



## FTIR, GC-MS and HPLC analysis of *Baliospermum montanum* (Willd.) Muell. Arg.

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### ABSTRACT

The present work is aimed to determine the chemical constituents in *Baliospermum montanum* methanolic extracts. An *in vitro* regenerated procedure was developed for the induction of callus from stem explant cultured on Murashige and Skoog (MS) medium fortified with various concentration and permutations of 2, 4-dichloro phenoxy acetic acid, 1-naphthalene acetic acid, 6-benzyl amino purine and gibberellic acid. FTIR & GC-MS analysis was done according to standard procedure. The quantitative estimation of  $\beta$ -sitosterol was done by HPLC method. Maximum fresh and dry weight of callus was estimated in the combination of GA<sub>3</sub> (0.5 mg/L) + NAA (2 mg/L) compared to other concentration. The FTIR analysis showed various functional compounds with different characteristic peak values in the extracts. Major bioactive constituents were recognized in the GC-MS analysis. Root extract revealed the existence of 1-hexadecanol, pentanoic acid, 2-(aminooxy)- and 1-hexacosanol. Leaf extract showed the presence of propanoic acid, 2-oxo-, trimethylsilyl ester, 9,12-octadecadienoic acid (z,z)-, trimethylsilyl ester, docosane, 1,22-dibromo- and pentatriacontane. Stem and stem derived callus exhibit the presence of 1,6,3,4-dihydro-2-deoxy-beta-d-lyxo-hexopyranose, n-hexadecanoic acid and pentanoic acid, 2-(aminooxy). The methanolic extract of leaf exhibited 0.2149 % of  $\beta$ -sitosterol content. There were no peaks observed in the root, stem and stem derived callus. Further studies are necessary for the isolation and characterization of bioactive compounds from *B. montanum*.



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### INTRODUCTION

Medicinal plants are a significant part of natural wealth. They act as vital curative agents as well as precious raw materials for producing several modern and traditional medicines (Rajiv *et al.*, 2017). Conventional medicine is the total of knowledge, skill and practice based on theory and experiences indigenous to different cultures that are used to maintain the physical and mental illness. Various types of traditional medicine and other complementary or alternative medicine are increasingly used in both developing and developed countries (Al-Huqail *et al.*, 2018). Plants play an important role in the treatment and prevention of diseases and can even reduce the adverse effects

of conventional treatments. They signify a rich resource of secondary metabolites to treat against antifungal and antibacterial activities. Many scientists have worked on pure active compounds and crude extracts isolated from plants species used in traditional and herbal remedies (Casuga *et al.*, 2016). Plants are the basis of chemical compounds for biological and pharmacological importance. Identification and characterization of phytochemical compounds present in the medicinal plants will provide information on the diverse functional groups accountable for their medicinal properties (Kumar and Ramaswamy, 2014). *Danti* has been botanically identified as *Baliospermum montanum* (*B. montanum*) (Willd.) Muell.-Arg. Belongs to family Euphorbiaceae, an erect herb - undershrub with perennial rootstock running horizontally and sending out new shoots. It is found throughout India from Kashmir eastwards to Arunachal Pradesh, up to an elevation of 1000 m and southwards into peninsular India, rising to an altitude of 1000 m in the hills of Kerala (Rout *et al.*, 2017). The plant parts are used medicinally due to the presence of secondary metabolites. The root sample contains phorbol ester belong to diterpene hydrocarbon viz., baliospermin, montanin, 12-deoxyphorbol-13-palmitate, 12-deoxy-16-hydroxy phorbol 13-palmitate and 12-deoxy-5B-hydroxyphorbol-13-myristate. Leaves contain hexacosanol,  $\beta$ -sitosterol and  $\beta$ D-glucoside (Pasqua *et al.*, 2003). *Danti* has been investigated by various scientists and found to have numerous phytochemicals like baliospermin (0.003%), montanin (0.018%) and 12-deoxyphorbol-13-palmitate (0.021%), 12-deoxy-16-hydroxyphorbol-13-palmitate (0.001 %) (Mali and Wadekar, 2008). It is used in constipation, calculus, abdominal pain, piles, helminthic manifestations, skin disorders, scabies, and jaundice (Sharma *et al.*, 2005). Therefore, the current study was designed to develop an efficient protocol for induction of callus from stem explant and to examine the chemical composition in methanolic extracts of stem, root and leaf derived callus of *B. montanum*.

