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Chemical and pharmacological evaluation of manglicolous lichen *Roccella montagnei* Bel em. D. D. Awasthi

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Abstract

Background: Lichen is a composite organism composed of fungus in association with algae or cyanobacteria. Particularly, lichen betide to mangroves are named as manglicolous lichens. From the folklore, lichen extracts were used in the management of many infections and diseases.

Results: The chemical investigation of acetone extract of manglicolous lichen *Roccella montagnei* (*RM-Ac*) yielded nine known metabolites namely divarinolmonomethylether (*1*), ethyl divaricatinate (*2*), divarinol (*3*), orcinol (*4*), methyl 2,6-dihydroxy-4-methylbenzoate (*5*), haematommic acid (*6*), atranol (*7*), ethyl haematommate (*8*) and ethyl orsellinate (*9*). Except 4, all are for the first reported from this species. The *RM-Ac* and its metabolites (*1*–*9*) were screened for antioxidant (DPPH, ABTS and superoxide free radical assays), anti-inflammatory, anticancer (SRB assay using A549, DLD-1, FADU, HeLa and MCF-7) and acute toxicity studies. The pharmacological results showed that compounds *6* and *8* depicted potent inhibitory profile against ABTS free radical (with IC_{50} value of 40.0 and 40.5 μg/mL, respectively) and protein denaturation (with IC_{50} value of 435 and 403 μg/mL, respectively). LD_{50} of *RM-Ac* was found to be above 2 g/Kg body weight. Moreover, the *RM-Ac* showed prominent inhibition of formalin-hind albino rat paw oedema at both the tested doses, i.e., 100 and 200 mg/Kg b.w than that of the standard drug (indomethacin). Furthermore, the compounds 6 and 8 exhibited significant degree of specificity against HeLa, FADU and A549; besides, they showed very little degree of specificity against NHME cell line specifying less toxicity to normal cells.

Conclusion: It can be concluded that the manglicolous lichen *R. montagnei* has an aptitude to act against free radicals, inflammation and cancer, and the main metabolites responsible for its biological activity are *6* and *8*.

Keywords: Antioxidant, Protein denaturation, Anti-inflammatory, Anti-cancer

Background

Lichen (a symbiotic organism) belongs to bryophytes which have an aptitude to persevere on any geographical region or any substratum [1, 2]. Due to their unique survival and mutualistic characteristics, lichens and their secondary metabolites are used for the treatment of several infections and diseases [2, 3]. The lichens particularly associated with the mangroves/mangals are termed as manglicolous lichens [2, 3]. As mangals persist in stressful environment such as high concentration of moisture and salt and low and high tidal water, lichens habituated on these groups of plants are also exposed to the aforementioned stressed conditions. As a result, they show a difference in phytochemical constituents than normal lichens due to stressed physiological adaptations [2–4]. Besides, there are very few chemical and pharmacological reports existing on manglicolous lichens due to their slow growth (1 cm/year) and the difficulty to collect a good amount of specimen from mangrove regions.

In recent times, the Roccella genus has been reviewed, reporting 24 main species which are constrained to coastal habitats. Among them, *Roccella montagnei* Bel em. D. D. Awasthi is one having a fructose growth form which commonly persists in mangroves of India [4, 5].

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In 1940, Rao and Seshadri reported novel metabolites namely erythrin, erythritol, lecanoric acid, orcinol and roccellic acid from *R. montagnei* [6, 7]. In 1942, occurrence of active montagnetol in *R. montagnei* was also reported by Seshadri's group [8]. In 1958 and 1959, Murty and Subramanian's group reported the carotene content of *R. montagnei* and also isolated carotene and ergosterol from *R. montagnei* [9–11].

In 2006, a biological report regarding the in vitro antimicrobial assay of *R. montagnei* from the mangrove tree, *Rhizophora* sp. in Pichavaram mangroves, Tamil Nadu, India, has been determined and found that methanolic extract of *R. montagnei* were active against microbes (bacterial and fungal) [12]. In 2016, the phytochemical and in vitro antioxidant assay of *R. montagnei* was quantified and it was reported that the species contains alkaloids, saponins, phytosterols, phenols, tannins, flavonoids and good inhibitory profile against DPPH and ferric radicals [13]. Furthermore, Mallavadhani and Sudhakar reported roccellatol along with nine known metabolites from *R. montagnei* [14]. In recent times, isomer of methyl-γ-orsellinate and roccellatol which were isolated from *R. montagnei* were screened for radical scavenging potential, anti-inflammatory and anti-arthritic activities [15], as well as, isolation of cytotoxic compounds along with docking evidences has been reported by Mishra's group [16]. Earlier, our group has established and reported the phytochemical analysis, antimicrobial, antimycobacterial, antioxidant and cytotoxicity activities of various extracts of *R. montagnei* [4]. In continuation of our research work to identify bioactive metabolites from manglicolous lichen *R. montagnei*, the present study was performed to identify the bioactive constituents present in manglicolous lichen *R. montagnei*.

Methods

Collection

From the twigs of mangrove plant *Excoecaria agallocha*, the specimens of manglicolous *Roccella montagnei* Bel em. D. D. Awasthi was collected from Godavari estuary, Andhra Pradesh, India, in December, 2016. The species was determined by Dr. D. K. Upreti, and a voucher specimen (14–027172) was deposited at Lucknow Lichen herbarium, National Botanical Research Institute, Lucknow, India [1].

