WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 4, Issue 3, 1255-1269. Research Article ISSN 2277– 7105

IN VITRO SUSCEPTIBILITY OF *ENTAMOEBA HISTOLYTICA* TO *FRAXINUS MICRANTHA* LEAF EXTRACTS AND ITS ISOLATED CONSTITUENTS (TRITERPENES AND COUMARINS)

Prince Firdoos Iqbal[1], Humaira Parveen[2], Abdul Roouf Bhat[2] and Amir Azam[2]*

[1]Department of Chemistry - Govt. Degree College (Boys) Pulwama, Kashmir, India.

[2]Department of Chemistry - Jamia Millia Islamia, Jamia Nagar, New Delhi 110025 India.

Article Received on
19 Dec 2014,

Revised on 13 Jan 2015,
Accepted on 07 Feb 2015

***Correspondence for Author**
Dr. Amir Azam
Department of Chemistry - Jamia Millia Islamia, Jamia Nagar, New Delhi 110025 India.

ABSTRACT

The activity of n-hexane, benzene, chloroform, ethyl acetate, acetone, n-butanol and crude methanolic extracts from the leaves of *Fraxinus micrantha* was tested against *HM1: IMSS* strain of *Entamoeba histolytica*. Results showed that benzene; ethyl acetate and n-butanol extracts were most active with an IC_{50} of 0.39µg/ml, 0.41µg/ml and 0.43µg/ml, respectively. Chromatographic separation of these extracts led to the isolation of a pentacyclic triterpene acid (3β-hydroxy-urs-12-en-28-oic acid), commonly known as ursolic acid, 6,7-dihydroxy-2H-1-benzopyran-2-one (esculetin) and 6-(β-D-glucopyranosyloxy)-7-hydroxy-2H-1-benzopyran-2-one (esculin). These compounds were derivatize to yield their methoxy and acetoxy derivatives and then subjected to *in vitro* antiamoebic activity. Ursolic acid, esculetin and esculin showed promising activity with an IC_{50} of 2.20 µg/ml, 2.25µg/ml and 2.97 µg/ml, respectively. Methyl ursolate (IC_{50} = 3.11 µg/ml) and acetyl ursolic acid (IC_{50} = 2.87 µg/ml) showed less activity than ursolic acid. Methoxy derivative (6,7-dimethoxycoumarin) (IC_{50} = 1.95 µg/ml) showed higher activity whereas acetoxy derivative (6,7-diacetoxycoumarin) (IC_{50} = 2.74 µg/ml) showed less activity than the parent compound esculetin. These findings support the traditional use of *Fraxinus micrantha* leaves for the treatment of amoebic dysentery.

KEYWORDS: Fraxinus micrantha; Triterpenes; Coumarins; Entamoeba histolytica.

1. INTRODUCTION

The genus *Fraxinus* (Oleaceae) is distributed mostly in the temperate regions and the subtropics of the Northern hemisphere.[1] *Fraxinus* species attract considerable attention for

their medicinal properties and find application in the folk medicine, as well as in the contemporary medicine.[2-4] The bark and the leaves of *F. excelsior* and *F. ornus* are applied in the Bulgarian and Polish folk medicine against various diseases, including wound healing, diarrhea and dysentery.[2,5,6] *Fraxinus micrantha* have been used in folk medicine in different parts of India for its diuretic and mild purgative effects as well as for the treatment of constipation, dropsy, arthritis, rheumatic pain, cystitis and itching scalp.[7] The leaves of *Fraxinus micrantha* L. (Oleaceae), are traditionally used in folklore medicine for the treatment of dysentery in different parts of India especially in Pauri-Garhwal region of Uttrakhand, India.[8]

Amoebiasis, a disease caused by *E. histolytica*, remains one of the major threats to public health in most parts of the globe and is considered to be the second or third leading cause of death amongst the parasitic diseases.[9] More than 50 million people worldwide are infected and up to 110,000 die every year due to amoebiasis.[10] Metronidazole is known to be 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.[11-13] In addition, metronidazole has several adverse effects for which the most common are gastrointestinal disturbances, especially nausea, vomiting and diarrhoea or constipation may also occur.[14] Due to its adverse effects and the emergence of drug resistance,[15,16] it is desirable to search new amoebicidal agents better than the actually available medication.

The use of medicinal plants by people in developing countries is popular 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. 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 leaves of *Fraxinus micrantha.* In this study we report the antiamoebic activity of extracts, isolated compounds and their derivatives from the leaves of *Fraxinus micrantha.*

2. CHEMISTRY

2.1. Plant material

Fraxinus micrantha leaves were 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- 278) has been deposited in the herbarium of the Department of Bioscience.

