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Effect of Leaf Extract of *Tridax procumbence* on Superoxide Dismutase and Lactate Dehydrogenase Concentration in Early Chick Embryo

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Abstract: Cellular injury takes place due to the toxicity caused by the oxygen radicals. The aging various cancers, heart diseases and liver disorders are detected due to free radicles (Harman,1981; Troll *et al.*, 1990). Superoxide dismutase (SOD, EC 1·5·1·1) is essential for biological defense against the superoxide anion (Fridovich,1983). Information on SOD in different tissues are least available. It would, therefore important to determine SOD activity in early chick embryo blood vessels during embryonic development. Since, there is evidence that oxygen radicals have a role in tumor formation (Troll *et al.*, 1990), it would also be essential to determine the SOD status in chick embryo blood vessels (Markland and Markland, 1974). In the present work, the author has collected the information by assaying the activities of marker enzymes Lactate dehydrogenase (LDH) concentration which increased after cellular damage in embryonic blood vessels of early chick embryo since it has been shown that the injury to the cell could be correlated and determined by assaying the level of activities of marker enzyme. The levels of LDH assayed in embryonic tissue to correlate the changes in the tissues (Akanji *et al.*, 2008; Kim *et al.*, 2010). In this communication the author has observed that there is significant rise in the level of SOD and LDH after *Tridax procumbence* (Linn.) leaf extract treatment in vascular tissue. It has been one of the feasible approaches for analyzing medicinal properties of *Tridax* leaf extract as herbal medicine.

Keywords: Chick embryo, *Tridax*, Superoxide dismutase, Lactate dehydrogenase, Blood vessels

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Introduction

In the field of developmental biology and toxicology, the chick embryo of *Gallus gallus domesticus* is an extremely valuable model organism because most of the development takes place within an egg. The embryonic chick occupies a privileged place among animal models in developmental biology, it's rapid development and accessibility for visualization and experimental manipulation are remarkable characteristics that have made it a vertebrate model of choice. A plant *Tridax procumbens* (Linn.) is very commonly used as traditional herbal medicine particularly in wounds healing (Petchi *et al.*, 2013; Kethamakka and Deogade, 2014). Superoxide dismutase

catalyzes the dismutation of the superoxide (O^{-}_{2}) radical into ordinary molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). SODs are enzymes of cells that live in the presence of O_2 (Bajaj *et al*., 1985). Superoxide is produced as a byproduct of oxygen metabolism and causes many types of cell damage if they are not regulated. Thus, superoxide dismutase is very essential part of defense in living cells. SOD out-competes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity. The reaction of superoxide with non-radicals is spin-forbidden. In biological systems, this means that its main reactions are with itself (dismutation) or with another biological radical such as nitric oxide (NO) or with a transition-series metal. The superoxide anion radical (O^{-2}) spontaneously dismutes to O_2 and hydrogen peroxide(H_2O_2) quite rapidly (Markland and Markland, 1974). The high efficiency of superoxide dismutase seems necessary even at the sub-nanomolar concentrations achieved by the high concentrations of SOD within cells. Superoxide inactivates the citric acid cycle enzyme aconitase, that can poison energy metabolism, and releases potentially toxic iron. Aconitase is one of several iron-sulfur-containing (de)hydratases in metabolic pathways shown to be inactivated by superoxide. Lactate dehydrogenase (LDH) is an enzyme found in all living cells. It catalyzes the conversion of lactate to pyruvate and reverse, as it converts NAD^+ to NADH and reverse. LDH is expressed extensively in body tissues, such as blood cells and heart muscle. Because it is released during tissue damage, it is a marker of common injuries and diseases such as heart failure. Therefore, the LDH test is generally used to screen for tissue damage (Wroblewski and Ladue, 1955). This damage may be acute as in the case of a traumatic injury or chronic i.e. due to a long-term condition such as liver disease or certain types of anemia. It is also used to monitor progressive conditions, such as muscular dystrophy. LDH is found in many of the tissues and organs, including the muscles, liver, heart, pancreas, kidneys, brain, and blood cells. The LDH test is prominently used to help identify the location and severity of tissue damage in the body. As new cells form in tissues, the body gets rid of older or dead cells. This normal process causes tissues to release LDH into bloodstream or other body fluids. Because of this, it is normal to always have some LDH in a blood or fluid sample (Wroblewski and Ladue, 1955).

Therefore in this study, efforts were made to study the effect of herbal medicinal plant leaf extract (*Tridax procumbence*) on the early chick embryo blood (48 h and 96 h incubation) vessels and estimated the SOD (superoxide dismutase) and LDH (Lactate dehydrogenase) to study the effect of extract. It is one of the feasible approaches for analyzing properties of *Tridax* extract as an herbal medicine.

