SYNTHESIS AND CYTOTOXIC ACTIVITY OF *N*-PHENYLATED DERIVATIVES OF 5-HALOPYRIMIDINE-2,4-DIONE

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ABSTRACT: The presented research project demonstrates new synthetic strategy for providing 5-halopyrimidine-2,4-dione derivatives comprising promising cytotoxicity. This study was carried out to explore the prospective future directions on the development of more effective yet specific 5-halopyrimidine-2,4-dione analogues effectively showing anticancer activity. Pyrimidine-2,4-dione is considered as privileged structures in drug discovery owing to marked medicinal potential and synthetic accessibility. As a result, a variety-oriented synthesis of multifaceted organic molecules from simple and readily available substrate has been carried out that has resulted in the derivatization of 5-halopyrimidine-2,4-dione predominantly giving N_1 substituted compounds. In addition, microwave-assisted solvent free conditions using solid support has proved to be of dynamic importance in term of energy efficacy and designing of an ecofriendly synthetic route with significantly reduced reaction time. Newly formed *N*-alkylated derivatives show greater anticancer activity determined by He La cell line.

Keywords: 5-halopyrimidine-2,4-dione, anticancer activity, He La cell line, solid support, microwave.

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INTRODUCTION

Halogenated phenstatin was synthesized from halogenated substituted benzoic acid and benzene carrying substituents. The analogues synthesized were characterized with H-NMR and C13 NMR, X-Ray diffraction and crystallographic analysis. Cytotoxicity was determined in vitro by using Huh-7 cell line (Wang *et al*., 2019).

Five different human cancer cell lines were used to determine the cytotoxicity of modified pyrimidine neucleoside analogues 2-deoxyuridine, thymidine and 5-flouro–2-deoxyuridine prepared by simple methods. These are very stable compounds (Michalska *et al*., 2019).

A solvent free methodology involves the contact of recyclable mineral supports such as alumina and silica on the neat reactants. In review article potash alum commonly known as alum has excellent catalytic activity and proved to be environment friendly low-cost catalyst in a number of organic reactions during the 2014-2018 (Brahmachan *et al*., 2019).

The traditional system of synthesis comprising several hours is too slow to control the fast paced spread of disease causing pathogens. The use of microwave in these protocols instead of the conventional method has increased the rate of synthesis to manifold (Quin *et al*., 2010, Sharma, 2015)

Heterocyclic moiety is the uracil containing compounds that are quite well known for possessing strong anti-microbial and anti-malignant properties due to which they often gain the focus of research projects (Gopalakrishnan *et al*., 2010, Tuker, 2010).

Amongst most of the compounds synthesized, that exhibit moderate to high level of bioactivity, 95% of these corresponds to those that consists of the heterocycle as the basic fragment in its diverse structure. Owing to this extraordinary feature of heterocycles, they are mostly embedded as the key element in numerous pharmacophores (Martin *et al*., 2008 and Jafri *et al*., 2011).

Moreover, it is of utmost importance that these developmental processes and their synthesis be speeded up so that the lead identification and optimization processes will ultimately be boosted so that the increasing demand to combat deadly microbials could be overcome (Lidstrom *et al*., 2001)

Taking into account the potency and extensive applicability of 5-halopyrimidine-2,4-diones, this study was organized to develop a new and convenient pathway for the fabrication of derivatives by the use of solvent free solid supported microwave assisted technology.

