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Research Article

POTENTIAL CYTOTOXIC AND ANTIANGIOGENIC PROPERTIES OF *EHRETIA MICROPHYLLA* LEAF ETHANOLIC EXTRACT THROUGH THE MODULATION OF MEK AND VEGFR 2

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ABSTRACT: *Ehretia microphylla* (*E. microphylla*) leaf is commonly consumed as herbal tea, known for its various benefits, such as anti-inflammatory, anti-mutagenic, anti-mitotic, antiproliferative, and anti-cancer. Two significant aspects of its anticancer potential, namely the intracellular signaling and angiogenic mechanism, remain elusive. This paper investigated the cell toxicity and antiangiogenic mechanism of *E. microphylla* leaf ethanolic extract (EMLEE). There were three experiments employed in this study. In the *in vitro* set-up, the human hepatoma cell line (HepG2) was examined after treatments with doxorubicin or various concentrations of EMLEE. For the *in-ovo* experiment, the 3-day-old duck embryo was observed for its degree of vascularization after injecting it with either EMLEE, 1% dimethyl sulfoxide (DMSO), or none. In the *in silico* study, the known compounds present in *E. microphylla* identified from various literatures available in PubChem were assessed for their predicted oral bioavailability. The candidate lead compounds were docked to selected proteins associated with intracellular signaling and angiogenesis. The top binding compounds were visualized along with their respective docked protein crystal structures. Findings show that the LC50 of EMLEE is about 1.1 mg/ml, diminishing blood vessel formation in the treated embryo by 57%. Out of the 58 known compounds, only 29 followed Lipinski's rule of five, indicating high predicted oral bioavailability. Additionally, only 8 of these compounds demonstrated high binding affinity with the selected proteins. Most of the compounds have a high binding affinity with MEK from intracellular signaling, whereas VEGFR2 for angiogenesis. Also, caffeic anhydride and apigenin bind to most of the selected proteins. Altogether, the high binding affinity of the lead compounds with the selected proteins, particularly MEK and VEGFR2, may explain the cytotoxic and antiangiogenic properties of EMLEE.

Keywords: : *Ehretia microphylla*, Cytotoxicity, Angiogenesis, Intracellular signaling, Molecular docking.

INTRODUCTION

Parts of medicinal plants are used in healthcare purposes since a very ancient day and many of their reported efficacies are proved by using modern research tools [1, 2, 3]. The plant *Ehretia microphylla* (Vahl.) Masam. (Boraginaceae) is locally known as the Philippine tea tree, originates from East Asia towards China, Japan, Korea, Taiwan, and further South towards Indonesia, Vietnam, Philippines, and New Guinea. In the Philippines, the leaves of this shrub were traditionally used as a substitute for tea and are considered a remedy for dysentery, diarrhea, and cough [4]. Leaf of this plant is traditionally used as anticancer medicine [5].

Evidence shows that *E. microphylla* roots and stems show antiangiogenic effects on the chorioallantoic membrane of fertilized duck embryos [6]. Quercetin isolated from leaves demonstrated cytotoxic activities on HepG2 human hepatoma cancer cell lines by MTT assay [7]. Previous studies also reported that due to the presence of triterpenoids in *E. microphylla*, results of anti-mitotic, antiproliferative, and DNA fragmentation might be a mechanism of action for the inhibition of cancer cell growth [8].

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However, the anticancer mechanisms involving *E. microphylla* are still poorly understood.

There are different pathways targeted to influence the tumor environment in drug development, and two of the most common mechanisms studied are intracellular signaling and angiogenesis, as shown in Figure 1. Some proteins associated with intracellular signaling in cancer tissues are RAS, RAF, MEK, and mTOR [9]. Studies have shown that RAS, RAF, and MEK modulation affect cell proliferation [10]. Likewise, down-regulating mTOR induces apoptosis in cancer cells [11]. Additionally, angiogenesis in cancer is typically associated with platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor (VEGF) family, which includes the VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2) [9].

RAS proteins (H-Ras, K-Ras 4A and 4B, and N-Ras) are involved in a significant fraction of human cancers. Mutations in KRAS alone account for approximately one million deaths per year worldwide [12], and the frequency of mutation of this protein accounts for 91% in the pancreas, 42% in the colon, and 33% in the lungs [13]. RAF is also involved in a wide range of human cancers through abnormal signaling of upstream proteins (*e.g.*, growth factor receptors and mutant RAS) and mutations by the protein itself [14]. A mitogen-activated extracellular signal-regulated kinase (MEK) is also triggered by growth factors or mutated oncogenic proteins (commonly RAS and RAF). The deregulation of the MEK pathway commonly involves melanoma, pancreatic, lung, colorectal, and breast cancers [15]. The mammalian or mechanistic target of rapamycin (mTOR) regulates cell survival, metabolism, and protein synthesis [16]. Abnormal mTOR signaling results from a mutation originating from different levels of the signal cascade, which is commonly seen in human cancers. This aberrant signaling results in cell proliferation and tumor metabolism, leading to their progression [16].

