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Study on Teratogenic Effects of Bhavana Panjankula Thailam, using Chick Embryo as a Model System

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ABSTRACT

Teratogenicity is the ability to cause developmental anomalies in fetus. Substances with teratogenicity effects can damage the DNA of a developing foetus. They may cause abnormal development of a limb malformation of an organ and the effects for the developing fetus can vary depending on the teratogen. Chicken eggs are used as a model system to test Bhavana panjankula thailam for its Teratogenic effect. Bhavana panjankula thailam is one of the Siddha drugs indicated for karuppachudu (a type of pitha disorder affecting uterus), urinary tract infections, dry skin and constipation. In this study five concentrations of drug Bhavana panjankula thailam were injected into the chicken embryos. Cadmium is used as a positive control in the same concentrations which affects limb deformities of the organ. 1:1 ratio of cadmium and drug were injected into the embryo for scrutinize the effect of drug. At the 12th day of incubation the eggs were dissected out and detect the malformation in the embryo. The results suggested that the lowest concentrations cof drug treated embryo has no malformation and highest concentrations of drug treated embryo has malformed like heart and other organs are distorted. The DNA was isolated from embryos of chick and run in agarose gel electrophoresis to confirm the DNA damage. Coct4 and cGATA4 genes which is responsible for chick embryogenesis. GATA and OCT family of transcriptional regulatory proteins, GATA-4 and OCT-4 thought to be involved in the regulation of cardio genesis and gut development. Functions for these factors are known in the heart, but relatively little is implicit concerning their possible roles in the regulation of gut-specific gene expression. In this study, we analyzed the expression of cGATA-4, and cOCT4. Further gene expression studies will be carried out for further authentication of teratogenic effect at gene level. Though the Bhavana panjankula thailam was shown teratogenic effect at the higher dose So detailed animal studies and clinical trials should be carried out for apposite remedy.

Introduction

Every year, an estimated 7.9 million infants (6% of worldwide births) are born with serious birth defects. Although some congenital defects can be controlled and treated, an estimated 3.2 million of these children are disabled for life(1). Birth defects are one of the leading causes of infant deaths, accounting for more than 20% of all infant deaths. Although some birth defects are inherited, others are a product of harmful environmental factors known as teratogens, and environmental influences.. Things that can cause developmental abnormalities are known as teratogens, and they include things like viruses, chemicals, and radiation(2).

About eight per cent of all pregnant women need to take ongoing medication for an existing health problem. For example, chronic health conditions such as epilepsy, high blood pressure, diabetes, thyroid conditions and asthma require management with medications the medicines may induce teratogenicity(3).

Teratogenicity is the ability to cause developmental anomalies in a fetus. Substances with teratogenic effects can damage the DNA of a developing fetus. They may cause anything from abnormal development of a limb to malformation of an organ, and the effects for the developing fetus can vary depending on the teratogen, the gestational age of the fetus, and other factors. Sometimes, prenatal exposure to teratogenic substances causes the death of the fetus, while in other instances, someone may be born with relatively mild anomalies like extra fingers or toes (4&5).

Substances with known teratogenicity must be handled carefully. Pregnant women are encouraged to avoid exposure to such substances. As researchers have learned, however, sometimes the danger of a substance is not known until it is too late. Thalidomide, for example, was widely used in pregnant women until medical experts realized that it was causing developmental abnormalities(6).

Siddha System

The Siddha system is a treasure-house of secret science, embodying the results of the ordent pursuit there of by the ancient Siddhars. The contribution of Siddhar to Siddha literature with its boundless therapeutics and wonderful pharmaceutical preparation of medicine is acclaimed par excellence (pre-eminent) even in this 20th century and worthy of its remarkable results(7).

Siddha medicine revitalizes and rejuvenates dysfunctional organs that cause the disease and to maintain the ratio of *Vaadham*, *Pitham* and *Kabam*. The equilibrium of humours is considered as health and its disturbance or imbalance leads to a diseased state. So proper diet, medicine and a disciplined regimen of life are advised for a healthy living and to restore equilibrium of humors in diseased condition.