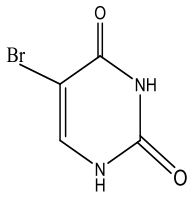
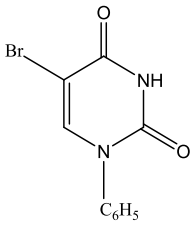
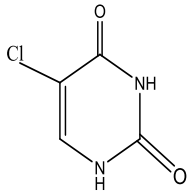
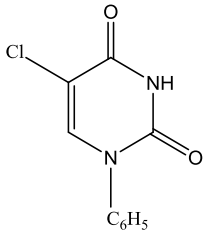
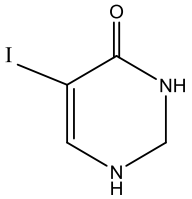
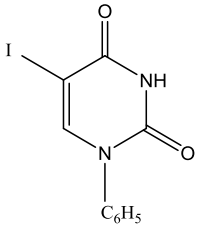
MATERIALS AND METHODS

Experimental: Chemicals used in this project purchased from Sigma and Merck showing the analytical grade
Instruments Used

MW oven (domestic), 2450 MHz with power of 950 W was used. Melting points determination was done with Gallenkamp M.P apparatus. The assembling of the compounds, **BU, BU-1, CU, CU-1, IU, IU-1** (Table-1)

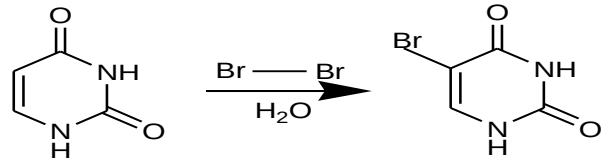
was carried out via conventional, microwave-assisted and solid support methodology.

Table-1: Systematic names and structures of the synthesized compounds

Name	Abbreviation	Structure
5-bromopyrimidine-2,4-dione	BU	
N-phenyl-5-bromopyrimidine-2,4- dione	BU-1	
5-chloropyrimidine-2,4-dione	CU	
N-phenyl-5-chloropyrimidine-2,4- dione	CU-1	
5-iodopyrimidine-2,4-dione	IU	
N-phenyl-5-iodopyrimidine-2,4- dione	IU-1	

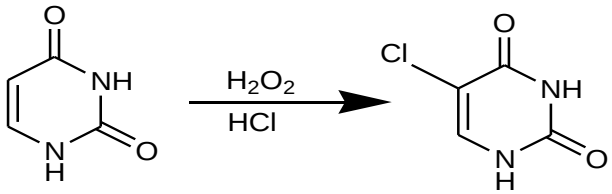
Synthesis of BU, CU and IU through microwave-assisted protocol (Scheme1,2 and Scheme3)

Synthesis of BU via microwave-assisted protocol: To a mixture of pyrimidinedione (1.0g/0.89mmol) in 8.0 ml of distilled water, liq.bromine (1.50ml) was added with constant stirring. Yellow coloured solution formed was irradiated for 60 s. White coloured products filtered and washed.



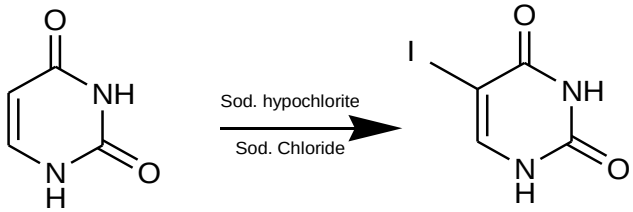
Scheme-1: showing 5-bromopyrimidinedione synthesis (B**U**).

Synthesis of CU via microwave-assisted protocol: To a mixture of uracil (0.89mmol/0.1050g) in H_2O_2 (1.0ml), conc. HCl was added drop wise with continuous stirring. It was then irradiated for 30s. The product was precipitated out which was separated through filtration and washed to obtain the pure product.



Scheme-2: 5-chloropyrimidinedione (C**U**).

Synthesis of IU via microwave-assisted protocol: Initially, 50 ml of distilled water was added to a mixture of pyrimidinedione (0.89mmole/0.1g),NaOH (0.2g) and I_2 (0.1g/ 0.78mmol).After stirring and irradiating, the contents of the flask were cooled to 20 ^{0}C followed by the addition of sodium hypochlorite solution (12%). It was again stirred for 60s at 20 ^{0}C. White precipitates obtained which were filtered and washed.



