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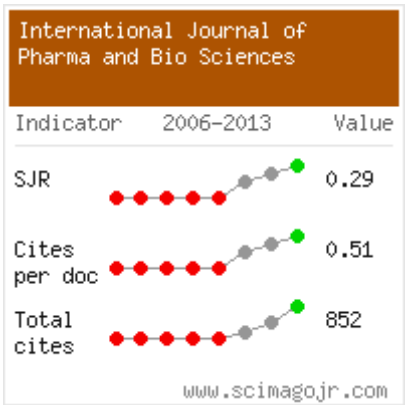

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ANTIOXIDANT, ANTIPROLIFERATIVE ACTIVITIES AND GC-MS ANALYSIS OF METHANOL EXTRACT OF LEAVES OF HERACLEUM SPRENGELIANUM LINN.

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ABSTRACT

The current study was aimed at evaluation of Antioxidant & Anti proliferative potential of the methanol extract of *Heracleum sprengelianum* leaves and to elucidate its phytochemical profile using GC-MS analysis. The methanol extract of leaves of *H. sprengelianum* as screened for its antioxidant potential by dot-plot assay, DPPH radical scavenging assay and hydroxyl radical scavenging assay. Since the results were quite encouraging, it stimulated the investigators to proceed further to screen the phytochemical profiles by qualitative and quantitative methods which were further extended to thin layer chromatography, GC-MS analysis and antiproliferative activity. The result indicates that the methanol extract of leaves of *H. sprengelianum* showed significant radical scavenging activity, potent toxicity to liver cancer cells and also less toxicity to normal cells. The GC-MS result showed the presence of important active metabolites such as fatty acids and phenolic acids. Since the results are promising, *H. sprengelianum* could be considered as a significant source of Antioxidant and Anti proliferative agents.

KEYWORDS: **Nilgiri Plant, DPPH, HepG2, TLC, PBMC, GC-MS.**

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INTRODUCTION

Latest trends have shown that there is an increasing demand for phyto drugs and some medicinal herbs have proven potential to cure dreadful diseases like cancer, paralysis, bronchial asthma etc. Medicinal herbs and their extracts are widely used in the treatment of liver disorders like hepatitis, cirrhosis and loss of appetite [1]. The medicinal plants contains a wide array of chemical compounds called as secondary metabolites which are responsible for curing various diseases and hence called as therapeutics agents [2]. Since the synthetic drugs pose a number of side effects currently, people developed confidence on herbal remedies to prevent or cure cancer. The side effects of synthetic drugs are remarkable because liver is the organ known to filter toxins and drug particle from blood. Hence it has become a great challenge for the synthetic drug manufacturers to find out an alternative and safe treatment for liver diseases, which resulted in exploring the herbal sources [3]. The Nilgiri district popularly known as "The Blue Mountains" is a vital place for medical, ethno-botanical as well as anthropological studies. It is located in the Western Ghats and the tribes of this district are Kotas, Kurumbas, Irulas, Paniyas, and Kattunayaks [4].Regarding the potent tribal drugs, they remain unexplored due to lack of scientific knowledge. *H. sprengelianum* has been reported to be effective in curing indigestion, sunburn, skin diseases and external tumors. Its leaves are 3 pinnate, 30 x 20 cm, ovate, hirsute; leaflets ovate, middle one often divided again; base rounded, apex acuminate, margin crenate-serrate; cauline leaves many. Umbels terminal or in upper axils; umbellules 16-18 flowered; calyx absent; petals small; ovary hairy; styles curved. Hence the present study is aimed to explore the efficacy of *H. sprengelianum* as anti-proliferative compounds. [5]

MATERIALS AND METHODS

MATERIALS [6]

The plant leaves of *H. sprengelianum* was collected from Kotagiri zone of Nilgiri district and identified by S. Aroumougame CAS in Botany University of Madras. Leaves of *H. sprengelianum* were washed with tap water, rinsed with distilled water and shade dried. The dried leaves were ground to obtain coarse powder and subjected to solvent extraction using methanol.

