CHAPTER 5
DISCUSSION

5.1 Genotoxicity of Arecoline and its Metabolites:

In an effort to understand the comparative genotoxic potential of major areca alkaloid ARC and its primary metabolites ARK, ARC-NO and NA, I studied the effect of these compounds with respect to CA, MN, SHA assay and total sperm count (TSC). The results show that ARC, ARK, ARC-NO and NA, significantly induced CA (Table 10, 11; Table 14, 15; Table 18, 19 and Table 22, 23) (Figure 35), and MN both after 24 hour and 48 hour in a dose dependent manner (Table 12, 16, 20 and 24) (Figure 36). ARC, ARK, ARC-NO and NA also induced SHA and TSC (Table 13, 17, 21 and 25) (Figure 37 and 38), after 35 days in a dose dependent manner. It was observed that both ARC3 and ARC-NO3 show similar percentage of CA (32.01 ± 0.23 and 32.33 ± 0.05 respectively). However, it was also observed that lowest dose used for ARC-NO is most potent in inducing CA among all the three metabolites tested and the parent compound ARC itself. In MN assay, the results indicate that ARC and ARC-NO in all tested doses exhibited marked increase in the frequency of MN-PCE as compared to ARK and NA. This observation is similar to CA study, as both CA and MN assays are sensitive cytogenetic end
points for genotoxicity assessment. In the present study, all the compounds tested, induced dose dependent increase in SHA. However, the extent of maximum damage to sperm head was observed with ARC treatment followed by ARC-NO. A dose dependent decrease in the total sperm count was observed in case of all the chemicals and tested dose range. The maximum reduction was observed in case of highest dose of ARC-NO (80 mg/kg/bw) and ARC (11.00 x10^6 ± 0.89) and (11.50 x10^6 ± 1.30) respectively when compare with equal dose of ARK and NA.

Induction of CAs and SCEs by arecoline has been already reported (Panigrahi and Rao, 1982, 1984; Stich et al., 1981). There are few reports confirming the genotoxic potentials of ARC in normal mice bone marrow cells (Chatterjee and Dave, 1999; Panigrahi and Rao, 1982). Using Chinese hamster ovary cells, arecoline yielded a dose-dependent increase in the frequencies of micronucleated cells (Lee et al., 1996). There are also reports regarding induction of SCEs and CAs in peripheral blood lymphocytes of areca nut chewers (Dave et al., 1992). Both ARC and ARK have been shown to cause cell transformation in vitro and CAs in mice (Panigrahi and Rao, 1982; Chatterjee and Deb, 1999). Therefore, this study once again confirms the genotoxic potential of ARC and ARK. All the cytogenetic end points were considered to be sensitive indicators of DNA damage which increases the risk of cancer (Chaganti et al., 1974; Buckton et al., 1978). In the present study, there is incidence of DNA damage as indicated by different kinds of chromosome abnormalities.
The role of metabolites in inducing cytotoxicity and genotoxicity is well established. Accordingly, both *in vitro* metabolic activation of a compound and *in vivo* assays were routinely utilized for genotoxic hazard identification of drug candidates for a complete genotoxic profile (Dobo *et al.*, 2009). Present work is the first report of ARC-NO and NA induced genotoxicity in mice test system. In a recent report, Lin *et al.*, 2011, reported mutagenic effect of ARC and ARC-NO in *S. typhimurium* TA 100 and TA 98 strains, in the absence or presence of rat liver S9 preparation. A low mutagenic effect was observed in S9 positive fraction. Also a dose dependent increase in the mutagenic effect was observed in ARC-NO. This report support the present finding that ARC-NO is most potent in inducing CA among all the three metabolites tested and the parent compound ARC itself. This indicates the possibility of formation of intermediate, more toxic N-oxide of ARC during metabolism of ARC which causes genotoxicity as well as may play important role in inducing DNA damage. It is also possible that a process called metabolic inter-conversion takes place *in vivo* which enhance the retention time of the parent compound before complete metabolism and urinary excretion (Nery, 1971; Giri *et al.*, 2007). The formation of N-methyl NA but not ARC-NO was also detected as major metabolite in ARK treated mouse. Earlier study established the formation of ARC-NO by human xenobiotic metabolizing enzyme Flavin monooxygenase enzyme (FMO1 and FMO3 isoforms but not FMO5) in a dose and time dependent manner both *in vitro* and *in vivo* (Giri *et al.*, 2007). In general, CYP is the major contributor to oxidative xenobiotic metabolism. However, FMO activity is significantly playing role in ARC metabolism. FMO and CYP have overlapping substrate specificities, but often yield distinct metabolites with
potentially significant toxicological/pharmacological consequences. In general, metabolites produced by FMO-mediated N-oxygenation, are highly lipophilic, easily excreted, and typically exhibit markedly less pharmacological and/or toxicological properties than the parent amine or CYP mediated metabolites. For this reason, FMO-mediated N-oxygenation of tertiary amines usually represents detoxification. However, there are also exceptions where FMO mediated toxic metabolite generation. It appears that FMO induced N-oxidation of ARC may produce reactive species which is more toxic.

