in silico Computational Studies of phenolic compounds from Pinaropappus roseus Less leaves extract against BCL-2 and BCL-XL proteins Associated with Cancer Cell Survival and Resistance

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Short Report

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Abstract

The aim of this study was to investigate the phenolic compound from *Pinaropappus roseus*, and its human breast anticancer properties. The phenolic molecules were isolated from the aqua-ethanoic extract isolated from the leaves of *P. roseus* by solid-phase extraction (SPE). The total phenolic content was determined by the Folin-Ciocalteu technique. The profile of phenolic compounds was analyzed by mass spectrometry (LC-ESI-MS^2). The quantification of phenolic molecules identified by mass spectrometry was carried out by UV-spectrophotometry (LC-PDA). The *in vitro* cytotoxicity assay was carried on MCF-7 and HMEC cell lines using the MTT assay method. The docking simulation was carried out in anti-apoptotic proteins, Bcl-2 and Bcl-xL. Six phenolic compounds were identified of which the apigenin (37.5 $mg_{CAE}g_{db}^{-1}$) was the most abundant compound. In the *in vitro* anti-cancer assay, the IC50 for the MCF-7 cells was of 426.15 µg g_{db}^{-1} at 24h and 297.40 µg g_{db}^{-1} at 48 h for the maximum evaluated phenolic extract concentration. The rutin and the chlorogenic acid showed the higher binding energies in the docking simulation for the active sites of the Bcl-2 and Bcl-xL proteins respectively. The phenolic compounds of *P. roseus* have cytotoxic activity against human breast cancer (MCF-7 cell line) and a low cytotoxic activity against normal human epithelial cells (HMEC cell line).These results suggest that the phenolic extract of *P. roseus* may have therapeutic potential against human cancer pathologies.

1 Introduction

There is an increasing in the use of the ethnopharmacological knowledge (the use of various tissues of plants, algae or fungi for medical proposes) for the treatment/prevention of human diseases, and especially for the chronic degenerative pathologies. The secondary metabolites are the biologically actives molecules of these *traditional drug*s, and they can be used for the development of new drugs (chemotherapeutic molecules).

One of the most studied secondary molecules are the phenolic compounds (phenolic acids, flavonoid family, xanthones, stilbenes and ligands). Several investigations have reported that the phenolic compounds exhibit different biological activities in human health, as anti-allergenic, anti-bacterial, anti-viral, anti-inflammatory, vasodilator and/or anti-carcinogenic activity [1]. The flavonoid, in particular, have been described as a family of molecules with a high anti-carcinogenic activity [2, 3].

The global incidence of breast cancer in 2020 was of 2.3 million diagnosed women and 685 000 deaths worldwide [4]. For the male incidence, the breast cancer represent close to the 1% of the diagnosed cases, and the incidence in men is experiencing an increase of up to 15% of diagnosed cases in some oblations [5] .

Breast cancer refers to the uncontrolled growth and proliferation of cells in the breast tissue beyond the ability of intrinsic cell control mechanisms (cell arrest and apoptosis) and from the immune system itself to suppress them.

The breast cancer has been correlated to several factors (*e. g.* female gender primordially, age, the age at which a woman bears her first full-term child, the age at which menopause occurs, diet, viruses, adrenal steroids, the presence of carcinogens, aberrant DNA mutations and the prolonged exposure to a variety of hormones such as the prolactin, the human growth hormone, the progesterone, the estrogen and the exogenous estrogens in oral contraceptive drugs) [6, 7, 8].

The treatment of breast cancer depends on the hormone response subtype (estrogen positive, progesterone positive and hormone response negative) and how much it has spread outside of the breast tissues to the lymph nodes (stage II or III) or to other parts of the body (stage IV) [9]. The medical treatment combines the surgery, the radiation and the use of multiple drugs. The breast cancer pharmacological treatment approach includes hormonal therapies, chemotherapy and/or targeted biological pharmacotherapy [10].

It has been proposed that the phenolic molecules, covered for carriers (e.g. NO_3Ag) to increase its solubility, membrane permeability, and avoid their microbial and (or) phase II metabolism); can act as chemotherapeutic molecules to prevent the breast cancer carcinomas (breast cancer phase 0) and as coadjutant of the traditional breast cancer pharmacological therapies. Their anti-cancer proprieties are generally attributed to their anti-oxidant activity, acting as and exogenous antioxidant preventing the apparition of aberrant mutations due the oxidation damages of DNA and RNA and stimulating the immune system [11].

Several investigations have reported that the phenolic compounds are able to modulate various biomarkers correlated with the cancer such as the transcriptional factors asociated with the cell proliferation inhibition (*e.g.* by the regulation of the extra-cellular

signal-regulated kinases), the angiogenic factors (*e.g.* the modulation of the vascular endothelial growth factor); and the apoptosis of cancer cell (*e.g.* by the inactivating the anti-apoptotic protein bcl-2, Bcl-xL) [9].

The *Pinaropappus roseus* is listed in the atlas of the plants of traditional Mexican medicine as a specie used in the treatment of chronic inflammatory pathologies (atopic dermatitis); using the stems and leaves as a cataplasm and/or as aqueous infusions [12]. Although there is no documented information about the use of *P. roseus* as an alternative/complementary therapy for any type of cancer, although in some rural communities in Mexico it is used in infusions as a complementary therapy for various types of cancer

The aim of this study was to identify the phenolic compound profile of the aqua-ethanolic extract of *Pinaropappus roseus* leaves and evaluate its *in vivo* and *in silico* anti breast cancer activity.

