RESEARCH ARTICLE

Sterols from *Cyathea bicrenata* rhizome and their anti human breast cancer activity, in vitro and in silico (docking) evaluation

APPEARANCE OF MORPHOLOGY AND CYTOLOGY OF SHALLOT (*Allium ascalonicum* L) VARIETY OF TRISULA RESULTS FROM POLYPLOIDY INDUCTION WITH VARIOUS CONCENTRATIONS AND SOAKING TIME OF COLCHICINE

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ABSTRACT *(Times New Roman 12, Bold)*

Breast cancer is the most commonly diagnosed cancer in women worldwide. The access to adequate pharmacological therapy is commonly in a high-income country; the opposite is true in most low/middle-income countries. The aim of this research was evaluate the cytotoxic effect of the sterols isolated from *Cyathea bicrenata* Liebm rhizome against human breast carcinoma cells (MCF-7 cell line) and simulate the effect of the sterols on anti-apoptotic BCL-2 proteins. The sterols were isolated from non-polar extract of *C. bicrenata* rhizome by solid phase extraction. The identification of the sterol profile was carried out by mass spectrometry. The evaluation of the cytotoxicity of the sterols on MCF-7 carcinoma cells was carried out by the MTT technique. The identified sterols were evaluated *in silico* (docking and ADMET evaluation). In the computational docking evaluation, the sterols were docked to anti-apoptotic proteins in cancer cells, B-cell lymphoma and B-cell lymphoma extra-large protein (Bcl-2 and Bcl-xL); and the results, Gibbs energy, were compared with their native ligands. Seven sterols were identified. The sterol fraction showed anti cancer activity in the MCF-7 cell line, IC50-24h=157.79 µg/ml, IC50- 48h=120.23 µg/ml. All the sterols showed interaction with the Bcl-2 and Bcl-xL proteins with different binding energy values. The brasiscasterol showed the highest binding energy for the Bcl-2 protein. The ergosterol showed the highest binding energy for the Bcl-xL protein. The sterols from *Cyathea bicrenata* rhizome exhibit proprieties to be considered as an alternative pharmacological treatment of human breast cancer

Keywords: **BCL-2 proteins, *Cyathea bicrenata* sterols, cytotoxicity evaluation, docking simulation.**

Introduction

The breast is the most commonly diagnosed cancer in women worldwide, and the deadliest malignant neoplasm; with 2.26 million new cases in 2020 (Ferlay et al. 2021).

The breast cancer has been correlated with multiple endogenous and exogenous factors (*e.g.* age, family history, race/ethnicity, breast tissue density, pregnancy and breastfeeding, aberrant DNA mutations, prolonged estrogen exposure) (Hankinson et al. 2004; Key et al. 2013; Zhang et al. 2013).

The synthetically derived chemotherapeutic drugs used to treat cancer showed toxic effects against the cancer cells, but healthy cells were also affected (WHO, 2016), becoming an undesirable secondary effect. It has been hypothesized that an alternative pharmacological treatment for cancer is the use of some secondary metabolites due their selective cytotoxicity to cancer cells and a low toxicity to normal cells (Ferlay et al. 2021). The selection of plant species to be evaluated in the search for a secondary metabolite of interest is usually based on the available ethnomedical information (Singh et al. 2019).

The BCL-2 protein family includes pro-apoptotic and anti-apoptotic proteins (de Santis et al. 2015). For human cells, six BCL-2 anti-apoptotic protein family members, Bcl-2, Bcl-xL, Bcl-W, MCL1 (myeloid cell leukemia sequence 1), A1 (also known as BFL1 in humans) and Bcl-B (also known as Bcl2L10) has been described.

