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Effect of Ethanolic Extract of Annona Squamosa Plant Seed on Male Rats' Infertility

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Abstract
The antifertility potential of a number of plants has been investigated in an effort to create a human contraceptive. Therefore, the purpose of this work is to assess how the ethanolic extract of the seeds of the plant Annona squamosa, also known as custard apple, affects the amount of protein, glycogen, and phosphatase activity in the rat reproductive organs. In comparison to control rats, the treated rats' testis, caput epidydymis, cauda epidydimis, vas deferens, and seminal vesicles showed a marked reduction in protein, glycogen, and phophatase activity throughout time and dose. Changes in these measures demonstrate that the plant has antiandrogenic properties and show interference of the active chemicals on the reproductive physiology..
Keywords: *Glycogen, protein, seminal vesicles, testis, caput epidydymis, cauda epidydimis, Annona squamosa, and acid phosphatase and alkaline phosphatase.*

I. Introduction

The Annonaceae family's "custard apple," or *Annona squamosa* Linn., is native to the West Indies and South America and is grown all throughout India, primarily for its edible fruits (Morton J, 1987). Because the seed and bark of this plant contain strong, physiologically active annonaceous acetogenins, it has been investigated for a variety of pharmacological and biological functions. Alkaloids, terpenoids, and saponins are also found in (A. squamosa) seeds, according to analysis. In mice and rats, this plant is said to have important medicinal qualities, such as anti-fertility, anti-spermatogenic, anti-tumor, and antidiabetic effects (Kritiker and Basu, 1975, Leboeuf et al. 1982, Gupta et al. 2005, Gupta et al. 2010 and Kaleem et al. 2006). Therefore, this article aims to assess the impact of an ethanolic extract of A. squamosa seeds on the biochemistry of male albino rats' reproductive organs.

II. Materials And Methods

The powdered seeds bark and leaves (50g) were extracted with ethanol for 48 hours. This ethanolic extract was dried at controlled temperature (40-500c) to yield solid powder that was further used for experiments. Healthy colony bred male albino rat weighting 120-150 grams were used for the experiments. The animals were housed in polypropylene cages and these cages were cleaned regularly to avoid rat smell and to maintain standard hygienic conditions (12h light / 12h dark cycles and 25°C ± 5°C at room temperature). The rats were acclimatized to laboratory conditions for 10 days and fed with standard diet and water was provided *ad libitum*. The animals were divided into three groups, containing 16 animals in each group. A group of rats was administered 200mg/kg body weight /rat/day of the ethanolic extract of seed of *A. squamosa* (EEAS) and other group was administered 300mg/kg bodyweight/rat/ day every morning for 28 days. The remaining third group was fed with vehicle of similar dilution without test material and kept as control group. After every 7th, 14th, 21st and 28th days both control and treated groups four animals from each group were autopsied under light chloroform anesthesia. The reproductive organs Testis, caput epdidymis, cauda epidydymis, vas defrense and seminal vesicles were dissected out, weighed and homogenized for further biochemical estimations

Biochemistry of reproductive organs

Estimation of glycogen: Glycogen was measured by the Anthrone method of Vander Vies (1954). Glycogen present in submicroscopic form in tissues, yield an amorphous product on reacting with KOH which produces a greenish brown colour with anthrone reagent. 50 mg tissue was homogenized with 5ml of 5% TCA and filtered. To 1.0 ml of filtrate, one ml of 10 N KOH was added and the mixture was boiled for exactly 60 minutes. Excess alkali was neutralized with 0.5 ml of glacial acetic acid and distilled water was added to make a final volume of 10 ml. 1.0 ml of the above mixture was added to 2.0 ml of freshly prepared anthrone reagent (2 mg anthrone/ml of 36 N H2SO4), shaken laterally and heated in a boiling water bath for 10 minutes. A greenish brown colour developed which was measured calorimetrically at 650 nm using visible spectrophotometer (Systronic, India) against blank, prepared simultaneously by using 1.0 ml of 5% TCA instead of 1.0 ml of tissue filtrate. The opti-

cal density was compared with a set of glucose standard of varying concentration (0.01 mg/ml to 0.04 mg/ml). Appropriate calculations were made to compute glycogen. Results have been expressed as µg glycogen/mg tissue.

