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Bioassay based discovery of anti-cancer drugs from fruits of Noni, *Morinda citrifolia* L.

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Foreword

The technical bulletin is the culmination of project, *"Bioassay based discovery of anti cancer drugs from fruits of Noni, Morinda citrifolia L.",* which envisaged to evaluate the extracts of noni fruits for anti-tumor properties and also to check chemopreventive effect. The Principal Investigator has systematically carried out the project. Fractionation of bioactive compounds from crude extract was not found to have any profound increment in cytotoxicity. The scopoletin was found to have cytotoxicity to all the cell lines tested, especially, WM115 and Saos 2 while rutin did not show any cytotoxicity. The noni fruit juice, the ethanolic extract had superior chemopreventive property. These observations are quite interesting and may warrant further studies to understand more about the action of noni fruit juice against any given cancer cell lines. The Principal Investigator proposes that the chemopreventive property could be exploited as they potentiate the efficacy of chemotherapy or radiotherapy.

I congratulate the Principal Investigator for having opened a new avenue to further the knowledge on cancer treatment using noni fruit juice. I also place on record our appreciation to the editorial team, Dr. P. Rethinam, Dr. T. Marimuthu and Dr. K. V. Peter for bringing out the 15th Technical Bulletin in time. The assistance rendered by Mr. T. Thanigai kumar and Mr. A. Arunachalam in computer setting and printing is appreciated.

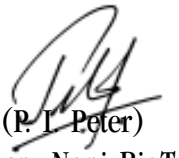
Chennai
September, 2014

(Kirti Singh)
Chairperson

About the Project

World Noni Research Foundation continues to support research project on Noni envisaging crop improvement and management, plant protection, clinical and pharmacological research besides food science aspects. The outcome of the project studying the efficacy of noni fruit juice as anticancer drug is quite interesting as the results indicated that noni juice could be better used as a chemopreventive agent as it potentiates the conventional therapies.

I appreciate the sincere efforts of Dr. K. Satyamoorthy and his team for taking up a challenging project working on several cancer cell lines studying the effect of noni fruit juice on the cytotoxic effect on these cell lines.



Chennai
September, 2014

(P. T. Peter)
Chairman, Noni BioTech

Preface

Chemopreventive agents are ideally non-toxic and exert their action by blocking or metabolizing carcinogens and thus inhibiting tumor growth. These qualities are reflected in Noni crude extracts with their reduced toxicity and superior chemopreventive potential. This quality can promote Noni crude extract as an excellent adjuvant for chemotherapeutics or radiotherapy, which opens a new avenue for cancer prevention. Current study proven by alkaline comet assay and neutral comet assay indicated that both extracts (aqueous and ethanolic) at 100µg/ml concentration are able to significantly bring down the OTM values after exposure to EMS and UV radiation respectively. Moderate reduction in the SSF × (-1) value was noticed after pretreatment with Noni extract in FADU assay. Studies with single strand break assay indicated that both aqueous and ethanol crude noni extracts help protect lymphocytes against single strand breaks induced by hydrogen peroxide when subjected to a half an hour treatment at 50 and 500µM concentrations. DPPH assay performed with Noni extracts and standard *Morinda* compounds exhibited antioxidant property which was tested further by flow cytometry. Thus Noni can emerge as a potent chemopreventive adjuvant which can be employed in targeted therapeutic strategies against cancer in future.

K. Satyamoorthy

1. Introduction

Morinda citrifolia L., commonly known as Noni or Indian mulberry is a native from Southeast Asia to Australia and is now distributed throughout the tropics. It belongs to the coffee family, Rubiaceae. Noni is known to have a long tradition with its use as a source of food and for its medicinal properties, both in India and the pacific Islands. Noni is reported to have many therapeutic properties some of which include antibacterial, antiviral, antifungal, antitumor, anthelminthic, analgesic, hypotensive, anti-inflammatory and immune enhancing effects. One of its profound effects being studied is its ability to act against cancer by the prevention of carcinogen-DNA adduct formation and the antioxidant activity of noni which may contribute to the cancer preventive effect of *Morinda citrifolia* L. Noni is a fruit spanning a wide range of classes of compounds, such as anthraquinones and their glycosides, carotenoids, coumarins, flavonoids, iridoids, lignans, polysaccharides, steroids and triterpenes. Some of its active principles include scopoletin, alizarin, octoanoic acid, β-sitosterol, *L*-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin and a putative proxeronine.

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 lack of sufficient information on the cytotoxic properties of the Noni fruit. Hence, the first part of 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, we 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.

Our preliminary studies on Noni extracts over a panel of six human tumor cell lines clearly indicated that Noni fruit extracts, neither crude nor fractionated were cytotoxic to tumor as well as normal cell lines. Our results were in consonance with other reports proven elsewhere 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 with the participation of macrophages, natural killer cells and T cells (Furusawa *et al.,* 2002). Noni juice has become popular health drink and dietary supplement (Kinghorn *et al.,* 2011), which would be plausible only if Noni juice is not cytotoxic. To test this alternative hypothesis the second part of the study was undertaken. Here, we hypothesized that, two chosen *Morinda* extracts namely aqueous and ethanolic might exhibit significant chemopreventive effect against damage induced by UV radiation, X-rays, hydrogen peroxide and ethyl methane sulfonate.

2. State of Knowledge

Morinda citrifolia L., commonly known as Noni or Indian mulberry is a native from Southeast Asia to Australia. Noni is reported to have many therapeutic properties some of which include antibacterial, antiviral, antifungal, antitumor, anthelminthetic, analgesic, hypotensive, anti-inflammatory and immune enhancing effects. One of its profound effects being studied is its ability to act against cancer by the prevention of carcinogen-DNA adduct formation and the antioxidant activity of noni may contribute to the cancer preventive effect of *Morinda citrifolia*L. Noni is a fruit spanning a wide range of classes of compounds, such as anthraquinones and their glycosides, carotenoids, coumarins, flavonoids, iridoids, lignans, polysaccharides, steroids and triterpenes. Some of its active principles include scopoletin, alizarin, octoanoic acid, β-sitosterol, *L*-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin and a putative proxeronine.

3. Objectives

The primary goals of the project include:

i. Chemical fingerprinting of Noni fruit extracts
ii. Activity guided fractionation of ripe noni fruit
iii. Evaluation of bio-efficacy of extracts and checking for anti-tumor properties of Noni extracts
iv. Checking for the chemopreventive effect of *Morinda* extracts using various assays

v. Carry out mitochondrial function assays using *Morinda* extracts

4. Experimental details

Fruit sample

Ripe fruit samples of Noni were provided by the World Noni Research Foundation, Chennai, India.

Maintenance of cell lines

Lymphocytes transformed with Epstein-Barr virus (EBV) obtained from marmoset cell lines were used as control cells to check for chemopreventive effect of Noni extracts. This cell line was cultured in RPMI augmented with 12% foetal bovine serum and was maintained in suspension culture. Fibroblast cells derived from human foreskin samples were maintained in DMEM containing with 10% fetal bovine serum and antibiotics and used. Fibroblast cells were maintained as monolayers. The cells were kept at 37°C in an incubator of humidified air with 5% CO_2 and were regularly sub-cultured every three days.

Preparation and extraction of fruits

The fruits were dried for three days at 60°C in a hot air oven and then ground to a fine powder. The powder was then mixed with ethanol and water in the ratio 1:10 and stirred overnight at 4°C to obtain ethanolic and aqueous extracts respectively. The solvents after the overnight extraction were lyophilized to powder form.

The Noni fruit was processed in three different ways to obtain the extract tested in the cytotoxicity assays. Two types of starting material were used for the extraction, fresh and dry fruits.

a)Extraction from fresh fruit

The fresh ripe fruit was processed immediately after collection. The fruit was ground to a fine pulp using a mixer grinder. The juice obtained was then centrifuged at 20,000 rpm for 20 min at 4 °C to remove suspending particles. The clear supernatant was then freeze dried till a fine clear powder was

obtained. The clear powder was then aliquoted into microfuge tubes and then stored at -20°C till further use.

b) Extraction from dry fruit

1) Soxhlet

The fruit was dried for three days at 60ºC in a hot air oven. All traces of water found were removed by this process. The dry fruit was then ground to a fine powder using a mixer grinder. Out of the powder obtained 10g was used for Soxhlet extraction (Franz, 1879). The individual extraction was done separately using three solvents namely water, ethanol and hexane. The process was continued till the solvent getting refluxed became colorless ensuring the complete extraction of the active principles have into the respective solvents, leaving the residue behind in the filter paper. This residue was dried and re-weighed. The solvent in the round bottom flask with the active principles was then collected and stored for further processing.

The sequential extraction was done sequentially using three solvents hexane followed by ethanol and finally with water. The process was repeated similar to the method carried out for individual extraction. The residue obtained was dried and re-weighed.

By using the above procedures a total of five extracts were obtained :

1) Ethanol extract (sequential and individual)
2) Aqueous extract (sequential and individual)
3) Hexane extracts (Individual)

The hexane and ethanol samples were first evaporated and then subjected for lyophilization whereas, the aqueous extract was directly subjected for lyophilization. The extracts obtained after lyophilization were then sealed and stored at -20°C until use.

2) Extraction using solvents

a) The dry fruit powder which was procured by the aforementioned method was dissolved in three different solvents: hexane, ethanol and water separately and kept overnight for stirring at 4°C using a magnetic stirrer. The solvent

containing the dissolved compounds was then centrifuged at 20,000 rpm for 20 min at 4 °C to remove suspending particles. The clear supernatant was then freeze dried and the clear powder was aliquoted into microfuge tubes and then stored at -20°C till further use.

Chemical finger printing using thin layer chromatography (TLC)

To determine the stage of the fruit to be used for extraction of the crude extracts, chemical finger printing was carried out using thin layer chromatography. Three stages of the noni fruit was used for this purpose. Amongst the three groups of compounds tested, largest number of compounds appeared in the ripe fruit in all three groups namely, alkaloids, phenylpropanoids and terpenoids, hence it was chosen as the stage for further extraction.

WHO guidelines suggest the use of finger printing methods to meet the global standards of quality control of the herbal formulations. Chemical finger printing is usually carried out by chromatographic and spectroscopic evaluations. The initial step of chemical finger printing was done by thin layer chromatography. Three main classes of compounds were screened using TLC based methods described by Egon Stahl in the hand book of thin layer chromatography (Fig.1).

