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In vitro Anticancer Potential and Molecular Modelling Study of Flavanol Glucoside from Graviola (*Annona muricata*) Fruit: A Potential Inhibitor of Antiapoptotic Proteins

Pattilthodika Suhail [1,2,*], Velappan Venkatachalam Venkatachalam [2], Sheron Joseph [1], Radhakrishnan Murali [2], Arvind Babu Lalpet Renganathan [3]

1. Department of Pharmacology, Al Shifa College of Pharmacy, Perinthalmanna, Kerala
2. Department of Pharmacy, Annamalai University, Annamalai Nagar, Chidambaram, Tamilnadu
3. Department of Computer Science, Annamalai University, Annamalai Nagar, Chidambaram, Tamilnadu

* Correspondence: ptsuhl@gmail.com;

Scopus Author ID 57887581500

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Abstract: The tropical plant *Annona muricata* Linn (Graviola), a member of the *Annonaceae* family, is well recognized for having anticancer effects. Molecular modeling and design are essential methods in pharmaceutical research for defining, producing, and analyzing biological and chemical compounds for effective treatments. This study aimed to link the *Annona muricata* fruit's chemical or phytochemical profile with its *in vitro* and *in silico* anticancer efficacy. In molecular modeling, one of the isolated components of *A. muricata* fruit, quercetin-3-O-β-D-glucoside (flavonol glucoside), was therefore intended to be docked with the proteins, phosphoinositide 3-kinase, topoisomerase II and cAMP-dependent protein kinase as well as their inhibitors. In Q-TOF LC/MS analysis, the different peaks were obtained at different retention times, and the highest peak was at the retention time of 2.77, followed by 2.07 and 2.61 minutes, belonging to the compounds Procyanidin B2, Quinic acid, and Quercetin-3-O-β-D-glucoside. The extract showed remarkable anticancer activity against MCF-7, T47D, HCT-15, and PC3 cancer cell lines. The molecular modeling study showed the most effective inhibition on phosphoinositide 3-kinase, topoisomerase II, and cAMP-dependent protein kinase proteins, indicating the flavanol glucoside's effectiveness in apoptosis. Determining how flavanol glucoside from *A. muricata* interacts with anti-apoptotic proteins is, therefore, important in understanding the anticancer effect of this substance.

Keywords: *Annona muricata*; quercetin; phosphoinositide 3-kinase; topoisomerase II; cAMP-dependent protein kinase; anticancer activity.

1. Introduction

Natural compounds are a crucial source of therapeutic medicines. The importance of natural products in discovering and developing novel medications cannot be emphasized by any current drug discovery methodologies, including combinatorial chemistry and computer-based molecular modeling design [1]. The metabolites found in medicinal plants can prevent the unwanted effects of synthetic medications because many synthetic drugs have significant side effects that are only acceptable as last-resort therapies for terminal diseases like cancer [2].

The extrinsic pathway is activated when cytokine ligands connect to tumor necrosis factor receptor family cell-surface death receptors. This binding sets off a chain of events that ultimately catalyzes the conversion procaspase-8 into caspase-8. Apoptosis enters its execution phase when caspase-8 is activated. When faced with internal stressors like heat, virulence agents, oxidative stress, growth factor deficiency, or DNA damage, the intrinsic pathway will be activated.

Many signaling pathways, including PI3K/AKT/mTOR, EGF/RAS/RAF/MEK /ERK, and RAF/MEK/ERK, are considered crucial in oncogenesis and the development of cancer [3]. For cells to grow and survive, the phosphoinositide-3-kinase (PI3K) signaling pathway is essential [4]. Activation of PI3K and malignant transformation can come from various modifications of the PI3K/AKT/mTOR pathway. Lipid kinases, or PI3Ks, can be divided into three types according to their structure and work. Human cancer is most strongly associated with class IA PI3K [5]. Depending on the specific enzyme, Topoisomerase II's catalytic activity is mediated by a unidirectional strand-passage mechanism that forces one DNA segment through a DNA cut, resulting in various activities such as DNA relaxing, negative DNA supercoiling, knotting/unknotting, and catenation/decatenation. Topoisomerase II inhibitors stop the catalytic enzyme process from fixing an intermediate that might later cause cell apoptosis by causing a DNA strand to be cut and covalently bonded to the enzyme [6]. The cAMP-dependant PKA phosphorylates several key substrates and inhibits all agonis-induced cellular activation pathways, including calcium release, and it can also induce pro- and anti-apoptotic effects [7].

