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Manglicolous lichen *Parmotrema tinctorum* (Despr. ex Nyl.) Hale: Isolation, characterization and biological evaluation

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The chemical examination of the ethanolic extract of manglicolous lichen *Parmotrema tinctorum* **(Pt-Et)** has resulted in isolation of six known metabolites, *i.e.*, methyl-β-orcinolcarboxylate **(1)**, isolecanoric acid **(2)**, methyl-2,6-dihydroxy-4-methylbenzoate **(3)**, haematommic acid **(4)**, ethyl haematommate **(5)**, and atranorin **(6)**. All the isolates **1-6** and **Pt-Et** have been screened against DPPH and superoxide free radicals, six different cancer cell lines (MDA-MB-231, SW620, HeLa, FADU, A549, SKOV3) and one normal human cell line (NHME). Compound **3** exhibits prominent inhibition of superoxide free radical, which appears to be better than that of the standard (ascorbic acid) with an IC_{50} value of 26.0 µg/mL. From the SRB assay results, it is observed that the better IC_{50} values have been obtained from **4** and **5** on HeLa, FADU, and A549, respectively. In addition, all the tested samples show low cell lysis on NHME, which indicates low toxicity. Consequently, the outcomes revealed that *P. tinctorum* could be a new source to treat oxidative stress and cancer. This work is the first report of antioxidant and cytotoxicity studies on the isolated metabolites **1-6**.

Keywords: Manglicolous lichen, toxicity studies, antioxidant, anticancer, Sulforhodamine B assay

A symbiotic organism known as the lichen belongs to the group of epiphytes, which have an aptitude to persist on any substratum or geographical region[1,2]. Manglicolous lichens are one of the types of lichen, which, mainly, survived on the mangroves[2,3]. Throughout ages, lichens are used for the treatment of several diseases due to their unique survival and their bioactive secondary metabolites. Lichens with their secondary metabolites exert a diverse range of pharmacological actions that include analgesic, antibiotic, anti-inflammatory, antimycotic, antipyretic, antiviral, and cytotoxic effects[3,4]. Chiefly, manglicolous lichens display differences in their biological constituents and actions than normal lichens do. It is due to the fact of their physiological adaptation at the intertidal zone (environment in which having both the marine and freshwater)[5]. This led the researchers to gain interest in the biological screening of particular manglicolous lichens. There are very few pharmacological evaluation reports that exist, to date, on the manglicolous lichens[2,4].

As per the general studies, *Parmotrema tinctorum* (Despr. ex Nyl.) Hale belongs to family *Parmeliaceae*, which is the most prominent family of foliose lichen. Pharmacologically, the crude extracts of *P. tinctorum* possess antioxidant, antiglycation, α-amylase, α-glucosidase, aldose reductase, tyrosinase, and carbohydrate digestive enzymes inhibitory activities[3,6]. Earlier the experiment which we had performed has established the phytochemical analysis along with total flavonoid and phenolic contents, antimicrobial, antioxidant and hypoglycaemic and, antihyperglycemic activities of this mangrove associated lichen *P. tinctorum*[3]. In addition, we reported the chemical examination of ethyl acetate extract from *P. tinctorum,* which yielded two known metabolites – atranorin and ethyl everninate[3]. In continuation of our research on chemical and pharmacological evaluation of some Indian mangrove lichens and also based on the aforementioned properties of *P. tinctorum*, we examined by recollecting the *P. tinctorum* specimens (in October 2018) for its chemical and pharmacological properties which are reported on this research paper.

Material and Methods

Collection

The specimens of mangrove associated lichen *Parmotrema tinctorum* (Despr. ex Nyl.) Hale was collected on the twigs of mangrove plant *Excoecaria agallocha* from Bhitharkanika Island, Rajnagar,

Orissa, India (20°74'N and 86°87'E with 0 m elevation) on 18 October 2018. The *P. tinctorum* was authenticated by Dr. D. K. Upreti, CSIR-National Botanical Research Institute (NBRI), Lucknow with accession number 15-027176 had been deposited at Lichen herbarium, CSIR-NBRI, Lucknow, India.

