

Effect of Aqueous Extract of Leaf of *Aegle marmelos* on Testicular Activities in Rats

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ABSTRACT

The aqueous extract of leaf of *Aegle marmelos* (Bael) at the dose 50 mg/100 g body weight resulted a significant diminution in the activities of key testicular steroidogenic enzymes along with low levels of plasma testosterone and relative wet weights of sex organs in respect to control without any significant alteration in general body growth. Germ cells numbers in different generation at stage VII of seminiferous epithelial cell cycle were diminished significantly after the treatment of the above extract. The above mentioned dose did not exhibit any toxicity in liver and kidney. Therefore, it may be predicted that the aqueous extract of leaf of *Aegle marmelos* has a potent antitesticular effect at a specific dose.

Keywords: *Aegle marmelos*, Steroidogenic key enzymes, Plasma testosterone, Germ cells

Since population explosion is a leading cause of poverty and pollution in developing countries, the World Health Organization (WHO) has constituted a population control programme, which includes studies having traditional medical practices. At present global attempt has been taken to search out the effect of herbal product for contraceptive purposes [1, 2]. Few herbal contraceptives have been developed but the potentiality of these contraceptives is very minimum and mode of action beyond our knowledge till now. It has been noted that the leaves of *Aegle marmelos* (*A. marmelos*) are used for contraceptive purpose in males in different tribal areas. This plant is tree type and grows in almost all districts in West Bengal, India. This is under the family of Rutaceae. The height of this plant is 20-30 ft and leaves are pinnately compound in type.

From Ayurvedic medicine, it has been claimed the leaf of *A. marmelos* posses contraceptive efficacy [3]. Proper scientific research on the contraceptive effects of *A. marmelos* and its mode of action has not been carried out till now. On this back ground the present study has been conducted to investigate the effects of aqueous extract of leaf of *A. marmelos* on parameters such as testiculo-somatic, seminal vesiculo-somatic, prostatic-somatic, epididymal-somatic indices, activities of testicular steroidogenic key enzymes, plasma testosterone

level, seminiferous tubular diameter (STD) and quantification of germ cells at stage-VII of seminiferous epithelial cell cycle. From trial and error the effective dose was selected to search out whether it has any antitesticular effect and to delineate the possible mode of action for such effect. To determine whether the aqueous extract of leaf of *A. marmelos* at the applied dose has any toxic effects on metabolic organs like liver and kidney, the activities of transaminases and phosphatases were studied in both the organs. Therefore, the aim of the present study is to find out whether the aqueous extract of leaf of the plant has any contraceptive potency or not and to focus a light on the possible ways of its contraceptive activities.

MATERIALS AND METHODS

Plant Material

Leaves of *A. marmelos* were collected from Midnapore in the month of May-June. The material was identified by taxonomist of Central National Herbarium (Kolkata), Botanical Survey of India (BSI), Shibpur, Howrah and Department of Botany, Vidyasagar University, Midnapore. The voucher specimen was deposited in the Department of Botany of Vidyasagar University having voucher specimen number HPCH No. 2.

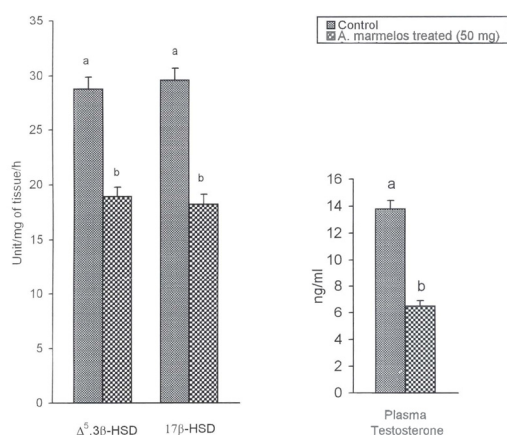


Fig 1. Inhibitory effect of aqueous extract of leaf of *A. marmelos* on testicular $\Delta^5, 3\beta$ -HSD, 17 β -HSD activities and plasma level of testosterone in mature albino rats (Mean \pm SEM, n=8) as compared with matched control (Students two tail "t" test). Bars with different superscripts (a, b) differ from each other significantly, $p < 0.05$

Preparation of Aqueous Extract of Leaf of *A. Marmelos*

Aqueous extract of leaf of *A. marmelos* (Bael) was prepared according to the method of National Institute of Health and Family Welfare (NIHFW), New Delhi, India. Fresh leaves were dried at 40°C for 48 hours and crushed in an electrical grinder. An aqueous extract was prepared by refluxing 200 g of leaf powder with 1.0 litre double distilled water. Extract was dried at reduced pressure and finally lyophilized.