The Siddha treatment deals not only as a curative but also as a preservative, taking care of the external body with its internal being - soul. We don't know about siddha drug fully for the any side effects, so analyse the siddha medicine to confirm the safety. Teratogecity is the one assay to identify the drugs which

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have the any defects to fetus in pregnancy period of humans. so there is a need for an hour to perform teratogenicity study of the drug which will be more efficient to our society(8).

Bhavana panjankula thailam

Bhavana panjankula thailam is one of the siddha drug indicated for karuppachudu (a type of pitha disorder affecting uterus) urinary tract infection, drug skin and constipation. Bhavana panjankula thailam give to pregnant women.Bhavana panjankula thailam (SKM siddha and ayurvedha) is one of the Siddha drugs indicated for karuppachudu (a type of pitha disorder affecting uterus), urinary tract infections, dry skin and constipation. Bhavana panjankula thailam given to pregnant women.

Dosage and Adjuvant

2 to 5ml with milk or ghee twice a day after food or directed by the physician.

Indication

Ganachudu (A type of Pitha disorder), Karuppachudu (A type of Pitha disorder affecting Uterus), Siruneerpathai Thabitham (Urinary tract infections), SariraVaratchi (Dry Skin), MarunthukalinUshnam (Body heat due to drugs), Malachikkal (Constipation). Advised for Pregnant ladies from the first month to the ninth month completion till delivery with dose of 5 ml. in the early morning empty stomach and 2.5 ml. at bed time by mixing with sugar candy which results in normal delivery and Child with good Physical and mental health.

Materials and Methods

Study of Teratogenic activity

Materials for teratogenic activity on fertile chick egg Drug

Bhavana panjankula thailam (SKM siddha and ayurvedha)

Equipments:

1. Laminar flow hood
2. Automatic Egg incubator
3. Autoclave
4. Freezer
5. Shaker

Reagents and Chemicals

1. PBS
2. Cadmium
3. Ethanol
4. distilled water

Glasswares

1. Petriplates
2. Conical flask (250 ml)
3. Test tubes
4. Beaker (250 ml)

Dissection Tools

1. Scissor
2. Forceps
3. Needle
4. Seal Tape
5. Dissection blade.

Method for Teratogenic activity

Collection of Eggs

Brown leghorn eggs were collected from government poultry station, potheri, Chennai. They were wiped with ethanol and kept for overnight incubation at temperature of 37 ºC in an egg incubator.

Treatment with Drug

1. Zeroth day Fertile white leghorn chicken eggs were obtained from poultry and wiped with ethanol and was stored at 6ºC.
2. These eggs were separated into 4 groups
3. Group-1 as control untreated egg
4. Group-2 as positive control induced with cadmium
5. Group-3 treated with Bhavana panjankula thailam in various concentrations(0.5µg/ml, 50 µg/ml, 250 µg/ml and 500 µg/ml)
6. Group-4 treated with 1:1 ratio of Bhavana panjankula thailam and Cadmium
7. The eggs were then transferred and maintained in a forced draft egg incubator at 37.5°C with relative humidity of 46%.
8. After incubation period, eggs were sacrificed and observed for its malformations. Photographs were taken.

Isolation of DNA

Materials for isolation of DNA

Equipments

1. Laminar flow hood
2. Freezer
3. Incubator
4. Shake
5. Centrifuge

Reagents and chemicals:

1. PBS
1. Sodium Chloride
2. Potassium Chloride
3. Disodium Hydrogen Phosphate
4. Potassium Dihydrogen Phosphate

2. Phenol
3. Chloroform
4. Isoamyl alcohol
5. DNA Digestion Buffer
   - Tris HCL (50mM)
   - EDTA (10mM)
   - NaCl (100mM)
   - SDS (1%)
6. Proteinase K (0.5mg/ml in DNA Digestion Buffer)
7. 100% Ethanol
8. 70% Ethanol
9. TE Buffer
   - TrisHCL (10mM)
   - EDTA (1mM)

Glasswares

1. Conical flask
2. Beaker

Method for isolation of DNA

Isolation of tissue from Chick Embryo

1g of tissue was collected from the limb part of the chick embryo. It was then homogenized using Pestle and Mortar. The homogenized tissue was collected and stored in PBS.

