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# Biochemical and toxicity evaluation of *Retama sphaerocarpa* extracts and *in-silico* investigation of phenolic compounds as potential inhibitors against HPV16 E6 oncoprotein

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ABSTRACT

Cervical cancer is a type of cancer which affects the cervix cells. The conventional treatments for cervical cancer including surgery, chemotherapy, and radiotherapy are only effective in premature stages and less effective in late stages of this tumor. Therefore, the therapeutic strategies based on biologically active substances from plants are needed to develop for the treatment of cervical cancer. The aim of the present study was to assess in vivo toxicity, hematological and biochemical blood parameters in Wistar rats fed *Retama sphaerocarpa* aqueous leaf extract (RS-AE), as well as to perform in silico molecular docking studies and dynamic simulation of phenolic compounds against HPV16 oncoprotein E6 in order to identify potential inhibitors. RS-AE was found not to induce acute or sub-acute oral toxicity or significant alterations in hematological and biochemical blood parameters in Wistar rats. A total of 11 phenolic compounds were identified in RS-AE, including dihydrodaidzein glucuronide, chrysoperiol pentoside, genistin and vitexin, which turned out to have the highest binding affinity to HPV16 oncoprotein E6. Based on these results, these RS-AE phenolic compounds could be used as natural drugs against the HPV16 E6 oncoprotein.

## 1. Introduction

Validation and use of three prophylactic vaccines (Cervarix®2, Gardasil®4, Gardasil®9) for persistent viral infections and HPV-associated cervical lesions [1], as well as the treatment of cervical cancer implies the use of chemotherapeutic agents and/or radiation

*Abbreviations:* AERS, *Retama sphaerocarpa* leaves; EDTA, Ethylene Diaminotetraacetic Acid; RBC, Red Blood Cells; RCDW, Red Cell Distribution Width; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; PLT, Platelet Count; Crea, Creatinine; TC, Total Cholesterol; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase.

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therapy, surgical and ablative techniques. As a consequence, anticancer drug research and development brought to the approval of over 100 anticancer drugs (cytotoxic and targeted) by the FDA [2,3]. Cytotoxic drugs act at the level of the mitosis and/or DNA replication processes; on the other hand targeted drugs interact with molecular targets involved in the pathways of cancer growth [4]. Cytotoxic drugs are less effective due to chemical resistance and the effect of non-target cells with undesirable side effects [5]. HR-HPV16 infection has been determined as a major etiological factor in the majority of cervical cancer cases [6]. The expression of HPV-encoded E6 and E7 oncogenes are key factors in HR-HPV-induced carcinogenesis, they are synergistically involved in the malignant transformation of infected cells due to their ability to degrade p53 and Rb, respectively. Through the cooperative interaction of the HPV E6 oncoprotein with different cellular proteins various pathways are activated ontogenically [7]. HR E6 oncoproteins which regulates the p53 gene transactivation can suppress the transcriptional transactivation activity of p53 [8]. HPV E6 proteins are quite small (comprising 150 amino acids, including cysteines, with two zinc-binding domains, namely E6N and E6C [9]. The amino-terminal zinc-binding domain of E6 and the carboxy-terminal zinc-binding domain has a conserved fold globally in the crystal [10]. The two zinc domains, along with an alpha helix tube connecting them, form a deep pocket in which the LXXLL peptide enters into a close relationship [11]. LxxLL is a 20 amino acid peptide in E6AP, which directs the recruitment and polyubiquitination of p53. The LxxLL peptide isolated from E6AP is large enough to make E6 susceptible to interaction with p53 [12]. The central pocket of E6 binds to the LxxLL motif of the E6AP ubiquitin ligase, which causes a conformational change in the HR proteins of E6 and allows the formation of a complex with p53 (E6/E6AP/p53) [13,14]. Herbal medicine has become a very safe and easily accessible source of anticancer compounds. Their effects on different types of cancers have also been identified as they neutralize the effects of diseases in the body due to various characteristics they possess [15], among which the extracted metabolites are used to induce apoptosis in cancer cells, It also plays a vital role in preventing malignant transformation and cancer development [16].

The aim of the work was to evaluate in vivo toxicity, hematological and biochemical blood parameters in Wistar rats fed with an aqueous extract of *Retama sphaerocarpa* leaves (RS-AE) grown in the Drâa-Tafilalet region of Morocco, as well as to characterize the phenolic compounds from RS-AE and investigate their in silico, molecular docking, dynamic simulation studies.

## 2. Materials and methods

### 2.1. Plant collection and preparation of the aqueous extract

*Retama sphaerocarpa* leaves were harvested fresh from a local in the Draa-Tafilalet region (semi-arid zone) in Morocco in October 2022. The leaves were washed under running tap water to remove soil debris and dirt and air-dried at 40 °C. A voucher specimen was deposited at the Faculty of Sciences and Techniques Errachidia (RS2022).

Plant material was prepared according to the traditional method used in Morocco (decoction), as described previously [17]. Finally, the extract was filtered and lyophilized in a lyophilizator (Labconco, G. Boyer, Casablanca).

### 2.2. Chemicals and reagents

LC-MS grade water ($H_2O$), acetonitrile (ACN), formic acid, vitexin 7-glucoside, glycitin, verbascoside, genistin, luteolin 7-*O*-glucoside, vitexin were obtained from Merck Life Science (Merck KGaA, Darmstadt, Germany). Kaempferol-3-O-(6-malonyl-glucoside) was attained from Axios Research (Toronto, Canada).

