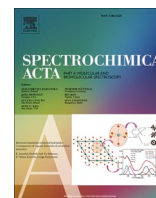




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A chemoinformatics study to prioritization of anticancer orally active lead compounds of pearl millet against adhesion G protein-coupled receptor

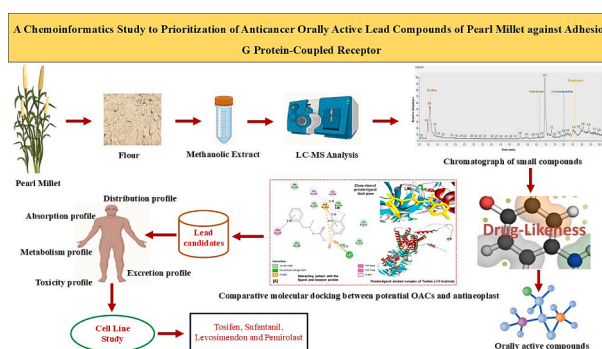
Adarsh Kumar Shukla¹, Neeru, Ashwani Kumar^{*}

Department of Nutrition Biology, Central University of Haryana, Mahendergarh, Jant-Pali 123031, Haryana, India

HIGHLIGHTS

- 55 small compounds identified from methanolic extract of pearl millet flour via LC-MS.
- ADGRF5 protein predicted as a promising target of potential OACs of pearl millet.
- Tosifen showed the good binding affinity (−7.9 kcal/mol) with ADGRF5 among 15 OACs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Adhesion G protein-coupled receptor F5
Colorectal cancer
LC-MS
Molecular docking
Pharmacokinetics

ABSTRACT

Metastasis is the main cause of colon cancer deaths worldwide. However, due to the disease's complexity, understanding its spread and finding natural treatments remain major challenges. The study aimed to identify therapeutic compounds in pearl millet and test them on metastatic colon cancer cell lines. The study used LC-MS and FTIR spectroscopy to identify small compounds and their functional groups. Further, theoretical chemistry, computational techniques, and ADMET analysis were applied for virtual screening and evaluating the pharmacokinetics of potential bioactive candidates of pearl millet. The 55 compounds of pearl millet were identified through the LC-MS, whereas 37 compounds were reported in PubChem database. Out of these, 15 compounds were predicted as potential orally active leads of pearl millet. Adhesion G protein-coupled receptor F5 was found as a potential target of these lead candidates. Further, molecular docking exhibited Tosifen (−7.9 kcal/mol), Sufentanil (−6.4 kcal/mol), Pemirolast (−6.3 kcal/mol), and Levosimendan (−6.3 kcal/mol) have similar binding affinity to the antineoplastics against the targeted protein. The “root mean square fluctuations (RMSFs)” analysis and deformability graph validated the docked complexes. Additionally, a comparative cell lines study against colon adenocarcinoma highlights Pemirolast as a promising candidate, demonstrating better activity compared to standard antineoplastics. This computational study predicted potential anticancer compounds in pearl millet and recommended further investigation through in vitro and in vivo studies.

^{*} Corresponding author at: Department of Nutrition Biology, Central University of Haryana, Mahendergarh, Jant-Pali 123031, Haryana, India.
E-mail addresses: ashwanindri@gmail.com, ashwanindri@cuh.ac.in, ashwanindri@rediffmail.com (A. Kumar).

1. Introduction

Colorectal cancer (CRC) is most frequent in Western nations and increasing day by day. CRC, the globally spread disease, affects both men and women among the other types of cancer. Mostly colorectal cancers are irregularly originated due to genetic and epigenetic variations. The colon becomes cancerous due to oncogene expression or tumor suppressor gene suppression, sometimes both caused by genomic abnormalities (stromal organization as predictive biomarker) [1].

The “adhesion G protein-coupled receptor F5 (ADGRF5)”, also known as a “G protein-coupled receptor 16 (GPCR16)”, is one of the family members of “VI adhesion GPCRs”. It includes extracellular fragments with sperm protein, enterokinase, and agrin domains as well as “immunoglobulin-like and immunoglobulin I-set domains” [2]. Kang et al. [2] reported that the overall survival and disease-free survival of patients was considerably lower in CRC patients with high ADGRF5 expression. Some cancer types undergo neoplastic transformation, which is mediated by ADGRF5 [2]. Consequently, they predicted that ADGRF5 was a potential therapeutic candidate for CRC. Studies reported that ADGRF5 is not only involved in CRC but also causes a lowered immune response, insulin resistance, adipose malfunctioning, glucose intolerance, and angiogenesis [3]. Additionally, ADGRF5 influences CRC formation and progression in addition to lung, breast, and other malignancies [4]. However, only limited attempts have been made to dock the receptor protein ADGRF5 in studies related to the development of CRC and the neoplastic transformation of the colon.

G-protein couple receptors (GPCRs) are essential for various cellular and physiological processes, including apoptosis, neurotransmission, neurodifferentiation, and cell proliferation [5]. GPCRs are known to play a significant role in the initiation, progression, and metastasis of cancer, as shown by several independent studies [6]. Several researchers suggested that the adhesion GPCR family and expression analysis identified GPR116 as a novel regulator of breast cancer metastasis [6].

Pearl millet is a rich source of polyphenols and other drug-like compounds that exhibit therapeutic activity in various pathophysiological conditions, including maintaining gut health [7,8], diabetes mellitus, hypertension, vascular fragility, cancer, and hypercholesterolemia [9]. Recent studies have highlighted its anticancer potential, attributing this to bioactive compounds that induce apoptosis, inhibit cell proliferation, and modulate oxidative stress pathways. These properties make pearl millet a promising candidate for developing functional foods or nutraceuticals targeting cancer prevention and therapy [8].

The metabolites-protein interactions are evaluated based on their binding affinity, reflecting the energetically favorable interactions between small molecules and target proteins [9]. Despite the vast diversity of phytochemicals, only a small fraction exhibits favorable pharmacokinetics and progress to clinical trials for drug development [10]. Pre-assessment of “absorption, distribution, metabolism, excretion, and toxicity (ADMET)” behaviors are crucial in the drug discovery process, ensuring candidate compounds are suitable for therapeutic use [11]. An ideal drug candidate should demonstrate efficient absorption, targeted distribution to the site of action, minimal metabolic conversion into toxic byproducts, effective elimination, and minimal or no adverse effects [12,13]. Furthermore, the functional groups present in a compound significantly influence its pharmacokinetics by affecting its binding interactions with target proteins, as well as its metabolic and excretory pathways [13]. This study focuses on a comparative molecular docking analysis of pearl millet metabolites identified through liquid chromatography–mass spectrometry (LC-MS) and known antineoplastic agents to explore their potential interactions and anticancer properties.

2. Materials and methods

2.1. Preparation of methanolic extract from pearl millet

Pearl millet (“Pioneer 9444 hybrid”) was purchased from the local market in Mahendergarh. Only healthy and visibly mature seeds were selected, and they were thoroughly cleaned to remove impurities, broken seeds, and plant debris. The cleaned seeds were then ground into a fine powder using an electric grinder. Obtained powder was added to methanol (Merck) in the ratio of 1:10 into a polypropylene vial and mixed properly by shaking thoroughly. The vial was placed in the incubator shaker “(Orbitek) (180 RPM)” at 37 °C for 4 h to extract desired compounds. The mixture was filtered by using a Whatman filter paper no.1 to remove solid particles from the extract and stored at –20 °C (Euronova).

2.2. Metabolite identification in pearl millet flour using LC-MS (mass to charge ratio)

Dissolved the residue in methanol to prepare a sample solution with the desired concentration. Injected 1 ml of the sample solution into a high-performance liquid chromatography (HPLC) system having an attached reverse-phase column. The separation of the compounds was based on differences in polarity, hydrophobicity, physical and chemical properties. The mobile phases A (water + 0.1 % formic acid) and B (Acetonitrile + 1 % formic acid) were used to elute the compounds. The flow rate of both the mobile phases (A and B) was maintained at 300 micro-litres/min. HPLC system was attached to a mass spectrometer (MS) for detection of the separated compounds on the basis of mass to charge ratio by gradient elution of small compounds. The MS detected the ions produced and generated a mass spectrum, which provided information about the molecular weight, structure, and identity of the compounds. The identification of compounds was performed using the LC-MS inbuilt software’s Compound Discoverer version 3.0 and X-Calibur. The mass error of the compounds was calculated in parts per million (ppm) by Compound Discoverer version 3.0.

