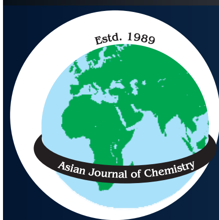

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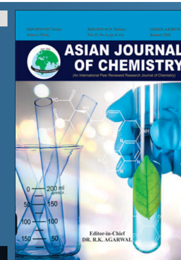

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Identification of Novel Bioactive Compounds, Neurosporalol 1 and 2 from an Endolichenic Fungus, *Neurospora ugadawe* Inhabited in the Lichen Host, *Graphis tsunodae* Zahlbr. from Mangrove Ecosystem in Puttalam Lagoon, Sri Lanka

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Endolichenic fungi (ELF) have been recognized as a promising source of bioactive secondary metabolites. Sri Lanka, a biodiversity hotspot, harbours a remarkable diversity in mangrove ecosystems. In the present study, endolichenic fungi obtained from mangrove lichens of Puttalam lagoon were studied for their secondary metabolites. An endolichenic fungus, *Neurospora ugadawe,* isolated from the lichen host *Graphis tsunodae* Zahlbr. was identified up to the species level. Secondary metabolites of *N. ugadawe* were extracted into ethyl acetate and crude extract showed promising antioxidant and anti-inflammatory activities. Bioassay guided separation on silica gel column chromatography and preparative TLC were performed to isolate the bioactive pure compounds. Two novel bioactive pure compounds were identified as Neurosporalol 1 and Neurosporalol 2. Neurosporalol 1 showed the highest antioxidant activity compared with the positive control BHT. Further, this compound showed very low anti-inflammatory activity compared to that of the positive control aspirin. Neurosporalol 2 showed comparable antioxidant and moderate anti-inflammatory activities.

Keywords: Endolichenic fungus, *Neurospora ugadawe*, Neurosporalol 1, Neurosporalol 2, Bioactivities.

INTRODUCTION

Over the years, natural products have been documented to be of great importance to the health of individuals and comm-unities. In many developing countries and rural communities, natural product based medicines have played an important role for many years in their primary health care. Natural product based medicines are not only the main resources of traditional medicines but most of the modern medicines have also been isolated or derived from plants, fungi, lichens and animals. World Health Organization (WHO) also has recognized that herbal medicines serve the health needs of about 80% of the world's population; especially for millions of people in the vast rural areas of developing countries [1]. It should be highlighted that therapeutic use of plants is as old as 4000-5000 B.C. and Chinese used the first natural herbal preparations as medicines [1]. Natural product derivatives constitute as much as 25% of the total drugs in developed countries, while in fast developing coun-tries such as China and India, the contribution is as much as 80% [2]. However, drug discovery using natural products is a challenging task. It constitutes several steps *e.g.* isolation of bioactive compounds from natural resources, chemical analysis, characterization and pharmacological investigation including animal testing. The success of these resources mainly depends on the search for the correct source of natural products and the process is more or less similar to finding a needle in a hay stack [3]. However, natural product studies are inclining into micro-organisms because they serve as treasures for bioactive comp-ounds and unlike plants, microorganisms are easily cultured on selective media. With slight modifications in culture media

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and conditions or inducing stress in the culture media, there is a possibility to obtain novel bioactive compounds [4]. Among various microorganisms, fungi have taken a prominent place in the natural product chemistry [5]. For example, penicillin and the cephalosporins have been isolated from *Penicillium chrysogenum* and *Cephalosporium acremonium*, respectively. An antifungal drug griseofulvin has been isolated from *P. griseofulvum.* Another medicinally useful fungal metabolite is lovastatin, which belongs to the group of drugs called statins also originated from *Aspergillus terreus* [6].

The endolichenic fungi (ELF) have been identified lately as a promising treasure trove of novel secondary metabolites [7]. Endolichenic fungi are a diverse group of non-mycobiont fungi inhabited the lichen thali. Lichen thallus is not a luxurious environment for endolichenic fungi. Lichens found in mangrove ecosystems encounter more habitat stress than lichens found in other terrestrial ecosystems. Mangrove ecosystems are often challenged with anaerobic soils, rapid variation in tidal currents, high salinity, high temperature (30-35 ºC) and strong winds [7]. Regardless these challenges, lichens are often found in association with mangrove plants and these lichen as well as the ELF species might be rich in secondary metabolites to meet these challenges. For example, Paranagama *et al*. [8] studied *Corynespora* sp., an ELF isolated from the lichen host, the cavern beard lichen, *Usnea cavenosa* from Arizona, USA. In this study, two new hepatketides, corynesporol and 1-hydroxydehydroherbarin along with herbarin were isolated from the ELF *Corynespora* sp. Chemical derivatization of these compounds has given six other compounds and all of them have been tested for their cytotoxic and cancer cell migration inhibitory activity. Dehydroherbarin, a chemically transformed product of the herbarin, has inhibited migration of cell lines, MDA-MB-231 and PC-3M with no cellular toxicity. Though herbarin showed similar activity, the effective concentration was very high [8]. Since then, many researches have conducted on ELF and have isolated remarkable bioactive compounds [9].

Sri Lanka being a bio diversity hotspot inhabits more than 1000 lichen species [8], there is a high chance of discovering novel secondary metabolites from ELF of lichens found in mangroves. Therefore, the main objective of the current study was to isolate and characterize secondary metabolites of the ELF species isolated from a lichen host *Graphis tsunodae* Zahlbr. collected from mangrove ecosystem.