METHODS

(i) Qualitative Phytochemical Analysis [6]

The phytochemical profile of methanol extract of leaves of *H.sprengelianum* (MELH) was studied by following standard procedures**.**

Table 1
Qualitative analysis of methanol extract of leaves of H. sprengelianum

S.No	Phytochemical Constituents	Test	Result
1.	Phenol	Ferric Chloride test	+
2.	Flavonoids	a) Mg filaments test b) NaoH test	+ +
3.	Alkaloids	Mayer's test	+
4.	Terpenoids	Salkowski test	+
5.	Saponins	Foam test	+
6.	Glycosides	Born trager's test	+

(ii) Quantitative Phytochemical Estimations

Total Phenolic Content [15]

The total phenol content was determined by Folin-Ciocalteu reagent method with slight modifications. 1 ml of MELH was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 3 min, 1 ml of Na_2CO_3 (7%) was added to the mixture and it was made up to 10 ml by adding

deionized water. The mixture was kept for 30 min at room temperature in dark. The absorbance was measured at 725 nm against the blank. The total phenolic content was expressed as mg of Gallic acid equivalents (GAE) per gram of dry extract.

Total Flavonoids Content [15]

The total flavonoid content was determined by aluminum chloride reagent method. 1 ml of MELH was mixed with 100 µl of 5% $NaNO_2$ solution and after 6 min, 500 µl of 10% $AlCl_3$ and H_2O were added. After 5 min, 0.5 ml of 1M NaOH was added. The absorbance was measured immediately against the prepared blank at 510nm. Quercetin was used as a standard and the results were expressed as mg of quercetin equivalents (QE) per g of dry extract.

Table 2
Quantitative analyses of methanol extract of leaves of H. sprengelianum

S. No	Phenol GAE/g	Flavonoids QE/g
1	95.23	27.77

ANTIOXIDANT ACTIVITY

The antioxidant activity of the MELH was assessed by dot-plot assay as well as radical scavenging assays.

(iii)Dot-Plot Rapid Assay [14]

Aliquots of MELH (3µl) of various concentrations were spotted carefully on TLC plates and dried for 3 minutes. The sheet bearing the dry spots was placed upside down for 10 sec in a 0.4 mM DPPH methanol solution and the layer was dried. The stained silica gel plate containing active compounds was observed for any significant color change from purple to yellow.

(iv) DPPH Scavenging Effects Of Extracts [15-17]

The DPPH radical scavenging activity of MELH was determined according to the method of Hossain *et al*, (2012). Various concentrations of MELH (1 ml) were mixed with 0.1 mM of DPPH solution in methanol (1 ml). The mixture was shaken vigorously and left t o stand for 30 min, at room temperature in dark and the absorbance was measured at 517nm. The percentage inhibition was calcul ated by the following formula. % RSA = {control – sample / control} X 10.

Table 3
DPPH radical scavenging assay of methanol extract of leaves of H. sprengelianum

S. No	Concentration (µg/ml)	% RSA
1.	10	23.87
2.	20	36.78
3.	30	48.31
4.	40	55.28
5.	50	67.52
6.	60	75.43

(v) Hydroxyl Radical Scavenging Assay [15-16]

The hydroxyl radical scavenging activity the MELH was determined according to the method of Chang W. Choi *et al*, (2002). Various concentrations of the MELH were taken in different test tubes and evaporated to dryness. One milliliter of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA (0.018%), and 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added to these tubes, and the

reaction was initiated by adding 0.5 ml of 0.22% ascorbic acid. Test tubes were capped tightly and heated on a water bath at 80-90 °C for 15 min. The reaction was terminated by the addition of 1 ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added to all of the tubes and left at room temperature for 15 min for color development. The intensity of the yellow color formed was measured spectrophotometrically at 412 nm against reagent blank. The percentage hydroxyl radical scavenging was calculated by the following formula,

% HRSA = {control – sample / control} X 100

Table 4
Hydroxyl radical scavenging assay of methanol extract of leaves of H. sprengelianum

S. No	Concentration (µg/ml)	% HRSA
1.	10	14.94
2.	20	27.58
3.	30	35.63
4.	40	51.72
5.	50	60.92
6.	60	63.85

Table 5
IC_{50} of methanol extract of leaves of H. sprengelianumon radical scavenging assay

S. No	Assay	IC_{50} (µg/ml)	
		H. sprengelianum	Standard value
1.	DPPH	31.05	4.58 (quercetin)
2.	OH·	38.67	5.14 (ascorbic acid)

(vi) Cytotoxic Activity On HepG2 & PBMC Cell Line [18-19]

The cytotoxic activity of MELH was carried out by MTT assay method. HepG2 cell line and PBMC cell line were used and effective doses were calculated from dose-response curve.

