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Multifunctional properties of *Phlomis aurea* extracts: In-vitro antioxidant, antimicrobial, anticancer, potent repellency against two mosquito vectors and molecular docking studies

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Abstract
To develop economically viable and environmentally benign methodologies for organic reactions and reveal the practical utility of transitional natural compounds and their derivatives. In addition, a new research method to conduct docking studies against nuclear factors sheds light on the theoretical mechanism of action of *Phlomis aurea* extracts as antioxidant, antimicrobial, anticancer, and repellent. The pharmacological potential of *Phlomis aurea* is investigated in this research by analysing its aqueous and petroleum ether extracts. So, to evaluate antioxidant activity, the DPPH scavenging test was used and compared against ascorbic acid; aqueous extract showed noteworthy activity. Both extracts demonstrated noteworthy efficacy against various pathogens, such as *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans*. The anti-cancer activity was also assessed using in-vitro assay on a standard cell line (Wi38) and two cancer cell lines (MDA and HepG2). The sensitivity of starving female *An. pharoensis* to the studied extracts was higher than that of *Cx. pipiens*, suggesting that these extracts may have potential applications in vector control. Docking study against nuclear factor erythroid 2–related factor 2 (Nrf2) (PDB ID: 3wn7), topoisomerase IV (PDB ID: 7lhz), COX protein (PDB ID: 6y3c), and Odorant Binding Protein 7 (OBP7) (PDB ID: 3r1o), to shed light on the theoretical mechanism expected as anti-oxidant, anti-microbial, anti-cancer and repellent effects against mosquitoes respectively, for galic acid as most significantly quantifying compounds on both extracts; highlighting the predicted mechanism of the proposed in-vitro assay, and confirming the present result.

Keywords *Phlomis aurea*, *Culex pipiens*, *Anopheles pharoensis*, Antibacterial activity, Cytotoxicity

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Introduction

Phlomis aurea, a member of the Lamiaceae family, has been historically recognised for its medicinal properties. The potent phenolic and flavonoid constituents of *Phlomis aurea* are the main contributors to its antioxidant effects [1]. Owing to their propensity to eliminate free radicals, these chemicals effectively alleviate oxidative stress, a significant factor in developing numerous chronic diseases. A direct association was established between the phenolic compounds and the reported antioxidant activity using sophisticated spectrophotometric techniques to assess the antioxidant capacity [1].

Extract of *Ph. aurea* extracts demonstrate antibacterial activity against various bacterial species with a noteworthy inhibitory effect against Gram-positive bacteria. This activity was ascribed to the presence of lipophilic chemicals in the extract; Phlomis genus promises a significant; Phlomis genus promises significance in an era where antibiotic resistance is on the rise [2]. In addition, the anticancer properties of hold potential anti-cancer action the Phlomis genus hold potential anti-cancer action, which is attributed to the induction of apoptosis and suppression of cell proliferation. This potentially serves as a vital asset in of advancing innovative anti-cancer treatments [1].

Mosquitoes are the most important group of insects because of their importance to public health [3, 4]. Mosquitoes are responsible for the transmission of many pathogens, such as Western Nile Virus (WNV) filariasis, malaria, Rift Valley Fever (RVF), dengue, and encephalitis [5–8]. Over and unwise use of chemical insecticides in vector control has led to the environmental hazards through the accumulation of toxic non-biodegradable compounds in the ecosystem, developing insecticide resistance in mosquito species, and toxic effects on human and non-target organisms [9, 10]. To eliminate the hazards of chemical insecticides, plant-derived materials are considered safer and easily environmentally biodegradable [11]. In addition, several plant species were confirmed to possess activity as larvicidal and repellent agents against different mosquito species [12–14].

Consequently, the principal objective of this study is to undertake a comprehensive inquiry into the pharmacological characteristics of *Ph. aurea.* These botanical species have long been acknowledged for their therapeutic advantages.

Materials and methods

Phlomis aurea extraction

The present study is based on dried materials of *Phlomis aurea* (Lamiaceae) Decne. Which was investigated by Cairo University Herbarium (CAI), Azhar University Herbarium and Saint Catherine Protectorate Herbarium. The whole mature plant was ground into fine powder. Using a Soxhlet apparatus, 75 g of the dry powder was extracted with petroleum ether and water. Each of the following extracts was filtered using Whatman No. 1 filter paper, dried in a water bath to a semisolid state, and its yield was recorded before being stored in a refrigerator at 40 °C until further use [15–17].

High-performance liquid chromatography (HPLC)

The HPLC analysis was performed with an Agilent 1260 series instrument. Using a Zorbax Eclipse Plus C8 column (4.6 mm x 250 mm, i.e., five µm), the separation was accomplished. At a flow rate of 0.9 ml/min, the mobile phase comprised water (A) and 0.05 per cent trifluoroacetic acid in acetonitrile (B). The mobile phase was sequentially coded in the following linear gradient fashion: 0 min (82% A); 0–1 min (82% A); 1–11 min (75% A); 11–18 min (60% A); 18–22 min (82% A); 22–24 min (82 per cent A). At 280 nanometers, the multiwavelength detector was observed. A volume of 5 µl was injected for each of the sample solutions. At 40 °C, the column temperature was maintained [18, 19].

Evaluation of antioxidant activity by DPPH radical scavenging method

The activity of leaf extracts in scavenging free radicals was assessed using 1,1-diphenyl-2-picryl hydrazyl (DPPH). 0.1 mM DPPH solution in ethanol was, in brief, made. One millilitre of this solution was added to 3 millilitres of various ethanol extracts at the following concentrations: 3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml. The extracts that solubilize in ethanol are solely utilised in this study; their concentrations were determined by dilution. 30 min after aggressively shaking the mixture, it was left to rest at room temperature. Following this, absorbance at 517 nm was determined using a spectrophotometer (UV-VIS milton roy). The ascorbic acid reference standard was utilised, and the experiment was conducted in triplicate. Using a log dosage inhibition curve, the IC_{50} value of the sample, which represents the concentration of sample needed to inhibit 50% of the DPPH free radical, was determined. A lower absorbance of the reaction mixture showed a greater degree of free radical activity.

Scavenging effect was calculated by using the following equation

$$DPPH\ scavenging\ effect\ (\%)\ or\ Percent\ inhibition = A_0 - A_1 / A_0 \times 100.$$

A0 represented the absorbance of the control reaction, while **A1** denoted the absorbance when the test or standard sample was present.

In-vitro assay of anti-microbial activity

Disk or well diffusion (Agar-Based) approaches

Differential diffusion in agar when testing plant or microbial extracts for antimicrobial activity, the agar well diffusion method is commonly employed. Like the disk-diffusion approach, this technique involves distributing a volume of microbial inoculum over the entire agar surface to inoculate the agar plate surface. The next step is to accurately measure the volume (20–100 mL) of the antimicrobial agent or extract solution at the correct concentration. A sterile cork borer or tip is used to aseptically punch a hole with a diameter of 6 to 8 mm. After that; the test microbe dictates the parameters that the agar plates are cultured in which the agar plates are cultured. The tested microbial strain is inhibited in its growth as the antimicrobial drug diffuses across the agar media [20].

CLSI disk or well diffusion testing guidelines

To determine if microbes are susceptible to a specific particular antibiotic, the well-sound diffusion test is the gold standard. This method provides numerical data (in the form of scalemillimetre zones) and qualitative information for interpretation (e.g., susceptible or resistant). Regarding testing for mould susceptibility, the CLSI has suggested a good diffusion approach. It is possible to get data from this disc diffusion approach after 16 to 48 h of incubation, which is a significant advantage (a shorter incubation time than the M38) [21, 22].

Mueller-Hinton agar plates from a regular bacteriology lab or those without supplements are used in the CLSI disc method. These plates have a pH of 7.2 to 7.4 after gelling, ideal for mould growth at 24–48 h. Some batches of Mueller-Hinton medium may not support sufficient growth of certain organisms; hence, it is essential to assess the appropriateness of each new batch according to CLSI standards. Therefore, well (disc) diffusion test results will often be, more significant than expected, and possibly surpass the permissible control limits [21].

