

IN VITRO ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF ISOLATED COMPOUNDS ON ROOTS OF CLERODENDRUM PHLOMIDIS LINNSATHISH M^{1*}, NIRAIMATHI V¹, NOORULLA KM¹, PALANIMUTHU A²¹Department of Pharmaceutical Chemistry, College of Pharmacy, Madras Medical College, Chennai, Tamil Nadu, India. ²Ramachandra Innovis, Sri Ramachandra University, Chennai, Tamil Nadu, India. Email: sathish.mmc08@gmail.com

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ABSTRACT

Objective: The main aim of the study was to screen the isolated compounds of *Clerodendrum phlomidis* roots for its *in vitro* antioxidant and anticancer activity and its efficacy against HeLa cell lines.

Methods: Pet ether, chloroform, ethyl acetate and ethanol extracts was prepared and assayed for the presence of phytochemicals. Three compounds were isolated from ethanol extract of *C. phlomidis* by column chromatography such as ET1 (phenyl acetic acid), ET2 (ethyl-2-hydroxy-4-methylbenzoate), ET3 (3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one) characterized by IR (KBr), ¹H-nuclear magnetic resonance (NMR), ¹³C-NMR and Gas chromatography-mass spectrometry. The above isolated compounds were subjected to *in vitro* antioxidant activity against 2,2-diphenylpicrylhydrazyl (DPPH) radical, superoxide radical scavenging assay and iron chelating activity. The effect of isolated compounds on HeLa cancer cell line was also evaluated by 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide colorimetric assay.

Results: ET2 has good *in vitro* antioxidant activity against DPPH radical, super oxide radical and iron chelating activity with an inhibitory concentration 50% value of 360 µg/ml, 150 µg/ml and 130 µg/ml respectively. ET1 showed significant cytotoxic activity than the other two compounds on HeLa cells with a percentage cell growth of 21.9% and a growth inhibition of 50% value of 180 µg, respectively.

Conclusion: On the basis of obtained results, ET1 and ET2 isolated from the ethanolic extract of *C. phlomidis* represent a new group of cytotoxic against HeLa cell line and antioxidant agents.

Keywords: Column chromatography, *Clerodendrum phlomidis*, *In vitro* antioxidant activity, 2,2-diphenylpicrylhydrazyl, Cytotoxicity, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assay.

INTRODUCTION

Cancer is the abnormal growth of cells in our bodies that can lead to death. Cancer is a general term applied of series of malignant diseases that may affect different parts of the body. These diseases are characterized by a rapid and uncontrolled formation of abnormal cells, which may mass together to form a growth or tumor, or proliferate throughout the body, initiating abnormal growth at other sites. If the process is not arrested, it may progress until it causes the death of the organism [1].

According to World Health Organization, 80% of the people living in rural areas depend on medicinal herbs as primary healthcare system. A great deal of pharmaceutical research done in technologically advanced countries like USA, Germany, France, Japan and China has considerably improved quality of the herbal medicines used in the treatment of cancer. Some herbs protect the body from cancer by enhancing detoxification functions of the body. Some herbs reduce the toxic side effects of chemotherapy and radiotherapy [2,3]. The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity. Of the 92 anticancer drugs commercially available prior to 1983 in the US and among worldwide approved anticancer drugs between 1983 and 1994, 60% are of natural origin [4]. Colorectal cancer (CRC) also known as colon cancer, is due to the abnormal growth of cells that have the ability to invade or spread to other parts of the body symptoms may include blood in the stool, a change in bowel movements, weight loss, and feeling tired all the time [5]. Globally more than 1 million people get CRC every year, resulting in about 715,000 deaths as of 2010 up from 490,000 in 1990. As of 2012 it is the second most common cause of cancer in women (9.2% of diagnoses) and the third most common in men (10.0%) with it being

the fourth most common cause of cancer death after lung, stomach, and liver cancer [6]. CRC was 10% of cancers in men and 9.2% of cancers in women. It is more common in developed than developing countries. Globally incidences vary 10-fold with highest rates in the Australia, New Zealand, Europe and the US and lowest rates in Africa and South-Central Asia [7].

