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Research Article

ANTIPROLIFERATIVE ACTIVITY OF N-BUTANOL FLORAL EXTRACT FROM *BUTEA MONOSPERMA* AGAINST HCT 116 COLON CANCER CELLS; DRUG LIKENESS PROPERTIES AND *IN SILICO* EVALUATION OF THEIR ACTIVE COMPOUNDS TOWARD GLYCOGEN SYNTHASE KINASE-3β/AXIN AND β-CATENIN/T-CELL FACTOR-4 PROTEIN COMPLEX

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ABSTRACT

Objective: The aim was to study the inhibitory effect of n-butanol fraction of *Butea monosperma* floral extracts (NBF-BMFE) against HCT116 cells. Moreover, the drug-likeness properties and *in silico* evaluation of their active compounds toward glycogen synthase kinase-3β (GSK-3β)/Axin and β-catenin/T-Cell factor-4 (Tcf-4) complex proteins.

Methods: The three-dimensional protein structures were incurred from RCSB protein data bank, and their active site amino acids predicted using CASTp server. Similarly, the NBE-BMFE phytochemicals were retrieved from PubChem Database then their absorption, distribution, metabolism, excretion, and toxicity (ADMET)-related descriptors were calculated by using the admetSAR along with ACD/i-lab software. The docking analysis was performed by using AutoDock 4.2. Concurrently, the NBF-BMFE were experimentally characterized by using liquid chromatography/mass spectrometry (LC/MS) besides their anticancer activity was assessed against HCT-116 human colon cancer cells.

Results: The docking studies results showed that the NBF-BMFE phytochemicals showed good hydrogen bond interaction against GSK-3β/Axin (4B7T) and β-catenin/Tcf-4 (1JPW) complex proteins. Moreover, the *in silico* results of ADMET factors were also satisfying correspondingly. The LC/MS results revealed that the NBF-BMFE contains isocoreopsin, butrin and isobutrin as major compounds, and it has significant anticancer activity (>100 μM) against HCT-116 human colon cancer cells.

Conclusion: Overall our results concluded that all the NBF-BMFE had significant inhibitory effect on HCT-116 cells plus good binding interaction with 4B7T and 1JPW, in specific isocoreopsin, butein and butin showed promising agents to develop as potent drug molecules against colorectal cancer.

Keywords: Colorectal cancer, *Butea monosperma,* Absorption; distribution; metabolism; excretion and toxicity, Molecular docking, Glycogen synthase kinase-3β/Axin, β-catenin/T-Cell factor-4.

INTRODUCTION

The colorectal cancer (CRC) is the fourth leading cause of cancer death in worldwide and diagnosed as third most common form of cancer in men and second in women [1]. There is more than 90% of CRC caused by active mutation of canonical Wnt signaling pathway. This believes to be a starting event for colorectal carcinogenesis if it sustains persistently [2]. Their Wnt proteins can transduce signaling by distinct intracellular routes through canonical or non-canonical Wnt pathways [3] and it is important for normal cell development, cell fate specification, polarity and migration of cells [4].

By the absence of Wnt, β-catenin is conjoined with the multi-protein complex of axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β) and casein kinase 1α [5]. In specific, the axin is a vital element for canonical Wnt signaling pathway, which acts as a scaffolding protein to form multi-protein complex. This further facilitates the β-catenin phosphorylation and degradation through ubiquitin-proteasomal pathway [6]. While there is the presence of Wnt, the canonical Wnt signaling pathway started to activate when Wnt ligands get bind with Frizzled (Fz) receptor and low-density lipoprotein related receptor 5/6 complex. Thereby, axin is translocate into the cell membrane to inhibit the multi-protein complex [7]. Thus, β-catenin accumulates in the cytoplasm and undergoes translocation into the nucleus, where it interacts with transcription factors such as T-cell factor (Tcf)/lymphoid enhancing factor (TCF) to activate the target genes transcription. From this, nuclear localization of β-catenin is a key feature for CRC cells, and there is a continuous activation of downstream target genes for tumor cells sustenance [8]. Hence, the encirclement of GSK-3β/Axin and β-catenin/Tcf-4 complex is becoming a vital target to treat CRC.

Recently, herbal medicines and their derived phytocompounds are being arises as a complementary treatment for cancer. In this series, *Butea monosperma* (Bm) Lam. Kuntze (Fabaceae), commonly known as flame of the forest has well-documented medicinal properties that includes anti-inflammatory, anti-cancer, anti-diabetic, antiulcer activities and also widely used in the treatment of hepatic disorders and viral hepatitis [9,10]. The ethanol extract of *B. monosperma* flowers has been shown as hepatoprotective and anti-tumorigenic activity [9,11]. In the previous study, we depicted the n-butanol fraction of Bm floral extract (NBF-BMFE) phytochemical compounds were effectively inhibited the mutated wnt/β-catenin destruction complex proteins such as β-catenin, APC and GSK-3β against CRC [12]. As continue of this, in the present study we docked NBF-BMFE with Wnt signaling proteins such as GSK-3β/Axin and β-catenin/Tcf-4 complex and also we studied anti-cancer activity against HCT116 human colon cancer cells. After, we validated the pharmacological properties of absorption, distribution, metabolism, excretion and toxicity (ADME-T) prediction to evaluate their ability to use as an orally active compound. Moreover, the characterization of NBF-BMFE were done by liquid chromatography/Mass spectrometry (LC/MS).