Materials and Methods

Fresh *Tridax procumbens* plants were collected from Fergusson College (Autonomous) campus, Pune, India. *Tridax* leaves were cleaned and dried on filter paper for three to four days, grounded with mortar and pestle and then used for Soxhlet extraction in 95% ethanol (Bhagwat *et al*., 2008). The proportion considered for extraction was 1g of dried leaves powder in 100 ml of solvent. After extraction the extract was concentrated using rotary vacuum evaporator. The 1 ppm solution of extract was prepared after dilution in dextrose with normal saline and stored in in refrigerator at 4 ˚C for further use (Pendharkar, 2021). Chick eggs were incubated at 37.5 ˚C up to 48 h and 96 h (HH stage 12-13 and HH stage 21-22), respectively and then treated with 1 ppm extract solution for 24 h. In aseptic condition the vascular tissue of an embryo was separated and homogenized in 0.25M sucrose solution (1:5 w/v) in physiological buffer. The homogenates were transferred into specimen bottles and kept frozen for 24 h before being used for analysis.

Lactate dehydrogenase (EC. 1.1.1.27) activity was determined by method of Wroblewski and Ladue (1955). Lactate dehydrogenase catalyzes the reversible reaction: L-lactate + NAD+ in equilibrium pyruvate + NADH. The bidirectional

reaction was monitored spectrophotometrically by measuring the increase in NADH at 340 nm produced in the lactate-to-pyruvate reaction (Wroblewski and Ladue, 1955). All measurements were done using spectrophotometer.

For SOD assay, the tissue homogenate (1:9 for vascular tissue, w/v) was prepared in ice-cold 0·25 M sucrose solution containing 0·5% Triton X-100. The crude homogenate 34.880 g was centrifuged at 30000 rpm for 30 min and the supernatant was used. Sucrose (0·25 M) and Triton X-100 (0·5%) did not interfere with the SOD. The amount of tissue homogenate needed for SOD assay by the pyrogallol method was 2-5 µl. When SOD was assayed by the X/XOD/Cyt c^{3+} and X/XOD/NBT methods, the 34.880 g supernatant was dialyzed against 20 volumes of 10 mM potassium phosphate buffer, pH 7·2, for 8 h with 8 changes of buffer at 4 °C. SOD assay by the pyrogallol autoxidation method was carried out following the procedure of Markland and Markland (1974). In this process the autoxidation of pyrogallol was studied with the EDTA at the pH 7.0 -11.0. It was observed that the process of autoxidation increases when the pH increases. At pH 7.0 to 8.0 the reaction is inhibited at high level by SOD (Markland and Markland, 1974). The assay mixture was transferred to a 1·5 ml cuvette and the rate of increase in the absorbance at 420 nm was recorded for 2 min from 1 min 30 s to 3 min 30 s in a spectrophotometer. The lag of 1 min 30 s allowed for steady state of autoxidation of pyrogallol to be attained. The concentration of pyrogallol was so adjusted that the rate of change of absorbance per min was approximately 0·020-0·023. The increase in the absorbance at 420 nm after addition of pyrogallol was inhibited by the presence of SOD. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 3 ml of assay mixture (Nandi and Chatterjee, 1988; Akanji *et al.*, 2008). Results have been expressed in units per g tissue or per mg protein for tissue homogenate.

All determinations were done in duplicate and means were calculated. Three replicates were estimated from Control and Experimental groups (*Tridax* leaves extract treated).

Statistical Analysis:

Data are the mean of 5 replicates ± SD. Duncan Multiple Range Test was used to account for the differences among different means as well as the interaction between the variables. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

The 48 h incubated chick embryo HH 12-13 has16–19 somites (Hamburger and Hamilton, 1992). The dextral-looping phase of cardiac looping is completed at stage HH 12-13. These late-migrating cardiac neural crest cells (HH 12–13-) are restricted to the proximal part of the pharyngeal arch arteries and late CNC ablations show that their primary role is in pharyngeal arch artery remodeling and not in AP septum formation (Boot *et al.*, 2003). However, they are not developmentally restricted and can contribute to the AP septum when transplanted into a younger environment (Boot *et al.*, 2003). The outflow attachment to the ventral pharynx shows a dynamic relationship with the pharynx. At stage HH 12, the outflow region joins the pharynx ventral to pharyngeal arch/artery 1, but by stage HH 24 it is located ventral to pharyngeal arches/arteries 4-6 (Hamburger and Hamilton, 1992). Endocardial cushions are first seen as an expansion of the cardiac jelly in the outflow tract and the AV canal. This precedes the actual formation of the cushion mesenchyme. Endocardial cushion development has been separated from valve formation in this reference guide to better identify the stages of cushion growth during the septation of the chick outflow tract, but valve development occurs in specific regions of the endocardial cushions as outlined in subsequent stages. By considering these features HH stage 12-13 was considered for this study.

