

DOXORUBICIN HYDROCHLORIDE INDUCES DEVELOPMENTAL DEFECTS IN CHICK EMBRYOS VIS-A-VIS AMELIORATIVE ROLE OF *VITEX NEGUNDO* LINN. ON BIOCHEMICAL CONSTITUENTS IN AMNIOTIC FLUID

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ABSTRACT

The detrimental effect of Doxorubicin hydrochloride in the rat, mouse and recently in the chick model, concomitantly triggered interest among embryologists to understand the role that the drug plays in the developing embryos. The drug caused embryonic death, stunted growth and gross morphological malformations in developing chick embryos in the present study. The study demonstrates that doxorubicin is toxic and teratogenic during the stage of organogenesis in the developing chick embryos and significant alteration ($P \leq 0.01$); ($P \leq 0.05$) was observed in the biochemical constituents in the amniotic fluid, which radically reduced malformations, when pretreated with alcoholic leaf extract of *Vitex negundo* Linn. It was further observed that there is significant ($P \leq 0.01$) ($P \leq 0.05$) elevation in proteins, glucose, amylase and lipase in the amniotic fluid of 12 days old chick embryo in 48 hrs.

of administration of doxorubicin, while levels of triglyceride, cholesterol and G₆PD reduced. Protective action of the alcoholic leaf extract of *Vitex negundo* Linn. was significantly evident ($P \leq 0.01$); ($P \leq 0.05$) in the said model in all the parameters except triglyceride and the same was observed to be dose dependent in case of proteins, cholesterol, amylase and lipase, which may be due to the protective effect of herbal drug containing rich amount of phenolics and flavonoids augmenting the antioxidant effect.

KEYWORDS: Doxorubicin hydrochloride, Teratogenicity, Chick embryos, *Vitex negundo* Linn.

INTRODUCTION

Anthracyclines are mutagenic, carcinogenic and also cardiotoxic. Concern has been shown over the use of anthracycline anticancer drugs during pregnancy as these may be teratogenic to the human fetus. Most anticancer drugs are teratogens, merely because they target vital cellular functions. Targets of standard and experimental anticancer drugs include DNA, topoisomerases, microtubules, histone deacetylases and essential protein kinases such as CDK9 (Blagosklonny, 2004). Aimed at targets that are present in all normal cells, anticancer drugs cause side effects, thus limiting the therapy of cancer. For example, paclitaxel causes mitotic arrest in proliferating cells leading to neutropenia, anaemia, mucositis, colitis, diarrhoea, etc. It also causes painful neuropathy due to inhibition of microtubule transport in long axons (Rowinsky et al., 1995). The most common side effect of anticancer drugs is nausea and vomiting. Several notorious teratogen, including arsenic and thalidomide are used as anticancer drugs. For example, arsenic is approved for therapy of promyelocytic leukemia (Zhu et al., 2002). A combination of two teratogen (namely retinoic acid and arsenic) is especially effective in therapy of promyelocytic leukemia (Shen et al., 2004). Teratogenic effect of Doxorubicin hydrochloride (Adriamycin) was first documented by Thompson (1978). Mortell et al. (2003) reported anophthalmia & exencephaly in chick embryos treated with Adriamycin. Shingadia Hitesh (2015) has reported hepato-protective effects of *Vitex negundo* Linn. on Adriamycin induced toxicity in developing chick embryos. Doxorubicin hydrochloride (DOX) is an anthracycline, anti-neoplastic drug with known teratogenic effects on foetal rats in what is known as the Adriamycin Rat Model (ARM). This includes conditions similar to those in newborn humans, known collectively as the VACTERL association. This comprises vertebral (V), anorectal (A), cardiac (C), tracheoesophageal (TE), renal (R) and limb (L) anomalies. We designed this study to test the hypothesis that the administration of Doxorubicin hydrochloride (Adriamycin) to developing chick embryos would cause similar anomalies to those in the VACTERL association seen in the ARM.

