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Antiproliferative Activities of the Lipophilic Fraction of *Eucalyptus camaldulensis* against MCF-7 Breast Cancer Cells, UPLC-ESI-QTOF-MS Metabolite Profile, and Antioxidative Functions

Yanping Huang, Mei An, Anning Fang, Opeyemi Joshua Olatunji, and Fredrick Nwude Eze*

Cite This: *ACS Omega* 2022, 7, 27369−27381

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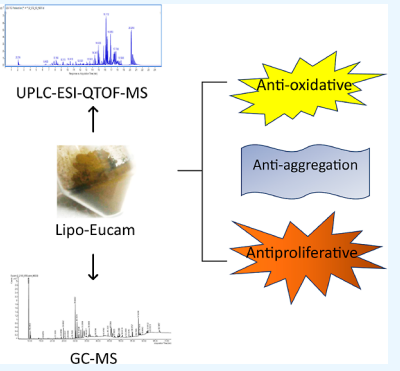
ABSTRACT: Although a number of pharmacological properties have been linked to *Eucalyptus camaldulensis* leaf essential oil and extracts, the biological attributes of the lipophilic fraction remain unknown. Moreover, only a limited number of active compounds have so far been identified. This work aimed to investigate the anti-oxidative, anti-aggregation, and cytotoxic properties as well as profile the secondary metabolites in the lipophilic fraction of *E. camaldulensis* leaf extract (Lipo-Eucam) using UHPLC-ESI-QTOF-MS and gas chromatography−mass spectrometry (GC−MS). It was found that Lipo-Eucam possessed potent antioxidant properties against DPPH, ABTS, and FRAP with IC_{50} values of 31.46, 32.78, and 10.12 μg/mL, respectively. The fraction was able to attenuate metal-catalyzed oxidation of bovine serum albumin (BSA) in a dose-dependent manner ($p < 0.05$) and abrogated the aggregation of amyloidogenic BSA as revealed by the Congo red assay and transmission electron microscopy. Furthermore, Lipo-Eucam demonstrated potent cytotoxic effects against MCF-7 (IC_{50} 7.34 μg/mL) but was noncytotoxic at used concentrations against HEK-293 cells (IC_{50} > 80 μg/mL), suggestive of its selective anticancer properties. Spectrophotometric, UHPLC−MS, and GC−MS analysis revealed that Lipo-Eucam is rich in phenolics, flavonoids, terpenoids, volatile constituents, and a plethora of active metabolites, probably responsible for the observed activities. These findings indicate that Lipo-Eucam is endowed with pharmacologically relevant active principles with strong potential for use in the amelioration of disease conditions related to oxidative stress, protein aggregation, and breast cancer and therefore worthy of further investigations.



1. INTRODUCTION

The demand for functional ingredients from natural origin for healthcare, food, and pharmaceutical applications is increasing at an unprecedented rate. In particular, the desire for antioxidants from natural sources over their synthetic counterparts has been steadily growing aided by rising concerns of the potential toxicity of chemically synthesized agents. Also, the increasing incidence of intractable chronic conditions and cancers justifies the search for novel bioactive agents to either prevent, counter, and/or ameliorate these conditions. Plants remain one of the most reliable sources for the identification and development of important bioactive principles.

Eucalyptus camaldulensis Dehnh (river red gum) is an evergreen perennial tree belonging to the Myrtaceae family. *E. camaldulensis* is one of the most widely distributed *Eucalyptus* spp. and one of the most planted trees in the world, having immense commercial and pharmacological value. Its dense wood has made *E. camaldulensis* very valuable for pulping.[1] During processing of the log, vast quantities of waste byproducts are often generated in the form of stem bark, small branches, and leaves. In view of closing the loop and enhancing the sustainable use of this plant, these byproducts represent a cheap and potentially valuable bioresource which can be valorized for various purposes. Traditionally, the plant leaves and stem bark had been used for the treatment of various ailments including fevers, flu, cold, general sickness,[2] as anesthetic, astringent, and antiseptic agent.[3] Recent reports on the pharmacological and biological properties of *E. camaldulensis* leaf extract have pointed to the antimicrobial, antioxidant, antifungal, anti-inflammatory attributes, and antitumor, among others. These activities were mostly attributed to the leaf essential oils[4] as well as other bioactive agents present in the leaf extracts, such as flavonoids, phenolic acids, and tannins.[5,6] Despite an increasing number of studies highlighting the potential bioactivity of *E. camaldulensis*, only a limited number of compounds have been identified to date.

Apparently, the composition and bioactivity of *E. camaldulensis* extract is influenced not only by the source material but also by the adopted method of preparation. In the

Received: April 17, 2022
Accepted: July 15, 2022
Published: July 29, 2022

https://doi.org/10.1021/acsomega.2c02389
ACS Omega 2022, 7, 27369−27381

Table 1. Chemical Profile of Bioactive Compounds Present in *E. camaldulensis* Extract Obtained by UPLC-ESI-QTOF-MS Analysis[a]

