



Research Article

Phytochemical Analysis and Cytotoxic Activity of Hexane Extract from *Lasiosphaera nipponica*

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Abstract: *Lasiosphaera nipponica* finds therapeutic applications as a hemostatic in the traditional Japanese and Chinese medicines. This study aimed to analyse the phytochemical compositions of two fractions from the *n*-hexane extract of *L. nipponica* by GC-MS analysis and evaluate the cytotoxic activity of these two fractions and a pure compound viz. ergosterol peroxide by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay). A hexane extract from *L. nipponica* was obtained by the maceration technique and silica gel column chromatography of this hexane extract yielded two fractions and three pure compounds. The two fractions were analysed for phytochemical compositions by GC-MS and the presence of fatty acids, esters of fatty acids and ethyl 9-oxononanoate were identified. Additionally, these two fractions were evaluated for cytotoxic activity against P388, HL60, MCF7, HepG2 and J82 cancer cell lines in culture by MTT assay. Fraction 1 showed good activity against P388 and HL60 cancer cell lines with IC₅₀ values of 9.8 and 18.2 µg/mL, respectively. Fraction 2 also showed good activity against P388 and HL60 cancer cell lines with IC₅₀ values of 19.4 and 16.2 µg/mL, respectively. However, these two fractions did not show any appreciable activity against MCF7, HepG2 and J82 cancer cell lines. The three pure compounds were characterised as *n*-hexadecanoic acid (3), *n*-octadecanoic acid (4) and ergosterol peroxide (5) by spectroscopic and spectrometric means. Of these three pure compounds, only compound 5 was evaluated for cytotoxic activity against HL60, HepG2 and J82 cancer cell lines. Ergosterol peroxide showed an IC₅₀ value of 82.2 µM (35.1 µg/mL) against HL60 cancer cell lines but it did not show any appreciable activity against HepG2 and J82 cancer cell lines. *n*-Hexadecanoic acid and *n*-octadecanoic acid were not evaluated for cytotoxic activity since they were obtained in small quantity. From this study, we concluded that the fractions from the *n*-hexane extract of *L. nipponica* have therapeutically important phytochemicals and exhibit promising cytotoxic activity against P388 and HL60 cancer cell lines. Further studies on *L. nipponica* are required to explore its therapeutic applications.

Keywords: *Lasiosphaera nipponica*, hexane extract, GC-MS analysis, cytotoxic activity, MTT assay

1. Introduction

Lasiosphaera nipponica is a mushroom species belonging to the Lycoperdaceae family of the Fungi Kingdom.¹ Mushrooms species such as chanterelle mushrooms, oyster mushrooms, sulfur mushrooms and related species are

edible forms.²⁻⁴ However, the emetic mushrooms and their near relatives have been reported as poisonous.²⁻⁴ *L. nipponica* has been used as a hemostatic in the traditional Japanese and Chinese medicines.⁵ Our literature search showed that *L. nipponica* has not been extensively studied for phytochemical compositions and pharmacological activities and only a few reports on *L. nipponica* have been available.^{1,6} For example, the isolation of steroids such as 3 β ,14 α ,17 α ,20,24,25-hydroxyergosta-6-one-7,22-diene, ergosterol peroxide, ergosterol, cerevisterol and a calvatic acid derivative from a methanolic extract of *L. nipponica* have previously been reported.⁶ Additionally, an aqueous extract of *L. nipponica* inhibited the HIV-1 induced CPE in MT-4 cells at a concentration of 250 μ g/mL.¹ To the best of our knowledge, the analysis of phytochemical compositions and the evaluation of the cytotoxic activity of a hexane extract from *L. nipponica* has not been reported previously. Therefore, the objective of the present study was to analyze the phytochemical compositions of fractions from the *n*-hexane extract of *L. nipponica* by GC-MS. Additionally, the cytotoxic activity of these fractions was evaluated in murine and human cancer cell lines such as murine lymphocytic leukaemia cells (P388), human leukaemia cells (HL60), human breast cancer cells (MCF7), Lewis lung carcinoma cells (LL2), hepatocellular carcinoma cells (HepG2) and bladder transitional carcinoma cells (J82) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2. Materials and methods

2.1 Chemicals, solvents and reagents used

Unless otherwise specified, Analytical Reagent (AR) grade of solvents, reagents and chemicals purchased from Merck and/or Sigma-Aldrich were used in this study. Silica gel 60 (Merck, 0.063-0.200 m) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60F 254, 0.25 mm or Baker Si250F, 0.25 mm) were used for thin layer chromatography (TLC). Spots in the TLC plates were detected using UV light or staining with iodine or spraying with 50% H₂SO₄ followed by heating at 110 °C for 5 min. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640 medium, DEME medium and trypan blue (all from Sigma-Aldrich) were used for cytotoxic assay.

