Inhibition of mouse embryonic stem cell proliferation and induction of differentiation by natural products isolated from *Rhazya stricta* Decne

Tanveer Ahmed[1], Zunaira Raiz[2], Laila Jafri[3], Rashid Mahmood[1], Ihsan ul Haq[4], Leng Chee Chang[5] and Bushra Mirza[1*]

[1]Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan
[2]Institutes of Biochemistry and Biotechnology University of the Punjab, Lahore, Pakistan
[3]Department of life sciences, Abasyn University, Islamabad Campus, Islamabad, Pakistan
[4]Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan
[5]Department of Pharmaceutical Sciences, Daniel K. Inouye College of Pharmacy, University of Hawaii at Hilo, Hilo, HI, US

Abstract: Embryonic stem cells provide an ideal system to study various therapies for serious human diseases such as juvenile diabetes, neurodegenerative diseases, heart diseases and cancer. Synthetic or natural compounds that affect cell proliferation and/or differentiation of embryonic stem cells are of great value. Focus of the current project was upon the isolation and evaluation of natural components from a medicinal plant; *Rhazya stricta* on proliferation/ differentiation potential of embryonic stem cells. For this purpose, after a series of fractionation and purification steps, 7 compounds named as RS1-RS7 were isolated from aerial parts of the plant. The effects of these compounds were evaluated on the morphology and rate of cell proliferation of mouse naive embryonic stem cells. Only RS7 inhibited the proliferation of cell and reduced the induction of differentiation of cell. The qPCR analysis confirmed that the expression of the selected pluripotency markers (Oct4, Nanog and Sox2) was down regulated by RS7 treatment as compared to control. Furthermore, upon withdraw of Leukemia inhibitory factor (lif) from medium; effect of RS7 to promote differentiation was enhanced. Through structure elucidation studies, RS7 was found to be ursolic acid. This study first time shows the effect of natural compounds of *Rhazya stricta* Decne. on mouse embryonic stem cells.

Keywords: Embryonic stem cell, natural products, *Rhazya stricta,* ursolic acid

INTRODUCTION

Embryonic stem cells are the pluripotent stem cells (PSCs) established in the short window of early mammalian development. The PSCs can produce any cell type excluding extra embryonic tissues. The successful establishment of mouse ESCs from blastocysts inner cell mass (ICM) was a breakthrough in the early 1980s (Evans, Kaufman, 1981). They express pluripotency markers like Sox2 SRY (Sex Determining Region Y) -box (Sox), Nanog, Rex1 (reduced expression 1) and Oct4 (Octamer-Binding Transcription Factor 4), (Loh *et al.*, 2006; Nichols, Smith, 2009; Ng, Surani, 2011). The transcription factors, Nanog, Oct4 and Sox2, are among the pluripotency-associated network that play key role for the undifferentiated state of ESCs, cell proliferation and cell survival by repressing the genes which stimulate differentiation (Loh, Lim, 2011; Loh *et al.*, 2006; Ng, Surani, 2011; Masui *et al.*, 2007; Boiani, Scholer, 2005). The special features of self-renewal and differentiation of ESCs have been used in various fields' i.e. clinical applications, specifically, in regenerative medicine, the tissue engineering and cell therapy (Liras, 2010). Furthermore, as these cells can be maintained *in vitro* in the Petri dish and cultivated for an unlimited period of time in the mESCs medium, they are used as model to study early development, drug screening and disease modeling *in vitro*.

During cell culture studies, Leukemia inhibitory factor (Lif) has been identified to be important for maintenance of mouse ESCs by regulating signaling mainly by the signal transducers and activator of transcription factor 3 (Stat3) pathway (Nichols, Smith, 2009). On removal of the lif from medium, cell undergoes through spontaneous differentiation. These cells have been used in research to determine the toxicity and efficiency of new lead compounds and to repair the damaged cells (Avasthi *et al.*, 2008).

