SMALL MOLECULES FROM *Vernonia amygdalina* (Del.) AS PROMISING AGENTS FOR KRAS-DRIVEN TUMORS

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Abstract

In spite of advances in medicine, cancer remains the second leading cause of death, accounting for 1 in every 6 deaths globally. Numerous oncogenes have been identified to be constitutively active in cancer due to genetic alterations. A notable and hard-to-hit one is the Kras (Kirsten rat sarcoma). Howbeit, resistance and toxicity from synthetic drugs have reduced the survival rate, causing most cancer patients to seek natural remedies with fewer side-effects as alternatives. This study was conducted to investigate the Kras inhibitory effect of small molecules from *Vernonia amygdalina*, utilizing in *silico* approach. A G12C mutated KRas protein was retrieved from Protein Data Bank while the structures of the compounds were collected from PubChem database. Both structures were prepared using the protein preparation and ligprep wizard of the Schrodinger Suite. The castP (Computed Atlas of Surface Topography of proteins) server was employed to identify pockets in KRas before the docking studies with the Glide, Schrodinger Suite. The docking protocol was validated with a RMSD of 0.1724 and binding orientation, similar to the co-crystal ligand. The docking poses and orientations were taken for each compound and the forces of interactions were all considered. From the results, compounds from *V. Amygdalina*, showed good binding interactions with active amino acids reported in mutated Kras inhibition. These binding stabilized the protein in the off state, reducing undue proliferation and downstream signaling. It is therefore promising, that these compounds could restore the lost intrinsic GTPase activity of KRas driven tumors, if taken into further studies.

Keywords: *In silico*; *Vernonia amygdalina*; Kras ;Schrodinger; molecular docking.

Introduction

Mutational activation of the Ras oncogene products (H-Ras, K-Ras, and N-Ras) is frequently observed in human cancers, making them promising anticancer drug targets. Among the isoforms of Ras, Kras (Kirsten rat sarcoma 2 viral oncogene homolog) is the most frequently mutated isoform, constituting 86% of all RAS mutations (Liu *et al*., 2019) and one of the most-hard-to-hit cancer-related proteins. Wild type KRas functions as molecular switches by cycling between guanosine triphosphate (GTP) bound active and guanosine diphosphate (GDP) bound inactive forms in intracellular signaling pathways regulating cell growth, differentiation, and apoptosis (Castagnola and Giaretti, 2005). Activation of Ras is facilitated by guanine nucleotide exchange factors (GEFs) and inactivation by GTPase-activating proteins (GAPs). Upon oncogenic mutation, Kras gets stuck in the ―always on conformation, leading to uncontrolled cellular proliferation. KRas mutation occurs in approximately 30 percent of all human cancers, including more

than 90 percent of pancreatic cancers, 35 to 45 percent of colorectal cancers and approximately 25 percent of lung cancers. The mutations often occur at amino acid positions 12, 13, or 61 that impair GAP-catalyzed GTP hydrolysis.

GTP-Ras can exist in two major conformational states, 1 and 2 (Spoerner *et al*., Araki *et al*., 2011). When in state 1, Ras harbors open pockets and has reduced affinity for effector molecules (Muraoka *et al*., 2012), whereas in state 2, it binds effector molecules effectively (Shima *et al., 2010*). Small-molecule inhibitors that can selectively stabilize the Ras-GTP state 1 conformation have the potential to inhibit Ras signaling by either interfering with effector or exchange factor binding. A few successes have been recorded so far; sotorasib (Lumakras) the first Kras G12C inhibitor has shown so much promise in the fight against cancer. Howbeit, the hunt for an Achilles 'heel continues especially in plants, which have had a long history of use in the treatment of cancers (Bahmani, 2016; Etti *et al*., 2016).

Vernonia amygdalina, commonly called bitter leaf has been used in traditional medicine for various medicinal purposes including: treatment of fever, malaria, diarrhea, stomach ache, scabies, hepatitis, cough and gastro intestinal disorders (Bihonegn *et al.*, 2019). It has also been used as a laxative and as fertility inducer (Francis *et al.*, 2013). *Vernonia amygdalina* has been found to contain sesquiterpenes lactones, glaucolides and elemanolides, some of which have shown tumor inhibiting activities (Jepkorir, 2016;Owoeye et al., 2010). Wong *et al.*, (2013) earlier reported a caspase-dependent breast cancer cell death observed in MCF-7 and MDA-MB 231-*Vernonia amygdalina*-treated cell lines. We anticipated that *Vernonia amygdalina* might possibly have an inhibitory potential towards Kras-driven tumors especially as findings implicate its role in the inhibition of ER-alpha and its downstream player, Akt. Thus, in this work, small molecules from *Vernonia amygdalina* were probed for their inhibitory potential towards G12C mutated Kras protein, using an *in silico* approach.

Materials and methods

The softwares used in this study included: Lenovo Precision work station 6.1.7600 running Intel® Core™ i5 Duo Processor, 4.0GB RAM, 436 GB hard disk and AMD Radeon graphics card, ChemSketch professional software version 15.1, Discovery studio visualizer version 4.5 and the Schrodinger molecular docking suites version 2018-4.