Isolation of DNA from the tissue sample

Procedure:

Quantification of DNA

1. 0.1g tissue (grinds well using homogenizer/mortar and pestle/frozen with liquid Nitrogen)
2. Add 0.5ml of DNA Digestion buffer with Proteinase K (can be stored at 4 C for several days)
3. Incubate overnight at 50 C-55 C with gentle shaking (if overnight cannot be done 1-2 hrs
Incubation/overnight for best results)
4. Spin tubes for 5 seconds at 500 rpm to collect mix in bottom of tube.
5. Add 0.7ml of P: C: I (phenol: chloroform: is amyl alcohol)
6. Mix by inversion for 1 hour (do not vortex).
7. Centrifuge at 10000 rpm for 5 mins and transfer 0.5 ml of upper phase to new tube.
8. Add 1ml of 100% ethanol at Room temperature and gently invert until DNA Precipitate.
9. Centrifuge at 1200 rpm for 5 mins and discard supernant.
10. Add 1ml of 70% ice cold ethanol and invert several times.

11. Centrifuge at1000 rpm for5 mins and discard supernatant
12. Air dry at room temperature for 10-15 mins
13. Re-suspend in 100µl TE buffer and incubate at 65c for 15 mins to dissolve DNA.

Materials Required

Equipments

Nanodrop 2000 – ThermoScientific

Materials:

Micropipette – eppendroff
Microtips - Tarsons
Tissue paper

Method of quantifying DNA

DNA was quantified using Nanodrop 2000.

AGAROSE GEL ELECTROPHORESIS

Materials Required

Equipments

Electrophoresis Power Pack - Genie
Electrophoresis Gel tank - Genie

Reagents and Chemicals

1. Tris Borate EDTA (TBE Buffer) (1X) - Merk
2. Agarose (1.2%)
3. Bromophenol Blue
4. Ethidium Bromide

Glasswares

Beaker
Conical flask

Method for Agarose gel Electrophoresis

Procedure

1. Agarose prepared by 1X TBE buffer (1.2%) (0.48 g in 60ml d.h20).
2. 8µl of EtBr (for 60 ml) added in agarose.
3. Agarose was heated in oven and pour into mold, the comb was fixed.
4. After that solidification of agar the DNA sample was loaded (2ul tracking dye and 10 ul DNA)
5. Pour 0.5 X TBE buffer casted to wells
6. The electrode was set and 100 volts run the gel
7. After 2 hrs remove the gel and observe under UV illuminator for DNA damage.

Polymearse Chain Reseation

Materials required

Equipments

PCR- G-Storm
Autoclave

Chemicals and reagents

1. Master Mix
   PCR Water-Genei
   PCR Buffer
   Magnesium Chloride - Merk
   dNTP - Invitrogen
   Taq DNA Polymerase-Biolabs
2. Primers
   cGATA4 – Sigma
   cOCT4 – Sigma

Materials

1. PCR tubes- Sorenson
2. Microtips - Tarsons
3. Micropipette – eppendorff research

Method for PCR

1. Add 10µl of master mix
2. Add Forward primer 1µl
3. Add Reverse primer 1µl
4. Add DNA 3µl
5. Add PCR Water 5µl.

Three steps of PCR:

Denaturation - 95ºC for 2 minutes.
Annealing - 55ºC for 59 seconds
Extension - 68-72ºC for 45 seconds

PCR run for 40 cycles. Thus the DNA was amplified and agarose gel electrophoresis was carried out as above procedure.

Result and Discussion

Evaluate tertogenic effect using five concentration (50,100,250, 100 µg/ml(1;1) of BPT form the stock (10mg).cadmium used as positive control & untreated eggs used as negative vs control.