One report claims that ARC is cytotoxic to human gingival fibroblasts in culture by virtue of glutathione depletion and inhibition of mitochondrial activity (Valenzuela, 1991). Similar effects in both fibroblasts and keratinocytes were also observed (Benzie, 1996). GSH, NAC, Cystine or methionine could revert the Salmonella typhimurium test, indicating their active role in N-oxide of ARC induced genotoxicity. Titenium chloride is a specific reducing agent for N-oxide and it could inhibit the reaction (Lin et al., 2011). Several studies were carried out on mechanism of ARC induced transformation altering the cellular pathways in different types of cells both in vivo and in vitro. The toxicity induced by metabolites throws light on in vitro metabolite formation and its role in carcinogenesis. Therefore, the ARC-NO may play a very crucial role in ARC induced toxicity leading to cellular transformation among people with betel nut chewing habit, which needs further investigation.

In one of the study already reported that the damage induced by ARC could be due to DNA adduct formation (Nery, 1971). This can be one of the important factors in induction of tumours in betel nut chewers. It is possible
that the damage induced by ARC and its metabolites are due to DNA adduct formation, oxidative stress, generation of ROS as well as the genotoxic potentialities of individual compounds acting together playing a role in tumor formation in areca nut chewers. Intermolecular cross linkages are formed in double stranded DNA molecules and as a result the likelihood of the appearance of CA is increased (Shalumashvili and Sigidin, 1976). Clastogenicity may arise from disturbances of many cellular process and not mere from direct DNA/drug interaction. It was reported that DNA topoisomerase II inhibition may produce clastogenic effect too. Topoisomerase/DNA/ drug ternary complex forms stable DNA-double strand break which acts as a site for recombination, mutagenic and chromosomal fragmentation event. In the study by Snyder (2000) in Chinese hamster V79 cells it was observed that topoisomerase II poisons induced clastogenicity via non-topoisomerase dependent mechanisms when catalytic inhibitors of topoisomerase II was used. Because of the close relation between mutagens and carcinogens and extensive human exposure to betel nut, the present study on ARC and three metabolites ARK, ARC-NO and NA induced CA, MN and SHA assays could give light on the possible carcinogenic effect of ARC through its mutagenic activity.

To test somatic mutagens in vivo, bone marrow MN assay has been used extensively (Krishna and Hayashi, 2000; Giri et al., 2002a; Engelhardt, 2006; Lee and Lee, 2007). The significant increase in the frequency of MN in the bone marrow cells following treatment observed in the present study further indicate the mutagenic potential of ARC and its metabolites, ARK,
ARC-NO and NA. PCE counts in bone marrow is one of the most popular and convenient method of monitoring erythropoiesis. Decreases in the proportion of immature PCE to mature or NCE gives us a view of mutagen-induced cytotoxicity (Suzuki et al., 1989). There is a dose-dependent decrease in the PCE/NCE ratio indicating the mutagenic potential of test chemicals and ARC-NO show the highest decrease in PCE/NCE ratio indicating its most potent nature and its ability to induce maximum cytotoxicity. ARC-NO shows highest degree of genotoxicity when MN assay is concerned, followed by parent compound ARC and metabolites ARK and NA respectively.

The SHA assay is a sensitive and reliable parameter and is widely used to identify germ cell mutagens (Wyrobek and Bruce, 1978, Giri et al., 2002a, b; Nahas and Ashwamy, 2004). It is of utmost importance to evaluate genotoxic effects of any agent in an organism in somatic as well as in germinal cells as the later provides us with information on transmissible genetic damage from one generation to the next (Au and Hsu, 1980; Wyrobek et al., 1983). This is also reported that chemicals that induced SHA also proved to be carcinogenic. There are few reports confirming the genotoxic potentials of ARC in germ cells (Sinha and Rao, 1985). In present study, all the compounds tested, induced dose dependent increase in SHA and decrease in total sperm count, indicating
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their carcinogenic potential which causes point mutation in Y-chromosome in testicular DNA resulting into the formation of abnormal sperm head or change in sperm morphology and reduction in total sperm count in exposed individuals. The exogenous substances upon getting accumulated in germ cell pool, induce alterations in sperm morphology mainly by point mutations (Chauhan et al., 2000), small alterations in testicular DNA (Topham, 1980), structural abnormality in Y-chromosome (Styrna et al., 1991), or interference of the test substance with the genetically controlled differentiation of the sperm cells (Bruce et al., 1974; Rai and Vijaylaxmi, 2001). Induced sperm abnormalities indicate point mutation in germ cells (Narayana et al., 2002, 2005), which should have triggered structural changes in cell organelles involved in head and tail formation, leading to sperm abnormalities.