The tumor tissue requires oxygen and nutrients from surrounding blood vessels [17]. To form new blood vessels, endothelial cells from surrounding blood vessels activate and break down the underlying basement membrane, then migrate through the damaged areas [18]. Different growth factors stimulate these endothelial cells. VEGFR-1 is mainly expressed in bone marrow progenitor cells, myelomonocytic inflammatory cells, and vascular smooth muscle cells and binds to VEGF-A with an affinity approximately 10-fold higher than that of VEGFR-2 [19, 20]. VEGFR-2, on the other hand, stimulates not only angiogenic signals but also the secretion of other proteins such as the von Willebrand factor from endothelial cells [21]. Additionally, VEGFR-2 initiates the PLCγ-PKC-MAPK pathway, which plays a pivotal role in pro-angiogenic signaling [22]. PDGFR has been observed to increase vascularity and maturation of the vascular wall and was identified in a large variety of human tumor cells [23]. The inhibition of PDGFR reduces interstitial fluid pressure, which increases drug uptake and the effectiveness of chemotherapy [24].

In this paper, the researchers hypothesized a possible mechanism of action for the cytotoxic and antiangiogenic properties of EMLEE using *in silico* methods like oral bioavailability prediction and virtual molecular docking.

MATERIALS AND METHODS

Plant preparation and ethanolic extraction

The *E. Microphylla* leaves were collected in San Marcelino, Zambales, in 2019 and were authenticated by Forester Apriliza Sagadraca (License No. 10385) from the Subic Bay Metropolitan Authority Ecology Center, Zambales, Philippines. The preparation and extraction of *E. microphylla* leaf follow the protocol from a previous study but with some modifications [25]. The leaves were sun-dried for 2-3 days before grinding. About 100 grams of ground leaves were soaked in 1 L of 95% ethanol for 48 hours. The suspension was filtered with Whatman No. 41 filter paper. After, the filtrate was evaporated in a rotary evaporator to remove the solvent. The extract was kept at 4°C until used. In preparing the various concentration of the extract, the maximum amount of extract soluble in 1% dimethyl sulfoxide (DMSO) solution was used as the starting concentration. Similar to the extract, the reconstituted EMLEE solution was stored at 4°C until used.

Cell viability assay (MTT assay)

Mosmann's method was followed in this *in vitro* experiment but with minor modifications [26]. HepG2 cells were seeded at 104 cells/mL in sterile 96-well microtiter plates. These plates were incubated at 37°C, 5% CO2, and 98% humidity to achieve the log phase of their growth curve. Varying concentrations of EMLEE were prepared to achieve a final concentration of 3.2 mg/ml, 1 mg/ml, 0.32 mg/ml, 0.1 mg/ml, 0.032 mg/ml, 0.01 mg/ml, 0.0032 mg/ml, 0.001 mg/ml, and 0.00032 mg/ml. Doxorubicin, a cancer drug, was used

as the positive control, while DMSO, the extract's solvent, served as the negative control. For each concentration, three replicates were used in this experiment. The plates were incubated for 72 hours at 37 °C and 5% CO2. After incubation, the medium was removed, and 20 mL of MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] dye was added to each well at a concentration of 5 mg/mL phosphate-buffered saline. The cells were then incubated for another 4 hours at 37°C with 5% CO2. About 150 mL DMSO was added to each well after that. The Ledetect reader was used to measure absorbance at 570 nm. To determine the IC50 of the extract, the linear regression of the absorbance versus concentration graph was calculated. The IC50 depicts the concentration potent enough to kill 50% of the cell population.

CAM assay

The duck eggs were candled first to confirm fertility. A total of 15 fertile eggs were selected and assigned five eggs per treatment. In this experiment, the CAM assay follows the protocol from a previous experiment but with some modifications [27]. The treatments were LC50 EMLEE, 1% DMSO, and untreated. The LC50 EMLEE was prepared by diluting the extract to 1.1 mg/mL in 1% DMSO. A small hole was punctured on the shell of the egg particularlyon the air sac portion. About 0.1 mL of either LC50 EMLEE or 1% DMSO was injected into each egg. Meanwhile, the untreated eggs were not punctured. The hole was covered with parafilm to prevent contamination.

After three days, the eggs were cracked open to observe the vascularization on the chorioallantoic membrane of the egg. Angiogenesis was evaluated through the degree of vascularization, which can be measured using branch point density [28]. The branch point density was computed using the formula:

Branch point density = No. of branchpoint in a blood vessel segment/Length of blood vessel segment.

Literature search strategy

Guided by the qualitative phytochemical screening, the identity of the compounds present in EMLEE was gathered through various research articles in different indexing databases, such as PubMed, Google Scholar, Scopus, EBSCO host, and ResearchGate [29]. The identity of the compounds collected was counter-checked in PubChem (https://pubchem.ncbi.nlm.nih.gov) for the availability of the 3D structure. Compounds with unavailable 3D structures were not considered in the *in silico* experiment.