Fig. 1. Thin layer chromatography of aqueous extract fractions. When we observed the TLC plate under UV various fluorescent spots were noticed. We noted that fractions 6 and 7 as a whole, displayed spots of higher intensity compared to the individual sub fractions obtained on further fractionation, indicating the possible loss of activity occurring at each step. 1=40-42, 2 = 40-45, 3 = 45-50, 4 = 42-44, 5=44-46, 6=46-48, 7=48-50.

HPLC profiling and activity guided fractionation of Noni extract

The analyses were performed using Waters series Alliance 2695 HPLC system and detection was carried out using Waters 2487 dual wave length 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 µm). The fractions which showed activity were further separated using a Phenomenex Prodigy series C8 column (15 cm × 4.6 mm i.d., 5 µm) 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 detection wave length was 254nm 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 µm). Using analytical C18 column total of seven fractions were collected over a period of sixty minutes. (Fig. 2-13).

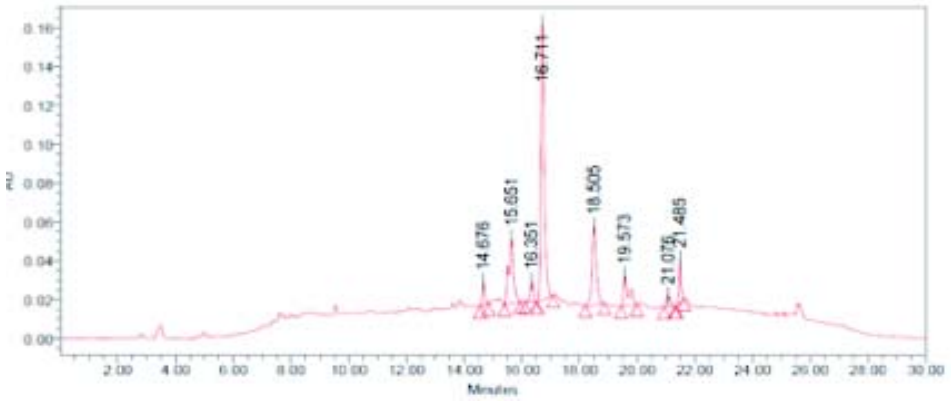


Fig.2. HPLC chromatogram of the fourth fraction of the freeze dried extract at 280nm on analytical C8 column

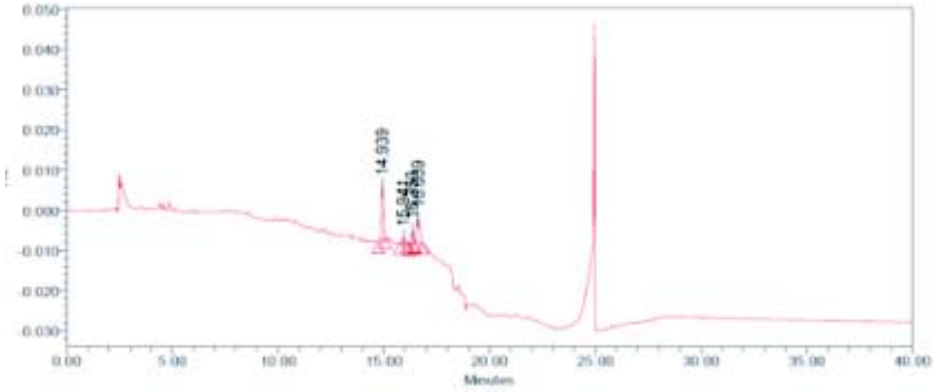


Fig.3. HPLC chromatogram of the fifth fraction of the freeze dried extract at 280nm on analytical C8 column

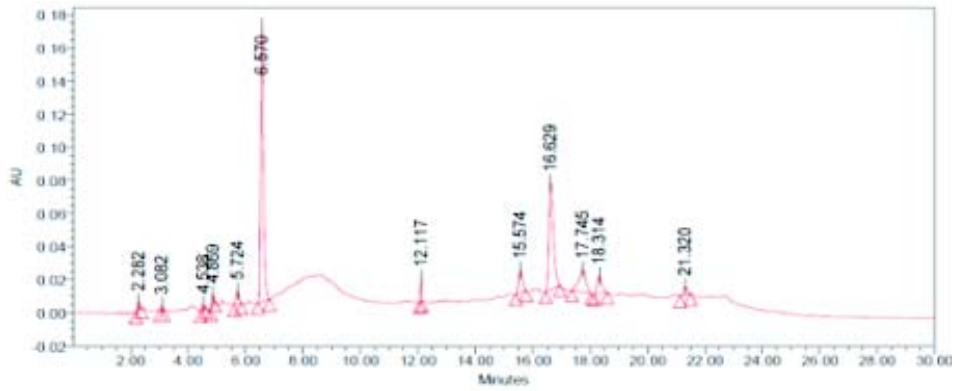


Fig.4. HPLC chromatogram of the first fraction of the freeze dried extract at 280nm on analytical C8 column

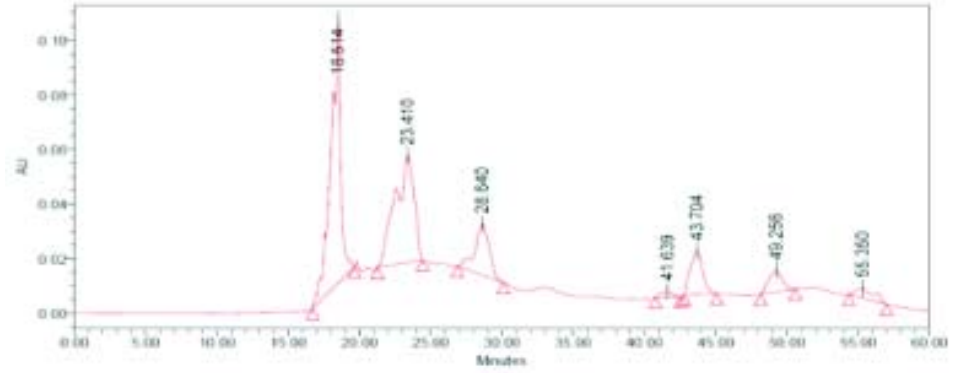


Fig. 5 : HPLC chromatogram of the aqueous extract at 254nm on semi prep C18 column. Fractions were collected every five minutes starting from the fifteenth minute till the end of sixty minutes. The similarity in the retention time of rutin and peak three in the aqueous extract indicates that this peak could be the compound rutin

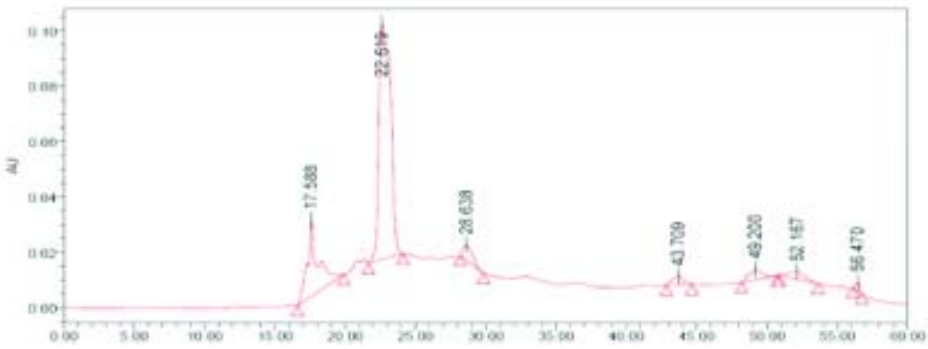


Fig. 6 : HPLC chromatogram of the aqueous extract at 280nm on semi prep C18 column.

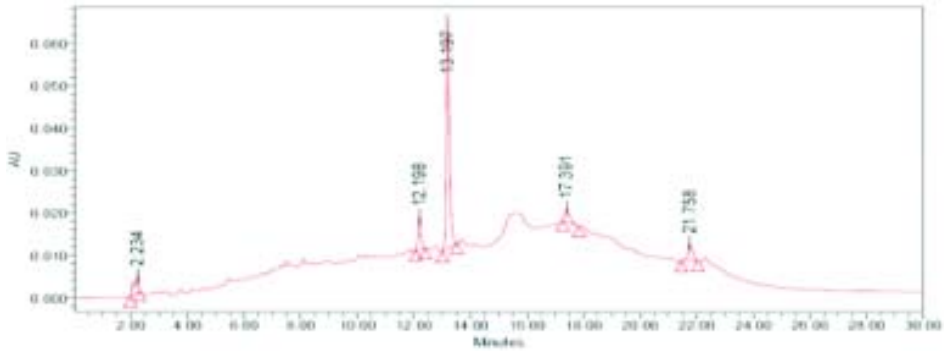


Fig. 7 : HPLC chromatogram of the aqueous extract fraction 6 280nm on analytical C18 column

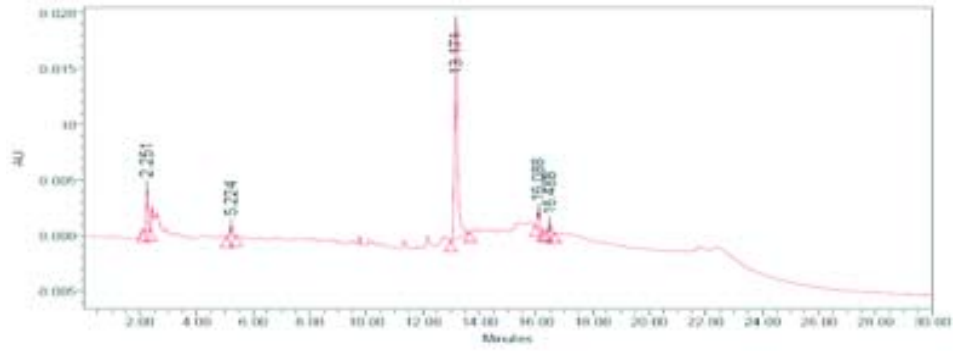


Fig. 8 : HPLC chromatogram of the aqueous extract fraction 7 280nm on analytical C18 column

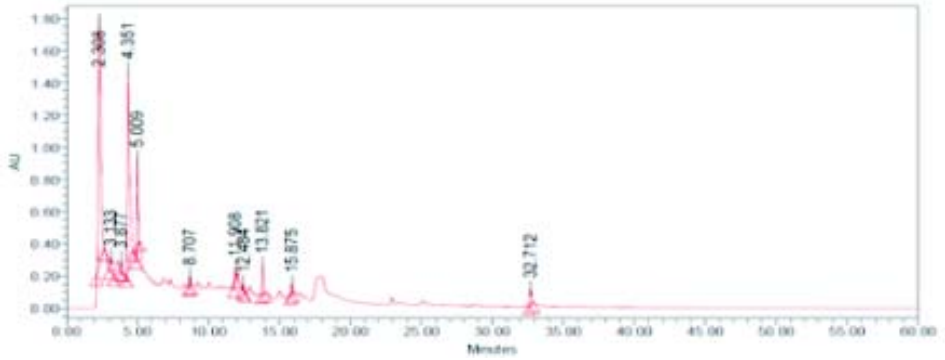


Fig. 9 : HPLC chromatogram of the ethanol extract at 280nm on analytical C18 column. Fractions were collected at an interval of seven minutes till the forty ninth minute.

