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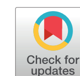
Antioxidant, anticancer and antimicrobial effects of *In vitro* developed protocorms of *Dendrobium longicornu*



Mukti R. Paudel[a,*], Pusp R. Joshi[b], Krishna Chand[b], Anil K. Sah[b], Sameer Acharya[b], Basant Pant[b], Bijaya Pant[a]

[a] *Central Department of Botany, Tribhuvan University, Kathmandu, Nepal*
[b] *Annapurna Research Center, Kathmandu, Nepal*

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ABSTRACT

In vitro seed germination and protocorms formation were successfully established in traditionally used *Dendrobium longicornu*. Fresh protocorms (178.34 g – 183.90 g) were produced on the elicitor of *Alternaria* sp, *Bacillus subtilis* and *Fusarium solani* supplemented MS-medium. Methanol extract of *D. longicornu* protocorms has scavenged 94.31 % of DPPH radicals at 1000 μg/mL. Its 117.56 μg/mL concentration has scavenged 50 % DPPH radical (IC_{50}). Similarly, it inhibits 25.39 % and 27.80 % HeLa and U251 cells at 500 μg/mL. The IC_{50} was found as 350.06 μg/mL and 507.22 μg/mL for HeLa and U251 cells respectively. Further, it inhibited the growth of *E. coli*, *K. pneumoniae* and *E. cloacae* with the zone of inhibition 4, 2 and 2 mm respectively. In conclusion, protocorms developed through *in vitro* seeds culture have accumulated and synthesized bioactive secondary metabolites. Therefore, protocorms could be utilized to the isolation of compounds for formulation of herbal drugs without damaging natural populations.

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1. Introduction

Dendrobium longicornu is widely used in traditional medicine for the treatment of fever and cough, as well as in tonic to produce the body fluid and nourish stomachache [1–3]. The whole plant of *D. longicornu* largely contains bibenzyl and phenanthrene, as well as minimal amounts of monoaromatics, steroid and flavonoid derivatives [4–7]. Due to the presence of such compounds, its extract has been shown the antioxidant, antimicrobial and cytotoxic effects [8,9]. Its wild resource has been depleted by overexploitation to meet the demand in traditional medicine. There is also difficulty in the germination of its seeds in nature without prior symbiosis with special mycorrhizal fungi [10–12]. Hence, it has to be found as threatened species in Appendix II of the Convention on International Trade in Endangered Species (CITES). Plant tissue culture technique provides a way forward to conserve its natural population [13] and production of bioactive secondary metabolites [14,15]. Several chemical compounds of pharmaceutical significance like alkaloids, polyphenols, anthocyanins and carotenoids are synthesized and accumulated on *in vitro* developed tissues [16]. Protocorm is a special organ induced from seed culture that belongs to the somatic embryo and undergo growth and differentiation into plants. They are highly proliferated tissues which accumulate high content of secondary metabolites [17–19]. Recent studies have focused on the biomass production of protocorms and callus for harvesting bioactive compounds through *in vitro* culture *via* suspension and bioreactor [20–23]. *In vitro* developed protocorms of *D. longicornu* were used as the main material source in the bioactivity assays. The objectives of the present study were to establish *D. longicornu* protocorm culture for the production of bioactive secondary metabolites and examine the effect of an extract isolated from protocorms on DPPH free radicals, bacterial growth and cancer cell proliferation.

2. Materials and methods

2.1. Plant materials and establishment of protocorm culture

A mature and healthy green pod of *D. longicornu* was collected from the natural habitat at Chitlang of Makawanpur district, central Nepal. The seeds of the sterilized pod were transferred to the full strength of MS (Murashige and Skoog) medium supplemented with endophytic elicitors. MS medium without any additives was used as control medium [12]. Cultures were incubated in the culture room at 25 ± 2 °C, with 16 h of light.

* Corresponding author.
E-mail addresses: mr.paudel@cdbtu.edu.np, muktiram.paudel@cdb.tu.edu.np (M.R. Paudel).

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Protocorms were induced from the seeds on the medium. All the cultures were maintained for 3 months for the maturation of protocorms and then used for the biomass and extraction.