Selection of Animal

In this experiment sixteen healthy and fertile adult male albino Wistar strain rats (Chakraborty Brother, Midnapore, India), 3 months of age, weighing 150 \pm 10 g were selected for acclimation for a period of 15 days in our laboratory condition (12 h dark: 12 h light, 28 \pm 2°C) prior to experimentation. They were provided with standard food and water *ad libitum*. Animals were divided into two equal groups according to the mode of treatment.

Treatment

Eight animals were forcefully fed by gavage at a dose of 50 mg of aqueous extract/0.5 mL of distilled water/100 g body weight/day for 28 days. The remaining eight animals were considered as control and they were treated with vehicle only by forceful feeding (0.5 mL of distilled water/100 g body weight/day for 28 days). The gavage treatment was performed at 6-8 AM and at fasting condition regularly and animal food was provided at 10 AM in each day.

On 29th day, body weights of all animals were recorded. For blood collection from dorsal aorta by heparinized syringe, all the animals were anaesthetized by application of light ether exposure. The animals were killed by decapitation. The testis, prostate, seminal vesicle, epididymis, liver and kidney of each animal were dissected out, and their wet weights were recorded. The

right testis of each animal was kept at -20°C for enzymatic studies. The left testis of each animal was placed in Bouin's fluid for histological studies. Plasma was separated from the collected blood by centrifugation at 3000 g for 5 minutes and was kept at -20°C for assay of testosterone by ELISA.

Assay of Testicular $\Delta^5, 3\beta$ -Hydroxysteroid Dehydrogenase ($\Delta^5, 3\beta$ -HSD) Activity

Testicular $\Delta^5, 3\beta$ -hydroxysteroid dehydrogenase activity was measured biochemically [4]. The right testis of each animal was homogenized in 20% spectroscopic grade glycerol (BDH Chemical Division, Bombay, India) containing 5 mM potassium phosphate (Loba Chemical Company, Bombay, India) and 1 mM EDTA (Organon, Kolkata, India) at a tissue concentration of 100 mg/mL homogenizing mixture and it was centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant at a volume of 1 mL was mixed with 1 mL of 100 μ M of sodium pyrophosphate buffer, pH=8.9 (Loba Chemical Company, Bombay, India), 40 μ L of ethanol containing 30 μ g of dehydroepiandrosterone (Organon, Kolkata, India) and 960 μ L of 25 mg% of bovine serum albumin (BSA) making the incubation mixture a total of 3 mL. Enzyme activity was measured after addition of 100 μ L of 0.5 μ M nicotinamide adenine dinucleotide (NAD)(SRL, Bombay, India) to the tissue supernatant mixture in a spectrophotometer cuvette at 340 nm against a blank (with out NAD). One unit of enzyme activity was the amount causing a change in absorbance of 0.001/min at 340 nm.

Assay of Testicular 17 β -Hydroxysteroid Dehydrogenase (17 β -HSD) Activity

The activity of testicular 17 β -HSD was measured biochemically [5]. The part of the supernatant prepared for the assay of $\Delta^5, 3\beta$ -HSD (above) was used for assaying 17 β -HSD activities. The supernatant (1 mL) was mixed with 1 mL of 440 μ M sodium pyrophosphate buffer, pH=10.2 (Loba Chemical Company), 40 μ L ethanol containing 0.3 μ M testosterone (Sigma Chemical Company, St Louis, MO. USA) and 960 μ L of 25% of BSA that make the incubation mixture a total of 3 mL. Enzyme activity was assessed after the addition of 100 μ L of 0.5 μ M NAD to the incubation mixture in a spectrophotometer cuvette at 340 nm against a blank with out NAD. One unit of enzyme activity was equivalent to a change in absorbance of 0.001/min. at 340 nm.