Scheme-3: Synthesis of 5-iodouracil (I**U**).

Synthesis of BU-1, CU-1, IU-1 through conventional protocol: Initially, BU/ CU/ IU (0.476g/ 2mmol) were added in DMSO (5.00ml/ 70.51mmol). In this solution, K_2CO_3 (0.138g/ 1mmol) was dissolved by continuous stirring at 80 ^{0}C. After adding bromobenzene (0.312g/ 2mmol) ,reaction mixture was heated for forty eight hours at a temperature of 80 ^{0}C to form mono phenylated compound.

Synthesis of BU-1, CU-1, IU-1 through microwave-assisted protocol: Initially, BU/ CU/ IU (2mmol) were added in DMSO (5.00ml/ 70.51mmol). In this solution, K_2CO_3 (0.138g/ 1mmol) was dissolved by continuous stirring at 80 ^{0}C. After fifteen minutes, bromobenzene (0.312g/ 2mmol) was also added in the reaction mixture. It was then heated in microwave for sixty seconds. After filtration and washing pure product was obtained.

Synthesis of BU-1, CU-1, IU-1 through solid supported microwave-assisted protocol: Initially, BU/ CU/ IU (2mmol) were added to solid support (celite/alumina2.0 g). To it, K_2CO_3 (0.138g/ 1mmol) and few drops of dist. water was added and mixed. The reaction mixture was then stirred at 80 ^{0}C for 15 min. after which phenyl bromide (0.312g/ 2mmol) was added. The contents of the flask were then irradiated with microwave for 60 s which resulted in the formation of product. In order to recover the final product, a mixture of DCM/methanol (1:1) was used to filter out the inorganic solid support and the leftover solvent was evaporated to get the pure product.

UV/VIS spectroscopic analysis was carried out using "Hitachi U-2800" spectrophotometer. The absorption peak of about 298-300 nm depicted by the compound BU, CU and IU in the UV spectra were a result of π-π* transition. In the case of **BU-1,CU-1** and **IU-1,** a slight red shift was observed due to the presence of a phenyl ring that extended the value of UV in the range of 312-315nm.

FTIR spectral analysis was done on Midac FT-IR spectrometer (M2000) applying KBr disc method. In the synthesized compounds, C-H stretching frequencies were observed in the region of 3204-3283 cm^{-1} which is characteristic of the aromatic ring. Other than this, the peaks appeared at 3500-3600 cm^{-1} showed aromatic N-H stretch. Similarly, the peak signal at 1350-1450 cm^{-1} was due to the aromatic C-N stretch. A characteristic sharp peak at about 1670 cm^{-1} was also observed owing to the C=O stretch of amides. The C-H in plane bending and stretch of aromatic halide was also confirmed by the presence of two peaks that were at 1380-1400 cm^{-1} and other at 700 cm^{-1}.

The HeLa cell line was used to study the anticancer activity of all synthesized compounds. The measurements of neutral red uptake essay depicted cell toxicity and cell viability. It provided a quantitative estimation of the amount of viable cells present in the culture as it is the capability of these cells that incorporate and bind the supra-vital dye neutral red within lysosome. 24 well plates were taken and seeded with Hela cellsncer with 5000 cells per well and cultured for two days. Then the samples were added on these cells after dissolving them in water at a concentration of 10mg/ml and autoclaving it. 150µM was the working concentration and then media was changed with new complete media supplemented with 150µM of all samples concentration. Cells were incubated at 37 ^{0}C and 5 % CO_2 for 48 hours. Once incubation has been done, the cells were removed from media and washed with phosphate buffer saline solution. They were then again incubated for 2 hours after the addition of Neutral red media. The cells were then again washed with PBS followed by the addition of freshly prepared de–staining solution and incubated for 10 min. As a result, the cells released their color .Then there OD (optical density) was taken at 570 nm. The control well had the highest OD which indicated higher cell density. The samples showing lower values than the control values showed cytotoxic effect on cells.

