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Exploring the Effectiveness of *Lepidium sativum* for Inhibiting Human Hepatocellular Carcinoma Cell Lines: In vitro Analysis, Molecular Docking, and Pathway Enrichment Analysis

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THE GROWING increase in the burden of hepatocellular carcinoma (HCC) across the world emphasizes the need for new modes of therapy. Therefore, *Lepidium sativum* has recently garnered increased attention for various medicinal uses. This study aimed to identify the chemical constituencies of garden cress seeds (methylene chloride and ethyl acetate extracts) and their impact on two HCC cell lines by measuring some apoptotic genes' expression. In addition, evaluation of their antimicrobial and antifungal activities. Regarding *L. sativum*'s chemical composition, GC–MS analysis of the Methylene chloride extract led to identifying twenty-eight compounds, including hydrocarbons, terpenoids, and other volatile compounds. At the same time, the ethyl acetate extract identifies twelve compounds, including 3,4-dihydro-2H-1,5-(3"-T-butyl) benzodioxepine, cetane, linolenic acid, and oleic acid. However, both extracts revealed a weak antibacterial effect and no antifungal activity. The gene expression results revealed that all genes (TGFβ, SMAD3, BAX, and P53) showed upregulation trends in the HuH-7 and HEPG2 cells compared to the untreated control cells in both extracts of *Lepidium sativum*. Among Methylene chloride compounds, Molecular docking computations revealed higher binding affinities of compound 17 (Eugenol) against active EGFR (docking scores of −30.46 kcal/mol). Furthermore, the Pathway Enrichment Analysis results demonstrated that Neutrophil degranulation and Interleukin-4/Interleukin-13 signaling pathways were found to be the most significant pathways targeted by Eugenol, which may explain its potential as a therapeutic agent for cancer therapy.

Keywords: GC-MS analysis, Gene expression, Hepatocellular carcinoma, *Lepidium sativum,* Molecular docking, Pathway enrichment analysis.

Introduction

The universal burden of hepatocellular carcinoma (HCC) is escalating, thus mandating the development of new treatment modalities (Sayiner et al., 2019; Lin et al., 2020). *Lepidium sativum* is an annual herb that belongs to the Brassicaceae family; it is rich in phytochemicals that support health (Zhan et al., 2009); including folic acid, vitamins, flavonoids, coumarins, glycosides, triterpenes, sterols, glucosinolates and numerous imidazole alkaloids mainly lepidine (Malar et al., 2014). Total phenolic constituents are the most common natural antioxidants (Kasabe et al., 2012). In addition, garden cress seeds have numerous medicinal uses (Singh & Paswan, 2017; Shah et al., 2021).

Hepatocellular carcinoma is one of the greatest health problems and the primary cause of morbidity and mortality due to cancer in Egypt (Rashed et al., 2020). The efficacy of chemotherapeutic

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drugs has been proven in numerous oncological conditions, with severe side effects (Cragg & Pezzuto, 2016; Kuruppu et al., 2019). It is well documented that combining phytochemicals and chemotherapeutic agents can exhibit synergistic actions and attenuate some side effects related to chemotherapy (HemaIswarya & Doble, 2006). Recently, garden cress was reported to have an anti-cancer effect on the endometrium and colon cancer (Selek et al., 2018), squamous cell carcinoma (AlObaidi, 2014), neuroblastoma, and lung cancer (Alqahtani et al., 2019). It is documented that the anti-cancer activity of garden cress is mediated by apoptotic induction and its antioxidant activity (Mahassni & Al-Reemi, 2013; Selek et al., 2018). Additionally, the antimicrobial effect of garden cress extracts against contagious bacteria and fungi has been demonstrated (Indumathy & Aruna, 2015). However, the role of garden cress in hepatoprotection remains unclear (Chengaiah et al., 2010). A previous study suggested that *L. sativum* extract exerted the hepatoprotective effect by decreasing the expression of caspase 3 and increasing the BCl2 expression (Raish et al., 2016).

As solvent extraction is the commonest method used in natural products studies, several factors were found to increase and modify the diffusion and solubility of extracted compounds, such as polarity, particle size, solvent-to-solid ratio, and intermiscibility. Moreover, the efficient extraction of various products depends on the type of solvent used and the conditions of the extraction process.

Accordingly, this study was conducted as an extension of Nazir et al. (2021) study to identify other novel chemical components of garden cress seeds using another two different organic solvents (Methylene chloride and ethyl acetate extracts) using gas chromatography-mass spectrometry. Furthermore, to assess their cytotoxic activities against two HCC cell lines (HEPG2 and HuH-7) and study their impact on different oncogenes and apoptotic genes. Ultimately, evaluating the antimicrobial potentials of the two extracts..

Materials and Methods

Method of extraction

Garden cress seeds were purchased from the private market in Cairo, Egypt. The seeds used in this study were taxonomically authenticated and deposited under voucher specimen number 10001 at the Department of Botany, Faculty of Science, Cairo University. A kilogram of garden cress seeds grounded in fine powder and then extracted using 80% aqueous methanol at room temperature. The extracts were evaporated at 45˚C, and consecutive liquid-liquid extraction of the crude extract was performed using ethyl acetate and methylene chloride.

Cell Lines culture & cytotoxicity assay

The two HCC cell lines (HEPG2 and HuH-7) were obtained from the American Type Culture Collection (ATCC), USA. The cells were conserved by serial sub-culturing in 75cm^3 flasks containing Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) enhanced with fetal bovine serum, L-Glutamine (Gibco), sodium pyruvate, and penicillin G/streptomycin (Gibco). Subsequently, the cells were conserved in monolayer cultures at 37°C under a humidified atmosphere of 5% CO_2 using a water-jacketed incubator (Revco, RCO 3000 TVBB, USA). Successive sub-cultures were applied to maintain exponential growth. Strict sterility was attained by equipped laminar flow (Microflow Laminar flow cabinet, Hamsphire SP105AA, U.K.).

