Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Nardostachys jatamansi Root Extract Modulates the Growth of IMR-32 and SK-N-MC Neuroblastoma Cell Lines Through *MYCN* Mediated Regulation of MDM2 and p53

Snehal Suryavanshi, Prerna Raina, Rashmi Deshpande, Ruchika Kaul-Ghanekar

Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Katraj-Dhankawadi, Pune, Maharashtra, India

Submitted: 20-04-2016 Revised: 11-07-2016 Published: 06-01-2017

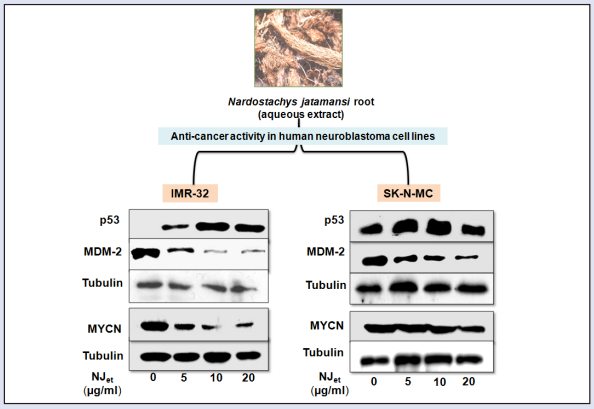
ABSTRACT

Aim: The present study evaluated the effect of ethanolic extract of *Nardostachys jatamansi* roots (NJ_{et}) on MYCN mediated regulation of expression of MDM2 and p53 proteins in neuroblastoma cell lines, IMR-32 and SK-N-MC. **Materials and Methods:** The effect of NJ_{et} on cell viability was determined by MTT; and on growth kinetics was evaluated by trypan blue dye exclusion method and soft agar assay. The expression of p53, MDM2 and MYCN proteins in response to NJ_{et} treatment was evaluated by immunoblotting. **Results:** NJ_{et} decreased the viability of neuroblastoma cells without affecting the viability of non-cancerous, HEK-293 cells. It altered the growth kinetics of the cancer cells in a dose-dependent manner. NJ_{et} down regulated the expression of MYCN and MDM2 proteins with a simultaneous increase in the expression of tumor suppressor protein p53. **Conclusions:** The present data demonstrated that NJ_{et} regulated the growth of IMR-32 and SK-N-MC through reduction in MYCN expression that lead to down regulation of MDM2 protein and increase in p53 expression. These preliminary results warrant further in depth studies to explore the therapeutic potential of *Nardostachys jatamansi* in the management of neuroblastoma.

Key words: Neuroblastoma; *Nardostachys jatamansi*; MYCN; p53; MDM2

SUMMARY

- NJ_{et} reduced the viability of human neuroblastoma cell lines without affecting the viability of non-cancerous, HEK-293 cells.
- NJ_{et} regulated the growth kinetics of the cancer cells.
- NJ_{et} decreased the expression of MYCN and MDM2 proteins and simultaneously increased the expression of tumor suppressor protein p53.



Abbreviation used: NJ_{et}: Ethanolic extract of *Nardostachys jatamansi* MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide HPTLC: High performance thin layer chromatography

Correspondence:
Dr. Ruchika Kaul-Ghanekar,
Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth Deemed University, IRSHA, Pune-Satara Road,
Katraj-Dhankawadi, Pune, Maharashtra, India.
E-mail: ruchika.kaulghanekar@gmail.com; kaul_r@yahoo.com