5.2 Genotoxicity of Areca Nut and Sadagura Extracts:

The present study was undertaken in an attempt to understand the genotoxic potential of AN, SG and AN+SG, by using parameters like CA, MN, SHA assay and TSC. In this study, the results indicate that there was a significant increase in the incidence of CA (Table 26, 27; 30, 31; 34 and 35) (Figure 51) and MN (Table 28, 32 and 36) (Figure 52) in the bone marrow cells of mice after 24 hour and 48 hour in a dose dependent manner. The extracts also significantly induced SHA and TSC (Table 29, 33 and 37) (Figure 53 and 54) respectively in germ cells of mice after 35 days in a dose dependent manner. It was clearly observed that for CA assay, out of the three extracts, the highest dose of AN+SG (80 mg/kg/bw) showed maximum level of CA.
(24.33 ± 0.05) compared to AN (19.33 ± 0.78) and SG (15.33 ± 0.78) treated alone. The result of MN assay indicates that AN+SG extract exhibited increase in the frequency of MN-PCE as compared to AN and SG treated alone, which indicates that among the three extracts tested AN+SG was showing highest degree of genotoxicity. This observation is similar to CA study described in previous section. There are reports regarding induction of CA in peripheral blood lymphocytes of areca nut chewers (Dave et al., 1992). The findings also show that AN and AN+SG treated group of mice, there was increase induction of CA. For SHA assay, the highest dose of AN+SG generated maximum frequency of abnormal sperms (13.46 ± 1.10) when compared to AN and SG treated alone. It was also observed that AN treated alone was showing the minimum level of toxicity. A dose dependent decrease in the TSC was observed in case of all the extracts and tested dose range and maximum reduction in sperm count was seen in highest dose of AN+SG (17.98 x10^6 ± 0.76).

In India areca nut along with tobacco chewing in various formulations are very popular. In southern part of Assam, consumption of a unique preparation a smokeless tobacco ‘sadagura’ is very popular among the local population. The highest incidence of oral cancer in India is reported in Assam in the North-East region, where it is the second leading cancer among men and third among women (Phukan et al., 2001). Areca nut along with tobacco chewing habit has been strongly associated with the causation of oral pre-malignant and malignant diseases (IARC, 1985). There has been sufficient evidence from epidemiological studies to show that BQ chewing, together with
tobacco chewing or smoking is associated with the increased risk of oral cancer (Sanghvi, 1981). There was a considerable inter-individual difference regarding dose consumed, duration of chewing and occurrence of the disease. Hence, it was difficult to rule out the exact dose and duration of chewing and formation of tumor. There is not much information available for sadagura and its association with causation of cancer. Consumption of other forms of smokeless tobacco, there is a higher exposure of a very limited mucosal surface occurs as the users habitually place these products repeatedly in the same location and the process continues for years (Rodu and Jansson, 2004). However, in case of sadagura, the entire oral cavity is exposed during chewing. Thus, intake of sadagura for years become a matter of concern regarding induction of cytotoxicity as well as genotoxicity which ultimately leading to the causation of different forms of pre-cancerous and cancerous lesions. Despite its toxic effects, little is known about the metabolism of sadagura and in combination with areca nut consumption. To date only Kausar et al. (2009) report that, sadagura, is as harmful in inducing genotoxicity as any other smokeless tobacco preparation, and most harmful when taken along with betel quid (areca nut important constituent). Their finding support the present result that AN+SG is more potent in inducing genotoxicity when compare with AN and SG treated alone. The combination study AN+SG3 is induced the highest frequency of CA, MN, SHA and reduction in TSC which might be due to conversion of certain potent mutagens and carcinogens during metabolism inside body. Nicotine is a major tobacco alkaloid, is by itself is a weak clastogen (Riebe and Westphal, 1983). Sadagura contains tobacco leaf which indicates the presence of nicotine, already reported as a clastogen.
Tobacco specific alkaloids along with areca nut alkaloids may further react and induced the cytotoxic and genotoxic effects leading to the induction of certain kind of precancerous lesions and/or tumor. It is interesting to know that tumor growth depends on a balance between cell death via apoptosis and cell proliferation (Wyllie, 1985). Many of the tobacco-areca nut specific nitrosamines are detected in the saliva of chewers (Wenke et al., 1984) and some of these are potent mutagens and carcinogens (IARC, 1985).

In this study, it was observed that AN, SG as well as AN+SG induced a decreased in MI in dose dependent manner. The decreased in MI could be due to either cell cycle blocking in G1, suppressing DNA synthesis (Schneiderman et al., 1971) or blocking in G2 preventing the cell from entering mitosis (Van’t Hof, 1968). Areca nut ingredients and metabolites may cause cytotoxicity by deregulation of cell cycle control, GSH homeostasis, mitochondrial function or reactive oxygen species production (Chang et al., 2001). Lime taken along with the tobacco may stimulate the clastogenic effect by creating an alkaline environment in the target site and is likely to generate free radicals in combination with the polyphenols (Nair et al., 1992). As can been seen in the present study, there is a time dependent decreased in the frequency of CA which might be due to repair mechanisms. Despite body's own repair mechanisms try to counter the effects, repeated and prolong used of it become a risk factor in the exposed population.