Extraction

The collected manglicolous lichen *Roccella montagnei* was shade dried, and about 250 g of dried lichen material was exhaustively extracted with acetone. The attained acetone extract of *R. montagnei* (*RM-Ac*; 5.45 g, 2.18%, percentage based on total lichen material) was exposed to column chromatography (CC) (#230–400) by *n*-hexane in ethyl acetate (EA) (increasing polarity) as eluent, which eventually resulted in three fractions. Fraction I was re-treated with CC (#230–400) by *n*-hexane in dichloromethane (DCM) (increasing polarity) yielded *1* (30 mg, 0.012%, percentage based on total lichen material) as pale yellow oil and *2* (18 mg, 0.007%, percentage based on total lichen material) as sharp colorless needles. Fraction II was re-treated with CC (#230–400) by *n*-hexane in EA (increasing polarity) as eluent, yielded *3* (12 mg, 0.005%, percentage based on total lichen material) as pinkish powder, *4* (15 mg, 0.006%, percentage based on total lichen material) as colorless needles, *5* (675 mg, 0.027%, percentage based on total lichen material) as pale yellow crystals and *6* (35 mg, 0.014%, percentage based on total lichen material) as pale yellow needles. Fraction III was re-treated with CC (#230–400) by DCM in EA (increasing polarity) as eluent, yielded *7* (12 mg, 0.005%, percentage based on total lichen material) as yellowish powder, *8* (15 mg, 0.006%, percentage based on total lichen material) as greenish solid and 9 (35 mg, 0.014%, percentage based on total lichen material) as pale yellow solid.

Antioxidant activity

DPPH assay

The metabolites (*1–9*) and *RM-Ac* was subjected to 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay in triplicate, and results are reported as percentage of inhibition of DPPH free radicals [17]. Initially, to the known concentrations of the sample added 0.004% DPPH dissolved in methanol and incubated for half-an-hour at 37 °C. By using UV-Visible spectrophotometry (Spectra MAX plus 384, USA), all the samples were noted with absorbance at 517 nm against blank. IC_{50} values of the metabolites (*1–9*) and *RM-Ac* were determined by plotting concentrations against their percentage inhibition.

ABTS radical scavenging assay

The metabolites (*1–9*) and *RM-Ac* was exposed to 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay in triplicate, and results are reported as percentage of inhibition of ABTS free radicals [18]. To 7 mM $ABTS^{+}$, added potassium persulfate (2.45 mM) in water at room temperature and standardized. Then, to know the concentrations of the sample, added 1 mL of above standardized solution and incubated for half-an-hour. In later incubation, all the samples were observed to have absorbance at 750 nm against blank. IC_{50} values of the metabolites (*1–9*) and *RM-Ac* were determined by plotting concentrations against their percentage inhibition.

Superoxide radical scavenging assay

The metabolites (1–9) and *RM-Ac* was exposed to superoxide radical scavenging assay in triplicate, and results are reported as percentage of inhibition of superoxide free radicals [19]. To NADH (73 μM), added 15 μM of PMS and NBT (50 μM) in 20 mM phosphate buffer (pH 7.4)

and standardized. Then, to know the concentrations of the sample, added 1 mL of above standardized solution and incubated for half-an-hour. After incubation, all the samples were observed to have absorbance at 562 nm against blank. IC_{50} values of the metabolites (*1–9*) and *RM-Ac* were determined by plotting concentrations against their percentage inhibition.

In vitro anti-inflammatory activity

The in vitro anti-inflammatory activity for metabolites (*1–9*) and *RM-Ac* were determined by using protein denaturation method [20] in triplicate, and results are reported as percentage of inhibition of protein denaturation against blank. Bovine serum albumin protein (1%) was solubilized in sodium phosphate buffer (50 mM, pH 6.4). Then, to know the concentrations of the sample, added 0.2 mL of above protein solution and make up to 5 mL with sodium phosphate buffer and incubated for 20 min at 37 °C. Later, all the samples were boiled for 20 min in steam bath at 95 °C and set to room temperature. After incubation, all the samples were observed to have absorbance at 562 nm against blank. IC_{50} values of the metabolites (*1–9*) and *RM-Ac* were determined by plotting concentrations against their percentage inhibition.

Animals

Albino rats (either sex) weighing 190–200 g were utilized for the current experimental study, and the experimental procedure were performed as per OECD regulations (Regd No. 516/01/A/CPCSEA).

Acute toxicity studies

A week before the acute toxicity study, five male albino rats were kept on standard diet under room temperature. Orally, *RM-Ac* at 2000 mg/kg body weight (b.w) were administered to the selected albino male rats and kept under observed for 24 h [21]. The mortality number triggered by the *RM-Ac* within this time duration was observed, from which log dose response plots were calibrated and median lethal dose (LD_{50}) of the sample was determined.

In vivo anti-inflammatory activity

The *RM-Ac* was subject to in vivo anti-inflammatory assay by plethysmographic measurement of formalin-induced hind albino rat (either sex; $n = 6$) paw oedema [2, 20]. Group I is for normal control (dosed with 0.5% CMC), group II for indomethacin (Indo, 100 mg/Kg b.w) and group III and group IV assisted for *RM-Ac* at 100 and 200 mg/Kg b.w, respectively. After 30 min of samples administration intraperitoneally to albino rats, 1% w/v formalin (0.1 mL) was dosed in the plantar area of albino rat left paw and paw volume was measured at 2 and 4 h by using right paw (non-inflammated) as a reference. The % variation in albino rat paw oedema was calculated using below formula.

$$\text{Percentage reduction} = \frac{(\text{Volume of paw oedema in controlled animals} - \text{Volume of paw oedema in treated animals})}{\text{Volume of paw oedema in controlled animals}} \times 100$$

Statistical analysis

The outcomes are stated as mean ± SEM values of three and six independent tests of in vitro and in vivo assays, respectively. The results of in vivo anti-inflammatory assay were deliberate by one-way ANOVA followed by Dunnett's test. The $p < 0.05$ value between the experimental groups is statistical significance.