2.2 Instruments used

Buchi B-540 melting point apparatus was used to determine the melting point. Bio Rad, Class II Laser product was used to record IR spectra. Bruker 300 MHz spectrometer was used to record NMR spectra of pure compounds. Finnigan/MAT MAT 95 XL-T or VG Micromass 7035 mass spectrometer was used to obtain electron impact ionisation mass spectra (EIMS). The chemical shift values (δ) and coupling constants (*J*) were reported in parts per million (ppm) and hertz (Hz), respectively. Agilent GC-MS instrument was used to analysis phytochemical compositions of fractions from the hexane extract. The following instruments were used at the Pharmacology Laboratory: Water incubator (Everbloom Medical & Scientific Pte. Ltd., Singapore), Beckman Avanti J-251 Centrifuge (Fullerton, CA, USA), -86 °C Freezer (Forma Scientific), Beckman Optima L-90K and Ultracentrifuge (Fullerton, CA, USA), ELX 800 Haemocytometer (Fortuna, Germany), Leitz Fluovert microscope (Ernst Leitz Wetzlar GMBH, Germany), Microplate reader (Bio-Tek Instruments Inc., USA) and Biological safety cabinet (NUAIRE, Plymouth, USA).

2.3 Samples of *L. nipponica*

Approximately, 600 g of dried *L. nipponica* was purchased from a local market in Singapore. A voucher specimen (KManoLN2002) was deposited at the Herbarium at the Department of Biological Sciences, National University of Singapore (NUL), Singapore.

2.4 Extraction and fractionation of *L. nipponica*

The powdered *L. nipponica* was macerated with *n*-hexane at room temperature (28-30 °C) for 12 hours. The extract was concentrated under vacuum using a Buchi-Rotavapour at 53-55 °C. The crude hexane extract thus obtained was kept in a beaker and the procedure was repeated three times with same materials. Approximately, 20 g of brown residue

of combined hexane crude extract was obtained after the removal of solvent. Silica gel column chromatography of this *n*-hexane extract yielded two fractions (fraction **1**, *ca.* 850 mg and fraction **2**, *ca.* 400 mg). Additionally, three pure compounds *viz.* *n*-hexadecanoic acid (palmitic acid) (< 10 mg) (**3**), *n*-octadecanoic acid (stearic acid) (< 10 mg) (**4**) and ergosterol peroxide (*ca.* 100 mg) (**5**) were also isolated.

2.5 Phytochemical analysis and identification of phytochemical compositions

Fraction **1** was analysed for phytochemical compositions on an Agilent GC-MS instrument using a DB-5 column of the dimension 50 m length, 0.25 mm internal diameter and 0.25 µm film thickness and a Flame Ionisation Detector (FID). The oven temperature was fixed initially at 50 °C for 2 min, increased by 10 °C/min until 180 °C and maintained for 2 min and then increased 5 °C/min up to 280 °C and maintained for a further 3 min. Fraction **2** was analysed for phytochemical compositions in the same instrument. In this case, the oven temperature was fixed initially at 50 °C for 2 min, increased by 10 °C/min until 180 °C and maintained for 2 min and increased by 5 °C/min up to 300 °C and maintained for a further 4 min. Helium was used as carrier gas and was pumped through the column at a constant flow rate of 1 mL/minute. Approximately, 0.05 mg of each fraction was dissolved separately in 5 mL of hexane. To remove the insoluble contaminants and other particulate matters, each of this solution was filtered separately through a membrane filter (MF Millipore, pore size is 0.45 µm). An aliquot of 1.0 µL of each of this solution was injected separately into the GC-MS column. The phytochemical compositions of fractions **1** and **2** were identified by comparison of their MS spectra data with NIST/EPA/NIH/NIST08 library data. On the other hand, the three pure compounds (**3-5**) were characterised as *n*-hexadecanoic acid (**3**), *n*-octadecanoic acid (**4**) and ergosterol peroxide (**5**) by spectroscopic and spectrometric means.

2.6 Cancer cell lines used

Murine lymphocytic leukaemia cells (P388), human leukaemia cells (HL60), human breast cancer cells (MCF7), Lewis lung carcinoma cells (LL2), hepatocellular carcinoma cells (HepG2), bladder transitional carcinoma cells (J82) and murine fibrosarcoma cells (WEHI1640) cancer cell lines were used in this study and were obtained from American Type Cell Culture (ATCC) (Manassas, VA, USA).

