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Original Article

Antioxidant, cytotoxic activity, chemical composition and molecular docking of the stembark of Breonadia salicina Hepper and J. R. I. Wood

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Abstract

The study aimed to evaluate the antioxidant properties, chemical composition, and total phenolic and flavonoid contents of the stembark from Breonadia salicina. Furthermore, lead compounds were studied for their possible antioxidant and anticancer effects using in silico analysis. Antioxidant activity was measured using the ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid) and DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical scavenging assays. The chemical composition was analyzed with LCMS/LC-HRM (Liquid Chromatography-High Resolution Mass Spectrometry) technique. The ethyl acetate (EAS) fraction derived from Stembark exhibited significant antioxidant properties, as evidenced by its total phenolic content (146.8 ± 1.2 mg GAE/g), flavonoid contents (84.7 ± 4.6 mg Rutin/g), DPPH and ABTS radical scavenging activity, yielding values of 279.7 and 259 µg/mL, respectively. A total of twenty-one compounds were identified within the EAS fraction. Notably, compounds 7, 18, and 19 recorded the highest docking scores of -9.2, -9.2, and -9.3 (kcal/mol, respectively. Compounds 1, 5, 8, 9, and 13 demonstrated significant cytotoxicity against ovarian cancer, with a probability (Pa) greater than 0.92. Furthermore, compounds 1, 2, 3, 6, 8, 9, 10, 12, 15, 19, and 21 exhibited strong IC50 values of less than 1 µM. Among these, compounds 1 and 19 received the highest predictive scores from the three in silico assessment tools employed. Anacardic acid (compound 1) and SSR161421 (compound 19) achieved the best results, being recognized by two of the three anticancer in silico tools. These lead compounds may serve as promising candidates for the development of therapeutic agents aimed at specifically targeting cancer cells.

Keywords: Cancer, Medicinal plants, Phytochemicals, Antioxidants, Protein docking, Inhibitors.

1. Introduction

Throughout history, humans have relied on plants to support their health and overall well-being. Plants possess the remarkable ability to offer numerous health benefits to humans through the active compounds they produce for their growth, development, and protection [1]. The utilization of alternative therapies as a therapeutic approach is on the rise. The use of medicinal plants for treating various ailments holds significant importance in this regard [2]. Folk medicine is widely practiced in many parts of the world, with over 80% of individuals in Asia and Africa relying on plants and plant extracts to address a range of health issues [3]. Due to the unique chemical compounds present in them, which exert known physiological effects on both humans and animals, plants are considered to have medicinal properties [4].

The secondary metabolites produced by different parts of plants find extensive applications in various aspects of human life [5]. Whether in their crude or refined forms, these substances are recognized for their diverse biological significance. Phenolic compounds, particularly phenolic acids and flavonoids, are primarily found in plants and

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contribute to enhancing overall health [1]. Plants are endowed with antioxidant properties by them [6], inhibiting conditions caused by free radicals [7], due to their ability to scavenge and neutralize free radicals [6]. Researchers have increased their efforts significantly in drug discovery and development from plants in order to validate and explore ethnomedicinal plants [8].

The *Breonadia salicina* tree can reach a height of 20 metres and a diameter of 80 centimetres [9]. *Breonadia salicina* is a member of the Rubiaceae family. There are 10,700 species in 637 genera that make up the Rubiaceae family, making it one of the biggest angiosperm groups [10]. Wounds, arthritis, inflammation, ulcers, cancer, fevers, headaches, diabetes, and fungal and bacterial infections are traditionally treated with *B. salicina* [8].

Elevated environments, like high escarpments, are ideal habitats for *B. salicin*, which can reach a height of 40 meters and a diameter of 2 meters. Typically, you may spot these trees between 500 and 2000 meters above sea level, either on the sides of rivers or in the middle of the water where they're running [11]. Many medical conditions, such as cancer, gastrointestinal difficulties, fevers, headaches, arthritis, diabetes, inflamed wounds, ulcers, bacterial infections, and fungal infections, have been treated with the tree's valuable medicinal and economic properties [12]. Its antidiarrheal effects are also well-known [9]. Astringent qualities are a hallmark of *B. salicina* bark [9]. There have been limited studies on the plant's leaves, stems, bark, and roots, with most of those studies focusing on phytochemical and biological analyses [8, 10, 13, 14].

Modern mass spectrometry [15] has been useful in many areas, including the identification of specific molecular fragments, the discovery of new natural products, and the characterization of components in mixtures of plant extracts [16]. Leads compounds for ADMET criteria drug-likeness, adopting the traditional method for each of the compounds is time-consuming, laborious and expensive [17]. Consequently, computational applications assist in managing time and resources effectively [17]. *In silico* application helps in screening the number of compounds to be subjected to the wet laboratory. Based on our extensive literature search, no data have been published on the chemical composition of the stembark of *B. salicina.*

The objectives of this study were to identify the phytochemicals present in *B. salicina* and examine their antioxidant activity through *in vitro* and *in silico* analysis. Furthermore, the study aimed to predict the potential of these phytochemicals to act as anticancer agents by predicting the IC_{50} values. The research thus offers detailed insights into the antioxidant properties, chemical composition, and molecular docking of the analysed compounds through the use of LC-MS.

2. Materials and methods

2.1. List of chemicals

The chemicals purchased from Sigma-Aldrich, St. Louis, MO, USA include Aluminium chloride, Ferric chloride, Folin-Ciocalteu reagent, Gallic Acid, Hydrochloric acid, Sodium Hydroxide, Sulphuric acid, Quercetin and Rutin Diethyl ether, n-Butanol and n-Hexane. Ethyl acetate was purchased from Fisher Scientific, UK. While Ethanol was purchased from Park Scientific Ltd, Northampton, UK.

2.2. Plant collection and identification

Taxonomic classification was used to identify *B. salicina* specimens that were collected from their natural habitat and then deposited in the herbarium. The identification of the plant was thoroughly validated by a botanist, Dr. Sunusi Namadi from Ahmadu Bello University in Zaria, Kaduna State. ABU 900383 was the voucher number given to the specimens (Figure 1). The World Flora Online (WFO) at https://www.worldfloraonline.org/ was examined in order to verify the species name.

2.3. Extraction of the stembark of *B. salicina*

After drying and grinding the 300 g of *B. salicina* stem bark, a powder was produced. Next, it was macerated with 1L of 95% ethanol for 6 hours at 25°C and 200 rpm in a shaker (Stuart Scientific Great Britain) [7]. A Whatman filter paper No. 1 was used to filter the resultant extracts, and then a rotary evaporator was used to dry them (Buchi Labortechnik) under reduced pressure and at a temperature of 50 °C. Lastly, the extract was carefully transferred to a sealed container and then placed in a desiccator to be used later [7]. The formula for calculating the mass of crude yield is $F1/F2\times100$. The mass of the crude extract is represented by F1 in this equation, whereas the mass of the sample is represented by F2 [18].

