ISSN : 0973-3337
CODEN : IJNRAV

International Journal of Noni Research

Accent on Clinical Science and Pharmacology

Vol. 7 No. 1 January 2012

isns

International Journal of Noni Research

Volume 7 Number 1 January 2012

Accent on Clinical Science and Pharmacology

International Society for Noni Science

12, Rajiv Gandhi Road, Chennai - 600 096.

Phone : 044-4901 1111 Fax : 044-4901 1149

E-mail : E-mail: intl.sns2010@gmail.com Visit : www.isns.in

Contents

International Journal of Noni Research

Volume 7 Numbers 1 January 2012

Accent on Clinical Science and Pharmacology

CONTENTS

P. Selvam[1], K. S. Praveen[2], Hitesh Jagani[2], J. Venkata Rao[2]*

Studies on cytotoxicity of Divine Noni *(Noni Garcinia cambogia mix)* against human liver cancer cells (HepG2 Cells)

Authors' affiliation :
P. Selvam[1*]
[1]Nova College of Pharmaceutical Education and Research, Jupudi, Ibrahimpatnam, AP
K. S. Praveen[2]
Hitesh Jagani[2]
J. Venkata Rao[2]
[2]Manipal College of Pharmaceutical Sciences, Manipal University, Manipal - 576104, Karnataka

Keywords: *Morinda citrifolia, Garcinia cambogia*, HepG2 cells, MTT, SRB

Abstract : Divine Noni *(Noni Garcinia cambogia mix)* with the concentrations of 40%, 20%, 10% and 5% were investigated for cytotoxicty on human liver cancer cells (HepG2 Cells) by MTT and SRB methods. 5-20 % concentration showed significant cytotoxicity against human liver cancer cells (HepG2 Cells) by both MTT and SRB methods 30 and 40 % Divine Noni (*Noni Garcinia cambogia mix)* cause complete cell death of Human liver cancer cells. Cytotoxicity effect of Divine Noni may be due to the additive effect of *Morinda citrifolia* and *Garcinia cambogia* mix. This *in vitro* study has proved the selective cytotoxicity of Divine Noni against human liver cancer cells.

Introduction

Morinda citrifolia L Noni is a versatile medicinal plant and wide spectrum of biological activities (Pawlus *et al.,* 2007). The Polynesians utilized the whole Noni plant (*Morinda citrifolia*) in various combinations for herbal remedies and reported to possess wide spectrum biological activities to manage arthritis, diabetes, high blood pressure, muscle aches and pains, menstrual disorders, headaches, heart disease, AIDS, cancers, gastric ulcers, sprains, mental depression, senility, poor digestion, atherosclerosis, blood vessel problems and drug addiction (Wang *et al.,* 2002 and Mc Clatchey *et al.,* 2002). Recently much attention has been devoted for searching potential and safe herbal remedies from natural products for the treatment of cancer and *Morinda citrifolia* is used for the treatment of a variety of cancer and tumors (West *et al.,* 2006).

Correspondence to :
P. Selvam
Nova College of Pharmaceutical Education and Research, Jupudi, Ibrahimpatnam, Andhra Pradesh
Email :
periyasamy_selvam@yahoo.co.in

Garcinia cambogia is a small or medium size tree, with drooping branches and ovoid fruits, native to Southeast Asia. Several compounds have been isolated from certain species of *Garcinia*, including xanthones, xanthones derivatives, and (-)-Hydroxycitric acid (Duke, *et al.,* 2002). *Garcinia cambogia* extract has been used traditionally in Indian medicine to treat tumors, ulcers, hemorrhoids, diarrhea, dysentery, fever, open sores, and parasites (Tharachand *et al.,* 2013). It has been reported to be indicated for constipation, rheumatism, dyspepsia, obesity, and high levels of triglycerides and cholesterol (Tharachand *et al.,* 2013). (-)-Hydroxycitric acid is present in the pericarp of the fruit of *Garcinia cambogia* up to 30% by

weight. Commercially available *Garcinia cambogia* extracts are prepared from the fruit rind and contain 50% (-)-Hydroxycitric acid (Onakpoya *et al.,* 2011). Benzophenones derivatives Garcinol and Isogarcinol were isolated from *G.cambogia* and reported to possess wide spectrum anticancer activity (Padhye *et al.,* 2009) . Based on the fact, the present research work was undertaken to study the cytotoxicity activity of Divine Noni *(Noni Garcinia cambogia mix),* against HepG2 (Human liver cancer) cells by MTT and SRB assays.

Materials and Methods

Divine Noni Treatment

Divine Noni *(Noni Garcinia cambogia mix)* was obtained from Noni Biotech Pvt. Ltd., Chennai. Divine Noni was diluted v/v with complete DMEM in various concentrations- 40%, 20%, 10% and 5% after the treatment, the cells were incubated for 24 hours after MTT assay and SRB assays were performed.

Cell lines and growth media

HepG2 (Human liver cancer) cells were cultured in MEM (Minimum Essential Medium) and DMEM (Dulbecco's Modified Eagles Medium) respectively. The medium also contains 10% fetal calf serum, penicillin (100 IU) and streptomycin (100 µg).

In vitro cytotoxicity screening

The ability of the cells to survive a toxic insult is the basis of most cytotoxicity assays (Francis *et al.,* 1986; Philip *et al.,* 1990). The monolayer cell culture was trypsinized and the cell count was adjusted to $1.0x10^5$ cells/ml using medium containing 10% new born calf serum. To each well of the 96 well microtiter plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours partial monolayer was formed, the supernatant liquid was flicked off washed the monolayer once and 100ml of different Divine Noni concentrations were added to the cells in micro titer plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded at every 24 hours. After 72 hours, the Divine Noni solutions in the wells were discarded, MTT and SRB assay performed. Cytotoxicity data of Divine Noni, *(Noni Garcinia cambogia mix)* is presented in Table 1.

Table 1 : Determination of CTC_{50} by using MTT and SRB assay in HepG2 cells

Drug Name	Dilution	MTT assay			SRB assay		
		% cell death					
		24 h	48 h	72 h	24 h	48 h	72 h
Divine Noni	1:5	60.12± 1.3	70.25± 2.11	72.43± 1.43	68.27± 2.3	75.0± 3.1	80.2± 4.3
	1:10	70.65± 0.33	74.66± 0.77	78.90± 3.33	74.77± 3.4	82.34± 0.76	85.44± 2.5
	1:20	80.34± 2.34	85.55± 1.2	97.54± 0.65	82.11± 0.9	86.42± 1.4	90.12± 0.8
	1:30	90.50± 0.4	95.24± 0.9	98.76± 0.2	94.67± 1.2	97.6± 2.3	100± 0.0
	1:40	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0

*Cytotoxic 50% concentration; *Average of four independent determinations, values is mean ± S.E.M.

Results

Divine Noni *(Noni Garcinia cambogia mix)* with the concentrations of 40%, 20%, 10% and 5% were investigated for cytotoxicity studies including cellular toxicity on human liver cancer cells (HepG2 Cells) by MTT and SRB methods. Graphical representation of cell proliferation as detected by MTT and SRB assay on human liver cancer cells were treated with different concentrations of Divine Noni is given in Fig .1 and 2. 5-20 % *Noni Garcinia cambogia mix* exhibits potent cytotoxicity against human liver cancer cells (HepG2 Cells) by both MTT and SRB methods (Table1: Fig.1 and 2). 30% and 40 % Divine Noni completely promote the cell death of HepG2 cells. This *in vitro* study has proved the selective cytotoxicity against human liver cancer cells by Divine Noni.

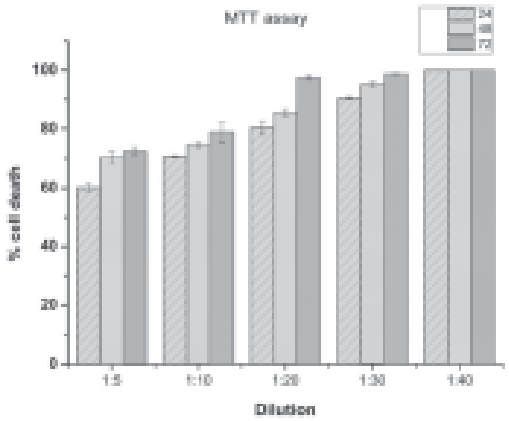


Fig. 1 : MTT assay for different dilutions of Divine Noni at 24, 48 and 72 h time interval

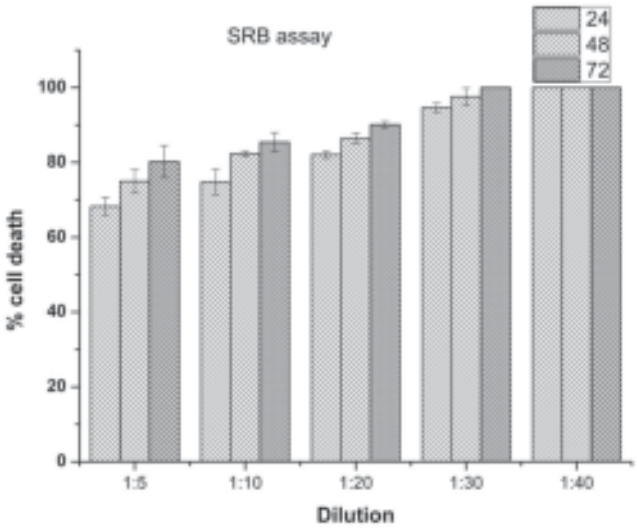


Fig. 2 : SRB assay for different dilutions of Divine Noni at 24, 48 and 72 h time interval

Discussions

Morinda citrifolia (Noni) has been extensively used in folk medicine by Polynesians for over 2,000 years. It has been reported to have broad therapeutic effects, including anticancer activity, in both clinical practice and laboratory animal models (Wang, and Su, 2001). Review of literature revealed that Divine Noni exhibits hepatoprotective (Wang *et al., 2008 a,b*), anticancer (Akihisa *et al.,* 2008), wound healing (Nayak *et al.,* 2007), antioxidant (Su *et al.,* 2005), immunomodulatory and anti-inflammatory (Palu *et al.,* 2007). Ethanolic extracts and their fractions showed cytotoxicity against human liver cancer cells (Selvam *et al.,* 2009 and 2010) and extract of Noni also demonstrated for hepatoprotective activity against CCl_4 induced

hepatotoxicity in human liver cells (Selvam *et al.,* 2011). *Garcinia cambogia* reported to possess wide spectrum of anti cancer activity due to presence of Garcinol (Prasad *et al.,* 2010; Matsumoto *et al*., 2003; Ahmad *et al*., 2011; Hong *et al*., 2007; Tanaka *et al.,*2000; Arif *et al.,* 2006; Balasubramanyam *et al.,* 2004). In the present investigation, cytotoxicity of Divine Noni *(Noni Garcinia cambogia mix)* against HepG2 (Human cancer cells) from liver origin due to additive effect of *Morinda citrifolia* Noni fruit and *Garcinia cambogia*. Divine Noni showed significant cytotoxicity against human lung cancer cell. This *in vitro* study has proved the selective cytotoxicity of Divine Noni against liver cancer cells.

References

Ahmad, A., Wang, Z., Wojewoda, C., Ali, R., Kong, D., Maitah, M. Y., Banerjee, S., Bao, B., Pandhye, S and Sarkar, F. H. 2011. Garcinol induced apoptosis in prostate and pancreatic cancer cells is mediated by NF Kappa B signaling, *Frontiers in Bioscience,* 3:1483-1492.

Akihisa, T., Matsumoto, K., Tokuda, H., Yasukawa, K., Seino, K.I., Nakamoto, K.H., Kuninaga, H., Suzuki, T and Kimura, Y. 2008. Anti-Inflammatory and Potential Cancer Chemopreventive Constituents of the Fruits of *Morinda citrifolia*. *Journal of Natural Product,* 71:1322-25.

Arif, M., Pradhan, S. K., Thanuja, G. R., Vedamurthy, B. M., Agarwal, S, Dasgupta, D and Kundu, T. K. 2006. Mechanism of p300 Specific Histone Acetyltransferase Inhibition by Small Molecules, *J. Med. Chem*., 52(2):267-277

Balasubramanyam, K., Altaf, M, Varier, R. A., Swaminathan, V., Ravindran, A., Sadhale P. P., Kundu., T. K. 2004. Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. *J. Biol. Chem.,* 279(32): 33716-33726.

Bina S Siddiqui, Fouzia A. Sattar, Sabira Begum, Tahsin Gulzar, and Fayaz Ahmad. 2007. Chemical constituents from the stems of *Morinda citrifolia* Linn. *Achieve Pharmaceutical Research,* 30: 793-798,

Cheng, A., Tsai M, Liu C, Lee M, Nagabhushanam, K, Ho C and Pan M. 2010. Garcinol inhibits cell growth in hepatocellular carcinoma Hep3B cells through induction of ROS-dependent apoptosis. *Food Functions,* 1: 301-307.

Duke, J.A., Bogenschutz-Godwin, M.J., duCellier, J. & Duke, P.-A.K. 2002. *Handbook of Medicinal Herbs*. 2nd ed., Boca Raton, FL, CRC Press, p 481

Francis, D. and Rita L. 1986. Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods*, 89: 271-277.

Hong, J., Kwon, S. K., Sang, S., Ju, J., Zhou, J., Ho, C., Huang, M and Yang ,C. S. 2007. Effects of Garcinol and its derivatives on intestinal cell growth: Inhibitory effects and autoxidation-dependent growth-stimulatory effects, *Free radical biology & medicine,* 42: 1211-1221.

Matsumoto, K., Akao, Y., Kobayashi, E., Ito, T., Ohguchi, K., Tanaka, T., Iinuma, M and Nozawa, Y. 2003.Cytotoxic benzophenone derivatives from *Garcinia* species display a strong apoptosis-inducing effect against Human Leukemia Cell lines, *Biol. Pharm. Bull,* 26(4):569-571.

McClatchey, W. 2002. From Polynesian healers to health food stores changing perspectives of *Morinda citrifolia* (Rubiaceae). *Integrated Cancer Therapy*, 1 :110-20.

Nayak, B.S, Isitor, G.N., Maxwell, N.A, Bhogadi, V. and Ramdath, D. 2007. Wound-Healing Activity of *Morinda Citrifolia* Fruit Juice on Diabetes-Induced Rats. *Journal Wound Care*, 16 (2) :83-7.

Onakpoya, I., Hung, S. K., Perry, R., Wider, B. and Ernst, E. 2011. The Use of Garcinia Extract (Hydroxycitric Acid) as a Weight loss Supplement: A Systematic Review and Meta-Analysis of Randomised Clinical Trials. *Journal of Obesity,* 2011: 1-10.

Padhye, S., Ahmad, A., Oswal, N. and Sarkar, F. H, 2009. Emerging role of Garcinol, the antioxidant chalcone from *Garcinia indica* Choisy and its synthetic analogs, *Journal of Hematology and Oncology,* 2(38):120-125

Palu, A.K., Kim, A.H., West, B.J., Deng, S., Jensen, J .and White, L. 2008. The Effects of *Morinda Citrifolia* L. (Noni) on the Immune System, Its Molecular Mechanisms of Action. *Journal of Ethnopharmacology*, 115 (3): 502-507.

Palu, A.K., Kim, A.H., West, B.J., Deng, S., Jensen, J .and White, L.2007. Anti-Inflammatory and Potential Cancer Chemopreventive Constituents of the Fruits of *Morinda Citrifolia* (Noni). *Journal of Natural Product*, 70 (5): 754-760.

Pawlus, A.D. and Kinghorn, D. 2007. A Review of the Ethnobotany, Chemistry, Biological Activity and Safety of the Botanical Dietary Supplement *Morinda Citrifolia* (Noni). *Journal of Pharmacy and Pharmacology,* 59 (12): 1587-1592.

Periyasamy Selvam, Paul Pandi ,T. and Vasanth Raj, P. 2009. Studies of anticancer activity of ethanolic extract of Noni fruit *Morinda citrifolia* L. *Noni Clinical Research Journal,* 3 (1-2): 41-46.

Periyasamy Selvam., Paul Pandi, T. and Vasanth Raj, P. 2010. Studies of cytotoxicity activity of ethanolic fractions of Noni fruit *Morinda citrifolia* L. *International Journal of Noni Research.,* 5: 1-2; 17-21

Philip, S., Rista, S., Dominic, S., Anne, M., James., David, V., Jonathan,. T.W, Heidi, B., Susan, K. and Michale R.B. 1990. New colorimetric cytotoxic assay for anti cancer drug screening. *Journal of National Cancer Institute,* 82: 1107-1112.

Prasad, S., Ravindran, J., Sung, B., Pandey, M. K and Agarwal B. B. 2010. Garcinol potentiates TRAIL-Induced Apoptosis through Modulation of death receptors and antiapoptotic proteins, *Mol Cancer Ther,* 9(4): 856-868.

Rakesh Kumar Gupta, Ayan Banerjee, Suajta Pathak, Chandresh Sharma and Neeta Singh. 2013. *Induction of mitochondrial-mediated apoptosis by Morinda citrifolia (Noni) in human cervical cancer cells. Asian Pacific J Cancer Prev.,* 14 (1), 237-242.

Su, B.N., Pawlus, A.D., Jung, H.A., Keller ,W.J., McLaughlin, J.L and Kinghorn, A.D. 2005.Chemical Constituents of the Fruits of *Morinda citrifolia* (Noni) and their Antioxidant Activity. *Journal of Natural Product*, 68 (4): 592-598.

Tanaka, T., Kohno, H., Shimada, R., Kagami, S., Yamaguchi, F., Kataoka, S., Ariga, T., Murakami, A., Koshimizu, K .and Ohigashi, H. 2000. Prevention of colonic aberrant crypt foci by dietary feeding of garcinol in male F344 rats, *Carcinogenesis,* 21(6): 1183-1189.

Wang, M.Y., Anderson, G., Nowicki, D. and Jensen, J. 2008a. Hepatic Protection by Noni Fruit Juice against CCl_4-Induced Chronic Liver Damage in Female SD Rats. *Plant Foods Human Nutrition,* (3): 141-147.

Wang MY, Nowicki D, Anderson G, Jensen J. and West B. 2008b. Liver Protective Effects of *Morinda citrifolia* (Noni). *Plant Foods Human Nutrition,* 63 (2): 59-63.

Wang, M.Y., West, B.J., Jensen, C.J., Nowicki, D., Su, C., Palu, A.K. and Anderson, G. 2002 *Morinda citrifolia* (Noni) a literature review and recent advances in Noni research. *Acta Pharmacologica Sinica*, 23: 1127-1141.

West, B.J,, Jensen, C.J. and Westendorf, J. 2006. Noni Juice is Not Hepatotoxic. *World Journal of Gastroenterology*, 12 (22): 3616-3620.

Wang, M.Y. and Su C. 2001. Cancer preventive effect of *morinda citrifolia* L Noni *Ann. N Y Acad Sci.,* 952:161-168.

Sridevi Nagalingam
Devanand Shanmugasundaram
Changam Sheela Sasikumar
Kotturathu Mammen Cherian

Effect of *Morinda citrifolia* aqueous fruit extract against ROS induced oxidative DNA damage in human lymphocytes - An *in vitro* study

Keywords: Oxidative DNA damage, DNA fragmentation free radicals, Reactive Oxygen species, redox state, *Morinda citrifolia*, Medicinal plant, Aqueous extract, Antioxidant, Lymphocytes.

Authors' affiliation :
Sridevi Nagalingam
Devanand Shanmugasundaram
Changam Sheela Sasikumar
Kotturathu Mammen Cherian
Department of Biochemistry
Frontier Lifeline Pvt Ltd,
R-30-C Ambattur Industrial Estate
Road, Mogappair,
Chennai 600 101, India
*E-mail: sheelsasic@yahoo.co.in

Abstract : A pilot study was undertaken to investigate the protective effect of *M. citrifolia* aqueous extract against ROS induced in vitro lipid peroxidation and DNA damage in human lymphocytes. The results infer that the aqueous extract of *M. citrifolia* pre-treated cells, showed lesser oxidative DNA damage and fragmentation when compared to post-treated cells. This may be due to the direct modulation of the antioxidant defense system of *M. citrifolia*. This study will highlight *M. citrifolia*, as a potent fruit with antioxidant capabilities, for overcoming ROS induced DNA damage and fragmentation.

Introduction

Oxidative stress, due to ionizing radiation and other exogenous toxins are the inevitable consequence of cellular metabolism that targets the healthy cells in our body. They modulate the structure of DNA and proteins by generating free radicals, which could contribute to many diseases. To protect this, our biological system is equipped with a sophisticated antioxidant protective system. In some conditions excess ROS in our body causes an imbalance in redox state, increase the oxidant load resulting in poor quenching or scavenging activity thus decreasing the life span of the cells. In order to counteract the imbalanced oxidant levels in our body we require alternative natural antioxidants found in many fruits and vegetables.

Correspondence to :
Sridevi Nagalingam
Department of Biochemistry
Frontier Lifeline Pvt Ltd,
R-30-C Ambattur Industrial Estate
Road, Mogappair,
Chennai 600 101, India
*E-mail: sheelsasic@yahoo.co.in

DNA is one of the most important key targets for highly reactive substances capable of attacking normal cells, damaging cellular macromolecules (Mantena *et al*., 2008). Under normal circumstances, DNA is constantly subjected to chemical modifications. Various types of DNA damage like SSB (single strand break), DSB (double strand break), CPDs (cyclobutane pyrimidine dimers), 6-4PPs (6-4 photoproducts) and their Dewar valence isomers have been identified, as a result of alkylating agents, hydrolytic deamination, free radicals and reactive oxygen species formed by various photochemical processes including UV radiation (Langseth., 1993).

Under normal conditions, variety of organisms ranging from bacteria to human are well equipped with complex antioxidant system. This system includes antioxidant

enzymes and non-enzymatic antioxidants, which could interactively deactivate or neutralize the free radical before it damages the cells (Halliwell., 1994). Exposure to toxins, cancer and chronic inflammatory diseases generate ROS as part of the pathophysiologic mechanism which cause an imbalance in redox state. This leads to migration of ROS from mitochondria to nucleus causing single or double stranded DNA breaks, purine, pyrimidine, or deoxyribose modification and DNA cross-links. (Jacob., 1995). Oxidative DNA damage can results either in arrest or induction of transcription, signal transduction pathways, replication errors and genomic instability all of which are associated with pathological condition (Devasagayam *et al.*, 2005)

Hence, to minimize the effect of oxidation present research efforts are directed towards natural antioxidants present in fruits and vegetables which are known for its own antioxidant and pharmacological significance (Glaser., 1998 and Akinmoladun *et al.*, 2007). One such traditional and Polynesian medicinal plant is *Morinda citrifolia* Linn Rubiaceae commonly known as Noni or Indian mulberry is increasingly being screened for their role in modulating the activity of environmental genotoxicants (West *et al.*, 2006) .The fruit of this plant has been used as food, drink, medicine, colorful dye, cosmetics purpose and has a high demand in medicines. (Duke *et al.*, 2002).

M. citrifolia is a potential source of natural antioxidant, containing more than 120 nutraceutical and 160 phytochemicals. They are reported to have antibacterial, antiviral, antifungal, antitumor, anti tubercular, antihelminthic, analgesic, hypotensive and anti- inflammatory activity. It has also been reported to enhance the immune system and improve mental health. Due to its beneficial effects the fruit juice of *M. citrifolia* is widely distributed throughout the world as a dietary supplement (Duke *et al.*, 2002; Mc Clatchey, 2002).

The aim of this study is to elucidate the protective role of *M. citrifolia* aqueous fruit extract against *in vitro* induced DNA damage in human lymphocytes.

Material and Methods

The fruits of *M. citrifolia* were collected from World Noni Research Foundation, Perungudi, Chennai.