Medicinal plants are known to be the source of a large number of active compounds of synthetic drugs (Harlev *et al.*, 2012). Hence a vast majority of today's popμlation is dependent on plants for their healthcare (Inoue *et al.,* 2015). Extensive studies have shown highly active nature of the plant secondary metabolites like as flavonoids, lignans and glycosides (Zhang *et al.*, 2009). Screening of natural compounds has resulted in isolation of several bioactive molecules from medicinal plants including lignin (Kim *et al.*, 2014) naringin (Rhizome drynariae) (Liu *et al.*, 2007) and garcinol (Garcinia indica) (Kim *et al.*, 2008) that affect stem cell proliferation and differentiation.

**Corresponding author:* e-mail: bushramirza@qau.ed.pk

Rhazya stricta (family Apocynaceae) is a small evergreen shrub (Akhgari, 2015). It is commonly used to prepare traditional herbal drugs for various diseases in South Asia and Middle East (Gilani *et al*., 2007; Baeshin *et al*., 2008). Its fruit has been shown to have antimicrobial and enzyme inhibition activities (Sultana and Khalid *et al*., 2010). Alagrafi *et al*., (2017) reported that the stem extract of *R. stricta* can induce cell differentiation in carcinoma cell lines (NT2). The compounds that can affect the pluripotency and induce differentiation are of great therapeutic interest. Keeping in view this and the various pharmacological activities of *R. stricta*, we selected this plant for fractionation, purification and isolation of bioactive compounds. Furthermore, the isolated compounds were screened on the mouse embryonic stem cells (mESCs) for their ability to check their effects on the cell proliferation and differentiation.

MATERIAL AND METHODS

Plant collection

Rhazya stricta Decne (family Apocynaceae) plant's aerial part was collected from Karak, Pakistan. It was identified by the taxonomists of herbarium of Quaid-i-Azam University Islamabad and a voucher specimen numbering PHM-523 was deposited in Herbarium of Medicinal Plants of Pakistan, Quaid-i-Azam University, Islamabad 45320, Pakistan.

Extraction, fractionation and isolation

Leaves and stem of the plant were cleaned, dried and crushed to yield 8 kg of plant material (RS). Crude plant extract (650g) was prepared by maceration in 24L of methanol and ammoniacal chloroform (1:1) for 7 days at room temperature. After concentrating the filtrate with a rotary evaporator (40°C), fractionation was performed by acid-base method. Crude extract was suspended in 2600 mL of distilled water and NH_4OH was added to obtain basic pH and shook continually until completely mixed together. Then equal volume of chloroform was added. Then upper aqueous layer was separated by using separating funnel and was termed as aqueous layer (RS-AQ (100g)), while to the remaining chloroform layer equal volume of acidified distl.H_2O was added. This time layer of chloroform was separated and was labeled as RS-CLF (400g). To the remaining aqueous layer equal volume of chloroform was added and lower ammoniacal-chloroform layer was separate (RS-ALK (4.5g)) and lower dark layer was left behind (RS-AQ (50g)). All these fractions were concentrated at 35°C and kept at -20°C for further processing.

Solid phase extraction

RS-CLF was selected for further fractionation. A total of 215 subfractions (RS-CLF1- RS-CLF215) were collected. The fraction with similar Rf values on TLC were combined to yield 21 (RS-A to RS-U) subfractions.

Isolation and purification of RS1-RS7 compounds:

The scheme of purification and isolation of seven pure compounds i.e. RS1, RS2, RS3, RS4, RS5, RS6 and RS7 by a series of normal and reversed-phase low pressure liquid column chromatography is given below in fig. 1.

Embryonic stem cell culture

Mouse E14 embryonic stem cells were maintained on feeder cells in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% serum (biowest), 1% GlutaMax (Gibco), 1% Non-essential amino acids, 1% sodium pyruvate (Corning), 50 unites/ml Penicillin and Streptomycin (HyClone), 0.1mM β. Mercaptoethanol (Sigma) and 100 unites/ml lif (Millipore) at 37^oC in 5% CO_2 incubator. Briefly, cells were digested with 0.25% trypsin (Gibco) in the 5 % CO_2 incubator for 3-5 minutes. 5-10ml fresh medium was added and centrifuged at 200 g for 3 minutes. Cells were counted by using cell counter (Count Star 1C1000) and the morphology of the cells was studied under microscope (ZEISS, microscope 3831001069). In total 50,000 cells were seeded in one well of 6 well plate and passaged after every 3-4 days. The medium was replaced with fresh medium daily.