2.4. Ethanol extract fractionating of the *B. salicina* Stembark

To prepare the stembark extract, 2.5 grams were soaked in 500 milliliters of aqueous and then sonicated for 10 minutes at 20 degrees Celsius. After that, 300 milliliters of n-Hexane were added, and the mixture was rocked for half an hour at 20 degrees Celsius using a Stuart Scientific Flask Shaker made in Great Britain [19]. We transferred the final mixture to another funnel and let it settle. The hexane fraction was evaporated using a Buchi Labortechnik rotary evaporator set to 50°C until it was entirely dry. Subsequently, 300 mL of ethyl acetate was used to extract the watery portion in the same manner as previously. The process was then repeated with n-butanol. Aqueous, ethyl acetate and n-butanol fractions were combined in a water

Fig. 1. *Breonadia salicina* (Vahl) Hepper & J.R.I. Wood growing in its natural habitat, showcasing its typical morphological features and environment.

bath and then moved to sample bottles where they were stored for further use [19]. Fractions Obtained from *B. salicina* ethanol extract: four fractions were acquired through a series of fractionations using three solvents, namely Hexane (HXS), ethyl acetate (EAS), aqueous (AQS) and n-butanol (NBS). The choice of hexane (HXS) is based on the reason that it is non-polar and therefore it is excellent in extracting non-polar substances such as lipids. Ethyl acetate (EAS) is a medium-polar solvent that can dissolve moderately polar substances such as flavonoids and alkaloids. Aqueous (AQS) solvents are highly polar and readily extract sugars, proteins, and ionic compounds. N-butyl alcohol (NBS) is amphiphilic, allowing semipolar compounds to be extracted, such as saponins and tannins, and is partially water-miscible. The combination of these solvents enables the systematic step-wise separation of a variety of chemical compounds in terms of solubility.

2.5. Total Phenolic Content (TPC)

Each sample (1,200 mg) of Stembark Ethanol Extract (SEE) and fractions of hexane (HXS), ethyl acetate (EAS), aqueous (AQS), and n-butanol (NBS) was combined with 25 µL of standard (gallic acid) and negative control (methanol), followed by the addition of 125 µL of Folin-Ciocalteu reagent (diluted 10 times in distilled water), and incubated at room temperature for 5 minutes. A saturated solution of NaHCO3 (100 µL of 7.5%) was added to the reaction mixture in a 96-well plate and thereafter incubated for 2 hours. All experiments were conducted in duplicate at varying concentrations. The fractions were mixed with different concentrations of gallic acid (0–600 µg/mL) [20]. Absorbance was spectrophotometrically measured at 725 nm using a microplate reader (Synergy H1, Biotec, USA). A gallic acid solution (1 mg/mL) was utilised to construct the standard curve, and the results were expressed as gallic acid equivalents (GAE) [20]. Standard curve equation of the gallic acid, $y= 0.0038x + 0.0673$ (Figure 2).

2.6. Total Flavonoid contents (TFC)

4 mL of filtered water, 0.3 mL of a sodium nitrate solution with a concentration of 5%, and 1 mL of extract were combined to create the mixture. After that, 0.3 mL of a 10% aluminum chloride solution, 2.4 mL of water, and 2 mL of 1 M sodium hydroxide were added. After that, the mixture was incubated for a full fifteen minutes. A spectrophotometer (Synergy H1, Biotec, USA). A wavelength of 510 nm was used to measure the absorption [20]. The test samples were composed of 50 milliliters of ethanol and 25 milligrams of standard rutin, ranging in concentration from 0 to 120 mg/g [20]. The generated data was expressed based on the equation (Figure 3), according to the equivalent of quercetin.

2.7. DPPH radical scavenging assay

The radical scavenging activity (DPPH) of the stembark ethanol extract (SEE), ethyl acetate stembark fraction (ESL), n-butanol stembark fraction (NBS), and the aqueous stembark fraction (AQS) was evaluated. Approximately 100 µL of extracts or fractions were combined with a 0.004% w/v DPPH solution in methanol. Extracts or fractions were produced at concentrations ranging from 0 to 800 µg/mL. After 30 minutes of storage in a dark atmosphere at 37°C, the mixture's DPPH reduction was measured using absorbance at 517 nm [21]. The formula to determine the radical scavenging activity is as follows:

$$\boldsymbol{Inhibition}\ \% = \frac{B - control - B - sample}{B - control} \times 100$$

where B-control is the control absorbance and B-sample is the test extract absorbance [21].

2.8. ABTS radical cation scavenging assay

Based on the methodology described by [21], the ABTS radical cation scavenging activity of the SEE, EAS, NBS, and AQS was ascertained. After mixing the ABTS (7 mM) and potassium persulfate (2.45 mM) solutions, they were left to incubate in darkness for 8 hours [21]. At 30°C, the absorbance was adjusted to 0.900 (±0.02) at 745 nm after diluting the stock solution with methanol. After mixing each sample (300 µL) with the ABTS working solution, the absorbance was measured. The concentrations of the samples ranged from 0 to 800 µg/mL in methanol. The following formula was used to determine the samples' and the standard's percentage scavenging property [21] :

$$\boldsymbol{The\ scavenging\ effect}\ \% = \frac{Control\ absorbance\ (ABTS) - sample\ absorbance}{control\ absorbance} \times 100$$

2.9. Liquid Chromatography Mass Spectroscopy of the stembark extracts of *B. salicina*

Sample Preparation

The master stock [15] and working stock (WS) for analysis were prepared by dissolving 1 mg of the stembark extracts of *B. salicina* in 1 mL of LC-MS grade methanol. The working stock concentration was adjusted to 10 µg/mL in methanol [16]. Prior to analysis, samples were filtered using a 0.22 µm PTFE membrane filter and transferred to 2 ml vials.

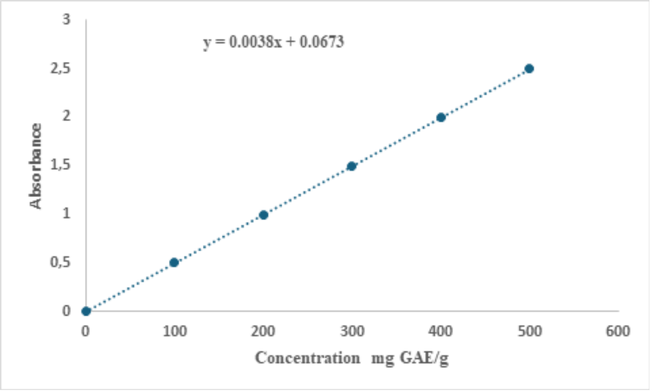

Fig. 2. Standard curve for Total Phenolic Content.

