CHAPTER IV

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The efficacy of papaya seed extracts viz. Methanol alone, Butanol alone, mixture of Aqueous:Benzene (1:1) and mixture of Aqueous:Alcohol:Benzene (4:2:1) as a contraceptive agent was tested on female albino mice (*Mus musculus*) of Swiss strain. These extracts were administered orally at a dose of 20 mg/kg body weight for 15 and 30 days respectively. Recovery of the induced effects, if any, was studied after 30 days withdrawal of treatment to investigate reversibility.

The papaya seed extracts were administered orally to groups of animals everyday for 15 and 30 days respectively. In our laboratory various routes for administration of the extracts i.e. intramuscular, subcutaneous, gastric intubation and oral have been tried on both sexes of rats and mice (Chinoy and Sam George, 1983; Chinoy and Geetha Ranga, 1984; Chinoy et al., 1984/85, 1994, 1995a, 1995b; Chinoy and Padman, 1996; Joshi and Chinoy, 1996) and all routes of administration were found to be effective in both males as well as on females for inducing fertility. However, in the present study, the oral route was selected for the treatment which is an easy and feasible way to administer the extract as well as keeping in mind its future use in humans.

The various parameters studied at the end of treatment were estrogenicity checking of methanol and butanol papaya seed extracts, LD<sub>50</sub> studies, body and organ weights, histology and ultrastructure of reproductive organs. Certain specific parameters viz., Cholesterol, activities of 3β and 17β hydroxysteroid dehydrogenases, serum FSH, LH, E2 and progesterone levels were investigated to study the impact of
papaya seed extracts on ovarian functions. In addition, the concentrations of glycogen, activity of phosphorylase were studied in uterus. The levels of DNA, RNA and protein were investigated in ovary and uterus to study the effects of treatment on nucleic acid metabolism in these organs. Ovarian ascorbic acid and glutathione levels as well as some selected parameters in liver, kidney and serum were also investigated to study toxicological effects if any. The fertility rate and cyclicity of the treated animals were studied during the course of the investigation.

In a different set of experiments, the treatment was withdrawn for 30 days after respective 15 and 30 days of treatments to study the reversible effects if any, upon withdrawal of treatment.

**Estrogenicity checking of papaya seed extracts:**

The methanol and butanol papaya seed extracts were tested for estrogenicity according to WHO Protocol MB-70/71 prior to further studies. Ovariectomised, extract-treated immature female mice did not reveal any estrogen dependent changes such as an increase in body weights or absolute uterine and adrenal weights, or the presence of vaginal opening. The study revealed that the extracts were non-estrogenic in mice. Moreover, the earlier work from our laboratory and elsewhere has elucidated that the aqueous, alcohol, benzene and hexane papaya seed extracts are devoid of any estrogenic activity (Chinoy et al., 1994; Chinoy and Padman, 1996; Joshi, 1995; Keshri et al., 1993.) Lohiya and Goyal (1992) showed that crude chloroform extract was mildly estrogenic in nature. However, this study was carried out by an estrogenic bioassay and not by standard WHO Protocol.

LD$_{50}$ studies revealed that both methanol and butanol papaya seed extracts were
non-toxic. The LD$_{50}$ does was 18 and 20g/kg body weight which is much higher than the LD$_{50}$ dose of 5g/kg body weight recommended by the WHO as of non toxic nature for a plant product. Earlier studies have also reported similar results in aqueous, alcohol and benzene papaya seed extracts (Chinoy et al., 1994; Joshi, 1995; Padman, 1995) in corroboration with the present data.

The results revealed that the body as well as organ weights of the experimental animals remained unaffected throughout the treatment. This indicated that the extracts do not promote weight gain causing obesity and water retention. Similar result was also obtained by Das (1980) in male rats by treatment with papaya seed extract. Moreover, the earlier work from our laboratory has also elucidated that during administration of different papaya seed extracts in male as well as female rodents, the body and organ weights were not affected (Chinoy and Sam George, 1983; Chinoy and Geetha Ranga, 1984; Chinoy et al., 1984/85, 1994, 1997a, Chinoy and Padman, 1996). Lohiya and his associates confirmed our data on ethanolic and aqueous extracts in male rodents (Lohiya et al., 1992, 1994).

Maintenance of electrolyte balance is important as changes in the level of sodium and potassium may cause loss of water from the cells and tissues leading to reduction of body weight. However, in the present study, no alterations in serum Na$^+$, K$^+$ and Ca$^{2+}$ ions occurred. This indicates that neither water retention occurred in the body nor electrolyte balance was disturbed. The extracts did not promote body weight gain nor affect the various calcium dependent functions of the body. The maintenance of ionic balance is important, since studies on an earlier contraceptive agent, viz., gossypol was found to cause chronic hypokalemia in humans (Prasad and Diczfalusy, 1983).
The osmotic and electrolyte balance was maintained which is in concomitance with earlier work (Chinoy and Padman, 1996) in male rodents. Kidney protein, creatinine levels and its histology were not affected by the extract treatments suggesting that kidney functions were not affected.