There have already been clear outcomes from the addition of novel targeted medicines with improved selectivity to traditional chemotherapy, availability for long-term treatment, and tolerability. Many elements of these survival pathways, therefore, have the potential to serve as molecular targets for cancer therapy. A rapidly emerging area called molecular docking seeks to explain and anticipate potential modes of interaction between a ligand and a target biomolecule [8-10]. At the same time, a feasible method for determining the dynamic stability and binding energetics of a protein-ligand complex was provided by molecular dynamics simulations [11,12]. This study aimed to link the *Annona muricata* (*A. muricata*) fruit's chemical or phytochemical profile with its *in vitro* and *in silico* anticancer efficacy. In molecular modeling, one of the isolated components of A. muricata, quercetin-3-O-β-D-glucoside (flavonol glucoside), was therefore intended to be docked with the proteins, phosphoinositide 3-kinase, topoisomerase II and cAMP-dependent protein kinase as well as their inhibitors.

2. Materials and Methods

2.1. Collection and authentication of the plant.

Fruits of *A. muricata* were collected from different parts of rural areas of Malappuram, Kerala, and authenticated by Mr. Harinarayanan CM, Scientist, Pharmacognosy Division, Arya Vaidyasala, Kottakkal, Malappuram, Kerala, India. A dried plant sample was recorded in the Herbarium of the Centre for Medicinal Plants Research (CMPR), Arya Vaidyasala, Kottakkal (No.10045).

2.2. Preparation of plant extract.

The fruits were dried in an oven at a temperature of 60°C after being rinsed with distilled water, and then they were crushed into a rough powder with a diameter of around 1

mm. One hundred grams of ground material was mixed with 500 ml of 96% ethanol in a conical flask and kept intermittent shaking for 72 hours. A rotary evaporator was used to evaporate the resulting extract at 50 rpm and 40°C. The concentrated extract was then stored in a refrigerator until further usage at 4°C to prevent damage.

2.3. Q-TOF LC/MS analysis.

The phytochemicals present in the ethanolic extract of the *A. muricata* fruit were initially identified, and LC-MS then validated their identities. The analysis was then carried out using Mariner Bio spectrometry, which is fitted with a binary pump. An ESI (Electrospray Ionisation) source-equipped Q-TOF mass spectrometer (Agilent 1260 Infinity I) was connected to the HPLC. A 140°C source temperature was selected for full-scan operation from m/z 100 to 1200. For the analysis, a Phenomenex 5 µ C8 HPLC column (150 × 2 mm) was employed. Ethanol was the solvent, while formic acid was 0.3%. The overall flow rate of the solvent delivery was 0.1 mL/min. Isocratic elution was employed to operate the solvent. The positive ion technique was used to acquire the MS spectra [13].

2.4. In vitro anticancer activity by MTT assay.

The National Centre for Cell Science (NCCS), Pune, India, provided breast cancer cell lines (MCF 7, T47D), colorectal cancer cell lines (HCT-15), and prostate cancer cell lines (PC3). These cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium enriched with 10% fetal bovine serum (FBS), streptomycin (100 g/mL), and penicillin (100 U/mL). The conditions for all cells were kept at 37°C, 5% CO_2, 95% air, and 100% relative humidity. These cell lines are used for anticancer activity of ethanolic extract of *A. muricata* using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay [14]. A 12-well plate with medium was seeded using approximately 1×10^5 cells and incubated for 24 hours at 37°C. The cells received various extract concentrations at the same temperature and time. Each well received 100 µL of MTT, which was then incubated for 4 hours. The 1 mL solubilization solution containing isopropanol, HCl, and Triton X 100 was used to dissolve the dark blue formazan crystals by continuous aspiration and re-suspension. At 570 nm, the colored product's absorbance was measured. The cytotoxicity was investigated by comparing the proportion of the treated cell population that died with the untreated control, which was demonstrated by their relative absorbance measured using the MTT test. Duplicate runs of three experiments with equivalent outcomes were made.