2.2. Determination of protocorm biomass

The protocorms biomass was separated from the medium by filtering through the mesh and the fresh weight was measured after surface drying. The protocorms biomass was dried at room temperature and dry weights were recorded.

2.3. Extraction of protocorm biomass

The dried protocorm biomass was extracted using MeOH in the ratio of 1:10 of the weight of biomass and volume of solvent (w/v) by 72 -h maceration. The solvent was evaporated under the reduced pressure and MeOH extract was stored at 4 °C.

2.4. Determination of antioxidant effect

Antioxidant effect of the extract was determined using a DPPH (2,2-diphenyl-1-picrylhydrazyl) free-radical scavenging assay [24]. The extract was diluted in methanol to prepare a series of concentrations (125, 250, 500 and 1000 μg/mL). A stock solution of 0.25 mM DPPH free-radical was also prepared in methanol. The reaction mixture was prepared by mixing 1.5 mL of DPPH solution and 1.5 mL of an extract of various concentrations. The reaction mixture was shaken well and incubated for 30 min in dark at room temperature. The rate of change of purple DPPH into yellow by the extract was determined quantitatively by measuring absorbance at 517 nm using Genesys UV–vis spectrophotometer. The ascorbic acid was used as the positive control compound for which the above-mentioned procedure was followed.

2.5. Determination of anticancer effect

Anticancer effect of the extract toward human cervical cancer (HeLa) and brain tumour (U251) cell lines was determined using MTT colourimetric assay [24]. From the cell suspension, 5×10^3 cells in 100 μl of RPMI-1640 medium supplemented with L-glutamine, penicillin/streptomycin and 10 % fetal bovine serum (FBS) were dispensed into the well of a 96-well cell culture plate and incubated under the cell culture conditions at 37 °C and 5% CO_2 for 48 h to allow for adherence and growth of cells. The medium was replaced by 100 μl of four cytotoxic concentrations (100, 200, 400 and 800 μg/mL) of extract and re-incubated for 24 h. Then, 10 μl of MTT (5 mg/mL) was added to every well and re-incubated for next 4 h. The formazan crystals of living cells were dissolved by the addition of 100 μl of dimethyl sulfoxide (DMSO). The plate was read in a microplate reader (iMarkTM, Bio-Rad) at 595 nm. Commercially available cisplatin was used as the positive control for which above-mentioned procedure was followed.

2.6. Determination of antimicrobial effect

MRSA, ATCC and clinical isolate bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Citrobacter freundii* and *Enterobacter cloacae*) were used for antimicrobial effect. Microorganisms were concentrated by comparing with a 0.5 McFarland standard. They were spread over the Muller Hinton Agar (MHA) nutrient medium plate using a sterilized cotton wool swab and incubated at 37 °C for 24 h. The antimicrobial effect was carried out using the well-diffusion method [9]. Three wells were filled by 100 μl of stock solution of plant extract (1 mg/mL in 4% DMSO), one well was filled by DMSO as a negative control, and gentamicin was used as a positive control. The plates were incubated at 37 °C for 24 h. After incubation, the clear zone of inhibition (diameter) around the point of application of each sample solution was measured in millimetre (mm).

2.7. Identification of compounds

Gas chromatography-mass spectrometry (GC–MS; Shimadzu Europa GmbH, Germany) was used to identify the compounds [24]. Spectroscopic detection has involved an electron ionization system which utilized high-energy electrons (70 eV). Pure helium gas (99.99 %) was used as the carrier gas with a column flow rate of 0.95 mL/min. The initial temperature was set at 100 °C and increased at a rate of 3 °C/min after a holding time of about 10 min. In the end, the temperature was increased to 300 °C at 10 °C/min. One microliter of 1% methanol extract was injected in a splitless mode. The relative quantity of the chemical compounds present in the extract was expressed as a percentage based on the peak area produced in the chromatogram. Compounds were identified based using GC retention time and by matching the spectra with standard values (literature) using computer software.

2.8. Statistical analysis

Dunnett's test was applied to compare the protocorm biomass produced on the fungal elicitor medium and the MS control medium, percentage antioxidant activity and IC_{50} of extract and ascorbic acid, IC_{50} for anticancer capacity of extract and cisplatin, and zone of inhibition of extract and gentamicin at the $p \leq 0.05$ significant level.

