



Phytochemical Profile and Cytotoxic Activity of Selected Organs of *Sambucus nigra* L. via Enzyme Assay and Molecular Docking Study



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Abstract

The purpose of this study is to shed light on the phenolics content and the presence of different classes of constituents in the flowers, leaves, and stems of *Sambucus nigra* L. cultivated in Egypt along with screening out their cytotoxic activity. Colorimetric investigation revealed that all tested samples were rich in phenolics. HPLC qualitative analysis revealed the presence of different phenolics including chlorogenic acid and rutin that were isolated and identified using ¹H and ¹³C NMR. The ethanolic extracts of the three organs, together with the two isolated phenolics, were tested for their cytotoxic activity against hepatocellular carcinoma, human lung adenocarcinoma, and human colon carcinoma. All tested samples were able to reduce the viability of the tested cell lines in a dose-response manner comparable to staurosporine as a reference drug. Molecular docking studies were performed for chlorogenic acid and rutin. Their ability to interact with the key amino acids in B-cell lymphoma 2 (Bcl-2) protein binding site rationalizes their pronouncing activity as proven by its docking pattern and docking score. The cytotoxic activity of the flowers, leaves, and stems of *Sambucus nigra* L. has been attributed to the synergetic effects of phenolic compounds, which affords the basis for investigations as promising candidates for discovering new anticancer drugs.

Keywords: *Sambucus nigra*; phenolics; chlorogenic acid; rutin; cytotoxic; MTT; molecular docking.

1. Introduction

Ancient Egyptians found that *Sambucus nigra* L., also known as black elderberry or black elder, can be beneficial in improving the body's wellbeing and healing of wounds and burns [1]. Black elder is a rich source of two biologically active components; rutin and chlorogenic acid [2] and has a very long history of useful medicinal attributes including, diuretic, diaphoretic, purgative, expectorant, and hemostatic properties [3], [4]. Besides, it was advocated as an effective traditional remedy for the treatment of chest complaints and diabetes [5].

Most potential chemotherapeutic agents were

derived from natural sources that have been regarded as a structural platform for discovering new anticancer drugs. Since 1960, over 50% of anticancer drugs approved by the Food and Drug Administration (FDA) have originated from the natural resources, and based on cytotoxicity bioassay, over 400 compounds have been isolated from plants and microorganisms [6].

Molecular docking is a molecular modeling technique used to predict the binding pattern and predict the binding affinity of a compound to its target. So, molecular docking can be used as a tool for lead optimization [7].

Though black elder has been utilized as early as ancient Egypt, the phenolic profile and the cytotoxic

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activity of the Egyptian cultivar has yet to be studied. The current study was undertaken to investigate the phenolic profiles of the flowers, leaves, and stems of *Sambucus nigra* L. and to evaluate the possible presence of cytotoxic components. Molecular docking studies were performed to find out the possible binding mode and interaction of chlorogenic acid and rutin with human anti-apoptotic B-cell lymphoma 2 protein (Bcl-2) with the aim of explaining their cytotoxic activity.

2. Material and methods

2.1. Plant material

The flowers, leaves, and stems of *Sambucus nigra* L. were collected from El-Orman Botanical Garden, Giza, Egypt, and identified by Mrs. Therese Labib, senior botanist, El-Orman botanical garden. Specimens (No.9.12.18.2) were kept in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

2.2. Tumor cell lines

Three human tumor cell lines, namely; human lung adenocarcinoma A549, hepatocellular carcinoma HePG2, and human colon carcinoma Caco2 were selected. The cell lines were purchased from American Type Culture Collection via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt.

2.3. Chemical reagents

The reagents used were RPMI (Roswell Park Memorial Institute)-1640 medium, MTT, DMSO and 5-fluorouracil (Sigma-Aldrich, St. Louis, MO, USA), Fetal Bovine serum (GIBCO, UK).

2.4. Preparation of the extracts

The flowers, leaves, and stems (1kg, each) were air-dried, powdered, and macerated till exhaustion in ethanol (70%). Each extract was then collected, filtered, and evaporated to dryness under reduced pressure. The residue obtained was saved for phytochemical and cytotoxic studies.

2.5. Preliminary Phytochemical screening

The air-dried powdered flowers, leaves, and stems were separately subjected to preliminary phytochemical screening for the presence of different classes of constituents. [8][9][10].