The cells' (HEPG2 and HuH-7) vitality was evaluated by conducting the MTT assay according to the protocol of Swapana et al. (2018). The culture media was aspirated, and fresh medium (without serum) was added to the cells with various concentrations (0, 3.125, 6.25, 12.5, 25, 50, and 100µg/mL) of each plant extract and incubated for 48h. Cell-free controls (blank) were used in each plate that retained MTT plus compounds in culture media to rectify any reduction of MTT in the absence of cellular mitochondria (Mosmann et al. 1983).

Gas chromatography

The garden cress extracts were analyzed via gas chromatography-mass spectrometry combined with a thermal mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The complete procedure includes sample preparation, instrument conditions, and sample injection performed according to Abd El-Kareem et al. (2016).

RNA extraction and quantitative real-time-PCR

RNA extraction was performed from treated HuH-7 and HEPG2 cells with two extracts (ethyl acetate and methylene chloride) as well as the control cells (untreated) using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The reverse

transcription was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA). Quantitative Real-Time-PCR (qRT-PCR) was conducted using the GoTaq® qPCR Master Mix (Promega, Madison, Wisconsin, USA) to assess the mRNA expression levels of TGFβ, SMAD3, EGFR, BAX, BCL2, and P53 on a Quantstudio5 Real-Time PCR System (Applied Biosystems, USA). The primers are listed in Table 1. Quantitative data analysis was performed using the ΔΔCt method (Livak & Schmittgen, 2001). β-Actin was used to normalize the values and expressed as the relative expression levels.

Antimicrobial activity

The antimicrobial effect of *L. sativum* extracts (Methylene chloride and ethyl acetate) were evaluated using the modified Kirby-Bauer disk diffusion method (Bauer et al., 1966; Pfaller et al., 1988). Standard disks of Ampicillin (antibacterial agent) and Amphotericin B (antifungal agent) were used as positive controls, whereas the two organic solvents used in this study served as negative controls. The diameters of the Zone of inhibition were measured using slipping calipers (Wikler, 2006).

Molecular Docking and pathway enrichment analysis (PEA)

Prediction of the binding affinities and modes of the major metabolites with EGFR was performed using BindScope Online Tool (Skalic et al., 2019). First, both active-inactive configurations of EGFR were considered based on deep convolutional neural networks. Then, the resolved crystal 3D structures of active EGFR (AlphaFoldDB: Q96AW1) were downloaded and prepared for docking calculations. The docking parameters were kept as the default. Furthermore, to explore all potential target–function interrelations based on biological network mining for the 20 most stimulated genes by the best compound exhibiting the highest binding affinity, then, pathway enrichment analysis (PEA) was accomplished using Cytoscape 3.8.2 (Shannon et al., 2003); finally, the ReactomeFIViz online tool was used for the modeling and illustration of all target interactions (Blucher et al., 2019).

Statistical analysis

Data were analyzed using IBM SPSS® Statistical version 23 (IBM® Corp., Armonk, NY, USA). Numerical data were presented as mean ± standard deviation. Comparisons between diverse groups were conducted using analysis of variance and confirmed using the post-hoc Dunnett's t-test. All tests were two-tailed, and a P value of <0.05 was considered significant.

Results

Extraction and gas chromatography of Lepidium sativum extracts

A 1kg of *Lepidium sativum* seeds powder was used for extraction as follows: the aqueous solution of 80% methanol was successively fractionated via liquid–liquid techniques to afford two different solvents, i.e., Methylene chloride (43g), and ethyl acetate (15g), containing compounds with varying polarity.

The The GC–MS analysis of the methylene chloride extract showed a predominant constitution of methyl-eugenol, linoleic acid, linolenic acid, oleic acid, palmitic acid, docosenoic acid, tocopherol, cis-methyl 11-eicosenoate, dichloroiodomethane, anisole, and estragole. Meanwhile, the ethyl acetate extract contained 3,4-dihydro-2H-1,5-(3"-T-butyl) benzodioxepine, cetane, oleic acid, and linolenic acid. The two extracts contained other constituents presented in Fig. 1(A, B) and Supplementary Tables.

TABLE 1. Different sequences of the primers for RT-PCR

Gene	Forward	Reverse
β-Actin	5'-TTC CAG CCTTCC TTC CTGG-3'	5'-TTG CGC TCA GGAGGA GCA AT-3'
TGFβ	5'-GGA CAC CAACTA TTG CTT CAG-3'	5'-TCC AGG CTC CAA ATG TAGG-3'
Smad3	5'-GCCTGTGCTGGAACATCATC-3'	5'-TTGCCCTCATGTGTGCTCTT-3'
EGFR	5'-GCAATATCAGCCTTAGGTGCGGCTC-3'	5'-ATAGAAAGTGAACATTTAGGAT-3'
BAX	5'-CTACAGGGTTTCATCCAG-3'	5'-CCAGTTCATCTCCAATTCG-3'
BCL2	5'-GTGGATGACTGAGTACCT-3'	5'-CCAGGAGAAATCAAACAGAG-3'
P53	5'-GTATTTCACCCTCAAGATCC-3'	5'-TGGGCATCCTTTAACTCTA-3'

