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Comparison of the Protective Effect of Ethanolic Extracts of Four Plants Against Damages Induced by Gamma Radiation in *Tetrahymena pyriformis*

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Abstract: This study investigated the ability of *Cistus ladanifer*, *Inula viscosa*, *Lavandula stoechas*, and *Nerium oleander* ethanolic extracts (EEs) to restore injuries caused by gamma radiation in *Tetrahymena pyriformis*. After chemical characterization of the 4 EEs by gas chromatography-mass spectrometry analysis and radical scavenging assays, their radiation protective effects on cell growth and morphology, as well as on certain metabolic and antioxidant enzymes, were assessed in *T. pyriformis* exposed to cobalt-60 as a radiation source. The addition of EEs at non-toxic concentrations significantly improves the growth of *T. pyriformis* under irradiating conditions. Morphological analysis showed that cells cultured under irradiating conditions in the presence of the 4 EEs were able to achieve their normal shape. Our results also show that the 4 EEs allowed the recovery, partially or completely, of the activities of glyceraldehyde-3-phosphate dehydrogenase and succinate dehydrogenase. Furthermore, the presence of EEs decreased the lipid peroxidation level and reduced the catalase and superoxide dismutase activities, augmented by exposure to gamma radiation. A protective effect was more markedly noted for *L. stoechas*, *N. oleander*, and *C. ladanifer* EEs compared to *I. viscosa* EE. Our results show, for the first time, that the studied EEs are promising sources of natural antioxidants that could protect cells against damage induced by gamma radiation and can, therefore, be useful in the medical field.

Keywords: gamma radiation; plant ethanolic extracts; protective effect; antioxidant activity.

1. Introduction

Ionizing radiation (IR) has an important role in the fields of industry, agriculture, and medicine (imaging and radiotherapy). A widely used source in radiotherapy is gamma beams from a radioactive cobalt-60 (^{60}Co) [1]. Gamma rays emitted by ^{60}Co are commonly used in many applications due to the relevance of this radioisotope as a significant dose contributor to

organisms, the great penetrating capacity of its radiation, and the ease of adjusting external conditions to gamma exposure [2]. Despite these great benefits, exposure to IR can inevitably cause direct or indirect radiation damage to the cell when not used properly. IR causes many side effects in cells, such as interfering with chemical bonds (breaking or crosslinking) and ionizing different essential macromolecules, such as membrane lipids, proteins, and nucleic acids [3]. Additionally, IR has indirect effects via the generation of reactive oxygen species (ROS), like hydrogen peroxide, superoxide, hydroxyl radicals, etc. [4], generated through water radiolysis [5]. These free radicals are highly oxidizing and react with some essential components of the cell (enzymes, membranes, RNA, and DNA), causing cellular dysfunction [6]. Gamma radiation-induced ROS can also affect some metabolic functions, such as glycolysis [7], and antioxidant defense systems, such as glutathione peroxidase, superoxide dismutase (SOD), and catalase (CAT) [8]. The radiation injury is aggravated with the increase in the absorbed doses [9]. However, several natural antioxidants have been described as reducing the risks of ROS-induced damage [10]. A number of phytochemicals (alkaloids, terpenes, flavonoids, phenolic acids, etc.) in medicinal plants can have a key role in the management and prevention of several diseases caused by ROS-induced stress due to their antioxidant activities [11]. These bioactivities can, therefore, be used to protect living organisms against radiation damage. In this context, only a few studies are available on medicinal plant extracts for the protective effect against radiation [12]. In addition, ethanolic extracts (EEs) from medicinal plants are rich in flavonoids, terpenes, and phenolic compounds [13], which may have great potential applications in reducing ROS levels to prevent damage caused by IR.

In our previous study, we examined radiation consequences on *Tetrahymena pyriformis* using gamma sources [14]. This ciliate was used as a eukaryotic unicellular model for a number of cytotoxicity, cell division, cell morphogenesis, conjugation, and protein regulation studies [15]. When the protozoan cells were cultured in the presence of gamma radiation, growth and morphological modifications were reported. Also, gamma radiation induced both an increase in lipid peroxidation and CAT and SOD activities and a decrease in succinate dehydrogenase (SDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activities [14]. The changes observed in enzymatic activities demonstrate a probable involvement of the studied enzymes in the defense system to protect the cell against damage induced by gamma radiation [16]. For that, *T. pyriformis* appears to be useful for assessing the protective effect of natural products against IR-induced injury in cells.

In this study, we sought to assess and compare the protective properties of 4 EEs against injury caused by *T. pyriformis* cultured under irradiating conditions. EEs used here are from *Cistus ladanifer*, *Inula viscosa*, *Lavandula stoechas*, and *Nerium oleander*, plants known for their therapeutic virtues [17-20]. To our knowledge, no study is available in the literature concerning the protective effect of these 4 EEs against radiation-induced damage by using a protozoan as a eukaryotic unicellular model. After chemical characterization of these EEs by gas chromatography-mass spectrometry (GC-MS) analysis and radical scavenging assays, the purpose of this study was to evaluate the cytotoxicity of these EEs and then to investigate their ability to repair the observed modifications on growth and morphology, as well as on the activities of certain metabolic and antioxidant enzymes in the ciliate *T. pyriformis* exposed to a gamma-radiation source.

2. Materials and Methods

2.1. Plant material.

Aerial parts of *C. ladanifer*, *I. viscosa*, *L. stoechas*, and *N. oleander* were collected during February 2019 from Ain Hjar Beni Mansour (35° 9’ 34” N; 4° 54’ 35” W), Ain Atiq (33° 54’ 16” N; 6° 56’ 52” W), Taounate (34° 32’ 20” N; 4° 38’ 3” W), and Rabat (33° 56’ 47” N; 6° 51’ 17” W), respectively. Plants were authenticated by Pr. Mohamed Fennane, a botanist from the Scientific Institute of Rabat, and deposited in the Herbarium Institute at the University of Mohammed V (Rabat, Morocco). After washing with distilled water, the vegetal material was air-dried in the shade at room temperature, ground to a fine powder using a grinder (IKA M20, Germany), and then stored at 4°C.

