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SPERMATOTOXIC EFFECT OF OCHRATOXIN AND ITS AMELIORATION BY *EMBLICA OFFICINALIS* AQUEOUS EXTRACT

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Abstract: The present study was carried out to evaluate the spermatotoxic effect of ochratoxin and it's amelioration by *Emblica officinalis* aqueous extract. When male albino mice were treated with ochratoxin (50 and 100 µg/0.2 mL of olive oil/animal/day for 45 days, orally) alterations in various reproductive parameters were observed (sperm count, sperm motility, sperm viability and fertility rate), when further treated with the aqueous extract of *Emblica officinalis* (2 mg/animal/day for 45 days) amelioration was noted in ochratoxin-induced spermatotoxic effect. Oral administration of ochratoxin for 45 days caused, as compared to vehicle control (Group 2), dose-dependent significant ($p < 0.05$) reduction in cauda epididymal sperm count, sperm motility, sperm viability and fertility rate (Groups 4, 5). Oral administration of aqueous extract of *Emblica officinalis* alone did not cause any significant changes in above mentioned parameters (Group 3). However, *Emblica officinalis* aqueous extract along with ochratoxin treatment caused significant recovery in all the sperm parameters as well as in fertility rate (Groups 6, 7) in comparison with ochratoxin alone treated animals (Groups 4, 5). Amelioration was higher in high dose ochratoxin plus extract treated animals than that of respective low dose. When normal human sperm cell suspension was treated with ochratoxin (*in vitro*), various morphological alterations were observed. These were mitigated further, when treated with aqueous extract of *Emblica officinalis*.

Keywords: ochratoxin; spermatotoxic; *Emblica officinalis* aqueous extract; cauda epididymis; human spermatozoa

Mycotoxins are produced in cereals either during pre- or post-harvest conditions by the growth of toxigenic fungi. The most significant mycotoxins are contaminants of agricultural commodities, food and feeds. The Academy of Grain Technology (1). has been analyzing cereals and other food samples for mycotoxins for over a decade. There are over 200 recognized mycotoxins, however, the study of mycotoxins and their health effects on human is in its infancy and many more are waiting to be discovered (2, 3). Generally, mycotoxins are a group of low molecular weight organic compounds characterized by their diversity, their frequent specificity with regard to the taxonomy of the producing organisms and their production during the stationary phase of batch cultures. Male reproductive health has been deteriorated in many countries during the last few decades. A number of toxins in environment have been suspected to affect reproductive system in male, and ochratoxin is considered to be one of them.

Ochratoxins are one of the natural mycotoxins which are secondary toxic fungal metabolites produced mainly by *Aspergillus ochraceus* and *Penicillium verrucosum* (4). Human exposure to ochratoxin is a widespread problem in certain European countries and in India too. The contamination of various food products by this group of mycotoxins has lead to ochratoxicosis in both human beings and animals (5).

Lipid peroxidation and oxidative stress is believed to play an important role in ochratoxin-induced toxicity. During the last two decades scientists are in search for new plant products having antioxidative properties and their role in the prevention of various diseases associated with oxidative stress, such as cancer, cardiovascular, reproductive and neurodegenerative diseases. So far 119 secondary metabolites have been isolated from higher plants, which are globally used as drugs. Free radicals with other reactive oxygen species (ROS) are normal by-products of the oxidative processes in cells and large numbers of naturally occurring components in various fruits, vegetables and spices have an ability to dispose off free radicals and limit their tissue damaging effects. Recently, plant-derived products have gained much attention in different pharmaceutical preparations due to their pharmacological properties.

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Emblica officinalis commonly known as amla (synonym – Indian gooseberry) is one of the fruits which contains an array of bioactive components showing antioxidative property and is widely used in India as a traditional medicine (6, 7), ayurvedic herbal formulation and also in Unani medicines. The fruits of *Emblica officinalis* have been used in treatment of vomiting, hemorrhage, fever, cough, dyspnea, eye inflammation, ulceration, anorexia, emaciation, scurvy, diabetes, jaundice, menorrhagia, leucorrhoea, common cold, heart diseases, cancer, hepatotoxicosis and renotoxicosis (8 – 10).

In the present study we have evaluated the possible ameliorative effect of *Emblica officinalis* extracts on ochratoxin-induced spermatotoxicity in mice.