Effect of compounds on embryonic stem cells proliferation and differentiation

The stock solution of pure compounds was prepared by dissolving 50mg/mL in Dimethyl sulfoxide (DMSO). Different concentrations (12.5, 25 and 50μg/mL) were used to access the effects of compounds on mESCs. 0.1% of DMSO was used as a control. The effect of RS7 was further studied at concentrations 15, 18, and 20μg/mL.

RNA extraction and RT-PCR analysis

For qPCR analysis, the cells were cultured on gelatin coated plate in the presences and absences of treatment and samples were collected on third day of treatment. Growing cells were washed with DPBS twice. 500μL of RNAzol@RT reagent was added and mixed. 200μL of RNase free H_2O was added, and samples were incubated for 15 minutes on ice. Centrifugation was done at 12,000 RPM for 15 minutes at 4^oC. The supernatant was taken in EP tube and an equal volume of isopropanol was added and incubated for 15 minutes on ice. Centrifugation was done at 12,000 rpm for 10 minutes at 4^oC. The supernatant was removed, and palate washed twice with 75% ethanol. Centrifugation was done at 4,000 rpm for 3 minutes. The supernatant was removed, and palate was left to air dry for 20 minutes. H_2O was added according to the quantity of palate and samples were incubated at 65^oC for 10 minutes and concentration was measured by using Nano-drop (TMPLEN N60). 2μg of RNA was taken and 0.5μl of oligo dT primer was added and volume was raised with RNase free H_2O up to 13μl. For RT-PCR, 4μl of 5x RT buffer, 2μL of dNTP, 0.4μL of RT Ace and 0.6μL of RRI were mixed and 7μL was added and loaded into PCR machine. The program was set as, 42^oC for 65 minutes, 95°C for 5 minutes, and hold at 16°C. Both forward primer (F.P) and reverse primers (R.P) were diluted with RNase free water up to 100µM. 2.5µl F.P and 2.5µl R.P were taken and 95µl ddH_2O was added 2µL of primers from primer mixture one was taken and 10µL of SYBR green (Roch) was added. 1µl of 50 times diluted cDNA was taken and 49µL of ddH_2O was added. 8µl of cDNA was taken and 12µL of primer mixture stage 2 was added. Samples were mixed and centrifuged. Samples were loaded into 7500 Real Time-PCR machine. After completion of RT-PCR, data were analyzed.

STATISTICAL ANALYSIS

Values obtained from experiments were expressed as mean S.E.M. and further analyzed using one-way ANOVA with Tukey's test for comparison and statistical significance was accepted for p values ≤ 0.05. Analyses were performed using GraphPad Prism software version 5.01.

RESULTS

Effect of seven compounds on proliferation and induction of the differentiation in the mESCs

The natural products isolated from *Rhazya Stricta* plant were dissolved in DMSO and were tested for their anti-proliferative ability on the mouse embryonic stem cells (mESCs). The cells were treated for 3 days continuously. Cell viability analysis showed that compounds RS1-RS6 did not have any significant effect on the morphology of mESCs (fig. 2A) and hence, have not shown any promising cytotoxic effect on mESCc as compared to control cells (fig. 2B). However, RS7 showed the highest cytotoxic inhibitory effect on proliferation of mESCs at 50 and 25µg/mL concentration (fig. 2B). Consequently, the active compound RS7 was further tested at lower concentrations i.e. 20, 18 and 15µg/mL concentration to testify its potential to inhibit the proliferation of mESCs. The result showed that R7 was cytotoxic at 20 and 18 µg/mL concentration but not at 15µg/mL dose as compare to control (fig. 2C&D). The above-mentioned results reveal that RS7 induced the cell death significantly at higher doses as compared to lower dose. Moreover, along with cell proliferation, the morphology of cell colonies changed from dome to flat shape and more spikes were observed on the edges of colonies under microscope. These results suggested that the RS7 induces differentiation in mESCs.

