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NON-TARGETED ANALYSIS AND CYTOTOXIC ACTIVITY OF *HAMELIA PATENS* JACQ.

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ABSTRACT: In the present study we have done non-targeted analysis of extract from *Hamelia patens* Jacq. by GC/MS and estimated the inhibitory concentration (IC_{50}) of the extract in three human tumor cell lines MCF-7, H-460 and SF-268. *Hamelia patens* is an herbaceous perennial plant of family Rubiaceae. Parts of the plant are used in Peruvian and Mexican folk-medicine. Dried leaves of *Hamelia patens* were extracted for alkaloid. The extract was named HPAE and the yield was 1.76% (w/w). Non-targeted GC/MS analysis of HPAE was performed using Shimadzu QP 2000 GC, equipped with a ULBON-HR-5 capillary column and mass spectrometer as detector. The cancer cell lines were obtained from National Centre for Cell Science, Pune. *In-vitro* cytotoxic activity of HPAE on cancer cell lines were conducted by MTT assay. The GC/MS analysis of the HPAE enabled the identification of 44 compounds and IC_{50} of HPAE in MCF-7, H-460 and SF-268 were found to be 8.42 ± 1.16, 90.40 ± 18.48 and 91.47 ± 19.74 µg/ml, respectively. The HPAE was found to be a potential cytotoxic agent against the above cell lines.

INTRODUCTION: *Hamelia patens* Jacq. is a herbaceous perennial plant of family Rubiaceae, commonly known as coffee family [1]. This plant is popular as Hamelia, Scarlet bush, Fire bush, Hummingbird bush, Polly red head, Texas firecracker and grows in various tropical regions like West Indies, Florida, Costa Rica and Argentina [2]. Parts of the plant are used in Peruvian and Mexican folk-medicine in skin diseases, wound healing, insect bites and menstrual disorders and also as anti-malarial, anti-inflammatory and anti-rheumatic.

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Extracts of different parts of the plant have also shown antidiarrheal, antibacterial, vasorelaxant, antifungal, cytotoxic and antinociceptive properties [2]. *Hamelia patens* has been proved to contain various biologically active chemicals like flavonoids and alkaloids like pteropodine, isopteropodine, (-)-hameline, aricine, speciophylline, rumberine, palmirine, stigmast- 4- ene- 3, 6- dione, rosmarinic acid, maruquine, isomaruquine, rutin and 5, 7, 2', 5'-tetrahydroxyflavanone 7- rutiroside [3].

Detailed analysis of constituents of a sample by hyphenated techniques such as gas chromatography - mass spectrometry (GC/MS) is called profiling. With the help of these techniques, a comprehensive chromatographic profile of a sample with the relative or absolute quantification of all compounds of the sample could be obtained [4]. It is estimated

that in plant kingdom approximately 200,000 compounds may be present and of these, about 10,000 are known [5]. With the help of GC/MS both targeted and non-targeted analysis can be performed. In targeted analysis, relatively small number of pre-defined compounds can be analyzed, for example, secondary metabolites of *Gaultheria procumbens* L. [6]. Methods for targeted analysis have been established to detect a few selective compounds while all other signals are neglected [5]. Such methods have high accuracy and precision which is further increased with the use of stable isotope labelled internal standards. But, stable isotope labelled standards are highly expensive and are commercially available only for a limited number of compounds [4]. In non-targeted analysis all compounds which show peaks above certain intensity, can be characterized on the basis of their GC retention indices and mass spectral patterns. Majority of compounds identified in such non-targeted analysis are not formally identified however, their relative measurement could be reliably made even though their structure is not known [7].

Conventional GC/MS employ the electron ionization technique to generate and measure only abundant positively charged ions. Here, constant energy (70 eV) is provided to obtain a reproducible mass spectrum by inducing fragmentation of the parent ion. Because of the use of constant energy, investigators can share libraries of such spectra as reference with each other and several commercial libraries are also available for reference [8].

In this study we have done non-targeted analysis of extract of leaves of *Hamelia patens* by utilizing GC/MS. The IC50 of the extract was estimated in three human tumor cell lines, MCF-7 (breast adenocarcinoma), H-460 (non-small cell lung cancer), and SF-268 (anaplastic astrocytoma).

MATERIALS AND METHODS:

Plant Material: The leaves of *Hamelia patens* were collected in month of August - September from New Delhi, India and were shade dried for 20 - 25 days. The was authenticated at CSIR-NISCAIR, New Delhi, India by Dr. Sunita Garg, Chief Scientist, Raw Materials Herbarium and Museum, and a specimen was deposited in the Museum, CSIR-NISCAIR, New Delhi, India.

