafish early life stages and adults. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152, 338–345.

Durak, I., Yurtarslanl, Z., Canbolat, O., Akyol, O., 1993. A methodological approach to superoxide dismutase (SOD) activity assay based on inhibition of nitroblue tetrazolium (NBT) reduction. Clin. Chim. Acta 214, 103–104.

Estêvão, D., Costa, N.R., Gil da Costa, R.M., Medeiros, R., 2019. Hallmarks of HPV carcinogenesis: the role of E6, E7 and E5 oncoproteins in cellular malignancy. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1862, 153–162.

Félix, L.M., Vidal, A.M., Serafim, C., Valentim, A.M., Antunes, L.M., Monteiro, S.M., Matos, M., Coimbra, A.M., 2018. Ketamine induction of p53-dependent apoptosis and oxidative stress in zebrafish (Danio rerio) embryos. Chemosphere 201, 730–739.

Ferreira, T., Nascimento-Gonçalves, E., Macedo, S., Borges, I., Gama, A.M., Gil da Costa, R., Neuparth, M.J., Lanzarin, G., Venâncio, C., Félix, L., Gaivão, I., Alvarado, A., Pires, M.J., Bastos, M.M.S.M., Medeiros, R., Nogueira, A., Barros, L., Ferreira, I.C.F.R., Rosa, E., Oliveira, P.A., 2021. Toxicological and anti-tumor effects of a linden extract (Tilia platyphyllos Scop.) in a HPV16-transgenic mouse model. Food Funct. 12, 4005–4014.

Ganapathy-Kanniappan, S., Geschwind, J.-F.H., 2013. Tumor glycolysis as a target for cancer therapy: progress and prospects. Mol. Cancer 12, 152.

Gil da Costa, R.M., Aragão, S., Moutinho, M., Alvarado, A., Carmo, D., Casaca, F., Silva, S., Ribeiro, J., Sousa, H., Ferreira, R., Nogueira-Ferreira, R., Pires, M.J., Colaço, B., Medeiros, R., Venâncio, C., Oliveira, M.M., Bastos, M.M.S.M., Lopes, C., Oliveira, P.A., 2017. HPV16 induces a wasting syndrome in transgenic mice: amelioration by dietary polyphenols via NF-κB inhibition. Life Sci. 169, 11–19.

Habig, W.H., Jakoby, W.B., 1981. [51] Assays for differentiation of glutathione S-Transferases. Methods Enzymol. 398–405. Academic Press.

Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74, 214–226.

Ismail, T., Calcabrini, C., Diaz, A.R., Fimognari, C., Turrini, E., Catanzaro, E., Akhtar, S., Sestili, P., 2016. Ellagitannins in cancer chemoprevention and therapy. Toxins 8, 151.

Jimenez-Lopez, C., Fraga-Corral, M., Carpena, M., García-Oliveira, P., Echave, J., Pereira, A.G., Lourenço-Lopes, C., Prieto, M.A., Simal-Gandara, J., 2020. Agriculture waste valorisation as a source of antioxidant phenolic compounds within a circular and sustainable bioeconomy. Food Funct. 11, 4853–4877.

Kiraz, Y., Neergheen-Bhujun, V.S., Rummun, N., Baran, Y., 2016. Apoptotic effects of non-edible parts of *Punica granatum* on human multiple myeloma cells. Tumor Biol. 37, 1803–1815.

Krishnamurthy, P., Wadhwani, A., 2012. Antioxidant enzymes and human health. In: El-Missiry, M.A. (Ed.), Antioxidant Enzyme. IntechOpen, pp. 1–17.

Kumar, M., Dandapat, S., Sinha, M.P., 2018. Hepatoprotective activity of *Punica granatum* leaf extract against carbon tetrachloride induced hepatotoxicity in rats. Balneo Research Journal 9, 24–27.