### 2.3. Phenolic compounds analysis by HPLC-PDA/ESI-$MS^2$

LC analyses were performed on a Nexera-e liquid chromatograph (Shimadzu, Kyoto, Japan), consisting of a CBM-20 A controller, two LC-30 CE dual-plunger parallel-flow pumps, a DGU-20A5R degasser, a CTO-20 AC column oven, a SIL-30 AC autosampler, an SPD-M30A photo diode array detector and an LCMS-8050 mass spectrometer, through an ESI source (Shimadzu, Kyoto, Japan). Separations were carried out on an Ascentis Express RP C18 column (2.7 µm, 150 mm, and 4.6 mm) (Merck Life Science, Merck KGaA, Darmstadt, Germany), by using the experimental conditions were reported in a previous work [18].

### 2.4. In vivo toxicity studies

#### 2.4.1. Preparation and grouping of experimental animals

Animal ethics committees at the Moulay Ismail University approved all the animals and experimental protocols used in the study. Healthy male and female Wistar albino rats (Wistar strain) weight about 150–250 g were obtained from the experimental center of Missour Morocco. The animals (each sex differently) were kept in plastic cages and maintained in standard condition, and the animals were fed ad libitum with a normal laboratory chow standard pellet diet. A total of 12 female rats containing three female rats per group were used for the acute toxicity study for each dose level [19], whereas 12 rats of both sexes containing 6 rats per group (three female and three male) were used for the sub-acute toxicity study [20].

#### 2.4.2. Acute oral toxicity study

The acute oral toxicity study on the aqueous extracts of *Retama sphaerocarpa* leaves (AERS) was conducted according to OECD guidelines [19]. Normal females, nulliparous and nonpregnant were fasted overnight before administration. Afterwards, all animals were weighed, and the doses were calculated based on their body weight. The extracts were prepared in distilled water. Firstly, one overnight fasted rat was administered a starting dose of 200 mg/kg body weight and observed for a period of 48 h. The animal was observed continuously for the first 30 min post-administration, every 1 h for 4 h, then once daily for 48 h for signs of toxicity including bodyweight variation, tremors, convulsions, salivation, diarrhea, mucous membranes, fur, and changes in the skin, or death before the next doses of 500 and 1000 kg body weight was administered. The control group received distilled water. In addition, the animals were monitored thereafter once every 24 h for the next 14 days for delayed signs of toxicity and mortality. The body weights were taken on day 0, day 7, and day 14. Finally, the $LD_{50}$ was determined.

#### 2.4.3. Sub-acute oral toxicity study

A subacute oral toxicity study was performed according to the OECD guidelines, by repeated dose toxicity method for 28 days. Twelve rats were randomly distributed into two groups (I and II,) each consisting of six rats (three female and three male) per group. Group I (the control group) received distilled water. Group II received the aqueous extract of AERS orally at a dose of 500 mg/kg body weight, the treatments were given by oral gavage once daily for 28 days. The clinical observation was taken out for 28 days and their weight was measured weekly for four weeks. On day 29, the animals fasted overnight with free access to water and then anesthetized under diethyl ether in a closed chamber. Blood was collected from each animal from the retro-orbital sinus into anticoagulant of ethylene diaminotetraacetic acid (EDTA) coated tubes and non-EDTA tubes for hematological and biochemical analyses, respectively. After collection of blood samples, the rats were sacrificed by cervical dislocation, and the organs (heart, lungs, liver, kidneys, and spleen) were carefully removed from the dissected rats, washed with distilled water, weighed and macroscopically examined for any gross changes and gross lesions. Organs were weighed and their index in relation to body weight was calculated as described in [21]:

$$\text{Relative organ weight (ROW) (g)} = \frac{\text{weight of organ}}{\text{body weight of rats on the day of sacrifice}} \times 100$$

#### 2.4.4. Evaluation of hematological and biochemical blood parameters

Blood samples in the test tubes containing EDTA were used and examined using an Automated Hematology Analyzer (Symex KX-21, Japan) to determine the hematological parameters, notably total white blood cell (WBC) count, Lymphocyte, Red Blood Cells (RBC), Red Cell Distribution Width (RCDW), Hemoglobin (HGB), Hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelet count (PLT). For biochemical analyses, Blood samples in the test tubes without anticoagulant were coagulated at room temperature, and then centrifuged at 3000 rpm for 10 min using an electrical centrifuge, and were obtained for biochemical analysis. Urea, Creatinine (Creat), total cholesterol (TC), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were analyzed using a chemistry analyzer (Erba XL-600, Germany).

#### 2.4.5. Statistical analysis

The results of the study are expressed as the mean ± SEM. Statistical differences among the means studied were assessed by one-way ANOVA followed by Tukey's multiple comparisons test with GraphPad Prism 8 software. Differences in the mean between the treatment groups were considered significant at $p < 0.05$.

### 2.5. In silico studies

#### 2.5.1. Data set

*Retama sphaerocarpa* is a broom-like, almost leafless, pioneer shrub with photosynthetic evergreen stems; this shrub is found in the Mediterranean zone of northeast Africa [22,23]. Although extracts of other *Retama* species, namely *Retama. monosperma*, were found to have anticancer activity against cervical cancer cells [24]. The 11 polyphenols, belonging to the leaves were selected [25], and the three-dimensional structure of these compounds was extracted from PubChem [26] in SDF format. The molecules were then transformed to PDB format. The PDB file was then used for molecular docking.

#### 2.5.2. Virtual screening

High-speed virtual screening was performed using the iGEMDOCK (Generic Evolution Method for Docking) program [27]. The in silico screening of 11 polyphenols was performed using the PDB [28] code of the targets (PDB ID: 4GIZ) chain "D".

#### 2.5.3. Molecular docking

The ligands Dihydrodaidzein glucuronide, Chrysoeriol pentoside, Genistin, Vitexin were docked to the 'D' chain of the unit cell of the crystal structure (PDB: 4GIZ) [29,30]. The SYBYL-X Surflex-Dock module v2.0, 21 [31], was used to generate bioactive binding positions of ligands in the E6HPV16 active site. After completing docking, the ligand pose gave the minimum binding energy. Discovery studio [32] and PyMOL [33] were used to visualize the results. The type of interactions established by each molecule inside the active site was also compared.

