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ANTICANCER POTENTIAL OF *AMPHIOCTOPUS AEGINA* INK: AN INTEGRATED *IN-VITRO* AND *IN-SILICO* STUDY

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ABSTRACT

Colorectal cancer is one of the major health challenges for man-kind. The genetic modifications, leading to altered signaling pathways has increased the severity of the disease and hence increased mortality. In this study aqueous ink extract of *Amphioctopus aegina*, commonly known as marbled octopus, has been investigated for the presence of bioactive compounds and its anticancer potential using experimental and computational approach. Cytotoxicity of the ink extract used on colorectal cancer cell lines HT-29, showed a dose-dependent reduction in cell viability with an IC_{50} of 82.68 ± 0.05 µg/mL. Further, the bioactive compounds were identified using LC-MS. Based on high-confidence spectral scores (≥95 %) 34 compounds were short-listed. ADMET profiling was carried out and eight compounds with favorable pharmacokinetic properties were considered for molecular docking studies on KRAS4B (G12V) and E17K-AKT1, the most commonly mutated proteins in colorectal cancer. Most of the compounds showed good binding affinity, notably dihydro-3-coumaric acid showed stronger interactions with both the proteins, while indole-3-carbinol showed good binding affinity with E17K-AKT1. The stability of these complexes was validated using Normal mode analysis (NMA). Collectively, the findings suggest that *A. aegina* ink extract contains bioactive compounds with potential anticancer activity, showing predicted interactions with KRAS and AKT pathways. This study highlights the therapeutic potential of marine-derived natural products as promising candidates for the development of novel anticancer agents.

Keywords: Colorectal cancer, KRAS4B mutation, PI3K/AKT pathway, *In-silico* analysis, Marine bioactive compounds

INTRODUCTION

Cancer is one of the major challenges for human health, with a steady increase in cases and mortality index every year. There has been an increase of around 6 million new cases over the past decade **(Wei et al., 2024)**. Colorectal cancer is the 4th leading cause of all the cancer deaths worldwide. Its drug resistance to most of the chemotherapeutic drugs used for the cancer treatment is the major contributing factor **(Cerrito & Grassilli, 2021)**.

Colorectal cancer tumorogenesis majorly involves genetic alterations caused by chromosomal instability (CIN). Around 85% of adenocarcinoma transitions are due to CIN and this leads to mutations in key oncogenes such as KRAS **(Malki et al., 2021)**. KRAS is one of the most common types of RAS molecules which plays a crucial role in multiple signaling pathways involved in tumor development. AKT1 is also an important signaling molecule which plays a central role in cell proliferation and cancer development. Hyper activation of AKT1 has been reported in many cancers including colorectal cancer **(Ortega et al., 2020)**. Targeting these mutations is the major therapeutic challenge.

The marine ecosystem is diverse with at least 0.5 million different species. Their interaction leads to several unique adaptive conditions. This makes the ocean a rich source of bioactive compounds. Cephalopods are one such species that use their inking ability to escape from predators. This has fascinated humans for a very long time **(Derby, 2014)**. Many studies have reported cephalopod ink to contain many bioactive compounds such as flavonoids, alkaloids, phenols, amino acids and proteins **(Sabry et al., 2023)** exhibiting antioxidant, anti-inflammatory and also anticancer activity **(Hernández-Zazueta et al., 2021)**.

Cephalopod ink is a combined secretion of two glands- ink gland, majorly produces melanin imparting thick black colour to the ink and funnel gland produces mucous. The melanin is a polymer of 5,6-dihydroxyindole (DHI) and 5,6-dihodroxyindole-2-carboxylic acid (DHICA). Melanin is produced in the ink gland by the tyrosinase enzyme which catalyzes the precursor molecules, tyrosine and L-DOPA **(Derby, 2014)**.

Many molecules belonging to these pathways are reported to be present in the ink. A study by Madaras et al, emphasizes that homogenizing ink sac itself would result in diverse bioactive compounds as it contains chemicals from the ink producing cells as well. These might as well have many pharmaceutical properties **(Madaras et al., 2010)**.

Different cephalopods such as squid, cuttlefish and a few species of Octopus have demonstrated significant antioxidant, anti-inflammatory and anticancer potential **(Nadarajah et al., 2017; Shaju et al., 2022; Šimat et al., 2020)**. But many species remain unexplored. In our previous study, we have investigated the antioxidant and anti-inflammatory potential of *Amphioctopus aegina* ink and identified the bioactive compounds composition **(Sahana & Joseph, 2026)**. With this preliminary work, in this study, we investigate the anticancer potential of *Amphioctopus aegina* ink sac extract. The identified bioactive compounds were evaluated for their binding affinity against mutant oncogenic proteins KRAS4B and AKT1 using *in-silico* approaches. Furthermore, normal mode analysis was performed to assess the stability of protein–ligand interactions, providing insights into their potential as therapeutic candidates for colorectal cancer.