Chemicals

All the chemicals used in the present experiment were of analytical grade. 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, Sulforhodamine B (SRB) was from Sigma Aldrich (Mumbai, India); doxorubicin from the Avantis Pharma Ltd (Mumbai, India).

Extraction of manglicolous lichen material

The lichen specimens were carefully handpicked from the twigs of respective mangrove plants and allowed to shade dry. The dried lichen then (of about 10 g) were powdered, suspended in ethanol for a week, and evaporated under reduced pressure to obtain an ethanolic extract from *P. tinctorum* (**Pt-Et,** 2.10 g, 21%w/w) as a dark blackish solid. The **Pt-Et** (1.5 g) was then subjected to column chromatography by the increasing polarity of hexane and ethyl acetate (as a solvent system, which affords five fractions). Fraction I (90 mg) obtained in 10% hexane in ethyl acetate yielded **1** as colorless crystals (40 mg), fraction II (150 mg) obtained in 20% hexane in ethyl acetate yielded **2** and **3** as a yellowish solid (50 mg) and as pale yellow crystals (75 mg), respectively, fraction III (60 mg) obtained in 30% hexane in ethyl acetate yielded **4** as pale yellow needles (35 mg), fraction IV (160 mg) obtained in 40% hexane in ethyl acetate yielded **5** as a greenish solid (15 mg), and fraction V (150 mg) obtained in 50% hexane in ethyl acetate yielded **6** as colorless crystals (90 mg) (Figure 1).

Antioxidant activity

DPPH assay

The antioxidant activity was assessed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay[7,8] in a triplet. Initially, 100 µL of 100 µM of DPPH in ethanol was prepared and reacted with known concentrations, *i.e.*, 50, 100, 150 and 200 µg/mL for extracts and 25, 50, 75 and 100 µg/mL for the isolated metabolites and standard (ascorbic acid) samples, incubated for 30 mins. The absorbance was noted at 517 nm on the UV-Visible spectrometer (Electron 420 series spectrophotometer). The percentage inhibition was calculated using the below formula. Simultaneously, the IC_{50} was calculated using a linear graph section between percentage inhibition and concentration of the tested sample.

Superoxide radical scavenging assay

In the radical method[8,9], the superoxide radicals generated from non-enzymatic phenazine methosulfate/ Nicotinamide adenine dinucleotide (PMS/NADH) reduces nitro blue tetrazolium (NBT) to purple formazan. To 1 mL of reaction mixture contained 20 mM phosphate buffer (*p*H 7.4), 73 µM NADH, 50 µM NBT, 15 µM PMS added various concentrations, *i.e.*, 50, 100, 150 and 200 µg/mL for extracts and 25, 50, 75 and 100 µg/mL for isolates/ascorbic acid and incubated for 10 min at room temperature and the absorbance was noted at 562 nm against the blank. The experiment was triplicated, and the data were expressed as percentage inhibition.