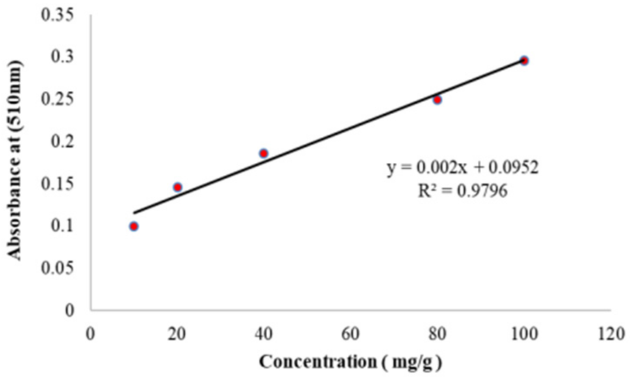

Fig. 3. Standard curve for the Total Content of Flavonoids.

2.10. LC-MS procedure and operating conditions

Liquid chromatography–mass spectrometry (LC-MS/LC-HRM) was used to profile the compounds in the EAS fraction. The reverse phase chromatography technique with solvent gradients of varying strength was utilized [16]. A complex technique for the search of the identified compounds was utilized, known as cloud search, to predict the compositions according to the full MS high resolutions and Chemspider searching to potentially identify the compounds. The spectra from MSn and MS/MS, ranging from different types of collision energy, are potentially selected from the available HRAM library fragmentation online. The sensations from ChemSpider were confirmed against the MS2 data using the built-in FISH scoring [22]. Thermo Scientific Compound Discoverer software version 3.1 was utilized to analyze the LC-MS/LC-HRMS results for matching compounds with online databases via cloud and ChemSpider [23].

2.11. Molecular docking

A docking study was conducted to examine the antioxidant effects of the 21 phytochemicals found in *B. salicina* against the human NADPH oxidase 5 enzyme (PDB: 8U85). The protein structure was obtained from the Protein Data Bank (PDB) website in PDB format. Using Chimera software tools, Chains B, C, and D were removed from the protein's PDB structure, leaving only Chain A for the docking analysis. The structures of the 21 phytochemicals were obtained from the PubChem Search database as 2 or 3D SDF files. These files, along with the receptor and ligand input files, were uploaded to the CB-Dock server and automatically optimized as described by Shityakov and Förster [24]. The docking procedure can be succinctly described as comprising three distinct phases: Search Cavities, View Results, and BlindDock. CB-Dock serves as a docking tool specifically designed for analyzing protein-ligand interactions. It autonomously identifies the binding sites, calculates their central coordinates and dimensions, modifies the docking box parameters in accordance with the specified ligands, and ultimately performs molecular docking utilizing the AutoDock Vina software, version 1.2.5. Before docking, all ligands and ions were removed by the server. Among the five cavities identified by CB-Dock, the C3 pocket was chosen as it aligned with the biologically significant active site where the co-crystallized FDA ligand was attached. This selection guaranteed that the interactions between the ligand and protein were assessed within the functionally critical catalytic area of NADPH oxidase 5. The docking outcomes were prioritized mainly according to binding free energy (ΔG) and inhibition constant (Ki), with the most energetically advantageous poses being chosen for additional examination. The docking process involved three steps: Search Cavities, View Results, and BlindDock. The parameters of the active site used for docking were a Cavity Volume of 1216 Å3 and a Center position of X = 147, Y = 118, Z = 144. The generated poses were evaluated and visualized using the CB-Dock server and Chimera software tools. The inhibition constant (Ki) was calculated using the formula: $Ki = exp(\Delta G/RT)$, where ΔG represents the binding energy, R is the universal gas constant (1.985 × 10-3 kcal mol-1 K-1), and T is the temperature (298.15 K). Super Pose Version 1.0 (http://superpose.wishartlab.com/) employs a modified quaternion method to compute the superposition of proteins. The discrepancies in structural variations are assessed based on both local and global root mean square deviation (RMSD) values.

2.12. Anticancer activity prediction

Cytotoxic effect of the 21 compounds was calculated using CLC-Pred (Cell Line Cytotoxicity Predictor) 2.0 [25]. The server is based on experimental data to assess cytotoxicity towards cancer cell lines. The compounds were uploaded to the server in SMILES format, with a *Pa* value of >0.9 to apply significant probability. *Pa* varies from zero, indicating no activity, to 1, signifying full activity.

2.13. IC_{50} for breast cancer cell lines

IC_{50} (Half-maximal inhibitory concentration) values of the 21 compounds were predicted for nine breast cancer cell lines (T47D, ZR-75-1, MX1, Hs-578T, MCF7-DOX, MCF7, Bcap37, MCF7R, BT-20) using BC CLC-Pred server [25]. BC CLC-Pred is an online tool that utilizes QSAR (quantitative structure-activity relationship) models developed with GUSAR software based on experimental IC_{50} values in nM (nanomolar) concentrations. The conversion of pIC50 to IC50 was carried out through the utilization of the software "PIC50". The software "PIC50" was employed to convert pIC_{50} into IC_{50} values. This particular software is the only open-source tool available for converting IC_{50} values to PIC_{50} values and vice versa, covering a range from millimolar to picomolar [26].

2.14. Estimation of pharmacokinetics properties

The estimation of pharmacokinetic properties was performed using the online platform http://www.SwissADME.ch. The evaluation of the enzyme inhibitory bioactivity of all the ligands was conducted by utilizing the Molinspiration server, https://molinspiration.com/cgi/properties. The ProTox-II Server was employed to calculate the cytotoxicity, https://tox.charite.de/protox3/index.php?site=compound_input.

2.15. Statistical analysis

Data were analyzed by Statistical Analysis System (SAS) software, University edition, 9.4. The experiment was conducted using a Completely Randomized Design (CRD) with three replications. A one-way repeated ANOVA was used in the study; the means were next compared using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ to assess the significance [7].

3. Results

3.1. Yields of extraction

The efficiency of the solvent in extracting specific components from the original material is quantified by the extraction yield. The extraction solvents used had a significant impact on the yield. Aqueous stembark fraction yields a higher recovery at 55.5 % (Figure 4). The ethanol extracts have shown a high yield, which can be attributed to the solvent's ability to extract a greater number of compounds from the samples (Table 1).

3.2. Quantification of total phenolic and flavonoid content

The quantification of TPC and TFC was presented in Tables 1 and 2, respectively. All the fractions present a

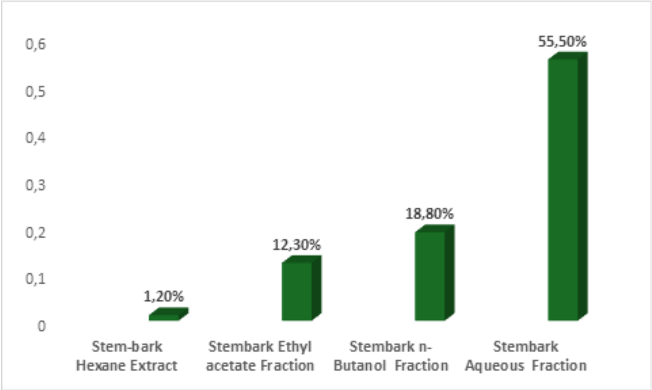

Fig. 4. Extraction yield % of the fractions.

significant amount of TPC and TFC. All the fractions revealed a trend of increasing TPC with rising concentration, with EAS having the highest value of 199.2 mg GAE/g extract (Table 1). Ethyl acetate fraction also recorded the highest TFC at 84.7±0.18 mg Rutin/g extract at 800 concentration, while other extracts had lower TFC values that significantly increased with concentration (Table 2).

