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CHEMOPROTECTIVE ACTIVITY OF *MORINDA CITRIFOLIA* (NONI) JUICE AGAINST ANTICANCER DRUGS INDUCED TOXICITY – A MECHANISTIC APPROACH

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ABSTRACT

The fruit juice of Noni obtained from *Morinda citrifolia* is documented for various pharmacological activities, including anti-cancer activity, anti-malarial, anti bacterial, antihelminthic, anti-inflammatory and chemoprotective activity due to its abundant phytochemical constituents. Based on the evident commercial use of the Noni juice which is available as Divine Noni Gold® (DNG),the present work was planned to investigate the potential benefits of DNG as an adjuvant to chemotherapy through invivo studies marily focusing on testicular toxicity. Initially the cytotoxicity of DNG and anticancer drug was performed on both normal cells (BEAS -2B) and cancer cells (MDA-MB-468) by SRB assay. The percentage of growth inhibition was calculated and Apoptotic fluorescent imaging study was also performed to assess the percentage of live cells. Later, *invitro* chemoprotective effect of DNG was performed against doxorubicin (DOX) induced toxicity using normal cells BEAS-2B by SRB assay followed by *in-vivo chemoprotective* study of DNG against DOX-induced testicular toxicity in healthy swiss albino mice. Estimation of serum Anti-Mullerian Hormone (AMH) and histology of testes, epididymis, seminiferous tubules, and prostate gland were examined. In *in-vitro* cytotoxicity study, Noni showed no cytotoxicity at the tested concentration whereas DOX showed cytotoxicity to normal cells (BEAS-2B). DNG showed significant cytoprotective effect against DOX-induced cytotoxicity on normal cells. Whereas on cancer cells (MDA-MB-468) DNG showed cytotoxicity and the anticancer efficacy ofDOX was enhanced in the presence of DNG. In the *in-vivo s*tudy, DNG showed a protective effect against DOX-induced testicular toxicity that was confirmed by the serum AMH levels and histopathological evaluation of testes, epididymis, seminiferous tubules, and prostate gland.

INTRODUCTION

Cancer is one of the most dreadful diseases of humans as well as animals. It stands as the second leading cause of death in economically developed countries (following heart diseases) and the third leading cause of death in developing countries (following heart and diarrheal diseases)[1]. Given the expected increase in incidence and prevalence of cancer, emergency medicine physicians are likely to encounter patients receiving chemotherapy. The spectrum of

chemotherapeutic agents is extensive and adverse effects are common. Due to the complexity of the disorder, most patients receive multidrug regimens. Distinguishing disease pathology from adverse chemotherapeutic effects remains a challenge. The termination of exposure is often required to control significant toxicity[2]. Due to advances in anticancer treatments, the survival rate of cancer patients has improved markedly, but the quality of life (QOL) following successful treatment is still of great concern. Among the major problems caused by chemotherapy are spermatogenetic disorders in male patients. Doxorubicin (DOX) is one of the most potent broad spectrum antitumor anthracycline antibiotics widely used to treat a variety of cancers, including severe leukemias, lymphomas, and solid tumors. The clinical use of DOX is limited due to its serious toxicity to various organs, including the heart, liver, lung, kidney and testis[3]. For centuries, plants and plant-based products have been used as a valuable and safe natural source of medicines for the treatment of various diseases. The therapeutic potential of most of these plants could be attributed to their anticancer, antidiabetic, hepatoprotective, cardioprotective, antispasmodic, analgesic and other pharmacological properties[4]. In the present study, wehave focused on the herbal approach of the fruit juice of *Morinda citrifolia* on the doxorubicin induced testicular toxicity. The genus *Morinda*(Rubiaceae) including the species *Morinda citrifolia* is made up of around 80 species. *Morinda citrifolia* commonly known as Indian Mulberry or Noni is a small tropical evergreen shrub or a tree indigenously found in open coastal regions at sea level and in forest areas up to about 1300 feet above sea levels[5,6].” The noni plant has been found to contain amino acids, anthraquinones, fatty acids, flavonoids, iridoids, lignans, polysaccharides, sterols, sugars, and terpenoids[7]. It has been reported to have a broad range of therapeutic effects including antibacterial,antifungal, antiviral, antitumor, anthelminthic, analgesic, hypotensive, anti-inflammatory, and immune-enhancing effects. The present study is carried out to determine the effect of *Morinda citrifolia*(Noni) juice against anticancer drugs induced toxicity.

MATERIALS AND METHODS:

Noni Formulation

Divine Noni Gold (DNG)® concentrate was procured from Noni Biotech Private Limited, Tamil Nadu, India. DNG was used for both *in-vitro* and *in-vivo* studies.

Cell lines

For the *In-vitro* studies, following cell lines were used to estimate the cytotoxicity and chemoprotective efficacy of Divine Noni Gold® and its beneficial use as an adjuvant. Human bronchial epithelial normal cells (BEAS-2B), Human breast cancer cells (MDA-MB-468) were procured from ATCC, and maintained at the Centre of Excellence in Molecular Biology and Regenerative Medicine. Normal BEAS-2B and Cancer MDA-MB-468 were cultured by using DMEM F-12 complete media. Cells were maintained in a CO2 incubator at 5% CO2 and 95% air
with the temperature at 37˚C, and the cells were cultured and treated in a sterile culture hood.

