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Antiproliferative and photoprotective effects of *Hamelia patens* phenolic extracts

A thesis presented by

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Dedication

I want to dedicate this work to the most important person in my life, my mother Erendira. Without her I could not stand where I am today.

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To my family, especially to those who lookout for me. To my “other” parents María, Teofilo and Pati. To my brothers Jose Angel, Ilse and Karla, whom I hoped to make them proud of me.

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Abstract

There is a growing need for novel targeted therapeutic drugs against cancer with new molecular targets. Moreover, UV-induced skin carcinogenesis and photoaging concerns are gaining strength among the population. Plants are pools of phytochemical compounds, including polyphenols, with many potential therapeutic effects. Here, the antioxidant activity, chemotherapeutic and photoprotective properties of two phenolic *Hamelia patens* leaves extracts (Hp2019 and Hp2021) from different harvest dates were evaluated. Hp2021 showed the highest total phenolic content and antioxidant activity compared to Hp2019. However, Hp2019 has the greatest antiproliferative effect in prostate cancer cells. The effect was more pronounced in the androgen-sensitive cell line LNCaP compared to the androgen-independent PC3 cell line, pointing to an androgen-disrupting mechanism. Both extracts showed a photoprotective effect from UV-induced damage in murine cells and had no cytotoxic effect against these normal cells. However, the molecular mechanisms behind the biological and therapeutic properties of *H. patens extracts* remain unknown; thus, further research should be conducted.

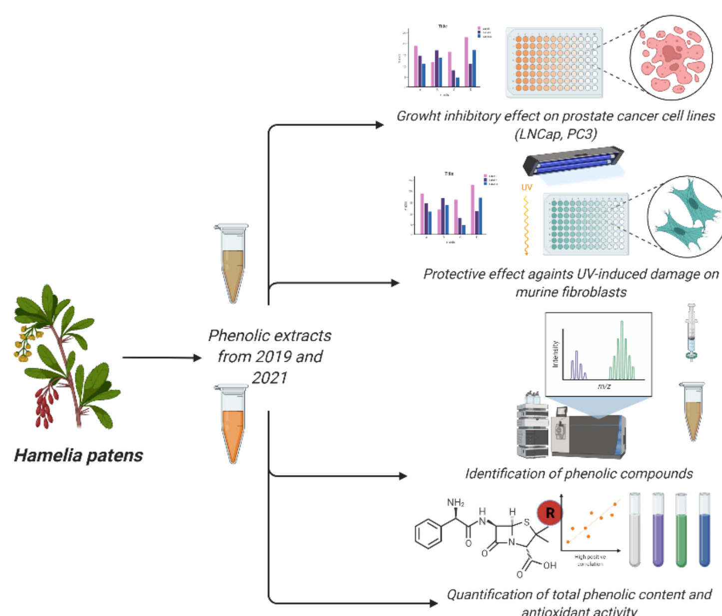

Fig. 1 | Graphical abstract. Created with BioRender.com

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1. Introduction

1.1. Problem statement

Globally, cancer is the leading cause of death, despite the COVID-19 pandemic. In Mexico, it is the third cause of death; the most common cancer, both by incidence and mortality, is prostate in men and breast in women. There are different treatments for cancer, surgical removal of the tumor and cytotoxic chemotherapies being the most used. The latter also damages healthy cells, leading to widespread undesirable adverse effects such as nausea, diarrhea, bleeding, hair loss, fatigue, pain, vomiting, and mental health issues derived from the mentioned side effects.

Over the last decades, therapeutic drugs (small molecules) have been developed to target specific proteins in aberrant pathways associated with proliferation, apoptosis inhibition, angiogenesis, metastasis, and immune evasion. Nevertheless, the population of low- and middle-income countries, like Mexico, have access problems to state-of-the-art therapies, such as targeted therapy, even the most essential and common chemotherapies fall short in supply sometimes.

Furthermore, the rational and timely use of targeted therapies is hindered by effective oncology services, including the use of biomarkers and imaging (Cortes et al., 2020). The lack of access to basic chemotherapies was particularly noted during the COVID-19 pandemic in Mexico due to the strain on supply chains worldwide (Das, 2021). It becomes evident that there is a need for new targeted therapeutic drugs, affordable and accessible for the most vulnerable populations worldwide.

Mexico is a megadiverse country with flora of over 20,000 species, half of them are thought to be endemial, of which 4,500 are medicinal plants used by 80% of the Mexican people with frequency (Alamilla & Neyra, 2020). However, barely 300 plants used for empirical cancer treatment have been scientifically recorded, of which only 181 have been tested experimentally. Moreover, only 88 extracts of

such plants have demonstrated inhibitory activities on at least one cancer cell line. Lastly, only 5 of 187 compounds isolated from those plants have been tested in vivo (Alonso-Castro et al., 2011).

The use of plants to treat a disease (termed phytotherapy) or as a means to discover novel molecules is a potential therapeutic strategy. In the past decades, plants have been exploited as pools of therapeutic drugs for many illnesses, mainly cancer (Newman & Cragg, 2016). Long-used medicinal plants and their phytochemicals, mainly phenolic compounds, represent such pools of drugs, with the added benefit of a long history of usage with relative safety over the centuries in traditional medicine practices among different cultures.

Lastly, other global health concerns are skin cancer and photoaging linked to oxidative stress and inflammation, mainly provoked by UV radiation. Recent evidence indicates that air pollution contributes to inducing skin oxidative stress (McDaniel et al., 2018); with this in mind, it is of great importance to develop new photoprotective products, such as sunscreens that contain antioxidant molecules. Polyphenols have great antioxidant power; therefore, research has focused on studying their potential applications against UV-induced skin damage as well as anti-aging ingredients in clinical and cosmetical formulations (Roh et al., 2017).

Hamelia patens is one of the plants employed in Mexico, especially by ethnic groups of the southeast region, as traditional medicine. It is widely used to treat several diseases empirically, including but not limited to wounds, diabetes, colitis, and cancer (Reimers et al., 2019). It has many therapeutic properties reported in scientific literature, such as antibacterial, anti-inflammatory, hypoglycemic activity, antioxidant, and antineoplastic properties.

1.2. Hypothesis

Hamelia patens' leaves extracts will inhibit the proliferation of cancerous cell lines while having no toxic effect in normal fibroblast cells. The extracts will prevent UV-induced damage in murine fibroblasts. The biological activities mentioned above will be mediated through their phenolic compounds and antioxidant activity.

1.3. Objectives

1.3.1. General objective

The main objective of the present work was to evaluate cell proliferation of cancerous cell lines and non-cancerous cells treated with *H. patens* extracts and evaluate the photoprotective effect of the extracts on murine fibroblasts. And to characterize the chemical properties of the extracts.

1.3.2. Specific objectives

- To obtain two ethanolic extracts from *H. patens* leaves harvested on two different yeats (2019 and 2021).
- Primarily, to evaluate the inhibitory activity of the extracts on prostate cancer cell growth (PC3, LNCaP)
  - To analyze cell proliferation of breast cancer (MCF7) and colorectal adenocarcinoma (Caco-2) after treatment with the extracts.
  - To analyze the effect of the extracts on the cell viability of normal fibroblast cells.
- To evaluate the photoprotective effect of the extracts on murine fibroblasts against UV-induced damage.
- To characterize the extracts and their antioxidant activities, specifically:
  - Total phenolic content
  - Antioxidant activity (DPPH, ABTS, FRAP)
- To identify and quantify the principal phenolic compounds present in the extracts by analytical methods.

2. Background

2.1. Cancer overview

The term “cancer” comprises a group of several non-communicable diseases that start in any organ of the body. The principal characteristic of cancer is abnormal and uncontrolled cell growth beyond their physiological limits, invading other tissues (metastases), forming new tumors, and disrupting the proper function of organs, eventually leading to death (WHO, 2021). Cancer is a multifactorial disorder and a multi-stage process caused by a complex interaction of factors, including genetic factors, lifestyle factors (alcohol, tobacco, diet), health factors (overweight, chronic diseases), environmental factors (physical and chemical carcinogens, infections).

Currently, cancer is the leading cause of death worldwide (Sung et al., 2021). In 2020, cancer positioned as the third cause of death in Mexico (INEGI, 2021). There are more than 36 types of cancer, classified according to the origin site; female breast cancer is the most diagnosed cancer worldwide (11.7), followed by lung (11.4%), colorectal (10.0%) and prostate (7.3%), and stomach (5.6%) cancers.

The hallmarks of cancer (**Fig. 2**), firstly reviewed by Hanahan & Weinberg (2000)and updated in 2011 (Douglas Hanahan & Weinberg), form the current conceptual framework to describe the biological and molecular processes involved in cancer cell development, tumorigenesis, and metastases. In addition to the current hallmarks, the heterogeneous mass of cancer and healthy cells, secreted factors, extracellular matrix proteins, and overall interactions, known as the tumor microenvironment, will shape the outcome of the tumor (Fane & Weeraratna, 2020).

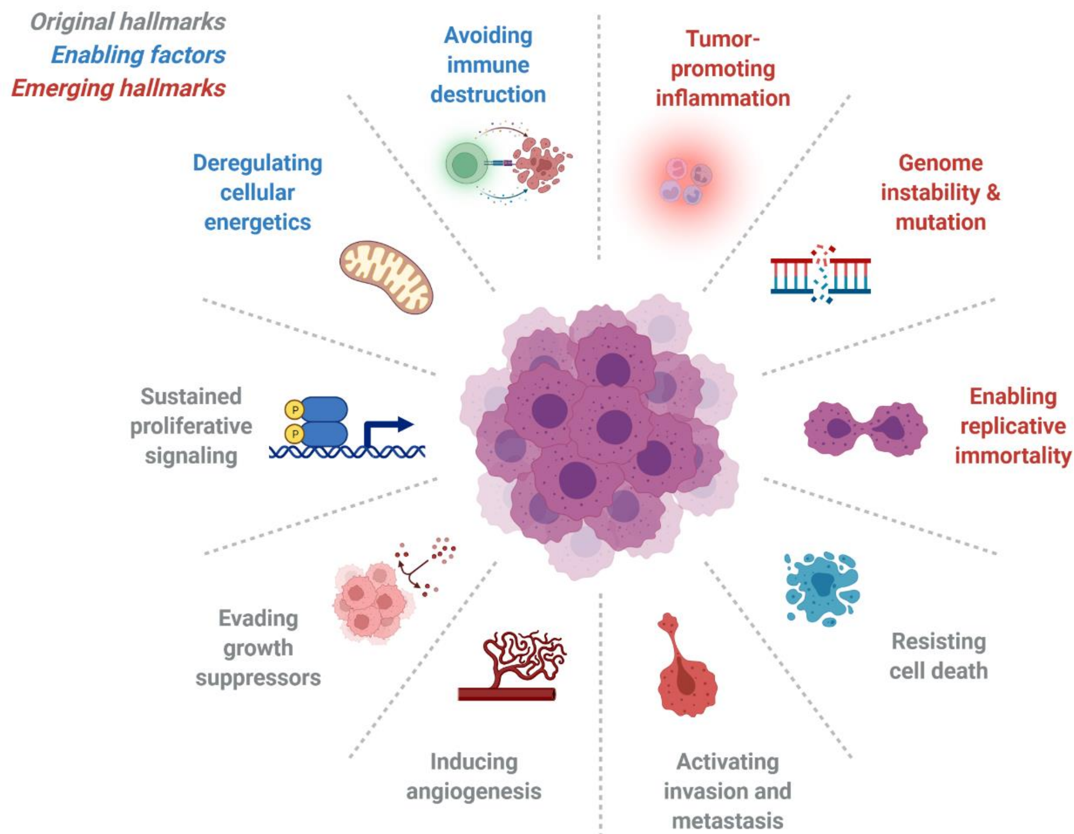


Fig. 2 | Current hallmarks of Cancer. Adapted from "Hallmarks of Cancer: Circle" by BioRender.com (2021)

Each hallmark comprises a myriad of metabolic pathways and molecular networks, interacting with other hallmarks and normal cellular processes. It is not the scope of this work to review each one of them. However, they serve as the basis for therapeutic research. Chemotherapeutic agents, including phytochemicals, target one or several pathways of the hallmarks, inhibiting cancer growth and spread. Furthermore, understanding the cellular and molecular processes of cancer will help researchers develop novel and specific therapies.

Only six approved types of treatments exist surgery, radiation therapy, cryotherapy, hormone therapy, immunotherapy, and chemotherapy (WHO, 2021). Chemotherapy therapies using cytotoxic drugs are considered general or unspecific, carrying several adverse and undesirable effects to patients; nevertheless, there are 61 drugs approved by the US FDA under this category(L.

Zhong et al., 2021). Cytotoxic drugs usually are alkylating agents, anti-microtubule agents, topoisomerase inhibitors targeting cell replication in fast-growing cells and are not cell-specific (J. Sun et al., 2017).

Over the last years, the focus has been shifted to targeted therapies and precision medicine such as immunotherapy and small molecules. From 2001 to 2021, only 103 small molecule-targeted compounds have been approved by the US FDA to treat tumors (G. Sun et al., 2021). These molecules work by interacting with aberrant pathways involved in different aspects of cancer biology inhibiting cancer cell growth, halting angiogenesis, or inducing apoptosis, while sparing healthy cells. Chemotherapeutic small-molecules are classified according to the protein family or pathways they target kinase inhibitors, epigenetic inhibitors, BCL-2 inhibitors, hedgehog pathways inhibitors, proteasome inhibitors, and PARP inhibitors (L. Zhong et al., 2021).

Despite all cancer types share general hallmarks (**Fig. 2**), each type has distinct genetic and metabolic alterations; therefore, therapies cannot be used interchangeably, especially targeted therapies. Next is detailed the biology of some common cancers worldwide and in the Mexican population.

2.1.1. Prostate cancer

The prostate gland tissue frequently gives rise to tumors after the mid-stage of life, and its risk is associated with age and family genetic predisposition (Kohestani et al., 2018; Siegel et al., 2018). The prognosis and survival of a patient are highly variable, and it largely depends on the tumor grade and stage at the first diagnosis (Buhmeida et al., 2006). Prostate cancer is the second most common cancer in men globally. In México, it is the most common cancer by incidence (41.2 per 100,000 per year ASR), only followed by breast cancer. By 2040, the incidence and mortality are expected to rise by 88.6% and 106.5%, respectively (Ferlay et al., 2020)

The progression of the disease is slow, and, in most cases, it is organ-confined (prostate gland) at detection. Nevertheless, it becomes metastatic at later stages spreading to the brains, lungs, bones, spinal cord, and lymph nodes (Gandaglia et al., 2014). Treatment election will depend on the stage of the tumor. When the tumor is confined during the initial stages, surgery, radiotherapy, and androgen deprivation therapy (ADT) are the recommended treatments (W. P. Harris et al., 2009). When these treatments fail, or the tumor becomes metastatic, immunotherapy (androgen receptor-targeting drugs) and chemotherapy are the last alternatives (Beer et al., 2014).

Prostate cancer onset is complex, and like all cancer types, starts with somatic mutations in oncogenes and tumor suppressor genes, leading to deregulate homeostasis. Most genetic changes are associated with gene rearrangements and duplication or copy number of genes (Baca et al., 2013; Ciriello et al., 2013; Hieronymus et al., 2014). The most common alterations are fusions of androgen receptor (AR)-regulated promoter regains with other transcription factors and oncogenes (Carver et al., 2009).

Prostate cancer tumors can be divided into two groups according to their androgen and AR dependence, castration-sensitive prostate cancer (CSPC) and castration-resistant prostate cancer (CRPC). In normal prostate epithelium and CSPC, androgen stimuli (testosterone) is transformed to dihydrotestosterone (DHT) which binds to the AR ligand-binding domain (**Fig. 3**). AR is released from heat shock proteins (HSP) and forms a dimer before translocating itself to the nucleus, where it binds to androgen-response elements (AREs) that regulates gene pathways with many functions, including cell growth, homeostasis regulation, and proteases for prostate functions (Tan et al., 2015).

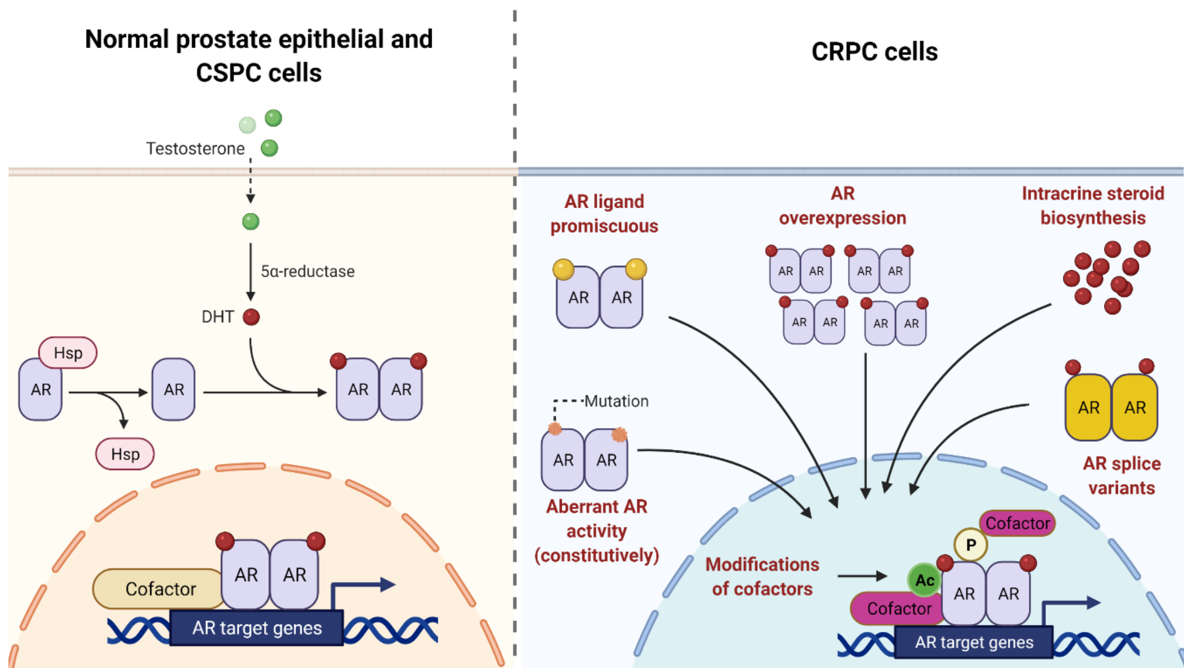


Fig. 3 | Androgen receptor dependence in normal prostate epithelial and CSPC cell and mechanism of androgen resistance in CRPC cells. Adapted from Chandrasekar et al. (2015). Created with BioRender.com

Lastly, CRPC develops after prolonged ADT (W. P. Harris et al., 2009); in CRPC, there is an aberrant upregulation in the AR signaling pathway due to the mechanisms shown in **Fig. 3**. The high activation of the AR axis leads to uncontrolled cell proliferation and suppression of apoptosis (Mills, 2014). Given the importance of AR, they have become one of the most important therapeutic targets in prostate cancer research and current treatments.