MATERIAL AND METHODS

Sample collection

The ink glands of *A. aegina* were collected from "Fresh Katch" industry, Madurai and transported to the laboratory in chilled condition. The obtained ink gland was homogenized in distilled water and lyophilized as described by Madaras et al **(Madaras et al., 2010)**. The sample was further extracted using sterile distilled water in 1:10 (w/v) ratio, as described in our previous study **(Sahana & Joseph, 2026)**.

In-vitro Anticancer activity

Cytotoxicity studies of the aqueous extract of *A. aegina* ink on human colorectal adenocarcinoma cell line HT-29 (Procured from NCCS, Pune) was done by MTT Assay. The cells (1 × 10^4 cells/well) were cultured in 96 well plate for 24 h in Dulbecco's Modified Eagle Medium (DMEM -AT149-1L- HiMedia) supplemented with 10 % Fetal Bovine Serum (FBS- HiMedia-RM 10432) and 1 % antibiotic solution (Penicillin-Streptomycin-Sigma-Aldrich P0781) at 37°C with 5 % CO_2. After incubation, cells were treated with different concentrations of the samples (0-1000 µg/mL). Stock solution of samples was prepared in 0.1 % Dimethyl Sulfoxide (DMSO –SRL- Cat no.- 67685) and further diluted to get different concentrations in serum free medium. After incubation for 24 h, MTT Solution (5 mg/mL) was added to cell culture and incubated (Air- jacketed CO_2 incubator-Heal force-HF90) for 2 h. Cells without treatment were considered as control and the media without cells were considered blank. At the end of the experiment, culture supernatant was removed, and 100 µL DMSO added to the cell

layer matrix to dissolve the formazan crystals. Absorbance was read in a microplate reader (iMark, Bio-Rad, USA) at 540 nm. Cell viability at each concentration was calculated from technical triplicate wells and expressed as mean ± standard deviation (SD). Dose-response curves were fitted using a four-parameter logistic (variable slope) nonlinear regression model in OriginPro 8.5, and the IC_{50} value was estimated from the fitted curve. The uncertainty reported for the IC_{50} corresponds to the standard error (SE) of the nonlinear regression estimate generated by the software. **(van Meerloo et al., 2011)**.

% Viable cells = (A_{test} / $A_{Control}$) *100

A_{test} = Absorbance of test sample

$A_{Control}$ = Absorbance of Control

LC-MS analysis

LC-MS analysis of the aqueous extract of *A. aegina* ink was carried out using an Agilent Q-ToF G6540B connected to Agilent 1260 Infinity II HPLC. The 15 µL of sample was injected to the Agilent Eclipse XDB-C18 (3X150 mm, 3.5 µm) with column temperature maintained at 40˚C. The Mobile phase was (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in acetonitrile, with a gradient elution ranging from 5-95 % (B). Total run time was 30 min with a flow rate of 0.3 mL/min.

Mass spectrometric analysis was done in both positive and negative ionization mode, with m/z ratio ranging from 100-1700. The optimized instrument parameters were as follows: gas temperature, 300°C; sheath gas temperature, 350°C; drying gas flow 8 L/min; nebulizer pressure 35 psi; sheath gas flow 11 L/min; capillary voltage 3500 V; and nozzle voltage 1000 V. The system recorded total ion chromatograms (TIC), retention times, and mass-to-charge ratios (m/z) for identification of phytochemical constituents.

Molecular Docking

The obtained compounds' chemical structure was retrieved from Pubchem database and the structures were redrawn using RDKit **(Landrum, 2024)**. Further, the pharmacokinetic properties and drug-likeness of compounds were evaluated through ADMET analysis using the SwissADME online tool **(Daina et al., 2017)** and ProTox 3.0 online tool **(Banerjee et al., 2024)**. Further, the molecular docking of compounds with mutant proteins KRAS4B and E17K-AKT1 were conducted using a combination of computational tools. The 3D structures of all the compounds (ligand) were prepared using UCSF Chimera **(Pettersen et al., 2004)**, where the ligand was energy-minimized and optimized for docking. The mutant KRAS4B (PDB ID: 8B69) and E17K-AKT1 (PDB ID: 8UW9) protein structures were retrieved from the Protein Data Bank and processed using PyMOL Molecular Graphics system version 3.1.0 **(Schrödinger, 2026)** by removing water molecules, ligands, and non-essential heteroatoms, and in case of KRAS4B bound effector molecule was also removed, followed by the addition of polar hydrogen. Docking simulations were carried out using AutoDock Vina **(Trott & Olson, 2010)** with PyRx Python prescription 0.8 **(Dallakyan & Olson, 2015)**, with exhaustiveness 32 and multiple binding poses were generated. Visualization and analysis of molecular interactions were performed using PyMOL Molecular Graphics system version 3.1.0 and Discovery Studio Visualizer v24.1.0.23298 to identify key interactions such as hydrogen bonds and hydrophobic contacts **(BIOVIA, 2024; Schrödinger, 2026)**.

