CHAPTER – 5

ALTERATIONS IN ANTIOXIDANT PROFILE IN *JATROPHA CURCAS* L. SEEDS UPON AGING

5.1. INTRODUCTION

Seed deterioration in the form of either depletion of seed quality or seed death is the major concern in seed aging especially during long term storage. Seeds, especially the orthodox ones are equipped with several mechanism for resistance against the unfavourable storage conditions. This facilitates the longevity of the seed and rejuvenation of it from the detrimental effects of free radicals produced during aging (Arc et al., 2011). Among the many purposes of storage, breaking of dormancy and germplasm preservation dominate seed storage while keeping the metabolic activities low and retaining the ability to germinate for substantial periods during storage (Buitink and Leprince, 2008). Seed drying is imperative prior to seed storage for the maintenance of moisture content well within the needed amount to prevent seed deterioration. During long term storage low moisture content is a highly desirable trait and acts as a restraining factor to inhibit biochemical processes in order to limit cellular damage for fostering seed viability and vigour (Arc et al., 2011). On the other hand, low moisture content is also primarily responsible for auto-oxidation which is inevitable especially for the orthodox seeds leading to the formation of free radicals and reactive oxygen species in organelles of the cells within seeds (McDonald, 1999; Kibinza et al., 2006).

There are two pools of antioxidants, the enzymatic and non-enzymatic antioxidants. They play a crucial role in protecting the macromolecules and the membrane in dry seeds during long term storage. Low molecular weight non-enzymatic anti-oxidants such as ascorbate, Vitamin E, glutathione, phenolic compounds and tocopherol have the ability to scavenge ROS and free radicals directly (Fotouo-M et al., 2018). Superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione-S-transferase (GST), and glutathione reductase (GSR) are the enzymatic antioxidants that maintain a perfect balance between free radicals produced and antioxidants in the aging seeds. Previous

studies support the fact that there is a decline in seed viability and germination and increased level of MDA, electrolyte leakage and hydrogen peroxide content associated with the dwindling activity of both enzymatic and non-enzymatic antioxidant molecules in seeds kept for long term storage (Tommasi et al., 2006; Pukacka and Ratajczak, 2005; Choudhury and Mandi, 2012).

5.1.1. Oxidative stress and oxidative damage

Superoxide anion (O_2*), hydroxyl radical (OH*), singlet oxygen radical (O_2^{-1}), nitric oxide radical (NO*), peroxy radical (ROO*) and lipid peroxy radicals (LOO*) are the major ROS that build up the oxidative stress by enhancing the concentration of each reactive species. Free radicals induced cell toxicity actually stems from the generation of superoxide anion or singlet oxygen radical. This becomes a platform for the production of other free radicals such as ROS and reactive nitrogen species (RON) (Ighodaro and Akinloye, 2017). With respect to seed storage, especially for oleaginous seeds lipid peroxidation because of the accumulation of hydrogen peroxide is the main source of reactive oxygen species. Unfavourable condition and prolonged time period of storage can strongly support the lipid peroxidation in oil rich seeds.

5.1.2. Generation of oxygen radicals

Oxygen is susceptible to attack by free radicals and is subsequently reduced to superoxide anion and singlet oxygen. Either through endogenous processes or chain reactions the superoxide anion (O_2*) is produced. This species of reactive oxygen can directly form hydroxyl radicals: $O_2^* + 2e^- \rightarrow OH^*$. By the action of superoxide dismutase (SOD) there is a formation H_2O_2 from OH*: $OH^* \rightarrow H_2O_2 + e^-$. OH* is extremely harmful in bringing about the cell toxicity (Devasagayam et al., 2004; Lobo et al., 2010). OH* and O_2* directly targets the lipids on the membrane forming lipid radicals (L*). These lipid radicals combining with oxygen forms lipid peroxy radical (LOO*) (Figure 5.1). This is the initiator molecule of the most devastating process of lipid peroxidation targeting the lipid membrane of the cells. Lipid peroxidation culminates in serious consequences such as loss of structural and functional integrity of the cell manifested as altered membrane permeability (Cheeseman, 1993), dysfunctionality of proteins at the membrane (Farooqui et al., 1998), decreased activity

of antioxidant enzymes and membrane bound enzymes (Wills, 1971) and increased membrane rigidity thus decreasing the membrane fluidity (Byung et al., 1992).

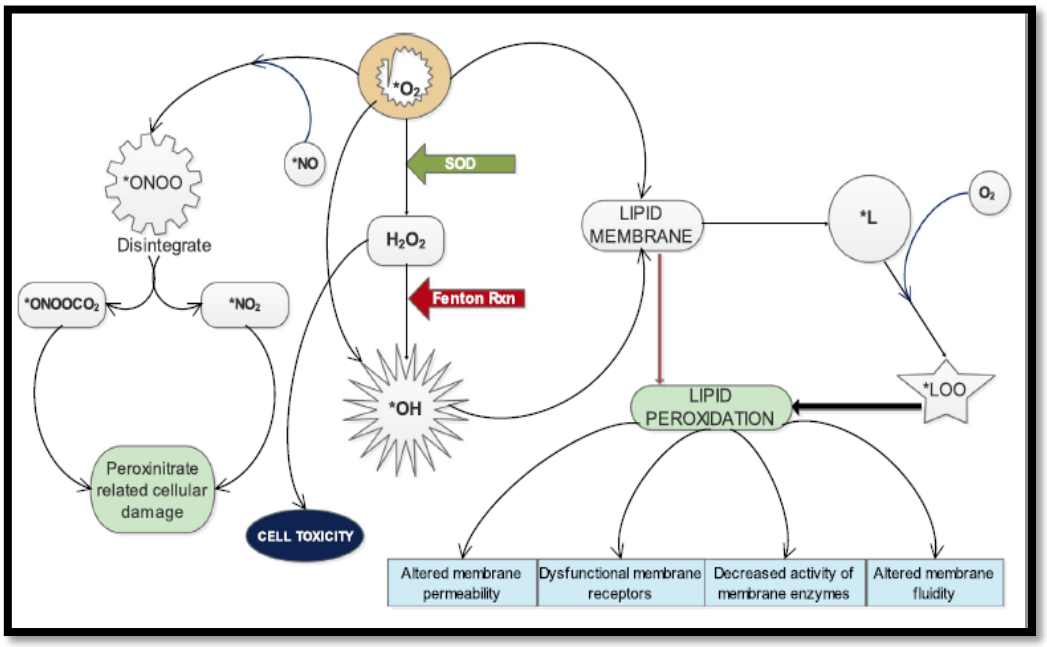

Figure 5.1: Generation of reactive species from superoxide anion (Ighodaro and Akinloye, 2017).

These reactive species formed from superoxide anion are capable of involving in cross linking directly between lipid-lipid, protein-lipid and protein-protein by attacking the lipid and membrane proteins.

5.1.3. Level of antioxidant enzymes actions

The primary purpose of antioxidant enzymes is to nullify the detrimental effect of reactive oxygen species. Antioxidants achieve this purpose by functioning at various levels: radical scavenging, radical preventive and radical induced damage repair. Based on their ability to participate in any of the above-mentioned line of defence, the antioxidants are classified into four categories (Ighodaro and Akinloye, 2017).

A. First-line defence antioxidants

Primary purpose of these antioxidants is to supress or prevent the free radicals and the formation of other reactive species. They are known for the quick action in neutralizing any molecule with the ability to develop into a free radical. It also neutralizes any free radical with the potential to induce other radical formation. Superoxide dismutase, catalase and glutathione peroxidase are the leading enzymes of

this category. Basically, they dismutate superoxide radical into a less toxic molecule of H_2O_2 and breakdown the hydrogen peroxides and hydroperoxides into less harmful molecules such as alcohol and O_2.

B. Second-line defence antioxidants

They function as scavengers of free radicals. Their scavenging property either inhibits the initiation of chain reaction or breaks the chain propagation reactions. By donating electrons to free radical they themselves become free radicals with lesser harmful effects. This type includes both hydrophilic antioxidants such as ascorbic acid, glutathione and uric acid and lipophilic antioxidants such as tocopherol (Vitamin E) and ubiquinol etc.