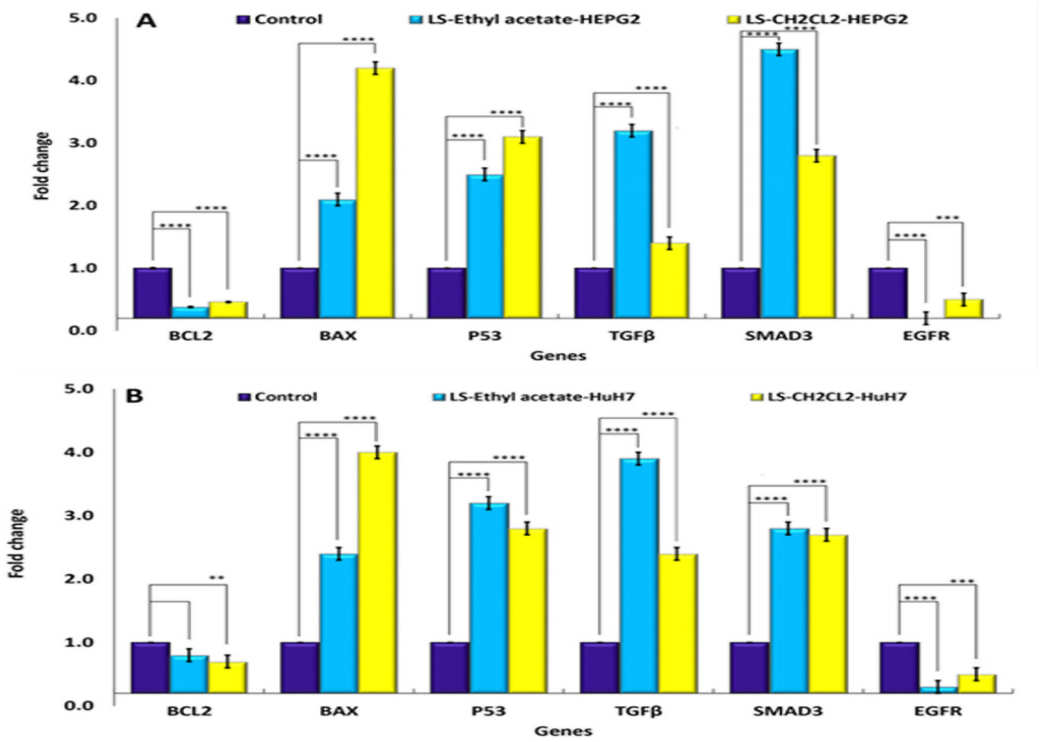


Fig. 1. Gene expression levels after treating hepatocellular carcinoma cells with *L. sativum* methylene chloride, and ethyl acetate extracts. (A): Expression of BCL2, BAX, P53, TGF-β, SMAD3, and EGFR in HEPG2 cells. Blue: untreated control, cyan: ethyl acetate, and yellow: methylene chloride extract. (B): Expression of BCL2, BAX, P53, TGF-β, SMAD3, and EGFR in HuH-7 cells. Blue: untreated control, cyan: ethyl acetate, and yellow: methylene chloride extract [Statistical analysis was performed using analysis of variance (ANOVA) followed by post-hoc test, and Dunnett t-test was used for comparison with the control group. All tests were two-tailed. A P value < 0.05 was considered significant ** P≤ 0.01, *** P ≤ 0.001, ****P ≤ 0.0001)]

Gas chromatography for different Lepidium sativum extracts

The GC–MS analysis of the Methylene chloride extract of *L. sativum* revealed the identification many compounds that represented 99 % of the total mass, including hydrocarbons, terpenoids and other volatile compounds. The hydrocarbons were characterized as abundant compounds in the methylene chloride extract of this plant, with 28 identified compounds. From the characteristic hydrocarbons, linolenic acid, methyl ester (25.72%), oleic acid, methyl ester (14.14%), cis-Methyl 11-eicosenoate (6%), and Dichloroiodomethane (5.04%) were identified as the major compounds, while à-Terpinyl acetate (0.64%) was identified as a minor one. Meanwhile, the second most identified class of compounds was terpenoids, including mono and sesqui-terpenoids, with the complete absence of diterpenoids. Anisole, p-propenyl-, cis- (2.56%) Methyl-eugenol (1.94%) and were identified as the main monoterpenes. In addition to the most aboundent classes of compounds (hydrocarbons and terpenes), other volatile compounds were identified at minor concentrations (Table 2).

On the other hand, the ethyl acetate extract contained 12 identified compounds. The major compounds were as follow: 3,4-dihydro-2h-1,5-(3”-t-butyl)benzodioxepine (53.76%), Cetene (9.14%), 1-nonadecene (7.62%), and Linolenic acid, methyl ester (7.22%) were identified as the major compounds, while Caryophyllene (1.52%) was identified as a minor one (Table 3). The two extracts contained other constituents presented in Fig. 1(A, B) and Supplementary Tables.

Antimicrobial effects of the different Lepidium sativum extracts

Ethyl acetate, and methylene chloride extracts of *Lepidium sativum* showed a weak antibacterial mainly against *Bacillus subtilis* and *Escherichia coli* with inhibition zone diameter ranged from 9 to 10mm. On the other hand, no antifungal activity was observed on the tested microorganisms as presented in Table 4.

