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Short communication

Antiamoebic coumarins from the root bark of *Adina cordifolia* and their new thiosemicarbazone derivatives

Prince Firdoos Iqbal, Abdul Roouf Bhat, Amir Azam*

Department of Chemistry, Jamia Millia Islamia, Jamia Nagar, New Delhi 110025, India

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Abstract

In continuation of our search for potential antiamoebic agents from folklore Indian medicinal plants, we found that the benzene and ethyl acetate extracts from the root bark of *Adina cordifolia* exhibited strong antiamoebic activity with IC_{50} values of 2.92 and 2.50 μg/ml, respectively. Bioassay-guided fractionation of benzene and ethyl acetate extracts led to the isolation of 7-hydroxycoumarin (umbelliferone **1**) and 7-β-D-glucosylcoumarin (skimmin **2**), respectively. Umbelliferone **1** was converted into 7-acetoxycoumarin **1a**, which on treatment with aluminium chloride afforded 7-hydroxy-8-acetylcoumarin **2a**. A new series of thiosemicarbazones **3a**–**e** of 7-hydroxy-8-acetylcoumarin with different thiosemicarbazides were synthesized. Umbelliferone was also converted into its methoxy derivative (7-methoxycoumarin **4**). Subsequently, all the compounds were assessed for antiamoebic activity against HM1:IMMS strain of *Entamoeba histolytica*. Umbelliferone and skimmin were found to possess a very good activity with IC_{50} values of 6.38 and 4.35 μM/ml, respectively. The activity drastically increased on converting compound **2a** into its thiosemicarbazone derivatives **3a**–**e** with IC_{50} values ranging between 1.06 and 4.46 μM/ml. Compounds **3b**,**c** and **e** with IC_{50} values of 1.49, 1.56 and 1.06 μM/ml, respectively, exhibited even higher antiamoebic activity than the standard drug metronidazole ($IC_{50} = 2.62$ μg/ml). The activity of 7-methoxycoumarin ($IC_{50} = 8.92$ μM/ml) was less than umbelliferone. Compounds **3b**, **c** and **e** were tested for toxicity using H9c2 cardiac myoblasts cell line. The compounds exhibit >80% viability at 3.125–200 μg/ml. It is apparent from these results that umbelliferone and skimmin may be a useful lead for the development of new antiamoebic drugs.

Keywords: *Adina cordifolia*; Umbelliferone; Skimmin; Thiosemicarbazones; *Entamoeba histolytica*; MTT assay

1. Introduction

Amoebiasis, a disease caused by ingestion of contaminated food or water containing cysts of *Entamoeba histolytica* remains one of the major threats to public health in most parts of the globe. It infects over 50 million people per annum leading to 50,000–100,000 deaths annually, making it the second leading cause of death among parasitic diseases [1]. Though ubiquitous in distribution, this parasite is more prevalent in tropical and subtropical regions with poor sanitary conditions and contaminated drinking water [2]. Moreover, in recent years there has been a rise in the number of reports with amoebic brain abscess [3–7]. Metronidazole is known to be a highly effective amoebicide and is considered to be the drug of choice for the treatment of amoebiasis, but this drug has been shown to be mutagenic in a microbiological system and carcinogenic to rodents [8–10]. The treatment with metronidazole is known to be associated with several side effects that include nausea, vomiting, dry mouth, metallic taste, abdominal pain, headache and diarrhoea or constipation may also occur [11]. In addition, the drug recipients exhibit lower immune response both cell and humoral mediated [12]. Moreover, there is possibility of drug resistance as demonstrated in other protozoan parasites [13]. Some recent reports also have described the *in vitro* generation of strains resistant to metronidazole [14,15]. In spite of the tremendous use of metronidazole, currently used therapies for treating disease

* Corresponding author. Tel.: +91 11 2698 1717/3253; fax: +91 11 2698 0229/1232.
E-mail address: amir_sumbul@yahoo.co.in (A. Azam).

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caused by *Entamoeba* provide inadequate protection and hence continuous research for discovering and developing newer antiamoebic agents even today is required so that the limitation as presented by available therapeutic agents can be minimized and more safer, effective antiamoebic drugs or vaccines can come into existence.

During the last 50 years a number of compounds have been isolated from plants and/or synthesized that have been screened *in vitro* against *E. histolytica* as a result of which numerous antiamoebic compounds have been identified. In developing countries many people rely on traditional preparations from various medicinal plants to treat this disease because these products are safe, widely available at low cost and easy to access. Thus, scientific validation and *in vitro* and/or *in vivo* evaluation of these traditional remedies are needed to prove their claimed effectiveness against the disease.

Adina cordifolia (Roxb.) Hook.f.ex Brandis (Rubiaceae), Syn. *Haldinia cordifolia* (Roxb.) Ridsd. is found scattered in deciduous forests throughout the greater part of India, ascending to an altitude of 900 m in the sub-Himalayan tract. It is also common in the forests of South India. The plant *A. cordifolia* has been used in oriental medicine since ancient times as an essential component of various antiseptic and febrifuge prescriptions [16]. The bark is acrid and bitter and is used in biliousness [17]. The roots are used as an astringent in dysentery [17]. The *A. cordifolia* stem has been evaluated for its antiulcer potential and enzyme assay-guided fractionation of the chloroform extract yielded 7-hydroxycoumarin which showed interesting H^+/K^+ ATPase inhibitory activity [18]. Antifertility properties of the leaf extract of *A. cordifolia* have been also examined [19].

