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Isolation of 3-(Hydroxyacetyl)indole and Indole-3-carboxylic acid from Red Alga *Halymenia durvillei*: Their Anti-lung Cancer Cell and *in vivo* Anti-aging Activity

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This study aimed to evaluate the bioactivity and phytochemical investigation in red algae *Halymenia durvillei*. The polarity based solvent partition (hexane, ethyl acetate, butanol and water) of *H. durvillei* ethanolic crude were used for characterization. The present results of the ethyl acetate extract of red alga *H. durvillei* generated a 3-(hydroxyacetyl)indole (**1**), indole-3-carboxylic acid (**2**) as well as two fatty acids *viz*. palmitic acid (**3**) and α-linoleic acid (**4**). The viability against lung cancer cells of compounds **1** and **2** showed moderate activities against the A549 cell line with inhibition percentages of 12.97% and 25.81%, respectively. Analysis of the dose response of *C. elegans* anti-aging assay indicated that the survival rate of compound **1** was 12.62% while it was 11.04% for compound **2**. Furthermore, *H. durvillei* isolation was used as an antioxidant, anti-cancer cell and anti-aging resource.

Keywords: 3-(Hydroxyacetyl)indole, *Halymenia durvillei*, Anti-aging activity, Anti-cancer cell.

INTRODUCTION

The red alga genus Halymenia (*Rhodophyta*) includes a total of 63 different species and has a broad distribution in different zones of Asia as well as the Indian and Pacific Oceans [1]. Studies have indicated that this genus has possible biological activities, which were shown to inhibit antioxidants and anticoagulants [2,3]. In a recent study, Halymenia genus reported an isolation of secondary metabolites [4]. The red alga *Halymenia durvillei* is one of the most common Halymenia species. Identificiation of this alga was performed by Bory de Saint Vincent in New Ireland [1]. The colour of *H. durvillei* is dark red and frequently has greater than five orders of non-uniform branching and multiple surface proliferation in addition to a flexible structure formed of cartilage [5]. It has also been shown that this alga is rich in polysaccharides [6].

Various researchers [7,8] have isolated indole derivatives and related tryptophan metabolites from the red marine alga. *Prionitis lanceoluta* and *Rhodophyta*, which contain indole derivatives after the subsequent isolation from the methanol extract. Tetrabromoindole exhibited the antimicrobial activities [9-11]. It has been shown that standard indole alkaloids have considerable biological activity, including the anti-inflammatory activity of *Nauclea officinalis* [12], antimicrobial activity of *Cladosporium* sp. [13] and anti-tumour activity of *Tetradium ruticarpum* [14].

The present study reports on the bioactivity, isolation and structure elucidation of 3-(hydroxyacetyl)indole (**1**) and indole-3-carboxylic acid (**2**) along with two fatty acids, namely palmitic acid (**3**) and α-linoleic acid (**4**) of *Halymenia durvillei*. Furthermore, elucidation of the structures was performed on the basis of MS and NMR analysis. Additionally, anti-lung cancer activities towards the A549 cell line of the indole alkaloids **1** and **2** along with the lifespan assay as an indicator of the anti-aging properties *C. elegans* were performed.

EXPERIMENTAL

NMR spectra (1D and 2D) were recorded on Bruker 400 AVANCE spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). The high resolution mass spectra (HRMS) were obtained using Bruker micrOTOF-Q II mass spectrometer. Column chromatography was carried out using Merck silica gel 60 *(*finer than 0.063 mm*)* and Pharmacia Sephadex LH-20. For TLC, Merck precoated silica gel 60 F_{254} plates were used. Spots on TLC were detected under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating.

Authentication of red algae *Halymenia durvillei* (voucher specimens No. SPFR16040) was performed by Dr. Montakan Tamtin, Department of Fisheries, Coastal Aquatic Feed Research Institute, Coastal Fisheries Research and Development Bureau, Petchaburi, Thailand.