Animals

Swiss albino mice of weight range (25-30gm) were used in the present study, and the animals were procured from CPSCEA registered breeder (Reg No:971/bc/06/CPCSEA) Biogen, Laboratory Animal Facility, Bangalore. The animal handling and care were done in accordance with CPCSEA. IAEC approval was obtained for the present study (IAEC Approval No.: 290/2018) before the commencement of the study. The experiments were conducted as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Animals were acclimatized forone week before the experimental study.

Drugs and chemicals

Chemicals: Acridine orange, ethidium bromide were procured from Sigma-Aldrich, Fetal Bovine Serum from Life Technologies, Dulbecco's Phosphate Buffered Saline 1X from Hi-Media, Trypsin-EDTA Solution 1X(0.25% Trypsin & 0.2% EDTA), Ethanol, Dulbecco's Modified Eagle Medium/Nutrient Mix F-12, DMSO (Molecular grade) were procured from HIMEDIA.

Drugs: Doxorubicin was procured from Sigma Aldrich, and Penicillin-Streptomycin (10,000 U/mL) from Life Technologies.

***In-vitro* cytotoxicity of DNG on normal and cancer cell lines by SRB Assay.**

This assay is used to measure cell viability and proliferation. It relies on the ability of SRB dye to bind cellular protein components and measure of the total biomass. A fixed number (10,000 cells/well) of normal BEAS-2B cells and triple-negative breast cancer MDA-MB-468 cells were seeded in each well of 96-well plates. They are stored in a CO2 incubator (5% CO2 + 95% Air) at 37˚±2˚C for adherence of the cells for 18-24 hr. After adherence, both the plates containing the cells were treated with various concentrations of DNG (0.3125 – 10mg) to derive the dose-response curve for specific treatments for 24 h and 48 hr. The concentrations of used for DOX were in the range of (0.635µM - 20µM), each concentration was tested in triplicates. Following the treatment cells were washed with phosphate-buffered saline (PBS),fixed with 10 % (w/v) trichloroacetic acid and stained with 100µl (0.4 % (w/v)in Acetic Acid) SRB dye for 30 min, after repeatedly washing with 1 % (v/v)acetic acid. The protein-bound dye was dissolved in 10 mMTris base solution of pH 10.5. The optical density was read at 540nm using a Microplate reader; the experiment was done in triplicates. Percentage inhibition of both normal and cancer cell growth by DNG and DOX and was calculated using the formula.

$$\% \text{ of Inhibition} = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

Anticancer efficacy of DNG on breast cancer line (MDA-MB-468)

Triple-negative breast cancer MDA-MB-468 cells were used for assessing the anticancer activity of DNG by SRB assay at 24hr and 48hr. For assessing the anticancer efficacy of DNG in combination with anticancer drugs, the combination study was performed on cancer cells at 24 and 48 hr by SRB assay. The selected lower and higher doses of DNG and DOX were the same as of the doses selected for the chemoprotective study. Fixed number of BEAS-2B (10,000 cells/well) and were seeded in each well of 96well plates (concentrations in triplicates). They are stored in a CO2 incubator (5% CO2 + 95% Air) at 37˚±2˚C for adherence ofthe cells for 18-24 hr. After adherence, cells were treated with various concentrations of DNG (0.3125-10mg for Noni), DOX (0.635µM – 20µM), Each concentration was tested in triplicates. Following the treatment cells were washed with phosphate-buffered saline (PBS), fixed with 10 % (w/v) trichloroacetic acid and stained with 100ul (0.4 % (w/v) in Acetic Acid) SRB for 30 min, after repeatedly washing with 1 % (v/v) acetic acid. The protein-bound dye was dissolved in 10 mMTris base solution of pH 10.5.The optical density was read at 540 nm using a Microplate reader, and the experiment was done in triplicates. The percentage inhibition of cancer cell growth by DNG and DOX was calculated using the below-stated formula.

$$\% \text{ of Inhibition} = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

A mechanistic approach to explore the chemoprotective action of DNG:

Fluorescent imaging studies (Apoptosis) using Acridine orange and Ethidium bromide dual staining method[8].

The Apoptosis was detected using acridine orange and ethidium bromide staining method on both normal and cancer cell lines. 0.3 x 10^6 BEAS-2B and MDA-MB-468 cells were plated into 6-well plates.After 24-36h the plates were exposed to DNG (1.25mg), DOX (20µM), DNG (1.25mg/ml) + DOX (50µM) for about 48h. The control and treated cells were trypsinized and mixed thoroughly to obtain a single-cell suspension. Trypsin was neutralized by the addition of complete medium of 4.0mL.20.0µL cell suspension was incubated with 10.0µL ethidium bromide(100.0µg/mL in PBS) and 10.0µL acridine orange (100.0µg/mL in PBS) mixture for 5 minutes. The cells were imaged using a fluorescence microscope with TRITC and FITC filters. The images obtained using two different channels were later merged to obtain acombined image, which showed green (live) and orange-red (apoptotic) cells. At least five different fields were considered for quantifying the live and apoptoticcells and the percentage of cells undergoing apoptosis measured.

