Journal of Functional Foods 117 (2024) 106249

Contents lists available at ScienceDirect

Journal of Functional Foods

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

Short communications

Hydroxytyrosyl punicate: A first overview of a novel phenolipid with antiproliferative and antitrypanosomal activity

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ARTICLE INFO

Keywords:
Phenolipid
Hydroxytyrosol
Punicic acid
Cytotoxicity
Antiproliferative
Antitrypanosomal

ABSTRACT

Polyunsaturated fatty acids and phenolic compounds are two families of natural products that have been widely investigated for their health benefits. The aim of this study was to prepare the novel phenolipid hydroxytyrosyl punicate (HT-PA) and evaluate its antiproliferative and antiparasitic properties. HT-PA was synthesized from hydroxytyrosol (HT) and punicic acid (PA) in a two-step chemical synthesis. HT-PA had an EC_{50} of 8.93 μM against non–small cell lung carcinoma A549 cells and was more active than HT or PA. It achieved a selectivity index of 11.20 for tumor cell line A549 over non-tumor cell line MCR-5. HT-PA displayed 80-fold and 60-fold greater activity against *Trypanosoma brucei* parasites (EC_{50} of 0.95 μM) compared with HT and PA, respectively, and > 100-fold selectivity for *T. brucei* over healthy MRC-5 cells.

1. Introduction

The natural phenolic compound hydroxytyrosol (HT) is one of the most effective natural antioxidants, around ten-fold more potent than green tea antioxidants (Martínez et al., 2018). Its health benefits have been acknowledged by the European Food Safety Agency (EFSA, 2017), and *in vitro* studies have demonstrated effects against colorectal, skin, digestive, breast, and lung tumor cells (de Pablos et al., 2019).

Among lipophilic HT derivatives, HT conjugated fatty acids have attracted increasing interest for their health-promoting effects, especially omega-3, −6 and −9 polyunsaturated fatty acids (Candiracci et al., 2016; Plastina et al., 2019; Caroleo et al., 2021). Oleic, linoleic, linolenic, docosahexaenoic, and eicosapentaenoic acids have been covalently linked to HT by chemical and enzymatic synthesis (Torres de Pinedo et al., 2005; Bernini et al., 2015; Zhou et al., 2017) and have exhibited enhanced antioxidant, antiproliferative, antiparasitic, and anti-inflammatory properties (Bernini et al., 2015; Belmonte-Reche et al., 2016). However, few data have been published on the synthesis of HT with omega-5 polyunsaturated fatty acids. In this regard, around 80 % of the content of pomegranate (*Punica granatum L.*) seed is punicic acid (PA) (Verardo et al., 2014), and extracts of PA and other parts of this fruit have demonstrated potential in the prevention of neurodegenerative diseases (Guerra-Vazquez et l., 2022) and the treatment of metabolic diseases (Shabbir et al., 2017). PA has been found to exert potent growth inhibitory activity against androgen-dependent prostate carcinoma LNCaP cells (Gasmi and Sanderson, 2010), and ethanolic

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https://doi.org/10.1016/j.jff.2024.106249
Received 7 March 2024; Received in revised form 2 May 2024; Accepted 16 May 2024
Available online 22 May 2024

extracts of whole pomegranate seed (PA content > 78 %) displayed antiproliferative activity against LNCaP and human breast cancer cells (Lucci et al., 2015).

The hypothesis of this study was that the therapeutic effects obtained by the combination of HT and PA would be greater than those obtained individually by HT or PA. The objectives were to synthesize a novel phenolipid formed by HT and PA and to explore its potential as antiproliferative and/or antiparasitic agent.

Thus, we studied the effects of HT-PA on cell proliferation in MDA-MB-231 triple-negative breast, A549 non-small lung, HT-29 colorectal, and MiaPaca2 pancreatic human cancer cell lines since the most common causes of cancer death in 2022 included lung, colon, breast, and pancreas cancer (Bray et al., 2024). In addition, *Trypanosoma brucei* are protozoan parasites that cause the deadly human disease African sleeping sickness, which threatens 65 million people worldwide. There is no vaccine, and approved therapies for the most severe, late-stage disease are toxic, impractical, and ineffective (Dean et al., 2021). Leishmaniasis is a neglected tropical disease caused by Leishmania species with a broad spectrum of clinical manifestations including cutaneous, visceral, and mucocutaneous presentations. *L. major* is a trypanosomatid parasite that causes most cases of cutaneous leishmaniasis (1–1.5 million cases per year). Many drugs are used for treatment, but results remain unsatisfactory (Garza-Tovar et al., 2020).