DOI: 10.4103/0973-1296.197645

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Nardostachys jatamansi (Valerianaceae), commonly known as muskroot, is indigenous to the Himalayan regions of India.[1] In Ayurveda, *N. jatamansi* has been used traditionally since centuries for treating various neurological disorders. It has been used as a sedative, an antispasmodic, for mind rejuvenation, promotion of sleep, alleviating mental diseases and as a brain tonic.[2] It has also been used for treating headache, insomnia, epilepsy; disorders of cardiovascular system; flatulence and intestinal colic as well as menopausal symptoms.[2] *N. jatamansi* has been shown to possess anti-inflammatory,[3] anti-oxidant,[4] anti-bacterial,[5] anticonvulsant[6] and hepatoprotective activities.[7] It has been shown to exhibit anti-tumour activity in lung, liver, ovary, breast and prostate cancers.[4,6] Recently, the higher doses of alcoholic and n-hexane extract of *N. jatamansi* were shown to significantly inhibit the growth of neurobalstoma cell lines, IMR 32 and SK-N-SH.[4]

In the present study, we have shown that ethanolic extract of *N. jatamansi* roots (NJ_{et}) significantly altered the growth kinetics of IMR-32 and SK-N-MC neuroblastoma cells at lower doses through down regulation of *MYCN* and MDM2 proteins and upregulation of p53.

Cite this article as: Suryavanshi S, Raina P, Deshpande R, Kaul-Ghanekar R. *Nardostachys jatamansi* root extract modulates the growth of IMR-32 and SK-N-MC neuroblastoma cell lines through MYCN mediated regulation of MDM2 and p53. Phcog Mag 2017;13:21-4.

MATERIALS AND METHODS

Drugs and chemicals

Tissue culture plasticware was purchased from BD Biosciences (CA, USA). DMEM powder, penicillin and streptomycin were obtained from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylthiazolium bromide (MTT), Valerinic acid and primary antibody against *MYCN* were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibody against p53 (DO-1), MDM2, tubulin (B-7) and donkey anti-mouse IgG antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Plant material, extract preparation and preliminary phytochemical analysis

N. jatamansi roots were purchased from Green Pharmacy (Pune, Maharashtra, India). The sample was authenticated and validated macroscopically and microscopically at the Department of Botany, Agharkar Research Institute (ARI), Pune (Maharashtra, India) (ref no.1045). Voucher specimen sample (Number R-154) has been deposited at the Department.

N. jatamansi roots were weighed, powdered and extracted in ethanol using soxhlet apparatus. The resulting extract was centrifuged at 13000 rpm for 15 min to remove the particulate matter. The supernatant was filter-sterilized using swiney filter (pore size, 0.45 µm) and the resultant filtrate was stored in aliquots at -80°C until use.

Cell culture

The cell lines (IMR-32, SK-N-MC and HEK-293) used in the present study were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2 mM L-glutamine supplemented with 10% fetal bovine serum and 20 U/ml of penicillin-streptomycin and incubated in a humidified 5% CO_2 incubator at 37°C.

Cell viability

IMR-32, SK-N-MC and HEK-293 were seeded at a density of 1×10^5 cells/ml in a 96-well plate. The cells were treated with different concentrations of NJ_{et} (0-160 µg/ml) in triplicates for 24 h. Cell viability was determined by MTT assay as described previously.[8,9]

Cell growth analysis

IMR-32 and SK-N-MC were seeded at a density of 5 × 104 cells/well in 24-well plate. The cells were treated with different concentrations of NJ_{et} (0-20 µg/ml) in triplicates for 24, 48 and 72 h. The effect of NJ_{et} on cell growth was determined by trypan blue dye exclusion method as described previously.[8,9]

Soft agar assay

IMR-32 and SK-N-MC cells (5×10^3 cells/ml) treated with different concentrations of NJ_{et} (0-20 µg/ ml) were mixed with culture medium containing 0.35% agarose and plated over a previously gelled layer of 0.5% agarose as described previously.[8,9] The colonies were manually counted and photographed directly using an Axiovert 200 M microscope (Carl Zeiss, Germany).