Preparation of Ligands

The ligand structures used in the docking study were obtained from the National Centre for Biotechnology information Pubchem database (www.ncbi.nlm.nih.gov/pccompound) in SDF format and prepared with Maestro, using ligprep version 3.6 (LigPrep 2015), an interface of the Schrodinger software suite. Accurate and high quality 3-dimensional molecular models can be generated with Ligprep from either 1-dimensional like the SMILES or from a 2-dimensional representation like the SDF presentation. Ligprep is a collection of tools designed to produce high quality, low energy 3D structure. The Ligprep employed an applied force field of energy minimization with optimized potentials for liquid simulations-2005 (OPLS_2005) and filtered the ligands for computational studies. It adds hydrogen, neutralize charged groups, generate tautomers, specify chiralities or retain default chiralities, generate possible ionization states at 25 the target pH and remove unwanted structures by desalting before generating low energy ring conformation. The output

structures generated after the whole ligand preparation process was finally written to a file in Maestro format.

Molecular Docking

Selection of Drug Target

A G12C mutated KRas protein with PDB ID 4LYF was chosen as the target for this study. This choice was based on the resolution, species of interest and the bound drug. Identification of binding pockets and preparation of the target protein regions in a protein molecule involved in specific interactions are often conjoined with structural pockets and cavities, within which a ligand can also bind to provoke response. To identify the pockets in KRas, the castP (Computed Atlas of Surface Topography of proteins) server was employed (Binkowski et al., 2003). The castP measures the number and area of mouth openings, circumference of mouth lips and quantitates the number of amino acids within the cavities (Dundas et al., 2006). Upon acquisition of data on amino acids in the ligand binding domain of the KRas protein, the protein was retrieved from the RCSB protein data bank with the PDB ID 4LYF. The Discovery studio visualizer, a suite of software developed by Accelrys (Brooks et al., 2009) was used to visualize the complete protein X-ray structure (Figure 1A) and to label its active site amino acids (Figure 1B). The protein preparation was done using the protein preparation wizard of the Schrodinger suite workflow option. It was done with the application of the force field, optimized potentials for liquid simulations-2005. The water molecules that accompanied the crystallographic structure were deleted and the missing side-chains updated according to the protein preparation protocol. Hydrogen atoms were added and optimized. Minimization of the complex was done using OPLS_2005 force field with Polack-Ribiere Conjugate Gradient (PRCG) algorithm after the energy gradient converged below 0.05 kcal/mol (Kaminski *et al.,* 2001). Grid, representing the identified active site needed for the ligand-docking was generated for the receptor using glide module, Glide version 11.0, (Schrodinger software release 2018). Briefly, the co-crystallized ligand was selected in the ligand binding domain of the glide grid and replaced during the docking process, with the native ligand (during the validation of docking protocol) and with each of the studied ligand during the docking studies of the tested compounds.

Validation of the Docking Protocol

To ascertain the predictive ability of the docking protocol, a native ligand, compound 21C, which by default accompanied the target protein, was redocked into the ligand binding domain (LBD) of the KRas protein with a careful attention to the obtained root mean square deviation (RMSD) and binding orientation. This information indicates the validity of the molecular docking protocol and the reproducibility of results obtained from the studies (Chandna *et al*., 2015; Hocker *et al*., 2013). The docking protocol was tested by redocking the co-crystal structure, compound 21C to the mutated KRas, 4LYF. The binding sites and ligand pose were identified and the root means square of deviation calculated after superimposing the two structures (Figure 2).

Docking Studies Using Schrodinger Software Suite

The docking study was carried out using the Schrodinger Suite (Maestro Version 11.8 and Glide version 8.0, 2018-4). The glide grid was selected and each ligand was individually docked onto the LBD of the KRas using Glide extra precision (XP) mode. In the course of the docking, several binding poses were generated for each ligand and the best binding pose was selected at the end of the docking process.