Anticancer activity

Cancer cell lines

Lung (A549), colon (DLD-1), cervical (HeLa), breast (MCF-7), head & neck (FADU), cancer cells and normal human mammary epithelial (NHME) cell lines were attained from National Centre for Cell Science, Pune. All the cancer cell lines were preserved in minimal essential medium MEM media, which contain 5% mixture of streptomycin (100 μg/mL) and penicillin (100 units), and fetal calf serum (10%), in presence of CO_2 (5%) incubator with humidity (90%) for 72 h at 37 °C.

Cell growth medium

Three days earlier to assay, selected cancer cell lines were maintained in MEM and grown on 10% FBS supplemented with trypsin (0.25%). In a sterilized polypropylene tube, final suspension of cancer cells were taken, and concentration of the cells in each well was calculated by using 0.4% trypan blue solution using hematocytochameter chamber under a microscope. The minimal concentration of 1×10^4 cells per well was noted as nominal seed density.

Sample preparation

The samples were dissolved in DMSO, which is used as a control and doxorubicin as a standard. Primary screening of the samples were performed at 100 μg/mL for *RM-Ac*, 30 μg/mL for compounds *1–9* and 10 μg/mL for standard drug, i.e. doxorubicin. Active samples were further screened at different concentrations for *RM-Ac* (25, 50, 75 and 100 μg/mL), compounds *1–9* (5, 10, 20 and 30 μg/mL) and doxorubicin (2.5, 5.0, 7.5 and 10 μg/mL) against particular cancer cells.

Sulforhodamine B (SRB) colorimetric assay

The anticancer activity of *RM-Ac* and compounds *1–9* were performed by SRB assay in triplicate ($n = 3$) [3]. In 96-well plate, added 190 μL screened ideal cancer cells

suspension and test samples and incubate with relative humidity (90%), 5% CO_2 for 3 h at 37 °C. Formerly, to each well, 100 μL cold TCA was added and incubated for 1 h at 4 °C. Thereafter, 96-well plate was gently washed with water, air-dried at 25 °C. Next, to each well, SRB solution (100 μL of 0.057%) was added, incubated for half an hour and stained with CH_3COOH (1%). Then, Tris base (200 μL of 10 mM at pH 10.5) solution was added to each well, agitated for few minutes and observed the optical density at 510 nm. The control contains only cancer cells, whereas blank contains only MEM medium. The % growth inhibition was determined by

$$\%\text{Growth inhibition} = 100\text{-}[\text{Absorbance of Sample}/\text{Absorbance of Control}] \times 100$$

IC_{50} values of the active samples were determined by plotting concentrations against their percentage inhibition.

Results

Chemistry

Chemical investigation of acetone extract from *Roccella montagnei* (*RM-Ac*) yielded compounds *1–9*, which are illustrated in Fig. 1. The chemical structures of metabolites *1–9* were characterized by using elemental analysis, ^1H & ^{13}C NMR, and mass spectral data and correlating with the existing literature data [22].

Divarinolmonomethylether

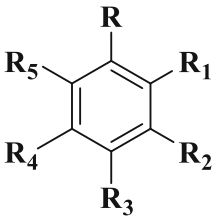
Pale yellow oil, R_f: 0.6 (Hex:DCM, 9:1), UV (λ_{max}): 273 in methanol, Mol. For.: $C_{10}H_{14}O_2$; ^1H NMR (400 MHz, DMSO-d_6): δ 0.74–0.82 (*m*, 3H), 1.41–1.52 (*m*, 2H), 2.66–2.74 (*m*, 2H), 3.76 (brs, 1H), 4.39 (*brs*, 3H), 6.24 (*d*, 1H, *J* = 2.1 Hz), 6.49 (*s*, 1H), 6.61 (*d*, 1H, *J* = 3 Hz); ^{13}C NMR (400 MHz, DMSO-d_6): δ 12.40 (C-10), 23.11 (C-9), 35.21 (C-8), 52.81 (C-7), 101.49 (C-2), 108.59 (C-6), 111.23 (C-4), 141.81 (C-5), 155.76 (C-3), 162.12 (C-1). Elemental analysis found C-72.33, H-8.42(%), calcd. C, 72.26, H, 8.49(%). ESI-MS: *m/z* 166.9 ([M-H^+], 100%).