2 Materials and methods

2.1 Reagents and equipment

All the solvents used for the leaves cleaning, the phenolic extraction, MCF-7/HMEC cell culture, cytotoxic assays and liquid chromatography and mass spectrometry were purchased from Sigma Aldrich Mexico and JT Baker Mexico.

The Oasis HLB SPE cartridges were purchased from Waters Mexico.

The MCF-7 (human mammary carcinoma) and HMEC (human mammary epithelial) cell lines were obtained from the Facultad de Medicina de la Universidad Autónoma Benito Juárez de Oaxaca, Oaxaca, Mexico.

Spectrophotometric evaluations were performed in a Thermo Fisher UV-Vis spectrophotometer. The cell evaluation (UV spectrophometer) was carried out in a Biorad microplate.

The mass spectrometric analyses were carried out in a Bruker (LC-MS) mass spectrometer using an Agilent Zorbax Eclipse Plus C18 column (1.8 µm, 150 mm × 4.6 mm).

2.2 Plant material

Pinaropappus roseus was collected at Oaxaca, Mexico (16.921210, -96.736804; 1520 ASL) and it was identified/preserved in the herbarium of the Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, unidad Oaxaca - Instituto Politécnico Nacional (OAX-FLO-129-0402).

2.3 Plant extract procedure

The rhizome of *Pinaropappus roseus* was removed. The leaves were cleaned by washing with a 1% (v/v) of sodium hypochlorite - distilled water solution.

The leaves were ground and then dried to constant weight (RH < 10%). The material was stored at 25°C.

Five grams of the material was macerated with 25 mL of methanol:water (80:20; v:v) solution. The mixture was sonicated (40 kHz and 160 W) at 20°C in continuous mode for 15 min, followed by a centrifugation process (3000 rpm for 20 min at 20°C) to remove any remaining suspended solids.

The liquid phase, raw extract, was dried under nitrogen steam and stored in dark place at 25°C.

2.4 Purification of phenols

The SPE cartridge was preconditioned by elution of 3 mL of methanol, 3 mL of acidified distilled water, and 3 mL of neutral distilled water. The raw extract (5 mL) was loaded into the pre-conditioned cartridge and eluted at a drop-wise flow rate to ensure efficient adsorption of phenolic compounds. The SPE cartridge was washed with 5 mL of acidified distilled water, followed by 5 mL of neutral distilled water to remove any residual compounds.

The elution of C_{18} cartridge-bound phenolic compounds was performed by adding 5 mL of methanol drop-wise.

The collected eluent, phenolic fraction, was stored in dark place at 25°C.

2.5 Evaluation of total phenolic content

The total phenolic content of the phenolic fraction was determined by the Folin-Ciocalteu reagent technique. A 0.5 mL of the phenolic fraction was mixed with 0.4 mL of Folin-Ciocalteau reagent (2N) and incubated for 30 minutes. 4 mL of Na_2CO_3 (7%, p/v) was added. The reaction mixture was diluted to the volume (10 mL) with distilled water.

The reaction mixture was incubated for 2 hours and the absorbance was measured at 765 nm.

The phenolic concentration was expressed as milligrams of gallic acid equivalents per gram in dry basis ($mg_{GAE}\ g_{db}^{-1}$) of the extract.

2.5 Characterization of compounds by liquid chromatography coupled to a PDA detector - quadrupole time-of-flight mass spectrometer.

1 mL of the phenolic fraction was dried under nitrogen steam. Then, the dried phenolic fraction was re-suspended in 1 mL of water-formic acid (0.1%, v/v) and acetonitrile (85:15, v/v) and passed through 0.25 µm PDVF filter before entering to the chromatograph.

The chromatography was carried out using a gradient methodology as follows: 0–5 min of 15–25% B; 5–10 min of 25–35% B; 10–28 min of 35–60% B; 28–28.01 min of 60–15% B; and 15% B for 35 min and a flow rate of 0.5 mL min^{-1}. The separation was carried out using a Perkin Elmer Brown Lee C_{18} column (1.8 µm, 150 mm × 4.6 mm) for UV spectrophotometric evaluation (PDA detector) and an Ascentis HPLC Express C_{18} column (2.7 µm, 150 mm × 4.6 mm) for spectrometric evaluation (mass spectrometer)

The electro spray ionization (ESI) interface was used in negative mode, considering a mass range of 50 to 3000 m/z. The mass spectrometry conditions were set as follows: sheath gas temperature at 250°C with a flow rate of 11 L min^{-1}, nitrogen gas temperature at 300°C with a flow rate of 5 L min^{-1}, and a nebulizer gas pressure of 310.2 kPa. The capillary and nozzle voltages were set at 3.5 kV and 500 V, respectively. 1 µL of the sterol fraction was introduced to the mass spectrometer. The identification was carried out comparing the pattern of the mass fragmentation spectra and the exact mass of the molecular ion with the MassBank database [13], and the NIST database [14]. Diagnostic ions were used to confirm the compound identification.

Each compound identified by mass spectrometry was quantified by evaluation of the PDA-UV areas. A regression model (area vs concentration) was constructed using caffeic acid as a standard.

The concentration of the phenolic compounds was expressed as milligrams of caffeic acid equivalents per gram on dry basis ($mg_{CAE}\ g_{db}^{-1}$)

2.6 Cell culture conditions

Breast cancer cells (MCF-7) were cultured in Roswell Park (ROMI 1640) supplemented with glutamine (2 mM), sodium pyruvate (1 mM), no essential amino acids (0.1 mM), sodium bicarbonate (1.5 g L^{-1}), 10% (v/v) fetal calf serum, 50 µg mL^{-1} gentamycin and 50 µg mL^{-1} amphotericin-B.

Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere, and maintained in a logarithmic growth phase. The cell growth (by Neubauer chamber), division, and morphology (by inverse microscopy) of the cells were monitored periodically.

Human mammary epithelial growth medium (HMEC) were cultured in serum-free mammary epithelium growth medium (MEGM) containing sodium pyruvate (0.11g L^{-1}), L-glutamine (1.461 g L^{-1}), dextrose (1.0g L^{-1}), 10% (v/v) fetal calf serum, 50 µg mL^{-1} gentamycin and 50 µg mL^{-1} amphotericin-B.

Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere, and maintained in a logarithmic growth phase. The cell growth (by Neubauer chamber), division, and morphology (by inverse microscopy) of the cells were monitored periodically.

2.7 *in vitro* cytotoxic activity assay

The cancer cells viability were carried out by the MTT technique according to the modified method of Razak et al [15]

The MCF-7 and HMRC cells, at a concentration of $5x10^6$ cells mL^{-1}, were maintained in 0.2 mL of HepG2's growth medium in a humidified atmosphere at 37°C for 24 h.

Cells were treated with increasing doses of the sterol fraction, 0 -500 µg mL^{-1}; and monitored at 24 and 48 h using a microplate reader at 570 nm. A 0.75% DMSO solution was used as the negative control.

The cytotoxic activity was expressed as the half maximal inhibitory concentration (IC50).

2.8 *in silico* simulation. Docking evaluation

To evaluate the interactions of the sterol profile, the crystallized structures of the Bcl-2 (PDB id 4IEH) and the Bcl-xL (PDB id 3ZK6) were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank [16].

The PubChem database [17] was used to obtain the phenolic compound (ligand), the $C_{42}H_{44}ClN_7O_6S_3$ (Blc-2 inhibitor) and the $C_{31}H_{30}N_4O_4S_2$ (Blc-xL inhibitor) structures.

The pretreatment of the evaluated ligands (adjust physical structure) was carried on using the Avogadro software [18].

The pretreatment of the proteins (dimer structure, bounded ions and water molecules removal) was carried on using the USCF Chimera ver. 1.17.3 software [19].

AutoDockTools ver. 1.5.7 software [20] was used for the *in silico* evaluation.

The docking results from the *in silico* evaluation were reported as the maximum negative scoring function (Gibb´s energy).

2.9 *in silico* simulation. Simulation of pharmacokinetic properties

The SmissADME software [21] was used to evaluate the drug properties of the phenolic compound profile.

3 Results

3.1 Phenolic compound profile.

The total phenolic content was of 68 ± 0.05 $mg_{GAE}\ g_{db}^{-1}$. A total of six phenolic compounds were identified in the phenolic extract. The phenolic compound profile consisted of caffeic acid, ferulic acid, chlorogenic acid, naringenin, rutin and apigenin (Table 1). The most abundant phenolic acid was the cafeico acid (7.2 ± 0.05 $mg_{CAE}\ g_{db}^{-1}$). The most abundant flavonoid was the apigenin (37.5 ± 0.01$mg_{CAE}\ g_{db}^{-1}$)

Table 1
Phenolic compounds of *Pinaropappus roseus*

Compound	Molecular ion $[M-H]^-$	Concentration ($mg_{CAE}\ g_{db}^{-1}$)
Cafeic acid	179.035	7.2 ± 0.05
Ferulic acid	193.0506	0.12 ± 0.02
Chlorogenic acid	353.0878	0.12 ± 0.01
Naringenin	271.0612	6.0 ± 0.02
Rutin	609.1461	9.0 ± 0.05
Apigenin	268.0528	37.5 ± 0.01

3.2 Cytotoxicity assay

The phenolic fraction significantly inhibited the proliferation of MCF-7 cells in a concentration- and time-dependent manner.

For MCF-7 cells, the IC50 was 426.15 µg g_{bd}^{-1} at 24 h and the IC_{50} was 297.40 µg g_{bd}^{-1} at 48 h

For HMEC cells, the evaluated concentration reached a maximum cytotoxicity of 4.72% for 24 h and 6.72% for 48h

The light microscopic observation of the morphological changes of untreated MCF-7 cells and MCF-7 cells treated with the phenolic fraction (Fig. 1) showed that the MCF-7 cells displayed and formed of membrane-bound vesicles (apoptotic bodies), condensed cytoplasm and nucleus; all typical morphological characteristics of cell apoptosis process.

3.3 Docking evaluation.

The *in silico* evaluation of the binding energy of the phenolic molecules against the Bcl-2 and Bcl-xL proteins are listed in Table 2 and Table 3 respectively.

The native ligands, $C_{42}H_{44}ClN_7O_6S_3$ for Blc-2 and $C_{31}H_{30}N_4O_4S_2$ for Blc-xL, showed binding energies of -8.9 kcal mol^{-1}.

Among the phenolic acids evaluated, the chlorogenic acid showed the highest binding energy for the Bcl-2 and Bcl-xL proteins (-6.9 kcal mol^{-1} and – 8.1 kcal mol^{-1} respectively).

Among the flavonoids evaluated, the rutin showed the highest binding energy for the Bcl-2 protein (-7.6 kcal mol^{-1}). For the Bcl-xL protein, all the flavonoids showed the same binding energy (-7.6-kcal mol^{-1}) respectively.