Preparation of aqueous extract

Fresh fruits were washed with running tap water repeatedly chopped with a sterile knife and dried in a Hot air oven at 45°C for 48 h. Air dried fruit were ground into powder; the ground powder was extracted with water and incubated for 72 h in shaker, and it was boiled for 20 to 30 minutes till the volume was reduced to half its original. The solvent was then separated by filtration. The extracts were condensed using rotary vacuum evaporator and stored at 0-4°C. The aqueous extract was dissolved in water and used for further purpose.

Blood collection

Fresh venous blood was obtained through venipuncture of healthy female donors using sterile disposable syringe and transferred into EDTA coated tube to avoid coagulation. The lymphocytes were isolated as follows.

Isolation of Lymphocytes

Lymphocytes were isolated by the method of (Boyum *et al*., 2002) with slight modifications. The Fresh defibrinated venous blood (2 ml) was added to 0.9 percent of saline (2 ml) in equal ratio (1: 1). The LSM (Lymphocyte separation medium) at room temperature was thoroughly mixed by inverting the bottle gently and aseptically transferring 3ml of LSM to a 15ml centrifuge tube. The diluted blood was carefully layered on top of the LSM, creating a sharp blood and LSM interphase (the content were not allowed to be mixed). Centrifuge the tubes at 400xg at room temperature for 15 – 20 minutes. Aspirate the top layer of clear plasma to within 2-3 mm above the lymphocyte layer and discard. Aspirate the lymphocyte layer and half of the LSM layer into the centrifuge tube and centrifuge for 10 minutes at room temperature at a speed sufficient to sediment the cells without damage. The cells were then washed with PBS or saline and resuspend in the RPMI-1640 medium at a concentration of ~ 2 x 10^6 cells/ml.

Cell Count and Cell Viability

The WBC diluted with WBC diluting fluid and visualized under a microscope using a Neubauer chamber. The viability of the cells was analyzed using trypan blue dye exclusion method (Phillips., 1973) before and after the treatment to check the viability.

Treatment

Two sets (pre treatment and post treatment) were prepared to assess the antioxidative potential of *M. citrifolia* aqueous fruit extract. The isolated lymphocytes were distributed into various concentrations of (~2 x 10^6 cells/ml) and subjected to Hydrogen peroxide (100mM/L), Ferrous sulfate ($FeSO_4$. $7H_2O$) (0.1 mM/L) and EDTA (0.1 mM/L) treatment to induce oxidative stress and lipid peroxidation in the lymphocytes. Freshly prepared *Morinda citrifolia* aqueous fruit extract was added with different concentrations such as 25, 50, 75, 100,125,150,175 and 200 µl to assess the pre antioxidative potential. The volume was adjusted to 1 ml with RPMI-1640 medium and incubated for 2 h at 37°C. After 2 hr incubation the second set was treated with the above mentioned concentration of *Morinda citrifolia* aqueous fruit extract for post treatment. Again the tubes were incubated for 30 minutes at 37°C. Lymphocytes with RPMI-1640 medium only acts as a Negative Control, with Hydrogen peroxide (100mM/L), Ferrous sulfate ($FeSO_4$. $7H_2O$) (0.1 mM/L) and EDTA (0.1 mM/L) as a Positive control. Lymphocytes with RPMI-1640 medium and

Morinda citrifolia aqueous fruit extract as a *M.citrifolia* Control (MC) and Lymphocytes with Ascorbic acid, RPMI-1640 medium, Hydrogen peroxide (100mM/L), Ferrous sulfate ($FeSO_4$. $7H_2O$) (0.1 mM/L) and EDTA (0.1 mM/L) labeled as a Standard Control. The experiments were performed in triplicates for each treatment and controls. After the treatment period, all tubes were centrifuged at 400xg for 5 minutes. The supernatant collected were used for biochemical assay analysis and pellets containing genomic DNA was isolated and assessed for DNA damage and DNA fragmentation.

The details of the treated cells are described below.

1. Group-1 : Pre treated with *M. citrifolia* aqueous extract before incubation period,

- **Negative Control (NC) –** Untreated Lymphocytes at the concentration of (~2 x 10^6 cells/ml)
- **Positive Control (PC) –** Lymphocyte with (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L)
- ***Morinda citrifolia* Control (MC) –** Untreated Lymphocyte and *M. citrifolia* Aqueous fruit extract (100µl)
- **Standard Control (SC)** - Lymphocyte with (400 µg) Ascorbic acid and (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L)
- **Treatment 1** - Lymphocyte with (25 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L)
- **Treatment 2** - Lymphocyte with (50 µl *M, citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L)
- **Treatment 3** - Lymphocyte with (75 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L)
- **Treatment 4** - Lymphocyte with (100 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L)
- **Treatment 5** - Lymphocyte with (125 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L)
- **Treatment 6** - Lymphocyte with (150 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L)

- **Treatment 7** - Lymphocyte with (175 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L)
- **Treatment 8** - Lymphocyte with (200µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).

2. Group-2: Post treated with *M. citrifolia* aqueous extract after incubation period,

- **Negative Control (NC) –** Untreated Lymphocytes at the concentration of (~2 x 10^6 cells/ml).
- **Positive Control (PC) –** Lymphocyte with (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).
- ***Morinda citrifolia* Control (MC) –** Untreated Lymphocyte and *M, citrifolia* Aqueous fruit extract (100µl).
- **Standard Control (SC)** - Lymphocyte with Ascorbic acid (400 µg) and (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).
- **Treatment 1** - Lymphocyte with (25 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).
- **Treatment 2** - Lymphocyte with (50 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).
- **Treatment 3** - Lymphocyte with (75 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).
- **Treatment 4** - Lymphocyte with (100 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).
- **Treatment 5** - Lymphocyte with (125 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).
- **Treatment 6** - Lymphocyte with (150 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).
- **Treatment 7** - Lymphocyte with (175 µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).
- **Treatment 8** - Lymphocyte with (200µl *M. citrifolia* Aqueous fruit extract, (100 µl of H_2O_2 (100mM), 60 µl of $FeSO_4$. $7H_2O$ (0.1 mM/L), 60 µl of EDTA (0.1 mM/L).

Isolation of Genomic DNA

Genomic DNA was isolated from the pellet of the treated groups using *Lahiri et al*., method (Dcbomoy *et al*., 1993) and the DNA damage was assessed using 3% Agarose gel electrophoresis.

DNA fragmentation

The pellets obtained after treatment were subjected to DNA fragmentation by *Hermann et al*., method (Herrmann *et al*., 1994). The pellets were washed with PBS and lysed with buffer containing 50mM Tris HCL (pH 7.5) 20mM EDTA and 1% NP-40. Then RNAse A (5mg/ml) and SDS (1%) were added and incubated for 2 h at 56°C and again incubated for 2 hrs at 37°C followed by the addition of Proteinase K (2.5mg/ml). DNA from the lysate was precipitated with 0.5 volumes of 10M glycine and 2.5 volumes of 70% ice cold ethanol at -80°C overnight. After centrifugation DNA was suspended in Tris EDTA and fragmented DNA was assessed using 1.5% Agarose gel electrophoresis

Biochemical Parameters

Total flavanoid content (Mervat *et al*., 2009), Total phenol content (Louli *et al*., 2004), DPPH free radicals assay (Dinis *et al*., 1994) and Metal chelating activity of *M. citrifolia* aqueous fruit extract were determined by standard methods. In addition, antioxidant activity of *M. citrifolia* against ROS- induced lymphocytes was estimated by Thio barbituric Acid Reaction (TBARS) (Rush *et al*., 1989), and the reduced glutathione (GSH) (Ohkawa *et al*., 1979) was estimated by their standards. Total protein content (Lowry., 1951) was determined spectrophotometrically at 595 nm and concentrations were compared to a calibration curve of bovine serum albumin.

All the data was evaluated using SPSS statistical package. Hypothesis testing method included one way analysis of variance (ANOVA) followed by Tukey post hoc t test. $p<0.05$ was considered to indicate statistical significance. All the results were expressed as mean ± S.D for each sample.

Results

The potential significance of *M. citrifolia* aqueous fruit extract to prevent DNA damage was tested by performing 3% Agarose gel electrophoresis. The treated groups of DNA showed maximal DNA (shearing) damage in Positive control, with minimum damage in NC and MC. whereas, DNA (shearing) damage is significantly decreased in *Morinda citrifolia* pre treated cells than post treated cells and are dose dependent. However, subminimum DNA (shearing) damage was noted in SC. The results were illustrated in (Fig. 1; 2)

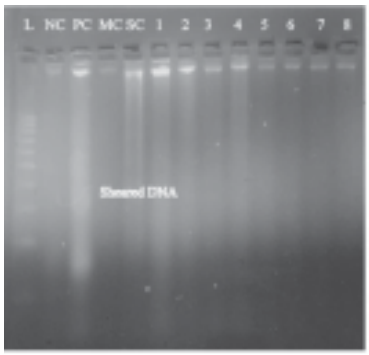


Fig. 1

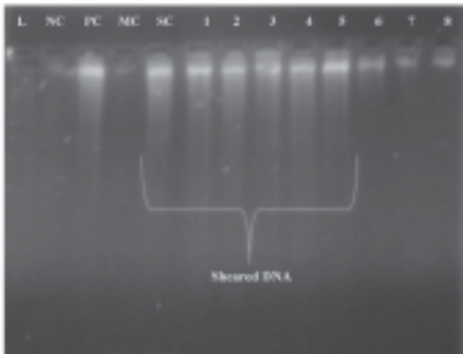


Fig. 2

Fig. 1 : Pre treated effect of *Morinda citrifolia* aqueous fruit extract against ROS induced Oxidative DNA damage in human lymphocytes

Fig. 2 : Post treated effect of *Morinda citrifolia* aqueous fruit extract against ROS induced Oxidative DNA damage in human lymphocytes

L- DNA Ladder, **NC**- Negative control, **PC**- Positive control, **MC**– *M. citrifolia* control, **SC**- Standard control (Ascorbic acid), **Lane 1**: 25 µl of *M. citrifolia* treated lymphocytes, **Lane 2**: 50 µl of *M. citrifolia* treated lymphocytes, **Lane 3**: 75 µl of *M.citrifolia* treated lymphocytes, **Lane 4**: 100 µl of *M. citrifolia* treated lymphocytes, **Lane 5**: 125 µl of *M. citrifolia* treated lymphocytes, **Lane 6**: 150 µl of *M.citrifolia* treated lymphocytes, **Lane 7**: 175 µl of *M. citrifolia* treated lymphocytes and **Lane 8**: 200 µl of *M. citrifolia* treated lymphocytes.

Incubation of *M. citrifolia* aqueous fruit extract with DNA showed a partial protection of the fragmented DNA (Fig. 3, lane: 4, 5 6) when compared to the (Fig. 3, lane: 1, 2 and 3). Whereas, DNA with genotoxicants showed maximum DNA fragmentation in (PC). However, the fragmentation was absent in NC, MC and SC (Fig. 3 and 4). Similar results were observed in post treated *Morinda citrifolia* aqueous fruit extract (Fig. 4 lane: 4, 5 6) and (Fig. 4 lane: 1, 2 and 3) but the protective effect was slightly lower than pre treatment.

Fig. 3

Fig. 4

Fig. 3 : Pre treated effect of *M. citrifolia* aqueous fruit extract against ROS induced DNA fragmentation in human lymphocytes

Fig. 4 : Post treated effect of *M. citrifolia* aqueous fruit extract against ROS induced DNA fragmentation in human lymphocytes

NC- Negative control, **MC**– *M. citrifolia* control, **PC**- Positive control, **SC**- Standard control (Ascorbic acid), **Lane 1**: 25 µl of *M. citrifolia* treated lymphocytes, **Lane 2**: 75 µl of *M. citrifolia* treated lymphocytes, **Lane 3**: 125 µl of *M. citrifolia* treated lymphocytes, **Lane 4**: 175 µl of *M. citrifolia* treated lymphocytes, **Lane 5**: 225 µl of *M.citrifolia* treated lymphocytes, **Lane 6**: 275 µl of *M. citrifolia* treated lymphocytes**.**

Total flavanoids were expressed as Gallic acid (80.41 mg), Total phenol with quercetin (0.327 mg), DPPH (2.66 mg) and Metal chelating activity in aqueous extract of *Morinda citrifolia* fruit was estimated using EDTA as (94.30 mg) equivalents per gram of formulation.

The antioxidant and scavenging activity of pre treated and post treated *Morinda citrifolia* aqueous fruit extract was determined by GSH and LPO. The concentration of GSH decreased significantly (<P 0.05) in EDTA, $FeSO_4$ and H_2O_2 treated lymphocytes (PC) relatively to controls (NC). Whereas, GSH level improved significantly (<P 0.05) with increase in concentration of *Morinda citrifolia* aqueous fruit extract. However, it is higher in (MC), and moderate in (SC) (Table 1).

Table 1: *In vitro* potential of *M. citrifolia* aqueous fruit extract on Glutathione in human lymphocytes

Parameters	Group 1	Group 2
	Morinda citrifolia **aqueous fruit extract Pre- treated cells (n moles MDA/ mg protein)**	*Morinda citrifolia* **aqueous fruit extract Post- treated cells (n moles MDA/ mg protein)**
Negative control	0.704 ± 0.503	0.704 ± 0.503
Positive control	0.535667 ± 0.01680	0.535667 ± 0.01680
M.c Control	0.976333 ± 0.22502	0.976333 ± 0.22502
Standard control	0.857 ± 0.03651	0.857 ± 0.03651
Treatment 1	0.5695 ± 0.023445[a]	0.24775 ± 0.013841[b]
Treatment 2	0.5945 ± 0.004796[a]	0.42925 ± 0.022187[b]
Treatment 3	0.67075 ± 0.0234[a]	0.4905 ± 0.00995[b]
Treatment 4	0.68825 ± 0.008221[a]	0.5645 ± 0.013379[b]
Treatment 5	0.77125 ±0.026158[a]	0.67425 ± 0.028052[b]
Treatment 6	0.845 ± 0.031937[a]	0.756 ± 0.008287[b]
Treatment 7	0.85275 ± 0.020759[a]	0.8005 ± 0.009[b]
Treatment 8	1.06675 ± 0.109097[a]	0.81425 ± 0.010046[b]

Values are expressed as mean ± S.D. Values are statistically significant at *P< 0.05.[a] *Morinda citrifolia* aqueous extract of pre-treated cells vs *Morinda citrifolia* aqueous extract of post treated cells;[b] by one way analysis of variance (ANOVA) followed by Student Newman Keuls post hoc test. P < 0.05 was considered significant.

Similarly when proceeded with TBARS, Lipid peroxidation assay, the level of LPO was significantly higher (P<0.05) in EDTA, $FeSO_4$ and H_2O_2 treated lymphocyte (PC) than control (NC) and the level of LPO significantly decreases as the concentration of *Morinda citrifolia* aqueous extract increases which is dose dependent. However, LPO is lesser in (MC), and moderate in (SC) (Table 2).

Table 2: ***In vitro* potential of *M. citrifolia* aqueous fruit extract on Lipid peroxides in human lymphocytes**

Parameters	**Group 1**	**Group 2**
	Morinda citrifolia **aqueous fruit extract Pre-treated cells (µ moles/ mg protein)**	*Morinda citrifolia* **aqueous fruit extract Post-treated cells (µ moles/ mg protein)**
Negative control	0.146667 ± 0.022189	0.146667 ± 0.022189
Positive control	0.379667 ± 0.061076	0.379667 ± 0.061076
M.c Control	0.249667 ± 0.079752	0.249667 ± 0.079752
Standard control	0.173333 ± 0.055076	0.173333 ± 0.055076
Treatment 1	0.193333 ± 0.003055[a]	1.16 ± 0.001732[b]
Treatment 2	0.186333 ±0.005033[a]	1.151333 ± 0.001155[b]
Treatment 3	0.176667 ± 0.003786[a]	1.143333 ± 0.003215[b]
Treatment 4	0.164667 ± 0.004726[a]	1.132333 ± 0.001528[b]
Treatment 5	0.156667 ± 0.006429[a]	0.791667 ± 0.59082[b]
Treatment 6	0.152333 ± 0.005859[a]	0.119333 ± 0.000577[b]
Treatment 7	0.136667 ± 0.003055[a]	0.104667 ± 0.004041[b]
Treatment 8	0.128 ± 0.003[a]	0.098667 ± 0.00057[b]

Values are expressed as mean ± S.D. Values are statistically significant at *P< 0.05. [a] *M. citrifolia* aqueous extract of pre-treated cells vs *M. citrifolia* aqueous extract of post treated cells;[b] by one way analysis of variance (ANOVA) followed by student Newman Keuls post hoc test. P < 0.05 was considered significant.

Discussion

The over production of highly reactive substances implicated in many types of diseases are found to damage the cellular compounds and interact with DNA. In normal biological system the balance between oxidants and antioxidant are maintained

by the antioxidant defense system, which is important for maintaining optimal physiological condition (Moron *et al.,* 1979). Herbal drugs with high content of polyphenols and flavanoids are intended for the treatment of diseases by preventing the damage of DNA caused by environmental genotoxins. Epidemiological studies have also strongly supported that, increased consumption of flavanoids prevent several pathological conditions by preventing DNA damage (Temple, 2000).

M. citrifolia is one such medicinal plant rich in antioxidants, phytochemicals, nutraceuticals and pharmacological substances reputed to have many medicinal properties (Duke *et al*., 2002; Mc Clatchey, 2002).

In this present study we investigated the antioxidant and scavenging potential of pre treated and post treated *M. citrifolia* aqueous fruit extract on oxidative DNA damage in human lymphocytes.

Lymphocytes are known for its redox and free radical scavenging system and also used to screen a variety of toxicants. In this study lipidperoxidation is induced by H_2O_2 (100mM), $FeSO_4$. $7H_2O$ (0.1 mM/L) and EDTA (0.1 mM/L) which could enhance an oxidative stress with transition metals that results in damage to cellular components like protein, lipid and principally to DNA (Asad *et al*., 2004). In the similar way Iron ($FeSO_{4)}$ and salt (EDTA) stimulate lipid peroxidation by decomposing lipid peroxides to form alkoxyl (LO•), peroxyl (LO_2•) by directly reacting with molecular oxygen to produce hydroxyl radicals (OH•) which in turn provides an oxidative stress to the cells (Gutteridge, 1977). These oxidative stress inducers were pre and post treated with *M. citrifolia* aqueous extract to assess its protective effect on DNA damage and the results were demonstrated.

Therefore, (Fig.1, 2, 3 and 4) the cells which were pre treated with *M. citrifolia* aqueous extract showed minimal DNA damage than cells with post treated *M. citrifolia* aqueous fruit extract. Interestingly we observed that as the concentration of the *M. citrifolia* increased the amount of flavanoids, polyphenols in the fruit increased thus mounting more antioxidants and scavenging activity which could neutralize the oxidizers that may directly modulate the antioxidant defense system.

Glutathione (GSH) is an antioxidant crucial for biotic and abiotic stress management and prevents DNA damage by reducing poisonous hydrogen peroxide and chelate heavy metals. High degree of oxidative stress could overwhelm any endogenous protective antioxidant system; Glutathione is one of the major non protein thiol that actively participates in many cellular function and amino acid transports. GPx and GST conjugate with GSH and scavenges ROS and detoxify many xenobiotics, carcinogens and free radicals that are generated by carcinogenic chemicals (Ashokkumar *et al.,* 2008).

Glutathione content were found to be decreased in positive control as compared to Negative controls. Whereas, *M. citrifolia* treated lymphocytes showed maximum level

of GSH and the increase is observed to be dose dependent. Similar observation was seen in Post-treated cells with *M. citrifolia* aqueous fruit extract but lesser when compared to pre-treated *M. citrifolia* cells (Table 1) which may be due to defensive effect of *M. citrifolia* against free radicals that may directly involved in the elimination of reactive oxygen metabolites.

In lipid peroxidation, the formation of aldehydes called malondialdehyde (MAD) a "marker of free radicals" that can react with proteins and nucleic acids to dysfunction the immune system. The imbalance in reactive oxygen species against antioxidative defense may create an oxidative stress that amplifies the DNA damage by initiating lipid peroxidation and prooxidative reactive iron that may erode the protective antioxidant system (Lee *et al*., 2004).

We observed (Table 2) an increased level of TBARS in (PC) due to the activation of lipid peroxidation than (NC). Whereas, the cells treated with *M. citrifolia* shows decreased level of TBARS due to the absence of free radicals that activate lipid peroxidation. Besides we also observe the increased concentration of *M. citrifolia* aqueous fruit extract gradually decreases the level of LPO. This same outcome was observed in Post-treated cells with *M. citrifolia* aqueous extract but it was considerably lesser than pre-treated *M. citrifolia* lymphocyte. Hence, the data provided us an idea that phenol, flavanoid, antioxidant and scavenging potential effect of *M. citrifolia* might inhibit the binding of lipid to MAD and that might possibly protect the dysfunction of immune system, consequently there could be a significant variation in the *M. citrifolia* treated cells.

Conclusion

It is concluded that *M. citrifolia* aqueous fruit extract under selected dosage could completely protect the lipid peroxidation but was moderately effective to improve the DNA damage. The previous studies have also demonstrated the protective effect of *M. citrifolia* fruit extract against anticancer drug. Additionally, these results will shed light on pre treatment of *M. citrifolia* which could provide a shield over the DNA and reduces the consequence of genotoxins. Therefore, these data further suggests the possible nutraceutical role of *M. citrifolia* against the lipid peroxidation involved human aliments. However, the concentrations of *M. citrifolia* selected in this *in vitro* experiment cannot be correlated with that required to render protection *in vivo*. Further detailed *in vivo* experimental models are required to establish the exact mechanism and nutraceutical role of *M. citrifolia*.

Acknowledgement

The authors are grateful to World Noni Research Foundation (WNRF) Perungudi Chennai, for providing *M. citrifolia* fruit for this study. The help rendered by Dr. N. Mathivanan, Assistant Professor. Centre for Advanced Studies (*CAS*) in Botany,

University of Madras, Maraimalai campus, Guindy, Chennai 600 025 is gratefully acknowledged.

The authors specially thank Mr. Anbarasan Chakarapani, Principal Scientist, Dr. Santhosh Mathapati., Scientist, Mr. Anand Duraiswamy., Ph.D Research Scholar, Mr. Theodore Lamuel Madhuram., Ph.D Research Scholar, Miss. Valli Pannerselvam, Mrs. Vimala Amulraj and Mrs. Lakshmi Pasupathi, Frontier Life line Pvt. Ltd., Chennai – 600 101 for their kind and enthusiastic help during this study.

References

Akinmoladun, A. C., Ibukun, E.O., Afor, E., Obuotor, E.M., Farombi, E.O. 2007. Phytochemical constituents and antioxidant activity of extract from the leaves of the Ocimum graticcimum. *Sci. Res. Essays*, 2:163-166.

Asad, N.R., Asad, L.M.B.O., Almeida, C.E.B., Felzenszwalb, I., Cabral-Neto,J.B., Leitão, A.C. 2004. Several pathways of hydrogen peroxide action that damage the *E. coli* genome. *Genet Mol Biol.,* 27:291-303.

Ashokkumar, P and Sudhandiran, G. 2008. Protective role of Luteolin on the status of lipid peroxidation and antioxidant defense against Azoxymethane induced experimental colon carcinogenesis. *Biomed Pharma*, 62: 590 – 597.

Boyum, A., Fjerdingstad, H.B., Martinsen, I., Lea, T., Løvhaug, D. *2002.* Separation of human lymphocytes from citrated blood by density gradient (NycoPrep) centrifugation: Monocyte depletion depending upon activation of membrane potassium channels. *Scand. J. Immunol.*, 56-1:76-84.

