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Antarvedisides A-B from Manglicolous Lichen *Dirinaria consimilis* (Stirton) and their Pharmacological Profile

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The chemical examination of acetone extract of *Dirinaria consimilis* resulted in isolation of six depsides of which two novel metabolites namely antarvediside A **(1)** and antarvediside B **(2)** and four known metabolites *i.e.* sekikaic acid **(3)**, atranorin **(4)**, divaricatic acid **(5)** and 2'-*O*-methyl divaricatic acid **(6)**. From the pharmacological screening of the isolates **(1-6)**, it was found that **1** and **2** exhibited better inhibition of ABTS and superoxide free radicals than that of the standard and compound **4** showed significant inhibition of protein denaturation with IC_{50} value of 390 mg/mL with respect to indomethacin with 110 mg/mL. From the SRB assay results, the better IC_{50} values was determined by compound **2** of 10.5, 11.50 and 12.50 µg/mL on HeLa, MCF-7 and FADU cancer cell lines, respectively. Thus, the outcomes revealed that the *D. consimilis* is a new source to treat free radicals, inflammation and cancer.

Keywords: Antioxidant, Protein denaturation, Inflammation, Cancer, Sulforhodamine B assay.

INTRODUCTION

Dirinaria consimilis (Stirton) D.D. Awasthi is a foliose lichen belongs to the *Dirinaria* genus and the chemical tests of thalli of *D. consimilis* depicted the existence of mainly sekikaic acid and traces of 3β-acetoxyhopane-1β,22-diol, 4-*O*-demethylsekikaic acid, atranorin, chloroatranorin, homosekikaic acid, whereas the thallus of other species of *Dirinaria* (*D. applanata*) contains atranorin and divaricatic acid. In 2009, a non-reducing polyketide synthase gene have been isolated and characterized from *D. applanata* [1]. The LC-HRESIMS analysis of methanolic extract of *D. applanata* noticed to have ellagic acid 3,3'-di-*O*-methyl ether, 5,5'-dehydrodiferulic acid, 3,3',4-tri-*O*-methylellagic acid, 5'-methoxydehydrodiconiferyl alcohol, gaigrandin, terpecurcumin Q, ergosteryl acetate, taxuspine C and dichrostachine F [2].

In 2013, studies related to polycylic aromatic hydrocarbon analysis of *Dirinaria picta* revealed the presence of naphthalene, acenaphthylene, acenaphthene, anthracene, phenanthrene, fluoranthene, pyrene, benzo anthracene and chrysene [3]. The metabolic profile using GC-MS, LC-QTOF and MS/MS analysis of *D. picta* showed existence of 5-methyluridine, ethyl syringate, δ-guanidinovaleric acid, orsellinic acid, ethyl ester, 3,4-dihydroxyphenylvaleric acid, usnic acid, ethyl everninate, 2-*O*-methylatranorin, carbamic acid, propionic acid, glycolic acid, 1,3-hydroxy-3-methylglutaric acid, ribonic acid, gluconic acid, β-amyrone and various sugar and amino acid moieties [4].

Earlier, the methanolic extract of *D. applanata* exhibited good antioxidant, antimicrobial, cytotoxic and larvicidal effects with good content of phenols [1,2], while dichloromethane and methanolic extracts of *D. confluens* showed only larvicidal and antimicrobial activities [3]. In 2016, the pharmacological studies on *D. aspera* showed activity against B16-F10 melanoma and UACC-62 cells and 3T3 normal cells [4]. In 2015, the biological studies of methanolic extract from the *D. consimilis* showed significant inhibitory activities against different bacterial and fungal stains [5]. In 2016, the *in vitro* anticaries activity of ethanolic extract of *D. consimilis* showed moderate activity against *Streptococcus mutans* [5]. Earlier, our group has reported the anti-inflammatory and cytotoxicity studies of different extracts of *D. consimilis* [6,7].

Keeping in mind of the aforementioned the chemical and biological profile of *D. consimilis* as well as *Dirinaria* genus,

we have established complete chemical and pharmacological profile of manglicolous lichen (lichen associated particularly on mangrove plants [8] *D. consimilis*.

The structures of novel and known depsides from *Dirinaria consimilis* are shown in Fig. 1.

EXPERIMENTAL

The specimens of manglicolous lichen *Dirinaria consimilis* (Stirton) was collected on the bark of mangrove plant *Excoecaria agallocha* from Vainateya Island, Godavari estuary, Andhra Pradesh, India (16°44'48"N and 81°98'19"E with 0 m elevation) in February, 2015 [8]. This species were authenticated by Dr. D.K. Upreti, CSIR-National Botanical Research Institute (NBRI), Lucknow and deposited at Lichen herbarium, CSIR-NBRI, Lucknow, India with accession numbers 15-027173.

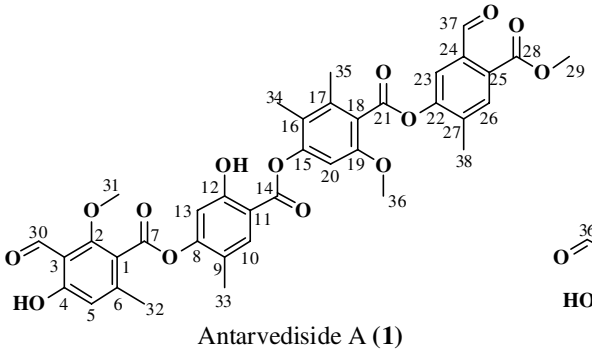
Extraction and isolation: The lichen specimen were gently collected from the bark of *E. agallocha* and shade dried. The dried lichen material (50 g) was powdered, suspended in acetone for 1 week and evaporated under reduced pressure and dried over anhydrous sodium sulphate and concentrated to obtain dry acetone extract of *D. consimilis* (DC-Ac, 1.44 g). The DC-Ac was subjected to column chromatography by using silica gel (#230-400) resulted in four bioactive fractions. Fraction I (100 mg) was obtained as a pale pink colour solid and re-chromatographed over silica gel (#230-400) using hexane and dichloromethane, which yields compound **1** (8 mg) as white solid and compound **2** (19 mg) as a pale pink colour substance, respectively. Fraction II (90 mg) was obtained as a white solid, which on purification by column chromatography (#230-400) using DCM in ethyl acetate as eluent to obtain compound **3** (69 mg) as white solid. Fraction III (281 mg) was white solid material is subjected to column chromatography (#230-400) using DCM in ethyl acetate as eluent to obtain compound **4** (90 mg) as colourless needles and compound **5** (162 mg) as a white solid, respectively. Fraction IV (20 mg) was white solid material is subjected to column chromatography (#230-400) using hexane in ethyl acetate as eluent to obtain compound **6** (11 mg) as colourless solid.

