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Research Article

In silico elucidation, toxicity prediction and *in vitro* analysis of *Andrographis paniculata* on hepatocellular carcinoma[#]

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Abstract

A study was conducted to evaluate *in silico* binding affinity, toxicity prediction and *in vitro* analysis of *Andrographis paniculata* (AP) on hepatocellular carcinoma (HepG2) cell line. *In silico* studies were conducted to determine the interaction between target proteins and ligands. The study revealed satisfactory binding affinities between ligands of AP and biomarkers involved in hepatocellular carcinogenesis. The toxicity analysis of the four main active ingredients of AP showed that they were non-mutagenic, non-carcinogenic and non-teratogenic. The *in vitro* cytotoxicity study was conducted for sorafenib, andrographolide and *Andrographis paniculata* extract (APE) on HepG2 cells using 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) dye. The IC_{50} values of andrographolide and APE were found to be comparable with sorafenib. The *in silico* elucidation, toxicity prediction and *in vitro* analysis of AP and its active ingredients revealed drug-likeness properties against hepatocellular carcinoma.

Keywords: *Andrographis paniculata*, Cytotoxicity, HepG2, Three dimensional, Two dimensional

Highlights

- *In silico* studies revealed satisfactory binding affinities between ligand of AP and biomarkers involved in hepatocellular carcinogenesis.
- The biomarkers identified in this study were α-fetoprotein, arginase 1, glypican 1, Bcl-2, HDAC-1, caspase 9, TNF-α, NF-κB and CEA.
- The docking interactions between ligand and target proteins were analysed as binding affinity, interacting amino acid residues, number of H-bond interactions, bond length of various ligands and protein interactions.
- Toxicity analysis of the active ingredients of AP showed that they were non-mutagenic, non-carcinogenic and non-teratogenic.
- The *in vitro* cytotoxic (IC_{50}) potential of andrographolide and APE were found to be comparable with sorafenib.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the 6th most common cancer and 3rd leading cause of cancer-related mortality worldwide in 2021 (Konyn *et al.*, 2021). Globally, around 6.3 lakh hepatocellular carcinoma patients are diagnosed annually, and they are mostly associated with poor prognosis (De Minicis *et al.*, 2013). Unfortunately, the incidence nearly equates to the death rate, and more than 80% of the patients are affected by underlying diseases. The recurrence rate of this cancer might reach up to 50% within 2 years (Li and Martin, 2011). Hepatocellular carcinoma is routinely treated by liver transplantation, surgical resection and chemotherapy (Ting *et al.*, 2015). The palliative therapies for HCC included trans arterial chemo-embolization, systemic chemotherapy, interferon and hormonal therapies (Li and Martin, 2011). Dismal results of both curative and palliative therapies against HCC attracted the development of novel well-tolerated treatments to improve the survival of the patients. At present, sorafenib is the only effective drug available to treat advanced stages of HCC, but it is associated with high cost and substantial side effects (Ting *et al.*, 2015).

Herbal medicine is emerging as an intriguing choice

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due to its multi-level, multi-target and coordinated intervention against HCC (Ting *et al.*, 2015). *Andrographis paniculata* is the most popular herbal plant used as a chief constituent of at least 26 ayurvedic formulations in Indian pharmacopoeia (Engels and Brinckmann, 2021). It is acting against various types of cancers by exhibiting cytotoxicity, cell cycle arrest, apoptosis, immunostimulant, anti-inflammatory, anti-angiogenic and chemo-protective potentials (Varma *et al.*, 2011). Andrographolide is the major constituent extracted from leaves of AP used to treat various disorders including cancer with more efficacy than plant (Okhuarobo *et al.*, 2014). The discovery of novel drugs against HCC-related medical interventions is lacking. Hence, the present study was undertaken to evaluate the *in silico* binding affinity, toxicity prediction and *in vitro* anti-cancer analysis of AP against hepatocellular carcinoma.

MATERIALS AND METHODS

***In silico* study:** The *in silico* studies were conducted to determine the interaction between the target proteins and ligand. The targets identified for the HCC pathway of this study were α-fetoprotein, arginase 1, glypican 1, Bcl-2, HDAC-1, caspase 9, TNF-α, NF-κB, CEA, and the ligand was andrographolide. The docking interaction between ligand and target proteins was done using Auto Dock Vina 1.1.2 software.

