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Research Article

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Turmeric and Black Cumin essential oil alone and in combination: *In vitro* and *in silico* molecular docking studies against inflammation and cancer

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ABSTRACT

Hepatocellular carcinoma (HCC) is the fifth most common disease globally. Obesity, long-term hepatitis virus infection, and other factors are among those that lead to the development of HCC, which has a poor survival rate. In Asia and Africa, essential oil-bearing plants like black Cumin and Turmeric are used for various traditional medical purposes. To date, no sufficient information has been provided on the biological properties of the herbal Turmeric-black Cumin combination. To compare the essential oils (EOs) of the Turmeric-black Cumin combined spice with those of the individual ingredients, we looked at *in silico* molecular docking studies, *in vitro* anti-inflammatory and anti-cancer characteristics, and the formulation's potential phytocompounds. The herbal materials were hydro-distilled on a Clevenger apparatus for their essential oils and further characterized using the GC-MS method. *In vitro* anti-inflammatory activity of the oils was based on the egg albumin denaturation (EAD) assay, while anti-cancer activity was based on the MTT cell viability assay against the HepG2 (human liver cancer cell line) cells. Lastly, the major compounds identified in the extract were molecular docked against the IL-6, TNF-α, and IGF-1R proteins. GC-MS analysis revealed sixty-three major compounds, with ar-Turmerone showing the highest composition in the combined oil (34.45%). The combined oil gave the best anti-inflammatory activity with an IC_{50} value of 51.33±2.22 µg/mL followed by individual EOs. The result showed that at 100 µg/mL, three tested oils treated RAW 264.7 normal cells exhibited more viability (≥60% growth). In contrast, the combined oil showed the best activity against the HepG2 cell line, which suggests that the combined oil extract is selectively cytotoxic to the cancer cells. The phytochemical alpha-Phellandrene and β-Turmerone showed excellent binding affinity against the three proteins in the *in-silico* study. As a result of the current research, it appears that combination oil can effectively combat both inflammation and cancer.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most frequent disease worldwide, accounting for approximately 75-85% of all primary liver cancer cases (McGlynn *et al.*, 2001; El-Serag *et al.*, 2003). In the USA, Europe, and Asia, the incidence of HCC is sharply rising. This is most likely because of the rising prevalence of toxins, obesity, liver cirrhosis, incorrect fatty acid metabolism, alcoholic liver disease, and chronic hepatitis virus infection (Bray *et al.*, 2018; Galle *et al.*, 2018). Owing to the rise in hepatitis virus (types A, B, C, D, and E) infection, primarily B and C types, there is a burden on global public health and a half-million-yearly death toll occurs from hepatocellular carcinoma (HCC) (WHO, 2017). Evidence has shown that oxidative stress and inflammation are two interrelated processes that contribute to the initiation and spread of cancer (Catalani *et al.*, 2017). Around the world, a wide range of therapeutic approaches is available for HCC treatment, including local tumor annihilation, resection, transplantation, ablation, molecular targeted therapy, chemoembolization, systemic radiation, and chemotherapy, which shows overall poor survival rates (Llovet *et al.*, 2003; Yang *et al.*, 2019). Due to this reason, plant-derived essential oils are now extensively used as a biological therapeutic approach and are also a topic of extensive research (Langseth & Andersen, 1999). Turmeric, or *Curcuma longa* L. (Family: Zingiberaceae), and Black cumin,

or black seeds, *Nigella sativa* L. (Family: Ranunculaceae), are also known to be essential oil-bearing plants. In Asian ethnomedicines, the herbal infusions from these plants and the essential oils are used as natural remedies for respiratory ailments, hypertension, diabetes, arthritis, wounds, cancer, fever, headaches, and memory loss, whereas, herbal infusions from these plants and their essential oils are used to treat digestive, circulatory, respiratory, and reproductive ailments and ailments related to dermatology in African ethnomedicines (Khan *et al.*, 2011; El-Bahr *et al.*, 2014). Turmeric and black cumin alone are claimed to have a variety of ethnomedicinal uses, however, the chemical makeup and ethnomedicinal uses of the herbal turmeric-black cumin combination are currently poorly understood. This prompted us to extract essential oils from the herbal formulation of turmeric-black cumin because no prior research has thoroughly examined the anti-inflammatory and anti-cancer properties. In this work, we examined the *in silico* molecular docking studies, *in vitro* anti-inflammatory and anti-cancer properties of turmeric-black cumin herbal formulation's essential oils as well as compared it with the turmeric and black cumin essential oils separately.

MATERIALS AND METHODS

Collection of Herbal Raw Materials

The powdered rhizomes of Turmeric (*Curcuma longa* L.) and the seeds of black Cumin (*Nigella sativa* L.) were purchased from the traditional herbal market in Ile-Ife, Osun State, Nigeria (latitude 7°33′00″N and longitude 4°33′00″E) under the registered name SpicYem RC: 875523 and RC: 875523 respectively. The two plants were confirmed with the plant list catalogue (http://www.theplantlist.org) as kew-235249 and kew-2381679, respectively.

Extraction

The extraction of essential oils (EOs) from Turmeric rhizome powder and the black Cumin seed separately (200 g each) and as an equal combination (100 g each), was carried out on a Clevenger apparatus by hydro-distillation method (Nodola *et al.*, 2024). The extraction solvent was 1000 mL of distilled water. The extraction temperature was set at 70-100 °C for 4 hrs., while the EOs were collected in *n*-hexane layered on distilled water. The oils were dried over anhydrous sodium sulfate. Physicochemical properties of the oils such as the colour, odour, and percentage yield, were recorded. They were stored in amber vials and refrigerated until needed for further analysis.

GC-MS Analysis

Separation of the EOs was performed on a gas chromatograph (6890N, Agilent technologies network) coupled to Agilent technology inert XL EI/CI Mass Selective Detector (MSD) (5975B, Agilent Technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics PAL autosampler. A 50 µL of the sample was diluted with 950 µL of acetone before injection on the GC-MS. A 1 µL of the sample was injected on the GC operated at a 50:1 split ratio. Separation of the EOs was performed on a 5% phenyl dimethylpolysiloxane fused-silica non-polar ZB-5Ms (30 m, 0.25 mm ID, 0.25 µm film thickness) capillary column Model Number: Zebron 7HG-G010-41. Helium was used as the carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 240 °C. The oven temperature was programmed as follows: 50 °C for 6 minutes and ramped at a rate of 5 °C/min until 320 °C and held for 2 minutes. The MSD was operated in a full scan mode, and the source and quad temperatures were maintained at 230 °C and 150 °C, respectively. The transfer line temperature was maintained at 250°C. The mass spectrometer was operated under electron impact (EI) mode at an ionization energy of 70eV, scanning from 35 to 650 m/z. The NIST 14 was used to identify the compounds.