Antarvediside A (1): $C_{38}H_{34}O_{14}$; Yield: 8 mg (0.56 %); R_f: 0.7 (Hexane:DCM, 9:1); m.p.: 191-192 °C; IR (KBr, ν_{max}, cm^{-1}): 576.98, 702.11, 824.89, 1023.35, 1083.37, 1204.44, 1273.79, 1356.55, 1395.78, 1456.68, 1519.46, 1646.32, 2928.97, 3426.57, 3747.67, 3858.20; UV (Ethanol) λ_{max} (log ε) 222 nm; CHNS analysis: anal. C-63.76, H-4.82 (%), calcd. C, 63.86, H, 4.80 (%); ESI-MS $[MH]^+$ *m/z*: 715.00.

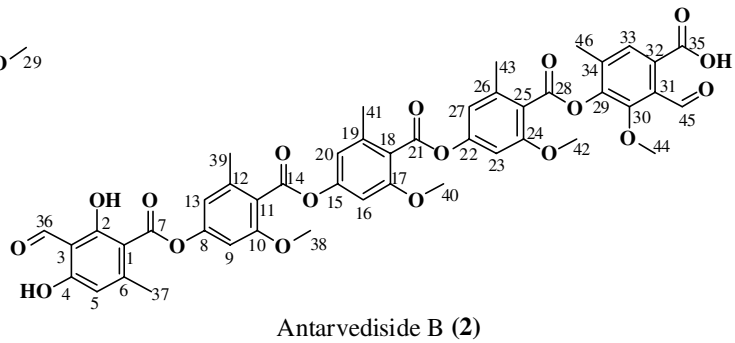
Antarvediside B (2): $C_{46}H_{40}O_{18}$; Yield: 19 mg (1.32 %); R_f: 0.2 (Hexane:Ethyl acetate, 7:3); m.p.: 203-204 °C; UV (Ethanol) λ_{max} (log ε) 219 nm; CHNS analysis: anal. C-62.76, H-4.54 (%), calcd. C, 62.73, H, 4.58 (%); ESI-MS $[MH]^+$ *m/z*: 881.21.

Sekikaic acid (3): $C_{22}H_{26}O_8$; Yield: 69 mg (4.79 %); R_f: 0.6 (DCM:Ethyl acetate, 1:1); m.p.: 220-221 °C; UV (Methanol) λ_{max} (log ε) 219 nm; IR (KBr, ν_{max}, cm^{-1}): 596.02, 660.91, 727.63, 756.03, 798.43, 861.89, 894.62, 953.06, 1018.59, 1039.09, 1138.46, 1203.15, 1241.04, 1283.58, 1311.71, 1353.07, 1425.20, 1458.95, 1610.97, 1644.68, 1667.87, 2588.20, 2874.03, 2956.55, 3440.69. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.97-1.05 (m, 6H, $2CH_3$), 1.69-1.72 (m, 4H, $2CH_2$), 2.95-3.04 (m, 4H, $2CH_2$), 3.85 (s, 3H, $1OCH_3$), 3.91 (s, 3H, $1OCH_3$), 6.40-6.42 (dd, *J* = 4, 8 Hz, 2H, $2CH_{ar}$), 6.66-6.67 (d, *J* = 3.2 Hz, 1H, $1CH_{ar}$), 6.77-6.78 (d, *J* = 3.8 Hz, 1H, $1CH_{ar}$), 10.39 (s, 1H, 1OH), 11.40 (s, 2H, 2OH); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 14.18, 14.23, 24.84, 25.28, 38.53, 39.18, 55.43, 99.03, 103.60, 108.55, 111.58,116.22, 148.11, 149.53, 154.92, 164.88, 165.33, 166.60, 169.40, 174.34; CHNS analysis: anal. C-63.16, H-6.24 (%), calcd. C, 63.15, H, 6.26 (%); ESI-MS $[MH]^+$ *m/z*: 419.33.

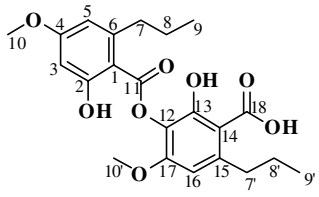
Atranorin (4): $C_{19}H_{18}O_8$; Yield: 90 mg (6.25 %); R_f: 0.4 (DCM:Ethyl acetate, 1:1); m.p.: 196-197 °C; UV (Methanol) λ_{max} (log ε) 210 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.12 (s, 3H, $1CH_3$), 2.57 (s, 3H, $1CH_3$), 2.71 (s, 3H, $1CH_3$), 4.01 (s, 3H, $1OCH_3$), 6.43 (s, 1H, CH_{ar}), 6.54 (s, 1H, CH_{ar}), 10.39 (s, 1H, 1CHO), 11.97 (s, 1H, 1OH), 12.52 (s, 1H, 1OH), 12.58 (s, 1H, 1OH); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 9.37, 24.02, 25.58, 52.34, 102.85, 108.56, 110.27, 112.87, 116.02, 116.08, 139.88, 152.00, 152.44, 162.89, 167.50, 169.10, 169.71,



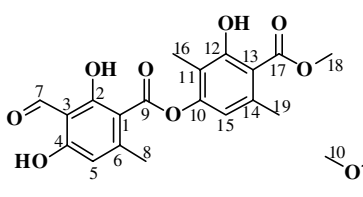
Antarvediside A (**1**)



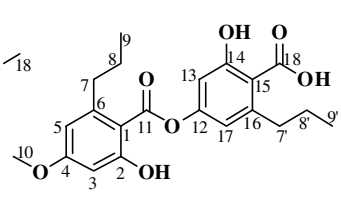
Antarvediside B (**2**)



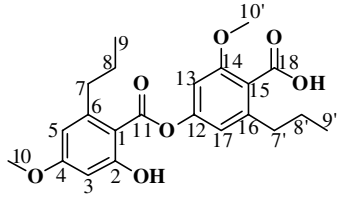
Sekikaic acid (**3**)



Atranorin (**4**)



Divaricatic acid (**5**)



2'-*O*-Methyldivaricatic acid (**6**)

Fig. 1. Structures of novel and known depsides from *Dirinaria consimilis*

172.21, 193.36; CHNS analysis: anal. C-60.76, H-4.74 (%), calcd. C, 60.96, H, 4.85 (%); ESI-MS $[MH]^+$ *m/z*: 375.11.