Normal Mode analysis

Stability and flexibility of the selected ligand-protein complex were analysed by Normal Mode Analysis (NMA) using the online software iMODS (https://imods.iqf.csic.es/) **(López-Blanco et al., 2014)**. The software detected the deformability, Eigen values and also elasticity of the complexes.

RESULTS AND DISCUSSION

Anticancer potential of Aqueous extract of *A. aegina's* ink

The cytotoxicity of the aqueous extract of *A. aegina* was analysed using MTT assay. The extract reduced the viability of colorectal adenocarcinoma cell line HT-29 in a dose-dependent manner. At 1000 µg/mL concentration, cell viability was reduced to 15.12±0.714%, corresponding to 84.88% inhibition of cell viability. The extract exhibited an IC_{50} value of 82.68±0.05 µg/mL, indicating potential cytotoxic activity (Fig 1). Although further studies on non-cancerous colon epithelial cells are required to evaluate the selectivity of the extract towards colon cancer cells.

Studies by Zazueta MSH, et al. 2021 have reported anti-proliferative capability of *Octopus vulgaris* ink extract on human prostate cancer cell line 22Rv1 **(Hernández-Zazueta et al., 2021)**. *Octopus vulgaris* ink has also been shown to have a good cytotoxic effect on breast cancer cell line and liver cancer cell line as reported in the study conducted by Hamdi S A, et al. 2024 **(Hamdi et al., 2024)**. Many studies have reported presence of many bioactive compounds such as flavonoids, alkaloids, phenols, melanin derivatives in the ink extract of cephalopods. These compounds are known to have many bioactivities such as antioxidant, anti-inflammatory properties **(Sabry et al., 2023)**. These compounds have also shown good anticancer activity **(Al-Khayri et al., 2022)** . Presence of these compounds in the ink extract of *A. aegina* as reported in our previous study **(Sahana & Joseph, 2026)** might be the reason for good anticancer activity against colorectal cancer cell line HT-29.

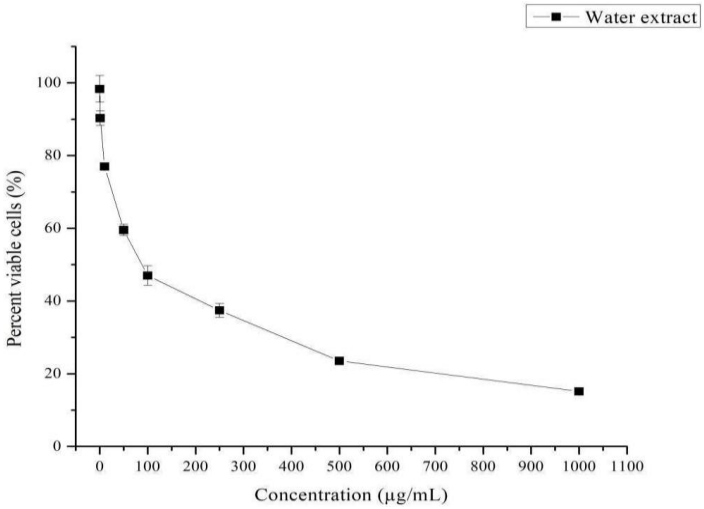


Figure 1 Cytotoxic effect of the aqueous extract of *Amphioctopus aegina* ink on HT-29 colorectal cancer cells, as determined by MTT assay after 24 h treatment. Data is presented as mean ± SD of technical triplicates. Dose-response curves were fitted using a four-parameter logistic nonlinear regression model. Estimated IC_{50} = 82.68 µg/mL (95% CI: 60.86–112.3 µg/mL).

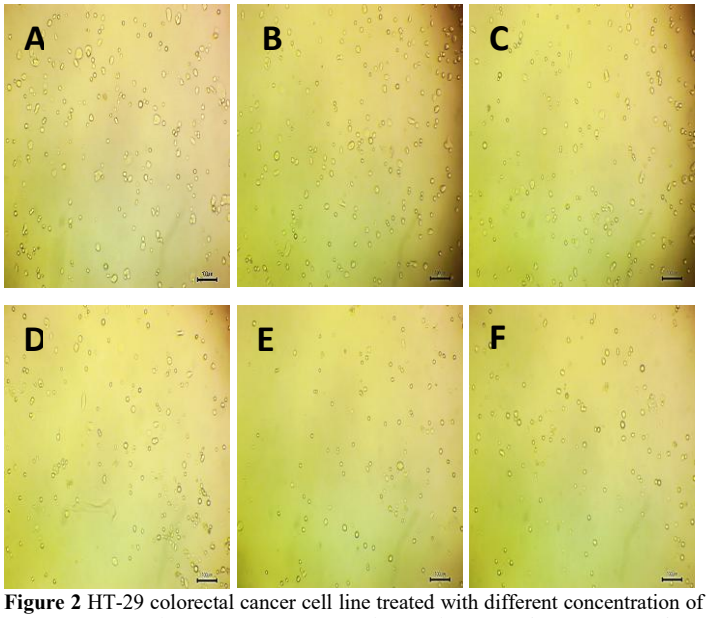


Figure 2 HT-29 colorectal cancer cell line treated with different concentration of aqueous extract of *A. Aegina.* A: Untreated control; B:50 µg/mL; C: 100 µg/mL; D: 250 µg/mL; E: 500 µg/mL; F: 1000 µg/mL;