In vitro Anti-inflammatory Study

The *in vitro* anti-inflammatory properties of essential oils of Turmeric, Black-Cumin, and Turmeric-Black Cumin herbal mixture were determined by their inhibitory effect on protein denaturation, using the egg albumin denaturation assay (Oriola *et al.*, 2023) with slight modification. A 0.2 mL of albumin from a fresh chicken egg, 2.8 mL of phosphate buffer saline at pH 6.4, and 2 mL of Turmeric, Black-Cumin, and Turmeric-Black Cumin herbal mixture essential oils at 12.5, 25, 50, 100, and 200 µg/mL concentrations, were mixed in three replicates. The reaction mixture was incubated at 37 °C for 15 minutes away from direct light. Then, it was boiled at 70 °C for 5 minutes in a thermostatic water bath. The resulting mixture was allowed to cool before the absorbance was measured at 655 nm wavelength. Diclofenac was used as the reference anti-inflammatory drug. The percentage inhibitory effect of essential oils on egg albumin denaturation (EAD) was calculated according to the following equation 1:

$$\%Inhibition of EAD = \frac{ABScontrol - ABSsample}{ABScontrol} x100 \quad (1)$$

Where, *EAD* = egg albumin denaturation, *ABS* = absorbance

Cytotoxicity Study

Cell culture

The South African company Highveld Biologicals (Pty) Ltd., located in Lyndhurst, provided the RAW264.7 and HepG2 cell lines. The cells were maintained using Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F12). 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, 100 units/mL penicillin, 0.14% sodium bicarbonate, and 0.1 mM sodium pyruvate were added as supplements. For a duration of 24 hours, cells were cultured on 35 mm petri dishes at 37 °C, 5% CO_2, and 95% humidity in a CO_2 incubator.

MTT assay

A standard MTT test was used to assess cell viability, following Denizot & Lang's (1986) instructions. The effects of different

doses of essential oils (Turmeric, Black-Cumin, and Turmeric-Black Cumin herbal mixture) on the inhibition of cancer cell proliferation were examined using the RAW264.7 normal cell line and the HepG2 human liver cancer cell line. The cells were grown to confluence in a DMEM complete medium that was enhanced with 10% FBS and 1% penicillin-streptomycin solution. The medium was kept at 37 °C in an incubator with 5% CO_2 humidity and a humidified environment. Trypsinized and counted exponentially developing cultured cells were sown at a density of 2×10^4 cells/well in a 96-well plate. The cells were treated with escalating doses of CT extract (10, 25, 50, 75, 100 µg/mL) for 48 hours following a 24-hour period of adherence. Following the above-mentioned conditions of incubation, 10 µL of MTT solution (5 mg/mL) was applied to each well, and the wells were left in the dark for three hours. After the medium was carefully removed, 50 µL of isopropanol was used to dissolve the formazan that had developed in the wells, and the plates were left on a plate shaker for five minutes. The absorbance was determined at 595 nm with an iMarkTM Microplate Absorbance Reader (Bio-Rad, USA). Every experiment was run via four duplicates.

In silico Molecular Docking

Preparation and refinement of the protein and ligand structures

The PDB structures of IL-6 (PDB ID 1ALU; 1.90 Å resolution) (Somers *et al.*, 1997), TNF-α (PDB ID 2AZ5; 2.10 Å resolution) (He *et al.*, 2005) and IGF-1R (PDB ID 3D94; 2.30 Å resolution) (Wu *et al.*, 2008) were retrieved from the Protein Data Bank (http://www.rcsb.org). The associated inhibitor and water molecules were removed from the appropriate protein structures prior to analysis. To get ready for docking in AutoDockTools, polar hydrogen atoms, and Kollman charges were added to the protein structures. The concerned phytocompounds were downloaded from the NCBI PubChem (https://pubchem.ncbi.nlm.nih.gov/). The Open Babel Server was then used to transform the downloaded.sdf structures into.pdb structures (O'Boyle *et al.*, 2011). The ligand structures underwent energy minimization using the Gromos 96 force field after the PRODRG server was used to optimize their energy (Schüttelkopf & van Aalten, 2004).

Molecular docking

The ligands of interest were molecularly docked at the active binding sites of the relevant proteins utilizing a stiff protein receptor and a flexible ligand docking methodology through the use of a grid-based molecular docking technology (Kar *et al.*, 2021, 2022a, b). A grid box involving the active site residues of the IL-6 and TNF-α protein was created, with center_x=8.57, center_y=-19.55, center_z=24.24, size_x=40, size_y=50, and size_z=40, for TNF-α x=-19.4, center_y=74.7, center_z=33.8, size_x=19.1, size_y=18.2, and size_z=18.5. Similarly, a grid box covering the active binding pocket of IGF-1R was employed, with center_x=24.7, center_y=17.9, center_z=-5.7, size_x=16.1, size_y=21.9, and size_z=24.4. After Autodock Vina finished the molecular docking procedure, the docked complexes were visualized using the Discovery Studio visualization tool (Kar *et al.*, 2021, 2022a, b).

Statistical Analysis

To compare the activity of CT extract and references, Student's t-Test integrated with the KyPlot program (version 5.0) was used for statistical analysis.

RESULTS AND DISCUSSION

Physicochemical and Chemical Characterization of Essential Oils

The International Organization for Standardization (ISO) demands the use of physicochemical and chemical analyses for the standardization and characterization of essential and non-essential oil substances, to ensure that products are safe, reliable, and of high quality (Lal *et al.*, 2021; ISO, 2024); hence, the ISO standards were adhered to in this study.

Based on sensory assessment (Table 1), the three afforded oils were generally earthy (herbaceous) and mildly spicy, indicating their aroma attribute as essential oil-bearing plants. Turmeric gave the highest oil yield after 4 hrs of hydro-distillation with 3.05% yield, while black Cumin was the lowest in oil yield with 0.33%. The observed low EO yield of black Cumin agrees with the report of Hajhashemi *et al.* (2004) in which low (0.4%) oil yield was obtained from fresh black Cumin cultivated in Iran. Furthermore, the black Cumin oil from this study appeared colourless, which is unlike the yellowish coloration reported for the Iranian oil (Hajhashemi *et al.*, 2004). Perhaps the disparity may be attributed to variations in the processing of the herbal raw materials and the geographical origin (Zgheib *et al.*, 2020; Hubert *et al.*, 2023).