METHODS

Preparation of BMFE

The Bm flowers were collected from the Bharathidasan University campus, and a voucher specimen was deposited in the Department of Plant science, Bharathidasan University (Tiruchirappalli, Tamil Nadu, India). The 500 g dried powdered parts of Bm flowers were extracted with methanol in a soxhlet for about 20 hrs. As a resultant, 145 g of orange colored powder was obtained by removing of solvent under reduced pressure in a rotatory evaporator. The total methanol extract of 100 g was partitioned thrice between water and ethyl acetate. After removal of ethyl acetate fraction, the remaining water phase also partitioned thrice with n-butanol. The obtained solvent was removed under reduced pressure to yield 2.7 g butanol fraction (yellow powder) [13].

LC/MS methodology

LC/MS analysis of NBF-BMFE was performed on LC system (SHIMADZU) equipped with photodiode array detector and electron spray ionization source. The mobile phase buffer used for this analysis was 0.1% formic acid in water (Pump A) and 0.1% formic acid in acetonitrile (Pump B). The sample was dissolved in 1 ml of water and filtered by using 0.22 µ syringe filter. Sample 20 µl was injected into C18 reversed phase column (Phemomenax, Luna, 5 µm particles size, 250 mm length, 4.6 mm internal diameter) by ambient conditions. A linear gradient has settled at 0 min, 10% B-26% B at 20 min, then to 65% B at 35 min and finally to 100% B at 36 min. The flow rate at 0.5 ml/min and the high-performance liquid chromatography flow was directed into mass spectrophotometer, operated in both positive and negative mode. The fall out spectra was recorded from 200 to 800 nm and scanned over a mass range of 100-800.

Identification of protein target

The structural and functional characteristics of Wnt/β-catenin signaling proteins such as GSK-3β complex with axin peptide [14-16] and β-catenin/TCF4 complex [17] are important to determine the cellular sensitivity of CRC. Three-dimensional (3D) structures of these complex were predicted using X-ray crystallography, which incurred from RCSB protein databank are GSK-3β/Axin (PDBID: 4B7T, resolution factor: 2.77Å) and β-catenin/TCF4 complex (PDBID: 1JPW, resolution factor: 2.50Å). It is existing as a good sources to predict structure-based pharmacophore analysis. The integration of 3D structures with stereochemical activity of least energy was predicted using PROCHECK v.3.0. Then, the environment profile was done by ERRAT graph and the results were drawn using Ramachandran plot. The theoretical model of active site amino acids was predicted using CASTp calculation server, identifies and measures pockets of ligand binding amino acids within cavities of surface area and surface volume.

Pharmacophore analysis

The medically important Indian traditional plant BMFE compounds such as butrin, isobutrin, butein, butin, coreopsin, isocoreposin, monospermoside, and isomonospermoside ligand molecules were retrieved from PubChem compound database. By using Hyperchem 7.5 professional and molinspiration (http://www.molinspiration.com/cgi-bin/properties), the drug-likeness properties along with the pharmacophore and biological activity against different enzymes has been calculated. It has used to evaluate drug-likeness and to describe whether a chemical compound has certain pharmacological activity as an orally active drug to human.

Calculation of absorption, distribution, metabolism, excretion and toxicity (ADMET)-related descriptors

A set of ADMET-related descriptors were calculated by using the admetSAR (http://www.admetexp.org) [18] and ACD/i-lab. The prediction of physical properties and molecular descriptors of top docking hits and quercetin were analyzed by brain/blood partition coefficient (QPlogB/B), human intestinal absorption (logHIA), P-glycoprotein inhibition (logPGI substrate and logPGI inhibitor), aqueous solubility (PlogS) and Caco-2 cell permeability (QPPCaco) to obtain the ADME properties of the compounds. In addition, the probability of health effects and physicochemical properties was predicted using lethal dose$_{50}$ (LD_{50}) value. The comparative analysis of ligand molecules LD_{50} values were studied in mouse by intraperitoneal, oral, intravenous, subcutaneous and the probability of toxic health effects were examined in blood, cardiovascular system, gastrointestinal system, kidney, liver, and lung tissues.

Molecular docking

Molecular docking studies were carried out using Autodock 4.2 and Autodock Tools 1.5.4 from the Scripps Research Institute, http://www.scripps.edu/mb/olson/doc/autodock. The Lamarckian genetic algorithm was used for ligand conformational searching. The local search algorithm, which builds a population of individuals (genes), each being a different random conformation of the docked molecule [19]. The grid was generated around the active site at 80 × 80 × 80 to calculate molecular simulation using AMBER tools, showed auto grid of active site residues around the complex structure. There were 150 populations with a mutation rate of 0.02, crossover rate of 0.8 and default grid spacing 0.375Å were used as parameters settings for docking. Consequently, these simulations were performed using up to 2.5 million energy evaluations with a maximum of 27,000 generations and each simulation was performed by 10 times that yielded 10 docked conformations. At last, the lowest energy conformations were regarded as the binding conformations between ligands and the protein.

Determination of cytotoxic activities of NBF-BMFE

Cell preparation and culturing

The β-catenin expressing HCT-116 human colon cancer cell line was obtained from NCCS Pune, India. The cells were grown in 25 cm × 25 cm × 25 cm tissue culture flasks containing RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO). The cells were grown at 37°C under a humidified atmosphere of 95% air and 5% CO_2.

