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Research article

Molecular docking and biochemical insights into the pro-apoptotic cytotoxicity of *Breonadia salicina*

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Abstract: The investigation of phytochemicals in plants has garnered substantial interest because of their potential therapeutic applications and pharmaceutical industries. These compounds can interact with biological systems, influencing critical biochemical mechanisms such as apoptosis, cell signaling, and oxidative stress. Therefore, understanding the mechanisms of action of these compounds is crucial to unlock their potential for developing novel agents for pharmaceuticals and health-promoting supplements. *Breonadia salicina* is a native species distributed throughout tropical and subtropical countries. This plant is used to treat of wounds, ulcers, fevers, headaches, gastrointestinal illness, cancer, arthritis, diabetes, inflammation, and bacterial and fungal infections. The roots were extracted via successive solvent extractions and evaluated for their antioxidant, phytochemical, and anticancer properties, with an emphasis on their apoptotic and underlying potential mechanisms. The ethanolic (REE), aqueous (AQR), ethyl acetate (EAR), and n-butanol (NBR) fractions significantly inhibited

HT-29 cancer cells ($p < 0.05$). The EAR fractions displayed the strongest inhibition (IC50 of 22.5 µg/mL) and promptly triggered early and late apoptosis and cell necrosis ($p < 0.001$). The EAR fractions demonstrated powerful TFC (19.4 Quercetin/g) activity, with IC_{50} values of 22 and 25 mg/ml for ABTS and DPPH, respectively. LC–MS/LC–HRM analysis revealed several phytochemicals in the EAR fractions that contributed to the potential properties of the plants, including polyphenols, coumarin derivatives, 4,5-dicaffeoylquinic acid, chlorogenic acid, glycosides, and geniposidic acid. Notably, the in-silico cytotoxicity of these phytochemicals revealed the significant cytotoxicity of four potent compounds against six colon cancer cell lines, which presented the greatest interaction with the CDK2 enzyme (− 5.0 kcal/mol). These findings underscore the potential of the identified phytochemicals as promising anticancer or nutraceutical candidates for drug development and in the pharmaceutical industry.

Keywords: medicinal plants; anticancer; apoptosis; antioxidants; cytotoxicity; phytochemicals; molecular docking

1. Introduction

The identification of novel natural sources with unique extraordinary compounds for pharmaceuticals is ongoing. Natural phytochemicals from plants can be used as alternative therapeutic agents in the development of new drugs, pharmaceuticals, nutraceuticals, and food products [1]. These compounds provide compelling opportunities for developing dietary supplements with the aim of enhancing human health [2]. Plant phytochemicals hold tremendous and remarkable status in many fields, especially in cancer research. Numerous studies have been conducted to identify phytochemicals with significant roles in the prevention or treatment of diseases, including cancer [3]. Despite the availability of cancer treatments, this disease is the second leading cause of death worldwide [4]. These treatments have several drawbacks, including pain, low specificity, non-selectivity, surgical complications, and other adverse effects [5]. In addition, the increasing cost of contemporary medicine has made it unattainable for 80% of populace [1]. Therefore, ensuring the safety and accessibility of treatments currently stands as a primary focal point in research endeavors [6]. In recent years, natural products have garnered attention as potential candidates for cancer therapy [3]. The role of these compounds as remedies has expanded because of their varied chemical compositions and their possible therapeutic uses with proven anticancer effects. Researchers have explored this avenue and investigated the role of natural bioactive compounds in treating several diseases, including cancer [1]. Moreover, the use of nutraceuticals and natural products in clinical practice as anticancer therapies has increased and has reached 60% [7]. Preclinical models have demonstrated the significant anticancer effects of natural phytochemical compounds against various malignancies [8]. Additionally, advancements in the use of natural products in the form of synthetic derivatives and nano formulations have shown promise in enhancing bioavailability and drug delivery [9,10]. Therefore, investigating novel chemical compounds from natural sources with potent properties is highly desirable, as they are considered promising candidates for pharmaceutical and therapeutic applications.

Breonadia salicina is a native species that is distributed throughout a wide range of tropical and subtropical regions of Africa as well as in the Arabian Peninsula, Saudi Arabia, Sudan, and Yemen. *B. salicina* is a medium to tall tree, approximately 2 m in diameter and 40 m in height, and grows primarily

in the seasonally dry tropical biome near banks or waters of rivers and permanent streams [11,12]. Historically, it has stood out as a significant medicinal remedy in various ethnic and cultural traditions. Traditionally, *B. salicina* tree stem bark, leaves, and roots have been widely used throughout Africa to treat illnesses, including pneumonia, headaches, arthritis, heart arrhythmia, vomiting, ulcers, cramps, digestive ailments, wound healing, antiparasitic, antimicrobial, and anticancer remedies [13−17]. Its traditional use in treating diseases has been supported recently by few experimental studies that have been conducted on the biological activity of *B. salicina* tree parts. *B. salicina* leaves cultivated in South Africa have been found to have antioxidant, antifungal, cytotoxic, antidiabetic, anti-inflammatory, antidiarrhetic, and antimicrobial effects, with an MIC of 10.89 mg/ml [12,17−19]. The leaf antibacterial property of *B. salicina* cultivated in Saudi Arabia has an MIC of 3 mg/ml [11], which is much lower than that of South Africa species. This is attributed to regional climate conditions such as temperature, humidity, and precipitation, which strongly affect the properties of *B. salicina* species in different geographical regions [16]. The medicinal properties of plants play crucial roles as preventive agents and remedies for several illnesses, including cancer [1]. However, the properties of *B. salicina* species in Nigeria are underrepresented, particularly their roots. No data has been reported concerning the phytochemical and biological studies of *B. salicina* roots. Therefore, there is a need to explore the phytochemical and biological activities of root extracts. These properties make this plant a valuable source for developing pharmaceutical agents and finding novel drug leads.

2. Materials and methods

2.1. Plant collection and identification

B. salicina specimens (Figure 1) were collected from their natural native habitat in Nigeria and then placed in the herbarium for taxonomic classification. A botanist from Ahmadu Bello University in Zaria, Kaduna State, Nigeria, thoroughly verified and identified the plant's identity. A voucher number (ABU 900383) was assigned to the samples. To confirm the species name, reference was made to the World Flora Online (WFO) at https://www.worldfloraonline.org/.

Figure 1. *B. salicina.*

2.2. Root extraction of B. salicina

Approximately 300 g of root was ground into a powder after drying. The mixture was then macerated in a shaker for six hours at 25 °C and 200 rpm in 1 liter of 95% ethanol (Stuart Scientific Great Britain) [20]. Whatman filter No. 1 was used to filter the extracts, which were then dried at 50 °C in a rotary evaporator (Buchi Labortechnik) at low pressure. Using the extract later, it was carefully placed in a sealed container and placed in a desiccator. The mass of the crude yield was calculated via the following formula:

$$\text{Yield (\%)} = F1/F2 \times 100$$

In this equation, the mass of the sample is represented by F2, whereas the mass of the crude extract is denoted by F1.

2.3. Fractionation of B. salicina roots

The root extract was prepared by soaking 2.5 grams in 500 ml of distilled water (a solvent-to-sample ratio of 1: 10) and sonicating it for 10 min at 20 °C. Subsequently, 300 ml of n-hexane was added to the mixture, which was subsequently shaken for 30 minutes via a British-made Stuart Scientific Flask shaker at 20 degrees Celsius [21]. The finished mixture was filtered through Whatman filter paper No. 1 and allowed to settle. Using a Büchi Labortechnik rotary evaporator set to 50 °C, the hexane fraction was evaporated until it was completely dry. The water fraction was then sequentially extracted with 300 mL of ethyl acetate, followed by extraction with n-butanol, with the same procedure being applied for both extractions. All the fractions were subsequently transferred to sample bottles and kept for later use.