## MATERIALS AND METHODS

### Collection of plant material and sterilization

Seedlings were collected from Western Ghats, Karnataka and were maintained in the Department of Botany (greenhouse condition). The plant material was authenticated at Botanical Survey of India, deposited in the herbarium No. BSI/WRC/100-1/IDEN.CER./2018/77. The explants were washed under running tap water for 30 min, followed by

sterilants for 20 min (tween- 20 and bavastin). Surface sterilization of the explants was done with 70 % alcohol for 30 sec, followed by sterile water wash. Then they were treated with HgCl<sub>2</sub> (0.1 %) for 2-3 min for mature explants and 1 min for young ones and were washed with sterile water for 3-5 times under aseptic condition to remove the traces of sterilants. 1 cm length excised explants were cultured on an MS medium fortified with various concentration of growth regulators (Murashige and Skoog, 1962).

### Callus induction

Stem explant (1cm) were inoculated on MS media fortified with different concentration and combination of growth hormones like 2, 4-D (0.2 mg/L), 2, 4 D (1.0 mg/L) + BAP (4.0 mg/L), GA<sub>3</sub> (0.5 mg/L) + NAA (2.0 mg/L) and NAA (0.5 mg/L) + GA<sub>3</sub> (4.0 mg/L). Cultures were maintained at 25 ± 2 °C in a culture room with 70 % relative humidity and 16 h of photo period.

### Preparation of extracts

The plant extracts were prepared following the procedure of Samyudurai and Saradha (2016) by soxhlet extraction method. Briefly, 10 g of dried root, stem, leaf and stem derived callus were finely powdered using a blender and extracted with methanol (100 ml) for 24 h. Then the extracts were concentrated by evaporation. The dried extract was stored at 4 °C until further analysis.

### Fourier transform infrared spectrophotometer (FTIR)

FTIR is the most powerful tools for detecting the functional groups present in compounds. The wavelength of light absorbed is the main feature of the chemical bond, as can be seen in the annotated spectrum. By interfering the spectrum, the chemical bonds in a compound can be identified. About 10 mg different samples were dissolved in methanol. A drop of each extract was encapsulated in KBr (100 mg) pellet, to prepare translucent sample disc by using pelletizer. The pellet of each sample was loaded in FTIR Spectroscopy, with a scanning range from 400 to 4000 cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup> (Shimadzu, IR, Japan) (Maobe and Nyarango, 2013).

### GC-MS analysis

Methanolic extract of *B. montanum* was examined with the help of GC-MS analyzer. The Clarus 680 GC was employed for analysis in a fused silica column, packed with Elite-5MS (5v % biphenyl 95v % dimethyl polysiloxane, 30 m × 0.25 mm ID × 250 μm df) and the constituents were separated using helium carrier (1 ml/min). The injector tempera-

ture was set at 260 °C during the chromatographic run. The 1  $\mu$ l of the extract was injected, and the oven temperature was maintained as follows: 60 °C (2 min), 300 °C at the rate of 10 °C min<sup>-1</sup> followed by 6 min at 300 °C. The mass detector conditions were: 240 °C transfer line temperature; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, scan time 0.2 sec and scan 0.1 sec. The spectrums of the constituents were related to the database of the spectrum of known components stored in the GC-MS NIST (2008) library (Sheng and Chen, 2009).

### HPLC analysis

#### Preparation of standard solution

$\beta$ -Sitosterol were procured from Sigma Aldrich was used for purification of calibration curve and retention time. Standard stock solutions were prepared in 100 % methanol and were stored at -20 °C until further analysis. A stock solution (100  $\mu$ g/ml) was prepared by suspending 10 mg of  $\beta$ -Sitosterol in 100 ml of HPLC grade methanol in each.