Therefore, in the light of the present findings, it can be concluded that consumption of areca nut and sadagura are harmful in inducing genotoxicity,
as well as become more harmful and genotoxic when consumed in combination with areca nut.

5.3 Lipid Peroxidation Induced by Arecoline and its Metabolites:

In an attempt to evaluate the cellular dysfunction lipid peroxidation level was measured as other toxicological parameter in the liver tissue of mice. LPO level was measured in terms of MDA concentration expressed in n mol/g wet weight. Present findings show that ARC, ARK, ARC-NO and NA, significantly induced LPO (Table 38, 39; Table 40; Table 41 and Table 42) (Figure 55, 56; Figure 57; Figure 58 and Figure 59), respectively after 24 hour exposure time in a dose dependent manner. Both i.p and oral (p.o) treatment of ARC induced LPO, after 24 hour, 48 hour and 72 hour exposure time. It is very interesting to note that (Table 38), ARC1 (20 mg/kg bw) when administered i.p induced LPO which is significantly high for 24 hour (approximately three and half times more as compare to oral route) and 48 hour (P<0.001) and 72 hour (P<0.05) study regimes when compared to the untreated control (Figure 55). Among the tested chemicals, ARC-NO gave the maximum LPO suggesting more cytotoxic potential. A decline in the LPO level with increased in exposure time was also observed, which may be due to body’s own defense mechanisms.

LPO, an indicator of damage to cell membranes, occurs when free radicals react with lipids, and is a source of cytotoxic products that may damage DNA and enzymes (Kehrer, 1993; Yu, 1994). LPO has been established as a major mechanism of cellular injury in many biological systems induced by
ROS. The present study collected evidence to indicate that exposure of ARC and its metabolites, ARK, ARC-NO and NA to liver tissue of mice leads to LPO, indicating the generation of free radicals which react with lipids present in the liver tissue cell membranes. The cellular sources of free radicals and lipid peroxidation products are shown in Figure 69, and the mechanism of LPO is given in Figure 70.

**Figure 69:**
Schematic representation of cellular sources of free radicals and lipid peroxidation products (from Jason and Marrow, 2002).

In conditions associated with high oxidative stress such as neurodegenerative diseases, or cancer, high plasma MDA concentrations have been reported (Berliner and Heinecke, 1996; Kanabrocki et al., 2002; Taysi et al., 2003). Plasma MDA concentration is generally used as an index of *in vivo* lipid peroxidation (Valenzuela, 1991; Esterbauer et al., 1991). The increase in plasma FFA concentrations was accompanied by an increase of plasma MDA levels, which is in accordance with experiments *in vivo* (Lind et al., 2000) and
vitro where polyunsaturated fatty acids but not other fatty acids served as MDA precursors (Allen and Cierzan, 1988). MDA is formed during oxidation of polyunsaturated fatty acids containing three or more double bonds (Valenzuela, 1991) and react with primary amine groups of biological molecules. In vivo MDA acts as a free aldehyde or as an adduct to other components, forming mutagenic and carcinogenic substances, or even provoke cell death (Marnett, 2002; Berliner and Heinecke, 1996; Dib et al., 2002).

Figure 70:
Mechanism of lipid peroxidation (from Jason and Marrow, 2002).

Extensive toxicological investigations have now established that increase in lipid peroxidation, along with decreased level of glutathione actually denote cytotoxicity and hepatocellular dysfunction (Plaa et al., 1991; Comporti, 1985; Deboyser et al., 1989). It is established that the lipid peroxidation increases with severity of the disease reflecting the extent of tissue injury (Halliwell and Chirico, 1993).
Oxidative stress is known to induce apoptotic cell death via p53 in several cells, including rat astrocytes and ovarian cancer cells (Bonini et al., 2004; Wu and Ding, 2002). Previous study provides evidence for the involvement of stress-responsive signaling pathways that could be a possible mechanism of ARC action leading to inflammatory oral diseases, including oral submucous fibrosis (Thangjam and Kondaiah, 2009).

Earlier report suggested that thiol depletion, but not attack by oxygen free radicals, could be the mechanism for ARC cytotoxicity (Deb and Chatterjee, 1998). However, another group reported involvement of reactive oxygen species, such as hydroxyl radicals, that are formed in the human oral cavity during areca quid chewing might cause oxidative DNA damage to the surrounding tissues (Chen et al., 2002). Present findings reveal the involvement of ROS in the induction of LPO by ARC and its metabolites which is in agreement with the already reported work of Chen et al., 2002. There may be more than one pathway involved but it further need a thorough investigation.