Extraction and isolation: Successive maceration of the dried red algae of *H. durvillei* (1.1 kg) was performed at room temperature with ethanol (5 L) for a period of 7 days. Rotary evaporation was used to evaporate the solvent at 40 ºC to yield ethanol crude extract. Subsequently, partitioning of the ethanol extract (44.5 g) was performed with hexane, ethyl acetate, *n*-butyl alcohol and distilled water. The hexane fraction (2.1 g) was implemented on silica gel column chrom-atography and then eluted with the mobile phase *n*-hexane-CH_2Cl_2 utilizing a gradient mechanism. The hexane fraction was then fractioned further to yield eight primary fractions (A-H). Fraction B (0.16 g) was re-columned using silica gel column chromatography with a gradient mechanism of *n*-hexane-CH_2Cl_2 to yield four subfractions (B1-B4). Purifications of subfractions B1 and B2 was achieved with Sephadex LH-20 utilizing CH_2Cl_2-MeOH (1:1) in order to acquire yields of 4.5 mg of compound **3** and 2.5 mg of compound **4**. Ethyl acetate fraction (3 g) was implemented on silica gel column chromatography and then eluted using the mobile phase *n*-hexane:ethyl acetate (4:1) to generate six primary fractions (A-F). Fraction-ation of fraction was performed with Sephadex LH-20 in methanol utilized as the effluent to produce three subfractions (E1-E3). Purification of subfraction E3 was performed with column chromatography utilizing CH_2Cl_2-MeOH (5:0.1) to yield novel compounds **1** (15 mg) and compound **2** (5.9 mg).

Compound **1** was isolated as a yellow powder, HRETMS (ESI) *m/z* 198.0504 $[M+Na]^+$; calcd. for $C_{10}H_9NO_2Na$, 198.0525; ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.93 (1H, brs, H-2), 8.21 (1H, dd, *J* = 5.2, 1.6 Hz, H-4), 7.28 (1H, dt, *J* = 5.2, 1.6 Hz, H-5), 7.26 (1H, dt, *J* = 5.2, 1.6 Hz, H-6),7.43 (1H, dd, *J* = 5.2, 1.6 Hz, H-7), 4.74 (2H, s, H-9), ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 131.5 (C-2), 113.6 (C-3), 136.5 (C-3a), 121.6 (C-4), 122.7 (C-5), 123.6 (C-6), 111.9 (C-7), 125 (C-7a), 193.2 (C-8), 65.0 (C-9) [7].

Compound **2** was obtained as a white amorphous solid, HRETMS (ESI) *m/z* 184.0313 $[M+Na]^+$; calcd. for $C_{10}H_9NO_2Na$, 184.0369; ^{1}H NMR (400 MHz, CD_3OD): δ_H 8.13 (1H, d, *J* = 6.8 Hz, H-4), 7.93 (1H, s, H-2), 7.42 (1H, d, *J* = 6.8 Hz, H-7), 7.18 (1H, m, H-5), 7.14 (1H, m, H-6), ^{13}C NMR (100 MHz, CD_3OD): δ_C 170 (C-8), 136.7 (C-4a), 131.1 (C-2), 126.4 (C-7a), 121.8 (C-5), 120.8 (C-4), 120.5 (C-6), 111.2 (C-7), 109.6 (C-3) [7].

Screening 2D anti-lung cancer assay: Seeding of human lung A549 cancer cells was performed at 1×10^4 cells per well on 96-well plates followed by culturing with Ham's F12k with the addition of 10% FBS and 1% penicillin/streptomycin. Incubation of the cells was performed at 37 ºC and 5% CO_2 for 24 h. A high throughput liquid handling system was used to screen the compounds to identify the active compound that has anti-cancer cell proliferation effects. The compounds were placed on cell plates at 50 µg/mL (for primary screening) and then incubated for another 24h at 37 ºC and 5% CO_2. The culture media was then extracted and then media without serum including MTT was introduced to the same well, followed by a further 3 h of 37 ºC, 5% CO_2. Finally, the media without serum including MTT was extracted and DMSO was introduced to the well, then the MTT absorbance was measured at 570 nm using a Multi-Mode Microplate Reader (ENVISON).