2.7 Evaluation by MTT assay and determination of IC₅₀ values

MTT assay and determination of IC₅₀ values in this study were as per the details given in the literature.^{2,7-8} Briefly, a stock solution of each fraction at a concentration of 100 µg/mL was prepared using dimethyl sulphoxide (100% DMSO) and two-fold further dilutions such as 50, 25, 12.5 and 6.25 µg/mL were obtained from this stock solution. For compound **5**, a stock solution at a concentration of 234.0 µM was prepared and the serial further dilutions such as 117.0, 56.5, 28.25 and 14.13 µM were obtained from this stock solution. The test samples consisted of solutions of fractions or pure compounds or positive controls at various concentrations prepared in DMSO together with cancer cell lines. The negative control consisted of DMSO at various concentrations with cancer cell lines and without fractions, pure compounds, or positive controls. The blank consisted of only DMSO at various concentrations and without cancer cell lines and without fractions or pure compounds or positive controls. Solutions of 6-mercaptopurine and doxorubicin in DMSO at similar concentrations served as positive controls for P388 and MCF7 cancer cell lines, respectively. Positive controls were not maintained for HL60, HepG2 and J82 cancer cell lines due to non-availability at the time of experiment. The absorbance of the resulting mixture was measured at 570 nm using a microplate reader. The average absorbance of eight replicate values for each concentration was taken for calculating the percentage growth inhibition for each cancer cell lines. The percentage growth inhibition was calculated using the formula given below.⁹

$$\% \text{ Growth inhibition} = 100 - \frac{[OD_{570} \text{ test} - OD_{570} \text{ blank}]}{OD_{570} \text{ control} - OD_{570} \text{ blank}} \times 100$$

Where, OD₅₇₀ test = Absorbance of test sample, OD₅₇₀ control = Absorbance of control and OD₅₇₀ blank =

Absorbance of blank.

The IC₅₀ value for each cancer cell lines was obtained from a dose-response curve by plotting various concentrations of the test sample in abscissa *versus* percent growth inhibition in ordinate. The IC₅₀ value is defined as the concentration of the extract that inhibits the growth of cancer cell lines by fifty percent. A lower value of IC₅₀ represents higher inhibition of growth and *vice versa*. An IC₅₀ value ≤ 20 $\mu\text{g/mL}$ is considered as cytotoxic and IC₅₀ values of 21-40 and > 40 $\mu\text{g/mL}$ are considered weak and non-cytotoxic, respectively.¹⁰








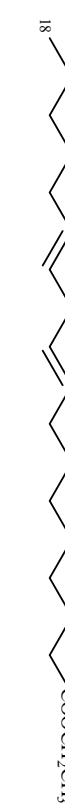



3. Results and Discussion

3.1 The GC-MS phytochemical analysis of fractions 1 and 2 from the hexane extract of *L. nipponica*

Two fractions (fractions 1 and 2) and three pure compounds *viz.* *n*-hexadecanoic acid (3), *n*-octadecanoic acid (4) and ergosterol peroxide (5) were obtained from an *n*-hexane extract of *L. nipponica* by silica gel column chromatography. The phytochemical compositions of fraction 1 was analysed by GC-MS and the presence of eight compounds were identified from this fraction 1 *viz.* i) octanoic acid, ii) *n*-tetradecanoic acid, iii) *n*-hexadecanoic acid, iv) *n*-heptadecanoic acid, v) 11(*Z*)-hexadecenoic acid, vi) methyl octadecanoate, vii) *n*-octadecanoic acid and viii) ethyl linoleate. These eight compounds are listed in Table 1 in the order of their elution in the column.