3.3. Antioxidant activity of B. salicina fractions

The inhibitory activity against radical scavenging activities was demonstrated by all fractions (SEE, EAS, NBS, and AQS). The order of free radical scavenging capacity was 200 > 400 > 800 mg/g. The highest free radical scavenging capacity was seen at 800 µg/mL concentrations, although there were significant differences at ($P > 0.05$) (Table 3). The results demonstrated a substantial difference among the treatments. The sequence of free radical scavenging capacity was 200 > 400 > 800 µg/mL. The highest free radical scavenging capacity was observed at 800 concentrations, although there were notable variances at ($P > 0.05$) (Table 4).

3.4. Chemical profiling of *B. salicina* ethyl acetate Stembark fraction using LC/MS analysis

A total of twenty-one (21) chemical compounds were

Table 1. Total Phenolic Content (TPC) of the Stembark.

mg GAE/g Extract ± SD				
Sample	0	200	400	800
SEE	0.0	65.2±3.2^{a}	101±2.3^{a}	199.2 ± 3.6^{a}
EAS	0.0	40.6±1.5^{c}	73.5±0.6^{c}	146.8 ± 1.2^{c}
NBS	0.0	43.7±2.9^{b,c}	86.9±1.7^{b}	171.1 ± 1.6^{b}
AQS	0.0	25.2±3.2^{d}	49.2±2.3^{d}	96.4 ± 5.1^{d}

Values are expressed as means ± SD of three replicates. Values sharing the same letter are not significantly different at $p \leq 0.05$.

Table 2. Total Flavonoid Content (TFC) of the Stembark.

mg Rutin/g Extract ± SD				
Sample	0	200	400	800
SEE	0.0	5.1 ±1.6^{b,c}	8.4 ±1.5^{b}	14.1 ± 3.7^{b}
EAS	0.0	22.6 ±1.3^{a}	45.8±1.9^{a}	84.7 ± 4.6^{a}
NBS	0.0	4.2 ±3.3^{c}	5.9 ±1.2^{c}	8.8 ± 4.3^{c}
AQS	0.0	1.8 ±2.1^{d}	2.1 ±3.1^{d}	2.9 ± 2.5^{d}

Values are expressed as means ± SD of 3 replications. Values with the same alphabet do not differ significantly at the $p \leq 0.05$ level.

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

Sample	Concentration µg/mL				IC$_{50}$ µg/mL
	0	200	400	800	
SEE	0.0	63.2±2.5^{b}	122.5±2.1^{b}	243.1 ± 11.7^{b}	28.14^{c}
EAS	0.0	72.2±1.5^{a}	145.7±0.6^{a}	279.7 ± 0.5^{a}	25.16^{d}
NBS	0.0	46.5±2.5^{c}	98.4±1.5^{c}	198.7 ± 0.3^{c}	30.13^{b}
AQS	0.0	21.2±2.1^{d}	32.3±1.9^{d}	43.4 ± 12.3^{d}	39.12^{a}

Values are expressed as means ± SD of 3 replications. Values with the same alphabet do not differ significantly at the $p \leq 0.05$ level.

Table 4. ABTS Radical Cation Scavenging of *B. salicina* fractions.

Sample	Concentration µg/mL				IC$_{50}$ µg/mL
	0	200	400	800	
SEE	0.0	47.1±1.5^{b,c}	92.4±2.4^{c}	171.7 ± 8.4^{b}	26.24^{b}
EAS	0.0	62.7±3.5^{a}	135.0±1.9^{a}	259.3 ± 0.5^{a}	22.53^{d}
NBS	0.0	46.7±2.5^{c}	97.6±3.1^{b}	159.2 ± 6.8^{c}	24.18^{c}
AQS	0.0	28.3±1.6^{d}	49.5±1.9^{d}	97.4 ± 4.6^{d}	28.12^{a}

Values are expressed as means ± SD of 3 replications. Values with the same alphabet do not differ significantly at the $p \leq 0.05$ level.

detected through LC-MS/MS analysis, aided by Thermo Scientific Compound Discoverer software version 3.1 for real-time compound database comparison via cloud and ChemSpider, as shown in supplementary file Table S1. These compounds, spanning various classes such as flavonoids, glycosides, phenolic acids, triterpenoids, amides, and sulphonamides, are detailed in Table S1.

3.5. Molecular docking

The docking results showed that all 21 compounds had binding affinities greater than -5.0 (kcal/mol), as shown in Table 5. Out of the 21 compounds, SSR161421 demonstrated the highest binding affinity with the targeted NADPH oxidase 5 enzyme Figure 5. The SSR161421 compound exhibited a binding affinity of -9.3 (kcal/mol) in the active site of the receptor, as illustrated in Figure 6. The docking screening results indicated that all 21 compounds gave binding affinities higher than -5.0 (kcal/mol), as shown in Table 5. The pi-cation (cation−π) interaction represents an additional form of noncovalent binding observed in the interaction between SSR161421 and NADPH oxidase 5 via the two Arginine residues 251 and 463.

3.5.1. Cytotoxic effect of the compounds

Prediction of anticancer activity of the 21 compounds was calculated using the CLC-Pred server to evaluate cytotoxicity against cancer cell lines (Table 6). The statistical probability *Pa* utilized for the prediction was greater than 0.9 to ensure specific outcomes. *Pa* varies from zero, indicating no activity, to 1, indicating definite activity. The results showed that out of the 21 compounds, five compounds showed significant cytotoxic effect against ovarian carcinoma, A2780cisR (Cisplatin-resistant ovarian carcinoma). Using lower *Pa* values (e.g. > 0.5) resulted in cyto-

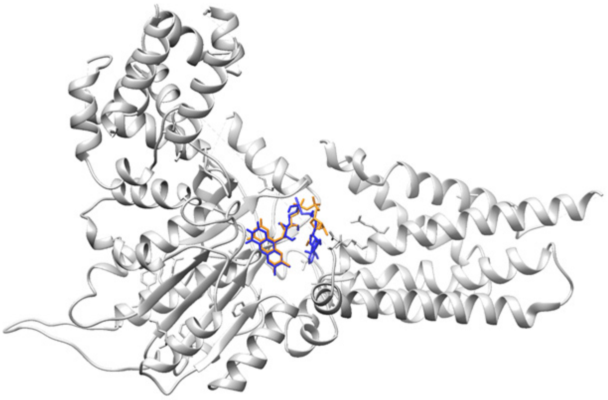

Fig. 5. Validation of the docking protocol. The PDB structure of NADPH oxidase 5 (gray) with the co-crystallized FAD (orange), re-docked FAD (blue).