The inoculum suspension used in the adjustment good method should be adjusted in the same way as the one used in the broth dilution standard method; then, the same way as the one used in the broth dilution standard method, within 15 min of making the adjustment adjusting, the agar plates should be infected. Streaks in three different directions cover the whole surface of the dried agar. After the agar has dried for at least 15 min, a sterile cork borer or a pointed object is used to aseptically punch a 6 to 8 millimeter hole in the surface. Volumes ranging from 20 to 100 µl of the antimicrobial agent or extract solution adjusted to the desired concentration and added to the well. It is recommended that the plates be incubated within 15 min of discarding the extract solution.

Intervals when growth noticeably decreases, measure the inhibition zone diameters (in mm) surrounding the wells to the nearest whole millimetre after incubation. If growth is not evident at the specified intervals, re-incubate the plates to read them later [22].

In-vitro assay of anti-cancer activity

Determination of sample cytotoxicity on cells

100 µl of 1×10^5 cells/ml were added to each well of the 96-well tissue culture plate and left to incubate at 37 °C for 24 h to form a full, complete monolayer. After a confluent layer of cells had grown, the growth material was removed from the 96-well microtiter plates. The cell monolayer was then washed twice with wash media. The tested material was diluted in RPMI medium with 2% serum times twice (maintenance medium). Separate wells were examined with 0.1 ml of each dilution, with three wells serving as controls and receiving only maintenance media. Once the plate had been incubated at 37 °C, it was examined. The cells were examined for any outward manifestations of toxicity, such as granulation, rounding, shrinkage, or loss of monolayer. A 5 mg/ml MTT solution in PBS was made (BIO BASIC CANADA INC). Each well was supplemented with 20 µl of MTT solution. After adding the MTT to the medium, shake the mixture at 150 rpm for 5 minutes. Metabolisation of the MTT can occur after four hours of incubation at 37 °C with 5% CO_2. In 200 µl of DMSO, dissolve formazan, a metabolic product of MTT. To ensure that the formazan and solvent are well mixed, shake at 150 rpm for 5 minutes. Density of light Reeded at 560 nm and subtract background at 620 nm. The quantity of cells has a direct correlation with optical density.

Morphological assay

Cell viability was followed by and related to large-scale morphological changes on the cell surface or in the cytoskeleton. Deterioration is defined by drastic reductions in volume as a result of protein and intracellular ion losses caused by changes in sodium or potassium permeability. Nuclear enlargement, chromatin flocculation, and diminished nuclear basophilia are hallmarks of necrotic cells. Cell death in apoptotic cells includes nuclear condensation and disintegration as well as cell shrinkage.

Repellency activity

Collection, DNA extraction, and identification of tested mosquitos' species

Larvae of *Culex pipiens* and *Anopheles pharoensis* were obtained from their natural habitat (Faiyum Governorate, Egypt; 29°18×53.4″ N, 30°39×19.2″ E) via netting.

A DNA barcode was applied to the collected mosquito and the specimens were placed in 1.5 µL Eppendorf tubes for identification purposes. The DNA was extracted using

PureLink® Genomic DNA Kits (Invitrogen, Waltham, Massachusetts, USA). In short, 180 to 250 µL of tissue lysis buffer was added to each sample, and then 10 µL of proteinase K was added for every 180 µL of tissue lysis buffer. The mixture was then incubated at 56 °C for 4 h. Following the directions provided by the manufacturer (Invitrogen, Waltham, Massachusetts, USA), the liquid above the sediment was carefully moved to another tube. Vortexing was performed after adding 200 µl of ethanol and 200 µl of Lysis/Binding Buffer to the lysate. After that, the mixture was spun in a centrifuge at 10,000 xg for one minute. The DNA was extracted using 50 µl of elution buffer after two washes with wash buffers. It was then stored at -20 °C until needed.

A forward primer, LCO1490:5′-GGTCAACAAATCAT AAAGATATTGG-3′, and a reverse primer, LCO1490:5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3', were utilised to amplify the mitochondrial DNA of the cytochrome oxidase C.O.I (COI) [23]. A final reaction volume of 50 µl was used for the PCR amplification. This amount included 25 µl of 2X master mix solution (i-Taq, iNtRON, Seongnam, Korea), 2 µl of each primer, 4 µl of template DNA, 0.2 mg/ml of BSA, and 14.5 µl of nuclease-free water. The concentration of each primer was 0.2 µM. Tick thermal cycling consisted of a 10-minute denaturation step followed by 40 denaturation, annealing, and extension cycles at 95 °C, 46 °C, and 72 °C, respectively. Ten minutes of the final extension was performed at 72 °C. Using the transilluminator, the PCR amplicon was run on a 1% agarose gel stained with ethidium bromide to ensure the product's purity and yield (U.V. transilluminator, Spectroline, Westbury, USA). Using a Macrogen reagent, the PCR products were purified (Seoul, Korea). Before aligning nucleotide sequences of M. domestica COI, single-strand DNA sequencing was carried out.

Mosquitos rearing

A conventional method was utilized to procure the required female *Cx pipiens* for the experiment, with some small minor adjustments made [24]. Adult mosquitoes were housed in wooden cages of 30 × 30 × 30 cm and fed pieces of sponge soaked in a 10.0% sucrose solution every day for two days following hatching. Subsequently, the practice of egg-laying females consuming a blood meal was legalised (an autogeny). A 15 × 15 centimetres plastic cup with distilled water was set in the cage for oviposition. The finished egg rafts were removed from the plastic container and placed into three 25 × 30 × 15 cm plastic pans with three litres of distilled water. Dog biscuits were given to the hatching larvae every day for their food.

The larvae of *An. pharoensis* were reared according to a previously standard procedure [10]. The adults were housed in wooden cages of 60 × 60 × 60 cm and given a 10% sucrose solution day. The plastic bowls that held the larvae measured 25 × 30 × 15 cm and contained 3 L of distilled water. A consistent temperature of 27 ± 2 °C, relative humidity of 70 ± 10%, and a light-dark cycle of 12:12 were all maintained for the colony.

Repellent activity of tested extracts

To determine the repellent activity of the studied extracts against *Cx. pipiens*, standard wooden cages measuring 30 × 30 × 30 cm were utilised and utilised. To determine the repellent activity against *An. pharoensis*, starving females were examined in wooden cages measuring 60 × 60 × 60 cm. To create different doses, various weights of extract were dissolved in 1 mL of either water or petroleum ether in 4 × 4 cm glass beakers. The mixture was then applied directly onto 5 × 6 cm of the ventral surface of a pigeon after its abdomen had been stripped of feathers. The repellency against *Cx. pipiens* and *An. pharoensis* was tested, and commercial repellent (DEET) served as a positive control. The pigeons were treated for 10 min and then kept in cages with 50 females of the *Cx. pipiens* or *An. pharoensis* species that had been deprived for five to seven days for three hours. Along with the treatments utilizing petroleum ether and water, control experiments were conducted. The average repellent value was determined by repeating each test three times [25]. The number of fed and unfed females was counted three hours after treatments, and the repellency percentage was estimated using Abbott's method [26].

Preparing protein for molecular docking research

Molecular docking is a potent computer method used to assess a ligand's potential energetic and geometric fit into the active site of a protein. Gaussian 09 was used to generate a file containing the structures of the compounds in the PDB format. From the protein data bank (http://www.rcsb.org.pdb), we obtained the crystal structures of the following proteins: Nrf2, topoisomerase IV, COX protein, Odorant Binding Protein 7, and topoisomerase IV (**PDB ID**: 3wn7, 7lhz, 6y3c, and 3r1o, respectively). The MOE 2015 program was used to conduct molecular docking investigations. Re-docked the co-crystallized ligand in its original structure using the program's default parameters. Amino acid interactions, hydrogen bond lengths in Ao, affinity measured in Kcal/mol, and the docking energy score were all visualised.