MATERIALS AND METHODS

All chemicals used in the present study were of analytical grade procured from Hi-Media Laboratories Pvt. Ltd., Mumbai.

Production and analysis of ochratoxin

A pure toxigenic strain of *Aspergillus ochraceus* (ITCFF NO-1456) was obtained from the Indian Agricultural Research Institute, New Delhi, India and was maintained on potato dextrose agar medium. Ochratoxin was obtained in the purest form as described earlier (11).

Preparation of plant extract and phytochemical analysis

The ripe fruits of Emblica officinalis were obtained from local market and confirmed with the help of Botany Department, School of Sciences, Gujarat University, Ahmedabad, India. The extract was prepared according to WHO protocol [19]. Preparation of extract and quantitative as well as qualitative estimations of the bioactive components was done, which has been described in earlier paper (11).

Animal experimentation

Young adult inbred Swiss strain male albino mice (*Mus musculus*) weighing approximately 30-33 g were obtained from Zydus Research Centre, Ahmedabad, India. Animals were provided with animal feed and water *ad libitum* and maintained in 12 h light/dark cycles at 26 ± 2°C. Animal feed was prepared according to the formulation given by the National Institute of Occupational Health, Ahmedabad, India and was confirmed to be free of mycotoxins. Guidelines for care and use of animals in scientific research published by the Indian National Science Academy, New Delhi, India (1991), were followed.

Seventy animals were divided into seven groups and caged separately. Group 1 (untreated control) animals were maintained without any treatment. Animals of groups 2 and 3 received olive oil (0.2 mL/animal/day) and *Emblica officinalis* aqueous extract (2 mg/animal/day), respectively, for 45 days and served as pre-treatment controls. Animals of groups 4 and 5 were orally administered with ochratoxin 50 and 100 µg in 0.2 mL olive oil/animal/day (1.5 and 3.0 mg/kg body weight/day) for 45 days. Groups 6 and 7 animals were orally treated with ochratoxin as mentioned for groups 4 and 5 along with aqueous extract of *Emblica officinalis* (2 mg/animal/day) for 45 days (Table 1).

Table 1. Experimental protocol

Experimental groups	No. of animals	Treatments	Days of treatment	Day of autopsy
1.	10	Untreated control	45	46th
2.	10	Vehicle control-olive oil (0.2 mL/animal/day)		
3.	10	Antidote control-*Emblica officinalis* aq. extract (2 mg /animal/day)		
4.	10	Low dose ochratoxin (50 µg/0.2 mL olive oil/animal/day) treated		
5.	10	High dose ochratoxin (100 µg/0.2 mL olive oil/animal/day) treated		
6.	10	Low dose ochratoxin (50 µg/0.2 mL olive oil/animal/day) + *Emblica officinalis* aq. extract (2 mg/animal/day) treated		
7.	10	High dose ochratoxin (100 µg/0.2 mL olive oil/animal/day) + *Emblica officinalis* aq. extract (2 mg/animal/day) treated		

Table 2. Effect of aqueous extract of *Emblica officinalis* on ochratoxin-induced changes in the sperm parameters of mice

Parameters	Experimental groups*						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Sperm count (million/mL)	43.92 ± 1.86	41.08 ± 0.99	40.51 ± 0.34	31.50 ± 0.92^{abce}	12.58 ± 0.50^{abcdfg}	39.09 ± 0.70^{e}	35.27 ± 1.03^{ae}
Sperm motility (%)	79..50 ± 1.48	78.80 ± 0.95	78.90 ± 0.97	43.70 ± 1.09^{abcf}	22.30 ± 1.02^{abcfg}	74.10 ± 1.18^{de}	54.20 ± 1.10^{ae}
Sperm viability (%)	81.80 ± 2.04	79.50 ± 1.27	82.00 ± 1.12	52.30 ± 1.01^{abcef}	32.30 ± 1.0^{cdfg}	73.90 ± 0.88^{abcde}	55.80 ± 1.26^{e}
Fertility rate (%)	100 (+ve)	100 (+ve)	100 (+ve)	50.00 ± 1.23^{abcefg}	23.40 ± 0.79^{abcdfg}	84.30 ± 0.95^{deg}	75.20 ± 0.82^{abcdef}

* See Table 1. Values are the means ± SEM; n = 10. [a]As compared to group 1: $p < 0.05$. [b]As compared to group 2: $p < 0.05$. [c]As compared to group 3: $p < 0.05$. [d]As compared to group 4: $p < 0.05$ [e]As compared to group 5: $p < 0.05$. [f]As compared to group 6: $p < 0.05$. [g]As compared to group 7: $p < 0.05$

Olive oil was obtained from Figaro, Madrid, Spain. Ochratoxin was dissolved in olive oil, hence it was used as a vehicle in group 2. The dose of ochratoxin was based on the report (12). The dose of *Emblica officinalis* aqueous extract was based on earlier studies (13). All the treatments were given orally using a feeding tube attached to a hypodermic syringe for 45 days.