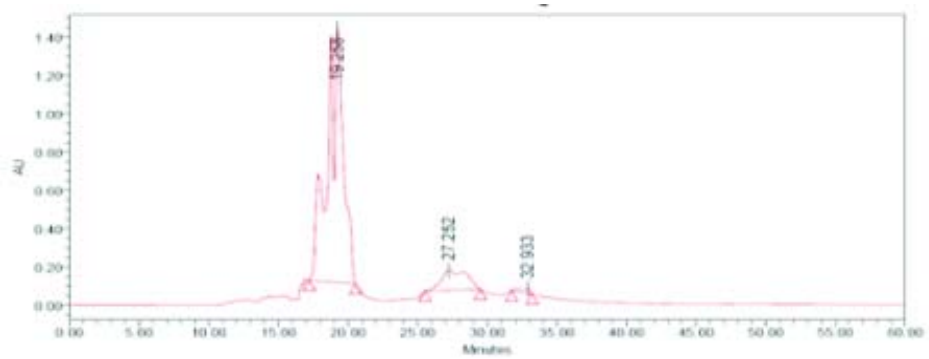


Fig. 10 : HPLC chromatogram of the ethanol on semi prep C 18 Column at 254 nm

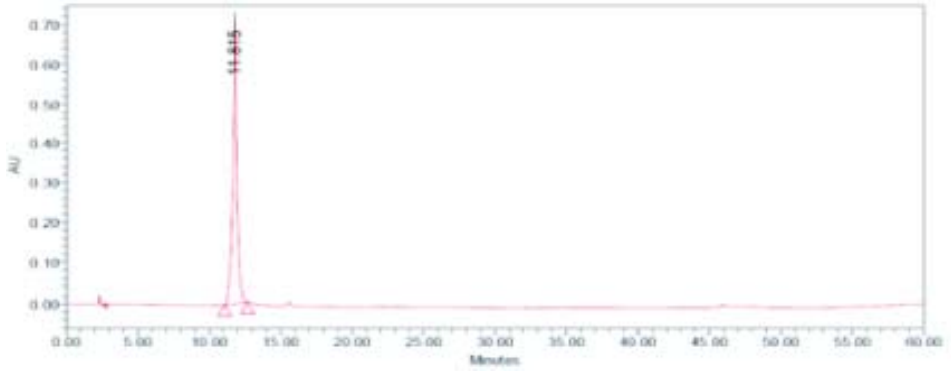


Fig. 11 : HPLC chromatogram of the standard scopletin at 280nm on analytical C18 column

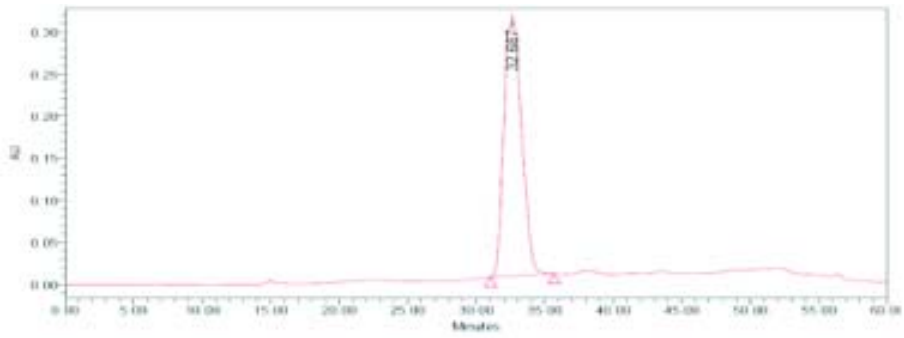


Fig. 12 : HPLC chromatogram of the standard scopoletin at 280nm on semi prep C18 column

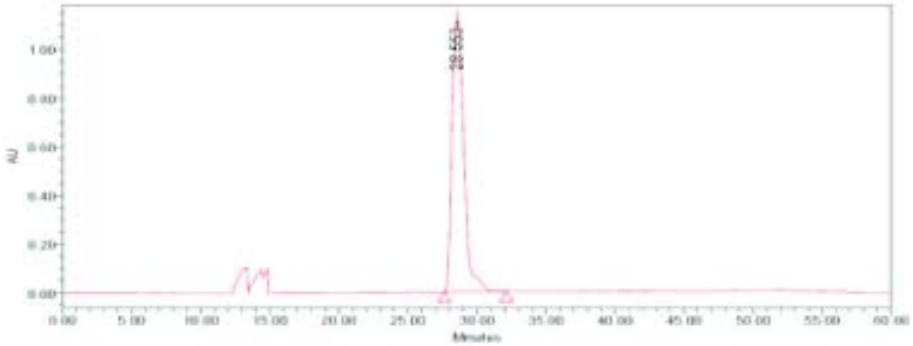


Fig. 13 : HPLC chromatogram of the standard rutin at 280nm on semi prep C18 column

A fraction was collected at every seventh minute; hence seven fractions were collected till the 49th minute. The fractions collected were then kept for lyophilization to obtain a clear powder. The powder obtained is then stored at -20°C till further use. Each of the seven fractions was then tried on the entire panel of cell lines to check for the fractions with possible cytotoxicity. The fractions showing cytotoxicity was further fractionated and analyzed.

Evaluation of bio-efficacy of extracts and checking for anti-tumor properties of Noni extracts

Cytotoxicity of the drug obtained after extraction was checked by performing MTT assay as described by Mosmann, 1983. The six cancer cell lines (Table 1) were chosen to perform the assay. In case of the suspension cell line Colo 205, WST was the assay of choice to test the anti-proliferative activity (Ishiyama, 1995).

Table 1: Information about the cell lines chosen for MTT assay

S. No	Cell line	Origin	Normal Gene	Defective Gene
1	A 549	Human lung carcinoma	APC, Braf, CDH1, CTNNB1, H RAS, MADH4, N RAS, PIK3CA, PTEN, RB1, TP53, BRCA 1,	KRAS, STK11, CDKN2A
2	Caski	Human cervical epidermoid carcinoma	APC, Braf, CDH1, CDKN2A, CTNNB1, H RAS, K RAS, MADH4, N RAS, PIK3CA, PTEN, RB1, STK11, TP53, EGFR, VHL	P53
3	COLO 205	Human colon adenocarcinoma	BRCA1, BRCA2, CTNNB1, CDH1, CDKN2A, EGFR, ERBB2, FLT3, HRAS, KRAS, NRAS, PDGFRA, PTEN, PIK3CA, RB1, STK11, VHL	MADH4, TP53, APC, BRAF
4	MDA MB 231	Human breast carcinoma	APC, BRCA1, BRCA2, CDH1, CTNNB1, EGFR, ERBB2, FLT3, HRAS, NRAS, MADH4, PDGFRA, PIK3CA, PTEN, RB1, STK11, VHL	KRAS, TP53, CDKN2A, BRAF
5	Saos 2	Human osteosarcoma	APC, BRAF, CDH1, CTNNB1, CDKN2A, EGFR, ERBB2, HRAS, KRAS, NRAS, MADH4, TP53, PIK3CA, PTEN, STK11, VHL	RB1
6	WM 115	Human skin melanoma	APC, CDH1, CDKN2A, CTNNB1,EGFR, H RAS, K RAS, MADH4, N RAS, PIK3CA, RB1, STK11, TP53, VHL	BRAF, PTEN

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 5µg/ml to 1000µg/ml in a series of MTT assays. However, in case of the freeze dried juice extract two additional concentrations of 5000 µg/ml and 10,000µg/ml were also tested. Standard compounds were tested for concentrations ranging from 1 to 100µg/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 48hrs. 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.

Comet Assay

Alkaline Comet Assay

The protective effect of the *Morinda* extracts against the carcinogen ethyl methanesulfonate (EMS) purchased from Sigma-Aldrich (Bangalore, India) were evaluated by single cell gel electrophoresis (comet assay). This assay was performed under alkaline conditions according to the procedure of Singh *et al.* (1988) with minor modifications of Collins *et al.* (1997). Exponentially growing lymphocytes were segregated into two groups. The former group was pre-treated with both ethanolic and aqueous extracts at a concentration of 100µg/ml for a period of 24 hours. The cells were subsequently subjected to post treatment with EMS at a concentration of 2.5mM for 24 hours. The latter group was treated with EMS (2.5mM) along with the Noni extracts (100µg/ml) for a period of 24 hours. After the two aforementioned treatments the comet slides were prepared. The comets captured were then analyzed using Komet software (Version 5.5, Kinetic Imaging Ltd., Bromborough, UK). The mean olive tail moment (OTM) was selected as the parameter that best reflected DNA damage.

Neutral Comet Assay

Noni extracts were assayed for their protective effects against UV radiation by single cell gel electrophoresis (comet assay). The assay was performed under neutral conditions according to the procedure described by Wilkins *et al.* (2002) with minor modifications. Exponentially growing lymphocytes were incubated with ethanol and aqueous extracts at a concentration of 100µg/ml for 24 hours, prior to damage with UV rays at 1 and 2 J/cm^2. The comets were captured using Komet software. OTM was calculated to assess protective effect of the extracts.