Lifespan assays: Caenorhabditis elegans strain Bristol N_2 (wild-type) was utilized in the lifespan assays. Preparation of N_2 synchronized L1 larvae was achieved by bleaching gravid adult worms in a bleaching solution that contained NaOH, NaClO and distilled water. The L1 larvae produced were then moved to NGM plates with OP50 followed by incubation at 20 ºC for 40 h and transformed into L4 larvae [15]. These synchronized L4 larvae were then moved to NMP plates that contained FUDR for the purpose of inhibiting the production of progeny. A total of 40 worms where then treated with OP50 combined with 10, 50 or 100 µg/mL of compounds **1** and **2**. Selection of the extracts was guided by tests that had previously been performed on the compounds in terms of their toxicity and effective concentration [16]. Furthermore, the experiment was repeated three times. Daily counts of survived, dead and censored worms were recorded from day one until no worms were alive. Scoring of dead worms was based on their lack of reaction when touched with a platinum wire and when they exhibited no pharyngeal pumping. Worms were defined as being censored when the progeny hatched internally and excluded from the analysis. Statistical analysis was performed on the amount of survived and deceased worms.

Statistical analysis: The inhibition percentage of cancer cell viability was utilized to select the compound capable of killing or inhibiting the proliferation of cancer cells to the same level or greater than cisplatin. The cancer cell viability % was calculated using the following equation:

$$\text{Inhibition of cancer cell viability (\%)} = 100 - \left(\frac{OD_{compound}}{OD_{DMSO}}\right) \times 100$$

In terms of lifespan assay, statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., USA). All findings were reported in the form of mean ± standard error of means (SEM). Calculation of the *p*-value for the survival curves in the lifespan assays was performed with the log-rank (Mantel-Cox) test.

RESULTS AND DISCUSSION

Phytochemical profiles: Collection of red algae *Halymenia durvillei* was extracted with ethanol through maceration. The obtained yields was 4.47% for the raw extract (Table-1). Next,

TABLE-1
INVESTIGATED PLANTS: EXTRACTION YIELDS AND TOTAL PHENOLIC AND FLAVONOID CONTENTS

Part extracts	Yield (%)	Total phenolic content	Total flavonoid content
HDET-c	4.47	2.15 ± 0.34	0.58 ± 0.01
HDHE	0.24	14.18 ± 2.82	3.09 ± 0.02
HDEA	0.07	24.88 ± 0.23	5.45 ± 0.02
HDBU	2.02	21.49 ± 0.11	0.02 ± 0.00
HDAQ	1.92	15.21 ± 0.19	N/S

Data are expressed as mean SE (n = 3). Results were expressed as mg of gallic acid or quercetin equivalent per g of dry plant material, respectively.

a fraction of ethanolic crude extract (HDET-c) was then successively extracted with solvents with different polarity, *n*-hexane (HDHE) (yield 0.24% for the plant species, referred to dry the red algae plant), ethyl acetate (HDEA) (0.07%), butanol (HDBU) (2.02%). Remaining aqueous fraction (HDAQ) (1.92%) was investigated as well.

Antioxidant activity of crude extracts: The *in vitro* antioxidant capacities of crude extracts were first evaluated by means of the DPPH radical scavenging assay. Almost all the samples demonstrated radical scavenging effectiveness. The relation between percentage inhibition and concentration was showed by non-linear regression models. Thus, a strong association between concentration and percentage inhibition was observed. As regards the four crude extracts, the best activity was revealed by the HDEA fraction (1 mg/mL) with an % inhibitor in value of 58.29%. The HDEA fraction of *Halymenia durvillei* showed an interesting antioxidant activity (Table-2).

TABLE-2
in vitro DPPH RADICAL SCAVENGING ACTIVITY OF FRACTIONS

Samples	Inhibitor (%)
HDET-c	12.09
HDHE	38.20
HDEA	58.29
HDBU	11.55

Data are expressed as mean SE (n = 3). Results were expressed as mg of vitamin C equivalent per g of dry plant material, respectively.

Anti-aging assay of crude extract: Investigation of the longevity characteristics of five crude extracts involved their application on N_2 wild-type worms at 25 ºC in concentrations of 100, 500 and 1000 µg/mL, where a comparison was made with a 1% DMSO control was also performed. The findings indicated that the average longevity of N_2 worms using HDEA at 10 µg/mL increased significantly 12.84 days which was more as compared to the equivalent control (log-rank, $p < 0.0001$) (Table-3). This HDEA fraction of *Halymenia durvillei* showed an interesting anti-aging activity.