The result in Table 2 shows the essential oil composition of Turmeric and black Cumin when hydro-distilled individually and in combination. A total of sixty-three (63) compounds were identified in the three oils. The EO compounds identified in Turmeric are thirteen (13) in number, forty (40) compounds for black Cumin oil, and twenty-five (25) compounds present in the Turmeric-black Cumin combined spice. Aromatic (ar)-Turmerone (54.88%) from Turmeric showed the highest composition among the characterized compounds and across the three oil samples. In the Turmeric oil alone, ar-Turmerone

Table 1: Physicochemical properties of essential oil from Turmeric and black Cumin alone and in combination

Essential oil	Colour	Odour	% yield
Turmeric	Bright yellow	Earthy, mildly aromatic or spicy and pungent	3.05
Black Cumin	Colourless	Mildly spicy, earthy, woody, nutty, roasty and smoky	0.33
Turmeric-black Cumin combine	Yellow	Earthy, mildly aromatic, woody and smoky	2.85

Table 2: Essential oil composition of Turmeric (TU) and Black Cumin (BC) alone and combined

S. No.	Compound	*RT*	*m/z*	% composition		
				TU	BC	TU-BC Combined
1.	Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	8.56	136.23		0.40	
2.	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-, or β-Pinene	10.49	136.23		0.12	
3.	Furan, 2-pentyl-	11.18	138.21		0.10	
4.	α-Phellandrene	11.60	136.23			0.18
5.	o-Cymene	12.36	134.22	1.13		
6.	p-Cymene	12.38	134.22		8.38	
7.	D-Limonene	12.51	136.23		0.13	
8.	Eucalyptol	12.59	154.25		0.13	
9.	γ-Terpinene	13.63	136.23		0.22	
10.	Trans-4-methoxy thujane	15.68	168.28		0.99	
11.	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-, or (-)-4-Terpineol	17.58	154.25		2.26	
12.	Benzenemethanol, alpha, alpha, 4-trimethyl-, or 8-Hydroxy-p-cymene	17.81	150.22		0.27	
13.	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-, or cis-Geraniol	19.14	154.25		0.13	
14.	Benzaldehyde, 4-(1-methylethyl)-, or Cuminal	19.45	148.20		0.10	
15.	D-Carvone	19.57	150.22		0.45	
16.	Thymoquinone	19.75	164.20		0.17	
17.	2-Caren-4-ol	19.83	152.23		0.02	
18.	Anethole	20.79	148.20		0.28	
19.	3-Methyl-4-isopropylphenol, or p-Thymol	21.27	150.22		1.27	
20.	2-Pentanone, 4-methyl-4-phenyl-, or Vetikon	23.36	176.25	0.76	0.17	
21.	Cyclopropanemethanol, 2-methyl-2-(4-methyl-3-pentenyl)-	25.09	168.28		0.24	
22.	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-, or α-Curcumene	26.02	202.34			1.37
23.	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [S-(R*, S*)]-, or (-)-Zingiberene	26.33	204.35	0.50	1.04	2.33
24.	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*, S*)]-, or β-Sesquiphellanderene	27.02	204.35	1.69	1.00	
25.	(*Z, Z*)-α-Farnesene	27.12	204.35		0.10	
26.	3-((1S,5S,6R)-2,6-Dimethylbicyclo[3.1.1]hept-2-en-6-yl) propanal	27.12	178.27			0.19
27.	(1R,4R,5S)-1,8-Dimethyl-4-(prop-1-en-2-yl) spiro[4.5]dec-7-ene, or β-Acoradiene	27.74	204.35			0.26
28.	2-Cyclohexen-1-one, 3,4,4-trimethyl-	27.86	138.21		0.13	0.15
29.	(*Z*)-γ-Atlantone	28.29	218.34			0.44
30.	Benzene, 1-methyl-4-(1-methylpropyl)-	28.34	148.25		0.65	
31.	7-epi-cis-Sesquisabinene hydrate	28.59	222.37		0.27	
32.	(*E*)-γ-Atlantone	28.68	218.34			0.27
33.	Benzene, 1-(1,5-dimethylhexyl)-4-methyl-, or isodihydro-ar-curcumene	28.92	204.35	2.46		2.29
34.	Cyclopentane, 1,3-bis (methylene)-	29.05	94.15		0.63	
35.	1,3-Cycloheptadiene	29.06	94.15			1.00
36.	(1R,4R)-1-methyl-4-(6-Methylhept-5-en-2-yl) cyclohex-2-enol, or Zingiberenol	29.15	222.37		0.30	0.73
37.	1H-3a, 7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3R-(3.alpha.,3a. beta.,7.beta.,8a.alpha.)]-, or 2-epi-α-Funebrene	29.43	204.35		0.36	
38.	β-Bisabolene	29.55	204.35		0.78	0.15
39.	Benzenemethanamine, alpha, 4-dimethyl-	29.62	135.21	0.68		
40.	Cyclopropyl phenylmethanol	29.63	148.20		0.81	1.62
41.	2-Pyrazoline, 1-allyl-	29.93	110.16			0.53
42.	Benzene, 1,2,3-trimethyl-, or Hemellitol	30.18	120.19		0.29	
43.	aR-Turmerone	30.39	216.32	54.88		34.45
44.	Tumerone	30.57	216.32	15.99		7.13
45.	(4R,6S)-2-Methyl-6-((S)-4-methylenecyclohex-2-en-1-yl) hept-2-en-4-ol	30.91	220.35			0.99
46.	Naphtho[2,1-d] thiazole, 2-methyl-	31.04	199.27			0.25
47.	Curlone	31.15	218.34	17.37		
48.	2-Methyl-6-(4-methylenecyclohex-2-en-1-yl) hept-2-en-4-one, or β-Turmerone	31.24	218.33			20.80
49.	(*Z*)-alpha-Atlantone	31.41	218.33	0.53		
50.	5-Methyl-2-(6-methylhept-5-en-2-yl) phenol	31.44	218.33			0.66
51.	(S)-3-Methyl-6-((R)-6-methylhept-5-en-2-yl) cyclohex-2-enone, or (6R,7R)-Bisabolone	32.07	220.35	0.90	0.62	
52.	Benzene, 1,2,3,5-tetramethyl-	32.54	134.22		0.73	
53.	(E)-Atlantone	32.68	218.34	2.46		2.19
54.	Cumenyl angelate, o-	32.82	218.29			0.22
55.	2-Butenoic acid, 3-methyl-, methyl ester, or Methyl β-methylcrotonate	33.47	114.14		0.38	0.94
56.	1-Isopropenyl-3,3-dimethyl-5-(3-methyl-1-oxo-2-butenyl) cyclopentane	34.50	220.35			0.15
57.	Hexadecanoic acid, methyl ester, or Methyl palmitate	35.69	270.45		0.13	
58.	n-Hexadecanoic acid or Palmitic acid	36.85	256.42	0.63	39.21	
59.	3-Methyl-2-butenoic acid, 2,7-dimethyloct-7-en-5-yn-4-yl ester	36.93	234.33			0.54
60.	9,12-Octadecadienoic acid (Z, Z)-, methyl ester or Cis-9, trans-12 methyl linoleate	38.88	294.47		0.39	
61.	11-Octadecenoic acid, methyl ester or Methyl trans-vaccenate	38.99	296.49		0.52	
62.	9-Octadecenoic acid, (E)- or trans-Oleic acid	39.83	282.46		7.80	
63.	Octadecanoic acid or Stearic acid	40.17	284.48		1.43	
	Total			99.98	73.40	79.83

was followed by Turmerone (15.99%), Curlone (17.37%), Isodihydro-ar-curcumin (2.46%), and (*E*)-Atlantone (2.46%) in terms of the major compounds detected. In the black Cumin, the major compounds were Palmitic acid (39.21%), p-cymene (8.38%), trans-oleic acid (7.80%), and (-)-4-Terpineol (2.26%). Zingiberene was present in the three analyzed oils, affording a considerably higher (2.33%) composition in the combined spice. The most dominant compound in the combined spice was ar-Turmerone (34.45%), which was contributed by Turmeric. Additionally, Zingiberene and Methyl β-methylcrotonate from black Cumin notably contributed to the EO composition of the combined spice, while β-Turmerone (20.80%) and α-Curcumene (1.37%) were among the newly found compounds in the combined spice. This addition may be attributed to a possible ring (cis-to-trans) rearrangement of the conjugated bond of Turmerone, which can occur under certain reaction conditions of temperature, pH, and enzymes, leading to the transformation of Turmerone from its α-isoform to β-Turmerone and vice versa (Fujiwara *et al.*, 2011). The highest level of ar-Turmerone in the EO from Turmeric-black Cumin equal combination, as revealed by this study, marks the first report for major detection of ar-Turmerone in a combined spice. However, the high compositional levels of ar-, α-, and β-Turmerones have been reported in Turmeric oils obtained from different regions (Ajaiyeoba *et al.*, 2008; Erdoğan, 2022), while our findings on the significant level of p-Cymene in Black Cumin oil is also in consonance with available literature (Hajhashemi *et al.*, 2004).