Fast micromethod DNA Single Strand Break Assay

To assess the ability of Noni extracts to protect against single stranded breaks caused by hydrogen peroxide (H_2O_2), fast micromethod DNA single-strand-break assay (SSBR) was performed (Schroder *et al.,* 2006). Lymphocytes were pre-incubated with ethanol and aqueous extracts at a concentration of 100µg/ml for 24 hours after which cells were exposed to H_2O_2 at concentrations 50 and 500µM. Single stranded breaks (SSBs) induced by H_2O_2 was quantified using Pico green double strand DNA quantitation reagent. Readings were taken using fluorescence micro plate reader at 480 nm excitation and 520 nm emissions. DNA damage was expressed as a function of strand scission factor (SSF).

Fluorescent Analysis of DNA unwinding

Morinda extracts were checked for their protective effect against double stranded breaks caused by UV rays using fluorescent analysis of DNA unwinding (FADU) assay (Khan *et al.,* 2000). Similar to the previous assays, lymphocytes were pretreated with ethanol and aqueous extract at 100µg/ml for 24 hours. Cells were then exposed to UV rays at a dose of 2 J/cm^2. Double stranded breaks were estimated using Hoechst 33258 dye. Readings were taken using spectroflurometer at 350 nm (excitation) and 455 nm (emission). Breaks were expressed as a value of strand scission factor (SSF).

Neutral Filter Elution Assay

Lymphocytes were pretreated with ethanol and aqueous extract, at 100µg/ml for 24 hours. 4×10^5 cells treated with X-ray irradiation at the dose 5 Gy were taken and transferred on to polycarbonate membrane filter mounted in the filtration assembly.

The pre-assembled filtration apparatus was placed in 15 ml conical centrifuge tubes and centrifuged at 1000g for 2 minutes. After the centrifugation, the filtrate was discarded and the cells were lysed with 300 µl of lysis reagent. Cells were lysed for 1 hour at 55ºC and followed by centrifugation at 30g for 4 minutes to remove the lysate. The damaged double stranded DNA fractions was eluted from the membrane by adding 300 µl of lysis reagent and centrifuged after 5 minutes at 50g for 6 minutes. Simultaneously, 4×10^5 cells were taken in a 1.5 ml centrifuge tube and lysed with 300 µl of lysing solution for 1 hour at 55ºC. The initial DNA damage was analyzed 30 minutes after irradiation for 5 Gy and the rejoining of the DSBs was evaluated after 180 minutes. The fraction of DSB containing DNA eluted was assessed in Nunc 96-well black microplates by fluorescence measurements. A 100µl of the fraction eluted was added with 100 µl of Pico green (500-fold diluted) and kept for 10 minutes at room temperature. Fluorescence readings were taken at excitation of 480 nm and emission of 520 nm using Tecan Infinite M200 series Microplate reader (Salzburg, Austria).

DPPH Assay

Antioxidant properties of Noni extracts were evaluated by testing their free radical scavenging potential using 2,2-diphenyl-1-picrylhydrazyl (DPPH) purchased from Sigma-Aldrich (Bangalore, India). The assay was performed using the method described by Ates et al., 2008 with minor modifications. The radical scavenging ability of the extracts was compared with known antioxidant, N-acetylcysteine (NAC) which was used as a positive control. In addition to this two standard compounds kaempferol and rutin identified in Morinda fruits were also assessed for their antioxidant potential in a similar fashion. Thirty minutes after the addition of the test compounds to DPPH, the absorbance was measured at 517nm. DPPH scavenging potential was expressed as IC50 and compared with that of the standard NAC.

Detection of Intracellular Reactive Oxygen Species

Production of reactive oxygen species (ROS) was measured by flow cytometry using the cell-permeable dye 2', 7'-dichlorfluorescein-diacetate (DCFHDA) based on a method performed by Zhang et al, 2011 with minor modifications. Fibroblast cells were first treated with DCFHDA for 15 minutes at 37ºC and then subjected to various treatments for a period of ten minutes. The protective effects of Morinda extracts at 1000 µg/ml concentration and standard Noni compounds at 10 µg/ml concentration were tested against hydrogen peroxide induced oxidative stress at a concentration 100µM, using NAC as a positive control at a concentration of 816 µg/ml (5mM). Standard compounds kaempferol and rutin dissolved in DMSO were tested alongside appropriate vehicle controls. The cells were acquired by FACS using the BD FACSCalibur flow cytometer (excitation at a wavelength of 488 nm and emission at 525 nm) and analyzed with BD Cell Quest analysis software.

5. Experimental findings

Extraction

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

TLC using fresh noni juice

Thin Layer Chromatography was performed using fresh noni juice. The results are described in the Fig. 14,15,16,17

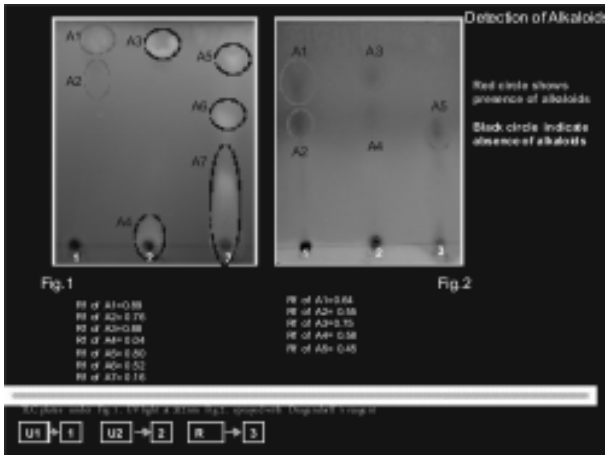


Fig. 14 : Detection of Noni juice on TLC plates. The solvent system and spray reagent used is specific to Alkaloids. Common spots were detected when visualized, both under UV light and using the specific visualizing spray reagent - *this indicates that they are alkaloids.* All extracts were found to have alkaloid specific spots. It can also be observed that as the fruit ripens new compounds are synthesized as seen on the TLC plate U1= Unripe fruit stage 1, U2= Unripe fruit stage 2, R= Ripe fruit

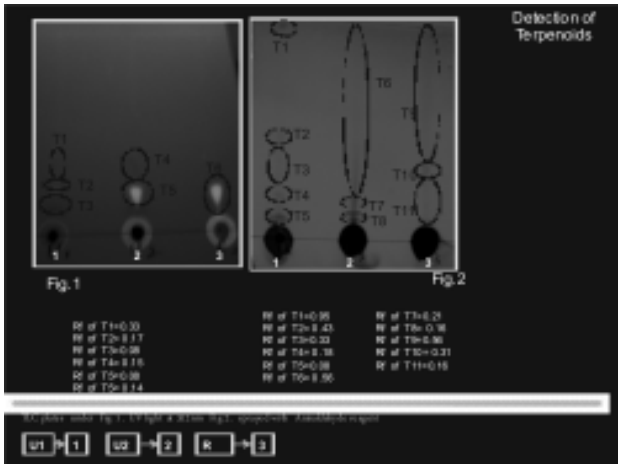


Fig. 15: Detection of Noni juice on TLC plates. The solvent system and spray reagent used is specific to terpenoids. Common spots were detected when visualized, both under UV light and using the specific visualizing spray reagent - *this indicates that they are terpenoids.* All extracts were found to have phenylpropanoid specific spots. It can also be observed that as the fruit ripens new compounds are synthesized as seen on the TLC plate. Also in this case it can be noticed that some of the reddish spots (possibly chlorophyll) seem to disappear as the fruit ripens.U1= Unripe fruit stage 1, U2= Unripe fruit stage 2, R= Ripe fruit

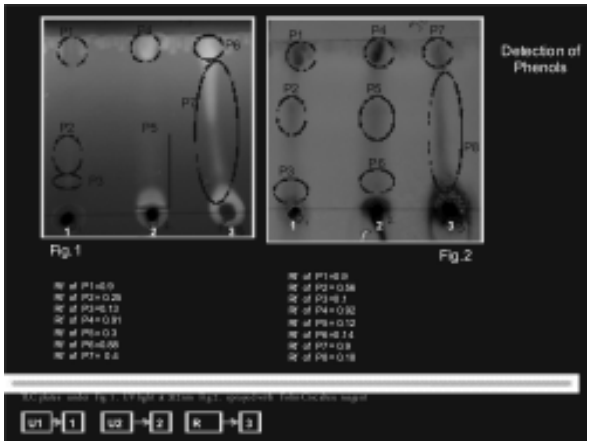


Fig. 16: Detection of Noni juice on TLC plates. The solvent system and spray reagent used is specific to phenylpropanoids. Common spots were detected when visualized, both under UV light and using the specific visualizing spray reagent - *this indicates that they are phenyl propanoidss.* All extracts were found to have phenylpropanoid specific spots. It can also be observed that as the fruit ripens new compounds are synthesized as seen on the TLC plate.
U1= Unripe fruit stage 1, U2= Unripe fruit stage 2, R= Ripe fruit

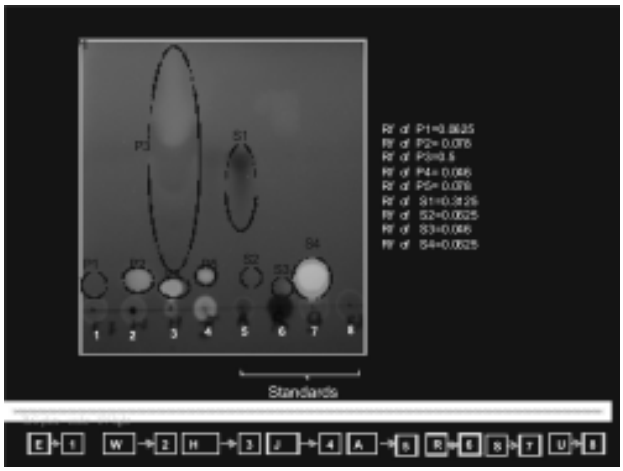


Fig. 17. : Detection of Noni extracts on TLC plates along with standard compounds.