Predicting the oral bioavailability of the compounds

The oral bioavailability of the compounds can be accessed through their absorption, distribution, metabolic, excretion, and toxicity (ADMET) properties using Lipinski's rule of five (RO5) [30]. The following physicochemical properties of the compounds were gathered through Swiss ADME (http://www.swissadme.ch/), namely molecular mass, octanol-water partition coefficient (LogP), H bond donor, and H bond acceptor [31].

Virtual molecular docking

The PDB crystal structures of different enzymes associated with intracellular signaling and angiogenesis were downloaded from Protein Data Bank (www.rcsb.org). There were four proteins identified associated with intracellular signaling, namely Ras (PDB: 1LFD), Raf (PDB: 6UAN), mek (PDB: 3SLS), and mTOR (PDB: 4JSV). Meanwhile, there were three angiogenesis-associated proteins of interest, particularly VEGFR1 (PDB: 4CL7), VEGFR2 (PDB: 3VNT), and PDGFR (PDB: 6JOK). The crystal structures of the various proteins were modified by combining hydrogen and Gasteiger charges, then deleting non-polar hydrogens, lone pairs, water molecules, and non-standard residues [32]. Each protein molecule's crystal structure was docked with the 3D structure of the candidate compounds. Mcule (Mcule Inc., USA) and Autodock Tools (The Scripps Research Institute, USA) were used to prepare the protein molecules as well as dock the ligand. Redocking a known ligand to the protein established the ligand's docking to the protein molecule. Superpose v.1.0 (Wishart Lab, University of Alberta, Edmonton, Canada) was used to superimpose the docked protein molecule with the original crystal structure. The root means square deviation (RMSD) of the atomic positions of the overlaid structures was verified to be less than 1.2Å [33]. The re-docked crystal structure's crystal structures are identical to the original structure if the RMSD value is less than 1.2 Å. PLIP was used to visualize the best-docked crystal structures with binding energies ranging from -8.0 to -11.71 kcal/mol (BIOTEC TU Dresden, Saxony, Germany) [33].

RESULTS AND DISCUSSION

EMLEE demonstrates cell toxicity and anti-angiogenic property

HepG2 cells were treated with various concentrations of EMLEE ranging from 0-3.2 mg/ml,

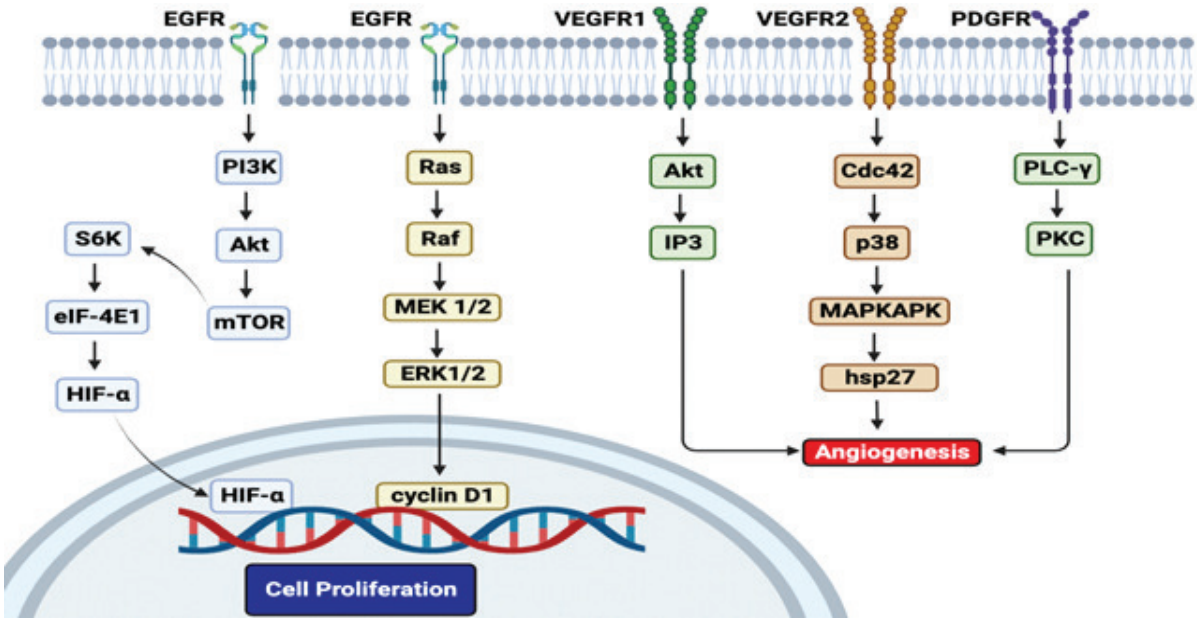

Fig. 1. Mechanism of angiogenesis and cell proliferation driving cancer progression.

as shown in Fig. 2A. The LC50 of EMLEE was computed to be around 1.1 mg/ml. Meanwhile, the highest concentration used in the experiment was found to have an LC value of about 95%. The LC50 and the highest concentration used were compared with the untreated and doxorubicin to visualize better its effect on the %viability of the HepG2 cells, as shown in Fig. 2B. This finding suggests that the cytotoxic potential of EMLEE has a dose-dependent response. Furthermore, the LC50 EMLEE was injected into a fertilized duck egg to observe its effect on the vascularization of the developing embryo. In Fig. 2C, LC50 EMLEE exhibited a 57% decrease in vascularization compared with the untreated and vehicle control. In addition, with its cytotoxic property, EMLEE exhibited anti-angiogenic properties, as well.

