Pro-Neurogenic Potential of *Daphne mucronata* Royle

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Summary: The petroleum ether soluble and insoluble fractions of the methanol extract of air dried stem extract of *Daphne mucronata* and a pure compound daphnin obtained from the more active petroleum ether insoluble fraction were evaluated for the pro-neurogenic activity on cortical and hippocampal primary cell cultures prepared from the neonatal mice brain. It showed significant proliferation of the cells as assessed by MTT assay.

Keywords: *Daphne mucronata*, Coumarin glycoside, Daphnin, Proneurogenic potential.

Introduction

Daphne mucronata is a medicinal plant used traditionally for the treatment of various ailments including problems of nervous system [1], muscles disorders, infections, gynecological problems, constipation, allergies, eye pain [2], skin disorders, ulcer, rheumatism, purgatives, toothache, bone disorders, inflammation, flue, arthritis [3]. It has 50 genera and 500 species. It belongs to the family Thymelaeaceae, found in temperate and subtropical regions of Australia, South Africa, Europe, South America, China [4], Saudi Arabia, Oman, Iran, Iraq, Turkey and Northern areas of Pakistan [5]. Phytochemical studies carried out on many *Daphne* species showed the occurrence of flavonoids, triterpenes, coumarins and lignins [6].

The present paper reports the neurogenic potential of fractions and a pure compound of *D. mucronata*.

Experimental

General Experimental Procedures

The research work was done at H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi. Distilled solvents were used in Column chromatographic techniques for separation and purification of the isolated compound Melting point was determined in glass capillary by using Yanaco (MP-S3) apparatus. The ultraviolet (UV) spectra were obtained on Thermo Scientific UV-Visible spectrophotometer. To measure the IR spectra VECTOR spectrophotometer was used. JEOL 600 MS Route instrument was used for electron ionization mass spectrometry (EI-MS). FAB (Fast atom bombardment) spectra were recorded on JEOL-600 HH-2 mass spectrometer with glycerol as matrix. The ^{1}H NMR spectra were obtained on Bruker AV, 600 instruments at 500 MHz cryo probe and 600 MHz respectively and internal standard was tetramethylsilane. ^{13}C NMR spectra were obtained on Bruker avance AV-600 CRYO PROBE and internal reference was TMS.

Chemicals and reagents

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye was purchased from SERVA (USA). The dulbecco's modified eagle's medium (DMEM) provided by Gibco (Thermo Fisher Scientific, USA) and was modified by addition of the fetal bovine serum (FBS, SERVA, USA), sodium pyruvate(Sigma-Aldrich, USA), penicillin-streptomycin(Gibco, Thermo Fisher Scientific, USA) and amphotericin B (Biowest, USA). Trypsin-EDTA, phosphate buffer saline (PBS), trypan blue dye and DMSO were obtained from the Gibco (Thermo Fisher Scientific, USA), Carl Roth (Germany), MP Biomedical (USA) and SERVA (USA), respectively.

Animals

Wistar rat pups (1-2 days old) were supplied by the Animal House Facility located at the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi. The study was performed according to the ethical guidelines developed by the

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Scientific Advisory Committee on Animal Care, Use, and Standards, ICCBS (protocol no. 0012-2018).

Plant material

The aerial parts of the plant were collected on 16 August, 2016 from Khaltarow Haramosh valley of Gilgit-Baltistan, Pakistan and identified by Dr. Sher Wali Khan. A voucher specimen (No.Dm-21) was deposited in the Department of Biological Sciences, Karakorum International University, Gilgit, Pakistan.

Extraction and isolation

The air dried ground stem (10 kg) was extracted three times with methanol by soaking at room temperature for three days. The combined extract was concentrated on a rotary evaporator and the syrupy extract (1.39 kg) obtained was partitioned between ethyl acetate and water. The ethyl acetate phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The gummy residue (0.205 kg) was divided into petroleum ether soluble (55.77 g) and petroleum ether insoluble (148.86 g) fractions. Solvent fractionation of petroleum ether insoluble fraction (138g) was carried out with dichloromethane, dichloromethane-methanol (1:1) and methanol successively. Dichloromethane-methanol fraction (1:1, 107.7g) was subjected to VLC over silica gel and eluted with dichloromethane, dichloromethane-methanol and methanol (100:00→00:100) and 41 fractions were collected. Similar fractions were added on the basis of TLC to yield 3 sub-fractions. Fraction 2 (10 g) of VLC was subjected to gravity column chromatography over silica gel and elution with dichloromethane, dichloromethane-methanol and methanol in the increasing order of polarity and 169 fractions were collected. On mixing similar fractions on the basis of TLC 17 fractions wereresulted. Sub-fraction 10 (26 mg) was a semi pure compound (Fig. 2). On washing with cold methanol a pure compoundwasidentified as daphnin (Fig. 1) on the basis of NMR data.