2.6. Colorimetric determination of the total phenolics content

Total phenolic contents were estimated by assessing the color intensity developed by mixing the extract with Folin-Ciocalteu reagent using gallic acid as standard [11]. Five ml of Folin-Ciocalteu reagent (1mg/10 ml) was mixed with one ml of extract, methanol, or gallic acid (5-50 $\mu\text{g}/\text{ml}$), and left for 10 minutes then 4 ml of sodium carbonate solution (75 mg/ml) were added. Aliquots (200 μl) were transferred to a clear 96-well plate, and absorbance was measured after 30 minutes at 760 nm using a microplate reader (Infinite F50, Tecan, Switzerland).

2.7. Colorimetric determination of the total flavonoids content

Total flavonoid contents were estimated by assessing the color intensity developed by mixing the extract with AlCl_3 in acidic medium using quercetin as standard [12]. Aqueous 2% AlCl_3 (250 μl) was mixed with 500 μl of extract, methanol or quercetin (5-50 $\mu\text{g}/\text{ml}$), then 1 M HCl (250 μl) was added and the mixtures were vortexed. Aliquots (200 μl) were transferred to a clear 96-well plate, and absorbance was measured at 420 nm using a microplate reader (Infinite F50, Tecan, Switzerland).

2.8. Qualitative HPLC analysis

HPLC analysis were performed for qualitative evaluation and peak identification of phenolic compounds using Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array Detector, column C18 thermo: 4.6x250 mm, 5 μm , using mobile phase consisting of Buffer (0.1 % phosphoric acid in water) and Methanol in gradient mode and flow rate: 1 ml/min.

2.9. Isolation of chlorogenic acid and rutin

Chlorogenic acid and rutin were isolated from the ethanolic extract (70%) of *Sambucus nigra* L. flowers. Isolation was carried out with a PuriFlash 4100 system (Interchim; Montluçon, France). For system controlling and process monitoring, Interchim

Software 5.0 was used. Sample (10 gm) was dissolved in 50mL of Methanol then introduced into the column (80gm - flash - NP column) *via* dry load using 50 gm silica.

2.10. Cytotoxicity assay (MTT assay)

Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Antibiotics were added, 100µg/ml streptomycin and 100units/ml penicillin, in a humidified 5% (v/v) CO₂ atmosphere at 37°C. The inhibitory effects of the tested compounds on cell growth, compared to Staurosporine, were examined on the aforementioned cell lines using the MTT assay in multiwell plates [13], [14]. The key component in this assay is that mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, converting the yellow tetrazolium bromide (MTT) to a purple formazan derivative. The cells were seeded in a 96-well plate at a density of 1.0x10⁴ cells/well at 37 °C for 48 h under 5% CO₂. After incubation, the cells were treated with (0.39,1.56, 6.25, 25,100) µg/mL concentrations of tested compounds. Triplicate wells were prepared for each dose and were incubated for 24 h. After 24 h of drug treatment, 20 µL of MTT solution at 5 mg/mL was added and incubated for 4 h. DMSO (100 µL) was added into each well to dissolve the formed purple formazan. Colour intensity was measured and recorded at a wavelength of 570 nm using a plate reader (EXL 800, USA).

2.11. Molecular docking study

All the molecular modeling studies were carried out using Molecular Operating Environment (MOE, 2010.10) software. All minimizations were performed with MOE until an RMSD gradient of 0.05 kcal·mol⁻¹Å⁻¹ with MMFF94x force field and the partial charges were automatically calculated. The X-ray crystallographic structure of human anti-apoptotic B-cell lymphoma 2 protein (Bcl-2) co-crystallized with a tetrahydroisoquinoline phenyl pyrazole inhibitor (J1Q) (PDB ID: 6QGK) was downloaded from the protein data bank ([http://www.rcsb.org](http://www.rcsb.org;); (2019) ACS Omega 4: 8892-8906, 10.1021/acsomega.9b00611). Water molecules and ligands that are not involved in binding were first removed. Then, the protein structure was prepared for docking study using Protonate 3D protocol in MOE with default options. The co-crystallized ligand was used to define the binding site for docking. Triangle Matcher placement method and Affinity dG scoring function were used for docking. Docking setup was first validated by self-docking of the co-crystallized

ligand J1Q in the vicinity of the binding site of the protein. Self-docking validation proved the suitability of the used docking protocol for the current study as indicated by the minor deviation between the docking pose and the co-crystallized structure binding mode as indicated by their RMSD of 0.856 Å and with the ability of the docking protocol to reproduce all the key interactions accomplished by the co-crystallized ligand with the key amino acids in the binding site.