The regulation of the function of anti-apoptotic BCL-2 family members appears to be critical for the therapeutic efficacy of most (perhaps all) conventional cytotoxic agents and inhibitors of oncogenic kinases. The anti-apoptotic proteins Bcl-2 and Bcl-xL are involved in maintaining mitochondrial integrity and the membrane potential in cancer cells The and the inactivation of Bcl-2 and Bcl-xL proteins, by small molecules, triggers the apoptotic mechanism of cancer cells (Sankaranarayanan et al. 2015; Czabotar and García-Saez, 2023).

Several species of the genus *Cyathea* are used for therapeutic purposes against chronic-degenerative human pathologies (Andrade-Cetto et al. 2005; Martínez-Moreno and

Heinrich, 2005; Romero-Cereser et al. 2009). Several secondary metabolites reported for the *Cyathea* genus, where the sterols (mainly β-sitosterol) and the phenolic compounds stand out (Brighente et al. 20007; Talukdor et al. 2010; Jiang et al. 2012; Andrade et al. 2014; Kale et al. 2015; Kekrasov et al. 2019).

Cyathea bicrenata is used against renal and anti-cancer pathologies; however, to the best of the authors´ knowledge, the identification and the biological evaluation of their active secondary metabolites has not been carried out.

The aim of this research is to analyze, *in vitro* and *in silico*, the biological effect of the sterols present in the rhizome of *Cyathea bicrenata* on breast cancer cells (MCF-7).

Methods *(Times New Roman 12, Bold)*

Reagents and equipment

All the solvents used for the rhizome cleaning, the sterols extraction, MCF-7/HMEC cell culture, cytotoxic assays and liquid chromatography coupled to mass spectrometry were purchase from Sigma Aldrich México and JT Baker México.

The C8/SAX SPE cartridges were purchased from Finetech Co.

The MCF-7 (human breast 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 ThermoFisher UV-Vis spectrophotometer. The cell evaluation (UV spectrophometer) was carried out in a Biorad microplate reader

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

Plant material

Cyathea bicrenata was collected at Oaxaca, Mexico (17.523913, -96.509311; 25670 meters above sea level, ASL). The identification/ safeguard was carried out at the herbarium of the

Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, unidad Oaxaca - Instituto Politécnico Nacional (OAX-FLO-129-0402).

Plant extract procedure

The leaves and roots of *Cyathea bicrenata* were removed. The rhizome was cleaned by a wash of 1% (volume-volume; v/v) of water:sodium hypochlorite solution-, followed by a washing with distilled water.

The rhizome of *Cyathea bicrenata* was grounded and then dried to constant weight (relative humidity, RH<10%). The material was stored at 25 Celsius degrees (°C).

Five grams of the material was macerated with 25 milliliters (ml) of hexane for 24 hours (h) – 25 °C; then the material was sonicated for 30 minutes (min). The resulting suspension was centrifuged at 3000 revolutions per minute (rpm) for 2 min, and the solid material was discarded. The solution was filtered through Whatman# 4 paper to remove any remaining suspended solids.

The solution, called fern extract, was dried under nitrogen steam and stored at 25 °C in a dark place.

Purification of sterols

The purification of the fern extract was carried on according to the methodology of Jenner and Brown (2017). C18/SAX SPE cartridge was pre-condition eluting 2 ml methanol followed by 2 ml 40 micro molar (mM) formic acid (pH 4.5).

The fern extract was re-suspended in 2 ml hexane, and was eluted through the SPE cartridge. Finally, the cartridge was washed with 2 ml of a solution of 40% (v/v) of methanol – formic acid solution (40 mM - pH 4.5).

The preconditioning, loading, and washing eluates were discarded. The SPE cartridge was dried in nitrogen flow for 1–2 min.

The sterol fraction was eluted passing 2 ml hexane through the loaded-dried cartridge. The solution, called sterol fraction, was dried under nitrogen steam and stored at 25 °C in a dark place.