When we observed the TLC plate under UV lamp, various fluorescent spots were noticed. Also most of the standards gave specific fluorescent spots which had Rf values comparable to the Noni extracts. Out of all the compounds; scopoletin seems to be present in almost all the extracts. However, its concentration seems to be very low in ethanol extract. Rutin and Alizarin also seem to be present in the extracts at a very low concentration. The standard Ursolic acid is devoid of any fluorescence under the UV lamp. E= Ethanol extract, W= Aqueous extract, H= Hexane extract, J= Noni juice, A=Alizarin, R=Rutin, S=Scopoletin, U= Ursolic acid

Effect of Soxhlet ethanol extract

The Fig. 17 represents the effect of the ethanol extract obtained by the soxhlet procedure on the panel of cell lines mentioned above. From the data it could be interpreted that the cell line WM115 seems to be most sensitive to this particular extract. Based on the trend observed at 500µg/ml the sensitivity to the extract follows the pattern:

WM115>A549>MDA MB 231>Saos 2>Colo 205>Caski.

When we consider the trend at 1000µg/ml it is found to follow the following pattern:

WM115> A549>Saos 2>MDA MB 231>Colo 205>Caski.

Hence, it could be concluded that WM115 as suggested before is most sensitive and Caski is most resistant to the ethanol extract. The IC 50 value of WM 115 was found to be close to 500µg/m and in the case of A549 it was found to be close to 1000µg/ml. whereas, the rest of the cell lines seem to have an IC 50 above 1000µg/ml. It can also be observed that fibroblast cells show a higher percentage of viability at all concentrations when compared to the tumor cell lines, indicating that Noni extracts might have a selective action against tumor cells Fig. 18. The soxhlet ethanol extract did not show a significantly higher cytotoxic effect after HPLC fractionation. Hence, it was not re-fractionated the second time.

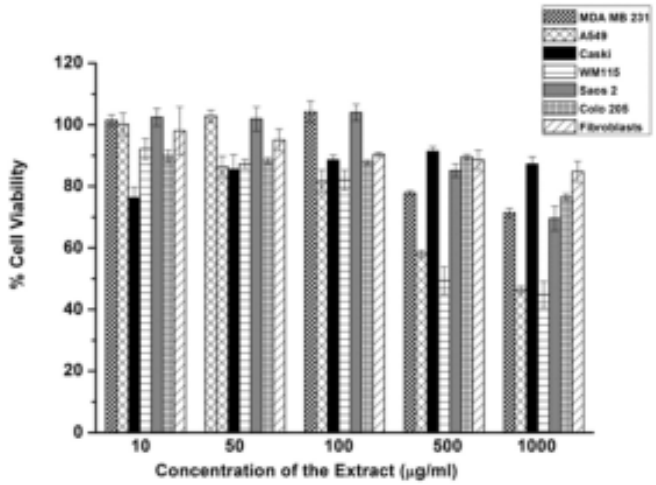


Fig. 18 : The graph represents the effect of soxhlet ethanol extract on six tumor cell lines using fibroblast as control

Effect of Soxhlet aqueous extract

The Fig. 19 depicts the effect of the aqueous extract obtained by the soxhlet procedure on the cell lines mentioned in the panel above. From the data above it could be interpreted that the cell line Saos 2 seems to be most sensitive to this particular extract. Based on the trend observed at 500µg/ml the sensitivity to the extract follows the pattern:
Saos 2>Caski>WM115> A549> MDA MB 231>Colo 205
When we consider the trend at 1000µg/ml it is found to follow the following pattern:
Saos 2> A549>Caski> MDA MB 231>WM115>Colo 205

At a higher concentration (1000µg/ml) A549 seems to have increased sensitivity compared to some other cell lines at 500µg/ml. However, Saos2 and Colo205 prevail to maintain the same pattern all through. Hence, it could be concluded that Saos 2 as suggested before is most sensitive and Colo 205 is most resistant to the aqueous extract. From the data above the IC 50 value of Saos 2 was found to be close to 500µg/m and in the case of A549 it was found to be close to 1000µg/ml. Whereas, the rest of the cell lines seem to have an IC 50 above 1000µg/ml. As observed in case of soxhlet ethanol extract the fibroblast cells show a higher percentage of viability at all concentrations when compared to the tumor cell lines, indicating that Noni extracts might have a selective action against tumor cells (Fig. 19). The soxhlet aqueous extract did not show a significantly higher cytotoxic effect after HPLC fractionation. Hence, it was not re-fractionated the second time.

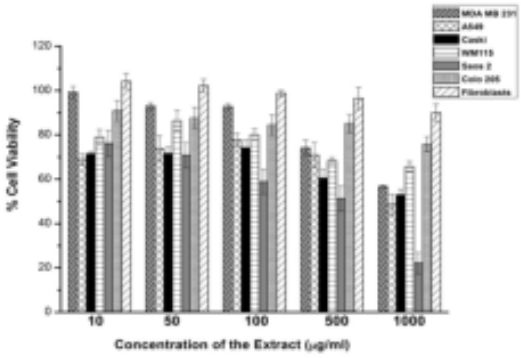


Fig. 19 : The graph shows the effect of soxhlet aqueous extract on six tumor cell lines using fibroblast as control.

Effect of freeze dried juice extract

The Fig. 20 depicts the effect of Noni juice powder on six chosen cell lines. Based on the trend seen it can be concluded that Colo 205 is most susceptible to this particular extract. When the values are compared at 5000µg/ml of the extract the sensitivity shows the following pattern:

Colo 205> MDA MB 231>Caski>Saos> WM115> A549

However, at 10000µg/ml WM115 shows an increased sensitivity. IC 50 values of most cell lines such as MDA MB 231, Caski, Saos and Colo 205 range between 1000-5000µg/ml. whereas, that of A549 and WM 115 falls between 5000-10000µg/ml (Fig. 20). Again a similar pattern was seen in its action against fibroblast cells showing that Noni extracts might have a selective cytotoxic effect against tumor cells.

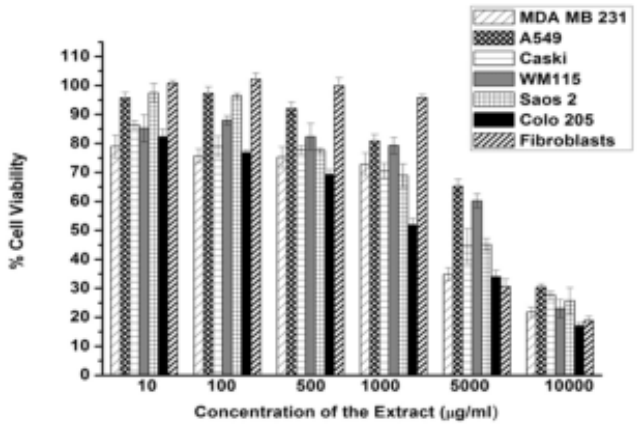


Fig. 20 : The graph represents the effect of Freeze dried extract on six tumor cell lines using fibroblast as control.

Effect of freeze dried juice extract HPLC Fractions

Schematic representation of the steps involved in the HPLC fractionation of the freeze dried extract is shown in Fig. 21.

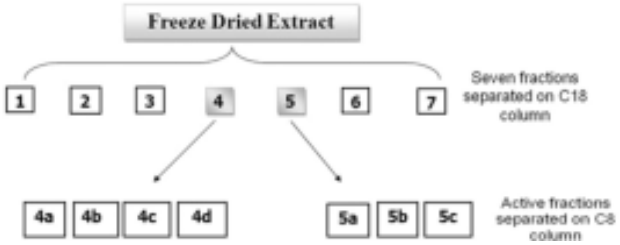


Fig. 21 : Schematic representation of the steps involved in the HPLC fractionation of the freeze dried extract

The seven HPLC fractions obtained were tested on the most sensitive cell line WM115, amongst six others in the panel. It was observed that out of the seven fractions, fraction four and five were found to be most effective with cell viability values of 48% and 20% respectively at 1000µg/ml concentration (Fig. 22). At 1000µg/ml concentration the HPLC purified fractions were found to be considerably more effective than the crude extract at the same concentration making it the method of choice for obtaining purer forms of the extract.

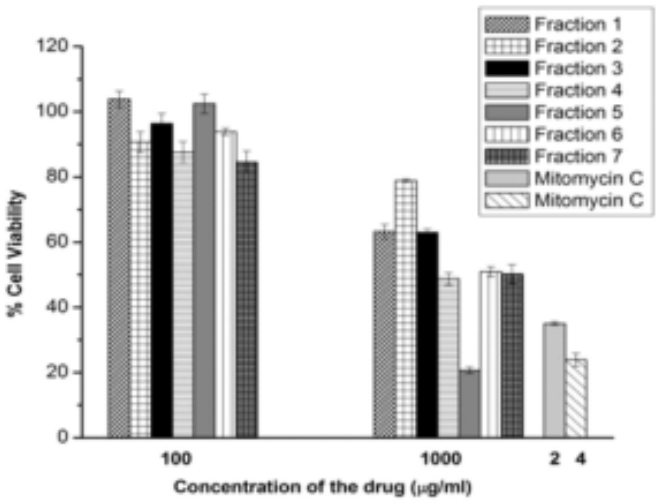


Fig. 22 : Effect of Freeze dried extract on WM115 cell line using Mitomycin C as positive control

Also, the fifth fraction was dissolved in DMSO instead of Milli-Q water and it was observed that it dissolved completely in DMSO. Moreover, it showed a higher cytotoxicity compared to the Milli-Q dissolved fraction at the same concentration (Fig. 23). Hence, based on the results in graph 4 and 5 fraction four and fraction five dissolved in DMSO were further sub-fractionated.