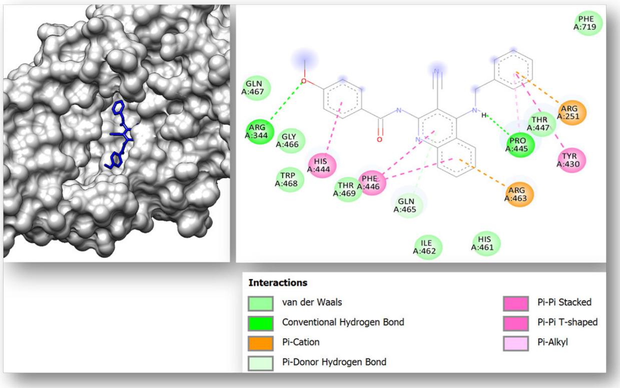

Fig. 6. On the left side, the cavity of NADPH oxidase 5 enzyme (gray), along with the presence of the SSR161421 compound (blue) inside the active site. On the right side, SSR161421-NADPH oxidase 5 enzyme interaction contacts.

Table 5. Docking scores of the 21 identified compounds with their inhibition constant.

Compound		PubChem CID	Docking Score (kcal/mol)	Inhibition Constant (µM)
FAD (Native Inhibitor)		643975	-10.5	0.01
Phytochemicals of *Breonadia salicina*				
1	Anacardic acid	167551	-6.2	27.5
2	Syringic acid	10742	-5.6	75.6
3	Prostaglandin A1 ethyl ester	12149511	-5.7	63.7
4	Genistein	5280961	-8.1	1.1
5	(+/-)19(20)-DiHDPA	35026912	-7.0	7.0
6	Cannabigerolic acid	6449999	-7.4	3.5
7	(2α,3β,19α)-2,3,19-Trihydroxyolean-12-en-28-oic acid	440025747	-9.2	0.1
8	5-[(Z)-Pentadec-8-enyl]benzene-1,3-diol	5281852	-5.6	75.6
9	11,12-Epoxy-(5Z,8Z,11Z)-icosatrienoic acid	5283204	-5.7	63.7
10	2,4,6-Trihydroxy-2-(4-hydroxybenzyl)-1-benzofu…….	103109950	-7.6	2.5
11	Quercetin	5280343	-8.6	0.5
12	Lariciresinol 4-O-glucoside	11972394	-8.8	0.3
13	Eriodictyol	440735	-9.0	0.2
14	Catechin	9064	-8.2	0.9
15	4-Dodecylbenzenesulfonic acid	8485	-6.6	13.9
16	Arjungenin	12444386	-8.1	1.1
17	Ursolic acid	64945	-8.6	0.5
18	18-β-Glycyrrhetinic acid	10114	-9.2	0.1
19	SSR161421	10201497	-9.3	0.1
20	Pelargonidin	440832	-7.7	1.9
21	Diisodecyl phthalate	33599	-7.1	5.9

Table 6. Cytotoxic effect of the compounds. *Pa* indicates the potential of an active compound, whereas *Pi* indicates the potential of an inactive compound as per the training set used in the IC_{50} prediction tool. *Pa* (probability to be active) ranges from zero, no activity, to 1, certainly active.

Compound	*Pa*	*Pi*	Cancer cell line	Tissue	Type
1	0.922	0.004	A2780cisR (Cisplatin-resistant ovarian carcinoma)	Ovarium	Carcinoma
5	0.932	0.003			
8	0.941	0.003			
9	0.947	0.003			
13	0.923	0.004			

Table 7. The IC_{50} of the compounds for breast cancer lines. pIC50 is a negative logarithmic expression of the half maximal inhibitory concentration (IC_{50}).

Compound	Breast cancer cell line	pIC_{50}	IC_{50} nM
1	MX-1	6.5895	257.3
2	MCF7-DOX	6.7488	178.3
	MX-1	6.5814	262.1
	T47D	5.1773	6648.1
3	MX-1	6.1637	685.9
4	MCF7-DOX	5.9778	1052.4
6	MX-1	6.0308	931.5
7	MX-1	5.7720	1690.4
	ZR-75-1	5.5706	2687.8
8	MX-1	6.6402	228.9
9	MX-1	6.3901	407.2
10	ZR-75-1	6.4226	377.9
12	MX-1	6.0422	907.4
	ZR-75-1	6.3500	446.6
15	MCF7-DOX	6.1440	717.7
16	MCF7R	5.7397	1820.9
	MX-1	5.7786	1664.9
	ZR-75-1	5.7312	1856.9
17	MX-1	5.5910	2564.4
18	MX-1	5.9164	2564.4
	ZR-75-1	5.4188	3812.4
19	MCF7-DOX	5.1259	7483.4
	ZR-75-1	6.1792	661.9
21	MX-1	6.5095	309.3

toxic activity against more cancer types, but *Pa* > 0.9 was selected for definitive or near-definite results. Compound 9, 11,12-Epoxy-(5Z,8Z,11Z)-icosatrienoic acid, exhibited the highest *Pa*, reaching a value of 0.947. Similar *Pa* values were observed by Anacardic acid, (+/-)19(20)-DiHDPA, 5-[(Z)-Pentadec-8-enyl]benzene-1,3-diol and Eriodictyol compounds, indicating their possible effect as anticancer against A2780cisR cancer cell line.

3.5.2. Prediction of IC_{50} values

The prediction of the IC_{50} values of the compounds against 9 types of breast cancer lines is presented in Table 7. Out of the 21 compounds that were identified, 16 of them exhibited inhibitory effects on 5 different breast cancer cell lines, namely MX-1, MCF7-DOX, T47D, ZR-75-1, and MCF7R. The significant number of compounds with anticancer properties underlines the extreme importance of *B. salicina* extract as a potential natural breast cancer treatment.

The majority of the compounds demonstrated significant IC_{50} values under 1000 nM Table 7. Out of the 16 compounds tested, 12 displayed inhibitory effects against the MX-1 cell line. Additionally, ZR-75-1 was affected by 6 out of the 12 compounds that showed inhibitory activity. Compound 2, Syringic acid, exhibited the most potent IC_{50} value of 178.3 nM. The following compounds exhibited strong IC_{50} values of less than 1µM against cancer cell lines: Anacardic acid, Prostaglandin A1 ethyl ester, Cannabigerolic acid, 5-[(Z)-Pentadec-8-enyl]benzene-1,3-diol, 11,12-Epoxy-(5Z,8Z,11Z)-icosatrienoic acid, 2,4,6-Trihydroxy-2-(4-hydroxybenzyl)-1-benzofuran-3(2H)-one, Lariciresinol 4-O-glucoside, 4-Dodecylbenzenesulfonic acid, SSR161421, and Diisodecyl phthalate as illustrated in Table 7.

