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Study of effect of *Tridax* leaf extract on mitochondrial activity of chick embryo hepatic cells

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Abstract
A plant Tridax procumbens (Linn.) is very commonly used as an herbal medicine in healing of wounds. Mitochondria are very important organelle of the cell, by considering the medicinal use of Tridax the purpose of research is to study the effect of leaf extract on mitochondria of the hepatic cell in early chick embryogenesis. The H.H stage 34, i.e., 8days;192hrs incubation was selected because of developed hepatic tissue in the stage 34. (Hamburger Hamilton) [4]. The embryonic characters of this stage ware observed and found that the mitochondrial activity is very important in chick embryo model. Therefore, the effect of extract was studied on the activity of mitochondria. The principle of this assay is succinate dehydrogenase converts succinate to fumarate and transfers the electron to an artificial electron acceptor (Probe), 2,6-dichlorophenolindophenol (DCPIP) ($C_{12}H_8NO_2C_{l2}$), which changes the colour from blue to a colourless product (Jared Rutter *et al*) [18]. Light transmittance measured using a spectrophotometer. The biochemical study of hepatic SDH activity under control and treated was performed to see the effect of Tridax extract at hepatic mitochondrial level. Our investigation reveals that the controlled sample shows gradual change in absorbance and *Tridax* extract treated sample showing more change in absorbance and high concentration of extract do not show the change in the absorbance. In this study, we propose a strategy that study of mitochondrial characterization using DCPIP and its oxidation and reduction to characterize aspects of mitochondria has been the feasible approach for analysing properties of *Tridax* extract as herbal medicine. (Ramesh Petchi *et al*) [15].

Keywords: chick embryo, *Tridax*, mitochondria, DCPIP, PMS

Introduction

Tridax procumbens (Linn.) is commonly known as coat buttons (Ramesh Petchi *et al*) [15]. It is well known as a widespread weed. *Tridax* occurs in mild temperate, tropical, subtropical regions worldwide. The *Tridax* easily available in the fields, meadows, croplands, lawns, and roadside area. *Tridax procumbens* is commonly being used in traditional medicine as hair tonic, antifungal herbal medicine, insect repellent, as antidiarrheal, on dysentery, and as effective medicine on wound healing. (Bhagwat D.A. *et al*.) [1]. By considering its medicinal property the *Tridax* weed collected from the Fergusson college campus. The fresh leaves of plant were cleaned and dried for three days the dried leaves were grounded with the help of mortar and pestle and powder used for extraction using Soxhlet extractor. Solvent used for the extraction was 95% ethanol (Manisha Mhaske *et al*) [11]. Mitochondria are membrane-bound cell organelles that generate most of the chemical energy needed to power the cytoplasmic biochemical reactions. Chemical energy produced by the mitochondria is stored in a small molecule called adenosine triphosphate (ATP). Mitochondria also perform some important functions like mitochondrial calcium exchange that is the flow of calcium in and out of mitochondria, this process is important in metabolic regulation and cell death, Innate immunity is the inborn system that recognizes and responds to various infections caused by pathogens, providing immediate and non-specific defence. The chick embryo model selected to study the interaction of Tridax leaf extract and mitochondria because chick embryogenesis model occupies its privilege role among animal models used in developmental studies. Its development and accessibility for visualization and experimental uses are some of the ideal characteristics. The progress in chick culture technologies and in imaging is advancing real-time visualization of developmental events, such as cell differentiation. tissue morphogenesis, angiogenesis, and cancer metastasis. As the chick egg is self-sufficient, and as its normal development takes place at 37°C this ensures consistent viability of animals without artificial support media and complex culture requirements. In mitochondria a large amount of energy produced which is required for cell maintenance and function, is processed in the form of ATP. ATP is consumed for energy in processes including ion transport, muscle contraction, nerve impulse propagation, substrate phosphorylation, and chemical synthesis.