**5.4 Lipid Peroxidation Studies of Areca Nut and Sadagura Extracts:**

To evaluate the cellular dysfunction, LPO level was measured as other toxicological parameter in the liver tissue of mice. In the present study, AN, SG and AN+SG show a significantly increase in LPO (Table 43; Table 44 and Table 45) (Figure 61; Figure 62 and Figure 63), after 24 hour exposure time in a dose dependent manner. It is interesting to note that the lowest dose (20 mg/kg bw) of AN was not able to induced LPO significantly as compare to the untreated control. However, in case of SG and AN+SG, a statistically significant
increase in LPO at (P<0.01) level of significance when compared to the untreated control was observed. The middle dose (50 mg/kg bw) and the highest dose (80 mg/kg bw) of SG as well as AN+SG show a highly significant increase at (p<0.001) in LPO compared to the untreated control (Figure 62 and 63). The highest frequency of LPO was observed in highest dose of SG and AN+SG showing LPO value (11.94± 0.22) and (11.57± 0.30) respectively.

Singh and Rao (1995b) reported in Swiss albino mice the introduction of areca nut in the diet showed significant increases in cellular MDA levels. Their work is a strong support to the present work; present findings also show a dose dependent increase in concentration of MDA (expressed in n mol/g wet tissue) in the liver of mice after AN, SG and AN+ SG treatment.

In the present study, it is observed that MDA levels, a lipid peroxidation product and a marker of oxidative stress, were elevated significantly in exposed mice. This clearly shows that mice, irrespective of the sex, were exposed to an increased oxidative stress via lipid peroxidation. The concept of "oxidative stress" is attributed to the German scientist Heimut Seis. Oxidative stress occurs when there is a higher concentration of oxidants than that of the opposing antioxidants. Basically our body uses oxygen for the maintenance of life. However, utilization of oxygen produces a long term risk. This risk is related to attack on our cellular macromolecules, e.g., protein, DNA, RNA, and lipid, from the myriad of different oxygen linked substances (Overview of Oxidative Stress by Knox Van Dyke).
Reactive oxygen species (ROS) is a term which encompasses highly reactive oxygen containing molecules, indicating free radicals. Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides (Percival, 1998). All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage. ROS are generated by a number of pathways, of which one of the important pathways is xenobiotic metabolism, i.e., detoxification of the toxic substance. There are different sources of hydrogen peroxide during xenobiotic metabolism. The majority of hydrogen peroxide-induced damage is thought to be due to transition metal ion-catalyzed production of the highly reactive hydroxyl radical, $\text{OH}^-$, by the Fenton reaction. As for example, in the mechanism of formation of ROS from polyphenols present in betel quid ingredients, areca nut and catechu, we can see the occurrence of Fenton reaction as shown in Figure 71 below:

![Chemical reaction diagram]

Adapted from Nair et al. (1996)

**Figure 71:**
Mechanism of formation of Reactive Oxygen Species from polyphenols present in betel quid ingredients, areca nut and catechu.
Experimental studies in mammalian cells have demonstrated that active oxygen radicals may contribute to clastogenesis directly (Ochi and Keneko, 1989) and indirectly through the production of lipid peroxides (Emerit et al., 1991). Increased production of lipid peroxides, malondialdehyde, as observed in the present study, strongly suggest that the extracts caused generation of ROS and oxidative stress in mice liver. This indicates that free radical-induced oxidative damage to DNA could be a factor in mediating the cytogenetic changes in liver cells with the extract exposure. Malondialdehyde, a major end product and biomarker of LPO, reacts with deoxyribonucleosides to produce DNA adduct and is documented as mutagenic in mammalian cells (Yau, 1979).

The SG is more potent than AN treated alone in the cytotoxicity in liver cells of mice as was evidenced by increase in LPO. Sadagura is more potent than areca nut which may be due to the presence of nicotine from the tobacco leaf used in its preparation. An in vitro study done by Chang et al., 2002 concluded that intracellular thiol depletion could be the mechanism for nicotine toxicity. Therefore, it can be conclude that LPO induced by AN, SG and AN+SG might be due to increase generation of ROS and reduced thiol level in the exposed individuals.

5.5 Vitamin C on Arecoline and its Metabolites Induced Lipid Peroxidation:

The experiments were carried out to evaluate the effect of Vit. C on LPO induced by ARC and its metabolites ARK, ARC-NO and NA. When same dose (50 mg/kg bw) of ARC, ARK, ARC-NO and NA was given to mice after 24 hour exposure time, a statistically significant increased in LPO was observed
when compare with untreated control. In the experiment undertaken, single
dose of antioxidant vitamin C (Vit. C = 500 mg/kg bw) was used. Vit. C was
administered p.o. It was interesting to note that in case of mice pre-treated
with Vit. C for five consecutive days, treated with ARC, ARK, ARC-NO and NA
fail to induce LPO significantly, and a marked (approximately two to three fold)
decline in LPO level was observed (Figure 60).

Vitamin C is one of the most widely studied dietary antioxidant. It is
considered the most important water-soluble antioxidant. The physiological
functions of ascorbic acid are largely dependent on the oxido-reduction
properties of this vitamin. Antioxidation (reduction) is the reverse of oxidation-
a gain of electrons, a gain of hydrogen, or a loss of oxygen. Most of the body’s
defense reactions (if not all) that are protective (defensive) are in fact
oxidations. However, there must be opposing forces that counter balance the
oxidation and this is called antioxidation. The body must be maintained in an
equilibrium or status quo, called homeostasis, where the two opposing forces
must be roughly balance if not weight toward antioxidants for protection
against the ravages of oxidation. The maximum oxidized state (at least when
compare to life) is probably death itself. But, as the body disintegrates even
more, continues oxidation must take place (perhaps because of continued
depletion of antioxidants) (Overview of Oxidative Stress by Knox Van Dyke).