Three-dimensional structures of human and rat α-fetoprotein, glypican 1, Bcl-2, HDAC-1, caspase 9, TNF-α and NF-κB along with the structures of arginase 1 and CEA were retrieved from RCSB Protein Data Bank and UniProt database, respectively. The three-dimensional structure of andrographolide was downloaded from the Chemical Database "PubChem". The ligand was made flexible using Auto Dock Tools-1.5.7 to facilitate flexible docking by cleaning the geometry.

Molecular docking: The docking interactions between ligand and target proteins were analysed using PyMol Visualization Tool 2.5.7. The binding affinity, interacting amino acid residues, number of H-bond interactions, bond length of various ligands and protein interactions to determine the ligand having a higher affinity towards the protein was observed. The two-dimensional diagram show option was used to view the interaction of the target proteins and ligand.

Toxicity analysis: The toxicity was analysed to find out the molecular properties, toxicity prediction, and detailed report, true and run options of the ligands using TOPKAT Discovery Studio 4.0.

***In vitro* assay:** The anti-cancer drug sorafenib was procured from M/s Granules India Ltd., Hyderabad, India. Andrographolide was procured from M/s Sigma Aldrich, Bengaluru, India. The ethanolic extract of AP was purchased from M/s Natural Remedies, Bengaluru, India. Human hepatocellular carcinoma (HepG2) cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India and maintained at 37°C in CO_2 incubator. The Eagle's Minimum Essential Medium (EMEM) was procured from M/s HiMedia, Mumbai, India. The thiazolyl blue tetrazolium bromide dye was purchased from M/s Sigma Aldrich, Chennai, India and stored in a refrigerator (2-8°C).

The *in vitro* assay was performed on HepG2 cells by using 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) dye. The cells were subcultured on EMEM supplemented with 10% FBS along with 1% antibiotic and antimycotic solution containing penicillin (100 IU/mL) and streptomycin (100 µg/mL). The cells were seeded into 96-well plates. The desired concentrations of sorafenib (0.10, 0.78, 3.33, 12.50, 50.00, 156.25, 312.50, 1250.00 µM), andrographolide (0.39, 1.56, 6.25, 25.00, 78.13, 312.50, 625.00, 2500.00 µM) and APE (10.34, 30.12, 100.90, 300.15, 600.30, 1000.00, 3000.00, 6000.00 µg) were added and incubated for 48 h. The MTT (5 mg/dL) dye was added to incubate for 4 h, and then the absorbance was read on a BIORAD iMax Microtitre Plate Reader at a wavelength of 550 nm. The cytotoxicity (IC_{50}) was determined based on the OD value obtained by using GraphPad Prism 5.02.

RESULTS

***In silico* study:** Andrographolide revealed satisfactory binding affinities with many proteins. The binding affinity, number of H-bond interactions, interacting amino acid residues and bond length of various ligands and proteins, interactions between andrographolide and biomarkers associated with hepatocellular carcinoma are presented in Table 1. The docking interaction for specific targets and andrographolide is presented in two-dimensional (Fig. 1) and three-dimensional (Fig. 2) views.

Toxicity: The toxicity properties of four active ligands, namely andrographolide, neoandrographolide, andrograpanin and didehydroandrographolide, are presented in Table 2. The toxicity prediction for these ingredients revealed non-mutagenic, non-carcinogenic and non-teratogenic potentials. Regarding the handler's safety, the active ingredients were non-toxic for skin sensitization, moderate skin irritancy, non-toxic for ocular irritancy and had biodegradable property.