Dcbomoy, K., Lahiri, P., Bill Schnabel.1993. DNA isolation by a rapid method from human blood samples: Effects of MgCI2, EDTA, storage time and temperature on DNA yield and quality. *Biochemical Genetics,* 31:7 8.

Devasagayam, T.P., Tilak, J.C., Boloor, K.K., Sane, K.S., Ghaskadbi, S.S., Lele, R. D. 2004. Review: Free radicals and antioxidants in human health. Current status and future prospects. *J. Assoc. Phys. India,*52:794 804.

Dinis, T.C.P., Madeira, V.M.C., Almeida, M.L.M.1994. Action of phenolic derivates (acetoaminophen, salycilate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.,* 315: 161-169.

Duke, J., Bogenschutz, M., Duke. 2002: Hand book of medicinal plants. *Boca Raton, FL*: *CRC Press*, pp: 529.

Glaser, J.L. 1988,. Maharishi Ayurveda: an introduction to recent research. *Modern Science and Vedic Science*. Vol. 2, no. 1, pp: 89-108.

Gutteridge, J. M. C. 1984. Ferrous ion-EDTA-stimulated phospholipid peroxidation. A reaction changing from alkoxyl-radical- to hydroxyl-radical-dependent initiation. *Anal. Biochem. J.,* 224:697-701.

Halliwell, B.1994. Free Radicals, Antioxidants, and Human Disease: Curiosity, Cause, or Consequence. *Lancet*., 344:721-724.

Herrmann, M., Lorenz, H.M., Voll, R., Grunke, M., Woith, W., Kalde, J.R. 1994. A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucleic Acids Res*., 22:5506-5507.

Jacob, R.A. 1995. The Integrated Antioxidant System. *Nutr Res*.,15 (5):755-766.

Langseth, L. From the Editor. 1993: Antioxidants and Diseases of the Brain. *Antioxidant Vitamins Newsletter,* 4: 3.

Lee, J., Koo, N., Min, D.B. 2004. Comprehensive review in food science and food safety. *Inst. Food Technol.*, 3: 21-33.

Louli, V., Ragoussis, N., Magoulas, K. 2004. Recovery of phenolic antioxidants from wine industry by-products. *Biores. Technol*., 92: 201.

Lowry, O.H., Rosenbrough, N.J., Farr, A. L. and Randall, R.J. 1951. Protein Measurement with the Folin's Phenol Reagent. *J Bio Chem.,* 193, pp. 265-275.

Mantena, R.K.R., Wijburg, O.L.C., Vindurampulle, C., Bennett Wood, V.R., Walduck, A ., Drummond, G.R. 2008. Reactive oxygen species are the major antibacterials against Salmonella Typhimurim purine auxotrophs in the phagosome of RAW 264. *Cell. Microbiol.,* 10 (5): 1058 1073.

Mc Clatchey, W. 2002. From the Polynesian healers to health food stores; changing perspectives of *Morinda citrifolia* (Rubiaceae). *Integrated Cancer Therapy*, 1 (2): 110-20.

Mervat, M. M.E.L Far., Hanan, A. A. and Taie. 2009. "Antioxidant activities , total anthrocynins, phenolics and flavonoids contents of some sweet potato genotypes under stress of different concentrations of sucrose and sorbitol". *Australian J Basic Applied Sci.,* 3, 3609-3616.

Moron, M.A. and Mannervick, B. 1979. Levels of glutathione, glutathione s transferase activities in rat liver. *Biochemical et Biophysica Acta.,* 582: 67 78.

Ohkawa, H., Ohishi, N. and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem.,* 95:351 358.

Phillips, H.J. 1973. Dye exclusion tests for cell viability. In: *(Eds.) Kruse, P.F., Patterson, M.J., editors. Tissue Culture. Methods and Applications. New York. Academic Press, pp: 406 – 408.*

Rush, C. R., Higgins, S. T., Hughes, J. R., Bickel, W. K. and Wiegner, M. S. 1989. Acute behavioral and cardiac effects of alcohol and caffeine, alone and in combination, in humans. *Behavioural Pharmacology,* 4: 562–572.

Temple, N.J. 2000. Antioxidants and disease: More questions than answers. *Nutr. Res.*, 20: 449-459.

West, B.J., Jensen, C.J. and Westendorf, J. 2006. A safety review of Noni juice (J). *J Food Sci.,* 71:100-105.

Satyanarayana Rentala[1]
Sarva Mangala Dhurjeti[1]
K. Sudhakar[1]
USN Murthy[2]

Role of Noni (*Morinda citrifolia* L.) fruit extract on oxidative stress induced cataract formation in lens epithelial cells

Keywords: Oxidative stress, Lens epithelial cells, Cataractogenesis, Apoptosis and Divine Noni

Authors' affiliation :
Satyanarayana Rentala[1]
Sarva Mangala Dhurjeti[1]
K. Sudhakar[1]
[1]Department of Biotechnology, GITAM Institute of Technology, GITAM University, Visakhapatnam- 530 045, India
E-mail:rsn@gitam.edu
USN Murthy[2]
[2]Department of Ophthalmology, Rajiv Gandhi Institute of Medical Sciences (RIMS), Srikakulam, India

Abstract : It is well established that various factors such as oxidative stress, ultraviolet radiations (UV) and other toxic agents could induce cataract formation in *in vivo* and *in vitro* conditions. The cataract patients may have deficient defence systems against factors such as oxidative stress and UV at the onset of the disease. Such stresses can trigger lens epithelial cell apoptosis, which may initiate cataract development. To investigate the role of "Divine Noni" in protecting against oxidative stress, chick lens epithelial cells were exposed to hydrogen peroxide (H_2O_2) with a concentration of 100 ìM over a time course of several hours, with and without pre-treatment of "Divine Noni". Cell viability and intracellular reactive oxygen species were determined. The results in the present investigation suggest that "Divine Noni" can preserve physiological functions of chick lens epithelial cells during oxidative stress suggesting "Divine Noni" could be effectively used for cataract patients.

Correspondence to :
Satyanarayana Rentala
Department of Biotechnology, GITAM Institute of Technology, GITAM University, Visakhapatnam- 530 045, India
E-mail:rsn@gitam.edu

Introduction

Apoptosis is a distinct form of programmed cell death characterized by chromatin condensation, DNA fragmentation, cell shrinkage, and membrane blabbing (Steinberg *et al*., 1993; Shibata *et al*., 1994). Apoptosis of lens epithelial cells by various factors can cause cataract formation. Cataract impose financial burden on health-care systems and there is an urgent need to develop effective therapeutic agents for the prevention or treatment of cataract. Cataract in adults may be due to the result of injury to the lens by oxidative stress, exposure to radiation such as X- rays, ingestion of toxic substances and or use of some of the drugs. In spite of volumes of work on the biological and biochemical changes that take place in the lens, the underlying cause of cataract formation is still unknown. Whatever may be the underlying biochemical changes, they result in an increasing clouding of the lens until the whole lens loses its normal transparency and becomes white and opaque (Shibata *et al*., 1994; Livingston *et al*., 1994; McCarty *et al*., 1999; Klein *et al*., 1992; Freeman *et al*., 2001).

Scientists found that people at high risk of developing advanced stages of cataract formation, a leading cause of vision loss, lowered their risk by about 25 per cent when

treated with a high-dose combination of vitamin C, vitamin E, beta-carotene and zinc. In the same high risk group which includes people with intermediate cataract formation in one eye but not the other eye, the nutrients reduced the risk of vision loss caused by advanced cataract formation by about 19 per cent. Noni is rich with vitamin A, beta carotenoids, vitamin E, vitamin C, vitamin B complex; trace minerals like Ca, Mg, K, Zn, Molybdenum *etc;* flavonoids is one of the potent and powerful antioxidant.

Materials and Methods

Lens Organ Culture: The lenses used in this investigation were isolated from chicks brought from slaughter houses. The eyes were removed and the lenses were carefully dissected by a posterior approach. Lens Epithelial Cells (LECs) were separated from each of the dissected lenses by incubating in 1× Trypsin-EDTA solution for 1-2 h at 37°C. Cells of 0.5×10^6 were cultured in 6-well culture plate containing 1.5 ml minimal essential medium (MEM199, M-3769; Sigma) containing 20% FBS for 6 days. Transparent lenses were selected for experimentation. All chick lens experiments (n=6) were performed in the MEM 199 containing 26 mM NaHCO3 as buffer. The MEM 199 was prepared with ion-exchange double-distilled water, sterilized by filtration through 0.22-ìm filter with a pH adjusted to 7.4. 50ìM and 100ìM H_2O_2 (Sigma). "Divine Noni" was made to final concentration of 10 mM and diluted to appropriate concentration in culture medium as required. Unless otherwise stated, steroid treatment to cell cultures involved a 2h pre-incubation followed by continued administration of the steroid in the presence of H_2O_2. Everyday 1 ml of the medium was replaced.

Viability of cells assay*:* Cells were plated 24 h before the initiation of the experiment, at a density of 5000 cells per well in 96 well plates. Cells were exposed to two doses of H_2O_2 (50 and 100ìM) from 1 to 24 h. After exposure to H_2O_2, cells were rinsed with 1×PBS (pH 7.4), and viability was assessed by the addition of 25 ìM calcein AM. Calcein AM fluorescence was determined at an excitation of 485 nm and an emission of 538 nm with the microplate reader (FL600; Biotek). Percentage viability was calculated by normalization of all values to the H_2O_2-free control group (100%). Qualitatively the effect of "Divine Noni" on oxidative stress induced lens epithelial cell apoptosis was figured through hematoxylin-eosin staining.

Measurement of Reactive Oxygen Species: Fluorescent dye DCFH-DA (Sigma) was used to estimate the extent of cellular oxidative stress and generation of reactive oxygen species (ROS). Cells were plated 24h before initiation of the experiment at a density of 5000 cells per well in 96 well plates. Cells were loaded with DCFH-DA at a final concentration of 50 ìM for 45 minutes. After incubation, DCFH-DA was removed and cells were washed twice with 1×PBS (pH 7.4) and incubated with MEM containing 20% FBS with a bolus dose of H_2O_2 (50 and 100 ìM) for 20 to 120 minutes; DCFH-DA fluorescence was determined at an excitation of 485 nm and an emission of 538 nm, by

microplate-reader. Values were normalized to the percentage in untreated control groups. It should be noted that, DCFH-DA will be taken up by cells, usually undergoing de acetylation by esterase enzymes. Oxidation of DCFH within cells leads to fluorescent dichloro fluorescein which can easily be visualized (strong emission at 525 nm with excitation at 488 nm). Control groups were maintained without addition of either H_2O_2 or "Divine Noni".

Results

Cell viability: H_2O_2 induced a time and dose-dependent decline in cell viability (Fig.1). After 12 h of exposure of cells to 50 ìM H_2O_2 concentration, cell viability decreased to 65% ± 2% of control. Exposure to 100 ìM H_2O_2 had a significantly greater effect on viability of LECs. At 100ìM H_2O_2 exposure, the cell viability decreased to 45% ± 4% after 2h and almost complete cell death after 8h with H_2O_2 treatment. Viability increased with the "Divine Noni" treatment both in 50 and 100 ìM H_2O_2 treated LECs. The qualitative study of "Divine Noni" on apoptosis of H_2O_2 insulted LECs was shown using hematoxylin and eosin staining (results not shown here). Hematoxylin-eosin staining indicated that the cells maintained their cellular integrity and organization in *in vitro* culture whereas an unorganized cellular integrity was shown when used 50 and 100 ìM H_2O_2 treatments. Cells retained their normal network with "Divine Noni" in 100 ìM H_2O_2 treated cultures.

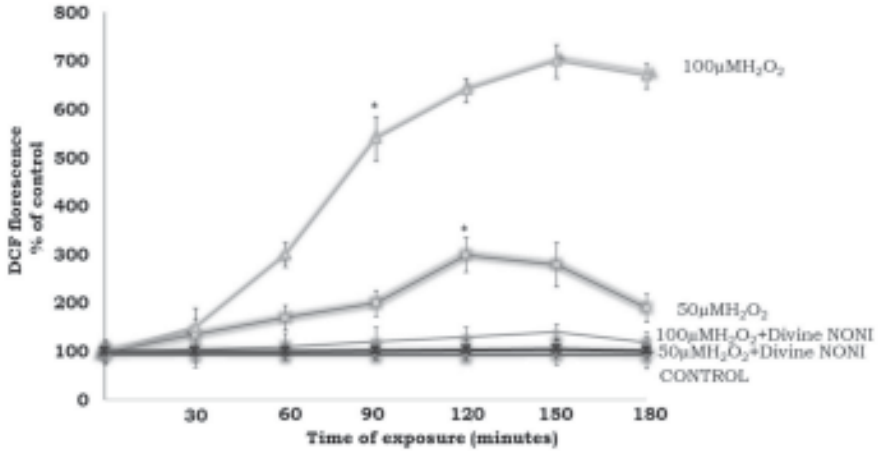


Fig. 1: Effects of H2O2 on ROS accumulation in lens epithelial cells

The control group was not treated with H_2O_2 after loading DCFH-DA. Depicted mean ± SEM (n = 6) percentages of DCF fluorescence normalized to the control (no H_2O_2) at each sampling time. Data is presented as amount of fluorescence (mean ± SEM; n=6). *p<0.05; using ANOVA followed by post hoc Bonferroni test.

ROS accumulation: As shown in Fig. 2 and as per the measured DCF fluorescence intensity, exposure to H_2O_2 caused an increase in intracellular ROS accumulation in the

cultured lens epithelial cells (n=6). 50ìM H_2O_2 initiated a modest but linear increase in ROS content (172% ± 11%) over control cells after a time period of 60 minutes. A higher dose of H_2O_2 (100 ìM) prompted a biphasic accumulation of ROS in the cultured cells. After 40 minutes, there was an ROS build up resulting in a moderate intracellular augmentation (200% of control), but accumulation reached to approximately 300% of control by 60 minutes with 100ìM H_2O_2. When 10mM of "Divine Noni" was used in the culture, the amount of ROS production decreased from 172% to 100%, as is in the normal controls. The per cent decrease was equal in cultures with 50ìM H_2O_2 and 100ìM H_2O_2.

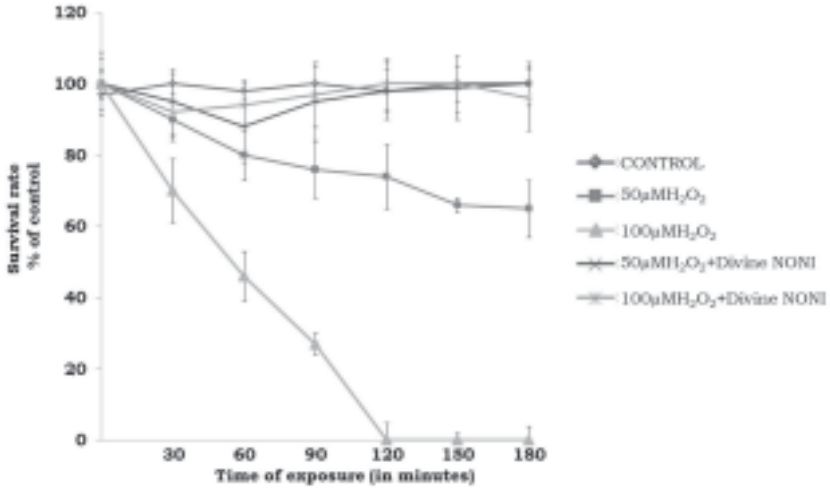


Fig. 2: Time course of the effects of H_2O_2 on viability of Lens epithelial cells

Data are expressed as a percentage of control group (non– H_2O_2- treated cells at each sampling time) and represent the mean ± SEM (n = 8). In all panels, when SEM bars are not shown, they are obscured by the symbol.

Discussion

The present study revealed that the cell death induced in chick lens epithelial cells by H_2O_2 is associated with production of intracellular ROS, decrease in cell viability and increase in late apoptotic cascade. In the present study LECs adaptively responded to a low concentration (50 µM) of H_2O_2 as evidenced by a moderate increase in ROS and relative resistance to cell death. In contrast, the higher (100 µM) concentrations of H_2O_2 were associated with a delayed but profound increase in ROS and nearly complete cell death within 2 to 3 h. The higher concentration of H_2O_2 is clearly a pathological insult from which cells failed to recover under the present study conditions. "Divine Noni" was effective in protecting LECs from death as evidenced form an increase in cell viability and decrease in production ROS with 10mM "Divine Noni".

Conclusions

These data suggest that at pharmacological concentration of "Divine Noni", cellular morphology and viability are preserved. The reported protection from cataracts during "Divine Noni" may be due to the cytoprotective effects of xeronine against H_2O_2 toxicities in lens epithelial cells by decreasing ROS production and increasing cell viability.

Acknowledgements

We are whole heartedly thankful to Dr. KV Peter, Dr. T. Marimuthu and Dr. Kirthi Singh of World Noni Research Foundation, Chennai for financial assistance for the entitled project.

References

Freeman, E.E., Munoz, B., Schein, O.D. and West, S.K. 2001. Hormone replacement therapy and lens opacities: the Salisbury Eye Evaluation project. *Arch Ophthalmol.*, 119: 1687–1692.

Klein, B.E., Klein, R. and Lee, K.E. 1998. Incidence of age-related cataract: the Beaver Dam Eye Study. Arch Ophthalmol., 116: 219–225.

Klein, B.E., Klein, R. and Linton, K.L.1992. Prevalence of age related lens opacities in a population- The Beaver Dam Eye Study. *Ophthalmology*, 99: 546– 552.

Livingston, P.M., Guest, C.S. and Stanislavsky, Y. 1994. A population-based estimate of cataract prevalence: the Melbourne Visual Impairment Project experience. *Dev Ophthalmol.*, 26: 1–6.

McCarty, C.A., Mukesh, B.N., Fu, C.L. and Taylor, H.R. 1999. The epidemiology of cataract in Australia. *Am. J. Ophthalmol.*, 128: 446–465.

Shibata, T., Sasaki, K., Katoh, N. and Hatano, T. 1994. Population-based casecontrol study of cortical cataract in the Noto area, Japan. *Dev Ophthalmol.*, 26: 25–33.

Steinberg, E.P., Javitt, J.C. and Sharkey, P.D. 1993. The content and cost of cataract surgery. *Arch Ophthalmol.*, 111: 1041–1049.

*Uday P. Pundir, Hannah P. Priyanka, Fariya Yasmine, Aparna Mohanty, RanVijay Singh, Miti Mishra, L. Hima and Srinivasan ThyagaRajan**

In vitro effects of Divine Noni on the age-related modulation of proliferation of lymphocytes, IL-2 production, IFN-γ production and antioxidant enzyme activities in draining lymph nodes of F344 rats

Authors' affiliation :
Uday P. Pundir,
Hannah P. Priyanka,
Fariya Yasmine,
Aparna Mohanty,
RanVijay Singh,
Miti Mishra,
L. Hima and
Srinivasan ThyagaRajan*
Integrative Medicine Laboratory,
Department of Biotechnology,
School of Bioengineering, SRM
University, Kattankulathur 603203,
Tamil Nadu
E-mail:
sthyagarajan@ktr.srmuniv.ac.in

Correspondence to :
Srinivasan ThyagaRajan*
Integrative Medicine Laboratory,
Department of Biotechnology,
School of Bioengineering, SRM
University, Kattankulathur 603203,
Tamil Nadu
E-mail:
sthyagarajan@ktr.srmuniv.ac.in

Keywords : Cytokine, Superoxide dismutase, Catalase, Glutathione peroxidise, Glutathione -S-transferase, TBARS, Draining lymph node, Noni

Abstract : *Morinda citrifolia* (Noni) or Indian mulberry has been widely used for the treatment of a variety of diseases in Ayurveda and folk medicine. It has been shown to possess antibacterial, antiviral, antifungal, antitumor, antihelminthic, analgesic, hypotensive, anti-inflammatory, and immune-enhancing properties owing to a wide range of potent plant phytochemicals. Our laboratory has established that the communication between the nervous system, endocrine system, and immune system is highly relevant in the maintenance of normal health and dys-regulations in this communication is responsible for the onset of various age-related diseases and cancer. The purpose of this study was to determine the effects of Divine Noni on the proliferation of lymphocytes, cytokine production and antioxidant enzyme [superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, and glutathione-s-transferase (GST)] activities in the lymphocytes isolated from the draining lymph nodes of young (3 months), middle-aged (8-9 months) and old (18-22 months) F344 rats. Lymphocytes isolated from the draining lymph node were treated with different doses of (Divine Noni 0.0001%, 0.01%, and 1%) in the presence and absence of Concanavalin A and lymphocyte proliferation and cytokine production (IL-2 and IFN-γ) were assessed. Divine Noni significantly increased Con A-induced proliferation and IL-2 production in the draining lymph lymphocytes from young, middle-aged, and old F344 rats in comparison with age-matched controls. Divine Noni alone significantly enhanced IFN-γ production in old rats, but significantly decreased in middle-aged (0.1%) and in young (0.0001%, 0.01%, and 1%) rats in comparison with age-matched controls. Divine Noni augmented antioxidant enzyme activities catalase in young (0.0001%) and old (0.0001% to 1%) F344 rats; increased GST activity in middle-aged (0.0001%) and old (0.0001% to 1%) F344 rats and significantly reversed age-related increase in lipid peroxidation in all age-groups. However, there was a decrease in the activity of GPx in all three age-groups while the activity of SOD decreased in young and middle-aged F344 rats.

Introduction

Various parts of the plant *Morinda citrifolia* (Noni) including the roots, barks, and fruits have been used for the treatment of a number of diseases for the past several centuries. The spectrum of its health benefits range from antibacterial, antiviral, antifungal, antitumor, antihelminthic, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects (Potterat and Hamberg, 2007). These effects are believed to be exerted through a number of phytochemicals such as anthraquinones in roots, and iridoids, fatty acid glycosides and alcohols in fruits. Several studies from others and our laboratories have provided a functional basis for the link between the three homeostatic systems namely, nervous system, endocrine system, and immune system (Wang, 2002). A number of studies have provided evidence for the existence of a bi-directional communication between the central nervous system (CNS) and the immune system (Wang, 2002). Many of the psychosomatic diseases have autoimmune phenomena in their etiology, a number of stressors precipitate the pathogenesis of neoplasms, and psychosocial factors such as depression, marital separation, divorce, are associated with immunosuppression. A number of areas in the brain, especially hypothalamus, respond to immune molecules through alterations in the metabolism of neurochemicals. However, the interaction and cross-regulation between these three systems are poorly understood. Although the beneficial effects of Noni have been reported through few studies (Palu *et al*., 2008), the mechanism(s) of action(s) from the perspective of the neuroendocrine-immune network have not been investigated. Therefore, the present study was conducted to assess the effects of *Morinda citrifolia* (Noni) on the age-related modulation of antioxidant status and proliferation of lymphocytes isolated from the draining lymph nodes of young, middle-aged, and old male F344 rats.

Materials and Methods

Animals

Young (3 months), middle-aged (8-9 months) and old (18-20 months) F344 rats were obtained from the National Institute of Nutrition at Hyderabad for the study. The animals were acclimatised to the animal house at SRM University for a period of one week. They were sacrificed by decapitation at 08:00 hrs. and the draining lymph nodes (axillary and inguinal lymph nodes) were dissected and kept in sterile tubes containing HBSS with sodium bicarbonate and HEPES.

Isolation of Lymphocytes

Lymphocytes were isolated by first dissociating the draining lymph nodes using thin forceps. The cell suspensions were then passed through a fine nylon mesh to remove large aggregates and washed once in HBSS. Cells were washed twice with

HBSS. After the final wash, the cells were resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2mM L?glutamine, 1mM sodium pyruvate, 0.01mM nonessential amino acids, $5x10^5$ M 2-mercaptoethanol, 100U/ml penicillin streptomycin solution, 24mM sodium bicarbonate and 10mM HEPES buffer for *in vitro* culture at 37°C in a humidified with 5% CO_2.