Results and Discussion

Active Amino Acids at the Ligand Binding Domain of the KRas Receptor

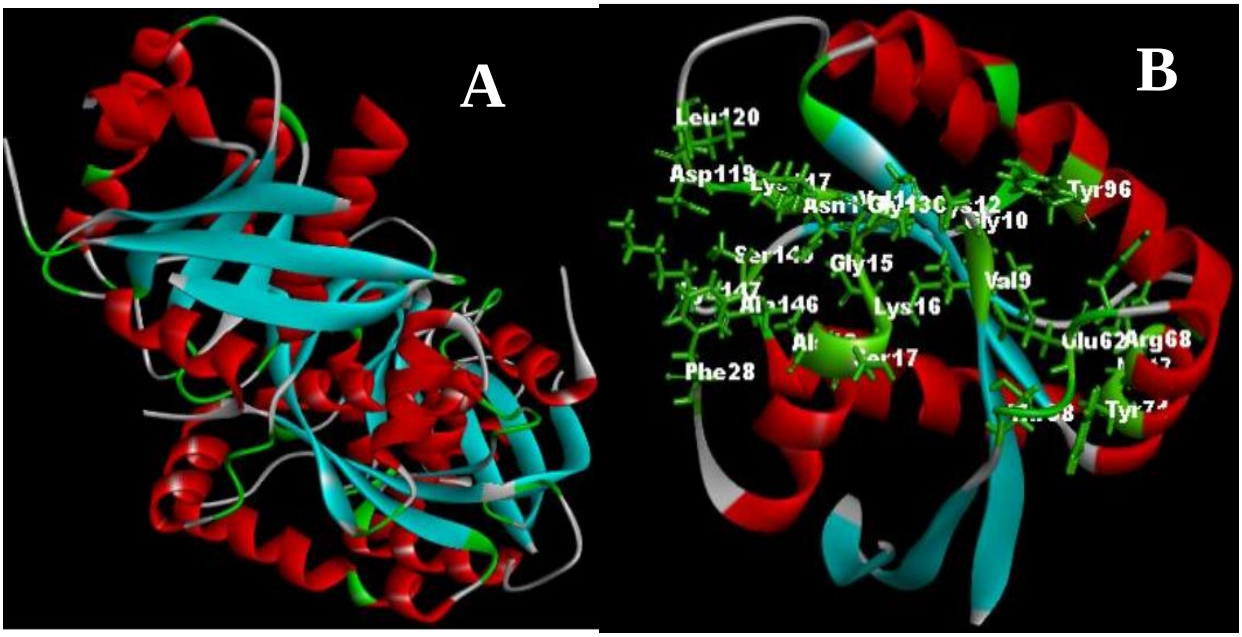


Fig 1. Complete X-ray structure of KRas receptor shown as a ribbons (A) and Active amino acids at the catalytic site of KRas, 4LYF (B)

The complete X-ray structure of KRas (PDB ID: 4LYF) at a resolution of 1.568 Å was visualized with Discovery studio visualizer suite, version 4.5 (Figure 1A). The active site which was earlier prediction with the CASTp server (Dundas et al., 2006) revealed active amino acids that participated in the inhibition of this mutated KRas.

Validation of Molecular Docking Protocol

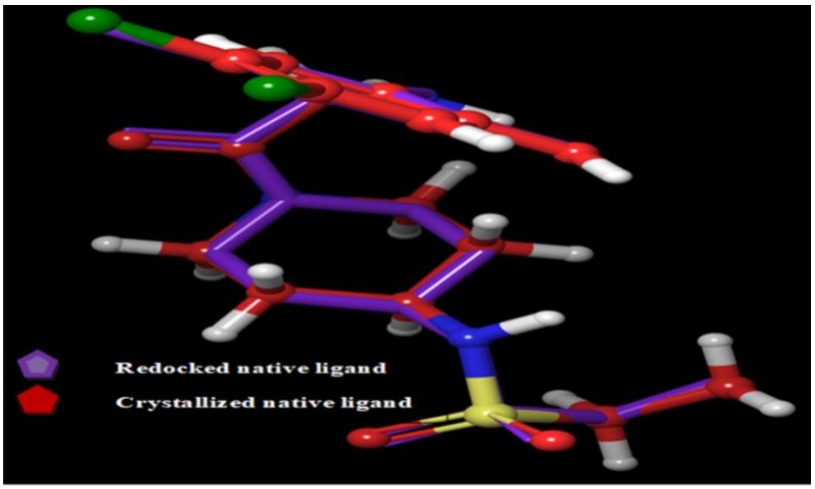


Fig 2. Superimposition of the redocked and co-crystalized native ligand

The docking protocol used in this study was validated by redocking compound 21C, the co-crystal structure of the KRas [Protein Data Bank (PDB) ID code 4LYF] into its original position. The appropriateness of the protocol was assessed by examining the binding pose

and orientation of the redocked co-crystal structure. The root mean square deviation calculated from the superimposition of the redocked co-crystal structure with the downloaded X-ray crystal structure (Figure 2) gave a root mean square deviation of 0.1724 which is within the 2Å limit for validation of a docking protocol (Chandna *et al*., 2015). The results showed that the binding sites and ligand pose were correctly identified. The binding position as well as the orientations of the native ligand within the receptors binding site was similar to the co-crystal structures with a root mean square of less than 2Å (0.1724Å), which indicated that the docking experiment is appropriately reproducible (Chandna *et al*., 2015).

Molecular Docking Analysis

The docking interactions of the active residues in the ligand-binding domain of KRas with the molecules from *Vernonia amygdalina* are shown in Figures 3 to 8 below. The glide or docking score (G Score) that approximates the ligand-binding free energy, was used to estimate the binding affinities of each ligand to the active site of the target receptor (Table 1) and to rank the best scorer (Friesner *et al*., 2006; Josh *et al*., 2014). Some key amino acids, which had been reported to be involved in the binding inhibition of the KRas receptor were used to investigate major non-covalent interactions of the ligands to the ligand-binding domain of KRas (Fatima and Yee, 2014; Ostrem *et al.,* 2013). The non-covalent interactions included: the coulomb interaction, Van der Waals interaction and hydrogen bond interaction. From the results, 11, 13 dihydrovermodalin formed hydrogen bond interaction with ASN116 and LYS117 at 1.87Å and 2.7Å respectively (Figure 3 A&B). The 3‘ and 4‘ hydroxyl groups of luteolin glycoside (Figure 4 A&B) formed hydrogen bonds with ASP119 at 1.82Å and 1.94Å respectively. The 3‘ hydroxy group also interacted with LYS147 at 2.44Å. Interaction with GLY13 and LYS16 at 2.67Å and 2.83Å respectively was also observed and this compound created a salt bridge with the mutated residue. There was also a pi-stacking interaction with PHE48. The 4‘ hydroxyl group of luteolin (Figure 4.C&D) interacted with ASP119 and LYS147 at 1.51Å and 2.35Å respectively while 3‘ hydroxy group also bonded with ASP119 at 1.93Å. A salt bridge with the mutated residue was formed and a pi-stacking interaction with PHE48 of the KRas protein. Oxygen and carbon atoms of Methyl 2-O-benzyl-D-arabinofuranoside (Figure 5 A&B) interacted with hydrogen atoms of LYS117 at 1.88Å and 2.57Å respectively. There was also an interaction with LYS147 and ASP119 at a distance of 2.02Å and 1.99Å. Other hydrogen bond interactions are as depicted in each figure as seen below (Figures 6 A-D).