Divaricatic acid (5): $C_{21}H_{24}O_7$; Yield: 162 mg (11.25 %); R_f: 0.2 (DCM:Ethyl acetate, 1:1); m.p.: 138-139 °C; UV (Ethanol) λ_{max} (log ε) 213 nm; IR (KBr, ν_{max}, cm^{-1}): 590.98 659.01, 727.03, 756.05, 798.33, 861.73, 893.24, 952.35, 1018.91, 1039.69, 1077.04, 1137.74, 1202.80, 1239.87, 1283.83, 1311.80, 1352.84, 1425.59, 1458.56, 1609.55, 1645.87, 1669.94, 2591.35, 2874.04, 2956.45, 3424.84; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.97-1.05 (m, 6H, $2CH_3$), 1.70-1.72 (m, 4H, $2CH_2$), 2.96-3.04 (m, 4H, $2CH_2$), 3.85 (s, 3H, $1OCH_3$), 6.40-6.42 (s, 2H, $2CH_{ar}$), 6.67 (s, 1H, CH_{ar}), 6.78 (s, 1H, CH_{ar}), 11.36 (s, 2H, 2OH); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 14.19, 14.23, 24.83, 25.28, 38.54, 39.18, 55.43, 99.03, 103.58, 108.50, 108.99, 111.59, 116.27, 148.11, 149.62, 154.98, 164.89, 165.35, 166.61, 169.39, 174.67; CHNS analysis: anal. C-64.76, H-6.34 (%), calcd. C, 64.94, H, 6.23 (%); ESI-MS $[MH]^+$ *m/z*: 387.00

2'-*O*-methyldivaricatic acid (6): $C_{22}H_{26}O_7$; Yield: 11 mg (0.76 %); R_f: 0.4 (hexane:ethyl acetate, 1:1); m.p.: 142-143 °C; UV (Ethanol) λ_{max} (log ε) 212.5 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.93-1.05 (m, 6H, $2CH_3$), 1.66-1.80 (m, 4H, $2CH_2$), 2.95-3.04 (m, 4H, $2CH_2$), 3.85 (s, 3H, $1OCH_3$), 3.91 (s, 3H, $1OCH_3$), 6.40 (s, 2H, $2CH_{ar}$), 6.46 (s, 1H, CH_{ar}), 11.18 (s, 1H, 1OH), 11.73 (s, 1H, 1OH); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 14.35, 14.36, 24.84, 25.15, 38.87, 39.10, 55.37, 56.05, 98.89, 104.45, 104.45, 104.65, 106.48, 110.97, 124.94, 147.31, 148.73, 156.40, 156.94, 164.42, 165.55, 168.91, 175.47; CHNS analysis: anal. C-65.66, H-6.62 (%), calcd. C, 65.66, H, 6.51 (%); ESI-MS $[MH]^+$ *m/z*: 403.05.

General procedure for the basic hydrolysis of compounds 1 and 2: The isolate **1** or **2** (2 mg) was dissolved in methanol and 2-3 drops of distilled water and 5 N potassium hydroxide were added to the solution. The reaction mixture was heated to reflux for 2-3 h. The thin layer chromatography confirmed the completion of the reaction and the reaction mass was subjected to gas chromatography-mass spectrometry (GC-MS) analysis.

Antioxidant activity

DPPH assay: The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay [9] is based on the reduction of $DPPH^{\bullet}$ radical in presence of natural/synthetic antioxidant turns to non-radical DPPH (yellow coloured). Initially, 0.004 % DPPH in methanol was prepared and reacted with known concentrations of the extract (50, 100, 150 and 200 µg/mL) and pure compound/standard (25, 50, 75 and 100 µg/mL), incubated for 30 min at 37 °C. Then the absorbance was noted at 517 nm on UV-visible spectrometer and the experiment was triplicated and the obtained optical density values were converted into percentage inhibition using the percentage inhibition formula.

ABTS radical scavenging assay: All the prepared extracts was subjected to 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radical scavenging assay [10]. Firstly, the stock solution was prepared by adding 7 mM of $ABTS^{+\bullet}$ to 2.45 mM potassium persulfate in water at 25 °C and standardized for 16 h. Then to each 1 mL of $ABTS^{+\bullet}$ solution added different concentrations of extract (50, 100, 150 and 200 µg/mL) and pure compound/standard (25, 50, 75 and 100 µg/mL), incubated for 6 min and the absorbance was measured at 750 nm using UV-visible spectrophotometer and the experiment was triplicated and the data was expressed as percentage inhibition.

Superoxide radical scavenging assay: In this radical method [9], the superoxide radicals generated from non-enzymatic phenazine methosulfate/nicotinamide adenine dinucleotide (PMS/NADH) reduces nitro blue tetrazolium (NBT) to a purple formazan. To 1 mL of reaction mixture contained 20 mM phosphate buffer (pH 7.4), 73 µM NADH, 50 µM NBT, 15 µM PMS added various concentrations of extract (50, 100, 150 and 200 µg/mL) and pure compound/standard (25, 50, 75 and 100 µg/mL) and incubated for 10 min at room temperature and the absorbance was noted at 562 nm against blank using UV-visible spectrophotometer and the experiment was triplicated and the data was data was expressed as percentage inhibition.

$$\text{Percentage inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

where A_c is the absorbance of the control, A_s is the absorbance of sample.

A graph is plotted between concentrations of the sample and their percentage inhibition to determine the IC_{50} values of particular sample.