2.4. Antioxidants study

2.4.1. Total phenols (TPC)

Approximately 200 mg of Folin-Ciocalteu solution was added to 1.5 mL of each root fraction, and the mixture was allowed to stand for two hours in a light-limited setting. The absorbance at 750 nm was determined via a spectrophotometer [22]. The fraction was combined with gallic acid at several doses (0–200 mg/mL). The gallic acid standard curve equation, $y = 0.0038x + 0.0673$, was used to quantify the data produced by calculating the equivalent gallic acid content per gram of weight by determining the coefficient (Supplementary Figure 1).

2.4.2. Total flavonoid content (TFC)

A total of 1 mL of extract was added to 0.3 mL of a 5% sodium nitrate solution and mixed with 4 mL of distilled water. Subsequently, 2.4 mL of water, 2 mL of 1 M sodium hydroxide, and 0.3 mL of aluminum chloride solution (10%) were added to the previous mixture and incubated for 15 minutes. The spectrophotometer wavelength was set to 510 nm, and the mixture absorption was measured [22]. The quercetin standard curve equation (Supplementary Figure 2) was used to quantify the data produced by calculating the equivalent quercetin content per gram weight by determining the

coefficient.

2.4.3. Root potential determination via a DPPH radical scavenging assay

All the obtained fractions (REE, EAR, NBR, and AQR) were tested for their ability to scavenge DPPH radicals following the methods of Dahham [23] in 2018. A microplate reader was used to calculate the absorbance of each fraction at 517 nm. The positive control GA was used as a reference point [24]. The formula for determining radical scavenging activity was as follows: *inhibition percentage = [(B-control – B-sample)/(B-control)]100*, where B-control represents the control absorbance and B-sample represents the absorbance of the test extract.

2.4.4. ABTS radical cation scavenging assay

The ABTS radical cation scavenging activity of the REE, EAR, NBR, and AQR fractions was determined via the approach outlined by Re et al. in 1999. Following the mixing of potassium persulfate (2.45 mM) and ABTS solutions (7 mM), the mixture was incubated for 8 hours in the dark [24]. After dilution with methanol, the absorbance was set to 0.900 (± 0.02) at 745 nm at 30 °C. The absorbance was measured after each sample (300 µl) was added to the ABTS working solution. Moreover, the methanol concentrations in the samples varied between 125 and 2000 mg/mL. The scavenging percentage of the standard and the samples was assessed via the following [control reading – sample reading)/(control reading)]/100.

2.5. Anticancer study of B. salicina root extracts

In this study, considering that *B. salicina* extract has anticancer and apoptotic effects, the HT-29 cell line, which originates from human colorectal adenocarcinoma, was chosen. HT-29 cells offer an appropriate platform for evaluating their potential cytotoxic and proapoptotic effects. The selection of HT-29 cells as the *in vitro* model for this investigation was based on their well-documented application in evaluating the anticancer properties of natural compounds. These cells are also characterized by dysregulated apoptotic pathways, making them a suitable model for investigating apoptosis-inducing agents. In addition, several studies have utilized HT-29 cells to assess the anticancer activity of plant-derived substances [23], which provides additional justification for their use in this study.

2.5.1. Sourcing of HT-29 cell lines

Human colorectal cancer (HT-29) (Company: ATCC, Cell line : HT-29, Product category: Human cells, Organism: Homo sapiens, human, Cell type: Enterocyte, Morphology: Epithelial, Tissue: Colon, Disease: Adenocarcinoma, Colorectal, Application: 3D cell culture, Cancer Research, Toxicology, Gastroenterology, Product format: Frozen, Storage condition: Vapor phase of Liquid Nitrogen cell lines) were obtained from the Molecular Medicine Laboratory, Institute of Bioscience, University Putra Malaysia. The cells were propagated in Dulbecco's modified Eagle's medium (DMEM-high glucose) supplemented with 10% fetal bovine serum (FBS). The cell cultures were maintained at 37 °C with 5% CO_2, 95% air and 100% relative humidity [23].

2.5.2. Cell treatment procedures

The anticancer effects of the extracts were assessed in HT-29 cells as described by [23]. Monolayer HT29 cells were detached via trypsin-EDTA to make a single-cell suspension. After viable cells were counted, approximately 100 µl of HT-29 cells (5 x 10^3 cells/well) were seeded, grown in a 96-well culture plate, and incubated for 24 hours at 37 °C and 5% CO_2. The supernatant was removed thereafter from the adherent cells, which were subsequently washed with phosphate-buffered saline (PBS). The fractions of the root along with the positive control (5-fluorouracil 5-FU) were added at different concentrations (0–1800 µg/mL) to the respective labeled wells and incubated further for 24 hours. The medium was used as a negative control, and all the tests were performed in triplicate for all the different concentrations.

2.5.3. Anticancer activity of *B. salicina* root extract

The assay was carried out using HT-29 cells following the method described by Daham et al. [23]. Following 24 hours of incubation, 20 µL of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5/mL) prepared in phosphate-buffered saline (PBS) was added to each well, and the plate was incubated for 4 hours at 37 °C and 5% CO_2. The medium supplemented with MTT was removed, and the formazan crystals formed were dissolved in 100 µL of DMSO. The absorbance was recorded via a microplate reader (BioTek) at 570 nm. Formazan formation is proportional to the number of existing active cells. To determine the IC_{50}, a nonlinear regression graph was plotted between the log concentration and the percentage of cell growth inhibition. The cell growth inhibition percentage was calculated as follows:

$$Cell\ Growth\ Inhibition\ \% = 100 - \frac{OD\ of\ cells\ treated}{OD\ of\ \ control\ cells}\ x\ 100$$

2.5.4. Qualitative apoptosis

Cultured cells treated with the ER fraction were centrifuged at 1000 RCF for 5 minutes after being trypsinized with trypsin-EDTA and collected. The pellets were washed twice with PBS, resuspended in PBS, and fixed with 4% methanol-free formaldehyde for 15 minutes at room temperature. The mixture was incubated at zero degrees Celsius for 30 minutes. Subsequently, 10 µl of a mixture containing 1 µg/ml propidium iodide and 10 µg/ml acridine orange was added to a 10 µl cell suspension and incubated in the dark for 15 min at room temperature on a glass slide. An inverted fluorescence microscope (Zeiss Axio Vert A1, Germany) equipped with an image acquisition system (AxioCam MRm, Germany) was used to observe the stained cells. Multiple sets of cell images were obtained in replicate experiments.

2.6. Liquid chromatography–mass spectrometry of the root extracts of B. salicina

2.6.1. Sample preparation

The master stock (MS) and working stock (WS) for analysis were prepared by dissolving 1 g of the root extracts of *B. salicina* in 1 mL of LCMS-grade methanol. The working stock concentration

was adjusted to 10 mg/mL in methanol [25]. The samples were filtered through a PTFE membrane filter (0.22 μm) and stored in 2 ml vials.

2.6.2. LC–MS procedure and operating conditions

Liquid chromatography was coupled with high-resolution mass spectrometry (LC-HRMS) to examine the chemical constituents of the ethyl acetate root (EAR) fraction. Chromatographic separation was performed on a reverse-phase C18 column (2.1 × 100 mm, 1.8 μm) using a binary mobile phase composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) [25]. A gradient elution of 5 to 95% B in 25 minutes at a constant flow rate of 0.3 mL/min was used to separate the products, and the temperature of the column was maintained at 40 °C. Mass spectrometric analysis in positive and negative ionization mode with a mass range of m/z 100–1500 was performed at high resolution. Data-dependent acquisition was used, in which the five most intense ions of each complete MS scan were chosen to undergo MS/MS fragmentation via stepped collision energies. The identification of compounds was performed via Compound Discoverer software (version 3.1, Thermo), which uses spectral matching to online compound databases (ChemSpider, mzCloud) and FISH scoring to validate MS/MS spectral matches. This broad annotation method allows the annotation of all phytochemicals with recognition of these compounds as tentative identifications awaiting verification by reference standards [26].