Keeping in view the medicinal importance of *A. cordifolia* and as a part of our programme to explore naturally occurring bioactive compounds from Indian folklore medicinal plants for the treatment of amoebic dysentery, we have investigated the root bark of *A. cordifolia*, which is traditionally used in folklore medicine for the treatment of dysentery in different parts of India, especially in Pauri Garhwal region of Uttrakhand [20]. In this study the *in vitro* antiamoebic activity of the extracts, isolated compounds and their derivatives from the root bark of *A. cordifolia* are reported.

2. Chemistry

2.1. Plant material

The stem bark of *A. cordifolia* was collected from the Hills of Pauri Garhwal district, Uttrakhand, India. The plant material was authenticated by Professor Tasneem Fatima, Department of Bioscience, Jamia Millia Islamia, New Delhi, India. A voucher specimen (TFU-537) has been deposited in the herbarium of the Department of Bioscience.

2.2. Analytical material and methods

IR spectra were recorded on Perkin–Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs. ^{1}H NMR, ^{13}C NMR and DEPT spectra were recorded on Bruker AVANCE 400 spectrometer using DMSO-d_6 as solvent with TMS as internal standard. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Chemical shift values are given in parts per million. Precoated aluminium sheets (Merck silica gel 60 F_{254}) were used for thin-layer chromatography (TLC) and spots were visualized under UV light. Analytical grade solvents were purchased from Merck (India).

3. Results and discussion

3.1. Identification of isolated compounds

n-Hexane, benzene, chloroform, ethyl acetate, acetone and *n*-butanol extracts from the stem bark of *A. cordifolia* were subjected to *in vitro* antiamoebic activity against HM1:IMSS strain of *E. histolytica* using metronidazole ($IC_{50} = 0.45$ μg/ml) as a reference drug. Results indicated that the benzene and ethyl acetate extracts ($IC_{50} = 2.92$ and 2.50 μg/ml, respectively) were the most active while the remaining extracts showed moderate activity against *E. histolytica* (Table 1). Therefore, benzene and ethyl acetate extracts were selected for the isolation of active constituents and were fractionated by column chromatography, which led to the isolation of umbelliferone **1** and skimmin **2** (Fig. 1). The structures of these compounds were elucidated by spectroscopic methods (IR, ^{1}H NMR, ^{13}C NMR, DEPT and ESI-MS) and compared with the literature data [18,21].

These isolated compounds were then assessed *in vitro* for antiamoebic activity and it was found that umbelliferone ($IC_{50} = 1.04$ μg/ml) and skimmin ($IC_{50} = 1.35$ μg/ml) exhibited better activity than their corresponding extracts. To the best of our knowledge, this is the first report that umbelliferone and skimmin inhibit the growth of *E. histolytica*.

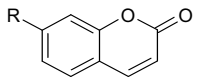
3.2. Pharmaco-modulation of umbelliferone

Umbelliferone (7-hydroxycoumarin) **1** was initially reacted with acetic anhydride to give 7-acetoxycoumarin **1a**, which was subjected to Fries rearrangement by treating with aluminium chloride to give 7-hydroxy-8-acetylcoumarin **2a** as the main product [22,23]. Various thiosemicarbazides **a–e** were prepared according to the literature procedure [24]. A series

Table 1
In vitro antiamoebic activity of the extracts HM1:IMSS strain of *E. histolytica*

Extract	IC_{50} (mg/ml)	SD[a]
n-Hexane	37.1	0.53
Benzene	2.92	0.32
Chloroform	20.33	0.21
Ethyl acetate	2.5	0.18
Acetone	42.02	0.25
n-Butanol	16.04	0.1

[a] Standard deviation.



1. R = OH (Umbelliferone)

2. R = OGlc (Skimmin)

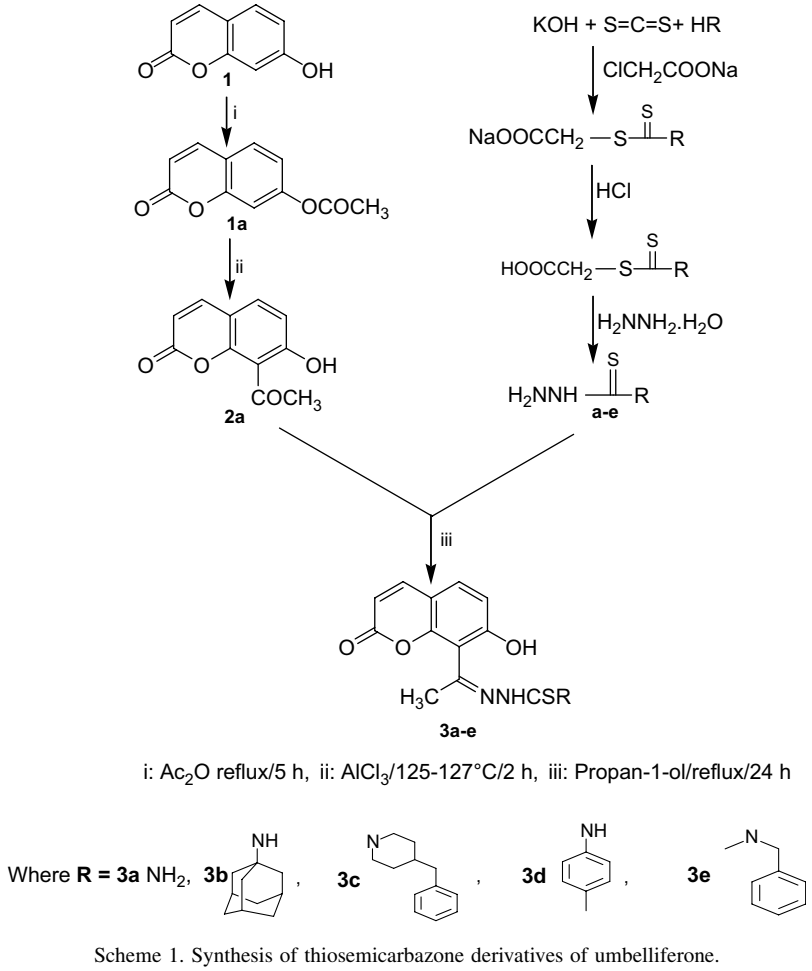
Fig. 1. Structure of isolated compounds.