EXPERIMENTAL

Field permit: Forest Department of Sri Lanka granted permission to collect lichen samples from mangroves. The study sites were managed by the forest Department in Sri Lanka. The samples were collected from the trees that grew wildly in the area, where specific permit was obtained for taking samples. The trees used for sampling were treated ethically and present study did not harm the local environment.

Collection of lichen samples: The lichen host was collected from Puttalam lagoon in Anawilundawa (7º42′41.03″N and 71º48′0.64″E). Puttalam lagoon has the largest extent of mangroves, spread across 36,426 hectares, in Sri Lanka. Annual rainfall is 1150 mm and average annual temperature is about 28 ºC. The samples were collected with stem bark of host plant, *Azadirachta indica* and immediately placed in acid free paper bags, labelled and transported to the Department of Chemistry, University of Kelaniya, Kelaniya, Sri Lanka. Samples were stored at 4 ºC and processed within 2 weeks. These lichens were used to isolate endolichenic fungi (ELF).

Lichen identification: The lichen samples (particle size 4 cm × 4 cm) were kept in a refrigerator for two weeks and air dried. Identification of the lichens was carried out at the Natural History Museum (BM) London, U.K. and photographs were taken using Olympus stereomicroscopes and Olympus compound microscopes with interference contrast, connected to a Nikon Coolpix digital camera. All measurements were made on sections mounted in tap water [10]. Voucher specimens were deposited in the Department of Chemistry, University of Kelaniya, Sri Lanka with a duplicate of each specimen.

Isolation and identification of endolichenic fungi (ELF): Healthy lichen thalli were cleaned with running tap water to eliminate suspended solids. Clean lichen thallus was then cut into 1-2 cm^2 segments and followed the protocol as discussed earlier [8] to remove surface microflora. The segments were dipped in 70% ethanol for 10 s, followed by 0.5% NaOCl for 3 min and then washed in sterilized distilled water for three times. The thalli were surface dried with sterile filter papers. After surface sterilization, five segments (approximately 3 mm × 3 mm) of each lichen sample was placed on 2% malt extract agar (MEA) plate supplemented with 0.01% streptomycin. Plates were sealed with parafilm and incubated under ambient light/dark condition at room temperature (30 ºC) for 14 days. The tissue segments were observed periodically and fungi growing out of them were scored, isolated and sub-cultured. Finally, based on the morphological differences, the pure cultures were made and further incubated on PDA at room temperature for one week [7]. Pure endolichenic fungal isolates were vouchered in sterile distilled water and deposited at the Department of Chemistry, University of Kelaniya under strain ID. Colony morphology on PDA plates and microscopic features, mycelial pigmentation, branching pattern and sporulating structures were observed under light microscope.

One of the most frequently isolated fungal isolate (83-02-05) was used for the species level identification. Isolate was inoculated into potato dextrose broth (PDB) and incubated at room temperature (30-32 ºC) for 7 days. The mycelia were separated from the broth and the DNA was extracted using the modified method described earlier [11]. Quality of the extracted DNA samples was tested using 1 % agarose gel electrophoresis and samples were stored at -20 ºC until use. Fungal rDNA-ITS region was amplified from the purified genomic DNA by using ITS1 and ITS4 universal primers [12]. The PCR reaction mixture comprised of 2-5 µL fungal DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 µM each forward and reserve primer, 1.25 units of Taq polymerase (Promega, USA) as mentioned by Maduranga *et al*. [7]. PCR protocol was as follows, an initial denaturation step at 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s with a final extension step of 72 ºC for 10 min. PCR was performed in a Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA,

USA). The amplification of ITS region was confirmed by separating the amplified product in 1 % agarose containing 0.2-0.5 µg/mL ethidium bromide using gel electrophoresis. Single PCR products were directly sequenced using Sanger dideoxy sequencing technology at Genetech, Colombo, Sri Lanka. DNA sequences were manually edited using BioEdit program [12] and compared with the sequences available in the GenBank using Basic Local Alignment Search Tool (BLAST). DNA sequence was deposited at the NCBI database and obtained an accession number.

Extraction of secondary metabolites: A seed culture of *N. ugadawe* was grown on a small PDA plate and this culture was used to inoculate 50 PDA plates (150 mm × 25 mm). After 2 weeks of inoculation, the PDA plates containing mycelia were cut into small pieces and extracted with EtOAc (6 × 500 mL). The combined EtOAc extracts were evaporated under reduced pressure to afford a dark brown semisolid (1.1 g) that was stored at low temperature (0-2 ºC) to be used in the bioassays given below [13,14]:

Screening for bioactivities

Free radical scavenging property: ABTS assay was carried out to screen free radical scavenging property of the test sample [15]. The radical scavenging ability assay was carried out in a flat bottom 96-well microtiter plate, according to the method described by Miller & Rice-Evans [14] with slight modifications. Different doses of the test sample and standard antioxidant, Butylated hydroxytoluene (BHT) (0.0, 15.62, 31.25, 62.5, 125.0, 250.0, 500.0, 1000.0 µg/mL) was added to 190 µL of freshly prepared ABTS solution in the 96-well plate. All reagents were mixed and incubated for 7 min at room temperature under dark conditions. The absorbance of each well was measured at 714 nm with a Microplate Reader (Biotek, USA). The percentage inhibition was calculated using the eqn. 1 [13] where, A_c is the absorbance of the control sample and the A_s is the absorbance of the treatment.