These processes, as well as others, create a high demand for ATP. Mitochondria are an energy source of ATP for cells and organs of developing embryo (Yoan Arribat *et al*.) [20]. Therefore, the mitochondria were isolated from the liver tissue during chick embryonic development under controlled and treated samples and characterized (Yoan Arribat *et al*.) [20] and (Tiwari U *et al*.) [19]. These early developmental studies eventually provide the information related to characterization of mitochondria. In the characterization the oxidation of succinate to fumarate is one of the steps in the TCA cycle. This reaction is catalysed by succinate dehydrogenase. An artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP) ($C_{12}H_8NO_2C_{l2}$) used as a redox dye electron acceptor. As DCPIP get reduced and becomes colourless, the resultant increase in light transmittance that measured using a spectrophotometer. This piece of research on characterization of mitochondria with the effect of *Tridax leaf* extract revealed that the leaf extract show effect on mitochondrial characterization and high concentration of extract blocking the activities of SDH reduction -oxidation and help in fast wound healing and. (SRP Kethamakka *et*

al.) [12]. These findings contribute role of herbal extract on mitochondrial activity.

Materials and methods

A sample of fresh Tridax procumbens plants was collected from Fergusson College campus in the month of February and March 2016. After cleaning and washing the sample the leaves ware dried on filter papers for three to four days. The leaves of dried plant were grounded on mortar and pestle and then the powder was packed in thimble and used for Soxhlet extraction in 95% ethanol (Manisha Mhaske *et al*.) [11]. Soxhlet extraction is also known as continuous extraction and provides an exhaustive extraction of the plant material. The proportion considered for extraction was 1gm of dried leaves powder in 100ml of solvent. After extraction the chemical extract was concentrated using rotary vacuum evaporator (Manisha Mhaske *et al*.) [11]. The 1mg of extract was dissolved in 1ml dextrose with normal saline and stored in small vials and stored in refrigerator at 4.0° C for further use. The fertilized eggs of *Gallus gallus* were incubated at 38.0 ^{0}C in humidified incubator with 70% relative humidity. The Hamburger and Hamilton stage 34 – [8days (192hrs incubation)], was selected for this experiment as H.H. stage 34 show development of liver tissue (Hamburger and Hamilton) [4]. The liver tissue of 0.036 gm was separated from embryo and washed with 01M potassium phosphate buffer, then minced and homogenized with 5ml isolation buffer (20% tissue homogenate) (Yuji Y. *et al*) [21]. The homogenate was filtered using muslin cloth and funnel. The differential centrifugation, a two-step centrifugation carried out at low speed (3000rpm) to remove intact cells, cell and tissue debris, and nuclei from whole cell extracts followed by high-speed centrifugation (10000rpm) to concentrate mitochondria and separate them from other organelles. Then the homogenate was centrifuged at 3000rpm for 5 min at 4^{0}C. The pellet was discarded because pellet contains nuclei and cell debris. The supernatant was decanted in another tube and centrifuged at 10000 rpm for 10 min at 4^{0}C. Pellet obtained contained mitochondria. The pellet was resuspended in 1ml 0.1M phosphate buffer. The half of the pellet was proceeded for SDH estimation as a control sample and remaining half pellet was treated with moderate concentration 10.0 µL and 25 µL high concentration of plant extract and then proceed for SDH activity. Estimation of succinate dehydrogenase (SDH) was performed by the catalytic conversion of succinate to fumarate. PMS oxidizes succinate in presence of SDH to release 2protons, which reduces DCPIP (an artificial electron acceptor) dye.

[Succinate+ PMS+DCPIP (oxidized) (Blue) → Fumarate + PMS + 2H+DCPIP (Reduced)].

Therefore, all the components except DCPIP were mixed. The reaction started by adding DCPIP and the change in absorbance measured spectrophotometrically at 600 nm. The objective of this is to evaluate the effect of the *T. procumbens* plant extract on mitochondrial activity of the hepatic cells. The composition of reaction mixture was prepared by addition of 0.1 M Phosphate buffer 1200 µl **,** 0.4 M Succinate 50 µl , 20 MM sodium azide 50 µl ,1.5 MM DCPIP ,100 µl, 60 MM PMS , 33 µl, Enzyme50 µl, Distilled wate,r490 µl and *Tridax* extract,10.0 µl. The total volume of mixture taken was1983 µl. Assessment of Mitochondrial function was performed by SDH activity that is reduction of DCPIP was performed. Therefore, all the components except DCPIP were mixed. The reaction was started by adding DCPIP to reaction mixture and SDH, the reaction activity noted at the interval of 30 sec for 3 minutes for each sample. Absorbance was noted at 600 nm for 3 min at interval of 30sec using UV spectrophotometer.

Observations

Table 1: Controlled and treated. Stage 34 (8days;192hrs.)