2.5. Protein and ligand structure retrieval and molecular docking.

The chemical structure of quercetin was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov) [15] and the X-ray crystal structure of phosphoinositide 3-Kinase (PDB ID: 1E8W), topoisomerase II (PDB ID: 1PVG) and cAMP-dependent protein kinase (PDB ID: 1CX4) was retrieved from Protein Data Bank (PDB). The protein preparation wizard in Epik version 3.4 processed the protein structure, and it was used to prepare the crystal structure for ionization, H-bond optimization, heterogeneous state creation, protonation, filling in missing loops and side chains (using Prime), and general minimization. The receptor grid generating panel of Glide version 6.9 generated the grid around active sites. The default grid size was 20 Å, and the grid points in the x, y, and z axes were maintained. The Glide version 6.9 in Schrodinger Maestro version 10.4 was used for all docking and scoring computations.

During this study, the active site of protein was set to adjust itself up to a distance of 5 Å for ligand accordingly.

2.6. ADMET prediction.

The pharmacokinetic features of the ligands, absorption, distribution, metabolism, excretion, and toxicity (ADMET) must be evaluated in order to determine their activity within the body. The ADMET properties of the ligands were analyzed using the QikProb module version 3.0 module of Schrodinger Maestro.

3. Results and Discussion

3.1. Q-TOF LC/MS analysis.

The preliminary phytoconstituent studies showed that the ethanolic extract of *A. muricata* fruit contains flavonoids and glycosides. The LC-MS/MS and important compounds identified are shown in Figure 1 It was observed that the different peaks were obtained at different retention times, and the highest peak is at the retention time of 2.77, followed by 2.07 and 2.61minutes, belonging to the compounds Procyanidin B2, Quinic acid, and Quercetin-3-O-β-D-glucoside.

Procyanidin B2 is a B-type proanthocyanidin with biological activities such as antioxidant, anti-inflammatory, and anticancer activity [16]. Quinic acid is a cyclohexane carboxylic acid found in extracts from several different medicinal plant parts [17]. Quinic acid has a variety of biological actions, including antioxidant, antidiabetic, anticancer, antibacterial, antiviral, anti-aging, protective, anti-nociceptive, and analgesic properties, according to pharmacological investigations [17]. Quercetin, a bioflavonoid that belongs to the flavanol group, is another major phytoconstituent discovered. It has several pharmacological properties, including antioxidant, anti-inflammatory, anticancer, atherosclerosis, and hypertension [18].