2.3. Functional group analysis of reported metabolites of pearl millet by Fourier Transform Infra-Red (FTIR)

The sample for FTIR analysis was prepared by grinding 2 mg of pearl millet into a fine powder and mixing it with an appropriate IR-transparent matrix KBr. Mounted the sample-matrix mixture on an IR-transparent plate, such as a KBr disc using a manual press. FTIR spectra of beverage samples were recorded using an “Alpha 2 compact FTIR spectrometer” facilitated with a diamond crystal cell. The sample was analyzed at room condition. Before analysis, the crystal was cleaned using wet tissue paper and then dried. Crystal was checked for the cleanliness of residues from the previously analyzed sample spectrally. Measurements were taken within the range of 4000 to 600 cm⁻¹ [14].

2.4. Drug likeness prediction of reported small compounds of pearl millet

The oral activity of metabolites was evaluated using molecular descriptors and widely accepted theoretical rules, including Lipinski’s Rule of Five, the Ghose rule, the Veber rule, and the Egan rule. The categorization of these small compounds was based on key chemical descriptors such as “molecular weight, log P value (octanol–water partition coefficient), hydrogen bond donors (HBDs), hydrogen bond acceptors (HBAs), molar refractive index, total number of atoms, molar refractivity, and the number of rotatable bonds” [13].

2.5. Bioactivity and target prediction of the orally active compounds (OACs)

Bioactivity of OACs against receptors like GPCRs, ion channels,

kinases, proteases, enzymes, and nuclear receptors was predicted using the “Molinspiration server (<https://molinspiration.com/>)” [13]. Target proteins for these potent compounds were identified through the “SwissTargetPrediction tool (<https://swisstargetprediction.ch/>)”.

2.6. Three-dimensional (3D) structure modeling and analysis of ADGRF5

The human ADGRF5 protein sequence was retrieved from the “National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>)”. This sequence was then used as a query in the “SwissModel web tool (<https://swissmodel.expasy.org/>)” for structural modeling. The template for homology modeling was selected based on the highest “Global Model Quality Estimation (GMQE)” score and percentage identity, following a similar approach to Bogra et al. [15]. The binding site of the modeled protein was identified using the probability-based online tool “PrankWeb (<https://prankweb.cz/>)”.

2.7. Molecular docking

Docking studies were carried out with an AutoDock Vina (version: Vina 1.2.5, a standalone tool). The series of steps as placed below were followed for the molecular docking protocol [15]. The 3D structure of the modeled protein was prepared in BIOVIA Discovery Studio 2021 (released by Dassault Systèmes) by removing water molecules, adding Kollman and Gasteiger charges, and assigning AD4 atom types. A grid encompassing the entire protein was defined for blind docking, and the receptor was saved as a rigid PDBQT file. The best-docked pose was visualized in BIOVIA Discovery Studio 2021 by analyzing receptor-ligand bonding pattern [12]. The receptor-ligand complex was saved in PDB format for further molecular dynamics simulations.

2.8. MD simulation

Root mean square fluctuations (RMSF) and normal mode analysis (NMA) were employed to explore the flexible states of the protein in the receptor-ligand docked complex, which are functionally significant [16]. The best-docked poses were analyzed using the “CABS-flex 2.0 server (<https://biocomp.chem.uw.edu.pl/CABSflex/about.php>)” with default parameters. It includes 10 ns simulation cycles for balanced accuracy and efficiency, with a temperature of 1.4 influencing flexibility. Force field weights for CA-CA distance constraints and secondary structure are 1.0, clustering uses a 3.0 Å threshold, forming 10–20 clusters, and the final ensemble comprises 10 representative models for structural analysis. Structural dynamics of the potential docked complex were further investigated using the “iMODS, (version:1.0) tool (<https://imods.iqfr.csic.es/>)”.

2.9. Cell line study

A freely available web-service “PASS CLC Pred (<https://www.way2drug.com/Cell-line/>)” was used to predict the cytotoxicity of potential compounds against colorectal cell lines. It consists key methods in modern drug discovery include QSAR, connectivity mapping, network pharmacology, and machine learning. QSAR links chemical structures to cytotoxicity, while connectivity mapping compares drug-induced gene expression profiles to identify potential targets. Network pharmacology unveils complex biological interactions, offering insights into therapeutic pathways. Machine learning amplifies these efforts by uncovering patterns and correlations between cytotoxicity and microarray data, boosting predictive accuracy for cancer treatment and drug development [17].

2.10. ADMET profiling

The ADMET properties of lead compounds are crucial for assessing

their pharmacokinetic and pharmacodynamic profiles during virtual screening [18]. This analysis was conducted using the “Swiss ADME (<https://www.swissadme.ch/>)” and “pkCSM (<https://biosig.lab.uq.edu.au/pkcsm/>)” online tools.

3. Result and discussion

3.1. LC-MS-based profiling of small compounds in pearl millet flour

Total 55 small compounds (Supplementary Table S1) of pearl millet extract were analyzed through the LC-MS. These were having different concentrations represented as a % relative area. Out of these, 16 compounds were present in higher concentrations, while others were present in small amounts. Benserazone was having maximum % area followed by Temodox, 2-Pyrrolidone, 4-Hydroxy-L-threonine, and Alttretamine. Other compounds like 2-Hexyl benzothiazole, 4-Amino-2-hydroxyamino-6-nitrotoluene, Pyrrolidine, 5-Hydroxy-2-furoic acid, Ruscopine, 2-Oxobutyric acid, Cartazolate, Temozolomide, Threonic acid, 3,5-Dihydroxy-2-[3-(3,4,5-trimethoxyphenyl) propanoyl] phenyl hexopyranosiduronic acid were 2 % to 3 %. The remaining compounds were less than 1 % area. All these compounds were categorized under the phenolic and flavonoid families (Supplementary Fig. 1). The mass error helps to assess the accuracy of mass measurements in high-resolution mass spectrometry has been given in Table 1.

3.2. Confirmation of functional group of identified small compounds through FTIR

The FTIR spectra of the methanolic extract of pearl millet revealed characteristic peaks within the range of 4000–400 cm^{-1} (Fig. 1). A prominent peak at 3400–3200 cm^{-1} corresponded to –OH stretching vibrations, indicating the presence of methanol and polyphenolic compounds. The functional region at 3000–2800 cm^{-1} showed peaks associated with CH_2 groups, while a smaller peak at 2400–2300 cm^{-1} suggested the presence of $\text{C}\equiv\text{N}$ bonds. A bend near 2200–2000 cm^{-1} was attributed to CH_3 groups, likely due to methanol. Sharp peaks in the range of 1700–1600 cm^{-1} were indicative of $\text{C}=\text{C}$ bonds in aromatic rings.

The fingerprint region (1600–600 cm^{-1}) displayed various peaks corresponding to functional groups such as $\text{C}-\text{O}$, $\text{C}=\text{O}$, CH_2 , and $\text{C}-\text{H}$, confirming the presence of hydroxy phenols and aromatic phenolic acids. Similar observations were reported by Gull et al. [19], who identified comparable peaks in pearl millet and finger millet flours. FTIR analysis of crystalline starch, as noted by Kaur and Gill [20], showed peaks between 1300–800 cm^{-1} . Additionally, Wani et al. [21] reported $\text{C}-\text{H}$ and $\text{O}-\text{H}$ stretching bands between 1600–1800 cm^{-1} and 2800–3000 cm^{-1} , respectively, in gamma-radiation-treated millets, alongside anhydrous $\text{C}-\text{O}$ stretching (990–1152 cm^{-1}) and amino region peaks (1628–1654 cm^{-1}).

The comparison of FTIR spectra of pearl millet and finger millet showed similar peaks, such as the –OH stretch at 3468.48 cm^{-1} for finger millet and 3466.95 cm^{-1} for pearl millet. The $\text{C}-\text{H}$ stretching peaks were at 2925.52 cm^{-1} for finger millet and 2925.84 cm^{-1} for pearl millet. Adebisi et al. [22] observed –OH peaks at 3285 cm^{-1} in non-fermented flour, 3298 cm^{-1} in malted flour, and 3300 cm^{-1} in fermented flour. Asymmetric stretching of $\text{C}-\text{H}$ bonds was identified in the 3328–2853 cm^{-1} range, while carbonyl stretching peaks appeared between 1744–1746 cm^{-1} . Peaks at 1158–1120 cm^{-1} indicated $\text{C}-\text{O}$ and $\text{C}-\text{N}$ stretching, with amide-associated peaks at 1653–1648 cm^{-1} .

This study confirms the presence of phenolic compounds in pearl millet by identifying the corresponding functional groups through FTIR analysis.