(vii) Cell Culture on Liver Cancer Cell

HepG2 cells obtained from NCCS (National Centre For Cell Science, Pune) were cultured in Rose well Park Memorial Institute medium (RPMI), supplemented with 10% fetal bovine serum, penicillin/streptomycin (250 U/ml), gentamycin (100µg/ml) andamphotericin B (1mg/ml) were 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.

Table 6
Cell viability of methanol extract of leaves of H. sprengelianum on HepG2 cell line

S. No	Concentration (µg/ml)	Cell viability (%)	Cytotoxicity (%)
1	100	67.41	32.59
2	200	57.14	42.86
3	300	45.44	54.56
4	400	24.38	75.62
5	500	19.33	80.67

(viii) Isolation of PBMC and Determination of Cytotoxicity

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood from healthy donors (aged 18–45) as described previously. Cells were obtained by Ficoll-Hypaque density centrifugation. PBMCs were suspended in DMEM medium and the cells were counted. 0.2×106 cells/0.2 ml/well seeded in 96-well cell culture plates.

Table 7
Cytotoxic activity of methanol extract of leaves of H. sprengelianum on PBMC cell line

S. No	Concentration(µg/ml)	Cell viability (%)	Cytotoxicity (%)
1	200	89.61	10.39
2	400	85.63	14.37
3	600	81.31	18.69
4	800	80.51	19.49
5	1000	78.62	21.38

(ix) 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, St. Louis.

(x) Cytotoxic Activity by MTT Assay Method

Cell viability was measured with the conventional MTT reduction assay, as described previously with slight modification. Briefly, HepG2 and PBMC cells were seeded at a density of 5×10^3 cells/well in 96-well plates for 24 h, in 200ul of RPMI with 10% FBS. Then culture supernatant was removed and RPMI containing various concentrations (0.11–100µg/ml) of test compound was added and incubated for 48 h. After treatment cells were incubated with MTT (10µl, 5mg/ml) at 37 ◦C for 4 h and then with DMSO at room temperature for 1 h. The plates were read at 595nm on a scanning multi-well spectrophotometer. Data represented the mean values for six independent experiments. The cell viability was calculated by the following formula.

Cell Viability (%) = 1-(OD of Treated Cells – OD of Control Cell) X 100

Table 8
IC_{50} of methanol extract of leaves of H. sprengelianum on cytotoxic activity in cell line

S. No	Cell line	IC_{50} (µg/ml)
1	HepG2	274.92
2	PBMC	635.97

(xi) Thin Layer Chromatography

TLC plate of Slica gel 60 F_{254} (5x1.5cm) was used to carried out thin layer chromatography method. The methanol extract of leaves of *H. sprengelianum* was spotted on the TLC plate 0.5 cm above from the bottom. The solvents chloroform and methanol were used as the mobile phase. Slica gel supported aluminum sheet was used as the stationary phase. The spotted silica gel plate was placed upside down in the TLC chamber containing mobile phase. The mobile phase was allowed to move through the silica gel. The TLC plate was removed from the TLC chamber after the mobile phase reached up to 80% of the TLC plate. The eluted spots were observed under UV (250 nm) as well as in iodine. [17]. R_f = {Distance travelled by the solute/Distance travelled by the solvent}