Table 2
Molecular docking of ligands and Bcl-2 protein

Protein ID	Compound	Energy value (Kcal mol^{-1})	Amino acid residues interactions
	Bcl-2-IN-13 ($C_{42}H_{44}ClN_7O_6S_3$, native ligand)	**-8.9**	TYR66, TYR161, ARG66, ALA59, PHE63, VAL107, GLY104,VAL107, GLY104, ALA108, ASN102, PHE71, ARG105, ASP70, LEU96, GLU95
	Cafeic acid	**-5.9**	ALA59, PHE63, VAL107, TYR161
	Ferulic acid	**-6.2**	ASP62, ALA59, GLY104, PHE63, TYR161
Bcl-2 (4IEH)	Chlorogenic acid	**-7.1**	ARG66, TYR161, TYR67,PHE63,GLY104,ALA108,LEU96
	Naringenin	**-7.0**	ARG106,ASP62, PHE63, GLY104, ASN102, ARG105
	Rutin	**-7.6**	GLN77, ARG105, ASP70, ALA108, ARG105, LEU96, MET74, GLN77, GLU95
	Apigenin	**-7.2**	ASP62, PHE63, VAL107, GLY104, TYR161

Table 3
Molecular docking of ligands and Bcl-xL protein

Protein ID	Compound	Energy value (Kcal mol^{-1})	Amino acid residues interactions
	Bcl-2-IN-13 ($C_{42}H_{44}ClN_7O_6S_3$, native ligand)	**-8.9**	ASP133, ARG132,GLU129, TYR195, SER106, ARG139, LEU130, PHE105, LEU108, GLY138, PHE97, ALA93
	Cafeic acid	**-6.1**	LEU130, LEU106, ASP107, PHE105, PHE97, ARG102
	Ferulic acid	**-6.2**	LEU130, LEU108, SER105, THR109, PHE105, ALA142, PHE67, ARG102, SER145
Bcl-2 (4IEH)	Chlorogenic acid	**-8.1**	ARG139, GLU120, LEU108,THR109, LEU130
	Naringenin	**-7.6**	TYR195, PHE97, TYR101, GLU96, ALA93, GLU92, ASN197
	Rutin	**-7.6**	ARG139, ARN136, TRP137, GLY138, PHE97, TY195, TYR101, VAL140, GLY96, ALA93
	Apigenin	**-7.6**	ARG139, LEU130, PHE105, ALA142, PHE97, TYR101, ALA93

For the Bcl-2 protein, all phenolic compounds interacted with the active site of the native ligand $C_{42}H_{44}ClN_7O_6S_3$ (Fig. 2), on the central hydrophobic α5 protein helix – hydrophobic groove [22]. The bonding is characterized by multiple hydrophobic/electrostatic interactions (Table 2). Only one hydrogen bond was observed for the native ligand (TYR66) on the hydrophobic groove of the BCL-2 protein. The naringenina (ARG106) and the rutin (GLN77, ARG 105) showed hydrogen bonds on the hydrophobic groove of the protein.

Based on the binding interactions between the Bcl-2 protein and the evaluated ligands; a common residue, the PHE63, interacted with all the phenolic compounds except with the rutin, via electrostatic interactions. For the phenolic acids TYR (161) is a common residue to.

For the Bcl-xL protein, all phenolic compounds interacted with the active site of the native ligand $C_{31}H_{30}N_4O_4S_2$ (Fig. 3), on the central hydrophobic groove. The binding is characterized by multiple hydrophobic/electrostatic bonds (Table 3). Only one hydrogen bond was observed for the chlorogenic acid (ARG 139) on the hydrophobic groove of the protein.

There is no binding common residue for the evaluated ligand. The LEU 130 residue interacted with all the phenolic acids and the Bcl-xL protein via electrostatic interactions.

3.5. Evaluation of pharmacokinetic proprieties.

The outcome of the ADMET results, Table 4, suggests that all the phenolic acids do not show important toxicity effects. The flavonoids show no desirable effect over C9450 enzymes, especially the apigenin.

The caffeic acid shows the best drug-like properties, with the highest bio ability scores. The rutin showed the lower bio ability scores

Table 4
ADME – Pharmacokinetics properties and physiological properties

Compound	ADME parameters								Drug likeness properties	
	GI-Absorption	BBB permeability	P-gp	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Lipinski rule	Bio ability score
Caffeic acid	High	No	No	No	No	No	No	No	Yes	0.56
Ferulic acid	High	No	No	No	No	No	No	No	Yes	0.85
Chlorogenic acid	Low	No	No	No	No	No	No	No	Yes	0.11
Naringenin	High	No	Yes	Yes	No	No	No	No	Yes	0.55
Rutin	Low	No	Yes	No	No	No	No	No	No	0.17
Apigenin	High	No	No	No	No	No	Yes	Yes	Yes	0.55

4 Discussion

There is little information about the characterization of the secondary metabolites of *Pinaropappus roseus* tissues. Harput *et al* [23] reported, for the methanoic extract of *Pinaropappus roseus* leaves, several rutinoside derivates, rhamnazin 3-O-rutinoside, rhamnocitrin 3-O-rutinoside, isorhamnetin 3-O-rutinoside, quercetin 3-O-rutinoside (rutin) and kaempferol 3-O-rutinoside. Arriaga et al [24] reported some phenolic acids as major phenolic compounds for water and ethanol.

At least 35 400 plants species are currently being used for medicinal purposes worldwide [25], but only a few of these species have been studied in a laboratory using modern pharmacological and pharmacognostic methods [26].