#### 2.5.4. Molecular dynamics

Dihydrodaidzein glucuronide, Chrysoeriol pentoside, Genistin, Vitexin are the best molecules that have higher affinity for the E6HPV16 active site. They were selected and analyzed by 50 ns molecular dynamics simulations using GROMACS 2018 (Groningen Machine for Chemical Simulations) [34]. Docking studies were the preparatory phase of protein-ligand complexes aimed at molecular dynamics modeling, which predicts the ligand binding state in static proportions. Molecular dynamics simulations aim to determine the atomic motions as a function of time by applying the classical Newtonian equation of motion. The binding state of the ligand in the physiological medium was predicted using simulations. Protein-ligand pairs were preprocessed using Maestro's Protein Preparation Wizard, which includes optimization and minimization of complexes. The System Builder tool was used to create all systems. TIP3P (Transferable Intermolecular Interaction Potential 3 Points) was selected as the solvent model with an orthorhombic box. The OPLS_2005 force field was used for the simulation [35].

## 3. Results and discussion

### 3.1. Phenolic profile by HPLC-PDA-ESI-MS$^2$

Phenolic profile analysis of the aqueous extract of RS was performed by HPLC coupled to PDA and ESI-MS$^2$ detection (Fig. 1). As seen in Table 1, a total of 11 polyphenolic compounds were detected in RS-AE, out of them, 10 were belong to flavonoids (kaempferol di-*O*-glucoside, vitexin 7-glucoside, glycitin, genistin, luteolin 7-*O*-glucoside, dihydrodaidzein glucuronide, derivatives, luteolin derivatives, dihydrodaidzein glucuronide, chrysoeriol pentoside chrysoeriol pentoside, vitexin, kaempferol 3-*O*-(6″-*O*-malonyl)glucoside, and apigenin malonyl hexoside), whereas one compound to phenylpropanoid (verbascoside). Identification was based on retention times, $\lambda_{max}$, MS and MS/MS data, co-standard injection (with the exception of dihydrodaidzein glucuronide and apigenin malonyl hexoside), and literature data.

Vitexin 7-glucoside was the most abundant compound (972.09 mg/Kg ± 5.23), followed by genistin (566.03 ± 3.25) and luteolin 7-*O*-glucoside (564.62 ± 9.27). Among the identified compounds, six out of them, namely glycitin, verbascoside dihydrodaidzein glucuronide, chrysoeriol pentoside, kaempferol 3-*O*-(6″-*O*-malonyl)glucoside and apigenin malonyl hexoside are here reported for the first time as constituents of the leaves of RS. Searching throughout literature vitexin 7-glucoside has been reported in the water extracts of Algerian RS fruits extracts [36], in the water/methanol extracts of Algerian RS fruits and grains [37] and in the water/ethanol extract of Moroccan *Retama sphaerocarpa* L. leaves [38]. Likewise, genistin has been reported, besides in Algerian RS fruits [36,38], also in water extracts of the aerial parts of Algerian RS [39].

### 3.2. In vivo toxicity studies

In terms of acute toxicity, the results of aqueous extract of AE-RS on female rats revealed that no mortality was noticed at the doses of 200,

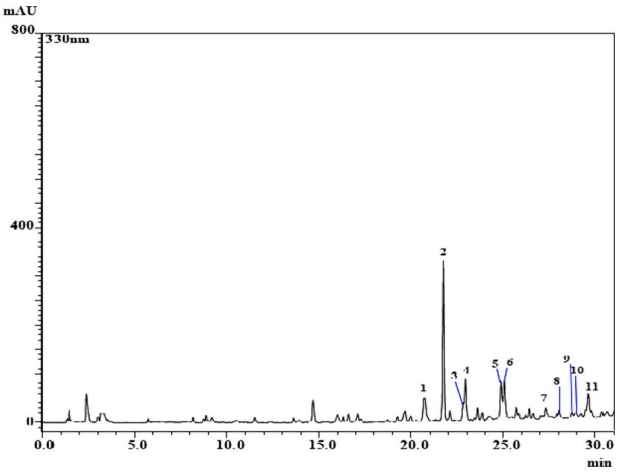


**Fig. 1.** Analysis of phenolic compounds by HPLC-PDA-ESI-MS$^2$ of the RS-AE extract (λ = 350 nm).

**Table 1**
Quantification of phenolic compounds in water extracts of RS-AE by LC-PDA/ESI-$MS^2$ analysis (mg/kg ± SD, $n = 3$).

| Peak No | Compound | $t_R$ (min) | $UV_{max}$ (nm) | [M-H]- | $MS^2$ | mg/kg ± SD | Ref. |
|---|---|---|---|---|---|---|---|
| 1 | Kaempferol di-O-glucoside | 20.66 | 207, 257, 356 | 609 | 285 | 379.12 ± 0.10 | [38] |
| 2 | Vitexin 7-glucoside | 21.71 | 216, 265, 340 | 593 | 431, 269 | 972.09 ± 5.23 | [36–38] Co-standard |
| 3 | Glycitin | 22.81 | 255, 320 | 445 | 285 | 393.04 ± 8.07 | Co-standard |
| 4 | Verbascoside | 22.91 | 233, 340 | 623 | 461 | 436.56 ± 6.60 | Co-standard |
| 5 | Genistin | 24.86 | 261, 335 | 431 | 270 | 566.03 ± 3.25 | [36,38,39] Co-standard |
| 6 | Luteolin 7-*O*-glucoside | 25.03 | 213, 256, 344 | 447 | 285 | 564.62 ± 9.27 | [37] Co-standard |
| 7 | Dihydrodaidzein glucuronide | 27.30 | 229, 268, 330 | 431 | 253 | 157.60 ± 2.55 | – |
| 8 | Chrysoeriol pentoside | 27.90 | 233, 336 | 431 | 299 | 123.64 ± 7.56 | Co-standard |
| 9 | Vitexin | 28.72 | 217, 270, 340 | 431 | 269 | 76.22 ± 0.33 | [37,38] Co-standard |
| 10 | Kaempferol 3-*O*-(6″-*O*-malonyl)glucoside | 28.93 | 233, 268, 331 | 533 | 489, 285 | 75.98 ± 2.53 | Co-standard |
| 11 | Apigenin malonyl hexoside | 33.09 | 197, 260, 332 | 517 | 269 | 324.82 ± 1.72 | – |