3. Result

3.1. Effect of elicitor supplemented MS-Medium on production of protocorms from seeds culture

The effect of endophytic elicitors supplemented MS-medium was tested for the proliferation and accumulation of protocorms biomass from the seeds culture of *D. longicornu*. The treatment of similar concentration of different endophytic elicitor supplemented in the full strength of MS-medium exhibits remarkable

Table 1
Response of MS medium with or without additives of different endophytic elicitors in the growth of protocorms from seed culture.

Medium Composition	Stage of Response	Fresh Biomass (g)	Dry Biomass (g)
MS (Control)	Swelled embryo, Embryo enlargement, Rupture	170.39^a	13.63^a
MS + *Agaricus bisporus*	of testa, The emergence of the first leaf,	13.35^b	1.13^b
MS + *Alternaria* sp.	Elongation of shoot	178.34^a	15.16^a
MS + *Bacillus subtilis*		179.25^a	15.24^a
MS + *Fusarium oxysporum*		10.80^b	0.92^b
MS + *Fusarium solani*		183.90^a	15.63^a

The value in a column with the same superscript alphabet are significantly similar at $p < 0.05$ (n = 6).

effects on the accumulation of protocorms biomass during the seed culture. The proliferation and accumulation of protocorms biomass were found varied on elicitor supplemented medium (Table 1). Seeds began swelling within 3 weeks after the inoculation (Fig. 1a), and emergence and elongation of first leaf/shoot commenced within 3 months of culture *via* the pathways of protocorms development on the tested medium (Fig. 1b). MS-medium (control) and MS-medium supplemented with elicitor of endophytes such as *Alternaria* sp, *Bacillus subtilis*, *Fusarium solani* induced the significant growth of protocorms biomass from the seeds. The fresh biomass of protocorms was significantly found higher on this medium from 170.39 to 183.90 g than the MS-medium supplemented with elicitor of *Agaricus bisporus* and *Fusarium oxysporum*.

3.2. Effect of protocorms biomass extract on radical scavenging activity

The methanol extract of *D. longicornu* protocorms was evaluated for its antioxidant effect by scavenging the DPPH free radicals. Extract has shown a capacity of scavenge 94.31 % of DPPH free radicals at 1000 μg/mL concentration. The scavenging capacity of extract was found statistically significantly similar to that of same concentration of ascorbic acid (96.19 %), a positive control. The concentration of extract required to scavenge 50 % DPPH free radicals (IC_{50}) was found as 117.56 μg/mL, however, IC_{50} value of ascorbic acid was 84.47 μg/mL. Statistically, IC_{50} values of extract and control were significantly different (Table 2).

3.3. Effect of protocorms biomass extract on growth of cancer cell lines

Anticancer effect of the methanol extract of *D. longicornu* protocorms was evaluated against HeLa and U251 cell lines by MTT colourimetric assay. The percentage of cancer cells growth inhibition by the action of extract was increased in both cell lines in a dose-dependent manner (Table 3). The extract has shown less percentage of HeLa cells growth inhibition (4.68–25.39 %). Similarly, it also showed less percentage of U251 cells growth inhibition (4.11–27.80 %). The concentration of extract required for the 50 % inhibition of cell growth (IC_{50}) was obtained as 350.06 μg/mL (for HeLa cells) and 507.22 μg/mL (for U251 cells). The IC_{50} value of extract on both cell lines was significantly different from that of the positive control, cisplatin (IC_{50}: 25.00 μg/mL) (Table 3). The result has shown the moderate anticancer effect of protocorms extract toward the cancer cell lines. The formazan crystals of the living cells were formed after the addition of MTT dye (Fig. 2a and c). They were dissolved by DMSO before reading the absorbance. The extract killed cells were became spherical or rounded shape not the formazan crystals (Fig. 2b and d).