Time	Absorbance at 600nm Controlled	Absorbance at 600nm Treated
30 Sec	0.535	0.651
60 Sec	0.528	0.648
90 Sec	0.523	0.644
120 Sec	0.518	0.641
150 Sec	0.511	0.638
180Sec	0.508	0.635

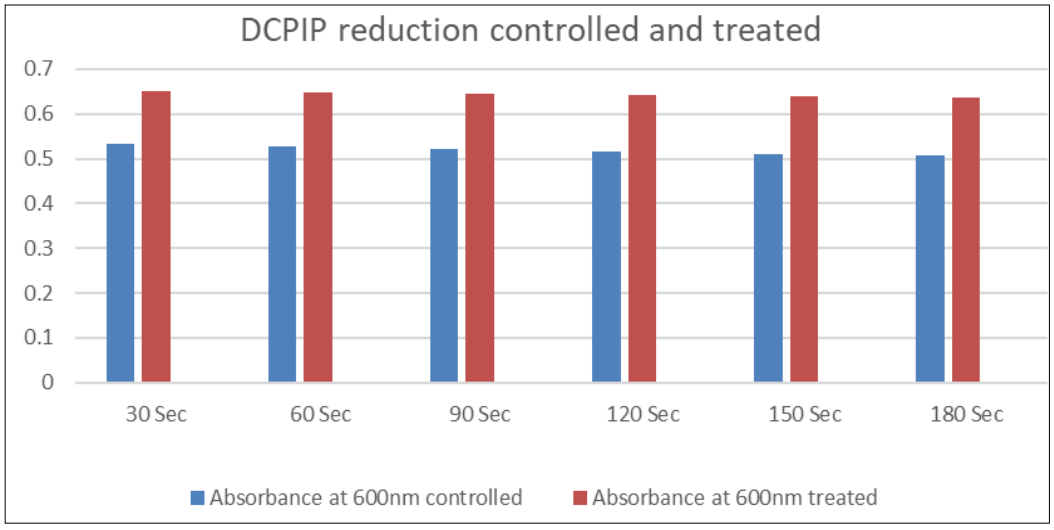

Fig 1: Graph 1- DCPIP reduction

Table 2: Controlled and Treated- H.H. Stage 34 (8days;192hrs.)

Time	Absorbance at 600nm controlled	Absorbance at 600nm treated
30 Sec	0.532	0.650
60 Sec	0.526	0.647
90 Sec	0.522	0.645
120 Sec	0.516	0.642
150 Sec	0.510	0.639
180 Sec	0.507	0.636

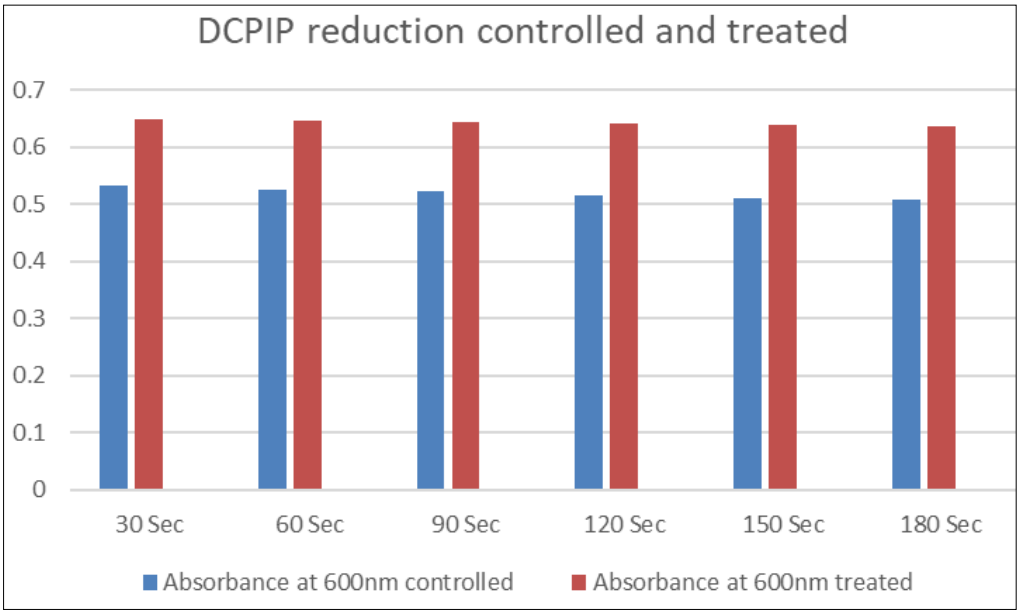

Fig 2: Graph 2- DCPIP reduction

Table 3: Controlled and treated- H.H. Stage 34 (8days;192hrs.)