2. Materials and methods

2.1. Chemistry

Analytical thin layer chromatography (TLC) was performed using Merck Kieselgel 60 F_{254} aluminum plates and was visualized with UV light or iodine. All evaporations were carried out *in vacuo* in a Büchi rotary evaporator, controlling the pressure with Vacuubrand CVCII equipment. Flash chromatography was conducted using Merck Silica gel 60 with particle size of 0.040–0.063 mm (230–400 mesh ASTM). Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz (^{1}H) and 600 MHz (^{13}C) on Bruker Nanobay Avance III HD and Varian Direct Drive, respectively. Chemical shifts (δ) are quoted in parts per million (ppm) and referenced to the residual solvent peak. Spin multiplicities are given as s (singlet), d (doublet), dd (double doublet) t (triplet), or m (multiplet). *J* values are expressed in Hz. High-resolution electrospray ionization (ESI-TOF) mass spectra were obtained using a Waters LCT Premier Mass Spectrometer. Anhydrous THF and CH_2Cl_2 were purchased from Acros Organics. Anhydrous conditions were maintained under argon. 3,4-dihydroxyphenylacetic acid, lithium aluminum hydride ($LiAlH_4$), and oxalyl chloride were purchased from Sigma Aldrich, dimethyl carbonate (DMC) from Merck, and punicic acid from Larodan AB.

2.1.1. HT synthesis

A solution of 3,4-dihydroxyphenylacetic acid (0.90 g, 5.35 mmol) in anhydrous THF (100 mL) was cooled at 0 °C under argon atmosphere. After adding $LiAlH_4$ (1.00 g, 26.35 mmol) in small proportions, the reaction mixture was stirred under reflux for 18 h. Next, the reaction mixture was cooled at 0 °C for 30 min and HCl 2 N (100 mL) was then added, followed by extraction with ethyl acetate (3 x 50 mL), drying (Na_2SO_4), and evaporation. The residue was purified by flash chromatography using hexane/ethyl acetate (1/1) as eluent, giving 659 mg of a colorless solid (80 % yield).

^{1}H NMR (400 MHz, CH_3OD): 6.69 (d, J = 8.0 Hz, 1H), 6.67 (d, J = 2.0 Hz, 1H), 6.54 dd, J = 2.0, 8.0 Hz, 1H), 3.69 (t, J = 7.2 Hz, 2H), 2.68 (t, J = 7.2 Hz, 2H).

2.1.2. Punicyl chloride synthesis

PA (25 mg, 0.09 mmol) was dissolved in anhydrous CH_2Cl_2 (0.7 mL) under argon, and a catalytic amount of DMF was added in 2 drops. The solution was cooled at 0 °C, oxalyl chloride (10 μl, 0.11 mmol) was added, and the reaction was stirred at 0 °C for 3 h. The reaction mixture was evaporated under reduced pressure to remove solvents and excess oxalyl chloride traces and was then used with no further purification steps.

2.1.3. HT-PA synthesis

A solution of punicyl chloride (27.9 mg, 0.09 mmol) in DCM (0.5 mL) was added to a solution of HT (15 mg, 0.10 mmol) in DMC (0.5 mL). The reaction mixture was stirred for 24 h at room temperature, solvents were evaporated under reduced pressure, redissolved in 5 mL of ethyl acetate, washed with H_2O (1 x 5 mL) and brine (2 x 5 mL), dried (Na_2SO_4), and evaporated. The residue was purified by flash chromatography DCM/MeOH (9.9/0.1) as eluent to give 11.5 mg of a colorless oil (37 % yield). The yield of the reaction was determined by considering the amount of HT-PA obtained after the two steps of synthesis without taking into account the amount of unreacted reagents.

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.81 (d, J = 8.0 Hz, 1H), 6.76 (d, J = 1.9 Hz, 1H), 6.66 (dd, J = 8.0, 1.9 Hz, 1H), 6.49 (dd, J = 5.9, 4.3 Hz, 2H), 6.15——6.01 (m, 2H), 5.50——5.43 (m, 2H), 4.26 (t, J = 7.1 Hz, 2H), 2.84 (t, J = 7.1 Hz, 2H), 2.30 (t, J = 7.5 Hz, 2H), 2.26——2.15 (m, 4H), 1.68——1.58 (m, 2H), 1.41——1.25 (m, 12H), 0.93 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.25, 143.75, 142.32, 132.93, 132.73, 130.94, 128.97, 128.88, 128.12, 127.99, 121.47, 116.09, 115.52, 65.06, 55.01, 34.62, 34.51, 32.00, 29.75, 29.27, 29.19, 27.99, 27.74, 25.07, 22.48, 14.10. HRMS (ESI-TOF) (m/z) calcd. for $C_{27}H_{39}O_6$ (M − H + HCOOH)$^-$ 459.2747, found 459.2726.

