CYTOTOXIC ACTIVITY OF ESSENTIAL OIL AND EXTRACTS OF *OCIMUM BASILICUM* AGAINST HUMAN CARCINOMA CELLS. MOLECULAR DOCKING STUDY OF ISOEUGENOL AS A POTENT COX AND LOX INHIBITOR

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The antiproliferative activity of the ethanolic extract and the essential oil of *O. basilicum*, cultivated in Greece, was evaluated *in vitro* against four different human cancer cell lines: the human cervix adenocarcinoma HeLa cells, human melanoma FemX cells, human chronic myelogenous leukaemia K562 cells and human ovarian SKOV3 cells. Qualitative analysis has been carried out with HPLC and LC/ESI-MS measurements and the prevalent constituents of the extract which were rosmarinic and caffeic acid and of the essential oil which were eugenol, isoeugenol and linalool have been tested with the above cell lines. All phytochemicals showed significant cytotoxic activity particularly against SKOV3 cell lines. Mild but definite inhibition was noticed regarding the extract and the essential oil. Remarkably, caffeic acid was found to be in the same range compared to cisplatin against the four cell lines exhibiting significant anticancer activity while isoeugenol is more cytotoxic than eugenol. *In silico* modelling has shown that isoeugenol can effectively inhibit cyclooxygenase and lipoxygenase enzymatic action.

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1. Introduction

*Ocimum basilicum* (sweet basil) is a common herb of the Lamiaceae family. The *Ocimum* genus has been classified into 64 species exhibiting a great variation in phenotype, oil content, composition and bioactivity. Within the species, several different chemical groups exist; the diverse environmental parameters such as the growing soil, climatic changes and the harvesting period influence the quantity and the composition of herbs and of the corresponding essential oils. *O. Basilicum* has been extensively studied for its medicinal properties which include antibacterial, anti-inflammatory, antiproliferative/anticancer, antioxidant, antiviral and antifungal activities [1-7]. Moreover, *O. basilicum* has exhibited inhibitory activity against HIV-1 reverse transcriptase and induced platelet aggregation by collagen and adenosine 5-diphosphate) [5]. Experiments carried out on rats have shown that the leaves of *O. basilicum* markedly increased glutathione S-transferase that partly protects from chemical carcinogens in the stomach, liver, and oesophagus [8]. These medicinal properties are mainly expressed through a wide range of non-volatile...
polyphenolic compounds known as phytochemicals. Two phytochemicals found in high concentrations in basil are rosmarinic and caffeic acid.

Rosmarinic acid is an ester of caffeic acid with 3,4-dihydroxyphenyl lactic acid and has antibacterial and anti-inflammatory properties [9]. Rosmarinic acid in humans and rats metabolizes to methylated rosmarinic acid, coumaric acid, ferulic acid and caffeic acid. The unconjugated form of rosmarinic acid decreases the effect of acetylcholinesterase which is correlated to the Alzheimer's disease [10]. Caffeic acid can inhibit the absorption of cholesterol in rats’ metabolism reducing the levels of phospholipids, free fatty acids, and triglycerides [11]. Furthermore, it exhibits immunomodulatory and anti-inflammatory activity [12]. The main components of the essential oil of basil are phenol derivatives, such as (iso)eugenol, methyl eugenol, chavicol, estragole, and linalool (Fig. 2).

The essential oil of sweet basil has shown potent antioxidant, anticancer, antiviral, and antimicrobial properties mostly by in vitro studies [13-18]. Secondary plant metabolites, such as polyphenols, exhibit antioxidant, antimitagenic, anticarcinogenic, antiinflammatory and antimicrobial effects[19]. Phytomolecules represent a wide source of natural antioxidants with increased anti-inflammatory activity while it is known that there is a definite correlation between cancer and chronic inflammation[20]. Laboratory studies and clinical trials relating cancer and chemotherapeutic drugs from medicinal plants support the notion that phytochemicals exhibit a promising action against carcinogenesis [21,22].