Treatment of cell line

Cells were trypsinized and seeded in 96 well plates at varying cell number according to the size and shape when their density in a culture flask reached 70-80% confluence. The HCT116 cells were seeded at the density of 3000 cells per well in 100 µl and incubated for 24 hrs at CO2 incubator. The NBF-BMFE and butein compound was prepared as 1 mg/ml stock by adding directly into the DMEM medium. The working stock of 2 × (2000, 600, 200, 60 and 10 µM) concentration to the cell in 100 µl volume and the final concentration range were made up to 1000, 300, 100, 30 and 10 µM/ml and the plates were further incubated for 48 hrs.

Cell viability assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide [MTT] assay)

MTT at 5 mg/ml in phosphate buffered saline (1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) was prepared, from this solution 50 µl was pipette out into each well to achieve 1 mg/mL as final concentration. The plate was further incubated at 37°C in 5% CO_2 for 2.30 hrs and the medium was carefully decanted. The formazan crystals were air dried in dark place and dissolved in 100 µL dimethylsulfoxide (DMSO), the plates were gently shake at room temperature and the optical density was measured using Synergy H4 micro plate reader at 570 nm [20].

Statistical analysis

The data were done in triplicate, and the results were expressed as mean±standard deviation. The experiments were analyzed using Graph Pad Prism software (Graph Pad Software Inc., CA 92037 USA).

RESULTS AND DISCUSSION

LC/MS analysis

The LC/MS chromatogram is a liable tool, which offers soft ionization to analyze flavonoids and identification [21] of NBF-BMFE that yielded two

peaks at the retention time of 6.9 min and 8.2 min with 94.7% purity, (Fig. 1). As correlative with earlier report of molecular weights (MW) of butrin (596.17), isobutrin (596.17), butein (272.25), butin (272.25), coreopsin (434.39), isocoreposin (434.39), monospermoside (434.39) and isomonospermoside (434.39) compounds in NBF-BMFE [10,22], our LC/MS analysis also confirmed with the presence of above bioactive compounds (Fig. 2). Interestingly, these compounds have also been exhibited highest binding (docking) interaction with Wnt/β-catenin signaling pathway proteins in CRC. Hence, our results pave the way to target the individual compounds, treat the CRC patients in the near future.

Pharmacophore analysis of lead molecules

The 3D structure of GSK-3β domain (green) with 350 amino acids was functionally associated with axin protein contained 18 amino acids (blue) that efficiently co-localized and thereby phosphorylate β-catenin. The another protein of Tcf-4 peptide with 18 amino acids (blue) has two active sites, interacted with region of 12 armadillo repeat in β-catenin (green) of 540 amino acids that functionally associated with transcriptional regulation of APC and cadherins (Fig. 3a and b). Therefore, the CRC causing by the association of these sequential events with Wnt/β-catenin signaling pathways.

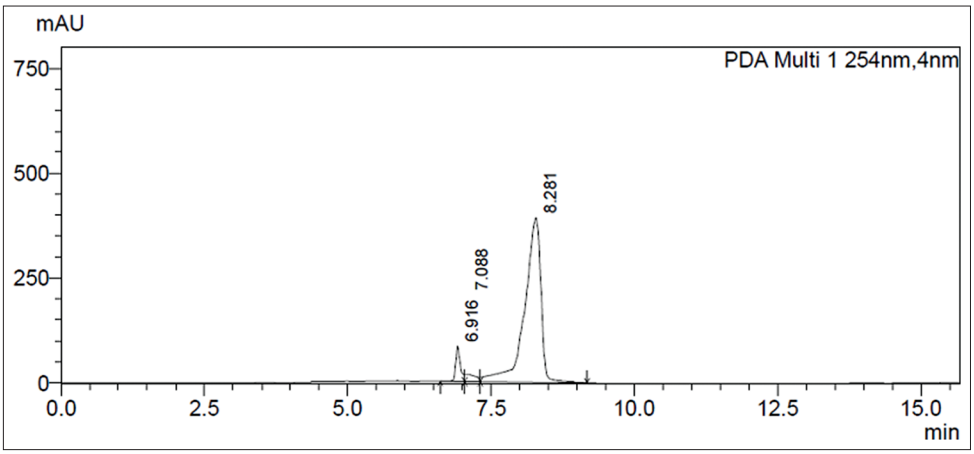

Fig. 1: High-performance liquid chromatography chromatogram of n-butanol fraction of *Butea monosperma* floral extracts

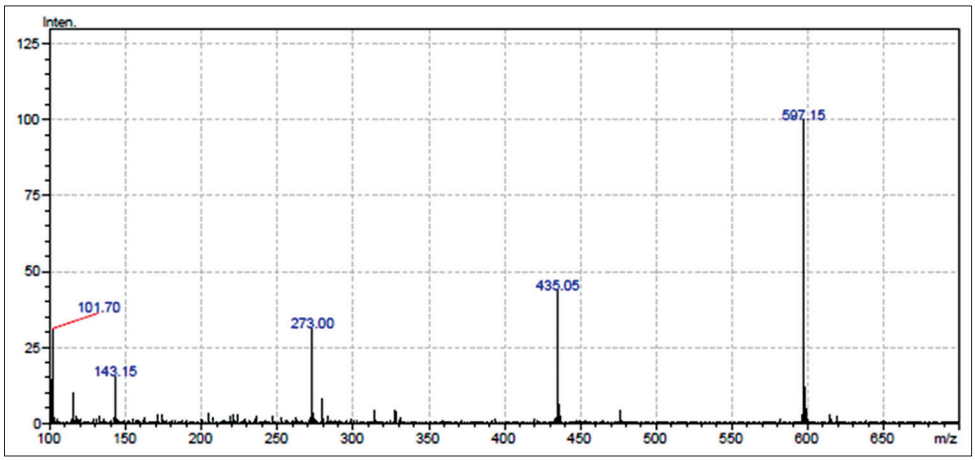

Fig. 2: Mass spectrum of n-butanol fraction of *Butea monosperma* floral extracts