The GC-MS phytochemical analysis of fraction 1 showed the presence of fatty acids and esters of fatty acids (Table 1). In general, fatty acids and their esters as a single molecular entity or in combination with other molecules have been shown to exhibit a variety of biological and pharmacological activities, which include antiasthma, antidiuretic, hepatoprotective, antieczemic, antimicrobial, antihistaminic, antiarthritic and cytotoxic activity in various murine and human cancer cell lines.¹¹⁻¹⁵ For example, octanoic acid has been shown to exhibit anticancer property on human colorectal, skin and mammary gland cancer cells in the MTT assay and reduced cancer cell viability by 70-90%.¹⁶ Additionally, the serum level of octanoic acid has been shown to serve as a biomarker or predictor for the prognosis of advanced colorectal cancer.¹⁷ *n*-Tetradecanoic acid exhibited cytotoxic activity in combination with other compounds. For example, the GC-MS analysis of chloroform extracts from *Ixora* species (Rubiaceae) showed the presence of *n*-tetradecanoic acid as one of the components together with other fatty acids and fatty esters. These extracts exhibited cytotoxic activity against HeLa, MCF-7 and NCI H-460 cancer cell lines with IC₅₀ and LC₅₀ of 15 ± 3.8 $\mu\text{g/mL}$ and 230 ± 3.9 $\mu\text{g/mL}$, respectively.¹⁸ Additionally, *n*-tetradecanoic acid together with other compounds showed antibacterial activity against several bacterial strains¹⁴ and *n*-tetradecanoic acid exhibited lubricant and nematicide activities.¹⁹ *n*-Hexadecanoic acid (palmitic acid) has been shown to exhibit cytotoxic activity against human leukemic cell lines (MOLT-4), human colorectal carcinoma cells (HCT-116) and human colon cancer lines (HT-29)²⁰⁻²¹ and it also exhibited favourable biological functions at the cellular and tissue level.²² Additionally, *n*-hexadecanoic acid exhibited antioxidant, antifungal, hypocholesterolemic and anti-inflammatory activities^{14,23} and it has been used as an emollient and 5 α -reductase inhibitor.^{15,23} *n*-Hexadecanoic acid together with other compounds exhibited antibacterial activity against a panel of bacterial strains.¹⁴ *n*-Heptadecanoic acid inhibited cell proliferation significantly and promoted apoptosis in PC9 and PC9/GR cells. *n*-Heptadecanoic acid has been shown to be an effective agent against non-small cell lung carcinomas cells (NSCLC). Therefore, consumption of food, which is rich in *n*-heptadecanoic acid, could be beneficial during the treatment of NSCLC.²⁴ 11-Hexadecenoic acid exerted cytotoxic potential on human cancer cell lines in culture.²⁵ Methyl octadecanoate (methyl stearate) in combination with other compounds exhibited cytotoxic activity in human cancer cell lines. For example, an *n*-hexane fraction from *Salsola oppositifolia* with methyl stearate as one of the constituents, exhibited an effective cytotoxic activity on the large lung carcinoma and amelanotic melanoma cell lines with IC₅₀ values of 19.1 and 24.4 $\mu\text{g/mL}$, respectively.²⁶ *n*-Octadecanoic acid (stearic acid) has been shown to exert cytotoxic activity against HT-1080 fibrosarcoma cells, Hs578T breast cancer cells, MDA-MB-231 breast cancer cells and various other human cell types.²⁷⁻²⁸ Ethyl linoleate exhibited tyrosinase activity in α -melanocyte stimulating hormone (α -MSH) B15F10 cancer cell lines.²⁹ Additionally, ethyl linoleate exerted a decrease in melanin production and was used as a biomarker for identifying Foetal Alcohol Syndrome (FAS).³⁰

Table 1. The GC-MS phytochemical analysis of fractions **1** and **2** from a hexane extract of *L. nipponica*

Fraction 1			Compounds identified	Structures of compounds
No	Rt. (min)	% of area		
1	9.217	4.56	Octanoic acid (Caprylic acid)	
2	16.911	1.18	<i>n</i> -Tetradecanoic acid	
3	20.562	11.62	<i>n</i> -Hexadecanoic acid (Palmitic acid)	
4	22.166	2.71	<i>n</i> -Heptadecanoic acid	
5	22.818	1.30	11(<i>Z</i>)-Hexadecenoic acid	
6	23.280	4.78	Methyl octadecanoate (Methyl stearate)	
7	24.052	12.43	<i>n</i> -Octadecanoic acid (Stearic acid)	
8	25.286	6.31	Ethyl linoleate	
Fraction 2				
1	13.239	2.26	Ethyl 9-oxononanoate	
2	13.350	5.84	Ethyl 11(<i>Z</i>)-hexadecenoate (Ethylpalmifolate)	
3	14.032	7.91	Ethyl hexadecanoate (Ethyl palmitate)	

