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Phytochemical screening and functional group analysis of various root samples of *Baliospermum montanum* (Willd.) muell. arg. by FTIR spectrum

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Abstract
Baliospermum montanum (Muell.) Arg. (Family Euphorbiaceae) is a well-known medicinal plant whose roots, stem, leaves and seeds are traditionally used for the treatment of different diseases. The phyto-components especially the bioactive terpenoids *viz*. 12-deoxy-5β-hydrophorbol-13-myristate, 13-palmitate, 12-deoxyphorbol-13-palmitate, baliospermin and montanin are reported in the roots. Since the harvested part is roots, the present study focused to determine various phytochemicals and the functional groups present in *in vivo* roots, *in vitro*-raised adventitious roots, hairy roots, cells harvested from root suspension samples. Among the solvents used for extraction, petroleum ether and methanolic extracts revealed more phyto-constituents than others and the preliminary phytochemical screening undertaken detected the presence of terpenoids, alkaloids, flavanoids and glycosides. The FTIR analysis showed different functional groups with various characteristic peak values. The entire samples showed the peaks at the wavelengths 3348.40, 3281.62, 3295.16, 3359.75 cm^{-1} that revealed the presence of polyhydroxy compounds (O-H stretch). The samples also showed the indication of ketones (C=O), phenols, esters and phosphate ions and these findings can be extended for the isolation and purification of active secondary metabolites from the normal and the various *in vitro* root-derived samples of this plant for therapeutic uses.

Keywords: *Baliospermum montanum*, phytochemical screening, hairy roots, FTIR spectroscopy, functional groups, esters

Introduction
Baliospermum montanum (Willd.) Muell. Arg. (Family Euphorbiaceae) is a rare, endangered, threatened (RET) medicinal species [1]. According to the comprehensive review of Rastogi and Mehrotra [2] about *B. montanum*, both Charaka and Sushrutha prescribed this one for jaundice, anaemia, constipation, disease of abdomen, piles, etc. In addition, the roots, stem, leaves and seeds are used by different tribal communities for the treatment of a variety of ailments. The seeds are externally used as stimulant and rubefacient, while the dried roots are considered antihelmenthic, diuretic and useful in treating enlarged spleen, abdominal tumors, etc. Moreover, pharmacological screening has revealed its antibacterial, hepatoprotective, anticancerous, free radical scavenging, immuno-modulatory and anthelmintic effects. The drug constitutes an important series of preparations like Dantyarishta, Kaisoraguggulu gulika, Dantiharitakileham, and others [3].
Several compounds have been isolated from this plant such as steroids, triterpenoids, diterpenes (baliospermin, montanin, phorbol-12-deoxy-13-O-palmitate, phorbo l-12-deoxy-5β-hydroxy-13-myristate) glycosides, saponins, alkaloids, flavanoids, and phenolic compounds. The presence of auxillarenic acid is in the seeds as well as the diterpene esters such as 12-deoxy-5β-hydrophorbol-13-myristate, 13-palmitate, 12-deoxyphorbol-13-palmitate, baliospermin and montanin in the roots are also reported [2]. Anti-leukaemic and cytotoxic activities have been demonstrated in the esters of both 12-deoxyphorbol and 12-deoxy-16-hydroxyphorbol, isolated from *B. montanum* [4]. Phorbol esters are ester derivatives of the tetra cyclic diterpenoid phorbol. The Euphorbiaceae family to which *B. montanum* belongs is prominent in ethnomedicine [5] and analyses of the extracts of a number of species in Euphorbiaceae have revealed that in many cases the biological activity is attributed to particular diterpenoids [6]. Most of these diterpenoids can only be sourced directly from the plant. However, many of the Euphorbiacean species are slow-growing and produce only minute quantities of the desired compound. Formulating alternative biotechnological interventions as tools for the bioproduction of these diterpenoids are inevitable in order to improve their supply. Though phytochemical analysis of *B. montanum* has been thoroughly reviewed by Rastogi [2], there is no information about the phytochemistry of hairy roots and *in vitro* adventitious roots of the same species. Hence the present investigation has been undertaken to conduct the phytochemical screening and to analyze the various functional groups present in them using FTIR analysis for the purpose of using these bioactive substances in different drug formulations so as to develop other *in vitro* scaling up methods to enhance the production of diterpenes.

