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# Antifertility studies of *Aegle marmelos* Corr., an Indian medicinal plant on male albino rats

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## Abstract

This experiment tested the antifertility effect of *Aegle marmelos*. Oral administration of aqueous extract to male rats brought about a highly significant decrease in the weights of testes, epididymis, seminal vesicle, ventral prostate and vas deferens. Sperm motility as well as sperm density in the cauda epididymis was reduced significantly: fertility was reduced by 70%. The sialic acid, protein, glycogen, fructose and ascorbic acid contents of the testes and other reproductive tissues were significantly decreased, while the testicular cholesterol content was significantly elevated. The assays of various hormones reflects the fact that serum testosterone levels reduced significantly in the experimental group, while no changes were observed in the levels of serum estradiol, LH and FSH. There were no significant changes in the total erythrocyte count, total leucocyte count, haemoglobin, haematocrit, blood sugar and blood urea in the blood, nor in acid phosphatase, alkaline phosphatase, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, total protein, cholesterol, triglyceride, phospholipid and bilirubin content in the serum, suggesting no ill effects on the physiology of the rats. We conclude that aqueous extract of the leaves of *Aegle marmelos* has an antispermatogenic/antifertility effect.

**Keywords**: sperm motility, sperm density, hormonal assays

## Introduction

Since ancient times, plants have been a source of drugs, but scientific medicines tend to ignore the importance of herbal medicine (Sofowora 1982). The World Health Organization suggested that effective, locally available plants be used as substitutes for drugs. Since the population explosion is a leading cause of poverty and pollution in developing countries, they created a population control programme, which includes studies of traditional medical practices.

The plant *Aegle marmelos* Corr. is indigenous to India, and belongs to the family Rutaceae: it is known as 'Vilvam' in Tamil, 'Bael' in Hindi, and 'Bilwa' or 'Sriphal' in Sanskrit. Various parts of this plant (mainly the leaves, fruits, stem and roots) have been used in ethnomedicine for several medicinal properties: they are said to be astringent, anti-diarrhoeal, anti-dysenteric, demulcent, anti-pyretic, anti-scourbutic, haemostatic, aphrodisiac and an antidote to snake venom (Kirtikar & Basu 1993). From Ayurvedic medicine, it has been claimed that the leaves of *Aegle marmelos* possess contraceptive efficacy (Bhattacharya 1982), and they are used for contraceptive purposes by men from different tribal areas of India. Proper scientific research on these contraceptive effects have not been carried out before.

Based on this background, we report the antispermatogenic/antifertility effect of aqueous extracts of the leaves by focusing on reproductive parameters such as organ weights, sperm dynamics, biochemistry of the reproductive tissues, assays of circulatory levels of reproductive hormones (testosterone, estradiol, LH and FSH), and haematological and serological profiles.

## Materials & Methods

Twenty adult male albino rats of the Wistar strain, 3-4 months old and weighing 190-210 gms were maintained under complete veterinary supervision in an animal facility with a 12L:12D schedule. Rat pellet diet (Aashirwad Ind. & Co., Chandigarh, India) and water were provided *ad libitum*. Only proven fertile male animals were used. The study was carried out with the approval of the Departmental Research Committee, and the guidelines (INSA 2000) were strictly followed throughout.

Leaves of *Aegle marmelos* were collected from the local area near the University Campus. The material was identified by a taxonomist at the Department of Botany, University of Rajasthan, Jaipur, and a voucher specimen deposited (specimen no. RUBL 20104). Aqueous extract of the leaves was prepared according to the method of the National Institute of Health & Family Welfare, New Delhi, India. Fresh leaves were shredded, dried and crushed in an electrical grinder and an aqueous extract prepared by soaking 200 gm of leaf powder with 1.0 liter of double-distilled water. The extract was dried at reduced pressure and finally lyophilized (drug yield = 6.0%).