It is known that serum protein level plays an important role in maintaining osmotic balance and any alterations would cause oedema. However, in the present study, no such changes were obtained in confirmation with earlier studies carried out on male mice (Chinoy et al, 1984/85, 1994).

The histological and ultrastructural studies on ovary of mouse treated with different papaya seed extracts revealed no structural alterations after 15 and 30 days as compared to control. All the papaya seed extract treated mice ovary showed normal histological features. The same observations were also recorded by earlier workers in our laboratory (Trivedi, 1982 and Joshi, 1995).

In the present study, the exploration of intermediary enzymes in the steroidogenic pathway after different papaya seed extract treatments revealed no alteration in the activities of 3β hydroxysteroid dehydrogenase (HSD) (which converts dehydroepiandrosterone to androstenedione) and 17β hydroxysteroid dehydrogenase (which converts testosterone to androstenedione). The ovarian cholesterol levels also remained unaffected suggesting that the ovarian steroidogenesis was unaltered in treated female mice. This data is further supported by the normal levels of serum FSH, LH, estradiol and progesterone in treated mice indicating that the hypothalamo-pituitary-gonadal axis, ovarian structure and functions as well as cyclicity of the animals remained unaffected. Earlier studies have also reported unaltered steroidogenesis in case of male and female rodents (Chinoy and Geetha Ranga, 1984;
Chinoy et al., 1984/85, 1994, 1995a, 1995b, 1997a; Chinoy and Padman, 1996; Joshi and Chinoy, 1996; Joshi, 1995) in corroboration with the present data. This finding is significant since the libido will not be affected and the extracts do not seem to possess any antigonadotrophic effects. Gopalakrishnan and Rajasekharasetty (1978) reported that the unripe papaya fruit and seeds mixed with normal diet interrupted the estrous cycle and induced abortions. However, in the present study the estrous cycle was not affected as the hormone levels were normal. This discrepancy might be due to differences in experimental protocol test material, animal model used. These authors had used pregnant females. The effect on cyclicity has to be tested on normal cycling animals and not in pregnant ones.

The total ascorbic acid (TAA) levels were enhanced in the ovary in all treated groups of mice, concurrent with a decrease in those of dehydroascorbic acid (DHA). This increase in TAA levels probably occurred to overcome the stress imposed by the treatment, as AA is known to be a potent reducing agent and its increase is an adaption to overcome the probable effects of the drug induced histamine which is toxic and known to be detoxicated by AA (Chinoy, 1978). Moreover, the decrease in glutathione levels in ovary after treatment could also contribute to the increase in reduced ascorbic acid levels. Similar results have been reported earlier which supports the present data (Chinoy et al., 1995b).

The RNA and DNA levels in ovary and uterus in all treated groups of mice were not affected suggesting that nucleic acid metabolism remained unaltered. This data is supported by the normal ultrastructural features of these organs which revealed a well developed nucleus bounded by nuclear membrane and well dispersed chromatin. Moreover, the seed extracts did not affect the frequency of sister chromatid exchange.
or cause chromosome aberrations in human leucocytes *in vitro* (Chinoy et al., 1997c).

The ovarian and uterine protein levels were decreased suggesting that protein metabolism was affected after treatments. Similar results were observed by others in agreement with the present data (Chinoy and Geetha Ranga, 1984; Chinoy et al., 1984/85, 1995a, 1995b, 1997a, 1997d; Chinoy and Padman, 1996; Joshi and Chinoy, 1996).

The above mentioned changes in uterus after different extract treatments might influence its internal milieu, growth of glands and hence secretion, as is evident from the decrease in protein, glycogen levels and alteration in phosphorylase activity. The glycogen concentration of the uterus has long been implicated as an important nutritional reserve for the many energy consuming reactions that take place in this dynamic proliferating tissue. The energy requirements of the uterine smooth muscle contractile process as well as the metabolic events surrounding nidation are believed to be satisfied in a major part by a rapid mobilization of glucose from the uterine glycogen reserve (Demers et al., 1972). Hence its decreased utilisation would affect the normal functioning of the uterus and would alter its internal milieu.

These investigations were supported by histological studies of uterus. The uterus is the site of implantation of the blastocyst. Therefore, it is necessary to maintain the internal milieu of the uterus in the normal state conducive for implantation of the blastocyst and its further development into the embryo. Papaya seed extract treatments caused only a decrease in the extent of serosa, myometrium, endometrium, its glands, lumen diameter and some vacuolisation in the endometrium which resulted in decline in uterine secretions. These results are in agreement with those of Chinoy et al. (1997a). However, the ultrastructural studies on uterus of treated
mice showed no alterations in the cytoplasmic organelles of the epithelial cell. Hence it is likely that the decrease in secretions might be related to the decline in number of uterine glands.