2.2. Biology

2.2.1. Cell culture

Breast carcinoma MDA-MB-231 (purchased from ATCC), non-small cell lung carcinoma A549, colorectal adenocarcinoma HT-29, pancreatic ductal adenocarcinoma MiaPaca2, and fetal lung fibroblast MRC-5 human cells were cultured in Dulbecco's Modified Eagle Medium (4.5 g/L glucose for MDA-MB-231, A549, HT-29, and MiaPaca2 cells; 1 g/L for MRC-5 cells) with 10 % inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin and L-glutamine (2 mM). Cells were then incubated in a tissue culture incubator at 37 °C and 5 % CO_2. Cells were plated and passaged according to ATCC recommendations and were used for the experiments while in exponential growth phase.

2.2.2. Trypanosoma brucei culture

'Single marker' (S16) BSF *T. brucei* (Lister 427, antigenic type MiTat 1.2, clone 221a) were cultured at 37 °C, 5 % CO_2 in HMI-9 medium supplemented with 10 % heat-inactivated FBS (hiFBS, Invitrogen), as previously described (Cabello-Donayre, 2016). *Leishmania major* (MHOM/IL/80/Friedlin) promastigotes were cultured at 28 °C in modified RPMI-1640 medium (Invitrogen, Carlsbad, CA) (Cabello-Donayre, 2016).

2.2.3. Cell viability assay

All compounds were dissolved in DMSO and stored at −20℃. For each experiment, stock solutions (100 mM) were further diluted in culture media to obtain desired concentrations. Cells were seeded in a 96-well plate (1,000 cells/well for MDA-MB-231 cells, 1,500 cells/well for A549 cells, and 5,000 cells/well for HT-29, MiaPaca2, and MRC-5 cells) and incubated for 24 h before treatment. Each well was then replaced with fresh medium containing increasing concentrations of HT, PA, or HT-PA and incubated for 72 h (HT-29, MiaPaca2, and MRC-5 cells) or 120 h (MDA-MB-231 and A549 cells). For the evaluation of the synergistic effect of PA and HT, A549 cells were plated as previously described and treated with PA (30 μM) and increasing concentrations of HT (3, 10, and 30 μM) for 120 h. Untreated cells were treated with DMSO (0.1 % v/v). Cytotoxicity was measured by using the PrestoBlue™ (MDA-MB-231 and A549 cells) or AlamarBlue® (HT-29, MiaPaca2, and MRC-5 cells) cell viability assay as previously reported

(Mosmann, 1983; Espejo-Román et al., 2023). All conditions were normalized to untreated cells (100 %), and curves were fitted using GraphPad Prism with a sigmoidal variable slope curve. Each point represents the mean ± SEM of three independent experiments done in triplicate.

2.2.4. Antiparasitic activity

The alamarBlue® assay (Thermo Fisher Scientific Inc.) was used to measure the trypanocidal activity of compounds (Larson et al., 1997). Briefly, 1 x 103 BSF *T. brucei* were incubated in 96-well plates alone or in the presence of increasing concentrations of compounds for 72 h in 5 % CO_2 at 37 °C, followed by the addition of 20 μL resazurin (110 ng/mL) and further incubation for 4 h at 37 °C. Finally, cells were lysed with 50 mL of 3 % SDS solution. Fluorescence was measured with an Infinite F200 plate reader (Tecan Austria, GmbH), exciting at 550 nm and recording the emission at 590 nm. Results are expressed as the concentration of compound that reduces cell growth by 50 % *versus* untreated control cells (EC_{50}) using SigmaPlot (4 parameter logistic curve). Data are expressed as the mean of three independent measurements done in triplicate (Raz et al., 1997). For the evaluation of the synergistic effect of PA and HT, parasites were plated as previously described, treated with PA and HT (100 μM of each) and serials dilutions of such starting concentrations for 72 h. The EC_{50} values were calculated as described above.