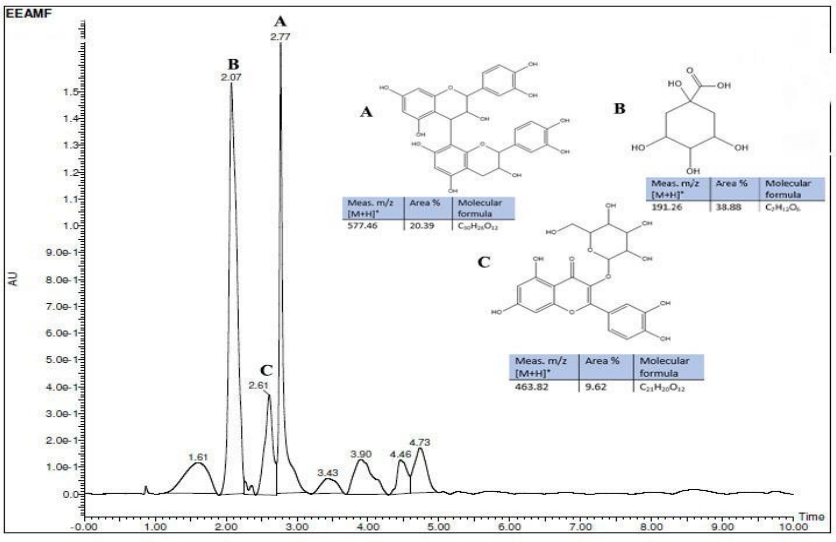

Figure 1. Chromatogram of active metabolite in ethanolic extract of *A. muricata* fruit, and the identified metabolites are (A) Procyanidin B2, (B) Quinic acid, and (C) Quercetin-3-O-β-D-glucoside. Meas m/z implies the measured m/z. The chemical structure of tentatively identified compounds is redrawn with ChemDraw®.

3.2. Anticancer activity of the extract.

Different growth factors can interact with one another and be modulated by several phytoconstituents, which can also activate or inhibit cytokine signaling pathways. Like

genistein, a phytoestrogen down-regulated the PI3K/AKT signaling pathway in MCF-7 breast cancer cells can cause apoptosis [19]. The AKT signaling pathway also prevents the activation of NF-kB in prostate cancer cells [20]. The extract with different concentrations showed cytotoxicity against the breast cancer cell lines MCF-7 and T47D in the MTT experiment, with IC_{50} values of 69.52 and 132.24 µg/mL, respectively. Figure 2 also showed dose-dependent cytotoxicity against the colorectal cancer cell line HCT-15 and the prostate cancer cell line PC3, with IC_{50} values of 52.62 and 164.48 µg/mL, respectively.

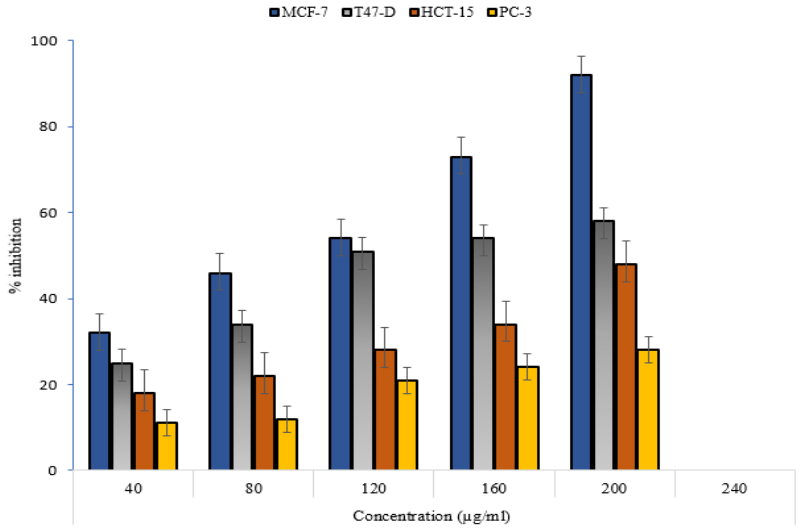

Figure 2. *A. muricata* fruit extract showed anticancer efficacy on MCF-7, T47-D, HCT-15, and PC-3 cell lines using the MTT test.

3.3. Molecular modelling.

The molecular docking and molecular dynamics of quercetin were performed on phosphoinositide 3-kinase (PI3K), topoisomerase II, and cAMP-dependent protein kinase (PKA), which are responsible for breast, colon, gastric, cervical, prostate, and lung cancer [21-23]. These enzymes activate different signaling cascades involved in the pathogenesis of different carcinomas [24]. The docking protocol was validated by redocking the native ligand to PI3K (PDB ID: 1E8W), topoisomerase II (PDB ID: 1PVG), and PKA (PDB ID: 1CX4). The root mean square deviation (RMSD) value of the ligand was found to be < 1.5 Å, which means the protocol used was prospectively validated [25,26]. The docking score of quercetin is shown in Table 1.

Table 1. Docking score and RMSD value of quercetin on PI3K (PDB ID: 1E8W), topoisomerase II (PDB ID: 1PVG), and cAMP-dependent PKA (PDB ID: 1CX4) proteins.

