Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(5): 1453-1457
Received: 13-07-2019
Accepted: 15-08-2019

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Anticancer activity and GCMS analysis of methanol leaves extract of *H. auriculata* (Schumach.) Heine

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Abstract
Cancer remains one of the leading causes of morbidity and mortality globally and is responsible for one in eight deaths worldwide. Amongst the non-communicable diseases, cancer is the second leading cause of death, after cardiovascular disease Chemotherapy is routinely used for cancer treatment. Since cancer cells lose many of the regulatory functions present in normal cells, they continue to divide when normal cells do not. This feature makes cancer cells susceptible to chemotherapeutic drugs. There are several factors for the continued popularity of traditional drugs and one is their ready availability as compared to the modern medicines besides the adverse effects of synthetic drugs. The aim of the present study was to evaluate the anticancer activity and GC-MS analysis of methanol extract of leaves of *H. auriculata*. MTT assay method was carried out for anticancer activity on A549 (lung) and HepG2 (liver) cancer cell lines. The maximum cell death of lung cancer cells was 70.54±0.004% and liver cancer cells was 72.37±0.002% at 160 µg/mL concentration of methanol leaves extract of *H. auriculata*. Antioxidant compound 5-Hydroxy-7-methoxy-2-phenyl-4H-1-Benzopyran-4-one was eluted by GC-MS, which neutralize free radicals, which causes oxidative stress to cells, leads to cancer.

Keywords: *Hygrophila auriculata*, anticancer activity, MTT assay GC-MS

1. Introduction
Medicinal and aromatic plants constitute a major role, which provides raw materials for the use in pharmaceuticals, cosmetics and drug industries. The indigenous systems of medicines, developed in India for centuries, make use of many medicinal herbs [1]. World Health Organization, estimated that 80 percent of the population of developing countries relies on traditional plant based medicines for their health requirements [2]. Even in many of the modern medicines, the basic composition is derived from medicinal plants and has become acceptable for many reasons that include easy availability, least side effects and low prices lasting curative property. Cancer is the second leading cause of death worldwide. Although great advancements have been made in the treatment and control of cancer progression, significant deficiencies still arises for the drug improvement. A number of undesired side effects sometimes occur during chemotherapy. Natural therapies, such as the use of plant-derived products in cancer treatment, may reduce adverse side effects. Currently, a few plant products are being used to treat cancer [3]. All traditional medicines have their potential in folk medicines and household remedies to treat various diseases. WHO has listed about 20,000 medicinal plants was used in different parts of the world for health problems. Plant derived products are present in 14 of the 15 therapeutic categories of pharmaceutical preparations, which are currently recommended to medical practitioners and an important part of health care system in the world [4]. The plant *Hygrophila auriculata* belongs to Acanthaceae family, has been traditionally used for the treatment of inflammation and is a promising medicinal plant with great economic potential for its health-promoting properties [5]. The plant is cultivated throughout India and the leaves and roots of *Hygrophila* have diuretic properties. This plant has been traditionally used in the preparation of several Ayurvedic medicines such as Aviltholadi Bhasmam, Shula Vinashini Vatika and Panaviraladi Bhasmam for the treatment of disorders of liver, spleen, oedema [6].

2. Materials and Methods
2.1. Collection of leaves and preparation of extract
The leaves of *H. auriculata* were collected from Madurai, Chennai, Tamilnadu, India. The leaves were washed, shade dried for 10 d and powdered in mechanical blender. About 10 g of leaves powder was soaked in methanol for 72 h. The greenish supernatant liquid was filtered by filter paper and condensed in rotor evaporator at 50 °C, which yields gummy extract.

Fig 1: Habitat of *Hygrophila auriculata*

2.2 Cytotoxicity Assay

2.2.1. Chemicals and reagents

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) invitrogen, USA. Acridine orange were obtained from Sigma, USA. All other fine chemicals were obtained from Sigma, Aldrich.

2.2.2. Cell culture

A549 and HepG2 cells were obtained from NCCS (National Centre For Cell Science, Pune) and cultured in Rose-well Park Memorial Institute (RPMI) medium, supplemented with 10% fetal bovine serum, penicillin/streptomycin (250 U/mL), gentamycin (100 µg/mL) andamphotericin B (1 mg/mL) obtained from Sigma Chemicals, MO, USA. All cell cultures were maintained at 37 ◦C in a humidified atmosphere of 5% CO_2. Cells were allowed to grow to confluence over 24 h before use [7].