These findings follow the results observed in a previous study that demonstrated that *E. microphylla* exhibits cytotoxic properties [7]. Additionally, it was reported that as low as 0.1 mg/ml exhibited about 96% cell death. This report is contrary to our findings, showing that even 0.1 mg/ml EMLEE was unable to decrease the number of viable HepG2 cells significantly. This discrepancy may be attributed to their purification method, where quercetin was isolated. The researchers came up with two hypotheses to explain these findings: (1) the amount of quercetin in 0.1 mg/ml EMLEE may not be enough to elicit cytotoxic activity; (2) there might be other compounds present in EMLEE which may have impeded the activity of quercetin.

The antiangiogenic property of EMLEE observed in this study is supported by previous studies using *E. microphylla* leaves methanolic and ethanolic extracts [34, 35]. Both studies used the paper disc to deliver the extracts. In the methanolic extract, 0.1 mg/disc of the extract delivered to the eggs exhibited 49.92% vascularization inhibition, which is lower than our findings [35]. Conversely, the ethanolic extract with unclear concentration demonstrated 89.05% inhibition [34]. These observations suggest that compounds present in *E. microphylla* may have impeded blood vessel formation in the embryo. However, there is inconclusive evidence that the ethanolic extract performed better than the methanolic extract, which warrants further investigation.

Predicting the oral bioavailability of known EMLEE compounds

There were 58 compounds present in EMLEE identified from previous literature, which have an available 3D structure in PubChem. The physicochemical properties of these compounds, namely molecular mass, octanol-water partition coefficient, H bond donor, and H bond acceptor, were assessed for compliance with RO5, as shown in Table 1. There were 29 compounds having violations with the RO5. Hence, only half of the identified compounds were docked with the different cancer-associated proteins. Furthermore, these compounds were predicted to have high oral bioavailability.

Table 1. Lead-likeness of the compounds found in EMLEE using Lipinski's rule of five (RO5).

Compounds	CID	Mol. mass ≤ 500g/mol	Log p≤5	H donor ≤10	H acceptor ≤5	RO5 violation
α-Amyrin	73170	426.7162	8.0248	1	1	1
β-Amyrin	225687	853.4323	16.1937	2	2	2
Bauerenol	111220	426.7162	8.0248	1	1	1
prenylbenzoquinone	276202	176.2111	1.9771	2	0	0
Anthraquinone	6780	208.211	2.462	2	0	0
Coumarin	323	146.1421	1.793	2	0	0
Tannins	250395	636.4668	-0.2769	18	11	3
Glutinone	10071029	424.7002	8.3771	1	0	1
Saponin	198016	1223.3483	-4.0693	27	15	3
Buddlenol B	21627696	584.6091	3.115	11	5	2
Caffeic acid	689043	180.1568	1.1956	4	3	0
Danshensu	11600642	198.1722	0.0858	5	4	0
Cinnamic acid	444539	148.158	1.7844	2	1	0
Ehletianol A	101928785	794.7046	-0.781	20	8	3
Ehletianol B	101928786	764.6786	-0.7896	19	8	3
Ehletianol D	92024104	520.5242	0.1188	11	6	3
(E)-ethyl caffeate	5317238	208.2099	1.6741	4	2	0
Caffeic anhydride	57506917	342.2983	2.3054	7	4	0
Icariside E5	91884923	522.5402	-0.0878	11	7	3
Lithospermic acid B	6451084	718.6113	3.3345	16	9	3
Methyl rosmarinate	6479915	374.3402	1.8497	8	4	0
p-hydroxybenzoic acid	135	138.1203	1.0904	3	2	0
Rosmarinic acid	5281792	360.3136	1.7613	8	5	0
Ehletianol C	101928787	556.5991	3.1356	10	5	1
Trans-ferulic acid	445858	194.1834	1.4986	4	2	0
Apigenin	5280443	270.2358	2.5768	5	3	0
Hyperoside	5281643	464.3749	-0.5389	12	8	2
Luteolin	5280445	286.2352	2.2824	6	4	0
Kaempferol	5280863	286.2352	2.2824	6	4	0
Quercetin	5280343	302.2346	1.988	7	5	0
Ovalifolin	15160715	346.3744	5.5512	4	0	1
Ehretinine	336435	275.3421	2.2726	4	0	0
Allantoin	204	158.1159	-0.431	7	4	0
Araneosol	5491522	374.3402	2.9056	8	2	0
Tetradecanoic acid 2, 3-dihydroxypropyl ester	79050	302.449	3.5839	4	2	0
(10E, 12Z, 15Z)-9-hydroxy-10, 12, 15-octadecatrienoic acid methyl ester	10244787	308.4548	4.7197	3	1	0
(9Z, 11E)-13-oxo-9, 11-octadecadienoic acid	5743409	308.4548	5.1519	3	0	1
(9Z, 11E)-13-hydroxy-9, 11-octadecadienoic acid	71684434	665.0813	12.7239	3	1	2
Ehretianone	10405602	350.4063	4.0077	4	1	0
Ehretiquinone	60201866	348.3903	3.7837	4	1	0
Simmondsin	6437384	375.3704	-2.5839	10	5	0
Ehretioside B	10425556	311.2866	-1.363	8	5	0
Betulin	72326	442.7156	6.9972	2	2	1
Bauerenol acetate	177801	468.7527	8.5956	2	0	1
Betulinic acid	64971	456.699	7.0895	3	2	1
Lupeol	259846	426.7162	8.0248	1	1	1
β-sitosterol	222284	414.7056	8.0248	1	1	1
Daucosterol	5742590	576.8459	5.849	6	4	2
Stigmasterol	5280794	412.6896	7.8008	1	1	1
α-spinasterol	5281331	412.6896	7.8008	1	1	1
Campesterol	173183	400.679	7.6347	1	1	1
Stigmastanol	241572	416.7215	8.1047	1	1	1
Ehretiolide	60201867	614.8517	8.8131	5	0	2
Ehreticoumarin	60201868	246.2577	2.7129	4	2	0
Ehretilactone A	60201869	204.2211	2.4076	3	0	0
Ehretilactone B	60201870	244.2406	-0.3931	6	1	0
Ehretiamide	17820828	181.1882	1.1289	4	2	0
Ehretiate	60202035	504.7403	9.0075	5	1	2