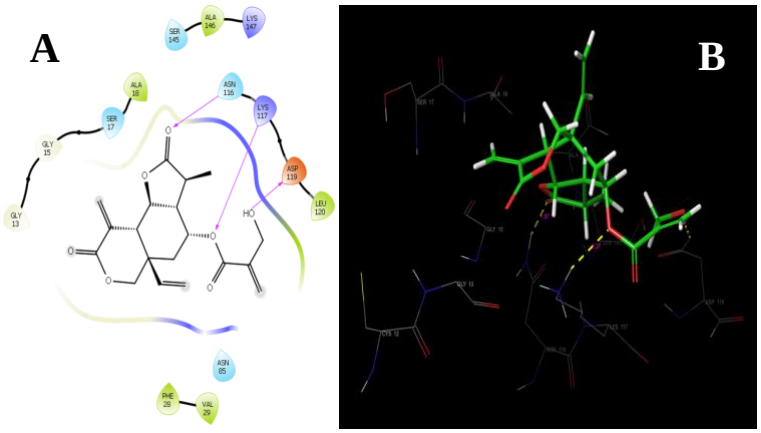


Fig 3 Docking interactions of of 11, 13 dihydrovermodalin with crucial amino-acids at the LBD of Kras

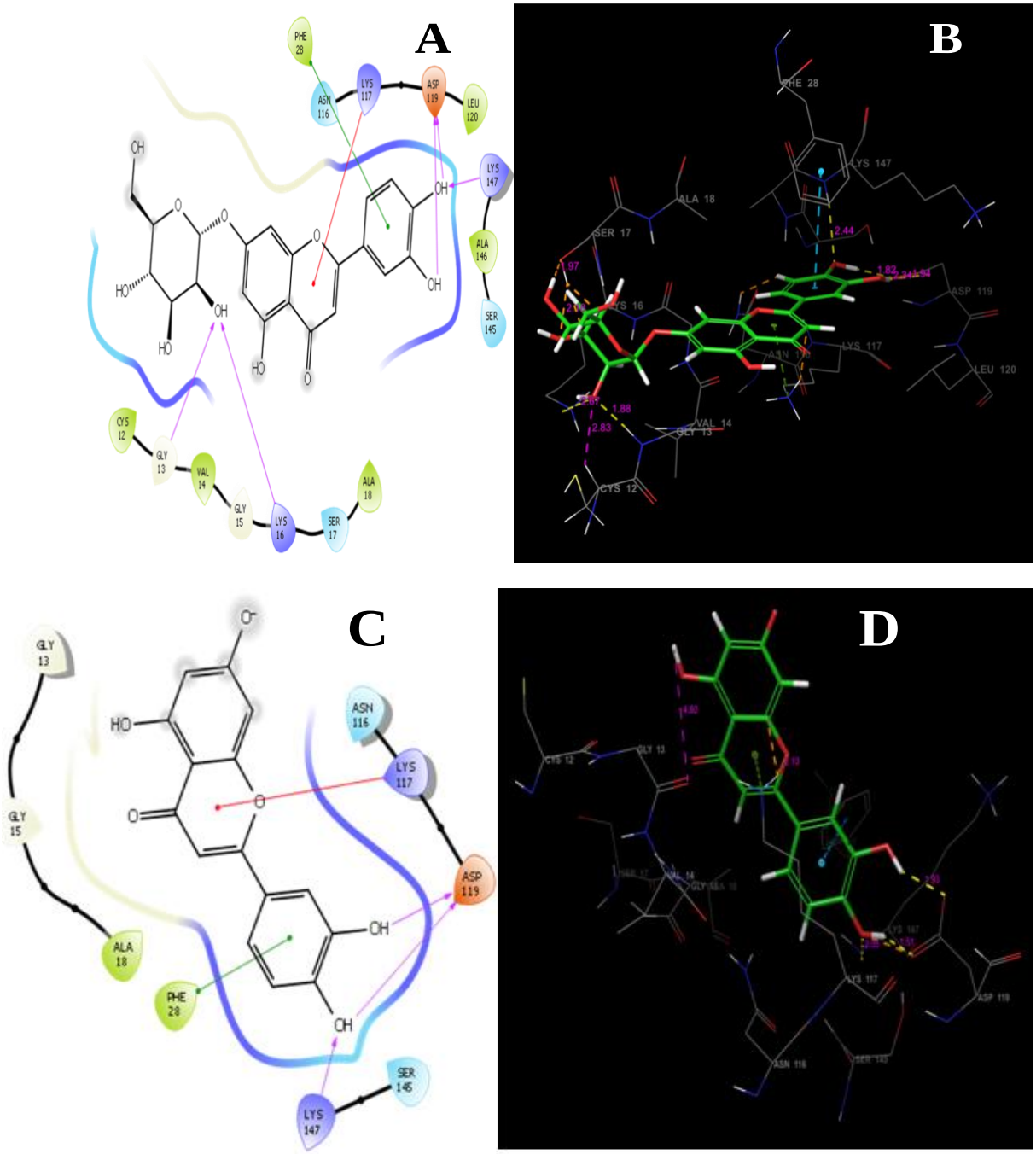


Fig 4. 2D and 3D docking interactions of luteolin glycoside (A&B) and