Ethyl divaricatinate

Sharp colorless needles, R_f: 0.4 (Hex:DCM, 9:1), m.p: 43–44 °C, UV (λ_{max}): 201 in methanol, Mol. For.: $C_{13}H_{18}O_4$; ^1H NMR (400 MHz, DMSO-d_6): δ 0.97–1.05 (*m*, 6H), 1.70–1.72 (*m*, 2H), 2.38–2.39 (*m*, 2H), 2.98–3.04 (*m*, 2H), 3.87 (*s*, 3H), 6.67 (*d*, 1H, *J* = 2.3 Hz), 6.79 (*d*, 1H, *J* = 2.8 Hz), 11.36 (*s*, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 14.50 (C-12), 14.60 (C-9), 24.87 (C-11), 31.17 (C-10), 55.75 (C-13), 56.53 (C-8), 99.46 (C-5), 106.21 (C-3), 108.59 (C-1), 144.12 (C-2), 162.92 (C-6), 166.74 (C-4), 172.65 (C-7). Elemental analysis found C-65.16, H-7.26(%), calcd. C, 65.53, H, 7.61(%). ESI-MS: *m/z* 239.0 ([M-H^+], 100%).

Divarinol

Pinkish powder, R_f: 0.8 (Hex:EA, 1:1), m.p: 51–52 °C, UV (λ_{max}): 274 in methanol, Mol. For.: $C_9H_{12}O_2$; ^1H NMR (400 MHz, DMSO-d_6): δ 0.97–1.04 (*m*, 3H), 1.65–1.76 (*m*, 2H), 2.95–3.04 (*m*, 2H), 3.53 (brs, 2H), 6.18 (*s*, 1H), 6.40–6.42 (*dd*, 1H, *J* = 8, 9.2 Hz), 6.66 (*s*, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 12.40 (C-9), 23.11 (C-8), 35.21 (C-7), 101.49 (C-2), 108.59 (C-4/C-6), 141.81 (C-5),



1 (R=C_3H_7; R_1=R_3=R_5=H; R_2=OH; R_4= OCH_3)
2 (R=C_3H_7; R_1=$COOC_2H_5$; R_2=OH; R_3=R_5=H; R_4= OCH_3)
3 (R=C_3H_7; R_1=R_3=R_5=H; R_2=R_4=OH)
4 (R=CH_3; R_1=R_3=R_5=H; R_2=R_4=OH)
5 (R=CH_3; R_1=R_5=H; R_2=R_4=OH; R_3= $COOCH_3$)
6 (R=CH_3; R_1=COOH; R_2=R_4=OH; R_3= CHO; R_5=H)
7 (R=CH_3; R_1=R_5=H; R_2=R_4=OH; R_3= CHO)
8 (R=CH_3; R_1=$COOC_2H_5$; R_2=R_4=OH; R_3= CHO; R_5=H)
9 (R=CH_3; R_1=$COOC_2H_5$; R_2=R_4=OH; R_3=R_5=H)

Fig. 1 Structures of secondary metabolites isolated from *Roccella montagnei*

155.76 (C-1/C-3). Elemental analysis found C-71.87, H-7.82(%), calcd. C, 71.03, H, 7.95(%). ESI-MS: *m/z* 153.0 ([M-H$^+$], 18.75%).

Orcinol

Colorless needles, R_f: 0.7 (Hex:EA, 1:1), m.p: 108–109 °C, UV (λ_{max}): 215.5 in methanol, Mol. For.: $C_7H_8O_2$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.46 (*s*, 3H), 3.34 (*s*, 2H), 6.23 (*s*, 2H), 6.30 (*s*, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 27.93 (C-7), 98.36 (C-4/C-6), 109.34 (C-2), 155.22 (C-5), 163.90 (C-3), 157.52 (C-1). Elemental analysis found C-67.59, H-6.52(%), calcd. C, 67.73, H, 6.50(%). ESI-MS: *m/z* 125.1 ([M-H$^+$], 28.88%).

Methyl-2,6-dihydroxy-4-methylbenzoate

Pale yellow crystals, R_f: 0.6 (Hex:EA, 1:1), m.p: 138–139 °C, UV (λ_{max}): 219.5 in methanol, Mol. For.: $C_9H_{10}O_4$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.23 (*s*, 3H), 3.75 (*s*, 3H), 6.12 (*d*, 2H, *J* = 1.2 Hz), 9.93 (*s*, 1H), 10.65 (*s*, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): 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-2/C-6), 170.59 (C-7). Elemental analysis: found C-59.66, H-5.62(%), calcd. C, 59.34, H, 5.53(%). ESI-MS: *m/z* 183.0 ([M-H$^+$], 68.81%).

Haematommic acid

Pale yellow needles, R_f: 0.4 (Hex:EA, 1:1), m.p: 172–173 °C, UV (λ_{max}): 219.5 in ethanol, Mol. For.: $C_9H_8O_5$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.54 (*s*, 3H), 6.42 (*s*, 1H), 9.68 (*s*, 1H), 10.59 (*s*, 1H), 11.46 (*s*, 1H), 13.75 (*s*, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): 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). Elemental analysis: found C-55.64, H-4.52(%), calcd. C-55.11, H-4.11(%). ESI-MS: *m/z* 198.3 ([M-H$^+$], 5.64%).

Atranol

Yellowish powder, R_f: 0.8 (DCM:EA, 7:3), m.p: 125–126 °C, UV (λ_{max}): 225 in methanol, Mol. For.: $C_8H_8O_3$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.23 (*s*, 3H), 6.12 (*s*, 2H), 9.94 (*s*, 1H), 10.66 (*s*, 1H), 11.29 (*s*, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 22.46 (C-8), 100.83 (C-1), 107.93 (C-5), 110.58 (C-3), 141.15 (C-4), 161.46 (C-2/C-6), 192.87 (C-7). Elemental analysis: found C-63.50, H-5.52(%), calcd. C-63.15, H-5.30(%). ESI-MS: *m/z* 153.1 ([M-H$^+$], 6.91%).