Characterization of compounds by liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry

The chromatography was carried out according to the methodology of Korne et al. (2006). The sterol fraction was resuspended in 1 ml of hexane and passed through 0.25 μm polyvinylidene difluoride (PVDF) filter before introduced to the chromatograph. The liquid chromatographic was carried out by gradient method using water:methanol (85:15, v/v) solution (phase A), and methanol:acetone:hexano (2:2:1,v/v/v) solution (phase B) at a flow rate of 0.4 milliliter per minute (ml/min).

The chromatographic gradient methodology was: 0–0.1 min, 100 % A; 0.1-0.3 min, 65 % A; 0.3-7.3 min, 65 % A; 7.3-7.4 min, 45 % A; 7.1–17.4 min, 45 % A; 17.4–17.5 min, 0 % A; 17.5-32.5 min, 0 % A. The separation was carried out using a C18 column.

The electrospray ionization interface in negative mode was used, considering a mass range of 50 to 3000 mass charge relation (m/z). The mass spectrometry conditions were set as follows: sheath gas temperature at 250 °C with a flow rate of 11 liter per minute (l/min), nitrogen gas temperature at 300 °C with a flow rate of 5 l/min, and a nebulizer gas pressure of 310.2 kilo Pascal (kPa). The capillary and nozzle voltages were set at 3.5 kilo volt (kV) and 500 volt (V), respectively. 50 microliter (μl) of the sterol fraction was introduced to the mass spectrometer. The identification was carried out comparing the mass fragmentation spectra pattern and the exact mass of the molecular ion with the MassBank database (Horai et al. 2010), and the NIST database (NIST 2023). Diagnostic ions were used to confirm the compound identification.

Cell culture conditions

Breast cancer cells (MCF-7) were cultured in Roswell Park growth medium (ROMI 1640) supplemented with glutamine (2 mM), sodium pyruvate (1 mM), no essential amino acids

(0.1 mM), sodium bicarbonate (1.5 gram per liter, g/l), 10% (v/v) fetal calf serum, 50 microgram per milliliter (μg/ml) of gentamicin and 50 μg/ml amphotericin-B.

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

Human mammary epithelial cells (HMEC) were cultured in serum-free mammary epithelium growth medium (MEGM) containing sodium pyruvate (0.11 gram per liter, g/l), phenol red (0.012 g/l), L-glutamine (1.461 g/l), dextrose (1.0 g/l), 10% (v/v) fetal calf serum, 50 μg/ml of gentamicin and 50 μg/m amphotericin-B.

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

in vitro cytotoxic activity assay

The viability of the cancer cells were carried out by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) technique according to the modified method of Razak et al (2019).

The MCF-7 and HMEC cells, at a concentration of $5x10^6$ cells per milliliter (cells/ml), were cultivated in 0.2 ml of HepG2 growth medium in a humidified atmosphere at 37 °C for 24 h.

Cells were treated with increasing doses of the sterol fraction, 0 -200 μg /ml; and monitored at 24 and 48 h using a microplate reader at 570 nanometer (nm). A 0.75% dimethyl sulfoxide (DMSO) solution was used as the negative control. The cytotoxic activity was expressed as the half maximal inhibitory concentration (IC50).

in silico simulation. Docking evaluation

To evaluate the interactions of the sterol profile, the crystallized structure 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 (PDB 2023).

The PubChem database (PubChem 2023) was employed to acquire the sterol (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 (Avogadro 2023). 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 (Sanner et al. 2011).

AutoDockTools ver. 1.5.7 software (AutoDock 2023) were employed to do the *in silico* evaluation. The docking results from the in silico evaluation were reported as the maximum negative scoring function (Gibb´s energy).

in silico simulation. Pharmacokinetics properties simulation

To evaluate the drug properties of the sterols profile, the SmissADMET software (SwissADME 2023) was used.

Data analysis

Results

Please report the results in subheadings. The figure and Table should be mentioned in the bracket.