Clerodendrum phlomidis is a common shrub of arid plains, low hills, and tropical deserts. They are distributed throughout the drier parts of India. *C. phlomidis* Linn, (Syn. *Clerodendrum multiflorum* (Burn.f) O.Kuntze, *Volkameria multiflorum* (Burn.f). (Verbenaceae) is an important and well-known medicinal plant extensively used in Ayurveda and Siddha system of medicine for treatment of various ailments. The popular therapies include on diseases like cancer, nervous disorders, asthma, rheumatism, digestive disorders. The medicinal values have been studied especially in leaf, flowers and roots of *C. phlomidis* are rich in polyphenols inhibit the proteolytic activity of the cancer cell proteosome and causes cell death [8]. In cell growth studies, 500 and 1000 mM Lev increased the toxicity of 5-fluorouracil in HCT 116 cell line. Human CRC cell lines are used widely to investigate tumor biology, experimental therapy, and biomarkers. However, to what extent these established cell lines represent and maintain the genetic diversity of primary cancers is uncertain [9].

METHODS**Collection of sample**

The root of *C. phlomidis* was collected in the month of the month July 2010 from Siddha herbal garden, Siddha Medical College, Arumbakkam, Chennai, India. The authenticity of the roots was confirmed by Dr. Sasikala Ethirajulu, Botanist, Govt. of India, Chennai.

Preparation of plant extracts

The dried powder of the root (1.2 kg) was extracted sequentially by hot continuous percolation method using Soxhlet apparatus, using different polarities of solvents from low polarity to high. The dried root powder was packed in Soxhlet apparatus and successively extracted with petroleum ether, chloroform, ethylacetate, and ethanol 48 hrs each. The extracts were concentrated using a rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained [10].

Equipment and apparatus

Melting points were determined by open end glass capillaries on Stuart SMP10 melting point apparatus and were uncorrected. The IR (KBr) spectrum was recorded on Perkin Elmer 1600 Fourier transform infrared spectrometer. The ¹H-nuclear magnetic resonance (NMR) and ¹³C-NMR spectra were recorded on a Bruker R32 (400 MHz) CDCL₃ with TMS as an internal standard (chemical shifts in δ, ppm). Thin layer chromatography (TLC) was performed with silica gel 60 G F254 and spots were visualized by ultraviolet light and iodine vapor and ammonia. Gas chromatography-mass spectrometry (GC-MS) model (GC-MS - QP-2010), Shimadzu Make. The sample (1 μl) was injected into a RTX-5 column (60 m × 0.25 mm i.d., film thickness 0.25 μm). Helium was used as a carrier gas at a constant column flow 1.2 ml/minutes at 173 kpa inlet pressure. All solvents were analytical reagent grade.

Phytochemical screening

The qualitative tests were carried out for the extracts of *C. phlomidis* using standard procedures. The extracts were analyzed for the presence of significant secondary metabolites viz. flavonoids, steroids, alkaloids, and tannins, lignans and phenolic compounds (Table 1) [11].

Isolation of compounds by column chromatography

The phytochemical screening suggests that the ethanol extract rich in phenolic compounds and flavonoids than other extracts. Hence, ethanol extract was subjected to column chromatography. A well-stirred suspension of silica gel (300 g in pet-ether 60-80°) was poured into the column (150 cm long and 50 mm in diameter). When the adsorbent was well settled, the excess of petrol-ether was allowed to pass through column. A volume of 15 g of ethanol extract was digested into well-stirred column and eluted with mobile phase of increasing polarity: Petrol-ether/chloroform/ethyl acetate/methanol.

Elution with pet ether: Chloroform led to the isolation of white crystals from fractions 15 to 18, and designated as ET1. Elution with chloroform: Ethyl acetate led to the isolation of green colored amorphous powder from fractions 26 to 30, and designated as ET2. Elution with ethyl acetate: Methanol led to the isolation of yellow colored crystals from fractions 50 to 54, and designated as ET3. TLC profile of isolated compounds has been shown in Table 2.