Result

Water extract yields about 7.5 g (10%), while petroleum ether yields about 1.5 g (2%). Table 1; Fig. 1 and supplementary data Table (S1) & Figure (S1) presents a quantitative analysis of phenolic and flavonoid compounds in aqueous and petroleum ether extracts of *Phlomis aurea*, measured using High-Performance Liquid Chromatography (HPLC). Compounds like Gallic acid, Chlorogenic

Table 1 Quantitative analysis of Phenolic and Flavonoid compounds in aqueous and eetroleum ether extract in HPLC profiles

Compounds	Aqueous extract			Petroleum ether extract		
	Area	Conc. (µg/ml)	Conc. (µg/g)	Area	Conc. (µg/ml)	Conc. (µg/g)
Gallic acid	1538.44	135.6	6780.4	1280.7	112.88	5644.44
Chlorogenic acid	206.22	27.33	1366.83	101.53	13.45	672.93
Catechin	78.32	17.5	875.01	9.95	2.22	111.18
Methyl gallate	87.66	4.51	225.81	50.16	2.58	129.23
Coffeic acid	581.97	46.16	2308.25	45.46	3.6	180.33
Syringic acid	193.15	14.41	720.6	270.25	20.16	1008.26
Pyro catechol	0	0	0	0	0	0
Rutin	10.03	1.55	77.5	21.87	3.37	168.89
Ellagic acid	40.64	4.18	209.05	34.59	3.55	177.92
Coumaric acid	150.71	5.5	275.18	526.75	19.23	961.76
Vanillin	365.97	13.9	695.07	922.66	35.04	1752.39
Ferulic acid	32.1	1.91	95.76	43.35	2.58	129.31
Naringenin	45.66	4.27	213.88	116.49	10.91	545.56
Rosmarinic acid	104.63	11.4	570.39	23.15	2.52	126.2
Daidzein	6.71	0.38	19.43	4.66	0.26	13.49
Querectin	17.01	2.26	113.11	40.57	5.39	269.73
Cinnamic acid	53.85	0.99	49.56	106.8	1.96	98.29
Kaempferol	7.1	0.45	22.9	2.38	0.15	7.68
Hesperetin	10.19	0.51	25.91	4.21	0.21	10.71

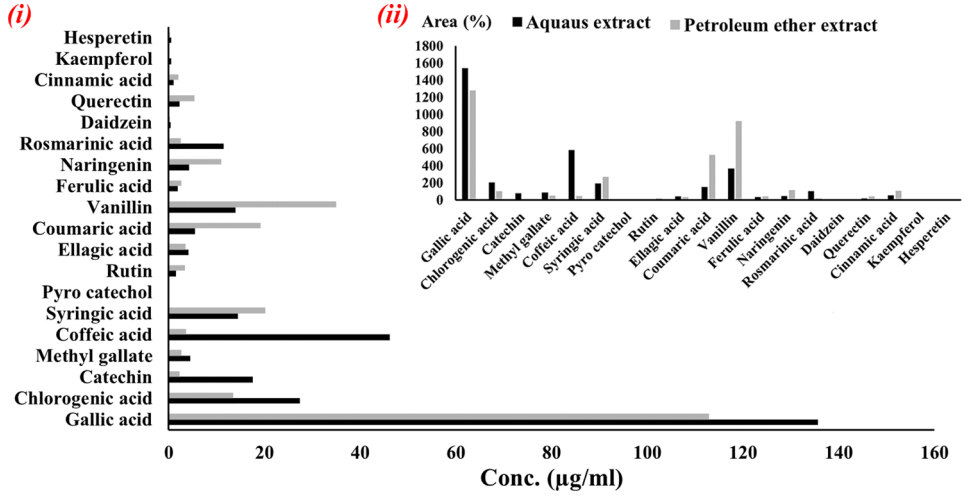

Fig. 1 (**i**) Phenolic and flavonoid compounds concentration (µg/ml) and (**ii**) Area % in aqueous and petroleum ether extract in HPLC profiles

acid, Catechin, and others are quantified in terms of their concentration in µg/ml and µg/g in both extracts. Notably, activity gallic acid shows a high concentration in the aqueous extract, which indicates its possible antioxidant activity, as described by Kumar and Pruthi [27]. Chlorogenic acid, found in moderate amounts, is known for its anti-inflammatory and neuroprotective effects, as mentioned by Tajik et al. [28]. Catechin, a well-known flavonoid, has been documented for its anti-cancer properties by Kozłowska and Szostak-Węgierek [29]. These compounds, along with others like Ferulic acid and Quercetin, which are present in varying concentrations, contribute to the overall pharmacological potential of the extracts, including anti-inflammatory, antioxidant, and anticancer activities.

In-vitro assay of antioxidant activity

A comparative examination of the antioxidant activity of aqueous and petroleum ether extracts of *Ph. aurea* and the standard ascorbic acid, was conducted using the DPPH scavenging assay (Table 2) and Fig. 2. The antioxidant capability is evaluated through the utilizationutilisation utilisation of optical density (OD), DPPH scavenging percentages, and IC_{50} values. With the lowest IC_{50} value, ascorbic acid exhibits the most significant antioxidant activity as expected due to its potent radical scavenging characteristics. Aqueous and petroleum ether extracts

Table 2 Optical density, DPPH scavenging % and IC_{50} of aqueous and petroleum ether extract of *Phlomis aurea* in comparison to stander ascorbic acid

Groups	Conc. (µg/ml)	OD ± SD	DPPH scavenging%	SD	SE	IC_{50} (µg/ml)
Stander Ascorbic Acid	1000	0.024 ± 0.001	98.5	0.002	0.001	2.85
	500	0.1 ± 0.0009	93.7	0.001	0	
	250	0.139 ± 0.002	91.3	0.003	0.001	
	125	0.205 ± 0.002	87.1	0.003	0.001	
	62.5	0.317 ± 0.001	80	0.002	0.001	
	31.25	0.433 ± 0.002	72.7	0.003	0.001	
	15.625	0.564 ± 0.002	64.5	0.003	0.001	
	7.8125	0.686 ± 0.003	56.8	0.004	0.001	
	3.9	0.791 ± 0.0009	50.1	0.001	0	
	1.95	0.831 ± 0.003	47.7	0.004	0.001	
	0	1.587 ± 0.002	0	0.003	0.001	
Aqueous extract	1000	0.061 ± 0.002	96.2	0.003	0.001	12.25
	500	0.143 ± 0.001	91	0.002	0.001	
	250	0.27 ± 0.002	83	0.003	0.001	
	125	0.394 ± 0.001	75.2	0.002	0.001	
	62.5	0.507 ± 0.004	68.1	0.005	0.002	
	31.25	0.639 ± 0.003	59.7	0.004	0.001	
	15.625	0.757 ± 0.0008	52.3	0.001	0	
	7.8125	0.873 ± 0.0008	45	0.001	0	
	3.9	0.989 ± 0.001	37.7	0.002	0	
	1.95	1.106 ± 0.001	30.3	0.002	0.001	
	0	1.587 ± 0.002	0	0.003	0.001	
Petroleum ether extract	1000	0.179 ± 0.003	88.7	0.004	0.001	24.94
	500	0.297 ± 0.001	81.3	0.002	0.001	
	250	0.406 ± 0.001	74.4	0.002	0.001	
	125	0.525 ± 0.001	66.9	0.002	0.001	
	62.5	0.643 ± 0.001	59.5	0.002	0	
	31.25	0.768 ± 0.001	51.6	0.002	0.001	
	15.625	0.886 ± 0.002	44.2	0.003	0.001	
	7.8125	0.98 ± 0.002	38.2	0.003	0.001	
	3.9	1.093 ± 0.002	31.1	0.003	0.001	
	1.95	1.208 ± 0.002	23.9	0.003	0.001	
	0	1.587 ± 0.002	0	0.003	0.001	