The sterol profile of *Cyathea bicrenata* rhizome was composed of seven molecules (Table 1). Some sterols, such as the ergosterol, the β–sitosterol, the campesterol, the lupeol and the daucosterol, have been already reported for the *Cyathea nilgirensis, Cyathea gigantea, Cyathea crinita*, *Cyathea latebrosa* and *Cyathea spinous* (Ikpa et al. 2000; Janakiraman et al. 2023). To the best of the authors´ knowledge, the presence of the brassicasterol, the

fucosterol, the stigmasterol, the ergosterol and stigmastanol has not been reported for any *Cyathea* species.

Cytotoxicity assay

The sterol fraction significantly inhibited the proliferation of MCF-7 cells in a concentration- and time-dependent manner (Table 2). The IC50 for the MCF-7 cells was 157.79 μg/ml at 24 h and 120.23 μg/ml at 48 h. The sterol fraction significantly inhibited the proliferation of HMEC cells in a concentration and time-dependent manner (Table 2). The IC50 for HMEC cells was 3239.84 μg/ml at 24 h and 706.06 μg/ml at 48 h.

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

Docking evaluation.

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 kilo calories per mol (kcal/mol) and -9.0 kcal/mol, respectively. Among the sterols evaluated, the brassicasterol and the ergosterol showed the highest binding energy for the Bcl-2 protein (-8.6 kcal/mol) and for the Bcl-xL protein (-8.8 kcal/mol) respectively (Table 3 and Table 4 respectively).

For the Bcl-2 protein, all sterols interacted with the active site of the native ligand $C_{42}H_{44}ClN_7O_6S_3$ (the hydrophobic α5 protein helix), which is characterized by multiple hydrophobic/electrostatic bonds (Table 3). Only one hydrogen bond was observed for the native ligand (asparagine, ASN 102) on the BCL-2 protein central hydrophobic groove).

The evaluated ligands (sterols) bind to the Blc-2 protein hydrophobic groove, and almost all of the bonds are of the hydrophobic/electrostatic type (the sitosterol showed a hydrogen bond with the glutamine, GLU 111 residue).

Based on the binding interactions between the Bcl-2 protein and the evaluated ligands; a common residue, the PHE 63, interacted with the sterols except with the fucosterol, via hydrophobic/electrostatic interactions.

For the Bcl-xL protein, all sterols are bound on the active site of the native ligand $C_{31}H_{30}N_4O_4S_2$ (on central hydrophobic α5 protein helix). The bond was characterized by multiple hydrophobic/electrostatic interactions (Table 4). Only one hydrogen bond was observed for the native ligand (arginine, ARG 139) on the BCL-2 protein central hydrophobic groove).

The evaluated ligands (sterols) bind to the hydrophobic groove. And almost all the bonds are of the hydrophobic/electrostatic type.

(The ergosterol and the stigmastanol showed a hydrogen bond with ARG 139 and alanine, ALA 93 residues, respectively).

Based on the binding interactions between the Bcl-xL protein and the evaluated ligands; two common residues, the glycine (GLY) 138 and phenylalanine (PHE) 97, interacted with all the sterols via hydrophobic or electrostatic interactions.

Evaluation of pharmacokinetics properties.

The outcome of the ADMET results suggests that all the evaluated sterols possess interesting pharmacokinetics properties and do not show important toxicity effects (Table 5).

Discussion

The evaluation of the cytotoxic effect of extracts from the *Cyathea* genus on MCF-7 cancer cell line was reported. Ethanolic extracts of the *Cyathea crinita*, *Cyathea nilgiriensis* and *Cyathea gigantea* showed a decrease in the cell viability and an increase in the growth inhibition in MCF 7 cells. *C. crinita* showed the maximum percentage cell inhibition, IC50 400 µg/ml; followed by *C. nilgirensis*, IC50 714.28 µg/ml and *C. gigantea,* IC50 806.45 µg/ml (Janakiraman and Johnson 2016). Aqueous extract of the *C. nilgiriensis* extract carried by silver nitrate ($AgNO_3$) nanoparticles against Dalton's lymphoma ascites cell line showed an inhibition of 56 % of the lymphoma cells (at a nanoparticle concentration of 200 µg/ml) (Johnson et al. 2018).