2.1.2. Breast cancer

Breast cancer is the second most common malignancy diagnosed in México and the first one in the female population; its incidence and mortality are expected to increase by 51.5% and 64.2% by 2040 (Ferlay et al., 2020).

The genetic drivers of breast carcinogenesis are diverse, and it is one of the cancers with the most biological and molecular heterogenicity (Stingl & Caldas, 2007); as such, classifications have been developed to group tumors and facilitate

treatments according to their histology and molecular genotype (Dai et al., 2015) as shown in **Fig. 4**.

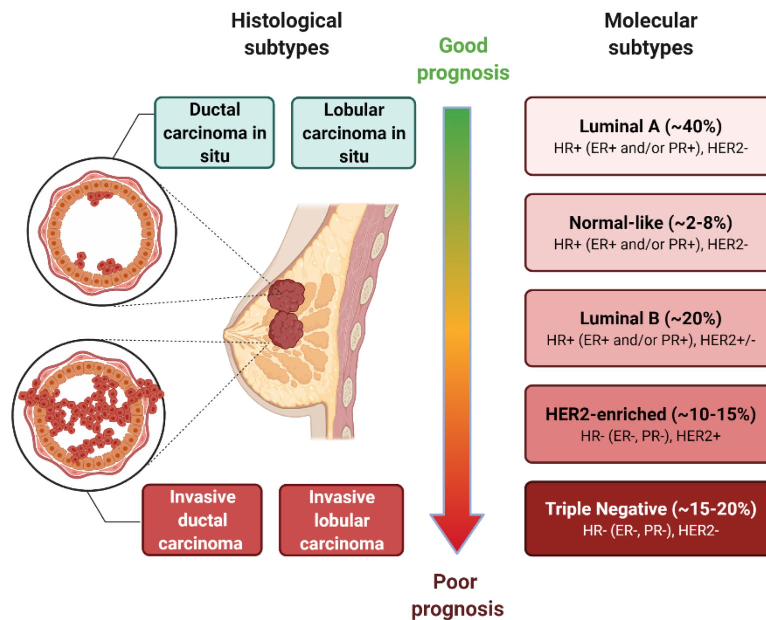


Fig. 4 | Breast cancer histological and molecular subtypes, prognosis, and prevalence. Data from Harbeck et al. (2019). Created with BioRender.com

The molecular classification is based on the expression of key receptors: oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER2). Additionally, the proliferation marker Ki67 can be detected; but it has limited utility in clinical settings until today (Nielsen et al., 2021). In general, the markers above serve as prognostic tools and as predictors for treatments (Plevritis et al., 2018).

Treatment will depend on the stage and cancer subtype. The main option at all stages is the surgical removal of the tumor and the associated tissue. At initial stages and good prognosis tumors subtype, the use of endocrine therapy is recommended; nevertheless, it will depend on the responsiveness of cancer cells (ER^+, PR^+) to estrogens (Korde et al., 2021). Finally, poor prognosis breast cancer subtypes will require chemotherapy combined with immunotherapy (Esteva et al., 2019).

2.2. UV radiation and skin damage

The sun is the natural and primary source of ultraviolet (UV) radiation, and humans are exposed to it on a daily basis; artificial sources include tanning beds, phototherapy, black-light lamps, and mercury vapor lamps. Exposure to solar UV is necessary for vitamin D synthesis, implied in many biological processes and beneficial effects. However, overexposure to UV radiation is harmful and correlated with detrimental health effects such as photoaging and skin cancer onset.

UV radiation can be divided based on its wavelength into three bands, namely UVA, UVB, and UVC. UVA (320-400 nm) is the principal UV radiation that reaches the earth and skin. It exhibits a higher penetration rate reaching the epidermis (**Fig. 5**) and contributes the most to photocarcinogenesis and photoaging (McDaniel et al., 2018). UVB (290-320 nm) is more energetic than UVA but exhibits a lesser degree of penetration, and its levels on earth are minor compared to UVA (Mohania et al., 2017). UVC (100-290 nm) is the more energetic and dangerous of all three. It is absorbed by the atmosphere and never reaches the skin, although humans might be exposed to UVC by artificial means (mercury-vapor lamps). Furthermore, it has germicidal activity and is used for disinfection purposes (Narita et al., 2018).

The skin is the largest organ by area surface in the body. It is divided into three layers (epidermis, dermis, and hypodermis), each comprising different types of cells, extracellular matrixes, and functions (**Fig. 5**). Skin is a complex organ, and current in vitro models to study it relies on cell lines to recreate the epidermal layer, with human keratinocytes HaCaT and the dermal layer with human fibroblasts HFF-1 or murine fibroblasts NIH/3T3 (Retting & Nguyen, 2018). Moreover, a 3D model can be constructed using polymers or extracellular matrix and both keratinocytes and fibroblasts.

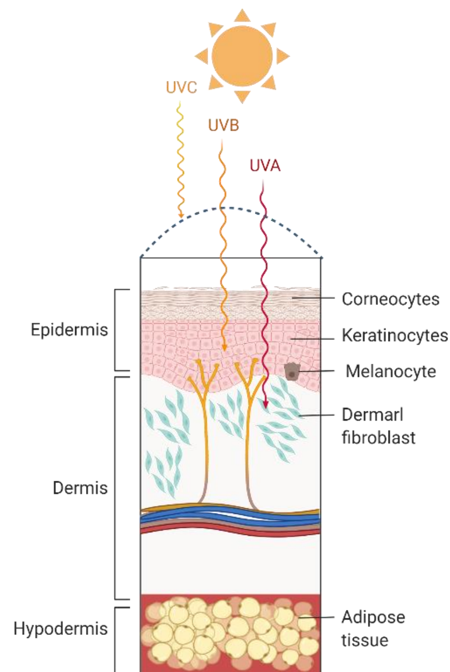


Fig. 5 | Different UV radiations bands and their penetration into the skin. Depiction of skin anatomy and their principal cells. Created with BioRender.com

The first concern of UV-induced damage is skin cancer, followed by immunosuppression, inflammation, and photoaging. Skin cancer has three categories: melanoma, basal cell carcinoma, and squamous cell carcinoma; the latter two are referred as non-melanoma skin cancers. Non-melanoma accounts for 6.2% of new cancer cases worldwide (Sung et al., 2021); their metastatic potential is low, associated with a good prognosis and low mortality (Madan et al., 2010). On the contrary, melanoma is the most aggressive skin cancer with higher mortality rates but only accounts for 1.7% of new cases (Sung et al., 2021).

UV-induced skin carcinogenesis is a complex and lengthy biological process, but the onset is given by DNA damage, inflected by two main mechanisms. (1) UV radiation absorbed by DNA induces the production of pyrimidine dimers and (2) increased ROS production, such as alkylating agents that damage DNA (Liu-Smith et al., 2017). Proteins (antioxidant enzymes) and molecules (porphyrins and melanin) related to redox pathways can be photo-oxidated through UV radiation, causing cross-linking and loss of their antioxidant function (Pattison et al., 2012);

moreover, they become aberrant and increase the production of ROS (Karran & Brem, 2016). In summary, DNA damages lead to mutations in proto-oncogenes and tumor suppressor genes, marking the onset of cancer.

The mechanism and intensity of damage will depend on the UV band. In comparison with UVA and UVB, UVC (254 nm) causes more serious to DNA (Goswami et al., 2013) and induces the production of ROS and oxidative damage, although to a lesser extent compared to the other UV wavelengths (Feng et al., 2012). Nonetheless, UVC is widely used to assess UV radiation damage at the cellular and molecular level (Tai et al., 2012).

2.3. Plants and phytochemicals

Recently, there has been an increasing trend, both at the research and market level, to develop novel pharmaceuticals and functional foods based on fruits, vegetables, spices, and medicinal plants. Indeed, medicinal plants have been safely consumed since immemorial times, and their uses are widespread in low and middle-income countries, such as China and Mexico.

Plants are sources of therapeutic molecules for several pathologies, including cancer. Over 37.5% of all categories of newly approved drugs by the FDA between 1981 and 2014 come from natural sources (plants, fungi, bacteria) and up to 38% of all anticancer drugs (Newman & Cragg, 2016). Most plants worldwide and their properties remain unexplored; just in Mexico, there are approximately 23,314 vascular plant species, half of which are endemial (Villaseñor, 2016), representing an excellent opportunity for therapeutic molecules discovery.

Plant chemicals, popularly known as phytochemicals, are secondary metabolites of plants with a wide range of functions, including growth, defense against pathogens and stress, and reproduction. Despite not being nutrients, phytochemicals have bioactive properties in other organisms aside from their hosts; the chronic and low daily intake of phytochemicals provides health benefits (Tungmunnithum et al.,

2018). Phytochemicals can be classified as phenolic compounds (polyphenols), alkaloids, terpenoids, phytosterols, and organosulfur compounds (Bayir et al., 2019).

2.3.1. Phenolic compounds and their biological activities

Phenolic compounds are one of the most abundant secondary metabolites in diverse plant matrices (fruits, vegetables, roots, leaves), with many bioactive functions in the plant (Albuquerque et al., 2021). They can be classified according to their chemical backbone (**Fig. 6**)

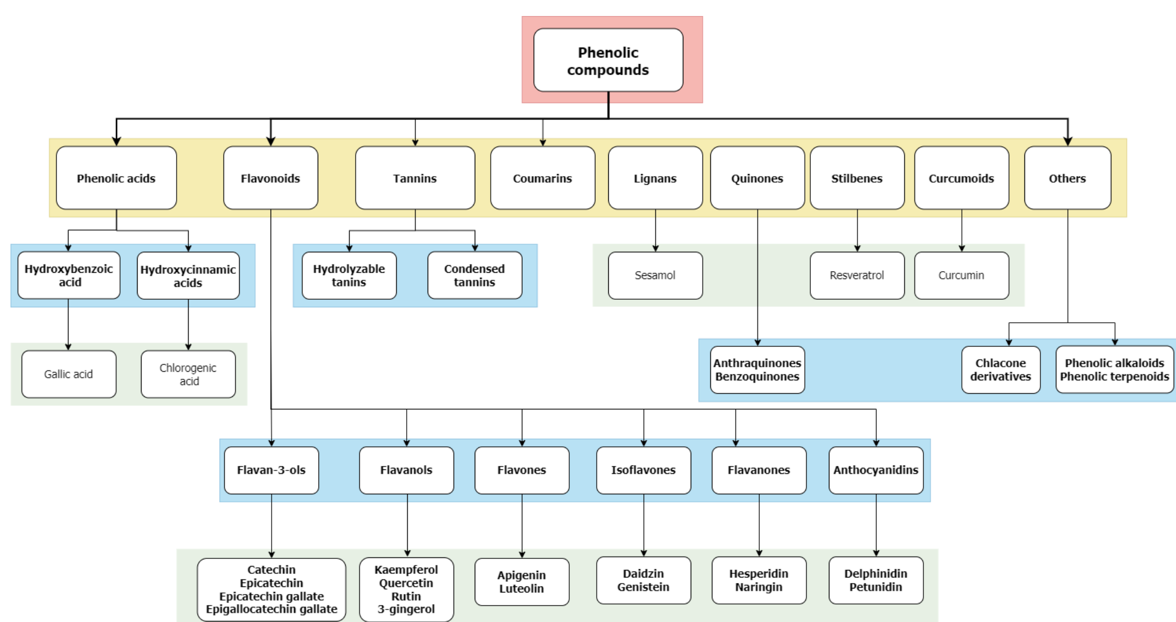


Fig. 6 | Classification of polyphenols according to their chemical structure and some representative examples. The major classes are highlighted, subclasses in blue and compounds in green. Adapted from Gan et al., (2019)

Polyphenols have a wide range of applications from food preservation to improve the health of animals and humans; some reported properties and potential applications are: increase shelf-life of foods (Papuc et al., 2017), an improved fatty acid profile of meat (Cimmino et al., 2018), increase susceptibility of bacteria to antibiotics (Smirnova et al., 2012), neuroprotective (Giacalone et al., 2011), antiviral (Mhatre et al., 2021), anticancer (K. W. Lee et al., 2011) and skin photoprotection (Nichols & Katiyar, 2010) properties. However, only the

antiproliferative and photoprotective properties of polyphenols are in the scope of this work.

2.3.2 Polyphenols in cancer

Several phytochemicals have antineoplastic effects, and many of them are used in current treatments; however, most of them are alkaloid compounds. The most known examples are paclitaxel and docetaxel derived from *Taxus brevifolia* and vincristine and vinblastine derived from *Catharanthus roseus* (K. W. Lee et al., 2011).

In the last decade, superfoods (vegetables and fruits) consumption has been attributed to antineoplastic properties due to their polyphenolic content and their ability to scavenge radicals. Indeed, a systematic review and meta-analysis revealed that a daily intake of 600 g of fruits and vegetables lowers the risk of cancer mortality (Aune et al., 2017). A long-term belief is that antioxidants can inhibit specific cellular processes such as aging, inflammation, and cancer. Indeed, polyphenolic antioxidants remove the excessive and damaging production of reactive oxygen species (ROS) involved in several underlying pathologies, including cancer (Singh et al., 2018).

However, a growing body of evidence suggests that cancer, oxidative stress, and the use of antioxidants have a complex relationship. Recently, there has been interest in a new research area termed redox signaling (Russo et al., 2017), which involves the study of oxidative protein modifications (induced by ROS) and their implications in cell signal transduction (Blaser et al., 2016). A significant player in redox signaling and oxidative stress is the nuclear factor erythroid 2 (NFE2)-related factor 2 (NRF2), a transcription factor (Ma, 2013).

Under homeostatic conditions, the transcription factor NRF2 is constantly ubiquitinated by the Kelch-like ECH-associated protein1 (Keap1) and cullin 3 (CUL3) complex for further degradation by the proteosome; this is critical to

regulate the intracellular levels of NRF2 (Iso et al., 2016). Upon oxidative stress sensing (ROS), KEAP1 becomes inactivated, and NRF2 is phosphorylated and further translocated to the nucleus where it binds to antioxidant response elements (ARE), initiating the transcription of antioxidant enzymes (**Fig. 7**).

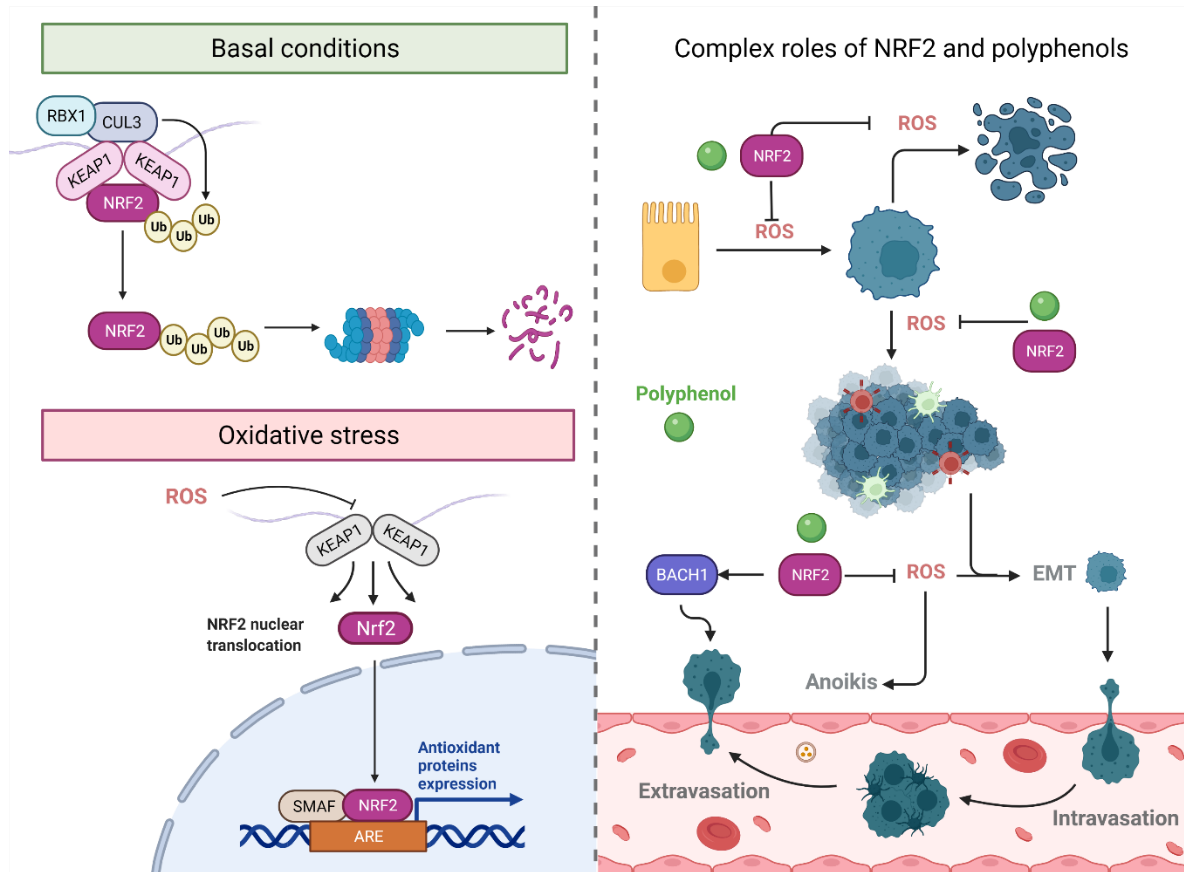


Fig. 7 | NRF2-KEAP1 pathways (left panel). Roles of phytochemicals (antioxidants) and NRF2 in oxidative stress signaling and cancer progression (right panel). Data from Rojo de la Vega et al. (2018). Created with BioRender.com

Due to their high replication rate, cancer cells are subject to high levels of ROS production. Excessive intracellular ROS concentrations work as molecular cues to induce cell apoptosis (Perillo et al., 2020); therefore, they need to increase their antioxidant defenses to survive. Both endogenous (NRF2, antioxidant enzymes) and exogenous (polyphenols) factors modulate redox signaling and influence cancer outcome.