Ethyl haematommate

Greenish solid, R_f: 0.6 (DCM:EA, 7:3), m.p: 112–113 °C, UV (λ_{max}): 209.5 in methanol, Mol. For.: $C_{11}H_{12}O_5$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.93–0.97 (*t*, 3H), 1.59–1.65 (*m*, 2H), 2.54 (*s*, 3H), 6.42 (*s*, 1H), 9.68 (*s*, 1H), 10.59 (*s*, 1H), 11.46 (*s*, 1H), 13.75 (*s*, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 14.28 (C-11), 20.14 (C-9), 68.63 (C-8), 106.38 (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). Elemental analysis found C-58.64, H-5.52(%), calcd. C-58.93, H-5.39(%). ESI-MS: *m/z* 224.9 ([M-H$^+$], 31.36%).

Ethyl orsellinate

Pale yellow solid, R_f: 0.4 (DCM:EA, 7:3), m.p: 131–132 °C, UV (λ_{max}): 219 in methanol, Mol. For.: $C_{10}H_{12}O_4$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.80–0.84 (*m*, 3H), 1.46–1.52 (*m*, 2H), 2.54 (*s*, 3H), 6.42 (*s*, 2H), 9.68 (*s*, 1H), 10.59 (*s*, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 14.28 (C-9), 20.14 (C-10), 68.63 (C-8), 105.25 (C-5), 109.34 (C-1), 114.78 (C-3), 155.22 (C-2), 163.90 (C-4/C-6), 173.42 (C-7). Elemental analysis found C-61.26, H-6.55(%), calcd. C-61.22, H-6.17(%). ESI-MS: *m/z* 197.1 ([M-H$^+$], 100%).

Antioxidant activity

In DPPH assay, reduction of the DPPH free radicals to a DPPH-H (non-radical) form by antioxidant capable substance takes place [17, 19]. As shown in Fig. 2, IC_{50} value of ascorbic acid on DPPH free radicals was found to be 27.0 μg/mL. Furthermore, the IC_{50} values of *1*, *2*, *3*, *4*, *5*, *6*, *7*, *8*, *9* and *RM-Ac* were determined to be 88.5, 58.0, 99.5, 84.0, 40.0, 53.0, 45.0, 50.5, 56.25 and 95.0 μg/mL, respectively.

In ABTS radical assay, radical cation $ABTS^{\cdot+}$ is decoyed [18]. As shown in Fig. 2, the IC_{50} value of ascorbic acid on ABTS free radicals was found to be 41.0 μg/mL. Among all samples, *6* and *8* showed better IC_{50} than that of the standard. The IC_{50} values of *Roccella montagnei* samples on ABTS radical were in the order *6* (40.0 μg/mL) > *8* (40.5 μg/mL) > *5* (43.5 μg/mL) > 7 (48.25 μg/mL) > *9* (65.0 μg/mL) > *2* (67.0 μg/mL) > *RM-Ac* (87.0 μg/mL) > *1* (91.0 μg/mL).

Generally, the superoxide free radicals ascend from biological metabolisms interrelate with chemical species, i.e. substrates in occurrence of metallic or enzymatic catalyzed routes to produce $^1O_2$ and OH radical [17, 19]. These superoxide radicals influence oxidative impairment in lipids, DNA, and, proteins. The superoxide free radical assay of all the prepared lichen samples was tabulated in Additional file 1: Table S3. As shown in Fig. 2, the concentration of *1*, *2*, *3*, *4*, *5*, *6*, *7*, *8*, *9* and *RM-Ac* required for 50% reticence of superoxide free radicals were found to be 81.5, 60.0, 98.0, 99.75, 40.0, 37.25, 38.0, 36.0, 62.5 and 99.75 μg/mL, respectively, whereas standard was 35.5 μg/mL.

In vitro anti-inflammatory activity

The route cause for inflammation is biological protein denaturation, which occurs by alkaline/acidic/radiation reactions and heat treatment, etc. [2, 3]. Therefore, in the

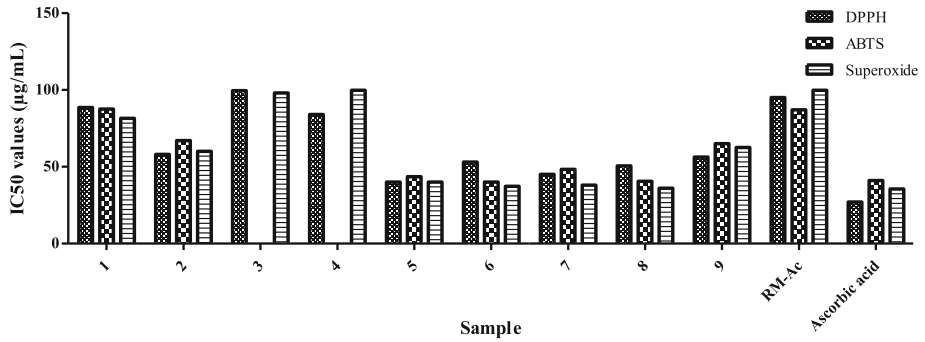


Fig. 2 IC_{50} values of acetone extract and all compounds of *Roccella montagnei* against DPPH, ABTS and Superoxide free radicals

current work, the acetone extract (*RM-Ac*) and isolates (*1–9*) from *Roccella montagnei* were experimented for reticence of albumin protein denaturation persuaded by heat. The results of in vitro anti-inflammatory assay were tabulated in Additional file 1: Table S4, which specified that almost all isolates showed significant anti-inflammatory activity. The IC_{50} values of *2, 5, 6, 7, 8, 9* and *RM-Ac* on protein denaturation were determined to be 529, 664, 435, 531, 403, 533 and 330 µg/mL, respectively, whereas standard drug (Indo) with 110 µg/mL (Fig. 3).