Time	Absorbance at 600nm controlled	Absorbance at 600nm treated
30 Sec	0.538	0.652
60 Sec	0.529	0.649
90 Sec	0.525	0.647
120 Sec	0.521	0.643
150 Sec	0.513	0.640
180 Sec	0.510	0.638

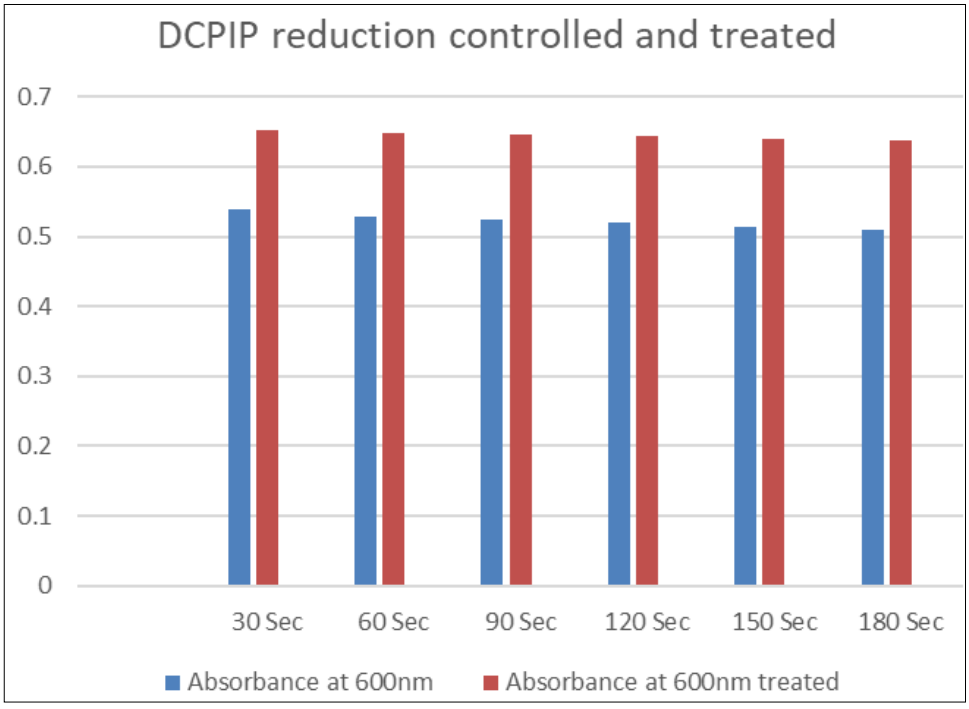

Fig 3: Graph 3 DCPIP reduction

Table 4: High concentration (25 µl) Treated- H.H. Stage 34 (8days;192hrs.)

Time	Absorbance at 600nm
30 Sec	2.500
60 Sec	2.500
90 Sec	2.500
120 Sec	2.500
150 Sec	2.500
180 Sec	2.500