Anti-inflammatory Activity

Egg albumin (protein) denaturation method was adopted in this study. Protein denaturation is a process whereby proteins lose their structure and biological function due to inflammation that is occasioned by factors of heat, stress, or some chemical compounds. Therefore, protein denaturation is regarded as a marker of inflammation (Altir *et al.*, 2021). As such, the EOs obtained from Turmeric and black Cumin was screened for their anti-inflammatory potentials using the inhibition method. The result showed a concentration-dependent rise in the inhibitory activities of the oils against protein denaturation from 6.25-100 µg/mL. The combined oil demonstrated better inhibition than each of the individual oils when evaluated across all the concentrations. It gave the best anti-inflammatory activity with an IC_{50} value of 51.33±2.22 µg/mL (Figure 1). In the case of the individual EOs, Turmeric alone (IC_{50}= 62.68±1.34 µg/mL) gave better anti-inflammatory activity than black Cumin oil, which exhibited an IC_{50} of 85.6±6.43 µg/mL. Therefore, the superior activity of EO from the combined spice may be attributed to the possible synergistic effect of active compounds that are present in both plants. In a related study where the protein denaturation method was used to assess the anti-inflammatory activity of non-oil, petroleum ether, and chloroform-methanol extracts from Turmeric, an activity of IC_{50} values of 106.21 and 212.52 µg/mL (Altir *et al.*, 2021), which are less active when compared to the result obtained in this study. A report on the assessment of the morphological parts of *Curcuma longa* has shown its rhizome anti-inflammatory assessment of Turmeric oil having majorly ar-Turmerone (61.79%), Curlone (12.48%), and ar-curcumene (6.11%), has been reported to show *in vivo*

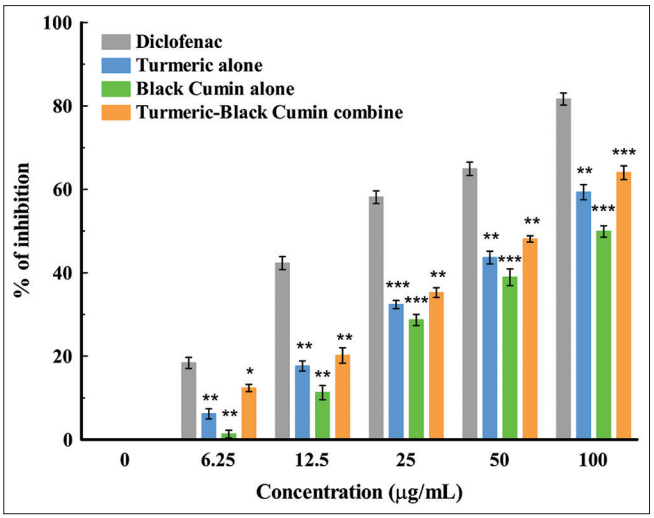

Figure 1: *In vitro* anti-inflammatory activity of the Turmeric, Black Cumin and Turmeric- Black Cumin mixture [Results are given as mean±S.D. (*n*=3). *p<0.05, **p<0.01, *** p<0.001]

dose-dependent inhibition of second phase of carrageenan-induced paw edema, suggesting its inhibition of inflammatory mediators (Liju *et al.*, 2011). Similarly, black Cumin extract has been reported to show *in vitro* protection from denaturation of albumin (Sherwani *et al.*, 2022). Freshly extracted oil of black Cumin with 33% Thymoquinone content has been shown in an *in vitro* model of low-grade inflammation in Simpson–Golabi–Behmel syndrome human pre-adipocytes to inhibit the activities of pro-inflammatory cytokines such as Interleukin (IL)-1α, IL-1β, and IL-6 (Bordoni *et al.*, 2019). Also, black Cumin oil at oral doses of 0.91 mL/kg and 1.82 mL/kg on arthritic rats showed a marked inhibition of the arthritis score, the percentage inhibition of paw edema and disease progression while also significantly inhibiting the expression of pro-inflammatory markers such as C-reactive protein (CRP) and interleukin 6 (Rashwan *et al.*, 2023). Thymoquinone in black Cumin oil has been reported to significantly lower the histological indices of joint inflammation and paw weight in pristane-induced arthritic rats (Faisal *et al.*, 2018). Additionally, Zingiberene, present in the three oil samples, has been reported to significantly reduce the level of inflammatory markers (TNF-α, IL-6, NF-κβ, and IL-1β) in isoproterenol-(ISO) induced myocardial infarction in rats (Li *et al.*, 2021). Conversely, palmitic acid in black Cumin oil has been shown to induce an upregulation of IL-6, TNF-α, and IL-1β secretions, accompanied by NF-κβ nuclear translocation and activation (Zhou *et al.*, 2013). Perhaps, the lowest anti-inflammatory activity shown by black Cumin in this study may be due to its high palmitic acid content, while on the other hand, the remarkable anti-inflammatory activity of EO from the combined spice may be a function of the synergistic effect of its putative anti-inflammatory compounds, notably ar-Turmerone and Zingiberene.

Anti-cancer Activity

The study evaluated the cytotoxic effect of Turmeric and black Cumin oils on normal and liver cancer cell lines. The MTT-based cell proliferation test was used in this study as a conventional bioassay technique for the assessment of the

cytotoxicity of the EOs against cell growth under standard culture conditions. MTT assay is widely used in cytotoxicity studies due to its accuracy, rapidity, and relative simplicity. It is a colorimetric viability assay based on enzymatic reduction of the MTT molecule to formazan when exposed to viable cells, and the outcome of the reduction is a color change of the MTT molecule (Ganot *et al.*, 2013).

The result in Figure 2 showed the percentage viability of the RAW264.7 normal cell line and the HepG2 human liver cancer cell line when treated with the EOs obtained from Turmeric, black Cumin, and the combined spice. The three tested oils were observed to be relatively non-cytotoxic to the RAW264.7 normal cell line across the concentrations 10-100 µg/mL, as the cell exhibited ≥60% growth even at the highest concentration of the oils. This may imply relative safety in terms of the biological application of the EOs for possible therapeutic use. It is worth mentioning that reported cases of turmeric toxicity are frequently related to curcumin, a Turmeric extract component that is not present in the oil (Balaji & Chempakam, 2010; Donelli *et al.*, 2020; Suhail *et al.*, 2020). Turmeric oil has been reported to show no sign of toxicity in terms of genotoxicity and mutagenicity (Liju *et al.*, 2013). In the same vein, reports have shown the non-toxicity profile of black Cumin oil, including its hepatic- and renal-protective effects in rats, and no histological changes in the kidneys, liver, heart, or pancreas or any alteration in liver enzymes (Zaoui *et al.*, 2002; Hamed *et al.*, 2013).