C. Third-line defence antioxidants

They have the potential to repair the damage caused by the free radicals previously. They are basically enzymes arise from *de novo* pathways which carry out the function of repair of damage occurred to biomolecules and cell membrane. Damage occurring to macro molecules such as DNA, proteins and lipids is reconstituted by these kinds of antioxidant enzymes. Damaged DNA and lipids are discarded from the cell by these enzymes in order to eliminate any toxicity at cellular level or tissue level. These enzymes include, (i) DNA repair enzyme system such as nucleases, polymerases and glycosylases, (ii) Proteolytic enzymes such as peptidases, proteases and proteinases which are located at cytosol and mitochondria.

D. Fourth-line defence antioxidants

They function on the principle of adaptation. They hold the ability to utilize the signals needed for formation or reaction of free radicals and thus prevent them from generation or reaction (Niki, 1993).

5.1.4. Responses of the enzymatic antioxidants against reactive species

Through metabolism or reactions taking place in the cell superoxide radicals or singlet oxygen gets generated. Superoxide dismutase (SOD) catalytically converts these radicals into hydrogen peroxide. Accumulation of hydrogen peroxide will impart toxicity to cell. In case of the presence of Fe^{2+} hydrogen peroxide is converted to hydroxyl radical which is deleterious causing cellular damage. In order to prevent the formation of hydroxyl radical, catalase which is present abundantly in peroxisomes,

breaks down hydrogen peroxide into water and molecular oxygen neutralizing the deleterious effect of hydrogen peroxide and hydroxyl radical. Since mitochondria does not contain catalase, the function of catalase is successfully carried out by glutathione peroxidase where hydrogen peroxide is reduced to lipid peroxide and water. This comprehended process to eliminate the reactive species constitutes the first line of defence.

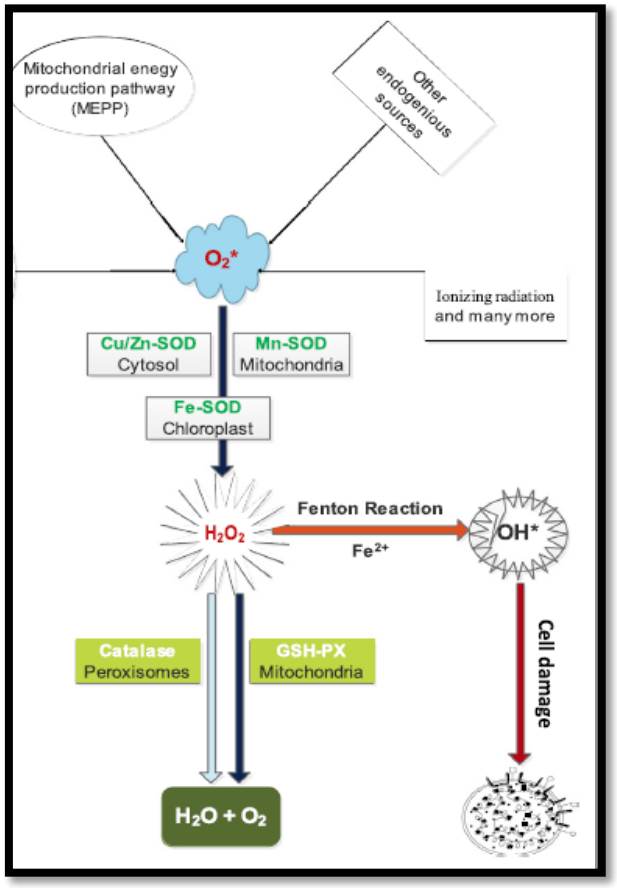

Figure 5.2: First line of defence (Ighodaro and Akinloye, 2017).

A. Superoxide dismutase

The most powerful and first antioxidant to plunge into action is superoxide dismutase (SOD). This endogenous antioxidant enzyme catalyses the dismutation of two superoxide anion molecules to hydrogen peroxide and oxygen making more potentially harmful molecule of superoxide anion into less harmful molecule of hydrogen peroxide (Figure 5.2). SOD always requires a metal as a cofactor for its activity since it is a metalloenzyme. Depending upon the type of cofactor used by SOD for its catalytic activity, there exist different forms of SOD (Fridovich, 1995; Dringen et al., 2005): (i) Fe-SOD predominates in prokaryotes and chloroplasts of some plants,

(ii) Mn-SOD found in prokaryotes and mitochondria of the eukaryotes, (iii) Cu/Zn-SOD commonly found in eukaryotes and localized in cytosol but also found in chloroplasts and peroxisomes (Gill and Tuteja, 2010; Karuppanapandian et al., 2011).

B. Catalase

Catalase is a tetrameric protein with molecular weight of 240 kDa. It contains four subunits. Gene ctt1 mapped to chromosome 11 encodes for catalase (Radi et al., 1991). All the living tissues that utilize oxygen contain catalase. It requires manganese or iron as a cofactor. It is directly involved in reduction or degradation of hydrogen peroxide into molecular oxygen and water, thus completing the process of detoxification initiated by SOD (Marklund, 1984; Chelikani et al., 2004). Catalytic efficiency of catalase is very high that millions of hydrogen peroxide can be broken down just in one second. Peroxisomes contain enormous amount of catalase but absent in mitochondria (Radi et al., 1991). The degradation or reduction of hydrogen peroxide formed by SOD is achieved in two steps by catalase. Heme is oxidized to an oxyferryl species by one molecule of hydrogen peroxide. One oxidation equivalent is removed from the porphyrin ring and one from iron thus generating porphyrin cation radical. The resting state enzyme is regenerated by a second molecule of hydrogen peroxide which acts as a reducing agent, producing water and molecular oxygen (Chelikani et al., 2004).

C. Glutathione peroxidases

Being an intracellular enzyme, glutathione peroxidases (GPX) is mainly present within mitochondria and also at times found in cytosol (Góth et al., 2004). It efficiently removes hydrogen peroxides generated by SOD and degrades them to water and lipid peroxides into respective alcohol. Selenium a micronutrient is a cofactor needed most of the times for its activity. Hence often GPX is known as selenocysteine peroxidase. Inhibition of lipid peroxidation thus protecting the cell oxidative damage is the primary function of GPX (Gill and Tuteja, 2010)

5.1.5. Responses of the non-enzymatic antioxidants against reactive species

Among many endogenous non-enzymatic antioxidants, ascorbic acids (Vitamin C) and tocopherol play a very important role in protecting the plant from oxidative damage.

A. Ascorbic acid

In scavenging the free radicals ascorbic acid undergoes oxidation to be converted to ascorbate free radicals or monodehydroascorbate. It is further converted to dehydroascorbate (DHA) in presence of ROS. DHA being unstable in nature gets hydrolysed irreversibly into 2, 3 – diketo-L-gulonic acid. This results in decreased level of ascorbic acids (Bode et al., 1990).

B. Tocopherol

Being lipid soluble in nature, lipid molecules are protected from lipid peroxidation and oxidative damage by the potential free radical scavenging capacity of tocopherol. Tocopherol is regarded as a major membrane bound antioxidant (Burton and Ingold, 1989). In scavenging the free radical tocopherol gets converted to tocopherol radicals by donating its hydrogen to lipid peroxyl radical generated by ROS. Vitamin C further reduces tocopherol radical into antioxidant tocopherol for encountering another free radical (Kojo, 2004).

Basically tocopherol is involved in the inhibition of chain propagation of generation of ROS which renders oxidative damage to the lipids. Two basic mechanisms of oxidation are involved in the elimination of free radical by tocopherol: (i) Tocopherol gets oxidized to become tocopherol radical, (ii) it forms hydroperoxides by reacting with singlet oxygen (Neely et al., 1988; Krieger-Liszkay and Trebst, 2006). In both the cases, tocopherol is retrieved through the re-reduction of tocopherol radical and hydroperoxides by ascorbic acid (Rodrigues et al., 2015).

Several experiments have demonstrated that the oxidative stability of the oil depends upon the tocopherol content present within oil (Emanuel and Lyaskovskaya, 1967; Reinton and Rogstad, 1981; Jung and Min, 1990; Fuster et al., 1998; Kamal-Eldin, 2006).