3. Results and Discussion

3.1. Preliminary Phytochemical screening

The preliminary screening revealed that carbohydrates and/or glycosides, flavonoids, sterols and/or triterpenes, saponins, as well as tannins were detected in all organs under investigation.

3.2. Determination of the total phenolics content

From figure 1: Flowers and leaves extracts recorded higher concentration rather than stem extract. The absorbance of the ethanolic (70%) extracts of *Sambucus nigra* L. flowers, leaves, and stem were 0.2982, 0.3036, and 0.2128, which corresponding to 42.08, 42.70, and 32.26 mg GAE/g respectively.

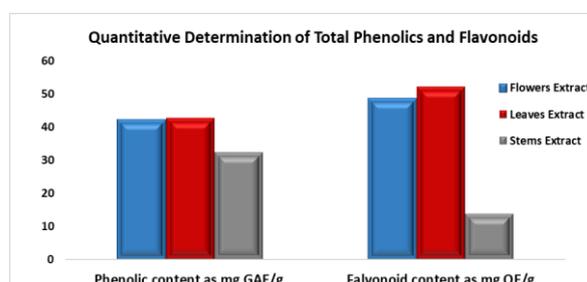


Figure 1. Quantitative determination of total

3.3. Determination of the total flavonoid contents

Both leaves and flowers extracts are rich with flavonoidal contents while stems extract recorded the lowest value (Figure 1). The absorbance of the ethanolic (70%) extracts of *Sambucus nigra* L. flowers, leaves, and stem were 0.3243, 0.3508, and 0.0702, which corresponding to 48.49, 52.12, and 13.68 mg QE/g respectively.

3.4. Qualitative HPLC analysis

HPLC chromatograms of the standards mixture together with the identified phenolics in the three extracts (Flowers, leaves, and stems) are shown in

figure 2. Among the identified phenolics were catechin, rutin, quercetin, chlorogenic acid, and kaempferol.