Assay of Plasma Testosterone

Plasma level of testosterone was measured following the immuno-enzymatic method by ELISA reader [6]. Horse reddish peroxidase was used as the enzyme labeled antigen that competes with unlabeled antigen for binding to a limited number antibody. After incubation, bound/free enzyme labeled antigen separation was performed by simple solid phase washing. The substrate of enzyme, H₂O₂ and chromogen were added as per scheduled and the enzyme reaction was stopped by a solution supplied by Equipar (Italy). The color intensity was

Table 1. Effect of aqueous leaf extract of *A. marmelos* on body weight, testiculo-somatic, seminal vesiculo-somatic, prostatico-somatic, epididymal-somatic, hepato-somatic and reno-somatic indices in albino rats (Mean \pm SEM, n=8) as compared with control (Student two tail "t" test). In each vertical column mean with different superscripts (a, b) differ from each other significantly $p<0.05$.

Condition	Initial body weight (g)	Final body weight (g)	Testiculo-somatic index (g/100g body weight)	Seminal -vesiculo somatic index (g/100 g body weight)	Prostato-somatic index g/100 g body weight	Epididymal-somatic index g/100 g body weight	Hepato-somatic index g/100 g body weight	Reno-somatic index g/100 g body weight)
Control	150.01 \pm 8.9 ^a	180.91 \pm 9.9 ^a	1.58 \pm 0.02 ^a	0.388 \pm 0.002 ^a	0.176 \pm 0.001 ^a	0.462 \pm 0.021 ^a	4.78 \pm 0.05 ^a	0.835 \pm 0.002 ^a
<i>A. marmelos</i> treated	150.01 \pm 8.9 ^a	178.02 \pm 8.8 ^a	1.19 \pm 0.01 ^b	0.175 \pm 0.001 ^b	0.083 \pm 0.001 ^b	0.260 \pm 0.023 ^b	4.85 \pm 0.50 ^a	0.830 \pm 0.005 ^a

inversely proportional to the testosterone concentration in the sample. Testosterone concentration in the sample was calculated based on 5 standards supplied by Equipper. The absorbance of standards and sample was monitored against the blank at 450 nm. The cross reaction of the testosterone antibody to dehydrotestosterone is 10% and intra-run precision had co-efficient of variation of 6.2%. The entire sample was run at the same time so there was no inter -run variation.

Measurement of Alkaline Phosphatase (ALP), Acid Phosphatase (ACP), Glutamate Oxaloacetate Transaminase (GOT) and Glutamate Pyruvate Transaminase (GPT) Activities in Liver and Kidney

Alkaline phosphatase activities of liver and kidney were estimated biochemically [7]. For quantitative estimation of ALP, the above organs were homogenized separately in a Potter Elvehjem homogenizer at a tissue concentration of 20 mg/mL using ice cold homogenizing medium (0.22 M Tris-Hcl buffer pH=7.5). Homogenate at a volume of 0.25mL was added in a centrifuge tube containing 1 mL buffer (1 mM p-nitrophenol phosphate in 1M Tris buffer, pH=8.0). The mixture was incubated at 37°C for 30 minutes in water bath. The assay was based on the formation of p-nitrophenol (pNP) in the hydrolysis of p-nitrophenol phosphate (pNPP). The activity was measured spectrophotometrically at 420 nm.

Acid phosphatase activities of liver and kidney were estimated biochemically [8]. For determination of acid phosphatase activity, the same homogenizing medium was used at the tissue concentration as in ALP. Activity of ACP was measured in acetate buffer at pH 4.5 using p-nitrophenol phosphate as substrate and OD was noted at 420 nm using spectrophotometer.

Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) are important indicators for the assessment of metabolic toxicity and we also measured GOT and GPT activities in those organs biochemically [9]. The above-mentioned tissues were homogenized separately at a tissue concentration of 50 mg/mL in 0.1M of ice-cold phosphate buffer (pH=7.4). The homogenates were centrifuged at 5000 rpm at 4°C for 15 min. The substrate GOT used here were 200 mM/L of DL-aspartate and 2 mM/L of α -ketoglutarate where the substrates of GPT were 200 mM/L DL-alanine and 2 mM/L of α -ketoglutarate (pH=7.4). The absorbance was noted at 505nm. The change in absorbance was determined by subtracting the blank reading from corresponding test reading. The en-

zyme activity was expressed in term of unit/mg of tissue.