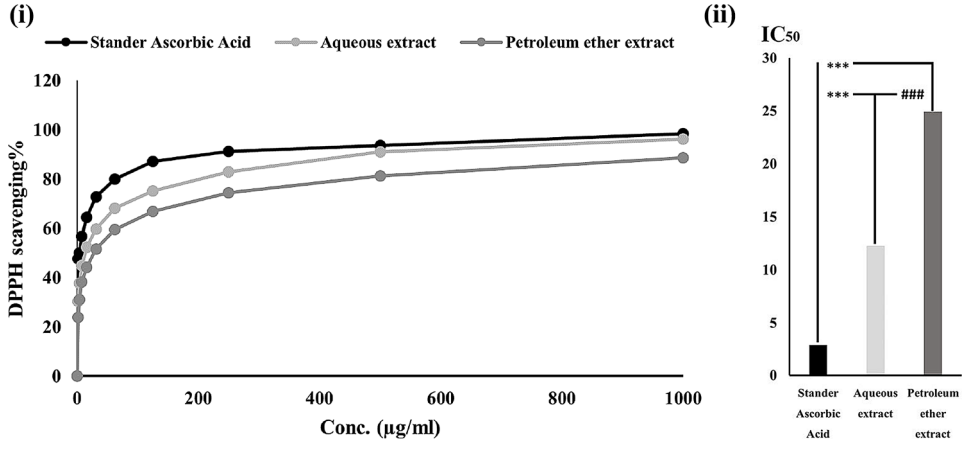

Fig. 2 (**i**) DPPH scavenging % and (**ii**) IC_{50} of aqueous and petroleum ether extract of *Phlomis aurea* in comparison to stander ascorbic acid. ***Note***: * or # = Sig. ($P < 0.05$); ** or ##= High Sig. ($P < 0.01$); *** or ###= Very high sig. ($P < 0.001$)

exhibited a statistically significant difference in antioxidant activity ($t = 224.77$, $P < 0.001$; $t = 394.86$, $P < 0.001$, respectively) compared to standard ascorbic acid. A comparable pattern was identified ($t = 170.09$, $P < 0.001$) wherein the aqueous extract exhibited a comparative advantage over the petroleum ether extract.

In-vitro assay of antimicrobial activity

In-vitro antibacterial assay was shown in Figs. 3 and 4 showcasing the effects of aqueous and petroleum ether extracts of *Phlomis aurea* against a range of pathogens. Both extracts had antibacterial activity; however, the efficacy of the aqueous extract was often higher. The aqueous extract demonstrates a more pronounced zone of inhibition against *Salmonella typhi*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans* than the petroleum ether extract and the positive control, the variation was not significant ($P > 0.05$) of both extracts in comparison to positive control which show remarkable antimicrobial activity. The increased efficacy in the aqueous extract may be ascribed to the elevated content of hydrophilic phytochemicals, recognized for their antibacterial characteristics. The lack of efficacy against *Mucor circinelloides* in both extracts indicates the presence of a selective antibacterial mechanism.

In-vitro assay of anti-cancer activity

The cytotoxic impacts of aqueous and petroleum ether extracts on a diverse array of cell lines two cancer cell lines (MDA representing adenocarcinoma breast cancer and HepG2 representing hepatocellular carcinoma) and a normal cell line (Wi38). In the case of the Wi38

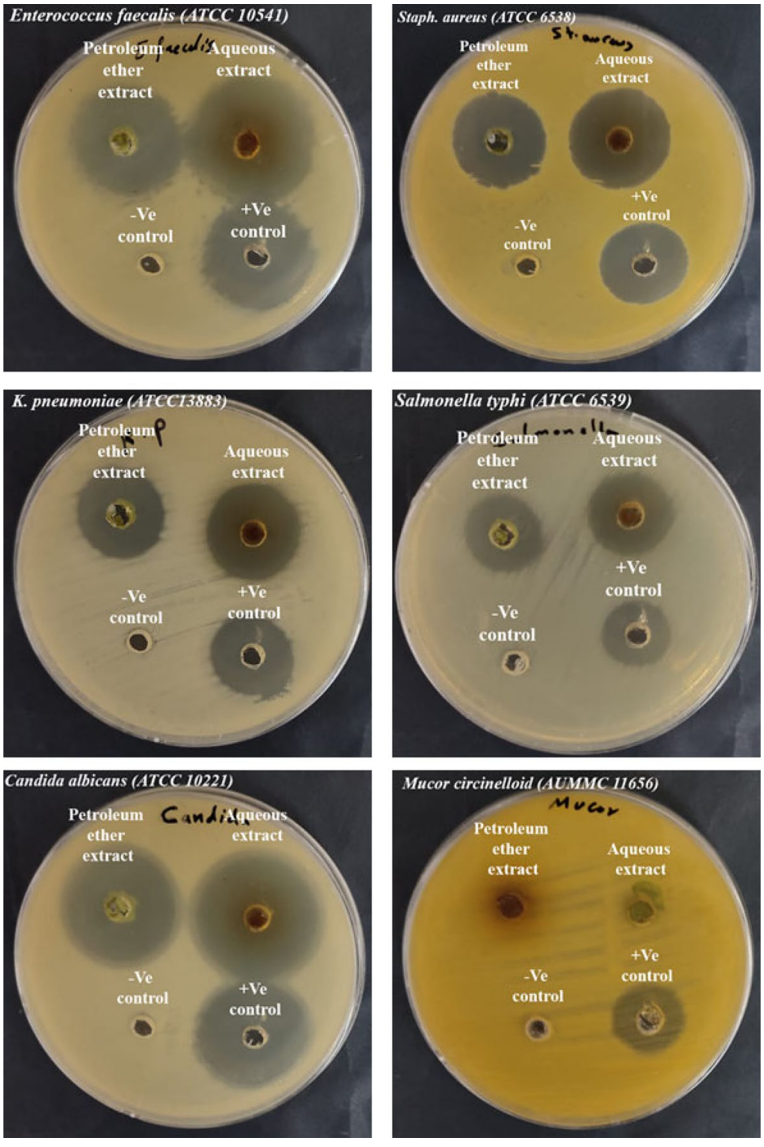

Fig. 3 In-vitro assay of anti-microbial assay for aqueous and petroleum ether extract of *Phlomis aurea*

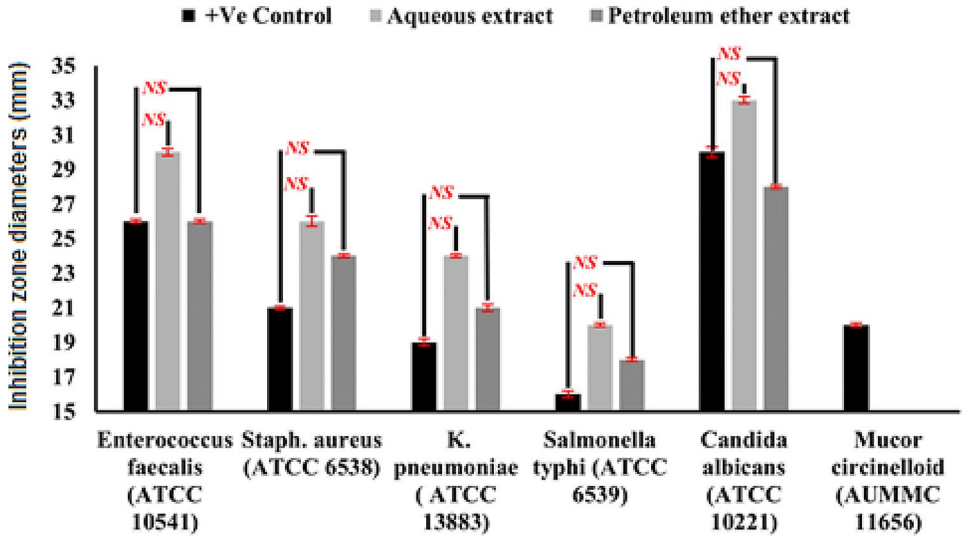

Fig. 4 In-vitro assay of anti-microbial assay for aqueous and petroleum ether extract of *Phlomis aurea*. ***Note***: *NS = non-significant*

(normal cell line), the IC_{50} values for the aqueous extract and petroleum ether extract were 378.46 ± 4.49 µg/ml and 417.17 ± 1.36 µg/ml, respectively, signifying moderate toxicity. Conversely, the IC_{50} values for the aqueous extract and petroleum ether extract were found to be 164.37 ± 0.85 µg/ml and 99.32 ± 1.49 µg/ml, respectively, for the MDA cancer cell line. These results indicate a greater degree of sensitivity and possible therapeutic utility of the extracts. In the same way, the sensitivity of the HepG2 cell line was higher in the aqueous extract, which exhibited an IC_{50} of 94.98 ± 0.77 µg/ml, in contrast to the petroleum ether extract, which demonstrated an IC_{50} of 188.76 ± 1.55 µg/ml.