***in vitro* Anti-inflammatory activity:** Protein denaturation method [6] was employed for the evaluation of *in vitro* anti-inflammatory activity for all compounds and extracts in three sets and the mean ± SD values are reported. Bovine serum albumin (BSA) protein was used in this study. The protein was solubilized to 1 % concentration using sodium phosphate buffer (50 mM, pH 6.4). To 0.2 mL of prepared protein, 0.1 mL of sample dissolved in DMSO (Sample size - 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL for sample) was added and final volume adjusted to 5 mL with buffer. Then the sample tubes are incubated at 37 °C for 20 min. Thereafter, the tubes are heated in steam bath at 95 °C for 20 min and then cooled to room temperature. Finally, the turbidity in the cooled tubes are measured at 660 nm by UV-visible spectrophotometer (Model SL 210, Elico India Ltd.). The percentage inhibition of serum albumin protein denaturation was determined as follows and IC_{50} values were calculated by plotting concentration *vs.* percentage inhibition.

$$\text{Percentage inhibition (\%)} = \frac{C - S}{C} \times 100$$

where C is absorbance of control, S is absorbance of sample.

A graph is plotted between concentrations of the sample and their percentage inhibition to determine the IC_{50} values of particular sample.

Anticancer activity

Cancer cell lines: MCF-7 (Breast), DLD-1 (colon), HeLa (cervical), FaDu (head & neck), A549 (lung) and normal human mammary epithelial (NHME) cell lines were kindly provided by National Centre for Cell Science, Pune. The cancer cells were maintained in MEM media (containing 10 % fetal calf serum, 5 % mixture of penicillin (100 units) and streptomycin (100 µg/mL) in presence of 5 % carbon dioxide incubator having 90 % humidity at 37 °C for 72 h.

Cell growth medium: All the cancer cell lines were maintained in minimal essential medium (MEM) (adjusted to 10 % (v/v) FBS, 1.5 g/mL $NaHCO_3$, 0.1 mM MEM non-essential

amino acids and 1 mM sodium pyruvate). Three days prior to performing assay, the cells were washed with sterilized PBS and grown using MEM media (supplemented with 0.25 % trypsin in versene-EDTA and 10 % FBS) and mixed to obtain homogeneous suspension of cells. The suspension was taken in a sterilized polypropylene tube and the cell concentration in each well was determined by hematocytochameter chamber under a microscope using 0.4 % trypan blue solution. The minimal seed density must be 1×10^4 cells per well.

Sample preparation: Initially, for all the extracts (at 100 µg/mL), compounds (at 30 µg/mL) and standard (doxorubicin at 10 µg/mL) were dissolved in DMSO and screened against selected cancer cell lines. The active samples *i.e*. more than 50 % inhibition were further screened at 25, 50, 75 and 100 µg/mL concentrations for extracts; 5, 10, 20 and 30 µg/mL concentrations for pure compounds; 2.5, 5.0, 7.5 and 10 µg/mL concentrations for doxorubicin, hence their IC_{50} values were determined by plotting percentage of inhibition *vs.* concentration. The DMSO were used as a control.

Sulforhodamine B (SRB) colourimetric assay: The SRB assay [7] is based on the estimation of cellular protein content. The samples were taken in 96-well tissue-culture plate and added 190 µL screened ideal cell suspension and mixed occasionally and incubate at 37 °C with 5 % CO_2 and 90 % relative humidity for 3 h. Then add 100 µL cold trichloro acetic acid to each well and incubate at 4 °C for 1 h. After that the plates were gently washed using water, dried using blow dryer and air-dried at room temperature. To each completely dried well, add 100 µL of 0.057 % SRB solution, kept aside for 30 min and quickly rinse with 1 % acetic acid. To the dried plate add 200 µL of 10 mM Tris base (pH 10.5) solution, shake for 5 min and measure the OD at 510 nm. The blank contains only medium while the control has only cancer cells with no test samples. The percentage of growth inhibition was calculated using below formula.

$$\text{Growth inhibition (\%)} = 100 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

RESULTS AND DISCUSSION

The compound **1** was isolated as a white powder with m.p. 191-192 °C and UV absorption at λ_{max} 222 in ethanol. The positive mode of FT-MS-ESI provided at *m/z* 715.00 [MH^+], corresponding to a molecular formula of $C_{38}H_{34}O_{14}$ (calcd. for $C_{38}H_{34}O_{14}$ *m/z* 714.68). Its FT-IR showed for the presence of phenol group (3426.57 cm^{-1}) and carbonyl group (1646.32 cm^{-1}). The ^1H and ^{13}C NMR (Table-1) of **1** indicated the presence of 5 methyl group protons at δ_H 2.09 (*s*, 3H), 2.17 (*s*, 3H), 2.55 (*s*, 3H), 2.62 (*s*, 3H) and 2.69 (*s*, 3H), with carbon signals at δ_C 9.39 (C-34), 23.46 (C-35), 24.05 (C-33), 25.60 (C-32) and 29.73 (C-38), respectively. The 2 phenolic group protons at δ_H 12.50 (*s*, 1H) and 12.55 (*s*, 1H), with carbons at δ_C 156.95 (C-12) and 169.71 (C-4), respectively and 2 aldehyde group protons at δ_H 10.36 (*s*, 1H) and 11.95 (*s*, 1H), with carbon signals at δ_C 182.41 (C-37) and 193.86 (C-30), respectively. The ^1H NMR spectrum also revealed the presence of aromatic protons at δ_H 6.41 (*s*, 2H), 6.52 (*s*, 2H) and 6.70-6.72 (*dd*, 2H), with carbon signals at δ_C 104.13 (C-5), 96.78 (C-13), 152.00 (C-10), 92.08 (C-20), 112.87 (C-23) and 139.89 (C-26). The ^1H NMR spectrum also depicted the existence of 3 methoxy at δ_H 3.90 (s, 3H), 3.93 (s, 3H) and 4.02 (s, 3H) with carbon signals at δ_C 52.36 (C-29), 55.72 (C-31) and 55.67 (C-36), respectively, including 6 carbonyl signals were observed at δ_C 165.82 (C-21), 167.50 (C-7), 169.10 (C-14), 172.22 (C-28), 182.41 (C-37), 193.86 (C-30).

The HMBC correlations (Fig. 2) revealed the relation between methyl protons at 32-H depicted correlation with C-1 & C-6, hence the placement of methyl group was confirmed at C-6 position as there is no possibility at C-1. Similarly, the methyl protons at 38-H also showed cross peaks with C-27 & C-26, which indicates the most possibility of methyl group at C-27 because of depisde correlation observed in C-6. Moreover, the methyl protons at 34-H showed strong cross peaks with C-16 & C-15 which justifies the presence of methyl group at C-16 position due to lack of possibility at C-15 and the 33-H showed cross peaks with C-9, which proves the presence of methyl group at C-9. In addition, the methoxy protons at 29-H depicted correlation with C-28, which indicates the presence of benzoate at C-25. Hence, the HMBC correlations were consistent with a rigid structure for **1**. Furthermore, the complete confirmation is attained by GC-MS analysis of basic hydrolyzed **1**.