The three tested oils showed a concentration-dependent reduction in liver cancer HepG2 cell line from 10-100 µg/mL (Figure 2). At 100 µg/mL, the Turmeric-black Cumin combined oil showed the best activity by significantly reducing the viability of the HepG2 cell line to 7.55±1.02%. At the same concentration, the Turmeric alone and black Cumin alone exhibited comparable activity, reducing the viable HepG2 cell line to 22%. In a related study to assess the anti-cancer activity of Turmeric oil alone and in combination with a notable cancer drug, Paclitaxel, it was found that Paclitaxel-Turmeric oil combination did not affect non-cancerous (WI-38) cell growth but was selectively more cytotoxic to prostate cancer PC-3 cell lines with IC_{50} values of 64.8 and 17.6 nM at 24 and 48 hrs post-treatment respectively (Jacob & Toloue, 2013). Thus, our report has further highlighted the importance of combination in the form of formulations for effective cancer management. Black Cumin oils having thymoquinone as its major oil component, have been reported to show cytotoxicity against cancer cell lines of the breast MCF-7, colon HCT116, pancreas PDA, liver HepG2 (88% inhibition at 50 mg/mL), lung LL/2, prostate LNCaP, C4-B, DU145, PC-3, and cervix HeLa (Farah & Begum, 2003; Huat & Swamy, 2003; Gali-Muhtasib *et al.*, 2004; Chehl *et al.*, 2009). Additionally, it may not be far-fetched that the considerable anti-HepG2 activity of the combined oil may partly be attributed to its high level of Turmerones and Zingiberene. Ar-Turmerone has been reported to show a repressive effect on P388D1 lymphocytic leukemia (Kim *et al.*, 2013), while also suppressing cathepsin B expression and P27 cleavage, thereby inhibiting the proliferation and mobility of U251, U87, and LN229 glioma cells *in vitro* (Cao *et al.*, 2023). Zingiberene has also been implicated as an anticancer compound based on the noticeably diminishing viability of MDA-MB-231 breast cancer cells via apoptotic cell death (Seshadri *et al.*, 2022). In summary, the considerable bioactivity of the Turmeric-black Cumin combined oil, as shown in this study, has added to the rekindled interest in polyherbal combination therapy for effective management of liver cancer and other chronic inflammatory diseases.

In silico Molecular Docking

In an attempt to identify potential inhibitory possibilities, we conducted a thorough molecular docking study using 63 essential oil components from turmeric, black Cumin, and Turmeric-black Cumin mixture against the human proinflammatory cytokines, IL-6 and TNF-α. Tables 3, 4, and 5 exhibit the phytocompounds' detailed binding affinity scores with these cytokines. It is interesting to note that we discovered a total of fifteen unique compounds were only detected in the Turmeric-black Cumin mixture. Whereas, four and five compounds were shared by the Turmeric-Turmeric black Cumin mixture and black Cumin-Turmeric black Cumin mixture,

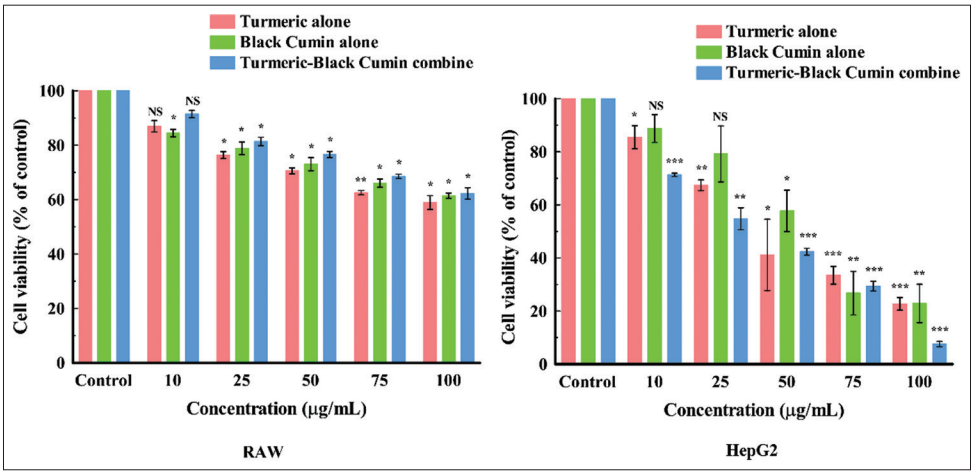

Figure 2: Graphical presentation of cell viability (%) of a) RAW and b) HepG2 cell line upon exposure to different concentrations of Turmeric, Black Cumin and Turmeric- Black Cumin mixture for 48 h. [Results are given as mean±S.D. (n=3). *p<0.05, **p<0.01, *** p<0.001, NS=non significant]

Table 3: Binding energy scores of the 13 Essential oil compounds from Turmeric with the proteins

S. No.	Phytocompounds	Binding energy scores (kcal/mol)		
		IL-6	TNF-α	IGF-1R
1.	o-Cymene	-5.0	-4.9	-5.8
2.	aR-Turmerone	-4.6	-5.2	-7.6
3.	Tumerone	-4.6	-5.2	-7.6
4.	Curlone	-4.9	-5.4	-7.6
5.	(Z)-alpha-Atlantone	-4.9	-5.0	-7.5
6.	(E)-Atlantone	-4.6	-5.1	-7.6
7.	n-Hexadecanoic acid	-3.0	-3.6	-5.3
8.	2-Pentanone, 4-methyl-4-phenyl	-4.5	-5.0	-6.4
9.	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [S-(R*, S*)]-	-4.8	-5.2	-7.3
10.	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*, S*)]-	-5.0	-5.3	-6.5
11.	Benzene, 1-(1,5-dimethylhexyl)-4-methyl-	-5.1	-4.9	-4.7
12.	Benzenemethanamine, alpha, 4-dimethyl-	-4.9	-4.5	-6.1
13.	(S)-3-Methyl-6-((R)-6-methylhept-5-en-2-yl) cyclohex-2-enone	-4.6	-5.0	-7.0

respectively. Many inflammatory mediators that stimulate tumor growth, including growth factors and cytokines like IL-6 and TNF-α, which either directly or indirectly contribute to the development of tumors, are often derived from pro-inflammatory cells (Schieber & Chandel, 2014). Therefore, for illnesses associated with inflammation, blocking this protein may be a good goal (Banerjee *et al.*, 2022). Acute inflammatory response is mediated by IL-6 via the signal transduction pathway (Del Valle *et al.*, 2020; Ragab *et al.*, 2020). The results of the research showed that Leu178, Arg179, and Arg182 formed the active site pocket in IL-6 (PDB ID 1ALU), whereas Tyr59, Tyr119, Leu120, Gly121, and Tyr151 were the key regions in TNF-α (PDB ID 2AZ5). According to our investigation, the phytocompound alpha-phellandrene exhibited the most persistent interaction with IL-6, yielding a binding energy score of -8.8 kcal/mol. The compounds (Z)-gamma Atlantone and β-Turmerone followed closely behind, both with -8.6 kcal/mol and -8.6 kcal/mol, respectively (Table 5). The protein IL-6 has interactions with the compound alpha-phellandrene through the hydrophobic interaction (Arg182) and Pi-Sigma interaction (Leu178) (Figure 3). TNF-α is a proinflammatory