Preparation of Extracts: Dried leaves of *Hamelia patens* were extracted for alkaloids with modified method of Harborne JB [9]. An accurately weighed 100 g dried powdered leaves were extracted with 70% ethanol using cold maceration method. The extract was filtered using Whatman filter paper Grade 1 and the collected filtrate was concentrated in Rotary evaporator below 40 °C. The percentage yield of the extract was 21.13% (w/w). About 18 g of the dried extract was re-dissolved in 100 mL of 70% alcohol and acidified with 2M H_2SO_4. The acidified content was extracted with chloroform and the aqueous acid layer was separated from the organic layer. Then the aqueous acid layer was basified with NH_4OH to pH 10. After that the basified content was extracted with a mixture of chloroform: methanol (3:1) and the organic layer was separated and concentrated in Rotary evaporator below 40 °C. The dried chloroform-methanol extract, named HPAE, was stored at 4 °C until further used.

GC/MS Analysis of HPAE: Non-targeted GC/MS analysis of HPAE was performed using a Shimadzu QP 2000 GC, equipped with a ULBON-HR-5 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) and a mass spectrometer as detector. The carrier gas was helium, at a flow rate of 1 mL/min. Column temperature was initially 100 °C for 6 min, then gradually increased to 250 °C at 10°C/ min. For GC/MS detection an electron ionization system was used with an ionization energy of 70 eV. Diluted samples (1.0 μL) were injected automatically in splitless mode. Injector and detector temperatures were set at 250 and 280°C, respectively.

Cell Culture: Three human cancer cell lines MCF-7, H-460 and SF-268 were obtained from National Centre for Cell Science (NCCS), Pune. These were grown as monolayer and routinely maintained in RPMI-1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL) at 37 °C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity Study: MTT (3- (4, 5-dimethyl-thiazol-2-yl)- 2, 5-diphenyltetrazolium) Assay, a colorimetric assay developed by Mosmann, 1983 was used to evaluate cell vitality [10]. Approximately,

1×10^4 cells per well were seeded on 96 well plate in RPMI-1640 medium supplemented with 2 mM glutamine, 10% FBS and antibiotics for overnight incubation at 37 °C in a humidified atmosphere containing 5% CO_2. Later, the medium was replaced with fresh medium containing different concentrations of HPAE (0.001, 0.01, 0.1, 1.0 and 10 µg/ml). Stock solution of HPAE was prepared by dissolving the extract in DMSO followed by filtration through syringe filters (pore diameter 0.2 µm). Stock solution was diluted in RPMI-1640 to obtain required concentrations for the assay.

The concentration of DMSO was kept below 0.1% in all experiments. After 48 h of incubation at 37°C in a humidified atmosphere containing 5% CO_2, media was replaced with fresh media and 20 µL of MTT solution (2 mg/mL in PBS) was added to each well and incubated for another 4 h. The media was replaced with 100 µL of DMSO and the plate was shaken for 15 min to solubilize the crystals of MTT formazan. The optical density for each well was determined using ELISA reader at 550 nm. The experiments were repeated at least three times and every reading was taken in triplicate. The effect of HPAE on the proliferation of cancer cells was expressed as the percent of cytotoxicity, using the following formula:

Percent of cytotoxicity = (A_{550} control - A_{550} sample) / A_{550} control × 100

Statistical Analysis: All data were expressed as Mean ± S.D.

RESULTS:

Extraction Yield: The extraction yield for HPAE was 1.76% (w/w).

GC/MS Analysis of HPAE: Gas chromatography mass spectroscopy analysis was carried out on alkaloid extract of *Hamelia patens*. The total ion chromatogram (TIC) of HPAE showing the GC/MS profile of the compounds identified is given in the **Fig. 1**. The peaks in the TIC were integrated and were compared with the spectrum of components stored in the NIST GC/MS library. The GC/MS analysis of the HPAE enabled the identification of 44 compounds of which mostly are nitrogenous compounds as per library reference. Detailed tabulations of GC/MS analysis of HPAE are given in **Table 1**.

Cytotoxicity Study: Cytotoxicity studies of HPAE was carried out by the MTT assay using MCF-7, H-460 and SF-268 cells treated with varying concentrations of the extract or positive control (doxorubicin) for 48 h. As shown in **Table 2**, alkaloid extract of *Hamelia patens* was cytotoxic to the three cancer cell lines tested and inhibited proliferation of the cell lines in a dose-dependent manner. HAPE showed considerable cytotoxicity in MCF-7 cell lines **Fig. 2**. However, in H-460 and SF-268 the extract showed a moderate inhibition of proliferation **Fig. 3** and **Fig. 4**. The IC_{50} of HPAE in MCF-7, H-460 and SF-268 were found to be 8.42 ± 1.16, 90.40 ± 18.48 and 91.47 ± 19.74 µg/ml, respectively **Table 2**.

