CHAPTER-2
MATERIALS AND METHODS

Chemicals

Trichloroacetic acid (TCA), Thiobarbituric acid, Ammonia solution, Phenolic compound, 4-Aminoantipyrine, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), Methanol, Potassium phosphate, Ethylenediaminetetraacetic acid (EDTA), Dithiothreitol (DTT), Trisaminomethane hydrochloride (TrisHcl), Pyrogallol, Polyvinylpyrolidone (PVP), Hydrogen peroxide (H_2O_2), Gamma tocopherol, Hexane, Petroleum ether, Potassium hydroxide, Phenolphthalein, Hydrochloric acid, Glacial acid, Chloroform, Potassium iodide, Sodium thiosulphate, Starch, Hanus Iodide solution, Formaldehyde, Acetic acid, Ethanol, Tertiary – butanol, Paraffin wax, Sudan Black B, Xylene, Alcohol, Sodium bicarbonate (Na_2CO_3), Acrylamide, Bis-acrylamide, Sodium dodecyl sulfate (SDS), Ammonium persulphate (APS), N,N,N′,N′- Tetramethylethylenediamine (TEMED), Tris HCl, Glycine, Bromophenol blue, 2-Mercaptoethanol, Glycerol, Glacial acetic acid, Cetyl Trimethyl Ammonium Bromide, Ethidium bromide, Agarose, 4, 6-Diamidino-2-phenylindole (DAPI). All the reagents were procured either from Sisco research laboratories (SRL) or HiMedia or Sigma. All the reagents were of analytical grade.

Seeds

Jatropha curcas L. fresh seeds were collected from in and around the area of Dahod, Gujarat, India. Collected seeds were dried under the sunlight for 48 hours to remove the initial moisture content. The seed lots were divided into three different groups. The fresh seeds were treated as control. Others were packed in polythene bags for natural aging (NA) or storage for 24 months. Naturally aged seeds of one month (NA1m), three months (NA3m), six months (NA6m), nine months (NA9m), twelve months (NA12m), fifteen months (NA15m), eighteen months (NA18m), twenty one months (NA21m) and twenty four months (NA24m) were used for the analysis. Third group of seed lots were subjected for aging treatment.

Aging treatment

Aging treatment for artificially aging the seeds comprises of accelerated aging and saturated salt accelerated aging (Powell and Mathews, 1984). Seeds were exposed

to controlled temperature (42°C - 46°C) and high moisture (80-100%) for accelerated aging (AA) and for saturated salt accelerated aging (SSAA), seeds were exposed to temperature (42°C - 46°C) and saturated salt (40%) to control the moisture content (Powell and Mathews, 1984 and Mendonca et al., 2008). Seeds were subjected to both types of artificial aging treatments for varying intervals such as 12hours, 1day, 2days, 3days, 4days, 5days, 7days, 10days, 12days and 15days and these seed lots were designated as AA12h, AA1d, AA2d, AA3d, AA4d, AA5d, AA7d, AA10d, AA12d and AA15d for accelerated aging and SSAA12h, SSAA1d, SSAA2d, SSAA3d, SSAA4d, SSAA5d, SSAA7d, SSAA10d, SSAA12d and SSAA15d for saturated salt accelerated aging.

Lipid peroxidation

Lipid peroxidation was determined by estimation of the Malondialdehyde (MDA) content following Heath and Packer method (1968) with slight modification. Plant material (0.5 g) was homogenized in 5 ml of 5% trichloroacetic acid (TCA) at 4°C to precipitate proteins. The homogenate was centrifuged at 14,000 rpm for 20 min. MDA was determined by adding 0.8 mL of 20% (w/v) trichloroacetic acid in 0.5% (w/v) thiobarbituric acid (TBA) to 0.2 mL of extracted sample (supernatant) and 3.0 mL of distilled water. The reaction was carried out at 95°C for 30 min and then terminated by soaking in ice-cold water and the absorbance of the supernatant was taken at 532 nm and 600 nm. The nonspecific absorbance at 600 nm was subtracted from the absorbance at 532 nm. The concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Electrical conductivity

Electrical conductivity assay was performed according to Halder and Gupta (1980) with a slight modification. Twenty seeds from each lot were soaked in 50 mL deionized water and kept for 24 hours and the electrical conductivity of the seed leachates was recorded using a direct reading conductivity meter. The conductivity was expressed as ms/cm.