The sterols are essential molecules for the cell membrane function controlling the membrane fluidity and permeability (Valitova et al. 2016).They have health-promoting effects in humans, such as anti-cancer activity, anti-inflammatory, anti-bacterial, and anti-fungal (Salehi et al. 2021). Furthermore, long-term studies in animal models and humans have shown no long term consumption toxic effects (Ling and jones 1995).

Several investigations have reported the biological activity of the identified sterols in *Cyathea bicrenata* rhizome extract against human breast cancer (MCF-7, MCF10A, MDA-MB-231, cell lines).

Several studies reported that the sterols (β-sitosterol, stigmasterol, campesterol, stigmastan-3,5-diene, γ-sitosterol, cholesterol margaret and 7- dehydrodiosgenin) isolated from plants induced apoptosis and decreased cell viability in human breast cancer, MCF-7 cell line (Mellanen et al. 1996; Young et al. 2004; Ayaz et al. 2019; Raju et al. 2021; AmeliMajorad et al. 2022). For the cytotoxicity effect of the stigmasterol and the campesterol in estrogen induced/related breast cancer cell has been correlated to the down-expression of some BCL-2 antiapoptotic proteins in the mitochondrial and death receptor-mediated pathways (Mellanen et al. 1996; Young et al. 2004; Awad et al. 2007 AmeliMojarad et al. 2022). The sterols activate the BCL-2 apoptotic initiator proteins (BIM, PUMA, BAD, NOXA, BIK, HRK, BMF, and tBID); at the same time, they inhibit the antiapoptotic BCL-2-like proteins (Bcl-2, BCL-xL), allowing the activation of the pro-apoptotic effectors BAX and BAK proteins. This pathway ends with the disruption of the mitochondrial outer membrane. These membrane disrupt release the cytochrome c from the mitochondria and promote the activation of effector caspases, caspase 3, caspase 7, and caspase 6 (Du et al. 2000; Verhangen et al. 2000; Awad et al. 2003; Subbiah and Abplanalp 2003; Harlozinska 2005; Riedl and Salvensen 2007; Pluchino et al. 2015; Hao et al. 2017; Jianjun et al. 2022).

In human breast cancer, the Bcl-2 and Bcl-xL proteins, have been described as important in the human breast cancer cell apoptotic signaling pathway (Rooswinkel et al. 2014). These proteins are usually over expressed in breast tumors, which inhibits the apoptosis pathway 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 (Qian et al. 2002).

The over expression of the Bcl-2 protein (sometime correlated with the presence of oncogene-induced events and/or the loss of micro-RNAs 15a and 16-1) prolongs the lifespan of normally short-lived cells, thereby allowing them to accumulate additional oncogenic mutations (Calin et al. 2005; Cimmino et al. 2005).

The over expression of the Bcl-xL promote several chromosomal translocations associated with uncontrolled expression of the C-myc oncogene (Horita et al. 2000; Cheung et al., 2004; Bilalovic et al. 2004; Linjawi et al. 2004).

The use of various molecules (peptides, oligonucleotides, small molecules and/or secondary metabolites) is employed as an anticancer targeted therapy strategy to specifically inhibit the anti-apoptotic proteins Bcl-2, Bcl-xL and BAX (Verdin and Walensky 2007; Qian et al. 2022).

The drugs ABT-737 and ABT-263 (navitoclax) are examples of this therapeutic approach (Oltersdorf et al. 2005; Tse et al. 2008); both drugs bind strongly to Bcl-2, Bcl-xL and Bcl-w proteins. The results are promising, especially in patients with chronic lymphocytic leukemia (Roberts et al. 2012). Recent preclinical studies indicate that the combination of these kind of targeted therapy with other anticancer drugs, either conventional agents, increases cancer survival rates (Manson et al. 2008; Tan et al. 2011).