S/N	name (tentative ID)	formula	precursor (m/z)	accurate mass (Da)	RT (min)	score (DB)	diff (ppm)
1	quinic acid	$C_7H_{12}O_6$	191.0561	192.0634	2.195	99.7	−0.02
2	4-glucogallic acid	$C_{13}H_{16}O_{10}$	331.0667	332.074	4.83	99.26	1
3	chlorogenic acid	$C_{16}H_{18}O_9$	353.0877	354.0949	6.525	99.82	0.41
4	gardoside	$C_{16}H_{22}O_{10}$	373.1137	374.1209	6.738	98.66	1.19
5	(±)-catechin	$C_{15}H_{14}O_6$	289.0716	290.0789	6.814	99.86	0.6
6	sonchuionoside C	$C_{19}H_{30}O_8$	431.1922	386.1943	6.914	90.44	−0.61
7	isosyringinoside	$C_{23}H_{34}O_{14}$	533.1871	534.1944	6.939	98.58	0.92
8	hydrojuglone glucoside	$C_{16}H_{18}O_8$	337.0928	338.1001	7.24	99.61	0.35
9	pantoyllactone glucoside	$C_{12}H_{20}O_8$	351.1295	292.1156	7.303	99.25	0.7
10	isoorientin 6″-*O*-glucoside	$C_{26}H_{28}O_{16}$	595.1299	596.1371	7.366	98.45	1.06
11	(*S*)-rutaretin	$C_{14}H_{14}O_5$	261.0769	262.0841	7.541	99.27	0.05
12	dihydroferulic acid 4-*O*-glucuronide	$C_{16}H_{20}O_{10}$	371.0982	372.1054	7.558	98.93	0.55
13	quercetin 3-(2-galloylglucoside)	$C_{28}H_{24}O_{16}$	615.0987	616.1057	7.642	96.32	1.2
14	isovitexin	$C_{21}H_{20}O_{10}$	431.0984	432.1058	7.667	96.84	−0.25
15	quercetin 7-(6″-acetylglucoside)	$C_{23}H_{22}O_{13}$	505.098	506.1048	7.767	91.86	2.47
16	quercetin 3-galactoside	$C_{21}H_{20}O_{12}$	463.0879	464.0952	7.881	98.58	0.61
17	quercetin 3′-*O*-glucuronide	$C_{21}H_{18}O_{13}$	477.0671	478.0744	7.881	99.33	0.66
18	rumexoside	$C_{20}H_{22}O_{10}$	421.1138	422.121	8.119	99.15	0.74
19	tricetin 3′-xyloside	$C_{20}H_{18}O_{11}$	433.0777	434.0849	8.194	98.56	−0.08
20	kaempferol 4′-glucoside	$C_{21}H_{20}O_{11}$	447.0934	448.1004	8.32	95.31	0.4
21	methyl (3x,10R)-dihydroxy-11-dodecene-6,8-diynoate 10-glucoside	$C_{19}H_{26}O_9$	397.1504	398.1576	8.42	98.89	0.19
22	6-*C*-*β*-d-xylopyranosylluteolin	$C_{20}H_{18}O_{10}$	417.0826	418.0898	8.571	98.82	0.54
23	epigallocatechin 3-*O*-*p*-coumarate	$C_{24}H_{20}O_9$	451.1034	452.1106	8.571	99.56	0.3
24	hesperetin	$C_{16}H_{14}O_6$	301.0711	302.0785	10.654	97.1	1.91
25	phloretin	$C_{15}H_{14}O_5$	273.0767	274.084	8.747	97.6	0.49
26	okanin 4-methyl ether 3′-glucoside	$C_{22}H_{24}O_{11}$	463.1246	464.1318	8.847	99.15	0.09
27	gallocatechin-(4*α*->8)-epigallocatechin	$C_{30}H_{26}O_{14}$	609.1245	610.1319	9.023	99.05	0.66
28	maritimetin 6-*O*-(3″,4″,6″-tri-*O*-acetylglucoside)	$C_{27}H_{26}O_{14}$	573.1244	574.1316	9.073	98.41	1.09
29	dalpaniculin	$C_{25}H_{28}O_{13}$	535.1457	536.1529	9.123	98.05	0.19
30	okanin 3,4-dimethyl ehter 4′-glucoside	$C_{23}H_{26}O_{11}$	477.1401	478.1482	9.148	71.75	−1.38
31	2,3-dihydro-5,5′,7,7′-tetrahydroxy-2-(4-hydroxyphenyl)[3,8′-bi-4*H*-1-benzopyran]-4,4′-dione	$C_{24}H_{16}O_9$	447.0722	448.0795	9.374	98.18	−0.11
32	medicagenic acid 3-*O*-*β*-D-glucuronide	$C_{36}H_{54}O_{12}$	677.3534	678.3609	9.449	96.88	0.99
33	3,5-dicaffeoyl-4-succinoylquinic acid	$C_{29}H_{28}O_{15}$	615.135	616.1423	9.525	99.27	0.92
34	(*R*)-rutaretin 1′-(6″-sinapoylglucoside)	$C_{31}H_{34}O_{14}$	629.1872	630.1945	9.588	99.55	0.64
35	2a-hydroxygypsogenin 3-*O*-*β*-D-glucoside	$C_{36}H_{56}O_{10}$	693.3847	648.3871	9.65	83.11	0.34
36	glucosyl passiflorate	$C_{37}H_{60}O_{12}$	695.4008	696.408	9.889	98.74	0.67
37	9,12,13-trihydroxy-10,15-octadecadienoic acid	$C_{18}H_{32}O_5$	327.2175	328.2248	10.353	99.53	0.63
38	2,8-di-*O*-methylellagic acid	$C_{16}H_{10}O_8$	329.03	330.0372	10.504	99.11	0.97
39	corchoionoside B	$C_{19}H_{28}O_9$	399.1655	400.1728	10.755	98.68	1.22
40	11,12,13-trihydroxy-9-octadecenoic acid	$C_{18}H_{34}O_5$	329.233	330.2403	10.78	98.73	0.91
41	lethedoside B	$C_{25}H_{28}O_{12}$	519.1507	520.1579	10.931	99.17	0.37
42	luteolin	$C_{15}H_{10}O_6$	285.0403	286.0476	11.081	99.11	0.57
43	5-hydroxy-7,2′,5′-trimethoxyflavone	$C_{18}H_{16}O_6$	327.0873	328.0945	11.232	98.81	0.54
44	matteuorienate B	$C_{29}H_{34}O_{13}$	589.1924	590.1997	11.433	97.9	0.43
45	5,3′,4′-trihydroxy-3-methoxy-6,7-methylenedioxyflavone	$C_{17}H_{12}O_8$	343.0457	344.053	12.11	98.51	0.73
46	(7′*S*,8′*S*)-4,7′-epoxy-3,8′-bilign-7-ene-3′,5-dimethoxy-4′,9,9′-triol 4′-glucoside	$C_{26}H_{32}O_{11}$	565.1929	520.1945	12.337	98.11	−0.05
47	cnidilin	$C_{17}H_{16}O_5$	299.0925	300.0998	12.437	97.59	0.07
48	luteolin 7-*O*-*β*-D-glucoside	$C_{21}H_{32}O_{10}$	443.1925	444.1996	12.613	98.75	−0.19
49	bayogenin	$C_{30}H_{48}O_5$	487.3429	488.3501	12.65	97.41	
50	methoxyhydroxymethyl hydrocinnamic acid	$C_{11}H_{14}O_4$	209.0818	210.0891	13.102	98.96	0.55
51	2,2,4,4,-tetramethyl-6-(1-oxopropyl)-1,3,5-cyclohexanetrione	$C_{13}H_{18}O_4$	237.1131	238.1203	13.654	98.76	0.87
52	bolegrevilol	$C_{28}H_{40}O_4$	439.2851	440.2922	13.868	96.77	0.98
53	quillaic acid	$C_{30}H_{46}O_5$	485.3269	486.334	13.993	77.95	1.16
54	aspidinol	$C_{12}H_{16}O_4$	223.098	224.1052	14.056	98.42	−1.73
55	*α*-peroxyachifolide	$C_{20}H_{24}O_7$	375.145	376.1523	15.199	97.48	−0.39
56	5,7,4′-trimethoxyflavone	$C_{18}H_{16}O_5$	311.0923	312.0995	15.248	99.6	0.77
57	3-trans-*p*-coumaroylrotundic acid	$C_{39}H_{54}O_7$	633.3798	634.3869	15.374	98.55	0.05

https://doi.org/10.1021/acsomega.2c02389
ACS Omega 2022, 7, 27369−27381

Table 1. continued

S/N	name (tentative ID)	formula	precursor (m/z)	accurate mass (Da)	RT (min)	score (DB)	diff (ppm)
58	anhydrocinnzeylanine	$C_{22}H_{32}O_7$	453.2128	408.2147	15.65	99.16	0.23
59	maslinic acid	$C_{30}H_{48}O_4$	471.3478	472.3551	15.738	99.34	0.26
60	macrocarpal I	$C_{28}H_{42}O_7$	489.2858	490.293	15.977	99.08	0.03
61	12-hydroxy-11-methoxy-8,11,13-abietatrien-20-oic acid	$C_{21}H_{30}O_4$	405.2283	346.2144	16.83	98.33	0.01
62	artecanin	$C_{15}H_{18}O_5$	277.1084	278.1157	17.081	96.42	−0.85
63	anhydrocinnzeylanine	$C_{22}H_{32}O_7$	453.213	408.2148	17.182	98.86	−0.08
64	eucalypcamal F	$C_{20}H_{22}O_6$	357.1344	358.1418	17.257	96.55	−0.56
65	pseudolaric acid B	$C_{23}H_{28}O_8$	431.1724	432.1797	17.521	90.14	−3.07
66	eucalypcamal B	$C_{23}H_{28}O_6$	399.1814	400.1886	17.709	91.61	−0.79
67	eucalypcamal N	$C_{23}H_{30}O_7$	417.1921	418.1992	18.663	98.4	−0.51
68	eucalypcamal D	$C_{23}H_{32}O_4$	371.2225	372.2301	20.596	99.03	0.9
69	laxiflorin	$C_{23}H_{26}O_7$	459.1661	414.1679	20.872	99.07	−0.01
70	eucalypcamal M	$C_{23}H_{30}O_6$	401.1967	402.2042	21.575	95.16	0.4

[a]Rt: retention time.