3.3. Retrieval of “molecular descriptors” of the reported compounds

The 15 reported compounds viz. “4-amino-2-hydroxyamino-6-

Table 1

Mass error of the identified compounds calculated in parts per million (ppm) using the LC-MS inbuild software Compound Discoverer version 3.0 and drug-likeness assessment of identified small compounds from pearl millet based on Lipinski's, Ghose, Veber, and Egan rules. Compounds marked in red indicate potent oral activity, while those in green signify oral activity compliance.

Sr. No.	Compounds	Molecular weight in Dalton (Da)	Mass error in ppm	Lipinski violations	Ghose violations	Veber violations	Egan violations
1	2-Amino adenosine	282.10701	-2.27	0	1	1	1
2	2-Hexylbenzothiazole	210.15957	+0.98	0	0	0	0
3	2-Oxobutyric acid	102.03124	-4.41	0	3	0	0
4	2-Pyrrolidane	85.05236	-4.72	0	4	0	0
5	3-Hydroxynonanoic acid	174.1263	0	0	0	0	0
6	4-Hydroxy-L- threonine	135.05306	-0.75	0	4	0	0
7	4-Trifluoro methyl phenol	162.02994	0	0	2	0	0
8	5-Hydroxy-2-furoic acid	128.0117	+5.81	0	3	0	0
9	Acipimox	154.03786	-0.11	0	3	0	0
10	Ascorbyl palmitate	414.2627	+2.28	0	0	1	0
11	Benserazide	257.09996	-4.7	1	1	1	1
12	Biotin Sulfone	276.07906	+3.86	0	0	0	0
13	Cartazolate	290.17527	+3.41	0	0	0	0
14	Edetol	292.23689	-2.35	0	1	1	0
15	Levosimendan	280.10677	-1.75	0	0	0	0
16	L-Threonic acid	136.03705	-0.90	0	4	0	0
17	Pemirolast	228.0761	0	0	0	0	0
18	Phosphopentothenic acid	299.07588	-3.74	0	1	1	1
19	Pyrrolidone	71.07331	-2.63	0	3	0	0
20	Ruscopine	306.20621	+2.28	0	0	0	0
21	Tosifen	332.1183	0	0	0	0	0
22	Tetrahydrofuran	72.05733	-2.58	0	3	0	0
23	Triethyl phosphate (TEP)	182.07115	0	0	0	0	0
24	Temozolomide	194.05596	+2.75	0	1	0	0
25	Temodox	264.07552	0	0	0	0	0
26	Sufentanil	386.2025	-0.77	0	0	0	0
27	Spectinomycine	332.15862	+0.80	0	1	0	0
28	Altretamin	210.15957	+1.29	0	0	0	0
29	2-Amino-6-hydroxyamipurine	166.06108	+4.66	0	2	0	0
30	Colcemide	371.17262	-1.75	0	0	0	0
31	Tiadenol	294.17006	+4.55	0	0	1	0
32	Asp-lys	261.1335	+3.94	0	1	1	1
33	3-Methylcyclopentene	246.16644	-4.85	0	3	0	0
34	2-Octyl-4-propyl-1,3-thiazole	239.17056	-0.86	0	0	0	0
35	2-Cyclohexyl-4,6-dinitrophenol	266.09108	0	0	0	0	0
36	4-Amino-2-hydroxyamino-6-nitrotoluene	183.06395	-3.07	0	1	0	0
37	Epsilon-(gamma-Glutamyl)-lysine	275.14888	0	1	1	1	1

nitrotoluene; 3,5-Dihydroxy-2-[3-(3,4,5-trimethoxyphenyl)propanoyl]phenyl hexopyranosiduronic acid; 4-Oxo-4-(1H-purin-6-ylamino)butanoic acid; (5E)-5-[(5-Methyl-2-thienyl)methylene]-2(5H)-furanone; 6-chloropyridin-3-amin; (1aR,2R,3S,11cS)-6,11-Dimethyl-1a,2,3,11c-tetrahydrotetrapheno[1,2-b]oxirene-2,3-diol; 1-[(4-Amino-2-methyl-5-pyrimidinyl)methyl]pyridinium; 3-(Methylthio)hexyl hexanoate; (2R)-1-[[[(2-Aminoethoxy)(hydroxy)phosphoryl]oxy]-3-hydroxy-2-propanyl (13Z,16Z)-13,16-docosadienoate; (17R)-23-Amino-20-hydroxy-20-oxido-14-oxo-15,19,21-trioxa-20lambda ~ 5 ~ -phosphatricosan-17-yl (9Z)-9-tetradecenoate; 6-Amino-3-(3-methyl-2-buten-1-yl)-7H-purin-3-ium; (3r,4s)-Tofacitinib; [(4Z,6E,10Z,13Z)-1-Carboxy-4,6,10,13-nonadecatetraen-8-yl]dioxidanyl; (2S,3R,3aR,4aS,4bS,6aS,8aR,11-aR,15bS,15cR,17aS)-3,4b-Dihydroxy-2-(2-hydroxy-2-propanyl)-9,9,11,11,15b,15c-hexamethyl-3,3a,5,6,6a,8a,9,11,11a,15,15b,15c,16,17,17a-hexadecahydro-2H,4bH-[2]benzofuro[5,6-e]oxireno[4',4a']chromeno[5',6':6,7]indeno[1,2-b]indol-12(7H)-one; 4-Amino-5-fluoro-1-[5-(hydroxymethyl)tetrahydro-2-furanyl]-2(1H)-pyrimidinone" were not found in the PubChem database. The molecular descriptors for the remaining compounds, as outlined in the Materials and Methods section, are provided in [Supplementary Table S2](#).

3.4. Study of drug likeness

Lipinski's Rule of 5, along with the other theoretical rules of Ghose, Veber, and Egan, were applied to screen for OACs among the 37 well-characterized compounds of pearl millet, based on their molecular

descriptors.

The Ghose rule has the following criteria such as log P values (-0.4-5.6), the molar refractive indexes (40-130), the molecular weights (160-480 Dalton), and a total atom count between (20-70) [13]. Most of the compounds "2-Oxobutyric acid, 2-Pyrrolidane, 4-Hydroxy-L- threonine, 4-Trifluoro methyl phenol, 5-Hydroxy-2-furoic acid, Acipimox, Benserazide, Edetol, L-Threonic acid, Phosphopentothenic acid, Pyrrolidone, Tetrahydrofuran, Temozolomide, and Spectinomycin" have violated the Ghose rule. The compounds have followed rule of RO5, which includes molecular weight of less than 500 Dalton, a log p value of less than 5 (which indicates how hydrophobic it is), no more than five HBDs, and no more than 10 (HBAs sites) [17].

Veber added two extra criteria in the rule of five for the drug similarity i.e. polar surface area (PSA) should be less than 140 Å² and it should have less than 10 rotatable bonds [23]. 8 compounds such as "2-Amino adenosine, Ascorbyl palmitate, Benserazide, Edetol, Phosphopentothenic acid, Tiadenol, Asp-lys, and Epsilon-(gamma-Glutamyl)-lysine" have violated the Veber Rule.

Based on the physical procedures involved in a small molecule's membrane permeability, the Egan rule for drug likeness advocated the oral medication of the compounds based on absorption [24]. The 5 compounds viz. 2-Amino adenosine, Benserazide, Phosphopentothenic acid, Asp-lys, and Epsilon-(gamma-Glutamyl)-lysine have violated the Egan rule (Table 1).

In summary, out of these 37 selected compounds, 14 compounds (2-Oxobutyric acid, 2-Pyrrolidane, 4-Hydroxy-L- threonine, 4-Trifluoro methyl phenol, 5-Hydroxy-2-furoic acid, Acipimox, Ascorbyl