2.2. Ethanolic extracts (EEs) preparation.

The powdered plants were extracted with 60% ethanol (10 g of lyophilized powder / 100 ml) at 45°C for 18 h using the maceration method [21]. The mixture was then filtered through Whatman N°1 filter paper (Whatman, UK). The filtrate was dried at 40°C under a reduced pressure of about 100 mbar using a rotary evaporator (Laborota 4003, Heidolph, Germany) to obtain the ethanolic extract (EE). The dry weight of each extract was measured to determine its yield and then stored at 4°C in the dark until use.

2.3. Gas chromatography-mass spectrometry (GC-MS) analysis of EEs.

The compositional analysis of the EEs was performed using a Shimadzu GC-2010 Plus gas chromatograph equipped with a Shimadzu QP2010 Plus mass spectrometer, a BP-5 capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness, SGE Ltd.), and a split-splitless injector. The oven temperature was programmed to follow a specific profile: an initial ramp from 60 to 280°C at a rate of 10°C/min, followed by a 10 min isothermal hold at 280°C. The temperatures of the injector and detector were set to 250°C, while the transfer line temperature was set to 300°C and the ion source temperature to 200°C. The carrier gas used was helium, with a linear velocity of 36.5 cm/s. The ionization energy was 70 eV, and the scan range was from 40 to 400 amu (atomic mass units), with a scan time of 1 s. Samples of 2 µl were injected using the split sampling technique, with a split ratio of 1:40. The identification of constituents was performed by comparing their retention indices and recorded mass spectra with those of the NIST05 and Shimadzu mass spectral libraries, a local library built based on analyses of commercially available standards, and other data from the literature [22].

2.4. Evaluation of the radical scavenging activity of EEs.

The EEs were evaluated for antioxidant activity through the two most common radical scavenging assays using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS). The DPPH assay was performed according to Cuendet et al. [23]: Solutions of EEs at different concentrations were prepared in methanol. 50 µl of each solution was added to 5 ml of DPPH solution in methanol (0.004%). After incubating for 30 min at room temperature in the dark, the absorbance was measured at 517 nm against a blank. The ABTS assay was performed as described by Re et al. [24]: ABTS dissolved in distilled water (7 millimoles per liter (mmol/l)) was mixed with potassium persulfate (2.45 mmol/l). The reaction mixture was left in the dark at room temperature overnight. The solution

was then diluted with ethanol until the absorbance at 734 nm was approximately 0.70 ± 0.02. After adding 10 µl of EE to 1 ml of the diluted ABTS solution and stirring, the absorbance at 25°C was taken at different time intervals (0, 1, 2, 4, and 8 min).

2.5. Microorganism and culture method.

Tetrahymena pyriformis strain E (ATCC 30005) was cultured without shaking at 28°C to the exponential phase (about 72 h) in proteose peptone and yeast extract medium (PPYE) according to the method described by Pousada et al. [25]. Cultures of 200 ml of sterile PPYE medium were prepared in 500 ml Erlenmeyer flasks. Culture media were inoculated with 1% (v/v) of a 3-day starter culture (approximately 10^5 cells/ml) grown under the same conditions cited above.

2.6. Cytotoxicity of EEs.

The cytotoxicity of the 4 studied EEs used at different concentrations (0.05, 0.10, 0.20, 0.40, 0.80, 1.60, and 3.20 mg/ml) on *T. pyriformis* growth was determined after 72 h of culture. This time interval corresponds to the exponential phase of growth of the protozoan under normal culture conditions, as previously reported [14]. A control without adding EE was carried out under the same conditions. During incubation, 500 µl aliquots were taken from cultures, diluted in phosphate-buffered saline (PBS), and fixed by 10% glutaraldehyde in PBS. Then, the number of cells was calculated by counting all cells in each of the six 50 µl subsamples using the Malassez counting plate and the optical microscope (Optika, Italy).

2.7. Irradiation protocol.

The irradiation protocol of cells in culture was carried out for 72 h at 28°C using a ^{60}Co source at different dose rates (from 5 to 40 cGy/h in 5 cGy/h steps) according to Ziyadi et al. [14]. The 50%-inhibitory dose rate (ID_{50}) was estimated from the dose-response curve using the Probit analysis [26] and showed to be 21.8 ± 2.2 cGy/h. Even if this dose rate is not lethal for the protozoan, it nevertheless causes numerous modifications to its growth, its structure, and its physiology, as has been previously reported [14]. To evaluate the protective effect of the EEs against gamma radiation, cultures of *T. pyriformis* containing EEs at different concentrations (0.05, 0.10, 0.15, and 0.20 mg/ml) were irradiated for 72 h at the ID_{50}. Controls, non-irradiated and irradiated, but without EEs, were performed under the same conditions.

2.8. Growth evaluation and morphological analysis.

500 µl aliquots were taken aseptically from both control and irradiated cultures with and without EEs at the beginning and after 72 h of incubation. They were diluted in PBS and fixed with 10% glutaraldehyde in PBS. Then, the number of cells was calculated by counting all cells in each of the six 50 µl subsamples using the Malassez counting plate and the optical microscope. At the same time as cell counting, morphological observations of cells were performed under the optical microscope at a magnification of 100 ×. Aliquots of 100 µl were taken and examined under the microscope to check if the cells were intact and active. Photos were taken with a monochrome camera (Hitachi, Japan), and the area and W/L ratio of the shortest (W) and longest (L) axes of cells were calculated using the software ScopeImage 9.0. Approximately 100 cells were measured for each aliquot.