TABLE 2. GC of Methylene chloride extract

Peak No.	RT (Min)	Compound Name	Formula	M.W	Area %
1	24.71	Linolenic acid, methyl ester	C19H32O2	292	19.35
2	24.13	Oleic acid, methyl ester	C19H36O2	296	14.14
3	24.35	Linoleic acid, methyl ester	C19H34O2	294	6.37
4	26.58	cis-Methyl 11-eicosenoate	C21H40O2	324	6
5	4.49	Dichloroiodomethane	CHCl2I	210	5.04
6	21.48	Palmitic acid, methyl ester	C17H34O2	270	4.86
7	25.29	Oleic Acid	C18H34O2	282	4.14
8	35.9	ç-Tocopherol	C28H48O2	416	3.39
9	11.43	2-Decenal, (E)-	C10H18O	154	3.24
10	28.86	13-Docosenoic acid, methyl ester, (Z)	C23H44O2	352	3.24
11	33.35	Methyl 5,13-docosadienoate	C23H42O2	350	3.23
12	13.21	Anisole, p-propenyl-, cis-	C10H12O2	148	2.56
13	15.74	Methyl-eugenol	C11H14O2	178	2.52
14	28.61	1-dimethylamino-3,3-diphenyl-4-hexanone	C20H25NO	295	2.17
15	13.53	Aromandendrene	C15H24	204	2.04
16	30.73	16-Trimethylsilyloxy-9-octadecenoic acid, methyl ester	C22H44O3Si	384	1.97
17	14.7	Eugenol	C10H12O2	164	1.94
18	27.71	Linolenin, 1-mono-	C21H36O4	352	1.89
19	29.19	1,2-15,16-Diepoxyhexadecane	C16H30O2	254	1.82
20	31.17	cis-10-Nonadecenoic acid	C19H36O2	296	1.58
21	24.52	Dibutyl phthalate	C16H22O4	278	1.43
22	23.96	2-Nonadecanone	C19H38O	282	1.37
23	28.54	5,8,11-Eicosatriynoic acid, tert-butyldimethylsilyl ester	C26H42O2Si	414	1.29
24	11.23	Estragole	C10H12O	148	1.26
25	15.93	Isothiocyanic acid, benzyl ester	C8H7NS	149	1.07
26	32.26	Glycidyl oleate	C21H38O3	338	0.74
27	26.32	E-8-Methyl-9-tetradecen-1-ol acetate	C17H32O2	268	0.7
28	13.01	à-Terpinyl acetate	C12H20O2	196	0.64

TABLE 3. GC of ethyl acetate extract

Peak No.	RT (Min)	Compound Name	Formula	M.W	Area %
1	16.3	3,4-dihydro-2h-1,5-(3”-t-butyl)benzodioxepine	C13H18O2	206	53.76%
2	15.14	Cetene	C16H32	224	9.14%
3	18.31	1-nonadecene	C19H38	266	7.62%
4	24.74	Linolenic acid, methyl ester	C19H32O2	292	7.22%
5	21.19	3-Eicosene	C20H40,	280	4.37%
6	11.67	1-Hexadecanol	C16H34O	242	4.13%
7	24.15	Oleic acid, methyl ester	C19H36O2	296	4.12%
8	23.83	1-Pentacosene	C25H50	350	2.31%
9	24.54	1-methyl-2-cyano-3-ethyl-4-pivaloyl-2-piperidiene	C14H22N2O	234	2.28%
10	26.6	11-Eicosenoic acid, methyl ester	C21H40O2	324	1.86%
11	24.38	9,12-octadecadienoic acid	C20H36O2	308	1.67%
12	13.54	Caryophyllene	C15H24	204	1.52%

TABLE 4. Antimicrobial effect of methylene chloride, and ethyl acetate extracts of *Lepidium sativum*

Sample		Inhibition zone diameter (mm / sample)					
		Bacterial species				Fungi	
		(Gram reaction$^+$)		(Gram reaction$^-$)			
		Bacillus subtilis	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Aspergillus flavus***	***Candida albicans***
Standard	Ampicillin	28 ± 0.3	23 ± 0.5	27 ± 0.6	28 ± 0.4	--	--
	Amphotericin B	--	--	--	--	19 ± 1	23 ± 0.9
Ls methylene chloride		9± 0.5	0.0	9 ± 0.2	0.0	0.0	0.0
Ls Ethyl acetate		10± 0.3	10 ± 0.6	9 ± 0.4	10 ± 0.2	0.0	0.0

Data is presented as mean of triplicate ± standard deviation

Effect of Lepidium sativum on the expression levels of various genes

The expression levels for all genes (TGFβ, SMAD3, BAX, and P53) showed upregulation trends in the HuH-7 and HEPG2 cells compared to the untreated control cells in both extracts of *Lepidium sativum*. Exposure of HEPG2 cells to ethyl acetate and methylene chloride extracts resulted in increased p53 expression with a fold-change of 5.52 ± 0.1 and 3.11 ± 0.1, respectively; the corresponding values in the HuH-7 cells were 4.96 ± 0.1 and 2.94 ± 0.1, respectively. Likewise, the expression of Bax in the HEPG2 cells was increased following treatment with the ethyl acetate and methylene chloride extracts (fold-change, 6.3 ± 0.1 and 4.27 ± 0.1, respectively); the corresponding values in the HuH-7 cells were 4.35 ± 0.1 and 3.95 ± 0.1, respectively. On the other hand, the expression level of Bcl-2 was downregulated following treatment with the ethyl acetate and methylene chloride extracts in the HEPG2 (fold-change, 0.41 ± 0.01 and 0.46 ± 0.01, respectively) and HuH-7 (fold-change, 0.55± 0.1 and 0.71 ± 0.1, respectively) cells (Table 3 and Fig. 1).