Herbal medicine is the oldest form of healthcare known to mankind and it will not be an exaggeration to say that use of herbal drug for human healthcare is probably as ancient as human civilization. A perfect example of medicinal plant credited with innumerable medicinal qualities validated by modern science and used since ancient times is *Vitex negundo* Linn. (Family: Verbenaceae). The genus consists of 250 species of which about 14 species are found in India and some have commercial and medicinal prominence. *Vitex negundo* Linn. is credited with innumerable medicinal activities like analgesic, anti-

inflammatory, anticonvulsant, antioxidant, bronchial relaxant, hepatoprotective, etc. Leaves are aromatic, bitter, acrid, astringent, anodyne, anti-inflammatory, antipyretic or febrifuge, tranquillizer, bronchial smooth muscle relaxant, anti-arthritic, antihelmintic and vermifuge. Leaves contain an alkaloid, flavonoids like flavones, luteolin-7, glucoside, casticin, etc. The present investigation is an attempt to show whether Doxorubicin hydrochloride causes similar defects if any in the developing chick embryos, an alternative animal model to rat and mice and also to study ameliorative role of alcoholic leaf extract of *Vitex negundo* Linn. on vital biochemical constituents and related enzymes.

MATERIALS AND METHODS

The fresh leaves of *Vitex negundo* Linn. were collected locally from in & around Mumbai & authenticated (59971). The leaves were shade dried for a week & powdered using a grinder. The sieved powder of leaves was subjected to soxhlet extraction using ethanol that gave highest extractive value. The extract was filtered & ethanol was vacuum evaporated at 55°C. The alcoholic extract was then stored at -20°C until further use. Antineoplastic drug, Doxorubicin hydrochloride (Adriamycin) from Pfizer was used. All other chemicals & reagents were of analytical grade & procured from Merck, Lobachem & Qualigens. The diagnostic kits for biochemical assays were procured from Span Diagnostics & Biolabs Pvt. Ltd.

Incubation & maintenance of eggs: The zero-day old freshly laid fertilized Hens eggs were procured from Central Poultry Farm (WR), Government of India, Aarey Milk Colony, Mumbai, India. The eggs were cleaned with distilled water & 70% alcohol & placed in an incubator set at 37°C with relative humidity of 58-60% maintained by keeping tray filled with water inside the incubator. The eggs were rotated manually & examined through candling every day for the proper growth & viability. Eggs were candled to locate the injection site for the administration of drug, avoiding membrane bound blood vessels that were marked 2.0 cm below the air sac (Schrott et al., 1999).

Embryo toxicity study: The 12 days old chick embryos were selected after candling for the toxicity studies. These eggs were divided into four groups containing six embryos in each group. Based on the LD₅₀ values, embryos were administered with Doxorubicin hydrochloride (50, 60 70 & 100 µg) on 12th day. Simultaneously normal saline (0.9% Sodium chloride) was administered in embryos of control group by injecting into air sac. After administration, the injection site was sealed with molten paraffin & eggs were further

incubated. The embryos were scored for morphological alterations; change in volume of amniotic fluid, body weight & length if any at the end of 48 hours of incubation by comparing the Doxorubicin hydrochloride treated groups with the respective control.

Experimental design: To study embryo protective effect of alcoholic leaf extract of *Vitex negundo* Linn. in Doxorubicin hydrochloride induced toxicity in chick embryos experimental set up was divided into five groups of six embryos each.

Group I (Control): Chick embryos administered with 100µL normal saline per egg.

Group II: Chick embryos administered with 100µL containing 70µg of DOX per egg.

Group III (Extract control): Chick embryos administered with 100µL containing 200µg of leaf extract of *Vitex negundo* Linn. per egg.

Group IV: Chick embryos administered with 50µL containing 100µg of leaf extract of *Vitex negundo* Linn six hrs. prior to administration of 50µL containing 70µg of DOX per egg (Dose 1).

