Exp Oncol 2011
33, 4, 212–215

Experimental ONCOLOGY

NUTRIENT MIXTURE INHIBITS *IN VITRO* AND *IN VIVO* GROWTH OF HUMAN ACUTE PROMYELOCYTIC LEUKEMIA HL-60 CELLS

*M.W. Roomi, N.W. Roomi, B. Bhanap, M. Rath, A. Niedzwiecki**
Dr. Rath Research Institute, Cancer Division, Santa Clara, CA-95050, USA

Aim: **Untreated acute promyelocytic leukemia is the most malignant form of acute leukemias, with median survival of less than one month. We investigated *in vitro* and *in vivo* synergistic effects of a nutrient mixture (NM) containing ascorbic acid, lysine, proline, and green tea extract, on acute promyelocytic leukemia HL-60 cells.** ***Methods*****: *In vitro*, the HL-60 cells were cultured and exposed to NM at doses 0–1000 μg/ml. Cell viability was assessed by Trypan blue dye exclusion test, matrix metalloproteinases (MMP) expression by gelatinase zymography, invasion through Matrigel and apoptosis by live green Poly Caspase Detection Kit. *In vivo* studies were carried out in athymic nude mice subcutaneously inoculated with HL-60 cells. *Results: In vitro*, NM exhibited a dose dependent reduction in cells viability. Zymography revealed matrix MMP-2 and phorbol 12-myristate 13-acetate induced MMP-9 expression. NM inhibited expression of both MMP in a dose dependent manner. Similar step-wise reduction in the Matrigel invasion by HL-60 cells was also observed by this combination with incremental doses. Gradually increasing doses of NM induced significant apoptosis in HL-60 cells. *In vivo*, NM inhibited tumor growth by 50%. *Conclusion:* The results indicate that NM significantly suppresses tumor growth, decreases cell viability, inhibits MMP expression, Matrigel invasion and induces apoptosis in HL-60 cells.**
Key Words**: HL-60, APL, tumor growth, MMPs, Matrigel invasion.**

Acute promyelocytic leukemia (APL) is a unique subtype of acute myelogenous leukemia (AML) with distinct cytogenetic, clinical features and biological characteristics including abnormal accumulation of promyelocytes. APL is the most malignant form of AML and without treatment has median survival of less than a month [1, 2].

In our previous studies we have used a combination of nutrients (NM) containing several bioactive agents, such as ascorbic acid, lysine, proline and green tea extract, combined with other supporting nutrients on multiple cancer cell lines. The nutrient mixture has exhibited synergistic anticancer activity *in vivo*, *in vitro* in a number of cancer cell lines through inhibition of cancer cell growth, MMP expression, invasion, angiogenesis and metastasis [3–5].

Considering the efficacy of NM on other cancer cell lines, we aimed to analyze if NM had any effects on HL-60 cells, a cell line which was originally obtained from an APL patient. The current study is designed to examine the *in vitro* effect of NM on viability of HL-60 cells, MMP expression, Matrigel invasion and apoptosis. In addition, we also studied effects of NM *in vivo,* on growth of HL-60 cell line xenografted into athymic nude mice.

MATERIALS AND METHODS:

Leukemia cell line and cell culture. APL cell line HL-60, obtained from ATCC (American Type Culture Collection, Rockville, MD), was cultured in modified Dulbecco's medium supplemented with 20% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. The media and sera used were obtained from ATCC, and penicillin, streptomycin were obtained from Gibco, BRL (Long Island, NY).

Cell proliferation study. The cells were treated with the NM, dissolved in media and tested at 0, 10, 50, 100, 500 and 1000 μg/ml in triplicate at each dose. Cell viability and cytotoxicity was assessed by Trypan blue dye exclusion test after 24 h as described in previous studies [6, 7].

Gelatinase zymography. Gelatinase zymography was used to determine the MMP activity in conditioned media. Zymography was performed in 10% Novex precast SDS-polyacrylamide gel (Invitrogen Corporation, Carlsbad, CA) in the presence of 0.1% gelatin under non-reducing conditions. Phorbol 12-myristate 13-acetate (PMA), 100 ng/ml, was added to the cells to induce MMP-9 secretion. Other steps were followed as described in previous publications [6, 7].

Invasion using Matrigel. Invasion studies were conducted as described in the prior studies [6, 7] using Matrigel™ (Becton-Dickinson, Franklin Lakes, NJ) inserts in 24-well plates.