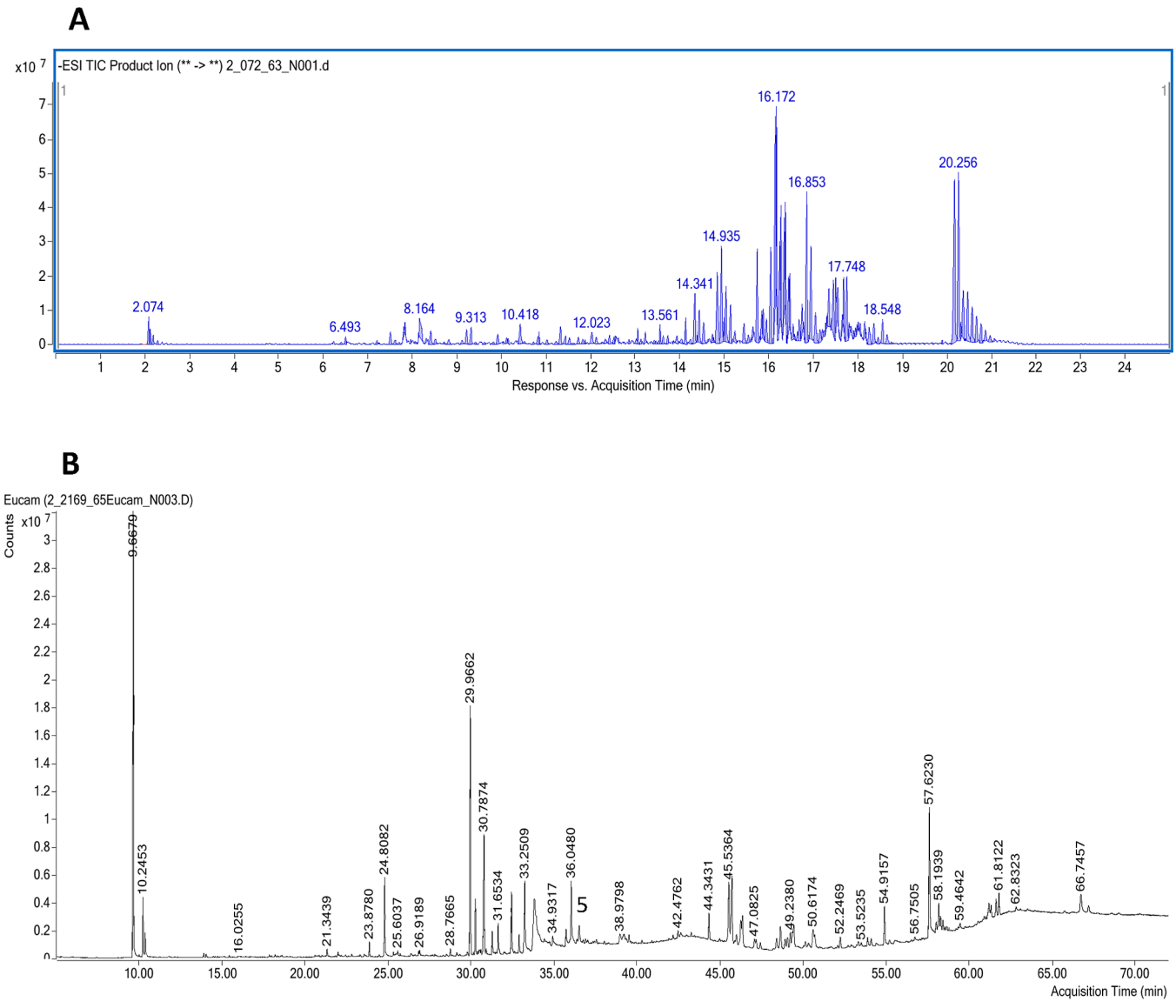


Figure 1. (A) Total ion chromatogram of secondary metabolites found in Lipo-Eucam by UPLC-ESI-QTOF-MS and (B) GC−MS chromatogram of Lipo-Eucam.

past, there had been reports on the activity of the aqueous,[3] aqueous-acetone,[7] methanol, ethyl acetate, *n*-butanol,[8] chloroform and hexane,[9] ethanol,[10] dichloromethane, and petroleum ether[11] prepared via conventional approaches. The limitations

https://doi.org/10.1021/acsomega.2c02389
ACS Omega 2022, 7, 27369−27381

of conventional extraction approaches such as requirements for high energy, enormous quantity of organic solvents which are sometimes toxic, and the tedious and time-consuming nature together pose considerable challenges to large-scale extract preparation, health and environmental concerns, as well as use of the extract in food, nutraceutical, cosmetics, or pharmaceutical applications. Consequently, there had been increasing push toward more benign and greener extraction techniques as previously reported.[12] Nonetheless, there is currently an absence of reports on the lipophilic extract of *E. camaldulensis* leaves prepared via a "green" route nor an assessment of its activity as potential antioxidative, anticancer, or anti-aggregation agent.

The current work aimed to elucidate some important biological activities of lipophilic fraction of *E. camaldulensis*. Specifically, the study is centered on evaluating the antioxidative, anti-aggregation, and anti-proliferative effects of the lipophilic fraction of *E. camaldulensis* leaf extract (Lipo-Eucam) prepared without using any noxious organic solvent. Consequently, the chemical profile of the secondary metabolites present in Lipo-Eucam will be ascertained by spectrophotometry and using UHPLC-ESI-QTOF-MS and gas chromatography−mass spectrometry (GC−MS) analysis. The impact of Lipo-Eucam on oxidation will be evaluated using DPPH, ABTS, and FRAP assays as well as metal-catalyzed protein oxidation system. Then, the influence of the fraction on protein aggregation will be monitored using Congo red assay and transmission electron microscopy (TEM). Finally, the cytotoxic effect will be examined using human breast cancer (MCF-7 and MDA-MB-231) cell lines and human embryonic kidney (HEK-293) cell line via the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay.

2. RESULTS AND DISCUSSION

2.1. Chemical Constituents of Lipo-Eucam. The leaves of *Eucalyptus* spp are well known for their poor nutritional attributes such as very low amounts of available protein;[13] however, these plant samples are equally renowned for their high content of secondary metabolites, including polyphenols, tannins, terpenes, phloroglucinol compounds, and essential oils, among others.[14] These bioactive compounds have been implicated in the plants' ability to fend off herbivores. More importantly, they are now widely recognized for the numerous pharmacological properties associated with *Eucalyptus* spp, such as antimicrobial, antifungal, antioxidant, anti-inflammatory, analgesic, antiulcer, and antidiabetic.[15,16] The number and content of secondary metabolites present in any particular *Eucalyptus* spp leaf extract are influenced by several factors including agronomic, environmental, genetic (species), and certainly, the manner in which the extract was prepared,[17] that is, extraction method, time, solvents, and ratio of solvent to solid material, among others. This in turn could play a significant role in their pharmacological and nutraceutical application. Thus, it is imperative to evaluate the content as well as determine the particular individual bioactives present in *Eucalyptus* species prepared under different conditions. Particularly, in *E. camaldulensis* leaves, the paucity of information on the chemical profile of the lipophilic fraction further accentuates the importance. Ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry qualitative analysis of the lipophilic fraction of *E. camaldulensis* leaf extract (Lipo-Eucam) enabled the identification of 70 compounds (Table 1) eluted between 2 and 22 min (Figure 1A).

The majority of these compounds were found to belong in major classes, such as terpenes and terpenoids, tannins, flavonoids, phenolic acid and derivatives, fatty acids, and phloroglucinol derivatives. Terpenes and terpenoid derivatives found in Lipo-Eucam included sonchuionoside C, medicagenic acid 3-*O*-β-d-glucuronide, 2α-hydroxygypsogenin 3-*O*-β-D-glucoside, glucosyl passiflorate, bayogenin, bolegrevilol, quillaic acid, α-peroxyachifolide, maslinic acid, artecanin, and pseudolaric acid B. The oleanane pentacyclic triterpene, maslinic acid, had previously been reported in *Eucalyptus* spp leaves.[18] Along with the terpenes, many flavonoid compounds were also detected in Lipo-Eucam. Notably, these were mostly quercetin (compounds #13, #15, #16, and #17), luteolin (compounds #22, #42, and #48), apigenin (compound #14), or kaempferol (compound #20) derivatives, as well as the methylated flavonoid 8-demethyleucalyptin,[19] which is consistent with past reports from other studies.[6,12]

Chlorogenic acid was found as compound #3 by examining the theoretical mass versus the experimental mass of the deprotonated parent ion $[M - H]^-$ (353.0878 vs 353.0877 Da) as well as the fragment at m/z 191.0560 which was consistent with the mass of quinic acid, the main fragment of chlorogenic acids. Other hydroxycinnamate derivatives uncovered included dihydroferulic acid 4-*O*-glucuronide, 3,5-dicaffeoyl-4-succinoylquinic acid, and methoxyhydroxymethylhydrocinnamic acid.

Eucalyptus spp. leaves are also known to harbor a great diversity of tannin compounds.[7] In Lipo-Eucam, several tannins were found to be present such as (±)-catechin (*5), epigallocatechin 3-*O*-*p*-coumarate, gallocatechin-(4-α->8)-epigallocatechin, 2,8-di-*O*-methylellagic acid. Phenolic compounds such as (±)-catechin, hesperitin, phloretin, tricetin, and chlorogenic acid found in Lipo-Eucam had previously been observed in *E. camaldulensis* leaves.[20] Phloroglucinol derivatives located in Lipo-Eucam included aspidinol and macrocarpal I. While this is the first report of the presence of macrocarpal I in the *E. camaldulensis* leaf sample, these compounds are known to be present in other *Eucalyptus* spp.[18] In addition, several phloroglucinol-meroterpenoids eluted between R_t 17.25−21.57 min were also present in Lipo-Eucam such as eucalypcamal F, eucalypcamal B, eucalypcamal N, eucalypcamal D, and eucalypcamal M. The strong presence of these compounds in Lipo-Eucam is consistent with recent reports according to Daus et al., wherein the authors noted that phloroglucinol-meroterpenoids constitute the major bioactive components in dichloromethane extract of the *E. camaldulensis* leaf extract.[21] The identity of most of these compounds was proposed on the basis of their UPLC−MS/MS data by comparing with chemistry databases as well as published literature on the phytochemistry of *E. camaldulensis* and related species of the same genus.[7,14,21] Thus, further corroboration is still warranted via the use of other spectroscopic techniques and compound standards to confirm the position of glycosylation as well as stereochemistry of the compounds. While consistent with previous reports on the class and identity of compound in *E. camaldulensis*, this study provides the first and extensive list of compounds in the lipophilic fraction of the leaves sample.