Groups	Treatment Dose, Duration & Route
Normal	Saline 10 Ml/Kg P.O. For 14 Days
Control (Dox)	2 Mg/Kg I.P. On 1st, 7th And 14th Day
Test (Dng)	0.35ml/B. Wt.P.O. For 14 Days
Test (Dng) + Control (Dox)	0.35ml/B. Wt. P.O. For 14 Days + 2 Mg/Kg I.P. On 1st, $7_{th}$and 14^{th} Day

Cytotoxicity of DNGon normal cell line by SRB Assay – 24hr

BEAS-2B-24hr			
DNG		DOX	
Conc.(mg/ml)	% inhibition	Conc.(µM/ml)	% inhibition
0.3125	2.89±2.14	0.635	12.85±1.39
0.625	3.20±2.74	1.25	23.64±0.72
1.25	5.00±1.80	2.5	27.05±1.67
2.5	9.75±3.96	5	30.08±2.45
5	27.23±2.53	10	32.41±1.58
10	29.53±1.96	20	33.86±2.62

Cytotoxicity of DNG on normal cell line by SRB Assay – 48hr

BEAS-2B 48hr			
DNG		DOX	
Conc. (mg/ml)	% inhibition	Conc. (µM/ml)	% inhibition
0.3125	3.34±2.60	0.635	18.58±4.34
0.625	4.12±0.91	1.25	21.47±10.01
1.25	6.30±0.52	2.5	24.88±0.51
2.5	13.32±12.86	5	28.17±0.55
5	19.20±5.50	10	31.68±0.85
10	28.66±1.10	20	32.61±1.38

Cytotoxicity of DNG on cancer cell line by SRB Assay – 24hr

MDA-MB-468 24hr			
DNG		DOX	
Conc. (mg/ml)	% inhibition	Conc. (µM)	% inhibition
0.3125	1.49±0.80	0.635	5.87±1.86
0.625	6.04±1.29	1.25	12.49±0.56
1.25	9.13±0.86	2.5	20.49±1.86
2.5	13.68±0.22	5	22.51±1.27
5	18.44±0.68	10	25.40±0.23
10	31.09±1.58	20	33.47±2.54

Cytotoxicity of DNG on cancer cell line by SRB Assay – 48hr

MDA-MB-468 48hr			
DNG		DOX	
Conc. (mg/ml)	% inhibition	Conc. (µM)	% inhibition
0.3125	4.10±2.04	0.635	13.49±0.66
0.625	10.75±2.28	1.25	26.37±3.42
1.25	12.49±3.10	2.5	26.81±4.02
2.5	16.14±6.75	5	28.92±4.56
5	20.04±3.90	10	31.73±2.09
10	38.26±0.99	20	37.38±4.54

***In- vitro* chemoprotective effect of DNG and DOX-inducedcytotoxicity by SRB assay.**
***In-vitro* chemoprotectiveeffect of DNG against DOX-induced cytotoxicity by SRB assay (24hr)**.

Concentrations	%inhibition at 24hr
0.625mg DNG	2.78±1.14
1.25mg DNG	3.71±1.03
2.5µM DOX	30.44±0.24
20µM DOX	37.69±3.29
0.625mg DNG + 2.5µM DOX	18.20±0.13[b1]
0.625mg DNG + 20µM DOX	23.00±0.14 [b2]
1.25mg DNG + 2.5µM DOX	12.68±1.06[b1]
1.25mg DNG + 20µM DOX	16.39±1.98 [b2]

Data represent mean ± SEM (n=3), One-way Analysis of Variance (ANOVA) followedbyTukey post hoc test, b1p<0.05 vs. 2.5µM DOX, b2p<0.0001 vs. 20µM DOX.

***In-vitro* chemoprotectiveeffect of DNG against DOX-induced cytotoxicity by SRB assay (48hr)**.

Concentrations	**%inhibition at 48hr**
0.625mg DNG	5.51±2.27
1.25mg DNG	6.98±1.77
2.5µM DOX	41.70±4.40
20µM DOX	45.42±2.37
0.625mg DNG + 2.5µM DOX	38.18±5.00
0.625mg DNG + 20µM DOX	44.63±0.52
1.25mg DNG + 2.5µM DOX	30.59±0.76
1.25mg DNG + 20µM DOX	36.62±1.73

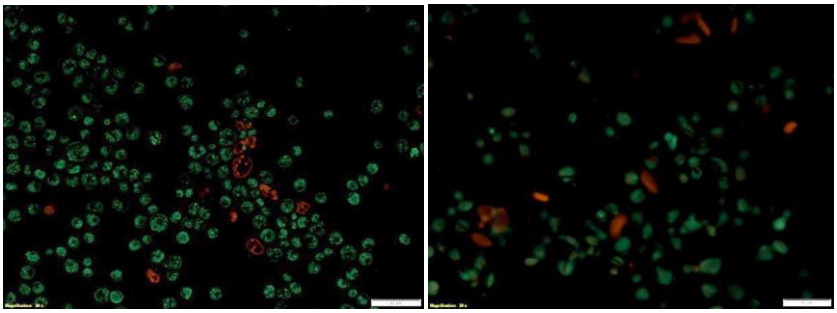
Data represent mean ± SEM (n=3), One-way Analysis of Variance (ANOVA) followed by Tukey post hoc test.