Estimation of hydrogen peroxide (H_2O_2)

H_2O_2 extraction and estimation from seeds of *Jatropha curcas* was carried out as described by Zhou et al., (2006). Using liquid nitrogen, fresh seeds of 0.5g were frozen and ground to powder in a mortar and pestle, along with 5 ml of 5%

Trichloroacetic acid (TCA) and 0.15 g of activated charcoal. The mixture was centrifuged at 12000g for 20 minutes at 4ºC. Supernatant taken was adjusted to pH 8.4 with 17 M ammonia solution before subjecting to filtration. Reaction mixture contained 1ml filtrate and 1ml colorimetric regent (Phenolic compound and 4-aminoantipyrine) and incubated at 30ºC for 10min. In a similar way, 1 mL of distilled water was used as a blank instead of filtrate. The activity was monitored at 505nm. (Aebi et al., 1974 and Patterson et al., 1984)

H_2O_2 + Phenolic compound + 4-aminoantipyrine

↓ peroxidase

Pink coloured compound + H_2O

Germination assays

Germination assays were performed according to Kibinza et al., (2006), at normal room temperature of 20°C to 25°C on the three replicates of 40 seeds placed in petri dishes on a layer of adsorbent cotton wool moistened with distilled water. Germination counts were made after 7days. Germination was scored as the emergence of radicle from the covering structures. Radicle length was counted after seven days of germination. Results were presented correspond to the means of the germination percentages and radicle length obtained after 7 days of germination.

Determination of free radical scavenging activity

This consists of preparation of seed extract using methanol as solvent and subjecting the seed extract to 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay to estimate the free radical scavenging capacity (Larrauri et al., 1998).

Preparation of seed extract by methanol

The *Jatropha curcas* seeds were crushed and a crude extract was prepared with methanol as solvent. 4 g of the fresh seeds were macerated in about 200 mL of solvent and subjected to extraction for 24 hours. The extracts were filtered by Whatman filter paper. The solvent was completely removed by rotary evaporator and further removal of water was carried out by freeze drying. The dry extracts were weighed, respectively, stored in clean sample bottles in freezer at -20°C. This was further used for DPPH radical-scavenging activity.

Determination of DPPH radical-scavenging activity

In our study 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was used to estimate the total DPPH free radical-scavenging activity of the methanol extract of *Jatropha curcas* seeds. Scavenging activity of seed extract against DPPH radicals was assessed according to the method of Larrauri et al., 1998. 1ml of methanol extract (mg^{-1}/ml^{-1}) of *Jatropha curcas* seeds was mixed with 1 mL of 0.1mM DPPH methanol solution. After the solution was incubated for 30 min at 25º C in dark, the decrease in the absorbance at 517 nm was measured. Control contained methanol instead of antioxidant solution while blanks contained methanol instead of DPPH solution in the experiment. Ascorbic acid was used as positive control. The inhibition of DPPH radicals by the samples was calculated according to the following equation:

DPPH-scavenging effect (%) = [(A0- A1) / A0 x 100]

Where, A0 is the absorbance of control with methanol and A1 is the absorbance of sample.