LC-MS analysis

To identify the bioactive compounds, present in extract that might be responsible for Anticancer activity against colorectal cancer cell line, LC-MS analysis was carried out (Fig 3). Around 34 compounds were shortlisted based on high-confidence spectral scores (≥95 %). These compounds have previously been reported as natural metabolites. Some of the compounds are also reported as marine metabolites. Most of these compounds were phenols, amino acids and alkaloids. Our previous study also reported the presence of phenolic compounds and alkaloids abundantly **(Sahana & Joseph, 2026)**. Madaras et al, also reported the presence of phenolic compounds, amino acids and alkaloids in the crude extract of ink gland of squid. In addition, they have also reported the presence of epinephrine, and norepinephrine in ink gland extracts **(Madaras et al., 2010)**. Few of the compounds identified in LC-MS such as 3,4-Methylenedioxybenzoic acid, Paeonol, 2,3-dihydrobenzofuran, Indole-3-carbinol and Caffeic acid have been reported to possess anticancer activity **(Dapkekar et al., 2022; Wang et al., 2025; Yang et al., 2023)** and might be responsible for the anticancer activity exhibited by the extract against colorectal cancer cell line. Few of the compounds like Caffeyl Alcohol, Apocynin, 3,4-Dimethoxybenzaldehyde and 3,4-Dihydroxymandelic acid are known to be good antioxidant molecules and few of them have also shown anti-inflammatory activities **(Ikram et al., 2024; R. H. Liu,**

2013; Nesovic Ostojic et al., 2025). Few compounds like phloretic acid, Ethyl 2-aminobenzoate and Benzyl glycinate are precursor molecules for many anticancer drug candidates **(X. Liu et al., 2022; Mudiganti Ram Krishna Rao1*, 2018)**. Although most of these compounds share a common phenolic group, their biological activities and interactions with biomolecules might differ, hence all the compounds were subjected to ADMET analysis before considering molecular docking.

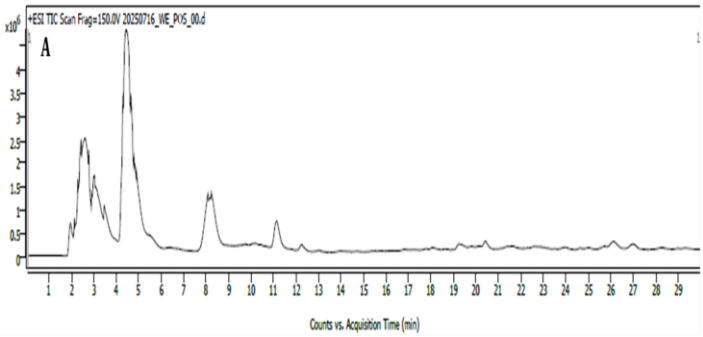
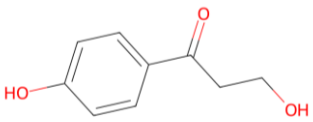
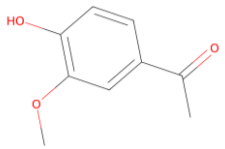
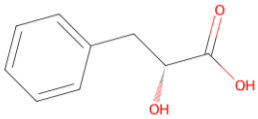
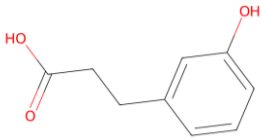
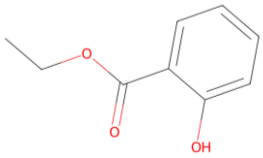
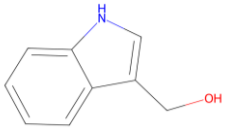
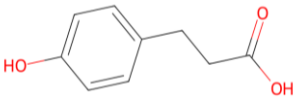
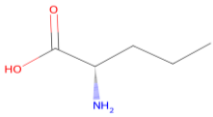


Figure 3 LC-MS Chromatogram of the aqueous extract of *A. aegina* ink

Drug-likeliness

ADMET analysis was carried out and 8 compounds with favorable ADMET profiles were selected (Table 1). These compounds were satisfying Lipinski's rule of five and had good gastrointestinal absorption. As the boiled egg- graph depicts most of the compounds were having lower Topological Polar Surface Area (TPSA) making it easier for gastrointestinal absorption. None of these compounds where substrates for glycoprotein hence cellular absorption might not be affected (Fig 4). These compounds were also predicted to be non-cytotoxic or non-carcinogenic to normal cells based on the online tool ProTox 3.0. Overall, the selected compounds were good drug candidates as per the ADMET analysis. But few of the compounds like 3',4'-Dihydroxyacetophenone, 3,4-Dimethoxybenzaldehyde, 3,4-Methylenedioxybenzoic acid, 2,3-dihydrobenzofuran, trans-2-Hydroxycinnamic acid, Caffeic acid, 3,4-Dihydroxymandelic acid, Methylnoradrenaline, etc. were predicted to be carcinogens and few of them were toxic to kidney cells, neurons and liver cells as well. Hence those compounds were not considered for further studies.