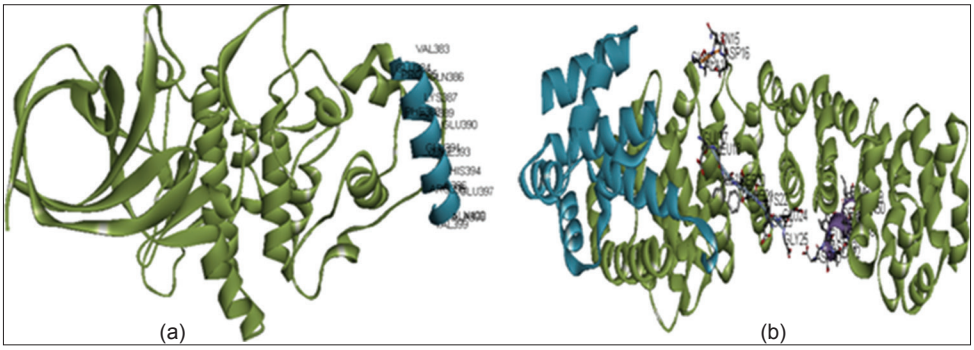

Fig. 3: Structure of the glycogen synthase kinase-3β (GSK-3β)/Axin and T-Cell factor-4 (Tcf-4)-β-catenin complexes, (a) Axin protein (blue) of 348-433 amino acids has GSK-3β interaction site (green), (b) Tcf-4 (blue) peptides of 13-25 and 40-50 amino acids interacts with β-catenin of 151-549 and 560-662 amino acids (green)

These pharmacophore properties of these 3D protein models were further validated by using stereo chemical analysis of root mean square deviation (RMSD), picked up the values of 4B7T protein were RMSD - 0.1Å, Procheck - 84.82%, quality factor - 79.037%, Ramachandran plot - 90.0% and the stereo chemical bonds of protein 1JPW were RMSD - 0.12Å, Procheck - 90.89%, quality factor - 93.874%, Ramachandran plot - 92.3%, which strongly enhanced the 3D structure formation. The potential ligand binding energy surface in active sites of the complex protein structures were calculated based on cavity surface and volume. The active site surface and volume of 4B7T were 658A^3 and 522.6A^3 which was close to Ile62, Gly63, Ala83, Lys85, Asp133, Tyr134, Val135, Leu188 and Asp200. Succeeding, the active site surface and volume of protein 1JPW was 990.6A^3 and 1830.6A^3, was close to Gly13, ala14, Glu17, Leu18, Ile19, Ser425, Asn426, Asn430, Glu462, Arg474, Val511 and Val613. The amino acids prevailed were actively used as a ligand binding sites.

Using molinspiration, the pharmacophore analysis of eight ligand molecules with reference of quercetin were generated, had different scaffolds and activities. The parameter of Lipinski rule of five includes total polar surface area (TPSA) for NBF-BMFE and quercetin has been shown in Table 1. There were different statistically significant parameters such as LogP of - 5 to 5, MW ≤500, number of hydrogen bond acceptors ≤10, number of hydrogen bond donors ≤5, rotatable bonds ≤12, number of atoms ≤40, molecular volume ≤500 and TPSA ≤1000 has been demonstrated (Table 1).

The observed results declared that the butrin and isobutrin have not been accepted drug-likeness properties, had poor permeation due to high hydrogen bond acceptors and hydrogen bond donors. Whereas, the resting compounds were strongly accepted since it had strong binding affinity against complex protein structure. Moreover, it has more permeable within membrane and blood cells when compared to quercetin. By improving the pharmacological properties of butrin and isobutrin, needed molecular modification of compounds.

ADMET analysis

Normally, many compounds failed at early stage due to poor pharmacokinetics properties and toxicity problems. If these problems could be analyzed early, it will be great advantageous for drug development process. In view of these, computer-based methods like ADMET tool plays a vital role in the studies of molecular descriptors and drug-likeness properties [23,24]. The pharmacokinetic properties results herein reported in Table 2. The efficiency of the blood/brain partition co-efficient (logB/B) used as a predictor for central nervous system (CNS). This predicted CNS activity was calculated from −3 (inactive) to +1 (active) scale, displayed all the molecules were come within the acceptable range. This clearly demonstrated butrin showed good results when compared to quercetin. The blood-brain barrier plays a main role to maintain homeostasis of CNS by separating the circulating blood from the brain [25]. The drug's intestinal permeability has been estimated through the Caco-2 cell permeability (PCaco), whereas the recommended range for this parameter is from −1 (poor) to +1 (good), explicit all the molecules had good absorption in the intestinal membrane. The HIA of drug metabolism and drug permeability with intestinal membrane was measured using Log_{HIA} and formed the range from 0 (poor) to 1 (great). Rather than quercetin, the molecules butein and butin was completely metabolized and easily permeable within HIA. The functional groups of these compounds had great absorption within the human intestine and also had a strong involvement in drug metabolism. The understanding of P-glycoprotein (P-gp) substrate and inhibitors study were based on drug-drug interaction within different tissues. Commonly, P-gp offers main role to trash out the xenobiotics from various organs to reduce the drug intestinal absorption and enhance the elimination of drugs, where pass into bile (liver) and urine (kidney) [26]. The recommended range for P-gp substrate was from −5 (poor) to +1 (good) and P-gp inhibitor was from 0 to 1. In which, the results of these molecules displayed within the acceptable range when compared with quercetin. The aqueous solubility prediction was mainly calculated the drug concentration, present in the target area for the establishment of therapeutic level and prevention of toxicity [27]. The recommended range for aqueous solubility (PlogS) was −6.5 to −0.5 and probability of