3.5.3. Pharmacokinetic properties

The physicochemical characteristics of the 21 compounds were analysed as shown in Table 8. None of the compounds exhibited cytotoxic effects. Each compound met the Lipinski criteria for being considered a drug except compound 12, Lariciresinol 4-O-glucoside. The best enzyme inhibitor scores were 0.70 for 18-β-Glycyrrhetinic acid and 0.69 for Ursolic acid, respectively. Molecules with a polar area exceeding 140 Å in Topological Polar Surface Area (TPSA) exhibit the least capability to penetrate cell membranes. All the compounds that underwent

Table 8. Physicochemical and biological properties of the compounds.

Compound	PubChem CID	Lipinski	GI absorption	TPSA (Å²)	Enzyme inhibitor	Cytotoxicity
1	167551	Yes; 1 violation: MLOGP>4.15	High	57.53	0.20	Inactive
2	10742	Yes; 0 violation	High	75.99	-0.15	Inactive
3	12149511	Yes; 0 violation	High	63.60	0.37	Inactive
4	5280961	Yes; 0 violation	High	90.90	0.13	Inactive
5	35026912	Yes; 0 violation	High	77.76	0.45	Inactive
6	6449999	Yes; 1 violation: MLOGP>4.15	High	77.76	0.59	Inactive
7	440025747	Yes; 0 violation	High	97.99	0.60	Inactive
8	5281852	Yes; 1 violation: MLOGP>4.15	High	40.46	0.19	Inactive
9	5283204	Yes; 0 violation	High	49.83	0.56	Inactive
10	103109950	Yes; 0 violation	High	55.12	-0.08	Inactive
11	5280343	Yes; 0 violation	High	131.36		Inactive
12	11972394	No; 3 violations: MW>500, NorO>10, NHorOH>5	Low	167.53	0.36	Inactive
13	440735	Yes; 0 violation	High	107.22	0.21	Inactive
14	9064	Yes; 0 violation	High	110.38	0.47	Inactive
15	8485	Yes; 1 violation: MLOGP>4.15	High	62.75	0.24	Inactive
16	12444386	Yes; 1 violation: MW>500	High	118.22	0.61	Inactive
17	64945	Yes; 1 violation: MLOGP>4.15	Low	57.53	0.69	Inactive
18	10114	Yes; 1 violation: MLOGP>4.15	High	74.60	0.70	Inactive
19	10201497	Yes; 0 violation	High	87.04	0.07	Inactive
20	440832	Yes; 0 violation	High	94.06	-0.02	Inactive
21	33599	Yes; 1 violation: MLOGP>4.15	Low	52.60	-0.03	Inactive

testing demonstrated the ability to cross cell membranes except compound 12, Lariciresinol 4-O-glucoside. All compounds except compounds 12, 17 and 21 showed high drug absorption from the gastrointestinal tract.

In order to present a more comprehensible summary of the study results, the principal findings have been encapsulated in two graphical formats. Figure 7 depicts the antioxidant activities of the *B. salicina* fractions, with the ethyl acetate fraction (EAS) exhibiting the lowest IC_{50} values in both DPPH and ABTS assays, thereby affirming its enhanced radical scavenging ability relative to the other fractions. As illustrated in Figure 8, various phytochemicals exhibited strong binding affinities that are comparable to the native FAD ligand, with SSR161421, 18-β-Glycyrrhetinic acid, and Eriodictyol emerging as the most promising candidates. Their advantageous docking

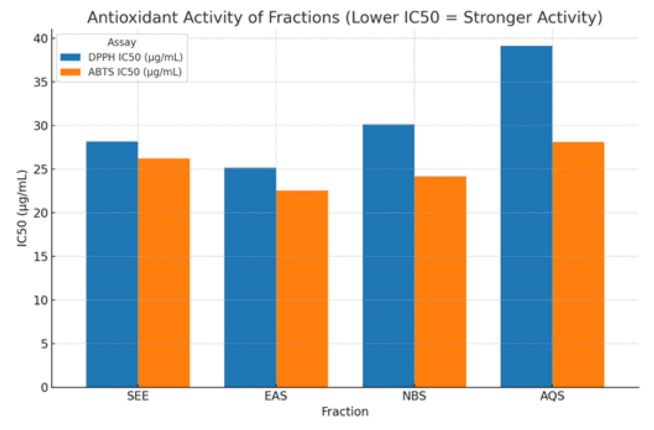

Fig. 7. A bar graph illustrating the IC_{50} values (μg/mL) for four fractions (SEE, EAS, NBS, and AQS) in relation to DPPH and ABTS radicals.

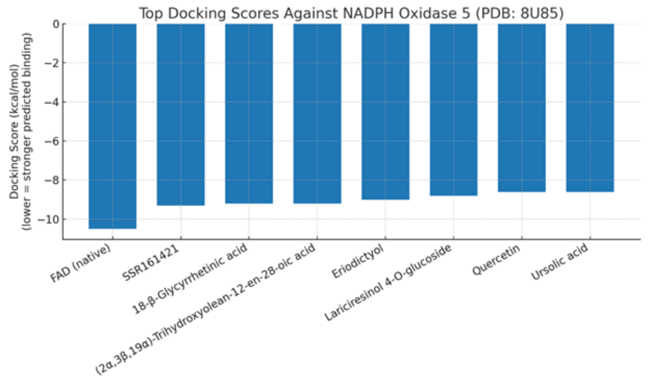

Fig. 8. Docking scores of key phytochemicals against NADPH oxidase 5.

scores and low inhibition constants indicate that these compounds could function as effective inhibitors of NADPH oxidase 5, thereby reinforcing their potential contribution to the antioxidant activity observed in vitro.

4. Discussion

The variation in the effectiveness of the solvent selection can be attributed to the polarity of the solvents and the nature of the compounds extracted by each solvent. The higher recovery of the aqueous stembark fraction (55.5%) suggests that water effectively solubilizes polar compounds. Poojary, Vishnumurthy and Adhikari [27] have reported a high yield of 10.43% from the root and bark of an aqueous extract of *Mammea suriga*, supporting this observation. Furthermore, Javadi, Abas, Hamid, Simoh, Shaari, Ismail, Mediani and Khatib [28] have reported a higher yield percentage from the aqueous extract compared to the ethanolic extract. However, Ramli, Radu, Shaari and Rukayadi [29] contradicted these findings by reporting a high yield of 8.21% from the ethanolic leaves extract of Indonesian *Syzygium polyanthum*. The extraction technique, solvent type, chemical type, and metabolite polarity all have a significant impact on the diversity in extract yield across medicinal plant sections. Many factors affect the yields, such as the weather, the plant's genetics, the portion of the plant used, how fresh it is, how long it takes to dry, and the method of extraction. Because of how complicated these aspects are, it is not feasible to make direct comparisons [18]. Variations in the fractions of various solvents are due to solubility, polarity, composition and sample matrix property differences. These elements determine the efficiency of the extraction of secondary compounds and can lead to differences in the observed results in analytical methods.