Compound	**Docking score (Kcal/mol)**			**RMSD(A^0)**
	1E8W	**1PVG**	**1CX4**	
Quercetin	-12.26	-12.66	-8.84	0.196

3.4. Docking study on 1E8W protein.

Most of the characteristics of cancer, such as cell cycle, survival, metabolism, motility, and genomic instability, are under the control of the signaling network described by PI3K/AKT [27]. The PI3K pathway is the most commonly changed in human malignancies, and the PIK3CA gene, which codes for the PI3K catalytic isoform p110, is the second most frequently mutated oncogene [28]. It is important to note that quercetin demonstrated positive interactions with all three protein targets linked to significant cytotoxicity levels in cancer cells. Quercetin

showed lower docking energy (-12.26 Kcal/mol) on the PI3K protein, indicating a higher binding affinity of quercetin.

Quercetin was found to interact with the catalytic residues of ASP964, LYS807, ASP 950, and ALA 885 by hydrogen bonding and van der Waals interaction with TRP 832 and TYR 869, showing hydrophobic interaction with some amino acids in the hydrophobic pockets of PI3K, as shown in Figure 3.

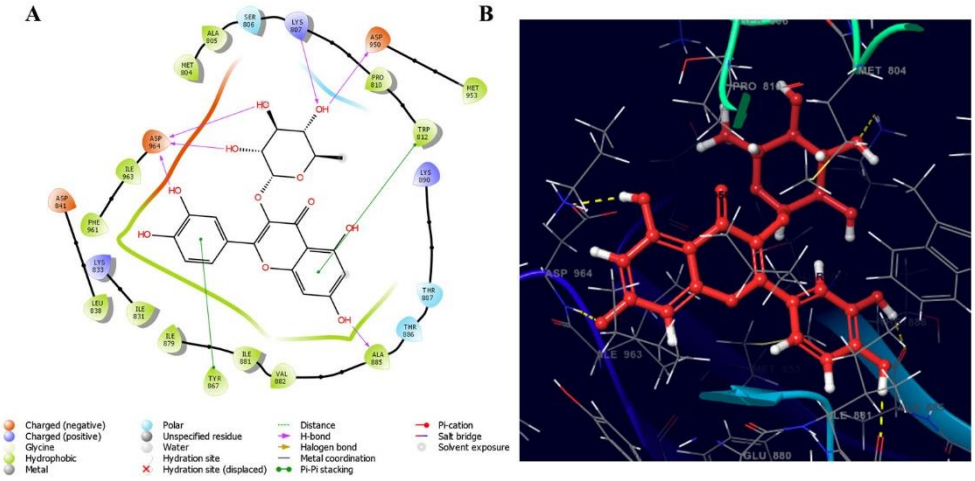

Figure 3. Molecular interaction of quercetin on the active site of PI3K **(A)** 2D; **(B)** 3D structures of quercetin against the 1E8W protein as potent anticancer activity.

3.5. Docking study on 1PVG protein.

Topoisomerases are crucial enzymes that facilitate changes to the DNA's tertiary structure. The human topoisomerases fall into two distinct types. While topoisomerase II uses double-strand breaking [29], topoisomerase I only affects one DNA strand at a time [30]. These enzymes assist in reducing DNA twisting and supercoiling and are necessary for replication, transcription, and recombinant repair. In cells that divide rapidly, topoisomerase II is expressed at high levels [31].

The hydrogen bonding interactions of ligands with topoisomerase II protein (PDB ID: 1PVG) are shown in Figure 4. Quercetin was found to interact with the catalytic residues of ASN 99 and SER 128 by double hydrogen bonding and van der Waals interaction with PHE 121. It showed a better docking score (-12.66 Kcal/mol), meaning quercetin interacted more with topoisomerase II, indicating better anticancer activity.