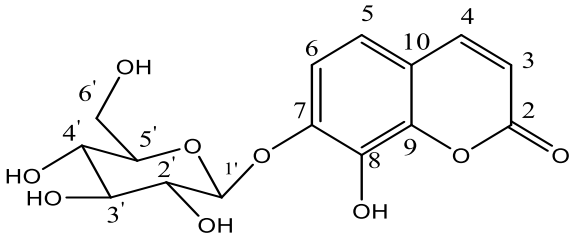

Fig. 1: Chemical structure of daphnin.

Isolation and culturing of cortical and hippocampal cells

Whole brain samples were isolated from the rat pups under sterile environment and immediately washed with sterile PBS. Hippocampus and cortex were dissected from whole brain (Fig. 3). Hippocampus was triturated into fine cell suspension followed by cell count and cell viability test. The cells were finally incubated at 37°C with 5% CO_2 in 25 cm^2 flasks containing high glucose complete DMEM (10% FBS, 1 mM sodium pyruvate, 100 U/mL penicillin-streptomycin and 0.25 μg/mL amphotericin B). The cortex was cut into small pieces and then placed in 0.05% trypsin-EDTA solution for 1-2 minutes at 37ºC and 5% CO_2 in a humidified chamber to digest the tissues. Following trypsinization, cells were washed with 10% DMEM and triturated into dissociated cell suspension which was then centrifuged at 241 rcf (1200 rpm) for 4 minutes. The cell pellets were re-suspended in complete media and total cell count and cell viability test was performed. The cells were re-suspended in high glucose DMEM and cultured in 25 cm^2 flasks incubated at 37°C with 5% CO_2. Both the hippocampal and cortical cell cultures were examined daily and media was changed 2-3 times weekly.

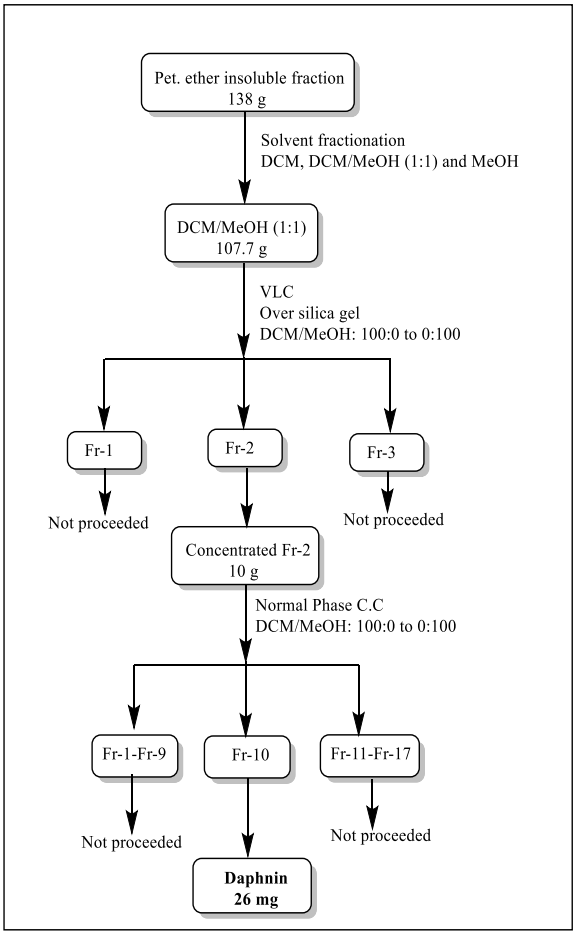

Fig. 2: Extraction Scheme.