Rt. (min) = Retention time in minutes

The phytochemical composition of fraction **2** was analysed by GC-MS and the presence of three compounds was identified from this fraction **2** viz. i) ethyl 9-oxononanoate, ii) ethyl 11(*Z*)-hexadecenoate and iii) ethyl hexadecanoate. These three compounds are listed in Table 1 in the order of their elution in the column. It has been noted that the presence of ethyl 9-oxononanoic acid was identified as one of the components in this fraction **2**. The presence of esters of 9-oxononanoic acid in natural resources is not uncommon and particular, their presence in the fungal resources has frequently been encountered. For example, ethyl 9-oxononanoate has been reported in *Epichloe typhina* (fungal endophytes).³¹ Previous reports from other studies revealed that ethyl 9-oxononanoate exhibited antifungal activity against *Cladosporium herbarum* in a TLC plate bioassay and the minimum amounts for inhibition was determined to be 10 µg/spot.³² Ethyl 11(*Z*)-hexadecenoate was also identified in fraction **2**. Ethyl 11(*Z*)-hexadecenoate has been reported to serve as a female sex pheromone of insect species viz. *Syndipnus rubiginosus*.³³ This compound even at nanogram scale attracted male insects of the same species³³ and approximately, 100 ng of ethyl 11(*Z*)-hexadecenoate was enough to attract the male insects in the field.³³ Fraction **2** also contained ethyl hexadecanoate (ethyl palmitate), which has been reported to exhibit promising anti-inflammatory effects on local and systemic experimental rat models.³⁴ Ethyl hexadecanoate has been used as an alcohol biomarker in hair.³⁵ As an emollient, ethyl hexadecanoate together with other fatty acid esters has also been used as skin and hair conditioning agents in the cosmetic industry.^{36,37}

3.2 Characterisation of 3-5 by spectral data

The structures of compounds **3-5** are shown in Figure 1 and their structural elucidations by spectral data are also detailed below.

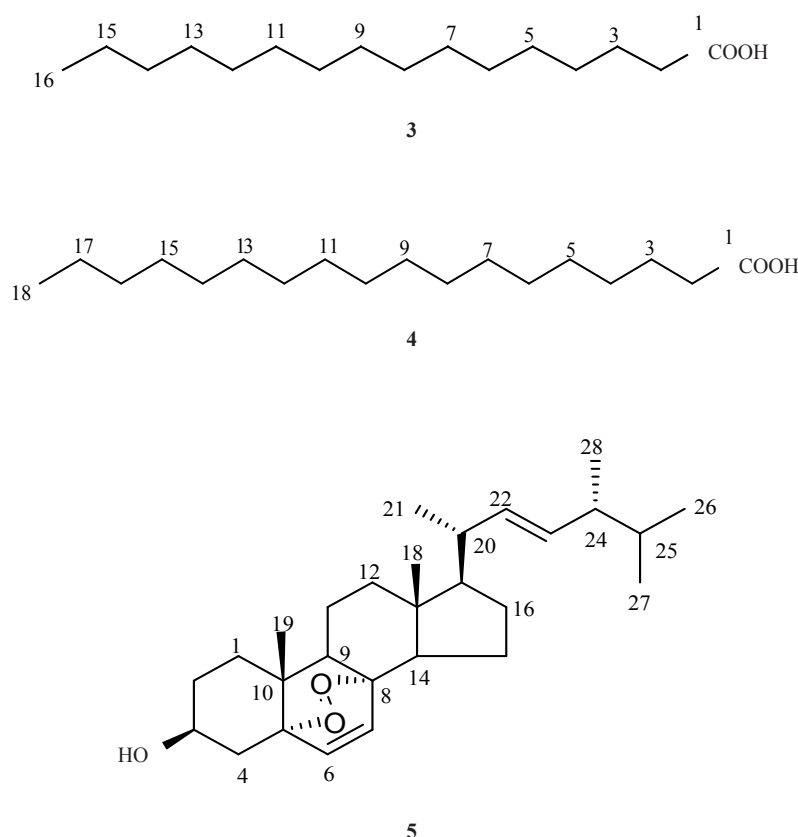


Figure 1. Structures of compounds **3-5**

Compound **3** was obtained as colourless crystals. Its electron impact ionisation (EI, 70 eV) mass spectrum showed molecular ion peak at m/z 256 (95) and other fragment ions at m/z 227 (24), 213 (70), 185 (38), 171 (35), 143 (15), 129 (74), 97 (48), 73 (100), 55 (90), 43 (86). The molecular formula of **3** was determined to be $C_{16}H_{32}O_2$ from its molecular ion peak. In the 1H NMR spectrum, **3** displayed only two peaks at high-field region. One of the peaks resonating at 0.90 ppm was assigned to the methyl protons. The other peak was very intense and resonated at 1.24-2.36 ppm that was assigned to all methylene protons that resonated in the same region. In the ^{13}C NMR spectrum, **3** displayed only three peaks. Two of these peaks resonated in the high-field region *i.e.* at 16.80 ppm for the methyl carbon and at 25.3-36.7 ppm for the methylene carbons. The third peak resonated at 182.7 ppm due to the presence of highly deshielding carbonyl carbon from the carboxylic group (Table 2).