Cell viability percentages exhibit a negative correlation with toxicity percentages; thus, as predicted by dose-response relationships, these percentages decline as extract concentration increases. Significantly, the viability percentage of all cell lines is consistently greater in the aqueous extract at lower concentrations than in the petroleum ether extract. This suggests that the aqueous extract may possess a less hazardous profile at those particular levels. An increase in extract concentration results in a drop in optical density (O.D) values, which indicate cell viability. This further substantiates the cytotoxic properties exhibited by the extracts.

For aqueous extract, cancer cell lines (MDA and HepG2) varied significantly in comparison to normal cell line Wi38 ($t = 57.69$, $P < 0.001$ & $t = 71.57$, $P < 0.001$ respectively). The same pattern was observed for the petroleum ether extract (t = 108.80, $P < 0.001$ & t = 47.42, $P < 0.001$ for MDA and HepG2 when compared to Wi38) (Table S2; Figs. 5 and 6). Aqueous or petroleum ether extracts exhibit cytotoxic characteristics. Notably, the impact on cancer cell lines is more pronounced when compared to the standard cell line, which is a good sign for the therapeutic potentiality of the extracts.

Figure (S2) illustrates the morphological alterations observed in various cell lines (MDA, Wi38, HepG2) to treatment with aqueous and petroleum ether extracts. These morphological changes are clear indicators in cytotoxicity assays, as they mirror the cellular reactions to both extracts. The observed images depict discrepancies in cell density, shape, and integrity. These alterations, which encompass cell shrinkage, adherence loss, and granularity modifications, and indicate cellular responses to the chemicals in both extracts.

Data in Table 3 and Fig. 7 showed the repellent activity of aqueous and petroleum ether extracts of *Ph. aurea* against *Culex pipiens* and *Anopheles pharoensis* starved females. The petroleum ether extract was more effective than the the aqueous extract during the entire testing period of 3 h post treatment aqueous extract during the entire testing period of 3 h post-treatment aqueous extract during the entire testing period of 3 h post-treatment and 3 h post-treatment testing period. Also, *An. pharoensis* starved females exhibited more sensitivity to tested extracts than *Cx. pipiens*. The repellent activity recorded 81.20 and 85.10% against *Cx. pipiens* and *An. pharoensis*. In comparison, phronesis starved females with *Ph. aurea* petroleum ether extract at the highest dose (1.67 mg/cm^2). At the same time, it recorded 67.82 and 93.91% by aqueous extract at the same dose, compared with 100.0% repellency recorded by positive control (DEET) at 1.80 mg/cm^2, respectively.

Also, *Ph. aurea* petroleum ether extracts recorded RD_{50}, RD_{75} and RD_{90} of 0.550, 1.429, and 1.956 mg/cm^2 against *Cx. pipiens* females, vs. 0.063, 1.099, and 1.585 mg/cm^2 against *An. pharoensis* females (Table 4).

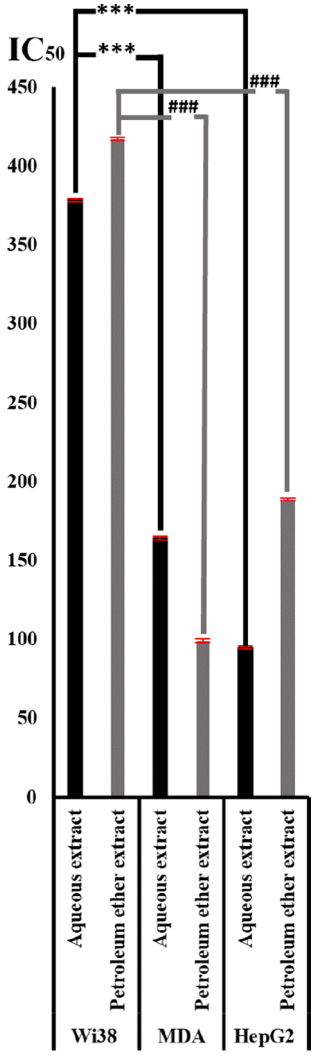

Fig. 5 IC_{50} for in-vitro assay of *Phlomis aurea* aqueous and petroleum ether extracts. **Note**: * or # = Sig. ($P < 0.05$); ** or ##= High Sig. ($P < 0.01$); *** or ###= Very high sig. ($P < 0.001$)

The High-Performance Liquid Chromatography (HPLC) provided information offers significant insights into the potential pharmacological properties of the investigated plant. Notably the concentration and presence of compounds such as chlorogenic acid, catechin, and gallic acid. The antioxidant capabilities of gallic acid, which is present in significant amounts in the aqueous extract, have been extensively reported. The significance of gallic acid as a potent antioxidant, capable of eliminating free radicals and so aiding in the mitigation of oxidative stress inside the body, has been underscored by Kumar and Pruthi [27]. Considering the correlation between oxidative stress and a multitude of chronic ailments, such as cancer and cardiovascular disease, this activity is of the utmost importance.

Nrf2 (Nuclear factor erythroid 2–related factor 2) (PDB ID: 3wn7) utilized to test the antioxidant activity due to essential role for cellular protection against oxidative stress. It controls the expression of antioxidant proteins, which are responsible for safeguarding against oxidative harm inflicted by inflammation and injury [30]. In terms of antibacterial action, bacterial topoisomerase IV (**PDB ID**: 7lhz) facilitated for its vital role in bacterial DNA replication which targeted frequently by antibiotics (Hardy, 2022). To validate in-vitro assay of the anticancer activity, COX protein (PDB ID: 6y3c) was chosen to evaluate the theoretical mechanism of Galic acid, it has been chosen for its crucial involvement in inflammation associated with cancer, its classified as a therapeutic target, and the possibility of selective targeting that may result in reduced adverse effects (Mahesh et al., 2021). Lastly, to elicit repellent effects against mosquitoes, Odorant Binding Protein 7 (OBP7) (**PDB ID**: 3r1o), a class of small, soluble proteins that are ubiquitous in insect olfactory systems and are essential for the recognition of odour compounds [31].

Molecular docking for the proposed Gallic acid compound as it the most quantifying compound on both aqueous and petroleum ether extract by significant margin to the nearest second abandoned compound. The docking study was performed against active site of nuclear factor erythroid 2–related factor 2 (Nrf2) (**PDB ID**: 3wn7), topoisomerase IV (**PDB ID**: 7lhz), COX protein (**PDB ID**: 6y3c), and Odorant Binding Protein 7 (OBP7) (**PDB ID**: 3r1o), to highlight light the mechanism expected as Antioxidant, Anti-Microbial, Anti-cancer and repellent effects against mosquitoes respectively. The crucial amino acids' binding mechanisms and interactions were identified by docking studies. Docking the co-crystallized ligands acetate ion, (4s)-2-methyl-2,4-pentanediol, citrate anion, and palmitic acid against the pocket's active site proved the technique was correct. The tested Gallic acid gave good energy scores (S) = -4.6622, -4.2821, -4.4849, and – 5.9359 kcal/mol respectively, which was higher or nearly equal to the ligands score= -3.6765, -4.168, -4.5458, and – 6.649 kcal/mol respectively (Table 5). The proposed binding pattern for Gallic acid is represented in Fig. 8. The obtained results indicated that the tested compound Gallic acid has good energy scores which align to the in-vivo assays, confirming the proposed activity mentioned earlier.