In order to evaluate the biological activity of molecules from plant species with potential pharmacological activity, the first step is accurate documentation with respect to their traditional application. The subsequent step is attempting to isolate pharmacologically active secondary metabolites, followed by an evaluation, *in vitro*, *in silico* and *in vivo* of the molecules to evaluate the biological effect, the interactions in environment similar to the human's cells, the undesirable effects, the pharmacokinetics [27, 28].

The BCL-2 family proteins have been classified into three groups: i) Pro-survival/anti-apoptotic activity: Bcl-2, Bcl-xL, Bcl-W, MCL-1, BFL-1, BCL-B and A1 [29, 30]. ii) Proteins with pro-apoptotic activity: Bax, Bak, Bid and Bok; both Bax [31] iii) Proteins with an indirect apoptotic activity: Bid, Bad, Bik, Bim, BMF, HRK, NOXA and PUMA [32, 33].

The anti-apoptotic proteins targeted in this study, Bcl-2 and Bcl-xL, have been described as important in the apoptotic pathway of cancer cells [34]. The over expression of the Bcl-2 protein prolongs the lifespan of normally short-lived cells, thereby allowing them to accumulate additional oncogenic mutations [35]. The over expression of Bcl-2, the presence of oncogene-induced events and/or the loss of micro-RNAs 15a and 16 – 1, have been correlated with the development of an early neoplasm pre-malignant state [36, 37].

The Bcl-xL protein has been proposed as a promoter of cancer cell survival through chromosomal translocations such as those associated with uncontrolled expression of the C-myc oncogene [38, 39].

High expression of Bcl-2 protein has been observed in estrogen receptor positive breast cancer as well as in progesterone receptor positive breast cancer [40, 41]; Bcl-2 or Bcl-xL, is usually expressed at high levels in breast tumor tissues, which inhibits the apoptosis of tumor cells and promotes an explosive cell growth. If the Bcl-2 and Bcl-xL proteins can be functionally blocked, the apoptosis of tumor cells can be restored [42].

Several investigations report that the identified phenolic compounds show inhibition/cytotoxic activity on MCF-7, MDA-MB-435, MDA-MB-231, MDA-MB-468, MDA-MB-231 and MCF-10, SK- BR-3 human breast cell lines [43, 44, 45, 46, 47, 48, 49].

The ferulic, caffeic and chlorogenic acids are able to modulate the apoptosis pathway of cancer cells [50, 51, 52]. The available information indicates that these phenolic acids induce the apoptosis down-regulating the expression of BCL-2 anti apoptotic Bcl-2

and Bcl-xL proteins, at the same time that it up-regulating the expression of the BCL-2 pro-apoptotic proteins Bax and the caspase-3 protein. [50] Bao et al indicate that the cellular apoptosis mediated by the action of phenolic acids (ferulic acid) occurs by up-regulating the expression of P53, reducing the expression of cyclin D1 and cyclin-dependent kinase (CDK) 4/6, up-regulating the caspase 9, 3 (unequivocal characteristic of the activation of the cellular apoptosis process). At the same time, the expression of Mir-43a RNA-m was up-regulated, which inhibited the expression of Bcl-2 anti-apoptotic protein. Other pathway of activation of cellular apoptosis; the phenolic acids up-regulate the JNK c-Jun N-terminal kinase, ERK extracellular signal-regulated kinase, C-fox forkhead box C, STAT signal transduction and transcriptional activator, JAK Janus kinase, which finally up-regulate the expression of Bax pro apoptotic protein.

For the flavonoids Rahman et al [1] review several investigations on the correlation between flavonoids (flavonols, flavones, isoflavones, flavanones, chalcones, anthocyanins, and catechins) and their cytotoxic effect on several cancer cell lines. The mechanism of action of flavonoids appears to be the same as that of phenolic acid: the up-regulation of the p53 expression followed by the induction of apoptosis by activation of Bax protein thereby the inactivation of Bcl-2 protein and the over-expression of the production of apoptotic mediators (caspase 3/9 and cytochrome C).

The breast cancer therapies include chemotherapy, targeted therapy, and immunotherapy. All of these therapies intend to promote the apoptosis of the tumor cells.

In the targeted therapy, the active molecules can be catalogued in: antisense oligonucleotide preparations [53], peptide inhibitors [54], and small-molecule inhibitors [55]. The small-molecule inhibitor drugs bind specifically to anti-apoptotic proteins (Bcl-2, Bcl-xL and BAX) inhibiting or inactivating them [56,57].

Identifying the right compound with the desired pharmacological activity is critical to the pharmaceutical industry. Computer aided drug design software (CADD) has helped to reduce the cost and time associated with the evaluation of promissory drug type molecules by directing experimental research and the optimization process of the chosen molecules. Within the CADD, the molecular docking and the virtual screening are the most commonly used techniques [57].

In the case of molecular docking, the simulation predicts the best position, orientation, bond energy and conformation of a small molecule (drug candidate) when bound to a protein. The information helps to rationally design changes to optimize protein-ligand interaction, improve activity, and avoid changes that could lead to protein-ligand collisions [58]. The bond energy is an estimate of the strength of a protein-ligand complex related to the intermolecular interactions between these binding partners, solvent effects, and dynamics.