2.9. Biochemical assays.

2.9.1. Crude extract preparation.

After 72 h of incubation under different conditions, the protozoan cells were collected by centrifugation at 5000 × g for 15 min at 4°C. Then, the pellets were washed with 20 mmol/l Tris-HCl (pH 7.5) and resuspended in 50 mmol/l Tris-HCl (pH 7.5) containing 10 mmol/l 2-mercaptoethanol, 2 mmol/l ethylenediamine tetraacetic acid (EDTA), 1 mmol/l dithiothreitol, 2 mmol/l phenylmethylsulfonyl fluoride, and 1% (v/v) glycerol in a ratio of approximately 3 ml/g (wt. humid). Then, cells were cold burst by sonication using the sonifier Bandelin Sonoplus (30 s, 90%, 20×). The supernatant obtained after centrifugation at 15,000 × g for 45 min at 4°C was considered the cell-free crude extract (soluble protein fraction).

2.9.2. Protein concentration.

The concentration of proteins was determined by the Bradford method with albumin from bovine serum as a standard [27].

2.9.3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity.

The GAPDH activity was determined spectrophotometrically by monitoring the NADH production at 340 nm [28]. The reaction was started by adding the crude extract to the mixture containing 1 mmol/l D-glyceraldehyde-3-phosphate (D-G3P), 1 mmol/l NAD^+, 10 mmol/l sodium arsenate, and 50 mmol/l tricine buffer (pH 8.5).

2.9.4. Succinate dehydrogenase (SDH) activity.

The SDH activity was measured at 625 nm, as described by King [29]. The reaction mixture consisted of 100 µg of protein, 0.053 mmol/l dichlorophenolindophenol, 0.3 mmol/l EDTA, and 100 mmol/l potassium phosphate buffer (pH 7.4). The mixture was pre-incubated for 10 min at 25°C before adding 50 µl of KCN-succinate (3.25 mg/ml KCN in 500 mmol/l succinate).

2.9.5. Lipid peroxidation level.

Lipid peroxidation was assessed by thiobarbituric acid reactive substances (TBARS) formation as described by Samokyszyn and Marnett [30]. Thus, it was measured in terms of malondialdehyde (MDA) equivalents. One ml of the crude extract was added to 1 ml of a solution containing 15% trichloroacetic acid and 0.375% thiobarbituric acid in 250 mmol/l HCl. The mixture was heated for 15 min at 100°C and transferred to an ice bath in order to stop the reaction. The absorbance of the supernatant recovered after centrifugation at 1000 × g for 10 min was measured at 535 nm. Data were represented as nmoles of MDA/mg of protein using the molar extinction coefficient of MDA (1.56×10^5 M^{-1} cm^{-1}).

2.9.6. Catalase (CAT) activity.

The CAT activity was determined as described by Aebi [31]. Ten µl of the crude extract was added to a solution containing 7.5 mmol/l H_2O_2 in 50 mmol/l potassium phosphate buffer (pH 7). The decomposition of H_2O_2 was monitored spectrophotometrically at 240 nm and 25°C.

2.9.7. Superoxide dismutase (SOD) activity.

The SOD activity was measured according to Paoletti et al. [32]. The NADH oxidation by superoxide radicals in the reaction mixture containing 3.9 mmol/l 2-mercaptoethanol, 2.5 mmol/l $MnCl_2$, 5 mmol/l EDTA, and 10 µl of the crude extract in 50 mmol/l potassium phosphate buffer (pH 7) was monitored at 340 nm. The reaction was started by adding NADH to a final concentration of 0.27 mmol/l.

2.10. Statistical data analysis.

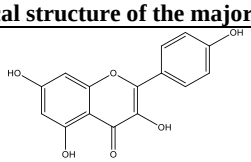
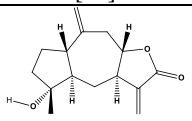
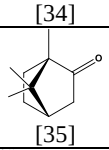
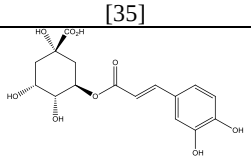
The experimental results (enzyme activities, number of cells, and cell dimensions) are reported as means ± standard deviations of 3 separate experiments carried out in triplicate. These results were compared using the Student's t test and variance analysis (ANOVA). Significant differences in means were evaluated using the Tukey test with a probability level of 5%.

3. Results and Discussion

3.1. Composition of EEs.

The EEs obtained by maceration with yields of 10.8, 12.4, 11.1, and 10.2% (w/w) for *C. ladanifer*, *I. viscosa*, *L. stoechas*, and *N. oleander*, respectively, were analyzed by GC-MS in order to determine their chemical compositions. The results showed the presence of several compounds corresponding to different retention times and percentages (Supplementary Table S1). A total of 25 compounds have been identified in the EE of *C. ladanifer*, 36 in the EE of *I. viscosa*, 31 in the EE of *L. stoechas*, and 24 in the EE of *N. oleander*. The major compounds, which were identified by their retention time with molecular weight and percentage area, are illustrated in Table 1. Thus, Kaempferol (42.6%), Inuviscolide (30.2%), L-Camphor (34.3%), and Chlorogenic acid (39.5%) were the main compounds identified in *C. ladanifer*, *I. viscosa*, *L. stoechas*, and *N. oleander* EEs, respectively. The structures of these main compounds are shown in Table 1.

Table 1. Major constituents of the 4 EEs tested against damages induced by gamma radiation in *T. pyriformis*.

Plant specie	Common name	Details of plant EE	
		Main compounds (area, %)[1]	**The chemical structure of the major compound**
C. ladanifer	Gum rockrose	Kaempferol (42.6) Carvacrol (24.1) α-Pinene (5.7)	 [33]
I. viscosa	False yellowhead	Inuviscolide (30.2) Tomentosin (29.1) Isocostic acid (19.7)	 [34]
L. stoechas	French lavender	L-Camphor (34.3) Fenchone (12.6) Linalool (8.2)	 [35]
N. oleander	Oleander	Chlorogenic acid (39.5) Rutin (17.8) Oleandrin (14.8)	 [36]

[1] According to the GC-MS analysis.