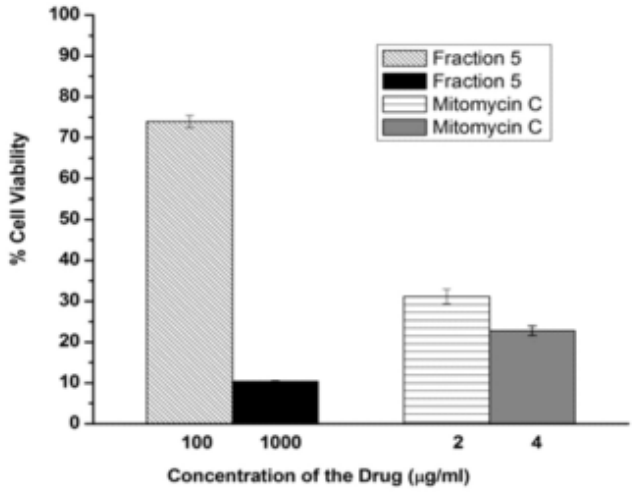


Fig. 23 : Effect of Freeze dried fraction five dissolved in DMSO on WM115 cell line using Mitomycin C as positive control

The sub- fractionated fractions of fraction four and five were tested at two concentrations 100 and 1000µg/ml. Fraction 4b was noted to cause a 40-50% cell viability at 500µg/ml in comparison to the 1000µg/ml concentration of whole fraction four which was necessary to produce the same effect (Fig. 24a). Hence, we can conclude that purification by fractionation definitely has the ability to cause increased cytotoxicity. However, in case of fraction five there was no considerable increase in the level of cytotoxic effect even after fractionation (Fig. 24b).

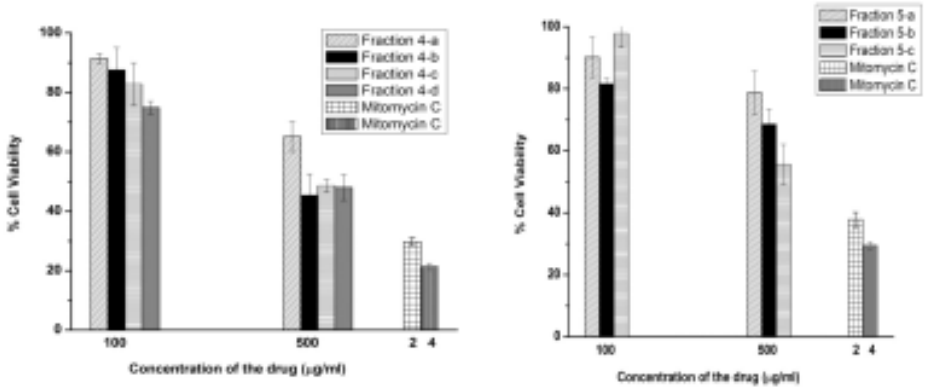


Fig. 24 : Effect of freeze dried extract sub-fractionated fractions a) Fraction four sub fractions (a,b,c,d) and b) Fraction five sub-fractions (a,b,c) dissolved in DMSO on WM115 cell line using Mitomycin C as positive control.

Effect of aqueous extract HPLC fractions

Schematic representation of the steps involved in HPLC fractionation of the aqueous extract. Based on the data represented in Fig. 25 it could be interpreted that there is a definite increase in the level of cytotoxicity of the extract after fractionation when compared to the level of cytotoxicity of the crude extract. Out of the six fractions tested fraction one showed the highest level of cytotoxicity with a per cent cell viability ranging close to 30 % at a concentration of 1000µg/ml.

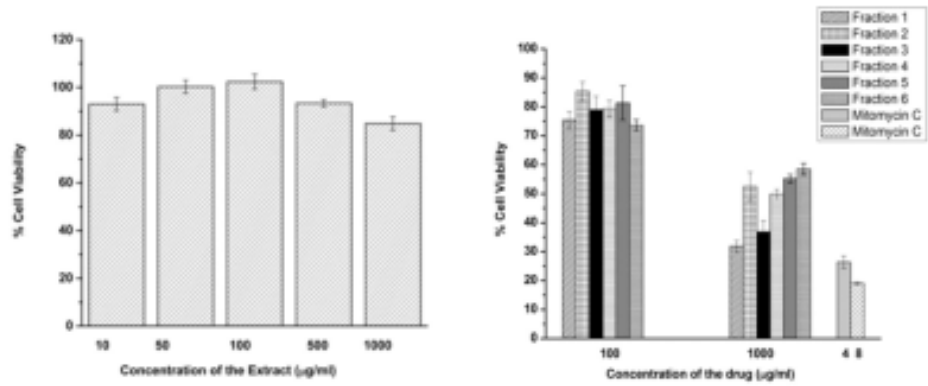


Fig. 25 : Effect of aqueous extract on the cell line WM115 a) Effect of crude extract b) Effect of HPLC fractions 1-6 on WM115 cell line using Mitomycin C as positive control

Aqueous extract when separated using an analytical column showed a good activity and hence it was isolated in larger amounts using a C18 semi-prep column. Based on the profile obtained fractions were collected every five minutes starting at the fifteenth minute till the sixtieth minute. Out of the fractions tested fraction six and seven showed a good activity (Fig.26).

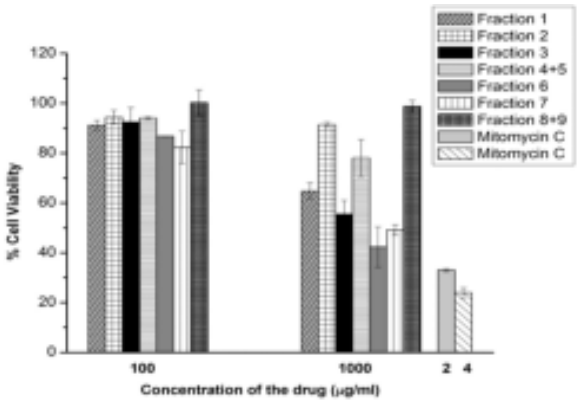


Fig. 26 : Effect of aqueous extract fractionated using Semi-prep C18 column on the cell line WM115

Based on the profound activity, fraction six and seven were further fractionated at an interval of two minutes starting at the 40th minute till the 50th minute, collecting a total of five fractions which were again tried in MTT. However, no profound increase in cytotoxicity was observed using the sub-fractions (Fig. 27).

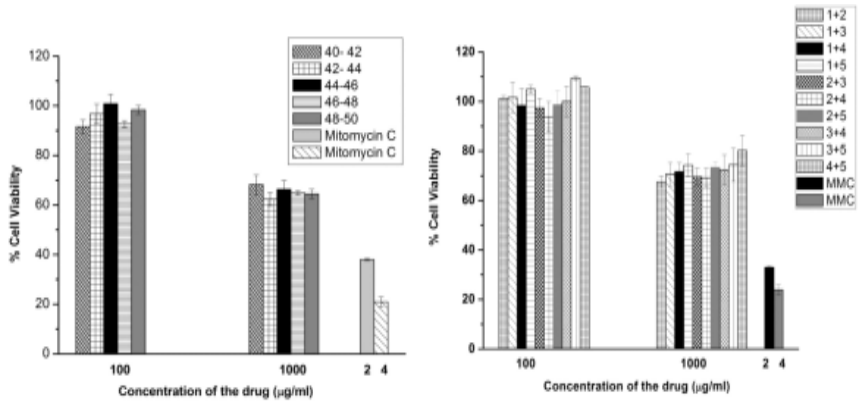


Fig. 27 : Effect of aqueous extract sub-fractions of fraction 6 and 7 on the cell line WM115 a) Effect of sub-fractions separately b) Effect of sub-fractions in combination

Effect of ethanol extract HPLC fractions

Schematic representation of the steps involved in fractionation of the ethanol extract (Fig. 28).

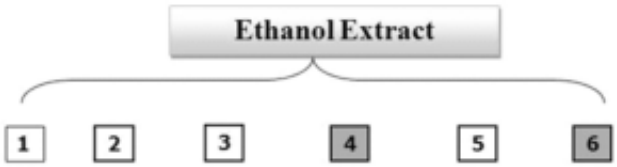


Fig. 28 : Schematic representation of the steps involved in fractionation of the ethanol extract. Active fractions have been highlighted

The data represented in Fig. 29 helps us conclude that even in case of ethanol extract there is a drastic increase in the extent of cytotoxicity after fractionation when compared to the cytotoxic effect produced at 1000µg/ml concentration of the crude extract. Out of all the fractions tested fraction four and six seemed to show the highest activity causing a per cent cell viability ranging between 20-30% at 1000µg/ml.

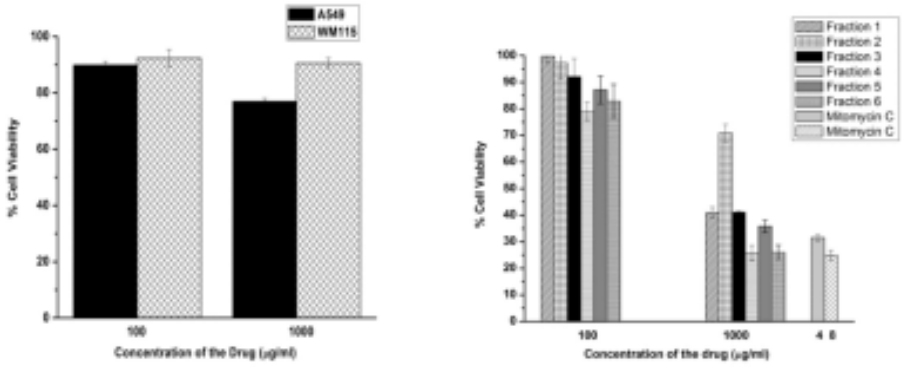


Fig. 29 : Effect of ethanol extract a) Effect of crude extract on cell line A549 and WM115b) Effect of HPLC fractions 1-6 on WM115 cell line using Mitomycin C as positive control

The results obtained after fractionation in case of all three extracts showed that fractionation helped to obtain compounds in purer forms which ultimately leads to a better cytotoxic effect at considerably lower concentrations. Ethanol extract was also fractionated using semi-prep column. However, none of the fractions showed any profound post fractionation cytotoxicity.

Effect of standard Noni compounds on tumor cell lines

a) Effect of scopoletin on the panel of tumor cell lines.

Based on the trend observed it can be suggested that the cell lines WM115 and Saos 2 are most sensitive to this compound with IC50 values of 5and 10µg/ml respectively.

c)Effect of rutin on the panel of tumor cell lines.

Based on the trend observed it can be suggested that the compound rutin is not very cytotoxic when compared with scopoletin.

Based on the data represented in Fig. 30 it could be observed that the standard scopoletin was cytotoxic to all cell lines, especially WM115 and Saos 2 with IC50 values of 5 and 10µg/ml respectively and it is likely that scopoletin might influence the pathways related to defective genes in them *viz.,* PTEN, RB1 and MADH4 /APC respectively. On the other hand, rutin did not show profound cytotoxicity in any of the cell lines tested. However, these standard compounds are present in very minute quantities in the crude extracts which would justify its low level of cytotoxicity in the crude form.