The leishmanicidal activity of the compounds was evaluated in previously described experiments on the susceptibility of *L. major* (MHOM/IL/80/Friedlin) (Wirtz et al., 1999). Briefly, 4 x 106/mL promastigotes were incubated for 72 h at 28 °C in 96-well plates in modified RPMI-1640 medium supplemented with 10 % hiFBS containing increasing concentration of the compounds. Cell proliferation was determined with an MTT-based assay (Perez-Victoria et al., 2011). EC_{50} values were calculated as described above.

2.2.5. Statistical analysis

One-way ANOVA and the Student's *t* test were carried out using GraphPad 8.0 software.

3. Results and discussion

3.1. Synthesis of HT and HT-PA

HT was obtained by reduction of the corresponding carboxylic acid (Roche et al., 2005). The novel phenolipid HT-PA (Fig. 1) was synthesized utilizing a methodology previously described for HT oleate (Bernini et al., 2017). Punicyl chloride was prepared by treating PA with oxalyl chloride using a catalytic amount of DMF. Finally, the esterification reaction between HT and punicyl chloride was carried out in DMC to afford HT-PA. The structure of the new compound was characterized by ^{1}H- and ^{13}C NMR and by HRMS (see supporting information).

3.2. Biological assays

3.2.1. Antiproliferative activity

The effects of HT, PA, and HT-PA on cell proliferation were studied in MDA-MB-231 triple-negative breast, A549 non-small lung, HT-29 colorectal, and MiaPaca2 pancreatic human cancer cell lines (Table 1) and in the non-cancerous human MCR-5 lung fibroblast cell line. The selectivity index (SI) was calculated according to the formula: EC_{50} (MRC-5)/ EC_{50} (A549).

All three compounds had similar antiproliferative activity against breast cancer MDA-MB-231 cells, with EC_{50} values ranging from 24.42 μM to 39.30 μM (Table 1 and Fig. 2A). Accordingly, the antiproliferative effects of HT and PA were not increased by their combination in the phenolipid HT-PA. In contrast, the compounds substantially differed in their antiproliferative activity against lung cancer A549 cells, with EC_{50} values of 116.65 μM for PA, 64.36 μM for HT, and 8.93 μM for HT-PA (Table 1 and Fig. 2B). Hence, the antiproliferative effect of HT-PA was 13-fold higher than the effect of PA and 7-fold higher than that of HT. To assess whether the antiproliferative activity observed for compound HT-PA is due to a synergistic effect, A549 cells were treated with PA alone (30 μM) or in combination with increasing concentrations of HT (3, 10, and 30 μM), and cell viability was tested after 5 days of treatment. As observed in Figure S4, the combined effect of HT and PA is not superior to that obtained when both structures are administered independently, confirming that there is not a synergistic effect.

In addition, no compound had antiproliferative effects against colon HT-29 or pancreatic MiaPaca2 cancer cells except for some activity of HT against MiaPaca2 cells (Table 1).

These findings on the bioactivity of HT are in line with the results of multiple *in vitro* experiments. For instance, a study based on the MTT assay reported an EC_{50} of 147 μM for HT against A549 cells and 50.16 μM against MCF7 cancer cells after 48 h of treatment (Calderón-Montaño et al., 2013), while a study using the SBB assay described an EC_{50} of 0.6 mM for HT against MCF-7 cells after 16 h of treatment (Calahorra et al., 2018). Another *in vitro* experiment observed EC_{50} values against

Table 1
EC_{50} values (μM) obtained after treatment of MDA-MB-231, A549, HT-29, MiaPaca2, and MRC-5 cell lines with PA, HT, and HT-PA and the selectivity index (SI) over the A549 cell line.

Compound	MDA-MB-231	A549	HT-29	MiaPaca2	MRC-5	SI (MRC-5/ A549)
PA	24.42 ± 3.87	116.65 ± 0.78	>100	76.71 ± 7.37	67.37 ± 0.35	<1
HT	35.05 ± 3.66	64.36 ± 3.05	87.55 ± 2.50	>100	>100	> 1.56
HT-PA	39.30 ± 2.37	**8.93 ± 1.40**	90.18 ± 1.06	>100	>100	**> 11.20**