Table 2. Docking score (kcal/mol) of EMLEE compounds in various intracellular signaling and angiogenesis-related enzymes.

	Intracellular signaling				Angiogenesis		
Compounds	RAS	RAF	MEK	MTOR	VEGFR1	VEGFR2	PDGFR
PDB ID	1LFD	6UAN	3SLS	4JSV	4CL7	3VNT	6JOK
Prenylbenzoquinone	-6.5	-4.4	-6.4	-6.2	-4.4	-6.8	-6.9
Anthraquinone	-6.7	-5.1	-8.4	-7.5	-4.7	-7.5	-6.5
Coumarin	-6.5	-4.2	-6.3	-6.1	-3.9	-6.3	-6.5
Caffeic acid	-6.4	-5.2	-6.8	-5.8	-4.3	-6.6	-6.8
Danshensu	-6.7	-4.7	-6.8	-5.7	-4.4	-6.4	-6.2
Cinnamic acid	-5.4	-4.3	-6.2	-5.8	-4.3	-6.1	-6.0
(E)-ethyl caffeate	-6.4	-4.6	-6.5	-6.0	-4.5	-7.0	-6.5
Caffeic anhydride	-8.4	-5.7	-8.4	-6.8	-5.1	-8.9	-8.5
Methyl rosmarinate	-8.3	-5.8	-8.2	-6.5	-4.7	-8.3	-7.2
p-hydroxybenzoic acid	-5.3	-4.0	-5.7	-5.4	-4.0	-5.6	-5.5
Rosmarinic acid	-8.7	-5.6	-8.4	-6.8	-5.2	-8.8	-7.8
Trans-ferulic acid	-6.0	-4.6	-6.7	-6.1	-4.3	-5.6	-6.6
Apigenin	-8.2	-6.3	-8.5	-8.1	-5.3	-8.9	-7.4
Luteolin	-6.8	-6.6	-8.8	-8.0	-5.6	-8.9	-7.6
Kaempferol	-7.9	-6.3	-8.6	-7.2	-5.2	-8.0	-6.6
Quercetin	-8.9	-6.5	-9.0	-7.2	-5.5	-8.3	-7.5
Ehretinine	-7.3	-5.1	-7.3	-6.4	-4.6	-7.2	-6.8
Allantoin	-6.3	-4.3	-5.4	-4.8	-3.9	-5.3	-5.1
Araneosol	-6.9	-6.0	-7.8	-6.3	-4.6	-6.7	-6.2
Tetradecanoic acid 2, 3-dihydroxypropyl ester	-5.6	-4.0	-4.8	-4.7	-3.1	-6.1	-4.8
(10E, 12Z, 15Z)-9-hydroxy-10, 12, 15-octadecatrienoic acid methyl ester	-5.3	-3.5	-5.3	-4.5	-3.4	-6.8	-5.2
Ehretianone	-6.8	-6.5	-7.1	-7.3	-5.5	-7.8	-6.5
Ehretiquinone	-6.8	-6.5	-6.0	-6.5	-5.5	-6.4	-6.2
Simmondsin	-6.1	-5.6	-7.1	-5.9	-4.7	-7.1	-5.2
Ehretioside B	-7.9	-5.7	-7.7	-6.3	-4.7	-6.3	-5.2
Ehreticoumarin	-7.1	-5.4	-7.3	-6.8	-4.8	-7.1	-5.8
Ehretilactone A	-6.1	-4.9	-7.3	-6.4	-4.4	-6.6	-7.0
Ehretilactone B	-5.8	-4.6	-5.9	-4.4	-3.9	-4.9	-4.6
Ehretiamide	-6.2	-4.3	-6.0	-5.4	-4.4	-6.4	-6.3