Also, *E. camaldulensis* is reputed as a rich source of essential oils and volatile constituents.[22] Lipo-Eucam was expected to contain some essential oil constituents, as well as other semi-

https://doi.org/10.1021/acsomega.2c02389
ACS Omega 2022, 7, 27369−27381

Table 2. GC−MS Qualitative Analysis of Bioactive Compounds Present in Lipophilic Fraction of *Eucalyptus camaldulensis* Leaf Extract[a]

S/N	RT (min)	name (tentative ID)	formula	CAS RN	% oftotal	nature of compound
1	9.6679	α-phellandrene	$C_{10}H_{16}$	2000048-82-3	15.3	cyclic monoterpenes
2	10.2453	*o*-cymene	$C_{10}H_{14}$	527-84-4	1.74	aromatic hydrocarbon
3	10.3736	3-methylene-6-(1-methylethyl)-cyclohexene	$C_{10}H_{16}$	555-10-2	0.86	cyclic monoterpene
4	16.0255	benzaldehyde, 4-methyl-	C_8H_8O	104-87-0	0.16	benzoyl derivative
5	21.3439	caryophyllene	$C_{15}H_{24}$	87-44-5	0.28	bicyclic sesquiterpene
6	21.9919	isoledene	$C_{15}H_{24}$	95910-36-4	0.15	sesquiterpene
7	23.878	(1*S*,4*S*,4α*S*)-1-isopropyl-4,7-dimethyl-1,2,3,4,4α,5-hexahydronaphthalene	$C_{15}H_{24}$	267665-20-3	0.41	cadinane sesquiterpenoids
8	24.8082	1,6,10-dodecatrien-3-ol, 3,7,11-trimethyl-	$C_{15}H_{26}O$	7212-44-4	2.55	sesquiterpene
9	25.6037	hexadecane	$C_{16}H_{34}$	544-76-3	0.2	hydrocarbon
10	26.9189	2-naphthalenemethanol, 1,2,3,4,4a,5,6,8α-octahydro-α,α,4α,8-tetramethyl-,[2*R*-(2α,4a.α.,8a.β.)]-	$C_{15}H_{26}O$	473-16-5	0.19	cedrane and isocedrane sesquiterpenoids
11	28.7665	α-phellandrene, dimer	$C_{20}H_{32}$	7350-11-0	0.22	cyclic monoterpenes
13	29.9662	α-phellandrene, dimer	$C_{20}H_{32}$	7350-11-0	8.58	cyclic monoterpenes
14	30.2677	α-phellandrene, dimer	$C_{20}H_{32}$	7350-11-0	1.63	cyclic monoterpenes
15	30.4923	(2,6,6-trimethylcyclohex-1-enylmethanesulfonyl)benzene	$C_{16}H_{22}O_2S$	56691-74-8	0.21	benzenesulfonyl compounds
16	30.7874	3,7,11,15-tetramethylhexadec-2-en-1-yl acetate	$C_{22}H_{42}O_2$	76337-16-1	5.09	acyclic diterpenoids
17	30.9285	(2*E*)-3,7,11,15-tetramethyl-2-hexadecene	$C_{20}H_{40}$	14237-73-1	0.17	hydrocarbone
18	31.2942	phytol, acetate	$C_{22}H_{42}O_2$	2000646-53-2	0.84	acyclic diterpenoids
19	31.6534	phytol, acetate	$C_{22}H_{42}O_2$	2000646-53-2	1.19	acyclic diterpenoids
20	32.4554	methyl 5-*tert*-butyl-3-(chloromethyl)-2-thiophenecarboxylate	$C_{11}H_{15}ClO_2S$	252914-61-7	2.24	
21	33.2509	*n*-hexadecanoic acid	$C_{16}H_{32}O_2$	57-10-3	3.51	fatty acid
22	33.8347	2*H*-indol-2-one, 3-[(4-ethylphenyl)imino]-1,3-dihydro-	$C_{16}H_{14}N_2O$	2000365-27-0	4.38	
23	33.9309	eicosane	$C_{20}H_{42}$	112-95-8	1.21	alkane
24	36.048	phytol	$C_{20}H_{40}O$	150-86-7	2.48	acyclic hydrogenated diterpene alcohol
25	36.388	9,12-octadecadienoic acid (*Z*,*Z*)-	$C_{18}H_{32}O_2$	60-33-3	0.19	fatty acid
26	36.5227	9,12,15-octadecatrienoic acid, (*Z*,*Z*,*Z*)-	$C_{18}H_{30}O_2$	463-40-1	0.89	fatty acid
27	38.9798	1,4-naphthoquinone, 2-acetyl-3-hydroxy-5,6,8-trimethoxy-	$C_{15}H_{14}O_7$	14090-54-1	0.73	1,4-naphthoquinones
28	42.4762	octadecane, 3-ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	55282-12-7	0.19	hydrocarbon
29	45.895	1-heptacosanol	$C_{27}H_{56}O$	2004-39-9	3.86	fatty alcohol
30	45.9983	triacontane	$C_{30}H_{62}$	638-68-6	0.49	hydrocarbon
31	49.0584	squalene	$C_{30}H_{50}$	111-02-4	0.6	triterpene
32	50.6174	17-pentatriacontene	$C_{35}H_{70}$	6971-40-0	1.09	hydrocarbon
33	54.1138	stigmasta-3,5-diene	$C_{29}H_{48}$	79897-80-6	0.41	steroid
34	54.9157	α-tocopherol	$C_{29}H_{50}O_2$	59-02-9	1.76	tocopherol
35	55.5636	7-methoxy-3-(3,4-dimethoxyphenyl)-4*H*-chromen-4-one	$C_{18}H_{16}O_5$	1621-61-0	0.43	flavonoid
36	57.623	γ-sitosterol	$C_{29}H_{50}O$	83-47-6	6.18	steroid
37	61.3311	betulinaldehyde	$C_{30}H_{48}O_2$	13159-28-9	0.54	pentacyclic triterpenoids
38	61.639	(3β)-3-hydroxy-urs-12-en-28-oic acid, methyl ester	$C_{31}H_{50}O_3$	32208-45-0	0.72	ursolic acid derivatives
39	61.8122	ursolic aldehyde	$C_{30}H_{48}O_2$	19132-81-1	0.87	ursolic acid derivatives
40	62.8323	uvaol	$C_{30}H_{50}O_2$	545-46-0	0.18	triterpenoid
41	66.7457	3-(1,5-dimethyl-hexyl)-3a,10,10,12b-tetramethyl-1,2,3,3a,4,6,8,9,10,10a,11,12,12a,12b-tetradecahydro-benzo[4,5]cyclohepta[1,2-*E*]inde	$C_{30}H_{50}$	2000800-89-0	1.26	hydrocarbon
42	67.2076	oleanolic acid	$C_{30}H_{48}O_3$	508-02-1	0.44	pentacyclic triterpenoid

[a]Rt: retention time; CAS RN: CAS Registry Number.

and volatile organic compounds. To further verify the identity of these compounds, GC−MS qualitative analysis was employed to extend the coverage. GC−MS analysis of Lipo-Eucam revealed several semi- and volatile components (Table 2) eluted between 9 and 70 min (Figure 1B). Most of the compounds identified by GC−MS were monoterpene hydrocarbons, which was in line with previous report on *E. camaldulensis* leaves.[23] Other compounds also captured by the analysis include many sesquiterpenes, terpenoids as well as flavonoid [7-methoxy-3-(3,4-dimethoxyphenyl)-4*H*-chromen-4-one], steroids (stigmasta-3,5-diene and γ-sitosterol), α-tocopherol, and many fatty acids. Similar compounds were also found in the lipophilic extractives of Brazilian eucalypt hybrids.[24] It is notable that the major oil components in Lipo-Eucam were α-phellandrene, *o*-cymene, *n*-hexadecenoic acid, and phytol, which was in agreement with previously documented accounts of leaf essential oils of *E. camaldulensis*.[25,26] α-Phellandrene was the most abundant volatile oil

https://doi.org/10.1021/acsomega.2c02389
ACS Omega 2022, 7, 27369−27381

component, and this compound is known for its widespread occurrence in *Eucalyptus* spp.[27] 1,8-Cineole previously found in other reports was absent. It is conceivable that this difference in composition could be attributed to the source of the plant sample, variation in season of harvest, genetics as well as the extraction approach.[20,26,28] The extraction method adopted herein is not tailored specifically for essential oils. Rather the objective was to obtain a broad spectrum of lipophilic components in the leaves presumably including essential oils and many other bioactive secondary metabolites. In this regard, the adopted extraction method proved to be suitable indeed.