3.2. In Vitro Antioxidant Activity of EEs.

EEs' antioxidant activities were carried out using a DPPH scavenging assay and ABTS decolorization assay. The results showed that the tested EEs exhibited higher scavenging activity against DPPH and ABTS radicals (Figure 1 and Table 2).

The DPPH assay revealed a dose-dependent radical scavenging activity, which increased proportionally to the concentration of the plant extract (Figure 1). The results showed that the EE of *L. stoechas* exhibited the highest DPPH scavenging activity, with the lowest IC_{50} value of 0.36 ± 0.03 mg/ml, as calculated by linear regression analysis. The IC_{50} values of the other EEs tested were 0.42 ± 0.02, 0.47 ± 0.02, and 0.53 ± 0.02 mg/ml for *N. oleander*, *C. ladanifer*, and *I. viscosa*, respectively (Figure 1).

This was confirmed by the ABTS assay, which showed almost complete decomposition in less than 1 minute for the EE of *L. stoechas* (Table 2). The decomposition effect was almost complete within 1 minute for *N. oleander* and *C. ladanifer* EEs, and 2 minutes for *I. viscosa* EE (Table 2).

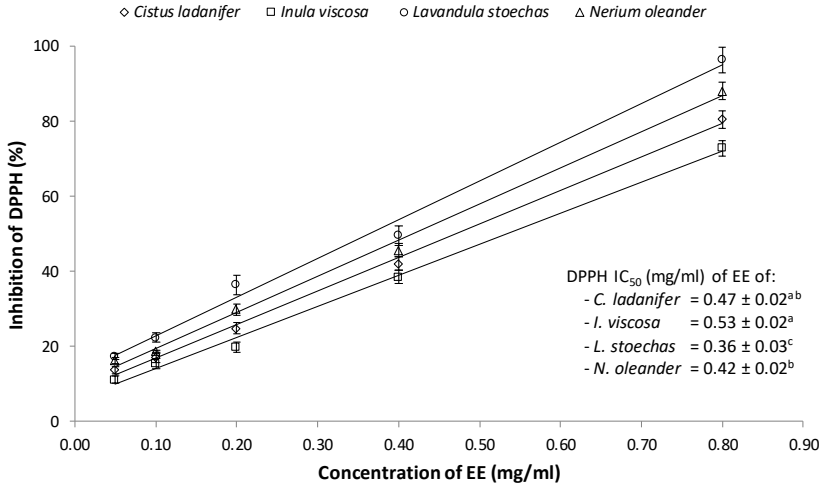


Figure 1. DPPH inhibition (%) of *C. ladanifer*, *I. viscosa*, *L. stoechas*, and *N. oleander* EEs. For each EE, 50 µl of solutions at different concentrations in methanol (from 0.05 to 0.80 mg/ml) were added to 5 ml of DPPH solution in methanol (0.004%). After 30 min of incubation in the dark at 25°C, the absorbance at 517 nm was read against a blank. DPPH 50%-inhibitory concentrations (IC_{50}) were calculated using linear regression. Values are represented as means ± standard errors of 3 separate experiments. Values with the same superscript letter (a, b, or c) do not differ significantly (Tukey test, $p < 0.05$).

Table 2. ABTS activity of EEs at different time intervals. After the addition of 1 ml of ABTS solution (Abs_{734nm} = 0.700 ± 0.020) to 10 µl of EE, the absorbance at 734 nm and at 25°C was read at 1 min intervals for 8 min.

	Absorbance at 734 nm				
	0 min	**1 min**	**2 min**	**4 min**	**8 min**
C. ladanifer	0.708 ± 0.016	0.073 ± 0.008^{b}	0.056 ± 0.007^{a}	0.045 ± 0.003^{a}	0.024 ± 0.002^{a}
I. viscosa	0.710 ± 0.014	0.114 ± 0.009^{a}	0.063 ± 0.008^{a}	0.050 ± 0.004^{a}	0.029 ± 0.003^{a}
L. stoechas	0.712 ± 0.015	0.054 ± 0.006^{c}	0.042 ± 0.005^{b}	0.027 ± 0.003^{c}	0.016 ± 0.002^{c}
N. oleander	0.709 ± 0.016	0.069 ± 0.007^{b}	0.051 ± 0.004^{a}	0.038 ± 0.004^{b}	0.020 ± 0.001^{b}

Values are represented as means ± standard errors of 3 separate experiments. Values with the same superscript letter (a, b, or c) do not differ significantly (Tukey test, $p < 0.05$).

3.3. Sensitivity of T. pyriformis to EEs.

To evaluate the cytotoxic effect of the studied EEs, *T. pyriformis* was cultured with various concentrations of each EEs (from 0.05 to 3.20 mg/ml). After 72 h of incubation, the number of cells was calculated and compared to that of cells cultured under similar conditions without EE. At concentrations below 0.10 mg/ml, the EEs of *L. stoechas* and *N. oleander*

activated the growth of *T. pyriformis*, while no significant variation in growth was observed for *C. ladanifer* and *I. viscosa* EEs (Figure 2 a). Beyond the concentration of 0.20 mg/ml, growth inhibition was observed for the 4 EEs tested. This inhibition is directly proportional to the concentration of each EE (Figure 2 a). All EEs completely or greatly inhibited *T. pyriformis* growth at the concentration of 3.20 mg/ml. For the 4 EEs, no significant inhibition of *T. pyriformis* growth was observed at concentrations below 0.20 mg/ml. For this reason, concentrations of EEs below 0.20 mg/ml were used to evaluate their protective effects against gamma radiation.