GC-MS analysis of HDEA fraction: The GC-MS technique determined the presence of many bioactive compounds in HDEA fraction of the red algae. Several compounds were identified in the non-polar portion of algae. In current study, only compounds with total peak area in total ion current (TIC%) greater than 1% were highlighted (Table-4). Twenty

TABLE-3
ANTI-AGING ASSAY OF CRUDE EXTRACTS

Samples	Treatments	Mean lifespan	% Increasing lifespan
HDET-c	1% DMSO	14.79 ± 2.76	–
	100	14.30 ± 2.58	9.86
	500	15.71 ± 2.26	9.44
	1000	15.77 ± 1.78	10.28
HDHE	1% DMSO	9.96 ± 2.10	–
	100	11.21 ± 3.61	12.55
	500	10.56 ± 2.65	6.02
	1000	10.84 ± 2.44	8.84
HDEA	1% DMSO	12.15 ± 1.58	–
	100	13.71 ± 1.63	12.84
	500	13.42 ± 1.63	10.45
	1000	13.17 ± 1.45	8.39
HDBU	1% DMSO	13.47 ± 1.40	–
	100	13.84 ± 1.47	2.75
	500	13.78 ± 1.19	2.30
	1000	13.83 ± 1.30	2.67
HDAQ	1% DMSO	12.38 ± 1.61	–
	100	12.37 ± 1.84	-0.08
	500	12.72 ± 1.91	2.75
	1000	12.09 ± 1.78	-2.34

five compounds, namely bicyclo[2.2.1]heptane, dodecanoic acid, tridecanoic acid, hexadecanal, clofibric acid, pentadecanoic acid, 2-undecanone, 2,2,2-trifluoroethyl-3-cyclohexylpropionate, palmitoleic acid, (*Z*)-7-hexadecene, *n*-hexadecanoic acid, bicyclo[2.2.2]oct-2-ene], 2-thiophenemethanol, Z-11-pentadecenol, shikimic acid, 1-naphthalenamine, 1-nonadecene, (Z,Z)-9,12-octadecadienoic acid, 2-butyl-5-hexyloctahydro-1*H*-indene, octadecanoic acid, 5-(1-*tert*-butyl-1*H*-pyrrol-2-ylmethylene)-1-(4-methoxyphenyl) pyrimidine-2,4,6-trione and benzidine were detected in all the plants, while others were specific to particular plants. In this study, the highest TIC% were 34.09% and 16.63% for *n*-hexadecanoic acid and 2-butyl-5-hexyloctahydro-1*H*-indene, respectively.

Identification of compounds from *H. durvillei*: Isolation of 3-(hydroxyacetyl)indole (compound **1**) yielded a non-crystalline powder with yellow colour, which was individually extracted using ethyl acetate. The IR spectrum exhibited an %T band of 2800, 1719 and 1069 cm^{-1} to hydroxyl and carbonyl fraction. Deduction of the molecular formula of compound **1** was perf-ormed with HREIMS and revealed a molecular ion peak at *m/z* 198.0504 $[M+Na]^+$ (calcd. for $C_{10}H_9NO_2Na$, 198.0525), which corresponds with the molecular formula $C_{10}H_9NO_2Na$ indicating seven degrees of unsaturation. The ^1H NMR spectrum exhibited four downfield signals aromatic spin system at δ_H 8.21 (1H, d, J = 9.2 Hz, H-4), 7.43 (1H, d, J = 9.2 Hz, H-7), 7.28 (1H, dd, J = 2.8, 2.4 Hz, H-5), 7.26 (1H, dd, J = 3.6, 2.0 Hz, H-6), in addition to a vinylic proton at 7.93 (1H, brs, H-2) [17]. A total of 10 signals were exhibited by the ^{13}C and DEPT 135 NMR spectrum of compound **1**, which included one ketone carbon (δ_C 193.4), four quaternary carbons (δ_C 113.6, 193.4, 136.5 and 125.1), five methine carbons (δ_C 131.62, 123.61, 122.73, 121.60 and 111.91) and one methylene carbon (δ_C 65). The above mentioned data showing the characteristics of compound **1** indicating the presence of 3-(hydroxyacetyl)indole skeleton, a finding which can be reinforced based