Similarly, compound **4** was also obtained as colourless crystals. Its electron impact ionisation (EI, 70 eV) mass spectrum showed molecular ion peak at m/z 284 (100) and other fragment ions at m/z 241 (52), 213 (10), 185 (42), 157 (8), 129 (54), 97 (28), 73 (74), 57 (55). From the molecular ion peak, the molecular formula of **4** was determined to be $C_{18}H_{36}O_2$. In the 1H NMR spectrum, **4** displayed only two peaks at high-field region. One of the peaks resonating at 0.88 ppm was assigned to the methyl protons. The other peak was very intense and resonated at 1.25-2.35 ppm that was assigned to all methylene protons that resonated in the same region. In the ^{13}C NMR spectrum, **4** displayed only three peaks. Two of these peaks resonated at high-field region at 16.8 and 25.3-36.7 ppm and were assigned to the methyl carbon and the methylene carbons, respectively. The third peak resonated at 182.8 ppm due to the presence of highly deshielding carbonyl carbon from the carboxylic group (Table 2). Overall, the 1H NMR and ^{13}C NMR spectra of **3** and **4** did not provide much information to distinguish between each other and their 1H NMR and ^{13}C NMR spectra appeared to be the same. However, their mass spectral data *viz.* molecular ion peaks (m/z 256 and m/z 284 for **3** and **4**, respectively), fragment ion peaks and the relative intensities of these ion peaks are easily distinguished these two compounds from each other. Additionally, the EIMS spectrum of this individual compound showed good agreement with the MS spectrum from the library data.

Compound **5** was obtained as colourless needles. Its melting point was determined to be 180.0-181.0 °C. The IR (KBr) spectrum showed absorptions at 3360, 1460, 1380, 1277, 1040, 1030, 960, and 940 cm^{-1} . The peaks at ν_{max} 3360 and 1460 cm^{-1} were attributed to the presence of a hydroxyl group and an olefinic bond, respectively. Its electron impact ionisation (EI, 70eV) mass spectrum showed a molecular ion peak at m/z 428 (22) and other fragment ions at m/z 410 (M-H₂O) (23), 396 (M-O₂) (65), 251 (83), 209 (38), 197 (45), 141 (35), 69 (100). From the molecular ion peak, the molecular formula of **4** was determined to be $C_{28}H_{44}O_3$. The fragment ion peak at m/z 410 was due to the loss of water molecule [M-H₂O]. The fragment ion peak at m/z 396 was due to the loss of two oxygen atoms [M-O₂] (peroxide moiety), which was present in one of the rings (Figure 1). The fragment ion peak at m/z 303 was due to the loss of side chain [M-side chain]. The 1H NMR spectrum of **5** gave a peak at 3.96 ppm (1H, m), which confirmed the presence of a hydroxyl group and this peak was assigned to the proton at H-3. The peaks resonating at 6.22 ppm (1H, d, $J = 8.5$ Hz, H-6), 6.50 (1H, d, $J = 8.5$ Hz, H-7) and 5.18 (2H, m, H-22 and H-23) were assigned to the protons of the double bonds between C6-C7 and C22-C23, respectively (Figure 1). The assignment of other 1H NMR peaks are given in Table 3. The ^{13}C NMR spectrum of **5** showed the presence of three oxygenated carbons and four carbons with double bonds. The peak resonating at 66.2 ppm was assigned to the oxygenated carbon at C-3 position. The other two oxygenated carbons resonating at 79.3 and 82.1 ppm were assigned to carbons at C-5 and C-8 positions, respectively. The peaks resonating at 135.0, 130.5, 135.3 and 132.1 ppm were assigned to the carbons with double bonds at C-6, C-7, C-22 and C-23 respectively. The list of 1H and ^{13}C NMR spectral data of **5** is given in Table 2. Comparison of spectral data of **5** with literature data had good agreement.³⁸

Table 2. ¹H and ¹³C NMR spectral data of compounds **3-5** in CDCl₃.