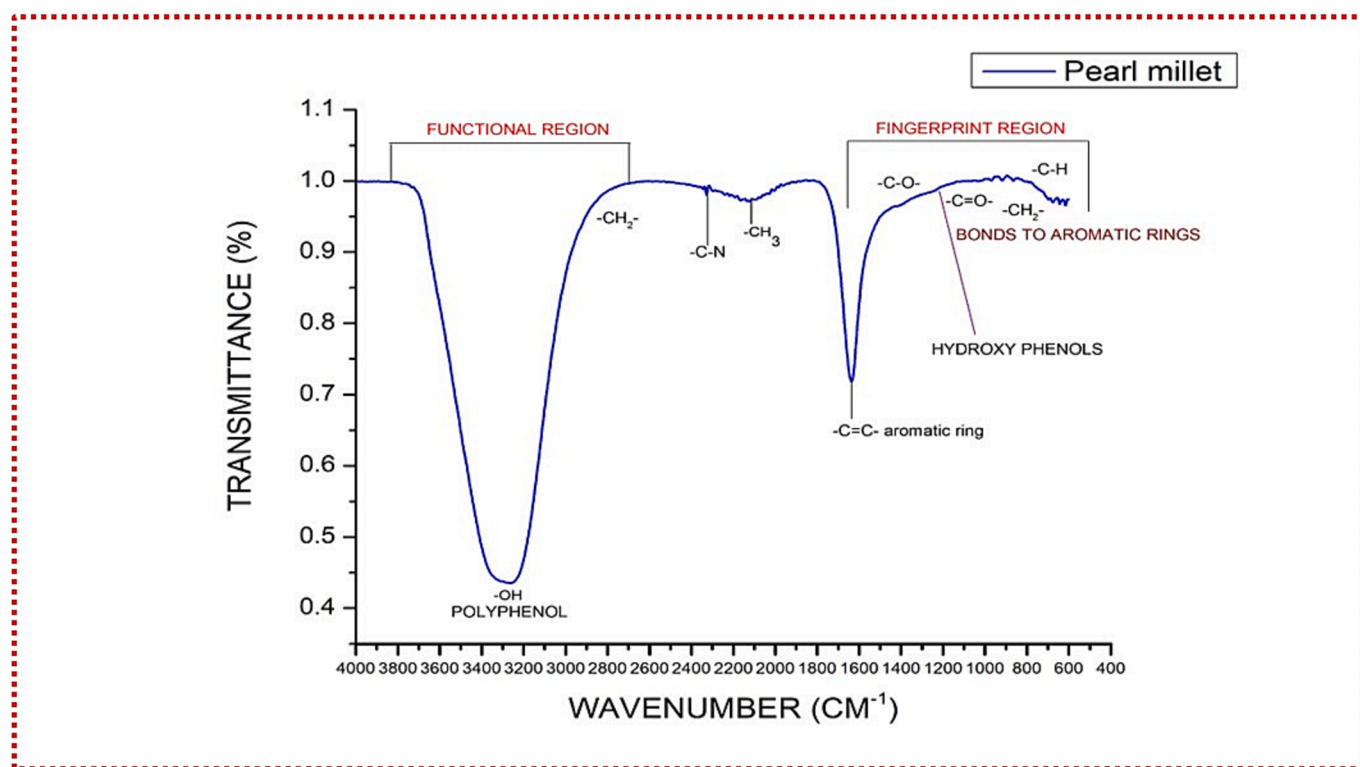


Fig. 1. Furrier Transform Infrared Reflection analysis of methanolic extract of pearl millet for the confirmation of functional group.

palmitate, L-threonic acid, Pyrrolidone, Tetrahydrofuran, Temozolomide, Spectinomycine, 5-Amino-2-Chloropyridine, and 2-Amino-6-hydroxyaminopurine) have violated any one theoretical rule. These compounds are considered as OACs [13]. The 4 compounds (2-Amino adenosine, Benserazide, Edetol, and Phospho-pantothenic acid) violated the 2 or > 2, theoretical rules; therefore, these compounds were categorized as the non-OACs. The 15 reported compounds such as 2-Hexyl benzothiazole, 3-Hydroxy nonanoic acid, Biotin sulfone, Cartazolate, Levosimendan, Pemirolast, Ruscopine, Tosifen, Triethyl phosphate (TEP), Temodox, Sufentanil, Altretramine, Colcemide, 2-Octyl-4-propyl-1,3-thiazole, and 2-Cyclohexyl-4,6-dinitrophenol have followed all the theoretical rules and were considered as highly potent OACs.

3.5. Bioactivity of OACs

The present study identified “Ascorbyl palmitate, Ruscopine, Tosifen, Sufentanil, and 2-Amino-6-hydroxyaminopurine” as potential ligands for “G-protein coupled receptor” activity. GPCRs have emerged as prominent targets in cancer therapy due to their role in regulating critical cellular processes such as proliferation, migration, and survival [6].

The compounds 2-Amino-6-hydroxyaminopurine and Ruscopine were identified as kinase inhibitors. Kinases, owing to their essential role in cellular signaling, have become prominent targets in modern pharmacological research, particularly in the context of cancer therapy [25].

This study has found that “Ascorbyl palmitate, Biotin sulfone, Ruscopine, Tosifen, and Spectinomycin as potential targets of the protease inhibition process. The metabolites; Acipimox, Ascorbyl palmitate, Ruscopine, Tosifen, TEP, Temozolomide, Spectinomycin, and 2-Amino-6-hydroxyaminopurine were predicted as enzyme inhibitors. Enzyme inhibitors targeting cancer-related enzymes are gaining significant attention for their ability to disrupt tumor growth and metastasis [26].

The findings indicate that these compounds show promise as potential leads for effective cancer therapies. Specifically, bioactivity predictions reveal that four compounds—Ascorbyl palmitate,

Ruscopine, Tosifen, and 2-Amino-6-hydroxyaminopurine—exhibited significant activity across multiple target types. This multi-target interaction potential underscores their capacity to modulate various receptors, enhancing their therapeutic value.

3.6. Prediction of target proteins of OACs

The present bioactivity study indicated that the majority of OACs would be a promising lead candidate for the family adhesion G-protein coupled receptor (AGPCR) protein, kinase protein, and enzymatic activity. The compounds such as 3-Hydroxynanoic acid (20.0 %), Altretramine (66.7 %), Biotin sulfone (6.7 %), Ruscopine (26.7 %), Colcemide (53.3 %), TEP (7.1 %), Tosifen (20.0 %), Sufentanil (40.0 %), Temodox (6.7 %), and Cartazolate (13.3 %) were found promising lead for the AGPCR protein.

The Levosimendan (13.3 %), 3-Hydroxynanoic acid (26.7 %), Biotin sulfone (40.0 %), TEP (7.1 %), and Sufentanil (26.7 %) are the promising lead compounds for the enzymatic activity. Several diseases such as cancer successfully targeted by kinase inhibitors, especially “Protein Kinase C (PKC)” family of kinases is one of the promising kinase targets for future therapeutic drug development approach [35]. Levosimendan (20.0 %), Pemirolast (13.3 %), Ruscopine (33.3 %), Tosifen (26.7 %), and 2-Cyclohexyl-4,6-dinitrophenol (20.0 %) has predicted the positive lead for the kinase. Pemirolast (20.0 %), Temodox (33.3 %), and Cartazolate (40.0 %) were predicted as promising lead candidates for the protease inhibitors which are the widely accepted therapeutic target in the drug development process.

The adhesion G-protein coupled receptor, a key therapeutic target for tumor suppression, is implicated in cancer initiation, progression, and metastasis. Multiple independent studies have highlighted the significant role of the ADGRF5 precursor protein in these processes [6]. The enzyme is thought to be a possible therapeutic target because it is linked to a number of illnesses including “cerebral hypoxia, azoospermia, endometrial and ovarian cancer, systemic lupus” etc. [27].

In summary, the target prediction validated the clues of bioactivity

study and sets the foundation for the receptor-ligand interaction through molecular docking and dynamics simulation for the potential OACs and AGPCR protein. The AGPCR, a key therapeutic target for tumor suppression, is implicated in cancer initiation, progression, and metastasis. Multiple independent studies have highlighted the significant role of the ADGRF5 precursor protein in these processes [2].

3.7. 3D structure modeling and analysis of adhesion G protein-coupled receptor F5 protein

The sequence of ADGRF5 precursor (*Homo sapiens*) was taken from the NCBI (accession no: NP_056049). The full length of the sequence (1346 aa) in FASTA format (Fig. 2[B]) was used as an input and then its 3D structure (Fig. 2[C]) was predicted based on homology modeling through the online server Swiss Model. The 3D structure of ADGRF5 (*Homo sapiens*) was obtained through the comparative modeling with the ADGRF5 (*Mus musculus*) protein as a template (Fig. 2[A]) with the higher GMQE score 0.78 and 73.22 % identity. The structural analysis of ADGRF5 found that the binding site pocket 1 (score: 44.04 and probability: 0.968) considers the following residues, A_1012, A_1013, A_1016, A_1017, A_1020, A_1065, A_1068, A_1071, A_1072, A_1075, A_1097, A_1158, A_1161, A_1163, A_1172, A_1176, A_1179, A_1180, A_1183, A_1226, A_1229, A_1230, A_1232, A_1233, A_1235, A_1236, A_1238, A_1239, A_1248, A_1251, and A_1255.