Lately, both a positive and negative role in cancer onset and progression have been attributed to NRF2. Oxidative damage (ROS) is prevented through NRF2 or the antioxidant activity of polyphenols; however, this promotes the viability and proliferation of cancerous cells in different stages (**Fig. 7**). NRF2 ablation or antioxidant power loss promotes epithelial-mesenchymal transition (EMT) via ROS, leading to migration and intravasation (I. S. Harris & DeNicola, 2020). On the other hand, NRF2 or polyphenols can interact with the transcription factor BACH1, promoting invasion and extravasation (Wiel et al., 2019). Finally, ROS promotes the death of detached cells (anoikis) but is prevented by antioxidants or NRF2. It is evident that there is a delicate interplay between ROS levels and cancer; equilibrium should be maintained to keep cells in homeostasis.

Although there are no polyphenolic compounds in clinical use, they have been reported to disrupt and modulate many transduction pathways. The antineoplastic activity of polyphenols occurs through direct interactions or binding with protein targets rather than through their antioxidant activity (Lee et al., 2011). They target different proteins involved in cancer metabolism, leading to cell apoptosis or growth inhibition. Some examples are shown in **Table 1**.

Table 1. Examples of molecular targets of selected polyphenolic compounds with antineoplastic activity

Binding polyphenol	Molecular target	Effect	Plant species	Reference
Sesamol	DNA minor groove binding	↑cell death in liver adenocarcinoma cells	*Sesamun indicum* (seeds)	Z. Liu et al., (2013)
Epigallocatechin gallate	IGF-IR	↓human breast and cervical cancer cells	*Camelia sinensis* (leaves)	Li et al., (2007)
Resveratrol	COX-2	↓anchorage-independent growth colorectal adenocarcinoma	*Vitis vinifera* (fruit)	Zykova et al., (2008)
6-gingerol	LTA(4)H	↓ anchorage-independent growth colorectal cancer	*Zingiber officinale*	Jeong et al., (2009)

		cells	(root)	
Kaempferol	SRC	↓UVB induced carcinogenesis skin epidermal cells	Several edible plants	K. M. Lee et al., (2010)

Overall, there is still a gap between in vitro and clinical research, especially regarding their low bioavailability, biotransformation, and absorption by specific tissues to exert any therapeutic effect (Russo et al., 2017). More clinical studies are urgently needed to fill this gap and assess the effectiveness of polyphenolic compounds.

2.3.3. Polyphenols in UV-induced carcinogenesis

As mentioned above, polyphenols are powerful antioxidants. Even though their role in tumorigenesis is complex, they can protect cells from oxidative damage and subsequently DNA damage through their radical scavenging capacities and interactions with other protein targets. Interestingly, they also reduce inflammation (Vicentini et al., 2011) and collagen matrix degradation (Kumar & Mandal, 2019), responsible for skin wrinkles, after prolonged UV exposure.

UV induces the formation of cyclobutene pyrimidine dimers (CPDs), which form dimers in the DNA, introducing mutations (Liu-Smith et al., 2017). Usually, cells repair CPDs through the nucleotide excision repair (NER) pathway; however, the formation of CPDs is UV dose-dependent, and the repair mechanisms are overrun (Britto et al., 2017). Punicalagin, a tannin, increases the expression and activation of related factors of the NER pathway, removing UV-induced CPDs (Chong et al., 2019).

Lastly, UV radiation depletes endogenous antioxidant enzymes and generates high intracellular levels of ROS. Polyphenols have radical scavenging capacity and reduce ROS concentration (NilamberLal Das et al., 2019). Furthermore, polyphenols have been noted to restore the levels of antioxidant enzymes

(glutathione peroxidase, catalase, and superoxide dismutase) in mouse skin cells after UV-induced damage (Vayalil et al., 2004).

2.4. *Hamelia patens*

Hamelia patens is an ornamental shrub, kwon in Spanish as "trompetilla", "coralillo", and "bayetilla"; "ix-canan" in Mayan and as "scarlet bush" or "read head" in English (Vibrans et al., 2014). It is native to the American continent, from Florida (USA) to Argentina and there are registers of its presence in the Mexican states of Campeche, Chiapas, Hidalgo, Jalisco, Morelos, Nayarit, Oaxaca, Querétaro, Quintana Roo, San Luís Potosí, Sinaloa, Tabasco, Tamaulipas, Veracruz, and Yucatán (Villaseñor, 2016).

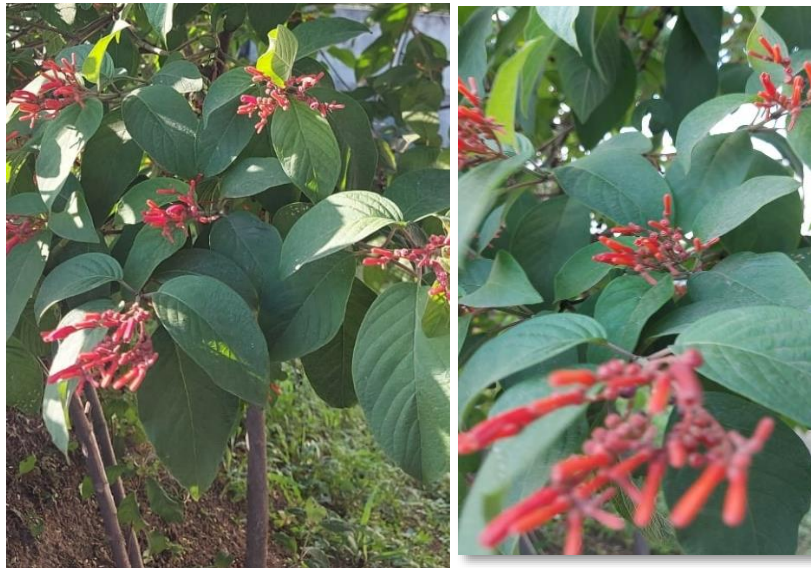


Fig. 8 | Aerial parts of *H. patens* (flowers and leaves).

H. patens grow as small evergreen and perpetual flowering shrub, but it can reach 7 m high. It has grey-green leaves (5-23 cm), petiolate, ovate, and opposite with reddish veins (**Fig. 8**). Their flowers are tubular orange-red or yellow-red, giving rise to small dark-purple berry fruits, which are eatable for humans and animals (Vibrans et al., 2014).

The aerial parts are used in traditional medicine and by indigenous communities in México. Ethnopharmacological studies report its widespread use among the following ethnic groups: Mayans in Yucatan state (Mena-Rejon et al., 2009) and Huastecs in Huasteca Potosina region (Alonso-Castro et al., 2012), and Totonacs in the state of Veracruz (Reimers et al., 2019). People use it as infusions or cataplasms to treat disorders like wounds, abscesses, skin problems, uterus and ovary illnesses, diarrhea, stomachache, anemia, diabetes, gastritis, colitis, menstruation, hypertension, and cancer.

H. patens extracts contain many compounds of different phytochemical classes, which depend on the harvested plant (due to environmental and genetic factors), part of the plant, extraction method, and solvent employed. **Table 2** shows the reported phytocompounds in several extracts. All of the retrieved articles in which compounds were identified by analytic means reported using leaves. Furthermore, most of them obtained the extracts through maceration are using methanol or ethanol. Many classes of chemical compounds are represented in the extracts (**Table 2**), including alkaloids, phenolic acids, flavonoids, triterpenes, steroids, glycosides, and amino acids.

Table 2. Reported phytochemical compounds in *H. patens* extracts.

Plant part (solvent)	Method	Compounds	Reference
L (MetOH)	Maceration	Isopteropodine, palmirine, and rumberine.	Reyes-Chilpa et al. (2004)
L (Ac)	Maceration	β-sitosterol, stigmasterol, ursolic acid, rotundic acid, arcine, catechin, oxindol arcine.	Rios & Aguilar-Guadarrama (2006)
L (MetOH)		Hameline, tetrahydroalstonine, aricine, pteropodine, isopteropodine, uncarine, speciophylline, palmirine, and rumberine.	Paniagua-Vega et al. (2012)
L (EtOH)	Maceration	Caffeic acid, quercetin, chlorogenic acid.	Andrade-Cetto et al. (2015)
L (EtOH)	Soxhlet	Malic acid, shikimic acid, hexadecanoic acid, caffeic acid phytol, α-linolenic acid, octadecanoic acid, dehydroabietic acid, squalene, catechin, y-tocopherol, mitraphylline, a-tocopherol, campesterol, β-sitosterol, 1-triacontanol, oleanolic acid, ursolic acid	Alonso-Castro et al. (2015)
L (MetOH)	Sonication	Arcine, arcine N-oxide, hameline, isopteropodine, palmirine, pteropodine, rumberine, acetic acid, aspartic acid, chlorogenic acid, p-coumaric acid, glutamic acid, glutamine, glucoso, loganic acid, strictosidine, sucrose, tryptophan.	Flores-Sanchez et al. (2016)
L (Chl)	Maceration	Methyl palmitate, eicosane, oleic acid, perivine, tetratriacontane, B-sitosteryl-D-glycoside. amides, saponins, esters, indole alkalaoids	Surana & Wagh (2017)
L (Hex)	Maceration	β-sitosterol, stigmasterol.	Jiménez-Suárez et al. (2016)
L (MetOH)	Maceration	chlorogenic acid, catechin, epicatechin	Rugerio-Escalona et al. (2018)
L (EtOH)	Maceration	Quinic acid, hydroxycinnamic acid, catechin, caffeoylquinic acid Procyanidin B2, epicatechin, catechin 3-O-glucose, quercetin, 3-O-rutinoside, laempferol 3-O-rutinoside.	Paz et al. (2018)
L (MetOH)	Microwave-assisted	Isoquercetin, rutin and soyasaponin b	Maamoun et al. (2019)

Leaves (L), methanol (MetOH), ethanol (EtOH), acetone (Ac), chloroform (Chl), and hexane (Hex).

Literature survey revealed that *H. patens* extracts have many bioactive functions. Methanolic extracts of leaves have shown antibacterial activity against *Escherichia coli, pseudomonas aeruginosa* (Camporese et al., 2003), while ethanolic extracts inhibited the growth of *Staphylococcus aureus, Salmonella typhi, Salmonella paratyphi* (Paz et al., 2018); moreover, they have antifungal properties against *Aspergillus niger* and *Candida albicans* (Okoye & Ezeogo, 2016). Antiviral activity of ethanolic extracts of leaves was reported against Herpes simplex virus 2 (HSV-2) but not against HSV-1 (Silva-Mares et al., 2019a).

The methanolic extract has relaxing properties, at least in the myometrium contraction in rats (Reyes-Chilpa et al., 2004), while butanol extracts have hepatoprotective properties (Perez-Meseguer et al., 2016). The ethanolic and methanolic fractions have hypoglycemic effects in diabetic rats (Andrade-Cetto et al., 2015; Rugerio-Escalona et al., 2018); additionally, the ethanol extract also has antinociceptive activity on thermal and chemical-induced oedemas in mice(Alonso-Castro et al., 2015).

Extracts with non-polar solvents have different properties; the dichloromethane fraction has leishmanicidal activity (Suarez et al., 2008); extractions with petroleum and alcohol mixtures have antipyretic properties (Khandelwal et al., 2011). Chloroform and hexane extracts have antidepressant (Surana & Wagh, 2017) and anti-inflammatory (Jiménez-Suárez et al., 2016) activities in mice respectively.

H. patens extracts have also shown antineoplastic properties in different cancer human cell lines, shown in **Table 3**. Furthermore, the methanolic extract of leaves(Silva-Mares et al., 2019a) and root bark(Mena-Rejon et al., 2009) were not toxic to non-cancerous kidney epithelial cells of monkeys (Vero) and Madin-Darby Canine Kidney (MDCK) cells, respectively.

Table 3. Reported antineoplastic and cytotoxic properties of *H. patens* extracts.

Extract	Cell line (Tissue represented)	IC_{50} (µg/ml)	Reference
Ethanol, flowers	PC3 (prostate), MCF7 (breast), HT-29 (colorectal), MDA-MB-231(breast)	41, 63, 34, 45, 31, 9	Taylor et al., (2013)
Methanol, leaves	HeLa (Cervix)	1450	Silva-Mares et al., (2019)
Methanol, root bark	HeLa (Cervix), SiHa (Cervix), Hep-2 (unknown), KB (papilloma)	13±1.2, 22±1.1, ND, ND	Mena-Rejon et al., (2009)
Methanol, leaves	HEPG-2 (liver), MCF7 (breast)	47, 23.8	Maamoun et al., (2019)

Extracts: the solvent and the part of the plant used are indicated. ND, non-detected, the extract did not affect cell growth.

Lastly, the median lethal dose (LD_{50}) has been determined in rats for the ethanolic extract of leaves: intraperitoneal LD_{50} 2964 mg/kg and oral LD_{50}>5000 mg/kg orally (Alonso-Castro et al., 2015). For the leaf's methanolic extract, the oral LD_{50} was >2000 mg/kg (Rugerio-Escalona et al., 2018). It is important to know the LD_{50} of the extracts; however, there is no information on the therapeutic or effective doses (ED_{50}). The therapeutic index is calculated with the LD_{50} and ED_{50}, and it is a more useful metric compared to only knowing one of them, as it measures the relative safety of a drug.

3. Materials and methods

3.1. Chemicals and reagents

Dulbecco′s Modified Eagle′s Medium (DMEM) (Cat No. D5030), RPMI-1640 Medium (Cat No. R8758), L-glutamine (Cat No. 49419), sodium bicarbonate (Cat No. 31437), trypsin-EDTA solution (Cat No. T4049), 2,2-diphenyl-1-picrylhydrahyl (DPPH) (Cat No. D9132), (R)-(+)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Cat No.391913), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Cat No. A1888), 3,5-dinitrosalicylic acid (DNS) (Cat No. D0550), Folin & Ciocalteu′s phenol reagent (Cat No. F9252), sodium hydroxide (Cat. No. S8045), and gallic acid (Cat No. G7384) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum, certified (FBS) (Cat No.16000-036), antibiotic-antimycotic (100x) (Cat No. 15240-096), and phosphate-buffered saline (PBS, pH 7.2) (Cat No. 20012-043) were acquired from Gibco (Grand Island, NY, USA). CellTiter 96® Aqueous (Cat No. G358A) was obtained from Promega (Madison, WI, USA). Ethanol (Cat No. 8006-05), methanol (Cat No. 9070-03), trichloroacetic acid, crystal (Cat No. 0414-01), sodium phosphate dibasic, anhydrous (Cat. No. 3828-01), and sodium phosphate monobasic, monohydrate, crystal (Cat No. 3818-01) were purchased from JT Baker (Phillipsburg, NJ, USA). D-Glucose (Dextrose), anhydrous (Cat 0188), and sodium carbonate, anhydrous (Cat No. 0585) were obtained from AMRESCO (Solon, OH, USA). Sodium sulfite (Cat No. 2510) and Ferric chloride (Cat No. 1330) were acquired from Meyer Chemical Reagents (Tlahuac, CDMX, Mexico). Potassium Sodium Tartrate Tetrahydrate (Cat No. 70851) was purchased from Fermont (Monterrey, NL, Mexico). Potassium ferrocyanide (Cat No. 13246-66-2) was bought from Química Mercurio (Puebla, Pue, Mexico). Phenol crystal (Cat No. 2651V) were acquired from Reproquifin (Ecatepec, Edo. Mex, Mexico). Potassium persulfate (Cat No. 0057567) was procured from Reasol (Iztapalapa, CDMX, Mexico). Gentian Violet, Crystal (Cat No. VC 1070) was obtained from High Purity (Iztapalapa, CDMX, Mexico).

3.2. Plant material and extract preparation

Healthy leaves of *H. patens* were harvested from two individual plants in February of 2019 and the other in February of 2021. Both plants were located in a common yard in the city of Poza Rica, Veracruz, México (20°32'31.6"N 97°28'25.6"W). The voucher specimens (ID: 81629) were deposited at the Jardín Botánico Universitario, Benemérita Universidad Autónoma de Puebla, Puebla, México and authenticated by Master Allen J. Coombes.

The leaves were immediately dried at 40°C in a commercial dehydrator (Hamilton Beach 32100A) and pulverized in a blender (Waring Z272221). The hydroalcoholic extracts were made by maceration of the powder. Briefly, 10 g of powder were mixed with 100 ml of an ethanolic solution (50%) and left for 24 h at 85°C and agitation (250 rpm) in an incubator (KS 4000i control, IKA, Janke & Kunkel, Staufen, Germany).

The resulting extracts were filtered with Whatman #4 paper; the ethanol was allowed to evaporate at 40°C for 24 hours. The remaining extract was stored at -80°C for 48 h and subsequently freeze-dried in a lyophilizer (FreeZone 4.5 Liter Benchtop Freeze Dry Systems, LABCONCO, Kansas City, MO, USA) for 48 h to remove the remaining water.

Stock solutions of both extracts were prepared by adding phosphate-buffered saline solution (PBS) to the lyophilized extract powder. The stock concentrations were the following: 9940 μg/ml for *H. patens* extract from 2019 (Hp2019) and 7588 ug/ml for *H. patens* extract from 2021 (Hp2021). The lyophilized extracts and the stock solutions were stored at -80°C until the experiments. The Hp2019 extract was stored for two and half years prior to the experiments under the same conditions.

3.3. Extract characterization

The total reducing sugar content, total phenolic content, and antioxidant capacity were analyzed using colorimetric methods. Phenolic compounds were identified using high-performance liquid chromatographic (HPLC) and liquid chromatography/time of flight mass spectrometer detection system (LC/MSD-TOF).