Group V: Chick embryos administered with 50µL containing 200µg of leaf extract of *Vitex negundo* Linn. six hrs. prior to administration of 50µL containing 70µg of DOX per egg (Dose 2).

The embryo protective role of leaf extract of *Vitex negundo* Linn. was assessed after 48 hrs. of incubation.

Collection of amniotic fluid: After experimental period of 48 hrs. embryos were sacrificed by opening air sac. Amniotic fluid were collected aseptically & stored at -20°C until further use.

Biochemical analysis of amniotic fluid: Amniotic fluid was centrifuged at 3000 rpm for 10 min. to remove suspended matter and cell debris. Clear supernatant was used for the assay of biochemical constituents. Protein (Lowry et al., 1951), Glucose (Sasaki and Matsui, 1972), Triglycerides (Schrott et al., 1999) & Cholesterol (Wybenga and Pileggi, 1925) were estimated. The concentration of enzymes like Amylase (Winn-deen et al., 1998), Lipase (Stoycheva et al., 2012) & G-6-Phosphate Dehydrogenase (G₆PD) (Burtis et al., 1999) were also estimated in the amniotic fluid using standard methods.

Statistical analysis: All the results were expressed as mean $\pm$ standard error for six embryos in each group & the difference between the groups were considered significant when P-value determined was less than 0.05 & 0.01.

RESULTS AND DISCUSSION

In the present study, chick embryo model was chosen since not much study has been carried out using Doxorubicin hydrochloride (DOX). The sensitivity of different organs varies according to the dose of DOX used & sometimes shown susceptibility at lower dose. The embryo toxicity of DOX in vitro, by exposing cultured rat embryos at low concentrations of DOX has shown a decrease in growth parameters; such as somite number, embryonic length, etc. As far as chick embryo model is concerned, some studies have been carried out in recent years by administering other antineoplastic agents such as Chlorambucil & Cytarabine also noticed malformations induced in chick embryos (Mastan and Parasarathy, 2005). The malformations in chick embryos in the present study substantiates with the result of study made by Mastan et al. (2007). Growth retardation & internal hemorrhage represent the most frequent malformation, evident in the present observation were demonstrated by some workers in past. Growing embryos needs a lot of energy as the cells multiply rapidly; however stunted growth of developing chick embryos might be result of reduced supply of energy and/or diversification of energy to mitigate DOX induced stress.

Morphological deformities observed in developing chick embryos exposed to Doxorubicin hydrochloride: The 12 days old chick embryos were examined for any malformation & mortality rate effects caused by DOX after 48 hrs. of treatment (Table 1). No mortality & deformities were observed in the control group administered with normal saline, while the spectrum of malformations in the embryos of treated groups was significantly observed; such as scanty feathers, limb deformities, short wings, beak deformities, hemorrhagic brain & fluids, hydrocephalous, thinning and twisting of neck (Fig. 1 & 2) & immediate death were observed (n=6). Stunted growth was the major malformation observed with teratogenic dose (DOX 70 μ g and 100 μ g). However it is worth noting that embryos from Group IV and V under the defensive mechanism of herbal drug did not show any significant defects. Gillick et al. (2002) reported that all newborns (100%) from the adriamycin-treated group injected during gestation demonstrated the typical abnormalities found in the ARM (Adrimycin Rat Model) i.e. oesophageal attretia, multiple gastrointestinal attretia, vertebral malformations, absent tails, ureterohydronephrosis, etc. According to Liu and Hutson (2007) all the treated fetuses of rat had no bladders and severe hydroureter/hydronephrosis on one or

both sides. Male fetuses had a proximal blind-ending urethra communicating with dilated ureters and giving rise to vasa. Female fetuses had a persistent urogenital sinus communicating with the ureters and cervix/uterus; 57% of the treated group had an imperforate anus and some had recto-urethral fistulae (males) or recto-urogenital fistulae (females). However no such anatomical defects were observed in our study on chick embryo probably due to span of exposure being 48 hrs. only. Birth defects such as syndactyly, cleft hands and absence of distal finger phalanges were associated with maternal exposure to chemotherapeutic agents (cyclophosphamide, 5-fluorouracil and adriamycin) during the first trimester of pregnancy (Paskulin et al., 2005).