luteolin (C&D) with crucial amino-acids at the LBD of Kras

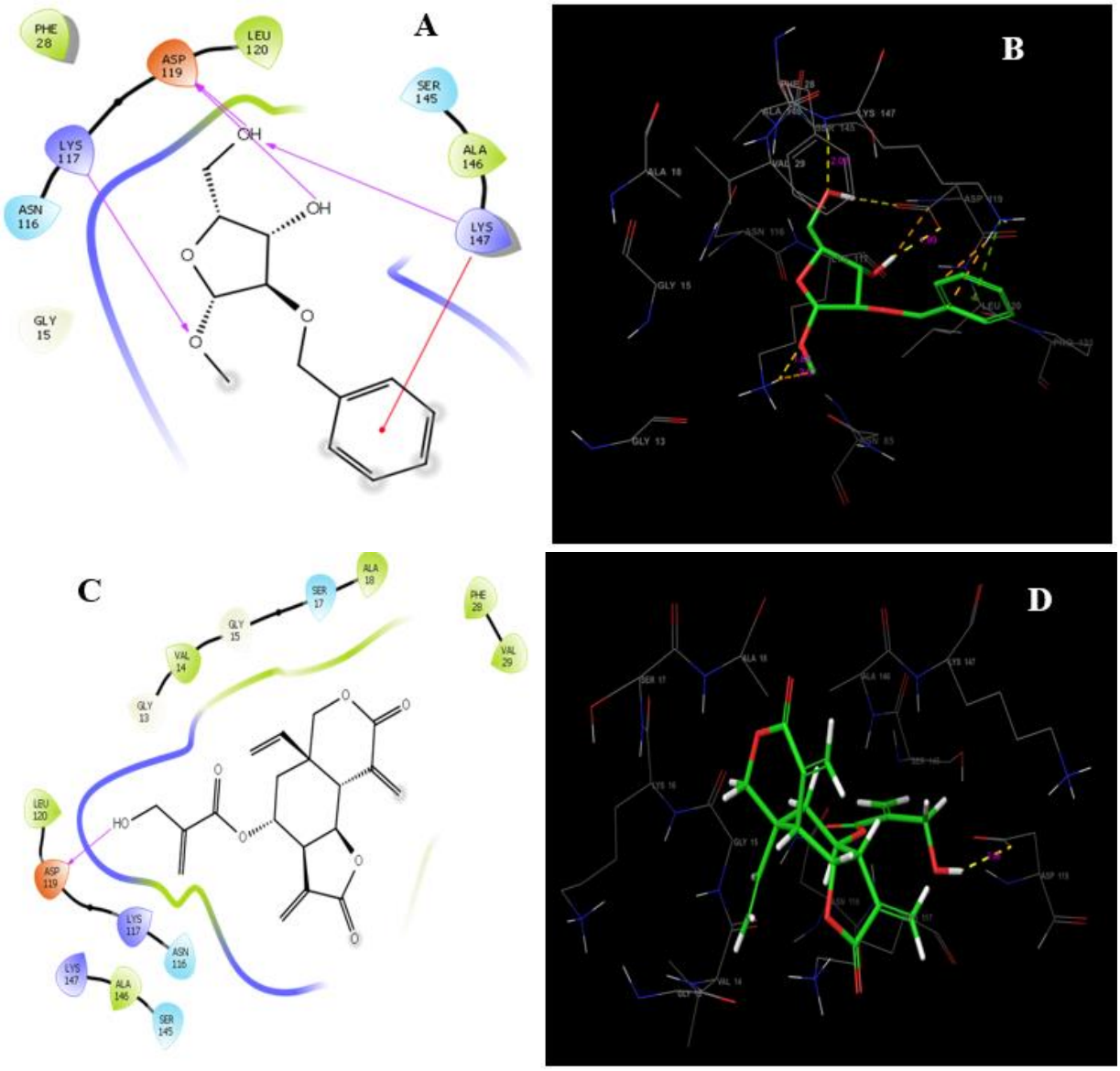


Fig 5. 2D and 3D docking interactions of Methyl-2-O benzyl-D-arabinofuranoside (A&B) and Vernodalin (C&D) with crucial amino-acids at the LBD of Kras