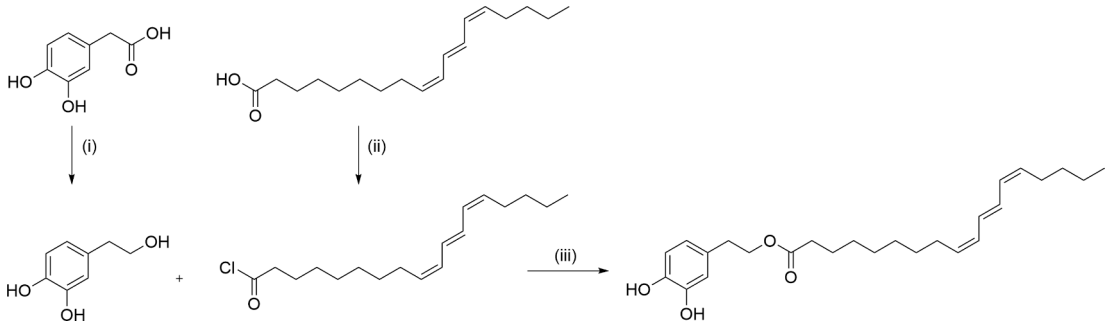

Fig. 1. Synthesis of HT-PA. *Reagents and conditions* (i) $LiAlH_4$, anhydrous THF, argon, 0 °C and then 18 h reflux; (ii) oxalyl chloride, DMF, anhydrous CH_2Cl_2, 0 °C, 3 h; (iii) DMC, rt, 24 h.

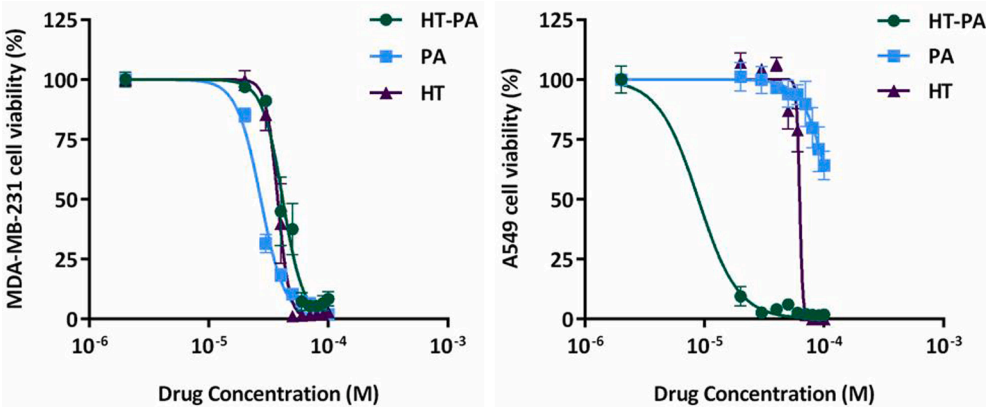

Fig. 2. Dose − response curves for HT-PA, PA, and HT against MDA-MB-231, and A549 cells after 5 days of treatment. Error bars: ±SD from n = 3.

human cervical cancer HeLa cells of 0.46 mM for HT and 0.33 mM for its oleyl ester (Bouallagui et al., 2011).

The anticancer effect of isolated PA has been reported in MDA-MB-231 cells (Grossmann et al., 2010) where proliferation was inhibited 92 % compared to untreated cells by 40 μM PA. In addition, a few studies have indicated that pomegranate seed oil, which is rich in PA (Costantini et al., 2014; Aruna et al., 2016; Sharma et al., 2017; Bassiri-Jahromi, 2018) may inhibit the migration of MDA-MB-468 cells (Mahmoudi et al., 2017). Cell viability of various colon, liver, prostate, and breast cancer cell lines was determined with the hydrophilic fraction of pomegranate seed oil, reaching EC_{50} at doses of 0.60 μL extract against MDA-MB-231 (Costantini et al., 2014). Pomegranate juice or a combination of its components (luteolin, ellagic acid, and PA) were found to enhance cancer cell adhesion and reduce cancer cell migration, without affecting normal cells (Rocha et al., 2012).

The SI of compounds was determined for lung cancer A549 and healthy lung MCR-5 cell lines, finding phenolipid HT-PA to be the most selective compound (SI = 11.6, Table 1).

3.2.2. *Antiparasitic activity*

The antiparasitic activities of HT, PA, and HT-PA were investigated against *T. brucei* and *L. major* (Table 2). Healthy human lung fibroblast MCR-5 cells were again used to establish their SI against the two parasites (Table 2).