Estimation of protein: Quantitative estimation of protein was made according to the procedure of Lowry, *et al*. (1957). The tissue was homogenized (1mg/ml, w/v) in 10% TCA using an electrical homogenizer for 5 minutes. The homogenate thus obtained was centrifuged at 6000 g for 20 minutes and the precipitate was collected. The precipitate was washed twice with 5% TCA and again centrifuged at 6000 g for 20 minutes in 4 ml of 5% TCA. The precipitate was dissolved in 4 ml of 1N NaOH. 1ml of dissolved solution was mixed with 5 ml of reagent C. Reagent C was prepared by addition of 50.0 ml of reagent A (2% sodium carbonate in 0.1 N NaOH) and 1.0 ml of reagent B (1% of sodium potassium tartarate, 0.5% copper sulphate, mixed with 1:1 ratio at the time of experiment). The reaction mixture was kept for 10 minutes at room temperature, the 0.5 ml of reagent D (freshly prepared phenol reagent/distilled water, 1:2 ratio) was added and mixed thoroughly. After 10 minutes a blue colour developed which was measured by visible spectrophotometer at 600 nm. Standard curves were prepared using different concentrations of bovine serum albumin. Results have been expressed as µg protein/mg tissue.

Estimation of phosphatase activity: Activity of acid and alkaline phosphatase in the tissue was determined according to method of Bergmeyer (1967) as modified by Singh and Agarwal (1989) using p-nitrophenyl phosphate as substrate. Homogenates of tissue (2% w/v) were prepared in ice cold 0.9% NaCl solution and centrifuged at 5000 g at 0oC for 20 minutes. The supernatant were used as enzymes.

Acid phosphatase: Acid phosphatase activity was determined by adding 0.2 ml of enzyme source, 1.0 ml of acid buffer substrate solution (prepared by dissolving 0.41 g citric acid, 1.125 g sodium citrate and 165 mg p-nitrophenyl phosphate sodium salt to 100 ml of double distilled water), the mixture was mixed thoroughly and incubated for 30 minutes at 37oC. Then 4.0 ml of 0.1 N NaOH was added to the incubated mixture. A yellow colour developed which was measured at 420nm using visible spectrophotometer. Standard curve was drawn using p-nitrophenol as substrate. Enzyme activity has been expressed as µ mole substrate hydrolyzed/30 minute/mg protein in supernatant.

Alkaline phosphatase: Alkaline phosphatase was determined by adding 0.1 ml of enzyme source to 1.0 ml of alkaline buffer substrate. Alkaline buffer substrate was prepared by addition of 375 mg glycine, 10 mg MgCl2.6H2O and 165 mg p-nitrophenyl phosphate sodium salt in 42 ml of 0.1 N NaOH. The mixture was made up to 100 ml with double distilled water. The mixture was mixed thoroughly and incubated for 30 minutes at 37oC. In the incubated mixture 10 ml of 0.02 N NaOH was added to stop the reaction. p-nitrophenol formed as a result of hydrolysis of p-nitrophenyl phosphate, gave a yellow colour with NaOH. Optical density was measured at 420nm using visible spectrophotometer. Standard curve was drawn with different concentrations of p-nitrophenol. Enzyme activity has been expressed as µ mole substrate hydrolyzed/30 minute/mg protein in supernatant.

III. Results and Discussion

A significant ($p<0.05$) dose and time dependent decrease in the level of glycogen and protein was observed in the testis, caput epidydymis, cauda epidydimis, vas deferens and seminal vesicles of rats treated with ethanolic extract of seeds of *Annona squamosa* in comparison to control rats. (Table 1 and 2).

A non-significant dose and time dependent decrease in the activity of acid phosphatase in the testis of treated rats after 7 and 14 day, while a significant ($p<0.05$) dose and time dependent decrease in the activity of acid phosphatase after 21 and 28 days in rats treated with 200 mg/kg body weight of rats treated with ethanolic extract of seeds of *Annona squamosa* in comparison to control. On exposure to 300 mg/kg body weight of ethanolic extract of seeds of *Annona squamosa* a significant ($p<0.05$) dose and time dependent decrease in the activity of acid phosphatase was observed in rats treated with ethanolic extract of seeds of *Annona squamosa* with respect to control (Table 3).