Disscusion

The High-Performance Liquid Chromatography (HPLC) provided information offers significant insights into the potential pharmacological properties of the investigated plant [32–35]. Notably the concentration and presence of chlorogenic acid, catechin, and gallic acid compounds. The antioxidant capabilities of gallic acid, which is present in significant amounts in the aqueous extract, have been extensively reported. The significance of gallic acid

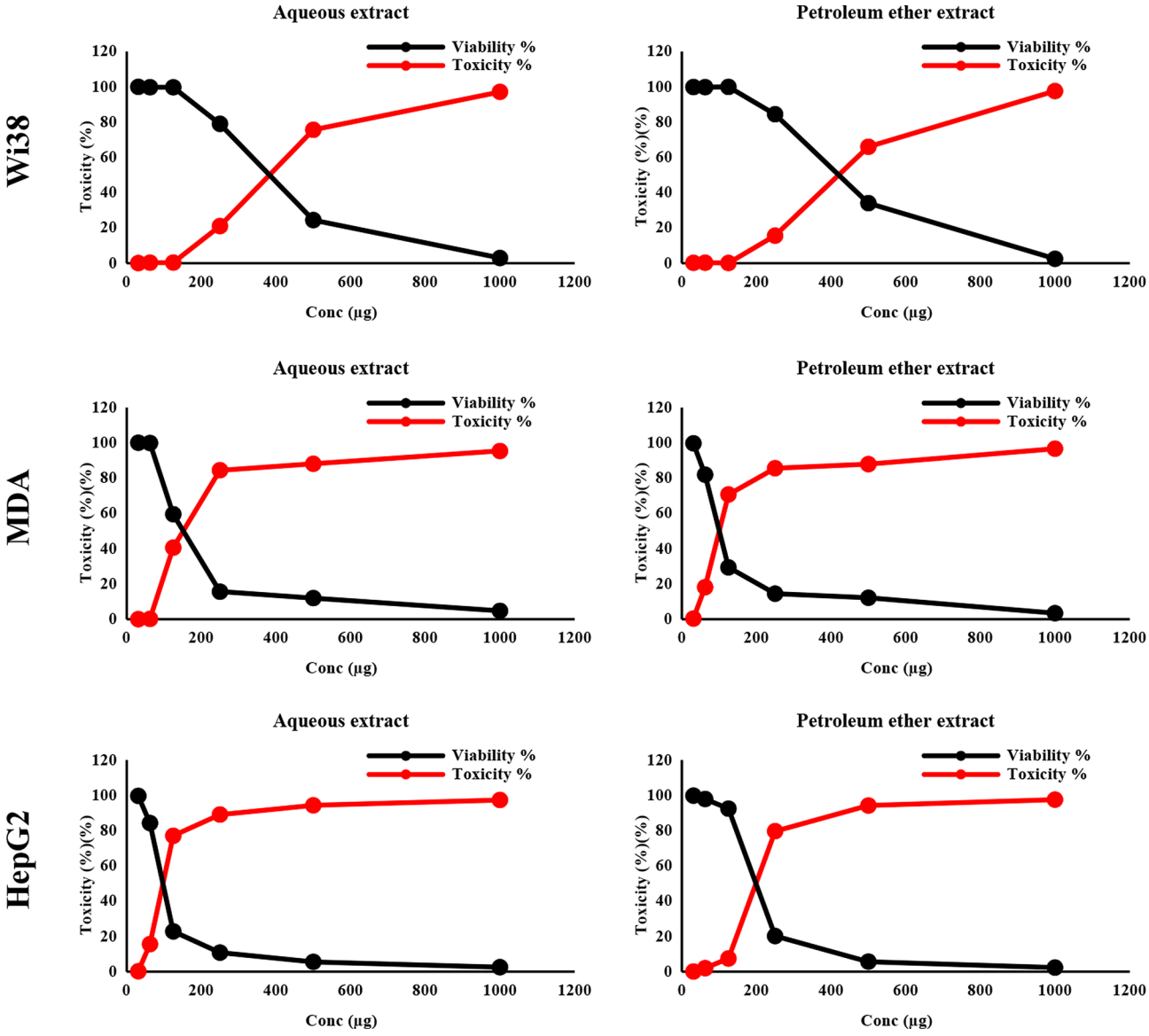

Fig. 6 Viability and toxicity of in-vitro assay of *Phlomis aurea* aqueous and petroleum ether extracts. ***Note***: * or # = Sig. ($P<0.05$); ** or ##= High Sig. ($P<0.01$); *** or ###= Very high sig. ($P<0.001$)

as a potent antioxidant, capable of eliminating free radicals and so aiding in the mitigation of oxidative stress inside the body, has been underscored [27]. Considering the correlation between oxidative stress and a multitude of chronic ailments, such as cancer and cardiovascular disease, this activity is of the utmost importance.

The moderate concentrations of chlorogenic acid found in the extracts have been identified as having anti-inflammatory and neuroprotective properties [28]. Several biological pathways implicated in inflammatory responses have been observed to be modulated by this compound, which has demonstrated potential as a neuroprotectant, specifically in relation to neurodegenerative disorders. Catechin, a widely recognized flavonoid proved to have anti-cancer benefits [29]. It has been demonstrated that catechin inhibits cancer cell proliferation and induces apoptosis, rendering it a substance of potential therapeutic significance in the field of cancer research. Additional components such as ferulic acid and quercetin, however, found in different amounts within the extracts, make substantial contributions to the therapeutic properties of *Ph. aurea*. A variety of biological actions, including anticancer, antioxidant, and anti-inflammatory, are attributed to these substances.

The in-vitro assay outcomes of present work, which utilised the DPPH scavenging assay to evaluate the antioxidant activity of extracts. It is not unexpected that ascorbic acid, renowned for its formidable antioxidant

Table 3 Repellent activity of *Phlomis aurea* extracts against *Culex pipiens* and *Anopheles pharoensis* starved females

Species	Tested Extracts	Dose mg/cm^2	Unfed Females %	Repellency %
Culex pipiens	aqueous	1.67	68.0 ± 5.29	67.82 ± 4.99
		0.83	56.67 ± 3.06	56.39 ± 2.61
		0.42	35.33 ± 5.03	34.93 ± 4.49
	Petroleum ether	1.67	81.33 ± 6.11	81.20 ± 6.16
		0.83	62.67 ± 4.16	62.44 ± 3.90
		0.42	42.67 ± 4.16	42.30 ± 3.75
Anopheles pharoensis	aqueous	1.67	94.0 ± 2.0	93.91 ± 2.08
		0.83	65.33 ± 6.43	64.83 ± 6.79
		0.42	54.0 ± 4.0	53.35 ± 4.52
	Petroleum ether	1.67	85.33 ± 6.43	85.10 ± 6.64
		0.83	72.0 ± 6.93	71.57 ± 7.30
		0.42	56.0 ± 3.46	55.41 ± 3.30
Positive Control (DEET)		1.80	0.0	100.0 ± 0.0
Negative Control		0.0	1.33 ± 1.16	0.0

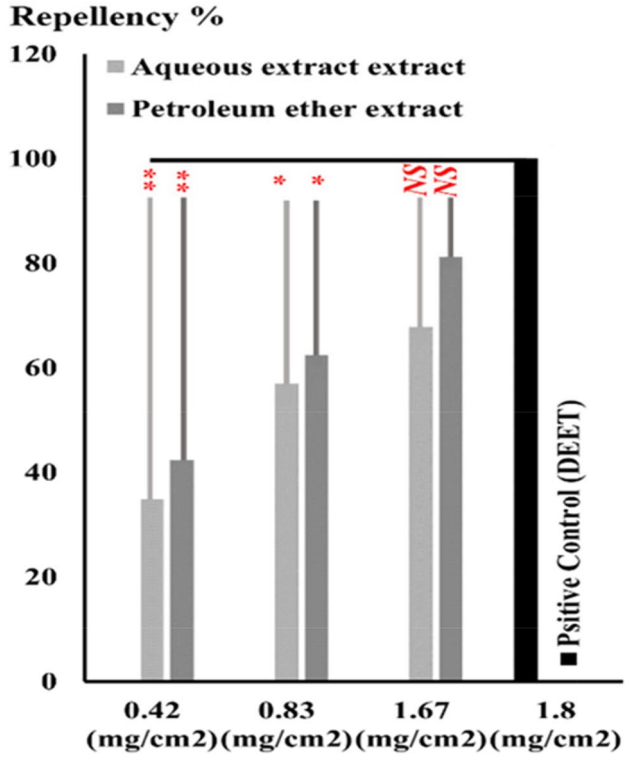

Fig. 7 Repellent activity of *Phlomis aurea* extracts against *Culex pipiens* and *Anopheles pharoensis* starved. ***Note***: * or # = Sig. ($P < 0.05$); ** or ##= High Sig. ($P < 0.01$); NS = non-significant

attributes, exhibits the greatest antioxidant activity in the present study while maintaining the smallest IC_{50} value. This discovery is consistent with the extensively documented effectiveness of ascorbic acid in scavenging free radicals, as documented in numerous studies, which provides further details on the mechanism of action and wide-ranging antioxidant capabilities of ascorbic acid [36].