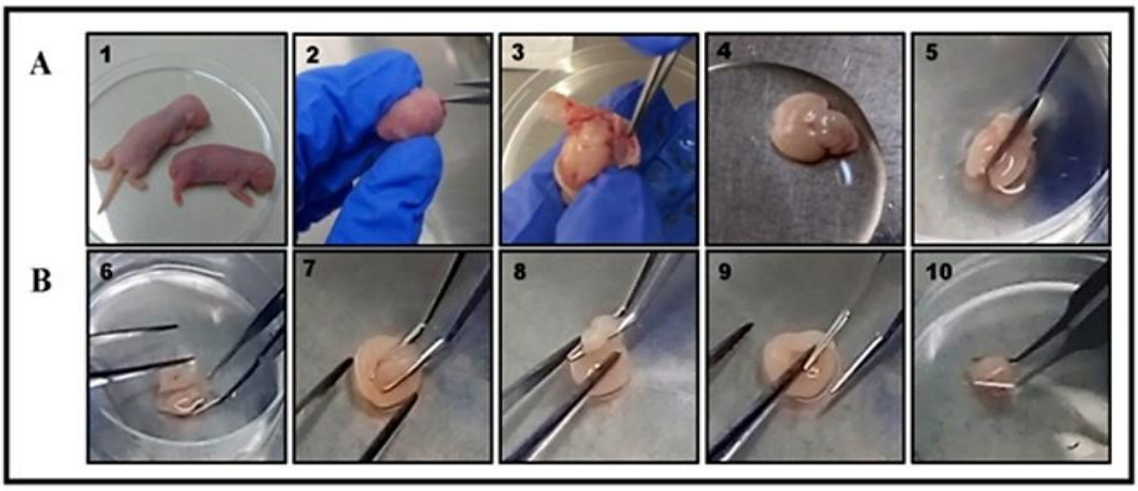

Fig. 3: Steps followed for isolation of the hippocampus and cortex from rat pup.
Panel **A** (1-5) indicates the isolation of whole brain from 1-2 days old rat pups. Panel **B** (6-9) representing the steps followed for isolation of the hippocampus. Where, Panel **B** (10) shows isolation of cortex.

Cell viability test (Trypan blue exclusion test)

Trypan blue exclusion test was used to determine cell viability in freshly prepared cell suspensions. The nonviable cells take trypan blue dye and thus appear blue whereas viable cells have clear cytoplasm. The viable cell suspension was diluted in a ratio of 1:1 with 0.4% trypan blue dye in PBS (pH 7.4) and loaded into hemocytometer. The cells were counted under the microscope at 20x magnification. The cell viability is calculated as:

Number of viable cells/mL = Total number of viable cells counted x dilution factor x 10,000

Passing of cultured cells and in vitro screening of compounds for cell proliferation activity

Once the cells attained 80% confluences, media was aspirated and cells were washed twice with PBS and then treated with 0.05% trypsin-EDTA solution and incubated for 3 minutes at 37°C. For neutralizing the trypsin, 3 mL of complete media was added in the culture flasks and the cell suspensions were centrifuged at 241 rcf for 5 minutes. The resultant cell pellets were re-suspended in 1 mL of complete DMEM and transferred into 25/75 cm^2 flask and re-incubated at 37°C in 5% CO_2.

The tetrazolium MTT [3-(4, 5-dimethyl thiazol-2-yl) - 2, 5-diphenyl tetrazolium bromide] assay

MTT assay is one of the most frequently used methods for the assessment of cell viability. The primary cultures of hippocampal and cortical cells (isolated and harvested from the pups of Wistar rat) were seeded 10,000/well in 96-wells plates and incubated for 24 hrs at 37°C with 5% CO_2. Following 24 hrs incubation, media was removed and cells were treated with 50, 100, 200, and 300 μM concentrations of daphnin. The culture plates were re-incubated for 24 hrs and 48 hrs at 37ºC in 5 % CO_2. As a vehicle control, cells were treated with 0.3% DMSO in complete media (10% FBS). The same volume of medium without cells was used as a blank. After incubation, sample solution in the wells was aspirated and 100 μL of 0.5 mg/mL MTT dye was added to each well and incubated at 37ºC in 5% CO_2 for 3 h. After 3 h incubation, medium was removed and 100 μL of DMSO was added to each well. The plates were shaken 1 minute to solubilize the formazan crystals and absorbance was recorded at the wavelength of 570 nm using Multiscan Go Spectrophotometer (Thermo scientific, USA).The percentage of viable cells following treatment was normalized to vehicle control. All assays were done in triplicates at least three times. The percent rate of proliferation was calculated using the following formula:

% rate of proliferation= {(At - Ab)/ (Ac - Ab)} x100

Where,

At = Absorbance value of test compound (cells + media + test compound / drug)
Ab = Absorbance value of blank (media + test compound / drug)
Ac = Absorbance value of control (cells + media + vehicle), [7].