Table 4: Binding energy scores of the 40 Essential oil compounds from Black Cumin with the proteins

S. No.	Phytocompounds	Binding energy scores (kcal/mol)		
		IL-6	TNF-α	IGF-1R
1.	p-Cymene	-4.3	-4.6	-6.4
2.	D-Limonene	-4.3	-4.6	-6.4
3.	Eucalyptol	-4.7	-5.0	-5.9
4.	gamma-Terpinene	-4.4	-4.6	-6.4
5.	trans-4-methoxy thujane	-4.5	-4.8	-6.0
6.	D-Carvone	-4.4	-4.7	-6.2
7.	Thymoquinone	-4.9	-4.6	-5.7
8.	2-Caren-4-ol	-4.7	-5.0	-6.2
9.	Anethole	-4.3	-4.4	-5.3
10.	beta-Bisabolene	-5.0	-5.2	-7.3
11.	(Z, Z)-alpha-Farnesene	-4.1	-4.3	-6.2
12.	7-epi-cis-sesquisabinene hydrate	-4.9	-4.2	-5.6
13.	1H-3a, 7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3R-(3.alpha.,3a.beta.,7.beta.,8a.alpha.)]-	-4.5	-6.8	-6.4
14.	2-Butenoic acid, 3-methyl-, methyl ester	-3.3	-3.3	-4.7
15.	Hexadecanoic acid, methyl ester	-3.1	-3.5	-5.7
16.	n-Hexadecanoic acid	-3.0	-3.6	-5.3
17.	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	-3.5	-4.2	-5.7
18.	11-Octadecenoic acid, methyl ester	-3.5	-3.2	-5.8
19.	9-Octadecenoic acid, (E)-	-3.6	-3.7	-6.0
20.	Octadecanoic acid	-4.1	-3.8	-6.4
21.	Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	-4.8	-4.6	-6.1
22.	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	-4.5	-4.9	-5.6
23.	Furan, 2-pentyl-	-3.8	-3.9	-5.3
24.	Cyclopropanemethanol, 2-methyl-2-(4-methyl-3-pentenyl)-	-4.0	-3.9	-5.1
25.	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	-5.0	-4.7	-6.1
26.	Benzenemethanol, .alpha.,.alpha.,4-trimethyl-	-5.1	-4.7	-6.3
27.	Benzaldehyde, 4-(1-methylethyl)-	-4.2	-4.6	-5.7
28.	(S)-3-Methyl-6-((R)-6-methylhept-5-en-2-yl) cyclohex-2-enone	-4.8	-4.7	-5.1
29.	3-Methyl-4-isopropylphenol	-4.5	-4.5	-6.2
30.	2-Pentanone, 4-methyl-4-phenyl-	-4.5	-5.0	-6.4
31.	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [S-(R*, S*)]-	-4.8	-5.2	-7.3
32.	(1R,4R)-1-methyl-4-(6-Methylhept-5-en-2-yl) cyclohex-2-enol	-5.1	-5.2	-5.6
33.	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*, S*)]-	-5.0	-5.3	-6.5
34.	2-Cyclohexen-1-one, 3,4,4-trimethyl-	-4.3	-4.7	-4.9
35.	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	-5.1	-4.5	-5.5
36.	Cyclopentane, 1,3-bis (methylene)-	-4.0	-3.7	-5.0
37.	Cyclopropyl phenylmethanol	-5.0	-4.8	-6.0
38.	Benzene, 1,2,3-trimethyl-	-4.6	-4.5	-5.6
39.	Benzene, 1,2,3,5-tetramethyl-	-5.0	-4.8	-6.0
40.	Benzene, 1-methyl-4-(1-methylpropyl)-	-4.4	-4.7	-4.3

Table 5: Binding energy scores of the 25 Essential oil compounds from Turmeric-Black Cumin mixture with the proteins

S. No.	Phytocompounds	Binding energy scores (kcal/mol)		
		IL-6	TNF-α	IGF-1R
1.	alpha.-Phellandrene	-8.8	-8.6	-9.8
2.	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	-5.0	-5.2	-7.3
3.	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [S-(R*, S*)]-	-4.8	-5.2	-7.3
4.	.beta.-Bisabolene	-5.0	-5.2	-7.3
5.	3-((1S,5S,6R)-2,6-Dimethylbicyclo[3.1.1]hept-2-en-6-yl) propanal	-4.4	-5.0	-5.9
6.	(1R,4R,5S)-1,8-Dimethyl-4-(prop-1-en-2-yl) spiro[4.5]dec-7-ene	-4.3	-5.0	-7.2
7.	(1R,4R)-1-methyl-4-(6-Methylhept-5-en-2-yl) cyclohex-2-enol	-4.8	-4.9	-5.2
8.	2-Cyclohexen-1-one, 3,4,4-trimethyl-	-4.8	-4.8	-6.5
9.	(Z)-.gamma.-Atlantone	-8.6	-8.2	-9.2
10.	(E)-.gamma.-Atlantone	-4.7	-5.2	-7.6
11.	Benzene, 1-(1,5-dimethylhexyl)-4-methyl-	-4.5	-5.0	-5.6
12.	1,3-Cycloheptadiene	-3.7	-3.5	-5.4
13.	Cyclopropyl phenylmethanol	-5.0	-4.8	-6.0
14.	2-Pyrazoline, 1-allyl-	-3.7	-3.2	-4.5
15.	Tumerone	-4.6	-5.2	-7.6
16.	aR-Turmerone	-4.6	-5.2	-7.6
17.	(4R,6S)-2-Methyl-6-((S)-4-methylenecyclohex-2-en-1-yl) hept-2-en-4-ol	-4.5	-5.4	-7.4
18.	Naphtho[2,1-d] thiazole, 2-methyl-	-4.9	-5.6	-5.1
19.	5-Methyl-2-(6-methylhept-5-en-2-yl) phenol	-4.8	-5.1	-6.9
20.	β-Turmerone	-8.6	-8.9	-9.0
21.	(E)-Atlantone	-4.6	-5.1	-7.6
22.	Cumenyl angelate, o-	-4.7	-5.0	-6.3
23.	2-Butenoic acid, 3-methyl-, methyl ester	-3.3	-3.3	-4.7
24.	1-Isopropenyl-3,3-dimethyl-5-(3-methyl-1-oxo-2-butenyl) cyclopentane	-4.8	-5.7	-6.7
25.	3-Methyl-2-butenoic acid, 2,7-dimethyloct-7-en-5-yn-4-yl ester	-4.4	-4.4	-5.9