Table 1. The docking interactions between active ligands and various proteins

Sl. No.	Target name	Species	PDB / UniProt ID	Affinity (Kcal/ mol)	No. of H-Bond	Interacting Residues	Bond length (Å)
1	AFP	Human	3MRK / P02771	-8.4	1	ARG A:48	2.75
		Rat	P02773	-7.8	3	GLY A:477; ASN A:321; ASN A:321	2.37; 2.49; 2.70
2	Arginase 1	Rat	1RLA / P07824	-7.4	0	-	-
3	Glypican1	Human	4ACR / P35052	-8.6	3	GLN C:225; ARG C:221; GLY C:154	2.34; 3.42; 3.73
4	Bcl 2	Human	2W3L / P10415	-7.4	2	GLU A:119; ARG B:66	2.24; 2.87
		Rat	P49950	-7.8	2	SER A:50; HIS A:183	2.61; 3.20
5	HDAC 1	Human	4BKX / Q13547	-7.8	2	ARG B:l36; TYR B:48	5.85; 2.18
		Rat	Q4QQW4	-7.0	4	HIS B:57; HIS B:57; GLN B:128; LYS B:123	2.21; 2.72; 2.77; 2.59
6	Caspase 9	Human	2AR9 / P55211	-8.6	4	GLN: D285; PRO :357; THR: D181; ARG: D178	2.08; 2.77; 2.42; 2.06
		Rat	Q9JHK1	-7.9	3	SER A:213; SER A:213; SER A:295	3.18; 2.76; 2.27
7	TNFα	Human	5MU8 / P01375	-7.7	3	ASN: d46; GLU d:135; ARG :138	2.16; 5.03; 5.94
		Rat	P16599	-8.8	4	LYS A:190; LYS A:190; CYS B:179; SER A:177	2.52; 2.13; 2.76; 2.77
8	NF-kB	Rat	Q63369	-6.5	1	HIS A:193	2.66
		Mouse	1NFK / P25799	-8.7	5	SER B:240; GLY B:52; ARG B:54; LYS B:241; ARG A:305	2.35; 2.54; 2.08; 2.36; 2.20
9	CEA	Rat	P16573	-6.7	1	ASN A:181	1.99

Table 2. TOPKAT analysis of active compounds of *Andrographis paniculata*

Sl. No.	Compound name	NTP prediction	WOE	Ames	DTP	Skin irritancy	Skin sensitization	Ocular irritancy	Biodegradability
1	Andrographolide	Non-Carcinogen	Carcinogen	Non-Mutagen	Non-Toxic	Moderate	Weak-sensitizer	None	Degradable
2	Neoandrographolide	Non-Carcinogen	Non-Carcinogen	Non-Mutagen	Toxic	Moderate	None	None	Degradable
3	Andrograpanin	Non-Carcinogen	Carcinogen	Non-Mutagen	Non-Toxic	Moderate	Weak-sensitizer	None	Degradable
4	Didehydroandro-grapholide	Non-Carcinogen	Carcinogen	Non-Mutagen	Toxic	Moderate	Weak-sensitizer	None	Degradable

***In vitro* study:** The HepG2 cells were incubated with various concentrations of sorafenib, andrographolide and APE. The proliferation inhibition potentials of these ingredients were proportional to their cytotoxicity. The cytotoxicity was severe in sorafenib, marked in andrographolide and moderate in APE treatments (Fig. 3). The IC_{50} values for sorafenib, andrographolide and APE are plotted in graphs as 18.21 µM, 33.79 µM and 353.00 µg/mL, respectively (Fig. 4).