The basic hydrolysis of **1** (**Scheme-I**) was analyzed by GC-MS showed four peaks at retention time 10.981, 16.271, 24.024 and 35.166 min, which were corresponding to *m/z* values of 194, 196, 210 and 168 respectively. Hence, from the GC-MS analysis the four components were confirmed as **1a** ($C_{10}H_{10}O_5$, 3-formyl-4-hydroxy-2-methoxy-6-methylbenzoic acid) with *m/z* 210.19 $[M]^+$, **1b** ($C_8H_8O_4$, 2,4-dihydroxy-5-methylbenzoic acid) with *m/z* 168.15 $[M]^+$, **1c** ($C_{10}H_{12}O_4$, 4-hydroxy-6-methoxy-2,3-dimethylbenzoic acid) with *m/z* 196.20 $[M]^+$ and **1d** ($C_{10}H_{10}O_4$,

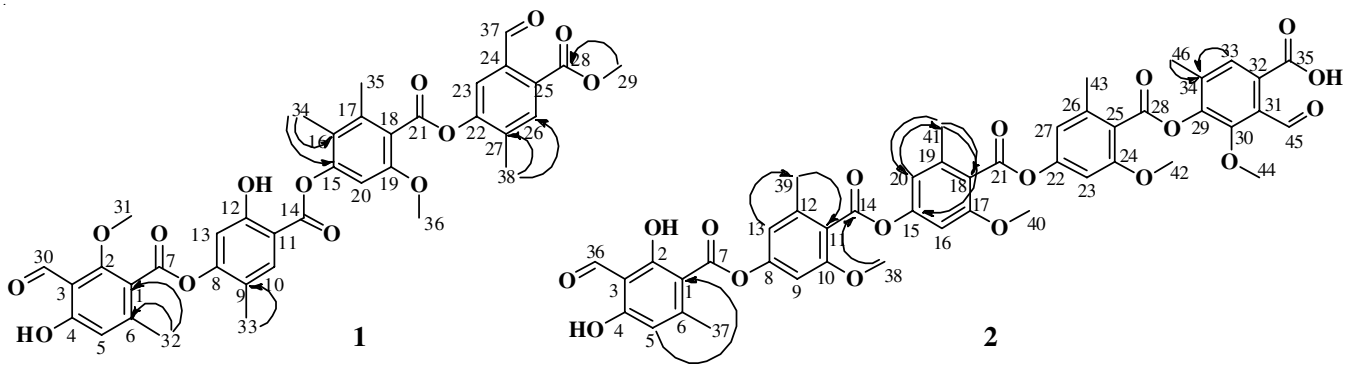


Fig. 2. HMBC correlations of compounds **1** and **2**

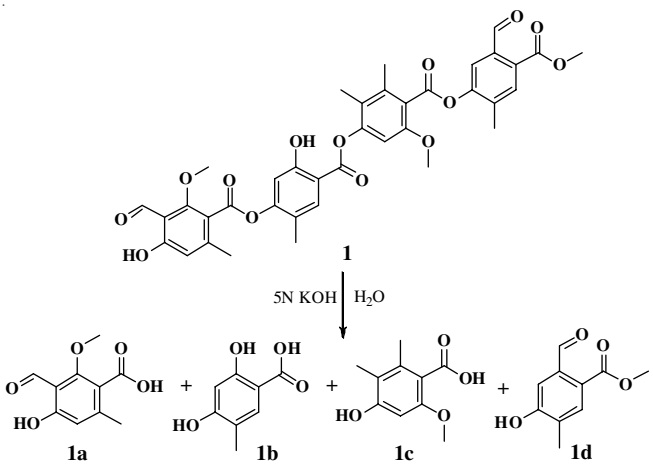
TABLE-1
^{1}H, ^{13}C NMR SPECTRAL DATA AND HMBC CORRELATIONS OF **1** AND **2**

Carbon No.	Compound **1** NMR data (400 MHz, $CDCl_3$)			Compound **2** NMR data (400 MHz, $CDCl_3$)		
	^{1}H	^{13}C	HMBC	^{1}H	^{13}C	HMBC
1	–	102.85	C-6, C-32	–	96.77	C-5
2	–	163.74	–	12.50 (*s*, 1H)	167.49	–
3	–	98.47	–	–	98.47	–
4	12.55 (*s*, 1H)	169.71	–	12.55 (*s*, 1H)	165.82	–
5	6.41 (*s*, 1H)	104.13	–	6.40 (*s*, 1H)	102.12	C-1
6	–	152.45	C-1, C-32	–	152.45	–
7	–	167.50	–	–	169.09	–
8	–	163.77	–	–	156.95	–
9	–	110.28	C-33	6.67-6.69 (*dd*, 1H)	92.07	–
10	6.52 (*s*, 1H)	152.00	–	–	159.42	–
11	–	108.56	–	–	112.86	C-39
12	12.50 (*s*, 1H)	156.95	–	–	130.02	–
13	6.41 (*s*, 1H)	96.78	–	6.30-6.34 (*dd*, 1H)	104.12	C-39
14	–	169.10	–	–	163.77	C-38
15	–	162.89	C-16, C-34	–	156.95	C-41
16	–	116.80	C-15, C-34	6.67-6.69 (*dd*, 1H)	92.07	–
17	–	143.47	–	–	159.42	–
18	–	110.53	–	–	112.86	C-41
19	–	159.42	–	–	130.02	-
20	6.52 (*s*, 1H)	92.08	–	6.30-6.34 (*dd*, 1H)	104.12	C-41
21	–	165.82	–	–	163.77	–
22	–	163.77	–	–	156.95	–
23	6.70-72 (*t*, 1H)	112.87	–	6.67-6.69 (*dd*, 1H)	92.07	–
24	–	112.96	–	–	159.42	–
25	–	115.46	–	–	112.86	–
26	6.70-72 (*t*, 1H)	139.89	C27, C-38	–	130.02	–
27	–	116.03	C-26, C38	6.30-6.34 (*dd*, 1H)	104.12	–
28	–	172.22	C-29	–	162.88	–
29	3.90 (*s*, 3H)	52.36	C-28	–	143.47	–
30	11.95 (*s*, 1H)	193.86	–	–	152.45	–
31	3.93 (*s*, 3H)	55.72	–	–	115.45	–
32	2.62 (*s*, 3H)	25.60	C-1, C-6	–	115.80	–
33	2.55 (*s*, 3H)	24.05	C-9	6.52 (*s*, 1H)	115.28	C-34
34	2.09 (*s*, 3H)	9.39	C-15, C-16	–	116.03	C-33, C-46
35	2.17 (*s*, 3H)	23.46	–	13.39 (*s*, 1H)	167.49	–
36	4.02 (*s*, 3H)	55.67	–	11.95 (*s*, 1H)	193.86	–
37	10.36 (*s*, 1H)	182.41	–	2.55 (*s*, 3H)	29.71	–
38	2.69 (*s*, 3H)	29.73	C-26, C-27	3.87 (*s*, 1H)	52.34	C-14
39	–	–	–	2.85 (*s*, 3H)	23.44	C-11, C-13
40	–	–	–	3.90 (*s*, 3H)	55.64	–
41	–	–	–	2.69 (*s*, 3H)	25.57	C-15, C-18, C-20
42	–	–	–	3.90 (*s*, 3H)	55.70	–
43	–	–	–	2.85 (*s*, 3H)	24.02	–
44	–	–	–	3.99 (*s*, 3H)	60.59	–
45	–	–	–	10.36 (*s*, 1H)	182.42	–
46	–	–	–	2.09 (*s*, 3H)	9.36	C-34