2.2.3. Cell growth inhibition studies by MTT assay

Cell viability was measured with the conventional MTT reduction assay, as described previously with slight modification. Briefly, A549 and HepG2 cells were seeded at a density of 5×10^3 cells/well in 96-well plates for 24 h, in 200µL of RPMI with 10% FBS. Then culture supernatant was removed and RPMI containing various concentrations (5–160 µg/mL) methanol extract of leaves of *H. auriculata* was added and incubated for 48 h. After treatment, the cells were incubated with MTT (10 µL, 5 mg/mL) at 37 °C for 4 h and then with DMSO at room temperature for 1 h. The plates were read at 595 nm on a scanning multi-well spectrophotometer. Data are represented as the mean values for three independent experiments [8].

$$\text{Cell viability (\%)} = \left[\frac{\text{Mean OD}}{\text{Control OD}}\right] \times 100$$

2.3. Gas chromatography–Mass Spectrometry (GC–MS)

The methanol extract of samples were injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Following chromatographic conditions were used: Helium as carrier gas, flow rate of 1 mL/min; and the injector was operated at 200 °C and column oven temperature was programmed as 50-250 °C at a rate of 10 °C/min injection mode. Following MS conditions were used: ionization voltage of 70 eV; ion source temperature of 250 °C; interface temperature of 250 °C; mass range of 50-600 mass units.

2.3.1. Identification of components

The database of National Institute Standard and Technology (NIST) having more than 62,000 patterns was used for the interpretation on mass spectrum of GC-MS. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library [9].

3. Results and Discussion

3.1. Anticancer activity by MTT assay method

Oxidative stress; the consequence of an imbalance of prooxidants and antioxidants in the organism and is a key phenomenon in chronic diseases. Oxidative stress is now recognized to be associated with more than 100 diseases, as well as with the normal aging process. Overproduction of free radicals can cause oxidative damage to biomolecules, (lipids, proteins, DNA), eventually leading to many chronic diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis [10]. Common treatments such as radiotherapy and chemotherapy cause some complications. Antioxidants are intimately involved in the prevention of cellular damage the common pathway for cancer, aging, and a variety of diseases. Herbal extracts have antioxidant compounds that can induce apoptosis and inhibit cell proliferation [11]. Cell cultures are widely used as *in vitro* cell-based models for various biological researches. Many medicinal and food plants contain large amounts of chemical components having broad spectrum of pharmacological activities like anticancer, antitumor and anti oxidant activities [12]. The anticancer activities are mainly due to phenolic acids, flavonoids, and phenolic diterpenes. Natural products are reportedly beneficial to physiological health. Various favonoids and nonflavonoids have been reported as showing anticancer, antitumor and antioxidant activities [13]. The extractive value, total polyphenolic content and anticancer activity was at its peak in methanol extract indicating that most of the active components are extracted with methanol [14]. The effect of methanol extract of leaves of *H. auriculata*, was performed on two different cancer cell lines and Vero cell line by MTT assay method. Dose response curve was plotted with the concentration ranges from 5-160 µg/mL of methanol extract. The maximum cell death of A549 cells by methanol extract of leaves of *H. auriculata* was 70.54±0.004% at 160 µg/mL concentration (Table 1 and Fig 2) and the IC_{50} was 68.70 µg/mL concentration. The maximum cell death of HepG2 cells by methanol extract of leaves of *H. auriculata* was 72.37±0.002% at 160 µg/mL concentration (Table 2 and Fig 3) and the IC_{50} was 67.08 µg/mL concentration. The maximum cell death of Vero cells by methanol extract of leaves of *H. auriculata* was 30.41±0.002% at 160 µg/mL concentration (Table 3 and Fig 4) and the IC_{50} was 263.07 µg/mL concentration.

Table 1: Anticancer activity of methanol extract of leaves of *H. auriculata* on A549 cell line

S. No	Concentration (µg/mL)	Cell death (%)
		A549 cell line
1	5	01.38 ± 0.005
2	10	02.67 ± 0.003
3	20	07.71 ± 0.004
4	40	28.58 ± 0.006
5	80	58.25 ± 0.005
6	160	70.54 ± 0.004

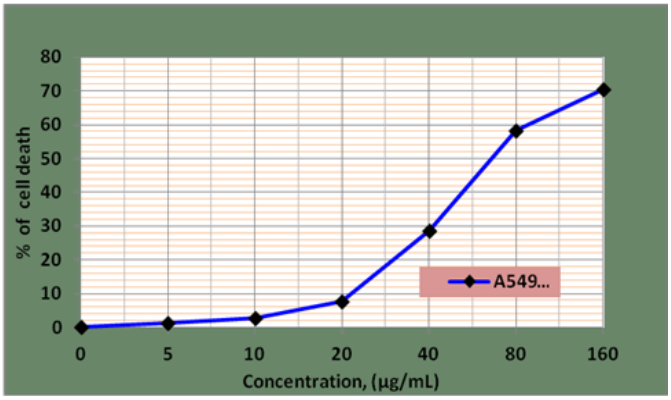


Fig 2: Anticancer activity of methanol extract of leaves of *H. auriculata* on A549 cell line