3.8. Molecular docking simulation

The comparative docking study between the antineoplastics and OACs of pearl millet observed that the Docetaxal has the minimum binding affinity (−13.3 kcal/mol). However, the other antineoplastics have interacted with ADGRF5 viz. Eupiribicin (−8.5 kcal/mol), Dauorubicin (−7.7 kcal/mol), Etoposide (−7.0 kcal/mol), Letrozole (−6.7 kcal/mol), Azathioprine (−5.9 kcal/mol), Flurouracil (−4.4 kcal/mol), Cyclophosphamide (−3.8 kcal/mol), and Busulfone (−3.6 kcal/mol). Muthiah et al. [28] has been compared the binding affinity of common

medications (generally used in cancer treatment) such as Doxorubicin, Neratinib malate, Lapatinib ditosylate, and 5-fluorouracil. They found Vicenin (−12.321 kcal/mol), Verbascoside (−11.002 kcal/mol), Cynaroside (−10.517 kcal/mol), Apigetrin (−9.69 kcal/mol), and Orientin (−9.596 kcal/mol) showed higher binding affinities than suggested medications.

In the present docking study, Tosifen (−7.9 kcal/mol) was identified as the most promising lead compound against ADGRF5 among the 15 OACs of pearl millet and selected antineoplastics, excluding Docetaxel and Epirubicin. Several other compounds showed good binding affinity, comparable to that of antineoplastics, including Sufentanil (−6.4 kcal/mol), Altretamine (−6.4 kcal/mol), Pemirolast (−6.3 kcal/mol), Levosimendan (−6.2 kcal/mol), and 3-Hydroxynonanoic acid (−6.2 kcal/mol). The binding affinity details for all OACs and antineoplastics are provided in Fig. 3.

3.9. Interaction pattern of receptor-ligand docked complex

In the present study ligand–protein binding pattern analysis revealed that the receptor protein ADGRF5 residues viz. HIS 1248, and ASP 1013, have interacted with Tosifen (3.53 Å) and Sufentanil (4.5 Å) through the H-bond. The H-bond formation in docked complex is also crucial bonding patterns in receptor-ligand docking [16]. The binding site prediction was also indicated that ASP 1013, and HIS 1248 have the binding residues of this receptor protein.

The receptor residues ARG 1158, TRP 1232, VAL 1239, THR 1238, SER 1016, and LEU 1012 were similarly to interact with Tosifen via van der Waals forces. Similarly, Sufentanil exhibited van der Waals interactions with key pocket residues, including GLN 815, TRP 811, GLN 914, LYS 812, ASP 912, LEU 996, ASN 917, PHE 918, GLU 915, and GLN 911.

The binding residues, including SER 861, GLN 892, LEU 891, ILE 858, GLU 864, HIS 862, and PRO 863, interacted via van der Waals forces with the ligand Pemirolast. Similarly, the residues ASP 988, THR 911, TRP 957, PHE 959, ASN 958, ARG 960, PHE 956, SER 971, and GLY

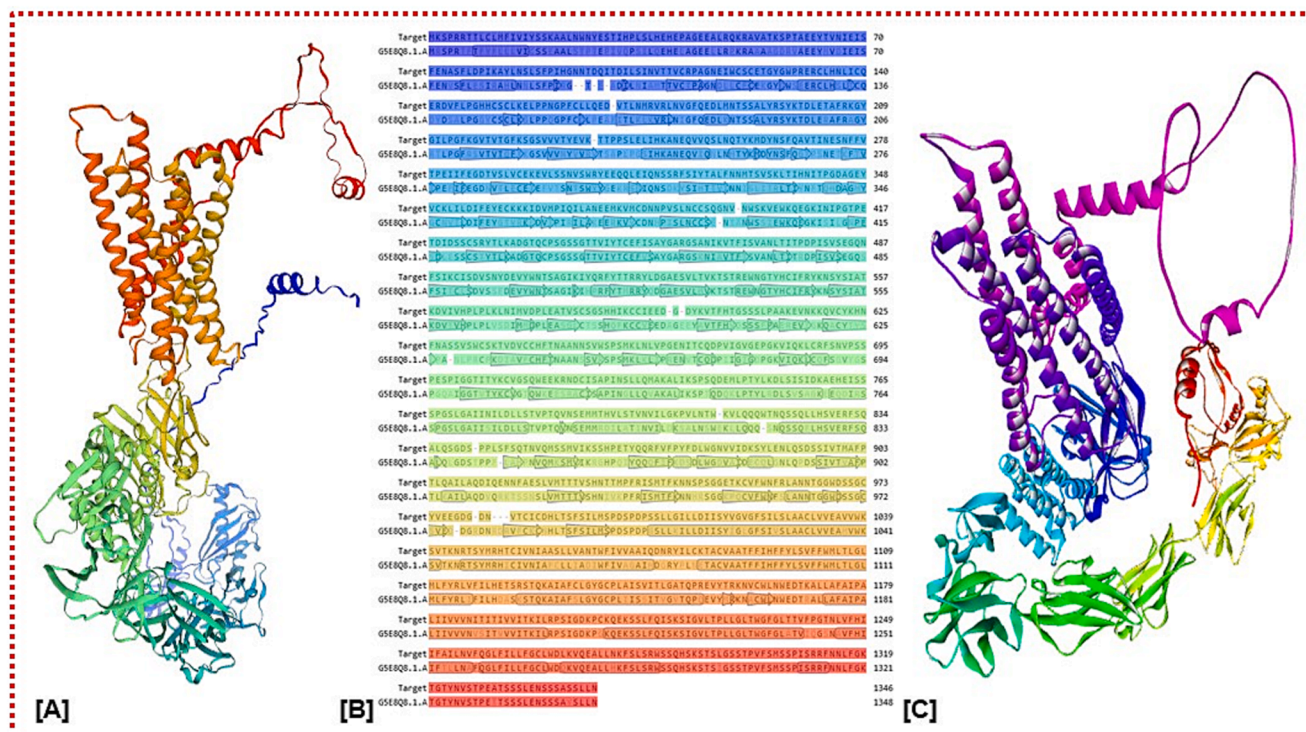


Fig. 2. The illustration of [A] the 3D structure of the template protein, [B] template-target sequence alignment, and [C] the 3D structure of target protein through the homology modeling by SWISS-Model online tool.

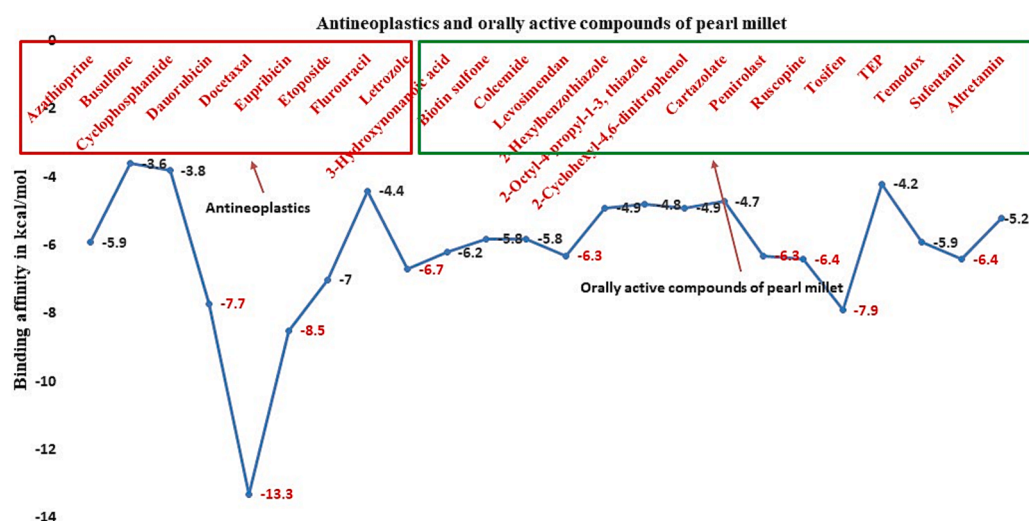


Fig. 3. Graph showing the binding affinity (kcal/mol) of OACs and antineoplastics, as determined through molecular docking simulations using AutoDock Vina.

972 interacted through van der Waals forces with the ligand Levosimendan.

The bonding patterns of the receptor protein with Tosifen, Sufentanil, Pemirolast, and Levosimendan are illustrated in Fig. 4[A], 4[B], 4[C], and 4[D], respectively.

3.10. RMSFs calculation

The RMSFs of the protein–ligand docked complexes were analyzed under default parameters, including protein rigidity set to 1.0, 50 cycles, and a cycle interval of 50, over a 10-nanosecond (ns) molecular dynamics (MD) simulation. This study observed that a significant number of amino acid residues in the target protein remained stable in the

docked complexes with Tosifen, Sufentanil, Pemirolast, and Levosimendan.