Quantitative estimation of antioxidant enzymes

Activity of Superoxide dismutase (SOD)

For SOD activity assay, known amount of plant seed tissue (0.5 g) was homogenized in 3-5 ml buffer pH 7.0, composed of 50 mM potassium phosphate, 0.1 mM EDTA and 1 mM Dithiothreitol (DTT) according to Dixit et al., (2001). Homogenate was filtered through nylon net and centrifuged (12,000 g for 10 minutes). The supernatant was used to determine SOD activity. 200 µL supernatant was added in a quartz cuvette containing 800 µL of 75 mM trisaminomethane hydrochloride (TrisHcl) buffer, pH 8.0 followed by mg^{-1}/ml^{-1} of pyrogallol (8 mM). Auto-oxidation of pyrogallol was monitored by recording the absorbance at 420 nm for 3 minutes. Specific activity of SOD was estimated by units of enzyme required for the 50% inhibition of auto-oxidation of pyrogallol mg^{-1} protein (Marklund and Marklund, 1974). Protein concentration was determined using Folin Lowry method.

Activity of Catalase (CAT)

0.5 g of seed was taken and homogenized in 50 mM potassium phosphate buffer (pH 7.2) containing 2% polyvinylpyrolidone (PVP) at 4°C. Homogenate was filtered with nylon net and centrifuged (12,000 g for 10 minute). Supernatant was taken for catalase activity. 100 µL of 500 mM of hydrogen peroxide (H_2O_2) solution was

taken. 50 mM (800ul) potassium phosphate buffer pH 7.8 was used as assay solution. 100 µL enzyme extract was added. The absorbance drop of H_2O_2 at 240 nm (ε240 = 43.6 mM-1 cm-1) was observed for 120 second. One unit of catalase activity was defined as the µmoles of H_2O_2 decomposed per min (Aebi, 1984). Catalase specific activity was expressed as µmoles of H_2O_2 degraded min^{-1} mg^{-1} protein. Protein concentration was determined using Folin Lowry method.

Activity of Peroxidase (POX)

For peroxidase activity assays, seed tissue from abscission zone region (0.5 g) was homogenized in 50 mM potassium phosphate buffer (pH 7.2) containing 2% PVP at 4ºC. Homogenate was filtered through nylon net and centrifuged (12,000 g for 10 minutes). The supernatant was used to determine peroxidase activity. 100 µL of 5% pyrogallol solution and 50 µL of 10 mM H_2O_2 solution in 750 µL of 100 mM potassium phosphate buffer, pH 6.0 was used as assay solution and 100 µL enzyme extract was added. Peroxidase activity was assayed by monitoring the degradation of pyrogallol into purpurogallin at 420 nm in 20 seconds mg^{-1} protein at 20ºC (Chance and Maehly, 1955). Protein concentration was determined using Folin Lowry method.

Determination of the gamma tocopherol content

Tocopherol present in oil extracted from control seeds and aged seeds were analyzed using high performance liquid chromatography (HPLC). The method and protocol prescribed by Cerchiara et al., (2010) was slightly modified and used. Gamma tocopherol was estimated using column C18 with mobile flow rate at 1.2 mL/min for 30 minutes. The mobile phase contained the mixture of methanol: water with the ratio of 96:4, v: v. *J. curcas* seed oil (100 µl = 0.1 g) was solubilized in 50 µl of hexane. The sample was then filtered and 20 µl of this filtered sample was injected into the system. Identification and quantification were done by comparison with the retention times and areas taken by the gamma tocopherol standard. The standard of gamma tocopherol was obtained from Sigma.

Extraction of *J. curcas* oil

The oil content (dry basis) of the seeds was determined by Soxhlet extraction method. 50 g of the seeds was milled to 1- mm particle size using standard methods (ASTM E11 2003) from each group of seed lots. The oil was extracted in using a Soxhlet extractor with petroleum ether as solvent and the milled seeds were wrapped in

extraction thimbles. The solvent to solid ratio was 7:1 and the extraction was carried out for 8 hours at a temperature of 75°C. The petroleum ether in the solvent-oil mixture was evaporated later. The oil was then collected and weighed. The percentage oil yield was calculated using the following expression:

$$Y = [M_o/M_m]*100$$

where, Y is the oil yield (%), M_o is the weight of oil expressed (g) and M_m is the weight of the sub-sample of milled *Jatropha curcas* L. seeds.