of thiosemicarbazones **3a–e** of 7-hydroxy-8-acetylcoumarin with different thiosemicarbazides were synthesized [25] (Scheme 1). Umbelliferone was also allowed to react with dimethyl sulphate and potassium carbonate in dry acetone to prepare its methyl derivative **4** (7-methoxycoumarin) (Scheme 2). All compounds **1a**, **2a**, **3a–e**, **4** were crystallized from appropriate solvents and were characterized by electronic, IR, ^{1}H and ^{13}C NMR spectra.

The formation of compound **1a** was confirmed by IR spectrum which showed the absence of OH group absorption around 3400 cm^{-1}, while strong bands were recorded in the spectrum at 1680–1780 cm^{-1} (C=O, lactone), and 1620 cm^{-1}, 1565 cm^{-1} (C=C, aromatic). The ^{1}H NMR spectrum showed the absence of a broad singlet at δ 8.40 due to OH group and the presence of a singlet at δ 2.02 due to CH_3 group. The structure was further supported by ^{13}C NMR spectra. A characteristic signal for acetyl (C=O) appeared at δ 169 and a signal at δ 19.7 revealed the presence of methyl group.

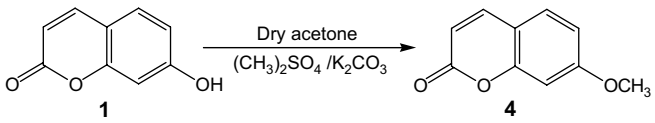
The formation of compound **2a** was confirmed by its IR spectrum which showed the presence of OH group absorption around 3400 cm^{-1} and usual strong bands were recorded in the spectrum at 1680–1780 cm^{-1} (C=O, lactone). The structure was further confirmed by ^{1}H NMR spectrum, which showed the presence of a signal at δ 12.3 due to (OH) proton and a singlet at δ 3.1 due to ($COCH_3$) protons.

The structure of the compounds **3a–e** was confirmed by the characteristic IR bands observed at 3380 and 3233–3115 cm^{-1} assigned to ν(OH) and ν(NH), respectively. A band at 1726 cm^{-1} was assigned to ν(C=O) of the lactone moiety. This band remained unchanged in the final products, indicating that the lactone oxygen was not involved in thiosemicarbazone formation. The appearance of ν(C=S) at 1110–1160 cm^{-1} and ν(C=N) at 1610–1680 cm^{-1} further confirmed the formation of the final products **3a–e**. The ^{1}H NMR spectrum of **3a–e** gave signals at δ 11.58 and 10.07 assigned to (NH–C=S) and (OH) protons, respectively, whereas a signal at δ 8.36 may be assigned to the (NHR) proton. The coumarin ring protons appeared as doublets centered at δ 6.12 and 7.98 and the methyl proton signal at δ 2.31. The formation of **3a–e** was further confirmed by ^{13}C NMR spectra. A characteristic



i: Ac_2O reflux/5 h, ii: $AlCl_3$/125-127°C/2 h, iii: Propan-1-ol/reflux/24 h

Scheme 1. Synthesis of thiosemicarbazone derivatives of umbelliferone.



Scheme 2. Methylation of umbelliferone.

signal for (C═S) appeared at 172 and a signal at δ 166 showed the presence of (C═N) of thiosemicarbazone.

The formation of compound **4** was confirmed by IR spectrum, which revealed the absence of (OH) band around 3400 cm^{-1}, while strong absorption band at 1680–1683 cm^{-1} was assigned to the coumarin ν(C═O). The ^1H NMR spectrum of compound **4** showed the absence of a broad singlet at δ 8.40 due to OH group, while the presence of a singlet at δ 3.25 due to CH_3 group showed the conversion of OH into OCH_3. The formation of compound **4** was further supported by the appearance of a signal due to (OCH_3) at δ 50 in ^{13}C NMR spectrum.