2.2. Analytical material and methods

IR spectra were recorded as KBr discs on Perkin-Elmer FT-IR spectrum RX1 spectrophotometer. ^{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. 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

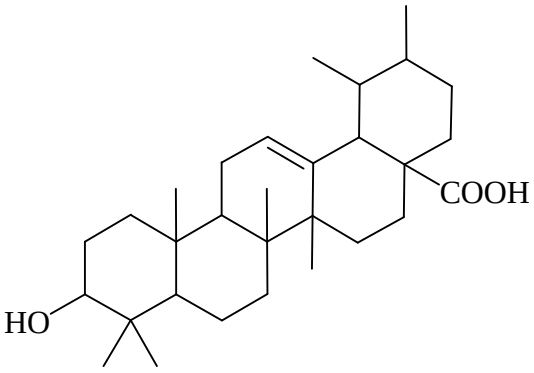
3.1. Identification of isolated compounds

Powdered and dried leaves of *Fraxinus micrantha* L. (Oleaceae) were exhaustively extracted with methanol. The extract was concentrated under *vacuo*. The residue was then fractionated successively. The fractions were subjected for *in vitro* antiamoebic activity against *HM1:IMSS* strain of *E. histolytica* using metronidazole as a reference drug. It was found that the benzene, ethyl acetate and n-butanol extracts (IC_{50} = 0.39 μg/ml, 0.41 μg/ml and 0.43 μg/ml, respectively) exhibited higher antiamoebic activity than the standard drug metronidazole (IC_{50} = 0.45 μg/ml) (**Table 1**) and were selected for the isolation of active constituents. Biologically monitored fractions benzene, ethyl acetate and n-butanol extracts were subjected to column chromatography, which led to the isolation of ursolic acid **1**, esculetin **2** and esculin **3**, respectively (**Fig. 1**). The identities of these compounds were established by comparisons of their spectroscopic (IR, ^{1}H NMR, ^{13}C NMR, DEPT and ESI-MS) data with those in the literature.[17-19] The structure of esculetin **2** was further confirmed by X-ray diffraction studies (**Fig. 2, Table 2 & 3**).

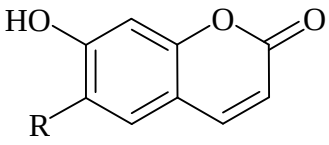
Table 1. In vitro antiamoebic activity of the extracts and isolated compounds/derivatives from the leaves of *F. micrantha* against *HM1:IMSS* strain of *E. histolytica*

Extract/Compound	IC_{50} (µg/ml)	SD[a]
Methanol	4.36	0.53
n-Hexane	12.5	2.2
Benzene	0.39	0.3
Chloroform	8.80	2.0
Ethyl acetate	0.41	0.7
Acetone	9.95	1.5
n-Butanol	0.43	0.61
Ursolic acid (1)	2.20	0.42
Esculetin (2)	2.25	0.23
Esculin (3)	2.97	0.22
1a	3.11	1.3
1b	2.87	1.6
2a	1.95	0.7
2b	2.74	2.2
Metronidazole	0.45	0.08

[a]Standard deviation

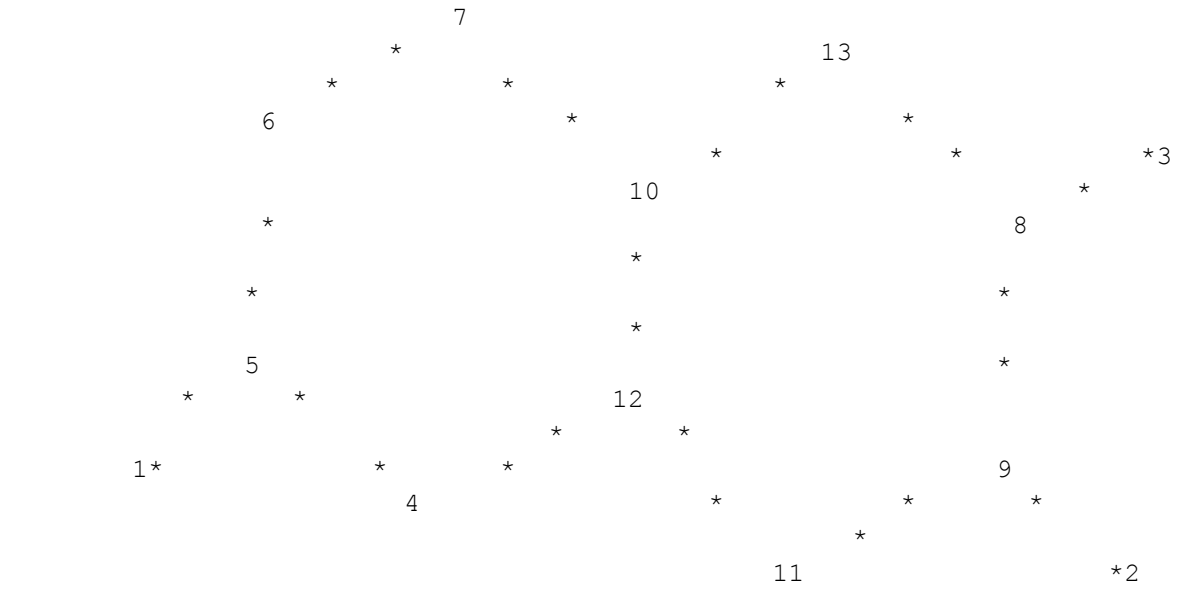


1. Ursolic acid



2. R = OH (Esculetin)
3. R = OGlc (Esculin)

Fig. 1. Structure of isolated compounds



fragment number 1 , 13 peaks included

peak	1	2	3	4	5	6	7	8	9	10	11	12	13
linked to	5	9	8	5	1	5	6	3	2	7	9	4	8
		12	4	7	10	9	8	12	12	10	10		
			6			13	11	13		11			
ring					*	*	*	*	*	*	*	*	*	*

Fig. 2. Crystal structure of esculetin.