Western blotting

IMR-32 and SK-N-MC cells were plated at a seeding density of 5×10^5 cells/well in a 6-well plate and incubated for 24 h at 37°C in CO_2 incubator. Next day, the cells were treated with different concentrations of NJ_{et} (0-20 µg/ml) for 24 h. The cells were lysed as previously described.[8,9] The protein was estimated using Bradford reagent (Biorad Laboratories Inc, CA, USA). Equal amount of protein was loaded on a 10% SDS-polyacrylamide gel and transferred electrophoretically to Amersham Hybond-P PVDF membrane (GE Healthcare, UK) in sodium phosphate buffer (pH 6.8). The membrane was blocked in 5% BSA in TBST and incubated at room temperature for 3 h with mouse monoclonal primary antibodies against p53, MDM2 and tubulin (Santacruz, CA, USA) at 1:2000 and *MYCN* (Sigma-Aldrich, St. Louis, MO) at 1:500 dilutions respectively. The blots were washed in TBST and incubated with donkey anti mouse IgG HRP conjugate at 1:4000 (for p53, MDM2, tubulin) and 1:2000 (for *MYCN*) dilutions. Proteins were visualized using a chemiluminescence kit (Amersham ECL Advance western blotting detection kit, GE Healthcare, UK) and densitometric analysis of X-ray films was performed using the Image J gel analysis tool.

Measurement of apoptosis

The cells were plated at a seeding density of 5×10^5 cells/well in a 6-well plate and treated with different concentrations of NJ_{et} (0-20 µg/ml). After 24 h of treatment, the cells were harvested and washed with PBS twice. Cells were stained with Annexin V-FITC following the manufacturer's instructions (Annexin V-FITC apoptosis kit #3, Invitrogen) and analyzed for apoptosis by FACS using Cell Quest Software.[8]

Statistical analysis

All the results were obtained from three independent experiments, each performed in triplicates and the values have been presented as mean ± SD. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA). The analyses were carried out using Graph-pad prism 5 software (San Diego, CA, USA). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ were considered to be statistically significant.

RESULTS

Chromatographic analysis of NJ_{et}

HPTLC chromatographic examination of NJ_{et} was carried out to authenticate *N. jatamansi* root extract based on its marker compound, valerinic acid [Figure 1]. The finger print profile was developed in toluene: ethylacetate: formic acid (8:2:0.5) solvent system and the peaks corresponding to valerinic acid were recorded at Rf 0.75. Similar peaks were found in chromatographic profile of NJ_{et} at the same Rf value of 0.75. The calibration curves were linear in the range of 200-1200 ng of valerinic acid. The amount of valerinic acid in NJ_{et} was found to be 41.58 mg/g.

NJ_{et} altered growth kinetics of neuroblastoma cell lines

In IMR-32 and SKNMC, NJ_{et} altered growth kinetics of neuroblastoma cell lines exhibited IC_{50} values of 61.43 and 63.42 µg/ml, respectively [Figure 2]. Contrarily, in non-cancerous HEK-293 cells, IC_{50} was not obtained upto 160 µg/ml concentration of NJ_{et} [Figure 2]. Thus, NJ_{et} significantly reduced the viability of neuroblastoma cells at lower doses without affecting the viability of the non-cancerous cells. Further studies were carried out at non-cytotoxic doses (0-20 µg/ml) of NJ_{et} to analyze its effect at cellular and molecular level.

In IMR-32, NJ_{et} reduced the cell proliferation at 20 µg/ml dose by ~2-fold at 24 h and by ~1.5-folds at 48 and 72 h ($p = < 0.001$) [Figure 3A]. On the other hand, in SK-N-MC, the proliferation was reduced by~2-($p = <0.001$), 3-($p = < 0.001$) and 2- folds ($p = < 0.001$), respectively at 24, 48 and 72 h compared to the untreated control cells [Figure 3B]. The effect of NJ_{et} on the cell growth was further evaluated by soft agar assay that showed a dose dependent decrease in the number of soft agar colonies. At 20µg/ml, there was ~2.5- ($p \leq 0.001$) and 3.5-folds