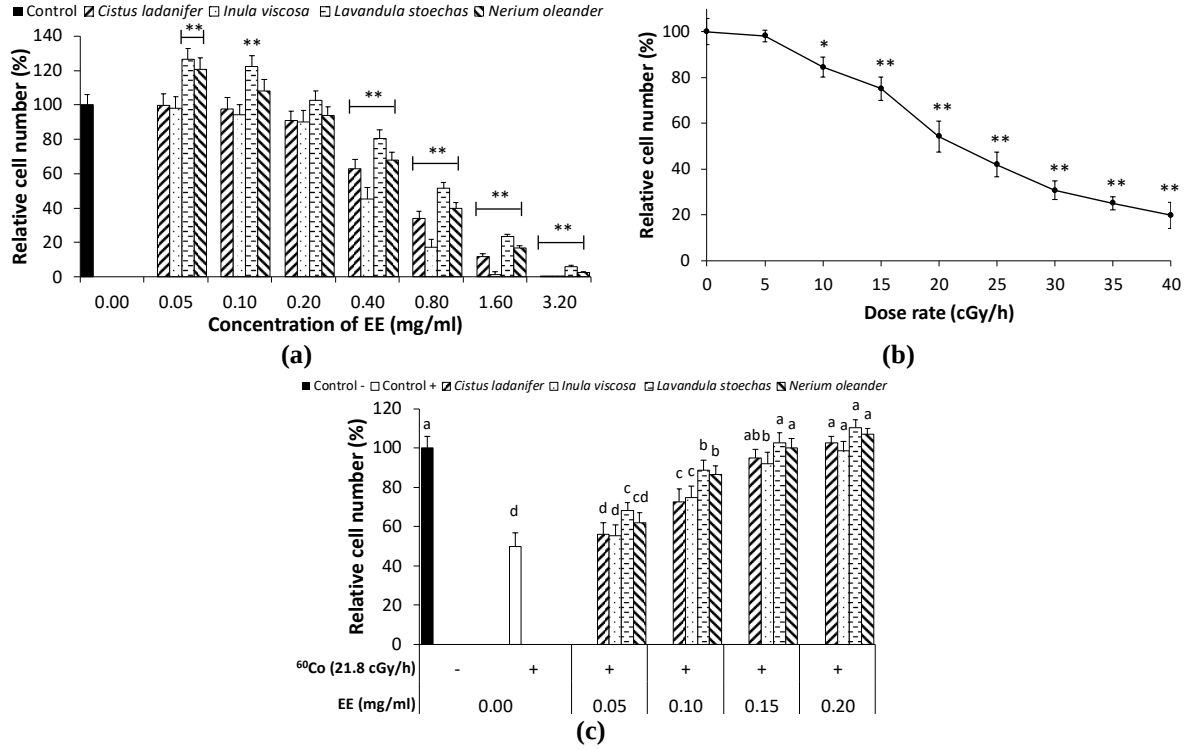


Figure 2. (a) Effects of *C. ladanifer*, *I. viscosa*, *L. stoechas*, and *N. oleander* EEs at different concentrations (0.05 - 3.20 mg/ml) on the cell number of *T. pyriformis* after 72 h of growth; **(b)** Dose-response effect of IR induced by ^{60}Co source at different dose rates (5 - 40 cGy/h) on the cell number of *T. pyriformis* after 72 h of growth; **(c)** Radiation protective effect of the 4 EEs at different concentrations (0.05 - 0.20 mg/ml) on the cell number of *T. pyriformis* after 72 h of growth in presence of ^{60}Co source (21.8 ± 2.2 cGy/h). Values are represented as means ± standard errors of 3 separate experiments. Values with the same superscript letter (a, b, c, or d) do not differ significantly (Tukey test, $p < 0.05$).

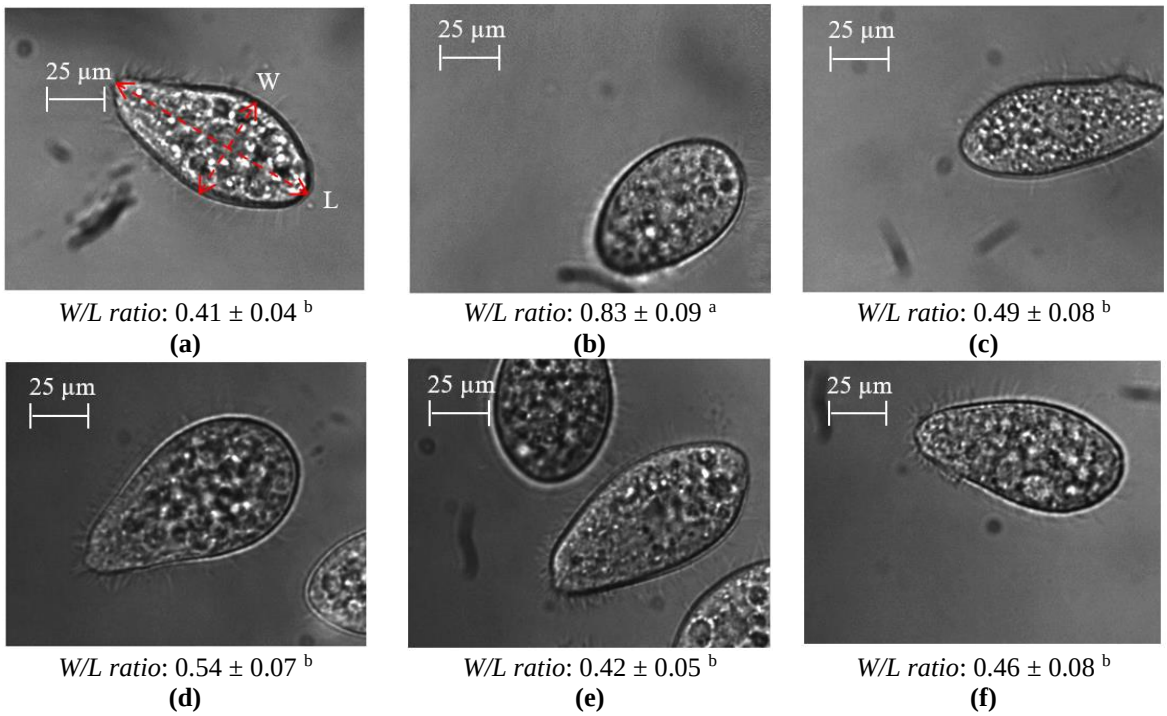
3.4. Protective effect of EEs on growth.