Acute toxicity studies

Acute toxicity studies of *RM-Ac* gave LD_{50} as above 2 g/Kg, and the low and high dose of *RM-Ac* was determined to be 100 and 200 mg/Kg b.w, respectively.

In vivo anti-inflammatory activity

Based on the in vitro bioassay and toxicological studies of *RM-Ac*, the *RM-Ac* at low and high dosage, i.e. 100 and 200 mg/Kg b.w, respectively, were exposed to formalin-induced albino rat paw oedema assay against Indo at single dose, i.e. 100 mg/Kg b.w. From the outcomes of in vivo assay, it is noticed that *RM-Ac* showed dosage-reliant reduction of albino rat paw oedema.

The albino rats treated with lower dosage of *RM-Ac* showed 34.87 and 41.89% reduction of albino rat paw oedema at both intervals of time, i.e. 2 and 4 h, respectively, whereas prominent result of 40.91 and 47.12%, respectively, reduction of albino rat paw oedema was noticed in albino rats administered with higher dosage of *RM-Ac* (Fig. 4). From the results, it can be assumed that *RM-Ac* are very effective in reducing albino rat paw oedema than that of the Indo.

Anticancer activity

In general, chronic inflammation is route cause for numerous lethal disorders and diseases including cancer. Additionally, compounds (*1–9*) displayed better anti-inflammatory activities, so we further evaluated *1–9* and acetone extract of *R. montagnei* (*RM-Ac*) for their anticancer activity. Primary screening of the samples were performed at 100 µg/mL for *RM-Ac*, 30 µg/mL for compounds *1–9* and 10 µg/mL for standard drug, i.e. doxorubicin against MCF-7, DLD-1, HeLa, FADU and A549

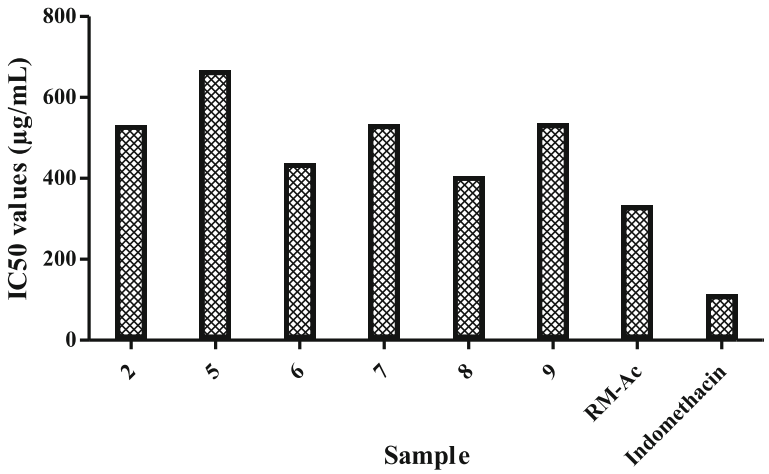


Fig. 3 IC_{50} values of acetone extract and all compounds of *Roccella montagnei* against albumin protein denaturation

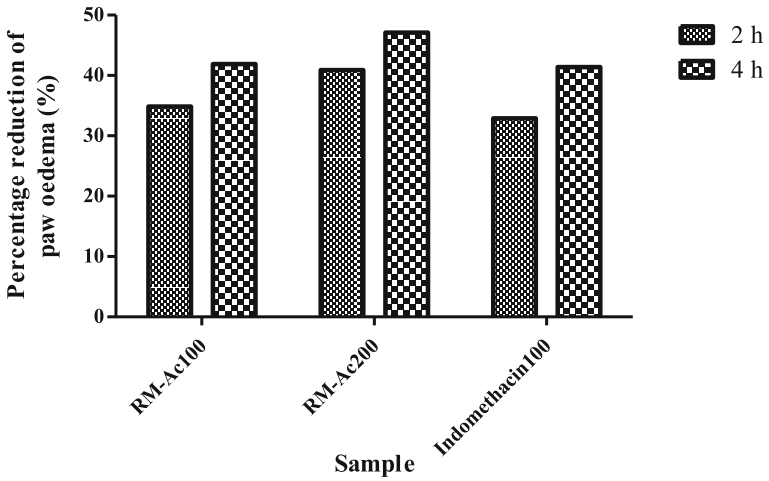


Fig. 4 In vivo anti-inflammatory assay of acetone extract of *R. montagnei* on formalin-induced hind rat paw oedema method

cancer cells and NHME cell lines, by using SRB assay, and the % cell growth inhibition was represented in Additional file 1: Table S4. Samples that are active against cancer cell lines were further screened at different concentrations for *RM-Ac* (25, 50, 75 and 100 μg/mL), compounds *1*–9 (5, 10, 20 and 30 μg/mL) and doxorubicin (2.5, 5.0, 7.5 and 10 μg/mL). The outcomes of percentage cell growth inhibition against concentrations are plotted to attain IC_{50} values. The poorer IC_{50} value directs improved inhibitory activity against cancer cells.