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100 \qquad (1)$$

The IC_{50} values of all the crude extracts were calculated using Prism 7 Release 2017, Statistical Software. The experiment was carried in triplicates.

Anti-inflammatory assay: The anti-inflammatory assay was carried using human red blood cells stability method (HRBS) as described by Shinde *et al*. [15]. Test sample (1 mg) was dissolved in 0.2 mL of DMSO and diluted to 5 mL by adding appropriate amount of normal saline. The reaction mixture was prepared with 5 mL of test solution and 0.5 mL of 10% RBC suspension. The negative control was prepared using equal amount of DMSO and normal saline. Aspirin was used as positive control in this study. All the centrifuge tubes containing reaction mixtures were incubated in a water bath at 56 ºC for 30 min and the tubes were cooled under running tap water. The reaction mixture was centrifuged at 3000 rpm for 10 min and the absorbance of supernatants was measured at 560 nm using UV-visible spectrophotometer (AquaMate 8000, Singapore). The test was performed in triplicates and the percentage stability was calculated using the eqn. 2. The percentage stability *versus* test concentrations was plotted in order to compare the anti-inflammatory activity of each sample with the standard compound separately [15].

$$\text{Membrane stability (\%)} = \frac{A_c - A_s}{A_c} \times 100 \qquad (2)$$

Tyrosinase inhibitory assay: Tyrosinase assay was carried out in a flat bottom 96-well microtiter plate, test extract and positive control Kojic acid were dissolved in 10% DMSO in phosphate buffer (pH 6.5) and different doses were prepared for the test sample and positive control (0.0, 15.62, 31.25, 62.5, 125.0, 250.0, 500.0 and 1000.0 µg/mL) 70 µL of each dilution was combined with 30 µL of tyrosinase (333 U/mL) after incubate at room temperature for 5 min 110 µL of substrate (2mM L-DOPA) was added to each well, incubation was commenced for 30 min at room temperature and absorbance was measured at 492 nm with a Microplate Reader (Biotek, USA). The percentage inhibition was calculated using the eqn. 1 [16]:

Antibacterial activities: Antibacterial activity of test sample was tested against *B. subtilus*, *E. coli* and *S. aureus* using agar well diffusion method [17]. The flasks containing Nutrient Broth (NA, 60 mL) were inoculated using each bacterial species separately under aseptic conditions and allowed to incubate at room temperature (28 ± 2 ºC) for 24 h. After incubation period series of dilutions (10^{-2}) were prepared using the inoculums of three bacterial species. Four wells bored on each NA plate and stock solution of the test sample (5 mg/mL) was prepared in DMSO and loaded into two well as 100 µL to each. Parallel experiments were carried out using azithromycin (5 mg/mL, 100 µL) as the positive control and DMSO (100 µL) as the negative control. This process was repeated each sample and inhibition zone was measured.

Streptococcus mutans (ATCC 700610) was obtained from the American Type Culture Collection and cultured in Trypticase soy agar supplemented with 5% defibrinated sheep blood or Trypticase soy broth supplemented with hemin and vitamin K1 under anaerobic conditions in an anaerobic jar containing anaerobic sachets at 37 ºC.

Ethyl acetate extract of the fungus dissolved in 10% DMSO were tested antimicrobial effect for the highest dose (5 mg/mL) using agar well diffusion assay as described by Okeke *et al*. [17]. Briefly, a 3 mL aliquot of an overnight culture of *S. mutans* adjusted to 0.5 MacFarland standard (1.5 × 10^8 CFU/mL) was evenly spread on the surface of the agar plate and the excess was removed after 2 min. Then 9 mm wells were made on agar plates using a stainless steel cork borer. Afterwards, the wells were filled with 200 µL of fungal extract. Chlorhexidine (5 mg/mL) was used as the positive control and 10% DMSO and sterilized distilled water were used as negative control. After incubating the plates for 48 h, bacteria become confluent on the surface of the agar plates except in the areas of growth inhibition around the wells. The zones of inhibition were measured using a micrometer gauge. Extracts which showed clear zones around the wells were considered to have an inhibitory effect on the bacterium and those, which did not show clear zones without bacterial growth were considered to have no inhibitory effect on the bacterium.

Quantitative analysis of bioactive compounds: Since, ethyl acetate extracts of fungus, 83-02-05 showed significant antioxidant activity and moderate anti-inflammatory activity, 1.10 g of crude extract was partitioned with chloroform, hexane and MeOH in order to separate nonpolar, moderately polar and polar compounds respectively [8]. The antioxidant activity of each fraction was evaluated using the methods described above [13,14].