First, the IR effect on *T. pyriformis* growth was assessed by cell counting at the beginning and after 72 h of ^{60}Co-radiation-source exposure at different dose rates (from 5 to 40 cGy/h). Figure 2 b indicates that the IR exposure affects the growth rate in a dose-rate-dependent manner. The ID_{50} determined by the Probit analysis was about 21.8 ± 2.2 cGy/h (Figure 2 b). Thus, this dose rate was considered for the study of the radiation-protective effect of the EEs.

Adding EEs to the culture media improved the growth rate of *T. pyriformis* under irradiating conditions (Figure 2c). Thus, the EE of *L. stoechas* significantly ameliorated the growth rate of *T. pyriformis* at only 0.05 mg/ml. The improvement in the growth rate was significant in the presence of *N. oleander*, *C. ladanifer*, and *I. viscosa* EEs, but only from a concentration of 0.10 mg/ml (Figure 2c). At 0.20 mg/ml, all EEs were allowed to restore the growth rate of *T. pyriformis* to its initial state under normal culture conditions.

3.5. Protective effect of EEs on morphology.

The response of *T. pyriformis* to a cumulative exposure of 1570 cGy after 72 h of incubation in the presence of the ^{60}Co radiation source was marked by a change in the appearance of cells (Figures 3a and b). The value of the W/L ratio (shortest/longest axis) calculated was greater in irradiated cells (0.83 ± 0.09) than in non-irradiated cells (0.41 ± 0.04), suggesting rounding of cells due to exposure to gamma radiation. Results in Figure 3 show that cells cultured under irradiating conditions were able to achieve their normal shape in the presence of different plant EEs compared to the control. Thus, in the presence of *L. stoechas*, *N. oleander*, *C. ladanifer*, and *I. viscosa* EEs, the W/L ratio decreased from 0.83 ± 0.09 to 0.49 ± 0.08, 0.54 ± 0.07, 0.42 ± 0.05, and to 0.46 ± 0.08, respectively, when cells were cultured under irradiating conditions (Figure 3).



W/L ratio: 0.41 ± 0.04 [b] **(a)**
W/L ratio: 0.83 ± 0.09 [a] **(b)**
W/L ratio: 0.49 ± 0.08 [b] **(c)**
W/L ratio: 0.54 ± 0.07 [b] **(d)**
W/L ratio: 0.42 ± 0.05 [b] **(e)**
W/L ratio: 0.46 ± 0.08 [b] **(f)**

Figure 3. Microscopic images of *T. pyriformis* cells after 72 h of growth at 28°C (magnification 100 ×): **(a)** Non-irradiated cells; **(b)** Irradiated cells; **(c)** Irradiated cells + *C. ladanifer* EE; **(d)** Irradiated cells + *I. viscosa* EE; **(e)** Irradiated cells + *L. stoechas* EE; **(f)** Irradiated cells + *N. oleander* EE. W/L ratios (shortest/longest axis) were calculated for 100 cells of each group in 3 separate experiments. Values with the same superscript letter (a or b) do not differ significantly (Tukey test, $p < 0.05$).

3.6. Protective effect of EEs on metabolic enzymes.

The protective properties of the EEs against gamma radiation were also evaluated in *T. pyriformis* after 72 h of incubation under irradiating conditions by measuring the activities of certain metabolic enzymes (GAPDH and SDH). Thus, inhibition of GAPDH and SDH activities was noticed in irradiated cells compared to non-irradiated cells. This inhibition reached 43% of residual activity for GAPDH and 45% for SDH (Figure 4). The addition of *L. stoechas*, *N. oleander*, and *C. ladanifer* EEs to the culture medium of irradiated cells at 0.020 mg/ml allowed the activities of GAPDH and SDH to return to their initial values under normal culture conditions (0.84 ± 0.05 and 58.6 ± 4.2 µmol/min/mg for GAPDH and SDH, respectively). At the same concentration, *I. viscosa* EE also had a protective effect on the activities of metabolic enzymes, but to a lesser degree. It allowed the recovery of a considerable part of GAPDH and SDH activities (0.64 ± 0.05 and 39.2 ± 4.8 µmol/min/mg for GAPDH and

SDH, respectively) compared to irradiated cells (0.36 ± 0.03 and 26.5 ± 2.3 µmol/min/mg for GAPDH and SDH, respectively) (Figure 4).