#### Chromatographic procedure

The  $\beta$ -Sitosterol were quantified by HPLC method using a C<sub>18</sub> column (250  $\times$  4.60 mm). Methanol is employed as a mobile phase with a flow rate of about 1.5 mL/min. The chromatogram was monitored at 206 nm, oven temperature 30 °C with a total running time of 20 min. The identification of peak was based on the evaluation of retention time with a standard of total running time. Three distinct calibration levels were used to set the calibration plot. The calibration plot was drawn by estimating the peak area against the concentration of the compound. The standard and samples (20  $\mu$ l) were injected separately, and the chromatograms were analyzed (Mohan *et al.*, 2016). The concentration of standards was determined using the following formula,

Standard concentration =

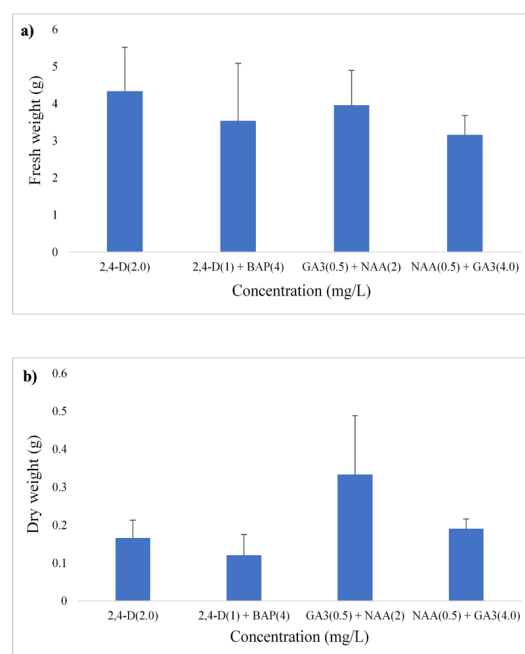
$$\frac{\text{Sample area}}{\text{Std. area}} \times \frac{\text{Std. weight}}{\text{Std. dilution}} \times \frac{\text{Sample dilution}}{\text{Sample weight}} \times \text{Purity}$$

## RESULTS AND DISCUSSION

### Callus induction

Induction of callus was estimated based on the percentage of explants responded. The time taken for callus initiation was noted, and the callus morphology was recorded. For growth measurement of calli, the proliferated callus was harvested and dried at room temperature, and the fresh weight (FW) and dry weight (DW) was recorded. The maximum FW 03.96  $\pm$  0.94g and DW 0.33  $\pm$  0.15 g was observed in stem derived callus with the combined treatment

of GA<sub>3</sub> (0.5 mg/L) + NAA (1.0 mg/L). Whereas in NAA 0.5 mg/L + GA<sub>3</sub> 4.0 mg/L showed moderate biomass (03.16  $\pm$  0.52 g FW and 0.19  $\pm$  0.02 g DW) compared to other concentration of growth hormones. While individual growth regulators (2 mg/L of 2, 4-D) recorded higher biomass (04.34  $\pm$  1.17g FW and 0.16  $\pm$  0.04 g DW). The lesser callus initiation was recorded in 1 mg/L of 2, 4-D and 4mg/L of BAP, and there was a significant decrease in callus induction rate in both auxin and cytokinins combinations (Figure 1). This may be due to activity of auxins and cytokinins, which stimulates cell division, cell elongation and shoot regeneration. A higher concentration of cytokinins has inhibited the effect of auxins in inducing callus initiation. The callus induction varies among different species depending on the plant growth regulators (Tan *et al.*, 2010). Studies by Gopi and Vatsala (2006), demonstrated that maximum FW and DW was recorded in 2,4-D (0.5 mg/L) from nodal explants of *Gymnema sylvestre* compared to other concentration of auxins and cytokinins.