Table 1: Lipinski rule of five filters including TPSA for the top poses

Ligand	xlogP	TPSA	n atoms	MW	HBA	HBD	RB	MV	n violations
Butrin	−2.092	245.293	42.0	596.538	15	9	7	494.504	3
Isobutrin	−1.523	256.287	42.0	596.538	15	10	9	498.166	3
Butein	2.28	97.983	20.0	272.256	5	4	3	233.924	0
Butin	1.711	86.989	20.0	272.256	5	3	1	230.261	0
Coreopsin	0.493	177.135	31.0	434.397	10	7	6	366.045	1
Isocoreposin	−0.076	166.141	31.0	434.397	10	6	4	362.383	1
Monospermoside	0.264	177.135	31.0	434.397	10	7	6	366.045	1
Isomonospermoside	−0.305	166.141	31.0	434.397	10	6	4	362.383	1
Quercetin	1.683	131.351	22.0	302.238	1	5	1	240.084	0

LogP: Logarithm of the octanol/water partition coefficient, TPSA: Topological polar surface area, natoms: Number of atoms, MW: Molecular weight, HBA: Number of hydrogen bond acceptors, HBD: Number of Hydrogen bond donors, RB: Number of rotatable bonds, MV: Molecular volume, nviolations: number of violations of the Lipinski's rule of five

Table 2: ADME and pharmacological parameters prediction for the selected ligands using admetSAR toolbox

S. No.	Ligand	PlogBB[a]	PCaco[b]	log_{HIA}[c]	logpGI (substrate)[d]	logpGI (non-inhibitor)[e]	PlogS[f]	logpapp[g]
1	Butrin	0.724	0.932	0.701	0.634	0.869	−2.196	−0.921
2	Isobutrin	0.539	0.881	0.621	0.617	0.859	−1.264	−0.577
3	Butein	0.586	0.607	0.970	0.536	0.919	−3.371	0.167
4	Butin	0.539	0.819	0.979	0.546	0.880	−2.275	0.472
5	Coreopsin	0.597	0.865	0.513	0.581	0.779	−1.528	−0.519
6	Isocoreposin	0.697	0.939	0.785	0.590	0.878	−2.448	−0.858
7	Monospermoside	0.597	0.865	0.513	0.581	0.779	−1.528	−0.519
8	Isomonospermoside	0.697	0.939	0.785	0.590	0.878	−2.448	−0.858
9	Quercetin	0.571	0.895	0.965	0.562	0.929	−2.994	0.224

[a]Predicted blood/brain barrier partition coefficient (concern value is −3.0 to 1.0), [b]predicted Caco-2 cell permeability in nm/s (acceptable range: −1 is poor, 1 is great), [c]predicted human intestinal absorption in nm/s (acceptable range: 0 poor, >1 great), [d]predicted P-gp substrate in nm/s (acceptable range of −5 is poor, 1 is great), [e]predicted P-glycoprotein inhibitor in nm/s (accepted range: 0-1), [f]predicted aqueous solubility, (concern value is −6.5 to −0.5). [g]predicted probability of Caco-2 cell permeability in cm/s (concern value is−1 to 1), P-gp: P-glycoprotein, HIA: Human intestinal absorption, ADME: Absorption, distribution, metabolism, excretion

Caco-2 cell permeability was −1 to 1. Indeed, the PlogS results showed all the molecules had good solubility and logpapp stated that butein and butin had good permeability on lipid absorption and metabolism. While resting compounds were came within acceptable range. On the whole, the NBF-BMFE recorded strong drug likeness properties.

The LD_{50} values of ligands were detected the cumulative potential of acute toxicity that has administered through oral, intraperitoneal, intravenous and subcutaneous on mouse models. A comparative analysis of LD_{50} mouse revealed that the NBF-BMFE had higher LD_{50} on oral and lower LD_{50} on subcutaneous when compared with quercetin (Fig. 4). The overall results suggested that all compounds had less toxic effect on internal tissues and no side-effect were observed in the tested dosages.

The toxicity were tested with different organs to check adverse effects of organs and their systems (blood, cardiovascular system, gastrointestinal system, kidneys, liver, and lungs) within the therapeutic dose range. The probability of health effects revealed that butein and butin had very less toxic effect on blood, cardiovascular, gastrointestinal, kidney, liver and lung. While other compounds had moderate toxic effect on all tissues except lung (Table 3). Hence, these inclusive results together suggested that the NBF-BMFE had no side-effects with corresponding organs.

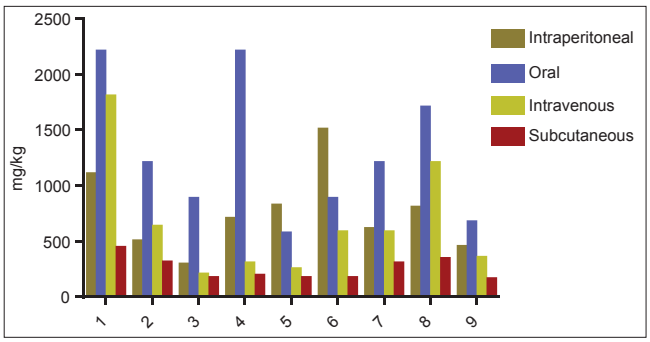

Fig. 4: Comparative analysis on lethal dose$_{50}$ mouse (intraperitoneal, oral, intravenous, subcutaneous) for n-butanol fraction of *Butea monosperma* floral extracts (1-8) and reference standard quercetin (9)

Molecular docking

The molecular docking results detected the position and orientation of the inhibitor or substrate within protein structure. The achievement of crystal 3D protein structures such as 4B7T and 1JPW had shown strong binding affinity with natural compounds extracted from Bm flowers. Herein, we mainly discussed our docking experiment on GSK-3β/Axin and β-catenin/TCF4 enzymes.