Apoptosis. Apoptosis was determined by the method described in the live green Poly Caspase Detection Kit at different doses of NM. Cells were challenged with the NM at 0, 50, 100, 500 and 1000 μg/ml concentration and incubated for 24 h. The culture was washed with PBS and treated with caspase reagent as specified in the manufacturer's protocol (Molecular Probes Image–IT™ Live green Poly Caspase Detection Kit 135104, Invitrogen). Cells were photographed under a fluorescence microscope and counted. Green colored cells represent viable cells, while yellow orange colored cells represent early apoptosis and red color represents late apoptosis.

In vivo study. Male athymic nude mice (NCr-nu/nu), approximately 6 weeks of age, were purchased from Simonsen Laboratories, Gilroy, CA and maintained

Received: June 29, 2011.
*Correspondence: E-mail: author@drrath.com
Fax: 408-567-5030

Abbreviations used: AML – acute myelogenous leukemia; APL – acute promyelocytic leukemia; ATRA – all-trans retinoic acid; MMP – matrix metalloproteinases; NM – nutrient mixture; SD – standard deviation.

in microinsulated cages under pathogen-free conditions on a 12h light/12h dark schedule for a week. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals. After housing for a week, the mice (n = 10) were inoculated with $3x10^6$ HL-60 cells in 0.2 ml of PBS and 0.1 ml of Matrigel (BD Bioscience, Bedford, MA) in the right flank. After injection, the mice were randomly divided in two groups; group A was fed regular diet while group B was fed the regular diet supplemented with 0.5% of NM. During the study, the mice consumed, on an average, 4 g of their respective diets per day. Thus, the supplemental mice received approximately 20 mg of NM per day. After 4 weeks, the mice were sacrificed and the tumors were excised and processed for histology. Tissue samples were fixed in 10% buffered formalin, embedded in paraffin and cut at 4–5 μm. Sections were deparaffinized through xylene and graduated alcohol series to water and stained with hematoxylin and eosin (H&E) for evaluation using a standard light microscope.

Statistical analysis. Results were expressed as means ± SD for the groups. Data was analyzed by independent sample "*t*" test.

RESULTS

***In vitro* studies.**

Cell proliferation study. NM was non-toxic to HL-60 acute promyelocytic leukemia cells at 10 μg/ml and exhibited a dose response toxicity with further increase in concentrations (Fig. 1).

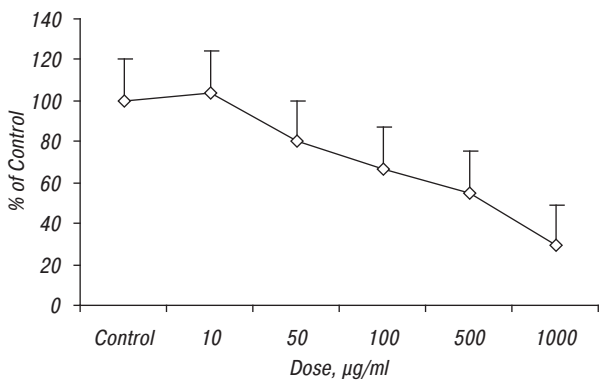

Fig. 1. Effect of NM on HL-60 viability

Gelatinase zymography. Zymography demonstrated a faint band corresponding to MMP-2 and enhanced secretion of MMP-9 by cells when treated with PMA (100 ng/ml). NM inhibited the expression of MMP-2 and MMP-9 in a dose dependent fashion, with virtually total inhibition at 1000 μg/ml as shown in Fig. 2, *a*, *c*. Densitometry analysis shows gradually decreasing MMP-2 expression with increasing concentration of NM (R^2 = 0.925) (Fig. 2, *b*) Similarly, expression of MMP-9 was inhibited in a dose dependent fashion with complete inhibition at 1000 μg/ml (R^2 =0.834) (Fig. 2, *d*).

Invasion through Matrigel. The NM significantly inhibited invasion of HL-60 cells through Matrigel. Compared with control, the NM showed increasing inhibition of invasion, starting at 100 μg/ml (18%) and 92% at 500 ug/ml. The invasion was totally blocked at 1000 μg/ml. (Fig. 3).