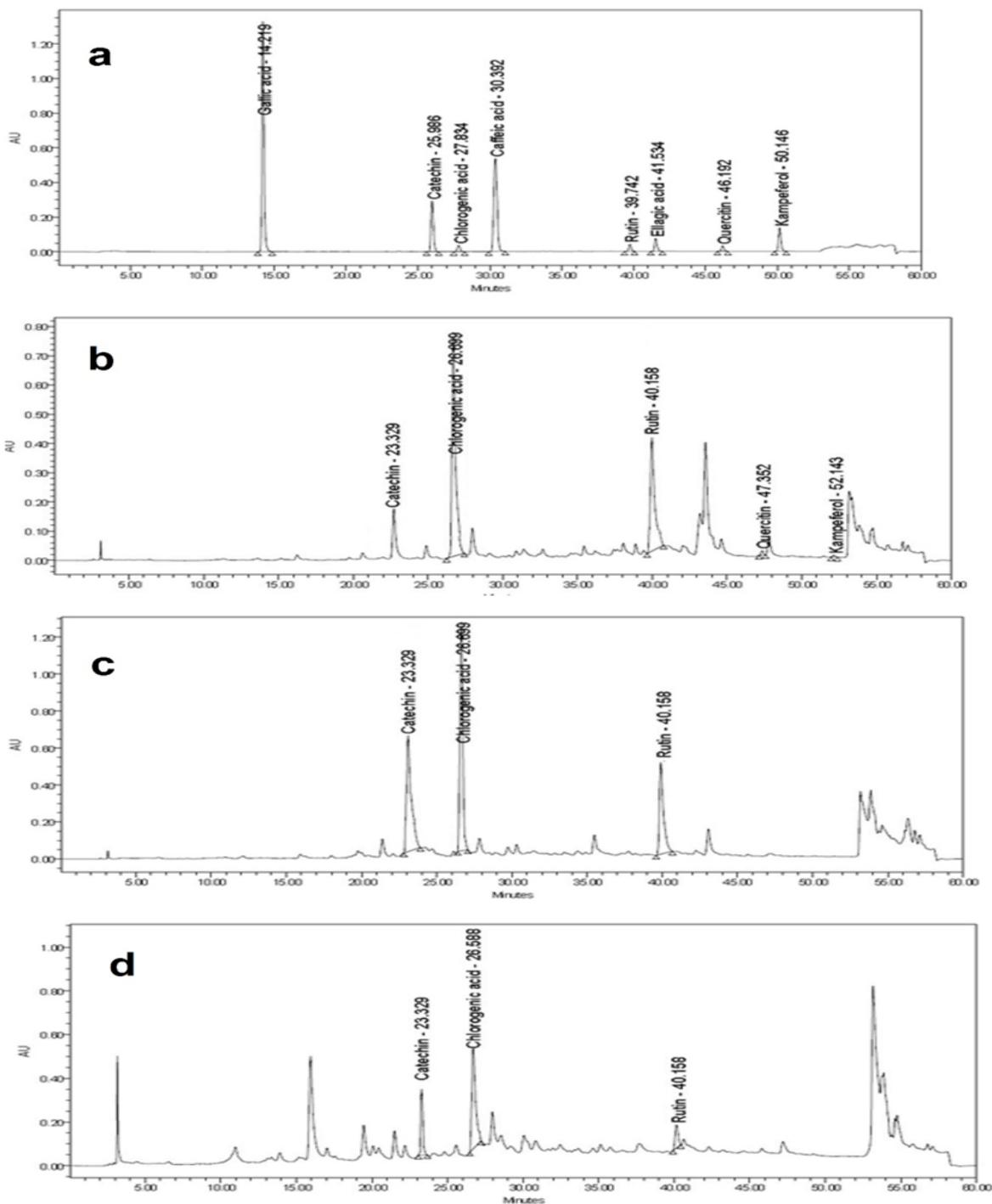


Figure 2. Qualitative HPLC Chromatogram of the standards mixture (a) and the identified phenolics in the flowers (b), leaves (c) and stems (d) of *Sambucus nigra* L.

3.5. Isolation of chlorogenic acid and rutin

Chlorogenic acid and rutin, the major identified phenolics in the three extracts, were targeted for isolation and identified using ^1H and ^{13}C NMR. NMR Spectral analyses were recorded at 400 MHz for ^1H and 100 MHz ^{13}C by Bruker NMR spectrometer using Methanol- d_4 , and chemical shifts were given on a δ (ppm) scale with tetramethylsilane as an internal standard.

3.6. Identification of chlorogenic acid

The ^1H -NMR spectrum showed doublet peak at 7.45 ppm, J value=16 (H-7') and doublet peak at 6.15 ppm, J=16 (H-8') indicated the presence of olefinic protons that is in conjugation with an aromatic ring; doublet peak at 6.67 ppm with J=8 which indicate the presence of aromatic double bond with ortho coupling (H-5'), doublet of doublet peak at 6.85 with J=8&2 which indicate ortho and meta coupling (H-6'), doublet peak at 6.94 with J=2 indicate meta coupling (H-2'), multiplet peak at 5.22 (H-5), multiplet peak at 4.06 indicate the presence of aliphatic protons (H-3), doublet of doublet peak at 3.62 with J=8&2 indicate the presence of aliphatic protons (H-4), multiplet peak at 2.03 indicate the presence of aliphatic methylene groups (H-2, H-6).

The ^{13}C -NMR spectrum showed the presence of sixteen carbon atoms, including two carbonyl groups at 175.6 and 167.2, corresponding to carbon 7 and 9', respectively; two aromatic carbons bonded to hydroxyl groups at 148.1 and 145.4 identified as C4', and C3'; two olefinic carbons at 145.6 and 113.8 corresponding to C7' and C8'; four aromatic carbons assigned to C1', C2', C5', and C6' at 126.4, 113.9, 115.1, and 121.6, respectively; three carbons bonded to hydroxyl groups at 74.7, 70.6, and 72.1 identified as C1, C3, and C4; one carbon bonded to ester group

at 70.6 attributed to C5; and two methylene identified as C2, and C6 at 37.4 and 36.8, respectively.

The ^1H and ^{13}C NMR spectral data of chlorogenic acid are in agreement with those reported in literature [15].

3.7. Identification of rutin

The ^1H -NMR spectrum revealed proton signals at δ 12.24 (1H, s, 5-OH), in the aromatic region at 7.53 (1H, dd, J=8 Hz, J=2, H-6'), 7.57(1H, d, J=2, H-2'), 6.78 (1H, d, J=8Hz, H-5') corresponding to B ring protons [16]. Resonances at 6.30 (1H, d, J=2 Hz, H-8), 6.11 (1H, d, J=2 Hz, H-6) are typical meta-coupling between H-6 and H-8 protons in ring A. Resonances at δ 5.13 (1H, broad S, H-1''), 4.54 (1H, broad S, H-1''') and protons of rhamnosyl CH_3 at 1.02 (3H, d, J=8 Hz) [16]. Resonance from 3.2-3.85 corresponds to sugar protons.