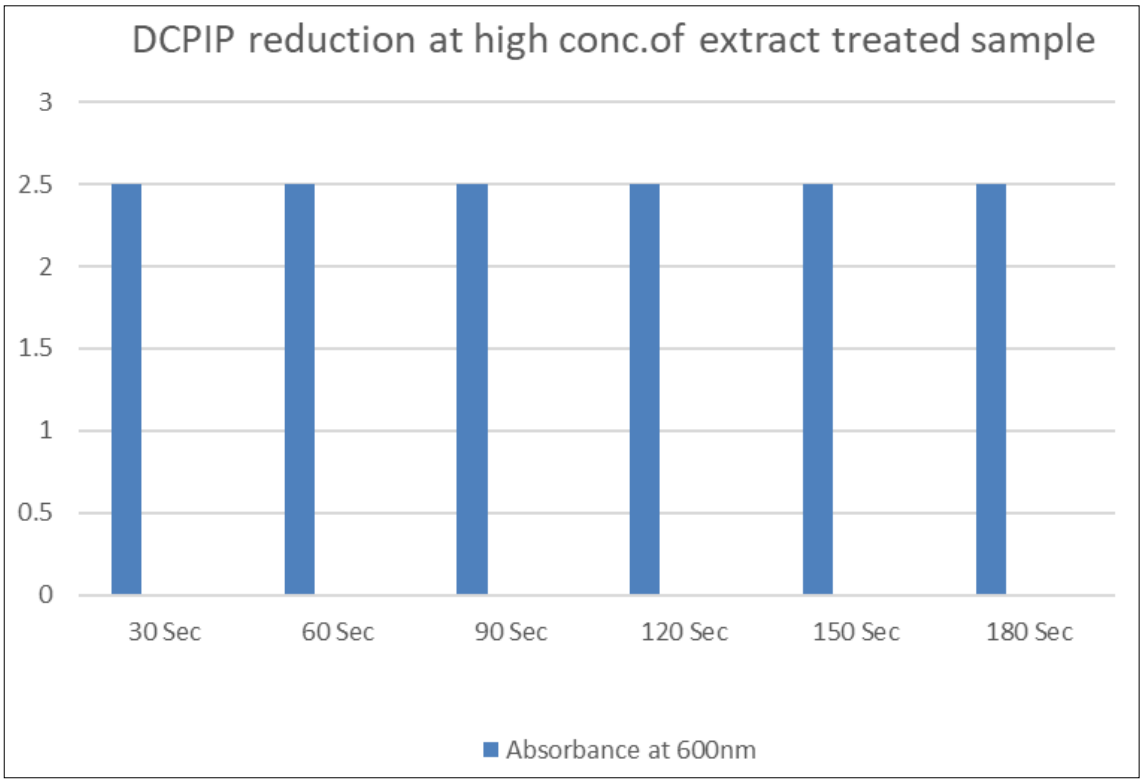

Fig 4: Graph VI- DCPIP reduction at high concentration of Tridax extract treated sample.

Calculations

µ moles of DCPIP reduced/min= ΔA/ Δt x V/v x 1/Ɛ0

Where-
ΔA/ Δt= change in absorbance after 3.0 mins.
V=Total volume of reaction
Ɛ0=321cm-1xmm-1(Coefficient of wavelength) =0.0031
V= Volume of sample (50 µL)

Statistical analysis

µ moles of DCPIP reduced/min for Set 1-sample1 (Control) = Result of calculations- µ moles of DCPIP reduced/min.
∴

Table 5

Set No.	Test Sample No	Test Sample	µ moles of DCPIP reduced/min
I	1	Controlled	5.833
	1	Extract treated	5.993
II	2	Controlled	5.858
	2	Extract treated	6.012
III	3	Controlled	5.821
	3	Extract treated	6.024
IV	4	High conc. extract treated	0.112

The chick embryo development was observer from 24 hrs,48 hrs and then further incubation till 192 hrs (8 days). The 192hrs incubation stage is considered for this study because it show small hepatic tissues development. The controlled chick embryo found healthy at H.H. Stage 34- (8 days) incubation; limbs differential growth of second digit and third toe found conspicuous.
Contours of webs between digits and toes were concave and arched. Lengthening of mandible and of neck was continues. Feather-germs on scapula, on ventral side of neck, on pro-coracoid, and posterior (flight) edge of wing, feather-germs were visible under illumination. Feather-germs next to dorsal midline, particularly at lumbo-sacral level, extend slightly over surface when viewed in profile. Feather-germs on thigh protrude conspicuously. One row on inner side of each eye. None around umbilical cord. Scleral papillae-thirteen or 14. Nictitating membrane extends halfway between outer rim of eye (eyelid) and scleral papillae. The embryonic liver originated from the ventral foregut endoderm, which becomes the hepatic diverticulum, this is the first morphological sign of the embryonic liver. At the earliest stage of liver development, the proliferated diverticulum (liver bud) is observed from the ventral foregut endoderm at the 14–20 somite stage. At this stage, the liver bud is separated from the septum transversum (ST) by the basement membrane (Hamburger and Hamilton) [4].