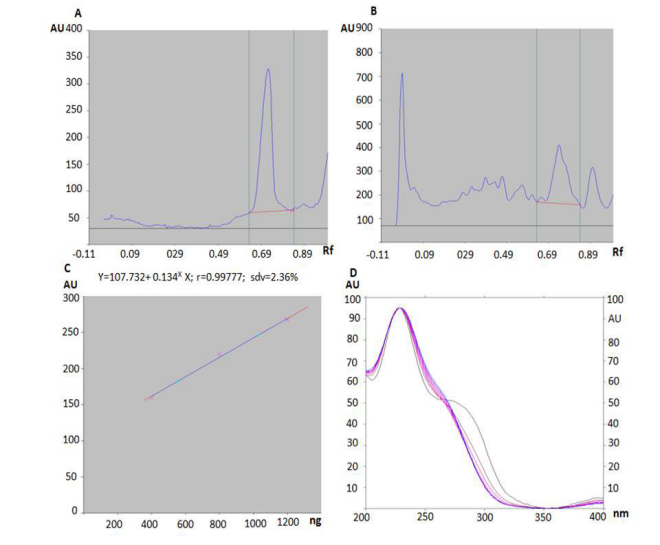


Figure 1: HPTLC images and standardization of NJ_{et}. HPTLC densitogram of (A) marker compound valerinic acid and (B) NJ_{et}. (C) Calibration curves in linear range of 200, 400, 800, 1000, 1200 ng of standard valerinic acid. (D) UV spectra of standard valerinic acid and samples of NJ_{et} obtained by HPTLC spot scanning from 200 to 400 nm.

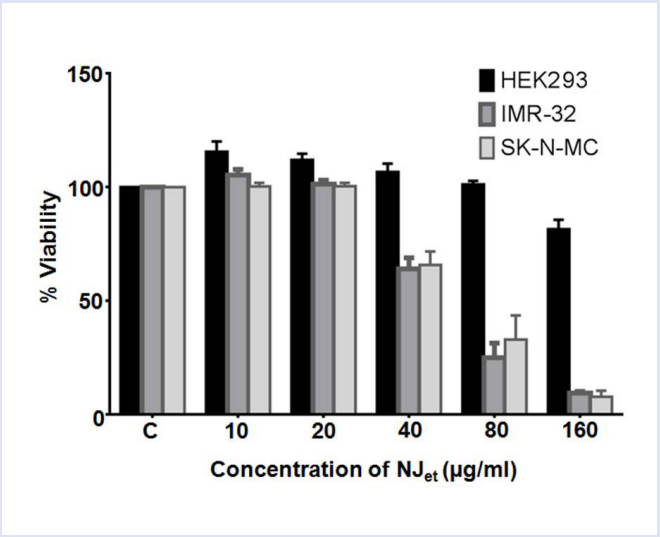


Figure 2: Effect of NJ_{et} on cell viability. IMR-32, SK-NMC and HEK-293 cells were treated with different concentrations (0-160 µg/ml) of *N. jatamansi* root extract. The cell viability was determined by MTT dye uptake method and the data represents mean ± SD of three independent experiments, each performed in triplicates.

($p \leq 0.001$) reduction in the number of colonies in IMR-32 and SK-N-MC, respectively, compared to the untreated control cells [Figure 3C]. These data demonstrated that NJ_{et} significantly regulated the proliferation and growth kinetics of IMR-32 and SK-N-MC cells.

NJ_{et} increased the expression of p53 through down regulation of *MYCN* and MDM2

It was found that NJ_{et} up regulated p53 expression in a dose-dependent manner in IMR-32 and SK-N-MC [Figure 4A and B, respectively] cells. At 20µg/ml, there was~1.5-($p < 0.05$) and 18.36-folds ($p < 0.05$) increase in p53 expression in IMR-32 and SK-N-MC cells, respectively, compared to the untreated control cells [Figure 4C]. Contrarily, a dose dependant decrease in the expression of MDM2 and *MYCN* proteins was observed in IMR-32 and SK-N-MC cells compared to the untreated control cells [Figure 4A and B, respectively]. In IMR-32 and SK-N-MC cells, at 20 µg/ml dose of NJ_{et}, there was ~4- and 9-folds ($p < 0.05$) decrease in the expression of MDM2 ($p < 0.05$) [Figure 4D], respectively, as well as ~1.7- and 4.4-folds decrease in *MYCN* expression, respectively, compared to the untreated control cells [Figure 4E].