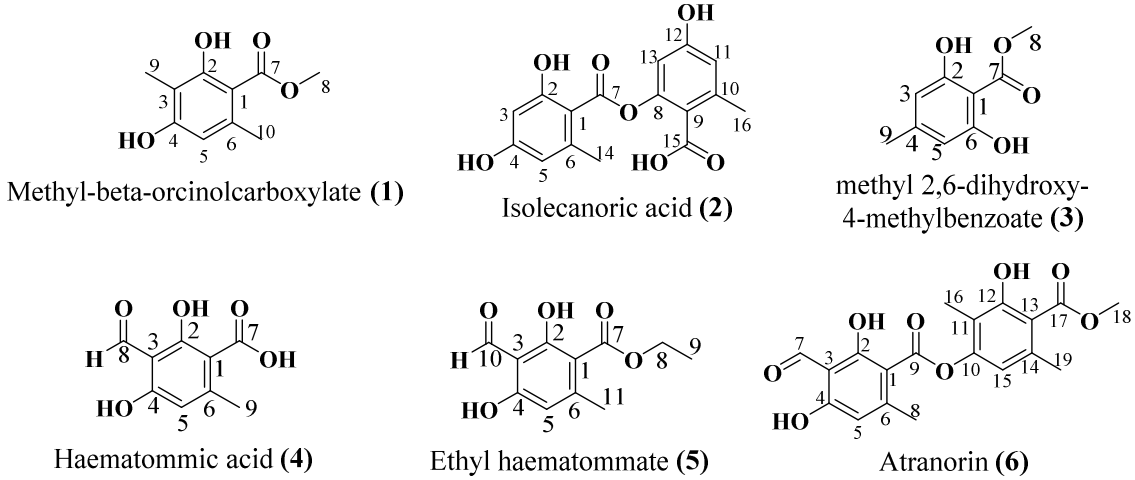

Figure 1 — Secondary metabolites **1-6** isolated from *Parmotrema tinctorum*

Percentage inhibition (%) = $(A_c - A_s)/A_c \times 100$

where A_c is the absorbance of the control.

A_s is the absorbance of sample.

Cytotoxicity assay

Cancer Cell lines

MDA-MB-231 (Breast), SW620 (Colon), HeLa (Cervical), FADU (Head and Neck), A549 (Lung), SKOV3 (Ovary) and Normal Human Mammary Epithelial (NHME) (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 the 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 the 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 a 0.4% trypan blue solution. The minimal seed density must be 1.9×10^4 cells per well.

Sample Preparation

All the crude extracts and standards were dissolved in DMSO to 100 mg/mL and 10 µg/mL, respectively. The doxorubicin and DMSO were used as a standard and control, respectively.

SRB Colorimetric assay

The Sulforhodamine B assay[10,11] is based on the estimation of cellular protein content. The prepared 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 TCA to each well and incubate at 4˚C for 1 h. After that, the plates were gently washed using water, dried using a 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 (*p*H 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 the below formula.

% Growth inhibition = $100 - [(S - B)/(C - B)] \times 100$

Where S is mean OD value of sample

B is mean OD value of blank

C is mean OD value of control

Results and Discussion

Chemistry

Methyl-β-orcinolcarboxylate, 1: Colorless crystals. Mol. Formula: $C_{10}H_{12}O_4$. Yield: 40 mg. R_f: 0.4 (Hexane:ethyl acetate, 1:4); m.p.144-145°C. UV (Ethanol) λ_{max} (log ε) 267 nm; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.25 (3H, s, 10-CH_3), 2.37 (3H, s, 9-CH_3), 3.95 (3H, s, 8-OCH_3), 6.01 (1H, s, 4-OH), 6.19 (1H, s, 5-ArH), 9.61 (1H, s, 2-OH); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 8.74 (C-9), 21.63 (C-10), 52.99 (C-8), 108.36 (C-1), 110.39 (C-3), 111.18 (C-5), 141.36 (C-6), 160.68 (C-2), 162.09 (C-4), 172.19 (C-7); ESI-MS positive mode: *m/z* 197.00 [M+H$^+$]. Calcd for $C_{10}H_{12}O_4$: *m/z* 196.07 (Figure 1). Anal. Found: C, 61.76; H, 6.14. Calcd for: C, 61.22; H, 6.17%.

Isolecanoric acid, 2: Yellowish solid. $C_{16}H_{14}O_7$. Yield: 50 mg. R_f: 0.6 (Hexane:Ethyl acetate, 3:7); m.p.367-368°C. UV (Ethanol) λ_{max} (log ε) 211 nm; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.49 (3H, s, 16-CH_3), 2.51 (3H, s, 14-CH_3), 4.01 (1H, s, 12-OH), 4.06 (1H, s, 4-OH), 4.48 (1H, s, 2-OH), 6.48 (1H, s, 3-ArH), 6.56 (1H, s, 5-ArH), 6.95 (1H, s, 11-ArH), 6.99 (1H, s, 13-ArH), 9.48 (1H, s, 15-COOH); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 21.92 (C-14/C-16), 103.34 (C-3), 106.18 (C-1), 106.58 (C-13), 112.26 (C-5), 115.18 (C-9), 118.01 (C-11), 142.78 (C-10), 144.92 (C-6), 155.31 (C-8), 162.64 (C-12), 163.72 (C-4), 164.75 (C-2), 168.00 (C-7), 171.86 (C-15); ESI-MS positive mode: *m/z* 319.9 [M+H$^+$]. Calcd for $C_{16}H_{14}O_7$: *m/z* 318.07 (Figure 1). Anal. Found: C, 60.71; H, 4.72. Calcd for: C, 60.38; H, 4.43%.