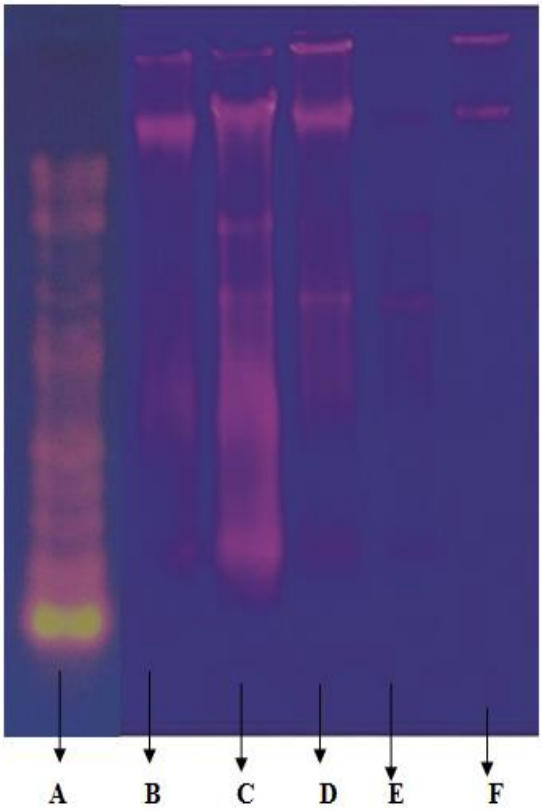
In this study 50 µg/ml concentration shows a good growth of embryo but in 250 µg/ml hear part oozing out and 50,100µg/ml concentration shows deformaties like strunded growth was observed. The positive control of cadmium result in 250µg/ml concentration internal organ was damage and 500µ/ml concentration malformation where observed like limb deformaties and one eye was not there in embryo .the negative control untreated egg shows embryo was observed the normal there was no deformaties.

Results and Discussion

Results of teratogenic effect of Bhavana panjankula thailam

Result Agarose Gel Electrophoresis

This of isolated various concentration of DNA was run in Agarose gel electrophoresis (1:1) ladder used for easy to identity DNA withbase pair .In the control have normal DNA band and cadmium shows heavy DNA forgementation .In 50 µg/ml concentration of DNA mild DNA fregamation was seen but it better than 50µg /ml concentration 'In100µg/ml(1:1) concentration shows verymild nucleotide feagementation was seen. So in this concentration drug was reading cadmium effect.



Well A-Ladder,
Well B-control
Well C-Lithium
Well D-50BP
Well E -250BP
Well F -100 BP (1:1)

Composition of Bhavana panjankula thailam

S.no	Siddha name	Scientific name
1	Amanakku vithai	Recinus communis
2	Chottrukattralai	Aloe barbadnesis
3	Seuilaneer	Cocosnucifera

Table 1. Group 1 Negative control

Concentration	Observation	Inference
Untreated egg	 Fig 3	Embryo growth was normal and there was no deformities observed.

Table 2. Group 2 Cadmium treated eggs

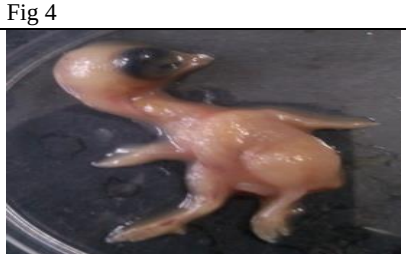
Concentration	Observation	Inference
0.5µg/ml	 Fig 4	Growth was normal and this indicates that at low concentration of cadmium,there was no malformations
50 µg/ml	 Fig 5	Embryo growth was normal and there was no malformations.
250 µg/ml	 Fig 6	*Internal organs were damaged. Hence, malformations observed.*
500 µg/ml	 Fig 7	Malformations were observed. (i)Limb Deformities (ii) One eye was not there in embryo.

Table 3 .Group 4 Bhavana panjankula thailam

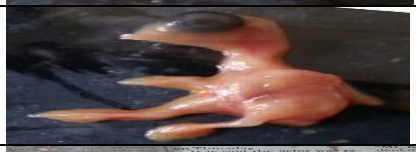
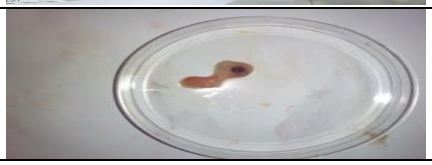
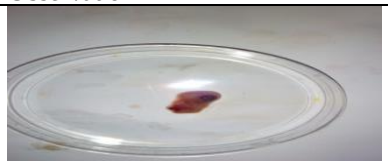
Concentration	Observation	Inference
0.5µg/ml		Normal growth was observed.
50µg/ml		Normal growth was observed. There was no deformities.
250µg/ml		Malformation was observed. Heart part of the embryo came out.
500µg/ml		Deformities like stunted growth was observed.