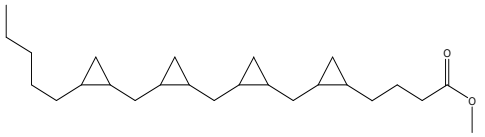
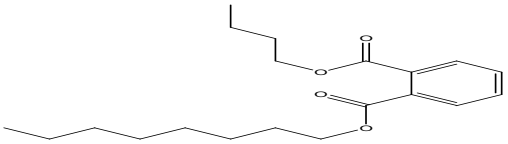
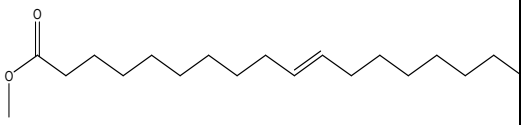
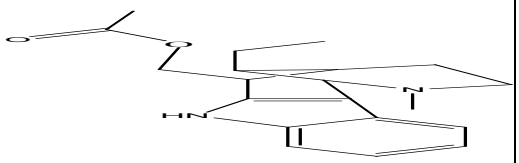
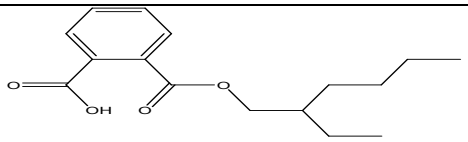
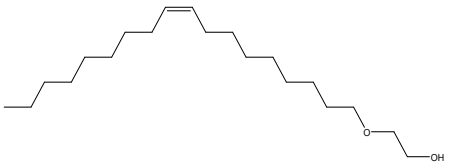
Table 9
R_f (Retention factor) value of methanol extract of leaves of H. sprengelianum by TLC method

S. No	Methanol : Chloroform (1:9)	
	UV	Iodine
1.	0.69	0.32
2.	0.79	0.41
3.	0.85	-

(xii) Gas Chromatography–Mass Spectrometry (GC–MS) [7-9]

The MELH was subjected to GC-MS analysis and the sample was 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. Chromatographic conditions are as follows: helium as carrier gas, flow rate of 1 ml/min, and the injector was operated at 200°C and column oven temperature was programmed as50-250°C at a rate of 10°C/min injection mode. MS conditions are as follows: ionization voltage of 70 eV; ion source temperature of 250°C,interface temperature of 250°C, mass range of 50-600 mass units. The active compounds were identified by the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The name, molecular weight and structure of the compounds of the MELH were ascertained.

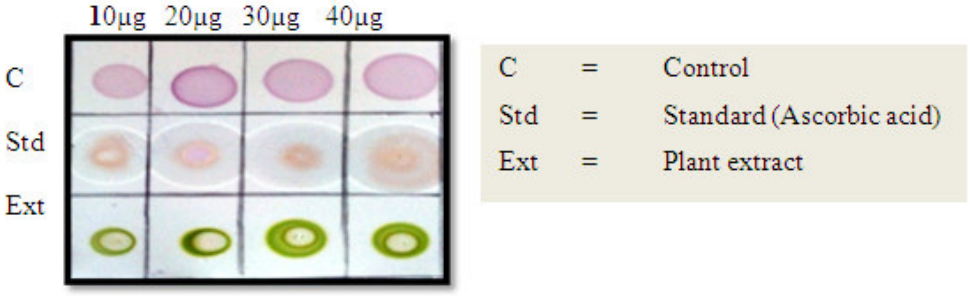
Table 10
GC-MS analysis of methanol extract of leaves of Heracleum sprengelianum

S. No.	Structure	Mol. formula	Mol. Weight (g/mol)	RT (min)
1.	 *Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl)methyl]cyclopropyl] methyl]cyclopropyl]methyl]-, methyl ester*	C_{25} H_{42} O_2	374.6	16.4
2.	 1,2-Benzenedicarboxylic acid, butyl octyl ester	C_{20} H_{30} O_4	334.45	16.95
3.	 10-octadecenoic acid, methyl ester	$C_{45}H_{96}O_2$		
4.	 Heptatriacontan-1-ol	$C_{45}H_{96}O_2$	669.24	27.23
5.	 Dasycarpidan-1-methanol, acetate (ester)	$C_{20}H_{26}N_2O_2$	326.43	24.65
6.	 *1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester*	$C_{16}H_{24}O_4$	278.34	22.48
7.	 2-[(Z)-9-Octadecenyloxy]ethanol	$C_{20}H_{40}O_2$	312.53	18.28