2.2. Anti-Oxidative Properties of Lipo-Eucam. Cellular oxidation is a complex biochemical process essential to normal physiological functions as well as the pathological mechanisms of many ailments. Reactive chemical intermediates are involved in signal transduction, inflammatory response, and cellular defense, among other vital physiological roles. On the other hand, unchecked production and accumulation of reactive species beyond cellular regulatory capacity could result in oxidative stress, under which important cellular components such as proteins, nucleic acids, and lipids are oxidatively modified leading to the loss of normal cellular functions and in some cases, acquisition of toxic functions. Oxidative stress has been implicated in the etiopathogenesis of several chronic conditions. There has been several reports suggesting that the use of natural antioxidants could modulate or ameliorate the incidence of deleterious oxidation. Plants have been recognized as vital sources of natural antioxidants, especially in the form of polyphenols and terpenoids. The UPLC−MS and GC−MS profile of Eucam indicated the presence of a multitude of natural phenolics and terpenoids with reported antioxidant function.

Spectrophotometric evaluation of the phenolic and flavonoid content of the sample revealed that Lipo-Eucam revealed a total phenolic content of 112.18 mg GAE/g DW and total flavonoid content of 34.56 mg QE/d DW (Table 3). To put

Table 3. Chemical and Antioxidant Properties of Lipo-Eucam

S/N	attribute	Lipo-Eucam
1	total phenolic content (mg GAE/g dw)	112.18 ± 5.81
2	total flavonoid content (mg QE/g dw)	34.56 ± 1.06
3	DPPH [IC_{50} μg/mL]	31.46 ± 1.32
4	ABTS [IC_{50} μg/mL]	32.78 ± 1.44
5	FRAP [IC_{50} μg/mL]	10.12 ± 0.35

these results in context, it is worth mentioning that many other authors have alluded to the fact that *Eucalyptus* spp is rich in phenolics, although the reports have been less consistent. For instance, Nasr et al. observed that the *E. camaldulensis* leaf extract yielded 28.44−38.21, 32.8−38.21, and 30.20−46.56 mg/g DW when prepared using ethanol, methanol, and ethyl acetate as extraction solvent. The authors also noted that when water was used as extractant under boiling, maceration, or sonication, the total phenolic content was less than 32 mg/g DW.[20] Similarly, Elansary et al. found that methanol extracts of *E. camaldulensis*, *E. camaldulensis var obtusa*, and *Eucalyptus gamphocephala* leaves displayed TPC of about 14−34 mg GAE/g DW.[5]

Similarly, Álvarez et al., in their recent study on the effect of six solvents on the phytochemical composition of leaves extract of six *Eucalyptus* spp including *E. camaldulensis* confirmed that the total phenolic content was greatly influenced by the solvent and extraction conditions, with the hexane extract of *Ectropis obliqua* presenting the lowest (1.57 mg/g DW) and methanol extract of *Eucalyptus nitens* presenting the highest (124.17 mg/g DW) values.[29] Compared to these accounts, Lipo-Eucam apparently displayed superior phenolic content against the majority of the extracts and this outcome can be attributed not only to the source of the material itself but also equally to the adopted mode of preparation. The ethanolic extraction allowed for a high phenolic content as suggested by Álvarez et al.[29] Also, cold fractionation further facilitated the enrichment of the extract, increasing the amounts of phenolic components with low aqueous solubility. Importantly, no toxic solvent was used in the preparation of Lipo-Eucam, indicating its potential suitability for various applications.

The in vitro anti-radical activity was evaluated against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. The results showed a concentration-dependent inhibition of both radical species by Lipo-Eucam with more than 90% inhibition recorded at 80 μg/mL of the sample (Figure 2A). Both assays are simple, reliable, and widely used in the evaluation of radical scavenging capacity of natural pharmacological agents. The DPPH assay is based on a hydrogen transfer system and therefore reflects the sample's ability to donate hydrogen atoms to the radical species, neutralizing them or breaking the radical chain reaction. Besides, electron transfer reactions have also been noticed in the DPPH reaction mechanism. Nonetheless, the ABTS assay was performed to cover this aspect of the anti-radical mechanism. The ABTS radical scavenging activity simulates the capacity of the antioxidant sample to transfer electrons to the radical species, facilitating their neutralization. The FRAP assay depicts the reducing antioxidant capacity of any sample and have been reported to strongly correlate with cellular antioxidant capacity. As indicated by the results of the assays, Lipo-Eucam demonstrated potent radical scavenging activity with low IC_{50} values of 31.46 and 32.78 μg/mL against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, respectively. Trolox, a standard antioxidant, reportedly exhibits antiradical activities with IC_{50} values of 113.12 and 93.83 μg/mL against DPPH and ABTS,[30] while the DPPH IC_{50} values for various *Eucalyptus* essential oils were 4.21−52.53 mg/mL.[31] In this context, the lower IC_{50} values of Lipo-Eucam suggest it is a stronger antioxidant than the synthetic antioxidant and an even more potent antioxidant compared to the essential oils, indicating its high prospects in combating oxidative stress and damage.

Protein carbonyls are one of the most prominent forms of protein oxidative modifications. These modifications do not only alter the structure of the proteins leading to loss-of-function but also trigger the formation of harmful adducts that have been implicated in inflammation stress, apoptosis, cytotoxic damage, and immunogenicity.[32] The formation of protein carbonyls occur during the early phase of oxidative stress, and in addition, these oxidative modifications are relatively stable and irreversible. Consequently, the measurement of the levels of protein carbonylation has come to be regarded as a reliable indicator of the global protein oxidation and the extent of oxidative stress within the context of cellular damage in aging as well as oxidative-stress related disorders such as chronic kidney disease, advance diabetes mellitus, cardiovascular diseases, and neurodegeneration among others.[33] In this work, bovine serum albumin (BSA) was subjected to metal-catalyzed oxidation (MCO) to simulate

https://doi.org/10.1021/acsomega.2c02389
ACS Omega 2022, 7, 27369−27381

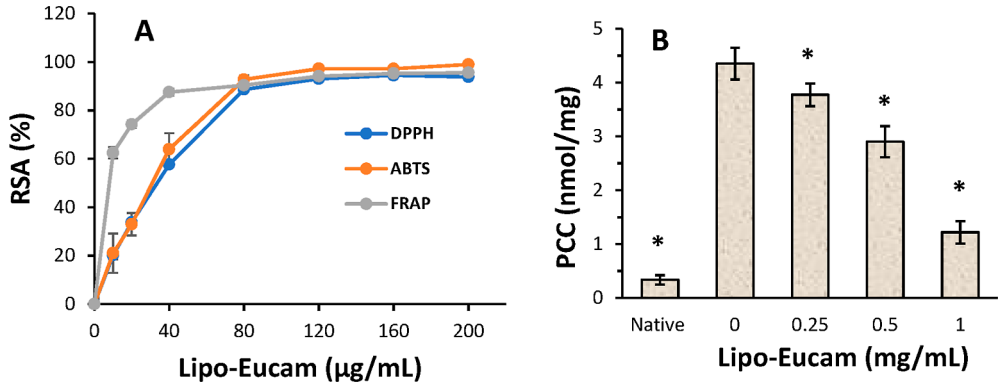


Figure 2. (A) In vitro radical scavenging activity and reducing antioxidant capacity of Lipo-Eucam. (B) The effect of Lipo-Eucam (0.25−1.0 mg/mL) on metal-catalyzed oxidation of BSA. Statistical significance was defined as $^*p < 0.05$ compared to oxidized BSA alone.