Rodrigues et al., (2015) has investigated that 42 days of storage of *Jatropha curcas* L. seeds lead to decrease in gamma tocopherol reduced the oxidative stability of the *Jatropha* oil. Among the various accessions, the seed oil that contained maximum gamma tocopherol content had the highest oxidative stability. Investigations by Gawrysiak- Witulska et al., (2009, 2016) have shown drying condition and storage significantly impacts the tocopherol content which is responsible for seed longevity. In

black rape seeds, low temperature seed drying induced a decline of 6 % to 11 % in tocopherol content. High temperature range of drying resulted in 4 % to 8 % decrease. At 10°C seeds stored for a year accelerated the decline of tocopherol very significantly. There are no reports on alterations in tocopherol levels in natural or aged Jatropha seeds.

Overall antioxidant status was assayed by the DPPH assay. Compared to hydrophilic antioxidants, antioxidant assays for hydrophobic compounds are limited. Mostly for plant extract, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay is frequently opted for antioxidant assay. In this assay, a weak A-H bonding of the antioxidant will react with a stable free radical DPPH (λ_{max}=517 nm) to cause the discolouration of the molecule. It is basically a reduction reaction (Moon and Shibamoto, 2009).

The DPPH assay is applied to estimate the free radical scavenging activity of the plant extract by calculating its capacity to inhibit the DPPH. The method requires short time and is easy to carry out. Stable molecule of DPPH (1, 1-diphenyl-2-picrylhydrazyl) reacts with compounds that can provide hydrogen atoms. This results in decolouration of DPPH methanol solution which is measured at 515 nm of UV–vis absorption. The reducing ability of antioxidants toward the free radical - DPPH is measured. The trolox equivalent antioxidant capacity (TEAC) assay, another widely used method is based on the principal discoloration of a preformed ABTS (2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical by antioxidant compounds. A limitation of this TEAC method is that the TEAC value indicates the capability of test sample to react with the ABTS radical rather than to inhibit the process of oxidation. The FRAP determination (ferric reducing antioxidant power) is another method available to estimate antioxidant power of plant extracts. It is based on reduction of Fe^{3+}-tripyridyltriazine to Fe^{2+}-tripyridyltriazine. Ferric salt potassium ferricyanide is used as an oxidant. In this reaction ferric 2, 4, 6-tripyridyl-*s*-triazine is reduced to the coloured ferrous form which is measured at 593 nm. The ORAC (oxygen radical absorbance capacity) assay is yet another method which uses AAPH (2, 2′-azobis (2-amidinopropane) dihydrochloride) and the fluorescent β-phycoerythrin (B-PE) (probe) to generate peroxyl radicals. However, under fluorescence plate-reader conditions B-PE is photobleached and due to nonspecific protein binding, it reacts with phenolic compounds. When antioxidants are added they compete with the substrate to capture the peroxyl radicals, thereby retarding fluorescein oxidation or inhibiting the free radicals. The substrate used gets (fluorescein) decomposed in the presence of peroxyl

radicals that are generated by controlled thermal decomposition of AAPH in an air-saturated solution. The emission wavelength of 525 nm with extinction at 485 nm is measured. In the ORAC reaction, its fluorescence intensity decreases as fluorescein is consumed. The ORAC assay has an advantage of measuring the antioxidant capacity both hydrophilic and lipophilic chain-breaking antioxidants (Ndhlala et al., 2010).

Also new methods of the estimation of total antioxidant activity are emerging constantly. Brainina et al., (2007) proposed a potentiometry method which is as good as other common methods of antioxidant activity assay like DPPH and TEAC assays.

In this chapter results of the investigations on the activity of enzymatic antioxidants (SOD, CAT and POD) along with non-enzymatic antioxidant (Gamma tocopherol) is reported.

5.2. RESULTS

5.2.1. Estimation of DPPH free radical scavenging capacity of seed extract of *Jatropha curcas*

The experimental conditions for DPPH assay are as described by Larrauri et al., 1998 (chapter 2).

The pattern in change of total antioxidant levels was similar for seeds exposed to natural or accelerated aging. There was an initial increase in DPPH scavenging activity indicating an increase in antioxidants, this increase however could not be sustained with prolonged aging. As seeds continued to age there was a decrease in antioxidant levels. Free radical scavenging capacity was significantly increased in the extract of seeds of NA3m, NA6m, NA9m and NA12m of natural aging compared to control. This trend was reversed and the free radical scavenging capacity significantly declined from NA18m onwards compared to control. The DPPH free radical scavenging capacity was observed to be the highest in NA9m seeds of natural aging (Table 5.1). In the accelerated and saturated salt accelerated aging groups too, a similar trend was observed. AA2d, AA3d, AA4d, SSAA2d, SSAA3d and SSAA4d also had a significantly increased level of DPPH scavenging capacity when compared to control. The activity was found to be non-significant from AA5d to AA10d and SSAA5d to SSAA10d compared to control. But as accelerated aging and saturated salt aging treatment were extended beyond 10days, significant decrease in DPPH scavenging

activity was observed (AA12d, AA15d, SSAA12d and SSAA15d when compared to control) (Table 5.2). Highest activity was found in AA4d and SSAA4d. DPPH free radical-scavenging activity found in AA3d, AA4d, SSAA3d and SSAA4d was same as that found in NA9m seeds.

Table 5.1: DPPH free radical scavenging activity of *Jatropha curcas* L. seeds subjected to natural, accelerated and saturated salt accelerated aging and ascorbic acid.

Percentage of inhibition of DPPH. (Mean ± SD)	percentage of inhibition of DPPH. (Mean ± SD) Natural aging (NA)	percentage of inhibition of DPPH. (Mean ± SD) Accelerated aging (AA)	percentage of Inhibition of DPPH. (Mean ± SD) Saturated salt accelerated aging (SSAA)
Ascorbic acid - 83.6 ± 1.19 **Control** - 60.7 ± 2.53	**NA1m** - 60.8 ± 1.84 **NA3m** - 64.0 ± 2.51 ***** **NA6m** - 65.0 ± 2.49 ****** **NA9m** - 68.9 ± 2.75 ******* **NA12m** - 65.0 ± 2.47 ****** **NA15m** - 59.3 ± 1.50 **NA18m** - 57.2 ± 1.93 ***** **NA21m** - 57.1 ± 2.54 ***** **NA24m** – 56.4 ± 3.10 ******	**AA12h** - 62.8 ± 5.58 **AA1d** - 63.1 ± 5.06 **AA2d** - 64.3 ± 3.74 * **AA3d** - 68.5 ± 5.27 * **AA4d** - 67.7 ± 6.61 ***** **AA5d** - 61.8 ± 5.57 **AA7d** - 61.8 ± 5.53 **AA10d** - 55.7 ± 5.19 **AA12d** - 52.5 ± 6.36 ***** **AA15d** - 51.0 ± 5.85 *****	**SSAA12h** - 63.2 ± 4.21 **SSAA1d** - 63.4 ± 4.80 **SSAA2d** - 65.6 ± 3.58 * **SSAA3d** - 68.4 ± 6.49 * **SSAA4d** - 68.7 ± 6.20 * **SSAA5d** - 63.0 ± 5.93 **SSAA7d** – 61.4 ± 5.75 **SSAA10d** - 59.9 ± 6.72 **SSAA12d** - 53.5 ± 2.82 ***** **SSAA15d** - 52.9 ± 4.21 *****

Values are mean of ± SD. *, **, * indicates significance at P ≤ 0.05, P ≤ 0.01 and P ≤ 0.001 respectively as compared to control, n = 3.**

5.2.2. Estimation of the activity of the antioxidant enzymes

Superoxide dismutase (SOD)

SOD activity was estimated by the method prescribed by Marklund and Marklund, (1974) (chapter 2). It was found to be significantly increased in NA6m, NA9m and NA12m seeds of natural aging compared to control. Significantly decreased activity was found in NA18m, NA21m and NA24m of natural aging compared to control. The highest activity was noted in NA9m and lowest was at NA12m (Table 5.2). In case of accelerated aging, AA2d, AA3d and AA4d expressed significantly increased SOD activity compared to control. As the accelerated aging treatment was accelerated, it resulted in the significant decline of SOD activity in AA7d, AA10d, AA12d and AA15d seeds compared to control. AA3d seeds outnumbered the other groups of accelerated aging for expressing the highest SOD activity. Lowest activity was found in AA15d (Table 5.2). With respect to saturated salt accelerated aging, SSAA2d,

SSAA3d and SSAA4d were marked with significantly increased activity of SOD compared to control. SSAA7, SSAA10d, SSAA12d and SSAA15d following the trend of accelerated aging expressed significantly decreased activity compared to control. SSAA4d seeds expressed the highest activity of SOD compared to control and lowest was recorded in SSAA15d (Table 5.2).