The ^{13}C -NMR spectrum revealed characteristic pattern at δ 157.9 (C-2), 134.2 (C-3), 178 (C-4), 161.6 (C-5), 98.4(C-6), 164.6(C-7), 93.5 (C-8), 157.1 (C-9), 104.2 (C-10), 121.6(C-1'), 116.3 (C-2'), 144.4(d, C-3'), 148.4 (C-4'), 114.7(C-5'), 122.1 (C-6'), 103.3(C1''), 74.3 (C2''), 75.8 (C3''), 70.4 (C4''), 76.8 (C5''), 67.1(C6''), 101(C1'''), 70 (C2'''), 70.8 (C3'''), 72.5(C4'''), 68.3 (C5'''), 16.5 (C6''').

The ^1H and ^{13}C NMR spectral data of rutin are in agreement with those reported in literature [17], [18].

3.8. Cytotoxic activity

Regardless of the efficacy of tested samples in the reduction of cell viability in a dose-response manner, each cell line was selectively sensitive to specific extract (Figures 3 and 4). In most cases, the IC_{50} values were under 30 $\mu\text{g/ml}$, except IC_{50} of leaves extract

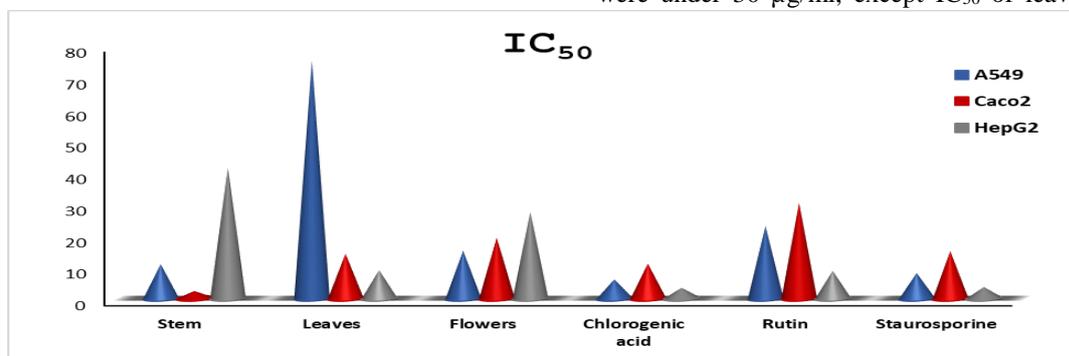


Figure 3. IC_{50} of the ethanolic extracts of the flowers, leaves, and stems of *Sambucus nigra* L. and two of the main phenolics; Chlorogenic acid and Rutin in comparison with Staurosporine against hepatocellular carcinoma HePG2, human lung adenocarcinoma A549, and human colon carcinoma Caco2

against human lung adenocarcinoma (75.09 ± 8.14 $\mu\text{g/ml}$) and IC_{50} of stems extract against hepatocellular carcinoma (41.32 ± 6.01 $\mu\text{g/ml}$), in agreement with NCI (National Cancer Institute, U.S.A.) recommendations to consider a crude extract promising for further purification [19], [20]. Most of tested samples (flowers, leaves, stems, and chlorogenic acid) recorded significant activities against colon carcinoma (IC_{50} values recorded 19.14 ± 2.14 , 14.11 ± 1.16 , 2.30 ± 0.08 , and 10.93 ± 0.8 $\mu\text{g/ml}$, respectively). Hepatocellular carcinoma was most sensitive to chlorogenic acid followed by rutin and leaves extract (IC_{50} values recorded 3.33 ± 0.11 , 8.65 ± 0.24 , and 8.89 ± 0.55 $\mu\text{g/ml}$, respectively) while lung adenocarcinoma was most sensitive to chlorogenic acid followed by stems extract (IC_{50} values recorded 5.97 ± 0.27 and 10.78 ± 0.61 $\mu\text{g/ml}$, respectively) comparable to staurosporine as reference drug (IC_{50}

7.98 ± 0.32 $\mu\text{g/ml}$). Flowers extract and Chlorogenic acid showed promising activities in all tested cell lines.