Table 1: SOD and LDH activity after treatment with *Tridax procumbens* leaf extract at 48 h and 96 h incubation

Incubation	Group	SOD (units/mg)	LDH (units/mg)
48 h	Control	602 ± 12	44± 1.1
	Control	601 ± 10	46± 1.2
	Control	600± 11	45± 1.2
	Treated	657 ± 8	56 ± 1.9
	Treated	659 ± 9	54 ± 1.8
	Treated	658 ± 8	52 ± 1.8
96 h	Control	698±10	52±1.6
	Control	697±10	57±1.5
	Control	698±10	56±1.6
	Treated	795±15	73±0.6
	Treated	796±14	75±0.7
	Treated	799±15	74±0.8

HH stage 21-23 (3.5–4 days, 43-44 somites) the epicardium has reached this surface of the right ventricle just prior to the apoptosis at stage HH 21, suggesting that the epicardium induces apoptosis within the cardiac myocardium. Also, proper timing of epicardial outgrowth is important for the ingrowth of coronary arteries into the aorta at later stages. Secondary myocardium is finished being added to the outflow tract. By considering these important features HH stage 21-23 is considered for the study and compared with 48 h. embryo tissue (Hamburger and Hamilton, 1992).

All the treated embryo remains alive after the treatment of 1 ppm conc. Of leaf extract. The measurement of the activities of enzymes (SOD and LDH) in tissues was used in assessing the degree toxicity of a chemical compound on tissues. Measurement of enzyme activities was used to indicate tissue cellular damage caused by a chemical compound long before histological changes. The significant loss of lactate dehydrogenase (LDH), an enzyme associated with the cytosol is quite understandable since it is near the plasma membrane (Akanji *et al*., 2008). Slight damage to the plasma membrane will easily lead to leakage of LDH from the cell interior to the extracellular environment. However, the increase in serum LDH activity in this study supported the reasoning of leakage of the cytosolic enzyme from the tissues into the serum due to labialized plasma membrane. SOD assay by the pyrogallol method is dependent on the pH of the assay mixture. The rate of increase in absorbance at 420 nm per min increased with increasing pH from 8·2–8·9. The author has observed that better sensitivity and reproducibility were obtained when the pH of the assay mixture was kept at 8·5. At this pH, the autoxidation of pyrogallol was inhibited about 88% by 2 pyrogallol units of vascular tissue SOD indicating that the reduction was mediated via $-O_2$ When applied to tissue homogenate, the

absorbance at 420 nm was linear with time up to 2-3 min.

In the present study treatment of *Tridax* leaf extract at 48 h and 96 h embryo showed variations in SOD and LDH concentrations. Possible cause for this may be to cope the stress induced by *Tridax* leaf extract.

In the control tissue mean concentration of SOD were 602±12 units/mg and 698±10 units/mg; 601±10 units/ mg and 697±10 units /mg and 600±11 units/ mg and 698±10 units /mg at 48 and 96 h, respectively (Table 1). The SOD in the treated tissue were 657 ±8 units/mg and 795±15 units/mg; 659 ±9 units/mg and 796±14 units/mg and 658 ±8 units/mg and 799±15 units/mg at 48 and 96 h, respectively (Table 1).

In the control tissue mean concentration of LDH were 44±1.1 units/ mg protein and 52±1.6 units/mg protein; 46± 1.2 units/ mg protein and 57±1.5 units /mg protein and 45± 1.2 units/ mg protein and 56±1.6 units /mg protein at 48 and 96 h, respectively (Table 1). The LDH in the treated tissue were 56 ± 1.9 units/mg protein and 73±0.6 units/mg protein; 54 ± 1.8 units/mg protein and 75±0.7 units/mg protein and 52 ± 1.8 units/mg protein and 74±0.8 units/mg protein at 48 and 96 h, respectively (Table 1).

Conclusion

The concentrations of SOD and LDH were not significantly different between the control tissue samples. Embryonic tissue revealed a significant increase in vascular LDH and SOD level in *Tridax* leaf extract treated group as compared to control. These results suggested that tissue concentrations of LDH may be useful as biomarkers for use of *Tridax* extract as herbal medicine. which is traditionally being used in wound healing. Overall observations suggested that *Tridax* leaf extract (1ppm conc.) is tolerable to Chick embryonic vascular tissue. In this study, author propose a strategy that studies of SOD and LDH using *Tridax* leaf extract has been the feasible approach for analyzing herbal medicinal properties in healing process by chick embryo model systems.

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