Several studies use RO5 as a primary tool in drug screening, mainly to predict the bioavailability of lead compounds [36, 37, 38]. Most bioactive compounds with ≤500 g/mol mass, ≤5 octanol-water partition coefficient, ≤10 hydrogen bond donor, and ≤5 hydrogen bond acceptor are pharmacologically active in terms of adsorption, distribution, metabolism, excretion, and toxicity throughout the system [38].

Molecular docking of candidate EMLEE compounds with cancer-associated proteins

The candidate EMLEE compounds were docked with two frequently targeted pathways in cancer, namely intracellular signaling, and angiogenesis, as shown in Table 2. There were four potent target proteins in the intracellular signaling pathway, such as the RAS, RAF, MEK, and MTOR. Meanwhile, three target enzymes were assessed in angiogenesis, which include VEGFR1, VEGFR2, and PDGFR.

The docking score, which has more negative binding energy (kcal/mol), was considered a high binding affinity to the respective protein [39]. Moreover, the usual binding energy of bioactive compounds is not lesser than -8.0 kcal/mol [40].

In the intracellular signaling enzyme RAS, only five candidate EMLEE compounds have a high binding affinity, namely caffeic anhydride, methyl rosmarinate, rosmarinic acid, apigenin, and quercetin. However, there was no candidate compounds present in EMLEE that exhibited high binding affinity with RAF. Meanwhile, eight candidate compounds show high binding affinity with MEK. These compounds were anthraquinone, caffeic anhydride, methyl rosmarinate, rosmarinic acid, apigenin, luteolin, kaempferol, and

Table 3. Binding interactions of EMLEE compounds docked in various enzymes associated with intracellular signaling and angiogenesis.