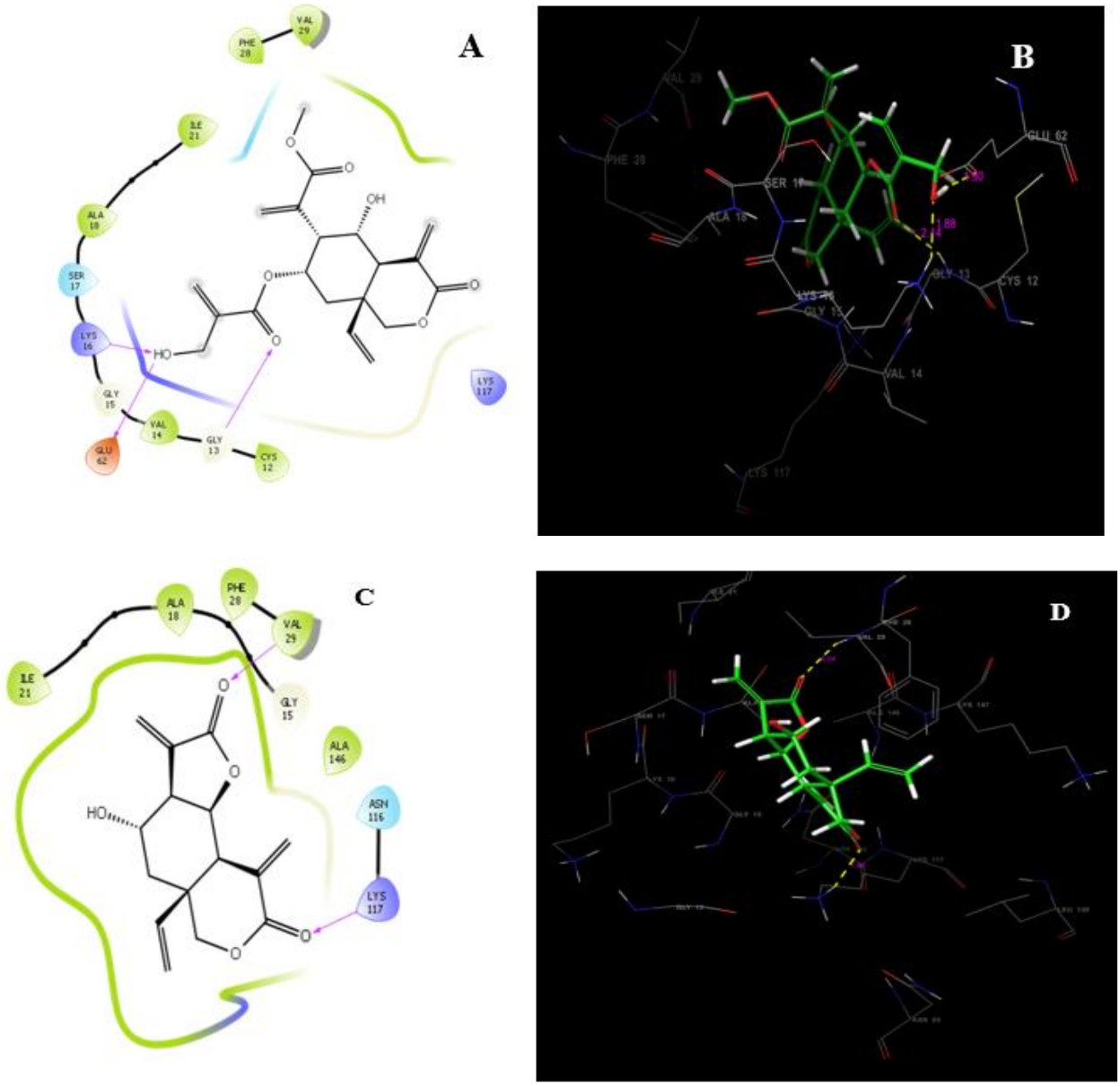


Fig 6. 2D and 3D docking interactions of Vernodalol (A&B) and Vernolepin (C&D) with crucial amino-acids at the LBD of Kras