The recovery yield was calculated with **equation 1**; the results were expressed as percentage of lyophilized extract (LE) over dry weight (DW) of plant material.

$$\boldsymbol{Recovery}\ (\%) = \frac{\boldsymbol{g\ of\ lyophilized\ extract}}{\boldsymbol{g\ of\ dry\ material}}\ \boldsymbol{x\ 100} \qquad \textbf{(1)}$$

3.3.1. Total reducing sugar content

The total reducing sugar content (TRSC) was determined with DNS (3,5-dinitrosalicylic acid) method (Miller, 1959). Briefly, 500 µl of DNS reagent (1% DNS, 1% NaOH, 0.2% phenol, 0.05% Na_2SO_3) were mixed with 500 µl and heated (100°C) for 15 minutes. The mixture was cooled to room temperature in an ice bath, and 166 µl of potassium sodium tartrate (40%) was added. The absorbance was determined at 540 nm with a spectrophotometer (DR600 HACH, Loveland, CO, USA). TRSC was calculated using D-glucose (40-240 µg/ml) as standard ($y = 0.0037x + 0.3834$, $R^2 = 0.9961$). The results were expressed mg of D-glucose equivalents per g of lyophilized extract (mg GE/ g LE).

3.3.2. Determination of total phenolic content and antioxidant activity

The total content of phenolic compounds (TPC) was determined with a colorimetric method (Ainsworth & Gillespie, 2007) with modifications. Samples of the lyophilized extracts (100 µl) were thoroughly mixed with 200 µl of Folin-Ciocalteu reagent at 10% (v/v). After 3 minutes, 800 µl of Na2CO3 (700 mM) was added and left in the dark for two hours at room temperature. Absorbance was measured at 765 nm using a spectrophotometer (DR600 HACH, Loveland, CO, USA). The TPC was calculated as gallic acid equivalents (GAE) using a standard curve (225-525

μM) of gallic acid (y = 0.0037x + 0.3834, R^2 = 0.9961). Results were expressed as mg GAE per g of lyophilized extract (mg GAE/ g LE).

The antioxidant capacity was measured using three different assays: 2,2-diphenyl-1-picrylhidrazyl (DPPH) radical assay, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assay, and the ferric reducing antioxidant power (FRAP) assay.

DPPH assay

The free DPPH radical scavenging method (Brand-Williams et al., 1995) was followed with modifications. DPPH (150 μM) stock solution was prepared with ethanol (80%) and sonicated for 20 minutes. Samples of extracts (92 μl) at different concentrations (Hp2019: 15.625-175 μg/ml, Hp2021: 6.25-100 μg/ml) were mixed with DPPH stock solution (908 μl) and left in the dark for 10 minutes at room temperature. Absorbance was recorded at 514 nm using a spectrophotometer (DR600 HACH, Loveland, CO, USA). The DPPH activity was expressed as a percentage of inhibition and calculated using **equation 2**. The IC_{50} was calculated with the regression equation (y = 0.0153x + 0.0376, R^2= 0.9803) from a standard curve of Trolox (5-55 μM). The results were expressed as μM of Trolox Equivalents per g of lyophilized extract (μM TE/ g LE).

$$\% \, \boldsymbol{Inhibition} = \frac{\boldsymbol{absorbance\ control - absorbance\ sample}}{\boldsymbol{absorbance\ control}} \, \boldsymbol{x\ 100} \qquad (2)$$

ABTS assay

The ABTS radical scavenging assay was employed as described by Re et al. (1999) with modifications (Ilyasov et al., 2020) and adapted to 96-well plate format. A stock solution of ABTS (7mM) was mixed with $K_2S_2O_4$ (2.4 mM) in a 1:1 ratio and incubated at room temperature for 16 hours, protected from the light. After incubation, the mixture was diluted with absolute ethanol (1:11). The antioxidant activity was evaluated by mixing 100 μl of each extract with 100 μl of ABTS+ dilution and allowed to react for 6 minutes. Absorbance was read at 415 nm in a

microplate reader (BIO-TEK ELx800, Winooski, VT, USA). The antioxidant was expressed as a percentage of inhibition and calculated using **equation 2**. The IC_{50} was calculated with the regression equation (y = 0.0311x + 0.0047, R^2= 0.998) from a standard curve of Trolox (4-28 µM). The results were reported as the IC_{50} in µM of Trolox Equivalents per g of lyophilized extract (µM TE/ g LE).

FRAP assay

The FRAP capacity was determined using the potassium ferricyanide–ferric chloride method (Shahinuzzaman et al., 2020) adapted for 96-well plates. Samples of the extracts (100 µl) were mixed with 250 µl of phosphate buffer (0.2 M, pH 6.6) and 250 µl of $K_3[Fe(CN)_6]$ solution (1%). The mix was incubated at 50° C for 20 minutes. After cooling to room temperature, 250 µl of trichloroacetic acid (10%) were added, mixed, and centrifuged for 10 minutes (10,000 g). Then, 250 µl of the mixture was recovered and diluted with 250 µl of distilled water and 50 µl of $FeCl_3$ (1%). The resulting mixture was allowed to stand for 15 minutes in the dark, and the absorbance was measured at 595 nm using a microplate reader (BIO-TEK ELx800, Winooski, VT, USA). The results were expressed as Trolox equivalents (TE) in µM Trolox per g of lyophilized extracts (µM TE/ g LE). TE was calculated by preparing a Trolox standard curve for the FRAP assay (y = 0.0107x + 0.0134, R^2 =0.9998) in the ranges of 6.25 µg/mL to 100 µg/mL of Trolox.

3.3.3. Analysis of phenolic compounds by HPLC and LC/MSD-TOF

HPLC coupled with a photodiode array detector (1260 Series, Agilent Technologies, Santa Clara, CA, USA) was performed o identify and quantify the compounds in the extracts. Chromatograms were obtained at 280, 320, and 365 nm after injection of 20 µL of the sample (Hp2019). The separation was performed on a Zorbax Eclipse XDB-C18 column (5 µm, 4.6 mm×150 mm, Agilent Corporation, MA, USA) operating at a temperature of 25 ° C with a flow rate of 0.45 ml/min. The mobile phases used were (A) HPLC grade water (Tedia, Fairfield, OH, USA) with 0.1% formic acid (Sigma, St. Louis, MO) and (B) HPLC grade methanol at 100% (Tedia, Fairfield, OH, USA). Separation was achieved starting with 35% B for the first 5 minutes, increasing to 60% B for up to 20 minutes, then to 90% B for

up to 25 minutes, and decreasing to 0% B for the next 5 minutes. Gallic acid, chlorogenic acid, and quercetin (Sigma, St. Louis, MO) were used as authentic standards.

The identification of the main compounds was confirmed by LC/MSD TOF (Agilent 1100, Santa Clara, CA, USA) following the same conditions described above. Ionization was achieved using positive mode electrospray with the spray voltage set to 4000 V. N_2 was used as the nebulizer gas, the pressure in the nebulizer was adjusted to 40 psi, and the nitrogen was heated to 350°C and delivered at a flow rate of 13 L/min.

Due to time constrictions, only the Hp2019 extract could be analyzed by HPLC and LC/MSD TOF. However, the absorbance spectrum (UV-Vis) was obtained as an alternative and qualitative approach to compare both extracts (Hp2019 and Hp2021). The spectra were recorded using a spectrophotometer (DR600 HACH, Loveland, CO, USA) in the ranges of 190-1100 nm.

3.4. Cell culture conditions

Human prostate cancer cell lines LNCaP (ATCC CRL-1740) and PC-3 (ATCC CRL-1435) were cultured in complete RPMI medium supplemented with 2 g/L D-glucose, 0.3 g/L L-glutamine, 5% fetal bovine serum (FBS), and 1% antibiotic-antimycotic 100x. Human breast cancer cell line MCF7 (ATCC HTB-22), human colorectal cancer cell line Caco-2 (ATCC HTB-37), and normal mouse embryo fibroblast cell line NIH/3T3 (ATCC CRL-1658) were culture in DMEM without phenol red supplemented with 1 g/L D-glucose, 0.584 g/L L-glutamine, 3.7 g/L $NaHCO_3$, 5% fetal bovine serum (FBS) and 1% antibiotic-antimycotic 100x. All cell lines were incubated (Memmert, Aeussere Rittersbacher, Schwabach, Germany) at 37° in a humidified environment with 5% CO_2.

3.5. Cell viability assays

LNCaP, PC3, MCF7, and Caco-2 cells were seeded in a 96-well plate at $5x10^3$ cells/well, while NIH/3T3 were seeded at $1x10^4$ cells/well. Each cell line was grown with their respective supplemented medium as specified in cell culture conditions. The plates were incubated for 24 h before the treatments, at 37°C in a humidified environment with 5% CO_2.

3.5.1. Treatments with extracts

Stock solutions of the extracts were diluted with complete media (RPMI 1640 or DMEM) to the appropriate concentrations. The assessed concentrations for Hp2019 and Hp2021 were 0.153, 0.610 and 2.44 µg/ml. LNCaP, PC-3, and MCF7 cell lines were incubated with the extracts for 24, 48, and 72 h, and Caco-2 and NIH/3T3 cell lines were only treated for 24 h. Cell viability was measured with two different assays: CellTiter 96® Aqueous One solution proliferation assay and the crystal violet method.

3.5.2. CellTiter 96® Aqueous method

After the designated duration of extract exposure, cells were incubated with 20 µl of CellTiter 96® Aqueous One solution reagent for one hour at 37°C and 5% CO_2. Absorbance was measured at 490 nm using a microplate reader (BIO-TEK ELx800, Winooski, VT, USA). Percentage cell viability was calculated compared to cell controls with **equation 3**.

$$\%\ \boldsymbol{cell\ viability} = \frac{\boldsymbol{absorbance\ treated\ cells - absorbance\ background\ control}}{\boldsymbol{absorbance\ cell\ control - absorbance\ background\ control}}\ \boldsymbol{x\ 100} \qquad \textbf{(3)}$$

Cell controls were cells incubated without extracts, and background controls were wells without extracts and cells.

3.5.3 Crystal violet method

Cells were stained with crystal violet solution (Feoktistova et al., 2016) with modifications (Hölzl-Armstrong et al., 2019). Briefly, the cell culture medium was aspirated from the plates. Cells were fixed with ice-cold absolute methanol (40 µl)

and let to rest for 10 minutes at 4°C. Methanol was removed, and cells were incubated with 50 µl of crystal violet solution (0.1% in 10% ethanol) at room temperature with agitation (50 rpm) for 20 minutes. Crystal violet solution was aspirated, and the plates were washed with PBS (150 µl) three times. The plates were left to dry for 24 hours. Before reading the plates, 150 µl of ethanol (50%) were added to each well and incubated for 20 minutes with agitation (150 rpm). The absorbance was read at 595 nm using a microplate reader (BIO-TEK ELx800, Winooski, VT, USA). Percentage cell viability was calculated compared to cell controls using **equation 3**.

3.6. Photoprotective activity assay

NIH/3T3 cells were seeded and treated as described above in the cell viability assay and treatment with extract sections. The UV exposure or photoprotective assay was performed as previously described (Varol, 2020) with some modifications. Cell culture medium was discarded after the 24 h treatment period and washed with PBS. A thin layer of PBS (50 µl) was added to the cells, and the plates were left without the lid in a type A2 biosafety cabinet (LABCONCO 3440001, Kansas City, MO, USA) with UV-C (254 nm) light on for 15, 30 and 45 minutes.

After the designated UV-C exposure time, the PBS was aspirated, and 200 µl of fresh and complete growth medium was added to each well. Plates were incubated for 4 h at 37°C and 5% CO_2. Cell survival or viability was measured with CellTiter 96® Aqueous and crystal violet as described above.

3.7. Statistical analyses

Three independent experiments with triplicates were performed for every assay or method. Data from the extract characterization section (TPC, DPPH, ABTS, FRAP, and TRSC) was subjected to a one-way analysis of variance (ANOVA). Tukey's multiple comparison tests were used to identify differences between groups (p ≤

0.001). Pearson correlation analysis was performed to determine the correlation among variables ($p \leq 0.001$).

Data from cell viability assays were analyzed using the general linear model to determine the influence of experimental factors. Normality (Anderson-Darling test, $p > 0.05$) and homogeneity of variances (Levene's test, $p > 0.05$) was confirmed prior to the analysis. One-way ANOVA was performed when significant interactions between factors were identified, and Tukey post-hoc test was used to identify differences between groups (extract and concentration) with $p \leq 0.05$.

Additionally, a general factorial regression ($p \leq 0.01$) was performed to identify the principal effects on the model: assay type, extract, incubation time, concentration, and their interactions. Pareto charts of the standardized effect, main effects, and interactions plots were graphed. The statistics mentioned above were also performed to the data of the photoprotective activity assay.

All statistics were performed using Minitab 19 Statistical Software (State College, PE, USA) Bar, and correlation plots were constructed using Origin(Pro) 2021 (Northampton, MA, USA).

4. Results and discussion

4.1 Total phenolic content and antioxidant activity

The extracts of *H. patens* leaves were performed with an aqueous mixture of ethanol (50%). **Table 4** summarizes the recovery yields, TPC, antioxidant activity, and TRSC of *H. patens* leaves extracts from 2019 and 2021.

Table 4. Extract characterization: recovery yield, phenolic contents, antioxidant activity, and reducing sugars contents

	Extract	
	Hp2019	Hp2021
Recovery yield* (%)	0.99	4.43
TPC (mg GAE/ g LE)	35.59±0.70 [a]	63.64±1.15 [b]
DPPH** (μmol TE/ g LE)	101.72±1.35 [a]	165.74±0.84 [b]
ABTS** (μmol TE/ g LE)	200.55±4.57 [a]	388.46±6.49 [b]
FRAP (μmol TE/ g LE)	4118.35±73.34 [a]	8342.30±50.80 [b]
TRSC (mg GE/ g LE)	85.51±0.39 [a]	175.78±0.11 [b]

Total phenolic contents (TPC), antioxidant capacity measured in DPPH, ABTS, and FRAP assays respectively (DPPH, ABTS, FRAP), total reducing sugars content (TRSC), *Hamelia patens* 2019 extract (Hp2019), *Hamelia patens* 2021 extract (Hp2021). Values are means ± standard error of mean (SEM). Different superscripts in the same row indicate statistical differences between extracts ($p < 0.001$). *The recovery yield (%) was calculated as g of LE over g of DW. ** DPPH and ABTS values were calculated as the IC50.

The choice of ethanol as solvent was due to two main reasons. (1) Most of the phenolic compounds reported in the literature (**Table 2**) were derived from ethanolic extracts. (2) Previous unpublished work showed that the ethanolic extract of *H. patens* leaves has antiproliferation activity on liver carcinoma cells (HepG2). A 0.994 μg/ml concentration reduced the viability to 54.7% (Huerta-Rodriguez. et al., 2019). In comparison, an aqueous extract of leaves inhibited cell proliferation

down to 63.0% with 1.466 µg/ml (Huerta-Rodriguez. et al., 2019). However, the previous work did evaluate the antioxidant activity nor the TPC.

Table 5. Phenolic contents and antioxidant activity expressed over a dry weight basis

	Extract	
	Hp2019	Hp2021
TPC (mg GAE/ 100g DW)	35.23±0.69 [a]	63.64±1.15 [b]
DPPH (µmol TE/ 100g DW)	101.72±1.35 [a]	165.74±0.84 [b]
ABTS (µmol TE/ 100g DW)	200.55±4.57 [a]	388.46±6.49 [b]
FRAP (µmol TE/ 100g DW)	4118.35±73.34 [a]	8342.30±50.80 [b]

Total phenolic contents (TPC), antioxidant capacity measured in DPPH, ABTS, and FRAP assays. Values are means ± standard error of mean (SEM). Different superscripts in the same row indicate statistical differences between extracts ($p < 0.001$).

The recovery rate of phenolic compounds and their overall antioxidant capacity depend on the chosen technique and the solvent of choice (Rodríguez-Pérez et al., 2015). Literature surveys suggest that the best solvents for phenolic compound recovery should have an intermediate polarity index (Herrera-Pool et al., 2021). Usually, aqueous mixtures with ethanol, methanol, or acetone are the best; nevertheless, ethanol is preferred as it has a good yield and is safe for human consumption (Do et al., 2014).

Several reports indicate that extractions with absolute ethanol or aqueous mixtures (50-85%) lead to a greater TPC compared to methanolic extract (Do et al., 2014; Sepahpour et al., 2018). Nevertheless, there is no rule with regards to recovery yield and the extraction method. For example, methanolic extracts of *H. patens* are reported to have between 335.26 ± 0.20 mg GAE/gDW (Rugerio-Escalona et al.,

2018) and 99.250 ± 1.39 mg GAE/gDW (Surana et al., 2016). Those extracts have a greater TPC than both Hp2019 and Hp2019 ethanolic extracts.

Rubio Fontanills et al. (2018) reported a content of 132.33 ± 2.03 mg of chlorogenic acid/ g DW for methanolic (90%) extracts of leaves. Different phenolic standards will result in different standard or calibration curves, and the estimation of TPC will not be completely comparable, such as gallic acid or chlorogenic acid, due to their structural differences (Bastola et al., 2017). Furthermore, mixtures of phenolic compounds will give different results, as each molecule interacts with all others.

Khandelwal et al. (2011) found that leaves contain as much as 104.6±1.12 mg/ g DW of TPC; however, they do not specify the standard or equivalent employed and, it is not possible to make an accurate comparison. PBS extracts of *H. patens* seeds had a content of 13.03 ± 0.1 mg GAE/ g DW (Mushtaq et al., 2017), which is low compared to Hp219 and Hp2021 extracts. Nonetheless, different organs or parts of plants (leaves, stems, seed, pulp, peel) often differ significantly in their phenolic composition (Jiménez-Aguilar et al., 2015). Overall, the phytochemical profile of different organs differs as much as their biological function due to chemical synthesis rates and transport phenoms (Feduraev et al., 2019).

H. patens extracts can be ranked in comparison with other medicinal plants, such as the ones used to treat cancer by the Totonac people according to their TPC, as follows: *A. muricata* 148 ± 4 (George et al., 2015) > *M. oleifera* 83.38 ± 1.2 (Oldoni et al., 2021) > Hp2021> *R. tetraphylla* 41.45 ± 3.90 (Nair et al., 2012) > Hp2019 > *A. linaria* 10.50 ± 0.27 (Sánchez-Gutiérrez et al., 2020). All results are expressed in mg GAE/ g of dried weight or dry extract of leaves; the solvents used vary by extract, hindering comparisons.