Catalase (CAT)

CAT activity was estimated by the method prescribed by Aebi, (1984) (chapter 2). Changes in CAT activity showed a similar trend as SOD. A significant high was recorded in NA6m and continued to increase till NA12m as compared to control. The trend was reversed when a decline in activity was observed from NA15m onwards compared to control. NA9m among all the other groups of natural aging expressed the highest activity and whereas NA24m had the lowest among all the other groups of natural aging (Table 5.2). With respect to accelerated aging, significantly increased activity was found as early as in AA1d and the increase continued till AA5d compared to control. By AA10d, the catalase activity began to decrease. During the study period AA3d had the highest activity and AA15d showed the lowest activity among all the other groups of accelerated aging (Table 5.2). In case of saturated salt accelerated aging, SSAA1d, SSAA2d, SSAA3d, SSAA4d and SSAA5d were observed with significantly increased activity compared to control. SSAA12d and SSAA15d showed a significantly declined activity compared to control. SSAA4d expressed the highest activity and SSAA15d registered the lowest activity among all the groups of saturated salt accelerated aging (Table 5.2).

Peroxidase (POD)

Peroxidase activity was estimated by the method prescribed by Chance and Maehly, (1955) (chapter 2). Peroxidase activity was seen significantly increased in NA3m, NA6m, NA9m and NA12m seeds of natural aging compared to control. Significant decrease was registered in AA12d and AA15d compared to control. NA9m expressed the highest activity and NA24 registered the lowest among all the other groups of natural aging (Table 5.2). In case of accelerated aging, AA1d, AA2d, AA3d, AA4d and AA5d were marked with significantly increased activity of peroxidase compared to control. In AA12d and AA15d there was a significant decrease in peroxidase activity compared to control. AA4d expressed the highest activity and

AA15d showed the lowest among the groups of accelerated aging (Table 5.2). With regard to saturated salt accelerated aging, SSAA1d, SSAA2d, SSAA3d, SSAA4d and SSAA5d registered significantly highest activity compared to control. Significant decline was observed in SSAA12d and SSAA15d. The highest activity was seen at SSAA3d and SSAA15d registered the lowest activity among the other groups of saturated salt accelerated aging (Table 5.2).

Table 5.2. Estimation of the antioxidant enzymes activity [Super Oxide Dismutase, Catalase, Peroxidase].

Groups	**Super Oxide Dismutase (SOD) activity. Units/mg (Mean ± SD)**	**Catalase (CAT) activity Units/mg (Mean ± SD)**	**Peroxidase (POD) activity Units/mg (Mean ± SD)**
Control	2.10 ± 0.069	0.240 ± 0.0221	0.133 ± 0.0114
Natural aging (NA)			
NA1m	2.22 ± 0.189	0.239 ± 0.021	0.149 ± 0.019
NA3m	2.19 ± 0.501	0.261 ± 0.041	0.167 ± 0.215 **
NA6m	2.51 ± 0.314 *	0.283 ± 0.027 *	0.215 ± 0.021 ***
NA9m	2.57 ± 0.239 **	0.310 ± 0.025 ***	0.230 ± 0.024 ***
NA12m	2.49 ± 0.245 *	0.276 ± 0.029 *	0.198 ± 0.016 ***
NA15m	2.16 ± 0.314	0.208 ± 0.016 *	0.146 ± 0.018
NA18m	1.92 ± 0.251 *	0.210 ± 0.014 *	0.120 ± 0.016
NA21m	1.74 ± 0.168 *	0.198 ± 0.020 **	0.094 ± 0.017 **
NA24m	1.65 ± 0.274 **	0.192 ± 0.025 ***	0.085 ± 0.019 ***
Accelerated Aging (AA)			
AA12h	2.35 ± 0.162	0.265 ± 0.019	0.165 ± 0.0285
AA1d	2.49 ± 0.196	0.272 ± 0.014**	0.180 ± 0.0281**
AA2d	2.52 ± 0.242*	0.308 ± 0.019***	0.220 ± 0.0173***
AA3d	2.81 ± 0.422***	0.312 ± 0.025***	0.231 ± 0.0321***
AA4d	2.54 ± 0.480***	0.311 ± 0.016***	0.233 ± 0.0282***
AA5d	2.31 ± 0.357	0.288 ± 0.013***	0.197 ± 0.0249***
AA7d	1.85 ± 0.221***	0.253 ± 0.019	0.164 ± 0.0233
AA10d	1.50 ± 0.192***	0.211 ± 0.007*	0.155 ± 0.0353
AA12d	1.39 ± 0.448***	0.204 ± 0.029**	0.097 ± 0.0275*
AA15d	1.04 ± 0.114***	0.185 ± 0.019***	0.093 ± 0.0210*
Saturated salt Accelerated aging (SSAA)			
SSAA12h	2.34 ± 0.118	0.249 ± 0.0249	0.149 ± 0.0154
SSAA1d	2.35 ± 0.137	0.274 ± 0.0368*	0.170 ± 0.0336**
SSAA2d	2.43 ± 0.341**	0.288 ± 0.0374**	0.223 ± 0.0173***
SSAA3d	2.49 ± 0.237**	0.305 ± 0.0121***	0.231 ± 0.0133***
SSAA4d	2.54 ± 0.138***	0.311 ± 0.0230***	0.217 ± 0.0103***
SSAA5d	2.17 ± 0.151	0.309 ± 0.0308**	0.199 ± 0.0157***
SSAA7d	1.72 ± 0.118***	0.269 ± 0.0138	0.156 ± 0.031
SSAA10d	1.53 ± 0.156***	0.254 ± 0.0237	0.136 ± 0.020
SSAA12d	1.22 ± 0.164***	0.207 ± 0.0250*	0.099 ± 0.018**
SSAA15d	0.87 ± 0.121***	0.190 ± 0.0146***	0.089 ± 0.018***

Values are mean of ± SD. *, **, * indicates significance at P ≤ 0.05, P ≤ 0.01 and P ≤ 0.001 respectively as compared to control, n = 3.**

5.2.3. Estimation of gamma – tocopherol (non-enzymatic antioxidants)

The quantification of tocopherol levels in naturally and artificially aged seeds was done by HPLC following the method prescribed by Cerchiara et al., (2010). Peak of gamma tocopherol was detected in HPLC chromatogram having the retention time between 18.2 and 20.1 min (Figure 5.4 A). Further using gradient concentration ranging from 1 to 25 µg/ml of gamma tocopherol, a calibration curve was constructed with linearity in nature (Figure 5.3). Overlay of chromatograms of gradient concentration of 2.5, 5.0, 7.5, 10.0 and 12.5 µg/ml of gamma tocopherol further confirmed the emergence of single peak with single retention time of 18.2 - 20.1 min (Figure 5.4 B). Diluted *Jatropha curcas* oil injected directly yielded a chromatogram with the peak at the retention time of 19.3 - 20.4 min indicating the presence of gamma tocopherol (Figure 5.4 C). The overlay of the chromatograms of *Jatropha curcas* oil sample and gamma tocopherol standard affirms the presence of gamma tocopherol in the given sample with slightly delayed retention time compared to standard (Figure 5.4 D).