Sperm parameters and fertility rate

On completion of the treatment, the animals were sacrificed by cervical dislocation. Cauda epididymis of all control and treated groups of animals were quickly isolated, blotted free of blood and utilized for the analysis of various reproductive parameters.

Sperm motility: The percentage of motile spermatozoa was measured by the method of Prasad et al. (14).

Sperm viability: The ratio of live : dead spermatozoa was determined using 1% trypan blue (supravital stain) as described by the method of Prasad et al. (14).

Sperm count: Sperm count of control and treated groups of animals were determined by the method of Prasad et al. using the Neubauer chamber of a hemocytometer (14).

Fertility rate: The fertility rates of control and treated groups of animals were assessed according to the WHO MB-50 protocol (15).

Effect of ochratoxin on human sperm cells: Effect of ochratoxin on sperm morphology was studied under *in vitro* condition. Papanicolaou staining was performed to study sperm morphology where a thin, wet smear of the semen sample was prepared and stained (16).

Statistical analysis

For each parameter at least 10 replicates were done. Results are expressed as the means ± SEM. The data (*in vivo* studies) were statistically analyzed using one way analysis of variance (ANOVA) followed by the Tukey test. The level of significance was accepted with $p < 0.05$. Student '*t*' test was done for the *in vitro* experiment.

RESULTS

Table 2 shows the effect of oral administration of ochratoxin and ochratoxin along with aqueous extract of *Emblica officinalis* on cauda epididymal sperm parameters of mice. No significant alterations were observed between the control groups (Groups 1, 2, 3). Oral administration of ochratoxin (Groups

Table 3. Effect of ochratoxin on sperm morphology *in vitro*

Ochratoxin (µg/mL)	Morphological alterations (%)						
	Normal cells	Swelled head	Swelled head + midpiece	Coiled tail	Decapitation	Head-tail agglutination	Head-head agglutination
0 (Control)	82.30 ± 0.78	4.40 ± 0.50^{c}	3.20 ± 0.30^{c}	2.60 ± 0.21	2.50 ± 0.21	2.45 ± 0.24	2.55 ± 0.37
1	67.05 ± 1.49^{c}	10.30 ± 0.66^{c}	7.75 ± 0.68^{c}	5.30 ± 0.50^{c}	4.05 ± 0.35^{b}	2.90 ± 0.36^{NS}	2.65 ± 0.30^{NS}
2	49.95 ± 1.37^{c}	20.80 ± 0.76^{c}	10.20 ± 0.82^{c}	6.85 ± 0.40^{c}	4.60 ± 0.41^{c}	4.05 ± 0.32^{c}	2.65 ± 0.30^{NS}
3	38.40 ± 1.92^{c}	28.05 ± 0.92^{c}	10.80 ± 0.55^{c}	7.70 ± 0.65^{c}	6.45 ± 0.64^{c}	4.55 ± 0.51^{b}	4.05 ± 0.41^{a}
4	25.23 ± 2.11^{c}	30.30 ± 1.42^{c}	13.20 ± 0.89^{c}	10.25 ± 0.53^{c}	8.40 ± 0.47^{c}	6.20 ± 0.47^{c}	6.30 ± 0.62^{c}
5	12.70 ± 2.09^{c}	32.25 ± 1.35^{c}	17.55 ± 1.29^{c}	12.00 ± 0.97^{c}	10.50 ± 0.91^{c}	9.00 ± 0.61^{c}	6.55 ± 0.61^{c}

Values are the means ± SEM; n = 10. Significant at the level: As compared with control of respective dose: $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$; ^{NS}: Non-significant

4, 5) brought about significant reduction in sperm count (LD : -23.33%; HD : -69.38%), sperm motility (LD : -44.55%; HD : -71.71%), sperm viability (LD : -34.22%; HD : -59.38%) and fertility rate (LD : -50.0%; HD : -76.60%).