Medeiros-Fonseca, B., Mestre, V.F., Colaço, B., Pires, M.J., Martins, T., Gil da Costa, R.M., Neuparth, M.J., Medeiros, R., Moutinho, M.S.S., Dias, M.I., Barros, L., Bastos, M.M.S.M., Félix, L., Venâncio, C., Ferreira, I.C.F.R., Antunes, L.M., Oliveira, P.A., 2018. Laurus nobilis (laurel) aqueous leaf extract's toxicological and anti-tumor activities in HPV16-transgenic mice. Food Funct. 9, 4419–4428.

Mega, C., Vala, H., Rodrigues-Santos, P., Oliveira, J., Teixeira, F., Fernandes, R., Reis, F., de Lemos, E.T., 2014. Sitagliptin prevents aggravation of endocrine and exocrine pancreatic damage in the Zucker Diabetic Fatty rat - focus on amelioration of metabolic profile and tissue cytoprotective properties. Diabetol. Metab. Syndrome 6, 42.

Mesquita, C.S., Oliveira, R., Bento, F., Geraldo, D., Rodrigues, J.V., Marcos, J.C., 2014. Simplified 2,4-dinitrophenylhydrazine spectrophotometric assay for quantification of carbonyls in oxidized proteins. Anal. Biochem. 458, 69–71.

Mestry, S.N., Gawali, N.B., Pai, S.A., Gursahani, M.S., Dhodi, J.B., Munshi, R., Juvekar, A.R., 2020. Punica granatum improves renal function in gentamicin-induced nephropathy in rats via attenuation of oxidative stress. J. Ayurveda Integr. Med. 11, 16–23.

Morosetti, G., Criscuolo, A.A., Santi, F., Perno, C.F., Piccione, E., Ciotti, M., 2017. Ellagic acid and Annona muricata in the chemoprevention of HPV-related pre-neoplastic lesions of the cervix. Oncol. Lett. 13, 1880–1884.

Nascimento-Gonçalves, E., Seixas, F., Silva, M., Fardilha, M., Ferreira, R., Neuparth, M.J., Faustino-Rocha, A.I., Colaço, B., Venâncio, C., Barros, L., Ferreira, I.C.F.R., Oliveira, M.M., Peixoto, F., Rosa, E., Oliveira, P.A., 2021. The influence of Castanea sativa Mill. flower extract on hormonally and chemically induced prostate cancer in a rat model. Food Funct. 12, 2631–2643.

Pal, A., Kundu, R., 2020. Human papillomavirus E6 and E7: the cervical cancer hallmarks and targets for therapy. Front. Microbiol. 10, 3116, 3116.

Patel, A.N., Bandawane, D.D., Mhetre, N.K., 2014. Pomegranate (Punica granatum Linn.) leaves attenuate disturbed glucose homeostasis and hyperglycemia mediated hyperlipidemia and oxidative stress in streptozotocin induced diabetic rats. Eur J Integr Med 6, 307–321.

Pottathil, S., Nain, P., Morsy, M.A., Kaur, J., Al-Dhubiab, B.E., Jaiswal, S., Nair, A.B., 2020. Mechanisms of antidiabetic activity of methanolic extract of Punica granatum leaves in nicotinamide/streptozotocin-induced type 2 diabetes in rats. Plants 9, 1609.

Puneeth, H.R., Chandra, S.S.P., 2020. A review on potential therapeutic properties of Pomegranate (*Punica granatum* L.). Plant Science Today 7, 9–16.

Rao, S.P., Krishnamurthy, V., 2019. Ethanol extract of Punica granatum L. Leaf in combination with gallic acid ameliorates liver dysfunction in high-fat diet-induced obese rats. Indian J. Pharmaceut. Sci. 81, 673–680.

Ross, I.A., 2005. Medicinal Plants of the World: Chemical Constituents, Traditional and Modern Medicinal Uses. Humana Press.

Sáez, G.T., Están-Capell, N., 2017. Antioxidant enzymes. In: Schwab, M. (Ed.), Encyclopedia of Cancer. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 288–294.