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, the ADMET evaluation and the virtual screening are the most commonly used techniques (Yu et al. 2017).

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 (Joseph et al 2007). 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 brassicasterol and the ergosterol showed a binding energy similar to that of the native ligand ($C_{42}H_{44}ClN_7O_6S_3$), indicating a high affinity of those molecules and the Bcl-2 protein. A similar behavior is observed between the ligands and the Bcl-xL protein

Gowtham et al. (2023) reported that the binding energy between the daucosterol and the Bcl-2 protein (PDB id 2W3L) was of -9.0 kcal/mol. Gideon et al. (2022) reported a binding energy of -9.4 kcal/mol for sitosterol and Bcl-2 protein (PDB id 4lvt).

Gideon et al. (2022) reported a binding energy of -7.8 kcal/mol for luteolin and Bcl-xL protein (PDB id 3zlr) Ayaz et al. (2019) reported blinding energies of -7.6 to -7.7 kcal/mol between tyrosine kinase and β-sitosterol and stigmasterol.

Raju et al. (2021) reported blinding energies of -6.2 to -10.0 kcal/mol between several phytosterols (γ-tocopherol, stigmastan-3,5- diene, α-sitosterol, cholesterol margarate, stigmastan-3,5-diene) and the estrogen receptor (ERα), progesterone receptor, and epidermal growth factor receptor correlated with human breast cancer.

Marisa et al. (2021) evaluated *in silico* the potential of phytosterols (α-tocopherol, β-sitosterol, campesterol, stigmasterol, fucosterol) as anti-human breast cancer drugs. The result suggests a possible inactivation of the estrogen receptor (ERα) by the phytosterols. The binding energies reported ranged from -6.8 to -8.9 kcal/mol.

In any docking evaluation, the position and orientation between the protein-ligand must be carefully considered, due that ligand can be bound in several parts of the protein surface far from the protein active site.

For the inhibition of BCL-2 protein family, the physical interactions between the ligands (sterols) and the anti-apoptotic proteins occur at the amphipathic helix groove mainly by the insertion of four hydrophobic residues along one face into hydrophobic pockets in the groove, and by the formation of a salt bridge between a conserved aspartame (Asp) residue and a conserved arginine (Arg) residue in α-1 helix (Sattler et al. 1997; Liu et al. 2003; Czabotar et al. 2007; Czabotar et al. 2014). However, mutational studies suggest that the bonds between the ligand and the BCL-2 protein occur across the entire amphipathic helix-helix-groove interface (Lee et al. 2008; Chen et al. 2013).

For the *in silico* evaluation of several sterols against proteins involved in the progression of breast cancer, Ayaz et al. (2019) reported multiple electrostatic bonds, and only one hydrogen bond, between the β-sitosterol and stigmasterol and the tyrosine kinase. Marisa et al. (2021) reported multiple bindings between estrogen receptor α (ER α) and α-tocopherol, β-sitosterol, campesterol, stigmasterol, fucosterol. The main binding interaction was of electrostatic type, even in the case of the evaluated native ligand (raloxifene). Raju et al. (2021) reported multiple binding residues (no hydrogen bonding was reported) with the Asp351, methionine, Met, 759 and ASP800 as the key residues for the evaluated protein.

Hydrogen bonds play an important role in the stabilization of the interaction structures between proteins-ligands due to their participation in the secondary structure elements (α helices and β sheets). The absence of hydrogen bonding in almost all of the evaluated ligands may indicate these bonds do not appear to be fully stable (Pantsar and Poso 2018; Bitencourt et al. 2019); although the information coming from the simulation indicates a good binding energy (values near of the exhibited for the evaluated natural ligands) and that the bonds are located in the same active region that for the natural ligands (the amphipathic helix groove region).