Biochemical changes in amniotic fluid of chick embryos exposed to Doxorubicin hydrochloride: Administration of DOX caused significant biochemical changes in amniotic fluid (Table 3-4). No noteworthy alterations in biochemical constituents were observed in Group 3 animals that were administered with herbal leaf extract of *Vitex negundo* Linn. when compared with control Group 1 chick embryos. In DOX treated Group 2, significant elevation ($P \leq 0.01$) in levels of protein, glucose, amylase and lipase was significantly observed. All these parameters were considerably normalized ($P \leq 0.01$), when pretreated six hours prior to treatment of DOX with herbal leaf extract of *Vitex negundo* Linn. (Group 4 & 5) in a dose-dependent manner at the end of 48 hrs. of incubation. The change in the level of glucose is an indication of alterations in carbohydrate metabolism & the increased levels of amniotic fluid might be due to changes in membrane permeability & diffusion of embryonic glucose into amniotic fluid (Dixit and Parasarathy, 2004; Mastan and Parasarathy, 2005).

The elevated levels of proteins in the amniotic fluid indicated leakage of erythrocytes into amniotic fluid. These levels were reversed to almost nearly normal by pretreatment with herbal leaf extract of *Vitex negundo* Linn. A significant increase ($P \leq 0.01$) in amylase & lipase activities was observed in DOX treated group of chick embryos. Increased levels of marker enzymes of amniotic fluid on DOX administration might be due to the effect of anticancer drug on normal hepatocytes & cardiac cell which cause possible leakage on these enzymes to amniotic fluid. The amount of these cellular enzymes in amniotic fluid reflects the alterations in plasma membrane integrity and/or permeability (Farvin et al., 2004). Shingadia and Dalvie (2014) reported alterations in the concentrations of Urea, Uric acid, Creatinine, Sodium, Potassium, Calcium and Inorganic phosphorus in amniotic fluid of chick embryo intoxicated with Adriamycin (ADR) and reported ameliorative role of herbal drug, *Vitex negundo* Linn.

in past study. Similarly, Shingadia and Vaidya (2015) have also recounted restorative outcome of herbal drug on ADR induced cardiotoxicity on the developing chick embryos.

A significant decrease ($P \leq 0.01$) in levels of Triglycerides, Cholesterol & G₆PD was observed in DOX treated Group 2. Pre-administration of herbal leaf extract of *Vitex negundo* Linn. (Group 4 & 5) significantly increased ($P \leq 0.05$), ($P \leq 0.01$) the levels of these parameters in Group 4 & 5, when compared with DOX treated Group 2. The decreased levels of Triglycerides & Cholesterol in DOX treated chick embryos reflect adverse effect on amniotic fluid vis-a-vis on the developing chick embryos as well. Thus the effective role of amniotic fluid involved in development of skeleton & embryonic growth during the period of development is significantly disturbed. The reversal in the levels was observed with herbal leaf extract of *Vitex negundo* Linn.

Table 1 Weight of 12 days old chick embryo, volume of amniotic fluid & length at 48 hrs. of Doxorubicin hydrochloride treatment

Parameter	Exposure period (12 th day)	Group 1 (Control)	Group 2 (DOX 50 μ g)	Group 3 (DOX 60 μ g)	Group 4 (DOX 70 μ g)	Group 4 (DOX 100 μ g)
Weight of embryo (G/egg wt.)	48 hrs.	9.28 $\pm$ 0.08	8.72 $\pm$ 0.03**	7.32 $\pm$ 0.04**	6.65 $\pm$ 0.11*	5.21 $\pm$ 0.18*
Volume of amniotic fluid (mL/embryo)	48 hrs.	5.5 $\pm$ 0.15	4.9 $\pm$ 0.20*	3.3 $\pm$ 0.12*	3.95 $\pm$ 0.09**	2.5 $\pm$ 2.26**
Length of embryo (mm)	48 hrs.	12.5 $\pm$ 1.73	11.4 $\pm$ 0.59*	9.8 $\pm$ 0.32*	7.9 $\pm$ 1.16**	7.1 $\pm$ 0.95**