Vitamin C is a known free-radical scavenger and has been shown to
inhibit LPO in liver and brain tissue of lead-exposed animals (Patra et al.,
2001). In lead-exposed rats, a minimal 500 mg/L concentration in drinking water
was able to reduce ROS levels by 40 percent (Hsu et al., 1998). Another
research work also reported that ingestion Vit.C causes reduction in the amount of products of LPO produced (Luqman et al., 2008). The reported works are in good accordance with the present finding. Present data reveal that in case of Vit. C, pre-treated mice the level of LPO were significantly lowered (Figure 64), indicating strong scavenging capacity of Vit. C-dependent antioxidant defensive system against elevated LPO processes in exposed mice. Since Vit. C is a potent reducing agent, it is capable of donating electron to ARC and its metabolite generated free radicals and stabilize the free radicals from ROS induced oxidative stress, thus protecting the cells membranes lipids from cellular injury. In addition, Vit. C can regenerate vitamin E (Sies et al., 1992; Percival, 1998) and other antioxidants such as α-tocopheroxyl, urate and β-carotene radical cation from their radical species (Halliwell and Gutteridge, 1986). Thus, ascorbic acid acts as co-antioxidant for α-tocopherol by converting α-tocopheroxyl radical to α-tocopherol and helps to prevent the α-tocopheroxyl radical mediated peroxidation reactions (Neuzil et al., 1997).

There was a matter of debate and controversies on health benefits of Vit. C (Akhilender, 2003). The protective role of Vit. C against oxidative DNA damage is a matter of much controversy (Riviere et al., 2006). However, various studies reported the protective role of Vit. C against oxidative DNA damage (Sardas et al., 2006; Ajey et al., 1992; Song et al., 2006). Vit. C significantly decreases the adverse effect of reactive species such as reactive oxygen and nitrogen species that can cause oxidative damage to macromolecules such as lipids, DNA and proteins which are implicated in chronic diseases including cardiovascular disease, stroke, cancer (Halliwell and Gutteridge,
Vit. C is a potent water soluble antioxidant capable of scavenging/neutralizing an array of reactive oxygen species viz., hydroxyl, alkoxyl, peroxyl, superoxide anion, hydroperoxyl radicals and reactive nitrogen radicals such as nitrogen dioxide, nitroxide, peroxynitrite at very low concentrations (Carr and Frei, 1999). Considerable biochemical and physiological evidence suggests that ascorbic acid functions as a free radical scavenger and inhibit the formation of potentially carcinogenic N-nitroso compounds (Schorah et al., 1991; Sobala et al., 1991; Drake et al., 1996). However, some group of researchers also believe that Vit. C prevents cancer by neutralizing free radicals before they can damage DNA and initiate tumor growth and or may act as a pro-oxidant helping body's own free radicals to destroy tumors in their early stages (Block, 1991; Frei, 1994; Uddin and Ahmad, 1995).

It has been reported that the distribution of Vit. C and its concentration in the organs are subjected to variations depending on introduction of different drugs (Linster and Schaftingen, 2007). Nevertheless, chronic treatment with Vit. C alters the dynamic equilibrium between production and elimination and thereby decrease MDA plasma levels (Desnuelle et al., 2001; Helen and Vijayammal, 1997). The importance of Vit. C as a plasma antioxidant is confirmed, by the existence of a strong inverse correlation between lipid peroxides and Vit. C plasma levels. The present study regarding LPO shows that the LPO induced by ARC and its metabolites could be reversed by Vit. C treatment, indicating the possible role of involvement of ROS in the underlying mechanism for ARC and its metabolites induced cytotoxicity.
5.6 Vitamin C on Areca Nut and Sadagura Extracts Induced Lipid Peroxidation:

The present study was undertaken in an effort to find out whether Vit. C had any protective role in the AN, SG and AN+SG induced LPO in the liver tissue of mice. When same dose (50 mg/kg bw) of AN, SG and AN+SG was given to mice after 24 hour exposure time, a statistically significant increased in LPO was observed when compare with untreated control. In the Vit. C supplementation study, it was observed that the LPO induced by extracts on liver tissue of mice was significantly (P<0.001) reduced by prior administration of the antioxidant Vit. C, showing level of protection after 24 hour exposure time (Figure 64). This clearly indicates the free radical scavenging capacity or antioxidant property of Vit. C.

Present findings are strongly supported by the other research works which suggest a role of Vit. C in reducing LPO (Glady et al., 2008) and supplementation of Vit. C reduced LPO (Huang et al., 2002). Abnormally-high levels of free radicals, lipid peroxidation and simultaneous decline in antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes. Antioxidant -dependent defenses play an important role in scavenging free radicals produced under oxidative stress.