The RMSF graph of the receptor chain without ligand (Fig. 5[A]) demonstrated a stable profile, indicating minimal structural fluctuations. In contrast, the receptor–ligand docked complex (Fig. 5[B]) exhibited higher fluctuations, particularly at the positions where the ligands interacted with the receptor. Notable ranges of amino acid residues with increased fluctuations were identified at 1–100, 685–710, and 1310–1346. These regions of greater RMSF values suggested enhanced flexibility in the receptor structure, which may facilitate stronger or more adaptable interactions with ligand molecules. Conversely, regions with lower RMSF values indicated reduced flexibility, potentially limiting their capacity to engage in interactions, as

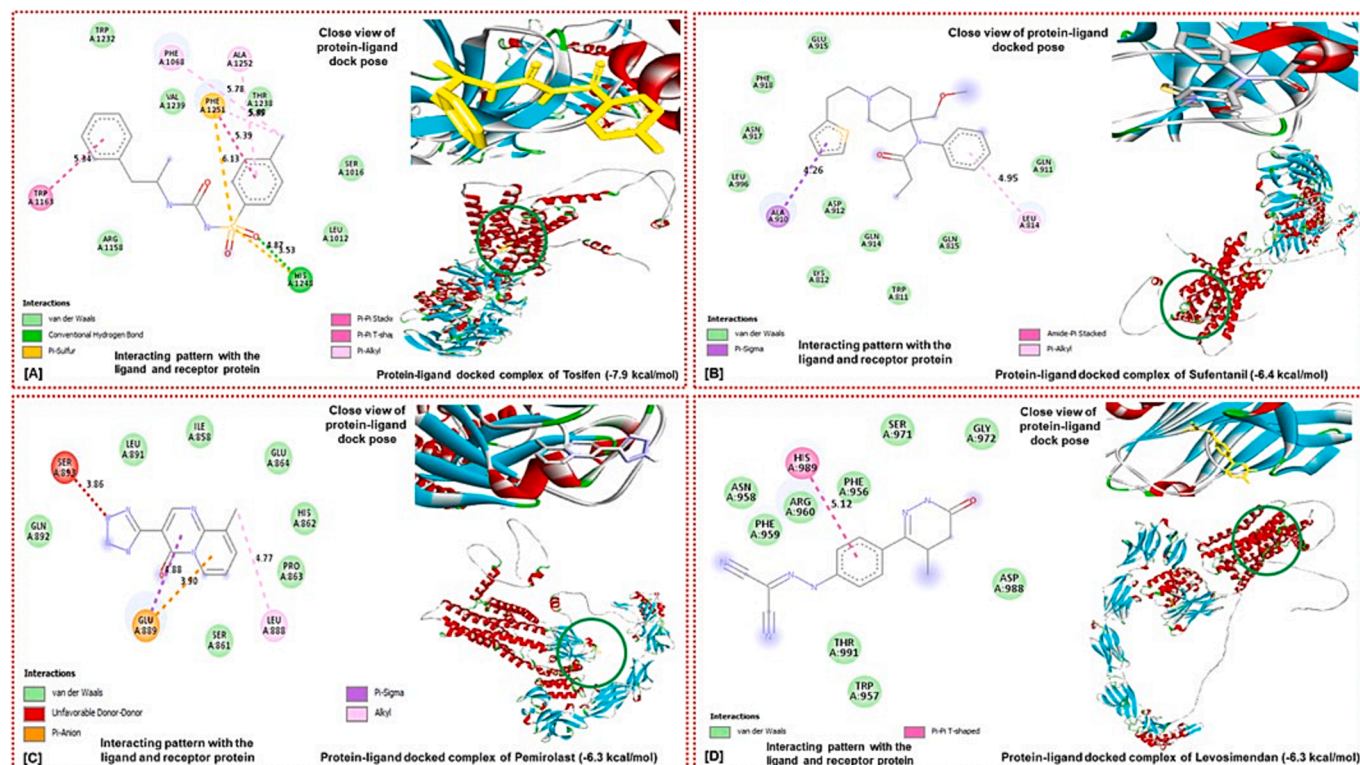


Fig. 4. Two-dimensional interaction mapping analysis of receptor–ligand docked pose of [A] Tosifen, [B] Sufentanil, [C] Pemirolast, and [D] Levosimendan through the BIOVIA Discovery Studio.

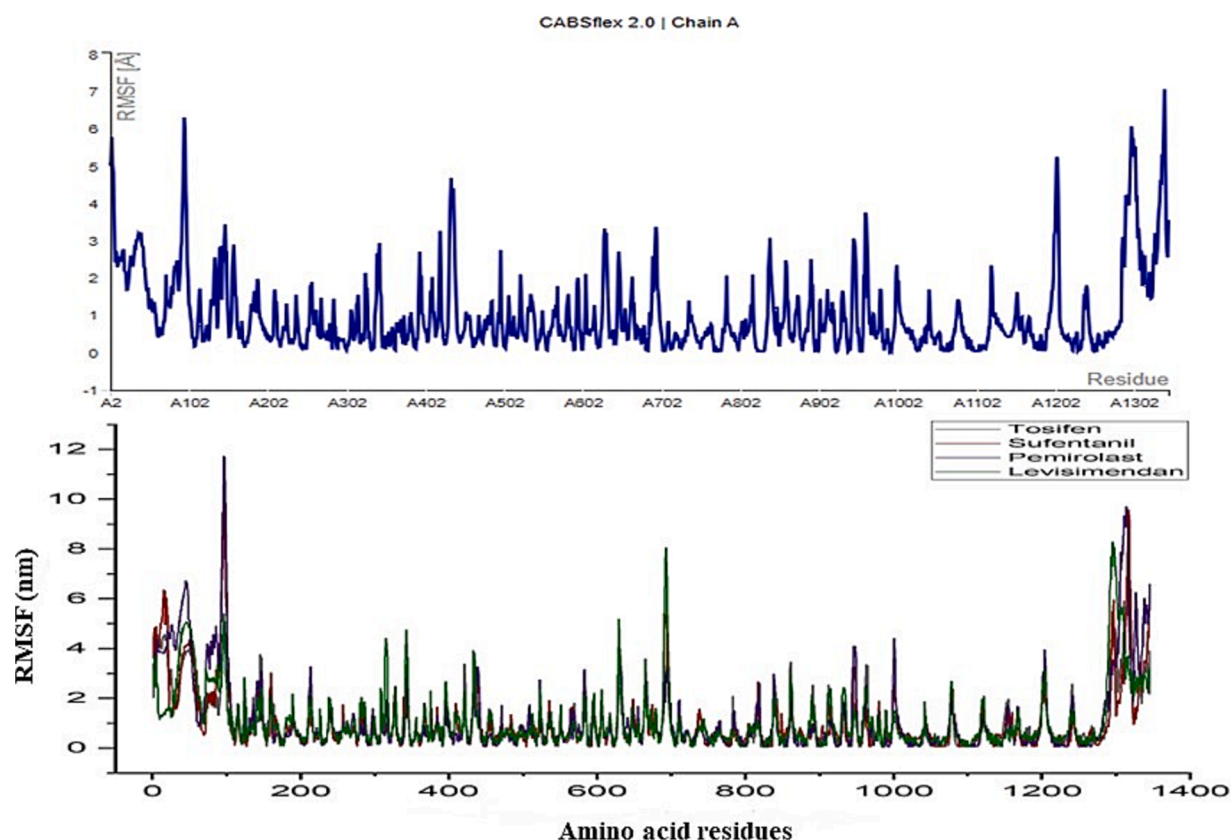


Fig. 5. RMSFs of the [A] receptor chain and [B] receptor-ligand docked complexes of Tosifen, Sufentanil, Pemirolast, and Levosimendan, calculated using the default parameters (protein rigidity: 1.0, number of cycles: 50, cycle between trajectory: 50 for 10 ns) on the CABS Flex 2.0 server.