The *in silico* emulation of the phenolic acids indicate that they bind to the bcl-2 and Bcl-xL proteins with the lower energy among the evaluated compounds. Praba and Kamalanathe [59] simulated the inactivation of the bcl-2 and Bcl-xL proteins by the caffeic acid and a natural ligand (adhatodine). For the caffeic acid, the bind energy was of -10.68 kcal mol^{-1} for bcl-2 and – 9.31 kcal mol^{-1} for bcl-2 and Bcl-xL respectively. For the natural ligand, the bind energy was of -10.68 kcal $mol^{-1}$for bcl-2 and – 9.31 kcal mol^{-1} for bcl-2 and Bcl-xL respectively. Manoj-Prabhakar et al [60] reported a bind energy of -4.66 kcal mol^{-1} between the bcl-2 protein and the ferulic acid.

Rezaei-Seresht et al simulated the inactivation between the cafeico and gallic acid with the estrogen receptor alpha (ERα) and reported binding energies of -4.47 to -4.2 kcal mol^{-1}.

The flavonoids have the higher energy of the phenolic compounds evaluated, but lower than the native ligands. Leelarani and Padmini [61] reported a bind energy of -8.23 kcal mol^{-1} between the bcl-2 protein and naringenin. Ghani [62] evaluated the inactivation of the bcl-2 and Bcl-xL proteins by the apigenin and reported a binding energy of -7.2kcal mol^{-1} for bcl-2 and – 98.6 kcal mol^{-1} for bcl-2 and Bcl-xL proteins respectively. Gowtham et al 63] reported a bind energy between the apigenina and bcl-2 protein of 7.7 kcal mol^{-1}.

Prasad et al [64] reported a binding energy between Bcl-xL protein and rutin of -3.27 kcal mol^{-1}.

Chen et al [65] reported a binding energy between bcl-2 protein and rutin of -9.49 kcal mol^{-1}.

Prasad et al [64] reported several hydrogen bonds ($^{H+}$), ASN128, PHE131, GLN160, VAL161 and SER164; and hydrophobic bonds, ARG132, ASP133, GLY134, VAL135, LEU162 and VAL163; between Bcl-xL protein and the rutin

Ghani et al [62] report several binding residues between the bcl-2 and Bcl-xL proteins and apigenin (ARG139^{H+} and PHE63 for bcl-2 and ARG139 $^{H+}$, ALA104 and PHE105 for Bcl-xL)

Ghani et al [62] report several binding residues between the Bcl-xL proteins and naringenin (GLN28 $^{H+}$, ARG40 $^{H+}$, UNK173^{H+} and PHE23).

The ADMET model predicts no mutagenic or central nervous system toxicity for any of the identified phenolic acids and the flavonoids. The most significant predicted effect was observed for the naringenin (inactivation of the CYP1A2 cytochrome) and the apigenin (inactivation of the CYP2D6 and CYP34 cytochromes).

Endogenous or exogenous chemicals are activated or detoxified by two metabolic steps catalyzed by Phase I and phase II liver enzymes. The cytochrome P450 (CYP) enzymes are a superfamily of hemoproteins primarily responsible for Phase I drug metabolism; they catalyze the modification of parent drugs by hydroxylation, dealkylation, dehalogenation, of parent drugs [66, 67]. Only about a dozen CYP enzymes belonging to the CYP1, CYP2, and CYP3 families are important for drug metabolism [68].

The CYP1A2 enzyme are abundantly expressed in the liver and it is involved in the metabolism of about 10% of clinically used drugs (clozapines, imipramines, cyclobenzaperines) that are metabolized by CYP enzymes [69].

The CYP2A6 enzyme expressed in the liver. It plays an important role in the metabolism of coumarin, letrozole, nicotine, and tegafur, and it contributes to the metabolism of other drugs, such as artemisinin, efavirenz, halothane, pilocarpine, and valproic acid [70].

The CYP3A4 enzyme is involved in the metabolism of approximately 50% of clinically used drugs (benzodiazepines, calcium channel blockers, cyclosporine, macrolide antibiotics, opioids) and it also contributes to the metabolism of steroid hormones [71].

5 Conclusions

To our knowledge, this is the first investigation reporting the evaluation of the phenolic compounds isolated from *Pinaropappus roseus* leaves as a cytotoxic compounds against human breast cancer (MCF-7 cell line)

Our results conclude that all identified phenolic compounds may act as potential inhibitors for the targeted Bcl-2 and Bcl-xL proteins. The flavonoids showed the high binding affinities in the in silico simulation.

However, further confirmation as well as more experimental studies are required to validate the docking results in anticancer drug development.

Declarations

6.1 Author contributions

Mass spectrometry experimental Ch. H., G.B.; methodology and writing M.L., R. R., G.B. *in silico* simulation S.T. , G.B.; *in vitro* experimental Ch. H.; All authors have read and approved to the published version of the manuscript.

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6.3 Institutional review board statement

Not applicable.

6.4 Informed consent statement

Not applicable.

6.5 Data availability statement

The ultraviolet spectral profiles and the mass data are available upon request, by email, to the authors

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6.7 Competing Interests

The authors declare that they no competing interests.