Table 1 List of compounds with favorable ADMET profile

Sl. No.	Compound Name	Formula	Mass	Structure	TPSA	LogP o/w
1	3-Hydroxy-1-(4-hydroxyphenyl)-1-propanone	$C_9H_{10}O_3$	166.17		57.53	0.98
2	Apocynin	$C_9H_{10}O_3$	166.17		46.53	1.28
3	D-(+)-3-Phenyllactic acid	$C_9H_{10}O_3$	166.17		57.53	1
4	Dihydro-3-coumaric acid	$C_9H_{10}O_3$	166.17		57.53	1.32
5	Ethyl salicylate	$C_9H_{10}O_3$	166.17		46.53	1.95
6	Indole-3-carbinol	C_9H_9NO	147.17		36.02	1.45
7	Phloretic acid	$C_9H_{10}O_3$	166.17		57.53	1.31
8	Norvaline	$C_5H_{11}NO_2$	117.15		63.32	-0.67

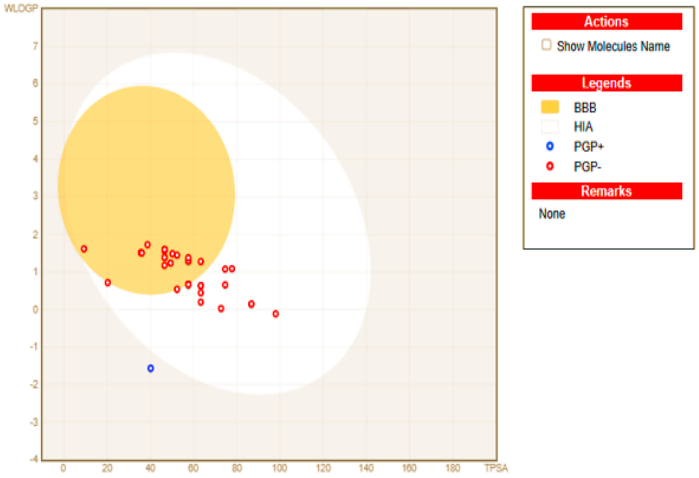


Figure 4 Boiled egg graph of the compounds

Molecular Docking with KRAS4B

KRAS4B is one of the common isoforms of RAS proteins. The catalytic domain region has P- loop region where the nucleotide binds and Switch I and II region where the effector molecule binds. The G12V is one of the common mutations that is localized in the P loop region, and this mutation strongly reduces the GTPase Activating Protein binding and stimulation, hence decreases the GTP hydrolysis. Since the GTP hydrolysis is decreased the GTP bound KRAS4B will have ordered switch I and II regions and hence effector binding is high. This keeps the protein constitutively active **(Erijman & M Shifman, 2016; Tariq et al., 2024)**. Targeting this protein inhibits the activation of many downstream proteins involved in cancer progression, especially G12V mutant is one of the most common mutants of KRAS4B and is highly difficult to target therapeutically **(Vasta et al., 2022)**.

All the compounds tested were able to bind to the catalytic domain of KRAS-4B (G12V) protein especially dihydro-3-coumaric acid showed lowest binding energy of -7.1 Kcal/mol followed by phloretic acid (binding energy of -6.7 Kcal/mol). Dihydro-3-coumaric acid is observed to form many hydrogen bonds with P-loop amino acids such as Gly13, Val14, Gly15, Lys16, and Ser17 and π-alkyl bond with Ala18. The core benzene ring of dihydro-3-coumaric acid also forms a π–π stacked interaction with Tyr32 and a hydrogen bond with Asp30 belonging to the switch I region. Phloretic acid as well has shown similar interactions.

Targeting KRAS4B protein is a challenge, especially G12V mutant, as no active drug has been identified for it yet. Although recently AMG510 and few more G12C inhibitors are in clinical trials, G12V mutants are still considered a challenging drug target. A probable reason could be that the valine sidechain is also hydrophobic in nature unlike cysteine, observed in G12C. Thus, obtaining ligand specificity towards valine is more challenging compared to cysteine, for which few inhibitors like AMG510 form covalent bonds with the reactive sidechain sulphur. Second major reason is, in G12V mutation GTP hydrolysis is strongly inhibited hence the protein is already in active state and switch I and II are in rigid form so any ligand to bind and inactivating it is difficult. In case of G12C mutatant, its inhibitors interact with GDP bound KRAS4B and keeps it in the inactive state and cycle will be inhibited, but this does not happen with G12V mutant **(Pantsar, 2020; Vasta et al., 2022)**. Our compounds have shown good binding affinity towards GTP bound G12V. However, these interactions do not confirm the inhibitory activity, further validation by molecular simulation and experimental studies has to be carried out to understand the therapeutic potential.