500, or 1000 mg/kg body weight. In addition, no sign of toxicity was observed in rats treated with 200 and 500 mg/kg. While the group treated with 1000 mg/kg shows some traces of urination after one hour of administration. The rats remained healthy throughout the 14-day study. In general, a gradual and normal increase in animal body weight within the study period (Fig. 2) was appreciated. According to the OECD 423 guideline, no death among animals treated with 200, 500, and 1000 mg/kg body weight of extract is reported implying that the $LD_{50}$ corresponds to a value higher than 1000 mg/kg. All the treated rats of both sexes at the dose of 500 mg/kg b.w survived throughout the 28 days of treatment with no observable toxicity signs.

It is known that the kidneys maintain essential compounds in the blood while allowing the elimination of toxins, and metabolic wastes as they control plasma osmolality by regulating the concentration of solutes and electrolytes in the blood [40]. Moreover, the single oral administration of aqueous extract of *Retama sphaerocarpa* with the doses of 200, 500, and 1000 mg/kg did not cause any significant change in body weight for 14 days in comparison to the control in female or male rats [41].

Subsequently, a sub-acute toxicity study was carried out with the aim of providing information on the general characteristics of toxicity, with accumulation in the body and effects on tissues and specific target organs. For this purpose, the aqueous extract of *Retama sphaerocarpa* was evaluated in rats at a dose of 500 mg/kg by measuring body weight, hematological and biochemical parameters, and calculating relative organ weights. During the subacute experimental period, there was a gradual and normal increase in mean body weight in rats treated with the 500 mg dose and in untreated rats (control). However, there were no significant variations ($p > 0.05$) mean body weights compared to the control group. (Fig. 3). Likewise, the sub-chronic oral administration of *Retama sphaerocarpa* extract resulted in no mortality and no behavioral changes in female and male rats throughout the 28-day study period. In

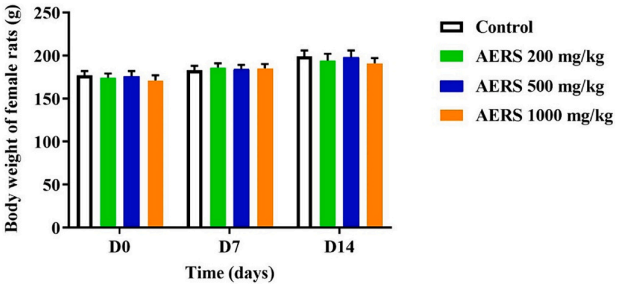


**Fig. 2.** Body weight of female rats, control group and treated with aqueous extract of *R. sphaerocarpa* at doses 200, 500, and 1000 mg/kg body weight in the acute toxicity study; Values expressed as mean ± SEM, $n = 6$.

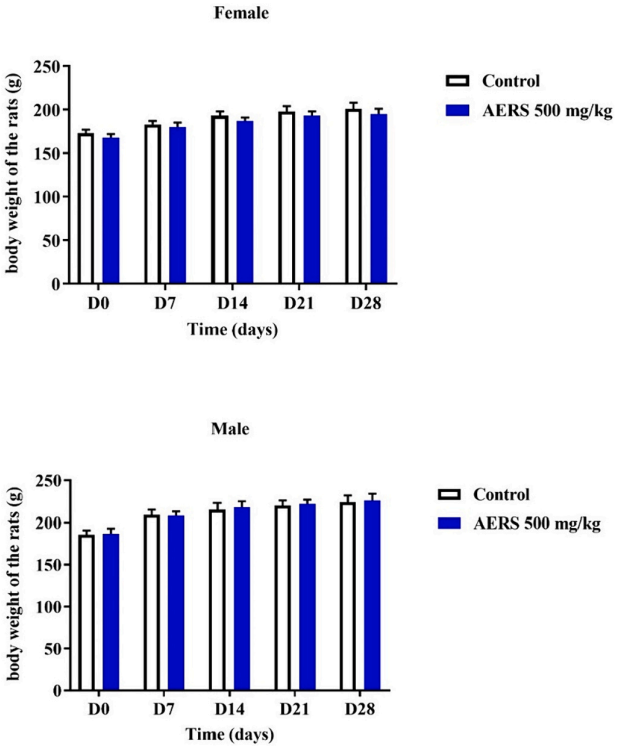


**Fig. 3.** Body weight of female and male rats, control group and treated with aqueous extract of *R. sphaerocarpa* at doses 500 mg/kg body weight in the sub-acute toxicity study; Values expressed as mean ± SEM, n = 6.

male or female rats, there were no significant ($p > 0.05$) variations in the relative weights of the heart, lungs, spleen, liver, kidney, ovary (Table 2).