Santos, S., Ferreira, T., Almeida, J., Pires, M.J., Colaço, A., Lemos, S., Gil da Costa, R.M., Medeiros, R., Bastos, M.M.S.M., Neuparth, M.J., Abreu, H., Pereira, R., Pacheco, M., Gaivão, I., Rosa, E., Oliveira, P.A., 2019. Dietary supplementation with the red seaweed porphyra umbilicalis protects against DNA damage and pre-malignant dysplastic skin lesions in HPV-transgenic mice. Mar. Drugs 17, 615.

Saratale, R.G., Shin, H.S., Kumar, G., Benelli, G., Kim, D.-S., Saratale, G.D., 2018. Exploiting antidiabetic activity of silver nanoparticles synthesized using Punica granatum leaves and anticancer potential against human liver cancer cells (HepG2). Artif. Cell Nanomed. Biotechnol. 46, 211–222.

Sarkar, S., Kotteeswaran, V., 2018. Green synthesis of silver nanoparticles from aqueous leaf extract of Pomegranate (Punica granatum) and their anticancer activity on human cervical cancer cells. Adv. Nat. Sci. Nanosci. Nanotechnol. 9, 025014.

Sethi, N., Palefsky, J., 2004. Transcriptional profiling of dysplastic lesions in K14-HPV16 transgenic mice using laser microdissection. Faseb. J. 18, 1243–1245.

Smith-McCune, K., Zhu, Y.-H., Hanahan, D., Arbeit, J., 1997. Cross-species comparison of angiogenesis during the premalignant stages of squamous carcinogenesis in the human cervix and K14-HPV16 transgenic Mice 1. Cancer Res. 57, 1294–1300.

Swilam, N., Nematallah, K.A., 2020. Polyphenols profile of pomegranate leaves and their role in green synthesis of silver nanoparticles. Sci. Rep. 10, 14851.

Tehranifar, A., Selahvarzi, Y., Kharrazi, M., Bakhsh, V.J., 2011. High potential of agro-industrial by-products of pomegranate (Punica granatum L.) as the powerful antifungal and antioxidant substances. Ind. Crop. Prod. 34, 1523–1527.

Toda, K., Ueyama, M., Tanaka, S., Tsukayama, I., Mega, T., Konoike, Y., Tamenobu, A., Bastian, F., Akai, I., Ito, H., Kawakami, Y., Takahashi, Y., Suzuki-Yamamoto, T., 2020. Ellagitannins from Punica granatum leaves suppress microsomal prostaglandin E synthase-1 expression and induce lung cancer cells to undergo apoptosis. Biosci., Biotechnol., Biochem. 84, 757–763.

Usha, T., Middha, S.K., Sidhalinghamurthy, K.R., 2020. Pomegranate peel and its anticancer activity: a mechanism-based review. In: Swamy, M.K. (Ed.), Plant-derived Bioactives: Chemistry and Mode of Action. Springer Singapore, Singapore, pp. 223–250.

Villalba, K.J.O., Barka, F.V., Pasos, C.V., Rodríguez, P.E., 2019. Food ellagitannins: structure, metabolomic fate, and biological properties. Tannins-Structural Properties, Biological Properties and Current Knowledge 26–46.

Viswanatha, G.L., Venkataranganna, M.V., Prasad, N.B.L., Ashok, G., 2016. Evaluation of anti-epileptic activity of leaf extracts of Punica granatum on experimental models of epilepsy in mice. J Intercult Ethnopharmacol 5, 415–421.

Wallin, B., Rosengren, B., Shertzer, H.G., Camejo, G., 1993. Lipoprotein oxidation and measurement of thiobarbituric acid reacting substances formation in a single microtiter plate: its use for evaluation of antioxidants. Anal. Biochem. 208, 10–15.

Yu, M., Gouvinhas, I., Barros, A., 2021a. Variation of the polyphenolic composition and antioxidant capacity of freshly prepared pomegranate leaf infusions over one-day storage. Antioxidants 10, 1187.

Yu, M., Gouvinhas, I., Rocha, J., Barros, A.I.R.N.A., 2021b. Phytochemical and antioxidant analysis of medicinal and food plants towards bioactive food and pharmaceutical resources. Sci. Rep. 11, 10041.