Materials and Methods
Sample collection
Iin vivo roots, *in vitro*-raised adventitious roots, hairy roots, cells harvested from root suspension culture were the plant sample used for the present study.

In vivo normal roots
Normal roots were selected from the green house maintained mother plants (Fig.1a).

In vitro adventitious root induction
Leaf segments of *B. montanum* selected from the green house-maintained plants were thoroughly washed under

running tap water and subsequently in a liquid detergent for 20-30 minutes followed by washing again in running tap water and rinsing in distilled water. They were then subjected to surface sterilization with 0.1% (w/v) $HgCl_2$ for 8 minutes and subsequent rinsing in sterile distilled water. The explants excised aseptically were inoculated on Murashige and Skoog nutrient medium supplemented with 0.5, 1.0 and 2.0 mgl^{-1} NAA either individually or in combination with 0.1 mgl^{-1} IAA and incubated under the standard culture conditions. Adventitious roots induced were used for further experiments (Fig.1b).

Hairy root induction

In vitro raised leaf segments of *B. montanum* were used as explants for the induction of hairy roots. 5.0-7.0 cm wide leaf discs were dissected out and transferred to pre incubation for 3-5 days. Scalpel method [7] was employed for infecting the leaf discs with over night-grown bacterial culture of *Agrobacterium rhizogens* A4 strain and then transferred to MS basal agar medium for 4 weeks for the induction of hairy roots. Then the induced roots were transferred to MS basal agar medium with antibiotic 500 mgl^{-1} sterptomycin for the disinfection. After 3 weeks, root lines were selected for multiplication, subsequently transferred to MS basal liquid medium and kept on gyratory shaker at 80 rpm. During this stage, fast growing roots with more number of branches were selected for the establishment of hairy roots. These established hairy roots were further used for the analysis in this study (Fig.1c).

Root derived cell suspension

Adventitious roots of *B. montanum* were transferred to liquid MS medium supplemented with 1.0 mgl^{-1} NAA in combination with different concentrations of IAA/IBA on a gyratory shaker at 120 rpm for 3- 4 weeks. The medium turns cloudy with dispensed root cells. Then the root cells were subcultured to MS liquid medium fortified with various concentrations of NAA, IBA or IAA. After 28 days of subculture root cells were harvested and used for analysis (Fig.1d).

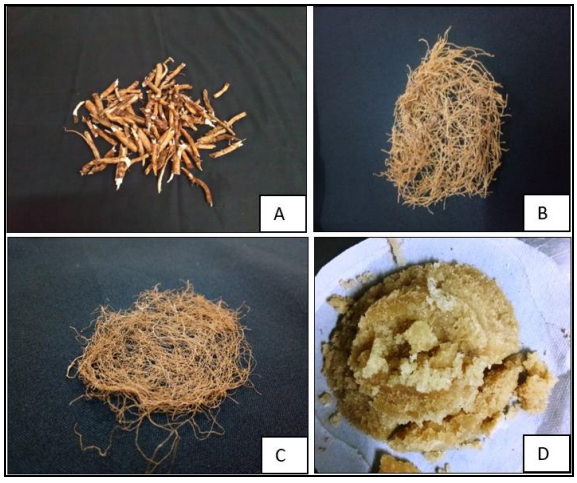

Fig 1: A. *In vivo* roots, B. *In vitro* adventitious roots, C. Hairy roots, D. Root derived cells of *B. montanum* harvested from suspension culture

Preliminary phytochemical screening

Chemical tests were carried out using the solvent extracts and on the powdered samples using standard methodology to identify the presence of different phytochemicals such as alkaloids, phenolic compounds, flavanoids, saponins, tannins, glycosides, terpenoids, steroids, anthraquinones, carbohydrates, fatty acids, proteins, amino acids, coumarins, phlobatannins based on the standardized procedures [8] [9].