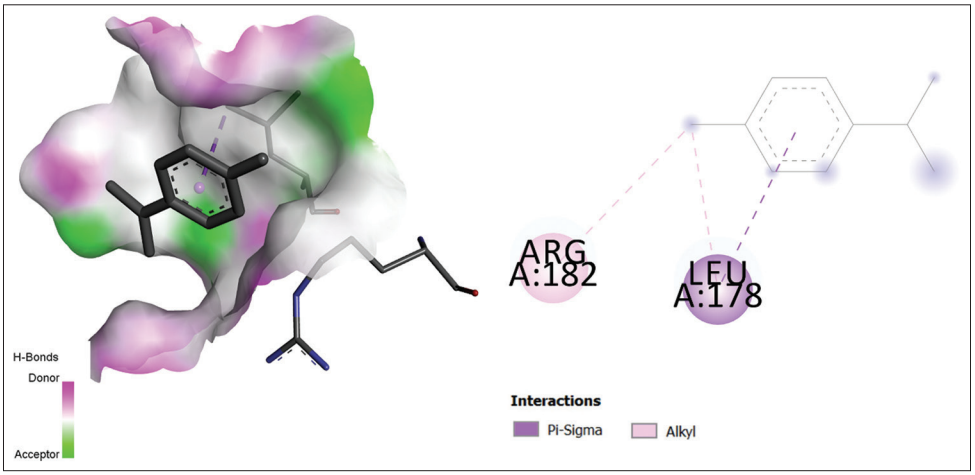

Figure 3: The mode of interaction and 3D and 2D visualization of alpha-Phellandrene from Turmeric-Black Cumin mixture with IL-6 protein (binding energy -8.8 kcal/mol)

cytokine that causes tissue damage and oxidative stress by activating the transcription pathway, cytokine shock, and nitric oxide production. It has been proposed that disorders linked to inflammation could result from blocking the TNF-α signaling pathways. According to our study, the molecule β-Turmerone had the most promising binding energy score of -8.9 kcal/mol with TNF-α followed by alpha-phellandrene (-8.6 kcal/mol) and (Z)-gamma Atlantone (-8.2 kcal/mol) respectively (Table 5). The interactions that included the active site residues of TNF-α and β-turmerone were Tyr59 (Pi-Pi stacking), Tyr119 (Pi-Sigma), and Tyr151 (hydrophobic interaction) (Figure 4). Remarkably, the findings indicate that these residues in the active site are linked to alpha-phellandrene and β-turmerone, compounds that have been demonstrated in this study to play a major part in the interaction between IL-6 and TNF-α and small molecule inhibitors. In an effort to find potential inhibitory candidates against the IGF-1R protein, 63 essential oil components from Turmeric, black Cumin, and Turmeric-black Cumin mixture were used in an extensive molecular docking investigation. Tables 3, 4, and 5 present the phytocompounds' comprehensive binding energy profile with this protein. It was discovered that the active residues in IGF-1R are Leu975, Val983, Ala1001, Met1052, Thr1053, and Asp1056. According to our findings, alpha-phellandrene (-9.8 kcal/mol) formed the most stable interaction, followed by (Z)-gamma atlantone (-9.2 kcal/mol) and β-turmerone (-9.0 kcal/mol) (Table 5). It was discovered

that alpha-phellandrene interacted with IGF-1R through the active site residues Leu975, Ala1001, Leu1051 (hydrophobic interactions), and Val983 (Pi-Sigma interaction) (Figure 5). Alpha-phellandrene and the IGF-1R protein interacted through the active site residues Leu975, Val983, and Ala1001 of IGF-1R, which is necessary for binding the potential inhibitor. The targeted active site and necessary method of interaction against IL-6, TNF-α, and IGF-1R receptors were revealed by this molecular docking investigation.

Hepatocytes release Kupffer cells when liver disorders brought on by hepatitis virus infection, obesity, alcohol, or toxins begin. Kupffer cells, sometimes referred to as liver macrophages, are essential for preserving liver function because they release more inflammatory cytokines, including IL-6, TNF-α, lysosomal enzymes, and ROS/RNS (Wardle, 1987). The establishment of cirrhosis in the hepatocytes is caused by the recruitment of tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) by the inflammatory cytokines. Liver cirrhosis can progress to hepatocellular carcinoma (HCC). In HCC, the liver experiences hypoxia and more TAMs and CAFs are drawn to the cancer microenvironment (Leonardi *et al.*, 2012). IL-6 secreted from Kupffer cells can also activate the IGF/IGF-1R signaling pathway. Insulin-like growth factors, or IGFs, are proteins that resemble insulin. Growth hormone (GH), which is secreted by anterior pituitary cells and has been shown to directly induce the transcription of IGFs in hepatocytes, mediates the production of IGF-1 and IGF-2 (Kadakia & Josefson, 2016). The insulin-like growth factor binding proteins (IGFBPs) control the IGF/IGF-1R signaling pathway. They bind to either IGF-1 or IGF-2 and stop IGF/IGF-1R signaling from being activated. Since IGFBP has a higher affinity for IGF-1/IGF-2 than does IGF-1/IGF-2 for IGF receptors (IGFR), the IGF/IGF-1R signal is inhibited once IGFBP-IGF-1/IGF-2 is produced (Clemmons *et al.*, 1995). The HCC microenvironment contains high levels of IGFBP proteases. IGFBP proteases have the ability to specifically cleave IGFBPs into small pieces, which release IGFs and activate IGF/IGF-1R signaling because of the decreased affinity of the IGFBP-IGF complex (Rajah *et al.*, 1995). The protein IGF-1R is a transmembrane heterotetrameric complex

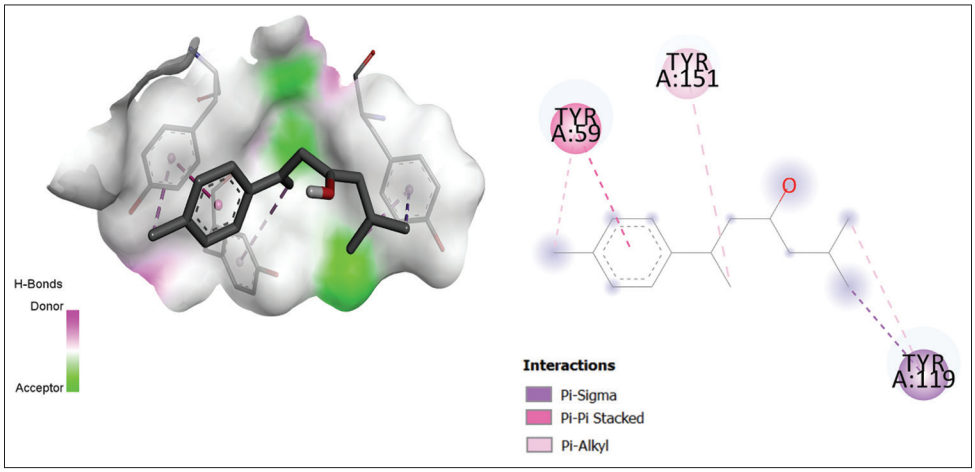

Figure 4: The mode of interaction and 3D and 2D visualization of β-Turmerone from Turmeric-Black Cumin mixture with TNF□α protein (binding energy -8.9 kcal/mol)