TABLE-4
CHEMICAL COMPOSITION OF ETHYL ACETATE FRACTION OF *H. durvillei*

Phytochemical compound	m.w.	Retention time (min)	Relative peak area (TIC) (%)
Indole	117.058	18.136	0.50
9-Decenoic acid	170.131	19.968	0.56
Bicyclo[2.2.1]heptane	136.125	24.728	1.31
Dodecanoic acid	200.178	24.982	0.64
Tridecanoic acid	214.193	27.233	0.73
Hexadecanal	240.245	28.396	2.45
Clofibric acid	214.04	29.889	1.14
Pentadecanoic acid	242.225	30.718	1.11
2-Undecanone	198.198	31.155	7.54
2,2,2-Trifluoroethyl 3-cyclohexylpropionate	238.118	32.615	2.98
Palmitoleic acid	254.225	33.100	3.75
(Z)-7-Hexadecene	224.250	33.237	2.21
n-Hexadecanoic acid	256.240	33.637	34.09
Bicyclo[2.2.2]oct-2-ene	192.115	34.503	1.04
2-Thiophenemethanol	114.014	34.663	0.58
Z-11-Pentadecenol	226.230	34.941	1.48
Shikimic acid	174.053	35.582	2.18
1-Naphthalenamine	143.073	36.246	0.84
1-Nonadecene	266.297	36.363	2.32
(Z,Z)-9,12-Octadecadienoic acid	280.24	36.561	0.71
2-Butyl-5-hexyloctahydro-1*H*-indene	264.282	36.938	16.63
Octadecanoic acid	284.272	37.211	4.60
5-(1-*tert*-Butyl-1*H*-pyrrol-2-ylmethylene)-1-(4-methoxyphenyl)pyrimidine-2,4,6-trione	367.1530	48.827	1.99
Benzidine	184.100	49.1	0.97

on evidence from ^{1}H-^{1}H COSY and HMBC experiments. The ^{1}H-^{1}H COSY data related to compound **1** indicated relationships of H-6/H-7; H-4/H-5. Likewise, the HMBC data for the same compound showed relationships between H2-8 (δ_H 4.72, s) with C-9; H-5 (δ_H 7.28, t) with C-4, C-4a, C-7 and C-7a; H-7 (δ_H 7.43, d) with C-5, C-6 and C-6; H-2 (δ_H 7.29, s) with C-3, C-4a and C-7a; H-4 (δ_H 8.20, d) with C-3, C-4a and C-7a (Table-5).

TABLE-5
^{1}H AND ^{13}C NMR DATA OF COMPOUND **1** IN $CDCl_3$

No	Δ_H (ppm), *J* (Hz)	δ_C (ppm)	HMBC
1	–	–	–
2	7.93 (s)	131.6, CH	C-3, C-3a, C-7a
3	–	113.6, C	–
4	8.21 (dd, *J* = 5.2, 1.6 Hz)	121.6, CH	C-3, C-3a, C-6
5	7.28 (dt, *J* = 5.2, 1.6 Hz)	122.7, CH	C-3a, C-4, C-6, C-7
6	7.26 (dt, *J* = 5.2, 1.6 Hz)	123.6, CH	C-4
7	7.43 (dd, *J* = 5.2, 1.6 Hz)	111.9, CH	C-5, C-6, C-7a
8	–	193.4, C=O	–
9	4.74 (s)	65.0, CH_2	C-8
3a	–	136.5, C	–
7a	–	125.1, C	–

As shown in Fig. 1, an isolation of four compounds from the red alga substances was performed. The isolation of compounds **3** and **4** was conducted from *n*-hexane extract and yielded colourless liquids. The ^{1}H NMR and GC-MS were used to identify the compounds as fatty acid of palmitic acid (**3**) [18] and α-linoleic acid (**4**) [19]. This represents the first study to report the isolation of the compounds in this plant. Furthermore, isolation of compounds **1** and **2** was achieved using ethyl