Activity of alkaline phosphatase level in testis, caput epidydymis, cauda epidydimis, vas deferens and seminal vesicles decreased significantly ($p<0.05$) on exposure to the ethanolic extract of the seed of *Annona squamosa* with respect to control rats. This decrease was observed to be dose and time dependent (Table 4).

Decreased level of glycogen and proteins in the reproductive organs of the treated rats suggest changes in the physiological changes. Glycogen particles are reported to be localized in the tunica albugínea, gonocytes, supporting cells, Leydig cells and in Sertoli cells. A constant supply of glucose is considered essential for proper functioning of the testis and the maturation of germ cells (Fabbrini, *et. al*. 1969 and Free 1970). The sertoli cells and spermatogonia contain glycogen and provide nourishments to the seminiferous tubular cells. The glycogen content is reported to be directly proportional to the steroid hormones (Rommerts *et. al*., 1974). A decrease in

glycogen content of the testis reduces the energy source for spermatogenic activity. Gunga *et. al.,* (1972) reported that the key sites of glucose metabolism in the rat testis are spermatocytes and spermatids. In degenerating tubules glycogen is less abundant than in normal tubules (Long and Engle, 1962). The reduced glycogen level in epididymis (cauda and caput), vas deferens and seminal vesicles may be due to severe necrobiosis. Leiderman and Mancini (1969) observed a relationship between the glycogen content and gonadal maturation. A significant decrease in the level of glycogen in testis, cauda and caput epididymis, vas deferens and seminal vesicles of rats after administration of EEAS observed in the present study might be due to impairment of carbohydrate metabolism. This also indicates degenerating tubules, disturbance in gonad maturation and decrease in the level of steroid hormones.

A fall in total protein after administration of EEAS noted in testis, epididymis (cauda and caput), vas deferens and seminal vesicles might be due to inhibition and marked reduction in germ cells as reported by Ramalingam (2006). Proteins are main component of the cell and in testes they are required for division and formation of new cells. A significant reduction in protein in the treated rats indicates inhibition of spermatogenesis (Dixit and Bhargava, 1983).

Similar result were also was reported by Vijaykumar *et. al*., (2003), Chinoy *et. al*., (2005), Chinoy and Mehta (1999). The reduced glycogen level could affect protein synthesis; because protein synthesis in spermatogenic cells is dependent on glucose (Dixit *et. al*., 1979). Reduced testicular and epididymal protein content can result in the absence of spermatozoa in the lumen (Zhen *et. al*., 1995, Patil *et. al*.,2010).

Acid and alkaline phosphatases regulate the secretory activity of the testis and are present in seminiferous tubules and Leydig cells (Blackshaw and Hemitton, 1970). The reduced activity of Acid phosphatase in testis, cauda and caput epididymis, vas deferens and seminal vesicles may be attributed to the increased spermatogenic destruction, as a consequence of inadequate androgen supply within these reproductive organs on exposure to EEAS (Blackshaw, 1973). Testicular acid phosphatase is dependent upon specific hormonal stimuli. The activity of this enzyme is an accurate biochemical marker of specific stages of spermatogenesis and it has been specifically correlated with androgenic stimulation (Males and Turkington, 1971). In testis, epididymis (Cauda and caput), vas deferens and seminal vesicles the decrease was observed due to severe impairment of testicular function, as there is a positive relationship between acid phosphatise activity and spermatogenesis in rats (Nair *et. al*., 1987).

Alkaline phosphatase is an enzyme, which acts as a marker for primordial germ cells (Mintz, 1957). It plays a critical role in protein synthesis (Pilo *et. al*., 1972). It also plays an important role in the transport of metabolite across the membrane (Vorbrodt 1959). Lundsgaard (1933) and Srivastava (1966) established that the alkaline phosphatase is concerned with phosphorylation and dephosphorylation processes during glucose absorption. Alkaline phosphatase helps in spermatogenesis, carbohydrate metabolism and in the synthesis of testicular hormones (Arzac 1954). The depletion of alkaline phosphatase activity in male genital organs after the treatment of seed of ethanolic extract of *Annona squamosa* may be due to some atrophic changes in testis and metabolic disturbances in the germinal and non germinal components of testis. The decrease in its level indicates arrest of spermatogenesis and absence of secondary spermatocytes and spermatids in seminiferous tubules.