FTIR (Fourier Transform Infra Red) profiling

For FTIR profiling, *in vivo*, *in vitro* and hairy roots sample were dried in an oven (Labline, India) for 2 days at 60 ºC. Tablets for FTIR spectroscopy were prepared in agate mortars by mixing the sample powder with (2 mg) with KBR (1:100 p/p). The absorbance spectra were measured between 300 and 4500 cm^{-1}. At least three spectra were obtained for each sample. A FTIR spectrometer (FTIR Schimadzu Prestige 21 was used to collect spectra [10].

Results and Discussion

***In vitro* adventitious root induction**

The induction of *in vitro* adventitious roots of *B. montanum* was achieved from leaf explants in MS medium supplemented with different concentrations of NAA, IBA and IAA. To induce *in vitro* roots, full strength MS semi solid medium containing 0.5% (w/v) agar supplemented with different concentrations of NAA, IBA and IAA were used in this study. It was found that highest percentage of root induction was obtained in the MS medium fortified with 1.0 mgl^{-1} NAA and 0.1 mgl^{-1} IBA. In agreement with this, a combination of 1.0 mgl^{-1} IBA and 1.0 mgl^{-1} NAA was very suitable for root induction in *Luffa acutangula* [11]. Similar observations were made earlier by Wadegaokar [12] in *Withania somnifera* and Praveen [13] in *Andrographis paniculata*. Adventitious root culture induction was also undertaken in medicinal plants *Psammosilene tunicoide* for the extraction of terpenoids [14].

Hairy root induction

The explants dissected out from established *in vitro* cultures and transferred to pre incubation medium subjected to bacterial infection by incision/ wounds through scalpel method exhibited the emergence of roots after three days of wounding. The hairy roots of *B. montanum* were induced from the wound sites after about 20 days of incubation on phytohormone-free MS medium, due to the high sensitivity to *A. rhizogenes* (A4 strain). After 4 weeks of root initiation, individual roots of 2-4 cm length were carefully dissected out individually from the infected site of the shoots and transfer to MS basal agar medium supplemented with the antibiotic 500 mgl^{-1} Streptomycin made the hairy root cultures free of bacteria cells. After 3 weeks, the selected root line was transferred again to MS basal agar medium for further establishment of root lines and such established root culture upon subculture in MS basal liquid medium when kept on Gyratory shaker at 80 rpm under constant agitation enhanced the biomass production. Similarly, such established hairy roots produced have been further investigated for the enhanced production of medicinally important secondary metabolites in *Solanum xanthocarpum* [15].

Root derived cell suspension

The study on plant cell suspension has been used as a useful method to enhance the production of secondary metabolites. In *B. montanum*, after 28 days of subculture the root cells were harvested from MS medium that was fortified with 0.1

mgl^{-1} IBA and 0.01 mgl^{-1} IAA. This hormonal combination was suitable for establishing cell suspension culture in *Gynura procumbens* [16]. These cells were subcultured again to MS liquid medium supplemented with various concentrations of NAA, IBA or IAA and the cells harvested after 28 days of subculture were subjected to phytochemical analysis in this study.

Phytochemical analysis of *in vivo* roots, *in vitro* roots, hairy roots and root derived cells from suspension culture of *B. montanum*