Methyl-2,6-dihydroxy-4-methylbenzoate, 3: Pale yellow crystals. Mol. Formula: $C_9H_{10}O_4$. Yield:

75 mg. R_f: 0.6 (Hexane:Ethyl acetate, 1:1); m.p.138-139°C. UV (Ethanol) λ_{max} (log ε) 219.5 nm; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.23 (3H, s, 9-CH_3), 3.75 (3H, s, 8-OCH_3), 6.12 (2H, d, *J*= 1.2 Hz, 3,5-ArH), 9.93 (1H, s, 6-OH), 10.65 (1H, s, 2-OH); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 22.46 (C-9), 52.16 (C-8), 100.83 (C-1), 107.93 (C-5), 110.58 (C-3), 141.15 (C-4), 161.46 (C-6/C-2), 170.59 (C-7); ESI-MS negative mode: *m/z* 183.0 [M-H$^+$]. Calcd for $C_9H_{10}O_4$: *m/z* 182.06 (Figure 1). Anal. Found: C, 59.66; H, 5.62. Calcd for: C, 59.34; H, 5.53%.

Haematommic acid, 4: Pale yellow needles. Mol. Formula: $C_9H_8O_5$. Yield: 35 mg. R_f: 0.4 (Hexane: Ethyl acetate, 1:1); m.p.172-173°C. UV (Ethanol) λ_{max} (log ε) 219.5 nm; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.54 (3H, s, 9-CH_3), 6.42 (1H, s, 5-ArH), 9.68 (1H, s, 4-OH), 10.59 (1H, s, 2-OH), 11.46 (1H, s, 7-COOH), 13.75 (1H, s, 8-CHO); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 17.10 (C-9), 105.25 (C-1), 109.34 (C-3/C-5), 155.22 (C-6), 163.90 (C-4), 167.04 (C-2), 173.42 (C-7), 191.73 (C-8); ESI-MS negative mode: *m/z* 198.30 [M-H$^+$]. Calcd for $C_9H_8O_5$: *m/z* 196.04 (Figure 1). Anal. Found: C, 55.12; H, 4.02. Calcd for: C, 55.11; H, 4.11%.

Ethyl haematommate, 5: Greenish solid. Mol. Formula: $C_{11}H_{12}O_5$. Yield: 15 mg. R_f: 0.6 (DCM: Ethyl acetate, 7:3); m.p.112-113°C. UV (Ethanol) λ_{max} (log ε) 209.5 nm; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 0.93-0.97 (3H, t, *J*= 8 Hz, 9-CH_3), 1.59-1.65 (2H, m, 8-CH_2), 2.54 (3H, s, 11-CH_3), 6.42 (1H, s, 5-ArH), 9.68 (1H, s, 4-OH), 10.59 (1H, s, 2-OH), 13.75 (1H, s, 10-CHO); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 14.28 (C-11), 20.14 (C-9), 68.63 (C-8), 105.25 (C-1), 109.34 (C-5), 114.78 (C-3), 155.22 (C-6), 163.90 (C-4), 167.04 (C-2), 173.42 (C-7), 191.73 (C-8); ESI-MS [M-H]$^+$: *m/z* 224.9. Calcd for $C_{11}H_{12}O_5$: *m/z* 224.07 (Figure 1). Anal. Found: C, 58.64; H, 5.52. Calcd for: C, 58.93; H, 5.39%.

Atranorin, 6 [3]: Colorless crystals. Mol. Formula: $C_{19}H_{18}O_8$. Yield: 90 mg. R_f: 0.4 (DCM:Ethyl acetate, 1:1) (Figure 1).

Antioxidant activity

In general, natural antioxidants maintain high tolerance to humankind without considerable side effects. The anti-radical assays of all the samples **(1-6** and **Pt-Et)** against DPPH and superoxide radicals were illustrated in Table SI and Table SII, respectively. The inferior IC_{50} values indicate higher inhibition of free radicals. From the antioxidant results, it is confirmed that the **Pt-Et** displayed to have promising anti-radical scavenging capacities. In both the antioxidant assays, the **1-6** and **Pt-Et** showed markedly higher radical scavenging activity.