Furthermore, there is a statistically significant ($p < 0.001$) variation of TPC between the extract from 2019 and 2021, the latter having almost the double. The harvesting date is a possible reason for the difference; the qualitative and

quantitative composition of phenolic contents and other phytochemicals diverge depending on their harvest date, years, and even months with no apparent pattern (Cezarotto et al., 2017; Dong et al., 2019). As phenolic compounds are secondary metabolites, their synthesis pathways are modulated to respond against environmental factors (climatic conditions, humidity, sun exposure) and human factors (soil modifications, fertilizers). Hence, the phytochemical variability between seasons and years is no surprise.

Hp2021 and Hp2019 extracts were obtained from different individuals (**Fig. 9**) located in the exact coordinates. Therefore, another reason for the TPC difference could be the intraspecies variability; Yaldiz and Camlica (2019) found significant variations in the phytochemical composition of extracts and essential oils of different genotypes of the *Foeniculum vulgare L*. growth and harvested under the same conditions.

Fig. 9 | *H. patens* plants used for the extracts of 2019 (left) and 2021 (right)

Correlation analysis was employed to measure the strength of association between the total phenolic (TPC) and reducing sugar contents (TRSC) and the antioxidant activity measured with three different methods (**Fig. 10**). A strong positive ($R^2 \approx 1$) and statistically significant ($p < 0.001$) relationship between the TPC and the antioxidant activity was found.

Generally, most of the antioxidant capacity comes from phenolic compounds in plant tissue infusions, per several studies working with a staggering amount of plant species (Deng et al., 2013; Dong et al., 2019; L. Fu et al., 2011; Gonçalves et al., 2013; Piluzza & Bullitta, 2011). Overall, data from (**Fig. 10**) suggest that the greater the phenolic content of *H. patens* extracts, the greater its antioxidant power.

Nonetheless, data must not be misinterpreted; the statistically significant correlations do not indicate that the phenolic compounds are the only ones responsible for the antioxidant activity observed (Csepregi et al., 2016). There are other non-phenolic phytochemical compounds antioxidant properties such as ascorbic acid, α-tocopherol, carotenoids (D. Liu et al., 2008), terpenoids (Baschieri et al., 2017), and polysaccharides (Chen & Huang, 2019), to mention some phytocompound classes. A negative or non-existing correlation can exist depending on the plant analyzed (Monteiro et al., 2020).

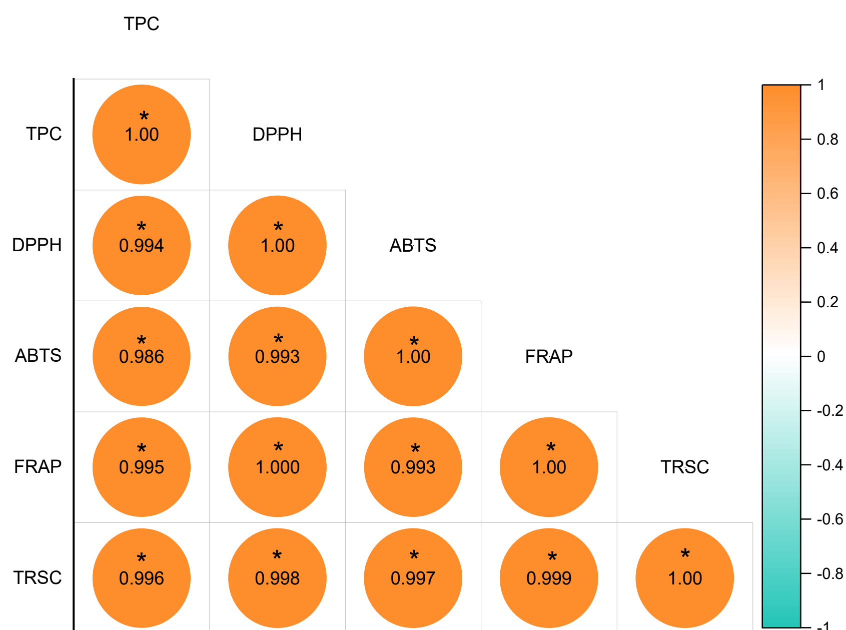


Fig. 10 | Pearson correlation coefficients plot of total phenolic content, antioxidant capacity measured in DPPH, ABTS, and FRAP assays, respectively (DPPH, ABTS, FRAP) and total reducing sugars content (TRSC) of *Hamelia patens* extracts. * Significantly different ($p < 0.001$)

Interestingly, as noted in **Fig. 10**, the TRSC also strongly positively correlates with antioxidant power. However, this does not mean that reducing sugars present in

the extracts have appreciable antioxidant activity since glucose and fructose (the most common reducing sugar found in plants) do not significantly react with DPPH, ABTS, and FRAP substrates (de Oliveira et al., 2017).

Antioxidant capacity is one of the main biological activities of extracts reported in the literature. Nevertheless, the process in which antioxidants interact with reactive or radical species is complex, especially in antioxidant mixtures such as extracts with synergistic or antagonistic interactions. Antioxidant activity is monitored with several assays, each with different mechanisms (single electron transfer or hydrogen atom transfer) and different substrates (peroxyl radical, ABTS, DPPH, Fe^{3+}, or Cu^{2+}); each has its limitations, and results are not interchangeable (Y. Zhong & Shahidi, 2015). Still, there is intra-assay variation, as assays are not entirely standardized, nor results are expressed in the same form; thus, they will differ from each other depending on the reaction conditions, like the ratio of antioxidant and radical concentration (Magalhães et al., 2008).

H. patens extract's antioxidant power was measured with three different methods (DPPH, ABTS, and FRAP) as it is advised to measure the antioxidant capacity of any given plant or food with more than one method since results may vary depending on the choice of assay and the sample (Csepregi et al., 2016). Interestingly. Results were highly correlated (**Fig. 10**) since they share the same dominant mechanism of action: single electron transfer. However, DPPH and ABTS are radical scavenging assays, as their methods rely on electron donation of the antioxidant molecules to neutralize the radical chromophores (Y. Zhong & Shahidi, 2015). FRAP belongs to the redox potential assays; the antioxidant capacity is measured by reducing higher valence elements to their lower valence state. Despite the mentioned differences, results of the three methods are usually strongly correlated in several studies (Deng et al., 2013; Dong et al., 2019; L. Fu et al., 2011; Gonçalves et al., 2013; Piluzza & Bullitta, 2011)

The difference in assays, expressed results, intra-assay variation of antioxidant measurements, coupled with the extraction conditions and the scarce literature of *H. patens* antioxidant activity, hampers comparisons. Jiménez-Suárez et al. (2016) reported that the IC_{50} of methanolic extracts was 158.2 ± 9.6 µg quercetin/gDW, Rugerio-Escalona et al. (2018) reported 50.7 ± 1.3 µg extract/ml and Surana et al. (2016) as 83.44 mg extract /ml; for the reasons mentioned before, it is not possible to make a valid comparison with the extracts in this work.

Unfortunately, *H. patens* extract antioxidant activity can only be compared with one plant used by the Totonac people, as the others were not reported with comparable units nor the same standard. Oldoni et al. (2021) reported that the antioxidant capacity of *M. oleifera* ethanolic extracts was 341.6 ± 42.3 and 928.5 ± 20.8 µmol TE/g in the DPPH and ABTS assay, respectively. As expected, the antioxidant power of *M. oleifera* extract is more significant than both Hp2019 and Hp2021 since the TPC of *M. oleifera* is also superior.

Interestingly, there is only a 1.3-fold increase in TPC between Hp2021 and *M. oleifera* extract, with only a 2-fold and 2.3-fold increase in the antioxidant capacity (DPPH and ABTS, respectively). TPC alone cannot be accounted for the difference of fold change with the antioxidant power. The phenolic profile of the plants might explain this variance, as the results also depend on the chemical structure of each phenolic molecule as well as their interactions between them (Bastola et al., 2017)

The reaction conditions of the assays also account for the difference in the results. In the case of the DPPH assay, the concentration of the radical varies from the ones used in this work (150 vs. 500 µM) and the reaction time (30 vs. 45 minutes). According to Fadda et al. (2014), the estimation of antioxidant activity is heavily influenced by the reaction kinetics of the samples; therefore, time is important for compounds with slow reaction times, while the initial DPPH concentration also led to changes in the kinetic rates of the reaction.

The wavelength used to determine the concentration of ABTS radicals concentration and their inhibition will directly influence the outcome (Re et al., 1999). ABTS radicals have the absorption maxima (λ_{max}) at wavelengths 415, 645, 734, and 815 nm, but researchers commonly use 415 and 734 nm. The λ_{max} employed in this work was 415 nm, while Oldoni et al. (2021) used 734 nm. Consequently, there is a variation in the estimation of the antioxidant activity not directly related to the TPC.

Carlsen et al. (2010) curated a database consisting of the antioxidant power of over 3100 typical foods worldwide, including fruits, vegetables, supplements, medicinal plants, and spices; the antioxidant capacity was measured with the FRAP assay. Remarkably, the FRAP values of Hp2019 (4118.35±73.34 µmolTE/g) and Hp2021 (8342.30±50.80 µmolTE/g) were above the highest mean FRAP values of the food categories listed in the database (supplements 985.58 µmolTE/g, medicinal plants 917.2 µmolTE/g, spices 290.2 µmolTE/g). Nevertheless, the antioxidant activity of *H. patens* extracts falls short compared to the highest value of the database, which is 28971 µmolTE/g, corresponding to a medicinal plant from Mexico and Peru: *Croton lechleri* (Carlsen et al., 2010).

4.2 Analysis of phenolic compounds by HPLC and LC/MSD-TOF

According to the chromatogram (**Fig. 11**) and the tentative identification using LC/MSD TOF, two main phenolic compounds, chlorogenic acid and epicatechin, were present in Hp2019 (**Table 6**) in agreement with previous research (Paz et al., 2018; Rugerio-Escalona et al., 2018). Nonetheless, chlorogenic acid was reported as the main constituent in ethanolic and methanolic leave extracts (respectively), whereas in Hp2019, it is the fourth compound (by concentration).

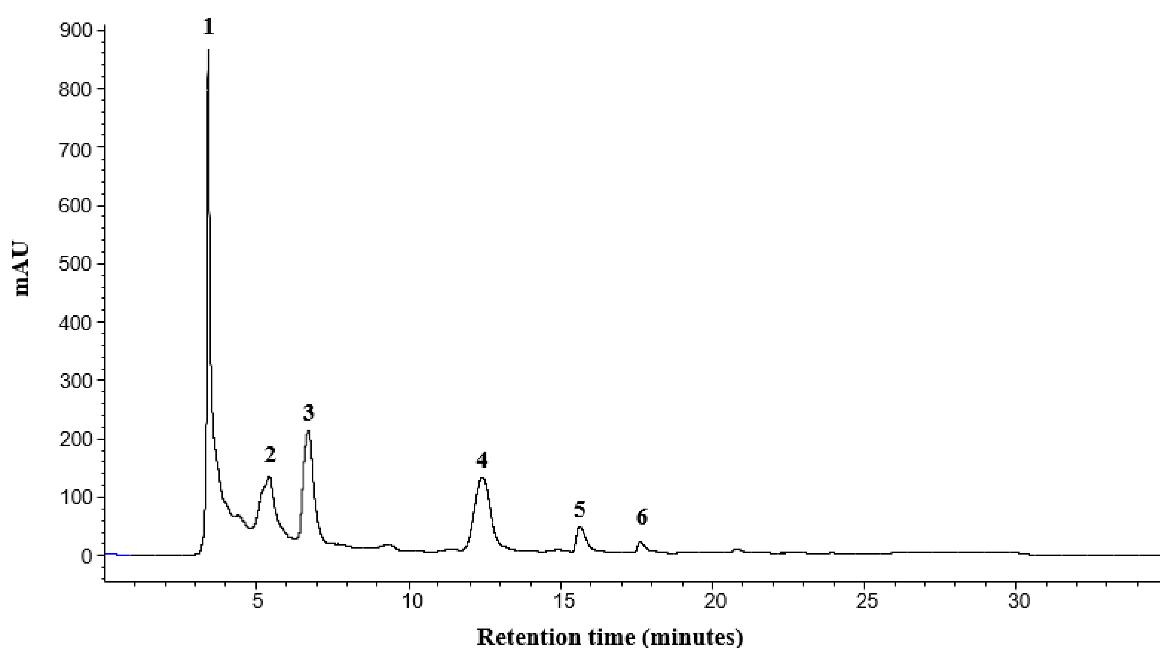


Fig. 11 | Chromatogram of Hp2019 extract.

To the author's knowledge, this is the first time that ascorbic acid has been reported in an *H. patens* extract. It is not surprising to find ascorbic acid on plant material, as it plays a vital role in many cellular processes, including photosynthesis, cell growth, and ROS scavenging (Davey et al., 2000). Still, sometimes, the ascorbic acid content is underestimated in extracts as it is readily degraded (Pereira et al., 2013).

Two more phenolic compounds were found in Hp2109: gallic acid derivatives, which are gallic acid fused with other substituents or moieties. Among these

derivatives, glucoside or glycoside-based are very common and of great pharmaceutical interest due to their anticancer properties (AL Zahrani et al., 2020).

Table 6. Characterization of phenolic compounds found in the extract of leaves of *H. patens* from 2019 (Hp2019)

Peak number	R.T. (min)	UV $_{\lambda max}$ (nm)	*m/z* $[M+H]^+$	Tentative Identification	Concentration (µg/ml)
1	3.432	239	177	Ascorbic acid	24.84
2	5.643	294, 326	355	Chlorogenic acid	9.38
3	6.699	278	291	Epicatechin	10.18
4	12.431	286, 306	399	Hameline	11.00
5	15.656	271	431	Gallic acid derivative	4.19
6	17.643	219, 271	645	Gallic acid glucoside	3.21

Interestingly, Hameline was the second compound (in concentration terms) found in Hp2019. *H. patens* commonly synthesizes many indol and oxindole alkaloid. Palmirine, isopteropodine, pteropodine have been reported in methanolic leaves extracts (Flores-Sanchez et al., 2016; Paniagua-Vega et al., 2012; Reyes-Chilpa et al., 2004). Hameline is a recently discovered monoterpenoid oxindole alkaloid in methanolic extracts from micropropagated plantlets (Paniagua-Vega et al., 2012), although their biological properties remain unexplored.

4.3 Viability of cancer cells

In order to determine the effect of *H. patens* extract on cancer cell lines, a fast screening of cell viability in cancer cell lines was performed, using three concentrations (0.153, 0.610, 2.44 µg/ml) and three incubation times (24, 48, and 72 h). Both extracts were tested since Hp2019 and Hp2021 had significant differences in their phenolic contents, antioxidant activities, and reducing sugars content. Cell viability was measured with two assays: CellTiter 96® Aqueous (CT) and crystal violet (CV).

In most cases, the results obtained did not allow to identify a clear trend with both methods. A Tukey's post hoc pairwise comparison was performed to assess a statistically significant difference between groups, specifically the effect of the combination of extract and concentration ($p<0.05$). This was done independently of the incubation time and the assay. However, there were not many differences among most of the treatments (extract and concentration).

A general factorial regression was performed to identify cell viability's principal variables, including the incubation time and the assay type. Pareto charts of the standardized effects, main effects, and relevant interactions graphs were plotted ($p<0.01$). Effect plots are graphical tools used to identify the impact of an input variable (method, extract, concentration, and incubation time) and its interactions on the output variable (% cell viability). The interaction plots can be found in Appendix A: supplementary figures.

4.3.1 Prostate cancer cells

The results showed that cell viability was reduced to 69.4±6.1% and 69.5±1.2% with 0.153 µg/ml of Hp2019 at 24 h and 72 h respectively measured with the CT assay (**Fig. 12a**). Hp2019 extract shows a more potent inhibitory activity compared to Hp2021 ($p<0.05$). Using the CV assay, the lowest viability was identified as 65.4±1.8% and 68.6±2.5% with 0.153 µg/ml of Hp2019 and Hp2021 for 72 h (**Fig. 12b**).

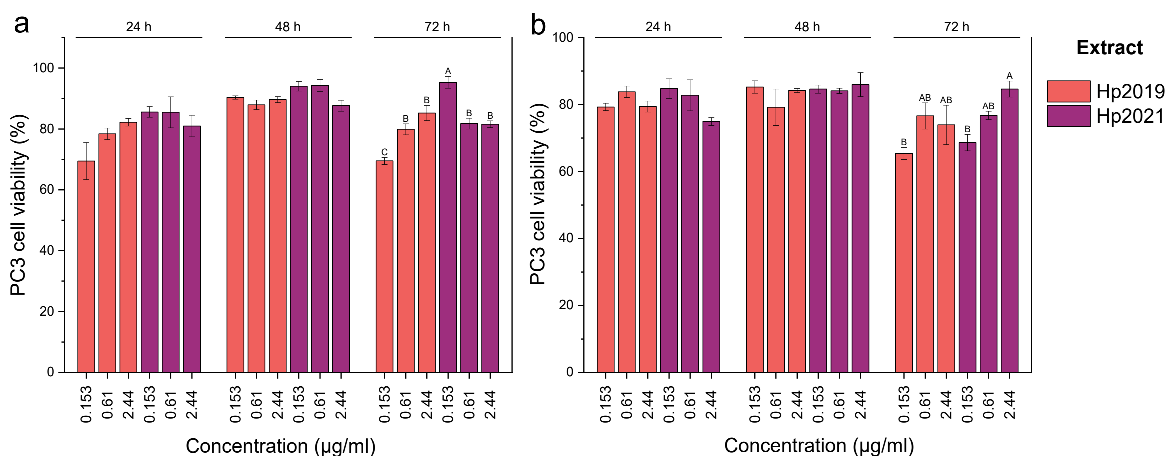


Fig. 12 | Effect of *H. patens* extracts on the viability of PC3 cells (%) compared to non-treated cells, quantified with CellTiter 96® Aqueous (a) and crystal violet (b). Values were expressed as mean % cell viability ± SEM Differences between treatments were determined with Tukey's post hoc test ($p < 0.05$). Different letters denote statistical differences between groups.