Structure elucidation of the compound RS7

Total seven compounds (RS1-RS7) were isolated from *R. stricta* and were investigated for their anti-proliferative potential against mESCs and for their ability to induce differentiation. Above mentioned results have shown that only RS7 was found active. Therefore, further structural elucidation was carried out to identify the RS7 compound. Mass spectrometry and NMR spectroscopy (Bruker AVANCE 400 MHz NMR) were used to identify the bioactive compound (RS7). The structure of the compound was confirmed by comparing its chemical and spectroscopic properties as reported earlier. A brief description is given below.

The RS7 (fig. 3) has chemical formula $C_{30}H_{48}O_3$, melting point 283-285°C and molecular weight 456.7 g/mol. Its UV spectrum shows maximum absorption at 474, 442, and 422 nm and the IR (ATR, cm-1) spectrum at 3562 (hydroxyl group), 2865 (C=C) and 1698 (C=O) [5], [6]. While a high resolution electrospray mass spectra in the positive ion mode showed *m/z* 455.3522 [M+H]+ (Gnoatta *et al*., 2008).

The results of the ^{13}C NMR spectrum of RS7 (able 2) along with that of ^{1}H-, DEPT, COSY and HSQC depicted that RS7 is Ursolic cid (UA) which is a pentacyclic triterpene (Gnoatta *et al*., 2008).

Ursolic acid inhibit the mESCs cell proliferation and induces the differentiation

The active compound i.e. Ursolic acid (UA), was further studied for its ability to trigger the differentiation of mESCs by detecting the expression level of Oct4, Sox2, and Nanog, pluripotency markers of mESCs after treatment at two different concentrations (15 and 18 µM/mL).

Ursolic acid was found to possess strong differentiation-inducing activity. The qPCR analysis depicted that UA treatment decreased the expression of Oct4, Nanog and Sox2 in mESCs in a concentration-dependent manner as compared to control (fig. 4).

Ursolic acid promotes the differentiation of E14 mESCs

The mESCs are maintained in serum plus lif based medium in naïve condition. Upon withdraw of lif, mESCs spontaneously undergo through differentiation on gelatin. We hypothesized that if UA induces the differentiation then this compound must promote the spontaneous differentiation of mESCs. The cells maintained on feeders in serum plus lif medium were taken and cultured on gelatin after withdraw of lif for 3 days. The morphology of cells in minus lif indicates that upon withdraw of lif cells undergo through spontaneous differentiations. The results have shown that in Ursolic acid treated cells, the colonies became flatter as compared to untreated upon withdrawal of lif. It suggested that the UA promotes the differentiation of mESCs upon lif removal. To further confirm these results, cells were lysed for RNA and qPCR analysis was done to assess the expression of pluripotency markers. The Oct4, Nanog, and Sox2 expression level dropped more quickly than control suggesting that the UA promotes differentiation of mESCs (fig. 5A&B).