In this study, the ethanolic extract and the essential oil of Ocimum basilicum, cultivated in in Greece, were considered both for their composition as well as their antiproliferative activity in vitro and in silico. High Performance Liquid Chromatography (HPLC-DAD) and liquid chromatography/electrospray ion trap tandem mass spectrometry (LC/ESI-MS) have been used in the experiments. The extract and the essential oil of Ocimum basilicum along with the main constituents of extract, rosmarinic and caffeic acid and of essential oil, eugenol, isoeugenol and linalool have been evaluated for antiproliferative activity in vitro against the cells of four human cancer cell lines: the human cervix adenocarcinoma HeLa cells, human melanoma FemX, human
chronic myelogenous leukaemia K562 and human ovarian SKOV3 cell lines. Furthermore, theoretical docking studies have been carried out towards cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) enzymes for isoeugenol.

2. Experimental

Chemicals
Caffeic acid (60020-10G-F), rosmarinic acid (536954-5G), Eugenol (E51791-100G), isoeugenol (05622BE) and linalool (L2602-5G) were purchased from Aldrich (Aldrich GmbH, Sternheim, Germany). Solvents used throughout the experiments were of analytical grade.

Plant Material
Fresh *O. basilicum* leaves were locally cultivated. Leaves were detached from the stems and washed with water. Small pieces were dried for 3 hours at 45 °C in an air dryer. A grinder was used to ground the samples to powder. Dry matter (4.5 g) and stored at -20 °C. *O. basilicum* essential oil was donated from a local greenhouse facility (BioArt Ltd).

Extraction procedure
*O. basilicum* extract was obtained by refluxing dried powder with 50 ml of ethanol and water (3:1, v/v) for 30 mins. The solution was filtered (125 mm filter paper) and the filtrate was collected and dried in a rotary evaporator. The resulted brown powder (1.2 g) was stored at 0 °C.

HPLC analysis
A reversed-phase liquid chromatographic method with gradient elution and UV detection was used identify the main polyphenolic compounds of *O. Basilicum*. HPLC separations were accomplished with the Agilent 1200 chromatographic system (Agilent, USA) equipped with a rhodynie valve with loop manual injector, a vacuum degasser, a quaternary pump and a variable wavelength UV-DAD diode array detector. Data collection and analysis were carried out using the ChemStation software. The analytical chromatographic columns were Eclipse XDB-C18 (15 cm x 4.6 mm, 5 μm) and Supelco (25 cm x 4.6 mm, 5 μm). The chromatographic assays were performed according to reference [23] with a mobile phase consisted of acetonitrile and water as follows: 50% acetonitrile at 0 min, 50% acetonitrile at 5 min, 60% acetonitrile at 15 min, 60% acetonitrile at 24 min and 16 min washing with 90% acetonitrile at 40 min. Identification was achieved by comparing the chromatograms of the samples and the corresponding reference standards. All samples and standards were dissolved in ethanol:water (1:1, v/v) solution of 40-60 ppm. An aliquot of 25 μL solution from each sample was injected for acquiring the chromatograms. Column temperature was set at 25°C.

LC/ESI-MS analysis
LC/ESI-MS experiments were performed on a quadrupole ion trap mass analyzer (Agilent Technologies, model MSD trap SL) retrofitted to a binary 1100 HPLC system equipped with an electrospray ionization source (Agilent Technologies, Karlsruhe, Germany) and controlled by Agilent Chemstation Software. Separation of basil extract and essential oil was carried out with a 25 cm x 4.6 mm i.d., 5 μm Altima C18 analytical column (Alltech, Deerfield, USA), at a flow rate of 0.7 mL/min, using solvent A, water/formic acid, 99.9: 0.1 v/v and solvent B, acetonitrile. Precursors and products ions of the phenolic compounds were monitored between m/z 100 – m/z 1,000 in positive and negative polarity. For the ionization source: capillary voltage, 3.5 kV; drying gas temperature, 349 °C; nitrogen flow, 12 L/min. Maximum accumulation time of ion trap was set to 3 ms. Three spectra were obtained for the MS average.