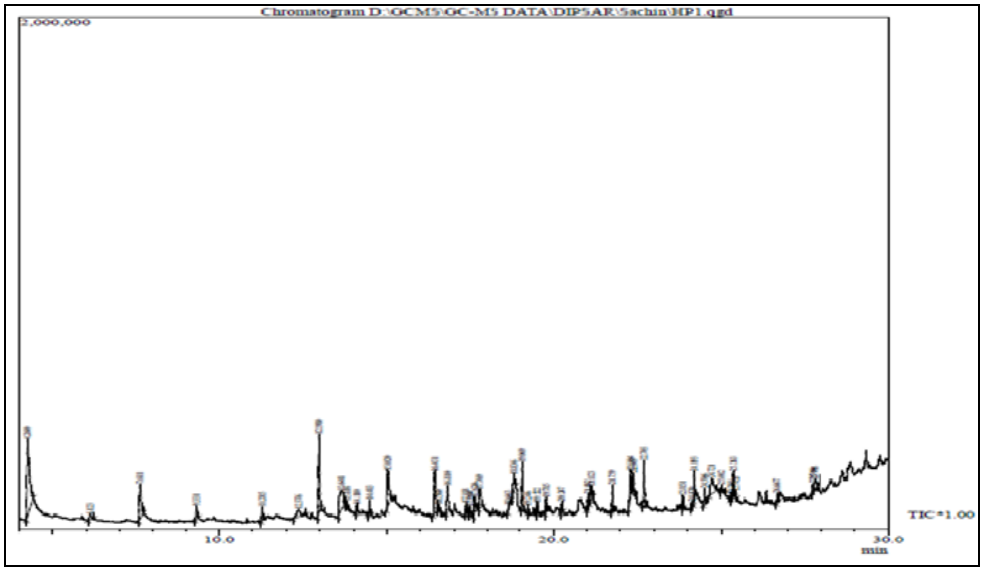

FIG. 1: TOTAL ION CHROMATOGRAM OF ALKALOID EXTRACT LEAVE OF *HAMELIA PATENS* JACQ.

TABLE 1: GC/MS ANALYSIS OF ALKALOID EXTRACT OF LEAVES OF *HAMELIA PATENS* JACQ.

Peak no.	RT	Area%	Name
1	4.260	14.66	Phosphoric acid, trimethyl ester
2	6.123	0.70	2, 4-dihydroxy-3, 3-dimethylbutanoic acid gamma lactone
3	7.611	4.86	Levoglucosenone
4	9.331	1.45	Benzoic acid, 2-hydroxy-, methyl ester
5	11.285	1.06	Benzyl 3-(3-methoxy-3-oxopropyl)-4-methyl-1H-pyrrole-2-carboxylate
6	12.376	2.08	Adamantan-1-yl-carbamic acid (1 azabicyclo[2.2.2]oct-3-yl) ester
7	12.980	6.52	l-Proline, N-methoxycarbonyl-, isohexyl ester
8	13.648	5.60	1-Methoxy-1-methyl-1-silacyclohexane
9	13.816	0.45	1H-Indole-2,3-dione, 1-(tert-butyldimethylsilyl)-5-isopropyl-, 3-[(O-tert-butyldimethylsilyl)oxime]
10	14.110	0.84	l-Proline, N-methoxycarbonyl-, isohexyl ester
11	14.483	1.16	3,8-Dimethylene-1-cyclooctene
12	15.020	5.42	Alpha-methylphenylalanine
13	16.431	5.81	3,3,4,6-Tetramethyl-1-benzofuran-2(3H)-one
14	16.560	0.47	Megastigmatrienone 4
15	16.810	3.27	4,7-Dimethoxy-2-methyl-1H-indene
16	17.358	1.16	Megastigmatrienone 4
17	17.460	1.07	4-oxo-alpha-damascone
18	17.620	2.20	1,4-Dioxa-7,9-diazacycloundecane-8-thione
19	17.769	3.00	2-(2-tert-butylphenoxy)-N'-[(2-nitrophenyl)methylidene]acetohydrazide
20	18.663	0.66	2-Methyl-2-(4-oxo-2-[(Z)-2-(2-thienyl)ethenyl]-4,5-dihydro-1,3-oxazol-5-yl)propyl acetate
21	18.836	2.35	12-Oxatetracyclo[5,2,1,1(2,6).1(4,10)]dodecan-11-one
22	19.069	3.86	3-(3,4-Dimethoxyphenyl)-1H-pyrazol-5-ol
23	19.246	0.56	4-([tert-Butyl(dimethyl)silyl]oxy)-3-methyl-2-butanone
24	19.522	0.92	Diethyl 2,6-dimethyl-3,5-pyridinedicarboxylate
25	19.785	1.20	n-Heptadecanol-1
26	20.247	0.73	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane
27	21.032	0.96	Phthalic acid, heptyl 2-(2-methoxyethyl)hexyl ester
28	21.123	2.25	1,4-Dimethoxy-6,7,8,9-tetrahydro-5H-benzo[a]cyclohepten-5-one
29	21.759	2.03	Methyleicosenate
30	22.304	3.08	Palmitic acid
31	22.389	1.22	1-butyl 2-(8-methylnonyl) phthalate
32	22.703	3.49	1-nonadecene
33	23.851	1.05	2-Cyclohexyleicosane
34	24.120	0.37	(R)-(-)-14-Methyl-8-hexadecyn-1-ol
35	24.193	2.32	Methyl (Z)-9-octadecenoate
36	24.506	1.37	Methyl 2-hydroxyarachidate 1TMS
37	24.721	3.90	2-Hexyl-1-decanol
38	25.012	0.86	Heptadecyl 3-chloropropanoate
39	25.293	0.34	1-[4-(3-hydroxyphenyl)-1-methyl-4-piperidinyl]-1-propanone
40	25.363	1.84	1-Heneicosanol
41	25.433	0.36	Octacosane
42	26.667	0.70	(2-fluorophenyl)methyl-1H-purin-6-amine
43	27.746	0.48	2-Bromotetradecane