An initial validation of the docking protocol was performed based on confirmations, positions and orientations of the ligand were obtained from docking with the experimentally determined 3D protein structures. The protein-ligand interaction energy was calculated based on the parameters such as hydrogen bond interaction, binding energy and RMSD of active site residues [28]. The NBF-BMFE was docked with GSK-3β/Axin complex protein, resulted isocoreopsin had a good dock score of −9.27 Kcal/mol with the formation of 6 hydrogen bonds than quercetin. It was strongly bound with active site amino acids of Ile62, Lys85, Asp133 and Val135, where other compounds also expressed good binding interactions with this protein complex (Table 4). The RMSD value of butrin and isobutrin were 0.18Å and 0.16Å, had strong interaction within active site cavity (Fig. 5). Whereas, the reference compound quercetin showed weak interactions with active site protein, which formed only one hydrogen bond with the dock score of −8.56 Kcal/mol.

The NBF-BMFE were docked with another protein complex β-catenin/Tcf-4, resulted isocoreopsin had a good dock score of −7.34 Kcal/mol with the formation of eight hydrogen bonds and strongly

Table 3: LD_{50} and probability of health effects of selected ligands using ACD/I-Lab 2.0

ADME-TOX Parameters	1	2	3	4	5	6	7	8	9
LD_{50} mouse[a] (mg/kg, intraperitoneal)	1100	500	290	700	820	1500	610	800	450
LD_{50} mouse[a] (mg/kg, oral)	2200	1200	880	2200	570	880	1200	1700	670
LD_{50} mouse[a] (mg/kg, intravenous)	1800	630	200	300	250	580	580	1200	350
LD_{50} mouse[a] (mg/kg, subcutaneous)	440	310	170	190	170	170	300	340	160
Probability of blood effect[b]	0.95	0.86	0.2	0.34	0.88	0.99	0.94	0.99	0.69
Probability of cardiovascular system effect[b]	0.98	0.97	0.57	0.78	0.98	0.89	0.91	0.85	0.27
Probability of gastrointestinal system effect[b]	0.97	0.71	0.36	0.48	0.98	0.99	0.71	0.99	0.45
Probability of kidney effect[b]	0.77	0.44	0.23	0.82	0.53	0.86	0.28	0.83	0.54
Probability of liver effect[b]	0.94	0.97	0.25	0.32	0.85	0.8	0.68	0.8	0.09
Probability of lung effect[b]	0.13	0.11	0.15	0.28	0.56	0.47	0.74	0.66	0.38

[a]Estimates LD_{50} value in mg/kg after intraperitoneal, oral, intravenous and subcutaneous administration to mice, [b]Estimates probability of blood, gastrointestinal system, kidney, liver and lung effect at therapeutic dose range, 1-8 represents the Bm derivatives (butrin, isobutrin, butein, butin, coreopsin, isocoreposin, monospermoside, isomonospermoside) and 9 represents quercetin, the drugs with moderate effect on reliability index (>0.5), the drugs with border line effect on reliability index (>0.3, <0.5), LD_{50}: Lethal dose$_{50}$, ADME: Absorption, distribution, metabolism, excretion, Bm: *Butea monosperma*

Table 4: Molecular docking of 4B7T protein with ligand molecules

Ligand	H-Bonds	H-Bond energy (Kcal/mol)	RMSD (Å)	Amino acids
Butrin	6	−7.58	0.18	Ser66, Asp133, Tyr134, Val135, Pro136, Glu137, Arg141, Lys183, Asn186, Asp200
Isobutrin	6	−7.27	0.16	Ser66, Lys85, Asp200, Gln185, Val135, Asp133, Cys199
Butein	6	−8.14	1.11	Lys85, Ile62, Asp200, Val135, Arg141
Butin	6	−7.93	0.02	Lys85, Asp200, Val135, Arg141, ILE62
Coreopsin	6	−7.43	1.96	Asp200, Lys85, Arg141, Thr138
Isocoreposin	6	−9.27	0.54	Ile62, Lys85, Asp133, Val135
Monospermoside	5	−7.77	1.10	Arg141, Asp200, Lys85, Ile62, Cys199,
Isomonospermoside	6	−9.13	1.72	Lys85, Asp133, Cys218, Ser203, Lys183
Quercetin	1	−8.56	0.13	Val155

RMSD: Root mean square deviation

Table 5: Molecular docking on 1JPW protein with ligand molecules

Ligand	H-bonds	H-bond energy (Kcal/mol)	RMSD (Å)	Amino acids
Butrin	6	−5.19	0.01	Ser20, Asp390, Thr393, Ser351, Asn353
Isobutrin	6	−6.26	1.36	Lys22, Asp23, Asp459, Ser20, Arg386
Butein	7	−5.43	0.01	Glu462, Asp459, Arg386, Lys22
Butin	4	−5.48	0.02	Thr393, Gln395, Asp390, Ser351
Coreopsin	8	−5.53	0.19	Glu462, Asp23, Gly25, Lys22,
Isocoreposin	8	−7.34	0.43	Glu17, Leu18, His470, Ser473, Lys435, Arg474, Asn430
Monospermoside	6	−6.41	1.87	Asn15, Asn16, Thr393, Asp390, Lys354, Ser351
Isomonospermoside	3	−4.81	0.01	Asp390, Asp16, Lys354, Ser20
Quercetin	5	−6.73	0.06	Thr393, Ala391, Gln395, Lys394