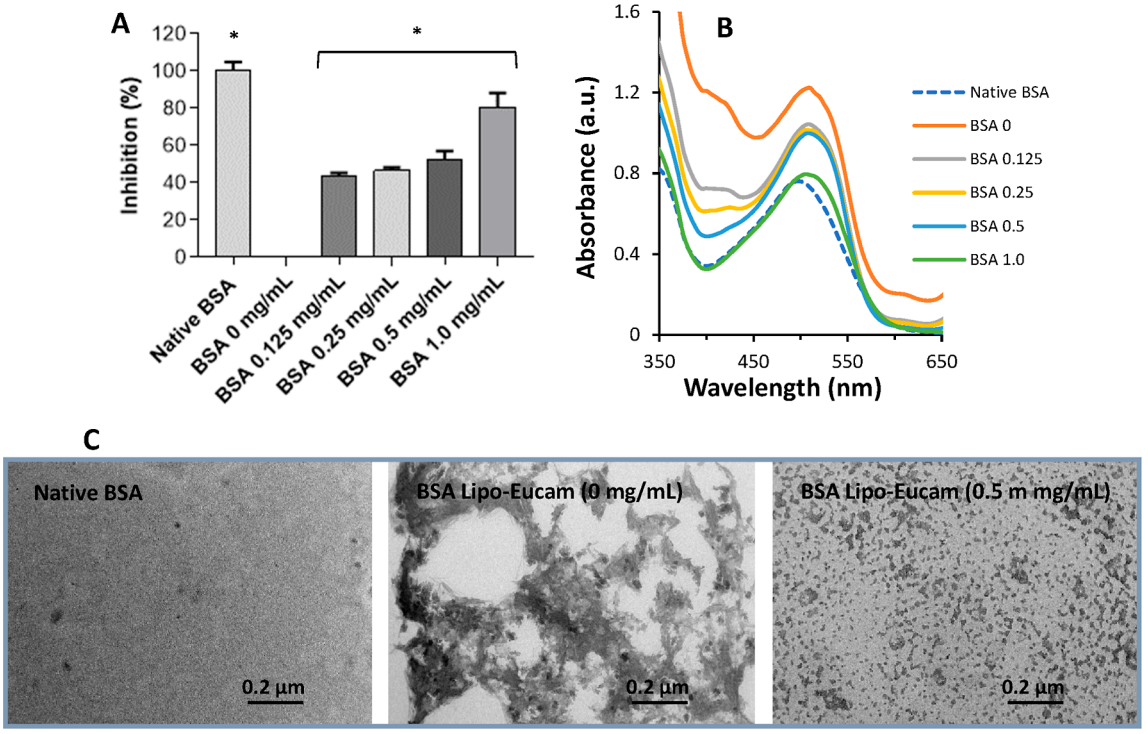


Figure 3. (A) Inhibition of aggregate formation by BSA subjected to aggregation stress with or without Lipo-Eucam determined by Congo red binding. Data are presented as the mean ± SD ($n = 3$) and statistical significance was defined as $^*p < 0.05$ compared to aggregated BSA alone. (B) Representative Congo red absorbance spectrum of BSA incubated with or without Lipo-Eucam. (C) TEM images of native and aggregated BSA with or without Lipo-Eucam.

oxidative stress in vitro. The content of protein carbonyls was measured spectrophotometrically via the DNPH assay, a simple technique widely used for the quantification of protein-bound carbonyls.[34] As revealed in Figure 2B, the content of protein carbonyls in the oxidized BSA sample (4.35 nmol/mg) clearly exceeded those of the native protein (0.34 nmol/mg), indicating the success of protein oxidation in the treated samples. This was consistent with other accounts in literature which had noted relatively modest amounts of serum albumin-bound carbonyls under MCO conditions compared to other oxidants such as hypochlorous acid, but a substantial increase nonetheless relative to the native protein.[34,35] Compared to oxidized BSA alone, BSA samples subjected to oxidation in the presence of Lipo-Eucam significantly retarded the formation of protein-bound carbonyls ($p < 0.05$). That is, the BSA samples presented bound carbonyl levels of 4.35, 3.77, 2.90, and 1.22 nmol/mg upon treatment with 0, 0.25, 0.5, and 1.0 mg/mL Lipo-Eucam. These results suggest that Lipo-Eucam could markedly suppress the formation of protein oxidation in a concentration-dependent manner. Le Guen et al. in a series of experiments involving chelators, catalase, and superoxide dismutase noted that hydroxyl radicals constitute the active principles in MCO.[36] As such, it is likely that the counteractive effect of Lipo-Eucam could be due to its capacity to scavenge these radical species, limiting the oxidation of susceptible lysine, proline, and arginine residues to glutamic and aminoadipic semialdehydes counterparts.[32] The suppressive effect of Lipo-Eucam on protein oxidation is an indication that this fraction could ameliorate oxidative stress and conditions related to oxidative damage.

2.3. Influence of Lipo-Eucam on Protein Aggregation.

Furthermore, the effect of Lipo-Eucam on protein aggregation,

https://doi.org/10.1021/acsomega.2c02389
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a process that is closely linked with oxidation and disease phenomenon in conditions such as Alzheimer's disease, Parkinson's disease, and type 2 diabetes mellitus was also evaluated. BSA was used as the model protein given that albumins are known to be amyloidogenic in vivo and thus have been extensively used to understand protein aggregation.[37] Four concentrations of Lipo-Eucam, vis: 0.125, 0.25, 0.5, and 1.0 mg/mL were investigated to ascertain their effect on BSA under aggregation stress. The extent of aggregation was probed using Congo red, an anionic diazo dye widely used for the quantification protein aggregation because of its propensity to intercalate between the β-strands of the amyloid morphology of aggregated proteins.[38] Native BSA and aggregated BSA without any treatment, respectively, served as positive and negative controls for the inhibition study. The effect of Lipo-Eucam on the formation of aggregated BSA as well as a representative Congo red absorption spectra of BSA samples incubated with or without treatment is presented in Figure 3. The Congo red spectra of the protein solutions revealed a red shift in the position of the λ_{max} of the native protein (~490 nm) relative to the protein solutions subjected to aggregation (~520 nm). Also, the peak intensity of the incubated protein solutions was largely higher than that of the native protein solution. Both observations were indicative and consistent with the presence of protein aggregates in the incubated protein solutions.[39] Upon binding with protein amyloids, the Congo red spectrum displays a red shift of the absorbance peak as well as an increase in the intensity of the λ_{max}. BSA co-incubated with Lipo-Eucam (BSA 0.125, 0.25, 0.5, and 1.0) revealed a consistent and marked decrease in peak intensity of the spectra relative to BSA without any treatment (BSA 0). Based on the absorbance at 520 nm, it was found that the presence of Lipo-Eucam induced a concentration-dependent inhibition of BSA aggregate formation ($p < 0.05$) (Figure 3A). The aggregate formation of BSA was suppressed by 43.67, 46.64, 52.37, and 80.47% as a consequence of adding 0.125, 0.25, 0.5, and 1.0 mg/mL Lipo-Eucam, respectively, to the aggregating protein solution. These data suggest a remarkable capacity of Lipo-Eucam to abrogate the aggregation of amyloidogenic proteins.

The morphological changes associated with BSA subjected to aggregation stress in the presence or absence of Lipo-Eucam was obtained via TEM microscopy. TEM images of the native protein solution revealed a clear solution, void of any aggregated proteins or amyloid fibrils (Figure 3C). In contrast, BSA exposed to aggregation conditions without any treatment exhibited a massive network of amorphous aggregates. Notably, when BSA was pre-incubated with 0.5 mg/mL of Lipo-Eucam, the presence of protein aggregates was greatly diminished. This result is consistent with the data from Congo red binding assay, indicating that the presence of Lipo-Eucam enabled the protein to resist morphological changes caused by aggregation. Phenolic and volatile compounds such as quercetin, isovitexin, luteolin, catechin, phytol, and caryophyllene found in Lipo-Eucam have been widely reported for their anti-aggregation activity[40−43] and are most likely responsible for the anti-amyloidogenic property of the lipophilic fraction.

2.4. In Vitro Cytotoxicity of Lipo-Eucam. *Eucalyptus* spp. remain of great interest owing to their versatile pharmacological attributes such as antioxidative, antimicrobial, antifungal, anti-nociceptive, and anticancer activities. The cytotoxic properties of Lipo-Eucam were evaluated against human breast cancer (MCF-7 and MDA-MB-231) cell lines as well as the normal human embryonic kidney (HEK-293) cell line. The antitumor drug, doxorubicin, was employed as a positive control. The results revealed a negligible decrease in the cell viability of HEK-293 upon exposure to Lipo-Eucam at every concentration tested (IC_{50} > 80 μg/mL, data not shown). Conversely, after MCF-7 cells were challenged with the sample, a remarkable, dose-dependent decrease in cell viability was observed (Figure 4). Similarly, the sample was

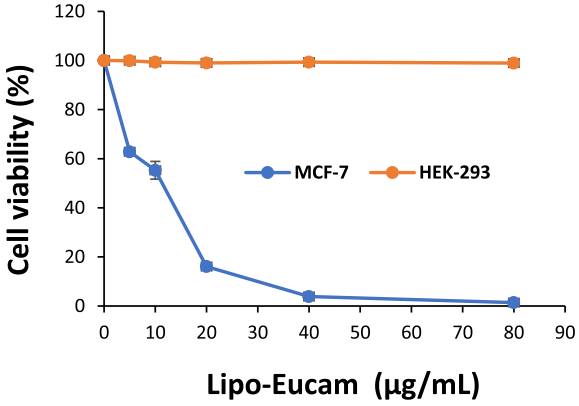

Figure 4. Effect of different concentration of Lipo-Eucam on the viability of human breast adenocarcinoma (MCF-7) and human embryonic kidney (HEK-293) cell lines using the MTT assay after 72 h.

very active against the MDA-MB-231 cell line. Between the cancer cells, MCF-7 cells (estrogen receptor-α positive) were more sensitive to the cytotoxic action of Lipo-Eucam compared to MDA-MB-231 (estrogen receptor-α negative). The cytotoxic activity of Lipo-Eucam against the cancer and normal cell lines is summarized in Table 4. The IC_{50} values of the extract/control obtained using GraphPad Prism denote the sample concentration required to reduce the viability of the cells to 50% relative to that of the untreated cells. Lipo-Eucam was found to exhibit an IC_{50} value of 7.34 μg/mL against the breast cancer cells and >80 μg/mL against HEK-293 cells. The US National Cancer Institute's guideline for in vitro screening of chemical agents and natural products for cytotoxic property recommended that for a pure compound, an IC_{50} value of ≤4 μg/mL should be considered cytotoxic. For natural products such as plant samples, the protocol stipulated that an IC_{50} value ≤20 is cytotoxic.[44] Accordingly, the high IC_{50} value of Lipo-Eucam against HEK-293 cells is an indication that this fraction is noncytotoxic and biocompatible to normal cells. In contrast, the very low IC_{50} value of the fraction against MCF-7 is indicative of its potent cytotoxic attributes against the breast cancer cells.