DISCUSSION

The present study reported the mechanism of anti-cancer potential of *N. jatamansi* root extract (NJ_{et}) in the human neuroblastoma cell lines, IMR-32 and SK-N-MC. NJ_{et} reduced the viability of neuroblastoma cell lines at lower doses (40 µg/ml onwards) compared to the non-cancerous cells. At 80 µg/ml dose, NJ_{et} reduced the cell viability of IMR-32 and SK-N-MC by 70 and 62%, respectively, with IC_{50} values of 61.43 and 63.42 µg/ml, respectively. It negatively regulated the growth kinetics of neuroblastoma cells in a significant manner and effectively reduced their proliferation. Previously, Monga and Kumar in 2013 had reported the anti- proliferative potential of alcoholic extract and n-hexane fraction of roots of *N. jatamansi* against neuroblastoma cell lines, IMR-32 and SK-N-SH.[2] They had shown that at 100 µg/ml dose, the alcoholic extract reduced the proliferation of IMR-32 and SK-N-SH by 71 and 85%, respectively, whereas at the same dose hexane fraction reduced the proliferation by 91 and 82% respectively. Interestingly, in our study NJ_{et} reduced the viability at much lower doses compared to that reported by Monga and Kumar, which is excellent for drug development point of view. The difference in these results could be due to time of collection of the roots, method of preparation of the extract and the method of cell proliferation assay. Various reports have shown anticancer activity of herbal formulations against neuroblastoma cell lines. PienTze Huang, a popular Chinese medicine for liver diseases, has been reported to decrease survival of SH-SY5Y neuroblastoma cell line.[10] The leaf extracts of *Holarrhena antidysenterica* have been reported to be cytotoxic to SK-N-MC neuroblastoma cells.[11] *Calotropisprocera*, *Ocimum sanctum* and *Cannabis sativa* have been shown to be cytotoxic to IMR-32.[12] However, neither of the studies has delineated the molecular mechanisms underlying the anticancer activity of these medicinal plants.

In the present work, we have shown the preliminary mechanism of action of NJ_{et} in neuroblastoma cell lines. We found that NJ_{et} significantly down regulated the expression of *MYCN*, the major negative prognostic marker in neuroblastoma with significant role in the pathogenesis and clinical onset of this aggressive malignancy.[13] *MYCN* has been reported to activate proliferative cellular pathways and inhibition of p53-mediated apoptosis has been considered as the prerequisite for MYC-driven tumorigenesis.[14] Interestingly, NJ_{et} not only decreased the expression of MYCN but simultaneously upregulated the expression of tumor suppressor protein p53. Moreover, NJ_{et} significantly reduced the expression of MDM2, the negative regulator of p53. MDM2 is amplified in a variety of human cancers, including neuroblastoma.[15] It has been reported to be a predictor for poor treatment outcome in neuroblastoma patients.[16] Thus, NJ_{et} regulated the growth of the neuroblastoma cells through disruption of *MYCN* and MDM2 expression and stabilization of p53. These results lead us to study the mechanism of cell death induced by NJ_{et}, which was found to induce apoptosis in IMR-32 and G2/M arrest in SK-N-MC cells (data not shown).

All these data suggested the potential of ethanolic extract of *N. jatamansi* (NJ_{et}) in regulating the growth of neuroblastoma cells.