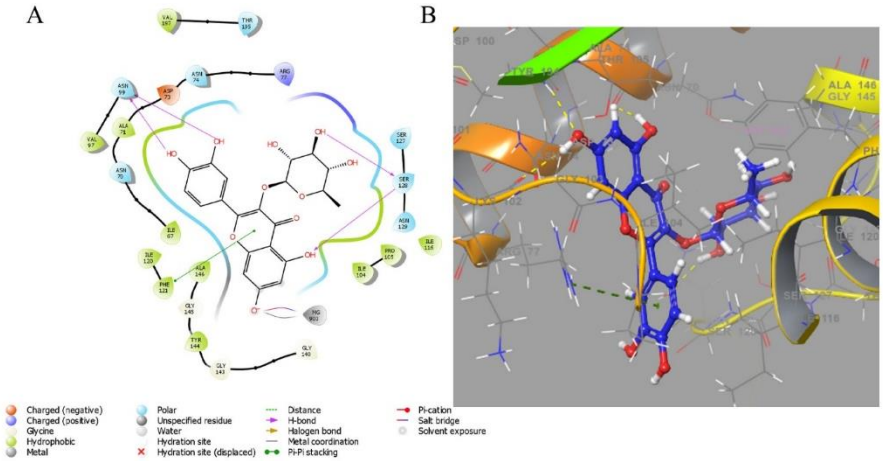

Figure 4. Molecular interaction of quercetin on active site of topoisomerase II (PDB ID: 1PVG) protein. **(A)** 2D; **(B)** 3D structure with potent anticancer activity.

3.6. Docking study on 1CX4 protein.

Individual cellular responses are prompted by the phosphorylation of substrate proteins. Different strategies exist for controlling protein kinases, and each one links a particular upstream signal to the kinase's catalytic activity [32]. With the help of Mg^{2+} ions, protein kinases catalyze the transfer of the ATP's -phosphoryl group to a selected serine, threonine, or tyrosine residue in their target proteins. The most physiologically, biochemically, and structurally characterized member of this enormous family of enzymes is the cAMP-dependent protein kinase (PKA). It distinguishes between various physiological substrates and regulates a variety of cellular activities [33]. The docking results revealed the binding of PKA with the lowest energy of -8.84 Kcal/mol. In addition, quercetin formed three hydrogen bonds with TYR 213, GLU 380, and VAL 210 of PKA amino acid residues Figure 5.

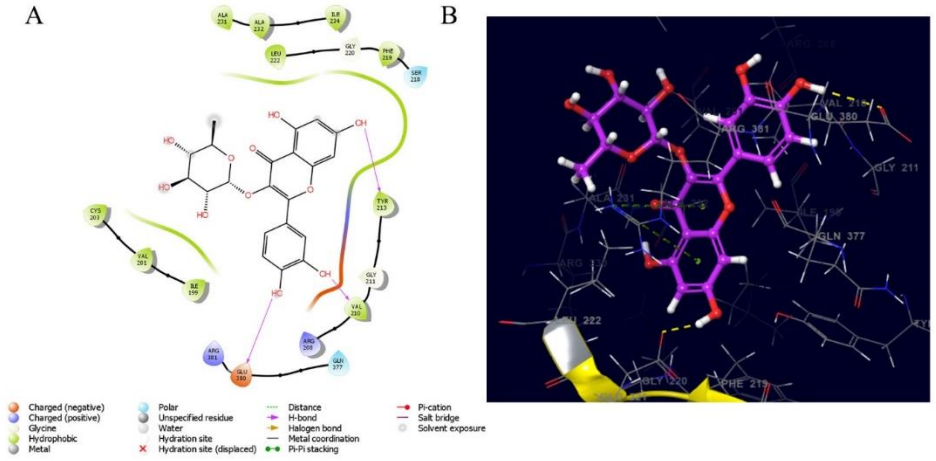

Figure 5. Molecular interaction of quercetin on the active site of cAMP-dependent protein kinase (PDB ID: 1CX4). **(A)** 2D; **(B)** 3D structures with potent anticancer activity.