Hence, the present investigation indicates ethanolic extract of the seeds of *Annona squamosa* is antiandrogenic and its exposure induces testes, epididymis (Cauda & Caput), vas deferens and seminal vesicles dysfunction in rat leading to a significant decrease in glycogen, protein and activity of phosphatases.

Table1: Glycogen content in reproductive organs of control and experimental mice following treatment with ethanolic extract of *Annona squamosa* seed.

Organ	Days	Control Rats Mean±	Treated rats	
			200mg/kg Body Weight Mean±	300mg/kg Body Weight Mean±
Testis	7	20.27±0.21	19.30±0.05	15.20±0.09
	14	20.76±0.41	17.57±0.16	9.45±0.17
	21	21.32±0.20	15.24±0.017	8.32±0.29
	28	21.47±0.40	8.085±1.22	5.26±0.35
Caput Epididy-mis	7	26.14±0.03	24.44±0.16	19.97±0.45
	14	28.13±0.03	23.15±0.32	19.20±0.40
	21	30.09±0.26	20.16±0.026	16.47±0.45
	28	33.35±0.17	15.18±0.024	13.15±0.023
Cauda Epididy-mis	7	18.06±0.02	16.32±0.18	12.64±0.22
	14	20.70±0.27	14.58±0.12	11.45±0.13
	21	22.33±0.04	11.72±0.38	10.515±0.25
	28	25.40±0.06	9.79±0.30	9.07±0.53
Vas deferens	7	20.25±0.073	18.70±0.23	16.52±0.26
	14	23.32±0.08	17.15±0.07	13.45±0.16
	21	25.45±0.22	15.31±0.08	12.40±0.18
	28	28.40±0.12	14.28±0.09	10.05±0.02
Seminal vesicles	7	21.59±0.24	19.3±0.12	18.60±0.18
	14	24.29±0.29	18.11±0.10	14.47±0.10
	21	26.02±0.23	16.85±0.20	12.51±0.21
	28	29.91±0.23	15.37±0.12	9.41±0.06

* indicates significant ($p<0.05$) difference between control and treated groups when student 't' test is applied between treated and control groups.
+ indicates significant ($p<0.05$) effect of variation in dose and time on treated rats when Two ways ANOVA was applied between control and treated groups.
(values are mean ±SE of 10 animals in each group).

Table 2: Protein content in reproductive organs of control and experimental mice following treatment with ethanolic extract of *Annona squamosa* seed.

Organ	Days	Control Rats Mean±	Treated rats	
			200mg/kg Body Weight Mean±	300mg/kg Body Weight Mean±
Testis	**7**	22.21±0.03	17.31±0.13	16.62±0.21
	14	22.26±0.04	16.69±0.44	16.23±0.31
	21	23.94±0.45	15.51±0.47	15.01±0.50
	28	27.17±0.03	13.95±0.42	13.37±0.61
Caput Epididy-mis	**7**	26.41±0.19	20.42±0.14	18.50±0.46
	14	23.32±0.39	17.87±0.50	16.22±0.44
	21	24.97±0.67	18.22±0.32	16.11±0.23
	28	27.15±0.72	17.05±0.48	12.72±0.42
Cauda Epididy-mis	**7**	20.54±0.11	18.60±0.13	15.49±0.12
	14	22.84±0.28	17.39±0.45	13.16±0.12
	21	24.99±0.36	15.36±0.14	11.85±0.19
	28	27.28±0.99	12.66±0.18	9.05±1.10
Vas deferens	**7**	19.53±0.08	17.95±0.25	15.97±0.26
	14	21.73±0.44	15.18±0.09	13.73±0.25
	21	23.41±0.16	14.15±0.13	12.50±0.25
	28	25.20±0.06	12.23±0.07	10.01±0.004
Seminal vesicles	**7**	20.72±0.10	19.03±0.29	16.60±0.11
	14	22.96±0.22	17.34±0.06	13.27±0.04
	21	24.56±0.61	14.82±0.09	10.40±0.13
	28	28.57±0.04	11.28±0.06	8.26±0.30

* indicates significant ($p<0.05$) difference between control and treated groups when student 't' test is applied between treated and control groups.
+ indicates significant ($p<0.05$) effect of variation in dose and time on treated rats when Two ways ANOVA was applied between control and treated groups.
(values are mean ±SE of 10 animals in each group**).**

Table 3: Activity of acid phosphatase in reproductive organs of control and experimental mice following treatment with ethanolic extract of *Annona squamosa* seed.