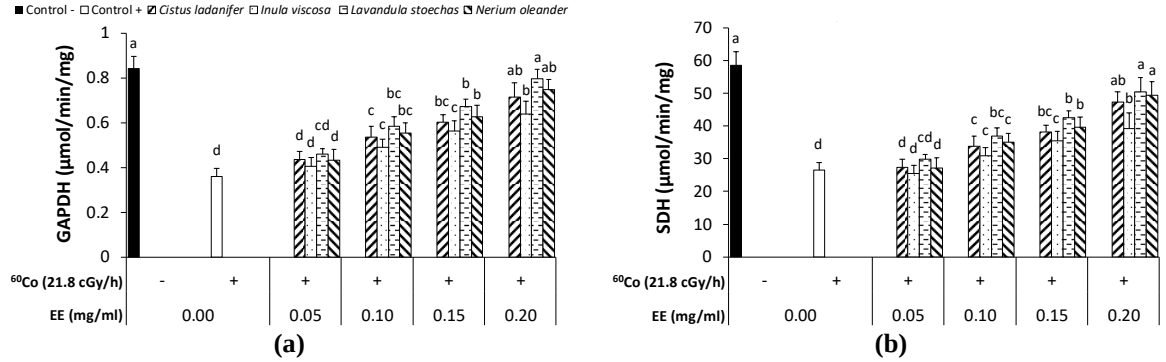
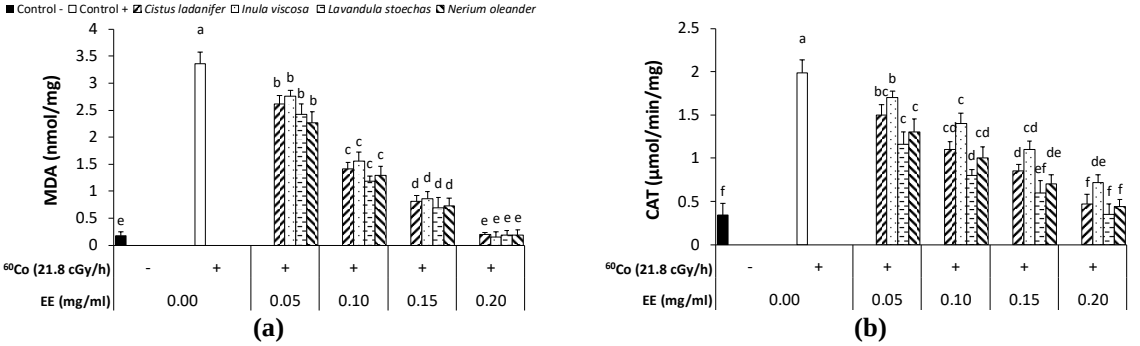
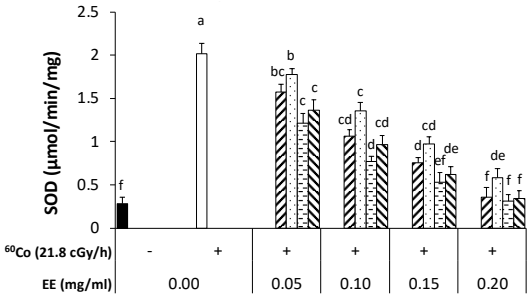


Figure 4. The protective effect of the 4 EEs tested against radiation on metabolic enzymes of *T. pyriformis*: **(a)** GAPDH and **(b)** SDH. Cells were grown for 72 h at 28°C under non-irradiating and irradiating conditions (21.8 ± 2.2 cGy/h) in the absence and in the presence of *C. ladanifer*, *I. viscosa*, *L. stoechas*, and *N. oleander* EEs at different concentrations (0.05, 0.10, 0.15, and 0.20 mg/ml). Values are represented as means ± standard errors of 3 separate experiments. Values with the same superscript letter (a, b, c, or d) do not differ significantly (Tukey test, $p < 0.05$).

3.7. Protective effect of EEs on antioxidant markers.

Antioxidant defense markers (lipid peroxidation, CAT, and SOD) were also investigated. For lipid peroxidation, the production of MDA was increased in cells cultured under irradiating conditions (3.36 ± 0.21 nmol/mg) compared to those cultured under non-irradiating conditions (0.17 ± 0.08 nmol/mg) (Figure 5a). However, MDA contents in cells cultured under irradiating conditions and in the presence of the 4 EEs decreased significantly and in direct proportion to the concentration of these EEs (Figure 5a). At 0.20 mg/ml of EEs, the MDA levels in exposed cells were similar to those in cells cultured under normal conditions (0.19 ± 0.08, 0.19 ± 0.09, 0.20 ± 0.04, and 0.15 ± 0.10 nmol/mg for *L. stoechas*, *N. oleander*, *C. ladanifer*, and *I. viscosa* EEs, respectively). Monitoring the activities of stress-defense enzymes CAT and SOD showed that they increased up to 5.8 and 7.2 fold, respectively, in response to a cumulative exposure of 1570 cGy compared to those of cells under normal culture conditions (Figures 5 b and c). The results shown in Figure 5 b indicate that the CAT activities in irradiated cells cultured in the presence of 0.20 mg/ml of *L. stoechas*, *N. oleander*, and *C. ladanifer* EEs decrease until returning to the normal value (0.34 ± 0.14 µmol/min/mg). Also, CAT activity measured in cells irradiated in the presence of the same concentration of *I. viscosa* EE showed a significant enhancement (0.72 ± 0.10 µmol/min/mg). As for CAT, the SOD activity was decreased in irradiated cells cultured in the presence of the 4 EEs (0.30 ± 0.09, 0.33 ± 0.10, 0.37 ± 0.10, and 0.56 ± 0.12 µmol/min/mg for *L. stoechas*, *N. oleander*, *C. ladanifer*, and *I. viscosa* EEs, respectively) compared to irradiated cells cultured without EEs (2.02 ± 0.12 µmol/min/mg) (Figure 5 c).





(c)

Figure 5. Protective effect of the 4 EEs tested against radiation on antioxidant defense markers of *T. pyriformis*: **(a)** MDA; **(b)** CAT; and **(c)** SOD. Cells were grown for 72 h at 28°C under non-irradiating and irradiating conditions (21.8 ± 2.2 cGy/h) in the absence and in the presence of *C. ladanifer*, *I. viscosa*, *L. stoechas*, and *N. oleander* EEs at different concentrations (0.05, 0.10, 0.15, and 0.20 mg/ml). Values are represented as means ± standard errors of 3 separate experiments. Values with the same superscript letter (a, b, c, d, e, or f) do not differ significantly (Tukey test, $p < 0.05$).