2.7. Docking study

In addition to their anticancer effects on cancer *in vitro*, the anticancer properties of phytochemicals derived from *B. salicina* were assessed through a docking study involving the human cyclin-dependent kinase 2 enzyme (CDK2) (PDB: 1HCK). The choice of the 1HCK PDB structure was based on multiple considerations, such as its origin from human sources, high resolution, absence of mutations, crystallization in the presence of an inhibitor for comparison with docked ligands, and identification of the active site. The protein structure was sourced from the Protein Data Bank (PDB) through a website (https://www.rcsb.org/) and was downloaded in PDB format. The molecular structures of the 9 identified compounds were retrieved from the PubChem search database in either 2D or 3D SDF file formats. Throughout the docking analysis, the protein and phytochemical PDB files were unequivocally submitted to the CB-Dock server for the docking procedure [27]. The receptor and ligand input files underwent rigorous automatic optimization via the CB-Dock server. The docking process was conducted using certain specifications for the active site, including a cavity volume of 1180 Å3, and the center coordinates were precisely set at X = 100, Y = 99, and Z = 80. The resulting conformations were subsequently analyzed and visualized via both the CB-Dock server and Chimera software tools. The inhibition constant (Ki) was accurately determined via the following formula:

$$Ki = exp\ (\Delta G/RT)$$

ΔG represents the binding energy, R represents the universal gas constant, and T represents the temperature. The drug likeness of the phytochemicals was rigorously evaluated via the SwissADME web server to assess adherence to Lipinski's rule of five.

2.7.1. Binding free energy computation via MM/GBSA

The rescoring method known as MM/GBSA was employed to validate the binding affinities derived from docking studies. The binding free energy of ligands to proteins is typically calculated via a combination of molecular mechanics with the generalized born and surface area solvation method (MM-GBSA). Binding free energy calculations are generally more accurate than molecular docking calculations because they consider solvation effects [6,28]. The free energies of the protein–ligand complexes were computed via fastDRH [29] alongside the MM/GBSA method. For the calculations, the force fields for the receptor and ligands were set to ff19SB, using the OPC water model for the receptor and GAFF2 for the ligands. In this approach, free binding energy is determined on the basis of the variance between the complex free energy and the free energies of its individual components. This relationship can be expressed as:

$$\Delta Gbind = \Delta Gcomplex - (\Delta Gprotein + \Delta Gligand)$$

2.7.2. Prediction of anticancer potential

The anticancer potential was predicted computationally via the software CLC-Pred (Cell Line Cytotoxicity Predictor, version 2.0) by assessing the cytotoxicity of the 9 compounds present in the extract. This server utilizes experimental data to determine the cytotoxicity of the compounds against various colon cancer cell lines. SMILES format was used to submit the compounds to the server with a *Pa* value exceeding 0.3. The *Pa* value ranges from zero, which indicates no activity, to one, which denotes complete activity.

2.8. Statistical analysis

A statistical analysis system (SAS) for data analysis (University version 9.4) and SPSS software were used to analyze the data. All the results are presented as the means ± SDs. Differences among groups were evaluated by one-way analysis of variance (ANOVA), and those with $p < 0.05$ were considered significant.

3. Results and discussion

The increasing demand for health-promoting natural substances has encouraged the exploration of plant species with exceptional chemical profiles and their application in pharmaceuticals. Here, we investigated for the first time the anticancer, apoptotic, antioxidant, and chemical compositions of successive extracts obtained from *B. salicina* roots. Different antioxidant properties, such as DPPH, TPC, TFC, and ABTS, were investigated. The anticancer assay was conducted via MTT on HT-29 cells, and the apoptotic potential was assessed via microscopy with acridine orange and propidium iodide. LCMS/LC-HRM was subsequently used to identify the principal chemical compounds of the most active fraction. Successive extraction has the potential to maximize the efficiency and recover all the active ingredients of the extract compared with single-solvent extraction [30]. Therefore, in this study, four solvents (ethanol, water, ethyl acetate, and n-butanol) were used in the extraction method. The results indicated that the extraction yields varied in accordance with the polarities of the solvents used in the extraction (Figure 2). The fraction with the highest yield was the water fraction (51.5%),

followed by the n-butanol fraction (17.1%) and ethyl acetate (14.8%), which resulted in the lowest yield of the ethanol fraction (2.2%).

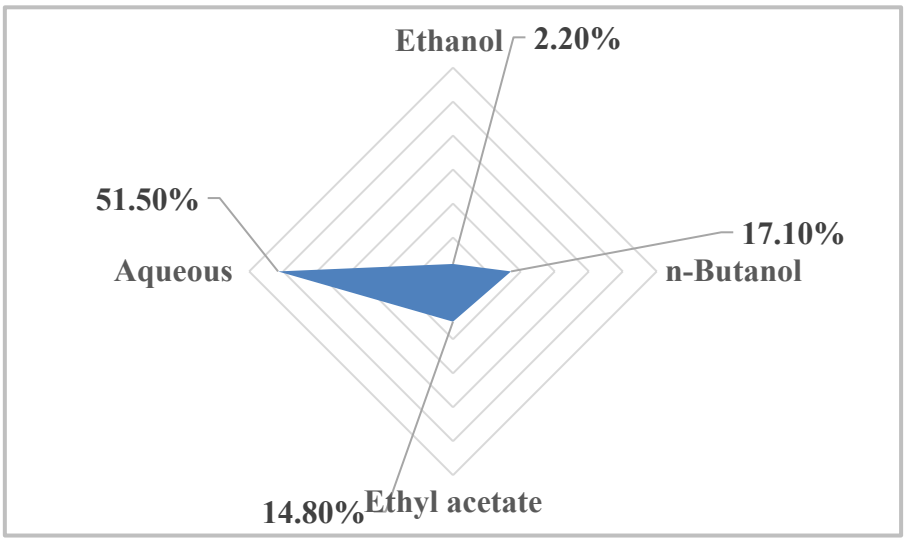

Figure 2. Percentage yield of extract from different solvents.

3.1. Total phenolic content of B. salicina root

The total phenolic content is considered one of the standard methods for verifying the antioxidant activity of plants [2]. The principle of this method is to quantify the total phenolic compounds in the plants [31]. Phenolic compounds constitute a significant main group of bioactive plant compounds with antioxidant properties that serve to terminate free radicals [32]. This warranted an investigation into the total quantity of these compounds in the *B. salicina* root extracts under investigation. The presence of phenolic compounds in the *B. salicina* fractions is illustrated in Table 1. The total amount of phenolics in the *B. salicina* root extracts was determined to be significantly different among all the fractions. Notably, the choice of solvent for extraction markedly influenced both the yield of extracted polyphenols and the antioxidant potency of the resulting extracts. The root ethanol extract (REE) presented the highest TPC, followed by the EAR and NBR fractions, whereas the water fraction presented the lowest TPC. This variation in the TPC among the fractions of *B. salicina* is due to the extraction solvent properties that were used. Each solvent used in the extraction significantly affects the yield of extracted TPC and the antioxidant properties of the extract. However, the results indicated that moderately polar solvents, such as ethanol, are more effective at solubilizing a greater number of phenolic compounds from *B. salicina* roots than other solvents are. This is due to the amphiphilic nature and solubility power of the ethanol solvent in obtaining both polar and nonpolar phenolic compounds from the extract. This finding was in line with prior studies suggesting that ethanol solvent extracts contain high amounts of phenolics and flavonoids [33,34]. Similarly, in a recent study, ellagic acid pentoside (polyphenol compound) and gallic acid (phenol) were identified in the root extract and fraction of *B. salicina* via UPLC–QTOF–MS analysis [18]. In addition, our results indicated that most of the phenols present are nonpolar aromatic rings, such as esters or glycosides [35]. Similarly, the water extract (which is highly polar) presented the lowest TPC, which indicates that fewer polar phenolic compounds are present in the roots of *B. salicina*. Similarly, other solvents, such as n-butanol