Organ	Days	Control Rats Mean±	Treated rats	
			200mg/kg Body Weight Mean±	300mg/kg Body Weight Mean±
Testis	7	0.028±0.002	0.0195±0.0005	0.016±0.0005
	14	0.031±0.002	0.018±0.0008	0.014±0.0007
	21	0.042±0.005	0.014±0.005	0.010±0.0006
	28	0.050±0.004	0.011±0.006	0.09±0.0005
Caput Epi-didymis	7	0.02±0.002	0.017±0.002	0.013±0.19
	14	0.031±0.001	0.014±0.001	0.011±0.0005
	21	0.041±0.002	0.012±0.002	0.010±0.001
	28	0.045±0.08	0.085±0.001	0.082±0.008
Cauda Epi-didymis	7	0.032±0.002	0.026±0.002	0.024±0.002
	14	0.053±0.004	0.024±0.0018	0.021±0.001
	21	0.071±0.003	0.021±0.002	0.019±0.001
	28	0.078±0.002	0.018±0.002	0.015±0.002
Vas defer-ens	7	0.026±0.006	0.022±0.001	0.02±0.0003
	14	0.037±0.002	0.021±0.003	0.018±0.001
	21	0.052±0.002	0.017±0.002	0.015±0.002
	28	0.069±0.002	0.012±0.002	0.011±0.002
Seminal vesicles	7	0.032±0.001	0.027±0.001	0.024±0.001
	14	0.053±0.003	0.025±0.10	0.022±0.001
	21	0.068±0.002	0.022±0.001	0.018±0.002
	28	0.092±0.003	0.019±0.003	0.015±0.003

indicates significant ($p<0.05$) difference between control and treated groups when student't' test is applied between treated and control groups.
(values are mean ±SE of 10 animals in each group**).**

Table 4: Activity of alkaline phosphatase in reproductive organs of control and experimental mice following treatment with ethanolic extract of *Annona squamosa* seed.

Organ	Days	Control Rats Mean±	Treated rats	
			200mg/kg Body Weight Mean±	300mg/kg Body Weight Mean±
Testis	**7**	0.069±0.002	0.053±0.0008	0.048±0.001
	14	0.064±0.048	0.048±0.001	0.045±0.001
	21	0.058±0.003	0.04±0.001	0.037±0.001
	28	0.055±0.04	0.034±0.003	0.029±0.003
Caput Epididy-mis	**7**	0.071±0.001	0.062±0.001	0.054±0.001
	14	0.068±0.001	0.054±0.001	0.037±0.002
	21	0.058±0.001	0.043±0.002	0.038±0.002
	28	0.053±0.001	0.033±0.001	0.018±0.0025
Cauda Epididy-mis	**7**	0.076±0.002	0.068±0.002	0.061±0.001
	14	0.069±0.002	0.060±0.002	0.048±0.003
	21	0.066±0.002	0.056±0.002	0.041±0.001
	28	0.064±0.001	0.05±0.0003	0.037±0.001
Vas deferens	**7**	0.023±0.0005	0.0215±0.0005	0.02±0.0003
	14	0.021±0.0003	0.018±0.0004	0.018±0.0007
	21	0.019±0.0005	0.015±0.0009	0.0145±0.0002
	28	0.016±0.018	0.07±0.029	0.011±0.0004
Seminal vesicles	**7**	0.028±0.001	0.025±0.001	0.022±0.0008
	14	0.025±0.000	0.021±0.001	0.019±0.001
	21	0.0215±0.001	0.018±0.001	0.015±0.0009
	28	0.0014±0.001	0.013±0.0007	0.010±0.0007

* indicates significant ($p<0.05$) difference between control and treated groups when student't' test is applied between treated and control groups.