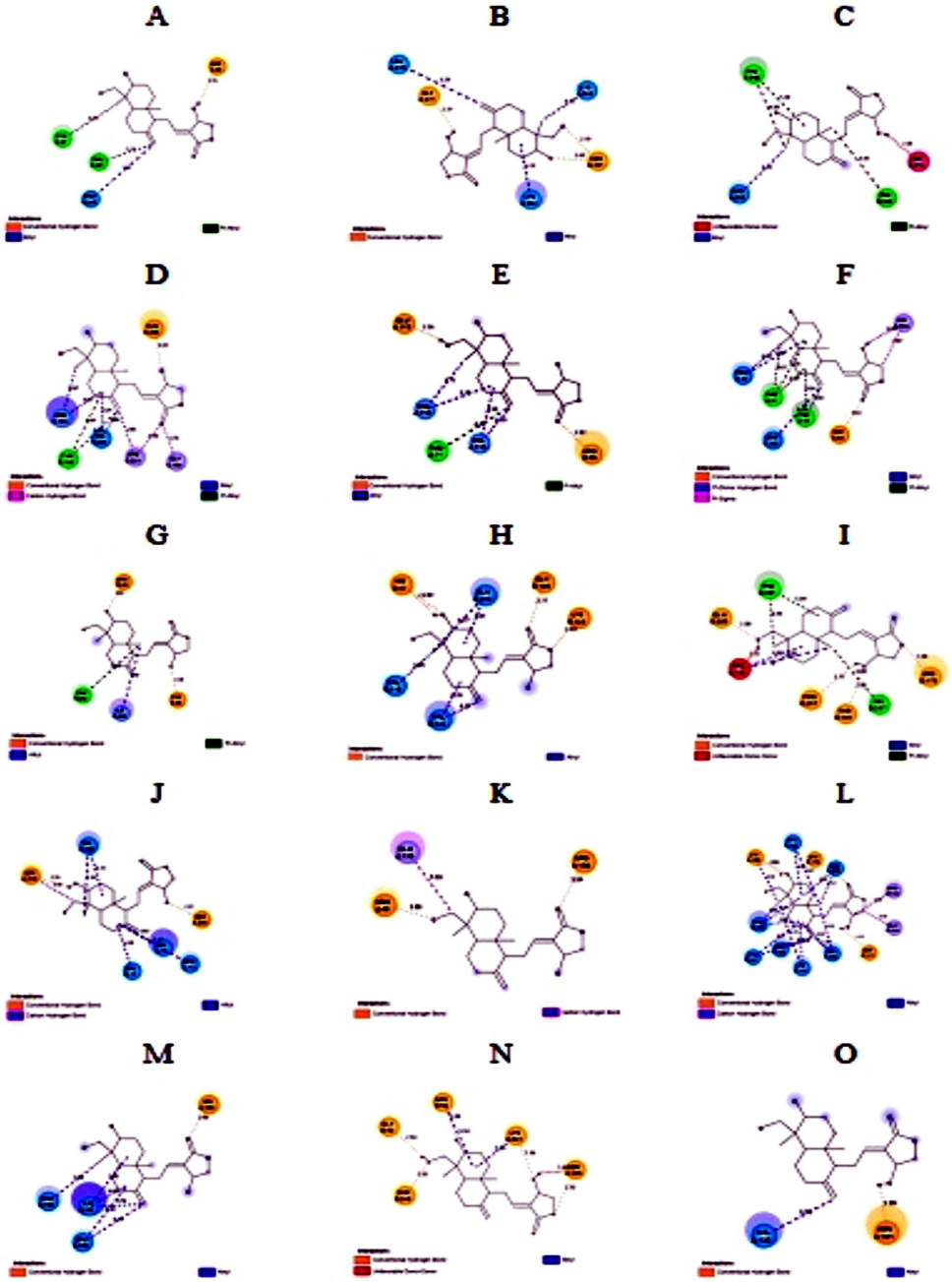

Fig. 1. Two-dimensional view of andrographolide interaction with (A) AFP in human, (B) AFP in rat, (C) arginase 1 in rat, (D) glypican 1 in human, (E) Bcl-2 in human, (F) Bcl-2 in rat, (G) HDAC-1 in human, (H) HDAC-1 in rat, (I) caspase 9 in human, (J) caspase 9 in rat, (K) TNFα in human, (L) TNFα in rat, (M) NF-κB in rat, (N) NF-κB in mouse and (O) CEA in rat