Furthermore, the expression level of TGF-β was increased in the ethyl acetate and methylene chloride extracts containing garden cress (fold-change in HEPG2 cells, 2.0 ± 0.1 and 1.45 ± 0.1, respectively; fold-change in HuH-7 cells, 1.7 ± 0.1 and 2.36 ± 0.1, respectively). Additionally, the expression level of SMAD3 was elevated after treatment with ethyl acetate and methylene chloride extracts in the HEPG2 cells (fold-change, 3.36 ± 0.1 and 2.76 ± 0.1, respectively) and HuH-7 cells (fold-change, 4.32 ± 0.1 and 2.74 ± 0.1, respectively). Moreover, the expression level of EGFR was decreased following treatment with ethyl acetate and methylene chloride extracts in the HEPG2 cells (fold-change, 0.23 ± 0.1 and 0.4± 0.1, respectively) and HuH-7 cells (fold-change, 0.35 ± 0.1 and 0.46 ± 0.1, respectively) (Table 5 and Fig. 1).

TABLE 5. *Lepidium sativum* extracts effect on the mRNA expression of BCL2, BAX, P53, TGF-β, Smad3 and EGFR genes in HEPG2 and HUH7 cells

Sample	Cellline	Fold-Change					
		BCL2	**BAX**	**P53**	**TGFβ**	**SMAD3**	**EGFR**
LS-methylene chloride	HEPG2	0.46	4.27	3.11	1.454	2.757	0.402
LS ethyl acetate		0.41	6.30	5.52	2.040	3.356	0.234
Control HEPG2		1	1	1	1	1	1
LS-methylene chloride	HUH7	0.71	3.95	2.94	2.364	2.740	0.460
LS- Ethyl acetate		0.55	4.348	4.96	1.707	4.318	0.353
Control HUH7		1	1	1	1	1	1

Molecular docking and molecular target prediction

Molecular docking was used to predict the binding modes and affinities of the ethyl acetate and methylene chloride extracts major metabolites with EGFR. Prior to *in silico* prediction, the binding modes of EGFR with the employed parameters was used as a reference. Predicted docking scores of the investigated metabolites revealed that metabolites showed relatively weaker docking scores towards active EGFR (AlphaFoldDB: Q96AW1) with values in the range −2.1 to −6.5 kcal/mol, compared to the inactive EGFR. Interestingly, among the investigated metabolites, compounds 17 (Eugenol) identified in the methylene chloride extract exhibited higher binding affinity with active EGFR (Rdock score of −30.46kcal/mol). The 3D representations of its binding mode inside the active site of EGFR protein was depicted in Fig. 2.

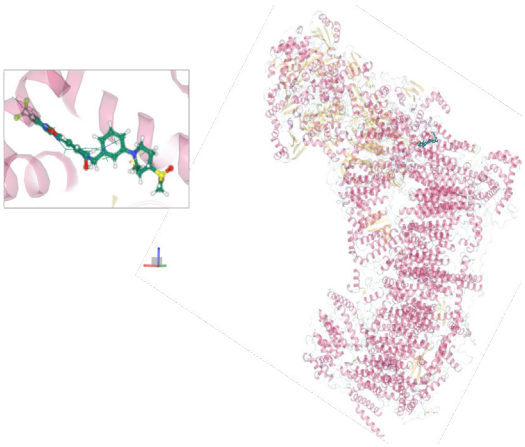


Fig. 2. 3D representations of the predicted (in yellow) and crystal (in ligh red) binding mode of Eugenol with the active conformation of EGFR (AlphaFoldDB: Q96AW1)

The Eugenol protein targets were primarily predicted and classified with the aid of SwissTargetPrediction (Fig. 3). The top 25 predicted targets of Eugenol compound, their common name, uniprot-ID, ChEMBL-ID and target class was listed in Table 6.

Pathway enrichment analysis

For better and deeper mining/dissection of Eugenol target-function interactions, a Foamtree map based on Boolean network modeling and Pathway Enrichment Analysis (PEA) analysis was developed. Notably, the Neutrophil degranulation, Interleukin-4 and Interleukin-13 signaling pathway and the G alpha (s) signalling events pathways, were found to be the most significant pathways targeted by Eugenol, with a false discovery rate (FDR) of <0.00001% (Table 7). The foamtree map was constructed to visualize the top targeted pathway influenced by the top 25 gene targets in response to Eugenol (Fig. 4a). Additionally, part of Interleukin-4 and Interleukin-13 signaling pathway was visualized to illustarte the two main stimulated genes/targets as a response to Eugenol within the human (Fig. 4b).

Discussion

The long-term consequences and adverse events associated with cancer chemotherapeutic agents are considered the main issues of cancer therapy (Mahassni & Al-Reemi, 2013). Combining natural remedies and chemotherapeutic agents can augment the anti-cancer effect and decrease its associated side effects (Greenwell & Rahman, 2015; Colafrancesco et al., 2017; Nurgali et al., 2018). This study aims to assess the possible anti-cancer activity of *L. sativum* ethyl acetate and methylene chloride extracts against HCC cell lines and determine the possible Antimicrobial activity.

Free radicals and reactive oxygen species are highly reactive molecules released during aerobic metabolism; they can damage the cell membrane or intracellular molecules unless removed by antioxidant defense mechanisms (Gowda et al., 2010). Free radicals contribute to several pathological processes, such as inflammation and cancer (Lobo et al., 2010). Previous studies supported by GC-MS analysis of garden cress reported the presence of different major compounds, including; antioxidants, proto-alkaloids, and alkaloids, which may enhance its antitumor effects (Basaiyye et al., 2019). Furthermore, various reports proved that *L. sativum* extracts displayed a cytotoxic effect alongside diverse types of cancer, including leukemia (Aslani et al., 2015) and breast cancer (Mahassni & Al-Reemi, 2013). In agreement with this, our recent study showed a concentration-dependent cytotoxic effect of methylene chloride and ethyl acetate (47ug/mL and 63.8ug/mL, respectively) extracts on HEPG2 cells; meanwhile, on HuH-7 cells, methylene chloride and ethyl acetate (59ug/mL and 63.5ug/mL, respectively), from *L. sativum* extracts were compared to the non-treated control.