Concentrations	% inhibition at 24hr	% inhibition at 48hr
0.625mg DNG	4.02±-.62	9.36±1.33
1.25mg DNG	8.68±0.67	12.39±127
2.5µM DOX	17.64±1.61	19.50±1.15
20µM DOX	28.12±0.89	29.60±0.86
0.625mg DNG + 2.5µM DOX	19.41±0.88	20.73±0.60
0.625mg DNG + 20µM DOX	30.22±0.88	32.54±1.25
1.25mg DNG + 2.5µM DOX	20.84±1.37	26.41±3.51
1.25mg DNG + 20µM DOX	33.39±1.09	39.49±1.46[b2]

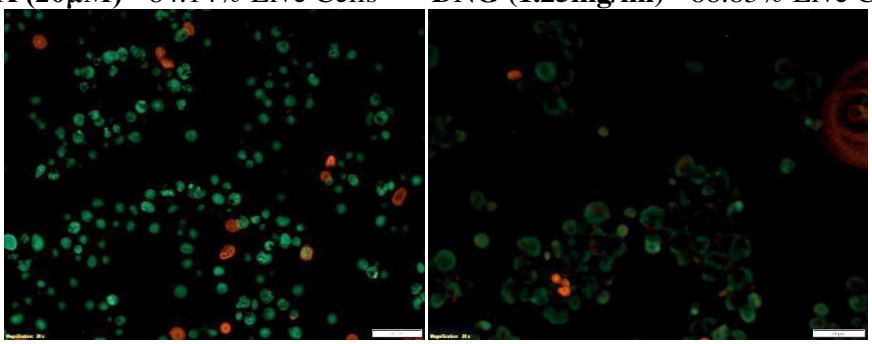
Data represent mean ± SEM (n=3), One-way Analysis of Variance (ANOVA) followed byTukey post hoc test; *b2p<0.01 vs. 20µM DOX (48hr)*.

BEAS-2B

CONTROL- 89.09% live cells

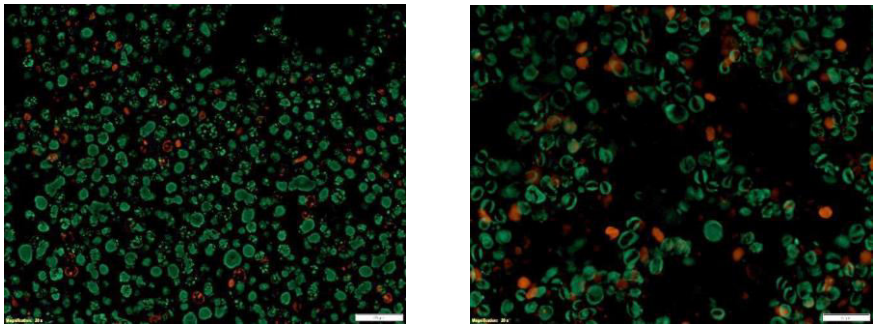


DOX (20µM) - 84.14% Live Cells **DNG (1.25mg/ml) -** 88.83% Live Cells

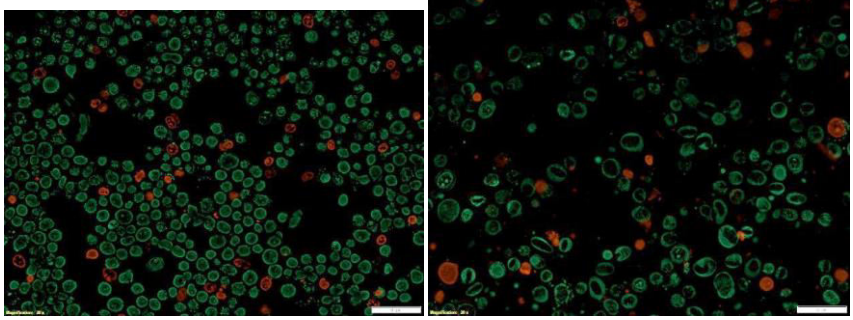


DNG (1.25mg/ml) + DOX (20µM) -80.22%Live cells **MDA-MB-468**

CONTROL - 87.37% Live Cells



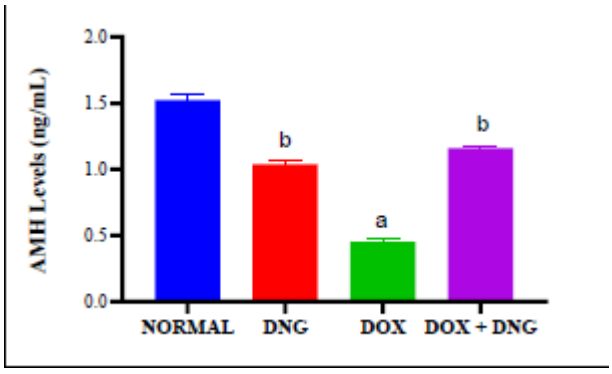
DOX (20µM) - 84.14% Live Cell **DNG (1.25mg/ml) -** 80.68% Live Cells



DNG (1.25mg/ml) + DOX (20µM) –70.94% live cells

Evaluation of the chemoprotective efficacy of noni against Doxorubicin-induced testicular toxicity in Swiss albino mice.

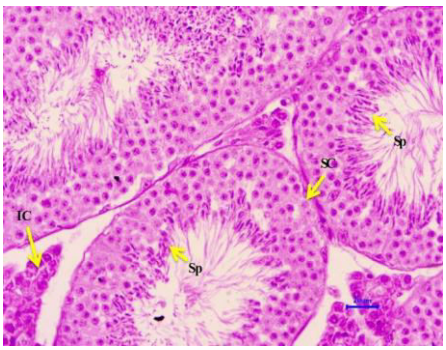
Serum AMH Estimation



Data represent mean ± SEM of 6 animals, One-way Analysis of Variance (ANOVA) followed by Tukey post hoc test; *ap<0.0001 vs. Normal, bp<0.0001 vs. DOX.* The DOX control showed significant (p<0.0001) decrease in serum AMH levels when compared with the normal group. DNG and DOX + DNG showed a significant increase in serum AMH levels when compared with DOX control.