(Average values of six observations, mean $\pm$ SE)

Statistically significant at ** $P \leq 0.01$, * $P \leq 0.05$

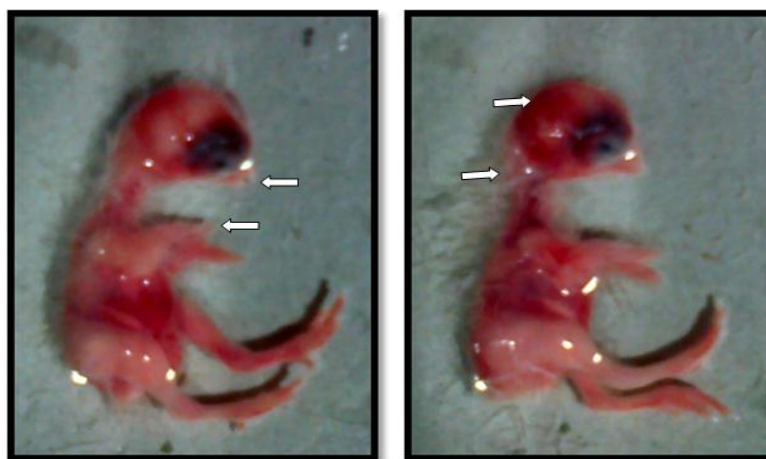


Fig. 1 & 2 Embryos from Group 3 (DOX treated) showing hemorrhagic head, defective limb and beak formation and twisting of neck

Table 2 Protective effect of alcoholic leaf extract (LE) of *Vitex negundo* Linn. in amniotic fluid of chick embryo at the end of 48 hrs. treatment of Doxorubicin hydrochloride

Groups	Protein (mg/dL)	Glucose (mg/dL)	Triglycerides (mg/dL)	Cholesterol (mg/dL)
Group 1 (Control)	42.56± 0.12	36.12 ± 0.05	64.55 ± 0.07	23.88 ± 0.27
Group 2 (DOX 70µg)	63.59± 0.09	52.07 ± 0.18	34.37 ± 0.04	14.57 ± 0.11
Group 3 (LE 200µg)	46.04± 0.22	38.95 ± 0.03	65.18 ± 0.08	25.19 ± 0.16
Group 4 (LE 100µg + DOX 70µg)	51.97± 0.05**	41.63 ± 0.24*	29.48 ± 0.23*	17.32 ± 0.06**
Group 5 (LE 200µg + DOX 70µg)	49.36± 0.07**	45.85 ± 0.21*	22.87 ± 0.17**	20.83 ± 0.02**

(Average values of six observations, mean ± SE)

Statistically significant at **P≤0.01, *P≤0.05

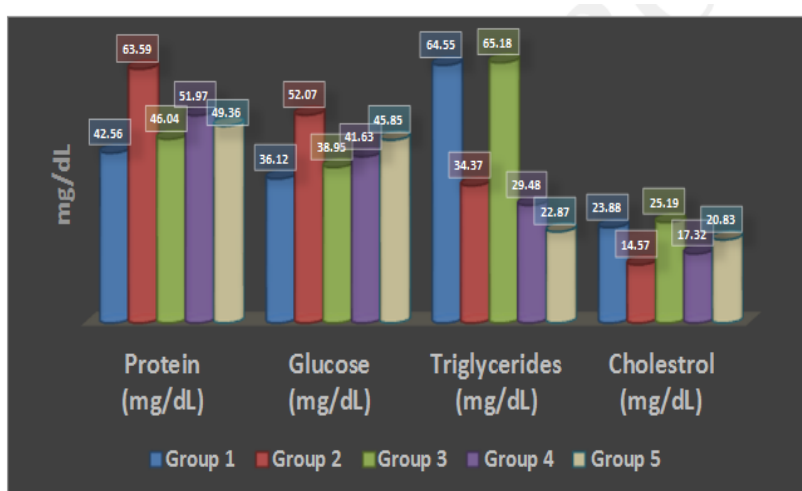


Fig. 3 Effect of leaf extract (LE) of *Vitex negundo* Linn. on vital constituents in amniotic fluid of chick embryo at the end of 48 hrs. treatment