Table 2. Crystal data and other experimental details

Crystal description	Colorless prism
Chemical formula	'C_9 H_6 O_4'
Molecular weight	178.14
Cell parameters (A°)	*a*=8.253(2), *b*=6.7809(17), *c*=13.198(3)
Cell angles (◦)	α= 90.00, β=103.525(4), γ=90.00
Unit cell volume (°A^3)	718.1(3)
Crystal system	Monoclinic
Space group	'P 21/c'
Density (calculated) (M gm^{-3})	1.648
No. of molecules per unit cell, *Z*	4
Radiation, wavelength (A°)	Mo K*α*, 0.71073
Temperature (K)	293(2)

Absorption coefficient (μ)	0.132
F (0 0 0)	368
θ range for entire data (◦)	$2.54<\theta<28.31$
Limiting indices	$-6 \le h \le 11$, $-8 \le k \le 8$, $-17 \le l \le 11$
Reflections collected/ unique	4561 / 1749 [R(int)= 0.0341]
Data/restraints/ parameters	1749 / 0 / 119
Final R indices	[I>2σ (I)] $R1$ = 0.0567, $wR2$ = 0.1266
Refinement method	Full-matrix least-squares on F^2
R indices (all data)	$R1$ = 0.790, $wR2$ = 0.1804
Largest diff. peak and hole	0.478 and -0.576 e.Å^{-3}
Weight	$1/[\sigma^2(Fo^2) + (0.0539P)^2 + 1.5982P]$ where $P=[Fo^2 + 2Fc^2]/3$
Goof (S) on F^2	1.183

Table 3. Selected bond lengths (A°) and bond angles (◦) for non-hydrogen atoms (e.s.d.s are given in parentheses)

C1 - O2	1.230(3)	O2 - C1 - O1	116.4(2)
C1 - O1	1.353(3)	O2 - C1 - C2	125.4(2)
C1 - C2	1.439(4)	O1 - C1 - C2	118.2(2)
C2 - C3	1.348(4)	C3 - C2 - C1	120.6(2)
C3 - C4	1.426(4)	C2 - C3 - C4	120.9(2)
C4 - C9	1.398(4)	C9 - C4 - C5	118.3(2)
C4 - C5	1.406(4)	C9 - C4 - C3	117.6(2)
C5 - C6	1.377(4)	C5 - C4 - C3	124.1(2)
C6 - O3	1.358(3)	C6 - C5 - C4	120.9(2)
C6 - C7	1.418(4)	O3 - C6 - C5	119.7(2)
C7 - O4	1.352(3)	O3 - C6 - C7	121.2(2)
C7 - C8	1.385(4)	C5 - C6 - C7	119.0(2)
C8 - C9	1.384(4)	O4 - C7 - C8	124.0(2)
C9 - O1	1.382(3)	O4 - C7 - C6	114.9(2)
		C8 - C7 - C6	121.1(2)
		C9 - C8 - C7	118.4(2)
		O1 - C9 - C8	117.1(2)
		O1 - C9 - C4	120.7(2)
		C8 - C9 - C4	122.2(2)
		C1 - O1 - C9	122.0(2)

The IR spectrum of ursolic acid showed a strong absorption band at 1692 cm^{-1} due to ν(C=O) and another band at 3432 cm^{-1} due to hydroxyl absorption. In ^{1}H NMR spectra a triplet at 5.25 ppm (J = 3.4 Hz) appeared due to H-12 proton suggested the presence of an olefinic double bond. A singlet at 11.2 ppm showed the presence of carboxylic proton. In ^{13}C NMR spectra a signal at 77.31 ppm (C-3) revealed the attachment of (OH) group at position 3 of the

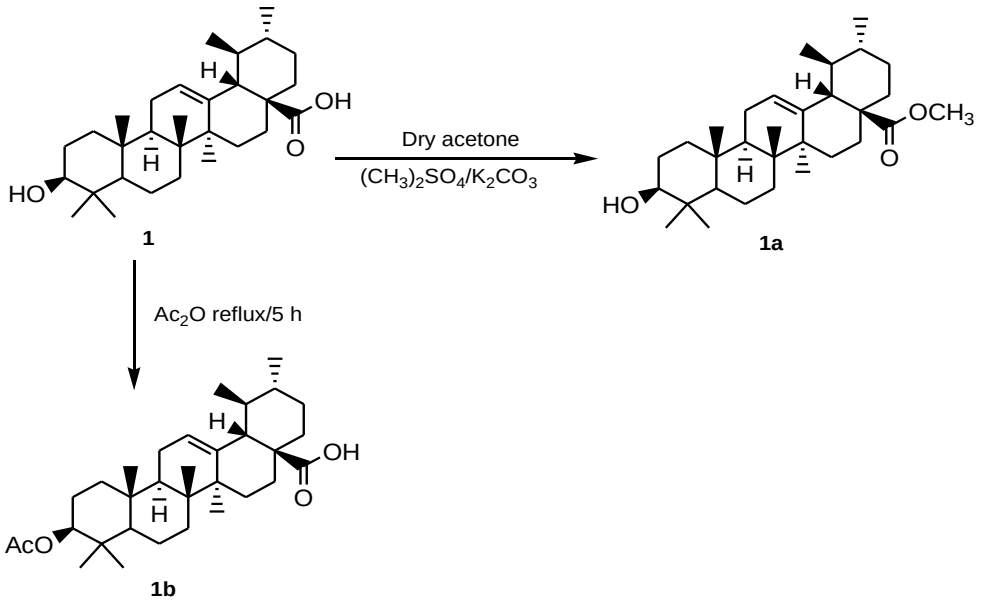
terpene. The signals at 125.04 ppm (C-12) and 138.62 ppm (C13) showed the presence of (C=C) double bond and a characteristic signal for (C=O) was found at 178.77 ppm (C-28).