3.8. Discussion.

IR can induce the production of ROS, leading to various diseases such as metabolic disorders, cancer, and neurodegenerative diseases [6, 8, 9]. Antioxidant defense systems can reduce IR-induced ROS effects [10]. The use of synthetic molecules as exogenous radioprotectors is constrained by factors such as elevated cost, adverse effects, and toxicity [37]. Consequently, there is a need to search for natural, safe, and powerful products that can protect against IR-induced ROS. Phytochemicals in medicinal plants, such as alkaloids and flavonoids, have antioxidant activities that can treat and prevent diseases [11, 38]. This study aims to investigate the ability of EEs from *C. ladanifer*, *I. viscosa*, *L. stoechas*, and *N. oleander* to restore gamma radiation-induced damages on *T. pyriformis* used as a unicellular eukaryotic model.

The composition of the four EEs was analyzed using GC-MS, and their antioxidant activities were evaluated using radical scavenging assays. The results showed that all EEs were rich in terpenes, flavonoids, and phenolic compounds (Table 1), which are known for their antioxidant activity [39-41]. The high composition of these compounds in the EEs, particularly for *L. stoechas* EE and to a lesser degree for *I. viscosa*, likely contributed to their antioxidant activity (Figure 1 and Table 2). However, high concentrations of these compounds can be toxic to cells [42, 43], and thus, the cytotoxic effect of the EEs on *T. pyriformis* growth was evaluated. The results showed that concentrations above 0.20 mg/ml inhibited growth (Figure 2 a). Therefore, concentrations lower than 0.20 mg/ml were used to evaluate the protective effect of the EEs against gamma radiation.

The addition of EEs at low concentrations to the culture media of *T. pyriformis* improved the growth rate (Figure 2c) and the morphology of cells cultured under irradiating conditions (Figure 3). At 0.15 mg/ml of *L. stoechas*, *N. oleander*, or *C. ladanifer* EE, or 0.20 mg/ml of *I. viscosa* EE, the growth rate of *T. pyriformis* was comparable to that of cells grown under non-irradiating conditions (Figure 2c). The EEs also prevented morphological changes in irradiated cells (Figure 3), such as reduction in size and rounding of cells, which are adaptations against stress [14, 15].

IR also affects the metabolism of *T. pyriformis* cells, inhibiting the activities of GAPDH and SDH enzymes (Figure 4), which are considered metabolic markers of many stressors [15]. However, the addition of EEs from *L. stoechas*, *N. oleander*, or *C. ladanifer* at a concentration of 0.20 mg/ml restored the activities of these enzymes to their initial values (Figure 4). The

EEs also protected cells from gamma radiation-induced damage by reducing lipid peroxidation and increasing the activities of defense system enzymes (CAT and SOD) (Figure 5). These defense mechanisms play an essential role in protecting key biological macromolecules from damage [44]. At a concentration of 0.20 mg/ml, all EEs decreased MDA levels in exposed cells to levels similar to those in non-irradiated cells. Additionally, the activities of CAT and SOD enzymes were restored to their initial values in the presence of *L. stoechas*, *N. oleander*, or *C. ladanifer* EE, but only partially restored with *I. viscosa* EE (Figure 5). The protective activities of the EEs were dose-dependent and correlated with their antioxidant activities. The EEs of *L. stoechas*, *N. oleander*, and *C. ladanifer* had the greatest protective activity, probably due to their content of L-Camphor, Fenchone, Linalool, 1,8-Cineole, flavonoids, and phenolic compounds, known for their great antioxidant properties [17, 20, 45].

4. Conclusions

The studied EEs from *C. ladanifer*, *I. viscosa*, *L. stoechas*, and *N. oleander* show significant abilities to protect *T. pyriformis* cells against damage induced by a cumulative exposure of 1570 cGy to a gamma-radiation source. This protective effect, which depends on the plant and the concentration of the extract, concerns various aspects of cellular physiology and metabolism. Specifically, the EEs were able to alleviate the slowing down of growth, changes in cell appearance, and the reduction in the activities of metabolic enzymes such as GAPDH and SDH, typically observed under irradiating conditions. Moreover, the EEs were also observed to prevent the activation of lipid peroxidation and the alteration of antioxidant enzymes, including CAT and SOD, essential for maintaining cellular homeostasis.

The antioxidant properties of the EEs, which are attributed to their rich composition of terpenes, flavonoids, and phenolic compounds, certainly play a crucial role in their protective effect against gamma radiation. Among the four studied EEs, those obtained from *L. stoechas*, *N. oleander*, and *C. ladanifer* EEs have shown the most pronounced protective effect against IR, followed by the EE derived from I. viscosa. This suggests that the specific composition and concentration of the EEs may have a significant impact on their ability to protect cells against the harmful effects of gamma radiation.

Our results demonstrate that these EEs can be regarded as natural sources of compounds that exert protective effects on cellular damage caused by gamma radiation. Consequently, further investigation and exploitation of these EEs is warranted to ascertain their potential for use in the prevention and protection against injuries associated with exposure to this radiation. In perspective, it is our intention to investigate the intracellular mechanisms involved in the protective effects of these studied EEs, in particular, their interactions with the ROS produced. Furthermore, we intend to investigate the potential synergistic effects of mixtures of these EEs as well as the effects of the active ingredients that compose them. These studies are currently underway in our laboratory.

Author Contributions

Conceptualization, A.M. and A.I.; methodology, A.M. and N.E.; formal analysis, A.M. and L.B.; investigation, A.M. and N.E.; data curation, A.I. and K.B.; writing—original draft preparation, A.M. and N.E.; writing—review and editing, A.M.; supervision, A.M. and M.E.; project administration, A.M. and A.I.; funding acquisition, A.I. and M.E. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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