When animals were treated with ochratoxin along with aqueous extract of *Emblica officinalis* (Groups 6, 7), significant ameliorations were observed as compared to animals treated with ochratoxin alone (Groups 4, 5). The amelioration was higher in case of high dose ochratoxin plus extract treated animals than that of their respective low dose.

Table 3 shows the effect of ochratoxin on sperm morphological features. Normal sperm cells show triangular head with distinct acrosomal envelope, the nucleus contained condensed chromatin material (Fig. 1, 2). Addition of ochratoxin to the sperm suspension *in vitro* caused, as compared with control (0.0 mg/mL), a significant, dose-dependent increase in various kinds of morphological abnormalities (swelled head, swelled head and midpiece, coiled tail, decapitation, head-tail agglutination and head-head agglutination). The effect was concentration- as well as dose-dependent. Maximal morphological abnormalities were encountered with 5 mg/mL of ochratoxin (Fig. 3 – 8). The morphological alterations were mitigated when the ochratoxin treated cells were treated with aqueous extract of *Emblica officinalis* (Fig. 9, 10).

DISCUSSION

The present study clearly indicates that oral administration of ochratoxin for 45 days caused adverse effects on male reproductive parameters in mice (Table 2). In ochratoxin-treated mice cauda epididymal sperm count was reduced significantly, along with a decrease in motility and viability, when compared with that of vehicle control (Group 2).

Various authors have reported similar kind of observations in different animals emphasizing ochratoxin as a reproductive toxicant (17). When ochratoxin was administered orally to mice for 45 days at a dietary dose of 1 µg/kg/bw/day, it induced chromosomal abnormalities and a decrease in spermatogenic numbers (18). The effect of the toxin on motility and longevity of breeding boar semen was also observed (19). The toxin was found in the testicles of laboratory animals impairing spermatogenesis and accumulation of premeiotic germinal cells (20). It was reported that the activities of some enzymes like α-amylase and alkaline phosphatase vary with the ochratoxin A poisoning in the

homogenate of the testicles of rats. Ochratoxin A was also found to inhibit testosterone secretion in isolated testicular interstitial cells of gerbils in *in vitro* condition (21).

A decrease in sperm viability may be because of alterations in membrane integrity of the sperm cells, believed to be caused due to the stress induced by ochratoxin, leading to lipid peroxidation (22). Decreases in the activities of ATPase and succinate dehydrogenase as well as sperm count and motility along with an increase in the numbers of non-viable spermatozoa were reported in fluoride (23) and aflatoxin (24) treated mice.

When human sperm cells were treated with ochratoxin *in vitro*, significant alterations in sperm motility, sperm viability and sperm morphology (swollen head, swollen head and mid-piece, coiled tail, decapitation, head-tail agglutination and head-head agglutination) in time-dependent and concentration-dependent manner were observed. Sperm motility is essential for successful fertilization. When the sperm cells become immobilized then they are unable to travel successfully in the female genital tract thus affecting the overall fertilization.

A decrease in human sperm motility might be due to mitochondrial disruption and/ or an increase in lipid peroxidation. The flagellar movement of the sperm cells decides the motility of cells and energy to flagella which is provided by the mitochondria (25). Wei et al. have suggested that ochratoxin A exerts direct effect on the mitochondrial respiration and oxidative phosphorylation impairing the mitochondrial membrane and inhibiting the succinate supported electron transfer activities of the respiratory chain (26). Meisner have observed that ochratoxin uptake is energy-dependent process resulting in a depletion of intramitochondrial ATP, in isolated rat liver mitochondrial cells (27).

Another possible cause of sperm immobilization might be due to oxidative damage leading to ultimate death of the cell. Rahimtula et al. have reported that ochratoxin A enhances lipid peroxidation under both *in vitro* and *in vivo* conditions and is responsible for the production of reactive oxygen species (ROS) (28). An increase in lipid peroxidation and a decrease in the activities of enzymatic antioxidants such as glutathione peroxidase, glutathione reductases, glutathione transferases, catalase and superoxide dismutase along with a decrease