In vitro antioxidant activity

2,2-diphenylpicrylhydrazyl (DPPH) scavenging activity

Methanolic solution of 0.5 ml of DPPH (0.4 mM) was added to 1 ml of the different concentrations of plant extract/compounds and allowed to react at room temperature for 30 minutes. Methanol served as a blank and DPPH in methanol without the compounds served as a positive control. After 30 minutes, the absorbance was measured at 518 nm and converted into percentage radical scavenging activity as follows [12]. Rutin was used as a standard.

$$\text{Scavenging activity (\%)} = \frac{A_{518}\text{Control} - A_{518}\text{Sample}}{A_{518}\text{Control}}$$

Where A_{518} control is the absorbance of DPPH radical + methanol; A_{518} sample is the absorbance of DPPH radical and compound/standard.

Super oxide anion radical scavenging activity

Superoxide radical (O_2^-) was generated from the photoreduction of riboflavin and was deducted by nitro blue tetrazolium dye (NBT)

Table 1: Phytochemical analysis of the extracts of *C. phlomidis*

Compounds	Pet ether	Chloroform	Ethyl acetate	Ethanol
Steroid	+	+	-	-
Glycosides	-	+	-	+
Phenolic compounds	-	-	-	+
Flavonoids	-	-	+	+
Saponins	-	+	-	+

+: Presence, -: Absent, *C. phlomidis*: *Clerodendrum phlomidis*

Table 2: TLC profiles of isolated compounds

Compound	Solvent systems	Number of spots	Detection in UV/iodine vapor/ammonia
ET1	Pet ether: Chloroform (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 25:75, 10:90)	1	Yellowish brown spot under iodine vapor
ET2	Chloroform: Ethyl acetate (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 25:75, 10:90)	1	Yellow spot under UV light
ET3	Ethyl acetate: Methanol (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 25:75, 10:90)	1	Dark brown spot under UV and ammonia

UV: Ultraviolet, TLC: Thin layer chromatography

reduction method [13]. Measurement of superoxide anion scavenging activity was performed. The assay mixture contained sample with 0.1 ml of NBT (1.5 mM NBT) solution, 0.2 ml of ethylenediaminetetraacetic acid (EDTA) (0.1 M EDTA), 0.05 ml riboflavin (0.12 mM) and 2.55 ml of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in dimethylsulfoxide (DMSO) was added instead of sample. The reaction mixture was illuminated for 30 minutes and the absorbance at 560 nm was measured against the control samples. Quercetin was used as a standard. All the tests were performed in triplicate, and the results averaged. The percentage inhibition was calculated by comparing the results of control and test samples.

Iron chelating activity

The principle is based on the formation of *O*-Phenanthroline-Fe²⁺ complex and its disruption in the presence of chelating agents. The reaction mixture containing 1 ml of 0.05% *O*-phenanthroline in methanol, 2 ml ferric chloride (200 μM) and 2 ml of various concentrations ranging from 10 to 1000 μg was incubated at room temperature for 10 minutes and the absorbance of the same was measured at 510 nm. The experiment was performed in triplicates [14]. EDTA a classical metal chelator was used as a standard.

In vitro cytotoxic activity

Cell culture

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science, Pune and grown in Eagles minimum essential medium containing 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 5% CO₂, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-EDTA to make single cell suspensions, and viable cells were counted using a hemocytometer and diluted with medium with 5% FBS to give final density of 1 × 10⁵ cells/ml. 10 μl per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO₂, 95% air and 100% relative humidity. After 24 hrs, the cells were treated with serial concentrations

of isolated compounds. They were initially dissolved in DMSO (M and B, England), and it was further diluted in serum free medium to produce five concentrations. At each concentration, 100 μ l per well was added to plates to obtain final concentrations of (0.1 μ g, 1 μ g, 10 μ g, 100 μ g and 300 μ g) to determine percentage cell growth and growth inhibition of 50% (GI_{50}). The final volume in each well was 200 μ l and the plates were incubated at 37°C, 5% CO_2 , 95% air and 100% relative humidity for 48 hrs. The medium containing without samples were served as control. Triplicate was maintained for all concentrations.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the formazan produced is directly proportional to the number of viable cells. After 48 hrs of incubation, 15 μ l of MTT (5 mg/ml) in phosphate buffered saline was added to each well and incubated at 37°C for 4 hrs. The medium with MTT was then flicked off, and the formed formazan crystals were solubilized in 100 μ l of DMSO and then measured the absorbance at 570 nm using micro plate reader. (MTT) manufactured from Sigma Aldrich, Mumbai, India. From the optical density (OD) values, the percentage growth of HeLa cells with the addition of the samples was calculated based on the formula: Percentage growth = $100 \times [(T-T_0)/(C-T_0)]$ if $T \geq T_0$. If $T < T_0$, percentage growth = $100 \times [(T-T_0)/T_0]$, where T was the OD of the test, T_0 was the OD at time zero and C was the OD of the negative control. From the percentage growth, a dose response curve was generated, and concentration at 50% GI_{50} values of the test samples was calculated [15-18].