Morinda citrifolia (Divine Noni)

Divine Noni was obtained from World Noni Research Foundation, Chennai. It was serially diluted in substituted RPMI medium from 1% to 0.01% and finally, to 0.0001 % and a dose response curve was generated. The RPMI medium had 5% fetal bovine serum, 2mM L?glutamine, 1mM sodium pyruvate, 0.01mM nonessential amino acids, $5x10^5$ M 2-mercaptoethanol, 100U/ml penicillin streptomycin solution, 24mM sodium bicarbonate and 10mM HEPES buffer.

Lymphocyte proliferation assay

The proliferation of lymphocytes treated with (0.0001%, 0.01%, and 1%) Divine Noni was assessed using the MTT assay in the presence and absence of different doses of Con A ranging from 0.5 ìg/ml to 5 ìg/ml by maintaining appropriate controls. The concentration of Noni was determined on the basis of preliminary studies conducted in the laboratory that enabled to establish the dose response curve for the different juices. A 96 well plate containing 100 µl samples having 90% confluence was treated with MTT reagent, incubated for 3 hrs and read at 620 nm after completely solubilising it in isopropanol containing 37% HCl. Triplicate wells were used for each experimental condition. Plated cells were incubated in the presence of 5% CO_2 at 37°C for 72 hrs. Replacement of medium containing Noni fruit dilutions was performed every day.

Con A-induced cytokine production

PBMCs were cultured with 1.25 mg/ml of Con A in supplemented RPMI medium at 37°C/ 5% CO_2. After 24 hours, supernatants were collected for cytokine assays (IL-2 and IFN-ã) and assayed by ELISA kits obtained from e Biosciences, San Diego, USA.

Antioxidant enzyme activities

Antioxidant enzyme activities were assessed according to the procedure mentioned below in PBMC lysates.

a) Estimation of SOD activity: The superoxide dismutase activity was measured in terms of percentage inhibition of epinephrine auto oxidation (Sun and Zigman, 1978). The tissue was homogenized in a 5:3 ice-cold mixture of ethanol and

chloroform, centrifuged and the supernatant thus obtained was used for the assay. The sample was diluted using 0.1M carbonate buffer, pH 10, added equal volumes of 0.6mM EDTA and 1.3mM Epinephrine in carbonate buffer, vortexes and read immediately at 480 nm The optical density was read immediately at 412 nm in a spectrophotometer at time intervals of 0', 30' and 60' respectively. The results were expressed in terms of units/ min/ mg of protein.

b) Estimation of CAT activity: The total catalase activity was estimated by the method of Goth L (Goth1991). The total CAT activity was measured by a hydrogen peroxide based assay where it forms a complex with ammonium molybdate. The sample was suspended in 60mM sodium phosphate buffer, pH 7.4, with 65mM hydrogen peroxide solution and incubated for 4 minutes after which the reaction was stopped using 32.4mM ammonium molybdate and the optical density was measured 405 nm. The total catalase activity was expressed in units/ min/ mg of protein.

c) Estimation of GPx activity: The GPx activity was measured using Ellman's Reagent (Ellman, 1959). Briefly, the sample was diluted in 0.4M phosphate buffer pH 7, with 10mM sodium azide, 4mM reduced glutathione, 2.5mM hydrogen peroxide and incubated for 0', 30' and 60' after which the reaction was stopped with 10% trichloroacetic acid and centrifuged. To the supernatant, added 0.3M disodium hydrogen phosphate solution and 1mM Di-thio-nitro-benzene in 1% sodium citrate. The optical density was read immediately at 412 nm in a spectrophotometer. Standard curve was obtained using serial dilutions of 0.1 Tri-nitrobenzene in water. The results were expressed in terms of units/min/ mg of protein.

d) Estimation of GST activity: The GST activity was measured using 1-chloro 2, 4-dinitrobenzene (CDNB) (Habig *et al*., 1974). Briefly, the sample was diluted in 0.5 M phosphate buffer pH 6.5, added 25 mM 1-chloro 2,4-dinitrobenzene in 95% ethanol and 20 mM reduced glutathione. The optical density was read immediately at 340 nm in a spectrophotometer at 30 seconds intervals for 1.5 minutes. One unit of GST activity is defined as the amount of enzyme producing 1 ìM of GS-DNB conjugate/min under the conditions of the assay.

Estimation of Lipid peroxidation:

The extent of lipid peroxidation was measured in terms of rate of formation of adducts with thiobarbituric acid according to the method of (Yagi, 1996). The extent of lipid peroxidation was measured in terms of formation of adducts with thiobarbituric acid (TBA). The sample was treated with ice-cold 10% trichloroacetic acid for precipitation of proteins, incubated for 15 min and centrifuged at 2200g for 15 min. The supernatants obtained were treated with equal volumes 0.67% TBA and incubated in a boiling water bath for 10 min. After cooling, the optical density

was measured at 532 nm using a spectrophotometer. Standard curve was obtained using serial dilutions of 1, 1, 3, 3- tetraethoxy-propane or malondialdehyde (MDA) in distilled water. The results were expressed in terms of MDA equivalents/min/mg of protein.

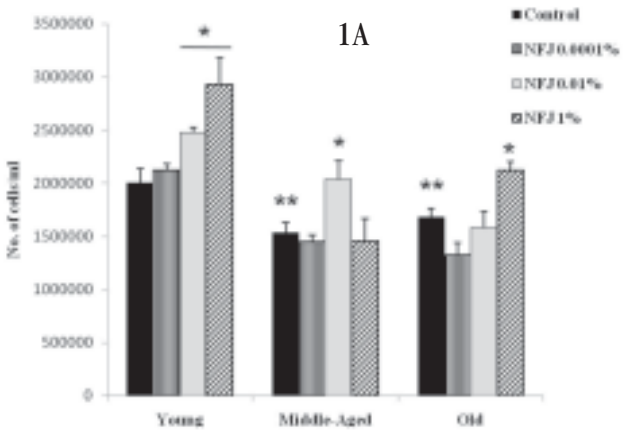
Statistical Analysis:

Data was analysed using ANOVA by SPSS software package. Parameters that attained significance with ANOVA ($P<0.05$) were further analyzed by post-hoc tests.

Results

1. Effects of Divine Noni on age-related alterations in lymphocyte proliferation and cytokine production

Proliferation of T-lymphocytes from the draining lymph showed significant ($P<0.05$) age-related decline (Fig. 1A). In young animals, there was a dose-dependent significant ($P<0.05$) increase in proliferation of lymphocytes isolated from draining lymph nodes 72 hours after treatment with Divine Noni (0.01% and 1%; Fig. 1A). In contrast, there was a significant increase in the proliferation of lymphocytes treated with 0.01% Divine Noni alone in middle-aged rats while lymphocyte proliferation significantly ($P<0.05$) increased when treated with 1% Divine Noni alone in old rats. There was a significant increase ($P<0.05$) in IL-2 production in young, middle-aged and old rats following treatment with all the doses of Divine Noni (Fig.1B). Similarly, IFN-γ production was increased in old (0.0001%, 0.01%, and 1%) rats thereby reversing the age-associated decline in the cytokine production, although in middle-aged (0.01%) and young (0.0001%, 0.01%, and 1%) rats there was a significant ($P<0.05$) decrease (Fig.1C).



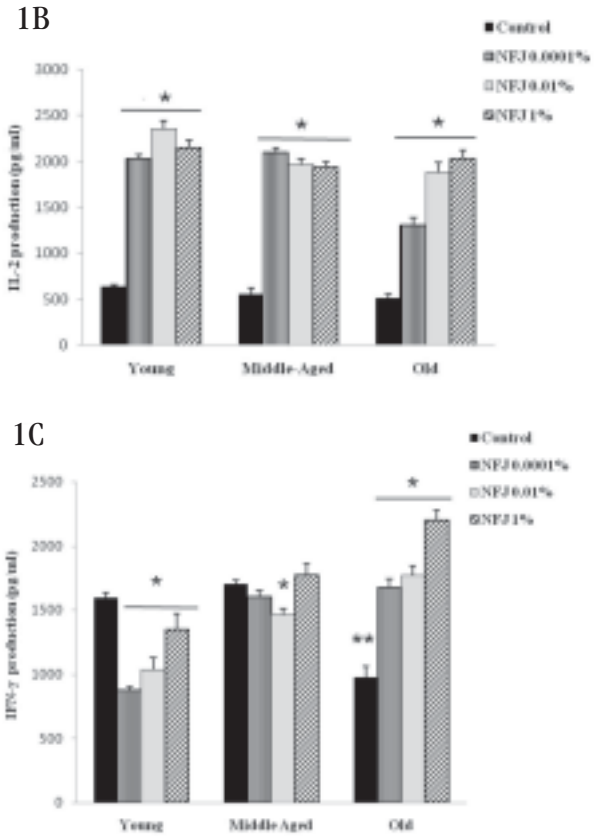


Fig. 1: Effects of Divine Noni on lymphocyte proliferation and cytokine production by draining lymph nodes lymphocytes

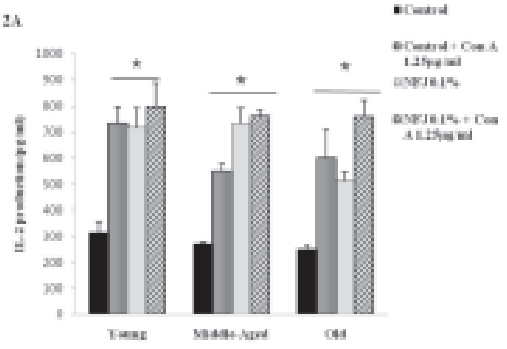
(A) Treatment with Divine Noni increased the proliferation of lymphocytes isolated from draining lymph nodes in young (0.01% and 1%), middle-aged (0.01%) and old (1%) F344 rats.

(B) Treatment with Divine Noni (0.0001%, 0.01%, and 1%) increased IL-2 production in young, middle-aged and old rats.

(C) Divine Noni treatment reversed the age-related decline in production in old (0.0001%, 0.01%, and 1%) rats, although in middle-aged (0.01%) rats and young (0.0001%, 0.01%, and 1%) rats there was a significant decrease in IFN-γ production.

*Significantly ($P<0.05$) different from age-matched control;

**Significantly ($P<0.05$) different from young control. (Divine Noni)



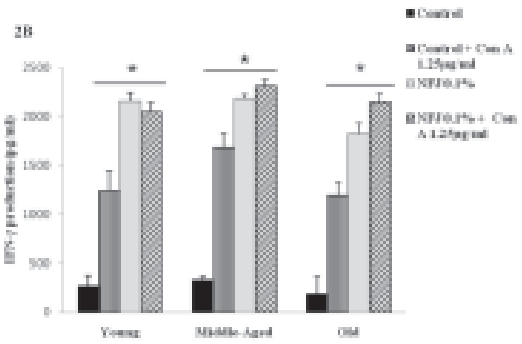
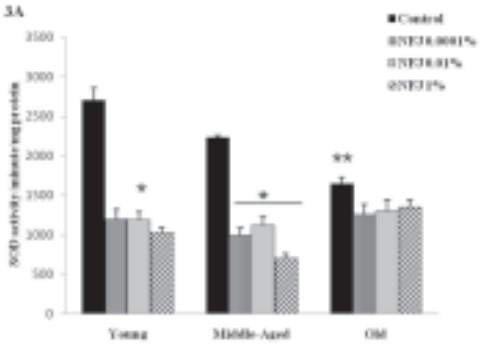
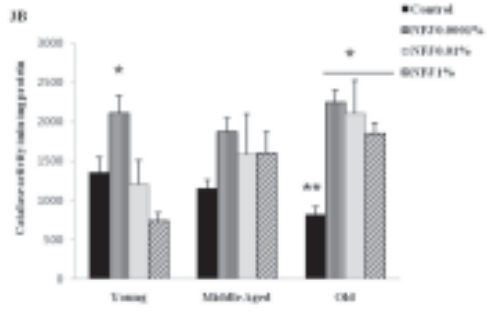


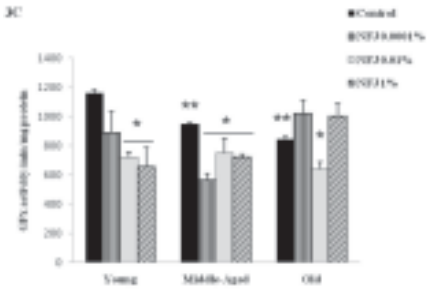
Fig. 2 : Effects of Divine Noni on Con A-induced cytokine production by lymphocytes from draining lymph nodes

Treatment with Divine Noni (0.1%) significantly increased (P<0.05) con A-induced IL-2 production (A) and IFN-γ production (B) in young, middle-aged, and old rats.

*Significantly (P<0.05) different from age-matched control. (Divine Noni)







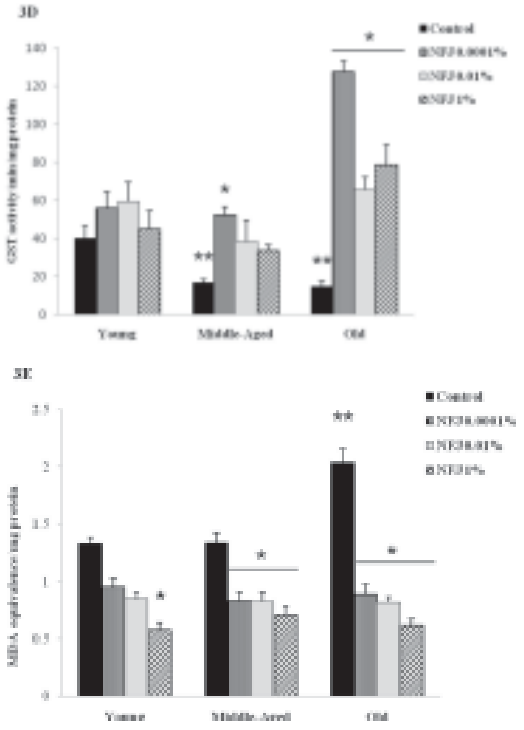


Fig. 3 : Effects of Divine Noni on antioxidant enzyme activities and extent of lipid peroxidation in the lymphocytes from draining lymph nodes

(A) Divine Noni treatment decreased the SOD activity in young (0.0001%, 0.01%, and 1%), middle-aged (0.0001%, 0.01%, and 1%), rats but there were no change in old F344 rats.

(B) Treatment with Divine Noni increased the catalase activity in young (0.0001%), and old (0.0001%, 0.01%, and 1%) F344 rats, but there were no change in catalase activity old F344 rats. (C) The GPx activity was also decreased following Divine Noni treatment in young (0.01%, and 1%), middle-aged (0.0001%, 0.01%, and 1%) and old (0.01%) F344 rats. A significant age-related decline in catalase activity (A) and GPx activity. (C) was also found.

D) Similarly, an increase in glutathione-S-transferase activity was found in middle-aged (0.0001%) and old (0.0001%, 0.01%, and 1%) rats following Divine Noni treatment.

(E) Divine Noni treatment reduced the age-related increase in lipid peroxidation in young (1%), middle-aged and old (0.0001%, 0.01%, and 1%) F344 rats.

*Significantly ($P<0.05$) different from age-matched control;

**Significantly ($P<0.05$) different from young control. (Divine Noni)

2. Effects of Divine Noni on Con A-induced cytokine production

Treatment with Divine Noni (0.1%) significantly increased ($P<0.05$) Con A-induced IL-2 production (Fig. 2A) and IFN-ã production (Fig. 2B) in young, middle-aged and old rats.

3. Effects of Divine Noni on age-related alterations in the antioxidant enzyme activities of lymphocytes from F344 rats :

Superoxide dismutase:

The activity of superoxide dismutase (SOD) did not show any age-related alterations (Fig. 3A). Divine Noni (0.0001%, 0.01%, and 1%) treatment significantly ($P<0.05$) decreased the SOD activity in young and middle-aged rats although there was no significant change in old F344 rats.

Catalase

The activity of catalase in the lymphocytes from draining lymph nodes was found to significantly decline ($P<0.05$) in old rats compared to young rats. However, treatment with Divine Noni significantly increased the catalase activity in young (0.0001%) and old (0.0001%, 0.01%, and 1%) F344 rats but there was no change in catalase activity in middle-aged F344 rats (Fig. 3B).

Glutathione peroxidase

A significant age-related decrease ($P<0.05$) in GPx activity (Fig. 3C) was also found. The GPx activity was also significantly ($P<0.05$) decreased following Divine Noni treatment in young (0.01% and 1%), middle-aged (0.0001%, 0.01%, and 1%) and old (0.01%) F344 rats (Fig. 3C).

Glutathione-S-transferase:

With age, there was a significant ($P<0.05$) age-related decrease in GST activity in the lymphocytes isolated from draining lymph nodes. Treatment with Divine Noni significantly ($P<0.05$) increased the glutathione-S-transferase activity in middle-aged (0.0001 %) and old (0.0001%, 0.01%, and 1%) F344 rats (Fig.3D).

Lipid Peroxidation:

With age there was a significant ($P<0.05$) increase in the extent of lipid peroxidation in the lymphocytes. Divine Noni treatment significantly ($p<.05$) reduced the age-related increase in lipid peroxidation in young (1%), middle-aged and old (0.0001%, 0.01%, and 1%) F344 rats (Fig. 3E).

Discussion

The results from the present study demonstrate that Divine Noni enhanced proliferation and cytokine production in lymphocytes isolated from draining lymph nodes in the presence and absence of Concanavalin A in young, middle-aged and old rats.

There was a concurrent increase in the IFN-γ production on treatment with Divine Noni in young, middle-aged and old rats in the presence and absence of con A. Further, IL-2 production was also increased in the lymphocytes isolated from the draining lymph node of young, middle-aged and old rats after treatment with Divine Noni when compared to age-matched controls treated with or without con A.

Unpublished data from our laboratory has shown that Noni juices increased the proliferation of lymphocytes in the case of young Wistar rats. The findings of the present study are also concurrent with these findings and other studies that have shown the immune-enhancing effects of the Noni fruit extracts (Palu *et al.*, 2008). Since plant extracts are known to have lectins that can stimulate proliferation in the lymphocytes, the increase in proliferation observed with the Noni extract could also be due to a lectin in the juice. The individual component (lectin and similar phytochemicals) that is responsible for the proliferative action must be ascertained and its selective activation of immune cells must be investigated.

Studies in lymphocytes isolated from the spleen conducted in our lab on F344 rats have also provided corroborative evidences. Divine Noni was found to reverse the age-associated decline in Catalase, and GST in middle-aged and old rats when compared with age-matched controls. Similarly, the age-associated increase in the extent of lipid peroxidation and glutathione peroxidase activity were significantly reduced in young, middle-aged and old rats following Divine Noni treatment. Treatment with Divine Noni significantly reduced the activity of superoxide dismutase activity in young and middle-aged groups.

Plant extracts are known to be rich in flavonoids and other free-radical scavengers that contribute to their antioxidant properties (Patterat and Hamsbuger, 2007). Thus Divine Noni may contribute to enhancing the immune function by increasing the antioxidant enzyme activities, reducing the age-dependent increase in free-radicals and through possible lectins that may also contribute to the same effect. Further there is a strong role played by the TH-1 cytokines as shown by increases in IL-2 and IFN-γ in contributing to this effect. It is important to examine the extent of influence exerted by Noni phytochemicals in modulating the bidirectional communication between the neuroendocrine system and immune system to alter the aging process (Palu *et al.*, .2008).

References

Ellman, G.L. 1959. Tissue sulfhydryl groups. *Arch Biochem Biophys.,* 82(1):70-77.

Goth, L. 1991. A simple method for determination of serum catalase activity and revision of reference range. *Clin Chim Acta*., 196(2-3): 143-151.

Habig, W. H., Pabst, M.J. and Jakoby, W.B. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem*., 249:7130-7139.

Palu, A.K., Kim, A.H., West, B.J., Deng, S., Jensen, J. and White, L.2008. The effects of *Morinda citrifolia* L. (Noni) on the immune system: its molecular mechanisms of action. *J. Ethnopharmacol*., 115(3):502-506.

Potterat, O., Hamburger, M. 2007. *Morinda citrifolia* (Noni) fruit—phytochemistry, pharmacology, safety. *Planta Med*., 73(3):191-199.

Sun, M. and Zigman, S. 1978. An improved spectrophotometric assay for superoxide dismutase based on epinephrine autoxidation. *Anal Biochem*., 90(1): 81-89.

ThyagaRajan, S. and Felten, D.L.2002. Modulation of neuroendocrine—immune signaling by L-deprenyl and L-desmethyldeprenyl in aging and mammary cancer. *Mech Ageing Dev.*, 123(8):1065-1079.

Wang, M.Y., West, BJ., Jensen, C.J., Nowicki, D., Su, C., Palu, A.K. and Anderson, G. 2002. *Morinda citrifolia* (Noni): A literature review and recent advances in Noni research. *Acta Pharmacol Sin.*, 23(12):1127-1141.

Yagi, K. 1996. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Res*., 15: 212-216.

Periyasamy Selvam[1]
T. Paul Pandi[1]
Nouri Neamati[2]
Tino Sanchenz[2]

Studies on LEDGF/P75-HIV-integrase inhibitory activity of isolated compounds of ethanol extract of *Morinda citrifolia* L.

Keywords : *Morinda citrifolia*, HIV-Integrase activity, HIV IN/LEDGF

Authors' affiliation :
Periyasamy Selvam[1]
T. Paul Pandi[1]
[1]Nova College of Pharmaceutical Education and Research, Jupudi Post, Ibrahimpatnam Mandel, Krishna dt, A.P
Nouri Neamati[2]
Tino Sanchenz[2]
[2]School of Pharmacy, University of Southern California, USA

Abstract : The development of antiviral drugs has provided crucial new means to mitigate or relieve the debilitating effects of many viral pathogens. A rich source for the discovery of new HIV infection inhibitors has been and continues to be, the 'mining' of the large diversity of compounds already available in nature and specifically those from botanical extracts. *Morinda citrifolia* is used in the Indian system of medicine for the treatment of variety of diseases including HIV/AIDS. Present work is to study HIV integrase and HIV Integrase/ Lens Epithelium Derived Growth Factor (LEDGF) inhibitory activity of compounds isolated from ethanolic extract of *Morinda citrifolia* L Noni. Compounds of ethanolic extract of *Morinda citrifolia* fruit have been studied against inhibition of HIV-1 integrase enzymatic activity by oligonucleotide based assay and HIV IN/LEDGF-P75 assay performed by Alpha Screen Technology, respectively. All compounds were investigated for both 3'processing and strand transfer process of HIV-1 integrase enzymatic activity and MCF C-14 displayed inhibitory activity against both step of HIV In enzymatic activity (3'P IC_{50}:94 µg/ml and ST IC_{50}:58 µg/ml). All the compounds (except MCF C-17 and C-18) exhibited inhibitory activity against HIV-1 integrase/LEDGF interaction (IC_{50}:0.54-36 µg/ml). and MCF C-14 exhibits potent inhibitory activity against the HIV IN/LEDGF protein-protein interaction at the concentration of 0.54 µg/ml.