The IR spectrum of esculetin and esculin showed a strong absorption band at 1666-1683 cm^{-1} which was assigned to the coumarin ν(C=O) and another band at 3328-3375 cm^{-1} was due to hydroxyl absorption. In ^{1}H NMR spectrum a doublet at 6.2-6.18 ppm and 7.87-7.82 ppm due to H-3 and H-4 protons respectively, suggested the presence of olefinic protons at α,β-position to the carbonyl group. A broad singlet at 5.5 ppm confirmed the presence of two hydroxyl groups in esculetin whereas a multiplet in the range of 3.29-4.8 ppm suggested the presence of a glucosyl moiety at (C-6) instead of a hydroxyl group in esculin. The structure was further supported by ^{13}C NMR spectra. A characteristic signal for coumarin (C=O) was found in the range of 161.08-161.25 ppm. The signals at 112.75-113.11 ppm (C-3) and 144.64-144.90 ppm (C-4) ppm revealed the presence of α,β-unsaturated keto function and a signal at 143.32-143.99 ppm supported the attachment of (OH) group at (C-7). A signal at 148.23-148.93 ppm supported the attachment of (OH/OGlc) group at (C-6). A bunch of peaks ranging from 61.16 to 101.36 suggested the presence of glucosyl moiety in case of esculin.

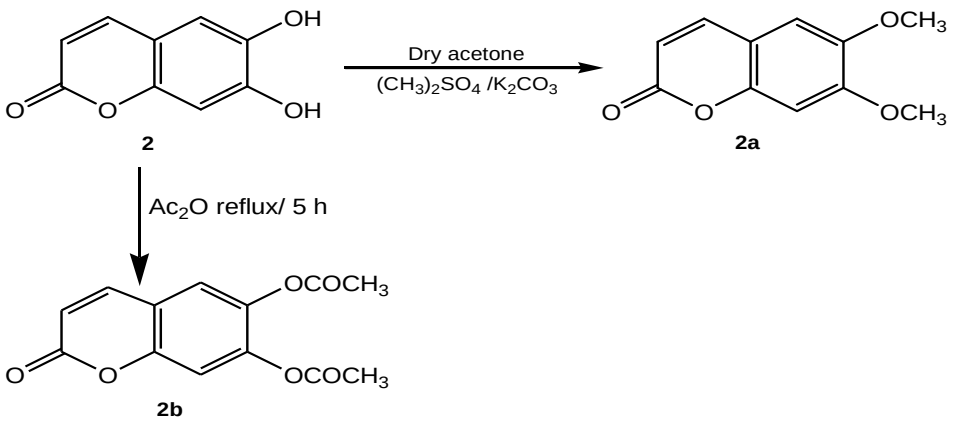
These isolated compounds were then assessed *in vitro* for antiamoebic activity and it was found that ursolic acid (IC_{50} = 2.20 μg/ml), esculetin (IC_{50} = 2.25 μg/ml) and esculin (IC_{50} = 2.97 μg/ml) exhibited moderate antiamoebic activity but less than that of their corresponding extracts.

3.2. Pharmaco-modulation of ursolic acid and esculetin

Methyl ursolate **1a** was prepared by treating ursolic acid **1** with dimethyl sulphate and potassium carbonate in dry acetone. Ursolic acid was refluxed with acetic anhydride to prepare acetyl ursolic acid **1b** (**Scheme 1**). Esculetin **2** was converted into its methyl derivative (6,7-dimethoxycoumarin) **2a** by treating with dimethyl sulphate and potassium carbonate in dry acetone and acetyl derivative (6,7-diacetoxycoumarin) **2b** by refluxing with acetic anhydride (**Scheme 2**). All the compounds were characterized by electronic, IR, ^{1}H and ^{13}C NMR spectra.