The main factors affecting cellular growth were identified by performing a general factorial regression on all PC3 cell line data ($\alpha=0.01$). PC3 cell viability is mainly affected by the incubation time (**Fig. 13a**), followed by the method employed, extract, and the combination of extract and concentration ($p<0.01$). In line with Hafner et al. (2016), IC_{50} values, or the viability (%), depend on drug treatment time due to the inherent characteristics of cellular growth of each cell line.

For PC3 cells, the CV assay detected a more significant reduction in cell viability (%). Overall, Hp2019 extract was more efficient at reducing cell proliferation with a mean value of 80% (**Fig. 13b**). Intriguingly, incubating the cells for 48 h increase their viability compared to 24 and 72 h. This effect could be due to a complex interplay between the biodisponibility of growth-stimulating compounds (e.g.,

reducing sugars, peptides) and inhibitory molecules (e.g., polyphenols, alkaloids) and the duration of the experiment; however, this was not analyzed in the present work. Furthermore, cells can present an adaptative response, where they respond early to the treatment, but lately, they recover their standard growth rate (Niepel et al., 2017). **Supplementary Fig 1** demonstrates that the most inhibitory combination (mean value of %) is the Hp2019 extract at 0.153 µg/ml regardless of incubation time and method.

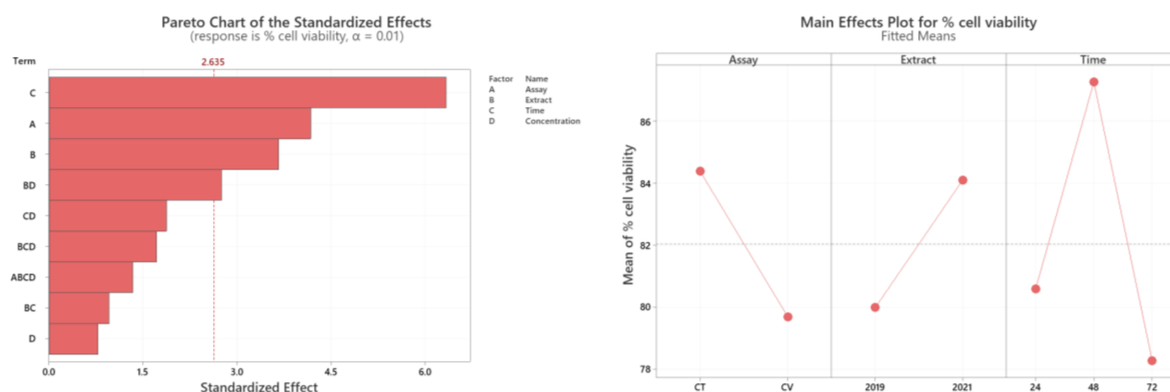


Fig. 13 | (a) Pareto chart of the standardized effects on the viability % of PC3 cells (%). The factors shown correspond to the adjusted model, p<0.01. (b) Main effects plot indicating how the assay type, extract, and incubation time affect cell viability of PC3 cells (%).

Taylor et al. (2013) reported that 41 µg/ml of an ethanolic extract of flowers led to a 50% growth inhibition (IG_{50}) in PC3 cells. There is almost a 300-fold difference in the concentrations of IG_{50} reported, and a minor concentration (0.153 µg/ml) tested in this work reduces cell viability the most, down to 69.4±6.1%. Even though the IG_{50} was not found, the ethanolic extract from leaves Hp2019 has a more pronounced inhibitory effect than the flowers extract (Taylor et al., 2013). Although there is no current research about the difference in the phytochemical profile of flowers and leaves extracts of *H. patens*, extracts from different plant organs displayed a variation in their IC_{50} (Maamoun et al., 2019). The extracts are expected to be distinct, as with other species (dos Santos Nascimento et al., 2021; F. Liu et al., 2018).

Additionally, the ethanolic extract of *H. patens* from leaves inhibits cell growth of DU-145 down to 51% and 77.8% with a concentration of 200 µg/ml during 24 h and 48 h respectively (Martínez Lara, 2016). The concentration is more than 1000-fold higher than the minimal working concentration used here, suggesting that Hp2019 and Hp201 extracts are more potent than those previously reported. Furthermore, they worked with a different prostate cancer line (DU-145) which share some similarities with PC3 (**Table 6**); both are androgen depletion independent (ADI) as they express aberrant nuclear androgen receptors (AR). However, PC3 cells retain the factors needed to activate the AR-dependent tumor suppressor pathway (Litvinov et al., 2006).

The LNCaP cell line was the most affected by the extracts. In the CT assay, there were no significant differences between treatments (**Fig. 14a**), Hp2019 reduced cell viability to 77.7±4.0% (0.153 µg/ml for 48 h) and Hp2021 to 80.6±5.4% (2.44 µg/ml for 72 h). Remarkably, there is a trend in the CV assay, particularly with the Hp2010 extract. Hp2019 at 0.153 µg/ml for 24 h showed the greatest effect, reducing the viability to 38.4±3.2%, while Hp2021 has a modest effect where viability declined to 64.1±9.6% (**Fig. 14b**).

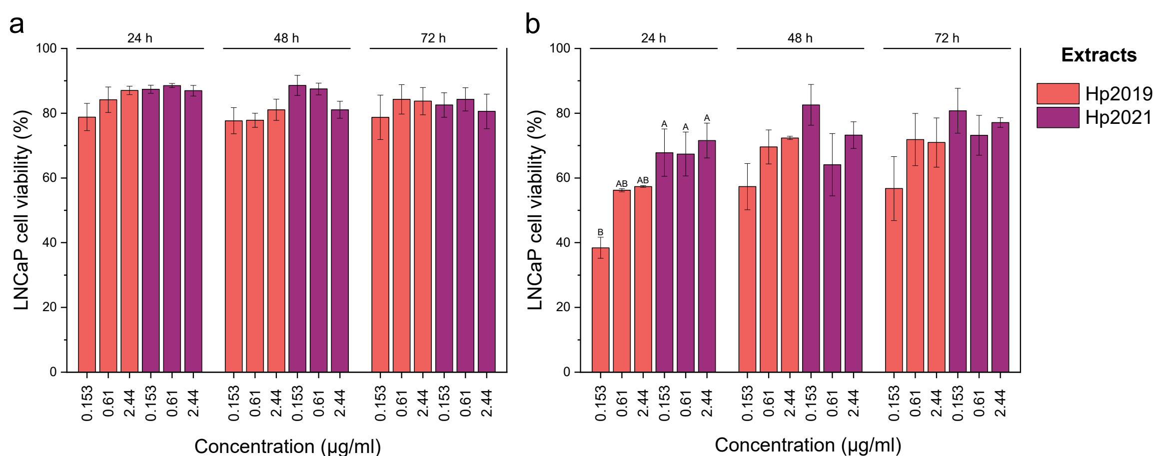


Fig. 14 | Effect of *H. patens* extracts on % viability of LNCaP cells compared to non-treated cells, quantified with (a) CellTiter 96® Aqueous and (b) crystal violet. Values were expressed as mean % cell viability ± SEM. Differences between treatments were determined with Tukey's post hoc test ($p < 0.05$). Different letters denote statistical differences between groups.

Hp2019 had a more significant inhibitory effect in LNCaP cells compared to Hp2021, as noted in the CV assay (**Fig. 14b**). Furthermore, the general factorial regression analysis shows that the main factor affecting the cell viability of LNCaP (α=0.01) was the assay employed, followed by the extract and the extract in combination with the concentration (**Fig. 15a**). Again, the maximum reduction in viability was detected with the CV assay and the Hp2019 extract (**Fig. 15b**). The best combination is Hp2019 at 0.153 μg/ml irrespective of time (**Supplementary Fig 2**).

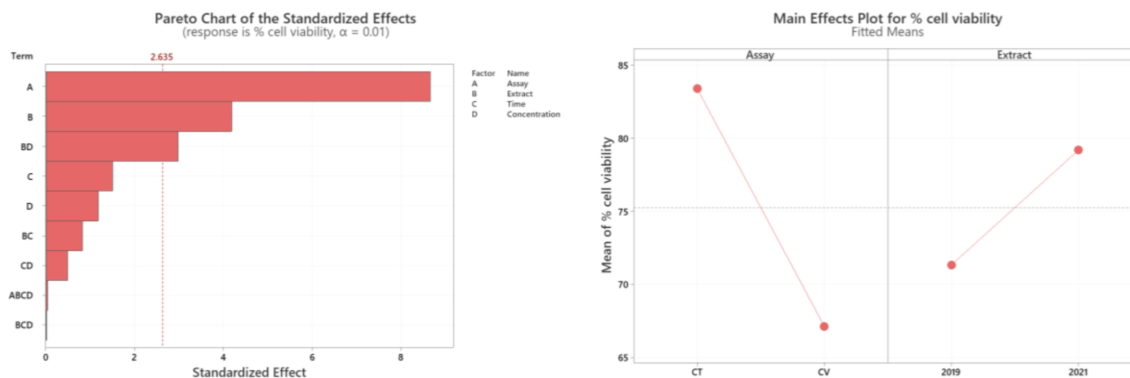


Fig. 15 | (a) Pareto chart of the standardized effects on the viability % of LNCaP cells (%). The factors shown correspond to the adjusted model, p<0.01. (b) Main effects plot indicating how the assay type and extract cell viability of LNCaP cells (%)

The inhibitory effect of Hp2019 was greater (p<0.01) compared to Hp2021 in both cell lines, despite having a lower content of total phenolic acids and antioxidant power (**Fig. 13b** & **Fig. 15b**), The relationship between radical scavenging activity and cell growth inhibition is complex. It is often not well correlated (Grigalius & Petrikaite, 2017); as mentioned above, antioxidants and their radical scavenging capacity may promote cancer and metastasis (I. S. Harris & DeNicola, 2020), leaving these molecules as a double-edged sword.

Based on these findings, the inhibitory effect of *H. patens* extracts cannot be attributed to the antioxidant power nor their TPC alone, but to specific compounds.

Each extract's phytochemical profile and the synergistic interaction of therapeutic molecules with their molecular targets might address their differential effects. Unfortunately, the compounds of Hp2021 could not be identified by HPLC LC/MSD TOF, leaving the issue unresolved. Some probable targets of polyphenols in prostate cancer cells are mentioned in **Fig. 16**.

A simple explanation for the differential effect of *H. patens* extracts in PC3 and LNCaP would be their divergent characteristics (**Table 6**); moreover, a study by (Sardana et al., 2008) of the proteomic profile of PC3 and LNCaP found that only 24% of the identified proteins (more than 1800) were common for both lines.

Table 7. Characteristics of PC3, LNCaP, and DU-145 prostate cancer cell lines

	Androgen sensitive	Doubling time	Derivation	Xenograft tumor
PC3	No	33 h	Bone (vertebrae)	Yes
LNCaP	Yes	60 h	Lymph node (liquid biopsy)	Yes
DU-145	No	34 h	Brain (epithelial)	Yes

Table adapted from Sobel & Sadar, (2005). All lines are derived from metastatic tumors.

One main difference between the two lines is the duplication time (**Table 7**). Cell division rates vary during and between assays, depending on the cell lines. Some slow down while density increases and the opposite happens in others. The unpredictable variation can change the IC50 by 100-fold or more, obscuring the effects of the drugs tested (Hafner et al., 2016).

H. patens extracts may have a cytotoxic effect in prostate cancer cells; as PC3 grows faster than LNCaP, the effect noted is less pronounced. It is important to note that neither the CT assay (an MTS tetrazolium reduction assay) nor the CV assay assesses cytotoxicity, apoptosis, or cell death. Indeed, there are methods to effectively measure cell death based on the loss of membrane integrity and the

penetration of compounds; the two most common methods being: trypan blue exclusion assay and fluorescent DNA-binding dyes (Riss et al., 2019).

LNCaP is a slow-growing line; there is barely one duplication cycle along with the duration of the assay (**Table 7**). For this reason, an actual cytostatic effect could not be perceived. Therefore, the marked reduction in cell viability during the first 24 h (**Fig. 15b**) may indicate a cytotoxic effect in LNCaP cells. As noted in both cell lines, growth rate response varies over treatment time depending on the cell line and the compound's inherent characteristics, including biodisponibility and half-life.

Some phenolic molecules have been deemed endocrine disruptors that interfere with hormone signaling (e.g., androgen and estrogen pathways) through several mechanisms, especially in prostate cancer cells. Androgen sensitivity of the cell lines is another source of variation. LNCaP cells have functional ARs and thus respond to androgen or androgen-like stimuli while PC3 cells do not. Green tea polyphenols have been found to disrupt the AR signaling; furthermore, they also interact with membrane androgen receptors (mARs), which, unlike AR, do not translocate to the nucleus to initiate a transcriptional response (Marilena Kampa et al., 2017).

Epicatechin, a flavonoid commonly found in the tea plant, is the most abundant phenolic compound in Hp2019 extract (**Table 6**). Kampa et al. (2000) reported a differential IC_{50} of epicatechin for LNCaP (0.64±0.20 pM) and PC3 (62.2±25.5 pM), highlighting for the first-time interaction of polyphenols and the AR axis in prostatic tissue. Furthermore, Lee et al. (2012) demonstrated that epicatechin suppressed agonist-dependent AR activation, reduced PSA expression, and induced apoptosis in LNCaP cells through inhibition of acetylation of AR (**Fig. 16**).

Transcription factors, such as AR, are regulated through acetylation by histone acetyltransferase (HAT). Acetylation of the AR is induced by ligand DHT and histone deacetylase inhibitors in the nucleus (Lavery & Bevan, 2011). Although it is

not completely necessary, acetylation increases the binding affinity to other cofactors, increasing the expression of specific genes involved in prostate cancer cell survival and growth (M. Fu et al., 2003).

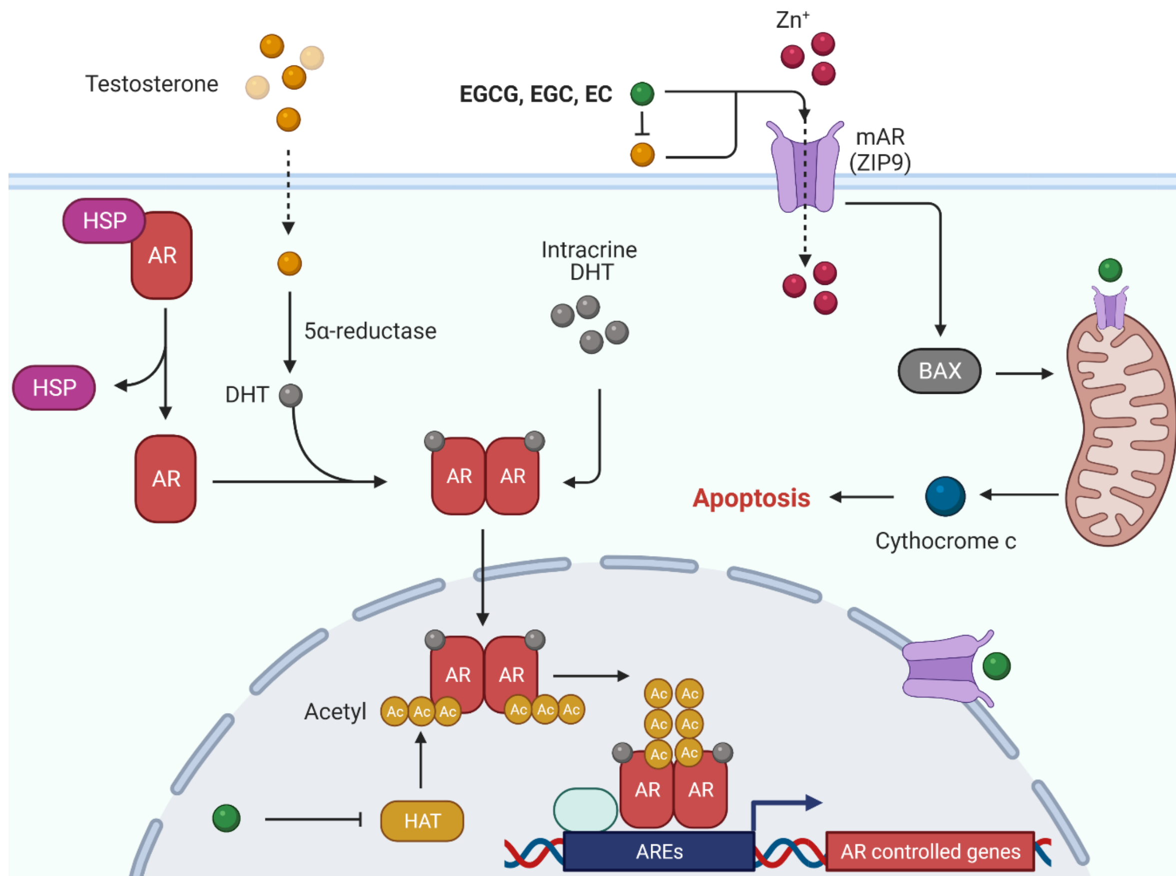


Fig. 16 | Green tea catechins mechanisms to inhibit cell growth and induce apoptosis in androgen-sensitive prostate cancer cells. Epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin (EC), bcl-2-like protein (BAX), heat shock protein (HSP), histone acetyltransferase (HAT), androgen response elements (AREs). Created with BioRender.com

ZIP9 is a recently discovered membrane androgen receptor and an SLC39A zinc transporter family member in breast and prostate cells (Thomas et al., 2018). Testosterone, but not DHT, can bind to the receptor and trigger a signaling cascade not completely known (Thomas et al., 2017). However, there is an increment in intracellular zinc, cAMP, and BAX levels; BAX2 translocates to the mitochondrial membrane forming pores and releasing cytochrome c, effectively inducing cell apoptosis through the intrinsic pathway (Westphal et al., 2011). ZIP9

(SLC39A) is also embedded in the nuclear and mitochondrial membrane (**Fig. 16**); however, it is unknown with which pathways they interact.

Polyphenols' direct interaction with mARs was first demonstrated in breast cancer cells (Nifli et al., 2005). Recently, Thomas & Dong (2021) found that epicatechin acts as an agonist in PC3, overexpressing the ZIP9 (SLC39A9) membrane AR, resulting in androgen-induced apoptosis (**Fig. 16**). In PC3-ZIP null cells, the polyphenol had no effect. Epicatechin acts as an analog of androgens, which have a biphasic behavior; in highly advanced prostate cancers, an increase in testosterone levels leads to dose-dependent growth inhibition (Song & Khera, 2014). Currently, there is no evidence of epicatechin binding to mARs in LNCaP cells; however, given that LNCaP holds functional mARs (Kampa et al., 2002), it is plausible that epicatechin induces apoptosis through this mechanism too, increasing its chemotherapeutic potential.