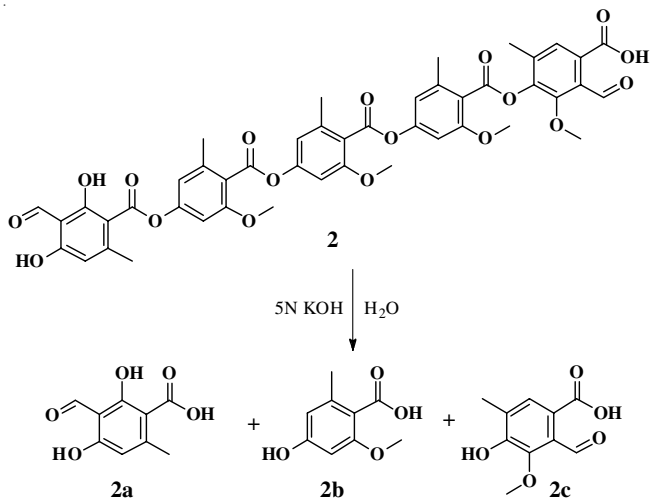
methyl 2-formyl-4-hydroxy-5-methylbenzoate) with *m/z* 194.19 $[M]^+$ in NIST library. Based on the correlations from NMR, Mass, HMBC and GC-MS spectral analysis, the linkage of the components of **1** was established and the structure and absolute configuration of **1** was assigned as 4-((4-((5-formyl-4-(methoxycarbonyl)-2-methylphenoxy)carbonyl)-5-methoxy-2,3-dimethylphenoxy)carbonyl)-5-hydroxy-2-methylphenyl-3-formyl-4-hydroxy-2-methoxy-6-methylbenzoate.

The compound **2** was isolated as a white powder with m.p. 203-204 °C and UV absorption at λ_{max} 219 in ethanol. The positive mode of FT-MS-ESI provided at *m/z* 881.21 $[MH^+]$ (calc. for $C_{46}H_{40}O_{18}$ *m/z* is 880.81), corresponding to a molecular formula of $C_{46}H_{40}O_{18}$.

The ^{1}H and ^{13}C NMR of **2** (Table-1) indicated the presence of 5 methyl group protons at δ_H 2.09 (*s*, 3H), 2.55 (*s*, 3H), 2.69 (*s*, 3H) and 2.85 (*s*, 6H), with carbon signals at δ_C 9.36 (C-46), 29.71 (C-37), 25.57 (C-41), 23.44 (C-39) and 24.02 (C-43). The 2 phenolic protons at δ_H 12.50 (*s*, 1H) and 12.55 (*s*, 1H), with carbon signals at δ_C 167.49 (C-2) and 165.82 (C-4), respectively and 2 aldehyde protons at δ_H 10.36 (*s*, 1H) and 11.95 (*s*, 1H), with carbon signals at δ_C 182.42 (C-45) and 193.86 (C-36), respectively. The 8 aromatic protons at 6.30-6.34 (*dd*, 3H), 6.40 (*s*, 1H), 6.52 (*s*, 1H) and 6.67-6.69 (*dd*, 3H). The 4 methoxy group protons at 3.87 (*s*, 1H), 3.90 (*s*, 6H) and 3.99 (*s*, 3H), with carbon signals at δ_C 52.34 (C-38), 55.64 (C-40), 55.70 (C-42) and 60.59 (C-44), including 7 carbonyl



Scheme-I: Basic hydrolysis of compound **1**



Scheme-II Basic hydrolysis of compound **2**

signals were observed at δ_C 162.88 (C-28), 163.77 (C-14/21), 167.49 (C-35), 169.09 (C-7), 182.42 (C-45) and 193.86 (C-36).

The HMBC (Fig. 2) confirmed the interaction of aryl protons *i.e*. 5-H with C-1 (one-two correlation), 13-H with C-39, 20-H with C-41 and 33-H with C-34, which confirm the presence of aromatic hydrogen at positions C-5, C-13, C-20 and C-33, respectively. The methoxy protons at 38-H showed cross peaks with C-14 *i.e*. one-two-three correlation, hence justifies the presence of methoxy group at C-38. Moreover, the methyl protons at 39-H depicted strong cross peaks with C-11 *i.e*. one-two correlation, which confirms the methyl group at C-12. Furthermore, the methyl protons showed one-three correlation at 41-H with C-15, C-18 and C-20, hence this support the presence of methyl group at C-19. Likewise, strong correlation from methyl protons at 46-H to C-34 was also noticed, which confirms the presence of methyl group at C-34 (Table-1). In addition, the complete confirmation is attained by GC-MS analysis of basic hydrolyzed **2**.