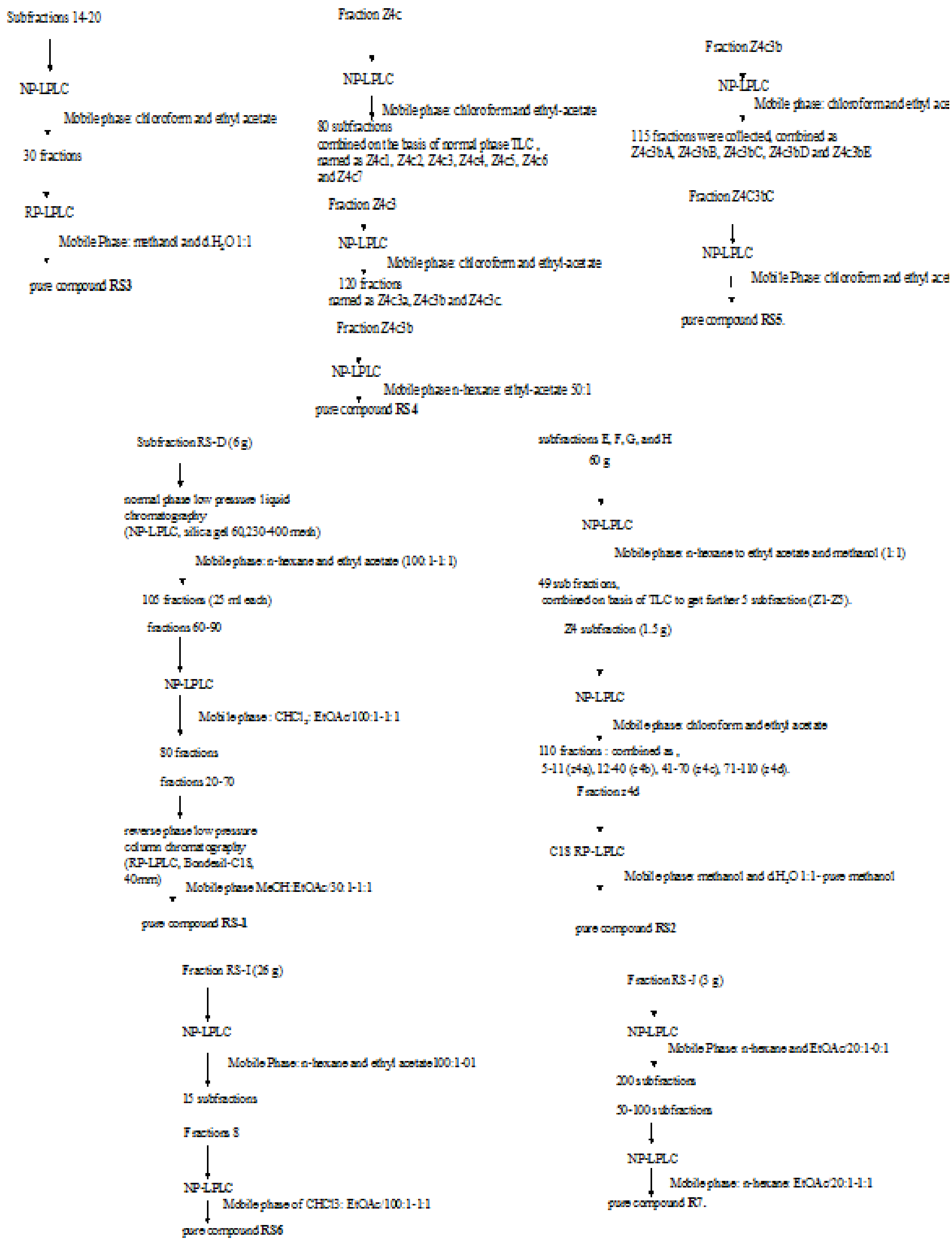

Fig. 1: The scheme of purification and isolation of seven compounds

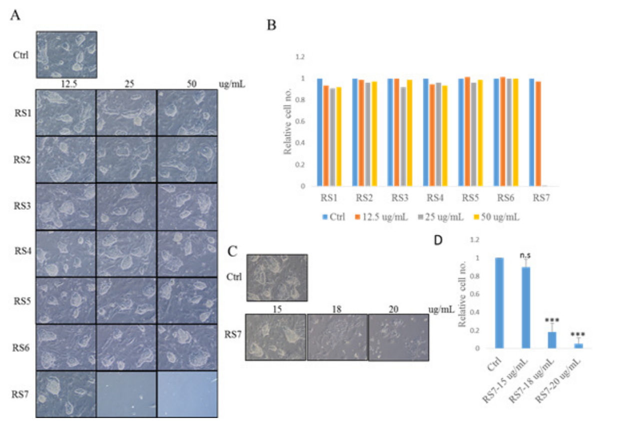

Fig. 2: Screening of compounds on mouse naïve embryonic stem cells. (A) Cell morphology after treatment with RS1-RS7 at doses 12.5, 25 and 50µg/mL. (B) The cell proliferation rate after treatment with RS1-RS7 compared to control. (C) Cell morphology of mESCs treated with RS7 corresponding to doses 15, 18 and 20µg/mL. (D) Cell proliferation rate after treatment with RS7 compared to control. Mean ± s.e.m. from n=3 independent experiments are shown *P<0.05, **P <0.01, ***P <0.001.