**Figure 1: Effect of different concentration and combination of auxins, cytokinins and GA<sub>3</sub> on a) fresh weight and b) dry weight of callus induced from stem explants**

### FTIR analysis

The FTIR was used to determine the functional groups of the active constituents present in extract based on the peaks values in the IR region (Karpagasundari and Kulothungan, 2014). Methanolic

**Table 1: FTIR spectra interpretation of methanolic extract of *B. montanum***

Sl. No	Wavenumber (cm <sup>-1</sup> )	Functional groups	Phyto Components	Wavenumber (cm-1)			
				R	S	L	SC
1	3570-3200	O-H stretch	Poly Hydroxy Compounds	3433	3432	3434	3452
2	2935-2915	-CH (CH <sub>2</sub> ) Asymmetric	Lipids, Proteins	2921	—	—	—
3	2865-2845	-CH (CH <sub>2</sub> ) Symmetric	Lipids, Proteins	2861	—	—	—
4	3500-2400	O-H stretch Acidic	Carboxylic acid	2457	2427	2427	2427
5	1650-1600	C=O stretching	Ketone group	—	—	1615	1614
6	1410-1310	O-H bend	Phenol	1382	1355	1385	1384
7	1140-1070	C-O stretch	Cyclic ether	1113	1111	1111	1111
8	1100-1000	Phosphate ion	Phosphate compound	1066	1068	1068	1068
9	995-850	P-O-C stretch	Aromatic phosphates	924	923	923	924
10	800-700	C-Cl stretch	Aliphatic Chloro compounds	718	721	721	721
11	700-600	C-Br stretch	Aliphatic Bromo compounds	620	617	614	614

R-Root, L-Leaf, S-Stem and SC-Stem Callus

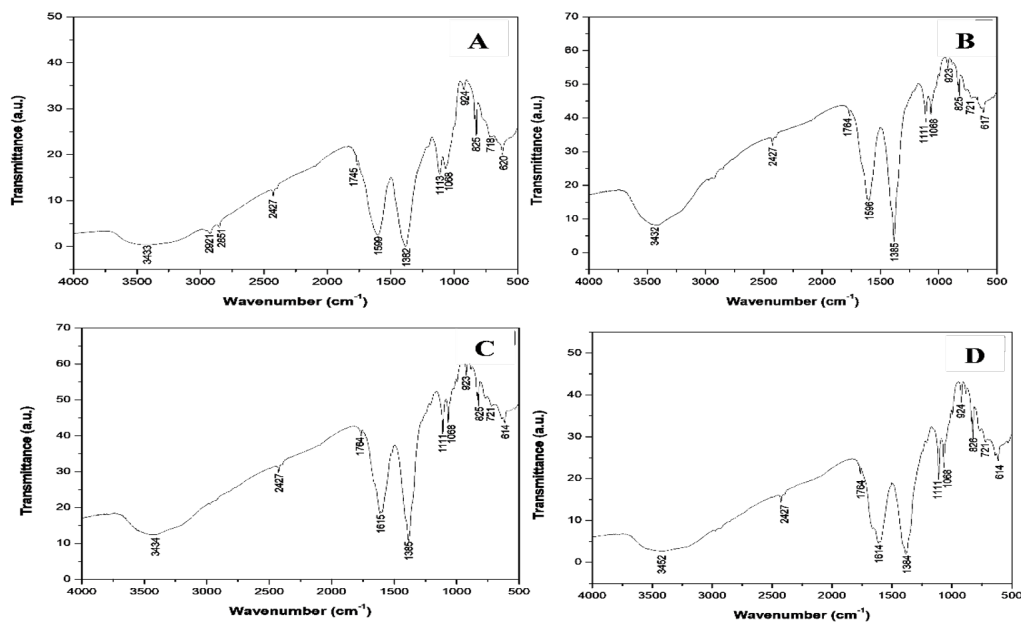
**Table 2: Compounds identified in methanolic extracts of *B. montanum* by GC-MS**