Cell lines - Treatment
HeLa, FemX, K562 and SKOV3 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). All cancer cell lines were maintained in the recommended RPMI-1640 medium supplemented with 10% heat-inactivated (56°C) fetal bovine serum, 1-
glutamine (3 mM), streptomycin (100 mg = mL), penicillin (100 IU = mL), and 25 mM HEPES and adjusted to pH 7.2 by bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Stock solutions (20 mM) of compounds, made in dimethylsulfoxide (DMSO), were dissolved in corresponding medium to the required working concentrations. HeLa cells (2000 cells per well), FemX cells (5000 cells per well), and SKOV3 cells (3000 cells per well) were seeded into 96-well microtiter plates, and 24 h later, after the cell adherence, five different, double diluted, concentrations of investigated compounds, were added to the wells. Final concentrations applied to target cells were 200, 100, 50, 25 and 12.5 μM, except to the control wells, where only nutrient medium was added to the cells. K562 cells (5000 cells per well) were seeded, 2h before addition of investigated compounds to give the desired final concentrations. Especially, cells were incubated with different concentrations of essential Basil oil and Sweet basil ranging from 12.5 to 200 μg/ml for 72h. Nutrient medium was RPMI 1640 medium, supplemented with l-glutamine (3 mM), streptomycin (100 lg/mL), and penicillin (100 IU/mL), 10% heat inactivated (56°C) fetal bovine serum (FBS) and 25 mM Hepes, and was adjusted to pH 7.2 by bicarbonate solution. The cultures were incubated for 72 hrs.

**Determination of cell survival (MTT test)**

The effect of extracts on cancer cell survival was determined by MTT test (microculture tetrazolium test), according to Mosmann[24] with modification by Ohno and Abe[25], 72 h upon addition of the compounds, as it was described earlier. Briefly, 20 μl of MTT solution (5 mg/mL PBS) were added to each well. Samples were incubated for further 4 h at 37 C in 5% CO2 and humidified air atmosphere. Then, 100 μl of 10% SDS were added to extract the insoluble product formazan, resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was then read in an ELISA plate reader at 570 nm. Absorbance (A) at 570 nm was measured 24 h later. To get cell survival (%), A of a sample with cells grown in the presence of various concentrations of the investigated extracts was divided with control optical density (the A of control cells grown only in nutrient medium), and multiplied by 100. It was implied that A of the blank was always subtracted from A of the corresponding sample with target cells. IC50 concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. As a positive control was used cis-diamminedichloroplatinum (cisplatin). All experiments were done in triplicate.

**Theoretical studies - Molecular Docking**

The three dimensional coordinates of COX-2 and 5-LOX were obtained from the Protein Data Bank (www.rcsb.org) (pdb IDs: 4COX and 3V99 respectively). The 4COX represents the x-ray crystal structure of COX-2 co-crystallized with indomethacin and the 3V99 the x-ray crystal structure of 5-LOX with arachidonic acid (AA) as the co-crystallized substrate located at the active site of the enzyme. All solvent molecules were removed from the protein structure. Molecular docking was performed with the grid based version of the MolDock Score function[26] as this is implemented in Molegro Virtual Docker software (www.molegro.com). The docking protocol was confirmed through validation tests which showed that the conformation of each co-crystallized ligand was successfully reproduced (RMSD < 1.5 Å). The docked derivatives were ranked according to the “rerank score” scheme which is a weighted linear combination of the intermolecular interactions (steric, van der Waals, hydrogen bonding, electrostatic) between the ligand and the protein, and intramolecular interactions (torsional, sp2-sp2, steric, van der Waals, hydrogen bonding, electrostatic) of the ligand. Prior to docking, ground state optimization on the X-ray structure of the ligand was carried out using the PM3 parametrization scheme[27].

**3. Results**

**HPLC Studies**

The concentration of caffeic and rosmarinic acid of the ethanolic extract and eugenol, isoeugenol and linalool was measured using the HPLC technique. The optimal conditions were met using as a mobile phase a gradient elution of MeCN/H2O at different proportions according to
the experimental section. All analytes were separated in less than 40 min. The UV/vis spectra were recorded in the range of 200–400 nm, and chromatograms were acquired at 254 and 280 nm. For basil extract the peaks of caffeic acid and rosmarinic acid were obtained at retention time of 4.1 and 4.9 mins respectively (Fig. 4). For the essential oil the retention time was 7.8 min for isoeugenol, 12.8 min for eugenol and 18.9 min for linalool (Fig. 4).