Correspondence to :
Periyasamy Selvam
Nova College of Pharmaceutical Education and Research, Jupudi Post, Ibrahimpatnam Mandel, Krishna dt, A.P
Email:
periyasamy_selvam@yahoo.co.in

Introduction

Acquired immunodeficiency syndrome (AIDS) is a life threatening and debilitating disease state caused by retrovirus HIV. Three different classes of chemotherapeutic agents are generally combined to block the replication of human immunodeficiency virus type 1 (HIV-1) responsible for AIDS and to prevent the occurrence of resistance: reverse transcriptase inhibitors (RTI), protease inhibitors (PRI), and fusion inhibitors. This widespread triple combination therapy is referred to as HAART [highly active antiretroviral therapy] (Richman, 2001). HAART effectively inhibits HIV replication to such an extent that the virus becomes undetectable in the blood. However, it fails to eradicate viruses that are integrated in the host genome or that persist in cellular and anatomical "reservoirs". In addition, prolonged drug exposure led to HIV drug

resistance, thus reducing patients' therapeutically available options (Cohen., 2002). The above considerations and the toxicity of a number of antiretroviral agents have fueled the discovery of drugs against additional targets.HIV-1 integrase (IN) is a vital enzyme which catalyzes the insertion of proviral DNA into host cell genome. This is an essential step in retroviral replication (Anthony 2004). HIV-1 IN comprises three structurally and functionally distinct domains: the amino terminal domain (residues 1-50), the catalytic core domain (residues 51-212) and the carboxyl terminal domain (residues 213-288). With these three domains, IN performs the integration process which consists of two subsequent steps: 3'-processing and strand transfer. Raltegravir which is the first US FDA-approved drug targeting HIV-1 IN on the strand transferring step would incur Raltegravir-resistant HIV shortly after administration (Malet *et al.,* 2008).

To overcome HIV-1 resistance, medications with novel mechanisms of action should be developed. It was found that human cellular cofactors play key roles in HIV-1 IN performing function (Van Maele *et al*., 2006). Among them, lens epithelial-cell derived growth factor (LEDGF, also referred to as p75) was identified in complex with HIV-1 IN and plays an essential role in the distribution of IN in the nucleus, which is the key procedure for viral replication (Llano *et al.,* 2006). Experiments confirmed that p75 bound to HIV-1 IN via a small, approximate 80- residue IN-binding domain (IBD) within its C-terminal region. IBD of p75 was mapped to residues 347–429 and interacted specifically with the IN core domain (Vanegas *et al*., 2005). p75-mediated chromatin tethering depended on specific interactions between the p75 IBD and the IN core domain. Therefore, it was speculated that disturbing or blocking p75-IN interaction would prevent the replication of the virus (Al-Mawsawi *et al.,* 2007; Poeschla E M 2008; De Rijck *et al.,*2006). Small molecule inhibitors of HIV IN/LEDGF have emerged as promising new class of antiviral agents for the treatment of HIV/AIDS.

Morinda citrifolia L (Noni) is a versatile medicinal plant with a broad spectrum of pharmacological activities. *Morinda citrifolia* is reported to possess hepatoprotective (Wang *et al.,* 2008a,b), anticancer (Akihisa *et al.,* 2008), immunomodulatory (Palu *et al.,* 2008), anti-inflammatory (Palu *et al.,* 2007), wound healing (Nayak *et al.,* 2007), antioxidant(Su *et al.,* 2005), anti-tubercular (Saludes *et al.,* 2002) and wide spectrum of biological activity (Pawlus and Kinghorn 2007) and safe herbal drug (West *et al.,*2006). Recently much attention has been devoted for searching potential antimicrobial agents from natural products and anti-HIV activity of *Morinda citrifolia* is relatively less explored. The present work is to study the HIV integrase and HIV IN/LEDGF inhibitory activity of compounds isolated from ethanolic extract of the fruit powder of *Morinda citrifolia.* HIV Integrase is a crucial enzyme for HIV replication and an attractive therapeutic target for designing novel anti-HIV agents. To understand the molecular mechanism for the antiviral efficacy of Noni we investigated its inhibitory activity against HIV-1 integrase and HIV IN/LEDGF interaction.

Preparation of Extracts: The fruit powder of *Morinda citrifolia* are dried under shade and powdered. The powder is extracted with ethanol for five days by cold maceration. It is then filtered to get the extracts evaporated to dryness under vacuum. The vacuum dried ethanolic extract is subjected to column chromatography for isolation of compounds and compounds MCF C-10, 12, 14, 15, 16, 17 and 18 were isolated and further purified by TLC.

Anti-HIV IN assays. Expression of the recombinant IN in *Escherichia coli* and subsequent purification of the protein were performed as previously reported (Marchand *et al.,* 2004) with addition of 10% glycerol to all buffers. Preparation of oligonucleotide substrates has been described.36 Integrase reactions were performed in 10 μL with 400 nM of recombinant IN, 20 nM of 52 -end [32P]-labeled oligonucleotide substrate, and inhibitors at various concentrations. Solutions of 10% DMSO without inhibitors were used as controls. Reactions were incubated at 37 °C (30 min) in buffer containing at a final concentration of 50 mM MOPS, pH 7.2 and 7.5 mM of divalent cations ($MgCl_2$ unless $MnCl_2$ is otherwise indicated). Reactions were stopped by addition of 20 μL of loading dye (10 mM EDTA, 98% deionized formamide, 0.025% xylene cyanol, and 0.025%bromophenol blue). Reactions were heated at 95 °C (1 min) then subjected to electrophoresis in 20% polyacrylamide-7 M urea gels. Gels were dried and reaction products were visualized and quantitated with a PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). Densitometric analyses were performed using ImageQuant from Molecular Dynamics, Inc. The concentrations at which enzyme activity was reduced by 50% (IC50) were determined using "Prism" software (GraphPad Software, San Diego, CA) for nonlinear regression to fit dose–response data to logistic curve model. The anti-HIV integrase inhibitory data are presented in Table 1.

Table 1: HIV Integrase and HIV IN/LEDGF inhibitory activity of Isolated compounds

Compound	3'P IC50a (µg/mL)	ST IC50b (µg/mL)	LEDGF IC50c (µg/mL)
MCF-ET-C-10-ME	>100	>100	14
MCF-ET-C-12-ME	>100	>100	10
MCF-ET-C-14-ME	94	58	0.53
MCF-ET-C-15-ME	>100	>100	36
MCF-ET-C-16-ME	>100	>100	31
MCF-ET-C-17-ME	>100	>100	>50
MCF-ET-C-18-ME	>100	>100	>50

a. Concentration required to inhibits 3' processing reaction, b. Concentration required to inhibits 3' processing reaction, a. Concentration required to inhibits HIV IN/LEDGF interaction.

AlphaScreen assay

The HIV IN-LEDGF/p75 AlphaScreen was developed as a 3-step procedure for high-throughput screening (Hou *et al.,* 2008;Selvam *et al.,*2012). For the assay, 2.5-µl aliquots of test compounds, resuspended in 10% DMSO, were first predispensed on a 384-well ProxiPlate. Then 5 µl of IN CCD, in 1.25 µ l assay buffer (25 mM HEPES, pH 7.3, 150 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 0.1% bovine serum albumin [BSA]) was added to a final concentration of 10 nM, after which the assay plates were incubated for 30 min at room temperature. The remaining components in 1.25 x assay buffer were then added in 5 µl and included p75 IBD protein (final 10 nM), glutathione donor beads (final 2.5 µg/ml), and Ni-chelate acceptor beads (final 2.5 µg/ml). Following a 60-min room temperature incubation under subdued light conditions (less than 100 Lux), the assay plates were analyzed with an EnVision multilabel plate reader (PerkinElmer, Boston MA). The anti-HIV integrase/LEDGF inhibitory data are presented in Table 2.

Table 2: HIV Integrase and HIV IN/LEDGF inhibitory activity of Ethanolic fractions

Compounds	3'Pa (µg/ml)	STb (µg/ml)	LEGDF-INc (µg/ml)
MCF-F1	>100	>100	>30
MCF-F2	49 ± 15	4 ± 1	57% at 30
MCF-F3	72 ± 25	9 ± 4	50
MCF-F4	30	27 ± 18	> 80
MCF-F5	63 ± 18	35 ± 21	80
MCF-F6	73 ± 39	56 ± 33	60% at 70

[a]Concentration required to inhibits 3' processing reaction, [b]Concentration required to inhibits 3' processing reaction, [a]Concentration required to inhibits HIV IN/LEDGF interaction.

Library screening for HIV IN–LEDGF/p75 interaction inhibitors

Screening was carried out on a BioCel Automated Workcell (Velocity11, Menlo Park, CA), allowing the timed transfer of plates between workstations using an integrated 360 high-speed robotic plate handler. The compound library (2 µg/ml individual compound concentrations) was formatted using an 8 x 8 orthogonal mixing protocol, with each compound appearing twice on each plate in separate mixtures; this provides an expedited process for hit identification.23 The reaction was carried out in buffer containing (final concentrations) 25 mM HEPES, 2 Mm MgCl2, 150 mM NaCl, 1 mM dithiothreitol, and 1 mg/ml BSA, pH 7.6. Assay plates were prepared containing 2.5 µl of test compound mixture (8 compounds per pool, 2 µg/ml compound concentration) in 2% DMSO. Purified HIV IN-CCD (1.99 ng in 5 µl) was

added, and the reaction was incubated for 30 min at room temperature. The binding reaction was started with the addition of a mix containing 5.18 ng purified p75 IBD plus 31.25 ng Nickel Chelate AlphaScreen acceptor beads plus 31.25 ng GSH AlphaScreen donor beads (in 5 µl). After incubation for 2 h at room temperature in the dark, the plates were analyzed using an EnVision multilabel plate reader.

Results

Compounds isolated from ethanol extract of *Morinda citrifolia* L (Noni) have been evaluated for HIV Integrase and HIV IN/LEDGF inhibitory activity (Table 1). All the compounds were investigated for inhibition of both 3' processing (3'P) and strand transfer process (ST) of HIV integrase enzymatic activity and also tested for the inhibition of HIV Integrase/ Cellular Co-factor Lens Epithelium Derived Growth factor (LEDGF) interaction by Alpha screen technique. All compound (except MCF C-17 and 18) exhibited inhibitory activity against HIV-1 integrase/LEDGF protein-protein interaction (IC_{50}: 0.54-36 ìg/ml). Compound MCF C-14 displayed inhibitory activity against both step of HIV In enzymatic activity (3'P IC_{50}:94 µg/ml and ST IC_{50}:58 µg/ml). All the compounds tested for the inhibition of HIV IN/LEDGF interaction and MCF ET C-14 demonstrated for inhibition of HIV IN/LEDGF interaction.

Discussion

The Polynesians utilized the whole Noni plant in various combinations for herbal remedies (Wang *et al.,* 2002; McClatchey 2002) such as arthritis, diabetes, high blood pressure, muscle aches and pains, menstrual difficulties, headaches, heart disease, AIDS, cancers, gastric ulcers, sprains, mental depression, senility, poor digestion, atherosclerosis, blood vessel problems and drug addiction. Review of literature revealed that only three studies were available for anti-HIV activity of Noni. 1) The compound isolated from Noni roots named 1-methoxy-2-formyl-3-hydroxyanthraquinone suppressed the cytopathic effect of HIV infected MT-4 cells, without inhibiting cell growth (Umezawa *et al.,* 1992). 2) Viral protein R (Vpr), one of the human immunodeficiency virus type 1 (HIV-1) accessory proteins, contributes to multiple cytopathic effects, G2 cell cycle arrest and apoptosis. The mechanisms of Vpr have been intensely studied because it is believed that they underlie HIV-1 pathogenesis. Damnacanthal (Dam), a component of Noni fruit, as an inhibitor of Vpr induced cell death (Masakazu *et al.,* 2006). 3) Ethyl acetate and aqueous phase of the methanolic extract of stems were subjected for screening of their in vitro HIV screening at NCI Bethesda, U.S.A (Bina *et al.,* 2007). The ethyl acetate phase showed confirmed moderate activity against cell line CEM-SS with IC_{50} >2.50×102 µg/mL, EC_{50} 2.36×102 µg/mL and TI50 (IC/EC) >1.06×100. Previously various crude extracts of fruit portion of *Morinda citrifolia* tested inhibition of HIV Integrase activity, all the exhibits significant HIV Integrase activity and acetone extract (AMC)

is potent inhibitor of HIV 1 integrase enzymatic activity (Selvam et al., 2010). Various fractions of ethanolic extract of Noni fruit (Table 2) also demonstrated for inhibitory activity against HIV IN-LEDGF/p75 interaction (Selvam *et al.,* 2012). From this study isolated compounds of ethanolic extract of *Morinda citrifolia* fruit exhibits significant inhibitory activity against HIV Integrase/LEDGF protein-protein interaction.

References

Al-Mawsawi, L.Q. and Neamati, N. 2007. Blocking interactions between HIV-1 integrase and cellular cofactors: an emerging antiretroviral strategy. *Trends Pharmacol Sci.,* 28:526–535.

Akihisa, T., Matsumoto, K., Tokuda, H., Yasukawa, K., Seino, K.I., Nakamoto, K. H., Kuninaga H., Suzuki, T. and Kimura, Y. 2008. Anti-Inflammatory and Potential Cancer Chemopreventive Constituents of the Fruits of *Morinda citrifolia*. *J. Nat.Prod*., 71(7): 1322-25.

Anthony, N. J. 2004. HIV-1 integrase: a target for new AIDS chemotherapeutics. *Curr. Top. Med. Chem.*, 4:979–990.

Bina, S., Siddiqui, Fouzia, A., Sattar, Sabira Begum, Tahsin Gulzar, and Fayaz Ahmad. 2007. Chemical Constituents from the Stems of *Morinda citrifolia* Linn, *Arch Pharm Res.,* 30 (7): 793-798.

De Rijck, J., Vandekerckhove, L., Gijsbers, R., Hombrouck, A., Hendrix, J., Vercammen, J., Engelborghs, Y., Christ, F. and Debyser, Z. 2006. Overexpression of the lens epithelium-derived growth factor/p75 integrase binding domain inhibits human immunodeficiency virus replication. *J Virol.,* 80:11498–11509.

Evering, T. H. and Markowitz, M. 2007. Raltegravir (MK-0518): an integrase inhibitor for the treatment of HIV-1. *Drugs Today,* 43, 865–877.

Hou, Y., McGuinness, DE., Prongay, AJ., Feld, B., Ingravallo, P., Lunn, CA. and Howe, JA. 2008. Screening for antiviral inhibitors of the HIV integrase - LEDGF/p75 interaction using the AlphaScreen (TM) luminescent proximity assay. *J Biomol Screening,* 13:406–414.

Llano, M., Saenz, DT., Meehan, A., Wongthida, P., Peretz, M., Walker, WH., Teo, WL. and Poeschla, EM. 2006. An essential role for LEDGF/p75 in HIV integration. *Science* 314:461–464.

Malet, I., Delelis, O., Valantin, MA., Montes, B., Soulie, C., Wirden, M., Tchertanov, L., Peytavin, G., Reynes, J., Mouscadet, JF., Katlama, C., Calvez, V. and Marcelin, AG. 2008. Mutations associated with failure of raltegravir treatment affect integrase sensitivity to the inhibitor in vitro. *Antimicrob Agents Chemother.,* 52:1351–1358.

Masakazu Kamata, Raymond, P., Wu, Dong Sung Anc, Jonathan, P., Saxe, Robert Damoiseauxd, Michael, E., Phelps, Jing Huang, and Irvin, S.Y. and Chena, 2006. Cell-Based Chemical Genetic Screen Identifies Damnacanthal as an Inhibitor of HIV-1 Vpr Induced Cell Death, *Biochem Biophys Res Commun.,* 348(3): 1101–1106.

McClatchey, W. 2002. From Polynesian healers to health food stores: changing perspectives of *Morinda citrifolia* (Rubiaceae). *Integr Cancer Ther.,* 1:110-20.

Nayak, B.S., Isitor, G.N., Maxwel,l N.A., Bhogadi, V. and Ramdath, D. 2007. Wound-Healing Activity of *Morinda citrifolia* Fruit Juice on Diabetes-Induced Rats. *J.Wound Care*. 16(2):83-7.

Palu, A K., Kim, A.H., West, B.J., Deng, S., Jensen, J. and White, L. 2007. Anti-Inflammatory and Potential Cancer Chemopreventive Constituents of the Fruits of *Morinda citrifolia* (Noni), *J. Nat. Prod.,* 70(5): 754-60.

Palu, A K., Kim, A.H., West, B.J., Deng, S., Jensen, J. and White, L. 2008. The Effects of *Morinda Citrifolia* L. (Noni) on the Immune System, Its Molecular Mechanisms of Action, *J. Ethnopharmacol*., 115(3): 502-7.

Pawlus, A. D. and Kinghorn, D. 2007. A Review of the Ethnobotany, Chemistry, Biological Activity and Safety of the Botanical Dietary Supplement *Morinda citrifolia* (Noni). *J Pharm. Pharmacol*., 59(12): 1587-92.

Periyasamy Selvam, Kasthuraiah Maddali and Yves Pommier. 2009. Studies on HIV Integrase inhibitory activity of *Morinda citrifolia* L. Noni fruit extracts. Noni search 2009-proceedings of fourth National Symposium on Noni for Empowerment and prosperity, Chennai, October 24-25, 193-201.

Periyasamy Selvam. 2012. Studies on HIV integrase-LEDGF/p75 interaction inhibitory activity of isatine derivative using the alpha screen luminescent proximity assay, *BMC Infectious Diseases*, 12 (1): 1-2.

Periyasamy Selvam, Chandramohan, M., De Clercq, E., Pannecouque, C., and Witrouw, M. 2001. Synthesis and anti-HIV activity of 4-[(1,2-dihydro-2-oxo-3H-indol-3-ylidene) amino]-N (4, 6-dimethyl-2-pyrimidinyl)-benzenesulphonamide and its derivatives. *Eur. J. Pharm. Sci.,* 14: 313-316.

Periyasamy Selvam, Paul Pandi, T. and Nouri Neamati. 2012. Studies on HIV Integrase and HIV Integrase/LEDGF inhibitory activity of ethanolic fractions (F1-F6) of Noni *(Morinda citrifolia* L.).In: (Eds) P. Rethinam and T. Marimuthu, Proceedings of Seventh National Symposium on Noni - The Tool for Wellness, Oct., 6-7, pp:110-120.

Poeschla, E. M. 2008. Integrase, LEDGF/p75 and HIV replication. *Cell Mol Life Sci.,* 65:1403–1424.

Saludes, J.P., Garson, M.J., Franzblau, S.G. and Aguinaldo, A.M. 2002. Antitubercular Constituents from the Hexane Fraction of *Morinda citrifolia* Linn. (Rubiaceae), *Phytother Res*., 16(7):683-8.

Su, B.N., Pawlus, A.D., Jung, H.A., Keller, W J., Mclaughlin, J.L. and Kinghorn, A.D. 2005. Chemical Constituents of the Fruits of *Morinda citrifolia* (Noni) and their Antioxidant Activity, *J. Nat. Prod*., 68(4): 592-8.

Umezawa, K. 1992. Isolation of 1-methoxy-2-foremyl-3-hydroxyanthraquinone from *Morinda citrifolia* and neoplasm inhibitors containing the same. *Japan Kokai Tokyo Koho JP*., 06 87, 736 (94-87, 736) Appl 1992; 92/264, 311 07.

Van Maele, B., Busschots, K., Vandekerckhove, L., Christ, F. and Debyser, Z. 2006. Cellular co-factors of HIV-1 integration. *Trends Biochem. Sci.,* 31:98–105.

Vanegas, M., Llano, M., Delgado, S., Thompson, D., Peretz, M. and Poeschla, E. 2005. Identification of the LEDGF/p75 HIV-1 integraseinteraction domain and NLS reveals NLS-independent chromatin tethering. *J. Cell Sci.,* 118:1733–1743.

Wang, M.Y., Anderson, G., Nowicki, D. and Jensen, J. 2008a. Hepatic Protection by Noni Fruit Juice against CCl_4-Induced Chronic Liver Damage in Female SD Rats, *Plant Foods Hum. Nutr*., (3), 141-47.

Wang, M.Y., Nowicki, D., Anderson, G., Jensen, J. and West, B. 2008b. Liver Protective Effects of *Morinda citrifolia* (Noni), *Plant Foods Hum. Nutr.,* 63(2): 59-63.

Wang, M.Y., West, B.J., Jensen, C.J., Nowicki, D., Su, C., Palu, A.K. and Anderson, G. 2002. *Morinda citrifolia* (Noni): a literature review and recent advances in Noni research. *Acta Pharmacol Sin.* 23:1127-41.

West, B.J., Jensen, C.J. and Westendorf, J. 2006. Noni Juice is Not Hepatotoxic, *World J. Gastroenterol*., 12(22): 3616-20.

P. Selvam[1],
S. Mahalingam[2]

Investigation on cytotoxicity of Divine Noni *(Noni Garcinia cambogia mix)* against human lung adenocarcinoma cell

Authors' affiliation :
P. Selvam[1]
[1]Nova College of Pharmaceutical Education and Research, Jupudi, Ibrahimpatnam, AP
S. Mahalingam[2]
[2]Dept of Biotechnology, Indian Institute of Technology-Madras, Chennai

Keywords: *Morinda citrifolia*, *Garcinia cambogia*, human lung adenocarcinoma cells.

Abstract : Divine Noni, *(Noni Garcinia cambogia mix)* with the concentrations of 40%, 20%, 10%, 7.5%, 5%, 2.5% and 0% were investigated for cytotoxicty studies including cellular toxicity, induction of cell apoptosis and inhibition of DNA synthesis. 40 % concentration showed significant cytotoxicity effect against A549, human lung adenocarcinoma cell and also induces cell apoptosis. 20 and 40 % concentration exhibits inhibition of DNA synthesis in human lung adenocarcinoma cell. Divine Noni, *(Noni Garcinia cambogia mix)* showed significant cytotoxicity against A549, human lung adenocarcinoma cell.

Introduction

Morinda citrifolia L Noni is a versatile medicinal plant with board spectrum of medicinal and therapeutic benefits (Pawlus *et al.,* 2007). The Polynesians utilized the whole Noni plant (*Morinda citrifolia*) in various combinations for herbal remedies and reported to possess wide spectrum biological activities to manage arthritis, diabetes, high blood pressure, muscle aches and pains, menstrual disorders, headaches, heart disease, AIDS, cancers, gastric ulcers, sprains, mental depression, senility, poor digestion, atherosclerosis, blood vessel problems and drug addiction (Wang *et al.,* 2002 and McClatchey *et al.,* 2002). Recently much attention been devoted for searching potential safe herbal medicines from natural products for the treatment of cancer and *Morinda citrifolia* was used for the treatment of a variety of cancer and tumors (West *et al.,* 2006).

Correspondence to :
P. Selvam
Nova College of Pharmaceutical Education and Research, Jupudi, Ibrahimpatnam, AP
Email:
periyasamy_selvam@yahoo.co.in

Garcinia cambogia is an yet another novel medicinal plant enriched with potential therapeutic values and several compounds have been isolated from certain species of *Garcinia*, including xanthones, xanthones derivatives, and (-)-hydroxycitric acid (Duke, *et al.,* 2002). *Garcinia cambogia* extract has been used traditionally in Indian medicine to treat tumors, ulcers, hemorrhoids, diarrhea, dysentery, fever, open sores, and parasites (Tharachand *et al.,* 2013). *Garcinia cambogia* has been reported to be indicated for constipation, rheumatism, dyspepsia, obesity, and high levels of triglycerides and cholesterol (Tharachand *et al.,* 2013). Commercially available *Garcinia cambogia* extracts are prepared from the fruit rind and contain 50% (-)-hydroxycitric acid (Onakpoya *et al.,* 2011). Benzophenones derivatives

garcinol and isogarcinol were isolated from *Garcinia cambogia* and reported to possess wide spectrum of anticancer activity (Padhye S, *et al.,* 2009). Based on the fact, The present work was undertaken to study the cytotoxicity activity of Divine Noni which contains *Morinda citrifolia* L (Noni) and Garcinia cambogia mix against human lung cancer cell lines by MTT assay method. This study also aimed at the apoptosis-inducing effects and inhibition of DNA synthesis of Divine Noni in human lung cancer cell lines.