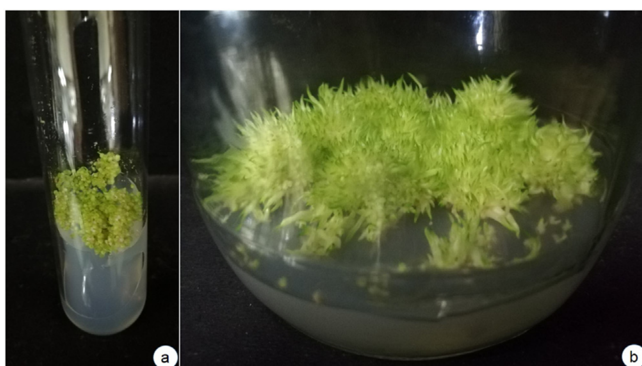

Fig. 1. *In vitro* protocorms formation from the seeds of *D. longicornu* (a), growth of the protocorms into the seedlings (b).

3.4. Effect of protocorms biomass extract on growth of Bacteria

Antimicrobial effect of methanol extract of *D. longicornu* protocorms was evaluated using the well-diffusion method against different bacterial strains. The zone of inhibition of *E. coli*, *K. pneumoniae* and *E. cloacae* growth was shown by extract as 4, 2 and 2 mm respectively (Fig. 3). However, the extract has not inhibited the growth of *S. aureus*, *P. aeruginosa* and *C. freundii* (Table 4). Moreover, the average zone of inhibition of growth of all the bacteria by gentamicin (a positive control) was found as 23 mm which was more than that of plant extract. The zone of inhibition of growth of bacteria by plant extract and gentamicin was statistically significantly different. It has proved that protocorms extract has moderate antimicrobial activity towards the bacterial strains.

3.5. Identified compounds in protocorms biomass extract

Twenty-two compounds were detected and identified in the extract as listed in the Table 5. The GC–MS graph of the detected compounds was shown in Fig. 4. The major compounds present in the extract are (Z)-7-Hexadecenoic acid (12.99 %), Hydroxyacetic acid (6.78 %), 14-methyl-pentadecanoic acid (6.39 %) and 8-Methyl-6-nonenoic acid (5.71 %). Besides, some phenol derivatives such as α-Cadinol (2.71 %), 6,10-Dimethyl-4-undecanol (2.95 %), 9-Hexadecyn-1-ol (3.27 %), 3-Heptadecanol (3.36 %), Nonadecatriene-5−14-diol (4.76 %) and Pentafluoropropionate-*trans*-2-dodecen-1-ol (7.05 %) were also identified.

4. Discussion

There are many nutrient medium and culture types that were successfully employed for the propagation of *Dendrobium* species from various explants [25,22]. And such nutrient medium supplemented with additives of exogenous supply has played a significant contribution to the protocorms culture of *Dendrobium* [19]. As a result, *Agaricus bisporus* and *Fusarium oxysporum* are not considered as compatible fungi for the germination of seeds and protocorms development. This is because of endophytic fungi isolated from one species does not necessarily stimulate the seed germination or further development of protocorms of the other species. The elicitors used in the present study were prepared in our laboratory from the endophytic fungi of *Dendrobium moniliforme* and *Vanda cristata* [11,12]. However, the supplementation of elicitors on MS-medium played a vital role in seed germination, production of protocorms biomass and plant growth of *Cymbidium aloifolium* and *Rhyncostylis retusa* [11,12]. We wanted to develop a protocol that will lead to the production of increased phenolic and other bioactive secondary metabolites in the cultured protocorms biomass. The biosynthesis of natural products is integral to plant immune responses activated in response to attempted cell and tissue culture [14]. During cell and tissue culture, the release of bioactive compound derivatives is very common as reported in much previous literature [26–28]. Many phytochemicals such as alkaloids, flavonoids, phenolic derivatives have been previously reported from its wild counterparts [4–7,29]. However, phytochemicals synthesized in the tissue cultured plant material (protocorms) are reported first time in this paper. Moreover, *in vitro* propagated protocorms or plants have less variation in the content of secondary metabolites than wild counterparts [23]. Also, *in vitro* propagated plant materials are found to contain a higher amount of active ingredients than the intact plants [20,30]. Therefore, the plant tissue culture technology is the alternative way to produce the bioactive secondary metabolites which may have found in the wild plants and also help to conserve the plants in the natural habitats.

Table 2
Percentage scavenging rate of DPPH free radicals by extract and ascorbic acid and their IC_{50} values.