![Graph](image1.png)

**Fig 3. Separation of the identified analytes via HPLC method**

**Mass spectrometry studies**

The peaks of the phenolic compounds under study of basil extract and basil essential oil were identified by MS data obtained from samples and standards. Fig 5 shows the mass spectra of the identified compounds which were the following for basil extract: caffeic acid ([M-H]⁻ 179 m/z) and rosmarinic acid ([M-H]⁻ 359 m/z) and the following for essential oil: linalool ([M+H]⁺ 157 m/z) and (iso)eugenol ([M+H]⁺ 163 m/z).
Fig 4: LC-MS spectra of the identified compounds present in the basil ethanolic extract and essential oil

By inspecting and comparing the spectra from the ethanolic extract and the essential oil, we conclude that all the analytes under study are well defined although the MS spectra in negative mode is more noisy due to the higher sensitivity of the technique in this mode[28].

Pharmacology
Antiproliferative activity in vitro

The results of cytotoxic activity in vitro are shown in Table 1 and are expressed as IC₅₀ which is the amount of a compound (in μg/mL) inhibiting cell survival by 50%, compared with a vehicle-treated control. As a positive control was used cis-diamminedichloroplatinum (cisplatin).

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>HeLa</th>
<th>FemX</th>
<th>K562</th>
<th>SKOV3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Eugenol</td>
<td>16.26±3.44</td>
<td>&gt;200</td>
<td>10.18±2.77</td>
<td>27.84±0.88</td>
</tr>
<tr>
<td>2</td>
<td>Isoeugenol</td>
<td>13.91±0.11</td>
<td>15.57±0.94</td>
<td>7.57±0.23</td>
<td>23.34±0.68</td>
</tr>
<tr>
<td>3</td>
<td>Linalool</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>24.37±2.67</td>
</tr>
<tr>
<td>4</td>
<td>Caffeic acid</td>
<td>4.17±0.26</td>
<td>5.46±1.91</td>
<td>13.11±0.12</td>
<td>4.89±3.43</td>
</tr>
<tr>
<td>5</td>
<td>Rosmarinic acid</td>
<td>41.29±1.28</td>
<td>53.24±3.24</td>
<td>29.51±0.72</td>
<td>59.24±1.06</td>
</tr>
<tr>
<td>6</td>
<td>Sweet basil</td>
<td>164.61±2.58</td>
<td>191.36±2.42</td>
<td>157.03±2.25</td>
<td>&gt;200</td>
</tr>
<tr>
<td>7</td>
<td>Basil Esential Oil</td>
<td>86.11±0.82</td>
<td>96.72±0.65</td>
<td>159.78±1.89</td>
<td>&gt;200</td>
</tr>
<tr>
<td>8</td>
<td>Cis-platin</td>
<td>0.68±0.22</td>
<td>1.10±0.35</td>
<td>2.72±0.35</td>
<td>9.62±0.52</td>
</tr>
</tbody>
</table>

Isoeugenol is 20 and 14 times less cytotoxic than cisplatin against HeLa and FemX cell lines and 2.8 and 2.4 times less cytotoxic than cisplatin against K562 and SKOV3 cancer cell lines indicating potent cytotoxicity and anticancer activity compared to the control. Eugenol exhibits very poor cytotoxic activity against FemX cancer cell line. Eugenol is 23.9, 3.7 and 2.9 times less cytotoxic than cisplatin against HeLa, K562 and SKOV3 cancer cell lines. Linalool has very poor
cytotoxic activity against. On the contrary, caffeic acid is in the same range or better compared to cisplatin against the four cell lines. Caffeic acid is 6.1 and 4.5 times less cytotoxic than cisplatin against HeLa and FemX cell lines, 4.8 times less cytotoxic than cisplatin against K562 cancer cell line but 2.0 times more cytotoxic than cisplatin against SKOV3 cancer cell line. Isoeugenol, eugenol, linalool and caffeic acid exhibit high cytotoxicity and anticancer activity against human ovarian SKOV3 cancer cell line. Since ovarian cancer is the most lethal gynecologic cancer in the Western world, and the fourth most common cause of cancer death in women, after breast, lung, and colorectal cancer, the findings are notable. Rosmarinic acid is 10.8 and 6.1 times less cytotoxic than cisplatin against K562 and SKOV3 cancer cell lines. The extract and the essential oil show mild but notable activity.