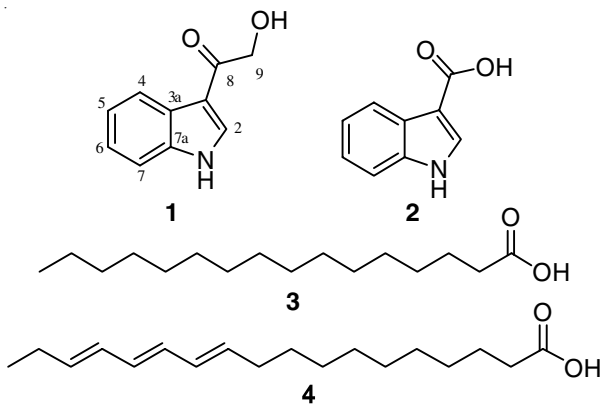

Fig. 1. Chemical structure of compounds **1-4** isolated from *Halymenia durvillei*

acetate extract and were in the form of a yellow powder. 1D NMR, 2D NMR and high-resolution electron ionization mass spectrometry (HRMS) data were employed to identify the compounds, which showed that they were indole alkaloid groups of 3-(hydroxyacetyl)indole (**1**) and indole-3-carboxylic acid (**2**) [7,8,20].

Screening anti-lung cancer activity: The potential activity of indole alkaloids (**1-2**) obtained by purification from the ethyl acetate extract of *Halymenia durvillei* against lung cancer was investigated using the MTT technique. Screening of the compounds was performed at a concentration of 50 μg/mL for primary screening with A549 cell line. Additionally, the positive control utilized was cisplatin. The results indicated that the cancer cell inhibition percentages of compounds **1** and **2** were 12.97% and 25.81%, respectively, which were lower compared to the positive control cisplatin (Table-6).

TABLE-6
SUMMARY OF COMPOUNDS **1** AND **2** SCREENING AGAINST ANTI-LUNG CANCER A549 CELLS

Compound	m.f.	Primary screening
		% Inhibition of cancer cell viability
Cisplatin	$Pt(NH_3)_2Cl_2$	87.91
Compound **1**	$C_{10}H_9NO_2$	12.97
Compound **2**	$C_9H_7NO_2$	25.81

Lifespan assays: Investigation of the longevity characteristics of compounds **1** and **2** involved their application on N_2 wild-type worms at 25 ºC in concentrations of 10, 50 and 100 µg/mL, where a comparison was made with a 1% DMSO control [15,16]. The findings indicated the average longevity of the N_2 worms that received treatment using compound **1** at 10 µg/mL and compound **2** at 100 µg/mL increased significantly 10.71 ± 1.70 and 10.56 ± 1.34 days, respectively, which were 12.64% and 11.04% more compared to the equivalent control (9.51 ± 1.23 days) (log-rank, $p < 0.0001$) (Table-7 and Fig. 2).

Conclusion

The present study involves to successfully isolate novel compounds 3-(hydroxyacetyl)indole (**1**), indole-3-carboxylic acid (**2**), which are alkaloid derivatives, in the form of natural compounds. Additionally, secondary metabolites (palmitic acid (**3**) and α-linoleic acid (**4**)) have been reported in *Halymenia durvillei*. Both compounds **1** and **2** were found to inhibit moderately A549 lung cancer cells and exhibited an increased proportion of *in vivo* anti-aging activity in *C. elegans*.