and ethyl acetate, that were used in the fractionation had a significant effect on the yield and on the extracted phenols or the antioxidants of the extracts. The researchers in [30] reported that using different solvents in successive extractions has the ability to extract relatively high amounts of phenolic compounds with varied antioxidant activities. In addition, the TPC almost doubled with increasing concentration. This is also due to the efficiency of the utilized solvents, as higher amounts of phenolic compounds and various activities can be obtained in successive extractions [36]. However, other antioxidant assays have also been conducted to assess other antioxidant activities of *B. salicina* roots and provide a holistic view of their overall effective properties.

Table 1. Total phenolic content (TPC) of *B. salicina* roots.

	Concentration GAE/g Extract ± SD				
Samples	0	200	400	800	p value *
REE	0.0	53.4 ± 3.2	99.5 ± 2.5	196.5 ± 2.7	< 0.005
EAR	0.0	47.4 ± 0.5	85.7 ± 1.6	167.6 ± 1.7	< 0.005
NBR	0.0	47.1 ± 1.7	92.8 ± 1.8	179.3 ± 5.3	< 0.005
AQR	0.0	20.2 ± 3.1	36.3 ± 0.7	71.5 ± 3.5	< 0.005

The values represent the means ± SDs of 3 replicates. REE = 95% Root Ethanolic Extract, EAR = Root Ethyl acetate Fraction, NBR = n-Butanol Root Fraction, AQR = Aqueous Root Fraction. *All concentrations of different extracts had significantly high Trolox/g equivalence, with $p < 0.05$, according to one-way ANOVA post hoc (Dunnett's test).

3.2. Total flavonoid content (TFC) of B. salicina roots

Phenolic and flavonoid compounds play crucial roles as primary antioxidants in plants and are responsible for the appealing colors of plants, fruits, and flowers. Its significant role in preventing and treating many diseases, including inflammation and cancer, has been well established [1]. These compounds can effectively interact with various radicals, i.e., hydroxyl, superoxide, and lipid peroxides [23]. Our TFC results revealed that the EAR fraction presented the highest TFC, followed by the root ethanol extract and n-butanol (Table 2). The water root extraction had the lowest TFC, which followed the same trend as the TPC presented earlier. In general, most flavonoid compounds are polar, but our results indicate that the flavonoid compounds present in *B. salicina*, such as flavanols, flavones, isoflavones, and flavanones, are of low polarity. These flavonoid compounds have affinity for solvents such as ethyl acetate [37]. Notably, this tendency might be due to the power of the extraction solvent polarity. Ethyl acetate has moderate polarity and can dissolve polar and nonpolar flavonoid compounds. Other factors, such as extraction temperature, selectivity, and pH, also affect flavonoid solubility [38]. The method of extraction and extraction temperature influence the solubility of a wide range of thermolabile bioactive compounds, such as flavonoids, and other nutritional components [39,40]. This finding indicates that the selection of solvent has an extreme influence on the solubility and extraction of phenols in successive fractionation. Moreover, the extraction of flavonoids, aglycones, and other methoxylated functional groups requires solvents with moderate polarity, such as ethyl acetate or hexane [41]. The solvent strongly influences the number and properties of extracted compounds [42]. Moreover, in succussive extraction, more flavonoid compounds are extracted than in pure solvent extraction [43]. In addition, this technique promotes the

presence of diverse phytochemicals in extracted fractions via the selection of a solvent that has high solubility to obtain compounds of interest while preserving their thermal stability.

Table 2. Total flavonoid content (TFC) of the root.

	Concentration Quercetin/g Extract ± SD				
Samples	0	200	400	800	p value*
REE	0.0	5.7 ± 2.1	9.2 ± 1.6	16.8 ± 3.6	< 0.005
EAR	0.0	7.4 ± 3.1	10.8 ± 2.2	19.4 ± 0.8	< 0.005
NBR	0.0	4.1 ± 1.4	5.9 ± 1.6	7.2 ± 2.6	< 0.005
AQR	0.0	2.2 ± 3.1	3.2 ± 0.9	4.6 ± 4.1	< 0.005

The values represent the means ± SDs of 3 replicates. REE = ethanol extraction, EAR = root ethyl acetate fraction, NBR = n-butanol root fraction, AQR = aqueous root fraction. *All concentrations of different extracts had significantly high Trolox/g equivalence, with $p < 0.05$, according to one-way ANOVA post hoc (Dunnett's test).

3.3. DPPH activity of B. salicina roots

In the pharmaceutical and food industries, natural antioxidants have been extensively investigated. Several techniques have been developed to evaluate the free radical-scavenging capacity of plant extracts and their overall antioxidant activity. The most widely used and reliable technique for measuring free radical reduction involves the use of the DPPH assay and a spectrophotometer. This assay accurately measures the antioxidant properties of plant extracts, individual compounds, and food products [44]. Therefore, in our study, a DPPH assay was employed to assess the antioxidants present in *B. salicina* root extracts and fractions. The results demonstrated that the *B. salicina* root extracts and fractions contained a substantial number of antioxidants. The highest DPPH activity (25 mg/ml) was detected in the ethyl acetate fraction, followed by the ethanolic extract of n-butanol (as shown by the $IC_{50\ values}$ in Table 3). The water extract had the least DPPH activity. This is attributed to the solvent power of ethanol and its ability to dissolve hydrophilic and lipophilic compounds. This result was in line with recent work from the authors in [18] in 2021, as they reported that the root extract showed a strong DPPH activity of 46.6 mg/ml. Moreover, the results indicated that the DPPH activity of the *B. salicina* extract, and fractions varied in accordance with the solvent polarity used. Work by the researchers in [1] confirmed that solvent polarity and conditions have a great impact on DPPH activity. The antioxidant activity of *B. salicina* roots is due to the presence of several active constituents, such as Asiatic acid (triterpenoid), hexadecane, and lupeol, which possess strong antioxidant activities [12,18].

Table 3. DPPH radical scavenging activities of *B. salicina* roots.

	Concentration Trolox/g Extract ± SD				IC_{50} mg/ml	p value *
Samples	0	200	400	800		
REE	0.0	52.4 ± 1.2	103.4 ± 1.2	209.1 ± 0.5	28.14	< 0.005
EAR	0.0	65.3 ± 1.5	129.6 ± 1.3	259.6 ± 1.6	25.16	< 0.005
NBR	0.0	48.5 ± 1.3	90.4 ± 1.4	178.4 ± 0.7	30.13	< 0.005
AQR	0.0	27.5 ± 6.1	56.4 ± 0.8	111.8 ± 1.6	39.12	< 0.005

The values represent the means ± SDs of 3 replicates. REE = Root Ethanolic Extract, EAR = Root Ethyl acetate Fraction, NBR = n-Butanol Root Fraction, AQR = Aqueous Root Fraction. *All concentrations of different extracts had significantly high Trolox/g equivalence, with $p < 0.05$, according to one-way ANOVA post hoc (Dunnett's test).