HISTOPATHOLOGY

Histology of Testes, Epididymis, Seminiferous tubules, and Prostrate gland was examined, and the results along with the respective reports, are as follows



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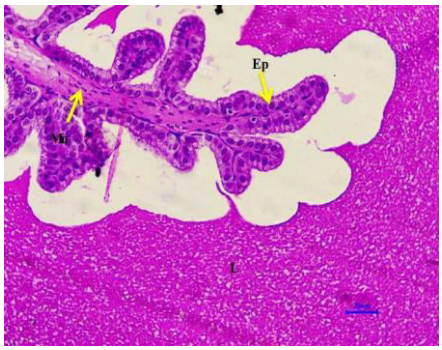


Fig:- 2

Fig:-3

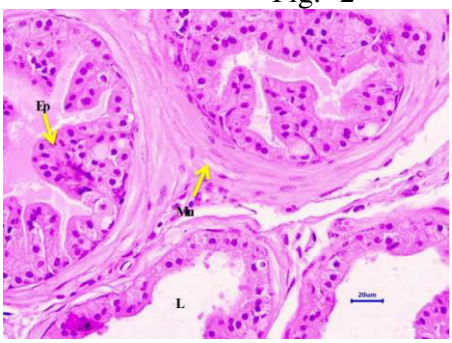
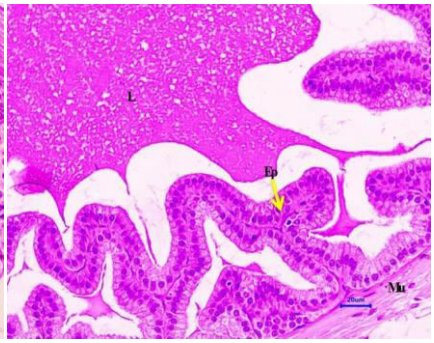


Fig:-4

Fig:-5



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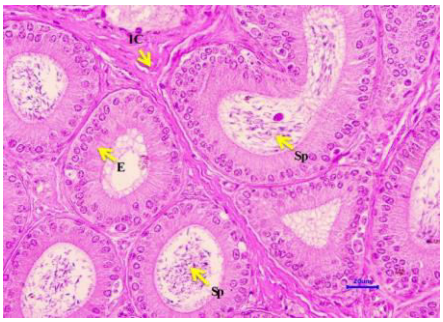

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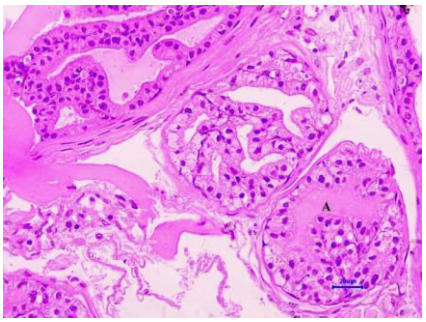

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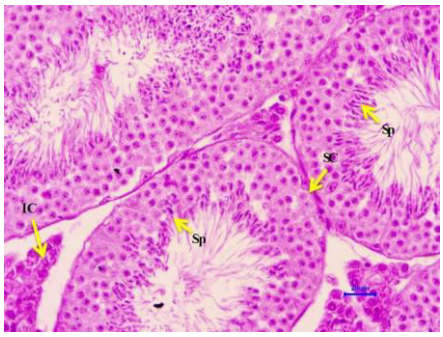

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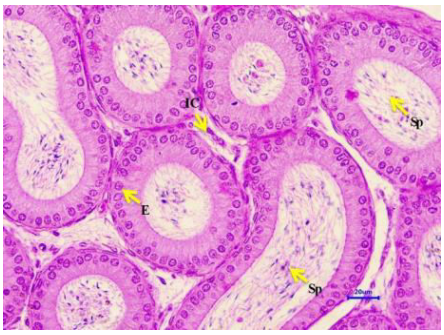

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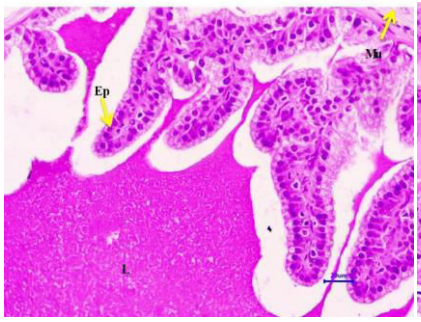

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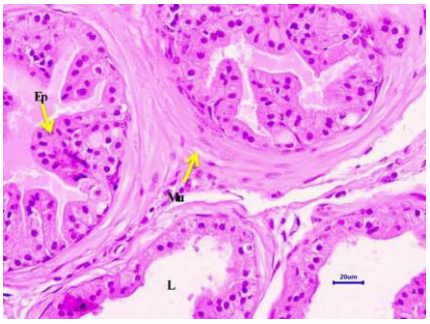

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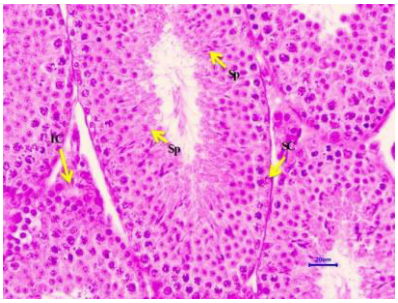