The *in silico* evaluation of the ADMET properties of the lupeol, the stigmasterol and the swertiamarin isolated from rhizomes and leaves of *Cyathea nilgirensis*, *Cyathea gigantea* and *Cyathea crinita* (Janakiraman et al. 2023) don´t showed significant differences compared to those reported for the sterol fraction isolated from the rhizome of *Cyathea bicrenata*. Janakiraman et al. (2023) evaluated the *in silico* binding energy of some sterols from *Cyathea nilgirensis, Cyathea gigantea* and *Cyathea crinita*; against proteins correlated with P450 cytochrome oxidation and proteins correlated with cell death pathways and concluded that the evaluated molecules can be considered as selective and non-toxic compounds with anticancer properties.

The action principles of the secondary metabolites and other natural products are likely able to exhibit inhibiting effects on various cytochromes leading to adverse reactions in humans and even some fatal interactions, particularly in the case in which the involved drugs have a

narrow therapeutic window (Lin and Lu 2001). Cytochrome P450 2C is the second most abundant subfamily of P450 enzymes and is responsible for metabolism of almost 20% of the drugs currently available in the market (Rendic and Jones 1997).

CYP2C9 is an important member of the subfamily, serving as the primary metabolic pathway of the narrow therapeutic index drugs warfarin and phenytoin as well as numerous other therapeutic entities (Rettie and di Carlo 2005).

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If possible, provide recommendations or suggestions for further research in this part. (*Times New Roman 12, spacing 1.5*).

Conclusion

Our study revealed that the sterol fraction of the *Cyathea bicrenata* rhizome is composed of six no-condensed sterol and one condensed sterol.

The sterols fraction showed cytotoxic activity against the human breast cancer cell line (MCF-7). The sterols fraction showed low cytotoxic activity against the HMEC human mammary epithelial cell line.

We declare that this is the first report of the binding simulation of the phytosterols of *Cyathea bicrenata* towards Bcl-2 and Bcl-xL proteins. The sterols bind on the allosteric-binding site of the proteins (helix α5), similar to the proteins known in activators.

These findings provide evidence that the *Cyathea bicrenata* can be further explored for its pharmacology activity against human breast cancer. Nonetheless, additional studies are still required to elucidate how the Bcl-2 and Bcl-xL are in-activated by the identified sterols.

It is important to note that further studies (*e.g.* virtual screening simulation, carrier design and evaluation, in vivo bioavailability assessment, etc.) are needed to consider the use of sterols as an alternative pharmacological therapy in the treatment of cancer

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Author contributions

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

Declaration of interest

The authors declare that none of them has any conflict of interest with any private, public or academic party related to the information contained in this manuscript

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References *(Times New Roman 12, Bold)*

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Example:

In the text: In the liver, alcohol is metabolized firstly to acetaldehyde by alcohol dehydrogenase (ADH), followed by oxidation to acetate by aldehyde dehydrogenase (ALDH) [1].

In the reference:

[1] Mackus M, Loo AJ van de, Garssen J, Kraneveld AD, Scholey A, Verster JC. The role of alcohol metabolism in the pathology of alcohol hangover. J Clin Med. 2020;9. doi:10.3390/jcm9113421

FIGURES AND TABLES

The picture caption must-have title. The title of the picture should be written in Arial, font size 12, center alignment, and should be placed exactly under the picture.

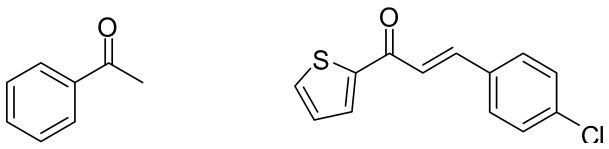

Figure 1. Molecule structure.....

The authors also have to prepare all Figures in a PPT file. One slide for one figure. Please download the PPT file (landscape style – for normal figure, portrait style – for the extensive figure).

Link landscape

Link portrait

The title of the Table should be written in Times New Roman, font size 12, single-spaced, center alignment, and should be placed exactly above the table.