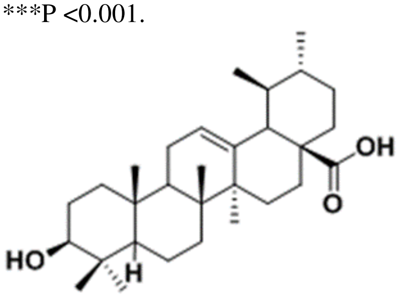

Fig. 3: Ursolic Acid (UA)

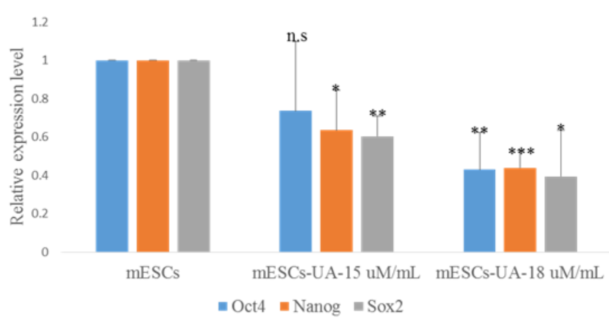

Fig. 4: Ursolic acid induces the differentiation in mESCs.

DISCUSSION

Natural products are used for the treatment of various ailments due to their less injurious effect on human as compared to synthetic drugs. Medicinal plants have been used throughout the world to cure different diseases including infections, diabetes, cancer, swelling, heart diseases etc (Awal *et al*., 2004; Jiang *et al*., 2006). To date, numerous groups of plants are used in herbal remedies in aboriginal structure of medicines, where the entire plant or its part or extract is utilized (Hamayun *et al.,* 2003). Now a day 80% of world's population depends on plant derived drugs to control diseases and people are realizing their benefits as it is reported that natural products have least side effects (Doughari, 2006).

Conventional cancer treatment requires chemotherapy and /or radiotherapy involving killing of cancer cells (Della *et al*., 2015; Dhermain 2014). Recently it is suggested that cancer cells can instead be transformed into normal cells by what is called differentiation therapy. Differentiation therapy works by reactivating endogenous differentiation programs of cancer cells so that they resume maturation process. The drugs which reactivate the differentiation program are believed to have less toxic effects than conventional cancer treatments. Now a day, it is therefore, desirable to discover more efficient natural products with the ability of induction of differentiation, which would create a new alternative for prevention and treatment of cancer disease. Keeping this in view, the present research work was designed to isolate active natural compounds from *R. stricta* having potential to inhibit the proliferation of mESCs and to induce differentiation in mESCs.

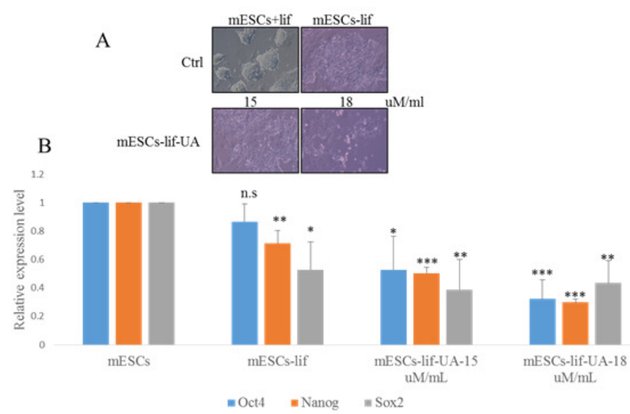

Fig. 5: Ursolic acid (UA) promotes the mESCs differentiation upon withdrawal of lif. (A) Cell images represents the cell morphology upon with draw of lif (B) q-PCR anylysis of pluripotency genes.