The evaluation of the total amount of phenolic compounds, well-known as TPC, is often performed using the Folin-Ciocalteu method. Similarly, the determination of TFC is done using the $AlCl_3$ method. The ethyl acetate fraction (EAS) superior performance in both TFC and TPC revealed it affinity for the extraction of medium-polarity phytochemicals. Phenols are known to be highly potent antioxidants that exhibit robust redox characteristics. The phenols possess the capacity of chelating metals, oxygen in the form of singlet or triplet is neutralized, and they also have the capacity of counteracting and absorbing free radicals [30]. Furthermore, flavonoids are class of polyphenol compounds, occur naturally and have the capacity to function effectively as donors of hydrogen and electrons [30]. Flavonoid compounds exhibit free radicals by electron delocalization and bonding of intramolecular hydrogen [30]. The observed total phenolic and total flavonoid fractions in the ethanol, ethyl acetate, n-butanol & aqueous solvent may be due to the solubility and polarity of the phenolic samples. Ethanol and ethyl acetate are more polar than the other solvents and would therefore produce higher yields of certain phenolic and flavonoid content, while n-butanol or aqueous would produce different yields and concentrations of the phenolic content.

Two standard techniques were used to assess the antioxidant power of *B. salicina*. The ABTS assay is used to determine the ability of *B. salicina* fractions to reduce the ABTS radical cation and, hence, their overall radical scavenging potential. The DPPH assay measures the ability to reduce the stable DPPH radical. As mentioned earlier by Dogara, Ibrahim, Mahmud, Danladi, Lema, Usman, Tahir and Tabti [1], the use of varied methods in assessment of antioxidant potential of extracts gives more effective and reliable data. Likewise, Tlhapi, Ramaite and Anokwuru [14] revealed stem crude extract of *B. salicina* had the highest antioxidant free radical scavenging activity, with IC_{50} value of 41.7 µg/mL, whereas the root crude extract showed the highest reducing power activity an IC_{50} value is 0.15 µg/mL. The inhibitory activity against ABTS radical cation scavenging was evident across all concentrations of the different fractions (SEE, EAS, NBS, and AQS). The results showed that the samples behaved in a dose-dependent way, with ABTS•+ rising as the sample concentration increased. Additionally, the EAS fraction demonstrated antioxidant activity in the ABTS•+ assay that was quite similar to the previously reported DPPH finding. The significant association pattern between ABTS and DPPH values was previously noticed by Dahham, Al-Rawi, Ibrahim, Majid and Majid [31].

The ethyl acetate fraction has the highest antioxidant activity, probably due to a higher level of active phytochemicals that might act as antioxidants since they complement each other. The positive correlation between the TPC, TFC and antioxidant assays (DPPH, ABTS) underscores the role of these compounds in scavenging free radicals. The radical cation scavenging effect and flavonoid content of the ethyl acetate stembark fraction extract are enhanced by the high concentrations of phenolic (1 GAE/g) and flavonoid (1 QE/g) components. Phenolic and flavonoid chemicals are essential because they interact with radicals such as hydroxyl, superoxide, and lipid peroxyl to form the main antioxidants. Many factors, including chemical makeup, polarity of metabolites, and properties of the extraction solvent, affect the efficacy of various plant components [1]. These results again indicate that ethyl acetate fraction is rich in antioxidant compounds because both DPPH and ABTS assays are consistent with fraction values. Ethyl acetate selectively solubilizes a specific set of phenolic and flavonoids with high radical scavenging ability, resulting in comparable antioxidant capacities obtained using different assays. The phytochemicals present in the EAS fraction, like the phenol and flavonoid compounds, help treat or delay different kinds of diseases in human. They are responsible for the fight against free radicals, cancer, heart disease, nerve damage, and inflammation and promote bone health [32]. Flavonoids are strong, water-soluble antioxidants that can scavenge free radicals, protecting cells from oxidative damage [33]. Therefore, based on these results, it can be presumed that Ethyl acetate is the best solvent for extracting phenolic, flavonoid compounds and other antioxidants from *B. salicina.*

Phytochemical study using the LC-MS/MS method revealed a complex phytochemical composition with high bioactive chemicals that could be used as therapy Table S1. The identification of a number of flavonoids and phenolic acid derivatives is highly correlated with known antioxidants and anticancer. This outcome was consistent with a previous study conducted by Tlhapi, Ramaite, Anokwuru and van Ree [12] in which they discovered a few related compounds. Research has established the following compounds playing a vital role against free radicals and cancer cells, making them valuable for therapeutic applications. The composition of compounds in an extract is influenced by various parameters, including temperature, drying period, geographic dispersion, plant species, freshness, and extraction method [18]. The following compounds were subjected to molecular docking research to evaluate their respective contributions to the biological activity of the EAS fraction. An integral component in the rational design of small molecules and the understanding of fundamental biological processes is the assessment of binding behavior of the EAS fraction.

The significant quantities of antioxidants present in the plant extract, coupled with their remarkable capacity to eliminate free radicals, motivated us to perform a thorough examination of the isolated phytochemicals as potential anticancer agents using an *in silico* investigation. In order to validate the docking protocol of the CB-Dock server, the co-crystallized ligand (FAD) was extracted from the binding site of NADPH oxidase 5 enzyme structure (PDB: 8U85). The CB-Dock server effectively re-docked the isolated ligand back into the active site of NADPH oxidase 5. The redocked ligand demonstrated a near-perfect alignment with the co-crystallized FAD (Figure 6), thereby affirming the accuracy of the docking protocol. The redocked ligand formed identical hydrogen bonds as those present in the native structure, involving TRP443, PRO445, THR447, HIS461, ARG463, GLN467, TRP468 and THR469. Furthermore, the redocked ligand also established two of hydrophobic interactions seen in the native structure, including PHE446 and ARG344. Moreover, the pose produced by re-docking the FDA ligand was evaluated for structural differences relative to the original PDB structure. The results revealed that the two structures had a local RMSD value of 0 Å, which confirms the reliability of the docking procedure.

The catalytic centre, which is present in all NOX enzymes, consists of two separate domains: the cytosolic dehydrogenase (DH) domain and the transmembrane [34] domain. The DH domain contains a region at the N-terminus which interacts with the flavin adenine dinucleotide (FAD) cofactor, and a lobe at the C-terminus which interacts with the NADPH [35]. NOXs utilize these domains to transport electrons, provided by NADPH, across biological membranes. Initially, two electrons are transferred from NADPH to FAD, converting it to FADH2. Subsequently, the electrons are transported from the inner to the outer heme, and eventually to the oxygen on the extracellular side, resulting in the reduction to superoxide anion. This anion can then be protonated and reduced to produce hydrogen peroxide (H_2O_2) or other reactive oxygen species (ROS) [36]. Hence, it is clear that the prevention of binding to FAD can be achieved by obstructing the active site of the DH domain. Keeping this principle in consideration, we commenced our docking analysis to predict prospective natural inhibitors for the DH domain. The optimal docked ligand molecules were chosen based on two criteria: the lowest binding energy and the lowest inhibitory constant (KI).