Samples	Name of the compound	Biological Activity	MW	MF	RT	Peak area %
Root	1-hexadecanol	Antimicrobial activity	242	C <sub>16</sub> H <sub>34</sub> O	20.14	100
	n-hexadecanoic acid	Anti-inflammatory, antioxidant, antiandrogenic, hypocholesterolemic, 5- $\alpha$ reductase inhibitor	256	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	18.79	29.50
Stem	1-hexacosanol	Antifungal activity	382	C <sub>26</sub> H <sub>54</sub> O	22.08	05.02
	1,6;3,4-dianhydro-2-deoxy-.beta.-d-lyxo-hexopyranose	Antimicrobial activity	128	C <sub>6</sub> H <sub>8</sub> O <sub>3</sub>	26.46	55.32
Leaf	Propanoic acid, 2-oxo-, trimethylsilyl ester	Antioxidant	160	C <sub>6</sub> H <sub>12</sub> O <sub>3</sub> Si	19.53	100
	9,12-octadecadienoic acid (z,z)-, trimethylsilyl ester	Anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, antihistaminic, antieczemic, antiarthritic, antimicrobial and 5- $\alpha$ reductase inhibitor	352	C <sub>21</sub> H <sub>40</sub> O <sub>2</sub> Si	20.41	97.56
Stem callus	Docosane, 1,22-dibromo-	Antibacterial activity	466	C <sub>22</sub> H <sub>44</sub> Br <sub>2</sub>	24.55	08.10
	Pentatriacontane	Antiseptic activity	492	C <sub>35</sub> H <sub>72</sub>	26.84	07.13
	Pentanoic acid, 2-(aminooxy)	Antioxidant activity	133	C <sub>5</sub> H <sub>11</sub> O <sub>3</sub> N	21.68	100

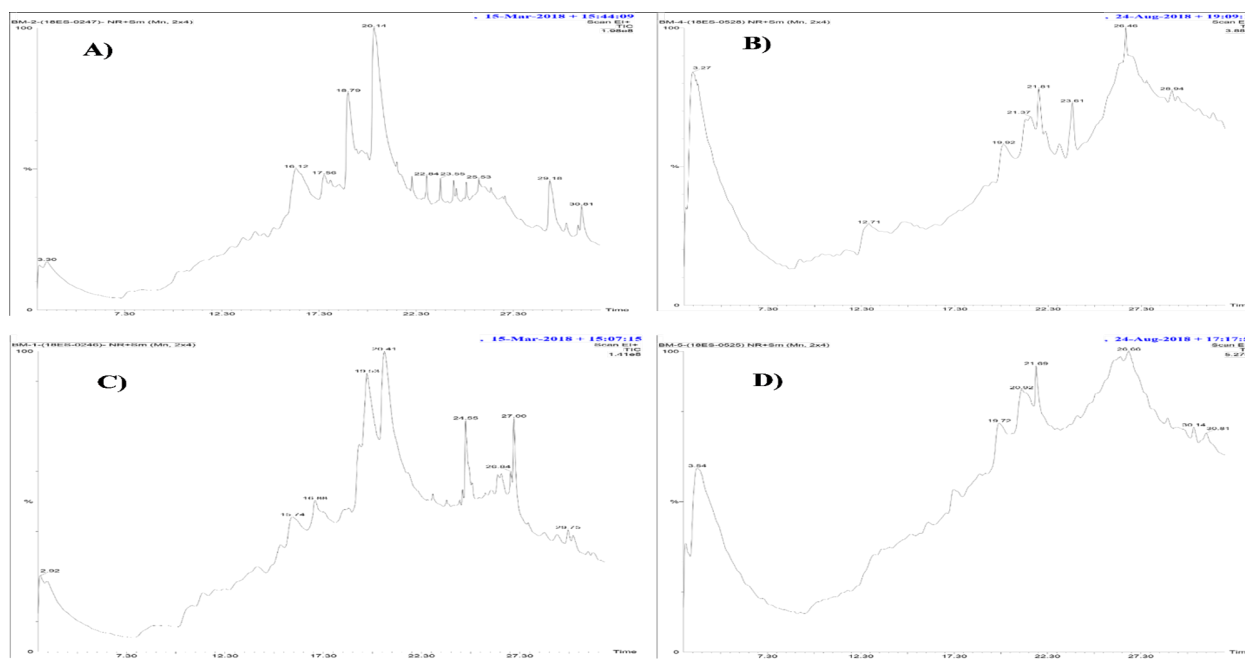
MF-Molecular Formula, MW-Molecular Weight and RT-Retention Time