TABLE 2: *IN-VITRO* CYTOTOXIC ACTIVITY OF ALKALOID EXTRACT OF LEAVES OF *HAMELIA PATENS* JACQ. (n = 3)

Cell Lines	HPAE	Doxorubicin
	IC_{50} (µg/mL)	
MCF-7	8.42±1.16	0.18±0.03
H-460	90.40±18.48	0.07±0.02
SF-268	91.47±19.74	0.08±0.01

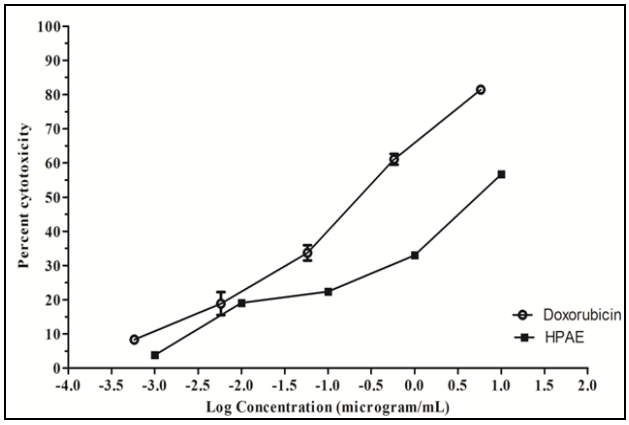

FIG. 2: CYTOTOXICITY OF ALKALOID EXTRACT OF LEAVES OF *HAMELIA PATENS* JACQ. IN MCF-7 CANCER CELL LINES

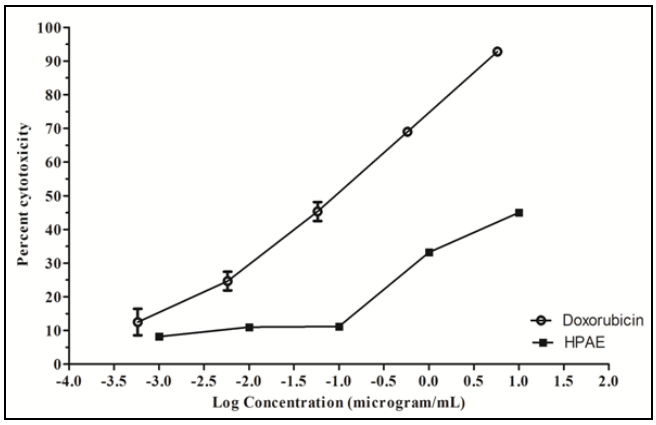

FIG. 3: CYTOTOXICITY OF ALKALOID EXTRACT OF LEAVES OF *HAMELIA PATENS* JACQ. IN H-460 CANCER CELL LINES

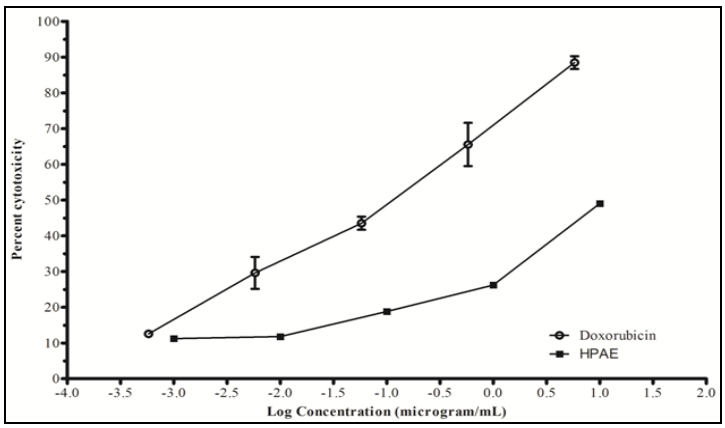

FIG. 4: CYTOTOXICITY OF ALKALOID EXTRACT OF LEAVES OF*HAMELIA PATENS* JACQ. IN SF-268 CANCER CELL LINES

DISCUSSION: Separation techniques like LC including HPLC and UPLC have wide adaptation capacity while, GC and CE have high resolution and are frequently used for separation of secondary metabolites of plant extract. For detection, NMR is convenient for highly abundant polar metabolites whereas MS is much better option for non-polar or semi-polar compounds present in low concentrations in samples [11]. GC/MS technique, now-a-days, is being utilized extensively to separate and identify compounds in plant extracts [12, 13]. Currently available GC/MS data systems have electron ionization mass spectral reference libraries as closely integrated component. The electron ionization mass spectral library of NIST/EPA/NIH provide spectra of over 200,000 compounds for reference [14]. The GC/MS analysis of HPAE was performed using ULBON-HR-5 capillary column and 44 compounds were identified **Table 1** with typical total ion chromatograms (TIC) of the extract was shown in **Fig. 1**.

Previous studies have reported the presence of pentacyclic oxindole alkaloids in the leaves of *Hamelia patens*. Borges *et al*., reported the isolation of palmirine and rumerine from the aerial parts of *Hamelia patens* [15]. Other pentacyclic oxindole alkaloids reported to be present in the leaves of *Hamelia patens* are isoteropodine, maruquine and alkaloid A [16]. Our data also suggested the presence of oxindole alkaloid (compound 