Concentration (μg/mL)	DPPH free radical scavenging rate by extract (%)	DPPH free radical scavenging rate by ascorbic acid (%)
125	70.25[b]	90.36[a]
250	84.95[b]	92.95[a]
500	86.13[b]	94.66[a]
1000	94.31[a]	96.19[a]
IC_{50} (μg/mL)	**117.56[b]**	**84.47[a]**

The value in a row with different superscript alphabet are significantly different at $p < 0.05$ (n = 3).

Table 3
Percentage growth inhibition of HeLa and U251 cells by extract and IC_{50} values of extract and cisplatin.

Concentration (μg/mL)	HeLa cells growth inhibition by extract (%)	U251 cells growth inhibition by extract (%)
62.5	4.68	4.11
125	10.64	16.74
250	20.57	26.67
500	25.39	27.80
IC_{50} of extract (μg/mL)	**350.06[b]**	**507.22[b]**
IC_{50} of cisplatin (μg/mL)	**25.00[a]**	**25.00[a]**

The value of IC_{50} in a column with different superscript alphabet are significantly different at $p < 0.05$ (n=3).

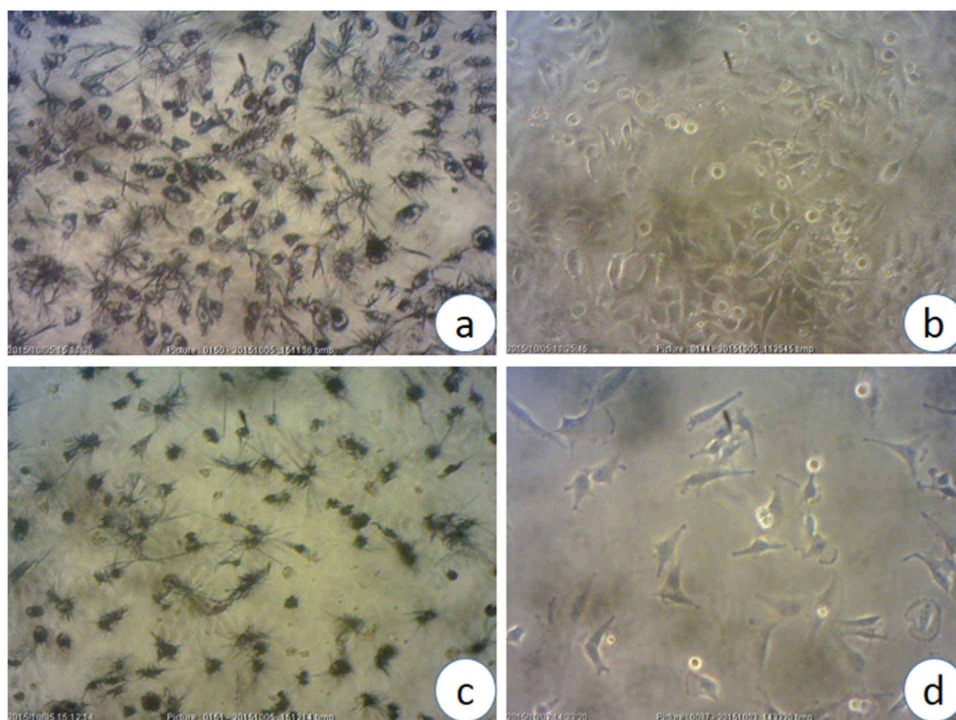

Fig. 2. Formazan crystals of living HeLa cells (a) and U251 cells (c) formed after addition of MTT dye, and floating, spherical and rounded extract-killed HeLa cells (b) and U251 cells (d).

Dendrobium longicornu exhibits the DPPH free radical scavenging capacity as it has many antioxidant-rich secondary metabolites [4–7,29]. We have already evaluated the antioxidant, cytotoxic and antimicrobial capacity of its wild counterparts [8,9]. Its secondary metabolites scavenged DPPH radicals that would be measured at 517 nm. The present study highlights the antioxidant effect of *in vitro* developed protocorms of *D. longicornu*. *In vitro* plant tissue cultures could be new sources of antioxidants because bioactive secondary metabolites could be biosynthesized in the plant materials [18,31]. Many research highlights the antioxidant activity of the *in vitro* developed plant materials of *Dendrobium* and other plant species, which support the present research [28,32,33]. Free radicals and reactive oxygen species generated in the human body induce the carcinogenesis [34,35]. Antioxidant-rich compounds present in the plant products scavenge such types of radicals that prevent cancer development [36,37]. Such compounds arrest the cell cycle and induced apoptotic bodies to prevent cancer. Similar types of the mechanism of action of antioxidant-rich compounds identified in protocorms were seen on the HeLa and U251 cell lines in this research. This could be explored that *in vitro* developed protocorms have potential source of anticancer