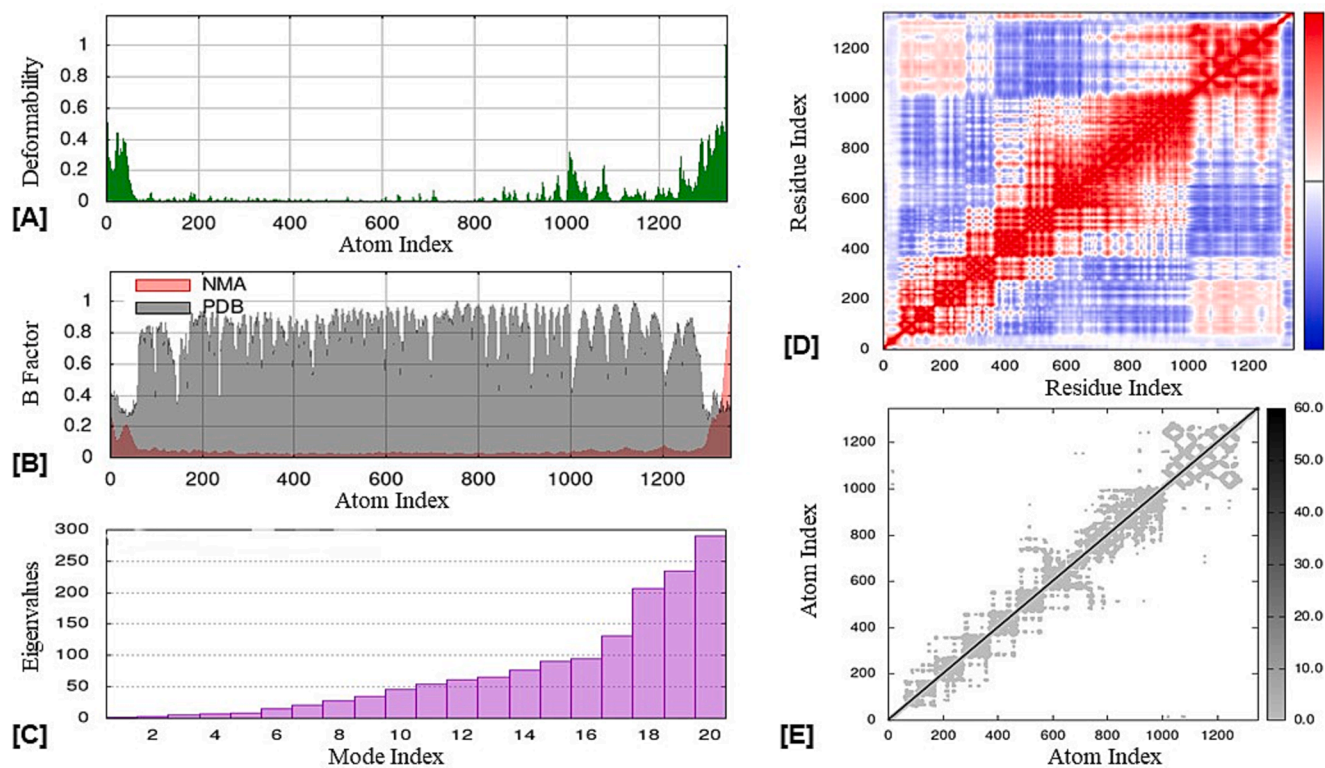


Fig. 6. NMA graph of [A] deformability, [B] B factor, [C] eigenvalues [D] covariance map, and [E] elastic network of the potential receptor-Tosifen docked complex by iMODS server.

previously reported [10].

In the context of the receptor protein ADGRF5, the higher RMSF values observed in the predicted binding sites correlate well with the structural adaptability required for ligand binding. This behavior highlights the dynamic nature of the receptor-ligand interaction, where flexibility within specific residues plays a critical role in accommodating and stabilizing ligand binding.

3.11. Normal mode analysis (NMA)

This NMA analysis determines the slow dynamics of the receptor-ligand complex as well as reveals the “large-amplitude conformational fluctuations” for potential docked complex ADGRF5 and Tosifen. The mobility profile of the receptor chain was determined by the deformability and B factor graph [13]. The deformability graph (Fig. 6[A]) of this docked complex illustrated that the starting residues (1–100) and the ending residues (1290–1346) have higher peaks. Therefore, the ligand has a good probability of deforming the structure of the receptor protein in the docked complex.

The comparison of the three-dimensional structural configuration of the docked pose between the predicted NMA and the PDB was determined through the B-factor graphs. This predicted B factor graph (Fig. 6[B]) of the docked complex has shown an identical structure that forecasts the mobility of the receptor chain.

The energy required to deform the structure is defined by the eigenvalues of the docked receptor-ligand complex. The eigenvalue graph (Fig. 6[C]) of the protein-ligand complex denoted the motion of stiffness. The lower eigenvalue (1.624079×10^{-8}) of this docked complex revealed that the receptor chain was more easily deformable [10].

The covariance map was defined as the interacting pattern between two molecules for the docked complex. In this covariance map, the correlated motion between the amino acid residues and ligand is indicated by the color red, whereas uncorrelated motion is indicated by the color white. Similarly, the anti-correlated motion is shown by the color blue (Fig. 6[D]). The larger molecule's assembly was represented for the receptor-ligand docked complex through the specialized elastic map (Fig. 6[E]), and the stiffer portions were denoted by the deeper grey color [13].

Overall, the MD analysis demonstrated good deformability, low eigenvalues, and strong molecular motion flexibility, underscoring the stability and adaptability of the ADGRF5-Tosifen complex.

3.12. Analysis of OACs targeting colorectal cancer cell lines

The analysis of 4 most potent OACs (Pemirolast, Levosimendan, Tosifen, and Sufentanil) targeting colorectal cancer cell lines revealed variable activity and apoptotic induction potential across different cancer subtypes. The variability in compound efficacy across different cell lines underscores the heterogeneity of colorectal cancer, emphasizing the need for tailored therapeutic approaches [29].

Pemirolast showed moderate potential activity ($P_a = 0.281$) and a low potential inactivity ($P_i = 0.088$), with a high intrinsic apoptotic potential (IAP) value of 0.875, indicating its effectiveness against KM12 colon adenocarcinoma cells. The latest studies [30] and [31], including molecular and in vivo validations, have confirmed that Pemirolast is a prominent anticancer compound.

Similarly, Letrozole an antineoplastics exhibited the lowest activity ($P_a = 0.153$) with a closely matched P_i of 0.150 but had the highest overall IAP value of 0.893, indicating its robust apoptotic activity in colorectal adenocarcinoma cells. While, Azathioprine showed moderate activity ($P_a = 0.200$) with a slightly elevated P_i (0.168) and an IAP of 0.890, suggesting significant apoptotic potential in HCT-116 colon carcinoma cells.

Tosifen displayed slightly lower activity ($P_a = 0.253$) with a P_i of 0.103 and an IAP value of 0.872, suggesting its promise in targeting HCC2998 colon adenocarcinoma cells. Levosimendan demonstrated

reduced activity ($P_a = 0.220$) and a slightly higher P_i (0.146) compared to Tosifen, but with the same IAP value (0.872), indicating comparable apoptotic potential in the same cell line. A latest finding of Ribeiro and Vale [32] shown that Levosimendan effectively inhibits cancer cell migration and enhances the sensitivity of hypoxic cells to radiation therapy, highlighting its potential as a complementary agent in cancer treatment.

Sufentanil, analyzed against RKO colon carcinoma cells, showed a relatively lower activity ($P_a = 0.204$) and higher P_i (0.163), but its IAP value of 0.879 was the highest among the compounds evaluated for carcinoma, highlighting its strong apoptotic induction potential. Zhang and Su also hypothesized that Sufentanil may alter oxaliplatin's cytotoxicity in CRC cells [33]. The details on the colorectal cell lines findings are provided in Table 2.

Overall, this comparative cell line study identified Letrozole and Azathioprine as antineoplastic agents with high apoptotic potential (Table 2). Similarly, Pemirolast, Levosimendan and Sufentanil, which are OACs derived from pearl millet, demonstrated a favorable balance between activity and inactivity, making them effective against specific colon adenocarcinoma cell lines.

3.13. Pharmacokinetics study

The comparative ADMET results of the OACs (Sufentanil, Tosifen, Pemirolast, and Levosimendan) and antineoplastics revealed notable differences in their absorption, distribution, Metabolism, excretion and toxicity properties. In absorption profiling, orally active leads-Sufentanil and Tosifen exhibited low water solubility (-4.442 and -3.845 , respectively) but relatively high intestinal absorption (91.783 % and 91.482 %, respectively). Pemirolast shows complete absorption (100 %) despite moderate solubility (-2.582). Levosimendan has the highest solubility (-1.501) but the lowest absorption (49.76 %). The gut-blood barrier's permeability is modeled using the apparent Caco-2 cell permeability [13]. Antineoplastics, Azathioprine and Letrozole demonstrate good absorption (78.64 % and 99.83 %) with moderate permeability. Sufentanil and Tosifen are P-glycoprotein substrates, whereas only Sufentanil inhibits both P-glycoprotein I and II. Skin permeability remains consistently low across all compounds.