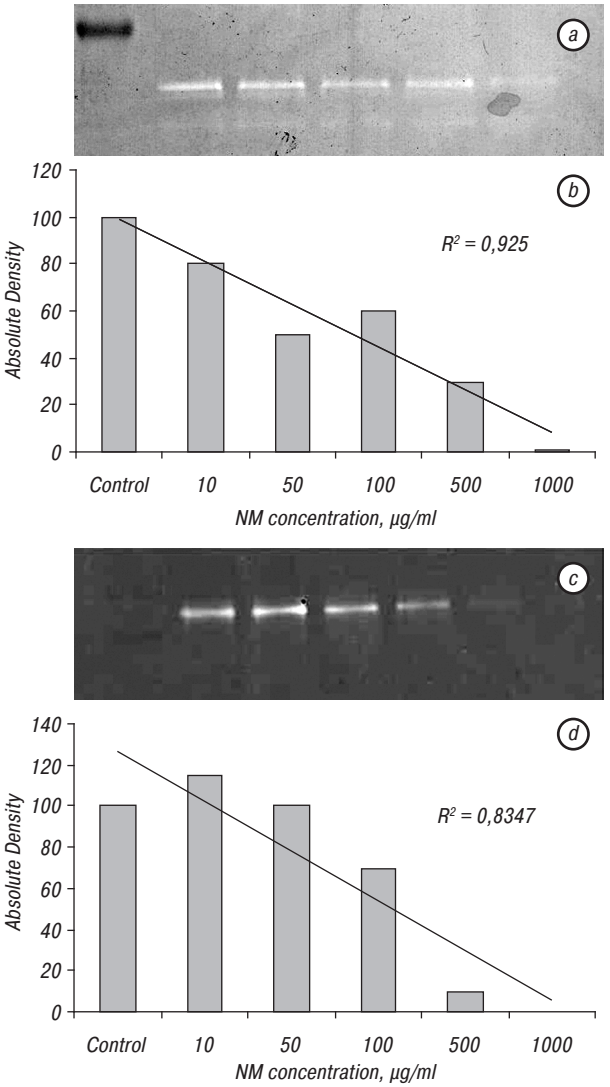

Fig. 2. Effect of NM on MMP-2 and MMP-9 expression by HL-60 cells: *a*, faint band corresponding to MMP-2 in untreated HL-60 cells; intensity of expression decreases with increasing concentrations of NM (1 — marker, 2 — control, 3–7 — NM doses 10, 50, 100, 500, 1000 μg/ml respectively); *b,* densitometry shows the effect of NM on relative activity of MMP-2 in HL-60 cells is reduced as the dose increases; *c,* effect of NM on PMA (100 ng/ml) treated HL-60 cells; NM exhibiting dose dependant decrease in MMP-9 expression (1 — marker, 2 — control, 3–7 — NM doses 10, 50, 100, 500, 1000 μg/ml respectively); *d,* densitometry shows the effect of NM on relative activity of MMP-9

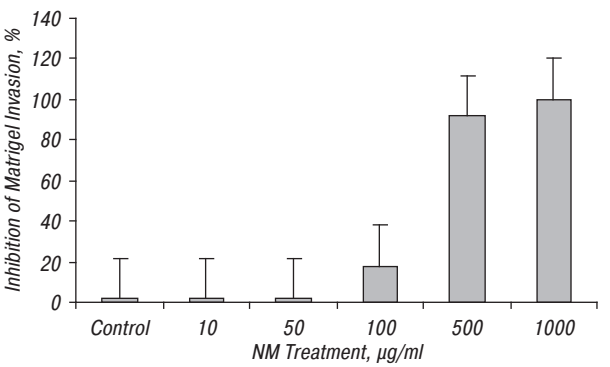

Fig. 3. Effect of NM on Matrigel invasion of HL-60 cells. Matrigel invasion significantly decreased by NM in dose dependent manner

Apoptosis (live green caspases detection kit). Dose dependent apoptosis of HL-60 cells was evident with

the NM challenge. The extent of apoptosis increased significantly with increasing doses of NM up to 1000 μg/ml. (Fig. 4, *a*, *b*). Quantitative analysis of live, early and late apoptotic cells is shown (Fig. 4, *c*). Moderate apoptosis with 64% live cells and 18% apoptotic cells was seen with exposure to 50 μg/ml NM. Percentage of dead cells gradually increased up to 58% at 1000 μml while the percentage of live cells decreased with increase in dose of NM from 65% to 29% at 1000 μg/ml.