Position	3			4			5		
	Groups	¹³ C NMR δ (ppm)	¹ H NMR δ (ppm), J (Hz)	Groups	¹³ C NMR δ (ppm)	¹ H NMR δ (ppm), J (Hz)	Groups	¹³ C NMR δ (ppm)	¹ H NMR δ (ppm), J (Hz)
1	COOH	182.7	-	COOH	182.80	-	CH ₂	34.7	1.88 (1H, m, H-1α) 1.63 (1H, m, H-1β)
2	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	CH ₂	30.0	1.75 (1H, m, H-2α) 1.45 (1H, m, H-2β)
3	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	CH	66.2	3.96 (1H, m, H-3)
4	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	CH ₂	36.8	2.05 (1H, dd, 13.6, 5.4, H-4α) 1.82 (1H, dd, 13.6, 11.4, H-4β)
5	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	C	79.3	-
6	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	CH	135.0	6.22 (1H, d, 8.4, H-6)
7	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	CH	130.5	6.50 (1H, d, 8.4, H-7)
8	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	C	82.1	-
9	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	CH	51.4	1.49 (1H, m, H-9)
10	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	C	39.3	-
11	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	CH ₂	20.6	1.53 (1H, m, H-11α) 1.33 (1H, m, H-11β)
12	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	CH ₂	39.3	1.70 (1H, m, H-12α) 1.26 (1H, m, H-12β)
13	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	C	44.5	-
14	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	CH	51.6	1.43 (1H, m, H-14)
15	CH ₂	25.3-36.7	1.24-2.36	CH ₂	25.3-36.7	1.25-2.35	CH ₂	23.4	1.39 (1H, m, H-15α) 1.13 (1H, m, H-15β)
16	CH ₃	16.8	0.90	CH ₂	25.3-36.7	1.25-2.35	CH ₂	28.6	1.86 (1H, m, 13.6, H-16α) 1.16 (1H, m, 13.6, H-16β)
17	-	-	-	CH ₂	25.3-36.7	1.25-2.35	CH	56.1	1.15 (1H, m, H-17)
18	-	-	-	CH ₃	16.8	0.88	CH ₃	12.8	0.81 (3H, s, H-18)
19	-	-	-	-	-	-	CH ₃	18.1	0.88 (3H, s, H-19)
20	-	-	-	-	-	-	CH	39.6	1.94 (1H, m, H-20)
21	-	-	-	-	-	-	CH ₃	20.8	1.02 (3H, d, 6.6, H-21)
22	-	-	-	-	-	-	CH	135.3	5.12 (1H, dd, 15.2, 7.4, H-22)
23	-	-	-	-	-	-	CH	132.1	5.18 (1H, dd, 15.2, 8.1, H-23)
24	-	-	-	-	-	-	CH	42.7	1.78 (1H, m, H-24)
25	-	-	-	-	-	-	CH	33.0	1.39 (1H, m, H-25)
26	-	-	-	-	-	-	CH ₃	19.6	0.82 (3H, d, 6.6, H-26)
27	-	-	-	-	-	-	CH ₃	19.9	0.83 (3H, d, 6.6, H-27)
28	-	-	-	-	-	-	CH ₃	17.5	0.94 (3H, d, 6.8, H-28)

3.3 Evaluation of cytotoxic activity of fractions 1 and 2 and compound 5 by MTT assay

Fractions **1** and **2** were evaluated for cytotoxic activity against P388, MCF7, HL60, HepG2 and J82 cell lines by MTT assay. Fraction **1** showed IC₅₀ values of 9.8, 18.2, 72.6, > 100 and > 100 µg/mL against P388, HL60, MCF7, HepG2 and J82 cancer cell lines, respectively (Table 3). The positive controls, 6-mercaptopurine (6-MP) and doxorubicin showed an IC₅₀ value of < 6.25 µg/mL for each against P388 and MCF7 cancer cell lines. Since an IC₅₀ value ≤ 20 µg/mL is considered as cytotoxic,¹⁰ fraction **1** was considered to show a significant activity against P388 and HL60 cancer lines with IC₅₀ values of 9.8 and 18.2 µg/mL, respectively. However, fraction **1** with IC₅₀ values of 72.6, > 100 and > 100 µg/mL against MCF7, HepG2 and J2 cancer cell lines, respectively, did not show any appreciable activity (Table 3).

Table 3. Cytotoxic activity of fractions **1** and **2** and compound **5** from an *n*-hexane extract of *L. nipponica*.

Fractions/ Compounds	Cancer cell lines and IC ₅₀ values in µg/mL				
	P388	HL60	MCF7	HepG2	J82
1	9.8	18.2	72.6	> 100	>100
2	19.4	16.2	> 100	64.8	> 100
5	NT	82.2	NT	> 234	> 234
Dox	-	-	< 6.25	-	-
6MP	< 6.25	-	-	-	-

Dox = Doxorubicin, 6MP = 6-Mercaptopurine, **5** = Ergosterol peroxide. NT = Not tested, (-) = Did not maintain positive control due to non-availability at the time of experiment. The IC₅₀ value ≤ 20 µg/mL is considered cytotoxic and IC₅₀ values of 21-40 and > 40 µg/mL are considered weak and non-cytotoxic, respectively. For **5**, the IC₅₀ values are expressed in µM.