The chloroform fraction (603.1 mg) showed the highest antioxidant activity and therefore, it was subjected to column chromatography using normal phase silica gel. The column (70-230 mesh, 19.0 g, 2 cm × 50 cm) was eluted with 100% CH_2Cl_2 containing increasing amount of MeOH and finally MeOH. Several fractions were collected and combined on the basis of their TLC profiles to obtained F_1 to F_7. Of which F_3 (50.0 mg) and F_4 (40.7 mg) fractions was found to contain significant radical scavenging activity against ABTS assay and they were further fractionated using normal phase silica gel column chromatography (3.0 g, 1 cm × 30 cm) separately using 100% CH_2Cl_2, 100% CH_2Cl_2 containing gradually increasing amount of MeOH and finally MeOH, similar fractions were combined according to the TLC pattern. The five fractions were obtained from F_3 and F_4 separately and antioxidant active fractions, F_3/F_4 and F_4/F_4 were separately purified by preparative thin layer chromatography (eluant: 8% MeOH in CH_2Cl_2) and isolate pure compounds. The compounds were isolated from F_3/F_4 and F_4/F_4 labelled as Neurosporalol 1 (5.4 mg; R_f = 0.48) and Neurosporalol 2 (8.4 mg; R_f = 0.43), respectively.

RESULTS AND DISCUSSION

Isolation and identification of endolichenic fungus: The distinctive morphological characters and molecular identification of the lichen sample was confirmed to be *Graphis tsunodae* Zahlbr., a novel Lichen record for Sri Lanka. Genus *Graphis* is the most speciose lichen genus in Sri Lanka. The host plant of *Graphis tsunodae* Zahlbr. was *Azadirachta indica*, a mangrove ecosystem associated plant [18].

Three fungal isolates were obtained from this lichen host, *Graphis tsunodae* Zahlbr. One of the isolated fungus, moderately growing strain was used in species identification and further bioassays. The isolate showed rapid growth on PDA covering a 9 cm diameter Petri-dish within two week. Colony color varied from dark brown to olive green on PDA after 2 weeks incubation period at room temperature (Fig. 1a) and mycelia were brown to dark brown. Spheroid macroconidia resulted from apical budding were observed after 14-day incubation period (Fig. 1b).

Fig. 1. Fourteen days old culture on the surface of PDA

Even after incubation of one month in PDA, no ascospores were observed. The nucleotide sequence of the isolate 83-02-05 matched with 97% similarity, with the highest total score of 983 and 100% query coverage to the existing *Neurospora ugadawe* type material (accession MH 862254) in the GenBank published by Vu *et al*. [19]. GenBank accession number of the ELF sample was MW161276.

Screening bioactivities of *Neurosepora ugadawe*

Free radical scavenging property: Antioxidant activity of the EtOAc extract of *N. ugadawe* (83-02-05) was determined in the ABTS assay. The standard, BHT showed a higher activity in the assay with IC_{50} at 4.50± 0.20 µg/mL than that of the crude extract with IC_{50} at 43.33± 1.25 µg/mL. The IC_{50} values were obtained for ABTS free radical using GraphPad Prism 7.0 software and IC_{50} values are presented in Table-1.

Anti-inflammatory assay: Anti-inflammatory activity of crude extract was determined using HRBCM stabilization method described by Leelaprakash & Dass [20] because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may well stabilize lysosomal membranes. The effect of the crude extract in inhibiting heat induced haemolysis at different concentrations (0.0, 2.84, 5.68, 11.36, 22.72, 45.45, 9.90 and 181.81 µg/mL) were plotted % cell membrane stability *vs.* concentration. The IC_{50}

TABLE-1
SUMMARY OF BIOACTIVITIES OF CRUDE EXTRACTS OF THE ENDOLICHENIC FUNGAL STRAIN OF *Neurospora ugadawe*

	ABTS assay (µg/mL) IC_{50}	Inflammatory assay, 50% cells stability dose (µg/mL)	Tyrosinase assay, max % inhibition for the highest dose (666.6 µg/mL)	Antibacterial assay Inhibition zone (mm) for highest dose 5 mg/mL			
				E. coli	*S. aureus*	*B. subtilis*	*S. mutans*
Neurospora ugadawe	43.33 ± 1.25	130.00 ± 0.81	7.00	–	–	–	–
Positive control	4.50 ± 0.20 (BHT)	24.16 ± 0.41 (Aspirin)	69.51 (Kojic acid)	26 (Azithromycin)	24 (Azithromycin)	25 (Azithromycin)	25 (Chlorohexidine)

value was obtained using GraphPad Prism 7.0 software and is presented in Table-1.

The anti-inflammatory activity of *N. ugadawe* (83-02-05) determined using HRBS method and the activity was compared with the anti-inflammatory drug, aspirin. According to the results, the IC_{50} for EtOAc extract was 130.00 ± 0.81 µg/mL suggesting a lower activity in the assay than that of aspirin with IC_{50} value of 24.16 ± 0.41 µg/mL showing a moderate activity.

Tyrosinase inhibitory assay: Tyrosinase inhibitory assay has been used as a method to study *in vitro* inhibitory activity of crude extract of *N. ugadawe* (83-02-05). The assay was carried out for different doses of extracts (10.41, 20.83, 41.66, 83.33, 166.60, 333.30, 666.60 µg/mL). The percentage inhibitions *versus* test concentrations were plotted in order to compare the inhibitory activity of *N. ugadawe* (83-02-05) crude with the standard compound Kojic acid.

The IC_{50} value was not calculated for *Neurospora ugadawe* (83-02-05) in this assay, since the maximum percentage inhibition value of crude extract didn't exceed 50%. Therefore, the maximum percentage values were used to compare the activity of *N. ugadawe* (83-02-05). Kojic acid was used as the positive control and maximum percentage inhibition was 69.51%. Maximum percentage inhibition was 7% for *N. ugadawe* (83-02-05), which is not a good activity compared to the positive control. A summary of activities is presented in Table-1.