Qualitative analysis of the oil

Different biochemical tests such as Iodine Value (IV), Saponification Value (SV), Peroxide Value (PV), Acid Value (AV), and Percentage of Free Fatty Acids (%FFA) were carried out as per standard methods and protocols prescribed by Cox and Pearson (1962); AOAC (1975); Thimmaiah (2006).

Determination of Acid Value (AV):-

0.1 g of oil sample was dissolved in 10 ml of neutral solvent. The mixture was titrated using 0.1N potassium hydroxide (KOH) solution after an addition of few (2-3) drops of 1% phenolphthalein indicator. The values were substituted in the following formula to estimate the acid value:

$$\text{Acid Value (mg KOH / g)} = (A \times 0.1 \times 56.1) / W$$

where, A = Titrated value (ml), 0.1 = Normality of KOH, W = Weight of sample (g) and 56.1 g/mol is the molecular weight of KOH.

Determination of Free Fatty Acids (FFA):-

Percentage of free fatty acids (%FA) was determined using following equation:

$$\% FA = K \times \text{Acid Value (AV)}$$

where, K = Constant (0.503).

Determination of saponification value (SV): -

0.5 g of sample was taken and dissolved in 12.5 ml of 0.5N alcoholic KOH solution. Using air condenser attached to the flask, the sample was boiled gently for about 30 minutes, which was then be cooled at room temperature. It was then titrated with 0.5N hydrochloric acid (HCl) with 1% phenolphthalein indicator. Besides, a blank

was also run to have precise comparison among duplicates and the mean results were recorded.

Saponification Value (SV) (mg KOH) = (A x 28.06 / W)

where, A= amount of HCL (ml) (Blank-Titrate value), W = weight of Sample (g).

Determination of Peroxide Value: -

1g of oil sample was taken and dissolved in 15ml of solvent mixture (2:1, glacial acid: chloroform). The tube was then placed in boiling water for 30 seconds to remove the liquid boils. This content was then transferred quickly to a conical flask containing 20ml of 5% potassium iodide solution. The mixture was titrated against 0.05N sodium thiosulphate, using starch as indicator. A blank was also set at the same time.

Peroxide Value (meq/kg sample) = (A x N x1000/W)

where A=Amount of $Na_2S_2O_3$ (ml), N=Normality of $Na_2S_2O_3$, W=Weight of sample (g).

Determination of Iodine Value: -

0.5 g of oil sample was taken and dissolved in 10 ml of chloroform. 12.5 ml of Hanus Iodide solution was added to this mixture and was kept in dark for 30 minutes. Later, 15 ml of KI solution was added. The mixture was then titrated with 0.1N sodium thiosulfate ($Na_2S_2O_3$) solution until yellow solution turns almost colorless. Few drops of 1% starch solution (indicator) was added and titrated until the blue color completely disappears. A blank was set at the same time.

Iodine Value (IV) = [(A x N x 0.1269 x 100) / W] g I_2 / 100 g of Oil

where, A = Amount of $Na_2S_2O_3$ (Blank-Titer), N = Normality of $Na_2S_2O_3$, W = Weight of sample (g).

Fatty Acid analysis of oil by Gas Chromatography

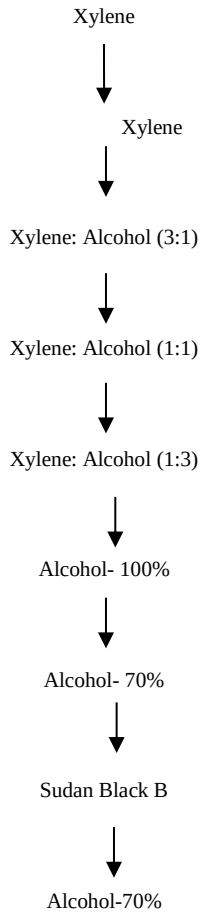
1-30 mg of oil was taken and added small volume of 0.4N sodium methylate. Cover of the mouth of the tube was sealed with aluminum foil and heated in water bath at 65˚C and later vortexed. Appearance of homogenous solution indicated the composition of esterification. An aliquot (1-2 µl) was injected into preconditioned GC in which the maintained temperature of injection port is 280˚C and column temperature 165˚C. After all the components of the sample have come out of the column, the

standard methyl esters were injected separately and noted the retention time of esters of different FAs. GC is equipped with SS packed column and flame ionization detector. The individual peaks of the samples were identified by comparing their relative position or retention time with the standards. GC automatically calculated the peak area of each acid sample and compared with that of standards (Kates 1986).