Table-2: Comparison of cytotoxic effects of synthesized compounds using different dilutions

Sample	Dilution factor	Avg OD at 570 nm
	0.5x	0.39
BU-1	0.25x	0.43
	0.12x	0.44
	0.5x	0.40
CU-1	0.25x	0.41
	0.12x	0.46
	0.5X	0.23
IU-1	0.25X	0.26
	0.12X	0.29
Control		0.49
Carrier (PBS)	0.5x	0.73

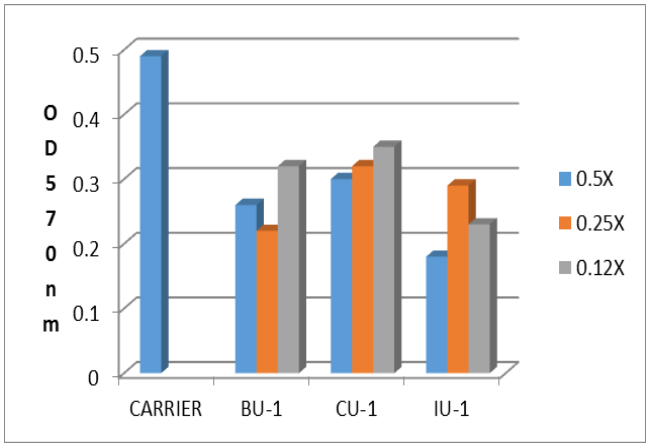


Fig. 1: Estimation of cytotoxic effect of *N*-phenylated analogues of BU-1, CU-1 and IU-1

RESULTS

Keeping in view the importance of all these bioactive analogues, following methods were used for their synthesis.

1. Conventional 2. Microwave 3. Solid support

Table-3: Reaction time, yield, mp of compounds synthesized by using different methods.

Compound	Conventional			Microwave assisted		
	Reaction Time Sec.	Yield %age	Melting Point ^{0}C	Reaction Time Sec.	Yield %age	Melting Point ^{0}C
CU	3600	70	283	60	77	285
CU-1	172800	15	186	60	18	186
BU	3600	65	296	60	75	298
BU-1	172800	10	200	60	12	202
IU	3600	70	297	60	86	297
IU-1	172800	12	210	60	19	211

Table-4: Comparison of synthesized compounds in term of yield and reaction time using different solid supports (Alumina and celite)

Compound abbreviation	Solid supported microwave assisted approach			
	Reaction time	Melting point	Yield by Using alumina	Yield by using celite
	Sec.	^{0}C	%age	%age
CU	40	285	81	87
CU-1	40	186	19	26
BU	40	300	80	85
BU-1	40	202	15	22
IU	40	297	89	90
IU-1	40	211	34	43

Spectral analysis of the compounds fabricated through solid-supported microwave-assisted protocol.

BU (5-bromopyrimidine-2,4-dione):
MS (m/z): 192 (M^+), 191 (M+1), 147, 119, 133; FT-$IR_{[KBr]}$ ν /cm^{-1}:3934, 3000, 1700, 1384, 999.834, 654.

BU-1 (5-bromo-1-phenylpyrimidine-2,4-dione)
MS (m/z): 266, 221, 120, 129, 104; FT-$IR_{[KBr]}$ ν /cm^{-1} 4784, 3128., 1515., 1391, 1100, 820., 575.

CU (5-chloropyrimidine-2,4-dione)
MS (m/z): 147, 131, 87, 78; FT-$IR_{[KBr]}$ ν /cm^{-1} 3850, 3204., 1676., 1425., 1224., 670.

CU-1 (5-chloro-1-phenylpyrimidine-2,4-dione)
MS (m/z): 225 (M^+), 207, 107, 120, 94; FT-$IR_{[KBr]}$ ν /cm^{-1}: 3560, 3065, 3214, 1669, 1430, 1225, 642.