Results and discussion

Daphnin was isolated as white powder. Its EI- MS spectrum displayed a peak at *m/z* 178 [M+H-sugar] $^{+}$, and its HRESI-MS spectrum showed molecular ion peak at *m/z* 340.0868 corresponding to the molecular formula $C_{15}H_{17}O_9$ (calcd. $C_{15}H_{17}O_9$: 340.0867). The IR spectrum of compound displayed bands at 3360 (O-H stretching), 1700 (C=O), 1665 (C=C), 1550 (C-H) and 1490 (C-O stretching) cm^{-1}. The UV spectrum displayed λ_{max} at 290, 330 and 332 nm. In the proton NMR spectrum a pair of doublets at δ 7.63 (1H, d, *J*= 9.5 Hz) and 6.21 (1H, d, *J*= 9.5 Hz) in low field region of the spectrum was attributed to H-4 and H-3 respectively of the aromatic ring A. Another pair of one-proton doublets showed up at *δ* 7.14 (1H, d, *J*= 8.6 Hz, H-5) and 6.87 (1H, d, *J*= 8.6Hz, H-6) suggesting that the compound is a disubstituted coumarin. A *ß*-D-glycopyranoside moiety was displayed by the signals at *δ* 4.50 (1H, d, *J*= 7.8 Hz, H-1'), 3.61 (1H, t, *J* = 9.1, H-2'), 3.45 (1H, t, *J* = 9.1, H-3'), 3.53 (1H, t, *J* = 9.2, H-4'), 3.24 (1H, m, H-5'), 3.76 (1H, dd, *J* = 12.5, 3.2 Hz, H-6'a), 3.81 (1H, dd, *J* = 12.5, 2.6 Hz, H-6'b), [8].

Table-1: Physical properties of daphnin.

Compound-1	
Color	**White**
Physical State	**powder**
MP	**223-224 °C**
UV	**290, 330, 332 nm**
IR (KBr)	**3360 (OH), 1700 (C=O), 1665 (C=C), 1550 (C-H), 1490 (C=O) cm^{-1}.**
HRESI-MS [M^{+}] *m/z*	**340.0868**

Table-2: NMR spectroscopic data ($CDCl_3$+MeOD) of daphnin

Position	δ_C	HSQC	δ_H (multiplicity, *J* in Hz)
2	161.8	-	-
3	113.8	CH	6.21 d(d,9.5)
4	144.7	CH	7.63 (d, 9.5)
5	124.6	CH	7.14 (d, 8.6)
6	112.5	CH	6.87 (d, 8.6)
7	147.6	-	-
8	131.8	-	-
9	153.6	-	-
10	111.5	-	-
1'	105.9	CH	4.50 (d, 7.8)
2'	73.5	CH	3.61 (t, 9.1)
3'	76.0	CH	3.45 (t, 9.1)
4'	68.9	CH	3.53 (t, 9.2)
5'	75.8	CH	3.24 (m)
6'	60.7	CH_2	3.76 (dd, 12.5, 4.5), 3.81 (dd, 12.5, 1.8)

The MTT assay demonstrated that both pet.ether soluble fraction (PESF) and pet.ether insoluble fraction (PEIF) supported the growth of the cortical cells in a dose dependent manner. However, the pet.ether insoluble fraction demonstrated higher rate of cell proliferation (Table 3).

Table-3: Proliferation of the cultured cortical cells following treatment with pet.ether soluble fraction (PESF) and pet.ether insoluble fraction (PEIF)

			Dm-PESF	Dm-PEIF
Only medium	**Only cells**	**DMSO**	**100 *µ*g/ml200 *µ*g/ml**	**100 *µ*g/ml200 *µ*g/ml**
0.079	0.683	0.649	0.897--1.147	1.133--1.580
0.080	0.669	0.673	0.749--0.909	0.926--1.425
0.077	0.639	0.652	0.700--1.045	1.175--1.420
		0.655	0.818--0.911	1.166--1.414
0.079	0.664	0.657	0.791--1.003	1.100--1.460
Average medium	0.585	0.579	0.712--0.924	1.021--1.381
% Proliferation	100	98.9031	121.7663818158.005698	174.5868946236.0826211

Table-4: Growth supporting activity of Daphnin on primary cultures of mice cortical and hippocampal cells.