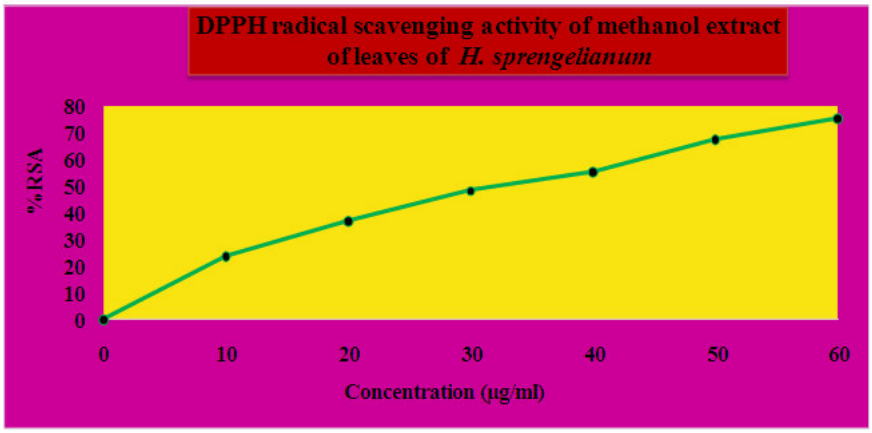
RESULTS

The qualitative phytochemical analysis of the MELH showed the presence of phenols, flavonoids, alkaloids, terpenoids, saponins and glycosides (Table 1). The quantitative phytochemical analysis of MELH showed the total phenolic content was 93.25 mg of GAE equivalents per gram and the total flavonoids content was 27.77 mg of QE equivalents per gram (Table 2). The result of dot- plot assay showed (Fig 1) that the MELH changed the purple background of DPPH to yellow, it indicates that the leaves of *H.sprengelianum* has antioxidant active compounds.

Figure 1
Dot Plot analysis of methanol extract of leaves of H. sprengelianum

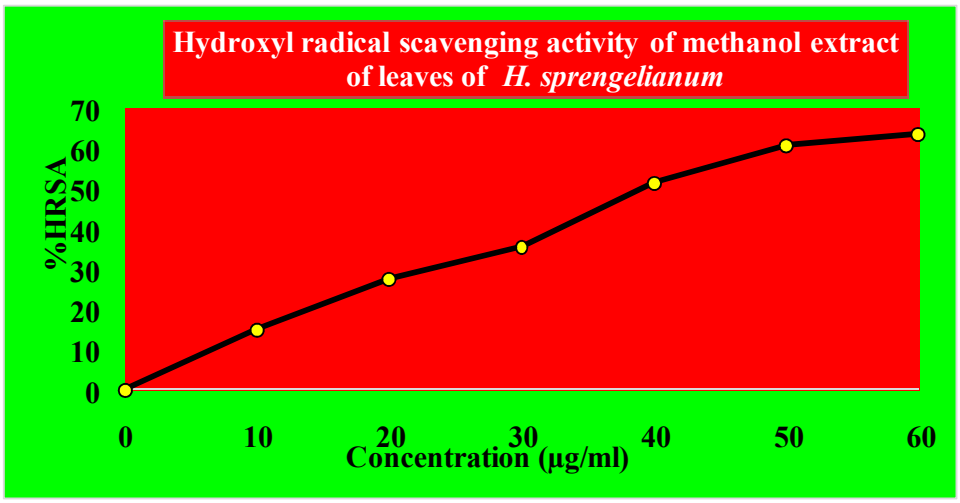


Graph 1
DPPH radical scavenging assay of methanol extract of leaves of H. sprengelianum



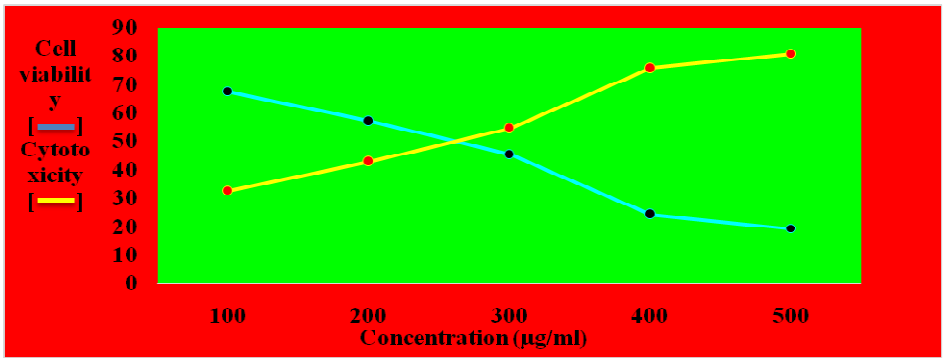
The DPPH radical scavenging activity of MELH showed (Table 3 & Graph 1) maximum activity of 75.43% at 60 µg/ml concentration. The degree of discoloration indicates that the MELH showed free radical scavenging potentials due to the hydrogen donating ability. The $OH^{\cdot}$ radical scavenging activity of MELH showed (Table 4 & Graph 2) maximum activity of 63.85% at 60 µg/ml concentration.