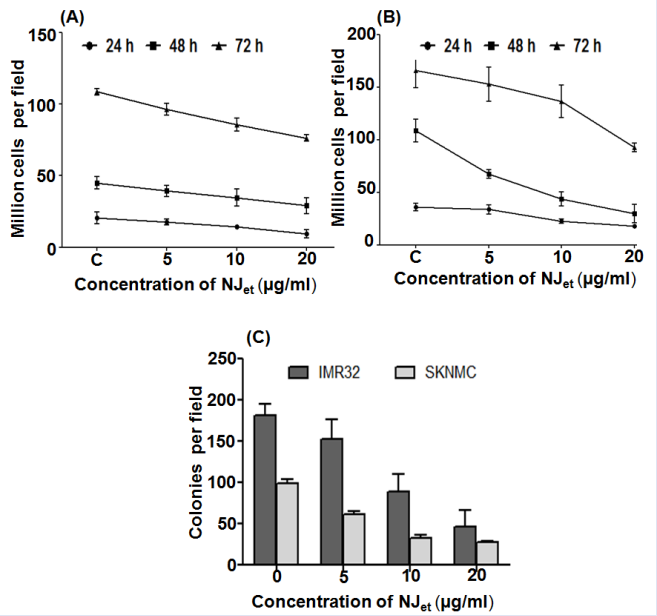


Figure 3: Effect of NJ_{et} on growth kinetics of neuroblastoma cells. (A) IMR-32 and (B) SK-N-MC cell lines were treated with NJ_{et} (0-20 µg/ml) for 24, 48 and 72 h and the number of viable cells were counted by trypan blue dye exclusion method. Data represent mean± SD of three independent experiments (C) IMR-32 and SK-N-MC cell lines were treated with NJ_{et} (0-20 µg/ml) and grown in soft agar for two weeks. Colonies were counted from at least 10 different areas and the average of each has been plotted. The data represents mean ± SD of five independent experiments.

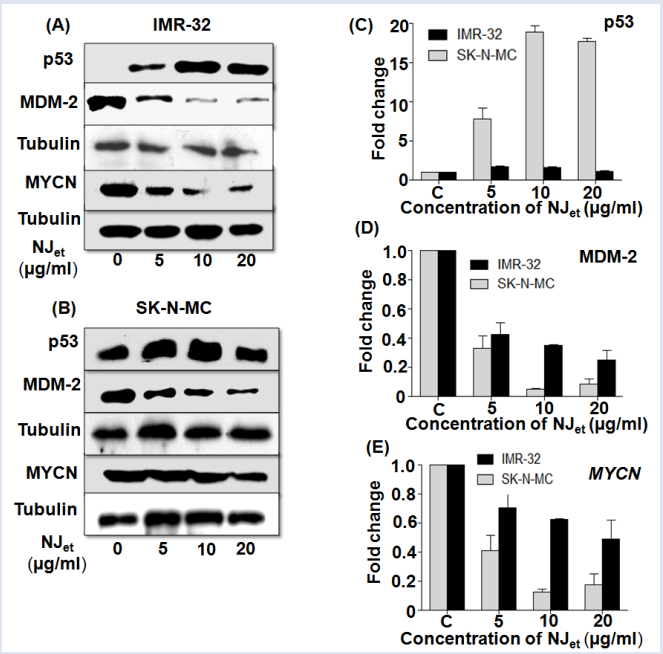


Figure 4: Effect of NJ_{et} on the expression of tumor regulatory markers. (A-E) IMR-32 and SK-N-MC cells were analyzed for the expression of p53, MDM2 and MYCN proteins by western blotting. Tubulin was used as a loading control.

CONCLUSION

The present study has evaluated the mechanism of anticancer activity of NJ_{et} in IMR-32 and SK-N-MC neuroblastoma cell lines. However, further in-depth studies are warranted against other neurablastoma cell lines as well as *in vivo* studies need to be done to establish the chemopreventive potential of *N. jatamansi*.

Acknowledgement

The authors thank Director of IRSHA for his generous support and encouragement. The authors would like to acknowledge Dr. Anand Zanwar, Department of Food and Nutrition Lab, for helping in HPTLC profiling of the extract.