Treatment	% Rate of Proliferation	
	Hippocampal Cells	Cortical Cells
Control (untreated cells in culture media only)	100.0± 0.000	100.0± 0.000
DMSO treated cells (1%)	98.96 ± 0.08	103.25± 1.77
Daphnin (24 h)		
50 *µ*M	107.66± 0.03	67.63± 0.06
100 *µ*M	101.5± 0.005	42.50±0.00
200 *µ*M	101±0.01	50.19±0.04
300 *µ*M	97.33± 0.009	65.28±0.00
Daphnin (48 h)		
50 *µ*M	87.01± 0.04	57.75± 0.06
100 *µ*M	83.23± 0.002	38.23± 0.01
200 *µ*M	89.39± 0.04	70.96±0.007
300 *µ*M	87.01± 0.009	61.44±0.03

Next, daphnin was targeted for its neurogenic ability of supporting the growth of neural progenitor cells (NPCs) or neurons cultured from cortex and hippocampus. A highest growth rate was observed following 24 h treatment of hippocampal cells with daphnin at the dose of 50 and 100 μM, however at the high dose the proliferation rate started to decline (Fig. 4, Table-4).

The morphological evaluation shows healthy neuronal cells following treatment with test compound. In addition, the synaptic connections between cells with the passage of culturing time were also observed. This sustained neuronal morphology might be due to true differentiation of NPCs with the daphnin. When the hippocampal cells were observed after 48 h incubation, it was noted that the rate of proliferation decreased. On observing under microscope, it was interesting to note that the supporting fibroblast cells drastically reduced however, the neuronal like cells were found to slowly proliferate further. This indicates that daphnin might be supporting the differentiation of the NPCs into neuron-like cells. The depletion of the supporting fibroblast cells and other non-neuronal cells might be the reason why we observed less value of proliferating cells after 48 h treatment. In case of cortical cultures, the rate of proliferation was less than hippocampus cultures. However, minor proliferation of the cortical cells was observed at higher dose of daphnin(Fig. 5).These observations are also supported by the studies affirming neurogenesis in the dentate gyrus (DG) of the hippocampus[9, 10, 11].

Statistical analysis

The experimental data of the study was expressed as mean ± standard error (S.E.M.) using IBM SPSS 20.0. The significant mean differences among different experimental group i.e. control and treatment groups were calculated by one-way analysis of variance (ANOVA) and the Posthoc Bonferroni's test for multiple comparisons. Each experiment was performed three times. The significant levels indicated as *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001.

It was observed that daphnin supported or enhanced the growth of hippocampal cells at 50, 100and 200 μM (Fig. 4) concentrations after 24 hrs treatments. Unlike hippocampal cells, no growth supporting activity of daphnin was observed in cortical cells (Fig. 5).It was also observed that the growth supporting activity of the compound decreases with the treatment initiated for 48 hrs.