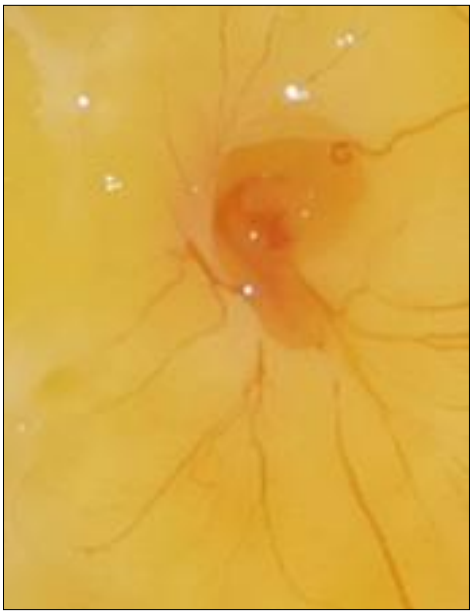

Fig 5: Fig-A, 48 hrs chick embryo W.M. showing cephalization

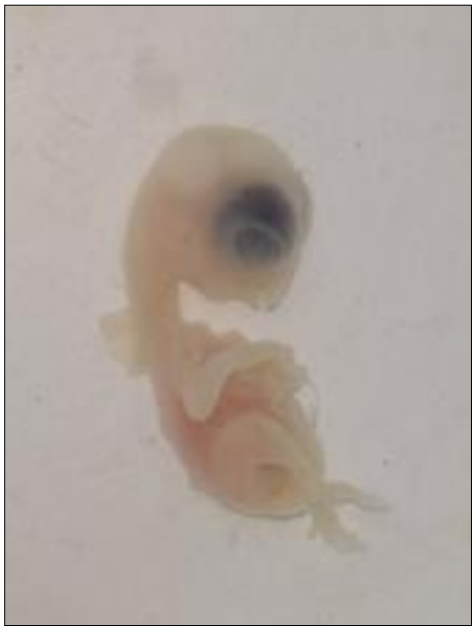

Fig 6: Fig-B, 8days (192 hrs incubation) chick Showing anatomical details

In this piece of work the effect of *Tridax* procumbens (L.) leaf extracts were studied on the characterization of mitochondria. We propose a strategy that study mitochondrial characterization using DCPIP its oxidation and reduction values under influence of *Tridax* leaf extract. This approach for analysing effect of extract on chick embryo model systems we utilized the strategy to study the SDH reduction -oxidation. The chick embryo hepatic mitochondria SDH activity studied and compared with Tridax extract treated mitochondrial activity this experimental model found to correlate with chemical properties of *Tridax procumbens* (Elena I. *et al*.) [2].

Conclusion

Our investigation reveals that in set I the controlled sample shows gradual change in absorbance from 0.532 to 0.508 and *Tridax* extract treated sample showing change in absorbance from 0.0651 to 0.635.

In set II the controlled sample shows gradual change in absorbance from 0.532 to 0.507 and *Tridax* extract treated sample showing change in absorbance from 0.0650 to 0.636. In set III the controlled sample shows gradual change in absorbance from 0.532 to 0.507 and *Tridax* extract treated sample showing change in absorbance from 0.0652 to 0.638. Set IV was exclusively designed to see the effect of *Tridax* extract in high concentration absorbance observed 2.500 constantly after every 30 seconds for 3 minutes. There was no change in absorbance noted. Therefore, we state that the high concentration of Tridax leaf extract inhibit SDH activity therefore no reduction was observed. The µ moles of DCPIP reduced/min for Set I- controlled sample is 5.833 and extract treated is 5.993, µ moles of DCPIP reduced/min for Set II- controlled sample is 5.585 and treated 6.012, µ moles of DCPIP reduced/min for Set III- controlled sample is 5.821 and treated 6.024, and for Set IV its merely 0.122 because hardly any reduction of DCPIP took place and then enzyme blocked. The Tridax leaf extract show fast wound healing effect by narrowing lumen of blood vessel. Angiogenesis playing a critical role in the growth and healing K. Pendharkar [9]. The Tridax leaf extract show fast wound healing effect by narrowing lumen of blood vessel. We delineate that the mitochondria predominantly composed of a small number of vastly abundant enzymes proteins involved in the principal processes of cellular survival and metabolism and the mitochondrial SDH are primarily dictated by its chemical environment Additionally, we determined that DCPIP, reflect the gradual diversification in the absorbance. Finally, this study provides a useful resource for future studies on mitochondrial biology and healing medicinal properties of *Tridax* extract (SRP Kethamakka *et al*.) [12].

In this study, we propose a strategy that study of mitochondrial characterization using DCPIP and its oxidation and reduction to characterize aspects of mitochondria has been the feasible approach for analysing properties of *Tridax* extract as herbal medicine in healing process by chick embryo model systems. (Yuji Y.) [21] (Yoan Arribat *et al*.) [20].

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