Secondary metabolites are non-nutritive phytochemicals which are produced under stress conditions at different developmental stages and plays important role in giving protection against pathogen attack. Medicinal values of plants lies in these phytochemicals that possess different medicinal properties like anti-tumor, anti-bacterial, anti-fungal, anti-oxidant, anti-inflammatory, anti-viral and so on. Due to these versatile natures of phytochemicals, researchers and industries are looking forward the phytochemicals as a rich source of succeeding drugs. The present study carried out on the *in vivo* roots, *in vitro* roots, hairy roots and root derived cells from suspension culture of *B. montanum*, revealed the presence of some medicinally active metabolites. A total of seven different solvent extracts of *B. montanum* were screened for extracting their active ingredients. Among these solvent systems, petroleum ether and methanolic extracts showed highest response on phytochemical screening. Hence we have selected petroleum ether as the best solvent for further experiments. Presence of alkaloids, flavanoids, phenols and terpenoids were noticed in all plant samples of petroleum ether extract (Table 1). In *Euphorbia hirta* also the presence of different phytochemicals has been revealed as the present study [17]. Preliminary screening of phytochemical analysis of *B. montanum* also showed the absence of glycosides, fatty acids, tannins, saponins, steroids, carbohydrates, coumarins, phlobatannins, anthraquinones, proteins and amino acids. This study demonstrates that the phytochemical profile of the petroleum ether extract of *B. montanum* is composed of flavanoids, phenolic compunds, alkaloids and terpenoids. On the other hand, it is devoid of tannins, saponins, steroids, carbohydrates, phlobatannins, anthraquinones, proteins and amino acids. Agreeing with this, Ramgopal [18] also reported the phytochemical screening of hairy roots of *Abrus precatorius*. Very recently the phytochemical screening of *in vitro* hairy roots of *Cucumis anguria* was also done [19].

Table 1: Qualitative analysis of Petroleum ether extract of *in vivo* roots, *in vitro* roots, hairy roots and root derived cells of *B. montanum*.

Samples				Tests	Phytochemicals
***In vivo* roots**	***In vitro* roots**	**Hairy roots**	**Cell suspension**		
+	+	+	+	Schinoda test	Flavanoids
-	-	-	-	Keller killiani test	Glycosides
-	-	-	-	Stain test	Fatty acids
+	+	+	+	Mayer's test	Alkaloids
+	+	+	+	Ferric chloride test	Phenols
-	-	-	-	Gelatin test	Tannins
-	-	-	-	Foam test	Saponins
+	+	+	+	Salkowski test	Terpenoids
-	-	-	-	Leibermann- Buchard test	Steroids
-	-	-	-	Molisch's test	Carbohydrates
-	-	-	-	Using NaOH	Coumarins
-	-	-	-	Using HCl	Phlobatannins
-	-	-	-	Brontrager's test	Anthraquinones
-	-	-	-	Ninhydrin test	Proteins and Amino acids

+ Present, - NIL

FTIR analysis of *B. montanum* samples

The FTIR spectroscopy method was used to identify the functional groups present in the extract of a number of medicinal plants by using the peak values in the IR region [20]. FTIR spectra of four different root samples of *B. montanum* are depicted in Figure 2. *In vivo* roots, *in vitro* roots, hairy roots and root cells from suspension culture showed peaks at 3348.40 cm^{-1}, 3281.62 cm^{-1}, 3295.16 $cm^{-1}$and 3254.89 cm^{-1} (Table 2). These peaks are indicating the presence of poly hydroxy compounds due to O-H stretch. All root samples showed the presence of ketone groups (C=O stretch) at 1636.30, 1624.06, 1638.69 and 1636.24 cm^{-1}. A peak of 1322.55, 1324.42, 1323.92 and 1410.87 cm^{-1} indicates the presence of phenols (O-H bend) and the presence of the same was detected in qualitative analysis also. The peaks of 1003.83, 1025.67, 1031.77 and 1023.81 cm^{-1} revealed the phosphate compounds (Figure 2). Alcohols, carboxylic acids, esters, ethers are seen in C-O stretch at the peak value 1003.83 cm^{-1}. Previously, Azhar [21] evaluated FTIR peaks in *Euphorbia lathyris* indicating the presence of alcohols, phenols, ethers and alkenes. The functional group analysis of *Alchornea cordifolia* leaves [22] also indicated the presence of various functional groups as similar to *B. montanum*. The roots of *Eclipta alba* also indicated the presence of carboxylic acid, amino acids, polysacharides etc. by FTIR analysis [23]. Here, the spectra showed broadly similar transmittance pattern for all the tested root samples of *B. montanum*. The indication of esters also confirms the presence of the most significant bioactive compound baliospermin and montanin, which belongs to the class of organic compounds known as phorbol esters in different root samples of *B. montanum*.