IU (5-iodopyrimidine-2,4-dione)
MS (m/z): 239 (M^+), 196, 169; FT-$IR_{[KBr]}$ ν /cm^{-1}: 667, 712, 845, 988.

IU-1 (5-iodo-1-phenylpyrimidine-2,4-dione)
MS (m/z): 315 (M^+), 169, 119, 104, 297; FT-$IR_{[KBr]}$ ν /cm^{-1}: 3208, 1499, 1391, 1099, 659, 580.

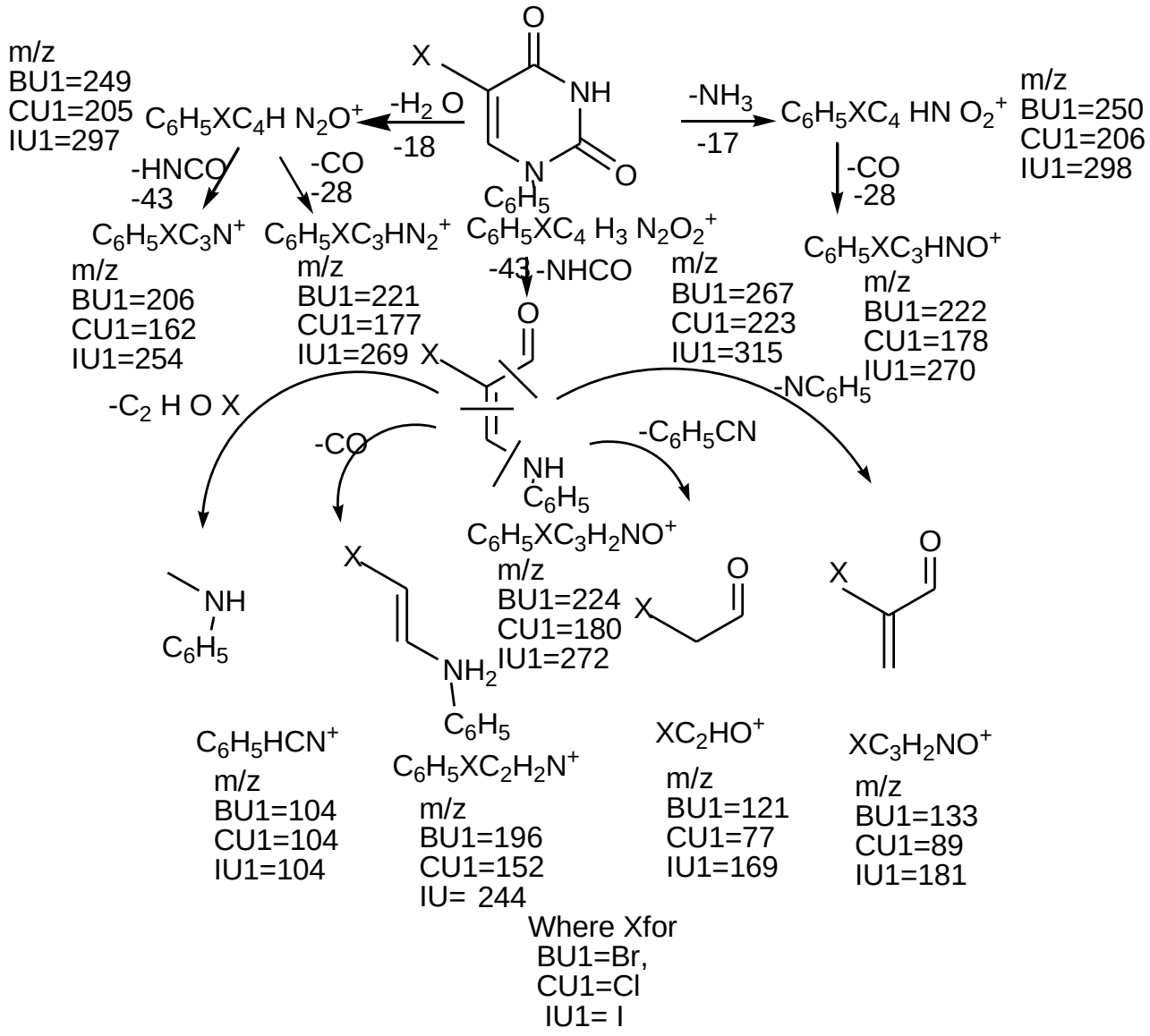


Fig. 2: scheme showing the fragmentation pattern of *N*-phenylated analogues of BU,CU and IU