Oil bodies staining by Sudan Black B

Paraffin sections of *J. curcas* seeds were taken. The seeds were fixed in FAA (formaldehyde-acetic acid- ethanol) for 48 hours. After several rinsing with distilled water, each fixed sample was dehydrated with a graded series of tertiary - butanol (TBA) from 10 to 100%. After dehydration, the sample was soaked in paraffin wax at 60˚ C overnight and finally embedded in paraffin wax at room temperature. Sections of 20µm were sliced by microtome and layered on glass slide (Ruzin, 1999).

Staining by Sudan Black B - The staining procedure is described by Krishnamurty (1988). The slides were dipped in the solvents mentioned below for 2 minutes each and in Sudan black for 10 minutes.



↓

Distilled water

↓

Mount with glycerol and observe under 100X.

Extraction and isolation of oil body protein Oleosins

Oleosins extraction was carried out according to Popluechai *et al*., 2010. 0.5 g of *Jatropha curcas* seed was ground in 5ml of 50 mM Na_2CO_3 pH 9. Aliquots of 200µl of this mixture were dispensed in 2 ml micro centrifuge tubes and added 9 volumes (1800µl) of chloroform/methanol (11/7) mix was added to it. The mixture was then vortexed and sonicated for 30 seconds in water bath sonicator and spun at 20,000g for 15min at 20˚C. The organic solvent was transferred to a new tube and evaporated under nitrogen for 1hour. The protein pellet was analyzed or kept at -20 ˚C until further use. Protein concentration was estimated by Lowry's method. Protein fractions (solvent extracted proteins) were separated on 15% polyacrylamide gel by SDS-PAGE.

Extraction and isolation of genomic DNA from *J. curcas* seeds

DNA was extracted from control and aged seeds as per the CTAB (Cetyl Trimethyl Ammonium Bromide) method of Doyle and Doyle (1990) using kit method.

Disrupt samples (≤100 mg wet weight or ≤20 mg lyophilized tissue) using the Tissue Ruptor, the Tissue Lyser II or a mortar and pestle. Add 400 µl lysis buffer and 4 µl RNase A. Vortex and incubate for 10 min at 65°C. Invert the tube 2–3 times during incubation. Note: Do not mix buffer AP1 and RNase A before use. Add 130 µl neutralization buffer. Mix and incubate for 5 min on ice. Recommended: Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm). Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20,000 x g. Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of wash buffer and mix by pipetting. Transfer 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at ≥6000 x g (≥8000 rpm). Discard the flow-through. Repeat this step with the remaining sample. Place the spin column into a new 2 ml collection tube. Add 500 µl wash buffer and centrifuge for 1 min at ≥6000 x g. Discard the flow through. Add another 500 µl wash buffer. Centrifuge for 2 min at 20,000 x g. Note: Remove the spin column from the collection tube carefully so that the column does not come into contact with the

flow-through. Transfer the spin column to a new 1.5 ml or 2 ml micro centrifuge tube. Add 100 µl elution buffer. Incubate for 5 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g. Collect the eluted sample as extracted DNA. DNA sample is further to electrophersis with 1% agarose gel made with TAE buffer and load the sample with bromophenol blue dye.

DAPI staining

Transverse hand-cut sections of embryonic axes were incubated in phosphate buffer (PBS) for 5 min at room temperature and an additional 10 min in 4, 6-diamidino-2-phenylindole (DAPI) with (10 mg/ml – stock solution and 0.5µg/mL – as working solution) before a final step of washing with PBS. Stained cells present in PBS were observed under fluorescence microscope (El-Maarouf-Bouteau et al., 2011).