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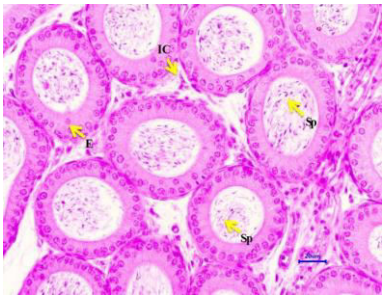

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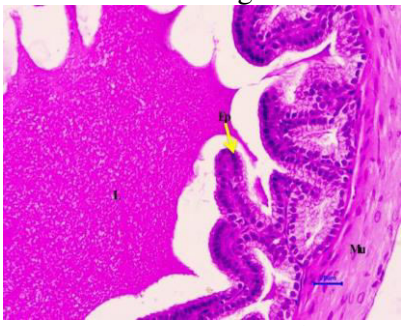

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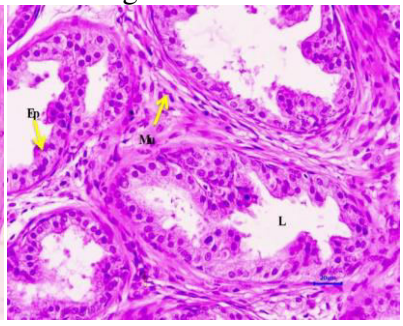

Fig:-16

Normal Group - Testes: Showing normal histology of seminiferous tubule, Spermatid (Sp), Interstitial cells (IC), Sertoli Cell (SC) {H & E, 400X} [As shown in fig. 1]**Normal Group - Seminal Vesicle:** Showing normal histology, Muscularis layer (Mu), Luminal secretion (L), Epithelium (Ep) {H& E, 400X}[As shown in fig. 2] **Normal Group - Epididymis:** Showing normal histology of Spermatid(Sp), Interstitial cells (IC), Epithelium (E) {H & E, 400X}[As shown in fig. 3] **Normal Group - Prostate:** Showing normal histology, Muscularis layer (Mu), Lumen (L), Epithelium (Ep) {H& E, 400X}[As shown in fig .4] **DOX Treated - Testes:** Showing degeneration of seminiferous tubule, Spermatid (Dg){H & E, 400X}[As shown in fig. 5] **DOX Treated - Epididymis:** Showing normal histology of Spermatid (Sp), Interstitial cells (IC), Epithelium (E) {H & E, 400X}[As shown in fig. 6] **DOX Treated - Seminal Vesicle:** Showing normal histology, Muscularis layer (Mu), Luminal secretion (L), Epithelium (Ep) {H& E, 400X}[As shown in fig. 7] **DOX Treated - Prostate:** Showing Atrophy of prostate(A) {H& E, 400X}[As shown in fig. 8] **DNG Treated - Testes:** Showing normal histology of seminiferous tubule, Spermatid (Sp), Interstitial cells (IC), Sertoli Cell (SC) {H & E, 400X}[As shown in fig. 9] **DNG Treated - Epididymis:** Showing normal histology of Spermatid (Sp), Interstitial cells (IC), Epithelium (E) {H & E, 400X}[As shown in fig. 10] **DNG Treated - Seminal Vesicle:** Showing normal histology, Muscularis layer (Mu), Luminal secretion (L), Epithelium (Ep) {H& E, 400X}[As shown in fig. 11] **DNG Treated - Prostate:** Showing normal histology, Muscularis layer (Mu), Lumen (L), Epithelium (Ep) {H& E, 400X}[As shown in fig. 12] **DNG + DOX Treated - Testes:** Showing normal histology of seminiferous tubule, Spermatid (Sp), Interstitial cells (IC), Sertoli Cell (SC) {H & E, 400X}[As shown in fig. 13] **DNG + DOX Treated - Epididymis:** Showing normal histology of Spermatid (Sp), Interstitial cells (IC), Epithelium (E) {H & E, 400X}[As shown in fig. 14] **DNG + DOX Treated - Seminal Vesicle:** Showing normal histology, Muscularis layer (Mu), Luminal secretion (L), Epithelium (Ep) {H& E, 400X}[As shown in fig. 15] **DNG + DOX Treated - Prostate:** Showing normal histology, Muscularis layer (Mu), Lumen (L), Epithelium (Ep) {H& E, 400X}[As shown in fig. 16]

Evaluation of chemoprotective efficacy of DNG against Doxorubicin-induced testicular toxicity in Swiss albino mice[9].

The animals were randomized based on weights and divided into four respective groups as shown in the table below. After 14 days of treatment, On the 15th day, the animals were anesthetized, and blood was collected by cardiac puncture and transferred into non-heparinized centrifuge tubes for the estimation of serum AMH. After blood collection, the animals were euthanized according to the norms of CPCSEA, followed by the collection of testes, epididymis, seminiferous tubules, and prostate gland for histology study.

PARAMETERS EVALUATED:

Estimation of serum AMH: Serum AMH was estimated via automated chemiluminescence immunoassay. The estimation of AMH was based on the combination of ruthenium electro chemiluminescence and streptavidin-biotin technology. The total assay time was 18 min, and the sample volume was 50µL of serum. The instrument used was Beckman Coulter Access Immunoassay Analyzer[10].