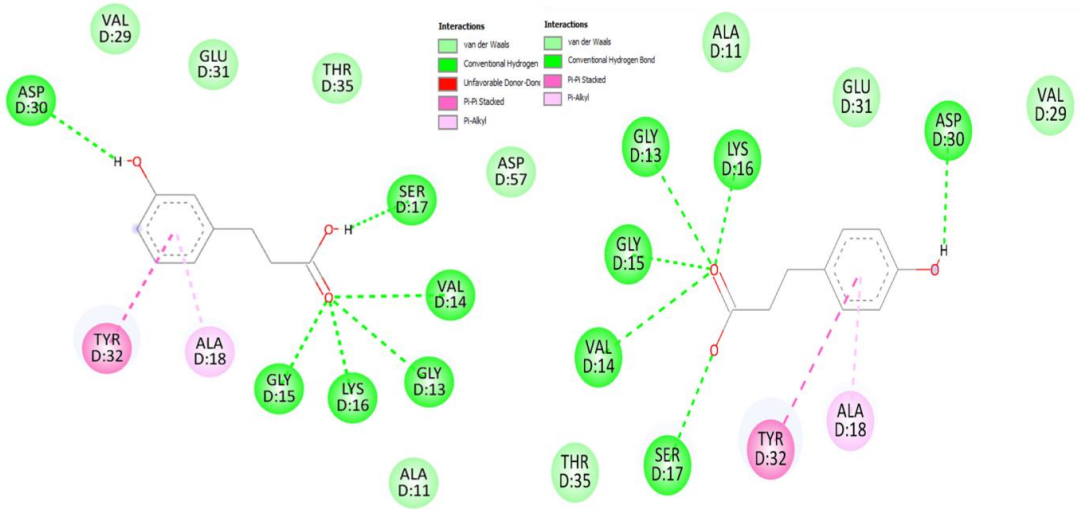


Figure 5 Binding pockets of Dihydro-3-Coumaric acid (a) and Phloretic acid (b) with KRAS4B (G12V)

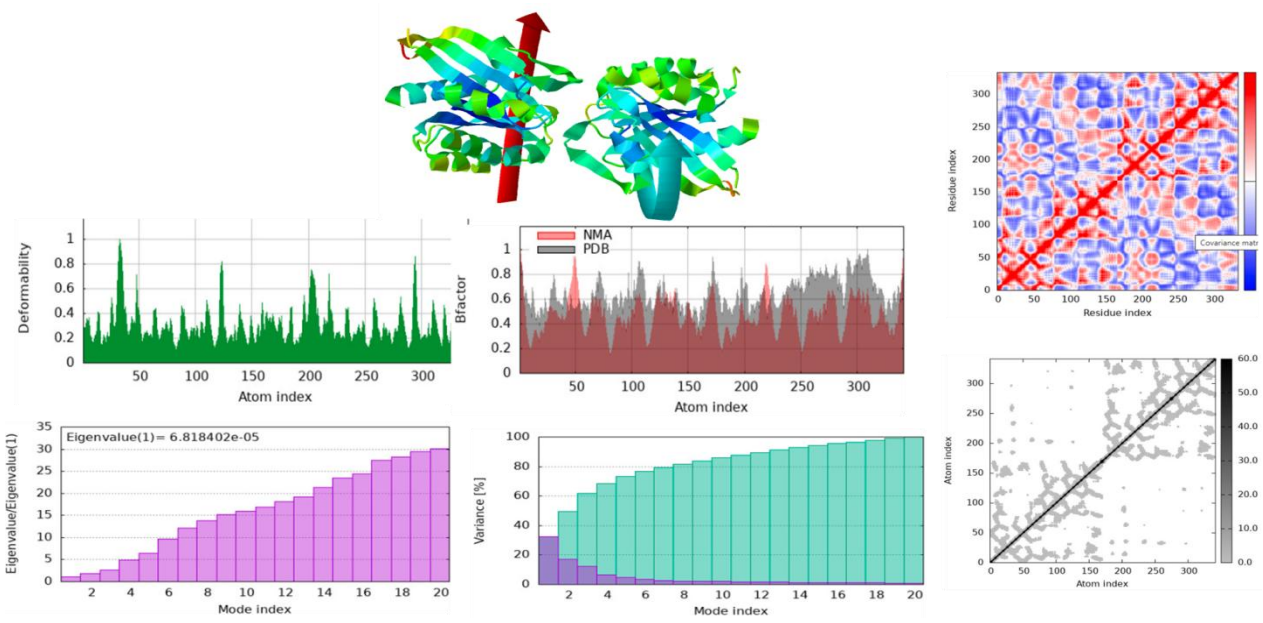


Figure 6 NMA of KRAS4B- Dihydro-3-Coumaric acid: NMA mobility of the complex (a); Deformability plot (b); B Factor plot (c); Eigen values for different modes (d); Variance plot (e); Covariance plot (f); Elasticity plot (g)

Nonetheless, we used normal mode analysis (NMA) as a preliminary tool to validate the stability of these complexes. From this study, the deformability graph and B-factor shows mobility in the switch I and II region, and the elasticity graph shows that switch I and II regions are more rigid. The co-variance graph suggests the correlated movement, which is observed in GTP bound KRAS4B. In sum, the NMA studies suggested that dihydro-3-coumaric acid binding to the P-loop and switch I region might not drastically alter the conformation.