The hematological parameters of the female and male rats revealed a slightly significant increase in platelet count at a dose of 500 mg/kg b.w (Table 3). During the subacute toxicity study, the hematological parameters of the female and male rats revealed a significant increase in serum AST at 500 mg/kg b.w to the control (Table 4). Considering the assessment of biochemical parameters, no significant differences in Urea, Creat, TC, and ALT values were appreciated except for serum AST which showed a significant increase compared to the control for both female and male rats. AST and ALT are principally produced by the liver cells and any assault on the liver may lead to an increase in the serum

**Table 2**
Effect of aqueous extract of *R. sphaerocarpa* on relative organ weights in female and male Wistar rats treated at a dose of 500 mg/kg for 28 days.

| Rats | Organ | Relative organ weight (%) | |
|---|---|---|---|
| | | 500 mg/kg b.w | 500 mg/kg b.w |
| Females | Heart | 0.49 ± 0.03 | 0.49 ± 0.03 |
| | Lungs | 0.78 ± 0.02 | 0.78 ± 0.02 |
| | Liver | 3.32 ± 0.12 | 3.32 ± 0.12 |
| | Kidneys | 0.74 ± 0.04 | 0.74 ± 0.04 |
| Males | Heart | 0.51 ± 0.05 | 0.47 ± 0.04 |
| | Lungs | 0.64 ± 0.03 | 0.55 ± 0.04 |
| | Liver | 2.92 ± 0.10 | 2.77 ± 0.11 |
| | Kidneys | 0.69 ± 0.03 | 0.58 ± 0.02 |
| | Spleen | 0.19 ± 0.02 | 0.23 ± 0.03 |

Data are expressed as means ± SEM, n = 6 rats per group.

**Table 3**
Hematological parameters of rats treated with aqueous extract of *R. sphaerocarpa* for 28 days in female and male rats.*

| Rats | Hematological parameters | 500 mg/kg b.w | Control group |
|---|---|---|---|
| Females | WBC (×109/L) | 9.44 ± 0.77 | 8.94 ± 0.42 |
| | LYM (×109/L) | 6.65 ± 0.70 | 4.21 ± 0.62 |
| | RBC (×1012/L) | 6.35 ± 0.32 | 6.07 ± 0.41 |
| | HGB (g/dL) | 13.21 ± 0.22 | 11.98 ± 0.32 |
| | HCT (%) | 33.67 ± 1.58 | 30.62 ± 1.78 |
| | MCV (fL) | 53.12 ± 2.43 | 46.8 ± 3.45 |
| | MCH (pg) | 20.3 ± 0.40 | 19.45 ± 0.22 |
| | MCHC (g/dL) | 35.82 ± 0.54 | 40.1 ± 0.82 |
| | RCDW (%) | 16.84 ± 1.85 | 18.31 ± 1.60 |
| | PLT (×109/L) | 501.21 ± 18.29 | 482.73 ± 23.12 |
| Males | WBC (×109/L) | 12.75 ± 1.40 | 8.44 ± 0.52 |
| | LYM (×109/L) | 8.01 ± 0.86 | 5.51 ± 0.53 |
| | RBC (×1012/L) | 7.67 ± 0.28 | 8.12 ± 0.47 |
| | HGB (g/dL) | 14.5 ± 0.63 | 15.32 ± 0.40 |
| | HCT (%) | 36.12 ± 1.82 | 39.16 ± 1,62 |
| | MCV (fL) | 46.11 ± 3.13 | 49.81 ± 2.83 |
| | MCH (pg) | 18.9 ± 0.50 | 19.8 ± 0.44 |
| | MCHC (g/dL) | 41.2 ± 0.73 | 39.13 ± 0.45 |
| | RCDW (%) | 20.4 ± 0.93 | 20.1 ± 1.02 |
| | PLT (×109/L) | 601.28 ± 19.14 | 587.34 ± 24.63 |

Data are expressed as means ± SEM, n = 6 rats per group.
* $p < 0.05$ when compared to the control.

**Table 4**
Biochemical parameters of rats treated with aqueous extract of *R. sphaerocarpa* for 28 days in female and male rats.

| Rats | Biochemical parameters | 500 mg/kg b.w | Control group |
|---|---|---|---|
| Females | Urea mg/dl | 41 ± 6.22 | 44 ± 4.72 |
| | Creat mg/dl | 0.49 ± 0.14 | 0.36 ± 0.10 |
| | TCmg/dl | 85 ± 9.70 | 78 ± 7.36 |
| | AST U/L | 352.1 ± 18.14* | 311.9 ± 15.52 |
| | ALAT U/L | 80.6 ± 12.16 | 77.6 ± 10.43 |
| Males | Urea mg/dl | 35 ± 4.12 | 39 ± 3.63 |
| | Creat mg/dl | 0.47 ± 0.08 | 0.35 ± 0.10 |
| | TCmg/dl | 73 ± 8.72 | 64 ± 6.98 |
| | AST U/L | 333.8 ± 7.11* | 292.7 ± 14.34 |
| | ALAT U/L | 93.5 ± 10.70 | 98.9 ± 13.12 |

Data are expressed as means ± SEM, n = 6 rats per group.
* $p < 0.05$ when compared to the control.

level of these enzymes. However, AST is also found in abundance in kidneys, testes, and cardiac and skeletal muscles [42]. This significant increase in the AST of both the male and female rats treated at 500 mg/kg may be due to the extract being mildly toxic.

### 3.3. In silico studies

In the human body, 30,000 genes were identified, and 6000 to 8000 are considered potential pharmacological target sites. Nevertheless, fewer than 400 encoded proteins are useful for drug development to date [43]. In recent years, cancer treatment has been considered a new field of research [44]. There are conventional techniques as well as very modern techniques applied against cancers, chemotherapy, radiotherapy, or surgery. The use of conventional chemicals leads to side effects and toxicities [45]. Therefore, it is necessary to develop new strategies for the prevention and cure of cancer to control the mortality rate from this disease. On the other hand, the use of medicinal plants has received much consideration for the treatment of various diseases. The role of plant metabolites in cancer treatment has been cited in several studies [46]. The occurrence of cervical cancer in women has been linked to HPV infection. The formation of complexes between the HPV E6 oncoprotein and the cellular ubiquitin ligase E6AP leads to many changes [47]. Furthermore, suppression of the creation of the E6-E6AP complex is one of the main strategies to inhibit the survival and proliferation of infected cells. This study is in line with the objective of valorization of natural resources and will highlight the antiviral properties of *Retama sphaerocarpa*.