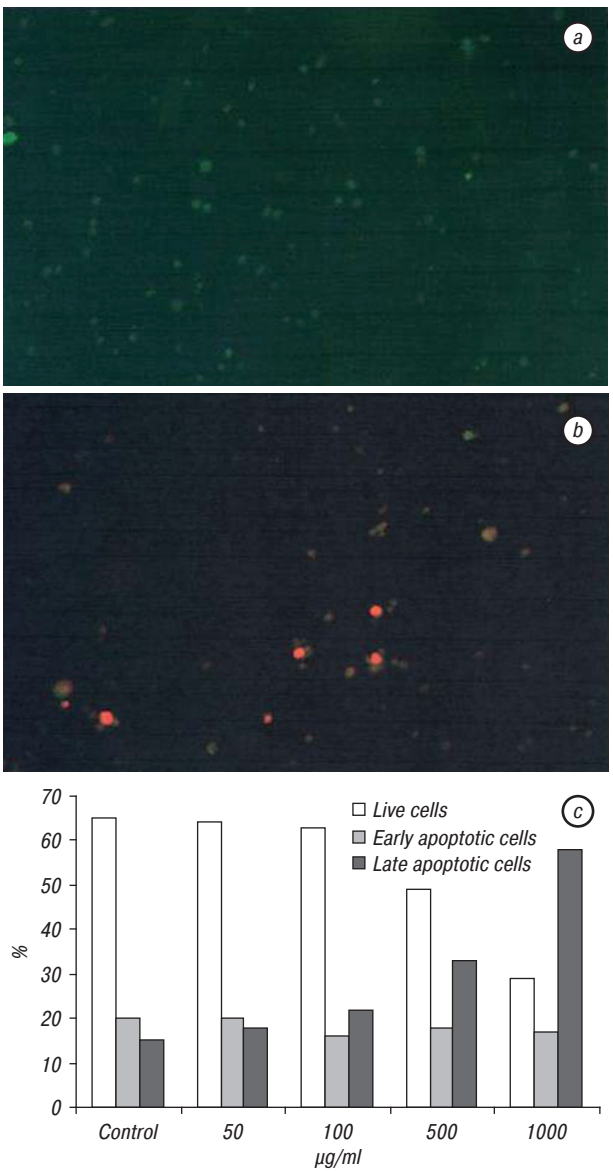

Fig. 4. Photomicrographs of apoptosis induction in HL-60 Cells by NM (Live green caspase detection kit): *a*, control; *b*, NM 1000 μg/ml; *c*, quantitative analysis of live, early and late apoptotic cells

In vivo studies. HL-60 xenografts developed in NM supplemented nude mice weighed 50% lower than those developed by the control group of mice. Nude mice from both groups showed no loss in body weight over the study period. The tumors from both groups were reported to be histologically similar. They were comprised of pleomorphic cells with abundant cytoplasm and vesicular nuclei. Abundant mitotic figures were noted. No capsule was noted. Tumor cells were forming small packets and sheets that were infiltrating within the surrounding tissues. Within control group, there were multifocal areas of necrosis that comprised less than 1% of the total area. Within the test group, there were multifocal areas of necrosis that comprised less than 10% of the tumor mass (Fig. 5).

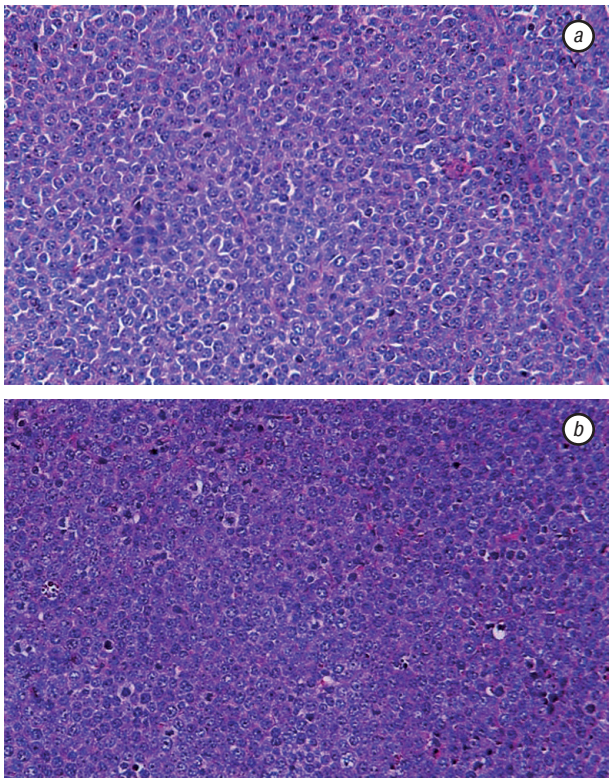

Fig. 5. Tumor histology: *a*, control group; *b*, NM supplemented group. Increased areas of necrosis seen within tumor tissue from animals in the NM supplemented group