TABLE-7
MEAN LIFESPANS OF WILD TYPE N_2 WORMS TREATED WITH COMPOUNDS **1** AND **2**

Compounds		Mean lifespan (days)	Increase lifespan (%)	log rank test
	1% DMSO	9.51	–	–
Compound **1**	10 µg/mL	10.71	12.64	$p < 0.0001$*
	50 µg/mL	9.74	2.42	$p = 0.1783$
	100 µg/mL	10.25	7.78	$p < 0.0001$*
Compound **2**	10 µg/mL	9.98	4.94	$p < 0.001$*
	50 µg/mL	10.50	10.41	$p < 0.00001$*
	100 µg/mL	10.56	11.04	$p < 0.0001$*

*Indicates significant increase of mean lifespan between the treated and the control groups at $p < 0.05$.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

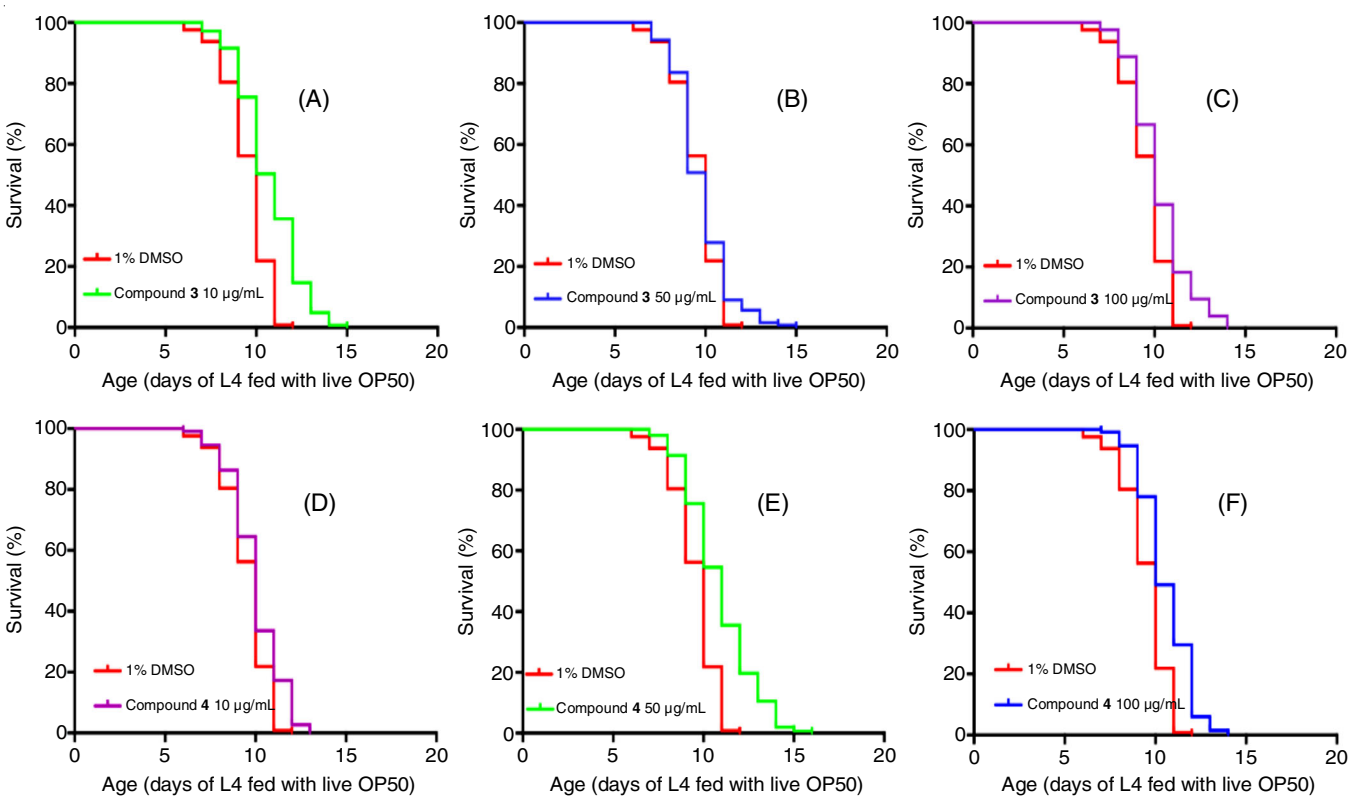

Fig. 2. Survival curves of the lifespans of worms treated with compound **1** at 10 µg/mL (A), 50 µg/mL (B) and 100 µg/mL (C) and compound **2** at 10 µg/mL (D), 50 µg/mL (E) and 100 µg/mL (F) compared to the control (1% DMSO)

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