Antibacterial activities: Antibacterial assay was performed for crude extract of *N. ugadawe* (83-02-05) against four bacterial species, *Escherichia coli*, *Estafilococos aureus*, *Billus subtilis* and *Estafilococos mutans.* Azithromycin was used as the positive control for bacterial species *E. coli*, *S. aureus* and *B. subtilis* whereas chlorohexidin was used as the positive control for *S. mutans.* Inhibition zone of the highest dose (5 mg/mL) of azithromycin for each test bacteria was 26 mm, 24 mm and 25 mm, respectively. Inhibition zone for the highest dose (5 mg/mL) of chlorohexidine was 25 mm for *S. mutans.* The exctracts of *N. ugadawe* did not show any activity against test bacteria (Table-1).

Biological activities of Neurosporalol 1 and 2

ABTS radical scavenging ability: Two pure compounds, Neurosporalol 1 and 2 of *Neurosepora ugadawe* (83-02-05) showed remarkable activities in ABTS assay and their IC_{50} values were 3.48 ± 0.33 µg/mL and 5.03 ± 0.15 µg/mL. Activity of Neurosporalol 2 was higher than that of the standard BHT (IC_{50} = 4.50 ± 0.20 µg/mL). Fig. 2 shows the ABTS radical scavenging activities of Neurosporalol 1 and 2 with BHT.

Anti-inflammatory activity: Pure Neurosporalol 2 showed the highest anti-inflammatory activity compared with Neurosporalol 1 (Fig. 3). Its IC_{50} was 129.03 ± 0.15 µg/mL. IC_{50} of Neurosporalol 1 was not calculated because its maximum % inhibition was less than 50%. Activity of Neurosporalol 2 did not show a significant value compared with the positive control aspirin (IC_{50} = 24.16 ± 0.41 µg/mL), even though it showed higher activity than Neurosporalol 1. Neurosporalol 2 had high antioxidant activity and moderate anti-inflammatory activity.

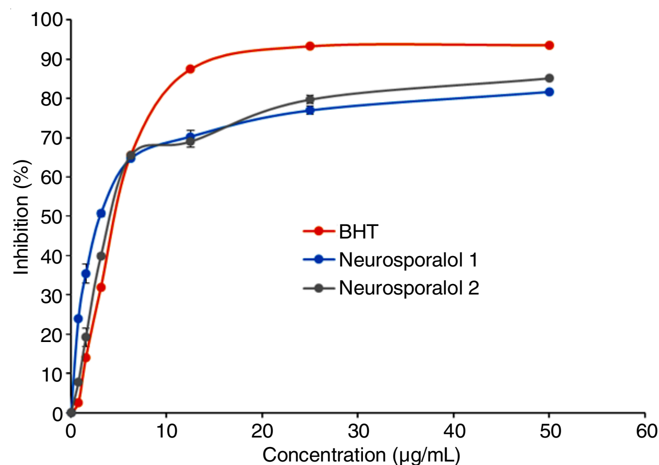

Fig. 2. ABTS radical scavenging activities of Neurosporalol 1 and 2 with BHT

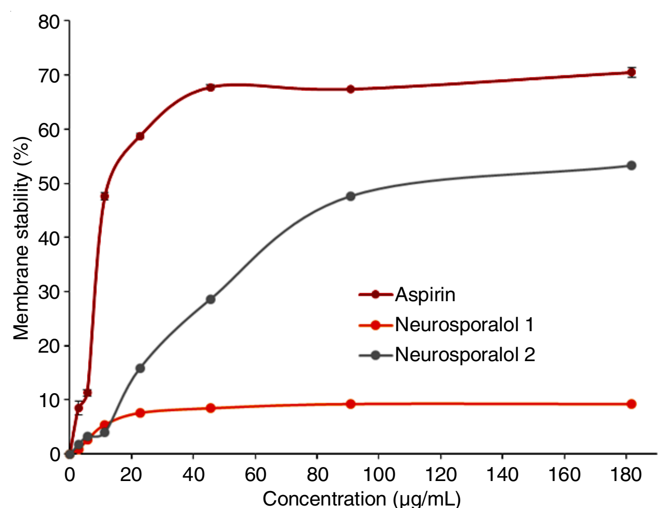

Fig. 3. Comparison of anti-inflammatory activities of Neurosporalol of 1 and 2 with aspirin

Structure elucidation of pure Neurosporalol 1 and 2 isolated from *Neurospora ugadawe*

Structure elucidation of Neurosporalol 1: Bioassay-guided fractionation of EtOAc extract of *N. ugadawe* involving solvent-solvent partitioning, silica gel column chromatography and preparative TLC furnished Neurosporalol 1 and 2. Neurosporalol 1 was obtained as a pale yellow solid. Its molecular formula, $C_{12}H_{16}O_4$, with five degrees of unsaturation, was determined by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) (*m/z* 225.1107 ([M + 1]; calcd. 224.1047). Its IR spectrum had absorption bands at 3645 (very broad peak), 1598, 1492 and 1045 cm^{-1}, suggesting the presence of OH groups, CO single bond and aromatic ring. Absorption bands at 200 and 254 nm in its UV spectrum were indicative of an aromatic ring.