The 11 polyphenols identified in the RS-AE were screened to test their efficacy in targeting E6HPV16 protein; for such a task, an in-silico approach with the aim to discover potential natural antiviral compounds. Molecular docking techniques are widely used to determine the binding mechanisms between the ligand and the receptor. The target ligand is docked into the active site to validate the accuracy of the molecular docking. 4 compounds out of them displayed a docking score between −7.4 kcal/mol and − 6.7 kcal/mol and were selected as promising E6HPV16 inhibitor candidates. The 4 candidates with the highest docking score are presented in Table 5. The positioning of these 4 molecules with HPV16 E6 was visualized. Dihydrodaidzein glucuronide was inserted into the binding pocket of the oncoprotein E6HPV16 (PDB code: 4GIZ), as shown in Fig. 4, docking results show hydrogen bonds with residues Gln(107), Tyr(70), as well as 9 Van der Waals bonds with residues Glu (75), Phe(125), Gln(123), Gly(134), Arg(117), and 1 stacked π divisions Tyr(79). Chrysoeriol pentoside was inserted into the binding pocket of the oncoprotein E6HPV16 (PDB code: 4GIZ), as shown in Fig. 5, the docking results show four hydrogen bonds with residues Arg(8), Tyr(70), Arg(55), Ser(71) and 4 pi-alkyl bonds with Val(31), Phe (45), Val(53), Val(62), as well as 8 Van der Waals bonds with residues Leu (52), Pro(5), Leu(50), Ser(74), Gln (107), Arg(131), Gln(107), Leu (61). Genistin, was inserted into the binding pocket of the oncoprotein E6HPV16 (PDB code: 4GIZ), as shown in Fig. 6, the docking results show one hydrogen bond with Arg(124), and 2 stacked pi-pi bonds with His (126), Tyr(79), as well as 8 Van der Waals bonds with His(118), Phe (125), Gln(123), Arg(117), and two alkyl bonds with His(126), Tyr(79). Vitexin was inserted into the binding pocket of the oncoprotein E6HPV16 (PDB code: 4GIZ), as shown in Fig. 7, the docking results show three hydrogen bonds with residues Gln(107), Ser(74), Tyr(32) and 2 alkyl bonds Leu(50), Val(62), as well as 12 Van der Waals bonds with residues Val(31), Val(53), Ala(61), Phe(45), Tyr(60), Cys(51), Leu(67), Arg(102);Arg(131),Ile(128), Ser(71), Tyr(70). These molecules had the highest stability and affinity (binding energy higher than −77.82 and lower than −90.55 kcal/mol). The docking results of these four molecules (Dihydrodaidzein glucuronide, Chrysoeriol pentoside, Genistin, Vitexin), applying SYBYL-X Surflex-Dock v2.0, 21, showed a higher docking score (−6.7 and − 7.4), and the four selected molecules

**Table 5**
Docking results highlighting binding affinities of phytocompounds and proven hydrogen interactions with amino acids.

| Ligand name | Total Energy | Binding affinity |
|---|---|---|
| Dihydrodaidzein glucuronide | −90.18 | −7.4 |
| Chrysoeriol pentoside | −85.33 | −6.8 |
| Genistin | −83.1 | −6.7 |
| Vitexin | −77.82 | −7.1 |

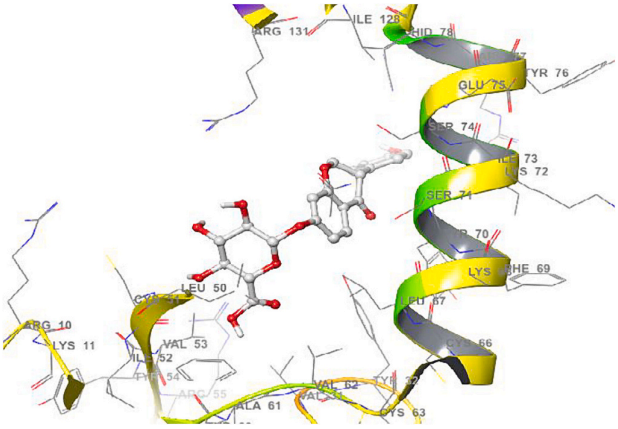


**Fig. 4.** 3D visualization of different interactions between the active site of E6HPV16 and selected Dihydrodaidzein glucuronide from *R. sphaerocarpa*.

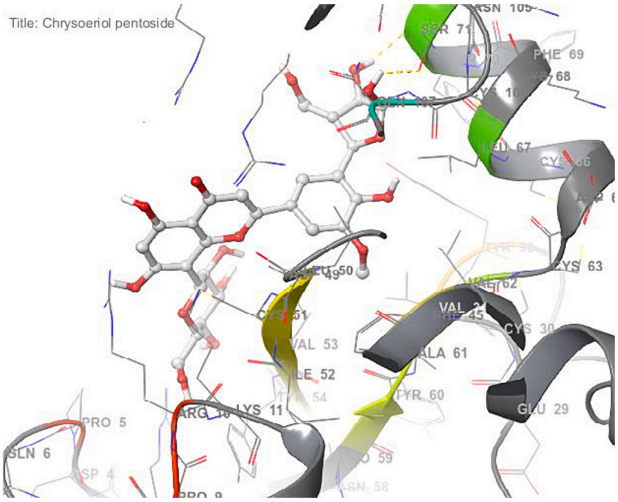


**Fig. 5.** 3D visualization of different interactions between the active site of E6HPV16 and selected Chrysoeriol pentoside from *R. sphaerocarpa*.