3.4. Radical ABTS cation scavenging properties of B. salicina roots

The ABTS Cation Radical Scavenging assay is widely used for evaluating the antioxidant properties of various samples [23]. In this assay, the overall antioxidant capacity of a compound is assessed via an electron transfer approach. This can be accomplished via spectrophotometric quantification after the dark blue ABTS•+ radical cation is converted to colorless ABTS [45]. In this work, an ABTS+ experiment was conducted to validate the antioxidant capacity of *B. salicina* roots. The results in Table 4 revealed that the ethyl acetate root fraction displayed a maximum ABTS•+ power of 58 mg/ml when 200 Trolox equivalents were used. This was followed by NBR 50.8 mg/ml and REE 45.6 mg/ml. Notably, the water extract had the lowest concentration of ABTS•+ (36.4 mg/ml). The results also indicated a dovely behavior of the samples, as ABTS•+ increased with increasing sample concentration. Following the earlier trend of the other antioxidant properties described in the previous section, the root ethyl acetate extracts displayed robust ABTS•+ with an IC_{50} of 22.5 mg/mL. In addition, when the highest REE concentration was used, the highest concentration of antioxidants (232.9 mg/ml) was also obtained among all the other fractions. Moreover, the ethanol extract exhibited antioxidant activity closely comparable to the DPPH results presented earlier. This has been noted by [23], who documented a high association pattern between the ABTS•+ and DPPH results. These results underscore the importance of antioxidant compounds in improving and increasing the phytochemical properties of the roots of *B. salicina*.

Table 4. Radical ABTS•+ scavenging ability of *B. salicina* root.

	Concentration Trolox Equivalent ± SD				IC_{50} mg/ml	P value*
Samples	0	200	400	800		
REE	0.0	45.6 ± 0.5	87.5 ± 1.4	175.5 ± 1.1	26.24	< 0.005
EAR	0.0	58.7 ± 1.5	118.9 ± 1.9	232.9 ± 1.3	22.53	< 0.005
NBR	0.0	50.8 ± 1.5	102.7 ± 2.1	200.4 ± 1.6	24.18	< 0.005
AQR	0.0	36.4 ± 1.7	72.5 ± 1.8	142.7 ± 1.4	28.12	< 0.005

The values represent the means ± SDs of 3 replicates. REE = Root Ethanolic Extract, EAR = Root Ethyl acetate Fraction, NBR = n-Butanol Root Fraction, AQR = Aqueous Root Fraction. *All concentrations of different extracts had significantly high Trolox/g equivalence, with $p < 0.05$, according to one-way ANOVA post hoc (Dunnett's test).

3.5. Anticancer effects of B. salicina root

In this study, the anticancer potential of different fractions of *B. salicina* was assayed for the first time. To assess the anticancer properties of *B. salicina* extract, an *in vitro* assay was conducted using the HT-29 human colorectal cancer cell line. The HT-29 cell line, which originates from human colorectal adenocarcinoma, is extensively employed in cancer research owing to its rapid proliferation and marked resistance to apoptosis. These attributes make HT-29 cells an ideal model for investigating the cytotoxic potential of prospective anticancer compounds. In addition, the water-soluble MTT salt, which is often used to determine cell viability, was employed in this study. In living cells, succinate dehydrogenase, a mitochondrial enzyme, catalyzes the conversion of MTT to insoluble purple formazan crystals by cleaving the tetrazolium ring. The more viable the cells are, the greater the degree of formazan production. The percentage of viable cells was determined as the percentage of purple formazan crystal absorbance. The anticancer effects of the extract and fractions of *B. salicina* are shown in Figure 3. These results indicate that the extract and fractions of *B. salicina* are considered active and have potent anticancer effects. Correspondingly, the results from the MTT analysis revealed that the viability of HT-29 cells decreased in a dose-dependent manner. All the extracts and fractions of the plants significantly ($P < 0.001$) inhibited HT-29 cell growth, with EAR accounting for the lowest IC_{50} at 22.5 µg/mL compared with the standard of 25.3 µg/mL (Figure 5). According to the American National Cancer Institute's classification system, plant extracts are evaluated for their cytotoxicity against human cancer cells on the basis of their IC_{50} values. Extracts with an IC_{50} of ≤ 20 µg/mL are regarded as potent anticancer extracts, extracts with an IC_{50} of 21−100 µg/mL are classified as active, extracts with an IC_{50} of 200−500 µg/mL are deemed moderately active, and extracts with an IC_{50} ≤ 1000 µg/mL are categorized as inactive [46]. In our study, the extract and fractions presented IC_{50} values ranging from 22.5 to 69.7 µg/mL, which indicated significant active power. These findings indicate that *B. salicina* is a potent anticancer fraction due to the presence of different antioxidants and powerful phytochemicals.

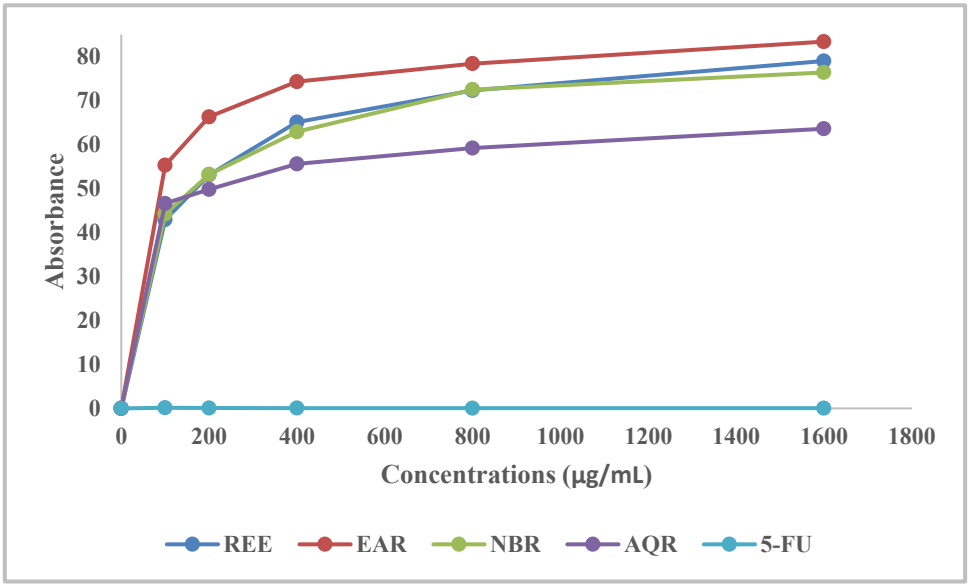

Figure 3. IC_{50} standard curve for the inhibition of HT-29 cells by the extract and the standard: REE = ethanolic extract, EAR = root ethyl acetate fraction, NBR = n-butanol root fraction, AQR = aqueous root fraction, 5-FU = 5-fluorouracil.