^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data is shown in Table-2. The presence of an aromatic ring with three Hs coupled with each other in Neurosporalol 1 was supported by its ^{1}H NMR signals (Fig. 4), at δ_H 6.57 (d, 8 Hz), 7.00 (t, 8 Hz) and 6.57 (d, 8 Hz) and the ^{13}C NMR signals for six aromatic carbons (δ_c 111.64, 113.48, 124.70, 128.77,

TABLE-2
^{1}H AND ^{13}C NMR DATA OF THE NEUROSPORALOL 1

Position	δ ^{13}C	δ ^{1}H (multiplicity, nH, J/Hz)	DEPT	HMBC
1	17.90	1.08 (d, 3H, 5)	CH_3	70.64, 72.11
2	39.05	1.72 (m, 2H)	CH_2	70.64, 81.24,144.26
3	69.96	4.95 (d, H, 5)	CH_2	151.4, 124.7
		4.85 (d, H, 5)		151.4, 124.7
4	70.64	3.52 (m, H)	CH	39.5, 72.11
5	72.11	3.61 (m, H)	CH	39.05
6	81.24	5.31 (d, H, 5)	CH	
7	111.64	6.57 (d, H, 8)		124.70,151.44
8	113.48	6.55 (d, H, 8)		81.24, 111.64
9	124.70 (quaternary)			
10	128.77	7.00, (t, H,8)	CH	124.70, 144.26
11	144.26 (quaternary)			
12	151.44 (quaternary)			

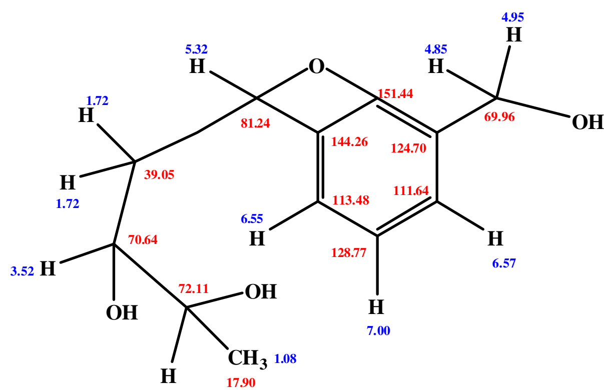

Fig. 4. The structure of Neurosporalol 1

144.26 and 151.44 ppm). An analysis of ^{13}C NMR spectrum of Neurosporalol 1 with the help of HSQC data revealed the presence of one methyl carbon (δ 17.9 ppm), two methylene carbons (δ 69.9 and 39.0 ppm) of which one is oxygenated, three methine carbons connected to oxygen (δ 72.1, 70.6 and 80.2 ppm) and six aromatic carbons (δ 151.4, 144.3, 128.8, 124.7, 111.64 and 113.48 ppm) of which three are quaternary carbons and other three carbons are CH groups. The ^{1}H-^{1}H correlations observed in the DQF-COSY spectrum (Fig. 5) suggested the presence of CH CH CH and CH CH_2 CH CH spin systems and it was also revealed that δ_H 5.32 ppm (d) coupled with protons at δ_H 1.72 ppm (m) indicating these protons are next to each other. The ^{1}H-^{1}H correlations observed in the DQF-COSY and ^{13}C NMR further indicated that two Hs of the CH_2OH (δ_H

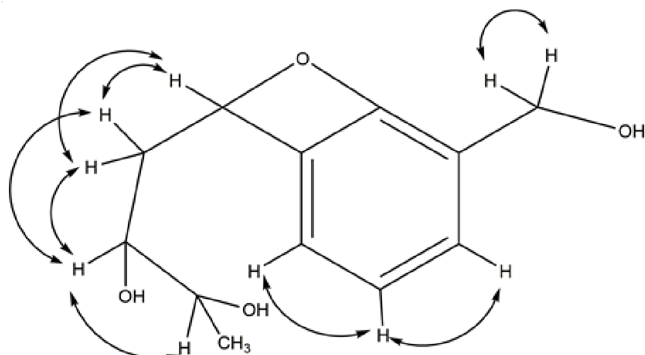

Fig. 5. $^{1}H^{1}H$ COSY correlations of Neurosporalol 1

4.85 and 4.95 ppm) are non-equivalent methylene protons connected to a *C*-bearing an oxygen atom.

In HMBC spectrum (Fig. 6), the aromatic protons showed correlations at δ_H 7.00 ppm with δ_c 144.3 and 124.7 ppm; δ_H 6.55 ppm with δ_c 80.2 and 111.6 ppm; δ_H 6.57 ppm with two quaternary aromatic carbons at δ_c 124.7 and 151.4 ppm indicating aromatic ring of Neurosporalol 1 is connected to both side chains as given in Fig. 3. The presence HMBC correlations from protons at δ 4.85 and 4.95 ppm with two aromatic carbons at δ_c 124.7 and 151.4 ppm unambiguously connected the CH_2OH group to C at δ_c 124.7 ppm. The HMBC correlations from protons at δ_H 1.08 to δ_c 72.1 and 70.6 ppm; fromδ_H 1.72 (d) to δ_c 70.6, 81.2 and 144.3 ppm; from δ_H 3.52 to δ_c 39.1 and 72.1 ppm; and δ_H 3.61 to δ_c 70.64 ppm constructed the side chain with 5 carbon atoms as given in Neurosporalol 1. The structure of Neurosporalol 1 was thus elucidated as 1-(5-(hydroxymethyl)-7-oxabicyclo[4.2.0]octa-1,3,5-trien-8-yl)butane-2,3-diol. This is the first report of isolation of a Neurosporalol 1 from a natural source.