Based on the cytotoxicity assay performed on a panel of six human tumor cell lines; it can be concluded that Noni fruit extracts are not highly cyototxic by nature. This conclusion is supported by the IC50 values obtained for all the crude extracts. Research elsewhere 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.

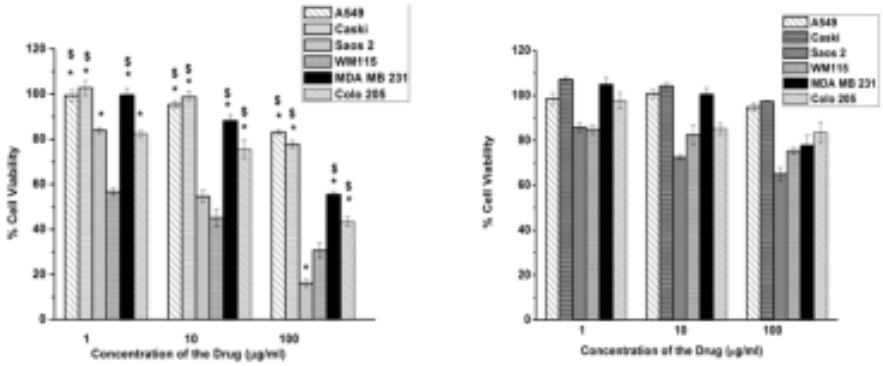


Fig. 30 : Cytotoxic profiles of standard drugs on the panel of six tumor cell lines, after a 48 hour incubation

*- 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.

The results obtained from the panel of six human tumor cell lines clearly indicated 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 immunostimulators of plant 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 be reasonable only if Noni juice or Noni fruit is indeed not very cytotoxic.

Checking for the chemopreventive effect of *Morinda* extracts

Alkaline Comet Assay

Cells treated with EMS alone showed profound DNA damage after 24 hours, with olive tail moment value (OTM) up to 6.6 in comparison to control cells with OTM value of 0.46 (Fig. 31A, 31B). However, ethanol and aqueous extracts alone did not show any DNA damage with OTM value equivalent to control cells. There was a significant reduction in the OTM values in both treatment groups using ethanolic extract, with OTM values of 3.3 ($p<0.01$) and 4.1 ($p< 0.05$) respectively. Aqueous extracts treated groups also showed a protection against EMS with OTM values of 5.5 and 4.9 respectively (Fig. 31C, 31D). However, ethanol extract was found to exhibit better protection against EMS in both the treatment groups.

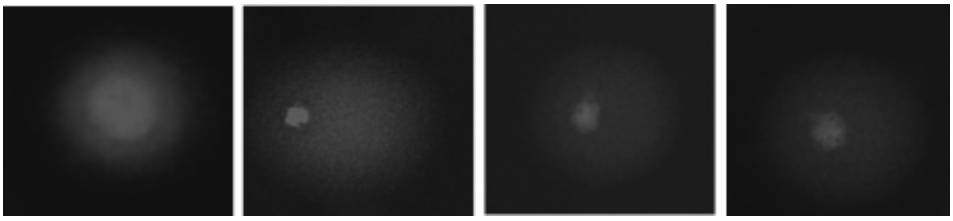


Fig. 31 : Images of lymphocytes in different treatment groups in the alkaline comet assay.
31A) Untreated lymphocyte cell displaying minimal DNA damage
31B) EMS treated cell with extensive DNA damage
31C) Cell pre-treated with ethanol extract (Pre-treatment) with moderate DNA damage, signifying the protective effect of the ethanol extract with $P<0.01$
31D) Cell treated with ethanol extract alongside EMS (co-treatment) with moderate DNA damage with $P<0.05$

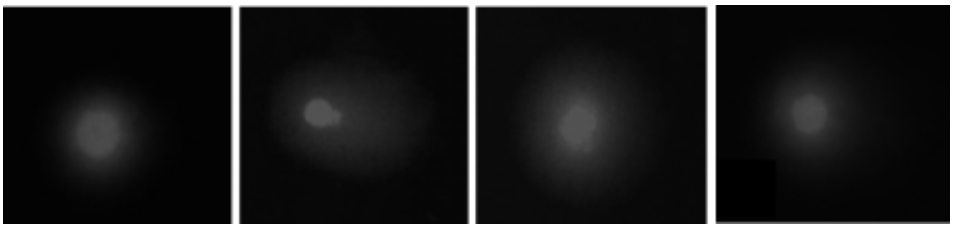


Fig. 32 : Images of lymphocytes in different treatment groups in the alkaline comet assay.
32A) Untreated lymphocyte cell displaying minimal DNA damage
32B) EMS treated cell with extensive DNA damage
32C) Cell pre-treated with ethanol extract (Pre-treatment) with moderate DNA damage, signifying the protective effect of the ethanol extract with $P<0.01$
32D) Cell treated with ethanol extract alongside EMS (co-treatment) with moderate DNA damage with $P<0.05$

Neutral Comet Assay

UV radiation induced significant double strand breaks at a dose of 2 J/cm^2, with an OTM value of 2.4, along with control cells with an OTM value of 0.5 (Fig. 32A, 32B). Ethanol and aqueous extracts displayed OTM values equal or less than that of control cells indicating that they do not induce double strand breaks. Both ethanol and aqueous extract displayed a considerable protection against UV rays at 2 J/cm^2, with OTM values of 0.7 ($p<0.001$) and 1.2 ($p<0.01$) respectively (Fig. 33B).

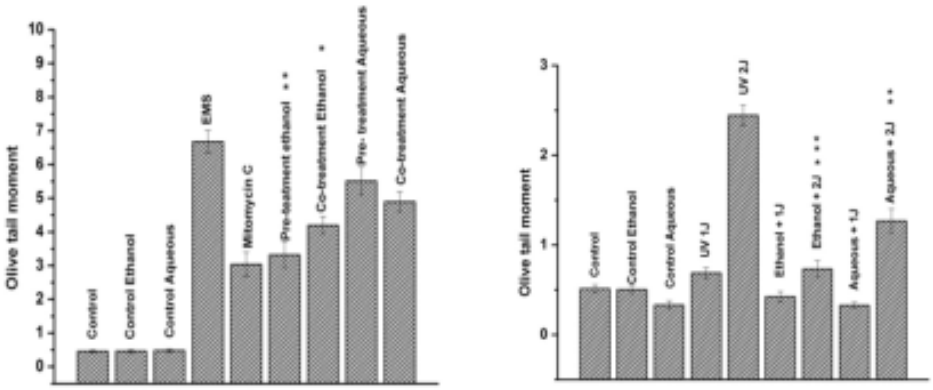


Fig. 33 : Results for alkaline comet assay used to check for chemopreventive effect of aqueous and ethanol extract. a) Depicts the percentage of tail DNA in each of the nine groups which is directly proportional to the extent of DNA damage caused b) Depicts the olive tail moment in each of the nine groups which can in turn be correlated to the damage caused to the DNAFigure

1A) Effect of Morinda extracts against EMS tested using alkaline comet assay. Based on the graph it can be observed that ethanol and aqueous extracts help protect against EMS, in both treatment groups. There is significant reduction in OTM value using ethanol extract

1B) Effect of Morinda extracts on UV radiation treated cells using neutral comet assay. Results suggest that both ethanol and aqueous extracts have a protective effect on UV treated cells. Significant reduction in the OTM values clearly indicated that both extracts have protective effect against UV damage

***- Statistically significant ($P<0.001$) difference was observed when OTM values of cells pre-treated with extract were compared with that of cells without the pre-treatment before exposure

**- Statistically significant ($P<0.01$) difference was observed when OTM values of cells pre-treated with extract were compared with that of cells without the pre-treatment before exposure

*- Statistically significant ($P<0.05$) difference was observed when OTM values of cells pre-treated with extract were compared with that of cells without the pre-treatment before exposure

However, of the two extracts, ethanol extract was found to show a comparatively higher protective effect against double strand breaks after UV treatment (Fig. 32C, 32D).

Fast Micromethod DNA Single-Strand-Break Assay

DNA damage due to SSBs was expressed in terms of SSF × (-1). A strong increase in DNA unwinding or SSF × (-1) is an indication of increased DNA damage. Treatment with hydrogen peroxide at both 50 and 500µM concentrations displayed DNA damage with SSF × (-1) values 1.5 and 1.7 respectively. There was a significant increase in SSF × (-1) value post H_2O_2treatment against control, with a SSF × (-1) of 0.26. None of these extracts exhibited any DNA damage after the pre-treatment with SSF × (-1) values 0.17 and 0.48 respectively. Cells pre-treated with the ethanol extracts showed a decrease in the SSBs at 50µM concentration of H_2O_2, with SSF × (-1) value of 1.4. However, no protection was noticed at 500µM concentration. Aqueous extract displayed a decrease in SSBs at both 50 and 500µM concentrations, with SSF X (-1) value of 1.46 and 1.48 respectively.

Fluorescent analysis of DNA unwinding (FADU)

FADU assay also estimates double strand breaks in terms of SSF × (-1) value. When exposed to UV rays the cells were found to have an SSF × (-1) value of 0.8 indicating damage caused. Noni extracts exhibited no DNA damage with SSF × (-1) values of -0.4 for ethanol extract and -0.3 for water extract. Cells when exposed to UV rays after pre incubation with ethanol extract displayed a reduction in the DNA damage with a value of 0.5. However, pretreatment with aqueous extract did not show any reduction in the SSF × (-1) value (Fig. 34B).