Materials and Methods

Cell Lines

A549, human lung adenocarcinoma cell line was used for the study. The cell line was maintained in DMEM (Dulbecco's modified Eagle's media) (GIBCO) supplemented with 10% Foetal Bovine Serum. The cell line was maintained in 5% CO_2 humidified at 37°C for growth. Cell apoptosis were also investigated by flow cytometric and DNA synthesis by using BrdU assay methods.

Divine Noni Treatment

Divine Noni *(Noni Garcinia cambogia mix)* was obtained from Noni Biotech Pvt. Ltd., Chennai. Divine Noni was diluted v/v with complete DMEM in various concentrations- 40%, 20%, 10%, 7.5%, 5%, 2.5% and 0%. After the treatment, the cells were incubated for 24 hours after which Apoptosis assay, MTT assay and BrdU incorporation assays were performed.

Apoptosis assay

A549 cells were trypsinized using 1X Trypsin-EDTA. The Trypsin was neutralised with complete DMEM with 10% FBS. The cells were counted using a TC10 cell counter (Biorad). Trypan blue was used to count viable cells. $2X10^5$ cells were added to each well of a 6 well plate and incubated for 24 hours. Different concentrations of Divine Noni were then added to the respective wells. 24 hours post-treatment, the extract was aspirated and washed with 1X PBS thrice. The cells were trypsinised after which it was neutralised with complete DMEM. The cells were spun and the pellet was washed gently twice with 1X PBS. The pellet was then re suspended in 100µl 1X Annexin binding buffer. 5µl of AnnexinV-APC (BD Pharmigen™) and 5µl of Propidium iodide were added. After 20 minutes of incubation, 400µl of 1X Annexin V binding buffer was added and analysed using the flow cytometer (BD Biosciences FACS Canto) and FACS Diva software. Apoptosis data are presented in Fig. 1.

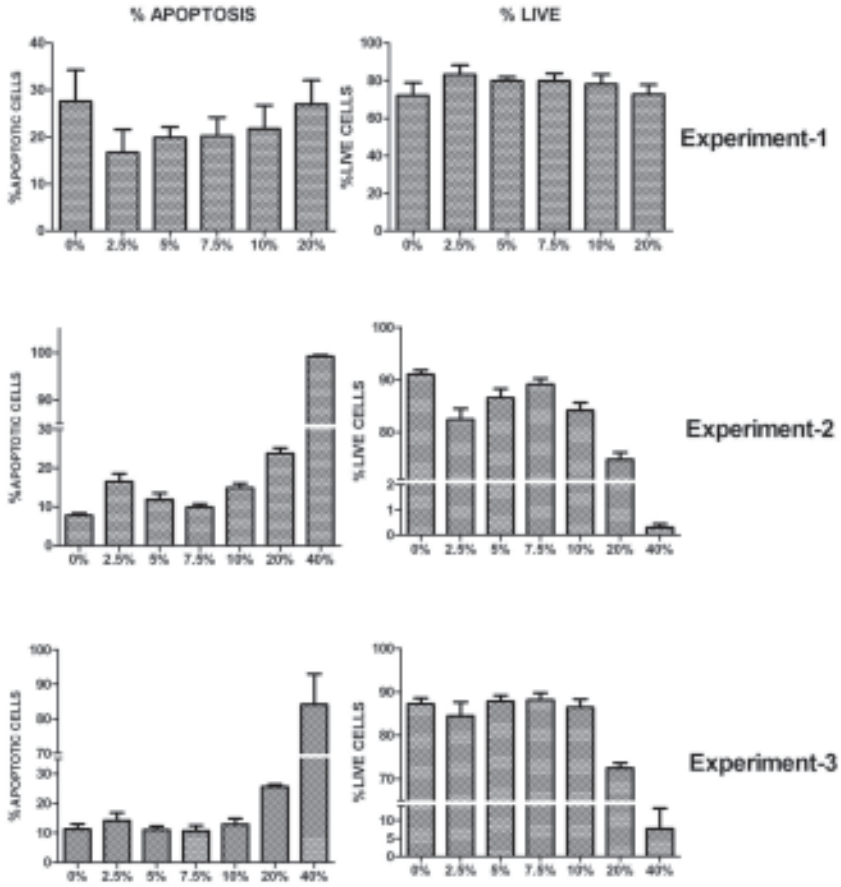


Fig. 1 : Graphical representation of the total apoptotic cells and live cells as determined by AnnexinV-APC/PI assay

(The experiments were done in triplicates and repeated thrice.)

MTT assay

A549 cells were trypsinized using 1X Trypsin-EDTA. The Trypsin was neutralised with complete DMEM with 10% FBS. The cells were counted using a TC10 cell counter (Biorad). Trypan blue was used to count viable cells. 10,000 cells were added to each well of a 96 well plate and incubated for 24 hours. Different concentrations of Divine Noni were then added to the respective wells. 24 hours post-treatment, the extract was aspirated and fresh media was added. Then 10µl MTT (Vybrant MTT Cell proliferation assay kit, Molecular probes) was added to each well and incubated at 37°C for 4 hours (Francis *et al.,* 1986; Philip *et al.,* 1990).. 50µl of DMSO was then added to each well to dissolve the Formazan crystals and incubated at 37°C for 10 minutes. Absorbance was measured at 540nm. MTT Cytotoxicity data are presented in Fig 2.

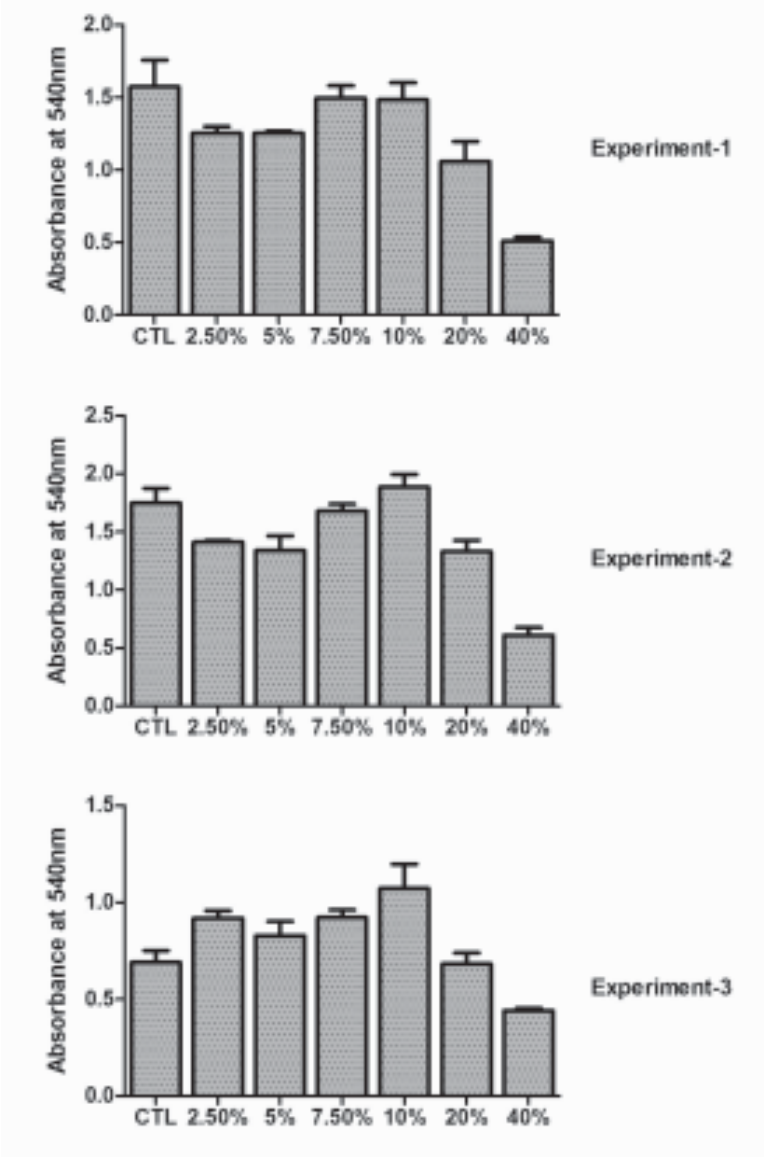


Fig. 2: Graphical representation of Cell proliferation as detected by MTT assay

(A549 cells were treated with different concentrations of Divine Noni and MTT assay was performed. Absorbance was measured at 540nm. Each experiment was done in triplicates. The experiment was repeated thrice.)

BrdU incorporation assay

A549 cells were trypsinized using 1X Trypsin-EDTA. The Trypsin was neutralised with complete DMEM with 10% FBS. The cells were counted using a TC10 cell counter (Biorad). Trypan blue was used to count viable cells. 10,000 cells were added to each well of a 96 well plate and incubated for 24 hours. Different concentrations of Divine Noni were then added to the respective wells. 24 hours post-treatment,

100µl Fixing/denaturing solution was added and incubated for 30 minutes at room temperature. 100µl of detection Antibody solution was added and incubated for one hour. After washings, 100µl 1X HRP-conjugated secondary antibody solution and 100µl of TMB substrate were added and incubated for 30 minutes. After adding stop solution, absorbance was measured at 450nm. Inhibition of DNA data are presented in Fig 3.

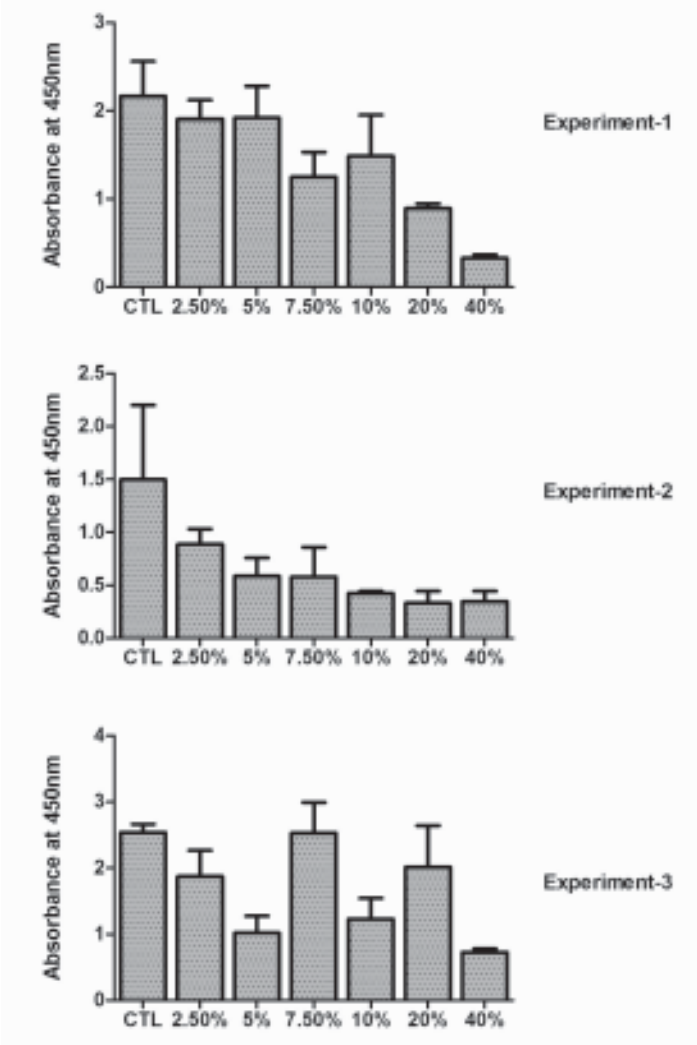


Fig. 3: Graphical representation of Cell proliferation as detected by BrdU assay

(A549 cells were treated with different concentrations of Divine Noni and BrdU incorporation assay was performed. Absorbance was measured at 450nm. Each experiment was done in triplicates. The experiment was repeated thrice.)

Results

Divine Noni containing Noni and *Garcinia cambogia* (40%, 20%, 10%, 7.5%, 5%, 2.5% and 0%) were investigated for cytotoxicty studies including cellular toxicity, induction of cell apoptosis and inhibition of DNA synthesis against human lung adenocarcinoma cell. Graphical representation of the total apoptotic cells and live cells as determined by AnnexinV-APC/PI assay is given in Fig.1. Graphical representation of Cell proliferation as detected by MTT assay.A549 cells were treated with different concentrations of Divine Noni and MTT assay is given in Fig .2. Graphical representation of Cell proliferation as detected by BrdU assay.A549 cells were treated with different concentrations of Divine Noni and BrdU incorporation assay in Fig.3. Forty per cent Divine Noni exhibits significant cytotoxicity against A549, human lung adenocarcinoma cell (Fig 2) and also inducer cell apoptosis (Fig 1). 20% and 40 % Divine Noni has potent inhibition of DNA synthesis in human lung adenocarcinoma cell (Fig 3). Divine Noni showed significant cytotoxicity against A549, human lung adenocarcinoma cell. This *in vitro* study has proved the selective cytotoxicity of Divine Noni against lung cancer cells.

Discussion

Morinda citrifolia (Noni) has been extensively used in folk medicine by Polynesians for over 2,000 years. It has been reported to have broad therapeutic effects, including anticancer activity (Wang, and Su, 2001.). Noni also reported to possess hepatoprotective (*Wang et al., 2008 a,b*), cancer chemopreventive *(Akihisa et al., 2008*), wound healing (Nayak *et al.,* 2007), antioxidant (Su *et al.,* 2005), immunomodulatory and anti-inflammatory (Palu *et al.,* 2007). Noni fruit extracts also reported to possess significant cytotoxicity against human liver cancer cells (Selvam *et al.,* 2009 and 2010). *Garcinia cambogia* also reported to possess wide spectrum of anticancer activity due to presence of Garcinol (Prasad *et al.,* 2010; Matsumoto *et al.,* 2003; Ahmad *et al.,* 2011; Hong *et al.,* 2007; Tanaka *et al.,* 2000; Arif *et al.,* 2006; Balasubramanyam *et al.,* 2004). *Morinda citrifolia* (Noni) **demonstrated for** *Induction of Mitochondrial-Mediated Apoptosis in Human Cervical Cancer Cells* (Rakesh *et al.,* 2013). In the present investigation, cytotoxicity of Divine Noni against human lung adenocarcinoma cell (A549) due to additive effect of *Morinda citrifolia* and *Garcinia cambogia* combination has been brought out.

References

Ahmad, A., Wang, Z., Wojewoda, C., Ali, R., Kong, D., Maitah, M. Y., Banerjee, S., Bao, B., Pandhye, S and Sarkar, F. H. 2011. Garcinol induced apoptosis in prostate and pancreatic cancer cells is mediated by NF Kappa B signaling, *Frontiers in Bioscience,* 3:1483-1492.

Akihisa, T., Matsumoto, K., Tokuda, H., Yasukawa, K., Seino, K.I., Nakamoto, K.H., Kuninaga, H., Suzuki, T and Kimura, Y. 2008. Anti-Inflammatory and Potential Cancer Chemopreventive Constituents of the Fruits of *Morinda citrifolia*. *Journal of Natural Product,* 71:1322-25.

Arif, M., Pradhan, S. K., Thanuja, G. R., Vedamurthy, B. M., Agarwal, S, Dasgupta, D and Kundu, T. K. 2006. Mechanism of p300 specific histone acetyltransferase inhibition by small molecules. *J. Med. Chem.*, 52(2):267-277

Balasubramanyam K, Altaf M, Varier R. A, Swaminathan V, Ravindran A, Sadhale P. P, Kundu T. K. 2004. Polyisoprenylated Benzophenone, Garcinol, a Natural Histone Acetyltransferase Inhibitor, Represses Chromatin Transcription and Alters Global Gene Expression. *J. Biol. Chem.,* 279(32): 33716-33726.

Bina S Siddiqui, Fouzia A Sattar, Sabira Begum, Tahsin Gulzar, and Fayaz Ahmad. 2007. Chemical Constituents from the Stems of *Morinda citrifolia* Linn. *Achieve Pharmaceutical Research,* 30: 793-798,

Cheng, A, Tsai, M, Liu, C, Lee, M, Nagabhushanam, K, Ho, C. and Pan, M. 2010. Garcinol inhibits cell growth in hepatocellular carcinoma Hep3B cells through induction of ROS-dependent apoptosis. *Food Functions,* 1: 301-307.

Duke, J.A., Bogenschutz-Godwin, M.J., duCellier, J. & Duke, P.-A.K. (2002) *Handbook of Medicinal Herbs*. 2nd ed., Boca Raton, FL, CRC Press, p 481

Francis, D and Rita L. 1986. Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods*, 89: 271-277.

Hong, J., Kwon, S. K., Sang, S., Ju, J., Zhou, J., Ho, C., Huang, M and Yang ,C. S. 2007. Effects of garcinol and its derivatives on intestinal cell growth: Inhibitory effects and autoxidation-dependent growth-stimulatory effects, *Free radical biology & medicine,* 42: 1211-1221.

Matsumoto, K., Akao, Y., Kobayashi, E., Ito, T., Ohguchi, K., Tanaka, T., Iinuma, M and Nozawa, Y. 2003.Cytotoxic benzophenone derivatives from *Garcinia* species display a strong apoptosis-inducing effect against Human Leukemia Cell lines, *Biol. Pharm. Bull,* 26(4):569-571.

McClatchey, W. 2002. From Polynesian healers to health food stores changing perspectives of *Morinda citrifolia* (Rubiaceae). *Integrated Cancer Therapy*, 1: 110-20.

Nayak, B.S, Isitor, G.N., Maxwell, N.A, Bhogadi, V and Ramdath, D. 2007. Wound-Healing Activity of *Morinda Citrifolia* Fruit Juice on Diabetes-Induced Rats. *Journal Wound Care*, 16 (2) :83-7.

Onakpoya, I., Hung, S. K., Perry, R., Wider, B and Ernst, E. 2011. The Use of Garcinia Extract (Hydroxycitric Acid) as a Weight loss Supplement: A Systematic Review and Meta-Analysis of Randomised Clinical Trials. *Journal of Obesity,* 2011: 1-10.

Padhye, S., Ahmad, A., Oswal, N and Sarkar, F. H, 2009. Emerging role of Garcinol, the antioxidant chalcone from *Garcinia indica* Choisy and its synthetic analogs, *Journal of Hematology and Oncology,* 2(38):120-125

Palu, A.K., Kim, A.H., West, B.J., Deng, S., Jensen, J and White, L. 2008. The Effects of *Morinda Citrifolia* L. (Noni) on the Immune System, Its Molecular Mechanisms of Action. *Journal of Ethnopharmacology*, 115 (3): 502-7.

Palu, A.K., Kim, A.H., West, B.J., Deng, S., Jensen, J and White, L.2007. Anti-Inflammatory and Potential Cancer Chemopreventive Constituents of the Fruits of *Morinda Citrifolia* (Noni). *Journal of Natural Product*, 70 (5): 754-60.

Pawlus, A.D and Kinghorn, D. 2007. A Review of the Ethnobotany, Chemistry, Biological Activity and Safety of the Botanical Dietary Supplement *Morinda Citrifolia* (Noni). *Journal of Pharmacy and Pharmacology,* 59 (12): 1587-92.

Periyasamy Selvam., Paul Pandi ,T and Vasanth Raj P. 2009. Studies of anticancer activity of ethanolic extract of Noni fruit Morinda citrifolia L. *Noni Journal of Clinical Research.,* 3: 1-2; 41-46.

Periyasamy Selvam., Paul Pandi, T and Vasanth Raj, P. 2010. Studies of cytotoxicity activity of ethanolic fractions of Noni fruit *Morinda citrifolia* L. International Journal of Noni Research., 5: 1-2; 17-21

Philip, S., Rista, S., Dominic, S., Anne, M., James., David, V., Jonathan,. T.W, Heidi, B., Susan, K. and Michale R.B. 1990. New colorimetric cytotoxic assay for anti cancer drug screening. *Journal of National Cancer Institute,* 82: 1107-1112.

Prasad, S., Ravindran, J., Sung, B., Pandey, M. K and Agarwal B. B. 2010. Garcinol potentiates TRAIL-Induced Apoptosis through Modulation of death receptors and antiapoptotic proteins, *Mol Cancer Ther,* 9(4): 856-868.

Rakesh Kumar Gupta., Ayan Banerjee., Suajta Pathak., Chandresh Sharma., Neeta Singh.2013. *Induction of Mitochondrial-Mediated Apoptosis by Morinda Citrifolia (Noni) in Human Cervical Cancer Cells. Asian Pacific J Cancer Prev, 14 (1), 237-242*

Su, B.N., Pawlus, A.D., Jung, H.A., Keller ,W.J., McLaughlin, J.L and Kinghorn, A.D. 2005.Chemical Constituents of the Fruits of *Morinda Citrifolia* (Noni) and their Antioxidant Activity. *Journal of Natural Product*, 68 (4): 592-8.

Tanaka, T., Kohno, H., Shimada, R., Kagami, S., Yamaguchi, F., Kataoka, S., Ariga, T., Murakami, A., Koshimizu, K and Ohigashi, H. 2000. Prevention of colonic aberrant crypt foci by dietary feeding of garcinol in male F344 rats, *Carcinogenesis,* 21(6): 1183-1189.

Wang, M.Y., Anderson, G., Nowicki, D and Jensen, J. 2008a. Hepatic Protection by Noni Fruit Juice against CCl_4-Induced Chronic Liver Damage in Female SD Rats. *Plant Foods Human Nutrition,* (3): 141-47.

Wang MY, Nowicki D, Anderson G, Jensen J and West B. 2008b. Liver Protective Effects of *Morinda Citrifolia* (Noni). *Plant Foods Human Nutrition,* 63 (2): 59-63.

Wang, M.Y., West, B.J., Jensen, C.J., Nowicki, D., Su, C., Palu, A.K and Anderson, G. 2002 *Morinda citrifolia* (Noni) a literature review and recent advances in Noni research. *Acta Pharmacologica Sinica*, 23: 1127-41.

West, B.J,, Jensen, C.J and Westendorf, J. 2006. Noni Juice is Not Hepatotoxic. *World Journal of Gastroenterology*, 12 (22): 3616-20.

Wang, M.Y and Su C. 2001. Cancer preventive effect of *Morinda citrifolia* L Noni *Ann. N Y Acad Sci* 952:161-8

D.R. Singh,*
Shrawan Singh
V. Shajeeda Banu

High Performance Liquid Chromatography (HPLC) analysis of different parts of *Morinda citrifolia* L.

Keywords : *Morinda citrifolia*, carotenoids, anthraquinones

Authors' affiliation :
D.R. Singh*,
Shrawan Singh
V. Shajeeda Banu
Division of Horticulture and Forestry, Central Agricultural Research Institute
Post Box No.181, Garacharma Post, Port Blair- 744 101, Andaman and Nicobar Islands
Email: drsingh66@gmail.com

Abstract : The present study was undertaken for identification of bioactives in ten anatomical parts (bark, branches, flowers, leaves, pulp, immature and mature fruit, stem, heartwood and root) of Noni (CARI TRA-1) using Reverse Phase – High Performance Liquid Chromatography (RP-HPLC). The mobile phase consisted of methanol (solvent A) and acetonitrile (solvent B) in 90:10 ratio at a flow rate of 1.0ml/min. The column temperature was 22°C and the absorbance was read at 450 nm. The UV-detection was done for phenolics and flavonoids at absorbance of 280 and 360 nm, respectively. Carotenoids identified at visible detection at 450 nm and anthraquinones at 254 nm. The HPLC analysis revealed that polydatin and physcion were dominant anthraquinone derivatives in majority of plant parts. Roots had rhein and resveratrol but no peak was recorded for anthraglycoside-B. Among the carotenoid, â-cryptoxanthin, zeaxanthin, rutin, quercetin and myrecitin were identified in Noni parts. Gallic acid was the major phenolics in all nine parts except immature fruit. Results indicated that mature fruits had maximum number of bioactives, therefore, suitable for therapeutic and industrial uses. The study indicates usefulness of every part of Noni for development of compound specific herbal products.