Molecular Docking with E17K-AKT1

AKT1 is a 480-amino acid protein with an N- terminal PH domain, kinase domain and C-terminal tail region. In the inactive state PH domain of the protein interacts with the kinase domain and autoinhibits the protein, when PI3K converts PIP2 to PIP3, it interacts with the PH domain and protein binds to membrane. Then gets phosphorylated at Ser473 and Thr308 positions, the PH domain opens up and the protein is activated. Glutamic acid in 17th position gives negative charge and allows only PIP3 to bind to the PH domain. When mutated to lysine, the positive charge removes the specificity hence AKT1 constitutively binds to the membrane. Studies have also shown the mutation causes reduced affinity of the inhibitors making it difficult to target **(Chu et al., 2020)**. The mutant protein used in this study has been co-crystallized with compound 4 by Carven et al, for their study **(Craven et al., 2025)**. Hence the protein used in this study is in an autoinhibited conformation.

All the compounds except norvaline have shown good binding affinity to E17K-AKT1, especially dihydro-3-coumaric acid and indole-3-carbinol with lowest binding energy of -7.9 Kcal/mol. Dihydro-3-coumaric acid has shown good interaction with the kinase domain of the protein, with possible hydrogen bond with Ser205 and Thr211; π-sigma bond with Leu264 and π-alkyl bond with Lys268 and Val270, but dihydro-3-coumaric acid has also shown possible unfavorable interaction with Trp80, whereas indole-3-carbinol binds well with Trp80 by π-π interaction. Indole-3-carbinol also binds with Ser205 and Thr211, Leu264, Lys268 and Val270 similar to dihydro-3-coumaric acid. Thus indole-3-carbinol possibly interacts with both PH domain and kinase domain of the protein; hence these interactions might be consistent with the autoinhibited conformation of the protein, however further computational and experimental studies are required to confirm its inhibitory activity.

In most of the studies, inhibitor interaction is evaluated with wild-type AKT1 protein, but few studies show these inhibitors have reduced affinity to mutant protein. Kumar A, 2013 reported, the known inhibitor AKT1/2 inhibitor VIII showed binding energy of -3.87 Kcal/mol with mutant protein **(Kumar et al., 2013)**. But in present study, the compounds have exhibited good, predicted binding affinity to the mutant protein. Hence these compounds especially indole-3-carbinol might be considered for further studies to evaluate their inhibitory activity against oncogenic AKT1, indole-3-carbinol is also known to have good anticancer activity as reported in different studies **(Wang et al., 2025)**.

Further in NMA analysis, both the ligand complexes show similar deformability pattern, Eigen values, B-factor and also covariance. As observed in the covariance plot, residue 1-100 (PH domain) shows correlated movement with 250-300 (Kinase domain) as the protein is in it autoinhibited conformation and moderate stability is as well observed. These results suggest that the protein-ligand complex is consitent to autoinhibited structure of the protein. Since, NMA alone cannot confirm the conformational stability, we cannot conclude, if these compounds alter the activity of the protein.