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Figures

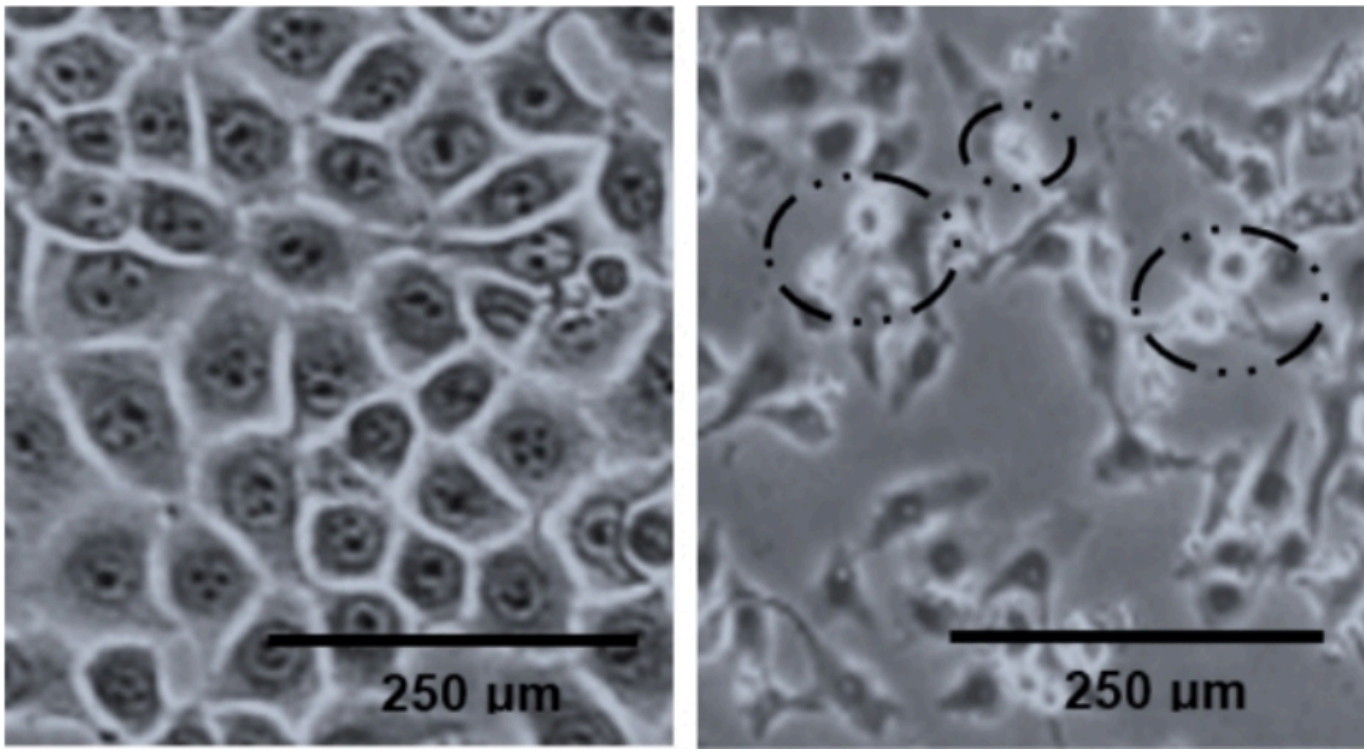

Figure 1

Morphological examination of MCF-7 cells by optical microscope. a) Untreated cells. b) Treated cells (the dying apoptotic cells are enclosed in circles).