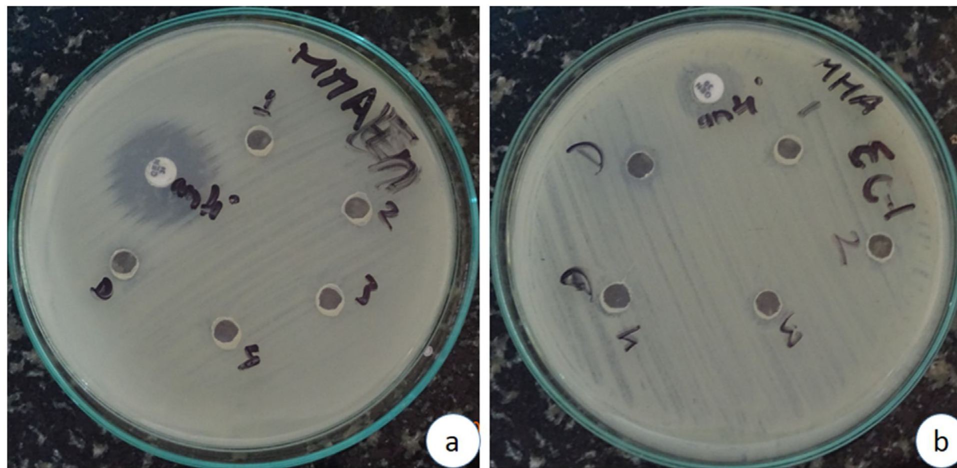

Fig. 3. Inhibition of growth of *E. cloacae* (a) and *E. coli* (b) by the extract and gentamicin.

Table 4
Zone of inhibition of growth of bacterial strains by the extract and gentamicin.

Bacteria Used	Zone of inhibition of extract (mm)	Zone of inhibition of gentamicin (mm)
S. aureus	0**	23
E. coli	4*	23
K. pneumoniae	2*	23
P. aeruginosa	0**	23
C. freundii	0**	23
E. cloacae	2*	23

*Significantly different with gentamicin at $p < 0.05$, and ** no response (n = 4).

Table 5
Compounds detected and identified in the extract by GC–MS.

S.N.	Name of Compound	RT (min)	Peak area%	Base (m/z)
1	Hydroxyacetic acid	3.533	6.78	32.05
2	4-Pyridinecarboxylic acid	3.573	3.82	32.05
3	Docosanoic acid	5.847	0.42	147.15
4	Cedrene	6.378	0.77	119.15
5	α-Cadinol	7.383	2.71	43.05
6	14-methyl-pentadecanoic acid	9.164	6.39	74.05
7	(Z)-7-Hexadecenoic acid	10.317	12.99	55.05
8	15-methyl-hexadecanoic acid	10.467	3.69	74.05
9	6,10-Dimethyl-4-undecanol	11.692	2.95	57.05
10	5-Isopentyl-4-methyl-2-(methylsulfanyl)-6-((trimethylsilyl)oxy) pyrimidine	11.806	3.12	298.05
11	9-Hexadecyn-1-ol	12.746	3.27	67.05
12	3-Heptadecanol	14.963	3.36	59.05
13	Hexahydro-2,5-Methano-2H-furo[3,2-b]-pyran-8-ol	15.503	3.09	207.00
14	Tetracosanoic acid	17.573	3.19	55.05
15	8-Methyl-6-nonenoic acid	17.673	5.71	67.00
16	E,E,Z,-1,3,12-Nonadecatriene-5−14-diol	17.831	4.76	81.05
17	(Z,Z,Z)-9,12,15-Octadecatrienoic acid	17.938	5.37	55.05
18	9-Methoxy-11-Oxatetracyclo [4.2.1.1(2,5).1(7,10)] undec-3-ene	19.016	2.81	71.10
19	8-Methyl-8-oxide-8-Azabicyclo [3.2.1] octan-3-ol	19.093	3.16	54.05
20	Pentafluoropropionate-*trans*-2-dodecen-1-ol	19.180	7.05	41.05
21	Cyclobutanecarboxylic acid	20.338	3.92	55.05
22	Methyl-6-methyl-3-pyridyl ketone-4-cyclohexyl-thiosemicarbazone	21.160	0.34	55.05