Tocopherols are considered to be one of the major groups of natural lipid soluble antioxidants involved in chain breaking. In the samples of oil extracted from *J. curcas* seeds subjected to natural aging, accelerated aging and saturated salt accelerated aging, there is a decrease in the content of gamma tocopherol due to both natural and artificial aging. Significant decrease in gamma tocopherol levels was observed when the storage period was beyond 12 months. This decrease got significantly intensified as storage period was prolonged up to 24 months (Figure 5.5). 12 hours of accelerated aging and saturated salt accelerated aging resulted in decrease of tocopherol levels significantly. This decreasing trend of gamma tocopherol was evident all through the artificial aging of both kinds (Figures 5.6 [AA] and 5.7 [SSAA]). The decrease seen in accelerated aging was more than in saturated salt accelerated aging. An obvious negative correlation was observed between MDA content and gamma tocopherol content in all types of aging such as natural aging (r = -0.964 ***), accelerated aging (r = -0.922 ***) and saturated salt accelerated aging (r = -0.959 ***) (Figure 5.8).

Table 5.3: Comparative analysis of gamma tocopherol between natural aging and accelerated aging and saturated salt accelerated aging.

Natural Aging (NA) γ – tocopherol (mg/kg) (Mean ± SD)	Accelerated Aging (AA) γ – tocopherol (mg/kg) (Mean ± SD)	Saturated salt Accelerated aging (SSAA) γ – tocopherol (mg/kg) (Mean ± SD)
NA6m - 151 ± 39.0 (ns)	----------	SSAA12h - 150 ± 12.5
NA9m - 137 ± 17.0 (ns)	AA12h - 137 ± 3.69	----------
NA12m - 134 ± 6.02 (ns)	----------	SSAA4d - 134 ± 10.8
NA18m - 98.9 ± 4.88 (ns)	AA2d - 99.5 ± 9.91	SSAA10d - 98.4 ± 10.3
NA21m - 76.9 ± 34.1 (ns)	AA3d - 77.4 ± 27.4	----------

Mean values of γ – tocopherol in NA6m, NA9m, NA12m, NA18m and NA24m found equivalent (~) with those groups in AA and SSAA. ns - Non-significant when SSAA12h, AA12h, SSAA4d, AA2d and SSAA10d, AA3d are compared to NA6m, NA9m, NA12m, NA18m and NA24m respectively. Values are mean of ± SD, n=3.

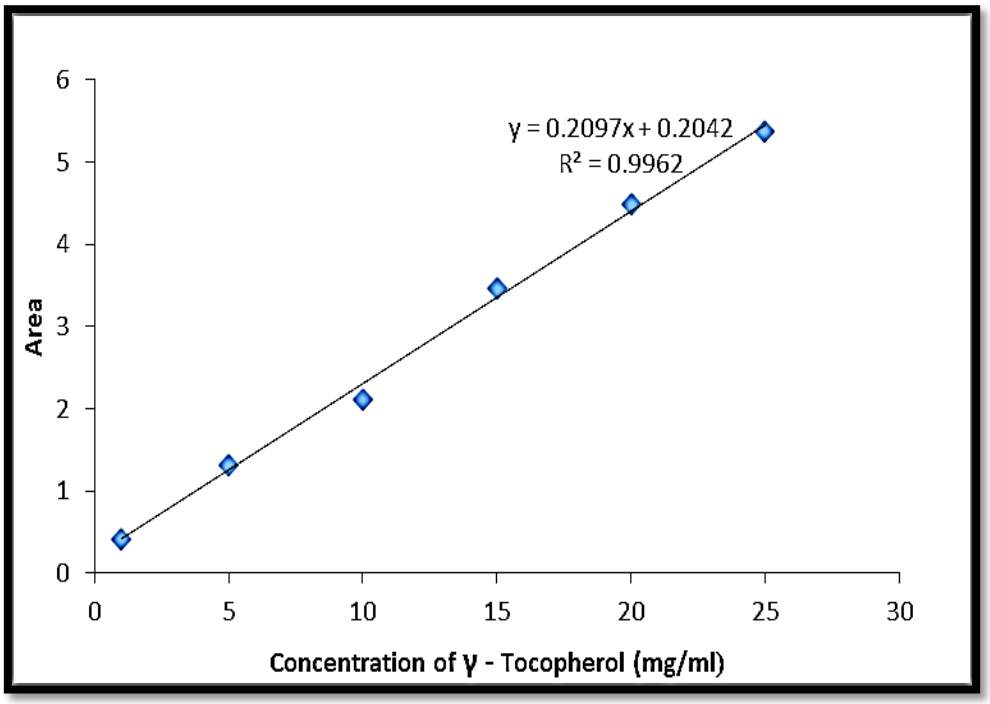

Figure 5.3: Calibration curve of standard - gamma tocopherol (1 - 25µg/ml).

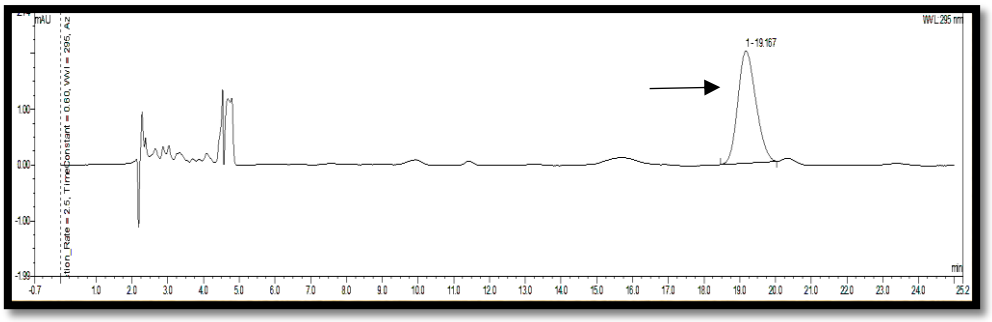

Figure 5.4 A. Chromatogram of gamma tocopherol standard (10µg/ml).

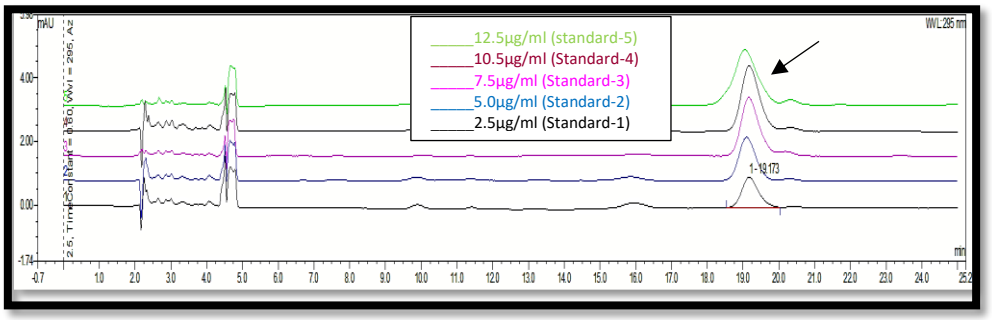

Figure 5.4 B. Chromatogram of the overlay picture of gradient concentration of standard (gamma tocopherol)

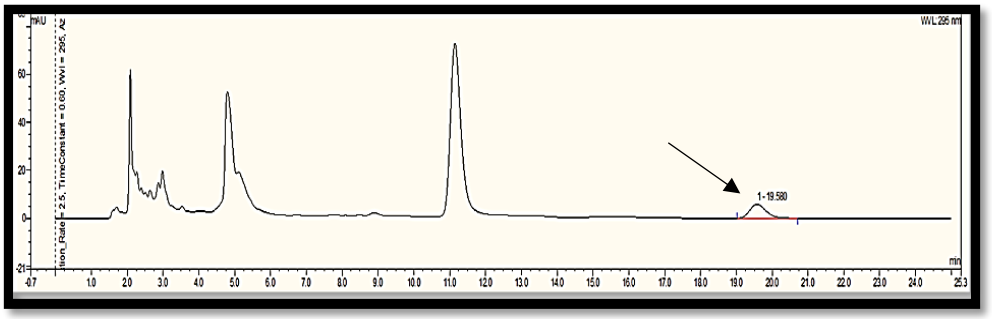

Figure 5.4 C. Chromatogram of the sample (*Jatropha curcas* oil).