**Molecular Docking Studies**

*In silico* tools and *in vitro* (or *in vivo*) methods can be applied synergistically for the successful study of the inhibitory effectiveness of a compound on a receptor. Natural compounds have been reported to exhibit anticancer properties although their action mechanism still remains vague. In an effort to elucidate the possible enzyme/receptor protein anticarginogenetic interaction mechanisms theoretical docking studies have been carried towards cyclooxygenase-2 (COX-2) and lipoxygenase-5 (LOX). COX-2 is the key enzyme in the biosynthesis of inflammatory mediators like prostaglandin E2. Because there is a definite correlation between inflammation and carcinogenesis, it is believed that potential COX-2 inhibitors can be also considered as cancer chemopreventive agents[29]. Lipoxygenases are iron-containing enzymes which catalyze the oxidation of polyunsaturated fatty acids and esters to hydroperoxy derivatives[30]. Lipoxygenase-5 is identified as a suitable target for reducing the production of leukotrienes which are associated with the process of inflammation, allergic responses and asthma[31,32]. Lipoxygenase-15 has been also linked with the evolution of certain cancers[33]. Literature shows that COX-2 and LOX-5 are two significant molecular targets for neoplasm progression[34,35]. Our *in vitro* results showed that the most potent cytotoxic compounds are eugenol, isoeugenol and caffeic acid. Leem et al.[36] have shown that the essential oil of *Eugenia caryophyllata*, which contains eugenol as then main constituent, exhibited strong inhibitory activity against COX-2 (58.15%) and 15-LOX (86.15%) enzymes at 10 μg/mL and 25 μg/mL, respectively. Moreover eugenol derivatives (not isoeugenol) have been evaluated as potential inhibitors of lipoxygenase-15 through molecular docking studies[37]. Caffeic acid is a known inhibitor of lipoxygenases and its action has been evaluated previously[38]. Through molecular docking calculations we have tested isoeugenol as a potent COX-2 and 5-LOX inhibitor. The docking conformation with the lowest “rerank score” was selected as the most feasible binding conformation.

Docking results of isoeugenol towards COX-2 (PDB ID: 4COX) reveal that the molecule resides into the active site with similar orientation with indomethacin. The pocket consists of Ala527, Gly526, Leu352, Leu384, Met522, Phe381, Phe518, Ser353, Ser530, Trp387, Tyr355, Tyr385, Val349, and Val523. The terminal carbon atom is heading towards Leu384 while the opposite hydroxyl- O atom is directed towards the entrance of this hydrophobic channel (Ser353). This oxygen atom forms a hydrogen bond of 3.1 Å with the oxygen atom of Val349. Significant steric and van der Waals interaction are also evident.
Fig 5. Isoeugenol docked in the active site of COX-2. Hydrogen bonding and steric interaction with amino acids are also shown.

The crystal structure of 5-LOX (3V99) contains arachidonic acid at the active site of the enzyme. The enzyme exhibits significant 15-LOX activity through a point mutation to mimic phosphorylation at Ser663[39]. Isoeugenol effectively binds into the cavity of the active site near arachidonic acid. It interacts through the hydroxyl-O atom forming H-bonds with the nitrogen atoms of Phe177 and Gln413 at 2.8 and 3.1 Å respectively. There are significant steric interactions with the adjacent residues (Ala405, Ala410, Gln413, Ile406, Lys409, Phe169, and Phe177). Hydrogen bonding contributes to almost 5% of the total protein ligand interactions (steric interactions PLP and LJ12-6).

Fig 6. Isoeugenol docked in the active site of 5-LOX. In same pocket AA is also present (grey wire). On the right, steric interactions and hydrogen bonds.