3.9. Molecular docking study

Molecular docking studies were performed to investigate the possible binding mode of chlorogenic acid and rutin in the anti-apoptotic protein Bcl-2. The X-ray crystallographic structure of the human Bcl-2 co-crystallized with J1Q as inhibitor (PDB ID: 6QGK) was used in this study. Docking setup was first validated by self-docking of the co-crystallized ligand (J1Q) in the vicinity of the binding site of Bcl-2. Self-docking validation step showed the suitability of the used docking protocol for the planned docking study. The docking protocol could reproduce the co-crystallized ligand pose as indicated by the low RMSD

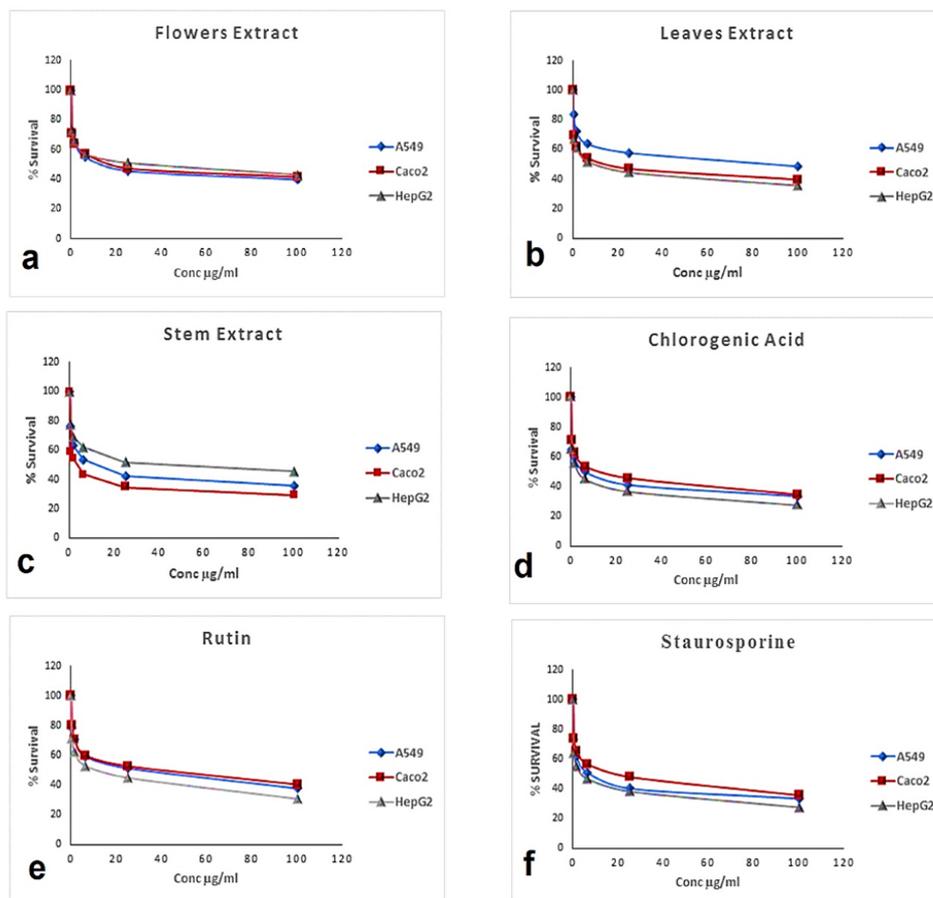


Figure 4. Cytotoxic activity of the ethanolic extracts of the flowers (a), leaves (b) and stems (c) of *Sambucus nigra* L. and two of the main phenolics; Chlorogenic acid (d) and Rutin (e) in comparison with Staurosporine (f) against hepatocellular carcinoma HePG2, human lung adenocarcinoma A549, and human colon carcinoma Caco2

of 0.856 Å between the docked pose and the co-crystallized ligand (energy score (S) = -10.25 kcal/mol) and by its ability to reproduce all the key interactions accomplished by the co-crystallized ligand with the key amino acids in the binding site. These interactions take place through ionic interaction with the side chain carboxylate of Asp111 and through hydrophobic interaction with hydrophobic side chains of Phe104, Phe112, Met115, Val133, Ala149 and Phe153 (Figure 5). The validated docking protocol was then used in predicting the ligand-target interactions at the binding site of Bcl-2 for the compound of interest.