Correspondence to :
D.R. Singh
Division of Horticulture and Forestry
Central Agricultural Research Institute (ICAR)
Post Box No. 181, Garacharma (Post), Port Blair-744 101
Andaman and Nicobar Islands, India.
Email: drsingh66@gmail.com

Introduction

Morinda citrifolia L. (Rubiaceae) known commercially as Noni (Nelson, 2006), is a tropical and subtropical plant native to Southeast Asia and to Australia (Blanco et al., 2006). Ranging from eastern Polynesia to India (Dixon et al., 1999), Noni grows widely throughout the Pacific islands (Yang *et al.,* 2006). Noni is one of the traditional folk medicinal plants that have been used for over 2000 years by Polynesians for treating diabetes, high blood pressure, cancer, eye problems and many other illnesses (Rethinam and Sivaraman, 2007; Ramamoorthy and Bono, 2007), with a broad range of therapeutic and nutritional value. It is reported to have antibacterial, anti fungal, analgesic, hypotensive, anti-inflammatory and immune enhancing effects (Mc Clatchy, 2002; Wang *et al.,* 2002; Mathivanan *et al.,* 2005). With its wide range of antioxidants, Noni can be a major source of natural or phytochemical antioxidants (Ramamoorthy and Bono, 2007).

Traditional cultures have been using various plant parts of Noni *viz.,* fruit, bark, leaves and roots of Noni to treat a broad range of diseases for over 2000 years (Dixon *et al,* 1999; Etkin, 1999; McClatchey, 2002; Baque et al., 2010). The therapeutic properties of Noni (*Morinda citrifolia* L.) are attributable to the presence of phytochemicals, which have been linked in reducing the risk of several major chronic diseases and about 160 phytochemical compounds have been identified in the Noni plant so far (Yang *et al.,* 2007).

A number of major components have been identified in the *M. citrifolia* L. plant, such as scopoletin, octanoic acid, potassium, vitamin C, terpenoids, alkaloids, anthraquinones (such as nordamnacanthal, morindone, rubiadin, rubiadin-1-methyl ether and anthraquinone glycoside), â-sitosterol, carotene, vitamin A, flavones glycosides, linoleic acid, alizarin, amino acids, acubin, L-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin and a putative proxeronine. Tee and Lim (1991) estimated á carotene content/µg/g edible portion in *Morinda citrifolia* as 31. Raweewan *et al.* (2005) has developed a reverse-phase high performance liquid chromatographic method for quantifying Vitamin E and beta-carotene in *Morinda citrifolia* L. and *Phyllanthus emblica* L. fruits using ODS (C18) column.

Noni (*Morinda citrifolia* L.) is known for manifold health benefits. Around 200 bioactive compounds were reported in Noni but their kind and concentration varies with plant parts, genotypes, environmental factors and estimation methods. The medicinal properties of Noni from Andaman and Nicobar Islands have not been documented comprehensively. The paucity in the information propelled the present investigation which deals with the estimation of carotenoid and phenolics in different stage fruits of *Morinda citrifolia* using Reverse Phase-High Performance Liquid Chromatography (RP-HPLC). The present study has been carried out to estimate the phytochemical profile of methanolic extracts of ten different anatomical parts *viz.,* bark, branches, flowers, leaves, pulp, immature and mature fruit, stem, heart wood and root has been carried out using Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) on a C_{18} column and the phytochemical substances *viz.,* anthocyanins, anthraquinones, carotenoids, flavonols and phenolic acids were estimated.

Materials and Methods

Study area

Andaman and Nicobar Islands (ANI) is one of the richest centers of diversity of Noni. Due to its wide range of adaptability, Noni grows abundantly throughout the coastal regions of ANI even in infertile, acidic, alkaline, sea inundated land and it also prefers to grow in dry and wet areas and it is attributable to the tropical humid climate which is very much suitable for its cultivation (Singh and Rai, 2005). The Nicobarese, one among the six aboriginal tribes of ANI have very long association with noni and used different parts as medicine to cure different ailments.

Collection of accessions

For the estimation of phytochemical profile of different anatomical parts, Noni from the germplasm (number of accessions= 33) of Central Agricultural Research Institute, Port Blair were used.

Extract preparation

Plant parts were collected and washed with Millipore water. 5 g of all the samples were grinded using 25 ml of methanol it was then passed through 0.45-ìm filter. The filtrate was further diluted using methanol and then used for further analysis using RP-HPLC on a C-18 column. The specifications of RP-HPLC were, Reverse phase HPLC (DIONEX, ultimate 3000 series), comprising a solvent rack (SRD-3200), a pump (HPG-3200SD), a column oven (TCC-3000SD), column: C18 5µm 120Å 4.6x 250mm and a diode array detector (variable wavelength detectors VWD-3100 and VWD-3400).

Phytochemical estimation

Separation of phenolic acid, anthocyanin and flavonol was carried out in the flow rate of 0.8ml/min and the injection volume was 10 µl. The mobile phase was a binary solvent system consisting of (A) methanol and (B) 1% acetic acid/ water and the gradient used was 0 min 40% B, 5 min 65% B, 10 min 90% B, 15 min 40% B until 60 min, at an ambient temperature and UV detection at 280, 520 and 360 nm for phenolic acids, anthocyanin and flavonols respectively. Separation of carotenoid was administered using an isocratic HPLC separation, 90:10 methanol/acetonitrile mobile phase, 1.0ml/min flow rate, at column temperature of 25^{o}C at visible detection at 450 nm. Anthraquinone separation was accomplished using a methanol-water-phosphoric acid (80:20:0.9, v/v/v). The sample injection volume was 10 µl at 40^{o}C. The flow rate was 1.0 ml/min and the absorption was recorded at 254 nm.

Results and Discussion

Analysis revealed significant disparity in phytochemical contents (Table-1). Among anthraquinone derivatives, polydatin was the most dominant in five parts (branch, flower, immature fruit, pulp and root) followed by physician present in four (bark, branch, heartwood and root) and the lowest were rhein and resveratrol present only in roots. Except anthraglycoside B, all the anthraquinone derivatives were present in the root, indicating that roots are rich in anthraquinones. Among the carotenoid derivatives, â- cryptoxanthin was reported in eight parts (bark, branches, heartwood, flower, immature fruit, leaf, root and stem) followed by zeaxanthin present in six parts (bark, flower, heart wood, mature fruit, leaf and pulp) and the lowest carotenoid derivative was found to be á- carotene present only in stem. Rutin was the dominant flavonol derivative present in four parts (heartwood, pulp, root and stem) followed by quercetin and myrecitin in two parts, where the former was present in bark and root and the latter

Table 1. HPLC analysis in different parts of *Morinda citrifolia*

Phyto-chemical group	Individual Compound	Bark	Branches	Flower	Heartwood	Immature fruit	Mature fruit	Leaf	Pulp	Root	Stem
	Ploydatin	-	+	+	-	+	-	-	+	+	-
	Rhein	-	-	-	-	-	-	-	-	+	-
	Resveratrol	-	-	-	-	-	-	-	-	+	-
Anthraquinone	Anthraglycoside B	-	-	-	-	-	+	+	-	-	-
	Emodin	-	+	-	+	-	-	-	-	+	-
	Chrysophanol	-	-		+	-	-	-	-	+	-
	Physcion	+	+	-	+	-	-	-	-	+	-
	Lutein	-	+	-	-	-	+	+	-	+	+
	Zeaxanthin	+	-	+	+	-	+	+	+	-	-
	α-Cryptoxanthin	+	+	+	+	+	-	+	-	+	+
Carotenoids	Echinenone	+	+	+	-	-	-	-	-	-	-
	α-Carotene	-	-	-	-	-	-	-	-	-	+
	β-Carotene	+	+	+	-	-	-	+	-	-	+
	Lycopene	+	-	-	-	-	-	+	-	-	-

contd...

Phyto-chemical group	Individual Compound	Bark	Branches	Flower	Heartwood	Immature fruit	Mature fruit	Leaf	Pulp	Root	Stem
Flavonols	Rutin	-	-	-	+	-	-	-	+	+	+
	Quercetin	+	-	-	-	-	-	-	-	+	-
	Myricetin	-	-	-	-	-	-	-	+	+	-
Phenolic Acids	Epicatechin	+	-	-	+	-	-	+	-	+	+
	Gallic Acid	+	+	+	+	-	+	+	+	+	+
	Catechin	-	-	-	+	-	-	-	-	-	-
	Naringin	-	-	-	+	-	-	-	-	-	-
	Syringic Acid	-	-	-	+	-	-	-	-	-	-
	P-Coumaric acid	-	-	-	+	-	+	-	-	-	-
	Ferulic Acid	-	-	-	-	-	-	-	+	+	+
	Trans-Resveratrol	+	-	-	-	-	-	-	+	+	-
	Epigallocatechin gallate	+	+	+	+	-	-	-	-	-	-
Anthocyanin	Delphinidin-3-glucoside	-	-	-	-	-	-	+	+	+	+
	Cyanidine-3-glucoside	-	-	-	-	-	-	-	-	+	-
	Petunidin-3-glucoside	-	-	-	-	-	-	-	-	+	-
	Peonidin--3-glucoside	-	-	-	-	-	-	-	-	-	-
	Malvidin--3-glucoside	-	-	-	-	-	-	-	+	-	-

'+' denotes presence of particular compound; '-' denotes absence of particular compound in the respective plant parts.

was present in pulp and roots. Gallic acid was the major phenolic acid derivative present in nine parts (bark, branch, flower, heartwood, mature fruit, leaf, stem, pulp and root) followed by epicatechin present in five parts (bark, heartwood, leaf, root and stem) and the lowest phenolic acid derivative were catechin, naringin and syringic acid present only in heartwood. Delpindin-3-glucoside was the major anthocyanin derivative present in four parts (leaf, pulp, root and stem) followed by cyanidine-3-glucoside, putunidin-3-glucoside present in root and malvidin-3-glucoside present in pulp. Results indicated that immature fruit is not ideal for therapeutic applications as only two phytochemical substances (polydatin and á-cryptoxanthin) were present. The present study is significant as it delineates the precise amount of phytochemicals in eight different plant parts which facilitates easier applications in the targeted study as well as in analyzing its therapeutic properties of this intriguing Divine plant.

Acknowledgment

The authors thank the Director, CARI, Port Blair for providing facilities and National Medicinal Plant Board, New Delhi for financial support for the study.

References

Ames, B. N., Shigenaga, M.K. and Hagen, T. M. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA Vol. 90, pp: 7915-7922, September 1993.*

Blanco, Y.C., Vaillant, F., Perez, A.M., Reynes, M., Brill, J.M and Brat, P. 2006. The Noni fruit (Morinda citrifolia L.): A review of agricultural research, nutritional .and therapeutic properties. *Journal of Food Composition and Analysis,* 19: 645-654.

Dixon, A.R., Mcmillen, H. and Etkin, N.L. 1999. Ferment this: the transformation of Noni, a traditional Polynesian medicine (*Morinda citrifolia*). *Economic Botany*, 53: 1: 51-68.

Dixon, A.R., Mcmillen, H. and Etkin, N.L. 1999. Ferment this: the transformation of Noni, a traditional Polynesian medicine (Morinda citrifolia). *Economic Botany*, 53: 1, 51-68.

Fatimah, A. M. Z., Norazian, M. H. and Rashidi, O. (2012). Identification of carotenoid composition in selected 'ulam' or traditional vegetables in Malaysia. *International Food Research Journal*, 19(2): pp527-530.

Mathivanan, N., Surendiran, G., Srinivasan, K., Sagadevan, E., and Malarvizhi, K. 2005. Review on the current scenario of Noni research: Taxonomy, distribution, chemistry, medicinal and therapeutic values of *Morinda citrifolia. Intl. J. Noni Res.*, 1(1)1-16.

Nelson, S.C. 2006. *Morinda citrifolia* (Noni), ver. 4. In: Elevitch, C.R. (ed.). Species Profiles for Pacific Island Agroforestry. Permanent Agriculture Resources (PAR), Hôlualoa, Hawai'i. http://www.traditional tree.org.

Ramamoorthy, P.K. and Bono, A (2007). Antioxidant Activity, total phenolic and flavonoid content of *Morinda citrifolia* fruit extracts from various extraction processes. *Journal of Engineering Science and Technology*, 2(1): pp70 – 80.

Rodriguez-Amaya, D. B. 1997. Important Food Sources of Provitamins A. Carotenoids and food preparation: The retention of pro vitamin A carotenoids in prepared, processed, and stored foods. Washington: OMNI Press.

Shu-Chuan Yang, Tsu-I Chen, Ken-Yuon Li and Tsun-Chung Tsai. (2007). Change in Phenolic Compound Content, Reductive Capacity and ACE Inhibitory Activity in Noni Juice during Traditional Fermentation. *Journal of Food and Drug Analysis*, 15(3):290 298.

Singh, D.R. Rai, R.B. and Singh, B. 2005. The Great Morinda– A potential underutilized fruits in Bay Islands. The Daily Telegrams, Port Blair, April 24, pp-2.

Siva, R. (2007). Status of natural dyes and dye-yielding plants in India. *Current Science,* 92:7 (10) 916-925.

Tee, E.S. and Lim, C. L. 1991. Carotenoid composition and content of Malaysian vegetables and fruits by the AOAC and HPLC methods. *Food Chem.,* 41:309-339.

Wang, M.Y., West, B., Jensen, C.J., Nowicki, D., Su, C., Palu, A.K., Anderson, G. 2002. *Morinda citrifolia* (Noni): a literature review and recent advances in Noni research. *Acta. Pharmacologica Sinica*, 23, 1127–1141.

K. S. Vidya[1],
U. Raghavendra[1],
A. Muthusamy[1],
S. B. Vidhu[1],
J. Subramani[2],
P. M. Gopinath[1],
K. Satyamoorthy[1]

Cytotoxic efficacy of Noni (*Morinda citrfolia* L.) fruit extracts and scopoletin on a preponderant panel of human tumor cell lines

Keywords : cytotoxicity, fractionation, HPLC, Morinda citrifolia, MTT, scopoletin

Authors' affiliation :
K. S. Vidya[1],
U. Raghavendra[1],
A. Muthusamy[1],
S. B. Vidhu[1],
P. M. Gopinath[1],
K. Satyamoorthy[1]
[1]Manipal Life Sciences Centre, Manipal University, Planetarium Complex, Manipal- 576104
J. Subramani[2],
[2]World Noni Research Foundation, 12 Rajiv Gandhi Road, Perungudi, Chennai- 600096.
*Email:
ksatyamoorthy@manipal.edu

Abstract : An exploratory analysis to mine the relationship with molecular defects in tumors and toxicity of known cytotoxic bioactive compounds from Morinda citrifolia was undertaken. The anti-proliferative potential of crude extracts and HPLC purified fractions of ripe Noni fruits were assayed on a panel of six human tumor cell lines and whose efficacy were compared with scopoletin. It was found that scopoletin and selected fractions of the extract exhibited a better anti-proliferative effect when compared to crude samples. Standard scopoletin was cytotoxic to all cell lines, especially to WM115 (melanoma cell line) and Saos 2 (osteosarcoma cell line) with an IC50 value of 5 and 10 µg/ml respectively. The results suggest that cytotoxic potential of Noni fruit extracts towards tumor cells may be dependent on scopoletin levels. Results further suggest that Noni fruit extracts may have potential chemo preventive effect against cancer.

Correspondence to :
K. Satyamoorthy
Manipal Life Sciences Centre, Manipal University, Planetarium Complex, Manipal- 576104
*Email:
ksatyamoorthy@manipal.edu

Introduction

Morinda citrifolia L. a medicinal plant belonging to the family Rubiaceae is of south Asian origin, with traditional medicinal uses and most commonly known by the name Noni or The Indian Mulberry (Kinghorn *et al.*, 2011). It has been reported to have a broad range of health benefits such as anti-bacterial, anti-fungal, anti-diabetic, anti-hypertensive, chemo preventive agent and analgesic to name a few. The roots, stems, bark, leaves, flowers and fruits of the Noni plant and its combinations are reported to be involved in almost 40 known and recorded herbal remedies (Wang *et al*, 2002). An ethanolic extract of Noni leaves were investigated and found to possess wound healing activity and reported to be safe in acute, sub-acute and sub-chronic oral toxicity tests on mice (West *et al.*, 2007). Recent research suggests its possible use as a food supplement and as a chemo preventive agent (Thani *et al.*, 2010). One particular anthraquinone viz., damnacanthal, isolated from the chloroform extract of Noni root, was shown to be capable of inducing normal phenotypes in ras-transformed cells. The same compound has also been found to inhibit the Epstein-Barr virus early antigen activation. Compounds isolated from roots have also been reported to suppress the cytopathic effect of HIV infected MT-4 cells, without inhibiting cell growth (Umezawa *et al.*, 1992).

An immune modulatory polysaccharide-rich substance (Noni-ppt) from the Noni fruit was found to suppress tumor growth through the regulation of the immune system in the host (Hirazumi *et al.*, 1992, 1994, 1996; Hirazumi and Furusawa, 1999). Two novel glycosides have been shown to possess inhibitory effect on AP-1 transactivation and cell transformation in the mouse epidermal JB6 cell line (Liu *et al.*, 2001). A number of major components have been identified in the Noni plant such as scopoletin, octoanoic acid, potassium, vitamin C, terpenoids, alkaloids, â-sitosterol, linoleic acid, alizarin, amino acids, acubin, *L*-asperuloside, caproic acid, caprylic acid, ursolic acid and rutin (Levand and Larson, 1979; Moorthy and Reddy, 1970; Simonsen, 1920). Numerous beneficial properties of Noni can be attributed to the presence of these active principles. Though, there is no dearth of information on the anti- cancer or anti-proliferative activity of the plant Noni as a whole or plant parts such as roots and leaves in particular; there is a lack of sufficient information on the cytotoxic properties of the Noni fruit. Hence, this study was an effort aimed at obtaining information about the holistic or the active principles of the Noni fruit against human tumor cells. Here it was attempted to identify anti-cancer properties especially the anti-proliferative properties, from the Noni fruit extracts, which could be used against human tumor cells whose molecular defects are well understood.

Materials and Methods

Fruit sample

Ripe fruits of Noni for experimental purposes were provided after authentication by the World Noni Research Foundation, Chennai.

Maintenance of cell lines

A panel of six human tumor cell lines for the experimentation were procured from National Centre for Cell Sciences, Pune, India. The cell lines human cervical epidermoid carcinoma (Caski) and human colon adenocarcinoma (Colo 205) were cultured in RPMI supplemented with 10% fetal bovine serum and antibiotics. Human lung carcinoma (A549), human osteosarcoma (Saos 2) and human skin melanoma (WM115) cells were cultured in DMEM fortified with 10% fetal bovine serum and antibiotics. Human breast sarcoma (MDA MB 231) was cultured in L-15 with aforementioned supplements. Fibroblast cells derived from human foreskin samples were used as controls and were maintained in DMEM containing with 10% fetal bovine serum and antibiotics and used as control cells. Cell lines were maintained as monolayers, except the non-adherent Colo 205 which was maintained in suspension cultures. All cells were kept in 5% CO_2 incubator at 37 ºC and were regularly sub-cultured every two to three days.

Preparation and Extraction of the Fruits

Freeze dried extract

The fresh Noni fruits were ground to fine pulp and the juice obtained was centrifuged at 20,000 rpm for 20 minutes at 4 °C to collect the supernatant. The supernatant was subsequently freeze dried until a fine clear powder was obtained. The clear powder was aliquoted into sterile microfuge tubes and then stored at -20°C until further use.

Soxhlet extraction procedure

The fruits were dried for three days at 60ºC in a hot air oven. The dry fruits were then ground into a fine powder of which 10gm was weighed and used for soxhlet extraction (Franz, 1879). The individual extraction was performed separately using water, ethanol and hexane. The sequential extraction was undertaken using three solvents such as with hexane followed by ethanol and finally with water. The solvents obtained after the soxhlet extraction were then subjected to lyophilization to obtain the respective extracts.

Extraction at 4ºC

The dry fruit powder was mixed with ethanol and water in the ratio 1:10 and stirred overnight at 4ºC to obtain ethanolic and aqueous extracts respectively. The solvents obtained after the overnight extraction were lyophilized to acquire the dry ethanol and aqueous extracts.

Identification and quantification of bioactive compounds

Chemical fingerprinting was carried out by high pressure liquid chromatography (HPLC) to identify the active principles. The analyses were performed using Waters series Alliance 2695 HPLC system and detection was carried out using Waters 2487 dual wavelength absorbance UV detector. Chromatographic separation of crude extracts was first carried out using a Phenomenex Aqua series C18 column (15 cm × 4.6 mm i.d., 5 ìm). The fractions which showed activity were further separated using a Phenomenex Prodigy series C8 column (15 cm × 4.6 mm i.d., 5 ìm) column to achieve higher resolution. The mobile phase consisted of 0.1% (v/v) Trifluoroacetic acid in water (A) and 100% Acetonitrile (B) with a linear gradient elution at a flow rate of 1.0 ml/min. The dual detection wave lengths used were 254 and 280 nm. The extract which showed a good activity was then fractionated and isolated in large quantities using Varian Pursuit series C18 Semi-prep column (25 cm × 212 mm i.d., 10 ìm).

The programmed mobile phase was consecutively in linear gradients as follows: 0 minutes, 100% of solution A, 45 minutes, 20% of solution A and 80% solution B and concludes with 55 minutes, 100% of solution A. Around six to seven fractions

were collected each time based on the peaks in the HPLC profile, over a period of sixty minutes. The fractions collected were then freeze dried to obtain a clear powder. All extracts were fractionated by HPLC. Aqueous extract and ethanol extract was further fractionated using semi-prep column. Standard compounds such as rutin and scopoletin were purchased from Sigma-Aldrich (Bangalore, India) and mitomycin C was obtained from Biochem pharmaceuticals limited (Mumbai, India).

Cell viability assay

Cytotoxicity assays were performed by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann *et al*, 1983) for adherent cells. The cells were seeded in a 96 well plate, 1×10^4 cells/well and incubated with all the extracts and the standard compounds for a period of 48 hours at concentrations ranging from 5ìg/ml to 1000ìg/ml in a series of MTT assays. However, in case of the freeze dried juice extract two additional concentrations of 5000 ìg/ml and 10,000ìg/ml were also experimented. Standard compounds were tested for concentrations ranging from 1 to 100ìg/ml. In case of the suspension cell line Colo 205, WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) was the assay of choice to test the anti-proliferative activity (Ishiyama, 1995). Cell suspensions growing in exponential phase were plated in a 96 well at plate a concentration of 5×10^3 cells/well. Preliminary experiments were conducted to estimate the number of cells to be seeded and a concentration of 5×10^3 was found to be in the linear range. Cells were treated with different concentrations of standard drugs and extracts for 48h. Mitomycin C was used as a positive control. All crude extracts, HPLC fractions, four standard compounds and standard compounds in combinations with each other were assayed to check the percentage viability at the indicated concentrations.

Statistical analysis

All the data were expressed as mean ± SEM. The statistical significance between the treatment groups was evaluated by one-way ANOVA and with Bonferroni's post-hoc test using GraphPAD InStat, Software, USA.

Results

Extraction

Post lyophilised sample yield after various extraction procedures was at an average of 1- 4 g dry wt.