Table 2: Anticancer activity of methanol extract of leaves of *H. auriculata* on HepG2 cell line

S. No	Concentration (µg/mL)	Cell death (%) HepG2 cell line
1	5	03.32 ± 0.002
2	10	11.51 ± 0.003
3	20	24.09 ± 0.002
4	40	44.16 ± 0.003
5	80	59.63 ± 0.002
6	160	72.37 ± 0.002

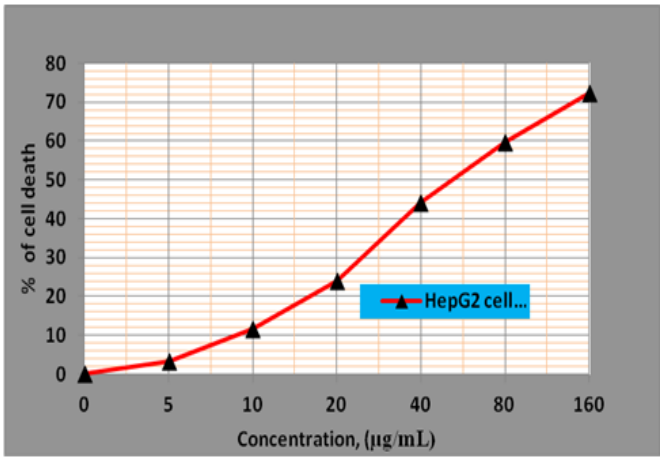


Fig 3: Anticancer activity of methanol extract of leaves of *H. auriculata* on HepG2 cell line

Table 3: Anticancer activity of methanol extract of leaves of *H. auriculata* on Vero cell line

S. No	Concentration (µg/mL)	Cell death (%) Vero cell line
1	5	02.16 ± 0.002
2	10	08.13 ± 0.004
3	20	15.07 ± 0.006
4	40	19.63 ± 0.005
5	80	21.93 ± 0.006
6	160	30.41± 0.002

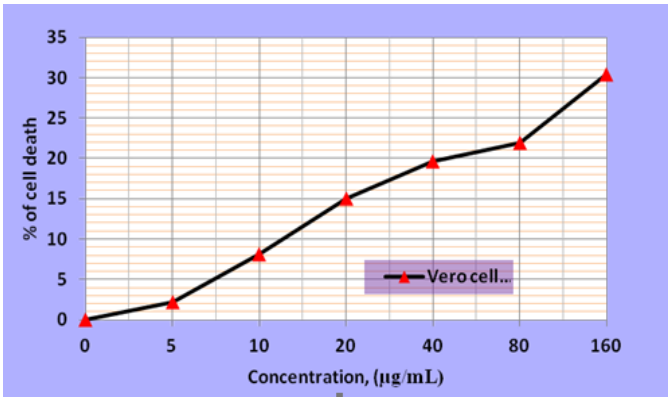


Fig 4: Anticancer activity of methanol extract of leaves of *H. auriculata* on Vero cell line

3.2. GC-MS analysis

The results of GC-MS analysis showed the presence of an antioxidant compound such as 5-hydroxy-7-methoxy-2-phenyl-4H-1-benzopyran-4-one in the methanol extract of leaves of *H. auriculata*, which neutralize free radicals, which injure tissues. The compound octahydroxanthen-1,9-dione,3,3,7,7-tetramethyl-10-propyl was eluted and the derivative of this compound reported as antibacterial, antiviral and antitumor effects [15]. The compound 8-Amino-5-p-chlorophenoxy-6-methoxy-2-methylquinoline was eluted and the derivative of this compound reported as nervous disorder and urinary disorder effects and thecompound 2-(p-Methoxyphenyl)-4-quinoline carboxamide was eluted, which the derivative was reported as CB1 and CB2 cannabinoid receptors (Fig 5 and Table 4).