Ten animals were forcefully fed by gavage at a dose of 200 mg of aqueous extract/ 0.5 ml of distilled water/ kg body weight/ day for 60 days. The remaining ten animals were considered as controls and were treated with vehicle only by forceful feeding of 0.5 ml of distilled water. The gavage treatment was performed in the morning hours and under fasting conditions. On the 61$^{st}$ day, the final body weights of all animals were recorded. For blood collection from heart by heparinised syringe, the animals were anesthetized by the application of light ether exposure. The reproductive organs (testes, epididymis, seminal vesicle, ventral prostate, vas deferens) and some vital organs (liver, heart, kidney and adrenal) of each animal were dissected out and wet weights recorded. Half of the tissues (testis, epididymis, seminal vesicle, ventral prostate and liver) were kept at -20°C for biochemical assays. One testis of each animal was placed in Bouin's fluid for histological studies. Serum was separated from blood by centrifugation at 3000 rpm for 10 minutes and kept at -20°C for the assay of testosterone and other serological parameters.

At the termination of the experiment (i.e. on days 55-60), males from each group were cohabited individually with two proestrous parous females. Successful mating on each case was confirmed by the presence of a copulation plug and spermatozoa in the vaginal smear. The day when spermatozoa were detected in the smear was designated as day zero of pregnancy. Such females were laparotomized on day 15 post *coitum*, and the number of implantation sites (if any) in the uteri was recorded (WHO Protocol 1983**).**

For sperm motility and density, a known amount of the cauda epididymis was teased in 2.0 ml of physiological saline. Within 5 min of sacrifice, one drop of evenly mixed sample was applied to a Neubaur's Chamber under a cover glass. The percentage motility was determined by counting both motile and immotile spermatozoa per unit area. Sperm density was then determined in the cauda epididymis and testis by routine procedures, and expressed as million/mm$^3$ of suspension (WHO 1999).

Sialic acid was assayed by the method of Warren (1959) in the testis, cauda epididymis, seminal vesicle and ventral prostate. Protein was estimated by the method of Lowry *et al.* (1951) in the testis, cauda epididymis, seminal vesicle and ventral prostate. Cholesterol was measured in testes and liver using the method of Zlatkis *et al.* (1953). Glycogen was estimated by the method of Montgomery (1957) in the testes

and liver, fructose in the testes and seminal vesicle (Foreman *et al.* 1973) and ascorbic acid in testes and cauda epididymis (Roe & Kuether 1943).

The serum concentration of testosterone was measured following the immunoenzymatic method in an ELISA reader (Merck, Japan) and according to the standard protocol given by National Institute of Health & Family Welfare (Srivastava 2000). Serum estradiol concentration was measured by radio immunoassay using commercial kits. The LH and FSH concentrations in the serum were measured using a double antibody radio-immuno assay, according to standard methods (Moudgal & Madhwa 1974). Whole blood was analyzed for total erythrocyte count, total leucocyte count, haemoglobin, haematocrit, blood sugar and blood urea according to standard procedures. The serum was analyzed to estimate acid phosphatase, alkaline phosphatase, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, total protein, cholesterol, triglycerides, phospholipids and bilirubin according to standard procedures.

Data are expressed as mean ± SD and analyzed for statistical significance by using one-way analysis of variance (ANOVA). Results were considered significant at the $p \leq 0.01$ level.

**Table 1:** Effect of leaf aqueous extract of *Aegle marmelos* on the body and organ weights of male albino rats. Each value is a mean ± SD of 10 animals

| | Body weight (gm) | | Reproductive organ weight (mg/100 gm b. wt.) | | | | | Vital organ weight (mg/100 gm b.wt.) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | Final | Testes | Epididymis | Seminal vesicle | Ventral prostate | Vas deferens | Liver | Heart | Kidney | Adrenal |
| **Control** | 190.0 ± 3.3 | 201.4 ± 2.5 | 1047.8 ± 8.3 | 459.8 ± 7.8 | 489.3 ± 2.4 | 306.8 ± 3.8 | 166.2 ± 4.7 | 3947.8 ± 28.9 | 504.4 ± 4.3 | 936.8 ± 8.4 | 27.6 ± 1.7 |
| **Treated** | 194.6 ± 2.8 | 211.0 ± 3.9 | 850.6 ± 6.8 [a] | 421.3 ± 8.6 [a] | 462.9 ± 1.8 [a] | 262.5 ± 4.2 [a] | 126.4 ± 3.8 [a] | 3950.9 ± 25.3 | 498.4 ± 3.8 | 920.4 ± 7.9 | 26.6 ± 1.4 |

Difference from control: a = highly significant ($p \leq 0.001$)

## Results

The treatment with *Aegle marmelos* Corr. for 60 days did not cause any significant change in their body weights (Table 1). However, the weights of reproductive organs (testes, epididymis, seminal vesicle, ventral prostate and vas deferens) declined significantly in the treatment group compared with control animals. The weights of vital organs remained unchanged.