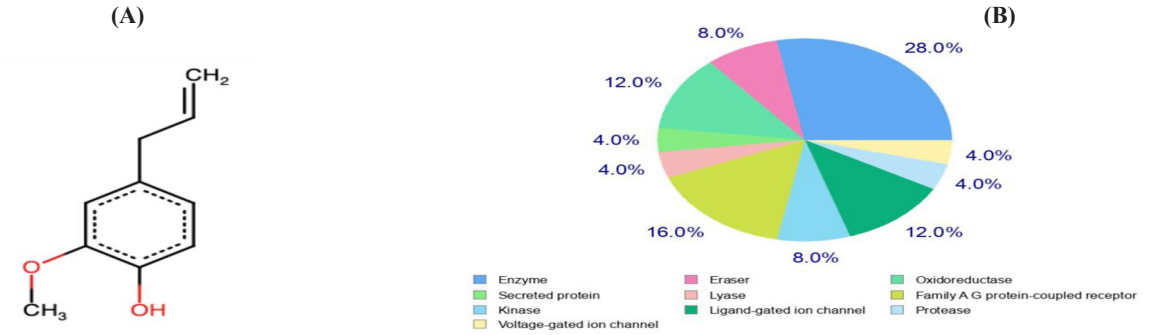


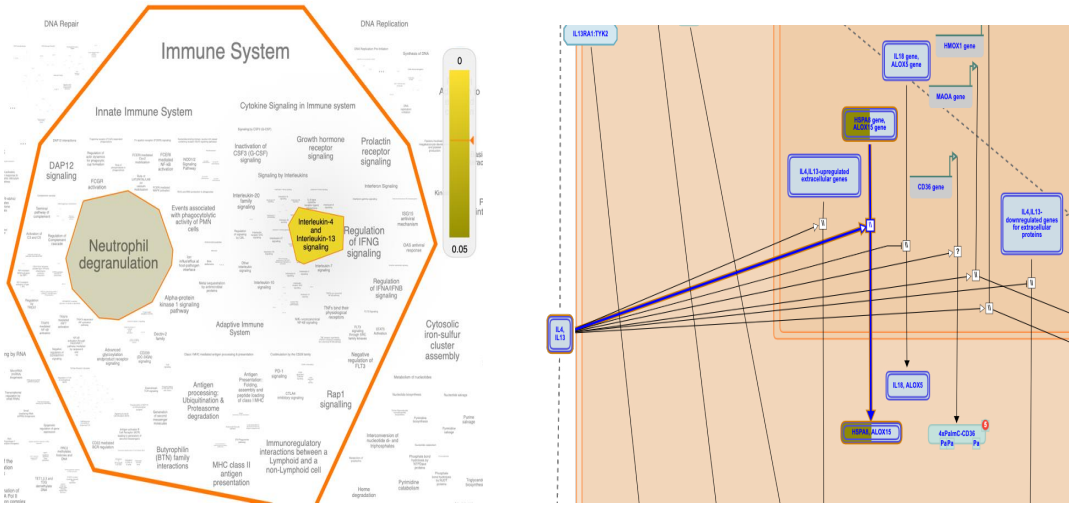
Fig. 3. A: Chemical structure of Eugenol , B: Target Classes of top 25 targets predicted by SwissTargetPrediction Toolbox

TABLE 6. List of the top 25 predicted targets of Eugenol compound, their common name, uniprot-ID, ChEMBL-ID and target class

Target	Common name	Uniprot ID	ChEMBL ID	Target Class
Fatty acid desaturase 1	FADS1	O60427	CHEMBL5840	Enzyme
Histone deacetylase 6	HDAC6	Q9UBN7	CHEMBL1865	Eraser
Egl nine homolog 1	EGLN1	Q9GZT9	CHEMBL5697	Oxidoreductase
Vascular endothelial growth factor A	VEGFA	P15692	CHEMBL1783	Secreted protein
Carbonic anhydrase II	CA2	P00918	CHEMBL205	Lyase
G-protein coupled receptor 84	GPR84	Q9NQS5	CHEMBL3714079	Family A G protein-coupled receptor
Cyclooxygenase-1	PTGS1	P23219	CHEMBL221	Oxidoreductase
D-amino-acid oxidase	DAO	P14920	CHEMBL5485	Enzyme
Poly [ADP-ribose] polymerase-1	PARP1	P09874	CHEMBL3105	Enzyme
Tyrosine-protein kinase SRC	SRC	P12931	CHEMBL267	Kinase
Adenosine A1 receptor	ADORA1	P30542	CHEMBL226	Family A G protein-coupled receptor
Adenosine A2a receptor	ADORA2A	P29274	CHEMBL251	Family A G protein-coupled receptor
Steroid 5-alpha-reductase 1	SRD5A1	P18405	CHEMBL1787	Oxidoreductase
Neuronal acetylcholine receptor subunit alpha-3	CHRNA3	P32297	CHEMBL3068	Ligand-gated ion channel
Neuronal acetylcholine receptor protein alpha-4 subunit (by homology)	CHRNA4	P43681	CHEMBL1882	Ligand-gated ion channel
Interleukin-8 receptor B	CXCR2	P25025	CHEMBL2434	Family A G protein-coupled receptor
dCTP pyrophosphatase 1	DCTPP1	Q9H773	CHEMBL3769292	Enzyme
Alkaline phosphatase, tissue-nonspecific isozyme	ALPL	P05186	CHEMBL5979	Enzyme
Methionine aminopeptidase 2	METAP2	P50579	CHEMBL3922	Protease
Carbonyl reductase [NADPH] 1	CBR1	P16152	CHEMBL5586	Enzyme
Calcium-activated potassium channel subunit alpha-1	KCNMA1	Q12791	CHEMBL4304	Voltage-gated ion channel
Arachidonate 15-lipoxygenase	ALOX15	P16050	CHEMBL2903	Enzyme
Neuronal acetylcholine receptor; alpha3/beta4	CHRNA3 CHRNB4	P32297 P30926	CHEMBL1907594	Ligand-gated ion channel
Vascular endothelial growth factor receptor 2	KDR	P35968	CHEMBL279	Kinase
Histone deacetylase 8	HDAC8	Q9BY41	CHEMBL3192	Eraser