3.7. In silico ADME prediction.

Lipinski's rule of five states that a chemical compound with a specific pharmacological or biological activity possesses those chemical and physical properties that would probably make it an effective treatment when taken orally by humans. The rule describes molecular properties, including ADME (absorption, distribution, metabolism, and excretion), essential for a drug's pharmacokinetics in the human body. ADME modeling has attracted the focus of pharmaceutical researchers for the drug development process due to its high throughput and cost [34]. Various physicochemical traits, that is, octanol/water partition coefficient, water/gas partition coefficient, brain/blood partition coefficient, donor HB, accept HB, and percent human oral absorption, were computed. These physicochemical properties are given in Table 2. The quercetin ADME findings showed significant results that were closely in accordance with the QikProp rule and Lipinski's rule of five.

Table 2. Physicochemical properties of quercetin.

Compound	QPlogP0/w[1]	QPlogPw	QPlogKp	QPlogBB[5]	DonorHB	AccptHB	Percent Human Oral Absorption
Quercetin	-0.48	23.474	-5.747	-2.976	6	12.05	96.237

Apoptosis is a tightly controlled and selective mechanism that is necessary for cellular development and equilibrium in multicellular organisms [35]. It is crucial for the early development of many body components and regulates the number and proliferation of cells to

maintain cellular equilibrium. Removing damaged cells with significant DNA damage that cannot be repaired also acts as a defensive mechanism [36,37]. In order to carry out apoptosis, cells use either the intrinsic or mitochondrial pathway with the activation of caspases, the extrinsic pathway with death receptors, or both [38]. Figure 6 shows possible mechanisms of flavanol glucoside from *A. muricata* fruit for the inhibition of PI3K, topoisomerase II and cAMP dependent protein kinase.

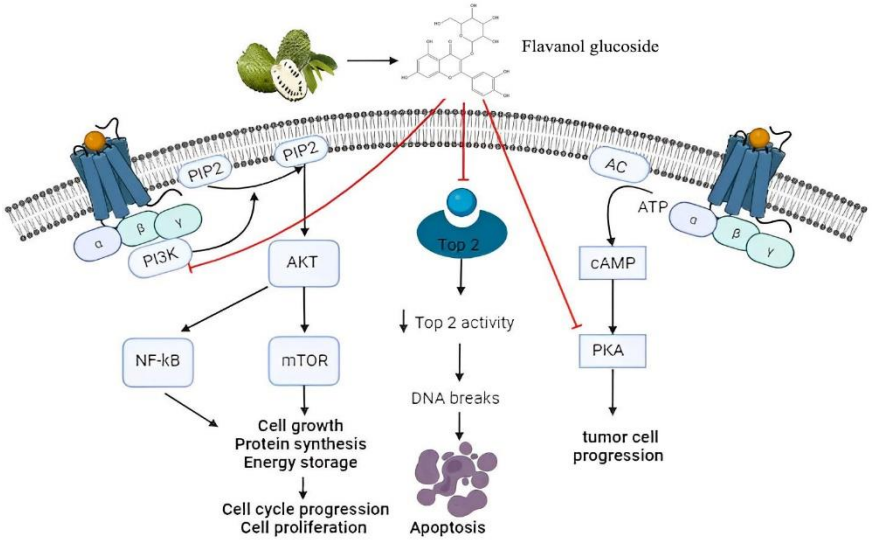

Figure 6. Mechanism of flavanol glucoside from *A. muricata* fruit for the inhibition of anti-apoptotic proteins.

4. Conclusions

The active phytoconstituents from the ethanolic extract of *A. muricata* fruit were a potential source for new drug and therapeutic leads. The result of the study revealed that *A. muricata* fruit contains pharmacologically active substances like flavonol glucoside with anticancer activity, particularly against breast, colorectal, and prostate cancer. It also showed the most effective inhibition on phosphoinositide 3-kinase, topoisomerase II, and cAMP-dependent protein kinase proteins, indicating the flavanol glucoside's effectiveness in apoptosis. Determining how flavanol glucoside from *A. muricata* interacts with anti-apoptotic proteins is important to understanding this substance's anticancer effect.

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Conflicts of Interest

The authors declare no conflict of interest.

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