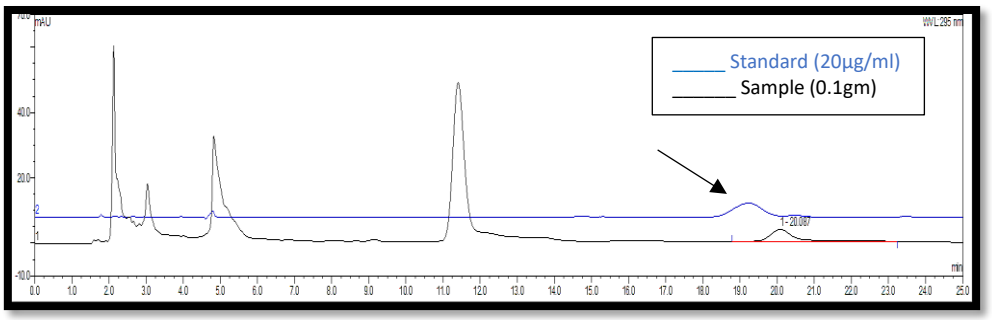

Figure 5.4 D. Chromatogram of the over lay of sample and standard (*Jatropha curcas* oil).

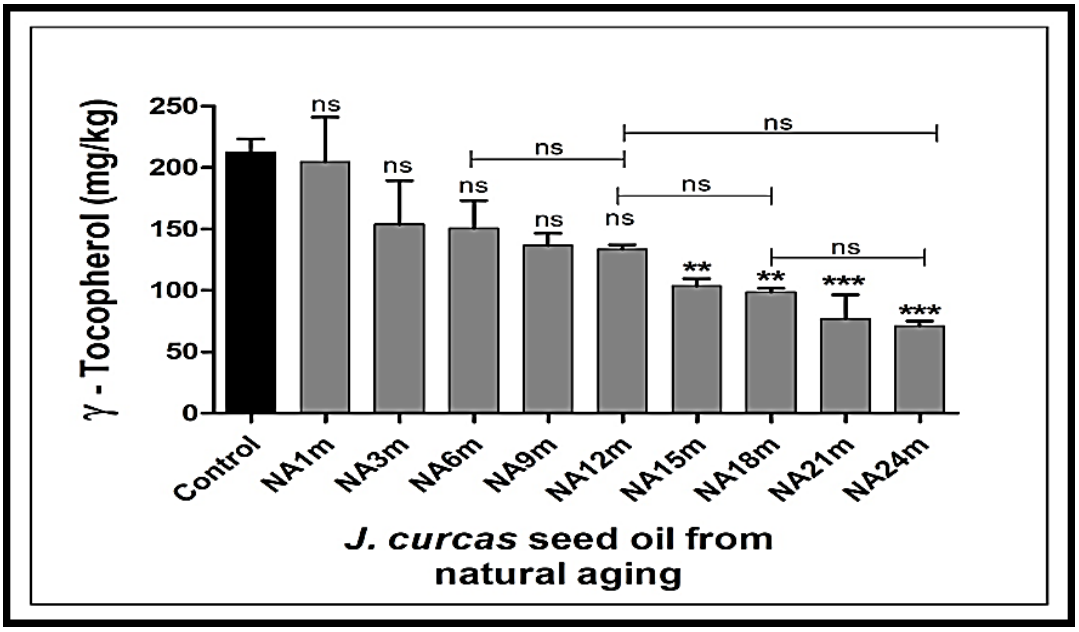

Figure 5.5: Gamma tocopherol levels in control seeds and in seeds of natural aging of 1, 3, 6, 9, 12, 15, 18, 21 and 24 months. Values are mean ± SE; *, **, * indicates significantly different at P<0.05, P<0.01 and P<0.001 as compared to the control, NA6m, NA12m and NA18m, n=3.**

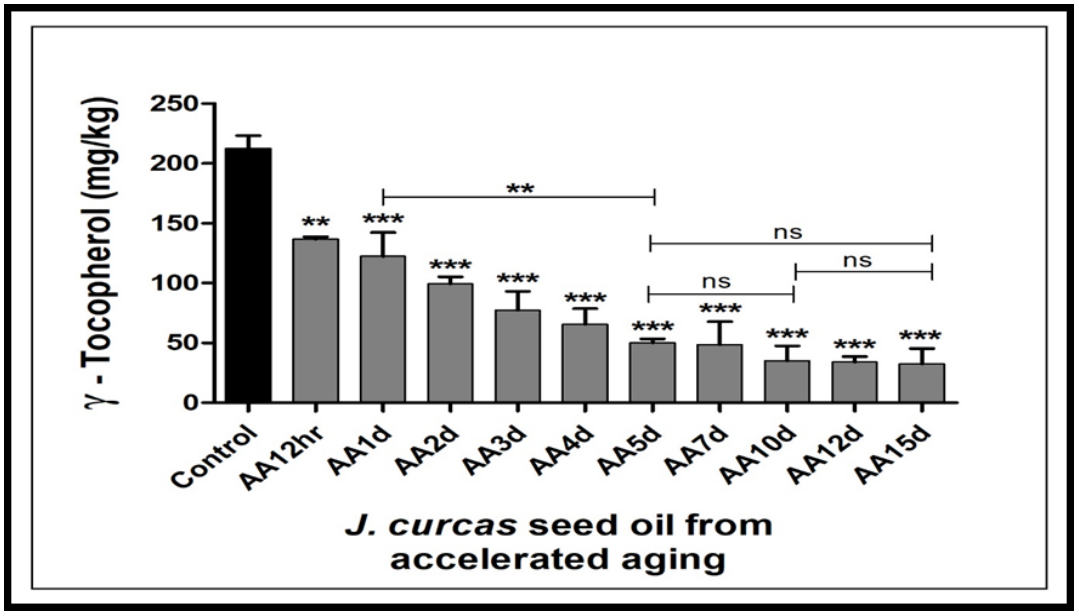

Figure 5.6: Gamma tocopherol levels in control seeds and in seeds of accelerated aging of 12hours, 1, 2, 3, 4, 5, 7, 10, 12 and 15 days. Values are mean ± SE; *, **, * indicates significantly different at P<0.05, P<0.01 and P<0.001 as compared to the control, AA1d, AA5d and AA10d, n=3.**

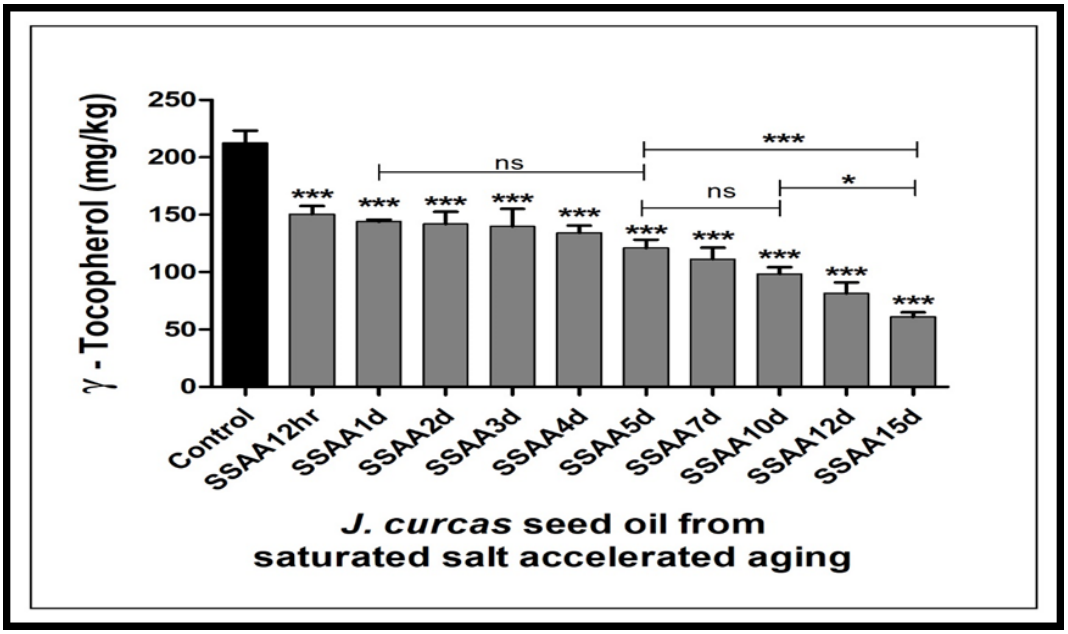

Figure 5.7: Gamma tocopherol levels in control seeds and in seeds of saturated salt accelerated aging of 12hours, 1, 2, 3, 4, 5, 7, 10, 12 and 15 days. Values are mean ± SE; *, **, * indicates significantly different at P<0.05, P<0.01 and P<0.001 as compared to the control, SSAA1d, SSAA5d and SSAA10d, n=3.**