DISCUSSION

The results of the present study clearly indicate the benefits of exposure of the NM formulated using several potential bioactive agents. *In vitro*, addition of NM resulted in decreased cell viability, inhibition of MMP expression, and invasion through Matrigel, as well as increased apoptosis in HL-60 cells. *In vivo*, the NM inhibited growth of HL-60 xenografts in athymic nude mice. The observation that the NM inhibited expression of MMP-2 and MMP-9 supports the argument that this could be one of the mechanisms by which the NM inhibits invasion of these cells into Matrigel.

Although, it is well accepted that MMPs are involved in tumor growth, angiogenesis, invasion and metastasis of solid tumors [8, 9], researchers continue to explore the role of MMPs in hematological malignancies. It has been suggested that an important aspect of favorable prognosis in myeloid leukemias is the cell capacity to store MMPs. Higher MMP storage may be linked to maturation of normal myelocytes [10]. Moreover, activation of MMP is a required initial step for malignant cells to cross the barriers within bone marrow and enter circulation before invading other tissues. Some studies indicate that myeloid leukemia patients with higher MMP-2 levels demonstrate significantly lower survival rate than the patients with lower MMP-2 levels [11]. In addition, increased level of MMP-2 activity was noted in leukemia patients with poor response to chemotherapy [12]. Similarly,

patients with lower MMP-9 levels were shown to have higher survival than otherwise [13]. Annabi *et al*. [14] have reported the specific role of epigallocatechin gallate in suppressing MMP-9 secretion and the significance of their angio-suppressive action in APL.

While researchers continue to explore synthetic compounds to target specific MMP enzymes in cancer therapies, we think that one of the most promising approaches to cancer treatment would be targeting multiple steps involved in cancer growth and progression. Thus, by encapsulating the tumor, strengthening of connective tissue, preventing angiogenesis, and inducing apoptosis; we believe that cancer progression could be arrested. Our previous research has proven that control of the proteolytic degradation of extracellular matrix using nutrient combination provides an opportunity to address these common mechanisms [15]. Many, if not all, these mechanisms could play a role in hematological malignancies as well. In this study we have used this approach to evaluate effects of NM on HL-60 cells representing APL. As results indicate, MMP-2 and MMP-9 expression and Matrigel invasion by HL-60 cells was inhibited by NM in a dose dependent fashion suggesting that the NM has significant bioactivity on HL-60 cells.

Most APL patients are treated with all-trans retinoic acid (ATRA) and arsenic trioxide in combination with chemotherapy. Although it increases the chance of favorable prognosis, it fails to achieve a complete and durable remission. ATRA resistance is known to develop in lab and clinical settings [16]. In addition, ATRA therapy is associated with its own unique and serious side effect called as retinoic syndrome or differentiation syndrome. It is characterized by pleural and pericardial effusion, edema, dyspnea and pulmonary infiltrates. And post remission therapy is crucial in ensuring disease-free survival. Despite all the measures, 20–30% of APL patients relapse and require further treatment with more arsenic oxide and stem cell transplant before palliative care is considered. Arsenic trioxide can cause dangerous arrhythmias [1, 2].

In the present study, exposure to 0.5% dietary supplement of the NM for four weeks neither affected the gain in body weight nor the food consumption by experimental mice. Further, in a previous *in vivo* study addressing the safety issues, we found that gavaging adult female ODS rats with the NM (at 30, 90, or 150 mg per day for seven days), had neither adverse effects on vital organs such as heart, liver, and kidney, nor on the associated functional serum enzymes. Therefore, indicating that the mixture is safe to use even at these higher doses, which exceed the normal equivalent doses of the nutrients [17].