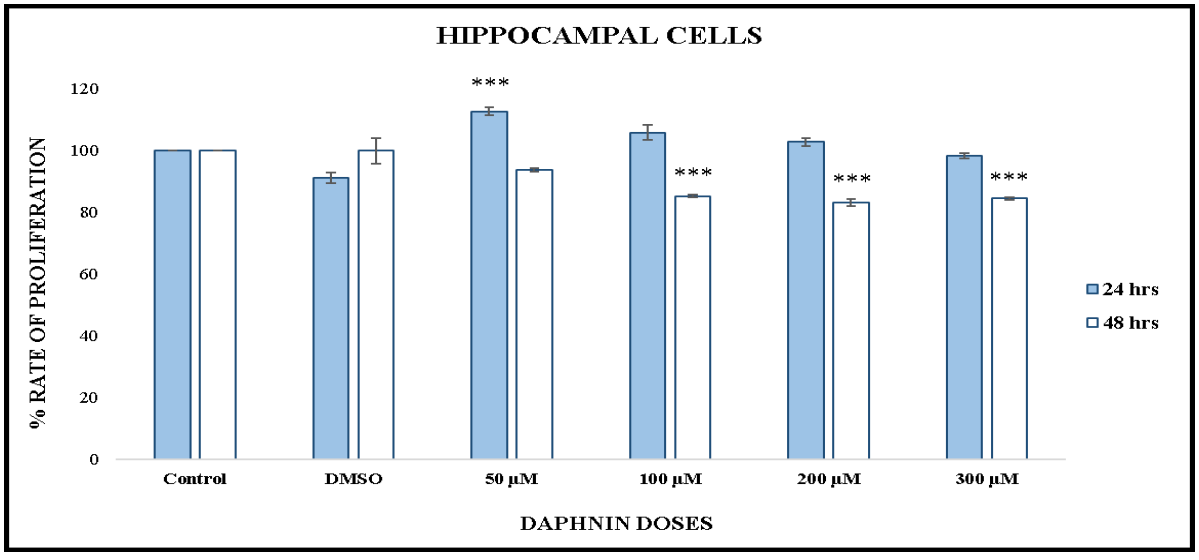

Fig. 4: Graphical representation of the effects of Daphnin on primary cultures of hippocampal cells. At 24 hrs, Daphnin significantly increased the rate of proliferation of hippocampal cells at the dose of 50 μM (maximum) whereas it decreased in dose-dependent manner as compared to the untreated control cells. At 48 hrs, Daphnin decreases the proliferation rate of the primary culture of hippocampal cells. The Bonferroni's post hoc analysis was applied to examine the mean difference between various doses of Daphnin. Each bar represents mean ± S.E.M of three individual experiments. The asterisk present on the bars indicates statistical significance level i.e. *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001.

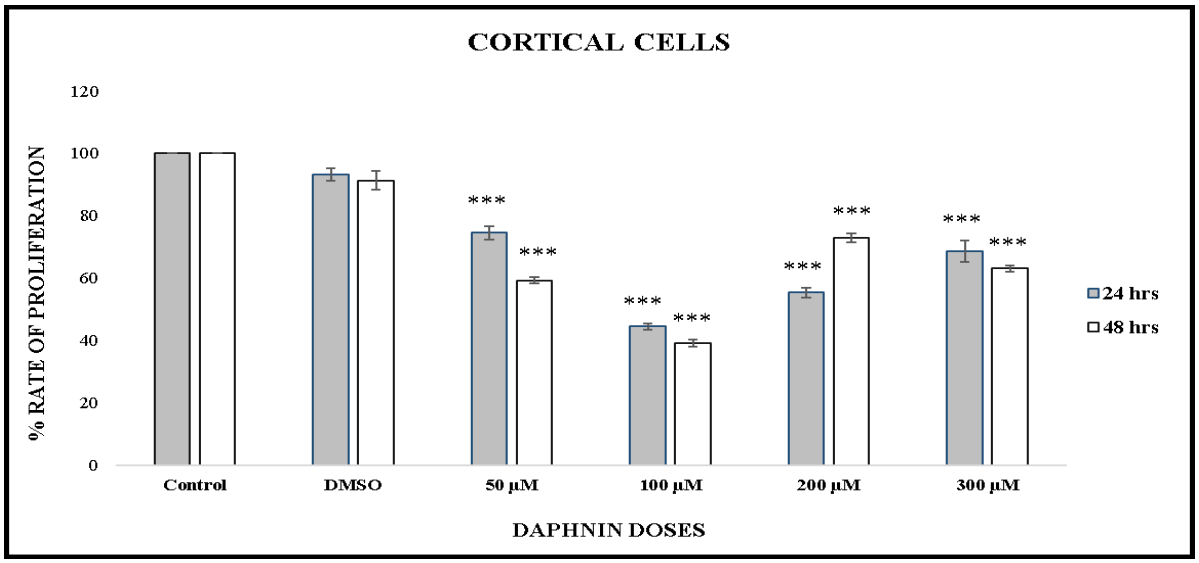

Fig. 5: Graphical representation of the effects of Daphnin on primary cultures of cortical cells. At 24 hrs, Daphnin significantly decreases the proliferation rate of cortical cells. At 48 hrs, it also significantly decreases proliferation rate of cortical cells indicating that the compound have no growth supporting effects on cortical cells. The Bonferroni's post hoc analysis was applied to examine the mean difference between various doses of Daphnin. Each bar represents mean ± S.E.M of three individual experiments. The asterisk present on the bars indicates statistical significance level i.e. *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001.

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Conclusion

In conclusion, two fractions of *D. mucronata* possessed potential neurogenic activity. The study was also supported by broad-spectrum activity of isolated compound, daphnin. Thus, it provided platform for further detailed studies on *D. mucronata* to get more efficient lead compound/s effective in neurogenesis.

Disclosure statement

There is no potential conflict of interest.

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