Docking scores above -5.0 suggest a relatively high binding affinity, while scores exceeding -7.0 indicate a strong interaction between the ligand and receptor [37]. According to these criteria, the extract of *B. salicina* may contain natural inhibitors for NADPH oxidase 5 enzyme. The compound SSR161421 established two conventional hydrogen bonds with ARG344 and PRO445. It has been documented that these conventional H-bonds, such as OH··O and OH··N, exhibit greater strength compared to other types of hydrogen bonds [38]. In addition to the hydrogen bonds, three aromatic residues, TYR430, HIS444, and PHE446, established pi-pi stacked noncovalent interactions by aligning their aromatic rings with the aromatic rings present in the compound SSR161421. The pi-pi interactions seem to play a crucial role in the structure of proteins and could also provide insights into their vital function in the creation and maintenance of biomolecular condensates [39].

The chemistry community has acknowledged the significance of the cation−π interaction as a key factor in molecular recognition. This interaction now stands alongside the hydrophobic effect, the hydrogen bond, and the ion pair in influencing the structure of macromolecules and interactions between drugs and receptors [40]. The cation−π interactions play a vital role in determining protein structure and protein-ligand binding [41]. After surveying the literature, no data were found linking NADPH oxidase 5 and *B. salicina*. The same lack of connection was observed between SSR161421 and NADPH oxidase 5.

Oxidative stress, resulting from the accumulation of free radicals and reactive oxygen species (ROS), has been linked to the development of numerous diseases, including cancer [42]. Research has shown that medicinal plants serve as valuable reservoirs of antioxidants, owing to their high content of phenolic compounds [43]. Antioxidants play a crucial role in defending biological molecules, such as DNA, from oxidation, thereby mitigating the probability of developing cancer and chronic ailments [44]. We attempted to explore the anticancer properties of the 21 identified compounds based on their notable antioxidant effects observed in our *in vitro* study. No information was found on *B. salicina* extracts and ovarian cancer. Some other studies have been done with *B. salicina* extracts on other types of cancer. Only a single study has examined the cytotoxicity towards the non-cancerous Vero cells, determining that the *B. salicina* extract exhibited a toxic impact with an LC_{50} of 82 µg/mL [10]. The research on *B. salicina* has not yet explored its anticancer and antioxidant properties, highlighting the significance of investigating this plant. Moreover, the absence of existing data underscores the distinctiveness of our study. Among the five compounds that exhibited potential anticancer effects on ovarian cancer, Eriodictyol compound underwent extensive investigation. Eriodictyol showed anticancer activity against two types of cancer cell lines, breast cancer cells (MCF7), and neuroectodermal cancer cell lines WAC2, with IC50 of 18 and 10 µmol/litter, respectively [45]. Eriodictyol exhibited a significant inhibitory effect on the growth of Hep-G2 liver cancer cells, with the extent of suppression varying based on the concentration. The IC50 value, indicating the concentration required for 50% inhibition, was determined to be 37.6 ÂµM [46]. A different study demonstrated that Eriodictyol exhibits an IC_{50} value of 50 µM when tested against human lung cancer cells [47]. Eriodictyol demonstrated cytotoxic effects and induced apoptosis in two types of ovarian cancer cell lines, CaoV3 and A2780 cells. The IC_{50} for CaoV3 cells after 24 and 48 hours was (229.74 ± 5.13) µM and (38.44 ± 4.68) µM, respectively. Conversely, the IC50 for A2780 cells after 24 and 48 hours was (248.32 ± 2.54) µM and (64.28 ± 3.19) µM, respectively [48]. The latter *in vitro* study aligns with our *in silico* prediction, wherein Eriodictyol exhibited a potent cytotoxic impact on A2780 ovarian cells.

This particular value indicates a considerable level of inhibition, falling into specific categories based on IC_{50} values. These categories include strong inhibition (IC_{50} < 1000 nM), moderate inhibition (IC_{50} between 1000 and 10000 nM), and minimal to negligible effect (IC_{50} > 10000 nM) [49]. According to the study by [47], the growth of breast cancer cells can be inhibited by syringic acid. This study is consistent with our computational results, which indicate that Syringic acid exhibits potent inhibition against breast cancer cell lines. Anacardic acid has also been documented as a compound with anti-proliferative and pro-apoptotic properties against breast cancers [50]. Subject to clinical trials, the lead compounds will serve as potential drugs against breast cancer cells.

There is currently a resurgence in interest in naturally occurring substances with biological activity in developed countries, and the utilization of medicinal plants remains a widely accepted form of treatment worldwide, especially in less developed regions. The leaves, stembark, and roots of *B. salicina* are frequently utilized in the treatment of gastrointestinal disorders, cancer, fevers, headaches, rheumatoid arthritis, diabetes, inflamed wounds, ulcers, and for the prevention of diarrhoea. The Stembark ethyl acetate (EAS) fraction exhibited strong antioxidant properties in relation to TPC (Total phenolic content), TFC (Total Flavonoid Content), DDPH radical scavenging activity, and ABTS radical cation scavenging assay at a level of 146.8 ± 1.2 mg GAE/g, 84.7 ± 4.6 mg Rutin/g, 279.7 ± 0.5 mg/g, and 259.3 ± 0.5 mg/g, respectively. A total of twenty-one (21) compounds were detected in the EAS fraction. Compounds 7, 18, and 19 exhibited the top docking scores of -9.2, -9.2, and -9.3 (kcal/mol), respectively. Compounds 1, 5, 8, 9, and 13 demonstrated significant cytotoxic effects against ovarian cancer with $Pa > 0.92$ of 1. Additionally, compounds 1, 2, 3, 6, 8, 9, 10, 12, 15, 19, and 21 displayed potent IC50 values of <1µM. Compounds 1 and 19, gave the best prediction score out of three *in silico* tools used in this study. Compounds 1 (Anacardic acid) and 19 (SSR161421) exhibited the highest prediction scores, being present in two out of the three anticancer *in silico* tools utilized in this research. Subject to clinical trials the lead compounds will serve as potential drugs against breast cancer cells.

Declaration section

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics consent

Not applicable

Funding

Not applicable

Data availability

Manuscript has no associated data.

Author Contributions

Authors' contribution statements: I.U., I.H., S.S., K.U.A., Y.H.M., and A. F. A.: conceptualized the idea, carried out laboratory experiments. A.A.A: *in silico* studies. A. M. D. and A.A.A. wrote the draft. All authors read and approved the final draft.

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