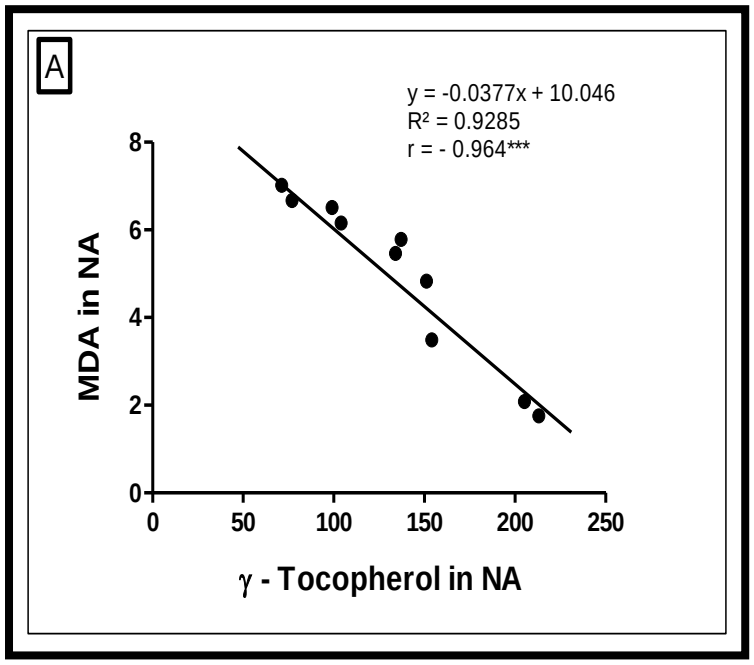

Figure 5.8 A: Correlation between gamma tocopherol content and MDA content found in seeds of natural aging (A), accelerated aging. *, **, * indicates significantly different at P<0.05, P<0.01 and P<0.001.**

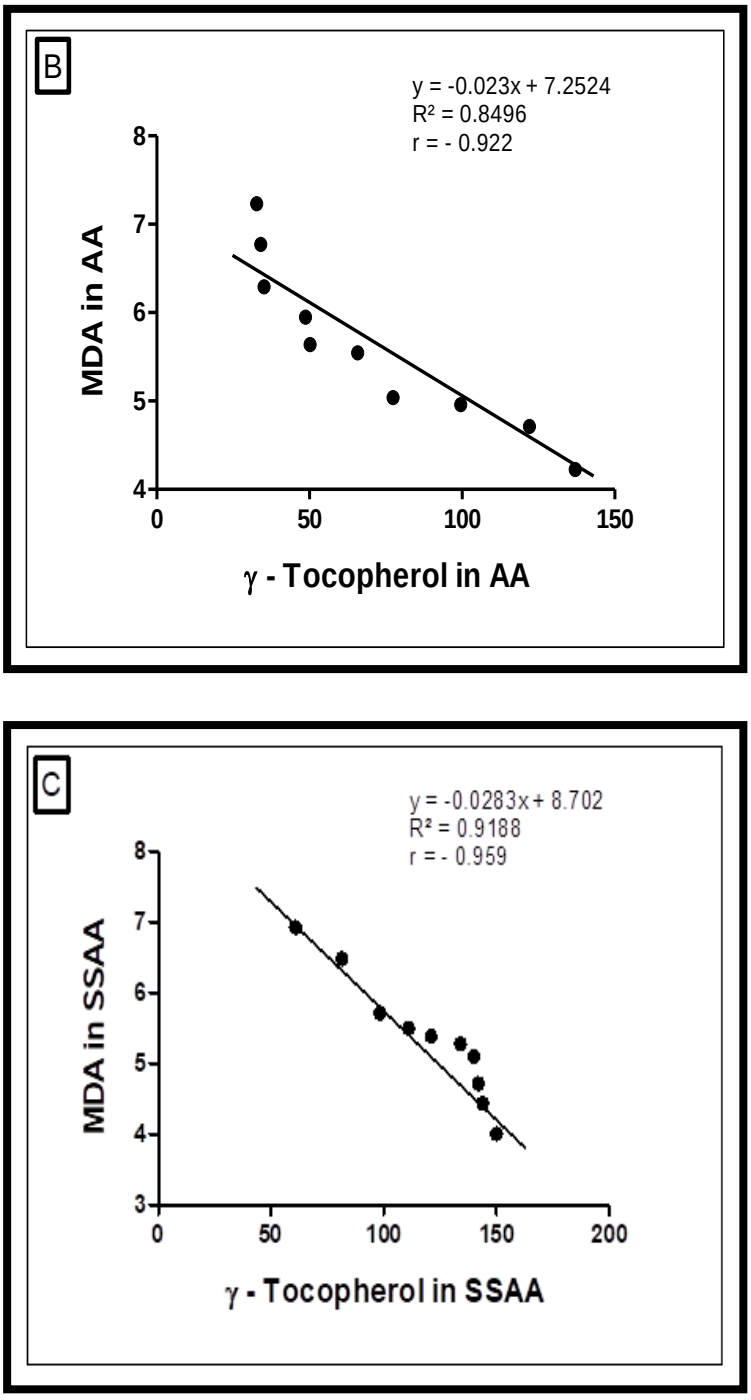

Figure 5.8 B and C: Correlation between gamma tocopherol content and MDA content found in seeds of accelerated aging (B) and saturated salt accelerated aging (C). *, **, * indicates significantly different at P<0.05, P<0.01 and P<0.001.**

5.3. DISCUSSION

5.3.1. Determination of DPPH free radical scavenging activity of the antioxidants

DPPH being a stable free radical is often used to estimate the free radical-scavenging capacity of any natural extract. An increase in the free radical-scavenging activity of the extract indicates the potentiality of the seed to combat free radicals and maintain a balance between ROS produced during storage and the antioxidant pool. Seeds of NA3m, NA6m, NA9m and NA12 expressed significant increase in free radical scavenging activity. This could be interpreted as an exhibition of an accelerated defence to neutralize the enormous ROS produced during these stags of storage. This significant increase in scavenging activity found in these group of seeds during natural aging gets reflected in the ability to germinate shown by these seeds. Same is true for AA12 h to AA3d where an increased scavenging activity complimented with germination potential of these group of seeds. The decreased length of the emerging radical in these groups of seeds may portray the detrimental and deleterious effect of free radicals generated from the extreme condition of accelerated aging. Decreased scavenging activity observed from NA18m to NA24m compared to control may indicate the beginning of seed deterioration which is evident through the failure of germination in these groups of seeds. The same is true for those seeds that were exposed to an extended period of artificial aging beyond the point of 3 days. A decrease in the activity designates the breakdown or degradation of the antioxidant system itself caused by elevated levels of ROS. The deleterious effects of artificial aging treatment beyond the point of 5 days, cause irreversible seed deterioration. In this present study, 3-4 days of accelerated or saturated salt accelerated aging was sufficient enough to bring about the same effect of free radical scavenging activity as seen in 9 months of natural aging.

Aging or environmental stress-related increase in free radicals happens in two phases. In first phase, upheaval in free radical generation during storage of oil seeds or extreme condition of stress stimulates the accelerated synthesis of antioxidants in order to successfully inhibit ROS to thwart any bio-molecular damage in the cell by free radicals. In the second phase there is a decline of antioxidants as the ROS production over takes antioxidants due to the continual negative effect of storage or stress. At this

level antioxidant synthesis is exceeded by its degradation giving way to the accumulation of ROS beyond the critical threshold (Munneé-Bosch, 2005; Shahidi et al., 2006).