4. Discussion

Our results indicate that the extracts of leaves and the essential oil of *O. basilicum* have noteworthy cytotoxic activity. Similar previous studies of pure plant-derived compounds such as flavones, flavonoles, phenoles, monoterpen and caffeic showed analogous results[1-4]. However,
marked differences are detected among different cell lines. Hence, the antiproliferative activity of essential oil from 17 Thai medicinal plants on human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines using MTT assay were previously investigated[18]. Sweet basil oil gave the highest anti-proliferative activity with the IC50 value of 0.0362 mg/ml or 36.2 μg/ml (12.7 times less potent than 5-FU) in P388 cell line. The essential oil of Ocimum basilicum Linn., cultivated in the Western Ghats of South India was found to mainly consists of methyl cinnamate (70.1%) and linalool (17.5%)[40]. These were investigated against the human cervical cancer cell line (HeLa), human laryngeal epithelial carcinoma cell line (HEp-2) and NIH 3T3 mouse embryonic fibroblasts and the IC50 values obtained were 90.5 and 96.3 μg/mL, respectively. The protective effect against carcinogenesis of essential oils from basil leaves on Swiss mice with induced neoplasia was shown by the significant increase of the glutathione-S-transferase activity[41]. Caffeic acid and its phenyl ester (CAPE) on hepatocarcinoma cells show a reversion of hepatoima growth and metastasis[42]. It was suggested that the anti-metastatic and anti-tumor effects of these compounds are mediated through the selective suppression of MMP-9 enzyme and the inhibition of NF-κB as well as MMP-9 catalytic activity. Eugenol is reported to have anticancer properties either alone or in combination with other chemotherapeutic agents. It was suggested that eugenol exerts its anticancer profile mostly via anti-inflammatory action[43]. Linalool showed strong activity against histiocytic lymphoma cells U937 (IC50: 3.51 μg/ml) and Burkitt lymphoma cells P3HR1 (IC50: 4.21 μg/ml)[44].

In our case, we have successfully identified the molecules of caffeic acid, eugenol, isoeugenol and rosmarinic acid which are the major phytochemicals of basil and therefore probably responsible for any anticancer potency against human cervix adenocarcinoma HeLa cells, human melanoma FemX, human chronic myelogenous leukaemia K562. It is interesting that although the plant derived compounds caffeic acid, eugenol, isoeugenol, linalool and rosmarinic acid are active against human ovarian SKOV3 cells, the extract and the oil are not. This may be due to a negative effect or influence of some other constituents of the extract and oil. Previous studies[45,46] have investigated the cytotoxic activity and the DNA synthesis inhibitory activity of eugenol and isoeugenol against salivary gland tumor cell line (HSG) and normal human gingival fibroblast (HGF) and have concluded that the higher cytotoxic activity of isoeugenol is due to lipophilic radicals and their interaction with cell membranes. In the present study, we have also shown that isoeugenol exhibits increased activity against HeLa, FemX, K562 and SKOV3 cells compared to eugenol. Isoeugenol has the highest activity from all the tested compounds against the K562 cells. Molecular docking studies revealed that isoeugenol has the potential to inhibit COX and LOX enzymes partly explaining its high cytotoxic activity. Caffeic acid is in the same range or better compared to cisplatin against the all four cell lines tested while its action against SKOV3 cancer cells is superior.

5. Conclusions

The aim of our study was to examine O. Basilicum and five pure plant-originated compounds for their possible effects on cell viability and cell death. The possible utilization of plant-derived compounds and extracts as chemopreventive and health-promoting agents in the future has focused increasing attention towards the understanding of their molecular mechanisms and targets of action. The use however, of medicinal plants as potential drugs is often hindered by the difficulty to classify the molecules responsible for such health improvement capacity. In this study we have identified the main constituents of Ocimum basilicum extract and essential oil, namely rosmarinic and caffeic acid and eugenol, isoeugenol and linalool using chromatographic and spectrometric techniques. All compounds were tested against four human cancer cell lines and have been found more effective than the corresponding extract or oil. Molecular docking studies of isoeugenol have shown that this major constituent of O. Basilicum essential oil can act as a potent inhibitor of COX-2 and LOX-5.
Acknowledgments

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Reference