Financial support and sponsorship

This work was supported by funding from the Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Raina AP, Negi KS. Essential oil composition of *Valerianajatamansi* jones from himalayan regions of India. Indian J Pharm Sci 2015;77:218-22.
2. Monga AK, Kumar S. A phytopharmacological review on Jatamansi. Pharm Res 2013;9:21-32.
3. Singh RK, Vaishali Panda SK, Murthy PN, Panigrahi G, Sharma PK, Gupta RK. Evaluation of anti-inflammatory potential of *Nardostachys jatamansi* rhizome in experimental rodents. Jcoastlife med 2014;2:38-43.
4. Chaudhary S, Chandrashekar KS, Pai KS, Setty MM, Devkar RA, Reddy ND, *et al.* Evaluation of antioxidant and anticancer activity of extract and fractions of *Nardostachys jatamansi* DC in breast carcinoma. BMC Complement Altern Med 2015;15:50.
5. Bengal Das MP, Banerjee A Anu. Comparative study on *In vitro* antibacterial and antifungal properties of five medicinal plants of west. Asian J Plant Sci 2013;3:107-11.
6. Bhagat M, Pandita RM, Saxena AK. *In vitro* and *In vivo* Biological Activities of *Nardostachys jatamansi* roots. MAP 2013;2:6.
7. Ali S, Ansari KA, Jafry MA, Kabeer H, Diwakar G. *Nardostachys jatamansi* protects against liver damage induced by thioacetamide in rats. J Ethnopharmacol 2000;71:359-63.
8. Choudhari AS, Suryavanshi SA, Kaul-Ghanekar R. The aqueous extract of *Ficusreligiosa* induces cell cycle arrest in human cervical cancer cell lines SiHa (HPV-16 Positive) and apoptosis in HeLa (HPV-18 positive). PLoS One 2013;8:e70127.
9. Koppikar SJ, Choudhari AS, Suryavanshi SA, Kumari S, Chattopadhyay S, Kaul-Ghanekar R. Aqueous cinnamon extract (ACE-c) from the bark of *Cinnamomum cassia* causes apoptosis in human cervical cancer cell line (SiHa) through loss of mitochondrial membrane potential. BMC Cancer 2010;10:210.
10. Lü L, Wai MS, Yew DT, Mak YT. PienTze Huang, a composite Chinese traditional herbal extract, affects survival of neuroblastoma cells. Int. J Neurosci 2009;119:255-62.
11. Sharma V, Hussain S, Bakshi M, Bhat N, Saxena AK. *In vitro* cytotoxic activity of leaves extracts of *Holarrhena antidysenterica* against some human cancer cell lines. Indian J Biochem Biophys 2015;1:46-51.
12. Sundaram S, Verma SK, Dwivedi PR. *In vitro* cytotoxic activity of Indian medicinal plants used traditionally to treat cancer. Asian J Pharm Clin Res 2011;4:27-9.
13. Slack A, Chen Z, Tonelli R, Pule M, Hunt L, Pession A, *et al.* The p53 regulatory gene MDM2 is a direct transcriptional target of MYCN in neuroblastoma. Proc Natl Acad Sci 2005;102:731-6.
14. Chen Z, Lin Y, Barbieri E, Burlingame S, Hicks J, Ludwig A, *et al.* Mdm2 deficiency suppresses MYCN-driven neuroblastoma tumorigenesis *in vivo*. Neoplasia 2009;11:753-62.
15. He J1, Gu L, Zhang H, Zhou M. Crosstalk between MYCN and MDM2-p53 signal pathways regulates tumor cell growth and apoptosis in neuroblastoma. Cell Cycle 2011;10:2994-3002.
16. Carol H, Reynolds CP, Kang MH, Keir ST, Maris JM, Gorlick R, *et al.* Initial testing of the MDM2 inhibitor RG7112 by the Pediatric Preclinical Testing Program. Pediatr Blood Cancer 2013;60:633-41.