RMSD: Root mean square deviation

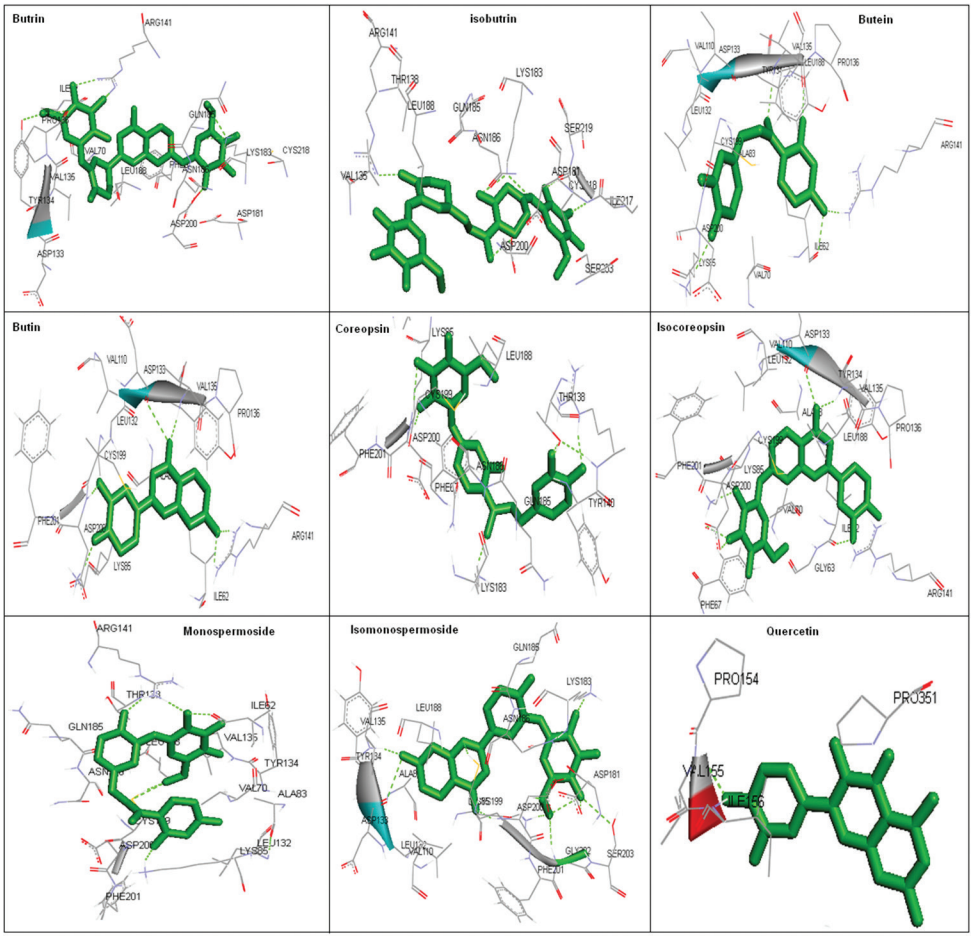

Fig. 5: Docking results of 4B7T with n-butanol fraction of *Butea monosperma* floral extracted compounds and quercetin (green dots denoted strong hydrogen bonds)

bound with the active site amino acids of Glu17, Leu18, His470, Ser473, Lys435, Arg474 and Asn430. The other compounds also evinced good binding interactions with this protein complex (Table 5). The RMSD value of butrin, butein and isomonospermoside scored 0.01Å, 0.02Å, 0.01Å respectively and displayed strong interactions with the active site cavity compared to quercetin (Fig. 6). The reference compound quercetin stated good interaction with active site protein by forming five hydrogen bonds with the dock score of −6.73 Kcal/mol. These docking results provide useful information to understand the structural characteristics of the target proteins that successfully determined the inhibitory of Wnt/β-catenin signaling pathways to control the CRC disease progression.

Cell growth inhibition property

The cytotoxic effects of NBF-BMFE and commercially available butein (Extrasyntahse, Genay, France) were determined by HCT116 cell line at time and dose dependent manner. The effectiveness of compounds were measured by half-maximal inhibitory concentration (IC_{50}) of cell death. The butein was used as a positive control and exhibited the IC_{50} values at >100 µM, but our previous report showed that the butein compound had 79% cell death at 100 µg on alpha mouse liver 12 cells [29] and the IC_{50} value of NBF-BMFE was >100 µM and the aqueous extract of BMFC revealed 50% cell death in 100 µg on human hepatoma cells [30]. The vehicle control DMSO had no inhibitory effect on the tested cell line, the statistical analysis was also showed that the significance results between butein and NBF-BMFE at time and dose-dependent manner (Fig. 7). The colorectal carcinoma cell line, HCT116, which had both mutated and wild-type β-catenin gene [31] and β-catenin mutations were detected in approximately 50% of the CRC [32]. By disrupting the β-catenin mutation in Wnt signaling pathway is a good therapeutic approach for the treatment of CRC. Our data clearly indicated that the