The ability of the compounds of interest to interact with the key amino acids in Bcl-2 binding site rationalizes their cytotoxic activity as indicated by its docking pattern and docking score compared to that of J1Q. (Figure 5) and (table 1). Chlorogenic acid interacts through several hydrogen bonds with the key amino acid Asp111, Gln118 and Asp140. (Figure 5). Rutin interacts by its sugar part through hydrogen bonding with the key amino acid Asp111 and Arg146. In addition, by its aglycon part through hydrogen bonding with Glu136 and through hydrophobic interaction with hydrophobic side chains of Phe104, Leu137, Ala149 and Phe153. (Figure 5).

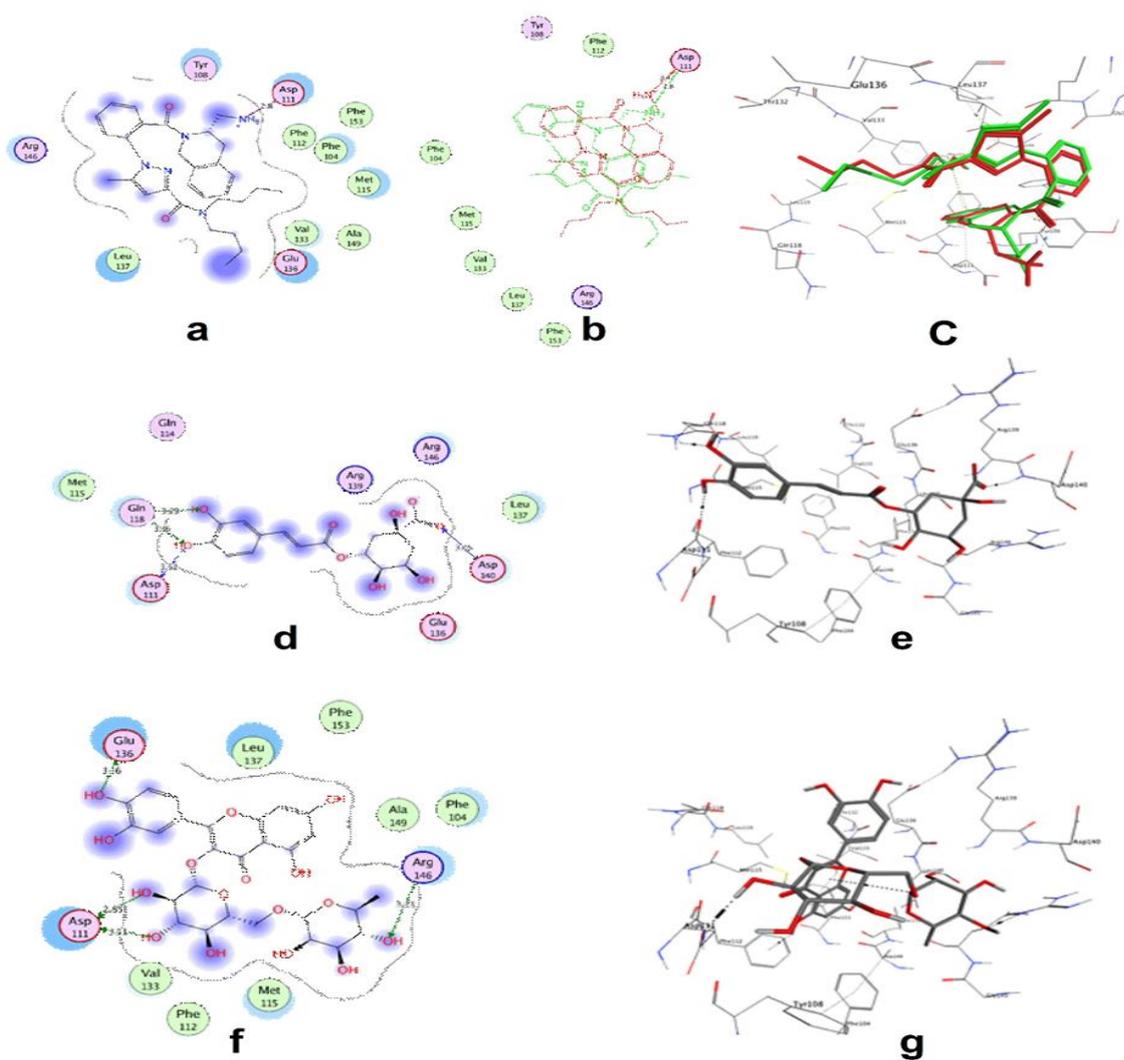


Figure 5. (a) 2D interaction diagram showing J1Q docking pose interactions with the key amino acids in Bcl-2 binding site. (b) 2D representation and (c) 3D representation of the superimposition of the co-crystallized (red) and the docking pose (green) of J1Q in the Bcl-2 binding site with RMSD of 0.856Å. (Ligand non-polar Hydrogen atoms were removed for clarity). 2D diagram (d) and 3D representation (e) of chlorogenic acid in Bcl-2 binding site. 2D diagram (f) and 3D representation (g) of rutin in Bcl-2 binding site.