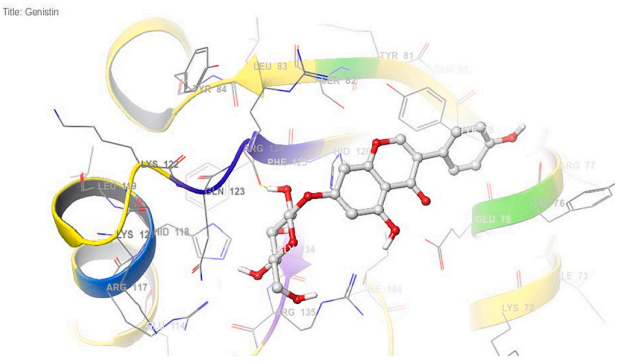


**Fig. 6.** 3D visualization of different interactions between the active site of E6HPV16 and selected Genistin from *R. sphaerocarpa*.

revealed several interactions with the E6HPV16 active site. These four molecules were selected in the virtual screening step as well as for an additional dynamic simulation study to verify their stability for 50 ns. The evolution of RMSD values with respect to time for the C-alpha atoms

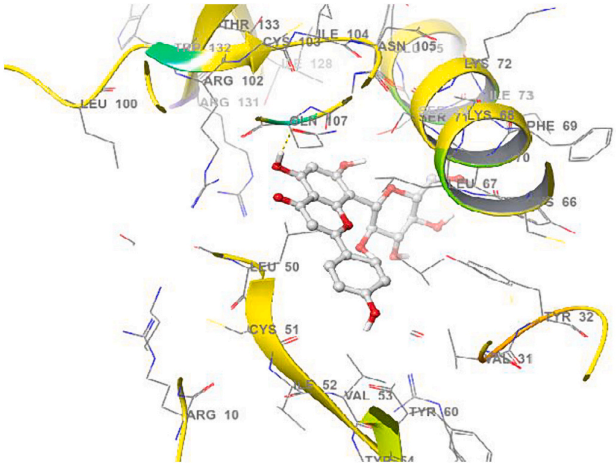


**Fig. 7.** 3D visualization of different interactions between the active site of E6HPV16 and selected Vitexin from *R. sphaerocarpa*.

of four complexes reaches stability at 10 ns, from this point on, the evolution of RMSD values remains within 1.5 for the target (1ea1) during the simulation time, which can be considered quite satisfactory. To provide evidence of retaining the same interactions after molecular docking studies, protein-ligand contact histograms are illustrated in Fig. 9.

Among the tools in the framework of computational modeling, the molecular dynamics simulation itself finds today many applications in biology and biomedicine. As a consequence, Dihydrodaidzein glucuronide, Chrysoeriol pentoside, Genistin, Vitexin were selected to evaluate their stability with E6HPV16 by the molecular dynamics simulation method. The Desmond simulation trajectories were analyzed, and the root mean square deviation (RMSD) (Fig. 8). The evolution of RMSD values as a function of time for the C-alpha atoms of four complexes reaches stability at 10 ns, from which point onwards the evolution of RMSD values remains within 1.5 for the target (4GIZ) for the duration of the simulation. The RMSD indices of protein-coupled ligands fluctuated between 1.5 and 50 ns after being stable, thus highlighting that the ligands are stably bound to the receptor binding site during the simulation period.

## 4. Conclusion

In this study, the phenolic profile, in vivo toxicity, hematology, biochemistry of the aqueous extract obtained from *Retama sphaerocarpa* leaves (RS-AE) collected in the Drâa-Tafilalet region of Morocco were evaluated, as well as the molecular docking and dynamic simulation studies of phenolic compounds were investigated. Results showed that the $LD_{50}$ values of the aqueous extract of *R. sphaerocarpa* leaves were 1000 mg/kg, and 500 mg/kg for acute oral toxicity, and subacute oral toxicity respectively. A total of 11 polyphenolic compounds attributed to flavonoids and phenylpropanoid were detected in RS-AE. Additionally, based on molecular docking and dynamic simulation studies, among of all phenolic compounds' studies, Dihydrodaidzein glucuronide, Chrysoeriol pentoside, Genistin, Vitexin were selected as a potent inhibitors against HPV16 E6 oncoprotein. Despite the promising results achieved, experimental verification of the computational predictions will be the subject of further incoming studies.

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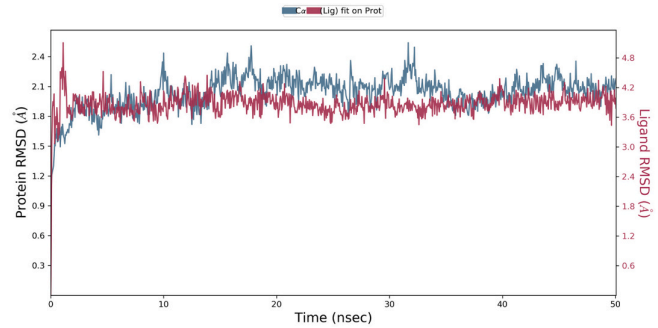
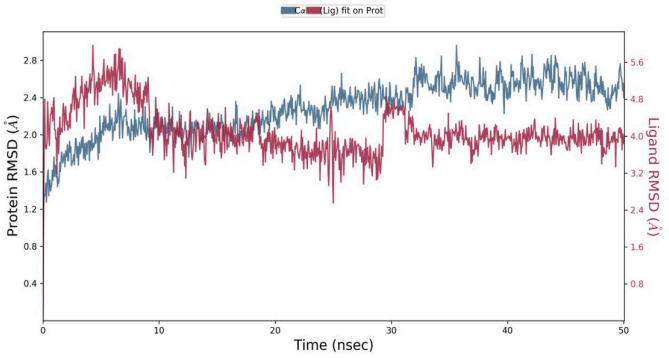
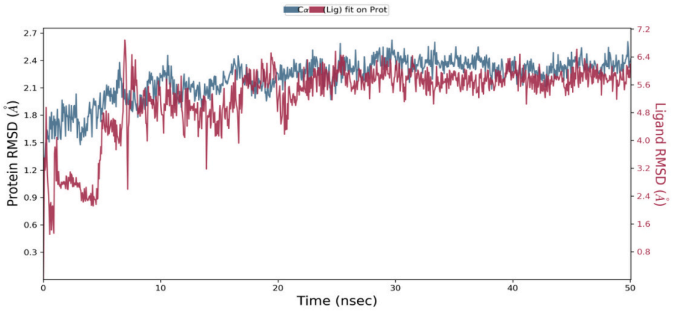