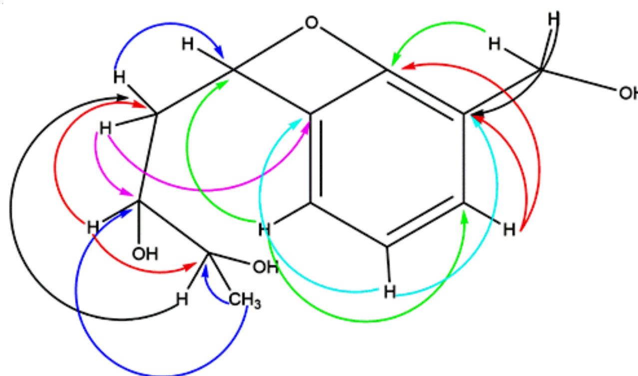

Fig. 6. HMBC correlations of Neurosporalol 1

Structure elucidation of Neurosporalol 2: Neurosporalol 2 was isolated as a white solid and determined to have the molecular formula, $C_{24}H_{28}O_6$ with 11 degrees of unsaturation by high resolution electrospray ionization mass spectrometry (HR-ESI-MS) (*m/z* 413.1974 ([M + 1]; calcd. 412.1886) with ^{1}H NMR (500 MHz, CD_3OD), ^{13}C NMR (125 MHz, CD_3OD) data. Its UV spectrum exhibited peaks at 360 nm, characteristic of conju-

gated double bonds, and its IR absorption bands at 3450 (broad peak), 1580 and 1272 cm^{-1} suggested the presence of OH, conjugated double bonds and C=O single bonds.

The ^{13}C NMR (Fig. 7) spectrum of Neurosporalol 2 (Table-3) when analyzed with the help of HSQC and DEPT confirmed the presence of seven CH groups, of which two were oxygenated (δ_C 70.3, 76.6) and four protonated olefinic carbons δ_C 113.9, 117.2, 128.3, 129.2 and 131.1 ppm), one oxygenated CH_2 group (δ_C 55.1 ppm), three quaternary carbon atoms (δ_C 156.9, 138.2 and 123.8 ppm) and methyl carbon (δ_C 17.5 ppm). The ^1H NMR spectrum of Neurosporalol 2 when analyzed with the help of DQF-$^1H^1H$ COSY (Fig. 8) suggested the presence two spin systems with $CH_3CHCHCHCH$ and CHCHCH. That also indicated occurrence of five olefinic protons, of which three protons at δ_H 6.75 (H, d, 10 Hz), δ_H 7.05 (H, dd 10, 2.5 Hz), δ_H 7.10 (H, t, 10 Hz) couple to each other with *cis*-isomerism and sandwiched in between two quaternary carbons C-6 and C-10 (138.2 and 156.9 ppm). The presence of $CH_3CHCHCHCH$ moiety is supported by $^1H^1H$ COSY spectrum and coupling constants (J = 12.5 Hz) as two protons attached to the olefinic carbons C-5 and C-6 (δ_C 131.1 and 117.2 ppm) should be *trans*-geometry. Further in its HMBC spectrum (Fig. 6), the proton at δ 6.25 (H-4) showed HMBC correlations with C-3 (δ 76.6 ppm) and C-6 (δ 138.2 ppm), unambiguously placed the CH=CH in

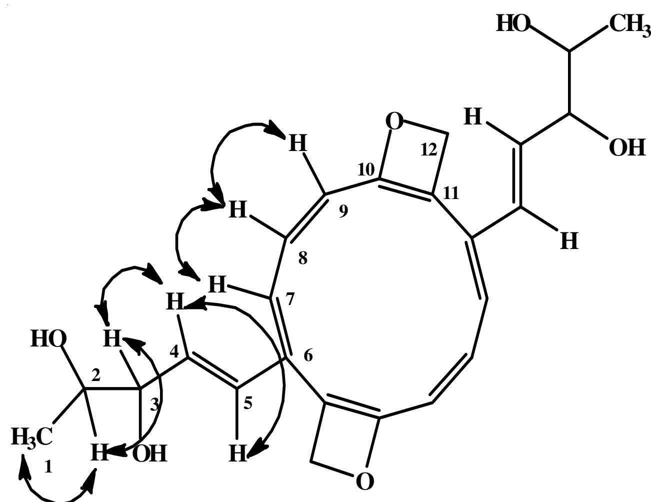

Fig. 8. ^1H-^1H COSY correlations of Neurosporalol 2

between C-3 and C-6. The cross-peaks between δ_H 1.22 (H-1) and δ_C 76.6 (C-2); δ_H 3.80 (H-1) and δ_C 117.2 (C-5); δ_H 4.10 (H-10) and δ_C 131.1 (C- 4); and δ_H 7.00 (H-5) and δ_C 138.2 (C-6) in the HMBC spectrum (Fig. 9) and the ^1H & ^{13}C NMR data for the remainder of the molecule established the presence