All the compounds **1a**, **2a**, **3a–e**, **4** were then assessed for *in vitro* antiamoebic activity and it was found that the activity of compounds **3a–e** with IC_{50} values of 4.46, 1.49, 1.56, 3.17 and 1.06 μM/ml, respectively, was lower than that of the parent compound **1** ($IC_{50} = 6.38$ μM/ml). The activity drastically increased on converting compound **2a** into its thiosemicarbazone derivatives **3a–e** with IC_{50} values ranging between 1.06 and 4.46 μM/ml. Compounds **3b**, **c**, and **e** with IC_{50} values of 1.49, 1.56, and 1.06 μM/ml, respectively, exhibited even higher antiamoebic activity than the standard drug metronidazole ($IC_{50} = 2.63$ μM/ml). The activity of 7-methoxycoumarin ($IC_{50} = 8.92$ μM/ml) was less than umbelliferone (Table 2).

In our previous work regarding the antiamoebic activity of thiosemicarbazone derivatives by using different amines, we herein give a comparative study of the present work with the previous one. The 3,7-dimethyl-pyrazolo [3,4e] [1,2,4] triazine-4-yl thiosemicarbazides derivatives substituted with adamantamine showed better activity whereas when substituted with *p*-toluidine showed less activity than metronidazole [26]. Similarly 1-*N*-substituted thiocarbamoyl-3,5-diphenyl-2-pyrazoline derivatives substituted with adamantamine showed better activity than metronidazole [27].

Table 2
In vitro antiamoebic activity of the isolated compounds and their thiosemicarbazone derivatives against HM1:IMSS strain of *E. histolytica*

Compound	IC_{50} (μM/mL)	SD[a]
Umbelliferone (**1**)	6.380	0.30
Skimmin (**2**)	4.15	0.11
1a	13.08	2.3
2a	6.51	2.1
3a	4.46	0.9
3b	1.49	0.27
3c	1.56	0.16
3d	3.17	0.19
3e	1.06	0.2
4	8.92	0.3
Metronidazole	2.63	0.08

[a] Standard deviation.

Palladium (II) complexes of 5-nitrothiophene-2-carboxaldehyde thiosemicarbazones substituted with adamantamine showed better activity whereas when substituted with *p*-toluidine and *N*-methyl benzyl amine showed less activity than metronidazole [28].

In the present work the coumarin thiosemicarbazone derivatives substituted with adamantamine, *p*-benzyl piperidine and *N*-methyl benzyl amine showed better activity whereas when substituted with *p*-toluidine showed less activity than metronidazole. These results thus indicated that the antiamoebic activity enhances by the introduction of adamantamine, *p*-benzyl piperidine and *N*-methyl benzyl amine.

Toxicity profile. To ensure the toxicity of the compounds **3b**, **c** and **e** with better IC_{50} values than metronidazole, they were tested against H9c2 cardiac myoblasts. A subconfluent population of H9c2 cells was treated with increasing concentrations of compounds **3b**, **c** and **e**, and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of compounds **3b**, **c** and **e** was 3.125–200 μg/ml. Fig. 2 depicted that compounds **3b**, **c** and **e** exhibited >80% viability at the concentration range of 3.125–200 μg/ml.

4. Experimental

4.1. Extraction and isolation of umbelliferone and skimmin from A. cordifolia

Dried and well-ground root bark (4 kg) was extracted by refluxing with methanol (5 × 10 L, 3 h each). The insoluble material was removed by filtration and the extract was concentrated under reduced pressure. The dried, crude methanolic extract (1.5 kg, 37.5%) was then fractionated successively by refluxing with *n*-hexane, benzene, chloroform, ethyl acetate, acetone and *n*-butanol (3 × 2.5 L, 3 h each) to give the hexane (20 g, 1.33%), benzene (8 g, 0.53%), chloroform (15 g, 1%), ethyl acetate (13 g, 0.86%), acetone (25 g, 1.66%) and *n*-butanol (23 g, 1.35%) extracts, respectively.

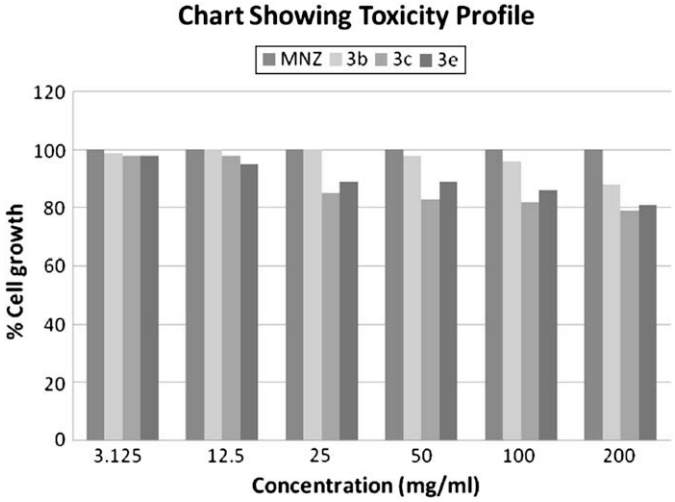


Fig. 2. ■

The benzene and ethyl acetate extracts showed inhibition of *E. histolytica* and were selected for the isolation of active constituents.