TABLE 7. Top 25 pathways for Eugenol targets resulting from the pathway enrichment analysis (PEA)

Pathway name	# Entities total	# Interactors total	Entities P value	Entities FDR
Neutrophil degranulation	480	0	0.02098132	0.34781745
Interleukin-4 and Interleukin-13 signaling	211	161	0.00795451	0.34781745
G alpha (s) signalling events	189	78	0.02031012	0.34781745
Arachidonic acid metabolism	168	29	0.0092154	0.34781745
Cellular response to hypoxia	86	145	0.01181697	0.34781745
Synthesis of Prostaglandins (PG) and Thromboxanes (TX)	58	18	0.01224441	0.34781745
Chemokine receptors bind chemokines	57	70	0.02127532	0.34781745
VEGFR2 mediated cell proliferation	31	78	0.00181029	0.09956618
Nucleotide-like (purinergic) receptors	23	25	0.00507807	0.25898142
Reversible hydration of carbon dioxide	17	0	7.91E-06	0.00176427
Acetylcholine binding and downstream events	17	11	3.48E-05	0.00258312
Postsynaptic nicotinic acetylcholine receptors	17	11	3.48E-05	0.00258312
Presynaptic nicotinic acetylcholine receptors	15	11	2.79E-05	0.00258312
Regulation of gene expression by Hypoxia-inducible Factor	15	98	0.02365505	0.34781745
Highly calcium permeable postsynaptic nicotinic acetylcholine receptors	13	11	2.20E-05	0.00258312
Highly calcium permeable nicotinic acetylcholine receptors	11	11	1.70E-05	0.00258312
COX reactions	9	0	0.01955331	0.34781745
Highly sodium permeable postsynaptic acetylcholine nicotinic receptors	9	0	1.19E-06	3.98E-04
VEGF ligand-receptor interactions	8	26	3.86E-05	0.00258312
VEGF binds to VEGFR leading to receptor dimerization	8	26	3.86E-05	0.00258312
Biosynthesis of DPAn-6 SPMs	8	1	0.01955331	0.34781745
Signaling by membrane-tethered fusions of PDGFRA or PDGFRB	7	0	0.01524076	0.34781745
Dopamine receptors	6	6	0.02384736	0.34781745
Adenosine P1 receptors	5	0	5.85E-05	0.0035693
UNC93B1 deficiency - HSE	2	5	0.01307752	0.34781745



(A) (B)

Fig. 4. (A) The FoamTree map of the top two pathways (Neutrophil degranulation and Interleukin-4 and Interleukin-13 signaling) influenced by the top 25 gene targets in response to Eugenol, and (B) part of Interleukin-4 and Interleukin-13 signaling pathway, illustarte the two main stimulated genes/targets as a response to Eugenol within the human

Additionally, recent reports demonstrated a cytotoxic effects of *L. sativum* seeds methanolic extract on the bladder cell line (ECV-304) (Al-Fatimi et al., 2005), K562 leukemia cell line (Aslani et al., 2015) and breast cancer cells (MCF-7) (Mahassni & Al-Reemi, 2013). Furthermore, *L. sativum* leaf extract showed a strong antiproliferative effect against CAL-27 cells, mediated through the apoptosis process (AlObaidi, 2014). Notebly, the existence of numerous antioxidants in *L. sativum* extracts, which might play potential roles in the antitumor activity was reported. These findings were comparable to those observed in the current study.

On the other hand, Adam et al. (2011) evaluated the antimicrobial effects of petroleum ether and methanol extracts of garden cress seeds and documented their mild effectiveness as antibacterial and antifungal agents. The ethanolic extract is reported to have a better antibacterial effect than the aqueous extracts (Hero & Jwan, 2012). In 2012, Sharma demonstrated the antifungal activity of the ethanolic extract of garden cress seeds against various fungi; all concentrations of the seed extract inhibited fungal growth (Sharma, 2012). These findings were contrary to results that revealed a weak antibacterial effect and no antifungal activity. This discrepancy might be attributed to the difference in the extraction method used in the two studies.

Methylene chloride and ethyl acetate extracts of garden cress significantly decreased the mRNA expression levels of EGFR and BCL2 and increased the expression levels of TGFβ, SMAD3, BAX, and P53 levels in both the HUH7 and HEPG2 cells when compared to the untreated controls. In agreement with our results, Basaiyye et al. (2019) reported the cytotoxic effect of *L. sativum* on leukemic cells and demonstrated significant upregulation in the apoptosis pathways (Basaiyye et al., 2019). Likewise, another study reported garden cress's anti-inflammatory and antioxidant effects (Alqahtani et al., 2019). A recent study reported the induction of caspase 3, p53, and Bax, but a reduction of Bcl2 genes in the HepG2 cell line after treatment with lectin extracted from garden cress (Yasin et al., 2020). On the contrary, another study reported that the chemical composition of garden cress prompts apoptosis without triggering caspase 3 activation (Greenblatt et al., 1994). In contrast to our obtained results, althgouh high concentration of *L. sativum* extract decreased the expression of P53; it increased apoptosis in colon cancer (Hussien & Alsulami, 2021).