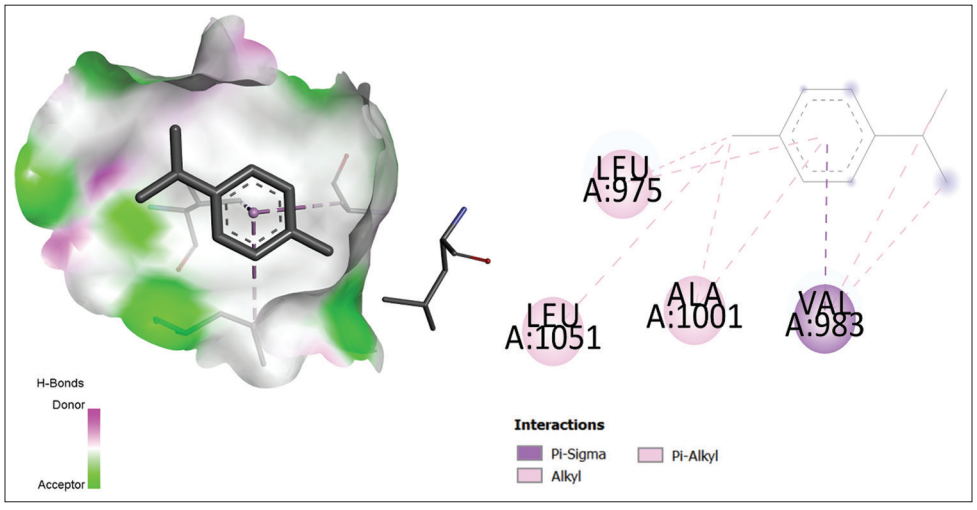

Figure 5: The mode of interaction and 3D and 2D visualization of alpha-Phellandrene from Turmeric-Black Cumin mixture with IGF-1R protein (binding energy -9.8 kcal/mol)

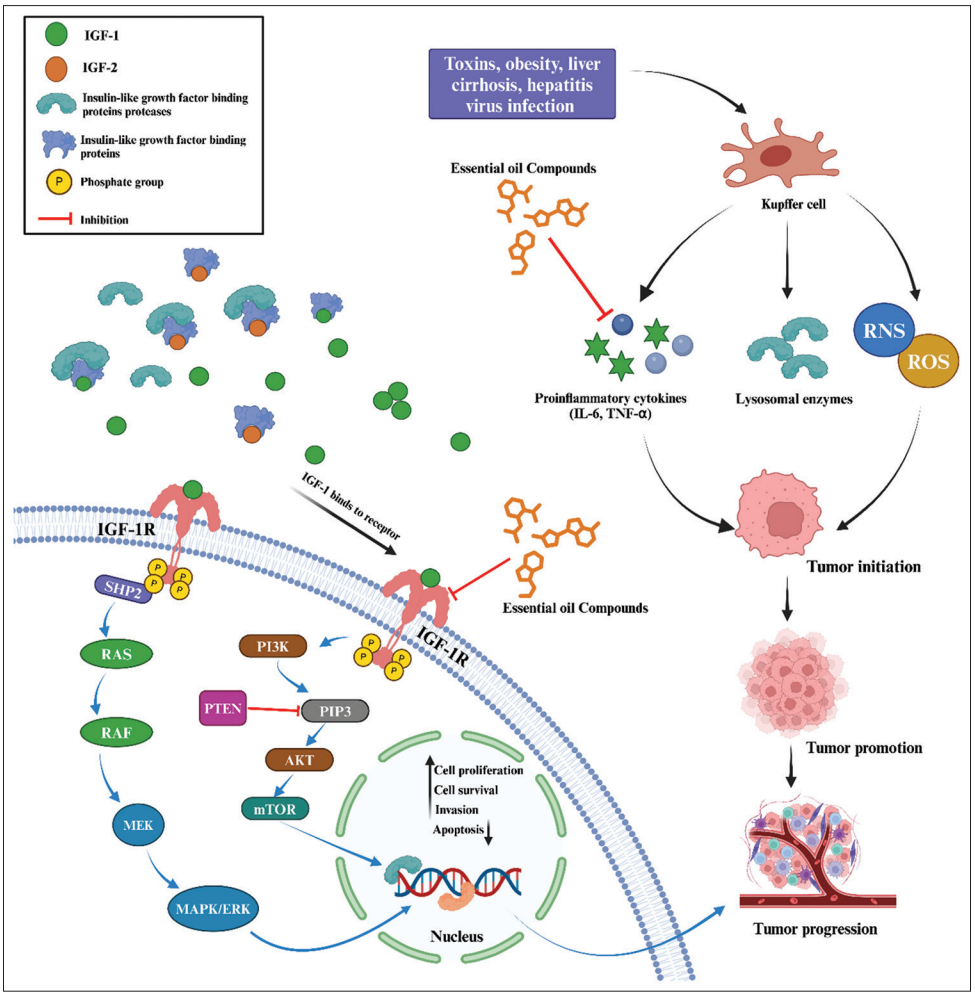

Figure 6: Schematic representation illustrating the antiproliferation mechanism of the essential oils from the turmeric-black cumin mixture and how it affects the development of liver disease by regulating IL-6, TNF-α and the insulin-like growth factor (IGF)/IGF-1R signaling pathway

(LeRoith *et al.*, 1995). When IGF-IR's ligands (IGF-1 and IGF-2) connect to its extracellular domains, its intracellular tyrosine kinase domain gets activated, which causes the receptor to become autophosphorylated. An activated IGF/IGF-1R signaling pathway initiates proliferative and antiapoptotic signal transduction pathways involving phosphatidylinositol-3-kinase (PI3K) pathway and mitogen-activated protein kinase (Ras-MAPK) pathway, activated IGF-IR phosphorylates its substrates (Kulik *et al.*, 1997). It is well known that IGFs stimulate different intracellular signaling pathways, which in turn cause numerous human cancers, including HCC, to exhibit mitogenic, transforming, and antiapoptotic features (Adams *et al.*, 2000). The idea that blocking IGF-1R function would cause tumor cells to selectively die off and stop growing makes IGF-1R an appealing therapeutic target (Baserga, 1995). In our study, the considered ligands inhibited IL-6, TNF-α, and IGF-1R proteins to suppress tumor initiation and progression (Figure 6).

CONCLUSION

This study has shown that the essential oil of Turmeric-black Cumin herbal combination possesses considerable *in vitro* anti-inflammatory and anticancer properties. The current molecular docking results revealed that alpha-Phellandrene and β-Turmerone proved to be the most promising of the twenty-five compounds identified in the essential oil of the Turmeric-black Cumin herbal combination. Our study provides new avenues to further evaluate the *in vivo* therapeutic potential of herbal combinations against hepatocellular carcinoma.

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