In both quantitative methods (figure 1) Flowers & leaves extracts recorded the highest concentration of total phenolics and flavonoids with a slight increase in the concentration of leaves extract.

HPLC results (Figures 2) recorded three identified phenolics in all tested samples (catechin, chlorogenic acid and rutin) while quercetin and kaempferol were identified in the flowers extract only. All detected phenolics in tested samples were higher valued in leaves and flowers extracts rather than stems extract. Chlorogenic acid and Rutin recorded the major concentration in all tested samples so these compounds were targeted for isolation and testing for their promising cytotoxic activities and support their cytotoxic activity results by molecular docking study of both compounds.

Concerning cytotoxic activity of tested samples (Figures 3 and 4) and the structural basis of this efficacy in molecular docking study (Figure 5) and (Table 1). Tested extracts and isolated compounds seem potentially attractive as cytotoxic and anticancer candidates as the interaction of both compounds with the target in the anti-apoptotic protein support cytotoxic results and specificity of compounds for cancer cells.

5. Conflicts of interest

There are no conflicts of interest.

6. References

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Table 1: Docking energy scores (S) in kcal/mol for the co-crystallized and tested compounds

#	Compound	Docking Score (Kcal/mol)
1	J1Q	-10.25
2	Chlorogenic acid	-9.36
3	Rutin	-9.98

4. Conclusion

The cytotoxic potential of the flowers, leaves, and stems of *Sambucus nigra* L. has been attributed to the synergetic effect of their phenolic compounds. Both chlorogenic acid and rutin are effector mediators for their cytotoxic potential. The ability of both compounds to interact with the key amino acids in Bcl-2 binding site rationalizes their cytotoxic activity as indicated by its docking pattern and docking score compared to that of J1Q. *In vivo* cytotoxic studies are still needed to verify of *Sambucus nigra* L. traditional use and further the underlying mechanisms, searching for the possible presence of other vital bioactive compounds acting as cytotoxic agents.

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- فحص فيتوكيميائي ودراسة التأثير على الخلايا السرطانية لبعض الأجزاء المختارة من نبات سمبوكس نيجرا ال من خلال فحص التأثير على الانزيم ودراسة الالتحام الجزيئي**
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- قسم النباتات الطبية و المنتجات الطبيعية - الهيئة القومية للرقابة و البحوث الدوائية - مصر. ٢
- قسم الكيمياء الصيدلانية والطبية - كلية الصيدلة - جامعة القاهرة - مصر. ٣
- تهدف هذه الدراسة الى عمل مسح كيميائي اولى لأجزاء النبات المختلفة (الأزهار و الأوراق و السيقان) و القاء الضوء على المحتوى الكلي للفينولات و التأثير على الخلايا السرطانية. ووجد ان جميع أجزاء النبات محل الدراسة غنية بالفينولات و الفلافونويدات. وتم فصل حمض الكلوروجينك و الروتين و التعرف عليهما بعد التحليل الكيفي للخلاصات باستخدام كروماتوجرافيا السائل ذات الكفاءة العاليه . وتم اختبار الخلاصة الكحولية لأجزاء النبات المختلفة و حمض الكلوروجينك و الروتين على الخلايا السرطانية لكل من الكبد و الرئة و القولون. تم دراسة الالتحام الجزيئي للمواد المفصولة مع بروتين بي سي ال ٢ و اثبتت الدراسة فاعلية جميع العينات التي تم اختبارها وذلك يوضح ان المواد الفينولية الموجودة بالنبات هي المواد الاساسية المسؤولة عن التأثير على الخلايا السرطانية.
- الكلمات الدالة: سمبوكس نيجرا ال , الفينولات , حمض الكلوروجين, روتين, التأثير السام للخلايا السرطانية, الالتحام الجزيئي