Proteins	Inhibitor	Binding interaction	Amino acid residues
RAS	GNP	Hydrogen bonds	gly213, gly215, lys216, ser217, ala218, asp230, lys231, tyr232, asp233, thr235, asn286, lys317
		Π-stacking	tyr232
		Salt bridges	asp230
	Caffeic anhydride	Hydrophobic interactions	lys231, tyr232, gln261, glu262, lys288
		Hydrogen bonds	lys48, gly212, asp230, tyr232, glu263, asn286
		Salt bridges	lys231. lys288
	Methyl rosmarinate	Hydrophobic interactions	ala218, lys231
		Hydrogen bonds	lys32, lys216, ser217, asp230, lys231, asp233, thr258, gly260
		Π-cation interactions	lys216
		Salt bridges	lys317
	Rosmarinic acid	Hydrophobic interactions	ala218, val229, lys231
		Hydrogen bonds	lys32, lys216, ser217, asp230, lys231, asp233, thr235, gly260
		Π-cation interactions	lys216
		Salt bridges	lys317
	Apigenin	Hydrophobic interactions	lys216, phe228
		Hydrogen bonds	lys216, ser217, ala218, thr235, gly260, lys317
	Quercetin	Hydrophobic interactions	asp233
		Hydrogen bonds	lys32, gly213, val214, gly215, lys216, ala218, val229, asp233, gly260
RAF	SEP	Hydrogen bonds	lys50, arg128, tyr129, glu730
		Salt bridge	lys50, arg57, arg128
MEK	ANP	Hydrogen bonds	ala76, lys97, met146, ser150, asp152, ser194, asp208
	Anthraquinone	Hydrophobic interactions	leu74, val82, leu197
		Hydrogen bonds	gln153
	Caffeic anhydride	Hydrophobic interactions	leu74, val82, ala95, leu197
		Hydrogen bonds	asn78, his145, met146, ser194
		Π-cation interactions	lys97
	Methyl rosmarinate	Hydrophobic interactions	leu74, val82, ala95, leu197
		Hydrogen bonds	ala76, lys97, glu144, glu144, met146, gln153, asn195, asp208
	Rosmarinic acid	Hydrophobic interactions	leu74, val82, ala95, leu197
		Hydrogen bonds	asn78, glu144, met146, ser150, lys192
		Π-cation interactions	lys97
	Apigenin	Hydrophobic interactions	leu74, val82, ala95, leu197
		Hydrogen bonds	lys97, met146, ser194, asn195, asp208
	Luteolin	Hydrophobic interactions	leu74, val82, met146, ley197
		Hydrogen bonds	ala76, met146, gly149, gln153, asp208
	Kaempferol	Hydrophobic interactions	leu74, val82, ala95, leu197
		Hydrogen bonds	ala76, lys97, met146, gln153, ser194, asn195, asp208
	Quercetin	Hydrophobic interactions	val82, met146, leu197
		Hydrogen bonds	leu74, met146, gly149, gln153, asn195, asp208
MTOR	ADP	Hydrogen bonds	ser2165, val2240, asn2343, asp2357
		Salt bridges	lys2187
	Apigenin	Hydrophobic interactions	ile2163, pro2169, leu2185, trp2239, val2240, ile2356
		Hydrogen bonds	ser2165, gly2238, trp2239, val2240
	Luteolin	Hydrophobic interactions	ile2163, pro2169, leu2185, trp2239, val2240, ile2356
		Hydrogen bonds	ser2165, gly2238, val2240
VEGFR1	EDO	Hydrogen bonds	thr166, thr168, thr210
VEGFR2	0JA	Hydrophobic interactions	pro839, leu840, arg842, leu1035, asp1052, asp1056
		Hydrogen bonds	arg842, asn923
		Π-cation interactions	phe1047
	Caffeic anhydride	Hydrophobic interactions	val848, ala866, lys868, val916, asn923, leu1035, phe1047
		Hydrogen bonds	glu885, cys919, asp1046, arg1051
		Π-cation interactions	phe1047
	Methyl rosmarinate	Hydrophobic interactions	leu840, val848, val916, phe918, leu1035, phe047
		Hydrogen bonds	lys868, glu885, phe921, asn923, asp1046
	Rosmarinic acid	Hydrophobic interactions	val848, lys868, val916, leu1035, phe1047
		Hydrogen bonds	lys868, glu885, cys919, asp1046, arg1051
	Apigenin	Hydrophobic interactions	val848, ala866, leu889, val899, val916, asp1046
		Hydrogen bonds	ala866, glu885, asp1046
		Π-cation interactions	lys868
	Luteolin	Hydrophobic interactions	val848, ala866, leu889, val899, val916, asp1046
		Hydrogen bonds	glu885, val914, asp1046
		Π-cation interactions	lys868
	Kaempferol	Hydrophobic interactions	leu840, val848, ala866, val916, leu1035, phe1047
		Hydrogen bonds	cys919
	Quercetin	Hydrophobic interactions	leu840, val848, lys868, val899, val916, leu1035, phe2047
		Hydrogen bonds	cys919, asp1046
PDGFR	B49	Hydrophobic interactions	leu840, val848, lys868, val899, val916, leu1035, phe1047
		Hydrogen bonds	cys919, asp1046
	Caffeic anhydride	Hydrophobic interactions	leu599, val607, phe837
		Hydrogen bonds	gly600, thr674, glu675, cys677, asp842

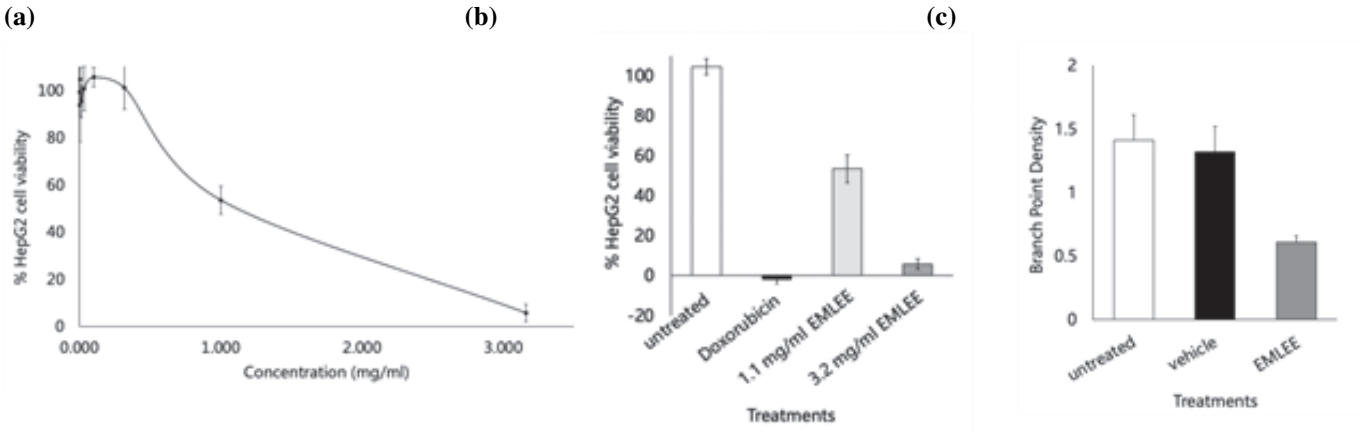

Fig. 2. Cell viability and antiangiogenic property of EMLEE *in vitro* and *in vivo*. [(a) Cell viability of HepG2 cells treated with various concentrations of EMLEE; (b) Cell viability of HepG2 cells under no treatment, doxorubicin, and EMLEE (LC50 and LC95) interventions; (c) Angiogenesis in 3-day duck embryo exposed to 1% DMSO in distilled water (vehicle), LC50 EMLEE, and no treatment].

quercetin. In addition, only two compounds demonstrated high binding affinity with MTOR, namely apigenin, and luteolin.