The basic hydrolysis of **2** (**Scheme-II**) was analyzed by GC-MS showed three peaks at retention time 6.141, 21.012 and 61.941 min which were corresponding to *m/z* values of 182, 210 and 196 respectively. Hence, from the GC-MS analysis the three components were confirmed as **2a** ($C_9H_8O_5$, 3-formyl-2,4-dihydroxy-6-methylbenzoic acid) with *m/z* 196.16 $[M]^+$, **2b** ($C_9H_{10}O_4$, 4-hydroxy-2-methoxy-6-methylbenzoic acid) with *m/z* 182.18 $[M]^+$ and **2c** ($C_{10}H_{10}O_5$, 2-formyl-4-hydroxy-3-methoxy-5-methylbenzoic acid) with *m/z* 210.19 $[M]^+$ in NIST library. Based on the correlations from NMR, Mass, HMBC and GC-MS spectral analysis, the linkage of the components of **2** was established and the structure and absolute configuration of **2** was assigned as 2-formyl-4-((4-((4-((4-((3-formyl-2,4-dihydroxy-6-methylbenzoyl)oxy)-2-methoxy-6-methylbenzoyl)oxy)-2-methoxy-6-methylbenzoyl)oxy)-2-methoxy-6-methylbenzoyl)oxy)-3-methoxy-5-methyl benzoic acid.

Antioxidant activity: Generally, natural antioxidants have great forbearance to humans and are sans of considerable side effects [7]. The inferior IC_{50} values indicates superior inhibition of free radicals. From the antioxidant results it is confirmed that the **1, 2** and **6** depicted to have promising antiradical scavenging capacities. In all the antioxidant assays, the **1, 2** and **6** showed markedly higher radical scavenging activity than other compounds.

In DPPH radical scavenging assay [9], the antioxidant capable substances are able to reduce the stable purple coloured DPPH radical to a yellow coloured non-radical DPPH-H form. Generally, the DPPH radical quenching activity of antioxidants are ascribed to their hydrogen donating capacities [9]. As shown in Fig. 3, the IC_{50} value for **1, 2, 3, 4, 5** and **6** on DPPH were calculated to be 30.5, 32.0, 68.0, 53.5, 43.0 and 39.0 µg/mL, respectively, while standard drug (ascorbic acid) with 27.0 µg/mL.

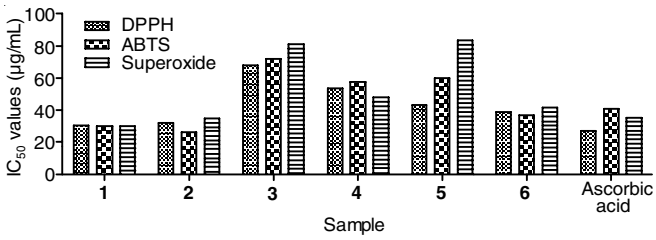


Fig. 3. IC_{50} values of isolates **(1-6)** against DPPH, ABTS and Superoxide free radicals

The principal of ABTS radical assay [10] is similar to that of DPPH assay in which decoy of the radical cation $ABTS^{+\bullet}$ takes place. Among all samples, the isolates **1, 2** and **6** showed better IC_{50} of 30.0, 26.0 and 37.0 µg/mL, respectively, than that of the standard (41.0 µg/mL). The IC_{50} values of **3, 4** and **5** on ABTS radical were noticed to be 72.0, 57.5 and 60.0 µg/mL, respectively (Fig. 3).

The superoxide radical generally arise from metabolic process/ROS, which interact with other substrates in presence of enzyme/metal catalyzed processes to generated hydroxyl radical, H_2O_2 and $^1O_2$. These radicals persuade oxidative damage in DNA, lipids and proteins. From the superoxide free radical assay [9], it was noticed that **1** and **2** showed better IC_{50} than that of the standard. The concentration of **1, 2, 3, 4, 5** and **6** needed for 50 % inhibition of superoxide radical were found to be 30.0, 35.0, 81.0, 48.0, 83.5 and 41.5 µg/mL, respectively, while ascorbic acid was 35.5 µg/mL (Fig. 3). Hence, the higher free radical quenching capabilities of the isolated metabolites may be due to the presence of oxygenated substituents like phenolics and carbonyls in the molecules.

***in vitro* Anti-inflammatory activity:** The denaturation of biological proteins causes inflammation. The denaturation pathways can be performed by acidic (or) alkaline reactions, heat treatment, radiation reactions, *etc*. Proteins lose their complex tertiary structure because of the externally induced stress under the above mentioned conditions thus leading to denaturation. Therefore, in the present work the isolates from *D. consimilis* were tested for inhibition of protein denaturation induced by heat [6]. Among the tested *D. consimilis* compounds, the compound **4** showed better inhibitory profile against protein denaturation with IC_{50} value of 390 µg/mL, which was far better than all other isolates of *D. consimilis*. The IC_{50} values of compounds **1, 2, 3, 5** and **6** on protein denaturation were found to be 878, 600, 632, 922 and 700 µg/mL, respectively, while indomethacin with 110.0 µg/mL (Fig. 4).

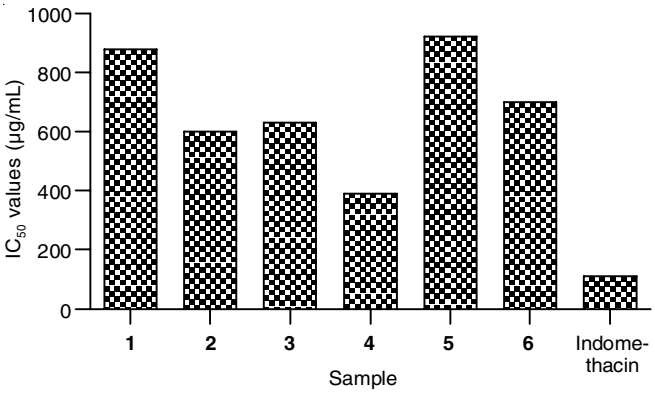


Fig. 4. IC_{50} values of isolates **(1-6)** against protein denaturation

Anticancer activity: As chronic inflammation is crucial in many deadly diseases like cancer. In addition, as the isolated metabolites showed good anti-inflammatory potentialities, we have screened the isolated secondary metabolites **(2-6)** for their anticancer activity by using Sulforhodamine B (SRB) assay [7] and calculated their IC_{50} values. Initially, the compounds **(2-6)** at 30 µg/mL concentration were screened against breast (MCF-7), colon (DLD-1), cervical (HeLa), head & neck (FaDu), lung (A549) and normal human mammary epithelial (NHME) cell lines using SRB assay and standard (doxorubicin, 10 µg/mL) and the percentage of inhibition of cell growth was tabulated in Table-2. Further, the active samples *i.e*. more than 50 % cell death were further screened at 5, 10, 20 and 30 µg/mL concentrations for pure compounds; 2.5, 5.0, 7.5 and 10 µg/mL concentrations for doxorubicin. The results are plotted to obtain IC_{50} values. The lower IC_{50} value indicates better inhibitory profile against cancer cell lines.