9, RT 13.816 min in **Table 1**). GC/MS data of HPAE also found to contain acetohyrdrazide compound (compound 19, RT 17.769 min **Table 1**). Series of acetohydrazide moiety containing compounds were synthesized and their anticancer activity against three different breast cancer cell lines *i.e.,* MCF-7, MDA-MB-231 and ZR-75 were evaluated. Several of the compounds had shown promising activity [17]. The mechanism of anticancer activity of acetohdrazide compound suggested were increase in BAX gene expression and decrease in BRAC-1 and CD 44 gene expression [18].

Study conducted by Mena-Rejon *et al.*, have found that methanolic extract of *Hamelia patens* have cytotoxic activity against cervix squamous carcinoma (SiHa) and cervix adenocarcinoma (HeLa) cell lines [19].

Another study has shown the cytotoxic effects of methanol and acetone extract of *Hamelia patens* on HeLa and CRL 1619 (skin) cancer cell lines [20]. The HPAE showed its highest cytotoxic activity against MCF-7 breast cancer cell lines. There is a possibility that the cytotoxicity in MCF-7 cancer cell might be because of 2-(2-tert-butylphenoxy)-N'-[(2 nitrophenyl) methylidene] acetohydrazide but similar level of cytotoxicity was not shown in H-460 and SF-268 cancer cell lines. The MCF-7 cancer cell lines are complexly affected by the antiestrogen like tamoxifen [21]. Therefore, HPAE must contain some constituents which affect the estrogen related pathways in the MCF-7 cancer cell lines.

CONCLUSION: This study adds on the existing information concerning cytotoxicity activity of *Hamelia patens*. According to data obtained from the present study, the HPAE was found to be a potential cytotoxic agent. Further studies are be required to pinpoint the active compounds that confer anticancer activity of the *Hamelia patens*. Identification of such compound(s) will further help in revealing the mechanism of by which the extract exert cytotoxic effects on cancer cell lines.

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CONFLICT OF INTEREST: Nil

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