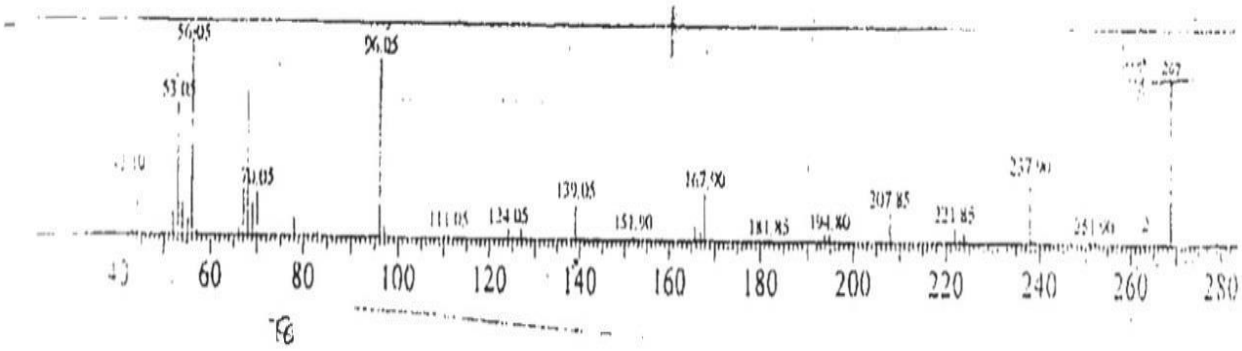


Fig. 2: Mass spectrum of BU-1

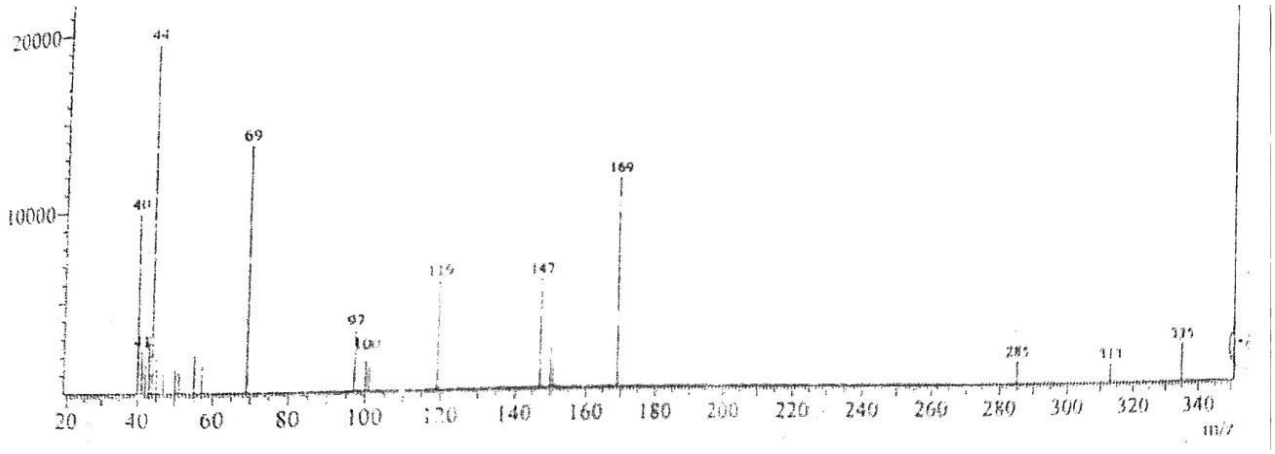


Fig. 3: Mass spectrum of CU-1

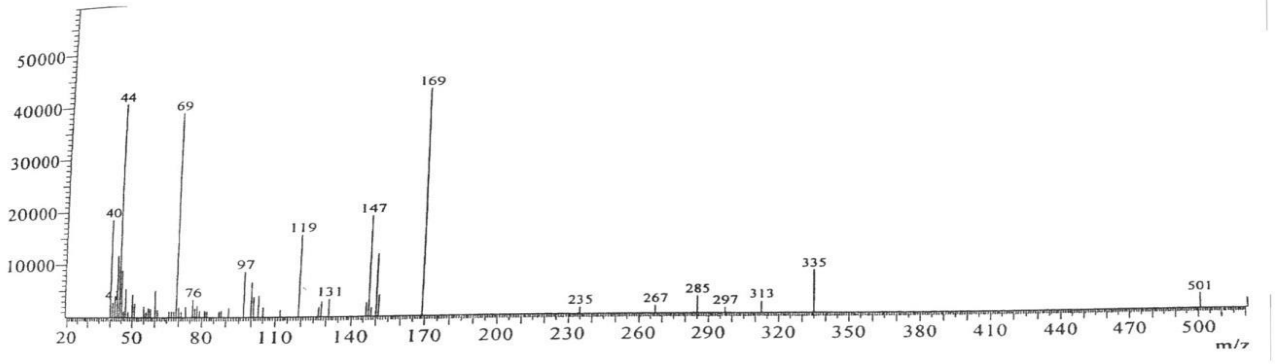


Fig. 4: Mass spectrum of IU-1

The GC-MS Schimadzo QP-2010 spectrometer was used for taking the spectra of all compounds synthesized. The spectra of the synthesized compounds showed that molecular ion peak was same as the base peak. Many other peaks were also observed according to the respective fragmentation of compounds that confirmed the synthesis of the compound.

DISCUSSION

The outcome of this research work demonstrated that the conversions obtained with microwave heating are higher when compared with conventional heating. The conventional heating technology renders more difficultly due to nonhomogeneous heating with large temperature ranges whereas microwaves heat the reaction mixture homogeneously and rapidly.

Moreover, the results obtained in case of solid-supported, microwave-assisted solvent free technique are better as compared to the conventional protocol having the additional feature of being carried out in a lot lesser time frame. Recent advances in their synthetic strategies through microwave-assisted organic synthesis was also reviewed (Elgemeie *et al*.,2019).

The cytotoxic activity carried out depicted that HeLa cell underwent cell lysis when they were exposed to various dilutions of BU-1,CU-1 and IU-1. As a result, their number reduced significantly in contrast to control or PBS cells. It was also observed that the higher the dilution was the lower stress the cells had to face. To summarize, in the given neutral red assay, the cells got impaired and were destroyed at three different concentrations of all analogues which proved their cytotoxic nature towards the tumor cells. (Sun *et al.*, 2019).

Conclusion: A facile, solid-supported microwave-assisted synthesis and cytotoxicity studies of the synthesized compounds were the basic focus of this research work. Evaluating the results in terms of higher percentage yield, lesser time consumed, high selectivity and easier work up signified the importance of this novel protocol. Moreover, best results in term of yield were given by celite when the yields of two solid supports were compared i.e. Alumina and celite.

This work will be useful for the rapid synthesis of bioactive compounds and may also have importance in pharmaceutical industry.Comparison of cytotoxicity shows that derivative of 5-iodo exhibited greater cytotoxic effects than 5-chloro and 5-bromo.This work has immense economic importance, which has been ignored for a long time.

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