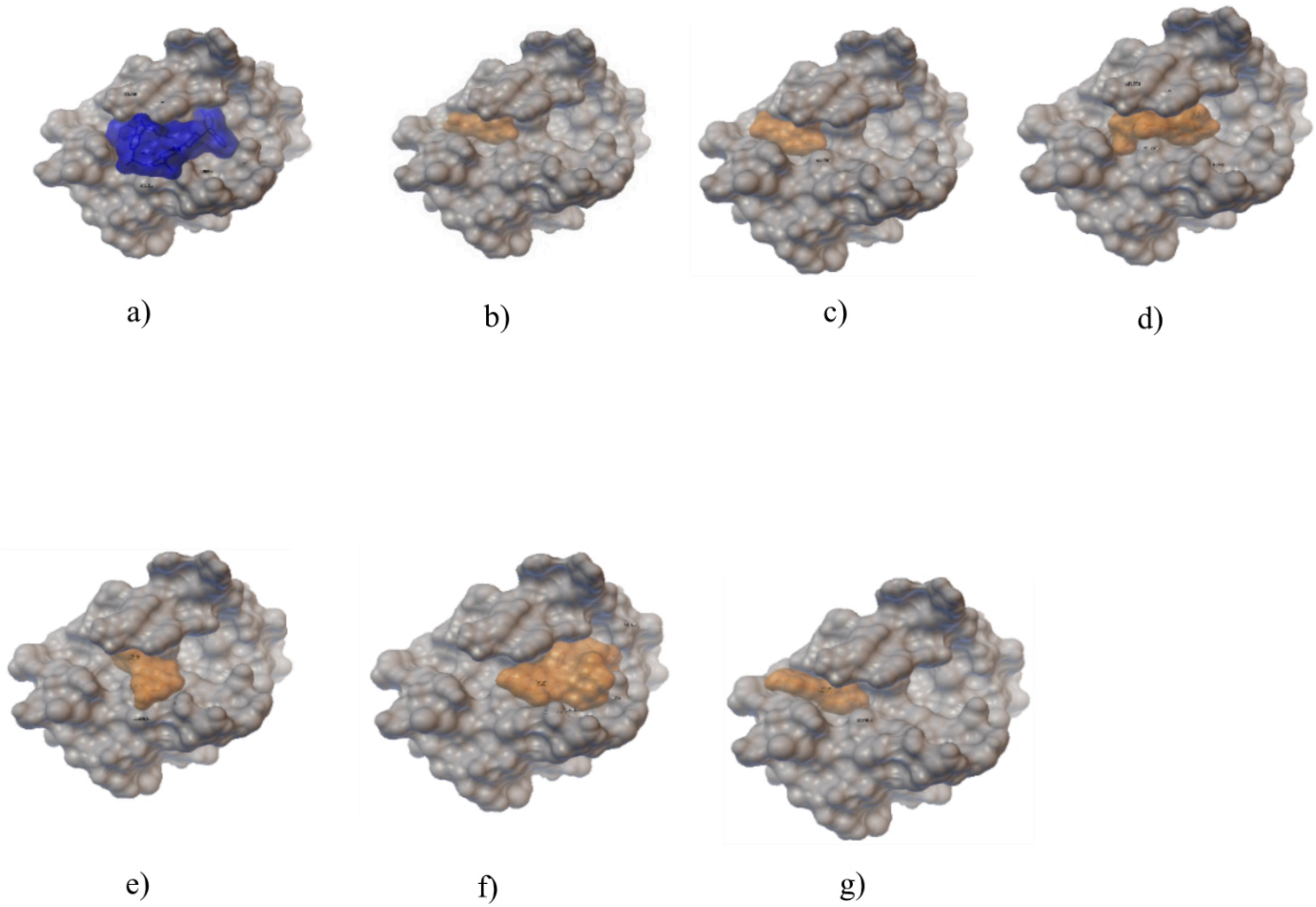

Figure 2

in silico ligand docking sites in Bcl-2 protein. (a) Bcl-2-IN-13 (native ligand). b) Caffeic acid. c). Ferulic acid. d) Chlorogenic acid. e) Naringenin. f) Rutin. g) Apigenin

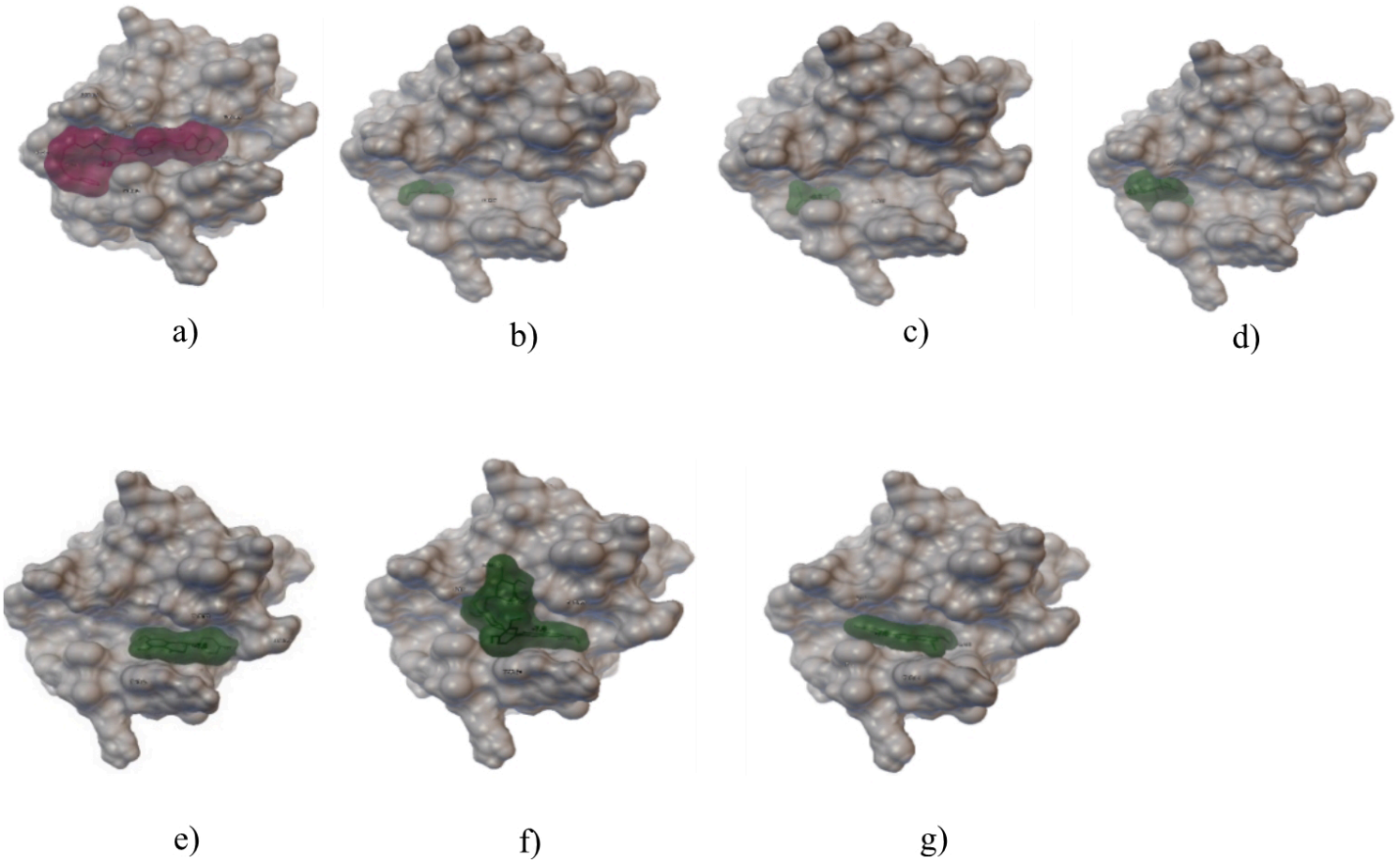

Figure 3

in silico ligand docking sites in Bcl-xL protein. b) Caffeic acid. c). Ferulic acid. d) Chlorogenic acid. e) Naringenin. f) Rutin. g) Apigenin