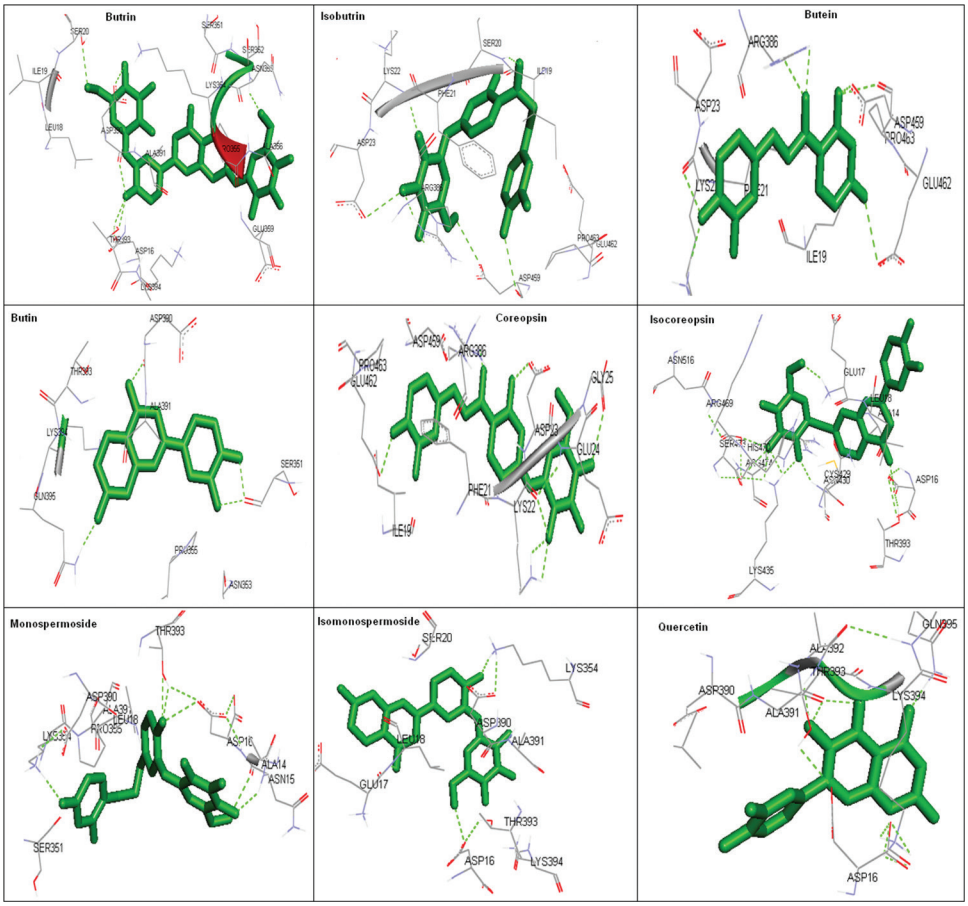

Fig. 6: Docking results of 1JPW with n-butanol fraction of *Butea monosperma* floral extracted compounds and quercetin (Green dots denoted strong hydrogen bonds)

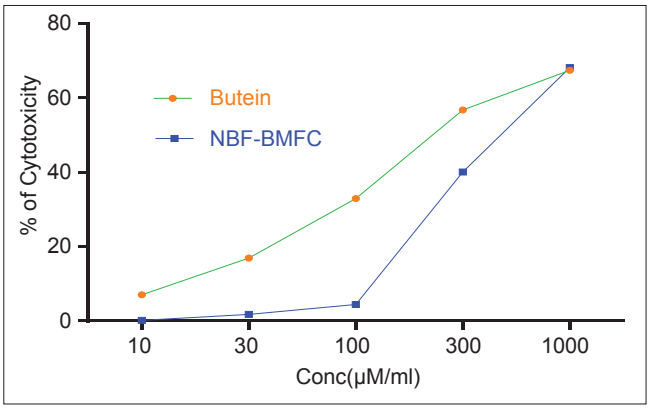

Fig. 7: *In vitro* cytotoxicity of n-butanol fraction of *Butea monosperma* floral extracts and butein in HCT-116 human colon cancer cells

butein and NBF-BMFE had effective cell growth inhibition on β-catenin expressing against HCT 116 colon cancer cells.

CONCLUSION

Pharmacophore models of X-ray crystal 3D structures of Wnt signaling proteins provide helpful information on protein-ligand interaction and further improvement on significant ligand binding affinity. While compare the binding interaction of docking studies with quercetin, the NBF-BMFE showed good hydrogen bond interaction against 4B7T and 1JPW. The pharmacophore properties of butrin and isobutrin compound does not obey the Lipinski rule of five parameters, other leading compounds were obeyed the Lipinski rule of five parameters. The ADMET prediction using admetSAR and ACD/i-lab revealed that the docked compounds were in the acceptable range whereas butrin and isobutrin require improving the permeability properties with suitable molecular modifications. A comparative analysis of LD_{50} and probability of health effects revealed the higher LD50 in oral and lower in intravenous with less toxic effect on blood, cardiovascular systems, gastrointestinal tracts, lungs, and liver. The reference compound quercetin showed good interaction with active site amino acids and better hydrogen bond energy, but less hydrogen interaction in 4B7T. The ADMET analysis of quercetin showed acceptable range but poor permeability. Also, the NBF-BMFE has significant anti-cancer activity against HCT-116 colon cancer cells. Overall our results concluded that all the NBF-BMFE had significant inhibitory effect on Wnt/β-catenin signaling pathway, in specific isocoreopsin, butein and butin showed promising agents to develop as potent drug molecules against CRC.

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