The benzene extract (8 g) was chromatographed over a silica gel column (60–120 mesh) eluting with C_6H_6–EtOAc mixtures of increasing polarity. Upon concentration, the fractions eluted with 9.5:0.5 (v/v) C_6H_6–EtOAc gave a cream coloured solid powder, which was identified as umbelliferone **1** (3 g, 37.5%). The ethyl acetate extract (13 g) was chromatographed over a column of silica gel (60–120 mesh), eluting with $CHCl_3$–MeOH mixtures of increasing polarity. The fractions eluted with 9.5:0.5 (v/v) $CHCl_3$–MeOH upon concentration gave a white powder, identified as skimmin **2** (2.2 g, 16.9%).

4.1.1. Umbelliferone *1*

M.p.: 234–237 °C; cream coloured solid; UV λ_{max} nm: 296, 256, 226; IR ν_{max} cm^{-1}: 3195, 1680, 1603, 1567; ^1H NMR (DMSO-d_6) δ (ppm): 7.70 (d, 1H, H-4, $J = 9.5$ Hz), 7.40 (d, 1H, H-5, $J = 10.3$ Hz), 6.83 (d, 1H, H-8, $J = 2$ Hz due to m-coupling with HC-6), 6.78 (dd, 1H, H-6, $J = 10.3$ Hz, 2 Hz), 6.20 (d, 1H, H-3, $J = 9.5$ Hz); ^{13}C NMR (DMSO-d_6) δ (ppm): 160.93 (C-2), 161.74 (C-7), 155.94 (C-9), 144.95 (C-4), 130.14 (C-5), 113.57 (C-3), 111.84 (C-10), 111.72 (C-6), 102.61 (C-8); ESI-MS *m/z*: 163 $[M + H]^+$.

4.1.2. Skimmin *2*

M.p.: 221–222 °C; white powder; UV λ_{max} nm: 294, 257, 224; IR ν_{max} cm^{-1}: 3370, 1683, 1615, 1562; ^1H NMR (DMSO-d_6) δ (ppm): 7.80 (d, 1H, H-4, $J = 9.5$ Hz), 7.52 (d, 1H, H-5, $J = 10$ Hz), 6.71 (d, 1H, H-8, $J = 2$ Hz due to m-coupling with HC-6), 6.55 (dd, 1H, H-6, $J = 10$ Hz), 6.45 (d, 1H, H-3, $J = 9.5$ Hz), 4.80 (d, 1H, H-1′, $J = 7.6$ Hz), 3.87 (dd, 1H, H-6b′, $J = 12.0$, 1.0 Hz), 3.68 (dd, 1H, H-6a′, $J = 12.0$, 5.0 Hz), 3.60 (t, 1H, H-3′, $J = 8.5$ Hz), 3.42 (dd, 1H, H-4′, $J = 9.5$, 8.5 Hz), 3.30–3.41 (m, 1H, H-5′), 3.29 (dd, 1H, H-2′, $J = 9.0$, 7.5 Hz); ^{13}C NMR (DMSO-d_6) δ (ppm): 160.73 (C-7), 160.67 (C-2), 155.48 (C-9), 144.73 (C-4), 129.90 (C-5), 114.11 (C-3), 113.73 (C-10), 113.60 (C-6), 103.60 (C-8), 100.40 (C-1′), 77.58 (C-5′), 76.35 (C-3′), 73.57 (C-2′), 70.07 (C-4′), 61.09 (C-6′); ESI-MS *m/z*: 325$[M + H]^+$, 347$[M + Na]^+$.

4.2. Synthesis of 7-acetoxycoumarin *1a*

Umbelliferone (0.005 mol) was refluxed with acetic anhydride for 5 h and the reaction mixture was cooled, poured onto the ice and left overnight. The precipitate was filtered, dried and crystallized by acetone to afford the acetoxy product 7-acetoxycoumarin **1a**. Yield: 88%; m.p.: 140 °C; white powder; IR ν_{max} cm^{-1}: 3184 (Ar-H), 2910 (C-H), 1780 (C=O, lactone), 1636, 1560 (C=C, aromatic), 1215 (C–O); ^1H NMR (DMSO-d_6) δ (ppm): 7.90 (d, 1H, H-4, $J = 8.8$ Hz), 7.50 (d, 1H, H-5, $J = 9.5$ Hz), 7.02 (d, 1H, H-8, $J = 2.2$ Hz due to m-coupling with HC-6), 6.95 (dd, 1H, H-6, $J = 9.5$ Hz, $J = 2.2$ Hz), 6.10 (d, 1H, H-3, $J = 8.8$ Hz), 2.95 (s, 3-H, CH_3); ^{13}C NMR (DMSO-d_6) δ (ppm): 172.00 (O–C=O), 160.95 (C-2), 156.73 (C-7), 152.63 (C-9), 144.95 (C-4), 126.42 (C-5), 119.86 (C-6), 117.11 (C-10), 113.5 (C-3), 106.90 (C-8), 19.79 (CH_3); ESI-MS *m/z*: 205 $[M + H]^+$.