Results of this DPPH free radical scavenging activity analysis are in conformity with the earlier reports shown by Fotouo-M et al., (2018). Long term storage of 24 months of *Moringa oleifera* oilseed resulted in increased level of MDA and decrease in the antioxidant activity. Elevated level of antioxidants activity observed in 6 - 18 months of *Moringa oleifera* oil seeds storage was followed by a decrease in antioxidant activity seen after 18 months of storage.

Kaewnaree et al., (2011) subjected sweet pepper seeds for 30 days of storage. In this investigation they revealed that first 10 days of storage has hiked the total antioxidant activity and further increase in storage period beyond 10 days declined the activity. This indicates that the increased level was a protective measure taken by cells aging deleterious effect of free radicals during storage and the decrease implied the imbalance between ROS and antioxidants and break-down of antioxidant system itself. This is the first time a detailed study of changes in antioxidant profile of aging *Jatropha curcas* seeds has been reported.

5.3.2. Estimation of the activity of the antioxidant enzymes

The changes in profile of antioxidant enzymes were monitored in all groups of seeds. An increased activity found in NA3m to NA12m was to maintain the cellular environment and viability when seeds were subjected to either long term storage or stressful conditions of high temperature and moisture content during artificial aging. Similar trend of significantly increased activity found in those groups of AA (AA2d, AA3d, AA4d for SOD, AA1d, AA2d, AA3d, AA4d and AA5d for CAT and POD) and of SSAA (SSAA2d, SSAA3d and SSAA4d for SOD, SSAA1d, SSAA2d, SSAA3d, SSAA4d and SSAA5d for CAT and POD) reaffirms the elevated level antioxidants are essential to insulate the seeds from deleterious effect of free radicals generated during extreme aging conditions. The significantly decreased activity of SOD observed from AA7d to AA15d and SSAA7d to SSAA15d, of CAT in AA12d to AA15d and SSAA12 to SSAA15d, and of POD in AA12 to AA15d indicates the inactivation and degeneration of antioxidant enzymes by the outburst of free radicals during artificial aging. In this study, reduced level of antioxidant enzymes activity could be negatively

correlated to the increased level of MDA and H_2O_2. Current results are in conformity with earlier studies done on maize seeds (Vashisth and Nagarajan, 2009). Depletion of antioxidant enzyme activity was also observed during seed aging of soybean (Murthy et al., 2002), cotton (Goel et al., 2003) and beech (Pukacka and Ratajczak, 2005).

Significantly increased DPPH free radical scavenging activity seen NA3m to NA12m, AA2d to AA4d and SSAA2d to SSAA4d can interpreted as combined venture of the entire antioxidants pool (both enzymatic and non-enzymatic) to combat the free radicals. The significantly increased activity of antioxidant enzymes and highest tocopherol content noted in all these above mentioned groups favours this argument.

Detoxification and maintenance of a cellular metabolic balance in orthodox seeds by antioxidant enzymes have been investigated in multiple species, including wheat (de Gara et al., 2003), Norway maple and European beech (Pukacha et al., 2003) and yellow lupine (Garnczarska et al., 2009).

In the present study, ROS scavenging enzymes especially SOD and H_2O_2 scavenging enzymes exhibited striking differences in their activity patterns during natural aging and artificial aging. Status of the antioxidant enzymes in response to prolonged storage or artificial aging can be summated in two phases. The first phase is comprised of the response of antioxidants characterized by defence to scavenge free radicals and the second phase is comprised of degradation characterised by break down of the antioxidants systems.

Antioxidant defence systems in plants include free radical and peroxide-scavenging enzymes such as SOD, CAT and POD (Foyer et al., 1994). SOD and POD play a significant role in maintaining the amount of superoxide radicals and peroxides well below the threshold level (Goel and Sheoran, 2003). Superoxide and hydrogen peroxide are scavenged by SOD and CAT, respectively. Therefore, lipid peroxidation is controlled by minimizing the free radicals produced through the scavenging activity of SOD and CAT (Irwin, 1995). The activity of SOD was higher than that of CAT and POD in this study, which conforms to previous study done on neem seeds (Sahu et al., 2017).

Aging seeds are associated with the reduced level of antioxidant enzymes with the accumulation of ROS (Pukacka and Ratajczak, 2005; Varghese and Naithani, 2008; Kibinza et al., 2011). But the higher activity of SOD seen in this study especially during

the early phase of natural and artificial aging is a surging response to the upsurge of superoxide free radicals, wherein superoxide is converted to H_2O_2, which is comparatively less harmful. Increase level of super oxide radicals, H_2O_2 and OH radicals beyond thresh hold level is associated with the impairment of SOD, CAT and POD during late phase of natural and artificial aging. These negative detrimental effects mount to molecular damage and eventual irreversible seed deterioration. Similar kind of results were obtained in neem seeds subjected to natural aging and controlled deterioration (Sahu et al., 2017).

Results of the present study on antioxidant activity support the fact that seeds of natural aging, especially between 0 and 12 months, possess seed vigour and viability and also express excellent antioxidant activity. These types of seeds are best suited for extraction of oil for biodiesel which will have maximum oxidative stability and also for seedling propagation.

5.3.3. Estimation of gamma - tocopherol (non-enzymatic antioxidant)

Among the many naturally available antioxidants, tocopherol is the most abundant one in vegetable oils that protects cellular membrane from oxidative damage and increase the shelf stability of oils. Eliminating the lipid peroxyl radicals, lipid peroxidation is kept at bay during storage by these types of antioxidants (Porter et al., 1995). Phenolic antioxidants called tocopherols are of four isomers: alpha, beta, gamma and delta. They possess basically same chemical structure: a hindered aromatic phenol is attached to a long chain phythyl group. Each fatty oil consists of unique amount and distribution of these four isomers of tocopherol. The gamma and delta occurs to be most effective in fatty oil rendering oxidative stability (Waynick, 2005).

Gamma tocopherol isoform has been reported to confer stronger oxidation stability than alpha tocopherol in tobacco (Abassi et al., 2007). Stronger desiccation tolerance attributed by gamma tocopherol is to protect polyunsaturated fatty acids from oxidation thus increasing seed longevity in lipid rich seeds (Sattler et al., 2004). Besides the role of being a potent antioxidant, gamma tocopherol is known to exert additional function of generation of plant resistance against osmotic stress or extreme desiccation of oil seeds (Falk and Munne´-Bosch, 2010).

Draganić et al., (2011) postulated that accelerated aging of sunflower seeds lead to decrease of the contents of α-, β- and γ-tocopherol. Decreased content reflects

the utilization of tocopherol for the protection against the detrimental effects of free radicals.

Rodrigues et al., (2015) investigated effect of storage on *J. curcas* seeds. They subjected the seeds to 42 days of storage with two different relative humidity (75% and 92%) conditions. The results reveal that both the types of storage resulted in the reduction of gamma tocopherol. Condition with 95% humidity was more pronouncing in reducing the gamma tocopherol than the lower humidity condition. There was also a negative correlation observed between reduction of tocopherol and increase of oxidation products.

Results of this investigation affirm that degradation of tocopherol during storage is inevitable. Our results are in conformity with the earlier reports done on rape seeds where decrease in gamma tocopherol was associated with storage (Gawrysiak-Witulska et al., 2016). At the initial phase tocopherol antioxidants try to maintain the lipid free radicals below the thresh hold level but as the storage is prolonged this threshold level crosses over to detrimental level where degradation of antioxidant itself is pronounced prominently. Greater rate of degradation of tocopherol seed in artificial aging is evidently points to the reality of seed deterioration and loss of seed longevity. In this study, just 12 h of saturated salt accelerated aging treatment was sufficient enough to bring down the content of tocopherol to a level which is equivalent to 6 months of natural aging (151 ± 39.0 mg/kg). With regard to accelerated aging, 12 h aging treatment reduced the level to the value which is equivalent to 9 months (137 ± 17.0 mg/kg). Since gamma tocopherols are the most effective antioxidant in any oil, significant decrease in their level during prolonged storage could be associated to being responsible for deterioration of seeds.