Histology

All the tissues were trimmed and processed routinely. Tissue processing was done to dehydrate in ascending grades of alcohol, clearing in xylene, and embedded in paraffin wax. Paraffin wax embedded tissue blocks were sections at 3-5µm thickness with the Rotary Microtome. All the slides of the brain were stained with Hematoxylin & Eosinstain. The prepared slides were examined under a microscope by Pathologist to notehistopathological lesion if any. The severity of the observed lesions was recorded as Minimal (<1%), Mild (1-25%), Moderate (26-50%), Moderately Severe (51-75%),Severe (76-100%).

RESULTS:

Cytotoxicity of DNG on normal cell line by SRB Assay.

Normal BEAS-2B cells were treated with different concentrations of DNG and DOX individually, and percent growth inhibition was determined by SRB assay. The percentage inhibition of normal cell growth

by DNG at a maximum concentration (10mg) was found to be 29.53%±0.60% at 24hr treatment. Standard drug DOX (20µM) exhibited dose-dependent inhibition of normal cell growth at 24hr treatment, with inhibition at a maximum concentration of 33.86%±2.62%.

DNG showed minimal cytotoxicity (dose limited) on normal cell lines even at 48hr treatment at the tested concentrations. The maximum tested concentration of 10mg DNG showed 28.66%±1.10% inhibition at 48hr. Standard drug DOX (20µM) exhibited dose-dependent inhibition of normal cell growth at 48hr treatment, with inhibition at a maximum concentration of 32.61%±1.38%.

5.1.2 Cytotoxicity of DNG on cancer cell line by SRB Assay[11].

Cancer cells MDA-MB-468 were treated with different concentrations of DOX, and inhibition of cancer cell growth by DNG was at a maximum concentration (10mg) was found to be 31.09%±1.58% at 24. The percentage inhibition of cancer cell growth by DNG was at a maximum concentration (10mg) was found to be 31.09%±1.58% at 24hr. Standard drug DOX exhibited dose-dependent inhibition of cancer cell growth at 24hr treatment, with inhibition at a maximum concentration of 33.47%±2.54%.

DNG (10mg) showed cytotoxicity on the cancer cell line at 48hr treatment. The maximum tested concentration of DNG (10mg) showed 38.26%±0.99% inhibition at 48hr. Similar to the 24hr study, DOX caused dose and time-dependent inhibition of cancer cell growth. The maximum tested concentration of DOX (20µM) showed 37.38%±4.54% inhibition at 48hr respectively.

Using DNG against DOX for 24hr and 48hr on the normal cell line.

From the cytotoxicity data, two doses of DNG (0.625mg/ml and 1.25mg/ml) and DOX (2.5µM and 20µM) were selected to assess the protective effect of DNG against DOX challenged normal cell line at 24hr.

Anticancer efficacy of combination treatment (DNG and DOX) on breast Cancer cell line (MDA-MB-468) at 24hr and 48hr:

For assessing the anticancer activity two doses of DNG (0.635mg and 1.25mg) and DOX (2.5µM and 20µM) were tested against each other in order to assess the synergism of DNG.

DOX and DNG on cancer cell line (MDA-MB-468) at 24hr and 48hr

In combination treatment, the lower dose of DNG (0.625mg DNG + 2.5µM DOX) and the higher dose of DNG (1.25mg DNG + 2.5µM DOX) enhanced the percentage of inhibition to 19.41±0.8 and 20.48±1.37 when compared to that of the lower dose of (2.5µM) DOX (17.41±1.12) alone at 24hr and were not significant.

Likewise, the lower dose of DNG (0.625mg DNG + 20µM DOX) and the higher dose of DNG (1.25mg DNG + 20µM DOX) increased the percent inhibition to 30.22±0.88 and 33.39±1.09 compared to that of the higher dose of (20µM) DOX alone at 24hr and found to be not significant.

Mechanistic studies to explore the chemoprotective action of Noni.

Toxicity to the normal cells was reduced by the combination 1.25mg DNG + 20µM DOX (80.22%) when compared to that of 20µM DOX (84.14%) alone respectively.

The anticancer drug potency was increased by the combination 1.25mg DNG + 20µM DOX (70.94%) when compared to that of 20µM DOX (73.01%) alone respectively.

DISCUSSION:

Cytotoxicity is one of the most important indicators for biological evaluation in *in-vitro* studies. *In-vitro* cell viability and cytotoxicity assays with cultured cells are widely used for cytotoxicity tests of chemicals and drug screening[12]. Initially, the cytotoxicity of the test substance (DNG) and standards (CIS and DOX) on normal cells was determined by SRB assay in order to select the optimum dose of DNG, CIS, and DOX for chemoprotective study. In the cytotoxicity study, 0.3125-10 mg/ml concentration range of DNG showed the least cytotoxicity (dose limited) to normal cell lines at both 24hr and 48 hr. The results of this study revealed that DNG did not show toxicity (dose limited) towards normal cells which are in agreement with Westendorf J (2002) who suggested in their in-vitro study that Noni fruit juice has no toxic effect on normal cells (V79).