The results of the present study show that supplementation with the NM formulated using multiple bioactive agents was effective by many parameters (decreased cell viability, inhibition of MMPs (2 and 9) expression, and Matrigel invasion, increased apoptosis of HL-60 cells, and 50% decrease in tumor weight in NM treated mice versus control group) and substantiate the potential of NM as a possible therapeutic combination.

ACKNOWLEDGEMENTS

Consulting pathologist Alexander DePoli (Idexx labs, Sacramento, CA) evaluated the histopathology slides. Dr. Rath Health Foundation, a nonprofit organization, provided research funding.

REFERENCES

1. Solomon S, Maklovska V. Acute Myelogenous Leukemia. Handbook of Clinical Hematology, Rodgers G.P., Young N.S. Lippicott Williams & Wilkins, USA, 2005; 135–150.

2. American Cancer Society (http://www.cancer.org/acs/groups/cid/documents/webcontent/003110-pdf.pdf)

3. Roomi MW, Ivanov V, Kalinovsky T, ***et al.*** *In vivo* and *in vitro* antitumor effect of ascorbic acid, lysine, proline, arginine, and green tea extract on human fibrosarcoma cells HT-1080; Med Oncol 2006; **23**: 105–11.

4. Roomi MW, Ivanov V, Kalinovsky T, ***et al.*** Suppression of human cervical cancer cell lines Hela and DoTc2 4510 by a mixture of lysine, proline, ascorbic acid, and green tea extract. Int J Gynecol Cancer 2006; **16**: 1241–7.

5. Roomi MW, Roomi N, Ivanov V, ***et al.*** Inhibitory effect of a mixture containing ascorbic acid, lysine, proline and green tea extract on critical parameters in angiogenesis. Oncol Rep 2005; **14**: 807–15.

6. Roomi MW, Bhanap BA, Roomi NW, ***et al***. Antineoplastic effects of nutrient mixture on Raji and Jurkat T cells: the two highly aggressive non Hodgkin's lymphoma cell lines. Exp Oncol 2009; **31**: 149–55.

7. Roomi MW, Roomi NW, Bhanap B, ***et al***. *In vivo* and *in vitro* antitumor effects of nutrient mixture in murine leukemia cell line P-388. Exp Oncol 2011; **33**: 71–7.

8. Duffy MJ. The role of proteolytic enzymes in cancer invasion and metastasis. Clin Exp Metastasis 1992; **10**: 145–55.

9. Kleiner D, Stetler-Stevenson W. Matrix metalloproteinases and metastasis. Cancer Chemother Pharmacol 1999; **43**: S42–51.

10. Kuittinen O. Matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) in hematological malignancies. 2003 ISBN: 951–42–6942-X.

11. Aref S, Osman E, Mansy S, ***et al***. Prognostic relevance of circulating matrix metalloproteinase-2 in acute myeloid leukemia patients. Hematol Oncol 2007; **25**: 121–6.

12. Song JH, Kim SH, Cho D, ***et al.*** Enhanced invasiveness of drug-resistant acute myeloid leukemia cells through increased expression of matrix metalloproteinase-2. Int J Cancer 2009; **125**: 1074–81.

13. Lin LI, Lin DT, Chang CJ, ***et al.*** Marrow matrix metalloproteinases (MMPs) and tissue inhibitors of MMP in acute leukaemia: potential role of MMP-9 as a surrogate marker to monitor leukaemic status in patients with acute myelogenous leukemia. Br J Haematol 2002; **117**: 835–41.

14. Annabi B, Currie JC, Moghrabi A, ***et al***. Inhibition of HuR and MMP-9 expression in macrophage-differentiated HL-60 myeloid leukemia cells by green tea polyphenol EGCG. Leukemia Res 2007; **31**: 1285–92.

15. Netke SP, Roomi MW, Roomi NW, ***et al.*** A specific combination of ascorbic acid, lysine, proline and epigallocatechin gallate inhibits proliferation and extracellular matrix invasion of various human cancer cell lines. Res Commun Pharmacol Toxicol Emerg Drugs 2003; **2**: 37–50.

16. Pelicano L, Brumpt C, Pitha PM, ***et al.*** Retinoic acid resistance in NB4 APL cells is associated with lack of interferon a synthesis Stat1 and p48 induction. Oncogene 1999; **18**: 3944–53.

17. Roomi MW, Ivanov V, Netke SP, ***et al***. Serum markers of the liver, heart, and kidney and lipid profile and histopathology