Quantitative Study of Spermatogenesis at Stage VII

Paraffin blocks of testis were serially cut at 5 μ m thickness and stained in haematoxyline and eosin. The quantitative analysis of spermatogenesis was carried out at stage VII of seminiferous epithelial cell cycle. Characteristics cellular associations of germ cells present at this stage are spermatogonia -A (ASg), preleptotene spermatocytes (pLSc), mid-pachytene spermatocytes (mPSc) and step 7 spermatids (7Sd). The relative number of each variety of germ cells at stage VII of seminiferous epithelial cells, i.e. ASg, pLSc, mPSc and 7Sd were counted. The different nuclei of germ cells (except step 19 spermatids, which cannot be enumerated precisely) were counted in 25 round tubular cross section at stage VII of the cycle in each rat. All the nuclear count (crude count) of the germ cells were corrected using the Abercrombie formula [10].

Histometric Study

The prepared slides were placed under high power objectives in a phase contrast microscope, and with the help of stage and ocular micrometer, the seminiferous tubular diameters (STD) were measure. For a single measurement of this parameter, 10 round or oval shaped seminiferous tubule were selected and the diameter was noted in each tubule by taking the upward and downward margin lines of the tubule as well as the left side and right side margin lines of the tubule from each rat [11]

Statistical Analysis

Student's two-tail "t" test was used for statistical analysis of the collected data [12]. Difference were considered significant when $p<0.05$.

RESULTS

Organo-Somatic Indices

In this experimental profile, there was no significant difference in body weight between two groups (Table 1). Testiculo-somatic, seminal vesiculo-somatic, prostatico-somatic and epididymal somatic indices were decreased significantly without any significant alteration in hepato-somatic and reno-somatic indices at the

Table 2. Inhibitory effect of aqueous leaf extract of *A. marmelos* on quantification of spermatogenesis at stage VII of seminiferous epithelial cell cycle and STD in mature albino rats (Mean±SEM, n=8) as compared with control (Student two tail "t" test). In each vertical column mean with different superscripts (a, b) differ from each other significantly $p<0.05$.

Group	ASg	pLSc	mPSc	7Sd	STD X 100 (μ m)
Control	1.25±0.03 ^a	17.30±0.36 ^a	19.26±0.25 ^a	61.50±1.04 ^a	249.55±2.5 ^a
<i>A. marmelos</i> treated	0.40±0.04 ^b	12.10±0.11 ^b	15.26±0.15 ^b	50.85±0.25 ^b	211.50±2.35 ^b

effective dose of aqueous extract treated rats in comparison to control (Table 1).

Testicular Δ^5 , 3 β -HSD, 17 β -HSD Activities and Plasma Level of Testosterone

Testicular Δ^5 , 3 β -HSD and 17 β -HSD activities were decreased significantly in treated group in comparison to control (Fig 1). Plasma testosterone level was decreased significantly in treated group in comparison to control (Fig 1).

Quantification of Germ Cells at Stage VII

Quantitative study of spermatogenesis at stage VII of seminiferous epithelial cell cycle revealed that leaf extract treatment resulted in significant reduction in the number of ASg, pLSc, mPSc and 7Sd in respect to the control (Table 2).

Histometric Study

Histometric studies indicated that STD was decreased significantly in leaf extract treated rats compared to the control (Table 2).

Phosphatase and Transaminase Activities in Liver and Kidney

Activities of ACP, ALP, GOT and GPT in the liver and kidney were not altered in the leaf extract treated rats in comparison to control (Table 3).

DISCUSSION

The present paper provides a number of observations regarding effects of aqueous extract of leaf of *A. marmelos* on testicular functions in male albino rats. From results, it has been revealed that there was no significant alteration in somatic growth in treated rats compared to the control. This suggests that this leaf extract has no general toxic effect on body growth. Diminution in the weight of testiculo-somatic, seminal vesiculo-somatic, prostatico-somatic and epididymal somatic indices in the treated group may be due to low plasma level of pituitary gonadotrophins and testosterone as these growth indices are controlled by plasma level of pituitary gonadotrophins and testosterone [13, 14].