Also, considering that one of the most important criteria for determining the anticancer potential of any agent in vitro is selectivity,[45] the selectivity index (SI) of Lipo-Eucam was equally determined to ascertain this parameter. For any anticancer agent to be meaningfully deployed as a pharmacotherapeutic agent, it is imperative that while the active agent inhibits the proliferation of cancerous cells, the harmful impact on normal cells should be negligible or minimal. A good number of prospective anticancer agents with strong in vitro cytotoxic activity against cancer cells failed in subsequent clinical trials for not satisfying this requirement. SI is regarded as the toxicity of the compound of interest toward cancer cell lines in comparison to normal cell lines. The SI of Lipo-Eucam was obtained as the average IC_{50} value in the

https://doi.org/10.1021/acsomega.2c02389
ACS Omega 2022, 7, 27369−27381

Table 4. In Vitro Cytotoxic Activities of Lipo-Eucam[a]

	in vitro cytotoxic activity ($IC_{50\ in}$ μg/mL)			SI[b]	
samples	MCF-7	MDA-MB-231	HEK-293	MCF-7	MDA-MB-231
Lipo-Eucam	7.34 ± 0.51	13.86 ± 0.89	>80	>10.9	5.77
DOX	0.45 ± 0.01	0.23 ± 0.01	0.31 ± 0.03	0.67	1.35

[a]The treatment period was 72 h. Data represent the mean ± standard deviation of triplicate. [b]The SI was obtained as the average of the IC_{50} value of the normal HEK-293 cell line divided by the IC_{50} value of the cancer cell line in obtained in each independent experiment.

normal (HEK-293) cell line divided by the average IC_{50} value in the cancer cell for each independent experiment.[45,46] The SI values of Lipo-Eucam were high and satisfactory, especially against MCF-7 cells (SI > 10 is indicative of an agent that display selective cytotoxic activity against cancer cells).[47] With regards to the anticancer drug, doxorubicin, while the IC_{50} value was very low (0.45 μg/mL), indicating strong antiproliferative activity against MCF-7 cell lines, the selectivity with respect to the normal kidney cell line was less encouraging.

A growing number of studies have indicated the antiproliferative properties of *Eucalyptus* spp extracts, fractions, essential oils, and bioactives against various cancer cell lines including colorectal (HCT-15), pancreatic (PANC-1), non-small cell lung cancer (NCI-H460),[48] hepatocellular carcinoma (HEPG2 and HU-7),[49] human clear renal cell carcinoma (Caki), kidney carcinoma (A495), prostate cancer (PC3), epithelial carcinoma (HeLa) Burkitt's lymphoma Raji, as well as breast adenocarcinoma (MCF-7 and MDA-MB-231).[7,8,50] According to Hrubik et al., the antiproliferative activity of *E. camaldulensis* methanol, ethyl acetate, *n*-butanol, and aqueous leaf extracts on MCF-7 and MDA-MB-231 breast cancer cells examined by the MTT assay after 72 h revealed that the extracts were cytotoxic to the cancer cell lines (IC_{50} 26.7−250.7 μg/mL).[8] They observed that the ethyl acetate extract was the most active, while water extract was the least. Similarly, Singab et al., observed that phenolic-rich aqueous acetone extract of *E. camaldulensis* leaves demonstrated growth inhibitory effects against MCF-7 (IC_{50} 36.5 μg/mL), Hep-2 (IC_{50} 57.7 μg/mL), HepG-2 (IC_{50} 38.7 μg/mL), HeLa (IC_{50} 49.0 μg/mL), HCT-116 (IC_{50} 33.3 μg/mL), and Caco-2 (IC_{50} 38.3 μg/mL).[7] Meanwhile, studies by Mubarak et al. found that essential oils of *E. camaldulensis* leaves exerted cytotoxic action against cancer cell lines, WEHI-3 (IC_{50} 16.1 μg/mL), HT-29 (IC_{50} 50.5 μg/mL), and HL-60 (IC_{50} 42.1), but were less cytotoxic against normal cells, RAW 264.7 macrophage (IC_{50} > 100 μg/mL).[23] Taheri et al., noted that the cytotoxic effect of *E. camaldulensis* aerial parts essential oils was likely mediated via the induction of the cellular apoptotic pathway,[51] which was consistent with past observations on Jurkat cancer cells[52] and on BJAB and Raji lymphoma tumor cell lines.[50] Given that most of the compounds in these earlier studies were represented in Lipo-Eucam, the cytotoxic action of the extract probably involves triggering cellular apoptosis. It is also important to underscore that compared to most of the aforementioned reports, Lipo-Eucam exerted superior cytotoxic activity against the human breast adenocarcinoma (MCF-7) cell line. Additionally, the presence of many bioactive compounds with demonstrated anticancer effects in Lipo-Eucam, including phenolic acids, polyphenols, flavonoids, phloroglucinols, phloroglucinol-meroterpenoids, 2,4,-naphthoquinones,[7,21,48,53] terpenes, sesquiterpenes, and terpenoids,[4,50,52,54−58] indicated that the potent inhibitory action against the viability of the breast cancer cell line may be related to entourage effect and synergism among the bioactives. These preliminary findings highlighted the fact that Lipo-Eucam holds encouraging prospects for anticancer therapeutic development. Further studies are required to elucidate the relevant mechanism(s) of action, the efficacy and safety in animal models as well as its bioavailability, in order to ascertain its effectiveness for clinical application.

3. CONCLUSIONS

In the present contribution, it was revealed that the lipophilic fraction of *E. camaldulensis* leaves extract (Lipo-Eucam) was rich in a plethora of bioactive compounds. The number of compounds putatively identified by LC−MS and GC extended the current list of known active compounds known to be present in *E. camaldulensis* leaves. Also, Lipo-Eucam was shown to exhibit superior anti-radical and anti-oxidative properties. Moreover, the lipophilic fraction was found to suppress the aggregation of amyloidogenic BSA. The selective cytotoxic property of Lipo-Eucam was very impressive, with high activity against MCF-7 cells, but negligible toxic effects against HEK-293 cells. These findings highlights the strong prospects of Lipo-Eucam as a rich bioactive natural agent with potential for combating oxidative stress, aggregation, and cancer-related conditions and therefore worthy of further investigations.

4. EXPERIMENTAL SECTION

4.1. Preparation of Lipophilic Fraction of *E. camaldulensis* Leaf Extract. The lipophilic fraction of *E. camaldulensis* leaf extract was prepared following cold extraction as previously described with minor modifications.[59] Briefly, the leaves were pulverized using an electric grinder into fine powder. The leaf powder was carefully weighed (100 g) and extracted with 80% ethanol (2 L) using an overhead stirrer at 300 rpm for 2 h at room temperature. The extract was then filtered using Whatman number 4 filter paper. The residue was re-extracted as before and filtered. The filtrate from both rounds of extraction were combined and filtered with Whatman number 1 filter paper. The clean extract was concentrated under reduced pressure at 35 °C to about 20 percent of the initial volume. This concentrate was left overnight at 4 °C to fractionate. Thereafter, the hydrophilic upper layer was carefully decanted while the congealed lipophilic precipitate at the bottom was collected and lyophilized, yielding the lipophilic fraction as a dark green powder (aka Lipo-Eucam). Lipo-Eucam powder was stored at −20 °C in airtight amber vials until further investigations.

4.2. Chemical Characterization of Lipo-Eucam. *4.2.1. UPLC-DAD-ESI-MS Analysis.* Briefly Lipo-Eucam was measured and dissolved in methanol: water (70:30 v/v). The solution was thoroughly vortexed and centrifuged (8000 rpm for 10 min). Thereafter, the Eucam solution was filtered through a 0.2 μm nylon membrane syringe filter. The clear Eucam solution was then analyzed by UPLC-DAD-ESI-

https://doi.org/10.1021/acsomega.2c02389
ACS Omega 2022, 7, 27369−27381

QTOF-MS as previously described[59] using liquid chromatograph-quadrupole time-of-flight mass spectrometer (LC-QTOF MS), 1290 Infinity II LC-6545 Quadrupole-TOF (Agilent Technologies, USA) with a Zorbax Eclipse Plus C18 2.1 × 150 mm, 1.8 μm column.