RESULTS AND DISCUSSION

Spectral characterization for isolated compounds

Compound ET1

ET1 was isolated as White crystals, melting point 75-77°C. The IR (KBr) shows that the stretching frequencies of aromatic C-H vibration occur in the range of 3071-2917/cm. There is an intense band at 1686/cm which correspond to the carbonyl group of the aromatic acids. 1H -NMR spectrum (400 MHz, $CDCl_3$) shows that, δ 8.11 (d, 2H, $J=7.65$ Hz, Ar-H), δ 7.480 (t, 2H, $J=7.65$ Hz, Ar-H), δ 7.62 (t, 1H, $J=7.64$ Hz), δ 11.00 (s, 1H, carboxylic-H), δ 1.30 ppm (s, 2H, $-CH_2-$ proton).

The ^{13}C -NMR spectra in $CDCl_3$ shows that, carbon of phenyl ring resonate at δ 128-130 ppm. Tertiary carbon of phenyl ring resonates at δ 133 ppm. Methylene carbon resonates at δ 76 ppm. Carbonyl carbon resonates at δ 172 ppm. GC-MS spectra shows that the mass of the molecular ion peak of compound ET1 is found to be m/z 136, corresponding to the molecular formula ($C_8H_8O_2$). Ion peaks were also observed at m/z 59, 78. Based on the above spectral data's the compound ET1 is found to be phenyl acetic acid (Fig. 1).

Compound ET2

ET2 was isolated as green colored amorphous powder with melting range 212-214°C. The IR (KBr) spectrum of compound ET2 shows that a broadband at 3422/cm is due to the phenolic -OH group. The carbonyl group of the ester occurs at 1654/cm. 1H -NMR spectrum (400 MHz, $CDCl_3$) shows that, δ 6.39 (d, 1H, $J=7.64$ Hz, Ar-H), δ 6.83 (d, 1H, $J=7.65$ Hz, Ar-H), δ 7.53 (d, 1H, $J=7.65$ Hz, Ar-H) δ 3.70-4.10 (m, 2H, $-CH_2-$) δ 1.10 (t, 3H, $J=7.64$ Hz, $-CH_3$ -proton associated with phenyl ring system) δ 2.50 (s, 3H, $-CH_3$ -) δ 12.60 (s, 1H, -OH-).¹⁸

The ^{13}C -NMR spectra in $CDCl_3$ Fig. 4 shows that, carbon (secondary) of aromatic ring resonates at δ 144-116 ppm. Tertiary carbon of aromatic ring resonates at δ 148 ppm. Carbon of methyl groups resonates at δ 76-70 ppm. Carbon carbonyl group resonates at δ 160 ppm. Carbon of the methylene group resonates at δ 98 ppm. GC-MS spectra shows that, the mass of the molecular ion peak of compound ET2 is found to be m/z 180, corresponding to the molecular formula ($C_{10}H_{12}O_3$). Ion peaks

were also observed at m/z 15, 73, 94. Based on the above spectral data's the compound ET2 is found to be ethyl-2-hydroxy-4-methyl benzoate (Fig. 2).

Compound ET3

ET3 was isolated as yellow colored crystals melting point 222-224°C. IR (KBr) of compound ET3 shows that, broadband at 3330-3612/cm is due to the phenolic OH group. 1655/cm (carbonyl group), Stretching frequencies of aromatic C-H vibration at 3089/cm. 2849/cm (C-H aliphatic). 1H -NMR spectrum (400 MHz, $CDCl_3$) shows that, δ 8.33 (s, 2H, Ar-H). δ 6.49 (d, 2H, $J=7.64$, Ar-H), δ 6.82 (d, 1H, $J=7.65$ Hz, Ar-H). δ 7.37 (t, 1H, $J=7.65$ Hz, Ar-H), δ 13.05 (s, 1H, -OH-), δ 10.72 (br s, 1H, -OH- proton), δ 9.60 (br s, 1H, -OH-) respectively. δ 3.75 (s, 3H, $-OCH_3$).