Scheme 1. Esterification and acetylation of ursolic acid



Scheme 2. Methylation and acetylation of esculetin

The structure of the compound **2a** was confirmed by IR spectra, which showed the absence of (OH) group absorption around 3400 cm^{-1}, while strong absorption band at 1680-1683 cm^{-1} was assigned to the coumarin ν(C=O). The ^{1}H NMR spectrum of compound **2a** showed the absence of a broad singlet at 5.5 ppm and that of the compound **1a** showed the absence of a singlet at 11.2 ppm due to (OH) group, while the presence of a singlet at 3.25 ppm due to (CH_3) group revealed the conversion of (OH) into (OCH_3). The structure of the compounds **1a, 2a** was further supported by ^{13}C NMR spectra due to the appearance of (OCH_3) signal in the range of 50-59 ppm. The structure of compound **1b, 2b** was confirmed by IR spectra which showed the absence of (OH) group around 3400 cm^{-1}, while strong bands were recorded 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 5.5 ppm due to (OH) group and

the presence of a singlet at 2.02-3.27 ppm due to (CH_3) group. The structure was further supported by ^{13}C NMR spectra. A characteristic signal for acetyl (C=O) was found in the range of 169-180 ppm and a signal at 19.7 ppm showed the presence of methyl group.

The derivatives **1a-2b** were assessed *in vitro* for antiamoebic activity and it was found that the methyl ursolate **1a** (IC_{50} = 3.11 µg/ml) and acetyl ursolic acid **1b** (IC_{50} = 2.87 µg/ml) showed less activity than ursolic acid **1** (IC_{50} = 2.20µg/ml). Methoxy derivative (6,7-dimethoxycoumarin **2a**) (IC_{50} = 1.95 µg/ml) showed higher activity whereas acetoxy derivative (6,7-diacetoxycoumarin **2b**) (IC_{50} = 2.74 µg/ml) showed less activity than the parent compound esculetin **2** (IC_{50} = 2.25 µg/ml) (**Table 1**).

4. EXPERIMENTAL

4.1. Extraction and Isolation of ursolic acid, esculetin and esculin

Dried and well-grounded leaves (5 kg) were extracted by refluxing with methanol (8 × 10 L, 4 h each). The insoluble material was removed by filtration and the extract was concentrated under *vacuo*. The dried, crude methanolic extract (1 kg, 20%) was then fractionated successively by refluxing with n-hexane, benzene, chloroform, ethyl acetate, acetone and n-butanol (5 × 2.5 L, 2 h each) to give after concentration the hexane (40 g, 4%), benzene (15 g, 1.5%), chloroform (10 g, 1%), ethyl acetate (20 g, 2%), acetone (13 g, 1.3%) and n-butanol (25 g, 2.5%) extracts, respectively. The benzene, ethyl acetate and n-butanol extracts showed inhibition of *E. histolytica* and were selected for the isolation of active constituents.

The benzene extract (15 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 9:1 (v/v) C_6H_6 - EtOAc gave a white powder, which was identified as ursolic acid **1** (900 mg, 6%). The ethyl acetate extract (20 g) was fractionated over a column of silica gel (60-120 mesh), eluting with $CHCl_3$ - EtOAc mixtures of increasing polarity. The fractions eluted with 9.5:0.5 (v/v) $CHCl_3$ - EtOAc upon concentration gave brown crystals, which were identified as esculetin **2** (3 g, 15%). The n-butanol extract (25 g) was subjected to column chromatography using $CHCl_3$ - MeOH as eluent. The fractions eluted with 9.5:0.5 (v/v) $CHCl_3$ - MeOH gave a shiny white powder, which was identified as esculin **3** (2.2 g, 8.8%).

4.1.1. Ursolic acid 1

M.p.:289-290 ^0C; white powder; UV λ_{max}nm : 381.0, 328.05, 322.50, 212.54; IR $\nu_{max}cm^{-1}$: 3432(OH), 1692(C=O); ^1H NMR(DMSO-d_6) δ(ppm): 5.25 (t, 1H, H-12, *J* = 3.4 Hz), 2.98

(dd, 1H, H-3, *J* = 5.0, 11.2 Hz), 2.63 (d, 1H, H-18, *J* = 11.3 Hz), 2.33 (m, 1H, H-15β), 2.14 (m, 1H, H-16β), 1.97 (m, 1H, H-22β), 1.82 (m, 1H, H-22α), 1.59 (m, 1H, H-7α), 1.57 (m, 1H, H-1β), 1.50 (m, 1H, H-20), 1.49 (m, 1H, H-19), 1.49 (m, 1H, H-21β), 1.45 (m, 2H, H-2), 1.44 (m, 1H, H-6α), 1.40 (m, 1H, H-21α), 1.39 (m, 1H, H-6β), 1.39 (m, 1H, H-7β), 1.36 (m, 1H, H-11α), 1.33 (m, 1H, H-16α), 1.24 (s, 3H, H-27), 1.22 (m, 1H, H-9), 1.22 (m, 1H, H-15α), 1.18 (m, 1H, H-11β), 1.04 (s, 3H, H-23), 1.02 (d, 3H, H-29), 0.97 (d, 3H, H-30), 0.92 (s, 3H, H-25), 0.88 (m, 1H, H-5), 0.87 (s, 3H, H-26), 0.83 (m, 1H, H-1α), 0.80 (s, 3H, H-24); ^{13}C NMR (DMSO-d_6) δ(ppm): 178.77 (C-28), 138.62 (C-13), 125.04 (C-12), 77.31 (C-3), 55.26 (C-5), 52.81 (C-18), 47.48 (C-9), 47.27 (C-17), 42.03 (C-14), 40.07 (C-8), 39.54 (C-19), 39.54 (C-20), 38.92 (C-4), 38.92 (C-10), 38.70 (C-1), 36.77 (C-22), 33.17 (C-7), 30.65 (C-21), 28.70 (C-23), 27.99 (C-15), 27.41 (C-2), 24.26 (C-16), 23.72 (C-27), 23.30 (C-11), 21.55 (C-30), 18.46 (C-6), 17.47 (C-26), 17.34 (C-29), 16.53 (C-24), 15.68 (C-25); ESI-MS m/z: 457 $[M+H]^+$; 479 $[M+Na]^+$.