In DPPH radical scavenging assay, the antioxidant substances can reduce the stable purple-colored DPPH radical to a yellow-colored non-radical DPPH-H form. In general, the DPPH radical quenching activity of antioxidants are assigned to their hydrogen (donating) capacities[9,12,13]. As shown in the Figure 2, the IC_{50} values remained 56.25, 60.0, 40.0, 53.0, 50.5 and 53.5, µg/mL for **1, 2, 3, 4, 5** and **6**, respectively, while standard ascorbic acid continued to exist at 27.0 µg/mL. In addition to that, the IC_{50} values for **Pt-Et** remained 95.0 µg/mL.

The superoxide radical generally shows up from metabolic process/ROS, which interacts with other substrates in the presence of enzyme/metal-catalyzed processes to generate hydroxyl radicals, H_2O_2, and $^1O_2$. These radicals persuade oxidative damage in DNA, lipids, and proteins[9,12,13]. In the case of superoxide radical-quenching assay of all the samples **(1-6** and **Pt-Et)** were tabulated in Table SII. From Figure 2, it was interesting to note that compound **3** showed pronounced inhibition of superoxide free radicals with an IC_{50} value of 26.0 µg/mL than standard (35.5 µg/mL). Compounds **4** and **5** revealed almost equivalent inhibition of superoxide free radical to that of the standard drug ascorbic acid with IC_{50} valuing of about 37.25 and 36.0 µg/mL, respectively. The concentration of **1, 2, 6,** and **Pt-Et,** which needed 50% inhibition of superoxide radicals, was found to be 62.5, 80.0, 48.0, and 99.75 µg/mL. Based on the aforementioned factors, it can be concluded that *P. tinctorum* has an aptitude to act against free radicals.

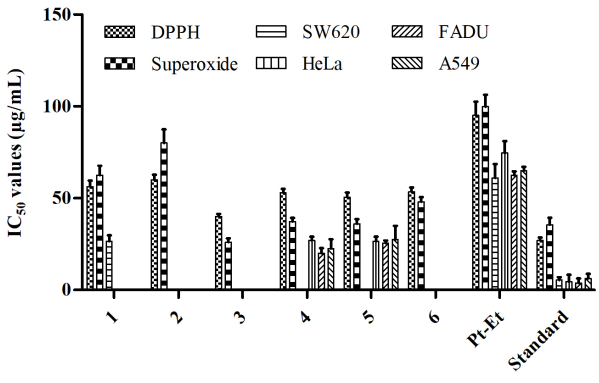

Figure 2 — IC_{50} values of **1-6** and **Pt-Et** against free radicals and tested cancer cell lines

Cytotoxicity studies

The cytotoxicity studies of ethanolic extract of *P. tinctorum* **(Pt-Et)** and its isolates **(1-6)** were screened against six different human cancer cell lines (MDA-MB-231, SW620, HeLa, FADU, A549, SKOV3) by SRB colorimetric assay[10,11] using doxorubicin as standard. The results of percentage growth inhibition were illustrated in Table SIII. Concurrently, the toxicity studies of all the samples **(1-6** and **Pt-Et)** were tested on normal human cell lines such as NHME.

Initially, extracts **Pt-Et** (at 100 µg/mL concentration), isolated metabolites **1-6** (at 30 µg/mL concentration) and doxorubicin (at 10 µg/mL concentration) were screened against selected panel of cancer cell lines. The results were tabulated in Table SIII. During the initial screening, **4, 5,** and **Pt-Et** showed a prominent degree of specificity against HeLa, FADU, and A549 moderate degree of specificity against MDA-MB-231, SW620, and SKOV3. Also, compound **1** showed a significant degree of specificity against SW620. In addition to that, all the samples exhibited a shallow degree of specificity against NHME cell lines, indicating that they are non-toxic against normal human cells (Table SIII). From Table SIII, it is compelling to notice that the **Pt-Et** (100 µg/mL concentration), as well as, the compounds **1** (30 µg/mL concentration) showed more pronounced growth by inhibiting profile against the SW620 than that of the doxorubicin (10 µg/mL concentration). Particularly, **Pt-Et** (100 µg/mL concentration) showed more pronounced growth by inhibiting profile against the A549 than that of the doxorubicin (10 µg/mL concentration).