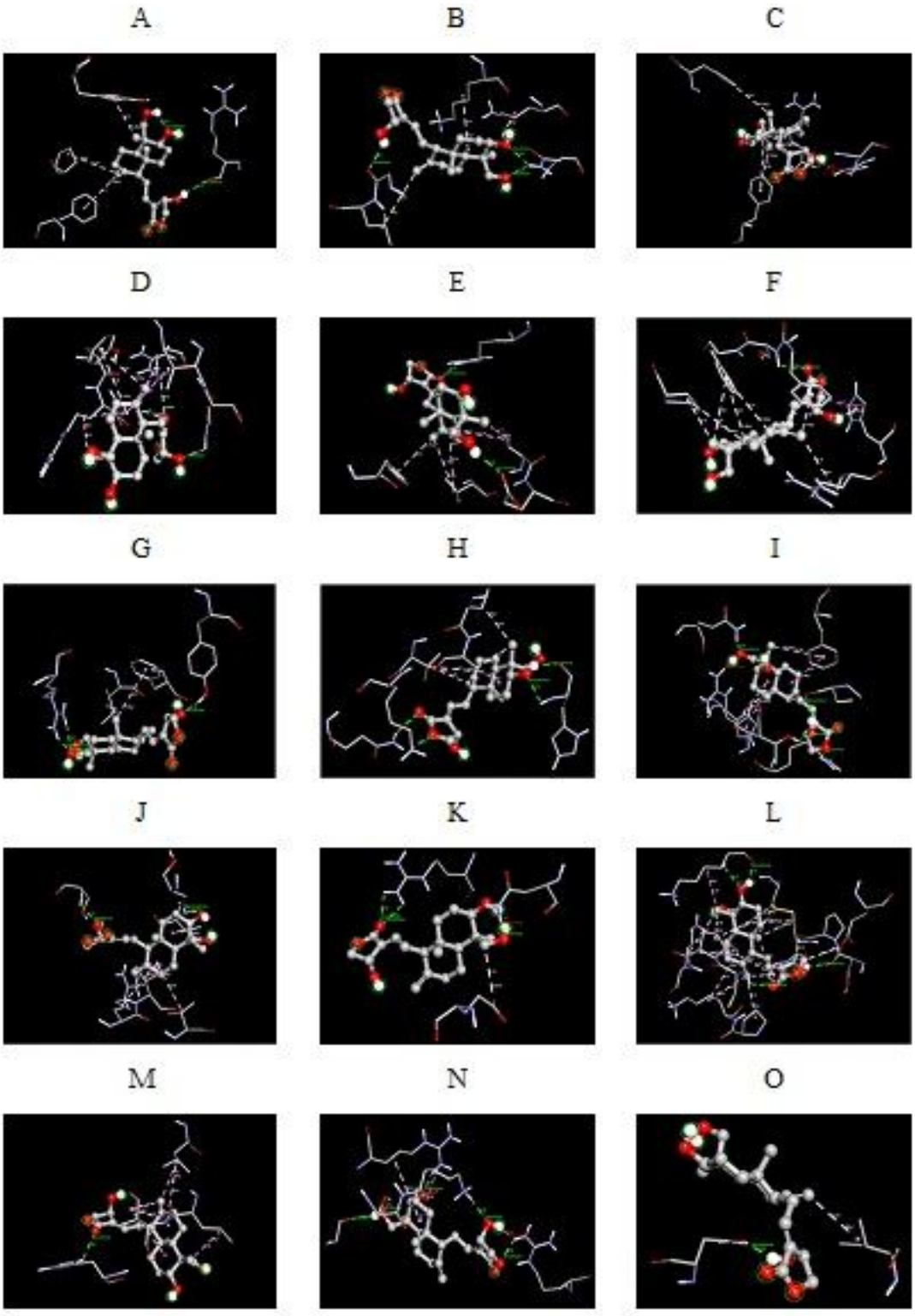

Fig. 2. Three-dimensional view of andrographolide interaction with (A) AFP in human, (B) AFP in rat, (C) arginase 1 in rat, (D) glypican 1 in human, (E) Bcl-2 in human, (F) Bcl-2 in rat, (G) HDAC-1 in human, (H) HDAC-1 in rat, (I) caspase 9 in human, (J) caspase 9 in rat, (K) TNFα in human, (L) TNFα in rat, (M) NF-κB in rat, (N) NF-κB in mouse and (O) CEA in rat

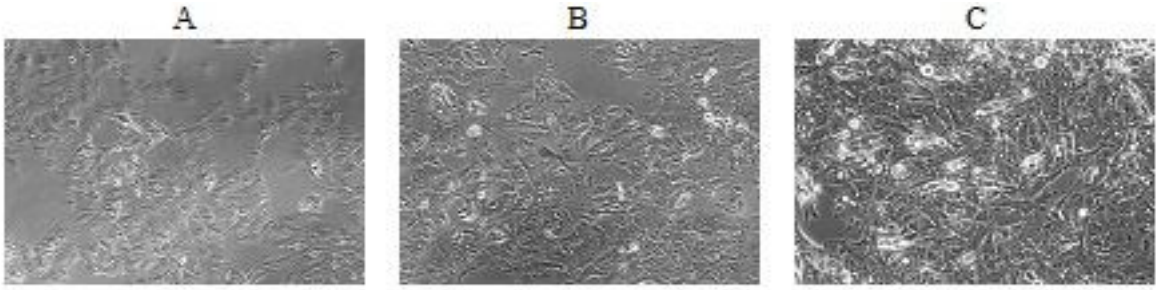

Fig. 3. (A) The HepG2 cells showing severe cytotoxicity at 12.5 µM of sorafenib in EMEM medium. x400. (B) The HepG2 cells showing marked cytotoxicity at 25.0 µM of andrographolide in EMEM medium. x400. (C) The HepG2 cells showing moderate cytotoxicity at 100 µg/mL of *Andrographis paniculata* extract in EMEM medium. x400