**Table 3: HPLC analysis detection of  $\beta$ -Sitosterol in *B. montanum***

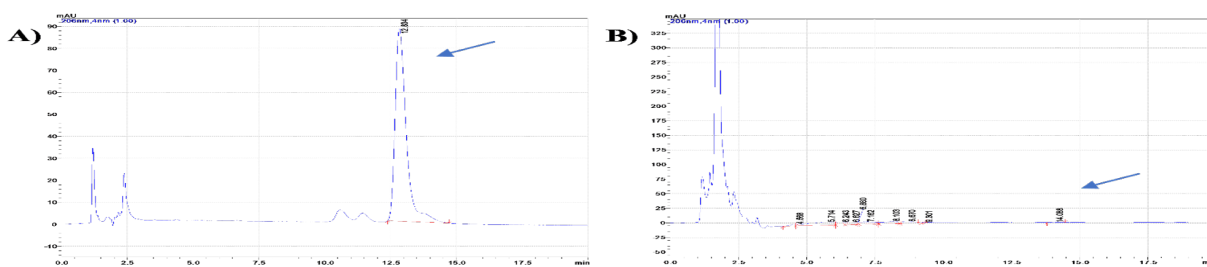
Sample	Retention time	Area	Concentration	Percentage of $\beta$ -sitosterol
Root	No peak	—	—	—
Stem	No peak	—	—	—
Leaf	14.088	14209	0.01498	0.2149 %
Stem callus	No peak	—	—	—



**Figure 2: FTIR spectrum of methanolic A) root, B) stem, C) leaf and D) stem derived callus extracts of *B. montanum***



**Figure 3: GC-MS chromatogram of methanolic A) root, B) stem, C) leaf and D) stem derived callus extracts of *B. montanum***



**Figure 4: HPLC analysis of A)  $\beta$ -sitosterol and B) methanolic leaf extract of *B. montanum***

extracts of root, stem, leaf, and stem derived callus observed the peaks at 3433, 3434, 3432 and 3452  $\text{cm}^{-1}$  revealed the presence of Polyhydroxy compounds (O-H stretch). Root extracts showed the presence of lipids and proteins at 2921 and 2861  $\text{cm}^{-1}$ . A peak of 2457 and 2427  $\text{cm}^{-1}$  indicated the occurrence of the carboxylic acid group (O-H stretch). The peaks of 1615 and 1614  $\text{cm}^{-1}$  indicate the ketone group (C=O stretching) in leaf and stem derived callus. The phenol groups (O-H bend) were identified at 1382, 1355, 1385 and 1384  $\text{cm}^{-1}$ . The peaks of 1113 and 1111  $\text{cm}^{-1}$  revealed the cyclic ether group (C-O stretch). Phosphate compounds (phosphate ion) were detected corresponding to the peaks of 1066 and 1068  $\text{cm}^{-1}$ . Peaks of 924 and 923  $\text{cm}^{-1}$  represent the presence of aromatic phosphates (P-O-C stretch). Aliphatic chloro compounds (C-Cl stretch) were detected in the peaks 718 and 721  $\text{cm}^{-1}$ . The peaks identified at 620, 617 and 614  $\text{cm}^{-1}$  show the existence of aliphatic bromo compounds (C-Br stretch) (Table 1 and Figure 2). Muruganatham *et al.* (2009) evaluated the root, stem and leaf extracts of *Eclipta alba* and *Eclipta prostrata*. They indicated that the presence of carboxylic acid, amines, amino acids, sulfonfyl, sulfonate, polysaccharides, nitrate, chlorate and carboxylic acids in the extracts by FTIR analysis. They reported that the very strong absorption band appearing in the range of 3400  $\text{cm}^{-1}$  and 2933–2922  $\text{cm}^{-1}$  may be due to the occurrence of amino acids and N-H stretching in both the plants.

### GC-MS analysis

GC-MS is a powerful technology which helps in identification and characterization of new compounds (Chauhan *et al.*, 2014). GC-MS analysis was done using the National Institute Standard and Technology (NIST) database, and the spectrum of unknown compounds was related with the library version NIST-2008. Table 2 represents the active constituents with their molecular formula (MF), molecular weight (MW), retention time (RT) and peaks areas percentage (%).