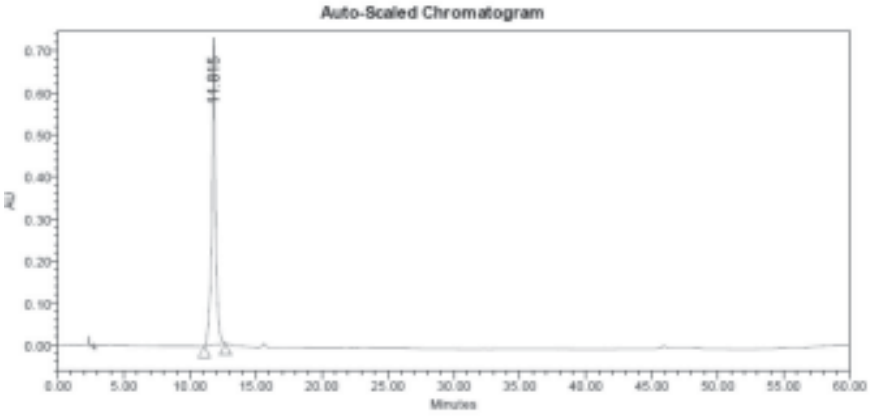


Fig. 1: HPLC chromatogram of standard compound Scopoletin

Scopoletin had a retention time of 11.815 minutes.

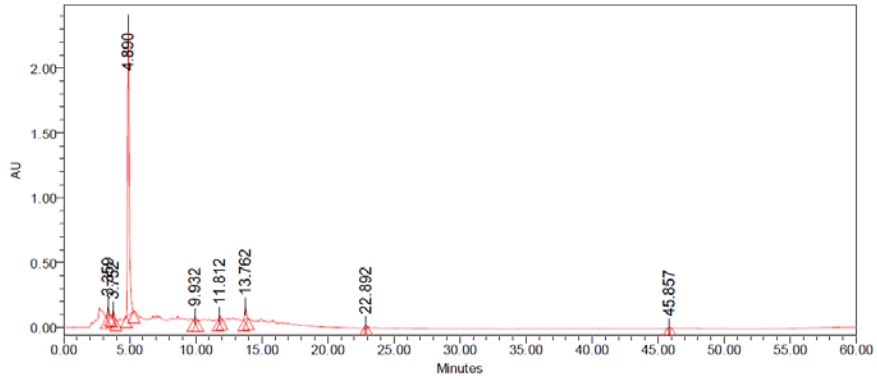


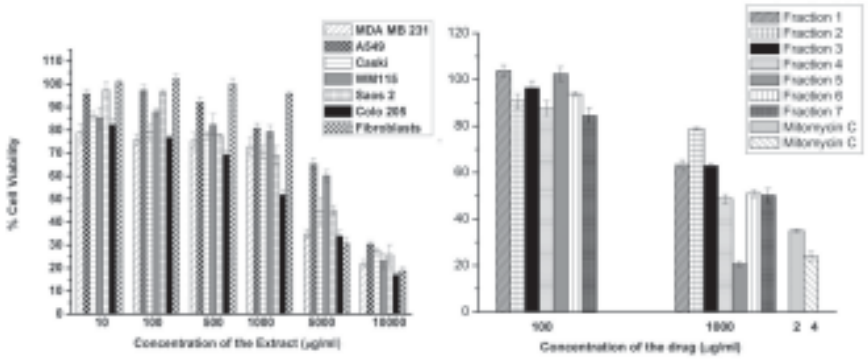
Fig. 2: HPLC chromatogram of the aqueous extract at 280 nm

HPLC chromatogram of the aqueous extract was obtained at the wavelength 280nm during a sixty minute run. Various peaks generated indicate the presence of numerous compounds. Specific peak at retention time 11.812 minutes suggests the presence of scopoletin.

Cytotoxicity assay

The results of MTT assays performed with crude extracts exhibited moderate levels of cytotoxicity. Out of the crude extracts that were fractionated; none of the soxhlet extracted fractions displayed a significant variation in the percentage viability compared to the crude drugs. Fractionated freeze dried samples were also attempted on the cell lines to check its cytotoxic effect. Fraction V of freeze dried extract showed higher cytotoxic effect when compared to crude extracts (Fig. 3A, 3B). On further fractionation using a C8 column, the active fraction did not show any profound increase in cytotoxicity. A similar trend was observed in case of aqueous extract (Fig.4A, 4B), where fraction VI showed greater cytotoxicity compared to the crude extracts. However, upon further fractionation it was found to lose its activity. Standard scopoletin was cytotoxic to all cell lines, especially in case of WM115 and Saos 2, where the IC50 values were 5 and 10 µg/ml respectively (Fig. 5A). The difference in cytotoxicity caused was found to be

statistically significant ($p<0.01$) at all concentrations assayed when compared with the cell lines Saos 2 and WM115. However, rutin did not show profound cytotoxicity in any of the cell lines tested (Fig. 5B). Fractionation of bioactive compounds from crude extract was not found to show any profound increment in cytotoxicity due to the presence of several compounds in the given fraction. The standard drugs when tested at various combinations on A 549 cell line did not show any significant increase in cytotoxicity (Fig. 3A, B, 4AB, 5AB, 6)

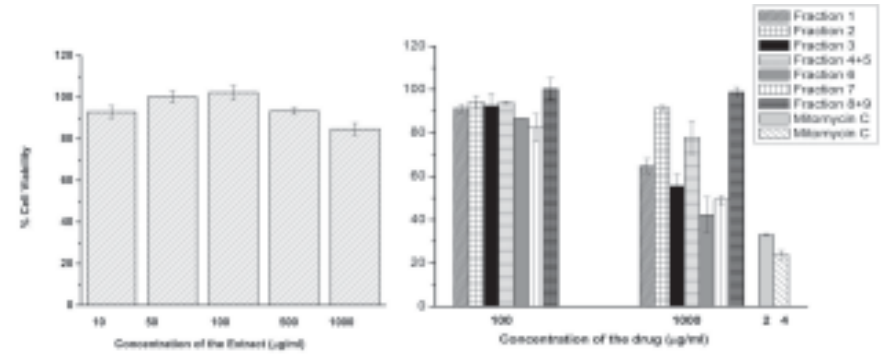


A **B**

Fig. 3: Cytotoxic profiles of freeze dried extracts after a 48 hour incubation

A) Effect of the freeze dried juice extract on the panel of six human tumor cell lines. Based on the trend seen it is suggested that the cell line Colo 205 is most susceptible to this particular extract with an IC50 of 1000µg/ml.

B) Effect of freeze dried extract HPLC fractions on the cell line WM115. Based on the trend observed it can be concluded that the freeze dried extract shows a higher cytotoxic effect post fractionation when compared to the crude extract at the same concentration.

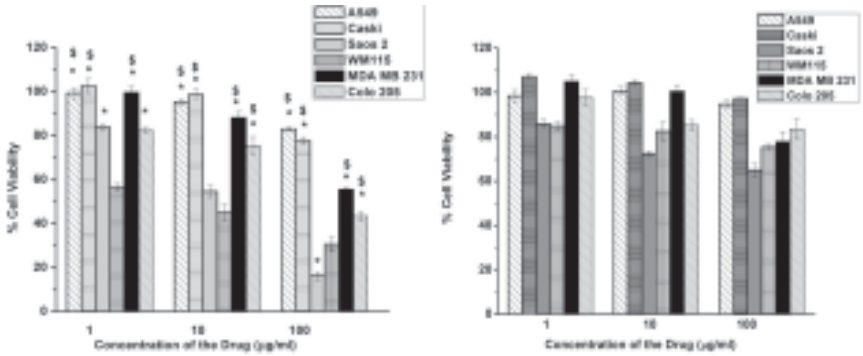


A **B**

Fig. 4: Cytotoxic profiles of aqueous extracts on tumor cells after 48 hour incubation

A) Effect of the crude aqueous extract on the most sensitive cell line WM115. Based on the data it is observed that it does not reach IC50 even at a concentration of 1000µg/ml.

B) Effect of aqueous extract HPLC fractions on the cell line WM115. Based on the results, it is observed that the aqueous extract shows a higher cytotoxic effect post fractionation when compared to the crude extract at the same concentration.



A **B**

Fig. 5: Cytotoxic profiles of standard drugs on the panel of six human tumor cell lines, after a 48 hour incubation

A) Effect of scopoletin on the panel of human tumor cell lines. It is shown that the cell lines WM115 and Saos 2 are most sensitive to scopoletin with IC50 values of 5 and 10µg/ml respectively. *- Based on results from ANOVA, statistically significant ($P<0.001$) difference was observed when values were compared with the cell line WM115. $- Based on results from ANOVA ($p<0.01$), statistically significant difference was observed when values were compared with the cell line Saos 2.
B) Effect of rutin on the panel of human tumor cell lines. Results suggested that the compound rutin is not very cytotoxic when compared with scopoletin in all the cell lines tested.

Discussion

Based on the cytotoxicity assay performed on a panel of six human tumor cell lines; it was concluded that Noni fruit extracts are more of a chemopreventive nature. This conclusion is supported by the IC50 values obtained for all the crude extracts. Researches elsewhere have showed that in some cases, fractionated samples have a more profound effect on cell lines than crude samples (Cassady *et al.,* 1979). However, in Noni fruit samples no such studies have been conducted till date correlating the pure sample and crude extract with cytotoxic effect.

The present study, in fact, compares the efficacy of the crude with the standard drug scopoletin which is one of the active principles of Noni. Scopoletin is a coumarin compound and a pharmacologically active agent that has been isolated from several plant species (Moon *et al*., 2007), (Cassady *et al*., 1979) have reported antitumor activity for scopoletin based on *in vivo* effect in mice in P388 lymphocytic leukemia cells. The antitumor efficacy and presence of the same in Noni was our rationale for selecting scopoletin as a standard for understanding the efficacy of crude extracts from fruit samples of Noni. When we closely observed the trend of action in sensitive cell lines to scopoletin such as WM115, Saos2 and Colo 205, we found that scopoletin might influence the pathways related to defective genes in them viz., PTEN, RB1 and MADH4 /APC respectively. There as a statistically significant ($p<0.05$) difference between the effect of scopoletin in all three concentrations tested in WM115, Saos 2 and Colo 205 cell lines.

It will be innovative to pursue the study in the manner described above which may convey better insights about the mode of action on human tumor cell lines. (Kim *et al*, 2005) have shown that scopoletin induces NF-8B activation, which in turn, causes the activation of caspase-3, degradation of PARP, and eventually leads to apoptotic cell death in a Human promyeloleukemic cells (HL-60). It is hypothesized that if scopoletin concentration would have been higher in fruit extracts, then it would have induced better cytotoxicity on cell lines than otherwise. The less cytotoxicity of the samples might be attributed to low levels of scopoletin contents in the same. However, it can be seen from literature that scopoletin is not found only in Noni but also found in other plants such as *Erycibe obtusifolia Benth, Aster tataricus, and Foeniculum vulgare* (Moon *et al*., 2007).

It is found that Noni samples have very different chemical profiling for leaf, root and fruit samples. This might be one of the reasons why the plant extracts of Noni has varied effect on cell lines. This pattern is not restricted just to plant parts but is reflected in different solvent based extractions as well. The freeze dried extract had very different chemical profile when compared to aqueous and ethanolic extracts. This is evident from results obtained from HPLC (with varied peaks and peak areas). The fruit juice of *M. citrifolia* contains a polysaccharides rich substance called Noni-ppt that has been reported to possess anti-tumour activity in Lewis lung peritoneal carcinoma model (Liu *et al*., 2001). Since Noni fruit is promoted as a health drink we wanted to investigate the bioactive compounds present in it and whether it has any toxic effects.

The results obtained from the panel of six human tumor cell lines clearly indicates that Noni fruit extracts neither crude nor in fractionated form are highly cytotoxic in nature to tumor and normal cells. Our results are in agreement with various studies conducted by (Furusawa *et al*, 2002), that Noni fruit juice is not cytotoxic in cell cultures (Lewis lung carcinoma cell line, sarcoma 180 cells, human KB carcinoma cell line or normal NIH / 3T3 and BALB / 3T3 cell lines) but the juice can indirectly kill the tumor cells via activation of cellular immune system involving macrophages, natural killer cells and T cells. Therefore Noni fruit juice is considered one of the most powerful anti-tumour immune stimulators of plant food origin. The fact that powdered fruits and fruit juice of this plant have become well established as health drink and as a popular dietary supplement, for its potential effects on arthritis, cancer, cardiovascular disease, inflammation, and as a general tonic (Kinghorn *et al.*, 2011), directs the thought that this could reasonable only if Noni juice or Noni fruit is indeed not very cytotoxic.

Conclusion

The study conducted in the panel of six human tumor cell lines is a preliminary exploratory analysis to mine the relationship with molecular defects and toxicity of known cytotoxic bioactive compounds from *Morinda citrifolia* which has not been reported elsewhere. It would be interesting to study the Noni extracts and their fractions in combination with other anticancer drugs, radiation, immunomodulatory drugs and vaccines on apoptosis, defective mutation mechanisms restricted to tumor cell lines and anti- tumorigenic effects in animal models to have conclusive results on efficacy of the Noni fruits. Finally, it is also interesting to exhaustively examine its effect as chemo preventive agent against cancer.

Acknowledgements

We are thankful to World Noni Research Foundation, Chennai for financial support, TIFAC-CORE and VGST Karnataka for infrastructural facilities. We are also grateful to Dr. T. Marimuthu for providing Noni fruits and for his constant support.

References

Cassady, J.M., Ojima, M., Chang, C.J. and Mc Laughlin, J.L. 1979. An investigation of the antitumor activity of Micromelum integerrimum (Rutaceae). Journal of natural proucts, 42:274-278.

Furusawa, E. 2002. Anti-cancer activity of Noni Fruit juice against tumors in mice. In: Proceedings of the 2002 Hawaii Noni Conference. University of Hawaii at Nanoa. College of Tropical Agriculture and Human Resources. Hawaii.

Hiramatsu, T., Imoto, M., Koyano, T. and Umezawa, K. 1993. Induction of normal phenotypes in ras -transformed cells by damnacanthal from *Morinda citrifolia*. *Cancer Letters*, 73: 161-166.

Hirazumi, A., Furusawa, E., Chou, S.C., Okano, C. and Ching, C. 1992. Antitumor activity of *Morinda citrifolia* on intraperitoneally implanted Lewis lung carcinoma in mice. *Proceedings of the American Association for Cancer Research*, 33: 515.

Hirazumi, A., Furusawa, E., Chou, S.C. and Hokama, Y. 1994. Anticancer activity of *Morinda citrifolia* on intraperitoneally implanted Lewis lung carcinoma in syngeneic mice. *Proceedings of the Western Pharmacology Society*, 37: 145–146.

Hirazumi, A., Furusawa, E., Chou, S.C. and Hokama, Y. 1996. Immunomodulation contributes to anticancer activity of *Morinda citrifolia* (Noni). *Proceedings of the Western Pharmacology Society,* 39: 7–9.

Hirazumi, A. and Furusawa, E. 1999. An immunomodulatory polysaccharide-rich substance from the fruit juice of *Morinda citrifolia* (Noni) with antitumour activity. *Phytotherapy research*, 13: 330–387.

Ishiyama, M. 1995. Advantages of WST-1 compared to MTT agents. *In vitro Toxicology*, 8:187-189.

Kim, E.K.,Kwona, K.B., Shinb, C.B, Seoc, E.A., Leed, Y.R., Kim J.S., Park J.W., Park B.K. and Ryu D.G. 2005. Scopoletin induces apoptosis in human pro myeloleukemic cells, accompanied by activations of nuclear factor 8B and caspase-3. *Life Sciences*, 77:824–836.

Kinghorn, A.D., Chai, H., Sung, C.K. and Keller, W.J. 2011. The classical drug discovery approach to defining bioactive constituents of botanicals. *Fitoterapia,* 82:71–79

Levand, O. and Larson, H.O. 1979. Some chemical constituents of *Morinda citrifolia*. *Planta Med*., 36: 186-187.

Liu, G., Bode, A., Ma, W.Y., Sang, S., Ho, C.T. and Dong, Z. 2001. Two novel glycosides from the fruits of *Morinda citrifolia* (Noni) inhibit AP-1 transactivation and cell trans formation in the mouse epidermal JB6 cell line. *Cancer Research*, 61: 5749-5756.

Moon, P.D, Lee, B.H., Jeong, H.J., An, H.J., Park, S.J., Kim, H.R., Ko, S.G., Um, J.Y., Hong, S.H. and Kim, H.M. 2007. Use of scopoletin to inhibit the production of inflammatory cytokines through inhibition of the IêB/NF-êB signal cascade in the human mast cell line HMC-1. *European Journal of Pharmacology,* 555:218–225.

Moorthy, N.K. and Reddy, G.S. 1970. Preliminary phytochemical and pharmacological study of *Morinda citrifolia* Linn. *Antiseptic*, 67: 167-171.

Mossman, T. 1983. Rapid colorimetric assay for cell growth and survival: application to proliferation and cytotoxicity assay. *The journal of Immunology, Methods*, 65:55–63

Shukla, S.S., Swarnlata, S. and Saraf, S. 2009. Approaches towards standardization and quality Assessment of herbals. *The journal of research and education in Indian medicine*, 15: 25-32

Simonsen, J.L. 1920.Note on the constituents of *Morinda citrifolia*. *Journal of chemical society*, 117: 561-564.

Soxhlet, F. 1879. Die gewichtsanalytische Bestimmung des Milchfettes. *Dingler's Polytechnisches Journal*, 232:461-465

Stahl, E. 1990.Thin-layer chromatography, A laboratory handbook, p. 857, 873, 878.

Thani, W., Vallisuta, O., Siripong, P. and Ruangwises, N. 2010. Anti-proliferative and antioxidative activities of Thai Noni/ yor (*Morinda citrifolia* Linn.) leaf extract. *Southeast Asian J trop med public health*, 41:482-489

Umezawa K. 1992. Isolation of 1-methoxy-2-foremyl-3-hydroxyanthraquinone from M citrifolia and neoplasm inhibitors containing the same. Japan Kokai Tokyo Koho JP, 736: 87-94.

Wang, M.Y., West, B.J., Jensen, C.J., Nowicki, D., Chen, S., Palu, A. and Anderson, G. 2002. *Morinda citrifolia* (Noni): A literature review and recent advances in Noni research. *Acta Pharmacol sin*., 23: 1127 -1141

West, B.J., Tani, H., Palu, A.K., Tolson, C.B. and Jensen, C.J. 2007. Safety tests and anti-nutrient analyses of Noni (*Morinda citrifolia* L.) leaf. *Journal of the Science of Food and Agriculture,* 87: 2583-2588.

INSTRUCTIONS FOR AUTHORS

AIMS AND SCOPE

International Journal of Noni Research (IJNR) publishes original research and review articles on all aspects on Noni (*Morinda citrifolia* L.) and other species of *Morinda*. All submissions will be reviewed by the editorial board or by external references. The journal covers: diversity, cultivation, phytochemistry and clinical research, etc. related to Noni.

Three categories of paper will be considered for publication in IJNR.

1) Reviews
2) Full-length papers
3) Short communications.

SUBMISSION OF MANUSCRIPT (HARD AND SOFT COPY)

Authors are advised to submit their manuscripts along with a covering letter to the Editor, International Journal of Noni Research, # 12, Rajiv Gandhi Road, Sreenivasa Nagar, Chennai - 600 096, India

E-MAIL SUBMISSION

Authors are encouraged to submit their manuscripts via e-mail as attachment file to the E-mail ID : mail@worldnoni.net, palms02@hotmail.com and marisorna2008@gmail.com (A covering letter to be sent with the manuscript).

PREPARATION OF MANUSCRIPT

Two typed copies of manuscripts using MS Word should be submitted. They should be typed on one side of the paper only, double-spaced with 1.2" margins in all the sides. All pages should be numbered.

All the accepted articles will be subjected to editorial revision. A singed statement from all the authors should be accompanied with the manuscript saying that

1. the content of the article has not been published in whole or in part elsewhere
2. the article is not currently being considered for publication elsewhere
3. all necessary ethical safeguards have been met regarding patient and animal experimentation, etc.

FULL-LENGTH PAPERS

There is no page restriction on overall length of article. It should be divided into

1) Abstract (100- 200 words) followed by up to six keywords
2) Introduction
3) Materials and Methods
4) Results
5) Discussion
6) Acknowledgements
7) References

Results and discussion may be combined. Title page should be prepared in a separate sheet, which should provide the title of paper, name(s) of the author(s), name(s) and address of the institution(s) where the work has been carried out and details of the corresponding author with telephone and fax numbers and e-mail ID.

SHORT COMMUNICATIONS

It should be up to 10 double-spaced manuscript pages with a short summary of 50 words. Short communications should not be divided into different headings. However, headings can be used for acknowledgements and references. Figures and Tables should be restricted to a maximum of 2 each. All other style should be as for Full-length papers.

FIGURES AND TABLES

Figures, figure legends and tables should be typed on a separate sheet and numbered consecutively in Arabic numerals. Photographs should be sharp with glossy prints. Micrographs, etc. should include a bar marker to provide an internal measure of scale or magnification should be mentioned in each microscopic photograph.

REFERENCES

References in the text should be cited as follows

Single author: Surendiran (2004) or (Surendiran, 2004)

Two authors: Surendiran and Mathivanan (2005) or (Surendiran and Mathivanan, 2005)

Three or more authors: Mathivanan *et al.* (2006) or (Mathivanan *et al*., 2006)

Where two or more than two references are quoted consecutively in the text, chronological order should be followed. If the references are within a year, alphabetical must be followed. Where references are made to papers by the same author(s) in the same year, it should be followed by a, b, c, etc.

References must be listed alphabetically by the name of the authors at the end of the manuscript. The following style must be followed to cite the references

Journal articles

Abbott, I.A. 1985. The geographic origin of the plants most commonly used for medicine by Hawaiians. Journal of Ethnopharmacology, 14: 213–22.

Surendiran, G. and Mathivanan, N. 2006. Antifungal activity of *Morinda citrifolia* and *Morinda pubescens*. International Journal of Noni Research, 2: 18-23.

Mathivanan, N., Surendiran, G., Srinivasan, K., Sagadevan, E. and Malarvizhi, K. 2005. Review on the current scenario of Noni research: Taxonomy, distribution, chemistry, and medicinal and therapeutic values of *Morinda citrifolia* L. International Journal of Noni Research, 1:1 – 9.

Books or monographs

Lalithakumari, D. 2000. *Fungal Protoplast: A Biotechnological Tool*. Oxford & IBH Publishing Co., Pvt., Ltd., New Delhi. p184.

Single author volumes

Mathivanan, N. 2004. Current scenario of the biocontrol potential of *Trichoderma* for the management of plant diseases. In: *Emerging Trends in Mycology, Plant Pathology and Microbial Biotechnology* (Eds. Bagyanarayana, G., Bhadraiah, B. and Kunwar, I.K.), BS Publications, Hyderabad, India. pp. 364-382.

Multi-authors volumes

Mathivanan, N., Bharati N. Bhat, Prabavathy, V.R., Srinivasan, K. and Chelliah, S. 2003. *Trichiderma viride*: Lab to land for the management of root diseases in different crops. In: *Innovative Methods and Techniques for Integrated Pest and Disease Management* (Eds). Mathivanan, N., Prabavathy, V.R. and Gomathinayagam, S.), Centre for Advanced Studies in Botany, University of Madras, Chennai, India. pp. 52-58.

Thesis / Dissertation

Surendiran, G. 2004. **Antimicrobial and Wound Healing Activity of Morinda tinctoria.** ***M. Sc. Thesis, University of Madras, Chennai, India.***

PROOFS

Proof will be sent to the corresponding author for checking and making essential corrections. At this stage, no extensive general revision or alteration will be allowed. Proof should be corrected and returned to the editor within 5 days of receipt.

OFFPRINTS

Corresponding author will receive the PDF format of the article by e-mail free of charge. A copy of the journal will be sent by post to the corresponding author after publication.

PAGE CHARGES

No page charge for publishing the paper in the International Journal of Noni Research.

COPYRIGHT