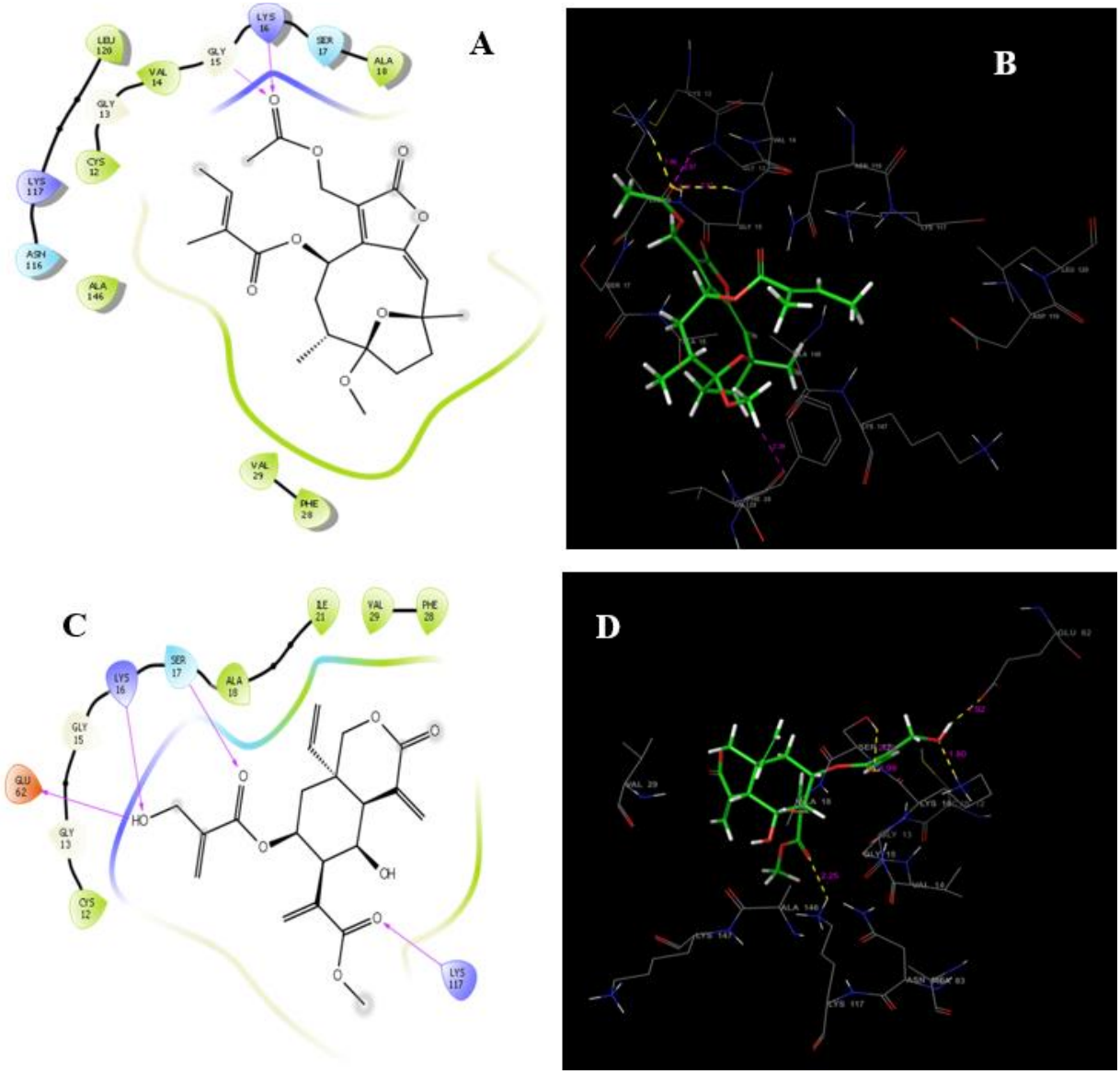


Fig 7. 2D and 3D docking interactions of Vernolide B (A&B) and Epivermodalol (C&D) with crucial amino-acids at the LBD of Kras