compounds synthesis. For example, presence of Tetracosanoic acid, 9-Hexadecyn-1-ol, 3-Heptadecanol, Pentafluoropropionate-*trans*-2-dodecen-1-ol, 6,10-Dimethyl-4-undecanol, α-Cadinol in the protocorms may have shown the antioxidant and anticancer effects [38,39]. The growth of *E. coli*, *K. pneumonia* and *E. cloacae* bacterial strains was inhibited by the extract. All these are Gram-negative bacteria whose outer membrane is thick that prevent the entering of antibiotic drugs so that they are highly resistivity to the antibiotic drugs [40,41]. The extract of protocorms can readily enter the bacterial cell and kill them because it has some compounds of strong antibiotic capacity and efficient permeability. Similar types of research has been carried out from the *in vitro* developed plant materials of orchid which supports our result [42]. However, in comparison to commercially available gentamicin the extract has less susceptible to bacterial strains, but it has capacity of antibiotic.

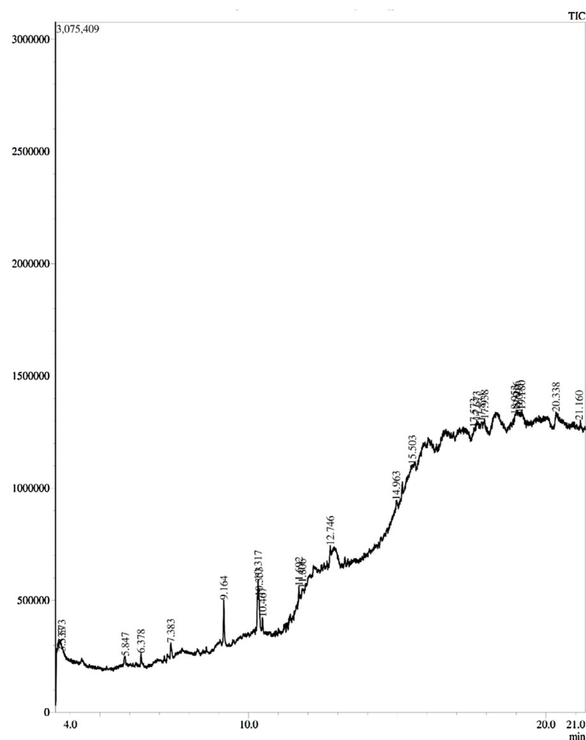

Fig. 4. GC–MS chromatograph of the extract.

5. Conclusion

In conclusion, this study is the first to report seed-derived protocorms of *D. longicornu* are the potential sources of successful plant regeneration and biological activities. The protocol described here could be applied in a propagation program for genetic resource conservation and commercial purposes. Protocorms synthesized the bioactive potential compounds which are rich in antioxidant, anticancer and antimicrobial activities. Due to the presence of bioactive compounds, protocorms extract scavenge the DPPH free radicals which have a potential antioxidant effect. The extract also inhibits the growth of HeLa and U251 cell lines and has moderate anticancer effect toward these cell lines. Further, the extract has a moderate antibacterial effect against *E. coli*, *K. pneumonia* and *E. cloacae*. Further works on isolation and purification of bioactive compounds from protocorms can reveal the exact potential leads to the discovery of drugs in future.

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CRediT authorship contribution statement

Mukti R. Paudel: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. **Pusp R. Joshi:** Investigation, Resources, Writing - original draft, Writing - review & editing. **Krishna Chand:** Investigation, Resources, Writing - original draft, Writing - review & editing. **Anil K. Sah:** Investigation, Writing - review & editing. **Sameer Acharya:** Investigation, Writing - review & editing. **Basant Pant:** Resources, Writing - review & editing. **Bijaya Pant:** Conceptualization, Methodology, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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