Molecular docking calculations revealed higher binding affinities of compound 17

(Eugenol) against active EGFR (docking scores of −30.46 kcal/mol). In line with our results, recent studies reported that Eugenol compounds exhibit anti-breast cancer properties due to their highest binding affinity energies towards the Mammalian target of rapamycin (mTOR) (Rasul et al., 2022; Abdullah et al., 2018; Al-Sharif et al., 2013).

Many studies reported that modulation/ overexpression of IL-4 and IL-13 receptors on cancer cells could provide potential targets for therapeutic agents for cancer therapy (Suzuki et al., 2015; Briukhovetska et al., 2021). Additionally, a recent study linked the type 2 cytokines IL-4 and IL-13 and their signaling via type I and type II IL-4Rs on neutrophils to inhibition of several neutrophil effector functions (Egholm et al., 2019). In line with these findings, our Pathway Enrichment Analysis demonstrated that Neutrophil degranulation and Interleukin-4/ Interleukin-13 signaling pathways were found to be the most significant pathways targeted by Eugenol, which may explain its potential as a therapeutic agent for cancer therapy.

Conclusion

Ultimately, *L. sativum* demonstrated potential for the treatment of HCC in the current study. However, additional studies are required to evaluate the possible mode of action. The cytotoxicity observed in this study might be attributed to the reduction in the expression levels of EGFR and BCL2 and an increase in the levels of TGF-β, Smad3, P53, and BAX. Eugenol compounds exhibit anti-cancer properties due to their highest binding affinity energies and their modulation of Neutrophil degranulation and Interleukin-4/Interleukin-13 signaling pathways.

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استكشاف فعالية حب الرشاد لتثبيط خلايا الكبد السرطانية: التحليل المختبري، الالتحام الجزيئي، وتحليل إثراء المسار

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يُعد سرطان الخلايا الكبدية (HCC) أكثر الأورام الخبيثة انتشارًا وهو سبب رئيسي للوفاة المرتبطة بالسرطان في جميع أنحاء العالم. ويكثر سرطان الكبد فى منطقة الشرق الأقصى والدول العربية (السعودية ومصر) وشمال أفريقيا فهناك أنواع لأورام الكبد: حميد وخبيث، فالخبيث قد يكون ناتجا من الكبد أو من عضو آخر منتشر إلى الكبد، فسرطان الكبد ينقسم إلى عدة أنواع حسب نوع الورم.

على الرغم من وجود العديد من العلاجات لسرطان الخلايا الكبدية إلا أن العلماء يبحثون في استخدام الاعشاب الطبيعية لعلاج هذا النوع من الاورام . و يرجع انجذاب العلماء لهذه العلاجات الطبيعية بسبب الآثار الجانبية المصاحبة للعلاج الكيميائي. من أشهر الاعشاب الطبيعية التي تستخدم في علاج بعض أنواع السرطان هو حَب الرّشاد لما يحتويه من مركبات الفيتوكيميكال (Phytochemical)، والسّتيرولات النباتيّة (Phytosterols) المعروفة بأثرها في محاربة السّرطان والوقاية منه. و لهذا فإن هذا البحث يهدف إلى تقييم الاستخدام المحتمل لحب الرشاد في سرطان الكبد في المختبر واستكشاف آليات عمله المحتملة.

أثبتت نتائج هذه الدراسة أن :

هناك تأثير إنتقائي سام ملحوظ لمستخلصات كلوريد الميثيلين ومستخلصات أسيتات الإيثيل على خلايا سرطان الكبد. يمكن أن ينسب ذلك إلى وجود العديد من مضادات الأكسدة في مستخلصات *Lepidium sativum*. علاوة على ذلك، أظهرت خلاصات الإيثيل أسيتات وكلوريد الميثيلين من *L. sativum* تأثير ضعيف ومتشابه كمضاد ات لبعض انواع البكتريا بشكل رئيسي ضد *Bacillus subtilis* و *Escherichia coli*. كما أظهر مستخلص الإيثيل أسيتات تأثير مضاد للميكروبات ضعيف ضد *Staphylococcus aureus* و *Pseudomonas aeruginosa* ليسجل أوسع طيف للنشاط. لم يلاحظ أي نشاط مضاد للفطريات على الكائنات الحية الدقيقة المختبرة. علاوة على ذلك ، قللت مستخلصات كلوريد الميثيلين وخلات الإيثيل من حب الرشاد بشكل كبير من التعبير الجيني لجينات EGFR و BCL2، على العكس من ذلك زادت مستويات TGFβ و SMAD3 و BAX و P53 mRNA في خلايا HUH7 وكذلك خلايا HEPG2 مقارنة باالخلايا غير المعالجة. كما كشفت حسابات الالتحام الطيفي ان المركب 17 (يوجينول) لمسخلص الميثيلين كلوريد ضد EGFR النشط جاذبية عالية (درجات الالتحام 30.46- كيلو كالوري / مول) علاوة على ذلك تم العثور على 13 مسارًا للإشارات لتكون من أهم المسارات التي يستهدفها Eugenol ، مما قد يفسر إمكانياته كعامل علاجي لعلاج السرطان.