During the initial screening, the isolates **2, 3, 5** and **6** depicted prominent degree of specificity against all the tested cancer cell lines. Besides, all the samples exhibited very low degree of specificity against NHME cell lines indicates they are non-toxic towards normal human cells (Table-2). The preliminary screening results revealed that the compound **2** (30 µg/mL) showed better inhibition of cell growth of MCF-7 and HeLa with 83.70 ± 2.40 and 90.90 ± 6.30 % cell growth inhibition, respectively, than the doxorubicin (10 µg/mL) with 81.25 ± 1.56 and 85.55 ± 1.31 % cell death, respectively (Table-2). Whereas, the **3** and **5** showed moderate degree of specificity against tested cancer cell lines. On the other hand, the **6** showed significant inhibitory profile against MCF-7, DLD-1, HeLa, FaDu and A549 (Table-2).

Considering the initial screening, the active samples *i.e*. compounds **2, 3, 5** and **6** were further evaluated of their IC_{50} values and illustrated in Fig. 5. The compound **2** depicted better IC_{50} values of 11.50 µg/mL on MCF-7 with respect to doxorubicin (5.50 µg/mL), whereas compounds **3** and **6** showed IC_{50} value of about 26.50 and 22.50 µg/mL, respectively (Fig. 5). Compounds **2, 3, 5** and **6** revealed significant IC_{50} values of 22.0, 15.50, 15.0 and 17.70 µg/mL, respectively on DLD-1, while doxorubicin with 5.4 µg/mL. Based on the SRB assay results of HeLa, it is observed that the IC_{50} values of tested compounds on HeLa were found to be in the order doxorubicin (4.5 µg/mL) < **2** (10.5 µg/mL) < **3** (16.5 µg/mL) < **5** (17.5 µg/mL) < **6** (18.5 µg/mL) (Fig. 5). Similarly, it is noticed that the concentration of **2, 3, 5** and **6** needed for 50 % inhibition of FaDu were found to be 12.5, 14.5 19.0 and 21.0 µg/mL, respectively, whereas doxorubicin with 3.8 µg/mL (Fig. 5). From the obtained data, it can be confirmed that the only compounds

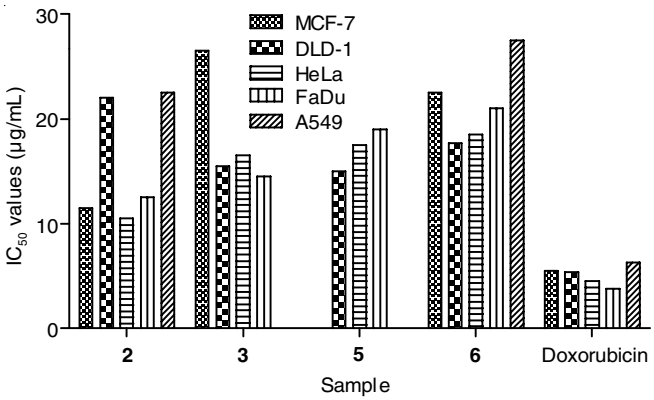


Fig. 5. IC_{50} of isolates **(1-6)** against test panel of cell lines

TABLE-2
PERCENTAGE OF INHIBITION AND IC_{50} VALUES OF ISOLATES **(2-6)** AGAINST SERIES OF CANCER CELL LINES AND NHME CELL LINE

Sample	Percentage inhibition (%) at 30 µg/mL concentration*					
	MCF-7	DLD-1	HeLa	FaDu	A549	NHME
2	83.70 ± 2.40	67.60 ± 7.90	90.90 ± 6.30	89.70 ± 6.80	66.50 ± 3.20	8.07 ± 0.60
3	56.21 ± 1.09	77.19 ± 0.57	75.89 ± 1.77	78.89 ± 5.82	40.75 ± 2.20	7.19 ± 0.45
4	10.91 ± 1.24	7.80 ± 1.15	2.55 ± 0.79	7.01 ± 0.93	8.68 ± 1.53	1.82 ± 0.46
5	44.95 ± 3.42	72.23 ± 3.20	70.17 ± 1.19	74.83 ± 4.01	30.78 ± 3.15	4.51 ± 0.73
6	64.69 ± 5.26	77.21 ± 1.06	73.97 ± 3.44	71.04 ± 2.38	55.82 ± 3.20	6.66 ± 1.33
Doxorubicin**	81.25 ± 1.56	72.67 ± 0.21	85.55 ± 1.31	98.50 ± 1.21	77.92 ± 0.41	1.20 ± 0.81

*n = 3; Mean ± SD; **10 µg/mL

2 and **6** are active against A549 with IC_{50} values of 22.5 and 27.5 µg/mL, respectively, while doxorubicin with 6.3 µg/mL (Fig. 5).

Hence, form the SRB analysis it can be concluded that the key agents responsible for the anticancer activity of *D. consimilis* were **2, 3, 5** and **6.** Furthermore, the low toxicity against NHME indicates that all the compounds are less toxic towards normal human cells. Hence, these active metabolites can be a benchmarks for designing potent anticancer agents.

Conclusion

In conclusion, we have established the chemical and pharmacological profile of manglicolous lichen *D. consimilis* by using standard methods. The chemical examination of the acetone extract revealed the presence of two new metabolites **(1 & 2)** along with four known metabolites **(3-6)**. The pharmacological evaluation of the pure isolated compounds **(1-6)** showed prominent inhibition of DPPH, ABTS, superoxide free radicals, protein denaturation, MCF-7, DLD-1, HeLa, FaDu and A549 cancer cell lines. This study confirms the biological importance of *D. consimilis* as a good source for antioxidants, anti-inflammatory and anticancer agents.

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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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