The diminution in the activities testicular Δ^5 , 3 β -HSD and 17 β -HSD, the key enzymes for androgenesis [15, 16], may be due to inhibition in pituitary gonadotrophins secretion [17, 18]. The low plasma level of testosterone reported here also supports this inhibition in steroidogenic enzyme activities.

Quantitative study of spermatogenesis was carried out at stage-VII because the cellular associations of germ cells in this stage were composed of elements positioned equidistant in the entire process of spermatogenesis. Therefore, counts of germ cells at this particular stage of the cycle are representative of the condition of spermatogenesis as a whole because all varieties of germ cell are present at this stage [19]. Reduction in the number of all the varieties of germ cells at stage VII of seminiferous epithelial cell cycle in the leaf extract treated rats may be due to low plasma level of pituitary gonadotrophins and testosterone, which are the major regulators of spermatogenesis [20, 21]. Moreover, diminution in STD in leaf extract treated rats also supports the low plasma level of testosterone as STD is one of the indicators of plasma level of testosterone [22].

To determine whether the effective dose of leaf extract of a *A. marmelos* that induces antitesticular effect have any metabolic toxicity, we measured ACP, ALP, GOT and GPT activities in the liver and kidney because these enzymes are the indicators of metabolic toxicity [23, 24]. Specific enzyme for toxicity study indicated that there was no significant alteration in ACP, ALP, GOT and GPT activities in liver and kidney of the leaf extract treated group. Therefore, it may be claimed that the applied dose of leaf extract of *A. marmelos* has anti-gonadal effect without induction of any toxic effects on metabolic organs.

In conclusion the data of our study suggest that the aqueous extract of the leaf of *A. marmelos* results in diminution in the activities of testicular androgenic key enzymes and plasma testosterone along with inhibition in spermatogenesis with out any induction of hepatic and renal toxicity. The actual mode of action of this leaf extract for its hypogonadal activities is not clear from this experiment and more information is necessary to better understand the effect of this leaf extract on the functional physiology of the reproductive system. This

Table 3. Effect of aqueous leaf extract of *A. marmelos* on ACP, ALP, GOT and GPT activities in liver and kidney in mature albino rats (Mean±SEM, n=8) as compared with control (Student two tail "t" test). In each vertical column mean with same superscript (a) did not differ from each other significantly, $p<0.05$.

Condition	ACP activities (μ g of PNP/mg of tissue/h)		ALP activities (μ g of PNP/mg of tissue/h)		GOT activities (Unit/mg of tissue)		GPT activities (Unit/mg of tissue)	
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
Control	11.00±0.13 ^a	16.15±0.21 ^a	13.25±0.91 ^a	14.25±0.42 ^a	52.35±1.85 ^a	28.00±1.20 ^a	50.25±1.65 ^a	20.23±0.75 ^a
<i>A. marmelos</i> treated	12.10±0.25 ^a	16.56±0.65 ^a	13.60±0.75 ^a	14.77±0.45 ^a	52.50±2.10 ^a	28.50±1.32 ^a	50.00±1.70 ^a	20.01±0.81 ^a

study creates a hope to develop a new drug from an herbal product in this new era of herbal drug technology.