Administration of *Aegle marmelos* caused a significant reduction in sperm motility of the cauda epididymis, and in sperm concentration/density of the testes and cauda epididymis. The fertility index of the males was inhibited by 70% (Table 2).

**Table 2**: Effect of leaf aqueous extract of *Aegle marmelos* on the sperm dynamics and fertility of male albino rats. Mean ± SD of 10 animals

| | mated males | mated females | pregnant females | implantation sites/rat | viable fetuses/rat | Sperm motility (cauda epididymis) (%) | Sperm density (million/ml) | | Fertility Test (% +ve) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | testes | cauda epididymis | |
| Control | 10 | 20 | 20/20 | 10.7 ± 1.3 | 10.0 ± 0.8 | 79.1 ± 1.7 | 5.1 ± 0.6 | 56.5 ± 1.6 | 100 |
| Treated | 10 | 20 | 6/20 | 7.1 ± 1.1 [b] | 6.0 ± 0.8 [b] | 54.0 ± 2.3 [b] | 3.8 ± 0.7 [b] | 40.2 ± 1.1 [b] | 30 |

Difference from control: b = significant ($p \leq 0.01$)

The treatment brought about the highly significant depletion in sialic acid contents of testis, cauda epididymis, seminal vesicle and ventral prostate (Table 3). The total protein content in the testis, cauda epididymis, seminal vesicle and ventral prostate decreased significantly (Table 3). The testicular cholesterol level was highly significantly elevated, whereas that of the liver remained unchanged. The level of glycogen in the testes was significantly lowered, while in liver it was at the normal value. The fructose content of the testes and seminal vesicle remained unchanged with respect to the control group. The level of ascorbic acid declined significantly in the testes and cauda epididymis of treated animals (Table 4).

**Table 3:** Effect of leaf aqueous extract of *Aegle marmelos* on sialic acid and total protein content of various tissues of male albino rats. Values are mean ± SD (n=10), units = mg/gm

| | Sialic Acid | | | | Total Protein | | | |
|---|---|---|---|---|---|---|---|---|
| | Testes | Cauda Epididymis | Seminal vesicle | Ventral Prostate | Testes | Cauda Epididymis | Seminal vesicle | Ventral Prostate |
| Control | 5.6 ± 0.03 | 5.3 ± 0.2 | 4.7 ± 0.1 | 5.2 ± 0.04 | 223.8 ± 20.1 | 263.0 ± 19.6 | 216.2 ± 13.9 | 192.3 ± 10.1 |
| Treated | 4.0 ± 0.1[a] | 4.2 ± 0.2[a] | 3.8 ± 0.3[a] | 4.6 ± 0.2[a] | 197.6 ± 19.3[b] | 243.9 ± 17.7[b] | 192.3 ± 14.8[b] | 170.6 ± 9.1[b] |

Difference from control: a = highly significant ($p \leq 0.001$), b = significant ($p \leq 0.01$)

**Table 4:** Effect of leaf aqueous extract of *Aegle marmelos* treatment on the cholesterol, glycogen, fructose and ascorbic acid content of various tissues of male albino rats. Means ± SD (n=10); units mg/gm

| | Cholesterol | | Glycogen | | Fructose | | Ascorbic Acid | |
|---|---|---|---|---|---|---|---|---|
| | Testes | Liver | Testes | Liver | Testes | Seminal Vesicle | Testes | Cauda Epididymis |
| Control | 8.0 ± 0.4 | 16.4 ± 0.3 | 4.9 ± 0.1 | 8.8 ± 0.2 | 5.4 ± 0.1 | 5.1 ± 0.1 | 0.35 ± 0.01 | 0.28 ± 0.001 |
| Treated | 8.3 ± 0.4 [a] | 16.4 ± 0.2 | 4.1 ± 0.2 [b] | 8.7 ± 0.2 | 5.2 ± 0.2 | 5.0 ± 0.1 | 0.28 ± 0.004 [b] | 0.23 ± 0.005 [b] |

Difference from control: a = highly significant (p≤0.001); b = significant (p≤0.01)

There was a highly significant decline in the level of serum testosterone in the treatment versus the control group, but the level of serum estradiol, LH and FSH remained unchanged (Table 5).