In our study, we isolated seven compounds from *R. stricta* using different column chromatography techniques. It has been reported that the compounds isolated from *R. stricta* induce apoptosis in cancer cells. It has also been reported that the phytochemicals of *R. stricta* areal part have potential to inhibit cell proliferation as well as to induce differentiation of embryonal carcinoma cells (Alagrafi, 2017). Among these seven compounds, only UA showed the blockade of proliferation and induction of differentiation in E14 cell line. E14 is a mouse naïve embryonic stem cell line. The transcription factors such as Oct4, Sox2, and Nanog, are known to be involved in cell differentiation. Down regulation of these genes results in initiating cell differentiation (Liu *et al*., 2007). In line with previous studies (Liu *et al*., 2007; Alagrafi, 2017), our results showed that the treated cells in this study had significantly lower level of gene expression of Nanog, Oct4 and Sox2 marker genes. Furthermore, as lif regulates the Stat3 pathway in mESCs and it is critical for the proliferation and maintenance of pluripotency, our results suggest that Ursolic acid might inhibit Stat3 pathway.

CONCLUSION

In accordance with our study ursolic acid isolated from *R. stricta* induces cell differentiation in mouse embryonic stem cells (mESCs, *in vitro* model). All the pluripotency markers, Sox2, Oct4 and Nanog were down regulated after treatment. Hence, Ursolic acid may be effective as a differentiating agent in treatment of cancer. Promising compound isolated from *R. stricta* can be further studied to develop into an effective cancer drug with minimum side effects.

Table 1: List of the primers used in this study

Primer	Sequence
m-Oct4-F	TAGGTGAGCCGTCTTTCCAC
m-Oct4-R	GCTTAGCCAGGTTCGAGGAT
m-Sox2-F	AGGGCTGGGAGAAAGAAGAG
m-Sox2-R	CCGCGATTGTTGTGATTAGT
m-Nanog-F	CTCAAGTCCTGAGGCTGACA
m-Nanog-R	TGAAACCTGTCCTTGAGTGC
m-Actin-F	TCCACCTTCCAGCAGATGTG
m-Actin-R	GCATTTGCGGTGGACGAT

Table 2: 1H – NMR and 13C – NMR chemical shifts for RS7 in solvent

Position	δ ^{13}C (ppm)	DEPT	δ ^{1} H (ppm)
1	38.9	CH_2	
2	28.1	CH_2	
3	78.8	CH	3.43 (1H, br *s*)
4	38.7	C	
5	55.3	CH	
6	18.8	CH_2	
7	33.6	CH_2	
8	40.0	C	
9	48.3	CH	
10	37.4	C	
11	23.6	CH_2	
12	125.6	CH	5.50 (1H, br *s*)
13	139.7	C	
14	42.5	C	
15	28.7	CH_2	
16	24.9	CH_2	
17	48.0	C	
18	53.5	CH	2.52 (1H, d, *J*= 11.0 Hz)
19	39.5	CH	
20	39.1	CH	
21	31.1	CH_2	
22	37.3	CH_2	
23	28.8	CH_3	1.24 (*s*)
24	15.7	CH_3	1.02 (*s*)
25	16.6	CH_3	0.93 (*s*)
26	17.4	CH_3	1.05 (s)
27	23.8	CH_3	1.22 (*s*)
28	180.0	C	
29	17.5	CH_3	0.97 (s)
30	21.4	CH_3	0.99 (d, *J* = 6.1 Hz)

ACKNOWLEDGMENTS

The authors thank Qin Baoming, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou China for providing the cell line and support to conduct this study.

REFERENCES

Alagrafi FS, Alawad AO, Abutaha NM, Nasr FA, Alhazzaa OA and Alharbi SN *et al* (2017). *In vitro* induction of human embryonal carcinoma differentiation by a crude extract of Rhazya stricta. *BMC Complement Altern. Med.,* **17**: 342.

Avasthi S, Srivastava R, Singh A and Srivastava M (2008). Stem cell: Past, present and future: A review article. *Internet J. Medical Update,* **3**: 22-30.

Baeshin NA, Qari SH, Sabir JSM, Qari SH and Alhejin AM (2008). Biochemical and molecular evaluation of genetic effects of *Rhazya stricta* (Decne) leafs extract on Aspergillus terreus. *Saudi J. Biol. Sci.,* **15**(1): 25-33.