Fraction **2** showed IC₅₀ values of 19.4, 16.2, > 100, 64.8 and > 100 µg/mL against P388, HL60, MCF7, HepG2 and J82 cancer cell lines, respectively (Table 3). This result indicated that fraction **2** showed a significant activity against P388 and HL60 cancer cell lines with IC₅₀ values of 19.4 and 16.2 µg/mL, respectively. Fraction **2** did not show any appreciable activity against MCF7, HepG2 and J2 cancer cell lines (Table 3). Compounds **3** and **4** were not screened for their cytotoxic activity since they were obtained in small quantity. However, previous reports from other studies revealed that **3** exhibited selective cytotoxicity to human leukemic cells at concentrations ranging from 12.5-50 µg/mL and induced apoptosis in the human leukemic cell line (MOLT-4) at a concentration of 50 µg/mL. Compound **3** also exhibited cytotoxicity against human colorectal carcinoma cells (HCT-116) and human colon cancer lines (HT-29) in the MTT assay and it showed IC₅₀ values of 0.8 and 36.04 µg/mL, respectively.²⁰⁻²¹ Compound **3** has been shown to exert favourable multiple fundamental biological functions at cellular and tissue levels.²² Therefore, it has been recommended to consume **3** to a minimal amount through diet²² and for the same reason **3** has been used as a food additive.³⁷ Furthermore, **3** in combination with other fatty acids has been used as an emollient in the cosmetic industry.^{37,39} Similarly, previous reports from other studies showed that **4** inhibited invasion of HT-1080 fibrosarcoma cells⁴⁰ and epidermal growth factor (EGF) receptor-mediated proliferation in Hs578t breast cancer cells.⁴¹ Compound **4** inhibited invasion and proliferation and induced apoptosis of MDA-MB-231 breast cancer cells⁴² and various other human cell types.⁴³ Additionally, compound **4** has been associated with a decrease in mammary tumour development and incidence in experimental animals.^{44,45}

Compound **5** was evaluated for cytotoxicity against HL60, HepG2 and J82 cancer cell lines and the IC₅₀ values of **5** were determined to be 82.2, > 234 and > 234 µM, respectively. These results revealed that **5** showed only a weak activity against HL60 cancer cell lines with an IC₅₀ value of 82.2 µM (35.1 µg/mL) and was inactive against HepG2 and J82 cancer cell lines with an IC₅₀ value > 234 µM (> 100 µg/mL) (Table 4). The cytotoxicity of **5** on various other cancer cell lines has previously been reported. For example, **5** is active against SNU-1, SNU-C4, SNU-354 and sarcoma-180

cancer cell lines with IC₅₀ values of 8.0, 67.7, 31.7 and 36.2 µg/mL respectively.⁴⁶ Compound **5** has also been shown to exhibit activity against HeLa, A549, J5, MCF7, Raw264.7 and Beas-2b cancer cell lines with IC₅₀ values of 31 ± 1.8, 23 ± 1.5, 33 ± 2.8, 29 ± 3.1, 174 ± 27.6 and 222 ± 22.6 µM, respectively.⁴⁷ Additionally, compound **5** has been shown to markedly inhibit the tumour-promoting effect of TPA in 7,12-dimethylbenz[a]anthracene-initiated mice² and showed a strong anticomplementary activity.²

4. Conclusion

Two fractions and three pure compounds were obtained from a *n*-hexane extract of *L. nipponica* by silica gel column chromatography. The GC-MS analysis of the two fractions showed the presence of fatty acids, esters of fatty acids and ethyl 9-oxononanoate. Additionally, these two fractions showed significant cytotoxic activity against P388 and HL60 cancer cell lines in the MTT assay. The three pure compounds were characterised as *n*-hexadecanoic acid (**3**), *n*-octadecanoic acid (**4**) and ergosterol peroxide (**5**) by spectroscopic and spectrometric means. *n*-Hexadecanoic acid and *n*-octadecanoic acid were not evaluated for their cytotoxic activity since they were obtained in small quantity, while ergosterol peroxide showed weak activity against HL60 cancer cell lines. Therefore, the *n*-hexane extract of *L. nipponica* has therapeutically important phytochemicals and further studies on *L. nipponica* are required to promote its therapeutic applications.

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Conflict of interest

The author declares that there is no conflict of interest.

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