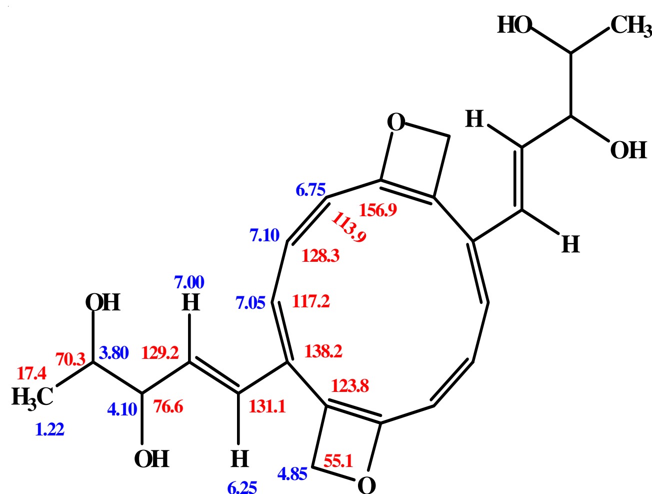

Fig. 7. The structure of Neurosporalol 2

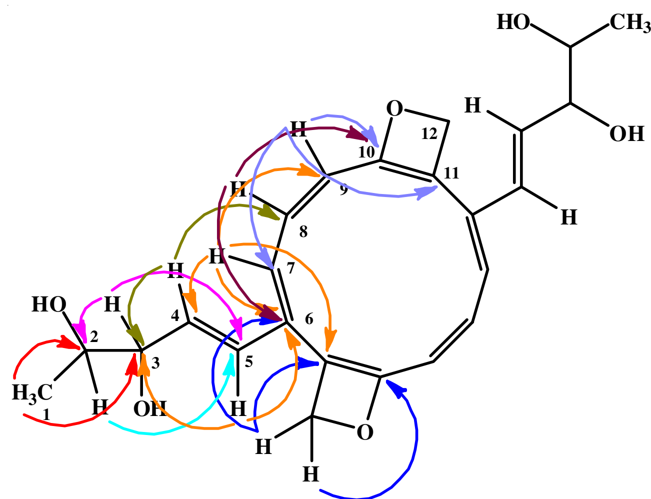

Fig. 9. Selected HMBC correlations of Neurosporalol 2

TABLE-3
^1H AND ^{13}C NMR DATA OF THE NEUROSPORALOL 2

Position	δ ^{13}C	δ ^1H (multiplicity, nH, J/Hz)	DEPT	HMBC
1	17.4	1.22 (3H, d, 5)	CH_3	70.3, 76.6
2	70.3	3.80 (H, m)	CH	131.1
3	76.6	4.10 (H, m)	CH	131.1, 70.3,
4	131.1	6.25 (H, dd, 12.5, 5)	CH	76.6, 138.2
5	117.2	7.05 (H, dd, 10, 2.5)	CH	138.2, 129.2, 123.8, 113.9
6	138.2 (quaternary)			
7	113.9	6.75 (H, d, 10)	CH	117.2, 123.8, 156.9
8	129.2	7.00 (H, d, 12.5)	CH	76.6, 117.2
9	128.3	7.10 (H, t, 10)	CH	138.2, 156.9
10	123.8 (quaternary)			
11	156.9 (quaternary)			
12	55.1	4.85 (2H, s)	CH_2	124.0, 138.2, 156.9

of partial structure $CH_3CHOHCHOHCHCH$ in Neurosporalol 2 (Fig. 6). The proton at δ 4.85 ppm (s) showed HMBC correlations with C-6 (δ 138.3 ppm); C-10 (δ_C 156.9 ppm) and connected to a oxygenated carbon atom C-12 (δ_C 55.1 ppm), placing this proton at C-12 in four membered ring.

On the basis of its HRMS, ^{13}C & ^{1}H NMR data, the carbon skeleton of Neurosporalol 2 was confirmed as a dimer and thus the structure of this Neurosporalol 2 was assigned as (4*E*,4′*E*)-5,5′-((2*Z*,4*Z*,10*Z*,12*Z*)-7,15-dioxatricyclo[12.2.0. 06,9]-hexadeca1(14),2,4,6(9),10,12-hexaene-2,10-diyl)*bis*-(pent-4-ene-2,3-diol).

Conclusion

Graphis tsunodae Zahlbr., a novel Lichen record for Sri Lanka confirmed by molecular identification was used for the isolation of *Neurospora ugadawe.* Two new compounds isolated and identified as Neurosporalol 1 and Neurosporalol 2 from *N. ugadawe.* Neurosporalol 1 showed remarkable antioxidant activity in ABTS assay and its IC_{50} value was 3.48 ± 0.33 µg/mL. That activity is higher than standard BHT (IC_{50} = 4.50 ± 0.20 µg/mL) but anti-inflammatory activity of this compound was very poor compared with positive control aspirin. Neurosporalol 2 showed comparable antioxidant activity (IC_{50} = 5.03 ± 0.15 µg/mL) in antioxidant assay compared with positive control BHT (IC_{50} = 4.50 ± 0.20 µg/mL). It also showed moderate anti-inflammatory activity (IC_{50} = 129.03 ± 0.15 µg/mL) compared with positive control aspirin (IC_{50} = 24.16±0.41 µg/mL).

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CONFLICT OF INTEREST

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

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