$^{+}$ indicates significant ($p<0.05$) effect of variation in dose and time on treated rats when Two ways ANOVA was applied between control and treated groups.

(values are mean ±SE of 10 animals in each group).

References

[1]. Morton J, (1987), Sugar apple In Fruits of warm climates, Miami Fl., pp 69-72.

[2]. Kritiker KR and Basu BD, (1975), In Indian Medicinal Plants, Singh Bishen, Singh Mahendra Pal (Eds), New Canaught Place, Dehradun, India, pp. 785–788.

[3]. Leboeuf M, Cave A, Bhaumik PK, Mukherjee B and Mukherjee R, (1982), The Phytochemistry of Annonaceae, Phytochemistry, 21(12), 2783-2813.

[4]. Gupta RK, Kesari AN, Murthy PS, Chandra R, Tandon V and Watal G, (2005), Hypoglycaemic and antidiabetic effect of ethanolic extract of leaves of *Annona squamosa* in experimental animals, J Ethnopharmacol, 99(1), 75-81.

[5]. Gupta RS, Sharma A and Kachhawa JBS, (2010), Evaluation of reversible contraceptive activities of *Annona squamosa* (Linn.) stem barks methanol extract in male rats, Plant Product Research Journal, 14, 28-31.

[6]. Kaleem M, Asif M, Ahmed QU and Bano B, (2006), Beneficial effects of *Annona squamosa* extract in streptozotocin induced diabetic rats, Singapore Med J, 47(8), 670-5.

[7]. Van Der Vies J, (1954), Two methods for the determination of glycogen in liver, Biochem. J., 57(3), 410-416.
[8]. Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ, (1951), Protein measurement with the folin phenol reagents, Journal of Biol. Chem., 193: 265-275.
[9]. Bergmeyer, U.H. (1967). Methods of enzymatic analysis. Acadmic Press, New York, 1129.
[10]. Singh DK and Agarwal RA, (1989), Toxicity of piperonyl butoxide carbaryl synergism on the snail *Lymnaea acuminate*, Int. Revue der Gesamten Hydrobiologie, 7496), 689-699.
[11]. Fabbrini A, Re M and Conti C, (1969), Glycogen in the normal human testis: A histochemical and histoenzymatic study, J Endocrinol, 43, 499-506.
[12]. Free MJ, (1970), Carbohydrate metabolism in the testis. In:The testis, Johnson AD, Gomes WR, Vendemark NL, (Eds.), Vol. II. The testis, Academic Press, London, pp 125-192.
[13]. Rommerts FFG, Cooke BA and Vander Molen HJ, (1974), The role of cyclic AMP in the regulation of steroid biosynthesis in testis tissue, J. Steroid. Biochem., 5, 279-285.
[14]. Gunga KP, Rao MC, Sheth AR and Rao SS, (1972), The role of glycogen during the development of the rat testis and prostate, J Reprod Fert., 29, 157-162,
[15]. Long ME and Engle ET, (1962), Cytochemistry of the human testis, Ann NY Acad Sci., 55, 619-628.
[16]. Leiderman B and Mancini RE, (1996), Glycogen content in the rat testis from post natal to adult ages, Endocrinology, 85, 607-609.
[17]. Ramalingam V, Paneerdoss S and Suryavathi V, (2006), Mercury toxicity and reproduction- A review, In Advances in reproductive toxicology, Joshi SC and Ansari AS (Eds.), Pointers Publishers, Jaipur, pp1-9
[18]. Dixit VP and Bhargava SK, (1983), Reversible contraception like activity of *Embelin* in male dogs (*Canis indicus* Linn), Andrologia, 15, 486-494.
[19]. Vijaykumar B, Sangamma I, Sharanabasappa A and Saraswati BP (2003), Antifertiltiy activity of various extracts of *Crotalaria juncea* Linn seeds in male mice, Philippine Journal of Science, 132, 39-46.
[20]. Chinoy NJ and Mehta D, (1999), Effects of protein supplementation and deficiency on fluoride- induced toxicity in reproductive organs of male mice, Fluoride, 32(4), 204-214.