The above mentioned changes resulted in disturbance in uterine milieu and caused lack of implantation and complete loss of fertility in treated females. Earlier reports have also revealed that the antifertility activity of papaya seed extracts in female rats was due to their anti-implantation and abortifacient effects (Chinoy et al., 1995b, 1997a; Joshi and Chinoy, 1996). Moreover, Garg and Garg (1970b) and Sareen et al. (1961) have also reported that antifertility effect of papaya unripe fruit and seed in female rat and mice respectively, were due to its anti-implantation activity.

Saha et al. (1961) showed that papaya fruit latex possesses oxytocic, emmenagogue and/or abortifacient properties. Kapoor et al. (1974) tested three different extracts of papaya seeds viz. petroleum ether, alcoholic and aqueous for their possible anti-ovulatory activity in rabbits. The petroleum ether extract of papaya seed inhibited ovulation in 20% of rabbit but the alcohol and aqueous extracts lacked antiovulatory activity in the rabbit (Kapoor et al., 1974). According to Gopalakrishnan and Rajasekharasetty (1978) the pulp of unripe fruits of papaya showed abortifacient activity in pregnant rats which was partially counteracted by exogenous progesterone. The surviving foetii were without any malformations. However, in the present study, progesterone levels remained unaffected. Hence, abortifacient and/or anti-implantation activity was not due to change in progesterone levels but related to the changes in uterine milieu and growth of its glands. Similarly, Kamboj (1988) has reviewed different Indian medicinal plants with interceptive activity and mentioned that unripe fruit of papaya showed (100%) and seeds (60%) showed abortifacient as well as anti-
implantation activity in female rodents.

The mechanism of action of papaya seed extracts at dose of 20mg/kg body to mice seems to be through a direct action on the uterine milieu rendering it non-conducive for implantation and hence resulting in loss of fertility.

The toxicological studies showed that histology of liver and kidney as well as their metabolism were not affected in all treated groups of animals. It is known that in a normal liver cell, glutamate oxaloacetate transaminase (GOT) is localised in the mitochondria and cytoplasm whereas, glutamate pyruvate transaminase (GPT) is localised in cytoplasm (Merck, 1974). Increase in the levels of these enzymes in the serum indicates hepatocellular death or damage caused by hepatotoxins which enhance the hepatic cell membranae permeability leading to the release of these enzymes in the blood stream (Hess, 1962). However, such changes were not found in the present study, since the SGPT and SGOT levels in the serum of the treated mice were within the normal range in support of the earlier work (Chinoy et al., 1984/85, 1994, 1997b, Lohiya et al., 1992; Lohiya and Goyal, 1992). The histology of the liver, its glycogen, phosphorylase and protein also revealed no alterations in papaya seed extract treated animals which suggest that the extract treatments had no adverse effects on the structure and metabolism of liver.

Total serum cholesterol level are known to be raised in cases of hypercholesterolemia, hyperlipidemia, hyperthyroidism and obstructive jaundice (Merck, 1974b). In the present study, serum cholesterol remained unchanged in all treated groups of mice in agreement with earlier work (Chinoy et al., 1984/85, 1994, 1997b) and elucidate that cholesterol metabolism was not affected. This observation is further supported by the fact that the ovarian cholesterol levels, circulating estradiol
and progesterone levels were also not altered.

The LD<sub>50</sub> dose was much higher than the dose of 5 g/kg body weight recommended by the WHO as of non-toxic nature for a plant product. This revealed that the papaya seed extracts used were non-toxic. Other workers (Lohiya and Goyal, 1992; Lohiya et al., 1994) have also reported that the papaya seed extracts (aqueous, alcohol and chloroform) was non-toxic in male rodents.

In order to investigate the reversibility of the induced effects by the treatments, a group of animals were treated for 15 and 30 days with the extracts and thereafter the treatment was withdrawn for another 30 days and the reversibility was studied. The findings revealed that all the induced effects were recovered to normal values after 30 days of withdrawal of treatment which also restored the fertility rate to 100% positive. Thus, the extract possesses reversible antifertility effects without any apparent toxic side effects. These extracts did not affect the ovarian function and structure, however, uterine secretions were altered thereby affecting the fertility of mice.

From the above data, it is evident that the oral treatment with papaya seed extracts, viz. Methanol, butanol, Aqueous:Benzene and Aqueous:Alcohol:Benzene manifested reversible anti-implantation and antifertility effects. The extracts were non-estrogenic, had no effect on hypothalamo-hypophysial gonadal axis and were non-toxic. Hence, functional sterility could be induced in female mice by the papaya seed extract treatments.