**Table 5:** Effect of leaf aqueous extract of *Aegle marmelos* treatment on the circulatory levels of hormones in male albino rats. Values are means ± SD (n=10)

| | Serum Testosterone (ng/ml) | Serum estradiol (pg/ml) | Testosterone/ estradiol ratio | Leuteinising hormone (ng/ml) | Follicle stimulating hormone (ng/ml) |
|---|---|---|---|---|---|
| Control | 4.4 ± 0.3 | 17.6 ± 0.3 | 0.25 ± 0.04 | 3.7 ± 0.5 | 10.2 ± 0.4 |
| Treated | 2.1 ± 0.4 [a] | 17.4 ± 0.2 | 0.12 ± 0.06 [a] | 3.7 ± 0.4 | 10.2 ± 0.5 |

Difference from control: a = highly significant (p≤0.001)

Total erythrocyte count, total leucocyte count, haemoglobin, haematocrit, blood sugar and blood urea did not show any changes (Table 6).

**Table 6:** Effect of leaf aqueous extract of *Aegle marmelos* treatment on haematological parameters of male albino rats. Values are means ± SD (n=10); treatment means are all not significantly different from control.

| | Total erythrocyte count (million/mm$^3$) | Total leucocyte count (per mm$^3$) | Haemoglobin (gm%) | Haematocrit (%) | Mean corpuscular volume (μm$^3$) | Mean corpuscular haemoglobin (Pg) | Mean corpuscular haemoglobin concn (%) | Blood sugar (mg/100 ml) | Blood urea (mg/100 ml) |
|---|---|---|---|---|---|---|---|---|---|
| Control | 5.9 ± 2.3 | 8894 ± 26 | 15.8 ± 0.8 | 49.0 ± 0.7 | 88.7 ± 5.4 | 26.6 ± 3.5 | 32.2 ± 1.9 | 90.8 ± 2.6 | 36.7 ± 1.3 |
| Treated | 5.2 ± 0.4 | 8769 ± 26 | 15.7 ± 0.4 | 48.1 ± 0.3 | 89.6 ± 6.7 | 25.6 ± 3.0 | 33.6 ± 0.2 | 89.9 ± 2.0 | 35.7 ± 1.4 |

Acid phosphatase, alkaline phosphatase, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, total protein, cholesterol, triglycerides, phospholipids and bilirubin content in serum did not change significantly (Table 7).

**Table 7:** Effect of leaf aqueous extract of *Aegle marmelos* treatment on serological parameters in male albino rats. Values are means ± SD (n=10); all treatment means are not significantly different from the control.

| | Acid phosphatase (mm/gm/hr) | Alkaline ohosphatase (mm/gm/hr) | Serum glutamic oxaloacetic transaminase (IU/L) | Serum glutamic pyruvic transaminase (IU/L) | Protein (mg/dl) | Phospholipids (mg/dl) | Triglycerides (mg/dl) | Cholesterol (mg/dl) | Bilirubin (mg/100ml) |
|---|---|---|---|---|---|---|---|---|---|
| Control | 3.0 ± 0.2 | 1.08 ± 0.04 | 64.9 ± 1.1 | 25.6 ± 2.0 | 14368 ± 109 | 127.0 ± 11.6 | 120.0 ± 15.6 | 116.0 ± 12.3 | 0.95 ± 0.06 |
| Treated | 2.9 ± 0.4 | 1.02 ± 0.09 | 63.9 ± 2.0 | 25.7 ± 3.7 | 14295 ± 128 | 125.5 ± 10.1 | 120.4 ± 14.4 | 114.3 ± 11.2 | 0.94 ± 0.08 |

## Discussion

Oral administration of *Aegle marmelos* to male rats at a dose level of 200 mg/kg b.wt. for 60 days caused a significant decrease in the testis weight which could be attributed to the loss of germ cell (D'Souza & Narayana 2001). Decreased weights of accessory sex glands indicate the atrophy of glandular tissue and diminished secretary ability reflects the decreased level of testosterone as these organs are androgen dependent (Reiter *et al.* 1995).