Remarkably, the aqueous extract exhibited substantial antioxidant activity, as evidenced by its moderate IC_{50} value. This implies that the aqueous extract is comprised of a significant quantity of bioactive compounds that can counteract free radicals. This is consistent with the results reported, which highlighted the antioxidant effects demonstrated by plant extracts on account of their abundant phenolic and flavonoid constituents [37].

Conversely, the petroleum ether extract has less antioxidant activity, as seen by its elevated IC_{50} value, which implies an alternative constituent or reduced concentration of active antioxidant chemicals. This finding aligns with other findings, where the extraction of antioxidant chemicals is substantially impacted by solvent characteristics [38].

It is typical for antioxidant experiments to see a dose-dependent antioxidant response in both extracts, with increasing scavenging percentages and lowering OD at higher doses. As illustrated, the concentration-dependent nature of the scavenging activity of phytochemicals [39]. The statistical analysis demonstrates that the aqueous extract has an advantage over the petroleum ether extract, which aligns with the findings. This finding is consistent with the overall tendency that aqueous extracts include more polar antioxidant compounds.

In-vitro assay results for the antibacterial activity of both extracts against various pathogens are consistent with previous research that has shown the antimicrobial characteristics of natural plant extracts. It is worth noting that the aqueous extract exhibits superior overall effectiveness against pathogens such as *Salmonella typhi*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans*. This result is consistent with the findings of Cowan, who observed that aqueous extracts containing hydrophilic chemicals frequently demonstrate potent antibacterial characteristics [40]. The observed greater zone of inhibition in the aqueous extract as opposed to the petroleum ether extract may be ascribed to the

Table 4 Effective repellent doses of *Phlomis aurea* extracts against *Culex pipiens* and *Anopheles pharoensis* starved females

Species	Tested Extracts	RD_{50} (LCL-UCL)	χ2	RD_{75} (LCL-UCL)	χ2	RD_{90} (LCL-UCL)	χ2
Culex pipiens	aqueous	0.85 (0.48–1.22)	0.61^{NS}	1.88 (1.40–2.36)	1.21^{NS}	2.50 (1.92–3.07)	1.64^{NS}
	Petroleum ether	0.55 (0.26–0.84)	0.43^{NS}	1.43 (1.15–1.71)	1.14^{NS}	1.96 (1.37–2.55)	1.35^{NS}
Anopheles pharoensis	aqueous	0.33 (0.11–0.78)	0.50^{NS}	1.19 (0.59–1.78)	0.82^{NS}	1.87 (0.96–2.78)	1.21^{NS}
	Petroleum ether	0.06 (0.04–0.16)	0.89^{NS}	1.10 (0.80–1.40)	0.67^{NS}	1.59 (1.44–1.73)	1.11^{NS}

Note: LCL = Lower 95% Confidential Limit; UCL = Upper 95% Confidential Limit; χ^2 = Chi square value; NS = non-significant ($P > 0.05$)

Table 5 Docking interaction data calculations of Galic acid against nuclear factor erythroid 2–related factor 2 (Nrf2) (PDB ID: 3wn7), topoisomerase IV (PDB ID: 7lhz), COX protein (PDB ID: 6y3c), and odorant binding protein 7 (OBP7) (PDB ID: 3r1o) active spots

In-vitro profile	Docked protein	Ligands	Energy score (S) (Kcal/mol)	Ligand	Amino acid	Interaction	Affinity Bond length (in A° from main residue)	Affinity Bond strength (Kcal/mol)
Antioxidant	Nuclear factor erythroid 2–related factor 2 (Nrf2) (**PDB ID: 3wn7**)	**Ligand (Acetate Ion)**	-3.6765	O 6	ARG 415	H-acceptor	2.95	-6.4
				O 7	ARG 415	H-acceptor	2.91	-7.7
				O 6	ARG 415	ionic	3.41	-2.3
				O 6	ARG 415	ionic	2.95	-4.8
				O 7	ARG 415	ionic	2.91	-5.1
				O 7	ARG 415	ionic	3.84	-0.8
		Galic acid	-4.6622	O 6	VAL 463	H-donor	3.03	-0.9
				O 12	VAL 604	H-donor	3.11	-2
				O 17	ILE 559	H-donor	3.02	-3.5
				O 16	ILE 559	H-acceptor	3.03	-1.9
Anti-Microbial	Topoisomerase IV **(PDB ID: 7lhz)**	**Ligand ((4s)-2-Methyl-2,4-Pentanediol)**	-4.168	O 17	SER 494	H-acceptor	3.04	-1.9
				O 17	TYR 1024	H-pi	4.21	-0.9
		Galic acid	-4.2821	O 6	SER 494	H-donor	2.75	0.1
				O 17	PRO 1076	H-donor	2.81	-4.4
Anti-Cancer	COX protein **(PDB ID: 6y3c)**	**Ligand (Citrate anion)**	-4.5458	O 13	ARG 469	H-acceptor	2.95	-8.7
				O 15	GLN 44	H-acceptor	3.06	-1.6
				O 13	ARG 469	ionic	3.57	-1.6
				O 13	ARG 469	ionic	2.95	-4.8
				O 14	ARG 469	ionic	3.11	-3.8
				O 17	ARG 469	ionic	2.97	-4.7
				O 17	ARG 469	ionic	3.81	-0.9
				O 18	ARG 469	ionic	3.77	-1
		Galic acid	-4.4849	O 6	TYR 39	H-donor	2.98	-0.7
				O 17	LYS 468	H-donor	2.95	-0.8
Repellent activity	Odorant Binding Protein 7 **(OBP7)**	**Ligand (Palmitic acid)**	-6.649	O 49	TYR 10	H-pi	4.79	-0.6
		Galic acid	-5.9359	O 17	CYS 35	H-donor	4.38	-1.3

enhanced extraction efficiency of hydrophilic phytochemicals in solvents based on water.

The lack of efficacy against *Mucor circinelloides* in both extracts indicates a selective antibacterial effect, which has been verified in previous study, which targets some infections with minimal or no impact on others [41]. The ability to selectively target antimicrobial medicines is of utmost importance due to the reduced likelihood of detrimental effects on beneficial microorganisms. It is noteworthy that both extracts exhibited antimicrobial activity and did not differ significantly from the positive control in terms of inhibition. This result is consistent with the conclusions drawn by Nascimento et al. who under specific circumstances similarly saw similar levels of effectiveness when using natural plant extracts versus conventional antibacterial drugs [42]. The aforementioned discoveries highlight the potential of natural extracts as supplementary or alternative therapies to conventional antimicrobial medications, particularly in light of the increasing prevalence of antibiotic resistance.

The in-vitro assay results pertaining to the anti-cancer properties of Phlomis aurea extracts are highly persuasive, specifically in regard to the discernible distinctions in effects between the normal cell line (Wi38) and cancer cell lines (MDA representing breast adenocarcinoma and HepG2 representing hepatocellular carcinoma). Consistent with prior investigations in the field, the results demonstrate that the aqueous and petroleum ether extracts of Phlomis aurea demonstrate substantial cytotoxicity towards cancer cell lines, as seen by their lower IC_{50} values in comparison to the normal cell line [43]. Significant is the fact that both extracts exhibit a moderate degree of toxicity towards the Wi38 normal cell line, as demonstrated by their higher IC_{50} values. This particular element of the investigation is consistent with the previously findings, where numerous natural compounds demonstrate some level of toxicity towards healthy cells, underscoring the importance of meticulous assessment and dosing optimization in therapeutic settings [44].