Table 2: Functional groups identified in FTIR analysis of *B. montanum*

Wave number (cm^{-1}) in samples				Functional groups	Phyto-components	Wave range (cm^{-1})
***In vivo* roots**	***In vitro* roots**	**Hairy roots**	**Cell suspension**			
3348.40	3281.62	3295.16	3254.89	O-H stretch	Poly hydroxy compounds	3570-3200
-	-	2921.69	-	$CH(CH_2)$	Assymetric lipids, proteins	2935-2915

1636.30	1624.06	1638.69	1636.24	C=O stretching	Ketone groups	1650-1600
-	-	-	1587.33	C=C stretching	Aromatic compounds	1580-1550
1322.55	1324.42	1323.92	1410.87	O-H bend	Phenol	1410-1310
-	-	1232.61	-	C-O stretching	Alkyl aryl ether	1275-1200
1003.83	1025.67	1031.77	1023.81	Phosphate ion	Phosphate compounds	1100-1000
-	-	825.05	-	Cl stretching	Aliphatic Chloro compounds	800-700

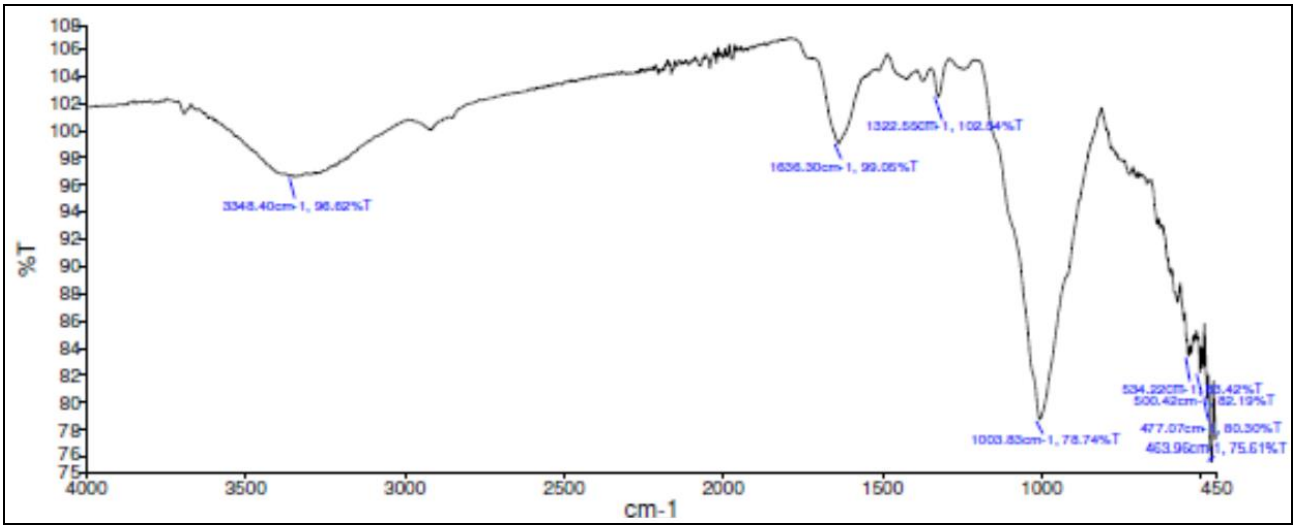

Fig 2: FTIR spectrum of *in vivo* roots of *B. montanum*

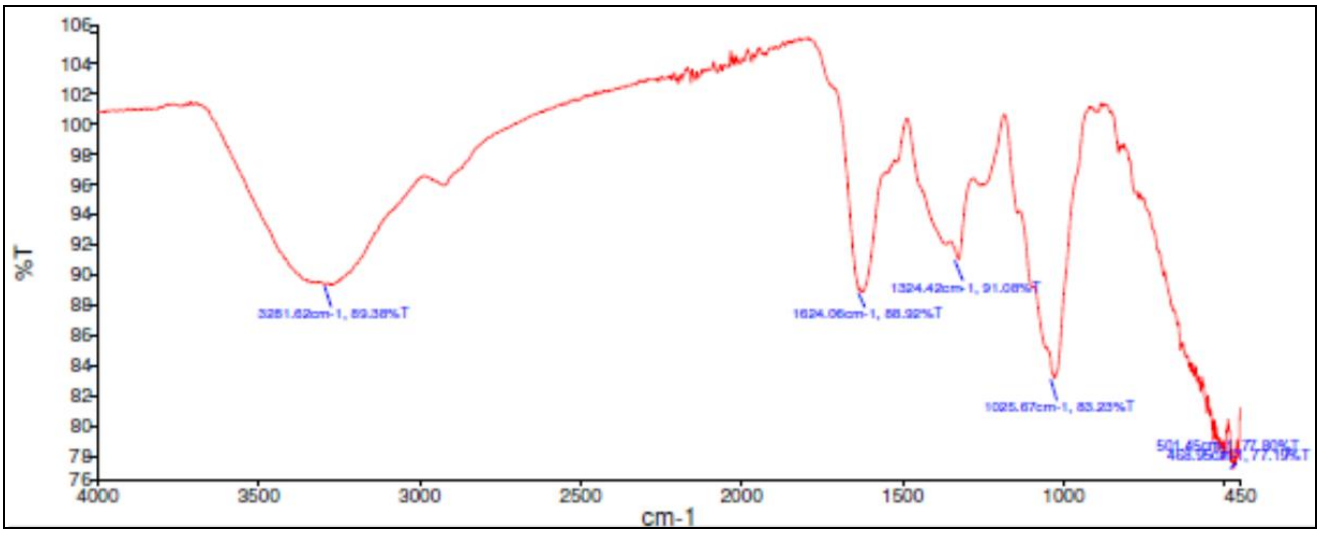

Fig 3: FTIR spectrum of *in vitro* roots of *B. montanum*.