For the disruption mechanisms mentioned above to be noticed in cellular assays, there must be androgen stimuli present in the medium, be it testosterone or DHT; in the present work, no androgen was supplemented. This issue is overcome as cells were grown with FBS, which provides the media with testosterone levels comparable to those of castrate levels in patients; still, this concentration is not enough to promote cell growth, LNCaP can metabolize testosterone into DHT to physiological intracellular levels to stimulate cell proliferation (Sedelaar & Isaacs, 2009).

Based on the previous reasoning, Hp2019 extract, through its epicatechin content, inhibits the growth of androgen-sensitive LNCaP cells compared to non-sensitive PC3 cells. Moreover, since the phytochemical composition of Hp2021 is unknown, it may lack compounds that disrupt the AR-axis, such as epicatechin.

A non-phenolic compound with many biological functions and chemotherapeutic properties is ascorbic acid, the most abundant compound in the Hp2019 extract. Administration of vitamin c to LNCaP and DU-145 resulted in a dose-dependent decrease in cell viability (Menon et al., 1997); 1 mM of ascorbic acid was enough to reduce viability to 12% (LNCaP) and 50% (DU-145). Moreover, vitamin C decreases cell growth and the expression of AR-induced genes in the androgen-responsive LAPC-4 cells (M. H. Wang et al., 2003). Nevertheless, the levels of ascorbic acid in the extract employed are insufficient, compared to the ones reported before, to induce a response in cellular proliferation.

Chlorogenic acid was the second most abundant polyphenol in the Hp2019 extract. (Iwamoto et al., 2019) found that chlorogenic acid in a concentration range of 5-50 µM did not inhibit PC3 and LNCaP growth. Whereas higher concentrations did halt cellular growth, the IC_{50} was 95 and 135 µg/ml for PC3 and LNCaP, respectively (Reddivari et al., 2010). There is not enough evidence to suggest that chlorogenic acid may interact with the AR-dependent tumor suppression pathway; moreover, considering the phenolic acid concentration in the extract (**Table 6**), the analyzed concentrations are low compared to the reported IC_{50} to have significant biological activity.

Additionally, the estimations of cell viability were different depending on the chosen assay (CT and CV). In both lines, the CV assay identified the highest reduction of cell proliferation (**Fig. 13a** & **Fig. 13b**). CellTiter 96® Aqueous One assay evaluates the mitochondrial metabolic activity as an indirect measure of viable cells (P. Wang et al., 2010); damaged or apoptotic cells may have viable mitochondria overestimating cell viability. In the CT assay, mitochondria reduce the MTS reagent into a chromogenic substance (formazan dye). Some phytochemicals can disrupt enzyme activity (Devika & Stanely Mainzen Prince, 2008) or directly interact with MTS, tampering with the results. It is known that certain phenolic compounds, such as catechins, increased the reduction of MTS and the viability measure, leading to erroneous results (Akter et al., 2019; P. Wang et al., 2010).

Considering the high content of phenolic compounds in the *H. patens* extracts, the CV assay was employed to verify the results. Crystal violet binds to DNA and proteins of adherent cells in the monolayer, and it is also an indirect measure since death cells are detached (Kueng et al., 1989); given that culture, media is removed before the staining, there is no interference with phenolic compounds and results represent better the inhibitory effect of the extracts.

The variation (showed as the SEM) in results was high and could be due to biological factors. Variations in the division rate of cells during an assay due to natural differences, growth conditions (cell density, media composition), genetic changes directly affect drug sensitivity (Hafner et al., 2016). Growth rates may be inconsistent between cell passes due to the unstable nature of cancerous cells genome (Hughes et al., 2007). Inter-assay inconsistencies are typical, even between large-scale studies, such as the Genomics of Drug Sensitivity in Cancer and Cancer Cell Line Encyclopedia (Safikhani et al., 2016); generally, reproducibility in cancer biology research is very low (Errington et al., 2014).

Finally, additional metrics for growth inhibition (GR values) should be established, as they are independent of the cellular division rates and produce more consistent results (Larsson et al., 2020), and the extract should be tested over a broader range of concentrations to find the IC_{50} (Niepel et al., 2017). The inhibitory effect must be elicited with very low concentrations so it can be translated to clinical studies with actual human disease outcomes since the dietary intake of phenolic compounds is chronic and at low doses.

4.3.2. Breast and colorectal cancer cells

H. patens extracts have a negligible effect on breast cancer MFC7 cells (**Fig. 17a**). Hp2019 only reduced cell proliferation to 86.0±2.1% (2.44 µg/ml for 72 h) and Hp2021 to 92.3±5.4% (2.44 µg/ml for 48 h) assessed with CT method. While in the CV assay, 0.154 µg/ml for 24 h of Hp2019 inhibited growth down to 86.5±4.0% and 87.4±4.9% with 0.610 µg/ml for 48h of Hp2021 (**Fig. 17b**).

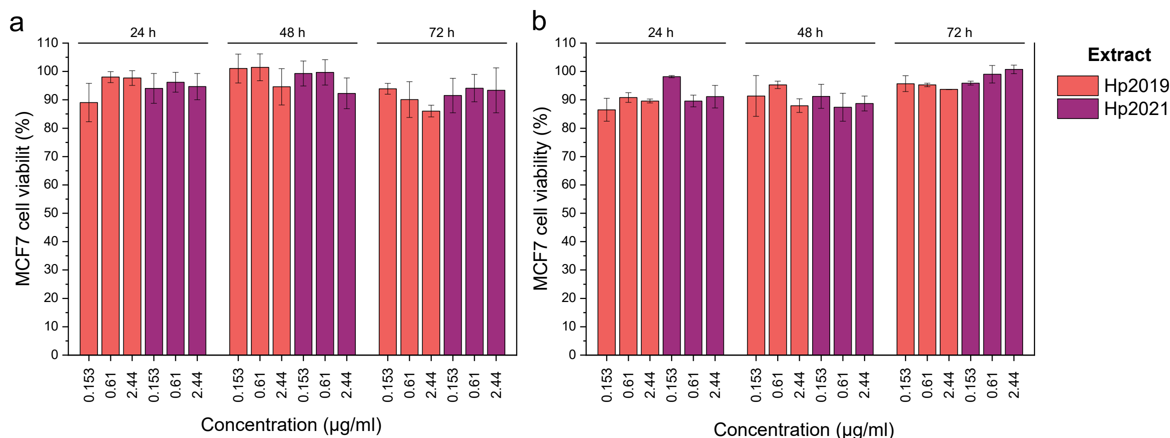


Fig. 17 | Effect of *H. patens* extracts on % viability of MCF7 cells compared to non-treated cells, quantified with (a) CellTiter 96® Aqueous and (b) crystal violet. Values were expressed as mean % cell viability ± SEM. Notice there are no statistically significant differences between groups (Tukey's post hoc test, $p < 0.05$).

The Pareto chart of the standardized effects (**Fig. 18**) shows that none of the studied variables or their combinations influence the MCF7 cell viability in a statistically significant way (α=0.01, α=0.05). Although not significant at the levels mentioned above, the primary variable would be the assay type (α=0.15).

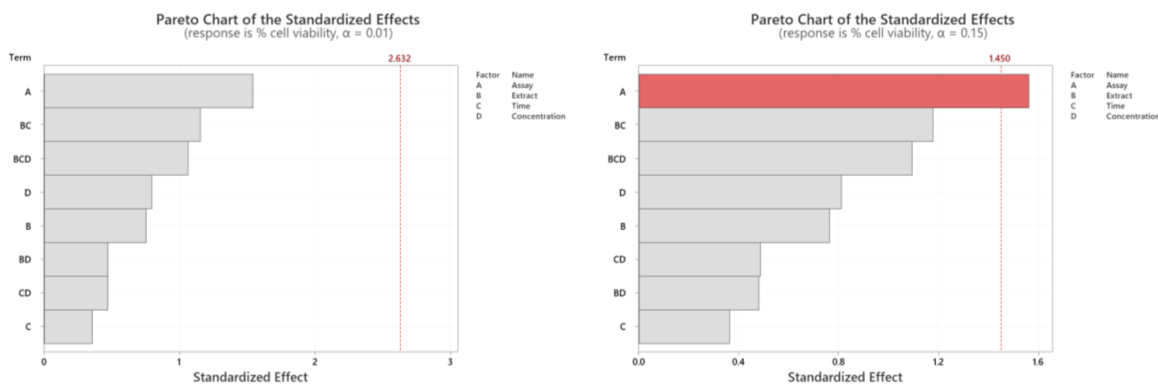


Fig 18. Pareto chart of the standardized effects on the viability % of Caco-2 cells (%) with α=0.01 and α=0.15. Notice that the conditions tested do not affect cell viability significantly (α=0.01)

Methanolic extracts of *H. patens* leaves and flowers had an inhibitory effect in MCF7 cells with an IC_{50} of 25.5 µg/ml and 23.8 µg/ml, respectively (Maamoun et al., 2019), while the extract from seeds had an IC_{50} of 63 µg/ml (Taylor et al., 2013). It is implicit that antiproliferation activity will be influenced by the part of the plant used for the extraction. Current research has not identified the precise phytochemical profile in *H. patens* extracts from different organs; however, it is known that the total content of soluble sugars, total phenolics, lipids, proteins, and chemotherapeutic between extracts varies (Khandelwal et al., 2011; Mena-Rejon et al., 2009).

Rutin, a glycoside flavonoid, has sensitizer and antiproliferative properties against MFC7 cells (Iriti et al., 2017; Saleh et al., 2019). Rutin was the main compound identified in the methanolic leaves extracts (Maamoun et al., 2019). Nevertheless, the compound was not detected in Hp2019, which may explain the small antiproliferative effect of this extract.

Chlorogenic have antineoplastic properties against breast cancer. (Bender & Atalay, 2018) tested the compound against five different breast carcinoma cell lines with different characteristics, including estrogen androgen-receptor positive MCF7. Chlorogenic acid displayed a dose-dependent inhibitory action against the five lines, and the IC_{50} in MCF7 cells was 953±32.5 µM. On the contrary, Hsu et al. (2021) reported an IC_{50} of 350 µM.

There is contradictory evidence about the inhibitory effect of epicatechin; (Kuban-Jankowska et al. (2020) reported that 125 µM of the flavonoid reduced the MCF-7 viability to 41%, while Phung et al. (2020) stated the same concentration did not affect cell viability. The seeding densities employed had a 10-fold difference; however, the plate density affects weakly the division rate and the drug sensitivity (Hafner et al., 2016). Additionally, Kuban-Jankowska et al. (2020) did not leave the cells to attach prior to the treatment while the others did, which may obscure the results.

According to Pereyra-Vergara et al. (2020), epicatechin showed an IC_{50} of 350 μM and induced apoptosis through the mitochondrial pathway. Moreover, the compound may act similarly as mentioned above with prostate cancer cells; epicatechin binds to mARs of breast adenocarcinoma MDA-MB-468 cells and induces apoptosis (Kuban-Jankowska et al., 2020); MFC7 also bears these receptors.

Concerning colorectal adenocarcinoma cells, only one incubation time (24 h) was proved in Caco-2 cells. *H. patens'* extracts also have a negligible effect on cell viability. Hp2019 inhibited cell growth down to 86.7±4.7% (0.153 μg/ml) and 92.8±2.0% (2.44 μg/ml) during the CT and CV assays respectively (**Fig. 19a** & **Fig. 19b**). Also, using the CT and CV method, 0.153 μg/ml of Hp2021 reduced cell viability to 88.9±3.6% and 87.7±1.3%. There were no significant differences between treatments ($p<0.05$).

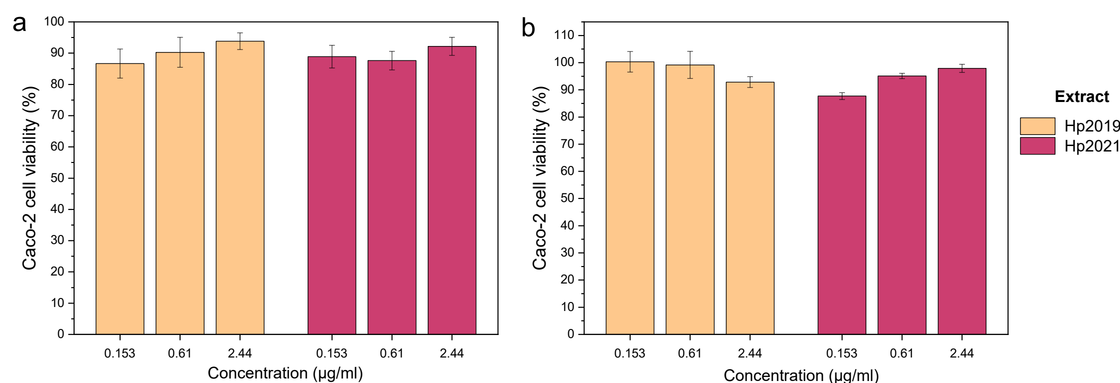


Fig. 19 | Effect of *H. patens* extracts on % viability of Caco-2 cells compared to non-treated cells, quantified with (a) CellTiter 96® Aqueous and (b) crystal violet. Values were expressed as mean % cell viability ± SEM Notice there are no statistically significant differences between treatment combinations (Tukey's post hoc test, $p < 0.05$).

The extract did not significantly affect cell viability, let alone the concentration or its combination with the extract (**Fig. 20a**). The assay type was the only variable that impacted the viability (**Fig. 20b**); the CV method identified the greatest reduction in cell viability (mean value %).

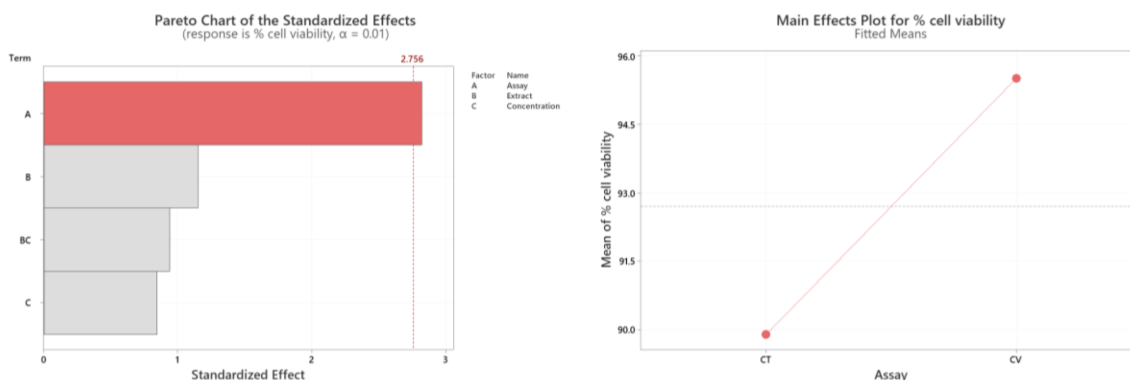


Fig. 20 | (a) Pareto chart of the standardized effects on the viability % of Caco-2 cells (%). The factors shown correspond to the adjusted model, p<0.01. (b) Main effects plot indicating how the assay affects cell viability of Caco-2 cells (%)

There are no reports about *H. patens* extracts cytotoxicity against Caco-2 cells; however, seed extracts displayed a GI_{50} of 34 µg/ml on HT-29 cells (Taylor et al., 2013). Both cell lines are derived from a colorectal adenocarcinoma; however, they display different markers and metabolic hallmarks. HT-29 grows as undifferentiated and unpolarized cells without characteristics of intestinal epithelial cells. They have high rates of glucose uptake and lactic acid production; meanwhile, Caco-2 have low rates, grow as enterocyte-like differentiated cells, and are cable of transepithelial transport like intestinal cells (Rousset, 1986).

Thus, comparisons between both lines are not equivalent, as illustrated by Gorlach et al. (2011). They demonstrate that a polyphenolic extract from the seeds of evening primrose inhibited the growth of Caco-2 and HT-29 cells in a dose-dependent manner; the IC_{50} of both cells was in a comparable range of 75-100 µM GAE and 125-150 µM GAE, respectively. Caco-2 cells were more sensitive, as the increase in membrane permeability and membrane asymmetry (characteristics of apoptosis) was more pronounced than HT-29.

Regarding the phenolic compounds in Hp2019 extract, chlorogenic acid inhibited Caco-2 proliferation, but the IC_{50} in the literature varies. Volstatova et al., (2019) reported an IC_{50} of 337.4 µM. Ekbatan et al. (2018) determined the concentration as 758 µM; additionally found that chlorogenic acid stopped cells at S-phase and

increased the expression of the inactive and the cleaved form caspase 3, involved in the completion of apoptosis.

Small concentrations of epicatechin (50 μM) did not affect Caco-2 viability (Ramos et al., 2011). Still, higher concentrations have a dose-dependent effect on proliferation; 500 μM was enough to inhibit cell growth down to 60% (Salucci et al., 2002). Curiously, epicatechin was readily uptake, and the intracellular content increased steadily over 72 h in Caco-2 cells; nonetheless, no apoptosis or disruption in the cell cycle was detected.

The concentration assessed with Hp2019 and Hp2021 extracts was far lower than those reported above for breast and colorectal cancer cell lines (at least a 10-fold difference). Notably, the levels of the phenolic compounds (chlorogenic acid and epicatechin) in the extracts did not reach the IC_{50} of the isolated compounds; as a result, the extract lacks a significant antiproliferative effect in MCF7 and Caco-2 cells, per the results found (**Fig. 17** & **Fig. 20**)

4.4 Photoprotective effect

None of the *H. patens* extracts were cytotoxic to NIH/3T3 cells after 24 h of incubation in a concentration range of 0.153 µg/ml to 2.44 µg/ml; there were no significant differences in cell viability between different concentrations, all were inside the 100.6±2.2% and 106.1±1.8% interval (**Fig. 21a** & **Fig. 21b**). *H. patens* extracts were selective against prostate cancer cell lines (PC3 and LNCaP), while they do not damage the healthy mouse fibroblast cell line NIH/3T3. However, further studies with non-cancerous human cell lines are required to evaluate this deduction properly.