Table 4. Group 5 Cadmium + Bhavana panjankula thailam

Concentration	Observation	Inference
100µg/ml (1:1)		Deformities like stunted growth was observed.

Result of Quantification of DNA

S.No	Sample	Nucleic acid Concentration (µg/ml)
1	Control	448
2	Bhavana panjankula thailam (50ug/ml)	823.2
3	Bhavana panjankula thailam (250 ug/ml)	417.9
4	Bhavana panjankula thailam + cadmium (100 ug/ml)	284.9
5	Cadmium	1243.1

Table 7. Interpretation

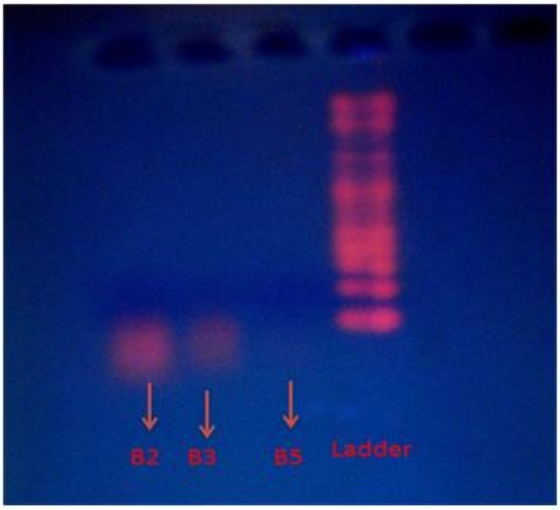
S.No	Sample	Interpretation
1	DNA Ladder (0.1- 10 kbp)	This is marker which observe various size of DNA.
2	Control	There is no DNA fragmentation observed in control which means (non induced) without treatment of drug.
3	Cadmium	DNA fragmentation and DNA damage is seen in cadmium teated band caused by cadmium.
4	bhavana panjankula thailam (50 ug/ml)	Mild DNA fragmentation is observed with thick dissorted band.
5	bhavana panjankula thailam (250 ug/ml)	DNA fragmentation is seen.but damage is better than 50µg of treated one.
6	bhavana panjankula thailam + cadmium (100ug/ml)	There is very mild DNA fragmentation is observed. because may be Bhavana panjankula thailam has reduced the DNA damage which caused by cadmium.

Table 8 . Interpretation

S.No	Sample	Interpretation
1	cGATA4 (Bhavana panjankula thailam 50ug/ml)	There is no traces of DNA.
2	cGATA4 (Bhavana panjankula thailam 250ug/ml)	There is no traces of DNA.
3	cGATA4 (Bhavana panjankula thailam+cadmium 100ug/ml)	There is no traces of DNA observed Cadmium damages the DNA
4	DNA Ladder	This is marker which observed various size of DNA
5	cOCT4 (Bhavana panjankula thailam 50ug/ml)	There is no traces of DNA
6	cOCT4 (Bhavana panjankula thailam 250ug/ml)	No traces of DNA found
7	cOCT4 (Bhavana panjankula thailam+ cadmium 100ug/ml)	DNA was not found in this gene. Cadmium damages the DNA

Result of PCR

Fig : c - GATA- 4 primer



Discussion

The teratogenic effect of BPT has shows in the table3&4 along with the chick embryo(9) .No malformation was observed in 0.5, 50µg/ml concentration & 250,500,100(1:1)µg/ml concentration of drug have deformaties .In 250µg/ml concentration heart part was oozing out and 500,100(1:1)µg/ml concentration have stund deded growth .the teratogenic effect of postoive control cadmium was observed at two concentration (250/500µg/ml). the result of molecular level onbservation where shows in table no7&8 there DNA fregamation or damage was observed in drug treated as well as cadmium treated chick embryo .The level of DNA Damage was more in cadmium and lovest DNA(10). Fregamnation compare than the concentration future the mechanism of activityof the drug analised by gene expersion study .the stadaration of protpol of the and analysis in PCR was bening.this may be the standaration future propective of the present study of get intesting result about of teratogenic effect ofBPT.

The further detail analysis of gene expersison as well as molecular mechanism study of drug will be evavluated as a future study.

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