Most of the protein targets in cancer studies are RAS inhibitors [41, 42, 43]. Inhibiting RAS impedes various cellular activities associated with cell growth, cell proliferation, and metastasis [44]. MEK belongs to the mitogen-activated protein kinase (MAPK) family, a downstream target of Ras [45]. MEK inhibitors are also utilized in cancer treatments [43]. Lastly, suppressing mTOR results in cytotoxicity, which is also used in cancer research [46].

Only VEGFR1 did not exhibit high binding affinity with the candidate ligands for the proteins involved in angiogenesis. Conversely, seven compounds, namely caffeic anhydride, methyl rosmarinate, rosmarinic acid, apigenin, luteolin, kaempferol, and quercetin, have a high binding affinity with VEGFR2. Lastly, only caffeic anhydride has a high binding affinity with PDGFR.

Both VEGFR2 and PDGFR affect angiogenesis-associated pathways in cancer. For instance, various VEGFR2 inhibitors modulate angiogenesis, metastasis, and tumor growth [47]. Additionally, MET/VEGFR2 complex suppresses cell invasion and mesenchymal transition [48]. Meanwhile, oncogenic activation of PDGFR shows a strong influence on vessel stability, metastasis, and cell migration [49, 50].

Binding interactions of top binding EMLEE compounds with intracellular signaling and angiogenesis related proteins

Only eight of the 29 compounds docked exhibited high binding affinity to the selected cancer-associated proteins. The binding interaction of these top binding compounds, namely caffeic anhydride, methyl rosmarinate, rosmarinic acid, apigenin, quercetin, anthraquinone, luteolin, and kaempferol, were visualized. Both caffeic anhydride and apigenin bind to most of the selected proteins. Both exhibited high binding affinity with RAS, MEK, and VEGFR2. However, only apigenin demonstrated high binding affinity with MTOR, whereas caffeic anhydride with PDGFR.

Compounds like anthraquinone, rosmarinic acid, and apigeninhave been reported to be cytotoxic [51, 52]. Meanwhile, anthraquinone has demonstrated antiangiogenic property [53]. Several compounds, like luteolin, echibited both cytotoxic and antiangiogenic properties [54, 55]. Quercetin, on the other hand, not only has its inherent cytotoxic property but it also can potentiate the cytotoxicity of luteolin [54, 56]. Similarly, kaempferol have also demonstrated both cytotoxic and antiangiogenic [57, 58]. However, no concrete findings directly confirm the cytotoxic and antiangiogenic properties of caffeic anhydride and methyl rose marinate.

Overall, the dominant non-covalent interactions between the EMLEE compounds and cancer-associated proteins were hydrogen bonding and hydrophobic interactions. In RAS, MEK, and PDGFR, most of the involved amino acids in these proteins formed hydrogen bonding with the compounds. Conversely, numerous hydrophobic interactions between the proteins and ligands were identified both in MTOR and VEGFR2. These findings suggest that numerous hydroxy groups in the candidate compounds may have contributed to

its high binding affinity with RAS, MEK, and PDGFR. In contrast, the large hydrophobic regions in these compounds may have influenced their high binding affinity with MTOR and VEGFR2.

CONCLUSION

In summary, about 1.1 mg/ml of *E. microphylla* leaf ethanolic extract (EMLEE) exhibited 50% cytotoxicity against HepG2 cells and 57% blood vessel formation in a 3-day-old duck embryo. Only 29 of the 58 known compounds present in *E. microphylla* have favorable ADMET properties. Likewise, only 8 of these 29 compounds displayed a high binding affinity with the selected proteins RAS, MEK, mTOR, VEGFR2, and PDGFR. These compounds exhibited a high number of hydrogen bonds with intracellular signaling-associated proteins, RAS and MEK, and angiogenesis-related protein PDGFR. Conversely, these compounds demonstrated numerous hydrophobic interactions with mTOR and VEGFR2, intracellular signaling, and angiogenesis-associated proteins, respectively. Overall, several studies support that most of the lead compounds assessed have a cytotoxic and antiangiogenic effect, which may explain the similarly observed properties of EMLEE. However, little is known about the cytotoxic and angiogenic properties of the other compounds, namely caffeic anhydride and methyl rosmarinate. Evidently, caffeic anhydride demonstrated a high binding affinity to most selected proteins, which warrants further investigation.

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