Graph 2
Hydroxyl radical scavenging assay of methanol extract of leaves of H. sprengelianum



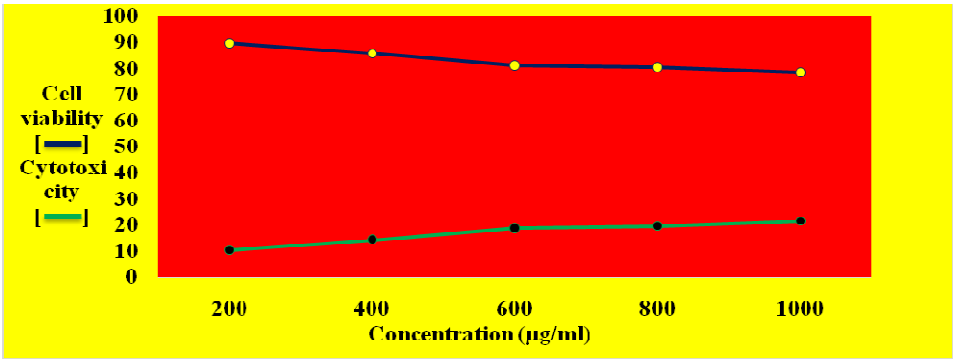
The IC_{50} value of MELH for DPPH radical scavenging activity was 31.05 µg/ml and $OH^{\cdot}$ radical scavenging activity was 38.67 µg/ml as shown in Table 5. The cytotoxic activity of MELH on liver cancer cell line (HepG2) showed (Table 6 & Graph 3) maximum cell death of 80.67% and the cell viability was 19.33% at 500 µg/ml.

Graph 3
Cytotoxic activity of methanol extract of leaves of H. sprengelianum on HepG2 cell line



The cytotoxic activity of MELH on normal cell line (PBMC) showed (Table 7 & Graph 4) maximum cell death of 21.38% and the cell viability was 78.62% at 500µg/ml. The IC_{50} of MELH was recorded as 274.92 µg/ml against HepG2 and 635.97 µg/ml against PBMC (Table 8).

Graph 4
Cytotoxic activity of methanol extract of leaves of H. sprengelianum on PBMC cell line



The R_f value of the eluted spots for the MELH were 0.69, 0.79 and 0.85 as exposed under UV at 254 nm, 0.32 and 0.41 as stained in iodine in the solvent ratio of 1:9 of methanol: chloroform showed in Table 9.GC-MS analysis revealed that (Table 10) MELH contains important secondary metabolites such as oxygenated hydrocarbons & phenolic hydrocarbons which were confirmed by GC-MS chromatogram and compared with NIST library.

DISCUSSION

The methanol extract of leaves of *H. sprengelianum* have shown significant antioxidant property. Based on the above mentioned phytochemical results evident that the presence of phenols, flavonoids, alkaloids, terpenoids, saponins and glycosides. The study is further extended to the anti-proliferative property and safety effective property of normal cell line. The methanol extract of the leaves of *H. sprengelianum* showed higher toxicity at lower concentration in liver cancer cell line and less toxicity at higher concentration in normal cell line, which indicates its safety in administration as drug.

CONCLUSION

Antioxidant studies of *H. sprengelianum* indicate its remarkable role in free radical scavenging activity also the possibilities of possessing anti-cancerous therapeutic property. The methanol extract of leaves of *H. sprengelianum* was effective in vitro against liver cancer cell lines. The results stimulate that further mechanistic studies is required to ascertain its anticancer property. The present study has provided a platform for further research to probe scientifically on *Heracleum sprengelianum* to bring out potent drug for liver cancer.

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