The GC-MS data of methanolic root extracts indicated the presence of three major peaks with retention time 20.14, 22.08 and 18.79 min. The first peak represents the 1-hexadecanol with 100 % peak area. The next peaks corresponded to 1-hexacosanol with 5.02 % peak area and n-hexadecanoic acid (29.50 %) with antioxidant, hypocholesterolemic, antiandrogenic, anti-inflammatory, 5- $\alpha$  reductase inhibitor (Jananie *et al.*, 2011). Methanolic extracts of stem revealed the presence of 1,6,3,4-dianhydro-2-deoxy-beta-d-lyxo-hexopyranose (55.32 %) with retention time 26.46 min. Whereas, stem derived callus showed the presence of pentanoic acid, 2-(aminooxy) (100 %) which shows antioxidant activity (Severin *et al.*, 2018) with a retention time of 21.68 min (Figure 3).

Methanolic leaf extract GC-MS analysis revealed the presence of five major peaks, and the corresponding compounds peaks were determined as follows. The first peak was identified to be propanoic acid, 2-oxo-, trimethylsilyl ester (100 %) with a retention time of 19.53 min. The second peak represents to be 9,12-octadecadienoic acid (z,z)-, trimethylsilyl ester (97.56 %) with retention time 20.41 min. The third and fourth peaks considered to be docosane, 1,22-dibromo- (08.10 %) and pentatriacontane (07.13 %) which shows antibacterial and antiseptic activity (Thirumalaisamy *et al.*, 2018; Kalsum, 2016) with retention time 24.55 and 26.84 min. Ganesh and Mohankumar (2017) reported the presence of 9,12-octadecadienoic acid (z,z)-, trimethylsilyl ester (23.89 %) in ethanolic extract of *Sida cordata*. It was proved with biological activity like anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, antihistaminic, antieczemic, antiarthritic, antimicrobial and 5- $\alpha$  reductase inhibitor. According to Sathya *et al.* (2016), root exudates of 15 days old seedlings of *Capsicum annum* treated with *Bacillus amyloliquefaciens* and polymer coating reported the presence of 1-hexadecanol (0.56 %) and 1-hexacosanol (0.50 %) with antibacterial and antifungal activ-

ity. Studies by Ara *et al.* (2013) identified 11 compounds by GC-MS analysis of a methanolic extract of *Juglans regia* tree bark. The main component was found to be 1,6,3,4-dianhydro-2-deoxy-beta-d-lyxo-hexopyranose (87.5 %) which reported to be an antimicrobial agent.

### HPLC analysis

HPLC is a column chromatography generally employed in biochemistry and analysis to separate, identify and quantify the active constituents (Malviya *et al.*, 2010). It is widely used for the separation of sterol compounds (Lagarda *et al.*, 2006).  $\beta$ -Sitosterol is known as a plant sterol which regulates cholesterol levels, reduce the cancer cell activity promotes prostate gland health and enhance immunity (Kakade and Magdum, 2012). HPLC analysis was carried out in root, stem, leaf and callus derived from stem segments. The leaf chromatogram of  $\beta$ -Sitosterol showed the peak with corresponding retention time. The results were compared using the standard  $\beta$ -Sitosterol with the retention time of 12.46 min. The percentage of  $\beta$ -Sitosterol in the plant samples were calculated. Methanolic extract of leaf exhibited 0.2149 % of  $\beta$ -Sitosterol content. There were no peaks recorded in the root, stem and stem derived callus (Table 3 and Figure 4). Till date, no studies are reported on  $\beta$ -Sitosterol content in *B. montanum*, and this is the first to report in this medicinal plant. Studies by Rajanandh and Kavitha (2010) isolated  $\beta$ -Sitosterol from hydroalcoholic extract of *Moringa oleifera* leaf, which exhibits hypolipidemic and as well as antioxidant properties.

### CONCLUSION

In the present investigation, essential bioactive compounds were recognized by FTIR and GC-MS analysis. The developed HPLC method was validated for the analysis of  $\beta$ -Sitosterol in methanolic leaf extract of *B. montanum* with a short analysis time of 14.088 min. Since  $\beta$ -Sitosterol has some health benefits, further analytical studies are required to identify and quantify major phytochemical compounds in elicitors treated callus of *B. montanum* for its potent antioxidant properties.

### Conflict of Interest

The authors declared that they have no conflicts of interest for this study.

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