The active samples, *i.e*., which remain more than 50% cell death were further screened at 25, 50, 75 and 100 µg/mL concentrations to extract **(Pt-Et)**; 5, 10, 20 and 30 µg/mL concentrations as for pure compounds **(1, 4** and **5)**; 2.5, 5.0, 7.5 and 10 µg/mL concentrations for standard (doxorubicin). The obtained results of percentage growth inhibition is plotted against concentrations to obtain IC_{50} values. The lower IC_{50} value indicates a better inhibitory profile against cancer cell lines. From Table SIV and Figure S16, it is evident that the **Pt-Et** showed a significant growth inhibitory profile against SW620 with an IC_{50} value of 61.0 µg/mL (Figure 2). Among the isolates of **Pt-Et,** compound **1** showed better IC_{50} values on SW620 with 26.5 µg/mL, while doxorubicin showed with 5.40 µg/mL (Figure 2).

From the outcomes of the SRB assay of HeLa, it is concluded that **Pt-Et** displayed a reasonable cell death rate of HeLa with an IC_{50} value of 74.5 µg/mL and, the metabolites **4** and **5** showed a reasonable degree of specificity against HeLa when compared to doxorubicin. The concentration needed for **4** and **5** for 50% growth inhibition of HeLa was found to be 27.0 and 26.5 µg/mL, respectively, while doxorubicin to be 4.5 µg/mL (Table SV, Figure S17, and Figure 2). Also, based on the cytotoxicity results of FADU, it is observed that **Pt-Et** revealed a significant degree of specificity against FADU with an IC_{50} value of 62.5 µg/mL (Figure 2). The IC_{50} value of **4** and **5** on FADU was found to be 20.0 and 25.5 µg/mL, respectively, while doxorubicin to be 3.8 µg/mL (Table SVI, Figure S18 and Figure 2).

From Table SVII and Figure S19, it is evident that the **Pt-Et** showed a significant growth inhibitory profile against A549 with an IC_{50} value of 64.9 µg/mL (Figure 2). Among the isolates of **Pt-Et,** compounds **4** and **5** showed better IC_{50} values on A549 with 22.5 and 27.5 µg/mL, respectively, while doxorubicin showed with 6.3 µg/mL (Figure 2). Additionally, from the SRB assay results, it can be concluded that the anticancer capabilities of **Pt-Et** were mainly due to the presence of compounds **1, 4,** and **5.** Also, compounds **4** and **5** showed better cancer cell lysis is due to the presence of higher levels of free oxygenated substituents in their chemical structure. Based on the outcomes, it can be concluded that *P. tinctorum* is a good source for antioxidants and anticancer agents.

Conclusion

To wind up, six known metabolites namely methyl-β-orcinolcarboxylate **(1)**, isolecanoric acid **(2),** methyl-2,6-dihydroxy-4-methylbenzoate **(3)**, haematommic acid **(4),** Ethyl haematommate **(5)** and atranorin **(6)** were isolated from the ethanolic extract of manglicolous lichen *P. tinctorum* **(Pt-Et)**, which were for the first time reported from *P. tinctorum*. From the pharmacological screening of the isolates **(1-6)**, it was found that **3, 4,** and **5** exhibited better inhibition of superoxide free radicals with an IC_{50} value of 26.0, 37.25, and 36.0 mg/mL, respectively. Correspondingly, these compounds **(4** and **5)** revealed a remarkable degree of specificity towards HeLa, FADU and A549 and delicate degree of specificity towards a normal human cell line, in which, it justifies the compounds containing the antioxidant properties have an aptitude to treat cancer. Hence, this study

represents the natural product researchers for further chemical and biological investigations of manglicolous lichens to discover new bioactive substances.

Supplementary Information

Supplementary information is available in the website http://nopr.niscair.res.in/handle/123456789/60.

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