These radical species are highly reactive and can trigger LPO reactions (Figure 71). Vit. C is capable of neutralizing ROS in the aqueous phase before LPO is initiated (Percival, 1998). Vit. C has the ability to sequester the singlet oxygen radical, stabilize the hydroxyl radical, and regenerate reduced vitamin E back to the active state. These functions work to halt peroxidation of cellular
lipid membranes (Kaminski and Boal, 1992). Vitamin C is a primary water-soluble antioxidant in plasma and within cells, but it can also interact with the plasma membrane by donating electrons to the α-tocopheroxyl radical and a trans-plasma membrane oxidoreductase activity (Luqman et al., 2008).

\[
\begin{align*}
AH^- + Fe^{3+} & \rightarrow A^- + Fe^{2+} + H^+ \\
AH^- + Cu^{2+} & \rightarrow A^- + Cu^+ + H^+ \\
H_2O_2 + Fe^{2+} & \rightarrow HO^+ + Fe^{3+} + \cdot OH \\
H_2O_2 + Cu^+ & \rightarrow HO^+ + Cu^{2+} + \cdot OH \\
LOOH + Fe^{2+} & \rightarrow LO^- + Fe^{3+} + \cdot OH \\
LOOH + Cu^+ & \rightarrow LO + Cu^++ \cdot OH \\
HO^*, LO^- & \rightarrow \text{Lipid peroxidation}
\end{align*}
\]

**Figure 72:**
Lipid peroxidation reactions triggered by free radicals (Adapted from Carr and Frei, 1999).

When we discuss about the functions of Vit. C in preventing or lessening oxidative stress a controversy arises in some findings. In one of the reported work, they observed that supplementation with two 500 g dosages of Vit. C for one day is associated with a decrease shift in prooxidant activity. The same dosage given over a two-week period did not elicit as great a change in prooxidation activity as a one-day supplementation provided (Alessio and Blasi, 1997). The difference may be related to the fact that one day dose, helped to regenerate the antioxidants vitamin E in the body. The authors hypothesize that a two-week period of Vit. C supplementation may replenish other antioxidants and then lead to prooxidant properties within body (Alessio and Blasi, 1997), likely via the Fenton reaction. The information regarding Vit C seems promising, but the need of further research in this area is also
required. Thus the question arises whether Vit. C acts as a pro-oxidant in \textit{in vivo} conditions? The answer appears to be "no" as though these reactions occur readily \textit{in vitro}, its relevance in \textit{in vivo} has been a matter of debate concerning ready availability of catalytically active free metal ions \textit{in vivo} (Halliwell and Gutteridge, 1986). In biological systems, iron is not freely available, but it is bound to proteins like transferrin, hemoglobin and ferretin. Mobilization of iron from these biomolecules may be required before it can catalyze LPO. The concentration of free metal ions in \textit{in vivo} is thought to be very low as iron and other metals are sequestered by various metal binding proteins (Halliwell and Gutteridge, 1986). Another factor that may affect pro-oxidant vs antioxidant property of Vit. C is its concentration. The \textit{in vitro} data suggest that at low concentrations Vit. C act as a pro-oxidant, but as an antioxidant at higher levels (Buettner and Jurkiewicz, 1996). Presently, high concentrations of Vit. C (500 mg/kg bw) was used, which might be the probable reason behind acting as an antioxidant. Moreover, Akhilender, 2003 reported and demonstrated that large doses of exogenous iron (200 mg) and Vit. C (75 mg) promotes the release of iron from iron binding proteins and also enhances \textit{in vitro} LPO in serum of guinea pigs. Their finding supported the hypothesis that high intake of iron along with Vit. C could increase \textit{in vivo} LPO and therefore could increase risk of disease (Chen \textit{et al.}, 2000). However, another study demonstrated that in iron over loaded plasma, ascorbic acid acts as an antioxidant and prevents oxidative damage to lipids \textit{in vivo} (Berger \textit{et al.}, 1997).
In conclusion it has been demonstrated that exposure of AN, SG and AN+SG is associated with increased MDA production, a LPO product and a marker of oxidative stress. The positive influences of Vit. C on MDA production or LPO suggests a potential need for Vit. C during exposure of areca nut and sadagura alone or in combination chewing habit in order to reduce LPO. Stress, smoking, alcoholism, fever, viral infections cause a rapid decline in blood levels of ascorbic acid. Therefore, it might be beneficial for those persons who chew or consume areca nut and/or sadagura for a long period of time to consume Vit. C rich diet in order to combat health hazards related with their exposure. There may be need for Vit.C to be included among the routine balance diets. Vit. C (Ascorbic acid) is widely distributed in fresh fruits and vegetables. It is present in fruits like orange, lemons, grapefruit, watermelon, papaya, strawberries, cantaloupe, mango, pineapple, raspberries and cherries. It is also found in green leafy vegetables, tomatoes, broccoli, green and red peppers, cauliflower and cabbage. The new average daily intake level that is sufficient to meet the nutritional requirement of ascorbic acid or recommended dietary allowances for adults (>19 yr) are 90 mg/day for men and 75 mg/day for women (Frei and Traber, 2001). Consumption of 100 mg/day of ascorbic acid is found to be sufficient to saturate the body pools (neutrophils, leukocytes and other tissues) in healthy individuals. Based on clinical and epidemiological studies it has been suggested that a dietary intake of 100 mg/day of ascorbic acid is associated with reduced incidence of mortality from heart diseases, stroke and cancer (Carr and Frei, 1999).
5.7 Tumorigenicity of Areca Nut and Sadagura Extracts:

The present experiment was undertaken in an effort to find out the effects of areca nut, sadagura and areca nut with sadagura extracts on Dalton's Lymphoma ascites bearing mice. Further, to see the trend with those groups of mice treated separately with extracts to normal mice without lymphoma for a long term. For in vivo tumorigenicity studies, mean survival time, body weight analysis and packed cell volume (PCV) were taken into consideration.

The effect of AN, SG and AN+SG on the mean survival time of with or without Dalton's Lymphoma bearing mice is presented in Table 46 and Figure 65. All the individual in the negative control group survive for 90 days i.e., for the whole period of the experiment. The group which was treated with AN (80 mg/kg bw/day for 14 days) survive for average of 85 days ± 1.23. However, in case of SG and AN+SG treated group duration of life span is found to be reduced significant (P<0.001) with an average life span of 60.16 ± 3.03 and 59 ± 2.71 days respectively. There was a mark decrease in mean survival time of DLA bearing mice which was statistically significant at P<0.001 when compare to the control group. Also is clearly indicted from the results of DLA model, out of the three different extracts used, AN+SG showed maximum decrease (27.00 ± 0.65) in mean survival time as compared to AN and SG treated alone. This clearly indicates the more toxic nature of areca nut when consume in combination with sadagura, this result is similar to the genotoxicity studies.

In the present in vivo cancer study of extracts of areca nut and sadagura alone or in combination was evaluated against a Dalton's lymphoma ascites.
The reliable criteria for judging the value of any cancer activity is the reduction of life span of animal. Hence, the mean survival time was studied. The intraperitoneal inoculation of DLA cells in the mice produces increased proliferation of cells. In DLA ascite model following the inoculation of tumor cell lines (DLA) a marked decrease (P<0.001) in life span and increase in body weight of mice were observed. Following the administration of AN, SG and AN+SG, proliferation of cells were observed. AN+SG treated groups had shown considerable decreased in MST and duration of life span and decrease in the body weight. The AN, SG and AN+SG treated mice survived upto 30, 31.33 and 27 days only respectively whereas the tumor control mice survived upto 35 days.

The effect of AN, SG and AN+SG on the body weight of with (Figure 67) or without (Figure 66) Dalton’s Lymphoma bearing mice is presented in Table 47. A regular rapid increase in ascites tumor volume was noted in tumor bearing mice. Linear progression in the body weight of tumor bearing mice with advancement of duration was observed. The body weight was found to have a significant increase (P<0.001) in cancer control (only DLA) group when as compared with the untreated control group (Figure 67). Such increase in body weight was not able to retard significantly following the extracts treatment. It showed that tumor regression was not very effective as was observed in tumor bearing mice receiving single dose of extracts for consecutive fourteen days. The changes in total body weight of tumor-bearing mice noted under different experimental conditions reveal definite changes in tumor growth and relate with the changes in host’s survivality rates. It was reported that the
presence of tumor in humans or experimental animals is known to affect many functions of the vital organs especially the liver, even when the site of the tumor does not interfere directly with organ functions (De Wys, 1982).

The tumor volume in terms of Packed Cell Volume of DLA bearing mice after thirty five days of AN, SG and AN+SG treatment is presented in Table 48 and Figure 68. Present result show that PCV was found to increase significantly at (P<0.001) in SG (80 mg/kg bw) alone treated group (5.25 ± 0.38) as well as in AN+SG extract (80 mg/kg bw) treated group (3.84 ± 0.08) respectively when compared with the cancer control (only DLA) group (1.16 ± 0.12).

The ascitic fluid is essential to tumor growth, since it constitutes the direct nutritional source for tumor cells (Gupta et al., 2004). PCV in cancer control mice increase significantly when compared with normal group. Moreover, the extracts treatment after thirty five days, increases packed cell volume content. The packed cell volume in the peritoneum was significantly increased in mice treated with the SG alone or in AN+SG treated groups in comparison with the tumor control group. These results could indicate either a direct cytotoxic effect of the extracts or indirect local effect, concerning macrophage activation and vascular permeability inhibition.

Therefore, in the light of the present finding, it can be concluded that the AN, SG and AN+SG treatments was not able to inhibit tumor cell growth and decreases the PCV. Furthermore, treatment with AN, SG and AN+SG reduces the survival time significantly and a decrease in body weight was observed in exposed mice.