3.6. Apoptotic effect of B. salicina

To investigate the effect of *B. salicina* extract on apoptosis, a qualitative analysis of HT-29 cells was conducted via the use of acridine orange and propidium iodide (PI) stains. The fluorescent nucleic acid dyes acridine orange (AO) and propidium iodide (PI) are commonly employed in combination to distinguish viable and nonviable cells via *in vitro* approaches. The AO dye stains the viable cells green, whereas the PI dye stains the apoptotic cells red. The most active fraction was screened for qualitative apoptosis via microscopy and upon staining with AO and PI dyes. Our results revealed that *B. salicina* root extract drastically induced the death of HT-29 cells. The images of the HT-29 cells revealed early and late apoptosis and necrosis in a timely manner, as shown in Figure 4. AO dye stains viable cells green, while PI dye stains apoptotic cells red. The intensity of the red coloration due to PI staining predicts whether the degree of apoptosis is early, late, or necrotic. A normal green nucleus indicates live cells, pale red cells indicate late apoptosis, and red coloration of the cells indicate late apoptosis, whereas intense red coloration of the cells indicates that the cells undergo necrosis, as shown in Figure 4. Figure 4 shows the apoptotic effects of *B. salicina* extract on HT-29 cells. Cytotoxicity can affect nucleic acids before the mitochondria, prior to the onset of apoptosis and necrosis [47]. Apoptosis is a physiological process that eliminates unregulated, abnormal, or damaged cells and is also known as programmed cell death. It is also a crucial endpoint for identifying anticancer drugs, as it targets any abnormal or damaged cells [23]. Promising anticancer agents frequently target this process during experimental evaluation. The use of apoptosis as a screening method allows scientists to exclude agents that can cause unspecific cytotoxicity, saving time and resources. A wide range of natural substances capable of inducing apoptosis have been discovered and well documented [48]. These valuable natural substances are frequently found in plant sources and are consumed by humans in small amounts [49]. Therefore, it is important to investigate apoptosis inducers from these resources.

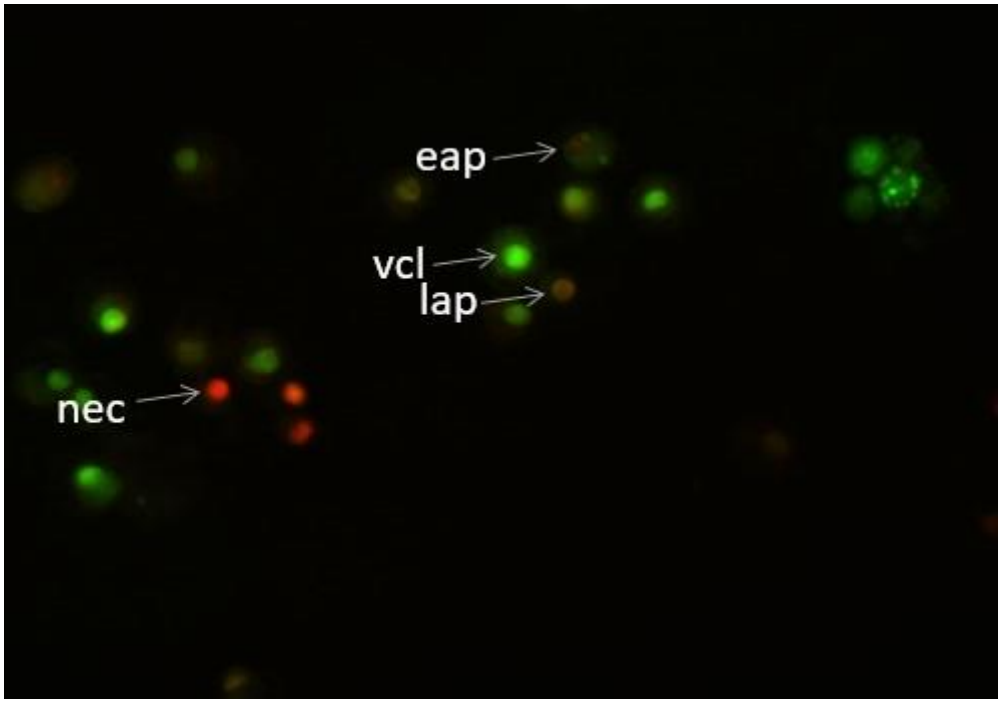

Figure 4. Morphological features of HT-29 cancer cells treated with the ER fraction and stained with AO/PI staining reagents and viewed under a fluorescence microscope; magnification X400 key: eap = early apoptosis, lap = late apoptosis, nec = necrotic cells and vcl = viable cells.

3.7. Chemical compounds present in B. salicina roots

The identification of the chemical composition of a plant can help in identifying potent bioactive compounds with nutritional and medicinal applications [50]. Therefore, the most active extract of *B. salicina* root, the EAR fraction, was subjected to quantitative chemical analysis via LCMS/LC-HRM to identify the potent constituents attributed to its antioxidant, anticancer, and apoptotic properties. The major chemical compounds present in *B. salicina* roots identified via LC–MS/LC-HRM analysis are presented in Supplementary Table 1. The analysis revealed that *B. salicina* root comprises groups of active compounds, such as esters, polyphenols, coumarin derivatives, glycosides, and other natural compounds. This result was in agreement with an earlier investigation in 2024 by Tlhapi et al., who identified several similar compounds [12,17−19]. Notably, all the identified chemical compounds in the extract of *B. salicina* roots have diverse potential biological and pharmacological activities. Chemical quantitative analysis revealed the presence of polyphenols, namely, 4,5-dicaffeoylquinic acid and chlorogenic acid, which are both derived from quinic acid and have antioxidant effects [51]. Dicaffeoylquinic acid is a natural polyphenolic compound that is present naturally in plants such as coffee and herbal teas and is used by many medicinal plants [52]. These compounds are implicated in mechanisms of action, such as anti-inflammatory and antidiabetes effects [53,54], and inhibit cancer cell growth through cell cycle arrest. Plant polyphenols are well known to reduce breast cancer metastasis [3]. In 2024, Tlhapi et al. identified the presence of Dicaffeoylquinic acid and other isomers in an extract of *B. salicina* [18]. Another phenolic compound with potential therapeutic applications was 3,4,5-trimethoxyphenyl-6-O-pentopyranosyl-β-D-glucopyranoside. This glycoside compound is

commonly found in many plant roots, and it has anti-inflammatory [55] with cytotoxic and apoptotic activities [56]. Similarly, polyphenols were identified in the root extract and fraction of *B. salicina* via UPLC-QTOF-MS analysis [18].

Similarly, two coumarin derivatives, 4-hydroxycoumarin and 7-hydroxycoumarin, were found in the *B. salicina* extract. Coumarin and its derivatives have significant stability and solubility in solvents, which has sparked significant interest in the field of medicinal chemistry. Coumarin analogs have demonstrated an extensive range of pharmacological actions, i.e., anticoagulant, antibacterial, antifungal, antiprotozoal, insecticidal, fungicidal, antimycobacterial, and antimutagenic properties [57]. The compound 4-hydroxycoumarin significantly contributes to the composition of many nutraceuticals and functional plants and has strong anti-inflammatory and microbial potential [58]. In addition, it plays a crucial role in the treatment of thromboembolic diseases (top cause of death worldwide) through its anticoagulant properties [59]. Coumarins are polyphenols that have been proven to have widespread biological actions, i.e., anticancer effects. 7-Hydroxycoumarin was found to have anticancer effects on numerous cancer cell lines [60]. Geniposidic acid (another compound of *B. salicina)* is a natural iridoid glucoside present in several plants, such as *Gardenia jasminoides* and *Eucommia ulmoides* [61]. Geniposidic acid has the potential to control hypertension, inflammation, diabetes, cancer, atherosclerosis, and oxidative stress in cells [61,62]. In addition, esters or phytosterols (which resemble cholesterol) are present in the extract and can be found in many plants. Another natural flavonoid named 2-oxo-2H-chromen-7-yl-6-O-β-xylopyranosyl-β-D-glucopyranoside was also identified. Thus, our results indicate that the presence of these compounds provides good justification for the anticancer and antiapoptotic activity of *B. salicina* roots. Moreover, these compounds have significant biological effects, i.e., antioxidant, antimicrobial, and anti-inflammatory effects. Therefore, *B. salicina* has potential for various industrial applications, such as pharmaceuticals, cosmetics, and food additives.