4.2.2. Identification of E. camaldulensis Secondary Metabolites by (GC−MS). The GC−MS analyses of Lipo-Eucam was conducted using GC-electron ionization/MS (GC-EI/MS) technique performed on GC/MS GC 7890 B, MSD 5977B, Agilent Technologies, USA. Helium was used as the carrier gas with Agilent HP-5MS column (flow rate 1 mL/min; pressure 7.0699 psi; average velocity 36.262 cm/s; holdup time 1.3789 min; post run 1 mL/min; 325 °C: 30 m × 250 μm × 0.25 μm). The sample (1 μL) was injected in the split mode. The initial oven temperature was 40 °C (hold time of 1 min) and then increased at 5 °C/min to 250 °C (hold time of 5 min) and further increased at a rate of 5 °C/min to 320 °C (hold time of 10 min), for a total run time of 72 min. The putative identities of the compounds in Lipo-Eucam were established using Wiley 10 and the National Institute of Standards and Technology 14 (NIST14) chemical libraries, and the match score criteria accepted was ≥90%.[60]

4.2.3. Total Phenolic Content. The total phenolic content of Lipo-Eucam was ascertained using Folin-Ciocalteu colorimetric assay as previously described.[59] Ethanol solution of Lipo-Eucam or gallic acid (100 μL) was added into 2 mL Eppendorf tubes. This was followed by 200 μL of 10% Folin−Ciocalteu reagent. In blank solutions, the Folin−Ciocalteu reagent was replaced by distilled water. The solutions were briefly vortexed. Thereafter, 800 μL of 0.7 M Na_2CO_3 solution was added to the mixtures, vortexed, and incubated in the dark for 2 h. The absorbance of the mixtures was obtained at 765 nm. The absorbance values of gallic acid (0−0.1 mg/mL) were used to prepare the standard curved from which the total phenolic content of Lipo-Eucam was extrapolated.

4.2.4. Total Flavonoid Content. The total flavonoid content was evaluated using the aluminum chloride colorimetric assay.[61] Briefly, various concentrations of Lipo-Eucam solution or quercetin (30 μL) were added into 2 mL Eppendorf tubes followed by 160 μL of methanol. Then, 30 μL of freshly prepared 10% aluminum chloride in methanol was added to the solutions and mixed. This was followed by 30 μL of 1 M sodium acetate solution and 850 μL of distilled water. The solutions were vortexed and incubated at room temperature for 30 min. Absorbance of the solutions were obtained ta 415 nm. The total flavonoid content was calculated and presented as milligram quercetin equivalent per gram of Lipo-Eucam in dry weight (mg QE/g dw).

4.3. Antioxidative Properties of Lipo-Eucam. *4.3.1. DPPH (2,2,-Diphenyl-1-picrylhydrazyl) Radical Scavenging Assay.* Sample solution (120 μL) was added to 96-well microplate. DPPH solution (0.1 mM, 120 μL) was subsequently added to the samples and incubated for 30 min in the dark at room temperature. The absorbance of the solutions was recorded at 515 nm and the anti-radical activity was expressed as percentage inhibition.[62]

4.3.2. ABTS Assay. ABTS reagent was prepared by mixing equal volumes of ABTS and potassium persulfate solution, followed by incubation for 12−16 h. The working solution was prepared by diluting the stock solution to an OD of 0.70 ± 0.01 at 730 nm. The sample (15 μL) was added to the ABTS reagent (180 μL) and incubated for 10 min. The absorbance of the solution was recorded at 730 nm and the anti-radical activity calculated as percent inhibition.[62]

4.3.3. Ferric Reducing Antioxidant Power. The reducing antioxidant capacity of Lipo-Eucam was assessed using the FRAP assay as previously described.[63] Succinctly, 10 μL of the sample was introduced to a 96-well plate. This was followed by 200 μL of pre-warmed and freshly prepared FRAP reagent. The blank solutions contained only sodium acetate buffer *in lieu* of the reagent. The plate was then incubated at 37 °C for 30 min. Thereafter, the absorbance was measured at 593 nm and the reducing capacity of the sample was presented as IC_{50} in μg/mL.

4.3.4. Determination of Protein Carbonyl Content. The influence of Lipo-Eucam on protein oxidation was evaluated using the MCO of the BSA system as previously described with minor modifications.[34,64] Briefly, oxidation was triggered via the addition of oxidation buffer into protein solutions preincubated with or without various concentrations of Lipo-Eucam. The solutions were incubated at 37 °C at a speed of 250 rpm for 12 h. Thereafter, oxidation was terminated by adding EDTA-Na_2 solution (final concentration of 1 mM). The levels of protein carbonyls in the various solutions was assessed spectrophotometrically using the 2,4-dinitrophenylhydrazine assay. The results were presented as protein carbonyl content in nmol per mg of protein.

4.4. Determination of Protein Aggregation. Oxidation-induced aggregation of BSA was triggered as previously described[65,66] with some modifications. Briefly, the protein solution in 20 mM phosphate buffer, pH 7.0 was incubated with varying concentrations of Lipo-Eucam at 37 °C for 30 min. Thereafter, hydrogen peroxide was added to the mixture and incubated at room temperature for 30 min. The final concentrations of BSA and hydrogen peroxide in the mixture were 4 mg/mL and 3%, respectively. The protein solutions were then adjusted with ethanol to a final concentration of 33% (v/v). The solutions were then incubated at 37 °C for 38 h. The reaction was stopped by introducing the solutions to −20 °C for 1 h. The impact of Lipo-Eucam on BSA aggregation was evaluated using the Congo red assay. Wherein, 2 mL of the protein solution was incubated with 1 mL of Congo red [100 μM in 10% ethanol/PBS (v/v)] for 30 min at room temperature. The absorbance was measured at 530 nm and used to calculate the inhibition of the aggregate formation.

$$\text{Inhibition} = [(\text{control}-\text{sample})/(\text{control}-\text{native})] \times 100$$

where control, sample, and native represent the absorbance of the BSA without any treatment, BSA treated with Lipo-Eucam, and native BSA, respectively.

4.5. Transmission Electron Microscopy. TEM was performed to examine the effect of Lipo-Eucam on the morphology of the aggregated sample. Sample (10 μL) was carefully placed on copper grids. Excess sample was blotted away. After about 2 min, the grid was rinsed twice with ultrapure water. Then, 2% uranyl acetate (10 μL) was used to fix the sample. After 3 min, excess uranyl acetate was removed from the sample and the grid was allowed to dry at room temperature. Subsequently, the grids were examined under TEM (JOEL Ltd., Tokyo, Japan) operating at 200 kV.

4.6. Cell Culture and Cell Viability. *4.6.1. Cell Lines and Culture Conditions.* Human breast cancer (MCF-7 and MDA-MB-231) cell lines and non-cancer (human embryonic kidney 293, HEK) were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in

https://doi.org/10.1021/acsomega.2c02389
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Dulbecco's modified Eagle medium containing 10% fetal bovine serum, at 5% CO_2 and 37 °C.

4.6.2. In Vitro Cytotoxicity Assay. The cytotoxic activity of Lipo-Eucam was evaluated using the MTT assay as previously described.[8] The cells were seeded in 96-well plates and exposed to various concentrations of Lipo-Eucam or doxorubicin (positive control) for a period of 72 h. Wells containing only the diluent served as negative control. The IC_{50} values, that is, half of the maximal inhibitory concentration of the extract or positive control were determined using GraphPad prism 7.0.

4.7. Statistical Analysis. Data analysis was performed on Graph Pad Prism version 7.0 for Microsoft windows (Graph Pad Software, San Diego CA, USA) using one-way analysis of variance, followed by post hoc analysis using the Tukey test. Statistical significance was defined as $^*p < 0.05$. All determinations were performed, at least, in triplicate and results represented as means ± SD.

AUTHOR INFORMATION

Corresponding Author

Fredrick Nwude Eze − *Drug Delivery Systems Excellence Center and Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai 90112, Thailand;* orcid.org/0000-0002-3742-9965; Email: fredrick.e@psu.ac.th

Authors

Yanping Huang − *Department of Human Anatomy, Histology & Embryology, Anhui Medical College, Hefei 230601, China*

Mei An − *Department of Human Anatomy, Histology & Embryology, Anhui Medical College, Hefei 230601, China*

Anning Fang − *Department of Human Anatomy, Histology & Embryology, Anhui Medical College, Hefei 230601, China*

Opeyemi Joshua Olatunji − *Traditional Thai Medical Research and Innovation Center, Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai 90110, Thailand;* orcid.org/0000-0002-6800-4919

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c02389

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work did not receive funding from any private, public, or not-for-profit agency or organization.

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https://doi.org/10.1021/acsomega.2c02389
ACS Omega 2022, 7, 27369−27381