The ^{13}C -NMR spectra in $CDCl_3$, (Fig. 6), Secondary carbon resonates at δ 115 ppm, aromatic C and C=C. Tertiary carbon resonates at δ 157 ppm, aromatic tertiary carbon. Carbonyl carbon resonates at δ 180 ppm. Methoxy carbon resonates at δ 59 ppm. GC-MS spectra shows that, the mass of the molecular ion peak of compound ET3 is found to be m/z 300.1, corresponding to the molecular formula ($C_{16}H_{12}O_6$). Ion peaks were also observed at m/z 75, 149.2, 207, 237, 295. Based on the above spectral data's the compound ET3 is found to be 3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one (Fig. 3).

The *in vitro* antioxidant activity was carried out for phenyl acetic acid (ET1), ethyl-2-hydroxy-4-methyl benzoate (ET2), 3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one (ET3).

DPPH radical scavenging assay

Table 3 depicts the percentage scavenging activity of the isolated compounds on DPPH radical. Among the isolated compounds, the maximum percentage scavenging of 73.64% was shown by ethyl-2-hydroxy-4-methyl benzoate at 1000 μ g/ml and the inhibitory concentration 50% (IC_{50}) value was found to be 360 μ g/ml. The standard Rutin shows the percentage scavenging of 80.65% at 1000 μ g/ml. The

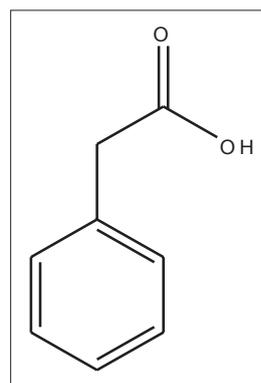


Fig. 1: ET1 (phenyl acetic acid)

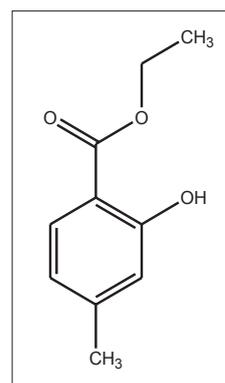


Fig. 2: Ethyl-2-hydroxy-4-methyl benzoate

IC₅₀ value of standard rutin was found to be 270 µg/ml. Phenyl acetic acid has weak antioxidant activity with an IC₅₀ value of 800 µg/ml against DPPH radical assay. From the results, it has been suggested that ethyl-2-hydroxy-4-methyl benzoate (ET2) has greater antioxidant activity against DPPH radical than other two compounds compared with the standard rutin.

Superoxide radical anion scavenging activity

Table 4 depicts the percentage of superoxide radical anion scavenging activity of the isolated compounds. Ethyl-2-hydroxy-4-methyl benzoate (ET2) shows maximum percentage scavenging of 85.61% at 1000 µg/ml and an IC₅₀ value of 150 µg/ml. 3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one (ET3) also shows better superoxide radical anion scavenging with a percentage scavenging of 78.77% at 1000 µg/ml and a IC₅₀ value of 200 µg/ml. The IC₅₀ value of standard quercetin was found to be 130 µg/ml respectively. Phenyl acetic acid has weak antioxidant activity with an IC₅₀ value of 1020 µg/ml. From the results, it has been suggested that ethyl-2-hydroxy-4-methyl benzoate has significant superoxide anion scavenging activity than other two compounds compared with standard quercetin.

Iron chelating activity

Iron is essential for life because it is required for oxygen transport, respiration and activity of many enzymes. However, iron is an extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components [19]. Table 5 depicts the iron chelating

Table 3: DPPH scavenging activity of isolated compounds of *C. phlomidis*

Concentration (µg/ml)	ET1	ET2	ET3	Standard (Rutin)
125	18.73±0.89	28.49±0.48	24.47±0.14	28.39±0.25
250	24.66±0.45	40.53±0.15	38.49±0.02	48.98±0.46
500	29.44±0.01	65.71±0.78	53.41±0.08	70.18±0.13
1000	37.64±0.78	73.64±0.44	61.73±0.77	80.65±0.20
IC ₅₀ (µg/ml)	800	360	460	270