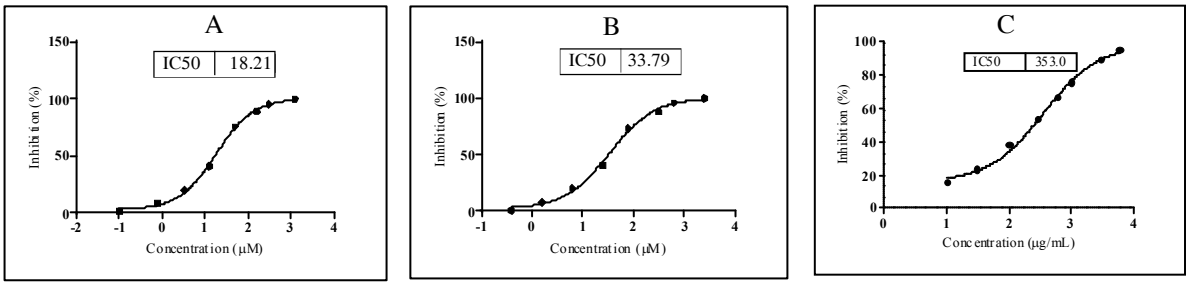

Fig. 4. The IC_{50} value of (A) sorafenib, (B) andrographolide and (C) *Andrographis paniculata* extract on HepG2 cell line

DISCUSSION

***In silico* study:** High binding affinities between andrographolide and human AFP, human glypican 1, human caspase 9, rat TNF-α and mouse NF-κB were observed in this study is agreed to the reported binding affinity of -8.27 Kcal/mol for NF-κB with andrographolide (Raghavan *et al.,* 2012). The binding affinity of -8.28 Kcal/mol between caspase 9 and andrographolide in this study is similar to the findings of previous workers (Wanandi *et al.*, 2020). The binding affinity of -8.8 Kcal/mol observed between TNF-α and andrographolide in this study agreed with the previous reports, which stated the affinity of -7.52 Kcal/mol (Rehan *et al.*, 2021).

The binding affinities for arginase 1, Bcl-2, HDAC-1 and CEA with andrographolide of this study are in accordance with the earlier reports, which revealed the affinity of -4.67 Kcal/mol between Bcl-2 and andrographolide (Kushmitha *et al.*, 2018). The higher binding affinity of these HCC pathway biomarkers with andrographolide indicated their role in apoptosis, inflammation, pain, immunomodulation and development of hepatocellular carcinoma.

Toxicity: The active components of AP revealed non-toxicity of this study supported their drug-likeness properties against various diseases including cancers, and these observations were also described by the previous workers (Chandramohan *et al.*, 2015; Zhong *et al.*, 2022).

***In vitro* study:** The IC_{50} value observed for the sorafenib of this study is in agreement with earlier workers who reported values of 6 µM and 12 µM (Wei *et al.*, 2015; Cervello *et al.*, 2012). The cellular cytotoxicity attributed to apoptosis and DNA fragmentation is concomitant with previous reports, which stated cytotoxicity at 10 µM of sorafenib in the HepG2 cell line (El-Ashmawy *et al.*, 2017).

The IC_{50} value observed for the andrographolide of this study is in agreement with previous workers who reported the value of 40.2 µM, which attributed to altered redox potential leading to cell cycle arrest (Li *et al.*, 2007). The IC_{50} value of this study is similar to the reported value of 11.3 µg/mL of andrographolide on human liver cancer cell line Huh-7 (Chen *et al.*, 2012).

The IC_{50} value observed for the APE of this study is in agreement with previous workers who observed 40% cytotoxicity at 250 µg/mL of APE (Therasa *et al.*, 2020; Kumar *et al.*, 2021). The IC_{50} value of this study contradicted the earlier reports, which stated 53.87% inhibition at 1 µg of ethanolic extract of AP (Mathankumar and Gani, 2018).

The *in silico* prediction showed satisfactory binding affinities between andrographolide and biomarkers of the HCC pathway. The toxicity prediction for the active ingredients of AP showed non-toxicity properties. *In vitro* study revealed the IC_{50} values of APE and andrographolide were found to be comparable with sorafenib on HepG2

cells. These findings indicated the drugable properties of AP against liver tumor without toxicity. However, further *in vivo* studies are required to establish APE as an anti-cancer drug against hepatocellular carcinoma.

Conflict of interest: The authors declare that there is no conflict of interest.

Author's contributions: MRD: Research work and preparation of the manuscript; RM: Overall responsible for planning and execution of the research work and preparation of the manuscript; GAB: Correction of the manuscript for the technical integrity; PS: Provided technical assistance for the interpretation of the data.

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