Sperm motility and density in the cauda epididymis was adversely affected after the treatment. The reduction in sperm motility in cauda epididymis is of importance with regard to fertilization (Bedford 1983). Inadequate concentration and immotility of the spermatozoa means they cannot penetrate the cervical mucus and thus fail to fertilize the ova (Lohiya & Goyal 1992).

The structural integrity of the acrosomal membrane is dependent upon sialic acid; alteration in its concentration may lead to changes in the motility and fertilizing ability of sperm (Riar *et al.* 1973; Levinsky *et al.* 1983). Reduced testicular and epididymal protein content could be correlated with the absence of spermatozoa in the lumen (Chinoy & Bhattacharya 1997) since the luminal fluid of epididymis contains a number of proteins (Brook & Higgins 1980) some of which remain bound to spermatozoa. Cholesterol is involved in steroidogenesis in the testes; an increased level of cholesterol is attributed to decreased androgen concentration, resulting in impaired spermatogenesis (Bedwal *et al.* 1994). A significant decrease in glycogen content after the treatment with *Aegle marmelos* possibly could be explained by an inhibition of glycolysis during spermatogenesis (Bone *et al.* 2000).

The significant decline in serum testosterone might be due to the adverse effects of treatment on the hormonal milieu of the testes. The reduced weights of seminal vesicle and ventral prostate further support the suppressed concentration of testosterone in circulation (Lohiya & Ansari 1999). The data do not explain the unchanged LH and FSH levels, and further studies will be required to clarify these effects. Blood and serum parameters were within the normal range in both the groups indicating non-toxic nature of the herbal drug from the leaves of *Aegle marmelos*.

In conclusion, *Aegle marmelos* induced antifertility effects in male albino rats without altering the general body metabolism, because the weight of vital organs as well as haematological and serological parameters remained unaltered. Further studies with this extract to achieve complete antifertility and its reversibility are in progress.

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## الملخص العربى

دراسات على تاثيرات نبات ايجيل مارميلوس – احد النباتات الطبية الهندية- على خصوبة الفئران البيضاء

الكا شاوهان – ميرا اجاروال – سوناليكا كوشواها – انشوى موترتشا

قسم علم الحيوان – جامعة راجاثان – جايبور – الهند

أجريت الدراسة بهدف معرفة تاثير مستخلص الاوراق لاحد النباتات الطبية الهندية " ايجيل مارميلوس" على الجهاز التناسلى للفئران البيضاء من خلال إعطاء الفئران المستخلص عن طريق الفم بالجرعات ٢٠٠مجم لكل كيلوجرام من الفئران وذلك لمدة ٦٠ يوماً. أظهرت النتائج ان المستخلص النباتى له تاثير مثبط على الجهاز التناسلى من خلال نقصان وزن الخصية والحوصلة المنوية والوعاء الناقل مع قلة عدد ونسبة الحيوانات المنوية خلال الخلايا. كانت نسبة التثبيط تصل إلى حوالى ٧٠% مع نقصان كمية حمض السياليك – البروتين – الجليكوجين – الفركتوز – حمض الاسكوربيك فى الخصية مع زيادة محتوى الكليسترول داخل جدران خلايا الخصية. لم تشاهد زيادة او نقصان معنوى فى عدد الخلايا الدموية الحمراء او كمية الهيموجلوبين او كمية السكر او كمية اليوريا فى الدم وغيرها من الهرمونات وبدت الفئران فى حالة صحية لاباس بها ولم تظهر اعراض المرض عليهم. ولذا يمكن القول ان مستخلص النبات له تاثير مثبط على خصوبة فئران التجارب.