All values are expressed as mean±SEM for three determinations. DPPH: 2,2-diphenylpicrylhydrazyl, SEM: Standard error mean, *C. phlomidis*: *Clerodendrum phlomidis*, IC₅₀: Inhibitory concentration 50%

Table 4: Superoxide radical anion scavenging activity of isolated compounds of *C. phlomidis*

Concentration (µg/ml)	ET1	ET2	ET3	Standard (quercetin)
125	11.47±0.11	52.43±0.74	22.43±0.08	49.44±0.48
250	18.86±0.36	69.82±0.08	58.49±0.01	61.88±0.49
500	29.69±0.07	73.04±0.45	80.81±0.03	78.39±0.18
1000	47.44±0.91	85.61±0.78	78.77±0.07	89.28±0.09
IC ₅₀ (µg/ml)	1020	150	200	130

All values are expressed as mean±SEM for three determinations, SEM: Standard error mean, *C. phlomidis*: *Clerodendrum phlomidis*, IC₅₀: Inhibitory concentration 50%

Table 5: Iron chelating activity of isolated compounds of *C. phlomidis*

Concentration (µg/ml)	ET1	ET2	ET3	Standard (EDTA)
125	19.83±0.11	56.58±0.06	15.89±0.14	58.68±0.01
250	25.64±0.36	63.63±0.84	30.47±0.99	65.87±0.02
500	43.71±0.07	67.04±0.70	64.97±0.49	83.83±0.01
1000	56.44±0.91	96.66±0.18	80.31±0.86	97.90±0.02
IC ₅₀ (µg/ml)	820	130	400	65

All values are expressed as mean±SEM for three determinations, SEM: Standard error mean, *C. phlomidis*: *Clerodendrum phlomidis*, IC₅₀: Inhibitory concentration 50%

activity of compounds of the isolated compounds. Ethyl-2-hydroxy-4-methyl benzoate (ET2) shows maximum percentage scavenging of 96.66% at 1000 µg/ml and an IC₅₀ value of 130 µg/ml. 3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one (ET3) also shows better superoxide radical anion scavenging with a percentage scavenging of 80.31% at 1000 µg/ml and a IC₅₀ value of 400 µg/ml. The IC₅₀ value of standard EDTA was found to be 65 µg/ml respectively. Phenyl acetic acid has weak antioxidant activity with an IC₅₀ value of 820 µg/ml. From the results, it has been suggested that ethyl-2-hydroxy-4-methyl benzoate has significant iron chelating activity than other two compounds. Ethyl-2-hydroxy-4-methyl benzoate was found to have more metal chelating activity than that of other compounds compared to the standards used. It indicates that the compound ET2 possess significant iron binding capacity which might be due to the presence of phenolic group that averts the cell from free radical damage by reducing of transition metal ions [20,21].

Based on the above antioxidant study of isolated compounds, ET2 (ethyl-2-hydroxy-4-methyl benzoate) possess good antioxidant activity when compared to other two compounds. This may be due to the presence of phenolic hydroxyl group of compound ET2.

In vitro cytotoxicity

The *in vitro* cytotoxic activity of phenyl acetic acid (ET1), ethyl-2-hydroxy-4-methyl benzoate (ET2) and 3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one (ET3) were performed. Table 6 shows that ET1, ET2 and ET3 at 300 µg the percentage growth of HeLa cells was found to be 22%, 86% and 38% respectively. The GI₅₀ value of ET1, ET2 and ET3 was found to be 184 µg, 300.0 µg and 209.4 µg respectively. Hence, ET1 and ET3 have good *in vitro* cytotoxic activity on HeLa cells. But ET1 has greater cytotoxic activity on HeLa cell comparing to other two compounds. ET2 has weak cytotoxic property on HeLa cell line. The effect of isolated compounds on percentage growth of HeLa illustrated in Fig. 4. Fig. 5a-o, depicts the cytotoxicity of the isolated compounds at different concentrations used against HeLa