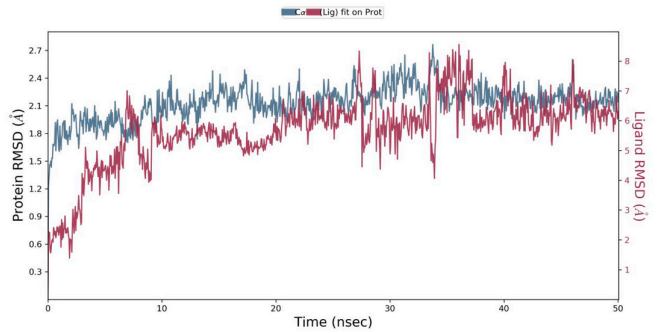

**Dihydrodaidzein glucuronide**




**Chrysoeriol pentoside**




**Genistin**




**Vitexin**

**Fig. 8.** Root means square deviation (RMSD) of the C-alpha atoms of protein and the ligand with time. The left Y-axis shows the variation of protein RMSD through time. The right Y-axis shows the variation of Ligand RMSD through time.

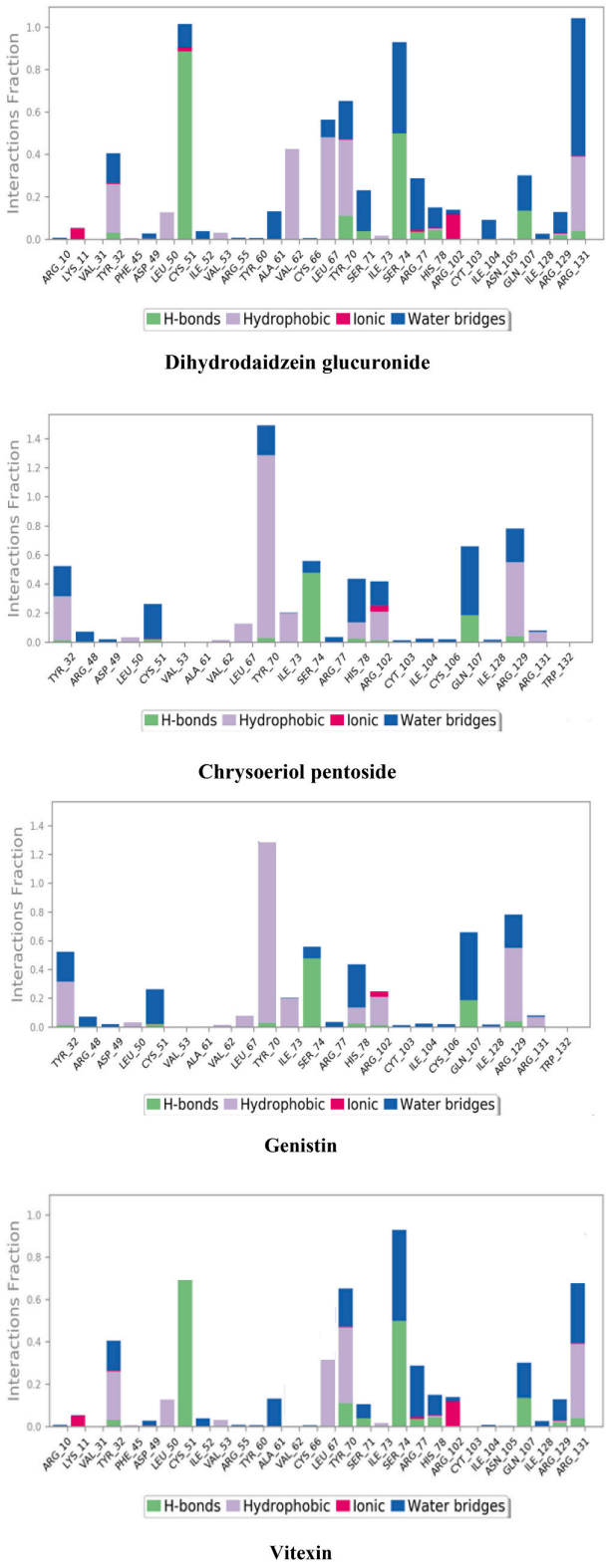



**Fig. 9.** Protein-ligand contact histograms.

## CRediT authorship contribution statement

**Soumia Moujane:** Conceptualization, Data curation, Methodology, Writing – original draft. **Ismail Bouadid:** Investigation. **Aziz Bouymajane:** Conceptualization, Data curation, Supervision. **Filali Zegzouti Younes:** Investigation. **Mohamed Benlyas:** Investigation. **Bouachrine Mohammed:** Investigation. **Francesco Cacciola:** Conceptualization, Supervision, Writing – review & editing. **Roberto Laganà Vinci:** Investigation. **Alessia Tropea:** Data curation. **Luigi Mondello:** Project administration. **Ammar B. Altemimi:** Writing – review & editing. **Mohamed Eddouks:** Supervision. **Benaissa Moualij:** Supervision.

## 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.

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