3.8. Molecular docking analysis

The significant findings obtained from this research on the effects of antioxidants in conjunction with their cytotoxic effects on the HT-29 cell line and their potential for inducing apoptosis using root extracts led us to perform in silico analysis of the identified phytochemicals as potential anticancer agents. The results of the chemical compound docking analysis are shown in Table 5. To evaluate the reliability of the docking protocol implemented by the CB-Dock server, the crystallized ligand (ATP) was extracted from the binding site of the cyclin-dependent kinase 2 (CDK2) enzyme (PDB: 1HCK). The CB-Dock server successfully positioned the ligand within the active site of CDK2. Compared with that of crystalline ATP, the process of ligand reloading demonstrated nearly perfect accuracy (Figure 5 A and B), confirming the effectiveness of the docking protocol. The same hydrogen bonds present in the native structure involve the residues THR14, ASP86, and ASN132. Furthermore, the interaction with the ligand resulted in the formation of several hydrophobic interactions that were also observed in the original structure, specifically with ILE10, ALA31, and GLU81. The redocking of the cocrystallized ATP ligand into CDK2 resulted in an almost perfect alignment with the native pose, successfully reproducing essential hydrogen bonds and hydrophobic interactions, thus validating the effectiveness of the CB-Dock protocol.

CDK2 was chosen as the docking target because of its role in regulating the G1/S phase transition and its overexpression in colorectal cancers, which corresponds with the HT-29 cell model utilized in our research.

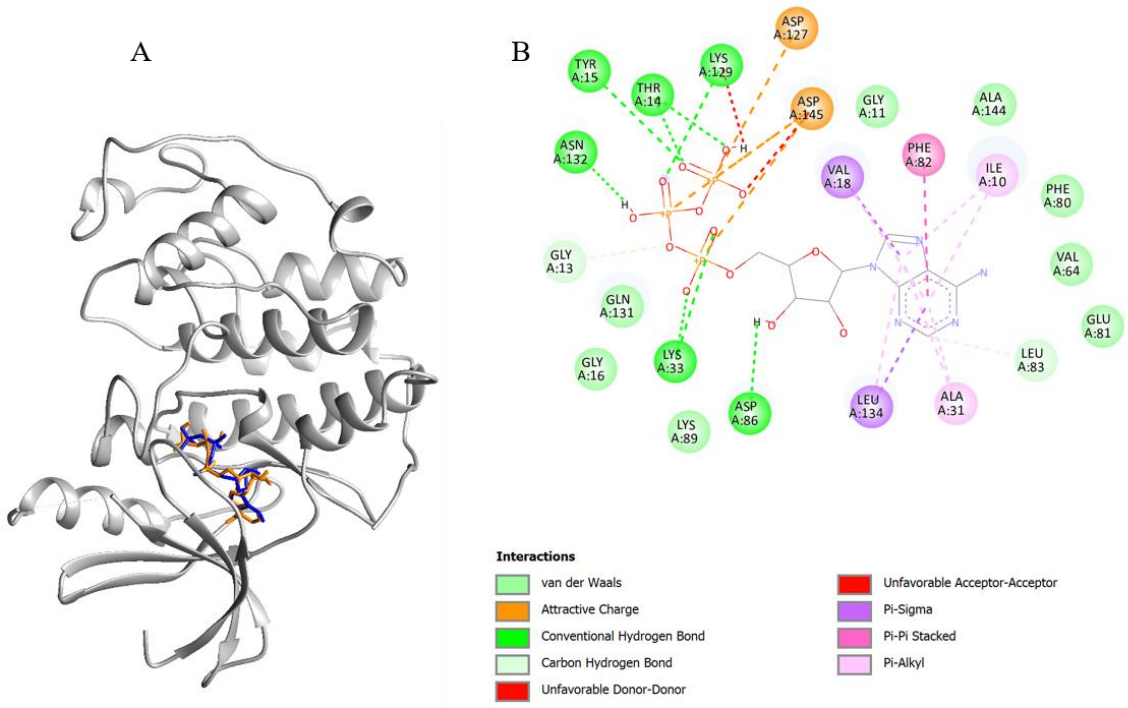

Figure 5. A: PDB structure of CDK2 (gray) alongside cocrystallized ATP (blue) and redocked ATP (orange). B: Contact interaction of the redocked ATP-CDK2 complex.

Table 5. Chemical compound docking analysis: No interaction; positive values, N/A; not applicable.

S/N	Compound	PubChem CID	Docking Score Kcal/mol	Inhibition Constant μM
Cocrystalized inhibitor	ATP	5957	− 8.3	0.78
Control 1	Flavopiridol	5287969	− 7.1	5.94
Control 2	Roscovitine	160355	− 8.0	1.29
Control 3	Dinaciclib	46926350	− 9.0	0.23
***Breonadia salicina* phytochemicals**				
1	4-Hydroxycoumarin	54682930	− 6.4	19.49
2	Geniposidic acid	443354	− 7.7	2.15
3	4,5-Dicaffeoylquinic acid	6474309	N/A	N/A
4	1-O-[(2alpha,3beta,5xi,9xi,18xi,19alpha)-2,3,19,23-Tetrahydroxy-28-oxoolean-12-en-28-yl]-beta-D-glucopyranose	45783065	− 7.8	1.81
5	Chlorogenic acid	1794427	N/A	N/A
6	2-Oxo-2H-chromen-7-yl-6-O-β-xylopyranosyl-β-D-glucopyranoside	10072745	− 8.9	0.28
7	7-Hydroxycoumarine	5281426	− 6.8	9.87
8	3,4,5-Trimethoxyphenyl-6-O-pentopyranosyl-β-D-glucopyranoside	23844027	− 2.0	33.74

3.9. MM/GBSA analysis

The binding free energy of the most favorable docked compound, number 6, was calculated via MM/GBSA to validate the findings from the docking study. The binding free energy for compound 6 and the reference ligand in association with the CDK2 enzyme was analyzed, as shown in Table 6. Figure 6 shows the contact interaction of compound 6 with the CDK2 complex. All six procedures demonstrated a negative binding free energy for the bond complexes. A negative value of $\Delta Gbind$ signifies a greater binding affinity between the two molecules, implying a more stable and energetically advantageous construction of the complex.

Table 6. Binding free energy (kcal/mol) of compound 6 and the reference ligand in association with the CDK2 enzyme.

Procedure	Compound 6	ATP (Control)
GB1	− 45.5	− 49.4
GB2	− 35.7	− 39.5
GB5	− 34.1	− 38.4
GB6	− 9.3	− 16.8
GB7	− 22.9	− 32.2
GB8	− 23.3	− 33.6
Mean	− 28.4	− 34.9