4.1.2. Esculetin 2

M.p.: 267-269 ^{0}C; brown crystals; UV λ_{max}nm: 298, 256, 228; IR ν_{max}cm^{-1} : 3328, 1666, 1615, 1565; ^{1}H NMR(DMSO-d_6) δ(ppm): 7.87 (d, 1H, H-4, *J* = 9.4 Hz), 6.97 (s, 1H, H-5), 6.74 (s, 1H, H-8), 6.18 (d, 1H, H-3, *J* = 9.5 Hz); ^{13}C NMR (DMSO-d_6) δ(ppm): 161.25 (C-2), 150.83 (C-9), 148.93 (C-7), 144.90 (C-4), 143.32 (C-6), 112.75 (C-3), 111.95 (C-5), 111.20 (C-10), 103.08 (C-8); ESI-MS m/z = 178 $[M]^+$, 179 $[M+H]^+$.

4.1.3 Esculin 3

M.p.: 204-206 ^{0}C; white powder; UV λ_{max}nm: 342, 288, 251, 226; IR ν_{max}cm^{-1}: 3375, 1683, 1612, 1560,1540; ^{1}H NMR(DMSO-d_6) δ(ppm): 7.82 (d, 1H, H-4, *J* = 9.5 Hz), 7.8 (s, 1H, H-8), 7.4 (s, 1H, H-5), 6.2 (d, 1H, H-3, *J* = 9.5 Hz), 4.8 (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): 161.08 (C-2), 149.93 (C-9), 148.23 (C-7), 144.64 (C-4), 143.99 (C-6), 113.89 (C-5), 113.41 (C-10), 113.11 (C-3), 103.75 (C-8), 101.36 (C-1'), 77.68 (C-5'), 76.26 (C-3'), 73.59 (C-2'), 70.23 (C-4'), 61.16 (C-6'); ESI-MS m/z :340 $[M]^+$, 363 $[M+Na]^+$.

4.2. General procedure for the preparation of methyl ursolate 1a and 6,7-dimethoxycoumarin 2a

Compound (200 mg) was refluxed in dry acetone (50 ml) with dimethyl sulphate (0.5 ml) and anhydrous potassium carbonate (5 gm) for 72 hrs 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 with several times by small portions of dry acetone. The washings and filtrate were combined and evaporate to dryness. A cream coloured solid was left which was washed with petroleum ether and then with water.

4.2.1. Methyl ursolate 1a

Yield: 72%; m.p.:172-174 °C; white powder; IR $\nu_{max}cm^{-1}$: 3433 (OH), 2895 (C-H), 1680 (C=O), 1616 (C=C); ^{1}H NMR(DMSO-d_6) δ(ppm): 5.31 (t, 1H, H-12, *J* = 3.6 Hz), 3.25 (s, 3H, OCH_3), 2.67 (dd, 1H, H-3, *J* = 5.2, 11.4 Hz), 2.61 (d, 1H, H-18, *J* = 11.3 Hz), 2.33 (m, 1H, H-15β), 2.18 (m, 1H, H-16β), 1.94 (m, 1H, H-22β), 1.58 (m, 1H, H-22α), 1.55 (m, 1H, H-7α), 1.55 (m, 1H, H-1β), 1.52 (m, 1H, H-20), 1.49 (m, 1H, H-19), 1.46 (m, 1H, H-21β), 1.44 (m, 2H, H-2), 1.42 (m, 1H, H-6α), 1.41 (m, 1H, H-21α), 1.38 (m, 1H, H-6β), 1.38 (m, 1H, H-7β), 1.35 (m, 1H, H-11α), 1.33 (m, 1H, H-16α), 1.28 (s, 3H, H-27), 1.22 (m, 1H, H-9), 1.22 (m, 1H, H-15α), 1.18 (m, 1H, H-11β), 1.05 (s, 3H, H-23), 1.01 (d, 3H, H-29), 0.99 (d, 3H, H-30), 0.93 (s, 3H, H-25), 0.88 (m, 1H, H-5), 0.85 (s, 3H, H-26), 0.83 (m, 1H, H-1α), 0.80 (s, 3H, H-24); ^{13}C NMR (DMSO-d_6) δ(ppm): 172.8 (C-28), 137.61 (C-13), 125.11 (C-12), 76.12 (C-3), 55.26 (C-5), 52.69 (C-18), 51.2 (OCH_3), 48.48 (C-9), 47.27 (C-17), 41.33 (C-14), 41.07 (C-8), 39.54 (C-19), 39.52 (C-20), 38.96 (C-4), 38.95 (C-10), 38.70 (C-1), 35.83 (C-22), 31.42 (C-7), 31.21 (C-21), 29.71 (C-23), 27.65 (C-15), 27.41 (C-2), 24.48 (C-16), 23.82 (C-27), 23.30 (C-11), 20.45 (C-30), 17.68 (C-6), 17.47 (C-26), 17.74 (C-29), 15.35 (C-24), 14.26 (C-25); ESI-MS m/z: 461 $[M+H]^+$.