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REFERENCES

1. Udoh P, Kehide A. Studies on antifertility effect of Pawapaw seeds (*Carica papaya*) on the gonads of the male albino rats. *Phytother Res*. 1999;13:226-8.
2. Hiremath SP, Badani S, Hunasagatta SK, Patil SB. Antifertility and hormonal property of flavones of strigaoroleanchioider. *Eur J Pharamcol*. 2000;391:193-7.
3. Bhattacharyay S. Medical importance Aegle marmelos. Bhattacharya S. (eds), In: Chiranjib Banushadi. Ananda Publisher, Kolkata, 1982; p. 341-6.
4. Talalay P. Hydroxysteroid dehydrogenase. Colowick SP, Kaplan NO. (eds), In: Methods in Enzymology. Academic Press, New York, 1962; p. 512-6.
5. Jarabak J, Adams JA, Williams Ashman HG, Talalay P. Purification of 17 β -hydroxysteroid dehydrogenase of human placenta and studies on its transdehydrogenase function. *J Biol Chem*. 1962;237:345-7.
6. Srivastava TJ. ELISA of steroid hormone. In: Orientation Training Course on Research Methodology in Reproductive Biomedicine, National Institute of Health and Family Welfare. New Delhi, 2002; p. 55-8.
7. Malamy M, Horecker BL. Alkaline phosphatase. Word WA. (eds), In: Methods in Enzymology, Academic Press, New York, 1966; p. 639-42.
8. Vanha-Perttula T, Nikkanen V. Acid phosphatase of the rat testis in experimental conditions. *Acta Endocrinol*. 1999;72:376-90.
9. Goel BK. Routine biochemical tests. Mukherjee KL, In: Medical Laboratory Technology. Tata McGraw-Hill Publishing Company Limited, New Delhi, 1988; p. 985-1079.
10. Abercrombie M. Estimation of nuclear population from microtome sections. *Anat Res*. 1945;99:239-47.
11. Burgos MH, Ladman AJ. The effect of purified gonadotrophins on the morphology of testis and thumb pads of the normal and hypophysectomized autumn frog (*Rana pipens*). *Endocrinology*. 1957;61:20-6.
12. Sokal RR, Rohle FJ. Introduction to Analysis of Variance. Sokal RR, Rohle FJ. (eds), In: Biometry, WH Freeman and Company, New York, 1997; p. 179-206.
13. Ghosh SP, Chatterjee TK, Ghosh JJ. Synergistic role of prolactin and testosterone in the regulation of acid phosphatase activity and isoenzyme pattern in accessory sex organs of adult male rats. *J Reprod Fertil*. 1983;67:235-40.
14. Barkeley MS, Goldmass BD. A quantitative study of serum testosterone. Sex accessory organ growth and the development of inter male aggression in the mouse. *Horm Behav*. 1979;8:208-18.
15. Maier DM. Species variations in testicular Δ^5 , 3 β -hydroxysteroid dehydrogenase activity: absence of activity in primate Leydig cell. *Endocrinology*. 1965;76:463-9.
16. Baillie AH, Mack WS. Hydroxysteroid dehydrogenase in normal and abnormal human testes *J Endocrinol*. 1966;35:239-48.
17. Hall PE, Eik-Nes KB. The influence of gonadotrophins in vivo upon the biosynthesis of androgens by homogenate of rat testis. *Biochem Biophys Acta*. 1963;71:438-47.
18. Muroto EP, Payne AH. Testicular maturation in the rat. In vivo effect of gonadotrophins on steroidogenic enzyme in the hypophysectomized immature rat. *Biol Reprod*. 1979;20:911-8.
19. Clermont Y, Harvey SC. Effects of hormones on spermatogenesis in the rat. Ciba Foundation *Colloquium Endocrinol*. 1967;16:173-96.
20. Spiteri-Grech J, Weimbauer GF, Bolze P, Chandolia, RK, Bartlett JM, Neieschlog E. Effects of FSH and testosterone on intratesticular insulin like growth factor-1 and specific germ cell population in rats treated with gonadotrophins releasing hormone antagonist. *J Ethnopharmacol*. 1993;137:81-9.
21. Itoh N, Nanbu A, Tachiki H, Akagashi K, Nitta T, Mikuma N, Tsukamoto T, Mumamoto Y. Restoration of testicular transferin, insulin like growth factor-1 (IGF-1), and spermatogenesis by exogenously administrated purified FSH and testosterone in medically hypophysectomized rats. *Arch Androl*. 1994;33:169-77.
22. Ghosh D, Jana D, Debnath J. Effect of leaf extract of *Stephania hernandifolia* on testicular gametogenesis and androgenesis in albino rats: a dose dependent response study. *Contraception*. 2002;65:379-84.
23. Akther MH, Mather M, Vhida NK. Skin and liver toxicity in experimental *Lantana camara* poisoning in albino rat *Ind J Physiol Pharmacol*. 1990;34:13-6.
24. Das KK, Dasgupta S. Influence of ascorbic acid on acid and alkaline phosphatase activities in some metabolically active tissue. *Ind J Physiol*. 1997;41:421-3.

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