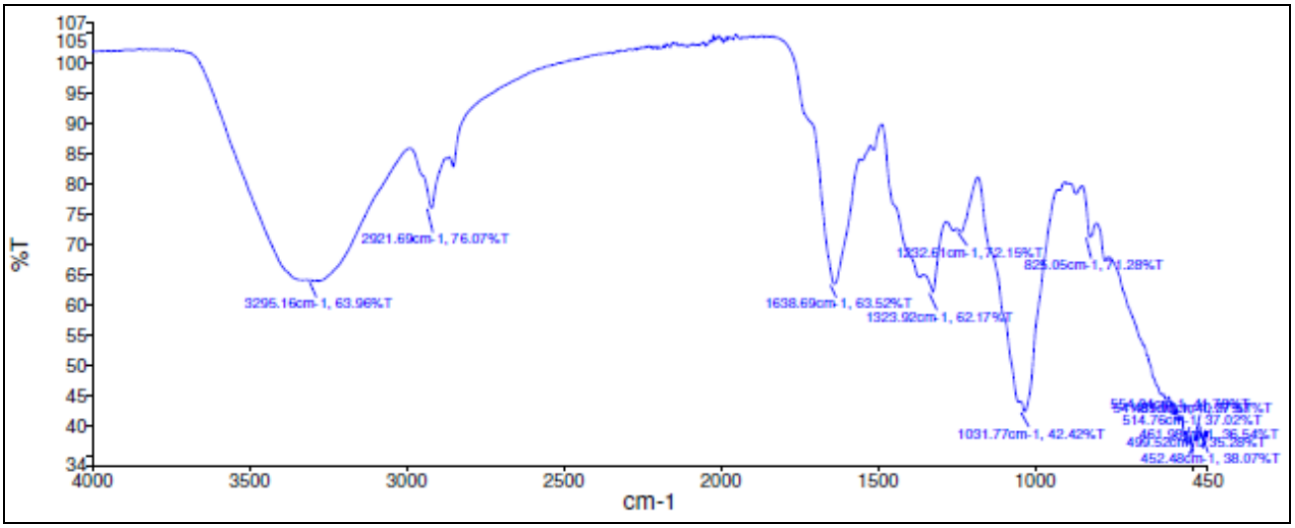

Fig 4: FTIR spectrum of hairy roots of *B. montanum*.

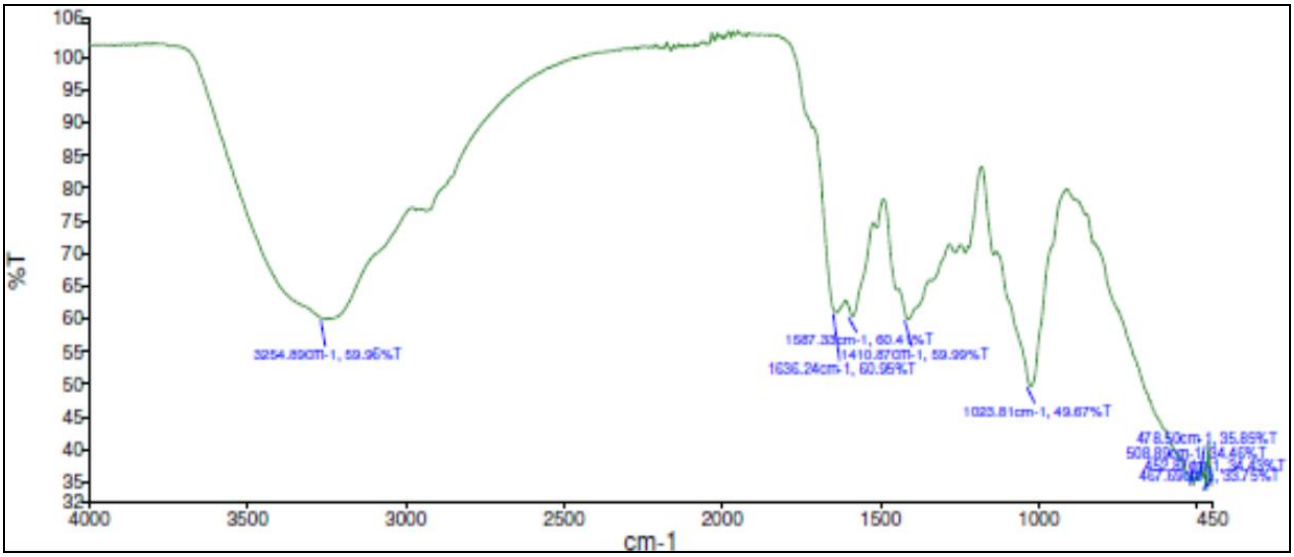

Fig 5: FTIR spectrum of root derived cells from suspension culture of *B. montanum*.

Conclusion

Thus the result obtained in the present study indicates petroleum ether extract of various root samples of *B. montanum* possess promising bioactive phytochemicals such as terpenoids, alkaloids, flavanoids and phenols which have the potential to act as a source of useful herbal formulations. The functional group analysis by FTIR on these extracts revealed the presence of various functional groups such as poly hydroxy groups, phenols, esters and ketones. The indication of esters in FTIR analysis in different root samples of *B. montanum* confirms the presence of the most significant bioactive compound baliospermin and montanin, that belongs to phorbol esters. Hence FTIR can be used as a diagnostic tool for detecting the bioactive phyto-constituents and the findings established here are the stepping stones for the isolation and purification of active secondary metabolites from the normal and the various *in vitro* root samples of *B. montanum* for drug formulation for therapeutic uses.

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