In the present study, the chemoprotective activity of DNG was assessed through *in-vitro* studies using normal cells (BEAS-2B). So, two doses of CIS (50µM and 100µM) and DOX (2.5µM and 20µM) were chosen for chemoprotective study as it caused up to 30% inhibition of normal cell growth. Based on the cytotoxicity study, two doses of DNG (0.635mg/ml and 1.25 mg/ml) were selected for the chemoprotective effect against anticancer drugs induced toxicity. DNG at the tested concentrations was effective in minimizing the CIS and DOX-induced toxicity. The *in-vitro* chemoprotective study revealed the protective effect of DNG against CIS and DOX anticancer drugs induced cytotoxicity and thus can be used as an adjuvant to chemotherapy. Since the anticancer mechanism of action of CIS and DOX is mediated through generation of free radicals, thus we wanted to investigate the anticancer efficacy of CIS and DOX in the presence of DNG owing to their potent antioxidant activity. The anticancer activity of DNG due to antioxidant activity associated with the presence of chemical compounds such as phenolics, flavonoids, damnacanthal and alkaloids[13]. Our present data suggest that DNG has anticancer activity. In the combination treatment, the anticancer activity of CIS (50µM) was enhanced up to 26.65% in the presence of DNG (1.25 mg/ml). Similarly, the anticancer activity of DOX (2.5µM) was enhanced up to 21.54% in the presence of DNG (1.25 mg/ml). Several preclinical studies have demonstrated that moderate or low dose of chemotherapy has advantages when combined with immunostimulant agents because of the synergistic antitumor effects without any accompanying toxicity[14]. Therefore, in similar lines, our present study demonstrates that the combination of Noni with lower doses of the aforesaid cytotoxic drugs shows increased anticancer activity with minimal toxicity to the normal cells. This preliminary in-vitro study helps to predict the combination of first-line chemotherapeutic agents (CIS and DOX) withDNG as a supplementing agent for future clinical applications to minimize the toxicity associated with chemotherapy[15]. An explanation for why Noni has shown cytoprotective effect to normal cells (BEAS-2B) and cytotoxic effect to cancer cells (MDA-MB-468) and not normal cells is yet to be understood[16]. DNG is a known compound to protect the normal cells as well as the toxic side effects of anticancer drugs. These observations are confirmed by SRB assay in our earlier results[17]. AMH might serve as the marker of Sertoli cell number, function and maturation of these in males especially[18]. DNG 1.04±0.03 helped the animal to maintain the significant closeness of values when compared with that of normal AMH (1.52±0.04) values and DOX (0.45±0.03). All the values obtained were highly significant. Not only DNG alone but the combination of it DOX+NONI (1.16±0.02) states that it was able to restore the loss compared to DOX (0.45±0.03) alone and were highly significant. Restoration of the levels of serum AMH (Anti-Mullerian Hormone) in DOX (2mg/ml) + DNG (0.35mg.ml) treated when compared to that DOX (2mg/ml) alone establishes the chemoprotective effect of DNG against testicular toxicity in particular. This is the first study to report the chemoprotective activity of DNG against testicular toxicity induced by DOX by considering the serum AMH as the testicular toxicity marker. In support with the above-discussed serum AMH biochemical levels, histopathological microscopic examination of testes, epididymis, seminal vesicles and prostate of healthy group, DOX + DNG and DNG alone mice did not reveal any lesion of pathological significance where that of doxorubicin-treated mice showed mild focal degeneration of seminiferous tubules and mild focal atrophy of prostate gland that is in agreement with the published literature, and the combination group with DNG also confirmed the protective activity of DNG . In conclusion, the present findings proved that Noni provided a significant protective effect against CIS and DOX and enhanced anticancer activity of CIS and DOX could be owing to the antioxidant as well as free radical scavenging actions of Noni. Therefore, our results suggest that Noni is a suitable herbal remedy to use with chemotherapy to prevent the toxic effect and to enhance the anticancer activity of existing anticancer drugs.

CONCLUSION:

In-vitro cytotoxicity studies on normal cell lines by SRB assay, DNG showed no toxic effect to normal human cells (BEAS-2B) at the tested concentration, indicating least toxicity to normal human cells. DNG at 2.5 mg/ml showed chemoprotective effect of 28.11% and 35.58% respectively against CIS 100µM induced toxicity. Similarly, DNG at 2.5 mg/ml showed protective effect of 30.77% and 35.07% respectively against DOX 20µM induced toxicity. Among the two, DNG showed more protective effect on DOX-induced toxicity. In the *in-vitro* anticancer activity of DNG combination treatment enhanced the anticancer efficacy of CIS and DOX. Where, in combination treatment with DNG at 0.625 mg/ml showed maximum enhancing anticancer efficacy (28.30%) with CIS 50µM at 24 h. While in combination treatment DNG at 0.625 mg/ml maximum enhanced anticancer efficacy (52.26%) to DOX-50µM at 48hr. So, DNG 0.6 mg/ml showed better anticancer effect and enhanced anticancer efficacy to CIS and DOX. Similar results were obtained in animal studies, where DNG protected the animals against anticancer drugs induced testicular toxicity induced by CIS, DOX. These toxic effects were evident by serum biochemicals levels i.e. AMH and histopathological studies. In the present study, the *in-vitro* findings corroborate with the *in-vivo* studies. Ours is the first study to demonstrate the chemoprotective mechanism of action of DNG through increased levels of serum AMH in DOX + DNG treated animals when compared to the levels of DOX alone. Thus, DNG may serve as a potential candidate to minimize the toxic effects of anticancer drugs and to enhance their anticancer efficacy. Therefore, DNG can be used as adjuvant therapy in cancer chemotherapy to prevent anticancer drug-associated toxicity and to enhance the QoL of cancer patients.

Conflict of Interest: None declared

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