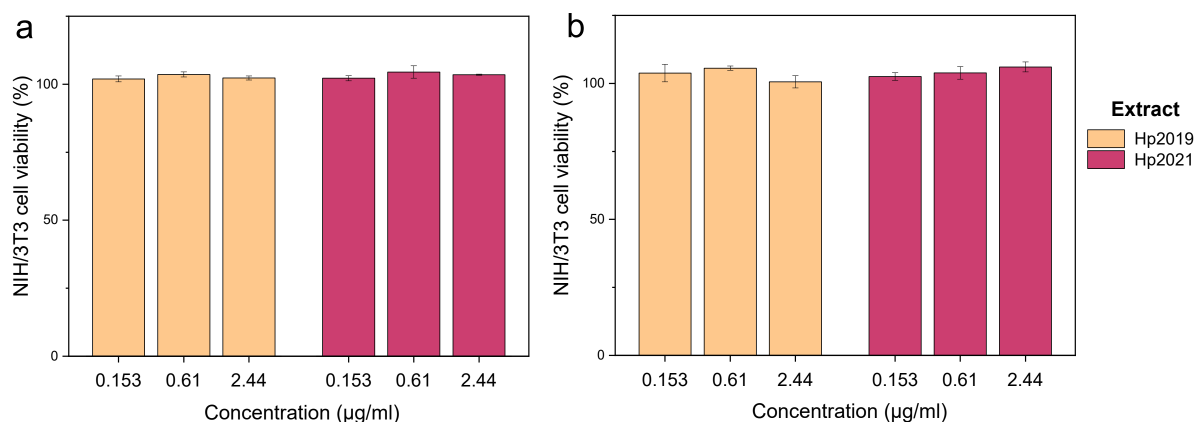


Fig. 21 | Effect of *H. patens* extracts on cell viability of NIH/3T3 (%) compared to non-treated cells (without extract) after 24 h of incubation, quantified with (a) CellTiter 96® Aqueous and (b) crystal violet. Values were expressed as mean % cell viability ± SEM. Notice there are no statistically significant differences between treatment groups (Tukey's post hoc test, $p < 0.05$).

Furthermore, *H. patens* extracts on UVC-induced damage were analyzed. **Fig. 22a** and **Fig.22b** show that treatment with *H. patens* extracts for 24 h before UVC exposure prevented UVC-induced cytotoxicity in NIH/3T3. Cell survival (%) was calculated using cells exposed to UVC without prior treatment. Compared with non-treated cells, cell survival of treated cells ranges in the 105.9±11.3%-126.9±10.9% interval. There were no statistical differences in cell survival (%) between different concentrations and extracts ($p<0.05$).

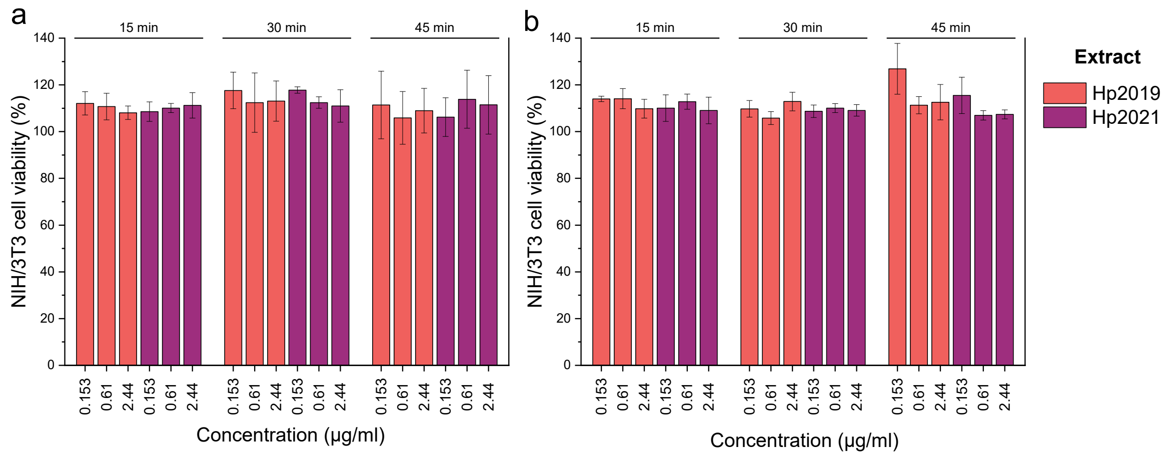


Fig. 22 | Effect of *H. patens* extracts NIH/3T3 cell survival (%) compared to non-treated cells after induced UV-damage, quantified with (a) CellTiter 96® Aqueous and (b) crystal violet. Values were expressed as mean % cell viability ± SEM. Notice there are no statistically significant differences between treatment combinations (Tukey's post hoc test, $p < 0.05$).

A general factorial regression was performed to analyze the assay type, extract, UVC-exposure time, and concentration standardized effects on cell survival (%) with an $\alpha=0.01$. As noted in **Fig. 23a**, UVC-exposure time was the only significant factor influencing cell survival during the tested conditions.

Interestingly, cell survival after 15, 30, and 45 minutes of UVC-induced damage (**Fig. 23b**) do not diverge significantly ($p<0.01$), the level of protection was equal after 45 minutes of UVC radiation. However, the viability between UVC-exposure and non-exposed cells indeed differs. Overall, prior incubations of cells with *H. patens* extracts prevent damage induced by UVC radiation, at least during 45 minutes of exposure. Further studies are needed to understand *H. patens* extracts mechanisms of photoprotection.

Additionally, evaluating cell survival with two different methods yield the same results. It was expected since there was no interference of polyphenolic compounds with the CT assay due to the media with extracts being aspirated before UVC- induced damage and end-point measurements; moreover, cells were washed with PBS to remove any remains of FBS and polyphenols to avoid ambiguous results.

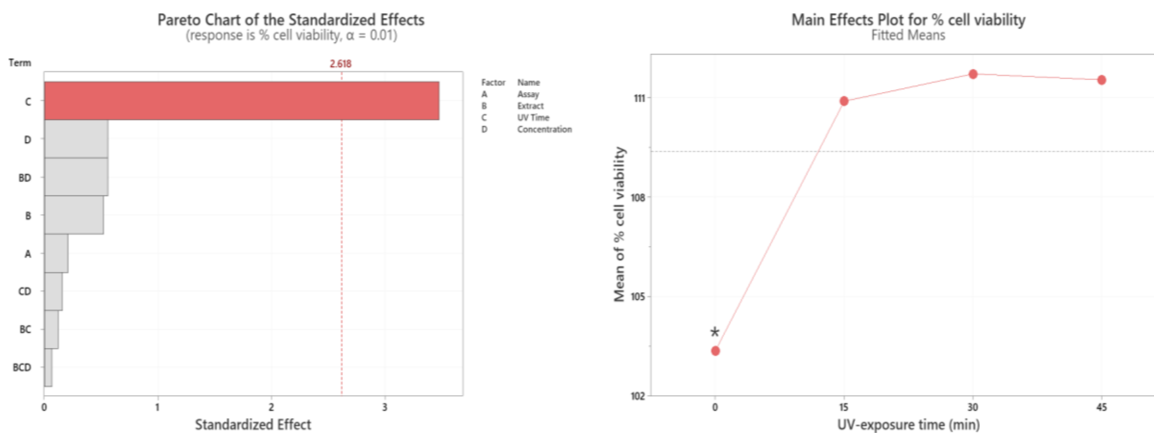


Fig. 23 | (a) Pareto chart of the standardized effects on the viability of NIH/3T3 cells (%). The factors shown correspond to the adjusted model, α=0.01. (b) Main effects plot indicating how UV-exposure times affects cell viability (%).

Currently, there are no reports of *H. patens'* photoprotective potential activities; nevertheless, there is growing evidence that plant extracts rich in polyphenols have great antioxidant power and a photoprotective effect in skin cells (dermal fibroblast and keratinocytes) against UV-induced damage across their spectra. As mentioned above, the extent of the protective effect is subject to the plant extract, UV-radiation type, and it is dose-dependent.

The molecular mechanisms by which they prevent cellular damage are not completely understood. However, polyphenolic extracts can reduce UV-induced DNA damage (Giampieri et al., 2012), protein oxidation, decrease the expression of matrix-degrading metalloproteinases (Vayalil et al., 2004), cyclooxygenase-2 (Cha et al., 2014), caspase 3, and reduce intracellular ROS content in (Pacheco-Palencia et al., 2008) in several skin cell lines both human and mouse-derived.

As noted in **Fig. 22**, there was no significant difference in cell survival between Hp2019 and Hp2021, despite using the same concentrations of each extract and having considerable differences in their TPC and total antioxidant power. It deserves further investigation as the photoprotective effect typically has a positive dose-dependent relationship (Pacheco-Palencia et al., 2008).

The antioxidant power also increases with the extract concentration (Harish Nayaka et al., 2010); thus, there is a positive relationship between photoprotective and antioxidant power. Additionally, plants synthesize more polyphenols in response to an increase in UV-radiation, increasing their antioxidant capacity to respond to damage (Ruhland et al., 2007; Surjadinata et al., 2017); consequently, a greater content of TPC and antioxidant capacity should also increase the photoprotective effect which is not observed in **Fig. 22**.

The photoprotective effect of *H. patens* may ought to the antioxidant capacity of the present compounds, such as phenolic acids and vitamin c. Pretreatment with 100 µM of ascorbic acid increased the viability of human fibroblasts after UVB exposure, although the difference was not significant (Gęgotek et al., 2020). Nevertheless, there was a significant reduction of UV-induced protein abduction and crosslinking formation compared to non-treated cells.

On the other hand, a concentration of 20 µM of chlorogenic acid suppressed UV-induced DNA damage and apoptosis of keratinocytes, probably through its intracellular ROS-scavenging activity (Cha et al., 2014). Epicatechin is also a photoprotective agent (Basu-Modak et al., 2003), doses of 2 µg/ml can reduce cellular damage and intracellular concentration of ROS to basal levels on dermal fibroblast after UV exposure (dos Anjos Oliveira Ferreira et al., 2020).

The results of the photoprotective effect of *H. patens* extracts are favorable, and it is suggested to conduct additional analyzes to address the molecular mechanisms involved. It would be of great interest to verify the photoprotective effect by assessing DNA damage, apoptosis induction, intracellular ROS levels, expression of metalloproteinases, pro-apoptotic proteins, and inflammatory factors (TNF-α, IL-6, IL-10, COX-2), among other markers.

5. Conclusions and future perspectives

The *H. patens* leaves extract from 2019 (Hp2019) showed the highest content of phenolic compounds and antioxidant activity compared to the one from 2021 (Hp2021). Both extracts have a superior antioxidant capacity than most foods, including vegetables, fruits, spices, and a myriad of medicinal plants. According to their biological activities, the most important phenolic compounds found in Hp2019 were epicatechin and chlorogenic acid.

Hp2019 showed a marked decrease in the proliferation of prostate cancer cells compared to Hp2021, despite the latter having a greater TPC and antioxidant activity. Both extracts have negligible effects on the viability of breast and colorectal adenocarcinoma cell lines. The inhibitory effect of Hp2019 may be mediated by the specific phenolic compounds found in the extract (e.g., epicatechin) and not by the overall concentration of phenolic molecules. Thus, identification of the compounds present in HP2021 is imperative to corroborate this hypothesis. Furthermore, the antiproliferative effect was more pronounced in the androgen-dependent line LNCaP cell line than in PC3 (androgen-independent), suggesting an androgen-disrupting mechanism.

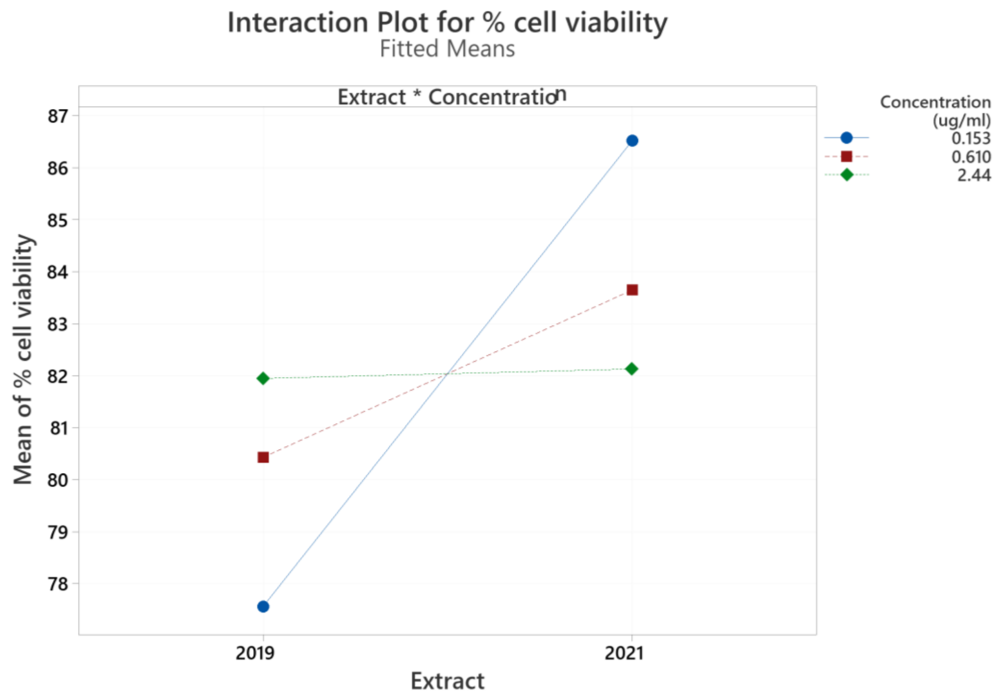
Further research should be carried with the Hp2019 extract, and the author strongly suggests the following steps. (1) Evaluate a broader range of concentrations to identify a dose-dependent effect (fitted to a hill-sloped curve) and the IC_{50}. (2) Confirm if there is an androgen-disruptive inhibitory mechanism using androgen-independent cell lines (PC3) transfected with functional AR or mARs and evaluate the proteomic and transcriptomic profile of both the transfected line and LNCaP upon stimuli with the extract. (3) The extract should be fractionated, and the potency of these fractions evaluated, for further selection, purification, and isolation of relevant biologically active compounds.

Lastly, both extracts showed photoprotective effects against UV-induced damage in a skin cell model (murine fibroblast); there was no difference between extracts.

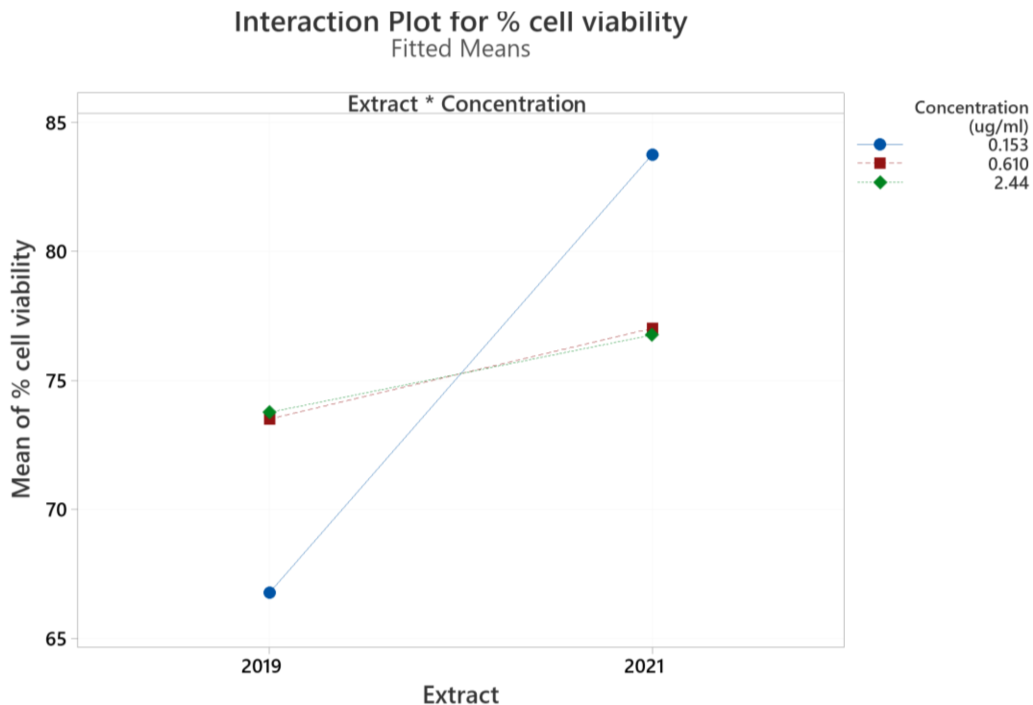
The extent of the photoprotection should be endorsed with other relevant biological data such as apoptosis and inflammation markers and total intracellular ROS and endogenous antioxidants content. Furthermore, the extracts should be tested with other carrier compounds (formulations) in human skin cell models to develop a novel photoprotective product, such as a sunscreen.

In summary, the present work demonstrated the feasibility of *H. patens* extracts as chemotherapeutic agents in prostate cancer cell lines, mediated through phenolic compounds, and a photoprotective agent against UV-induced damage in murine fibroblasts. New insights on the molecular mechanisms of the properties mentioned above and novel bioactive compounds can be obtained by extending this research.

Appendix A: supplementary figures



Supplementary Fig. 1 | Interaction plot indicating how the combination of extract and concentration affects cell viability of PC3 cells (%). The factors shown correspond to the adjusted model, p<0.01.



Supplementary Fig. 2 | Interaction plot indicating how the combination of extract and concentration affects cell viability of LNCaP cells (%). The factors shown correspond to the adjusted model, p<0.01.

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Congress certificate

El Tecnológico de Monterrey

otorga el presente reconocimiento a

Erick Huerta Rodríguez

por su participación con el trabajo "Cytotoxic Effect of Alcoholic and Aqueous Extracts of Terminalia catappa and Hamelia patens on Different Cancer Cell Lines", en los proyectos de investigación de profesional, en el **50 Congreso de Investigación y Desarrollo**

Dr. Neil Hernández Gress
Presidente del Comité Organizador del
50 Congreso de Investigación y Desarrollo

Monterrey, Nuevo León, febrero de 2020