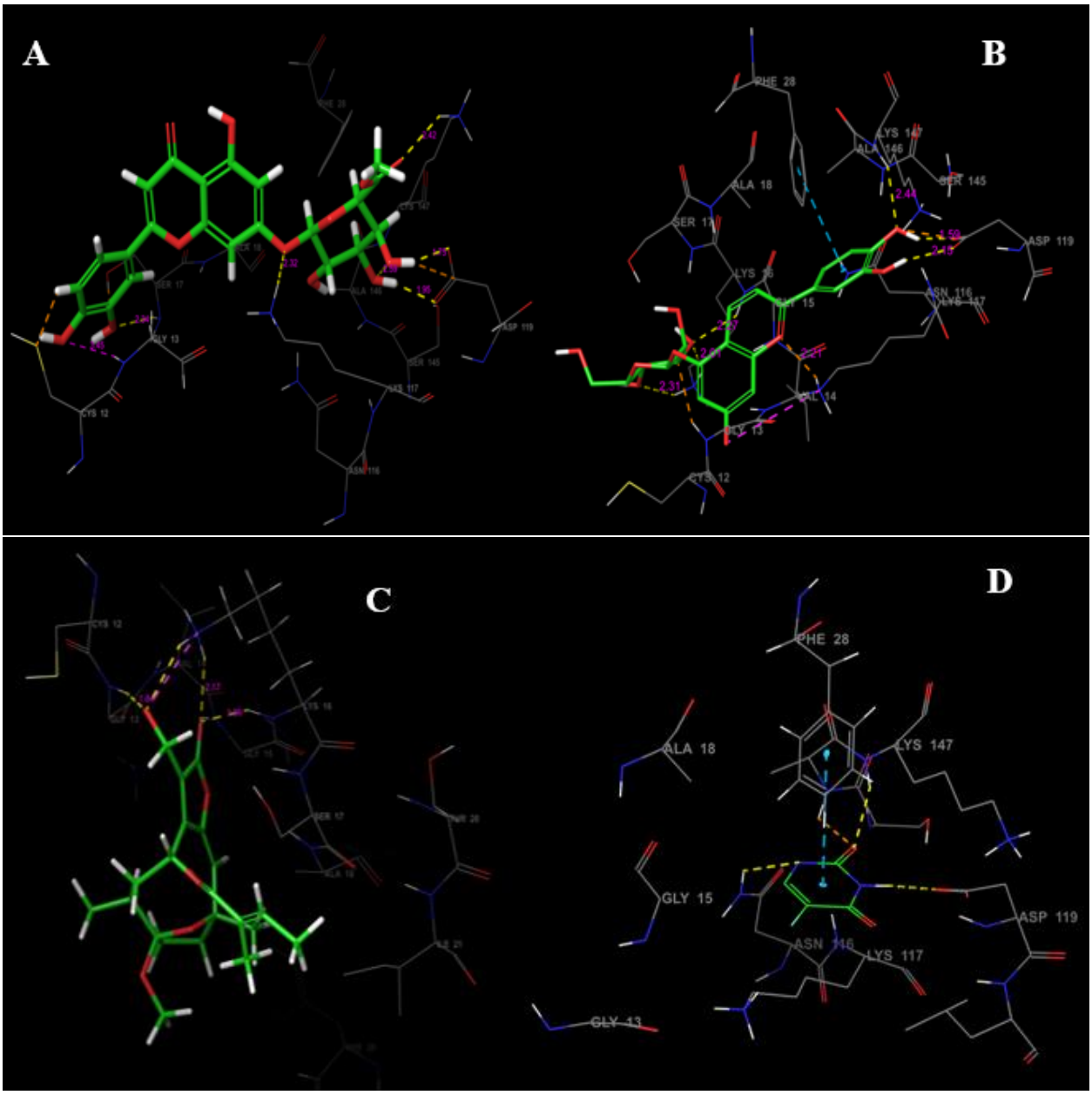


Fig 8. 3D docking interactions of Luteolin-7-O- glucoronide (A), Luteolin 3′-O-glucoside (B) and Vernolide A (C) with 5 Fluorouracil (D) with crucial amino-acids at the LBD of Kras

Vernolide B (Fig 7 A&B) had interaction with GLY15 and VAL29 at 2.43Å and 0.38Å respectively. Its oxygen atom bonded firmly with LYS16 at 1.96Å and 2.14Å respectively. Epivernodalol (Figure 7 C&D) formed a firm bond with SER17 at 2.29Å and 1.99Å. Its hydrogen atom also bonded firmly with GLU62 at 1.92Å, with LYS16 and LYS117 at 1.80Å and 2.25Å respectively. Like vernodalol (Fig 6 A&B), the binding orientation was similar to the native ligand. For luteolin-7-O-glucoronide (Figure 8A), a firm hydrogen bonding was observed with ASP119 at 1.75Å and 1.95Å and with LYS147 at 2.49Å/2.59Å and with LYS16, LYS17 and SER17. For luteolin 3′-O-glucoside (Figure 8B), a firm interaction was observed with LYS147, LYS16 and ASP119.

Like the native ligand, a firm interaction was observed in Vernolide A with LYS16 at 1.96Å, 2.17Å, 2.32Å and with GLY15 at 2.02Å and GLY13 at 1.84Å (Fig 8 C). For 5 Fluorouracil (Figure 8 D), interactions were found with ASP119, ASN116, ALA146, LYS147 with a pi-pi stacking interaction with PHE28. Generally, it was observed that the compounds from *V. Amygdalina*, had good binding interactions with vital amino acids which had been reported to play key role in the inhibition of the mutated Kras.

Table 1: Glide Energy Ranking of Studied molecules

Compounds	Glide Energy
5 Fluorouracil	-24
Leuteolin-7-glycoside	-43.9
Leuteolin-7-glucuronide	-47.8
Vernolepin	-22.1
Methyl 2-0-benzyl	-26.7
Leutolin	-34.1
Vernolide A	-37.0
Epivernodalin	-34.6
11,13-dihydrovermodalin	-31.7
Vernodalol	-36.8
21C	-37.9
Suphanyl butamide	-43.8
Vernolide B	-34.9
Gemcitabine	-34.1

Somatic mutations in the small GTPase K-Ras are the most common activating lesions found in human cancer, and are generally associated with poor response to standard therapies (Ostrem *et al.*, 2013). Oncogenic mutations result in functional activation of Ras family proteins by impairing GTP hydrolysis. The nucleotide state of Ras becomes more dependent on relative nucleotide affinity and concentration because of diminished regulation by GTPase activity. Thus, giving GTP an advantage over GDP and increases the proportion of active GTP-bound Ras. In this study, small molecules from *Vernonia amygdalina* showed good binding interactions with the oncogenic mutant, K-Ras(G12C). Noncovalent binding interaction was observed and involved all the usual molecular forces, like, hydrogen bonds, electrostatic and van der Waals forces, hydrophobic and cation–π interactions as shown in the results above. This combination of multiple noncovalent bonds reflects the

affinity of the tested molecules for the mutated Kras receptor.

Conclusion

It is promising that the binding of small molecules from *Vernonia amygdalina* to K-Ras(G12C) will disrupt both switch-I and switch-II, subverting the native nucleotide preference to favour GDP over GTP and impairing binding to downstream effector molecules. Thus. showing promise of inducing apoptosis in Kras - driven cancer cells.

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