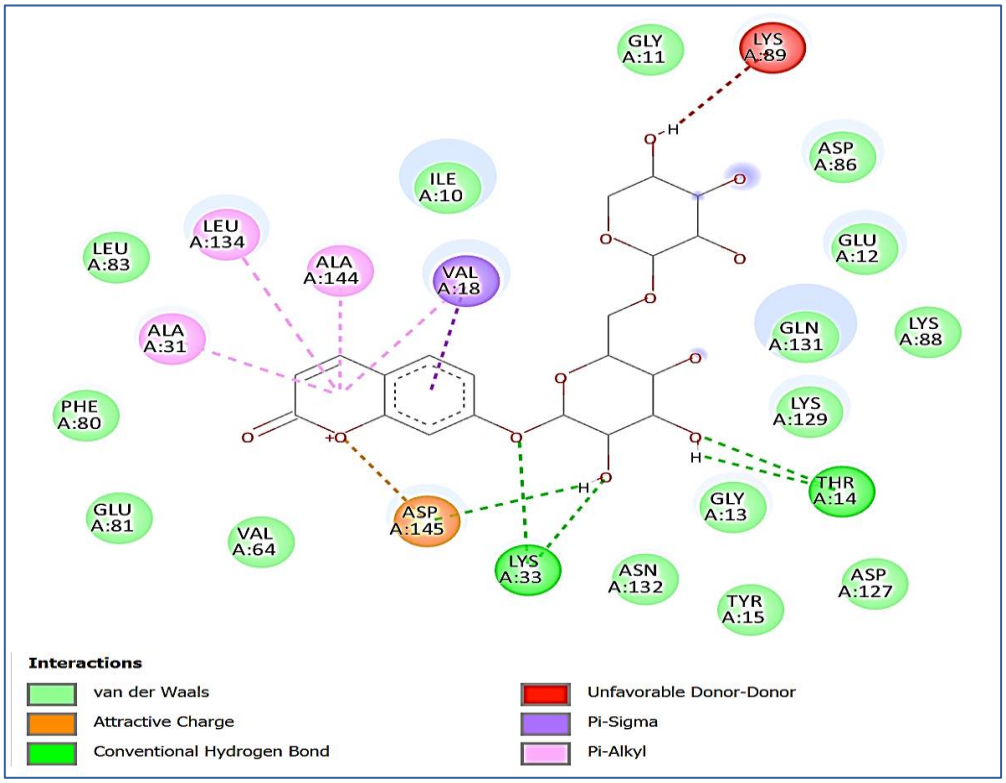

Figure 6. Contact interaction of the compound 6-CDK2 complex.

3.10. Assessment of compound cytotoxicity

The anticancer activities of the 8 compounds were assessed via the CLC-Pred server, which calculates their cytotoxicity toward several colon cancer cell lines (Table 7). The results indicated that four of nine compounds exhibited cytotoxic effects against six different colon cancer cell lines. Compound 4 yielded the highest predictive value, 0.478, against colon adenocarcinoma (HCT-15). The potential cytotoxic effects observed in colon cancer cell lines align well with the findings obtained from *in vitro* studies. The 8 phytochemicals that were docked exhibited significant inhibitory activity, as indicated by binding affinity scores that were lower than − 5.0 kcal/mol. A binding energy below − 5.0 kcal/mol signifies a robust binding affinity between the receptor protein and the ligand [29]. However, some research indicates that binding values below − 6.0 kcal/mol, and in certain instances below − 8.0 kcal/mol, are considered optimal thresholds for identifying potential candidates [63]. The docking analysis indicated that compound 6 achieved a noteworthy inhibition score of − 8.9 kcal/mol. This score exceeded that of two FDA-approved drugs, flavopiridol, and roscovitine, and was nearly equivalent to the score achieved by dinaciclib, as illustrated in Table 7. Upon reviewing prior research on any correlation between the nine compounds and CDK2, we found that no studies have been conducted on compound 6, which exhibited the most significant inhibition score against the CDK2 enzyme. The approach of targeting CDK2 shows immense potential in cancer therapeutics. CDK2 is essential for controlling the cell cycle, especially in facilitating the G1 to S phase transition and DNA synthesis. Overactivation of CDK2 is common in cancers, resulting in unregulated cell division and tumor development. Pharmaceutical agents targeting CDK2 inhibition can impede cancer cell multiplication by obstructing its associations with cyclins or its ATP-binding region. The suppression of CDK2 not only interrupts the cell cycle but also induces cancer apoptosis and increases the efficacy of complementary treatments. Numerous researchers have investigated medicinal plants and identified CDK2 inhibitors as potential anticancer agents. Compounds from plants such as *Aristolochia manshuriensis* have shown promise in preclinical cancer models through the inhibition of CDK2 [64,65]. Geniposidic acid (compound number 2), which achieved a notable docking score of − 7.7 kcal/mol, has been associated with the inhibition of CDK2 *in vitro*, thereby reducing the propagation and migration of human oral squamous carcinoma cells [66]. A previous computational analysis revealed that geniposidic acid has a significant inhibitory effect on CDK9, achieving a docking score of − 13.908 kcal/mol [64,65]. Therefore, the numerous bioactive compounds identified in our study, such as antioxidants, polyphenols, and flavonoids, could be used for health promotion and disease prevention. These plant materials, i.e., antioxidants, phytochemicals, and anticancer agents, can offer natural alternatives to synthetic compounds, which contribute to the development of new therapeutic agents in industry. In addition, the outcomes of this study can serve as a foundation for additional experimental research, potentially involving the assessment of the biological efficacy of the identified ligands through both *in vitro* and *in vivo* methodologies.

Table 7. The cytotoxic effects of the compounds were assessed through their respective potentials. *Pa* denotes the likelihood of a compound being active on the basis of the training set utilized in the IC_{50} prediction tool. The value of *Pa*, which indicates the probability of activity, varies from zero, indicating no activity, to one, indicating definite activity.

Compound	*Pa*	Cancer cell line	Tissue	Type
2	0.311	Colon adenocarcinoma (KM12)	Colon	Adenocarcinoma
4	0.478	Colon adenocarcinoma (HCT-15)	Colon	Adenocarcinoma
	0.395	Colon adenocarcinoma (HCC 2998)		
	0.389	Colon adenocarcinoma (DLD-1)		
8	0.366	Colon adenocarcinoma (Caco-2)	Colon	Adenocarcinoma
	0.354	Colon adenocarcinoma (HCC 2998)		
	0.339	Colon adenocarcinoma (DLD-1)		
	0.307	Colon carcinoma (RKO)		Carcinoma

This study was limited to *in vitro* and *in silico* analyses; therefore, the biological activities reported here should be interpreted as preliminary findings. The docking and MM/GBSA results provide theoretical support but require further validation through *in vitro* enzyme inhibition assays and *in vivo* models to confirm target specificity and pharmacological relevance. Additionally, differences in extraction efficiency, compound bioavailability, and experimental variability may influence the observed activities. In the future, researchers should also explore dose–response relationships, toxicity assessments, and comparative analyses with known CDK2 inhibitors to strengthen the mechanistic understanding.

4. Conclusions

This is the first investigation to report on the phytochemical composition, anticancer, and apoptotic activities of *B. salicina* roots, complemented by *in silico* toxicity studies. Our findings emphasize the potential phytochemicals of *B. salicina* as candidates for colon cancer treatment. The extract inhibited HT-29 cell line growth in a dose-dependent manner, demonstrating significant potential for apoptosis, which is attributed to its phytochemicals and antioxidant compounds that inhibit the CDK2 enzyme. The *in silico* study identified four new lead compounds for possible therapeutic applications. These findings offer a cornerstone for promoting the use of these phytochemicals in the advancement and development of medicines to enhance human health and well-being. These findings are also fundamental for promoting innovations in green chemistry to address pharmaceutical, environmental and industrial issues. In order to build these findings, further studies are needed to determine the individual compounds in the ethyl acetate fraction through advanced quantitative techniques. Furthermore, ethyl acetate fraction should be evaluated for its activity against a panel of cancer cell lines, employing multiple assays to assess its anticancer mechanisms.

Use of generative-AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Conflict of interest

All the authors declare and confirm that they have no conflicts of interest.

Author contributions

IU, SS, YY, SH, GMA: Carried out practical, AAA, HD; Molecular docking Analysis, SAR, AHI, AMD and AAH; Conceptualize the idea, analyze and drafted the manuscript. All authors read and approved the final version.

Availability of data and materials

The data that support the findings of this study are available in the article.

Ethical statement

The research was conducted in accordance with the protocol that was approved by the Health Research Ethical Clearance Committee of the College of Allied Health and Pharmaceutical Sciences, Kaduna State University, Kaduna, Nigeria (Ethical Approval No: Date 17/07/2020, A/CAP/KASU/20/259).

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