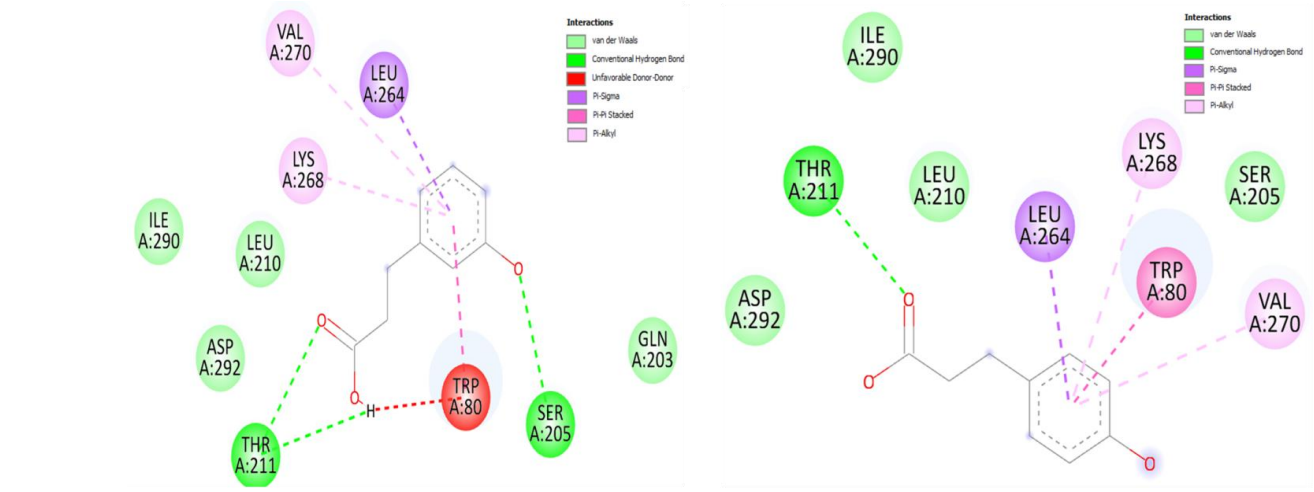


Figure 7 Binding pockets of Dihydro-3-Coumaric acid (a) and Indole-3-carbinol (b) with E17K-AKT1

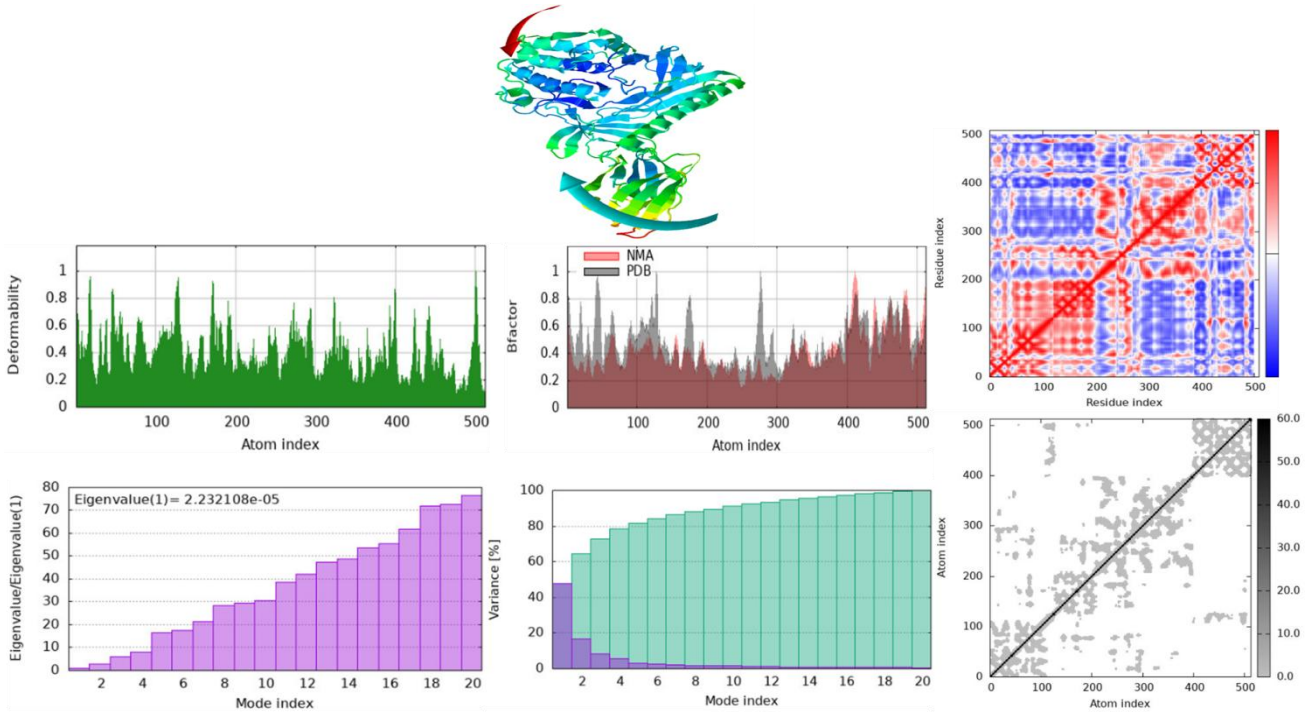


Figure 8 NMA of E17K-AKT1- Indole-3-carbinol: NMA mobility of the complex (a); Deformability plot (b); B Factor plot (c); Eigen values for different modes (d); Variance plot (e); Covariance plot (f); Elasticity plot (g)

CONCLUSION

The present preliminary study revealed the anticancer potential of aqueous extract of *A. aegina* ink on colorectal cancer cell line HT-29. LCMS analysis identified possible bioactive compounds present in the extract. The docking studies showed few of these bioactive compounds' possibility to bind to crucial oncogenic proteins KRAS4B (G12V) and E17K-AKT1. Dihydro-3-coumaric acid and indole-3-carbinol exhibited lower binding energy. The NMA also suggested stable binding protein-ligand complexes. But these studies are preliminary and are primarily based on computational predictions. The exploratory study suggests few potential candidates that might be considered for further *in-vitro* and *in-vivo* anticancer studies against colorectal cancer.

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