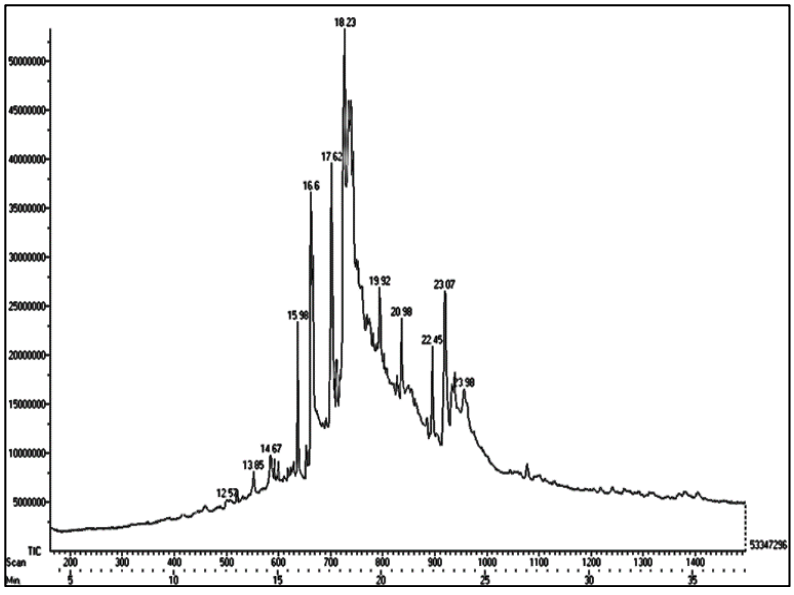
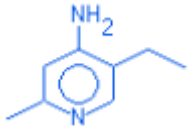
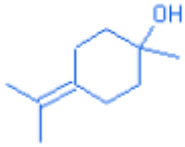
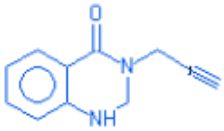
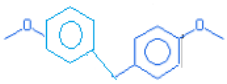
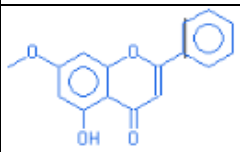
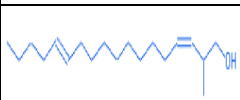
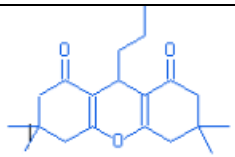
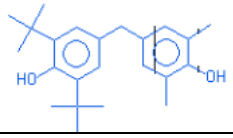
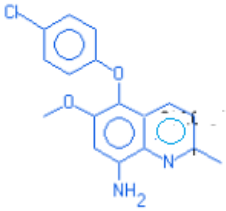
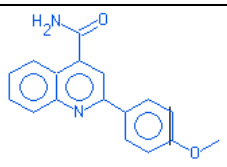


Fig 5: GC-MS chromatogram of methanol extract of leaves of *H. auriculata*

Table 4: GC-MS analysis of methanol extract of leaves of *H. auriculate*

S. No	Retention Time	Compounds	Structure	Mol. Wt. (g/mol)	Mol. Formula
1	12.57	5-Ethyl-2-methyl-pyridin-4-amine		136	$C_8H_{12}N_2$
2	13.85	Cyclohexanol,1-methyl-4-(1-methylethylidene)-		154	$C_{10}H_{18}O$
3	14.67	4(1H)-Quinazolinone,2,3-dihydro-3-(2-propynyl)-		185.52	$C_{11}H_{10}N_2O$
4	15.98	Dodecanoic acid,10-methyl,methyl ester		227.58	$C_{14}H_{28}O_2$
5	16.6	Methane, bis (p-methoxyphenyl)-		227.58	$C_{15}H_{16}O_2$
6	17.62	7-methoxy-2,2,4,8-tetramethyltricyclo[5.3.1.0(4,11)]undecane		235.58	$C_{16}H_{28}O$
7	18.23	5-hydroxy-7-methoxy-2-phenyl-4H-1-Benzopyran-4-one,		268	$C_{16}H_{12}O_4$
8	19.92	Z,E-2-Methyl-3,13-octadecadien-1-ol		280.65	$C_{19}H_{36}O$
9	20.98	Octahydroxanthen-1,9-dione,3,3,7,7-tetramethyl-10-propyl		315.60	$C_{20}H_{28}$
10	23.98	Phenol,2,6-bis(1,1-dimethylethyl)-4-[(4-hydroxy-3,5-dimethylphenyl) methyl]		339.68	$C_{23}H_{32}O_2$
11	23.07	8-Amino-5-p-chlorophenoxy-6-methoxy-2-methylquinoline		314	$C_{10}H_8N_2O_3$
12	22.45	2-(p-Methoxyphenyl)-4-quinoline carboxamide		277.50	$C_{16}H_{11}$

4. Conclusion

Several therapeutic methods are available for the treatment of cancer, and in most cases, undesirable side effects (gastrointestinal disorders, kidney damage) are associated with them. Compounds including alkaloids, phenol compounds, and monoterpenes have anticancer effects. Especially the terpenoids and polyphenolic compounds have antioxidant properties which inhibit damage of DNA by free radicals, cell cycle arrest, induction of apoptosis and inhibition of angiogenesis in tumor cells. The secondary metabolites present in the methanol extract of leaves of *H. auriculata* showed the significant cytotoxic effect on A549 and HepG2 cell lines. Further research work needed to find out the active compound present in the methanol extract of *H. auriculata*.

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