4.3. Synthesis of 7-hydroxy-8-acetylcoumarin *2a* (Fries rearrangement)

A mixture of 7-acetoxycoumarin (24.5 mmol), anhydrous aluminium chloride (74.9 mmol) and anhydrous sodium chloride (34.4 mmol) was heated at 170 °C for 1.5 h. The mixture was allowed to cool and dil. HCl (50 ml) was added. The reaction mixture was refluxed for 10 min, diluted with water (200 ml) and extracted three times (100 ml) with ethyl acetate, dried over sodium sulphate. The solvent was evaporated and solid residue was subjected to column chromatography and eluted with $CHCl_3$ yielded 7-hydroxy-8-acetylcoumarin (**2a**) free from the 6-isomer (7-hydroxy-6-acetylcoumarin). Yield: 60%; m.p.: 180 °C; light brown crystals; IR ν_{max} cm^{-1}: 3185 (Ar-H), 2890 (C-H), 1785 (C=O, lactone), 1640, 1555 (C=C, aromatic); ^1H NMR (DMSO-d_6) δ (ppm): 12.76 (s, 1H, OH), 7.76 (d, 1H, H-4, $J = 9.5$ Hz), 7.52 (d, 1H, H-5, $J = 8.6$ Hz), 6.78 (d, 1H, H-6, $J = 8.6$ Hz), 6.32 (d, 1H, H-3, $J = 9.5$ Hz), 2.95 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ (ppm): 195.50 (C=O), 161.4 (C-2), 159.9 (C-7), 152.31 (C-9), 144.60 (C-4), 136.42 (C-5), 120.21 (C-8), 116.80 (C-10), 113.71 (C-3), 111.51 (C-6), 27.90 (CH_3); ESI-MS *m/z*: 205 $[M + H]^+$.

4.4. General procedure for the synthesis of substituted thiosemicarbazides *a–e*

4.4.1. Preparation of substituted thioglycolic acid

Carbon disulphide (1 equiv.) was added drop wise to a solution of primary or secondary amines (1 equiv.) containing potassium hydroxide (1 equiv.) in water:ethanol (1:3) mixture. The temperature of the reaction was maintained below 10 °C. Sodium chloroacetate (1 equiv.) was added and the reaction mixture was left overnight at room temperature. Addition of conc. hydrochloric acid (pH ~ 1) precipitated substituted thioglycolic acid, which was crystallized by appropriate solvent.

4.4.2. Conversion of thioglycolic acid into thiosemicarbazides *a–e*

A solution of thioglycolic acid (1 equiv.) in water (15 ml) containing sodium hydroxide (1 equiv.) and hydrazine hydrate (1 equiv.) was refluxed for 2 h with continuous stirring. The compound separated out during the reaction or on cooling at 0 °C for 12 h. The product was filtered and crystallized from suitable solvent.

4.5. Preparation of thiosemicarbazones of 7-hydroxy-8-acetylcoumarin *3a–e*

A mixture of 7-hydroxy-8-acetylcoumarin **2a** (5 mmol) and the corresponding thiosemicarbazides, in the molar ratio 1:1 was refluxed in 1-propanol (20 ml) for 24 h. The mixture

was allowed to cool and the resulting precipitate was filtered and subsequently dried to afford the pure products **3a–e**.

4.5.1. 7-Hydroxy-8-acetylcoumarin-N(4) thiosemicarbazone **3a**

Yield: 55%; m.p.: 195 °C; brown solid; calculated for $C_{12}H_{11}O_3N_3S$: C, 51.9; H, 3.9; N, 15.2%. Found: C, 52.2; H, 3.7; N, 15.1%. IR ν_{max} cm^{-1}: 3380 (O–H), 3195 (N–H), 1726 (C=O), 1665 (C=N), 1145 (C=S); ^1H NMR (DMSO-d_6) δ (ppm): 12.18 (s, 1H, HN–C=S), 10.21 (s, 1H, OH), 8.36 (s, 2H, NHR), 7.57 (d, 1H, H-4, $J = 9.3$ Hz), 7.21 (d, 1H, H-5, $J = 8.4$ Hz), 6.45 (d, 1H, H-6, $J = 8.4$ Hz), 6.12 (d, 1H, H-3, $J = 9.3$ Hz), 2.23 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ (ppm): 180.6 (C=S), 170.21 (C=N), 160.90 (C=O), 158.51 (C-7), 151.42 (C-9), 144.43 (C-4), 133.91 (C-5), 121.50 (C-8), 117.57 (C-10), 113.32 (C-3), 112.70 (C-6), 18.41 (CH_3). ESI-MS *m/z*: 278 $[M + H]^+$.

4.5.2. 7-Hydroxy-8-acetylcoumarin-N(4)adamentamine thiosemicarbazone **3b**

Yield: 43%; m.p.: 210 °C; brown solid; calculated for $C_{22}H_{25}O_3N_3S$: C, 64.2; H, 6.1; N, 10.2%. Found: C, 64.5; H, 6.2; N, 10.1%. IR ν_{max} cm^{-1}: 3410 (O–H), 3205 (N–H), 1722 (C=O), 1676 (C=N), 1145 (C=S); ^1H NMR (DMSO-d_6) δ (ppm): 12.51 (s, 1H, HN–C=S), 10.05 (s, 1H, OH), 8.06 (s, 1H, NHR), 7.35 (d, 1H, H-4, $J = 9.2$ Hz), 7.11 (d, 1H, H-5, $J = 8.3$ Hz), 6.52 (d, 1H, H-6, $J = 8.3$ Hz), 6.22 (d, 1H, H-3, $J = 9.2$ Hz), 2.22 (s, 3H, CH_3), 1.03–2.31 (m, 15H, adamentyl ring); ^{13}C NMR (DMSO-d_6) δ (ppm): 179.80 (C=S), 168.71 (C=N), 161.30 (C=O), 158.70 (C-7), 152.31 (C-9), 144.62 (C-4), 130.91 (C-5), 120.11 (C-8), 116.20 (C-10), 112.51 (C-3), 110.21 (C-6), 25–47 (adamentyl ring), 16.70 (CH_3); ESI-MS *m/z*: 412 $[M + H]^+$.