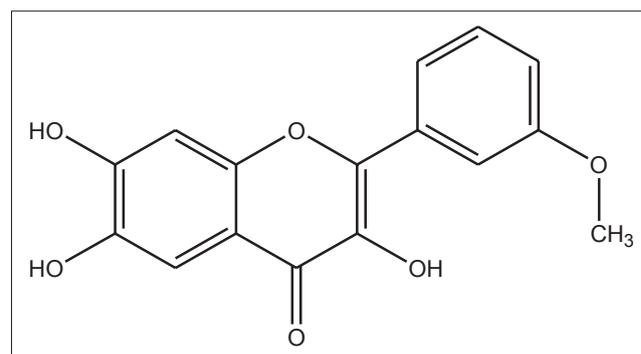


Fig. 3: 3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one

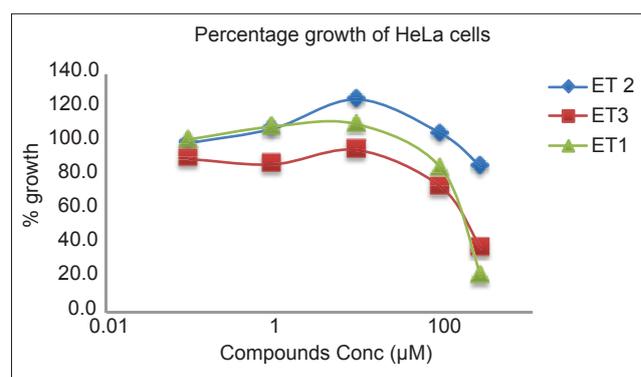


Fig. 4: Effect of isolated compounds on percentage cell growth of HeLa

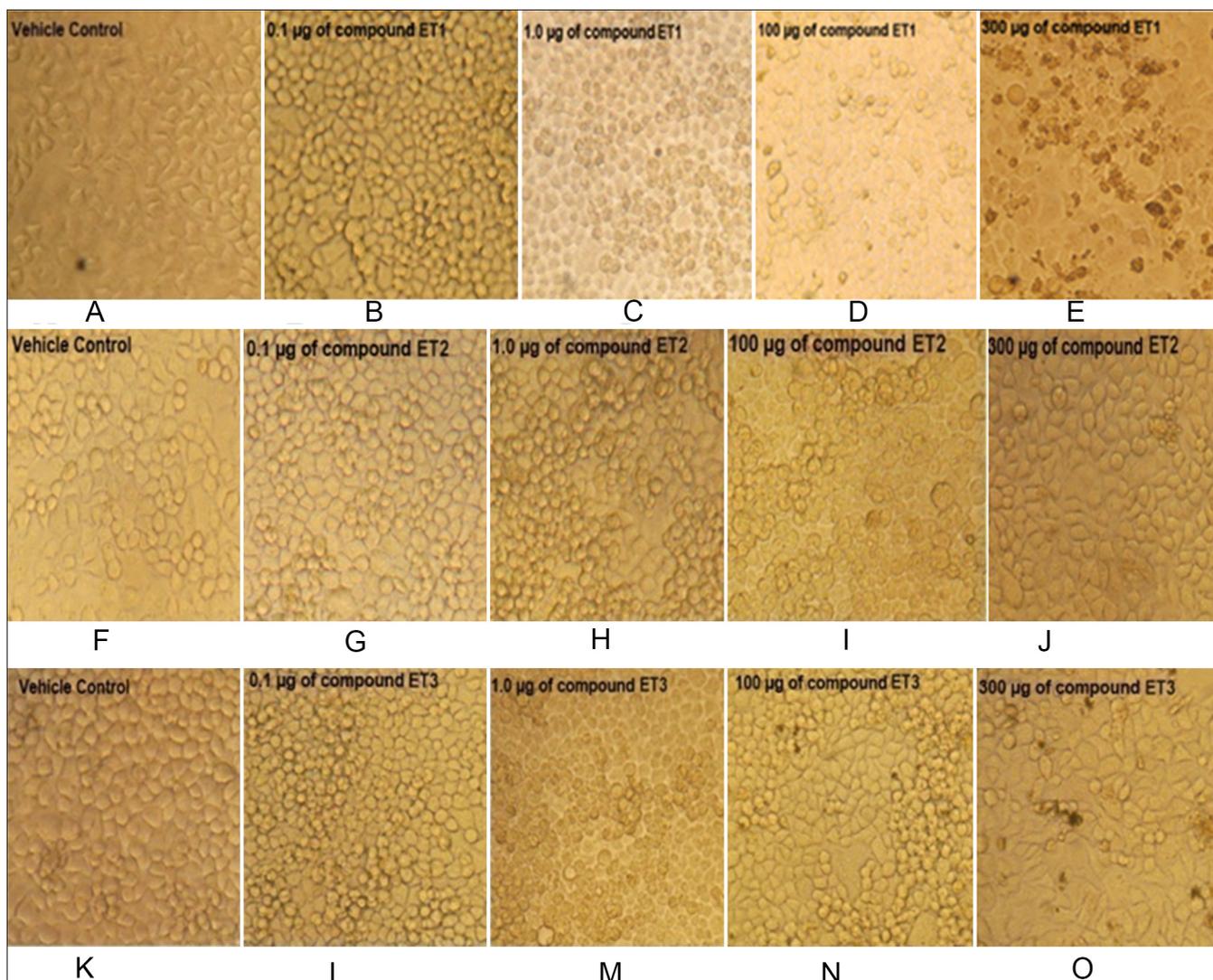


Fig. 5: Effect of cytotoxicity of isolated compounds on HeLa cell line, effect of cytotoxicity of ET1 (phenyl acetic acid) - B, C, D, E at (0.1, 1.0, 100 and 300 µg/ml) compared to vehicle control A. Effect of cytotoxicity of ET2 (ethyl-2-hydroxy-4-methyl benzoate) - G, H, I, J at (0.1, 1.0, 100 and 300 µg/ml) compared to vehicle control F. Effect of cytotoxicity of ET3 (3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one) - G, H, I, J at (0.1, 1.0, 100 and 300 µg/ml) compared to vehicle control K.

Table 6: Effect of isolated compounds on percentage growth of HeLa

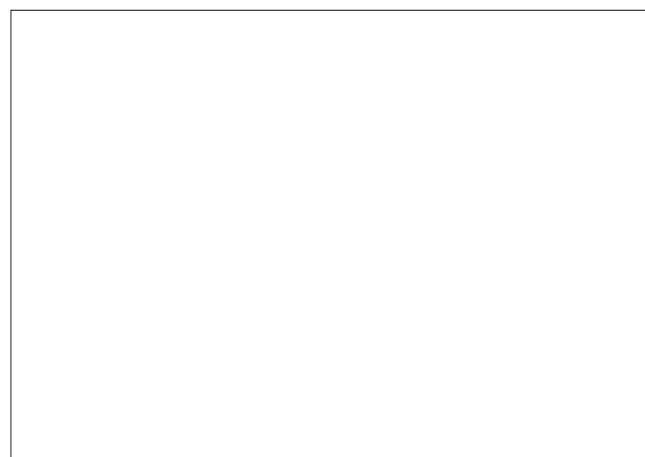
Concentration (µg/ml)	ET1	ET2	ET3
300	21.9±0.55	86.2±0.25	38.3±0.25
100	85.1±0.64	105.5±0.35	74.1±0.64
10	111.0±0.35	125.6±0.40	95.9±0.40
1	109.7±0.55	108.5±0.35	87.5±0.30
0.1	102.2±0.40	100.0±0.35	90.5±0.25
GI ₅₀	180.0 µg/ml	300.0 µg/ml	209.4 µg/ml

All values are expressed as mean±SEM for three determinations, GI₅₀: Growth inhibition of 50%, SEM: Standard error mean

cells. The results clearly indicate that ET1 shows significant *in vitro* cytotoxic activity against HeLa cell line than other two compounds. ET3 also shows better cytotoxic activity against HeLa cell line. ET2 shows weak cytotoxic activity.

CONCLUSION AND RECOMMENDATION

The investigated isolated analogs have shown to possess antioxidant and cytotoxic activity. Particularly ET2 (ethyl-2-hydroxy-4-methyl benzoate) possess a good *in vitro* antioxidant activity compared to



other two compounds. Furthermore ET1 (phenyl acetic acid) was the most active, displaying the most significant cytotoxic action compared to other two compounds.

Briefly the study shows that ET1 and ET2 represents a new group of cytotoxic and antioxidant agents. The isolated compounds also pave the way to explore and design some bioactives related to above leads.

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