HT and PA both exerted low activity against the two parasites, with EC_{50} values in the high micromolar range. Our results for HT are in line with previous findings for olive oil extracts against *T. brucei*, *L. infantum*, *L. donovani*, and *L. major* (Kyriazis et al., 2013; Koutsoni et al., 2018). No published data could be traced on the potential antiparasitic effects of PA. However, a review recently appeared on the therapeutic potential of pomegranate extracts against *Schistosoma mansoni*, *Giardia lamblia*, *Plasmodium falciparum*, *Eimeria tenella*, *L. infantum*, and other parasites (Dardona, 2023).

In contrast, HT-PA had an EC_{50} value of 0.95 μM against *T.brucei*, together with a striking SI (105-fold) and a marked increase in activity compared with HT (80.8-fold higher) and PA (60.7-fold higher). *T. brucei* parasites were treated with both, HT and PA serial dilutions starting from 100 μM of each compound and the EC_{50} value 72 h after the treatment was determined. A value of 12.9 ± 0.6 μM was calculated. The combined effect of HT and PA is slightly better to that obtained when both structures are administered independently (4–6 times), but still much worse than the effect of the HT-PA itself (13 times lower). Additionally, HT-PA stability in MRC5 and *T. Brucei* cell culture mediums was checked by HPLC and around 90 % and 60 % of compound remaining was detected after 72 h incubation, respectively (Figure S5). Taken both results together we might conclude that HT-PA is not acting as a prodrug of HT or PA but as a bioactive compound itself.

The antiparasitic efficacy of HT-PA was within the range of that previously reported for HT laurate and 2.5-fold higher than described for HT palmitate (Belmonte-Reche et al., 2016). This may indicate a potential effect of the three insaturations in the lipophilic chain of HT-PA. However, HT-PA showed only poor activity against *L. major*.

Table 2
Antiparasitic activity of HT-PA, HT and PA against *T.brucei* and *L.major* and the selectivity index (SI) over *T.brucei*.

Compound	*T. brucei* EC_{50} (μM)	*L. major* EC_{50} (μM)	MRC5 EC_{50} (μM)	SI (MRC5/T. brucei)
PA	57.66 ± 0.64	99.61 ± 1.94	67.37 ± 0.35	<1
HT	76.78 ± 13.64	>100	>100	> 1.30
HT-PA	**0.95 ± 0.10**	>100	>100	**> 105.32**

SI = Selectivity Index (EC_{50} MRC-5/ EC_{50} parasite).

4. Conclusions

In summary, we report here on the synthesis of the HT ester of PA and on its evaluation as antiproliferative and antiparasitic agent. HT-PA exhibited an EC_{50} of 8.93 μM against lung A549 cancer cells, demonstrating superior activity compared to HT or PA alone, and a selectivity index of 11.20 over non-tumor cell line MCR-5. Additionally, HT-PA displayed significantly increased activity against T. brucei parasites (EC_{50} of 0.95 μM), with 80-fold and 60-fold greater potency compared to HT and PA, respectively. Moreover, it demonstrated over 100-fold selectivity against *T. brucei* parasites over healthy MRC-5 cells. Much more comprehensive studies will be carried out to ascertain the biological targets affected by this compound and elucidate the mechanisms underlying its therapeutic effects on cancer and parasites. Further research will also focus on the synthesis of HT-PA using materials recovered from Mediterranean fruit by-products such as olive leaves and pomegranate seeds.

Funding

This work was funded by the project PID2021.128109OB.I00 financed by MICIU/AEI/https://doi.org/10.13039/501100011033 and for FEDER, UE. This research was also financed by the project TED2021-132047B-I00 funded by MICIU/AEI /https://doi.org/10.13039/501100011033 and UE NextGenerationEU/ PRTR.

Ethical Statements

There are no ethical questions for the study as there is no animal research or human clinical research. The study is based on cell lines.

CRediT authorship contribution statement

Olga Cruz-López: Writing – review & editing, Methodology, Formal analysis, Data curation. **Elixabet Díaz-de-Cerio:** Writing – original draft, Investigation, Formal analysis. **Belén Rubio-Ruiz:** Writing – review & editing, Formal analysis, Data curation. **Jose Manuel Espejo-Román:** Formal analysis. **Pablo Peñalver:** Formal analysis. **Juan Carlos Morales:** Writing – review & editing, Supervision, Resources, Methodology. **Maria Fiorenza Caboni:** Writing – review & editing, Supervision. **Ana Conejo-García:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Vito Verardo:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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

All data are reported in the paper

Acknowledgments

Elixabet Díaz-de-Cerio is grateful for the post-doctoral fellowship from “Fundación Alfonso Martín Escudero”. The authors thank Richard Davies M.A. for language assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2024.106249.

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