4.2.2. 6,7-Dimethoxycoumarin 2a

Yield: 84%; m.p.: 110-112 ^{0}C; yellow crystals; IR $\nu_{max}cm^{-1}$: 3175 (Ar-H), 2890 (C-H), 1682 (C=O), 1640, 1535 (C=C, aromatic); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.68 (d, 1H, H-4, *J* = 9.5 Hz), 7.20 (s, 1H, H-5), 6.43 (s, 1H, H-8), 6.11 (d, 1H, H-3, *J* = 9.3 Hz), 3.21 (s, 6H, CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 160.7 (C-2), 152.2 (C-9), 150.7 (C-7), 148.6 (C-6), 144.6 (C-4), 119.8 (C-10), 113.6 (C-3), 110.3 (C-5), 106.6 (C-8), 55.4 (CH_3); ESI-MS m/z: 207 $[M+H]^+$.

4.3. General procedure for the preparation of acetyl ursolic acid 1b and 6,7-diacetoxycoumarin 2b

Compound (0.81g, 0.005 mol) was refluxed with acetic anhydride for 5 hrs and the reaction mixture was cooled, poured onto ice and left over night. The formed precipitate was filtered, dried and crystallized by acetone to afford acetoxy product.

4.3.1. Acetyl ursolic acid 1b

Yield: 70%; m.p.:142 ^{0}C; white crystals; IR ν_{max}cm^{-1}: 2890 (C-H), 1660-1780 (C=O), 1622 (C=C); ^{1}H NMR(DMSO-d_6) δ(ppm): 11.29 (s, 1H, COOH), 5.22 (t, 1H, H-12, *J* = 3.3 Hz), 3.45 (s, 3H, OCH_3), 2.85 (dd, 1H, H-3, *J* = 5.5, 11.7 Hz), 2.79 (s, 3H, CH_3CO), 2.54 (d, 1H, H-18, *J* = 11.7 Hz), 2.63 (m, 1H, H-15β), 2.46 (m, 1H, H-16β), 1.87 (m, 1H, H-22β), 1.82 (m, 1H, H-22α), 1.58 (m, 1H, H-7α), 1.52 (m, 1H, H-1β), 1.50 (m, 1H, H-20), 1.48 (m, 1H, H-19), 1.46 (m, 1H, H-21β), 1.44 (m, 2H, H-2), 1.42 (m, 1H, H-6α), 1.40 (m, 1H, H-21α), 1.40 (m, 1H, H-6β), 1.39 (m, 1H, H-7β), 1.35 (m, 1H, H-11α), 1.30(m, 1H, H-16α), 1.25 (s, 3H, H-27), 1.24 (m, 1H, H-9), 1.21 (m, 1H, H-15α), 1.18 (m, 1H, H-11β), 1.05 (s, 3H, H-23), 1.01 (d, 3H, H-29), 0.94 (d, 3H, H-30), 0.91 (s, 3H, H-25), 0.89 (m, 1H, H-5), 0.85 (s, 3H, H-26), 0.83 (m, 1H, H-1α), 0.80 (s, 3H, H-24); ^{13}C NMR (DMSO-d_6) δ(ppm): 177.64 (C-28), 172.33 (O-C=O), 138.62 (C-13), 125.04 (C-12), 85.59 (C-3), 56.26 (C-5), 52.81 (C-18), 51.2 (OCH_3), 47.72 (C-9), 47.27 (C-17), 41.67 (C-14), 41.21(C-8), 40.55 (C-19), 39.54 (C-20), 38.85 (C-4), 38.82 (C-10), 38.70 (C-1), 36.62 (C-22), 31.17 (C-7), 30.65 (C-21), 29.34 (C-23), 28.90 (C-15), 28.41 (C-2), 25.81 (C-16), 24.72 (C-27), 23.50 (C-11), 20.95 (C-30), 20.82 (CH_3), 18.88 (C-6), 17.47 (C-26), 16.42 (C-29), 15.67 (C-24), 14.11 (C-25); ESI-MS m/z: 499 $[M+H]^+$.