From the primary evaluation, *RM-Ac* (72.23 ± 1.75) at 100 μg/mL concentration showed equivalent inhibitory profile against DLD-1 as that of the standard drug doxorubicin (10 μg/mL concentration, 72.67 ± 0.21). Among the metabolites of *RM-Ac*, only *2*, *6*, *8* and *9* displayed reasonable degree of specificity towards experimented series of cancer cells. Moreover, *RM-Ac* and its metabolites showed very little degree of specificity against normal cell lines, i.e. NHME indicates non-toxic (Additional file 1: Table S4).

From Fig. 5, it was clearly evident that the *RM-Ac* showed more pronounced degree of specificity against DLD-1, HeLa, FADU and A549 with IC_{50} values of 61.0, 74.5, 62.5 and 64.9 μg/mL, respectively. Further screening of the isolates obtained from this extract showed significant inhibitory profile against all the experimented cancer cells. Among all isolates, only *2* showed better IC_{50} value of 28.20 μg/mL on MCF-7, whereas standard with 5.5 μg/mL, *2* and *9* depicted IC_{50} value of 18.5 and 26.5 μg/mL on DLD-1, respectively, while standard with 5.4 μg/mL; *2*, *6* and *8* revealed IC_{50} value of 25.5, 27.0 and 26.5 μg/mL on HeLa, respectively, whereas standard with 4.5 μg/mL; *6* and *8* showed IC_{50} value of 20.0 and 25.5 μg/mL on FADU and 22.5 and 27.5 μg/mL on A549, respectively, while standard with 3.8 and 6.3 μg/mL, respectively.

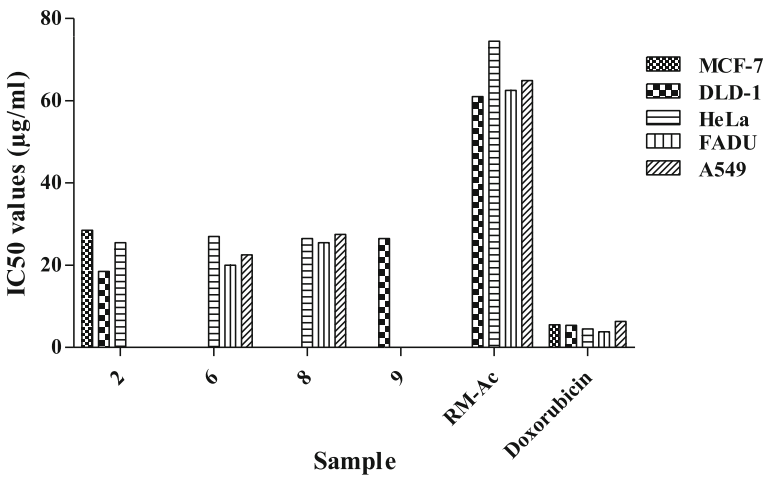


Fig. 5 IC_{50} values of acetone extract and all compounds of *Roccella montagnei* against panel of cancer cell lines

Discussion

In present study, the chromatographic examination of acetone extract of *Roccella montagnei* (*RM-Ac*) yielded nine monoaromatic compounds (*1*–*9*) substituted with hydroxyl group(s), which are confirmed by UV, NMR, mass spectral and elemental analysis (Fig. 1). All the isolated metabolites and *RM-Ac* were subjected to antioxidant activity using DPPH, superoxide and ABTS free radicals, and in vitro & in vivo anti-inflammatory by using protein denaturation method and formalin-induced albino rat paw oedema bioassay, and anticancer activity using SRB assay. In addition, the acute toxicity of *RM-Ac* was performed on male albino rats [20].

From the antioxidant and in vitro anti-inflammatory outcomes, it can be determined that *RM-Ac* and samples showed prominent inhibitory capabilities against DPPH, superoxide and ABTS free radicals, and albumin protein denaturation is due to the existence of freely available oxygenated substances namely phenolics, carboxylic acids, etc. in their chemical structure (Figs. 2 and 3).

The LD_{50} of *RM-Ac* was found to be above 2 g/Kg and low and high dosage i.e. 100 and 200 mg/Kg b.w, respectively *RM-Ac* was determined. As *RM-Ac* was active against in vitro anti-inflammatory assay, we prolonged the anti-inflammatory study for its in vivo model. The outcomes are denoted as the % reduction of albino rat paw oedema calculated to basal rat paw size (Fig. 4). From outcomes of in vivo assay, it is evident that *RM-Ac* showed dose-reliant reduction of albino rat paw oedema. In addition, higher dose of *RM-Ac* are extremely effective in reducing paw oedema in albino rat than that of the Indo (Fig. 4).

The illustrations of the current research proposed that the *RM-Ac* has pronounced anti-inflammatory capability against prolonged models of inflammation like formalin-induced assay [20], which is also used to measure anti-arthritic activity. Therefore, outcomes of current research explained the application of above lichen extract, i.e. *RM-Ac* in folklore support for the management of both types of inflammation, i.e. acute and chronic, and in predominantly allied with arthritis. Besides, the dominance consequence of the *RM-Ac* may be due to the blockade of the biosynthesis of thromboxane (TXA2), prostanoids (PGE2, PGF2α, PGD2, PGI2), and Interluekin-8 [2, 3, 20].