Boiani M and Scholer HR (2005). Regulatory networks in embryo-derived pluripotent stem cells. Nature reviews. *Mol. Cell Bio.,* **6**: 872-884.

Della pepa C, Tonini G, Pisano C, Di napoli M, Cecere SC and Tambaro R *et al* (2015). Ovarian cancer standard of care: Are there real alternatives? *Chin. J. Cancer.,* **34**(1): 17-27.

Dhermain F (2014). Radiotherapy of high-grade gliomas: current standards and new concepts, innovations in imaging and radiotherapy and new therapeutic approaches. *Chin J. Cancer.,* **33**(1): 16-24.

Evans MJ and Kaufman MH (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature,* **292**: 154-156.

Gnoatto SCB, Dassonville-Klimpt A, Da Nascimento S, Galera P, Boumediene K and Gosman G *et al* (2008). Evaluation of Ursolic acid isolated from *Ilex paraguariensis* and derivatives of aromatase inhibition. *European J. Med. Chem.,* **43**: 1865-1877.

Gilani SA, Kikuchi A, Shinwari ZK, Khattak ZI and Watanabe KN (2007). Phytochemical, pharmacological and ethnobotanical studies of Rhazya stricta Decne. *Phytother. Res*., **21**(4): 301-307.

Harlev E, Nevo E, Lansky EP, Lansky S and Bishayee A (2012). Anticancer attributes of desert plants. *Anti-Cancer Drugs*, **23**: 255-71.

Inoue Y, Hasegawa S, Yamada T, Date Y, Mizutani H and Nakata S *et al* (2013). Lignin induces ES cells to differentiate into neuroectodermal cells throµgh mediation of the Wnt signaling pathway. *PLoS One*., **8**(6): e66376.

Kim SJ, Son TG, Park HR, Park M, Kim MS and Kim HS *et al* (2008). Curcumin stimulates proliferation of embryonic neural progenitor cells and neurogenesis in the adult hippocampus. *J. Biol. Chem.,* **283**(21): 14497-14505.

Kim YY, Ku JB, Liu HC, Ku SY, Kim SH and Choi YM (2014). Ginsenosides may enhance the functionality of human embryonic stem cell-derived cardiomyocytes in vitro. *Reprod. Sci.,* **21**(10): 1312-1318.

Liras A (2010). Future research and therapeutic applications of human stem cells: general, regulatory, and bioethical aspects. *J. Transl. Med.,* doi: 10.1186/1479-5876-8-131

Liu JW, Tian SJ, De barry J and Luu B (2007). Panaxadiol glycosides that induce neuronal differentiation in neurosphere stem cells. *J. Nat. Prod.,* **70**(8): 1329-1334.

Liu N, Lu M, Tian X and Han Z (2007). Molecular mechanisms involved in self-renewal and pluripotency of embryonic stem cells. *J. Cell Physiol.,* **211**: 279-286.

Loh KM and Lim B (2011). A precarious balance: pluripotency factors as lineage specifiers. *Cell Stem Cell,* **8**: 363-369.

Loh YH, Wu Q, Chew JL, Vega VB, Zhang W and Chen X *et al* (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat., Genetics*, **38**: 431-440.

Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R and Takahashi K *et al*. (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat. Cell Bio.,* **9**: 625-635.

Ng HH and Surani MA (2011). The transcriptional and signalling networks of pluripotency. *Nat. Cell Bio.,* **13**: 490-496.

Nichols J, Smith A (2009). Naive and primed pluripotent states. *Cell Stem Cell*, **4**: 487-492.

Sultana N and Khalid A (2010). Phytochemical and enzyme inhibitory studies on indigenous medicinal plant *Rhazya stricta. Nat. Prod. Res.,* **24**(4): 305-314.

Zhang P, Dai KR, Yan SG, Yan WQ, Zhang C and Chen DQ *et al* (2009). Effects of naringin on the proliferation and osteogenic differentiation of human bone mesenchymal stem cell. *Eur. J. Pharmacol.,* **607**: 1-5.