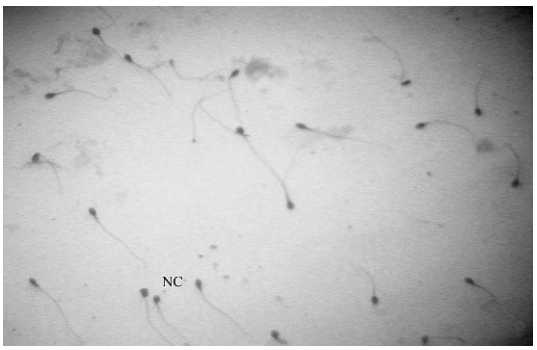

Figure 1. NC – Normal Cells

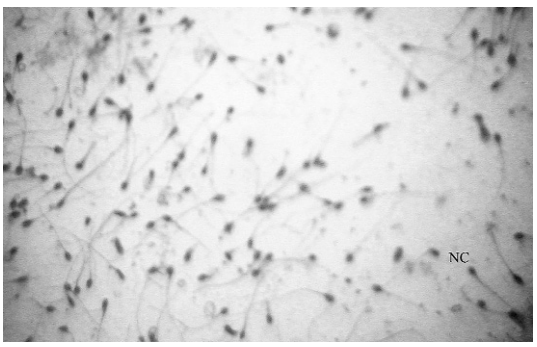

Figure 2. NC – Normal Cells

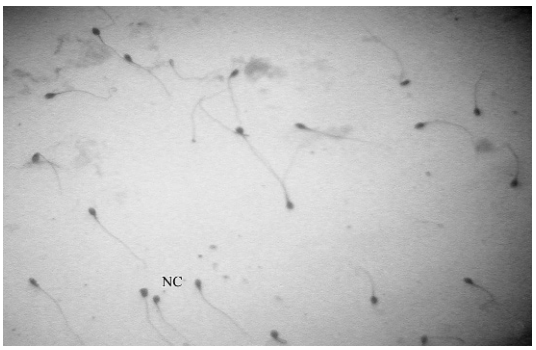

Figure 3. SH – Swelled Head

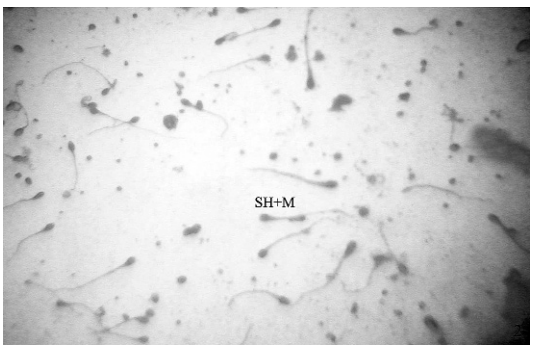

Figure 4. SH+M – Swelled Head+Midpiece

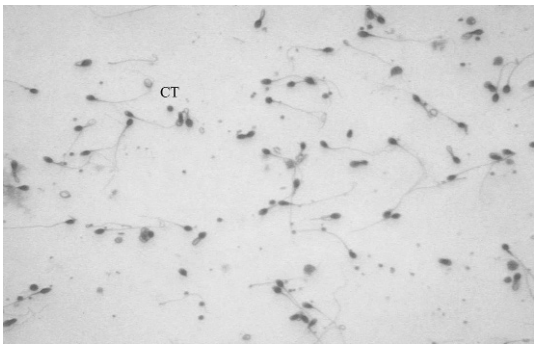

Figure 5. CT – Coiled Tail

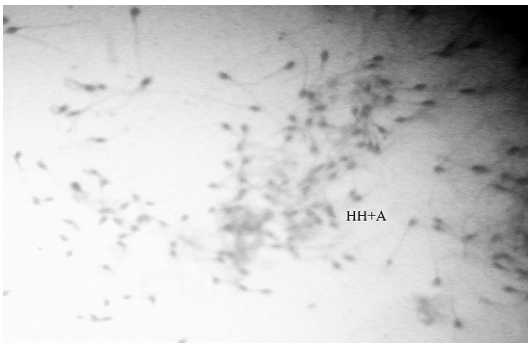

Figure 8. HH+A – Head-Head+Agglutation

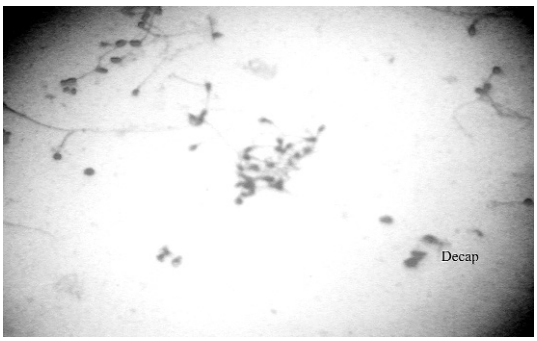

Figure 6. Decap – Decapitation

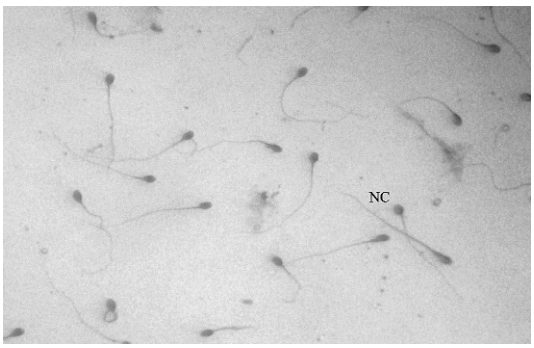

Figure 9. NC – Normal Cells

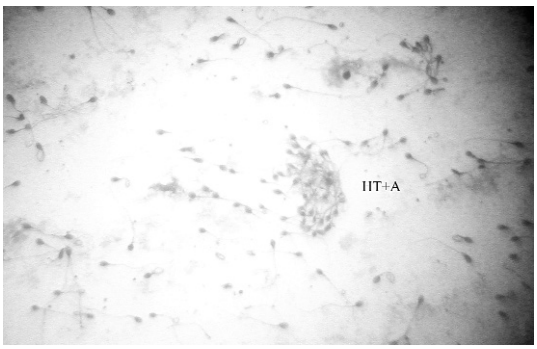

Figure 7. HT+A – Head-Tail+Agglutation

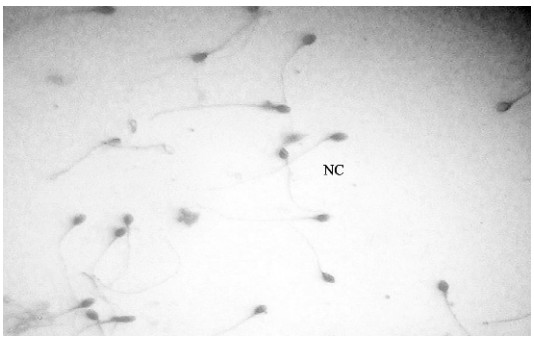

Figure 10. NC – Normal Cells

in the concentration of glutathione and ascorbic acid in the testis of ochratoxin treated mice has been reported earlier (11). Secondarily, a decrease in other enzyme protein involved in sperm cell activity may also affect the overall fertility. For instance, ochratoxin, being an analogue of phenylalanine amino acid, inhibits phenylalanine tRNA synthetase enzyme during protein synthesis and decreases a large content of protein in the cell.

The elevated ROS level is one of the main causes of sterility which mainly reduces the sperm motility (29). Spermatozoa are sensitive to acute stress under aerobic conditions. As Alverez et al. have explained, the peroxidized metabolites of fatty acids originated from the ROS damages phosphatides of cell membrane, directly damaging the sperm function and morphology (30). Tsuda et al. have observed a decrease in viability when AT1 (anion transporter-1) cells were treated with ochratoxin A (31).

Oral administration of aqueous extract of *Emblica officinalis* along with ochratoxin for 45

days significantly mitigates ochratoxin-induced alterations in reproductive parameters in mice. This might be due to the presence of radical scavengers in the extracts. The four main bioactive compounds, namely: emblicanin A, emblicanin B, punigluconin and pedunculagin were shown to provide protection against oxygen radicals in various *in vitro* studies (6). Vitamin C present in the fresh fruit pulp of *Emblica officinalis* maintains first natural antioxidants defense in plasma and can act as powerful inhibitor of lipid peroxidation. The fruit has shown a marked increase in the level of hematopoietic parameters and other tissue lipid peroxide levels. It also regenerates the major antioxidants tocopherol in lipoproteins of the cell membrane.

Oral administration of *Emblica officinalis* to dimethylbenzyl anthracene treated mice caused a significant increase in liver antioxidants mainly GSH, GRX, GPX and GST (32). The fruit is believed to contain small molecular weight bioactive compounds believed to have powerful antioxidizing activity, hence prevents the lipid peroxidation and ameliorates the spermatotoxic effect of ochratoxin.

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