As chronic inflammation is route cause for several deadly diseases including cancer. Moreover, as the *RM-Ac* and isolates depicted better anti-inflammatory properties, we evaluated metabolites (*1*–*9*) and acetone extract of *R. montagnei* (*RM-Ac*) for their anti-cancer activity on MCF-7, DLD-1, HeLa, FADU and A549 cancer cells and NHME cells. Form the SRB study it can be determined that key agents accountable for *RM-Ac* were *2*, *6*, *8* and *9*. In addition, the redox reactions (free radical production) and chronic inflammation are the key elements that cause deadly diseases like cancer (Fig. 5). By comparing the antioxidant and anti-inflammatory bioassay results, it is evident that the anticancer activity of these compounds were may be due to the presence of their antioxidant and anti-inflammatory capabilities. Moreover, *RM-Ac* and its metabolites showed very little degree of specificity against normal cells states non-toxic (Additional file 1: Table S4). Therefore, metabolites (*1*–*9*) can be a benchmarks for designing powerful anticancer agents.

Conclusion

This is a primitive study of chemical and biological evaluation of manglicolous lichen *R. montagnei*. Chemical investigation of acetone extract of *R. montagnei* results in the isolation of nine known metabolites namely divarinolmonomethylether (*1*), ethyl divaricatinate (*2*), divarinol (*3*), orcinol (*4*), methyl 2,6-dihydroxy-4-methylbenzoate (*5*), haematommic acid (*6*), atranol (*7*), ethyl haematommate (*8*) and ethyl orsellinate (*9*) which were confirmed by spectral data. The pharmacological evaluation revealed the inhibitory capabilities of *R. montagnei* against DPPH free radicals, superoxide free radicals, ABTS free radicals, albumin protein denaturation and rat paw oedema, MCF-7, HeLa, FADU, DLD-1 and A549. Moreover, key agents responsible for pharmacological activity of *R. montagnei* were also identified as *2*, *6*, *8* and *9*.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s43094-019-0009-6.

Additional file 1: Figure S1. ^{1}H NMR of 1. **Figure S2.** ^{13}C NMR of 1. **Figure S3.** Mass spectrum of 1. **Figure S4.** ^{1}H NMR of 2. **Figure S5.** ^{13}C NMR of 2. **Figure S6.** Mass spectrum of 2. **Figure S7.** ^{1}H NMR of 3. **Figure S8.** ^{13}C NMR of 3. **Figure S9.** Mass spectrum of 3. **Figure S10.** ^{1}H NMR of 4. **Figure S11.** ^{13}C NMR of 4. **Figure S12.** Mass spectrum of 4. **Figure S13.** ^{1}H NMR of 5. **Figure S14.** ^{13}C NMR of 5. **Figure S15.** Mass spectrum of 5. **Figure S16.** ^{1}H NMR of 6. **Figure S17.** ^{13}C NMR of 6. **Figure S18.** Mass spectrum of 6. **Figure S19.** ^{1}H NMR of 7. **Figure S20.** ^{13}C NMR of 7. **Figure S21.** Mass spectrum of 7. **Figure S22.** ^{1}H NMR of 8. **Figure S23.** ^{13}C NMR of 8. **Figure S24.** Mass spectrum of 8. **Figure S25.** ^{1}H NMR of 9. **Figure S26.** ^{13}C NMR of 9. **Figure S27.** Mass spectrum of 9. **Table S1.** Percentage inhibition and IC_{50} values of samples against DPPH. **Table S2.** Percentage inhibition and IC_{50} values of samples against ABTS. **Table S3.** Percentage inhibition and IC_{50} values of samples against Superoxide. **Table S4.** Percentage inhibition and IC_{50} values of samples against protein denaturation. **Table S5.** In vivo anti-inflammatory assay of RM-Ac. **Table S6.** Anticancer activity of samples against various cell lines. **Table S7.** Percentage inhibition of samples against MCF-7. **Table S8.** Percentage inhibition of samples against DLD-1. **Table S9.** Percentage inhibition of samples against HeLa. **Table S10.** Percentage inhibition of samples against FADU. **Table S11.** Percentage inhibition of samples against A549. **Figure S28.** Images of active samples against MCF-7. **Figure S29.** Images of active samples against DLD-1. **Figure S31.** Images of active samples against FADU. **Figure S32.** Images of active samples against A549.

Abbreviations

A549: Lung cancer cells; ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; b.w: Body weight; CMC: Carboxymethyl cellulose; DLD-1: Colon cancer cell lines; DMSO: Dimethyl sulfoxide; DPPH: 1,1-Diphenyl-2-picrylhydrazyl; FADU: Head & Neck cancer cells; HeLa: Cervical cancer cells; MCF-7: Breast cancer cells; NHME: Normal human mammary epithelial cells; *RM-Ac*: Acetone extract of *Roccella montagnei* Bel em. D. D. Awasthi; SRB: Sulforhodamine B

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Studies involving plants

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Authors' contributions

VBT is a research Scholar who carried out the isolation and biological evaluations and was a major contributor in writing the manuscript. GSV is the principal investigator of the MoES project, who guided in the isolation and characterization of the secondary metabolites from manglicolous lichens. AVSS is a professor in pharmacology, who proposed and monitored the pharmacological studies. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethics approval and consent to participate

All the experiment protocols were according to the OECD guidelines and regulations of Institutional Ethical Committee bearing registered number: 516/PO/c/01/CPCSEA. For ethical reasons, each animal was used only once and all animals were sacrificed at the end of the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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