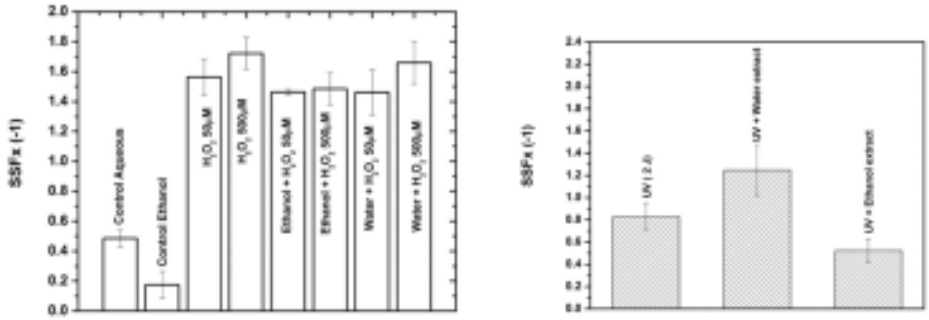


Fig. 34 : Effect of Morinda extracts tested by fast micromethod DNA single-strand-break assayand fluorescent analysis of DNA unwinding assay

A) Effect of Morinda extracts tested against hydrogen peroxide using single strand break assay.Aqueous extract displayed a decrease in SSBs at both 50 and 500µM concentrations. Ethanol extracts showed a reduction in SSBs at 50µM concentration. However, no reduction was observed at 500µM concentration of hydrogen peroxide

B)Effect of Morinda extracts tested against UV radiation using fluorescent analysis of DNA unwinding assay. Based on the graph it can be observed that ethanol extracts help reduce the damage caused by UV radiation

Neutral Filter Elution Assay

Neutral filter elution was performed to detect the ability of Noni extracts to protect against radiation induced double stranded breaks as described by Goutham *et al.* (2011).

It was observed that neither of the extracts helps in reducing the damage caused by X-rays when the assay was performed immediately after exposing to X-rays (Fig. 35).

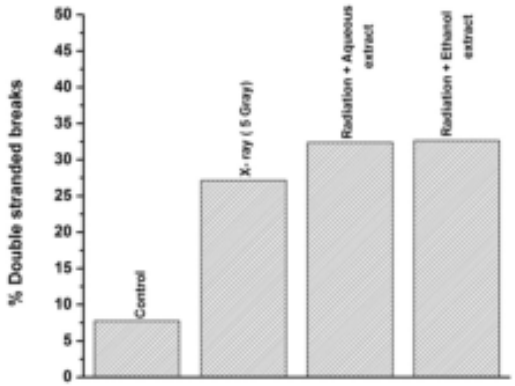


Fig. 35 : Effect of Morinda extracts tested against X-rays by neutral filter assay

DPPH Assay

The radical scavenging power of Noni compounds tested were evaluated based on their ability of reduce the DPPH radical to a more stable form. The antioxidant potential was expressed as IC50 values. Both extracts displayed IC50 values above a concentration of 1000µg/ml (Fig. 36A). Pure compounds kaempferol and rutin were found to have IC50 values close to 50 and 100µg/ml respectively (Fig. 36B). N-acetylcysteine which was used a positive control was observed to have an IC50 close to 100µg/ml (Fig. 36A, 36B).

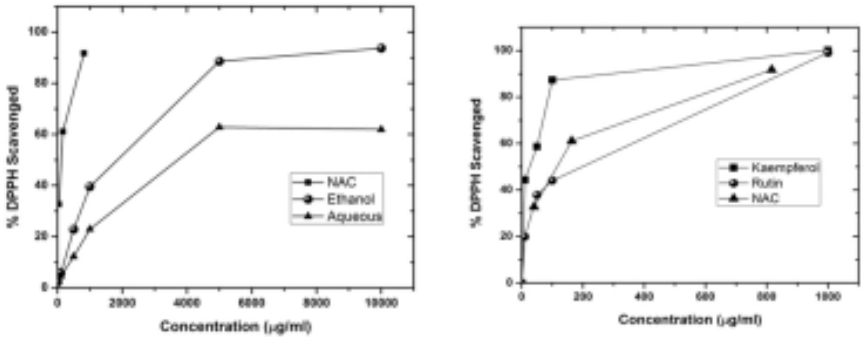


Fig. 36 : Antioxidant activity of Morinda extracts tested by DPPH assay.
A)Antioxidant of Morinda extracts tested by DPPH assay. Both aqueous and ethanol extracts displayed IC50 values above 1000µg/ml. NAC exhibited an IC50 value close to 100µg/ml
B)Antioxidant of standard Morinda compounds tested by DPPH assay. Pure compounds kaempferol and rutin, were found to have IC50 values close to 50 and 100µg/ml respectively. N-acetylcysteine which was used a positive control was observed to have an IC50 close to 100µg/ml

Detection of intracellular Reactive Oxygen Species

DCFH-DA fluoresces on its conversion to 2', 7'-dichlorofluorescein when it reacts with reactive oxygen species. The fluorescence intensity thus produced is measured to assess the oxidative stress in the cells which was expressed on terms of relative fluorescence intensity (RFI). The RFI of untreated stained cells was observed to be 60.9, with a significant increase in the RFI post hydrogen peroxide treatment with a value of 237.14. Cells co-treated with NAC during the hydrogen peroxide treatment were able to bring down the RFI value to 192.82. However, neither the *Morinda* crude extracts nor its standard compounds were able to reduce the RFI value when co-treated with hydrogen peroxide (Fig. 37A-37G).

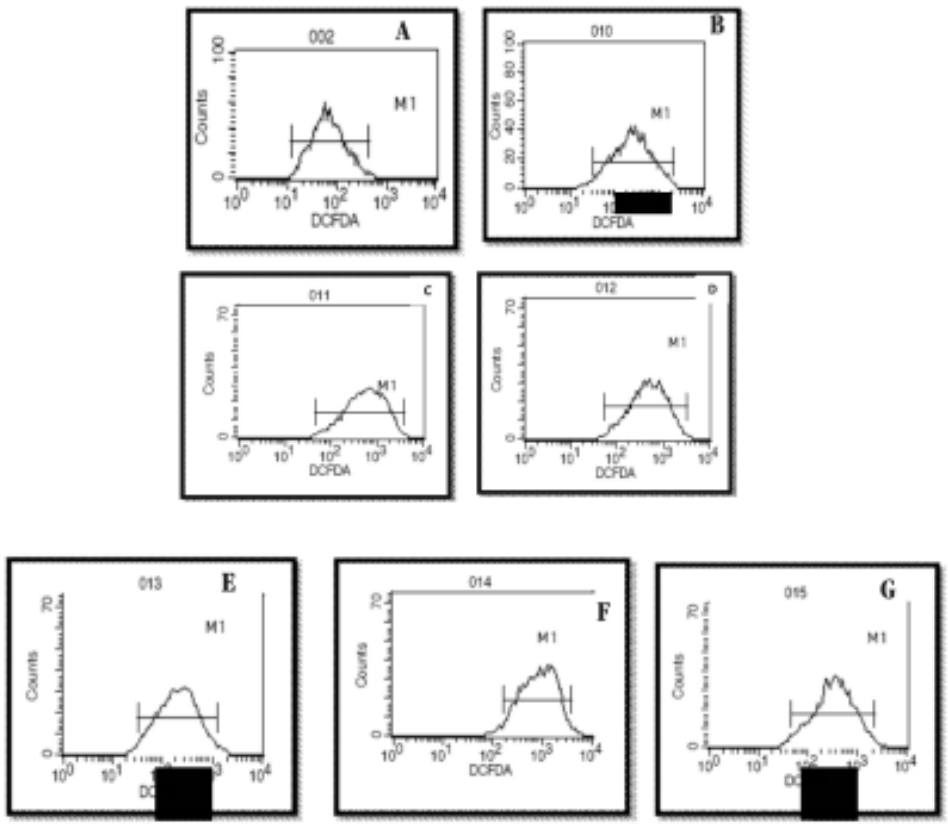


Fig. 37 : Analysis of intracellular reactive oxygen species using flow cytometry. A) Untreated cells B) Hydrogen peroxide (100µM) C)Hydrogen peroxide (100µM) cotreated with ethanol extract at 1000µg/ml D)Hydrogen peroxide (100µM) cotreated with water extract at 1000µg/ml E)Hydrogen peroxide (100µM) cotreated with NAC at 5mM concentration F)Hydrogen peroxide (100µM) cotreated with kaempferol at 10µg/ml G)Hydrogen peroxide (100µM) cotreated with rutin at 10µg/ml

6. Summary and conclusion

- Crude extracts exhibited mild levels of cytotoxicity
- None of the soxhlet extracted fractions displayed a significant variation in the percentage viability compared to the crude drugs
- Initial studies helped to establish that the cell line WM115 was relatively more sensitive to most extracts

- Fraction four and five of freeze dried extract showed higher cytotoxic effect when compared to crude extracts
- Fraction one of aqueous extract showed higher cytotoxic effect when compared to crude extracts
- Fraction four and six of ethanol extract showed higher cytotoxic effect when compared to crude extracts
- Fractionation of bioactive compounds from crude extract was not found to show any profound increment in cytotoxicity. This could be due to the loss of activity with each fractionation step. This is consistent with the literature survey which states that Noni extracts are more of the chemopreventive nature when compared to its cytotoxic nature.
- Standard scopoletin was cytotoxic to all cell lines, especially WM115 and Saos 2 with IC50 values of 5 and 10 µg/ml respectively
- Scopoletin might harness the pathways related to defective genes in them *viz.,* PTEN, RB1 and MADH4 /APC respectively
- Rutin did not show profound cytotoxicity in any of the cell lines tested
- Ethanolic extract was found to have a protective effect against EMS, hydrogen peroxide, X-rays and UV radiation, whereas aqueous extract was found to have a protective effect only against hydrogen peroxide, UV radiation and X-rays

Chemopreventive agents are non-toxic and might exert their effects either by blocking or metabolizing carcinogens or by inhibiting tumor cell growth (Sarkar *et al.,* 2007). These qualities are reflected in Noni crude extracts especially the ethanolic extract with their reduced toxicity and superior chemopreventive property. Thus Noni crude extract can be regarded as an excellent candidate and can be promoted as an adjuvant for chemotherapeutics or radiotherapy. This strategy opens a new avenue for cancer prevention. Growing evidence has shown that chemopreventive agents potentiate the efficacy of chemotherapy and radiotherapy through the

regulation of multiple signaling pathways, including Akt, NF-ºB, c-Myc, cyclooxygenase-2, apoptosis and others suggesting a multitargeted nature of chemopreventive agents (Sarkar *et al.*, 2007). Among the natural and synthetic chemopreventive agents Noni can emerge as a potent candidate which can be employed in targeted therapeutic strategies against cancer which are likely to make a significant impact to combat cancer.

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