Table 3 Protective effect of alcoholic leaf extract (LE) of *Vitex negundo* Linn. in amniotic fluid of chick embryos at the end of 48 hrs. treatment of Doxorubicin hydrochloride

Group	Amylase (IU/L)	Lipase (IU/L)	G6PD (IU/L)
Group 1 (Control)	26.07 ± 0.16	38.3 ± 0.07	83.5 ± 0.11
Group 2 (DOX 70µg)	44.07 ± 0.13	76.7 ± 0.03	40.23 ± 0.08
Group 3 (LE 200µg)	25.91 ± 0.09	36.8 ± 0.09	79.12 ± 0.22
Group 4 (LE 100µg + DOX 70µg)	36.99 ± 0.11**	55.2 ± 0.31*	65.51 ± 0.15*
Group 5 (LE 200µg + DOX 70µg)	24.48 ± 0.05**	49.01 ± 0.23**	55.85 ± 0.07**

(Average values of six observations, mean ± SE)

Statistically significant at **P≤0.01, *P≤0.05

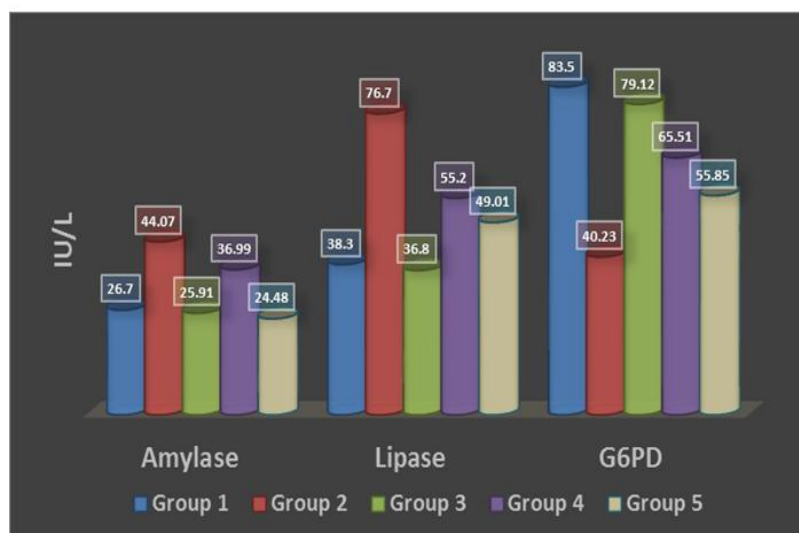


Fig. 4 Effect of leaf extract (LE) of *Vitex negundo* Linn. on vital constituents in amniotic fluid of chick embryo at the end of 48 hrs. treatment.

CONCLUSION

Due to the paucity of antioxidants for embryo protection against anticancer agents, newer and better herbal drugs need to be developed with antioxidant potentials that are efficient in upholding peroxidant/antioxidant stability. Hence in the present investigation pretreatment with leaf extract of *Vitex negundo* Linn. proved beneficial by restoring antioxidant balance and biochemical variables in developing chick embryos. A significant alterations in the levels of activity of marker enzymes as well as visible ameliorative effect clearly designates restorative role of the leaf extract of *Vitex negundo* Linn. Nevertheless further characterization of herbal drug necessitates the efficacy studies. Elaborate trials are obligatory before it is recommended for clinical exercise in future, even though it carries immense potential to be developed as drug for pharmaceutical diligence.

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