A common observation in cytotoxicity experiments is the decrease in cell viability percentages with increasing

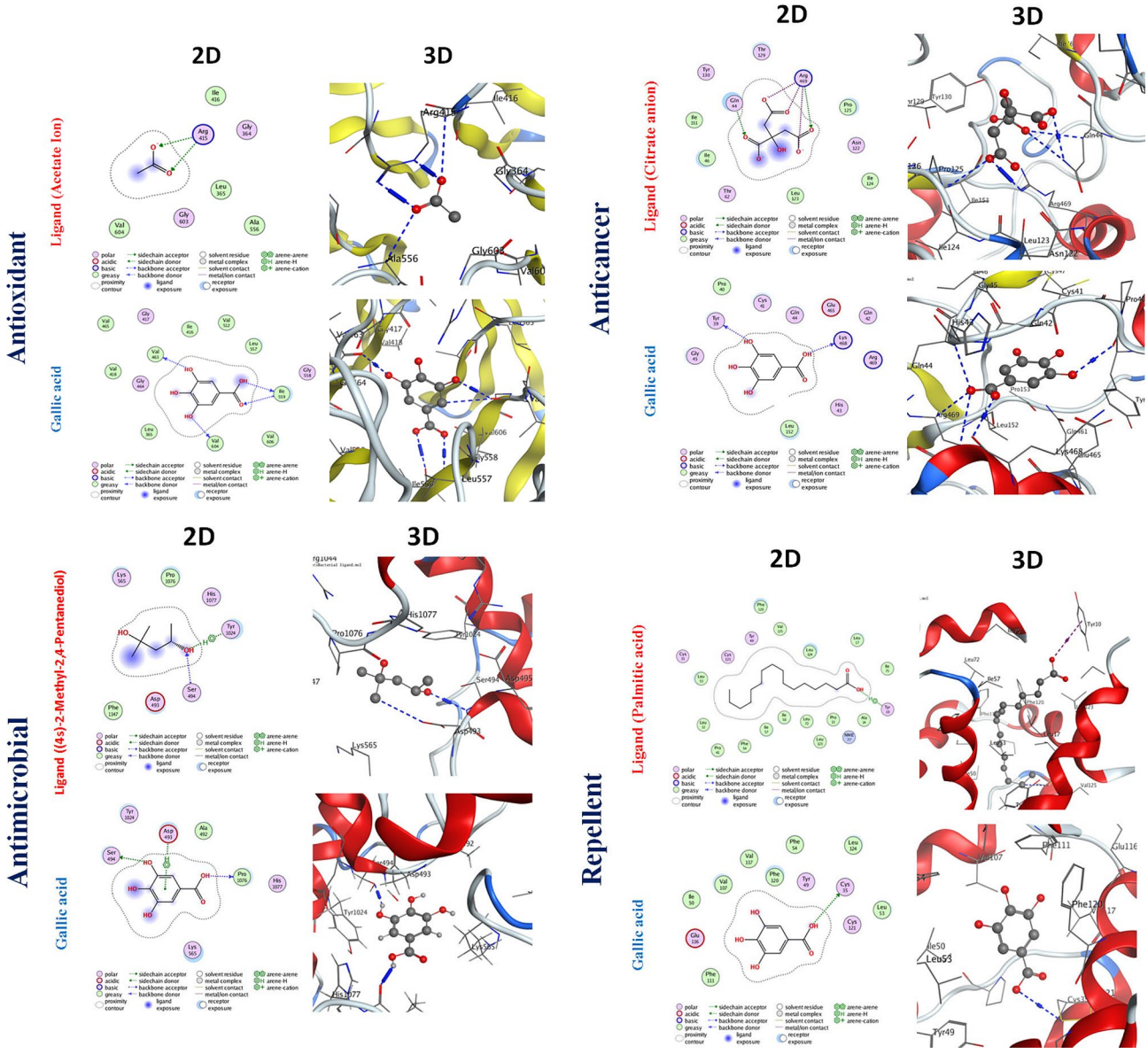

Fig. 8 2D& 3D interactions of Galic acid against nuclear factor erythroid 2–related factor 2 (Nrf2) (PDB ID: 3wn7), topoisomerase IV (PDB ID: 7lhz), COX protein (PDB ID: 6y3c), and Odorant Binding Protein 7 (OBP7) (PDB ID: 3r1o) active spots

extract concentration, which corresponds to the dose-response relationship documented in Kuete et al. study, who conduct research on comparable assays, which serves to underscore the significance of dose-dependency when assessing the potential safety and effectiveness of anticancer drugs [45]. Moreover, the finding that the aqueous extract consistently exhibits a greater proportion of viable cells at lower doses across all cell lines in comparison to the petroleum ether extract implies that the profile of toxicity may be comparatively lower at those concentrations. This discovery aligns with the previously results reported, which suggest that the toxicity profile of the resulting extracts might be substantially impacted by the solvent employed during extraction [46].

Obtained data revealed that aqueous and petroleum ether extracts of *Ph. aurea* exhibited a variable repellent activity against both *Culex pipiens* and *Anopheles pharoensis* starved females depending on solvent used in extraction and the dose of the extract. Generally, petroleum ether extract was more effective than aqueous extract. Also, *An. pharoensis* females showed more sensitivity to tested extracts than those of *Cx. pipiens.* The repellent activity of tested extracts reflects the complexity of their constituents and *Ph. aurea* chemical composition [47]. Thus, obtained repellent activity can be attributed to the presence of various compounds, including terpenoids, phenolics, and alkaloids which exist in *Ph. aurea*, and these compounds may jointly or

independently contribute to produce the repellent activity [48, 49]. Obtained results of repellent activity exhibited by *Ph. aurea* tested extracts confirms the previously results using *Delonix elata* extracts against *An. stephensi* and they reported that, both leaf and seed methanol extracts showed maximum efficacy at 5.0 mg/cm^2; different extracts of *Otostegia fruticosa* leaves against *Cx. pipiens* females where methanol and ethyl acetate extracts recorded 64.13 and 75.09% repellency at 3.33 mg/cm^2, respectively and hexane extract recorded repellency of 88.08, 79.01 and 75.50% at 3.33, 1.67 and 0.83 mg/cm^2; *Pyrus communis* hexane extract, which had to be more effective as repellant agent against *An. pharoensis* females than methanol extract, where at 5.0 mg/cm^2 potent repellency (95.5%) obtained by hexane extract, while methanol extract exhibited 80.0% repellency at the same dose [50–52].

The extensive examination of *Phlomis aurea* via a variety of in-vitro experiments reveals the plant's varied pharmacological potential. Significant progress is made in the comprehension of *Ph. aurea's* bioactive chemicals and their therapeutic implications as a result of the study's findings. The aqueous and petroleum ether extracts underwent high-performance liquid chromatography (HPLC) examination, which unveiled a substantial quantity of phenolic and flavonoid components, including chlorogenic acid, gallic acid, and catechin. The variability in the amounts of these components, with gallic acid being especially abundant in the aqueous extract, serves to emphasize the antioxidant properties of the plant. Additional evidence of the extracts of *Ph. aurea's* antibacterial properties against a range of pathogens, was provided by in-vitro assay. Particularly, the aqueous extract demonstrated enhanced antibacterial activity, which can be attributed to its elevated quantity of hydrophilic phytochemicals. Considered essential in light of the escalating issue of antibiotic resistance, this discovery proposes *Ph. aurea* as a prospective reservoir for the development of novel antimicrobial drugs. Within the domain of cancer research, the study yielded noteworthy findings, both extracts demonstrated discernible cytotoxic effects on cancer cell lines, although their toxicity towards the normal cell line was mild. The decreased toxicity profile of the aqueous extract at lower concentrations indicates its potential for therapeutic use, albeit requiring careful dosage to prevent damage to healthy cells. In addition, the study demonstrated that these extracts exhibited repellent properties against many kinds of mosquitoes, indicating their potential utility in vector control approaches. This feature presents *Ph. aurea* with novel opportunities in the realm of public health applications, specifically in areas where mosquito-borne diseases are prevalent. As a result of its varied bioactive components, Phlomis aurea possesses noteworthy antioxidant, antibacterial, anticancer, and repellant characteristics. These results provide encouraging possibilities for its incorporation into contemporary pharmacology. Nevertheless, the cytotoxicity that was found towards healthy cells underscores the need for additional investigation and meticulous deliberation when designing therapeutic interventions.

Supplementary Information

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Supplementary Material 1

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Data availability
The data supporting this article have been included as part of the Supplementary Information.

Declarations

Ethical approval
The study was carried out according to the guidelines of the declaration of Benha University and approved by the Faculty of Science Ethics Committee of Benha University (Code: BUFS-REC-2024-256Ent).

Consent to participate
Not applicable.

Consent to publish
Not applicable.

Competing interests
The authors declare no conflict of interest.

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