4.5.3. 7-Hydroxy-8-acetylcoumarin-N(4)4-benzylpiperidine thiosemicarbazone **3c**

Yield: 40%; m.p.: 160 °C; red solid; calculated for $C_{24}H_{25}O_3N_3S$: C, 66.2; H, 5.7; N, 9.6%. Found: C, 66.5; H, 5.4; N, 9.3%. IR ν_{max} cm^{-1}: 3385 (O–H), 3190 (N–H), 1740 (C=O), 1650 (C=N), 1077 (C=S); ^1H NMR (DMSO-d_6) δ (ppm): 11.95 (s, 1H, HN–C=S), 10.90 (s, 1H, OH), 7.36 (d, 1H, H-4, $J = 8.8$ Hz), 7.12 (d, 1H, H-5, $J = 8.2$ Hz), 7.02–7.75 (m, 5H, Ph), 6.33 (d, 1H, H-6, $J = 8.2$ Hz), 6.21 (d, 1H, H-3, $J = 8.8$ Hz), 2.68–3.16 (m, 4H, $-NCH_2$), 2.63 (d, 2H, CH_2, $J = 7.3$ Hz), 2.13 (s, 3H, CH_3), 1.60–1.86 (m, 1H, CH), 1.32–1.87 (m, 4H, CH_2); ^{13}C NMR (DMSO-d_6) δ (ppm): 179.30 (C=S), 169.21 (C=N), 160.60 (C=O), 159.51 (C-7), 150.00 (C-9), 144.22 (C-4), 132.51 (C-5), 140.41, 128.90, 124.72 (Ph), 118.00 (C-8), 116.61 (C-10), 112.55 (C-3), 108.93 (C-6), 52.62 ($2 \times CH_2$), 44.33 (CH_2), 33.12 (CH), 29.80 ($2 \times CH_2$), 17.72 (CH_3); ESI-MS *m/z*: 437 $[M + H]^+$.

4.5.4. 7-Hydroxy-8-acetylcoumarin-N(4)p-toluidine thiosemicarbazone **3d**

Yield: 43%; m.p.: 173 °C; white solid; calculated for $C_{19}H_{17}O_3N_3S$: C, 62.1; H, 4.6; N, 11.4%. Found: C, 62.3; H, 4.4; N, 11.3%. IR ν_{max} cm^{-1}: 3390 (O–H), 3154 (N–H), 1720 (C=O), 1660 (C=N), 1123 (C=S); ^1H NMR (DMSO-d_6) δ (ppm): 11.85 (s, 1H, HN–C=S), 10.03 (s, 1H, OH), 8.62 (s, 1H, NHR), 7.91 (d, 1H, H-4, $J = 9.5$ Hz), 7.62 (d, 1H, H-5, $J = 8.2$ Hz), 7.50 (d, 2H, $J = 7.8$ Hz, Ph), 7.07 (d, 2H, $J = 7.8$ Hz, Ph), 6.38 (d, 1H, H-6, $J = 8.2$ Hz), 6.11 (d, 1H, H-3, $J = 9.5$ Hz), 2.35 (s, 3H, CH_3), 2.22 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ (ppm): 182.51 (C=S), 172.41 (C=N), 160.23 (C=O), 159.80 (C-7), 152.42 (C-9), 146.31, 128.81, 126.22, 114.20 (Ph), 144.30 (C-4), 130.91 (C-5), 122.50 (C-8), 116.21 (C-10), 113.40 (C-3), 110.90 (C-6), 25.31 (CH_3), 16.80 (CH_3); ESI-MS *m/z*: 368 $[M + H]^+$.

4.5.5. 7-Hydroxy-8-acetylcoumarin-N(4,4)methylbenzyl thiosemicarbazone **3e**

Yield: 36%; m.p.: 187 °C; white solid; calculated for $C_{20}H_{19}O_3N_3S$: C, 62.9; H, 4.9; N, 11.0%. Found: C, 63.2; H, 4.7; N, 10.9%. IR ν_{max} cm^{-1}: 3405 (O–H), 3216 (N–H), 1734 (C=O), 1613 (C=N), 1121 (C=S); ^1H NMR (DMSO-d_6) δ (ppm): 12.13 (s, 1H, HN–C=S), 10.22 (s, 1H, OH), 7.79 (d, 1H, H-4, $J = 9.6$ Hz), 7.31 (d, 1H, H-5, $J = 8.8$ Hz), 7.04–7.24 (m, 5H, Ph), 6.42 (d, 1H, H-6, $J = 8.8$ Hz), 6.11 (d, 1H, H-3, $J = 9.6$ Hz), 5.21 (s, 2H, CH_2), 3.18 (s, 3H, CH_3), 2.12 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ (ppm): 180.20 (C=S), 169.51 (C=N), 160.80 (C=O), 159.31 (C-7), 153.41 (C-9), 144.62 (C-4), 140.51, 129.51, 126.12 (Ph), 132.90 (C-5), 121.71 (C-8), 117.54 (C-10), 112.51 (C-3), 108.42 (C-6), 59.00 (CH_2), 38.82 (CH_3), 16.41 (CH_3); ESI-MS *m/z*: 382 $[M + H]^+$.