4.3.2. 6,7-Diacetoxycoumarin 2b

Yield: 77%; m.p.: 132 ^{0}C; brown crystals; IR ν_{max}cm^{-1}: 3188 (Ar-H), 2936 (C-H), 1775 (C=O, lactone), 1620, 1580 (C=C, aromatic), 1225 (C-O); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.98 (d, 1H, H-4, *J* = 9.3 Hz), 7.31 (s, 1H, H-5), 6.83 (s, 1H, H-8), 6.12 (d, 1H, H-3, *J* = 9.4 Hz), 2.81 (s, 6H, CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 169.9 (O-C=O), 161.37 (C-2), 150.2 (C-9), 147.6 (C-7), 144.82 (C-4), 141.68 (C-6), 120.4 (C-10), 116.72 (C-5), 114.55 (C-8), 112.4 (C-3), 20.22 (CH_3); ESI-MS m/z: 263 $[M+H]^+$.

4.4. Organism culture and in vitro testing against E. histolytica

E. histolytica trophozoites were cultured in wells of 96-well microtiter plate by using Diamond TYIS-33 growth medium.[20] All the extracts, isolated compounds and their derivatives were screened in vitro for antiamoebic activity against *HM1:IMSS* strain of *E. histolytica* by microdilution method.[21] All extracts (10 mg) and compounds (1 mg) were dissolved in DMSO (40 µl, level at which no inhibition of amoeba occurs).[22,23] The stock solutions of the extracts and the compounds were prepared freshly before use at a concentration of 10 mg/ml and 1 mg/ml respectively. Two-fold 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 amoeba suspension was prepared from a confluent culture by pouring off the medium at 37 ^{0}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 number of amoeba/ml was estimated with a haemocytometer, using trypan blue exclusion to confirm the viability. The suspension was diluted to 10^5 organism/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 ^{0}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 ^{0}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 0.1N 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 are reported in Table 1.

ACKNOWLEDGEMENTS

This work was supported by Department of Science and Technology (Grant no. Vll-PRDSF/44/2004-05/TT), New Delhi, India.

REFERENCES

1. E. Wallander, V.A. Albert, Am. J. Bot, 2000; 87: 1827-1841.
2. N. Stoyanov, Nauka i izkustwo, 1973; 1: 321.
3. Y.C. Shen, C.H. Chen, K.H. Lee, Phytochemistry, 1993; 33: 1531-1533.
4. S. Von Kruedener, W. Schneider, E.F. Elsner, Drug. Res., 1995; 45: 169-171.
5. D.Yordanov, P. Nikolov, A. Boychinov, in: I. Ivanova, L. Spasova, (Eds.) Phytotherapy Sofia, Medizina i fizkultura, 1973; 150.
6. B. Kowalczyk, W .Olechnowicz-Stepien, Herba. Pol., 1988; 34: 7-13.
7. R.K. Joshi, D. Prasad, M.S.M. Rawat, G. Pant, Fitoterapia LXIX, 1998; 275-276.
8. R. D. Gaur, Flora of District Garhwal, North West Himalaya (with ethnobotanical notes). Transmedia Bhandari Bag, Srinagar Uttrakhand., 1999.
9. S. Ghosh, J.M.W. Chan, C.R. Lea, G.A. Meints, J.C. Lewis, Z.S. Tovian, R.M. Flessner, T.C. Lofuts, I. Bruchhaus, H. Kendrick, S.L. Croft, R.G. Kemp, S. Kobayashi, T. Nozaki, E. Oldfield, J. Med. Chem., 2004; 47: 175-187.
10. S.L. Stanly Jr, Lancet, 2003; 361: 1025-1034.
11. M.C. Conde-Bonfil, C. De la Mora-Zerpa, un desafio vigente. Salud Publica Mex**,** 1992; 34: 335-341.
12. R. Cedillo-Rivera, A. Tapia-Contreras, J. Torres, O. Munoz, Arch. Med., 1997; Res. 28: 295-297.
13. K. Kapoor, M. Chandra, D. Nag, J.K. Paliwal, R.C. Gupta, R.C. Saxena, Int. J. Clin. Pharmacol. Res, 1999; 19: 83-88.
14. S.C. Swetman, Martindale, in: The Complete Drug Reference, Pharmaceutical Press, London, 33rd Edn., 2002; 594.
15. E. Orozco, D.G. Perez, M.C. Gomez, P. Ayala, Parasitol. Today, 1995; 11: 473-475.
16. P. Sharma, J.D. Sharma, Phytother. Res., 2001; 15 1-17.
17. S. Werner, S. Nebojsa, W. Robert, S. Robert, K. Olaf, Magn. Reson. Chem., 2003; 41: 636-638.
18. L. Renmin, S. Qinghua, S. Ailing, C. Jichun, Journal of Chromatography A, 2005; 1072: 195-199.
19. Z. Teresa, P. Katarzyna, W. Iwona, Solid State Nucl. Magn. Reson., 2003; 23: 77-87.

20. L.S. Diamond, D.R. Harlow, C.C.R. Cunnick, Trans. R. Soc. Trop. Med. Hyg, 1978; 72: 431-432.
21. C.W. Wright, M.J. O'Neill, J.D. Phillipson, D.C. Warhurst, Antimicrob. Agents Chemother., 1988; 32: 1725-1729.
22. F.D. Gillin, D.S. Reiner, M. Suffness, Antimicrob. Agents Chemother., 1982; 22: 342-345.
23. A.T. Keene, A. Harris, J.D. Phillipson, D.C. Warhurst, Planta Med., 1986; 52: 278-284.