Drugs that are too polar cannot pass the BBB [11]. Sufentanil exhibits moderate distribution (volume distribution in steady-state (VDss): 1.152 log L/kg) with low unbound fraction (0.065) and good blood-brain barrier (BBB) permeability (0.458). The majority of the drug is present in tissues as opposed to binding to the blood plasma if the VDss is greater than the optimal value (>0.45 log L/kg) [12,33]. Tosifen shows poor distribution (-0.276 log L/kg) and low BBB permeability (0.145). Pemirolast has moderate distribution (0.431 log L/kg) and high unbound fraction (0.462), with poor CNS permeability. Levosimendan and antineoplastics (Azathioprine and Letrozole) shows low distribution, with Levosimendan having the highest unbound fraction (0.617) and moderate BBB permeability. Both types of compounds have low central nervous system (CNS) permeability, with Pemirolast and Levosimendan showing the poorest.

In metabolism, logPS value greater than -2 are considered capable of permeating the CNS and are classified as CNS-active, while those with a logPS of -3 or lower are classified as CNS-inactive [33]. Sufentanil is a substrate for both CYP2D6 and CYP3A4 and inhibits CYP2D6. Tosifen does not interact with CYP2D6 or CYP3A4 but inhibits CYP1A2 and CYP2C19. Pemirolast does not interact with CYP2D6 or CYP3A4 but inhibits CYP1A2. Levosimendan, and antineoplastics, Azathioprine, and Letrozole shows minimal metabolic interactions, with Levosimendan and Azathioprine not interacting with any CYP enzymes. Letrozole is a CYP3A4 substrate and inhibits CYP1A2, CYP2C19, and CYP3A4.

Sufentanil has moderate clearance (1.176 log ml/min/kg) and is a renal OCT2 substrate. Tosifen, Pemirolast, and Levosimendan have lower clearance rates, with Levosimendan showing the highest among them (1.198). Azathioprine shows the lowest clearance (0.148) and is

Table 2

Comparative analysis of the activity (Pa), inactivity (Pi), and apoptotic potential (IAP) of potential orally active compounds from pearl millet (Pemirolast, Tosifen, Levosimendan, and Sufentanil) and antineoplastics (Letrozol and Azathioprine) against colorectal cancer cell lines, as predicted by “PASS CLC Pred” tool.

Sr. No.	Compounds	Pa	Pi	Cell-line	Description	Tissue/Organ	IAP
1	Pemirolast	0.281	0.088	KM12	Colon adenocarcinoma	Colon	0.875
2	Tosifen	0.253	0.103	HCC2998	Colon adenocarcinoma	Colon	0.872
3	Levosimendan	0.22	0.146	HCC2998	Colon adenocarcinoma	Colon	0.872
4	Sufentanil	0.204	0.163	RKO	Colon carcinoma	Colon	0.879
5	Letrozol	0.153	0.15	WiDr	Colorectal Adenocarcinoma	Colon	0.893
6	Azathioprine	0.2	0.168	HCT-116	Colon carcinoma	Colon	0.89

not a renal OCT2 substrate. Letrozole has a clearance of 0.77 and is a renal OCT2 substrate. Additional details on the metabolism profiling are provided in Table 3.

3.14. Toxicity profiling of promising compounds

The present study evaluated the toxicity profiles of the selected compounds, including their “AMES toxicity, Oral Rat Acute Toxicity (LD₅₀), Oral Rat Chronic Toxicity (LOAEL), and Hepatotoxicity”. Among the promising leads, Sufentanil and Pemirolast exhibited higher LD₅₀ values of 2.933 mol/kg and 2.274 mol/kg, respectively, indicating lower

acute toxicity compared to Tosifen (1.613 mol/kg) and Levosimendan (2.153 mol/kg). Additionally, Pemirolast has the highest LOAEL value (2.467 log mg/kg bw/day), suggesting its potential for chronic toxicity at higher doses compared to the other lead compounds.

The mutagenicity, a critical factor in drug discovery and development, was assessed using the bacterial reverse “mutation assay (Ames test)” to evaluate the potential for carcinogenicity and/or teratogenicity. Substances predicted to test positive in the Ames assay could pose mutagenic risks, emphasizing the importance of these predictions in early-stage drug screening [34]. Sufentanil, Tosifen, Pemirolast, and Azathioprine (antineoplastics) do not show AMES toxicity but exhibit

Table 3

ADMET profiling of predicated lead OACs (Tosifen, Sufentanil, Pemirolast, and Levosimendan) of pearl millet and antineoplastics (Azathioprine and Letrozole).

1. Absorption							
	Water Solubility	Caco-2 Permeability (log Papp in 10 ⁻⁶ cm/s)	Intestinal Absorption (Human) (%) Absorbed)	Skin Permeability (log Kp)	P-glycoprotein Substrate	P-glycoprotein I Inhibitor	P-glycoprotein II Inhibitor
Sufentanil	−4.442	1.209	91.783	−2.372	Yes	Yes	Yes
Tosifen	−3.845	0.907	91.482	−2.876	Yes	No	No
Pemirolast	−2.582	1.022	100	−2.735	No	No	No
Levosimendan	−1.501	−0.079	49.76	−2.734	No	No	No
Azathioprine	−2.891	0.616	78.64	−2.735	Yes	No	No
Letrozole	−3.793	0.883	99.83	−2.492	No	No	No
2. Distribution							
	VDss (human) (log L/kg)	Fraction unbound (human) (Fu)	BBB permeability (log BB)		CNS permeability (log CS)		
Sufentanil	1.152	0.065	0.458		−1.489		
Tosifen	−0.276	0.06	0.145		−2.315		
Pemirolast	0.431	0.462	−1.379		−4.162		
Levosimendan	−1.165	0.617	−0.866		−3.635		
Azathioprine	0.138	0.396	−1.227		−3.509		
Letrozole	−0.031	0.164	−0.386		−2.05		
3. Metabolism							
	CYP2D6 substrate	CYP3A4 substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
Sufentanil	Yes	Yes	No	No	No	Yes	No
Tosifen	No	No	Yes	Yes	No	No	No
Pemirolast	No	No	Yes	No	No	No	No
Levosimendan	No	No	No	No	No	No	No
Azathioprine	No	No	No	No	No	No	No
Letrozole	No	Yes	Yes	Yes	No	No	Yes
4. Excretion							
	Total Clearance (log ml/min/kg)			Renal OCT2 substrate			
Sufentanil	1.176			Yes			
Tosifen	0.76			No			
Pemirolast	0.458			No			
Levosimendan	1.198			No			
Azathioprine	0.148			No			
Letrozole	0.77			Yes			
5. Toxicity							
	AMES toxicity	Max. tolerated dose (human) (log mg/kg/day)	hERG I inhibitor	hERG II inhibitor	Oral Rat Acute Toxicity (LD50) (mol/kg)	Oral Rat Chronic Toxicity (LOAEL) (log mg/kg.bw/day)	Hepatotoxicity
Sfentanil	No	−0.067	No	Yes	2.933	1.45	Yes
Tosifen	No	0.464	No	No	1.613	1.616	Yes
Pemirolast	No	0.594	No	No	2.274	2.467	Yes
Levosimendan	No	0.301	No	No	2.153	2.307	No
Azathioprine	Yes	0.491	No	No	2.486	1.515	Yes
Letrozole	No	−0.592	No	No	2.085	1.238	No

varying degrees of toxicity.

This toxicity profiling found that Sufentanil has the lowest maximum tolerated dose ($-0.067 \log \text{ mg/kg/day}$), and Tosifen, Pemirolast, and Azathioprine have moderate tolerated doses. Levosimendan and Letrozole showed lower tolerated doses compared to the others. Sufentanil is a hERG II inhibitor, while others are not. All compounds except Levosimendan and Letrozole showed hepatotoxicity, with Levosimendan showing no hepatotoxic effects. Letrozole has no hepatotoxicity but exhibited lower oral rat chronic toxicity. A details study of the Toxicity proofing has been given in Table 3.

4. Conclusion

The presence of small phenolic and aromatic compounds were analyzed using FTIR from the 55 compounds identified through LC-MS. This cheminformatics study has established a robust, effective, and straightforward pipeline for evaluating drug likeliness, structure-activity relationships (SAR), virtual screening, and pharmacokinetics of natural products.

The present findings suggested 4 OACs—Tosifen, Sufentanil, Pemirolast, and Levosimendan—as promising anti-cancer agents targeting ADGRF5, among all the small compounds derived from pearl millet. These findings highlighted the potential of these compounds as therapeutic candidates for cancer treatment.

Funding

The authors declare that no funds, grants, or other support were received for this work.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors wish to thank the Vice Chancellor of Central University of Haryana, Mahendergarh, Haryana, India, for providing the necessary infrastructure.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.saa.2025.125960>.

Data availability

The manuscript has no associated data.

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