4.6. Preparation of 7-methoxycoumarin **4**

Umbelliferone (200 mg) was refluxed in dry acetone (50 ml) with dimethyl sulphate (0.5 ml) and anhydrous potassium carbonate (5 g) for 72 h till it did not give any colour with $FeCl_3$. The reaction mixture was then allowed to cool at room temperature and filtered off insoluble potassium carbonate, which was washed several times with small portions of dry acetone. The washings and filtrate were combined and evaporated to dryness. A cream coloured solid was left which was washed with petroleum ether and then with water. Yield: 80%; m.p.: 90–92 °C; cream coloured crystals; IR ν_{max} cm^{-1}: 3165 (Ar-H), 2890 (C-H), 1680 (C=O), 1603, 1555 (C=C, aromatic); ^1H NMR (DMSO-d_6) δ (ppm): 7.82 (d, 1H, H-4, $J = 9.5$ Hz), 7.46 (d, 1H, H-5, $J = 9.2$ Hz), 6.94 (m, 1H, H-8, $J = 2.0$ Hz), 6.53 (m, 1H, H-6, $J = 10.11$ Hz), 6.12 (d, 1H, H-3, $J = 9.5$ Hz), 3.25 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ (ppm): 160.91 (C-2), 158.30 (C-7), 151.32 (C-9), 144.35 (C-4), 127.28 (C-5), 115.50 (C-10), 112.51 (C-3), 111.20 (C-6), 107.91 (C-8), 57.23 (CH_3); ESI-MS *m/z*: 177 $[M + H]^+$.

4.7. In vitro antiamoebic assay

E. histolytica trophozoites were cultured in wells of 96-well microtiter plate by using Diamond TYIS-33 growth medium [29]. All the extracts and isolated compounds/derivatives

were screened *in vitro* for antiamoebic activity against HM1:IMSS strain of *E. histolytica* by microdilution method [30]. All the extracts (10 mg) and compounds/derivatives (1 mg) were dissolved in DMSO (40 μl, level at which no inhibition of amoeba occurs) [31,32]. The stock solutions of the extracts and the compounds/derivatives were prepared freshly before use at a concentration of 10 and 1 mg/ml, respectively. Twofold serial dilutions were made in the wells of 96-well microtiter plate (costar). Each test includes metronidazole as a standard amoebicidal drug, control wells (culture medium plus amoebae) and a blank (culture medium only). All the experiments were carried out in triplicate at each concentration level and repeated thrice. The amoebal suspension was prepared from a confluent culture by pouring off the medium at 37 °C and adding 5 ml of fresh medium, chilling the culture tube on ice to detach the organisms from the side of the flask. The suspension was diluted to 10^5 organisms/ml by adding fresh medium and 170 μl of this suspension was added to the test and control wells in the plate so that the wells were completely filled (total volume, 340 μl). An inoculum of 1.7×10^4 organisms/well was chosen so that confluent, but not excessive growth, took place in control wells. Plates were sealed and gassed for 10 min with nitrogen before incubation at 37 °C for 72 h. After incubation, the growth of amoeba in the plate was checked with a low power microscope. The culture medium was removed by inverting the plate and shaking gently. Plate was then immediately washed with sodium chloride solution (0.9%) at 37 °C. This procedure was completed quickly and the plate was not allowed to cool in order to prevent the detachment of amoebae. The plate was allowed to dry at room temperature and the amoebae were fixed with methanol and when dried, stained with (0.5%) aqueous eosin for 15 min. The stained plate was washed once with tap water, then twice with distilled water and allowed to dry. A 200 μl portion of 0.1 N sodium hydroxide solution was added to each well to dissolve the protein and release the dye. The optical density of the resulting solution in each well was determined at 490 nm with a microplate reader. The % inhibition of amoebal growth was calculated from the optical densities of the control and test wells and plotted against the logarithm of the dose of the drug tested. Linear regression analysis was used to determine the best fitting line from which the IC_{50} value was found. The IC_{50} values in μg/ml and μM/ml are reported in Table 1 and Table 2, respectively.

MTT assay. H9c2 rat cardiac myoblasts were cultured and maintained as monolayer in Dulbecco's modified Eagle's medium (DMEM), high glucose, supplemented with 10% fetal bovine serum (heat inactivated), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B, at 37 °C in humidified incubator with 5% CO_2 [33]. Cells were incubated with different concentrations of metronidazole and compounds **3b**, **c** and **e**, for 48 h at 37 °C in 5% CO_2 humidified incubator together with untreated control sample. At appropriate time points, cells were washed in PBS, treated with 600 μl MTT solution (5 mg/ml, tetrazolium salt) and incubated for 45 min at 37 °C. After 45 min of incubation at 37 °C, the cell supernatants were discarded, MTT crystals were dissolved with acid isopropanol and the absorbance measured at 570 nm. All assays were performed in triplicate. Percent viability was defined as the relative absorbance of treated versus untreated control cells. Plates were analyzed in an ELISA plate reader (Labsystems Multiskan RC, Helsinki, Finland) at 570 nm with a reference wavelength of 655 nm.

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